Validation of Oral Fluid as a Matrix for Drug Detection

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This thesis is dedicated with great gratitude to my father Horst, my mother Conny, my sister Anne and my partner Tim for always encouraging me to be the best I can be.

Abstract

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New testing procedures used as an aid for law enforcement are subject to intensive scrutiny in the courts. In recent years in workplace drug testing there has been a shift away from using traditional specimens (i.e urine) for drug testing and monitoring and a move to employing less invasive testing using oral fluid.

Whilst it is now widely accepted that drugs can be detected in oral fluid and devices are now available to facilitate analysis of drugs in this matrix, our understanding of the behaviour of drugs in the mouth and oral secretions is far from complete. Since the introduction of oral fluid drug testing in the late 1990's it has been observed that some drugs appear to be present at higher than expected concentrations, often at concentrations that would be fatal if they were in blood. Clearly some extra process is occurring in addition to drugs entering the oral fluid by simple blood and saliva partitioning. Little is really known about the physiology of drugs in the mouth and limited understanding of drug elimination via the mouth poses a problem to forensic toxicologists with the interpretation of analytical results in relation to an individual's drug use or the possible effects that the drug may be exhibiting on that individual.

The work described in this thesis is aimed at increasing our understanding of the factors and processes concerning the deposition, secretion and detection of drugs in oral fluid and enhancing our ability to interpret the results of analysis in this matrix. The objective of this study was to explore how high drug concentrations can be deposited in the mouth tissues and oral fluid together with other factors that may influence drug detection in order to assist with the interpretation of testing results.

To test the hypothesis that drug depots form within the mouth, preliminary screening methods in combination with confirmatory techniques such as GC-MS and LC-MS/MS were employed. The development of an immunohistochemical method was successfully demonstrated for the detection and visualisation of cocaine and opiates (heroin and morphine) in porcine and mouse tissue.

The work undertaken in this thesis showed that elevated drug concentrations can be observed when drugs are consumed via oral administration either in form of an oral solution or smoking. Immunohistochemical analysis in combination with confirmatory techniques demonstrated that drugs such as cocaine and opioids can bind to oral tissue and be subsequently released over time, therefore has the potential to contribute to the drug concentration in oral fluid. Although this is not an issue under legislation that forbids the presence of drugs or as a check for drug compliance or abstinence, it must be considered in relation to the interpretation of results in more complex forensic cases.

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Abbreviations

6-MAM	6 - monoacetylmorphine
ABC	Avidin – biotin complex
AEME	Anydroecgonine methyl ester
BSA	Bovine Serum Albumin
BZE	Benzoylecgonine -
Ca2+	Calcium
CE	Cocaethylene
Cl	Chlorine
CNS	Central Nervous system
COC	Cocaine
CYP	Cytochrome 450
DAB	3, 3’ diaminobenzidinetrahydrochloride
DUID	Driving Under the Influence of drugs
EIA	Enzyme Immunoassay
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
EME	Ecgonine methyl ester
EWDTS	European Workplace Drug Testing Service
FIT	Field Impairment Test
GC-MS	Gas chromatography-Mass Spectrometry
GMA	glycol methylacrylate
hCE-1	Human carboxylesterase 1
hCE-2	Human carboxylesterase 2
HCl	Hydrochloric acid
IgA	Immunoglobulin A
IgG	Immunoglobulin G
K+	Potassium
LC-MS	Liquid Chromatography-Mass Spectrometry
MOR	Morphine
Na+	Sodium
NaOH	Sodium Hydroxide
pKa	Acid dissociation constant
S/P	Saliva/Plasma Ratio
SAMHSA	Substance Abuse and Methal Health Services Administration
TBS	Tris-buffered saline
TMB	3,3’,5,5’ - tetramethylbenzidine
UK	United Kingdom

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Chapter 1.0 - Background

Workplace drug testing originated in the U.S. Navy when, in 1980, the U.S. Department of Defence launched a survey within the armed forces to investigate the extent of drug use and its effects on performance in the workplace. The Survey revealed that 47% of US Navy personnel admitted marijuana use in the 30 days prior to testing (Manno 1986, Cangianelli 1989).

The enforcement of drug testing was considered following the 1981 crash of an aircraft on the aircraft carrier USS Nimitz resulting in several deaths. Post-mortem results carried out on those killed in crash revealed traces of marijuana in seven members of the crew (Li et al 2011). As a consequence of the results obtained from the US Navy personnel survey and this aircraft incident, the US military developed a drug testing programme in which random urine specimen collections were enforced and disciplinary action imposed on those found positive for controlled drugs (Peat 1995).

In 1986, this drug testing scheme was extended to all federal agencies with the setup of the Drug Free Workplace Programme (Jacobson 2013). Since then, workplace drug testing has become an established procedure worldwide, particularly in safety related industries. Verstraete et al (2001) reported that in the United Kingdom, 40% of workplace drug testing took place in the military, 35% in prisons and 25% in all other companies.

Urine was initially used for drug testing in the workplace because it is easy to obtain, is a relative simple matrix to analyse and drugs or their metabolites are normally present in significantly higher concentrations than alternative matrices such as blood. Following the widespread introduction of workplace drug testing, entrepreneurial

individuals manufactured and marketed a wide range of chemical agents to act as reagents to "Beat the Test". As these substances became widely available via the internet workplace drug testing authorities introduced a number of guidelines for controlled specimen collection and testing in order to minimise adulteration of urine samples (Manno 1986, Winecker and Goldberger 1998, EWDTS 2009). The mandatory guidelines included observed specimen collection procedures and the introduction of laboratory tests to detect adulterants. Observed urine collection is not only unpleasant for the donor, but is also costly, hence suppliers of workplace drug testing services began to explore the use of alternative matrices such as oral fluid.

Oral fluid is advantageous as a matrix for drug detection, as it is a fast and cost effective method for drug detection, but the technique is still in its relative infancy and there are many unknown factors, related to drugs in oral fluid that remain unresolved. One of the principal problems that remains to be tackled is related to explaining the exceptionally high drug concentrations that can be observed after use of some drugs and the implications that these may have on oral fluid testing and the interpretation of oral fluid drug concentrations. The work undertaken in this thesis aims to explore some of the issues mentioned above.

1.1 Oral fluid

Oral fluid, also commonly referred to as "whole saliva", is principally composed of saliva, gingival fluid, bronchial and nasal secretions, serum, traces of blood, bacteria, viruses, epithelial cells, cellular components and food debris (Malamud et al 1993) Oral fluid is critical to oral hygiene and therefore its composition has been widely investigated and published in the literature.

The term oral fluid is used by many authors interchangeably with saliva but for the purpose of work undertaken in this thesis my measurements will refer to oral fluid rather than saliva.

1.1.1 Composition of Oral Fluid

1.1.1.1 Saliva

Saliva is a clear, tasteless, odorless and viscous fluid formed in the salivary glands that plays an important role in maintaining dental health by removing bacteria and food from the mouth (van Niew Amerongen et al 2004). The main functions and constituents of saliva are summarised in Figure 1.1.

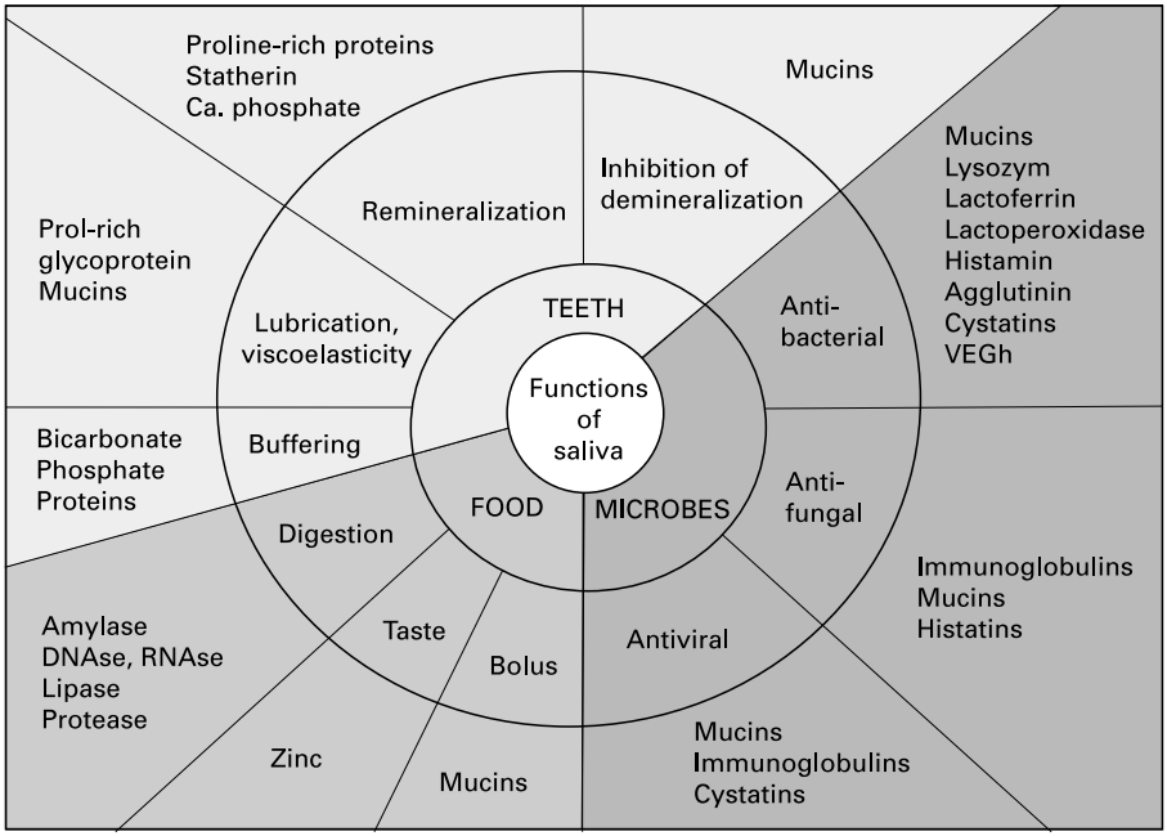


Figure 1.1 represents the main functions of saliva on the teeth, food and microbes within the mouth. It also shows the main constituents of saliva to achieve those functions and processes (Amerongen et al 2004)

1.1.1.1.1 Salivary Glands

Saliva is principally produced by the *glandular parotis* (parotid gland), *glandular submandibularis* (submandibular gland) and the *glandular sublingualis* (sublingual gland) (Figure 1.2). Saliva comprises approximately 99 % water and 1 % proteins and other constituents.

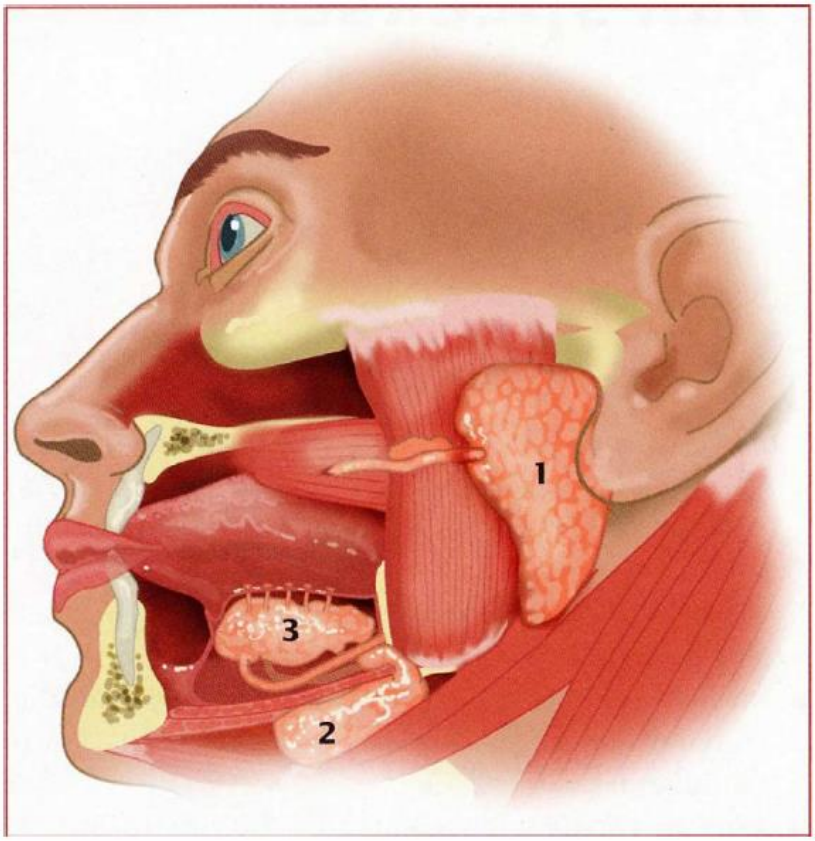


Figure 1.2 Diagrammatic representation of the mouth showing the location of the principal salivary glands 1) parotid gland 2) submandibular gland 3) sublingual gland (Aps and Martens 2004)

The parotid gland is the largest of the three main saliva glands. It is positioned in front of and below each ear (Figure 1.2). Controlled by the central nervous system, the parotid gland produces saliva at a flow rate of 0.3 mL/min, which is equivalent to around 65% of the total saliva secreted from all glands (Eisbruch et al 1999, Burlage et al 2005, Bhargava et al 2012). The submandibular gland is placed on the inner

sides of the lower mandible and produces saliva at a flow rate of around 0.26 mL/min. Located below the tongue, the sublingual gland produces the lowest amount of saliva at a flow rate of 0.012 mL/min (Schneyer and Levin 1955).

In addition to the three main salivary glands it has been estimated that there are between 450 and 750 minor accessory saliva glands situated on the tongue and buccal mucosa / palate which also produce saliva (Nieuw Amerongen et al 2007). Human salivary glands consist of acini consisting of small exocrine glands (Figure 1.3). Acini are made up of basophilic cytoplasm and large spherical nuclei. Acinar excretion glands produce the saliva which is then excreted from an intercalated duct via the striated duct (excretory duct).

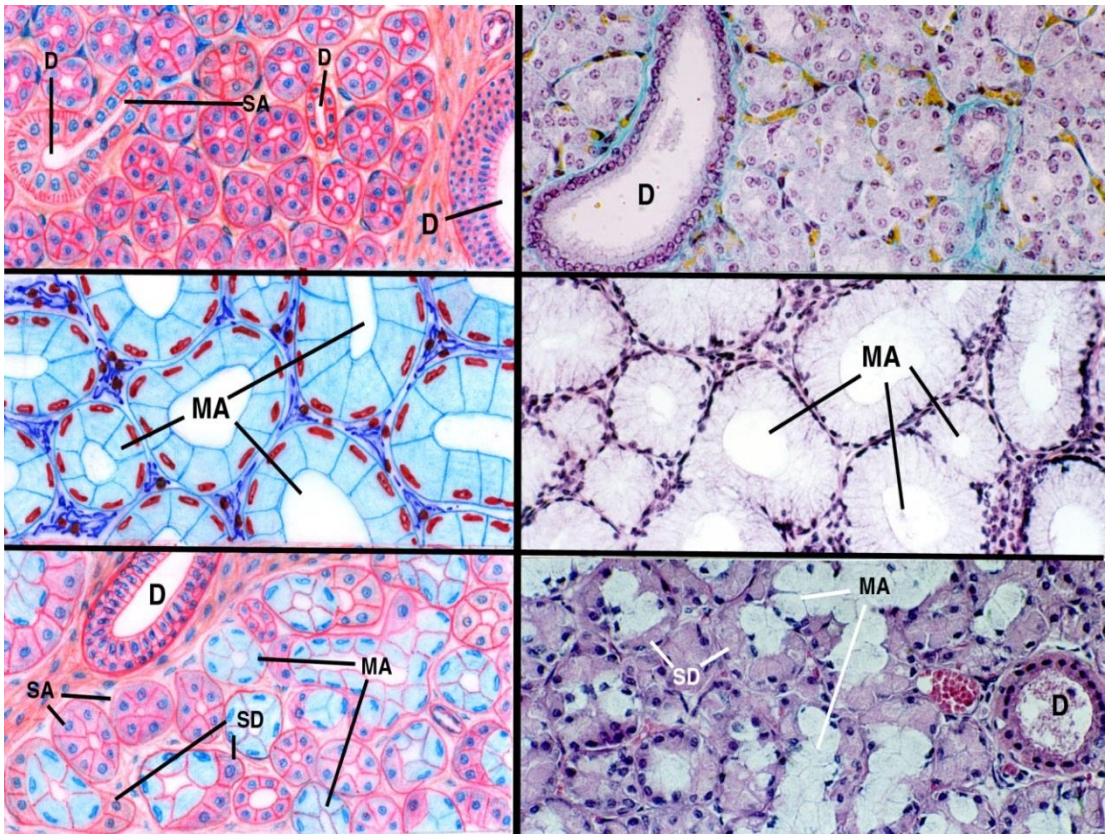


Figure 1.3 Images showing the structure of salivary glands – the left column represents drawings whereas the right are images taken using a microscope (image taken from www.vetmed.vt.edu)

Legend: SA =serous acini; MA = mucous acini; D= duct, SD = serous duct

1.1.1.1.2 Salivary pH

The pH of saliva varies between pH 6.2 – pH 7.4 however the pH of saliva can change following the consumption of food. Meurman et al (1987) showed that salivary pH was affected by drinks such as sugary energy drinks and orange juice, with the pH dropping to a low of pH 3.8. Coffee and Cola resulted in a pH of 5.26. A study by Sanchez and Fernandez De Preliasco (2003) investigating salivary pH in relation to soft drink consumption in children also revealed that following the consumption of coca cola®, Sprite® and chocolate milk the pH of oral fluid dropped from pH 7.4 to pH 6.35, pH 6.20 and pH 7.17, respectively.

Dawes and Macpherson (1992) investigated the effects of different chewing gums on salivary pH. As well as a tenfold increase in saliva flow rate, chewing gum containing organic acids resulted in decrease of pH 7.00 to pH 6.18 (unstimulated saliva is normally pH 6.95). Gum containing sugar as well as sugar free gum only resulted in a minor change in oral fluid pH.

1.1.1.2 Lipids in oral fluid

Larsson et al (1996) reported that the lipid concentration in saliva excreted from different salivary glands varied significantly. The paper showed that saliva excreted from the parotid gland contained lipids at a concentration of 0.21 mg/100 mL whereas submandibular and whole saliva displayed lipid concentrations of 0.89 mg/100 mL and 1.31 mg/100mL respectively (Table 1.1). Neutral lipids such as cholesteryl esters, cholesterol, triglycerides, diglycerides and monoglycerides, were shown to be 99 % in the parotid saliva, 98 % in submandibular saliva and 96.4 % in whole saliva. Neutral lipids have been reported to make up around 70 - 95 % of lipids.

Table 1.1 Non-polar lipids in human saliva from different glands, pooled from 10 individuals (Larsson et al 1996)

	Parotid Saliva (mg/ 100mL).	**Submandibular saliva (mg/ 100mL).**	**Whole saliva (Oral Fluid) (mg/100 mL).**
Cholesteryl esters	0.03	0.26	0.43
Triglycerides	0	0.19	0.29
Diglycerides	0	0.05	0.26
Cholesterol	0.09	0.17	0.13
Monglycerides	0.07	0.15	0.10
Total	0.19	0.82	1.21

Polar lipids, consisting of phosphatidylcholine, phosphatidylethanolamine and sulphatides that are found in the membrane only accounted for around 1 to 5 % of the total saliva lipid concentration (Table 1.2).

Table 1.2 Polar lipids as percentages of the total amount of polar lipids in parotid, submandibular and whole saliva (Larsson et al 1996)

	Parotid saliva	**Submandibular saliva**	**Whole saliva**
Phosphatidylcholine	14	9	1
Phosphatidylethanolamine	15	23	9
Sulphatides	8	28	7
Unidentified lipids	63	40	78
% of non - polar lipids	0.8	2.0	3.6

Lipids in oral fluid which result from contamination of food, consist of a wide range of neutral and polar lipids. Lipids in the mouth originating from food have been shown to alter drug absorption and bioavailability (Charman et al 1997, Humberstone and Charman 1997).

1.1.1.3 Proteins in oral fluid

The primary function of the proteins and glycoproteins in saliva is to facilitate the retention of moisture in the gums, to act as a protective barrier and lubricant and to exert antimicrobial activity (Levine 1993) (Table 1.3).

Table 1.3 Summary of the uses of the major salivary proteins (Levine 1993)

Function	**Salivary component**
Anti-bacterial	Amylases, cystatins, histatins, mucins, peroxidases
Anti-viral	Cystatins, mucins
Anti-fungal	Histatins
Tissue coating	Amylases, cystatins, mucins, proline-rich proteins, stratherins
Lubrication and viscoelasticity	Mucins, stratherins
Mineralisation	Cystatins, histatins, proline-rich proteins, stratherins
Digestion	Amylases, Mucins
Buffering	Carbonic anhydrase, histatins

The proteins, which are located primarily on the gum surfaces, fall into four categories: proline-rich proteins, stratherins, histatins and cystatins.

Proline-rich proteins make up around 70% of the secretory proteins and play an important role in oral hygiene and oral health. Stratherins and histatin enzymes inhibit crystal growth of calcium phosphate salts and inhibit precipitation of calcium

phosphate, resulting in a build up of plaque, whereas cystatin proteins inhibit protease enzymes (Lamkin and Oppenheim 1993).

Another vital protein present in saliva is amylase, which plays an important role in the digestion of food as well as conferring anti-microbial activity. Lee et al (2007) reported the mean concentrations of a selection of proteins and carbohydrates in unstimulated and stimulated oral fluid obtained from 30 healthy volunteers (15 men and 15 women) with an age range of 20 – 26 years (mean age 23.3). This is summarised in Table 1.4.

Table 1.4 Mean concentrations of total protein, total carbohydrate, Immunoglobulin A, lactoferrin and sialic acid in unstimulated and stimulated oral fluid (Ahn et al 2007, Lee et al 2007a, Lee et al 2007b)

	Mean Concentration (µg/mL) Unstimulated Oral Fluid	**Mean Concentration (µg/mL) Stimulated Oral Fluid**
Total protein	785.6	646.2
Total carbohydrate	26.3	23.2
Immunoglobulin A	173.2	107.6
Lactoferrin	1.3	1.0
Sialic Acid	4.6	4.4

Ferguson (1999) reviewed the composition of saliva produced by the human labial gland which is one of the many minor salivary glands and reported the organic components as summarised in Table 1.5. The concentrations for total protein and immunoglobulin A (IgA) reported in Fergusson's review fall within similar ranges to those reported by Lee et al (2007).

Table 1.5 Organic components of Labial gland saliva (Fergusson, 1999)

	Concentration	Concentration (µg/mL)
Total protein	0.4 – 5.35 g/L	400 - 5350
Amylase	1.0 – 12.0 mg/L	1.0 – 12
Lysozyme	3.0 – 4.0 mg/L	3 – 4
IgA	194 mg/L	194
IgG	5mg/L	5
IgM	<1mg/L	<1

1.1.1.4 Electrolytes in oral fluid

The main inorganic electrolytes of saliva are sodium, potassium, chloride, calcium, phosphate and bicarbonate. Calcium and phosphate are responsible for maintaining the integrity of the teeth by re-mineralising tooth surface following exposure to acids during consumption of foods and beverages.

Bicarbonate, phosphate, histatins and carbonic anhydrase play a role in the buffering capacity of saliva which is influenced by storage conditions and may change if carbon dioxide loss occurs (Schipper et al 2007). Neutral pH is optimal for the growth of micro-organisms associated with oral health (Bradshaw et al 1989, Marsh and Bradshaw 1990, Bradshaw and Marsh 1998). The concentration of electrolytes is dependent on variables such as age and health. Nagler and Hershkovich (2001, 2005b) compared the composition of electrolytes and proteins in a population of 23 healthy 18 to 25 years olds (mean age 21.2 years) against a population of 22 healthy 60-90 year olds (mean age 75.8 years) and reported significant differences in salivary composition (Table 1.6). Calcium secretion into saliva increases as the flow rate increases whilst potassium concentrations appear to be independent of flow rate.

Table 1.6 Salivary composition in young and elderly populations (Nagler and Hershkovich, 2005)

Concentration	**Young (n=23) Mean concentration**	**Elderly (n=22) Mean concentration**
Na^+ (mmol/L)	9.52	12.56
K^+ (mmol/L)	19.2	27.78
Cl (mmol/L)	19.7	29.50
Ca^{2+} (mg/100mL)	3.45	5.15
Protein (mg/100mL)	49.8	93.4
Amylase (mg/l)	748	1476
Lysozyme (mg/mL)	10	26.3
Albumin (mg/100mL)	3.97	13.5
IgA (mg/100mL)	21.12	27.58
IgG (mg/100mL)	0.24	0.5

The electrolyte concentrations in oral fluid reported by Nagler et al (2005a) and Shahar et al (2008) are compared against those summarised to be present in labial gland saliva (Ferguson 1999) in Table 1.7.

Table 1.7 Collated reported electrolyte concentrations in oral fluid

Electrolyte	Shahar et al, (2008)	Nagler (2005a)	Weismann et al (1972) (from Fergusson, 1999)	Dawes and Wood (1973) (from Fergusson, 1999)	Fergusson (1999)
Phosphate (mmol/L)	-	-	-	0.25 – 1.07	0.29 – 1.22
Phosphate (mg/100mL)	8 – 42.5	-	-	-	-
Na^+ (mmol/L)	-	9.5 – 12.6	4 – 15	3 – 38	30 - 125
K^+ (mmol/L)	-	19.2 – 27.78	12 – 30	10 – 30	4 – 20
Cl (mmol/L)	-	19.7 – 29.5	-	16 – 54	-
Ca^{2+} (mmol/L)	-	-	-	1.6 – 3.2	2.4 – 5.8
Ca^{2+} (mg/100mL)	0.7 – 2.9	3.45 – 5.15	-	-	-
Mg^{2+} (mmol/L)	-	-	0.5 – 0.6	0.4 – 1.2	-
Mg^{2+} (mg/100mL)	0.4 – 1.3	-	-	-	-

1.1.2 Salivary flow rate

The average person produces between 0.5 L to 1.5 L of saliva per day at a flow rate of approximately 0.6 mL/min (864 mL a per day) (Dawes 1987; Percival et al. 1994). However, the flow rate of saliva varies depending on different conditions such as smoking, drug use, climate and hydration. Smoking has been reported to cause a dry mouth by decreasing saliva flow rate and hence affecting oral fluid collection. (Anttila et al 1998, Axelsson et al 1998).

Shannon (1966) reported that the production of saliva can be influenced by temperature and climate. A flow rate of 0.46 ml/min was reported in the winter and 0.30 ml/min in warmer climates. A different study by Kavanagh et al (1998) who also investigated the effects of temperature and climate on saliva flow rate in great depth. Salivary flow rate can vary throughout the year with changing temperatures and that the flow rate increased with decreasing temperature (Shannon 1966, Kavanagh et al 1998). The change of the quantity of saliva production in warmer climates suggests that less saliva is produced as a result of dehydration. Additionally, salivary flow rate has been shown to decrease during exercise suggesting that this is a result of dehydration during exercise (Li and Gleeson 2004, Walsh et al 2004)

1.1.3 Drug entry into oral fluid

The original concept behind oral fluid drug testing was that drugs circulating in the blood pass into the oral cavity via the saliva leading to a relationship between the concentration of drugs in the circulating blood and those that pass into the oral fluid (Kidwell et al 1998; Aps and Martens 2005). The transfer of drugs into oral fluid is dependent on lipid solubility of the drug, pH and molecular weight. Following intravenous or tablet administration of many drugs it has been possible to calculate saliva:plasma (S/P) ratios (Spiehler 2011). S/P ratios differ between drugs as a result of the variability of properties of different drugs (Table 1.8).

Table 1.8 Factors influencing the S/P Ratio of diffusible compounds (Haeckel 1993)

1. Molecular Weight
2. Lipid solubility
3. pKa of ionized compounds
4. Protein binding in plasma and saliva
5. Flow rate of saliva
6. Phenomenon of fluctuating arterial – venous differences
7. Elimation kinetics

Diffusion depends partly on the solubility of a compound, therefore lipophilic penetrate tissue easier than hydrophilic compounds. A theoretical S/P concentration can be calculated using the Henderson-Hasselbalch equation by applying the drug's pKa, plasma and saliva pH and the amount of drug which has bound to the proteins within saliva and plasma (Haeckel 1993, Spiehler 2011). The legend is S=drug concentration in saliva; P=drug concentration in plasma; pK_d=log of the ionisation constant for basic drugs; pH_s=pH of saliva; pH_p=pH of plasma, f_p=fraction of drug protein bound in plasma and f_s=fraction of drug protein bound in saliva

$$S/P = \frac{\left[1 + 10^{(pK_d - pH_s)}\right]}{\left[1 + 10^{(pK_d - pH_p)}\right]} + \frac{f_p}{f_s}$$

This equation represents the calculation of the S/P ratio for acidic drugs.

$$S/P = \frac{\left[1 + 10^{(pH_s - pk_a)}\right]}{\left[1 + 10^{(pH_p - pK_a)}\right]} + \frac{f_p}{f_s}$$

The Henderson-Hasselbalch equation clearly shows that the S/P ratio is dependent on the saliva pH. In section 1.1.1.1.2 the pH of saliva was discussed and it was shown that the average pH of saliva ranges between pH 6.2 – pH 7.4, dependent on the type external contamination. The variation of pH in saliva means that the S/P ratio can vary for each drug depending on the properties of saliva and plasma at the time of collection (Spiehler et al 2000). Additionally the quantity of drug detectable in saliva is subject to the flow rate. Variations in flow rate (section 1.1.2) may result in a larger dilution of the drug within the oral fluid decreasing the concentration detected (Haekel 1993, Woyceichoski et al 2013).

1.1.4 Oral fluid collection

Oral fluid may be collected by draining or spitting oral fluid into a tube, chewing on an absorbent material or placing an absorbent collection pad in the mouth and allowing the oral fluid to be absorbed. Oral fluid drug testing currently involves three stages: 1) oral fluid collection, 2) immunoassay screening and 3) confirmatory analysis of immunoassay positive samples using either gas chromatography/ mass spectrometry (GC-MS) or high performance liquid chromatography linked to a mass spectrometer (LC-MS). It is therefore essential that for oral fluid testing to be successfully achieved a) a sufficient flow of oral fluid is available for collection, b) there is sufficient drug present to be detected by the immunoassay and subsequent

confirmatory technique, c) the analytical testing steps are not affected by any non-drug substances that might be present in the mouth e.g. foods or chemicals.

A number of different types of oral fluid collection devices are commercially available however these differ markedly in their efficiency, the volume of oral fluid collected and whether an indicator is incorporated within the device to inform collection personnel when a sufficient quantity of oral fluid has been collected. Most oral fluid collection devices currently employed for workplace testing are based on the presence of some form of absorbent collection pad however they differ significantly in performance depending upon design, composition of the pad and also whether a chemical has been added to the pad (e.g. citric acid) to act as an oral fluid stimulant. Suggested factors which possibly affect the production of oral fluid in sufficient amounts for drug testing include diurnal rhythm, season of the year, the presence or thought of food, foods adulterants, smoking and the use of medications (Spiehler 2011).

1.1.5 Workplace drug testing

In 1986 the drug testing extended to all federal agencies in the USA under the Drug Free Workplace Programme (Jacobson 2003). Since then workplace drug testing has become an established procedure worldwide, particularly in safety related industries and guidelines have been established to provide guidance and ensure consistency for workplace drug testing.

1.1.5.1 SAMHSA guidelines

The Substance Abuse and Mental Health Services Administration (SAMHSA) was the first agency to publish mandatory guidelines for federal workplace urine drug testing in 1988 (SAMHSA 1994). There have been several edits to the drug testing

guidelines and in 2010 SAMHSA published their most recent guidelines which included oral fluid as a matrix for workplace drug testing (SAMHSA 2010). The purpose of these guidelines was to ensure the uniformity and reliability when testing for illicit drugs in the workplace (Bush 2008).

1.1.5.2 UK workplace drug testing guidelines

Guidelines have since also been established in the United Kingdom for the use of oral fluid drug testing in the workplace (Cooper et al. 2011). These guidelines were based on the SAMHSA guidelines but adapted for different drug use patterns. The guidelines were introduced in order to ensure the following:

- consistency for workplace drug testing within the UK and between different workplace service providers
- capability of legal scrutiny
- providing a definition and guidance to laboratories for quality assurance and control
- accreditation for participating laboratories

The oral fluid workplace testing guidelines provide direction to UK based laboratories for oral fluid collection, laboratory confirmation, quality control and assurance and interpretation of results. Additionally they offer guidance on challenges to the oral fluid drug testing results.

1.1.5.2.1 Oral fluid collection guidelines

The UK guidelines specify that the oral fluid collection systems should allow for an observed oral fluid collection, privacy of the collection, identification of the donor,

prevention of tampering and adulteration as well as ensuring that consent is obtained prior to collection of the oral fluid sample from the donor.

1.1.5.2.2 Laboratory confirmation guidelines

The guidelines also recommend that it should be compulsory for preliminary immunoassay screening to be confirmed via a confirmatory analytical technique such as GC-MS or LC-MS. For this, agreed cut offs were introduced to provide uniformity when reporting results such that any samples with drug concentrations greater than the cut off are regarded as positive and those below the set cut off are reported as negative.

1.1.5.2.3 Quality control and assurance guidelines

All laboratories participate in workplace drug testing are recommended to be accredited using ISO 17025 accreditation which is provided by the United Kingdom Accreditation Service. The accreditation is designed to ensure that the correct chain of custody has been followed, correct screening, confirming, reporting and security of results. Additionally it is compulsory that all calibrators and controls are certified and all analytical methods fully validated before the use in workplace drug testing.

1.1.5.2.4 European workplace drug testing guidelines

In 2011, the European Workplace Drug Testing Society adapted the UK workplace drug testing guidelines for the use in all European laboratories (EWDTS 2011).

1.1.6 Oral Fluid in the UK legal system

Oral Fluid drug testing was first introduced into the UK Criminal Justice System in 2001 with the amendment of Section 57 under the Criminal Justice and Court Services Act 2000. The main purpose of the amendments was to monitor opiate and

cocaine related crime and identify drug users for direction into drug rehabilitation programs.

1.1.6.1 Roadside Testing of Oral Fluid

1.1.6.1.1 Early legislation

Established in 1930, the Road Traffic Act was the first statutory document that classed driving under the influence of drugs as an offence. Convictions were based on the level of impairment be shown in drivers. The Act was later amended in 1960 and 1962 to change the definitions of impairment to "under the influence drink or a drug to such an extent as to be incapable of having proper control of a motor vehicle". In addition to a change of the legislative wording, in 1962 the act allowed for the first time sample collections of urine, blood or breath for the identification of alcohol or other controlled substances (Road Traffic Act, 1962).

1.1.6.1.2 Current legislation

In order to enhance police officers ability to recognise and apprehend drivers whose performance may be impaired as a consequence of drug use, the UK Road Traffic Act 1988 was amended in 2003 by the Railways and Transport Safety Act 2003 to allow a preliminary oral fluid test to be carried out at the roadside. In a similar manner to roadside breath testing, oral fluid drug testing in a driving under the influence of drugs (DUID) scenario offers police officers an immediate indication of whether a driver may have used drugs and provides support to the officer to execute an arrest. As oral fluid testing becomes more available and more robust, there is the potential for significant cost savings to police forces in relation to the development of evidential oral fluid testing devices since these would significantly reduce the

requirement for a forensic medical examiner to attend and collect blood specimens and may even in time reduce the need for costly laboratory analysis.

Some other countries including Germany, Australia and the USA have already introduced oral fluid drug testing at the roadside as a preliminary screen in order to identify drivers that may be impaired by drug use. Oral fluid drug testing offers police officers the advantage of being rapid, easy to collect, less easy to adulterate and can be collected by individuals that require minimum training. Although oral fluid has been incorporated into the Road Traffic Act, it is not yet used at the roadside by police forces in the United Kingdom because a number of concerns exist in relation to the technique and the interpretation of oral fluid screening results. The UK parliament is scheduled to consider introducing the use of oral fluid testing of motorists in the near future. To date, following the collection of an oral fluid sample, a blood sample is collected for confirmation and in March 2014 the UK Department for Transport approved and published drug driving limits in blood for 16 drugs including eight illicit drugs and eight prescription drugs. The new drug driving limits means that it is an offence to drive over the recommended limit for each drug rather than relying on police officers to prove impairment by a drug in order to achieve a conviction (Department of Transport 2014).

1.1.7 Variation of drug concentrations in oral fluid - the problem!

The original belief underlying drug entry into oral fluid was based on a suggestion that drugs enter the oral cavity via the bloodstream and that this was dependent upon of the concentration of the drug not bound to proteins in circulating blood (section 1.1.3). Studies have shown that drug concentrations in oral fluid can vary widely and in some cases may be significantly higher than would be expected if the concept of a simple blood : saliva ratio accounted for the presence of drugs in oral fluid. This lead

to the postulation that drugs may form depots in mouth tissue subject to the mode of entry into the body (Osselton et al 2001, Crouch 2005, Drummer 2006, Samyn et al 2007).

Variation may be caused by a number of factors such as production and flow of saliva, contamination of the mouth cavity with drugs or other substances that may interfere with the drug test and the properties of the drugs consumed and their ability to cross from the blood into saliva i.e. is it parent drug or drug metabolite that enters the saliva and oral fluid. Additionally, deposition of drugs and their metabolites have been reported following exposure to drugs via nasal insufflation (snorting), inhalation (smoking) or oral consumption (Jenkins et al 1996, Osselton et al 2001). The problem may be illustrated by reference to two common classes of drugs that are tested for in the UK i.e. cocaine and opiates. Cocaine and opiates have subsequently been adopted as the principal compounds for study in this work.

1.1.7.1 Cocaine

Cocaine is an alkaloid principally sourced from the leaves of the *Erythroxylum coca,* but can also be synthesised from ecgonine. Incas and natives of South America have been using the leaves in religious ceremonies for over 2000 years. Leaves are widely chewed in the higher mountain regions of Peru to suppress and prevent altitude sickness and the low levels of cocaine within the leaves suppress hunger and thirst. In the lower non-mountainous regions of South American Countries such as Peru, the leaves are used to brew coca tea which is a well-practised social custom.

Medicinally, cocaine was first used as a local anaesthetic during surgery in 1884 when Carl Koller demonstrated the benefits of cocaine in ophthalmology (Grzybowski 2007). Cocaine was also applied for the use in local anaesthesia and nerve blocking during medical procedures. To date however, cocaine has decreased

in its popularity for medical use, as it showed signs of potentially harmful effects as well as risk of addiction.

1.1.7.1.1 Effects of cocaine administration

Cocaine is a stimulant producing effects such as euphoria, excitement, a profound sense of well-being, alertness and increased self-confidence. The increased self-confidence may result in increased sociability and sexual stimulation. However, adverse behavioural effects such as insomnia, disorientation, paranoia, and violent tendencies have been reported in relation to higher doses of cocaine (Boghdadi and Henning 1997, Moffat et al 2011). Cocaine also exerts several physiological effects on the body such as the heart, brain, lungs and kidneys. Pulmonary complications related to the use of cocaine are bronchiolitis, pulmonary oedema or haemorrhage (Ettinger and Albin 1986, Bailey et al 1994, Glauser and Queen 2007). Cocaine has also been linked to seizures as its can constrict blood vessels which increases blood pressure and results in cardiac dysrhythmia (White and Lambe 2003, Glauser and Queen 2007). Other cardiac effects from cocaine use include aortic aneurysms, infarction and arrhythmia (Boghdadi and Henning 1997, Pozner et al 2005).

1.1.7.1.2 Cocaine Purity

Cocaine as a street drug has decreased in purity over time. A report by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) in 2010 showed that the average purity of street samples seized in Europe ranged from 25 to 55 %. This report also stated that the reported purity decreased by 15% in comparison to the years 2005 – 2008. The report for 2011 showed that the average purity range in Europe had dropped to 25% -43%, with lowest values of 20% purity reported in England and Wales (EMCDDA 2010). In 2011 the Home Office also published data

on the purity of street drugs where the average purity of drug seizures analysed by the Forensic Science Service (FSS) reported increased from 21 % in 2009/2010 to 28 % in 2010 / 2011 (Coleman 2011). In 2012 the EMCDDA reported on the purity of cocaine in the United Kingdom to be 23.8 % (EMCDDA 2012).

1.1.7.1.3 Metabolism

Cocaine is primarily metabolised in the liver, where it is hydrolysed into ecgonine methyl ester and benzoylecgonine, both of which are pharmacologically inactive. Benzoylecgonine and ecgonine methyl ester are both formed by the action carboxylesterase. The carboxylesterase hCE-1 causes the hydrolysis of cocaine to benzoylecgonine by demethylation (Fleming et al 1990, Pindel 1997). Ecgonine methyl ester is formed via the separation from the benzoyl group which is catalysed by the carboxylesterase hCE-2 (Pindel et al 1997). Benzoylecgonine is further biotransformed into the minor metabolites norbenzoylecgonine, m-hydroxybenzoylecgonine and p-hydroxybenzoylecgonine (Figure 1.4).

A minor pathway results in the formation of norcocaine, which is part of the oxidative metabolism of cocaine. During norocaine formation which is catalysed by the cytochrome (CYP) 450, cocaine is undergoes oxidation to remove a methyl group. Norcocaine is then further metabolised by the CYP-450 enzyme to form N-hydroxynorcocaine. N-hydroxynorcocaine has been reported to be hepatotoxic targeting the liver and thus resulting in drug induced liver disease (Figure 1.4).

Anhydroecgonine methyl ester is a pyrolysis product of smoking “crack” cocaine and is formed by thermal degradation of cocaine. Anhydroecgonine methyl ester is then further metabolised into anhydroecgonine in the body.

Consumption of cocaine with alcohol results in the formation of cocaethylene, the ethyl ester of benzoylecgonine. Cocaethylene is formed as the addition of ethanol changes the path of the biotransformation of cocaine via *in vivo* transesterification catalyzed by hepatic carboxylesterase (hCE1) enzymes. During the formation of cocaethylene, the methyl ester group is replaced with an ethyl group. Cocaethylene is pharmacologically active and its pharmacological activity is similar to that of cocaine (Laizure et al 2003) (Figure 1.4).

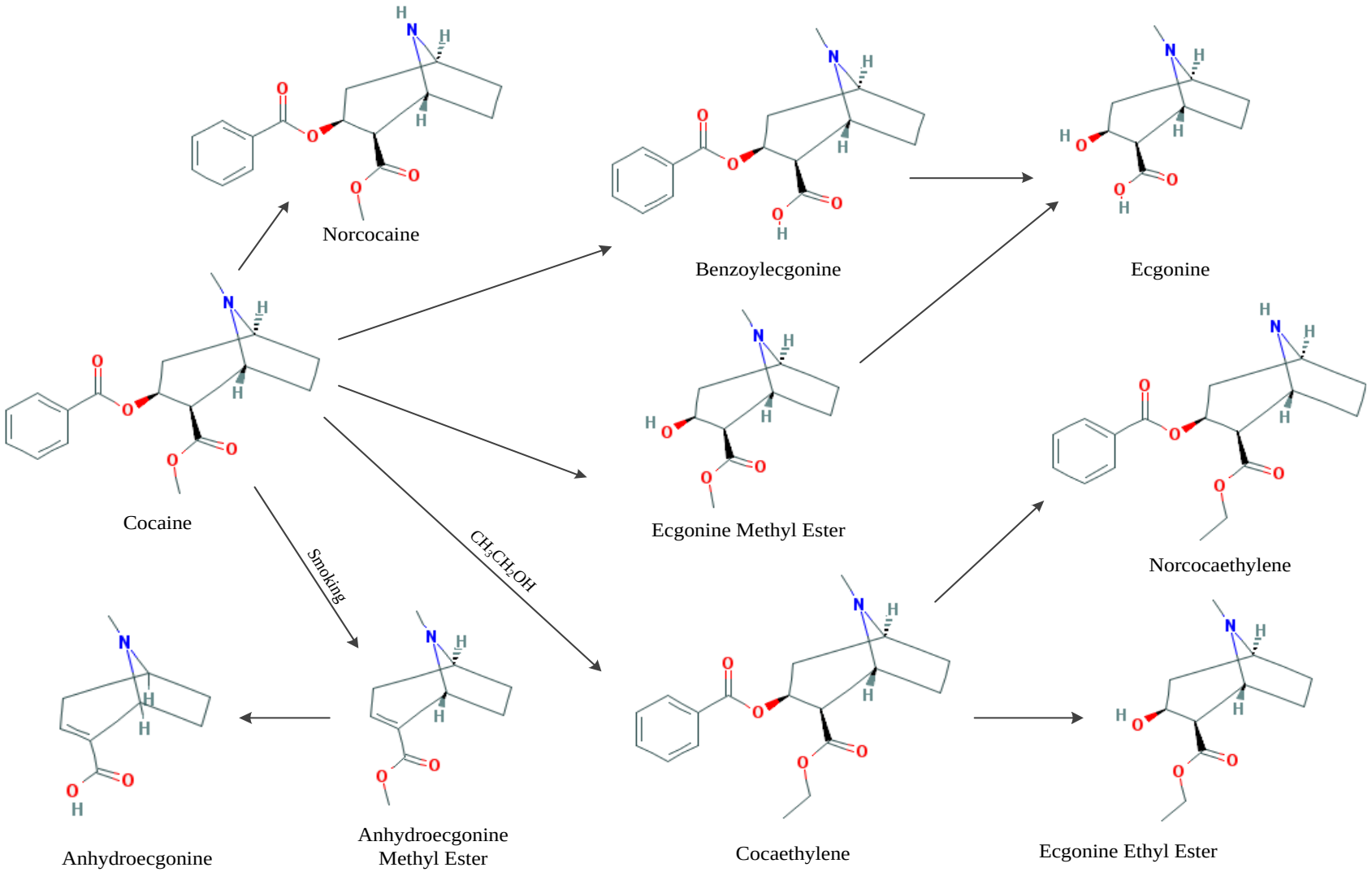


Figure 1.4. Metabolic pathway of cocaine (structures available on www.ncbi.nlm.nih.gov/strucutre [accessed 24/03/2014])

1.1.7.1.4 Route of Administration

Cocaine is consumed via three main routes of administration: intravenous injection, snorting or smoking.

1.1.7.1.4.1 Intravenous injection

Cocaine use via injection is the least common method of administering cocaine but can be achieved by combining cocaine powder with water, as cocaine is soluble 1 in 600 of water. The solubility of cocaine increases to 1 in 270 of water if the water temperature is increased to 80 °C (Hudgins et al. 1995; Middleton et al. 2004; Moffat et al. 2011).

Cocaine concentrations following intravenous use are dose dependant. Low doses such as a 32 mg and 40 mg resulted in peak plasma concentrations of 250 ng/mL (t = 7.3 min) and 523 ng/mL (t = 10 min), respectively (Chow et al 1985, Kumor et al 1989). Increased concentrations result in larger peak plasma concentrations. Following a 100 mg intravenous dose, (Barnett et al 1981) reported peak plasma concentrations of between 700 ng/mL and 1000 ng/mL (t = 5 min) whereas at the higher dose of 200 mg resulted in a peak plasma concentration of 2500 ng/mL (t = 5 min) (Barnett et al 1982). The elimination half-life for cocaine when injected intravenously has been reported to be 41 min (range= 19 min – 64 min).

1.1.7.1.4.2 Nasal insufflation (snorting)

Cocaine hydrochloride is generally used when snorting cocaine. Nasal insufflation or snorting of cocaine is relatively inefficient and results in low absorption of cocaine. The intranasal elimination half-life was shown to be 75 min (range = 70 min – 80 min) (Wilkinson et al 1980). There is a variation in the reported literature regarding time taken to reach peak plasma concentrations. Wilkinson et al (1980)

reported peak plasma concentration 35 min – 90 min whereas Javaid et al (1983) reported peak plasma concentrations between 120 min - 160 min post cocaine insufflation.

1.1.7.1.4.3 Oral ingestion

Orally ingested cocaine is most commonly administered via coca tea drinking or coca leaf chewing. Studies have shown that the concentration of cocaine within the leaf is dependent on the species of the coca plant with the average cocaine concentration ranging between 0.39% and 0.5% (Blejerpr 1965, Hamner and Villegas 1969, Hannah and Hornick 1977, Negrete 1978, Holmstedt et al 1979, Paly et al 1980). In addition to cocaine, the typical coca leaf contains a number of other structurally related compounds including benzoylecgonine and ecgonine methyl ester (Weil 1978, Penny et al 2009). The amount of cocaine and its metabolites present in coca leave can vary depending on species and origin of the *Erythroxylum* plant (Jenkins et al 1996). The Peruvian Erythroxylum species contains 5.11 mg of cocaine, 0.11 mg of benzoylecgonine and 1.15 mg of ecgonine methyl ester in 1 g of leaf material (Jenkins et al 1996).

Coca tea is commonly prepared from an infusion of the leaves obtained from the coca bush *Erythroxylum coca*. Coca tea is widely consumed in a number of South American countries, where its use is both legal and socially acceptable (Hamner and Villegas 1969, Hanna and Hornick 1977, Siegel et al 1986, Jenkins et al 1996, Turner et al 2005). Coca leaves have been used for over 2000 years and were widely used by the Incas of Peru in religious ceremonies (Martin 1970, Zapata-Oritz 1970, Allen 1981, Billman 1990).

In addition to the use of coca leaves during the preparation of coca tea, the custom of chewing coca leaves is still widely practiced today by people working at high altitude. Coca chewing alleviates adverse symptoms of pain, thirst, hunger and tiredness that are experienced by humans working or living at high altitude (Fuchs 1978). Chewing coca can be advantageous in colder environments (Hanna 1971a). Chewers have been reported to consume on average 62.4 g and 51.1 g of coca leaves per day, respectively (Hanna 1974). Coca leaf chewing resulted in a mean concentration of 150 ng/mL of cocaine in plasma following chewing of 4.4g of coca leaves (Holmstedt et al 1979).

1.1.7.1.4.4 Smoking

Smoking of cocaine generally occurs in the form of "crack" cocaine. Cone et al 1994 reported peak cocaine plasma concentrations immediately after smoking of "crack" cocaine before rapidly decreasing over time.

This was further supported in a study reported by Jenkins et al (1996) who reported that the mean plasma concentrations during "crack" smoking occurred after 2 minutes. The onset of physiological effects was reported 5 min after the smoking of "crack" cocaine. Studies by Haney et al (2001a, 2001b) also reported cocaine concentrations in plasma following "crack" cocaine smoking in 10 individuals. Smoking of a 12 mg of "crack" resulted in cocaine plasma concentrations ranging between 30.6 mg/L and 88 mg/L (mean = 59.3 µg/mL). In comparison, an initial dose of 50 mg of "crack" cocaine resulted in mean cocaine plasma concentrations ranging between 121.8 µg/mL and 166.4 mg/L (mean = 144.1 µg/mL).

1.1.7.1.5 Mechanism of action

Cocaine acts on the neurotransmitters dopamine, norepinephrine and serotonin in the central nervous system. Neurotransmitters are chemical signals, which travel across the synaptic cleft and are responsible for initiating of a new electric signal in the post synaptic cell. Cocaine inhibits the reuptake of these neurotransmitters by binding to the transporters which are responsible for removing excess neurotransmitter from the synaptic cleft (Ritz et al 1987, Volkow et al 1999).

Serotonin is most potent in binding to the receptors followed by dopamine and norephedrine (Ritz et al 1990, Carroll et al 1992). The increased amount of dopamine within the synaptic cleft results in most of the behavioural effects observed with cocaine, such as euphoria and increased locomotor behaviour (Uhl et al 2002). The blocking of the re-uptake of serotonin results in rewarding effects by enhancing the mood, similar to why serotonin blockers are commonly used during treatment for depression (Uhl et al 2002, Meyer and Quenzer 2005). However, the increased concentration of norepinephrine affects sensory functions, memory and can result in anxiety. As well as acting on neurotransmitters, cocaine reduces the sodium currents within nerve axons, which is the action responsible for the anaesthetic effects of cocaine (Crumb and Clarkson 1990, Warner 1993).

1.1.7.1.6 Tissue disposition

The amount of drug that distributes into tissue is dependent on its volume of distribution (V_d). The reported V_d of cocaine is between 1 and 3 L/kg (Moffat et al 2011). The disposition of cocaine in different body tissues has been thoroughly investigated. Poklis et al (1987a, 1987b) examined the disposition of cocaine in tissue in five cases of fatal poisoning. In two out of the five cases, additional drugs such as diazepam, nordiazepam and morphine were also detected and quantified. With the exception of urine, the highest concentrations of cocaine were observed in the kidney and spleen with mean concentrations of 39.4 µg/mL and 26 µg/mL respectively. An overview of the results in various tissues from Poklis et al (1987) can be seen in Table 1.9.

Table 1.9 Disposition of Cocaine in Fatal Poisoning (µg/mL) (Poklis et al 1987)

Specimens	***Cases***					
	1	**2**	**3**	**4**	**5**	**Mean**
Blood	1.8	6.9	31.0	13.0	3.9	11.3
Bile	10.0	18.0	-	25.0	3.2	14.1
Brain	4.0	24.0	59.0	83.0	6.4	35.3
Heart	6.1	-	-	-	5.3	5.7
Kidney	26.0	26.0	58.0	53.0	34.0	39.4
Liver	1.6	17.0	6.5	10.0	15.0	10.0
Lung	3.4	-	69.0	24.0	27.0	30.9
Spleen	22.0	25.0	42.0	-	15.0	26.0
Skeletal muscle	6.1	-	48.0	30.0	-	28.0
Adipose tissue	1.0	-	5.8	-	0.7	2.5
Urine	39.0	41.0	270.0	-	27.0	94.3
Vitreous	2.4	-	-	14.0	-	8.2

Spiehler and Reed (1985) reported cocaine concentrations in the liver to be an average of 6.7 µg/mL.

A different measure of lipophilicity is the partition coefficient (logP) of a drug. A log P value larger than 5, will favour sequestration into body fat. Therefore the lower the logP value, the easier the absorption into tissues. The reported logP value for cocaine is 2.3 whereas the logP value for heroin is 0.2 (Moffat et al 2011).

1.1.7.1.7 Elimination of cocaine

Wilkinson et al (1980) reported a plasma elimination half-life of 75 min (range = 70 min - 80 min) following the snorting of cocaine in comparison to when the drug is consumed orally where the reported elimination half-life was reported at 48 min (range = 45 min – 51 min). Intravenous cocaine has a reported elimination half-life of around 78 min (Jeffcoat et al 1989). The elimination half-life of benzoylecgonine and ecgonine methyl ester have been reported to be 5.1 hrs and 4.2 hrs, respectively (Ambre et al 1984).

Reports have shown that following an intravenous dose of 120 mg of cocaine between 1 % and 9 % is excreted in the urine unchanged (Moffat et al 2011) in addition to 35 % to 55 % of its main metabolite benzoylecgonine. Similarly it has been reported that following snorting of a 1.5 mg/kg dose of cocaine resulted in 4% of cocaine to be excreted unchanged within the urine with 16% - 36 % of the dose excreted as benzoylecgonine (Moffat et al 2011). Cone et al (1998) showed that following intravenous injection, snorting or smoking of cocaine 39 %, 30% and 16 % of the dose of cocaine was excreted via the urine. Additionally Cone et al (1998) reported that 18 %, 15 % and 8% of the administered dose was excreted as norcaine, benzoylnorcocaine, *m*-hydroxycocaine, *p*-hydroxycocaine, *m*-

hydroxybenoyzlecgonine and *p*-hydroxylbenzoylecgonine. Anydroecgonine methyl ester has also been detected in the urine in trace amounts (~0.02 % of the dose administered) following the smoking of "crack" cocaine (Cone et al 1998).

1.1.7.1.8 Toxicity

Toxicity depends on each individual as regular users can build up a tolerance to cocaine. Reports on the lethal dose of cocaine vary in the literature. Trouve and Nahas (1990) reported the lethal dose at 60 mg/kg. In comparison, a study by Hearn et al (1991) reported the lethal toxicity of cocaine to be on average 93 mg/kg. In 1998, Karch et al analysed blood concentrations in 48 cocaine related fatalities and reported a mean blood concentration of 1.12 mg/L (range 0.001 – 18.1 mg/mL) in comparison to a mean urine cocaine concentration of 40.1 mg/L (range 0.001 – 468 mg/L), with mean benzoylecgonine concentrations reported at 1.54 mg/L (range = 0.001 mg/L – 3.22 mg/L).

Lethal cases of cocaine overdose are commonly seen in drug smuggling as a result of the rupture of a package in the gastro intestinal tract. Following rupture of packages Furnari et al (2002) reported peak blood concentrations of 4 mg/L of cocaine and 17 mg/L of benzoylecgonine. Urine concentrations detected for cocaine and benzoylecgonine were 152 mg/L and 512 mg/L respectively. In comparison, Fineschi et al (2002) reported cocaine and benzoylecgonine concentrations following the rupture of a cocaine package of 98.1 mg/L and 86.1 mg/L in blood, and 10 mg/mL and 3.3 mg/L in urine, respectively.

The most prominent toxic effect of cocaine is exhibited on the cardiac system. Cocaine can induce cardiac arrhythmias and myocarditis as well as pulmonary oedema, all of which can result in sudden death. Cocaine also has major toxic effects

on other organs such as the kidneys. When taken repeatedly cocaine can result in kidney damage and acute renal failure.

Cocaine use also has toxic effects on the central nervous system such as psychosis, agitation and seizures. Cocaine users have been reported to be affected by cerebral aneurysms. Other toxic effects on the central nervous system include stroke and cardiovascular collapse.

Lesser toxic effects of cocaine especially, when snorting cocaine, include damage of the nasal septum e.g. septal and avascular necrosis, tumours in the nasal chambers and ulcers.

1.1.7.1.9 Elevated cocaine concentrations in oral fluid

Jenkins et al. (1996) investigated concentrations of cocaine derivatives in oral fluid following smoking and intravenous administration. Cocaine concentrations in oral fluid after smoking ranged from 15.85 mg/L to 504.88 mg/L whereas concentrations following intravenous injection were significantly lower (0.43 mg/L to 1.93 mg/L). Jenkins also reported concentrations of anhydroecgonine methyl ester in saliva ranging from 0.56 mg/L to 4.37 mg/L. Anhydroecgonine methyl ester could not be detected in plasma following smoking.

Although the phenomenon of elevated drug concentrations in oral fluid has been documented, little is known about the physiology of drugs in the mouth to explain the observations. The measurement of a high drug concentration in oral fluid could potentially be explained by recent drug use or by release of the drug from a drug depot. Limited understanding of drug elimination via the mouth poses a problem to forensic toxicologists since the toxicologist is required by the courts to interpret analytical results in relation to an individual's performance. Drug depot formation

has a clear implication on the interpretative value of oral fluid as a matrix in the workplace drug testing arena or for prosecuting motorists since lawyers will argue that positive test results produced by their clients will be a consequence of past drug use and not recent use that would be associated with impairment.

1.1.7.2 Heroin

Heroin or diacetylmorphine is synthesized from opium, a naturally occurring alkaloid. Opium is a product of the *Papaver Somniferum L*. Following the extraction of morphine, it undergoes acetylation with acetic anhydride to produce heroin. Heroin was originally produced as a commercial product to treat respiratory diseases (Hosztafi, 2001). This quickly changed when the addictive properties of heroin became apparent and drug users realised its euphoric properties. In the UK, heroin is listed and controlled as diamorphine under the Misuse of Drugs Act 1971 as a class A controlled drug.

1.1.7.2.1 Effects of heroin administration

The analgesic effects produced by heroin is on average three times more potent than morphine. Opioids, such as heroin, are narcotic analgesics that affect the central nervous system (CNS) and may produce pupillary constriction, euphoria and respiratory depression (Karch 2003). Short term effects of heroin include feelings of euphoria and "rush" followed by drowsiness. As a result of its suppressive effects on the CNS, heroin can lower heart and respiratory function which can ultimately result in death. In addicts/regular users, constant heroin use has been shown to cause liver and kidney disease as well as cardiac conditions (Paterna et al 1991). Additionally the repeated injection of heroin has been shown to result in collapsing of veins close to the injection site as well as causing abscesses.

1.1.7.2.2 Metabolism

Heroin (diamorphine) is metabolised quickly into 6-monoacetylmorphine (6-MAM) by deacetylation of the 3-acetyl group. 6-MAM is then hydrolysed into morphine. The deacetylation of-heroin to 6-MAM occurs at an estimated half-life between 3 minutes and 7.6 minutes whereas the hydrolysis to morphine is slower with the estimated half-life reported at 21.8 minutes (Rook et al 2006, Rentsch et al 2001, Moffat et al 2011). Heroin, metabolised in the liver is catalysed by carboxylesterases (Kamendulis 1996; Polettini et al 2005) – specifically human liver carboxylesterase form 1 (hCE-1) and human liver carboxylesterase from 2 (hCE-2). hCE-1 is responsible for removing the 3-acetyl group and has been shown to also the 6-acetyl link which results in the formation of 6-MAM from heroin (Pindel et al 1997, Redinbo et al 2003). hCE-2 catalyses the hydrolysis of the acetyl groups of heroin in the liver (Pindel et al 1997). hCE-2 has been shown to have a higher catalytic efficiency than hCE-1 for heroin (Kamendulis 1996, Pindel et al 1997, Satoh et al 2002). In the blood, heroin is also metabolised via hydrolysis which is catalysed by the hydrolytic esterases butyrylcholinesterase and acetylcholinesterase (AChE). AChE then further catalyses the metabolism of 6-MAM to morphine (Lockridge et al 1980, Salmon et al 1990) (Figure 1.5).

Morphine is further metabolised into morphine-3-glucuronide and morphine-6-glucuronide by the glucuronidation of a phenolic hydroxyl group at the third and sixth position, respectively. These glururonides are not further metabolised in the body but are excreted via the kidney. Morphine glucuronidation is catalysed by uridine – 5’ – diphosphate- glucuronosyltransferases (UGT), specifically UGT-2B7 (Antonilli et al 2012, Eissing et al 2012). Although the main pathway for morphine glucuronidation is a hepatic pathway, gluruonidation has also been reported in the

kidneys, intestines and brain (Brunk and Delle 1974, Gerostamoulos and Drummer 2000) (Figure 1.5).

Morphine also undergoes N-demethylation to a lesser extent in order to form normorphine, which is also an active metabolite (Meadway et al 2002) (Figure 1.7).

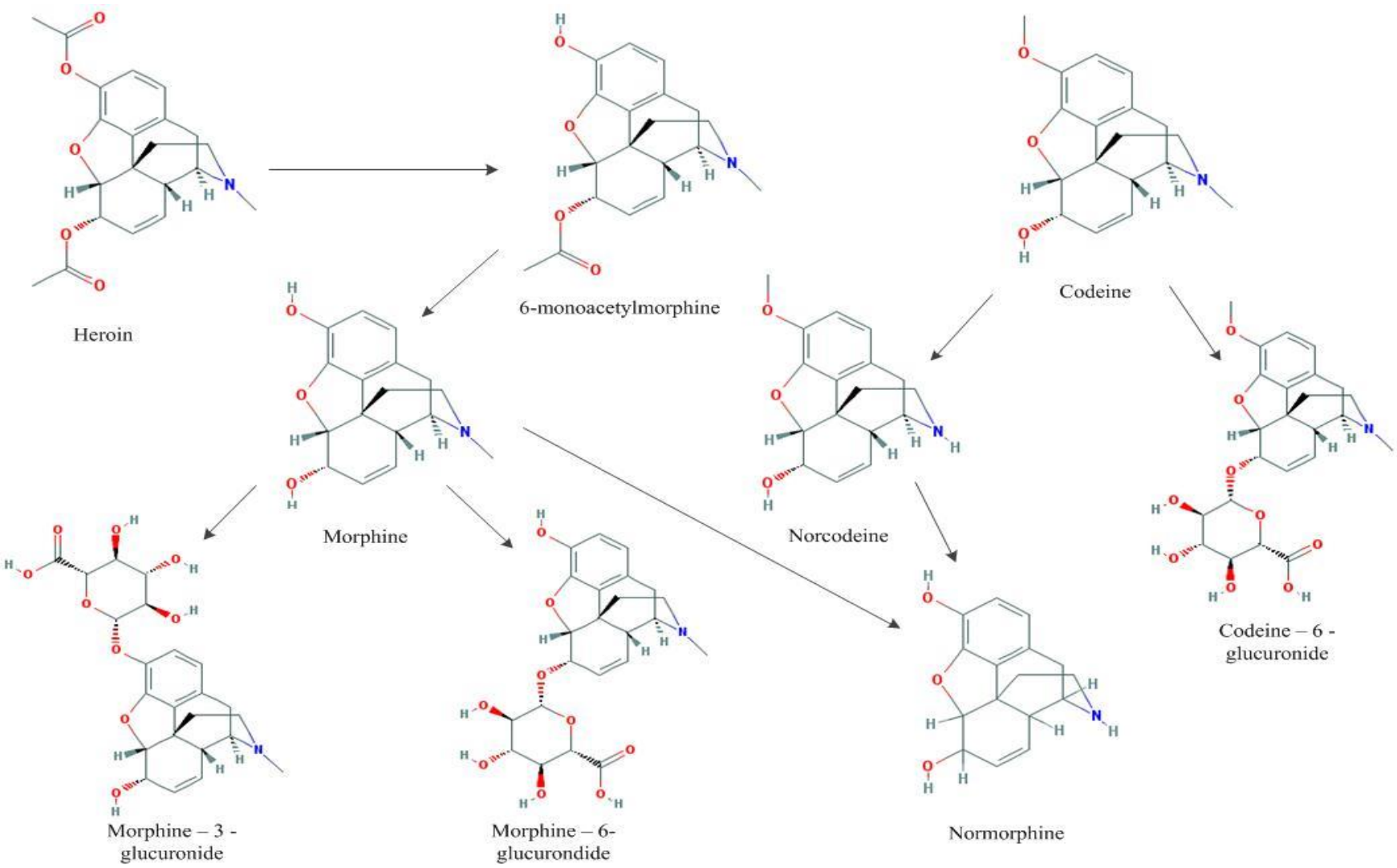


Figure 1.5 Metabolic pathway of heroin (structures available on www.ncbi.nlm.nih.gov/strucutre [accessed 24/03/2014])

1.1.7.2.3 Heroin Purity

The Forensic Science Service reported a decrease in heroin purity from 44 % in 2009/2010 to 30 % in 2010/2011. In 2011, heroin purity in the UK was reported by the EMCDDA to be much lower with a mean purity of 17.6 % (range = 1 % and 96 %, median = 16 %) (EMCDDA 2011). It is most commonly cut with caffeine and paracetamol. However less common adulterants such as phenobarbitone and diazepam have also been reported.

1.1.7.2.4 Routes of Administration

1.1.7.2.4.1 Intravenous injection

Intravenous injection is the most common route of administration of heroin during illicit drug use. However, as a result of a decrease in heroin purity over recent years and potential spreading of HIV and Hepatitis B, alternative routes of administrations increased (Strang et al 1992). Following intravenous injection of heroin, Jenkins et al (1994) reported the peak plasma concentration of heroin, morphine and 6-MAM. Heroin concentrations appeared in blood between 1 – 5 minutes post injection at concentrations of 141ng/mL but decreased rapidly. Morphine and 6-MAM concentrations resulted in concentrations of 151 ng/mL and 44 ng/mL, respectively.

1.1.7.2.4.2 Nasal insufflation (snorting)

The effects of nasal insufflation of heroin have been reported to be similar to effects when heroin is injected. However, as there is no trauma from a needle, snorting has become more popular. Cone et al (1996) reported peak blood concentration in plasma within 5 minutes of snorting of heroin. Following an intranasal dose of heroin of 6 mg in 6 volunteers peak morphine blood concentrations of 2821 ng/mL (range = 1174 ng/mL – 3455 ng/mL) and peak 6-MAM concentration of 34 ng/mL (range = 0

ng/mL – 81 ng/mL). Positive morphine and 6-MAM concentrations were detected for up to 23.9 hours and 1.8 hours respectively. In comparison, following a higher dose of 12 mg, morphine and 6-MAM were detected for 11.1 hours and 2.9 hours, respectively. Peak urine concentrations of morphine and 6-MAM were reported at 6371 ng/mL (range = 3085 ng/mL – 10425 ng/mL) and 73 ng/mL (range = 0 ng/mL - 183 ng/mL), respectively.

1.1.7.2.4.3 Oral Ingestion

Oral ingestion as a route of administration is not commonly reported for the use of heroin. Sawynok (1986) reported that orally ingested heroin is on average 1.5 times more potent than morphine. In comparison when heroin is administered via intravenous injection its potency is on average three times larger than that of morphine.

1.1.7.2.4.4 Smoking

Smoking heroin, commonly known as 'Chasing the Dragon', involves heating heroin on a foil and inhaling the smoke via a straw. Hendriks et al (2001) reported that the bioavailability of heroin was 35% - 45 % following smoking of 100 mg of heroin, Rook et al (2006) also reported the bioavailability of heroin following inhalation of smoke to be at a mean of 52 % (range = 44 % - 61%). Peak plasma concentrations following smoking were reported to be on average four times lower than following intravenous injection with a plasma mean concentration 1.70 mg/L (mean = 7.54 mg/L) (Rook et al 2006).

1.1.7.2.5 Mechanisms of action

Heroin crosses the blood-brain-barrier quickly and freely to then interact with opioid receptors present within the brain. There are three main opioid receptors: mu (μ),

delta (δ) and kappa (κ) (Kieffer 1995). The affinity to the μ-opioid binding sites is higher in comparison to the affinity to other receptors such as delta and kappa (Goldstein and Naidu, 1989, Pasternak 2001). These opioid receptors are present in the central nervous system as well as the gastrointestinal tract. Heroin acts as an antagonist at the μ-opioid receptor which, as a result, inhibits the release of gamma-aminobutyric acid (GABA) and hence increases the amount of dopamine produced. Increased dopamine activation then causes the effects felt during heroin use such as euphoria, feelings of wellbeing and intense pleasure.

Heroin itself has a low binding affinity for μ-opioid receptors. Heroin is quickly metabolised to 6-MAM and morphine, both of which have a high affinity to bind to opioid receptors and hence exert maximum effects (Inturrisi, et al, 1983, Rossi et al 1996, Selly et al 2001). The presence of μ-opioid receptors within the gastrointestinal tract inhibits the intestines secretory activity leading to constipation (Holzer 2009).

1.1.7.2.6 Tissue disposition

The volume of distribution of heroin is not very documented, as it is metabolised quickly to 6-MAM and morphine within the body. Heroin is lipophilic, which results in large estimated volume of distribution ranging between 25 L/kg and 33.8 L/kg (mean = 29.5 L/kg) (Rook et al 2006, Baselt, 2011, Urso et al, 2012). The higher volume of distribution of heroin suggests that a larger amount of drug travels into the tissue. However, as a result of its rapid metabolism, heroin is rarely detected and reported to be present in biological specimens. In addition to heroin, 6-MAM is also lipophilic indicating that the drug can also freely travel across the blood-brain

barrier. The volume of distribution for 6-MAM has been reported to be similar to that of heroin (Rook et al 2006).

The reported volume of distribution of morphine ranges between 3 - 5 L/kg (Moffat et al 2011). Morphine has been reported to cross the blood-brain barrier at a slower rate than heroin or 6-MAM. Moriya and Hashimoto (1997) reported a large quantity of morphine in the stomach contents, liver and lung at concentrations of 5220 ng/g, 4200 ng/g and 2270 ng/g following an overdose of self-injected heroin. Following intravenous morphine injection morphine concentrations were reported in blood, brain, lung, liver and bile at concentrations of 670 ng/mL, 40 ng/mL, 210 ng/mL, 110 ng/mL and 440 ng/mL, respectively (Cravey and Reed 1977). In comparison, Felby (1974) reported morphine concentrations following an intravenous overdose in 10 cases to range between 200 and 2300 ng/mL (mean= 700 ng/mL) in blood, 100 ng/mL and 2000 ng/mL (mean = 800 ng/mL) in muscle, 400 ng/mL and 18000 ng/mL (mean = 3000 ng/mL) in liver and 14000 ng/mL and 81000 ng/mL (mean = 52000 ng/mL) in urine.

1.1.7.2.7 Elimination

Heroin has a half-life of 10 - 15 minutes and thus is rapidly metabolised to 6-MAM and morphine in the body and is subsequently excreted as morphine-3-gluruondide and morphine-6-gluruconide. As a result of this, the detection of heroin in biological specimens is uncommon. A study by Elliot et al in 1971 investigated urinary excretion of heroin and its metabolites following an intravenous dose. Elliot et al showed that following injection of 70 mg of heroin, 45 % of the dose was recovered in the urine in the form of total morphine and 6-MAM over the course of 40 hours. Smith et al (2001) reported the excretion half-life for both, morphine and 6-MAM, to

be 3.11 hrs (±0.3 hrs). Peak morphine urine concentrations were observed at 1392 – 9250 ng/mL at 1.2 hrs – 6.2 hrs following an intravenous heroin dose of less than 7 mg. An intravenously administered heroin dose above 10 mg, resulted in peak morphine concentrations at 2.3 hrs – 9.3 hrs ranging between 2065 ng/mL and 29030 ng/mL. Peak 6-MAM concentrations in urine following a low dose (<7mg) were observed to be ranging between 6.1 – 298 ng/mL at 1.2 hrs- 5.1 hrs post injection. Following an intravenous dose larger than 10 mg, peak 6-MAM concentrations were observed 1.4 – 4.3 hours post injection at concentrations ranging between 13 – 598 ng/mL (Smith et al 2001).

Normorphine has also been reported to a lesser extent to be excreted in the urine (Yeh et al 1976). However, the amount of drug which is excreted via the urine is dependent on the route of administration of the drug. Orally consumed morphine undergoes extensive first pass metabolism resulting in a delayed absorption and hence slower excretion than morphine which is administered via injection (Brunk and Delle 1974, Rook et al 2006).

1.1.7.2.8 Toxicity

Heroin is three times more potent than morphine resulting in a lethal dose to average around 200 mg (Rob et al 1997, Moffat et al 2011). Regular drug users can build up a tolerance to opioids, resulting in a larger lethal dose. Users who use the drug regularly have a larger risk of overdose, as the difference between the heroin dose that achieves the desired effect and the dose that causes lethal respiratory depression is not great.

Rop et al (1997) reported a lethal overdose following exposure to heroin. Although the quantity of heroin which was consumed was unknown, the blood concentrations

were 109 ng/mL, 168 ng/mL and 1140 ng/mL for heroin, 6-MAM and morphine, respectively. In a separate fatality reported by Winek et al 1999, the concentrations of morphine in blood and urine following heroin injection were 680 ng/mL and 490 respectively. Following the death of eight body packers who transported heroin from Columbia, concentrations of morphine in blood ranged between 4.4 µg/mL and 52.6 µg/mL (Wetli et al 1997).

One of the most commonly reported toxic effects of heroin is pulmonary disease (Warner-Smith et al 2001, Meyer and Quenzer 2005). Pulmonary disease is a condition of the lungs in which the breathing can be restricted and exacerbated leading to respiratory depression (White and Irvine 1999). Heroin use has been associated with liver (hepatic) disease as a result of slower drug clearance from the liver (Torre, 1999, Warner-Smith et al 2001).

Heroin also induces cardiac toxicity which results in cardiac arrhythmias. Additionally, heroin usages results in muscular effects and neurological effects, however none of the three previously mentioned factors are a direct cause for death following an overdose (Darke et al 2000, Ghuran and Nolan 2000).

1.1.7.2.9 Elevated heroin and metabolite concentrations in oral fluid

Jenkins et al. (1996) investigated drug concentrations in oral fluid following the controlled consumption of smoked heroin. Both blood and oral fluid samples were collected and screened for heroin, 6-monoacetylmorphine and morphine in order to compare results from these matrices. Heroin concentrations ranged from 3.53 µg/mL to 20.58 µg/mL following smoking 2.6 mg and 5.2 mg heroin respectively. Concentrations could be detected for up to 24 hours. Significant 6-monacetylmorphine concentrations could be detected within a range of 1.12 µg/mL

(2.6 mg) and 3.58 μg/mL (5.2 mg) and could be detected for 30 minutes and 480 minutes respectively. In comparison, concentrations of heroin in oral fluid following intravenous injection, ranged from 6 ng/mL (10 mg) to 22 ng/mL (5 mg) and could be detected for 30 minutes. 6-monoacetylmorphine was only detected at very low concentrations in saliva following intravenous injection ranging between 18 ng/mL (5 mg) and 40 ng/mL (10 mg).

Data from the Forensic Science Service (Osselton et al 2001) has shown that oral fluid concentrations of opiates, with or without the combination of a second opiate, ranged from 0.023 μg/mL to 17.375 μg/mL for morphine, 0.003 μg/mL to 7.75 μg/mL for codeine, 0.002 μg/mL to 66.93 μg/mL for 6-monoacetylmorphine and 0.001 μg/mL to 274.27 μg/mL for dihydrocodeine A potential explanation for the elevated drug concentrations in oral fluid is that drug accumulate to form depots in mouth tissues.

Although the phenomenon of elevated drug concentrations in oral fluid has been well documented, little is known about the physiology of drugs in the mouth to help explain the observations. The measurement of a high drug concentration in oral fluid could potentially be explained by recent drug use or by release of the drug from a drug depot. Limited understanding of drug elimination via the mouth poses a problem to forensic toxicologists since the toxicologist may be required by the courts to interpret analytical results in relation to an individual's performance. Drug depot formation has a clear implication on the interpretative value of oral fluid as a matrix for prosecuting motorists since lawyers will argue that positive test results produced by their clients will be a consequence of past drug use and not recent use that would be associated with impairment.

Chapter 2.0 - Aims and Objectives

2.1 Aims

The research described in this thesis aimed to increase our understanding of the factors and processes concerning the deposition of drugs in oral fluid and to further enhance the ability of forensic toxicologists to interpret the results of analysis in this matrix.

The work undertaken in this thesis aimed to test the following hypotheses:

1. Foods and Adulterants can interfere with oral fluid collection systems and subsequently result in false positive screening results
2. Drugs which are consumed orally either in form of an oral solution, via smoking or snorting can result in elevated oral fluid drug concentration which can subsequently affect the interpretation of oral fluid drug concentrations
3. Microbleeding of the gums can contribute to elevated drug concentration by leaking blood, and hence drug, directly into the oral fluid
4. Drugs, such as cocaine and heroin, can bind to tissue within the oral cavity and subsequently form drug depots in oral tissues
5. Drug depots that formed within the oral cavity can subsequently be released into the oral fluid over time.

2.2 Objectives

The objective of this work was to enhance our knowledge and understanding of the behaviour of drugs in oral fluid and the mouth cavity by:

1. Identifying possible chemical agents and adulterants that may interfere with oral fluid drug testing systems in order to determine which collector will be used in the experiment designed for the work planned in this thesis

2. Investigating how high drug concentrations are formed in oral fluid, particularly with respect to opiates / opioids and cocaine by

    a. Detection and quantification of cocaine and its metabolites in oral fluid following the consumption of coca tea

    b. Investigating and comparing drug concentrations from volunteers who consumed Codeine Linctus or Collis Browne's Opium Tincture

    c. Investigating whether microbleeding may be the cause of elevated oral fluid drug concentrations. This will be achieved by

        i. Investigating a relationship between transferrin (a marker of oral bleeding) and drug concentrations in oral fluid samples collected from drug clinics in the UK

        ii. Comparing drug concentrations in human volunteers following controlled administration of codeine tablets and comparing the results with codeine concentrations in volunteers who stimulated microbleeding following consumption

    d. Designing laboratory models to mimic exposure of mouth tissues to drug solutions and smoking.

3. Investigate the nature of drug binding sites within the mouth (porcine tongue) by developing an immunohistochemical visualisation technique to demonstrate the localisation of drugs/components *in situ*. This was achieved by

    a. Developing an immunohistochemical staining technique using monoclonal antibodies for morphine and benzoylecgonine

b. Applying staining techniques to porcine tongues which were exposed to smoke or solution of cocaine or heroin in order to identify the distribution of drug throughout the tissue and identify binding sites in tissue

c. Performing i*n-vivo* studies in mice to identify a) whether drugs accumulate in mice salivary glands and b) are subsequently excreted during production of saliva

Chapter 3.0 - Investigation of the effects of common food and beverages on oral fluid drug detection

3.1 Introduction

Oral fluid drug testing is based on three main processes: 1) oral fluid collection, 2) immunoassay screening and 3) confirmatory analysis of immunoassay positive samples using either GC-MS or HPLC-MS. It is essential for oral fluid testing that a sufficient flow of saliva is available for collection and that there is sufficient drug present to be detected by immunoassay and subsequent confirmatory technique in order to achieve successful testing. A further factor to take in to consideration when interpreting results is whether the testing processes are affected by any non-drug substances which might be present within the mouth i.e. foods or chemicals.

The work described in this chapter investigates the effects of a variety of common foods, beverages and oral hygiene products on two oral fluid collection systems. The objective was to characterize the effects of common food and beverages on the oral fluid collection and screening systems, including their collection devices and immunoassays.

A number of different types of oral fluid collection devices are commercially available each differing in efficiency, oral fluid collection volume and presence of an incorporated indicator within the device to inform the user when a sufficient quantity of oral fluid has been collected. Most oral fluid collection devices currently employed for workplace testing are based on the presence of an absorbent collection pad the performance of which is depending upon design, composition of the pad and also whether a stimulant chemical has been added to the pad (e.g. citric acid) to act as a stimulant of secretion of saliva (Navazesh 1993, Crouch 2005).

The Orasure Intercept and the Alere™ Certus collection systems are the two devices which are the most commonly used devices in the UK. The purpose of investigating the effects of foods and beverages on the two collection systems was to establish which one of the two collectors is more suitable for use in further experimental studies.

3.1.1 Orasure Intercept collection system

The Orasure Intercept system (Figure 3.1) is a well-established collection device comprising a small pad that is wiped around the inside of the mouth and is claimed by the manufacturer to collect approximately 0.4 ml of oral fluid (Cone et al 2002). The device is 510K cleared by the FDA as a medical device. The collection device does not incorporate any mechanism to indicate when sufficient oral fluid has been collected, however the manufacturers advise that 0.4 ml of oral fluid is collected within two to five minutes. Following collection of the oral fluid specimen, the Intercept collection pad is immersed into 0.8 ml of buffer / preservative provided in a sealable tube.

Figure 3.1 Image showing the Orasure Intercept Collector

The performance of the Orasure Intercept device has been evaluated as reported in the literature and it was shown that the recovery of oral fluid ranged between of 82.91 % and 88.18 %. Although the device showed a good recovery of 80 %, the use of the device is not pleasant for the volunteer (Langel et al 2008). Volunteers commented on the bitter taste and hence gave negative feedback on the collection device.

3.1.2 Alere™ Certus collection system

The Alere™ Certus collector was introduced onto the market during 2010 and is a Class I medical device. It comprises a small absorbent foam cylinder attached to a handle that incorporates a built-in sample adequacy indicator. This turns blue when 1 mL of oral fluid has been collected (Figure 3.2). The Certus collector is provided with a sealable tube containing 3mL preservative / buffer into which the collected oral fluid specimen is placed for transport to the laboratory.

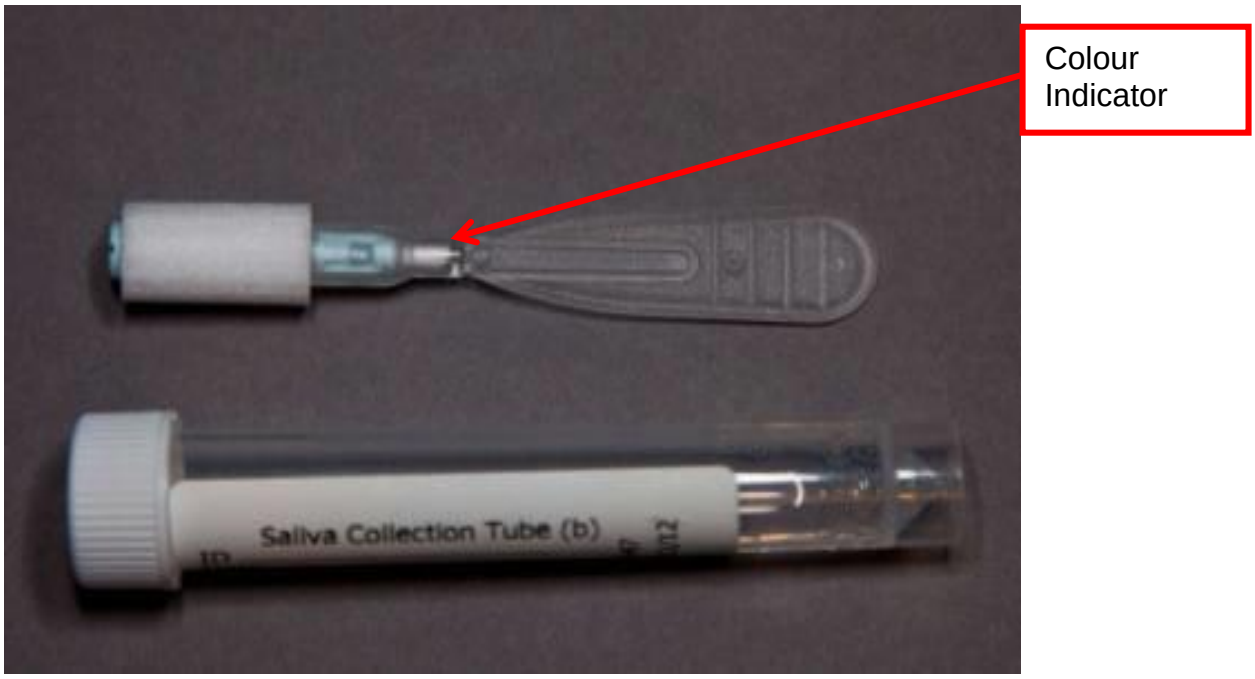


Figure 3.2 Image showing the Alere^{TM} Certus Collector with colour indicator

The operation of the Certus and Orasure collection devices differs significantly in that the Certus collection device collects a measured volume of oral fluid whereas the Orasure device collects an unspecified quantity of oral fluid by wiping the collector across the mucosal membranes inside the mouth. It is outside the scope of this thesis to discuss the detailed reasoning behind manufacturers' preference for different collection methods and the two collection devices are both widely used internationally by drug testing industry. It may be argued that if a measurement of drug concentrations in oral fluid is the desired outcome, which seems logical when positive and negative results are compared against agreed cut off concentrations, then there is a requirement to know the volume of oral fluid collected which is not possible with the Orasure collector.

Both collection devices are immersed in buffer solution after oral fluid has been collected. The composition of the buffer solutions used in both devices is

commercially restricted, however in general terms the buffer solution serves to a) extract drugs from the collection device into a matrix from which they can be subsequently extracted and analysed, b) control the pH of the medium to minimise drug breakdown and c) to act as a microbial inhibitor and preservative for the sample. Although no known fixed volume of oral fluid is collected using the Orasure device however the manufacturers of the Certus device state that samples should not be submitted if the volume indicator does not turn blue.

Although studies have been reported in relation to oral fluid drug detection and identification, little has been published in relation to the investigation of the effects of common beverages and food substances on oral fluid drug testing. In 2005, Wong et al investigated the effects of a range of foods on oral fluid testing and showed that Asian, Hispanic or American food did not interfere with the test results and neither did salt or sodium glutamate. Additionally, Wong et al (2005) showed that toothpaste and mouthwash also did not interfere with the analysis of the samples.

Other common household products and foodstuff have been investigated (Niedbala et al 2001). Sugar water, toothpaste, cranberry juice, baking soda, orange juice, cola, cough syrup and antiseptic water all resulted in no interference to the immunoassay testing system when added to negative oral fluid samples. Additionally these substances did not interfere with the detection of benzoylecgonine or its recovery (Niedbala et al 2001).

3.2 Materials and Methods

Two studies were performed that involved collecting oral fluid specimens. The first study involved volunteers consuming food, beverages and oral hygiene products. The second study focused on investigating the effect of a range of vinegar products

on the testing process. Informed consent was obtained from all participants and the experimental procedures were approved in compliance with the University of Bournemouth ethical guidelines (Appendix A). Volunteers undergoing treatment with any prescribed drugs or medications were excluded from the experiments. Volunteers were aged between 22 and 64. .

3.2.1 Materials

Orasure devices / kits were purchased from Orasure, Bethlehem, Pennsylvania, USA. Alere™ devices / kits were donated by Alere™, Abingdon, Oxfordshire, UK.

3.2.2 Methods

3.2.2.1 Collection Methods

3.2.2.1.1 Collection Method Study 1 – Effects of a range of foods and beverage on oral fluid collection methods

Non-drug using human volunteers were invited to consume one apple, coffee, cola, cranberry juice, fried food (plate of fish, chips and mushy peas), 5 grapes, a stick of chewing gum, milk, alcohol free mouthwash, one whole orange, Red Bull® , five sour candy sweets, spicy food, five sugar coated candy sweets, tea with milk, toothpaste, vinegar and yoghurt.

Ten volunteers sampled each test substance individually and in separate studies. Solid foods and beverages were totally consumed whilst liquids such as mouthwash and vinegar (n = 30 mL) were swirled around the mouth for 30 seconds and then discarded. Sufficient toothpaste to cover the brush of a toothbrush was dispensed. Oral fluid was collected on separate occasions using the Orasure Intercept and Alere™ Certus oral fluid collection devices a) immediately after mouth emptying and b) 10 minutes after mouth emptying. Oral fluid was collected in accordance with

each manufacturer's instructions. The Orasure Intercept oral fluid collection device was allowed to remain in the mouth for 3 minutes in accordance with the manufacturer's guidance after which it was removed and placed into the manufacturer's collection tube containing buffer and sealed.

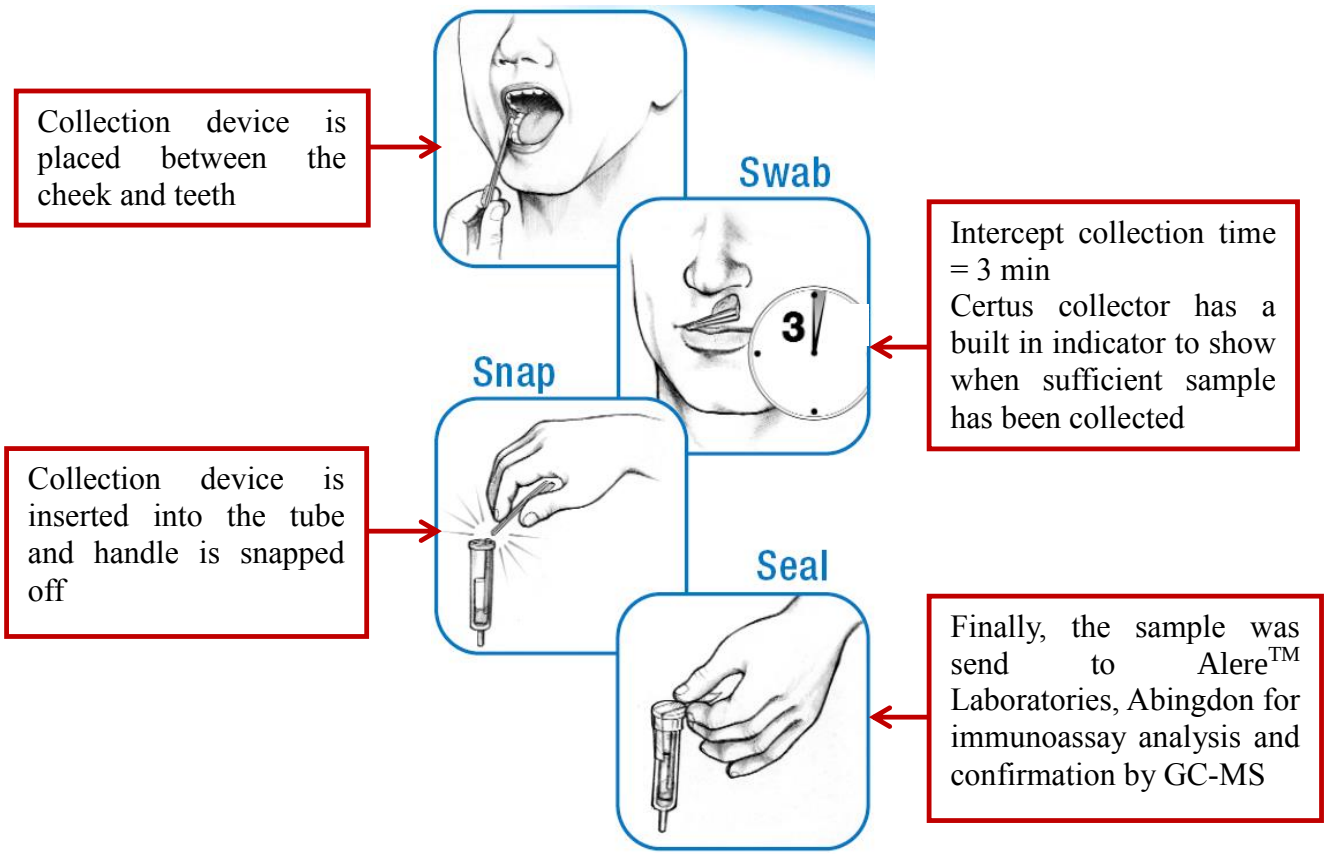


Figure 3.3. Summary of the oral fluid collection process using the Alere™ Certus and Orasure Intercept device (image available from http://www.orasure.com)

The Alere™ Certus oral fluid collection device was allowed to remain in the mouth until the dye indicator turned blue after which time the collector was removed and placed in the manufacturer's tube, containing buffer, and sealed. Collected oral fluid samples were stored at -20°C until analysis. Collections using each device were made alternatively and randomly. The volume of oral fluid collected, time for collection of

samples and pH were recorded. pH was measured using a Minilab ISFET electronic pH probe. The volume of oral fluid was determined gravimetrically by weighing the collection devices pre- and post-collection whilst assuming a specific gravity of 1. Oral fluid samples were subsequently analysed using their respective supplier's immunoassay reagents to observe whether the substances affected the immunoassay screening system.

The dilution factor for the Intercept device has been reported as an average of 1:4 (Niedbala et al 200). The Certus dilution factor is also 1:4. Cut off concentrations in neat oral fluid for opiates, cocaine, and methadone were the same for both Intercept and Certus at 40ng/ml. Intercept cut offs in neat oral fluid were higher than Certus for Amfetamines (100 versus 15 ng/mL) and methamfetamines (45 versus 15 ng/mL).

3.2.2.1.2 Collection Method Study 2 - Effects of vinegar on oral fluid collection methods

Drug-free human volunteers (n=10) were asked to swirl 5mL of a selected range of vinegars around the mouth for a period of 30 seconds and then discard the vinegar into a waste collecting receptacle. The vinegars included malt vinegar, white distilled vinegar, balsamic vinegar, red wine vinegar and white wine vinegar. After exposure, oral fluid was collected using the Orasure Intercept or the new Alere^{TM} Certus oral fluid collection devices a) immediately after mouth emptying and subsequently at 10, 20 and 30 minute intervals after mouth emptying. The sample collection procedure employed was the same as for Experiment one. Each volunteer provided samples using both devices for all vinegars tested. Oral fluid volume, pH and time of collection were recorded.

3.2.2.2 Analytical Method

Oral fluid samples were analysed for the presence of amfetamine, methamfetamine, cocaine, methadone and opiates using the Intercept and Certus oral fluid collection systems. Intercept samples were analysed in duplicate using Orasure microplates and the manufacturer's recommended cut offs. Intercept samples were analysed on Orasure microplates run on a Dynex microplate reader read at 450 nm whereas the Certus samples were analysed using the Alere^{TM} homogeneous assays on an Ilab 650 using the manufacturer's recommended cut offs. The screening cut offs for Intercept and Certus were the same for opiates, cocaine and methadone (10 ng/ml). They differed for amfetamine (100 ng/ml and 15 ng/ml) and methamfetamine (45 ng/ml and 15 ng/ml) for Intercept and Certus respectively. All samples that indicated a positive immunoassay response were confirmed by extraction (solid phase using Bond Elut Certify 3mL) with suitable internal standard and analysis by GC/MS or LC-MS-MS. Identification of each analyte for GC-MS was confirmed by three characteristic mass ions and for LC-MS-MS by two MS-MS transitions. The limit of detection (LOD) for the drug groups cocaine, opiates, amfetamines and metamfetamines was 1 ng/ml and for methadone was 3 ng/ml.

3.3 Results and Discussion

3.3.1 Study 1 - Effects of a range of foods and beverage on oral fluid collection methods

The pH of oral fluid prior to consumption of food and adulterants ranged from pH 6.8 to pH 7.3 (mean= 6.9; median= 6.9, SD = 0.2). Neither of the two devices showed large pH changes after the consumption of most foods or adulterants. Following the consumption of vinegar the pH of oral fluid decreased to a mean pH of 5.3 (range = pH 4.7 - pH 6, median = pH 5.2) (Table 3.1).

Table 3.1 Summary of oral fluid pH pre and post exposure of common foods, beverages and oral hygiene products

	Intercept						Certus					
	Mean pre-exposure pH	Standard Deviation pre-exposure pH	Mean pH post exposure	Standard Deviation post-exposure pH	pH post exposure		Mean pre-exposure pH	Standard Deviation pre-exposure pH	Mean pH post exposure	Standard Deviation post-exposure pH	pH post exposure	
					Min	Max					Min	Max
Apples	6.8	0.2	7.4	0.4	6.5	7.9	6.9	0.2	7.5	0.4	6.5	7.8
Coffee	6.9	0.3	7.1	0.3	6.2	8.2	6.9	0.3	7.2	0.5	6.2	8.2
Coke	6.9	0.2	6.8	0.5	5.9	8.1	6.9	0.2	6.7	0.6	5.7	7.7
Cranberry Juice	6.9	0.1	6.5	0.4	5.9	7.3	6.9	0.1	6.3	0.7	4.5	7.5
Fried Food	7	0.3	7.2	0.4	6.6	8.1	6.9	0.1	7.3	0.4	6.8	8
Grapes	6.9	0.2	7.4	0.6	5.9	8.6	6.9	0.2	7.6	0.5	6.8	8.6
Gum	6.9	0.3	7.6	0.5	6.5	8.1	7.1	0.2	7.7	0.5	6.7	8.1
Juice	7	0.2	6.2	0.8	4.8	7.7	7.2	0.2	6.6	0.8	5.2	7.7
Milk	6.9	0.2	7	0.3	6.5	7.3	6.9	0.2	6.9	0.3	6.2	7.3
Mouthwash	7	0.2	7.3	0.4	6.5	7.9	6.9	0.2	7.1	0.4	6.3	7.8
Oranges	6.9	0.2	6.6	0.7	5.1	7.9	7.1	0.3	7	0.6	5.9	7.9
Red bull	6.9	0.2	6.6	0.7	5.7	8.2	6.9	0.2	6.7	0.6	5.7	8.2
Sour Candy	7	0.3	7.2	0.5	6.2	7.9	6.9	0.2	6.9	0.9	4.7	8.4
Spicy Food	7	0.2	7.5	0.5	6.4	8.7	6.9	0.1	7.1	0.2	6.8	7.5
Sugar Coated Candy	7	0.2	7.4	0.4	6.3	8.5	7	0.3	7.8	0.7	6.6	7.9
Tea	7	0.1	7.2	0.2	6.6	7.4	6.9	0.1	7.3	0.3	6.6	7.8

	Intercept						Certus					
	Mean pre-exposure pH	**Standard Deviation pre-exposure pH**	**Mean pH post exposure**	**Standard Deviation post-exposure pH**	**pH post exposure**		**Mean pre-exposure pH**	**Standard Deviation pre-exposure pH**	**Mean pH post exposure**	**Standard Deviation post-exposure pH**	**pH post exposure**	
					Min	**Max**					**Min**	**Max**
Toothpaste	7	0.1	7.4	0.2	6.9	7.8	7	0.1	7.3	0.3	6.5	7.7
Vinegar	6.9	0.1	5.3	0.6	4.7	6	6.9	0.3	5.8	0.8	5.2	6.8
Yoghurt	7	0.2	6.9	0.4	5.3	7.8	6.9	0.2	6.9	0.4	6.1	7.7
Mean (n=380)	**6.9**	**0.2**	**7**	**0.5**	**6**	**7.9**	**6.9**	**0.2**	**7**	**0.5**	**6**	**7.8**
Median (n = 380)	**6.9**	**0.2**	**7.2**	**0.4**	**6.2**	**7.9**	**6.9**	**0.2**	**7.1**	**0.5**	**6.2**	**7.8**

Table 3.2 summarises the volume of oral fluid collected by the Orasure Intercept collection device using a fixed 3 minute collection time (as recommended by the manufacturer) ranged from 0.01 mL to 1.77 mL (mean = 0.58 mL, median = 0.57 mL, SD = 0.2 mL). In comparison the Alere^{TM} Certus device collected an average of 1.19 mL (range = 0.42 mL - 1.96 mL, median = 1.20 mL, SD = 0.3 mL). The collection time of the Certus device ranged from 1.1 min to 1.9 minutes (mean = 1.6 min, median = 1.5 min).

Table 3.2 Summary of the volume of oral fluid collected with the Orasure Intercept device and the Alere™ Certus device following the exposure to several substances

	Volume Collected (mL)							
	Intercept				**Certus**			
	Mean	**Standard Deviation**	**Min**	**Max**	**Mean**	**Standard Deviation**	**Min**	**Max**
Apples	0.57	0.2	0.18	0.86	1.18	0.3	0.84	1.68
Coffee	0.59	0.2	0.31	0.93	1.15	0.4	0.54	1.87
Coke	0.56	0.2	0.02	0.9	1.11	0.4	0.45	1.87
Cranberry Juice	0.56	0.2	0.04	0.8	1.15	0.3	0.73	1.71
Fried Food	0.49	0.2	0.15	0.79	1.26	0.3	0.84	1.59
Grapes	0.64	0.2	0.21	0.95	1.27	0.3	0.8	1.96
Gum	0.65	0.2	0.25	0.95	1.13	0.3	0.66	1.71
Juice	0.54	0.2	0.27	0.85	1.29	0.3	0.55	1.87
Milk	0.66	0.8	0.1	0.86	1.26	0.3	0.92	1.6
Mouthwash	0.58	0.2	0.14	0.83	1.2	0.3	0.66	1.54
Oranges	0.54	0.2	0.22	0.9	1.22	0.3	0.67	1.71
Red bull	0.65	0.4	0.01	1.77	1.27	0.3	0.82	1.96
Sour Candy	0.55	0.2	0.19	0.87	1.07	0.4	0.42	1.73
Spicy Food	0.61	0.2	0.13	0.94	1.14	0.3	0.43	1.63
Sugar Coated Candy	0.56	0.2	0.29	0.79	1.19	0.3	0.78	1.69
Tea	0.52	0.2	0.26	0.77	1.2	0.4	0.78	1.43
Toothpaste	0.65	0.2	0.3	0.86	1.22	0.2	0.86	1.66
Vinegar	0.56	0.2	0.16	0.89	1.24	0.3	0.78	1.82
Yoghurt	0.59	0.2	0.01	0.9	1.17	0.3	0.78	1.64
Mean (n=380)	**0.58**	0.2	**0.18**	**0.92**	**1.19**	**0.3**	**0.7**	**1.72**
Median (n = 380)	**0.57**	0.2	**0.18**	**0.87**	**1.2**	**0.3**	**0.78**	**1.71**

The buffer volume in the Intercept tube was 0.8 mL which yields a range of dilution factors from 1 in 81 for the smallest collected sample (0.01 mL) to 1 in 1.5 for the largest collected sample.

By comparison, the buffer volume in the Certus tube is 3 mL which yields a range of dilution factors from 1 in 8.1 for the smallest volume collected to 1 in 2.5 for the largest collected sample.

No presumptive positive results were obtained using the combination of Certus collector and Alere™ immunoassay. Several screen positive results were observed using the Orasure Intercept oral fluid collector in combination with the Orasure immunoassays. These were confirmed as drug free by chromatography - mass spectrometry and as such were deemed presumptive positives. It was noted however that these were mainly observed at time 0 and therefore represent a worst case scenario.

Intermittent presumptive positives (1 subject out of 10) were obtained with the Orasure combination after consumption of coffee, coke, fruit juice, oranges, spicy food and toothpaste (Table 3.3). In these cases the intermittent presumptive positive results were observed across the volunteers and not from one single subject. High numbers of presumptive positives were observed following the consumption of malt vinegar.

Table 3.3 Summary of false positive immunoassay results from Orasure combination following the exposure to a range of foods and beverages

Substance	Amfetamine	Methadone	Methamfetamine	Opiate	Cocaine
Apples	-ve	-ve	-ve	-ve	-ve
Coffee	**+ve (n=1, t=0)**	**+ve (n=1, t=0)**	-ve	**+ve (n=1, t=0)**	-ve
Coke	**+ve (n=1, t=0)**	-ve	-ve	-ve	-ve
Cranberry Juice	-ve	-ve	-ve	-ve	-ve
Fried Food	-ve	-ve	-ve	-ve	-ve
Fruit juice	-ve	-ve	-ve	**+ve (n=1, t=0)**	-ve
Grapes	-ve	-ve	-ve	-ve	-ve
Gum	-ve	-ve	-ve	-ve	-ve
Milk	-ve	-ve	-ve	-ve	-ve
Mouthwash	-ve	-ve	-ve	-ve	-ve
Oranges	**+ve (n=1, t=0)**	-ve	-ve	-ve	-ve
Red Bull	-ve	-ve	-ve	-ve	-ve
Sour Sweets	-ve	-ve	-ve	-ve	-ve
Spicy food	-ve	**+ve (n=1, t=0)**	-ve	-ve	-ve
Sugar Coated Sweets	-ve	-ve	-ve	-ve	-ve
Tea (with milk)	-ve	-ve	-ve	-ve	-ve
Toothpaste	**+ve (n=1, t=0)**	**+ve (n=1, t=0)**	-ve	-ve	-ve
Vinegar	**+ve (n=20, t=0,10))**	**+ve (n=20, t=0,10))**	-ve	-ve	**+ve (n=20, t=0,10))**
Yoghurt	-ve	-ve	-ve	-ve	-ve

Legend: +ve =positive immunoassay screening result, -ve = negative immunoassay screening results, n= number of positive samples out of 10, t= time after mouth emptying (min)

Vinegar, coffee, cola, fruit juice, oranges, spicy food and toothpaste are all capable of affecting the Orasure assay system to produce presumptive positive results. With the exception of vinegar positives occurred randomly in 1% of samples indicating that this result is not statistically significant. Since vinegar was shown to produce a significant effect on the Intercept device it was decided to explore this in more detail.

3.3.2 Study 2 - Effects of vinegar on oral fluid collection methods

Vinegar (all types) consistently produced positive screening results with the Orasure combination for amfetamine, methamfetamine and cocaine immunoassays (Table 3.4). No screening positives were observed with the Orasure combination for opiate and methadone assays. The majority of screening test positives were observed at the early time points although a significant number were also observed out to 30 minutes. All screen positives were submitted for GC MS confirmation and found to be confirmation negative (false positives) i.e. screening presumptive positive results.

Table 3.4 Binding (%) in different oral fluid samples after vinegar exposure using the Intercept oral fluid collection device

	Binding (%)					
	Time	**Amfetamine**	**Methadone**	**Methamfetamine**	**Opiate**	**Cocaine**
Cut Offs		**28**	**13**	**23**	**13**	**52**
Malt Vinegar	**0**	8	32	14	45	39
	10	4	31	22	46	43
	20	8	34	16	47	43
	30	27	46	43	62	64
White Wine Vinegar	**0**	4	25	7	36	24
	10	3	23	5	31	20
	20	5	30	10	39	32
	30	11	37	21	49	45
Red Wine Vinegar	**0**	3	25	6	34	26
	10	3	25	7	36	25
	20	7	34	15	45	37
	30	7	32	13	45	36
Balsamic Vinegar	**0**	2	22	5	29	14
	10	3	26	7	35	22
	20	12	38	22	54	44
	30	24	44	33	60	51

Legend: Binding (%) for the timed vinegar samples was calculated by dividing the optical density obtained for the sample by that of the kit's zero calibrator and multiplying by 100. Binding (%) for the cut off calibrator was calculated by dividing the optical density obtained for that calibrator by that of the kit's zero calibrator and multiplying by 100. As it is a competitive immunoassay, any binding value less than the cut off calibrator is deemed presumptive positive and any binding value greater than the cut off calibrator is deemed negative. Highlighted values in the table represent presumptive positives relative to the cut off binding for that particular assay. Non-highlighted values represent screen negative results. For clarity, the table shows the response from single donors each time point being post vinegar exposure.

Of note was the depression in the binding response for most of the vinegar samples using the Intercept/Orasure combination. Due to the wide displacement between negative and cut off for the opiate and methadone assays, the vinegar samples did not trigger a positive response. The other Orasure assays have less displacement between negative and cut off which resulted in higher numbers of presumptive positive results.

Depressed binding could clearly be shown for up to 75 minutes post vinegar exposure for methamfetamine, amfetamine and cocaine. Although samples were reported as negative and above the cut off for the percentage binding 30 minutes post exposure to a substance, binding was still found to be depressed post this period as can been in Figure 3.4, Figure 3.5 and Figure 3.6. This indicates that the vinegar still affects the Orasure collection system after 30 minutes post consumption. These results should be borne in mind when interpreting forensic data as should the large difference in dilution factors observed between the devices.

