Searching for Potential Markers for Monitoring the Presence of Opiates in Urine Exposed to Oxidising Adulterants

By

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Certificate of authorship and originality

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of the requirements for a degree except as fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all the information sources and literature used are indicated in the thesis.

Susan Luong

DATE

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Abbreviations

1D	one-dimensional
^{1}H-NMR	one-dimensional proton NMR
^{1}H-^{1}H COSY	two-dimensional correlation spectroscopy NMR
^{1}H-^{13}C HSQC	heteronuclear single quantum coherence spectroscopy
^{1}H-^{13}C HMBC	heteronuclear multiple bond correlation spectroscopy
2D	two-dimensional
2-nitro-M6G	2-nitro-morphine-6-glucuronide
2-nitro-MAM	2-nitro-6-monoacetylmorphine
2-nitro-MAM-TMS	trimethylsilyl derivative of 2-nitro-MAM
3-MAM	3-monoacetylmorphine
6-MAM	6-monoacetylmorphine
6-MAM-TMS	trimethylsilyl derivative of 6-MAM
AIDDC	Australian Illicit Drug Data Centre
APCI	atmospheric pressure chemical ionisation
AS/NZS 4308	Australian/New Zealand Standard™ 4308
BSTFA	*N,O*-bis(trimethylsilyl)trifluoroacetamide
C6G	codeine-6-glucuronide

$CDCl_3$	deuterated chloroform
CD_3OD	deuterated methanol
CEDIA	cloned enzyme donor immunoassay
CID	collision induced dissociation
CNS	central nervous system
DEA	Drug Enforcement Administration
DPC	diphenylcarbazide
EIC	extracted ion chromatogram
EI-MS	electron impact-mass spectrometer
ELISA	enzyme linked immunosorbent assay
EMIT	enzyme multiplied immunoassay
EPO	erythropoietin
ESI	electrospray ionisation
ESI-MS	electrospray ionisation-mass spectrometry
FPIA	fluorescence polarisation immunoassay
GC-MS	gas chromatography-mass spectrometry
h	hour(s)
HCl	hydrochloric acid

HPLC	high performance liquid chromatography
ICP-MS	inductively coupled plasma-mass spectrometry
KNO_2	potassium nitrite
LC	liquid chromatography
LC-MS	liquid chromatography-mass spectrometry
LC-MS/MS	liquid chromatography tandem mass spectrometry
LLE	liquid-liquid extraction
M3G	morphine-3-glucuronide
M6G	morphine-6-glucuronide
MALDI	matrix assisted laser desorption ionisation
MeOH	methanol
min	minutes
MRE	mean relative error
MRM	multiple reaction monitoring
MS	mass spectrometry
MSTFA	*N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide
m/z	mass-to-charge
NaOH	sodium hydroxide

NMI	National Measurement Institute
NMR	nuclear magnetic resonance
PCC	pyridinium chlorochromate
QQQ-MS	triple quadrupole-mass spectrometer/spectrometry
QTOF-MS	quadrupole time-of-flight mass spectrometer/spectrometry
R_f	retention factor
R_t	retention time
RIA	radioimmunoassay
RSD	relative standard deviation
SAMHSA	substance abuse and mental health services administration
SIM	selective ion monitoring
SPE	solid phase extraction
THC	Δ^9-tetrahydrocannabinol
THC-COOH	11-nor-9-carboxy-Δ^9-tetrahydrocannabinol
TIC	total ion chromatogram
TLC	thin layer chromatography
TMB	tetramethylbenzidine
TMCS	trimethylchlorosilane

UNODC	United Nations Office on Drug and Crime
WADA	World Anti-Doping Agency

Abstract

Urine is a long accepted biological matrix used for the detection of prescription and illicit drug use in the population. In today's society, there is still a social stigma attached to individuals that have been found to be using contraband drugs. Being labelled a "drug addict" or a "drug cheat" in sports can potentially be detrimental to a person's reputation. As such, it is not surprising to learn that they are motivated to discover and utilise new and ingenious ways of circumventing routine drug testing protocol. A very effective method for doing so is to purposefully tamper a urine specimen to invalidate the results of a drug test.

Currently, urine samples deemed to be tampered are not analysed further for drugs of abuse as the presence of the target analytes may be significantly deteriorated or even undetectable using routine testing methods. One pathway for the mechanism of action of commercially available urine adulterants is through oxidation.

The research carried out in this project has shown that following exposure of six opiates (6-MAM, morphine, codeine, codeine-6-glucuronide, morphine-3-glucuronide and morphine-6-glucuronide) to various oxidising adulterants (nitrite, PCC and hypochlorite), stable reaction products were identified in urine. The structures of 12 reaction products were elucidated using high resolution mass spectrometry and nuclear magnetic resonance spectroscopy, where possible. The reaction products were characterised to be: 2-nitro-MAM, 2-nitro-morphine, 2-nitro-M6G, codeinone, 14-hydroxycodeinone, 6-*O*-methylcodeine, 8-hydroxy-7,8-dihydrocodeinone, a lactone derivative of C6G, morphinone-3-glucuronide, 7,14-dihydroxy-6-MAM, a 7,8-di-keto analogue of 6-MAM and a 7,8-di-keto analogue of morphine.

In all cases, the original opiate abundances were found to be diminished or undetectable. However, the reaction products were found to be stable for at least seven days using LC-MS. Reaction mechanisms for the formation of the 2-nitro analogues and codeinone were also proposed. The formation of the 2-

nitro analogues was hypothesised to follow an electrophilic substitution reaction. The production of codeinone was suggested to be initiated by the chromium (VI) complex found in PCC.

It was discovered that both nitrite and PCC caused a decrease in the response of the CEDIA 6-AM and opiate assays, respectively. In addition, the morphine/codeine ratios (used during confirmation testing) were found to be affected by the presence of PCC, due to the loss of both native and internal standard species.

The exposure of the opiates to hypochlorite in water resulted in the detection of several potential reaction products. However, it is disadvantageous that they appear to be relatively unstable, only forming under narrow hypochlorite concentration ranges. Due to these reasons, further investigation was not pursued.

Finally, an in-house quantitative NMR procedure for the certification of reaction product material was demonstrated using 2-nitro-MAM and 2-nitro-morphine following their syntheses and isolation. This method can be used as a quick alternative to certifying material through commercial institutions when there are constraints with time and funding.

Overall, the research carried out in this project has laid the groundwork for future work concerning the use of the reaction products as markers for monitoring the presence of opiates in adulterated urine. Due to its relative stability, ease of formation and detection, the identified reaction products show potential for their incorporation into drug testing programs as a way of monitoring opiate positive urine specimens adulterated with nitrite.

Chapter 1:

Introduction

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1.1 The opium poppy- from the fields to the streets

The opium poppy (botanical name: *Papaver Somniferum* [1, 2]) is one of the most recognised plants throughout the history of human civilisation, with its cultivation dating back to 3000 BC [3]. It was used to relieve pain, induce a general sense of wellbeing and utilised in cooking [1, 3]. There are many subspecies of *Papaver Somniferum*; each variety differs in the shape of petals, abundance of buds and opium content. The characteristic bright and colourful flowers found in opium poppy plants means that it is often connected with beauty (Figure 1-1).

Figure 1-1: The opium poppy plant, Papaver Somniferum [1].

During the last several centuries, opium has been associated with two wars (the opium wars: 1839-1842 and 1856-1860 [4]) and has continually been the subject of political and social upheaval. In 1906, it was found that 41,624 tonnes of opium was produced worldwide; 85% was attributed to china and 12% was mapped back to British India [1]. The propagation of opium use from the east to the west was significantly brought to the attention of the US during the 1870's. Addiction to opium smoking was brought over by the Chinese during the gold rush, and became such a problem that Britain passed the Opium Act in 1878 in an effort to reduce opium consumption in its colonies [1].

Tracing its existence throughout time, it has been difficult to determine the exact geographical origin of the opium poppy. There appears to be a symbiotic relationship between human settlement and the presence of the plant, suggesting that its distribution is parallel with human migration. The growth of opium poppy colonies have spread across Asia, Europe, North and South America, Australia and Africa. Its ability to adapt to most ecological environments allows it to flourish in diverse climate and soils [1]. The difference in climatic conditions, harvesting time and soil composition results in variation of the alkaloid composition found in the opium poppy crop. However, the types of natural alkaloids found in the latex sap of *Papaver Somniferum* remain constant and include morphine (Figure 1-2a), codeine (Figure 1-2b), thebaine (Figure 1-2c), noscapine (Figure 1-2d) and papaverine (Figure 1-2e) [5-7]. Morphine is the major opiate alkaloid found in the opium poppy, with an abundance of 8-17%; codeine is commonly present at only 0.7-5% [3].

Regardless of its criminalisation through legislation in today's society, the cultivation and production of opium remain ongoing and are the stable income of choice for poverty stricken farmers [1]. The 'Golden Triangle' (Figure 1-3) and the 'Golden Crescent' (Figure 1-4) are the two principal regions known for their illicit opium production. The 'Golden Triangle' lies in South-East Asia and encompasses Burma, Vietnam, Laos and Thailand. The 'Golden Crescent' is found in South-West Asia and includes Afghanistan, Iran and Pakistan [8]. Regions in South America and Central America provide a relatively small contribution to the world's illicit opium production [9]. The largest opium poppy

fields are situated in the 'Golden Crescent' region. In 2011, 131362 hectares (ha) of opium poppy were found in South-West Asia, in Afghanistan (131000 ha) and Pakistan (362 ha). In South-East Asia, 47700 Ha of opium poppy fields were established for illicit opium cultivation, predominately in Burma (43600 ha) and Laos (4100 ha). Latin America contained 14341 ha of opium poppy fields, however, the exact distribution through Columbia and Mexico remains unknown. Finally, approximately 13300 ha of the world's illicit poppy crop originated in other regions, to give a total of 206703 ha of opium poppy fields worldwide [9].

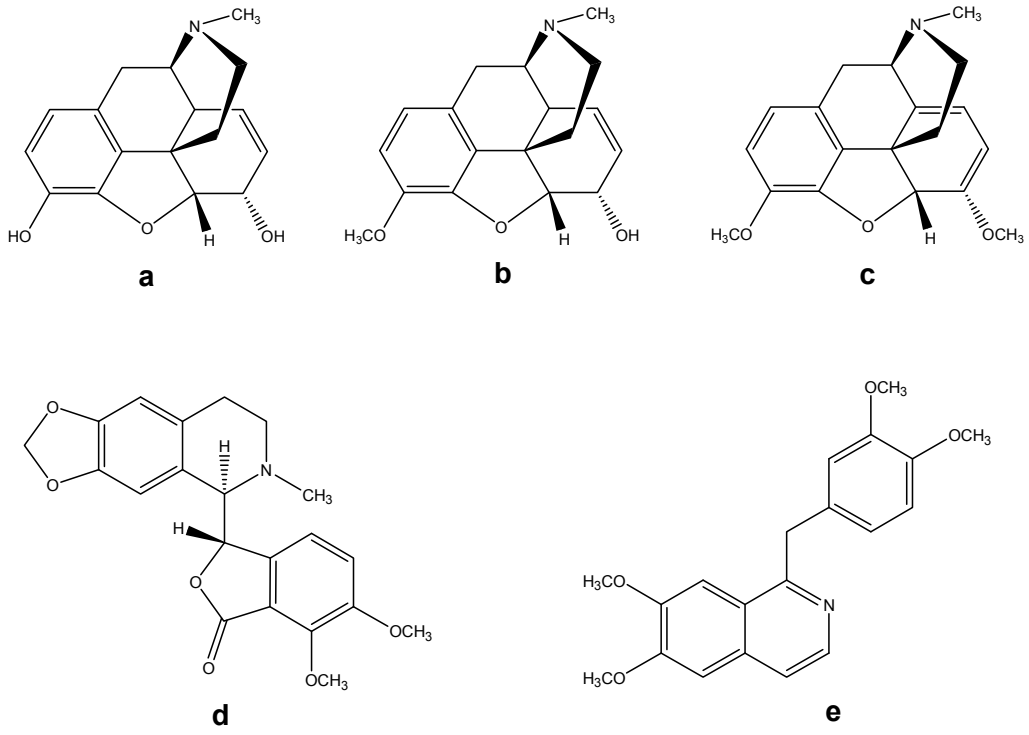


Figure 1-2: Opiate alkaloids (a) morphine, (b) codeine, (c) thebaine, (d) noscapine and (e) papaverine, found in the opium poppy.

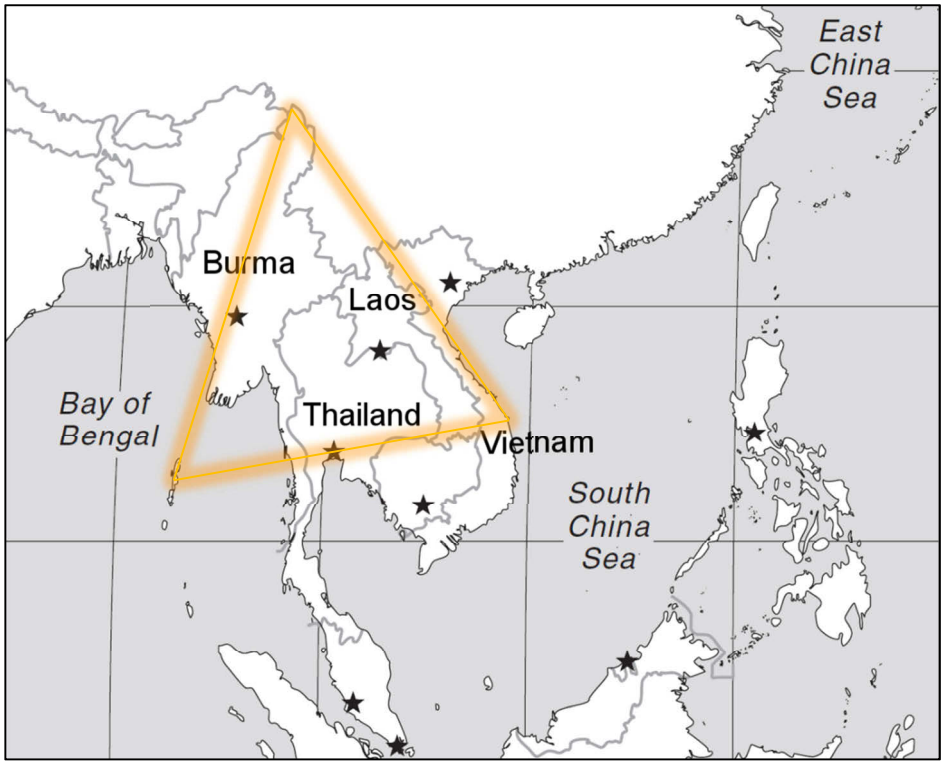


Figure 1-3: The 'Golden Triangle' (map adapted from [10]).

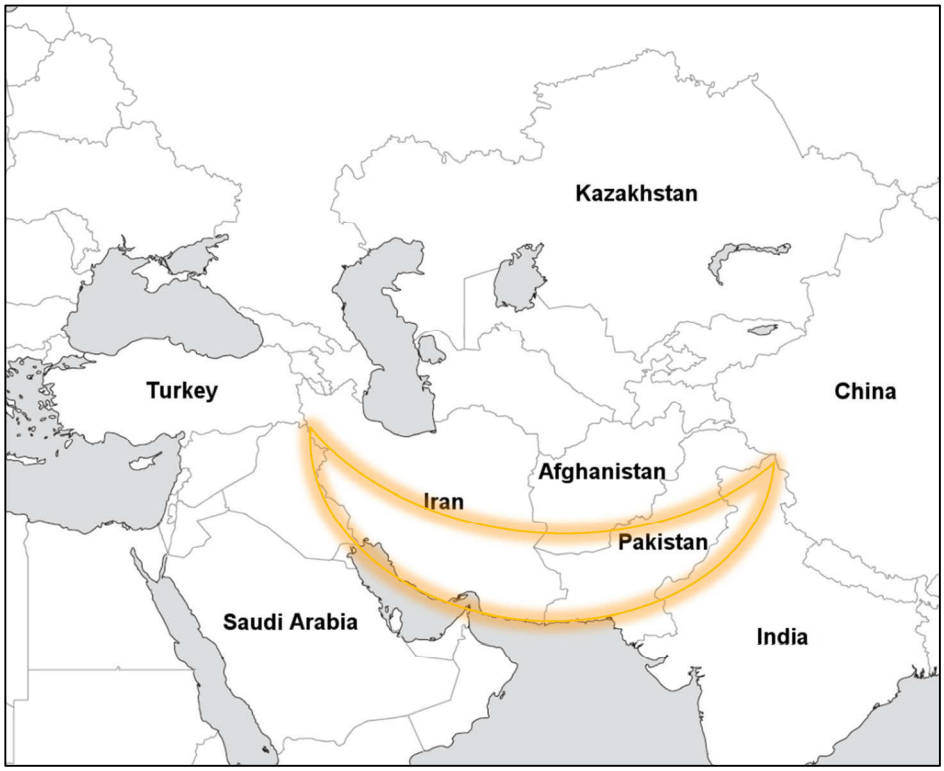


Figure 1-4: The 'Golden Crescent' (map adapted from [10]).

1.1.1 Manufacture of illicit heroin

Heroin (3,6-diacetylmorphine) was first synthesised in 1874 [11]. It is a two-stage process that involves the extraction and purification of morphine from the opium poppy plant, followed by its acetylation with acetic anhydride to produce heroin [5, 6]. During illicit heroin manufacture, the two main procedures used to isolate morphine are the lime method and the ammonia method. It was suggested that the lime method is commonly employed in the 'Golden Triangle' region, whereas production in the 'Golden Crescent' utilises the ammonia Method [5, 12]. However, research into authentic illicit heroin manufacturing processes in the last several years have shown that the lime method has also been adopted in Afghanistan, the world's largest area of opium poppy cultivation and greatest supplier of opium [13]. It has been discovered that raw opium originating from Afghanistan has a higher morphine content than opium found in other countries [14].

As both isolation methods are not specific to morphine, other alkaloids can also be co-extracted into the final sample. Both methods have shown to produce a similar yield of morphine, codeine and thebaine in the extracts; however, the content of papavarine and particularly noscapine has been found to be strikingly higher in the extracts obtained from the ammonia method [12]. Thus, the concentrations of both papaverine and noscapine can be used as markers to determine the morphine extraction method used to prepare the heroin sample [5, 15, 16]. Nevertheless, interpretation should be approached with caution; noscapine and papaverine have been detected at unusually high concentrations in heroin samples, likely added as an adulterant to bulk up the sample [12, 17].

1.1.1.1 Lime method- predominant procedure for the isolation of morphine

There are three classical procedures for the extraction of morphine dated back to the 1800's. They include the Merck process, the Robertson-Gregory process and the Thiboumery and Mohr process [18]. The lime method is adapted from

the Thiboumery and Mohr process and has the advantage of requiring minimal technical skills to carry out the extraction, with adequate separation between morphine and the other natural alkaloids [14, 18, 19].

Opium cultivation begins by lancing the pod of the opium poppy plant to produce a milky liquid latex (raw opium), which hardens upon standing [20]. The depth of scoring is significant and should be approximately 1 mm; an overly shallow score will cause the sap to coagulate at the scoring site, preventing the flow of sap. However, an excessively deep score may cause the sap to flow out too fast, resulting in loss of the raw opium [21]. The scoring process ideally begins in the late afternoon; this allows the white opium to oxidise overnight into a dark brown gum before it is scraped off the pods the next morning, ready for morphine extraction [21, 22]. The amount of opium per pod does vary depending on the size of the pod and the skill of the farmer, with an average of 50 mg of opium obtained per pod [23].

In South-East Asia, the arrangement of small clandestine laboratories close to the vicinity of the opium poppy fields has been documented. As only approximately one-tenth of the raw opium mass belongs to morphine base, it is desirable to extract the morphine prior to transportation of the product to a heroin laboratory [21].

For the lime method, the process of morphine extraction from raw opium begins with the addition of hot water to the raw material to form a suspension. This is typically carried out in oil drums or barrels, and with continual stirring, some components of the raw opium dissolves in the water to produce a brown suspension. Insoluble debris (such as leaves and twigs) floats on the surface and is scooped out. It is common to place the reaction vessel on bricks with a fire built underneath the vessel to maintain the heat for optimal dissolution of the opium. Otherwise, extra hot water can be added as required. Following this, lime is added to the suspension: calcium hydroxide (slaked lime) and calcium oxide (anhydrous lime) can both be used. Some clandestine laboratories also use readily available chemical fertilizer with high lime content. The lime converts the insoluble morphine into water soluble calcium

morphenate. Since the other alkaloids cannot form calcium salts, they are separated (for the most part) from the morphine and are insoluble in the solution. Codeine is slightly water soluble and so remains to a small degree in the aqueous fraction.

The morphine solution is then retrieved and filtered through sacks, which are squeezed using a press to maximise the collection of filtrate. Ammonium chloride is added to the filtrate, and the solution is heated but not boiled. This adjusts the alkalinity of the solution from pH 10-12 to pH 8-9. As a result, precipitation of the morphine base (and some codeine base) from the solution occurs, allowing crude brown morphine solid to be collected using cloth filters. In South-East Asia, crude morphine is further purified through the process of dissolution in hydrochloric acid (HCl) and addition of activated charcoal, with the solution re-heated and re-filtered. The morphine hydrochloride is then dried and transported as brick sized blocks to heroin manufacturing laboratories (Figure 1-5). In West Asian countries such as Afghanistan, crude morphine base is air dried and used directly for heroin manufacture (Appendix, Figure A-1) [13, 21, 22].

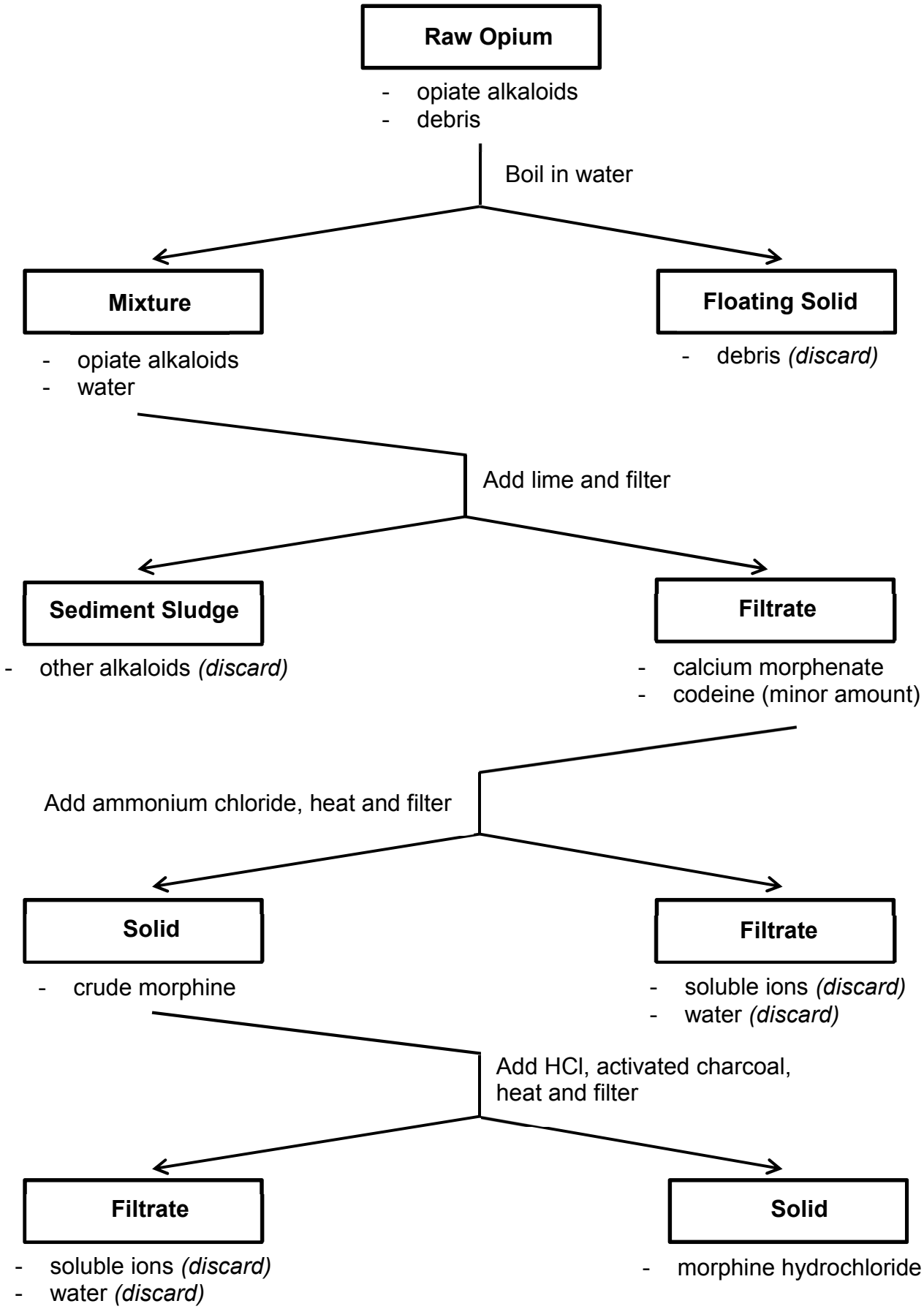


Figure 1-5: Flowchart outlining the lime method for the extraction of morphine.

1.1.1.2 Conversion of morphine to heroin

The synthesis of heroin from morphine is a two-step process and can be carried out using either crude morphine or purified morphine hydrochloride as the starting material. The procedure is relatively simple and requires the use of acetic anhydride as the acetylating agent (Figure 1-6). Common kitchen appliances can be used to 'cook' heroin, and it is only the sophisticated laboratories that use proper chemical glassware (such as Pyrex flasks and reflux condensers) as well as intricate exhaust systems. Due to the pungent vinegar-like odours exuded by acetic anhydride, heroin conversion laboratories are often established in rural areas to minimise the risk of exposure [21].

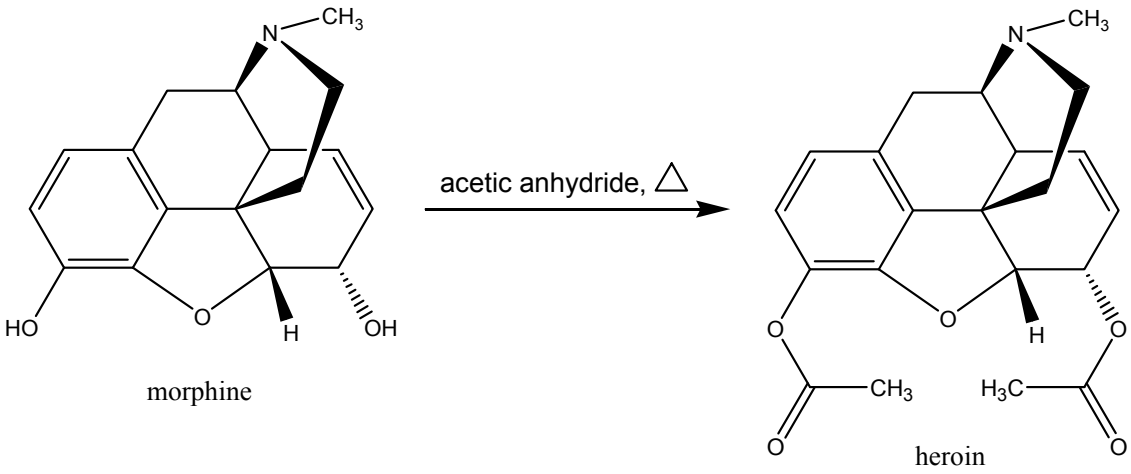


Figure 1-6: Reaction scheme showing the acetylation of morphine to heroin.

The first step involves the conversion of morphine to heroin and is typically a one pot reaction. Morphine is placed in a pot with excess acetic anhydride; the reaction mixture is heated and agitated as necessary to ensure that morphine is completely dissolved and acetylated to form heroin. If morphine hydrochloride bricks are used, it is necessary to pulverise it to powder form prior to reaction. During the synthesis, the reaction mixture is covered and heating is maintained at below boiling point to prevent overproduction of

fumes. Once the conversion is complete, steps to isolate and purify the heroin are commenced.

The second step begins with the isolation of heroin. The reaction mixture is diluted with hot water and filtered. The filtrate is collected and contains heroin in its ionised form. Sodium carbonate is added to precipitate out the heroin base. As a by-product of the reaction, carbon dioxide is produced (visualised as bubbles of gas). The ceasing of effervescence indicates maximum precipitation of heroin base. The precipitate is collected and washed numerous times with water, resulting in the isolation of crude brown heroin base solid [14, 21, 22].

In South-East Asia, activated charcoal is added to the filtrate prior to basification with sodium carbonate. This removes the coloured impurities and is filtered out before the heroin base precipitation. Once the crude heroin base is obtained, it is converted to either smoking heroin ("heroin no. 3") or injectable heroin ("heroin no. 4"). "Heroin no. 3" is produced by reacting the crude base with HCl to form heroin hydrochloride. The sample is then adulterated with caffeine and "flavourings" such as quinine and strychnine, resulting in a wet paste. Once it is dried (course lumps), it is passed through a sieve and the grains are pressed into blocks for shipping. For the production of "heroin no. 4", the crude heroin base is repeatedly dissolved in dilute HCl, treated with activated charcoal, precipitated and dried until a white solid is achieved. It is then converted to the hydrochloride salt using concentrated HCl, ethanol and ether. Upon visualisation of small crystals, the reaction mixture is covered and left to stand to promote precipitation of heroin hydrochloride. Heroin hydrochloride is collected by filtration and packaged for sale. Compared to "heroin no. 3", "heroin no. 4" is significantly purer and thus more suitable for injection [21]. A distinctive red 'Double UOGlobe' logo with two lions is commonly found on the packaging of heroin manufactured in Burma, however it is also found on heroin packages originating from other regions in South-East Asia (Appendix, Figure A-2) [24].

In Afghanistan, the processing of crude brown heroin base is similar to “heroin no. 4” produced in South-East Asia, where the base is purified into a white solid prior to conversion to its hydrochloride salt form. However, dilute ammonia (instead of the sodium carbonate that was used to isolate the crude brown heroin base in the previous step) is used to precipitate the white heroin base. Its conversion to heroin hydrochloride is carried out with concentrated HCl and acetone; the solution is then filtered, and the acetone solvent is evaporated on a water bath, leaving behind white heroin hydrochloride crystals (Appendix, Figure A-3) [14].

1.1.2 Major legal opium poppy fields for pharmaceutical and culinary uses

In accordance with the United Nations Single Convention on Narcotic Drugs [25], legal opium poppy fields are permitted to operate in many countries where crop cultivation and harvesting are tightly monitored by the government. Major legal poppy fields are found in Australia, India, Turkey, France and Japan. Morphine, thebaine and codeine are highly valued in the pharmaceutical industry and are retrieved from legal opium farms. They are the active ingredients in numerous prescription and non-prescription pain relieving medications worldwide [26]. As mentioned previously, codeine is a relatively minor product extracted from the opium poppy plant; however, codeine can also be synthesised from thebaine. Other synthetic opioids of significant pharmaceutical value can also be produced from thebaine. Additionally, the poppy seeds distributed to supermarkets and bakeries to be incorporated in baked goods are also derived from seeds of the crop grown in legal opium poppy fields [27].

The largest manufacturer of active pharmaceutical ingredients in Australia is Tasmanian Alkaloids, situated in north Tasmania. The Tasmanian poppy industry was pioneered by Glaxo Australia (now GlaxoSmithKline) in the early 1960’s [26]. It is also recognised as the largest exporter of codeine and thebaine in the world, producing approximately 40% of the world’s legal opiates [28]. At this crop handling facility, opiate alkaloids are extracted from

the opium poppy plant using a warm solvent percolation system. Both morphine and thebaine are removed from the poppy straw (a term used internationally to refer to the whole poppy plant, excluding the seeds [25]) as two final types of concentrate [27]. However, it has been documented that the extracted morphine practically all comes from the pods [29]. Another site for the extraction of medicinal alkaloids from the Tasmanian opium poppy crop is at the GlaxoSmithKline factory in Port Fairy, Victoria. Here, more than 95% of the refined product is exported worldwide [26]. Tasmanian Alkaloids operates as a subsidiary of Johnson and Johnson, a pharmaceutical company based in the United States [30, 31].

In India, legal opium production is carried out under the surveillance of Central Narcotics Bureau officials, an entity affiliated to the government's Ministry of Finance. To date, it is the only country that is authorised to grow and harvest raw opium for large scale export [32]. It is cultivated in three states: Uttar Pradesh, Madhya Pradesh and Rajasthan. Once harvested, the opium is shipped to one of two processing plants: Ghazipur in Uttar Pradesh or Neemuch in Madhya Pradesh. Most of the opium produced is exported to the United States, United Kingdom, France and Japan [33, 34].

Turkey is recognised by the International Narcotics Control Board (INCB) as a country permitted to carry out licit cultivation of opium poppies. As of 2010, Turkey's main opium poppy cultivating regions were in Ankara city and Afyon (Afyonkarahisar) city [35]. Crop processing takes place at the state run Bolvadin alkaloid factory, where the entire poppy plant is ground and the morphine extracted for international exportation, where there is high demand for morphine for medicinal purposes [36].

The legal opium poppy fields situated in France are considered to be one of the most valuable in the context of legalised opium poppy farming in the world, with an estimated crop value of $86.5 million. Although Australia possesses opium crops with an estimated value of $178 million, the crops belonging to Turkey and India are considered less prized, with an estimated $71 million and $46 million value, respectively [37]. On the contrary, the market for legal

cultivation of opium in Japan is extremely limited and is restricted for producing opium for local pharmaceutical purposes only [32].

1.1.3 Illicit heroin – the global picture

The use of illicit drugs (as a whole) is a global issue entrenched in current society: its existence undermines economic and social development, increases the rate of crime as well as significantly contributing to the spread of blood-borne diseases such as HIV and hepatitis C (through intravenous drug use). According to data presented in the World Drug Report 2012 (conducted by the United Nations Office on Drug and Crime (UNODC)), illicit drugs including heroin and cocaine kill about 0.2 million people each year, destroying families and causing hardship to all those involved. It was also documented that approximately 27 million people (0.6% of the world adult population) are classified as problem drug users [9].

The negative impact that the illicit drug market imparts on society is clear: high instances of violence, kidnapping, corruption and human trafficking [9] can be related to organised crime syndicates that are involved in illicit drug activity. There is no simple solution to reduce or eradicate the presence of illicit drugs in modern life. However, by monitoring its usage as well as the extent of its transnational importation and exportation, government bodies and policy makers can gain insight into the supply and demand of the illicit drugs. This provides invaluable intelligence which can be used to aid the global objective of reducing illicit drug demand through prevention, treatment, rehabilitation, reintegration and health [9].

1.1.3.1 Illicit heroin supply and demand

In 2011, 7000 tonnes of opium were produced worldwide. Afghanistan is still the largest producer of opium, responsible for approximately 63% of the total amount harvested. Burma and Laos account for over 20% and countries in Central America and South America (primarily Mexico and Columbia) account for nearly 7%. In the last decade, global opium production peaked in 2007 at

just below 9000 tonnes. Although it appears to be a decreasing trend from 2007 to 2011, it is a significant increase in production in comparison with 2010 (when almost half the crop was destroyed by plant disease in Afghanistan). Overall, although global opium production is shown to follow a decelerating trend from 2007 (Figure 1-7), the cause may not necessarily be due to a decrease in demand [9].

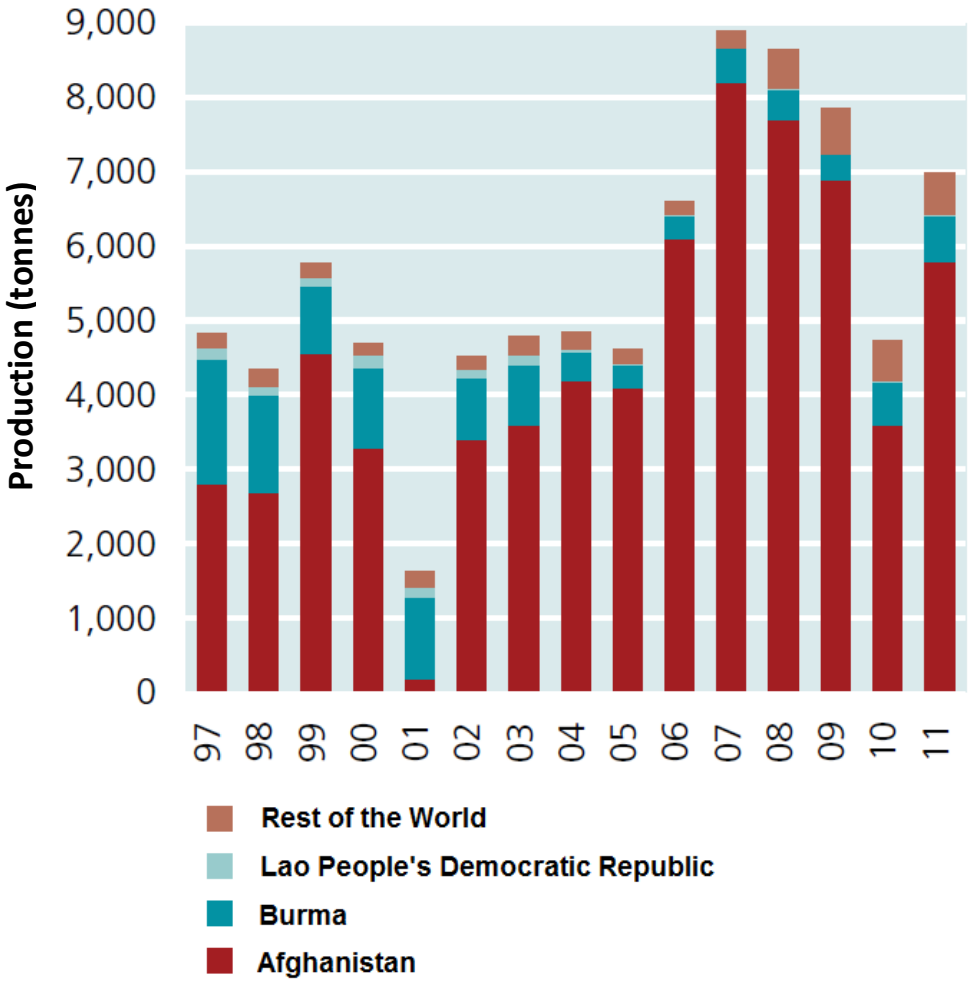


Figure 1-7: Global potential opium production, 1997-2011 [9]. Note: the 2011 estimate for the rest of the world is provisional.

For first world countries, the recreational use of illicit heroin has remained relatively stable when compared to previous years. It is estimated that the number of opiate users worldwide fall within the range of 12.9-21 million people, with health issues associated with opiate use such as infection and death shown to be undiminished. It has been reported that there are over one

million heroin addicts in the United States [38]. An earlier publication by Moeller and Mueller stated that heroin is the most important drug of abuse in Germany and possibly the rest of Europe [39].

In third world countries, it has been noted that there has been an increase in illicit heroin usage. Opium and heroin use is highly prevalent in Afghanistan and Iran, which is unsurprising, as they are the world's largest illicit cultivators of opium poppy. The situation in major opium producing countries such as Afghanistan and Burma can be used to provide further support that the demand for opium is continuing to rise. Despite an increase in opium production, the farm-gate prices have continued to increase (instead of plateauing or decreasing), possibly suggesting that that the demand is still high. However, another perspective for this rise in price is the intensified risk associated with cultivation and trafficking as a result of amplification of law enforcement activities [9].

Table 1-1 outlines how opium produced from 2004-2011 was utilised. Of the 7000 tonnes cultivated globally in 2011, an estimated 3400 tonnes was consumed or traded as raw opium. The remainder was used to manufacture an estimated 467 tonnes of heroin, and relative to total opium production of that year, demonstrates that the illicit heroin market is still thriving. The demand for raw opium can be explained by the illicit (yet unmeasured) market for other opiates such as morphine, operating parallel to the illicit heroin market [9].

Table 1-1: Potential illicit production of opium and manufacture of heroin of unknown purity, 2004-2011 (in tonnes, adapted from [9]).

	2004	2005	2006	2007	2008	2009	2010	2011
total potential opium production	4850	4620	6610	8890	8641	7853	4736	6995
potential opium not processed into heroin	1197	1169	2056	3411	3080	2898	1728	3400
potential opium processed into heroin	3653	3451	4555	5479	5561	4955	3008	3595
total potential heroin manufacture	529	472	629	757	752	667	384	467

Note: The proportion of potential opium production not converted into heroin could be estimated only for Afghanistan. For the purpose of this table for all other countries it is assumed that all opium potentially produced is converted into heroin. If total potential opium production in Afghanistan in 2011 were converted into heroin, total potential heroin manufacture would be 829 tonnes (Afghanistan) and 948 tonnes (global). The 2011 estimate of "opium not processed into heroin" in Afghanistan was based exclusively on regional seizure data, in contrast to previous years, when information from key informants was also taken into consideration. The 2011 estimate is not directly comparable with previous years.

1.1.3.2 Illicit heroin trafficking and seizures

Figure 1-8 reveals the regions where seizures of heroin and morphine took place in 2010. Overall, there was a small increase in total global seizures with 81 tonnes and 76 tonnes seized in 2010 and 2009, respectively. However, interesting observations can be made by looking at the seizure data for individual illicit markets, and comparing it to the heroin production data for the same period for South-East Asia, South-West Asia and South America. In general, seizures were prevalent in countries where the trafficked product originated from South-East Asia and/or Central and South America. This reflects the increase of heroin supply from these regions as a result of increased opium poppy production and cultivation, despite unfavourable growing conditions and forced eradication imposed by the government in principal heroin producing regions in South-East Asia [9, 40].

On the contrary, there is a marked decrease in heroin seizures in regions such as Russia and Western and Central Europe in 2010 when compared to the previous year. Two primary routes are used to smuggle heroin from Afghanistan: the Balkan Route and the Silk Route. The Balkan Route is mapped out through South-Eastern Europe and can be further divided into three sub-routes: the southern route, the central route and the northern route. The southern route transports illicit heroin through Turkey, Greece, Albania and Italy. The central route goes through Turkey, Bulgaria, Macedonia, Serbia, Bosnia, Croatia, Slovenia and finally to either Italy or Austria. The northern route exports heroin through Turkey, Bulgaria, Romania, Austria, Hungary and the Czech Republic, and is finally destined for either Poland or Germany. Turkey acts as an anchor point for Afghan heroin to be diverted into the European markets [40]. Thus, the decreasing trend in heroin seizures in regions of Europe can be explained by two phenomena. It is likely that the trafficking routes stemming from Afghanistan are monitored closely by law enforcement agencies, and therefore, acts as a deterrent for illegal drug distribution activities. Secondly, the decrease in seizures in these regions can also be explained by the opium shortage in Afghanistan. This was a result of the crops being destroyed by disease in 2010, as well as a secondary effect of decreased opium production after the 'peak' observed in 2007 (Figure 1-7) [9].

Over the centuries, China has remained a significant market for heroin, with 1.19 million registered heroin addicts [20]. The number of heroin and morphine seizures were documented to be relatively stable (Figure 1-8) with a slight decrease from 2009 (5.4 tonnes in 2010 compared to 5.8 tonnes in 2009). There appears to be a shift in the source of heroin being illegally imported into China. Once a major destination for heroin trafficked from South-East Asia, particularly Burma, it is now known that bulk quantities of heroin are being transported over the Chinese border from Afghanistan. The trafficking route detours to other countries including Pakistan. Nonetheless, illicit heroin from Burma is still being smuggled into China through Yunnan Province [9].

It is curious to note that there has been an increase in heroin seizures in both North America and Latin America, in addition to some regions in Africa (Figure

1-8). East Africa (mainly Kenya and Tanzania) has been discovered to be a main entry point for illicit importation from South-West Asia, supplying heroin to East and South Africa. In the United States, heroin is trafficked from Mexico and Columbia. Mexican heroin is destined for the western region of the United States whereas Columbian heroin is trafficked to the eastern region of the United States. The predominant route transporting illicit heroin from Columbia is through Venezuela, Argentina, Ecuador, Panama and Mexico [20, 40].

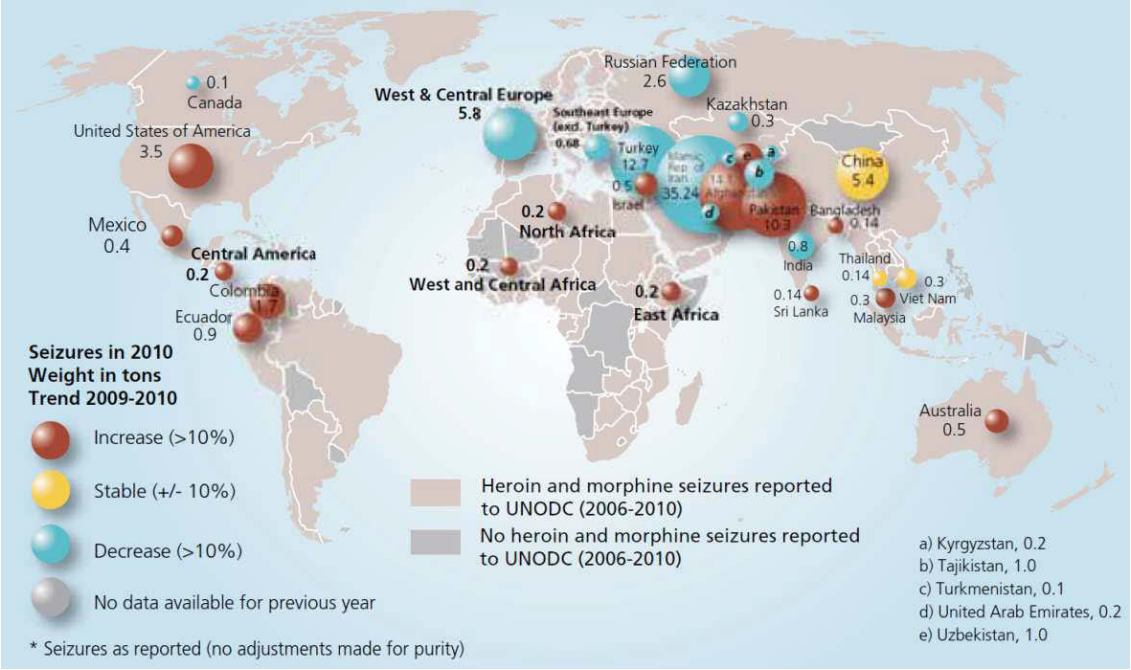


Figure 1-8: Global seizures of heroin and morphine in 2010 (countries and territories reporting seizures of more than 100 kg) [9].

1.1.3.3 Heroin- the situation in Australia

The illicit heroin that enters the Australian market varies in appearance, and can be either white or off-white in colour and finely powdered or granulated in texture [41]. Another common form of heroin is brown rock, a version of the latter with lower purity. It is relatively uncommon to find unrefined heroin base in Australia [42]. In a study conducted to investigate the purity of heroin sold on the black market in Sydney during 1997, 88 samples from 33 seizures were analysed for composition. The samples were obtained off the streets of Cabramatta, NSW; it was found that the purity of the majority of the samples were in the 61-80% range. All samples contained heroin as the hydrochloride

salt; no free base was detected. Furthermore, paracetamol and caffeine were used to adulterate the samples [43, 44].

Based on the Illicit Drug Data Report 2011-2012 presented by the Australian Crime Commission [20], illicit heroin supply into the country does not appear to be significantly slowing down. Although the number of heroin detections at the border has continued to decline from 2006-2007, the total weight of seized heroin remains the third highest weight reported in the past decade (Figure 1-9). Furthermore, the number of national heroin seizures has increased and is the highest reported in the last decade. Data from the Australian Federal Police's Australian Illicit Drug Data Centre (AIDDC) shows that the geographical origin of heroin samples originate predominately from South-East Asia and South-West Asia, with a minor proportion from unclassified origins [20].

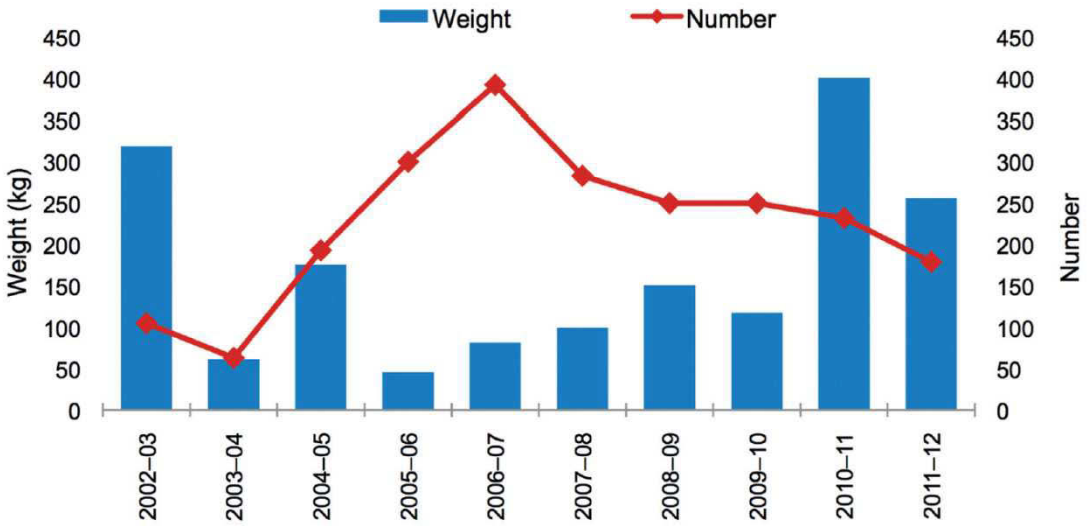


Figure 1-9: Number and weight of heroin detections at the Australian border, 2002-03 to 2011-12 [20].

Between 2011-2012, the cost of one gram of heroin ranged between $200 and $1000 [20]. In terms of national usage, a domestic study conducted in 2011 revealed that 53% of the responses of regular injecting drug users indicated that heroin was their drug of choice, with 62% of the respondents reporting recent heroin use [45]. Research into the drug use habits of police detainees in Australia have shown that heroin use from 2002-2012 has remained relatively

stable, with 10-15% of detainees testing positive for heroin through voluntary urinalysis. On the other hand, self-reported use (through voluntary surveys) indicated that heroin use was actually higher in the population than detected by urinalysis, however the same trend was followed from year to year [20].

1.1.3.4 Legislation for heroin, morphine and codeine

In regards to legislature, heroin is still currently legally prescribed in some parts of the world for pain management. However, its high potential for abuse has removed it from the therapeutic use category in many countries, including Australia [46]. Morphine, 2-3 times less potent than heroin [47], is still frequently prescribed for the management of moderate to severe pain, however its usage is monitored [48]. Codeine is found in over-the-counter cold and flu medications, however, the sale of these pharmaceuticals are now regulated as a measure for decreasing the incidence for its abuse.

In Australia, substances are classified according to schedules outlined in the Therapeutic Goods Administration Poisons Standard 2009 [49]. Heroin is classified as a Schedule 9-Prohibited Drug, with morphine as a Schedule 8-Controlled Drug. Codeine is classed in either the Schedule 2-Pharmacy Medication, Schedule 3-Pharmacist Only Medication or Schedule 8-Controlled Drug, depending on the type of preparation [49]. In the US, heroin is listed as a Schedule I Substance by the Drug Enforcement Administration (DEA) as having no current medical use (under the Controlled Substances Act of 1970) [11, 50].

1.2 Effect of Heroin, Morphine and Codeine on the Human Body

The literature set out in section 1.1.2 and section 1.1.3 has demonstrated that the use of heroin, morphine and codeine remains prevalent in society. As it forms an important class of analgesics, it is important to understand how these opiate alkaloids affect the human body.

1.2.1 Routes of administration

The intravenous route is the most common method for administering heroin [45]. However, smoking is the preferred route for some users in order to avoid risks associated with syringe sharing. The incidence of contracting human immunodeficiency virus, hepatitis B, hepatitis C and bacterial and fungal infections are likely to be lower when there is no use of syringes [20]. In addition, inhalation of heroin via the nasal passage is an alternate route of heroin delivery into the body [51].

In both the illicit and therapeutic setting, morphine and codeine can be administered intravenously or orally depending on the preparation.

1.2.2 Metabolism and excretion of heroin, morphine and codeine

Once administered into the body, heroin (Figure 1-10a) is rapidly metabolised to 6-monoacetylmorphine (6-MAM, Figure 1-10b) by enzymatic de-acetylation in the liver [52]. De-acetylation at the C-3 position to produce 3-monoacetylmorphine (3-MAM, Figure 1-10c) remains a questionable route of metabolism. Following this, 6-MAM is further hydrolysed more slowly to morphine (Figure 1-10d) [53-55]. 6-MAM is used as a unique marker for heroin use, since it can only be formed during biotransformation of heroin or by incomplete morphine acetylation during the heroin manufacturing process [6]. The half-lives of heroin, 6-MAM and morphine are approximately 3 minutes (min), 6-25 min and 2-3 hours, respectively. These half-lives are indicators of the stability of these species, and positively correlate with their windows of detection. Because heroin is converted so quickly to 6-MAM, it is rarely detected in urine following excretion unless the voided urine is collected immediately after its intake. For 6-MAM and morphine, the windows of detection are approximately eight hours and 1-2 days, respectively [6, 15, 47, 53, 56, 57]. Heroin, 6-MAM and morphine are all pharmacologically active species [58].

The primary elimination pathway of morphine (resulting from either heroin or morphine administration) involves its conjugation with glucuronic acid at the C-3 position (phenolic hydroxyl group) or at the C-6 position (alcohol hydroxyl group) of the molecule. This results in the formation of morphine-3-glucuronide (M3G, major metabolite, Figure 1-10e) and morphine-6-glucuronide (M6G, minor metabolite, Figure 1-10f), respectively [6, 48]. Pharmacologically, M3G is essentially inactive and has little analgesic effect. On the contrary, M6G has been proven to exhibit pain relieving properties which surpasses the potency of morphine [46, 48]. The conversion of morphine to normorphine (an active metabolite, Figure 1-10g) in the liver by cytochrome P450 enzymes has also been documented as a minor biotransformation route, resulting in the formation of normorphine-3-glucuronide (Figure 1-10h) and normorphine-6-glucuronide (Figure 1-10i) to aid its elimination [52]. Furthermore, there has been evidence for the formation of morphine-3,6-diglucuronide, however this di-glucuronidation has only been observed in urine [46]. Other reactions include *N*-demethylation, *O*-methylation, and *N*-oxide formation [47].

Codeine (Figure 1-10j) may enter the body as either the parent drug following its direct ingestion, or as acetylcodeine subsequent to heroin administration. Since small amounts of codeine are co-extracted into the morphine used for heroin manufacture, the codeine present is also acetylated to produce acetylcodeine. Acetylcodeine possesses a similar half-life to 6-MAM [15, 58] and is rapidly converted to codeine [59]. One metabolic pathway of codeine is its transformation to morphine by P450 CYP2D6 enzymes in the liver. Another reported biotransformation route is the *N*-demethylation of codeine, facilitated by CYP3A4 enzymes to produce norcodeine (Figure 1-10k). However, conjugation of both the parent drug and other metabolites as the glucuronide is the major metabolic pathway [47, 60, 61]. It is worth noting that the codeine glucuronidation to form codeine-6-glucuronide (C6G, Figure 1-10l) occurs at a much slower rate compared to morphine glucuronidation as there is only one position for conjugation (alcohol hydroxyl group at the C-6 position) [6].

Generally, 80% of the heroin dose is excreted in urine in 24 hours, mainly as the M3G metabolite. However, 5-7% of the dose is excreted as free morphine,

1% as 6-MAM and 0.1% as the unchanged drug. Trace amounts of other metabolites are also found [47]. Following an oral dose of morphine, 60% is excreted in urine in 24 hours as free morphine (approximately 10%), conjugated morphine (65-70%), normorphine (1%) and normorphine glucuronide (3%). Approximately 3% of the dose is excreted as free morphine in the 48 hours following morphine ingestion. The concentration of morphine in urine appears to be pH dependent. It has been found that the excretion of free morphine rises as the urine becomes more acidic. However, the excretion of the glucuronide conjugates increases when the urine becomes more alkaline [47]. After an oral dose of codeine, 80-90% is excreted in urine as codeine (10%) or codeine-6-glucuronide (C6G, 90%) [47, 60, 61].

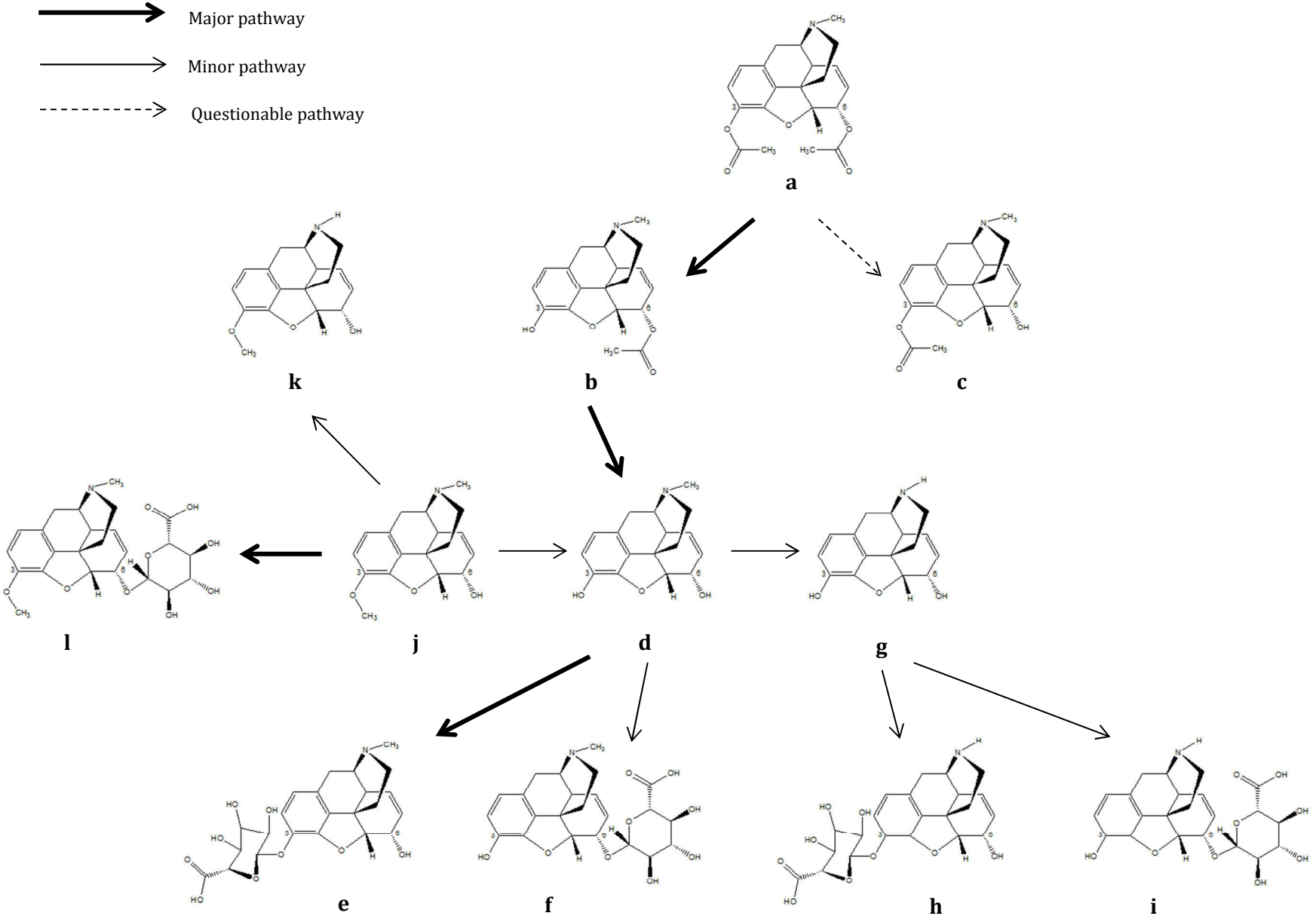


Figure 1-10: Metabolic pathway of (a) heroin in the human body, including the metabolic routes for (b) 6-MAM, (c) 3-MAM, (d) morphine, (e) M3G, (f) M6G, (g) normorphine, (h) normorphine-3-glucuronide, (i) normorphine-6-glucuronide, (j) codeine, (k) norcodeine and (l) C6G.

1.2.3 Mechanism of action of opiate alkaloids

The general mechanism of action of opiate analgesics is to mimic the function of endogenous endorphin neurotransmitters such as beta-endorphins and endorphin peptides. These opiate alkaloids bind to the mu, kappa and delta opioid receptors that are mainly found in the central nervous system (CNS). This process inhibits the release of pain inducing neurotransmitters in the body, resulting in the diminished sense of pain. The main neurotransmitter that is inhibited is substance P, which is ordinarily responsible for activating the main pathway for the generation of pain [62, 63].

1.2.4 CNS and peripheral effects associated with the use of opium alkaloids

The CNS and peripheral effects associated with the use of heroin, morphine and codeine are similar as they are all opiate analgesics. Within seconds of intravenous administration, a state of euphoria is achieved. A longer duration of time is required for the same euphoric sense to be reached if the opiates are administered using other routes. The onset of the drug's sedative effects are characterised by a feeling of relaxation; this feeling is associated with the 'high' experienced by opiate users. Once this 'high' feeling begins to wear off, some drowsiness, confusion and slowed cardiac function may set in. This is particularly pronounced in heroin users. Following exposure to opiates, miosis and constipation are commonly experienced reactions. Several short-term and long-term effects related to opiate use is summarised in Table 1-2 [51]. Furthermore, it is well documented that physical and psychological dependence can develop rapidly in opiate users, resulting in addiction [51].

Table 1-2: Short-term and long-term effects associated with opiate use.

symptoms of short-term use	**symptoms of long-term use**
nausea	hallucinations
itching (histamine release)	nightmares
muscle spasms and cramps	constipation
vasodilation	decreased sexual function
runny nose and eyes	impaired vision
slurred speech	decreased fertility
loss of appetite	collapsed veins
restlessness	Abscesses
diarrhoea	increased risk of lung and cardiovascular diseases

1.3 Detection of opiate alkaloids in urine

In many developed countries such as Australia, drugs-of-abuse urine testing begins with specimen collection, where a chain of custody is initiated. Following this, the specimen is transported to an accredited drug testing laboratory and is received for analysis [64]. At the laboratory, the specimen is screened for the target drugs of interest, including any metabolites produced by the parent drugs. Screening methods are adopted as the 'first line of detection' and provides a preliminary indication of whether the target analytes are present in the specimen. This is predominately carried out with an immunoassay based analysis; however, thin layer chromatography (TLC) has also been recognised as a preliminary test for drugs of abuse. The target drug analytes in "presumptive-positive" samples are subsequently identified and

quantified using a confirmatory technique such as Gas Chromatography-Mass Spectrometry (GC-MS) or Liquid Chromatography-Mass Spectrometry (LC-MS) [50, 65, 66]. GC-MS is considered the "golden standard" for unambiguous drugs of abuse confirmation testing. However, LC-MS is quickly emerging as an alternative technique for this purpose.

In Australia and New Zealand specifically, drugs of abuse urinalysis is carried out in accordance with the guidelines outlined in the Australian/New Zealand Standard™ 4308 (2008) (AS/NZS 4308). The general screening cut-off concentration for opiates is 300 ng/mL. The confirmatory test cut-off value for both morphine and codeine is also 300 ng/mL. However, for 6-MAM, the confirmatory test cut-off concentration is 10 ng/mL [67]. It is important to note that the relative concentrations of free and conjugated morphine and codeine in a specimen can also be used to determine the product that was most likely administered or ingested (whether it was illicit heroin, prescription medication or poppy seed consumption). The 6-MAM metabolite must be present in the sample above the confirmatory cut-off concentration to prove heroin use. It is noteworthy to mention that the screening and confirmatory cut-off concentrations for morphine and codeine in the United States is significantly higher (2000 ng/mL) to prove heroin use, to avoid the "poppy seed defence" used by individuals [64, 68].

1.3.1 Immunoassay techniques

The Cloned Enzyme Donor Immunoassay (CEDIA), Enzyme Linked Immunosorbent Assay (ELISA), Enzyme Multiplied Immunoassay (EMIT), Fluorescence Polarisation Immunoassay (FPIA) and Radioimmunoassay (RIA) are all immunoassay techniques used for the preliminary detection of opiates in urine. Sensitivity, efficiency, simplicity and ease of automation are all advantages provided by immunoassay analysis. However, the technique may lack specificity and suffer from cross-reactivity issues. pH and ion strength of the specimen can also interfere with the assay [53, 69]. Nevertheless, it is still employed as a screening assay, with the CEDIA DAU opiate assay and the CEDIA DAU 6-AM assay (Microgenics Corporation (Fremont, CA)) being the

leading technologies for the preliminary detection of opiates in human urine [15, 65, 70-73]. Both assays are homogeneous enzyme immunoassays, with the 6-AM assay used to quantify (in addition to qualify) the unique 6-MAM heroin metabolite. The assays may be performed with the Olympus AU600 and AU800 analysers or the Hitachi 717, 902, 911 and 917 analysers [50, 59, 66, 73, 74].The principle behind CEDIA is outlined in Figure 1-11.

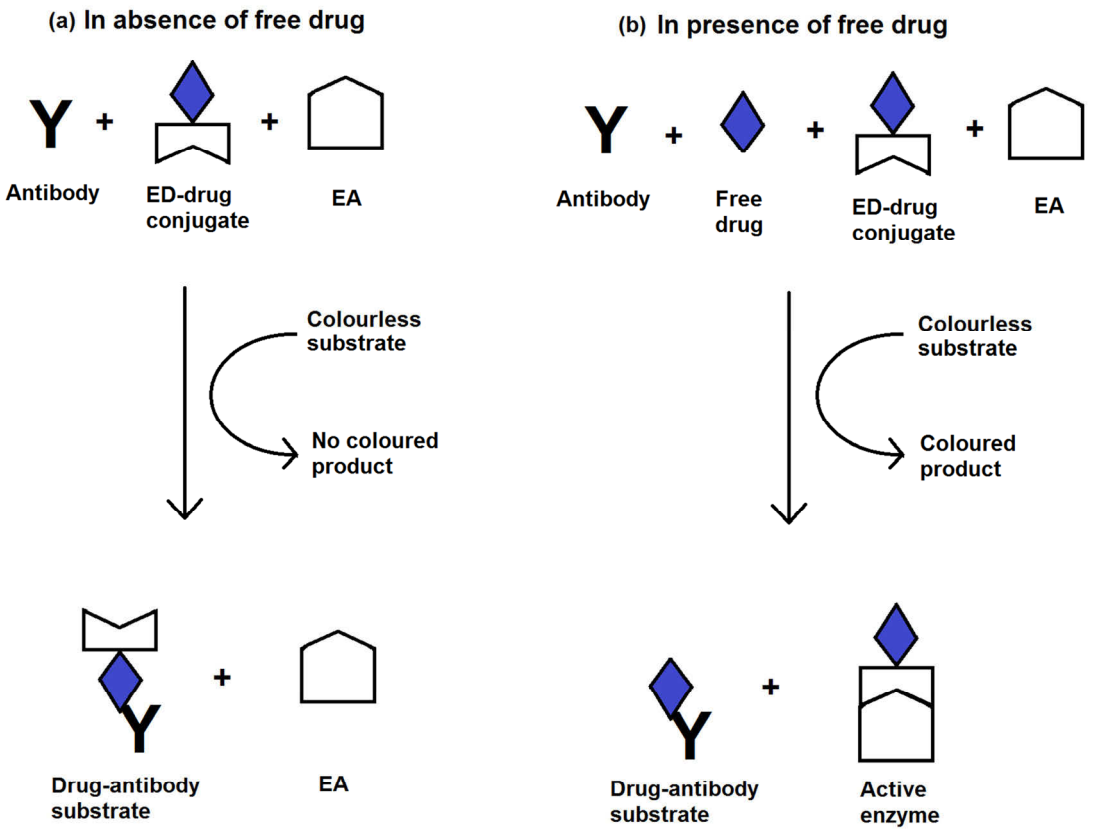


Figure 1-11: Principle behind the CEDIA immunoassay, where (a) in the absence of free drug, formation of a complete tetrameric enzyme is inhibited, and no coloured product is generated after addition of substrate to the reaction mixture; and (b) in presence of free drug, it competes with the enzyme donor (ED)-drug conjugate for anti-drug antibody binding sites. Complete active enzyme molecules are formed, which converts the colourless substrate into coloured product in proportional to the drug concentration. Note that EA is the enzyme acceptor (schematic obtained from [75]).

The consensus among the majority of opiate immunoassay studies is that there is good agreement between CEDIA screening and GC-MS confirmatory results [71]. It is especially useful in laboratories where large volumes of

specimens need to be analysed in a timely manner, such as in workplace drug testing programs. However, a disadvantage is that false positives for 6-MAM, morphine and codeine may be obtained if high concentrations of other opioid analogues are present in the urine specimen [72].

1.3.2 Thin layer chromatography

Once used as a confirmatory technique for drugs of abuse in urine, TLC is now used in some laboratories as a screening assay; however, it is not as widely implemented as immunoassay for this purpose. TLC is also integrated into the urinalysis protocol as a third tier, to complement the results obtained from immunoassay and confirmatory analysis. In general, sample preparation involves the "spotting" of extracted drug analytes at a controlled pH onto a silica plate. A suitable mobile phase separates the analytes based on its physicochemical characteristics, and the separation is expressed as a retention factor (r_f value). Theoretically, each compound possesses a definitive r_f value under a particular set of stationary and mobile phase conditions. Reagents may also be used to visualise the separated analytes on the TLC plate further. Among the chromatographic methods available, TLC is the most simple and inexpensive. However, it suffers from lack of sensitivity and specificity [47, 53, 65].

1.3.3 Gas chromatography-mass spectrometry

The sample preparation required for GC-MS analysis of opiate alkaloids such as 6-MAM, morphine and codeine begins with specimen hydrolysis, followed by extraction and derivatisation of the target analytes. The final reconstitute is injected into the GC-MS instrument for separation and detection of the target analytes.

1.3.3.1 Specimen hydrolysis

Often, total morphine and total codeine concentrations are reported. However, as both drugs are mainly excreted as their glucuronic acid conjugates,

recoveries are variable due to inconsistency in hydrolysis. Specimen hydrolysis is necessary to cleave the glucuronide moiety off M3G, M6G and C6G. Glucuronide bound analytes are highly hydrophilic, making their extraction into an organic solvent difficult (this is required for GC-MS analysis). Hydrolysis also increases the volatility of the derivatives formed later on in the sample preparation process, allowing it to be detected by GC-MS. For opiate hydrolysis, two main methods are used; enzymatic hydrolysis by β-glucuronidase and acid hydrolysis by HCl.

β-glucuronidase enzyme is commonly extracted from *Helix Pomatia* (type H-2) or *Escherichia Coli (E. Coli)* for hydrolysis. β-glucuronidase is also traditionally derived from limpets *(Patella Vulgata)* and bovine liver. More recently, a new product on the market is β-glucuronidase enzyme that is sourced from red abalone *(Haliotis Rufescens).* Red abalone enzymes have been reported to be of a superior quality compared to the enzymes found in traditional preparations [76]. Generally, β-glucuronidase is added to the urine aliquot, and the pH of the sample is controlled using an acetate buffer (approximate pH range of 4.5-5.2). For *E. Coli* extracted enzyme, neutral pH should be used for optimal enzyme performance. The sample is then incubated overnight, at temperatures ranging from 37°C to 60°C. This is dependent on the urine aliquot volume (often 2-4 mL is required for the assay) [15, 50, 55, 77]. Glucuronide cleavage occurs during this period, freeing any bound drugs present in the sample.

The procedure of acid hydrolysis is somewhat simpler, only requiring the addition of concentrated HCl to the urine aliquot, which is subsequently incubated. The ranges of HCl concentration (0.1 M – 11.6 M), incubation time (15-60 min) and incubation temperature (80°C -121°C) recommended for acid hydrolysis that has been found in the literature are extremely broad, however it appears to be proportional to the urine aliquot volume [6, 11, 59, 78, 79].

In comparison to acid hydrolysis, enzymatic hydrolysis is usually less destructive to de-conjugated products [65]. It is important to note that hydrolysis is not required when assaying 6-MAM as the process may destroy

the metabolite [50, 72, 73]. Acid hydrolysis in particular is destructive to 6-MAM and its use is not recommended. For C6G and M6G, harsher hydrolysis conditions are required to free the glucuronide bound morphine and codeine. HCl hydrolysis gives higher recoveries of free morphine and codeine compared to enzymatic hydrolysis. In regards to quantification, internal standards are added to the urine aliquot prior to specimen hydrolysis. Deuterium labelled morphine (morphine-d_3 or morphine-d_6), codeine (codeine-d_3 or codeine-d_6) and 6-MAM (6-MAM-d_6) are generally used [6, 50, 59, 71]. Two other internal standards commonly utilised for analysis are nalorphine and levallorphan [55, 66, 79].

1.3.3.2 Extraction of opiate analytes

Following hydrolysis, the target analytes are extracted from the urine matrix. The purpose of the extraction is to isolate the drug analytes from the endogenous urinary compounds, increasing the sensitivity of the method. Liquid-liquid extraction (LLE) [6, 55, 66] and solid phase extraction (SPE) [50, 72, 74, 80] are both used for opiate extraction. LLE is used to separate compounds based on their solubilities in two immiscible solvents, typically an aqueous and an organic liquid. However, it is common for drugs of abuse compounds to have two functional groups available for ionization, and so they can exist in either their neutral or ionised forms. This amphoteric nature is exhibited by both 6-MAM and morphine, but not codeine (Figure 1-12) [81]. Both 6-MAM and morphine possess two pKa values, accounting for both the acidic phenol and basic amine groups. For the extraction to be successful, the pH of the sample during extraction must be controlled. It is ideal to maintain the pH close to the average of the pKa values of the opiates, to ensure that they are predominately in their neutral (zwitterionic) forms. In the case of 6-MAM and morphine, the ideal pH for extraction is 9.0 (however, pH 9.5 is also commonly used with adequate recoveries). This allows the opiates to partition into the organic phase, isolating the drugs analytes of interest from urine endogenous compounds, which remain in the aqueous phase. Saturated ammonium chloride/ammonium hydroxide (NARCS buffer) [66], sodium hydroxide/phosphate buffer [55] and carbonate/bicarbonate buffer [6] can all

be used to achieve a pH of 9.0-9.5. In terms of organic solvents, dichloromethane, *n*-heptane and ethyl acetate are viable for opiate extraction; isopropanol and acetic acid can also be added for further fraction purification. A general flowchart for the LLE of 6-MAM, morphine and codeine from urine is shown in Figure 1-13.