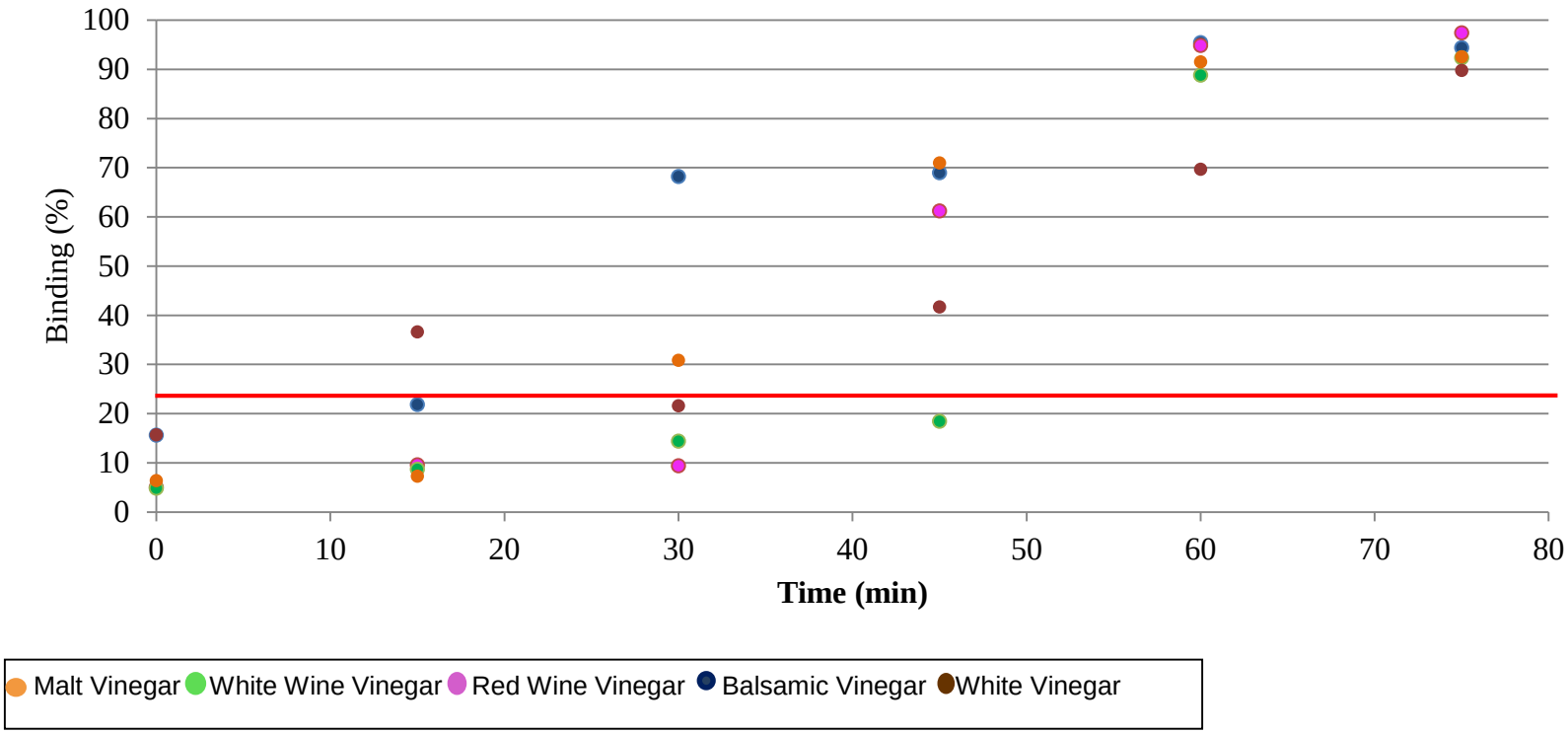


Figure 3.4 Binding (%) for methamfetamine in samples taken after the consumption of malt, distilled, balsamic and red and white vinegar over 75 minutes collected using the Orasure Intercept® device. The red line (---) indicates the percentage binding cut off for metamfetamine (23 %)

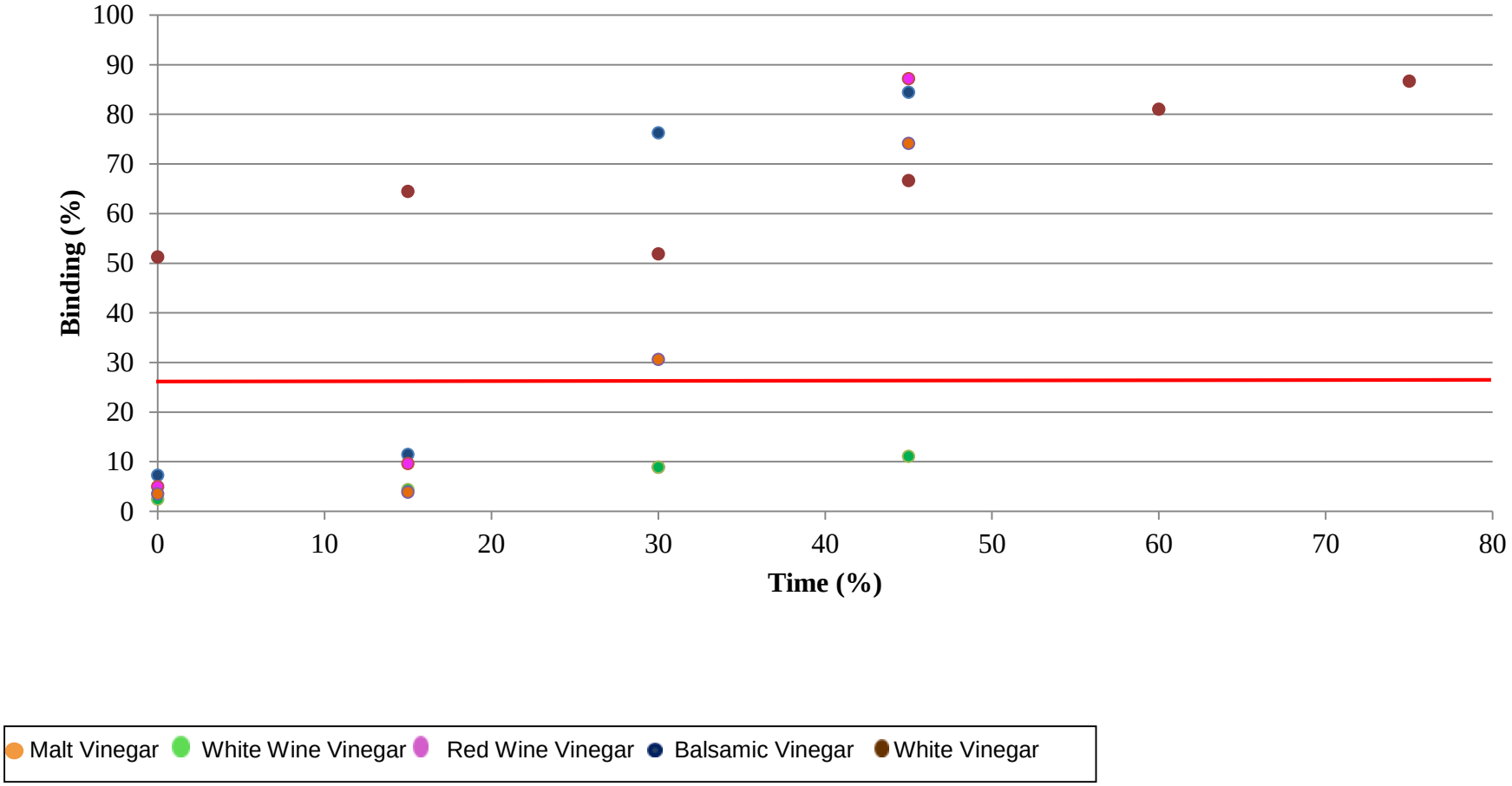


Figure 3.5 Binding (%) for Amfetamine in samples taken after the consumption of malt, distilled, balsamic and red and white vinegar over 75 minutes collected using the Orasure Intercept® device. The red line (---) indicates the percentage binding cut off for amfetamine (28 %)

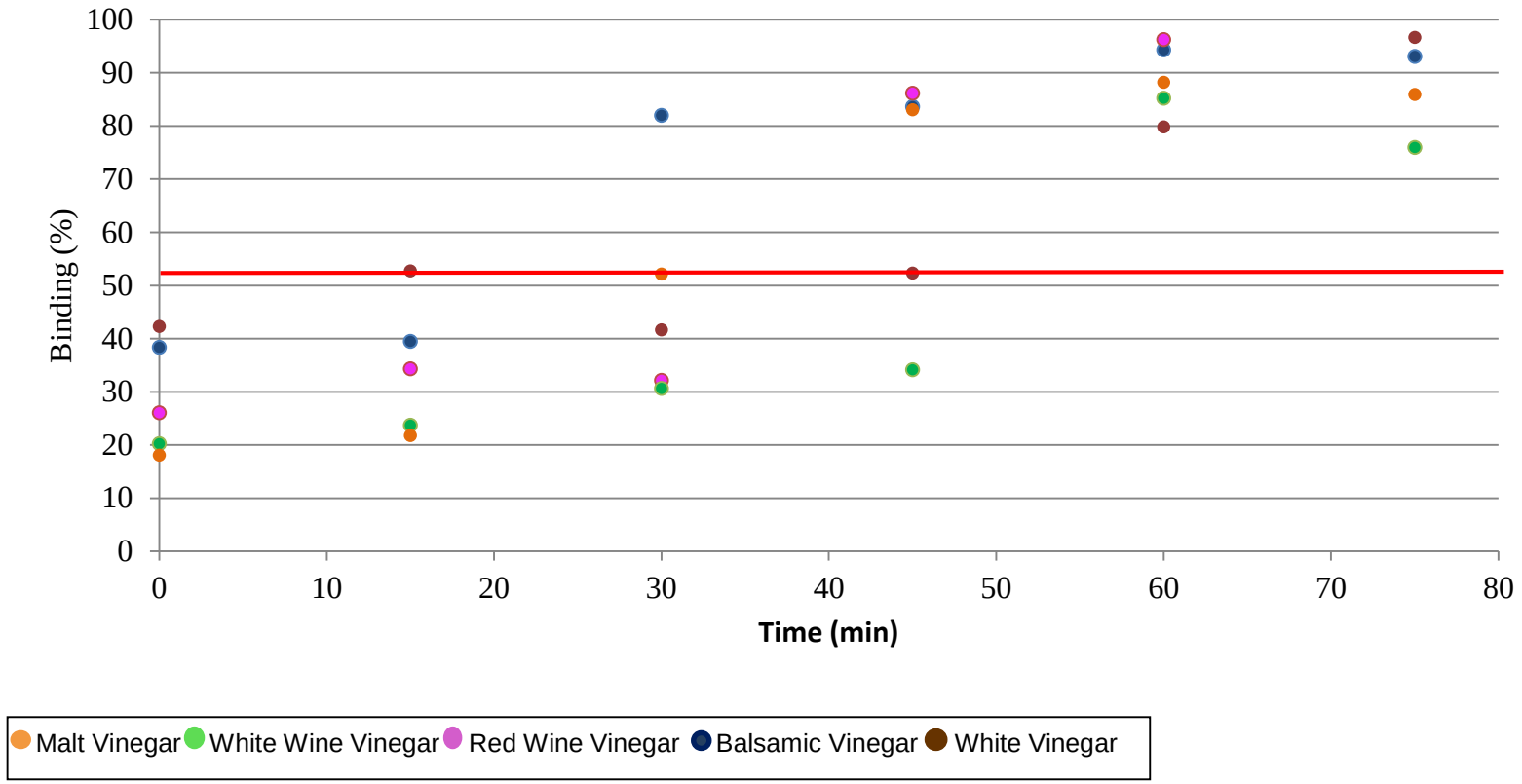


Figure 3.6 Binding (%) for cocaine in samples taken after the consumption of malt, distilled, balsamic and red and white vinegar over 75 minute collected using the Orasure Intercept® device. The red line (---) indicates the percentage binding cut off for cocaine (52 %)

Although opiate and methadone sample did not result in a false positive result, depressed binding can still be shown in all types of vinegar for up to 75 minutes post exposure. Percentage binding could clearly be observed to be above the cut off.

Opiate binding was initially depressed by 50 % but during the collections over 75 minutes, depression quickly decreased. However, at t = 75 min depression still ranged between 80 % and 90 %. Although results were all above the cut off for percentage binding, depressed binding could still affect the concentration detected and hence the interpretation of the results (Figure 3.7).

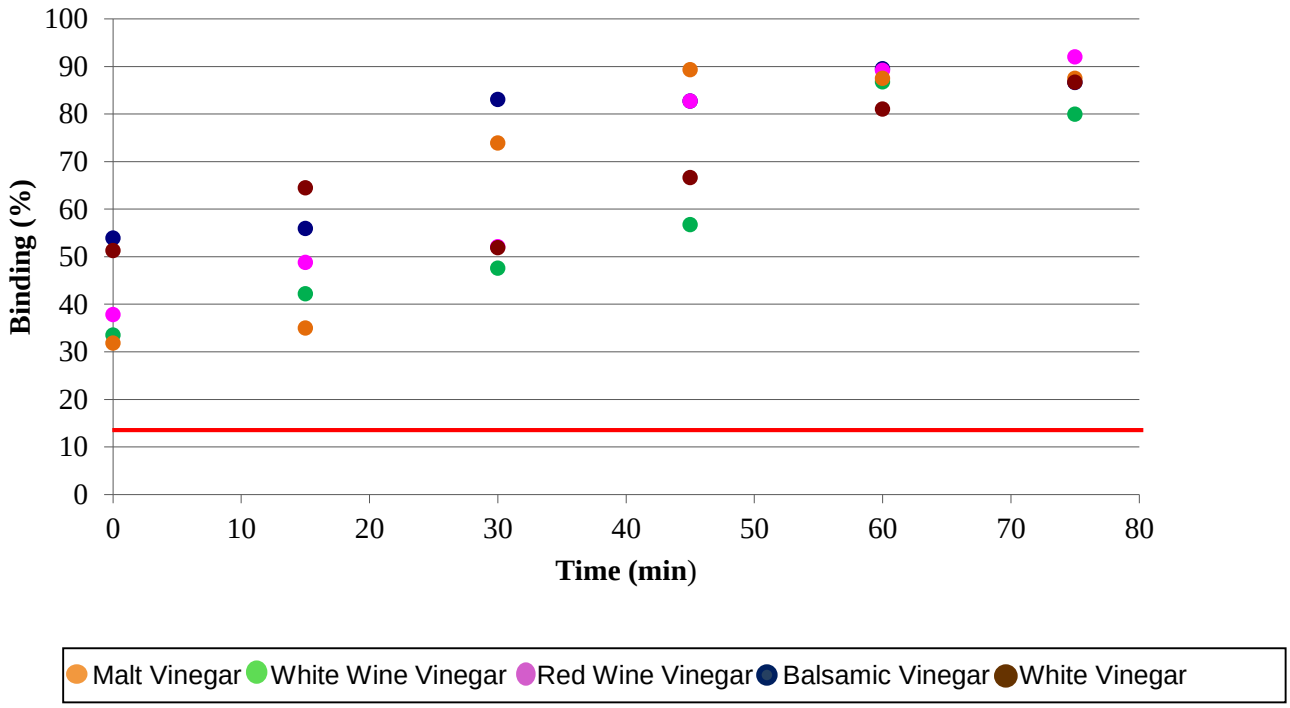


Figure 3.7 Binding (%) for opiates in samples taken after the consumption of malt, distilled, balsamic and red and white vinegar over 75 minutes collected using the Orasure Intercept® device. The red line (---) indicates the percentage binding cut off for opiates (13 %)

Similarly to opiate, methadone depression was observed in all oral fluid samples during the 75 minute collection. Binding was depressed by up to 70 % in the sample collected immediately post exposure. Depression only slowly decreased and in samples collected at t = 75 min depression was still observed ranging between 40 % and 60 % (Figure 3.8).

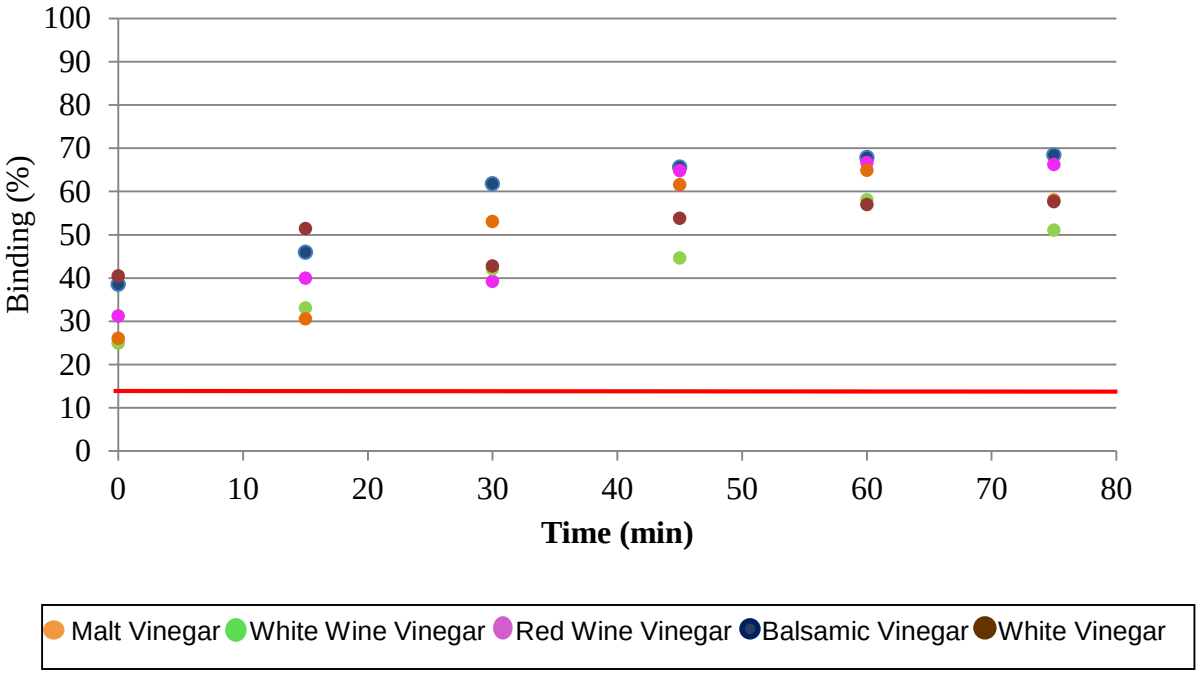


Figure 3.8 Binding (%) for methadone in samples taken after the consumption of malt, distilled, balsamic and red and white vinegar over 75 minutes collected using the Orasure Intercept® device.
The red line (---) indicates the percentage binding cut off for methadone (13 %)

By comparison the Certus / Alere^{TM} samples were negative for all vinegar types and time points. No visual changes to either buffer were observed in specimens that were tested following the use of vinegar.

Vinegar proved to be a major interference to the immunoassay. Most manufacturers of oral fluid drug tests indicate that the effects of foods and beverages have dispersed from the mouth after 10 - 15 minutes (Spiehler 2011) and that after this time has

elapsed an oral fluid sample can then be collected with minimum risk of contamination.

The results presented here clearly contradict this general guideline in relation to vinegar. Although no opiate or methadone presumptive positives results were observed, the antibody binding following the consumption of vinegar was reduced significantly (>50%) however results were still above the cut off and hence had to be reported as negative. There was no relationship between presumptive positive result and the variable dilutions.

Vinegar (pH = 4.2) was the most acidic of the substances tested and it is possible that the acid pH of collection. Following the consumption of vinegar the pH of oral fluid decreased to a mean pH of 5.3 (range = pH 4.7 - pH 6) when collected using the Orasure Intercept device. In sample collected with the Alere^{TM} Certus device the pH ranged between 5.2 and 6.8 (mean = 5.3).

In comparison to the Alere^{TM} Certus Collector, which is provided with 4 mL of buffer, the Orasure collection device contains a lower buffer volume of 3 mL. Each collection buffer is designed to ensure sample stability and minimize the side effects of sample pH to immunoassay screening (Ericson and Bratthall, 1989). The effectiveness of a buffer or buffer capacity is determined by the concentration of acid which is added to the buffer solution. The weak base in the buffer is designed to neutralize acidic solutions, however if the acid/base ratio is too large, it can lead to a buildup of acid and hence lower the pH of the sample solution. Therefore the dilution factor plays an important role, when investigating false positive results, following the consumption of vinegar.

The Intercept collector has a much greater variation in the dilution factor than the Certus system. The buffer volume in the Intercept tube was 0.8 mL, which resulted in a range of dilution factors from 1 in 81 for the smallest collected sample to 1 in 1.5 for the largest collected sample. Due to the lower dilution factor in the Intercept system, the acid/base ratio will be larger in comparison to the acid/base ratio in the Certus collector, hence lowering the pH of the solution and resulting in a false positive effect on the immunoassay.

Additionally, the hypothesis that pH can affect antibody binding and hence lead to unreliable results is further supported in the literature. Kim et al (2003) discussed large amounts of false positives in samples collected using a Salivette collector using the Cozart Microplate EIA Cocaine Oral Fluid Kit have been reported in the literature (Kim et al 2003). The Salivette system is based on a cotton swab which is treated with citric acid to stimulate saliva flow. The citric acid results in a lower sample pH (mean pH 2.8) which has been shown to increase the amount of false reactions in immunoassay testing systems. Niedbala et al (2001) also investigated the effects of pH on the Cozart Cocaine EIA kit by adjusting oral fluid (pH 7.07) with aqueous sodium hydroxide or hydrochloric acid solutions to achieve a range of pH 5.0 to pH 9.0 and applying this to the immunoassay. No interference from the pH on the Cozart Cocaine EIA kit was detected during testing.

3.4 Conclusion

The Orasure Intercept oral fluid collection device has limited buffering capacity and hence exhibited numerous presumptive positive immunoassay test results. This was particularly observed following the consumption of vinegar. With the exception of vinegar, interferences from foods, common beverages and oral hygiene products were not observable after 10 minutes providing support for the widely held view that

foods should not be a problem after 10-15 minutes has elapsed. The interference by vinegar was not related to the pH of the sample or dilution factor when applied to the immunoassay. Use of the Alere™ Certus collection device did not provide any presumptive positive results by immunoassay. As a consequence of the improved performance of the Certus oral fluid collection device, this was used for collecting oral fluid samples in the experiments described in the following sections.

Chapter 4.0 - Concentrations of cocaine, benzoylecgonine and ecgonine methyl ester in oral fluid following the consumption of coca tea

4.1 Introduction

It has been reported (Chapter 1) that orally consumed drugs can be deposited in the oral cavity and hence result in the detection of elevated concentrations in oral fluid above the contribution from drug passing into the blood stream and back into the mouth via saliva secretions (Cone 1996, Huestis and Cone 2004, Drummer 2006). Most scientific studies examining oral fluid cocaine appearance have been restricted to relatively small doses of drug for ethical and safety reasons. For the purpose of this study, oral fluid samples following the consumption of coca tea were collected in Lima, Peru.

Coca tea is widely consumed in a number of South American countries where its use is both legal and socially acceptable. The tea is prepared from an infusion of the leaves obtained from the coca bush *Erythroxylum coca* (Hamner and Villegas 1969, Hanna and Hornick 1977, Siegel et al 1986, Jenkins et al 1996, Turner et al 2005). Coca leaves have been used for over 2000 years and were widely used by the Incas of Peru in religious ceremonies (Martin 1970, Zapata-Oritz 1970, Allen 1981, Billman 1990).

In addition to the use of coca leaves during the preparation of coca tea, the custom of chewing coca leaves is still widely practiced today by people working at high altitude. Coca chewing alleviates adverse symptoms of pain, thirst, hunger and tiredness that are experienced by humans working or living at high altitude (Fuchs 1978). Chewing coca can be advantageous in colder environments (Hanna 1971a). In

addition to cocaine, the average coca leaf contains a number of other structurally related compounds including benzoylecgonine and ecgonine methyl ester (Weil 1978, Penny et al 2009). The amount of cocaine and its metabolites present in coca leave can vary depending on species and origin of the *Erythroxylum* plant (Jenkins et al 1996). The Peruvian *Erythroxylum* species, as used by Jenkins et al, contains 5.11 mg of cocaine, 0.11 mg of benzoylecgonine and 1.15 mg of ecgonine methyl ester in 1 g of leaf material (Jenkins et al 1996).

Elevated drug concentrations are reported to be caused by contamination of the oral mucosa following exposure to orally consumed drugs. Concentrations of cocaine in oral fluid following crack cocaine smoking ranged between 15.85 µg/mL to 504.88 µg/mL in comparison to cocaine concentrations following injection that ranged between 0.43 µg/mL to 1.93 µg/mL (Jenkins et al 1996). These concentrations greatly exceed normal levels thus indicating possible depot formation.

The experiments described in this section were designed to investigate the concentration of cocaine and related compounds that may be detected in oral fluid following consumption of coca tea. To identify whether elevated drug concentrations can be detected for extended time periods and hence interfere with the interpretation of results, oral fluid specimens were collected from volunteers following the consumption of coca tea over a 60 minute interval.

4.2 Materials and Methods

4.2.1 Materials

Alere™ Certus collectors were provided by Alere Toxicology, Abingdon, UK

Alere™ Microplate EIA Kits for cocaine were used to screen for the presence of cocaine and its derivatives and were provided by Alere Toxicology, Abingdon, UK.

Dried Coca leaves (500 g) were purchased in a local market in Lima, Peru.

4.2.2 Methodology

4.2.2.1 Preparation of coca tea

Following local custom, coca tea was prepared by soaking 10 whole coca leaves in boiling water for 5 minutes prior to straining to remove the leaf tissues (Figure 4.1).

Coca tea – prior to straining

Coca tea following straining of leaves

Figure 4.1. Coca tea preparation – Coca leaves were infused with boiling water (left), and subsequently strained (right) to yield a clear pale yellow infusion prior to drinking

4.2.2.2 Collection methods

Twenty-five healthy volunteers, ranging in age between 21 and 88 (12 male, 13 female) whom were resident in Lima, Peru, participated in this study. Informed consent was obtained from all participants prior to participation and the experimental procedures were undertaken in compliance with the University of Bournemouth ethical guidelines (Appendix B). All volunteers completed a questionnaire regarding prior use of cocaine/coca tea and stated that they had not consumed cocaine, coca tea or alcohol within the preceding 24 hours. Volunteers provided a blank control oral fluid sample immediately prior to drinking the coca tea for confirmation that no coca tea, cocaine or alcohol was consumed prior to participating in this study.

Volunteers drank 1 cup (~250 mL) of coca tea immediately post straining of the leaves over a period of approximately 5 minutes and afterwards provided a sample of oral fluid immediately following completion of tea drinking. Oral fluid was subsequently collected at 15, 30, 45 and 60 minute intervals. Oral fluid collection was facilitated using the Alere^{TM} Certus device and subsequently transferred to buffer of pH 4.5 buffer since acidic buffers have been shown to reduce the hydrolysis of cocaine to benzoylecgonine (Kiszka et al. 2000). The collected specimens were stored at –20^{o}C prior to transportation to the laboratory at Alere Toxicology, Abingdon, UK. A period of 14 days elapsed between collection of the specimens and their subsequent analysis. Oral fluid samples were screened using a cocaine immunoassay prior to confirmation using LC-MS.

4.2.2.3 Immunoassay

The Alere™ Microplate EIA Kit for cocaine metabolites was provided with four calibrators ranging between 0 ng/mL and 300 ng/mL. The manufacturers report cross reactivity of the assay with cocaine and cocaethylene as summarised in Table 4.1.

Table 4.1. Summary of compounds cross-reacting with the Alere™ Microplate EIA Kit for cocaine (Alere™, 2013)

Compounds Cross Reactants	Concentration ng/mL	Apparent Benzoylecgonine	Reactivity (%)
Cocaine	300	277.1	92.4
	50	46.4	92.8
	10	10.2	102.0
Cocaethylene	300	290.9	97.0
	50	59.9	119.7
	10	13.0	129.7

All non-cross reacting compounds are summarised in Appendix C.

The principal upon which this immunoassay is based relies on the fact that the wells of a microplate are coated with a benzoylecgonine antibody. Initially the sample is added to the wells in combination with an enzyme conjugate which is included in the immunoassay kit. The enzyme conjugate is a cocaine metabolite derivative labelled with horseradish peroxidase <0.1% (v/v) and diluted in a matrix with stabilers. During the incubation time, the analyte of interest competes with the enzyme conjugate for the binding sites of the antigen of the wells. The plates were then thoroughly washed using a DAS automated microplate washer to remove excess enzyme conjugate. A substrate solution which consists of a solution of <0.05% 3,3',5,5'-tetramethylbenzidine was then added. The substrate reagent is converted directly by the enzyme conjugate which has bound to the well antibody resulting in a

blue colour reaction. After a further incubation period, a stop solution of 1 mol/L sulphuric acid is added to stop the reaction and convert the colour reaction into a more readable yellow colour. The immunoassay plate is read at a wavelength of 450 nm and secondary wavelength of 630 nm on a DAS 8 channel ELISA photometer. The procedure is summarised in below in Figure 4.2.

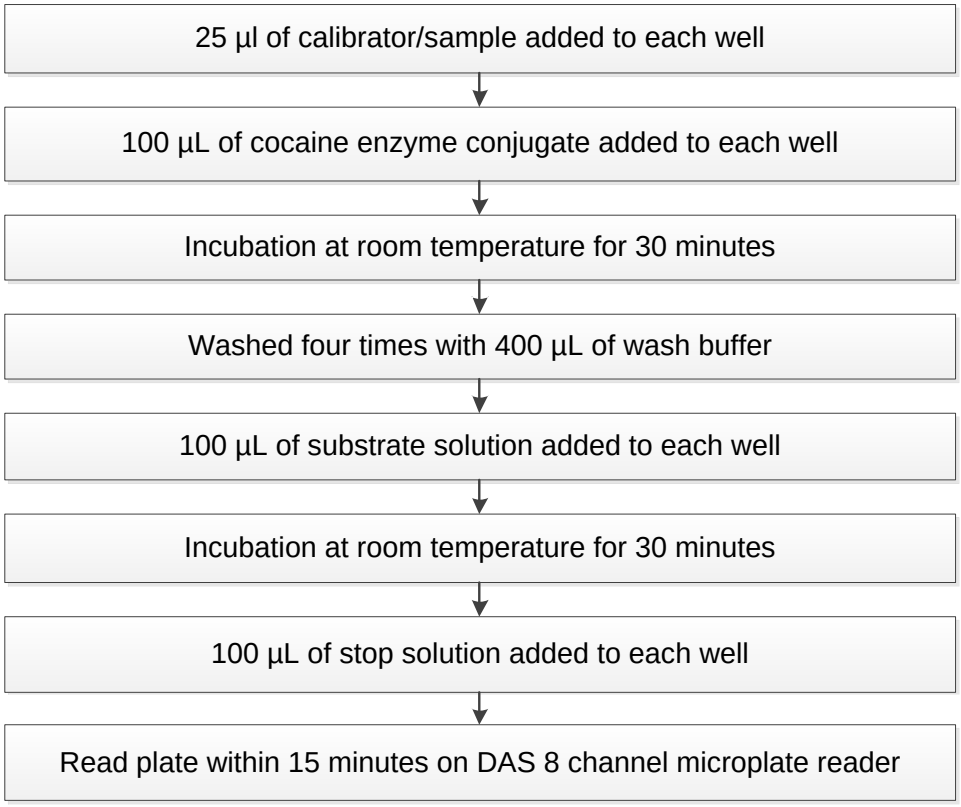


Figure 4.2. Outline of the procedure for using the Alere™ immunoassay method for cocaine

4.2.2.4 Solid Phase Extraction (SPE)

200 µL of 0.1M hydrochloric acid was added to 200 µL of the oral fluid samples. Extraction was performed using Oasis mixed-mode cation exchange (MCX) micro elution plates (Waters, Manchester, UK). Cartridges were conditioned with 200 µL of methanol and 200 µL of 0.1 M hydrochloric acid followed by addition of sample. The columns were washed with 200 µL of 0.1 M hydrochloric acid and 200 µL of 30% methanol in water (70/30 v/v) and dried. Elution was achieved using 50 µL of 5% ammonia in methanol. The sample was reconstituted in 50 µL of 0.1% formic acid in water and analysed on a Waters LC-MS system.

4.2.2.5 Liquid Chromatography – Mass Spectrometry

Extracted oral fluid samples were analysed at Alere Toxicology, Abingdon using LC-MS. Chromatographic analysis was carried out using a C18-ether column (3µ x 50 mm x 2 mm) (Phenomenex, UK). An elution gradient at a flow rate of 0.25 mL/min was used for a run time of 17 minutes. The Mobile Phase A was 0.1 % acetic acid and Mobile Phase B was 0.1 % acetic acid in methanol. The HPLC column temperature was maintained at 40°C. To ensure the assay performance is fit for purpose, the following parameters were determined to validate the quantitative chromatographic methods used within the laboratory (Table 4.2):

- Method linearity
- Limit of Quantitation (LOQ)
- Upper (ULOQ) and Lower (LLOQ) robustness of the assay
- Stability of the analytes of interest
- Recovery from collection device
- Extraction efficiency
- Matrix effects
- Specificity

- Carryover
- Bias (accuracy)
- Reproducibility (precision) – within and between batches and the measurement of uncertainty

Table 4.2 Parameters for the detection of cocaine and its metabolites by LC-MS

Compound	MRM transition (m/z)	Retention Time (min)	LOD (ng/mL)	LLOQ (ng/mL)	Analyte Bias (%) (n=79)	Measurement of Uncertainty (%)
Cocaine	304→105 304→182	12.36	0.1	0.8	- 9	± 46
Cocaine-D3	307→185.	12.36	--	--	--	--
Benzoylecgonine	290→168 290→105	11.43	0.1	0.8	+ 22	± 38
Benzoylecgonine- D3	293→171	11.43	--	--	--	--
Ecgonine Methyl Ester	200→182 200→82	2.45	0.33	0.8	- 13	± 48
Anhydroecgonine methyl ester	182→118 182→122	5.94	0.66	0.8	+ 25	± 59
Anhydroecgonine methyl ester – D3	185→125	5.94	--	--	--	--
Cocaethylene	318→150 318→196	13.41	0.33	0.8	- 1	± 36
Cocaethylene – D3	321→199	13.41	--	--	--	--

4.3 Results and Discussion

4.3.1 Immunoassay Results

In toxicology, immunoassay is adapted as a fast, preliminary screening technique commonly used as an indicator to identify the presence or absence of a drug group, rather than a specific compound. The immunoassay results generated are only qualitative or semi-quantitative and require confirmation, usually by chromatography-mass spectrometry. Due to the limited amount of sample, the immunoassay was used in qualitative mode reporting as positive (+ve) or negative (-ve) rather than perform multiple dilutions to obtain a value. The cut off recommended by the kit manufacturer was 50 ng/mL for the immunoassay below which the results were reported as negative. Table 4.3 summarises the results from the immunoassay screening of coca tea samples. None of the 25 volunteers indicated a positive result prior to participating in this study. All volunteers tested positive immediately post consumption of coca tea however, the immunoassay result showed that 20 volunteers out of the 25 tested did not test positive for cocaine or any of its cross-reacting compounds 60 minutes post consumption of coca tea.

Table 4.3. Summary of cocaine immunoassay results (ng/ml) in oral fluid obtained from 25 volunteers before and after the consumption of coca tea. -ve = negative result below the manufacturers recommended cut off (50 ng/mL) ; +ve = positive result above the manufactures recommended cut off for the immunoassay (50 ng/mL)

ID	Time (min)	Concentration (ng/ml)	ID	Time (min)	Concentration (ng/ml)	ID	Time (min)	Concentration (ng/ml)	ID	Time (min)	Concentration (ng/ml)	ID	Time (min)	Concentration (ng/ml)
28021	Pre - dose	-ve	28029	Pre - dose	-ve	280214	Pre – dose	-ve	1032	Pre – dose	-ve	28023 Day2	Pre - dose	-ve
	0	+ve		0	+ve		0	+ve		0	+ve		0	+ve
	15	+ve		15	+ve		15	+ve		15	+ve		15	-ve
	30	-ve		30	+ve		30	+ve		30	-ve		30	+ve
	45	-ve		45	+ve		45	+ve		45	-ve		45	-ve
	60	-ve		60	+ve		60	+ve		60	-ve		60	-ve
28024	Pre - dose	-ve	28029	Pre - dose	-ve	280215	Pre – dose	-ve	28022 Day1	Pre – dose	-ve	28023 Day3	Pre - dose	-ve
	0	+ve		0	+ve		0	+ve		0	+ve		0	+ve
	15	+ve		15	+ve		15	+ve		15	+ve		15	-ve
	30	-ve		30	-ve		30	+ve		30	-ve		30	+ve
	45	-ve		45	-ve		45	-ve		45	-ve		45	-ve
	60	-ve		60	-ve		60	-ve		60	-ve		60	-ve
28025	Pre - dose	-ve	280210	Pre - dose	-ve	280216	Pre – dose	-ve	28022 Day2	Pre – dose	-ve			
	0	+ve		0	+ve		0	+ve		0	+ve			
	15	-ve		15	+ve		15	+ve		15	+ve			
	30	-ve		30	-ve		30	+ve		30	-ve			
	45	-ve		45	-ve		45	+ve		45	-ve			
	60	-ve		60	-ve		60	+ve		60	-ve			

ID	Time (min)	Concentration (ng/ml)	ID	Time (min)	Concentration (ng/ml)	ID	Time (min)	Concentration (ng/ml)	ID	Time (min)	Concentration (ng/ml)	ID	Time (min)	Concentration (ng/ml)
28026	Pre - dose	-ve	280211	Pre - dose	-ve	280217	Pre - dose	-ve	28022 Day3	Pre – dose	-ve			
	0	+ve		0	+ve		0	+ve		0	+ve			
	15	+ve		15	+ve		15	+ve		15	+ve			
	30	+ve		30	+ve		30	-ve		30	+ve			
	45	-ve		45	+ve		45	-ve		45	+ve			
	60	-ve		60	-ve		60	-ve		60	-ve			
28027	Pre - dose	-ve	280212	Pre - dose	+ve	280218	Pre - dose	-ve	28023 Day 1	Pre – dose	-ve			
	0	+ve		0	+ve		0	+ve		0	+ve			
	15	-ve		15	+ve		15	-ve		15	+ve			
	30	-ve		30	+ve		30	-ve		30	-ve			
	45	-ve		45	+ve		45	-ve		45	-ve			
	60	-ve		60	+ve		60	-ve		60	-ve			
28028	Pre - dose	-ve	280213	Pre - dose	-ve	1031	Pre - dose	-ve						
	0	+ve		0	+ve		0	+ve						
	15	-ve		15	+ve		15	+ve						
	30	-ve		30	-ve		30	+ve						
	45	-ve		45	-ve		45	+ve						
	60	-ve		60	-ve		60	+ve						

4.3.2 LC-MS results

Mass spectra were unobtainable due to loss of data following a computer fault.

4.3.2.1 Cocaine

The SAMSHA guidelines for confirmatory analysis cut off for cocaine in oral fluid has been set at 8 ng/mL. Cocaine concentrations immediately after consumption of coca tea ranged between 14 ng/mL and 8,595 ng/mL (mean = 2,729 ng/mL, median = 1,807 ng/mL, SD = 2434 ng/mL) (Table 4.4). The interquartile range, which excludes low and high outliers within the results, was 2175 ng/mL.

Table 4.4. Summary of the mean, median and range of concentrations (ng/mL) of cocaine analysed by LC/MS in oral fluid specimens collected from human volunteers following the consumption of coca tea

n = 25	**Concentration (ng/ml)**					
Time (min)	**Mean**	**Median**	**Standard Deviation (SD)**	**Interquartile Range (IQR)**	**Range**	
					Min	**Max**
pre-dose	9	4	14	5	1	62
0	2729	1808	2434	2175	14	8595
15	368	184	444	365	25	1570
30	138.	33	205	100	6	768
45	44	25	52	35	5	186
60	22	12	37	19	2	176

Cocaine concentrations dropped rapidly to a mean concentration of 368 ng/mL after 15 minutes. Mean concentrations at 30 and 45 minutes after tea drinking were 128.0 ng/mL and 44 ng/mL respectively. In all 25 cases cocaine was still detectable at 60 minutes with concentrations ranging between 1.9 ng/mL and 176 ng/mL (Figure 4.3).

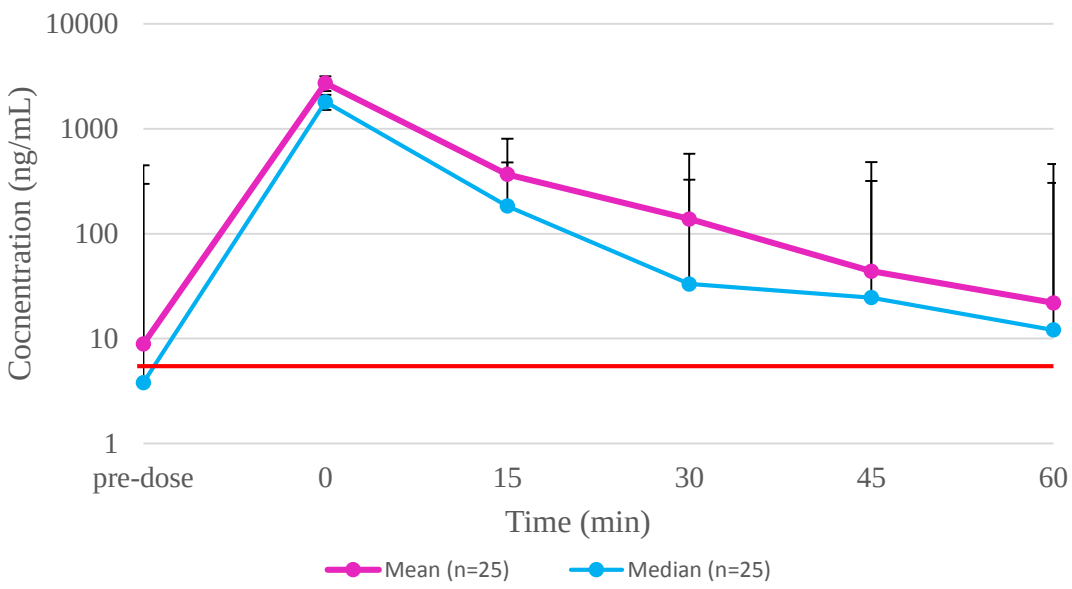


Figure 4.3. Mean and Median concentrations (ng/mL) of cocaine in oral fluid following the consumption of coca tea in samples collected over 60 minutes. Red line (---) indicates the SAMHSA cut off for cocaine in oral fluid (8 ng/mL) during confirmatory analysis

Of note was the maximum concentration of cocaine in samples collected prior to consumption. Whilst no positives were reported from the immunoassay analysis due to the higher cut off, the concentrations of cocaine in pre-dose samples during confirmatory analysis ranged from 1 ng/mL to 62 ng/mL (mean = 9 ng/mL, median = 4 ng/mL) (Figure 4.4). The difference between screening and confirmatory positives prior to consumption of tea is a result of the higher sensitivity of the LC-MS method.

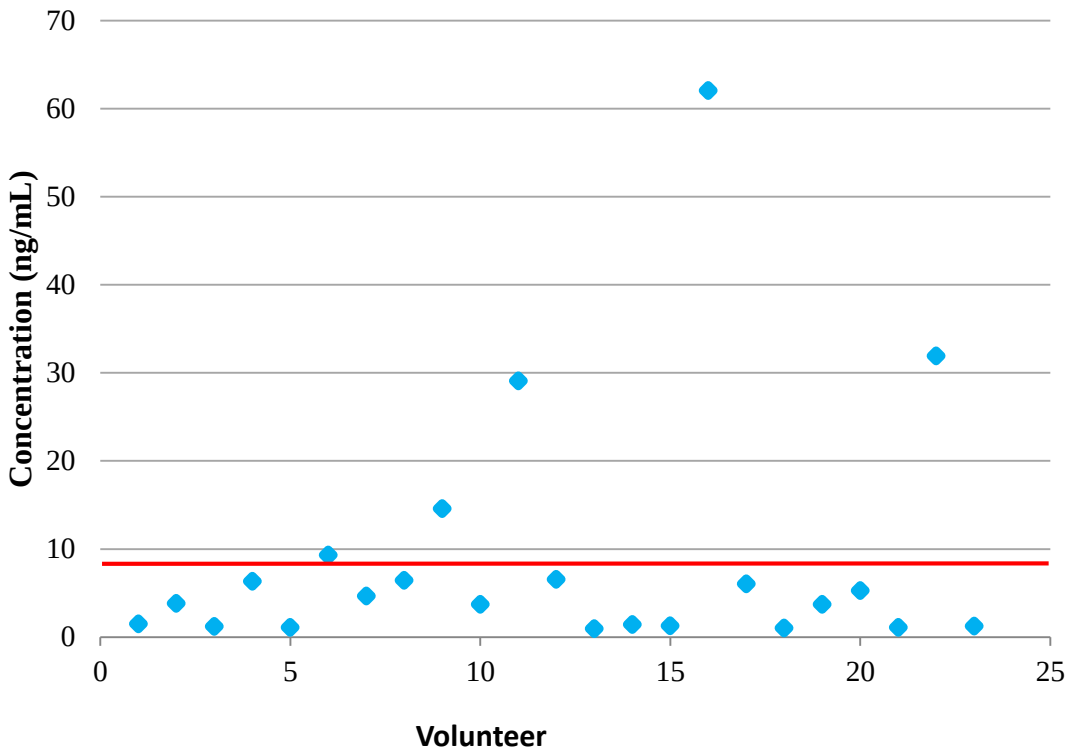


Figure 4.4 represents the concentrations of cocaine in oral fluid in samples collected prior to consumption of coca tea shown by LC/MS. The red line (---) indicates the recommended SAMHSA cut off for cocaine in oral fluid of 8ng/mL

Although volunteers were asked not to consume coca tea 24 hours prior to participating in this study, as with any human volunteer study, this could not be guaranteed. Drinking of coca tea prior to collection of the pre-dose sample may be the cause of positive results prior to drinking. Excluding the positive pre-dose samples which were above the 8 ng/mL cut off did not result in a significant change in mean, median, minimum or maximum concentrations. Concentrations observed when excluding anomalies are still above the recommended SAMHSA cut off of 8 ng/mL for up to 60 minutes post consumption (Table 4.5).

Table 4.5. Summary of the mean, median and range of concentrations (ng/mL) of cocaine analysed in oral fluid specimens collected from human volunteers post consumption of coca tea following the exclusion of positive volunteers pre-drinking above the recommended SAMHSA cut off of 8ng/mL

n = 20	**Concentration (ng/ml)**					
Time (min)	**Mean**	**Median**	**Standard Deviation (SD)**	**Interquartile Range (IQR)**	**Range**	
					Min	**Max**
pre-dose	3	3	2	4	1	7
0	2959	1808	2631	3261	14	8959
15	396	191	480	381	25	1570
30	160	49	224	131	6	768
45	50	25	57	51	5	186
60	24	11	41	20	2	177

4.3.2.2 Benzoylecgonine

Concentrations of benzoylecgonine were detected above the recommended SAMHSA cut off for benzoylecgonine in oral fluid (8ng/mL) for up to 60 minutes post consumption of coca tea (Table 4.6).

Table 4.6. Summary of the mean, median and range of concentrations of benzoylecgonine analysed in oral fluid specimens collected from human volunteers following the consumption of coca tea

n = 25	**Concentration (ng/ml)**					
Time (min)	**Mean**	**Median**	**Standard Deviation (SD)**	**Interquartile Range (IQR)**	**Range**	
					Min	**Max**
pre-dose	8	1	17	1	1	53
0	174	146	113	138	11	452
15	17	7	20	17	3	81
30	12	7	14	10	2	66
45	15	11	17	10	2	87
60	15	10	15	11	3	73

Concentrations of coca tea benzoylecgonine concentrations in oral fluid immediately post consumption of coca tea ranged between 11 ng/mL to 452 ng/mL (mean = 174 ng/mL, median = 146 ng/mL, SD = 113 ng/mL, IQR = 138 ng/mL). Although the tea itself was not analysed during this study, Jenkins et al (1996) reported presence of benzoylecgonine in Peruvian coca tea at concentrations of 440 ng/mL which indicated that immediately post consumption of coca tea, concentrations detected in oral fluid are likely to be reflecting the concentration in the coca tea rather than concentrations from oral fluid itself.

Initially the concentration of benzoylecgonine dropped rapidly within 15 minutes to a concentration of 17 ng/mL (range = 3.0 ng/mL – 81 ng/mL, median = 7.3 ng/mL, SD = 20 ng/mL). Following the initial 15 minutes the concentrations plateaued and no substantial concentrations change was observed over the next 45 minutes (Figure 4.5).

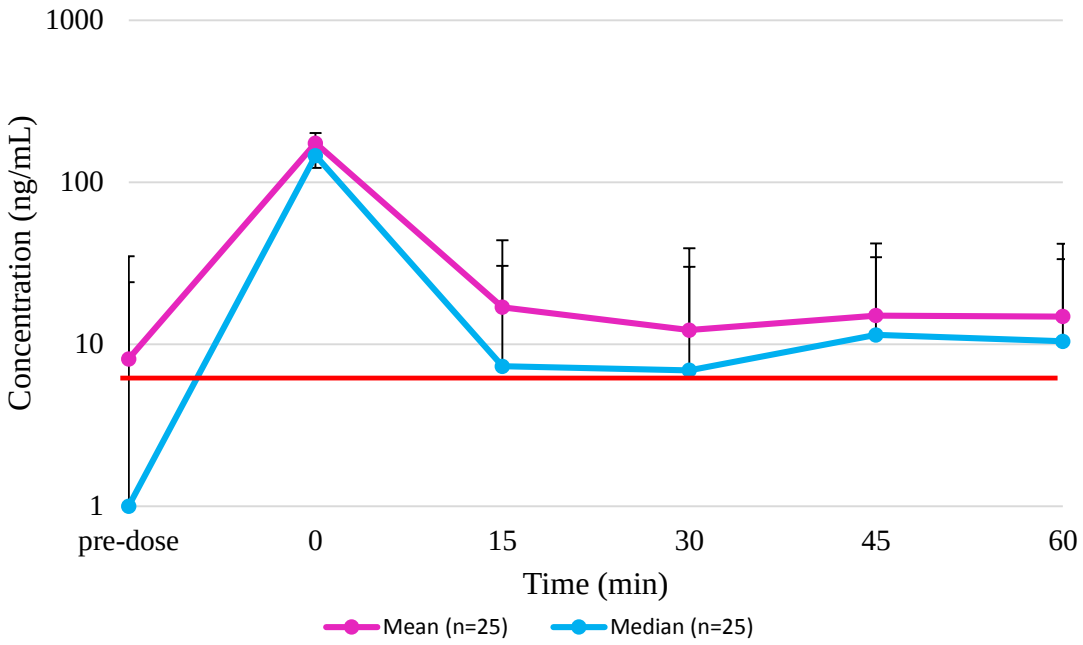


Figure 4.5. Mean and Median concentrations (ng/mL) of benzoylecgonine in oral fluid following the consumption of coca tea in samples collected over 60 minutes. The red line (---) indicates the recommended SAMHSA cut off for benzoylecgonine in oral fluid of 8ng/mL

At 60 minutes post consumption concentrations of benzoylecgonine were still above the recommended SAMHSA cut off of benzoylecgonine in oral fluid (8 ng/mL) ranging between 3 ng/mL and 73 ng/mL (mean = 15 ng/mL, median = 10 ng/mL, SD = 15 ng/mL, IQR = 11 ng/mL).

Similarly to cocaine, positive results above the SAMHSA recommended cut off of 8 ng/mL could be detected in samples from two volunteers collected prior to the consumption of coca tea. Concentrations ranged between 1 ng/mL and 53 ng/mL (mean = 8 ng/mL, median = 1 ng/mL, SD = 17 ng/mL). Only two volunteers were above the recommended SAMHSA cut off of 8ng/mL prior to participation in this study (Figure 4.6).

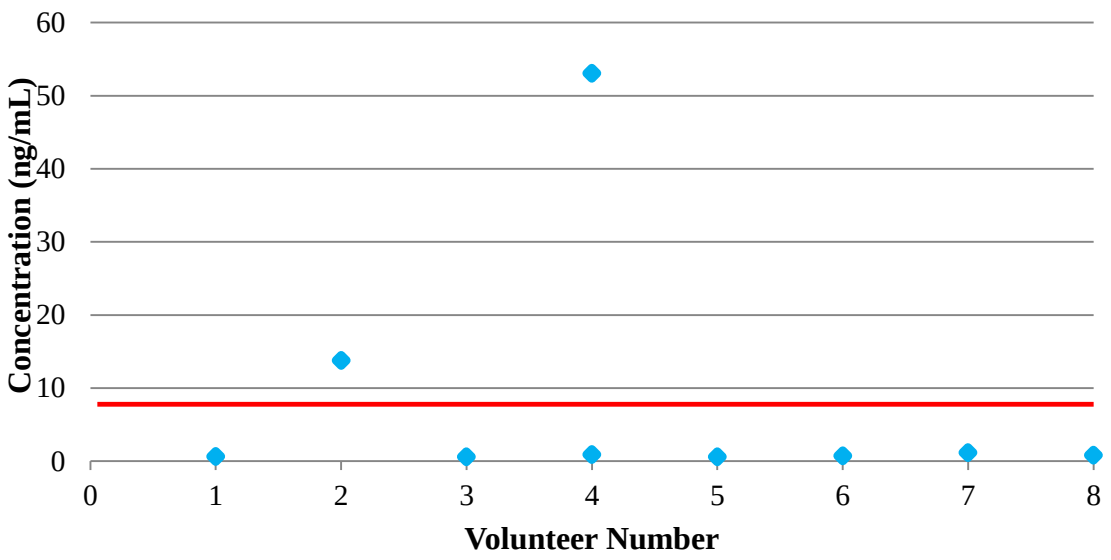


Figure 4.6. Concentrations of benzoylecgonine in oral fluid samples collected prior to consumption of coca tea. Benzoylecgonine was detected in 8 out of 25 samples, however only 2 were positive above the recommended SAMHSA cut off. The red line (---) indicates the recommended SAMHSA cut off for benzoylecgonine in oral fluid of 8ng/mL

In order to identify whether mean and median concentrations were raised artificially by the outliers pre-drinking, the two volunteers were excluded. Concentrations following the exclusion of the two volunteers did not show a significant difference to the initial results and results were still reported above the recommended SAMHSA cut off of 8 ng/mL with a mean concentration of 175 ng/mL (median = 146 ng/mL, range = 11 ng/mL – 452 ng/mL, SD = 115 ng/mL) immediately post consumption. Concentrations of benzoylecgonine in the final samples 60 minutes post consumption ranged between 3 ng/mL and 37 ng/mL (mean = 12 ng/mL, median = 10 ng/mL, SD = 8 ng/mL). The interquartile range 60 minutes post consumption was 10 ng/mL (Table 4.7).

Table 4.7. Summary of the mean, median and range of concentrations (ng/mL) of benzoylecgonine analysed in oral fluid specimens collected from human volunteers post consumption of coca tea following the exclusion of positive volunteers pre-drinking above the recommended SAMHSA cut off of 8 ng/mL

n = 23	**Concentration (ng/ml)**					
Time (min)	**Mean**	**Median**	**Standard Deviation (SD)**	**Interquartile Range (IQR)**	**Range**	
					Min	**Max**
pre-dose	1	1	0	0	1	1
0	175	146	115	117	11	45
15	16	7	20	16	3	81
30	13	7	14	10	2	66
45	12	11	7	7	2	29
60	12	10	8	10	3	37

4.3.2.3 Ecgonine Methyl Ester (EME)

Ecgonine methyl ester was the compound observed with the highest concentration immediately after consumption of the tea with a mean concentration of 3,239 ng/mL (range = 24 ng/mL – 16,685 ng/mL, median = 2,246 ng/mL, SD = 3602 ng/mL, IQR 1,946 ng/mL) (Table 4.8).

Table 4.8. Summary of the mean, median and range of concentrations of ecgonine methyl ester analysed in oral fluid specimens collected from human volunteers following the consumption of coca tea

	Concentration (ng/ml)					
Time (min)	**Mean (n=25)**	**Median (n=25)**	**Standard Deviation (SD)**	**Interquartile Range (IQR)**	**Range (n = 25)**	
					Min	**Max**
pre-dose	24	14	30	30	1	98
0	3,239	2246	3602	1946	25	16,686
15	257	144	280	219	39	1,209
30	141	85	129	115	20	533
45	137	92	143	100	17	675
60	72	56	59	61	2	241

The maximum concentration of ecgonine methyl ester observed in oral fluid samples immediately post consumption is at a four-fold increase above the reported concentration of 4,600 ng/mL found in peruvian tea by Jenkins et al (1996). It is well known that cocaine and benzoylecgonine can both convert to form ecgonine methyl ester hence this may offer an explanation for the elevated concentration of ecgonine methyl ester in oral fluid samples immediately after tea drinking (Chapter 1). Although an acidic buffer was used to slow down the degradation of cocaine to benzoylecgonine and ecgonine methyl ester during transportation, the samples underwent several freeze-thaw cycles prior to analysis, allowing a possible degradation to ecgonine methyl ester and resulting in increased concentrations of ecgonine methyl ester.

Concentrations of ecgonine methyl ester rapidly decreased within the first 15 minutes post consumption of coca tea. Mean concentrations decreased from 3,239 ng/mL (range = 25 ng/mL – 16,686 ng/mL, median = 2,246 ng/mL, SD = 3602 ng/mL) to

257 ng/mL (range = 39 ng/mL – 1,209 ng/mL, median = 144 ng/mL, SD = 280ng/mL). Concentrations of ecgonine methyl ester did not show a substantial decrease and plateaued resulting in concentrations ranging between 2 ng/mL and 250 ng/mL (mean = 72, median = 56 ng/mL, SD = 59 ng/mL) 60 minutes post exposure (Figure 4.7).

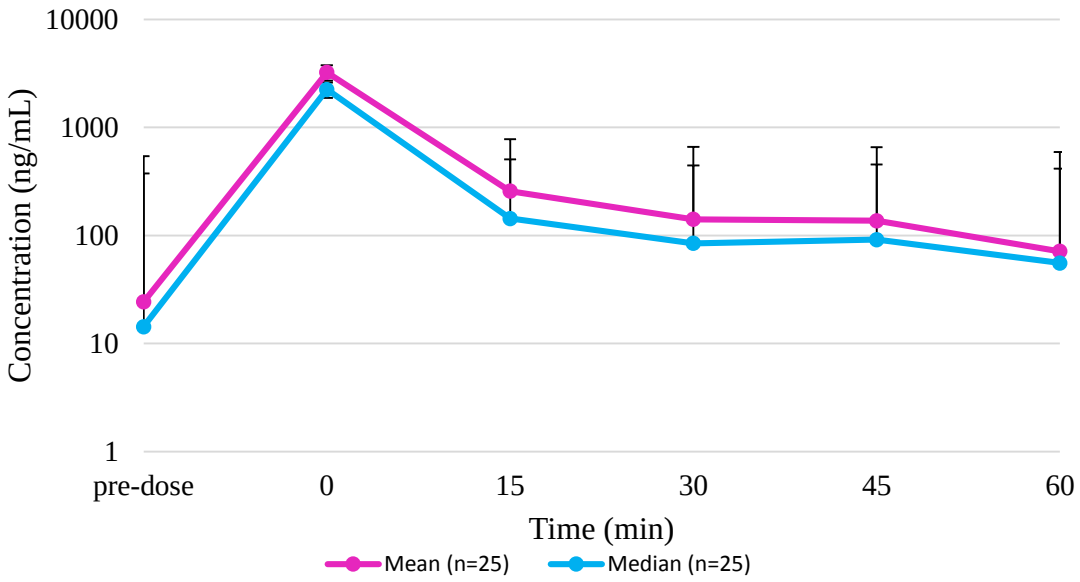


Figure 4.7. Mean and Median concentrations (ng/mL) of ecgonine methyl ester in oral fluid collected over 60 minutes following the consumption of coca tea

There is currently no SAMHSA cut off for ecgonine methyl ester in oral fluid, but the presence of ecgonine methyl ester in oral fluid samples prior to consumption of coca tea followed a similar trend to the pre-dose cocaine and benzoylecgonine samples with five samples showing high positive results in comparison to other volunteers (Figure 4.8).

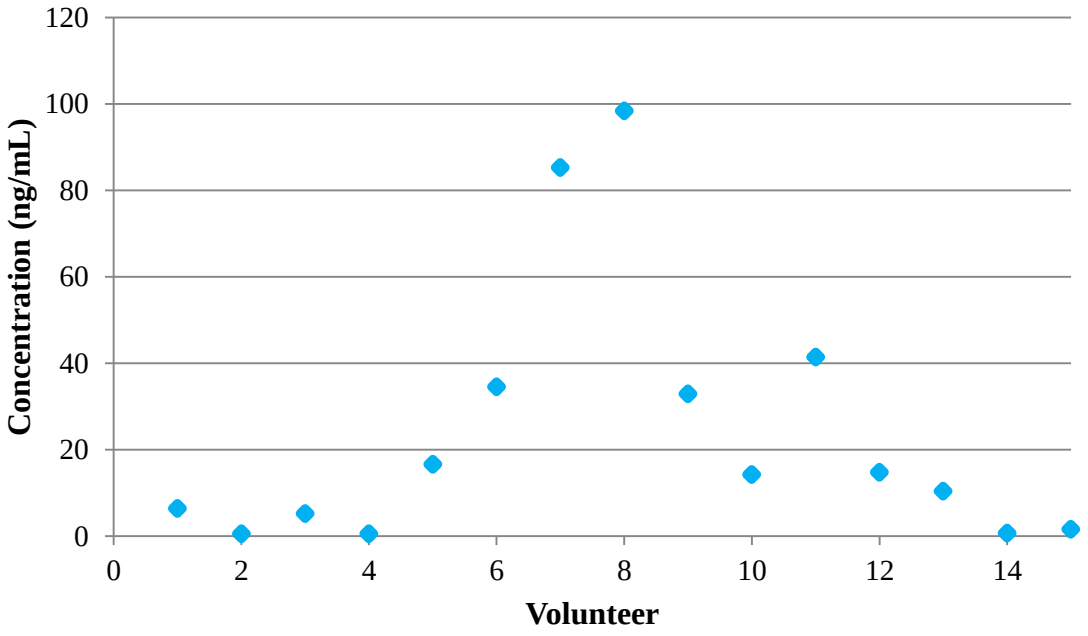


Figure 4.8 Concentrations of ecgonine methyl ester in oral fluid samples collected prior to consumption of coca tea. Ecgonine methyl ester was detected in 15 out of 25 samples. To date no cut off has been recommended by SAMHSA for the detection of ecgonine methyl ester in oral fluid

Although there is no cut off for ecgonine methyl ester in oral fluid to date, when excluding pre-dose concentrations at a hypothetically cut off of 8 ng/mL, no large change in the mean, median, minimum and maximum concentration was observed. Large concentrations were still observed 60 minutes post consumption of coca tea ranging between 16 ng/mL and 241 ng/mL (mean = 77 ng/mL, median = 61 ng/mL, SD = 62 ng/mL) (Table 4.9).

Table 4.9. Summary of the mean, median and range of concentrations (ng/mL) of ecgonine methyl ester analysed in oral fluid specimens collected from human volunteers post consumption of coca tea following the exclusion of positive volunteers pre-drinking above a hypothetical cut off of 8 ng/mL

n = 16	Concentration (ng/ml)					
Time (min)	Mean	Median	Standard Deviation (SD)	Interquartile Range (IQR)	Range	
					Min	Max
pre-dose	3	1	3	4	1	7
0	3,965	2,761	2306	2463	56	16,685
15	318	165	324	302	65	1209
30	161	80	151	179	20	533
45	160	87	169	132	17	675
60	77	61	62	51	16	241

4.3.2.4 Summary of cocaine, benzoylecgonine and ecgonine methyl ester concentrations

Concentrations of cocaine, benzoylecgonine and ecgonine methyl ester all indicate that there is a potential for drugs to accumulate in oral tissues. Concentrations were above the SAMHSA recommended cut off of 8 ng/mL (where applicable) up to one hour post consumption as measured by LC-MS/MS.

In order to be able to collect a specimen from a driver suspected to have been driving whilst impaired through drug use the current United Kingdom Road Traffic Act requires, police offices to demonstrate that an offender is impaired and unfit to drive and that any potential impairment may be attributable to drugs. Drugs can affect different people in different ways and may even affect the same person in different ways on different occasions making it difficult to correlate impairment to a specific concentration range.

Volunteers, who were undertaking their normal work duties during participation in this study, were asked whether any stimulant effects were felt post consumption of coca tea. None of the volunteers described any stimulant effects after drinking just one single cup of coca tea. As regular coca tea drinkers, they may well have developed tolerance to the effects of cocaine and require higher doses of drug to gain an effect. By contrast, many of the published papers describe the effects of cocaine from low dose clinical studies conducted in healthy, non-drug using individuals. The elevated concentrations detected in oral fluid in this study are the first to be observed in a coca tea using population.

Positive results in the blank control sample that was collected pre-drinking, resulted in cocaine, could be explained by drinking of coca tea prior to collection of the pre-dose samples. Although volunteers were asked not to consume coca tea 24 hours prior to participating in this study, as with any human volunteer study, this could not be guaranteed. When volunteers who tested positive for cocaine, benzoylecgonine or ecgonine methyl ester were excluded, no significant change in the mean, median, minimum or maximum concentrations were observed. Concentrations observed were still above the recommended SAMHSA cut off of 8 ng/mL for cocaine and benzoylecgonine for up to 60 minutes post consumption of coca tea.

4.3.3 Unexpected results

In addition to cocaine, benzoylecgonine and ecgonine methyl ester, the LC-MS/MS method used in this work to analyse the oral fluid samples collected in Peru also detects cocaethylene and anhydroecgonine methyl ester.

4.3.3.1 Cocaethylene

Cocaethylene forms in the human body following the co-consumption of cocaine and ethanol was detected in a number of oral fluid samples. All volunteers stated that they had not consumed alcohol for 24 hours prior to participating in this study. All pre-dose samples were confirmed to be negative for cocaethylene (Table 4.10).

Table 4.10. Summary of randomly distributed positive results for Cocaethylene (CE) in oral fluid following the consumption of coca (V = Volunteer, ND = not detected)

	Cocaethylene concentration (ng/ml)					
Time (min)	V 1	V 2	V 3	V 4	V 5	V 6
Pre-dose	*ND*	*ND*	*ND*	ND	*ND*	*ND*
0	*ND*	*ND*	*ND*	3	*ND*	*ND*
15	14	*ND*	17	*ND*	*ND*	*ND*
30	2	*ND*	*ND*	*ND*	*ND*	10
45	*ND*	29	86	*ND*	2	*ND*
60	*ND*	61	*ND*	*ND*	*ND*	*ND*

Concentrations of cocaethylene detected ranged between 2 ng/mL to 86 ng/mL and the presence of cocaethylene was randomly distributed amongst samples analysed. The random distribution of cocaethylene positives mediated against the proposition that its presence could be explained by alcohol consumption prior to participating in the experiment. Additionally, participants were monitored throughout the study and therefore potential alcohol consumption during the collection can be excluded as a cause for unexpected presence of cocaethylene.

4.3.3.2 Anhydroecgonine Methyl Ester

Anhydroecgonine methyl ester is a pyrolysis product formed during the smoking of crack cocaine and is commonly used as a biomarker for the use of crack cocaine.

Anhydroecgonine methyl ester results were randomly distributed in a similar manner to cocaethylene indicating that the concentrations did not originate from smoking crack cocaine (Table 4.11).

Table 4.11. Summary anhydroecgonine methyl ester concentrations detected in oral fluid following consumption of coca tea showing the random distribution of this compound (V=Volunteer, ND = not detected)

	Anhydroecgonine methyl ester concentration (ng/ml)								
Time (min)	V 1	V 2	V 3	V 4	V 5	V 6	V 7	V 8	V 9
Pre-dose	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*
0	257	*ND*	*ND*	322	215	*ND*	*ND*	118	*ND*
15	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	32.7	*ND*	*ND*
30	1	3	1	4	*ND*	1	40	5	20
45	*ND*	*ND*	*ND*	*ND*	5	*ND*	*ND*	*ND*	*ND*
60	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	2

Nine out of the twenty volunteers who participated in this study tested positive for anhydroecgonine methyl ester in at least one sample during the one hour collection period. Concentrations of anhydroecgonine methyl ester ranged between 1 ng/mL and 322 ng/mL with highest concentrations observed in samples collected immediately post consumption of coca tea.

Anhydroecgonine methyl ester has been reported to be present in some species of the *Erythroxylum coca* (Jenkins et al 1996). The hypothesis that anhydroecgonine methyl ester positives results from the presence of anhydroecgonine methyl ester in the leaf, can be dismissed due to the random manner at which the positives appear. In the event of a volunteer consuming coca tea containing anhydroecgonine methyl ester, positives for anhydroecgonine methyl ester should have been observed in all samples throughout the timed collection.

All samples were subsequently reanalysed for cocaethylene and anhydroecgonine methyl ester one year post collection of the sample in order to assist with the explanation for the presence of these two compounds in such a random manner. The reanalysis of samples was intended to assist with the explanation for the presence of anhydroecgonine methyl ester and investigate the possibility that anhydroecgonine methyl ester concentrations were formed as the result of an instability of compounds present within the samples.

Samples were analysed using the same method as in the initial analysis one year earlier (section 4.2.2.4, section 4.2.2.5).

4.3.3.3 Reanalysis of samples containing cocaethylene and anhydroecgonine methyl ester

4.3.3.3.1 Cocaethylene

During the second analysis with the same method, cocaethylene was not detected in any of the oral fluid samples collected following the consumption of coca tea. One explanation for the disappearance of cocaethylene positives, could be a possible instability of cocaethylene in oral fluid. Only limited information is available in the literature on the stability of cocaethylene in oral fluid, Clauwaert et al (2004) stated

that cocaethylene is stable in oral fluid at -20 ºC for a minimum of six months. Concentrations of cocaethylene in sweat, a compositionally similar matrix to saliva due to its high water content, has been reported to decrease by 17 % after two weeks at a temperature of 4 ºC (Follador et al 2004). Cocaethylene is an ester and therefore de-esterfication of cocaine to from cocaethylene can occur. Oral fluid contains esterases such as cholinesterases, pseudocholinesterase and cholesterol esterases (Finer and Santerre 2004). Therefore, a possible explanation is the de-esterification by salivary esterases of cocaine to from cocaethylene.

4.3.3.3.2 Anhydroecgonine methyl ester

An increased number of samples tested positive for anhydroecgonine methyl ester during the second analysis (Table 4.12). Samples positive during the initial analysis mostly decreased in concentration with some to the positives disappearing completely. An increased number of samples which had previously been confirmed to be negative, resulted in positive anhydroecgonine methyl ester concentration during the second LC-MS analysis one year post initial analysis.

Table 4.12. Concentrations (ng/mL) of anhydroecgonine methyl ester in oral fluid samples following consumption of coca tea. Samples were analysed initially in 2011 and then reanalysed one year later (2012) to investigate the presence of anhydroecgonine methyl ester

Time (min)	Volunteer ID	Analysis 2011	Analysis 2012
0	1031	ND	8.7
	1032	ND	98.4
	28021	256.8	59.4
	280210	ND	27.9
	280211	ND	25.2
	280212	ND	53.1
	280213	ND	53.1
	280214	ND	40.2
	280215	ND	60
	280216	ND	32.1
	280217	ND	58.2
	280218	ND	22.5
	28022	10.8	ND
	28022	ND	218.1
	28022	ND	35.1
	28022	ND	79.8
	28023	ND	66.9
	28023	ND	118.5
	28024	ND	21.6
	28025	ND	4.2
	28026	321.6	54
	28027	ND	14.7
	28028	ND	22.5
	28029	214.65	114.3
15	1032	ND	9
	28021	ND	5.1
	280211	ND	19.2
	280212	ND	12.9
	280213	ND	4.2
	280215	32.7	34.8
	280216	ND	3.6
	28022	ND	2.1
	28023	ND	51
	28029	ND	9.3
30	28021	1.23	ND
	280210	0.96	ND
	280215	40.2	17.4
	28023	ND	6.3
	28023	5.4	ND
	28024	2.52	ND
	28025	1.26	ND
	28026	4.38	ND
	28029	ND	8.1
45	280212	2.97	ND
	280215	ND	6.3
	28029	5.25	ND
60	28023	1.77	ND

Samples collected immediately post consumption of one cup of coca tea showed the largest difference in positive results between the first and second analysis of oral fluid samples. The amount of positives increased significantly between the initial analysis in 2011 and second analysis in 2012 (Figure 4.9). Concentrations immediately post consumption ranged from 4.2 ng/mL to 218 ng/mL (mean = 52.5 ng/mL, median= 37.64 ng/mL).

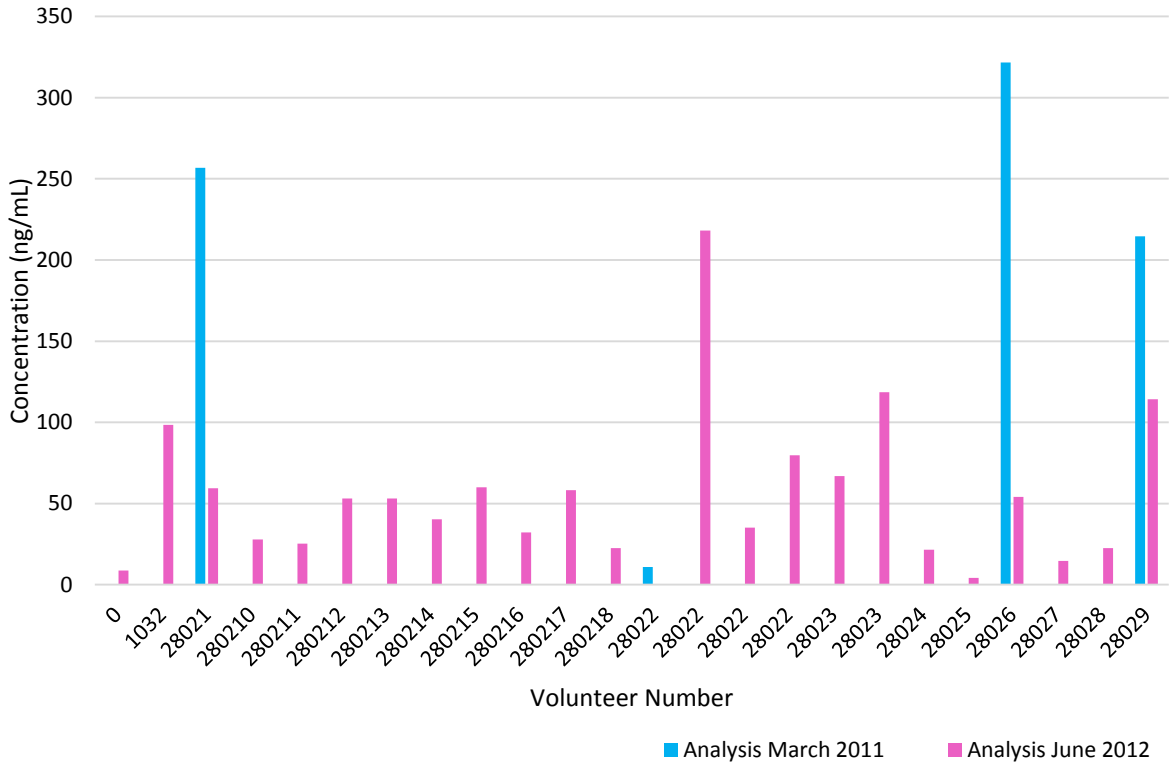


Figure 4.9 Chart showing the presence of anhydroecgonine methyl ester in oral fluid samples immediately post consumption of coca tea in the initial analysis in 2011 (t=0) and one year later (2012)

In comparison to the initial analysis where only one positive for anhydroecgonine methyl ester was observed for the 15 minute time point, the reanalysis resulted in ten anhydroecgonine methyl ester positives. Concentrations ranged from 2.1 ng/mL to 51 ng/mL (mean = 14.1 ng/mL, median = 9 ng/mL) (Figure 4.10).

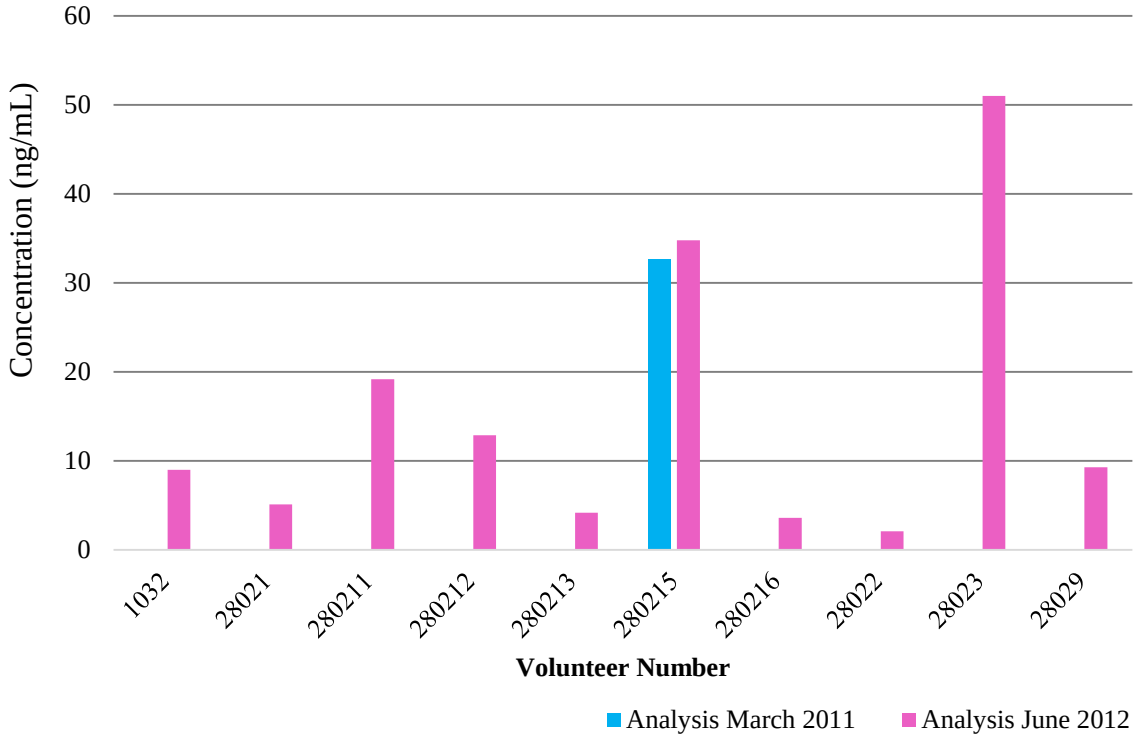


Figure 4.10. Comparison of the presence of anhydroecgonine methyl ester in oral fluid samples 15 minutes post consumption of coca tea in the initial analysis (2011) and one year later (2012)

The number of samples containing anhydroecgonine methyl ester 30 minutes post consumption also increased with a mean anhydroecgonine methyl ester concentration of 52.3 ng/mL (median = 42.15, range = 6.3 ng/mL to 118.5 ng/mL) (Figure 4.11).

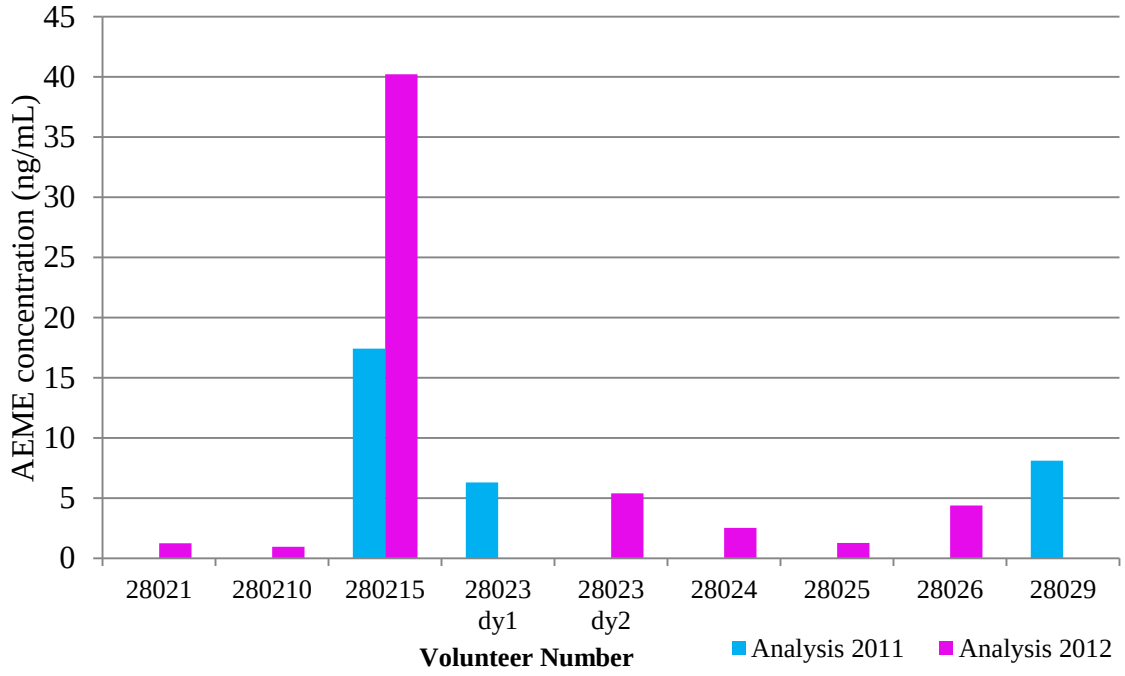


Figure 4.11. Comparison of the presence of anhydroecgonine methyl ester in oral fluid samples 30 minutes post consumption of coca tea in the initial analysis (2011) and one year later (2012)

At 45 minutes post exposure the two previously confirmed samples for anhydroecgonine methyl ester, tested negative for the anhydroecgonine methyl ester, whereas one volunteer showed an increase in concentration during the second analysis (Figure 4.12).

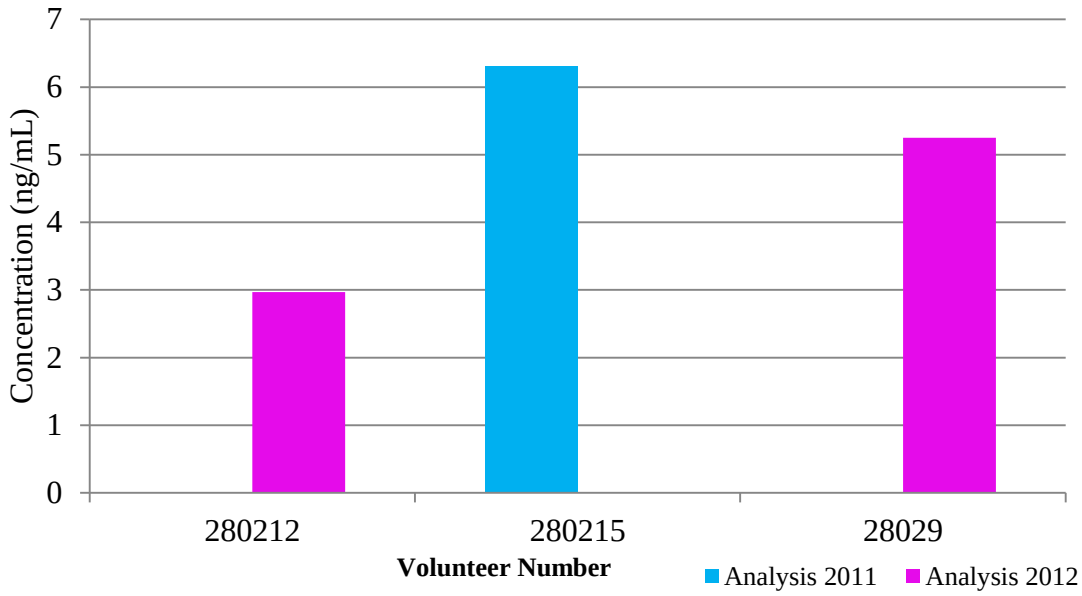


Figure 4.12. Comparison of the presence of anhydroecgonine methyl ester in oral fluid samples 45 minutes post consumption of coca tea in the initial analysis (2011) and one year later (2012)

Only one volunteer had tested positive for anhydroecgonine methyl ester in the first year at t = 60 min, however during the second analysis, no anhydroecgonine methyl ester positives were observed in the samples collected one our post consumption.

4.3.3.3.3 Summary of the results from the secondary analysis of samples to quantify anhydroecgonine methyl ester

The repeat analysis one year post the initial analysis resulted in an increased number of samples testing positive for anhydroecgonine methyl ester. This indicates that anhydroecgonine methyl ester was formed within the sample during the one year of storage. The stability of anhydroecgonine methyl ester in oral fluid has not been documented in the literature, however Fandino et al (2002) reported on stability of anhydroecgonine methyl ester in plasma. During his work, anhydroecgonine methyl ester had fallen by 50 % to anhydroecgonine within 5 days at room temperature and within 13 days at 4 °C.

In comparison to the study by Fandino et al (2002), the anhydroecgonine methyl ester positive results in the work described in this chapter showed an increase in the number of samples that tested positive, which suggests the increased amount of positive samples is due to a formation of anhydroecgonine methyl ester in oral fluid samples following the consumption of coca tea. Although anhydroecgonine methyl ester has been reported to form through thermal degradation of cocaine as a pyrolysis product of smoking crack cocaine, formation due to storage conditions or instability have not previously been reported in the literature (Jacob et al 1990, Kintz et al 1995, Kintz et al 1997, Toennes 1999).

When considering the structure of cocaine a carbon-oxygen bond can be observed. Thermal decomposition has been suggested to cause the scission of carbon-oxygen bonds (Chan et al 1976). This can be applied to the formation of anhydroecgonine methyl esters and ecgonine methyl ester. The decomposition of the carbon-oxygen as a result of the heating process during the preparation of the coca tea (highlighted at A

in Figure 4.13) would result in the formation of anhydroecgonine methyl ester from cocaine. Similarly, the scission the oxygen-carbon bond as highlighted at B in Figure 4.13 would result in the formation of ecgonine methyl ester which could provide an explanation why the ecgonine methyl ester concentrations within in the oral fluid were significantly higher than those within the coca tea which was prepared from the teabag (section 4.3.2.3).

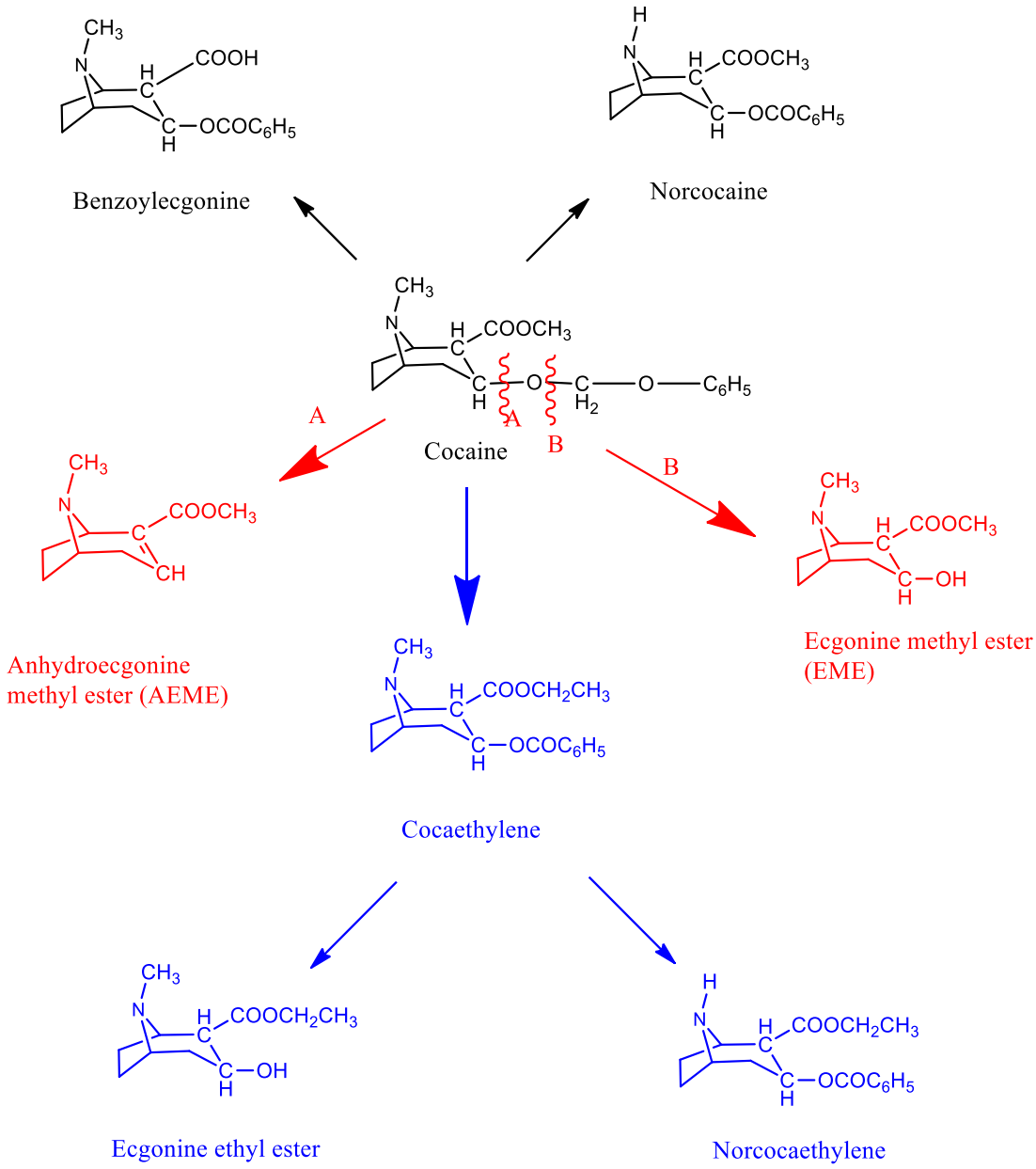


Figure 4.13 Metabolic pathway of cocaine highlighting the potential thermal decomposition of cocaine during the preparation of coca tea resulting in the formation of anhydroecgonine methyl ester (A) and ecgonine methyl ester (B)

A major drawback of this study was that it was not possible to analyses the coca leaves and tea itself due to importation restrictions of coca leaves and coca tea into the UK. Jenkins et al (1996) reported that 1 g of plant material contained 5.11 mg of cocaine, 0.11 mg of benzoylecgonine and 1.15 mg of ecgonine methyl ester which resulted in a cocaine dose of 4.14 mg per cup leading to concentration of 16.56 µg/mL cocaine, 0.44 µg/mL benzoylecgonine and 4.6 µg/mL ecgonine methyl ester present in the tea.

Following the anomalous results regarding cocaethylene and anhydroecgonine methyl ester, coca tea was prepared with tea bag which contained ground coca leaves and green tea. The Andes Spirit tea bag contained 1 g of *Erythroxlum coca* and green tea. There was no quantity provided on the amount of coca leaves present within the tea bag. The tea was prepared by adding a tea bag to 250 mL of boiling water and following soaking of 5 minutes, five samples were collected from the tea using the Alere^{TM} Certus collector. Samples were analysed using the LC-MS method described in section 4.2.2.5.

Results showed that cocaine detected in the five samples taken from the coca tea ranged between 16,404 ng/mL and 17,317 ng/mL (mean = 16,828 ng/mL). The mean benzoylecgonine concentration detected was 5,888 ng/mL (range = 5,539 ng/mL – 6211 ng/mL) (Table 4.13).

Table 4.13. Summary of results from five samples collected from coca tea prepared from a ready-to-use teabag that contained ground coca tea leaves as well as green tea powder.

	Concentration (ng/mL)				
	COC	**BZE**	**EME**	**AEME**	**CE**
Coca Tea 1	16404	6075	5947	54	*ND*
Coca Tea 2	16436	5599	6050	51	*ND*
Coca Tea 3	16745	5539	6476	66	*ND*
Coca Tea 4	17241	6015	6202	58	*ND*
Coca Tea 5	17317	6212	6005	54	*ND*
Mean	16829	5888	6136	57	*ND*

No cocaethylene was detected supporting the results from the second analysis of the oral fluid samples collected following consumption of one cup of coca tea. The analysis of the coca tea showed that anhydroecgonine methyl ester was present in all 5 samples collected. Anhydroecgonine methyl ester ranged between 51 ng/mL and 66 ng/mL (mean = 57 ng/mL). However the analysis had a number of limitations. The quantity of coca leaf within the tea bag was unknown and therefore it is difficult to compare the concentrations of cocaine and its metabolites within the tea from the tea bag with those observed in oral fluid following the consumption of coca tea. Additionally it is unknown whether the species of coca leaf was the same in both instances. Some species of the *Erythroxylum* coca plant have been reported to contain anhydroecgonine methyl ester within the leaf which could explain the presence of anhydroecgonine methyl ester within the analysed tea. Alternatively, the presence of anhydroecgonine methyl ester in the coca tea suggests that anhydroecgonine methyl ester is formed during the production of the coca tea, by the thermal degradation of cocaine during the boiling process The analysis of the tea

does not provide an explanation as to why anhydroecgonine methyl ester was detected at random time points in oral fluid throughout the initial analysis. However it does confirm that anhydroecgonine methyl ester is present within the tea which would subsequently be detected in oral fluid.

4.4 Conclusion

This experiment demonstrated that the consumption of coca tea can result in elevated concentrations of cocaine and its metabolites in oral fluid. High concentrations were detected as expected immediately after consumption of coca tea. However, the detection of positive concentrations of cocaine and its metabolites up to one hour post consumption of the tea supports the proposition that drug depots can be formed within mouth tissues.

Additionally, cocaethylene and anhydroecgonine methyl ester were detected and quantified. The appearance of cocaethylene and anhydroecgonine methyl ester in oral fluid samples at random time points has not yet been fully explained and offers scope for further investigation. Differences in concentrations between the first and second analysis could be explained by instability of cocaethylene and anhydroecgonine methyl ester in oral fluid and further research is required to investigate this further.

Chapter 5.0 - Oral fluid opiate concentrations following oral consumption of Codeine Linctus® and Collis Browne's® mixture

5.1 Introduction

The work described in Chapter 4 showed that elevated drug concentrations can be observed following the consumption of Peruvian coca tea with cocaine, benzoylecgonine and ecgonine methyl ester. Positive concentrations were still detected one hour post consumption of coca tea which strongly supported the hypothesis that drug depots can be formed within tissues when drugs are consumed orally i.e. via an oral solution.

Looking at different routes of administration, Jenkins et al (1995) further supported this proposition. Jenkins et al (1995) reported elevated oral fluid concentrations following heroin smoking with concentrations of 3.53µg/mL and 20.58 µg/mL between 2 – 5 minutes post smoking of 2.6 mg and 5.2 mg respectively. Measurable concentrations were reported for up to 24 hours post the smoking of heroin. In comparison, concentrations in oral fluid 2 – 5 minutes post intravenous injection were much lower from 6 ng/mL (from a 10 mg dose) to 22 ng/mL (5 mg dose) and were only detected in blood for 30 minutes (Jenkins et al 1995). 6-MAM is rarely detected in oral fluid following injection as it has short half-life and, when reported, has only been detected at very low concentrations; 18 ng/mL (dose = 5 mg dose) or 40 ng/mL (dose = 10 mg). In contrast, when heroin was smoked, large concentrations of 6-MAM were observed in oral fluid with concentrations up to 1.12 µg/mL and 3.58 µg/mL (Jenkins et al 1995). Additionally, Osselton et al 2001 showed that concentrations of opiates, with or without the combination of a second opiate, ranged

from 0.023 μg/mL to 17.375 μg/mL for morphine, 0.003 μg/mL to 7.75 μg/mL for codeine, 0.002 μg/mL to 66.93 μg/mL for 6-monoacetylmorphine and 0.001 μg/mL to 274.27 μg/mL for dihydrocodeine A potential explanation for the elevated drug concentrations in oral fluid is that drug accumulate to form depots in mouth tissues. The elevated concentrations of opiates in oral fluid following the smoking of heroin in comparison to lower concentration following injection of heroin suggest that deposition of drug in the mouth tissues can significantly contribute to opiate concentration measure in oral fluid.

The above studies show that depending upon the mode of exposure, drugs coming into contact with mouth tissue can cause contamination of the oral cavity resulting in the measurement of elevated concentrations in oral fluid leading to easier detection using point of care or laboratory tests. However, it can also lead to difficulty in interpretation of S/P ratios in drug pharmacology. The purpose of the work described in this chapter was to examine drug deposition in oral fluid and potentially the surface of the mouth cavity by investigating concentrations of opiates following oral consumption of Codeine Linctus® and Collis Browne's® ® mixture.

The elevated drug concentrations in oral fluid over a prolonged period suggest that drugs such as cocaine and opioids can bind to oral tissue. Collected oral fluid can contain cell and food debris which can be collected by the abrasion of the collector against the oral tissues. The presence of drug depots in tissues suggests that the cell debris present in oral fluid also contains drugs, which can potentially contribute to and hence increase oral fluid concentrations.

The work undertaken in this chapter aimed to investigate characteristics of deposition in the oral fluid using observed concentrations such as the length of time drug can be detected in oral fluid and the effect of cell abrasion during collection.

5.2 Materials and Methods

5.2.1 Materials

5.2.1.1 Collection devices

Alere™ Certus devices/kits were donated by Alere Toxicology, Abingdon UK.

Alere™ Microplate EIA Kits for opiates were provided by Alere Toxicology, Abingdon, UK.

5.2.1.2 Collis Browne's® mixture

Collis Browne's® mixture, manufactured by Thornton & Ross Ltd, Huddersfield, UK contains two principal active ingredients, morphine hydrochloride at a dose equivalent to 1.0 mg/5 mL anhydrous morphine and 1.5 µL/5mL of peppermint oil. Collis Browne's® mixture also contains ethanol, benzoic acid, sorbitol sucrose and fructose. Collis Browne's® mixture is available for purchase as an over-the-counter medication.

5.2.1.3 Codeine Linctus®

Codeine Linctus®, Care+, Thornton & Ross Ltd, Huddersfield, UK, contains only one active drug ingredient, Codeine phosphate at a dose of 15 mg/5 mL. Other ingredients include citric acid monohydrate, ethyl and propyl parahydroxybenyzoates, sodium methyl, ethanol, sugars, food colourings and flavouring. Codeine Linctus® is available for purchase as an over-the-counter medication.

5.2.1.4 Methodology

Informed consent was obtained from all participants prior to participation and the experimental procedures were undertaken in compliance with the University of Bournemouth ethical guidelines (Appendix D). All volunteers completed a questionnaire regarding prior use of medication which may interfere with the results. Volunteers provided a blank control oral fluid sample immediately prior to participating in this study for confirmation that medication – opioid based drug was consumed prior to commencing of the study.

5.2.1.5 Collection Procedure

5.2.1.5.1 Study 1 – Investigation into opiate oral fluid concentrations following the exposure to Collis Browne's® mixture and Codeine Linctus®

Volunteers were invited to swirl 5 mL of Collis Browne's® or Codeine Linctus® around the mouth for one minute before discarding the liquid from the mouth. The calculated dose for Codeine Linctus® was 15 mg and 5 mg for Collis Browne's® mixture. Oral fluid was subsequently collected at 15, 30, 45, 60, 90, 120, 150 and 180 minutes after mouth emptying using the Alere™ Certus oral fluid collection device. Oral fluid was collected in accordance with the kit manufacturer's instructions.

5.2.1.5.2 Study 2- Investigation into opiate oral fluid concentrations following the exposure to Collis Browne's® mixture and Codeine Linctus® with two different collection methods

Volunteers were invited to swirl 5 mL of Collis Browne's® or Codeine Linctus® around the mouth for one minute before discarding the liquid from the mouth. The calculated dose for Codeine Linctus® was 15 mg and 5 mg for Collis Browne's®

mixture. Two oral fluid samples were collected following exposure. One oral fluid sample was collected following the collection procedure in 5.2.1.5.1 at 15, 30, 45, 60, 90, 120, 150 and 180 minutes after mouth emptying. In addition to the collection according to the manufacturer's guidelines, a second Alere™ Certus collector was brushed against the oral cavity to identify whether the increased presence of cellular debris can increase the amount of drug detected in oral fluid. Brushing was undertaken until the adequacy indicator showed that sufficient oral fluid had been collected.

5.2.1.6 Immunoassay (Study 2)

The Alere™ Microplate EIA Kit is provided with five calibrators of 0 ng/mL, 10 ng/mL, 100 ng/mL and 500 ng/mL. Samples which were detected above the highest calibrator of 500 ng/mL calibrator following analysis were diluted 1 in 10 and reanalysed. The manufacturers report cross reactivity of the assay with several compounds that are summarised in Table 5.1. Non cross reacting compounds are presented in Appendix B.

Table 5.1. Summary of compounds cross-reacting with the Alere™ Microplate EIA Kit for opiates

Compounds Cross Reactants	ng/mL	Apparent Morphine	Reactivity (%)	Compounds Cross Reactants	ng/mL	Apparent Morphine	Reactivity (%)
6-Acetylmorphine	100,000	442.86	0.4	**Meperidine**	100,000	74.33	0.07
	1,000	381.32	38.1		10,000	8.17	0.08
	500	96.1	18.2		1,000	<5	-
	100	81.16	81.2	**Morphine - 3 - Glucuronide**	100,000	403.3	0.4
	10	7.1	71		1,000	8.95	0.9
Codeine	100	398.9	398.9		500	5.1	1
	10	95.37	953.7		100	<5	-
	5	80.97	1619.4	**Nalorphine**	100,000	95.89	0.1
Dextro-methorphan	100,000	31.26	0.03		1,000	38.63	0.4
	10,000	<5	-		500	<5	-
Dihydro-codeine	500	>500	-		100		
	100	196.7	196.7	**Norcodeine**	100,000	>500	-
	10	73.43	734.3		1,000	37.26	3.7
	5	39.49	789.8		500	20.02	4
Heroin	500	98.29	19.7		100	<5	-
	100	58.17	58.2	**Normorphine**	100,000	363.74	0.4
	10	<5	-		10,000	62.8	0.6
Hydro- codone	500	82.17	16.4		1,000	<5	-
	100	82.69	82.7	**Oxycodone**	100,000	429.67	0.4
	10	9.38	93.8		1,000	8.51	0.9
	5	7.23	144.6		500	<5	-
Hydro-morphone	100,000	>500	-	**Oxymorphone**	100,000	161.51	0.2
	1,000	245.05	24.5		10,000	32.8	0.3
	500	78.56	15.7		1,000	<5	-
	100	50.29	50.3				
	10	<5	-				

The immunoassay method used for the detection of opiates in oral fluid is summarised in Figure 5.1.

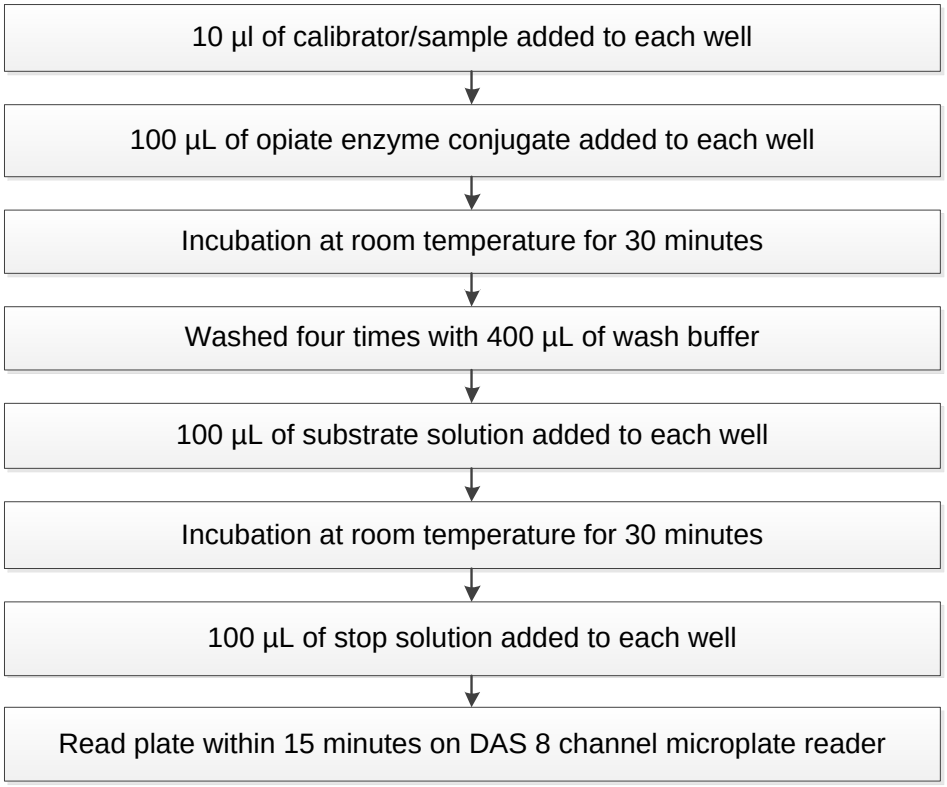


Figure 5.1. Outline of the procedure for using the Alere™ immunoassay method for opiates

5.3 Results and Discussion

5.3.1 Study 1 - Investigation into concentrations of opiates in oral fluid following the exposure to Collis Browne's® and Codeine Linctus®

5.3.1.1 Collis Browne's® mixture

Following exposure to Collis Browne's® mixture highest opiate concentrations (ng/mL) were observed in the first timed collection after exposure, 15 minutes post exposure, in all volunteers. Concentrations then decreased steadily over the collection time of 180 minutes (Figure 5.2).

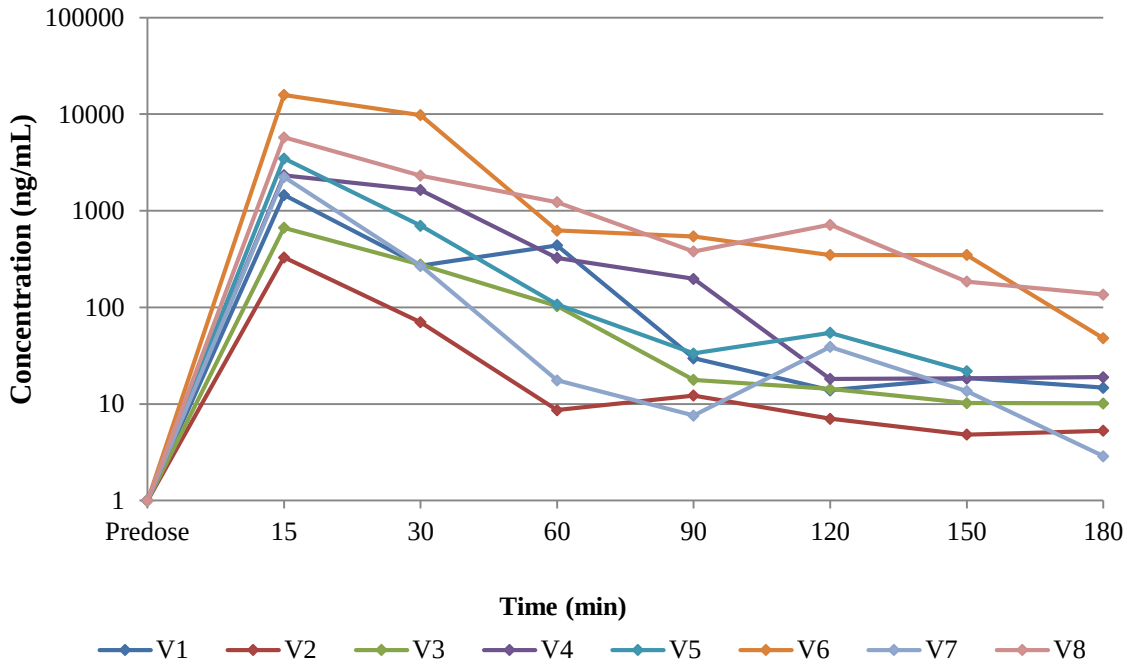


Figure 5.2 Opiate concentrations (ng/mL) in oral fluid following consumption of Collis Browne's® in eight volunteers

All pre-dose samples were negative. In the initial sample, 15 minutes following exposure, opiate concentrations ranged between 330 ng/mL and 15,808 ng/ml (mean = 4,008 ng/mL, median = 2,284 ng/mL, SD = 5067 ng/mL). Opiate concentrations rapidly decreased to an average of 1,916 ng/mL at t = 30 min (range = 70 ng/mL – 9798 ng/mL, median = 489 ng/mL, SD = 3277 ng/mL) and 357 ng/mL at t = 60 minutes (range = 9 ng/mL to 1230 ng/mL, median = 216 ng/mL, SD = 415 ng/mL) (Table 5.2).

Table 5.2 Summary of opiate concentrations (ng/mL) including mean, median and range in oral fluid in eight volunteers following a 5 mg dose of Collis Browne's® mixture

	Concentration (ng/mL)												
Time (min)	**V1**	**V2**	**V3**	**V4**	**V5**	**V6**	**V7**	**V8**	**Mean**	**Median**	**SD**	**Min**	**Max**
Predose	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
15	1456	330	666	2332	3484	15808	2236	5750	4008	2284	5067	330	15808
30	271.4	70	277	1644	700	9786	270	2310	1916	489	3277	70	9786
60	438	9	103	326	107	624	18	1230	357	216	415	9	1230
90	30	12	18	197	33	542	8	379	152	32	205	8	542
120	40	7	14	18	54	348	39	718	152	29	254	7	718
150	19	5	10	18	22	348	14	185	78	18	125	5	348
180	15	5	10	19		48	3	136	34	15	48	3	136

The opiate concentrations decreased even further at t= 90 ranging between 9 ng/mL and 542 ng/mL (mean = 152 ng/mL, median = 29 ng/mL, SD = 205 ng/mL). Following the last rapid decline, concentrations plateaued averaging 152 ng/mL (range = 7 ng/mL – 718 ng/mL, median = 29 ng/mL, SD = 254 ng/mL) at t = 120 min, 78 ng/mL (range = 5 ng/mL – 348 ng/mL, median = 18 ng/mL, SD = 125 ng/mL) at t = 150 min and 34 ng/mL (range = 3 ng/mL – 136 ng/mL, median = 15 ng/mL, SD = 48 ng/mL) at t = 180 min.

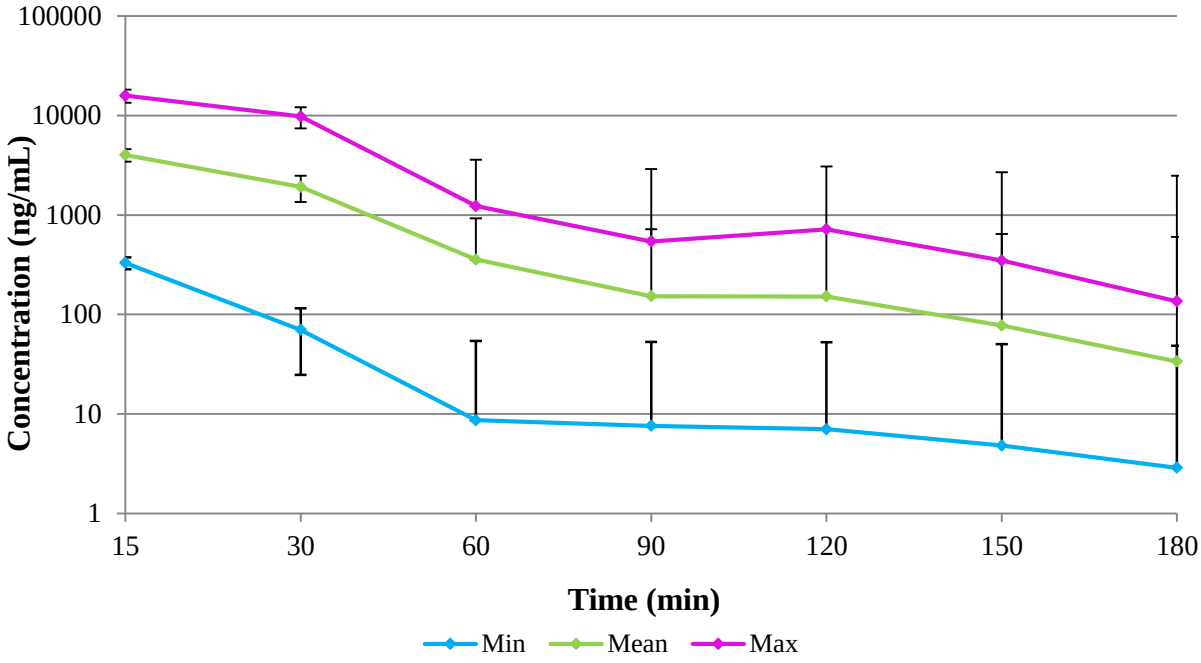


Figure 5.3. Minimum, Maximum and Mean concentrations (ng/mL) of opiates in oral fluid collected over 180 minutes following the consumption of Collis Browne's® (dose = 5 mg) in eight volunteers

5.3.1.2 Codeine Linctus®

The exposure to Codeine Linctus® resulted in highest opiate concentrations in oral fluid 15 minutes post exposure in all volunteers. Opiate concentrations then decreased steadily over 180 minutes (Table 5.4).

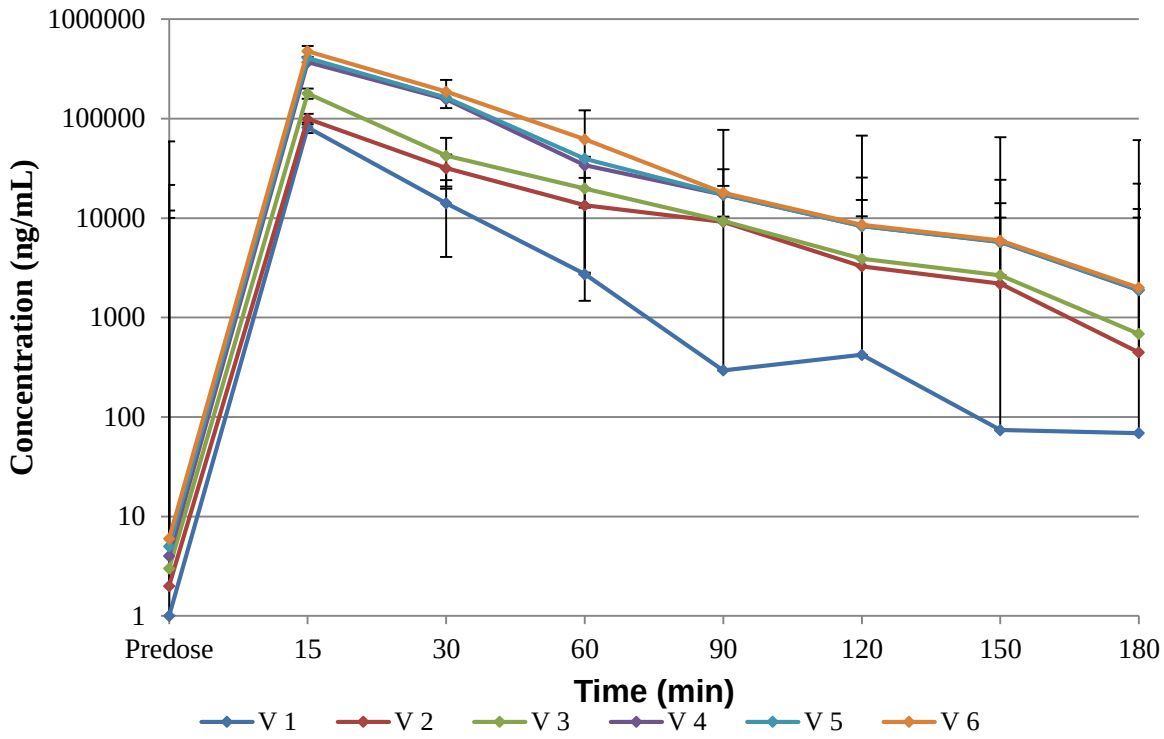


Figure 5.4. Oral fluid Concentrations (ng/mL) of opiates in six volunteers (V1 – V6) following a dose of 15 mg Codeine Linctus®

Samples of oral fluid collected prior to commencement of Codeine Linctus® swirling were all negative. Highest concentrations were observed at t = 15 min and t = 30 min with an average of 79,666 ng/mL (range = 17,876 ng/mL – 190,400 ng/mL, median = 74,630 ng/mL, SD = 59,776 ng/mL) and 31,161 ng/mL (range = 6104 ng/mL – 114,456 ng/mL, median = 15,863 ng/mL, SD = 41,256 ng/mL) respectively (Table 5.3).