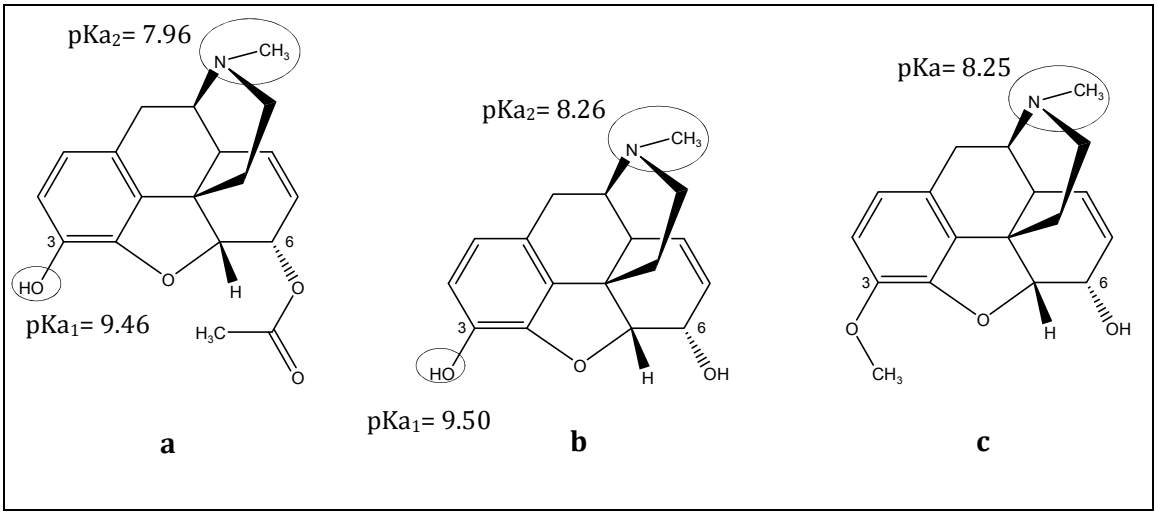


Figure 1-12: pKa values of (a) 6-MAM, (b) morphine and (c) codeine [81].

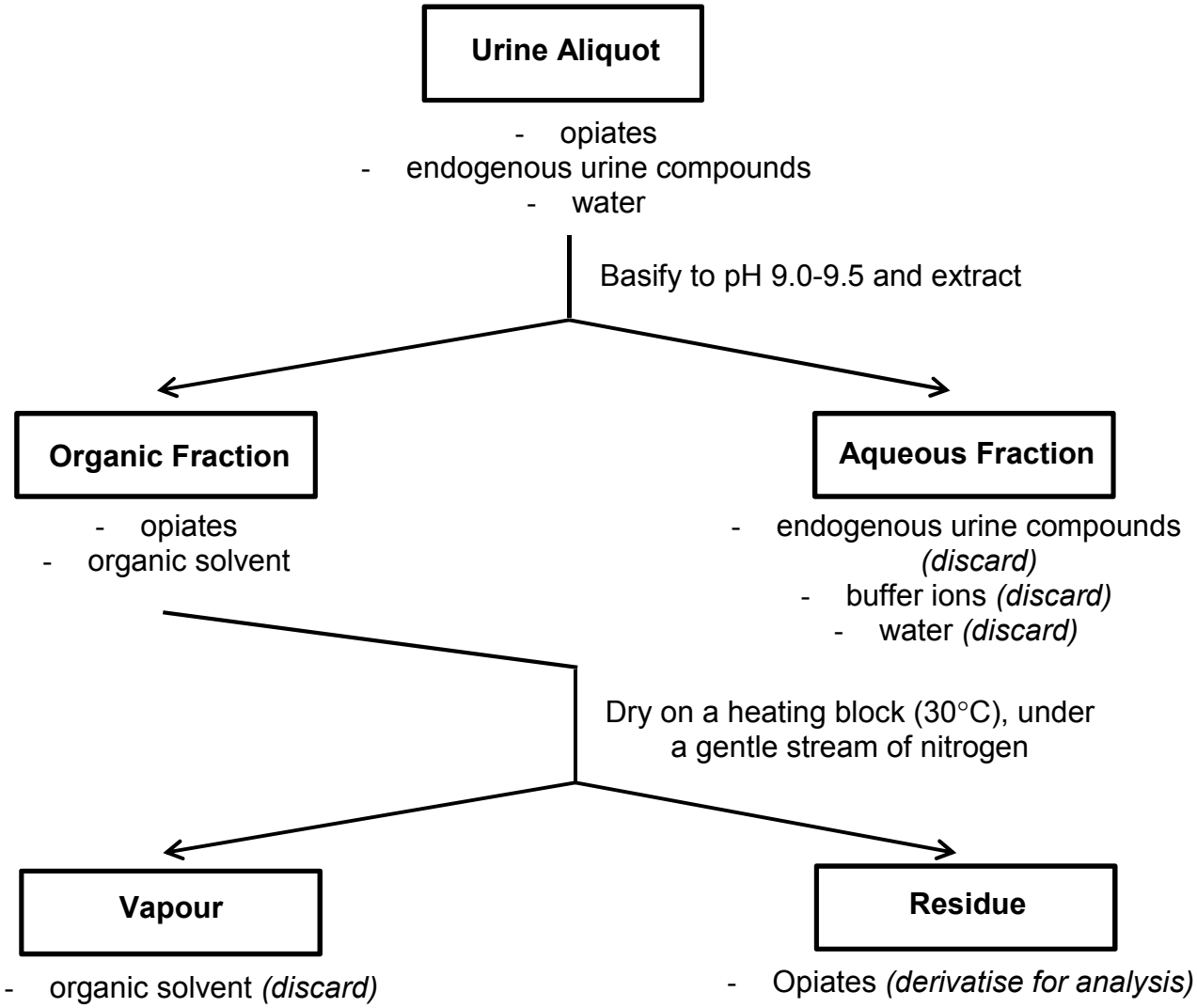


Figure 1-13: General procedure for the LLE of opiates from urine.

Traditionally, LLE was the preferred method adopted for drugs-of-abuse extraction, however in recent years the implementation of SPE in laboratories has surpassed that of LLE. Formation of emulsion, poor phase separation, low degree of automation and labour intensive sample work-up are well known drawbacks of LLE [81]. Several advantages are obtained through the use of SPE over LLE, including speed, reproducibility, selectivity, reduction of solvent usage and cleaner extracts. As the extracts are cleaner, the issue of ion suppression or enhancement experienced in LC-MS analyses that are caused by the matrix is significantly reduced. Furthermore, SPE offers the capability of fractionalisation so that the analytes can further be separated into classes of

compounds. The separation power of a typical SPE device is 50 times greater than a single, simple LLE procedure [82].

In essence, SPE is a cartridge-like device that adopts the use of chromatographic packing material (stationary phase or sorbent) that is supported by a base material to chemically separate the different components of a complex sample, based on the analyte's affinity for the sorbent. Examples of base materials and sorbents are found in Table 1-3. SPE method development begins with the conditioning of the sorbent to activate the sorbent ligands and equilibrate the sorbent bed. Once a sample is introduced into the column, solvent is passed through the SPE cartridge; the choice of solvent is dependent on the mode of SPE and the retention strategy that is used. If the analytes of interest are present in the sample at high concentrations, it is desirable to adsorb matrix interferences and allow the analytes to pass through the cartridge un-retained. Conversely, if the analytes of interest are found at low concentrations in the sample, it is ideal to adsorb the components of interest onto the stationary phase and allow the matrix interferences to elute from the cartridge without being retained. The analytes of interest can subsequently be concentrated. The latter strategy can also be employed when there are analytes with varying polarities that require isolation from the sample matrix. Following on from sample loading, the sorbent is washed with a suitable solvent and then dried; the final step involves the elution of analytes that are still retained in the cartridge [82-84].

Table 1-3: Base materials and sorbent functional groups commonly used in SPE cartridges [83].

base materials	sorbent functional groups
silica (sodium and potassium silicates)	silica
Fluorosil® (magnesium silicates)	diol
alumina	diethylamino
carbon	cyanopropyl
vinylbenzene polymer (polystyrene)	C2-C8
divinylbenzene polymer	C10, C12, C18, C20, C30
N-vinylpyrrolidone	phenyl
cellulose	cyclohexyl
hydroxyapatite	benzenesulfonic acid
fullerenes	propylsulfonic acid
cyclodextrin	carboxylic acid
agarose	amino (primary, secondary, quaternary, aminopropyl, diethylamino)

There are three main techniques or modes of separation in SPE: ion exchange, normal phase, and reversed phase SPE [82, 83]. Ion exchange SPE separates compounds based on electrostatic interactions between the analytes in the matrix and the sorbent. The matrix is often aqueous for ion exchange extractions. This mechanism requires manipulation of the pH conditions to ensure that both the sorbent ligands and the analytes of interest to be retained are in their charged (ionic) states. Ion exchange sorbents can be further divided into anionic and cationic sorbents. Cationic exchange sorbents have the capability for becoming negatively charged (benzenesulfonic acid, propylsulfonic acid and carboxylic acid from Table 1-3) and are used to retain positively charged analytes (produced by basic drugs, catecholamines and herbicides). Thus, basic elution solvents are required to neutralise the analytes. In contrast, anionic exchange sorbents have the capacity to become

positively charged (amino ligands from Table 1-3) and are utilised for the retention of deprotonated analytes (acidic drugs, organic and fatty acids and vitamins). Acidic elution solvents are required for the neutralisation and elution of these analytes [82-84].

In normal phase cartridges, the sorbent is hydrophilic (silica, diol, diethylamino and cyanopropyl from Table 1-3), and interacts strongly with polar compounds. Thus, it can be used to retain polar analytes from a non-polar matrix or retain polar matrix compounds while non-polar analytes of interest passing through the cartridge. Analytes are adsorbed onto the sorbent through π-π and dipole-dipole interactions (hydrogen bonding is a strong dipole-dipole interaction). Normal phase SPE is commonly used to extract compounds containing amino, hydroxyl, carbonyl and aromatic functional groups, in addition to compounds with heteroatoms (oxygen, sulphur, nitrogen and phosphorus), from non-polar matrices. In these cases, the medium to high polarity solvents are required for analyte elution from the sorbent [82-84].

On the contrary, reversed phase sorbents are manufactured from hydrophobic hydrocarbon chains. They are used to retain non-polar to moderately polar compounds (either analytes of interest or matrix contaminants). Retention is produced based on the intermolecular dispersion forces between sorbent and analyte C-H bonds. As a result, non-polar to moderately polar solvents are required for the displacement and elution of these compounds off the cartridge surface. Reversed phase SPE is commonly applied in areas of drugs-of-abuse and pesticide testing [82-84]. As with LLE, sample pH is very important for the adequate recoveries of the analytes of interest using SPE, and must be optimised for each class of compounds, and for each mechanism of separation. Both reversed phase and cationic exchange SPE (or a combination of these) has been employed for the extraction of opiates from urine. An example of a typical extraction procedure for opiates in urine using a mixed mode column is described by United Chemical Technologies [85] and begins with specimen pH adjustment to pH 5.5. Following column conditioning, the sample is slowly applied onto the column (the rate of loading is important; a slower rate allowing the opiate analytes to interact and adsorb

efficiently with the stationary phase of the column). The column is then washed and vigorously dried under vacuum before the opiates are eluted off the column with a strong eluting solvent, such as dichloromethane: isopropanol: ammonium hydroxide (78:20:2).

SPE may be automated or semi-automated, and most semi-automated systems rely on the use of a vacuum manifold to aspirate the fluid through the columns. Most laboratories utilising SPE use the vacuum manifolds, with fully automated computer controlled robotic arms more of a rarity. Full automation, does, however, provide higher drug recoveries and greater precision than the semi-automated procedure. This is because the fluid flow rate, sample application and elution rate can be controlled more consistently than laboratory staff manually adjusting the vacuum of the manifold. Nevertheless, semi-automated SPE provides more consistent results than manual LLE [86]. Furthermore, the relatively small volume of solvent required for SPE results in minimised exposure to toxic fumes, as well as decreased expenditure for laboratories. The procedure is approximately 12-fold less time consuming and also five-fold less costly than LLE. It is therefore not surprising that over the last two decades, there is an increasing shift towards the use of SPE in high throughput laboratories [53].

1.3.3.3 Derivatisation of opiate analytes

Following LLE or SPE extraction, the opiate analytes undergo chemical derivatisation prior to GC-MS analysis. The purpose of this step is to convert polar structures into less polar structures. As opiate molecules contain hydroxyl and amino functional groups, strong intermolecular hydrogen bonding is possible and so the structures need to be modified so that they are more volatile. Volatile compounds can be vaporised with greater ease and is a requirement for GC-MS analysis. The most common derivatisation procedures are trimethylsilylation, trifluoroacetylation, pentafluoropropionylation, heptafluorobutyrylation and methylation [15, 50, 54, 65, 72, 73, 80, 87].

For 6-MAM, morphine and codeine, a very common derivatising reagent is the use of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) or *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) for the formation of trimethylsilyl (TMS) derivatives. In this procedure, the hydroxyl -OH groups are converted to -OTMS moieties. Additionally, propionic anhydride can be used to carry out opiate derivatisation. However, the reagent may contain trace amounts of acetic anhydride as an impurity. It has been reported that morphine derivatives undergo acetylation in the presence of trace acetic anhydride, producing 6-MAM derivatives [71]. It is important to test the purity of the propionic anhydride prior to its implementation. Derivatisation is carried out at elevated temperatures (in the range of 40-90°C) [6, 55, 59, 66, 88]. Following incubation, the sample is dried under nitrogen and the opiate derivatives reconstituted in organic solvent, or injected directly into the GC-MS instrument (possible with BSTFA and MSTFA).

1.3.3.4 GC-MS separation and detection of opiates

Following injection of the sample into the GC-MS instrument, the high temperature of the injection port (typically 250°C) causes the opiate derivatives to be vaporised and swept into the capillary column via the flow of helium carrier gas. The fused silica capillary column is located in an oven, allowing its temperature to be carefully controlled. The column temperature can be operated in an isothermal manner or programmed with a temperature gradient. Traditionally, packed columns were favoured, however they have been replaced by capillary columns due to their high efficiency. A popular choice of stationary phase composition for the analysis of opiate alkaloids is 5% phenyl bonded with 95% dimethylpolysiloxane, such as the HP-5ms capillary column manufactured by Agilent Technologies [89]. Once in the column, the opiate analytes partition between the carrier gas and the stationary phase of the column. Under a particular set of experimental conditions, the extent of partitioning is dependent on the analyte; it determines the elution time, and thus its chromatographic separation [90].

As the opiate analytes leave the column, they are directed into the ion source. The temperature is extremely high at this region to produce vaporised analyte molecules. The target analytes are then bombarded with a beam of energetic electrons to induce ionisation. This mechanism is known as electron impact ionisation, and is the most common ionisation technique used. Despite excessive fragmentation leading to the loss of the molecular ion peak in some cases, this technique is capable of producing reproducible mass spectra for a large variety of analytes. The focusing lenses funnel the fragment ions into the mass analyser, where they are separated. The signals belonging to the fragment ions are then detected and amplified by an electron multiplier detector, which transmits the data to a data processing system [90].

In terms of acquisition mode, opiate analysis is generally carried out in selective ion monitoring (SIM) mode. The molecular ion, in addition to a qualifier ion, must be detected for the qualification of a particular opiate. However, the general criterion for its quantification is the presence of a third qualifier ion which must be present in the required ratio [15, 50, 79, 87].

1.3.4 Liquid chromatography-mass spectrometry

The potential for replacing GC-MS with LC-MS in urine drug testing programs was reported over 10 years ago. Although GC-MS is still considered by some as the ‘golden standard’ for confirmation testing, LC-MS provides an alternative avenue for the confirmatory testing of drugs of abuse in urine. LC-MS presents advantages over GC-MS, such as simplification of the sample preparation. With the advent of LC-MS, the need for sample hydrolysis to liberate glucuronide bound opiates is no longer required. Additionally, the tedious derivatisation step for the analysis of polar and thermolabile compounds such as opiates is also not required [73, 91].

With traditional GC-MS, the analysis of 6-MAM is performed using a separate, non-hydrolysed specimen (parallel to the analysis of a hydrolysed specimen for the detection of morphine and codeine), since the metabolite is easily destroyed by the hydrolysis procedure. This is no longer a concern with LC-MS

as free and conjugated morphine and codeine, in addition to 6-MAM, can be analysed in the same sample, in one single analysis [73]. This increases the sample throughput and turnaround time of the results. Furthermore, the ability of LC-MS to measure both free and conjugated morphine and codeine independently rather than collectively (as the total concentration) may lead to an improvement in the interpretation of heroin and morphine toxicity [58]. The limits of quantification for morphine and codeine have been found to be as low as 1 ng/mL for LC-MS, compared to 10 ng/mL for GC-MS [92].

Although the hydrolysis and derivatisation steps of the sample preparation procedure can be eliminated for LC-MS, sample clean-up is still a requirement in order to maintain the life of the chromatographic column and instrument. Additionally, the issue of analyte ion suppression or enhancement contributed by the endogenous compounds in the urine (commonly referred to as 'matrix effects') can be minimised if the matrix is removed. Ion suppression may occur when a compound from the matrix elutes at the same time as an analyte of interest, therefore reducing the area of the analyte peak. Sample clean-up involves the extraction of the opiate analytes from the urine matrix using LLE or SPE (section 1.3.3.2), and increases the overall sensitivity of the analysis method. Some laboratories employ a more basic sample pre-treatment method known as 'dilute and shoot'. It is cheaper and more rapid, and involves the addition of acetonitrile to precipitate the proteins from the urine specimen; the sample is then centrifuged and the supernatant collected and filtered (through a syringe filter unit) for analysis. However, the significant downfall of this method is the very observable ion suppression effects. Since both the endogenous urine compounds and analytes of interest are diluted using 'dilute and shoot', the ion suppression effect is proportional to a sample that was not extracted at all [87].

1.3.4.1 Chromatographic separation of opiates using LC

Following the injection of an opiate extract into the LC system, the analytes are carried onto the chromatographic column by mobile phases. A typical column for the analysis of opiates in urine is a silica packed column embedded with

hydrophobic C18 ligands (constituting the stationary phase). However, other alkyl or phenyl ligands capable of hydrophobic of π-π interactions provide adequate chromatography [93]. The interaction between the opiate analytes and the stationary phase of the column is similar to the mechanisms described for SPE carried out in reversed phase conditions (section 1.3.3.2).

LC mobile phases can be divided into two categories; aqueous and organic mobile phases. In reversed phase LC-MS, analyte retention and elution is controlled by the organic component of the mobile phase; the higher the organic content, the greater the eluent strength of the mobile phase. The greater the eluent strength of the mobile phase, the quicker the analyte displacement from the stationary phase of the column [93]. Purified water with a formate or acetate additive constitutes the aqueous phase, whereas acetonitrile or methanol is often used as the organic mobile phase for opiate analysis. Formic acid may also be added to improve the peak shape of the analytes. The mobile phase can be set to follow an isocratic elution or a gradient elution profile. Isocratic elution refers to the use of a single mobile phase composition throughout the analysis. Gradient elution refers to the change of composition of the organic and aqueous components of the mobile phase, as the analysis progresses. The flow-rate of the mobile phase is generally in the range of 0.2-0.5 mL/min, and is dependent on optimal column and chromatographic separation requirements [54, 69, 73, 87, 91, 92]. The chromatographic separation of the opiate analytes is also affected by the temperature of the column, and so controlling this parameter ensures the reproducibility of a method.

Once the opiate analytes are displaced from the column, the eluent is directed to the ion source for ionisation and subsequent mass spectrometric detection.

1.3.4.2 Ionisation interfaces and mass spectrometric detection for opiates

There are various ionisation sources that can be interfaced with an LC system for the analysis of opiates, and includes electrospray ionisation (ESI), matrix

assisted laser desorption ionisation (MALDI) and atmospheric pressure chemical ionisation (APCI). ESI is the most frequently used technique for the ionisation of opiates in urine [94].

Once the eluent is passed into the ESI source, it passes the ESI probe and is sprayed through a fine capillary tip. As the capillary is electrically charged, the eluent droplets that are sprayed out become charged. Nitrogen gas flows around the tip to aid nebulisation of the ionic droplets, which continually become smaller until they are small enough for ion desorption. The formation of small ions is crucial for the transfer of the analytes into the mass spectrometer, as charged entities are attracted and accelerated into the mass spectrometer [87, 94, 95]. Ionisation of opiate analytes occurs in positive ion mode, and so the compounds are detected as the protonated molecule, $[M + H]^+$ [69, 73, 78, 87, 91, 92].

Compared to electron impact ionisation utilised by GC-MS, ESI is considered a 'softer' method of ionisation, therefore reducing the instances of excessive fragmentation. However, it is susceptible to ion suppression; it is suspected that any polar endogenous urine compounds that may be present in the injected sample competes with the analytes for charge that is created in the ESI probe within the ionisation source, thus causing the suppression effect. Chromatographically, ion suppression can be identified as a drop in baseline when comparing the spectral response of the analytes of interest in both water and in urine [87, 96].The use of APCI for the ionisation of opiates has also been documented as an alternative for circumventing this issue, however it is not preferred due to the weaker analyte signal response obtained when compared to ESI [87, 91].

To fulfil a forensic level of identification of drugs of abuse analytes including opiates, there is an increasing number of laboratories which use liquid chromatography-tandem mass spectrometry (LC-MS/MS) in order to increase the specificity of the technique [73]. With tandem mass spectrometry, the mass analyser is divided into three sections: the first quadrupole (MS1), collision cell (MS2) and the second quadrupole (MS3). The opiate analytes enter the first

set of quadrupoles, where they are filtered according to their mass to charge (*m/z*) ratio by the voltages applied across the quadrupole. Any unwanted masses are drifted out of the quadrupole. The ions that pass through then enter the collision cell, where the ions collide with inert argon molecules, causing them to either dissociate or pass unhindered into the second quadrupole and into the detector. In terms of acquisition, LC-MS/MS analyses can be conducted in full scan mode, selective ion mode (SIM), product ion scan mode and multiple reaction monitoring (MRM) mode [69, 95, 97]. For MRM analyses, the majority of the literature advocates the monitoring of at least two transitions; precursor ion->quantifier ion and precursor ion->qualifier ion [46, 48, 73, 87, 91, 98].

1.3.5 Stability of opiate alkaloids in urine

Morphine and codeine are relatively stable in urine in comparison with 6-MAM. It is well documented that 6-MAM is not stable for long periods of time in aqueous environments such as urine. The acetyl functional group present in the 6-MAM structure may be affected by pH, causing the metabolite to undergo hydrolysis to produce morphine. This process is accelerated in acidic conditions, which is the expected pH range for normal urine [87, 91, 99]. Therefore, interpretation of the results based on the concentrations of the target analytes should be preceded with caution.

The stability of opiate alkaloids is also influenced by temperature. For instance, two studies found in the literature, in investigating the stability of 6-MAM, morphine and codeine in urine at both room temperature (22°C) and freezing temperature (-22°C), reached conclusive results. At both temperatures, 6-MAM was found to be noticeably more unstable than morphine and codeine under the experimental conditions employed. This observation was also true in cases where methanol (instead of urine) was used as the sample matrix. However, freezing appeared to have a stabilising effect on 6-MAM [91, 99]. It was also demonstrated that successive freezing and thawing of 6-MAM samples must be avoided. Two identical frozen samples were analysed after one was simply thawed, and the other was subjected to successive freezing and thawing.

Comparison of the two samples showed that 6-MAM was more stable in the sample that was simply thawed [99].

1.4 Sample adulteration in urine drug testing

Urine is a long accepted biological matrix used for the detection of prescription and illicit drug use in the population. Repercussions may exist if it is found that an individual is guilty of using a prohibited drug. These vary in severity and include incarceration, termination of employment, suspensions and fines [100]. In today's society, there is still a social stigma attached to individuals who have been found to be using contraband drugs. Being labelled a "drug addict" or a "drug cheat" in sports can potentially be detrimental to a person's reputation. As such, it is not surprising to learn that individuals are motivated to discover and utilise new and ingenious ways of circumventing routine drug testing protocol. The aim is to conceal a 'true positive result' by producing a 'negative result', thus masking drug use [100, 101]. A very effective method for doing so is to purposefully adulterate a urine specimen to invalidate the results of a drug test [102].

One definition for an adulterated urine sample is "a urine specimen containing a substance that is not a normal constituent or containing an endogenous substance at a concentration that is not a normal physiologic concentration" [64]. More simply, urine adulteration can be defined as "the tampering of specimens with the purpose of altering the test results" [103]. This is possible because the adulterants disrupt the mechanisms of the assays employed to detect the drugs.

Successful adulteration disproves drug use in cases where it should not be disproven, and is an ongoing issue for sports and workplace drug testing laboratories [103, 104]. To date, the extent of urine tampering is not conclusively known as investigation and documentation of such cases is not implemented in most laboratories. It has been estimated that nearly 1000 adulterated specimens are submitted to US drug testing laboratories per week [100, 104]. In the early 2000's, the prevalence of adulteration was also

assessed by the Substance Abuse and Mental Health Services Administration (SAMHSA). In an audit of 66 certified laboratories, the National Laboratory Certification Program identified a total of 6440 (0.05%) adulterated specimens among 12 million urine samples tested over a two year period. However, the actual number of cases of adulteration must be higher as this figure does not include specimens where the presence of an adulterant was suspected but unable to be confirmed [102].

The sophistication of drug testing protocol is paralleled by the intricacy of the methods available for urine adulteration. These methods are conceptualised by intelligent and creative individuals with knowledge of drug testing methodologies [74]. Although drug testing programs strive to achieve ways of counteracting new procedures available for adulteration, they are always marginally behind as their procedures are only good to prevent the effectiveness of adulterants currently known to them; this is where the issue lies.

In comparison with other relatively common biological matrices used for drug testing (such as blood, oral fluid and hair), urine is by far the easiest matrix to adulterate. Blood, oral fluid and hair specimens are obtained in the presence of a medical professional or a police officer, thus greatly limiting the opportunity for sample tampering. According to Dasgupta, the chances of adulterating oral fluid specimens are low to non-existent [64]. Nevertheless, several products are available for purchase over the internet such as shampoos for the concealment of drugs in hair, as well as commercial adulterants claiming to 'destroy' drugs present in oral fluid resulting in a negative drug test. No systematic study has been reported regarding the capabilities of the shampoos for passing a drug test. The commercial oral fluid adulterants have been found to be incapable of destroying drugs of abuse [64].

Regardless of the ease of adulteration of urine samples, it is the most commonly used biological matrix for drugs of abuse testing. Urine is relatively non-evasive to obtain compared to other biological matrices. It is also regularly

voided and has a less complicated matrix compared to blood and hair, making it easier for drug extraction.

1.4.1 Methods of urine adulteration

The techniques used to carry out urine specimen adulteration can be divided into three sub-categories: substitution adulteration, *in-vivo* adulteration and *in-vitro* adulteration.

1.4.1.1 Substitution

According to SAMHSA mandatory guidelines, substituted adulteration is defined as "a urine specimen with creatinine and specific gravity values that are so diminished and incongruent that they are not consistent with normal human urine" [105]. The use of water, saline and other liquids to replace urine has been documented [102]. However, the majority of individuals who choose substitution as their method of choice take a much more complex approach involving the use of commercially available synthetic urine. Otherwise, "clean" urine can be obtained from a drug free volunteer [100]. Synthetic urine such as "Quick Fix Synthetic Urine™" is a bottle of premixed urine with all the characteristics of endogenous human urine, such as correct pH, specific gravity and creatinine concentration [106]. To minimise suspicion during collection, the substituted urine can be taped next to a heating pad to maintain the correct physiological temperature of urine. More commercial methods for warming a substituted sample include the use of a "Butt Wedge™", which employs a wedged shaped container to store and warm urine between the users buttocks [100]. Similarly, "The Urinator™" utilises a flexible plastic container that is strapped to the body along with a heating pad; a small tube is placed in close proximity to the urethra so that the delivered stream appears realistic. Other commercial products are also available to make the delivery of urine even more realistic. For instance, 'The Whizzinator™" employs a prosthetic penis which is used to excrete the sample. It is available in five different skin tones, and it is unlikely that the supervisor present at the

collection will be able to detect its use without significantly intruding on an individual's privacy [106, 107].

A more extreme method of substitution is catheterisation. The individual first voids their urine, and a catheter is inserted up the urethra to fill the bladder with a synthetic or "clean" urine sample to ensure a negative outcome for the drug test. However, this practice may result in an increased risk of urinary tract infection [100, 107].

1.4.1.2 In-vivo adulteration

In-vivo adulteration is the intentional ingestion of a product designed to dilute the urine or to increase the metabolism and/or excretion of drugs in the body to avoid detection of recent substance use [100, 102]. Water can be classified as an effective *in-vivo* adulterant. Excessive consumption causes the concentration of urine constituents, including any drugs that may be present in the system, to be diluted to below drug cut-off concentrations. Therefore, a false-negative result on the drug test will be returned [64, 102]. In addition to excessive water consumption, commercial fluids or tablets can be used to flush out metabolites by inducing diuresis. Examples of commercial diuretics include: the "Absolute Detox XXL™" drink, "Fast Flush Capsules™" and "Ready Clean Gel Capsules™". It is also believed that drinking goldenseal tea helps to avoid a positive drug test result by diluting the urine at a faster rate than water consumption alone. However, the disadvantage of this method is the production of dark urine, which arouses suspicion upon visual inspection of the specimen [64].

1.4.1.3 In-vitro adulteration

In-vitro adulteration is the addition of foreign substances into the urine specimen after it has been voided, which work by either interfering with the analysis procedure or converting the target drug to compounds that cannot be detected during routine analysis. Common readily available household chemicals such as table salt, bleach (active constituent: sodium hypochlorite),

soap, concentrated lemon juice, vinegar, "Drano®" (active constituent: sodium hydroxide), ammonia and Visine® eye drops can be used to avoid a positive drug test. Bleach is one of the most effective urine adulterants and has been used to conceal the presence of cannabis metabolites in urine [100, 101]. However, the easy detection of these additives in a urine specimen has caused its use as a urinary adulterant to wane. Currently, more sophisticated commercial *in-vitro* chemicals are available for purchase. They cannot be detected by visual inspection, smell or routine integrity testing, where parameters such as specific gravity, temperature, creatinine and pH of the sample are checked. These commercial *in-vitro* chemicals contain active ingredients that possess very potent oxidising capabilities. It has been estimated that two thirds of urine adulteration cases involves the use of oxidising adulterants [64, 102].

1.4.2 Oxidising adulterants

Oxidising adulterants affect the results of routine urinalysis in two ways. Firstly, they may adversely interfere with the assays employed to identify the drugs of abuse, therefore creating a false negative result. For instance, an oxidising adulterant may disrupt the mechanism of action of reagents used in immunoassay analysis. This causes the drug analytes to be undetectable using this assay, despite its presence in the specimen. Secondly, oxidising adulterants have the ability to alter the molecular structures of the drug analytes. Since most of the assays employed in routine analysis screen for specific analytes of interest, they will be unable to detect compounds that were not previously selected. Drugs are rendered undetectable as they are present as analogues of the parent drugs (in this case, a true negative result occurs) [59].

Numerous commercial oxidising adulterants exist and although they each have their own mechanism of action, the overall effect is similar; they successfully mask the presence of drugs in a urine specimen. The active constituents of these products are detailed in section 1.4.2.1 to section 1.4.2.5.

1.4.2.1 Nitrite

Oxidising adulterants with nitrite as the active ingredient have been marketed since 1996. Commercial products such as Klear™, Whizzies™, Krystal Klean™ and Purafyzit™ all contain nitrite as either sodium or potassium salts [100]. Both Klear™ and Whizzies™ are supplied as a set of two vials, with each vial containing 500 mg of the white crystalline nitrite salt. As recommended by the manufacturers, one vial is required for the concealment of Δ^9-tetrahydrocannabinol (THC) in a urine specimen voided by a cannabis user. On the other hand, two vials should be utilised to ensure that the presence of other drugs of abuse are masked [64, 74, 101, 102, 104].

One study has found that oxidation of the cannabis metabolite, 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH), by nitrite in acidic urine went to completion after 16 hours of reaction [101]. A separate study demonstrated that nitrite leads to the decomposition of THC ions and its internal standard, and that the extent of "destruction" of THC-COOH in urine is not proportional to the nitrite concentration in the specimen. Hydrosulfite can be added to the specimen at the beginning of sample preparation to help reduce the nitrite (redox reaction), therefore eliminating its interference [104].

1.4.2.2 Pyridinium chlorochromate

Pyridinium chlorochromate (PCC) has been marketed as an adulterant since 1998 [100]. It was sold under the tradenames of Urine Luck™, LL 418™, Sweet Pee's Spoiler™ and even Klear II™. The concentration of PCC in the commercial products was typically determined to be 200 mmol/L (mM). The oxidative capability of this product lies with the hexavalent chromium (Cr^{6+}). It has been documented that Cr^{6+} interferes with drug screening tests for THC and morphine [64, 74, 101, 102, 108]. Interestingly, there are different perspectives on the actual mechanism of action of PCC. One study claimed that PCC causes the pH of urine samples to decrease, therefore interfering

with the drug testing assays. It has been found to lower the responses of morphine and THC when using AbuScreen assays [100]. On the contrary, the majority of studies demonstrated that its ability to mask the presence of drugs of abuse in urine stemmed from the oxidising capability of the active Cr^{6+} ion. One study has shown that THC-COOH in urine was considerably oxidised after 16 hours of exposure to PCC [101]. Other studies have substantiated this finding. It has been demonstrated that 60-100% of THC-COOH in urine samples were "inactivated" following treatment with 2 mM PCC. A high percentage of morphine was also lost when exposed to 2 mM PCC, however this was dependent on the pH of the urine [109, 110]. The concentration of free morphine and free codeine appeared stable in the pH range of 5-7 when the urine specimen was treated with PCC, however significant losses were observed at a lower pH [64].

1.4.2.3 Hypochlorite

Hypochlorite is the active ingredient of household bleach and is generally used as a disinfectant. As an adulterant however, bleach was found to oxidise opiates in urine specimens, rendering them undetectable by GC-MS testing [102]. Bleach also directly affects immunoassay reagents, resulting in erroneous test results. False negative responses were obtained when opiate positive urine specimens were screened with FPIA assays. In addition, it has been observed to significantly interfere with CEDIA assays [64, 100, 101]. Studies have shown that both opiates and cannabinoids are significantly susceptible to bleach adulteration, where actual degradation of the target analytes were confirmed by GC-MS analysis [64].

1.4.2.4 Peroxide/peroxidase

The commercial oxidising adulterant Stealth™ is sold as two vials. The first vial contains powdered peroxidase enzyme and the second vial contains liquid hydrogen peroxide. Upon the addition of both vials to a urine specimen, a strong oxidising potential is created (peroxide alone has been found to be an ineffective adulterant). This results in the efficient oxidation of most drug

analytes present in the specimen. As the target analytes are no longer present in the sample, they can no longer be detected during both presumptive and confirmatory stages of analysis. Stealth™ has been shown to decrease concentrations of most drugs of abuse using GC-MS and is particularly efficient in the concealment of THC-positive urine [64, 74, 101, 102]. The oxidation process cannot be reversed, although the addition of a reducing agent such as sodium hydrosulphite (like with nitrite adulterated samples) or sulfamic acid before the extraction process allows any unchanged target analytes to be detected. The peroxide/peroxidase oxidation system significantly interferes with the extraction processes used to isolate morphine and codeine for GC-MS testing, and so it is worthwhile to remove them prior to extraction [101, 102].

The disadvantage of using Stealth™ is that it causes the urine to rapidly change colour. A dark brown specimen may be easily noticeable by visual inspection of the sample, and so limits the use of this product as a urine adulterant [74, 100]. Further, the use of peroxidase extract obtained from red radish skin for adulteration purposes has also been documented. However, the red radish skin pigmentation causes the urine to become a distinct red colour. This visual anomaly explains why it is unlikely to be used a urine adulterant [101].

1.4.2.5 Glutaraldehyde

Glutaraldehyde solution is available in hospitals and clinics and is used as a cleaning and sterilisation agent. A 10% glutaraldehyde solution is available from pharmacies as over-the-counter medication for the treatment of warts [64]. Glutaraldehyde was first marketed in 1993 as one of the earliest commercially available adulterants [100]. These adulterants existed under the tradenames of Instant Clean ADD-IT-ive™, Urin-Aid™, Clear Choice™ and Clean-X™. Each product typically contained 4-5 mL of glutaraldehyde solution, to be added to 50-60 mL of urine for adulteration purposes. It was found that the glutaraldehyde concentration was 75% in Urin-Aid™. It has been documented that glutaraldehyde interferes with immunoassay screening of

drugs of abuse by decreasing the absorbance rates [100]. With a 1-2% glutaraldehyde solution, a decrease in sensitivity is observed for opiates when specimens are screened with EMIT assays. Glutaraldehyde interference is also witnessed when screening for drugs of abuse using CEDIA assays [64]. Although the price is reasonable at $US 20 to $US 30 per kit, it is not as popular as the other oxidising products employed for urine adulteration [64, 74, 102].

1.4.2.6 Factors affecting the effectiveness of oxidising urine adulterants

Overall, the extent to which oxidising adulterants are effective in masking target drug analytes is dependent on various factors, such as pH, storage time, temperature and presence of endogenous compounds found in the urine specimen. Generally, most oxidising adulterants are more effective in acidic pH conditions, with the loss of target analytes increasing with increasing exposure time to the adulterants. The addition of bicarbonate buffer to the collection vessel before or immediately after voiding has been suggested. This helps maintain a non-acidic pH in the urine, thus hindering the oxidation process [64, 101]. However, in Australia, the addition of bicarbonate or any other substance to a urine collection cup is prohibited. Certain endogenous compounds in urine may compete for reaction with the adulterants, possibly resulting in a smaller extent of reaction between the adulterants and the drug analytes (and therefore the adulterants appearing to be less effective at concealing the drugs).

1.4.3 Methodologies for the detection of urine adulteration

The first line of defence for the detection of sample tampering is the personnel at both the sample collection sites and drug testing laboratories. They are responsible for visual inspection of the sample, as well as monitoring simple parameters such as: temperature, pH, specific gravity and creatinine (routine integrity testing). The collection cup quite often incorporates panels to monitor these parameters, however its effectiveness is debatable. These parameters

assist in the identification of obviously invalid specimens, and in the cases where household chemicals were used for adulteration, the strong odour can also be detected by smell [102, 111]. The endogenous characteristics of freshly collected, normal human urine are outlined in Table 1-4.

Table 1-4: Physiological measurements of temperature, pH, specific gravity and creatinine in normal human urine.

parameter	**expected Range**
temperature	32.5-37.7°C [112]
pH	4.7-7.8 [113, 114]
specific gravity	1.003-1.035 g/mL [113, 115, 116]
creatinine concentration	80-200 mg/dL [116-118]

Unfortunately, many of the oxidising adulterants are odourless and can easily escape detection when assessed solely using routine integrity check parameters. At low level concentrations, the darker colour of urine after the addition of “Stealth” or a slightly more intense yellow colour subsequent to the addition of PCC are not obvious enough to arouse suspicion of adulteration due to the natural variation of urine in the human population. This variation can be attributed to diet, as well as physiological and pathologic conditions [74, 102]. However, when sample adulteration is suspected, the laboratories may test for the presence of the adulterants themselves. Table 1-5 outlines the criteria recommended by SAMHSA for the detection of adulterated specimens. A vast array of spot tests (section 1.4.3.1), analytical techniques (section 1.4.3.2) and dipstick detection devices (section 1.4.3.3) are available for the detection of oxidising adulterants.

Table 1-5: SAMHSA guidelines for adulteration and substitution testing [105].

test and cut-off limits	**interpretation**
creatinine < 5 mg/dL and specific gravity < 1.002	substituted
creatinine < 5 mg/dL and specific gravity ≥ 1.002	substituted
creatinine ≥ 5 and < 20 mg/dL and specific gravity < 1.003	diluted
creatinine ≥ 5 mg/dL and specific gravity = 1.000	invalid result
creatinine ≥ 5 and < 20 mg/dL and specific gravity ≥ 1.020	invalid result
pH < 3 or ≥ 11	adulterated, pH outside of endogenous range
pH ≥ 3 and < 4 or pH ≥10 and < 11	invalid result
nitrite ≥ 500 mg/L	adulterated, nitrite
nitrite ≥ 200 and < 500 mg/L	invalid result
chromate > the lab's limit of detection	adulterated, chromium (VI)
halogen > the lab's limit of detection	adulterated, halogen containing adulterant
glutaraldehyde > the lab's limit of detection	adulterated, glutaraldehyde
decreased GC-MS internal standard (≥ 70%)	invalid result

1.4.3.1 Spot tests

Spot tests are easily performed in well-plates and provide rapid results; however, the disadvantage of this technique is that it lacks specificity. Spot tests have been developed for the detection of PCC, nitrite and Stealth™, however false positive results may occur when other interfering substrates are present.

The PCC colour test involves the addition of two drops of 1,5-diphenylcarbazide (DPC) indicator solution (10 g/L in methanol) to 1 mL of urine. A reddish purple colour change indicates the presence of Cr^{6+}, though other ions such as $Fe^{3+,}$ $Cu^{2+,}$ $Ni^{2+,}$ $V^{4+,}$ $Pb^{2+,}$ $Cr^{3+,}$ $Sn^{2+,}$ $Mo^{6+,}$ Hg^{2+} and Cl^{-} can cause interference. Other alternative spot tests reagents for PCC include the use of acidified potassium iodide or hydrogen peroxide. A rapid colour change is indicative of a positive result using both these reagents, with the formation of a dark brown precipitate observed when using the hydrogen peroxide [64, 65, 106].

Acidified solutions of potassium iodide or potassium permanganate can be used to detect the presence of nitrite. Upon the addition of acidified permanganate, a pink colour should be observed due to the reagent. However, discolouration accompanied by effervescence occurs when nitrite is present. This reaction is instantaneous; if the reaction is found to occur slowly, this may be due to a high glucose concentration in the urine, causing a false positive result [64, 65, 106].

The peroxidase enzyme in Stealth™ can be detected using a tetramethylbenzidine solution buffered with 0.1 mol/L (M) phosphate. An immediate dark brown colour change indicates the presence of peroxidase. Additionally, acidified potassium dichromate solution can also be used to detect the presence of Stealth™ in a urine specimen. A positive result using this reagent is characterised by a deep blue colour change, which fades over time [64, 65, 106].

1.4.3.2 Instrumental techniques

Analytical instruments in the laboratory can be used to detect the active constituents of commercial oxidising adulterants. One technique developed is the immunoassay based Microgenics DRI® General Oxidant-Detect® Test that is specifically used for the detection of urine adulteration by oxidising compounds. This assay can be performed on an automated clinical chemistry analyser, and is based on the reaction between tetramethylbenzidine (TMB) reagent and the oxidant in the specimen, which forms a coloured complex that can be observed at 660 nm. However, this assay does not appear to be routinely implemented in drug testing laboratories, mainly due to cost.

Other instrumental techniques have been developed to test for specific components present in the oxidising adulterants. For instance, capillary electrophoresis has been used for the detection of the chromate and nitrite ions found in PCC and nitrite-based oxidants, respectively [53, 119, 120]. Also, electrospray tandem MS, GC-MS and inductively coupled plasma mass spectrometry (ICP-MS) has been shown to detect chromium species found in PCC [64, 108]. Furthermore, high performance liquid chromatography (HPLC) coupled with MS or a conductivity detector has demonstrated a discerning ability to detect the active Cr^{6+} and nitrite ions in commercial urine adulterants [64]. Finally, spectrophotometric analysis is an accepted analytical technique used to study peroxidase enzyme activity, and may be used to indicate the presence of Stealth® [121]. PCC in a urine solution can be detected by a colour reaction with 1,5-diphenylcarbazide (DPC) [109]. Six other spectrophotometric methods were developed to detect oxidants in urine including ferric, chromate, nitrite, permanganate, oxychloride, and hydrogen peroxide [122]. Like the DRI® General Oxidant-Detect® Test, the analyses of the active components of oxidising adulterants using these instrumental techniques are not part of routine testing protocols for drugs of abuse.

1.4.3.3 Dipstick detection devices

On-site adulteration detection dipsticks are commercially available, offering an advantage over other methodologies because its portability allows it to be used at the specimen collection site [64]. Every detection device possesses advantages and disadvantages; thus, the specific purpose(s) of the assay has to be known.

Widely available and designed for urinalysis, the Multistix® (Bayer) and Combur-Test® (Roche Diagnostics) reagent strips can be used to test for nitrite, pH, specific gravity and Stealth™. However, the detection of Stealth™ is indirect; specimens adulterated with this oxidant give a strong trio of positive readings for glucose, blood and nitrite. This is because the tests for glucose and blood are based on peroxidase activity. A disadvantage of these two reagent strips is their inability to determine the difference between substituted and diluted urine, as it has difficulty distinguishing specific gravity at the cut-off levels for these two types of urines. The nitrite pad on these reagent strips also only detects nitrite concentrations at a clinically significant range (patients with urinary tract infection or pathological conditions may have urine nitrite as high as 100-150 µg/mL). This is not helpful in the cases of adulteration, where Klear™ adulterated urine specimens have been found to contain 1900-15000 µg/mL nitrite [64, 123].

The Adultacheck® 4 and Adultacheck® 6 dipsticks are available from Sciteck® Diagnostics. They are to be used for forensic toxicology purposes only, and tests for creatinine, pH, nitrite, glutaraldehyde and PCC in urine specimens. They can detect a large range of creatinine and pH values at both ends of the spectrum, including abnormally low and high levels. Furthermore, the nitrite assay is designed to detect nitrite concentrations above clinical levels. The limitation of these devices however, is the difficulty in determining a precise reading for creatinine and pH [64, 100].

The MASK Ultrascreen (Kacey Inc.) is another popular on-site adulteration detection device. A greater range of adulterants can be detected using this

product when compared with the Adultacheck® strips. Assays for creatinine, pH, specific gravity, Stealth™, PCC, nitrite and glutaraldehyde are available on the testing panel. However, a disadvantage is that the adulterants will only be detected if they are present in concentrations well above the recommended usage [64, 100].

Intect7® (Branan Medical Corporation) was evaluated to be the most sensitive and economical adulteration test strip on the market. Each plastic strip is affixed with seven chemically treated pads for assessing the levels of creatinine, pH and specific gravity, in addition to exogenous bleach, PCC, nitrite and glutaraldehyde. It has been found to identify adulterants correctly, with even 10 µL of bleach/ mL of urine able to be detected. This is significant as only a miniscule amount of bleach is added to mask the presence of many drugs in urine; the practice of using copious amounts of bleach arouses suspicion during routine integrity testing, and so is avoided [64, 100, 124].

1.5 Aims and objectives of the PhD research

Currently, urine samples deemed to be “tampered” are not analysed further for drugs of abuse as the presence of the target analytes may be significantly deteriorated or even undetectable using routine testing methods. One pathway for the mechanism of action of commercially available urine adulterants is the ability of the active species to oxidise compounds. It is well documented that both presumptive and confirmatory opiate urinalysis is significantly affected by these adulterants. However, no research has been conducted to study the interactions between the target opiate analytes and the oxidising adulterants. Furthermore, although there is a vast selection of methodologies that can be employed to detect the presence of oxidising adulterants in urine, there are no alternative methods for the detection of the target opiate analytes in urine subsequent to the act of adulteration. Therefore, the aims and objectives of this research are to:

1. Investigate the effect of various oxidising adulterants on the concentration of heroin related opiate analytes in urine samples.

2. Monitor the formation of any stable reaction products resulting from exposure of the opiates to the oxidants in urine.

3. Isolate any stable reaction products and carry out structural elucidation using spectroscopic and spectrometric techniques.

4. Assess the effects of various oxidising adulterants on CEDIA immunoassay analysis.

5. Evaluate the viability of using the reaction products as markers for monitoring the presence of opiates subsequent to urine adulteration with oxidising adulterants.

It is hopeful that this research can contribute new knowledge to the field of urine drug testing, and can potentially be used to help overcome the issue of adulteration by oxidising adulterants. The individuals responsible for conceptualising and creating the commercial products that are on the market are extremely resourceful; they will invent new strategies when the old ones have been discovered. Because of this, they will always be one step ahead. Nonetheless, the findings of this research may help to narrow the gap between those individuals and the drug testing authorities, and provide an alternate avenue for analysis when routine methods cease to work.

Chapter 2:

Pilot study

Chapter 2: Pilot Study

2.1 Introduction

Literature has shown that drugs of abuse compounds, including opiates, are susceptible to oxidation by nitrite, PCC, hypochlorite, peroxide/peroxidase and gluteraldehyde. Due to the questionable relevance of peroxide/peroxidase and glutaraldehyde, they were chosen not to be further investigated in the project. The presence of peroxide/peroxidase has been reported to cause noticeable colour in urine, and the waning popularity of glutaraldehyde means that many laboratories have actually stopped testing for this adulterant [102]. One objective of the pilot study was to conduct a simple visual experiment to examine the preliminary potential for nitrite, PCC and hypochlorite to be used as a urine masking agent. It would be highly unlikely for individuals to use adulterants that would cause obvious change to the specimen, and accordingly, they have been removed from the list of adulterants that warrant further investigation.

An additional objective of the pilot study was to determine a suitable qualitative LC-MS method for the screening of opiate compounds in urine.

2.2 Selection of oxidants

2.2.1 Chemical reagents and materials

Potassium nitrite solid (KNO_2) was sourced from Sigma Aldrich (St. Louis, MO, USA). PCC solid, potassium permanganate solid and sodium hypochlorite solution (10-15% available chlorine) were also purchased from Sigma Aldrich (Castle Hill, NSW, Australia). Universal indicator strips (pH 0-14) were purchased from Merck (Darmstadt, Germany).

2.2.2 Donor urine

Human donor urine was obtained from healthy individuals (in yellow top polypropylene urine jars) and pooled (n=4) in a measuring cylinder. This allowed a representative blank urine matrix to be obtained. This collection process was carried out in all experiments requiring urine, unless otherwise specified.

2.2.3 Experimental method

Equal volumes (approximately 15 mL) of pooled urine were poured into five polypropylene jars. One third of a spatula of potassium nitrite was added to one jar of blank urine. This procedure was repeated for PCC; in the case of potassium permanganate, only a pinch (approximately several granules) was added. A 10 µL aliquot of hypochlorite solution was added to the fourth jar of blank urine. A fifth jar of blank urine was kept unmodified and used as a control specimen. All specimens were sealed and mixed using a vortex mixer. The pH, colour and smell of the specimens were recorded.

2.2.4 Results and discussion

This simple experiment was carried out to determine how inconspicuous each oxidant was. Potassium permanganate was also tested as it was available in the laboratory. Since it is easily accessible and can be readily purchased at pharmacies (used as a disinfectant and deodoriser), and is an oxidising chemical, it possesses potential to be used as a urine adulterant. Both subjective and objective parameters were measured and included olfactory detection, visual observation and pH level. Realistically, these are the tools available to personnel administering the urine drug tests; besides relying on their senses, a pH panel and temperature strip (to detect cases of substitution) are often integrated into the urine collection cup.

The quantity of oxidant solid added was representative of the amount of material available in commercial oxidising adulterant kits. In the case of

hypochlorite, a seemingly small volume was added. However, this was based on anecdotal evidence of how hypochlorite is used as a urine adulterant; a finger or drawstring is dipped into bleach and then swirled into the urine immediately after voiding. It is important to note that commercially available strong strength bleach such as 'White King' contains only 4% available chlorine (decreasing to 2% at the use-by date).

As Figure 2-1 reveals, nitrite, PCC and hypochlorite do not significantly alter the colour of the urine. Although the presence of PCC did appear to make the urine more 'orange', suspicion would not be raised due to the natural variation present in the population. In contrast, potassium permanganate drastically changed the colour of the urine to an unnatural dark brown. Based on this observation, potassium permanganate was discounted as a viable oxidising adulterant to be used in further studies. As Figure 2-1 further suggests, the presence of the oxidants in the urine specimens did not cause any major pH shifts. All specimens, including the control, are at pH 6 according to the universal indicator used. Moreover, under the experimental conditions employed, the presence of the oxidants could not be determined based on olfactory detection.

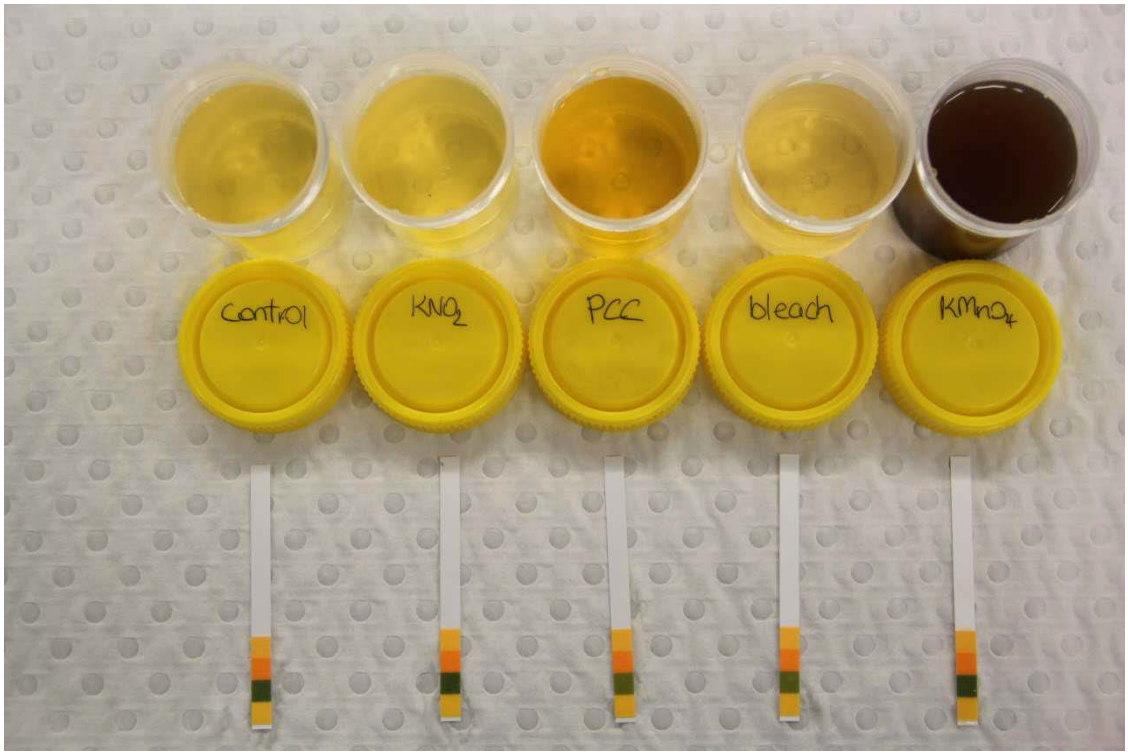


Figure 2-1: The effect of various oxidants on the appearance and pH of urine.

2.3 Qualitative LC-MS method for the screening of opiates in urine

The objective of this portion of the study was to develop a general full-scan, reversed phase LC-MS method for the detection of 6-MAM, morphine, codeine, M3G and M6G in aqueous matrices. A significant criterion of the method was to construct a broad gradient elution profile. This would ensure that any potential products forming as a result of adulteration in later studies can be detected with this method, and be adequately separated from their analogues. As the LC-MS method would be used for screening at the initial stages, a fully validated method was not required.

2.3.1 Chemical reagents and materials

Drug standards (1 mg/mL free base equivalent) of 6-monoacetylmorphine hydrochloride (in methanol), morphine monohydrate (in methanol), morphine-3-β-D-glucuronide (in methanol/water 1:1), morphine-6-β-D-glucuronide (in acetonitrile/water 1:1) and codeine (in methanol) were sourced from Lipomed (Arlesheim, Switzerland). These drug standards were purchased from Lipomed for all the studies, unless otherwise specified.

HPLC grade acetonitrile was sourced from Merck (Victoria, Melbourne, Australia). Ammonia formate solid and formic acid solution was obtained from Sigma-Aldrich (Castle Hill, NSW, Australia). Milli-q water was generated with a Sartorius Arium® 611 laboratory purification system equipped with a Sartopore 0.2 µm membrane filter (Göttingen, Germany). Hydrophilic 0.22 µm syringe filter units were purchased from MicroAnalytix Pty Ltd (Taren Point, NSW, Australia). Micro test tubes were sourced from Eppendorf (North Ryde, NSW, Australia). High recovery vials and septa lids were obtained from PM Separations (Capalaba, QLD, Australia).

2.3.2 Experimental method

2.3.2.1 Preparation of drug standards in water and urine

Individual 10 µg/mL (1 mL) opiate standards in addition to a mixed standard (containing all five opiates) were prepared in water, diluted from the 1 mg/mL stock standards. A parallel set of spiked urine standards were prepared and subjected to centrifugation (4500 g for 10 min) and filtration through syringe filter units prior to LC-MS analysis.

2.3.2.2 Preparation of mobile phases

The organic portion of the mobile phase consisted of 95% acetonitrile, and was prepared by mixing 475 mL acetonitrile with 25 mL Milli-q water. The solution was stored in a Schott bottle.

Two aqueous mobile phases were trialled; 0.1% formic acid and 5-20 mM ammonium formate. The 0.1% formic acid solution was prepared by adding 500 µL formic acid stock to 499.5 mL Milli-q water. The ammonium formate mobile phase solutions were diluted from a 2 M stock solution, prepared by dissolving 6.306 g of ammonium formate solid in Milli-q water in a 50 mL volumetric flask. Following filtration through a syringe filter unit, the 2 M stock was stored in a 50 mL falcon tube and refrigerated when not in use. Ammonium formate mobile phase solutions at 5 mM, 10 mM and 20 mM were prepared by spiking 1.25 mL, 2.5 mL, and 5 mL of 2 M stock, respectively, in water made up to 500 mL in a volumetric flask. An additional set of mobile phase solvents were also prepared and consisted of acetonitrile and 10 mM ammonium formate, each spiked with 0.05% formic acid. Ammonium formate mobile phase was prepared fresh on the day of LC-MS analysis.

2.3.2.3 LC separation

Chromatographic separation was achieved using an Agilent Technologies 1290 LC system. The opiate water standards were injected onto an Agilent Zorbax Eclipse Plus C18 Rapid Resolution HD column (2.1 mm $\times$ 50 mm $\times$ 1.8 µm) and the different mobile phases were trialled to determine the elution conditions required for adequate peak shape and peak separation. The effect of column temperature on analyte retention time was also briefly investigated. Once an adequate gradient elution profile was established, the opiate standards in urine were analysed to ensure that the retention times were reproducible.

As this portion of the study was predominately trial and error, more experimental detail is discussed in conjunction with the results (section 2.3.3.1).

2.3.2.4 MS detection

The opiate analytes were detected using both an Agilent Technologies 6460 Triple Quadrupole mass spectrometer (QQQ-MS) and a 6490 QQQ-MS,

connected to the LC system via an ESI interface utilised in positive ion mode. The optimal fragmentor voltages of the opiate analytes were determined in full scan acquisition mode (for the 6460 QQQ-MS), and the optimal collision energies for each of the analytes were determined in product ion scan mode (for both the 6460 and 6490 QQQ-MS). It is important to note that due to the iFunnel technology implemented in the 6490 QQQ-MS, the optimisation of fragmentor voltage parameters was not necessary. However, the optimal collision energy parameters were still required, and were found to be transferable from the 6460 QQQ-MS to the 6490 QQQ-MS. The methods used for the determination of optimal fragmentor and collision energy parameters are discussed detail in section 2.3.3.2. Default source parameters were utilised on both the 6460 and 6490 QQQ-MS, and is displayed in Table 2-1.

Table 2-1: Source parameters for the 6460 and 6490 QQQ-MS.

	6460 QQQ-MS	**6490 QQQ-MS**
gas temperature (°C)	300	200
nebuliser gas pressure (psi)	45	20
sheath gas temperature (°C)	250	250
sheath gas flow (L/min)	11	11
capillary voltage (V)	3500	3000
nozzle voltage (V)	500	1500

2.3.3 Results and discussion

2.3.3.1 Chromatographic separation of the opiate analytes

In reversed phase liquid chromatography, a typical gradient analysis commences with a mobile phase of low organic content, which is then

increased over time until all analytes of interest are eluted. As such, analyte retention is significantly dependent on the choice of mobile phase. Hydro-organic mixtures are typically used for reversed phase chromatography, where the composition, pH and concentration of the mobile phases all have contributing effects on the separation. Mobile phases are generally selected based on the eluent strength required, in addition to peak shape quality. Reproducible, sharp and symmetrical peaks are desirable. In order to achieve these results, the analytes of interest must be swept off the column at the same time, thus minimising the effect of band broadening. Having the analytes in one predominant ionisation state ensures that they will interact with the stationary phase in the same manner, and therefore will be displaced from the column at the same time by the mobile phase. This is achieved by selecting an aqueous mobile phase that is at least one unit away from the pK_a values possessed by the analytes [93].

For basic compounds such as opiates, the use of acetonitrile or methanol organic phase and a formic acid or ammonium formate aqueous phase are commonly adopted [93]. However, due to the small 1.8 µm particle size of the LC column chosen for this analysis, the risk of backpressure in the system is higher in comparison with LC systems where conventional 5 µm columns are used. Therefore, acetonitrile was selected as a more suitable option for the organic mobile phase rather than methanol, which is associated with high backpressure issues. Compared to methanol, acetonitrile also possessed a higher eluent strength [93].

Trials with 0.1% formic acid (pH = 3.25) and 5-20 mM ammonium formate (pH ≈ 6.2) as the aqueous component of the mobile phase showed that there were advantages and disadvantages with each of the solutions. It was expected that the use of both formic acid and ammonium formate would result in tall peak shapes and stable retention times. For both the 95% acetonitrile/0.1% formic acid solvent system and the acetonitrile (+0.05% formic acid)/ammonium formate (+0.05% formic acid) solvent system, sharp peaks were observed for the analysis of opiate analytes in water. However, disadvantages were noted, including slightly asymmetrical peak shapes and the elution of various analytes

at the beginning of the analysis. This was despite manipulation of the mobile phase gradient so that the organic composition was only 0-2%. This was particularly noted for morphine (Figure 2-2). This disadvantage would have a significant negative impact on the analysis of opiates in urine; since endogenous urinary compounds are highly water soluble, they tend to elute early in the chromatographic run as a result of the highly aqueous environment. As a result, early elution of the opiate compounds must be avoided to prevent co-elution with endogenous compounds. For this reason, the use of formic acid as the mobile phase additive was deemed unsuitable in this case.

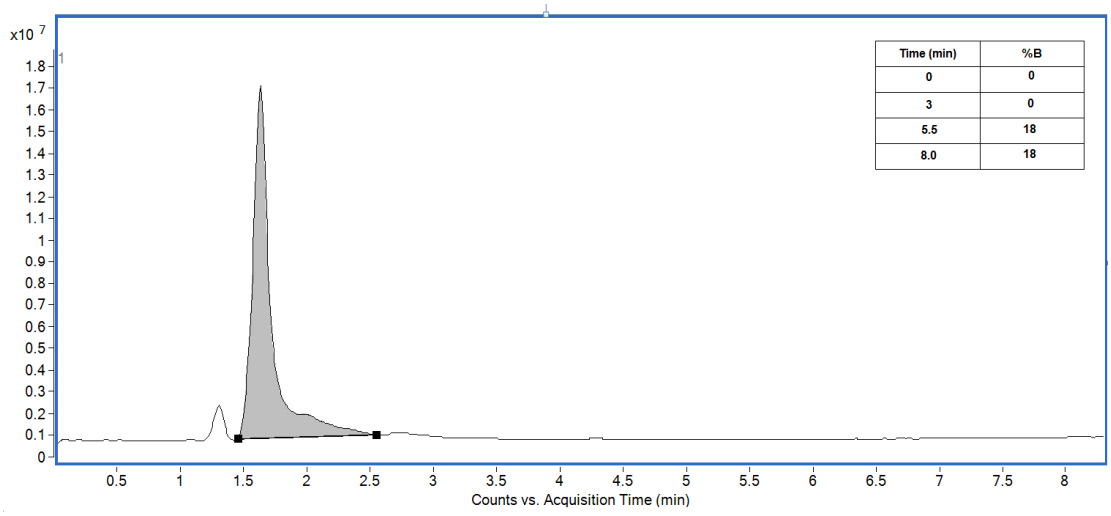


Figure 2-2: chromatogram of morphine (R_t = 1.6 min) in water obtained with the inset gradient, where solvent B = acetonitrile +0.05% formic acid, and solvent A = ammonium formate +0.05% formic acid.

The trials with 95% acetonitrile coupled with 2-20 mM ammonium formate yielded interesting results. The peak shapes of the opiates were sharp and were not noticeably different when comparing the chromatograms obtained with 5 mM, 10 mM and 20 mM formate solutions. However, it appeared that the 20 mM ammonium formate solution provided the most reproducible analyte retention times when comparing day-to-day runs. As such, the most suitable solvent system was chosen to be acetonitrile and 20 mM ammonium formate; the gradient elution profile is shown in Table 2-2. An analysis of a mixed opiate standard is displayed in Figure 2-3. Admittedly, M3G and M6G elute early in the run; they are known to be difficult to retain in reversed phase

chromatography, as they are very polar and are easily eluted off the column with high aqueous mobile phase composition. However, multiple injections indicated that the peak shapes and retention times of these glucuronide compounds remain relatively stable. It should be noted that as a comparison to gradient elution, isocratic conditions were trialled. However, a combination of long run times in addition to poor separation of the opiate analytes confirmed that gradient elution was necessary.

Table 2-2: gradient elution profile for the separation of opiates in aqueous matrices.

Time (min)	**% organic (95% acetonitrile)**
0	2
6	5
12	30
17	70
19	95
21	95
21.1	2
post-run column equilibration	4
total run time	**25.1**

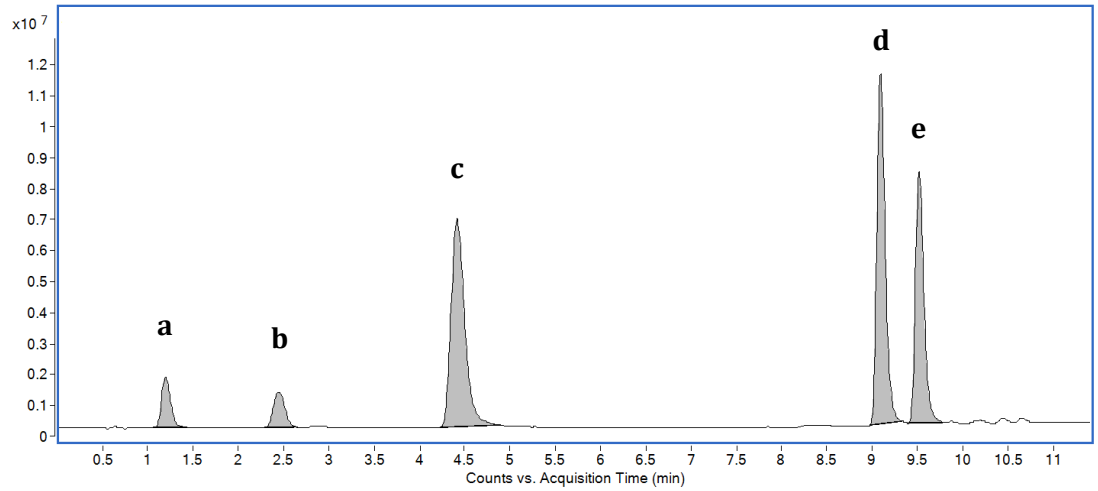


Figure 2-3: Total ion current (TIC) chromatogram of a mixed opiate standard in water obtained using the gradient outlined in Table 2-2, where the order of elution is: (a) M3G (R_t = 1.1 min), (b) M6G (R_t = 2.4 min), (c) morphine (R_t = 4.4 min), (d) codeine (R_t = 9.1 min) and (e) 6-MAM (R_t = 9.5 min).

The effect of column temperature was also briefly investigated. During analyses, the column was left at room temperature (thermostat not controlled), or adjusted to 30°C, 40°C or 50°C. No significant differences were observed in terms of peak shapes at the various temperatures, however a noticeable difference in backpressure was observed. It appeared that a higher column temperature resulted in a decrease in backpressure of the system. Taking into consideration that the maximum temperature recommended for the column is 60°C, a column temperature of 40°C was chosen and was implemented as part of the LC method. Finally, the important but often overlooked injection volume and auxiliary parameters were established in the LC method and are shown in Table 2-3.

Table 2-3: Injection volume and auxiliary parameters implemented in the LC method.

injection volume (µL)	1.0
draw speed (µL/min)	200
eject speed (µL/min)	200
equilibration time (sec)	5.0

2.3.3.2 Fragmentor voltage and collision energy optimisation

A fragmentor voltage range of 120-230 V was trialled for each of the opiates. The purpose for fragmentor voltage optimisation was to generate the greatest abundance of parent ions to aid its detection. Once trialled, the resulting TIC chromatograms were extracted to obtain extracted ion chromatograms (EIC's). For each opiate, mass spectral data were obtained from the EIC's and the mass-to-charge *(m/z)* peak belonging to the parent ion ($[M+H]^+$) was overlayed. In this case, the optimal fragmentor voltage provided the highest *m/z* peak. Alternatively, the peak areas of the EIC's were determined, with the greatest peak area given by the optimal fragmentor voltage. Both methods were used to determine the optimal fragmentor voltage for each of the opiate compounds, and were in agreement with each other. Figure 2-4 to Figure 2-8 shows the effect of fragmentor energy variation on the peak areas of the respective opiate analytes. Under the experimental conditions employed, slight deviation from the 'optimal' fragmentor voltage (± 20 V) does not appear to significantly affect the peak area obtained for each opiate analyte, and thus its detection. The protonated parent ions and optimal fragmentor voltages for each opiate analyte are tabulated in Table 2-4. Finally, a scanning mass range of 100-1000 Da and a scan time of 500 ms was integrated into the full scan method.

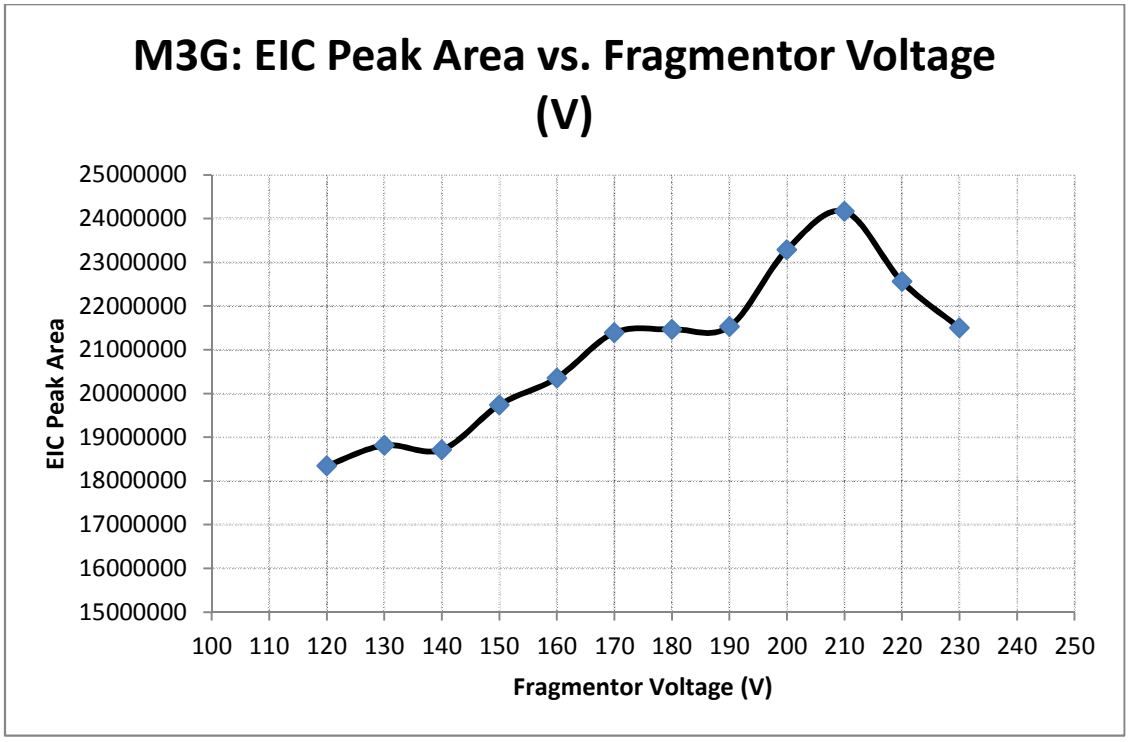


Figure 2-4: Plot of extracted M3G (m/z 462) peak area vs. fragmentor voltage.

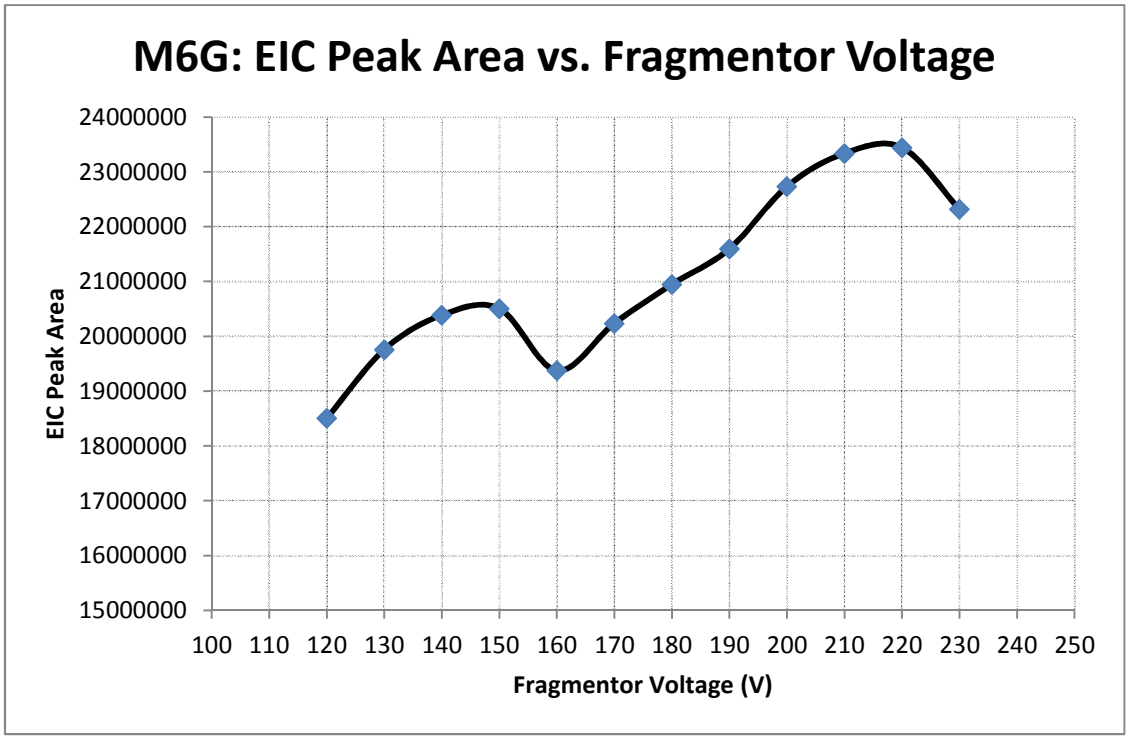


Figure 2-5: Plot of extracted M6G (m/z 462) peak area vs. fragmentor voltage.

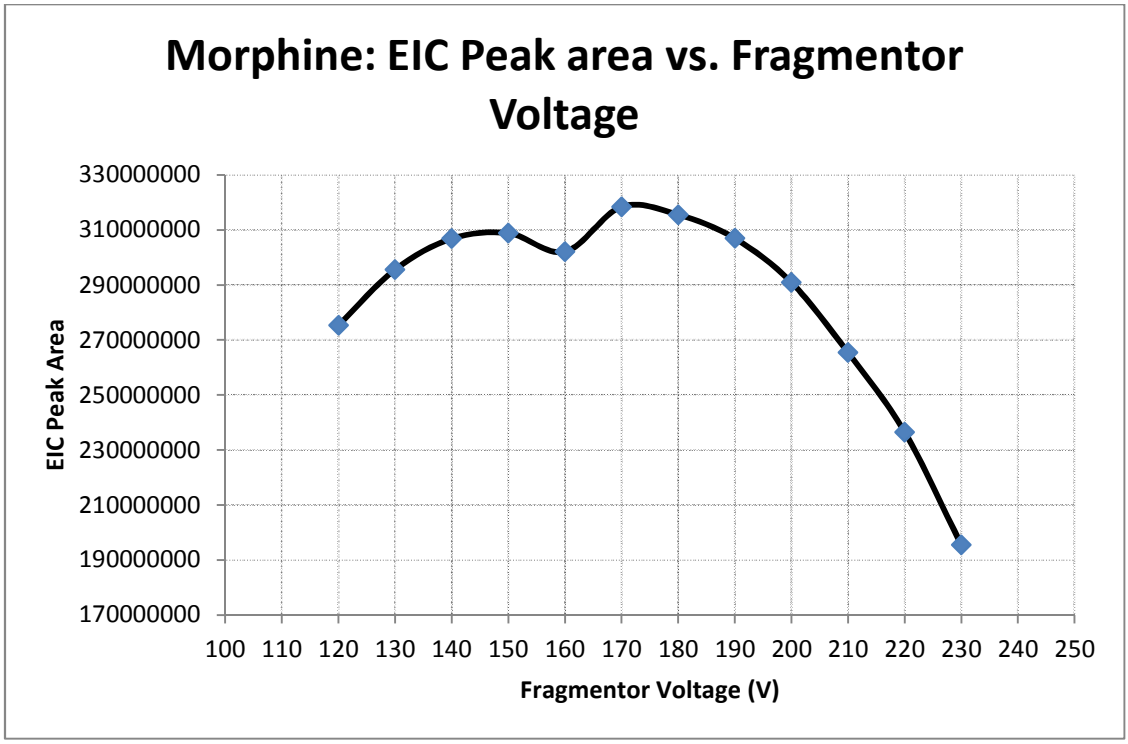


Figure 2-6: Plot of extracted morphine (m/z 286) peak area vs. fragmentor voltage.

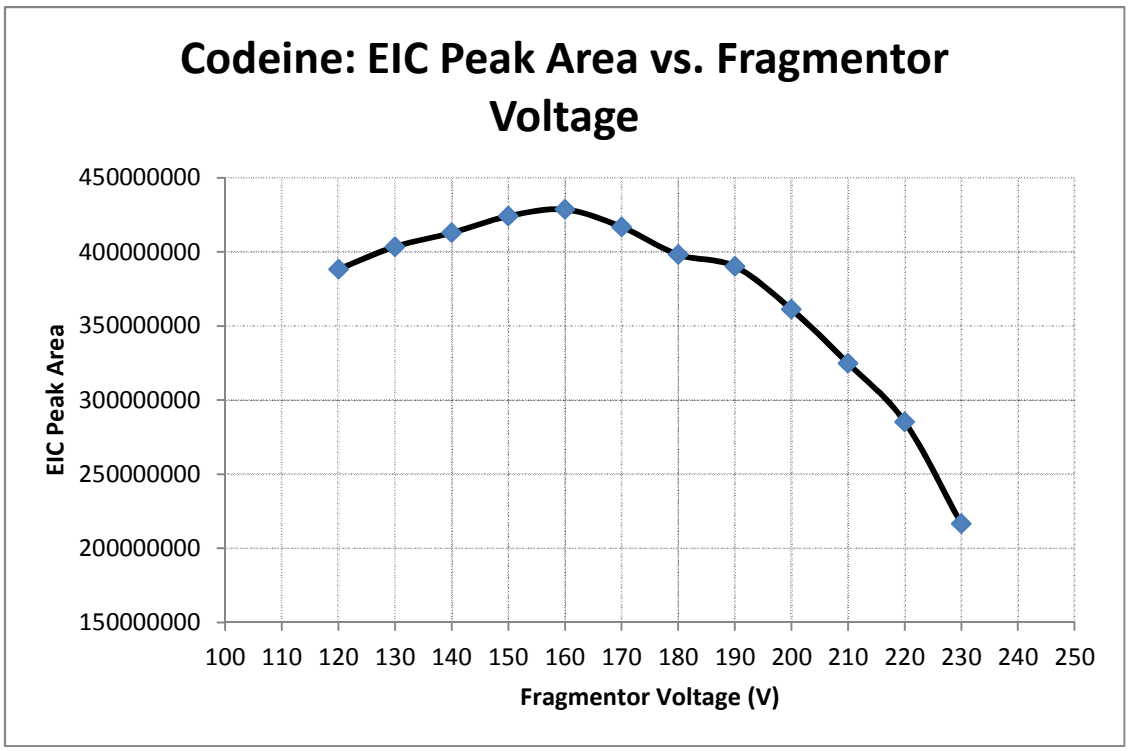


Figure 2-7: Plot of extracted codeine (m/z 300) peak area vs. fragmentor voltage.

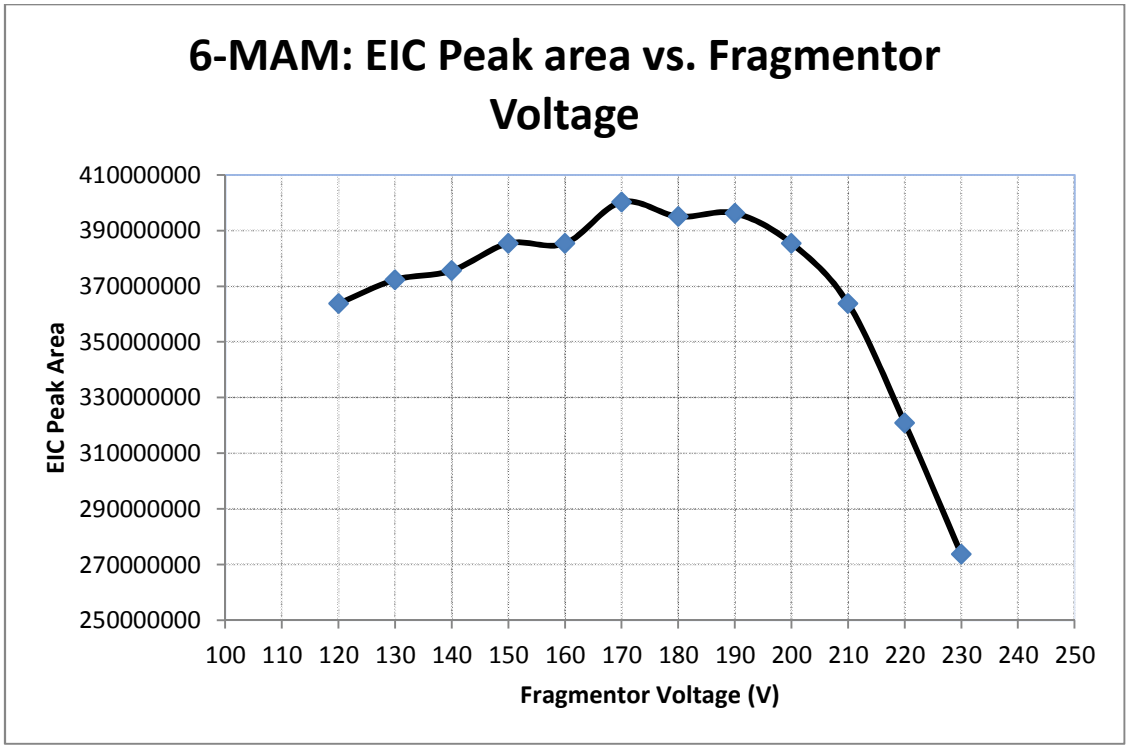


Figure 2-8: Plot of extracted 6-MAM (m/z 328) peak area vs. fragmentor voltage.

Table 2-4: Protonated parent ions and the optimal fragmentor voltage determined for each opiate analyte.

opiate analyte	$[M+H]^+$	optimal fragmentor voltage (V)
M3G	462	210
M6G	462	220
morphine	286	170
codeine	300	160
6-MAM	328	170

2.4 Conclusions for the pilot study

Based on the findings presented in section 2.2.4, nitrite, PCC and hypochlorite are relatively easy to conceal in a urine specimen, thus warranting further investigation into their effects as an oxidising adulterants in opiate-positive specimens. With the full scan LC-MS method developed in section 2.3, it is possible to monitor the presence of 6-MAM, morphine, M3G, M6G and codeine in urine specimens adulterated with these three chemicals. Any stable reaction products that form as a result should also be detected with this broad screening method. This LC-MS method is used for monitoring all reaction mixtures unless otherwise indicated.

Chapter 3:

Search for potential markers for monitoring the presence of 6-monoacetylmorphine in urine adulterated with nitrite

Chapter 3: Search for potential markers for monitoring the presence of 6-monoacetylmorphine in urine adulterated with nitrite

3.1 Introduction

As discussed in the introductory chapter, 6-MAM is monitored in urine drug testing programs as its presence is indicative of heroin use. It is included in the panel of drugs outlined in Australian/NZ Standard AS/NZS 4308. Most laboratories initially screen urine specimens using a CEDIA based assay, and if the result is positive for 6-MAM, the sample is further processed using GC-MS or LC-MS analysis. Currently, there is limited information on the specific effects of nitrite oxidant on the detection of 6-MAM using these preliminary and confirmatory techniques.

Therefore, the aims of this study were to observe the outcome of 6-MAM positive urine specimens following nitrite adulteration, and were two-fold: Firstly, to determine the effect of nitrite on the CEDIA® Heroin Metabolite (6-AM) assay (Microgenics Corporation), and secondly, to determine if nitrite is capable of oxidising 6-MAM. If the latter case is proven to be true, then the formation of stable oxidation product(s) are possible; these products may be useful for indirectly monitoring 6-MAM in nitrite adulterated urine.

3.2 Materials

3.2.1 Drug standards and reagents

6-MAM hydrochloride hemihydrate (1 mg/mL free base equivalent in methanol (MeOH)) and 6-MAM hydrochloride solid were sourced from Alltech-Applied

Science Labs (State College, PA, USA). *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) derivatising agent was sourced from United Chemical Technologies (Bristol, PA, USA). Two working solutions of KNO_2 were freshly prepared at 0.5 M and 6 M in water daily.

3.2.2 Urine specimens

Blank urine was obtained from healthy donors and pooled (n=4 per batch) to give a representative biological urine matrix. Urine was stored in polypropylene urine specimen collection containers and refrigerated at 4℃ for no more than three days prior to use. These storage conditions are in line with section 3.8 of AS/NZS 4308.

A urine sample positive for 6-MAM ('Authentic Urine 1') was obtained from the Drug Toxicology Unit, NSW Forensic and Analytical Scientific Service (Macquarie Hospital, Ryde). The sample belonged to an active heroin user and was supplied after removal of specimen identification. The sample was kept refrigerated by the testing laboratory for one week before it was used in this study.

3.3 Instrumentation

3.3.1 CEDIA® 6-AM assay

The CEDIA® Heroin Metabolite (6-AM) Semi Quantitative Assay (Microgenics Corporation, Fremont, CA, USA) was performed on an Olympus AU 2700 analyser (Olympus America Inc., Melville, NY, USA) located at the Drug Toxicology Unit. The assay utilised a cut-off calibrator (10 ng/mL), a high calibrator (20 ng/mL), a negative control (7.5 ng/mL) and a positive control (12.5 ng/mL).

3.3.2 LC-MS

LC-MS analysis was carried out on an Agilent 1290 LC system (see Appendix, Table A1 for sampler and auxiliary parameters) coupled with an Agilent 6460 Triple Quadrupole (QQQ-MS) detector or an Agilent 6510 Accurate Mass Quadrupole Time-of-Flight Mass Spectrometer (QTOF-MS) (Agilent Technologies, Forest Hill, VIC, Australia). An ESI interface operated in positive ion mode was used for both instrument configurations. For the QQQ-MS, data was collected in full scan (as described in section 2.3), product ion scan and multiple reaction monitoring (MRM) modes. On the other hand, high resolution QTOF-MS data was collected in MS and targeted MS/MS modes. The fragmentor voltages and collision energies used for the analytes were the same for both the QQQ-MS and QTOF-MS analyses. In addition, an Agilent reference mix (calibrant A) containing 10 µM purine and 2 µM HP-0921 in acetonitrile and water (AcN:H_2O = 95:5) was prepared and enabled during all QTOF-MS analyses.

3.3.3 GC-MS

GC-MS analysis was conducted on an Agilent 7890 AGC System coupled to an Agilent 5975C Inert XL electron impact-mass spectrometer (EI-MS). Samples were injected in splitless mode (1 µL) onto an Agilent HP-5MS capillary column (30 m × 250 µm × 0.25 µm). Helium carrier gas with a constant flow rate of 1.65 mL/min was used. The oven was initially set at 140℃ and held for 1.00 min; the temperature was then ramped up by 50℃/min until 300℃ was reached; finally it was held at 300℃ for 6.00 min for a total run time of 10.20 min. The inlet and auxiliary heaters were both set at 250℃. Both scan and selected ion monitoring (SIM) analyses were carried out. In regards to sample preparation, TMS derivatives were prepared by adding 50 µL BSTFA (with 1% TMCS) and 150 µL acetonitrile to analyte residues and heated at 75ºC for 30 min on a heating block.

3.3.4 Nuclear Magnetic Resonance (NMR) spectroscopy

NMR analysis was conducted on a Bruker Avance III 600 MHz NMR spectrometer (Nuclear Magnetic Resonance Facility, UNSW). The samples were dissolved in deuterated chloroform ($CDCl_3$). One-dimensional proton NMR (^{1}H-NMR) and two-dimensional correlation spectroscopy NMR (^{1}H-^{1}H COSY), heteronuclear single quantum coherence spectroscopy (^{1}H-^{13}C HSQC) and heteronuclear multiple bond correlation spectroscopy (^{1}H-^{13}C HMBC) experiments were performed using standard acquisition parameters for each type of experiment [125].

3.4 Experimental method

3.4.1 Exposure of 6-MAM to potassium nitrite

Exposure of 6-MAM to KNO_2 was initially conducted in water, followed by urine, in a similar manner. Blank urine samples were adjusted to pH 3, 4, 5, 6, 7 or 8 with either 2 M HCl or 2 M sodium hydroxide (NaOH). The urine samples were then spiked with the 6-MAM stock solution (1mg/mL in methanol) or its further diluted working solutions (also prepared in methanol) to yield specimens with a final 6-MAM concentration ranging from 5 to 10,000 ng/mL. The methanol concentrations in these fortified urine samples were less than 2.5% in any case. The urine samples were mixed with KNO_2 at a final concentration of either 0.05 M or 0.6 M by using the 0.5 M or 6 M KNO_2 solution, respectively. Unless otherwise specified, the samples were left to react at room temperature for 10 min.

For 'Authentic Urine 1', only 0.6 M KNO_2 was used. Two pH conditions were investigated: at pH 3, following adjustment with 2 M HCl and at the original pH (pH 6) without acidification or further manual adjustment.

These samples together with various control specimens were subject to both CEDIA® 6-AM assay and LC-MS analysis. For LC-MS analysis, urine samples

were centrifuged at 4500 *g* for 10 min, followed by filtration through 0.22 µm hydrophilic syringe filter units (MicroAnalytix Pty Ltd, Taren Point, NSW, Australia).

3.4.2 MRM method validation

An initial experiment was performed where a set of 6-MAM calibration standards in urine (1-10,000 ng/mL) was prepared and analysed to check the approximate linear range of the method. Following on from this, a set of 6-MAM calibration standards in urine (1-1000 ng/mL) were prepared on five separate days from working solutions in methanol (0.0001-0.1 mg/mL) (Table 3-1). The working solutions were prepared by serial dilution (Table 3-2). In addition, two quality control (QC) urine samples (5 ng/mL and 250 ng/mL, n= 5) were prepared from 6-MAM working solutions made up independently from the calibration standards (0.0005 and 0.025 mg/mL) (Table 3-3 and Table 3-4). The samples were analysed in duplicate.

Table 3-1: Preparation of the 6-MAM calibration standards in urine.

[6-MAM calibration standard] (ng/mL)	[6-MAM working solution] added (mg/mL)	Volume of working solution added (µL)	Volume of urine added (µL)	Total sample volume (mL)
1000	0.1	10	990	1
750	0.075	10	990	1
500	0.05	10	990	1
100	0.01	10	990	1
50	0.005	10	990	1
10	0.001	10	990	1
1	0.0001	10	990	1

Table 3-2: Preparation of the 6-MAM working solutions in methanol.

[6-MAM working solution] (mg/mL)	**[6-MAM working solution] added (mg/mL)**	**volume of 6-MAM working solution added (µL)**	**volume of MeOH added (µL)**	**total sample volume (µL)**
0.1	1[a]	25	225	250
0.075	0.1	150	50	200
0.05	0.075	120	60	180
0.01	0.05	40	160	200
0.005	0.01	100	100	200
0.001	0.005	40	160	200
0.0001	0.001	20	180	200

[a]stock 1 mg/mL 6-MAM standard in MeOH.

Table 3-3: Preparation of the 6-MAM QC samples in urine.

[6-MAM calibration standard] (ng/mL)	**[6-MAM working solution] added (mg/mL)**	**volume of working solution added (µL)**	**volume of urine added (µL)**	**total sample volume (mL)**
5	0.0005	10	990	1
250	0.025	10	990	1

Table 3-4: Preparation of the 6-MAM working solutions in methanol for the QC samples.

[6-MAM working solution] (mg/mL)	[6-MAM working solution] added (mg/mL)	volume of 6-MAM working solution added (µL)	volume of MeOH added (µL)	total sample volume (µL)
0.025	1[a]	25	975	1000
0.0005	0.025	10	490	500

[s]stock 1 mg/mL 6-MAM standard in MeOH.

3.4.3 Isolation of the 6-MAM/nitrite reaction product

For structural elucidation purposes, the 6-MAM/nitrite reaction was carried out as for the LC-MS analyses, with the reaction adjusted to accommodate 10 mg 6-MAM hydrochloride starting material. The reaction mixture was left at room temperature for one hour. A clean-up liquid-liquid extraction was subsequently performed with dichloromethane:isopropanol (9:1). The aqueous layer was basified with 1.5 M carbonate/bicarbonate buffer to pH 9.5, and then further extracted with dichloromethane in triplicate. The organic layers were recovered after each extraction, combined, and dried down using a gentle stream of nitrogen gas at 30℃. The remaining residue (approximately 6 mg) was dried overnight in a desiccator under vacuum and was subjected to LC-MS, GC-MS and NMR analyses.

3.5 Results and discussion

3.5.1 Effect of nitrite on CEDIA®

The CEDIA® Heroin Metabolite (6-AM) assay is used by the majority of urinalysis laboratories in Australia for the qualitative and semi-quantitative screening of 6-MAM in human urine. In line with AS/NZS 4308 [67], the cut-off level for this assay is 10 ng/mL. An objective of the study was to determine the effect of nitrite on the CEDIA® 6-AM assay.

At the beginning of the study, both solid and working solutions of KNO_2 were trialled. Solid KNO_2 was considered as the preferred form due to its easy application during the adulteration process. The KNO_2 concentrations of 0.05 M and 0.6 M were selected based on the concentrations detected in nitrite adulterated urine specimens (0.04-0.3 M or 1910-12200 µg/mL [123]). However, it was discovered that the same results were produced regardless of whether the KNO_2 was in solid or solution form. Further, it was interesting to note that the addition of excess nitrite resulted in a decreased abundance of both the starting materials and the oxidation products, however no additional degradation products were detected. Therefore, to investigate the effect of a broad concentration range of nitrite, solutions were prepared and used for adulteration for the remainder of the study. This allowed the concentrations of nitrite investigated to be more consistently delivered into the specimens.

The initial experiment focused on exposure of 6-MAM to nitrite in blank urine at pH 3, as nitrite is known to exert its optimal oxidising capability under acidic conditions [126]. Four concentrations of 6-MAM in urine were tested at 5, 10, 100 and 1,000 ng/mL. When nitrite was not involved, a good correlation was observed between the spiked 6-MAM concentration and the concentration determined by the immunoassay at 5 and 10 ng/mL. At 100 and 1,000 ng/mL spiked concentrations, the CEDIA® assay returned a reading at approximately 25 ng/mL for both specimens. These results were in agreement with the manufacturer's specification for the assay. The assay only ensures that a semi-quantitative relationship is followed if 6-MAM concentration in urine does not exceed 20 ng/mL [127]. When KNO_2 was added to these samples at 0.05 M and 0.6 M, all specimens yielded a 6-MAM result close to 0 ng/mL. The effect of pH on test results was investigated by adjusting the blank urine to pH 4, 5 and 6 before mixing with 6-MAM (100 ng/mL) and nitrite (0.6 M). The lower nitrite concentration (0.05 M) would also be suitable for this experiment, as both nitrite concentrations appeared to exhibit the same effects on the CEDIA assay. However, 0.6 M nitrite was chosen after considering the fact that the oxidant would likely be present in excess amounts in tampered specimens following authentic adulteration processes.

The pH 4 and pH 5 reactions showed a significant decrease in response when compared to the corresponding controls where no nitrite was present. A close to zero response was observed for the pH 4 condition. Similarly, when nitrite (0.6 M) was mixed with 'Authentic Urine 1', the masking effect was clearly observable when the urine pH was adjusted to pH 3. The results summarised in Table 3-5 clearly indicate that nitrite is an effective adulterant in masking the presence of 6-MAM in urine under acidic conditions when tested by the CEDIA® 6-AM assay.

Table 3-5: CEDIA® Heroin Metabolite (6-AM) assay responses for spiked and authentic urine samples following nitrite adulteration.

exp.	sample[a]	6-MAM conc. in urine (ng/mL)	0.5 M KNO_2 volume (µL)	6 M KNO_2 volume (µL)	pH of reaction mixture	6-AM assay response (ng/mL)
1	urine blank	-	-	-	6.5[c]	0.0
	KNO_2 control	-	100	-	3	-0.1
		-	-	100	3	0.1
	MAM control standards	5	-	-	6.5-7[c]	4.2
		10				8.0
		100				24.1
		1000				25.5
	MAM + KNO_2 reaction mixtures	5	100	-	3	-1.3
		10				-0.7
		100				-0.5
		1000				1.9
		5	-	100		-0.9
		10				-1.7
		100				0.0
		1000				0.6
2	MAM control standards	100	-	-	4	23.6
					5	24.1
					6	23.9
	MAM + KNO_2 reaction mixtures	100	-	100	4	-0.4
					5	13.4
					6	23.1
3	authentic urine 1	408[b]	-	-	7[c]	22.5
			-	100	6	17.2
					3	-5.8

[a]the total volume for each sample was 1 mL after addition of nitrite reagent solution.

[b]the testing laboratory (Drug Toxicology Unit) reported 'Authentic Urine 1' to contain 408, 9639 and 2720 ng/mL of 6-MAM, free morphine and codeine, respectively. The quantification method used was an in-house developed GC-MS method which involved an initial solid-phase extraction of opiates including 6-MAM from the urine matrix and a subsequent GC-MS analysis on the TMS derivatives of the analytes.

[c]pH of the urine specimen without manual adjustment.

3.5.2 LC-MS studies

LC-MS analysis was used to monitor the reaction mixtures to investigate whether exposure of 6-MAM to nitrite resulted in product formation. Water was used as the reaction medium instead of the blank urine during the method development phase to avoid any potential urine matrix effect. The full scan method was only used to do a general scan analysis of analytes present at 10 µg/mL concentrations of 6-MAM to develop the product ion scan method. The product ion scan method was used to determine fragmentation patterns for 6-MAM and the oxidation product for structural elucidation purposes. With the LC-MS conditions utilised, 6-MAM had a retention time of 9.5 min and yielded an expected protonated molecule $[M+H]^+$ at *m/z* 328. When the reaction mixture containing 6-MAM (10 µg/mL) and nitrite (0.05 M or 0.6 M) in the acidified water was analysed under the same conditions, 6-MAM was no longer observable in the TIC chromatogram. Instead, a major peak with a retention time of 12.0 min was noted, with a protonated molecule $[M+H]^+$ at *m/z* 373. This peak did not appear in any TIC of the various reagent and blank control specimens, suggesting that it was a reaction product of 6-MAM and nitrite. The reaction product produced a distinctive pattern of fragment ions when monitored by LC-MS operated in product ion scan mode. The same reaction product was observed in repeated experiments in which urine was used as the reaction medium. Representative TIC and product ion scan spectra of 6-MAM and the reaction product in urine are shown in Figure 3-1.

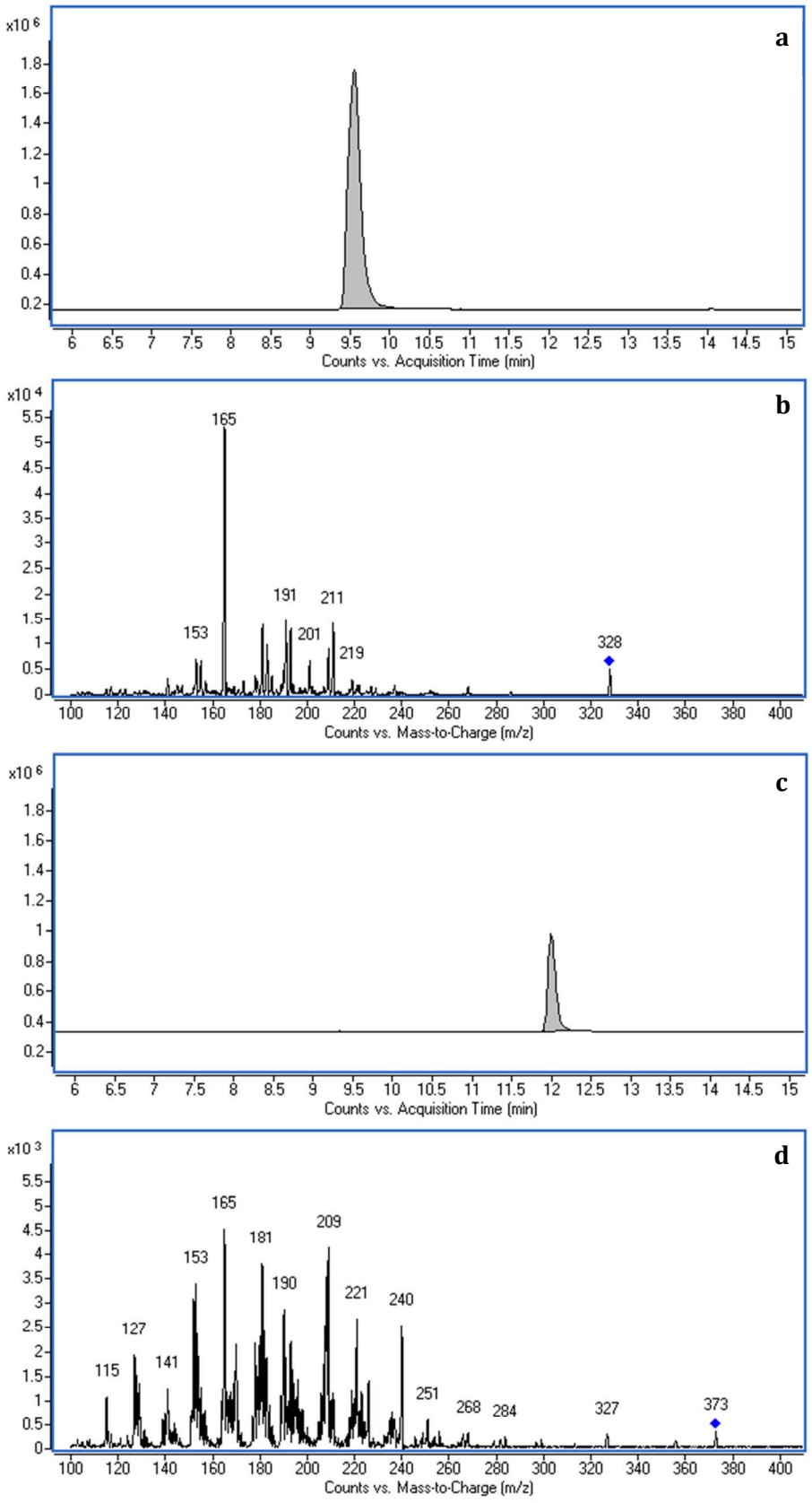


Figure 3-1: TICs and product ion scan spectra of 6-MAM and the reaction product in urine: (a) TIC of 6-MAM (R_t = 9.5 min), (b) MS of 6-MAM (fragmentor voltage 170 V, collision energy 35 eV), (c) TIC of the reaction product (R_t = 12.0 min) and (d) MS of the reaction product (fragmentor voltage 200 V, collision energy 45 eV).

In order to qualitatively assess the effect of pH on product formation and the stability of the formed product under various reaction conditions, an LC-MS method in MRM mode was developed. Two transitions were selected for each analyte, i.e. *m/z* 328→211 and *m/z* 328→165 for 6-MAM; and *m/z* 373→327 and *m/z* 373→209 for the reaction product. 6-MAM concentrations of 1-10,000 ng/mL in urine were analysed using this MRM method, and it was shown that the method was sensitive enough to detect low levels of 6-MAM in urine. The limit of detection was determined to be < 1 ng/mL. However, linearity was achieved over the range of 1-1000 ng/mL, with a correlation coefficient of 0.9999 (Figure 3-2; refer to Figure A4 in the appendix for an overlay of five calibration curves). For accuracy and precision determination, quality control samples spiked at 5 and 250 ng/mL (n=5) were analysed, along with calibration standards (1-1000 ng/mL). The intra-day and inter-day precision and accuracy data is displayed in the appendix (Table A2-Table A5), with results summarised in Table 3-6. Accuracy and precision are expressed as percentages of mean relative error (MRE) and relative standard deviation (RSD), respectively.

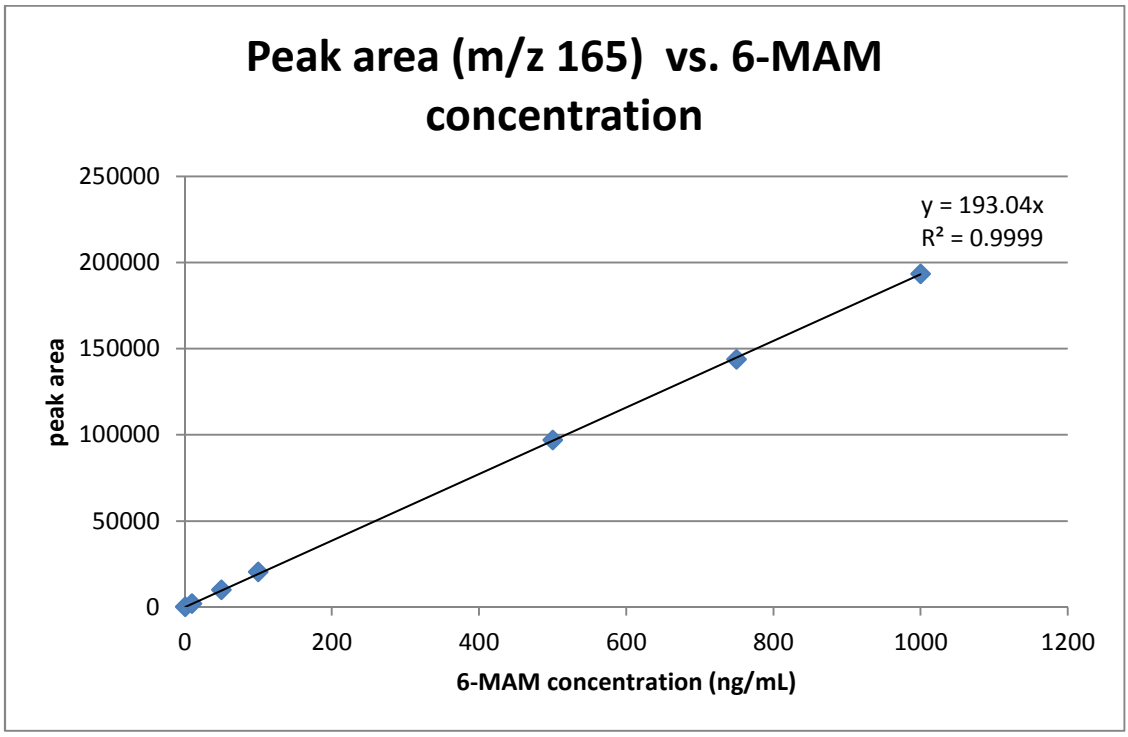


Figure 3-2: 6-MAM calibration curve displaying the linear response function between 1-1000 ng/mL for day four of the method validation.

Table 3-6: Accuracy and precision of the developed MRM method for monitoring 6-MAM in urine.

concentration (ng/mL)	intra-day accuracy (MRE)	intra-day precision (RSD)	inter-day accuracy (MRE)	inter-day precision (RSD)
5	2.3%	1.4%	4.9%	6.0%
250	0.3%	1.3%	1.8%	5.6%

At urine pH 7 and 8, no product formation was observed up until the end of the monitoring period (3.5 days). Most of the starting material (>95%) had disappeared and the reaction product detected within the first 15 min of the reaction at pH 3. Initial detection of the reaction product occurred within 15 min at pH 4, and within 2.4 hours (h) for pH 5 and pH 6. Disappearance of 6-MAM was found at 14 h, 16 h and 37 h at pH 4, 5 and 6, respectively. It was apparent that the more acidic the urine, the more rapid the product formation. These results are summarized in Table 3-7.

Table 3-7: Summary of the effect of urine pH on the formation of the reaction product.

urine pH	reaction observed	time taken for reaction product to be detected	time taken for 6-MAM to be undetectable
3	✓	within 15 min	5 h
4	✓	within 15 min	14 h
5	✓	within 2.4 h	16 h
6	✓	within 2.4 h	37 h
7	×	n/a	n/a
8	×	n/a	n/a

The reaction mixture at pH 3 was further monitored for up to 11 days and the product, although significantly lower in concentration compared to the first 15 min of the monitoring period, remained detectable. Given the relatively long

window of detection observed, it was speculated that the product had potential to serve as a marker for monitoring the presence of 6-MAM in nitrite-adulterated urine specimens.

To provide proof of concept, the 'Authentic Urine 1' sample prepared for the CEDIA® 6-AM assay was also analysed. The initial analysis was conducted one day after specimen exposure to nitrite. The product, but not 6-MAM, was detected in the non-acidified sample (pH 6) and the acidified sample (pH 3). In the corresponding control specimen where no nitrite was added, only 6-MAM was detectable under the same analysis conditions (Figure 3-3).

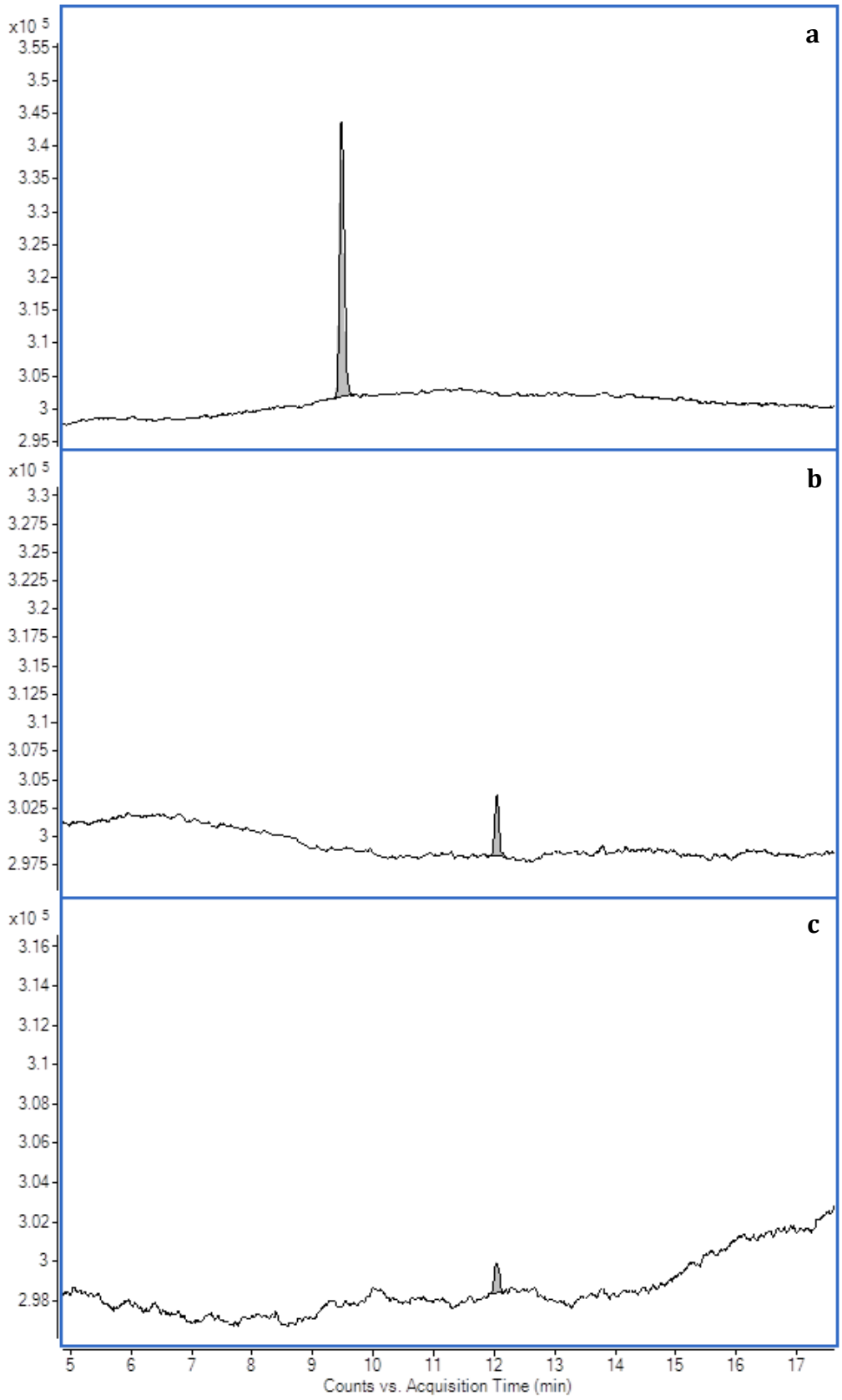


Figure 3-3: TICs of 'authentic urine 1' (a) prior to nitrite fortification, (b) subsequent to nitrite fortification (no acidification, pH 6) and (c) following nitrite fortification (pH 3). Retention time of 6-MAM is 9.5 min and the reaction product is 12.0 min.

3.5.3 Structural elucidation of the 6-MAM/nitrite oxidation product

Results from the LC-MS studies suggest that the oxidation product may be a nitrated species of 6-MAM. This hypothesis was based on firstly the observed protonated molecule $[M+H]^+$ at *m/z* 373 (45 Da greater than 6-MAM), which was consistent with a nitro ($–NO_2$) group substitution on the 6-MAM structure, and secondly the similarity of its product ions in the collision induced dissociation (CID) spectrum to those for 6-MAM (see Figure 3-1b and Figure 3-1d). High resolution QTOF-MS analysis of the oxidation product supported this hypothesis. It was found that the oxidation product had a $[M+H]^+$ at *m/z* 373.1393, corresponding well with the proposed molecular formula $C_{19}H_{20}N_2O_6$ ($[M+H]^+$ at *m/z* 373.1457). The difference between the actual mass and the calculated mass was only 0.27 ppm. Additionally, the loss of a mass unit of 45.9972 Da from the parent ion *m/z* 373.1393 to yield a product ion of *m/z* 327.1421 during the CID process was consistent with the loss of a $-NO_2$ group (calculated mass of 45.9929 Da).

There are two possible substitution sites: at the C-1 or C-2 positions of the 6-MAM structure. The C-2 position was considered more nucleophilic than the C-1 position due to the electron donating effect of the ortho phenolic group (C-3 position) and thus the preferred site of electrophilic substitution. It was therefore proposed that the nitration occurred at the C-2 position via an electrophilic substitution reaction to yield 2-nitro-6-monoacetylmorphine **(**or 2-nitro-MAM). The proposed reaction mechanism involving the generation of the NO_2^+ electrophile and the subsequent nitration of 6-MAM is provided in Figure 3-4.

(1) Formation of NO_2^+ from KNO_2

Solid potassium nitrite readily dissolves in aqueous solutions to form potassium and nitrite ions:

$$KNO_{2(s)} \longrightarrow K^+_{(aq)} + NO_2^-{}_{(aq)}$$

Under acidic conditions, the nitrite ion is protonated to form nitrous acid:

$$NO_2^- + H^+ \longrightarrow HNO_2$$

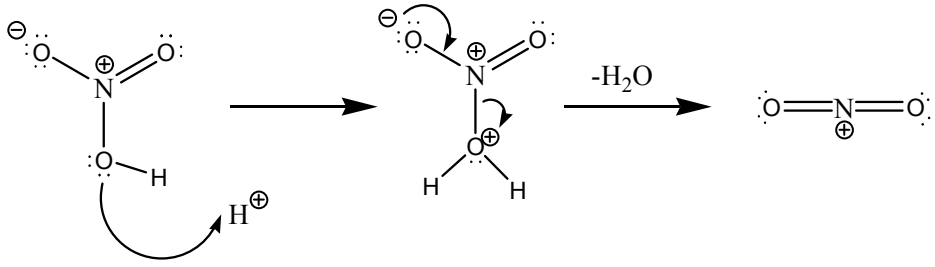
Due to instability, HNO_2 decomposes (via redox reaction) to nitrogen dioxide, nitric oxide and water:

$$2HNO_2 \longrightarrow NO_2 + NO + H_2O$$

Exposure of NO_2 to water results in the production of nitric acid, and the reformation of nitrous acid:

$$2NO_2 + H_2O \longrightarrow HNO_3 + HNO_2$$

Finally, protonation of nitric acid yields the nitronium ion:



(2) Reaction of NO_2^+ and 6-MAM to form 2-nitro-MAM

The nitration then proceeds, where 6-MAM reacts with the NO_2^+ electrophile. The OH group at the C-3 position activates the aromatic ring, causing the ortho (C-2) and para (C-11) positions to be the sites of electrophilic substitution. Since the para- position is blocked, the $-NO_2$ substitution occurs at the ortho position, resulting in the formation of 2-nitro-MAM.

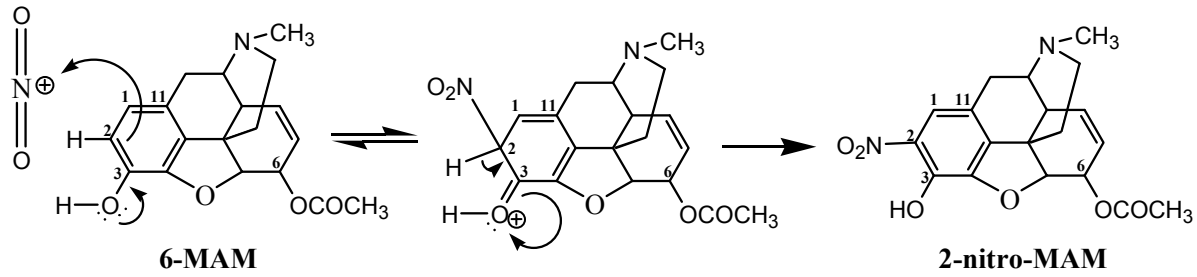


Figure 3-4: Proposed reaction mechanism for the formation of 2-nitro-MAM from reaction of 6-MAM with potassium nitrite under acidic conditions.

Unambiguous structural elucidation of the oxidation product as 2-nitro-MAM was obtained through high resolution 1D and 2D NMR studies [125, 128]. It was noted that the ^{1}H-NMR spectra of 6-MAM (Figure 3-5) and 2-nitro-MAM (Figure 3-6) showed great similarity in the 0-6 ppm region.

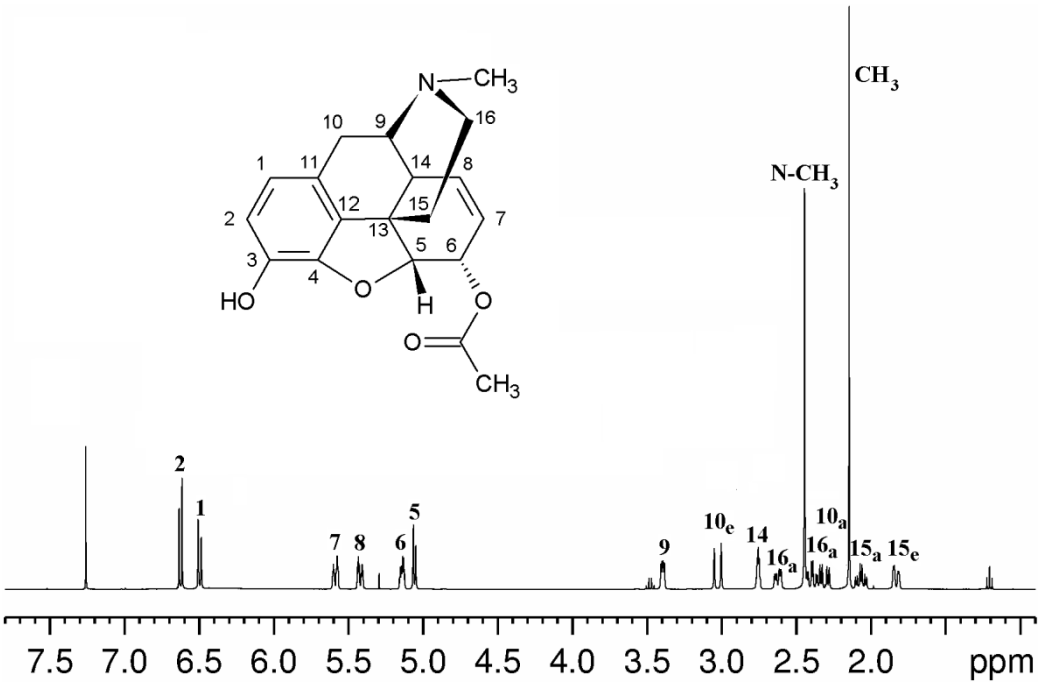


Figure 3-5: ^{1}H-NMR spectrum of 6-MAM in $CDCl_3$.

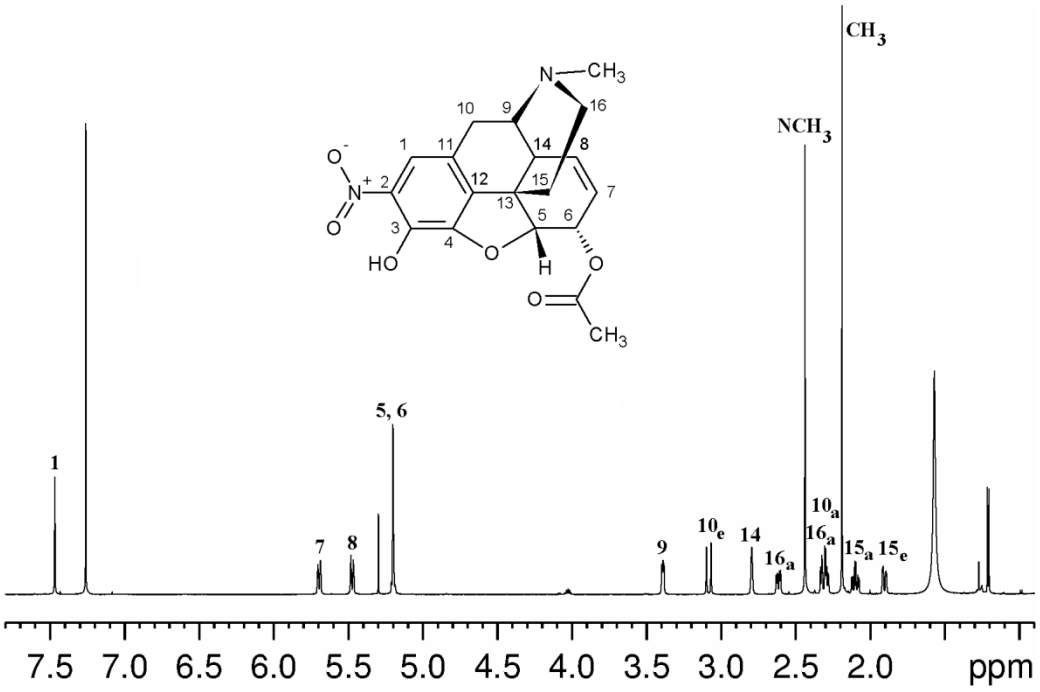


Figure 3-6: ^{1}H-NMR spectrum of 2-nitro-MAM in $CDCl_3$.

This region contained all proton signals excluding the aromatic ones. Successful assignment of the signals in the region was based on comparison with 6-MAM NMR data reported in the literature [129]. The data clearly indicates that 2-nitro-MAM retains the core structural feature of 6-MAM. However, a marked difference in chemical shifts was observed in the 6-8 ppm region in the ^{1}H-NMR spectra of 6-MAM and 2-nitro-MAM. The two aromatic protons on C-1 and C-2 in 6-MAM were observed as two apparent doublets ($J_{1,2}$ = 10 Hz) resonating at δ 6.5 and 6.6 ppm respectively (Figure 3-7a), consistent with what was reported in the literature [129]. In 2-nitro-MAM however, there was only one aromatic proton remaining due to the substitution by a nitro group on the aromatic ring. The aromatic proton was observed as an apparent singlet at δ 7.5 ppm (Figure 3-7b). The more downfield shift (by approximately 1 ppm) when compared to the chemical shifts of the corresponding aromatic protons for 6-MAM was in agreement with the introduction of a nitro group at the C-2 position of the aromatic ring. The nitro group, being strongly electron withdrawing, reduces the electron density of the aromatic ring and is known to cause a “de-shielding” effect on the remaining aromatic proton [128].

The key evidence to support the nitro group substitution on C-2 was the observation of long-range coupling between the aromatic proton on C-1 and the two protons on C-10. It has been well established that the C-1 proton, but not the C-2 proton, in morphine and its three *O*-acetyl derivatives experiences long-range coupling with the two C-10 protons with a coupling constant of approximately 1 Hz [129]. This long-range coupling was clearly revealed in the resolution-enhanced ^{1}H-NMR spectrum of 6-MAM (Figure 3-7c). Following resolution enhancement processing, the broad and shorter doublet for H-1 was resolved into a clear doublet of triplets ($J_{1,2}$ = 10.1 Hz, $J_{1,10}$ = 1.2 Hz), whereas the signal for H-2 remained as a sharper and taller doublet ($J_{1,2}$ = 10.1 Hz). When the same resolution enhancement process was applied to the δ 7.5 ppm signal present in the 2-nitro-MAM spectrum, a clear triplet was observed with a coupling constant of 1.2 Hz (Figure 3-7d). The triplet resembles the H-1 signals for 6-MAM, indicating strongly that the aromatic proton is at the C-1 position, making long-range coupling to C-10 protons possible.

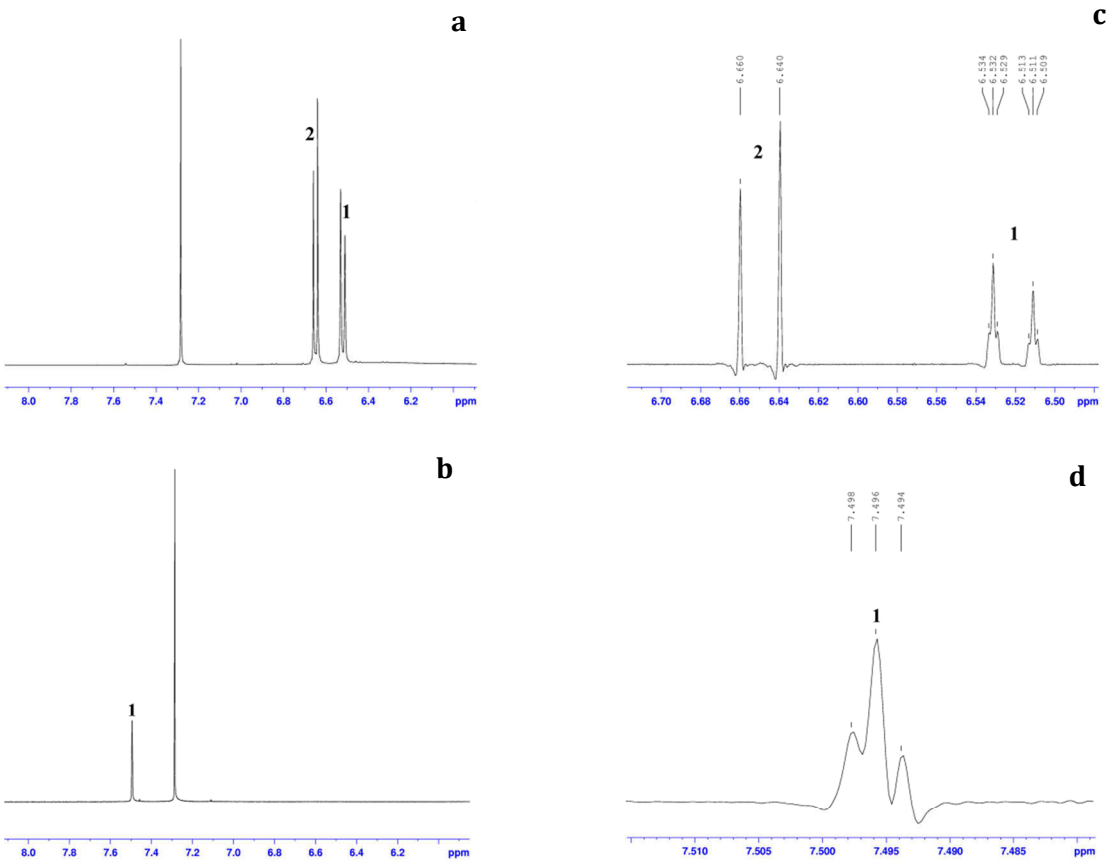


Figure 3-7: ^{1}H-NMR spectra of 6-MAM and 2-nitro-MAM (6.0-8.0 ppm region): (a) 6-MAM before resolution enhancement, (b) 2-nitro-MAM before resolution enhancement, (c) 6-MAM after resolution enhancement, with line broadening (lb) and Gaussian broadening (gb) set at -0.5 and 0.6, respectively, and (d) 2-nitro-MAM after resolution enhancement, with lb and gb set at -1 and 0.35, respectively.

The 2D COSY experiment further supports this argument. This experiment was used to reveal coupling between protons up to four bonds apart in a rigid system. As Figure 3-8 shows, there are correlation spots between the two H-10 protons and the proton resonating at δ 7.5 ppm, indicating that it is the H-1 proton.

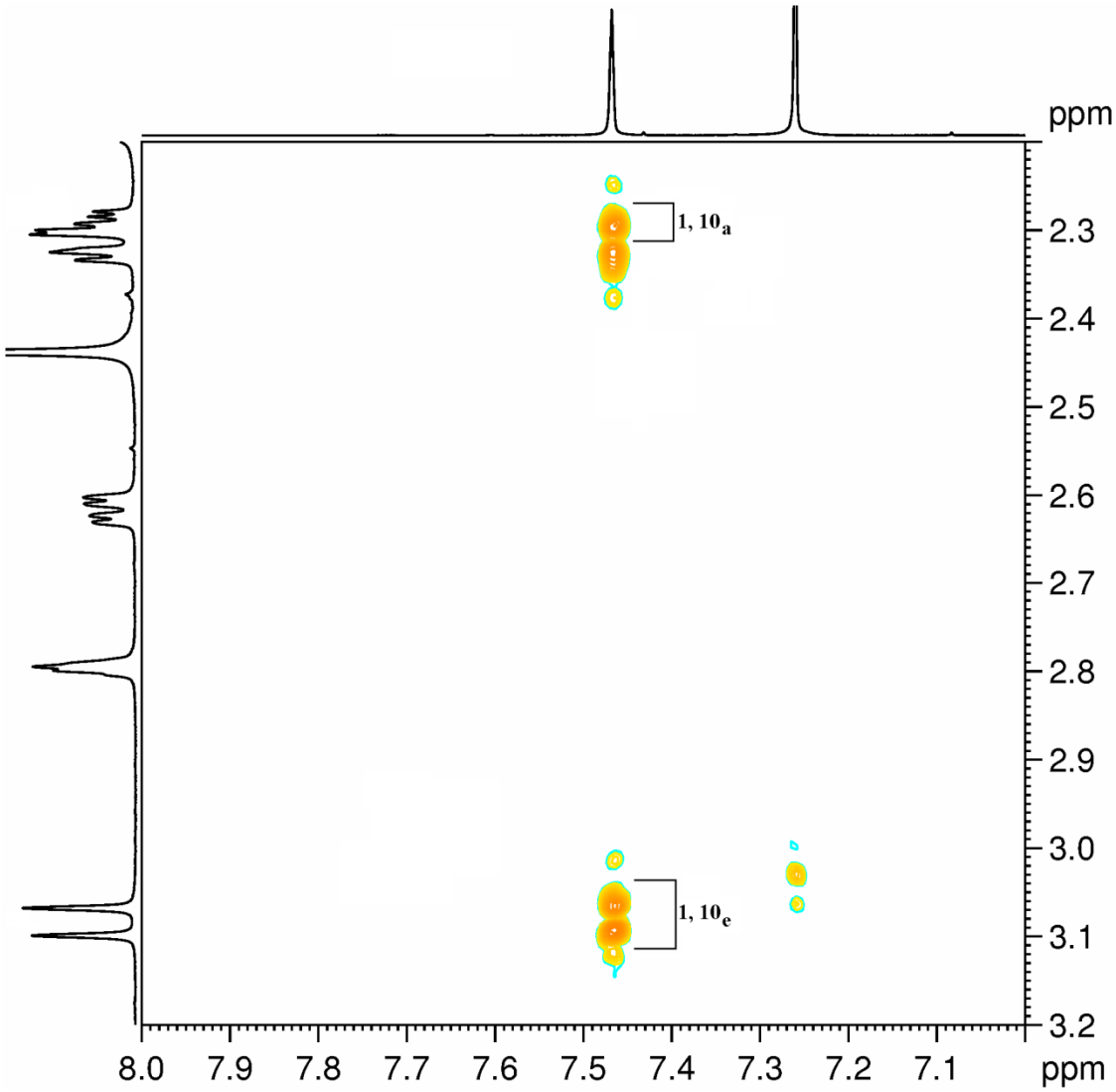


Figure 3-8: Selected region of ^{1}H-^{1}H COSY spectrum of 2-nitro-MAM.

Further evidence was obtained by conducting 2D HSQC and HMBC experiments for 2-nitro-MAM. The existence of the H-1 proton was confirmed by the HSQC experiment where correlation between H-1 and C-1 was observed (Figure 3-9). The HMBC experiment established the correlation between H-1 and C-10 [130] (Figure 3-10).

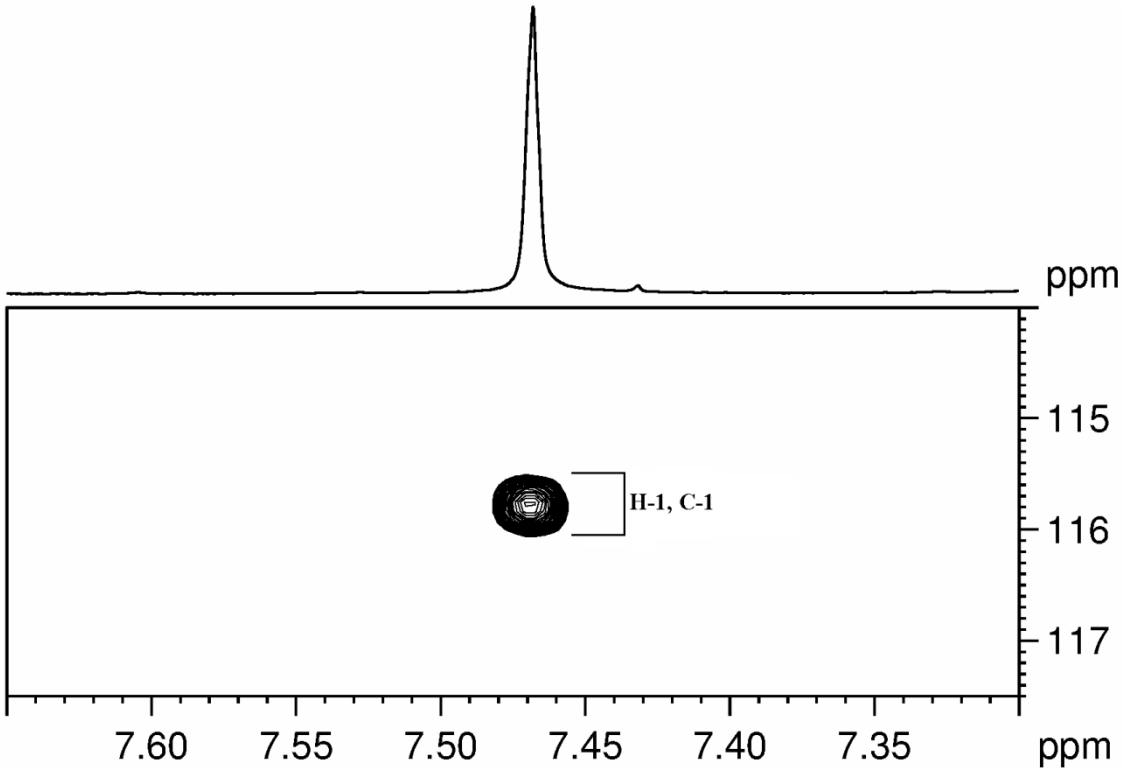


Figure 3-9: Partial ^{1}H-^{13}C HSQC spectrum of 2-nitro-MAM in $CDCl_3$.

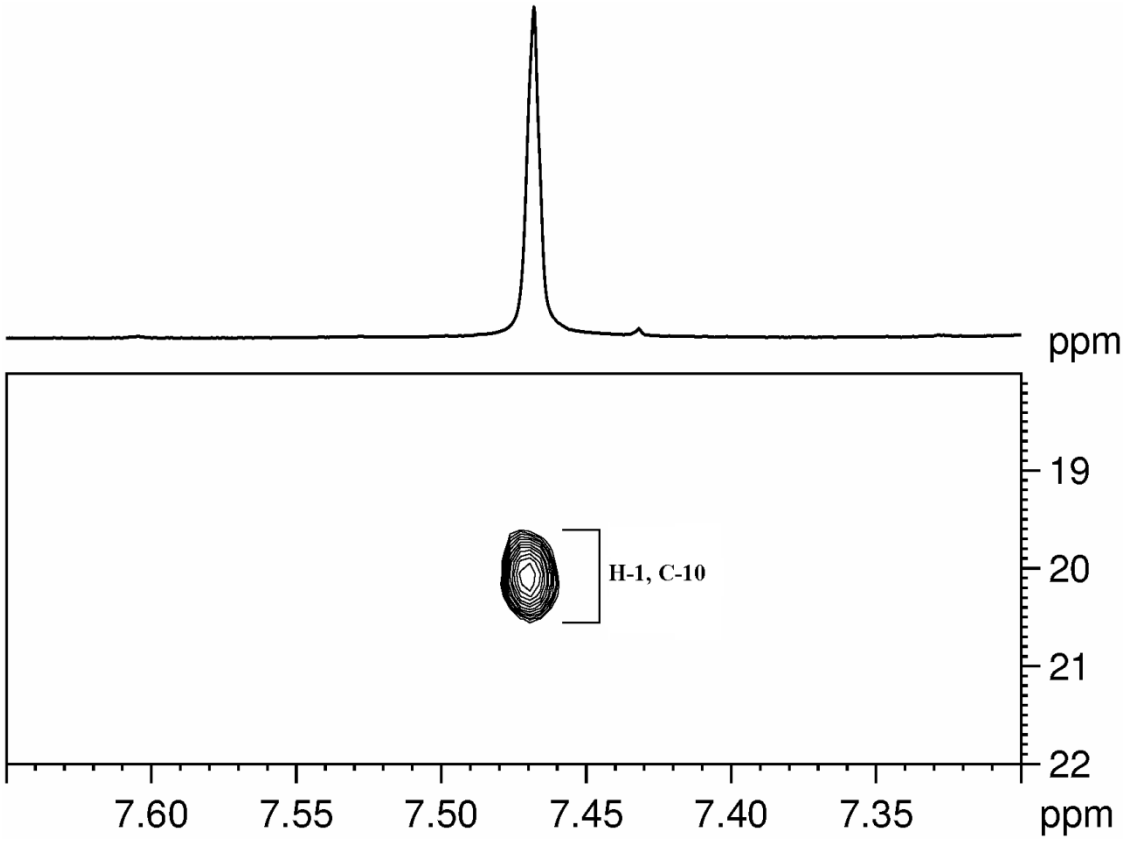


Figure 3-10: Partial ^{1}H-^{13}C HMBC spectrum of 2-nitro-MAM in $CDCl_3$.

GC-MS experiments were also performed to further confirm the structure elucidated, and to determine whether 2-nitro-MAM can be detected using this technique. Both 2-nitro-MAM and the TMS derivative of 2-nitro-MAM (2-nitro-MAM-TMS) were prepared and analysed concurrently with 6-MAM and 6-MAM-TMS samples. It was found that the non-derivatised analytes were not detectable by the GC-MS method used in the study. However, the TMS derivatives showed good chromatographic behaviour in the GC and distinct mass fragmentation patterns in the MS. Eluting at 5.12 min, 6-MAM-TMS possessed a molecular ion at *m/z* 399 and two characteristic fragment ions at *m/z* 340 and 287.On the other hand, 2-Nitro-MAM-TMS had a retention time of 6.73 min with a molecular ion at *m/z* 444 and some prominent fragment ions at *m/z* 385, 332 and 204. The corresponding representative TICs and mass spectra are given in Figure 3-11.

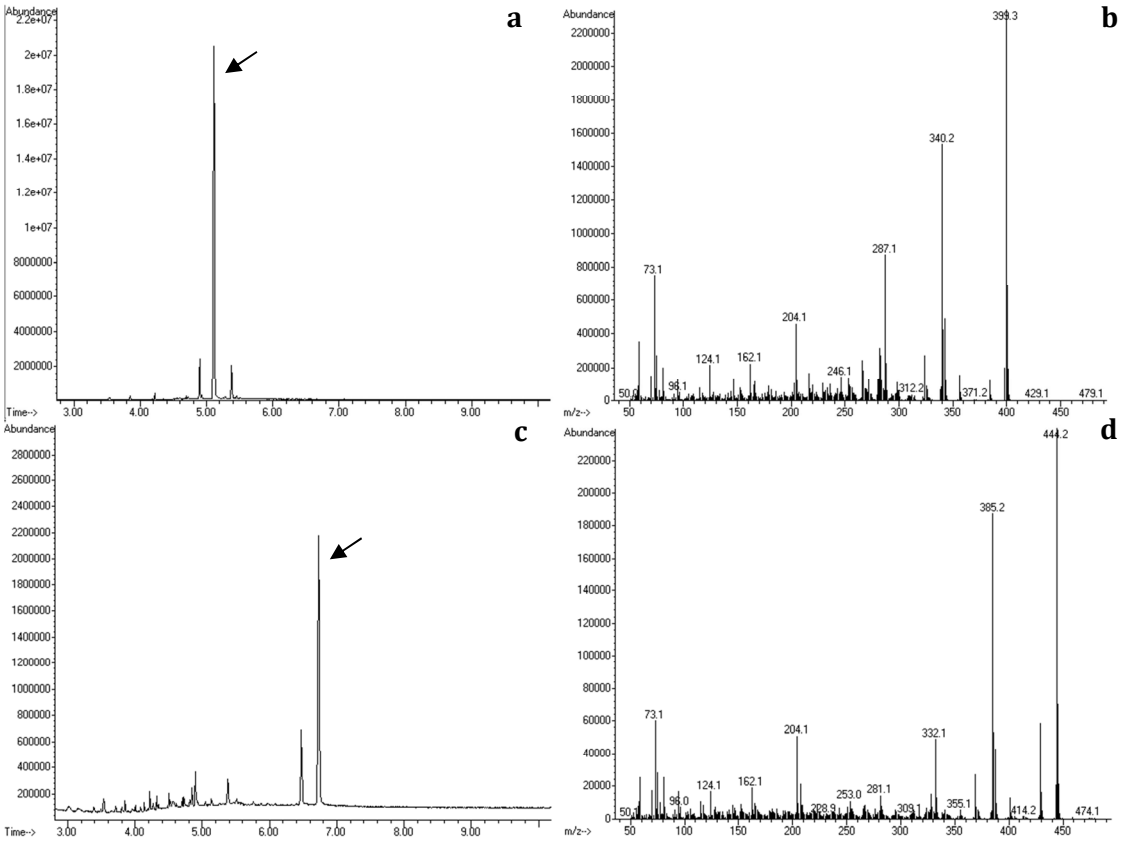


Figure 3-11: Total ion chromatograms (TICs) and mass spectra of the TMS derivatives of 6-MAM and 2-nitro-MAM: (a) TIC of 6-MAM-TMS eluting at 5.12 min, (b) MS of 6-MAM-TMS, (c) TIC of 2-nitro-MAM-TMS eluting at 6.73 min, and (d) MS of 2-nitro-MAM-TMS.

3.6 Conclusions

This study has shown that potassium nitrite can effectively mask the presence of 6-MAM in urine specimens, rendering the metabolite undetectable by the CEDIA® 6-AM screening and LC-MS confirmatory assays. However, it was demonstrated that 6-MAM was converted to 2-nitro-MAM in these specimens. There is currently nothing in the literature about 2-nitro-MAM. Aromatic nitration is not a route of metabolism of 6-MAM in the human body. Further, there is no literature to suggest that nitration of 6-MAM to produce 2-nitro-MAM as a by-product of reaction during the synthetic manufacture of heroin can

occur. Therefore, it appears that the only source of 2-nitro-MAM is by *in-vitro* oxidation. If it is found in urine, the probability of producing a false positive result are unlikely, making it a viable potential marker for the indirect monitoring of 6-MAM in such cases of adulteration.

2-Nitro-MAM appeared to remain detectable in urine for at least 11 days under the experimental conditions employed. Successful detection of 2-nitro-MAM as its TMS derivative by GC-MS provides a foundation for the development of a GC-MS method alternative to LC-MS/MS for the detection of 2-nitro-MAM.

Chapter 4: Detection and Identification of 2-nitro-morphine and 2-nitro-morphine-6-glucuronide in nitrite adulterated urine specimens containing morphine and its glucuronides

Chapter 4: Detection and identification of 2-nitro-morphine and 2-nitro-morphine-6-glucuronide in nitrite adulterated urine specimens containing morphine and its glucuronides

4.1 Introduction

In this study, opiates including morphine, codeine, morphine-3-glucuronide and morphine-6-glucuronide were exposed to potassium nitrite in water and urine to mimic the process of nitrite adulteration. Besides workplace drug testing, morphine is an analyte of interest in sports anti-doping testing. According to the 2012 world anti-doping code (international standard) released by the World Anti-Doping Agency (WADA), morphine is listed under class S7 in the prohibited list, with its use banned in-competition [131].

Although urine collection protocols are quite strict, particularly in sports drug testing, the act of adulteration is not impossible. Individuals motivated to circumvent a drug test can be very innovative, as demonstrated by the use of 'rice grains' that contain proteases placed into the urethra prior to urination to mask the use of certain peptide hormones such as erythropoietin (EPO) [132]. Furthermore, adulteration with the aid of corrupt doping control officers has also been documented [133]. In workplace drug testing, urine sample collection is supervised but not observed, in order to maintain the individual's privacy [67], making urine adulteration relatively easy to perform.

This study aimed to extend the search for reaction products that may be formed when urine specimens containing morphine, codeine, M3G and M6G were adulterated with nitrite. Any reaction products detectable by LC-MS and/or GC-MS were isolated for characterisation. The effect of enzymatic

hydrolysis commonly employed during urine sample pre-treatment for opiate analysis on the formation of reaction products was also assessed.