Table 5.3. Summary of opiate concentrations (ng/mL) including mean, median and range in oral fluid in six volunteers following a 15 mg dose of Codeine Linctus® mixture

	Concentration (ng/mL)										
Time (min)	**V 1**	**V 2**	**V 3**	**V 4**	**V 5**	**V 6**	**Mean**	**Median**	**SD**	**Min**	**Max**
Predose	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
15	81720	17876	79920	190400	38740	69340	79666	74630	59776	17876	190400
30	14118	17608	10704	114456	6104	23976	31161	15863	41256	6104	114456
60	2734	10702	6412	14088	5588	22352	10313	8557	7132	2734	22352
90	295	8874	195	7836	167	669	3006	482	4160	167	8874
120	422	2862	620	4394	58	230	1431	521	1779	58	4394
150	74	2112	481	3062	46	183	993	332	1280	46	3062
180	69	378	239	1198	22	88	332	164	444	22	1198

One hour post consumption concentrations of opiates decreased to range between 2,734 ng/mL and 22,352 ng/mL (mean = 10,313 ng/mL, median = 8,557 ng/mL, SD = 7,132 ng/mL). Over the course of the next hours concentrations further decreased, averaging at 3,006 ng/mL (range = 167 ng/mL – 8,874 ng/mL, median = 482 ng/mL, SD = 4,160 ng/mL) resulting in a mean concentration of 1,431 ng/mL (range = 58 ng/mL – 4,394 ng/mL, median = 521 ng/mL, SD = 1,779 ng/mL). In the final hour of collection, samples ranged from 49 ng/mL to 3,062 ng/mL (mean = 993 ng/mL, median = 332 ng/mL, SD = 1,280 ng/mL) resulting in a final opiate concentration range of 22 ng/mL to 1198 ng/mL (mean = 332 ng/mL, median = 164 ng/mL, SD = 444 ng/mL) (Figure 5.5).

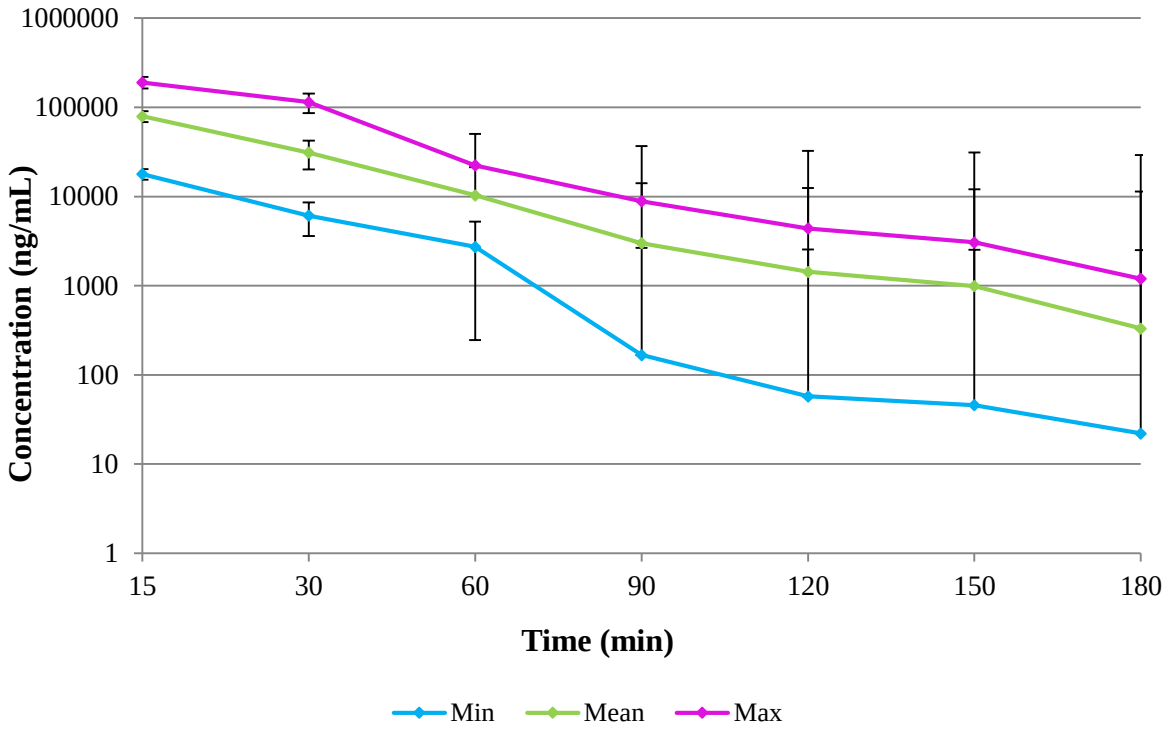


Figure 5.5. Minimum, Maximum and Mean concentrations of opiates in oral fluid collected over 180 minutes following the exposure to Codeine Linctus® (dose = 15 mg) in six volunteers

5.3.1.3 Summary of Study 1

Opiate concentrations in oral fluid observed were highest in the first (t = 15 min) sample collected post exposure to Codeine Linctus® and Collis Browne's® mixture, which was in agreement with the drug concentrations observed following consumption of coca tea (Chapter 4).

Mean opiate concentrations 15 minutes post exposure to Collis Browne's® mixture and Codeine Linctus® were 4,008 ng/mL to 79,666 ng/mL, respectively. Opiate concentrations following codeine use were much higher than morphine as a result of the difference in dose. This could be explained by the higher dose of 15 mg of Codeine Linctus® which was swirled around the mouth whereas Collis Browne's® was swirled at a dose of 5 mg. The cross reactivity of codeine, which was assessed against morphine, in the opiate immunoassay has been reported to be 398.9% in Table 5.1. The high cross reactivity of codeine also has to be borne in mind when explaining the higher oral fluid concentrations observed 15 minutes post exposure to Codeine Linctus®.

High opiate concentrations in oral fluid 15 minutes post exposure to either Collis Browne's® mixture or Codeine Linctus® resulted from this method of administration. Both solutions were swirled around the mouth and not swallowed which indicates that observed concentrations in oral fluid are a result of oral contamination and did not originated from the blood to saliva transfer. Although information on the length of exposure is limited in the literature, contamination of the oral mucosa is reported to affect the correlation between saliva and blood concentrations for 30 – 60 minutes (Bosker and Huestis, 2009).

The rate of elimination of opiates from oral fluid differs between individuals as a result of variations of saliva production and flow. A larger saliva flow rate, which is dependent on age, sex, climate, hydration state or stimulation will dilute the contamination faster and hence result in lowered concentrations (Chapter 1). This may account for the large variations observed between volunteers.

The principle of contamination suggests that an orally consumed solution lingers in the oral mucosa but also in cavities within the mouth. Participants were initially not requested to provide information on possible fillings or cavities. However, as a result of the variation of concentrations between volunteers, participants were asked retrospectively to provide this information. Out of six volunteers sampled post Codeine Linctus® exposure, only one reported to have numerous cavities and fillings. High codeine concentrations of 191,400 ng/mL at t=15 min in comparison to the mean concentration of 79,666 ng/mL, would appear to support the theory that cavities result in higher contamination concentrations, but more work with a larger sample population would be required to further test this hypothesis.

Additionally, manufacturers of Collis Browne's® and Codeine Linctus® solution listed ethanol in their list of ingredients. Ethanol intake can alter the distribution of drugs into tissue, which could hence affect the interpretation of results (Sellers and Holloway 1978). The manufacturers for Collis Browne's® and Codeine Linctus® did not provide information on the amount of alcohol present within each solution.

5.3.2 Study 2 - Investigation into concentrations in oral fluid following the exposure to Collis Browne's® and Codeine Linctus® with two different collection methods

5.3.2.1 Collis Browne's® mixture

During collection, the Alere™ Certus collector is inserted into the oral cavity and left there until sufficient oral fluid has been collected. For the purpose of this study, an additional collector was then brushed against the oral tissues in order to stimulate cell abrasion. Brushing did not result in significant larger concentrations in comparison to oral fluid collected with the conventional oral fluid method (Figure 5.6).

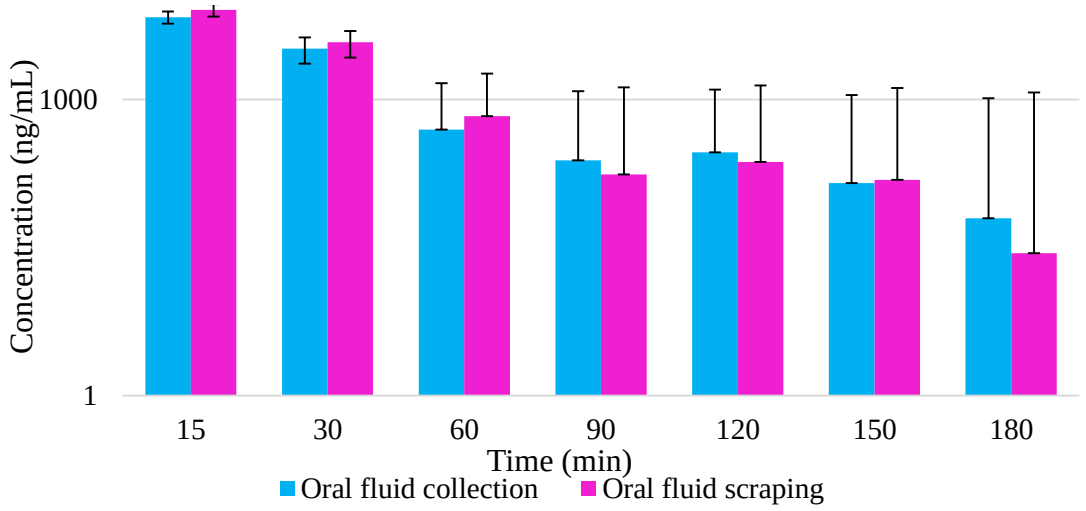


Figure 5.6. Comparison of mean oral fluid concentrations following exposure to Collis Browne's® mixture over three hours collected with the Certus collector via the manufacturer's recommended method and the scraping method (

The recommended collection method resulted in a mean oral opiate oral fluid concentration 15 minutes post exposure of 6,819 ng/mL (median = 2,617 ng/mL) in comparison to the mean concentrations of 8,070 ng/mL (median = 7,680 ng/mL) detected when using the collector to stimulate cell abrasion (Table 5.4).

Table 5.4 Mean and Median opiate concentrations (ng/mL) measured by immunoassay following the exposure of Collis Browne's® . Oral fluid was collected with two different methods a) as recommended by the manufacturer and b) whilst scraping the collector hard against mouth tissues.

	Concentration (ng/mL)			
	Oral fluid collection		**Oral fluid scraping**	
Time (min)	**Mean**	**Median**	**Mean**	**Median**
Predose	<5	<5	<5	<5
15	6,820	4617	8070	7680
30	3,267	1505	3798	1315
60	495	366	674	706
90	241	206	174	187
120	290	201	232	211
150	142	104	153	109
180	62	48	28	38

Concentrations decreased quickly in the first 30 minutes resulting in concentrations of 3,266 ng/mL (median = 1,505 ng/mL) and 3,798 ng/mL (median = 1,315 ng/mL) with the conventional and scraping collection method, respectively.

No large changes in concentrations were observed between the two methods of collection i.e. scraping or ordinary collection. Concentrations at the final concentrations decreased to 62 ng/mL (median = 48 ng/mL) with the manufacturers recommended method and 28 ng/ml (median = 38 ng/mL) when collected whilst scraping the collector. Results indicated that scraping the collector and hence increasing the amount of cellular debris within the sample did not cause a large difference in oral fluid opiate concentration following the swirling of Collis Browne's® solution.

5.3.2.2 Codeine Linctus®

As observed with the results following the exposure of Collis Browne's®, no significant changes were observed when applying the two different collection methods following the exposure to Codeine Linctus® (Figure 5.7).

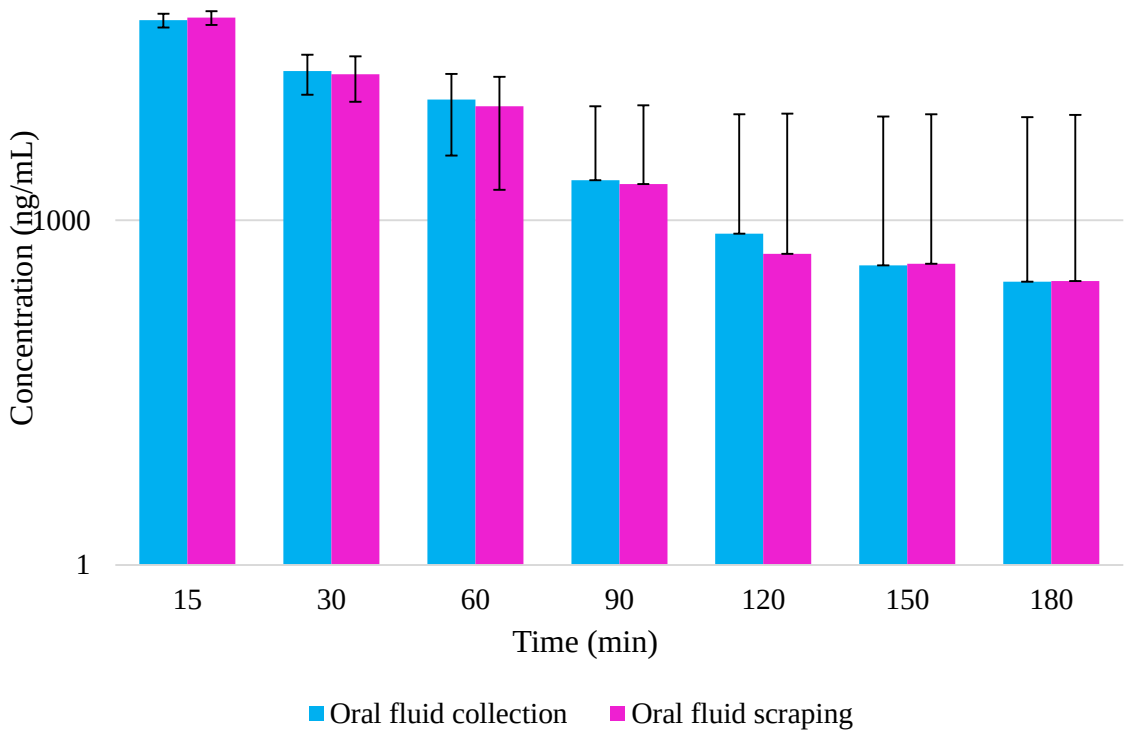


Figure 5.7. Comparison of oral fluid concentrations following exposure to Codeine Linctus® solution over three hours collected with the Certus collector via the manufacturer's recommended method and the scraping method

Opiate oral fluid concentrations were much higher following Codeine Linctus® which may be explained by the difference in dose each volunteer swirled around the mouth. The dose of Collis Browne's® mixture was 5 mg whereas the dose of Codeine Linctus® was a higher dose of 15 mg.

Mean opiate concentrations when collected with the manufacture recommended method and the scraping technique 15 minutes post exposure were detected at 55,406 ng/mL (median = 38,480 ng/mL) and 58,230 ng/mL (median = 51,112 ng/mL) respectively.

Table 5.5 Mean and Median opiate concentrations (ng/mL) following the exposure of Codeine Linctus® . Oral fluid was collected with two different methods a) as recommended by the manufacturer and b) whilst brushing the collector hard against mouth tissues.

	Concentration (ng/mL)			
	Oral fluid collection		**Oral fluid scraping**	
Time (min)	**Mean**	**Median**	**Mean**	**Median**
Predose	7	1	9	3
15	55406	38480	58230	51112
30	19998	23324	18746	20492
60	11277	6700	9840	5278
90	2240	2570	2066	2558
120	764	802	510	512
150	405	389	419	459
180	292	227	296	239

Concentrations decreased rapidly in the first 30 minutes although high concentrations are still observed up to 90 minutes post exposure. Concentrations at 90 minutes had a mean concentration of 2,240 ng/mL (median = 2,570 ng/mL) when collected using the manufactures recommended method of collection. In comparison opiate concentrations in samples that were collected 90 minutes using the scraping method, were at a mean concentration of 2,066 ng/mL (median = 2,558 ng/mL). Opiate concentrations during normal collection and brushing continued to decrease and were still above the manufacturers cut off at 292 ng/mL (median = 227 ng/mL) and 296

ng/mL (median = 239 ng/mL). Results indicated that brushing the collector against the oral mucosa and hence increasing the amount of cellular debris does not result in elevated drug concentration following the use of an oral opiate solution.

5.4 Conclusion

The results from work undertaken in this chapter suggest, that elevated opiate concentrations can be observed following the exposure to Collis Browne's® and Codeine Linctus® as a result of the deposition of drugs in the oral mucosa. Both solutions were discarded from the mouth prior to collecting oral fluid samples and hence were not swallowed. That means that observed oral fluid concentrations were a result of oral contaminations rather than originating from the transfer of blood to saliva. Oral fluid drug concentrations were observed for up to three hours post exposure to the oral solution, but may extend beyond the final collection time. Results indicate that the consumption of an oral solution such as Codeine Linctus® or Collis Browne's® mixture has the potential to artificially elevate oral fluid concentrations and hence interfere with the interpretation of oral fluid drug concentrations. Additionally, it was suggested from the results in this chapter that a large amount of cavities can increase the potential for drug depots when drugs are consumed orally as drugs can linger within the oral cavities.

The hypothesis that drugs bind to tissue and thus cells, suggests that the presence of cellular debris within the oral fluid could increase elevated drug concentrations. However, no large variation in opiate concentration were observed between concentrations in oral fluid samples collected via the recommended manufacturer's method and brushing the collector against the oral tissues indicating that increased

cellular debris within the oral fluid sample, does not contribute to elevated drug oral fluid concentrations.

Chapter 6.0- Effect of microbleeding of the gums on oral fluid drug detection

6.1 Introduction

The work described in Chapter 4 showed that elevated cocaine and metabolite concentrations could be observed for up to one hour post consumption of one single cup of coca tea. Similarly, research undertaken in Chapter 5 showed that the elevated opiate concentrations were observed following the exposure to Collis Browne's solution and Codeine Linctus. Oral fluid opiate concentrations were reported for up to three hours post exposure to either of the solution. The results presented in these previous sections suggest that drugs liquid formulations of drugs which are consumed orally have the potential to accumulate in mouth tissues.

Oral contamination however cannot only be caused by drugs but also by other factors such as microbleeding of the gums. This led to the question whether microbleeding of the gums could be a contributing factor to elevated drug concentrations in oral fluid. This propositions has previously been raised in the literature (Schartz and Granger 2004) but has not been fully investigated.

6.1.1 Microbleeding

Microbleeding is a natural phenomenon in which blood is released from the gums into the mouth and may subsequently be present in oral fluid. Several factors have been reported to cause microbleeding in the gums yet these factors do not appear to have been extensively explored in relation to oral fluid drug testing. The main causes for microbleeding are tooth brushing, chewing gum or gum disease (Fure et al 1998, Yaegaki et al 2002, Krause et al 2002, Pratibha et al 2006). The contamination of oral fluid by microbleeding could hypothetically increase drug concentrations in oral

fluid if the donor of the oral fluid specimen had consumed drugs and they were present in his / her circulating blood. Leao and Sheiham (1995) also reported that subjects with poor dental health, fillings and decaying teeth are prone to increased microbleeding of the gums.

The work described in this chapter was undertaken to investigate whether microbleeding in the gums can contribute to elevated oral fluid concentrations. The original concept (Chapter 1) was that drugs enter oral fluid via the saliva from the circulating blood (Kidwell et al 1998, Aps and Martens 2005). If an individual has drugs in the blood microbleeding of the gums could hypothetically contribute to the overall oral fluid drug concentration. At the present time there are no reports in the available literature on studies which investigate the hypothesis that microbleeding can contribute to or influence the oral fluid drug concentration.

6.1.2 Protein hormones in Saliva

During microbleeding, large blood proteins such as transferrin are released into the oral fluid. Transferrin is present in oral fluid in trace amounts as it is present in the gingival fluid (Asman et al 1981, Curtis et al 1988). It binds tightly to the glycoproteins in blood plasma and hence has become a useful biomarker for blood in saliva with concentrations ranging between 0 mg dL and 5.5 mg/dL (mean = 3.7 mg/dL) (Schwartz and Granger 2004). Transferrin concentrations in oral fluid are dependent on age and sex (Granger et al 2007). In 2004, a study by Kivlighan et al revealed that transferrin concentrations rise significantly when micro-injury occurs in the oral cavity. In comparison to healthy gums where mean transferrin concentrations were 0.37 mg/dL (range = 0.32 mg/dL – 0.49 mg/dL), concentrations

of transferrin immediately post injury rose to 0.75 mg/dL and then quickly decreased to 0.43 mg/dL over one hour.

Saliva contains a wide variety of hormones including steroids, thyroid hormones and protein hormones (Vining and McGinley 1986). Hormones are passed into saliva by transfer from surrounding tissues. Salivary glands express enzymes responsible for the conversion of steroid hormones such as converting unbound cortisol into inactive cortisone (Boumba et al 1995, Smith et al 1996, Morineau et al 1997). The main functions of salivary hormones are immune response and inflammatory processes which contributes to wound healing and cell repair. Additionally, salivary hormones have been used as diagnostic tumour markers (Gröschl 2009).

There are other methods for detecting blood in oral fluid. In addition to transferrin, haemoglobin has been proposed as a useful biomarker for blood in oral fluid (Hofman 2001, Kivlighan et al 2004), however, salivary peroxidases interfere with the haemoglobin test and can result in a false positive test for haemoglobin hence making it an unsuitable marker for blood in saliva (Schwartz and Granger 2004). Therefore, transferrin was selected as a method for the detection of blood in oral fluid for the following research undertaken in this chapter.

6.2 Materials and Methods

6.2.1 Materials

Transferrin immunoassay kits were purchased from Salimetrics Europe, Newmarket, UK. This immunoassay is optimised by the manufacturer to measure salivary transferrin, where there is a requirement to quantitate at low concentrations compared to analysis in blood where the levels are considerably higher. Elevated concentrations of transferrin in saliva indicate that contamination through blood has

occurred. The manufacturer, Salimetrics, recommends that saliva samples with transferrin concentrations higher than 0.5 mg/dL should be reported as contaminated. The kit has a mean recovery ranging between 96% and 117.7% with the lower limit of detection at 0.08 mg/dL. No cross reactivity has been reported with cortisol, testosterone, dehydroepiandrosterone, progesterone, melatonin, estradiol, secretory IgA and lactoferrin.

6.2.2 Methods

Two studies were performed during the investigation on the effect of microbleeding on oral fluid drug detection. The initial study focused on comparing oral fluid drug and transferrin concentrations in samples provided by drug using volunteers. The second study investigated the effects of the stimulation of microbleeding following the ingestion of a 15 mg dose of codeine. For the second study, volunteers undergoing treatment with any prescribed drugs or medications were excluded from this experiment. The experimental procedures were approved in compliance with the University of Bournemouth ethical guidelines.

6.2.2.1 Study 1 - Sample collection

Alere Toxicology Laboratories regularly screen oral fluid samples collected from regular drug users attending drug clinics as part of drug treatment programmes. Oral fluid samples were collected from 75 drug clinics throughout the UK and were then analysed by Alere Toxicology (Abingdon, UK) via immunoassay as part of their routine work. The samples were then anonymised and supplied to the University of Bournemouth for screening for transferrin. Prior to providing the oral fluid samples, drug users gave consent for additional testing on their provided samples as deemed appropriate by the laboratory.

6.2.2.2 Study 1 - Sample selection

Alere Toxicology, Abingdon, UK provided 3,000 samples where the screening resulted in a positive, for cocaine, opiates or 6-MAM. Because of the high cost of purchasing the transferrin assay it was necessary to limit the number of samples for analysis. For the purpose of identifying possible microbleeding, samples were categorised into four groups (Table 6.1) a) below 100 ng/mL b) low concentration range, c) medium concentration range and d) high concentration range.

Table 6.1. Concentration ranges (ng/mL) for each one of the four groups in which drug clinic samples were divided. Table summarises the ranges for cocaine, opiates and 6-MAM

	Concentration range (ng/mL)		
	Cocaine	**Opiates**	**6-MAM**
Group 1	<100	<100	<100
Group 2	100 – 150	100 - 200	100 - 250
Group 3	150 - 240	201 - 320	250 – 450
Group 4	>241	>321	>451

The number of samples analysed using the transferrin immunoassay was restricted due to the high cost of the Salimetrics transferrin kits. As a result 120 samples in total were analysed, 40 per drug and 10 samples of each drug and group were selected to be screened for transferrin using the Salimetrics' transferrin immunoassay. Samples were collected at random with every 15th sample in the list selected for analysis using a Salimetrics' transferrin immunoassay.

6.2.2.3 Study 1 - Transferrin Immunoassay

The manufacturer's recommended immunoassay cut off for transferrin in saliva is 0.5 mg/dL. Calibrators ranged between 0.08 mg/dL and 6.6 mg/dL. 10 µL of sample,

50 µL of enzyme conjugate and 50 µL of antiserum were added to each well and incubated for 40 minutes. Wells were then washed four times with 400 µL of wash buffer provided with the kit. Samples were then incubated with 100 µL of 3,3',5,5' - tetramethylbenzidine (TMB) solution for 10 minutes in the dark before addition of the 100 µL of stop solution at reading the absorbance at 450 nm. The transferrin immunoassay method is summarised in Figure 6.1.

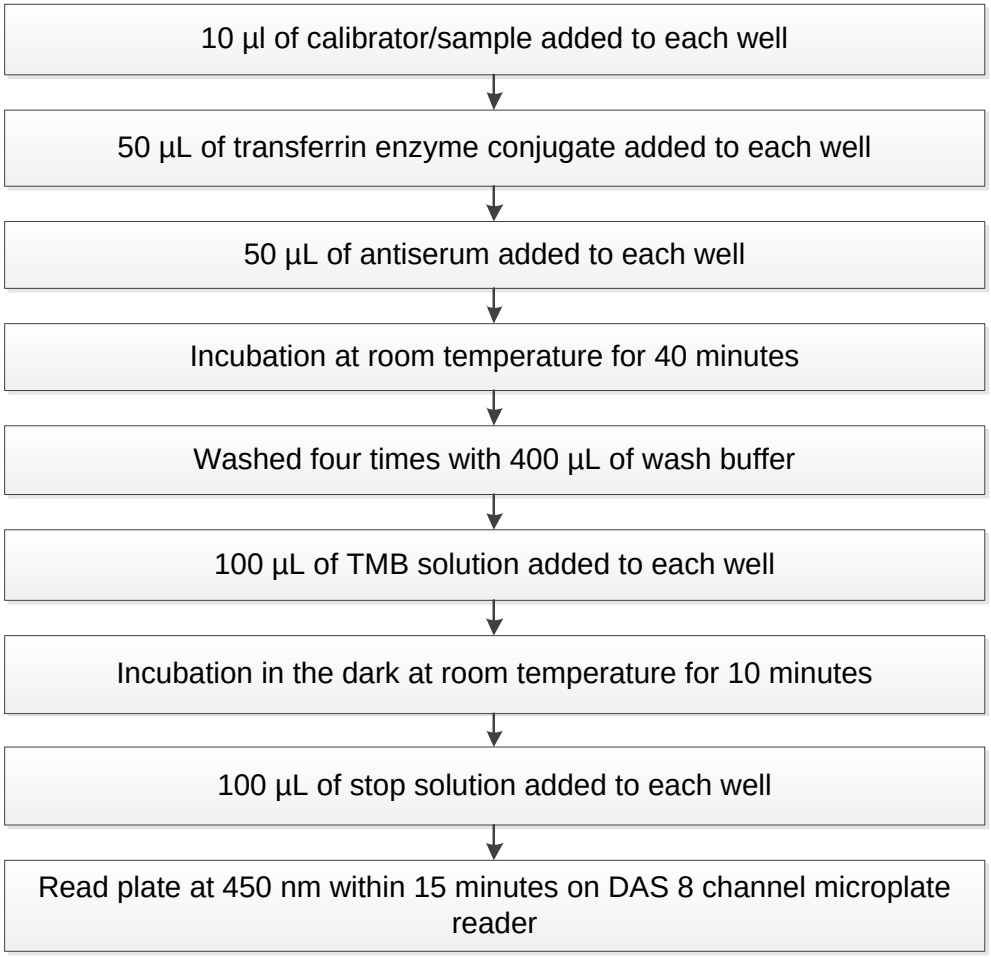


Figure 6.1. Summary of method for the analysis of transferrin in oral fluid using the Salimetrics' transferrin kit for blood contamination

6.2.2.4 Study 2 – Sample collection

Eight non-drug using human volunteers (4 male, 4 female) were invited to swallow a 15 mg codeine tablet. Volunteers ranged in age between 21 and 66. Volunteers were asked to provide a pre-dose sample of oral fluid immediately to participating in this study. Oral fluid was subsequently collected 15, 30, 45, 60, 90, 120, 150, 180 and 240 minutes after consumption of codeine using the Alere™ Certus oral fluid collection device. Two groups of volunteers were formed. The first group ingested the codeine tablet and subsequently provided oral fluid. The second group stimulated microbleeding three hours post ingesting the codeine tablet. Oral fluid was collected in accordance with the Alere™ manufacturer instructions for the Alere™ Certus collection device.

6.2.2.5 Study 2 - Immunoassay

For the detection of opiates, the Alere™ Opiate EIA kit was used as described in Chapter 5.

6.3 Results and Discussion

6.3.1 Study 1 - Results

Transferrin concentrations detected in samples in Group 1 (Table 6.1) ranged from 0.2 mg/dL to 6.2 mg/dL (mean = 2.1 mg/dL, median = 1.2 mg/dL, SD = 1.6 mg/dL, IQR = 1.1 mg/dL). In the low and medium drug concentration groups mean transferrin concentrations were detected at 1.9 mg/dL (range = 0.1 mg/dL- 6.5 mg/dL, median = 1.4 mg/dL, SD = 1.7 mg/dL, IQR = 1.3 mg/dL) and 1.7 mg/dL (range = 0.1 mg/dL – 6.2 mg/dL, median = 0.7 mg/dL, SD = 1.6 mg/dL, IQR = 1.6 mg/dL), respectively. Similar concentrations were observed in samples with high

drug concentrations ranging between 0.1 mg/dL and 6.2 mg/dL (mean = 1.7 mg/dL, median = 0.1 mg/dL, SD = 1.9 mg/dL, IQR = 1.2 mg/dL) (Table 6.2).

Table 6.2 Summary of transferrin concentrations (mg/dL) in cocaine, opiates and 6-MAM positive samples collected from drug clinics in the UK

	Transferrin Concentrations (mg/dL)					
	Min	**Max**	**Mean**	**Median**	**Standard Deviation (SD)**	**Interquartile Range (IQR)**
Group 1	0.2	6.2	2.1	1.2	1.6	1.1
Group 2	0.1	6.5	1.9	1.4	1.7	1.3
Group 3	0.1	6.2	1.7	0.7	1.6	1.6
Group 4	0.1	6.2	1.7	0.1	1.9	1.2

6.3.1.1 Cocaine

Transferrin positive results were observed in all of the four groups. In the cocaine group 1 below 100ng/mL, transferrin concentrations ranged from 0.3 mg/dL to 6.2 mg/dL (mean = 2.3 mg/dL, median = 1.6 mg/dL, SD = 2.0 mg/dL) with eight out of the ten samples in this group above the manufacturers recommended cut off (0.5 mg/dL).

In the cocaine group 2, six volunteers had extremely high levels of transferrin resulting in a mean concentration of 5 mg/dL (range = 0.1 mg/dL – 6.6 mg/dL, median = 4.2 mg/dL, SD = 2.3 mg/dL) (Table 6.3). Transferrin concentrations detected in cocaine group 3 were all above the manufacturers recommended transferrin cut off of 0.5 mg/dL cut off. Transferrin concentrations in this group

ranged between 1.0 mg/dL and 6.2 mg/dL (mean = 2.8 mg/dL, median = 1.8 mg/dL, SD = 1.9 mg/dL).

In group 4 the cocaine concentrations were the highest detected in the oral fluid samples, however only seven samples were positive for transferrin with a mean concentration of 1.8 mg/dL (range = 0.1 mg/dL – 5.6 mg/dL, median = 1.9 mg/dL, SD = 2.1 mg/dL) (Table 6.3).

Table 6.3 Summary of cocaine concentrations (ng/mL) compared to transferrin results (mg/dL) – (Samples reported as 'over', were above the highest calibrator provided with the kit)

	Concentration (ng/ml)	**Transferrin (mg/dL)**		**Concentration (ng/ml)**	**Transferrin (mg/dL)**
Below	59	1.4	**Med**	176	4.4
	17	4.0		187	6.2
	29	1.4		209	1.0
	20	over		191	3.0
	10	0.6		206	1.7
	12	0.3		190	over
	55	over		204	over
	25	3.0		195	1.2
	60	6.2		169	over
	82	1.8		160	1.8
Mean	36.9	2.3	Mean	188.7	2.8
median	27	1.6	median	190.5	1.8
Min	10	0.3	Min	160	1.0
Max	82	6.2	Max	209	6.2
SD	25.0	2.0	SD	16.2	1.9
R^2	0.0855		R^2	0.1047	
Low	100	0.7	**High**	249	0.7
	111	1.8		242	1.5
	110	0.1		246	0.1
	104	0.2		241	0.5
	109	over		236	5.4
	106	6.5		241	0.2
	110	0.8		234	2.7
	102	over		241	0.5
	108	over		244	5.7
	111	0.2		243	0.8
Mean	107.1	1.5	Mean	241.7	1.8
median	108.5	0.7	median	241.5	0.7
Min	100	0.1	Min	234	0.1
Max	111	6.5	Max	249	5.6
SD	3.9	2.3	SD	4.4	2.1
R^2	0.0085		R^2	0.1571	

This indicated that the concentrations of cocaine are not in correlation with the concentration of transferrin detected in these samples. Samples which were below the cut off showed similar results to the samples analysed from the high cocaine concentration group indicating that the microbleeding cannot be a cause for the high drug concentrations detected. This was also supported by the coefficient of correlation (R^2) which was 0.0855 for the concentrations below the cut off for cocaine. For the low, medium and high cocaine concentrations the R^2 values were 0.0085, 0.1047 and 0.1571, respectively (Figure 6.2). The closer the coefficient of correlation is to 1, the closer results correlate. However, the R^2 value for cocaine vs transferrin concentrations were low, indicating that there is no correlation between elevated cocaine concentrations and transferrin concentration in oral fluid samples.

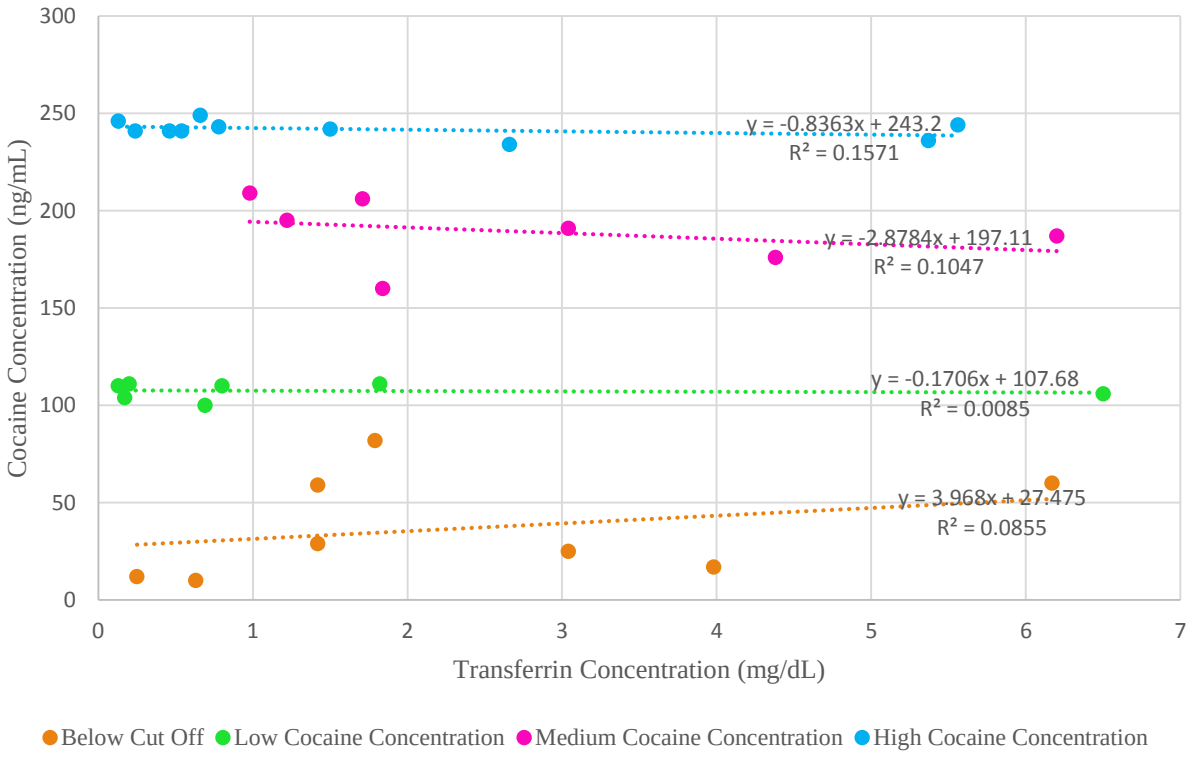


Figure 6.2 Scatter graph showing the correlation between the cocaine concentration (ng/mL) against the transferrin concentration (mg/mL) observed in oral fluid collected from drug clinics

6.3.1.2 Opiates

Transferrin concentrations in oral fluid samples which previously were confirmed to contain opiates also did not show good correlation between the opiate concentrations detected and the transferrin concentrations.

In the Group 1 for opiates, 50% of all samples screened for transferrin, gave a positive results above the recommended transferrin cut off. Transferrin concentrations ranged from 0.2 mg/dL to 6.0 mg/dL (mean = 1.0 mg/dL, median = 0.4 mg/dL, SD = 2.0 mg/dL). Transferrin concentration in Group 2 had a mean of 1.8 mg/dL (range = 0.4 mg/dL – 5.2 mg/dL, median 1.2 mg/dL, SD = 1.7 mg/dL) with eight out of ten volunteers confirmed above the transferrin cut off.

Similarly, medium and high opiate concentrations resulted in mean transferrin concentrations at 0.5 mg/dL (range = 0.1 mg/dL – 1.6 mg/dL, median = 0.3 mg/dL, SD = 0.5 mg/dL) and 2.4 mg/dL (range = 0.1 mg/dL – 6.2 mg/dL, median = 0.6 mg/dL, SD = 2.5 mg/dL). In both, the medium and the high opiate concentration group, only three volunteers tested positive for transferrin using the Salimetrics' transferrin kit (Table 6.4).

Table 6.4. Summary of immunoassay opiate concentrations (ng/mL) compared to immunoassay transferrin results (mg/dL) – (ND = not detected)

	Concentration (ng/ml)	**Transferrin (mg/dL)**		**Concentration (ng/ml)**	**Transferrin (mg/dL)**
Below	9	0.3	**Med**	315	0.2
	12	1.2		300	over
	31	0.2		310	0.7
	62	6.0		309	0.5
	9	0.7		293	1.6
	29	0.6		288	over
	10	0.7		310	0.2
	16	0.3		301	0.2
	46	0.2		279	0.4
	20	0.2		313	0.1
Mean	24.4	1.0	Mean	301.8	0.5
Median	18	0.4	Median	305	0.3
Min	9	0.2	Min	279	0.1
Max	62	6.0	Max	315	1.6
SD	18.9	2.0	SD	11.9	0.5
R^2	0.4557		R^2	0.0222	
Low	103	0.4	**High**	327	0.6
	103	2.9		332	0.1
	111	1.5		338	over
	104	0.6		329	over
	110	5.2		329	over
	110	0.5		353	0.2
	106	0.9		336	0.2
	104	0.6		333	4.1
	100	4.0		342	6.2
	113	1.8		342	5.3
Mean	106.4	1.8	Mean	336.1	2.4
Median	105	1.2	Median	334.5	0.6
Min	100	0.4	Min	327	0.1
Max	113	5.2	Max	353	6.2
SD	4.3	1.7	SD	8.0	2.5
R^2	0.0002		R^2	0.0851	

The results for opiates also indicate that there is no relationship between the amount of drug present in saliva and the presence of microbleeding, which was also supported by the low R^2 value obtained when comparing opiate concentration to transferrin concentration in oral fluid samples. . The R^2 value for samples which were below the opiate cut off was 0.4557. For the low, medium and high opiate concentrations the R^2 values were 0.0002, 0.0222 and 0.0851, respectively (Figure 6.3). The low R^2 value for opiate vs transferrin concentrations indicating that there is no correlation between elevated opiate concentrations and transferrin concentration in oral fluid samples.

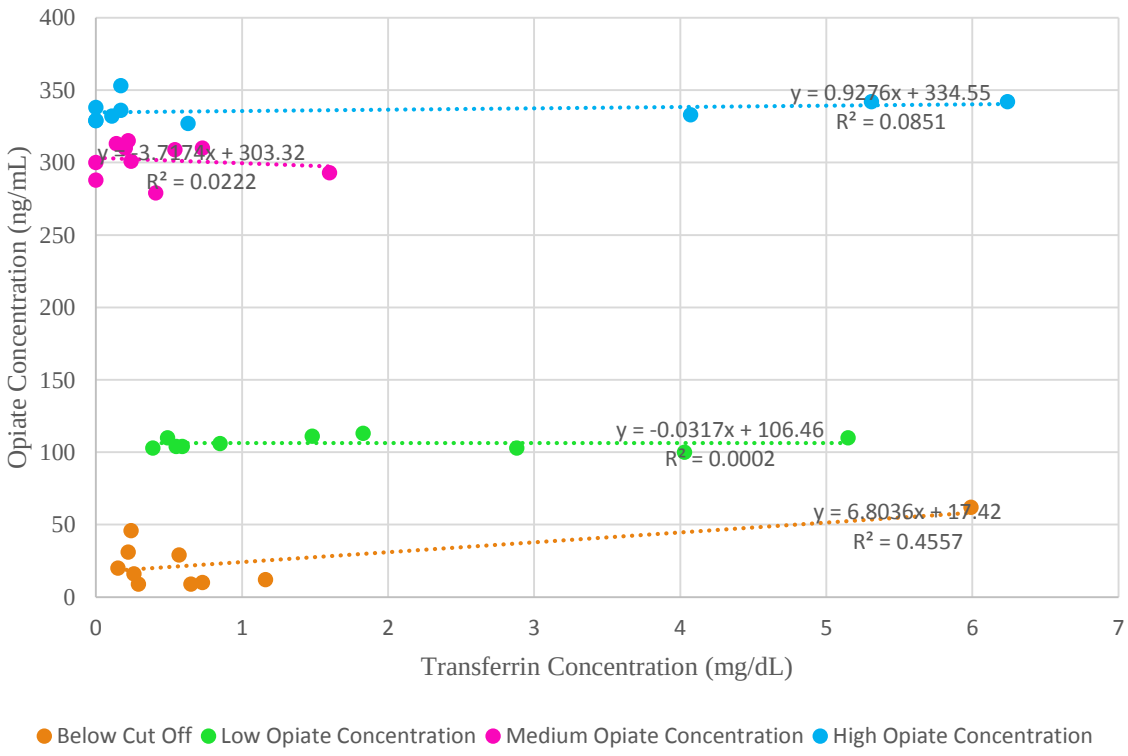


Figure 6.3 Scatter graph showing the correlation between the opiate concentration (ng/mL) against the transferrin concentration (mg/mL) observed in oral fluid collected from drug clinics

6.3.1.3 6-monoacetylmorphine

Transferrin concentrations in samples containing 6-MAM below 100 ng/mL ranged from 0.2 mg/dL to 1.7 mg/dL (mean = 0.9 mg/dL, median = 0.8 mg/dL, SD = 0.5 mg/dL). Only two samples with concentration below the 100 ng/mL of 6-MAM gave a negative result for transferrin.

Similarly, the analysis of group 2 and group 3 which contained 6-MAM resulted in negative results on the transferrin immunoassay. Low and medium drug concentrations mean at 1.4 mg/dL (range = 0.3 mg/dL – 5.0 mg/dL, median = 0.7 mg/dL, SD = 1.4 mg/dL) and 1.5 mg/dL (range = 0.3 mg/dL – 5.2 mg/dL, median = 0.7 mg/dL, SD = 1.5 mg/dL).

High 6-MAM concentrations were associated with the lowest positivity rate for transferrin with only five samples testing above the manufacturers recommended 0.5 mg/dL cut off. A mean transferrin concentration of 0.6 mg/dL (range = 0.1 mg/dL – 1.9 mg/dL, median 0.5 mg/dL, SD = 0.5 mg/dL) was detected in samples previously confirmed to contain a high 6-MAM concentration (Table 6.5).

Table 6.5. Summary of 6-monoacetylmorphine concentrations (ng/mL) compared to transferrin results (mg/dL) – (ND = not detected)

	Concentration (ng/ml)	**Transferrin (mg/dL)**		**Concentration (ng/ml)**	**Transferrin (mg/dL)**
Below	25	0.55	**Med**	349	2.18
	14	over		393	0.5
	13	0.18		383	0.34
	87	0.79		391	0.6
	10	0.71		393	5.16
	20	1.52		420	2.82
	16	0.87		394	0.68
	29	1.72		364	0.39
	81	0.52		423	0.68
	47	0.88		398	2.02
Mean	34.2	0.86	Mean	390.8	1.54
Median	22.5	0.79	Median	393	0.68
Min	10	0.18	Min	349	0.34
Max	87	1.72	Max	423	5.16
SD	28.3	0.5	SD	22.3	1.5
R^2	0.0122		R^2	0.0089	
Low	108	2.12	**High**	472	0.7
	109	0.65		458	0.53
	108	0.32		459	0.6
	107	0.28		475	0.09
	107	0.69		459	0.18
	110	0.72		459	1.87
	113	1.83		459	0.41
	103	0.67		460	0.12
	101	1.23		493	0.1
	110	5.01		489	0.86
Mean	107.6	1.35	Mean	468.3	0.55
Median	108	0.705	Median	459.5	0.47
Min	101	0.28	Min	458	0.09
Max	113	5.01	Max	493	1.87
SD	3.5	1.4	SD	13.4	0.5
R^2	0.0955		R^2	0.0309	

When 6-MAM concentrations in oral fluid increased, transferrin concentrations decreased leading the conclusion that blood contamination of saliva is not a contributing factor to the elevated oral fluid concentrations observed previously (Chapter 4, Chapter 5). Similarly, to cocaine and opiate concentrations, this was also supported by the low R^2 value obtained when comparing 6 MAM concentration to transferrin concentration in oral fluid samples. . The R^2 value for samples which were below the opiate cut off was 0.0122. For the low, medium and high 6 MAM concentrations the R^2 values were 0.0955, 0.0089 and 0.0309, respectively (Figure 6.4). The low R^2 value for 6 MAM vs transferrin concentrations indicating that there is no correlation between elevated 6 MAM concentrations and transferrin concentration in oral fluid samples.

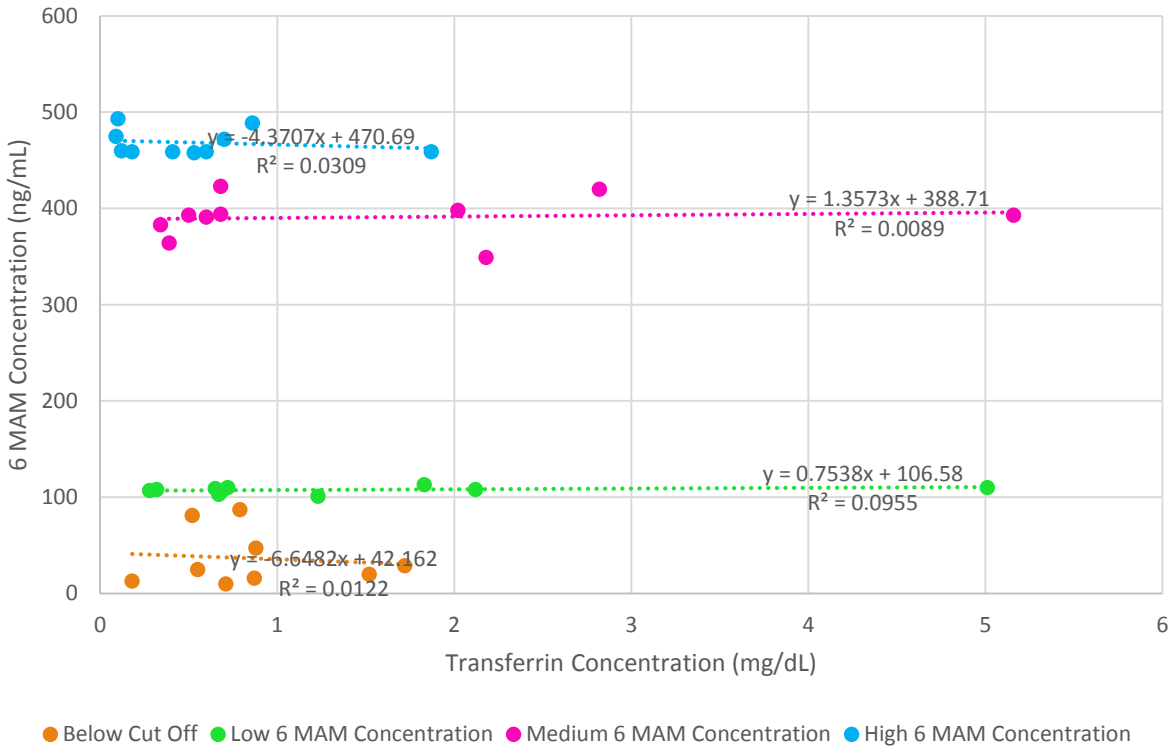


Figure 6.4 Scatter graph showing the correlation between the 6 MAM concentration (ng/mL) against the transferrin concentration (mg/mL) observed in oral fluid collected from drug clinics

6.3.1.4 Summary of Drug Clinic Data results

During analysis of the drug clinic data, results showed that for cocaine, opiates and 6MAM, high transferrin concentrations did not relate directly to high drug concentrations. Elevated transferrin concentrations were observed no matter what the concentration of the drug, i.e. below 100 ng/mL, low concentration, medium concentration or high concentration, which is supported by the low R^2 value between drug and transferrin concentration. This further indicates that there is no correlation between concentrations of drug and concentration of transferrin in oral fluid.

No information was available on the oral hygiene from oral fluid donors. However, it has been shown that periodontal disease can result in increased gingival fluid (Schenkein and Genco 1977, Genco et al 1985). As transferrin is present in gingival fluid, observed elevated transferrin concentrations could also be resulting as a cause of periodontal disease in the oral fluid donor.

Results suggested that microbleeding was not a contributing factor to elevating drug concentrations in oral fluid. However this study has a number of limitations, beyond control of this study as the samples were already collected before key information could be recorded such as:

6.3.1.4.1 Route of administration

As discussed in other chapters, the contribution to oral fluid drug levels is significant from smoking, intranasal and chewing routes of administration whilst intravenous/intramuscular use results in higher blood levels rather than oral fluid levels. It would have been advantageous to know the route of administration to further segregate the groups into oral versus injection routes. This would have

allowed to conclude whether observed elevated concentrations are related to the route of administration.

6.3.1.4.2 Drug use

It would have been beneficial to have known the time between last drug use and collection of sample. This information would have given some insight as to whether the sample was in early or late elimination phase. In early phase we would expect to see high oral fluid levels resulting from contamination of the mouth cavity whereas in late phase elevated concentrations might be relating to drug deposition within and release from the oral cavity.

6.3.1.4.3 Drug dose

The final missing information in this data set is dose of the drug consumed and drug purity. The missing detail on the dose hinders the correct interpretation of the oral fluid concentrations in relation to the drug consumed.

Because of the large number of unknowns associated with the drug clinic data, a study was undertaken involving oral fluid collection under controlled conditions following the controlled consumption of a codeine tablet.

6.3.2 Study 2 - Results

Following stimulation of microbleeding three hours post consumption of a 15 mg dose of codeine (as a tablet) by a group of eight volunteers, mean opiate concentrations 15 minutes post ingestion were 1,359 ng/mL (range = 70 ng/mL – 2,811 ng/mL, median = 1,185 ng/mL, SD = 909 ng/mL). Peak concentrations were observed at 60 minutes post ingestion with concentrations ranging between 441 ng/mL to 3,551 ng/mL (mean = 1,414 ng/mL, median = 1,098 ng/mL, SD = 1,002 ng/mL) (Table 6.6).

Table 6.6 Summary of immunoassay opiate concentrations results from volunteers who stimulated microbleeding 3 hours post consumption of a 15 mg codeine tablet

Time (min)	Concentration (ng/mL)												
	V1	**V2**	**V3**	**V4**	**V5**	**V6**	**V7**	**V8**	**Min**	**Max**	**Mean**	**Median**	**SD**
15	1244	1126	70	1362	2811	631	2523	1108	70	2811	1359	1185	909
30	102	1224	76	345	2904	474	1896	1463	76	2904	1060	849	1002
60	441	3551	1188	514	1514	621	2483	1007	441	3551	1415	1098	1090
120	608	700	197	588	451	471	1885	273	197	1885	647	530	528
180	323	447	40	296	494	34	137	230	34	494	250	263	173
180MB	463	290	27	41	571	31	125	21	21	571	196	83	219
195	371	312	36	45	27	58	231	28	27	371	138	51	143
210	345	298	36	51	40	106	423	38	36	423	167	78	161
240	190	227	21	55	25	36	143	41	21	227	92	48	82

Legend: V= Volunteer number, 180MB=Sample collected post stimulation of microbleeding at t=180 minutes

Microbleeding was stimulated at t = 180 min. Immediately before stimulation of microbleeding, the mean opiate concentration was 250 ng/mL (range = 34 ng/mL – 494 ng/mL, median = 263ng/mL, SD = 173 ng/mL). Microbleeding was stimulated until blood was visible in the sample. Microbleeding did not result in an immediate increase in opiate concentration with concentrations ranging between 21 ng/mL and 571 ng/mL (mean = 196 ng/mL, median = 83 ng/mL, SD = 219 ng/mL) at t = 180 min (post microbleeding). No significant change in concentration could be detected in samples immediately following microbleeding. However, a delayed rise could be observed at t = 210 min in 5 out of the 8 volunteers participating in this study. Mean concentrations at t = 210 min rose to 167 ng/mL (range = 36 ng/mL - 423 ng/mL, median = 78 ng/mL, SD = 161 ng/mL) from 138 ng/mL (range = 27 ng/mL – 371 ng/mL, median = 51 ng/mL, SD = 143 ng/mL) at t = 195 min (Figure 6.5).

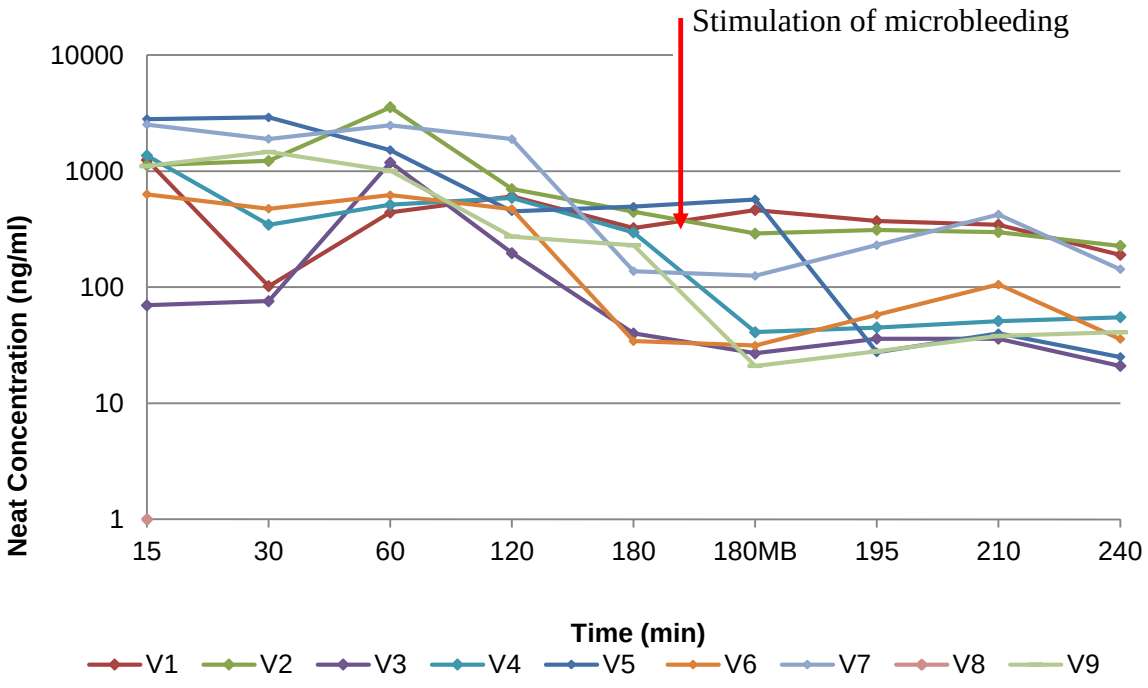


Figure 6.5. Comparison of codeine concentrations in eight volunteers following the consumption of a 15 mg dose of codeine. Microbleeding was stimulated 180 minutes post consumption and is indicated by the red arrow. V = Volunteer

Only two volunteers showed a slight increase in concentrations following microbleeding (Table 6.6 – V1, V5). However, concentrations then continue dropping at constant rate and showed no significant change in comparison to the other volunteers.

Results from controlled exposure experiments support the results from drug clinics data and indicated that micro-microbleeding is not a significant contributory factor to the elevated drug concentrations observed in oral fluid.

The amount of blood released during microbleeding has not been reported in the literature. Therefore calculations were based on an estimated volume of microbleeding ranging between a minimum volume of 5 µL and a maximum volume of 20 µL. The theoretical quantity of drug released from the blood into saliva can be calculated when applying the saliva/plasma ratio (S/P) for codeine. Although the S/P ratio for codeine has been variously reported, the mean S/P ratio applied during calculation in this study is 3.32 (Sharp et al 1983, Schramm et al 1992, Cone 1993, O'Neal et al 1999, Kim et al 2002) (Table 6.7).

Table 6.7 Summary of saliva plasma ratios for codeine as reported in the literature

Saliva/Plasma Ratio	**Source**
3.7	O'Neal et al 1999
3.3	Cone 1993
3.3	Kim et al 2002
3	Schramm et al 1992
3.3	Sharp et al 1983
3.32	Mean S/P ratio

Microbleeding during this study was stimulated three hours post exposure however, concentrations of opiates in the oral fluid in the literature are reported only at 2 hours or 4 hours. Following an oral dose of 30 mg of codeine, O'Neal et al (1999) reported a mean opiate concentration of 34 ng/mL at 2 hours post exposure and 18 ng/mL at 4 hours post exposure. When applying the mean S/P ratio to the plasma concentration at 2 hour and 4 hour, the amount of drug which would pass into oral fluid during microbleeding can be calculated.

Based on the plasma concentration reported by O'Neal et al (1999) the mean quantity of drug contaminating saliva at 2 hours with a minimum of a 5 µL microbleed would be 0.17 ng. At four hours post consumption the quantity of drug contaminating the saliva decreased to 0.09 ng. This is a minimal contamination of oral fluid through drug caused by microbleeding.

Even when considering the highest quantity of microbleeding reported, the amount of drug contaminating the oral fluid would only have a theoretical quantity of 0.68 ng and 0.36 ng at 2 hours and 4 hours post consumption, respectively. This quantity would also not elevate opiate concentrations in oral fluid significantly during this study when comparing to the observed mean oral fluid concentration of 250 ng/mL.

6.4 Conclusion

This chapter examined the hypothesis that microbleeding of the gums could contaminate the oral fluid. However, the work undertaken in this chapter showed that no relationship between elevated drug concentration and transferrin concentrations could be observed during the comparison of drug and transferrin concentrations in samples collected at drug clinics indicating that elevated drug concentrations are not a result of microbleeding of the gums.

Additionally, microbleeding did not result in an immediate rise of opiate oral fluid concentrations when bleeding was stimulated following the consumption of a 15 mg dose of codeine. A delayed response was however observed at t = 210 min, half an hour post stimulation of microbleeding. Concentrations then continued to decrease over the following 30 minutes.

The low response to microbleeding was explained by the low quantity of drug present within the microbleed when assuming that the average microbleed is between 5 µL to 20 µL. When applying the average S/P ratio of 3.32 to the average plasma concentration reported following a dose of 30 mg/mL, it results in the quantity of drug leaking into the oral fluid to range between 0.17 ng and 0.68 ng. This amount is insignificant when considering the much higher concentration of 250 ng/mL of opiates at three hours post consumption on one 15 mg dose of codeine. Therefore when considering both the results from the drug clinics and the results from the controlled codeine exposure study, microbleeding can be ruled out to be a contributing factor to elevated drug concentrations.

Chapter 7.0 - Development of an immunohistochemical method for the visualisation of cocaine and heroin in tissues with a benzoylecgonine or morphine antibody

7.1 Introduction

Immunohistochemistry (IHC) is a technique commonly used in pathology and morphological studies to identify specific cells or cultures. Although the technique is widely used for the visualisation of tumours, only a few papers are available where the technique has been applied to the visualisation of drugs in tissue. Kajitani et al (1989) demonstrated that immunohistochemical techniques could be applied in order to localise methamphetamine in salivary glands of mice. Additionally, Yotsu-Yamashita et al (2013) described the use of immunohistochemistry for the visualisation of puffer fish toxin in tissue and Wehner et al (2000) for visualising methadone in human brain. It was postulated that this method could be used to enable the detection of cocaine and opioids bound within cells and tissues to be visualised by the use of an antibody-antigen reaction.

The aim of the work undertaken in this chapter was to explore whether an immunohistochemical method could be developed to show the presence of cocaine and heroin and/or their metabolites in oral tissues after exposure to these drugs and the formation of drug depots. Immunohistochemical techniques are based on four main processes: a) fixation, b) processing, c) antigen retrieval methods and d) staining. The principals of these processes are described below.

7.1.1 Fixation

Fixation is a process that is designed to prevent tissue autolysis and bacterial decomposition. It also coagulates the tissue to prevent loss of easily diffusible substances and protects the tissue against from damage due to the effect of any subsequent stages in the tissue preparation (Bozzola and Russel 1999). Strong fixing can destroy protein based antigens and therefore compromise this technique. Additionally, fixation should leave the tissue in a condition which allows for subsequent staining (Karnovsky 1965).

7.1.2 Processing

Following fixation, the tissue sections need to be processed into an embedding medium. The two most common embedding media used during immunohistochemical staining are glycol methyl acrylate (GMA) resin or paraffin wax. Both embedding media differ in the approach needed for the immuno-reaction with antibody. GMA is a water miscible acrylic resin and has been reported to give the best resolution when locating cells. In comparison to techniques such as paraffin wax or freezing, GMA does not require dehydration of the tissue prior to staining, it prevents shrinkage and the resin does not need to be removed for subsequent staining procedures. GMA has a low viscosity, which allows easy penetration of the tissue. Other advantages of GMA include good antigen preservation, excellent morphology and localisation of antigens and no antigen retrieval is required. Good antigen preservation allows specialised fixation and low temperature processing which would be advantageous for drug detection in tissue. A major disadvantage of GMA resin as an embedding medium is that sections should be no larger than 2mm x 2mm.

GMA resin could be beneficial for the detection and visualisation of drugs as the high sensitivity allows for detection of low quantity of drugs in tissue.

In comparison to GMA resin, paraffin wax is commonly used to embed larger sections for immunohistochemical staining. However, tissue embedded in paraffin wax has been reported to lose some of its sensitivity and resolution as a result of de-paraffinisation and de-hydration steps during the following staining methods which are needed during the staining procedure.

7.1.3 Antigen retrieval methods

Fixation using neutral buffered formalin and embedding using paraffin wax causes links to form between tissue proteins. During the antigen retrieval step, the protein links are broken to unmask the antigen sites and enhance specific staining. The antigen retrieval methods vary depending on the antigens/epitopes of interest. For drug detection appropriate methods could include microwave treatment, pronase treatment or no pre-treatment.

7.1.4 Staining Principle

Tissue sections contain endogenous peroxidases and biotin in addition to the antigen of interest. Enzyme activity and biotin can interfere with the staining process by causing non-specific binding of the antibody. Thus, they must be inhibited during the staining process. Next, a culture medium needs to be applied consisting of amino acids, glucose, salts and vitamins to block additional non-specific binding sites.. Once all blocking steps are completed, a primary monoclonal or polyclonal antibody is applied which has been raised to bind directly to the antigen of interest. The working antibody concentration is calculated by dividing the antibody neat

concentration by an appropriate dilution factor. The formula for calculating the antibody working concentration is presented below:

$$\text{Antibody working concentration (mg/mL)} = \frac{\text{Antibody neat concentration (mg/mL)}}{\text{Dilution Factor}}$$

After a short incubation, any excess primary antibody is washed from the tissue and a secondary antibody added. This secondary antibody is one that has been raised against the primary antibody and is labelled with a moiety to allow it to act as a reporter antibody. Typically for a mouse monoclonal primary antibody (for example mouse anti-benzoylecgonine) a good choice of secondary antibody would be biotinylated rabbit-anti-mouse antibody. Biotinylation is the process of linking biotin directly onto the secondary antibody and has the benefit that it amplifies the signal.

Signal amplification comes from using avidin biotin-peroxidase complexes (ABC). ABCs were first developed for immunohistochemistry techniques in 1981 (Hsu 1990). Since then, they have become an established tool in chemistry, diagnostics and immunohistochemistry (Wilcheck and Bayer 1990). Avidin is a glycoprotein with great binding affinity for the vitamin biotin. When mixed together, avidin will bind to the biotinylated secondary antibodies (which are already bound to the drug within the tissue) and biotin-peroxidase forming a complex. Finally, 3, 3' diaminobenzidinetrahydrochloride (DAB) was applied as a substrate for the peroxidase enzyme bound within the ABC. Conversion of the substrate results in a brown chromagen and specific staining within the tissue sections. This process is shown in Figure 7.1. Sections are then rinsed and cover slips applied.

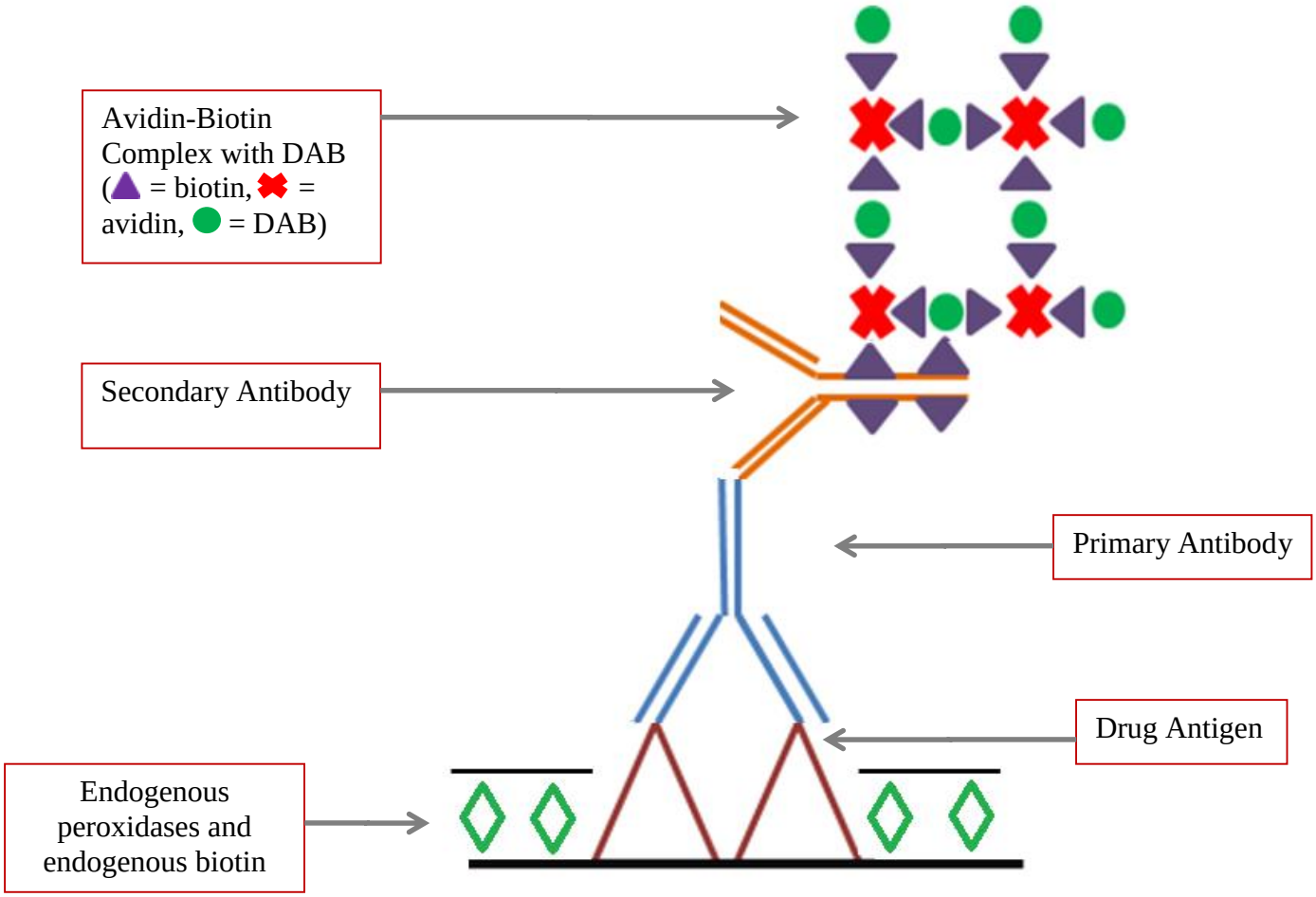


Figure 7.1 Avidin - Biotin staining principle applied during the immunohistochemical detection of drugs (

7.1.5 Tris-buffered saline (TBS) controls

Tris-buffered saline (TBS) controls act as blanks throughout the staining process. During staining of tissue sections, TBS is applied to a second tissue section instead of the primary antibody which acts a control to ensure that no non-specific binding occurs.

7.1.6 Isotype Controls

In addition to TBS controls, isotype controls are used. These are a type of negative control designed to show any signal due to non-specific binding and need to be included for each experimental test. Typically mouse primary antibodies will bind non-specifically to receptors present on the cell surface and this can be inhibited with an Immunogloblulin G (IgG) isotype control. Isotype controls are used to ensure the specificity of the primary antibody as well as ruling out any non-specific binding of the primary antibody to unwanted receptor or cellular proteins.

In order to prevent the tissue sections from drying out over night the sections are covered by microscope slides. TBS controls were incubated in TBS only. The appropriate isotype dilution is calculated by dividing the isotype neat concentration by the antibody working concentration. The formula for calculating the appropriate isotype dilution is presented below:

$$\text{Isotype dilution} = \frac{\text{Isotype neat concentration (mg/mL)}}{\text{Antibody working concentration (mg/mL)}}$$

7.2 Materials and Method Development

7.2.1 Materials

Artificial Salvia was prepared according to the Cozart biosciences protocol for the production of synthetic saliva (Appendix C).

In vitro experiments involved the use of porcine tongues due to its physiological and anatomical similarities to human tissue (Simon and Maibach 2000). Porcine tongues were purchased from The Village Butcher, Kingsclere, UK.

7.3 Method Development

7.3.1 Exposure and sectioning of tongues

Porcine tongues were exposed to solutions of cocaine or heroin at concentrations of 100 ng/mL and 10 µg/mL in artificial saliva for 10 minutes. This allowed the determination of the sensitivity of the antibody to a low and high concentration of drug. Following exposure to a drug solution, a 0.5 cm thick transverse cross section was removed from the porcine tongue (Figure 7.2).

Figure 7.2. Image representing the position of the transverse cross section which was removed for immunohistochemical analysis

7.3.2 Glycol methyl acrylate

7.3.2.1 Sectioning

Tissue sections used for GMA resin fixation should be no larger in size than 2mm x 2mm. Since the transverse cross sections of porcine tongue were significantly larger than 2mm x 2mm in size, 12 biopsy size samples were taken from a transverse cross

section of the tongue as depicted in Figure 7.3. The sections were removed using a biopsy stance to keep the size of the samples consistent as variable sample volume could affect the fixing stage.

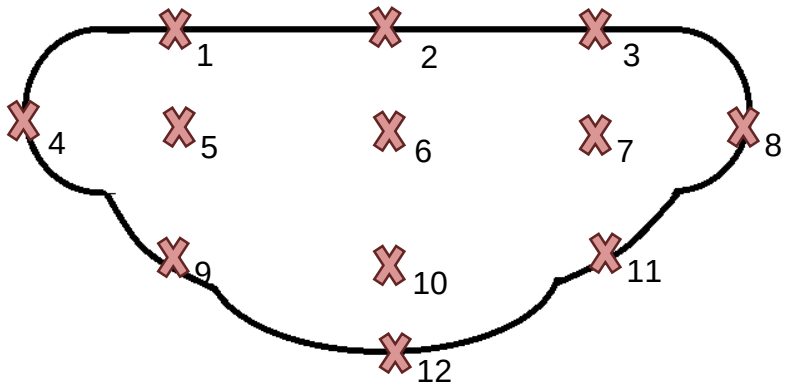


Figure 7.3 Transverse cross section of tongue showing the position of removal of 12 biopsy size samples for GMA immunohistochemical staining

7.3.2.2 Fixation

Initially, tissue sections were fixed overnight at -20°C in a solution of ice cold acetone (Fisher Scientific, UK) containing 2mM phenylmethylsuphonyl fluoride (Sigma and Aldrich, UK) and 20 mM iodoacetamine (Sigma and Aldrich, UK). Following overnight fixing of tissue sections, the fixative solution was replaced with acetone for 15 minutes prior to incubation in benzoate for a further 15 minutes. Next, the tissue sections were infiltrated with a 5% methyl benzoate in GMA solution A (JB-4 Embedding Kit; Polysciences, Inc.) at 4°C for 6 hours. The infiltration/processing solution was chosen to be similar to the final embedding solutions as it saturates the tissue and builds a supporting structure for the following processing steps. GMA Solution A (JB4 embedding Kit, Polysciences, Inc., UK), part of the final embedding solution which was also used in the previous processing step, comprises the following range of constituents (1,4 Dioxane, 2-Hydroxyethyl

methacrylate, 4-Methoxyphenol, Acetaldehyde, Ethylene oxide, Formaldehyde, Poly(ethylene oxide).

In addition, to GMA Solution A the embedding kit contains benzoylperoxide and GMA Solution B (JB-4 Embedding Kit; Polysciences, Inc.). Benzoylperoxide acts as a plasticiser whereas Solution B acts as the catalyst in this process. Solution B contains 2,4,6(1H,3H,5H)-Pyrimidinetrione,5-phenyl-1-(phenylmethyl) and Poly(ethylene oxide).

Tissue sections were placed in flat bottom tubes and the freshly prepared embedding solution (solution B) was added to the brim of the tube prior to placing the samples in the fridge to polymerise at 4°C. Following 48 hours polymerisation time, samples were then safely stored at -20°C until cutting.

7.3.2.3 Cutting of sections

Fixed tongue sections were cut at a thickness of 2 µm using a Reichert – Jung 2050 SuperCut Programmable Microtome and floated onto water containing 1 % ammonia solution for 1 to 1.5 minutes to avoid shrinking and creasing of the tissue sections. Floating sections were picked up onto labelled poly-L-lysine coated microscope slides. Samples were next dried for at least one hour at room temperature before commencing the staining process.

7.3.2.4 Primary antibody Titration

7.3.2.4.1 Cocaine

The antibody used to visualise the presence of cocaine in tissue was a monoclonal benzoylecgonine antibody at a stock concentration of 3.5 mg/mL (Alere^{TM}, Abingdon, UK). Several dilutions of the primary monoclonal antibody were prepared

to determine the best working antibody concentration. Dilution factors and working antibody concentrations were summarised in Table 7.1.

Table 7.1 Summary of dilution factors and working antibody concentrations used to determine best resolution within the tissue section

Neat antibody concentration	Dilution factor	Working antibody concentration
3.5 mg/mL	1 : 50	70 µg/ml
	1 : 100	35 µg/ml
	1 : 200	17.5 µg/ml
	1 : 400	8.75 µg/ml
	1 : 800	4.38 µg/ml
	1 : 1600	2.19 µg/ml

In addition to sections which were stained using antibodies, a second section of each tongue was covered using TBS which acted as control.

7.3.2.5 Isotype Controls

For the purpose of the work undertaken in this chapter, IgG was selected as the isotype antibody. The stock solution of the isotype IgG had a concentration of 1 mg/mL. Dilution factors, working antibody concentrations and subsequent isotype dilutions are summarised in Table 7.2.

Table 7.2. Summary of dilution factors, working antibody concentrations and isotype concentrations used during isotype testing in paraffin for porcine tongue sections previously exposed to cocaine

Neat antibody concentration	**Dilution factor**	**Working antibody concentration**	**Isotype dilution**
3.5 mg/mL	1 : 50	70 µg/ml	1 :14
	1 : 100	35 µg/ml	1 : 28
	1 : 200	17.5 µg/ml	1 : 57
	1 : 400	8.75 µg/ml	1 : 114
	1 : 800	4.38 µg/ml	1 : 228
	TBS control	Control	Control

7.3.2.6 Staining Method

The staining method applied for the detection of cocaine and heroin and their metabolites is summarised in Figure 7.4.

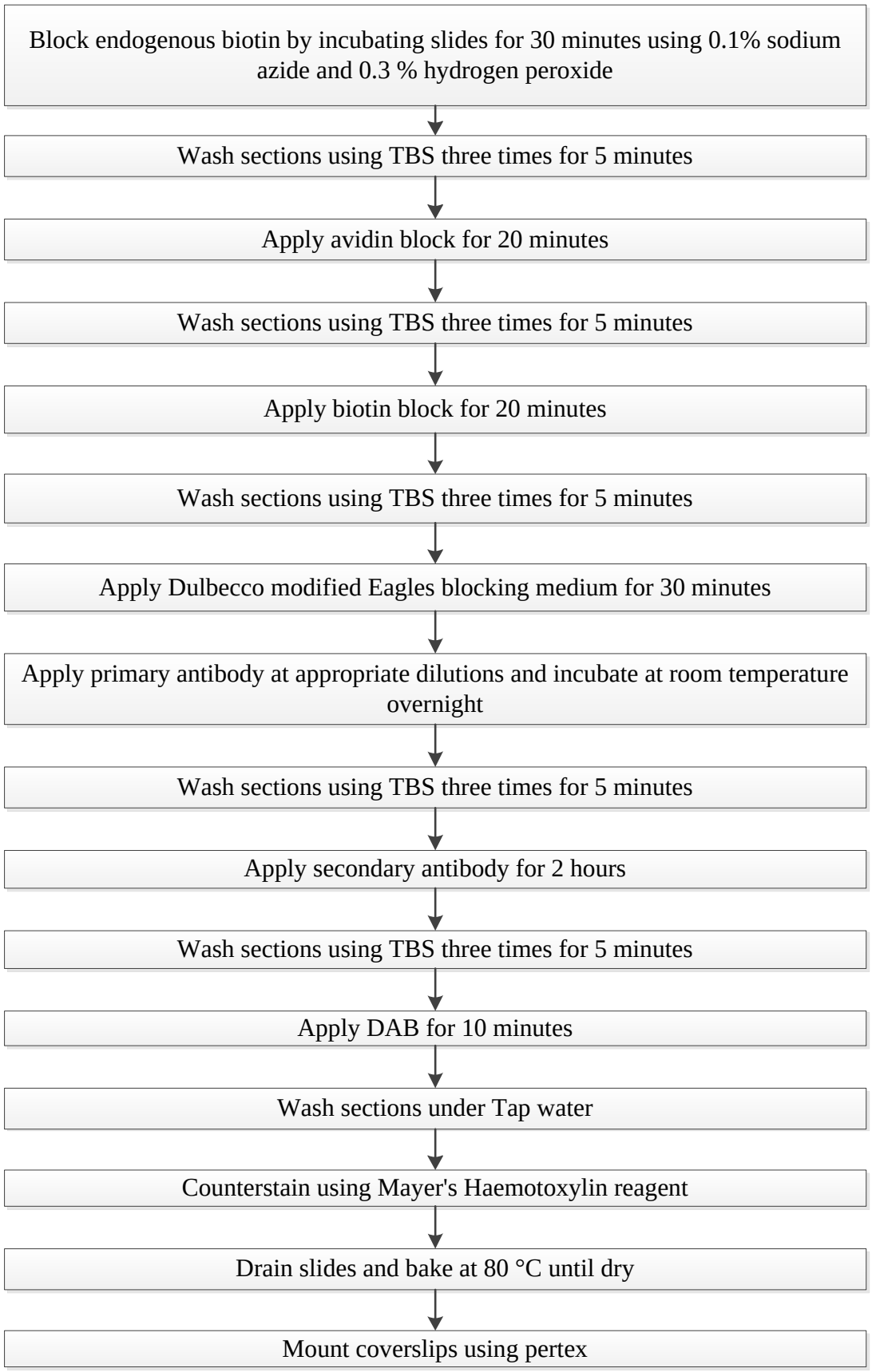


Figure 7.4. Summary of immunohistochemical staining method for the detection of cocaine and heroin in porcine tissue samples embedded in GMA resin

7.3.3 Paraffin Wax embedding

7.3.3.1 Sectioning

Full transverse cross sections were used for immunohistochemical staining when paraffin was used as an embedding medium.

7.3.3.2 Fixation

Initially, transverse cross sections were fixed in 10% formalin for 24 hours however it was observed that tissue sections were not completely fixed after 24 hours and that the centre of the sections were soft and unusable for processing into paraffin. The time and amount of fixation can vary depending on the density of the tissue. Muscle tissue is dense and hence the infiltration of the fixation agent was slower. The fixation time was subsequently extended to 48 hours allowing complete penetration of the fixative into the section of tongue. On completion of the fixation stage, tongue sections were processed into paraffin wax prior to sectioning.

7.3.3.3 Cutting of sections

In comparison to GMA resin, tissue sections embedded in paraffin wax were cut at a thickness of 4 µm using a Leica rotary microtome. Sections were floated on to a 40 ºC water bath for a minimum of one minute in order to avoid shrinking and creasing of the tissue sections. Floated sections were picked up using labelled 3-aminopropyltriethoxysilane (APES) coated microscope slides to improve adhesion of tissue to the slides. It is recommended that slides then need to be incubated for 24 hours at 37 ºC, however due the large size of the tongue sections used in the in-vitro studies, slides were incubated for 48 hours.

7.3.3.4 De-Paraffinisation

Prior to staining the sections needed to be de-waxed in clearene twice for five minutes. This removed the embedding media, as non-removal of excess media could result in non-specific binding and reduced resolution within the section. Sections are then re-hydrated in different strengths of alcohol (up to 70% v/v) for five minutes each.

7.3.3.5 Antigen Retrieval Methods

7.3.3.5.1 Microwave treatment

During microwave pre-treatment de-waxed sections were placed in a 0.01 M citrate buffer (pH of 6) then placed in an 800W SHARP microwave for 25 minutes at 50% power and heated to unmask the epitopes of interest.

7.3.3.5.2 Pronase treatment

During pronase pre-treatment samples were covered with a pronase solution which was prepared by adding 0.1 mL of 1% pronase stock to 1.9 mL of TBS. Slides were then incubated at room temperature for approximately 10 minutes.

7.3.3.5.3 No pre-treatment

Slides not undergoing pre-treatment were covered using TBS to prevent dehydration of the sections whilst performing pre-treatments on the remaining slides.

7.3.3.6 Primary antibody Titration

7.3.3.6.1 Cocaine

The antibody used to visualise the presence of cocaine and its metabolites in tissue was a monoclonal benzoylecgonine antibody at a stock concentration of 3.5 mg/mL

(Alere Toxicology, Abingdon, UK). The cross reactivity of the antibody is summarised in Appendix D. Several dilutions of the primary monoclonal antibodies were prepared to determine the best working antibody concentration. Dilution factors and working antibody concentrations for the optimization of the monoclonal benzoylecgonine antibody for use with paraffin sections are summarised in Table 7.3.

Table 7.3 shows the a summary of the working antibody concentrations tested during optimisation of monoclonal benzoylecgonine antibody for immunohistochemical staining with paraffin as the embedding medium

Antibody concentration	**Dilution factor**	**Working antibody concentration**
3.5 mg/mL	1 : 100	35 µg/ml
	1 : 200	17.5 µg/ml
	1 : 400	8.75 µg/ml
	1 : 800	4.38 µg/ml
	TBS control	Control

7.3.3.6.2 Heroin

The antibody used to visualise the presence of heroin and its metabolites in tissue was a monoclonal morphine antibody at a stock concentration of 5.47 mg/mL (Alere Toxicology, Abingdon, UK). The cross reactivity data of the morphine antibody is summarised in Appendix E. This was at a higher concentration than the monoclonal benzoylecgonine antibody and as a result the dilution factor of 1 in 50 was not tested during the antibody titration process. Dilutions factors and working antibody concentrations for the optimization of the monoclonal morphine antibody in paraffin are summarised in Table 7.4.

Table 7.4 Summary of the working antibody concentrations tested during optimisation of monoclonal morphine antibody for paraffin wax embedded sections

Antibody concentration	Dilution factor	Working antibody concentration
5.47 mg/mL	1 : 100	54.7 µg/ml
	1 : 200	27.4 µg/ml
	1 : 400	13.7 µg/ml
	1 : 800	6.8 µg/ml
	TBS control	Control

7.3.3.7 Isotype Controls

7.3.3.7.1 Cocaine

Benzoylecgonine antibody concentrations were calculated from the stock antibody concentration of 3.5 mg/mL (Table 7.5)

Table 7.5. Summary of dilution factors, working antibody concentrations and isotype concentrations in paraffin used during isotype testing in paraffin for porcine tongue sections previously exposed to cocaine

Antibody concentration	Dilution factor	Working antibody concentration	Isotype dilution
3.5 mg/mL	1 : 100	35 µg/ml	1 : 28
	1 : 200	17.5 µg/ml	1 : 57
	1 : 400	8.75 µg/ml	1 : 114
	1 : 800	4.38 µg/ml	1 : 228
	TBS control	Control	Control

7.3.3.7.2 Heroin

Monoclonal morphine antibody concentrations were calculated from the stock antibody concentration of 5.47 mg/mL (Table 7.6).

Table 7.6. Summary of dilution factors, working antibody concentrations and isotype concentrations used during isotype testing in paraffin for porcine tongue sections previously exposed to heroin

Antibody concentration	Dilution factor	Working antibody concentration	Isotype dilution
5.47 mg/mL	1 : 100	54.7 µg/ml	1 : 18
	1 : 200	27.35 µg/ml	1 : 36
	1 : 400	13.68 µg/ml	1 : 73
	1 : 800	6.84 µg/ml	1 : 146
	TBS control	Control	Control

7.3.3.8 Staining method

The staining method applied for the detection of cocaine and heroin and their metabolites is summarised in Figure 7.5.

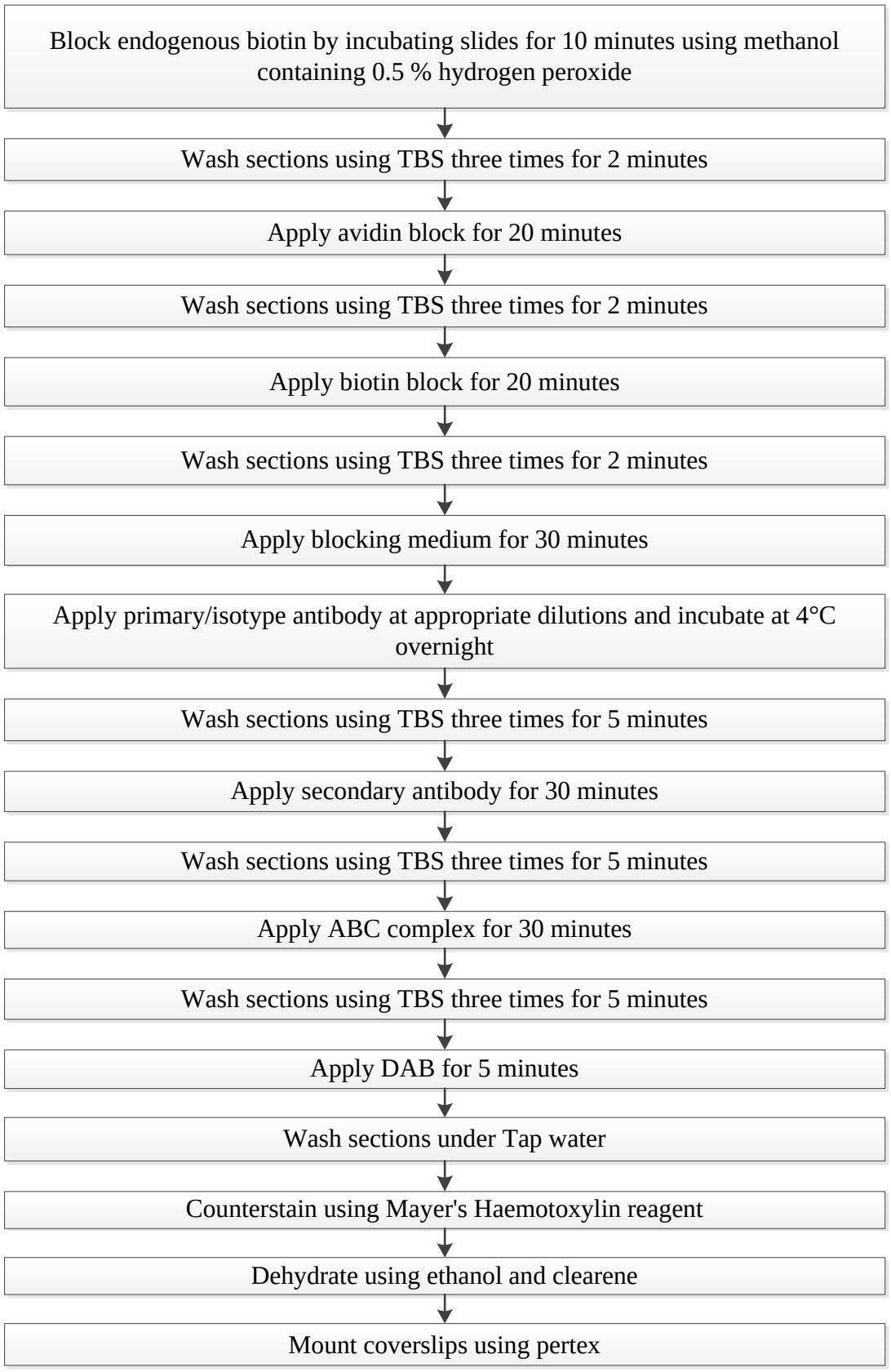


Figure 7.5. Summary of immunohistochemical staining method for the detection of cocaine and heroin in porcine tissue samples embedded in Paraffin wax

7.3.4 Visual Examination – GMA and Paraffin

Slides were visually examined on a Leica microscope which was fitted with a Nikon Coolpix camera for photographing transverse cross sections.

7.3.5 Quantification of staining – GMA and Paraffin

For the quantification of staining Alere Toxicology provided the Alere^{TM} DDS software which uses a colour filter to highlight all pixels given within the colour range brown before quantifying it as the ration of highlighted pixels over the total number of pixels within a section. The result is then given as a percentage staining within the section.

7.4 Results and Discussion

7.4.1 GMA

7.4.1.1 Antibody titration

The presence of drug was indicated by a brown colour which is a result of the reaction between DAB and the peroxidase from the avidin-biotin complex. Higher antibody concentrations resulted in non-specific binding of other cells whereas the lowest concentration showed weak staining that was barely visible under the microscope (Figure 7.6). No dark brown staining indicated samples were negative and do not show any binding.

Negative result for cocaine **Positive result for cocaine**

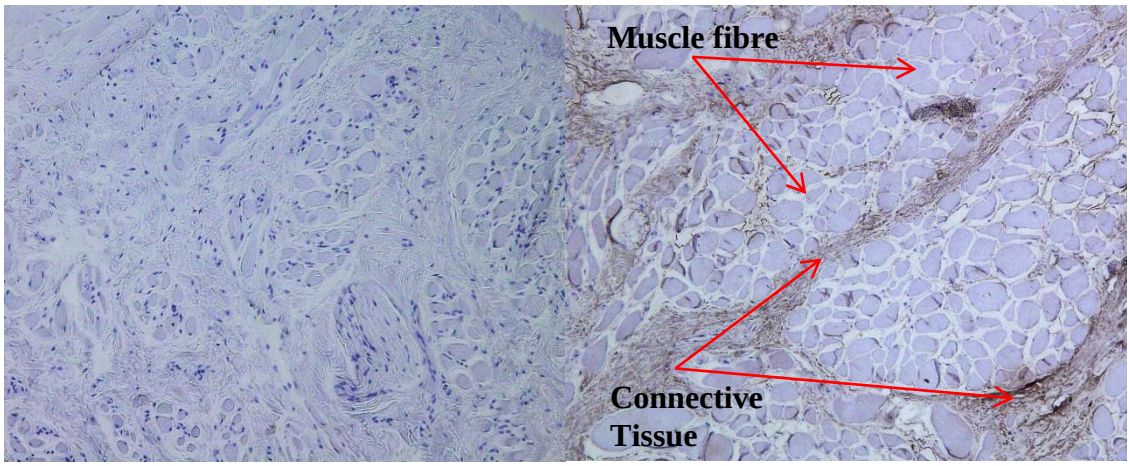


Figure 7.6. Comparison between negative and positive sections which were stained using a monoclonal benzoylecgonine antibody (8.75 µg/mL). A positive is indicated by brown pigmentation (magnification x25)

TBS controls were negative and hence did not show any brown staining which indicated that no non-specific binding occurred (Figure 7.7).

TBS Control

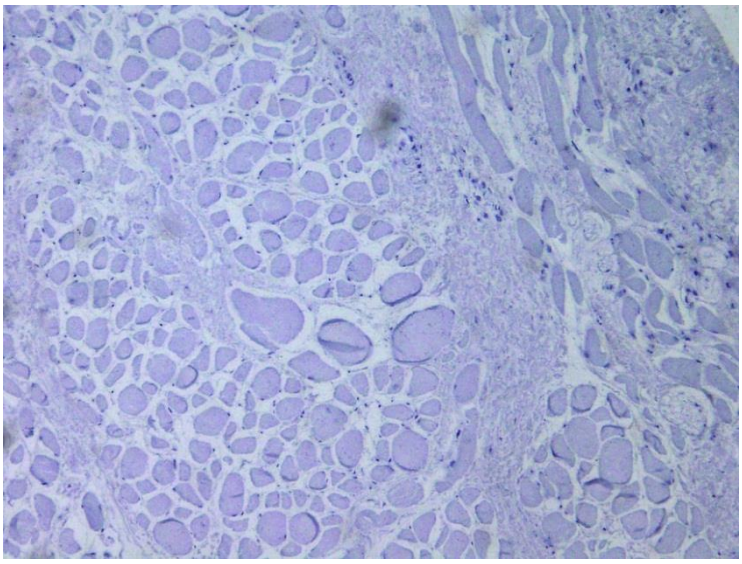


Figure 7.7 Visual results of TBS control in porcine tongue tissue (magnification x 25)

Staining results showed that in the largest dilution (1: 600) no drug was detected as the working antibody concentration (2.16 µg/mL) was too weak. Staining increased with decreasing dilution factor. In the lowest dilution (1:50) staining was observed to be present throughout the tissue indicating that the antibody concentration (70 µg/mL) was too high and that staining was non-specific. Optimal staining was observed in sections staining with an antibody working concentration of 8.75 µg/mL as staining was clear but specific to selected structures within the tissue (Figure 7.8).

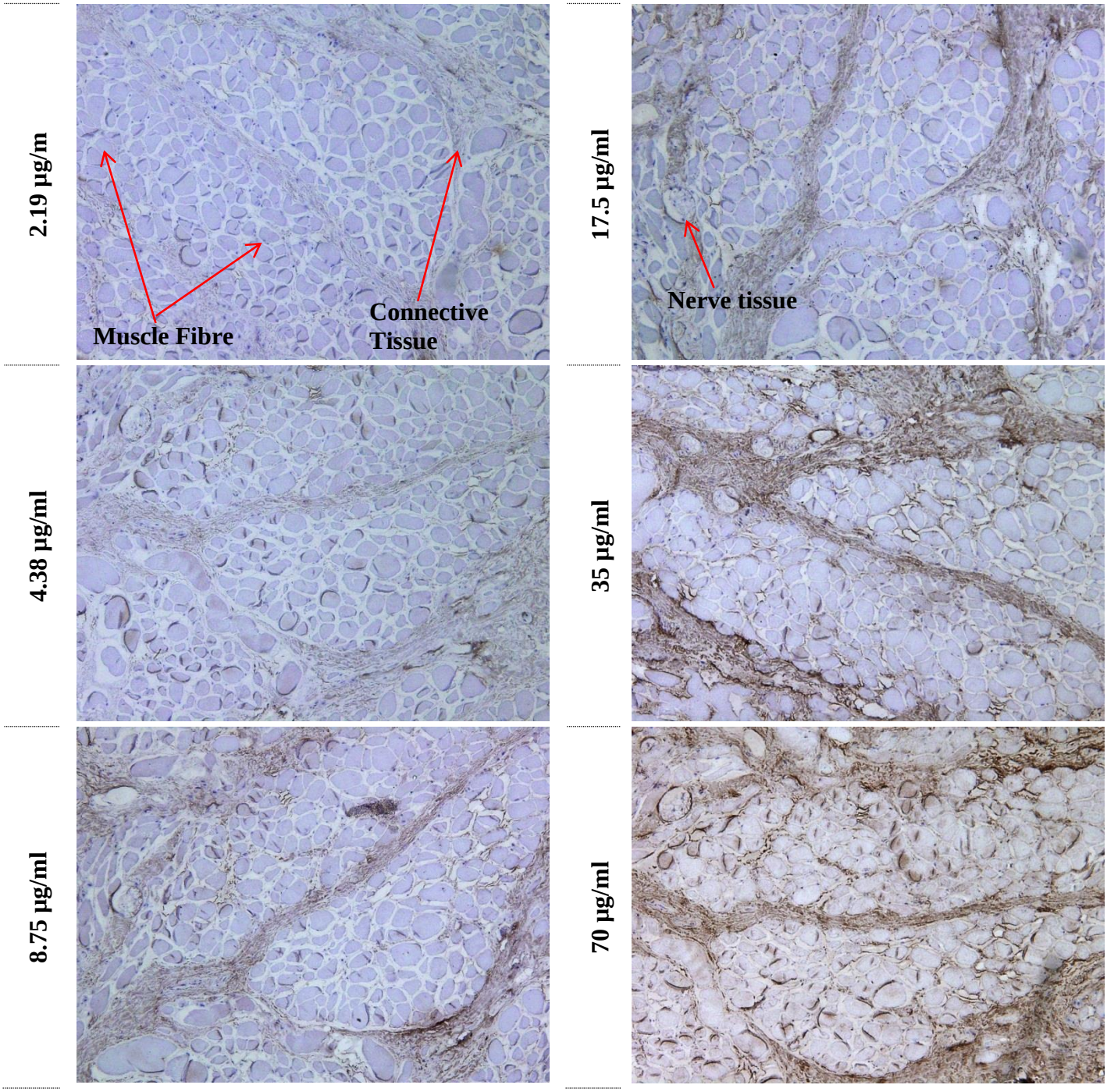


Figure 7.8 Visual results of porcine tongue tissue which was stained benzoylecgonine antibody at selected working antibody concentrations (magnification x25)

7.4.1.2 Isotype Controls

Sections of porcine tongues previously exposed to cocaine and stained using a monoclonal benzoylecgonine antibody showed positive brown staining for cocaine or cocaine related compounds. Isotype, as well as TBS controls, did not result in a positive stain for cocaine. Negative isotype controls confirmed that the primary antibody is specific and that positive staining observed is not a result of non-specific binding to unwanted receptor or cellular proteins. Therefore, the benzoylecgonine antibody was shown to be specific for the detection of cocaine and its metabolites when applied during the GMA immunohistochemical staining technique (Figure 7.9).

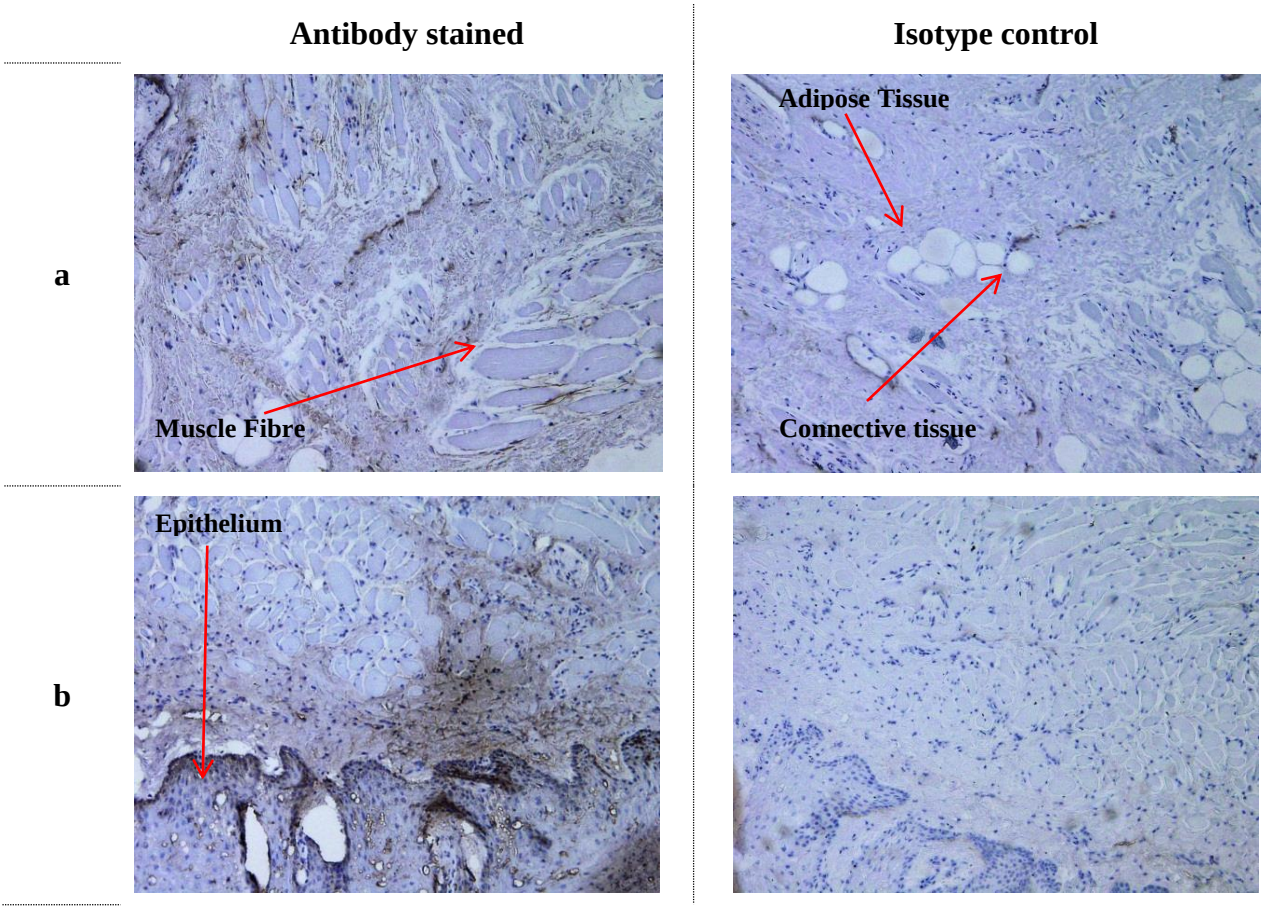


Figure 7.9 Summarisation of results for isotype controls and antibody stained sections in tongues exposed to 100 ng/mL (a) and 1000 ng/mL (b) of cocaine which were embedded in GMA resin (magnification x25)

7.4.1.3 Summary of results from the application of the staining method

The GMA method showed promising results when staining for cocaine ensuring the specificity of the antibody. Isotype controls and TBS controls were both negative, confirming that the positive staining which was observed within stained sections, was a result of the presence of the drug and was not non-specific binding. However, during visual examination of the slides, it was noted that important features, such as blood vessels and epithelium were destroyed during the removal of the biopsy samples from the transverse cross sections. Features, such as blood vessels were vital to identify whether the drug is able to easily travel across the blood vessel wall and hence contaminate the blood. Therefore, GMA was deemed an unsuitable embedding medium for the measurement of drug distribution.

7.4.2 Paraffin Wax

7.4.2.1 Antigen Retrieval methods

Best immunohistochemical results were achieved when no antigen retrieval methods were applied. The heating process during microwave treatment caused the sections to float off the slides. This was a result of the large size of the tissue section and the large surface area needed for the section to stick. Pronase pre-treatment resulted in clear staining similarly to sections not treated with any antigen retrieval method. As results did not differ significantly between the pronase method and when no pre-treatment was applied, for the ease of the method, in all later staining no antigen pre-treatment method was applied.

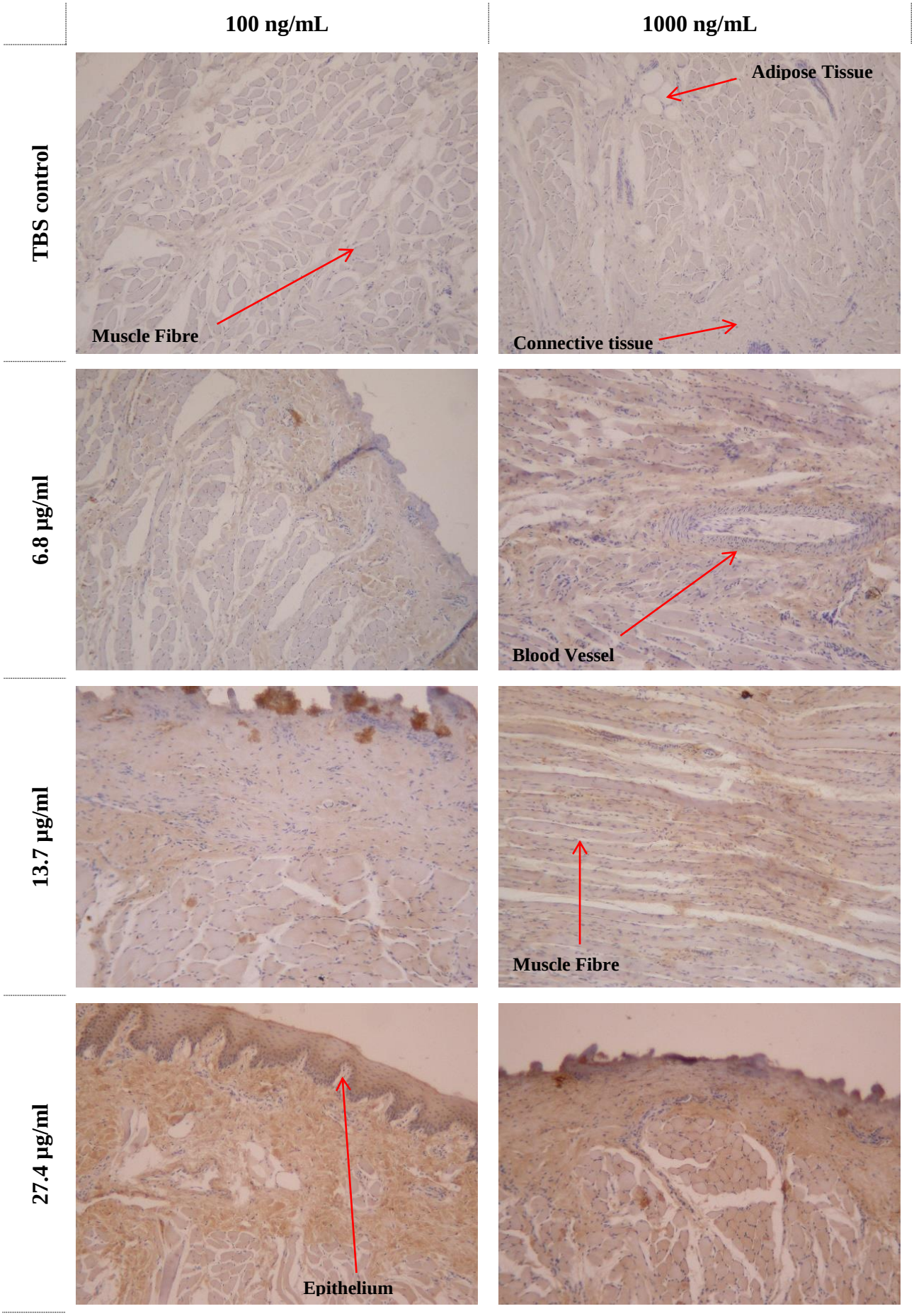
7.4.2.2 Antibody Titration

7.4.2.2.1 Cocaine

Best resolution of staining was achieved at an antibody concentration of 35 µg/ml (1 in 100 dilution).

Lowest antibody concentration resulted in weak staining in tongues exposed to a lower cocaine concentration of 100 ng/mL.

High antibody concentrations resulted in non-specific staining as excess antibody increases non-specific reactions within the tissue which therefore resulted in background staining. In the antibody titrations the highest antibody concentrations tested of 70 µg/ml, lost resolution and increased background staining at higher concentration of drug and hence making it an unsuitable dilution of antibody. Results are summarised in Figure 7.10.


100 ng/mL
1000 ng/mL
TBS control
6.8 µg/ml
13.7 µg/ml
27.4 µg/ml
Adipose Tissue
Muscle Fibre
Connective tissue
Blood Vessel
Muscle Fibre
Epithelium

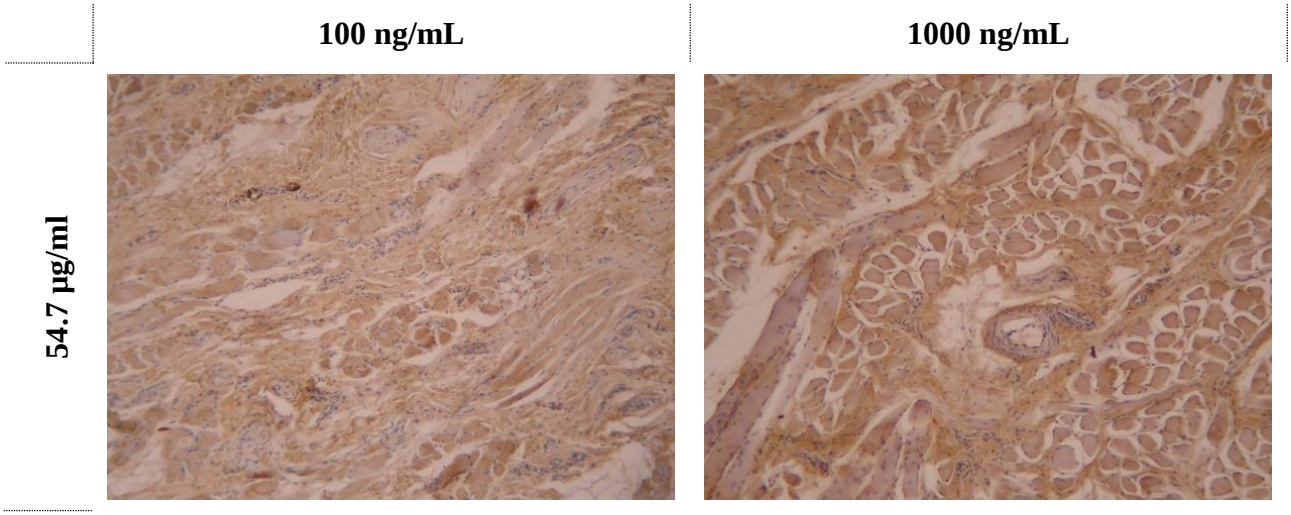
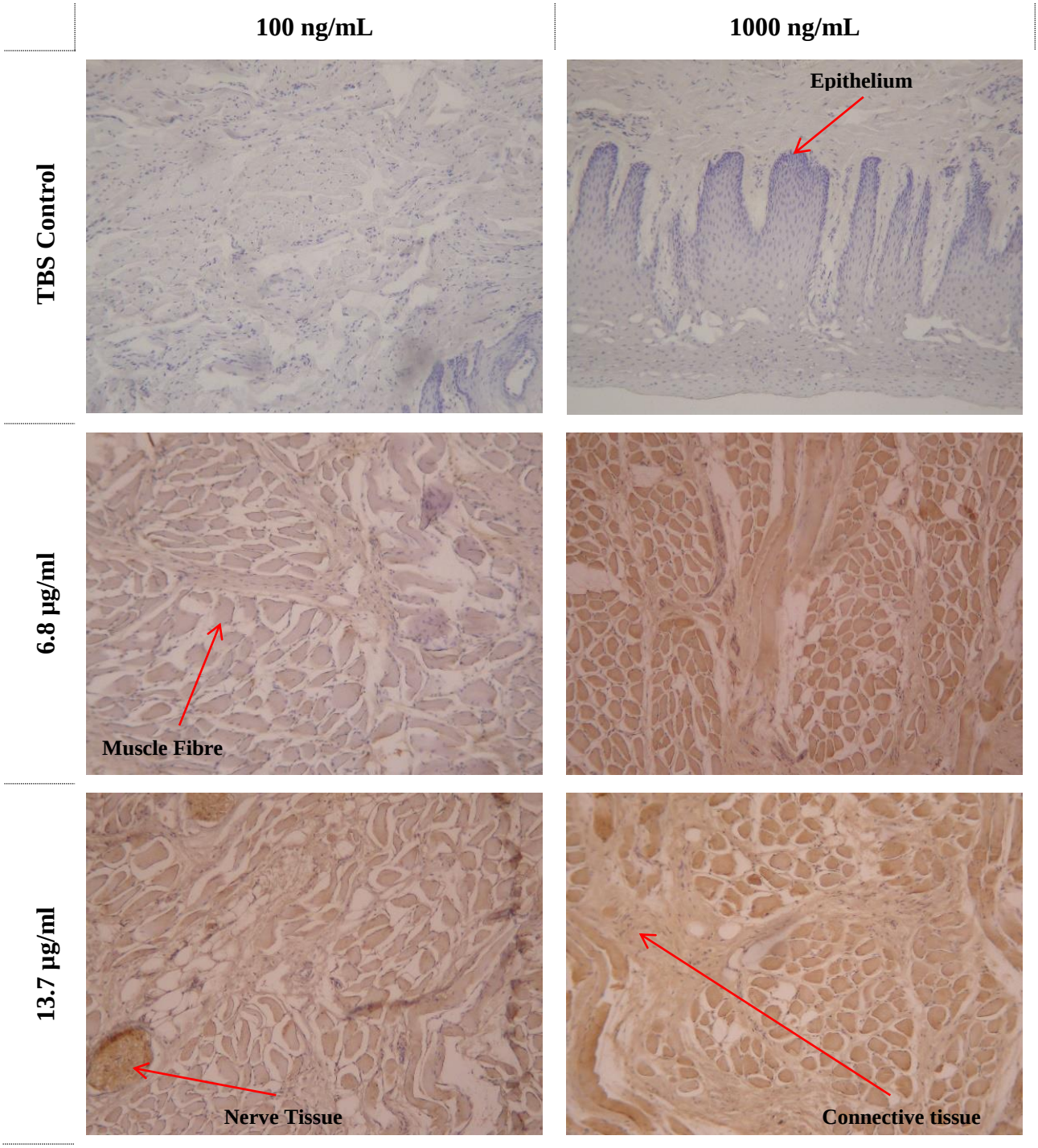


Figure 7.10 Visual summary of antibody titration of a benzoylecgonine antibody (3.5 mg/mL) for the detection of cocaine and its metabolites in porcine tongue tissues which were exposed to 100 ng/mL or 1000 ng/mL of cocaine (magnification x25)

7.4.2.2.2 Heroin

Opioid staining was observed to be more intense in color than the staining observed following exposure to cocaine. The optimum result of concentration for morphine in paraffin wax sections was achieved at a dilution of 1 in 800 resulting in a working antibody concentration of 6.8 µg/ml. Antibody titration results for heroin in paraffin wax immunohistochemistry are observed in Figure 7.11.