The overall purpose of this study was to further explore the possibility of the use of oxidation products for the detection and monitoring of opiates in urine specimens subsequent to nitrite adulteration.

4.2 Materials

4.2.1 Drug standards and reagents

Free base equivalent drug standards (1 mg/mL) of morphine monohydrate (in methanol), morphine-3-β-D-glucuronide (in methanol/water 1:1), morphine-6-β-D-glucuronide (in acetonitrile/water 1:1) and codeine (in methanol) were sourced from Lipomed (Arlesheim, Switzerland). Solid morphine hydrochloride was obtained from Macfarlan Smith Limited (Edinburgh, United Kingdom).

KNO_2 and *Helix Pomatia* β-glucuronidase Type H-3 crude solution (109996 units/mL) were sourced from Sigma Aldrich (St Louis, MO, USA). BSTFA with 1% TMCS was purchased from United Chemical Technologies (Bristol, PA, USA).

4.2.2 Urine specimens

Donor urine was obtained from healthy individuals and pooled (n=4) to obtain a representative blank urine matrix. Urine was collected in polypropylene urine specimen containers and used on the day of collection after testing negative for opiates by using the LC-MS methods developed for this study.

An authentic urine specimen that was tested positive for morphine, M3G and M6G was obtained from the Drug Toxicology Unit, NSW Forensic and Analytical Science Service (Macquarie Hospital, Ryde). The specimen was supplied subsequent to identification removal and stored at -20℃. It was thawed at room temperature on the day of analysis.

4.3 Instrumentation

4.3.1 LC-MS

LC-MS analysis was conducted on an Agilent 1290 LC system paired with either an Agilent 6490 QQQ-MS or an Agilent QTOF-MS, via an ESI interface (positive ion mode). Chromatographic separation was achieved by a gradient elution of 20 mM ammonium formate (pH 6.3, solvent A) and 95% acetonitrile in water (ACN, solvent B) and at a 0.25 mL/min flowrate. With initial conditions at 2% B, it was increased to 5% at six min, 30% at 12 min, 70% at 17 min and 95% at 19 min; held at 95% to 21 min, and decreased to 2% at 21.1 min, with a post-run column equilibration of four min. Samples prepared for LC-MS and enzymatic hydrolysis studies were injected in 1 µL volumes onto an Agilent Zorbax eclipse XDB-C18 column (2.1 mm × 50 mm × 1.8 µm), set at 40℃. Further sampler and auxiliary parameters for the LC system are detailed in the appendix, Table A1. Full scan, product ion scan and MRM analyses were utilised, with a fragmentor voltage of 380 V (QQQ-MS) and 170 V (QTOF-MS), and a collision energy range of 35-65 eV. The scanning mass range was set at *m/z* 100-1000 (scan time = 500 ms). The gas temperature and flow were adjusted to 200℃ and 14 L/min, respectively. The sheath gas temperature and flow (QQQ-MS) were set to 250℃ and 11 L/min, respectively. The capillary and nozzle voltages were 3000 V and 1500 V, respectively.

4.3.2 GC-MS

GC-MS analysis was carried out on an Agilent 7890 AGC system equipped with an Agilent 5975C Inert XL EI-MS, in full scan (scanning mass range of *m/z* 50-1000) and SIM modes. Samples prepared for the GC-MS studies were injected in 1 µL volume in splitless mode onto a HP-5MS capillary column (30 m × 0.250 mm × 0.25 µm). Helium carrier gas at a flow rate of 1.65 mL/min was used, with the inlet and auxiliary heaters both maintained at 250℃. The oven temperature program was set to begin at 115℃ and held for 0.5 min; ramped 20℃/min until 305℃, and held for 0.5 min; then increased 10℃/min

until 320℃ was reached, where it was held for 0.5 min for a run time of 12.5 min.

4.3.3 NMR

NMR characterisation was performed on an Agilent Technologies 500/54 (500 MHz/54 mm bore) premium shielded NMR spectrometer coupled to a 7510-AS autosampler. One dimensional ^{1}H-NMR and two dimensional ^{1}H-^{1}H COSY and ^{1}H-^{13}C HSQC data were obtained for morphine and the isolated reaction product. The samples were dissolved in deuterated methanol (CD_3OD). Table 4-1 outlines the key acquisition parameters for each experiment.

Table 4-1: Key NMR acquisition parameters for the analysis of morphine and the reaction product in CD_3OD.

acquisition parameter	NMR experiment		
	^{1}H-NMR	**^{1}H-^{1}H COSY**	**^{1}H-^{13}C HSQC**
spectral width (Hz)	8012.8	8012.8	8012.8
number of scans	1024	8[a]	12[a]
relaxation delay (s)	1.0	2.0	1.5
pulse angle (°)	60	n/a	
acquisition time (s)	4.089	0.250	0.150
t1 increments	n/a	512	512
F1 nucleus		n/a	C-13
C13 spectral width (ppm)			0-160
one bond J1 × h (Hz)			146.0

[a]per t1 increment.

4.4 Experimental method

4.4.1 LC-MS studies

Opiate drug standards of interest were spiked at 10 µg/mL in water (1 mL). For each opiate, two corresponding reaction mixtures were prepared whereby the

samples were adulterated with 0.5 M and 6 M KNO_2 working solutions, to give final oxidant concentrations of 0.05 M and 0.6 M in the samples, respectively. Sample adulteration was also carried out with solid KNO_2 (several grains added to each sample). All reaction mixtures were acidified to pH 3 with 2 M HCl and were analysed after one hour reaction at room temperature. KNO_2 reagent controls at the spiked concentrations in addition to a blank control were prepared and analysed.

The adulteration reactions were repeated with blank urine as the reaction medium instead of water. Samples were left to react for one hour at room temperature before analysis, with the exception of the preliminary stability study. During this study, analysis was carried out 25 min after the commencement of the reaction. Urine samples were analysed following centrifugation (4500 *g* for 10 min) and filtration through 0.22 µm hydrophilic syringe filter units (MicroAnalytix Pty Ltd, Taren Point, NSW, Australia).

For the authentic urine specimen, adulteration with KNO_2 (0.05 M and 0.6 M) was conducted at both pH 3 (adjusted by 2 M HCl) and natural urine pH (no pH adjustment, determined to be pH 6). The same procedure for sample pre-treatment prior to analysis was followed as for the spiked urine samples.

4.4.2 MRM method validation

The same methodology was followed as detailed in section 3.4.2, with several deviations due to instrumental availability. For morphine, M3G and M6G, QC samples were spiked at 250 (n=5 for morphine and n=3 for the glucuronide metabolites) and 800 ng/mL (n = 3 for all three analytes). Additional QC samples at 5 ng/mL (n=5) were prepared for morphine. The validation was carried out over four days.

4.4.3 Enzymatic hydrolysis studies

The enzymatic hydrolysis procedure consisted of the addition of 25 µL β-glucuronidase crude solution and 500 µL of 1 M sodium acetate buffer (pH 4.5)

to a 1 mL urine sample, followed by incubation at 50℃ for 18 hrs. Liquid-liquid extraction with dichloromethane/isopropanol (9:1) was carried out after the samples were basified with 1 mL carbonate/bicarbonate buffer (1 M, pH 9.5). The organic fractions were evaporated under a gentle stream of N_2 at 30℃. The residual analytes were reconstituted in 200 µL of methanol/ammonium formate (30:70) in high recovery vials (PM Separations, Capalaba, QLD, Australia) for LC-MS analysis.

4.4.4 GC-MS studies

Urine samples were prepared using the same method employed for the LC-MS studies. The analytes were isolated following the liquid-liquid extraction procedures outlined for the enzyme studies. TMS derivatives for GC-MS analysis were then prepared by combining 50 µL of BSTFA (with 1% TMCS) and 150 µL of acetonitrile to the analyte residues and heated at 75℃ for 30 min on a heating block.

4.4.5 NMR studies

To produce adequate material for NMR analyses, the morphine/KNO_2 reaction was scaled up in water with 6 mg of morphine hydrochloride starting material. The reaction mixture was left at room temperature for one hour prior to isolation of the reaction product using the above described liquid-liquid extraction, enabling the recovery of the reaction product residue (approximately 1 mg).

4.5 Results and discussion

4.5.1 Formation of 2-nitro-morphine

The exposure of morphine to KNO_2 at pH 3 resulted in the same observations at both oxidant concentrations, and in both water and urine matrices. Under the LC-MS conditions employed, morphine was found to elute at 4.1 min, with $[M+H]^+$ at *m/z* 286 (Figure 4-1a). The reaction was evidenced by the loss of

morphine, with a single and major reaction product peak found at 7.8 min corresponding to an analyte with $[M+H]^+$ at *m/z* 331 (Figure 4-1b).

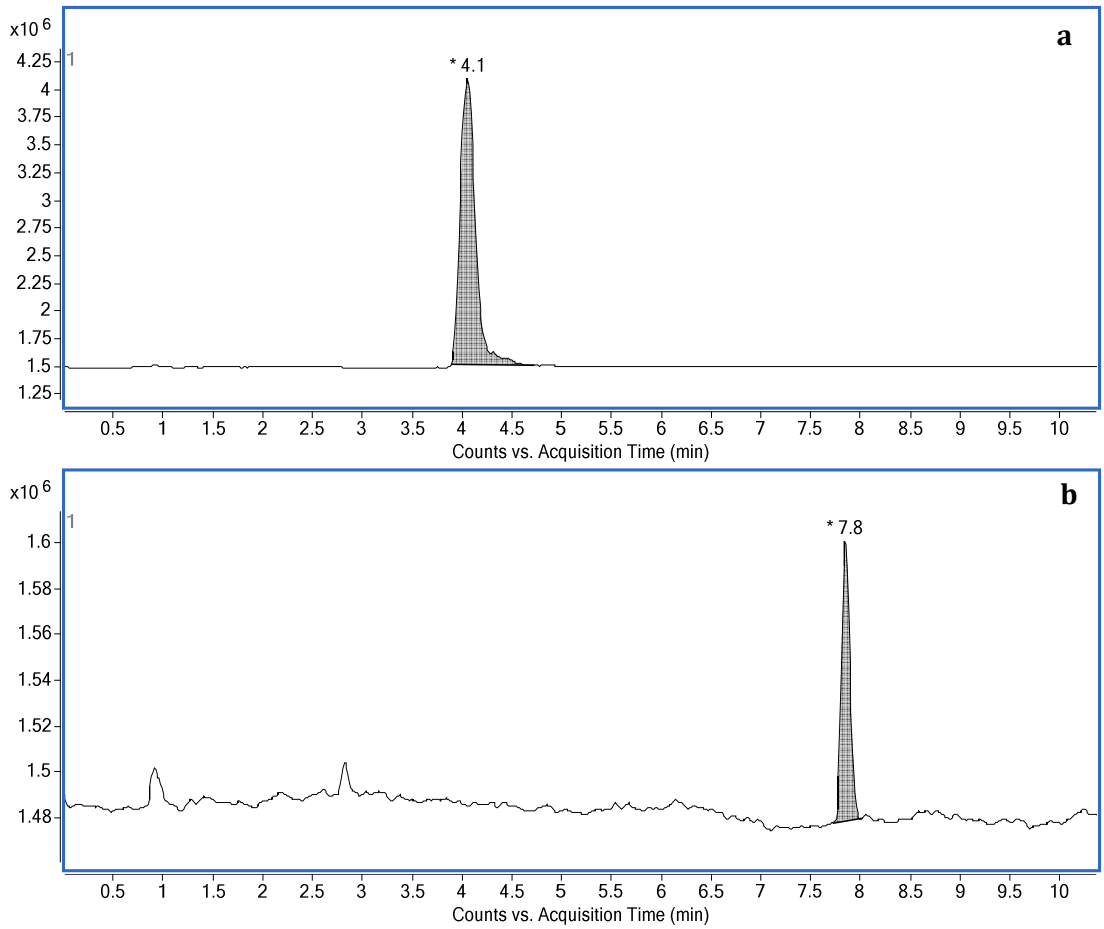


Figure 4-1: TIC chromatograms of (a) morphine, eluting at 4.1 min and (b) the major reaction product resulting from morphine reaction with 0.6 M KNO_2, eluting at 7.8 min.

Comparison of the CID fragmentation patterns of morphine and the reaction product revealed that they were different. However, fragmentation of the protonated molecule at *m/z* 331 for the reaction product resulted in a major ion at *m/z* 285. This was consistent with the mass of morphine, raising the possibility that both morphine and the reaction product shared a fundamental molecular structure. Furthermore, the mass difference between morphine and the reaction product was 45 Da; this was consistent with the substitution of a nitro ($–NO_2$) group onto the morphine structure, resulting in the formation of the reaction product.

Accurate mass experiments provided support for the formation of a nitro product. The MS fragmentation patterns of the morphine starting material (Figure 4-2a) and the reaction product (Figure 4-2b) were obtained. It was determined that the actual mass of the reaction product was found to be 330.1203 Da, matching the molecular formula $C_{17}H_{18}N_2O_5$. Comparison of this actual mass, to the molecular formula generated mass for $C_{17}H_{18}N_2O_5$ (330.1216 Da), showed an acceptable difference of 3.92 ppm. Furthermore, the difference of $[M+H]^+$ at *m/z* 331.1248 and the next major product ion of *m/z* 285.1326 was 45.9922 Da. The calculated mass for a $–NO_2$ group is 45.9929 Da, suggesting that the mass loss was due to the cleaving of the $–NO_2$ group from the molecule. Furthermore, the loss of a $–NO_2$ group would result in a radical cation of morphine, consistent with the major product ion observed at *m/z* 285.1326.

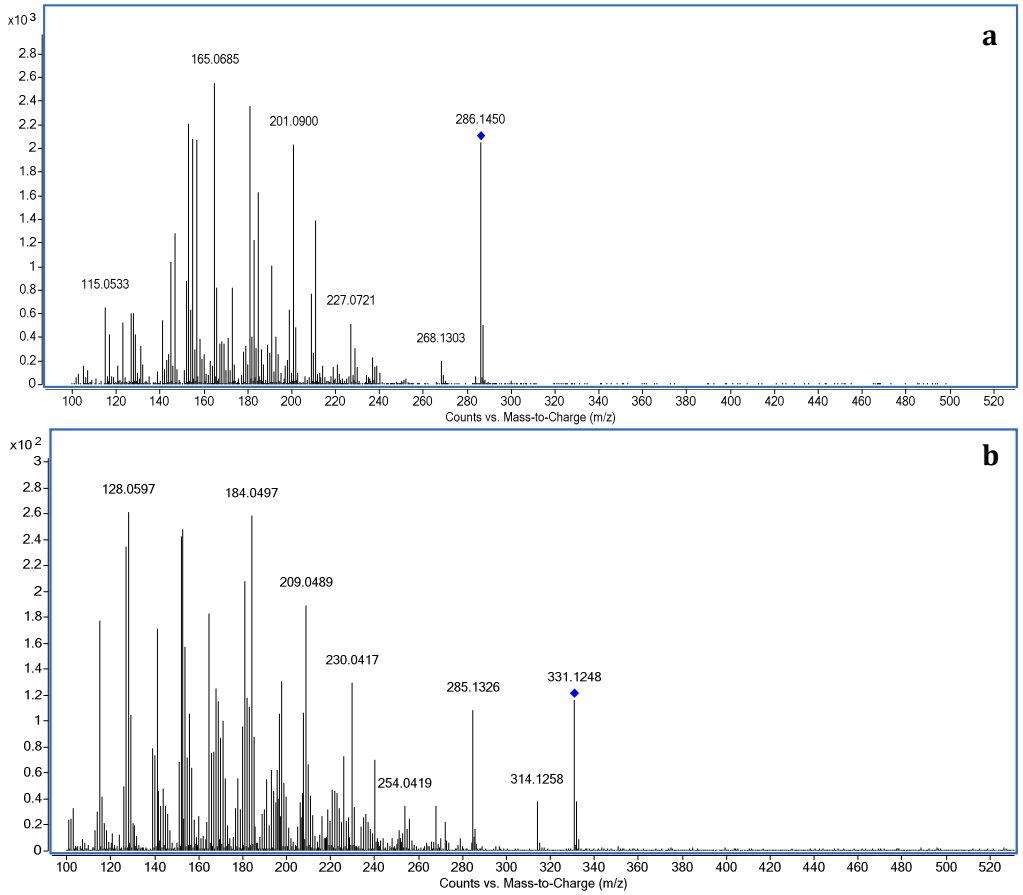


Figure 4-2: Accurate mass CID spectra for (a) morphine (FE = 170 V, CE = 45 eV) and (b) 2-nitro-morphine (FE = 170 V, CE = 45 eV)

Based on the fragmentation and accurate mass data obtained and the reaction mechanism proposed previously for the formation of 2-nitro-MAM upon exposure of 6-MAM to KNO_2 in aqueous matrices (see Section 3.5.3, Figure 3-4,), it was hypothesised that the morphine reaction product was 2-nitro-morphine (Figure 4-3). The isolated reaction product was found to be distinctly orange; this was consistent with the extended conjugation of the $–NO_2$ group with the aromatic ring, a visual feature also shared by 2-nitro-MAM.

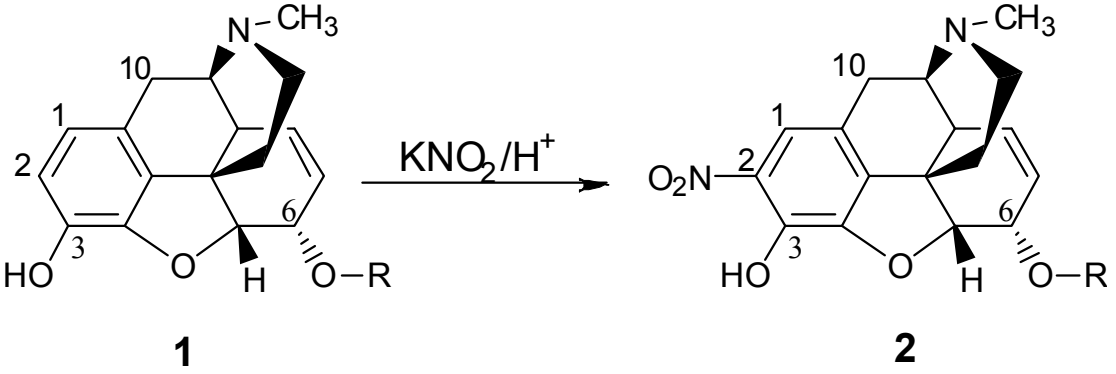


Figure 4-3: General reaction scheme depicting the conversion of (1) the opiate starting material to (2) the nitrated oxidation product, where R = H for morphine, and R = $C_6H_9O_6$ (glucuronide) for M6G.

High resolution NMR studies also supported this hypothesis. NMR signal assignment of the proton environments within morphine and the reaction product was carried out based on published values in the literature [129, 130]. Comparison of the ^{1}H-NMR spectra of morphine and the reaction product revealed the presence of corresponding signals from δ 0-6 ppm, indicating that both compounds shared a common core structure (Figure 4-4 and Figure 4-5). However, a marked difference was observed in the aromatic region. Figure 4-6a shows the two doublet signals resonating at δ 6.56 ppm and δ 6.49 ppm, corresponding to the aromatic H-2 and H-1 protons on the morphine structure, respectively. The doublet for H-1 is not as tall and sharp as the doublet for H-2 due to long range coupling between H-1 and the two neighbouring H-10 protons. On the contrary, it was observed that these two signals were no longer present in the ^{1}H-NMR spectrum of the reaction product. A singlet resonating at δ 7.20 ppm was found instead (Figure 4-6b); this downfield shift in signal was consistent with the substitution of an electron withdrawing group (such as a $–NO_2$ group) onto the aromatic ring. The singlet multiplicity also suggested that there was only one proton remaining on the aromatic ring.

2D COSY experiments confirmed that the remaining proton was H-1. Figure 4-6c shows the correlation observed between H-1 and both H-10 protons within the morphine structure. Figure 4-6d shows that there is a correlation between the remaining aromatic proton found in the reaction product, and the

two H-10 protons. Since COSY experiments can identify proton correlations up to four bonds apart in a rigid system, it can be concluded that H-1 is the remaining aromatic proton, with the $–NO_2$ substitution occurring at the C-2 position.

HSQC experiments were carried out to determine single ^{1}H-^{13}C bond correlations. As a reference, the single bond correlation between H-1 and C-1, and H-2 and C-2 for the morphine starting material is depicted in Figure 4-6e. HSQC data for the reaction product shows existing correlation between the remaining aromatic proton and the C-1 carbon (Figure 4-6f), indicating that the remaining proton was indeed H-1. Thus, the reaction product was unambiguously elucidated as 2-nitro-morphine.

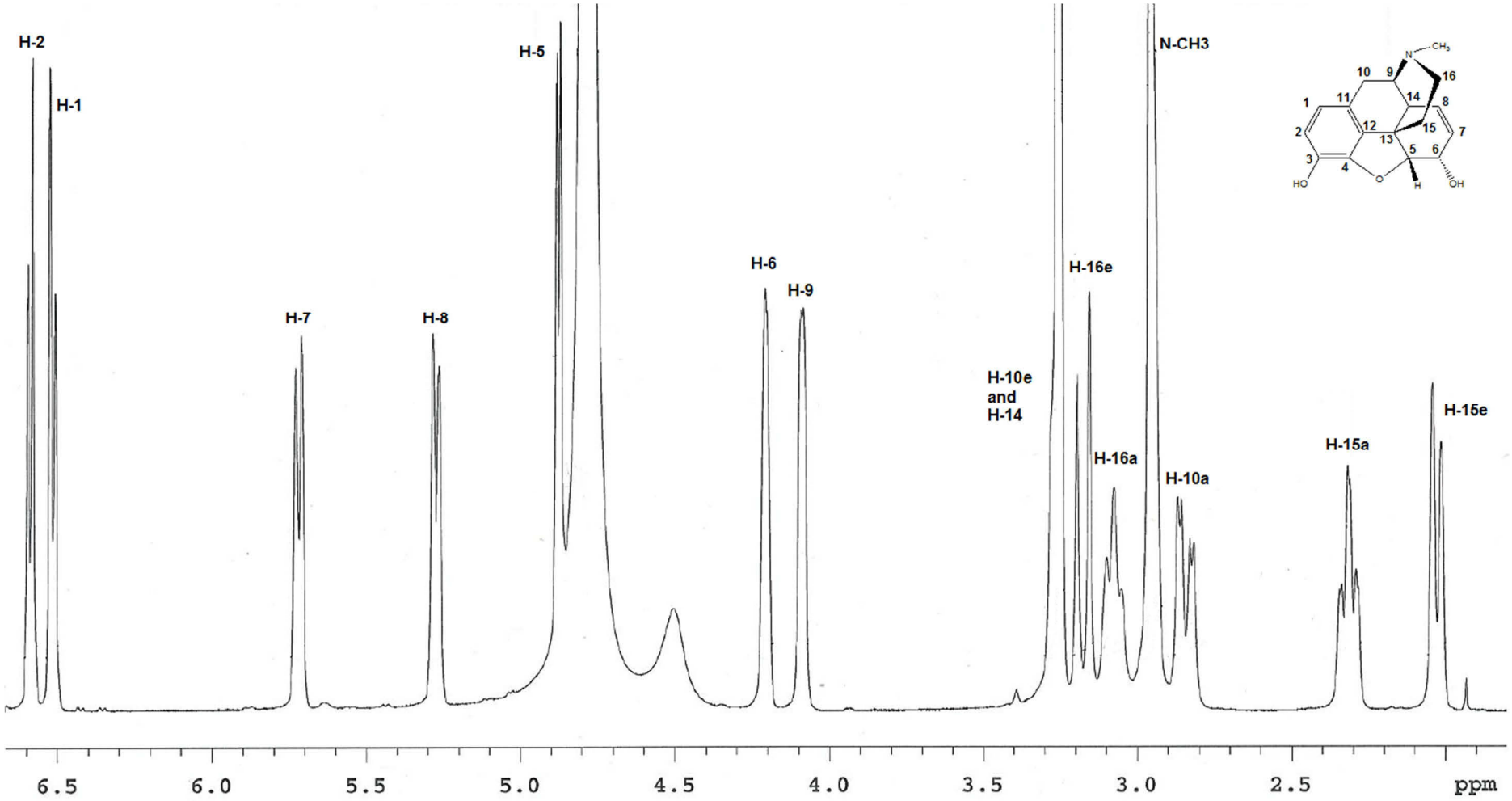


Figure 4-4: ^{1}H-NMR spectrum of morphine in CD_3OD.

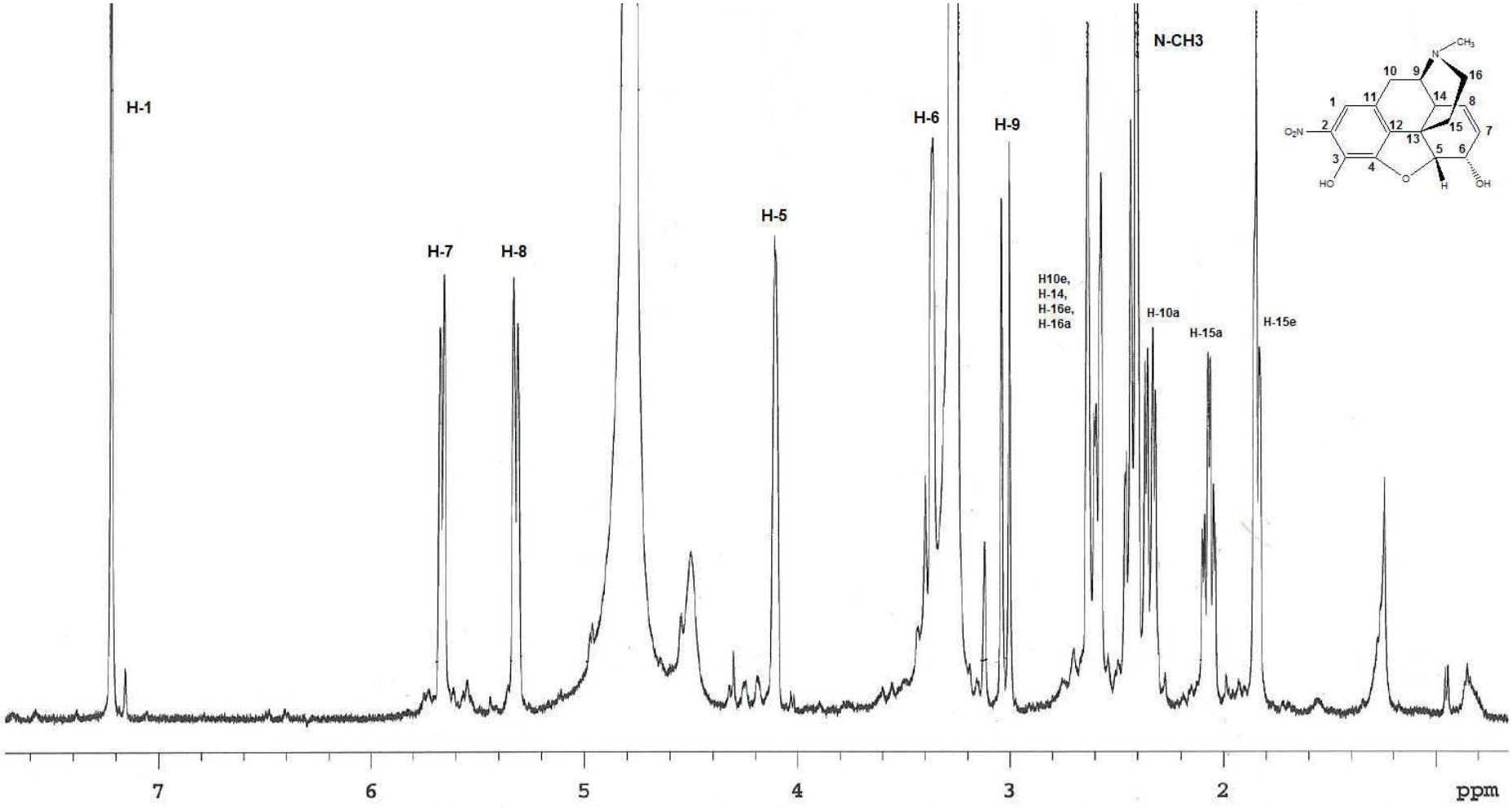


Figure 4-5: ^{1}H-NMR spectrum of the reaction product 2-nitro-morphine in CD_3OD.

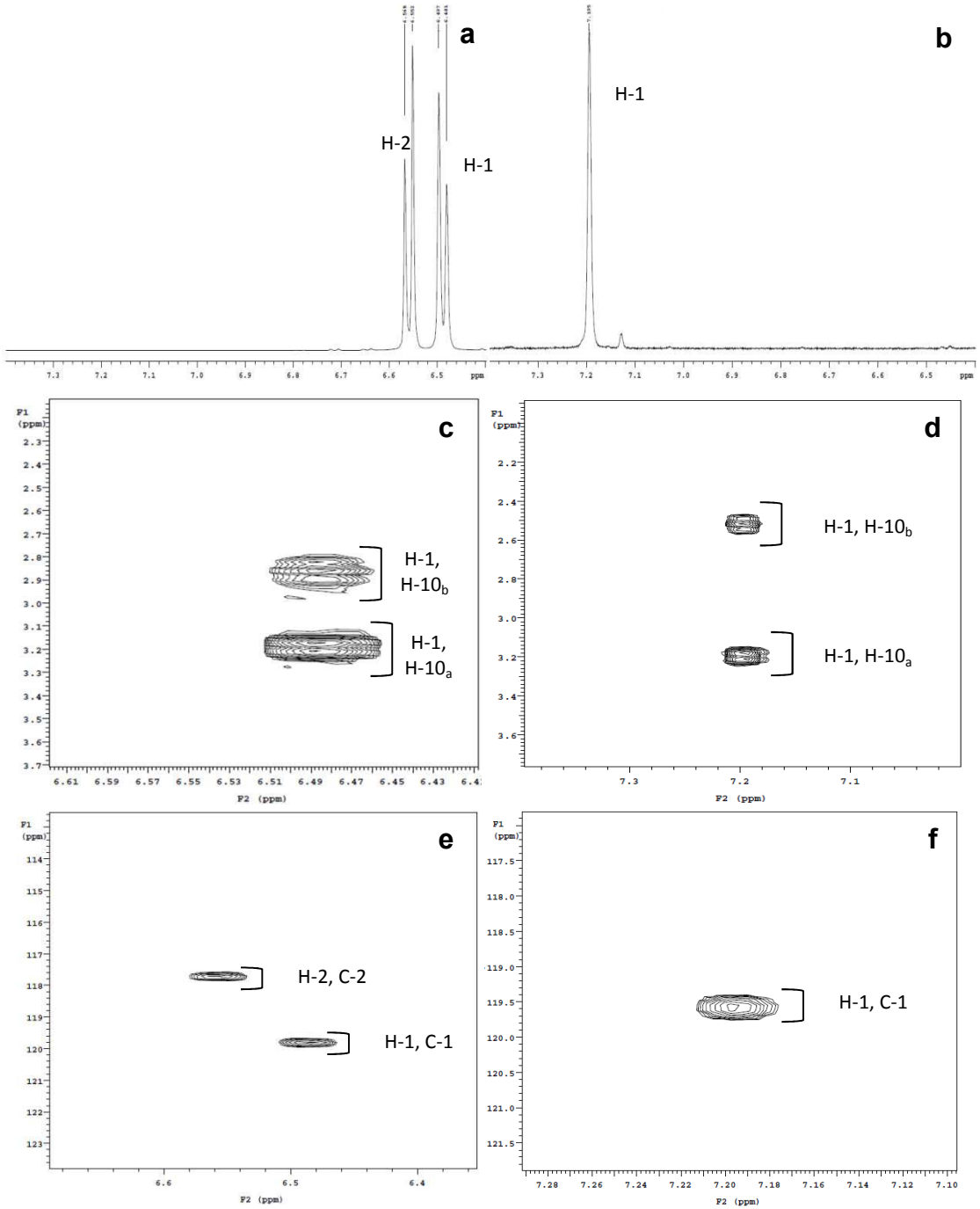


Figure 4-6: ^{1}H-NMR spectra of (a) morphine and (b) 2-nitro-morphine; ^{1}H-^{1}H COSY spectra of (c) morphine and (d) 2-nitro-morphine; and ^{1}H-^{13}C HSQC spectra of (e) morphine and (f) 2-nitromorphine. Spectra shown are for the aromatic region only.

4.5.2 Formation of 2-nitro-morphine-6-glucuronide

A similar finding was observed for the exposure of M6G to both 0.05 M and 0.6 M KNO_2 at pH 3, in both water and urine reaction matrices. The M6G starting

material eluted at 2.1 min, with $[M+H]^+$ at *m/z* 462 (Figure 4-7a). Upon KNO_2 fortification, a distinct reaction product was found to elute at 4.5 min, with $[M+H]^+$ at *m/z* 507 (Figure 4-7b). Depletion of M6G was also noted in these reaction mixtures.

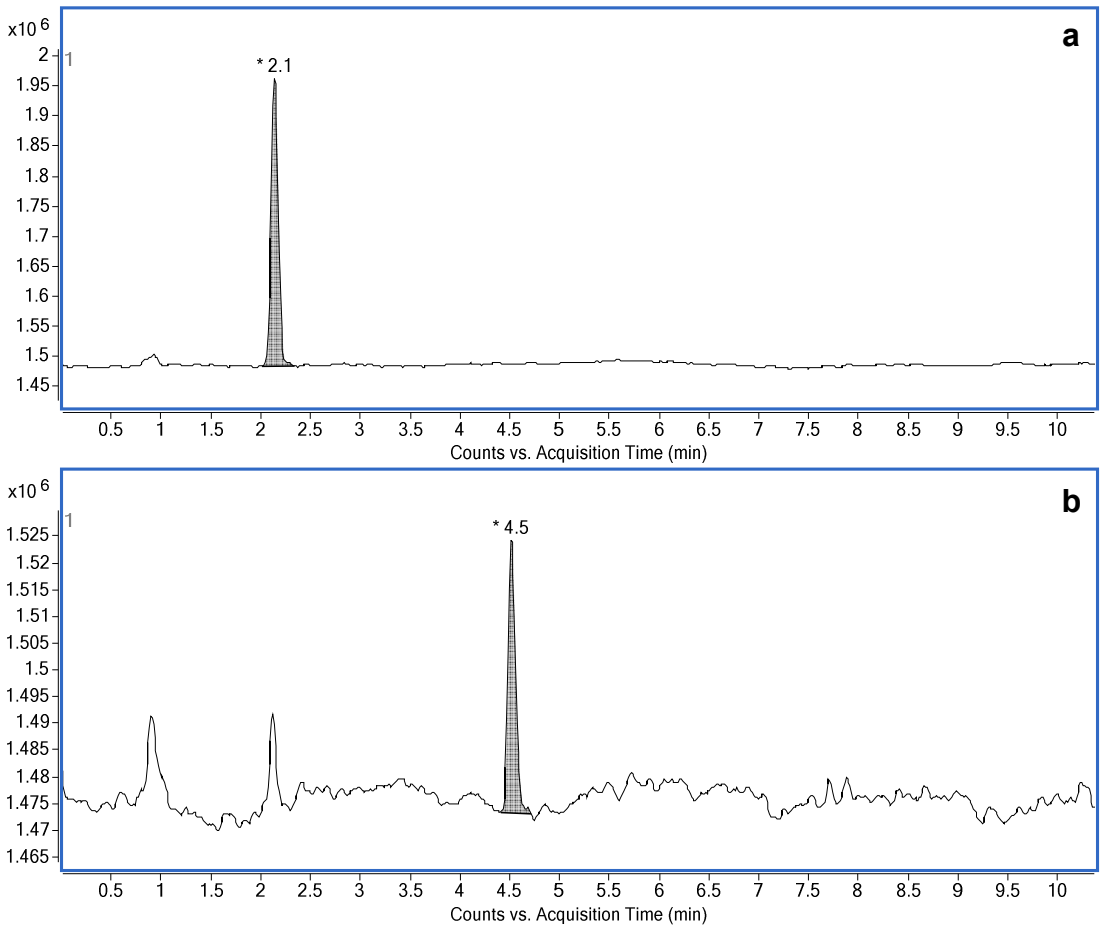


Figure 4-7: TIC chromatograms of (a) M6G, eluting at 2.1 min and (b) the major reaction product resulting from M6G reaction with 0.6 M KNO_2, eluting at 4.5 min (note: the small peak at 2.1 min is not M6G, and belongs to an unknown urinary compound).

Accurate mass analysis was conducted to investigate the structure of the M6G reaction product. Comparison of the product ions produced by the fragmentation of the protonated molecules of morphine, 2-nitro-morphine, M6G and the M6G reaction product showed encouraging results. As observed in Figure 4-8a, the induced fragmentation of the M6G reaction product ($[M+H]^+$ at *m/z* 507.1568) resulted in a base ion of *m/z* 331.1273. The protonated molecule $[M+H]^+$ at *m/z* 507.1568 corresponded to the molecular formula

$C_{23}H_{26}N_2O_{11}$, with a mass accuracy of 2.74 ppm. This formula was consistent with a nitrated species of M6G. Additionally, the fragment ion at *m/z* 331.1273 was in line with the cleaving of the glucuronic acid group situated at the C-6 position, leaving behind a protonated species of nitrated morphine ($C_{17}H_{19}N_2O_5$). In this case, the MS/MS mass accuracy was determined to be 4.53 ppm, within the acceptable range based on the instrument specifications [134]. An attempt to induce further fragmentation resulted in the detection of *m/z* 285.1256 (Figure 4-8b), which was consistent with the mass of morphine. Furthermore, it was found that the M6G reaction product shared other common product ions (*m/z* 127.05, *m/z* 154.06, *m/z* 181.06, *m/z* 209.04 and *m/z* 230.04) with 2-nitro-morphine (Figure 4-2b).

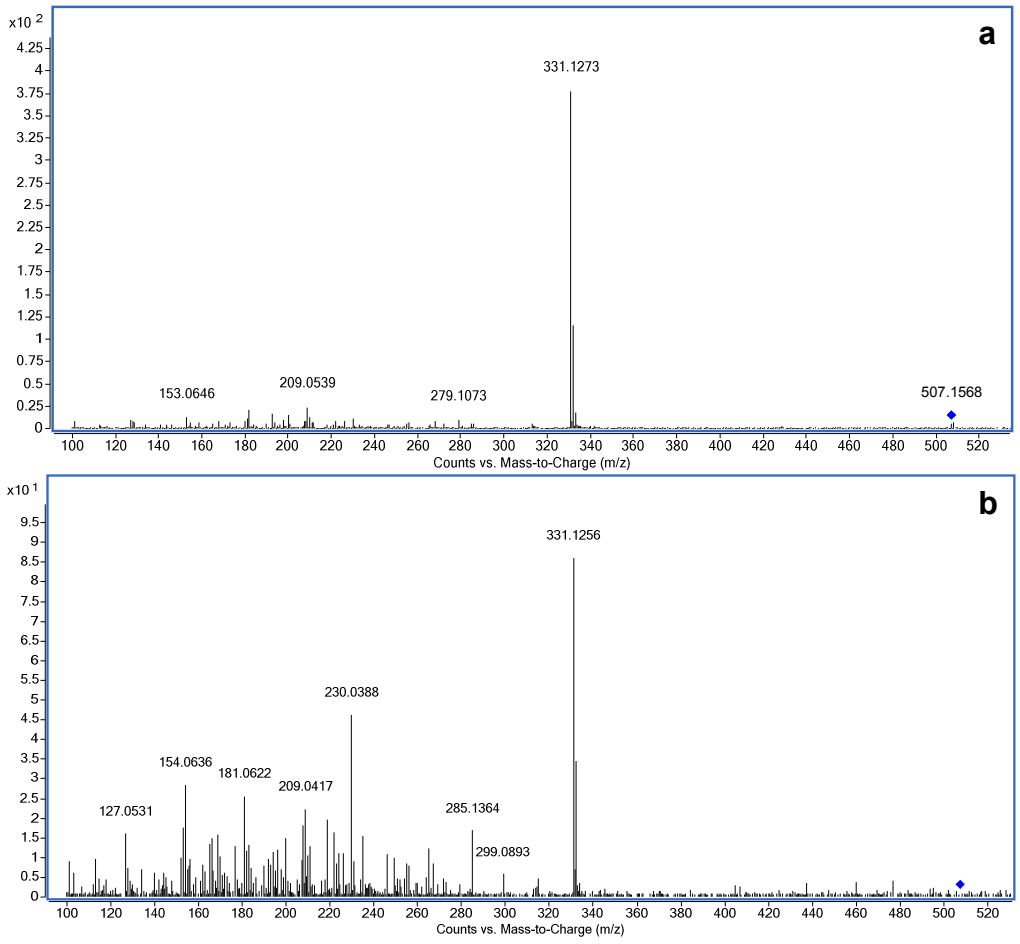


Figure 4-8: Accurate mass MS/MS data for (a) 2-nitro-M6G (FE = 170 V, CE = 45 eV) and (b) 2-nitro-M6G (FE = 170 V, CE = 60 eV).

Based on this data and the reaction pathway observed between morphine and KNO_2, the reaction product with $[M+H]^+$ at *m/z* 507 was hypothesised to be 2-

nitro-morphine-6-glucuronide (2-nitro-M6G, Figure 4-3). This elucidation was confirmed during enzymatic hydrolysis studies (section 4.5.7), whereby cleavage of the glucuronic acid group resulted in the detection of 2-nitro-morphine.

4.5.3 Inability of nitrite to react with codeine and morphine-3-glucuronide

On the contrary, exposure of codeine and M3G to KNO_2 in both water and urine did not result in the formation of any significant reaction products. These reactions were monitored over a nine day period for signs of product formation. Although there were very small peaks in the baseline that were not present in the corresponding reagent controls, comparison of codeine and M3G in the reaction mixtures with the corresponding standards over time showed that the peak intensities were similar. Both codeine and M3G were still the predominant analytes in the reaction mixtures. This finding was expected based on the aforementioned proposed reaction mechanism (Figure 3-4). The presence of the aromatic –OH functional group at the C-3 position of the opiate structure appeared to be a driving force for the reaction. The –OH group activates the ring at the ortho C-2 position, directing the NO_2^+ electrophile to substitute at this site. As the C-3 position is occupied by $–OCH_3$ and glucuronide functional groups in codeine and M3G, respectively, reaction with KNO_2 was not expected to occur with these two opiates. Although the $–OCH_3$ group does have a ring activating effect [130], the reaction may not proceed due to possible steric hindrance. Taking this argument into consideration, codeine-6-glucuronide (one of the major urinary metabolites of codeine) was not sourced for the study due to its lack of an –OH group at the C-3 position.

4.5.4 Development of an MRM method

An LC-MS/MS MRM method was developed to ensure that the starting materials and reaction products could be detected at low concentrations. The method monitored the presence of morphine, M3G, M6G, 2-nitro-morphine and 2-nitro-M6G. The data used to determine the accuracy and precision of the

method is detailed in the appendix for morphine (Figure A5 and Table A6-Tabel A9), M3G (Figure A6 and Table A10-Table A13) and M6G (Figure A7 and Table A14-Table A17); with the summary of the validation results displayed in Table 4-2.

Table 4-2: LC-MS/MS MRM method validation results for morphine, M3G and M6G.

analyte	transition 1 (quant.)	transition 2 (qual.)	linearity range (ng/mL)	correlation co-efficient (R^2)	LOD (ng/mL)	QC conc. (ng/mL)	intra-day accuracy (% MRE)	intra-day precision (% RSD)	inter-day accuracy (% MRE)	inter-day precision (% RSD)
morphine	286→152	286→165	1-1000	>0.99	<1	5	2.043	1.588	-4.292	13.23
						250	3.071	2.98	-4.531	12.77
						800	7.62	1.250	-12.30	10.65
M3G	462→286	462→201	5-1000	>0.99	1	250	-3.078	2.689	-7.180	4.536
						800	8.053	1.423	-9.643	1.987
M6G	462→286	462→201	5-1000	>0.99	1	250	-5.070	1.334	6.620	1.631
						800	7.741	1.878	-10.13	2.353

Note: Transitions monitored for the oxidation products were 331→115 (quantifying) and 331→152 (qualifying) for 2-nitro-morphine, and 507→331 (quantifying) and 507→285 (qualifying) for 2-nitro-M6G.

4.5.5 Detection of 2-nitro-morphine and 2-nitro-M6G in authentic morphine-positive urine following nitrite adulteration

The MRM method was used to analyse an authentic urine specimen tested positive for morphine, M3G and M6G prior and subsequent to adulteration with KNO_2. The results were consistent with findings established from studies with spiked standards and served as a means of proof of concept.

In all samples including the original specimen analysed as a control, the M3G responses (R_t = 1.0 min) remained constant, indicating that no significant analyte losses were detected. However, the converse was true for morphine (R_t = 4.1 min) and M6G (R_t = 2.1 min); there appeared to be losses > 99% of morphine in samples containing 0.05 M KNO_2, at both pH conditions (pH 3 and pH 6). In samples containing 0.6 M KNO_2, morphine was not detectable. M6G was undetectable in all KNO_2 fortified samples. However, the most significant finding was the detection of both 2-nitro-morphine (R_t = 7.7 min) and 2-nitro-M6G (R_t = 4.4-4.5 min) in all the KNO_2 fortified samples, at both pH conditions. These observations are depicted in Figure 4-9. The results of this study are particularly significant for doping control laboratories, where the determination of a positive or negative test result for morphine is complicated by the minor biotransformation of morphine (the WADA threshold for morphine is 1.0 µg/mL [135]). A doping violation can be determined more easily with the use of 2-nitro-morphine and 2-nitro-M6G, since these reaction products could only be a result of chemical manipulation of the specimen, an act which is also prohibited by WADA.

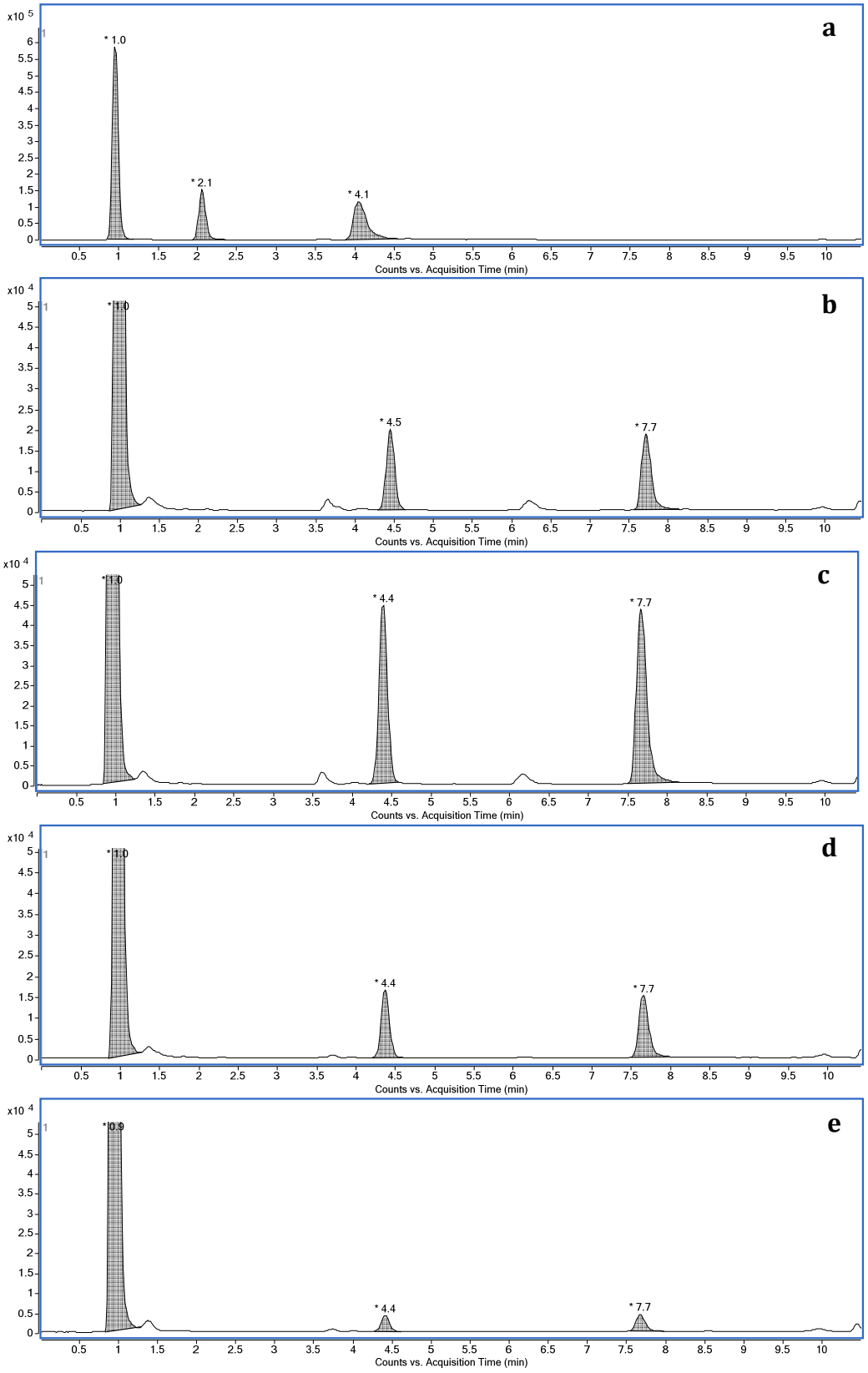


Figure 4-9: TIC chromatograms showing the major analytes detected in (a) the authentic specimen, (b) the authentic specimen spiked with 0.05 M KNO_2, (c) the authentic specimen spiked with 0.05 M KNO_2 (pH 3), (d) the authentic specimen spiked with 0.6 M KNO_2, and (e) the authentic specimen spiked with 0.6 M KNO_2 (pH 3). Retention times are: morphine = 4.1 min, 2-nitro-morphine = 7.7 min, M6G = 2.1 min, 2-nitro-M6G = 4.4-4.5 min and M3G = 1.0 min.

4.5.6 Preliminary stability assessment of the nitrated opiate species upon storage

A 12 day time course study of 2-nitro-morphine and 2-nitro-M6G was conducted using the MRM method; the results are summarised in Table 4-3.

Four samples were monitored, and consisted of morphine and M6G each exposed to 0.05 M and 0.6 M KNO_2 in urine (pH 3). The samples were refrigerated at 4℃ when not undergoing analysis. Injections were performed at 25 min, 10 and 19 h, and 1, 3, 8 and 12 days after commencement of the reaction. It was discovered that for all reactions, no morphine or M6G starting materials could be detected at 25 min. However, the respective reaction products were detectable in all samples. In the samples containing 0.6 M KNO_2, 2-nitro-morphine and 2-nitro-M6G were not detectable at day 12 after reaction commencement. On the eighth day, the percentage loss of 2-nitro-morphine and 2-nitro-M6G was found to be approximately 96% and 98%, respectively. This was estimated based on comparison of analyte peak areas at 25 min (highest analyte abundance for both 2-nitro-morphine and 2-nitro-M6G detected during the study period) and at eight days. In contrast, 2-nitro-morphine and 2-nitro-M6G in samples containing 0.05 M KNO_2 could still be detected 12 days after reaction commencement. At the end of the 12 day monitoring period, only losses of approximately 23% for 2-nitro-morphine and 32% for 2-nitro-M6G were observed in these samples. As expected, the absolute peak area responses of the same reaction products exposed to 0.05 M KNO_2 was larger when compared to reaction products exposed to 0.6 M KNO_2. The results suggest that an excess quantity of KNO_2 destroys 2-nitro-morphine and 2-nitro-M6G formed in urine.

Table 4-3: A summary of the analytes present in each urine sample monitored during the 12 day time course study.

time elapsed since start of the reaction	sample							
	morphine + 0.05 M KNO_2		morphine + 0.6 M KNO_2		M6G + 0.05 M KNO_2		M6G + 0.6 M KNO_2	
	morphine	2-nitro-morphine	morphine	2-nitro-morphine	M6G	2-nitro-M6G	M6G	2-nitro-M6G
25 min	×	✓	×	✓	×	✓	×	✓
10 h	×	✓	×	✓	×	✓	×	✓
19 h	×	✓	×	✓	×	✓	×	✓
1 day	×	✓	×	✓	×	✓	×	✓
3 days	×	✓	×	✓	×	✓	×	✓
8 days	×	✓	×	✓	×	✓	×	✓
12 days	×	✓	×	×	×	✓	×	×

Note: Relative to the product abundance at 25 min, the percentage losses were estimated at 96% (2-nitro-morphine) and 98% (2-nitro-M6G) with 0.6 M nitrite on day 8, and at 23% (2-nitro-morphine) and 32% (2-nitro-M6G) with 0.05 M nitrite on day 12.

4.5.7 Enzymatic hydrolysis studies

Enzymatic hydrolysis studies consisted of preparing two identical urine sample sets, each containing morphine, M3G and M6G standards, the corresponding reaction mixtures with 0.05 M and 0.6 M KNO_2 at pH 3, in addition to the urine matrix blank and reagent controls. One set was subjected to hydrolysis using *β*-glucuronidase, with the parallel set undergoing the same incubation and extraction procedures without addition of the enzyme. This latter set was used as the unhydrolysed controls, so that any observed changes due to the heating and extraction methods could be monitored and taken into account when assessing the results of the hydrolysed samples.

LC-MS/MS analysis confirmed the effectiveness of the hydrolysis protocol employed. A complete conversion of M3G and M6G into morphine and 2-nitro-morphine-6-glucuronide into 2-nitro-morphine was demonstrated by the disappearance of the glucuronides and the appearance of morphine and 2-nitro-morphine in the hydrolysed samples.

It was interesting to note that 2-nitro-morphine was detected in the samples containing M3G and KNO_2 following enzymatic hydrolysis. While M3G does not undergo reaction with KNO_2 readily due to the presence of the glucuronide group at the C-3 position, enzymatic cleavage of the glucuronide group yields free morphine which can undergo reaction with KNO_2 present in the hydrolysates, leading to the formation of 2-nitro-morphine. The extent of nitration in the sample containing 0.05 M KNO_2 was significantly less in comparison to the sample with 0.6 M KNO_2. Comparing the absolute peak areas of morphine in the hydrolysed reaction mixtures to the hydrolysed M3G standard without nitrite exposure, it was observed that less than 1% of morphine was lost in the sample with 0.05 M KNO_2. 2-Nitro-morphine was detectable, with the predominant peak identified as morphine. On the contrary, the absolute peak intensity of 2-nitro-morphine was four times greater than morphine in the sample containing 0.6 M KNO_2. The results suggested that KNO_2 is capable of nitrating morphine that has been released during enzymatic

hydrolysis of the morphine glucuronides. The reaction was positively correlated to the oxidant concentration used.

It was also worth noting that the incubation period contributed to the hydrolysis of the glucuronides to a small degree. Morphine was found in the unhydrolysed but incubated M3G and M6G standards. Although M3G and M6G remained the predominate analytes in these samples, morphine was detectable at approximately 2% of the absolute peak area response compared to the morphine response in the hydrolysed M3G and M6G samples. Furthermore, 2-nitro-morphine was detectable in the unhydrolysed but incubated reaction mixtures containing M3G and KNO_2. In terms of abundance, morphine and 2-nitro-morphine were at trace levels while M3G was prominent. A likely scenario for this observation would be the production of morphine from thermal degradation of M3G, which further reacts with KNO_2 present in the hydrolysate to form 2-nitro-morphine. Thermal degradation is also a plausible explanation for the detection of 2-nitro-morphine in the unhydrolysed but incubated samples containing M6G and KNO_2, where 2-nitro-M6G was formed. Heat may have caused the glucuronide cleaving at the C-6 position of 2-nitro-M6G, resulting in the formation of 2-nitro-morphine. However, the extent of hydrolysis by thermal degradation is so small (< 2% in any case) that it should not affect the interpretation of results attributed by the enzymatic hydrolysis procedure.

4.5.8 GC-MS studies

Given the popularity of GC-MS used by many drug testing laboratories in detecting opiates in urine, it is beneficial to determine if GC-MS is a viable technique for the detection of 2-nitro-morphine. Under the conditions employed, it was found that the TMS derivative of 2-nitro-morphine could be detected at 12.1 min, with characteristic and prominent product ions at *m/z* 474, 459 and 281 (Figure 4-10). The retention time and fragmentation pattern data was distinguishable from the starting material morphine (as a TMS derivative), which eluted at 10.0 min with prominent product ions at *m/z* 429, 414 and 287 (Figure 4-11).

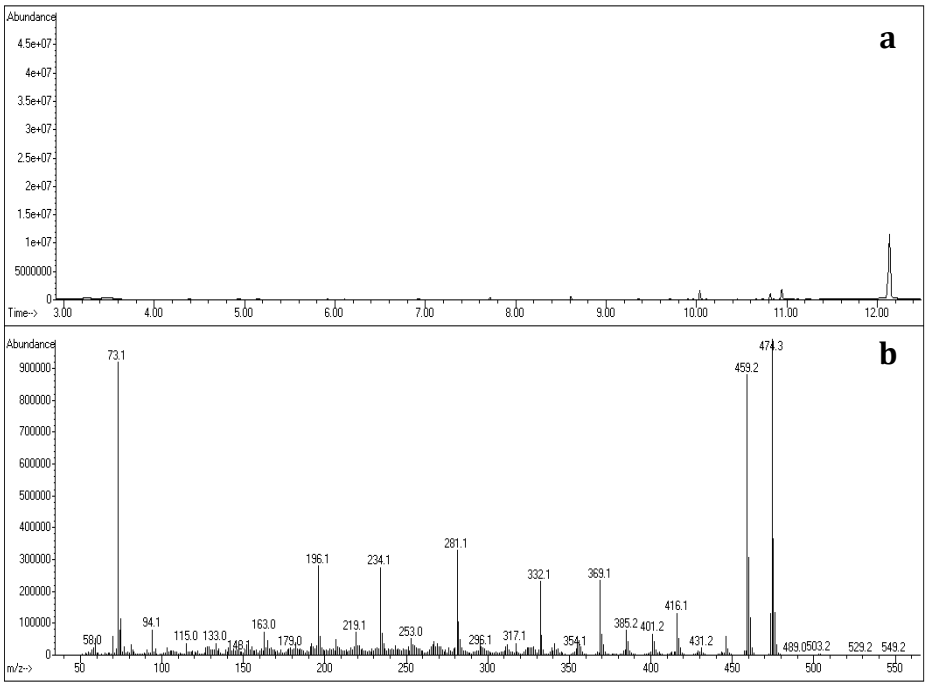


Figure 4-10: (a) GC trace and (b) MS fragmentation pattern of the TMS derivative of 2-nitro-morphine, in scan mode.

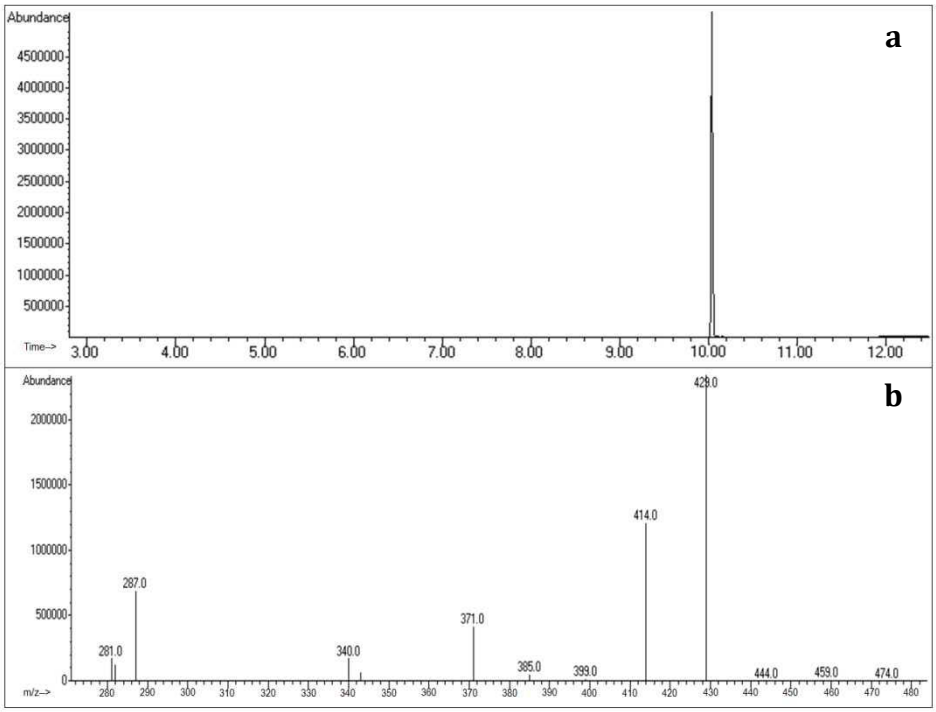


Figure 4-11: (a) GC trace and (b) MS fragmentation pattern of the TMS derivative of morphine, in scan mode.

4.6 Conclusions

Exposure of morphine and M6G to KNO_2 in urine resulted in the formation of two major reaction products, 2-nitro-morphine and 2-nitro-M6G, respectively. Both compounds were found to be detectable within the time frame required for urinalysis. Both reaction products are not naturally produced in the body or formed as by-products during opiate synthesis. Significant conversions from morphine and M6G to the reaction products were observed under relatively broad acidic pH and oxidant concentration conditions. Overall, it did not appear that the enzymatic hydrolysis procedure had detrimental effects on the detection of 2-nitro-morphine. On the contrary, it may potentially enhance the abundance of 2-nitro-morphine in the sample, by hydrolysing M3G and allowing nitration to occur. However, it is important to mention that there is a probability that laboratories conducting acid hydrolysis may inadvertently destroy 2-nitro-morphine and 2-nitro-M6G (and other drug product markers) due to the harsh hydrolysis conditions.

It can be concluded that 2-nitro-morphine and 2-nitro-M6G have the potential for proving the act of nitrite adulteration, and establishing that morphine and M6G were initially present in the urine specimen.

Chapter 5: Transformation of codeine and codeine-6-glucuronide to opioid analogues by urinary adulteration with pyridinium chlorochromate

Chapter 5: Transformation of codeine and codeine-6-glucuronide to opioid analogues by urinary adulteration with pyridinium chlorochromate

5.1 Introduction

In the human body, codeine is metabolised in the liver by P450 CYP2D6 enzymes to form morphine. The *N*-demethylation of codeine facilitated by CYP3A4 enzymes to produce norcodeine is also reported. However, conjugation of both the parent drug and the metabolites as the glucuronide remains a significant metabolic pathway. After an oral dose, 80-90% is excreted in urine as codeine or codeine-6-glucuronide (C6G); within this 80-90%, approximately 10% is codeine and 90% is C6G [47, 61].

The objective of this study was to determine the effect of PCC adulteration on codeine and C6G in urine. It has been reported that PCC contains the hexavalent chromium (Cr^{6+}) which significantly oxidises THC-COOH in urine [101, 102, 108]. On the contrary, it has also been suggested that the mechanism of interference by PCC appeared to be through the decrease of pH levels of the urine specimens, and not by chemically altering the target drug analyte [100]. Thus, the aims of our study were to expose codeine and C6G to PCC in urine and monitor the specimens over time. LC-MS allowed any stable reaction products that may be formed to be identified. Structural elucidation was also complemented by NMR spectroscopy when possible. The use of reaction products as a means for indirectly monitoring the presence of codeine and C6G in urine adulterated with PCC was also preliminarily assessed.

5.2 Materials

5.2.1 Drug standards and reagents

Codeine free base (1 mg/mL in methanol), codeine-6-β-D-glucuronide (1 mg/mL in acetonitrile/water 1:1), oxycodone hydrochloride monohydrate (1 mg/mL in methanol) and ethylmorphine (1 mg/mL in methanol) were sourced from Lipomed (Arlesheim, Switzerland). Codeine hydrogen phosphate solid was obtained from Macfarlan Smith Limited (Edinburgh, United Kingdom) and 6-O-methylcodeine solid was sourced from the National Measurement Institute (North Ryde, NSW, Australia).

PCC and oxalyl chloride were sourced from Sigma Aldrich (Castle Hill, NSW, Australia). Ammonium formate, acetic acid and $CDCl_3$ were sourced from Sigma Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile was purchased from Honeywell (Muskegon, MI, USA). Sodium acetate was obtained from Merck (Darmstadt, Germany). Disodium hydrogen orthophosphate, sodium dihydrogen orthophosphate, sodium hydrogen carbonate and sodium carbonate were sourced from Ajax Chemicals (Sydney, NSW, Australia).

The carbonate/bicarbonate buffer (pH 9.5, 1.5 M) was prepared by dissolving 63.6 g sodium carbonate and 75.6 g sodium hydrogen carbonate in water, and made up to a 1 L volume.

5.2.2 Urine specimens

Urine from healthy individuals were collected using polypropylene urine specimen containers and pooled (n=4) to create a representative blank urine matrix. Volunteers were selected randomly and had highly variable diets, both male and female, aged between 25-60 and from different ethnic backgrounds. The imposed condition was that they had not taken pain medication or eaten poppy seeds. The same combination of donors was not used for more than one experiment. Pooled urine was used on the day of voiding, and analysed

using the LC-MS methods developed in this study to ensure that it was negative for opiates prior to use.

Authentic urine specimens testing positive for codeine and C6G were supplied by the Drug Toxicology Unit, NSW Forensic and Analytical Science Service after removal of sample identification. The specimens were stored in a freezer at -18℃ before analysis.

5.3 Instrumentation

All samples were analysed using a 1290 LC system coupled to a 6490 QQQ-MS or a 6510 QTOF-MS for high resolution mass measurement. NMR data were recorded on a 500/54 premium shielded NMR spectrometer paired with a 7510-AS autosampler. These instruments were from Agilent Technologies (Forest Hill, VIC, Australia). The instrumental parameters are detailed in section 5.3.1 (LC-MS) and section 5.3.2 (NMR).

5.3.1 LC-MS

An ESI interface was utilised for LC-MS analysis. Analyte separation was achieved by injecting 1 µL of the sample onto an Agilent Zorbax Eclipse XDB-C18 column (2.1 mm × 50 mm × 1.8 µm), set at 40℃ with a 0.25 mL/min flow rate. Mobile phase A consisted of ammonium formate (pH 6.3, 20 mM), and mobile phase B was 95% acetonitrile in water. Mobile phase A was diluted from a 2 M ammonium formate stock solution that was prepared by dissolving ammonium formate solid in water. Each analysis had a starting gradient of 2% B, which was increased to 5% at six min, 30 % at 12 min, 70% at 17 min and 95% at 19 min; this gradient was held until 21 min was reached, and then decreased to 2% at 21.1 min. A four min post-run column equilibration at 2% B was the final step to ensure that the column was conditioned for the next analysis. Full scan MS (scan time = 500 ms) and product ion scan MS/MS analyses (scan time = 150 ms) were performed in positive ion mode. The protonated molecule of an analyte was used as the precursor ion for MS/MS experiment. For QQQ-MS, the default fragmentor voltage (380 V) was used,

with a collision energy range of 25-45 eV. The sheath gas temperature and flow were 250℃ and 11 L/min, respectively. For the QTOF-MS, Targeted MS/MS was carried out (scan time = 200-500 ms) with the fragmentor and collision energy ranges falling within 150-250 V and 20-40 eV, respectively. Mass correction was carried out using *m/z* 121.0509 and *m/z* 922.0098 reference ions. The gas temperature and flow were maintained at 200℃ and 14 L/min, respectively. The capillary and nozzle voltages were adjusted to 3000 V and 1500 V, respectively.

5.3.2 NMR

^{1}H-NMR (1024 scans; 1 sec relaxation delay) and ^{13}C-NMR (10,000 scans; 1 sec relaxation delay) were performed.

5.4 Experimental procedures

5.4.1 Exposure of opiates to PCC

To monitor the effect of PCC on codeine and C6G in aqueous environments, a series of samples were prepared and monitored over time using LC QQQ-MS. A 1 M PCC working solution was prepared by dissolving solid PCC in water. Another four PCC working solutions were then prepared (200, 20, 2 and 0.2 mM) by serial dilution. Codeine and C6G were spiked into water (at 10 µg/mL in the final 1 mL sample) and adulterated with each of the PCC working solutions to give final oxidant concentrations of 100, 20, 2, 0.2 and 0.02 mM PCC in the samples (100 µL PCC working solution per 1 mL sample). For C6G, only PCC concentrations of 100 mM and 20 mM were trialled. The reactions were allowed to proceed for one hour at room temperature (22℃) prior to analysis, and then subsequently refrigerated at 4°C when not analysed. The samples were monitored at one hour, one day and one week, unless otherwise specified. A codeine standard (10 µg/mL), reagent controls (opiate negative), and a water blank were prepared and analysed alongside the adulterated samples. The pH readings were measured for all samples

(including the control samples) and recorded. A relatively high concentration of codeine and C6G was used in this study to facilitate easy monitoring of product formation.

In a parallel sample set, the codeine/C6G reactions with PCC were also replicated in blank urine. Samples were fortified with 100 mM and 20 mM PCC working solutions. The urine samples were centrifuged at 4500 *g* for 10 min and filtered through 0.22 µm hydrophilic syringe filter units (MicroAnalytix Pty Ltd, Taren Point, NSW, Australia) prior to analysis.

One authentic urine specimen positive for codeine and C6G was also adulterated with 100 mM and 20 mM PCC, and processed in the same manner as the other specimens. The samples were monitored at one hour, one day and five days after adulteration.

5.4.2 Preparation of reaction products

To produce enough material for structural elucidation of the reaction products present in the codeine and PCC reaction mixture by NMR, the reactant ratios detailed in section 5.4.1 were proportionally scaled up in water to accommodate 5.62 mg codeine with 20 mM PCC. The reaction mixture was left for several weeks at room temperature and monitored by LC QQQ-MS prior to extraction. Solid phase extraction was performed to isolate the reaction products, which were adsorbed onto Clean Screen® CSDAU extraction columns (United Chemical Technologies, Bristol, PA, USA) [85]. Anhydrous potassium carbonate was used to dry the combined final eluate fractions, before being dried down under a gentle stream of nitrogen at 30℃. The remaining residue was also placed in a vacuum desiccator overnight prior to reconstitution in 600 µL $CDCl_3$ and analysis by NMR spectroscopy.

In addition, a codeinone reference sample was synthesised via the Swern oxidation using a method adapted from Huang et al. [136]. Codeine hydrogen phosphate (30 mg) was converted to the free base form by dissolving the solid in water, basifying the solution to pH 9.5 with carbonate/bicarbonate buffer and

extracting the aqueous fraction with dichloromethane (DCM). The organic solvent was evaporated under nitrogen and the codeine residue (19 mg) was re-dissolved in anhydrous DCM. Codeinone was then synthesised from the codeine base. The reaction was conducted under a nitrogen atmosphere at -78℃ (dry ice/acetone cooling bath). A solution of oxalyl chloride (100 µL) in anhydrous DCM (580 µL) was added dropwise to a solution of dimethylsulfoxide (170 µL) in anhydrous DCM (905 µL) in the reaction vessel over 20 min, with continual stirring. The reaction mixture was further stirred at -78℃ for 1.5 hrs. This was followed by a dropwise a ddition of the codeine base solution over 15 min with continuous stirring extending over another two hours. Finally, triethylamine (56 µL) and dry DCM (112 µL) were added and the reaction mixture stirred for another 10 min before being warmed up to room temperature. The sample was washed with six equivalent volumes of water. The organic fraction was isolated and dried using sodium sulphate (refer to Figure A8 in the appendix for the proposed mechanism for the formation of codeinone via codeine through the Swern reaction, adapted from Parashar [137]).

LC-MS analysis was carried out on this sample, in addition to the samples prepared in section 5.4.1 to further aid structural elucidation of the reaction products.

5.4.3 Immunoassay and GC-MS study

A batch of urine specimens consisting of two opiate negative blank samples and six opiate positive samples were adulterated with 100 mM and 20 mM PCC and left to react for 16 h (overnight) at 22℃. Each specimen was divided into two aliquots; the first aliquot was sent to the Drug Toxicology Unit for immunoassay screening and GC-MS confirmatory testing. The CEDIA® Opiate immunoassay (Microgenics Corp., Fremont, CA, USA) was performed on an Olympus AU 2700 analyser (Olympus America Inc., Melville, NY, USA). The GC-MS confirmatory testing was performed using an in-house validated method that involved enzymatic hydrolysis by β-glucuronidase, extraction on Clean Screen® CSDAU columns, derivatisation of the extract by BSTFA

containing 1% TMCS, and MS analysis in SIM mode. Quantification was based on the use of internal standards codeine-d_6 and morphine-d_6. The second aliquot was concurrently analysed on the LC-MS instrument using the conditions detailed in section 5.3.1.

5.5 Results and discussion

5.5.1 Exposure of codeine and C6G to PCC

Codeine and C6G samples prepared in water and urine were spiked with PCC at various concentrations to mimic adulteration conditions. The oxidant working solution concentrations were chosen based on the PCC concentration found in the commercial product 'Urine Luck', which has been reported to contain 200 mM PCC [138]. In a real life situation, the exact number of vials used is arbitrary and amount of urine voided is highly variable; therefore a concentration range was trialled. During each LC-MS analysis conducted in the exposure studies, fresh codeine/C6G standards and reagent controls were successively analysed with the adulterated specimens. This established that significant decreases in codeine and C6G peak area abundances were attributed to reaction progression, and not starting material degradation. The reagent controls ensured that any reagent peaks could be distinguished from peaks belonging to potential reaction products. Additionally, post-column infusion experiments did not indicate that PCC in the urine contributed any additional matrix effects in the analyte regions of interest (approximately 6-12 min) when compared to urine alone.

5.5.1.1 Reaction of codeine and PCC in water and in urine

The exposure of codeine ($[M+H]^+$ at *m/z* 300) to PCC in both water and urine resulted in the formation of multiple reaction products: one major product with $[M+H]^+$ at *m/z* 298 (product *m/z* 298), one minor product with $[M+H]^+$ at *m/z* 316 (product *m/z* 316) and another two minor products with $[M+H]^+$ at *m/z* 314 which is referred to as product *m/z* 314a and product *m/z* 314b in this study. It

appeared that the same four reaction products were detected in both water and in urine under the LC-MS conditions employed. However, their relative abundances appeared to be affected by three factors: the oxidant concentration, the type of aqueous reaction medium and the time elapsed since the codeine samples were adulterated with PCC. It was found that all four reaction products were still detected in the urine specimens one week subsequent to PCC adulteration, demonstrating that they are stable enough to be detected in the timeframe required for urinalysis. Furthermore, codeine is still detectable in the samples following PCC adulteration, albeit with a loss of abundance. This increases the difficulty in data interpretation of opiate test results. The observation of codeine and its analogues in the specimen may be an indication that the other reaction products may have originated from codeine modification.