100 ng/mL
1000 ng/mL
TBS Control
Epithelium
6.8 µg/ml
Muscle Fibre
13.7 µg/ml
Nerve Tissue
Connective tissue

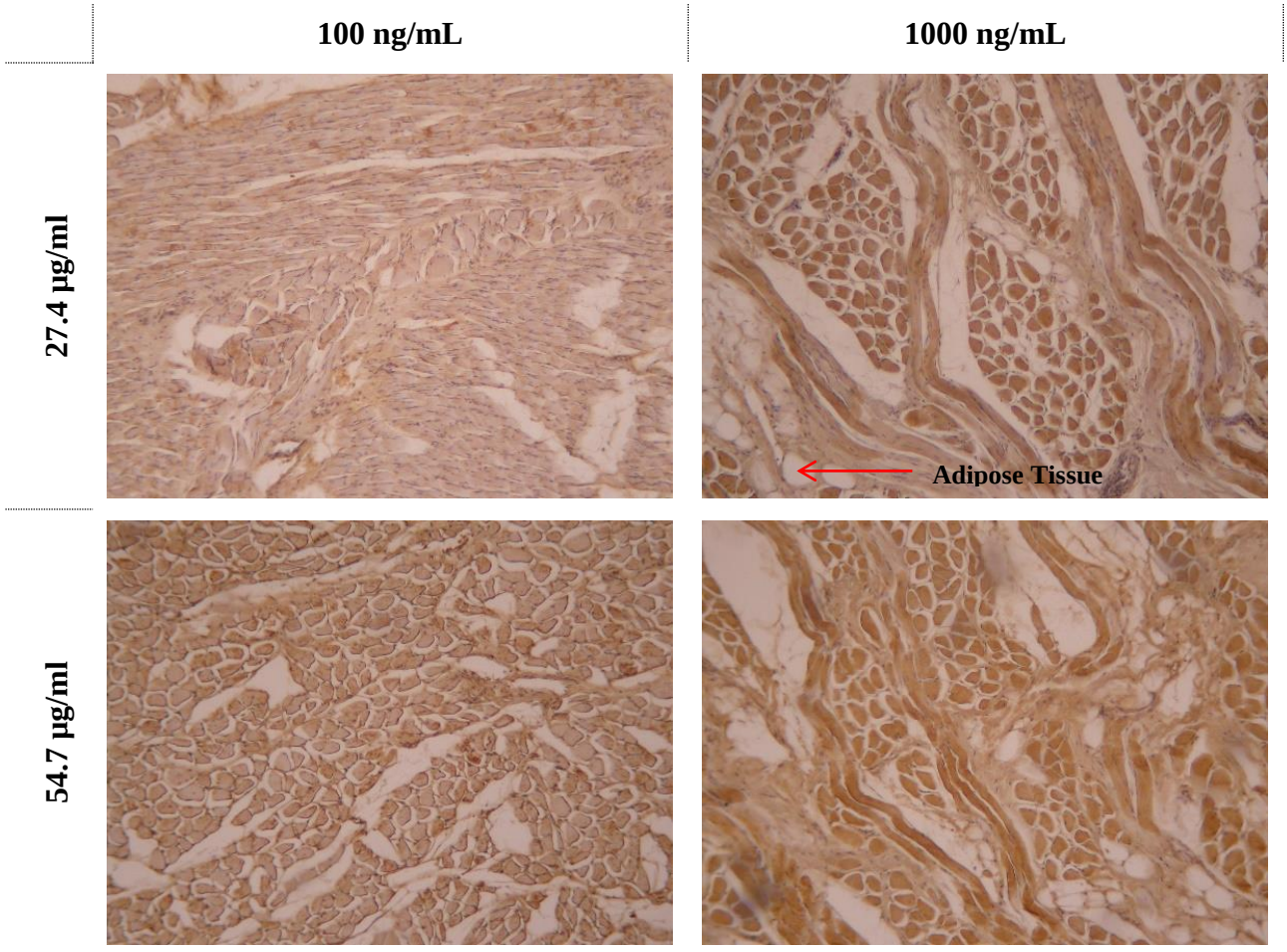


Figure 7.11 Visual summary of antibody titration of a monoclonal morphine antibody (5.47 mg/mL) for the detection of heroin and its metabolites in porcine tongue tissues which were exposed to 100 ng/mL or 1000 ng/mL of heroin (magnification x25)

7.4.2.3 Isotype Controls

Similarly to GMA, isotypes and TBS controls were negative when applied to porcine tongue sections which were embedded in paraffin wax for both, sections stained for cocaine or heroin. Therefore, the benzoylecgonine (Figure 7.12) and morphine (Figure 7.13) antibodies were shown to be specific for the detection of cocaine/ heroin and its metabolites when applied to during the paraffin wax immunohistochemical staining technique.

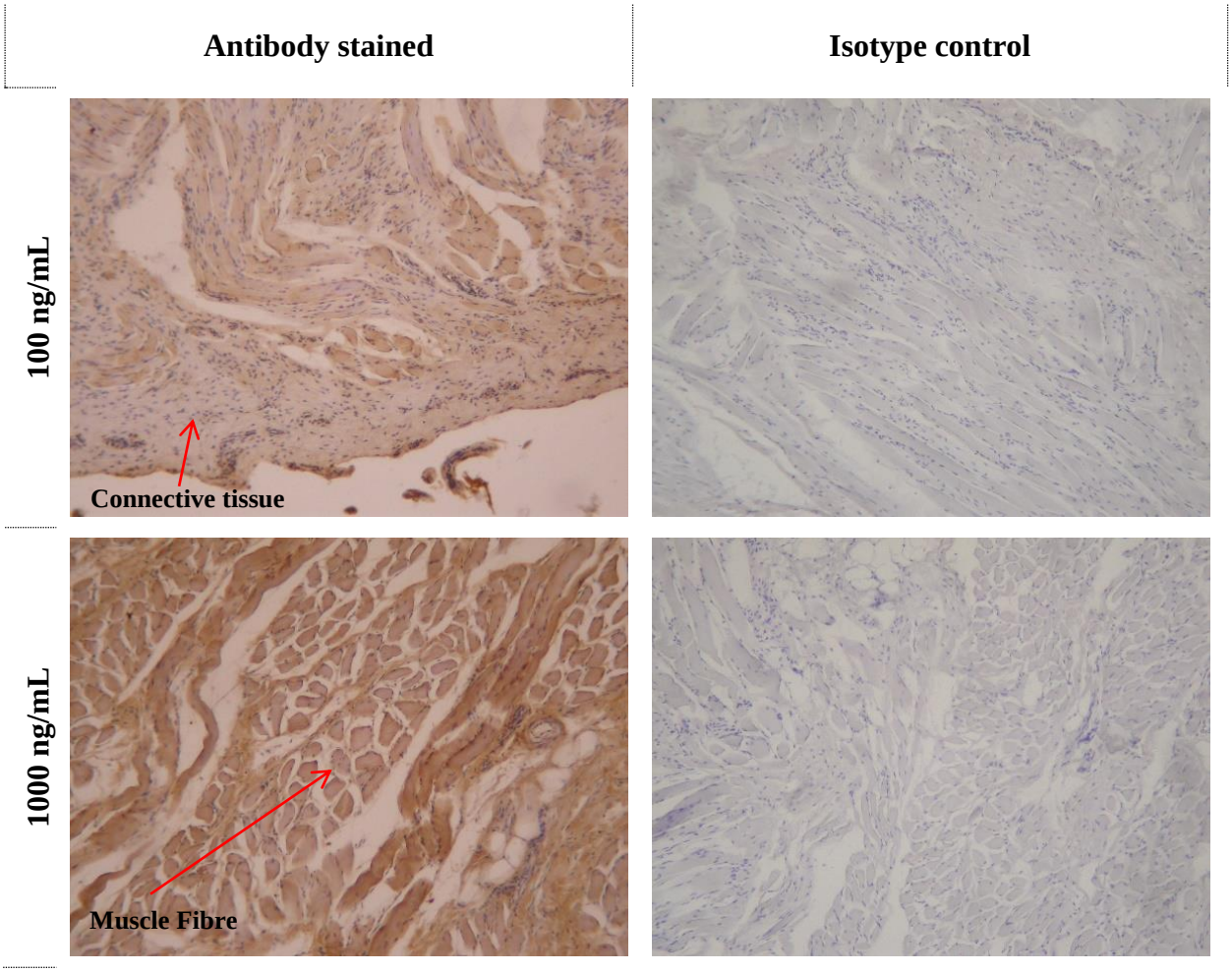


Figure 7.12 Summarisation of results for isotype and antibody stained sections in tongues exposed to different concentrations of cocaine which were embedded in paraffin resin (magnification x25)

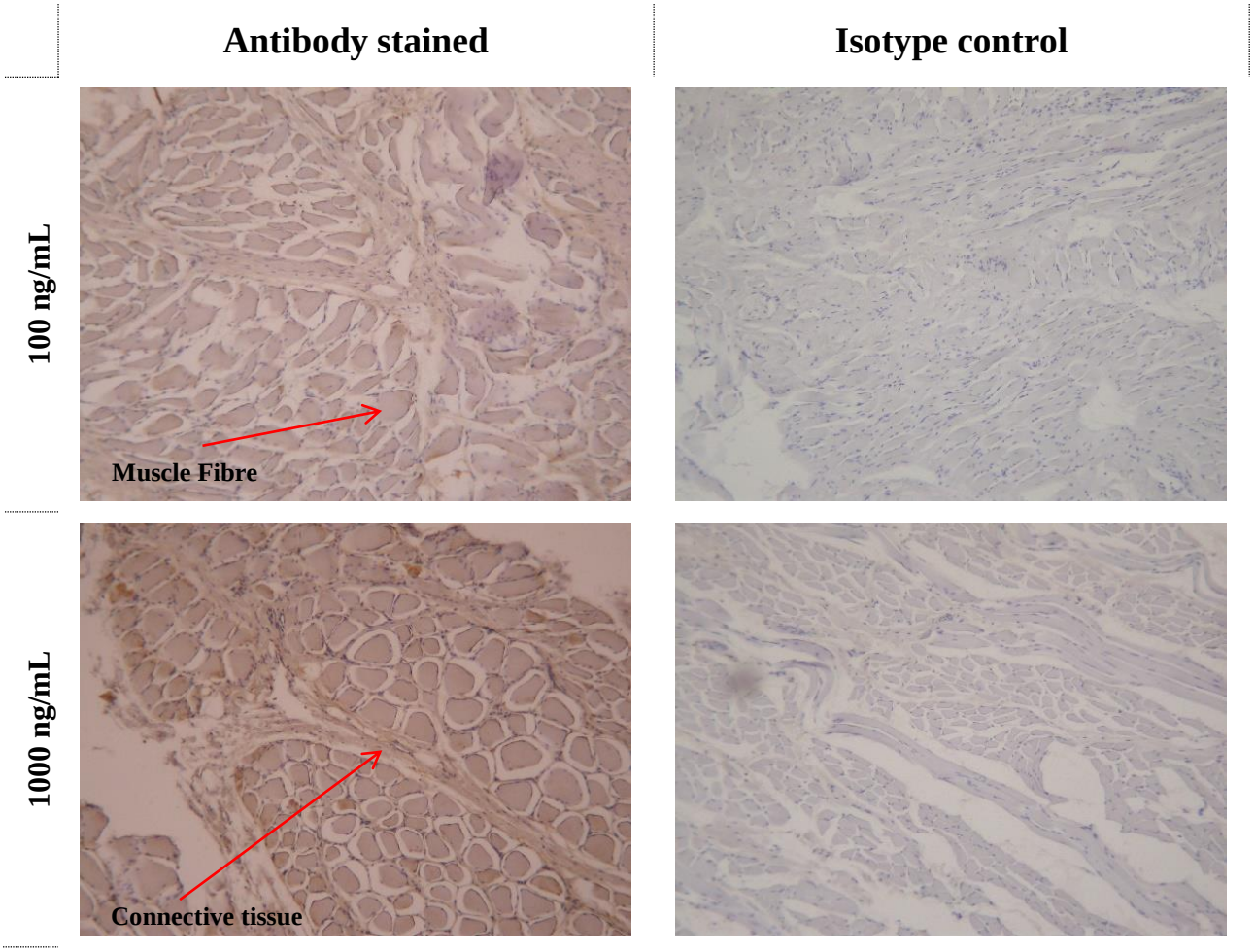


Figure 7.13 Summarisation of results for isotype and antibody stained sections in tongues exposed to different concentrations of heroin which were embedded in paraffin resin (magnification x25)

7.4.2.4 Summary of results from the application of the staining method

The paraffin wax staining method resulted in specific staining for both heroin and cocaine. Isotype controls and TBS controls were both negative, confirming that the positive staining which was observed within stained sections, was a result of the presence of the drug and was not non-specific binding. The main advantage for the use of paraffin wax as an embedding medium is that the entire transverse cross section of the porcine tongue tissue could be embedded allowing the drug distribution throughout the tongue to be observed. Paraffin wax has been reported to have less resolution during immunohistochemical analysis than GMA resin. However, the work undertaken in this chapter showed that for both cocaine and heroin, the paraffin immunohistochemical staining method was specific for tongues exposed to 100 ng/mL of either drug as well as the higher concentrations of 10 µg/mL. Overall, the paraffin wax immunohistochemical staining method was a more suitable matrix for the detection of drugs in porcine tongue tissue and was subsequently adopted for further investigation of oral tissues.

7.4.2.5 Quantification of staining

The staining could not be quantified as the staining counter-software was not specific enough to identify all the staining during image analysis. The software is based on a filter by which it quantifies the brown pixels in the image and simultaneously translates them into red pixels which are then identified by the software. However, the identification of the brown pixels is dependent on the light intensity used in the microscope, the magnification employed and intensity of staining. Additionally, the software accounts for staining at low and high intensity, and quantifies both in the same manner hence cannot differentiate between the low and the high drug

concentrations. These factors resulted in the quantification method being deemed unsuitable for use (Figure 7.14). Visual and non-quantitative analysis was subsequently adopted using a Leica DMLB microscope.

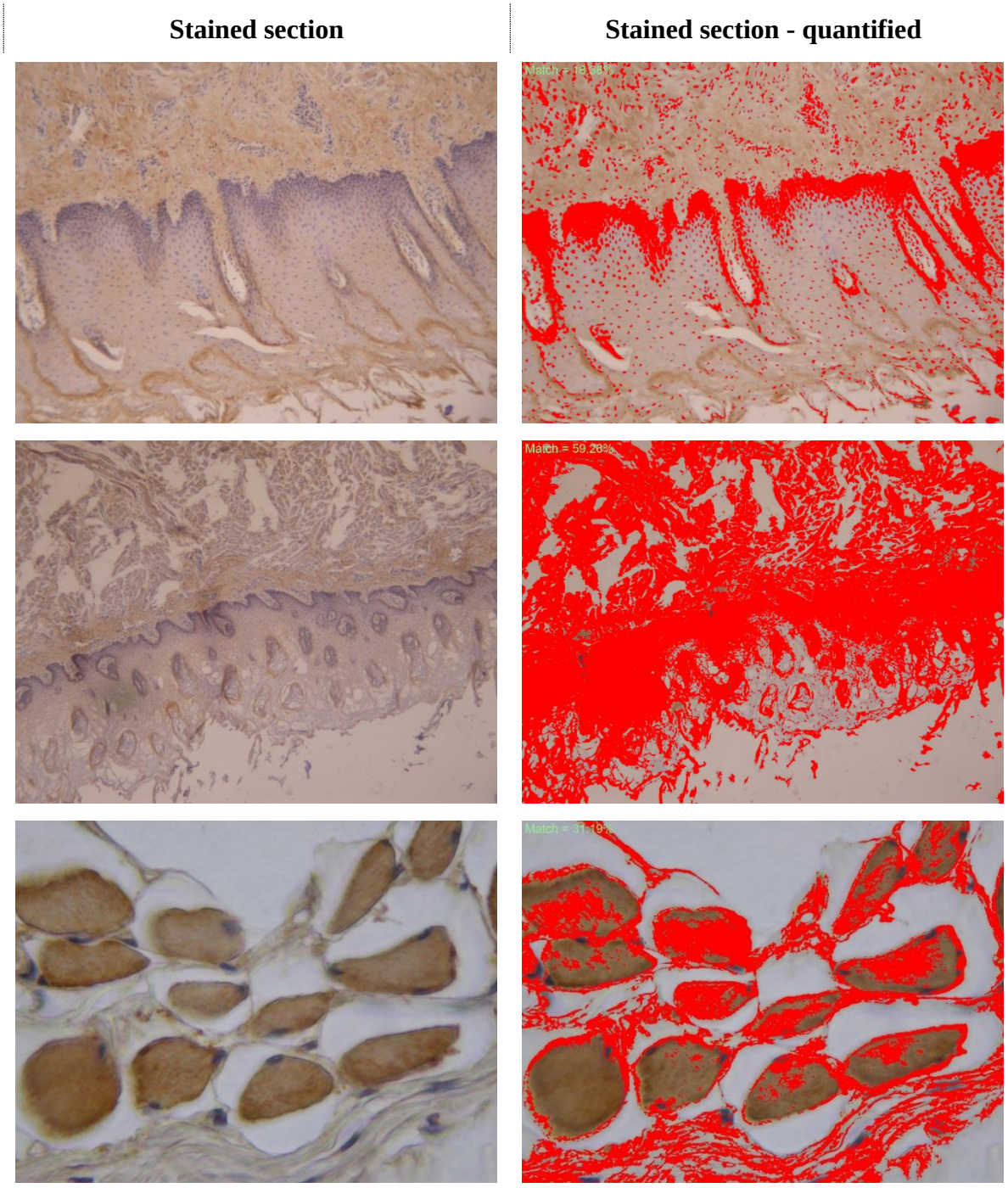


Figure 7.14 Images represent how the staining software cannot quantify the brown staining correctly due to factors such as microscope lighting, magnification and intensity (magnification x63)

7.5 Conclusion

The immunohistochemical methods for GMA resin and paraffin wax both resulted in good precise staining for cocaine and in the case of paraffin wax, heroin. However, quantification using the Alere^{TM} method was not suitable for this technique as results were not specific and hence unreliable. Although GMA immunohistochemistry staining was specific, important histological features such as blood vessels and epithelium were destroyed during the removal of the biopsy samples from the transverse cross sections. In comparison, paraffin wax allowed the embedding of whole transverse cross sections and hence should be the preferred method for any further analysis.

Chapter 8.0 - *In vitro* investigations of concentrations of cocaine and heroin in porcine tongue tissue

8.1 Introduction

Previous chapters of this thesis showed that contamination of the oral mucosa can occur following the oral consumption of drugs. High concentrations of cocaine and its derivatives have been shown to be present in oral fluid for 1 hour following the consumption of a single cup of coca tea (Chapter 4). Additionally, large concentrations of opiates were also observed when swirling Collis Browne's mixture and Codeine Linctus around the mouth for one minute (Chapter 5).

The work described in this chapter aimed to test the hypothesis that drugs which were consumed orally via solution or smoking can build up in oral tissue and form depots resulting in a slow release of drug over time.

Most manufacturers of oral fluid drug tests indicate that the effects of contamination have dispersed from the mouth after 10 - 15 minutes (Spiehler 2011) and that after this time has elapsed an oral fluid sample can then be collected with minimum risk of interference (Chapter 3). Previous results presented in this thesis clearly contradict this guideline following the exposure to oral solutions containing cocaine and opiates.

The amount of drug which distributes into tissue is dependent on its volume of distribution amongst other factors. The reported volume of distribution for cocaine is 1 -3 L/kg (Moffat et al 2011). The volume of distribution for heroin is not very well documented as it is rapidly metabolised to 6-MAM and morphine. Heroin is more lipophilic than cocaine resulting in a much higher estimated volume of distribution of

heroin 25 L/kg (Baselt 2011, Urso et al 2012). The higher volume of distribution of heroin suggests that a larger amount of drug travels into the tissue. The reported volume of distribution of morphine is between 3 - 5 L/kg (Moffat et al 2011). A second measure of lipophilicity is the partition coefficient (logP) of a drug. A log P value larger than 5 does not absorb easily into tissues. Therefore the lower the logP value, the easier the absorption into tissues. The reported logP value for cocaine is 2.3 whereas the logP value for heroin is 0.2 (Moffat et al 2011).

The experiments described in this chapter aimed to investigate the possible formation of drug depots in oral tissue by designing an *in vitro* model to model the exposure of drugs to porcine tongues either via solution or smoke.

8.2 Materials and Methods

8.2.1 Materials

Certus oral fluid collectors were provided by Alere Toxicology plc, Abingdon, UK.

Artificial saliva was prepared as described in Appendix D.

Street heroin and crack cocaine were provided by John Ramsey, TICTAC communications, St Georges Medical School, University London.

In vitro experiments involved the use of porcine tongues due to their physiological and anatomical similarities to human tissue (Simon and Maibach 2000). Porcine tongues were purchased from The Village Butcher, Kingsclere.

7.2.1.2. Porcine Tongue Histology

The tongue is the floor of the mouth and is made of skeletal muscles which is covered by mucous membrane. The primary function of the tongue is to detect taste and to assist chewing and swallowing of food.

The top/dorsal surface of the tongue is made of two parts which are the anterior part and posterior part of the tongue, also known as the body and root of the tongue and are separated by a membrane - the sulcus terminalis. The outside layer of the tongue is a layer of oral mucosa with the addition of taste buds. This special mucosa forms papillae. Papillae are projections of the lamina propria which are covered with stratified squamous epithelium. The tongue contains 4 different types of papilliae, the filiform, fungiform, circumvallate and foliate. As well as papillae the tongue contains taste buds, which contain receptor cells to communicate with the taste pore. The structure of porcine tongue is shown in Figure 8.1.

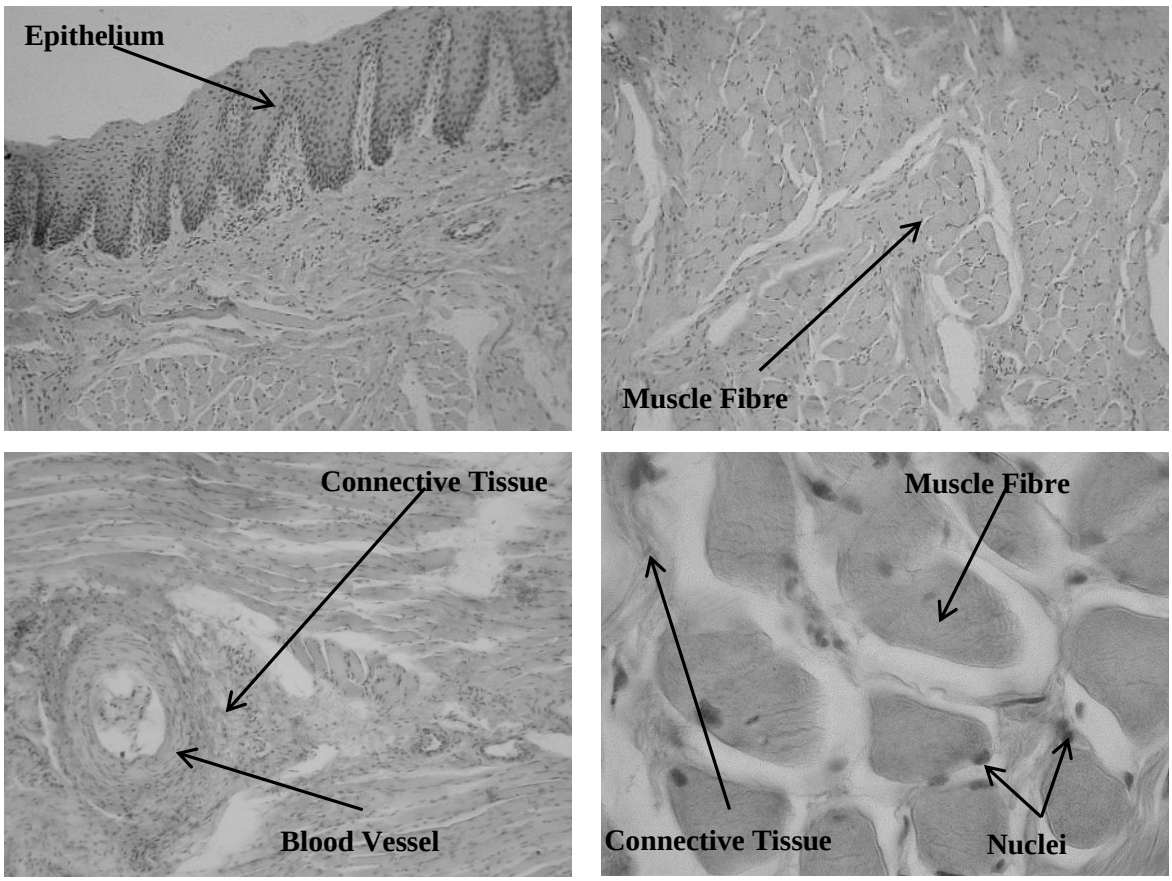


Figure 8.1 shows the structure of porcine tongue tissue

8.2.2 Methods

8.2.2.1 Tongue preparation methods

8.2.2.1.1 Tissue exposure to drug solutions of cocaine and heroin

Tongues were immersed in 50 mL artificial saliva containing cocaine or heroin at concentrations of 100 ng/mL, 250 ng/ml, 500 ng/mL, 1000 ng/mL and 10 µg/mL for 10 minutes whilst being stirred on magnetic stirrer (Figure 8.2).

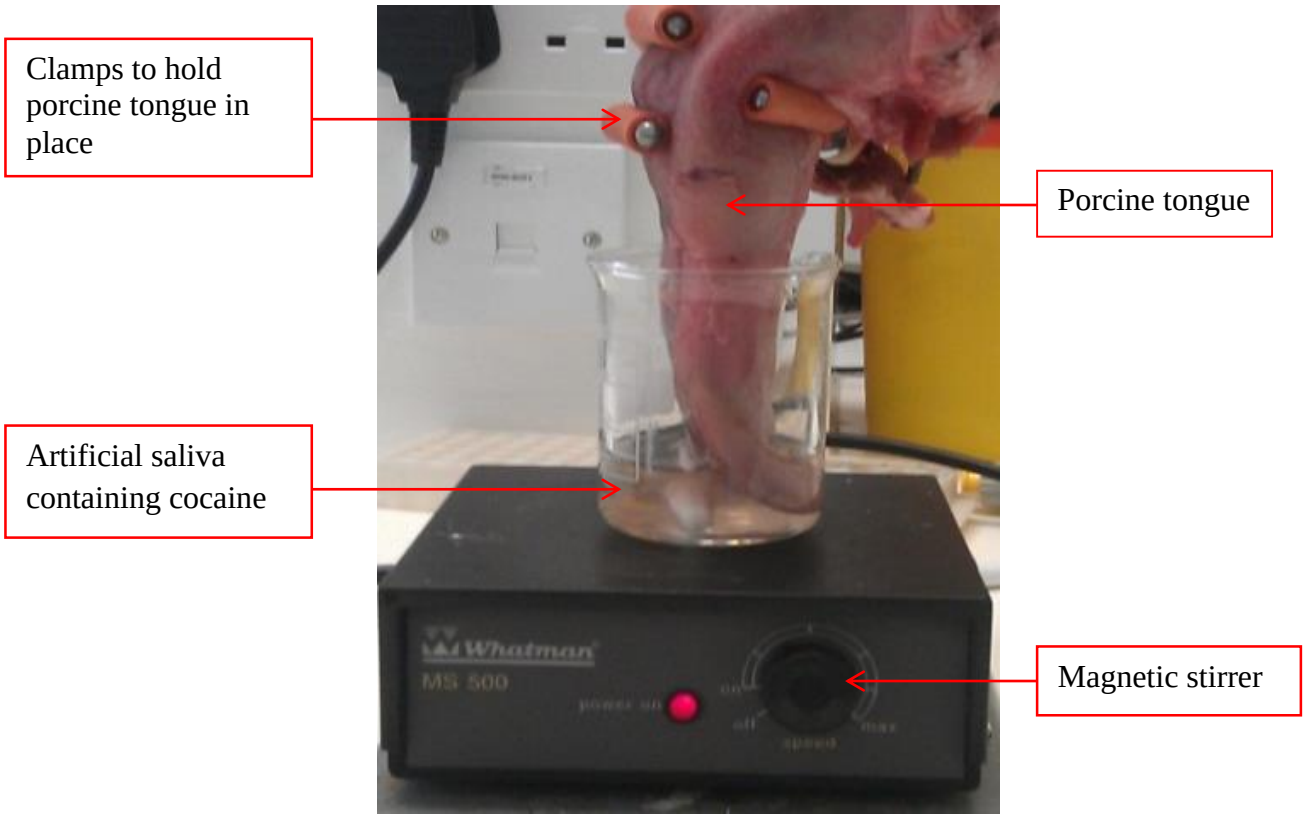


Figure 8.2 Model for exposure of porcine tongues to either cocaine or street heroin spiked into artificial saliva at concentration of 100 ng/mL, 250 ng/mL, 500 ng/mL, 1000 ng/mL or 10 µg/mL

After 10 minutes exposure to the drug solution, tongues were removed and washed in 50 mL of artificial saliva for 1 minute whilst being stirred on a magnetic stirrer. This process was repeated seven times. The wash steps were introduced in order to mimic the constant washing of the oral cavity with oral fluid in the mouth. Then a Certus collector was pre-wetted with artificial saliva and then brushed against the washed porcine tongue following the last wash.

8.2.2.1.2 Tissue exposure to smoking from either crack cocaine or street heroin

Tongues were individually suspended in a smoking chamber. In order to ensure that tongues were all treated the same way they were suspended at the same height during

each exposure run. Crack cocaine or street heroin was heated using a Bunsen burner whilst a vacuum at the other end of the smoking chamber provided sufficient suction to mimic smoking conditions. The vacuum created airflow was measured using a kestrel 3000 and was detected to be 0.5 m/s. Exposure was performed until smoking ceased, but for no more than 5 minutes. Following exposure to cocaine or heroin, samples were immersed in 10 % neutral buffered formalin in preparation for subsequent immunohistochemical analysis (Figure 8.3).

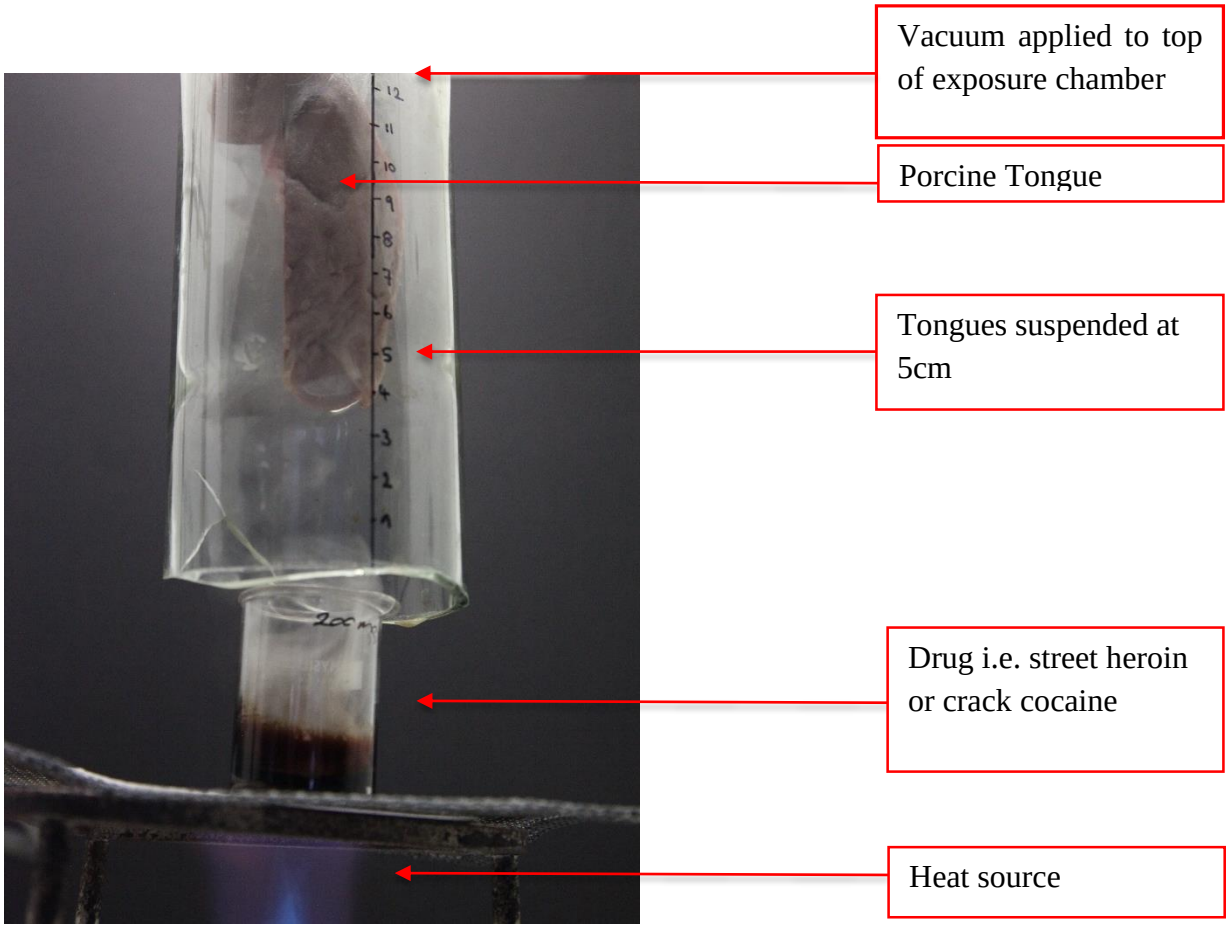


Figure 8.3. Model for the smoke exposure of crack cocaine or street heroin at a dose of either 100 mg or 200 mg

8.2.2.1.3 Investigation into drug release from tongue tissue over time

Tongues were immersed in artificial saliva containing cocaine or heroin at a low and high concentration of 100 ng/mL or 1000 ng/mL for 10 minutes with vigorous stirring on magnetic stirrer. This was in keeping with the previous laboratory model for the exposure of porcine tongues to a solution (Figure 8.2). Following exposure to either cocaine or heroin, tongues were suspended in artificial saliva for either 1 hour, 6 hours, 24 hours or 48 hours. Irrespective of the time exposed after the first hour, the artificial saliva wash was changed to prevent the tongues being exposed to potentially drug-rich artificial saliva resulting from the drug being initially washed off the tongue.

8.2.2.2 Immunohistochemical methods

The immunohistochemical method for paraffin embedding developed and described in Chapter 7 was applied for the work undertaken in this chapter (Figure 8.4).

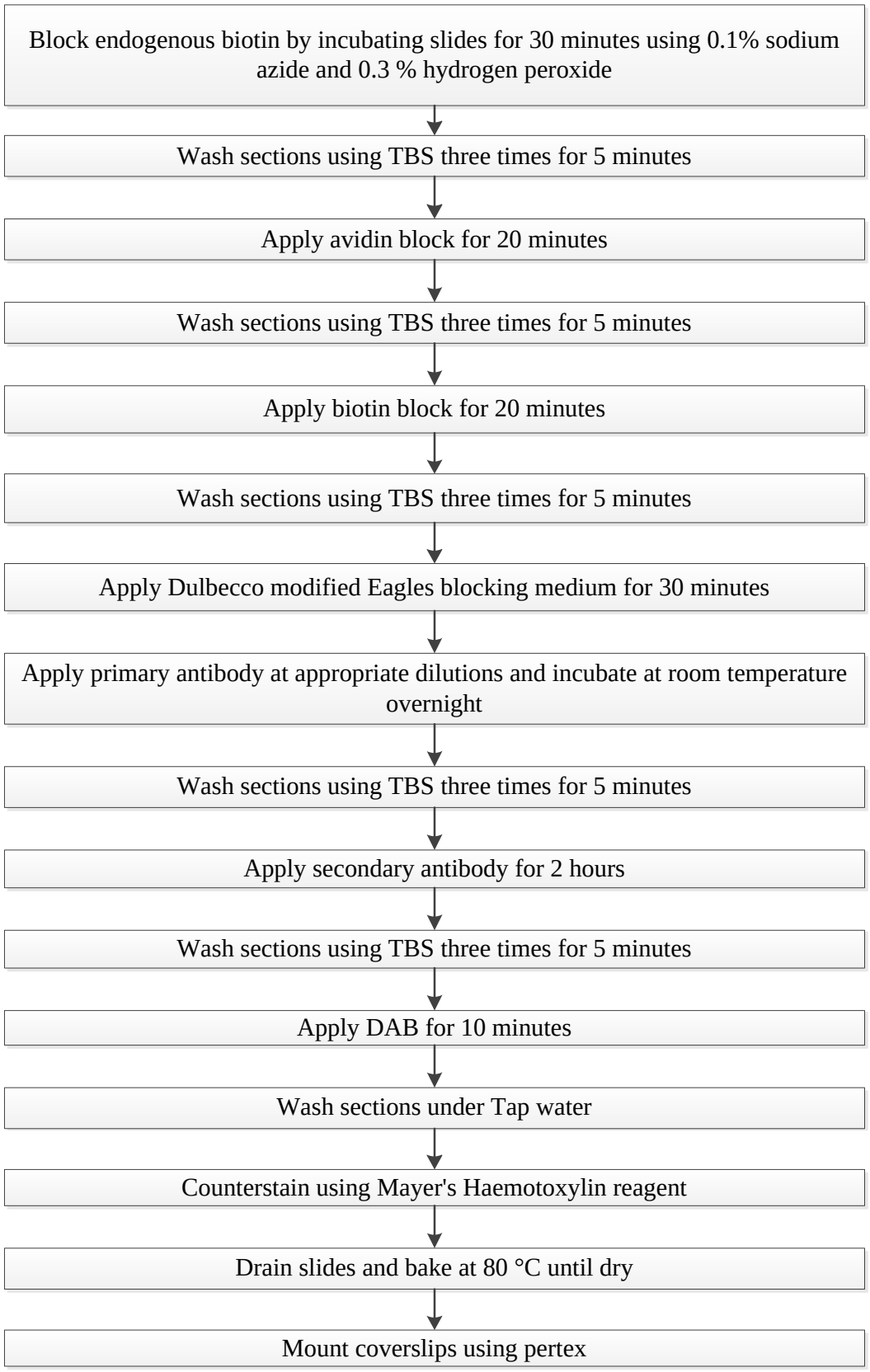


Figure 8.4 Summary of immunohistochemical staining method for the detection of cocaine and heroin in porcine tissue samples embedded in GMA resin

8.2.2.3 Analytical Methods

8.2.2.3.1 Homogenisation

Following removal of transverse tissue sections from the tongue, sections were divided further into five individual parts for homogenisation (Figure 8.5).

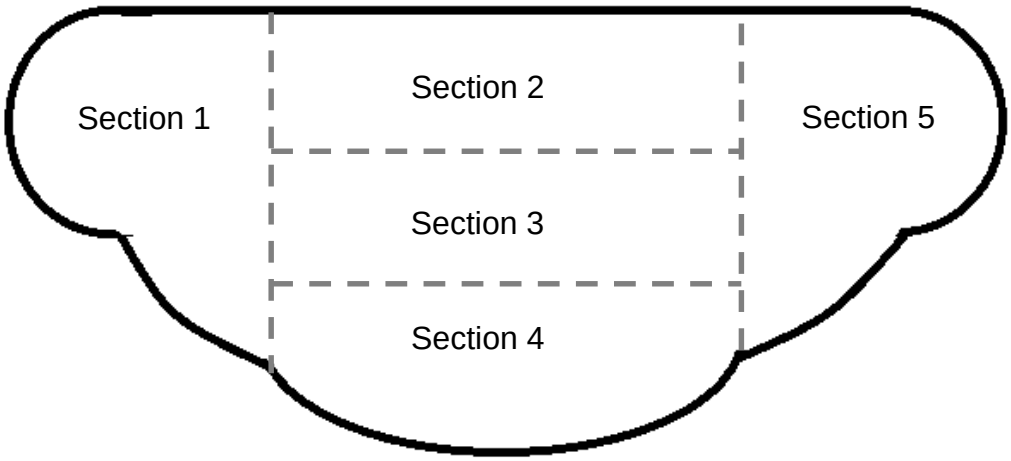


Figure 8.5. Diagram of sectioning of the transverse cross section of the tongue for homogenisation

Each tissue section was homogenised using a hand-held Potter homogeniser by adding 3 mL of pH 6 phosphate buffer to 1 g of tissue until the sample was smooth and homogenous.

8.2.2.3.2 Solid Phase Extraction

200 µL of 0.1M hydrochloric acid was added to 200 µL of the oral fluid samples or tissue extracts. Extraction was performed using Oasis MCX micro elution plates (Waters, Manchester, UK). Cartridges were conditioned with 200 µL of methanol and 200 µL of 0.1 M hydrochloric acid followed by addition of sample. The columns were washed with 200 µL of 0.1 M hydrochloric acid and 200 µL of 30% methanol

in water (70/30 v/v) and dried. Elution was achieved using 50 µL of 5% ammonia in methanol. The sample was reconstituted in 50 µL of 0.1% Formic Acid in Water and analysed on a Waters LC-MS/MS system.

8.2.2.3.3 LC-MS/MS method

Samples were analysed using LC-MS/MS at AlereTM Toxicology, Abingdon, UK.

After sample extraction 5 µL was injected onto the LC-MS/MS system (Waters Acquity® TQD) which consisted of a tandem quadrupole mass spectrometer fitted with a Z-Spray ion interface coupled to a Waters Acquity UPLC® system. Analytes were separated on an Acquity UPLCTM BEH C18 column (130Å, 1.7µm, 1mm x 100mm) (Waters, Manchester, UK) using gradient elution with 0.1% Formic Acid in Water (solvent A) and Acetonitrile (solvent B). The flow rate was 0.4 mL/min and the gradient is summarised in Table 8.1.

Table 8.1. Summary of the gradient for the elution for LC-MS/MS method

Time	Solvent A	Solvent B
0 min	98 %	2 %
1.5 min	90 %	10 %
3.5 min	80 %	20 %
5 min	60 %	40 %
6.5 min	20 %	80 %
6.55 min	98 %	2%
8 min	98 %	2%

Positive electrospray ionisation was used and all analyses were performed in multiple reaction monitoring (MRM) mode with 2 transitions for each analyte as shown below in table 8.2. To ensure the assay performance is fit for purpose the following

parameters were determined to validate the quantitative chromatographic methods used within the laboratory (Table 8.2):

- Method linearity
- Limit of Quantitation (LOQ)
- Upper and Lower robustness of the assay
- Stability of the analytes of interest
- Recovery from collection device
- Extraction efficiency
- Matrix effects
- Specificity
- Carryover
- Bias (accuracy)
- Reproducibility (precision) – within and between batches and the measurement of uncertainty

Table 8.2 Parameters for the detection of cocaine, heroin and their derivates by LC-MS/MS

Compound	MRM transition (m/z)	Collision energy (v)	Retention Time (min)	LOD (ng/mL)	LLOQ (ng/mL)	Analyte Bias (%) (n=40)	Measurement of Uncertainty (%)
Cocaine	304.1→105.1 304.1→182.1	30 20	4.21	0.031	0.5	- 17	± 10.6
Cocaine-D3	307.1→185.1	20	4.21	--	--	--	--
Benzoylecgonine	290.1→179.1 290.1→168.1	30 20	3.60	0.125	0.5	- 14	± 30.1
Benzoylecgonine- D3	293.1→171.1	20	3.60	--	--	--	--
Norcocaine	290.1→136.1 290.1→168.1	25 25	4.39	0.063	1	- 21	±17
Anhydroecgonine methyl ester	182.1→118.14 182.1→122.1	25 20	1.96	0.031	0.5	+ 1.5	±12.8
Anhydroecgonine methyl ester – D3	185.1→125.1	25	1.96	--	--	--	--
Cocaethylene	318.1→82.1 318.1→196.1	25 20	4.75	0.031	0.5	- 9.6	±7.7
Cocaethylene – D3	321.1→199.1	20	4.75	--	--	--	--

Compound	MRM transition (m/z)	Collision energy (v)	Retention Time (min)	LOD (ng/mL)	LLOQ (ng/mL)	Analyte Bias (%) (n=40)	Measurement of Uncertainty (%)
Heroin	370.1→211.1 370.1→268.1	25 25	4.15	0.5	0.5	+ 19	± 10
Heroin – D9	379.1→272.1	25	4.12	--	--	--	--
Morphine	286.1→153.1 286.1→201.1	40 24	1.96	0.063	0.5	- 2.97	± 9.5
Morphine –D3	289.1→153.1	40	1.96	--	--	--	--
6-monoacetylmorphine	328.1→165.1 328.1→211.1	40 30	2.82	0.063	0.063	- 27.3	± 23.9
6-monoacetylmorphine – D6	334.1→165.1	40	2.80	--	--	--	--
6-Acetylcodeine	342.1→165.1 342.1→225.1	40 25	4.08	0.063	0.5	- 25.2	± 14.5
Codeine	300.1→153.1 300.1→165.1	45 45	2.52	0.25	0.5	+ 12.1	± 12.1
Dihydrocodeine	302.1→199.1 302.1→245.1	35 25	2.47	0.031	0.25	+ 8.41	± 12
Dihydorcodeine – D6	308.1→202.1	35	2.46	--	--	--	--

8.3 Results and discussion

8.3.1 Immunohistochemical results

8.3.1.1 Results from controlled exposure to drug solutions of either cocaine or heroin

Transverse cross sections which were stained using immunohistochemical techniques were examined using a Leica DMLB microscope fitted with a Nikkon Coolpix camera at 12 points throughout the cross-section of the porcine tongue (Figure 8.6).

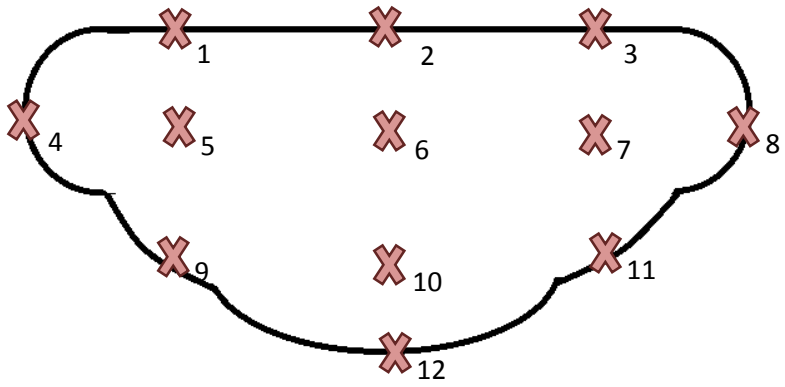


Figure 8.6 represents the position of each of the photographs taken of the stained cross section of each porcine tongue previously exposed to cocaine or heroin

Both cocaine and heroin staining resulted in brown staining when tissue was positive for opioids (heroin, 6-MAM, and morphine) and cocaine (cocaine and benzoylecgonine). Staining in porcine tongues exposed to lower concentrations of drug resulted in lower intensity staining in tissue sections than in tissue sections which were exposed to higher concentrations. Duplicate tongues were exposed to each cocaine or heroin solution in order to test the reproducibility of the exposure and staining methods. As a large number of images were taken throughout the analysis, only pictures of sections 2, 6, 10 and 12 are represented in this thesis.

However, all images taken were visually analysed and showed similar results to those represented in this thesis.

8.3.1.1.1 Cocaine

Sections were assessed visually, as the staining counter software proved to be unsuitable for quantifying the amount of staining present as described in chapter 7. Porcine tongues exposed to a lower concentration of cocaine (100 ng/mL cocaine) showed less intense staining than the tongues exposed to a higher concentrations (500 ng/mL, 1000 ng/mL, 10 µg/mL). The observed differences in intensity between staining in tongues exposed to 100 ng/mL and 250 ng/mL was not markedly different. A larger intensity increase can be observed at 500 ng/mL, 1000 ng/mL and 10 µg/mL (Figure 8.7, Figure 8.8, Figure 8.9, Figure 8.10, Figure 8.11).

Image - position 2	Image - position 6

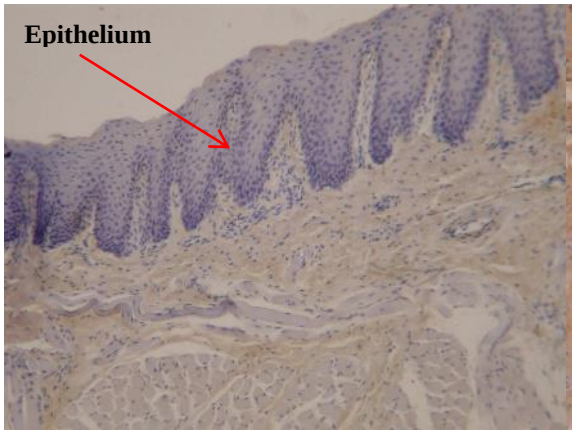
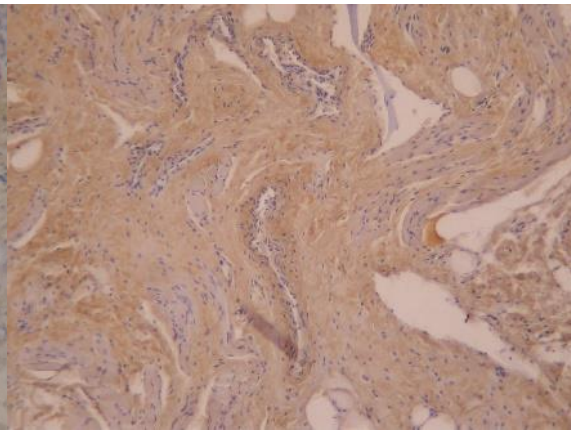

	

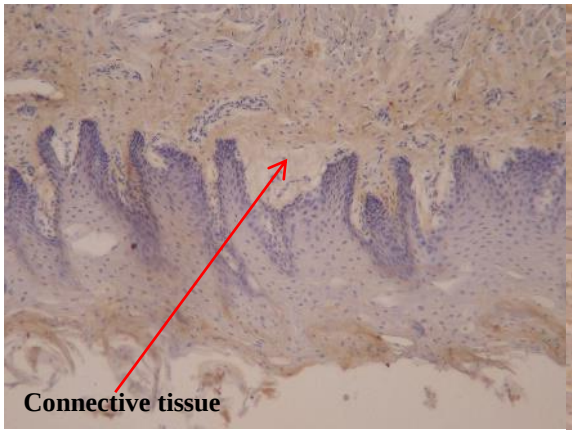
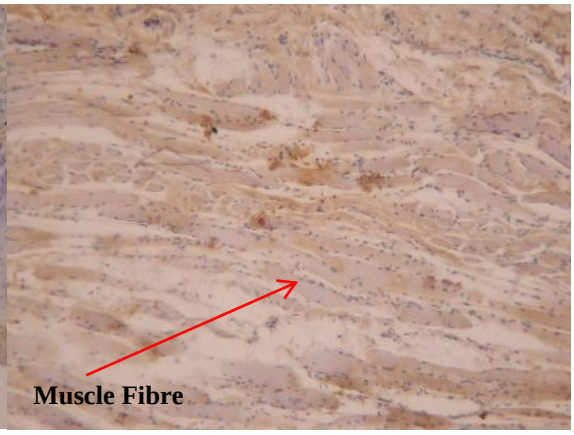
Image - position 12	**Image - position 10**
	

Figure 8.7 Images of porcine tongues exposed to 100 ng/mL of cocaine solution. Porcine tongues were exposed in duplicate (tongue 1 / tongue 2). Only images 2, 6, 10 and 12 are represented in this table (Figure 8.6) (magnification x25)

Image - position 2	Image - position 6

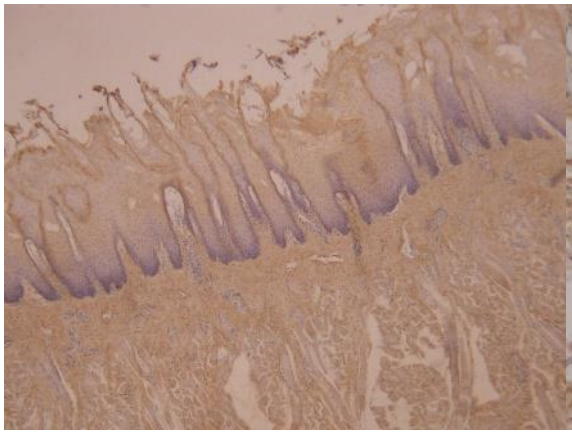
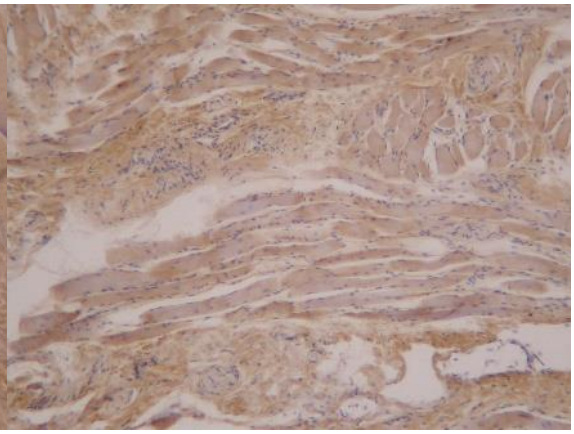

	

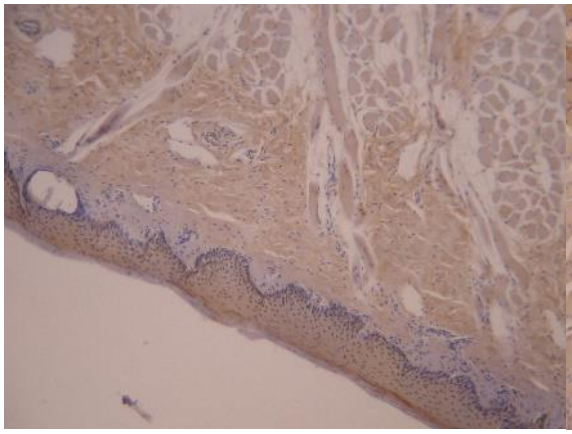
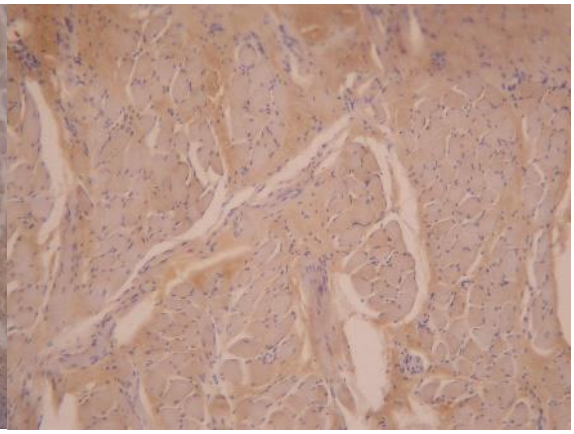
Image - position 12	**Image position 10**
	

Figure 8.8 Images of porcine tongues exposed to 250 ng/mL of cocaine solution. Porcine tongues were exposed in duplicate (tongue 1 / tongue 2). Only images 2, 6, 10 and 12 are represented in this table (Figure 8.5) (magnification x25)

Image - position 2	Image - position 6

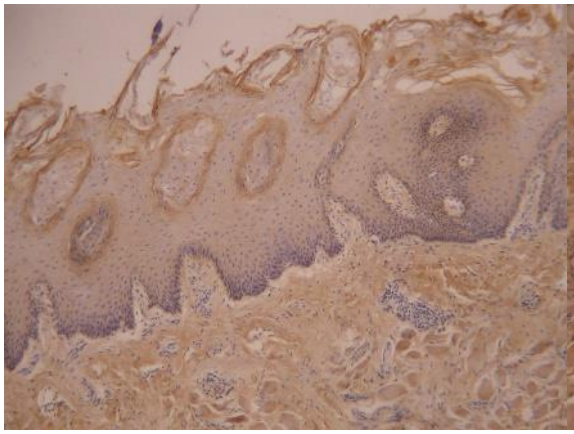
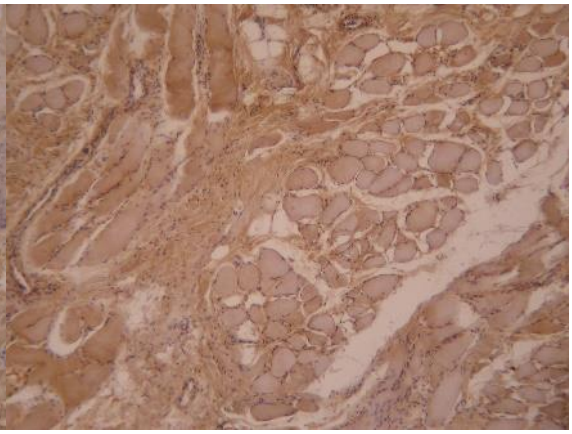

	

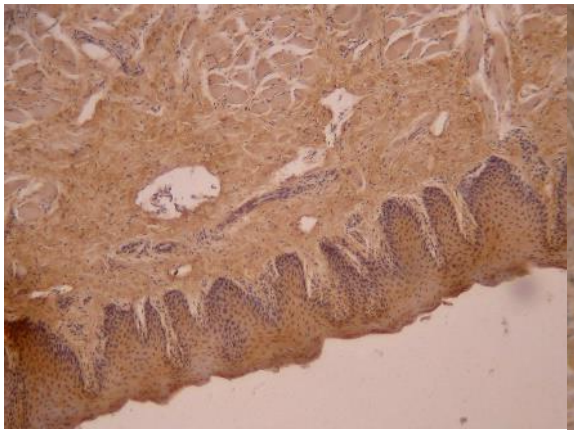
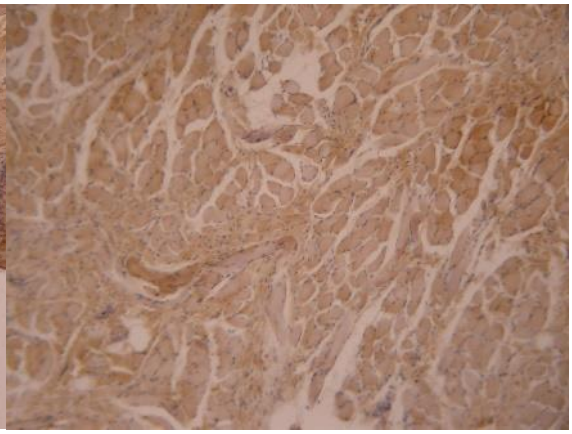
Image - position 12	**Image - position 10**
	

Figure 8.9 Images of porcine tongues exposed to 500 ng/mL of cocaine solution. Porcine tongues were exposed in duplicate (tongue 1 / tongue 2). Only images 2, 6, 10 and 12 are represented in this table (Figure 8.5) (magnification x25)

Image - position 2	Image - position 6

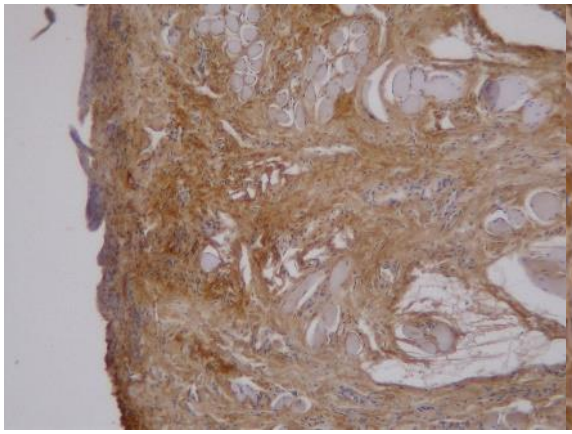
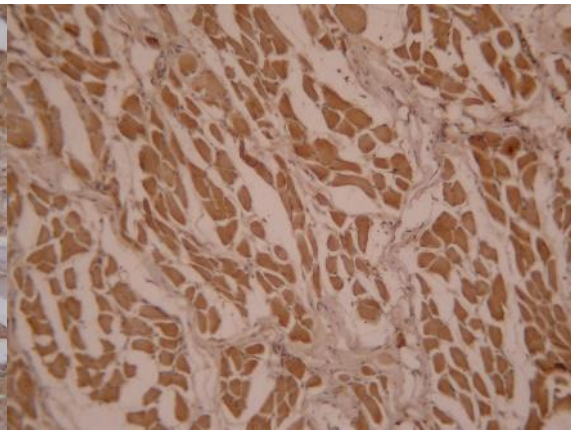

	

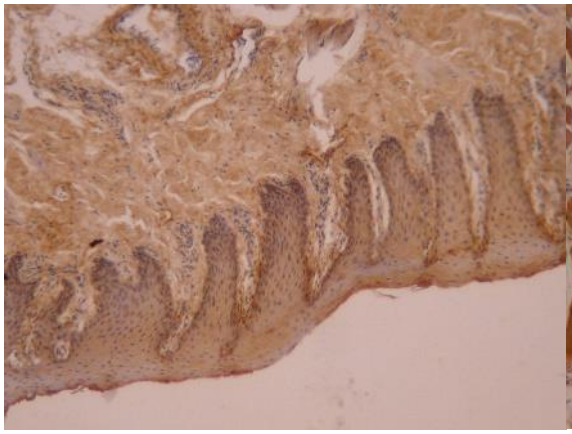
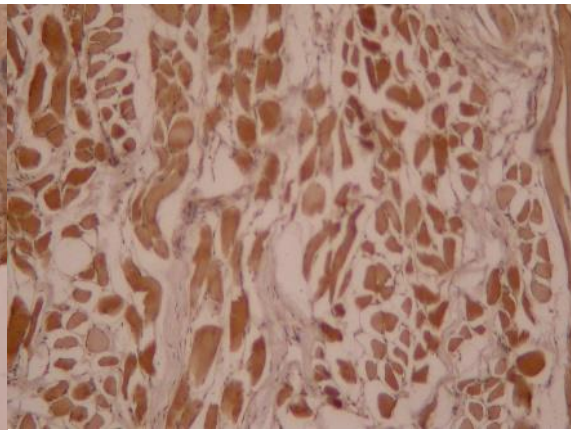
Image - position 12	**Image - position 10**
	

Figure 8.10 Images of porcine tongues exposed to 1000 ng/mL of cocaine solution. Porcine tongues were exposed in duplicate (tongue 1 / tongue 2). Only images 2, 6, 10 and 12 are represented in this table (Figure 8.5) (magnification x25)

Image - position 2	Image - position 6

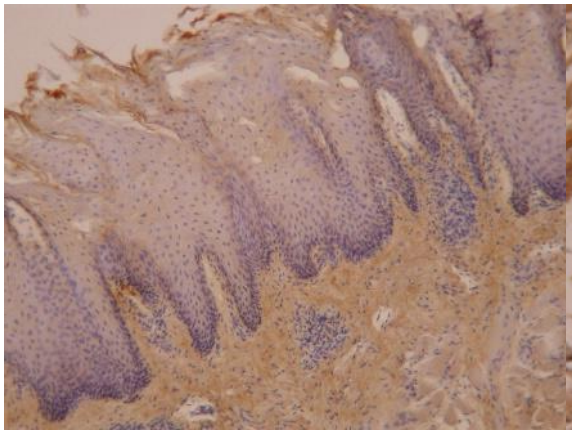
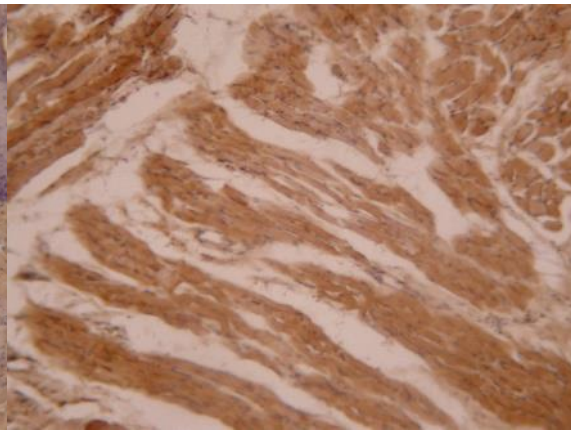

	

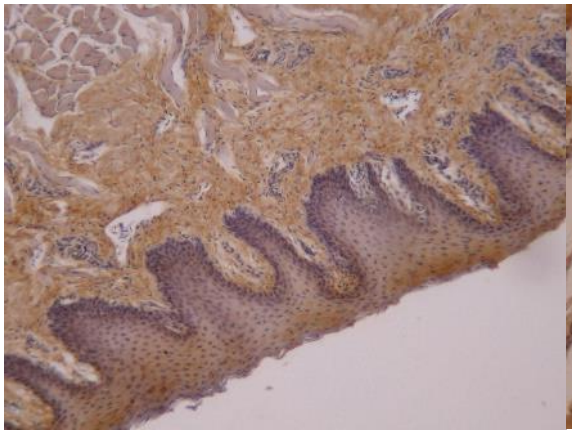
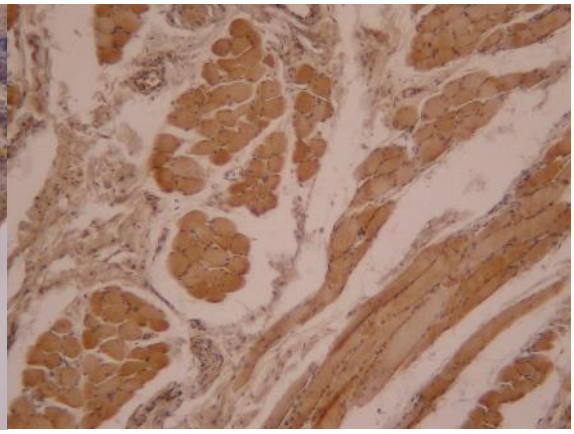
Image - position 12	**Image - position 10**
	

Figure 8.11 Images of porcine tongues exposed to 10 µg/mL of cocaine solution. Porcine tongues were exposed in duplicate (tongue 1 / tongue 2). Only images 2, 6, 10 and 12 are represented in this table (Figure 8.5) (magnification x25)

The positive staining in the porcine tissue which was observed following the exposure to solution of cocaine indicated that cocaine and/or benzoylecgonine can enter the oral tissue by ways other than via the circulating blood. When visualising the tissue sections with a higher power (x 63) magnification, it can be observed that with increasing concentration of cocaine the drug travels further into the muscle bundles (Figure 8.12).

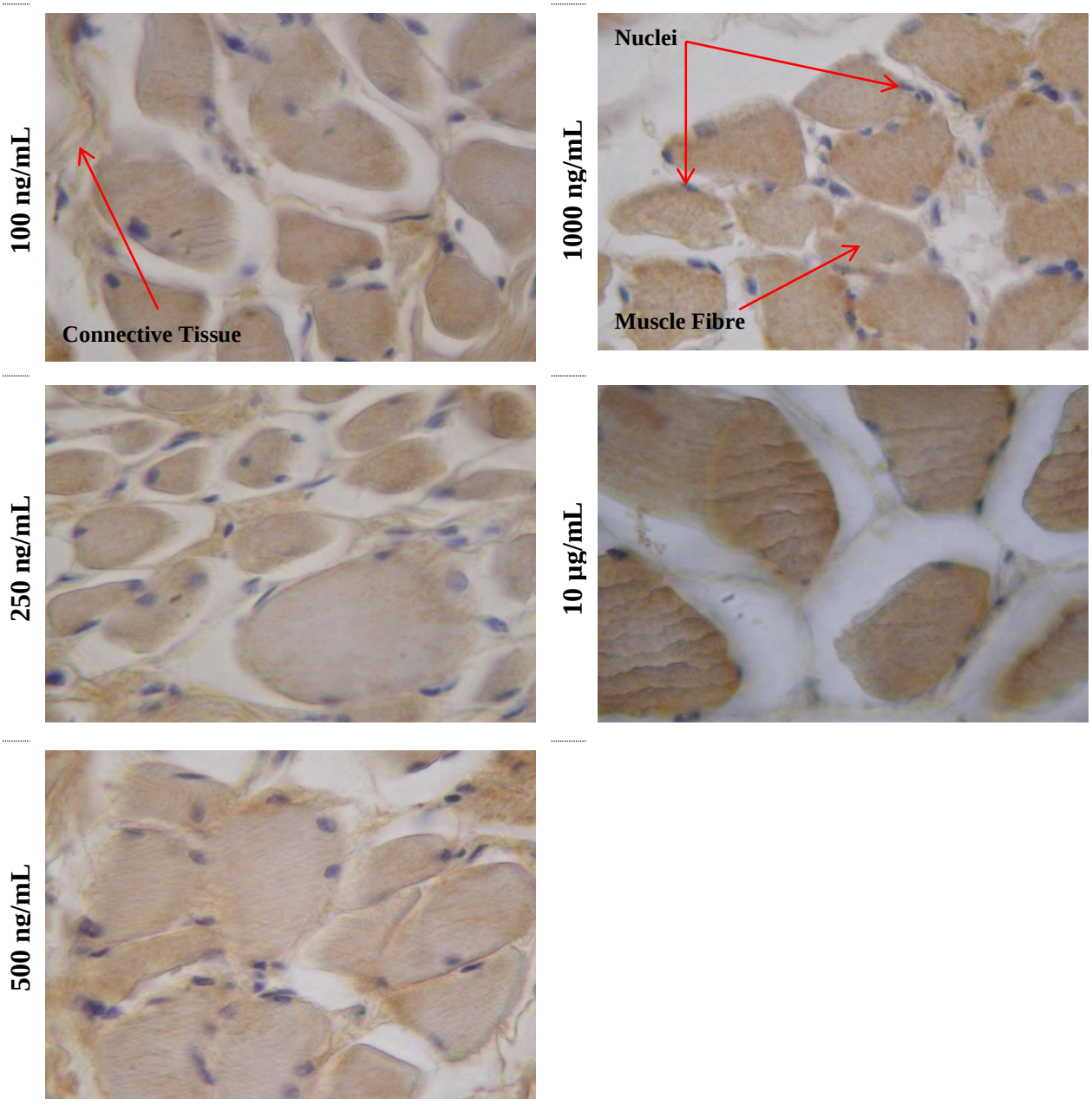


Figure 8.12 Images to representing the difference of penetration of cocaine into tongue muscle at a magnification of x 63 depending on the concentration to which each tongues were exposed

Only a small difference in staining intensity between tissue exposed to 100 ng/mL and 250 ng/mL was observed. However, over the concentration range, staining clearly increased and when directly comparing the lowest (100 ng/mL) and highest (10 µg/mL) cocaine exposure, the difference in staining intensity is more readily observed (Figure 8.13).

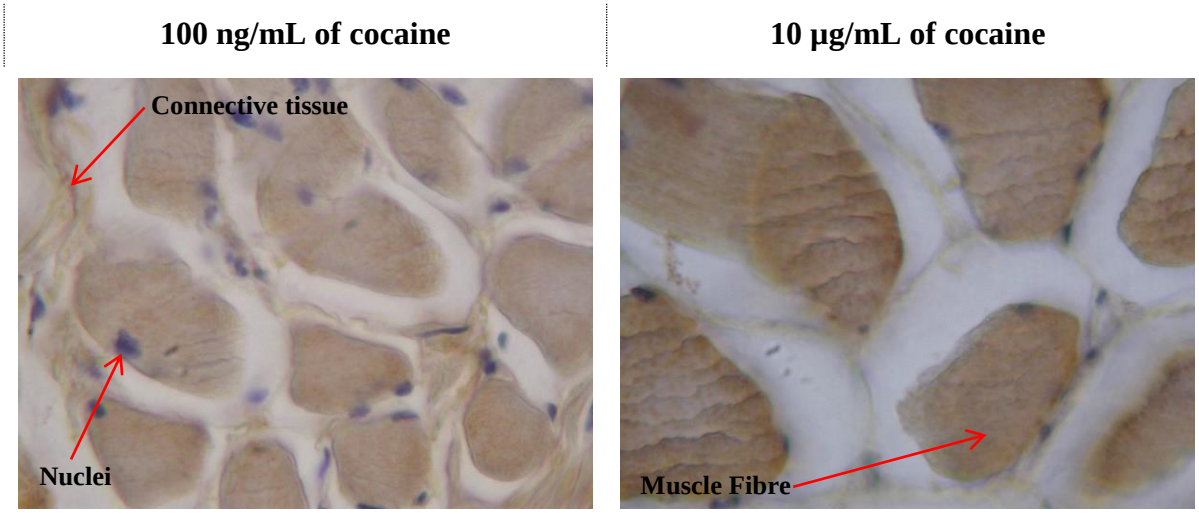


Figure 8.13 Direct comparison of tongues exposed to 100 ng/mL and 10 µg/mL of cocaine. Images taken at power x63 show the difference of penetration between the two different cocaine concentrations

Prior to embedding porcine tongues in paraffin and immediately post exposure to cocaine, porcine tongues were washed seven times to wash off any excess cocaine from the surface of the tongue. The presence of cocaine and/or benzoylecgonine in tongue tissue after the washes clearly indicates that drugs can enter and bind into tissue and form depots.

8.3.1.1.2 Heroin

In a similar manner to the results observed after cocaine exposure, tongues exposed to heroin also showed increased staining as concentrations of drug increased (Figure 8.14, Figure 8.15, Figure 8.16, Figure 8.17, Figure 8.18).

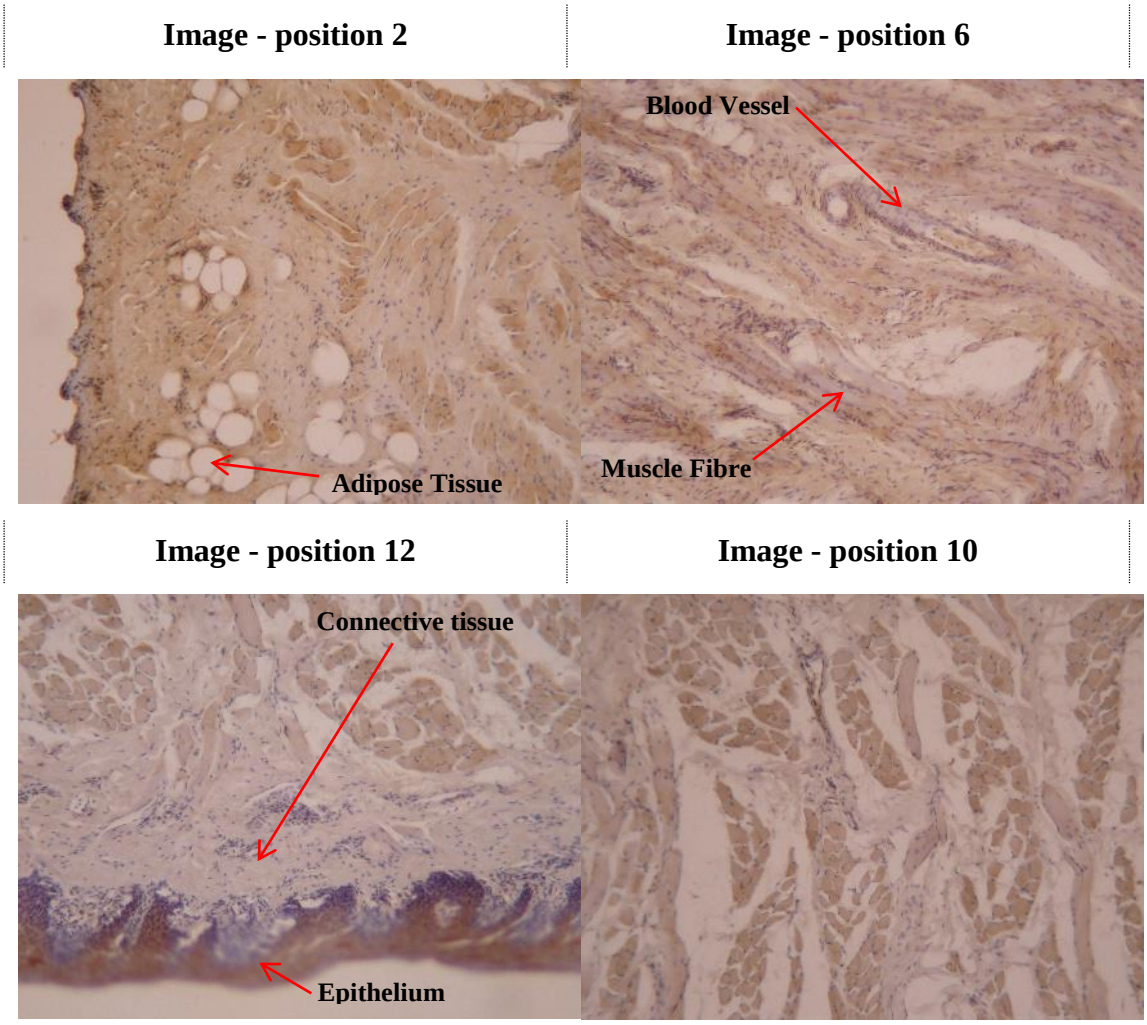


Figure 8.14 Images of porcine tongues exposed to 100 ng/mL of heroin solution. Porcine tongues were exposed in duplicate (tongue 1 / tongue 2). Only images 2, 6, 10 and 12 are represented in this table (Figure 8.5) (magnification x25)

Image - position 2	Image - position 6

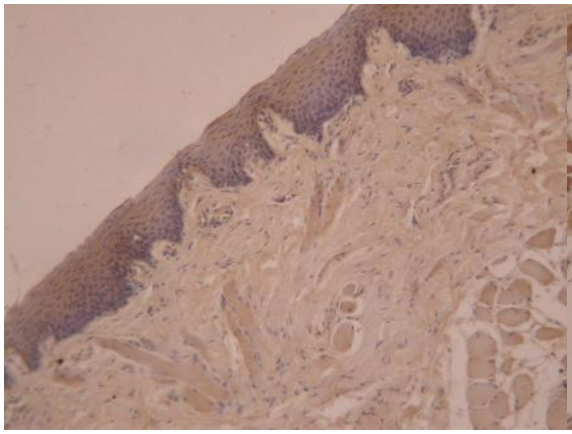
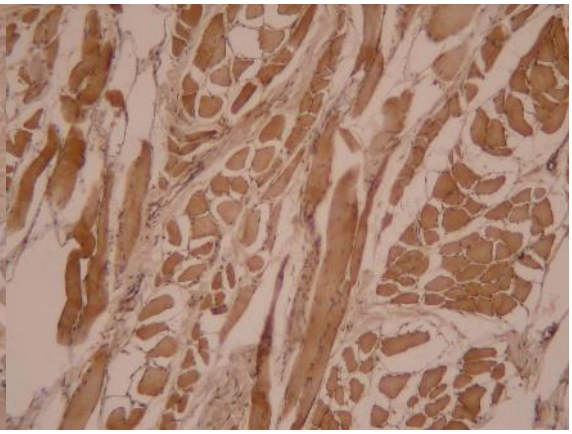

	

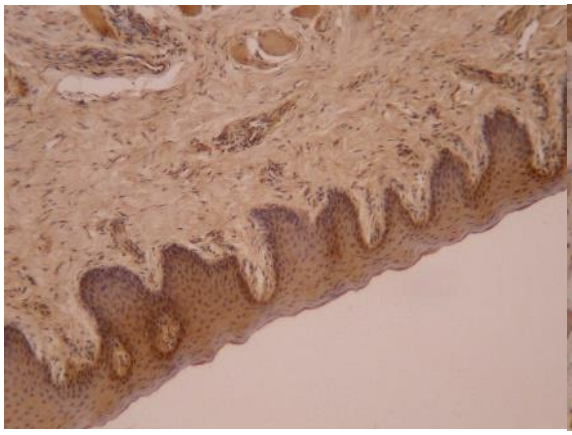
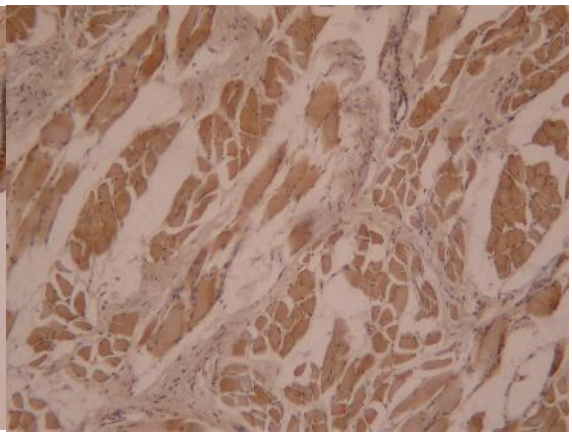
Image - position 12	**Image - position 10**
	

Figure 8.15 Images of porcine tongues exposed to 250 ng/mL of heroin solution. Porcine tongues were exposed in duplicate (tongue 1 / tongue 2). Only images 2, 6, 10 and 12 are represented in this table (Figure 8.5) (magnification x25)

Image - position 2	Image - position 6

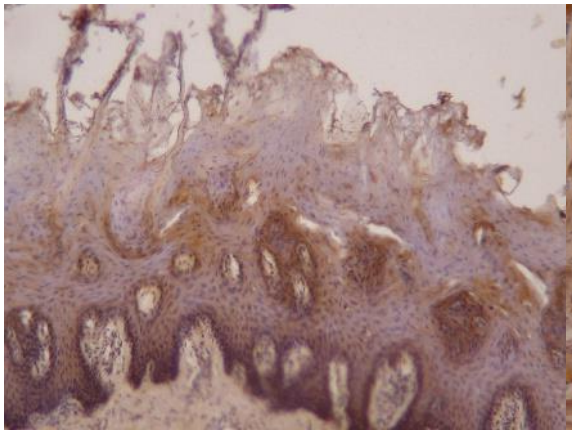
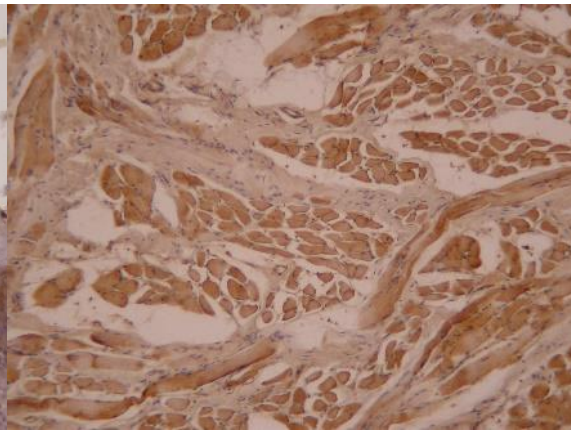

	

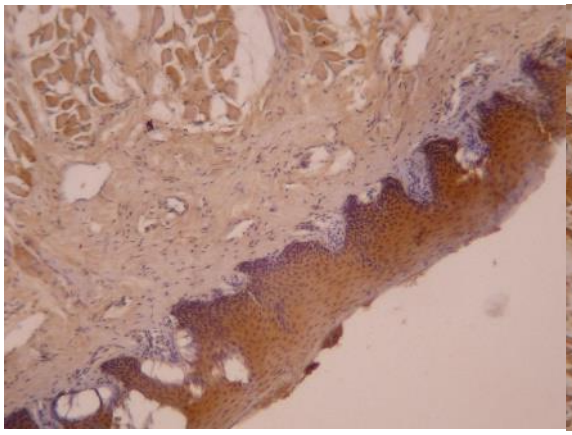
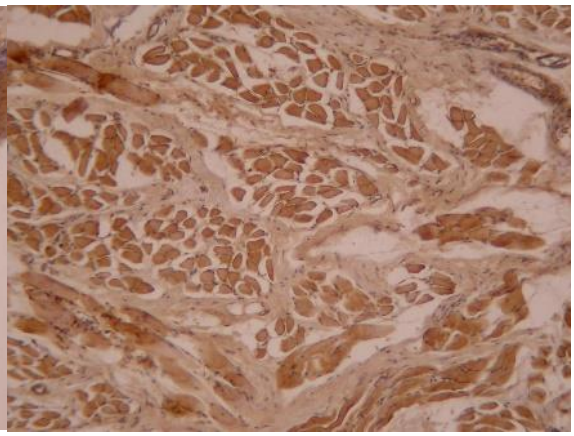
Image - position 12	**Image - position 10**
	

Figure 8.16 Images of porcine tongues exposed to 500 ng/mL of heroin solution. Porcine tongues were exposed in duplicate (tongue 1 / tongue 2). Only images 2, 6, 10 and 12 are represented in this table (Figure 8.5) (magnification x25)

Image - position 2	Image - position 6

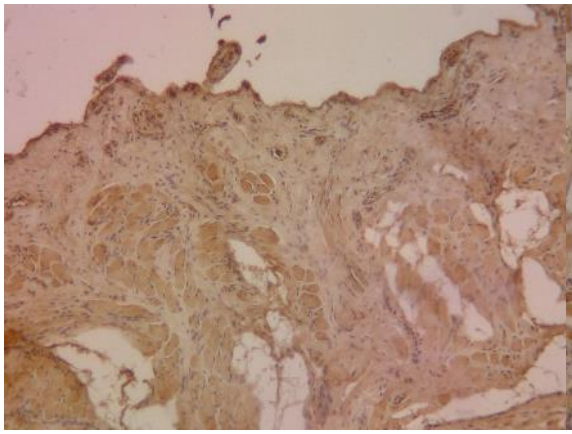
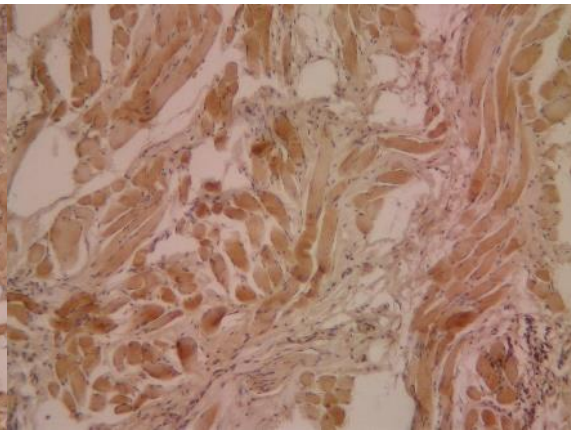

	

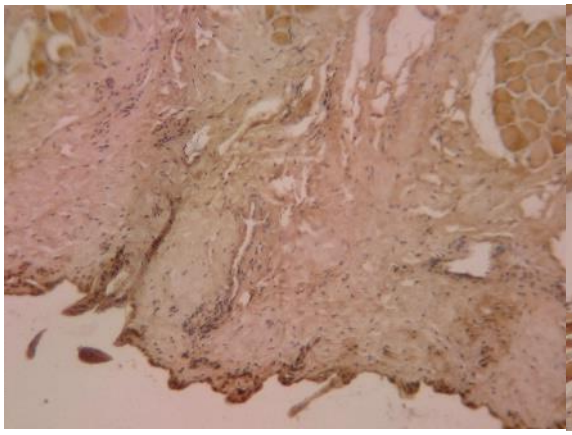
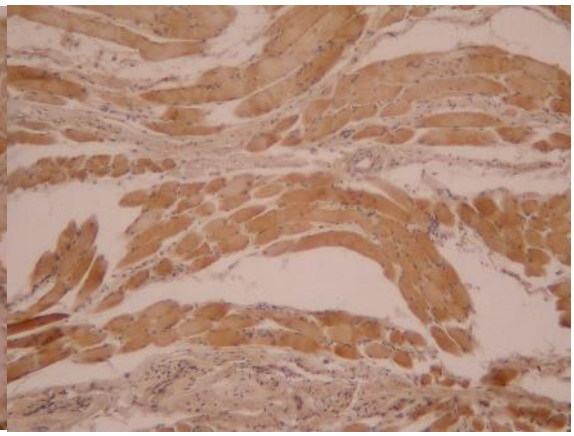
Image - position 12	**Image - position 10**
	

Figure 8.17 Images of porcine tongues exposed to 1000 ng/mL of heroin solution. Porcine tongues were exposed in duplicate (tongue 1 / tongue 2). Only images 2, 6, 10 and 12 are represented in this table (Figure 8.5) (magnification x25)

Image - position 2	Image - position 6

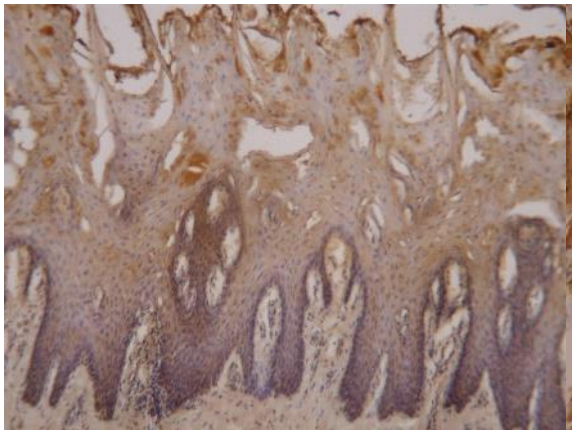
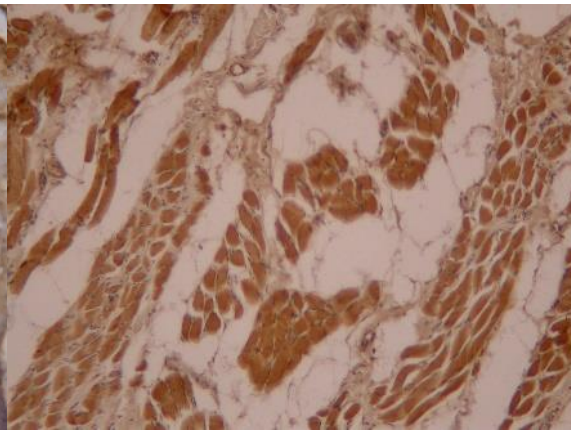

	

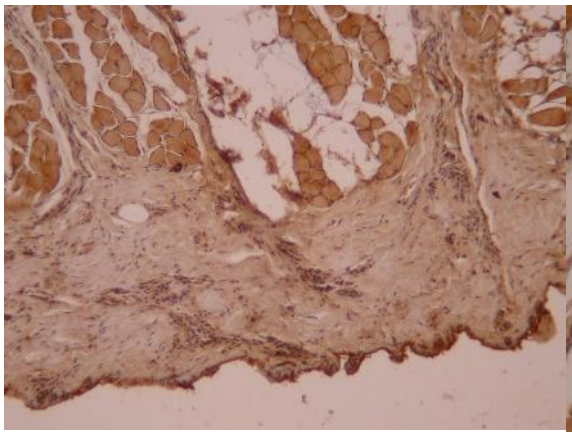
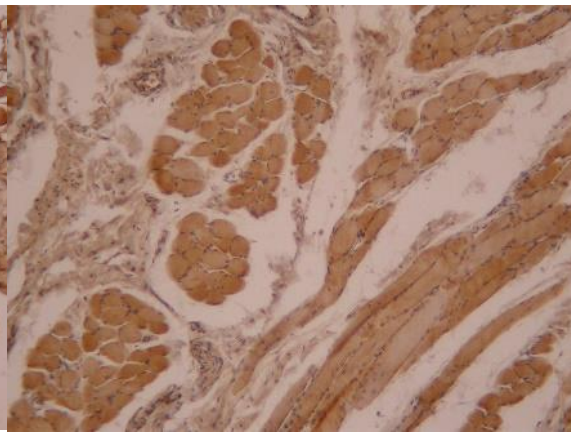
Image - position 12	**Image - position 10**
	

Figure 8.18 Images of porcine tongues exposed to 10 µg/mL of heroin solution. Porcine tongues were exposed in duplicate (tongue 1 / tongue 2). Only images 2, 6, 10 and 12 are represented in this table (Figure 8.5) (magnification x25)

Heroin and/or its metabolites were also shown to enter the tongue tissue. The intensity of staining was shown to increase with increasing heroin exposure concentration which indicated that more drug has entered the tissue.

Additionally when visualising the tissue sections with a higher magnification (x63), it can be observed that with increasing concentration of heroin, more drug appears to penetrate into the muscle bundles (Figure 8.19).

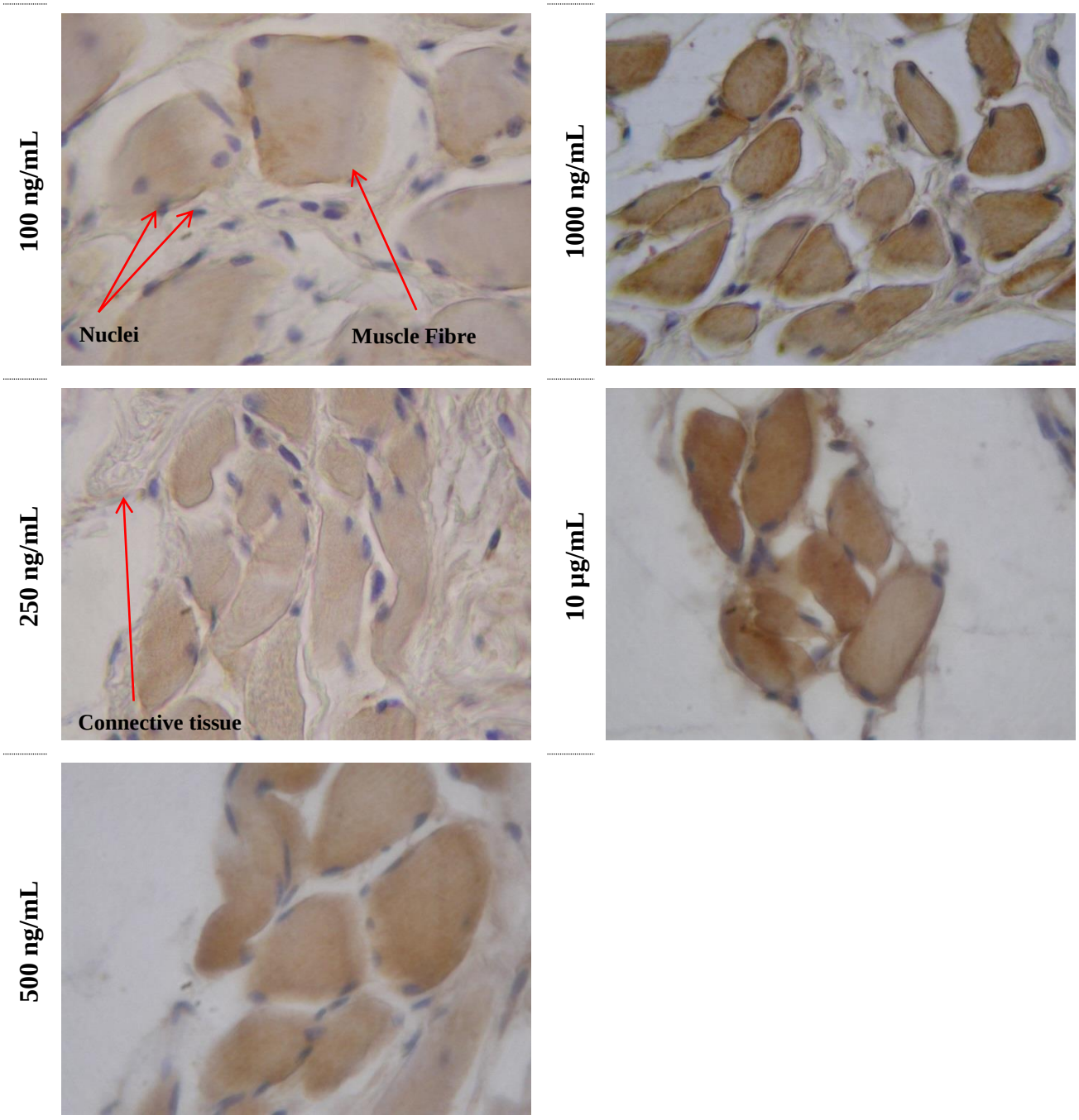


Figure 8.19 Images to representing the difference of penetration of heroin into tongue muscle at a magnification of x 63 depending on the concentration to which each tongues were exposed

Porcine tongues were washed seven times following a 10 minute drug exposure time; however drug was still present in the tissue following this extensive wash process. This indicates that drug can enter and bind to the tissue and hence form depots.

8.3.1.1.3 Summary of results from controlled exposure to drug solutions of either cocaine or heroin

The immunohistochemical methods developed (Chapter 7) resulted in successful staining for cocaine and heroin including their metabolites following the exposure of porcine tongues to cocaine and heroin solutions demonstrating that drugs can enter tissues and bind in a manner that could represent the formation of a tissue depot. The presence of cocaine or heroin in the tissue after sequential washing gave negative result for the presence of drugs adds further support to the proposition that drugs can be absorbed into, and subsequently be retained, in oral tissue. Drugs have been shown to enter the tissue for concentrations ranging between 100 ng/mL and 10 µg/mL.

In comparison to cocaine staining, the staining of heroin appeared to be significantly more intense in all tongues suggesting that the drug binds more readily to the tissue than cocaine. This was most noticeable in tissue sections which were exposed to higher concentrations of drug (10 µg/mL) (Figure 8.20, Figure 8.21).

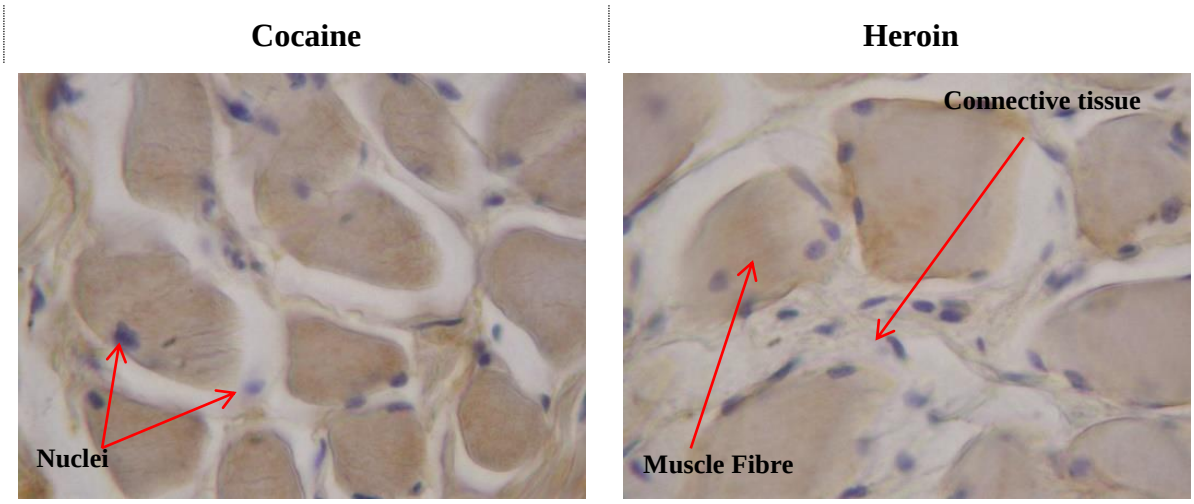


Figure 8.20 Comparison of images of tongues exposed to 100 ng/mL concentration of cocaine or heroin (magnification x63)

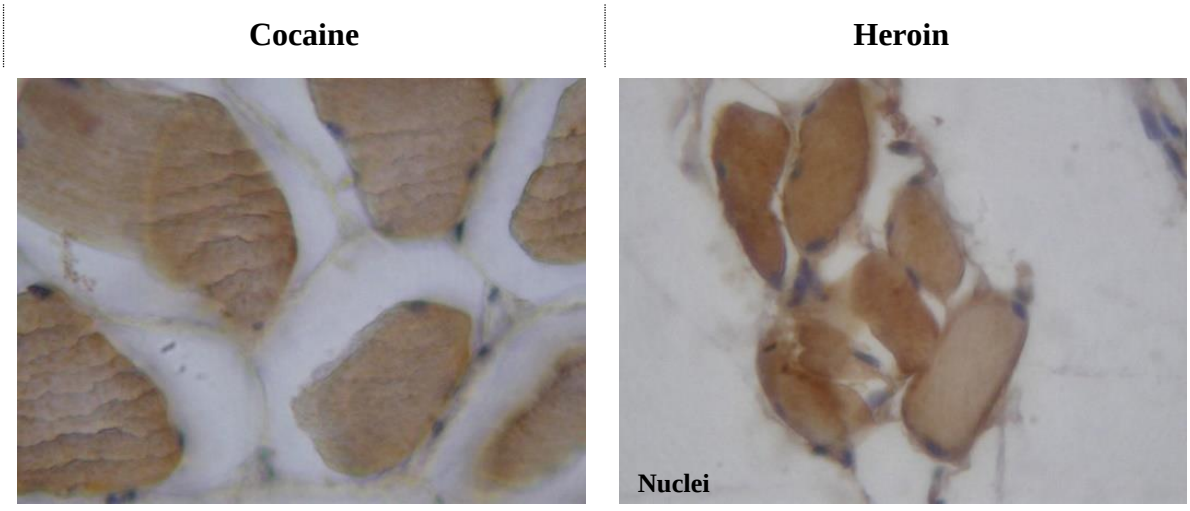


Figure 8.21 Comparison of images of tongues exposed to 10 µg/mL concentration of cocaine or heroin (magnification x63)

Additionally, it was observed that no positive staining was observed for either cocaine or heroin within the blood vessel wall tissue (Figure 8.22). The lack of staining in the walls of blood vessels indicated that neither cocaine nor heroin binds to these vessels. However some staining was observed in the centre of the blood vessels which could indicate that the drug did cross the blood vessel wall.

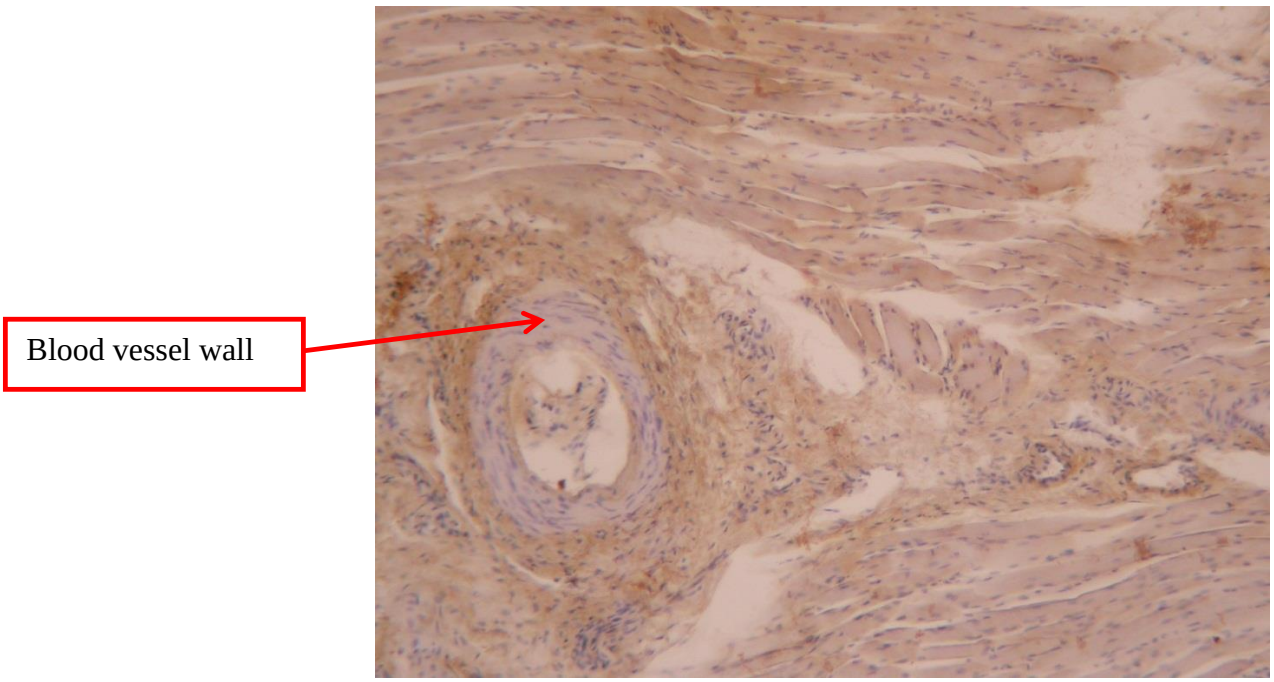


Figure 8.22. Image of a tongue exposed to 10 µg/mL of cocaine showing the staining around the blood vessel and the negative staining of the blood vessel wall (magnification x25)

Of note was the staining presence around the epithelium (Figure 8.23). The epithelium stained was positive on the outside of the tongue. However, no staining was observed on the inside of the epithelium in tongues exposed to either cocaine or heroin. The inside layer of the epithelium showed an increased number of nuclei. The cluster of nuclei, which were blue in colour, resulted in a negative appearance on the inside of the epithelium.

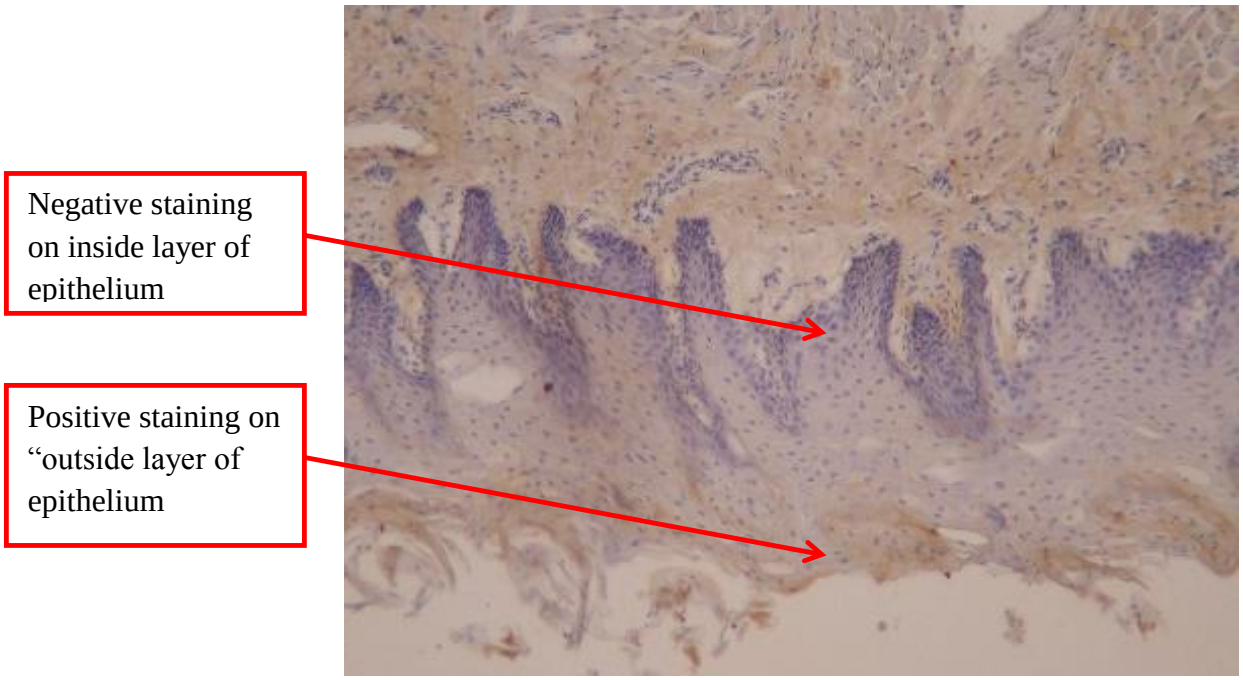


Figure 8.23. Image of epithelium from a porcine tongue exposed to a cocaine solution clearly showing the negative stain on the inside of the epithelium whereas the outside of the epithelium is positively stained (magnification x25)

8.3.1.2 Results from the prolonged washing study of porcine tongues to investigate the drug release from tongue tissue by immunohistochemical staining

Results presented in Chapter 5, have shown that contamination from orally consumed drugs can occur for up to four hours post consumption of either Collis Browne's mixture or Codeine Linctus. Additionally, it has been reported in the literature that drugs in oral fluid can be detected for up to 96 hours post consumption (Osselton et al 2001).

The development of the immunohistochemical method has showed that drugs can enter the oral tissue via external contamination, be incorporated into tissue and hence form depots within the tissue.

The drug which has bound to the tissue can subsequently be released over time. During the investigation into drug depots, porcine tongues were exposed to a concentration of 100 ng/mL and 1000 ng/mL of cocaine or heroin. Tongues were then soaked in artificial saliva for either 1 hour, 6 hours, 24 hours or 48 hours.

The staining procedure showed that drug was present in tongues washed for 1 hour, 6 hours, 24 hours and 48 hours following exposure to either 100 ng/mL or 1000 ng/mL of cocaine or heroin. Staining intensity weakened with increasing washing time. This was most noticeable around the epithelium of the porcine tongue. Staining in the muscle tissue was less intense but still prominent. Similarly to the previous exposure experiments, the staining for heroin appeared to be more intense in all tongues (Figure 8.24, Figure 8.25, Figure 8.26, Figure 8.27).

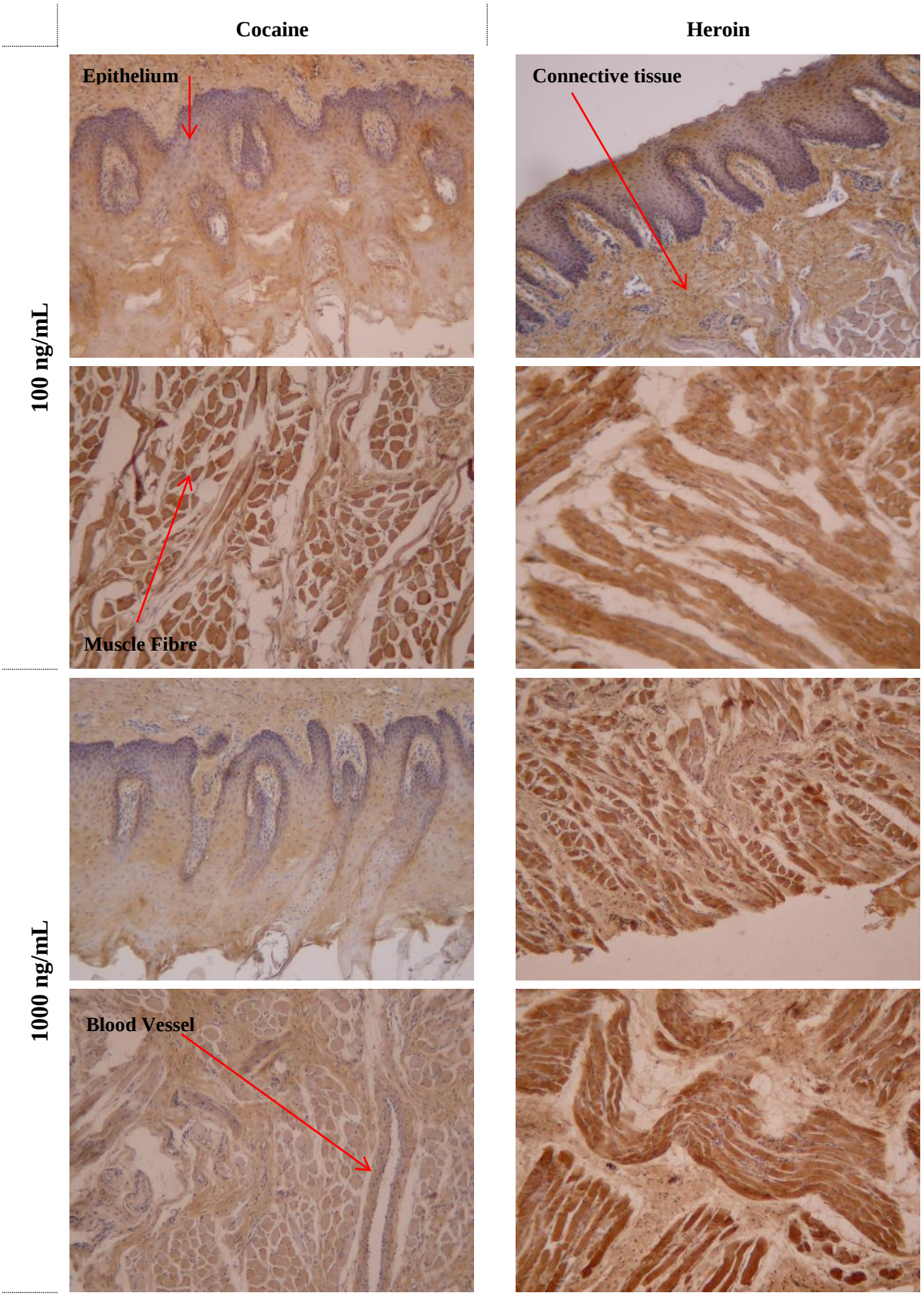


Figure 8.24 Images representing staining in tongues exposed to 100 ng/mL or 1000 ng/mL to either cocaine or heroin and followed by washing each tongue for 1 hour (magnification x25)

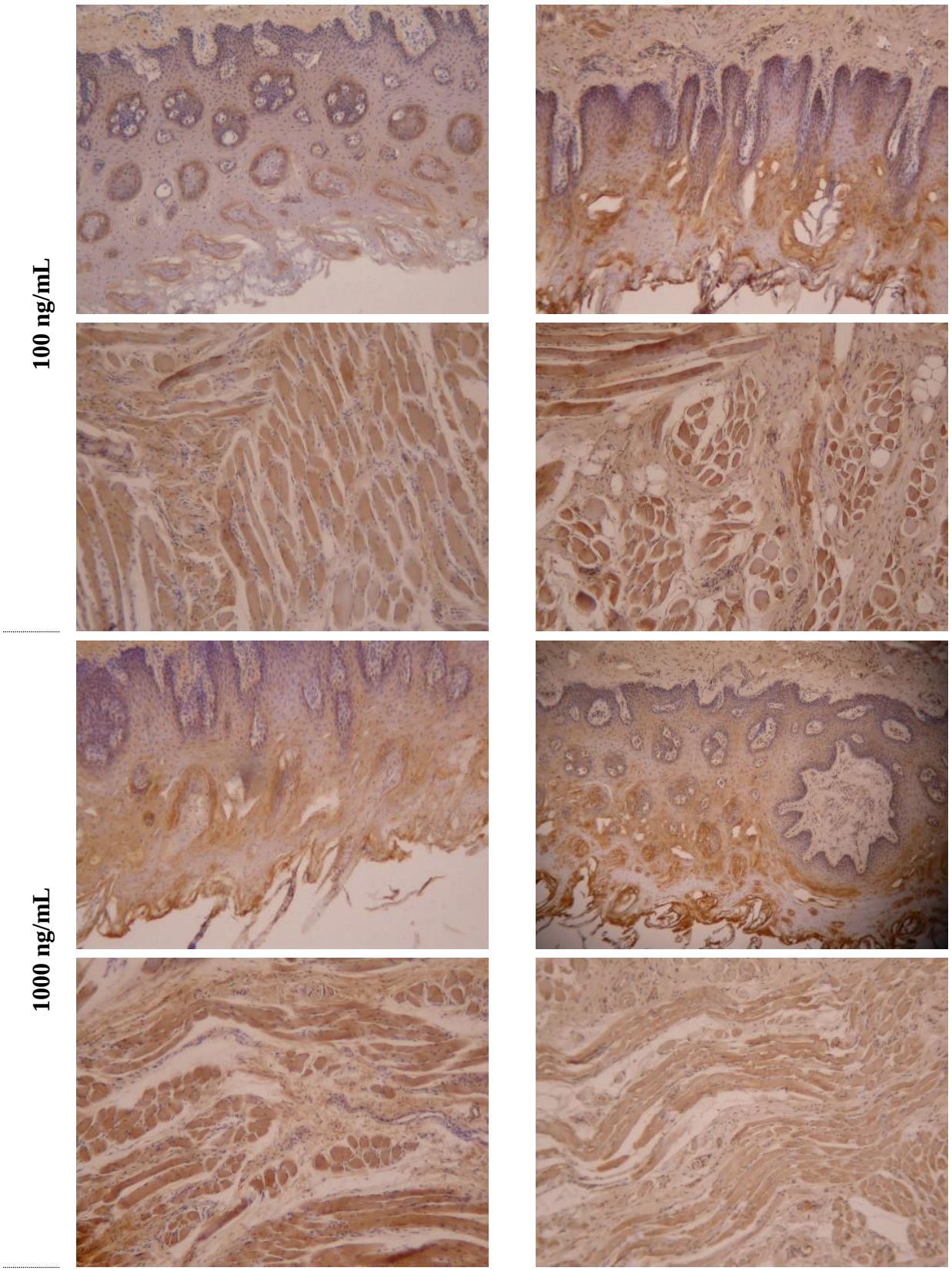


Figure 8.25 Images representing staining in tongues exposed to 100 ng/mL or 1000 ng/mL to either cocaine or heroin and followed by washing each tongue for 6 hours (magnification x25)

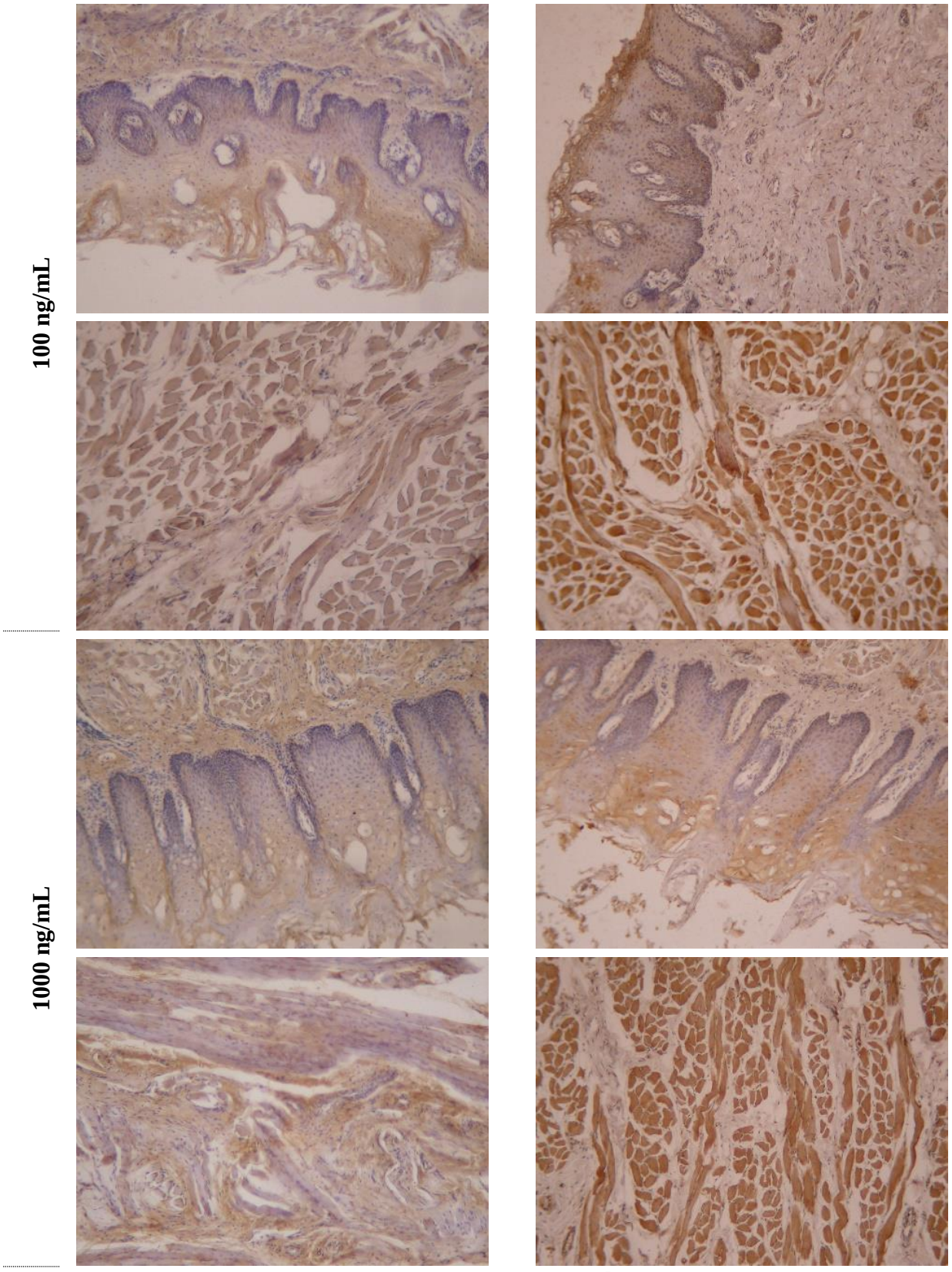


Figure 8.26 Images representing staining in tongues exposed to 100 ng/mL or 1000 ng/mL to either cocaine or heroin and followed by washing each tongue for 24 hours (magnification x25)

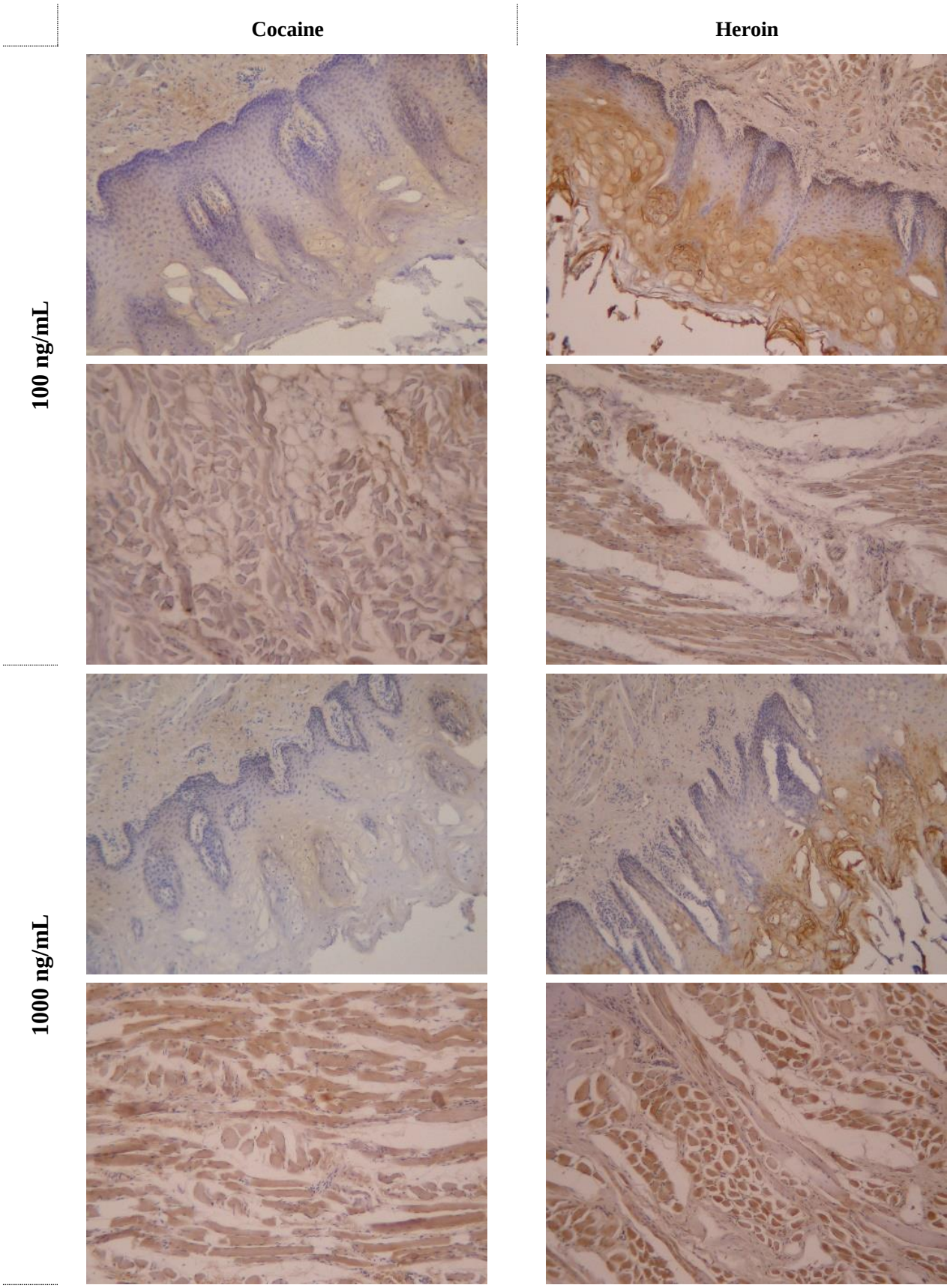


Figure 8.27 Images representing staining in tongues exposed to 100 ng/mL or 1000 ng/mL to either cocaine or heroin and followed by washing each tongue for 48 hours (magnification x25)

When sections were photographed using a higher magnification (x63) the decrease of the intensity of staining of drugs from porcine tongue muscle became more apparent. Previously, it was shown that the drug penetrated deep into the muscle bundles especially at higher concentrations. This was also shown when investigating drug release which also showed that results from the immunohistochemical staining method are reproducible. The most intense staining was observed following the 1 hour wash following exposure to 1000 ng/mL. A decrease in the intensity of staining could be observed in tongues exposed to heroin and cocaine with prolonged washing with artificial saliva (Figure 8.28, Figure 8.29, Figure 8.30, Figure 8.31).

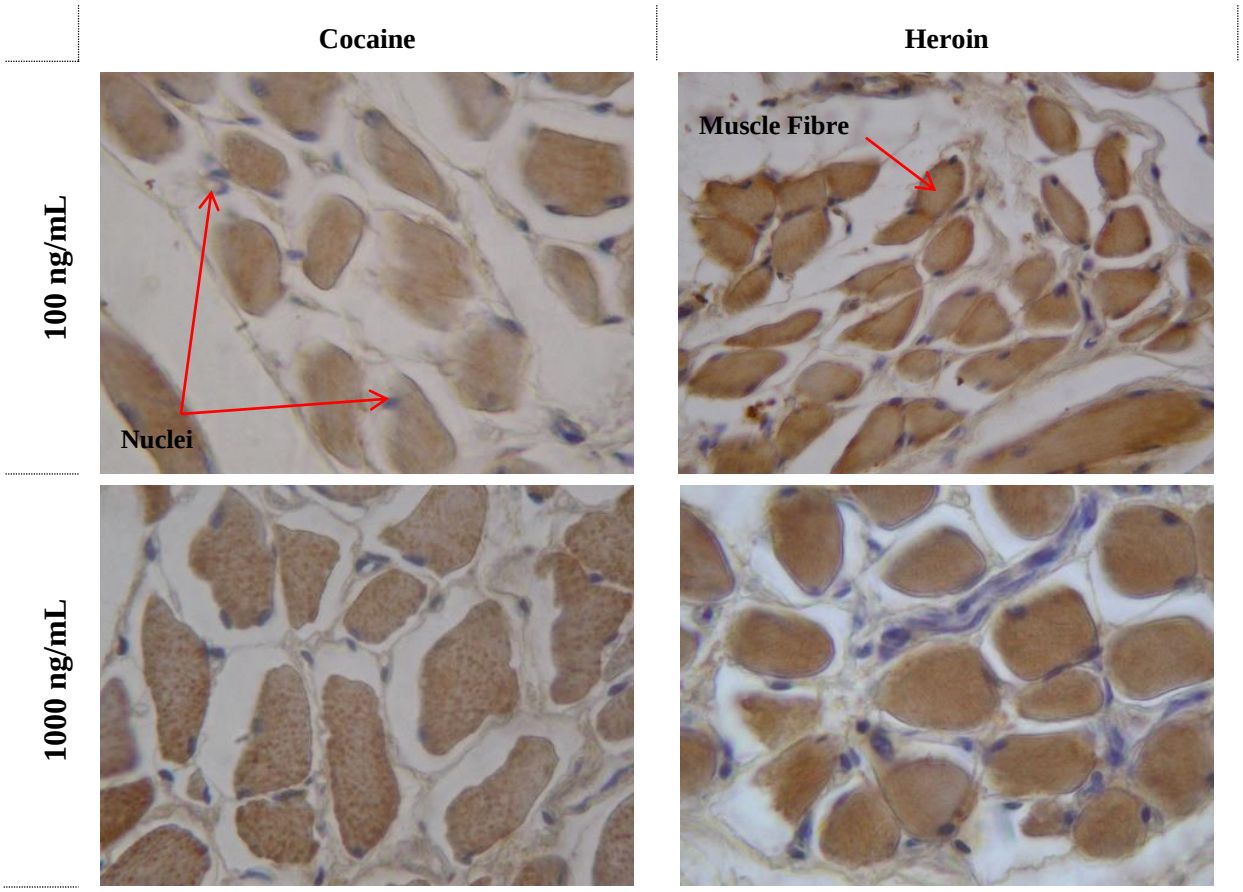


Figure 8.28 Summary of porcine tongues at magnification x 63 stained using an immunohistochemical technique following the exposure to either 100 ng/mL or 1000 ng/mL of either cocaine or heroin and washing for 1 hour

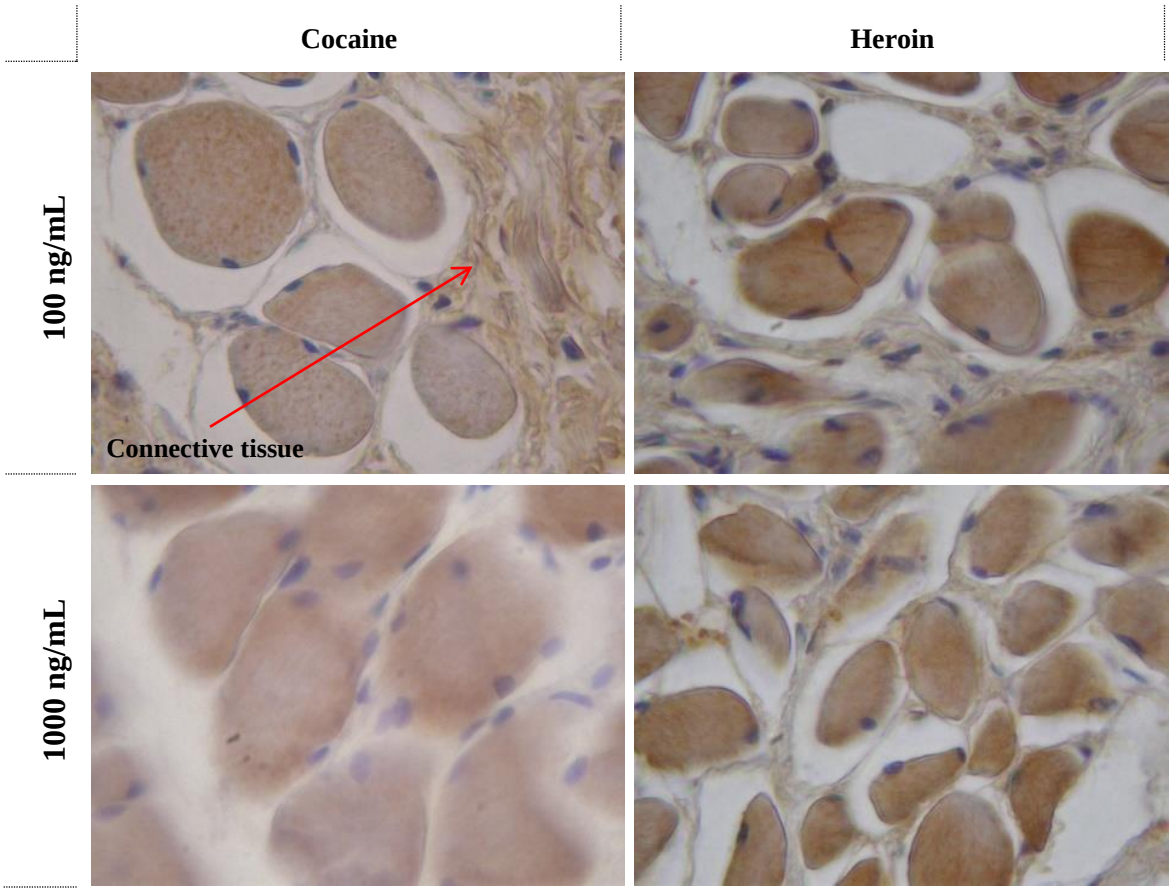


Figure 8.29 Summary of porcine tongues at magnification x 63 stained using an immunohistochemical technique following the exposure to either 100 ng/mL or 1000 ng/mL of either cocaine or heroin and washing for 6 hours

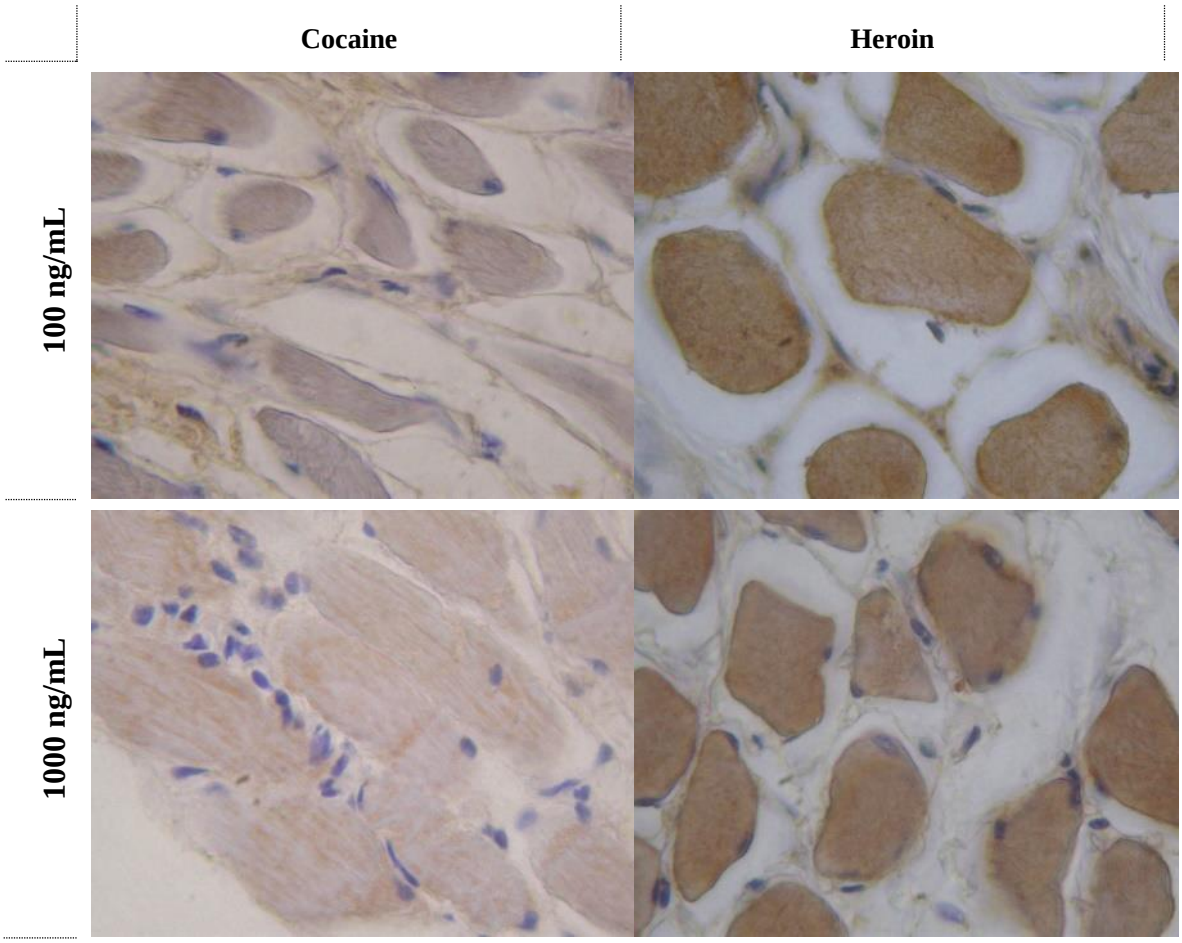


Figure 8.30 Summary of porcine tongues at magnification x 63 stained using an immunohistochemical technique following the exposure to either 100 ng/mL or 1000 ng/mL of either cocaine or heroin and washing for 24 hours

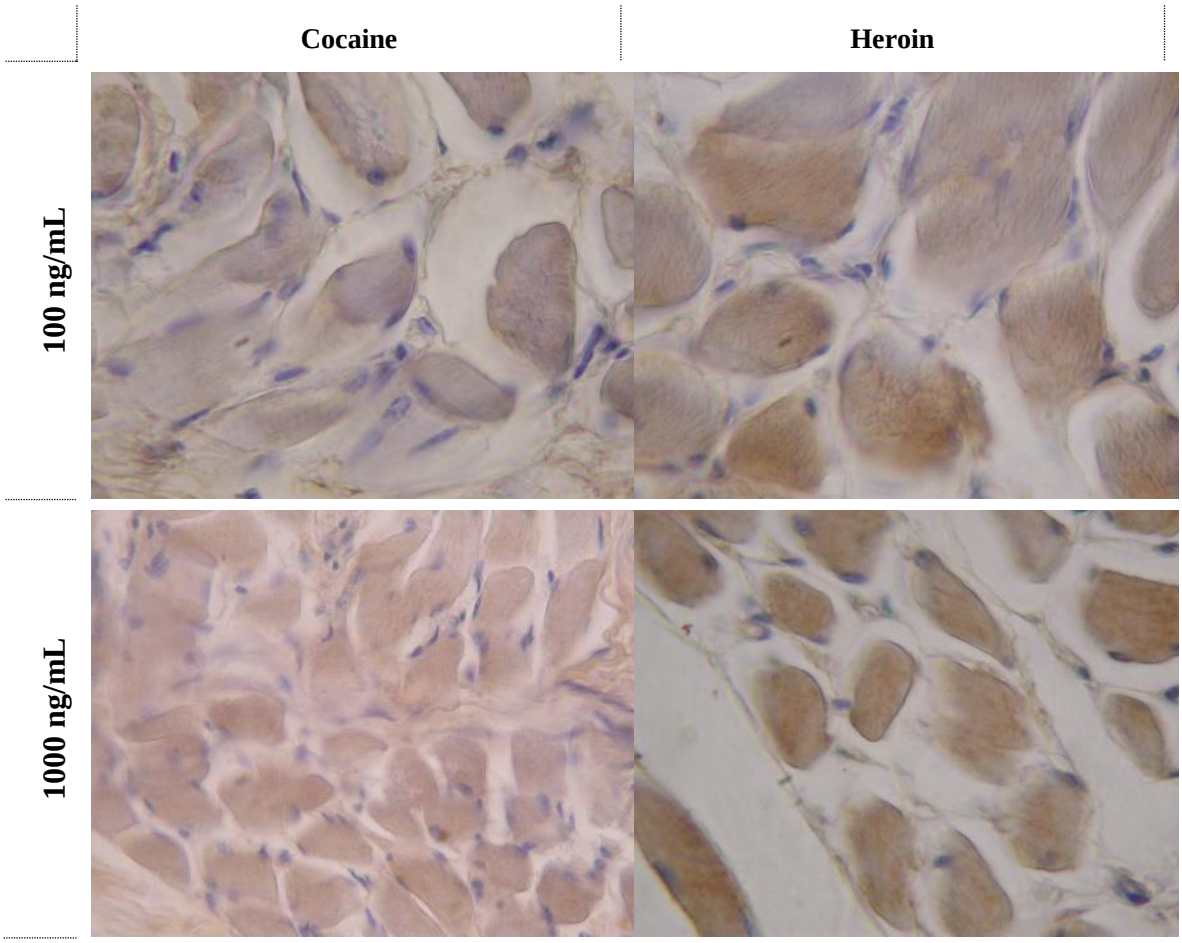


Figure 8.31 Summary of porcine tongues at magnification x 63 stained using an immunohistochemical technique following the exposure to either 100 ng/mL or 1000 ng/mL of either cocaine or heroin and washing for 48 hours

Drug can be visualised in muscle bundles for up tp 48 hours. Although the change between 1 hour and 6 hours was small, when tongues were soaked for 24 hours, cocaine showed loss of staining at both exposure to 100 ng/mL and 1000 ng/mL. A slight loss of staining following the 24 hours washing was also observed following the exposure of 100 ng/mL or 1000 ng/mL of heroin. However, in comparison to cocaine, the staining in the muscle bundles for heroin was more intense and was still detected throughout the muscle 48 hours post exposure.

Results indicated that drug depots are capable of being formed in the porcine tongue tissue, which may be slowly released over time. A slow release of drug over a time period could potentially interfere with oral fluid drug detection by elevating the concentration and prolonging the detection of drugs in oral fluid. Although this is an issue when applying to roadside laws as toxicologist would be required to state if someone's impairment was due to the drug concentration, it also allows for a greater detection window for drugs in oral fluid which could be beneficial in other areas such as workplace drug testing and drug treatment programmes. Both heroin and cocaine were still clearly visible 48 hours post exposure. Longer wash times would be beneficial to ascertain a point at which no drug staining could be seen. However when tongues were washed for 96 hours post exposure, the tongue tissue had started to decompose and when embedded in paraffin, the muscle structure had broken up and hence was not useable for immunohistochemical staining.

8.3.1.3 Detection of cocaine and heroin in porcine tissue following controlled exposure to crack cocaine and heroin smoke

Tongues were exposed to either crack cocaine or heroin smoke following heating of 100mg or 200 mg of each drug for 5 minutes. The presence of cocaine and heroin could be visualised using immunohistochemical staining techniques following smoking of either drug (Figure 8.32, Figure 8.33, Figure 8.34, Figure 8.35, Figure 8.36, Figure 8.37, Figure 8.38, Figure 8.39).

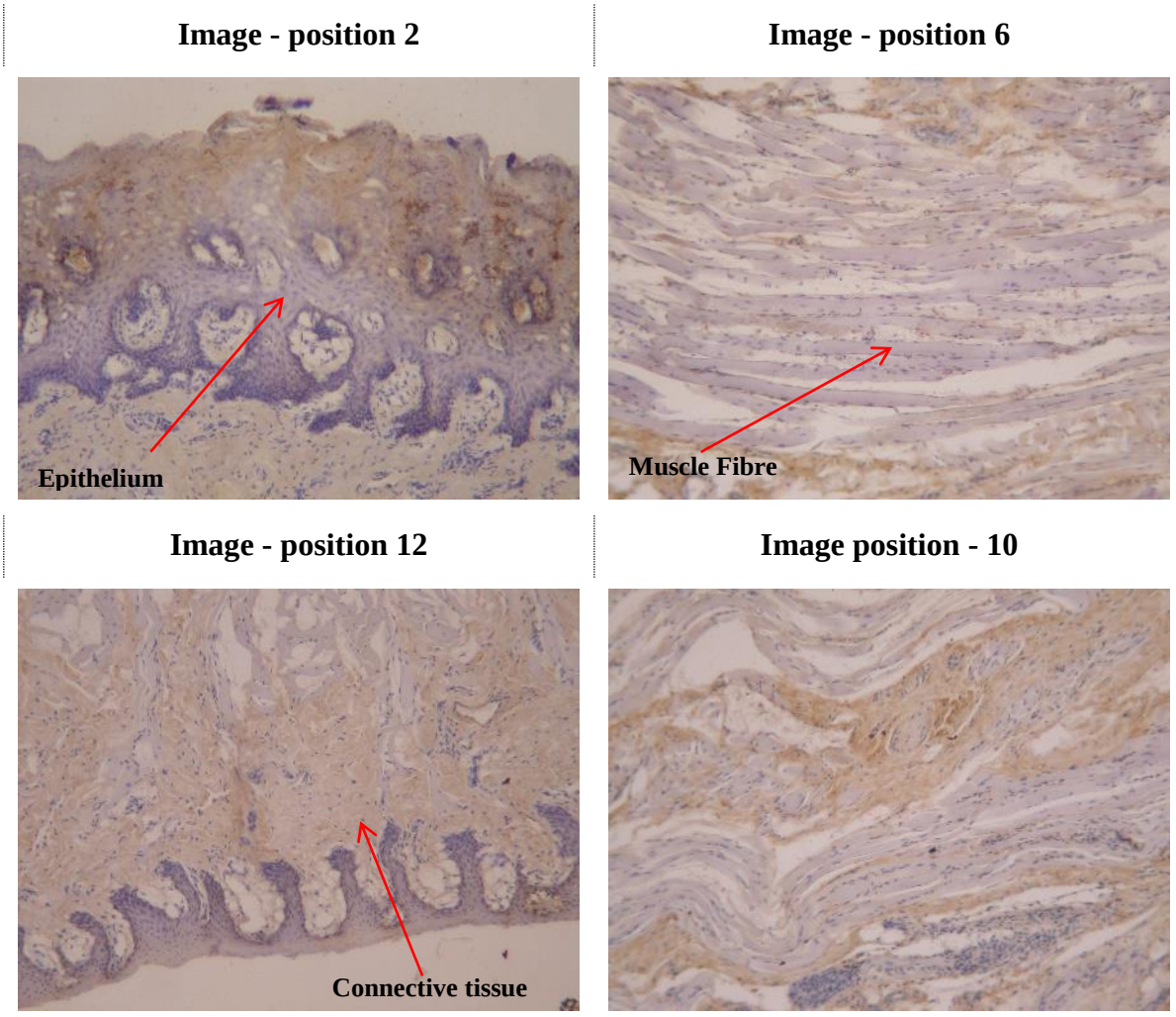


Figure 8.32 Images of porcine tongues (1) exposed to smoke from a dose of 100 mg cocaine. Only images from position 2, 6, 10 and 12 are represented in this figure (magnification x25)

Image - position 2	Image - position 6

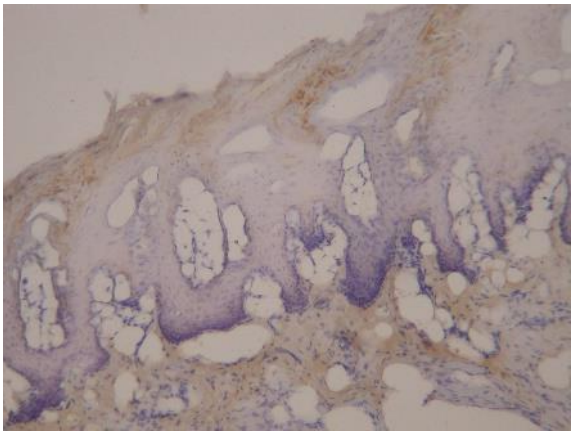
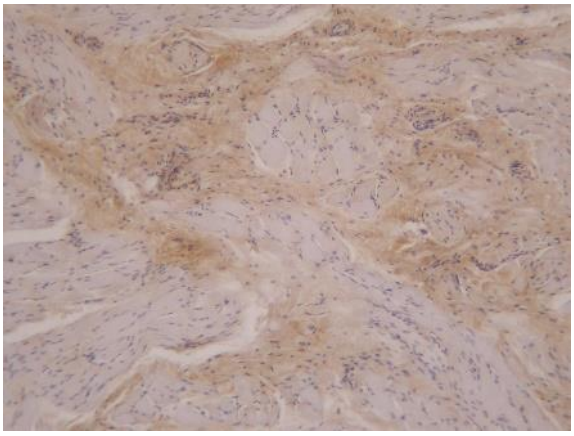

	

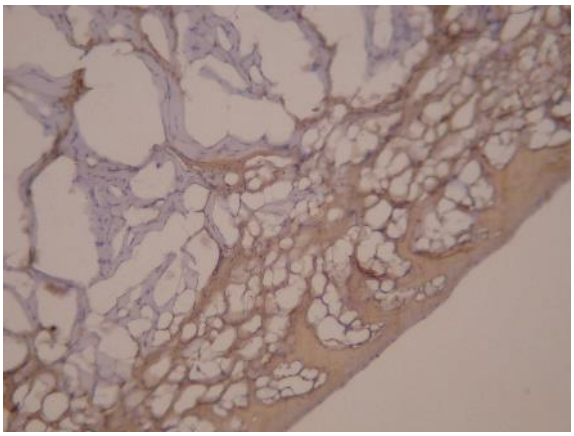
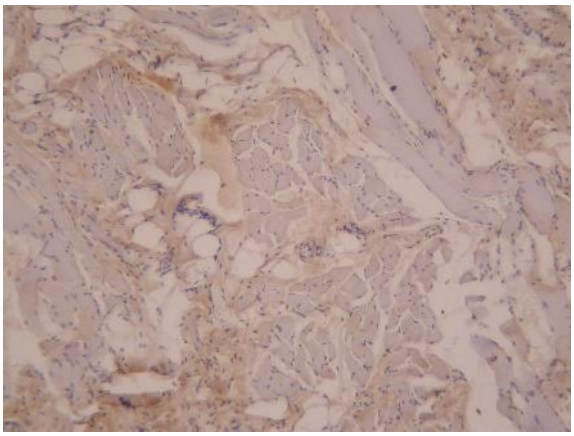
Image - position 12	**Image - position 10**
	

Figure 8.33 Images of porcine tongues (2) exposed to smoke from a dose of 100 mg cocaine. Only images from position 2, 6, 10 and 12 are represented in this figure (magnification x25)

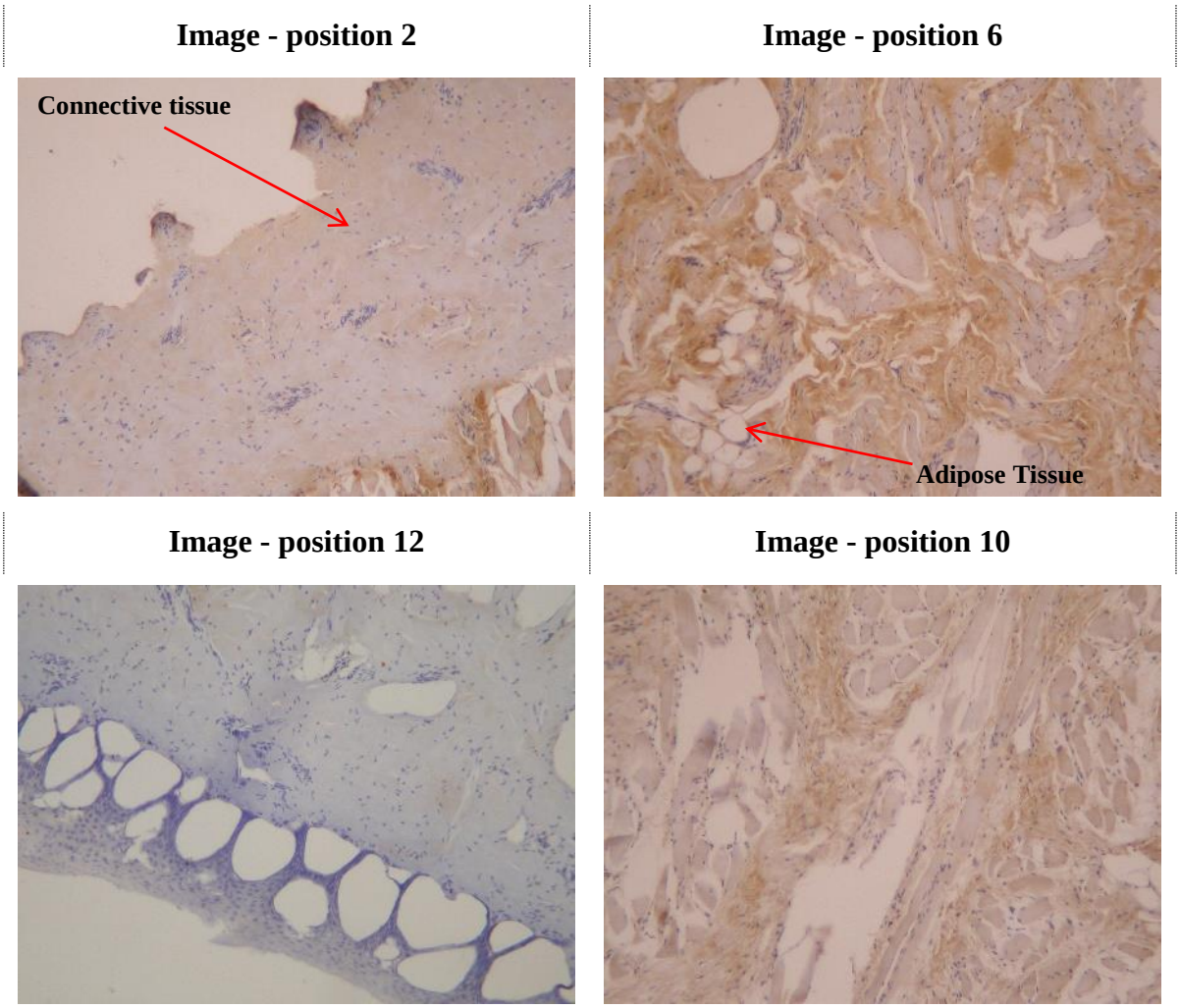


Figure 8.34 Images of porcine tongues (1) exposed to smoke from a dose of 200 mg cocaine. Only images from position 2, 6, 10 and 12 are represented in this figure (magnification x25)

Image - position 2	Image - position 6

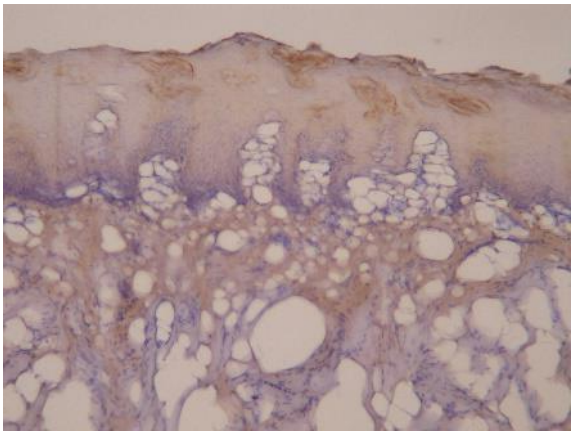
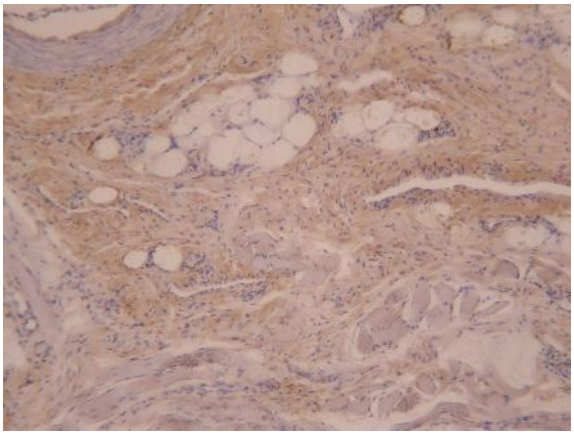

	

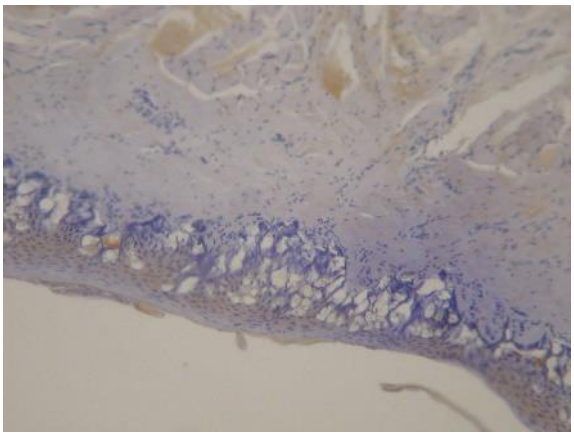
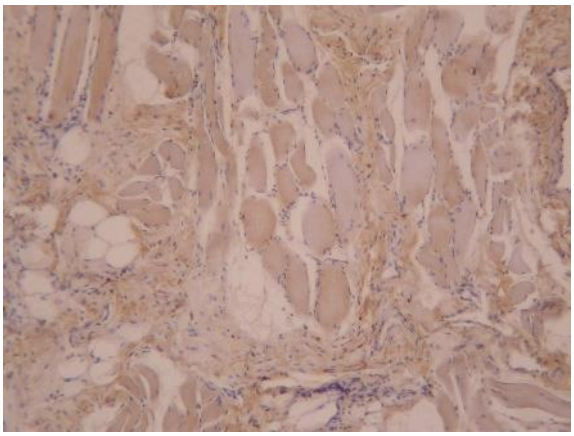
Image - position 12	**Image position 10**
	

Figure 8.35 Images of porcine tongues (2) exposed to smoke from a dose of 200 mg cocaine. Only images from position 2, 6, 10 and 12 are represented in this figure (magnification x25)

Image - position 2

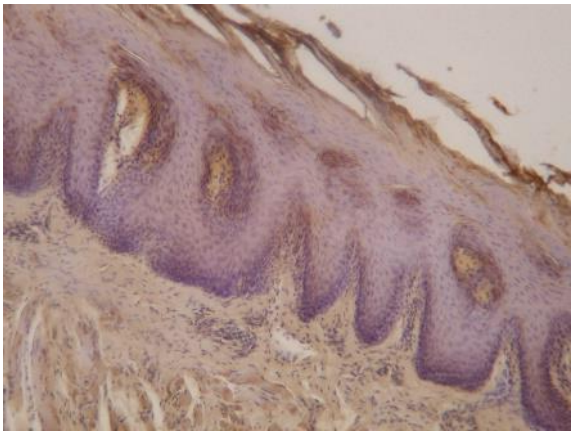


Image - position 6

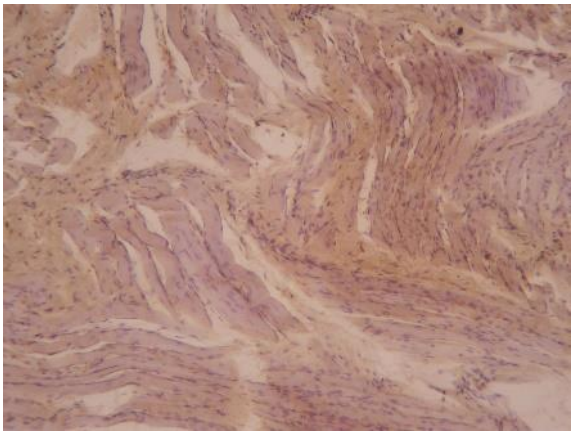


Image - position 12

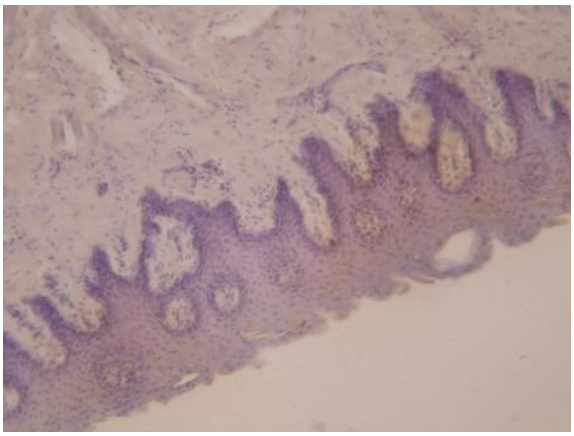


Image - position 10

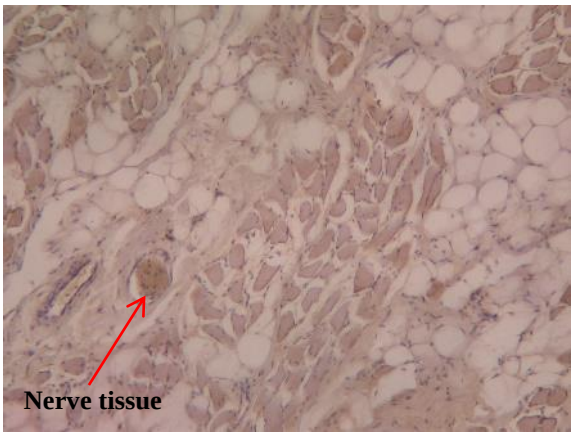


Figure 8.36 Images of porcine tongues (1) exposed to smoke from a dose of 100 mg heroin. Only images from position 2, 6, 10 and 12 are represented in this figure (magnification x25)

Image - position 2	Image - position 6

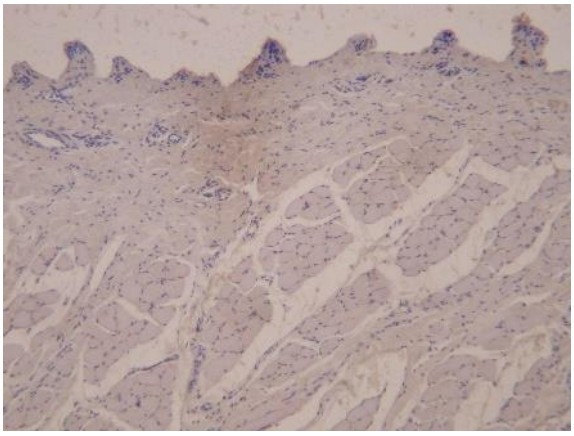
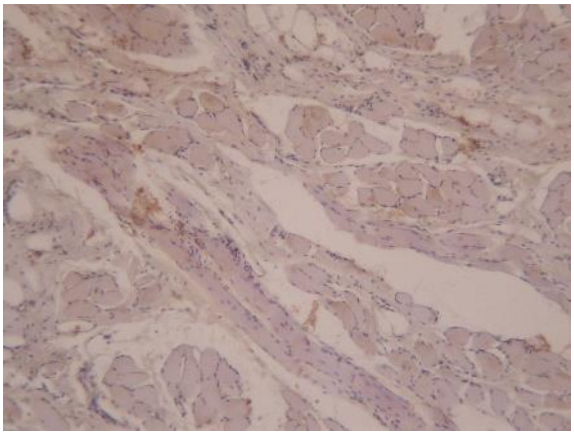

	

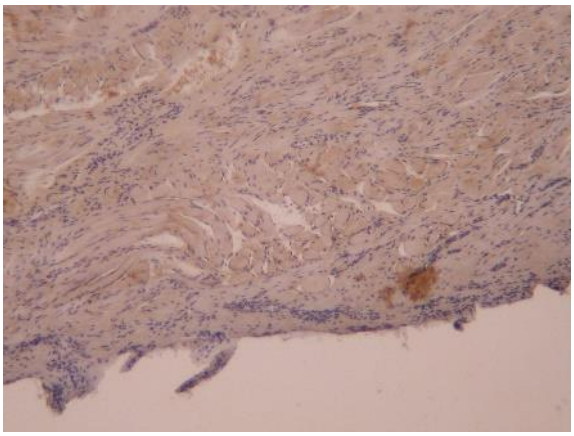
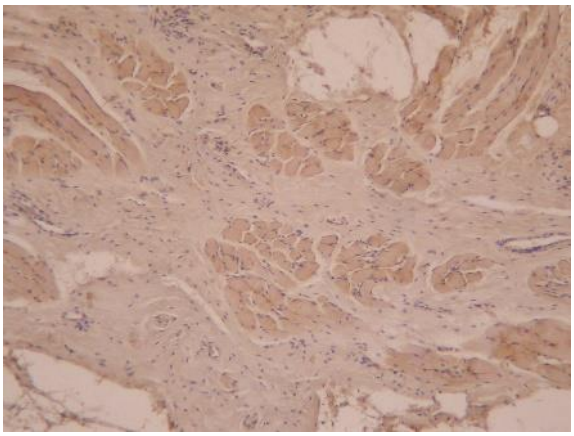
Image - position 12	**Image - position 10**
	

Figure 8.37 Images of porcine tongues (2) exposed to smoke from a dose of 100 mg heroin. Only images from position 2, 6, 10 and 12 are represented in this figure (magnification x25)

Image - position 2	Image - position 6

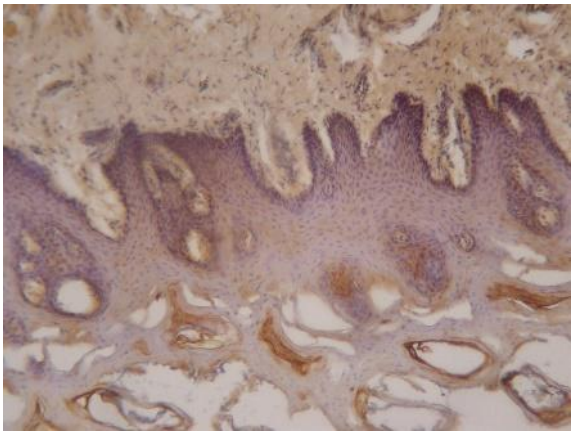
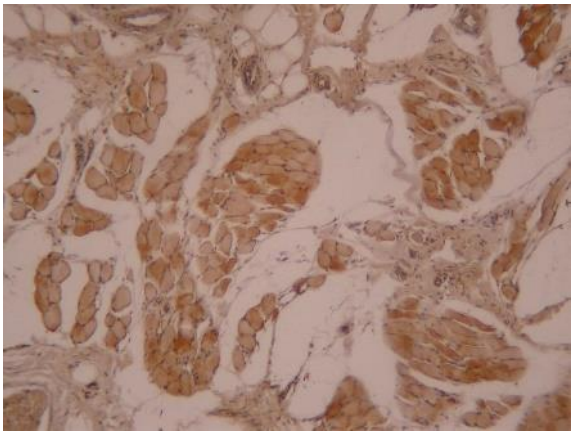

	

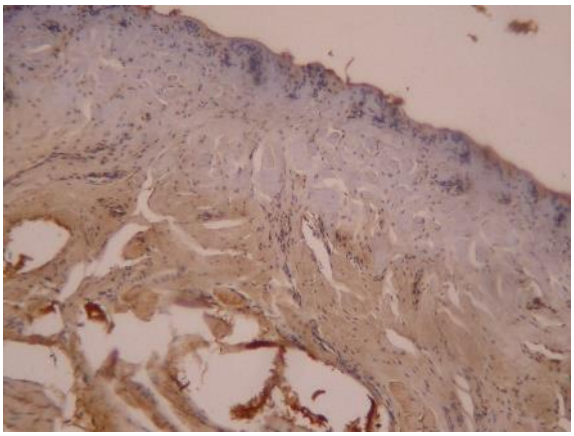
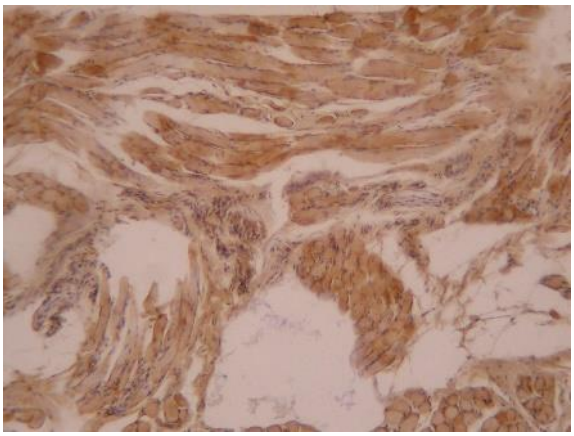
Image - position 12	**Image - position 10**
	

Figure 8.38 Images of porcine tongues (1) exposed to smoke from a dose of 200 mg heroin. Only images from position 2, 6, 10 and 12 are represented in this figure (magnification x25)

Image - position 2

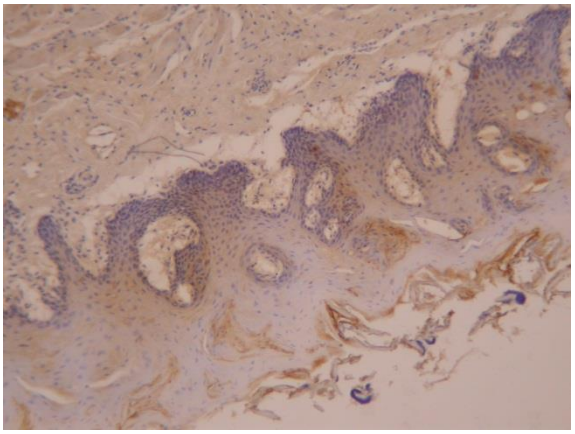


Image - position 6

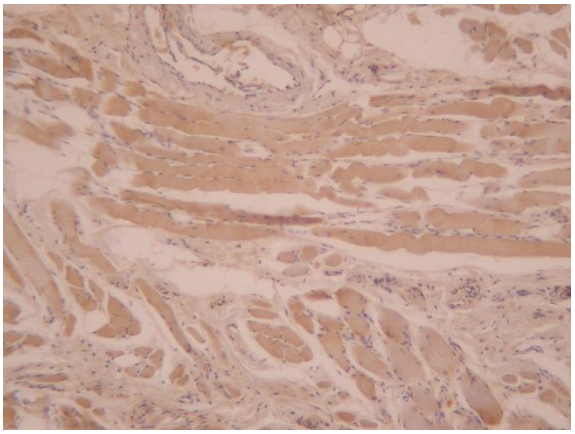


Image - position 12

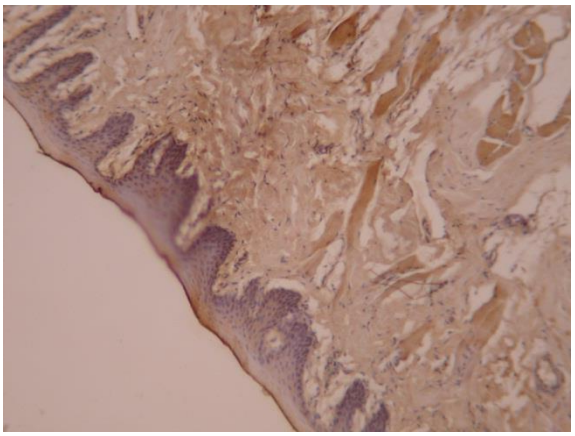


Image - position 10

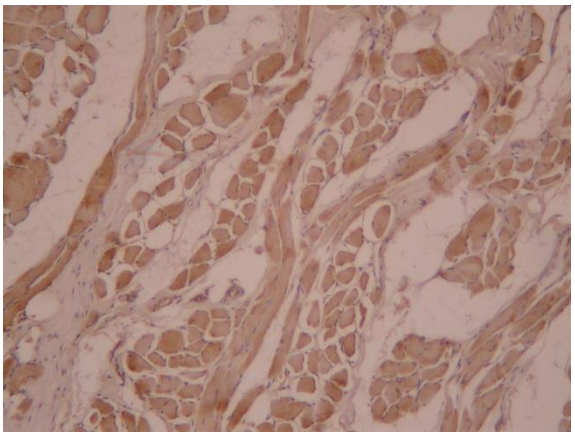


Figure 8.39 Images of porcine tongues (2) exposed to smoke from a dose of 200 mg heroin. Only images from position 2, 6, 10 and 12 are represented in this figure

Images presented in Figure 8.39 show that drug entered the tongue tissue following exposure to both heroin and cocaine smoke. Tongues exposed to smoke from 100 mg of crack cocaine were demonstrated to enter into the tongue. Staining for cocaine was present in the epithelium; however it was not obvious in the muscle bundles and was mainly retained in the connective tissue surrounding the muscle bundles.

Similarly heroin was detected in the epithelium following the generation of smoke from a 100 mg dose of street heroin. In comparison to cocaine, heroin appeared to have entered the muscle bundles as well as the connective tissue at the low dose.

The high dose of cocaine resulted in more intense staining which seemed to be starting to enter the muscle bundles but the largest amount of staining and the most intense staining was still observed within the connective tissue.

Higher doses of heroin showed more intense staining in the muscle of the tongue than exposure to lower doses of heroin. Heroin was clearly visible in the muscle bundles although a comparison at higher power magnification allows for a better comparison of the penetration of each drug.

When visualising the sections of porcine tongues with a higher magnification (x63) the results support the initial statement that tongues exposed to cocaine smoke from a 100 mg dose do not enter the muscle bundles at all and is only detected in the connective tissue (Figure 8.40). Following smoking of 200 mg dose of crack cocaine, it was seen to start to enter the muscle bundles with weak staining visual surrounding the muscle bundles. Cocaine was also visible in the connective tissue.

Crack cocaine (100 mg)	Crack cocaine (200 mg)

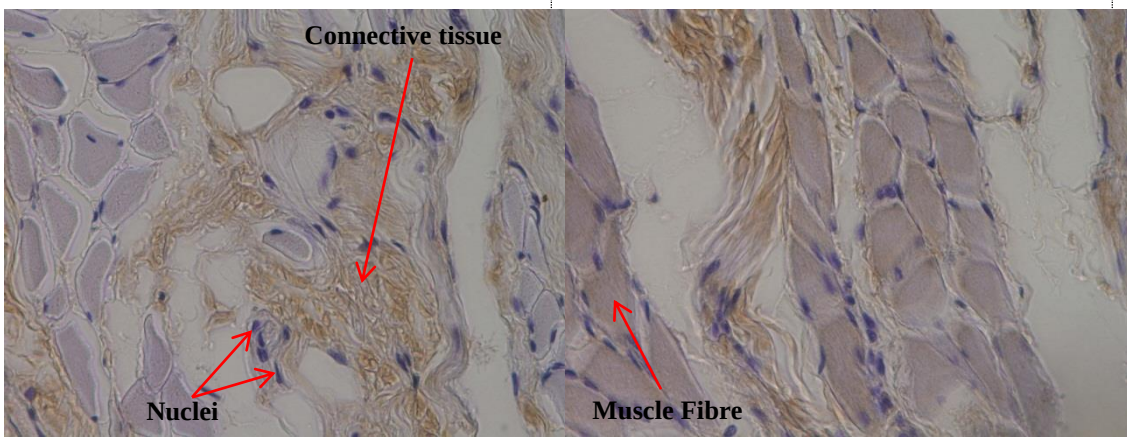


Figure 8.40 Image representing the presence of cocaine in porcine tongue tissue following exposure to smoke from a 100 mg or 200 mg dose of crack cocaine visualised by an immunohistochemical technique. (magnification x63)

In comparison following the exposure to smoke from 100 mg of heroin the drug can be seen to be weakly entering the muscle bundles with the outside of the bundles showing stronger binding.

Following smoke exposure from a dose of 200 mg of heroin the drug has entered the muscle bundles and staining can be clearly strongly observed all the way through the muscle bundles (Figure 8.41).

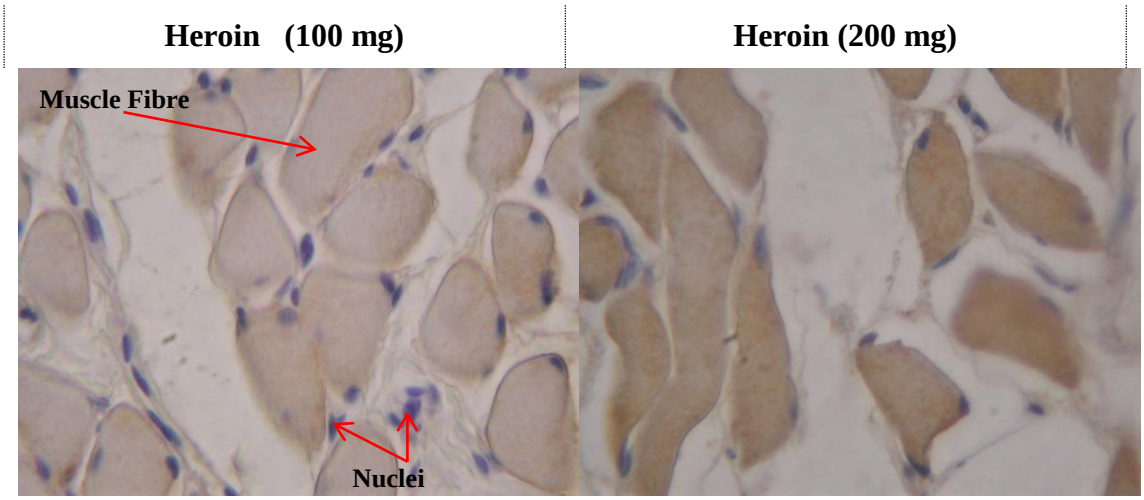


Figure 8.41 Image representing the presence of cocaine in porcine tongue tissue following exposure to smoke from a 100 mg or 200 mg dose of heroin visualised by an immunohistochemical technique (magnification x63).

The presence of drugs, visualised by immunohistochemical staining in tissue following smoking also strongly supports the hypothesis that drugs can enter the oral fluid via routes other than via the surrounding blood. Drugs enter the muscle and form depots within the tissue when smoked which indicates that they can linger in the tissue and subsequently be released over time. Due to time restrictions during this project, no studies were undertaking to investigate the release and its detecting time for smoked tongues.

8.3.2 Analytical results

8.3.2.1 Tissue exposure to drug solutions of cocaine and heroin

8.3.2.1.1 Cocaine

8.3.2.1.1.1 Cocaine and benzoylecgonine concentration detected in the artificial saliva washes using LC-MS/MS

LC-MS results showed how concentrations of cocaine and benzoylecgonine in artificial saliva washes decreased throughout the washes 1 –7 (W1 – W7) following exposure to cocaine at concentration in solution of either 100 ng/mL, 250 ng/mL, 500 ng/mL, 1000 ng/mL or 10 µg/mL. With the exception of the highest exposure concentration of 10 µg/mL, all washes were no longer detectable at wash 7. Analytical results for cocaine and benzoylecgonine are summarised in Table 8.3.

Table 8.3 Concentrations (ng/mL) of cocaine and benzoylecgonine in wash 1 to wash 7 (W1 – W7) from tongues exposed cocaine concentrations of either 100 ng/mL, 250 ng/mL, 500 ng/mL, 1000 ng/mL or 10 µg/mL (ND – not detected)

Concentration (dose)	Tongue No.	Wash No.	Concentration (ng/mL)	
			Cocaine	Benzoylecgonine
100 ng/mL	1	W1	*ND*	*ND*
		W2	*ND*	*ND*
		W3	*ND*	*ND*
		W4	*ND*	*ND*
		W5	*ND*	*ND*
		W6	*ND*	*ND*
		W7	*ND*	*ND*
	2	W1	*ND*	*ND*
		W2	*ND*	*ND*
		W3	*ND*	*ND*
		W4	*ND*	*ND*
		W5	*ND*	*ND*

Concentration (dose)	Tongue No.	Wash No.	Concentration (ng/mL)	
			Cocaine	Benzoylecgonine
		W6	*ND*	*ND*
		W7	*ND*	*ND*
250 ng/mL	1	W1	3	1
		W2	1	2
		W3	*ND*	1
		W4	*ND*	*2*
		W5	*ND*	*ND*
		W6	*ND*	*ND*
		W7	*ND*	*ND*
	2	W1	1	3
		W2	0	3
		W3	ND	0
		W4	ND	2
		W5	ND	1
		W6	ND	ND
		W7	ND	ND
500 ng/mL	1	W1	1	3
		W2	ND	1
		W3	ND	0
		W4	ND	1
		W5	ND	1
		W6	ND	ND
		W7	ND	ND
	2	W1	3	2
		W2	ND	0
		W3	ND	0
		W4	ND	1
		W5	ND	3
		W6	ND	ND
		W7	ND	ND
1000 ng/mL	1	W1	68	54
		W2	6	5
		W3	5	5
		W4	1	4
		W5	1	3
		W6	2	2
		W7	ND	ND

Concentration (dose)	Tongue No.	Wash No.	Concentration (ng/mL)	
			Cocaine	Benzoylecgonine
	2	W1	45	33
		W2	11	11
		W3	4	4
		W4	2	3
		W5	2	3
		W6	4	3
		W7	ND	ND
10 µg/mL	1	W1	109	65
		W2	54	39
		W3	23	24
		W4	9	9
		W5	9	11
		W6	16	11
		W7	8	9
	2	W1	198	104
		W2	60	29
		W3	26	18
		W4	11	10
		W5	10	8
		W6	15	9
		W7	13	3

Most manufacturers of oral fluid drug tests indicate that the effects of contamination have dispersed from the mouth after 10 - 15 minutes (Spiehler 2011) and that after this time has elapsed an oral fluid sample can then be collected with minimum risk of contamination. Tongues were washed during the work undertaken in this chapter as in the mouth saliva is constantly renewed and oral tissue washed with new fresh saliva at an average rate of 0.5 mL/min to 1 mL/min. The results supported the hypothesis that some drug had loosely bound to the tongue surface which was gently and progressively removed, potentially leaving significant binding and transfer to the tissue itself.

Immunohistochemical results of the tongue tissue which was processed following wash 7, showed significant staining in all tongue tissue which previously exposure to cocaine at concentration in solution of either 100 ng/mL, 250 ng/mL, 500 ng/mL, 1000 ng/mL or 10 µg/mL. This indicated that although no drug was being released during the wash steps, drug was still present within the tissue and hence bound to the oral tissue.

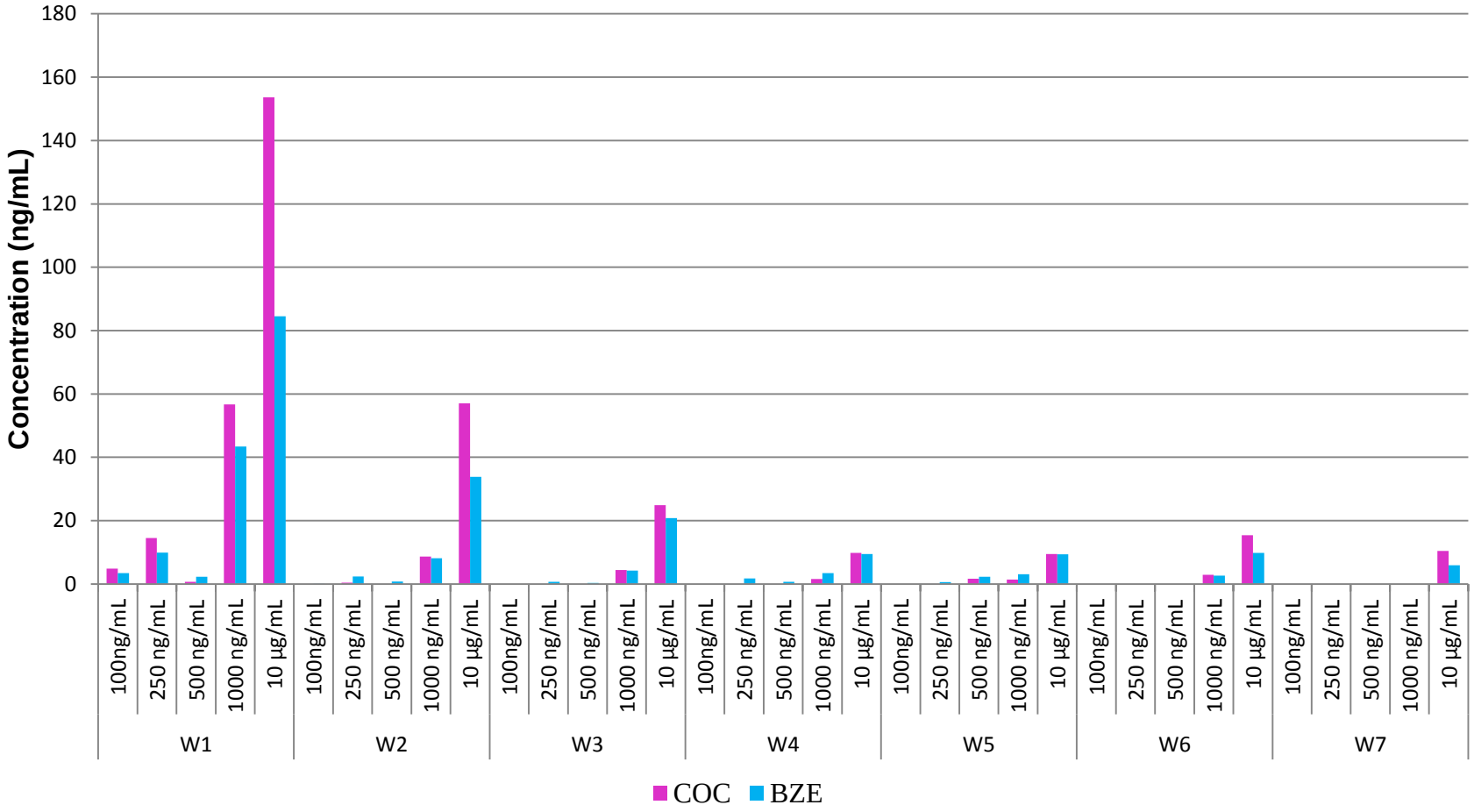


Figure 8.42 shows how concentrations of cocaine and benzoylecgonine in artificial saliva washes decreased throughout the washes 1 – washes 7 (W1 – W7) following exposure to cocaine at concentration in solution of either 100 ng/mL, 250 ng/mL, 500 ng/mL, 1000 ng/mL or 10 µg/mL

8.3.2.1.1.2 Collectors

After tongues were washed with artificial saliva a pre-wetted Alere™ Certus Collector was brushed against the tongue, in order to determine whether the use of the collector against the tongue would be able to collect cocaine from the tissue of the tongue. Previous results showed that during washing, no cocaine or benzoylecgonine could be detected in the last wash (wash 7), with the exception of tongues exposed to the highest concentration of cocaine of 10 µg/mL (Table 8.2).

When Certus collectors were brushed against the tongue, both cocaine and benzoylecgonine could be detected in the collected samples which had been exposed to either 100 ng/mL, 250 ng/mL, 500 ng/mL, 1000 ng/mL or 10 µg/mL (Table 8.4).

Table 8.4. Concentrations (ng/mL) of cocaine and benzoylecgonine that were brushed against the porcine tongues which had previously been exposed to cocaine at concentrations of either 100 ng/mL, 250 ng/mL, 500 ng/mL, 1000 ng/mL or 10 µg/mL and washes seven times. Samples were collected using a pre-wetted Alere™ Certus Collector.

Concentration (dose)	**Tongue No.**	**Concentration (ng/mL)**			
		Cocaine	**BZE**	**Mean Cocaine**	**Mean BZE**
100 ng/mL	1	2	10	4	12
	2	6	14		
250 ng/mL	1	22	24	10	16
	2	34	32		
500 ng/mL	1	8	14	28	28
	2	12	18		
1000 ng/mL	1	46	44	38	39
	2	30	34		
10 µg/mL	1	78	70	76	70
	2	74	70		

Mean concentrations of cocaine and benzoylecgonine from tongues surfaces which were exposed to 100 ng/mL of cocaine solution were 4 ng/mL and 12 ng/mL, respectively. Following exposure to 250 ng/mL of cocaine solution, the mean cocaine and benzoylecgonine concentration detected in samples, collected with the Alere™ Certus collector, was 10 ng/mL and 16 ng/mL. Exposure to 500 ng/mL of cocaine solution resulted in a mean concentration of 28 ng/mL for both cocaine and benzoylecgonine. Brushing the Certus collector against porcine tongues which had been exposed to 100 ng/mL of cocaine solution resulted in mean concentration of

cocaine and benzoylecgonine of 28 and 39 ng/mL, respectively. The highest cocaine solution of 10 µg/mL resulted in mean concentrations of 76 ng/mL and 70 ng/mL for cocaine and benzoylecgonine respectively, in samples collected by brushing a pre-wetted Alere™ Certus collector. Figure 8.43 summarises how cocaine and benzoylecgonine increased with increasing concentration of exposure.

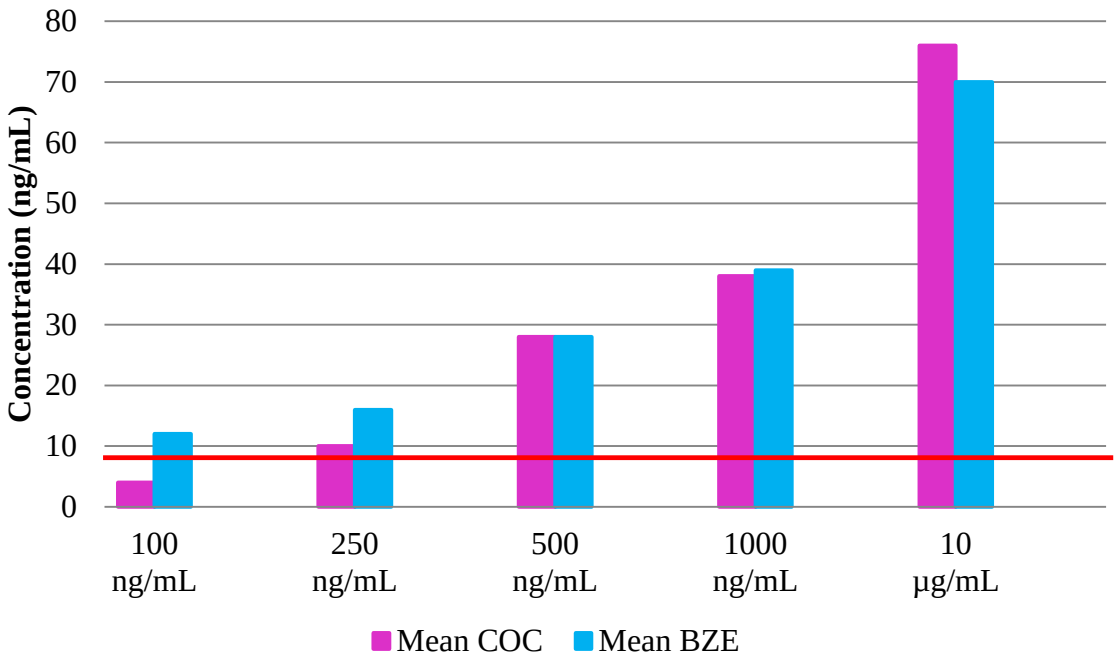


Figure 8.43. Mean cocaine and benzoylecgonine concentrations in samples collected by brushing the Alere™ Certus Collector against porcine tongues which were either exposed to 100 ng/mL, 250 ng/mL, 500 ng/mL, 1000 ng/mL or 10 µg/mL. The red line (---) indicates the SAMHSA cut off for cocaine of cocaine and benzoylecgonine in oral fluid)

The recommended SAMHSA cut off for cocaine and benzoylecgonine in oral fluid is 8 ng/mL (Chapter 4). The brushing of the collector against the tongue clearly removed cocaine and benzoylecgonine at concentrations which were significantly above the SAMHSA cut off. The presence of cocaine and benzoylecgonine in samples collected using a pre-wetted Alere^{TM} Certus collector following seven washes after the original exposure to various concentrations of cocaine solution, indicated that the brushing the collector against the tongue can potentially extract drug from the oral tissue. The seventh wash was negative for cocaine and benzoylecgonine when exposed to cocaine spiked into artificial saliva, with the exception of the 10 µg/mL dose, indicating that all excess external contamination of cocaine had been washed off by wash seven. Therefore the presence of cocaine and benzoylecgonine in samples collected with the Certus collector, are not a direct result of external contamination, but the drug must have been released from the oral tissue.

The extraction of cocaine and benzoylecgonine by simply brushing the collector against the porcine tongue has the potential to artificially elevate concentrations of cocaine and benzoylecgonine detected in an oral fluid drug test and hence can interfere with the interpretation of oral fluid testing results for toxicologist that look at saliva:plasma ratios. This could therefore affect oral fluid collection in individuals where samples were collected using any device that is designed to be rubbed or scraped over the mouth tissues. Expectorated samples may assist to fully avoid artificially elevated concentration through the use of a collector. On the other hand it may assist in cases where it is necessary to check for drug compliance or abstinence where it is not relevant where the drug has come from.

8.3.2.1.1.3 Tissue Drug Concentrations

Homogenates were prepared by analysing 1 g of tissue from five sections of tongue tissue (section 8.2.2.3.1).

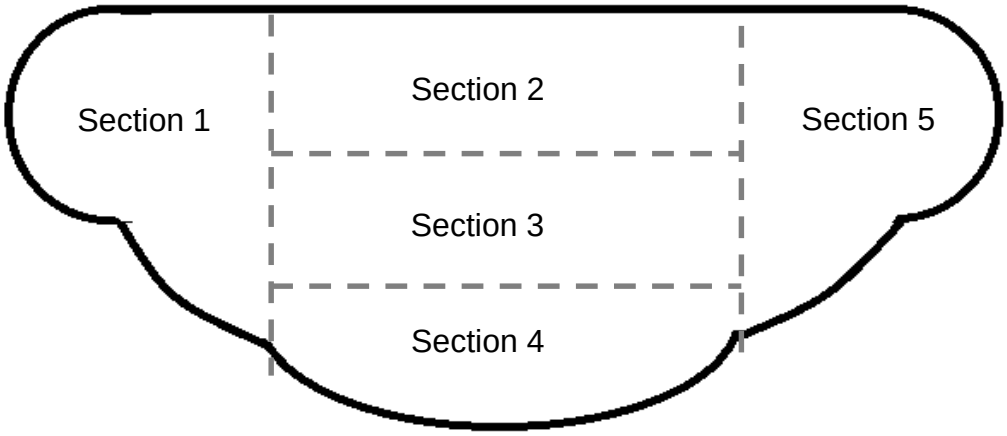


Figure 8.44. Diagram of sectioning of the transverse cross section of the tongue for homogenisation

Both cocaine and benzoylecgonine were detected in tongue tissue following exposure to either 100 ng/mL, 250 ng/mL, 500 ng/mL, 1000 ng/mL or 10 µg/mL of cocaine in artificial saliva. The mean cocaine concentration detected in tongues which were exposed to 100 ng/mL of cocaine solution was 12.8 ng/mL (range = 6–25 ng/mL) with the lowest cocaine concentration observed in the centre of the tongue (section 3).

Following exposure to a cocaine solution of 250 ng/mL in artificial saliva mean cocaine concentrations detected in tongue tissue ranged between 7 ng/mL and 39 ng/mL (mean = 19.8 ng/mL). The lowest mean cocaine concentration detected in

tissue was again in the centre of the tongue with a mean cocaine concentration between tongue 1 and tongue 2 of 7 ng/mL.

Concentrations of cocaine which were detected in the transverse cross section of the tongue ranged between 6 ng/mL and 44 ng/mL (mean = 27.2 ng/mL). Largest concentrations were observed in section 1 and section 5 with 36 ng/mL and 44 ng/mL, respectively. The lowest mean cocaine concentration was observed in section 3 in the centre of the tongue at 6 ng/mL (Table 8.5).

Table 8.5. Cocaine concentration (ng/mL) resulting from the analysis of homogenates from porcine tongues which were previously exposed to either 100 ng/mL, 250 ng/mL, 500 ng/mL, 1000 ng/mL and 10 µg/mL of cocaine in artificial saliva

Spiked Concentration	**Section**	**Cocaine Concentration (ng/mL)**		**Mean Cocaine concentration (ng/mL)**
		Tongue		
		1	**2**	
100 ng/mL	**S1**	4	22	13
	S2	10	8	9
	S3	2	10	6
	S4	6	44	25
	S5	2	20	11
250 ng/mL	**S1**	24	32	28
	S2	10	22	16
	S3	12	2	7
	S4	42	36	39
	S5	12	6	9
500 ng/mL	**S1**	34	38	36
	S2	12	46	29
	S3	4	8	6
	S4	30	12	21
	S5	34	54	44

Spiked Concentration	Section	Cocaine Concentration (ng/mL)		Mean Cocaine concentration (ng/mL)
		Tongue		
		1	2	
1000 ng/mL	S1	132	10	71
	S2	20	40	30
	S3	28	52	40
	S4	24	40	32
	S5	96	20	58
10 µg/mL	S1	88	322	205
	S2	14	96	55
	S3	34	34	34
	S4	360	188	274
	S5	354	190	272

Cocaine concentrations detected in transverse cross section of the tongue did not show a large increase between tongues exposed to 100 ng/mL, 250 ng/mL and 500 ng/mL which supported the immunohistochemistry staining result.

Following the exposure of porcine tongues to 1000 ng/mL of cocaine solution, the mean concentration observed in the transverse cross section was 46.2 ng/mL (range = 32 ng/mL – 71 ng/mL). The mean cocaine concentration in the centre of the tongue (section 3) was 40 ng/mL which was a large increase from cocaine concentration detected following exposure to 500 ng/mL cocaine solution indicating that cocaine has travelled further into the porcine tongue.

Exposure of porcine tongue to 10 µg/mL of cocaine solution resulted in large mean cocaine concentrations ranging between 34 ng/mL and 274 ng/mL (mean = 168 ng/mL). Lowest mean cocaine concentration was observed at 34 ng/mL in section 3 which was removed from the centre of the tongue.

Results showed that there was large variation in cocaine concentration (ng/mL) measured in each section between the duplicate tongues exposed to either 100 ng/mL, 250 ng/mL, 500 ng/mL, 1000 ng/mL and 10 µg/mL of cocaine in artificial saliva (Figure 8.45). Porcine tongues varied in size during this experiment as tongues are proportionate to the size of the animal it was removed from. As tongues were provided directly by the butcher there was no way to individually selecting each tongue depending on its size. Therefore the large variations in concentrations between two tongues exposed to the same cocaine concentrations could be a direct result of the difference in size between tongues. The butcher received the tongues from an abattoirs and it was therefore not known how long the tongues were stored frozen before the use in the work undertaken in this chapter. Immunohistochemical analysis however showed that the tissue structure was intact in all tongues, confirming that the tongues were suitable for the use in the work undertaken in this chapter.

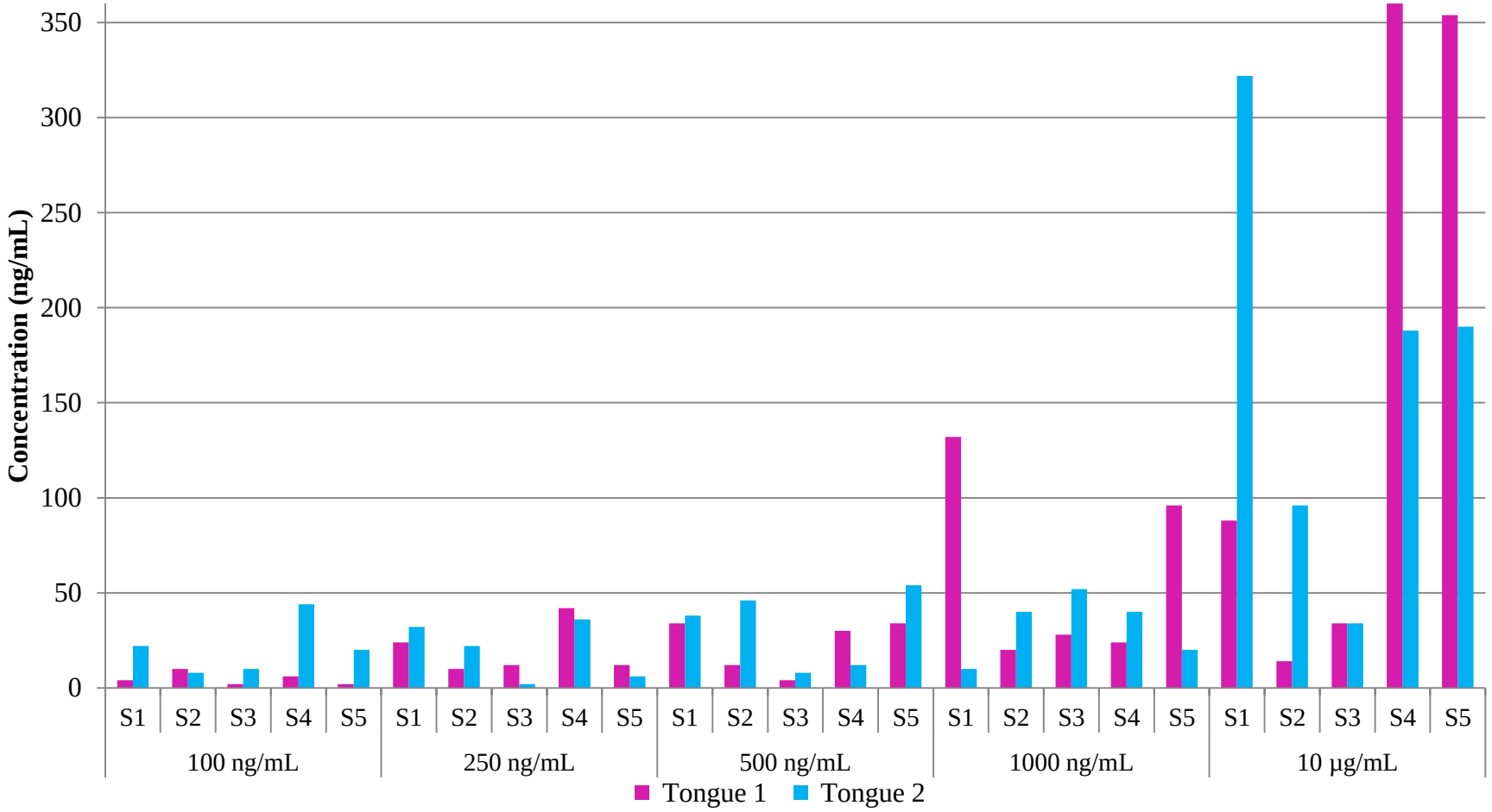


Figure 8.45. Comparison between cocaine concentrations (ng/mL) in duplicate tongues following exposure to either 100 ng/mL, 250 ng/mL, 500 ng/mL, 1000 ng/mL or 10 µg/mL of cocaine in artificial saliva

Following exposure to a cocaine solution of 100 ng/mL in artificial saliva mean benzoylecgonine concentrations detected in tongue tissue ranged between 20 ng/mL and 35 ng/mL (mean = 24.2 ng/mL). The lowest mean benzoylecgonine concentrations detected in porcine tongue tissue was in sections 2 and 3 at a concentration of 20 ng/mL. Only little difference was observed between concentrations observed following exposure to 100 ng/mL, 250 ng/mL and 500 ng/mL which supported the immunohistochemical results as no large difference in the intensity of staining had been observed between those concentrations (Table 8.6).

Table 8.6 Benzoylecgonine concentration (ng/mL) resulting from the analysis of homogenates from porcine tongues which were previously exposed to either 100 ng/mL, 250 ng/mL, 500 ng/mL, 1000 ng/mL and 10 µg/mL of cocaine in artificial saliva

Spiked Concentration	**Section**	**Benzoylecgonine Concentration (ng/mL)**		**Mean Benzoylecgonine concentration (ng/mL)**
		Tongue		
		1	**2**	
100 ng/mL	**S1**	22	22	22
	S2	22	18	20
	S3	18	22	20
	S4	30	40	35
	S5	18	30	24
250 ng/mL	**S1**	28	42	35
	S2	12	22	17
	S3	16	12	14
	S4	34	46	40
	S5	20	26	23
500 ng/mL	**S1**	28	26	27
	S2	12	28	20
	S3	8	16	12
	S4	36	30	33

Spiked Concentration	Section	Benzoylecgonine Concentration (ng/mL)		Mean Benzoylecgonine concentration (ng/mL)
		Tongue		
		1	2	
	S5	32	38	35
1000 ng/mL	**S1**	170	38	104
	S2	142	42	92
	S3	52	58	55
	S4	80	56	68
	S5	238	40	139
10 µg/mL	**S1**	158	146	152
	S2	50	60	55
	S3	52	22	37
	S4	156	102	129
	S5	276	118	197

Following the exposure of porcine tongues to 1000 ng/mL of cocaine solution, the mean benzoylecgonine concentration observed in the transverse cross section was 91.6 ng/mL (range = 55 ng/mL – 139 ng/mL). The mean benzoylecgonine concentration in the centre of the tongue (section 3) was 55 ng/mL which was a large increase from benzoylecgonine concentration detected following exposure to 500 ng/mL cocaine solution indicating that more drug has penetrated the tissue. Cocaine has been known to breakdown to benzoylecgonine in aqueous solution and the presence of benzoylecgonine suggests an instability of cocaine and hence the breakdown of the cocaine to benzoylecgonine.

Following exposure to a cocaine solution of 10 µg/mL in artificial saliva mean benzoylecgonine concentrations detected in tongue tissue ranged between 37 ng/mL and 197 ng/mL (mean = 114 ng/mL). The lowest mean cocaine concentration

detected in tissue was again in the centre of the tongue with a mean cocaine concentration between Tongue 1 and Tongue 2 of 37 ng/mL.

Benzoylecgonine concentrations, like cocaine concentrations, also showed large variation between the duplicate tongues were exposed to either 100 ng/mL, 250 ng/mL, 500 ng/mL, 1000 ng/mL or 10 µg/mL of cocaine in artificial saliva (Figure 8.46). This further supported the theory that variations in drug concentration in tongue tissue can be caused by a difference in size of the tongue and hence the amount of drug accumulating in tongue tissue is proportionate to its size.

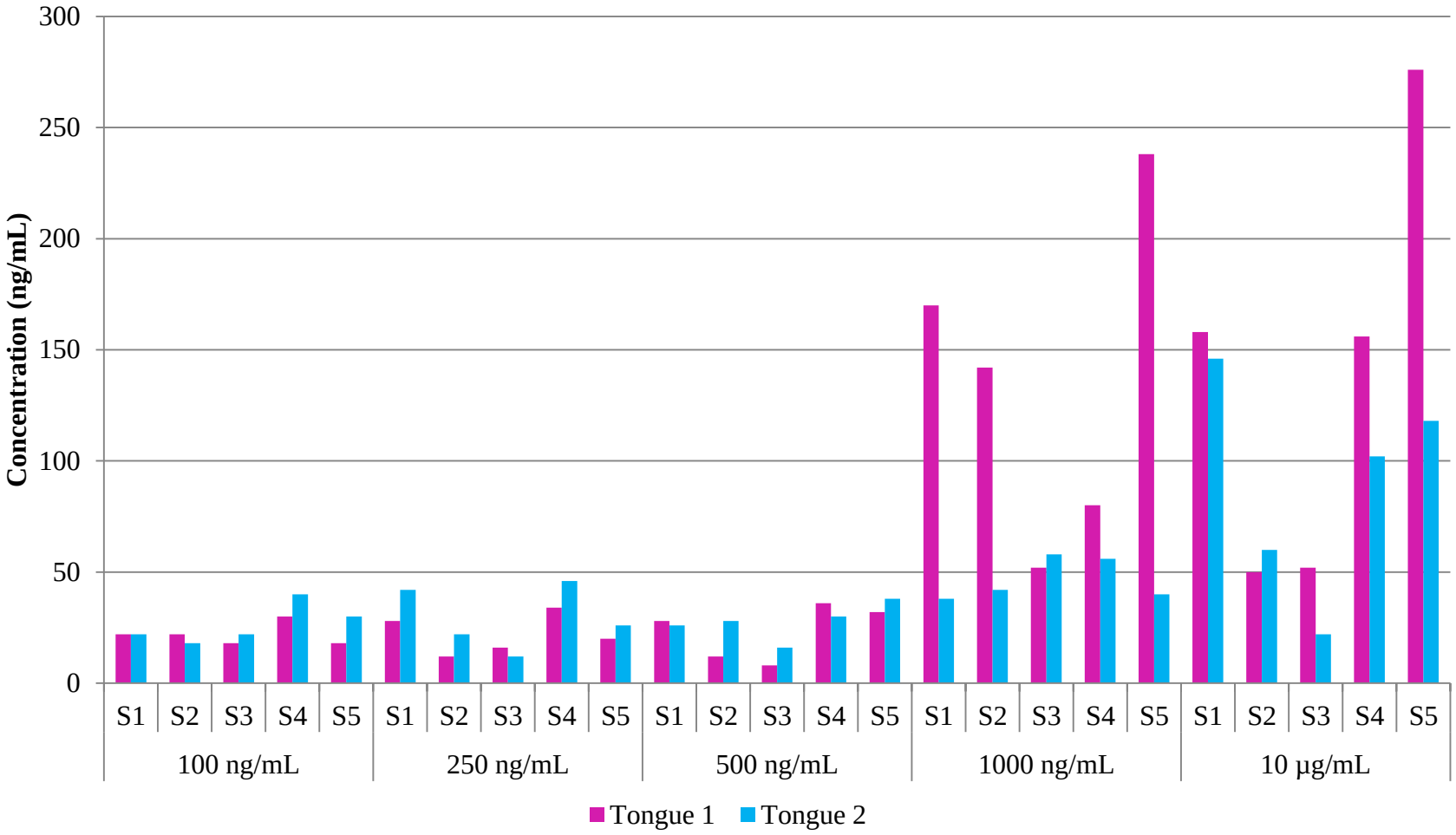


Figure 8.46. Comparison between benzoylecgonine concentrations (ng/mL) in duplicate tongues following exposure to either 100 ng/mL, 250 ng/mL, 500 ng/mL, 1000 ng/mL or 10 µg/mL of cocaine in artificial saliva

Mean concentrations of cocaine and benzoylecgonine (ng/mL) detected in transverse cross sections of tongues increased as the concentrations of drugs to which tongues were exposed increased. Both cocaine and benzoylecgonine concentrations detected were lowest when exposed to 100 ng/mL. Although there was small increase in cocaine and benzoylecgonine concentrations from 100 ng/mL to 250 ng/mL and 500 ng/mL, the largest increase was observed when porcine tongues were exposed to 1000 ng/mL and 10 µg/mL (Figure 8.47).

The increasing mean cocaine and benzoylecgonine concentrations with increasing exposure dose suggest that the larger the exposure concentration, the further the drug can travel into the tissue and hence the larger the potential that drug depots can be formed within the tissue.

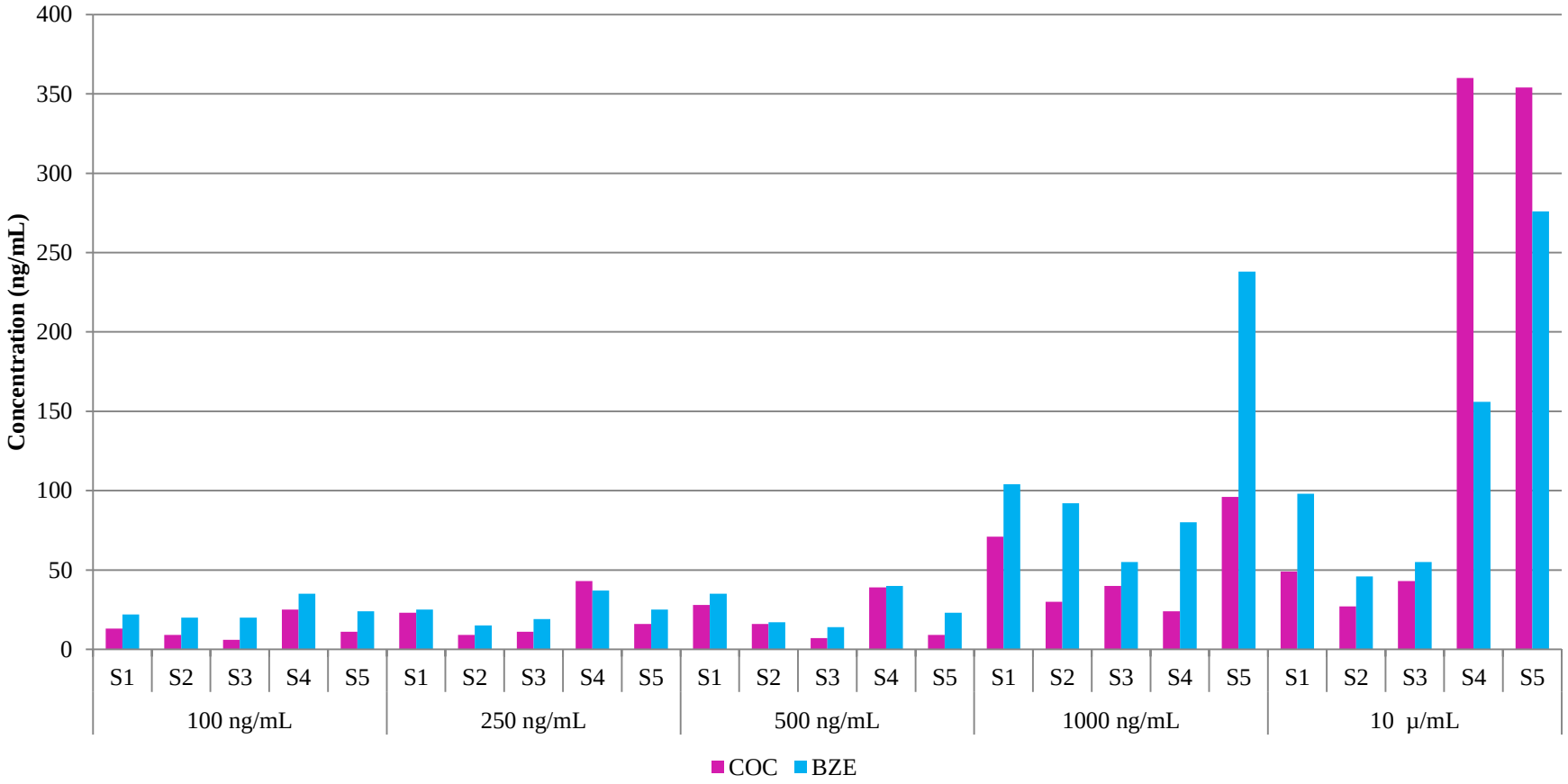


Figure 8.47. Mean concentrations of benzoylecgonine (ng/mL) detected in transverse cross sections of the tongue following the exposure to either 100 ng/mL, 250 ng/mL, 500 ng/mL, 1000 ng/mL or 10 µg/mL of cocaine in artificial saliva

Highest benzoylecgonine and cocaine concentrations were observed in homogenates of sections 1, 2, 4 and 5. Although sections 1 and 5 were removed from the same position of the tongue, a large difference of concentration of benzoylecgonine and cocaine could be observed in tongues which were exposed to cocaine concentrations of 1000 ng/mL and 10 µg/mL (Figure 8.48).

Mean concentrations of cocaine and benzoylecgonine were shown to be lowest in section 3 taken from the centre of the tongue (Figure 8.48).

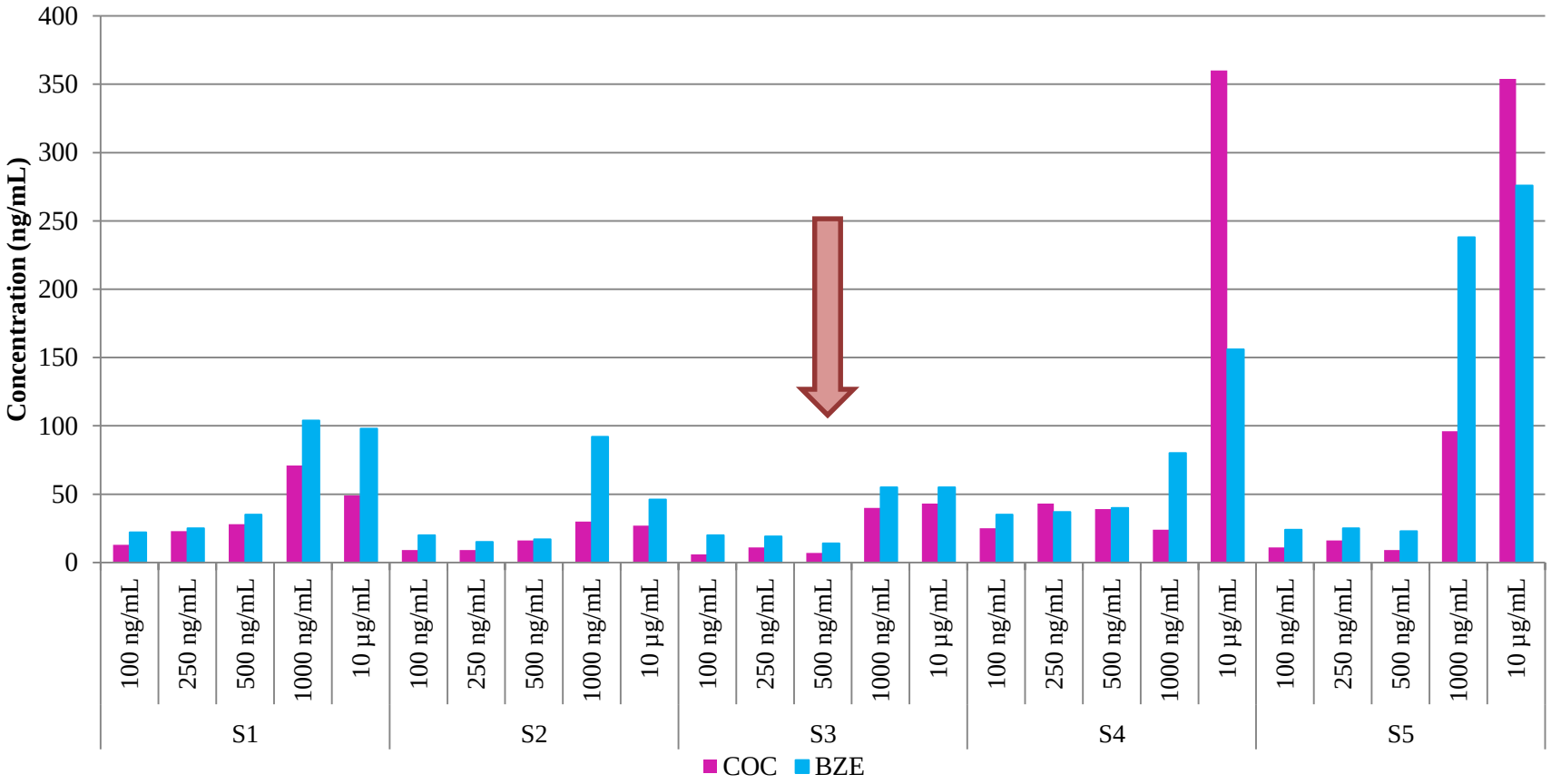


Figure 8.48. Mean concentrations of cocaine and benzoylecgonine (ng/mL) detected in transverse cross sections of the tongue following the exposure to either 100 ng/mL, 250 ng/mL, 500 ng/mL, 1000 ng/mL or 10 µg/mL of cocaine in artificial saliva. The red arrow represents the centre section (S3) of the transverse cross section of the tongue n, where observed concentration of cocaine and benzoylecgonine were lowest (n = 2)

8.3.2.1.2 Heroin

8.3.2.1.2.1 Washes

No heroin was detected in any of the 7 washes following exposure to heroin solutions from 100 ng/mL to 10 µg/mL. Morphine and 6-MAM could be detected in washes following exposure to 500 ng/mL of heroin. No positives were detected in wash 7 following exposure to any of the heroin concentrations (Table 8.6).

Table 8.7 Concentrations (ng/mL) of heroin, morphine and 6-MAM in wash 1 to wash 7 (W1 – W7) from tongues exposed heroin concentrations of either 100 ng/mL, 250 ng/mL, 500 ng/mL, 1000 ng/mL or 10 µg/mL (ND – not detected)

Concentration (dose)	Tongue No.	Wash No.	Concentration (ng/mL)		
			Heroin	Morpine	6-MAM
100 ng/mL	1	1	*ND*	*ND*	*ND*
		2	*ND*	*ND*	*ND*
		3	*ND*	*ND*	*ND*
		4	*ND*	*ND*	*ND*
		5	*ND*	*ND*	*ND*
		6	*ND*	*ND*	*ND*
		7	*ND*	*ND*	*ND*
	2	1	*ND*	*ND*	*ND*
		2	*ND*	*ND*	*ND*
		3	*ND*	*ND*	*ND*
		4	*ND*	*ND*	*ND*
		5	*ND*	*ND*	*ND*
		6	*ND*	*ND*	*ND*
		7	*ND*	*ND*	*ND*
250 ng/mL	1	1	*ND*	*ND*	*ND*
		2	*ND*	*ND*	*ND*
		3	*ND*	*ND*	*ND*
		4	*ND*	*ND*	*ND*
		5	*ND*	*ND*	*ND*
		6	*ND*	*ND*	*ND*
		7	*ND*	*ND*	*ND*

Concentration (dose)	Tongue No.	Wash No.	Concentration (ng/mL)		
			Heroin	Morpine	6-MAM
	2	1	*ND*	*ND*	*ND*
		2	*ND*	*ND*	*ND*
		3	*ND*	*ND*	*ND*
		4	*ND*	*ND*	*ND*
		5	*ND*	*ND*	*ND*
		6	*ND*	*ND*	*ND*
		7	*ND*	*ND*	*ND*
500 ng/mL	1	1	*ND*	**40**	**2**
		2	*ND*	*ND*	*ND*
		3	*ND*	*ND*	*ND*
		4	*ND*	*ND*	*ND*
		5	*ND*	*ND*	*ND*
		6	*ND*	*ND*	*ND*
		7	*ND*	*ND*	*ND*
	2	1	*ND*	**35**	**3**
		2	*ND*	*ND*	*ND*
		3	*ND*	*ND*	*ND*
		4	*ND*	*ND*	*ND*
		5	*ND*	*ND*	*ND*
		6	*ND*	*ND*	*ND*
		7	*ND*	*ND*	*ND*
1000 ng/mL	1	1	ND	**1**	**2**
		2	ND	**1**	**2**
		3	ND	*ND*	*ND*
		4	ND	*ND*	*ND*
		5	ND	*ND*	*ND*
		6	ND	*ND*	*ND*
		7	ND	*ND*	*ND*
	2	1	ND	**1**	**1**
		2	ND	**0**	**0**
		3	ND	*ND*	*ND*
		4	ND	*ND*	*ND*
		5	ND	*ND*	*ND*
		6	ND	*ND*	*ND*
		7	ND	*ND*	*ND*
10 µg/mL	1	1	ND	**43**	**41**

Concentration (dose)	Tongue No.	Wash No.	Concentration (ng/mL)		
			Heroin	Morpine	6-MAM
		2	ND	**45**	**12**
		3	ND	**1**	ND
		4	ND	*ND*	*ND*
		5	ND	*ND*	*ND*
		6	ND	*ND*	*ND*
		7	ND	*ND*	*ND*
	2	**1**	ND	**55**	**19**
		2	ND	**38**	**3**
		3	ND	**2**	*ND*
		4	ND	*ND*	*ND*
		5	ND	*ND*	*ND*
		6	ND	*ND*	*ND*
		7	ND	*ND*	*ND*

Following exposure to all concentrations of heroin solution, no opioids were detected in any of the washes after the 3rd wash however the drug had clearly been incorporated into the tissue as shown by immunohistochemical staining (section 8.3.1.1.2).

During immunohistochemical staining, it was observed that the staining for morphine in heroin exposed tongues was more intense in colour compared to the staining of cocaine, indicating that heroin binds more readily to the tissue. This is further supported by the analytical results of the washes. During the wash steps it was shown that cocaine was released and hence detected in several washes, indicating that some of the drug is released from the tissue. Heroin in comparison did not result in large concentration in the washes indicating that the drug was not released from the tongue

resulting in larger concentrations of drug in the tissue and hence resulting in more intense staining of the tissue during histochemical analysis.

In a living organism the amount of drug which distributes into tissue is dependent on its volume of distribution. The reported volume of distribution for cocaine is 1 -3 L/kg (Moffat et al 2011). The volume of distribution for heroin is not very well documented as it metabolises quickly to 6-MAM and morphine. Heroin is more lipophilic than cocaine resulting in a much higher estimated volume of distribution of heroin 25 L/kg (Baselt 2011, Urso et al 2012). The higher volume of distribution of heroin suggests that a larger amount of drug travels into the tissue. The reported volume of distribution of morphine is between 3 - 5 L/kg (Moffat et al 2011). This is further supported when comparing the logP value for cocaine and heroin. Heroin has a much lower logP value suggesting that the drug distributes more freely into the tissue than cocaine. Although the work undertaken in this chapter was carried out with *in vitro* tissue, the concept of the drugs crossing membranes still applies in that the microscopy of tissue sections showed the tissue to be intact and therefore drug still had the ability to cross membranes and accumulate in the tissues.

8.3.2.1.2.2 Collectors

After tongues had been washed seven times for 1 minute, a pre-wetted Alere™ Certus Collector that had been wetted using artificial saliva was brushed against the tongues. Analytical results of the seven artificial saliva washes, showed that no heroin was present in any of the washes and although morphine and 6 MAM were detected and quantified. Heroin and its metabolites were no longer detected after 6 washes.

No diacetylmorphine or morphine was detected when Alere™ Certus collectors were brushed against the tongue following completion of the washing process from tongues previously exposed to all heroin solutions (Table 8.8).

A trace of 6-MAM (2ng/mL) was detected in tongues exposed to heroin at a concentration of 1000 ng/mL in solution but not at lower concentrations. A slight increase was observed in tongues which were dipped into 10 µg/mL, with concentrations of 8 ng/mL (Tongue 1) and 6 ng/mL (Tongue 2).

Table 8.8. Concentrations (ng/mL) of heroin, morphine and 6-MAM in samples collected using the Alere™ Certus collectors that were brushed against the porcine tongues which had previously been exposed to heroin concentrations of either 100 ng/mL, 250 ng/mL, 500 ng/mL, 1000 ng/mL or 10 µg/mL and washes seven times. Samples were collected using a pre-wetted Alere™ Certus Collector. (ND – not detected)

Concentration (dose)	**Tongue No.**	**Concentration (ng/mL)**		
		Heroin	**Morphine**	**6-MAM**
100 ng/mL	1	ND	ND	ND
	2	ND	ND	ND
250 ng/mL	1	ND	ND	ND
	2	ND	ND	ND
500 ng/mL	1	ND	ND	ND
	2	ND	ND	ND
1000 ng/mL	1	ND	ND	2
	2	ND	ND	2
10 µg/mL	1	ND	ND	8
	2	ND	ND	6

The lack of heroin, morphine and 6-MAM in collectors suggest that the collector does not gather either of the compounds from the oral tissue and hence does not interfere with oral fluid interpretation. This further supports the proposition that opioids bind tightly to the tissue, as immunohistochemical staining resulted in intensive staining throughout the tissue (section 8.3.1.1.2). The recommendations (in draft) for the SAMHSA cut off for 6 MAM in oral fluid is 5 ng/mL. Although 6-MAM was detected and quantified when collectors were brushed against the tongue following exposure to 1000 ng/mL and 10 µg/mL of heroin, concentrations above the SAMHSA cut off were only observed following exposure to 10 µg/mL.