A steady decrease in codeine concentration was observed in specimens fortified with PCC, with codeine detected at 9307 ng/mL (one hour), 4301 ng/mL (one day) and 1330 ng/mL (one week) in urine containing 20 mM PCC. This diminishment was more pronounced in urine containing 100 mM PCC, where the codeine concentration was detected at 7895 ng/mL (one hour) and 1985 ng/mL (one day). At the end of the one week monitoring period, the codeine concentration was detected at less than 100 ng/mL.

Figure 5-1 depicts the TIC chromatograms of the codeine urine samples adulterated with 20 mM and 100 mM PCC over one week. Under the LC-MS conditions employed, the retention time (R_t) for codeine is 9.2 min, with major product ions at *m/z* 153, 165 and 181. Product *m/z* 314a (R_t = 10.3 min) has major product ions at *m/z* 239, 254 and 296, with product *m/z* 314b (R_t = 10.8 min) possessing major product ions at *m/z* 152, 165 and 181. Product *m/z* 316 (R_t = 9.5 min) and product *m/z* 298 (R_t = 11.3 min) have characteristic product ions at *m/z* 171,185 and 199, and *m/z* 153, 165 and 181, respectively.

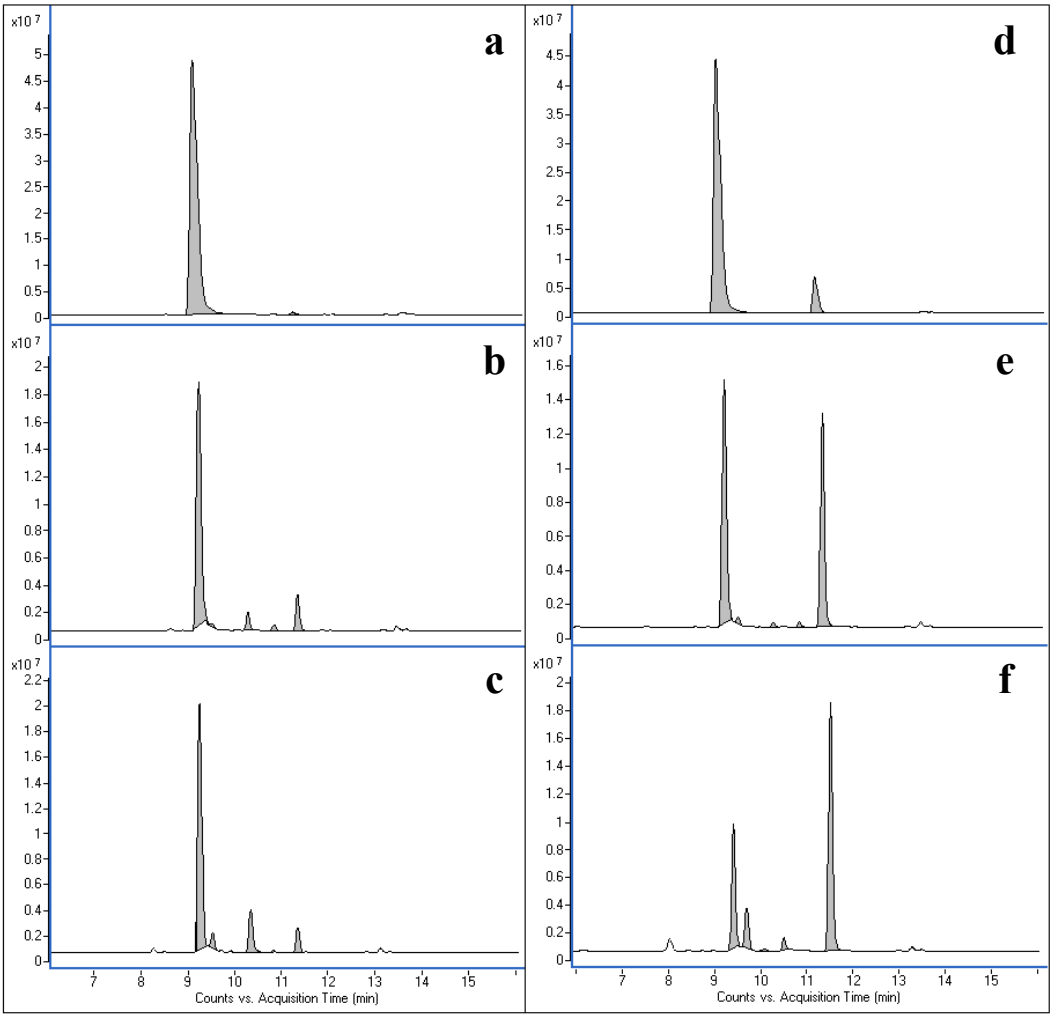


Figure 5-1: TIC chromatograms (product ion scan) of a codeine urine sample adulterated with 20 mM PCC (a) one hour, (b) one day and (c) one week after adulteration; and TIC chromatograms of a corresponding codeine urine sample adulterated with 100 mM PCC (d) one hour, (e) one day and (f) one week after adulteration (the peak at 8.1 min was also present in the control samples and therefore determined not to be a reaction product).

Nb: R_t for codeine is 9.2 min; R_t for products m/z 316, 314a, 314b and 298 are 9.5 min, 10.3 min, 10.8 min and 11.3 min, respectively.

Table 5-1 is a summary of the analytes that were detected in water and in urine over one week. Upon comparison of the codeine specimens adulterated with 20 mM PCC, it appears that the urine matrix facilitates the reaction pathway for the formation of product *m/z* 316 and product *m/z* 314a to a greater extent than water. This can be seen by the absence of both these products after one day of reaction, but present after one week, in water. In contrast, all four reaction products were detectable after one day and also one week in urine.

***Table 5-1:** Relative peak areas of analytes (normalised to the most abundant analyte (100%)) detected in the codeine adulterated samples after one hour, one day and one week elapsed since commencement of the reaction using LC-MS (product ion scan) analysis.*

	20 mM PCC					**100 mM PCC**				
	codeine	**product *m/z* 316**	**product *m/z* 314a**	**product *m/z* 314b**	**product *m/z* 298**	**codeine**	**product *m/z* 316**	**product *m/z* 314a**	**product *m/z* 314b**	**product *m/z* 298**
codeine in water										
1 h	100	-	-	-	1.11	100	-	-	-	25
1 day	100	-	-	2.34	38.17	15.1	2.85	-	0.61	100
1 week	83.21	5.3	0.25	2.02	100	-	22.06	1.07	-	100
codeine in urine										
1 h	100	-	-	-	0.63	100	-	-	-	10.78
1 day	100	0.06	5.72	1.99	11.55	100	1.58	1.68	1.93	79.55
1 week	100	4.98	15.86	0.87	8.39	48.65	14.08	0.77	4.45	100

The collision energy used for codeine is 40 eV. Collision energies used for products *m/z* 316, 314a, 314b and 298 are 40 eV, 25 eV, 25 eV and 40 eV, respectively. Reactions were allowed to proceed for one hour at room temperature (22°C) prior to analysis, and then subsequently refrigerated at 4°C when not analysed.

In terms of the effect of oxidant concentration on the formation of the major reaction product, the data suggested that higher PCC concentrations encouraged the formation of product *m/z* 298, relative to the lower concentrations trialled. This was evidenced by the relative peak areas of codeine to product *m/z* 298 at the same time period, when comparing the two different PCC concentrations (Table 5-1). This observation was further demonstrated with samples fortified with lower PCC concentrations (0.2-20 mM). In a simplified reaction system, it was expected that an increase in substrate (PCC) concentration would result in an increased rate of formation of product *m/z* 298.

Due to the numerous pathways of oxidation by PCC, it is quite difficult to determine the effect of the reaction matrix on the rates of the reactions, which is out of the scope of this study. However, the findings of the exposure studies suggested that formation of product *m/z* 298 was more favoured in water compared to urine. With adulteration using 20 mM PCC and sample monitoring after one day, abundances of codeine (7612 ng/mL and 4301 ng/mL in water and urine, respectively) were approximately three and nine times greater than product *m/z* 298, in water and in urine, respectively. After one week, the abundance of product *m/z* 298 became 1.2 times greater than codeine itself (2044 ng/mL) in water. On the other hand, codeine (1330 ng/mL) remained the predominant analyte in urine after one week, approximately 12 times greater than product *m/z* 298 (Table 5-1). The same trend was observed with samples adulterated with 100 mM PCC. This suggested that the endogenous compounds in urine were competing against codeine for reaction with PCC.

Additional information can be derived from monitoring all four of the reaction products of interest in the codeine specimens adulterated with 100 mM PCC. In general, it was apparent that the PCC was exerting its oxidising capabilities, with the depletion of the codeine starting material and formation of reaction products detected over time. The buffering capacity of the urine matrix appeared to stabilise the reaction products, which were all detected one day and one week after adulteration (Figure 5-1d-1f, Table 5-1).

A different scenario was observed in the corresponding specimen with water as the reaction matrix. After one day, the codeine peak area was seen to have significantly decreased (less than 100 ng/mL), with product *m/z* 298 being the major analyte in the sample (approximately seven times greater than codeine, Table 5-1). After one week, codeine was found to be undetectable under the conditions of analysis. It is important to note that product *m/z* 314a was not detectable in this specimen after one day, but was detectable after one week; the contrary was observed for product *m/z* 314b. These findings support the idea that urine possesses a buffering role in the multiple oxidation pathways of codeine, and stabilises the reaction products in the sample to some degree.

Since LC-MS monitoring showed that the same four reaction products were formed in both water and urine matrices, codeine fortified water samples were adulterated with PCC at a lower concentration range (0.02-2 mM). These experiments attempted to provide some insight on the oxidation route at lower concentrations of PCC, however still keeping in mind that the abundances of the products formed may be affected by the reaction matrix used. The reactions were monitored one hour, two days and 16 days after adulteration. It appeared that a pronounced trend was followed regardless of the three different PCC concentrations trialled. In general, only two of the four reaction products (product *m/z* 314b and *m/z* 298) detected in the exposure studies were found to have formed; no additional reaction products were detected. Product *m/z* 314b appeared to be formed readily, supported by its detection at all PCC concentrations trialled. It was the only reaction product detected in the sample adulterated with 0.02 mM PCC throughout the duration of the study. Product *m/z* 298 was detectable in the samples adulterated with 0.2 and 2 mM PCC during the monitoring period, with the exception of one hour after adulteration with 0.2 mM PCC. It was evident that the higher the PCC concentration or the longer the elapsed time since adulteration, the more likely the detection of product *m/z* 298. Product *m/z* 314b remained a minor analyte relative to codeine in the sample, whereas product *m/z* 298 became a major product. These observations were expected and consistent with the findings shown in Table 5-1. It can be inferred that the formation of product *m/z* 314a

and product *m/z* 316 may require a longer duration of time for the reaction to proceed, or a higher PCC concentration to be present in the sample.

The pH measurements of both the water and urine samples (codeine and reagent control samples) spiked with PCC demonstrated the acidifying effect of the oxidant; the higher the PCC concentration, the lower the pH. The addition of 20-100 mM PCC to urine specimens with pH 6 resulted in an increase in acidity of pH 4-5. With the corresponding water set, the pH dropped from pH 7 to pH 2 as expected, due to the absence of buffering capacity. It is possible that the oxidation process is facilitated by pH, and may account for the differences in the relative abundances of the reaction products observed in urine and water.

5.5.1.2 Reaction of C6G and PCC in water and in urine

Initially, the study involved the monitoring of reaction mixtures containing C6G and PCC in water. The samples were monitored over a one week period. It was found that the exposure of C6G to both concentrations of PCC in this matrix resulted in the formation of three reaction products of interest; codeine, product *m/z* 298 (same as the reaction product yielded from the codeine reaction with PCC) and a new analyte with a protonated molecule of *m/z* 416 (product *m/z* 416, R_t = 10.1 min). Analysis of the parallel urine sample set revealed that these reaction products were also forming in urine (Figure 5-2). Due to the detection of product *m/z* 298, the samples were also monitored for the minor reaction products (products *m/z* 314a, 314b and 316) which yielded from the reaction between codeine and PCC. Surprisingly, these analytes were not formed upon reaction of C6G with PCC in both water and in urine over this monitoring period.

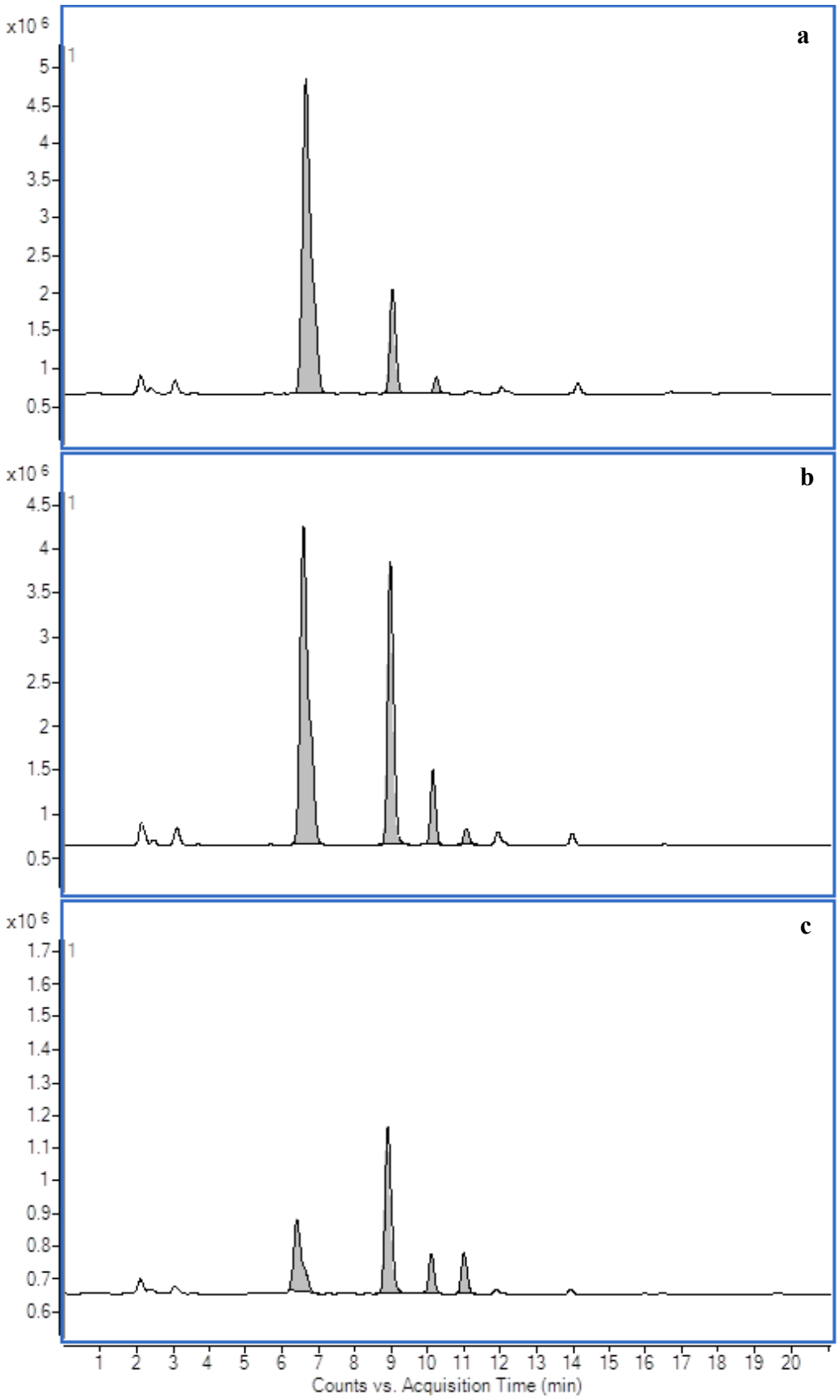


Figure 5-2: TIC chromatograms (product ion scan) of the C6G urine sample adulterated with 100 mM PCC (a) one hour, (b) one day and (c) one week after adulteration. Nb: R_t for C6G is 6.5 min; Rt for codeine and products m/z 416 and m/z 298 are 9.2 min, 10.1 min and 11.3 min, respectively.

Over the monitoring period, C6G concentration was found to decrease from 8468 ng/mL (one hour) to 5299 ng/mL (one week) following 100 mM PCC exposure in urine. Exposure of C6G to 20 mM PCC in urine resulted in a less marked decrease in analyte abundance, with C6G concentration at 9063 ng/mL (one day) and 8999 ng/mL (one week). The trends observed for reaction progression with C6G and PCC were parallel to those that were observed for codeine and PCC. It was apparent that the reaction between C6G and PCC does not go to completion in urine. Although the abundance of C6G does show a decrease over time in both water and urine, it was detectable at all time points of analysis, under the LC-MS conditions employed. On the other hand, the abundance of codeine was observed to increase within the first day after PCC adulteration in both aqueous environments. Therefore, it is quite possible that codeine is being formed via deconjugation of C6G by the PCC adulterant (relative peak area of free codeine in the C6G standard is < 3%). Based on the relative abundances of C6G and codeine shown in Table 5-2, cleavage of the C6G glucuronic acid functional group appeared to be the favoured route of reaction.

Table 5-2: Relative peak areas of analytes (normalised to the most abundant analyte (100%)) detected in the C6G adulterated samples after one hour, one day and one week elapsed since commencement of the reaction using LC-MS (product ion scan) analysis.

	20 mM PCC				**100 mM PCC**			
	C6G	**codeine**	**product *m/z* 416**	**product *m/z* 298**	**C6G**	**codeine**	**product *m/z* 416**	**product *m/z* 298**
C6G in water								
1 h	100	2.62	-	-	100	2.30	-	0.19
1 day	100	6.38	-	-	100	3.66	-	2.77
1 week	100	54.67	4.56	4.71	17.70	-	-	100
C6G in urine								
1 h	100	3.46	-	-	100	20.88	3.23	-
1 day	100	5.43	-	-	100	61.85	13.83	3.22
1 week	100	6.08	-	-	60.17	100	20.41	23.45

The collision energies used for C6G and codeine were 45 eV and 40 eV, respectively. The collision energy used for products *m/z* 416 and 298 was 40 eV. Reactions were allowed to proceed for one hour at room temperature (22°C) prior to analysis, and then subsequently refrigerated at 4°C when not analysed.

In general, an increase in PCC concentration and/or time was also found to contribute to the formation of product *m/z* 416 and product *m/z* 298. A comparison of the relative analyte abundances in the C6G urine specimens spiked with 20 mM PCC and 100 mM PCC showed that product *m/z* 416 and product *m/z* 298 were present in the latter specimen only. Upon adulteration with 100 mM PCC, product *m/z* 416 was formed within the first hour after adulteration; product *m/z* 298 was detectable one day after adulteration. This was also observed with the corresponding water specimens, whereby product *m/z* 298 was detectable one week in the specimen adulterated with 20 mM PCC, compared to one hour in the specimen adulterated with 100 mM PCC. Codeine and products *m/z* 416 and *m/z* 298 appeared to be more stable in urine than in water.

5.5.1.3 PCC adulteration of an authentic specimen

Exposure of an authentic specimen positive for codeine (235 ng/mL) and C6G (4880 ng/mL) to both concentrations of PCC resulted in the formation of products *m/z* 298 and *m/z* 416 within one hour. These reaction products were still found in the sample one day and five days subsequent to adulteration, in addition to codeine and C6G (Figure 5-3). It was also noted that the addition of 100 mM PCC did not appear to significantly alter the pH of the sample (pH 4-5). Likewise, the pH of the authentic specimen adulterated with 20 mM PCC did not cause a substantial pH change in the sample (pH 5-6), and yielded formation of products *m/z* 298 and *m/z* 314b within one day. These reaction products could still be observed in the sample on the fifth day of monitoring, in addition to product *m/z* 314a, codeine and C6G. The overall results were consistent with those obtained during previous exposure studies with spiked samples.

This study served as a proof of concept that addition of PCC to authentic codeine/C6G positive urine specimens does alter the codeine and C6G abundances, and also produces reaction products that may be used as markers for proving the act of adulteration with this oxidant.

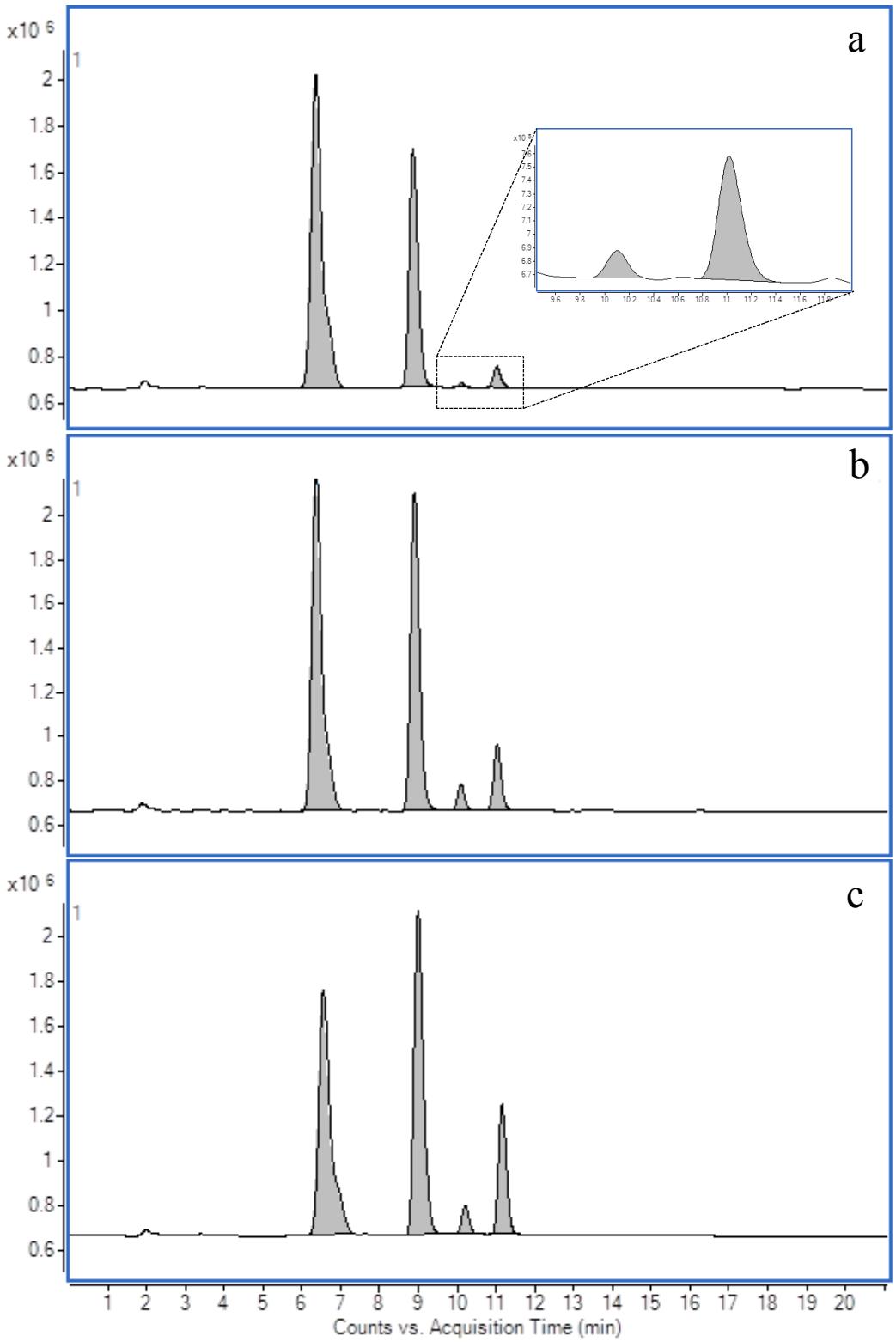


Figure 5-3: TIC chromatograms (product ion scan) of the authentic urine specimen adulterated with 100 mM PCC (a) one hour, (b) one day and (c) five days after adulteration. Nb: R_t for C6G, codeine and products m/z 416 and m/z 298 are 6.4 min, 9.0 min, 10.1 min and 11.1 min, respectively.

5.5.1.4 Immunoassay and GC-MS study

Following the analysis of 24 urine specimens (Table 5-3), immunoassay screening indicated that PCC consistently decreased the response of the CEDIA® Opiate assay. Both PCC concentrations caused a reduction in response when compared to the original specimen without PCC fortification. A greater decline in response was caused by the presence of 100 mM PCC when compared to 20 mM PCC. This observation was also reflected in the immunoassay readings for the blank specimens. All patient samples positive for opiates remained positive following PCC adulteration (with a 300 ng/mL cut-off concentration).

Morphine/codeine ratios are conventionally used to aid determination of heroin, morphine or codeine use of a test subject. However, such ratios are highly unreliable due to individual variations in metabolising these drugs and are subject to debate [139-144]. In this study, GC-MS analysis showed that PCC adulteration caused the morphine/codeine ratios to change in a sporadic manner (Table 5-4). This was partly due to the significant loss of morphine-d_6 and codeine–d_6 internal standards observed in the presence of PCC (Figure 5-4). LC-MS analysis of these specimens did indicate the presence of products *m/z* 298, 314a, 316 and 416, in addition to codeine and C6G.

Table 5-3: Immunoassay screening results for the unmodified blank (B1, B2) and opiate positive (U1-U6) urine specimens and the corresponding adulterated specimens with 20 mM and 100 mM PCC.

Sample	Opiate concentration (ng/mL)
B1	-10
B1 + 20 mM PCC	-30
B1+ 100 mM PCC	-80
B2	0
B2 + 20 mM PCC	-30
B2 + 100 mM PCC	-90
U1	2640[a]
U1 + 20 mM PCC	2380 [a]
U1 + 100 mM PCC	1400 [a]
U2	2700 [a]
U2 + 20 mM PCC	2440 [a]
U2 + 100 mM PCC	1100 [a]
U3	2650 [a]
U3 + 20 mM PCC	2260 [a]
U3 + 100 mM PCC	610 [a]
U4	2700 [a]
U4 + 20 mM PCC	2300 [a]
U4 + 100 mM PCC	1320 [a]
U5	2460 [a]
U5 + 20 mM PCC	2330 [a]
U5 + 100 mM PCC	1430 [a]
U6	2570 [a]
U6 + 20 mM PCC	2280 [a]
U6 + 100 mM PCC	1390 [a]

[a]above opiate screening cut-off concentration of 300 ng/mL.

Table 5-4: GC-MS confirmatory results for the unmodified blank (B1, B2) and opiate positive (U1-U6) urine specimens and the corresponding adulterated specimens with 20 mM and 100 mM PCC.

sample	morphine concentration (ng/mL)	codeine concentration (ng/mL	morphine/codeine ratio
B1	0	0	n/a[a]
B1 + 20 mM PCC	0	0	n/a[a]
B1 + 100 mM PCC	0	0	n/a[a]
B2	0	0	n/a[a]
B2 + 20 mM PCC	0	0	n/a[a]
B2 + 100 mM PCC	0	0	n/a[a]
U1	15443	1437	10.75
U1 + 20 mM PCC	910	836	1.09
U1 + 100 mM PCC	0	588	n/a[a]
U2	22770	2889	7.88
U2 + 20 mM PCC	0	3447	n/a[a]
U2 + 100 mM PCC	0	309	n/a[a]
U3	8399	918	9.15
U3 + 20 mM PCC	0	2357	n/a[a]
U3 + 100 mM PCC	0	61	n/a[a]
U4	30070	16675	1.80
U4 + 20 mM PCC	29264	14708	1.99
U4 + 100 mM PCC	248	1431	0.17
U5	3795	22450	0.17
U5 + 20 mM PCC	3614	25570	0.14
U5 + 100 mM PCC	41	5045	0.01
U6	4068	23496	0.17
U6 + 20 mM PCC	3489	24150	0.14
U6 + 100 mM PCC	204	21902	0.01

[a]no ratio given as morphine concentration was not able to be calculated due to complete loss of morphine-d_6.

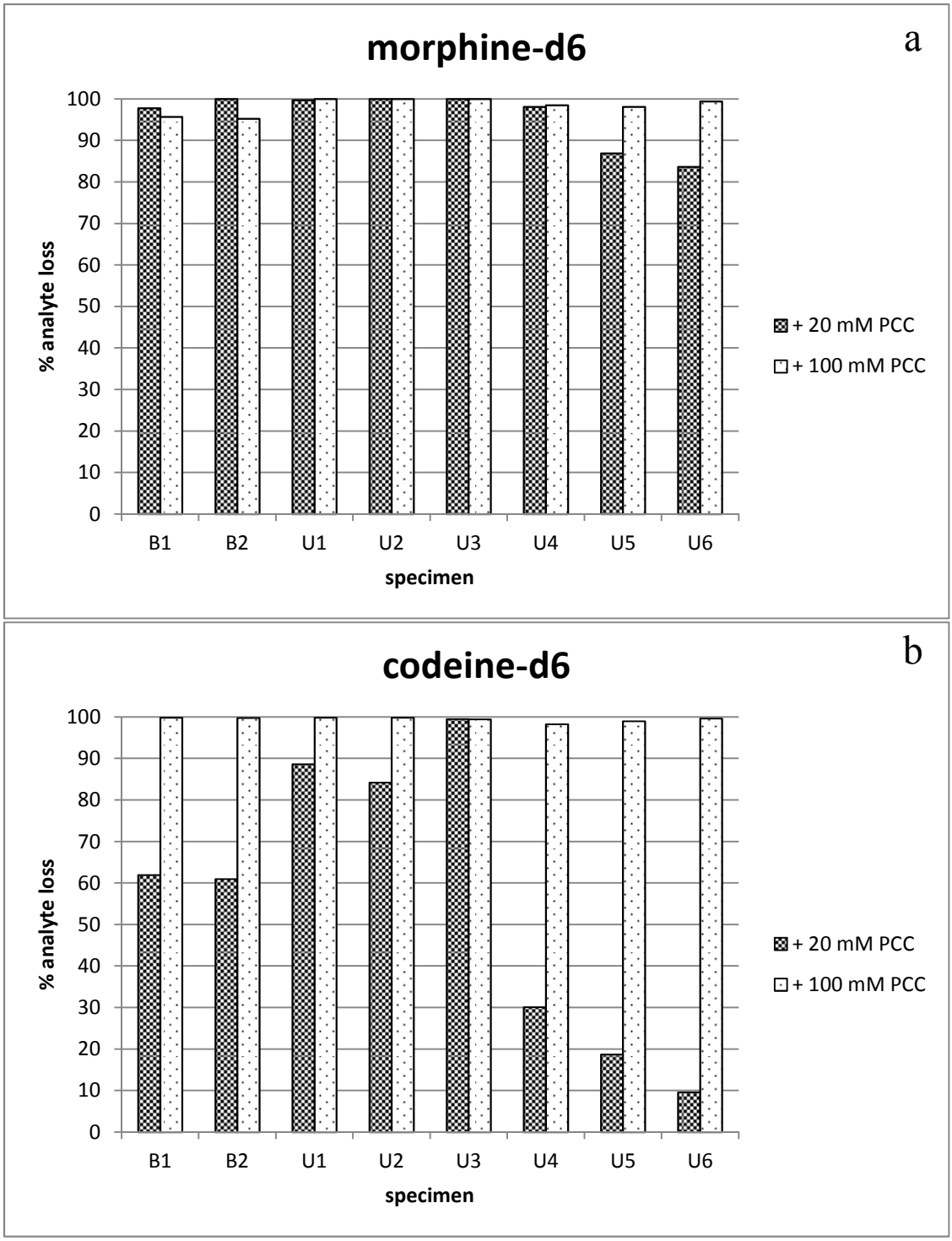


Figure 5-4: plot of (a) morphine-d_6 loss and (b) codeine-d_6 loss in two urine blank (B1, B2) specimens and six opiate positive (U1-U6) urines adulterated with PCC (analyte loss is expressed as a percentage relative to morphine-d_6 and codeine-d_6 abundance in the corresponding unadulterated specimen).

5.5.2 Structural elucidation of the reaction products

Structural elucidation of the reaction products of interest was based on high resolution MS data obtained from LC-MS analysis. In addition, NMR spectroscopy was also utilised to aid the identification of the major reaction product (product *m/z* 298). Various mechanisms of reactions documented in literature were also consulted to lend support to the molecular structures proposed for the reaction products.

5.5.2.1 Characterisation of product m/z 298

The most obvious difference observed between codeine and product *m/z* 298 was the loss of 2 Da in the latter product. One typical pathway for oxidation by PCC is through the conversion of primary and secondary alcohols to aldehydes and ketones, respectively [145, 146]. Since the structure of codeine contains an –OH functional group at the C-6 position (Figure 5-5a), it was hypothesised that product *m/z* 298 is codeinone (Figure 5-5b), an α, *β*-unsaturated ketone derivative of codeine. This was unambiguously confirmed by ^{1}H-NMR and ^{13}C-NMR analyses. Direct injection of the NMR sample prepared in section 5.4.2 into the LC QQQ-MS system showed that although codeine and the other reaction products were present, product *m/z 298* was the major analyte in the sample (Figure 5-6). Therefore, chemical shift signals of significant intensity were attributed to product *m/z* 298.

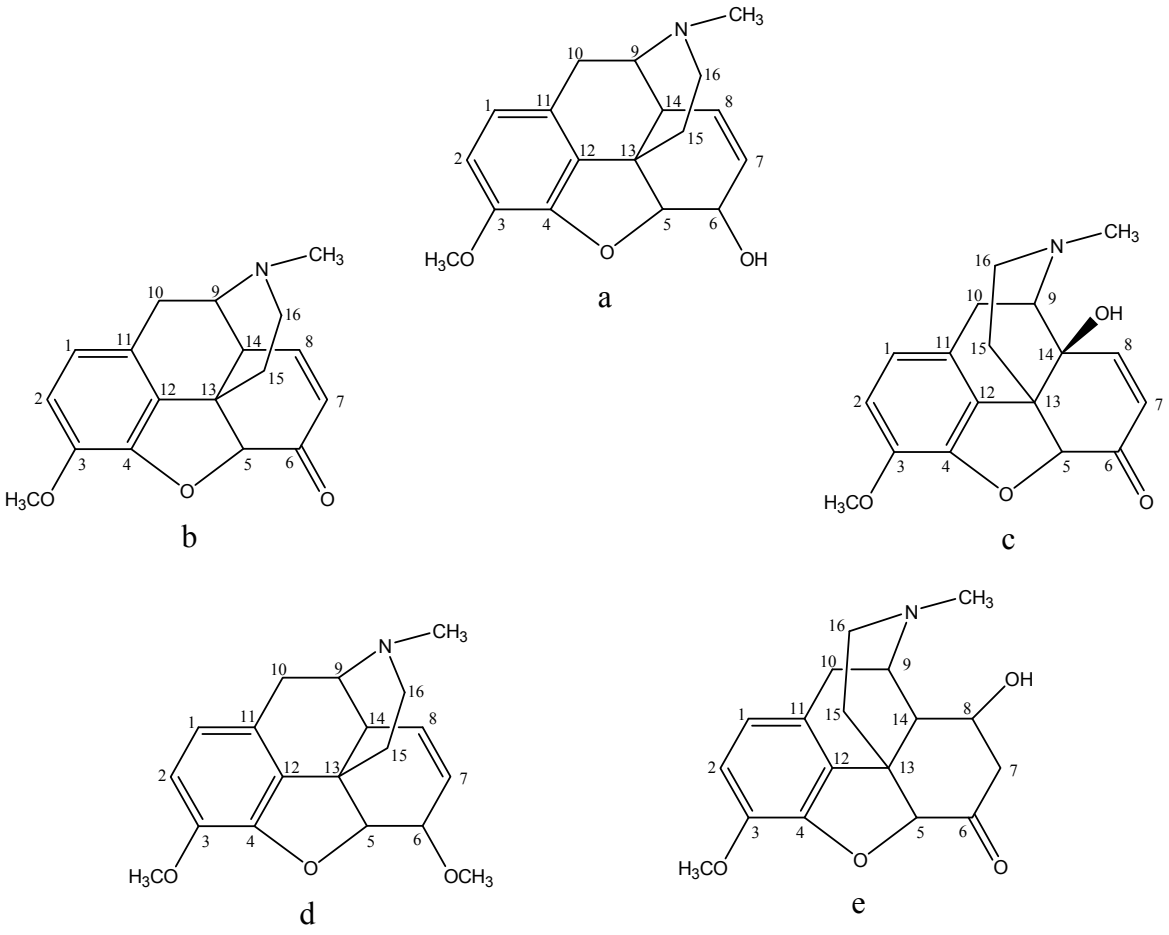


Figure 5-5: Molecular structures of (a) codeine, (b) codeinone, (c) 14-hydroxycodeinone (d) 6-O-methylcodeine and (e) 8-hydroxy-7,8-dihydrocodeinone.

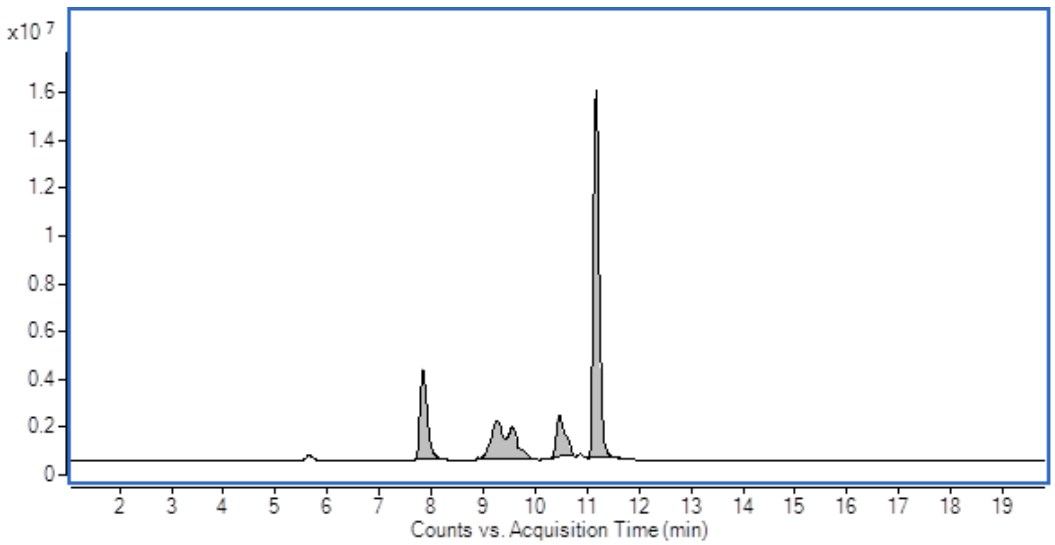


Figure 5-6: TIC chromatogram (product ion scan) of the NMR sample containing the extracted opiate derivatives in $CDCl_3$. Rt for codeine and products m/z 316, 314 (314a and 314b) and 298 are 9.2 min, 9.5 min, 10.6 min and 11.3 min, respectively.
Nb: The peak at 5.6 min is not an analyte of interest; however, the peak at 7.9 min also belongs to codeine.

The structural elucidation process for this compound was multifaceted. Firstly, it was expected that product *m/z* 298 shared the majority of its ^{1}H chemical shifts with the codeine starting material, except for the signals belonging to the H-5 to H-8 protons in product *m/z* 298. Comparison of the codeine ^{1}H-NMR chemical shifts to the chemical shifts belonging to product *m/z* 298 (Table 5-5) showed that this was the case.

Table 5-5: ^{1}H-NMR chemical shifts of literature codeine and codeinone compared to the codeine standard and product m/z 298 analysed in this study.

identification of proton[a]	literature ^{1}H chemical shifts for codeine[b] (ppm)	^{1}H chemicals shifts for the codeine standard (ppm)	literature ^{1}H chemical shifts for codeinone[c] (ppm)	^{1}H chemicals shifts for product *m/z* 298 (ppm)
1	6.57	6.57	6.59	6.58-6.71
2	6.66	6.66	6.67	6.58-6.71
3-OCH_3	3.84	3.84	3.85	3.86
5	4.90	4.89	4.68	4.65
6	4.19	4.18	n/a	n/a
OH	3.00	2.89	n/a	n/a
7	5.71	5.73	6.07	6.09
8	5.30	5.30	6.62	6.58-6.71
9	3.35	3.34	3.35-3.45	3.34
10_{α}	2.30	2.29	2.30	2.31
10_{β}	3.07	3.05	3.10	3.09
14	2.67	2.67	3.17-3.25	3.51
15_{a}	2.07	2.06	2.06	2.06
15_{e}	1.88	1.85	1.85	1.87
16_{a}	2.40	2.39	2.30	2.30
16_{e}	2.60	2.58	2.61	2.60
NCH_3	2.45	2.44	2.45	2.46

[a]numbering of nuclei shown in Figure 5-5a and Figure 5-5b.

[b]values obtained from [147] and [148].

[c]values obtained from [136].

The signal attributed to the H-5 proton for product *m/z* 298 was found to have shifted slightly upfield (δ 4.65 ppm) compared to the codeine H-5 proton (δ 4.89 ppm). This could be explained by the diamagnetic shielding effect, due to

the close proximity of the electron dense carbonyl bond (C=O) at the C-6 position found in the codeinone structure (Figure 5-5b). Furthermore, the H-5 signal for product *m/z* 298 was a singlet, unlike the distinct doublet observed for the H-5 proton of codeine. This was consistent with a lack of a neighbouring proton at the H-6 position in the codeinone molecule. On the other hand, the H-7 proton in product *m/z* 298 was observed to have exhibited a downfield shift (δ 6.09 ppm) when compared to the H-7 proton for codeine (δ 5.73 ppm). This deshielding effect was due to the anisotropy of the adjacent C=O group.

Similarly, the signal belonging to the H-8 proton for product *m/z* 298 was found to be further downfield (δ 6.65 ppm) than codeine (δ 5.30 ppm), and also coinciding with the aromatic region. This can be explained by the partial positive charge carried by the C-8 carbon in codeinone; the presence of a C=O group conjugated with a carbon-carbon (C=C) double bond, results in resonance within the structure [145] (Figure 5-7). Therefore, a partial positive charge was carried by the C-8 carbon (also known as the *β* carbon), causing the H-8 proton to be deshielded. The ^{1}H-NMR chemical shifts correlated well with those described for codeinone in the literature (Table 5-5).

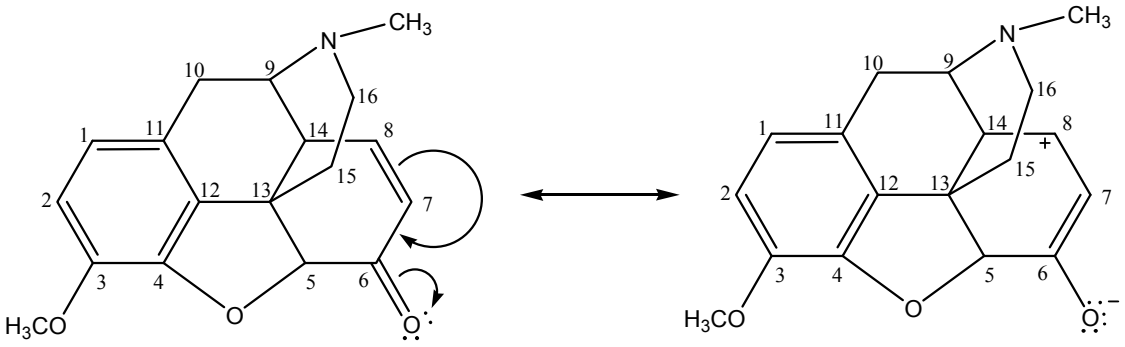


Figure 5-7: Resonance structures of codeinone demonstrating the presence of a partial positive charge on the C-8 carbon.

Complementary spectroscopic characterisation with ^{13}C-NMR analyses also supported the identification of product *m/z* 298 as codeinone. The only differences when comparing the ^{13}C-NMR chemical shifts of codeine and

product *m/z* 298 (Table 5-6) were the signals corresponding to the C-5 to C-8 carbons. This was expected for codeinone, especially the significant chemical shift of the C-6 carbon from δ 66.40 ppm to δ 194.51 ppm. This indicated the presence of a C=O group (aldehyde or ketone) within the structure of the compound. A comparison of the literature ^{13}C-NMR chemical shifts for codeinone and product *m/z* 298 showed that they were in good agreement.

MS/MS data was also obtained for codeine, product *m/z* 298 and the codeinone that was synthesised in section 5.4.2 as reference material for comparison (due to the incomplete oxidation of codeine to codeinone during this synthesis, there was not enough material for NMR analysis). In addition to the desired codeinone, a small amount of product *m/z* 314b was also present in the sample (Appendix, Figure A9).

The distinct fragmentation patterns of codeine (Figure 5-8a) and product *m/z* 298 (Figure 5-8b) showed that they share common product ions at *m/z* 153, 165, 181 and 223, an expected observation since they share a common core structure. The identification of product *m/z* 298 as codeinone was strongly supported by comparison of Figure 5-8b and Figure 5-8c. Both fragmentation patterns appeared to be the same under the same analysis conditions employed, with common product ions at *m/z* 153, 165, 181, 183, 198, 211, 223 and 239, in addition to the precursor ion at *m/z* 298.

Table 5-6: ^{13}C-NMR chemical shifts of literature codeine compared to the codeine standard analysed in this study.

identification of carbon[a]	literature ^{13}C chemical shifts for codeine[b] (ppm)	^{13}C chemical shifts for the codeine standard (ppm)	literature ^{13}C chemical shifts for codeinone[c] (ppm)	^{13}C chemical shifts for product *m/z* 298 (ppm)
1	119.39	119.56	119.39	119.56
2	112.81	112.90	112.81	112.90
3	142.12	142.22	142.12	142.22
4	146.17	146.29	146.17	146.28
5	91.15	91.33	115.1	114.89
6	66.18	66.40	194.00	194.51
7	133.39	133.40	127.80	129.07
8	127.83	128.29	142.80	142.58
9	58.76	58.90	58.76	58.89
10	20.38	20.40	20.38	20.40
11	126.71	127.25	126.71	126.09
12	131.10	131.09	131.10	131.10
13	42.90	42.96	38.10	37.13
14	40.33	40.83	43.40	42.97
15	35.40	35.86	35.40	35.85
16	46.28	46.46	46.28	46.83
NCH_3	42.76	43.14	42.76	42.97
3-OCH_3	56.18	56.35	56.18	56.90

[a]numbering of nuclei shown in Figure 5-5a and Figure 5-5b.

[b]values adapted from [130].

[c]values adapted from [130] and [149].

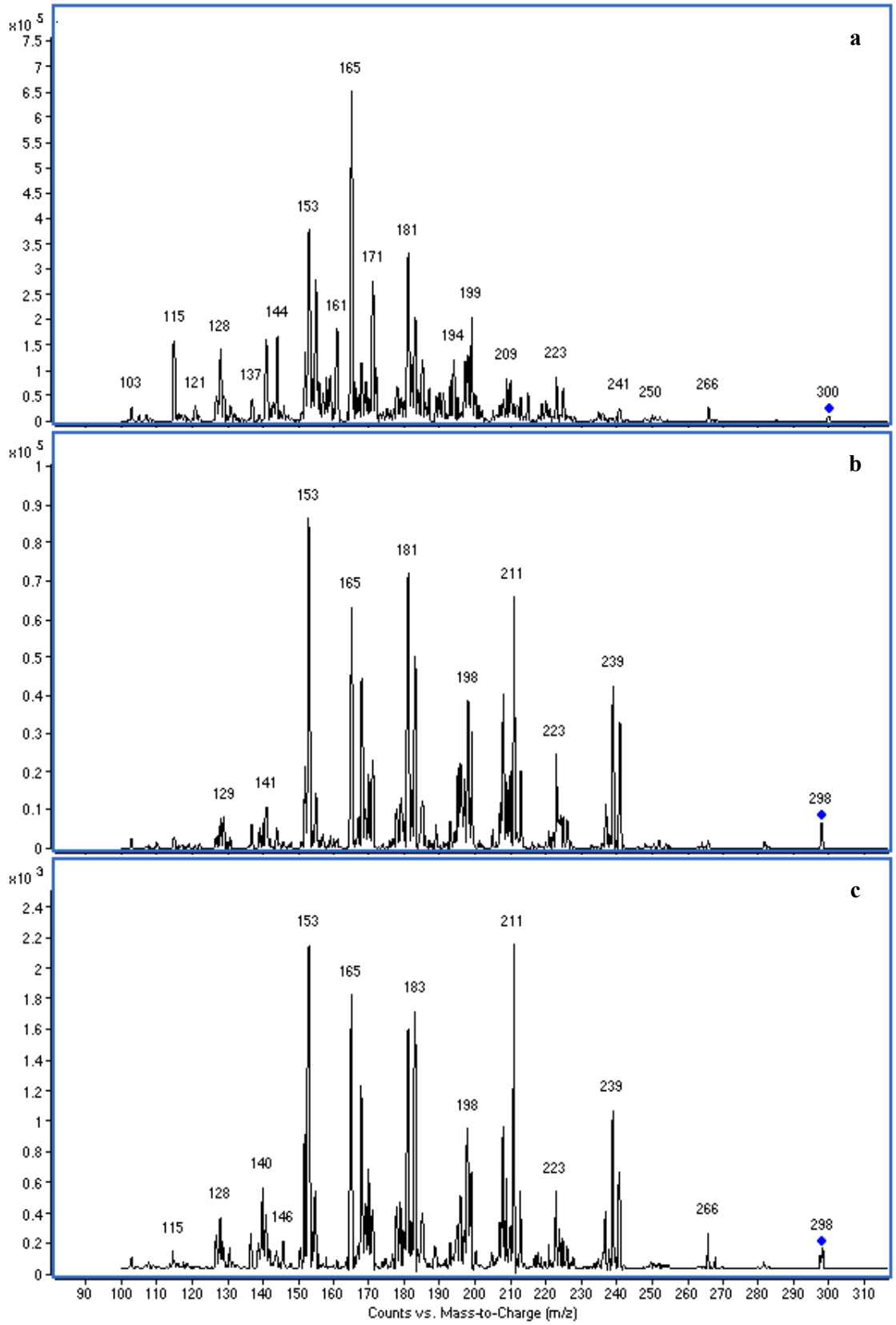


Figure 5-8: QQQ-MS fragmentation patterns of (a) codeine (CE = 45 eV), (b) the m/z 298 product (CE = 40 eV) and (c) the codeinone reference material synthesised via the Swern oxidation route (CE = 40 eV).

It is worthwhile noting that a constitutional isomer of codeinone, known as neopinone (Figure 5-9), was also considered as a possible structure for product *m/z* 298. In aqueous environments, codeinone exists in equilibrium with neopinone (3:1 ratio) in both acidic and alkali conditions (acid and alkali catalysed isomerism) [150]. Although their fragmentation patterns are very similar, they can be distinguished by the different intensity pattern. More importantly, it appears that the product ion at *m/z* 198 is present in the ESI-MS/MS spectrum of codeinone, but not neopinone [151]. Since this ion is quite prominent in the CID spectrum of product *m/z* 298, the reaction product (at least the major isomer) was determined to be codeinone. Furthermore, the presence of the C=O and C=C conjugation found in codeinone, but not neopinone, was confirmed with NMR analyses.

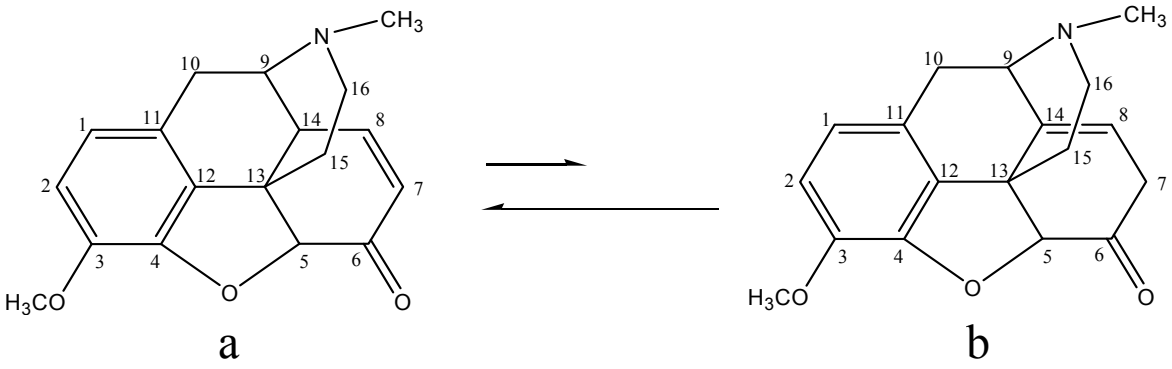


Figure 5-9: Equilibrium between (a) codeinone and (b) neopinone.

Finally, the CID spectrum of the product from high resolution MS, in addition to the proposed fragmentation pathways are exhibited in Figure 5-10 and Figure 5-11, respectively. The $[M+H]^+$ at *m/z* 298.1423 was found to correlate with the molecular formula for protonated codeinone ($C_{18}H_{20}NO_3$, -2.35 ppm mass accuracy error). Further, the MS peaks corresponding to the sodium and potassium adducts of codeinone were also found at *m/z* 320.1250 ($[M+Na]^+$, -2.19 ppm mass accuracy error) and *m/z* 336.0989 ($[M+K]^+$, -2.38 ppm mass accuracy error). Overall, the MS data correlated well with literature [151, 152], with the mass accuracy determination within the dynamic range in line with the instruments' specifications [134].

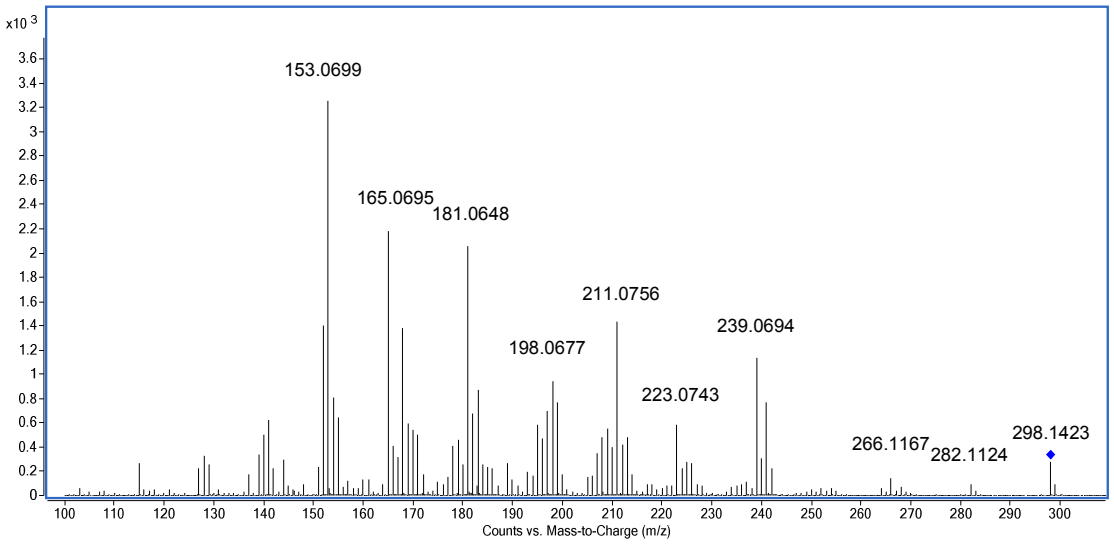


Figure 5-10: High resolution CID spectrum of product m/z 298, codeinone (FE = 250 V, CE = 40 eV).

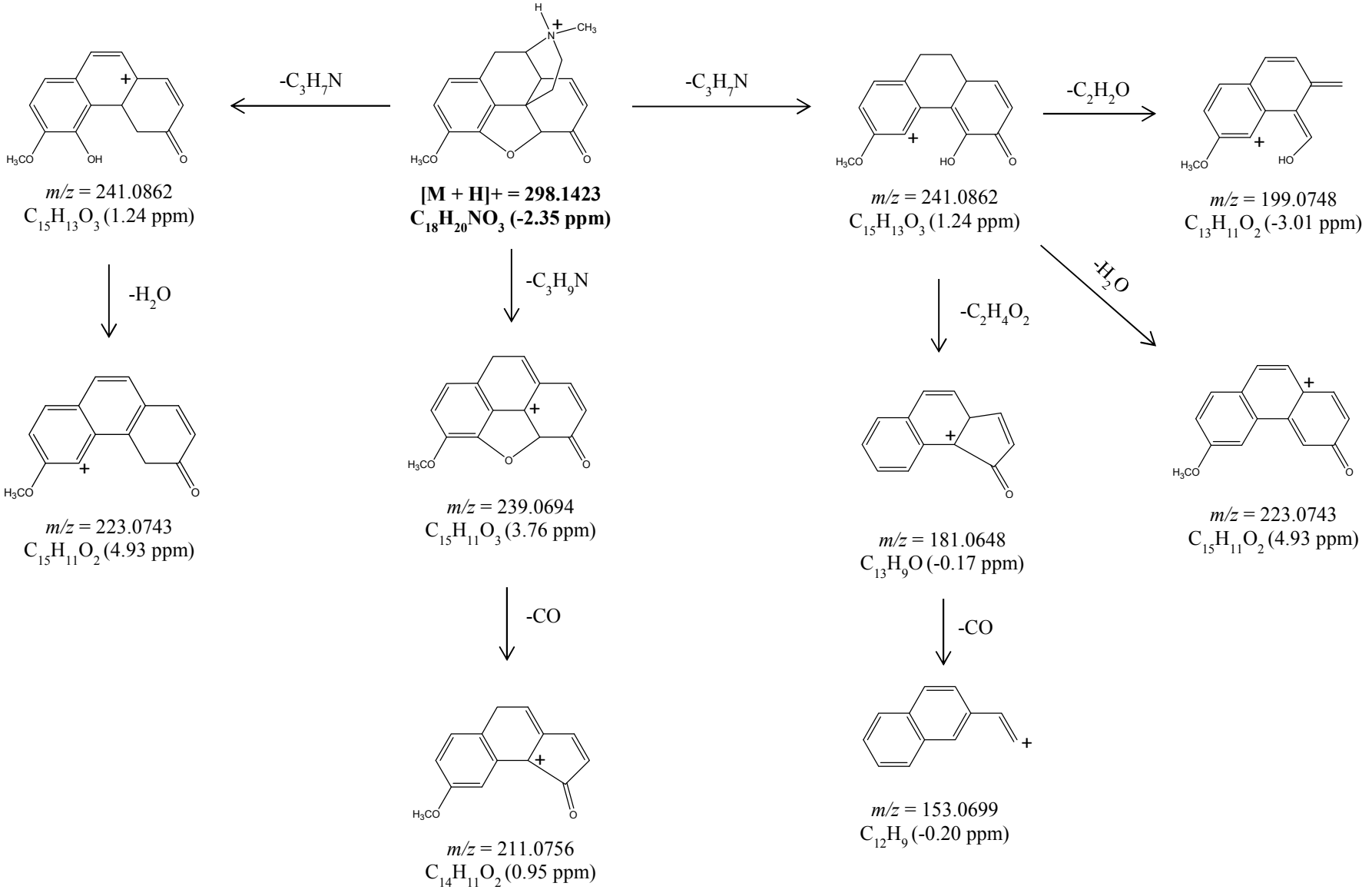


Figure 5-11: Proposed MS fragmentation pathways of the product m/z 298, codeinone.

5.5.2.2 Characterisation of product m/z 314a

High resolution MS identified product *m/z* 314a to be 14-hydroxycodeinone (Figure 5-5c). The protonated molecule had a *m/z* 314.1380, with major product ions at *m/z* 296.1276, *m/z* 281.1038, *m/z* 264.1013, *m/z* 254.1168 and *m/z* 239.0942 (Figure 5-12). It was determined that the mass of the protonated molecule corresponded to the molecular formula $C_{18}H_{20}NO_4$ (-3.82 ppm mass accuracy error). This indicated that there was an additional oxygen atom within the structure of product *m/z* 314a in comparison to codeinone.

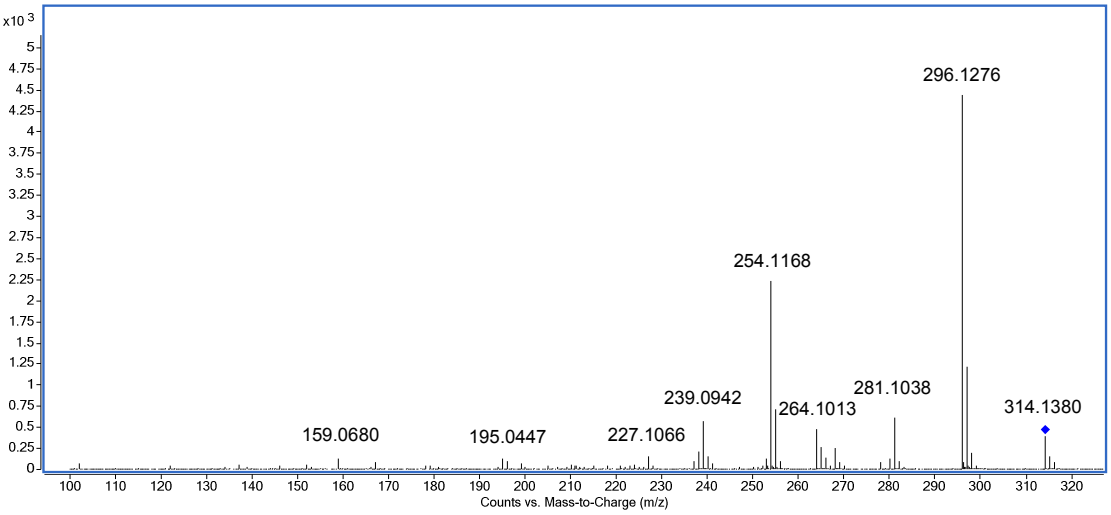


Figure 5-12: High resolution CID spectrum of product m/z 314a, 14-hydroxycodeinone (FE = 150 V, CE = 20 eV).

Such an observation could be explained by the substitution of an –OH group at the C-10 or C-14 carbons of codeinone, or the formation of an N-oxide of codeinone. It has been reported that the C-10 carbon is a viable site for reaction, with 10α-hydroxy analogues of codeine able to be recovered following chromium trioxide oxidation [153]. On the other hand, the production of 14-hydroxycodeinone from codeinone has also been documented, despite the relatively unreactive nature of the tertiary C-14 carbon found in morphine alkaloid structures. This hydroxylation reaction has been observed through the direct oxidation of codeinone to 14-hydroxycodeinone using various oxidising agents [136, 154-156], as well as in biological systems whereby codeine

undergoes biotransformation by *Pseudomonas Putida* M10 to produce 14-hydroxycodeinone [157].

Product *m/z* 314a was identified to be 14-hydroxycodeinone based on the major product ions and its intensities observed in the CID spectrum. The proposed fragmentation pathways of 14-hydroxycodeinone can be found in Figure 5-13, and is consistent with the product ions observed. Fragmentation of the protonated molecule at *m/z* 314.1380 resulted in the loss of water to yield the prominent ion at *m/z* 296.1276. The removal of a $-CH_3$ radical from this latter product ion produced the distinctive ion at *m/z* 281.1038. Following this, the observation of the *m/z* 264.1013 ion could be explained by a loss of CH_3OH or an –OH radical from *m/z* 296.1276 or *m/z* 281.1038, respectively. The *m/z* 296.1276 ion could also be alternatively fragmented to yield a C_2H_2O neutral loss, resulting in the observation of the ion at *m/z* 254.1168. The appearance of the *m/z* 239.0942 ion could be explained by the removal of a $-CH_3$ radical from the *m/z* 254.1168 ion. Furthermore, the sodium and potassium adducts of 14-hydroxycodeinone could be observed at *m/z* 336.1220 (2.38 ppm mass accuracy error) and *m/z* 352.0939 (-3.41 ppm mass accuracy error), respectively. Finally, ESI-MS/MS data for 14-hydroxycodeinone found in literature [152] was consistent with the accurate mass data obtained in this study, further supporting the hydroxylation of codeinone at the C-14 site to produce product *m/z* 314a.

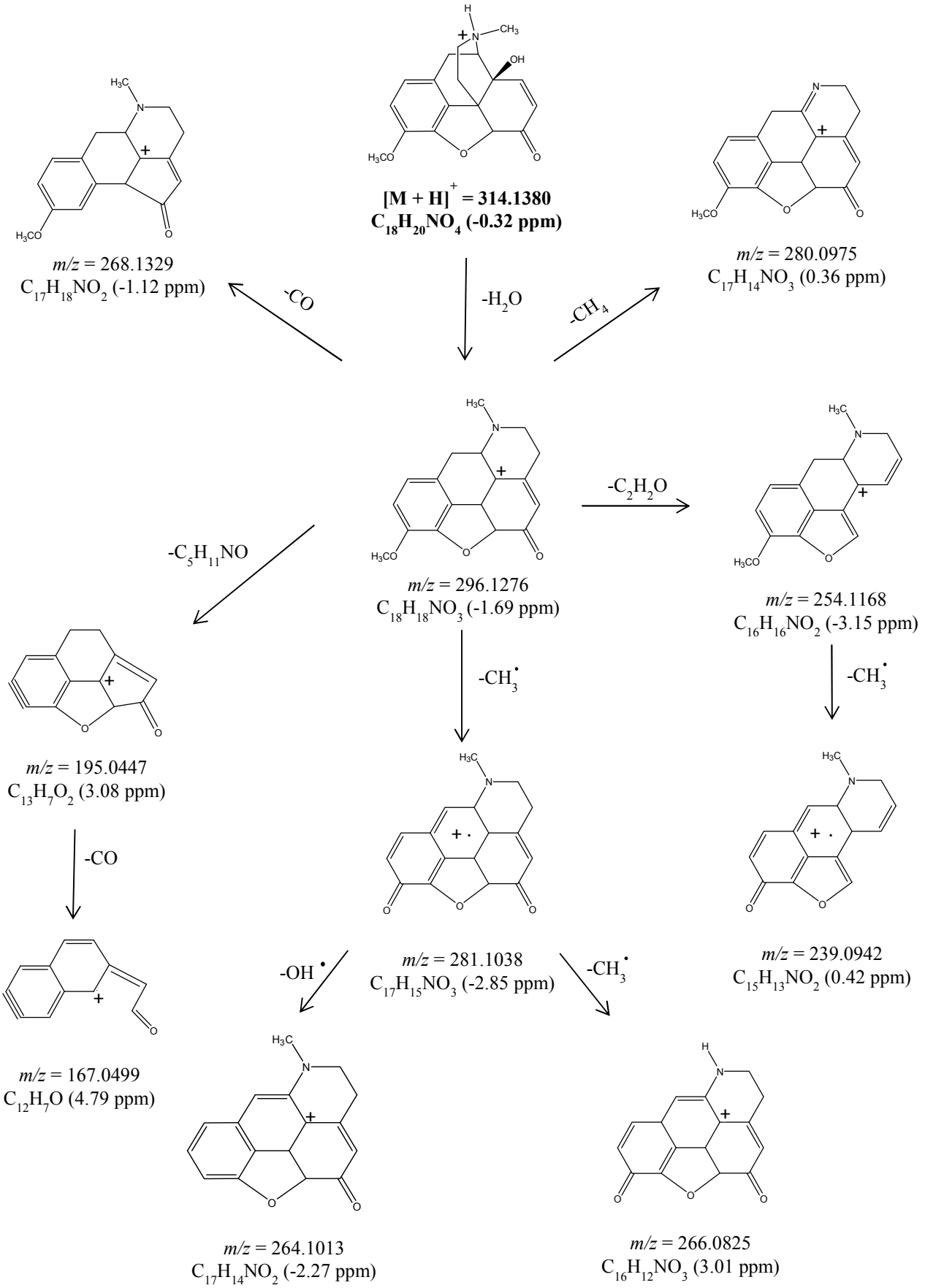


Figure 5-13: Proposed MS fragmentation pathways of product m/z 314a, 14-hydroxycodeinone.

5.5.2.3 Characterisation of product m/z 314b

Product *m/z* 314b had a $[M+H]^+$ at *m/z* 314.1751, corresponding well with $C_{19}H_{23}NO_3$ (0.10 ppm mass accuracy error). Compared to codeine, it appears that there is an addition of a $–CH_2$ group in the structure, an unexpected finding based on the oxidative mechanism of PCC. Nevertheless, two likely possibilities for product *m/z* 314b given the structure of the codeine starting material were 6-O-methylcodeine (Figure 5-5d) and ethylmorphine (Figure 5-14). LC-MS analysis of commercial standards identified product *m/z* 314b to be 6-*O*-methylcodeine, with both product *m/z* 314b and the 6-*O*-methylcodeine standard sharing the same retention time and characteristic mass fragmentation pattern (Figure 5-15).

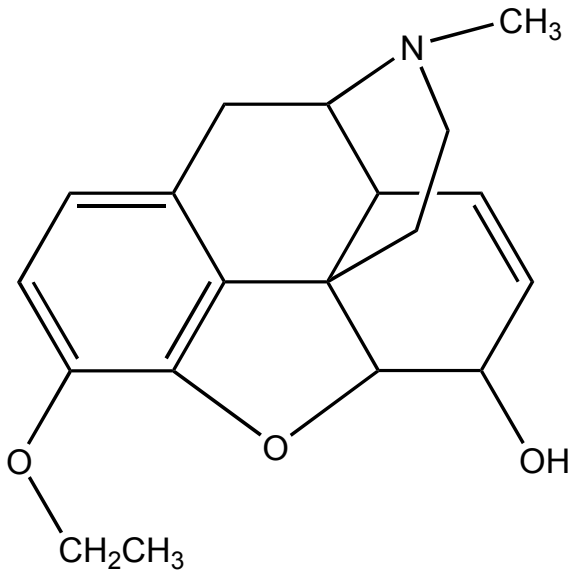


Figure 5-14: Structure of ethylmorphine.

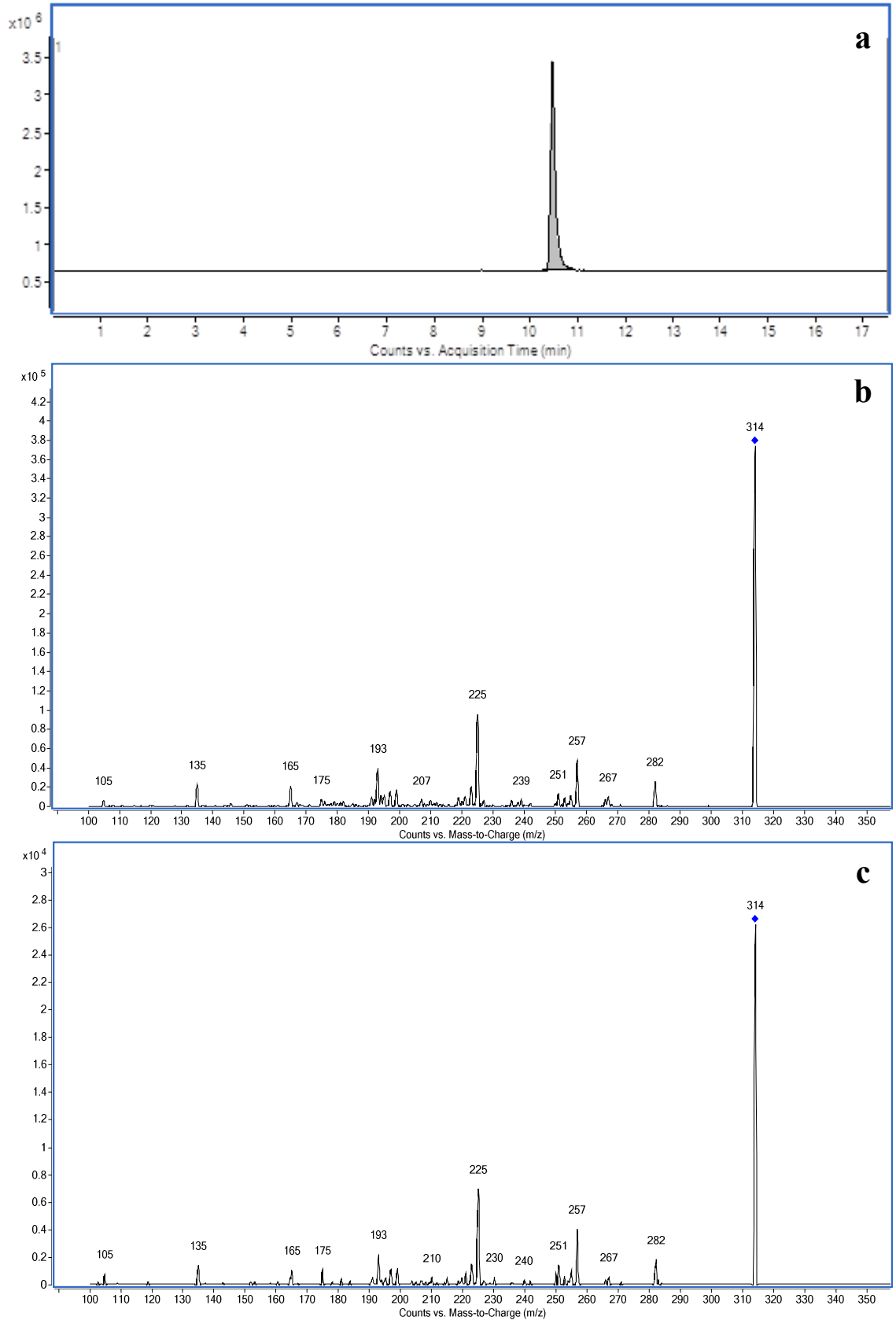


Figure 5-15: TIC chromatogram (product ion scan) of (a) a 6-O-methylcodeine standard, and QQQ-MS CID spectra of (b) a 6-O-methylcodeine standard (CE = 25 eV) and (c) the m/z 314b product (CE = 25 eV).