8.3.2.1.2.3 Tongue Homogenates

Both morphine and 6-MAM were detected in tongue tissue which had previously been exposed to heroin. Concentrations of morphine are summarised in Table 8.9.

Table 8.9. Morphine concentration (ng/mL) resulting from the analysis of five sections of porcine tongue homogenates which were previously exposed to either 100 ng/mL, 250 ng/mL, 500 ng/mL, 1000 ng/mL and 10 µg/mL of heroin in artificial saliva

Spiked Concentration	**Section**	**Morphine concentration (ng/mL)**		**Mean Morphine concentration (ng/mL)**
		Tongue		
		1	**2**	
100 ng/mL	**S1**	39	43	41
	S2	16	16	16
	S3	8	20	14
	S4	23	86	55
	S5	44	39	42

Spiked Concentration	Section	Morphine concentration (ng/mL)		Mean Morphine concentration (ng/mL)
		Tongue		
		1	2	
250 ng/mL	S1	94	63	78
	S2	39	43	41
	S3	47	41	25
	S4	64	70	67
	S5	47	42	44
500 ng/mL	S1	99	74	87
	S2	35	90	62
	S3	12	16	14
	S4	87	23	55
	S5	99	106	102
1000 ng/mL	S1	411	390	401
	S2	87	78	83
	S3	62	102	82
	S4	75	78	76
	S5	299	200	250
10 µg/mL	S1	351	330	340
	S2	136	188	162
	S3	56	66	61
	S4	143	368	255
	S5	422	371	397

Morphine concentrations detected in transverse cross section of the tongue did not show a large increase between tongues exposed to 100 ng/mL, 250 ng/mL and 500 ng/mL which supported the immunohistochemistry staining result. Morphine concentrations then increased following exposure to 1000 ng/mL and 10 µg/mL of heroin. Lowest morphine concentrations were observed in section 3 which was removed from the centre of the tongue.

Similar to cocaine results, concentrations showed that there was large variation in morphine concentration (ng/mL) measured in each section between the duplicate tongues which were exposed to heroin solutions. Porcine tongues varied in size during this experiment as tongues are proportionate to the size of the animal it was removed from. As tongues were provided directly by the butcher there was no way to individually selecting each tongue depending on its size. Therefore the large variations in concentrations between two tongues exposed to the same morphine concentrations could be a direct result of the difference in size between tongues.

In addition to morphine, 6-MAM was also detected in tongue tissue which was exposed to heroin solutions at different concentrations. Concentrations of 6-MAM are summarised in Table 8.10.

Table 8.10. 6-MAM concentration (ng/mL) resulting from the analysis of homogenates from porcine tongues which were previously exposed to either 100 ng/mL, 250 ng/mL, 500 ng/mL, 1000 ng/mL and 10 µg/mL of heroin in artificial saliva

Spiked Concentration	Section	6-MAM Concentration (ng/mL)		Mean 6-MAM concentration (ng/mL)
		Tongue		
		1	2	
100 ng/mL	S1	68	73	70
	S2	68	59	64
	S3	53	64	59
	S4	93	132	113
	S5	56	99	77
250 ng/mL	S1	87	139	113
	S2	37	73	55
	S3	34	25	29
	S4	105	152	129
	S5	62	86	74
500 ng/mL	S1	87	86	86
	S2	47	92	70
	S3	23	53	38
	S4	112	99	105
	S5	99	125	112
1000 ng/mL	S1	264	251	257
	S2	220	277	249
	S3	81	383	232

Spiked Concentration	Section	6-MAM Concentration (ng/mL)		Mean 6-MAM concentration (ng/mL)
		Tongue		
		1	2	
	S4	124	370	247
	S5	369	264	317
10 µg/mL	**S1**	490	482	486
	S2	155	198	177
	S3	109	73	91
	S4	242	337	289
	S5	428	390	409

Lowest 6-MAM concentrations were detected in sections 3 which had been removed from the centre of the tongue.

The presence of morphine and 6-MAM in tissue which was exposed to a heroin solution, suggest that heroin was broken down to morphine and 6-MAM during the preparation of the samples either during exposure, storage, extraction or analysis.

The large variation between the duplicate tongues which were exposed to heroin in artificial saliva further supported the theory that variations in drug concentration in tongue tissue can be caused by a difference in size of the tongue and hence the amount of drug accumulating in tongue tissue is proportionate to its size (Figure 8.49).

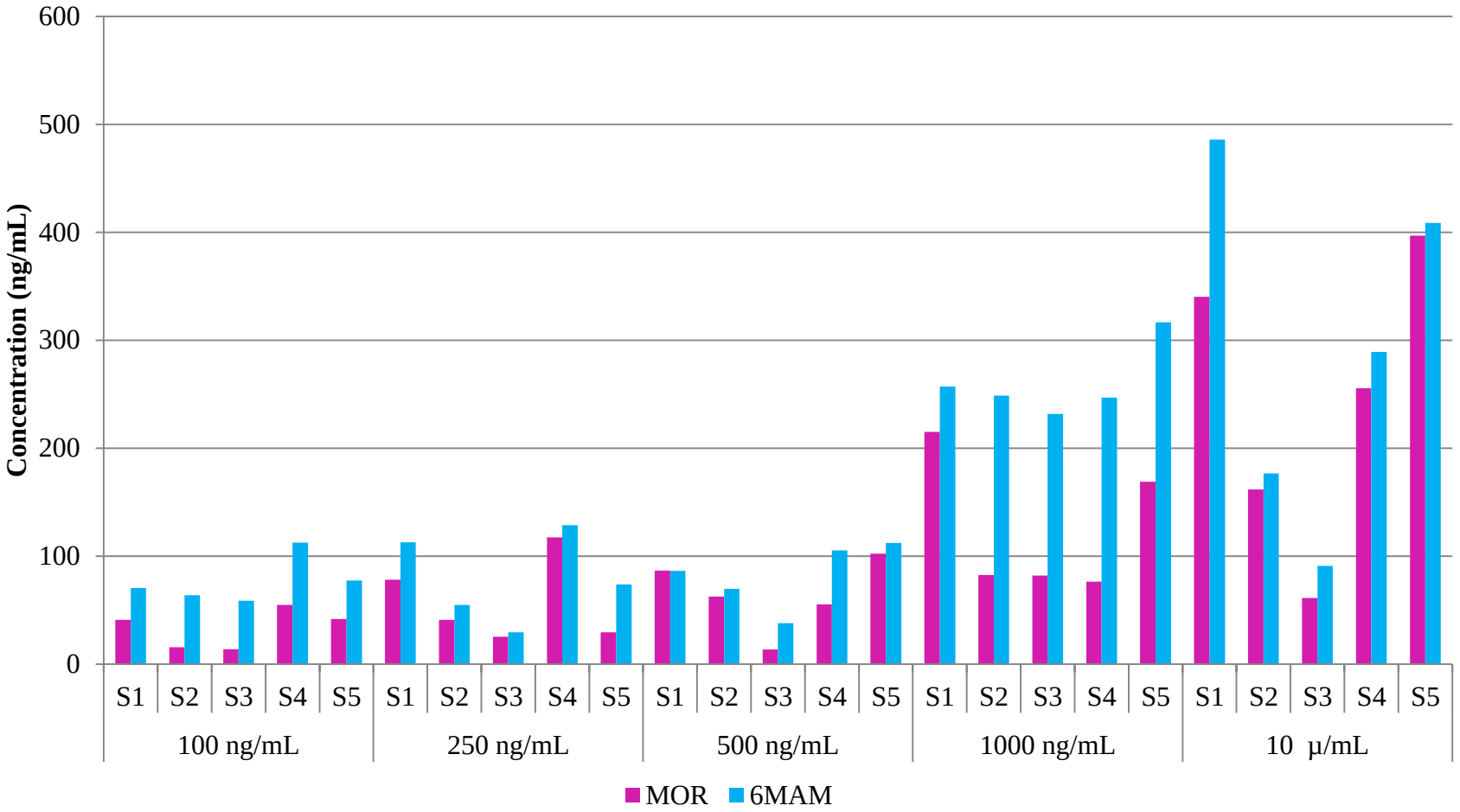


Figure 8.49. Mean concentrations of morphine and 6-MAM (ng/mL) detected in transverse cross sections of the tongue following the exposure to either 100 ng/mL, 250 ng/mL, 500 ng/mL, 1000 ng/mL and 10 µg/mL of heroin in artificial saliva (n =2)

Highest morphine and 6-MAM concentrations were observed in homogenates of sections 1, 2, 4 and 5. Although sections 1 and 5 were removed from the same position of the tongue, a large difference of concentration of morphine and 6-MAM could be observed in tongues which were exposed to heroin concentrations of 1000 ng/mL and 10 µg/mL (Figure 8.50). Similar, to the difference in concentration of morphine and 6-MAM between duplicate tongues in section 1 and Section 5 can be explained by the variations of size in each tongue.

Mean concentrations of morphine and 6-MAM were shown to be lowest in section 3 as indicated by the arrow in Figure 8.50. Section 3 was the removed from the centre of the tongue indicating that the tongue travels to the centre of the tongue, however is not as concentrated in the centre of the tongue.

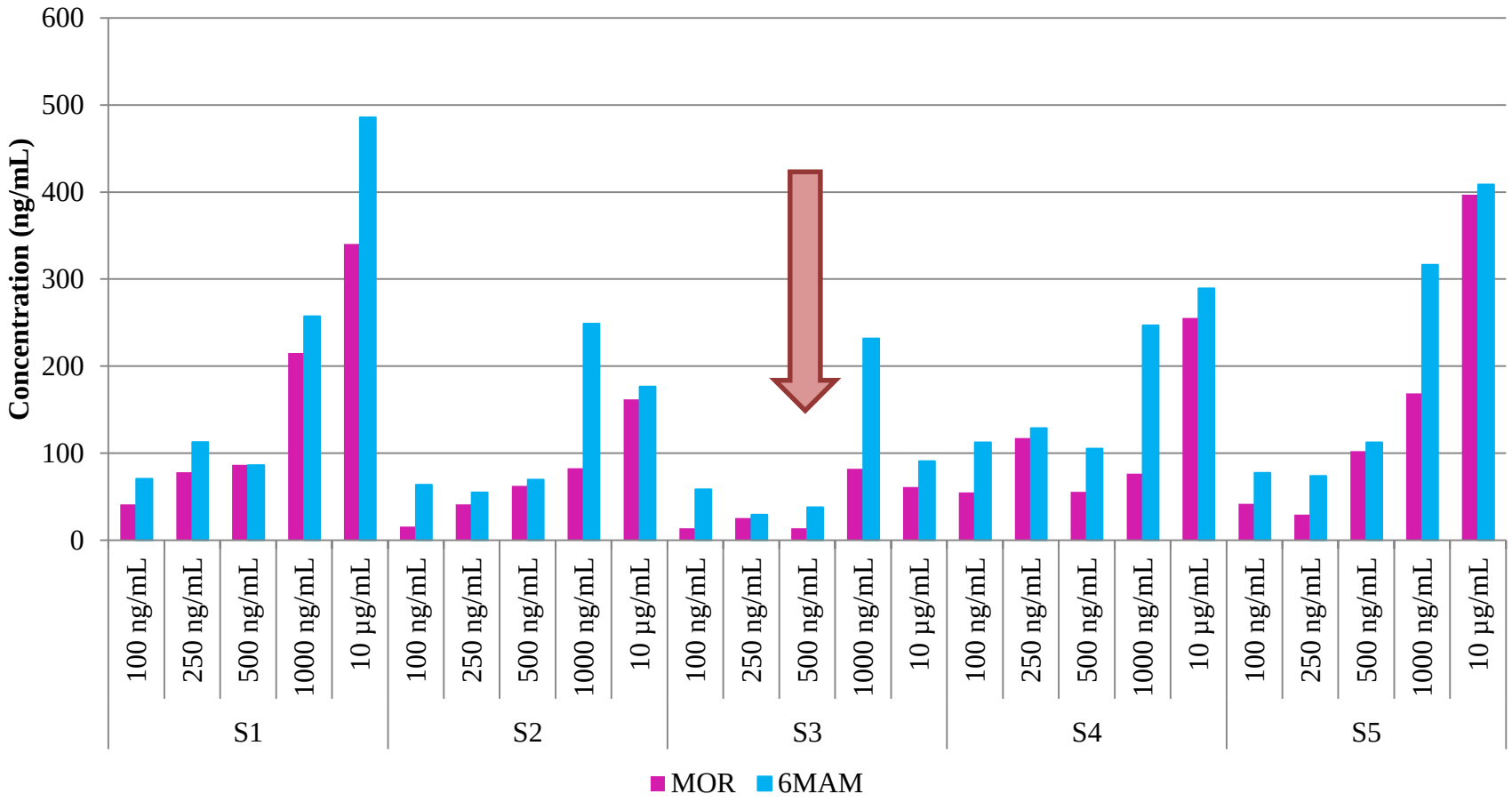


Figure 8.50. Mean concentrations of morphine and 6-MAM (ng/mL) detected in transverse cross sections of the tongue following the exposure to either 100 ng/mL, 250 ng/mL, 500 ng/mL, 1000 ng/mL and 10 µg/mL of heroin in artificial saliva. The red arrow represents the centre section (S3) of the transverse cross section, where observed concentration of morphine and 6-MAM were lowest. (n=2)

8.3.2.2 Tissue exposure to crack cocaine and exposure to street heroin smoke

8.3.2.2.1 Crack cocaine smoke

Cocaine, benzoylecgonine and anhydroecgonine methyl ester were detected in all five sections which were homogenised from a transverse cross section of the tongue.

Following the exposure to smoke from a 100 mg dose of crack cocaine, mean concentrations of cocaine, benzoylecgonine and anhydroecgonine methyl ester were 187 ng/mL, 1082 ng/mL and 4574 ng/mL, respectively, in section 1 of the samples taken for homogenisation of the transverse cross section of the tongue. The largest increase in concentration following smoke from a 200 mg dose of crack cocaine was observed with cocaine and anhydroecgonine methyl ester where the mean concentration detected were 870 ng/mL and 8902 ng/mL, respectively. benzoylecgonine showed a small decrease in concentration in section one dropping from 1,082 ng/mL to 836 ng/mL following smoke from a 100 mg and 200 mg dose of crack cocaine, respectively (Table 8.11).

Section 2 of the tissue samples taken from porcine tongues did not show a large increase between the 100 mg dose or the 200 mg dose with mean concentrations observed of 484 ng/mL and 494 ng/mL respectively. Similarly to concentrations in Section 1, concentrations of benzoylecgonine decreased from 1,383 ng/mL when exposed to a 100 mg dose to 662 ng/mL following a 200 mg dose of crack cocaine. Anhydroecgonine methyl ester concentrations did not increase significantly from section 1 with mean concentrations detected of 4,396 ng/mL and 8,899 ng/mL following exposure to smoke from a 100 mg or 200 mg dose respectively (Table 8.11).

Table 8.11. Concentrations (ng/mL) of cocaine, benzoylecgonine and anhydroecgonine methyl ester detected in five sections (s1 – s5) the tongue following the exposure to smoke from either a 100 mg or 200 mg dose of crack cocaine

Section	Dose	Tongue No.	Concentration (ng/mL)			Mean concentration (ng/mL)		
			COC	BZE	AEME	COC	BZE	AEME
S1	100 mg	1	184	124	4468	187	1082	4575
		2	190	2040	4682			
	200 mg	1	426	454	3180	870	836	8902
		2	1314	1218	14624			
S2	100 mg	1	468	648	5678	484	1383	4396
		2	500	2118	3114			
	200 mg	1	14	194	6780	494	662	8899
		2	974	1130	11018			
S3	100 mg	1	6	68	1190	25	111	1124
		2	44	154	1058			
	200 mg	1	156	152	1162	115	127	2036
		2	74	102	2910			
S4	100 mg	1	160	128	4210	230	248	4772
		2	300	368	5334			
	200 mg	1	126	344	5830	415	689	7355
		2	704	1034	8880			
S5	100 mg	1	932	590	6594	722	516	8008
		2	512	442	9422			
	200 mg	1	140	320	10278	80	242	20652
		2	20	164	31026			

Lowest concentrations of cocaine, benzoylecgonine and anhydroecgonine methyl ester were observed in section 3 which was removed from the centre of the tongue. Following the exposure to smoke from a 100 mg dose of crack cocaine, mean concentrations of cocaine, benzoylecgonine and anhydroecgonine methyl ester were 25 ng/mL, 111 ng/mL and 1124 ng/mL in section 3 of the samples taken for homogenisation of the transverse cross section of the tongue. Concentrations for all three compounds (cocaine, benzoylecgonine, anhydroecgonine methyl ester) increased with the increasing dose of crack cocaine (200 mg) to mean concentrations of 115 ng/mL, 127 ng/mL and 2036 ng/mL, respectively (Table 8.11).

Concentrations of cocaine, benzoylecgonine and anhydroecgonine methyl ester also increased in section 4 when exposed to smoke from a dose of 200 mg rather than 100 mg from 230 ng/mL, 248 ng/mL and 4,772 ng/mL to 415 ng/mL, 689 ng/mL and 7,355 ng/mL respectively.

In section 5, both concentrations of cocaine and benzoylecgonine were lower in tongues which were exposed to smoke from a 200 mg dose rather than a 100 mg dose of crack cocaine. Cocaine and benzoylecgonine concentrations following a 100 mg dose were 722 ng/mL and 516 ng/mL respectively, whereas concentrations of cocaine and benzoylecgonine following a 200 mg dose were 80 ng/mL and 242 ng/mL. Anhydroecgonine methyl ester showed a larger concentration when exposed to smoke from 200 mg of crack cocaine rather than smoke from 100 mg. The mean concentration of anhydroecgonine methyl ester increased from 8,008 ng/mL to 20,652 ng/mL following a 200 mg dose rather than 100 mg dose.

Lowest concentrations for cocaine, benzoylecgonine and anhydroecgonine methyl ester were observed in section 3, which was removed from the centre of the tongue

indicating that although the drug penetrated the tissue section all the to the centre of the tongue, less drug reached the centre than on the external sections of the tongue.

Although sections 1 and 5 were removed from the same position from the left and right side of the tongue, a large difference in concentration for cocaine, benzoylecgonine and anhydroecgonine methyl ester could be observed in tongues which were exposed to smoke to a dose of 100 mg or 200 mg. (Figure 8.51). There are two possible explanations for this phenomenon. The size of the porcine tongues throughout the smoking of crack cocaine could not be guaranteed. Therefore some drug may infiltrate slimmer tongue sections faster than porcine tongues which were larger. Additionally, when tongues were suspended in the smoking chamber, it was observed that the smoke travelled upwards in a spiral motion, which could hence result in potentially uneven exposure to crack cocaine smoke. No noticable difference was observed between the exposure to smoke from either a 100 mg or a 200 mg dose of crack cocaine.

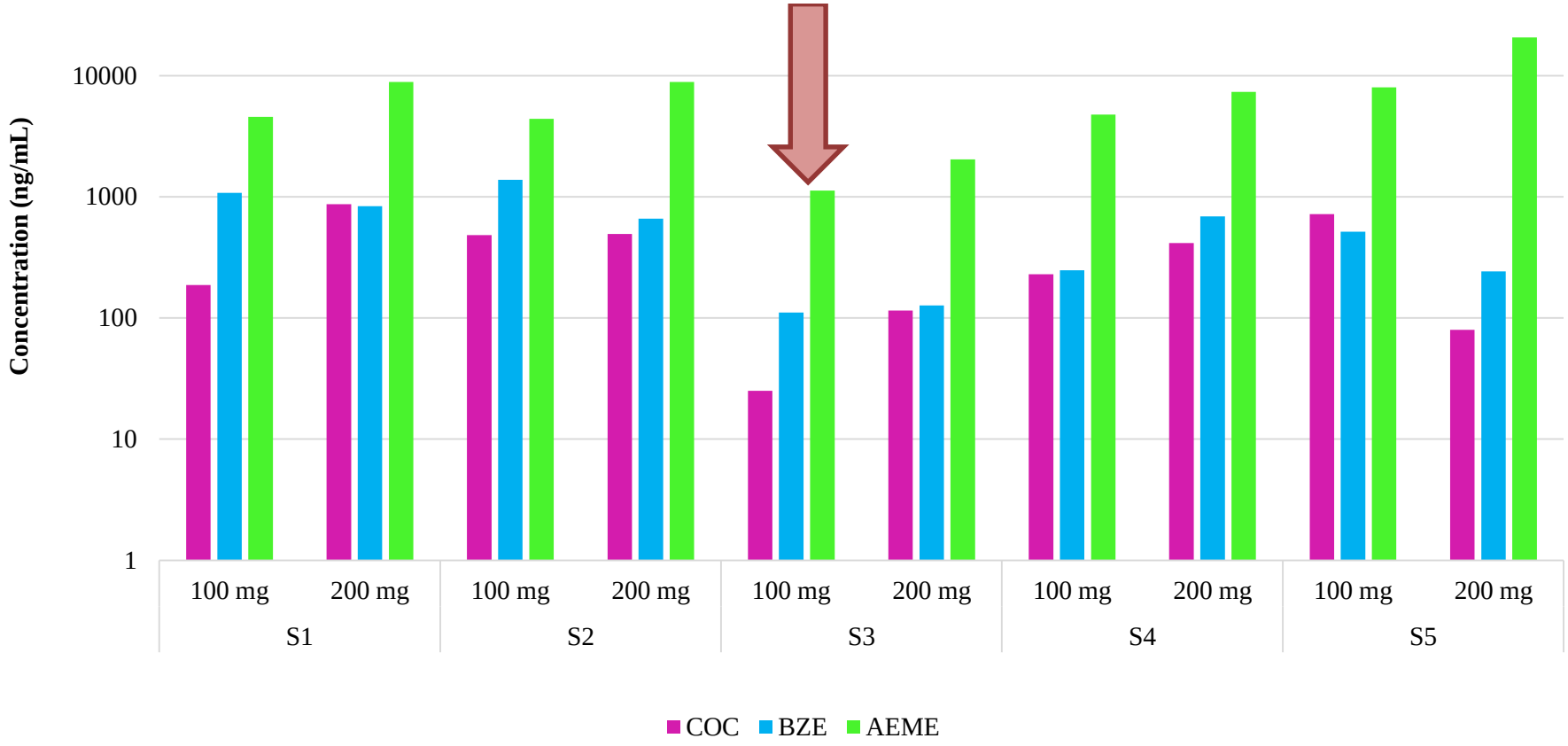


Figure 8.51. Mean concentrations of cocaine, benzoylecgonine and anhydroecgonine methyl ester (ng/mL) detected in five sections (s1 – S5) the tongue following the exposure to smoke from either a 100 mg or 200 mg dose of crack cocaine. The red arrow represents the centre section (S3) of the transverse cross section of tongue, where observed concentration of cocaine, benzoylecgonine and AEME were lowest. (n=2)

8.3.2.2.2 Heroin Smoke

Sections removed from tongues which were exposed to smoke from either 100 mg or 200 mg of street heroin were positive for both morphine and 6-MAM.

When tongues were expose to smoke from 100 mg of heroin, mean concentration of morphine and 6-MAM were 360 ng/mL and 290 ng/mL in comparison to concentrations following the exposure to 200 mg of heroin which were 569 ng/mL and 367 ng/mL, respectively (Table 8.12).

Mean morphine concentrations in section 2 were 391 ng/mL and 872 ng/mL following tongue exposure to either 100 mg or 200 mg of heroin, respectively. Mean concentrations of 6-MAM were 872 ng/mL when exposed to smoke from 100 mg heroin and 781 ng/mL following exposure to smoke from 200 mg heroin.

Table 8.12. Concentrations (ng/mL) of morphine and 6-MAM detected in five sections (s1 – S5) the tongue following the exposure to smoke from either a 100 mg or 200 mg of heroin

Section	Dose	Tongue No.	Concentration (ng/mL)		Mean concentration (ng/mL)	
			MOR	6-MAM	MOR	6-MAM
S1	100 mg	1	336	222	369	290
		2	402	357		
	200 mg	1	447	375	569	367
		2	691	358		
S2	100 mg	1	855	535	391	599
		2	889	662		
	200 mg	1	350	742	872	781
		2	432	820		
S3	100 mg	1	65	192	73	237
		2	80	281		
	200 mg	1	285	342	228	310
		2	172	277		
S4	100 mg	1	411	618	419	645
		2	428	672		
	200 mg	1	860	1213	862	1551
		2	863	1888		
S5	100 mg	1	1046	743	236	775
		2	935	807		
	200 mg	1	256	904	990	954
		2	217	1003		

Lowest concentrations of morphine and 6-MAM were observed in section 3 which was removed from the centre of the tongue. Following the exposure to smoke from a 100 mg heroin, mean concentrations of morphine and heroin were 73 ng/mL and 237 ng/mL in section 3 of the samples taken for homogenisation of the transverse cross section of the tongue. Concentrations for morphine and 6-MAM increased with the increasing dose of heroin (200 mg) to mean concentrations of 228 ng/mL and 310 ng/mL, respectively (Table 8.12). Concentrations of morphine and 6-MAM also increased in section 4 when exposed to smoke from a dose of 200 mg rather than 100 mg from 419 ng/mL and 645 ng/mL to 862 ng/mL and 1551 ng/mL respectively. In section 5 concentrations of morphine and 6-MAM in tissue following exposure to smoke from 100 mg of heroin were 236 ng/mL and 775 ng/mL whereas concentrations following exposure to smoke from 200 mg of heroin were 990 ng/mL and 954 ng/mL, respectively.

Similarly to exposure to cocaine, lowest concentrations for morphine and 6-MAM were observed in section 3, which was removed from the centre of the tongue indicating that although the drug penetrated the tissue section all the way to the centre of the tongue, less drug reached the centre than on the external sections of the tongue (Figure 8.52).

Just like sections removed from tongue exposed to smoke from crack cocaine, variations in concentration between sections 1 and 5 were observed, although sections 1 and 5 were removed from the same position of the tongue. Similarly to tongues which were exposed to solutions of cocaine or heroin, the size of the porcine tongues throughout the smoking of heroin could not be guaranteed. Therefore some drug may infiltrate slimmer tongue sections faster than porcine tongues which were

larger. No significant difference was observed between the exposure to smoke from either a 100 mg or a 200 mg of heroin.

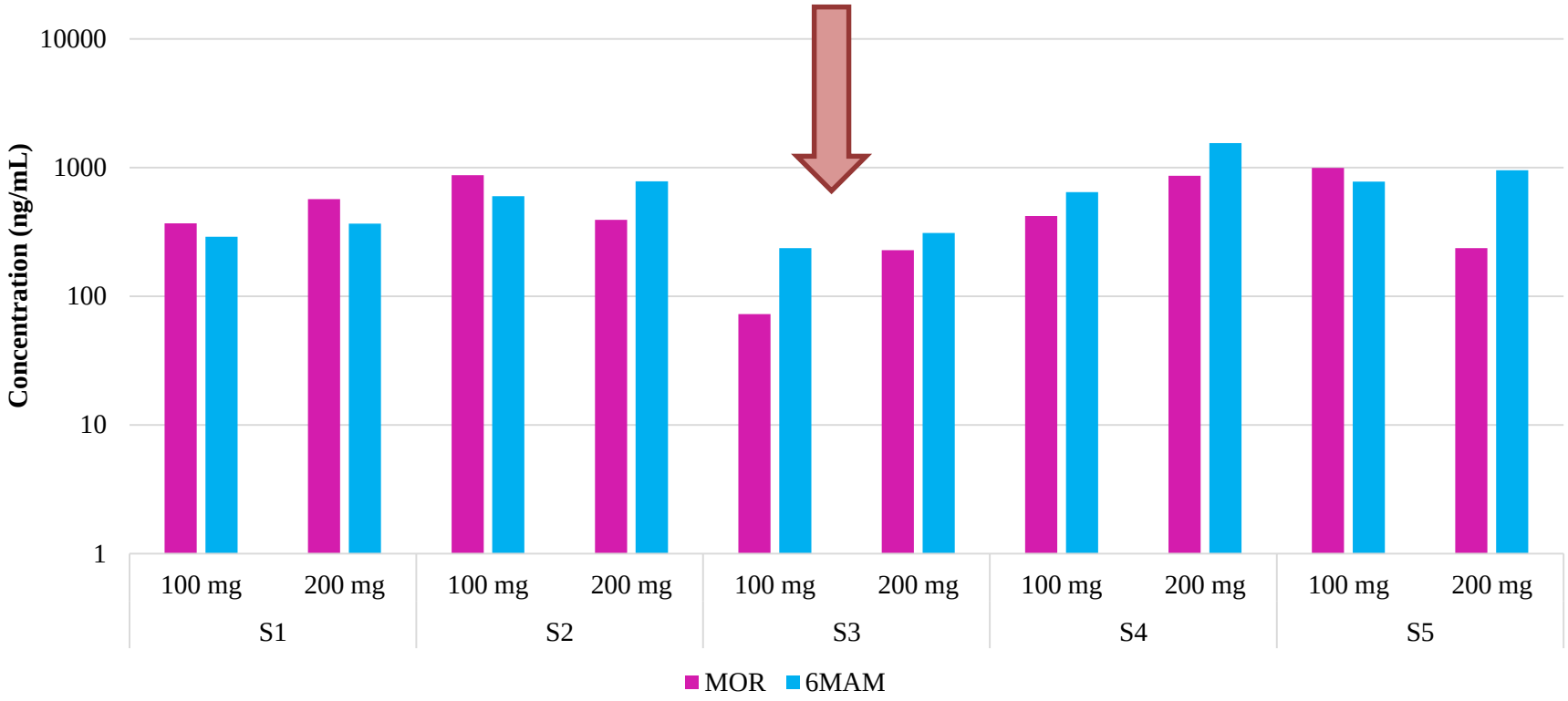


Figure 8.52. Mean concentrations of morphine and 6-MAM (ng/mL) detected in five sections (s1 – S5) the tongue following the exposure to smoke from either a 100 mg or 200 mg of heroin. The red arrow represents the centre section (S3) of the transverse cross section, where observed concentration of morphine and 6-MAM. (n = 2)

8.3.2.3 Prolonged washing of porcine tongues to investigate the drug release from tongue tissue using LC-MS/MS

8.3.2.3.1 Cocaine

Following exposure to concentration of 100 ng/mL or 1000 ng/mL of cocaine in artificial saliva, tongues were soaked in artificial saliva for 1 hour, 6 hours, 24 hours or 48 hours prior to homogenisation and extraction for analysis using LC-MS/MS (section 8.2.2.3).

After 1 hour soaking of the tongue cocaine concentration ranged between 8 ng/mL and 15 ng/mL after the exposure to 100 ng/mL and 13 ng/mL and 28 ng/mL following exposure to 1000 ng/mL. Benzoylecgonine concentrations detected were lower ranging between 3 ng/mL to 7 ng/mL for a dose of 100 ng/mL and 3 ng/mL and 9 ng/mL for exposures to 1000 ng/mL (Table 8.13).

When tongues were soaked for a longer time (6 hours), no significant difference concentrations were observed. Concentrations in tongues which were exposed to 100 ng/mL ranged between 11 ng/mL and 28 ng/mL for cocaine and 6 ng/mL and 8 ng/mL for benzoylecgonine. Exposure to cocaine of 1000 ng/mL resulted in higher concentrations which ranged between 12 ng/mL and 63 ng/mL for cocaine and 6 ng/mL and 11 ng/mL for benzoylecgonine.

Table 8.13. Results from prolonged washing of porcine tongues to investigate the drug release from tongue tissue by immunohistochemical staining following the exposure to cocaine at concentrations of either 100 ng/mL and 1000 ng/mL and washing for either 1 hour, 6 hours, 24 hours or 48 hours

Time	Concentration (ng/mL)	Section	Concentration (ng/mL)	
			Cocaine	Benzoylecgonine
1 hour	100	S1	15	6
		S2	12	5
		S3	8	3
		S4	9	6
		S5	15	7
	1000	S1	27	8
		S2	20	6
		S3	13	3
		S4	14	7
		S5	28	9
6 hours	100	S1	20	8
		S2	16	7
		S3	11	6
		S4	13	6
		S5	22	8
	1000	S1	60	11
		S2	39	9
		S3	12	6
		S4	21	7
		S5	63	11
24 hours	100	S1	12	6
		S2	18	6
		S3	1	6

Time	Concentration (ng/mL)	Section	Concentration (ng/mL)	
			Cocaine	Benzoylecgonine
		S4	6	6
		S5	8	6
	1000	**S1**	29	8
		S2	12	7
		S3	8	2
		S4	13	6
		S5	25	7
48 hours	**100**	**S1**	ND	3
		S2	ND	2
		S3	ND	ND
		S4	ND	3
		S5	ND	4
	1000	**S1**	ND	5
		S2	ND	5
		S3	ND	5
		S4	ND	5
		S5	ND	5

Following 1 hour soaking of the tongue cocaine concentrations ranged between 1 ng/mL and 18 ng/mL after the exposure of 100 ng/mL and 8 ng/mL and 29 ng/mL following exposure to 1000 ng/mL. Benzoylecgonine concentrations detected were 6 ng/mL for a dose of 100 ng/mL and 2 ng/mL and 7 ng/mL for exposures to 1000 ng/mL (Table 8.13).

Tissue samples removed from tongues exposed for 48 hours to 100 ng/mL of cocaine solution did not result in any detectable cocaine and only concentrations of benzoylecgonine were observed.

Overall, when tongues were exposed to 100 ng/mL of cocaine in artificial saliva, cocaine and benzoylecgonine did not result in significant changes with different wash times (Figure 8.53). Although cocaine was not detected following 48 hours post exposure, benzoylecgonine was still detected at low concentrations. This was in keeping with the immunohistochemical results which showed that, although weak, positive staining was still observed 48 hours post exposure to 100 ng/mL of cocaine in solution.

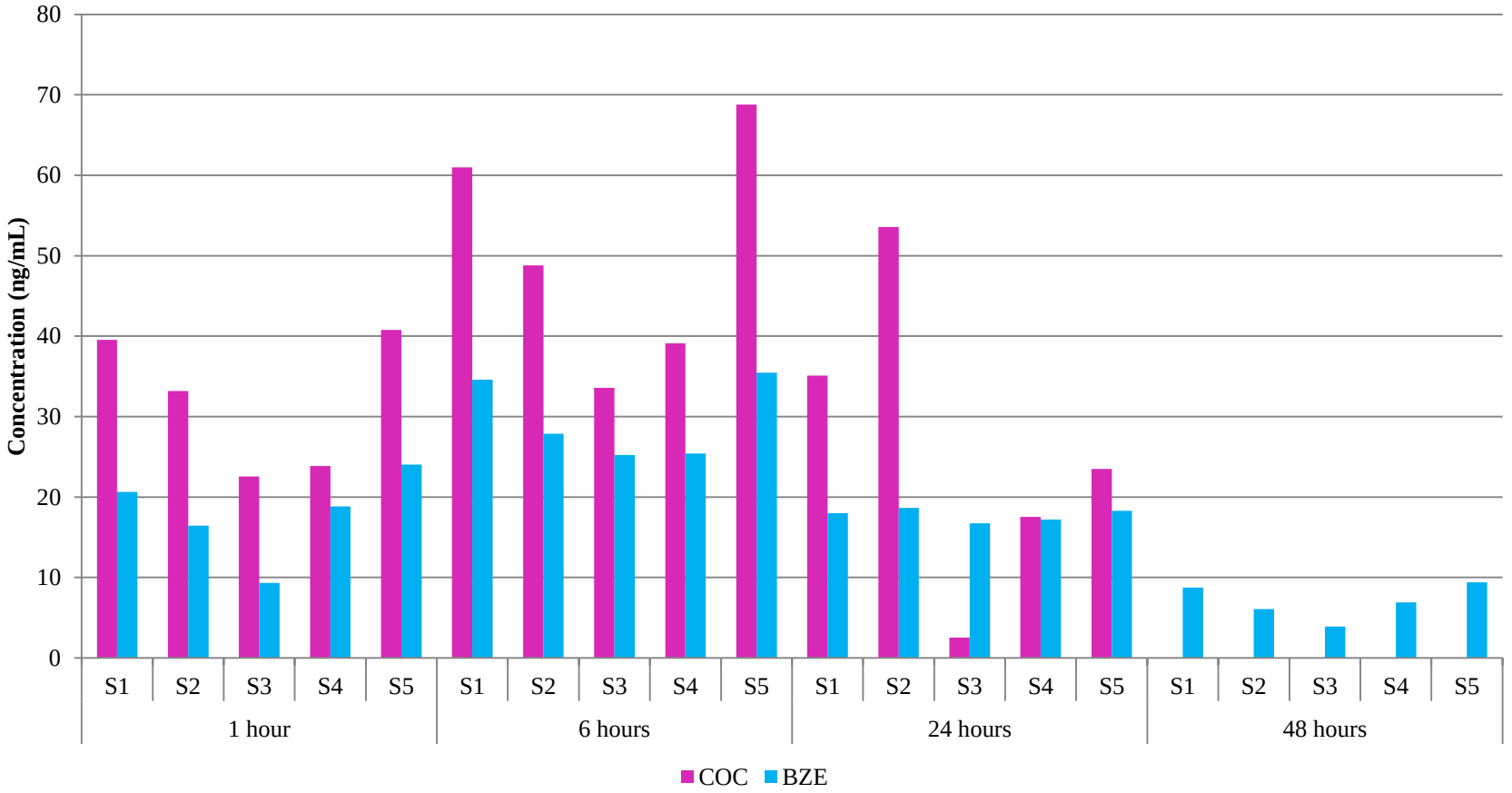


Figure 8.53. Concentrations of cocaine and benzoylecgonine in sections 1 – 5 of tongues which were exposed to a 100 ng/mL solution of cocaine in artificial saliva and washed for either 1 hour, 6 hours, 24 hours or 48 hours

In comparison, tongues exposed to cocaine solution of 1000 ng/mL resulted in positive concentration of cocaine and benzoylecgonine for up to 48 hours.

When tongues were exposed to 1000 ng/mL, no significant difference in benzoylecgonine concentrations were observed between the different wash times (Figure 8.54). Cocaine concentrations did not result in significant changes in the first 24 hours, but decreased following 48 hours of washing. Similarly to cocaine concentration in tissue following exposure to 100 ng/mL, results following exposure to 1000 ng/mL were in keeping with the immunohistochemical results which showed that positive staining was still observed 48 hours post exposure to 1000 ng/mL of cocaine in solution (Figure 8.23).

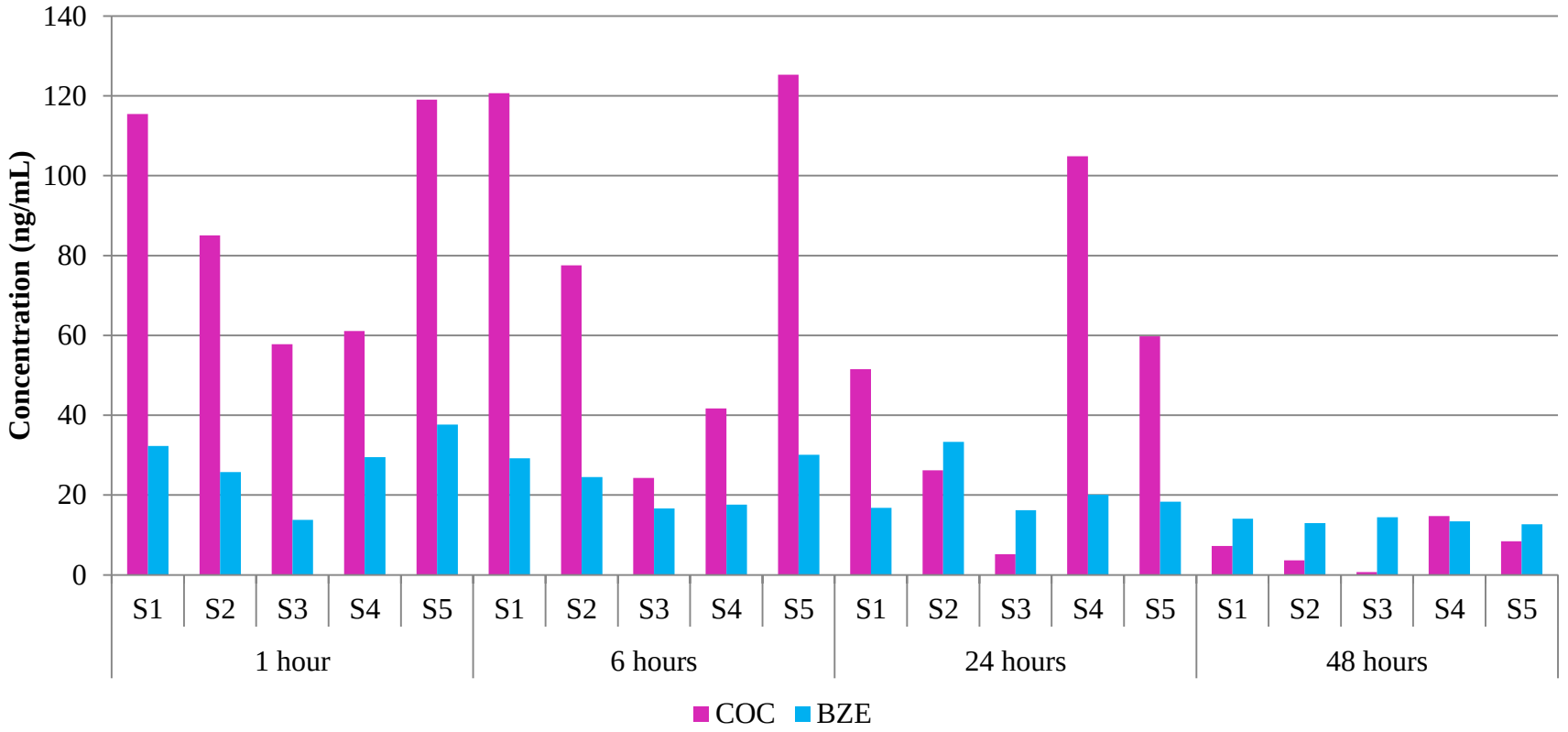


Figure 8.54. Concentrations of cocaine and benzoylecgonine in sections 1 – 5 of tongues which were exposed to a 1000 ng/mL solution of cocaine in artificial saliva and washed for either 1 hour, 6 hours, 24 hours or 48 hours

8.3.2.3.2 Heroin

Following exposure to concentration of 100 ng/mL or 1000 ng/mL of heroin in artificial saliva, tongues were soaked in artificial saliva for 1 hour, 6 hours, 24 hours or 48 hours and homogenized and extracted for analysis using LC-MS/MS (section 8.2.2.3).

Results showed that morphine and 6-MAM was detected in all tongues for up to 48 hours. When tongues were soaked for 1 hour post exposure to solutions of heroin, morphine concentration detected ranged between 23 ng/mL and 41 ng/mL after the exposure to 100 ng/mL of heroin and 58 ng/mL and 113 ng/mL following exposure to 1000 ng/mL. 6-MAM concentrations detected were lower ranging between 9 ng/mL to 24 ng/mL for a dose of 100 ng/mL and 14 ng/mL and 38 ng/mL for exposures to 1000 ng/mL of heroin (Table 8.14).

Following soaking of exposed tongues for 6 hours, no significant changes in concentration for morphine or 6-MAM were observed. Concentrations in tongues which were exposed to 100 ng/mL ranged between 34 ng/mL and 69 ng/mL for morphine and 25 ng/mL and 35 ng/mL for 6-MAM. Exposure to heroin of 1000 ng/mL resulted in higher concentrations which ranged between 24 ng/mL and 125 ng/mL for morphine and 17 ng/mL and 30 ng/mL for 6-MAM.

Table 8.14. Results from prolonged washing of porcine tongues to investigate the drug release from tongue tissue by immunohistochemical staining following the exposure to heroin at concentrations of either 100 ng/mL and 1000 ng/mL and washing for either 1 hour, 6 hours, 24 hours or 48 hours

Time	Concentration (ng/mL)	Section	Concentration (ng/mL)	
			Morphine	6 MAM
1 hour	100	S1	40	21
		S2	33	16
		S3	23	9
		S4	24	19
		S5	41	24
	1000	S1	115	32
		S2	85	26
		S3	58	14
		S4	61	30
		S5	119	38
6 hours	100	S1	61	35
		S2	49	28
		S3	34	25
		S4	39	25
		S5	69	35
	1000	S1	121	29
		S2	78	25
		S3	24	17
		S4	42	18
		S5	125	30
24 hours	100	S1	35	18
		S2	54	19
		S3	3	17

Time	Concentration (ng/mL)	Section	Concentration (ng/mL)	
			Morphine	6 MAM
		S4	18	17
		S5	23	18
	1000	**S1**	52	17
		S2	26	33
		S3	5	16
		S4	105	20
		S5	60	18
48 hours	**100**	**S1**	19	9
		S2	15	6
		S3	9	4
		S4	11	7
		S5	21	9
	1000	**S1**	19	14
		S2	10	13
		S3	2	14
		S4	39	13
		S5	22	13

Following 24 hour soaking of the tongue morphine concentration ranged between 1 ng/mL and 20 ng/mL after the exposure of 100 ng/mL and 2 ng/mL and 39 ng/mL following exposure to 1000 ng/mL. 6-MAM concentrations detected ranged between 4 ng/mL and 9 ng/mL for a dose of 100 ng/mL of heroin in solution and 2 ng/mL and 7 ng/mL for exposures to 1000 ng/mL (Table 8.14).

Tongues which were exposed to 100 ng/mL of heroin in artificial saliva did not result in large changes in the morphine and 6-MAM concentrations in tissue. Both compounds were still detected 48 hours post washing with artificial saliva. This was in keeping with the immunohistochemical results which showed that, although weak, positive staining was still observed 48 hours post exposure to 100 ng/mL of cocaine in solution. Detected morphine and 6-MAM concentrations were higher following exposure to 100 ng/mL of heroin than cocaine concentrations observed following exposure to 100 ng/mL of cocaine solution which further supports the results from the immunohistochemical staining as observed staining of heroin was more intense than positive staining for cocaine (Figure 8.55).

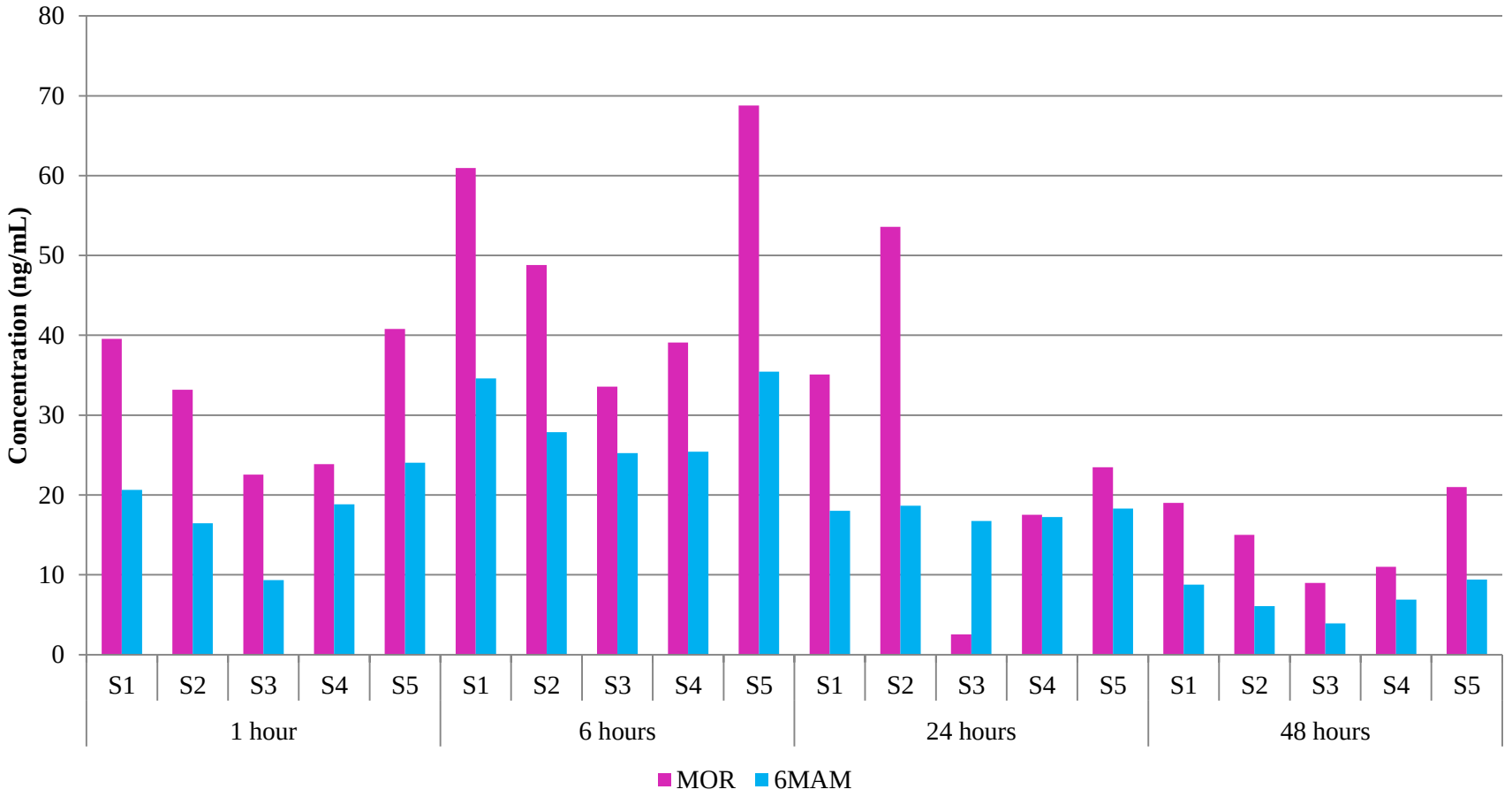


Figure 8.55. Concentrations of morphine and 6-MAM in sections 1 – 5 of tongues which were exposed to a 100 ng/mL solution of heroin in artificial saliva and washed for either 1 hour, 6 hours, 24 hours or 48 hours

In comparison, tongues which were exposed to a higher concentration of heroin solution of 1000 ng/mL resulted in the detection more significant concentrations of morphine and 6-MAM for up to 48 hours.

Exposure to 1000 ng/mL of heroin in artificial saliva resulted in detection of morphine and 6-MAM for up to 48 hours although over the first 24 hours no significant changes were observed between different wash times (Figure 8.56). Similarly to morphine and 6-MAM concentration in tissue following exposure to 100 ng/mL, results following exposure to 1000 ng/mL were in keeping with the immunohistochemical results which showed that positive staining was still observed 48 hours post exposure to 1000 ng/mL of heroin in solution (Figure 8.23).

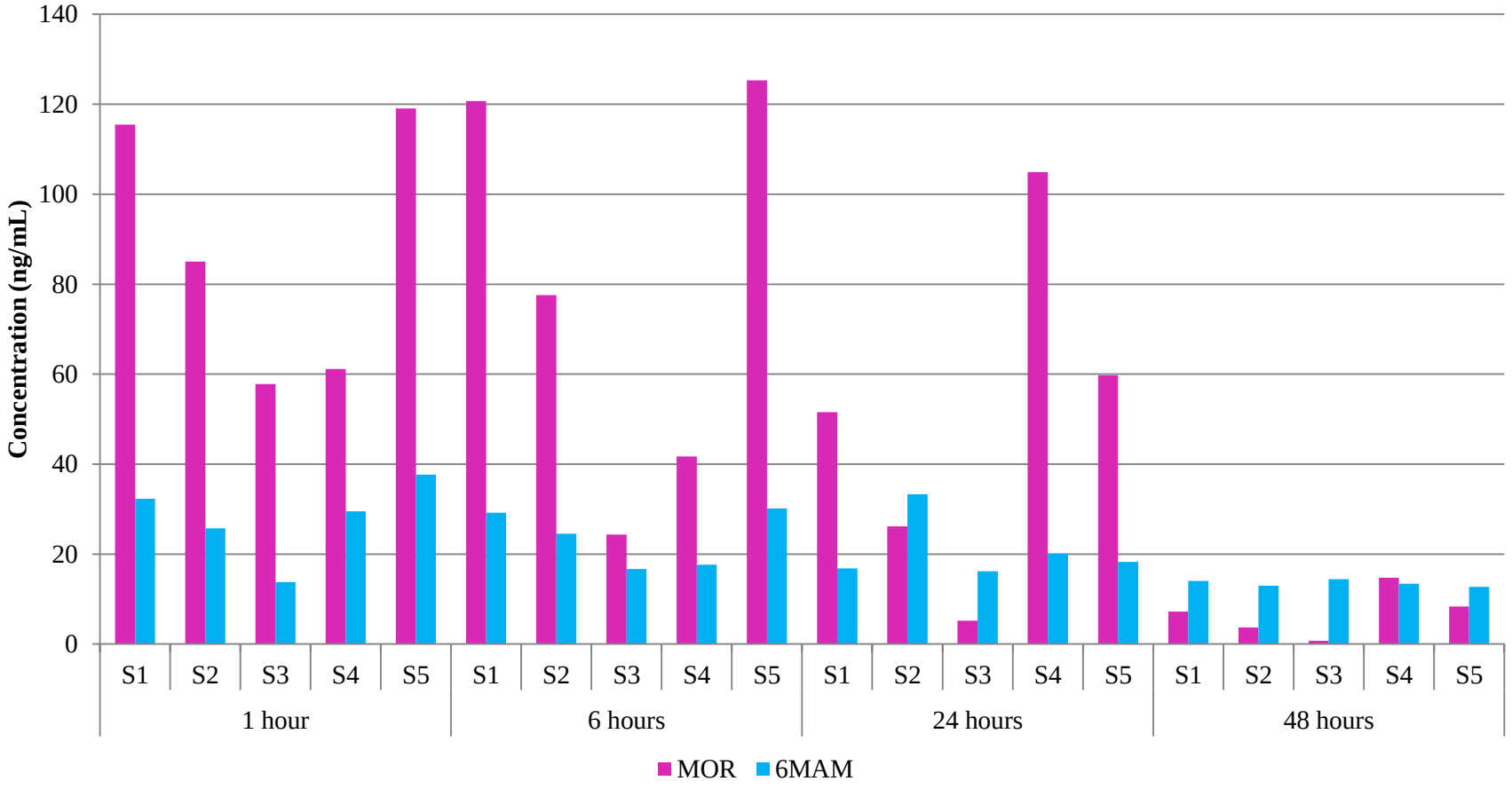


Figure 8.56. Concentrations of morphine and 6-MAM in sections 1 – 5 of tongues which were exposed to a 1000 ng/mL solution of heroin in artificial saliva and washed for either 1 hour, 6 hours, 24 hours or 48 hours

Results indicated that drug (i.e. cocaine and its metabolites and heroin and its metabolites) which enters the tongue, can penetrate and bind tightly to the tissue. Drug was still visible via immunohistochemical staining and detected in tissue using LC-MS for up to 48 hours of washing using artificial saliva. Although the amount of drug detected decreased with prolonged washing, benzoylecgonine, morphine and 6-MAM were still detected 48 hours post the exposure to either cocaine or heroin solution at concentrations of either 100 ng/mL or 1000 ng/mL. Drug which has bound to the tissue can subsequently be released over time and hence affect the interpretation of drug concentrations in oral fluid.

8.4 Conclusion

The work undertaken in this chapter has demonstrated the successful application of the immunohistochemical staining method developed in Chapter 7. Immunohistochemical staining in combination with toxicological analysis demonstrated that cocaine and heroin may penetrate into porcine tongue tissue following exposure to either drug in solution or following simulated smoking. Analysis of transverse cross sections of the tongue demonstrated that following exposure of tongues to cocaine, both cocaine and benzoylecgonine were observed in the analysed sections. Following exposure to heroin, morphine and 6-MAM were detected in tissue sections. Repeated washing of tongues exposed to cocaine and heroin was capable of removing surface contamination however after repeated washes were shown to be negative for drugs, immunochemical staining and tissue analysis revealed that drugs had accumulated well into the tissue offering evidence that the formation of drug depots is possible. More cocaine and its metabolites were observed in washes than heroin and its metabolites, indicating that heroin binds more tightly into the oral tissue. Wiping tongue tissue with an oral fluid collection device

demonstrated that cocaine and benzoylecgonine, but not morphine or 6-MAM could be released from washed tongue tissue. Sufficient cocaine and benzoylecgonine could be removed by an oral fluid swab from washed tongue surfaces to exceed the positive cut-off concentrations recommended by SAMHSA. Immunohistochemical staining results showed that the staining intensity in tongue sections increased with increasing drug exposure concentration. Staining following heroin exposure was observed to be more intense than following cocaine exposure indicating that more heroin had entered into and bound to tissue than cocaine. The increased amount of cocaine within the washes suggested that less cocaine had entered the tissue and hence less intense staining is observed during immunohistochemical staining. Lowest concentrations of cocaine, benzoylecgonine, 6-MAM and morphine were detected in the centre of the tongue showing that the drugs penetrated the centre of the tongue following both routes of exposure.

In a separate study the release of drugs from tongue tissue was investigated. Tongues were exposed to either 100 ng/mL or 1000 ng/mL of cocaine or heroin and then washed for 1 hour, 6 hours, 24 hours or 48 hours in artificial saliva with continuous mechanical stirring. Cocaine was detected for 24 hours post exposure whereas benzoylecgonine, 6-MAM or morphine were detected for up to 48 hours post exposure. This was in keeping with the immunohistochemical results of the transverse cross sections of the tongues which showed that positive staining was observed for up to 48 hours following exposure to cocaine or heroin at concentrations of either 100 ng/mL or 1000 ng/mL.

To summarise, the work undertaken in this chapter showed that drugs have the ability to enter the oral tissue and form drug depots. Drugs which have previously

bound to tissue can subsequently be released over time and hence interfere with the interpretation of drug concentrations in oral fluid when investigating saliva:plasma ratios. However, the vast majority of oral fluid testing is carried out for two reasons a) under legislation that forbids the presence of drugs in oral fluid or b) as a check for drug compliance or abstinence. In both oral fluid testing procedures it is not necessary to identify whether the drug resulted from contamination, contribution from depots or from the salivary gland in which case the interpretation of oral fluid drug concentrations would not be affected by the work undertaken in this chapter.

Chapter 9.0- *In Vivo* and *in vitro* investigation into the detection of morphine in salivary gland and tongue tissue in mice using immunohistochemical staining techniques

9.1 Introduction

The *in vitro* investigation of opioid and cocaine distribution in porcine tongues (Chapter 7) discussed the application of an immunohistochemical technique to visualise the presence of opioids and cocaine in tongue tissue. The technique demonstrated extensive incorporation of drugs into tongue tissue that had previously been exposed to cocaine or heroin either via exposure to a solution of the drug or to simulated smoking. Results from work undertaken in Chapter 8 indicated that drugs which were administered orally either via smoke or solution, have the potential to enter the mouth tissue by mechanisms other than via the circulating blood thus providing support for a proposition that drugs can enter tissue via external contamination and may subsequently form depots in oral tissue from which they may be released over time.

The challenge with any *in vitro* study is determining whether the propositions being tested would also be applicable in an *in vivo* model. The research described in this chapter investigated the application of the immunohistochemical staining method developed in chapter 7 on an *in vivo* model whereby mice were injected with morphine prior to removal of the tongue and subsequently comparing the result to *in vitro* mice tongues and salivary glands that were exposed to heroin smoke.

The experiments described in this chapter aimed to investigate whether morphine can be detected in oral tissues (tongue and salivary glands) from circulating blood following entry into the body via peritoneal injection.

9.1.1 Anatomy of the mouse mouth: salivary glands

Mice, like humans, have three main salivary glands – the parotid gland, the submandibular gland and the submaxillary gland. The histological structure of mouse salivary glands is similar to that in humans such that all glands consist of small acini, a structure consisting of small exocrine glands (Chapter 1, section 1.1.1.1.1). Acini are made up of basophilic cytoplasm and large spherical nuclei. Acinar excretion glands produce the saliva which is then excreted from an intercalated duct via the striated duct (excretory duct). The excretory ducts join up to form one single excretory duct via which saliva is excreted (Figure 9.1).

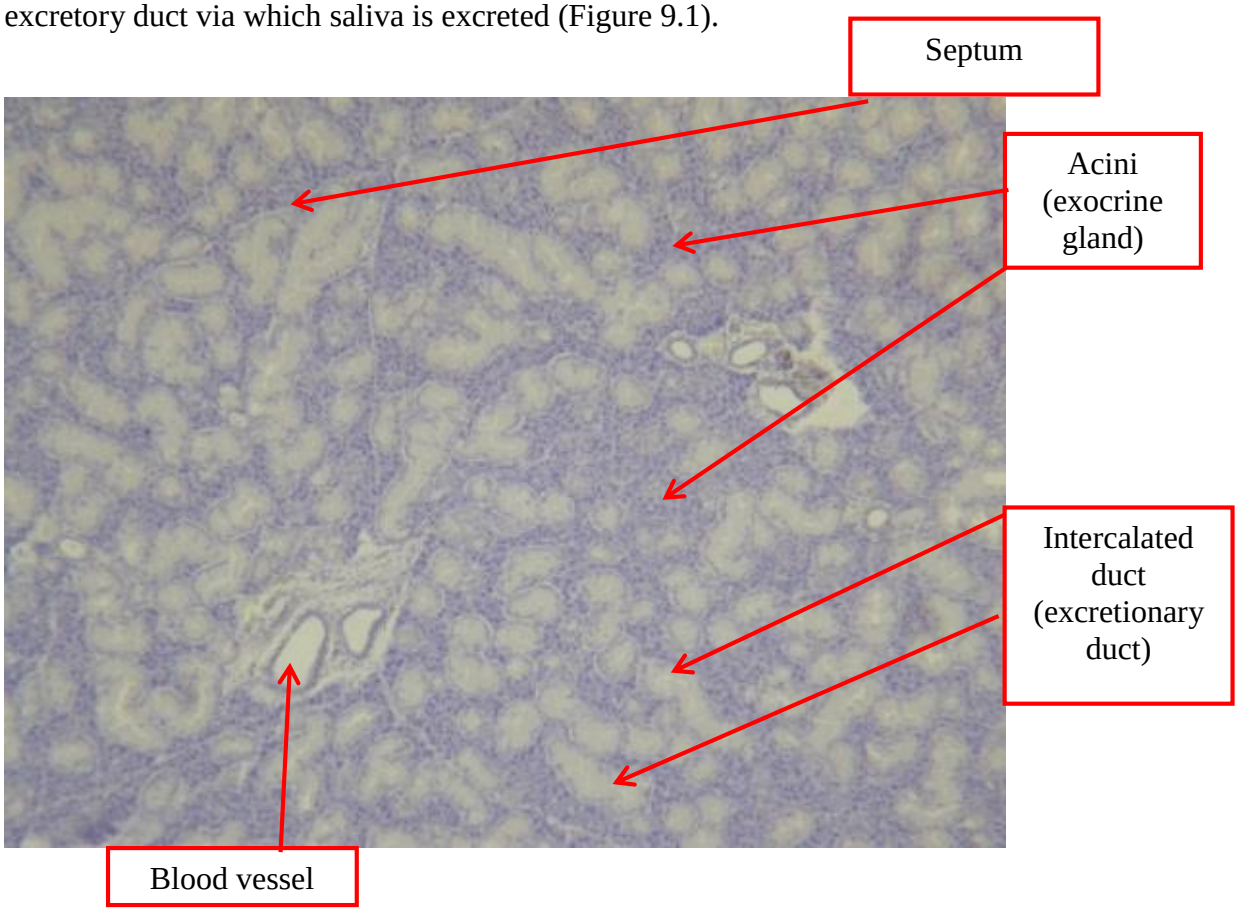


Figure 9.1. Images showing the main structures including exocrine gland, blood vessels, ecretionary duct within mice salivary gland tissue

9.2 Materials and Methods

9.2.1 Materials

In vivo: Live mice and morphine solution used for the experiments were provided and conducted at the School of Physiology & Pharmacology, University of Bristol.

In vitro: Frozen mice used for the exposure of tongues to simulated heroin smoking were obtained from Pets Corner, Pet supplies, Wallisdown, Bournemouth.

For the purposes of staining for morphine in mouse salivary glands and tongues, a polyclonal morphine-3-glucuronide antibody (Ig-Innovations) was selected. This antibody was raised by repeated immunisation of sheep with purified antigen of interest and had been conjugated to keyhole limpet haemocyanin (KLH). KLH is a carrier protein responsible for carrying the antibody to the morphine.

9.2.2 Methods

9.2.2.1 Preparation of mice injected with morphine solution

9.2.2.1.1 Dose calculation and preparation of mice

Mice were divided into three groups each comprising of 6 mice as follows: a) a saline control group b) a group injected with a low dose of morphine and c) a group injected with a high dose of morphine. The three groups of mice averaged at 29.8 g, 30.1 g and 30.3 g in body weight, therefore the average animal weight was taken to be 30 g. The intended dose for each group was 10 mg/kg for the low group and 30 mg/kg for the high group with saline injections acting as a control.

The concentration of drug needed for injection was calculated with the animal weight, intended dose and volume of injecting in mind. The formula for calculating the volume of injection is summarised below:

$$\text{Volume of injection (mL)} = \frac{\text{animal weight (kg)} \times \text{intended dose (mg/kg)}}{\text{concentration of drug (mg/mL)}}$$

The injection volume for all mice was 0.1 mL resulting with an injection concentration of 3 mg/mL of the low group of mice and 9 mg/mL for the high group of mice.

9.2.2.1.2 Dissection

Mice were killed humanely by cervical dislocation (Cook 1965) 30 minutes post injection prior to the removal of the tongue, submandibular, submaxillary and the parotid glands. (Figure 9.2).

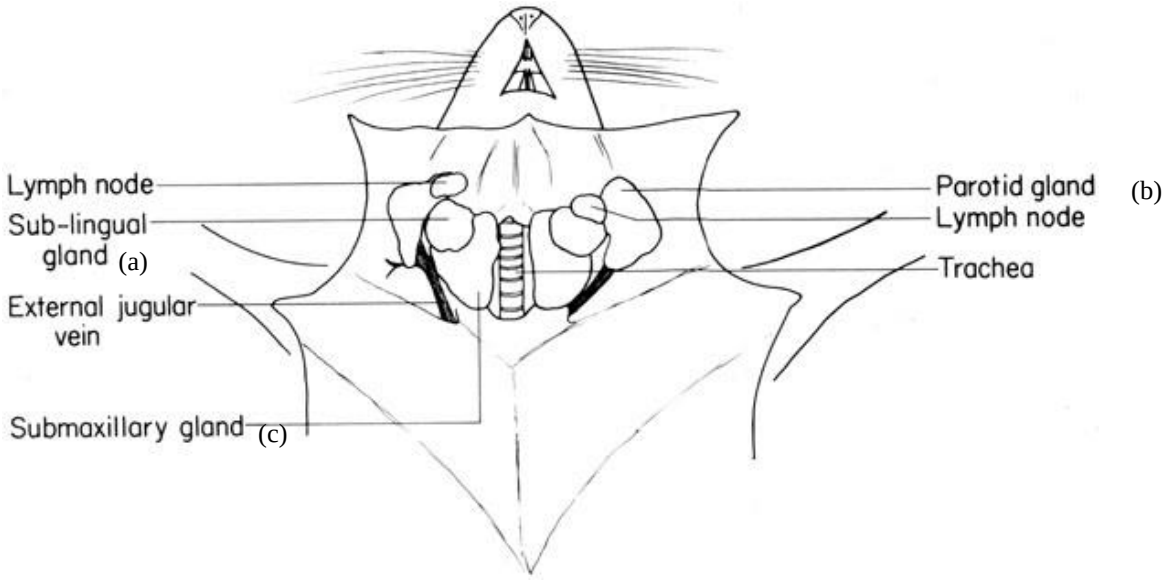


Figure 9.2 shows the position of the individual salivary glands in mice (Cook 1965). For the purpose of this study the sub-lingual gland (a), parotid gland (b) and submaxillary gland (c) were removed.

All glands are located in clusters below fat tissues and submandibular lymph nodes. Upon removing all fat tissues the salivary gland, the cluster becomes clearly visible. Following removal of salivary gland, teeth were removed from the oral cavity to allow for the removal of the mice tongues. Salivary glands and tongues were then fixed and processed for immunohistochemical analysis as described in section 9.2.2.3.

9.2.2.2 Preparation methods of mice tongues exposed to heroin smoke

Tongues were removed from frozen mice (Pets at Home), after thawing, in the same manner as from live mice (section 9.2.2.1.2). Following removal, tongues were immediately suspended in the smoking chamber using micro fishing hooks (Figure 8.3). Mice tongues were exposed to smoke from a 100 mg dose of heroin for either 30 seconds or 60 seconds and then fixed immediately for further analysis using immunohistochemical methods.

9.2.2.3 Immunohistochemical methods

9.2.2.3.1 Fixation and Processing

Mice salivary glands and tongues were placed into 10 % formalin immediately post removal from each animal in the case of injected mice or after exposure to heroin smoke in the smoke exposure experiment. In comparison to the porcine tongues, mice salivary glands and tongues were much smaller and were therefore only fixed in 10 % formalin for 24 hours to avoid over-fixing of the tissue. Following complete fixation, samples were processed at Southampton Histochemistry Research Unit using the methods previously described in chapter 7.

9.2.2.3.2 Antibody selection

Tissue used for the *in vivo* study originated from a mouse donor hence the use of the monoclonal antibodies employed in the porcine studies was not viable (Chapter 7, Chapter 8), since they had been raised as mouse monoclonal antibodies. The use of a mouse monoclonal antibody would result in a larger amount of non-specific binding as the secondary anti-mouse antibody can bind to endogenous mouse tissue and other compounds (Lu and Partridge 1998). Therefore the monoclonal antibody used in chapter 8 could no longer be used and a polyclonal antibody was sourced. The polyclonal antibody was a morphine-3-glururonide antibody. The Morphine-3-glucuronide antibody had not yet been tested for the use in any particular technique and therefore needed to be titrated to establish the optimum antibody working concentration.

9.2.2.3.3 Titration

For the purposes of titration, one section of the salivary gland from each group (low and high dose of morphine and saline control) of mice was selected. A saline control, low and high concentration was exposed a range of dilutions of morphine-3-glucuronide antibody as summarised in Table 9.1.

Table 9.1. Summary of dilution factor and working antibody concentration for the titration of the morphine-3-glucuronide stock antibody of 5mg/mL

Neat antibody concentration	Dilution factor	Working antibody concentration
5 mg/mL	1 : 50	100 µg/ml
	1 : 100	50 µg/ml
	1 : 200	25 µg/ml
	1 : 400	12. 5 µg/ml
	1: 600	8.3 µg/ml
	1 : 800	6.25 µg/ml
	TBS	Negative control

9.2.2.3.3.1 Titration results

All TBS controls were negative indicating that no non-specific binding had occurred. An optimum antibody concentration was observed at a dilution of 1 in 600 which resulted in a primary antibody concentration of 8.3 µg/ml. Lower dilution factors resulted in non-specific binding whereas a larger dilution factor resulted in staining with very little intensity. Results are summarised in Figure 9.3 and Figure 9.4.

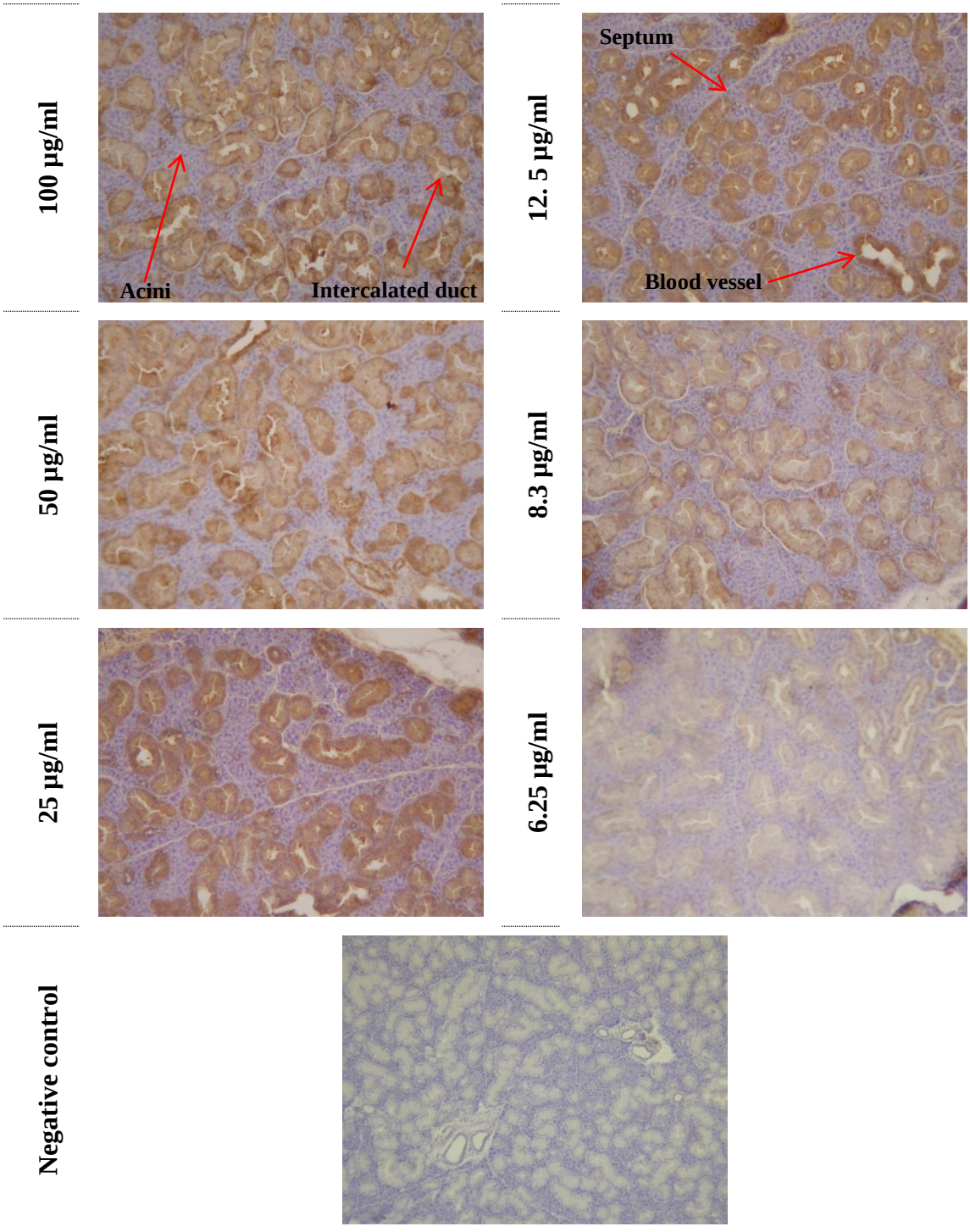


Figure 9.3 Summarised visual results from antibody optimisation at different dilution factors from mice injected with a low (3 mg/mL) concentration of morphine (magnification x25)

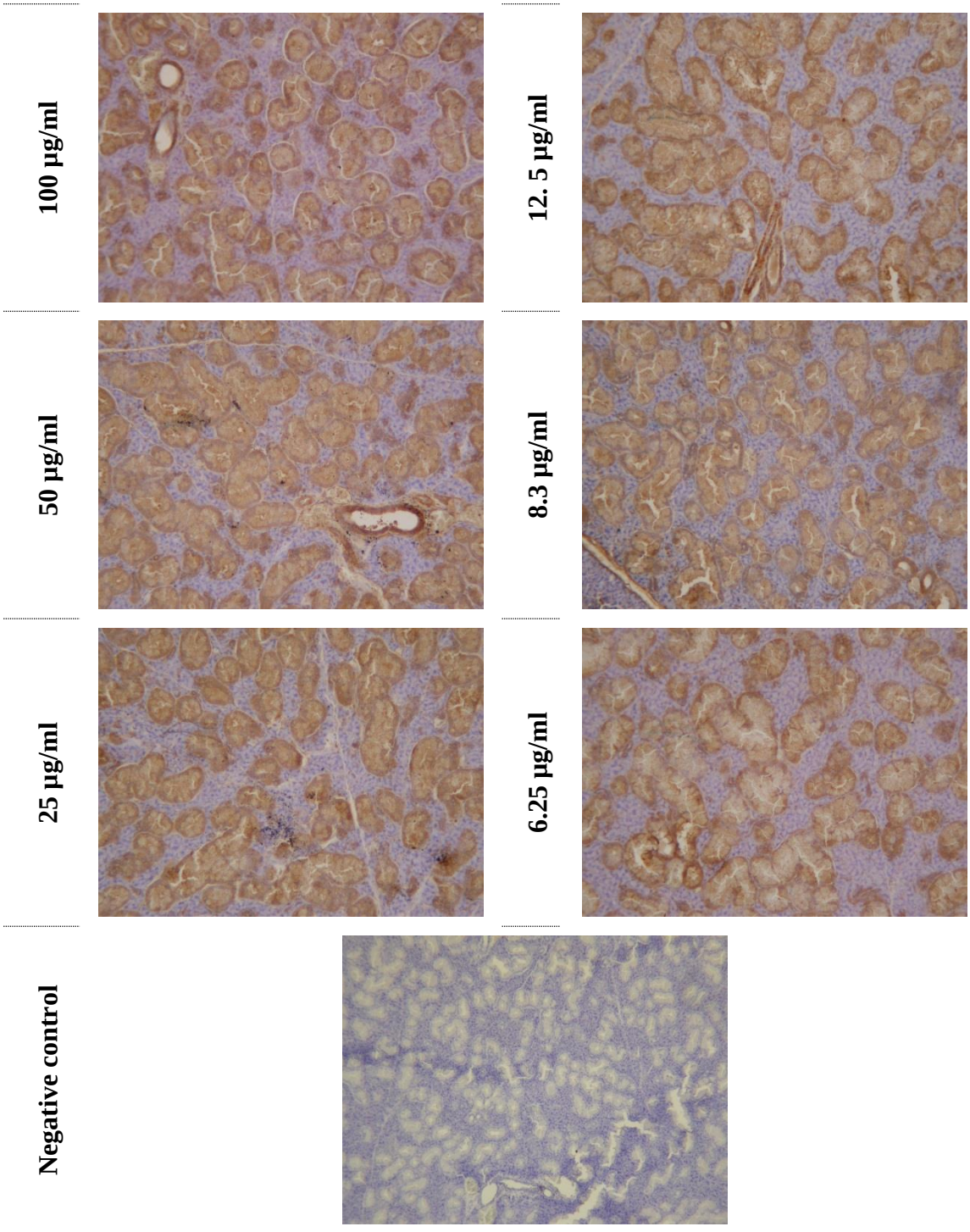


Figure 9.4 Summarised visual results from antibody optimisation at different dilution factors from mice injected with a high (9 mg/mL) concentration of morphine (magnification x 25)

9.2.2.3.4 Secondary antibody

The secondary antibody also had to be adapted to match the polyclonal antibody. As this antibody was raised in sheep, the secondary antibody selected was a biotinylated rabbit-anti-goat antibody (Dako). The secondary antibody was applied at a concentration of 2.67 µg/mL.

9.2.2.3.5 Staining method

The principle staining method in paraffin from Chapter 7 was adapted as a consequence of the nature of secondary antibody. As rabbit anti-goat can generate more non-specific binding, 1% of Tween 20 detergent was added to all TBS washes performed during the staining procedure. Tween 20 is designed to promote more effective washings as it promotes reagent spreading, resulting in a decrease of non-specific background staining.

Following blocking of endogenous peroxidases by applying a solution of 0.5 % hydrogen peroxide to the slides for 10 minutes, all slides were washed with TBS/ Tween 20 three times for two minutes. Slides were then drained and an avidin blocking solution was applied and incubated for 20 minutes each prior to washing with TBS / Tween 20 three times for two minutes. Biotin was then applied to all sections and incubated for 20 minutes. Together, the avidin and biotin blocking steps block all of the endogenous avidin and biotin binding sites which can be present in tissues and thus prevent non-specific binding at those sites.

On completion of the avidin and biotin steps, a further wash step was performed three times for 2 minutes. The rabbit-anti-goat antibody had a tendency to cause non-specific binding and therefore the Dulbecco's Culture Medium (Chapter 6) was

ineffective to prevent non-specific binding. Instead of the Dulbecco's culture medium, the slides were incubated in a 5% rabbit serum solution for 30 minutes.

Following incubation with the primary antibody (in TBS containing 1% BSA) overnight at 4 ºC the slides were washed three times using TBS for five minutes. The slides were then drained and the biotinylated secondary antibody was applied at a concentration of 2.67 µg/mL and incubated for 30 minutes. Following another three washes for five minutes each, the avidin biotin-peroxidase complexes (ABC) were applied for 30 minutes before repeating the wash steps. The solution should only be used if a sufficient amount of time was given for these complexes to form. Slides were then drained and the DAB substrate applied for five minutes. After rinsing in water for 5 minutes to remove the DAB, sections were counterstained with Mayer's haematoxylin before being washed in running tap water.

Finally sections were dehydrated through graded alcohols, cleared in clearene solvent (Leica Biosystems) and mounted in Pertex. The method is summarised in Figure 9.5.

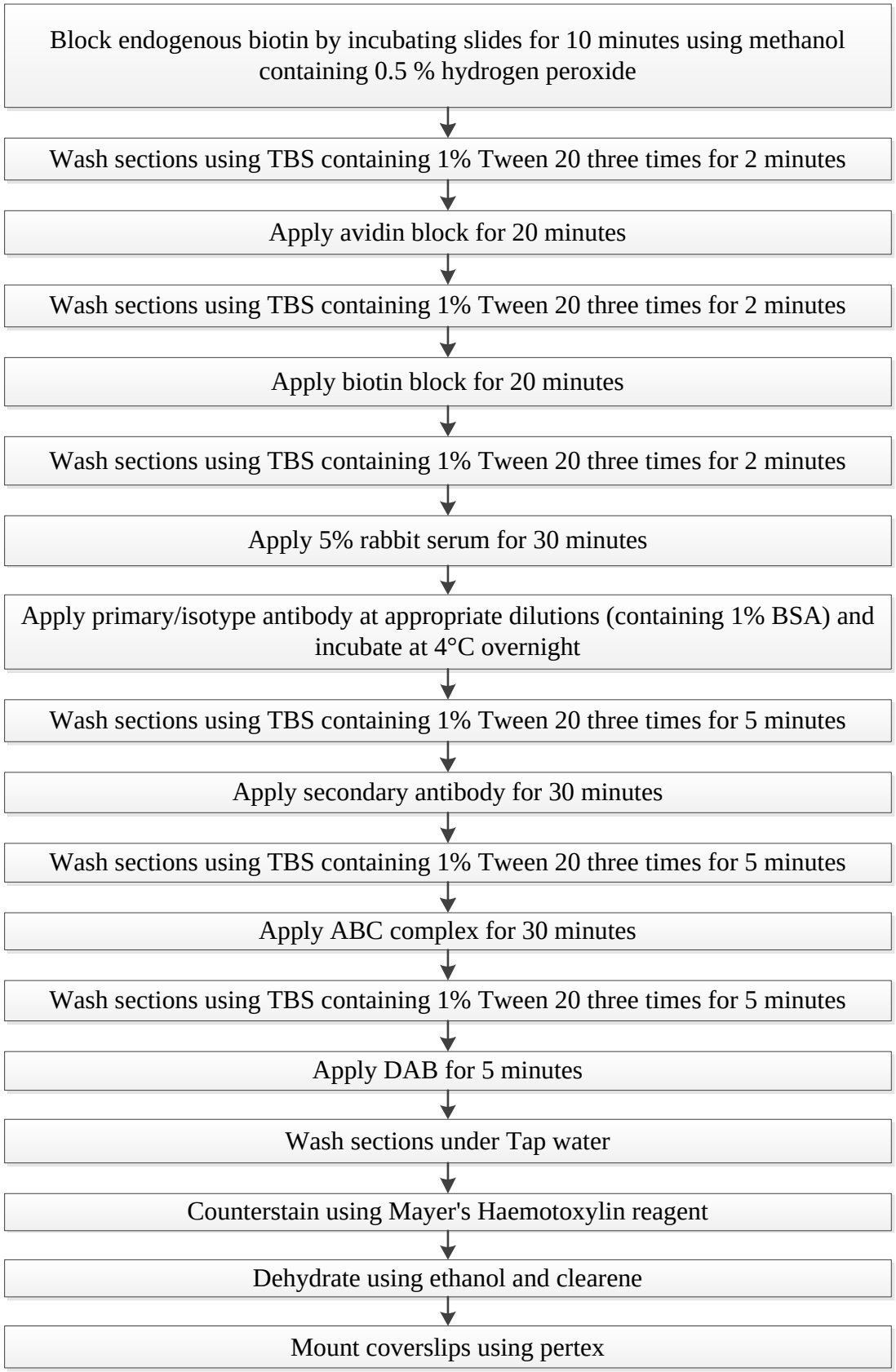


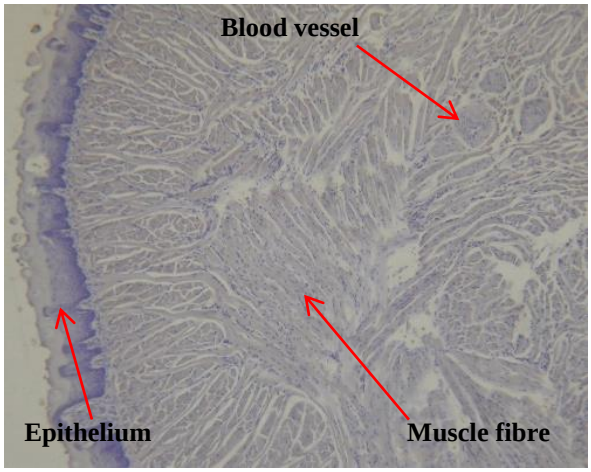
Figure 9.5 Summary of immunohistochemical staining method for the detection of morphine in mouse tissue samples embedded in GMA resin

9.3 Results and Discussion

9.3.1 Results mice injected with a saline solution

Six mice which were injected with a saline solution to act as a negative control, did not result in any positive brown staining confirming a lack of non-specific binding (Figure 9.6). A full set of images for each of the mice is available in Appendix D.

Mouse tongue (magnification x10):



Mouse salivary gland (magnification x 25):

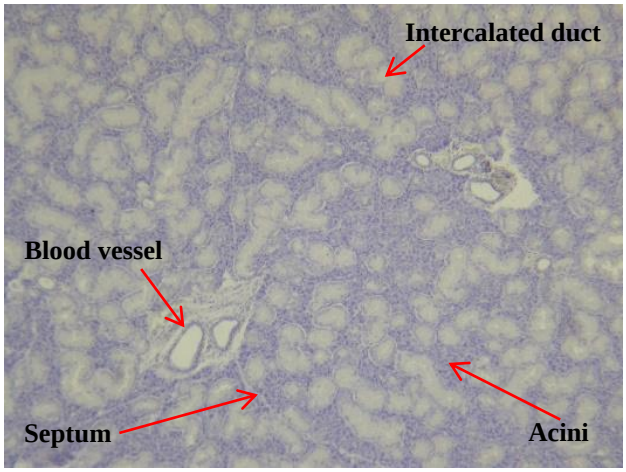


Figure 9.6. Image showing the negative staining in salivary glands in mice which were injected with a saline solution to act as a control

9.3.2 Results from salivary glands and tongues from mice injected with a low dose (3 mg/mL) of morphine

9.3.2.1 Mice Tongues

Specific staining was observed in tongues from mice injected with a low concentration of morphine. Clear staining was observed around the connective tissue, and in all muscle tissue (Figure 9.7, Figure 9.9, Figure 9.10, Figure 9.11, Figure 9.12, Figure 9.12). Staining was uniform throughout the section with no visual difference observed in the intensity of staining between the outside of the muscle tissue to the centre of the tongue.

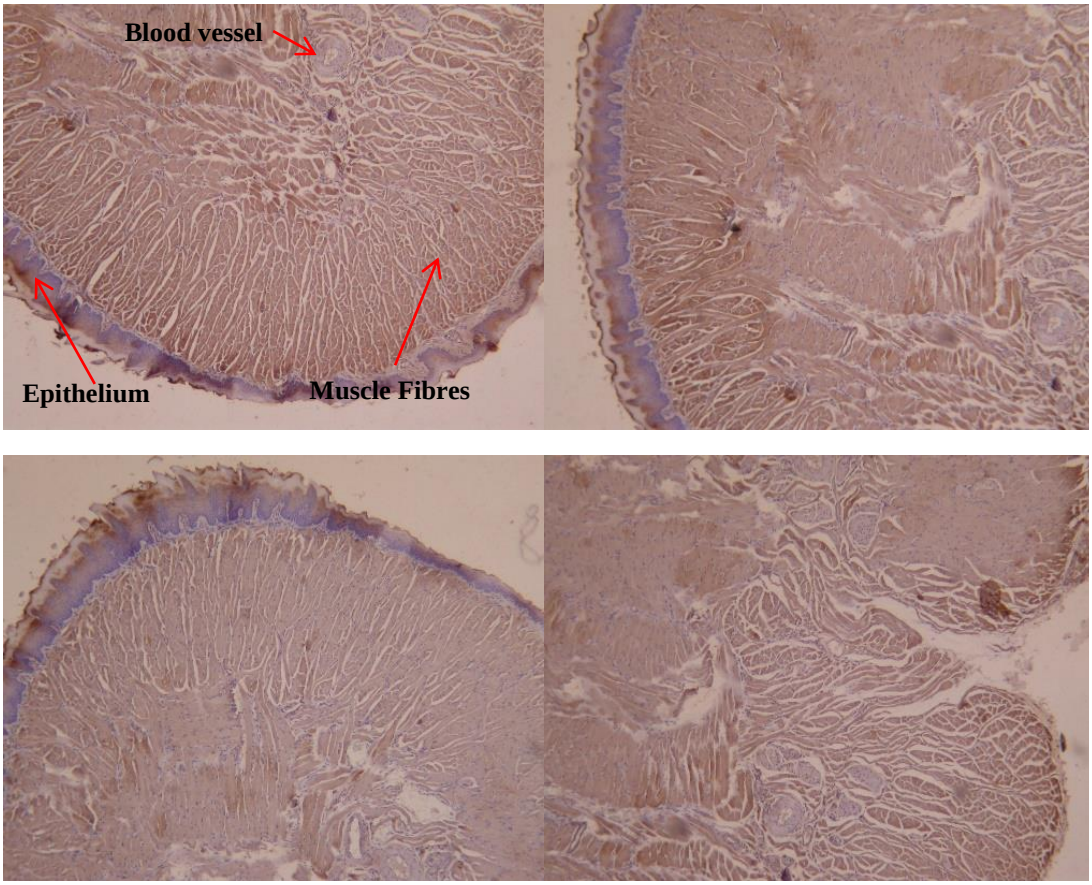


Figure 9.7 Images of stained slides of a mouse tongue from mice injected with a 3mg/mL (low) dose of morphine using an immunohistochemical technique (Mouse 1 of 6) (magnification x10)

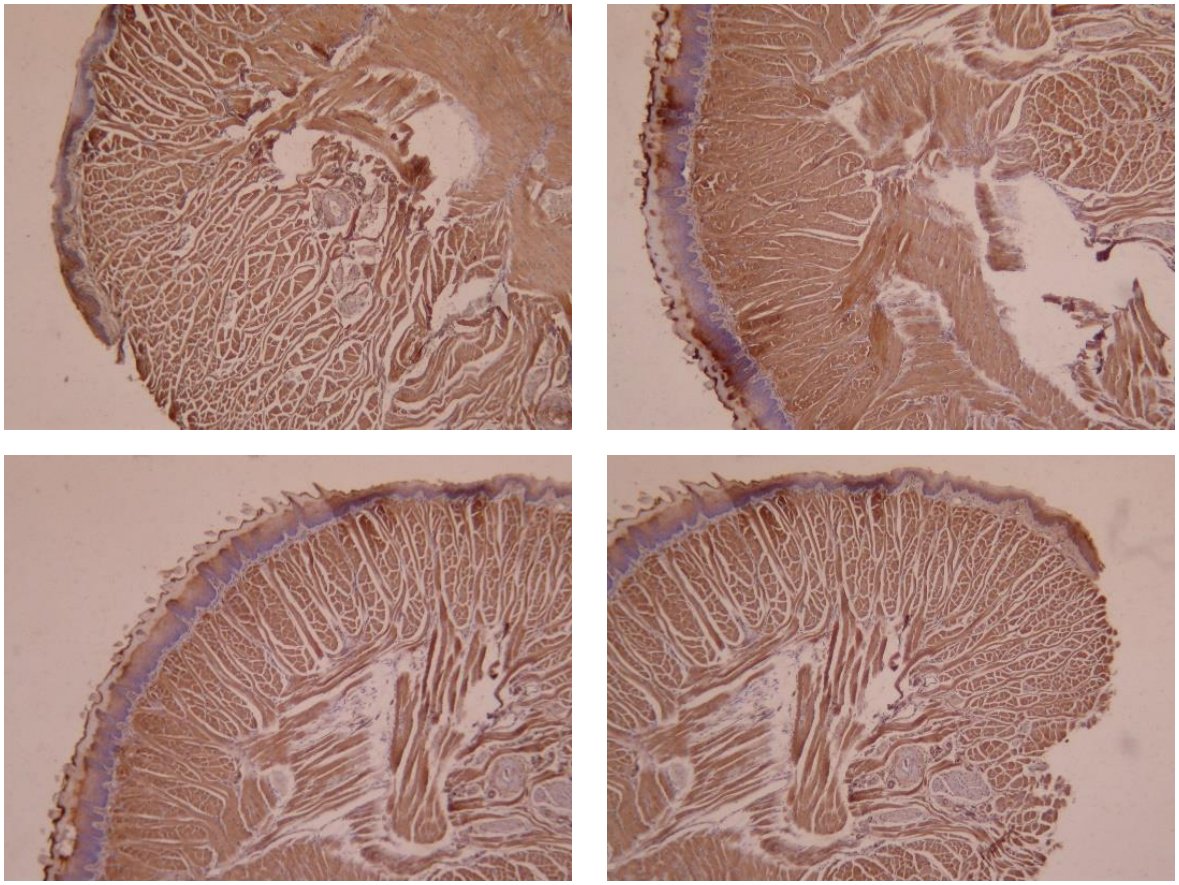


Figure 9.8 Images of stained slides of a mouse tongue from mice injected with a 3mg/mL (low) dose of morphine using an immunohistochemical technique (Mouse 2 of 6) (magnification x10)

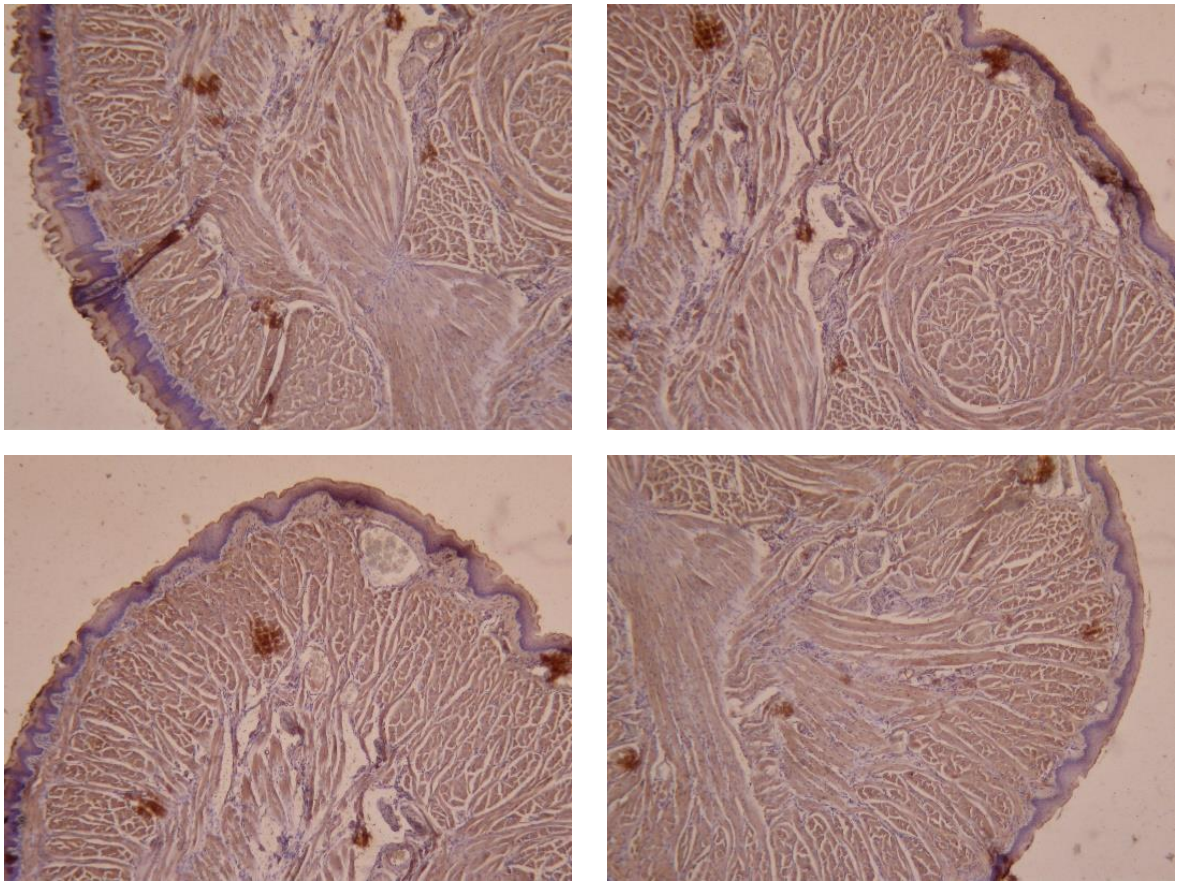


Figure 9.9 Images of stained slides of a mouse tongue from mice injected with a 3mg/mL (low) dose of morphine using an immunohistochemical technique (Mouse 3 of 6) (magnification x10)

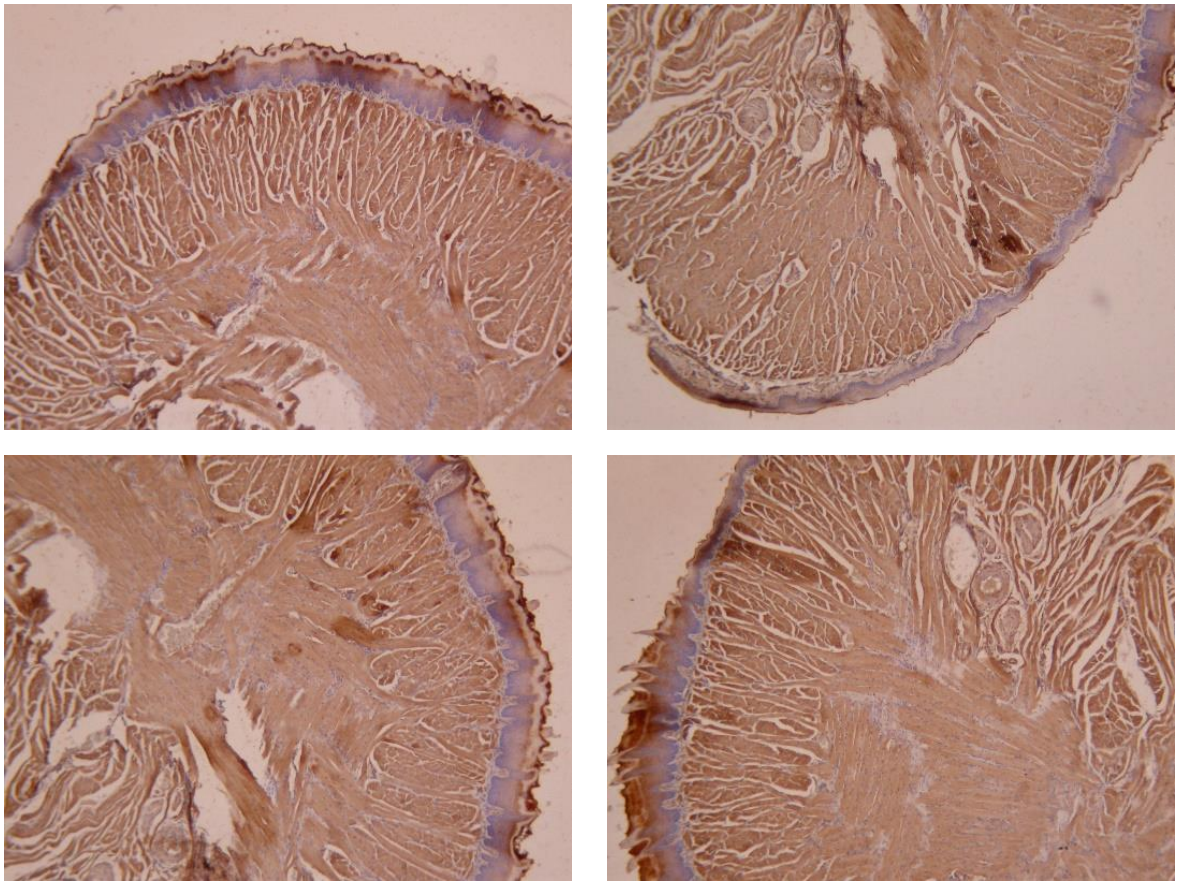


Figure 9.10 Images of stained slides of a mouse tongue from mice injected with a 3mg/mL (low) dose of morphine using an immunohistochemical technique (Mouse 4 of 6) (magnification x10)

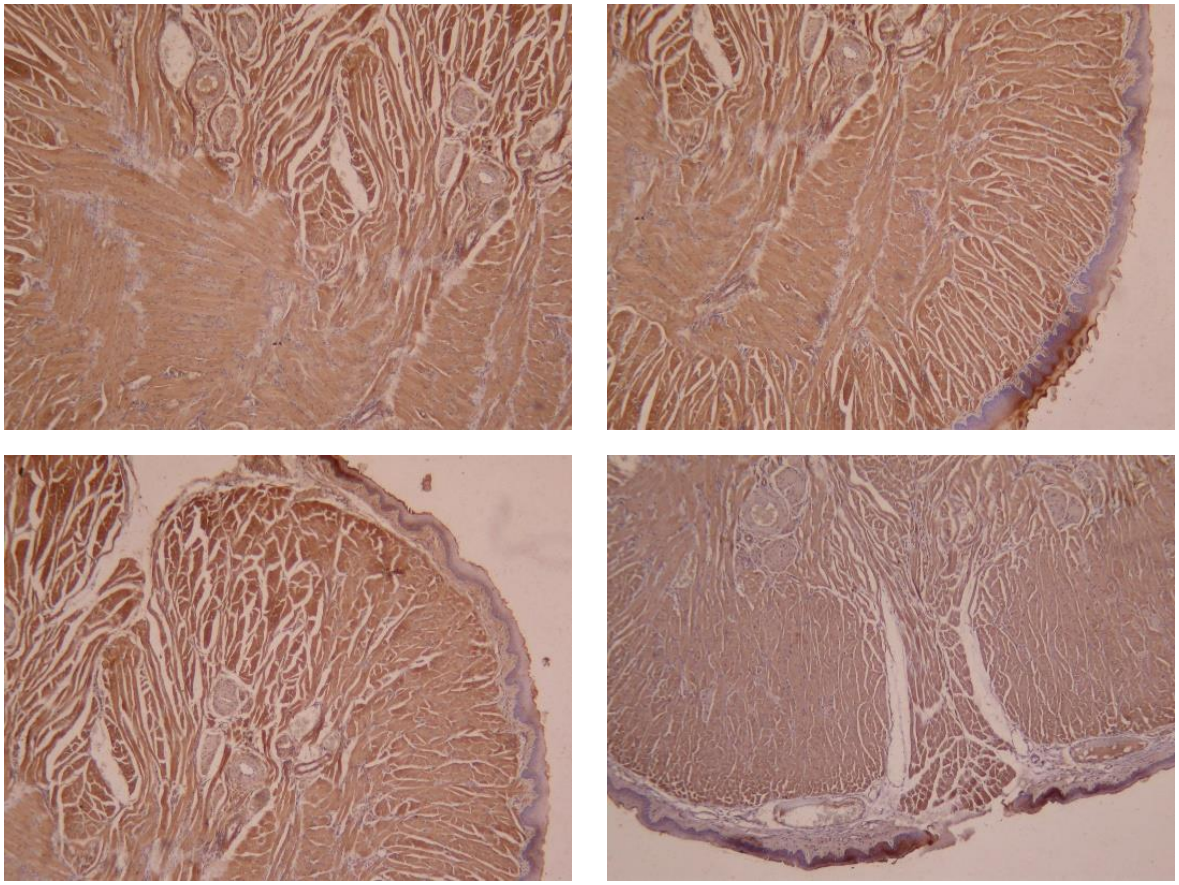


Figure 9.11 Images of stained slides of a mouse tongue from mice injected with a 3mg/mL (low) dose of morphine using an immunohistochemical technique (Mouse 5 of 6) (magnification x10)

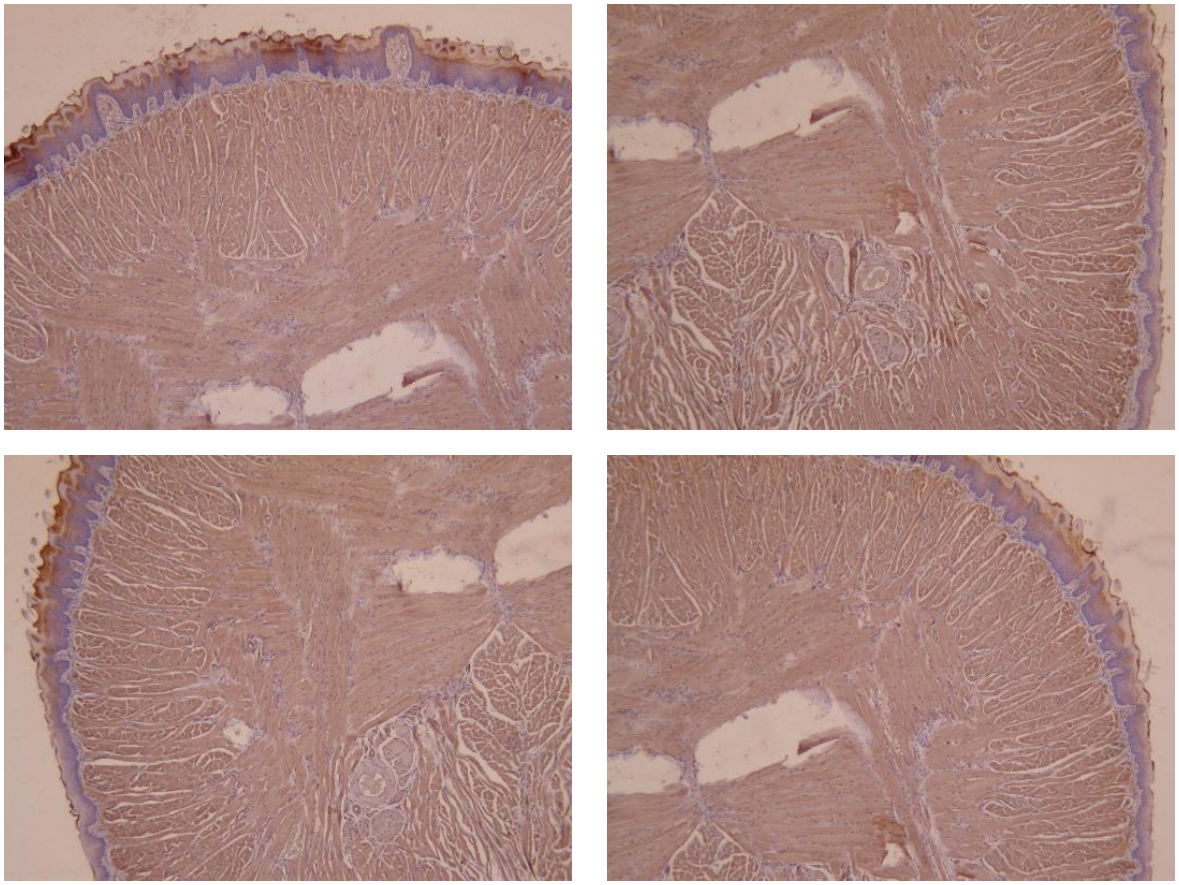


Figure 9.12 Images of stained slides of a mouse tongue from mice injected with a 3mg/mL (low) dose of morphine using an immunohistochemical technique (Mouse 6 of 6) (magnification x10)

Staining was observed throughout the sections and at a higher power (x 63) visual analysis it was observed that morphine was present throughout the tongue tissue penetrating the muscle bundles fully (Figure 9.13).

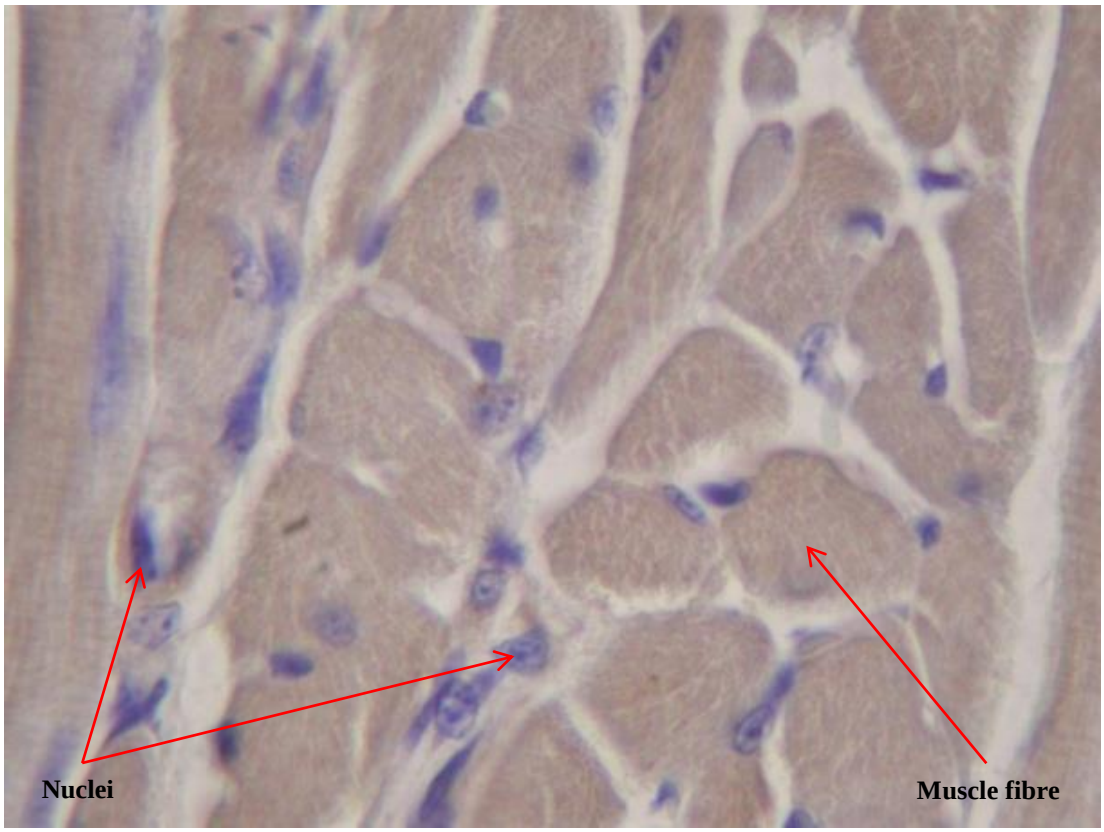


Figure 9.13. Image showing staining in muscle bundles of mice tongues following injection of mice with a low (3 mg/mL) dose of morphine (magnification x63)

The epithelium was stained positively on the outside of the tongue; however it showed negative staining on the inside of the epithelium in all six mice which were injected with a low concentration of morphine. Staining of the epithelium in porcine tongue, showed areas of non-drug staining that were attributed to the epithelium rate of repair and renewal of tissue in response to abrasion in the mouth (Chapter 8). A similar pattern of staining was also observed in mouse tongue tissue (Figure 9.14). Light positive staining was also observed in the blood vessel walls indicating that the drug travelled from the blood through the blood vessel wall into the tissue.

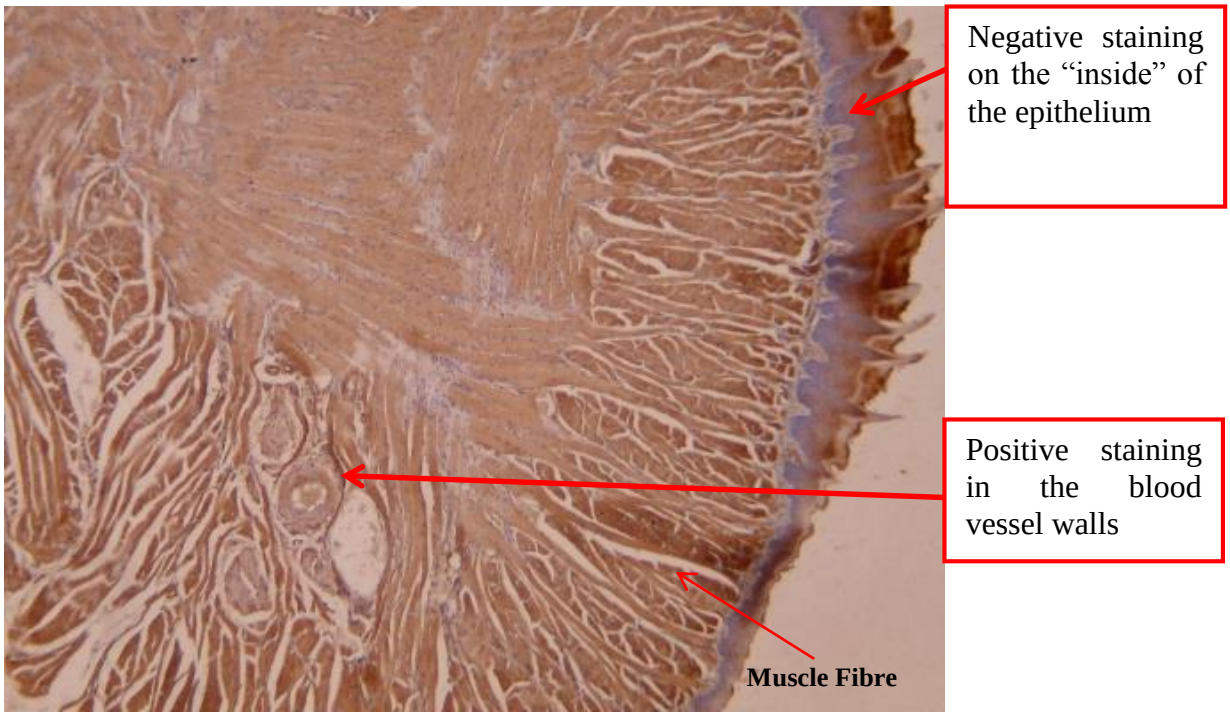


Figure 9.14. Image shows a stained section of mouse tongue following injection of low dose morphine (3 mg/ml). Negative staining on the "inside" of the epithelium is clearly illustrated as also observed in porcine tongues (magnification x25)

9.3.2.2 Salivary glands

Following injection of 3 mg/mL of morphine, all three salivary glands resulted in positive staining for morphine (Figure 9.15, Figure 9.16, Figure 9.17, Figure 9.18, Figure 9.19, Figure 9.20).

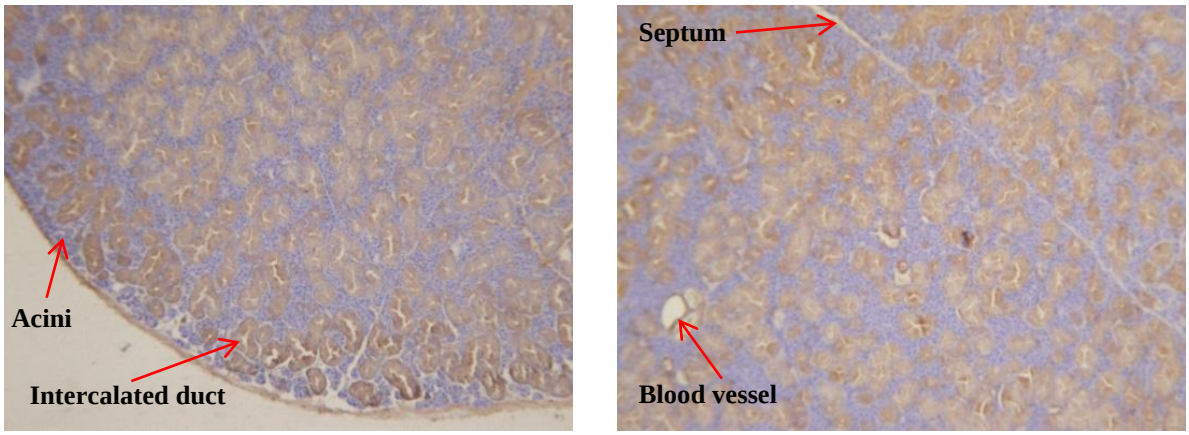


Figure 9.15 Images of stained slides of mice salivary glands injected with a 3 mg/mL (low) dose of morphine (Mouse 1 of 6) (magnification x10)

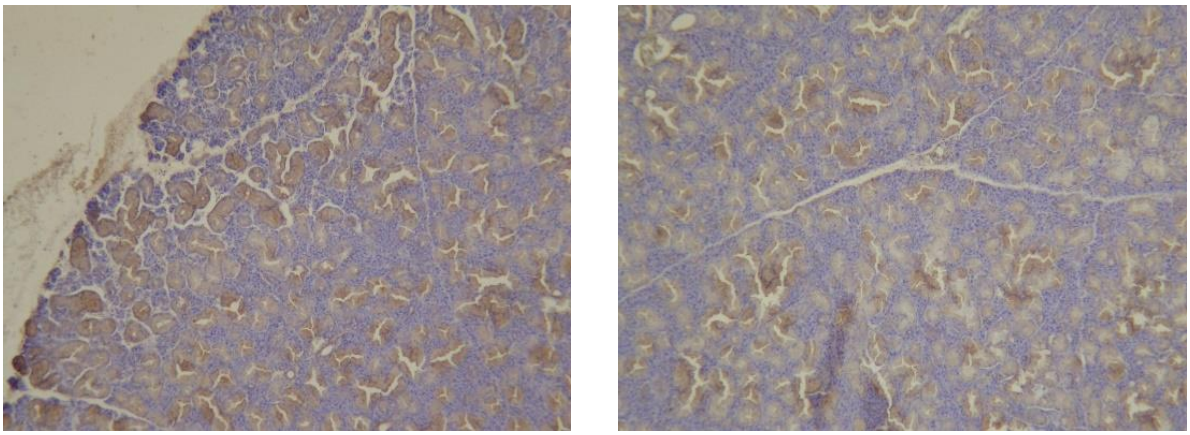


Figure 9.16 Images of stained slides of mice salivary glands injected with a 3 mg/mL (low) dose of morphine (Mouse 2 of 6) (magnification x10)

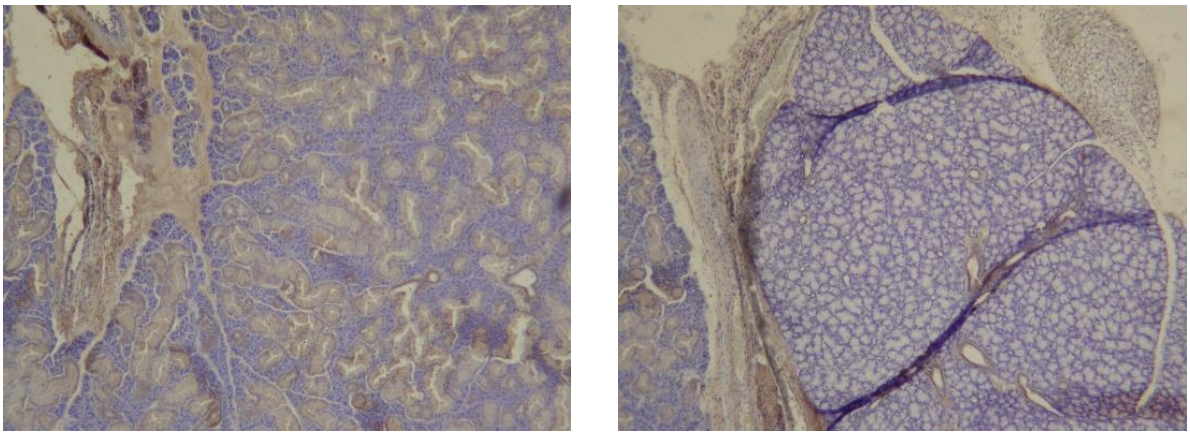


Figure 9.17 Images of stained slides of mice salivary glands injected with a 3 mg/mL (low) dose of morphine (Mouse 3 of 6) (magnification x10)

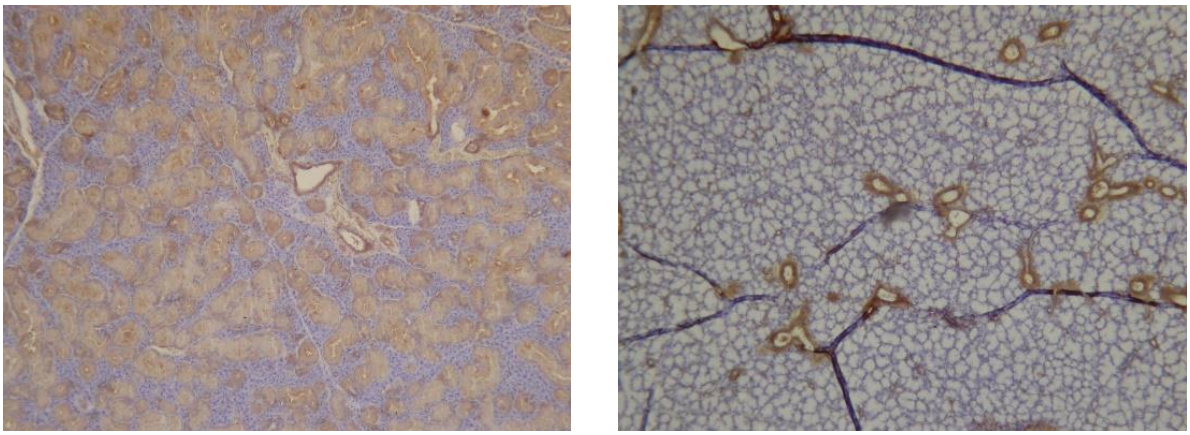


Figure 9.18 Images of stained slides of mice salivary glands injected with a 3 mg/mL (low) dose of morphine (Mouse 4 of 6) (magnification x10)

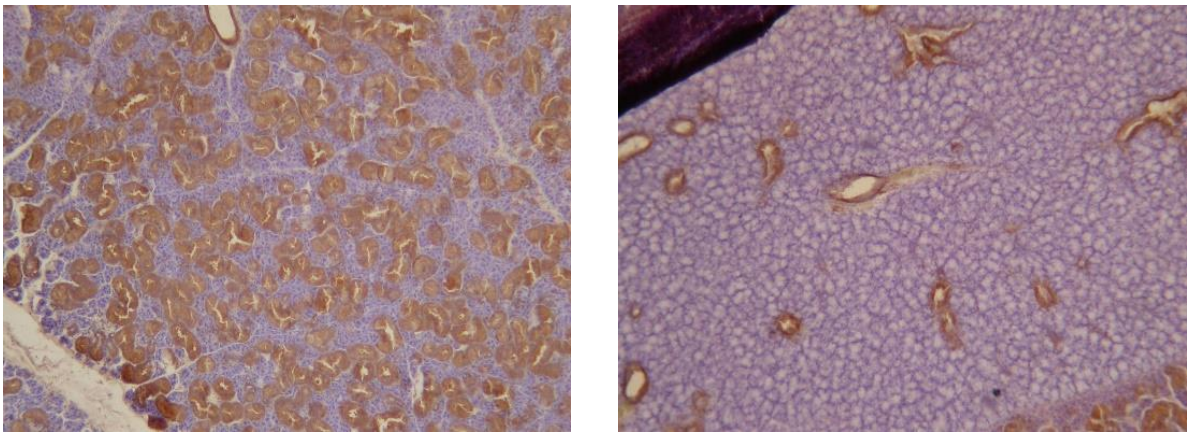


Figure 9.19 Images of stained slides of mice salivary glands injected with a 3 mg/mL (low) dose of morphine (Mouse 5 of 6) (magnification x10)

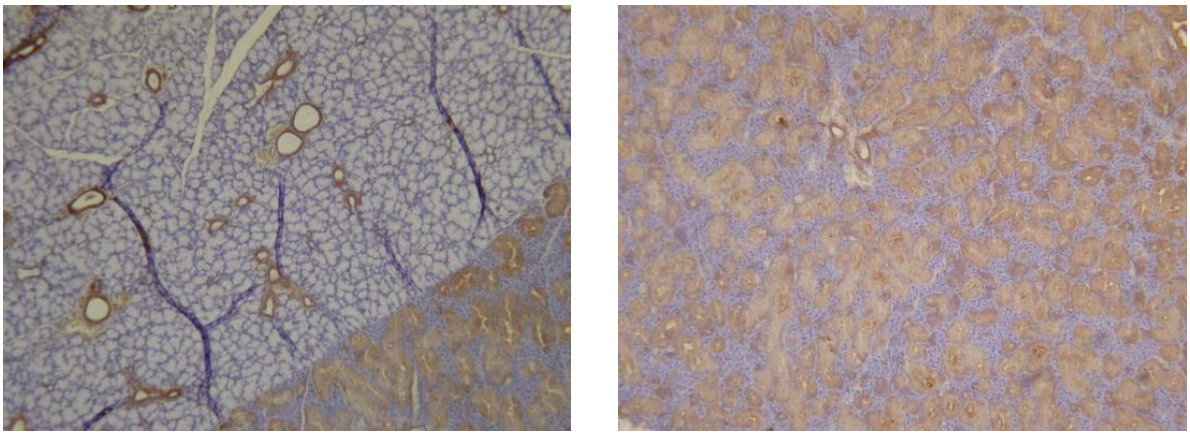


Figure 9.20 Images of stained slides of mice salivary glands injected with a 3 mg/mL (low) dose of morphine (Mouse 6 of 6) (magnification x10)

Staining was present in parotid, submandibular and sublingual gland. Although the composition of the glands differ slightly, applying the immunohistochemical technique resulted in staining in the intercalated and interlobular ducts as well as the blood capillaries and vessel in each gland. However, no staining could be detected in the acini of each of the glands (Figure 9.21).

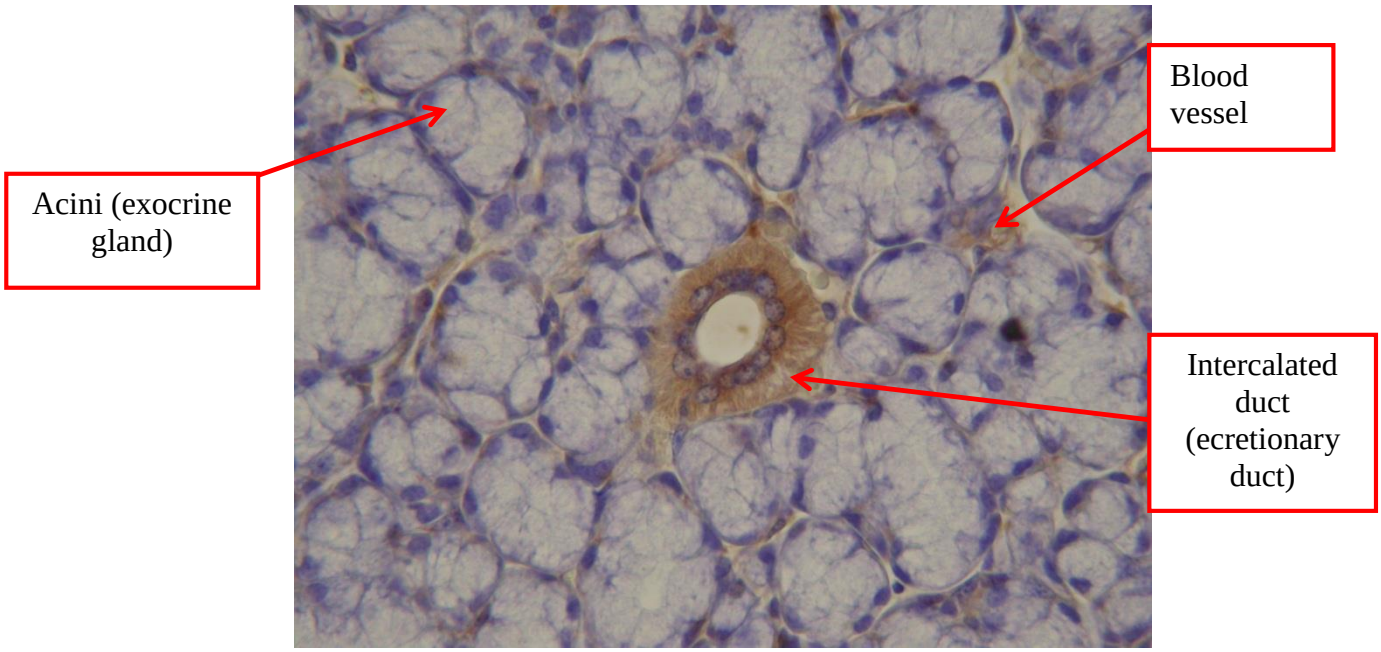


Figure 9.21. Image showing the positive staining about the excretion ducts and blood vessels following an injection of 3 mg/mL of morphine (magnification x63)

9.3.3 Results from salivary glands and tongues from mice injected with a high dose (9 mg/mL) of morphine

9.3.3.1 Tongues

Significant positive staining was also observed following a much higher dose of 9 mg/mL. In comparison to the tongues from the mice injected with the low concentration of morphine, the tongues from the higher concentration showed staining that was more intense in colour. All six mice showed positive staining in tongue tissue following an injection of 9 mg/mL of morphine (Figure 9.22, Figure 9.23, Figure 9.24, Figure 9.25, Figure 9.26, Figure 9.27).

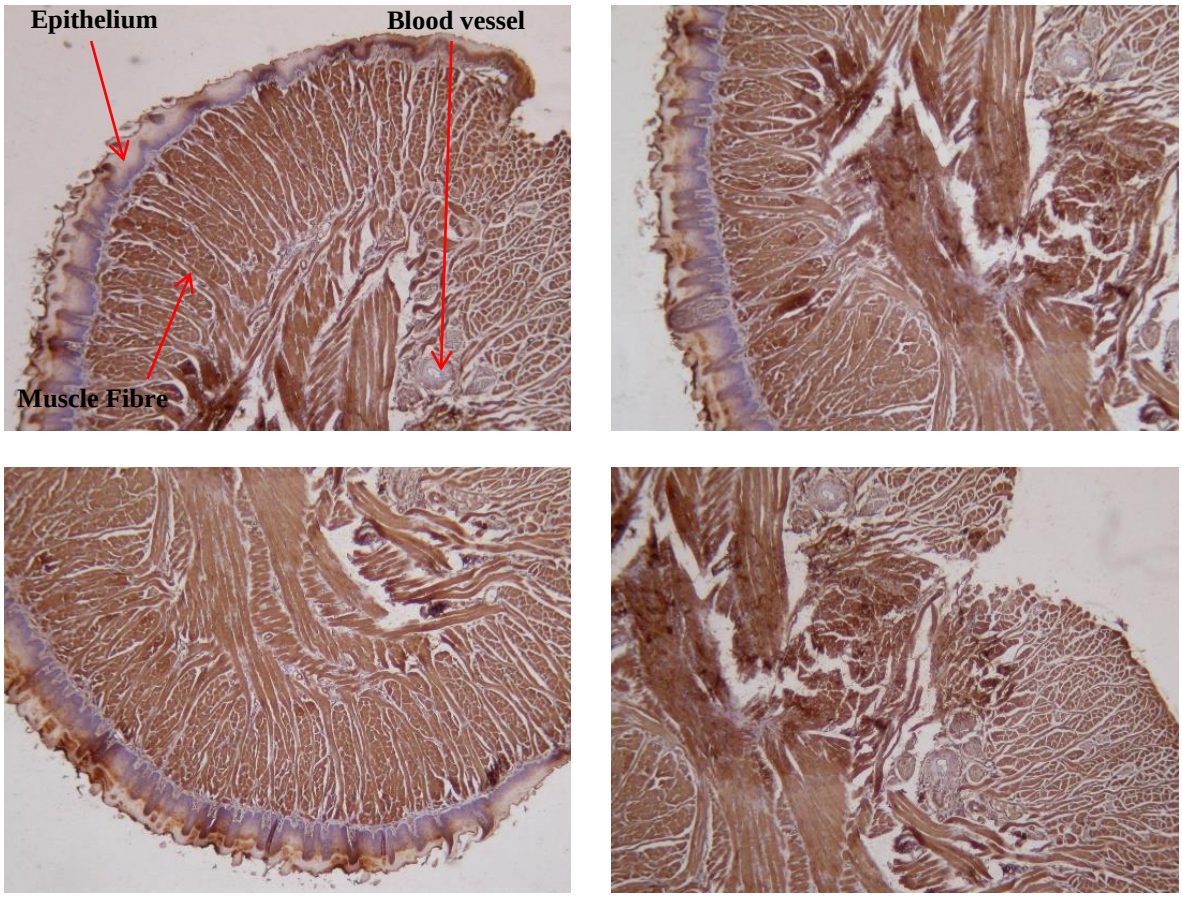


Figure 9.22 Images of stained slides of a mouse tongue injected with a 9 mg/mL (high) dose of morphine using an immunohistochemical technique (Mouse 1 of 6) (magnification x10)

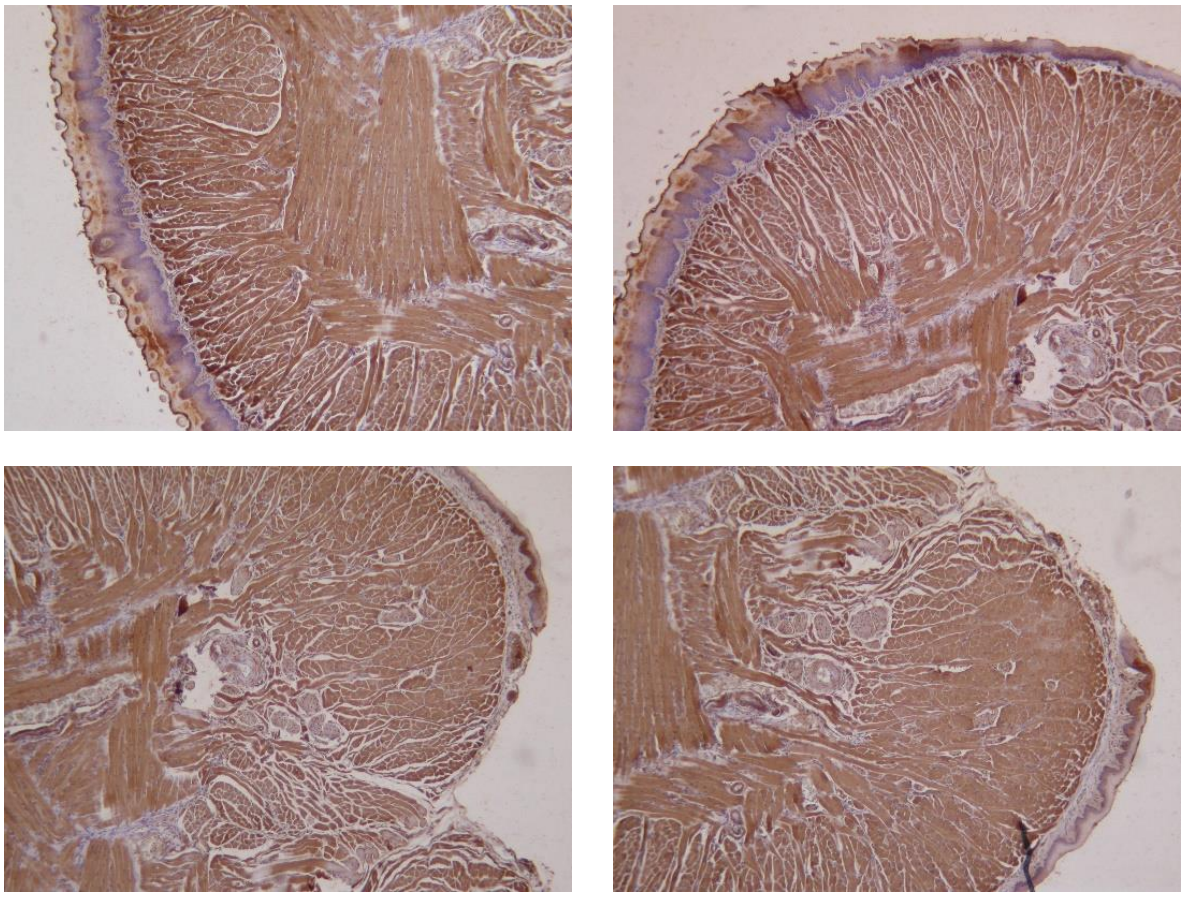


Figure 9.23 Images of stained slides of a mouse tongue injected with a 9 mg/mL (high) dose of morphine using an immunohistochemical technique (Mouse 2 of 6) (magnification x10)

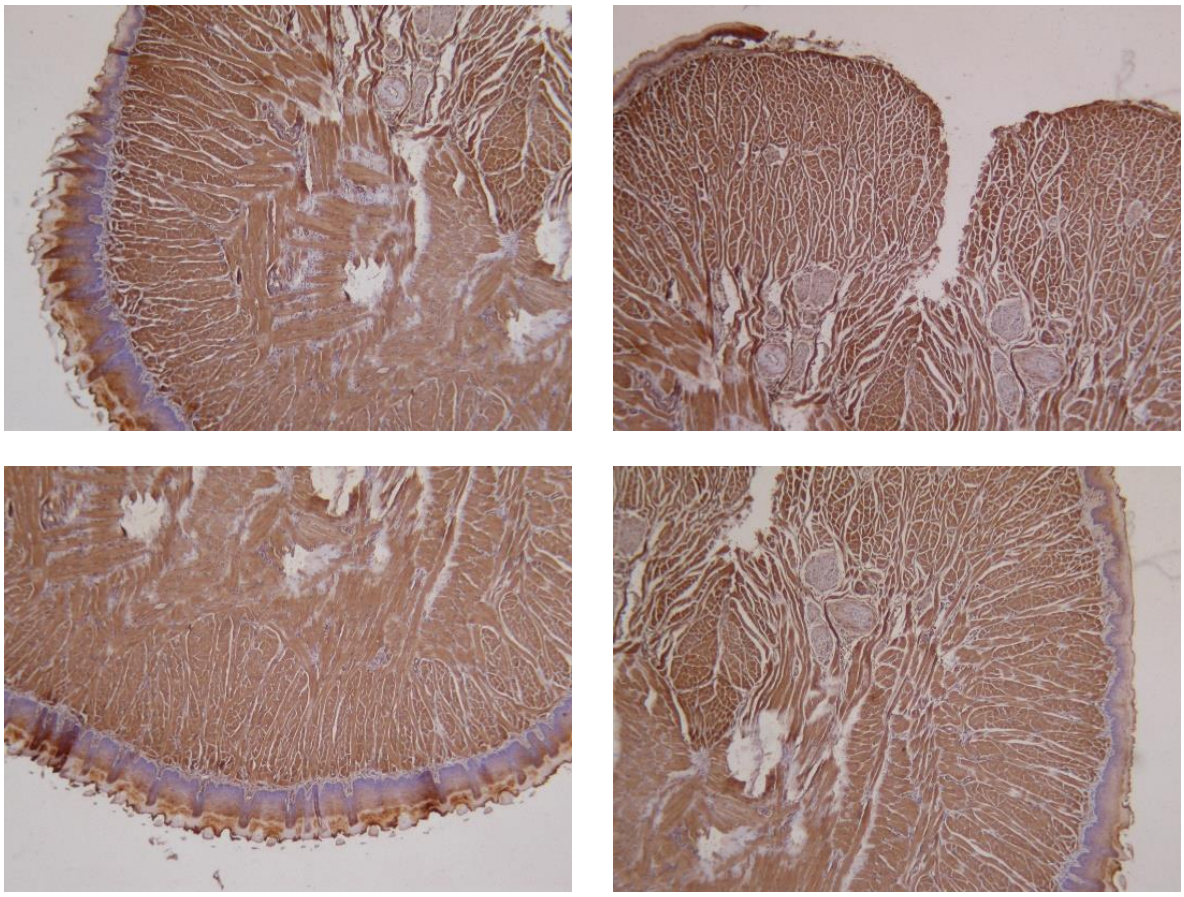


Figure 9.24 Images of stained slides of a mouse tongue injected with a 9 mg/mL (high) dose of morphine using an immunohistochemical technique (Mouse 3 of 6) (magnification x10)

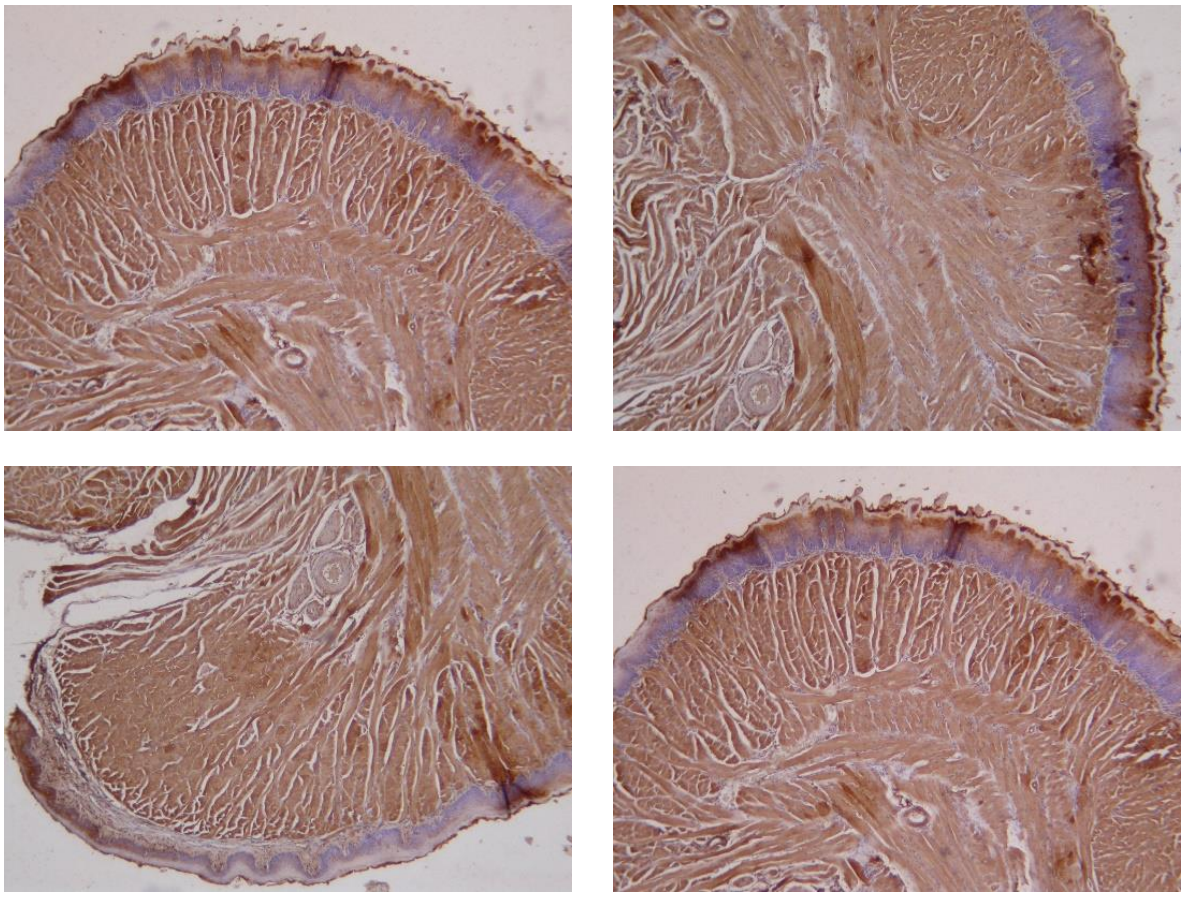


Figure 9.25 Images of stained slides of a mouse tongue injected with a 9 mg/mL (high) dose of morphine using an immunohistochemical technique (Mouse 4 of 6) (magnification x10)

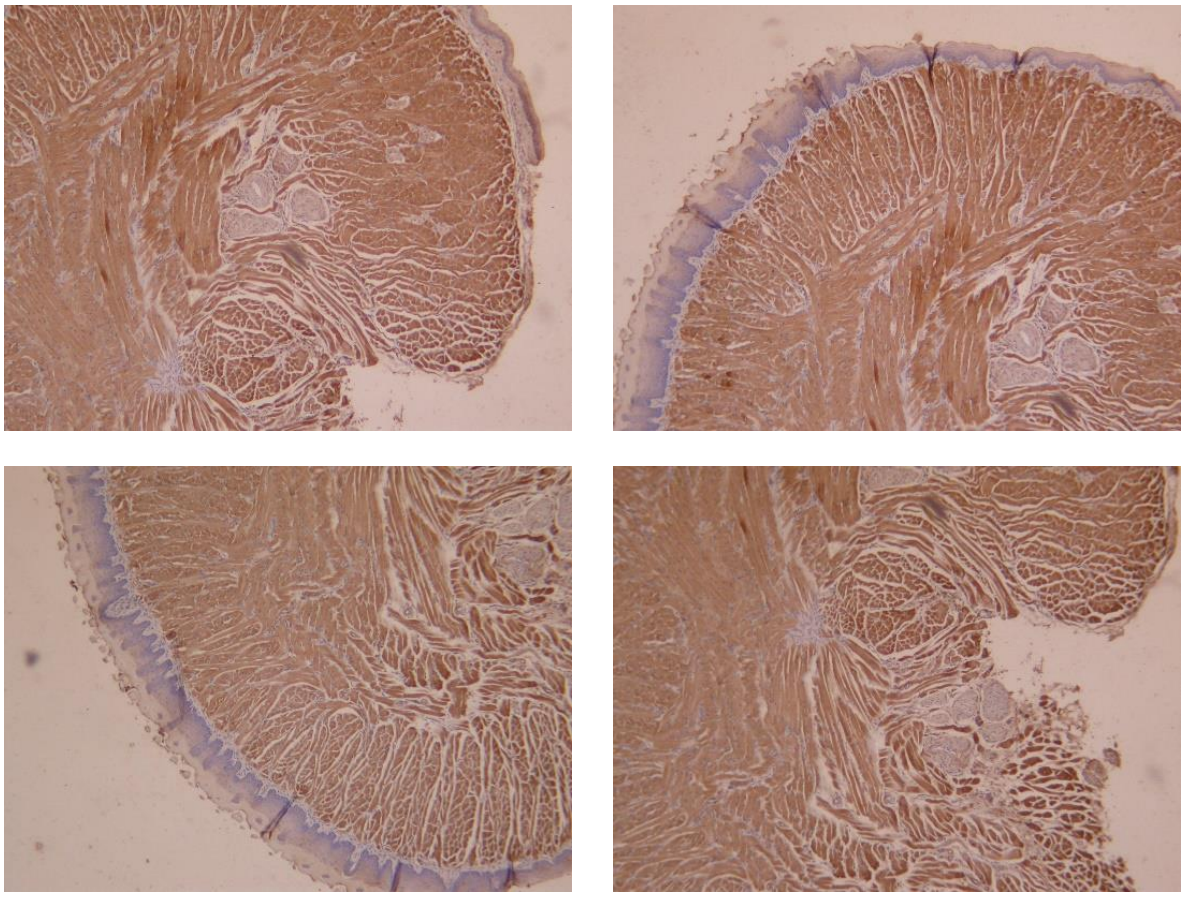


Figure 9.26 Images of stained slides of a mouse tongue injected with a 9 mg/mL (high) dose of morphine using an immunohistochemical technique (Mouse 5 of 6) (magnification x10)

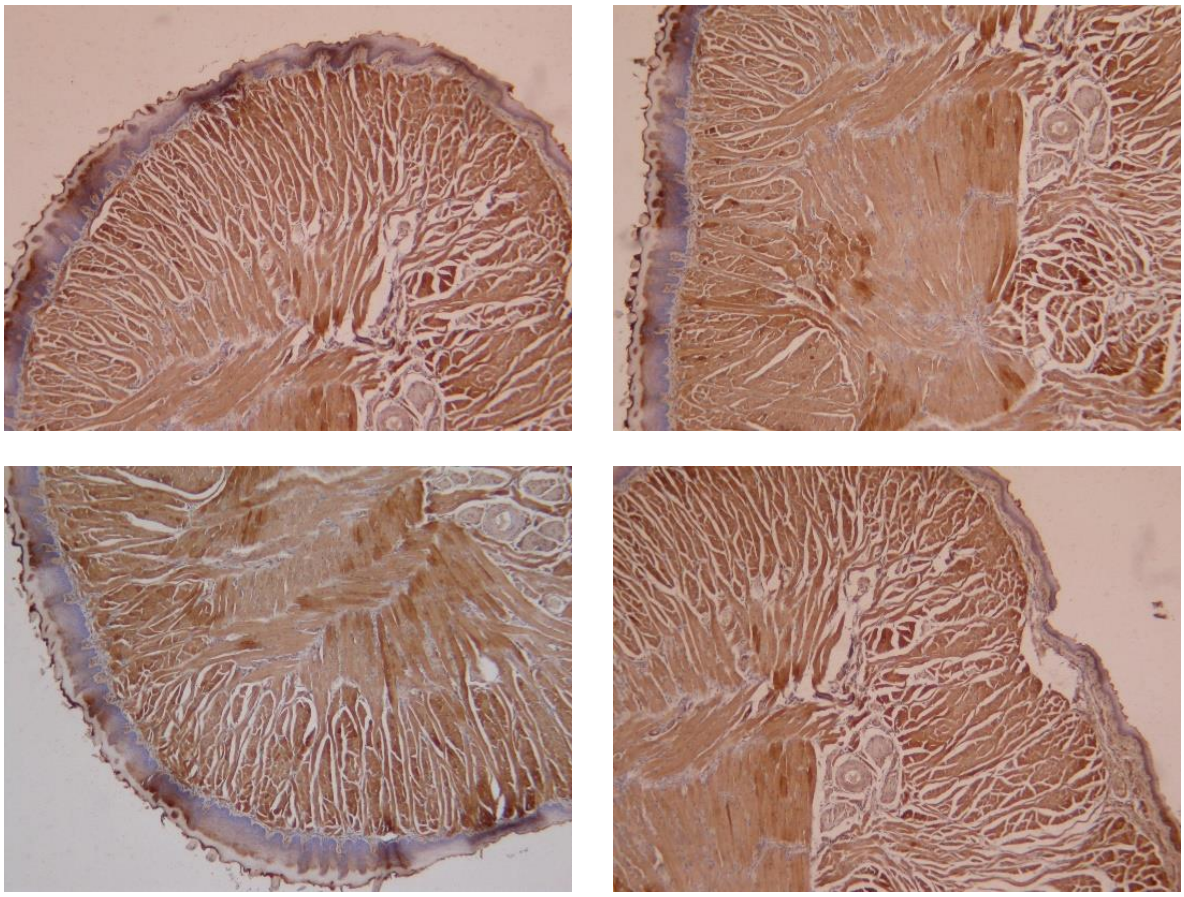


Figure 9.27 Images of stained slides of a mouse tongue injected with a 9 mg/mL (high) dose of morphine using an immunohistochemical technique (Mouse 6 of 6) (magnification x10)

When the drugs are visualised with a higher powered microscope (x63) it can be observed that the drug penetrated the muscle bundles fully (Figure 9.28). Staining was intense in colour suggesting high concentrations of morphine present in the muscle.

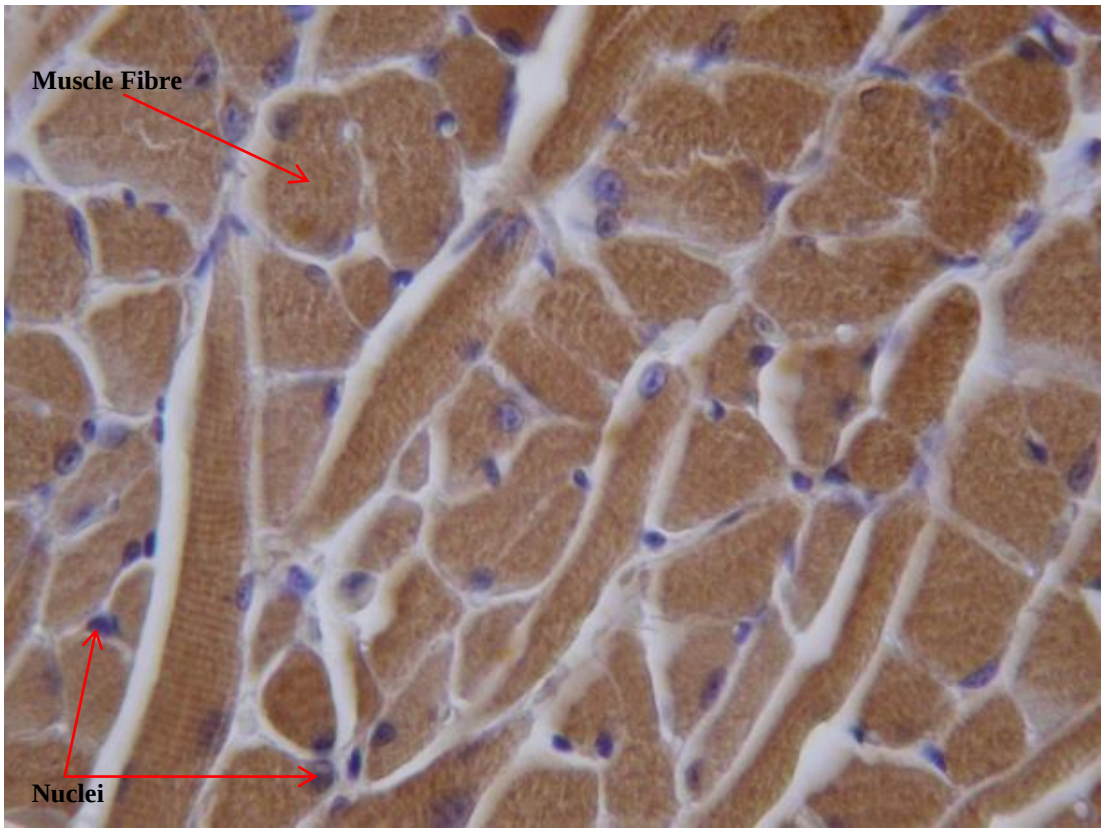


Figure 9.28. Image showing staining in muscle bundles following injection of mice with a 9 mg/mL dose of morphine (magnification x63)

Tongues from mice that had been injected with a 9 mg/mL of morphine showed a similar effect to the one injected with a 3 mg/mL in that the epithelium showed a negative stain on the inside (muscle side). The staining on the outside of the epithelium is stained more intensely than in the low dosed mice tongues. Staining can also be observed in the blood vessel walls (Figure 9.29).

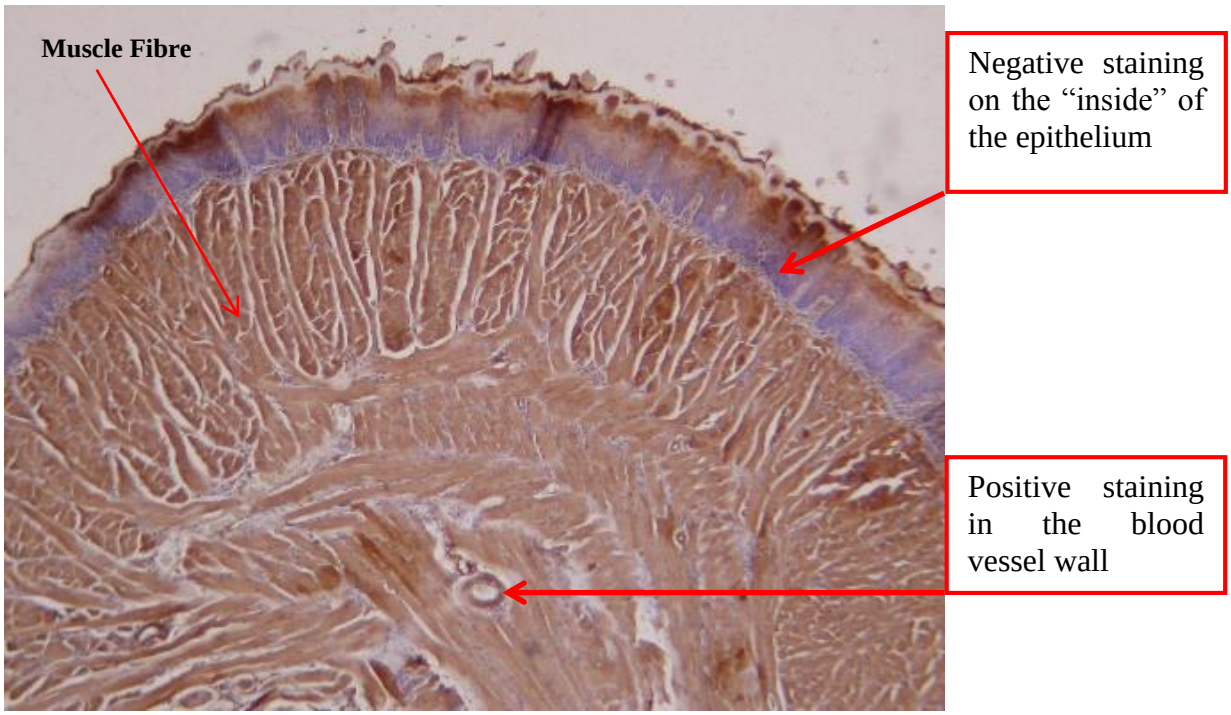


Figure 9.29. Image shows a stained section of mouse tongue following a high dose injection of morphine. This picture highlights negative staining on the "inside" of the epithelium and positive staining in the blood vessels (magnification x25)

9.3.3.2 Salivary glands

Following injection of 9 mg/mL of morphine, all three salivary glands resulted in positive staining for morphine (Figure 9.30, Figure 9.31, Figure 9.32, Figure 9.33, Figure 9.34, Figure 9.35). Staining observed was more intense in colour than in salivary glands which originated from mice injected with a lower dose of morphine.

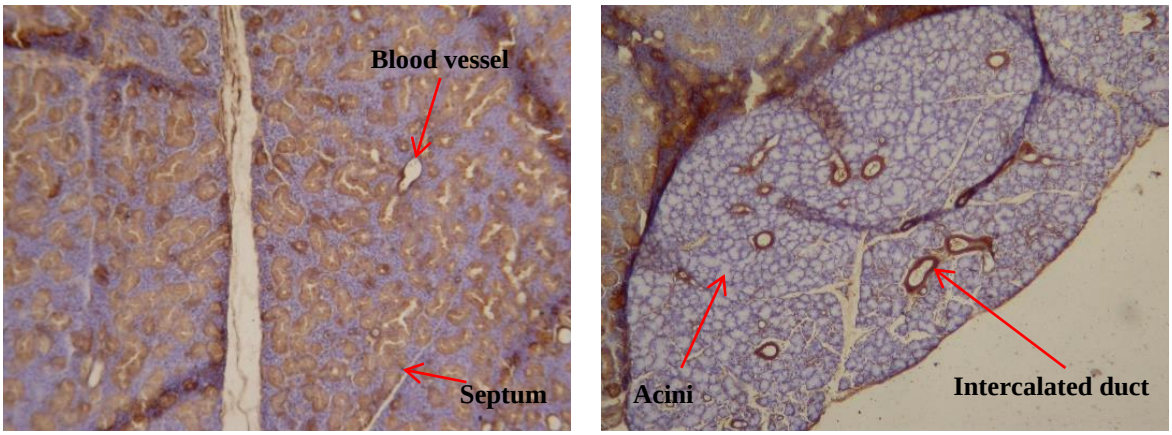


Figure 9.30 Images of stained slides of mice salivary glands injected with a 9 mg/mL (high) dose of morphine using an immunohistochemical technique (Mouse 1 of 6) (magnification x10)

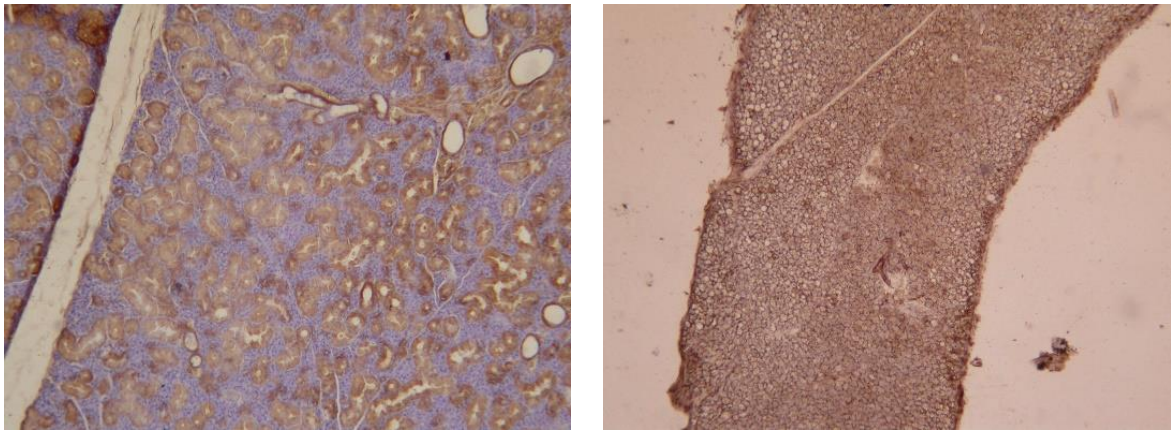


Figure 9.31 Images of stained slides of mice salivary glands injected with a 9 mg/mL (high) dose of morphine using an immunohistochemical technique (Mouse 2 of 6) (magnification x10)

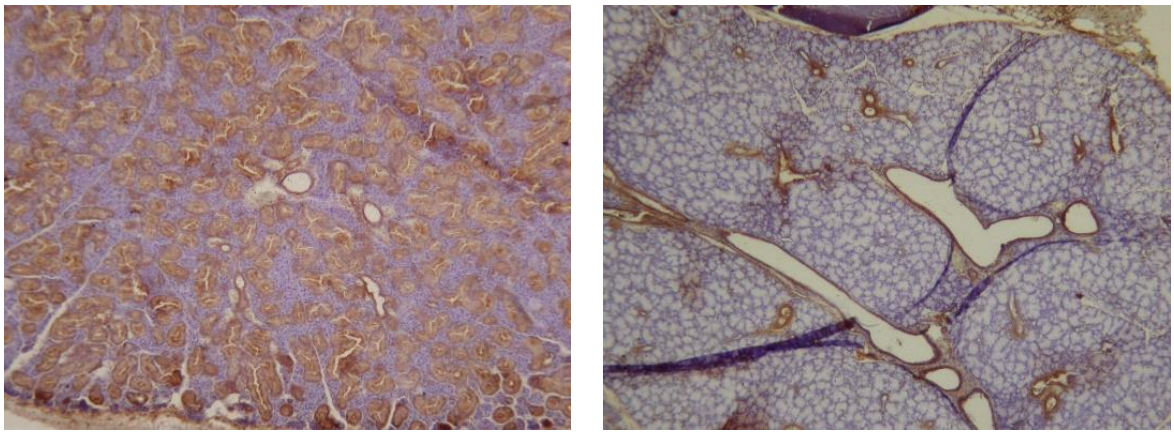


Figure 9.32 Images of stained slides of mice salivary glands injected with a 9 mg/mL (high) dose of morphine using an immunohistochemical technique (Mouse 3 of 6) (magnification x10)

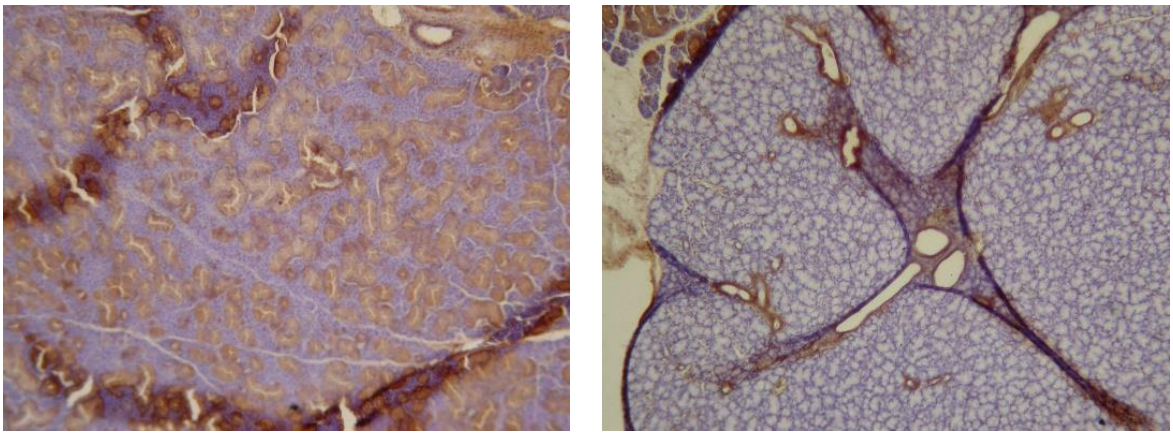


Figure 9.33 Images of stained slides of mice salivary glands injected with a 9 mg/mL (high) dose of morphine using an immunohistochemical technique (Mouse 4 of 6) (magnification x10)

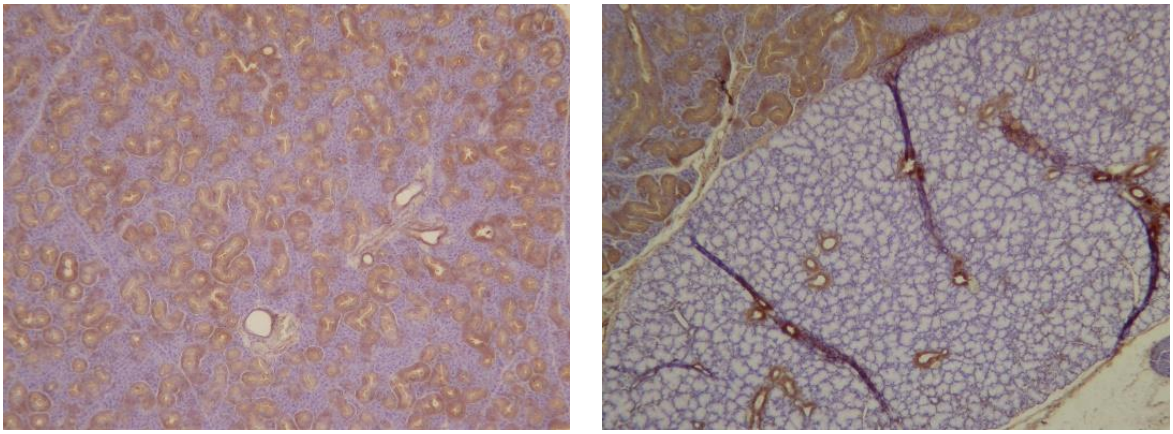


Figure 9.34 Images of stained slides of mice salivary glands injected with a 9 mg/mL (high) dose of morphine using an immunohistochemical technique (Mouse 5 of 6) (magnification x10)

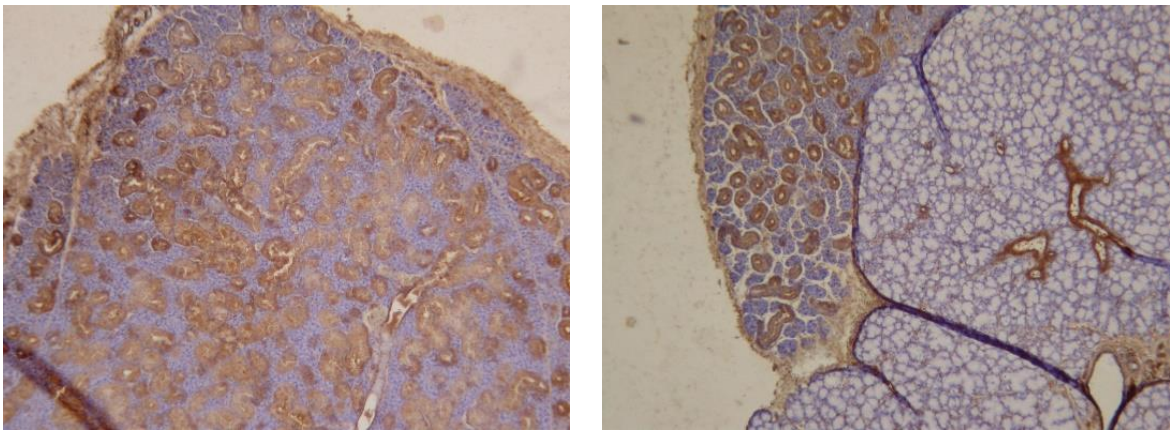


Figure 9.35 Images of stained slides of mice salivary glands injected with a 9 mg/mL (high) dose of morphine using an immunohistochemical technique (Mouse 6 of 6) (magnification x10)

Mice injected with a low dose of morphine, salivary glands obtained from mice injected with a higher dose of 9 mg/mL of morphine resulted in staining in the intercalated and interlobular ducts as well as the blood vessel. However, no staining was observed in the acini of any of the salivary glands (Figure 9.36).

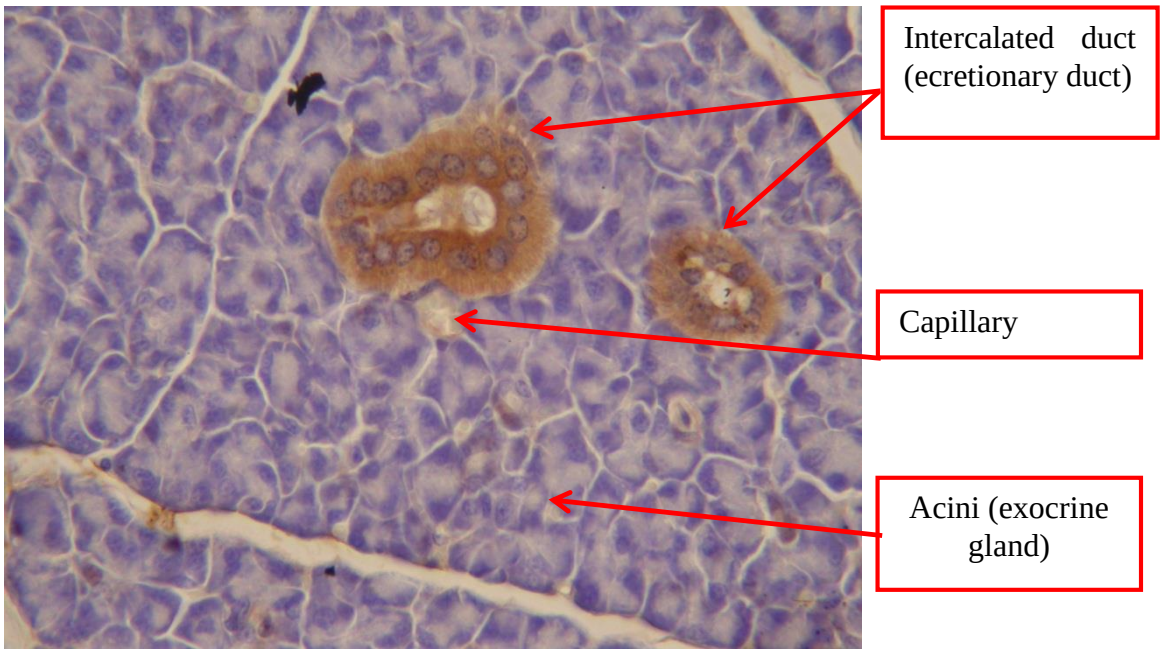


Figure 9.36. Image showing the positive staining about the excretion ducts and blood vessels following an injection of 9 mg/mL of morphine (magnification x63)

9.3.4 Summary of *in vivo* study results

The morphine-3-glucuronide polyclonal antibody was a suitable antibody for the use as a stain to visualise the presence of drugs in mouse tissues. Binding was specific for morphine and TBS controls did not show any staining. Mice injected with a saline solution acted as a second control and did not result in any positive brown staining.

In the low (3 mg/mL) and the high (9 mg/mL) morphine dose group, significant staining could be observed in the tongue tissue. Tongues showed staining in the muscle bundles and blood vessels. As mice were injected with morphine, no external

oral contamination occurred. Therefore positive staining for morphine can only have originated via diffusion from the blood into muscle tissue.

In both concentration groups the epithelium had a negative stain on the inside layer. The epithelium in the mouth is continuously exposed to abrasions. Therefore, it has a fast rate of repair, which results in a constant reproduction of cells on the inside layer of the tongue to repair the damaged cells. This is seen as a dense layer of nuclei in developing tissue. Nuclei did not stain positive and therefore a large amount of nuclei on the inside layer of developing epithelium cells creates the appearance of total negative stain.

As mice were killed 30 minutes post injection, drug may have entered the saliva during that time from the surrounding blood and that re-absorption could have occurred from excreted saliva.

When tissue sections were examined using a magnification of x63, it was observed that following injection of a low dose of morphine the staining within the muscle bundle was less intense than the staining in mice that were injected with a higher dose, suggesting that more drug has penetrated into the muscle and bound to the tissue (Figure 9.37, Figure 9.38).

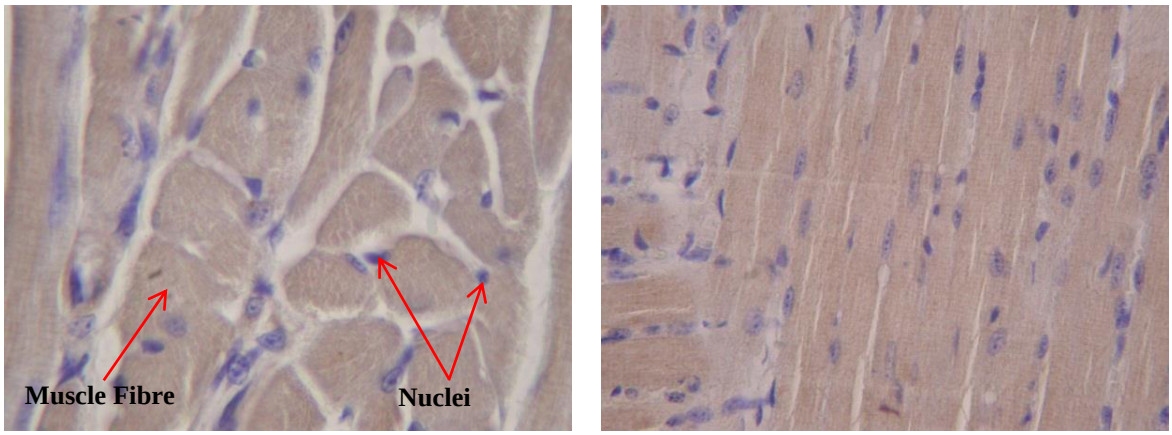


Figure 9.37 Images representing morphine penetration into the tissue following a 3 mg/mL (low dose) of morphine in live mice (magnification x63)

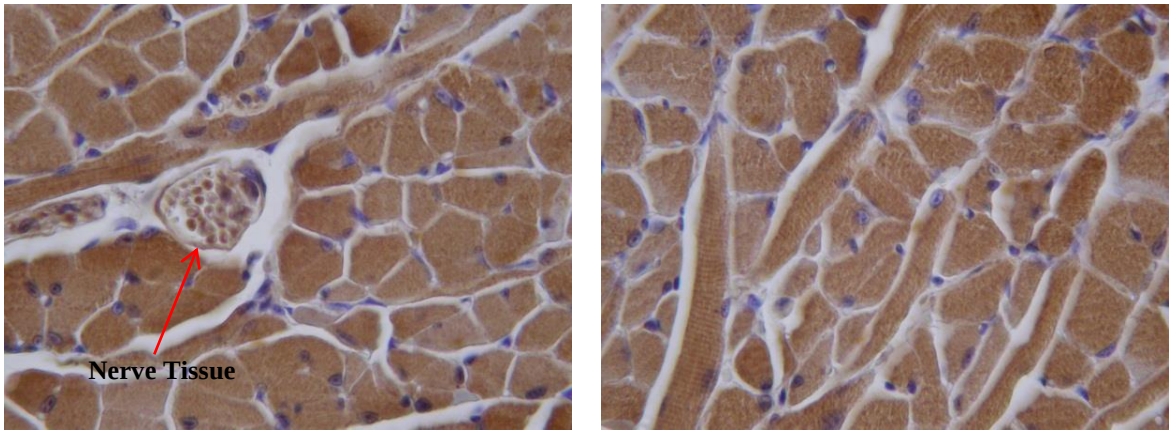


Figure 9.38 Images representing morphine penetration into the tissue following 9 mg/mL (high) dose of morphine in live mice (magnification x63)

The higher the dose consumed, the more likely drug depots are in oral tissues and suggests that when drugs travel further into tissue, it subsequently takes longer for the drug to be released / eliminated from the tissue.

Additionally staining for morphine was observed in the blood vessel walls for both concentration groups, indicating that the drug travels through the blood vessel walls to the connective and muscle tissue. In comparison, in chapter 8.2 it was shown that when oral contamination occurred through external contact such as solution or smoking the blood vessel walls are negative and hence to do not penetrate the blood vessels and migrate into the blood.

The parotid, submandibular and submaxillary glands were successfully removed from the mice and the immunohistochemical method applied to salivary glands. Opioid staining was observed in all three salivary glands for mice injected with either 3 mg/mL (low) or 9 mg/mL (high). Staining was present in the excretory ducts as well as the blood vessels. However, no staining could be observed in the acini of the salivary glands. Acini are the secretory units mainly consisting of epithelial cells.

The presence of morphine in the salivary ducts strongly supports a hypothesis that drug is excreted from the salivary gland during production and excretion of saliva into the oral cavity hence contributing to the elevated drug concentrations observed in oral fluid.

9.3.5 Exposure or mice tongues to smoke produced from heating 100 mg heroin

Mice tongues were exposed to smoke produced by heating 100 mg of heroin. In comparison to the porcine tongues (Chapter 7), mice tongues were only exposed to

the smoke for 30 seconds and 60 seconds in order to prevent overheating and hence deterioration of the tissue as tongues were significantly smaller in size.

Two tongues were exposed to heroin smoke for 30 seconds. Both tongues showed significant staining within the tissue; however staining appeared to be weak which was noticeable in the muscle and the epithelium (Figure 9.39, Figure 9.40).

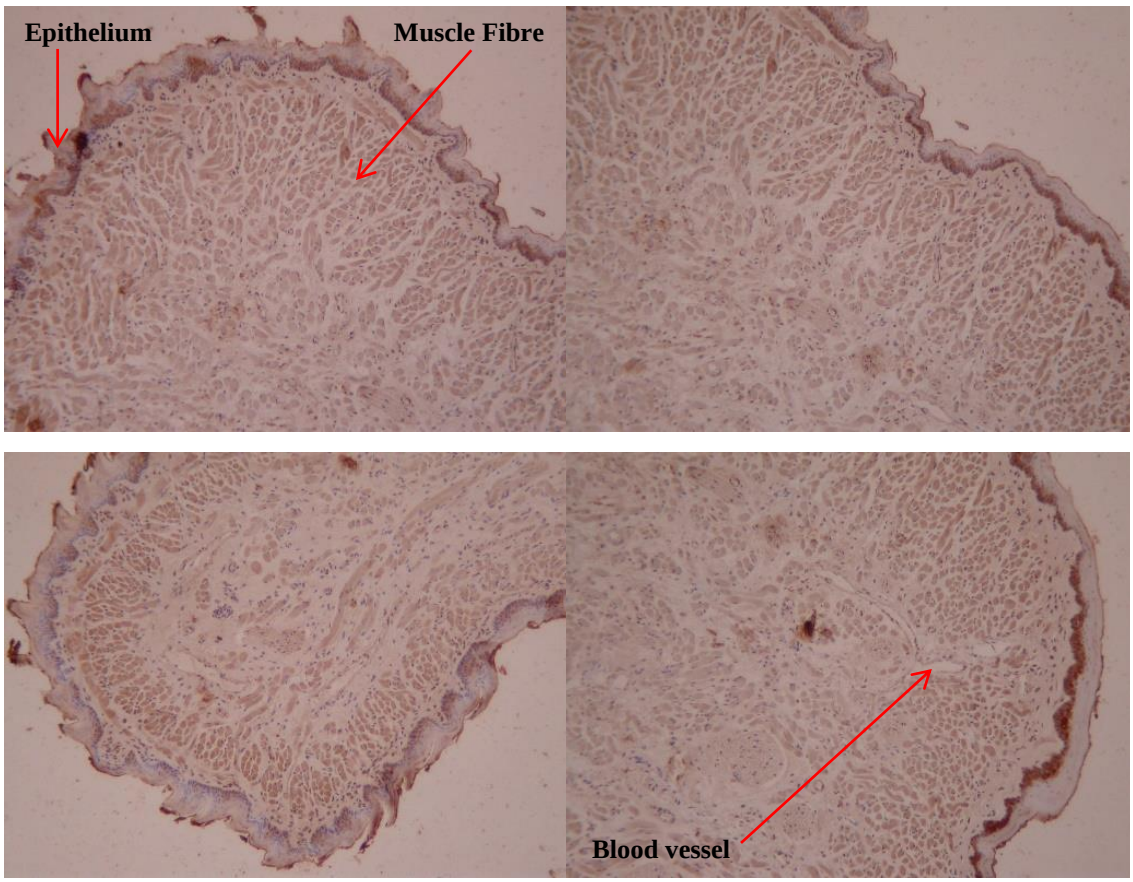


Figure 9.39 Images show the staining in mice tongues exposed to smoke from 100 mg of heroin for 30 seconds (Tongue 1 of 2) (magnification x10)

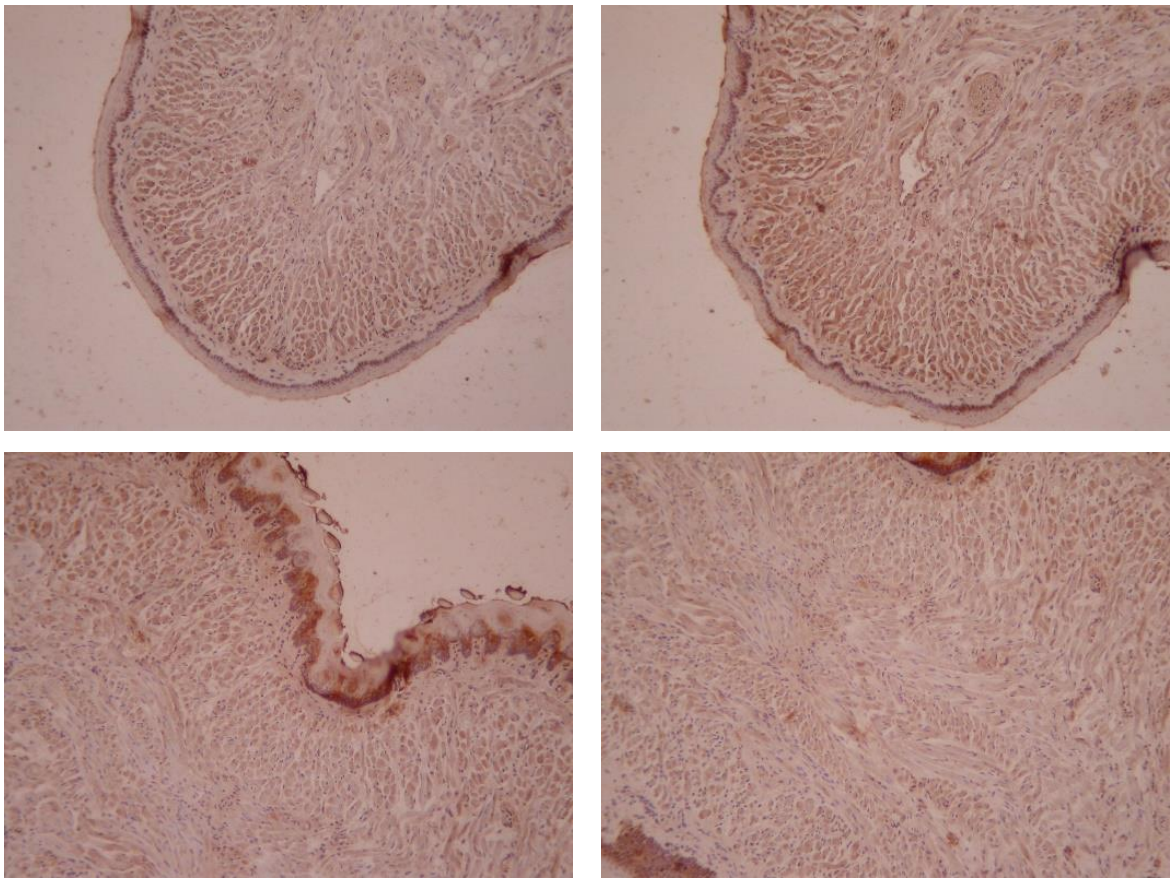


Figure 9.40 Images show the staining in mice tongues exposed to smoke from 100 mg of heroin for 30 seconds (Tongue 2 of 2) (magnification x10)

When examining the tissue sections which were exposed to heroin smoke at a magnification of x 63, it was observed that the muscles bundles were fully penetrated (Figure 9.41). However, staining was weak, indicating that little drug was present within the bundles.

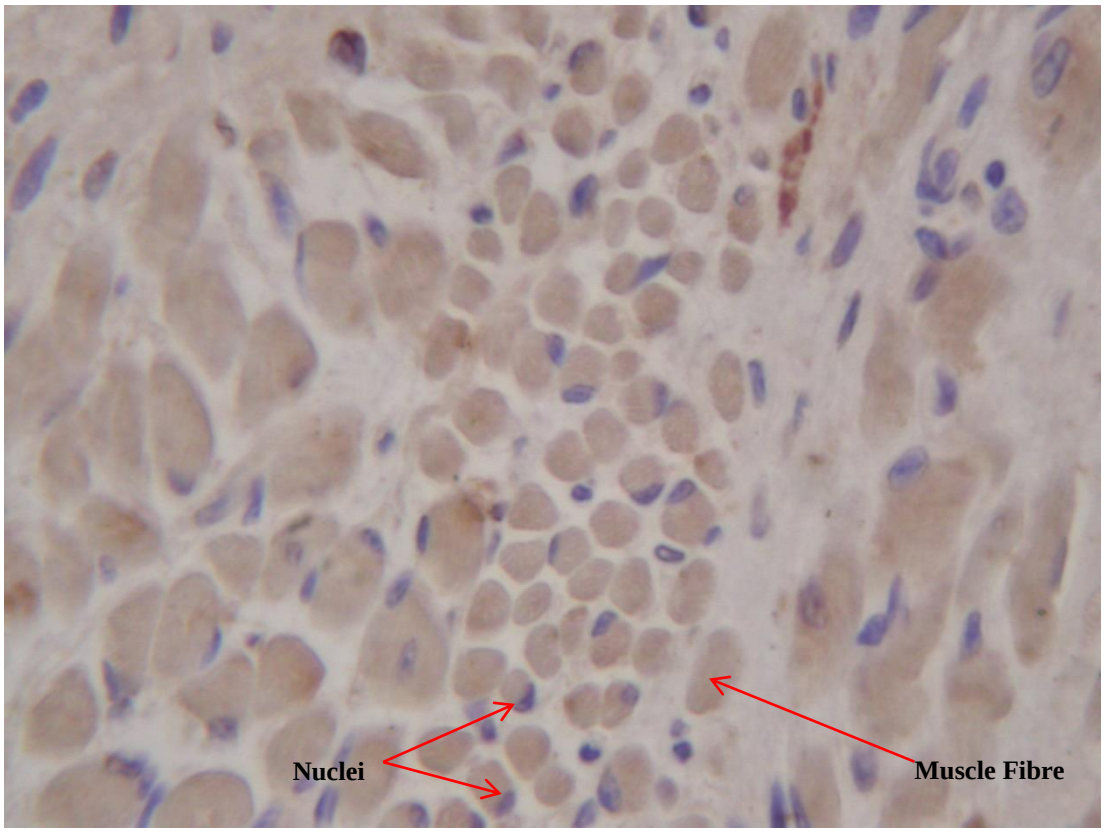


Figure 9.41. Image showing the staining in mice tongues following the expose to smoke from a 100 mg dose of heroin (magnification x63)

Tongues exposed to smoke from a 100 mg dose of heroin for 60 seconds showed a greater density of staining in comparison to tongues exposed for 30 seconds (Figure 9.42, Figure 9.43). Staining was observed throughout the entire cross section of the tongue.

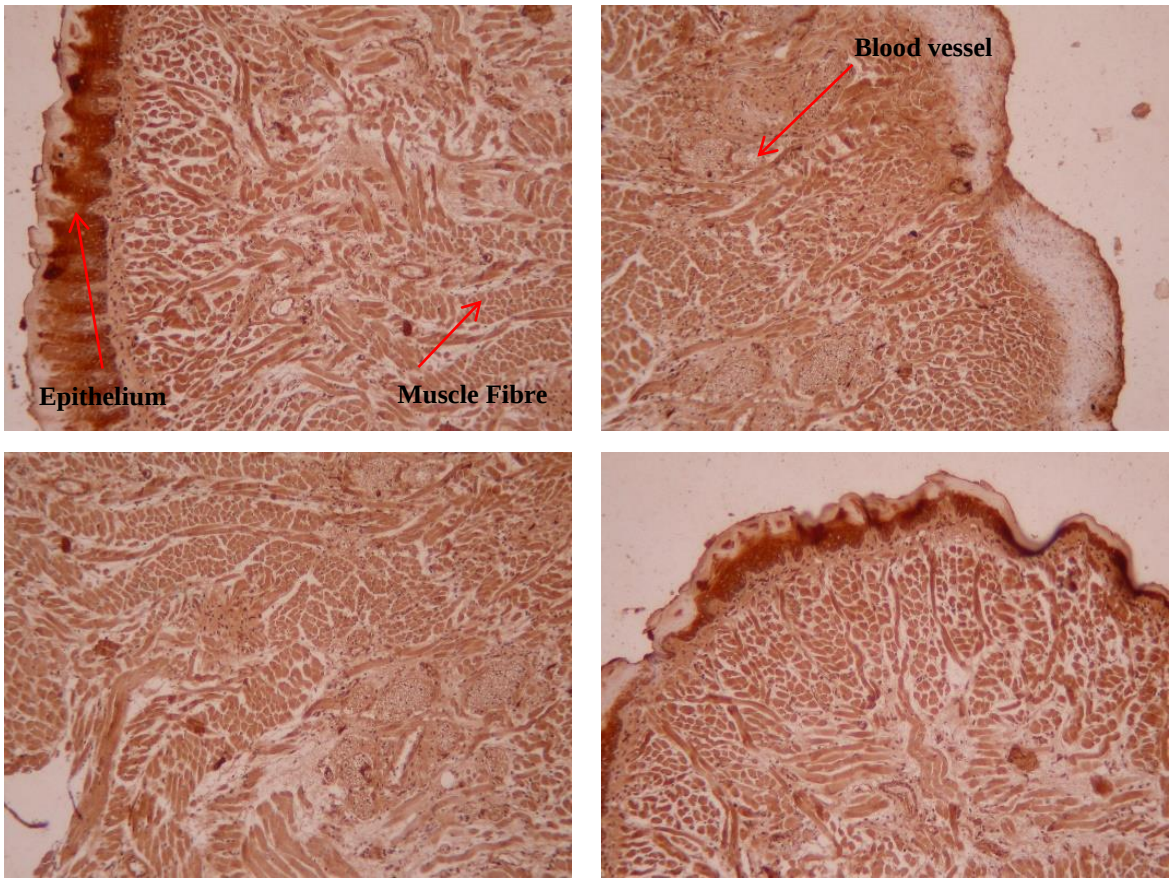


Figure 9.42 Images show the staining in mice tongues exposed to a dose of 100 mg heroin smoke for 60 seconds (Tongue 1 of 2) (magnification x10)

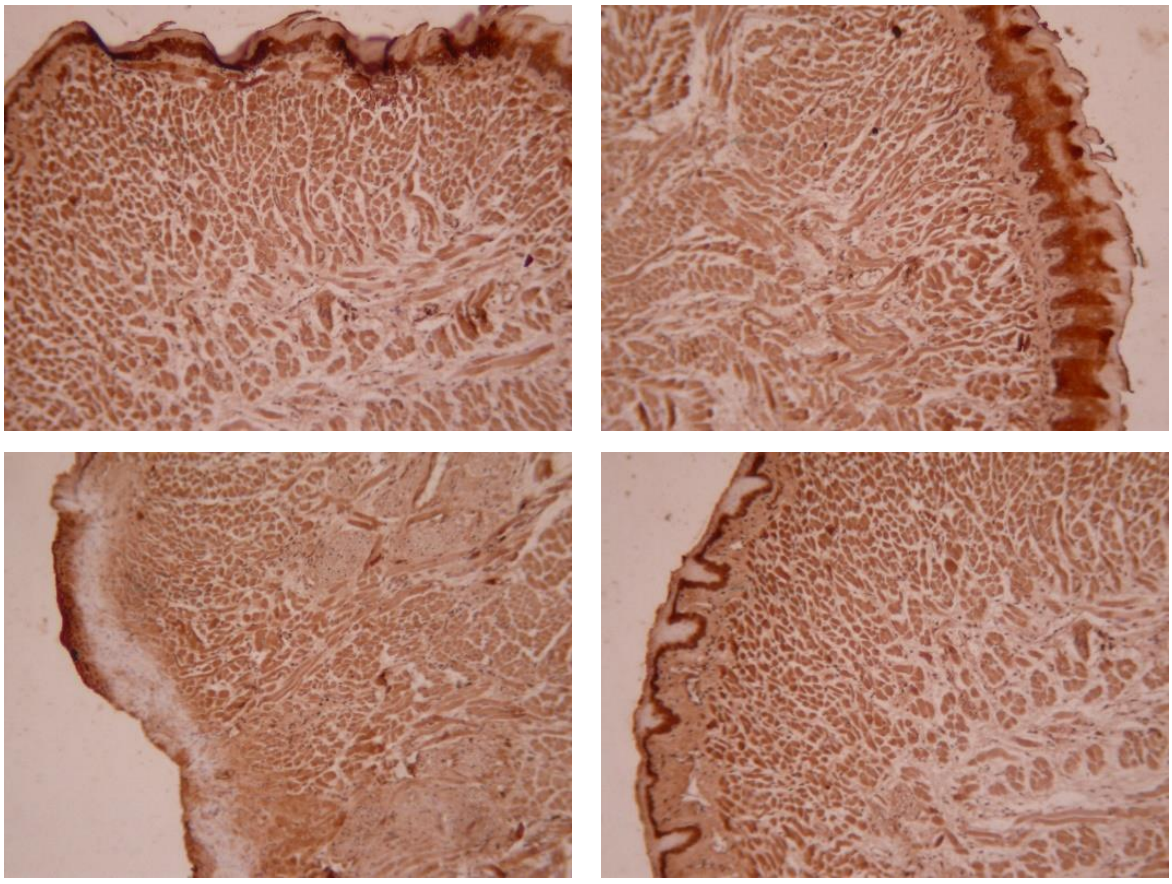


Figure 9.43 Images show the staining in mice tongues exposed to a dose of 100 mg heroin smoke for 60 seconds (Tongue 2 of 2) (magnification x10)

When visualising the sections at a magnification of x 63, staining was intense and muscle bundles were fully penetrated. This suggested that the significant amount of drug travelled into the muscle bundles (Figure 9.44). The staining pattern indicates that drug depots can be formed within tissue and that dose and exposure time can contribute to the amount of drug incorporation into tissue.

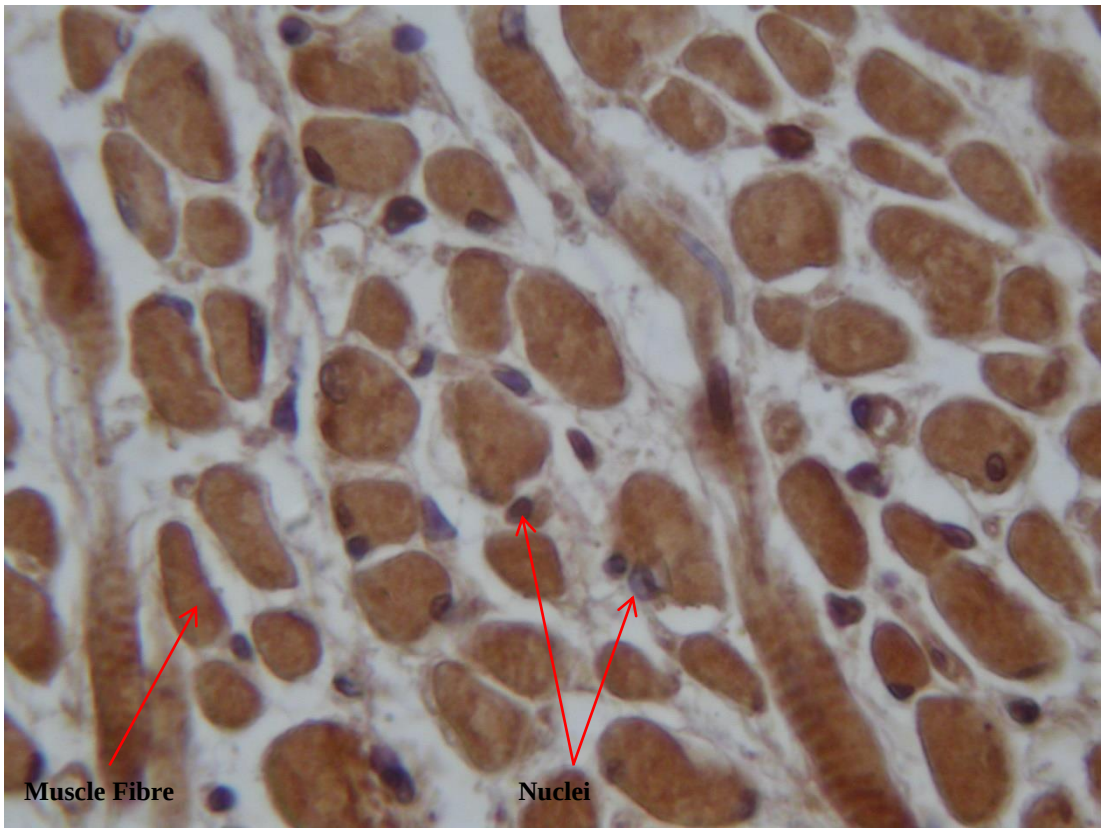


Figure 9.44. Image showing the staining in mice tongues following the exposure to smoke from a 100 mg dose of heroin for 60 seconds (magnification x63)

When the tongues were exposed to smoke from heating 100 mg of heroin for 60 seconds, the epithelium was fully saturated with drug and in comparison to mice which were injected with morphine, no negative staining was observed on the inside of the epithelium (Figure 9.45).

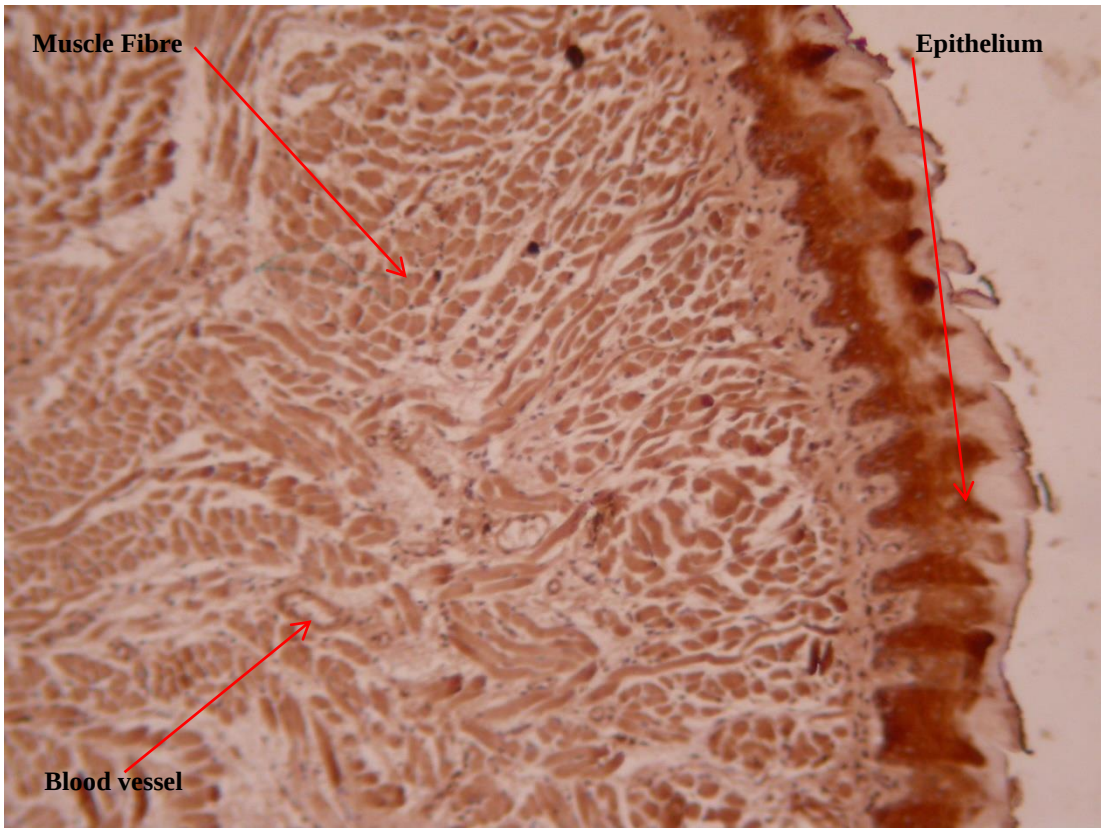


Figure 9.45. Image showing the staining in epithelium of mice tongues following the exposure to smoke from a 100 mg dose of heroin for 60 seconds (magnification x25)

Tissue exposed to smoke under conditions that simulated smoking showed that heroin can fully penetrate mice tongues. Tongues exposed for 30 seconds only showed weak staining whereas tongues exposed for 60 seconds showed intense staining. The intensity of staining following smoking of heroin was similar to that of mice injected with a high dose of morphine (Figure 9.46).

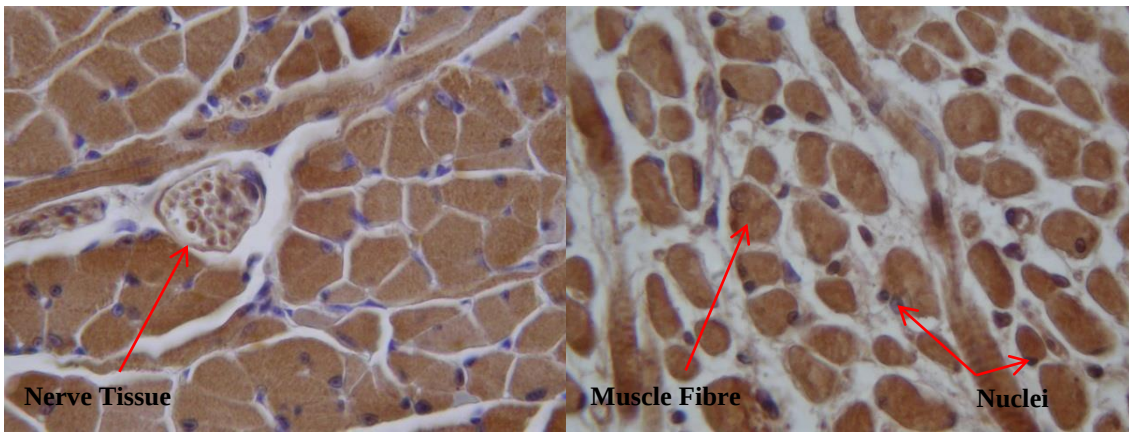


Figure 9.46. Images comparing staining in a stained tongue section from mice injected with a high dose of morphine (9 mg/mL) (left image) and a low dose of heroin smoke (100 mg) (right image) (magnification x63)

Morphine has a volume of distribution of between 3 - 5 L/kg. Heroin is more lipophilic than cocaine resulting in a much higher estimated volume of distribution of heroin 25 L/kg (Moffat et al 2011, Urso et al, 2012). The higher the volume of distribution the larger the amount of drug which travels into the tissue offering an explanation why a low dose of smoked heroin resulted in similar staining to a high injected dose of morphine.

9.4 Conclusion

The research described in this chapter showed that the proposition tested in the *in vitro* studies in chapter 7 and chapter 8 is also applicable in this in *vivo study*. Morphine administered to mice via peritoneal injection at concentrations of 3 mg/mL and 9 mg/mL was demonstrated to enter into tongue and salivary gland tissue. In comparison to porcine tongues exposed to external contamination *in vitro*, drug was detected in the walls of the blood vessel suggesting it diffuses from the blood into the tissue. The presence of drug was shown in all tongues as well as in the parotid, submandibular and submaxillary gland. The presence of morphine in the excretion ducts of the salivary glands, strongly supports a hypothesis that drug can enter the

salivary glands and be excreted simultaneously to saliva. This is a major source for oral fluid drug concentrations that do not rely on oral contamination such as intravenous, intramuscular, trans-dermal and suppository.

Chapter 10.0 - Discussion and Conclusion

Oral fluid is gaining popularity as a matrix for drug testing as it is relatively simple and quick to collect and is non-invasive. Testing procedures for on-site and laboratory screening are inexpensive and no specially trained personnel are required for the collection of oral fluid.

Oral fluid sampling is commonly used in the workplace, hospitals and drug treatment programmes. Recently, countries such as Germany, Belgium, Holland, France, Spain, Italy and Australia have adopted oral fluid sampling at the roadside as a preliminary drug screening method. In the United Kingdom, the Road Traffic Act 1988, Section 5A was amended to allow police officers to employ roadside testing devices although this requires devices to have met Type Approval (Department for Transport 2014). The first device for testing within police stations, the Draeger Drug Test 5000, was approved in December 2012 for tetrahydrocannabinol (THC) only (Home Office 2013). The implementation of a point of care (PoC) testing system enables an inexpensive and rapid way to obtain a preliminary result at the roadside. The UK government is now planning to introduce oral fluid collection on the roadside as an initial screening by the autumn 2014, later followed by evidential testing on a blood sample. Whilst PoC testing systems are not suitable for evidential drug testing, they can act as an aid for law enforcement, supporting a Police Officer's Drug Recognition test. In March 2014 the UK Department for Transport approved and published drug driving limits for 16 drugs including 8 illicit drugs and 8 prescription drugs in blood. The new drug driving limits means that it is an offence to drive over the recommended limit for each drug rather than relying on police officers to prove impairment due to a drug in order to achieve a conviction

(Department of Transport 2014). The embryonic issue facing the UK system is the assumption that a drug positive oral fluid screen will always result in a drug positive blood sample. Type approval for roadside screening for oral fluid cocaine has been set at 30 ng/mL whilst the recommended threshold limit in blood (without alcohol present) is 80 ng/mL (Home Office 2012). The saliva:plasma concentration ratio for cocaine is generally accepted to be 3 which would indicate that using blood as a confirmation method, would need a threshold of 90 ng/mL or an oral fluid cut off at 240 ng/mL. The work presented in this thesis has shown the potential contribution of drug from tissues - depending on route of administration, this will contribute to a better understanding in modifying these cut offs. In addition to roadside drug testing, the potential build-up of drugs in the oral tissue can affect workplace drug testing as well as testing as part of drug treatment centres. The vast majority of oral fluid testing is carried out for two reasons: a) under legislation that forbids the presence of drugs or b) as a check for drug compliance or abstinence. For both of these processes, it is not relevant where the drug has come from, just that the drug is present.

Oral fluid drug testing is based on three main processes i.e. 1) oral fluid collection, 2) immunoassay screening and 3) confirmatory analysis of immunoassay positive samples. For oral fluid collection, numerous collection systems are commercially available. One of the questions raised in this thesis was whether the testing systems are affected by common foods, beverages and oral hygiene products and whether those can result in false results during the testing process (Chapter 3). Most manufactures recommend that the effect of foods and beverages have dispersed from the mouth after 10 -15 minutes following exposure and that after this time an oral

fluid sample can be collected with a minimum risk of contamination. These effects were investigated on the Orasure Intercept and Alere™ Certus collection devices.

The Orasure Intercept device yielded several positive results in combination with its matched immunoassay screen (the Orasure Microplate system), which were confirmed to be false positives when analysed by GC-MS. Coffee, Coca Cola, fruit juice, oranges, spicy food and toothpaste were all capable of interfering with the Orasure testing system, whereas no interferences were observed with the Alere™ Certus collector and associated immunoassay screen. The Orasure false positives appeared immediately post consumption and no positives were observed 10 minutes post consumption of each substance (Table 3.4). Therefore, when adhering to the manufacturing guidelines most of the substances tested should not interfere with the oral fluid drug test systems when collected 10 - 15 minutes post consumption/exposure.

This general guideline was invalidated when vinegar was tested. Vinegar resulted in numerous false positives in samples collected with the Orasure Intercept device for up to 30 minutes post exposure to malt, distilled, balsamic, red and white wine vinegars. Vinegar was shown to interfere with the Orasure Microplate testing system for methamfetamine, amfetamine and cocaine. No false positives were observed when oral fluid was analysed on the methadone or opiate assays. Although no false positives were observed after 30 minutes post exposure, the percentage binding was still shown to be significantly depressed for methamfetamine, amfetamine and cocaine for up 75 minutes post exposure. Similarly, although no opiate and methadone positive were observed on the Orasure Microplate testing system, a depression of the percentage binding of up to 50% could still be observed for

samples up to 75 minutes post collection (Table 3.5). Although results were all still above the recommended manufacturer's cut off for percentage binding, depressed binding can still affect the concentration detected and hence also interfere with the interpretation of the results. Vinegar was the most acidic of the substance tested and it is possible that the buffer capacity of the Orasure device is too low as the Orasure device yielded a much larger dilution factor range with the largest being 1 in 81 and the lowest 1 in 1.5. This indicated that the pH of the oral fluid samples can interfere with the Orasure Intercept system and hence affect the interpretation of results. Therefore, for the purpose of investigating drug depots during this thesis, the Alere™ Certus collector were used as it did not yield any false positives following the exposure to foods, beverages and oral hygiene products.

Elevated drug concentrations have been reported in combination with contamination of the mouth as a result of the exposure of drugs to the oral cavity during consumption. Literature information is limited on elevated drug concentrations as a result of drug depots and length of detection window. During the investigation into concentrations of cocaine and its metabolites in oral fluid following consumption of coca tea, concentrations for cocaine and its metabolites were above a recommended SAMHSA cut off (8 ng/mL) and were observed for up to 60 minutes post consumption of coca tea (Table 4.3, Table 4.4, Table 4.7). The detection of elevated concentrations for cocaine and its derivatives over the course of an hour supports the proposition that drug depots can be formed within mouth tissues. In addition to cocaine, benzoylecgonine and ecgonine methyl ester, cocaethylene and the pyrolysis product anhydroecgonine methyl ester were also identified and quantified following the consumption of coca tea. Cocaethylene is only formed when cocaine and ethanol are consumed simultaneously (Table 4.9, Table 4.10).

The presence of cocaethylene in oral fluid samples collected over the course of one hour following the consumption of coca tea was randomly distributed and therefore could not be explained by alcohol consumption (Table 4.9). Samples were therefore reanalysed one year post collection of coca tea oral fluid samples. During the second analysis, no cocaethylene positives were observed in any of the oral fluid samples collected. This could be explained by a potential instability of cocaethylene in oral fluid. Studies in the literature only report stability in oral fluid at -20 °C for up to 6 months (Clauewaert et al 2004). Stability of cocaethylene in oral fluid for a longer time period has not been reported in the literature and should be researched further. A secondary cause for the presence of cocaethylene could be the presence of esterases in oral fluid such as cholinesterases, pseudocholisterase and cholesterol esterase. Cocaethylene can be formed by the transesterfication of cocaine and hence has the potential to be formed by the esterases present in oral fluid. However, although the presence of esterases in oral fluid has been reported in the literature, the effects on cocaine and its potential formation to cocaethylene have not yet been reported.

In addition to cocaethylene, anhydroecgonine methyl ester was also quantified during the investigation into concentrations of cocaine and its derivatives in oral fluid following the consumption of coca tea (Table 4.10). Anhydroecgonine methyl ester is a pyrolysis product of smoking crack cocaine as it is formed by the thermal degradation of cocaine. Although it has been reported to be present in some species of the *Erythroxylum coca,* similarly to cocaethylene, the presence of anhydroecgonine methyl ester positives was randomly distributed throughout collected samples. This indicated that positive samples did not result from the presence of anhydroecgonine methyl ester within the coca leaf as the results would

be less random and instead be detected throughout each volunteers 60 minute collection timeline. Additionally, the production of anhydroecgonine methyl ester as a result of thermal degradation of cocaine by boiling of the leaf during preparation of coca tea could also be disregarded as a cause for the presence of anhydroecgonine methyl ester in oral fluid samples. If anhydroecgonine methyl ester was produced as an artefact throughout the production of coca tea, again the positives would have been expected to appear at a less random pattern.

When samples were reanalysed one year post collection of the oral fluid samples, the amount of anhydroecgonine methyl ester positives increased significantly (Table 4.11). This indicated that anhydroecgonine methyl ester was formed during storage of oral fluid samples which were collected following the consumption of coca tea. There is no information available on the stability of anhydroecgonine methyl ester in oral fluid neither is there any information on the stability of coca tea. Therefore more research should be undertaken to investigate the formation of anhydroecgonine methyl ester and its stability further.

The investigation into concentrations of cocaine and its derivatives strongly supported the hypothesis that drugs consumed orally either via solution (tea), smoked or snorted can result in elevated concentration of drug in oral fluid. This information needs to be kept in mind depending on the application to which the result is being applied. In order to further research the effects of oral deposition of drugs and the effects it has on its subsequent collection and analysis of oral fluid, research was undertaken to investigate the concentrations of opiates following swirling of codeine linctus and Collis Browne's solution. In the work undertaken for this thesis, high concentrations of opiates were observed in oral fluid following the swirling of either

of the two solutions. Both, Codeine Linctus and Collis Browne's solution were discarded from the mouth following swirling (without swallowing) and therefore concentrations can be explained by oral contamination rather than originating from blood to saliva gland transfer. Elevated concentrations were observed for up to 3 hours post exposure to either of the both solution. Previous studies in the literature have reported oral deposition to affect the correlation between saliva and blood concentrations for 30 to 60 minutes post consumption (Bosker and Huestis 2009). The research undertaken in this thesis clearly indicates that drugs which are exposed to the oral cavity as a solution have the potential to contribute to oral fluid drug levels for up to three hours post exposure (Table 5.2, Table 5.3).

Additionally, results indicated that a large amount of cavities can increase the deposition through when drugs are consumed orally via solution. The principle of contamination suggests that the drug solution can linger in the cavities within the mouth in addition to the increased presence of drug in the oral mucosa. Only one volunteer who participated in the Collis Browne's / Codeine Linctus exposure study had reported to have a large amount of dental work and cavities. A large increase in opiate concentration, in comparison to volunteers with less dental work, was observed in samples collected from this volunteer. Although the validity of data from one volunteer being different to that from the other subjects is questionable, results suggest that increased oral fluid concentrations can be observed following exposure to a drug solution especially in volunteers with large amounts of cavities. This strongly supported the hypothesis that drugs can accumulate in oral tissues, however the effects of such a build-up of drug on the oral fluid collection has not yet been widely investigated. When oral fluid is collected, the collection device is placed in the mouth according to each manufacturer's instructions. In addition to trace

amounts of lipids and proteins, oral fluid contains food residues and cellular debris. Drug depots in oral tissue could lead to an increased amount of drug within the cellular debris and hence result in elevated drug concentrations in oral fluid. However, when oral fluid collectors were scraped firmly around the oral cavity during oral fluid collection following exposure to Collis Browne's mixture or Codeine Linctus, no significant increase in opiate concentration were observed in comparison to samples which were collected according to the manufacturer's collection instructions (Figure 5.6).

The investigation of elevated drug concentrations in oral fluid following administration via snorted, smoked or oral routes has the potential to accumulate drugs in mouth tissues. Elevated drug concentrations can be observed following the consumption of coca tea (Chapter 4) and Codeine Linctus and Collis Browne's (Chapter 5). Both studies strongly support the hypothesis that oral deposition can occur when drugs are consumed orally i.e. via solution.

In addition to deposition by external drug consumption, the hypothesis that microbleeding of the gums could contaminate the oral fluid was raised in this thesis. As a result of the microbleeding, drug may be released into oral fluid and potentially interfere with the interpretation of oral fluid drug concentrations. (Chapter 6). During the investigation into the effects of microbleeding on oral fluid drug detection, transferrin was utilised as a marker for microbleeding. The study looked to determine whether increased microbleeding correlates to elevated drug concentrations. Transferrin is a large blood glycoprotein (molecular weight 80,000) that transports iron. It would not normally be present in oral fluid, (trace amounts) and hence is a useful marker for blood presence in saliva.

The research undertaken showed that there was no relationship between transferrin concentration and drug concentrations. Additionally, microbleeding did not result in an immediate increase in oral fluid drug concentration when bleeding was stimulated following the consumption of 15 mg of codeine. These results can be explained when considering that only minute amounts of drug would leak into oral fluid during microbleeding. Assuming that the average microbleed is between 5 µL to 20 µL, the quantity of drug entering into oral fluid would be minimal in comparison to the concentration detected in oral fluid following codeine consumption. When applying the average S/P ratio of 3.32 to the average plasma concentration reported following a dose of 30 mg/mL, it results in the quantity of drug being released into the oral fluid to range between 0.17 ng and 0.68 ng (Scharp et al 1983, Schramm et al 1992, Cone 1993, O'Neal et al 1999, Kim et al 2002). This amount is insignificant when considering the much higher concentration of 250 ng/mL of opiates at three hours post consumption on one 15 mg dose of codeine. Therefore when considering both the results from the drug clinics and the results from the controlled codeine exposure study, microbleeding can be excluded to be a contributing factor to elevated drug concentrations.

The work undertaken in chapters 4 and 5 demonstrated that drugs which can be consumed orally, i.e. coca tea, Collis Browne's solution or Codeine Linctus can contribute to the oral fluid concentration and hence influence the interpretation of results. With elevated concentrations reported for up to one hour following the consumption of coca tea and four hours following the consumption of Codeine Linctus or Collis Browne's, the question of how deep drugs can penetrate into the tissue and whether there are subsequently released over time was investigated.

The work undertaken in this thesis successfully showed the development of an immunohistochemical method for the detection and visualisation of cocaine, heroin and their metabolites. The immunohistochemical method utilised an antigen-antibody detection system and was successfully developed for cocaine and heroin in porcine and mouse tissue. The immunohistochemical method (Chapter 6) was applied to visualise drugs in *in vitro* and *in vivo* tissue. Porcine tongues exposed to a range of concentrations of cocaine or heroin showed that the presence of drug was observed following tissue exposed to concentrations ranging between 100 ng/mL and 10 µg/mL. The immunohistochemistry revealed that the degree of drug entry was concentration dependent. Staining in tongues which were exposed to cocaine was weaker in intensity in comparison to that of heroin. Heroin is more lipophilic than cocaine resulting in a much higher estimated volume of distribution of heroin (~ 60 – 100 L/kg) in comparison to the volume of distribution of cocaine (~ 1 – 3 L/kg) (Moffat et al 2011, Urso et al, 2012). The higher the volume of distribution, the more the drug which travels into the tissue, which is clearly supported by the result of the immunohistochemical staining. This is further supported when comparing the logP value for cocaine and heroin. Heroin has a much lower logP value suggesting that the drug distributes more freely into the tissue than cocaine.

No staining was observed in the blood vessel walls of the treated porcine tongue sections following external exposure to cocaine or heroin solution indicating that the drugs do not cross the blood vessels walls and hence would not elevate blood concentrations. Of note was the staining around the epithelium in porcine tongue tissue. The epithelium showed positive staining on the outside layer. However the inside of the epithelium (closest to the muscle) the staining appeared to be negative. This is yet to be explained but the hypothesis is that this is a result to the epitheliums

fast rate of repair and renewal of tissue in response to abrasions in the mouth which results in a cluster of nuclei around the inside layer of the epithelium. A high concentration of nuclei in close proximity to each other resulted in a negative appearance for cocaine or heroin.

The presence of cocaine and heroin in tissue suggests that they have the ability to enter into and subsequently bind to tissue to form depots which can subsequently be released over time. This also indicates that drugs can enter the oral fluid by mechanisms other than via the surrounding blood.

In addition to immunohistochemical analysis, the artificial saliva washes and homogenates of tongues were analysed using LC-MS. Positive results of artificial saliva washes from tongues exposed to cocaine concentrations showed that the presence and concentration of cocaine and its metabolite benzoylecgonine increased with increasing concentration. No cocaine or benzoylecgonine were detected in washes from tongues which were exposed to the lowest concentration of 100 ng/mL of cocaine. With the exception of the highest exposure dose of 10 µg/mL of cocaine, washes were negative by the last wash, suggesting that all external contamination had been removed. When relating the analytical results to the immunohistochemical staining, it was noted that although no drug was detected during the wash steps, significant staining was still observed in tongues. The positive staining suggested, that cocaine had entered and bound to the tongue tissue to form a depot and could subsequently be released over time.

In comparison to cocaine, heroin was not detected throughout the washes and 6-MAM and morphine were only detected in washes 1 and 2 following exposure to 1000 ng/mL or 10 µg/mL. In comparison to the analytical results, immunohistochemical results of tongues exposed to heroin solutions showed

significant staining in all tongue tissue. This indicated that although no drug is being released during the wash steps, drug is still present within the tissue and has hence bound to the oral tissue from where it subsequently could be released over time. Tongues were washed during the work undertaken in this chapter as in the mouth saliva is constantly renewed and oral tissue washed with new fresh saliva at an average rate of 0.5 mL/min to 1 mL/min. The results support the hypothesis that some drug had loosely bound to the tongue surface which was gently and progressively removed, potentially leaving significant binding and transfer to the tissue itself.

Furthermore, a section from each tongue was removed at the end of the seventh wash step for homogenisation and analysis using LC-MS. The transverse cross section was divided into five parts (Chapter 7). Cocaine and benzoylecgonine were detected in all homogenates from tongues which were exposed to cocaine in artificial saliva. The observed concentrations were highest in the S1, S2, S4 and S5, which made up the outside of the tongue transverse cross section. Lowest concentrations for cocaine and benzoylecgonine were observed in section 3, which was removed from the centre of the tongue indicating that although the drug penetrated the tissue section to the centre of the tongue, less drug reached the centre than on the external sections of the tongue.

Homogenates from tongues exposed to heroin showed that morphine and 6-MAM concentrations were detected in all sections removed from tongues. Similarly to cocaine, morphine and 6-MAM concentrations observed were lowest in section 3 which was removed from the centre of the tongue. Concentrations of morphine and 6-MAM observed in tissue were higher in comparison to cocaine and benzoylecgonine concentrations which further supported the hypothesis that heroin

penetrates the tissue easier than cocaine in addition to the more intense staining observed following exposure to heroin.

Following completion of wash steps pre-wetted Alere^{TM} Certus oral fluid collectors were brushed against the tongue to identify whether the contact between collector and tongue tissue could result in a contamination of oral fluid by extraction drug from the mouth tissue.

When collectors were brushed against tongues exposed to cocaine, it was observed that cocaine and benzoylecgonine were detected in samples collectors with the Alere^{TM} Certus collector. The presence of cocaine and benzoylecgonine in samples collected using a pre-wetted Alere^{TM} Certus collector following seven washes after the original exposure to various concentrations of cocaine solution, indicated that the brushing the collector against the tongue can potentially extract drug from the oral tissue. The seventh wash was negative for cocaine and benzoylecgonine when exposed to cocaine spiked into artificial saliva, with the exception of the 10 µg/mL dose, indicating that all excess external contamination of cocaine had been washed off by wash seven. Therefore the presence of cocaine and benzoylecgonine in samples collected with the Certus collector, are not a direct result of external contamination, but the drug must have been released from the oral tissue.

In contrast, heroin was not detected in samples collected with the Alere^{TM} Certus device. The absence of heroin, morphine and 6-MAM in collection devices suggest that the collector does not extract either of the compounds from the oral tissue. This further supports that heroin binds tightly to the tissue, as immunohistochemical staining resulted in intensive staining throughout the tissue. The lack of drug when swiping the collector against the tongue, also supports the results in chapter 5, where no change in drug concentrations were observed following scraping of the Alere^{TM}

Certus device against the tongue following consumption of Codeine Linctus or Collis Browne's solution. Results from chapter 5 further indicated that opiates such as morphine and codeine can bind to oral tissue and subsequently be released over time.

In a separate study the release of drugs from tongue tissue was investigated. Tongues were exposed to either 100 ng/mL or 1000 ng/mL of cocaine or heroin and then washed for either 1 hour, 6 hours, 24 hours or 48 hours in artificial saliva. Immunohistochemical results of the transverse cross sections of the tongue showed that following cocaine exposure, specific staining was still observed 48 hours post exposure. Although staining weakened with prolonged washing, significant staining was still observed after washing 48 hours. Additionally, when homogenate sections were analysed, it was observed that concentrations of cocaine and benzoylecgonine decreased with prolonged washing time and 48 hours post exposure, only Benzoylecgonine were detected.

Similarly, following heroin exposure, as with the initial exposure study, the staining observed following immunohistochemical staining, was more intense in comparison to cocaine. Analysis from homogenates showed that concentrations of morphine and 6-MAM were higher than cocaine and benzoylecgonine which hence further supported the hypothesis that following the exposure to heroin, drug enters the tissue more easily. Positives for morphine and 6-MAM were still detected following 48 hours of washing which indicated that the drug binds tightly to the tissue and can subsequently be released over time.

Additionally, in a separate study, porcine tongues were exposed to smoke from a dose of 100 mg or 200 mg of either crack cocaine or street heroin and stained using the same immunohistochemical methods. Results showed, that drug was present in all of the tongues exposed to drug via the smoking route. Similar to the porcine

tongues exposed to drug solutions, porcine tongues showed a difference in intensity depending on whether they were exposed to crack cocaine or street heroin. This further supports that heroin penetrates the tissue more than cocaine does as a result of its lipophilicity.

Cocaine was only present in the connective tissue following exposure to smoke from a 100 mg dose of crack cocaine and did not penetrate the muscle bundles at all. At a higher dose, cocaine began to surround the muscle bundles and was seen to start to enter the muscle bundles. In comparison, heroin started entering the muscle bundles at a lower dose of 100 mg and had nearly fully penetrated the muscle bundles at the higher dose of 200 mg.

Blood vessel walls also did not show any positive staining in concurrence with porcine tongues exposed to a drug solution rather than drug smoke. The same pattern of staining in the epithelium was observed with the inner layer of the epithelium showing no positive staining whereas the outside layer was positively stained.