Further, the high resolution MS/MS spectrum obtained for product *m/z* 314b is displayed in Figure 5-16; a protonated molecule at *m/z* 314.1751 was produced, followed by major product ions at *m/z* 280.0974, *m/z* 266.1167, *m/z* 252.1032, *m/z* 239.0943, *m/z* 225.0909, *m/z* 210.0923, *m/z* 193.0654, *m/z* 181.0655, *m/z* 165.0694 and *m/z* 152.0624. The proposed fragmentation pathways (Figure 5-17) are consistent with the product being 6-*O*-methylcodeine.

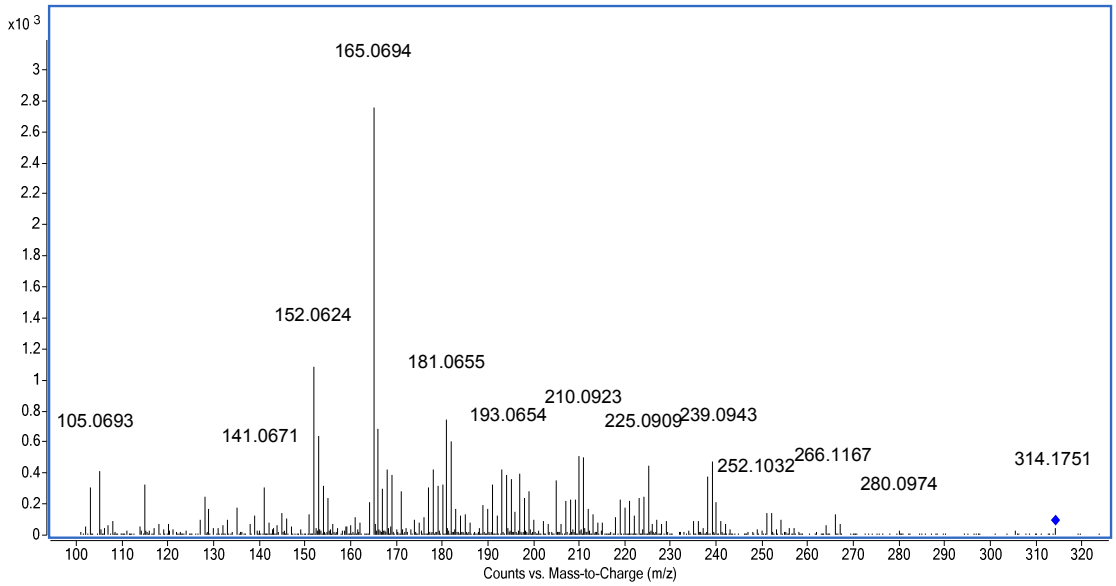


Figure 5-16: High resolution CID spectrum of product m/z 314b, 6-O-methylcodeine (FE = 220 V, CE = 45 eV).

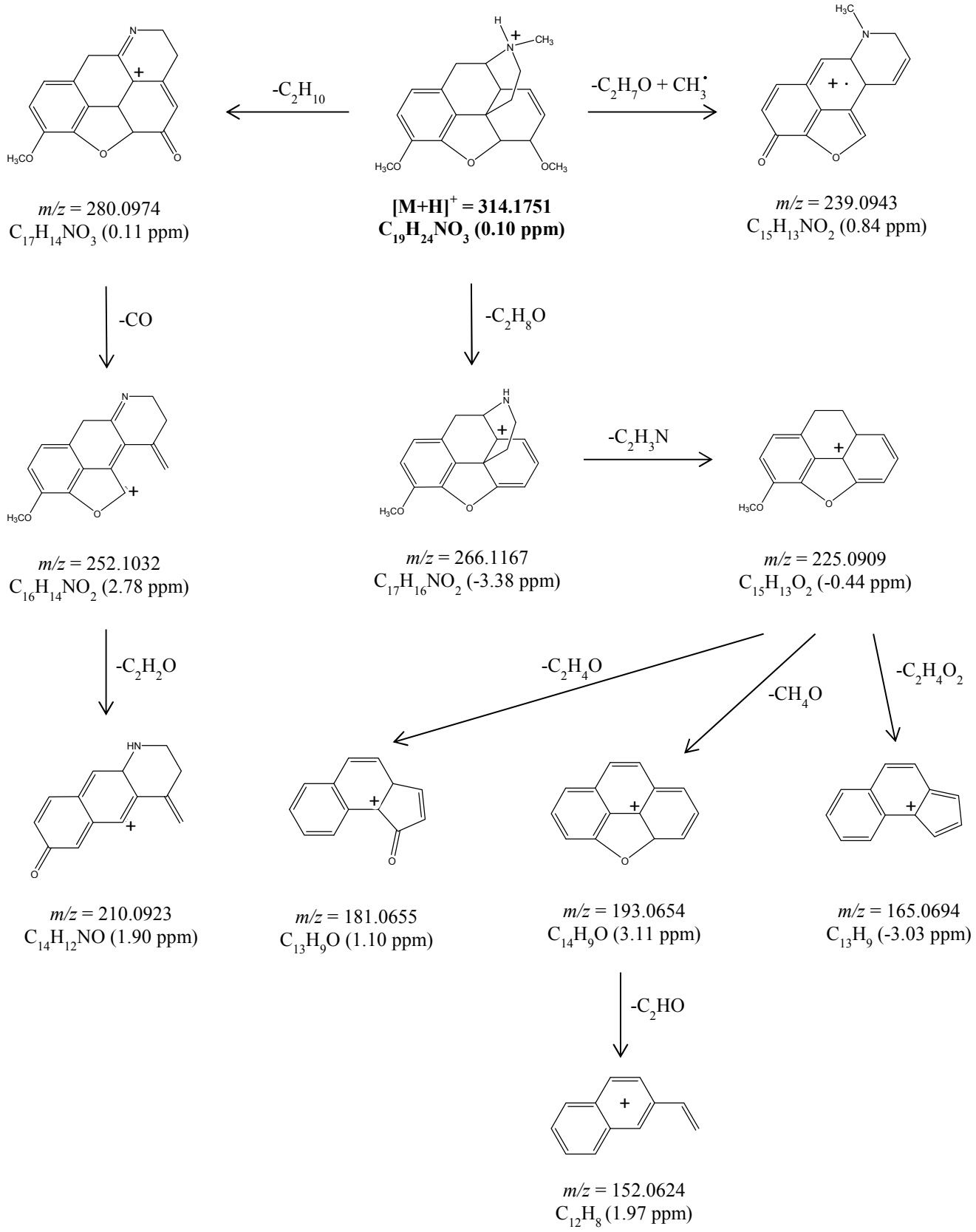


Figure 5-17: Proposed MS fragmentation pathways of product m/z 314b, 6-O-methylcodeine.

5.5.2.4 Characterisation of product m/z 316

High resolution MS of product *m/z* 316 revealed a protonated molecule of *m/z* 316.1546. This was consistent with the molecular formula $C_{18}H_{22}NO_4$ (0.95 ppm mass accuracy error). Initially, the reaction product was hypothesized to be 14-hydroxy-7, 8-dihydrocodeinone (commonly known as oxycodone). The preparation of oxycodone from codeine starting material has been achieved as an alternate process for the production of oxycodone from thebaine [136, 155, 156].

However, LC-MS/MS analysis of a commercial oxycodone standard under the same parameters employed for product *m/z* 316 indicated that they were not the same product (Figure 5-18). Codeine-N-oxide and 14-hydroxycodeine were also considered as potential molecular structures, however the fragmentation pathways proposed for these two compounds could not account for the observed product ions at *m/z* 270 and *m/z* 213, respectively (Figure 5-19). Additionally, the CID spectrum for the reaction product did not correlate with those documented for 14-hydroxycodeine [152].

The first product ion in Figure 5-19 at *m/z* 298.1445 indicated a neutral loss of water from the precursor ion, also observed for 14-hydroxycodeinone; this supported the hypothesis that the reaction product was hydroxylated. Thus, it is proposed that product *m/z* 316 is likely to be 8-hydroxy-7, 8-dihydrocodeinone (Figure 5-5e). The major product ions (*m/z* 213.0542, 199.0763, 185.0595 and 171.0815) observed in the CID spectrum appeared to correlate with those proposed for 8-hydroxy-7,8-dihydrocodeinone in literature (Figure 5-20, [152]).

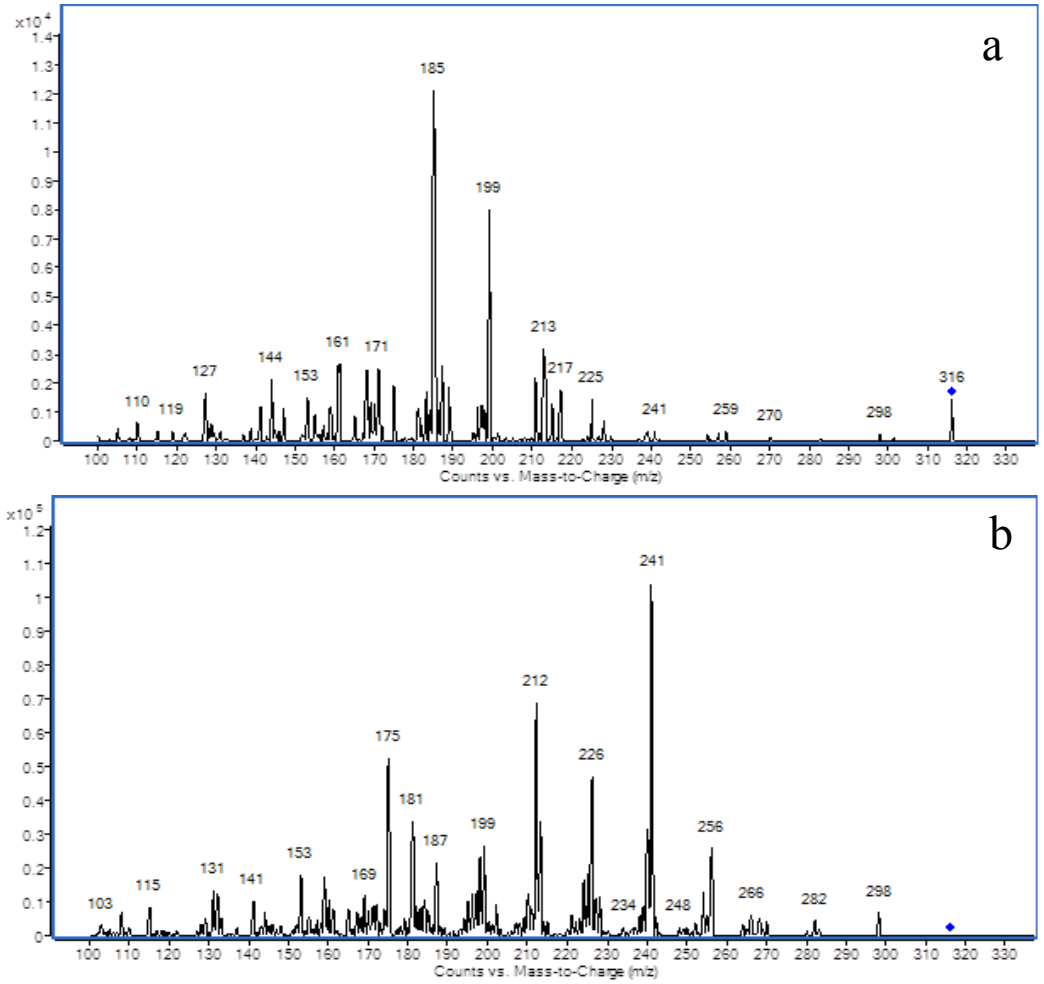


Figure 5-18: QQQ-MS fragmentation patterns of (a) the product m/z 316 (CE = 40 eV) and (b) oxycodone (CE = 40 eV).

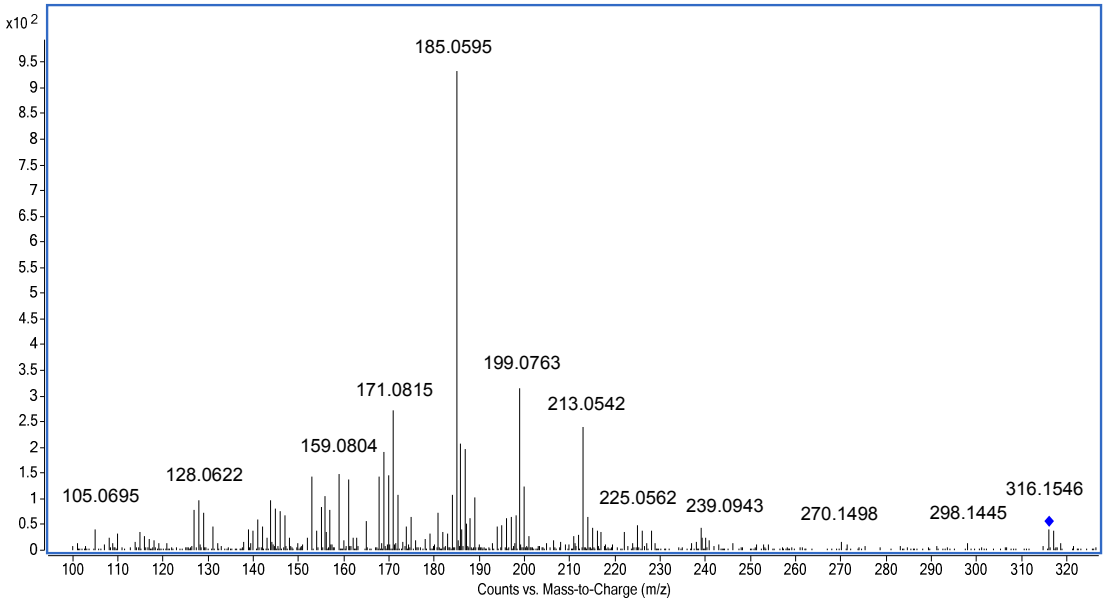


Figure 5-19: High resolution CID spectrum of product m/z 316, 8-hydroxy-7,8-dihydrocodeinone (FE = 240 V, CE= 40 eV), resulting from the reaction of codeine and PCC in urine.

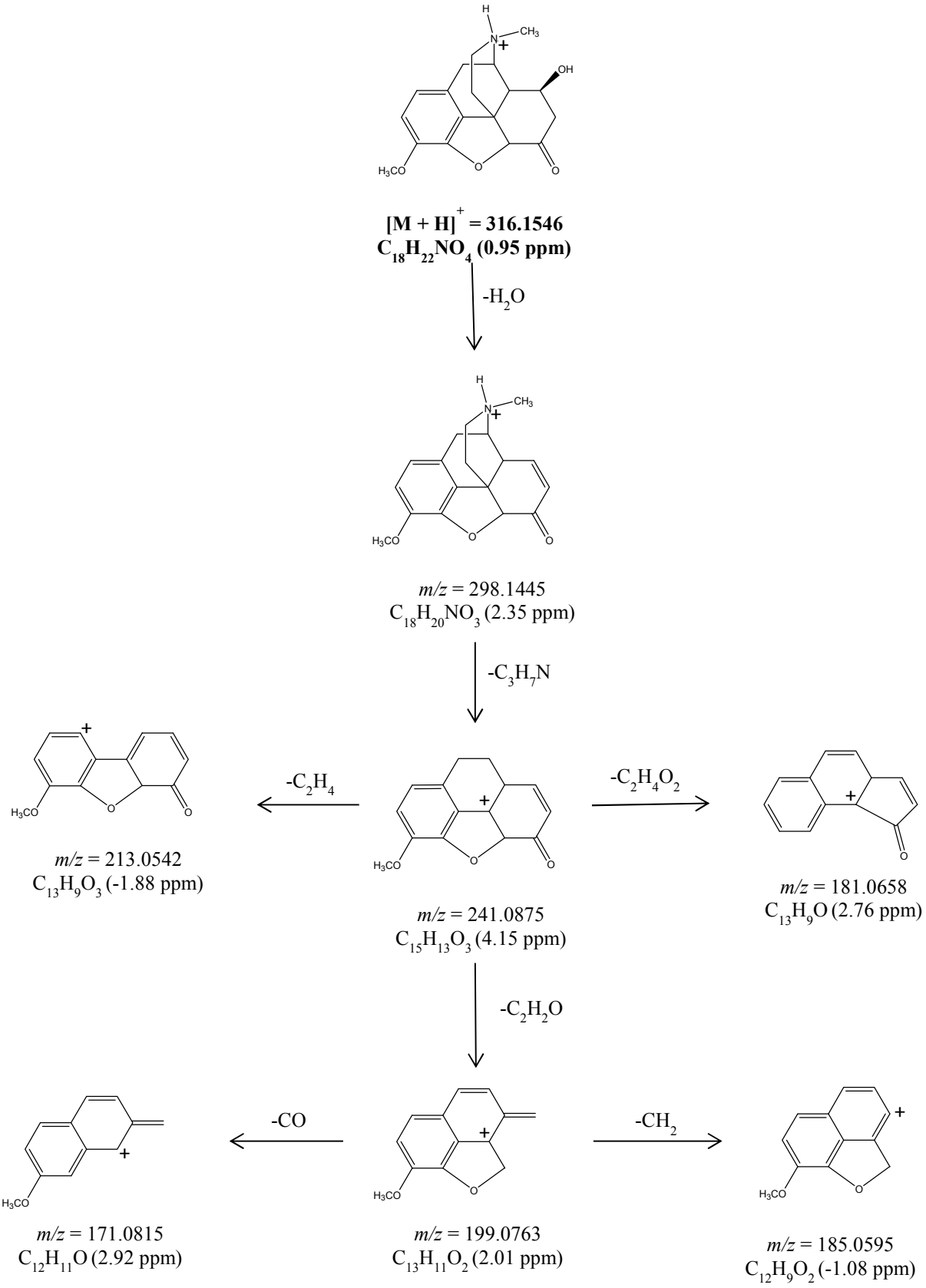


Figure 5-20: Proposed MS fragmentation pathways of product m/z 316, hypothesized to be 8-hydroxy-7,8-dihydrocodeinone.

5.5.2.5 Tentative structure for product m/z 416

It was interesting to note that the MS fragmentation pattern of product *m/z* 416 bore resemblance to the fragmentation pattern belonging to C6G, with major product ions at *m/z* 300, 225 and 215 (Figure 5-21). Comparison of the CID spectra obtained by QQQ-MS for the C6G starting material (Figure 5-21a) and product *m/z* 416 (Figure 5-21b) suggested that the latter structure had incurred a mass loss of 60 Da. Closer inspection of the fragmentation pattern belonging to product *m/z* 416 showed that it possessed the same prominent product ions as detected in the C6G fragmentation pattern, including *m/z* 300, 282, 266, 243, 225, 215, 209, 193, 183 and 165. Since the formation of *m/z* 300 (product ion) from *m/z* 476 (precursor ion) observed for C6G is due to the loss of the glucuronide entity from the structure, it appears that product *m/z* 416 shares a common molecular skeleton as the starting material, with the PCC reaction occurring somewhere on the glucuronic acid moiety. Thus, it is hypothesized that the transformation of C6G (Figure 5-21c) to the reaction product is carried out via ring cleavage at the C-O epoxy bond within the glucuronic acid group with a loss of $–C_2O_2H_2$, followed by ring closure. Additionally, an –OH group on the glucuronide is also oxidized to a carbonyl (C=O) group, with the position of the carbonyl group likely to be adjacent to the remaining oxygen (in line with lactone formation, (Figure 5-21d)).

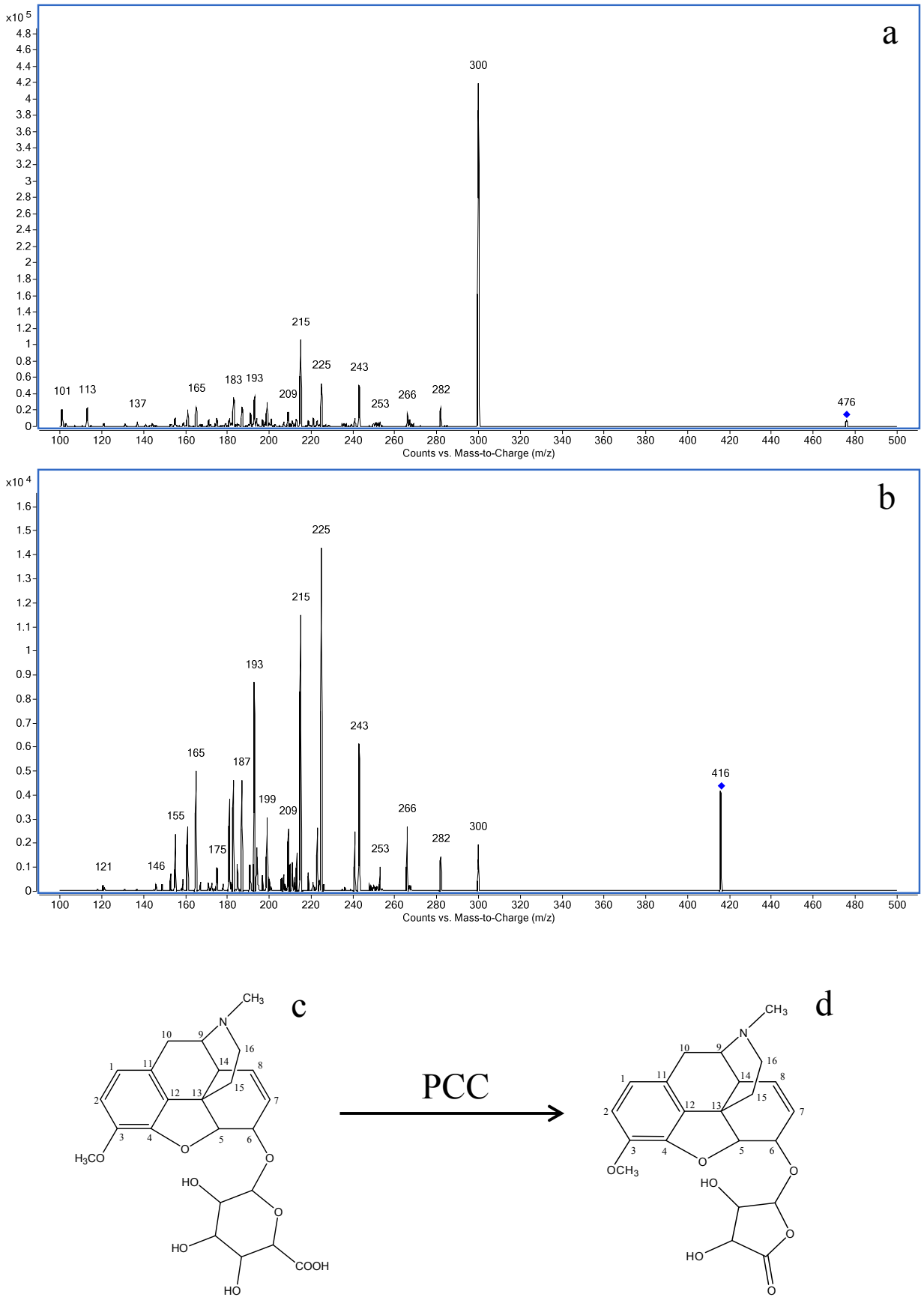
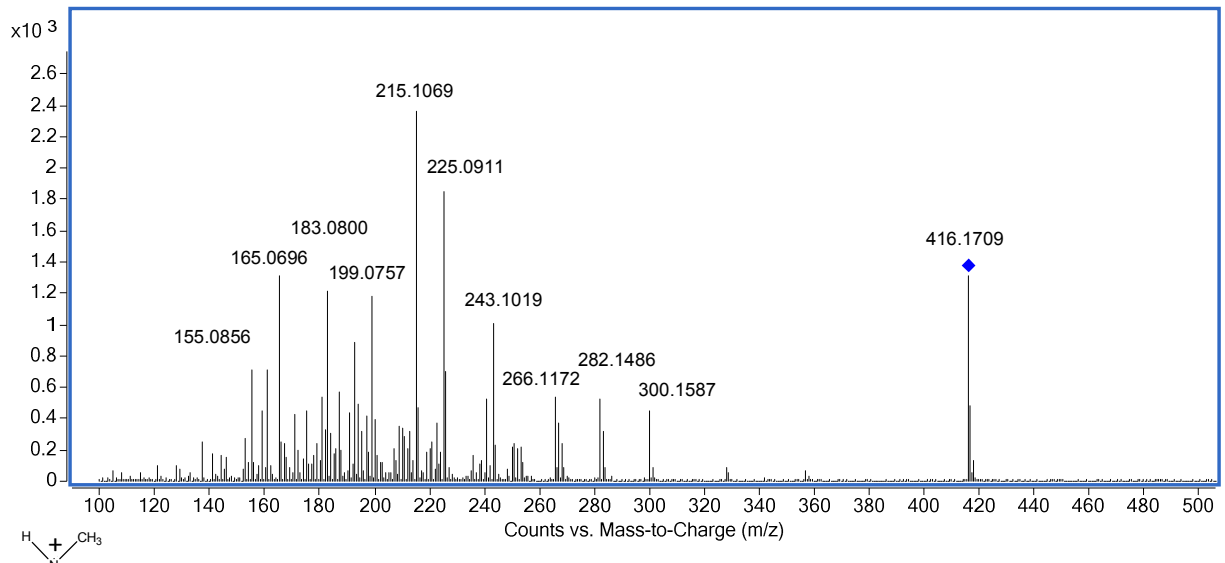


Figure 5-21: QQQ-MS CID spectra of (a) C6G (CE = 45 eV) and (b) product m/z 416 (CE = 40 eV), with a reaction scheme depicting the proposed conversion of (c) C6G to (d) the tentative reaction product by PCC.

Further structural elucidation with high resolution MS analysis supported this hypothesis. The $[M+H]^+$ for product *m/z* 416 was measured to be *m/z* 416.1709, corresponding well with the formula of $C_{22}H_{26}NO_7$ (1.25 ppm mass accuracy error). The next major ion is found at *m/z* 300.1587, which corresponds with the mass of protonated codeine ($C_{18}H_{21}NO_3$, -2.41 ppm mass accuracy error). This corroborates with the suggestion that the site of oxidation is on the glucuronic acid moiety. Further proof that the codeine portion of the structure remains intact was obtained through examination of the remaining product ions; they are in agreement with the high resolution MS data published for codeine [152]. Figure 5-22 illustrates the high resolution MS/MS data and the proposed structures for the product ions for product *m/z* 416. Attempts at fragmenting the glucuronic acid entity to obtain further structural information proved difficult. However, as lactones are relatively stable, and based on the data obtained thus far, product *m/z* 416 is tentatively characterized as a C6G lactone derivative.



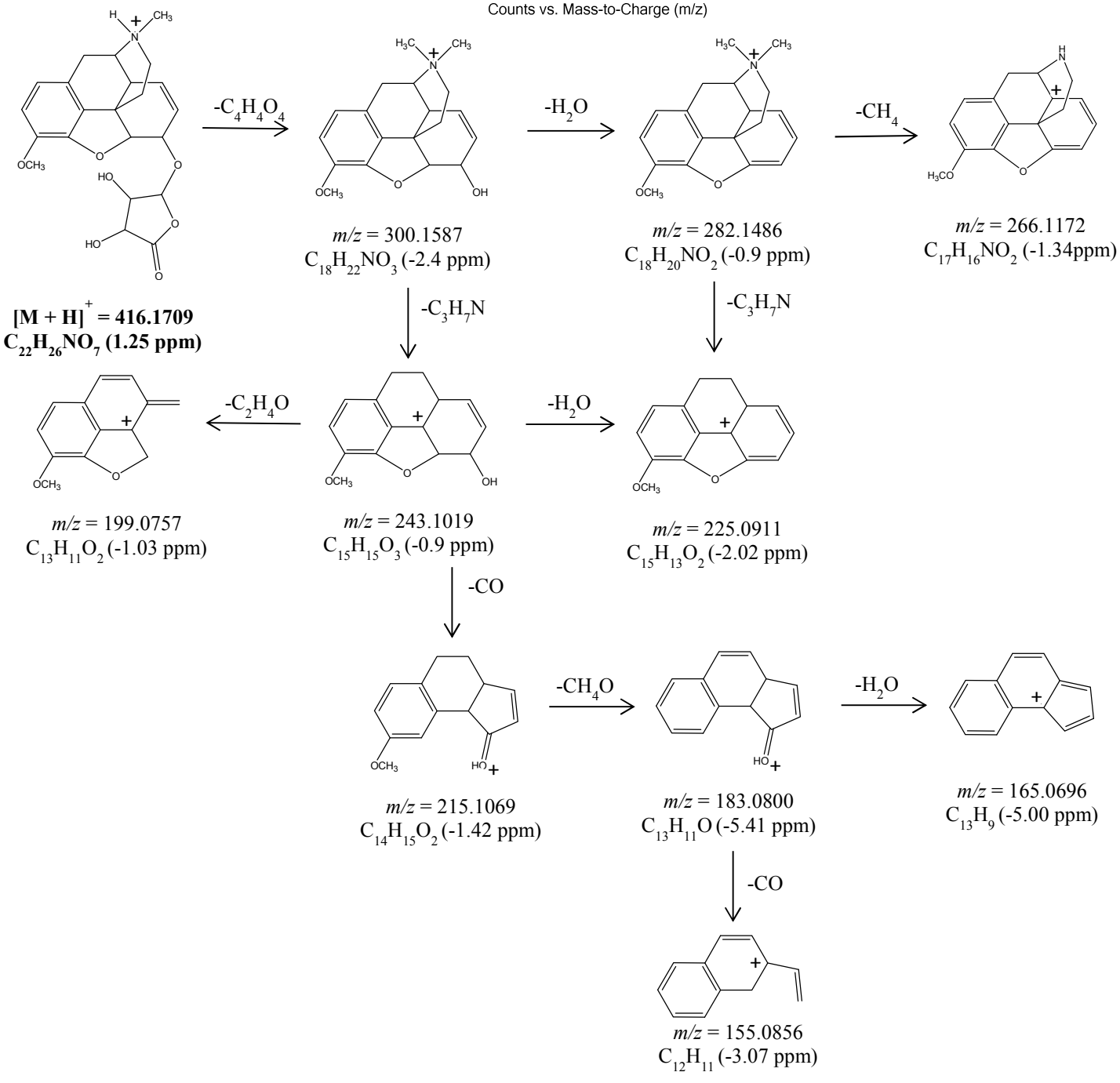


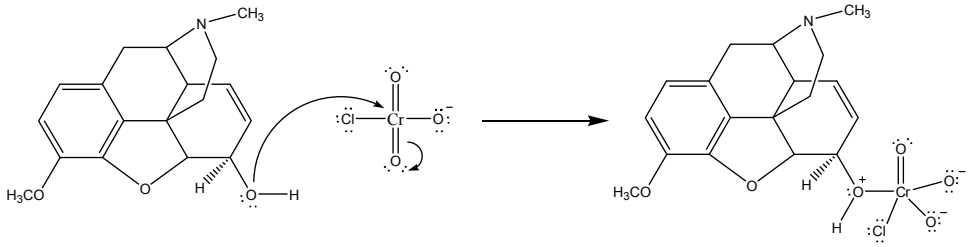
Figure 5-22: High resolution CID spectrum (FE = 170 V, CE = 40 eV) and the proposed structures for the product ions for product m/z 416, tentatively identified to be a C6G lactone derivative.

5.5.2.6 Proposed mechanism of action of PCC as a urine adulterant

Although pH was found to be slightly lowered by PCC, this study has shown that its main mechanism of action is through the oxidation of codeine and C6G in urine to various other analogues. Since these analogues exhibit different chromatographic and mass spectrometric behaviour, they are not detected using LC-MS parameters employed for the parent compounds.

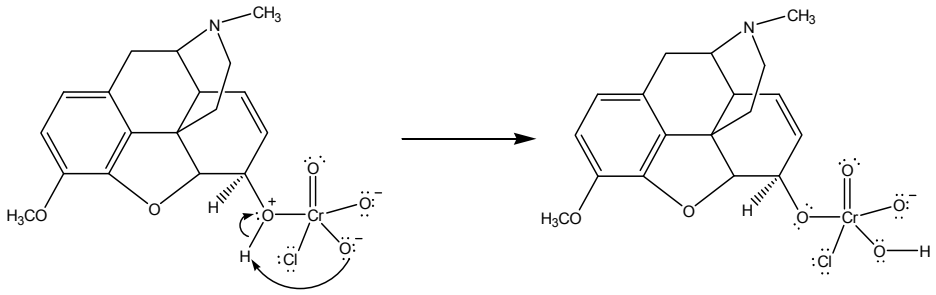
Upon exposure of codeine to PCC, the major route of oxidation results in the formation of codeinone. The proposed reaction mechanism is initiated by the co-ordination of chromium (VI) (existing as the chlorochromate ion) and the codeine –OH group (C-6 position), to form a chromium (VI) acid ester. Following intermolecular re-arrangement, ultimately resulting in the removal of the codeine H-6 proton, codeinone is produced, with the reduction of chromium (VI) to chromium (IV) (Figure 5-23, adapted from Bruckner [158]). For the formation of 14-hydroxycodeinone, it is viable to propose that it is produced by further oxidation of codeinone. This route (codeine -> codeinone -> 14-hydroxycodeinone) has been documented in literature by Cr^{6+} and hydrogen peroxide, however the yield is not significant [136, 154, 157]. Finally, the Michael addition [159] is suspected as a possible pathway of reaction for the relatively minor production of 6-*O*-methylcodeine and 8-hydroxy-7,8-dihydrocodeinone.

1. Formation of a chromium (VI) acid ester from codeine and the chlorochromate ion:

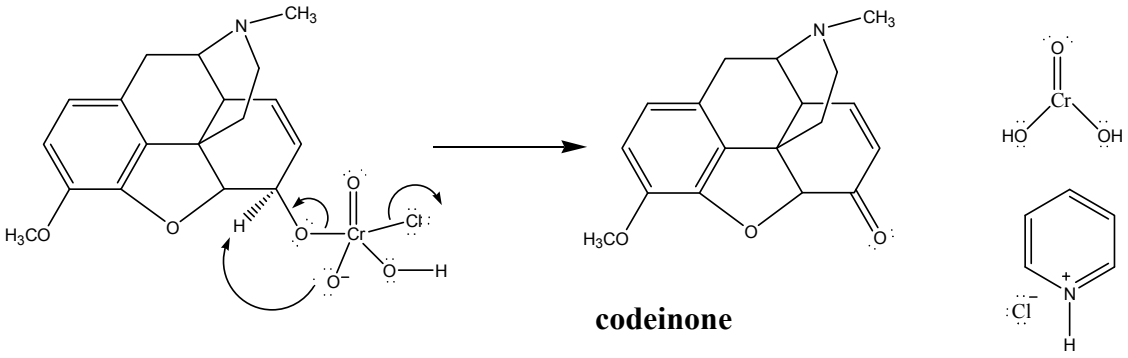


codeine

2. Proton transfer step:



3. Elimination of the chloride ion and removal of H-6 to produce codeinone:



codeinone

Figure 5-23: Proposed mechanism for the oxidation of codeine to codeinone by PCC.

5.6 Conclusions

The adulteration of codeine and C6G positive urine specimens with PCC results in the conversion of the parent drugs to various reaction products. Although codeine and C6G are still detectable in these samples, ambiguity may be introduced to the interpretation of the results; the presence of PCC decreases the concentrations of codeine and C6G, therefore altering the drug to metabolite ratios. Morphine/codeine ratios were also found to be affected by the presence of PCC. Consequently, the use of morphine/codeine ratios in result interpretation should be excised with care. It was also determined that although PCC did alter the urine specimen pH, its ability as an adulterant predominately lies with its oxidative capabilities. Furthermore, this study has shown that the presence of codeinone in a urine specimen may be due to adulteration with PCC and not as an impurity of hydrocodone synthesis from codeine. As these reaction products are stable for approximately one week after its formation, more investigation is warranted to further determine their potential for use as markers for monitoring the presence of codeine and C6G in urine specimens adulterated with PCC.

Chapter 6: Exposure of 6-MAM, morphine, morphine-3-glucuronide and morphine-6-glucuronide to PCC in urine

Chapter 6: Exposure of 6-MAM, morphine, morphine-3-glucuronide and morphine-6-glucuronide to PCC in urine

6.1 Introduction

In the previous study, it was concluded that codeine and its major metabolite C6G are converted to various analogues following exposure to PCC in urine. It was found that the morphine to codeine ratios were significantly affected by PCC however the changes did not follow a definitive trend. This suggests that morphine may be undergoing a similar conversion to at least one other reaction product. Therefore, in this study, the effect of PCC on 6-MAM, morphine, M3G and M6G were determined. A similar workflow to the experimental designs used for previous studies was followed. Initially, the opiate analytes were exposed to PCC in water and for the reaction mixtures where reaction products were produced, the same reactions were replicated in urine. Once determined to be forming in urine and stable enough to be detected by LC-MS several days following adulteration, the reaction products were then structurally elucidated using high resolution mass spectrometry. The effect of PCC adulteration on immunoassay and GC-MS analyses of morphine positive authentic urine specimens was also assessed.

6.2 Materials

6.2.1 Drug standards and reagents

Morphine monohydrate (1mg/mL free base in methanol), morphine-3-β-D-glucuronide (1mg/mL free base in methanol/water:1/1), morphine6-β-D-glucuronide (1mg/mL free base in acetonitrile/water:1/1) and 6-monoacetylmorphine hydrochloride (1mg/mL free base in methanol) were

sourced from Lipomed (Arlesheim, Switzerland). PCC, ammonium formate and acetonitrile were sourced from the same suppliers as indicated in section 5.2.1.

6.2.2 Urine specimens

Blank urine matrix was obtained from random volunteers as outlined in section 5.2.2. Authentic urine specimens testing positive for morphine (following enzymatic hydrolysis and GC-MS analysis) were supplied by the Drug Toxicology Unit, NSW Forensic and Analytical Science Service after removal of sample identification. The specimens were stored in a freezer at -18℃ before analysis.

6.3 LC-MS instrumentation

LC-MS analyses (QQQ-MS and QTOF-MS) were carried out using the parameters outlined in section 5.3.1. Chromatographic separation of drug analytes was achieved using an Agilent Zorbax Eclipse XDB-C18 column (2.1 mm × 50 mm × 1.8 µm) in section 6.4.1. For all subsequent experiments in this study, an Agilent Zorbax Eclipse XDB-C18 column (2.1 mm × 50 mm × 3.5 µm) was used. This was due to column degradation of the former column with the smaller particle size.

6.4 Experimental methods

6.4.1 Exposure of morphine analogues to PCC in water

A 1 M PCC working solution was prepared by dissolving solid PCC in water. Another four PCC working solutions were then prepared (200, 20, 2 and 0.2 mM) by serial dilution. Morphine, M3G, M6G and 6-MAM were fortified into water at a concentration of 10 µg/mL (final sample volume = 1 mL). These drug standards were then spiked with 100 µL of each of the five PCC working solutions to give final concentrations of 100 mM, 20 mM, 2 mM, 0.2 mM and 0.02 mM PCC in the final water samples. All samples were analysed alongside

water blank samples, reagent control samples (opiate negative) and 10 µg/mL drug standards, with a monitoring period of up to three weeks. Samples were left at room temperature (22℃) during the first hour and then refrigerated (4℃) at all times unless removed for sampling.

6.4.2 Exposure of morphine analogues to PCC in urine

Morphine, M3G and 6-MAM were individually spiked in blank urine at 10 µg/mL concentrations. The samples were fortified with PCC whereby each drug analyte was exposed to two final concentrations (100 mM and 20 mM). Samples were left at room temperature (22℃) for the first hour of reaction, and then analysed one hour, five hours, one day, two days, four days and seven days following PCC adulteration. With the exception of the first hour, all samples were refrigerated at 4℃. Prior to analysis , an aliquot of each sample was removed from the urine jar, centrifuged at 4500 *g* for 10 min and filtered through 0.22 µm hydrophilic syringe filter units (MicroAnalytix Pty Ltd, Taren Point, NSW, Australia). In addition to the adulterated specimens, drug standards and reagent controls (opiate negative) were prepared in urine and sequentially analysed.

6.4.3 Structural elucidation of the reaction products

Reaction mixtures containing either M3G, morphine or 6-MAM with 100 mM PCC in urine were prepared as detailed in section 6.4.2 and refrigerated for two days prior to analysis using high resolution QTOF-MS. The analysis procedure was based on characterisation studies that had been previously carried out for other reaction products.

6.4.4 Analysis of PCC-adulterated authentic specimens by a routine toxicology laboratory

A batch of urine specimens consisting of two opiate negative blank samples and four opiate positive samples were adulterated with 100 mM and 20 mM PCC and left to react for 16 h (overnight) at 22℃. The samples were then sent

to the Drug Toxicology Unit for immunoassay screening and GC-MS confirmatory testing as described in section 5.4.3.

6.5 Results and discussion

6.5.1 Exposure of morphine analogues to PCC in water

6.5.1.1 Reaction of M3G to PCC in water

M3G was initially exposed to PCC at the four lower concentrations (0.02-20 mM). After one hour subsequent to PCC fortification of M3G drug solutions, a potential reaction product was detected (using full scan mode) in the sample containing 20 mM PCC. Eluting at 1.2 min with a protonated parent molecule of *m/z* 460, this product was considered minor, with an M3G: reaction product ratio of 31:1. On the second day of analysis, product *m/z* 460 was detected in the samples spiked with 2 mM and 20 mM PCC. This product remained detectable in these samples three weeks since the PCC fortification was carried out (Figure 6-1). Product *m/z* 460 was not detectable in the M3G samples adulterated with 0.02 mM and 0.2 mM PCC, with the opiate peak remaining predominant in the TIC trace. No other potential products were identified in these samples. Upon collection of the CID spectrum for product *m/z* 460, it was found that a good fragmentation pattern was difficult to obtain just like it was for M3G. The CID spectrum is shown in Figure 6-1f, with its precursor ion at *m/z* 460 and product ions at *m/z* 284 *and m/z* 227*.*

To ensure that product *m/z* 460 is robust enough to be formed under higher oxidant concentrations, M3G was exposed to 100 mM PCC in water. Sample monitoring over a five day period revealed that this product was detected one hour, one day, three days and five days following PCC fortification.

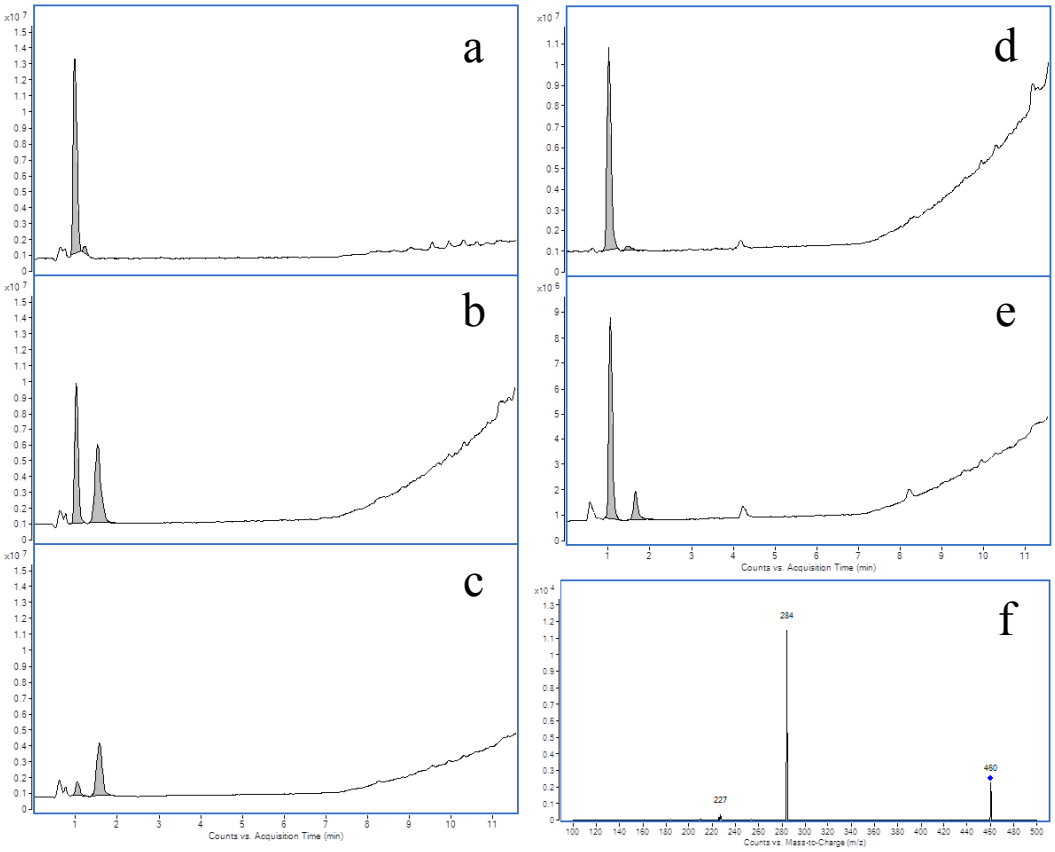


Figure 6-1: TIC chromatograms of M3G spiked with 20 mM PCC in water (a) one hour, (b) two days and (c) three weeks after reaction commencement, and TIC chromatograms of M3G with 2 mM PCC in water (d) two days and (e) three weeks following fortification, with (f) the CID spectrum of product m/z 460.

Note that R_t of M3G and product m/z 460 is 1.0 min and 1.2-1.5 min, respectively.

6.5.1.2 Reaction of morphine to PCC in water

The exposure of morphine to 0.02-2 mM PCC in water did not yield any identifiable reaction products. On the contrary, LC-MS analyses of the morphine samples containing 20 mM and 100 mM PCC indicated the presence of a reaction product eluting at 8.9 min with a precursor ion of *m/z* 316. This product was detected in samples containing both 20 mM and 100 mM PCC concentrations one day following oxidant fortification, and still detectable at three and five days (Figure 6-2). Product *m/z* 316 was still detectable in the sample fortified with 20 mM PCC after three weeks. It can also be observed

that morphine itself (TIC and CID spectrum shown in Figure 6-3a and Figure 6-3b, respectively) appears to be absent in the adulterated samples at the analysis time points. Unlike product *m/z* 460 that was previously discussed, a characteristic CID spectrum was obtained for product *m/z* 316 (Figure 6-3c).

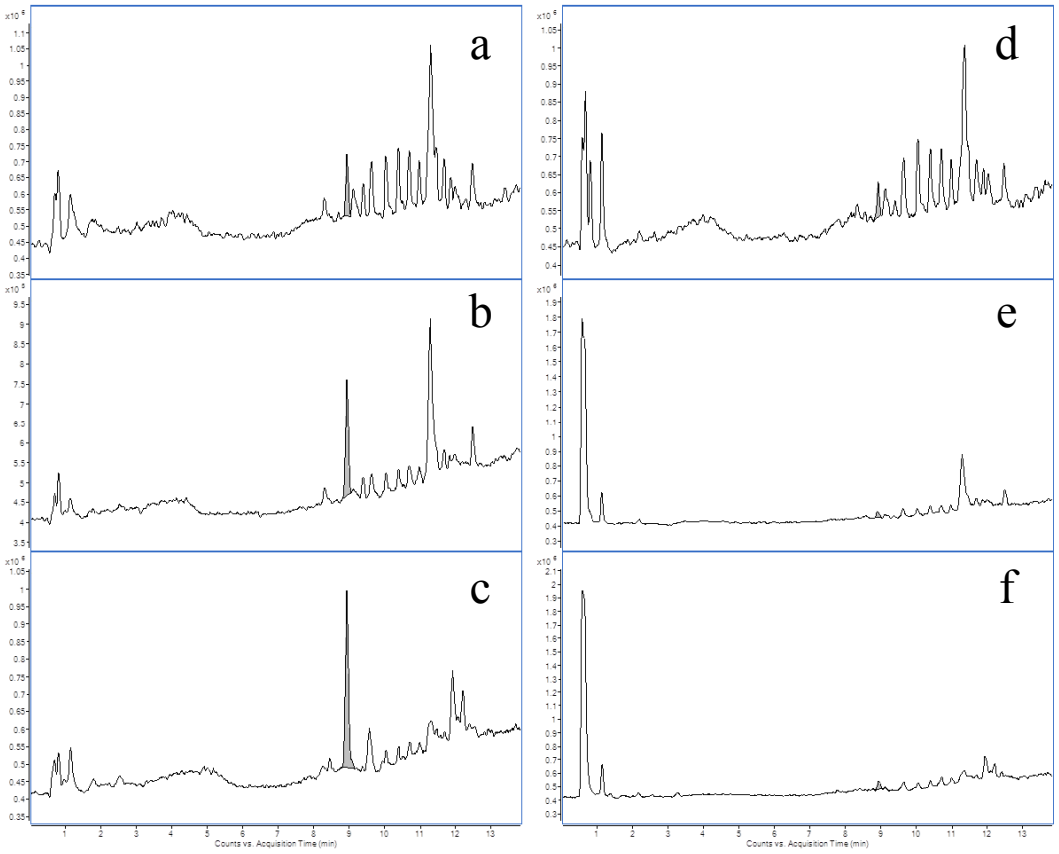


Figure 6-2: TIC chromatograms of morphine spiked with 20 mM PCC in water (a) one day, (b) three days and (c) five days after reaction commencement; and TIC chromatograms of a parallel set of samples containing 100 mM PCC (d) one day, (e) three days and (f) five days following adulteration. R_t of product m/z 316 is 8.9 min.

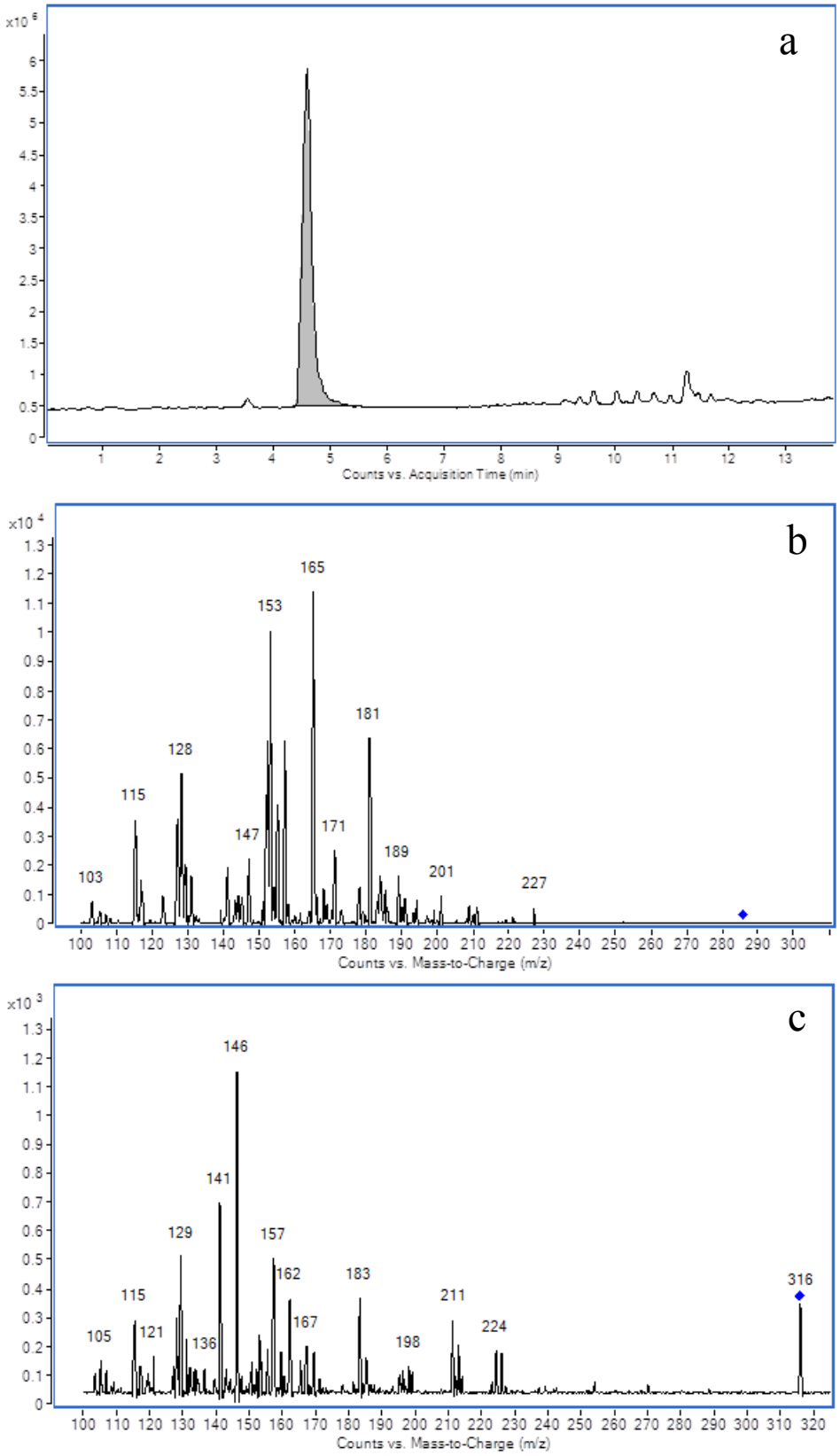


Figure 6-3: (a) TIC chromatogram and (b) CID spectrum of morphine (Rt = 4.6 min), and (c) CID spectrum of product m/z 316.

6.5.1.3 Reaction of 6-MAM to PCC in water

In line with the findings obtained for morphine and PCC, no reactions between 6-MAM and the lower concentrations of PCC trialled (0.02-2 mM) were observed. On the other hand, exposure of 6-MAM to 20 mM PCC resulted in its conversion to two products; *m/z* 360 and *m/z* 358 eluting at 6.2 min and 12.7 min, respectively. These products were detected one hour subsequent to 6-MAM exposure to the oxidant, and remained detectable in the samples at least to five days. Product *m/z* 358 was found in a 6-MAM sample exposed to 20 mM PCC three weeks later.

Comparison of the CID spectra of 6-MAM and products *m/z* 360 and *m/z* 358 showed that they are quite distinct and different from one another (Figure 6-4). However, it is interesting to note that product *m/z* 358 shares a similar fragmentation pattern to product *m/z* 316 (resulting from morphine exposure to PCC). The difference between *m/z* 358 and *m/z* 316 is 42 Da, which accounts for the acetyl group found on the 6-MAM structure. This suggests that product *m/z* 360 and *m/z* 358 may share a common fundamental molecular structure.

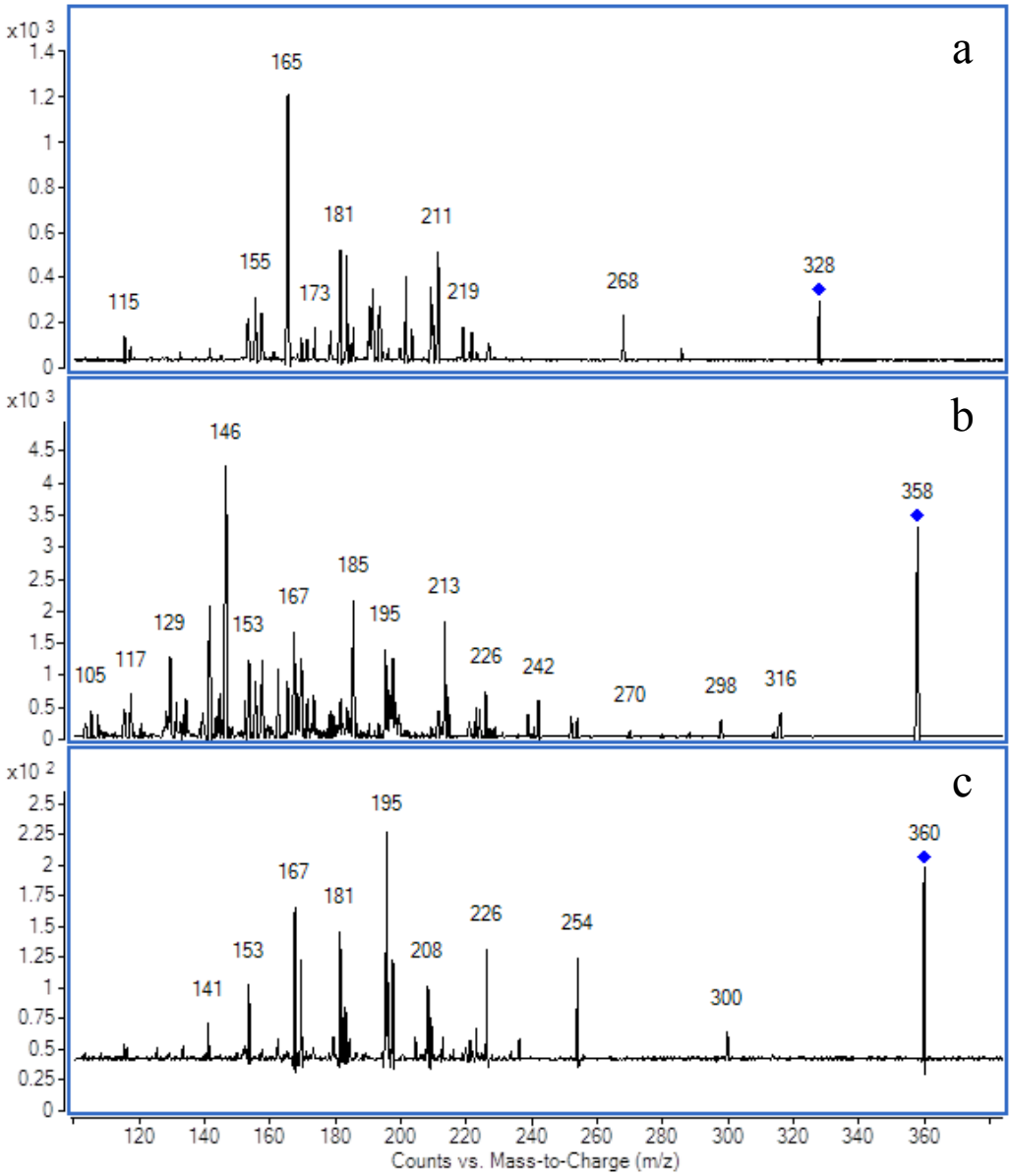


Figure 6-4: CID spectra of (a) 6-MAM, (b) product m/z 358 and (c) product m/z 360.

6.5.1.4 Effect of PCC on M6G in water

The trend of stable product formation observed for M3G, morphine and 6-MAM when exposed to PCC was not followed by M6G. Following repeated experiments, it was shown the reaction between M6G and PCC was erratic, with the detection of potential products not able to be reproduced on different days. The general trend was that no degree of reaction was found to have

taken place when the oxidant was spiked at the lower concentrations (0.02 and 0.2 mM). These samples were monitored over three weeks, and at the end of this period, M6G was still found to be in a significant abundance with no additional analyte peaks in the TIC chromatogram spectra. With 2 mM PCC, no reaction was observed two days following adulteration. However, analysis of the samples at three weeks showed that M6G was no longer detectable using the LC-MS conditions employed, with no other analyte peaks of interest present. By spiking M6G samples with 20 mM PCC, the opiate abundance was significantly diminished after the first hour, with no potential reaction products found. All samples were additionally analysed in negative ion mode in an attempt to detect reaction products. Nonetheless, no useful reaction products were located.

With the analyses of M6G specimens fortified with 0.02-2 mM PCC, the presence of an analyte peak consistent with the retention time and protonated mass of morphine was present in the traces. It was initially believed that morphine was produced via acid facilitated cleavage of the glucuronic acid entity off M6G. This idea was viable since PCC causes a significant decrease of pH in water (from pH 6-7 to pH 3). However, experiments where M6G standards were acidified using HCl (pH 3) and the samples analysed by LC-MS did not indicate M6G hydrolysis had occurred to produce morphine. Therefore, it is hypothesised that morphine was produced via reaction between M6G and PCC (in a similar manner observed for C6G and PCC). Since the extent of possible conversion of M6G to morphine appears to be quite insignificant, and with no additional reaction products able to be detected, no further investigation was justified into the effect of PCC on M6G.

6.5.2 Exposure of M3G, morphine and 6-MAM to PCC in urine

Based on the findings of section 6.5.1, MS parameters required for the acquisition of CID spectra for the opiates (M3G, morphine and 6-MAM) and the reaction products of interest were established (Table 6-1). Product ion scan analyses were used to ascertain if the same reaction products formed in water was also formed in urine.

Table 6-1: MS parameters used for the acquisition of CID spectra for the opiates and their respective reaction products.

Analyte	Precursor ion (*m/z*)	Collision energy (eV)
M3G	462	45
Product *m/z* 460	460	30
Morphine	286	50
Product *m/z* 316	316	40
6-MAM	328	40
Product *m/z* 358	358	40
Product *m/z* 360	360	35

6.5.2.1 Search for product m/z 460 in urine spiked with M3G and exposed to PCC

The observations following the exposure of M3G positive urine specimens to PCC in urine were expected, and in line with what were found to occur in water. Product *m/z* 460 was formed at both 20 mM and 100 mM PCC concentrations trialled, with a greater extent of transformation with the latter concentration. Figure 6-5 (a-c) shows the TIC chromatograms obtained for the analysis of M3G positive urine fortified with 20 mM PCC over the one week period. Although M3G remains the predominant analyte in the mixture, product *m/z* 460 was also present as a minor product with its initial detection at one day after reaction commencement. At 20 mM PCC fortification, it appears as though the abundance of product *m/z* 460 is at its maximum at approximately four days after adulteration (Table 6-2).

On the contrary, adulteration of M3G positive urine with 100 mM PCC resulted in a quicker, more extensive conversion of M3G to product *m/z* 460. Product *m/z* 460 was initially detected one hour following adulteration, with its presence as a major analyte commencing at four days (Figure 6-5 (d-f) and Table 6-2). Once it has formed, this reaction product was shown to be adequately stable in PCC adulterated urine for at least approximately one week. Therefore, its relative stability increases its potential to be used as a marker for monitoring M3G in PCC adulterated urine specimens.

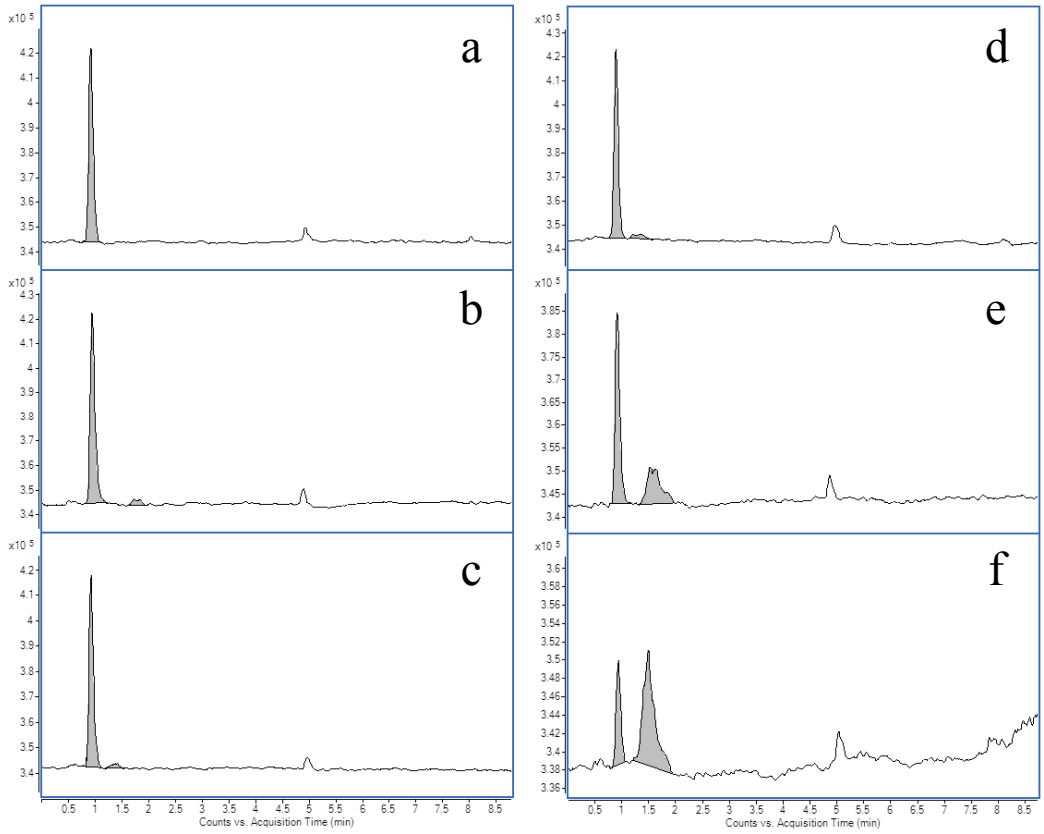


Figure 6-5: TIC chromatograms (product ion scan) of M3G exposed to 20 mM PCC in urine (a) one hour, (b) one day and (c) one week elapsed since the commencement of the reaction; and M3G reaction to 100 mM PCC in urine (d) one hour, (e) one day and (f) one week following adulteration.

Note: Rt ranges for M3G and m/z 460 are 0.90-0.92 min and 1.3-1.7 min, respectively.

Table 6-2: Relative peak areas of analytes (normalised to the most abundant analyte (100%)) detected in the M3G adulterated samples after one and five hours and one, two, four and seven days elapsed since the commencement of the reaction using LC-MS (product ion scan) analysis.

	20 mM PCC		**100 mM PCC**	
	M3G	Product *m/z* 460	M3G	Product *m/z* 460
1 h	100	-	100	6.55
5 h	100	-	100	10.59
1 day	100	3.46	100	61.68
2 days	100	5.20	100	68.54
4 days	100	7.46	66.83	100
7 days	100	6.35	39.27	100

6.5.2.2 Search for product m/z 316 in urine spiked with morphine and exposed to PCC

PCC adulteration of morphine positive urine resulted in the formation of the *m/z* 316 product, however only with the higher concentration of PCC. At a final concentration of 100 mM PCC, morphine was not detectable at one hour after reaction. At this point, no product was observed to have formed. At five hours, neither morphine nor product *m/z* 316 was detected in the specimen. At the one day time point subsequent to adulteration, only product *m/z* 316 was detected; this remained the case by the end of the one week monitoring period. Similar to product *m/z* 460, its abundance was found to peak at four days following PCC adulteration (Figure 6-6). During this time, spiked mixed standards were concurrently analysed at each time point of analysis to ensure that the instrument response did not fluctuate significantly (to allow for a qualitative comparison of peak area abundances from day to day).

A possible reason for the delay (which was found to sometimes vary from urine to urine) in observing product *m/z* 316 in the specimen could be due to the conversion of morphine to an intermediate reaction product prior to the formation of product *m/z* 316. This explains why neither morphine nor the reaction product of interest could be detected until one day after the reaction had occurred. Revisiting the results of the corresponding experiments where water was used as the matrix, numerous minor products were observed during this timeframe. However, they were deemed not suitable as ‘markers’ as they

appeared to be quite transient; they did not always form and when they did, repeated experiments subsequently carried out did not result in the same combination of possible minor products. Product *m/z* 316 was of further interest because it was always formed.

Another possible explanation for the delay in detection of product *m/z* 316 in urine is that the conversion of morphine to its reaction products by PCC may follow different reaction mechanisms in urine when compared to water. This idea is further highlighted by the monitoring of the morphine positive urine specimen fortified with 20 mM PCC. The morphine abundance was found to steadily decrease over the first two days, and was no longer detectable coincidently on day four (coincidental because the abundance of product *m/z* 316 was observed to peak at day four). However, no formation of product *m/z* 316 was found to occur at all during the one week monitoring period.

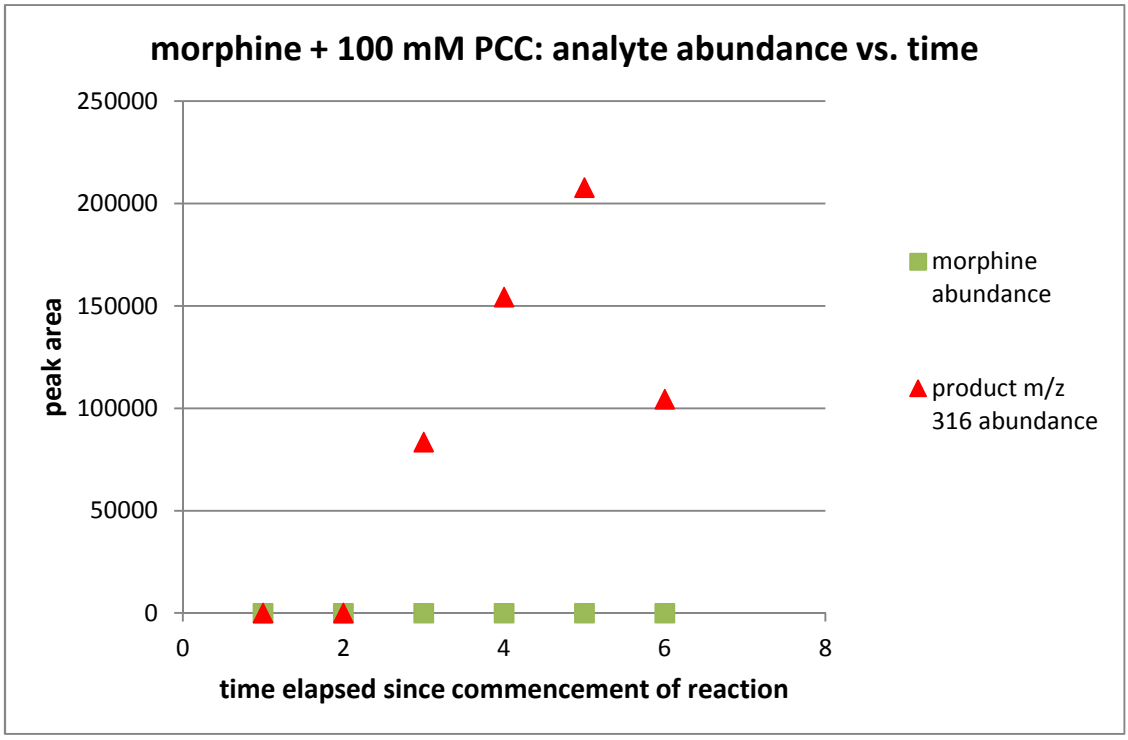


Figure 6-6: Plot of morphine and product m/z 316 abundance in urine adulterated with 100 mM PCC.

6.5.2.3 Search for product m/z 358 and m/z 360 in urine spiked with 6-MAM and exposed to PCC

The observations for the reaction of 6-MAM with PCC in urine further demonstrated the hypothesis that the route of formation of reaction products may be different in water compared to urine. In the 6-MAM positive urine specimen fortified with 20 mM PCC, a decrease in 6-MAM abundance was observed over the first four days, yet no reaction product of interest was detected until the fourth day. However, another perspective is that there are endogenous species in the urine competing for reaction with PCC. The reaction over the one week period does appear to be progressing slower, with 6-MAM still the predominant analyte at the fourth day and only product *m/z* 360 detectable in the specimen (Table 6-3).

On the contrary, the exposure of 6-MAM to 100 mM PCC in urine resulted in the detection of both products *m/z* 358 and *m/z* 360, as expected. From the first time point of analysis of one hour after reaction commencement, 6-MAM was no longer detectable in the specimen. The two reaction products of interest were detected, with product *m/z* 360 as the major analyte. Over the course of the one week period this remained the case, with the abundance of product *m/z* 358 peaking on the second day (Table 6-3 and Figure 6-7).

One observation of notable interest was the detection of a second reaction product with a protonated molecule at *m/z* 360. Eluting at 6.48 min, this reaction product was present at significant abundance at both one and five hours following 6-MAM reaction with PCC (Figure 6-8a). However, it was no longer detectable at one day. Comparison of its mass spectrum and fragmentation pattern with that belonging to product *m/z* 360 at 5.17 min indicates that they are different compounds (Figure 6-8 (b-c)). It is likely that product *m/z* 360 at 6.48 min is another reaction product, however further investigation was not pursued since it cannot be detected after one day into the monitoring period. In addition, this reaction product was not detected in the corresponding experiments conducted in water. This once again suggests that the reaction kinetics of 6-MAM and PCC may be different in water and in urine.

Table 6-3: Relative peak areas of analytes (normalised to the most abundant analyte (100%)) detected in the 6-MAM adulterated samples after one and five hours and one, two, four and seven days elapsed since commencement of the reaction using LC-MS (product ion scan) analysis.

	20 mM PCC			**100 mM PCC**		
	6-MAM	**product *m/z* 358**	**product *m/z* 360**	**6-MAM**	**product *m/z* 358**	**product *m/z* 360**
1 h	100	-	-	-	7.80	100
5 h	100	-	-	-	7.86	100
1 day	100	-	-	-	36.25	100
2 days	100	-	-	-	88.45	100
4 days	100	-	17.42	-	52.19	100
7 days	-	-	100	-	41.93	100

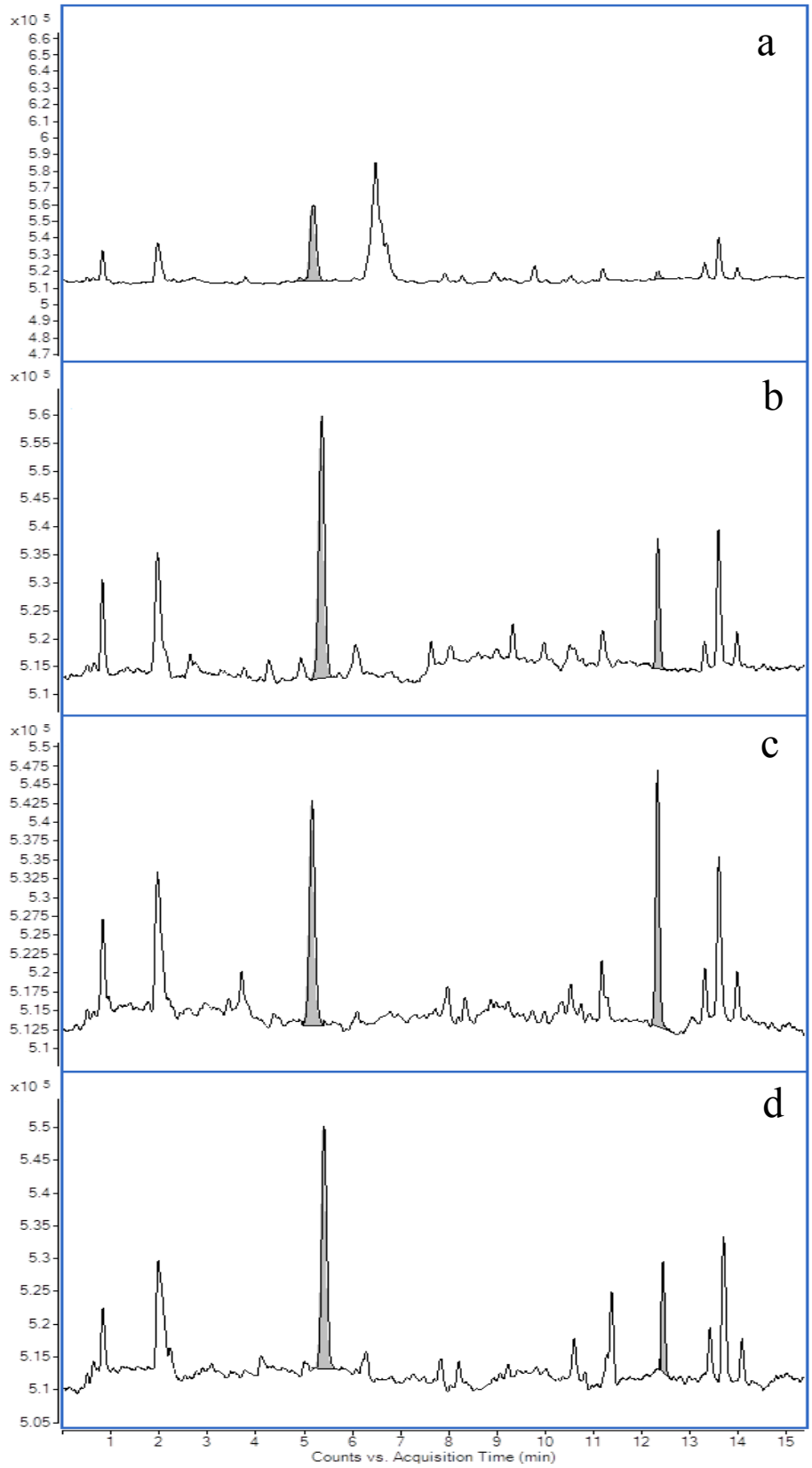


Figure 6-7: TIC chromatograms (product ion scan) of 6-MAM exposed to 100 mM PCC in urine (a) one hour, (b) one day (d) two days and (d) seven days following adulteration.