The presence of drugs in tissue following exposure to smoke from either crack cocaine or street heroin, further supports the hypothesis that drugs with are consumed by either snorting, smoking or solution have the potential to form depots in tissue which can subsequently be released from the tissue over time.

In-vitro studies in porcine tissues gave promising results for the visualisation of drugs in oral tissue. However, the challenge with any *in vitro* study is to determine how this would apply to an *in vivo* study. Therefore an *in vivo* was designed in order to test the immunohistochemical staining technique and identify whether morphine could be visualised in oral tissue such as tongue and salivary glands after injection in mice.

Mice were injected with either a low (3 mg/mL) or a high dose of morphine (9 mg/mL) and humanely killed 30 minutes post injection. Staining was observed tongues for both low and high concentrations of morphine. Staining was observed in the connective tissue, muscle bundles and epithelium although no difference was observed between the centre of the tongue and the outside of the tongue indicating that morphine was evenly distributed throughout the tongue. Both injection doses resulted in positive staining of epithelium. However in the inside layer (closest to muscle) a negative stain was observed indicating that this was negative whereas the outside layer of the epithelium was specifically stained. Similar to the in-vitro study this could be explained by the epitheliums rate of repair and renewal of tissue in response to abrasion in the mouth.

As mice were exposed to morphine via injection rather than oral ingestion, no external oral contamination occurred. Therefore an additional difference observed between *in vitro* porcine tongues, which were exposed to solution, and *in vivo* mice tongues, which were from mice injected with morphine, was that the specific staining was observed in the blood vessel walls indicating that the drug travelled from the blood through the blood vessel wall into the tissue.

Overall the specific staining in tongues for mice which were injected with the higher dose of morphine (9 mg/mL) was more intense in colour than the staining observed in following an injection with a lower dose (3 mg/mL) suggesting that more drug has entered the tongue tissue.

In addition to the tongues, salivary glands were removed from mice which were injected with a saline control, low concentration or high concentration of morphine. Mice, like humans have three salivary glands – the sublingual gland, parotid gland and submaxillary gland. All three glands are located in clusters below fat tissues and

submandibular lymph nodes. Salivary glands were removed 30 minutes post injection of morphine and fixed immediately for immunohistochemical staining. Immunohistochemical analysis confirmed that all three salivary glands managed to be collected during the procedure. Following application of an immunohistochemical staining method that utilised a morphine-3-glururonide antibody, it was visually observed that specific staining appeared to be present in all three salivary glands. Specific staining did not occur in the acini of the glands, but was only detected in the salivary ducts of the gland as well as the blood vessels. All saline controls, were negative for drug staining.

Although staining was present in salivary glands of both groups, from mice injected with low or high concentrations of morphine, staining was more intense when mice were injected with a higher dose of 9 mg/mL indicating that more morphine has travelled into the structures of the salivary gland. This further supports the hypothesis that the larger the dose of the drug consumed, the greater is the potential that drug depots can form within the oral tissue.

Drug was also visualised in the blood vessels / capillaries confirming that the drug travels across the blood vessel wall into the salivary gland. The presence of morphine in the salivary ducts strongly support the hypothesis that drug is excreted simultaneously to the excretion of saliva into the oral cavity hence leading to a contamination of drug and hence potentially contributing to the elevated drug concentrations observed in oral fluid. Although, similar to the acini, the salivary duct consist of epithelium, the epithelium is the thinnest layer of epithelium present in the salivary gland and hence allowing an easy transfer across the epithelium.

To sum up, the research described in this thesis enhanced the understanding of the factors and processes concerning the depositions of drugs in oral tissue and oral

fluid. Results of this thesis showed that elevated drug concentrations were observed where drugs are consumed via oral routes such as solution or smoking. Immunohistochemical staining in combination with analytical analysis demonstrated that drugs such as cocaine and opioids can bind to oral tissue and be subsequently released over time and hence have to potential to interfere with the interpretation of drug concentrations in oral fluid. This is not an issue under legislation that forbids the presence of drugs or as a check for drug compliance or abstinence as for these it is not relevant where the drug has come from, just that the drug is present. However, the formation of drug depots and the subsequent slow release of drugs from tissue must be considered in relation to the interpretation of results where cut off concentrations are used to determine whether a person is positive or negative in an oral fluid drug test.

Chapter 11.0 - Limitations

Table 11.1 Summary of limitations to the work undertaken in this research

Limitation:	**Comment:**
Due to time constraints, the work undertaken in Chapter 3 focussed solely on the potential that foods and adulterants can affect the immunoassay screening system and subsequently cause false positives	A secondary study to investigate the potential of food and adulterants to cause false negatives would have been beneficial as drug users often try to adulterate samples with substances such as lemon juice, tea, milk or vinegar in order to mask a positive result in the drug test. However, due to time constraints for the research undertaken, it was not possible to investigate this further.
Additional samples of coca tea could not be collected due to limitation of time during this research and lack of collaborators within Peru. Additionally, it was not possible to import coca tea leaves due to the lack of a Home Office Import licence (Chapter 4).	Samples were only collected for one hour post consumption of coca tea. Results showed that coca tea samples were positive for cocaine and its derivatives for up to 60 minutes. Therefore it would have been beneficial to repeat the collection process in Peru and collect sample for a longer time period in order to determine the time point at which concentrations dropped below the SAMHSA cut off for cocaine and its derivatives. Additionally the presence of anhydroecgonine methyl ester and cocaethylene has yet to be explained fully. However, due to a lack of a Home Office Import licence it was not possible to import coca tea leaves within the UK and hence prepare the coca tea

Limitation:	**Comment:**
	in the same manner than when samples were originally collected in Peru.
Mass spectra were unobtainable for results of cocaine and its derivatives detected in oral fluid following a consumption of coca tea	Mass spectra were unobtainable due to loss of data following a computer fault.
Limited information available during the analysis of the coca tea which was prepared from a ready-to-use teabag that contained ground coca tea leaves as well as green tea powder (Chapter 4)	The quantity of coca leaf within the tea bag was unknown and therefore it is difficult to compare the concentrations of cocaine and its metabolites within the tea from the tea bag with those observed in oral fluid following the consumption of coca tea. Additionally it is unknown whether the species of coca leaf was the same in both instances. Some species of the coca plant have been reported to contain anhydroecgonine methyl ester within the leaf which would explain its presence within the tea.
Samples for transferrin analysis were collected by Alere Toxicology as part of their routine analysis before key information could be recorded. Additional information such as route of administration, drug use and drug dose	**Route of administration**: As discussed in other chapters, the contribution to oral fluid drug levels is significant from smoking, intranasal and chewing routes of administration whilst intravenous and intramuscular routes result in higher blood levels compared to oral fluid. It would have been

Limitation:	**Comment:**
would have benefitted with the interpretation of the results (Chapter 6)	advantageous to know the route of administration to further segregate the groups into oral versus injection routes. This would have provided further evidence of whether observed elevated concentrations are related to the route of administration. **Drug Use**: It would have also been beneficial to have known the time of between last drug use and collection of sample. This information would have given some insight as to whether the sample was in early or late elimination phase. In early phase we would expect to see high oral fluid levels resulting from contamination of the mouth cavity whereas in late phase elevated concentrations might be relating to drug deposition within and release from the oral cavity. **Drug dose**: The final missing information in this data set is dose of the drug consumed and drug purity. The missing detail on the dose hinders the correct interpretation of the oral fluid concentrations in relation to the drug consumed. Because of the large number of unknowns associated with the drug clinic data, a study was undertaken

Limitation:	**Comment:**
	involving oral fluid collection under controlled conditions following the consumption of a codeine tablet. (Chapter 6 – Study 2)
The amount of blood released during microbleeding has not been reported in the literature (Chapter 6)	All calculations on the estimated quantity of drug released into oral fluid during microbleeding were based on an estimated volume of microbleeding ranging between a minimum volume of 5 µL and a maximum volume of 20 µL.
Although the development of a method for the use of GMA resin was successfully achieved, the method was deemed unsuitable for the use in any further study due to the small sample size that could be embedded (Chapter 7)	Important features, such as blood vessels and epithelium were destroyed during the removal of the biopsy samples from transverse cross sections. These features were vital to identify whether the drug is able to easily travel across the blood vessel wall and hence contaminate the blood.
The staining could not be quantified as the staining counter-software was not specific enough to identify all the staining during image analysis (Chapter 7, Chapter 8, Chapter 9)	The software is based on a filter by which it quantifies the brown pixels in the image and simultaneously translates them into red pixels which are then identified by the software. However, the identification of the brown pixels is dependent on the light intensity used in the microscope, the magnification employed and intensity of staining. Additionally, the software

Limitation:	**Comment:**
	accounts for staining at low and high intensity, and quantifies both in the same manner hence cannot differentiate between the low and the high drug concentrations. These factors resulted in the quantification method being deemed unsuitable for use.
Porcine tongues varied in size during the work undertaken in Chapter 7 and Chapter 8	Porcine tongues varied in size during this experiment as tongues are proportionate to the size of the animal it was removed from. As tongues were provided directly by the butcher, there was no way to individually selecting each tongue depending on its size. Therefore large variations in concentrations between two tongues exposed to the same drug concentrations could be a direct result of the difference in size between tongues. For any future work, the weight and size of the tongues should be recorded to aid with the interpretation of results.
Tongues were stored frozen prior to treatment and immunohistochemical analysis (Chapter 7, Chapter 8)	The butcher received the tongues from an abattoir and it was therefore not known how long the tongues were stored frozen before the use in the work undertaken in Chapter 7 and Chapter 8. Therefore it was difficult to assess the damage that could have occurred during the freezing and thawing process. Histological analysis however showed that the tissue structure was intact in all tongues, suggesting that tongues were suitable for

Limitation:	**Comment:**
	the use in the work undertaken in those chapters.
Due to time restrictions during this project, no studies were undertaken to investigate the release of drug and its detection time for smoked tongue (Chapter 8)	The presence of drugs, visualised by immunohistochemical staining (Chapter 8) in tissue following smoking also strongly supports the hypothesis that drugs can enter the oral fluid via routes other than via the surrounding blood. Drugs enter the muscle and form depots within the tissue when smoked which indicates that they can linger in the tissue and subsequently be released over time.
The secondary antibody for the use during immunohistochemical staining was a biotinylated rabbit-anti-goat antibody	Although the primary antibody was raised in sheep, the secondary antibody selected was biotinylated rabbit-anti-goat antibody. This anti-goat antibody can generate more non-specific binding than an anti-sheep antibody; however no anti-sheep antibody was available/accessible during this study.
The sample size in Chapter 9 was limited as the study was undertaken in collaboration with the School of Physiology & Pharmacology, University of Bristol.	A larger sample size would allow analysing tongue tissue using LC-MS/MS in keeping with Chapter 7 and Chapter 8, to show some statistical evidence in addition to the visual examination of the sections. Due to time restrictions during this research, it was not possible to repeat the experiments in chapter 9 to obtain additional tongue and salivary gland tissue.

Chapter 12.0 - Future recommendations

12.1 Adulterants and Food contaminants

The work undertaken in this thesis showed that foods such as vinegar have the potential to affect the immunoassay screening system and cause false positives. Future research should be focussed on whether these foods or adulterants can also mask positive results and hence result in false negatives. Drug users have claimed that foods such as lemon juice, vinegar, tea and milk can invalidate an oral fluid drug test (Mikkelsen and Ash 1988) and hence mask a positive test result. This hypothesis should be further tested to follow on from the adulterant and contaminant work undertaken in Chapter 3.

12.2 Microbleeding of the gums

Microbleeding of the gums was shown not to be a contributing factor to elevated drug concentrations following controlled codeine exposure. However, for further research this should be further investigated for drug such as benzodiazepines which are highly protein bound. Benzodiazepines have reported S/P ratios between 0.01 and 0.1 which suggests that any microbleed would significantly add to the oral fluid concentration.

12.3 Oral contamination

The research undertaken in this thesis clearly showed that drugs which have been consumed orally have the ability to contaminate the oral cavity and hence affect the interpretation of oral fluid drug results. Elevated concentrations of cocaine and its metabolites above the recommended SAMHSA cut off were observed for up to one hour post consumption of one single cup of coca tea. Further work should extend the time of collection to identify how long concentrations are reported positive before

dropping below the recommended SAMHSA cut off. Additionally, the work undertaken showed that concentrations of anhydroecgonine methyl ester and cocaethylene following consumption of one cup of coca tea which were detected at random time points throughout the collection timeline. Samples were reanalysed one year post the initial analysis. During the second analysis no cocaethylene was detected and the number of positive samples containing anhydroecgonine methyl ester increased. Possible explanation for the disappearance of cocaethylene positives could be instability of cocaethylene in oral fluid or the presence of esterase enzymes. Only limited information is available in the literature on the stability of cocaethylene in oral fluid, hence it should be further investigated to fully explain the disappearance of cocaethylene from the oral fluid samples.

The repeat analysis one year post the initial analysis resulted in an increased number of samples testing positive for anhydroecgonine methyl ester. This indicates that anhydroecgonine methyl ester was formed within the sample during the one year of storage. The stability of anhydroecgonine methyl ester in oral fluid has not been documented in the literature. Although anhydroecgonine methyl ester has been reported to form through thermal degradation of cocaine as a pyrolysis product of smoking crack cocaine, formation due to storage conditions or instability have not previously been reported in the literature (Jacob et al 1990, Kintz et al 1995, Kintz et al 1997, Toennes 1999). Therefore the formation and stability of anhydroecgonine methyl ester should be further researched in order to identify whether storage conditions can result in the formation of anhydroecgonine methyl ester as a result of instability.

Additionally, during the research undertaken throughout this thesis it was not possible to analyse the coca tea as a result of restrictions for the import of coca tea or

coca tea leaves. Future work to investigate drug concentrations in coca tea would provide essential information of the presence and concentration of constituents. This information could assist with the explanation of the presence of anhydroecgonine methyl ester and the subsequent interpretation of results.

12.4 Drug depots

The work described in this thesis showed the successful development and application of an immunohistochemical technique for visualising cocaine and opiates in tongue tissue and salivary glands. The presence of staining could not be quantified in transverse cross sections of porcine tongue tissue. The results of the application of the DDS staining software showed that staining could be identified but that the quantification of staining was depending on the light intensity used in the microscope, the magnification employed and the intensity of staining. For any future research in this field it would be beneficial to develop a quantification method which is able to account for low and high intensity of staining and quantify accordingly. The quantification of staining would enhance our understanding of the distribution and facilitate more informed interpretation of oral fluid drug analysis.

The immunohistochemical staining technique was only developed for cocaine and opiates but could be further expanded to other commonly used drugs to enhance our understanding of how drugs are excreted into oral fluid. Cannabis is one of the most commonly smoked drugs and thus should be a prime candidate for further work. The *in vivo* study in mice successfully demonstrated that drug can travel into the tongue tissue and salivary gland following peritoneal injection of morphine. The study was undertaken in collaboration with Bristol University, School of Pharmacology. The *in vivo* study in mice was hence limited to morphine because Bristol University only had a licence for injecting morphine and not cocaine. Future research should be

expanded to explore the application of more drugs in *in vivo* studies using immunohistochemical staining.

Bibliography

Ahn, R.-S., Lee, Y.-J., Choi, J.-Y., Kwon, H.-B., and Chun, S.-I., 2007, Salivary cortisol and DHEA levels in the korean population: Age-related differences, diurnal rhythm, and correlations with serum levels, *Yonsei Medical Journal*, 48 (3), 379-388.

Alere™, 2013a, Cocaine Metabolite, Alere™ Microplate EIA KIT, *Alere Toxicology PLC*

Alere™, 2013b, Opiate Metabolite, Alere™ Microplate EIA KIT, *Alere Toxicology PLC*

Allen, C.J., 1981, To be Quechua: The symbolism of coca chewing in highland Peru, *American Ethnologist,* 8 (1), 157 – 171

Ambre, J., Fischman, M., Ruo, T.I., 1984, Urinary Excretion of Ecgonine methyl Ester, a major metabolite of Cocaine in Humans, *Journal of Analytical Toxicology,* 8 (1), 23 – 25

Antonilli, L., De Carolis, L., Brusadin, V., Togna, A.R., Dovizio, M., Togna, G.I., Patrignani, P., Nencini, P., 2012, Repeated exposure to codeine alters morphine glucuronidation by affecting UGT gene expression in rat, *European Journal of Pharmacology,* 693 (1 – 3), 7 -14

Anttila, S. S., Knuuttila, M. L. E., and Sakki, T. K., 1998. Depressive symptoms as an underlying factor of the sensation of dry mouth. *Psychosomatic Medicine*, 60 (2), 215-218.

Aps, J. K. M., and Martens, L. C., 2005. Review: The physiology of saliva and transfer of drugs into saliva. *Forensic Science International*, 150 (2-3), 119-131.

Asman, B., Bergström, K., Söder P., 1981, Ratio of alpha-l-antitrypsin to transferrin in gingival fluid and in blood from patients with periodontal disease, *European Journal of Oral Sciences*, 89 (5), 407 – 411

Axelsson, P., Paulander, J., and Lindhe, J., 1998. Relationship between smoking and dental status in 35-, 50-, 65-, and 75-year-old individuals. *Journal of Clinical Periodontology*, 25 (4), 297-305.

Bailey, M.E., Fraire, A.E., Greenberg, S.D., Barnard, J., Cagle, P.T., 1994, Pulmonary histopathology in cocaine abusers, *Human Pathology*, 25 (2), 203 – 207

Barnett, G., Hawks, R., and Resnick, R., 1981. Cocaine pharmacokinetics in humans. *Journal of Ethnopharmacology*, 3 (2-3), 353-366.

Barnett, G., Chiang, C. W. N., Perezreyes, M., and Owens, S. M., 1982. Kinetic-study of smoking marijuana. *Journal of Pharmacokinetics and Biopharmaceutics*, 10 (5), 495-506.

Baselt, R.C., 2011, *Disposition of toxic drugs and chemicals in man*, Biomedical : California

Bhargava, S., Motwani, M.B., Patni, V.M., 2012, Effect of handheld mobile phone use on parotid gland salivary flow rate and volume, *Oral surgery, oral medicine, oral pathology and oral radiology*, 114 (2), 200 - 206

Billman, G.E., 1990, Mechanisms responsible for the cardiotoxic effects of cocaine, *The Journal of the Federation of American Societies for Experimental Biology*, 4 (8), 2469 – 2475

Blejerpr.H. 1965. Coca leaf and cocaine addiction - some historical notes. *Canadian Medical Association Journal*, 93 (13), 700 - 704

Boghdadi, M.S. and Henning, R.J., 1997, Cocaine: Pathophysiology and clinical toxicology, *Heart & Lung: The Journal of Acute and Critical Care,* 26 (6), 466 – 483

Bosker, W.M. and Huestis, M.A., 2009, Oral fluid testing for drugs of abuse, *Clinical Chemistry,* 55 (11), 1910 – 1931

Boumba, D., Skopouli, F.N., and Moutsopoulos, H.M., 1995, Cytokine mRNA expression in the labial salivary gland tissues from patients with primary Sjogren's syndrome, *British Journal of Rheumatology,* 34 (4), 326 – 333

Bozzola, J.J. and Russell, L.D., 1999, *Electron Microscopy: Priniples and Techniques for Biologists,* 2nd edition, Jones and Bartlett: Sudbury, MA

Bradshaw, D. J., Mckee, A. S., and Marsh, P. D., 1989. Effect of pH on the stability of a microbial community. *Journal of Dental Research*, 68 (SPEC. ISSUE JUNE), 930-930

Bradshaw, D. J., and Marsh, P. D., 1998. Analysis of ph-driven disruption of oral microbial communities in vitro. *Caries Research*, 32 (6), 456-462.

Brunk, S.F., and Delle, M., 1974, Morphine metabolism in man, *Clinical Pharmacology and Therapeutics,* 16 (1), 51 – 57

Burlage, F.R., Pijpe, J., Coppes, R.P., Hemels, M.E.W., Meertens, H., Canrinus, A., Vissink, A., 2005, Variability of flow rate when collecting stimulated human parotid saliva, *European Journal of Oral Sciences,* 113 (5), 386 - 390

Bush, D.M., 2008, The U.S. Mandatory Guidelines for Federal Workplace Drug Testing Programs: Current status and future considerations, *Forensic Science International*, 174 (2 – 3), 111 – 119

Cangianelli, L. A., 1989, The effects of a drug testing program in the navy, *NIDA research monograph*, 95, 211-217

Carroll, F., Lewin, A.H., Boja, J.W., Kuhar, M.J., 1992, Cocaine Receptor: Biochemical Characterization and Structure-Activity Relationships of Cocaine Analogues at the Dopamine Transporter, *Journal of Medicinal Chemistry*, 35 (6), 969 - 981

Chakko, S., and Myerburg, R. J., 1995. Cardiac complications of cocaine abuse, *Clinical Cardiology*, 18 (2), 67-72.

Chan, H.W., Prescott, Frank, A.A., Swoboda, P.A.T., 1976, Thermal decomposition of individual positional isomers of methyl linoleate hydroperoxide: Evidence of carbon-oxygen bond scission, *Journal of the American Oil Chemist's Society,* 53 (9), 572 - 576

Charman, W. N., Porter, C. J. H., Mithani, S., and Dressman, J. B., 1997, Physicochemical and physiological mechanisms for the effects of food on drug absorption: The role of lipids and pH, *Journal of Pharmaceutical Sciences*, 86 (3), 269-282.

Chow, M. J., Ambre, J. J., Ruo, T. I., Atkinson, A. J., Bowsher, D. J., and Fischman, M. W., 1985, Kinetics of cocaine distribution, elimination, and chronotropic effects, Clinical Pharmacology & Therapeutics, 38 (3), 318-324.

Clauwaert, K., Decaestecker, T., Mortier, K., Lambert, W., Deforce, D., Van Peteghem, C., Van Bocxlaer, 2004, The Determination of Cocaine, Benzoylecgonine, and Cocaethylene in Small-Volume Oral Fluid Samples by Liquid Chromatography-Quadrupole-Time-of-Flight Mass Spectrometry, *Journal of Analytical Toxicology*, 28 (8), 655 – 659

Coleman, K., 2011, Seizures of drugs in England and Wales, 2010/2011, *Home Office Statistical Bulletin*, available online at https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/115735/hosb1711.pdf (accessed 28/02/14)

Cone, E.J., Dickerson, S., Paul, B.D., Mitchell, J.M., 1993, Forensic drug testing for opiates. V. Urine testing for heroin, morphine, and codeine with commercial opiate immunoassays, *Journal of Analytical Toxicology*, 17 (3), 156 – 164

Cone, E. J., Hillsgrove, M., and Darwin, W. D., 1994a, Simultaneous measurement of cocaine, cocaethylene, their metabolites, and crack pyrolysis products by gas-chromatography mass-spectrometry, *Clinical Chemistry*, 40 (7), 1299-1305.

Cone, E.J., Hillsgrove, M.J., Jenkins, A.J., Keenan, R.M. and Darwin, W.D., 1994b, Sweat Testing for Heroin, Cocaine, and Metabolites, *Journal of Analytical Toxicology,* 18 (6), 298 - 305

Cone, E.J., Jufer, R., Darwin, W.D., 1996, Forensic Drug Testing for Opiates. VII. Urinary Excretion Profile of Intranasal (Snorted) Heroin, *Journal of Analytical Toxicology*, 20 (6), 379 – 392

Cone, E.J., Tsadik, A., Oyler, J., Darwin, W.D., 1998, Cocaine metabolism and urinary excretion after different routes of administration, *Therapeutic Drug Monitoring*, 20 (5), 556 – 560

Cone, E.J., Presley, L., Lehrer, M., Seiter, W., Smith, M., Kardos, K.W., Fritch, D., Salamone, S. and Niedbala, R.S., 2002, Oral fluid testing for drugs of abuse: positive prevalence rates by Intercept immunoassay screening and GC-MS-MS confirmation and suggested cutoff concentrations, *Journal of Analytical Toxicology,* 26 (8), 541 -546

Cook, M.J., 1965, *The Anatomy of the Laboratory Mouse,* Academic Press : University of Michigan, USA

Cooper, G., Moore, C., George, C., and Pichini, S., 2011, *Guidelines for European workplace drug testing in oral fluid. Drug Testing and Analysis*, 3 (5), 269-276.

Cozart Bioscience Ltd, 2008, Production of Synthetic Saliva, *Health and Safety Information,* PR., 716 (2), 1 - 2

Cravey, R.H. and Reed, D., 1977, The distribution of morphine in man following chronic intravenous administration, *Journal of Analytical Toxicology,* 1 (3), 166 – 167

Crouch, D.J., 2005, Oral fluid collection: the neglected variable in oral fluid testing, *Forensic Science International*, 150 (2-3), 165 -173

Crumb, W.J., and Clarkson, C.W., 1990, Characterization of cocaine induced block of cardiac sodium channels, *Biophysical Journal,* 57 (3), 589 – 599

Curtis, M.A., Griffiths, G.S., Price, S.J., Coulthurst, S.K., Johnson, N.W., 1988, The total protein concentration of gingival crevicular fluid, *Journal of Clinical Periodontology,* 15 (10), 628 – 632

Darke, S., Sims, J., McDonald, S., Wickes, W., 2000, Cognitive impairment among methadone maintenance patients, *Addiction*, 95 (5), 687 – 695

Dawes, C. and Wood, C.M., 1973, The composition of human lip mucous gland secretions, *Archives of Oral Biology,* 18 (3), 343 – 350

Dawes, C., 1987, Physiological factors affecting salivary flow-rate, oral sugar clearance, and the sensation of dry mouth in man, *Journal of Dental Research*, 66, 648-653.

Dawes, C., and Macpherson, L. M. D., 1992, Effects of 9 different chewing-gums and lozenges on salivary flow-rate and pH, *Caries Research*, 26 (3), 176-182.

Department for Transport, 2014, Public approval for driving limits for 16 drugs, *Making roads safer,* available online from https://www.gov.uk/government/news/public-approval-for-driving-limits-for-16-drugs [accessed: 01/04/2014]

Drummer, O. H., 2005, Review: Pharmacokinetics of illicit drugs in oral fluid, *Forensic Science International*, 150 (2-3), 133-142.

Drummer, O.H., 2006, Drug testing in oral fluid, *The Clinical Biochemist Reviews*, 27 (3), 147 – 159

Eisbruch, A., Ten Haken, R. K., Kim, H. M., Marsh, L. H., and Ship, J. A., 1999, Dose, volume, and function relationships in parotid salivary glands following conformal and intensity-modulated irradiation of head and neck cancer, *International Journal of Radiation Oncology Biology Physics*, 45 (3), 577-587.

Eissing, T., Lippert J., Willmann, S., 2012, Pharmacogenomics of Codeine, Morphine, and Morphine – 6- Glururonide – Model-Based Analysis of the Influence of CYP2D6 Activity, UGT2B7 Activity, Rnal Impariment, and CYP3A4 Inhibition, *Molecular Diagnosis & Therapy*, 16 (1), 43 - 53

Elliott, H.W., Parker, K.D., Wright, J.A., Nomof, N., 1971, Actions and metabolism of heroin administered by continuous intravenous infusion to man, *Clinical pharmacology and therapeutics*, 12 (5), 806 – 814

EMCDDA, 2010, Cocaine and crack cocaine, *Annual report 2010: the state of the drugs problem in Europe*, available online at http://www.emcdda.europa.eu/online/annual-report/2010/cocaine/2 (accessed 28/02/14)

EMCDDA,2011, Heroin, *Annual report 2011: the state of the drugs problem in Europe*, available online at http://www.emcdda.europa.eu/online/annual-report/2011/heroin (accessed 15/02/20140

EMCDDA, 2012, Cocaine and Crack cocaine, *Annual report 2012 : the state of the drugs problem in Europe*, available online at http://www.emcdda.europa.eu/publications/annual-report/2012 (accessed 28/02/14)

Ericson, D. and Bratthall, D., 1989, Simplified method to estimate salivary buffer capacity, *European Journal of Oral Sciences,* 97 (5), 405 -407

Ettinger, N.A. and Albin, R.J., 1989, A review of the respiratory effects of smoking cocaine, *The American Journal of Medicine,* 87 (6), 664 – 668

European Workplace Drug Testing Society (EWDTS), 2011, *Guidelines for Legally Defensible Workplace Drug Testing Specimen Collection Procedures*, Version 2.0, 1 – 4

EWDTS. 2009. Guidelines for legally defensible workplace drug testing specimen collection procedures: European Workplace Drug Testing Society.

EWDTS, 2011, Guidelines for Oral Fluid, Version 001, available online at http://www.european-accreditation.org/publication/ewdts-ta-fluid [accessed 01/04/2014

Fandino, A.S., Toennes, S.W., Kauert, G.F., 2002, Studies on in vitro degradation of anhydroecgonine methyl ester (methylecgonidine) in human plasma, *Journal of Analytical Toxicology,* 26 (8), 567 – 570

Felby, S., Christensen H., Lund, A., 1974, Morphine concentrations in blood and organs in cases of fatal poisoning, *Forensic Science International,* 3 (1), 77 - 81

Ferguson, D. B., 1999, The flow rate and composition of human labial gland saliva, *Archives of Oral Biology,* 44, 11-14.

Finer, Y. and Santerre, J.P., 2004, Salivary Esterase Activity and Its Association with the Biodegradation of Dental Composites, *Journal of Dental Research,* 83 (1), 22 – 26

Fineschi, V., Centini, F., Monciotti, F., and Turillazzi, E., 2002, The cocaine "Body stuffer" Syndrome: A fatal case, *Forensic Science International*, 126 (1), 7-10

Fleming, J. A., Byck, R., and Barash, P. G., 1990, Pharmacology and therapeutic applications of cocaine, *Anesthesiology*, 73 (3), 518 - 531

Follador, M.J.D., Yonamine, M., Moreau R.L, Silva, O.A., 2004, Detection of cocaine and cocaethylene in sweat by solid-phase microextraction and gas chromatography/mass spectrometry, *Journal of Chromatography B*, 811 (1), 37 – 40

Fowler, J. S., and Volkow, N. D., 1995, Multi-tracer pet studies of cocaine pharmacokinetics and pharmacodynamics, *Medicinal Chemistry Research*, 5 (2-3), 193-207

Fuchs, A., 1978, Coca chewing and high-altitude stress – possible effects of coca alkaloids on erythropoiesis, *Current Anthropology*, 19 (2), 227 – 291

Fure, S., Lingstorm, P., and Birkhed, D., 1998, Effect of three months' frequent use of sugar-free chewing gum with and without urea on calculus formation¸ *Journal of Dental Research*, 77, 1630 – 1637

Furnari, C., Ottaviano, V., Sacchetti, G., and Mancini, M., 2002, A fatal case of cocaine poisoning in a body packer, *Journal of Forensic Sciences*, 47 (1), 208-210

Genco, R., Zambon, J.J., Murray, P.A., 1985, Serum and Gingival Fluid Antibodies as Adjuncts in the Diagnosis of *Acinobacillus actinomycetemcomitans* Associated Periodontal Disease, 56 (11), 41 -50

Gerostamoulos, J., and Drummer, O.H., 2000, Postmortem redistribution of morphine and its metabolites, *Journal of Forensic Sciences*, 45 (4), 843 – 845

Ghuran, A., and Nolan, J., (2000), Recreational drug misuse: issues for the cariologist, *Heart*, 83 (6), 627 – 633

Glauser, J. and Queen, J.R., 2007, An overview of non-cardiac cocaine toxicity, *The Journal of Emergency Medicine*, 32 (2), 181 – 186

Goldstein, A. and Naidu, A., 1989, Multiple opioid receptors: ligand selectivity profiles and binding site signatures, *Molecular Pharmacology*, 36 (2), 265 - 272

Granger, D.A., Cicchetti, D., Rogosh, F.A., Hibel, L.C., Teisl, M., and Flores, E., 2007, Blood contamination in children's saliva: Prevalence, stability, an impact on the measurement of salivary cortisol, testosterone, and dehydroepiandrosterone, *Psychoneuroendocrinology*, 32 (6), 724 - 733

Gröschl, M., 2009, The physiological role of hormones in saliva, *BioEssays*, 31 (8), 843 - 852

Grzybowski, A., 2007, The history of cocaine in medicine and its importance to the discovery of the different forms of anaesthesia, *Klinika oczna*, 109 (1-3), 101 - 105

Gyr, E., Brenneisen, R., Bourquein, D., Lehmann, T., Vonlanthen, D., Hug, I., 2000, Pharmacodynamics and pharmacokinetics of intravenously, orally and rectally administered diacetylmorphine in opioid dependents, a two-patient pilot study with a heroin-assisted treatment program, *International journal of clinical pharmacology and therapeutics*, 38 (10), 486 – 491

Haeckel, R., 1993, Factors Influencing the Saliva/Plasma Ratio of Drugs, *Annals of the New York Academy of Sciences*, 694 (1), 128 - 142

Hamner, J. E., and Villegas, O. L., 1969, Effect of coca leaf chewing on buccal mucosa of aymara and quechua indians in Bolivia, *Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontics*, 28 (2), 287-295

Haney, M., Ward, A. S., Foltin, R. W., and Fischman, M. W., 2001, Effects of ecopipam, a selective dopamine d1 antagonist, on smoked cocaine self-administration by humans, *Psychopharmacology*, 155 (4), 330-337.

Haney, M., Ward, A. S., Gerra, G., and Foltin, R. W., 2001, Neuroendocrine effects of d-fenfluramine and bromocriptine following repeated smoked cocaine in humans, *Drug and Alcohol Dependence*, 64 (1), 63-73.

Hanna, J.M., 1971a, Responses of Quechua Indians to coca ingestions during cold exposure, *American Journal of Physical Anthropology*, 34 (2), 273 – 275

Hanna, J.M., 1971b, Ecological considerations of coca use in a high altitude populations, *American Journal of Physical Anthropology*, 35 (2), 282 – 286

Hanna, J.M., 1971c, Further studies on effects of coca chewing on exercise, *Human Biology*, 43 (2), 200 – 209

Hanna, J. M., 1974. Coca leaf use in southern-peru - biosocial aspects, *American Anthropologist*, 76 (2), 281-296.

Hanna, J. M., and Hornick, C. A., 1977, Use of coca leaf in southern peru - adaptation or addiction, *Bulletin on Narcotics*, 29 (1), 63-74.

Hearn, W.L., Rose, S., Wagner, J., Ciarleglio, A., Mash, D.C., 1991, Cocaethylene is more potent thatn cocaine in mediating lethality, *Pharmacology Biochemistry and Behaviour*, 39 (2), 531 – 533

Hendriks, V.M., Van den Brink, W., Blanken, P., Bosman, I.J., Van Ree, J.M., 2001, Heroin self-administration by means of 'chasing the dragon': pharmacodynamics and bioavailability of inhaled heroin, *European Neuropsychopharmacology*, 11 (3), 241 – 252

Hofman, L.F., 2001, Human saliva as a diagnostic specimen, *Journal of Nutrition*, 131 (5), 1621 – 1625

Holmstedt, B., Lindgren, J. E., Rivier, L., and Plowman, T., 1979, Cocaine in blood of coca chewers, *Journal of Ethnopharmacology*, 1 (1), 69-78.

Holzer, P., 2009, Opioid receptors in the gastrointestinal tract, *Regulatory Peptides*, 155 (1-3), 11 – 17

Home Office, 2012, Preliminary Drug Testing Devices, *A Guide to Type Approval Procedures for Preliminary Drug Testing Devices Used for Transport Law Enforcement in Great Britain*, pp 1 -24

Home Office, 2013, Home Office Type Approved Drug Testing Devices, *August 2013 UPDATE*, available online from https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/275543/DrugTestingDevices.pdf [accessed: 01/04/2014]

Hosztafi, S., 2001, The history of heroin, *Acta pharmaceutica Hungarica*, 71 (2), 233 – 42

Hsu, S., Immunohistochemistry, In: Wilcheck, M., Bayer, E., 1990, *Methods in Enzymology*, 184, 357 – 363

Hudgins, R., Mccusker, J., and Stoddard, A., 1995, Cocaine use and risky injection and sexual behaviors, *Drug and Alcohol Dependence*, 37 (1), 7 – 14

Huestis, M.A. and Cone, E.J., 2004, Relation of Delta 9-tetrahydrocannabinol concentrations in oral fluid and plasma after controlled administration of smoked cannabis, *Journal of Analytical Toxicology*, 28 (6), 394 – 399

Humberstone, A. J., and Charman, W. N., 1997, Lipid-based vehicles for the oral delivery of poorly water soluble drugs, *Advanced Drug Delivery Reviews*, 25 (1), 103-128

Inturrisi, C.E., Schultz, M., Shin, S., Umans, J.G., Angel, L., Simon, E.J., 1983, Evidence from opiate binding studies that heroin acts through its metabolites, *Life Sciences*, 33 (1), 773 – 776

Isenschmid, D. S., Fischman, M. W., Foltin, R. W., and Caplan, Y. H., 1992, Concentration of cocaine and metabolites in plasma of humans following intravenous administration and smoking of cocaine, *Journal of Analytical Toxicology*, 16 (5), 311-314.

Jacob, P, Lewis, E.R., Elias-Baker, B.A., Jones, R.T., 1990, A pyrolysis product, anhydroecgonine methyl ester (methylecgonidine), is in the urine of cocaine smokers, *Journal of Analytical Toxicology*, 14 (6), 353 – 357

Jacobson, M., 2003, Drug testing in the trucking industry: The effect on highway safety, *Journal of Law & Economics*, 46 (1), 131-156.

Javaid, J.I., Musa, M.N., Fischman, M., Schuster, C.R., Davis, J.M., 1983, Kinetics of cocaine in humans after intravenous and intranasal administration, *Biopharmaceutics & Drug Disposition*, 4 (1), 9 – 18

Jeffcoat, A.R., Perez-Reyes, M., Hill, J.M., Sadler, B.M., Cook, C.E., 1989, Cocaine disposition in humans after intravenous injection, nasal insufflation (snorting), or smoking, *Drug Metabolism and Disposition*, 17 (2), 153 – 159

Jenkins, A.J., Keenan, R.M., Henningfield, J.E., Cone, E.J., 1994, Pharmacokinetics and pharmacodynamics of smoked heroin, *Journal of Analytical Toxicology*, 18 (6), 317 – 330

Jenkins, A.J., Oyler, J.M., Cone, E.J., 1995, Comparison of heroin and cocaine concentrations in saliva with concentrations in blood and plasma, *Journal of Analytical Toxicology*, 19 (6), 359 – 374

Jenkins, A. J., Llosa, T., Montoya, I., and Cone, E. J., 1996, Identification and quantitation of alkaloids in coca tea, *Forensic Science International*, 77 (3), 179-189.

Kajitani, A., Kaiho, M., Okada, Y., and Ishiyama, I., 1989, Immunohistochemical study on the excretion of a drug (methamphetamine) by salivary glands, *The Japanese Journal of Experimental Medicine*, 59 (5), 197 - 202

Kamendulis, L.M., Brzsezinski, M.R., Pindel, E.V., Bosron, W.F., Dean, R.A., 1996, Metabolism of cocaine and heroin is catalysed by the same human liver carboxylesterases, *The Journal of Pharmacology and Experimental Therapeutics*, 279 (2), 713 – 717

Karnovsky, M.J.A., 1965, A formaldehydegluteraldehyde fixative of high osmolarity for use in electron microscopy, *Journal of Cell Biology*, 27, 137 - 138

Karch, S. B., Stephens, B., and Ho, C. H., 1998, Relating cocaine blood concentrations to toxicity - an autopsy study of 99 cases, *Journal of Forensic Sciences*, 43 (1), 41-45.

Kavanagh, D. A., O'mullane, D. M., and Smeeton, N., 1998, Variation of salivary flow rate in adolescents, *Archives of Oral Biology*, 43 (5), 347-352.

Kidwell, D. A., Holland, J. C., and Athanaselis, S., 1998, Testing for drugs of abuse in saliva and sweat. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*, 713 (1), 111-135.

Kieffer, B.L., 1995, Recent Advances in Molecular Recognition and Signal Transduction of Active peptides: Receptors for Opioid Peptides, *Cellular and Molecular Neurobiology*, 15 (6), 615 - 635

Kim, I., Barnes, A.J., Schepers, R., Moolchan, E.T., Wilson, L., Cooper, G., Reid, C., Hand, C. and Huestis, M.A., 2003, Sensitivity and specificity of the

Cozart microplate EIA cocaine oral fluid at proposed screening and confirmation cutoffs, *Clinical Chemistry,* 49 (9), 1498 – 1503

Kim, I., Barnes, A.J., Oyler, J.M., Schepers, R., Joseph Jr, R.E., Cone, E.J., Lafko, D., Moolchan, E.T., Huestis, H.A., 2002, Plasma and oral fluid pharmacokinetics and pharmacodynamics after oral codeine administration, *Clinical Chemistry*, 48 (9), 1486 – 1496

Kintz, P., Cirimele, V., Sengler, C., Mangin, P., 1995, Testing human hair and urine for anydroecgonine methyl ester, a pyrolysis product of cocaine, *Journal of Analytical Toxicology,* 19 (6), 479 – 482

Kintz, P., Sengler, C., Cirimele, V., Mangin, P., 1997, Evidence of crack use by anhydroecgonine methylester identification, *Human & Experimental toxicology,* 16 (2), 123 – 127

Kiszka, M., Buszewicz, G., Mydro, R., 2000, Stability of cocaine in phosphate buffer and in urine, *Z Zagadnien Nauk Sadowych,* XLIV, 7 – 23

Kivlighan, K.T., Granger, D.A, Schartz, E.B., Nelson, V., Curran, M., and Shirtcliff, E.A., 2004, Quantifying blood leakage into the oral mucosa and its effects on the measurement of cortisol, dehydroepiandosterone, and testosterone in saliva, *Hormones and Behaviours*, 46 (1), 39 - 46

Krause, W., Mueller, U. and Mazur, A., 2002, Measurement of steroid levels in saliva in a population-based survey of liefestyle, medican conditions, marriage, sex life and hormone status in aging men: a feasibility study, *The agining male: the official journal of the International Society for the Study of the Aging Male*, 5, 203 – 15

Kumor, K., Sherer, M., Thompson, L., Cone, E., Mahaffey, J., and Jaffe, J. H., 1988, Lack of cardiovascular tolerance during intravenous cocaine infusions in human volunteers, *Life Sciences*, 42 (21), 2063-2071.

Lamkin, M. S., and Oppenheim, F. G., 1993, Structural features of salivary function, *Critical Reviews* in Oral Biology & Medicine, 4 (3-4), 251-259.

Langel, K., Engblom, C., Pehrsson, A., Gunnar, T., Ariniemi, K. and Lillsunde, P., 2008, Drug testing in oral fluid-evaluation of sample collection devices, *Journal of Analytical Toxicology,* 32 (6), 393 – 401

Larsson, B., Olivecrona, G., and Ericson, T., 1996, Lipids in human saliva, *Archives of Oral Biology,* 41 (1), 105-110.

Laizure, S.C., Mandrell, T., Gades, N.M., Parker, R.B., 2003, Cocaethylene Metabolism and Interaction with Cocaine and Ethanol: Role of Carboxylesterases, *Drug Metabolism and Disposition,* 31 (1), 16 – 20

Leao, A., and Sheiham, A., 1995, Relation between clinical dental status and subjective impacts on daily living, *Journal of Dental Research*, 74 (7), 1408 – 1413

Lee, B. K., Glass, T. A., Mcatee, M. J., Wand, G. S., Bandeen-Roche, K., Bolla, K. I., and Schwartz, B. S., 2007a, Associations of salivary cortisol with cognitive function in the Baltimore memory study, *Archives of General Psychiatry*, 64 (7), 810-818.

Lee, J. Y., Chung, J. W., Kim, Y. K., Chung, S. C., and Kho, H. S., 2007b, Comparison of the composition of oral mucosal residual saliva with whole saliva, *Oral Diseases,* 13 (6), 550-554.

Levine, M. J., 1993, Development of artificial saliva, *Critical Reviews in Oral Biology & Medicine*, 4 (3-4), 279-286.

Li, G., Baker, S. P., Zhao, Q., Brady, J. E., Lang, B. H., Rebok, G. W., and Dimaggio, C., 2011, Drug violations and aviation accidents: Findings from the us mandatory drug testing programs, *Addiction*, 106 (7), 1287-1292.

Li, T. L., and Gleeson, M., 2004, The effect of single and repeated bouts of prolonged cycling and circadian variation on saliva flow rate, immunoglobulin a and alpha-amylase responses, *Journal of Sports Sciences*, 22 (11-12), 1015-1024.

Lockridge, O., Mottershaw-Jackson, N., Eckerson, H.W., La Du., B.N., 1980, Hydrolysis of diacetylmorphine (heroin) by human serum cholinesterase, *Journal of Pharmacology and Experimental Therapeutics*, 215 (1), 1 - 8

Qi L. Lu and Terry A. Partridge (1998) A New Blocking Method for Application of Murine Monoclonal Antibody to Mouse Tissue Sections, *Journal of Histochemistry and Cytochemistry*, 46, 977 - 984

Malamud, D., Davis, C., Berthold, P., Roth, E., and Friedman, H., 1993, Human submandibular saliva aggregates HIV, *Aids Research and Human Retroviruses*, 9 (7), 633-637.

Manno, J. E., 1986, Specimen collection and handling, *NIDA research monograph*, 73, 24-29.

Marsh, P. D., and Bradshaw, D. J., 1990, The effect of fluoride on the stability of oral bacterial communities *in vitro*, *Journal of dental research*, 69 Spec No, 668-671; discussion 682-663.

Martin, R.T., 1970, The role of coca in the history, religion, and medicine of South American Indians, *Economic Botany*, 24 (4), 422 -438

Meadway, C., George, S., Braithwaite, R., 2002, A rapid GC-MS method for the determination of dihydrocodeine, codeine, norcodeine, morphine, normorphine and 6-MAM in urine, *Forensic Science international*, 127 (1 – 2), 136 - 141

Meurman, J. H., Rytomaa, I., Kari, K., Laakso, T., and Murtomaa, H., 1987, Salivary ph and glucose after consuming various beverages, including sugar-containing drinks, *Caries Research*, 21 (4), 353-359.

Meyer, J.S., Quenzer, L.F., 2005, *Psychopharmacology: Drugs, The Brain, and Behaviour*, Sunderland MA: Sinauer Associates

Middleton, L. S., Cass, W. A., and Dwoskin, L. P., 2004, Nicotinic receptor modulation of dopamine transporter function in rat striatum and medial prefrontal cortex, *Journal of Pharmacology and Experimental Therapeutics*, 308 (1), 367-377.

Moffat, A. C., Osselton, M. D., Widdop, B., and Watts, J., 2011, *Clarke's Analysis of Drugs and Poisons*, 4th ed, London: Pharmaceutical Press.

Morineau, G., Boudi, A., Barka, A., Gourmelen, M., Degeilh, F., Hardy, N., al-Halnak, A., Soliman, H, Gosling, J.P., Julien, R., Brerault, J.L., Boudou, P., Aubert, P., Villette, J.M., Pruna, A., Galons, H., Fiet, J., 1997, Radioimmunoassay of cortisone in serum, urine, and saliva to assess the status of the cortisol-cortisone shuttle, *Journal of Clinical Chemistry*, 43 (8 pt 1), 1397 -107

Moriya, F. and Hashimoto, Y., 1997, Distribution of free and conjugated morphine in body fluids and tissues in a fatal heroin overdose: is conjugated morphine stable in post-mortem specimens?, *Journal of Forensic Sciences*, 42 (4), 736 – 740

Nagler, R. M., Gez, E., Rubinov, R., Laufer, D., Ben-Aryeh, H., Gaitini, D., Filatov, M., and Kuten, A., 2001, The effect of low-dose interleukin-2-based immunotherapy on salivary function and composition in patients with metastatic renal cell carcinoma, *Archives of Oral Biology*, 46 (6), 487-493.

Nagler, R. M., and Hershkovich, O., 2005a, Age-related changes in unstimulated salivary function and comvosition and its relations to medications and oral sensorial complaints, *Aging Clinical and Experimental Research*, 17 (5), 358-366.

Nagler, R. M., and Hershkovich, O., 2005b, Relationships between age, drugs, oral sensorial complaints and salivary profile, *Archives of Oral Biology*, 50 (1), 7-16.

Navazesh, M., 1993, Methods for collecting saliva, *Annals of the New York Academy of Sciences,* 694 (20), 72 -77

Negrete, J. C., 1978, Coca leaf chewing - public-health assessment, *British Journal of Addiction*, 73 (3), 283-290.

Niedbala M., Kardos, K., Fries, T., Cannon, A. and Davis, A., 2001, Immunoassay for detection of cocaine/metabolites in oral fluids, *Journal of Analytical Toxicology*, 25 (1), 62 -68

Nieuw Amerongen, A.V., Bolscher, J.G. and Veerman, E.C., 2004, Salivary proteins: protective and diagnostic value in cariology, *Caries Research,* 38 (3), 247 – 253

Niew Amerongen, A.V., Ligtenberg, A.J., Veerman, E.C., 2007, Implications for diagnostics in the biochemistry and physiology of saliva, *Annals of the New York Academy of Science*, 1098, 1 - 6

O'Neal, C.L., Crouch, D.J., Rollins, D.E., Fatah, A., Cheever, M.L., 1999, Correlation of saliva codeine concentrations with plasma concentrations after oral codeine administration, *Journal of Analytical Toxicology*, 23 (6), 452 – 459

Osselton, M.D., Cox, A.R., Robinson, S.T., Reddick, S.R., 2001, An evalutation of Oral Fluid Drug Testing for the detection of Opiate and Cocaine use in a population of Drug Users, *Soft annual meeting abstracts*, P45

Paly, D., Jatlow, P., Van Dyke, C., Jeri, F.R., Byck, R., 1982, Plasma cocaine concentrations during cocaine paste smoking, *Life Sciences,* 30 (9), 731 – 738

Pasternak, G.W., 2001, Insights into the mu opioid pharmacology: The role of mu opioid receptor subtypes, *Life Sciences*, 68 (19 -20), 2213 – 2219

Peat, M. A., 1995, Financial viability of screening for drugs of abuse, *Clinical Chemistry*, 41 (5), 805-808.

Penny, M.E., Zavaleta, A. and Lemay M., 2009, Can coca leaves contribut to improving the nutrional status of the Andean population, *Food and Nutrion Bulletin*, 30 (3), 205 - 216

Percival, R. S., Challacombe, S. J., and Marsh, P. D., 1994, Flow-rates of resting whole and stimulated parotid-saliva in relation to age and gender, *Journal of Dental Research*, 73 (8), 1416-1420.

Pindel, E.V., Kedishvili, N.Y., Abraham, T.L., Brzezinski, M.R., Zhang, J., Dean, R.A., Bosron, W., 1997, Purification and Cloning of a Broad Substrate Specificity Human Liver Carboxylesterase That Catalyzes the Hydrolysis of Cocaine and Heroin, *The Journal of Biological Chemistry*, 272 (23), 14769 – 14775

Poklis, A., Maginn, D., and Barr, J. L., 1987a, Drug findings in driving under the influence of drugs cases - a problem of illicit drug-use, *Drug and Alcohol Dependence*, 20 (1), 57-62.

Poklis, A., Maginn, D., and Barr, J. L., 1987b, Tissue disposition of cocaine in man - a report of 5 fatal poisonings, *Forensic Science International*, 33 (2), 83-88.

Polettini, A., Poloni, V., Groppi, A., Stramesi, C., Vignali, C., Politi, L., Montagna, M., 2005, The role of coaine in heroin-related deaths: Hypothesis on the interaction between heroin and cocaine, *Forensic Science International*, 153 (1), 23-28

Pozner, C.N., Levine, M., Zane, R., 2005, The cardiovascular effects of cocaine, *The Journal of Emergency Medicine*, 29 (2), 173 – 178

Pragst, F., and Balikova, M. A., 2006, State of the art in hair analysis for detection of drug and alcohol abuse, *Clinica Chimica Acta*, 370 (1-2), 17-49.

Pratibha, P.K., Bhat, K.M. and Bhat, G.S., 2006, Oral malodor:a review of the literature, *Journal of dental hygiene: JDH/ American Dental Hygienists' Association*, 80 (3), 8

Redinbo, M.R., Bencharit, S., Potter, P.M., 2003, Human caroxylesterase 1: from drug metabolism to drug discovery, *Biochemical Society Transactions,* 31 (3), 620 – 624

Rentsch, K.M., Kullak-Ublick, G.A., Reichel, C., Meier, P.J., Fattinger, K., 2001, Arterial and venous pharmacokinetics of intravenous heroin in subjects who are addicted to narcotics, *Clinical Pharmacology & Therapeutics*, 70 (3), 237 – 246

Ritz, M.C., Lamb, R.J., Goldberg, S.R., Kuhar, M.J., 1987, Cocaine receptors on dopamine transporters are related to self-administration of cocaine, *Science,* 237 (4819), 1219 – 1223

Ritz, M.C., Cone, E.J., Kuhar, M.J., 1990, Cocaine inhibition of ligand binding at dopamine, norepinephrine and serotonin transporters:a structure-activity study, *Life Sciences,* 46 (9), 635 – 645

Roberts, J.R., Price, D., Goldfrank, L., Hartnett, l., 1986, The bodystuffer syndrome: A clandestine form of drug overdose, *The American Journal of Emergency Medicine,* 4 (1), 24 – 27

Rook, E.J., Van Ree, J.M., Van den Brink, W., Hillebrand, M.J.X., Huitema, A.D.R., Hendriks, V.M., Beijnen, J.H., 2006a, Pharmacokinetics and Pharmacodynamics of High Doses of Pharmaceutically Prepared Heroin, by Intravenous or by Inhalations Route in Opioid-Dependent Patients, *Basic & Clinical Pharmacology & Toxicology,* 98 (1), 86 – 96

Rook., E.J., Huitema, A.D.R., van den Brink, W., Van Ree, J.M., Beijnen, J.H., 2006b, Population Pharmacokinetics of Heroin and its Major metabolites, *Clinical Pharmacokinetics*, 45 (4), 401 – 417

Rop, P.P., Fornaris, M., Salmon, T., Burle, J., Bresson, M., 1997, Concentration of heroin, 06-Monoacetylmorphine, and morphine in a lethal case following an oral heroin overdose, *Journal of Analytical Toxicolgy*, 21 (3), 232 -235

Rossi, G.C., Brown, G.P., Leventhal, L., Yang, K., Pasternak, G.W., 1996, Novel receptor mechanisms for heroin and morphine-*6ß*-glucuronide analgesia, *Neuroscience Letters*, 216 (1), 1 – 4

Salmon, A.Y., Goren, Z., Avissar, Y., Soreq, H., 1999, Human Erythrocyte but not brain acetylcholinesterase hydrolyses heroin to morphine, *Clinical and Experimental Pharmacology and Physiology*, 26 (8), 596 - 600

SAMHSA (Department of Health and Human Services), 1994, Mandatory Guidelines for Federal Workplace Drug Testing Programs, *Federal Register*, 59 (110), available online at http://www.gpo.gov/fdsys/pkg/FR-1994-06-09/html/94-13940.htm [accessed 01/04/2014]

SAMHSA (Department of Health and Human Services), 2010, Mandatory Guidelines for Federal Workplace Drug Testing Programs, *Federal Register*, 75 (83), 22809 - 22810

Samyn, N., De Boeck, G., and Verstraete, A. G., 2002, The use of oral fluid and sweat wipes for the detection of drugs of abuse in drivers, *Journal of Forensic Sciences*, 47 (6), 1380-1387.

Sanchez, G. A., and Fernandez De Preliasco, M. V., 2003, Salivary ph changes during soft drinks consumption in children, *International journal of paediatric dentistry / the British Paedodontic Society [and] the International Association of Dentistry for Children*, 13 (4), 251-257.

Satoh, T., Taylor, P., Bosron, W.F., Sanghani, S.P., Hosokawa, M., La Du, B.N., 2002, Current Progress on Esterases: From Molecular Structure to function, *Drug Metabolism & Disposition*, 30 (5), 488 – 493

Sawynok, J., 1986, The therapeutic use of heroin: a review of the pharmacological literature, *Canadian Journal of Physiology and Pharmacology*, 64 (1), 1 – 6

Schenkein, H., and Genco, R., 1977, Gingival Fluid and Serum in Periodontal Diseases: I. Quantitative Study of Immunoglobulins, Complement Components, and Other Plasma Proteins, *Journal of Periodontology*, 48 (12), 772 – 777

Schipper, R. G., Silletti, E., and Vinyerhoeds, M. H., 2007, Saliva as research material: Biochemical, physicochemlical and practical aspects, *Archives of Oral Biology*, 52 (12), 1114-1135.

Schneyer, L. H., and Levin, L. K., 1955, Rate of secretion by exogenously stimulated salivary gland pairs of man, *Journal of Applied Physiology*, 7 (6), 609-613.

Schramm, W., Smith, R.H., Craig, P.A., Kidwell, D.A., 1992, *Journal of Analytical Toxicology*,16 (1), 1 – 9

Schwartz, E.B. and Granger, D.A., 2004, Transferrin enzyme immunoassay for quantative monitoring of blood contamination in saliva, *Clinical Chemistry*, 50 (3), 654 - 656

Sellers, E.M. and Holloway, M.R., 1978, Drug Kinetics and Alcohol Ingesting, *Clinical Pharmacokinetics*, 3 (6), 440 – 452

Selly, D.E., Cao, C.C., Sexton, T., Schwegel, J.A., Martin, T.J., Childers, S.R., 2001, μ Opioid receptor-mediated G-protein activation by heroin metabolites: evidence for greater efficacy of 6-monoacetylmorphine compared with morphine, *Biochemical Pharmacology*, 62 (4), 447 – 455

Shahar, E., Pollack, S., Kedem, E., Hassoun, G., and Nagler, R., 2008, Effect of haart on salivary composition and oxidative profile in HIV-infected patients, *Current HIV Research*, 6 (5), 447-451.

Shannon, I. L., 1966, Climatological effects on human parotid gland function, *Archives of Oral Biology*, 11 (4), 451.

Sharp, M.E., Wallace, S.M., Hindmarsh, K.W., Peel, H.W., 1983, Monitoring saliva concentratins of methaqualone, codeine, secobarbital, diphenhydramine and diazepam after single oral doses, *Journal of Analytical Toxicology*, 7 (1), 11 – 14

Siegel, R.K., Elsohly, M.A. and Plowman, T., 1986, Cocaine in Herbal Tea, *Journal of the American Medical Association*, 255 (1), 40

Simon, G.A. and Maibach, H.I., 2000, The pig as an experimental animal model of percutaneous permeation in man: qualitative and quantitative observations -- an overview, *Skin Pharmacology and applied skin physiology*, 13 (5), 229 - 234

Smith, R.G. and Burtner, A.P., 1994, Oral side effects of the most frequently presribed drugs, *Special care in dentistry: official publication of the American Association of Hospital Dentists, the Academy of Dentistry for the Handicapped and the American Society for Geriatric Dentistry*, 14 (3), 96 – 102

Smith, R.E., Maguire, J.A., Stein-Oakley, A.N., 1996, Localisation of 11 beta-hydroxysteroid ehydrogenase type ll in human epithelial tissues, *Journal of Endocrinological Metabolism*, 80 (7), 2203 - 2209

Smith, M.J., Shimomura, E.T., Summers, J., Paul, B.D., 2001, Urinary Excretition Profiles for Total Morphine, Free Morphine, and 6-Acetylmorphine Following Smoked and Intravenous Heroin, *Journal of Analtytical Toxicology*, 25 (7), 504 – 514

Spiehler, V.R., and Reed, D., 1985, Brain concentrations of cocaine and benzoylecgonine in fatal cases, *Journal of Forensic Sciences,* 30 (4), 1003 – 1011

Spiehler, V., 2011. Drugs in saliva. In: Moffat, T., Osselton, M. D., and Widdop, B. eds. *Clarke's analysis of drugs and poisons 4th ed*., Vol. 1. London: Pharmaceutical Press, 109 - 123.

Strang, J., Griffiths, P., Powis, B., Gossop, M., 1992, First use of heroin: changes in route of administration over time, *BMJ,* 304 (6836), 1222 – 1223

Strang, J., Griffiths, P., Gossop, M., 1996, Heroin Smoking by 'chasing the dragon':origins and history, *Addiction,* 68 (2), 221 – 228

Tegeder, I., Lotsch, J., Geisslinger, G., 1999, Pharmocokinetics of opioids in liver disease, *Clinical Pharmocokinetics,* 37 (1), 17 – 40

Toennes, S.W., Fandino, A.S., Kauert, G., 1999, Gas chromatographic-mass spectrometric detection of anhydroecgonine methyl ester (methylecgonidine) in human serum as evidence of recent smoking of crack, *Journal of chromatography B, Biomedical sciences and applications,* 735 (1), 127 – 132

Torre, R. and Cami, J., 1999, More on opioid overdose, *Addiction,* 94 (7), 976 - 977

Tortora, G. J., and Derrickson, B., 2009, *Principles of anatomy and physiology*, 12 ed. Vol. 2. Hoboken, N.J.: Wiley.

Trouve, R. and Nahas, G.G., 1990, Antidotes to lethal cocaine toxicity in the rat, *Archives Internationales de Pharmacodynamie et de Therapie,* 305, 197 – 297

Turner, M., McCrory, P., Johnston, A., 2005, Time for tea, anyone?, *British Journal of Sports Medicine,* 39 (10), 37

Uhl, G.R., Hall, F.S., Sora, I., 2002, Cocaine, reward, movement and monamine transporters, *Molecular Psychiatry*, 7 (1), 21 - 6

Urso, R., Montefrancesco, G., Rigato, M., 2012, A compartmental model for the pharmacokinetic of heroin and its metabolites in man, *Heroin Addiction & Related Clinical Problems*, 14 (1), 35 – 58

Verstraete, A. G., and Pierce, A., 2001, Workplace drug testing in Europe, *Forensic Science International*, 121 (1-2), 2-6.

Vining, R.F. and McGinley, R.A., 1986, Hormones in Saliva, *CRC Critical Reviews in Clinical Laboratory Sciences*, 23 (2), 95 – 146

Volkow, N. D., Ding, Y. S., Fowler, J. S., Wang, G. J., Logan, J., Gatley, J. S., Dewey, S., Ashby, C., Liebermann, J., Hitzemann, R., and Wolf, A. P., 1995, Is methylphenidate like cocaine - studies on their pharmacokinetics and distribution in the human brain, *Archives of General Psychiatry*, 52 (6), 456-463.

Volkow, N. D., Fowler, J. S., Logan, J., Gatley, S. J., Dewey, S. L., Macgregor, R. R., Schlyer, D. J., Pappas, N., King, P., Wang, G. J., and Wolf, A. P., 1995, Carbon-11-cocaine binding compared at subpharmacological and pharmacological doses - a pet study, *Journal of Nuclear Medicine*, 36 (7), 1289-1297.

Volkow, N.D., Fowler, J.S., Wang, G.J., 1999, Imaging studies on the role of dopamine in cocaine reinforcement and addiction in humans, *Journal of Psychopharmacology*, 13 (4), 337 - 345

Volkow, N. D., Wang, G. J., Fischman, M. W., Foltin, R., Fowler, J. S., Franceschi, D., Franceschi, M., Logan, J., Gatley, S. J., Wong, C., Ding, Y.-S., Hitzemann, R., and Pappas, N., 2000, Effects of route of administration on cocaine induced dopamine transporter blockade in the human brain, *Life Sciences*, 67 (12), 1507-1515.

Walsh, N. P., Laing, S. J., Oliver, S. J., Montague, J. C., Walters, R., and Bilzon, J. L. J., 2004, Saliva parameters as potential indices of hydration status during acute dehydration, *Medicine and Science in Sports and Exercise*, 36 (9), 1535-1542.

Warner, E.A., 1993, Cocaine Abuse, *Annals of internal medicine* , 119 (3), 226 – 235

Warner-Smith, M., Darke, S., Lynskey, M., Hall, W., 2001, Review: Heroin overdose: causes and consequences, *Addiction*, 96 (8), 1113 -1125

Wehner, F., Wehner, H., Schieffer, M.C., Subke, J., 2000, Immunohistochemical detection of methadone in the human brain, *Forensic Science International*, 112 (1), 11 – 16

Weil, A.T., 1978, Coca Leaf as a therapeutic agent, *American Journal of Drug and Alcohol Abuse*, 5 (1), 75 – 86

Wetli, C.V, Rao, A., Rao, V.J., 1997, Fatal heroin body packing, *American Journal of Medicine and Pathology*, 18 (3), 312 - 318

White, J., and Irvine, R., 1999, Mechanisms of fatal opioid overdose, *Addiction*, 95 (7), 961 – 972

White, S.M. and Lambe, C.J.T., 2003, The pathophysiology of cocaine abuse, *Journal of Clinical Forensic Medicine*, 10 (1), 27 – 39

Wiesmann, U.N., Boat, T.F., Di Sant'Agnese, P.A., 1972, Flow-rates and electrolytes in minor-salivary-gland saliva in normal subjects and patients with cystic fibrosis, *Lancet*, 2 (7776), 510 – 512

Wilcheck, M., Bayer, E., 1990, Colorimetric enzyme assays for avidin and biotin, *Methods in Enzymology*, 184, 217 - 223

Wilkinson, P., Van Dyke, C., Jatlow, P., Barash, P., Byck, R., 1980, Intranasal and oral cocaine kinetics, *Clinical Pharmacology and Therapeutics*, 27 (3), 386 -394

Winecker, R., and Goldberger, B., 1998. Urine specimen suitability for drug testing. In: Karch, S. B. ed. *Drug of abuse handbook,* Boca Raton, FL: CRC Press, 764 - 772.

Winek, C.L., 1997, Heroin fatality due to penile injection, *American Journal of Medicine and Pathology,* 20 (1), 90 – 92

Wong, R.C., Tran, M. and Tung, J.K., 2005, Oral fluid drug tests: effects of adulterants and foodstuffs, *Forensic Science International*, 150 (2-3), 175 -180

Wood, G.S., and Warnke, R., 1981, Suppression of endogenous avidin-binding activity in tissues and its relevance to biotin-avidin detection systems, *Journal of Histochemistry & Cytochemistry,* 29 (10), 1196 – 1204

Woyceichoski, I.E.C., Costa, C.H., de Araujo, C.M., Brancher, J.A., Resende, L.G., Vieira, I., de Lima, A.A.S., 2013, Salivary buffer capacity, pH , and stimulated flow rate of crack cocaine users, *Journal of Investigative and Clinical Dentistry*, 4 (3), 160 -163

Yaegaki, K., Coil, J., Kamemizu, T. and Miyazaki, H., 2002, Tongue brushing and mouth rinsing as basic treatment measures for halitosis, *International Dental Journal*, 52 (3), 192 – 96

Yeh, S.Y., Gorodetzky, C.W., McQuinn, R.L., 1976, Urinary excretion of heroin and its metabolites in man, *The journal of pharmacology and experimental therapeutics*, 196 (2), 249 - 256

Yotsu-Yamashita, M., Okoshi, N., Watanbe, k., Araki, N., Yamaki, H., Shoji, Y., Terakawa, T., 2013, Localization of pufferfish saxitoxin and tetrodotoxin

binding protein (PSTBP) in the tissues of the pufferfish, *Takifugu paralis,*analyzed by immunohistochemical staining, *Toxicon,* 72, 23 – 28

Zapata-Ortiz, V., 1970, The Chewing of Coca Leaves in Peru, *Substance Use & Misuse,* 5 (2), 287 – 294

Appendix A

Bournemouth University

Research Ethics Checklist

Status	Approved

Researcher Details

Name	Eva Reichardt
School	Faculty of Science & Technology
Status	Postgraduate Research (PhD, MPhil, DProf, DEng)
Course	Postgraduate Research
Have you received external funding to support this research project?	No

Project Details

Title	Investigation of the effects of common food and beverages on oral fluid drug detection
Proposed Start Date	February 2011
Proposed End Date	June 2012

Summary (including detail on background methodology, sample, outcomes, etc.)

As part of this study each volunteer will be requested to consume, or swirl, each of the adulterants listed below around in the mouth. An oral fluid sample will then be collected immediately. The pH (acidity or alkalinity) of the oral fluid sample will be measured. A second oral fluid sample will be collected after 10 minutes. Again, the pH of this sample will be measured.
As part of this study the time it takes for each device to collect a sufficient amount of oral fluid will recorded. Samples will then be stored and send to Cozart Bioscience Ltd. for analysis.

The Intercept device follows a simple process. Firstly, in order for the device to absorb a sufficient amount of oral fluid the device will be kept in the mouth for around 2 to 5 minutes. The pad is then inserted into the vial containing a buffer and the wand is snapped. The sample will then be re-sealed and send to Cozart Bioscience Ltd. for further analysis. The following adulterants have been chosen for this study:

Mouthwash
Toothpaste
Chewing Gum
Sour Sweets (Lemon Sherberts)
Sugar Coated Sweets
Spicy Food
Fried/Oily Food
Apples
Oranges
. Grapes
. Yoghurt
. Fruit Juice (unsweetened)
. Coke
. Coffee
. Tea
. Fruit juice (sweetened)
. Cheese
. Milk
. Vinegar
. Cranberry Juice
Red Bull

External Ethics Review

Does your research require external review through the NHS National Research Ethics Service (NRES) or through another external Ethics Committee?	No

Research Literature

Is your research solely literature based?	No

Human Participants

Will your research project involve interaction with human participants as primary sources of data (e.g. interview, observation, original survey)?	Yes
Does your research specifically involve participants who are considered vulnerable (i.e. children, those with cognitive impairment, those in unequal relationships—such as your own students, prison inmates, etc.)?	No
Does the study involve participants age 16 or over who are unable to give informed consent (i.e. people with learning disabilities)? NOTE: All research that falls under the auspices of the Mental Capacity Act 2005 must be reviewed by NHS NRES.	No
Will the study require the co-operation of a gatekeeper for initial access to the groups or individuals to be recruited? (i.e. students at school, members of self-help group, residents of Nursing home?)	No
Will it be necessary for participants to take part in your study without their knowledge and consent at the time (i.e. covert observation of people in non-public places)?	No
Will the study involve discussion of sensitive topics (i.e. sexual activity, drug use, criminal activity)?	Yes

Are drugs, placebos or other substances (i.e. food substances, vitamins) to be administered to the study participants or will the study involve invasive, intrusive or potentially harmful procedures of any kind?	Yes
Please explain why your research project does not require ethical review by a	
Solid foods and beverages are totally consumed whilst liquids such as mouthwash and vinegar (n = 30 mL) are swirled around the mouth for 30 seconds and then discarded. All substances for testing are not harmful to the volunteer. Volunteers will provide informed consent and can withdraw from the study at any time. Volunteers can also choose not to have a certain substance if they feel in any way uncomfortable in doing	

Will tissue samples (including blood) be obtained from participants? Note: If the answer to this question is 'yes' you will need to be aware of obligations under the Human Tissue Act 2004.	Yes

Please explain why your research project does not require ethical review by a NHS REC.
Samples will be collected using the Intercept Orasure or Concateno Certus Oral Fluid collection device. Both devices are non-invasive and samples will be fully anonymised. Volunteers will be provided with consent. Participation in this research study is voluntary. If volunteers chose to take part, they have the right to stop at any time.

Could your research induce psychological stress or anxiety, cause harm or have negative consequences for the participant or researcher (beyond the risks encountered in normal life)?	No
Will your research involve prolonged or repetitive testing?	No
Will the research involve the collection of audio materials?	No
Will your research involve the collection of photographic or video materials?	No
Will financial or other inducements (other than reasonable expenses and compensation for time) be offered to participants?	No

Please explain below why your research project involves the above mentioned criteria (be sure to explain why the sensitive criterion is essential to your project's success). Give a summary of the ethical issues and any action that will be taken to address these. Explain how you will obtain informed consent (and from whom) and how you will inform the participant(s) about the research project (i.e. participant information sheet). A sample consent form and participant information sheet can be found on the Research Ethics website.
The non-invasive sample collected will be fully anonymised. Before participating in the study, participants will be provided with a patient information sheet that details the purpose of the study, why it is important to undertake the work, what will happen to them and how the information they provide will be used. If the participants are willing to volunteer for the study they will be asked to sign a consent form. The participants in this study will all be over 18 and able to read and write English. It is important to. Any information the participants provide will be kept strictly confidential and all data will be fully anonymised. (Consent form attached below)

Final Review

Will you have access to personal data that allows you to identify individuals OR access to confidential corporate or company data (that is not covered by confidentiality terms within an agreement or by a separate confidentiality agreement)?	No
Will your research involve experimentation on any of the following: animals, animal tissue, genetically modified organisms?	No
Will your research take place outside the UK (including any and all stages of research: collection, storage, analysis, etc.)?	No

Please use the below text box to highlight any other ethical concerns or risks that may arise during your research that have not been covered in this form.

Consent Form for Collection of Oral Fluid

Bournemouth University, United Kingdom

Title of Project: Investigation of the effects of Adulterants and Food on Oral Fluid Drug Test Devices (Intercept and Cozart devices)

Principal Investigators:

- Professor David Osselton, Director of the Centre for Forensic Sciences, University of Bournemouth.
- Eva—Maria Reichardt, Bournemouth University
- Dr Dene Baldwin, Technical Director, Cozart Bioscience Ltd.

This is a research study. Research studies include only people who want to take part and you do not have to give consent for participation if you do not wish to do so. Your oral fluid sample is being collected to act as a control from someone who does not participate in recreational drug use. This form provides information about this research, which will be discussed with you. It may contain words or procedures that you do not understand. Please ask questions about anything that is unclear to you. Discuss it with your family and friends if you wish and take your time to make your decision.

1. **Purpose of the Research**

   Oral fluid drug testing has become increasingly popular over the past years. Oral fluid drug testing is a non-invasive process which can be administered quickly anytime and anywhere. The process of oral fluid testing is reduces risk of tampering as the process can be monitored directly. Adulterants such as food are used as dilution tactics in order to beat the oral fluid drug tests. This study will look at the effects of common foods and adulterants on oral fluid drug testing procedures. In this study the effects of the adulterants on the "Intercept" and "Cozart" oral fluid collection devices.

   As part of this study each volunteer will be requested to consume, or swirl, each of the adulterants listed below around in the mouth. An oral fluid sample will then be collected immediately. The pH (acidity or alkalinity) of the oral fluid sample will be measured. A second oral fluid sample will be collected after 10 minutes. Again, the pH of this sample will be measured.

As part of this study the time it takes for each device to collect a sufficient amount of oral fluid will recorded. Samples will then be stored and send to Cozart Bioscience Ltd. for analysis.

The Intercept device follows a simple process. Firstly, in order for the device to absorb a sufficient amount of oral fluid the device will be kept in the mouth for around 2 to 5 minutes. The pad is then inserted into the vial containing a buffer and the wand is snapped. The sample will then be re-sealed and send to Cozart Bioscience Ltd. for further analysis. You will also be requested to provide details of your age, sex and whether you are taking any drugs or medications.

The following adulterants have been chosen for this study:

21. Mouthwash
22. Toothpaste
23. Chewing Gum
24. Sour Sweets (Lemon Sherberts)
25. Sugar Coated Sweets
26. Spicy Food
27. Fried/Oily Food
28. Apples
29. Oranges
30. Grapes
31. Yoghurt
32. Fruit Juice (unsweetened)
33. Coke
34. Coffee
35. Tea
36. Fruit juice (sweetened)
37. Cheese
38. Milk
39. Vinegar
40. Cranberry Juice
41. Red Bull

2. Discomforts and Risks:

One risk of giving samples for this research may be the release of your name that could be linked to the stored samples and/or the results of the tests run on the samples. To prevent this, these samples will be given a unique identification code. Only the study staff will know the code. The name that belongs to the code will be kept in a locked file or in a computer with a password. Only authorized members of the research team will have access to your name.

3. Statement of Confidentiality:

a. Privacy and confidentiality measures

- Each sample will be given a unique code for which the samples will hereby be known.
- Samples are going to be analysed at Cozart Bioscience Ltd. The information provided throughout this study i.e. (sex, health, drug use) will not identify you by name.

The results of this research study may be presented at scientific / medical meetings or in scientific / medical publications. The identity of donors will not be disclosed in those presentations.

If you agree, samples and results will be stored along with information about yourself obtained in the course of this research study (age, sex, health, drug / medication use). The information stored will not identify you by name.

If you agree to participate in this study you will receive a signed and dated copy of this consent form for your records.

In the event of any publication or presentation resulting from the research, no identifiable information will be shared.

We will keep participation in this research study confidential to the extent permitted by law. However, it is possible that other people may become aware of study participation. For example, the following people/groups may inspect and copy records pertaining to this research.

- The Bournemouth University Institutional Ethics Review Board (a committee that reviews and approves research studies)

The possibility of identification is very low and every effort will be made to keep your personal information in the research record private and confidential but absolute confidentiality cannot be guaranteed.

4. Costs for Participation:

a. Costs:

There are **NO** costs for participation in this research

b. Rights:

Signing this form will lose no legal rights.

c. Compensation:

No payment or compensation will be received for participation in this research study.

5. Voluntary Participation / Withdrawal:

Participation in this research study is voluntary. You do not have to participate in this research. If you choose to take part, you have the right to stop at any time. If it is decided not to participate or if it is decided to stop participation in the research at a later date, there will be no penalty.

6. Contact Information for Questions or Concerns:

You have the right to ask any questions you may have about this research. If you have questions, complaints or concerns about this research, contact Professor David Osselton, Centre for Forensic Sciences, Bournemouth University.

Consent:

I have read the information in this consent form. All my questions about this study and my participation in it have been answered to my satisfaction.

I authorize the collection of oral fluid samples for analysis for the purpose of the study by the investigation team at Bournemouth University.

By signing this consent form, no legal rights have been waived, which would otherwise have been had for participation in a research study.

Please initial the following option:

I would like scientists to study my oral fluid sample only to complete the study outlined above and then to discard the oral fluid sample so that other studies cannot be done.

Signature_____ Date_____

Name (please print) _____

Signature of Person Conducting
Informed Consent Discussion_____

Date_____

Name (please print) _____

Signature of Witness _____ Date _____

(Required)

Name (please print) _____

Appendix B

Research Ethics Checklist

Status	Approved

Researcher Details

Name	Eva Reichardt
School	School of Applied Sciences
Status	Postgraduate Research (PhD, MPhil, DProf, DEng)
Course	Postgraduate Research
Have you received external funding to support this research project?	No

Project Details

Title	Concentrations of cocaine, benzoylecgonine and ecgonine methyl ester in oral fluid following the consumption of coca tea
Proposed Start Date	February 2011
Proposed End Date	December 2012

Summary (including detail on background methodology, sample, outcomes, etc.)

Most scientific studies examining oral fluid cocaine appearance have been restricted to relatively small doses of drug for ethical and safety reasons. For the purpose of this study, oral fluid samples following the consumption of coca tea will be collected in Lima, Peru. Coca tea is widely consumed in a number of South American countries where its use is both legal and socially acceptable. The tea is prepared from an infusion of the leaves obtained from the coca bush *Erythroxylum coca* . Following local custom, coca tea will be prepared by soaking 10 whole coca leaves in boiling water for 5 minutes prior to straining to remove the leaf tissues. Volunteers will be asked to drink 1 cup (~250 mL) of coca tea immediately post straining of the leaves over a period of approximately 5 minutes and afterwards provide a sample of oral fluid immediately following completion of tea drinking. Oral fluid will subsequently collected at 15, 30, 45 and 60 minute intervals. Oral fluid collection was facilitated using the Alere^{TM} Certus device

External Ethics Review

Does your research require external review through the NHS National Research Ethics Service (NRES) or through another external Ethics Committee?	No

Research Literature

Is your research solely literature based?	No

Human Participants

Will your research project involve interaction with human participants as primary sources of data (e.g. interview, observation, original survey)?	Yes
Does your research specifically involve participants who are considered vulnerable (i.e. children, those with cognitive impairment, those in unequal relationships—such as your own students, prison inmates, etc.)?	No
Does the study involve participants age 16 or over who are unable to give informed consent (i.e. people with learning disabilities)? NOTE: All research that falls under the auspices of the Mental Capacity Act 2005 must be reviewed by NHS NRES.	No
Will the study require the co-operation of a gatekeeper for initial access to the groups or individuals to be recruited? (i.e. students at school, members of self-help group, residents of Nursing home?)	No

Will it be necessary for participants to take part in your study without their knowledge and consent at the time (i.e. covert observation of people in non-public places)?	No
Will the study involve discussion of sensitive topics (i.e. sexual activity, drug use, criminal activity)?	Yes

Are drugs, placebos or other substances (i.e. food substances, vitamins) to be administered to the study participants or will the study involve invasive, intrusive or potentially harmful procedures of any kind?	Yes
Please explain why your research project does not require ethical review by a NHS REC.	
Coca tea is widely consumed in a number of South American countries where its use is both legal and socially acceptable. The tea is prepared from an infusion of the leaves obtained from the coca bush *Erythroxylum coca. The consumption of coca tea is legal in Peru and tea only contains low concentrations of cocaine which should not result in impairment.* The amount of cocaine and its metabolites present in coca leave can vary depending on species and origin of the *Erythroxylum* plant (Jenkins et al 1996). The Peruvian *Erythroxylum* species, as used by Jenkins et al, contains 5.11 mg of cocaine, 0.11 mg of benzoylecgonine and 1.15 mg of ecgonine methyl ester in 1 g of leaf material. The tea will be prepared by a Peruvian colleague, Karina Gerdau-Radonic, which will ensure that the tea is prepared correctly.	

Will tissue samples (including blood) be obtained from participants? Note: If the answer to this question is 'yes' you will need to be aware of obligations under the Human Tissue Act 2004.	Yes
Please explain why your research project does not require ethical review by a NHS REC	
Samples will be collected using the Concateno Certus Oral Fluid collection device. Both devices are non-invasive and samples will be fully anonymised. Volunteers will be provided with consent. Participation in this research study is voluntary. If volunteers chose to take part, they have the right to stop at any time.	

Could your research induce psychological stress or anxiety, cause harm or have negative consequences for the participant or researcher (beyond the risks encountered in normal life)?	No
Will your research involve prolonged or repetitive testing?	No

Will the research involve the collection of audio materials?	No
Will your research involve the collection of photographic or video	No
Will financial or other inducements (other than reasonable expenses and compensation for time) be offered to participants?	No

Please explain below why your research project involves the above mentioned criteria (be sure to explain why the sensitive criterion is essential to your project's success). Give a summary of the ethical issues and any action that will be taken to address these. Explain how you will obtain informed consent (and from whom) and how you will inform the participant(s) about the research project (i.e. participant information sheet). A sample consent form and participant information sheet can be found on the Research Ethics website.
The non-invasive sample collected will be fully anonymised. Before participating in the study, participants will be provided with a patient information sheet that details the purpose of the study, why it is important to undertake the work, what will happen to them and how the information they provide will be used. If the participants are willing to volunteer for the study they will be asked to sign a consent form. For those volunteers who do not speak and understand fluent English, the consent form will be translated to Spanish. Karina Gerdau-Radonic will be present at all times throughout this study in order to be able to translate and assist in answering any questions or concerns. The participants in this study will all be over 18. Any information the participants provide will be kept strictly confidential and all data will be fully anonymised. (Consent form attached below)

Final Review

Will you have access to personal data that allows you to identify individuals OR access to confidential corporate or company data (that is not covered by confidentiality terms within an agreement or by a separate confidentiality agreement)?	No
Will your research involve experimentation on any of the following: animals, animal tissue, genetically modified organisms?	No
Will your research take place outside the UK (including any and all stages of research: collection, storage, analysis, etc.)?	No

Please use the below text box to highlight any other ethical concerns or risks that may arise during your research that have not been covered in this form.

Consent Form for Collection of Oral Fluid following Consumption of Coca Tea

Bournemouth University, Inglaterra, Reino Unido

Investigadores Principales:

- Professor David Osselton, Director, Centro de Ciencias Forenses y Biologicas, Bournemouth University
- Eva—Maria Reichardt, Bournemouth University
- Benni Juckes, Bournemouth University

Este es un proyecto de investigación y su participacion es voluntaria. No tiene que hacerlo si asi no lo desea.

El siguiente documento expica la naturaleza del proyecto y el por que se estan colectando muestras de saliva. Si contiene palabras o expresiones que no entienda, por favour pida una aclaracion.

Proposito de la Investigacion

La hoja de coca contiene varios alcaloides, entre elleos "cocaine". Estudios con otras substancias han demonstrado que estas se pueden acumular en los tejidos bucales despues de su consume oral, lo que llevaria a indicar que se pueden formar depositos de estas substancias en la boca. Este studio busca investigar si el consume de mate de coca conlleva la formacion de depositos de "alcaloides" en los tejidos bucales.

Metodo

Este studio se lleva a cabo de forma anonima. Cada voluntario recibira un codigo de identificacion unico para proteger su identidad. Se les pedira solamente sue dad, sexo e historial medico y de drogas. Toda informacion sera tratada de manera confidencial.

1. Las muestras bucales se tomaran por medio de un hisopo
2. Una muestra se tomara antes del consume de mate de coca
3. Despues se ofrecera el mate de coca

4. Luego de beber el mate se tomaran muestras a los 0,15,30, 45 y 60 minutos despues del consume

Las muestras seran analizadas por la empresa Concateno, ubicada en Abingdon, Inglaterra, Reino Unido.

Confidencialidad

- Cada muestra llevara un codigo unico de identificacion
- Ningun dato proporionado (sexo, edad, historical medico, consume de drogas) estara relacionado con su nombre
- Los resultados de este studio pueden ser presentados en conferencias profesionales pueden ser publicados. En ningun momento se identificara a los participantes por sus nombres.

Contactenos

Eva Reichardt, Centre for Forensic and Biological Sciences, Bournemouth University, Inglaterra, Reino Unido (ereichardt@bournemouth.ac.uk)

Autorizacion

He leído este document y mis preguntas han sido respondidas.

Autorizo que mis muestras de saliva sean usadas en el marco de este proyecto de investigación, y que no sirvan para otros estudios.

Nombre (en letra de imprenta) _____

Firma _____ Fecha _____

Appendix C

Non-cross reacting compounds for the Alere™ Cocaine Metabolite EIA Kit (Chapter 4)

Benzoylecgonine		
6 Monoacetylmorphine	Fentanyl	Norpropoxyphene
Alprazolam	Heroin	Oxymorphone
Amobarbital	Hexobarbital	Pentazocine
Barbital	Ibuprofen	Pentobarbital
Butabarbital	Ketamine	Phentermine
Caffeine	LAAM	(+) Pseudoephedrine
Carbamazepine	Lidocaine	(-) Pseudoephedrine
Chlordiazepoxide	Lorazepam	Phenylpropanolamine
Clonazepam	MDEA	PCP
Codeine	MBDB	Ranitidine
Cyclobenzaprine	MDMA	Salicylate
D9-THC	MDA	Secobarbital
Dextromethorphan	Meperidine	Temazepam
Dextropropoxyphene	Medazepam	Tramadol
Diazepam	Mephobarbital	Tyramine
Diphenhydramine	Methaqualone	Venlafaxine
(+) Ephedrine	Morphine	Zolpidem
(-) Ephedrine	Naltrexone	
EMDP	Naloxone	
EDDP	11-nor-9-Carboxy-D9-THC	

Appendix D

Bournemouth University

Research Ethics Checklist

Status	Approved

Researcher Details

Name	Eva Reichardt
School	School of Applied Sciences
Status	Postgraduate Research (PhD, MPhil, DProf, DEng)
Course	Postgraduate Research
Have you received external funding to support this research project?	No

Project Details

Title	Oral fluid opiate concentrations following oral consumption of Codeine Linctus® and Collis Browne's® mixture
Proposed Start Date	February 2011
Proposed End Date	December 2012

Summary (including detail on background methodology, sample, outcomes, etc.)

The purpose of the work described in this was to examine drug deposition in oral fluid and potentially the surface of the mouth cavity by investigating concentrations of opiates following oral consumption of Codeine Linctus® ® and Collis Browne's® ® mixture. The elevated drug concentrations in oral fluid over a prolonged period suggest that drugs such as cocaine and opioids can bind to oral tissue. Collected oral fluid can contain cell and food debris which can be collected by the abrasion of the collector against the oral tissues. The presence of drug depots in tissues suggests that the cell debris present in oral fluid also contains drugs, which can potentially contribute to and hence increase oral fluid concentrations. The work undertaken in this chapter aimed to investigate characteristics of deposition in the oral fluid using observed concentrations such as the length of time drug can be detected in oral fluid and the effect of cell abrasion during collection. Volunteers will be invited to swirl 5 mL of Collis Browne's® or Codeine Linctus® around the mouth for one minute before discarding the liquid from the mouth. The calculated dose for Codeine Linctus® was 15 mg and 5 mg for Collis Browne's® mixture. Oral fluid was subsequently collected at 15, 30, 45, 60, 90, 120, 150 and 180 minutes after mouth emptying using the Alere™ Certus oral fluid collection device. Oral fluid was collected in accordance with the kit manufacturer's instructions. In addition to the collection according to the manufacturer's guidelines, a second Alere™ Certus collector was brushed against the oral cavity to identify whether the increased presence of cellular debris can increase the amount of drug detected in oral fluid. Brushing was undertaken until the adequacy indicator showed that sufficient oral fluid had been collected.

External Ethics Review

Does your research require external review through the NHS National Research Ethics Service (NRES) or through another external Ethics Committee?	No

Research Literature

Is your research solely literature based?	No

Human Participants

Will your research project involve interaction with human participants as primary sources of data (e.g. interview, observation, original survey)?	Yes

Does your research specifically involve participants who are considered vulnerable (i.e. children, those with cognitive impairment, those in unequal relationships—such as your own students, prison inmates, etc.)?	No
Does the study involve participants age 16 or over who are unable to give informed consent (i.e. people with learning disabilities)? NOTE: All research that falls under the auspices of the Mental Capacity Act 2005 must be reviewed by NHS NRES.	No
Will the study require the co-operation of a gatekeeper for initial access to the groups or individuals to be recruited? (i.e. students at school, members of self-help group, residents of Nursing home?)	No
Will it be necessary for participants to take part in your study without their knowledge and consent at the time (i.e. covert observation of people in non-public places)?	No
Will the study involve discussion of sensitive topics (i.e. sexual activity, drug use, criminal activity)?	Yes

Are drugs, placebos or other substances (i.e. food substances, vitamins) to be administered to the study participants or will the study involve invasive, intrusive or potentially harmful procedures of any kind?	Yes
Please explain why your research project does not require ethical review by a NHS REC.	

Collis Browne's mixture and Codeine Linctus are available over the counter in most UK pharmacies. Collis Browne's® mixture, manufactured by Thornton & Ross Ltd, Huddersfield, UK contains two principal active ingredients, morphine hydrochloride at a dose equivalent to 1.0 mg/5 mL anhydrous morphine and 1.5 µL/5mL of peppermint oil. Collis Browne's® mixture also contains ethanol, benzoic acid, sorbitol sucrose and fructose. Collis Browne's® mixture is available for purchase as an over-the-counter medication. Codeine Linctus®, Care+, Thornton & Ross Ltd, Huddersfield, UK, contains only one active drug ingredient, Codeine phosphate at a dose of 15 mg/5 mL. Other ingredients include citric acid monohydrate, ethyl and propyl parahydroxybenyzoates, sodium methyl, ethanol, sugars, food colourings and flavouring. Codeine Linctus® is available for purchase as an over-the-counter medication. Both Collis Brownes mixture and Codeine Linctus will be administered at a dose as recommended by the manufacturer. Additionally, both substances will be discarded from the mouth prior to oral fluid collection.

Will tissue samples (including blood) be obtained from participants? Note: If the answer to this question is 'yes' you will need to be aware of obligations under the Human Tissue Act 2004.	Yes
Please explain why your research project does not require ethical review by a NHS REC.	
Samples will be collected using the Concateno Certus Oral Fluid collection device. Both devices are non-invasive and samples will be fully anonymised. Volunteers will be provided with consent. Participation in this research study is voluntary. If volunteers chose to take part, they have the right to stop at any time.	

Could your research induce psychological stress or anxiety, cause harm or have negative consequences for the participant or researcher (beyond the risks encountered in normal life)?	No
Will your research involve prolonged or repetitive testing?	No
Will the research involve the collection of audio materials?	No
Will your research involve the collection of photographic or video	No
Will financial or other inducements (other than reasonable expenses and compensation for time) be offered to participants?	No
Please explain below why your research project involves the above mentioned	

criteria (be sure to explain why the sensitive criterion is essential to your project's success). Give a summary of the ethical issues and any action that will be taken to address these. Explain how you will obtain informed consent (and from whom) and how you will inform the participant(s) about the research project (i.e. participant information sheet). A sample consent form and participant information sheet can be found on the Research Ethics website.
The non-invasive sample collected will be fully anonymised. Before participating in the study, participants will be provided with a patient information sheet that details the purpose of the study, why it is important to undertake the work, what will happen to them and how the information they provide will be used. If the participants are willing to volunteer for the study they will be asked to sign a consent form (consent form attached below). The participants in this study will all be over 18. Any information the participants provide will be kept strictly confidential and all data will be fully anonymised.

Final Review

Will you have access to personal data that allows you to identify individuals OR access to confidential corporate or company data (that is not covered by confidentiality terms within an agreement or by a separate confidentiality agreement)?	No
Will your research involve experimentation on any of the following: animals, animal tissue, genetically modified organisms?	No
Will your research take place outside the UK (including any and all stages of research: collection, storage, analysis, etc.)?	No

Please use the below text box to highlight any other ethical concerns or risks that may arise during your research that have not been covered in this form.

Consent Form for Collection of Oral Fluid and Buccal Swabs

Bournemouth University, United Kingdom

Title of Project: Investigation of the effects of oral contamination on Interpretation of Opiate Oral Fluid Results

Principal Investigators:

- Professor David Osselton, Director of the Centre for Forensic Sciences, University of Bournemouth.
- Eva—Maria Reichardt, Bournemouth University
- Benni Juckes, Bournemouth University

This is a research study. Research studies include only people who want to take part and you do not have to give consent for participation if you do not wish to do so. Your oral fluid sample is being collected to act as a control from someone who does not participate in recreational drug use. This form provides information about this research, which will be discussed with you. It may contain words or procedures that you do not understand. Please ask questions about anything that is unclear to you. Discuss it with your family and friends if you wish and take your time to make your decision.

2. Purpose of the Research

Oral fluid drug testing has become increasingly popular over the past years. This is largely due to the advantages it has over conventional matrices such as blood and urine. Oral fluid collection is an observed, non-invasive proves that can be undertaken in any location and is less susceptible to adulteration. Oral fluid results are extremely elevated by the presence of oral contamination caused by smoked, insufflated or orally administered drugs. This contamination is thought to cause formation of drug depots in cheek tissue. Furthermore, blood contamination through induction of microbleeding will also be investigated as this also has the potential to increase oral fluid drug concentrations. This study will look at the effects of drug depots and blood contamination on oral fluid drug test results and their interpretation.

As part of this study volunteers will be requested to consume a 15 milligram codeine tablet, swill liquid codeine and swill J. Collis Browne's mixture in their mouth and spit it out. Attendance at multiple sessions will be required

and volunteers are requested not to eat within the half hour preceding each session. An oral fluid sample will be collected 15 minutes prior to administration and consecutive samples along with buccal swabs from the inside of the cheek will be taken following administration. These will be collected at the following time points: 15, 30 minutes, 1, 1.5, 2, 2.5 and 3 hours.

The Concateno Certus device will be used for collection of oral fluid. This process is non-invasive and will involve the device being kept in the mouth for approximately 2 minutes or until the detector turns blue indicating a sufficient volume has been collected. The pad is then inserted into a vial containing buffer. Samples will then be stored and analysed at the University laboratory.

You will also be requested to fill in a questionnaire providing details of your age, sex and whether you are taking any drugs or medications. The concurrent use of two or more central nervous system depressant drugs can increase drowsiness and reduce alertness. These drugs include opioid analgesics, alcohol, antidepressants, antiepileptics, antihistamines antipsychotics, anxiolytics, hypnotics and skeletal muscle relaxants. Volunteers currently taking medication containing opiates or any of the drugs mentioned above and who have allergies to morphine or codeine will not be eligible to take part in this study.

7. Discomforts and Risks:

One risk of giving samples for this research may be the release of your name that could be linked to the stored samples and/or the results of the tests run on the samples. To prevent this, these samples will be given a unique identification code. Only the study staff will know the code. The name that belongs to the code will be kept in a locked file or in a computer with a password. Only authorized members of the research team will have access to your name.

Another potential risk is the ingestion of codeine. Codeine is a medication available over the counter for use in mild to moderate pain relief and as a cough suppressant. Volunteers will be required to take a single 15 milligram tablet. Participants are warned that these medicines may cause drowsiness. If affected do not drive or operate machinery. Alcohol should be avoided. This low dosage and route of administration will minimise any potential adverse drug effects. J. Collis Browne's is an opium tincture containing morphine that for the purpose of

this study, along with the codeine linctus, will not be swallowed therefore risk of adverse effects should be minimal.

8. Statement of Confidentiality:

a. Privacy and confidentiality measures

- Each sample will be given a unique code for which the samples will hereby be known.
- Samples are going to be analysed at Bournemouth University. The information provided throughout this study i.e. (sex, health, drug use) will not identify you by name.

The results of this research study may be presented at scientific / medical meetings or in scientific / medical publications. The identity of donors will not be disclosed in those presentations.

If you agree, samples and results will be stored along with information about yourself obtained in the course of this research study (age, sex, health, drug / medication use). The information stored will not identify you by name.

If you agree to participate in this study you will receive a signed and dated copy of this consent form for your records.

In the event of any publication or presentation resulting from the research, no identifiable information will be shared.

We will keep participation in this research study confidential to the extent permitted by law. However, it is possible that other people may become aware of study participation. For example, the following people/groups may inspect and copy records pertaining to this research.

- The Bournemouth University Institutional Ethics Review Board (a committee that reviews and approves research studies)

The possibility of identification is very low and every effort will be made to keep your personal information in the research record private and confidential but absolute confidentiality cannot be guaranteed.

9. Costs for Participation:

a. Costs:

There are **NO** costs for participation in this research

b. Rights:

Signing this form will lose no legal rights.

c. Compensation:

No payment or compensation will be received for participation in this research study.

10. Voluntary Participation / Withdrawal:

Participation in this research study is voluntary. You do not have to participate in this research. If you choose to take part, you have the right to stop at any time. If it is decided not to participate or if it is decided to stop participation in the research at a later date, there will be no penalty.

11. Contact Information for Questions or Concerns:

You have the right to ask any questions you may have about this research. If you have questions, complaints or concerns about this research, contact Professor David Osselton, Centre for Forensic Sciences, Bournemouth University.

Consent:

I have read the information in this consent form. All my questions about this study and my participation in it have been answered to my satisfaction.

I authorize the collection of oral fluid samples for analysis for the purpose of the study by the investigation team at Bournemouth University.

By signing this consent form, no legal rights have been waived, which would otherwise have been had for participation in a research study.

Please initial the following option:

I would like scientists to study my oral fluid sample only to complete the study outlined above and then to discard the oral fluid sample so that other studies cannot be done.

Signature_____ Date_____

Name (please print) _____

Signature of Person Conducting

Informed Consent Discussion_____

Date_____

Name (please print) _____

Signature of Witness _____ Date _____

(Required)

Name (please print) _____

Appendix E

Non-cross reacting compounds for the Alere™ Opiate Metabolite EIA Kit (Chapter 5)

Methadone		
Alprazolam	Fentanyl	Norburprenorphine
Amobarbital	Hexobarbital	Norpropoxyphene
Barbital	Ibuprofen	Pentazocine
Benzoylecgonine	Ketamine	Pentobarbital
Butabarbital	LAAM	Phenobarbial
Caffeine	Lidocaine	Phentermine
Carbamazepine	LSD	(+) Pseudoephedrine
Chlordiazepoxide	Lorazepam	(-) Pseudoephedrine
Clonazepam	Medazepam	Phenylpropanolamine
Cocaethylene	Mephobarbital	PCP
Cocaine	Methaqualone	Salicylate
D9-THC	MDEA	Secobarbital
Dextropropoxyphene	MBDB	Temazepam
Diazepam	MDA	Tramadol
(+) Ephedrine	MDMA	Tyramine
(-) Ephedrine	Naltrexone	Venlafaxine
EMDP	Naloxone	Zolpidem
EDDP	11-nor-9-Carboxy-D9-THC	

Appendix F

Protocol for the production of 1000 mL synthythic saliva (Cozart Bioscience 2008) (Chapter 7).

Materials Required	Quantity	Supplier
De-ionised water	800 mL	Fisher Scientific
Di-Sodium hydrogen orthophosphate anhydrous	2.3 g	BDH
Potassium dihydrogen orthophosphate	0.4 g	BDH
Sodium Chloride	8.77 g	BDH
Bovine Serum Albumin (BSA) (1.0 % w/v)	3.0 g	Sigma - Aldrich
Glucose	0.01 g	Fisher Scientific
Mucin	0.5 g	Sigma - Aldrich
Amylase Type II - A	0.25 g	Sigma - Aldrich
Sodium Azide (0.05 %)	0.5 g	BDH
5-Bromo-5-Nitro-1,3 Dioxane	0.1 g	Sigma - Aldrich
Polyvinyl Alcohol	4.5 g	Sigma - Aldrich
De-ionised water	Make up to 1000 mL	Fisher Scientific
Preparation instructions: 1. Add the above ingredients but allow the BSA to dissolve before adding additional ingredients 2. Mix on a magnetic stirrer until the PVA has dissolved		

Appendix G

Cross reactivity data for the monoclonal benzoylecgonine antibody used for the detection of cocaine during immunohistochemical staining (Chapter 7)

Compounds Cross Reactants	Concentration ng/mL	Apparent Benzoylecgonine	Reactivity (%)
Cocaine	300	277.1	92.4
	50	46.4	92.8
	10	10.2	102.0
Cocaethylene	300	290.9	97.0
	50	59.9	119.7
	10	13.0	129.7

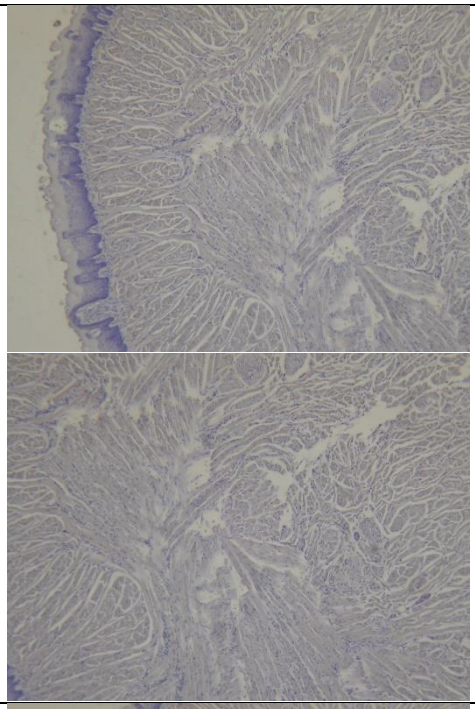
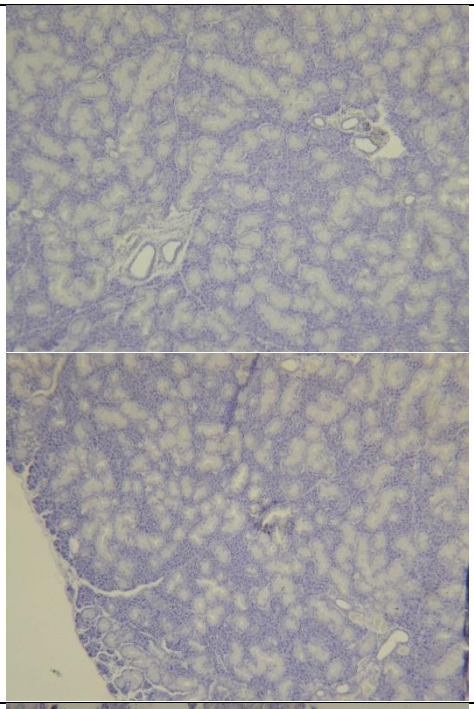
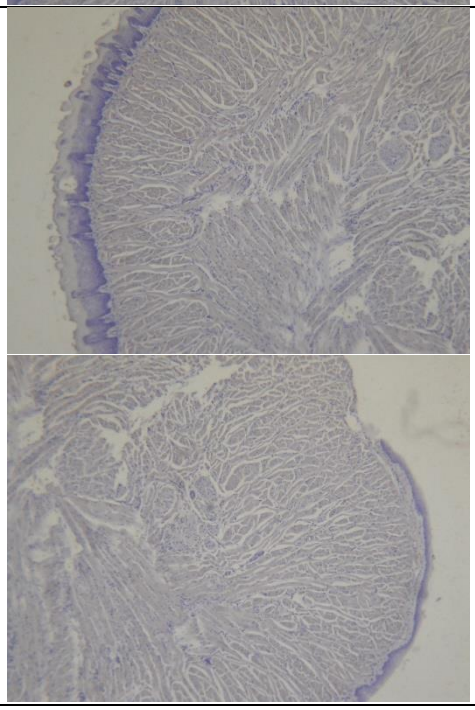
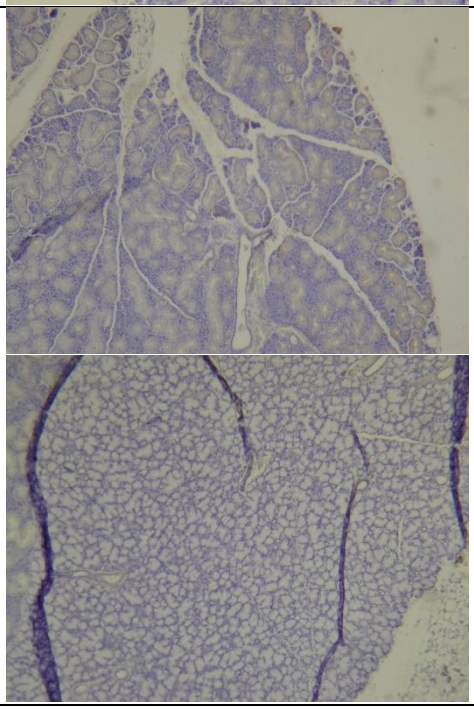
Appendix H

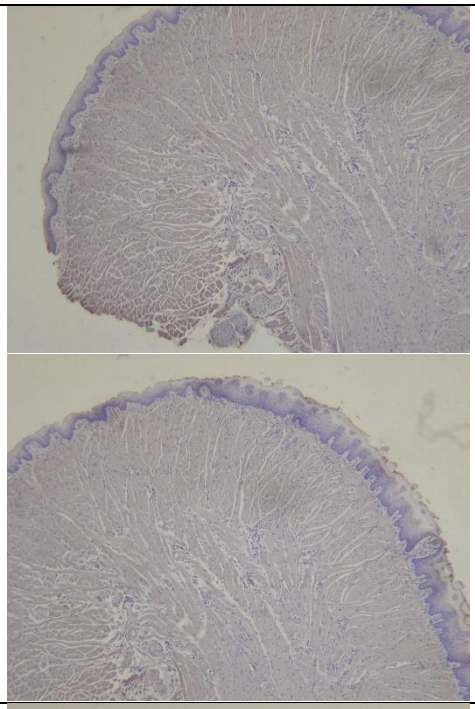
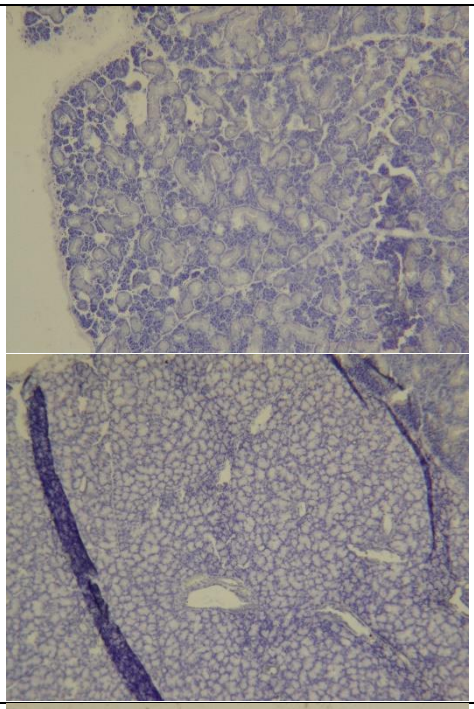
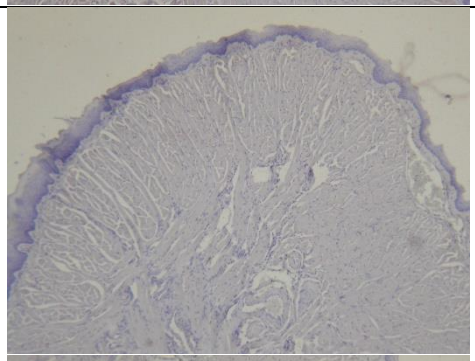
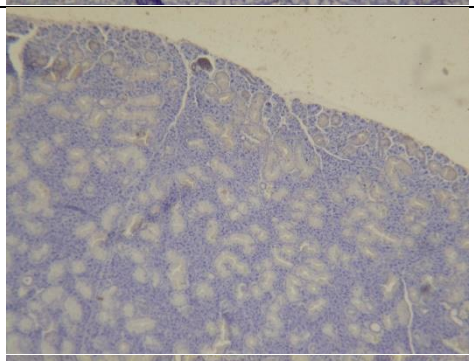
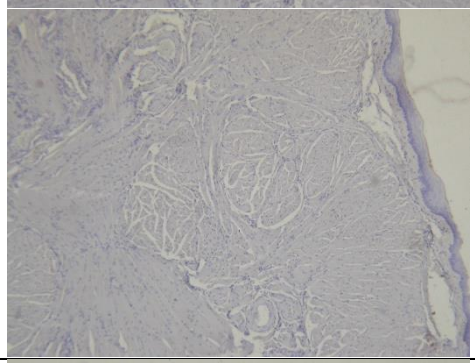
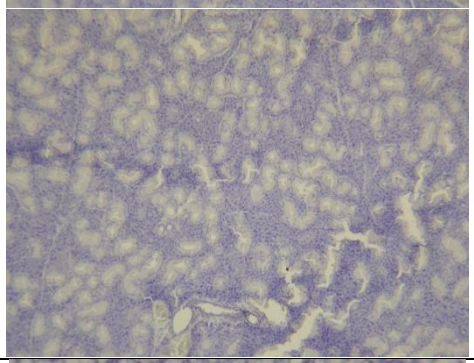
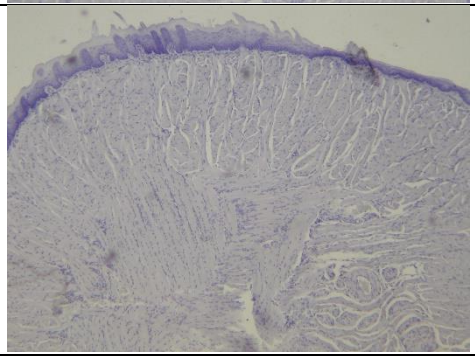
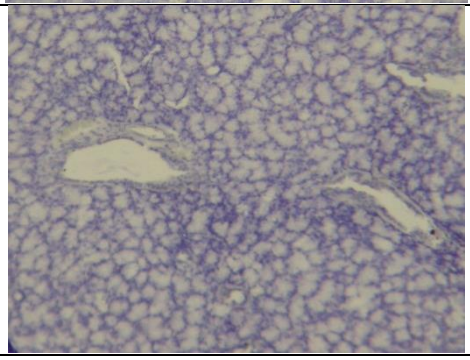
Cross reactivity data for the monocolonal morphine antibody used for the detection of cocaine during immunohistochemical staining (Chapter 7)

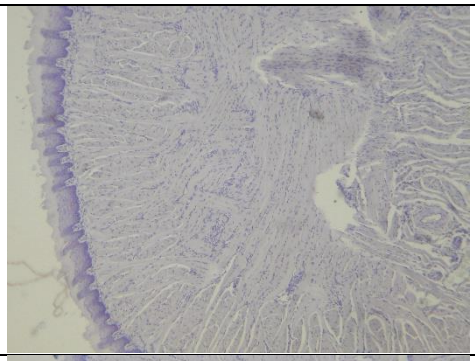
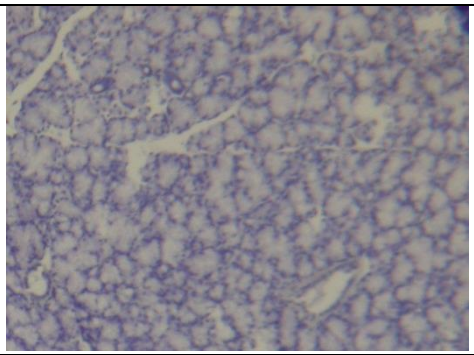
Compounds Cross Reactants	Concentration ng/mL	Apparent morphine	Reactivity (%)
6 MAM	500	434	86.8
	100	88	88.0
	10	9.3	93.0
	5	4.7	94.0
Codeine	100	186	186.0
	10	18	180.0
	5	9	180.0
	1	2	200.0
Dihydrocodeine	500	432	86.4
	100	93	93.0
	10	10	100.0
	5	5	100.0
Heroin	500	623	124.6
	100	81	81.0
	10	8	80.0
	5	5	100.0
	1	2	200.0
Morphine 3 glucuronide	500	532	106.4
	100	150	150.0
	10	10	100.0
	5	5	100.0

Appendix I

Images of stained slides of mice injected with a saline control using an immunohistochemical technique (Chapter 9)

Mice	Tongue of Saline group	Stained section (salivary gland)
1		
2		

Mice	Tongue of Saline group	Stained section (salivary gland)
3		
4		
		
5		

Mice	Tongue of Saline group	Stained section (salivary gland)
		
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