Note: Rt ranges for products m/z 360 and m/z 358 are 5.14-5.38 min and 12.33-12.45 min, respectively.

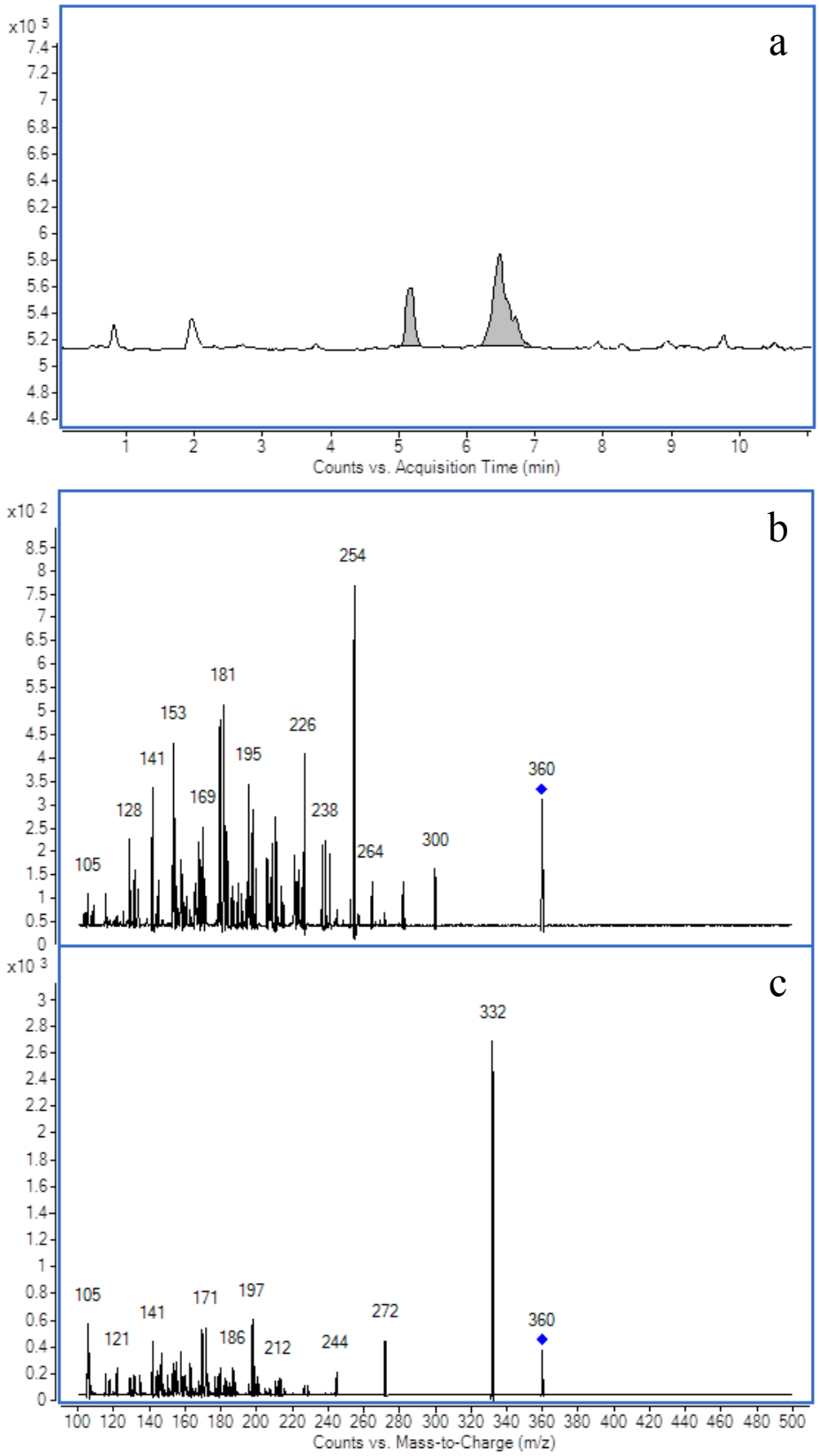


Figure 6-8: (a) Close-up of the TIC chromatogram in Figure 6-7a of products m/z 360 at 5.17 and 6.48 min at one hour after reaction of 6-MAM and PCC in urine; (b) CID spectrum of product m/z 360 at 5.17 min and (c) CID spectrum of product m/z 360 at 6.48 min.

6.5.3 Structural elucidation of the reaction products

6.5.3.1 Characterisation of product m/z 460

Based on previous findings regarding the formation of codeinone, a major reaction product resulting from the exposure of codeine to PCC, product *m/z* 460 was hypothesised to be morphinone-3-glucuronide (Figure 6-9). This is the most likely scenario, and is consistent with the mass loss of 2 Da when comparing the formula masses of the M3G starting material and the reaction product of interest.

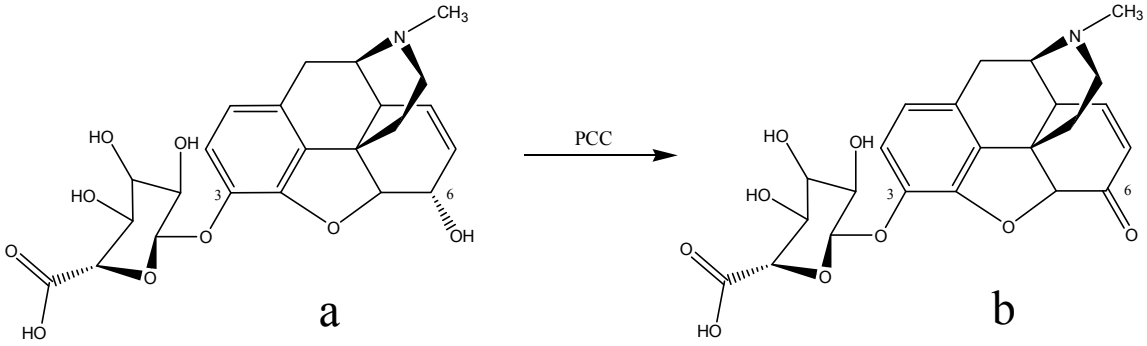


Figure 6-9: Reaction scheme showing the conversion of (a) M3G to (b) morphinone-3-glucuronide in the presence of PCC in urine.

High resolution mass spectrometry analysis of product *m/z* 460 supported the formation of morphinone-3-glucuronide. The protonated parent molecule at $[M+H]^+$ = 460.1600 matched a protonated molecule with a formula mass of $C_{23}H_{25}NO_9$ (-0.43 ppm). The ammonium and potassium adducts were also found at *m/z* 477.1863 (1 ppm) and *m/z* 498.1156 (1.07 ppm), respectively. The CID spectrum of product *m/z* 460 was not abundant with peaks, with only three observed at *m/z* 460.1600, *m/z* 284.1287 and *m/z* 227.0711 (Figure 6-10). The first mass loss from *m/z* 460.1600 to *m/z* 284.1287 corresponded to the loss of $C_6H_8O_6$, which is characteristic of the loss of the glucuronic acid group from the C-3 position. This confirms that the site of oxidation is not on the glucuronic acid entity, because if this had occurred, a loss of $C_6H_6O_6$ would be expected. The proposed product ions are shown in Figure 6-11 with

protonated morphinone suggested for the *m/z* 284.1287 product ion. Following the subsequent loss of the amine bridge (C_3H_7N) from the protonated morphinone molecule, the *m/z* 227.0711 product ion is formed.

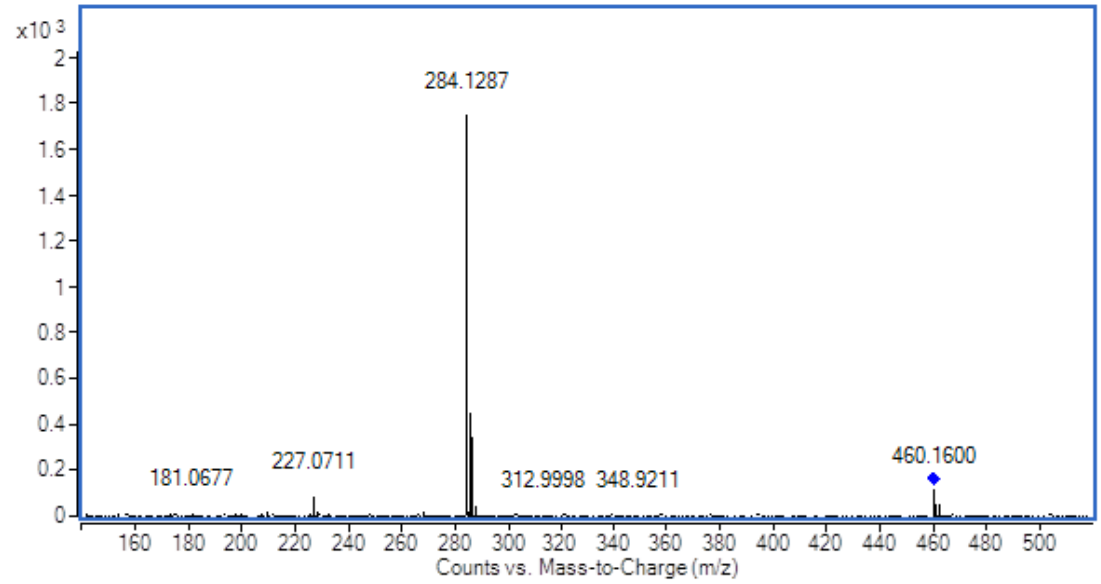


Figure 6-10: High resolution CID spectrum of product m/z 460, morphinone-3-glucuronide (FE = 200 V, CE = 30 eV).

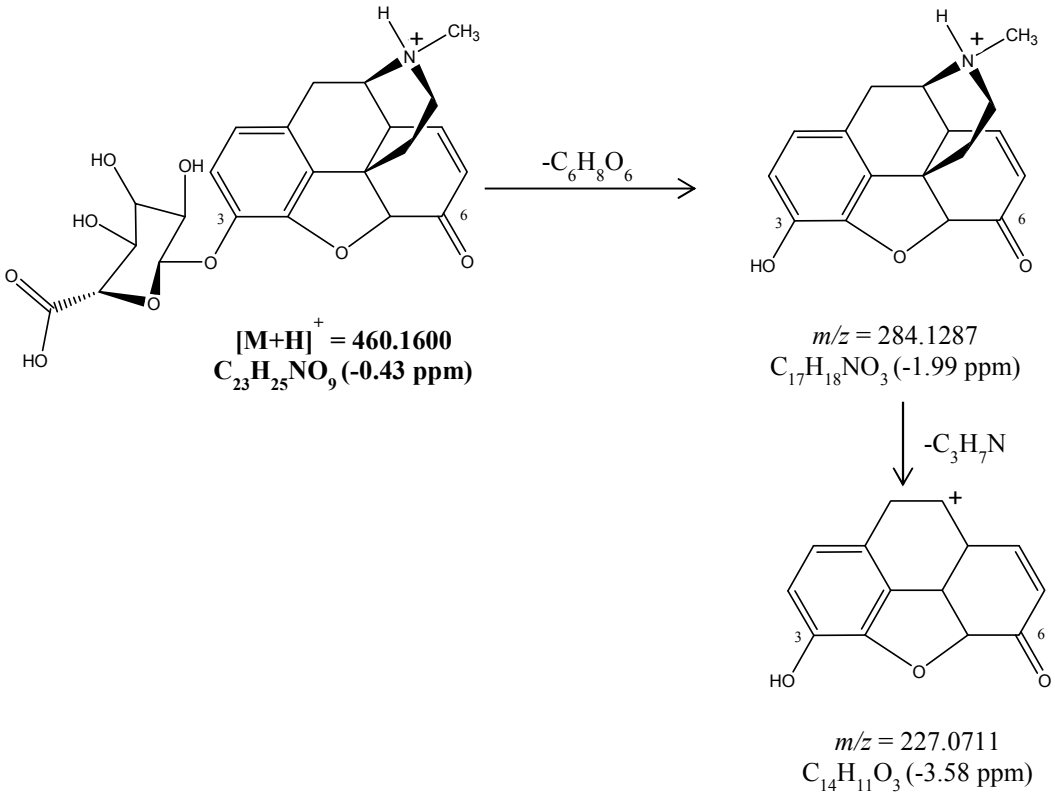


Figure 6-11: Proposed mass fragmentation pathways for product m/z 460, morphinone-3-glucuronide.

6.5.3.2 Characterisation of product m/z 358 and product m/z 360

At the initial stages of the investigation, product *m/z* 360 was hypothesised to be a hydroxylated species of 6-MAM (Figure 6-12a), with the hydroxylation occurring at two sites on the structure (most likely sites being at C-7, C-8 and C-14). With information derived from the high resolution mass spectrometry analysis experiments and previous elucidation of one of the codeine reaction products to be 14-hydroxycodeinone, product *m/z* 360 is tentatively assigned as 7,14-dihydroxy-6-monoacetylmorphine (7,14-dihydroxy-6-MAM, Figure 6-12b).

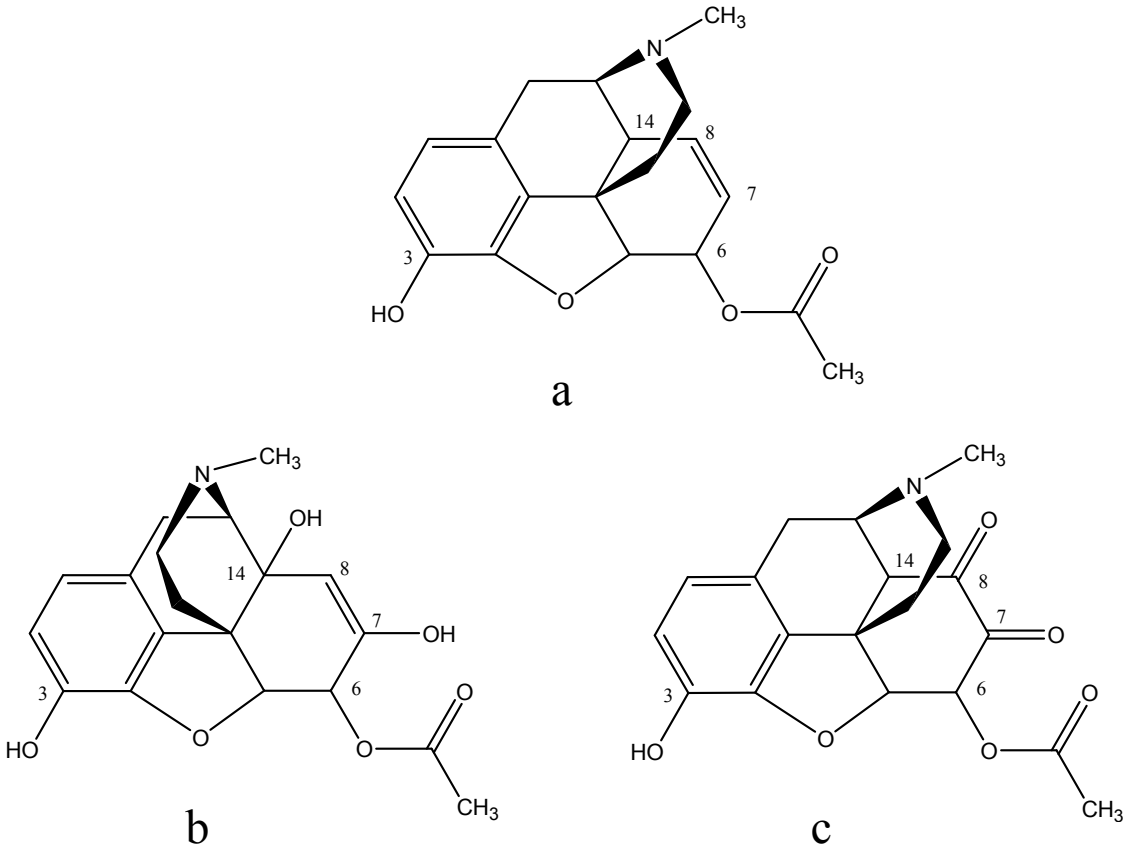


Figure 6-12: Molecular structure of (a) 6-MAM and the proposed structures for (b) product m/z 360 (7,14-dihydroxy-6-MAM) and (c) product m/z 358.

The formula mass of the protonated molecule of product *m/z* 360 was found to be 360.1441, corresponding to a protonated species with a molecular formula of $C_{19}H_{21}NO_6$ (-0.32 ppm). Subsequent to the loss of the characteristic acetyl

group (from *m/z* 360.1441 to *m/z* 300.1235), there is a loss of water observed. This is similar to what was seen for 14-hydroxycodeinone, supporting the presence of an –OH group at the C-14 position. The second –OH group is proposed to be substituted at the C-7 carbon to minimise steric hindrance in the molecule. The literature has shown that formation of 8,14-dihydroxy codeine analogues are possible, however the double bond between C-7 and C-8 is no longer present in these cases [152]. This is unlikely for product *m/z* 360 based on MS results. Figure 6-13 shows the distinct fragmentation pattern belonging to product *m/z* 360, with product ions in Figure 6-14 shown to be consistent with the fragmentation pathways expected for 7,14-dihydroxy-6-MAM.

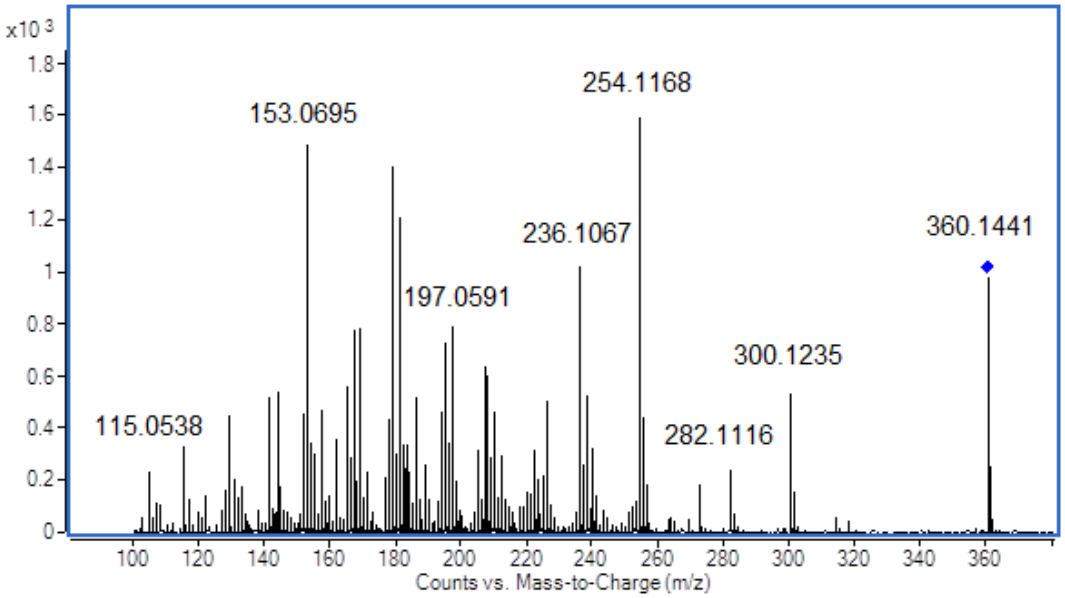


Figure 6-13: High resolution CID spectrum of product m/z 360 (FE = 170 V, CE = 35 eV).

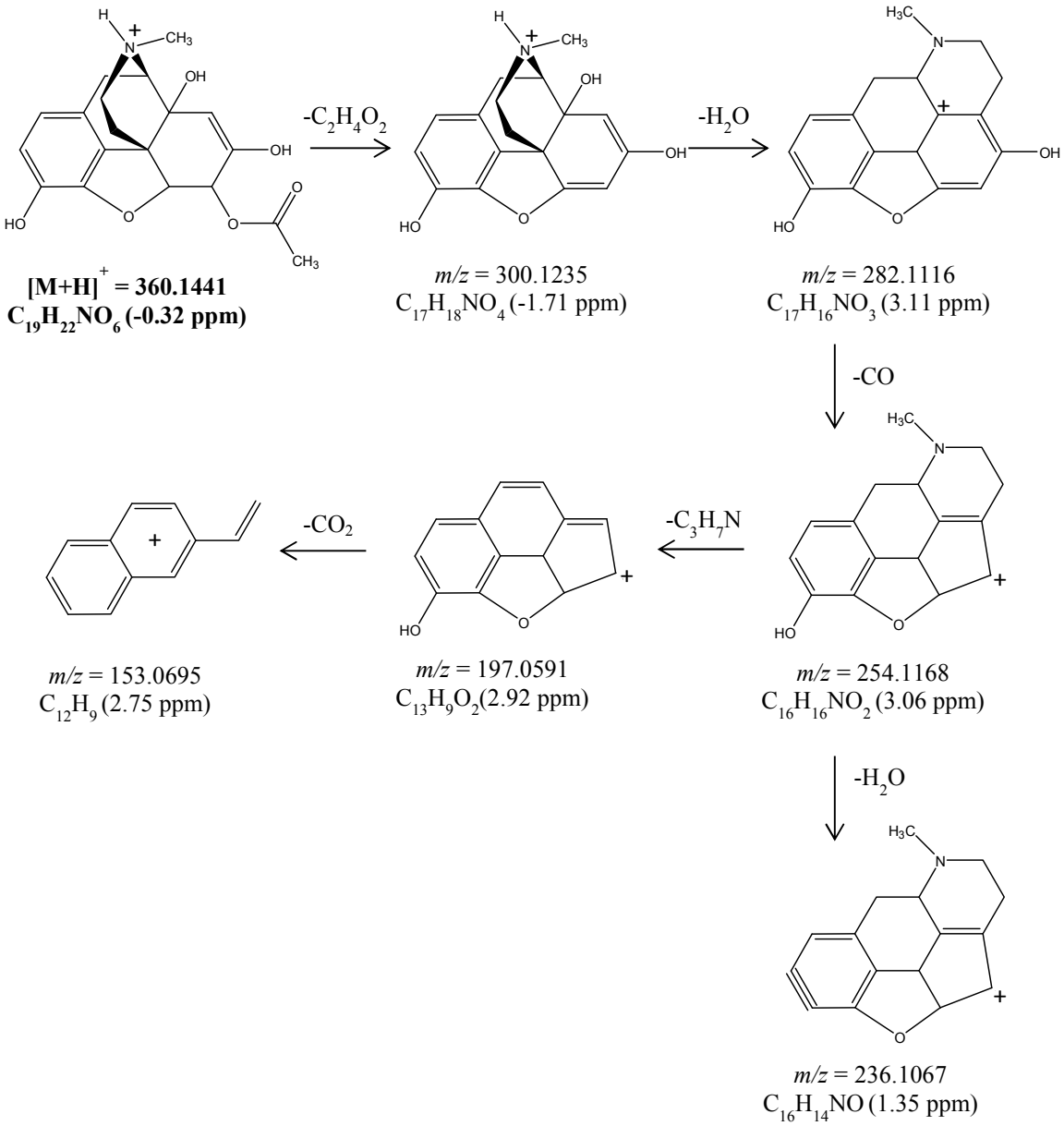


Figure 6-14: Proposed MS fragment ions and fragmentation pathways for product m/z 360, 7,14-dihydroxy-6-MAM.

High resolution mass spectrometric analysis of product *m/z* 358 indicated a strong match to a protonated molecule with the formula $C_{19}H_{19}NO_6$ (-0.53 ppm). Compared to the molecular formula of the 6-MAM starting material ($C_{19}H_{21}NO_4$, structure shown in Figure 6-12a), product *m/z* 358 contains two additional oxygen atoms with a loss of two hydrogen atoms from the structure. The most likely case would be the formation of the reaction product that is

proposed in Figure 6-12c, with the sites of reaction for carbonyl (C=O) formation at C-7 and C-8. The corresponding CID spectrum (Figure 6-15) and the resulting proposed fragmentation pathways (Figure 6-16) are consistent with the molecular structure hypothesised for product *m/z* 358.

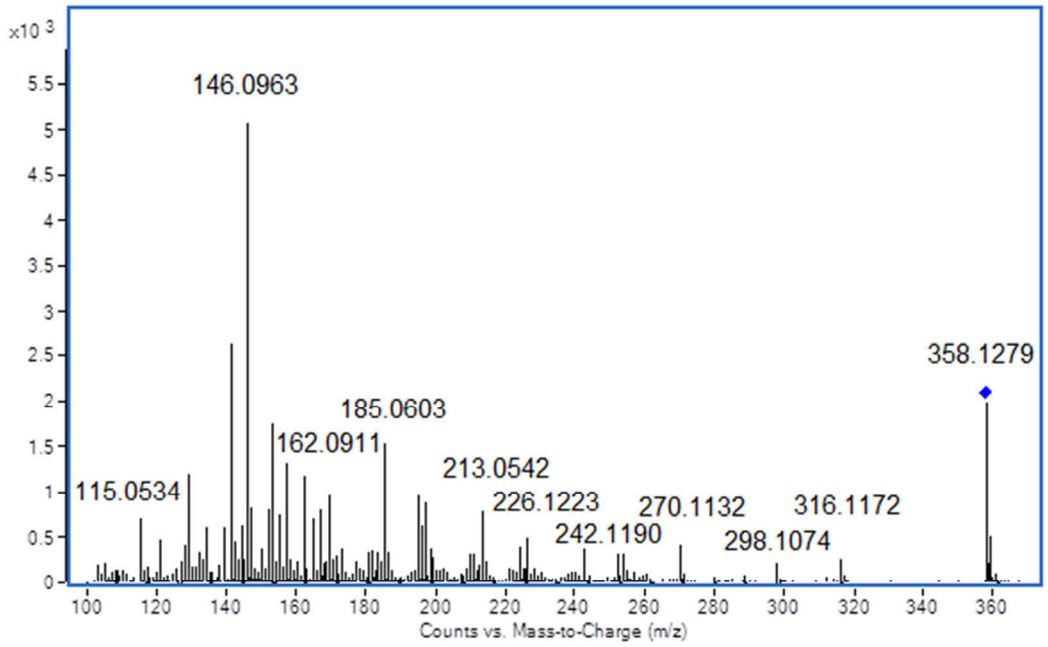


Figure 6-15: High resolution CID spectrum of product m/z 358 (FE = 170 V, CE = 40 eV).

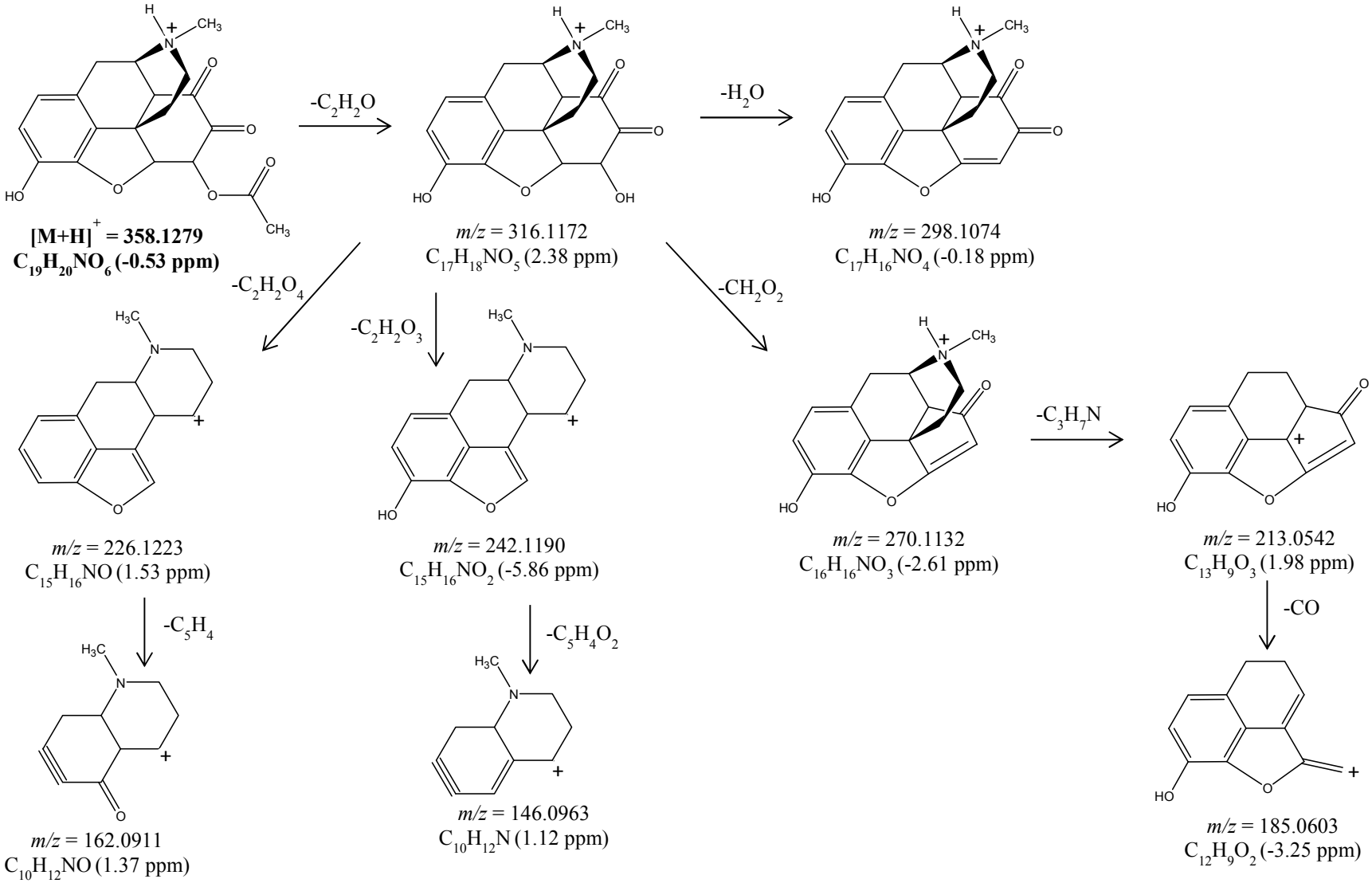


Figure 6-16: Proposed MS fragment ions and fragmentation pathways for product m/z 358.

6.5.3.3 Characterisation of products m/z 316

Inspection of the CID spectrum obtained for product *m/z* 316 (Figure 6-17) showed that the product ion peaks (position and relative ratios) were very similar to those observed in the CID spectrum for product *m/z* 358 (see section 6.5.3.2). Therefore, it is proposed that morphine (Figure 6-18a) is converted to its keto analogue (Figure 6-18b). The proposed product ions are the same as those suggested in Figure 6-16 however with *m/z* 316 as the protonated parent molecule. Table 6-4 displays the mass accuracy differences between the actual mass of the product ions observed, compared to the calculated mass of the proposed fragments.

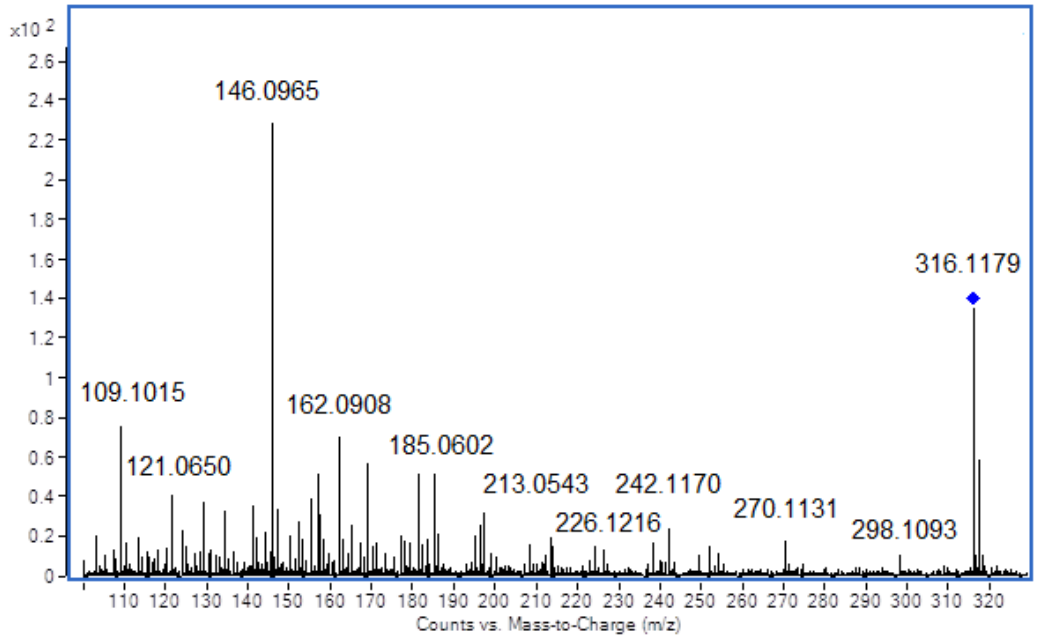


Figure 6-17: High resolution CID spectrum of product m/z 316 (FE = 170 V, CE = 35eV).

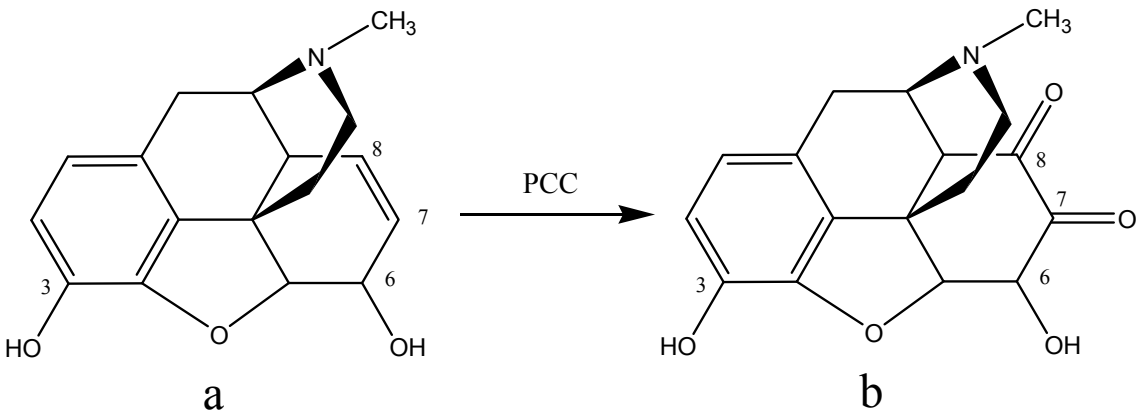


Figure 6-18: molecular structure of (a) morphine and (b) the proposed structure for product m/z 316.

Table 6-4: Proposed product ions and corresponding mass accuracy differences for the peaks observed in the CID spectrum of product m/z 316.

	molecular formula of proposed product ion	**mass accuracy difference (ppm)**
$[M+H]^+$ = 316.1179	$C_{17}H_{18}NO_5$	-1
***m/z* 298.1093**	$C_{17}H_{16}NO_4$	-6.4
***m/z* 270.1131**	$C_{16}H_{16}NO_3$	-2.2
***m/z* 242.1170**	$C_{15}H_{16}NO_2$	2.31
***m/z* 226.1216**	$C_{15}H_{16}NO$	4.57
***m/z* 213.0543**	$C_{13}H_9O_3$	1.58
***m/z* 185.0602**	$C_{12}H_9O_2$	-2.58
***m/z* 162.0908**	$C_{10}H_{12}NO$	3.59
***m/z* 146.0965**	$C_{10}H_{12}N$	-0.84

6.5.4 Analysis of PCC adulterated authentic specimens using a routine toxicology laboratory

The overall outcome of this study reflects the results obtained in section 5.5.1.4. PCC was found to decrease the CEDIA immunoassay response (Table 6-5). In each case, the specimen adulterated with 20 mM PCC was found to have a decrease of approximately 300-400 ng/mL when compared to the corresponding unmodified specimen. Similarly, the immunoassay response determined for the specimen adulterated with 100 mM PCC was found to decrease by approximately half (compared to the unmodified specimen).

The GC-MS results tabulated in Table 6-6 shows that the morphine/codeine ratio decreases following PCC fortification of the specimens. The greater decrease is associated with the higher PCC concentration used. The morphine concentrations are also observed to be decreasing as the PCC concentration is higher. However, the discrepancy in morphine and codeine concentrations used to calculate the morphine/codeine ratios could have stemmed from the reaction of both native and internal standards of morphine and codeine with PCC. It is clear from the results in Figure 6-19 that morphine-d_6 in particular is susceptible to degradation or transformation by PCC. Analyte losses of up to 100% were observed in specimens containing either 20 mM or 100 mM PCC. On the contrary, codeine-d_6 losses were more variable in the PCC adulterated urine specimens. The specimens containing 20 mM PCC experienced less codeine-d_6 analyte loss when compared to the corresponding specimens fortified with 100 mM PCC, ranging from as low as 17% up to 99%. On the other hand, within the group of specimens containing 100 mM PCC, the percentage loss was observed to be more constant (greater than 98%).

Table 6-5: Immunoassay screening results for the unmodified blank (B1, B2) and opiate positive (U1-U4) urine specimens and the corresponding adulterated specimens with 20 mM and 100 mM PCC.

sample	opiate concentration (ng/mL)
B1	-10
B1 + 20 mM PCC	-30
B1 + 100 mM PCC	-90
B2	-10
B2 + 20 mM PCC	-30
B2 + 100 mM PCC	-70
U1	2620[a]
U1 + 20 mM PCC	2390 [a]
U1 + 100 mM PCC	1180 [a]
U2	2740 [a]
U2 + 20 mM PCC	2360 [a]
U2 + 100 mM PCC	1490 [a]
U3	2640 [a]
U3 + 20 mM PCC	2240 [a]
U3 + 100 mM PCC	1470 [a]
U4	2680 [a]
U4 + 20 mM PCC	2340 [a]
U4 + 100 mM PCC	1370 [a]

[a]above opiate screening cut-off concentration of 300 ng/mL.

Table 6-6: GC-MS confirmatory results for the unmodified blank (B1, B2) and opiate positive (U1-U4) urine specimens and the corresponding adulterated specimens with 20 mM and 100 mM PCC.

sample	morphine concentration (ng/mL)	codeine concentration (ng/mL)	morphine/ codeine ratio
B1	0	0	n/a
B1 + 20 mM PCC	0	0	n/a
B1 + 100 mM PCC	0	0	n/a
B2	0	0	n/a
B2 + 20 mM PCC	0	0	n/a
B2 + 100 mM PCC	0	0	n/a
U1	13757	993	13.85
U1 + 20 mM PCC	5108	1446	3.53
U1 + 100 mM PCC	162	665	0.24
U2	22594	10994	2.06
U2 + 20 mM PCC	1239	6203	0.20
U2 + 100 mM PCC	206	2875	0.07
U3	9177	757	12.12
U3 + 20 mM PCC	6175	773	7.99
U3 + 100 mM PCC	0	446	n/a[a]
U4	2948	18367	0.16
U4 + 20 mM PCC	3028	20851	0.15
U4 + 100 mM PCC	247	6275	0.04

[a]no ratio given as morphine concentration was not able to be calculated due to complete loss of morphine-d_6.

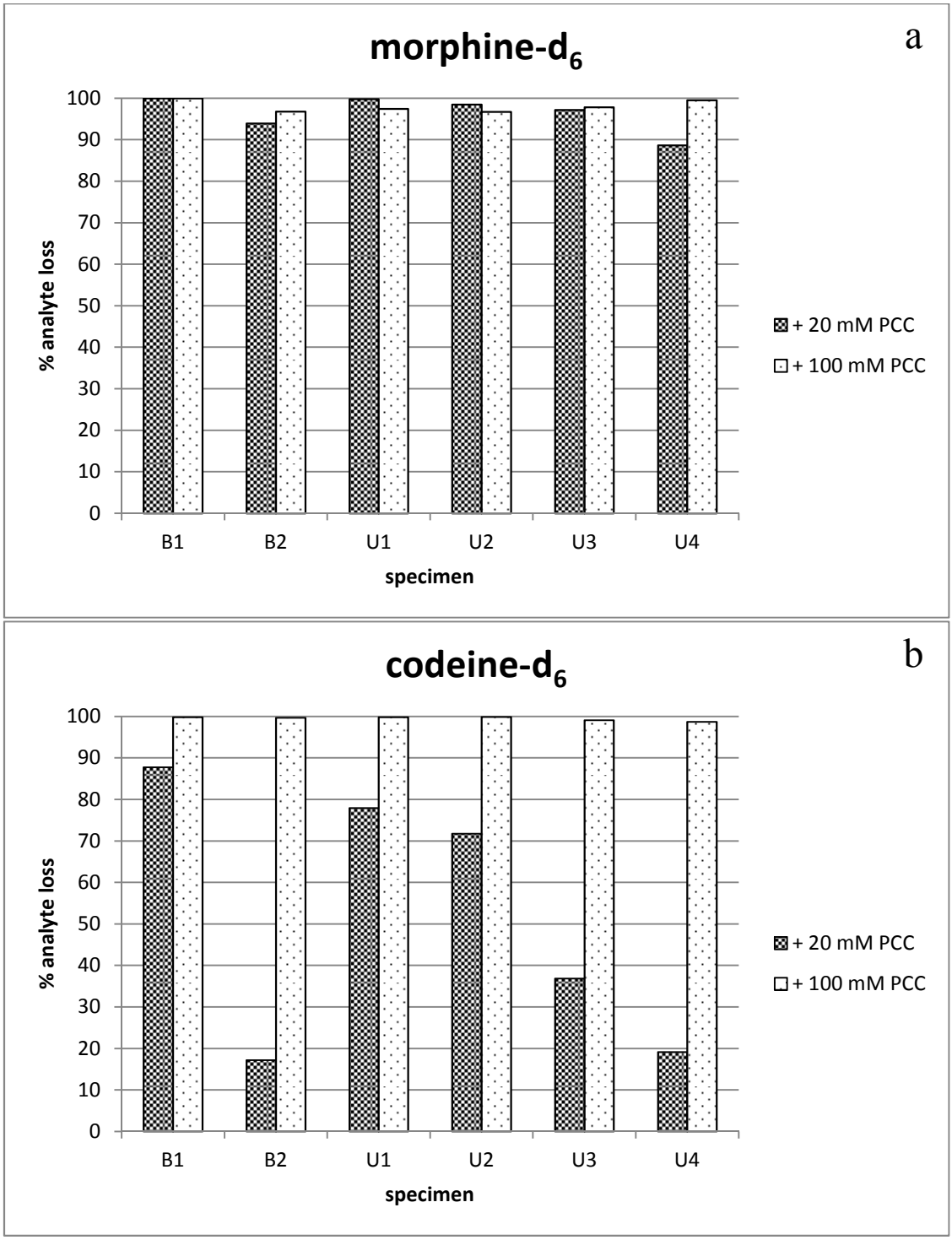


Figure 6-19: Plot of (a) morphine-d_6 loss and (b) codeine-d_6 loss in two urine blank specimens and four opiate positive urines adulterated with PCC (analyte loss is expressed as a percentage relative to morphine-d_6 and codeine-d_6 abundance in the corresponding unadulterated specimen).

6.6 Conclusions

The adulteration of opiate positive (M3G, morphine and 6-MAM) urine specimens with PCC (20 and 100 mM) resulted in the formation of numerous reaction products, with only four of them chosen for structural elucidation. These products were stable enough to be monitored by LC-MS, and once formed, detectable for at least approximately one week. However, the time taken for product formation appears to be quite variable between water and urine. In addition, there appears to be some disparity between different urine specimens as well.

With previous studies conducted using the same experimental design, the outcomes were always reproducible in the sense that reaction products either formed or did not; if formation did occur in a certain matrix, then they always formed with respect to that matrix regardless of its source or age. Additionally, the trend appearing with previous similar studies is that the lower the oxidant concentration, the longer it takes for reaction product formation to occur. Furthermore, the rate of reaction appeared to always be slower in urine due to the competing endogenous species that are present. Irrespective of these two influencing factors, the same route of reaction still occurred. This did not appear to be the case for the reaction of M3G, morphine and 6-MAM with PCC in urine, and the reason for this remains unknown.

The outcome of this study does suggest that the reaction kinetics observed for these substrates may be more complicated, and that the experimental design used so far may not be adequate. Therefore, further research is warranted to investigate the possibility of additional reaction products that are formed in urine, but not in water. Also, the cause for the loss (degradation of oxidation) of morphine-d_6 and codeine-d_6 when exposed to PCC should be explored. It can be deduced that should adulteration of opiate positive urines occur with PCC, it is likely that immunoassay testing would detect the drugs. However, the interpretation of the results obtained by GC-MS confirmatory testing may be quite difficult due to the loss of both native and internal standard species.

Chapter 7: Exposure of opiates to hypochlorite

Chapter 7: Exposure of opiates to hypochlorite

7.1 Introduction

Sodium hypochlorite is an easily attainable oxidising agent in the form of commercial bleach, and is widely sold at supermarkets. Bleach has a characteristic odour and is very alkaline, and so can easily be detected by smell and pH testing if added in copious amounts. However, it is suspected that only a very small amount of bleach is required for the effective concealment of drugs in a urine specimen, without arousing suspicion. Anecdotal evidence has suggested that individuals dip a finger or the drawstring of their trousers into bleach prior to the drug test, which is then swirled into the urine after it has been voided in the collection cup.

This study aims to expose morphine, codeine, 6-MAM, M3G and M6G to hypochlorite and determine if the oxidant is an effective masking agent for these opiates, and if so, to conclude whether any stable reaction products can be observed within the confines of the experimental design.

7.2 Materials

7.2.1 Drug standards and reagents

Morphine, codeine, 6-MAM, M3G and M6G opiate stock standards (1 mg/mL) were sourced from the same location as described in section 2.3.1.

Reagent grade sodium hypochlorite stock solution (10-15% w/v available chlorine) was purchased from Sigma Aldrich (Castle Hill, NSW, Australia). Three additional working solutions were prepared by serial dilution resulting in solutions with 5-7.5%, 2.5-3.75% and 1.25-1.875% w/v available chlorine.

7.2.2 Urine specimens

Opiate negative 'blank' urine from healthy donors were obtained and pooled as outlined in section 5.2.2.

7.3 Sample preparation

Each of the five drug standards was spiked in water to give a final concentration of 10 µg/mL in the 1 mL samples. A corresponding set of reaction mixture samples were prepared (n = 6 for each drug), with each of the opiates standards spiked with the four hypochlorite solutions to give mixtures with final oxidant concentrations of 1-1.5%, 0.5-0.75%, 0.25-0.375%, 0.125-0.1875%, 0.075-0.1125% and 0.025-0.0375% (w/v free chlorine; the latter two concentrations were spiked using the 2.5-3.75% (w/v free chlorine) hypochlorite working solution). In addition, four hypochlorite reagent controls were prepared where an equivalent volume of methanol was spiked into the sample instead of the opiate stock standard. The pH of the opiate reaction mixtures and the reagent controls were measured using Merck universal indicator paper (Darmstadt, Germany).

7.4 Analysis of opiate positive urine specimens spiked with hypochlorite by LC-MS

All samples prepared in section 7.3 were analysed one hour subsequent to oxidant exposure. This was carried out on the LC QQQ-MS in full scan mode using the chromatographic and MS parameters detailed in section 5.3.1 for this instrument. In addition, analyses of the samples were also conducted with the mass spectrometer configured in negative ion mode.

7.5 Results and discussion

As a starting point for the selection of a suitable hypochlorite concentration range for this study, the literature was initially consulted to determine the

hypochlorite concentration reported for bleach adulterated urine specimens. However, this information was quite difficult to obtain from a peer reviewed source and so the strength of commercial bleach was used as a guide. According to the commercial label for the strong strength bleach 'White King', the concentration is 4% w/v available chlorine, which decreases to 2% w/v at the expiry date. Other commercial disinfectant bleach products such as Jasol™ and Clorox® contain 12.5% w/v and 1-12% w/v (overall range for various products), respectively [160, 161]. Therefore, the overall hypochlorite concentration range of the working solutions used in this study was chosen to overlap with the ranges found in various commercial bleach products. The pH measurements of both the opiate positive samples and the respective reagent controls were found to be the same, and is summarised in Table 7-1. As these pH measurements were taken of reaction mixtures in water, they do not reflect the pH effect of hypochlorite on urine. These pH readings were only used as a guide for how hypochlorite affects the pH of the water samples relative to each other, at the various spiked concentrations.

Table 7-1: pH of the reaction mixtures with various concentrations of fortified hypochlorite.

	hypochlorite concentration (% w/v available chlorine)					
	1-1.5	**0.5-0.75**	**0.25-0.375**	**0.125-0.1875**	**0.075-0.1125**	**0.025-0.0375**
pH	11	11	10	10	10	9-10

In general, the exposure of morphine, codeine, 6-MAM, M3G and M6G to hypochlorite resulted in the loss of the opiate analytes in varying degrees at the hypochlorite concentrations trialled. Although several analyte peaks were observed in positive ion mode and suspected to belong to stable reaction products in some of the reaction mixtures, the results were not always

reproducible when the experiments were subsequently repeated. For all reaction mixtures, no reaction products were detected in negative ion mode.

The effect of hypochlorite on 6-MAM and morphine was found to be similar. Following exposure of these opiates to all hypochlorite concentrations, no 6-MAM or morphine were found in the samples under the analysis conditions employed. At spiked hypochlorite levels of 0.125-1.5% (w/v free chlorine), a possible reaction product was detected at 0.9 min, with *m/z* 328 (Figure 7-1 and Figure 7-2). Interestingly, this product was not observed in the 6-MAM reaction mixtures containing hypochlorite at the two lower concentrations spiked (0.075-0.1125% and 0.025-0.0375% (w/v free chlorine)). In these cases, two other reaction products were observed at 0.8 min (*m/z* 334) and 3.0 min (*m/z* 364), as shown in Figure 7-3. The mass of the latter product indicates that it may have been formed following oxidation of 6-MAM, with a gain of the equivalent of two water molecules (H_4O_2). On the other hand, the exposure of morphine to the lower hypochlorite concentrations resulted in the detection of the product eluting at 0.9 min (*m/z* 328) in addition to another product at 3.0 min (*m/z* 354, Figure 7-4).

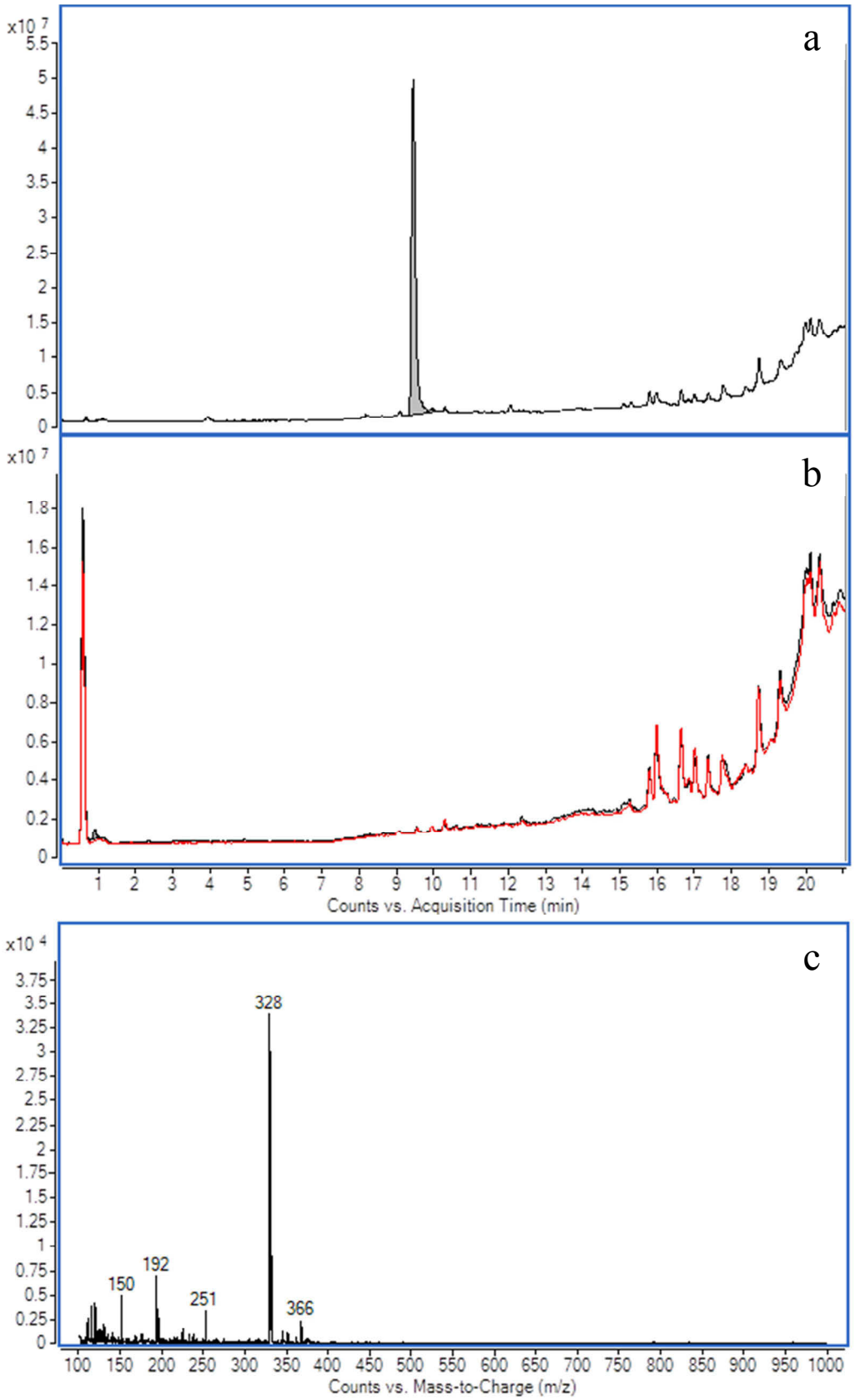


Figure 7-1: (a) TIC chromatogram of 6-MAM in water and (b)TIC chromatogram overlay of the 6-MAM + 0.5-0.75% (w/v available chlorine) hypochlorite solution and the corresponding reagent control, with the possible reaction product eluting at 0.9 min and (c) MS spectrum of the product eluting at 0.9 min.

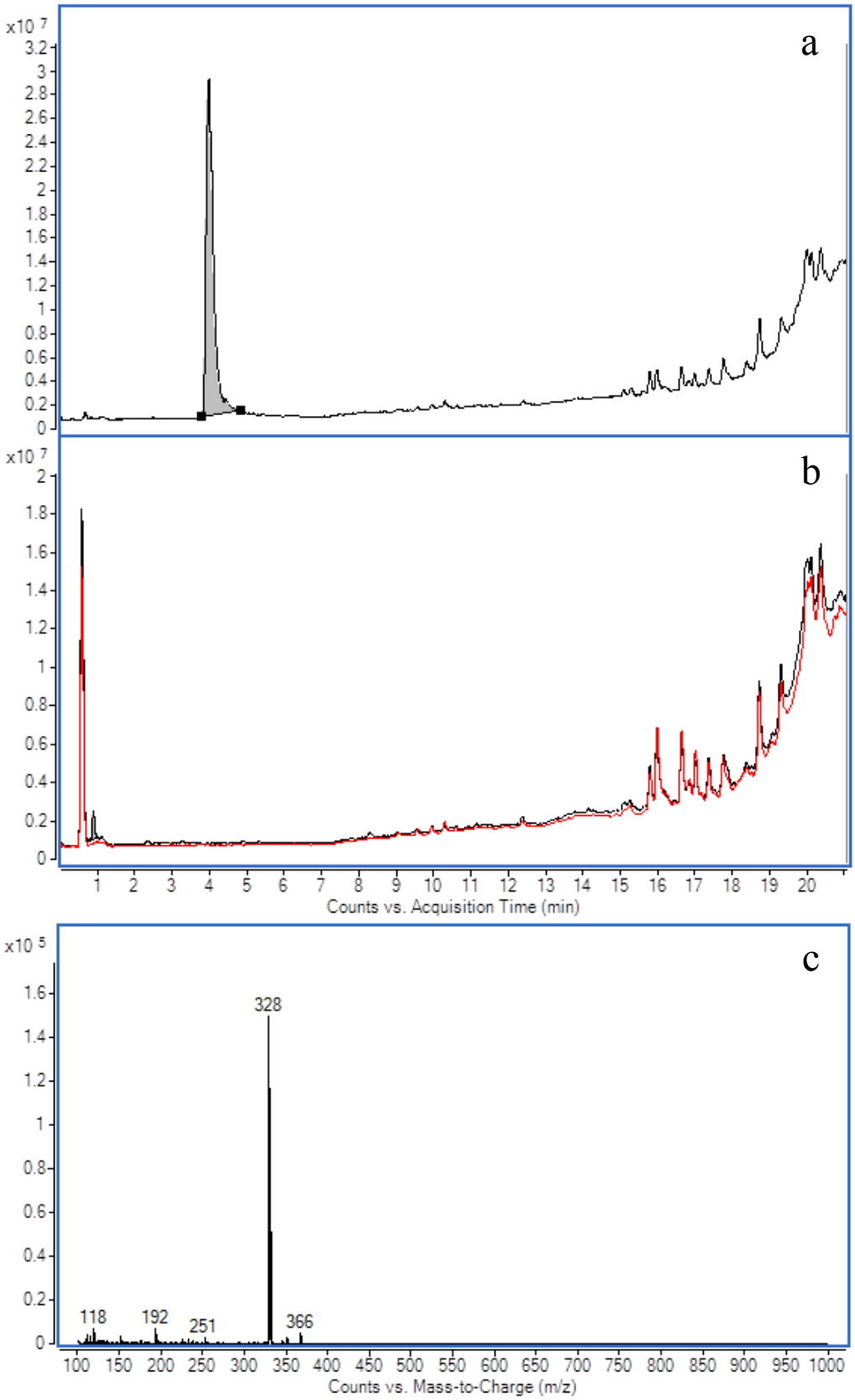


Figure 7-2: (a) TIC chromatogram of morphine in water and (b)TIC chromatogram overlay of the morphine + 0.5-0.75% (w/v available chlorine) hypochlorite solution and the corresponding reagent control, with the possible reaction product eluting at 0.9 min and (c) MS spectrum of the product eluting at 0.9 min.

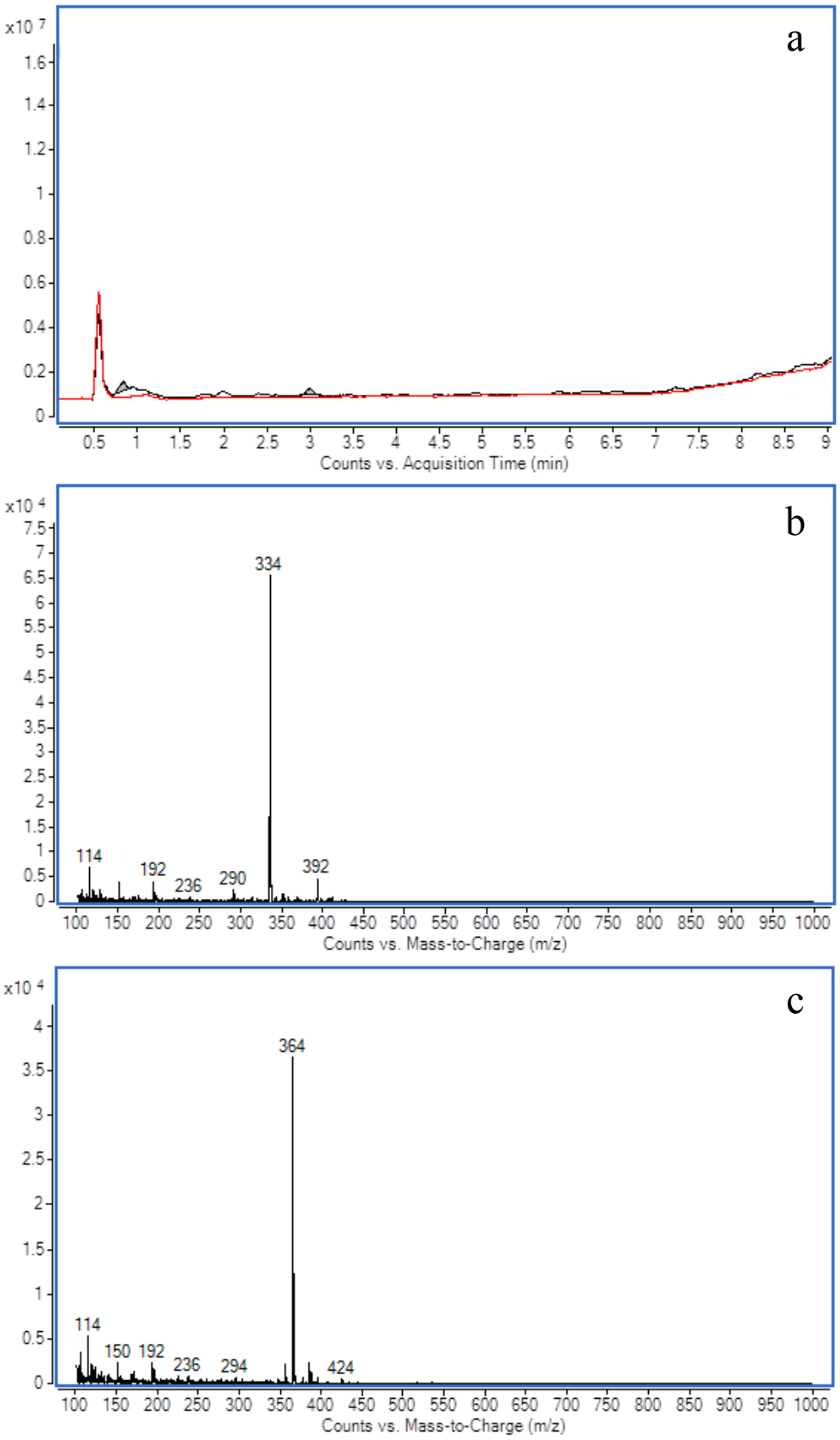


Figure 7-3: TIC chromatogram overlay of the 6-MAM + 0.025-0.0375% (w/v available chlorine) hypochlorite solution and the corresponding reagent control, with the possible reaction products eluting at 0.8 min and 3.0 min, (b) MS spectrum of the product eluting at 0.8 min and (c) MS spectrum of the product eluting at 3.0 min.

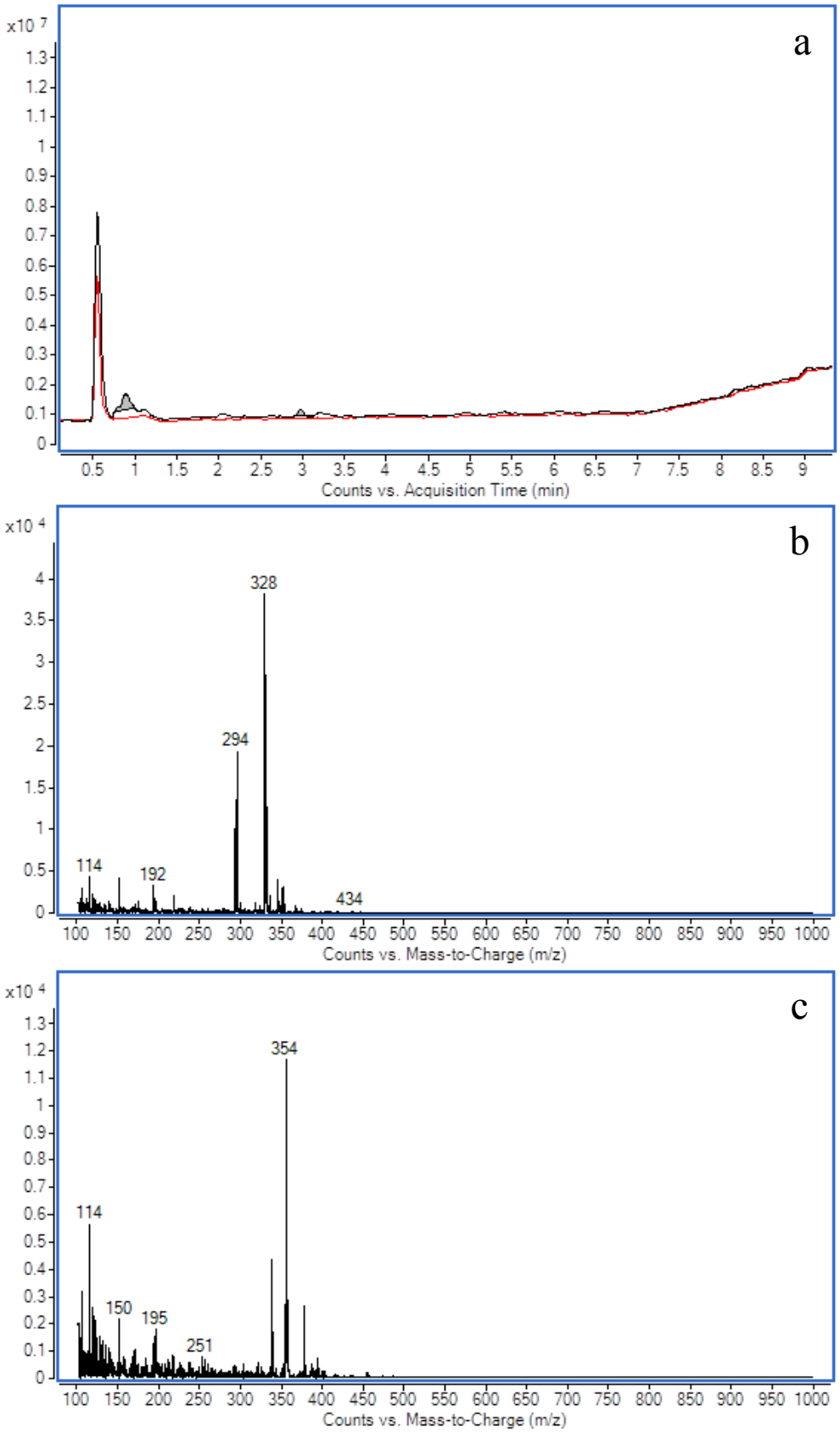


Figure 7-4: TIC chromatogram overlay of the morphine + 0.025-0.0375% (w/v available chlorine) hypochlorite solution and the corresponding reagent control, with the possible reaction products eluting at 0.9 min and 3.0 min, (b) MS spectrum of the product eluting at 0.9 min and (c) MS spectrum of the product eluting at 3.0 min.

Upon spiking codeine and M6G samples with the hypochlorite solutions (0.125-1.5% w/v available chlorine), it was found that these opiates became unstable in the mixture and could no longer be detected using the LC-MS conditions employed. In addition, no subsequent reaction products could be detected either. This was particularly noticeable for M6G, as nothing of interest was observed even in the samples spiked with the two lowest hypochlorite concentrations trialled.

Although the codeine samples spiked with the two lowest hypochlorite concentrations no longer contained codeine, there were two possible reaction products that may have been yielded; one at 11.8 min (*m/z* 334) and the other at 14.2 min (*m/z* 418) (Figure 7-5). More weight is particularly lent to the suggestion that the analyte at 11.9 min is a reaction product; comparison of the peak areas of the analyte at both hypochlorite concentrations indicates that it is more abundant in the sample with less oxidant (Figure 7-5b). This also indicates how prone codeine is to hypochlorite oxidation, as so little was found to mask its presence. Furthermore, the instability of the possible reaction product at 11.9 min is also highlighted, with small increases in hypochlorite concentration rendering it undetectable.

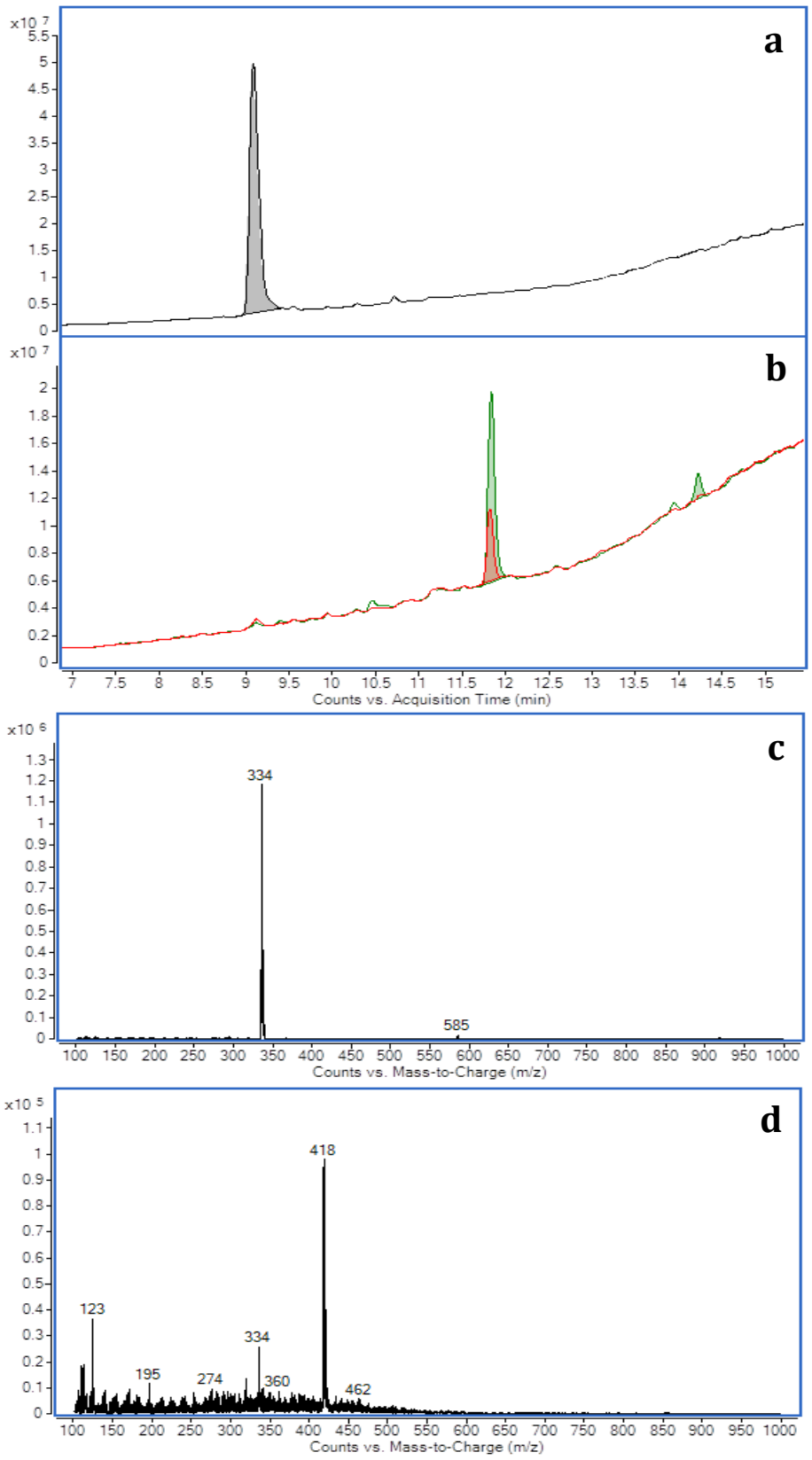


Figure 7-5: (a)TIC chromatogram of codeine in water, (b) TIC chromatogram overlay of the codeine + 0.025-0.0375% (w/v available chlorine) hypochlorite (green trace) and the codeine + 0.075-0.1125% (w/v available chlorine) hypochlorite (red trace), with the possible reaction products eluting at 11.9 min and 14.2 min (c) MS spectrum of the product eluting at 11.9 min and (d) MS spectrum of the product eluting at 14.2 min.

As with the other opiate analytes, the exposure of M3G to hypochlorite at the higher concentrations (0.25-1.5% w/v available chlorine) resulted in a significant decrease in its abundance in the reaction mixtures, with no potential reaction products detected. However, samples spiked with lower concentrations of hypochlorite did indeed yield potential reaction products. Following the fortification of an M3G standard in water (Figure 7-6a) with 0.125-0.1875% (w/v available chlorine) hypochlorite solution, the opiate analyte could no longer be detected in the sample using full scan mode analysis. Instead, the presence of two additional analyte peaks not observed in either the standard or the reagent controls were noted; the potential reaction products were found to elute at 3.9 min and 8.1 min (Figure 7-6b).

Interestingly, the analyte eluting at 3.9 min possessed a precursor ion $[M+H]^+$ of *m/z* 496 (Figure 7-6c), indicating that this product could have formed via chlorine substitution of M3G (the mass difference between product *m/z* 496 and the precursor ion of M3G (*m/z* 462) is 34 Da; the molecular mass of chlorine is 35.45 Da). An enlargement of the *m/z* 496 peak supports the idea that the reaction product is chlorinated- *m/z* 496 is approximately three times larger than the neighbouring *m/z* 498 peak. This observation is in line with the natural abundance of the chlorine-35 (^{35}Cl) and chlorine-37 (^{37}Cl) isotopes occurring in nature, where ^{35}Cl: ^{37}Cl is 3:1 (Figure 7-7). Product *m/z* 496 was also detected in the reaction mixtures fortified with the lower hypochlorite concentrations (0.025-0.075% w/v available chlorine); in these cases, M3G starting material was also detected in the samples (Figure 7-8).

Further to this, an additional potential reaction product at 8.1 min with a precursor ion of *m/z* 130 (Figure 7-6d) was detected. This finding was unusual as the molecular mass corresponding to this product was significantly lower than the M3G starting material. This ordinarily indicates that it is a peak contributed by the reagent; however an overlay of the TIC of both the reaction mixture and the corresponding reagent control showed that this was not the case. It was also suspected that product *m/z* 130 was a partial structure resulting from the cleavage of M3G following exposure to hypochlorite.

However, no complementary structures in both positive and negative ion mode analyses could be observed.

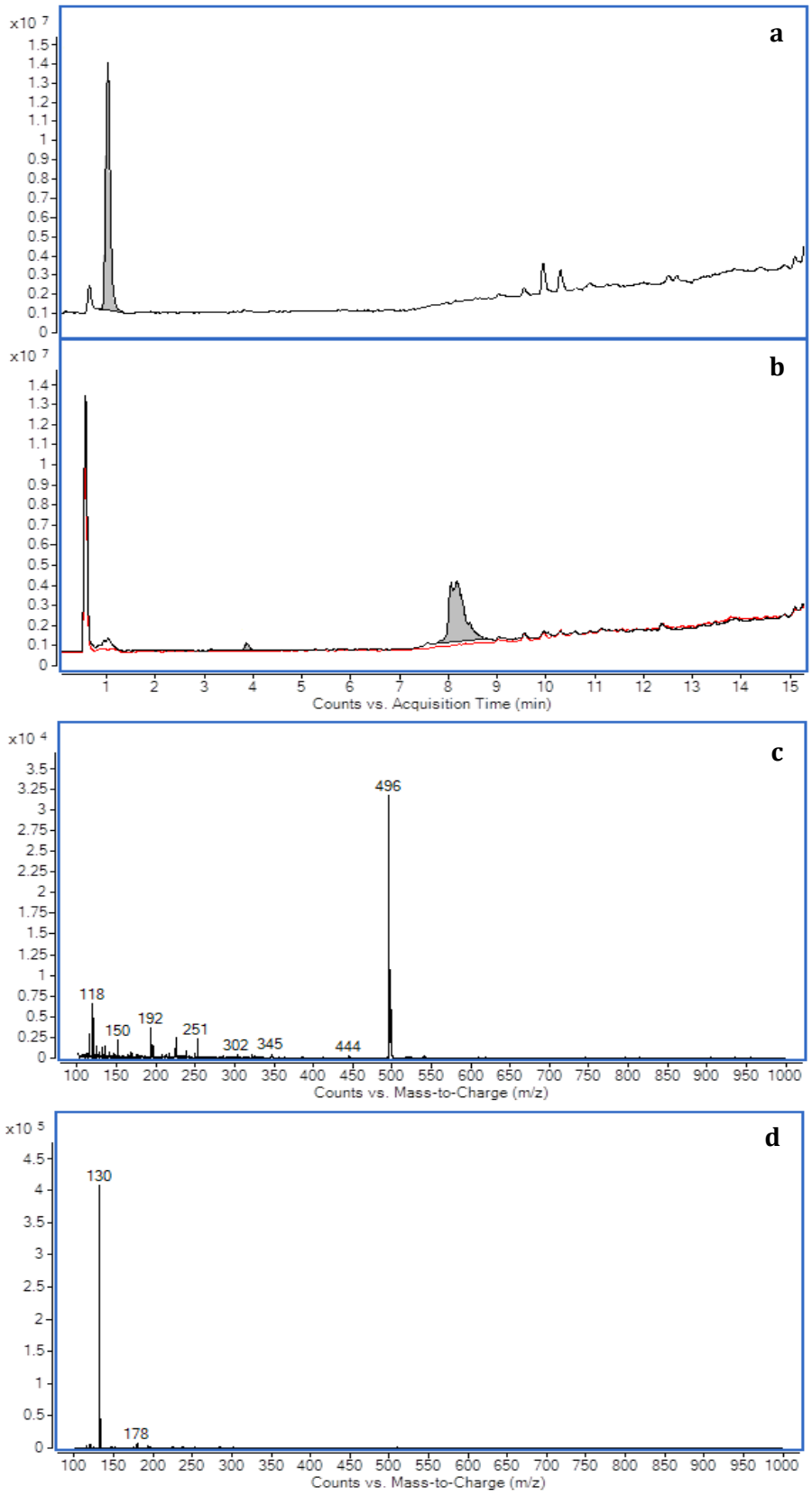


Figure 7-6: (a)TIC chromatogram of M3G in water, (b) TIC chromatogram overlay of the M3G + 0.125-0.1875% (w/v available chlorine) hypochlorite solution and the corresponding reagent control, with the possible reaction products eluting at 3.9 min and 8.1 min, (c) MS spectrum of the product eluting at 3.9 min and (d) MS spectrum of the product eluting at 8.1 min.

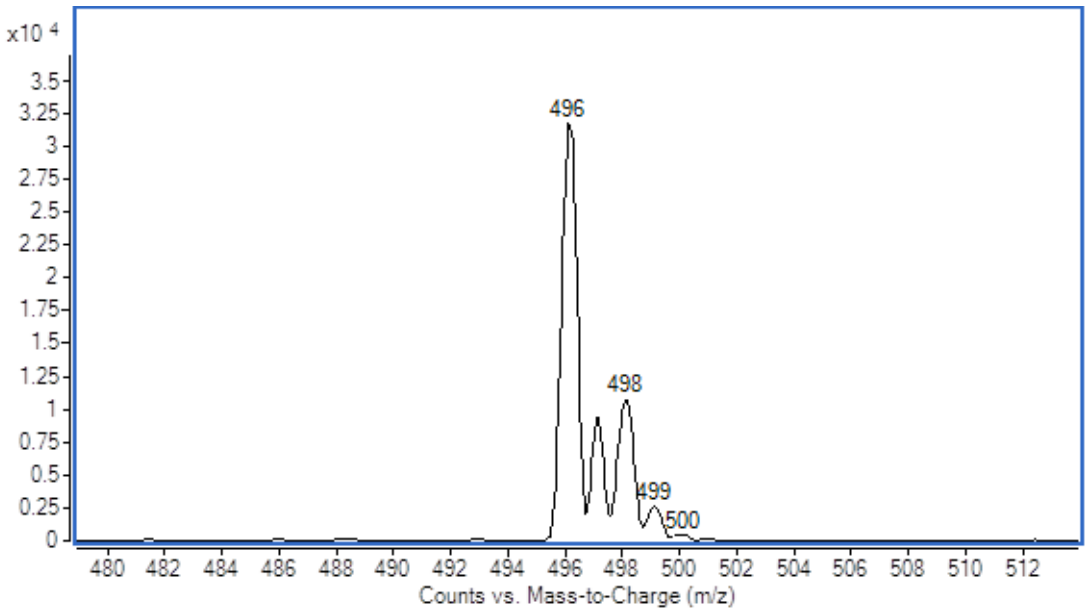


Figure 7-7: enlargement of the m/z 496 peak belonging to the potential reaction product eluting at 3.9 min, demonstrating the 3:1 ratio of m/z 496 to m/z 498.

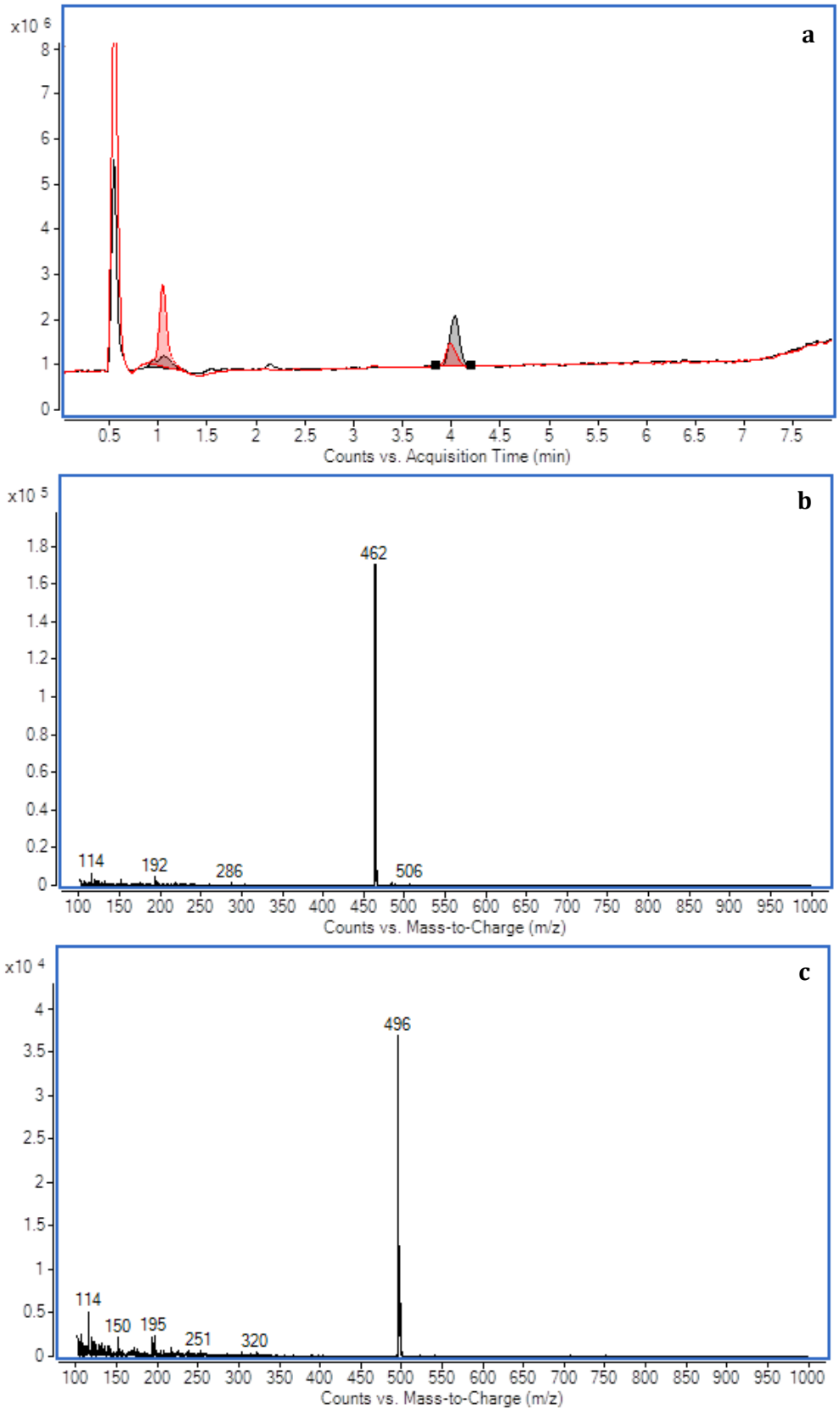


Figure 7-8: (a) TIC chromatogram overlay of the M3G + 0.025-0.0375% (w/v available chlorine) hypochlorite (black trace) and the codeine + 0.075-0.1125% (w/v available chlorine) hypochlorite (red trace), with M3G eluting at 1.0 min and product m/z 496 at 3.9 min,, (b) MS spectrum of M3G (c) MS spectrum of product m/z 496.

7.6 Conclusions

The exposure of 6-MAM, morphine, codeine, M3G and M6G in water resulted in the detection of several potential reaction products. However, it is disadvantageous that they appear to be relatively unstable, only forming under narrow hypochlorite concentration ranges. During authentic cases of *in-vitro* urine adulteration, it is unlikely that the same amount of oxidant is added each time. If the reaction products are only formed under confined conditions and are easily further oxidised or are unstable (as alluded by this study), they are not likely to be viable markers to be used for the purpose of monitoring opiates in adulteration cases. Although the reaction kinetics may be very different in a urine matrix, it is difficult to detect the reaction products in urine without the reaction being initially successful in water. The endogenous urinary compounds will add ambiguity during the identification stage, when products resulting from the reaction between opiate and hypochlorite are recognised.

Based on these considerations, no further investigations were undertaken in regards to the exposure of opiates to hypochlorite. Product *m/z* 496 in particular does appear to be a genuine reaction product resulting from M3G reaction with hypochlorite. However, the time taken to isolate and fully characterise the product could not be justified due to the reasons aforementioned. Instead, the time was spent on isolating and characterising reaction products yielding from opiate reaction with other oxidising adulterants. These other reaction products were more stable and thus have a greater chance for detection in authentic adulteration cases.

Chapter 8: Quantitative NMR analyses of 2-nitro-MAM and 2-nitro-morphine

Chapter 8: Quantitative NMR analyses of 2-nitro-MAM and 2-nitro-morphine

8.1 Introduction

To develop a validated GC-MS or LC-MS method for the detection of the reaction products, certified reference standards of the materials are required. However, it is potentially difficult to source commercial standards of these products due to high cost or lack of availability. The latter is particularly an issue for 2-nitro-MAM and 2-nitro-morphine. Although certification of standards can be carried out by institutes such as the National Measurement Institute (NMI), it may require a significant amount of time. Therefore, the objective of this study was to develop an in-house method for the quantification of 2-nitro-MAM and 2-nitro-morphine using quantitative NMR. These two reaction products were chosen as model analytes to trial the method as the final products were found to be relatively pure. As observed with LC-MS, the syntheses of both products yielded a complete reaction, with no starting material or by-products present. The quantitation is aided by the use of 1,3,5-triazine (Figure 8-1) that is added to each sample. Due to its molecular symmetry, the three protons on 1,3,5-triazine will result in a singlet peak in the ^{1}H-NMR spectrum. Knowing the moles of 1,3,5-triazine and the corresponding integration of this peak, this can then be used to determine the mass of 2-nitro-MAM and 2-nitro-morphine in the sample.

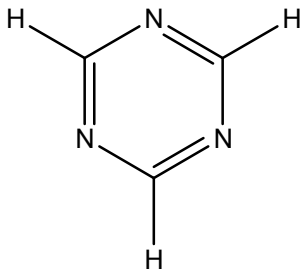


Figure 8-1: structure of 1,3,5-triazine.

8.2 Materials

Drug standards and reagents required for the synthesis and NMR analyses of 2-nitro-MAM and 2-nitro-morphine were sourced from the suppliers previously mentioned in sections 3.2 and 4.2, respectively. In addition, 1,3,5-triazine (97% purity) was obtained from Sigma-Aldrich. A Hamilton 1000 µL syringe was used for all liquid (solvent and solution) deliveries.

8.3 Instrumentation

A Bruker Spectrospin 300 MHz NMR instrument was used to collect one dimensional ^{1}H-NMR spectra for this study. The acquisition parameters are presented in Table 8-1.

Table 8-1: Acquisition parameters used for the collection of ^{1}H-NMR data.

pulse program (PULPROG)	Zg30
time domain (TD)	65536
number of scans (NS)	128
dummy scans (DS)	2
spectral width (SWH)	6172.839 Hz
FID resolution (FIDRES)	0.094190 Hz (per point)
acquisition time (AQ)	5.3084660 sec
receiver gain (RG)	812.7
dwell time (DW)	81 µsec
prescan delay (DE)	20 µsec

8.4 Experimental procedures

Two 1,3,5-triazine standards were prepared by dissolving approximately 10 mg of triazine in 1 mL of $CDCl_3$ and 1 mL of CD_3OD. Following the syntheses of 2-nitro-MAM and 2-nitro-morphine as described in sections 3.4.3 and 4.4.5, respectively, each sample was transferred to an amber GC vial and 1 mL of deuterated solvent ($CDCl_3$ for 2-nitro-MAM and CD_3OD for 2-nitro-morphine) was added. For each sample, 500 µL of the solution was transferred to an NMR tube. Each sample was analysed twice, initially with no triazine to ensure that the correct product was synthesised, and then again following the addition of 100 µL triazine standard with the corresponding solvent. All transfers were accounted for using mass.

8.5 Results and calculations

Based on the weighing data, it can be shown that the transfer volumes using the Hamilton syringe was consistent with the masses expected for the solvents. The weighing data obtained is presented in the appendix, Table A18 and Table A19.

From the weighing data collected for triazine in $CDCl_3$ (Appendix, Table A20), the mass of triazine dissolved in the solvent was found to be 0.01088 g.

Using $n = \frac{m}{fm}$ and accounting for the 97% purity,

$n\ (triazine) = \frac{0.01088}{81.084} \times \frac{97}{100} = 1.3015\ldots \times 10^{-4}$ moles

Therefore, there are $1.3015\ldots \times 10^{-4}$ moles of triazine in 1 mL of $CDCl_3$.

Since 100 µL of this solution was added to the 2-nitro-MAM NMR sample,

$$n\ (triazine\ in\ NMR\ sample) = \frac{1}{10} \times 1.3015\ldots \times 10^{-4}$$

= 1.3015… × 10^{-5} moles

Given that there are three protons in one triazine molecule, the number of moles of protons attributed = 3 × 1.3015… × 10^{-5} moles

= 3.9045… × 10^{-5} moles

As shown in Figure 8-2, the triazine singlet peak resonates at approximately 9.2 ppm. Thus, a peak with area assigned as 1.0000 is given by 3.9045… × 10^{-5} moles of protons in this analysis.

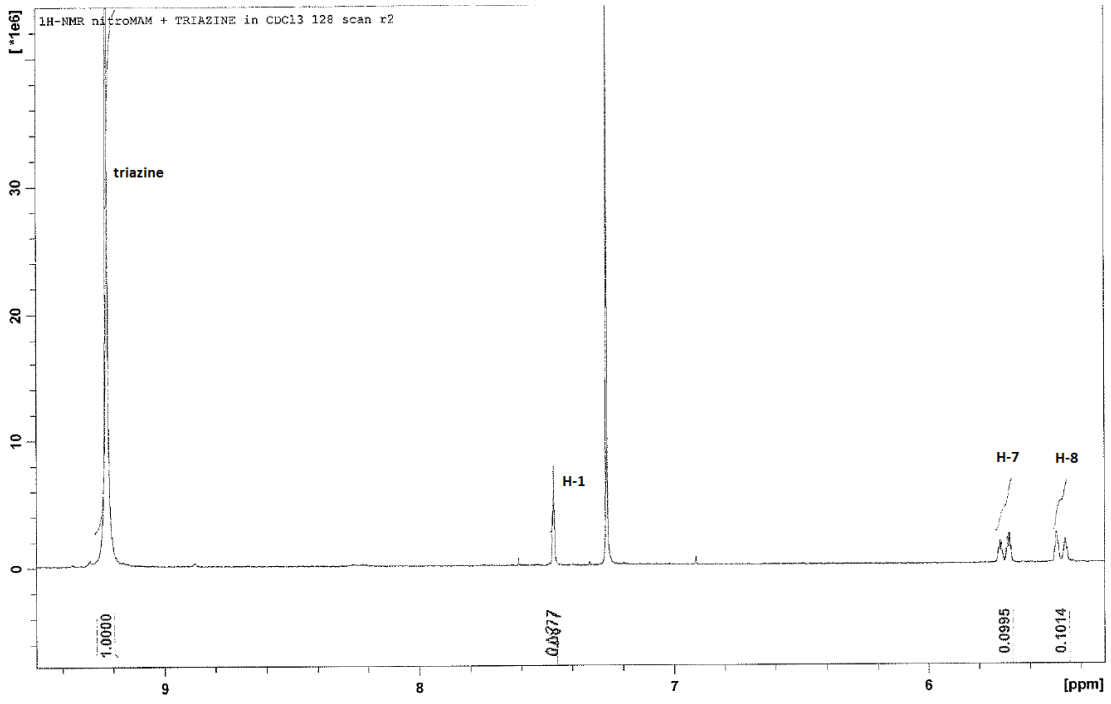


Figure 8-2: Integrated ^{1}H-NMR spectrum of 2-nitro-MAM with spiked 1,3,5-triazine in $CDCl_3$ (δ 5.4-9.4 ppm region).

From Figure 8-2, the average signal of a 2-nitro-MAM proton (using the peak areas of H-1, H-7 and H-8) = $\frac{0.0877+0.0995+0.1014}{3}$

= 0.0962

Therefore, moles of 2-nitro-MAM = $0.0962 \times 3.9045 \ldots \times 10^{-5}$

$= 3.7563\ldots \times 10^{-6}$ moles

Using $m = n \times fm$,

Mass of 2-nitro-MAM = $3.7563\ldots \times 10^{-6} \times 372.37$

$= 1.3987 \times 10^{-3}$ g

= 1.3987 mg

The same method was applied to determine the mass of 2-nitro-morphine reaction product in the NMR sample. For the triazine used in this analysis, the mass of triazine was found to be 0.01067 g.

Using $n = \frac{m}{fm}$ and accounting for the 97% purity,

$n\ (triazine) = \frac{0.01067}{81.084} \times \frac{97}{100} = 1.2764\ldots \times 10^{-4}$ moles

Therefore, there are $1.2764\ldots \times 10^{-4}$ moles of triazine in 1 mL of CD_3OD.

Since 100 µL of this solution was added to the 2-nitro-morphine NMR sample,

$n\ (triazine\ in\ NMR\ sample) = \frac{1}{10} \times 1.2764\ldots \times 10^{-4}$

$= 1.2764\ldots \times 10^{-5}$ moles

Given that there are three protons in one triazine molecule, the number of moles of protons attributed = $3 \times 1.2764\ldots \times 10^{-5}$ moles

$= 3.8292\ldots \times 10^{-5}$ moles

From Figure 8-3, the average signal of a 2-nitro-morphine proton (using the peak areas of H-1, H-7 and H-8) = $\frac{0.0248+0.0296+0.0296}{3}$

= 0.028

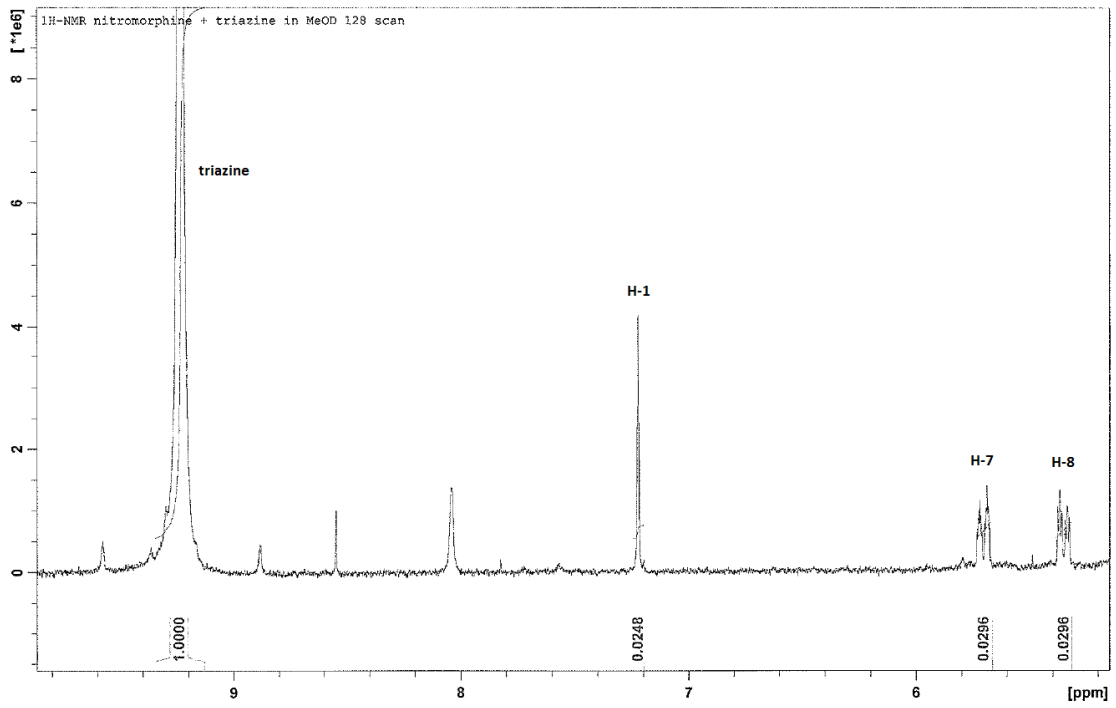


Figure 8-3: Integrated ^{1}H-NMR spectrum of 2-nitro-morphine with spiked 1,3,5-triazine in CD_3OD (δ 5.2-9.8 ppm region).

Therefore, moles of 2-nitro-morphine = $0.028 \times 3.8292 \ldots \times 10^{-5}$

= 1.0722… × 10^{-6} moles

Using $m = n \times fm$,

Mass of 2-nitro-MAM = 1.0722… × 10^{-6} × 330.33

= 3.5419 × 10^{-4} g

= 0.35419 mg

Through the use of quantitative NMR, the mass of 2-nitro-MAM and 2-nitro-morphine in each sample were found to be 1.3987 mg and 0.35419 mg, respectively.

8.6 Conclusions

An in-house method for the quantification of 2-nitro-MAM and 2-nitro-morphine using 1,3,5-triazine was carried out. The masses of 2-nitro-MAM and 2-nitro-morphine in the NMR samples were found to be 1.3987 mg and 0.35419 mg, respectively. The availability 2-nitro-MAM and 2-nitro-morphine reference standards will allow its quantification using both GC-MS and LC-MS methods, which can be compared to determine the more feasible analytical technique for routine analysis. Further, quantitative kinetic and pH studies can also be conducted to further assess the viability of 2-nitro-MAM and 2-nitro-morphine to be used in urinary drug testing programs.

Overall, the quantitative NMR method described in this study can be used as a quick alternative to certifying material through commercial institutions when there are constraints with time and funding. The method can be applied to mixtures as well; however issues may arise if there is significant overlap of the chemical shifts belonging to the reaction product requiring mass certification. The triazine peak must also not overlap with other signals from the reaction products, as this would interfere with its integration.

Chapter 9:

Overall conclusions and recommendations for future work

Chapter 9: Overall conclusions and recommendations for future work

9.1 Overall conclusions

The exposure of the 6-MAM, morphine and M6G to nitrite in urine resulted in the formation of their nitro analogues, 2-nitro-MAM, 2-nitro-morphine and 2-nitro-M6G. Enzymatic hydrolysis facilitates the formation of 2-nitro-morphine from M3G by removing the glucuronic acid functional group from the C-3 position. 2-Nitro-MAM and 2-nitro-morphine can be detected by both LC-MS and GC-MS (as TMS derivatives) due to their relative stability and ease of derivatisation. There is no literature to suggest that it can be formed via metabolic reactions or as a by-product of drug manufacturing processes. As such, these 2-nitro analogues show potential for their incorporation into drug testing programs as a way of monitoring opiate positive urine specimens adulterated with nitrite. All three analytes can be integrated into pre-existing LC-MS methods, with 2-nitro-morphine and 2-nitro-M6G able to be included in GC-MS methods.

The reaction of the opiates with PCC was significantly more complex. Although this oxidant was found to have an acidifying effect on urine, its main mechanism of action is through the oxidation of the opiate analytes. Numerous reaction products were detected for each opiate analyte, which in addition to the small amount of product formed, made it difficult for NMR analysis. Upon fortification of codeine positive urine with PCC, four reaction products were formed; codeinone, 14-hydroxycodeinone, 6-*O*-methylcodeine and 8-hydroxy-7,8-dihydrocodeinone. C6G was found to be transformed to codeine and codeinone, with one additional product tentatively assigned as a lactone derivative of C6G. The adulteration of 6-MAM, morphine, M3G and M6G with PCC resulted in the formation of four detectable reaction products proposed to be morphinone-3-glucuronide, 7,14-dihydroxy-6-MAM, and two 7,8-di-keto

analogues of 6-MAM and morphine. All reaction products were detectable by LC-MS.

Should adulteration of opiate positive urines occur with PCC, it is possible that immunoassay testing would be able to detect the drugs (since some original opiate analytes may still be present). However, the morphine/codeine ratios (used during confirmation testing) were found to be affected by the presence of PCC, due to the loss of both native and internal standard species. Consequently, the use of morphine/codeine ratios in result interpretation should be excised with care. In addition, several of these reaction products resulting from PCC adulteration are potentially pharmacologically active. Because of this, its source must be determined and its presence in a urine specimen cannot be simply interpreted as drug administration alone. For instance, codeinone is a by-product of hydrocodone production from codeine, and may be present in the final drug sample that is administered. Similarly, morphinone is a by-product of hydromorphone manufacture from morphine starting material. During metabolism, it is likely that morphinone undergoes phase II glucuronidation to form morphinone-3-glucuronide. Therefore, determining the source of both codeinone and morphinone-3-glucuronide may be difficult as it could be due to both administration and adulteration.

The exposure of the opiates to hypochlorite in water resulted in the detection of several potential reaction products. However, it is disadvantageous that they appear to be relatively unstable, only forming under narrow hypochlorite concentration ranges. Due to these reasons, further investigation was not pursued.

Finally, an in-house quantitative NMR procedure for the certification of reaction product material was demonstrated using 2-nitro-MAM and 2-nitro-morphine following their syntheses and isolation. It can be used as a quick alternative to certifying material through commercial institutions when there are constraints with time and funding.

9.2 Recommendations for future work

The aims and objectives that were outlined in section 1.5 for this project have been fulfilled and have laid the groundwork for future work concerning the use of the reaction products as markers for monitoring the presence of opiates in adulterated urine.

In regards to the identified reaction products, further immunoassay cross-reactivity studies and stability studies are required to further assess their viability. In order to do so, quantitative and fully validated LC-MS and GC-MS methods targeting the reaction products need to be established to allow their exact concentrations to be determined. This requires certified standards of the reaction products to be made available. Some identified products such as codeinone are already available (at high cost), however the 2-nitro analogues have not been able to be sourced commercially. Therefore, they must be synthesised, purified and their masses properly certified before they can be used to develop methods. These certified standards can also be used to carry out further immunoassay experiments, where the level of cross reactivity of the reaction products to the opiate and 6-AM CEDIA assays can be accurately measured. In addition, stability studies involving the use of a large range of opiate concentrations and oxidant concentrations over a longer period of time (one to six months) should be carried out. Experiments using fresh and aged urine should also be trialled to determine the effect of the endogenous compounds on product formation.

Once methods are developed for the quantification of the reaction products, they can be used to further investigate the reaction between the opiates and PCC. It would be ideal to establish the relative concentration ratios of the analytes present in a range of authentic adulterated specimens. This data can then be used as a guide to hopefully aid the analyst in determining the source of the drugs in the specimen.

In the cases of morphine, 6-MAM and M3G reaction with PCC, data supported the idea that the reaction kinetics between the opiate and the oxidant may be

different in water and in urine. A suggestion that may help the study of product formation in urine (without performing the reaction in water first, as the pathways for the formation of the products in both matrices may differ) is the use of isotopic labelling. This involves replacing specific atom(s) found in the opiate molecule with their isotope, and tracking its movement following opiate reaction with the oxidant to see what resulting molecules (if any) are produced. Isotopes of oxygen and carbon are recommended as deuterated hydrogen often undergoes deuterium exchange in aqueous environments, which is unfavourable.

For unambiguous confirmation of the tentative structures proposed in this research, the reactions mixtures can be separated using HPLC and fractions of the elute can be collected and concentrated for NMR analysis. Otherwise, investigation into NMR analysis of the reaction products in urine without (or minimal) sample pre-treatment would also be beneficial for its characterisation. This includes the use of LC-NMR (where the elute from the LC component is directly analysed by the NMR without deuterated solvents) or freeze drying the urine specimen and dissolving it directly into deuterated solvent for analysis.

Due to the significant negative effect of PCC on the morphine/codeine ratios, similar experiments carried out in this research should be used to investigate the reaction of the deuterated species of the opiates with the oxidising agents.

It has been demonstrated that at acidic pH, nitrite and PCC appear to be more effective in converting the opiate compounds to their analogues. However, there is no significant value in measuring urine pH at the time of specimen collection. The pH of nitrite- and PCC-adulterated urine was typically found to be pH 6, which is within the range expected for normal urine. However, the measurement of pH to identify specimens where nitrite adulteration is not effective (that is, when urine pH > 7) may be a useful tool for laboratories. The incorporation of the reaction products into routine LC-MS and GC-MS assays is a significant step for the identification of urine adulteration by oxidising adulterants. In the case where a urine test is “negative” for opiates and there are no obvious signs of adulteration, the use of confirmatory assays to detect

the reaction products will provide conclusive results that adulteration has occurred and the specific opiate(s) that were originally in the specimen.

Appendix

Appendix

Figure A1: Crude morphine base collected using cloth lined filtering baskets [13].

Figure A-2: 'Double UOGlobe' logo found on heroin packaging originating from South-East Asia [162].

Figure A3: Heroin hydrochloride produced from purified white heroin base [13].

Table A1: Sampler and auxiliary parameters for the Agilent 1290 LC pump.

Injection	With needle wash (10 sec in flush port)
Flexcube	Enable needle seat backflush (2.0 mL/min for 5 sec)
Draw speed	200 µL/min
Eject speed	200 µL/min
Draw position	0 mm
Equilibration time	5 sec
Sample flush-out factor	5
Vial/well bottom sensing	enabled

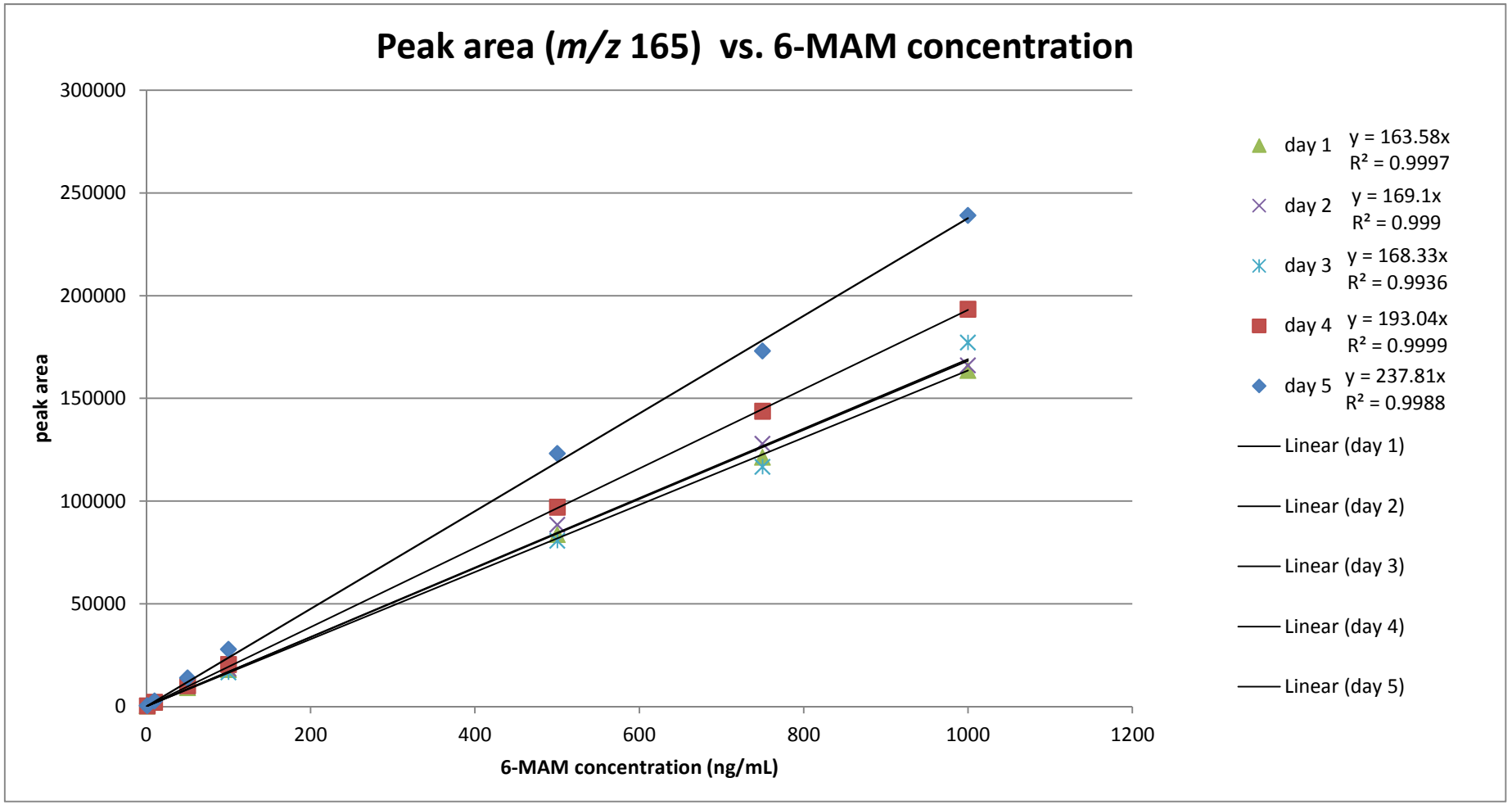


Figure A4: Overlay of calibration curves used for the 6-MAM MRM method validation.

Table A2: Intra-day precision data for the validation of the 6-MAM MRM LC-MS method.

Day	[QC] (ng/mL)	QC 1 peak area[a]	QC 2 peak area[a]	QC 3 peak area[a]	QC 4 peak area[a]	QC 5 peak area[a]	[QC 1] calc (ng/mL)	[QC 2] calc (ng/mL)	[QC 3] calc (ng/mL)	[QC 4] calc (ng/mL)	[QC 5] calc (ng/mL)	Mean	Std Dev	%RSD
1	5	817.25	827.20	830.70	856.55	851.70	5.00	5.06	5.08	5.24	5.21	5.11	0.10	2.00
2	5	890.30	907.20	899.95	912.10	923.65	5.26	5.36	5.32	5.39	5.46	5.36	0.07	**1.39**
3	5	817.60	812.30	799.40	806.55	784.70	4.86	4.83	4.75	4.79	4.66	4.78	0.08	1.59
4	5	1035.40	1028.50	1034.45	1060.95	1006.55	5.36	5.33	5.36	5.50	5.21	5.35	0.10	1.88
5	5	1202.25	1404.00	1364.40	1360.70	1352.45	5.06	5.90	5.74	5.72	5.69	5.62	0.33	5.82
1	250	39485.5	41221	41004	40850.5	41119	241.38	251.99	250.66	249.72	251.36	249.02	4.35	1.75
2	250	46921.5	43591	43345	43633.5	43401	277.47	257.78	256.32	258.03	256.65	261.25	9.09	3.48
3	250	37475	37853.5	38209.5	37613	37246.5	237.74	240.14	242.39	238.61	236.29	239.03	2.34	0.98
4	250	45863	46525.5	47402	47258	47022.5	237.58	241.01	245.55	244.80	243.58	242.51	3.24	**1.34**
5	250	59165.5	59529.5	59094	61042	59392.5	248.79	250.32	248.49	256.68	249.74	250.80	3.36	1.34

[a]Peak areas for 6-MAM were determined using the *m/z* 165 quantifier ion.

Table A3: Intra-day accuracy data for the validation of the 6-MAM MRM LC-MS method.

Day	[QC] (ng/mL)	QC 1 peak area[a]	QC 2 peak area[a]	QC 3 peak area[a]	QC 4 peak area[a]	QC 5 peak area[a]	[QC 1] calc (ng/mL)	[QC 2] calc (ng/mL)	[QC 3] calc (ng/mL)	[QC 4] calc (ng/mL)	[QC 5] calc (ng/mL)	Mean	Actual conc (ng/mL)	Abs. error	Rel. error	%MRE
1	5	817.25	827.20	830.70	856.55	851.70	5.00	5.06	5.08	5.24	5.21	5.11	5	0.11	0.02	**2.30**
2	5	890.30	907.20	899.95	912.10	923.65	5.26	5.36	5.32	5.39	5.46	5.36	5	0.36	0.07	7.23
3	5	817.60	812.30	799.40	806.55	784.70	4.86	4.83	4.75	4.79	4.66	4.78	5	-0.22	-0.04	-4.46
4	5	1035.40	1028.50	1034.45	1060.95	1006.55	5.36	5.33	5.36	5.50	5.21	5.35	5	0.35	0.07	7.04
5	5	1202.25	1404.00	1364.40	1360.70	1352.45	5.06	5.90	5.74	5.72	5.69	5.62	5	0.62	0.12	12.42
1	250	39485.5	41221	41004	40850.5	41119	241.38	251.99	250.66	249.72	251.36	249.02	250	0.97	0.00	0.39
2	250	46921.5	43591	43345	43633.5	43401	277.47	257.78	256.32	258.03	256.65	261.25	250	11.26	0.05	4.50
3	250	37475	37853.5	38209.5	37613	37246.5	237.74	240.14	242.39	238.61	236.29	239.03	250	10.96	0.04	4.38
4	250	45863	46525.5	47402	47258	47022.5	237.58	241.01	245.55	244.80	243.58	242.51	250	7.49	0.03	3.00
5	250	59165.5	59529.5	59094	61042	59392.5	248.79	250.32	248.49	256.68	249.74	250.80	250	-0.81	0.00	**-0.32**

[a]Peak areas for 6-MAM were determined using the *m/z* 165 quantifier ion.

Table A4: Inter-day precision data for the validation of the 6-MAM MRM LC-MS method.

[QC] (ng/mL)	Day 1 peak area[a]	Day 2 peak area[a]	Day 3 peak area[a]	Day 4 peak area[a]	Day 5 peak area[a]	[Day 1] calc (ng/mL)	[Day 2] calc (ng/mL)	[Day 3] calc (ng/mL)	[Day 4] calc (ng/mL)	[Day 5] calc (ng/mL)	Mean	Std Dev	%RSD
5	836.68	906.64	804.11	1033.17	1336.76	5.11	5.36	4.78	5.35	5.62	5.25	0.32	**6.05**
250	40736	44178.4	37699.5	46814.2	59644.7	249.03	261.26	223.96	242.51	250.81	245.51	13.80	**5.62**

[a]Peak areas for 6-MAM were determined using the *m/z* 165 quantifier ion.

Table A5: Inter-day accuracy data for the validation of the 6-MAM MRM LC-MS method.

[QC] (ng/mL)	Day 1 peak area[a]	Day 2 peak area[a]	Day 3 peak area[a]	Day 4 peak area[a]	Day 5 peak area[a]	[Day 1] calc (ng/mL)	[Day 2] calc (ng/mL)	[Day 3] calc (ng/mL)	[Day 4] calc (ng/mL)	[Day 5] calc (ng/mL)	Mean	Actual conc (ng/mL)	Abs. error	Rel. error	%MRE
5	836.68	906.64	804.11	1033.17	1336.76	5.1148	5.3616	4.7770	5.3521	5.6211	5.25	5.00	0.25	0.05	4.91
250	40736	44178.4	37699.5	46814.2	59644.7	249.028	261.256	223.961	242.510	250.808	245.51	250.00	-4.49	-0.02	-1.79

[a]Peak areas for 6-MAM were determined using the *m/z* 165 quantifier ion.

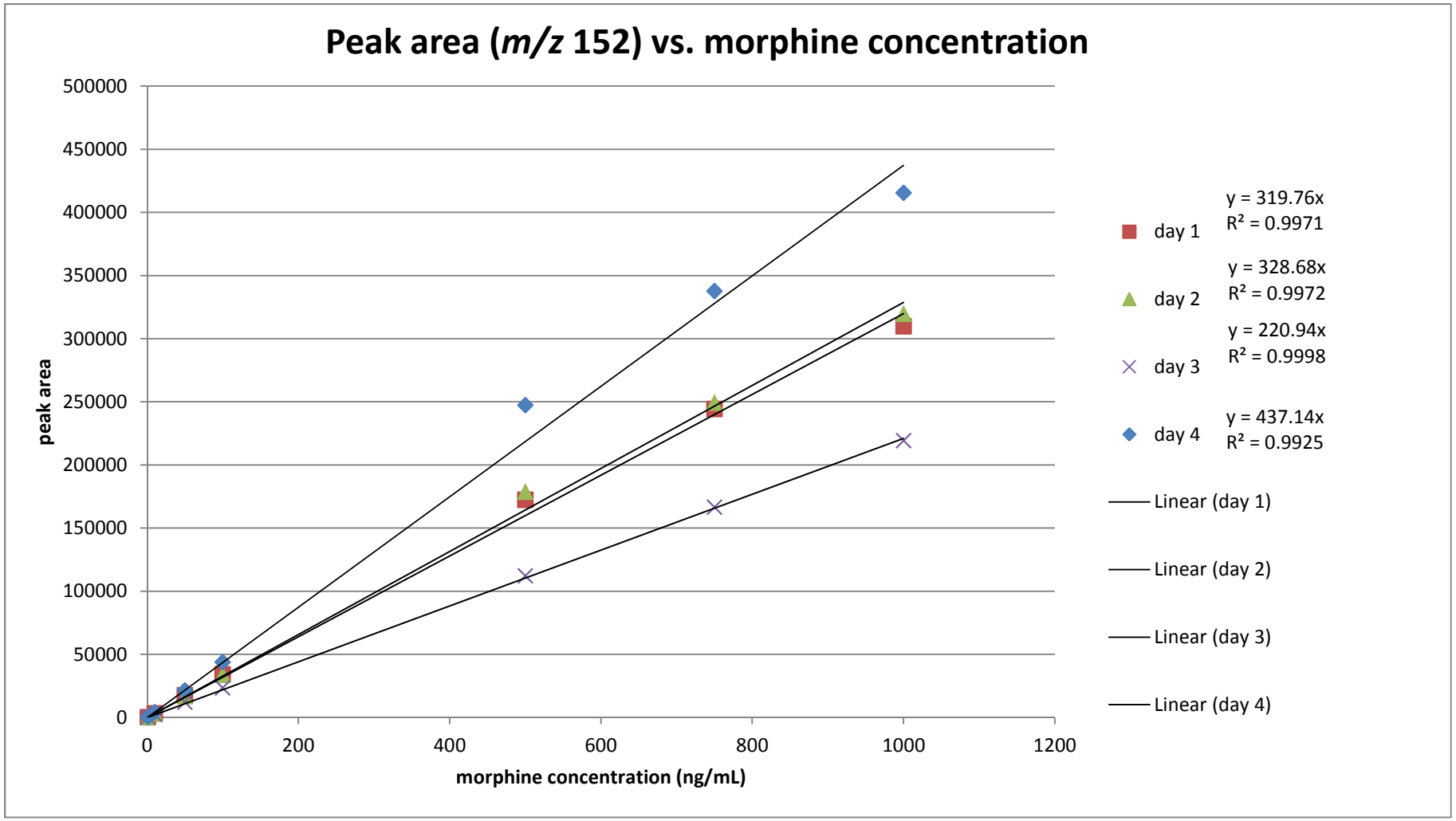


Figure A5: Overlay of calibration curves used for the morphine MRM method validation.

Table A6: Intra-day precision data for the validation of the morphine MRM LC-MS method.

Day	[QC] (ng/mL)	QC 1 peak area[a]	QC 2 peak area[a]	QC 3 peak area[a]	QC 4 peak area[a]	QC 5 peak area[a]	[QC 1] calc (ng/mL)	[QC 2] calc (ng/mL)	[QC 3] calc (ng/mL)	[QC 4] calc (ng/mL)	[QC 5] calc (ng/mL)	Mean	Std Dev	%RSD
1	5	1665	1641	1687	1617.5	1645	5.21	5.13	5.28	5.06	5.14	5.16	0.08	**1.59**
2	5	1657	1760	1753	1748	1655.5	5.04	5.35	5.33	5.32	5.04	5.22	0.16	3.12
3	5	1184.5	1105.5	1100.85	1160	1085.5	5.36	5.00	4.98	5.25	4.91	5.10	0.19	3.78
4	5	1669.5	1688.5	1707	1719	1751	4.64	4.69	4.74	4.78	4.87	4.74	0.09	1.81
1	250	79505.5	84232	82708.5	85833	-	248.64	263.42	258.66	268.43	-	259.79	8.43	3.25
2	250	80614	84596	85950	88205	84101	245.27	257.38	261.50	268.36	255.88	257.68	8.46	3.28
3	250	53583	56056	52825.5	56391	55970.5	242.52	253.72	239.09	255.23	253.33	248.78	7.41	**2.98**
4	250	87201.5	85825.5	86855.5	87676.5	86716.5	242.33	238.51	241.37	243.65	240.99	241.37	1.90	0.79
1	800	264144	263514	270107	-	-	734.06	732.31	750.63	-	-	694.16	62.04	8.94
2	800	195645	206207	204850	-	-	543.70	573.05	569.28	-	-	562.01	15.97	2.84
3	800	293851	294855	294871	-	-	718.32	720.78	720.81	-	-	719.97	1.43	0.20
4	800	297740.6667	305030.6667	303122	-	-	681.11	697.79	693.42	-	-	690.77	8.65	**1.25**

[a]Peak areas for morphine were determined using the *m/z* 152 quantifier ion.

Table A7: Intra-day accuracy data for the validation of the morphine MRM LC-MS method.

Day	[QC] (ng/mL)	QC 1 peak area[a]	QC 2 peak area[a]	QC 3 peak area[a]	QC 4 peak area[a]	QC 5 peak area[a]	[QC 1] calc (ng/mL)	[QC 2] calc (ng/mL)	[QC 3] calc (ng/mL)	[QC 4] calc (ng/mL)	[QC 5] calc (ng/mL)	Mean	Actual conc (ng/mL)	Abs. error	Rel. error	%MRE
1	5	1665	1641	1687	1617.5	1645	5.21	5.13	5.28	5.06	5.14	5.16	5.00	0.16	0.03	3.27
2	5	1657	1760	1753	1748	1655.5	5.04	5.35	5.33	5.32	5.04	5.22	5.00	0.22	0.04	4.34
3	5	1184.5	1105.5	1100.85	1160	1085.5	5.36	5.00	4.98	5.25	4.91	5.10	5.00	0.10	0.02	**2.04**
4	5	1669.5	1688.5	1707	1719	1751	4.64	4.69	4.74	4.78	4.87	4.74	5.00	-0.26	-0.05	-5.12
1	250	79505.5	84232	82708.5	85833	-	248.64	263.42	258.66	268.43	-	259.79	250.00	-9.79	-0.04	-3.92
2	250	80614	84596	85950	88205	84101	245.27	257.38	261.50	268.36	255.88	257.68	250.00	7.68	0.03	**3.07**
3	250	53583	56056	52825.5	56391	55970.5	242.52	253.72	239.09	255.23	253.33	248.78	250.00	1.22	0.00	0.49
4	250	87201.5	85825.5	86855.5	87676.5	86716.5	242.33	238.51	241.37	243.65	240.99	241.37	250.00	8.63	0.03	3.45
1	800	264144	263514	270107	-	-	734.06	732.31	750.63	-	-	739.00	800.00	61.00	0.08	**7.62**
2	800	195645	206207	204850	-	-	543.70	573.05	569.28	-	-	562.01	800.00	237.99	0.30	29.75
3	800	293851	294855	294871	-	-	718.32	720.78	720.81	-	-	719.97	800.00	80.03	0.10	10.00
4	800	297740	305030	303122	-	-	681.11	697.79	693.42	-	-	690.77	800.00	109.23	0.14	13.65

[a]Peak areas for morphine were determined using the *m/z* 152 quantifier ion.

Table A8: Inter-day precision data for the validation of the morphine MRM LC-MS method.

[QC] (ng/mL)	Day 1 peak area[a]	Day 2 peak area[a]	Day 3 peak area[a]	Day 4 peak area[a]	[Day 1] calc (ng/mL)	[Day 2] calc (ng/mL)	[Day 3] calc (ng/mL)	[Day 4] calc (ng/mL)	Mean	Std Dev	%RSD
5	1651.1	1714.17	1127.2	1707	5.16	5.22	5.10	4.74	4.79	0.63	13.23
250	83069.75	84693.2	54965.2	86855.1	259.79	257.68	248.78	241.37	238.67	30.49	12.77
800	249787.6	202234.5	294525.666	301964.444	694.16	562.01	719.97	690.77	666.73	71.02	10.65

[a]Peak areas for morphine were determined using the *m/z* 152 quantifier ion.

Table A9: Inter-day accuracy data for the validation of the morphine MRM LC-MS method.

[QC] (ng/mL)	Day 1 peak area[a]	Day 2 peak area[a]	Day 3 peak area[a]	Day 4 peak area[a]	[Day 1] calc (ng/mL)	[Day 2] calc (ng/mL)	[Day 3] calc (ng/mL)	[Day 4] calc (ng/mL)	Mean	Actual conc (ng/mL)	Abs. error	Rel. error	%MRE
5	1651.1	1714.2	1127.2	1707.0	5.16	5.22	5.10	4.74	4.79	5.00	-0.21	-0.04	-4.29
250	83069.8	84693.2	54965.2	86855.1	259.79	257.68	248.78	241.37	238.67	250.00	-11.33	-0.05	-4.53
800	249787.6	202234.5	294525.7	301964.4	694.16		719.97	690.77	701.64	800.00	-98.36	-0.12	-12.30

[a]Peak areas for morphine were determined using the *m/z* 152 quantifier ion.

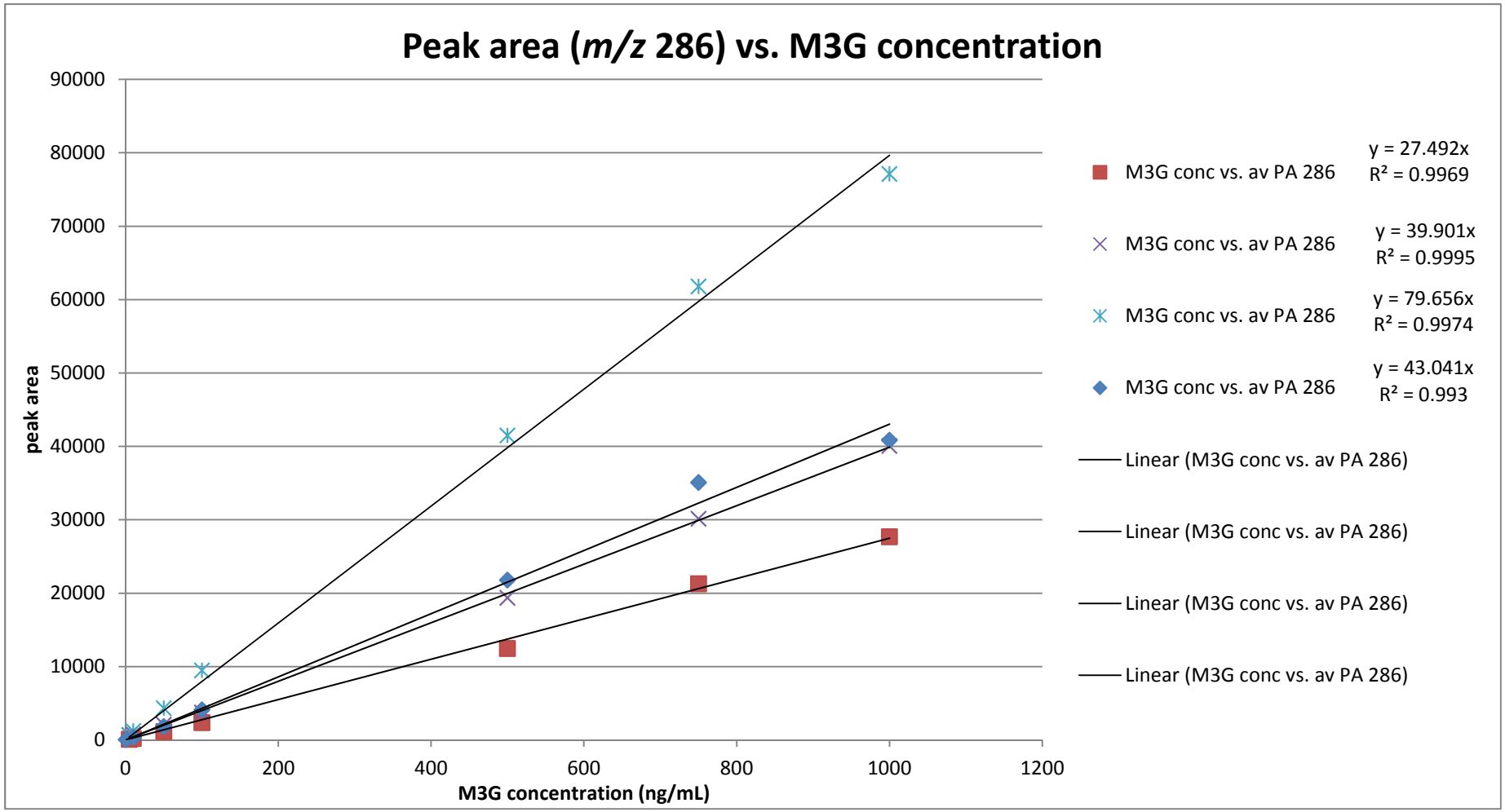


Figure A6: Overlay of calibration curves used for the M3G MRM method validation.

Table A10: Intra-day precision data for the validation of the M3G MRM LC-MS method.

Day	[QC] (ng/mL)	QC 1 peak area[a]	QC 2 peak area[a]	QC 3 peak area[a]	[QC 1] calc (ng/mL)	[QC 2] calc (ng/mL)	[QC 3] calc (ng/mL)	Mean	Std Dev	%RSD
1	250	5885	6311.5	6097.5	214.06	229.58	221.79	221.27	5.95	**2.69**
2	250	6332	6330	6331	230.32	230.25	230.29	230.29	0.04	0.02
3	250	9375.5	9694.5	9934.5	234.97	242.96	248.98	242.30	7.03	2.90
4	250	23292.5	23186.5	21625	292.41	291.08	271.48	284.99	11.72	4.11
1	800	19181.5	19588	19738.5	697.71	712.50	717.97	707.28	10.07	**1.42**
2	800	18830.5	20547.5	20476	684.94	747.40	744.80	725.71	35.33	4.87
3	800	29795.5	29447	28808	746.74	738.00	721.99	735.57	12.55	1.71
4	800	33806	40430	76248	424.40	507.56	957.22	629.72	286.65	45.52

[a]Peak areas for M3G were determined using the *m/z* 286 quantifier ion.

Table A11: Intra-day accuracy data for the validation of the M3G MRM LC-MS method.

Day	[QC] (ng/mL)	QC 1 peak area[a]	QC 2 peak area[a]	QC 3 peak area[a]	[QC 1] calc (ng/mL)	[QC 2] calc (ng/mL)	[QC 3] calc (ng/mL)	Mean	Actual conc (ng/mL)	Abs. error	Rel. error	%MRE
1	250	5885	6311.5	6097.5	214.06	229.58	221.79	221.27	250.00	-28.73	-0.11	-11.49
2	250	6332	6330	6331	230.32	230.25	230.29	230.29	250.00	-19.71	-0.08	-7.89
3	250	9375.5	9694.5	9934.5	234.97	242.96	248.98	242.30	250.00	-7.70	-0.03	**-3.08**
4	250	23292.5	23186.5	21625	292.41	291.08	271.48	284.99	250.00	34.99	0.14	14.00
1	800	19181.5	19588	19738.5	697.71	712.50	717.97	707.28	800.00	92.72	0.12	11.59
2	800	18830.5	20547.5	20476	684.94	747.40	744.80	725.71	800.00	-74.29	-0.09	-9.29
3	800	29795.5	29447	28808	746.74	738.00	721.99	735.57	800.00	64.43	0.08	**8.05**
4	800	33806	40430	76248	424.40	507.56	957.22	629.72	800.00	170.28	0.21	21.28

[a]Peak areas for M3G were determined using the *m/z* 286 quantifier ion.

Table A12: Inter-day precision data for the validation of the M3G MRM LC-MS method.

[QC] (ng/mL)	Day 1 peak area[a]	Day 2 peak area[a]	Day 3 peak area[a]	Day 4 peak area[a]	[Day 1] calc (ng/mL)	[Day 2] calc (ng/mL)	[Day 3] calc (ng/mL)	[Day 4] calc (ng/mL)	Mean	Std Dev	%RSD
250	6083.2	6394	9668.166	14260.33	221.2716	232.5767	242.3038	179.023[b]	232.0508	10.52597	4.536066
800	19444.5	19951.33	29350.16	50161.33	707.2784	725.7141	735.5747	629.724[b]	722.8558	14.36304	1.986985

[a]Peak areas for M3G were determined using the *m/z* 286 quantifier ion.
[b]Outlier value and not used for the determination of %RSD.

Table A13: Inter-day accuracy data for the validation of the M3G MRM LC-MS method.

[QC] (ng/mL)	Day 1 peak area[a]	Day 2 peak area[a]	Day 3 peak area[a]	Day 4 peak area[a]	[Day 1] calc (ng/mL)	[Day 2] calc (ng/mL)	[Day 3] calc (ng/mL)	[Day 4] calc (ng/mL)	Mean	Actual conc (ng/mL)	Abs. error	Rel. error	%MRE
250	6083.2	6394	9668.166	14260.33	221.2716	232.5767	242.3038	179.023[b]	232.05	250.00	-17.95	-0.07	-7.18
800	19444.5	19951.33	29350.16	50161.33	707.2784	725.7141	735.5747	629.724[b]	722.86	800.00	-77.14	-0.10	-9.64

[a]Peak areas for M3G were determined using the *m/z* 286 quantifier ion.
[b]Outlier value and not used for the determination of %MRE.

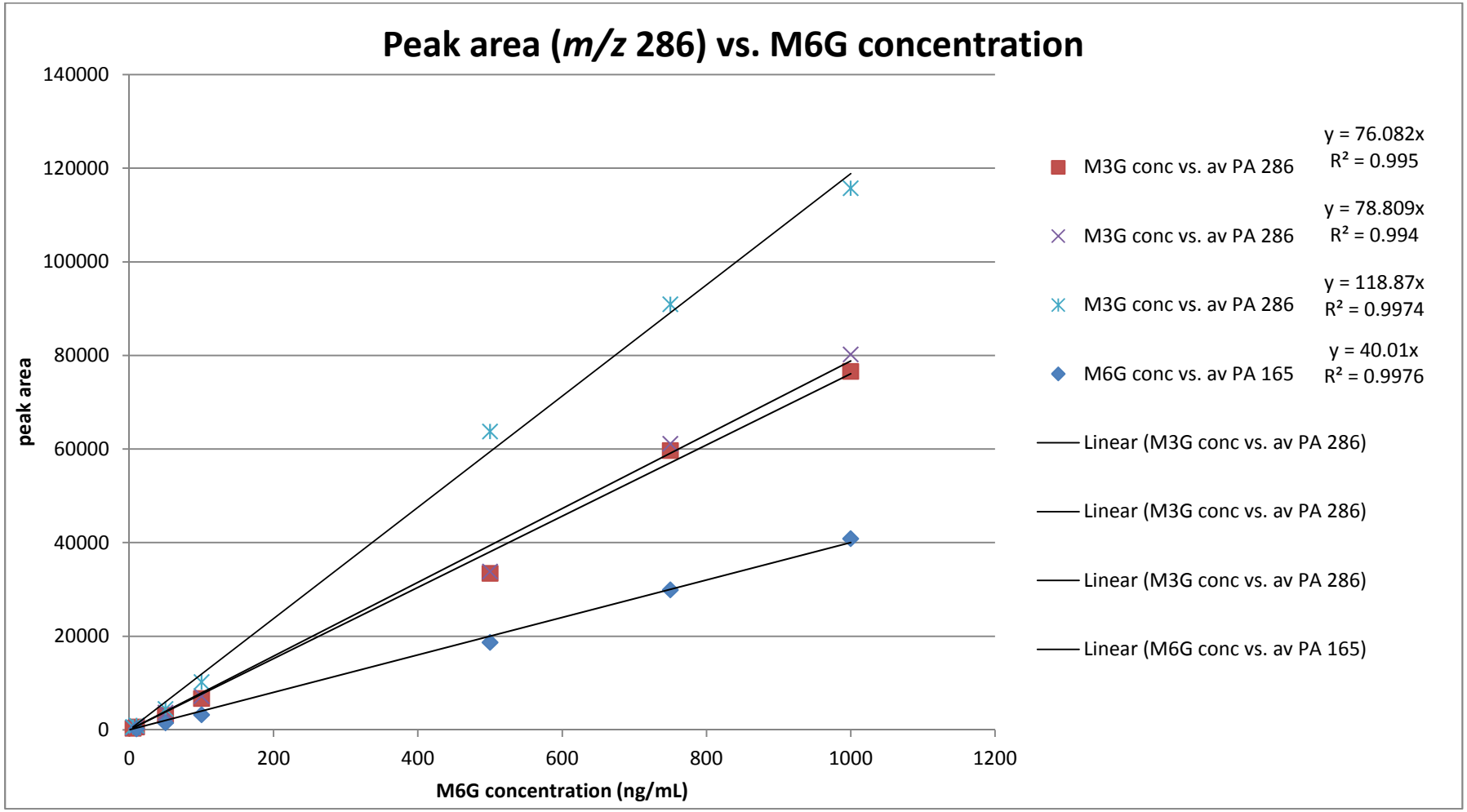


Figure A7: Overlay of calibration curves used for the M6G MRM method validation.

Table A14: Intra-day precision data for the validation of the M6G MRM LC-MS method.

Day	[QC] (ng/mL)	QC 1 peak area[a]	QC 2 peak area[a]	QC 3 peak area[a]	[QC 1] calc (ng/mL)	[QC 2] calc (ng/mL)	[QC 3] calc (ng/mL)	Mean	Std Dev	%RSD
1	250	17908.5	18090	18301.5	235.38	237.77	240.55	237.33	3.17	**1.33**
2	250	13304.5	13617.5	13230.5	174.87	178.98	173.90	175.92	2.70	1.53
3	250	18066	18513	18582	229.24	234.91	235.79	233.31	3.55	1.52
4	250	26545	25633	26343	223.31	215.64	221.61	220.19	4.03	1.83
1	800	56737.5	58171.5	57896.5	745.74	764.59	760.98	705.84	71.69	10.16
2	800	39242.5	44909	42097.5	515.79	590.27	553.32	553.13	37.24	6.73
3	800	58532	59029.5	56938.5	742.71	749.02	722.49	738.07	13.86	**1.88**
4	800	86813	84613	82856	730.32	711.81	697.03	713.05	16.68	2.34

[a]Peak areas for M6G were determined using the *m/z* 286 quantifier ion.

Table A15: Intra-day accuracy data for the validation of the M6G MRM LC-MS method.

Day	[QC] (ng/mL)	QC 1 peak area[a]	QC 2 peak area[a]	QC 3 peak area[a]	[QC 1] calc (ng/mL)	[QC 2] calc (ng/mL)	[QC 3] calc (ng/mL)	Mean	Actual conc (ng/mL)	Abs. error	Rel. error	%MRE
1	250	17908.5	18090	18301.5	235.38	237.77	240.55	237.33	250.00	-12.67	-0.05	**-5.07**
2	250	13304.5	13617.5	13230.5	174.87	178.98	173.90	175.92	250.00	-74.08	-0.30	-29.63
3	250	18066	18513	18582	229.24	234.91	235.79	233.31	250.00	-16.69	-0.07	-6.68
4	250	26545	25633	26343	223.31	215.64	221.61	220.19	250.00	-29.81	-0.12	-11.93
1	800	56737.5	58171.5	57896.5	745.74	764.59	760.98	705.84	800.00	94.16	0.12	11.77
2	800	39242.5	44909	42097.5	515.79	590.27	553.32	553.13	800.00	-246.87	-0.31	-30.86
3	800	58532	59029.5	56938.5	742.71	749.02	722.49	738.07	800.00	61.93	0.08	**7.74**
4	800	86813	84613	82856	730.32	711.81	697.03	713.05	800.00	86.95	0.11	10.87

[a]Peak areas for M6G were determined using the *m/z* 286 quantifier ion.

Table A16: Inter-day precision data for the validation of the M6G MRM LC-MS method.

[QC] (ng/mL)	Day 1 peak area[a]	Day 2 peak area[a]	Day 3 peak area[a]	Day 4 peak area[a]	[Day 1] calc (ng/mL)	[Day 2] calc (ng/mL)	[Day 3] calc (ng/mL)	[Day 4] calc (ng/mL)	Mean	Std Dev	%RSD
250	18056.2	13384.16667	18387	27306.33333	237.3255172	175.92[b]	233.31	229.72	233.45	3.81	1.63
800	53701.5	42083	58166.66667	84760.66667	705.8371231	533.99[b]	738.07	713.05	718.99	16.92	2.35

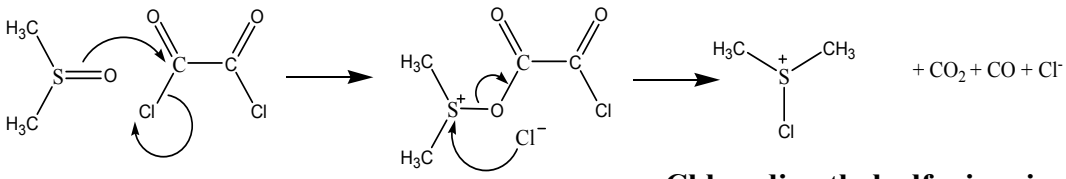
[a]Peak areas for M6G were determined using the *m/z* 286 quantifier ion.
[b]Outlier value and not used for the determination of %RSD.

Table A17: Inter-day accuracy data for the validation of the M6G MRM LC-MS method.

[QC] (ng/mL)	Day 1 peak area[a]	Day 2 peak area[a]	Day 3 peak area[a]	Day 4 peak area[a]	[Day 1] calc (ng/mL)	[Day 2] calc (ng/mL)	[Day 3] calc (ng/mL)	[Day 4] calc (ng/mL)	Mean	Actual conc (ng/mL)	Abs. error	Rel. error	%MRE
250	18056.2	13384.16667	18387	27306.33333	237.33	175.92[b]	233.31	229.72	233.45	250.00	-16.55	-0.07	-6.62
800	53701.5	42083	58166.66667	84760.66667	705.84	533.99[b]	738.07	713.05	718.99	800.00	-81.01	-0.10	-10.13

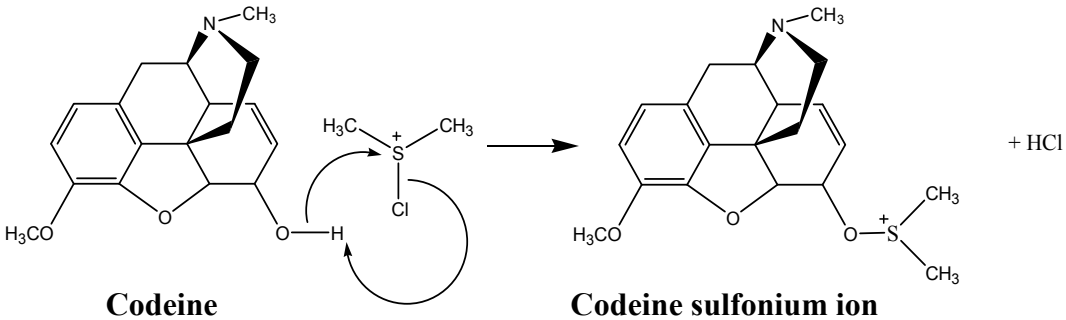
[a]Peak areas for M6G were determined using the *m/z* 286 quantifier ion.
[b]Outlier value and not used for the determination of %MRE.

1. The reaction of DMSO and oxalyl chloride generates the chlorodimethylsulfonium ion:



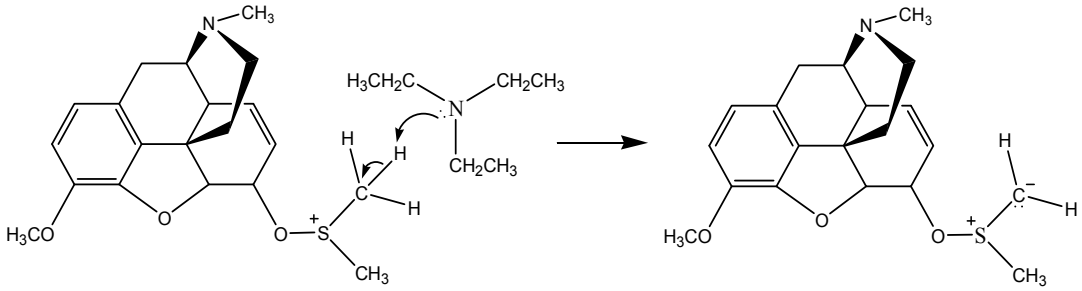
Chlorodimethylsulfonium ion

2. The chlorodimethylsulfonium ion then reacts with codeine to form a codeine sulfonium ion:



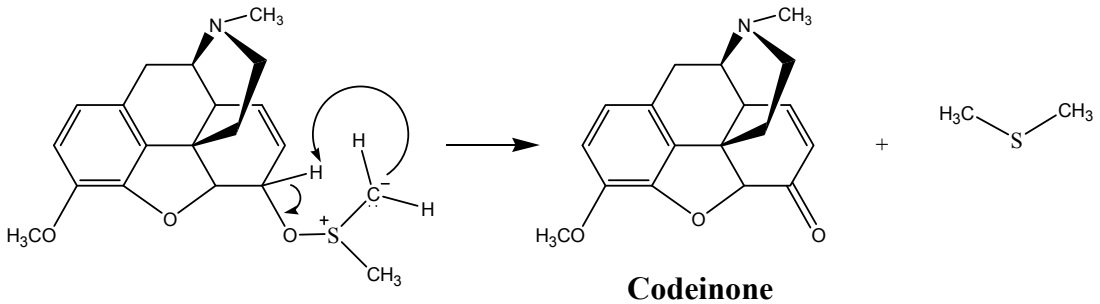
Codeine **Codeine sulfonium ion**

3. The addition of triethylamine deprotonates the codeine sulfonium ion, resulting in the formation of a sulfur ylide:



Sulfur ylide

4. Intermolecular deprotonation yields codeinone and dimethylsulfide, (characteristic odour):



Codeinone

Figure A8: Proposed mechanism for the formation of codeinone via the Swern reaction.

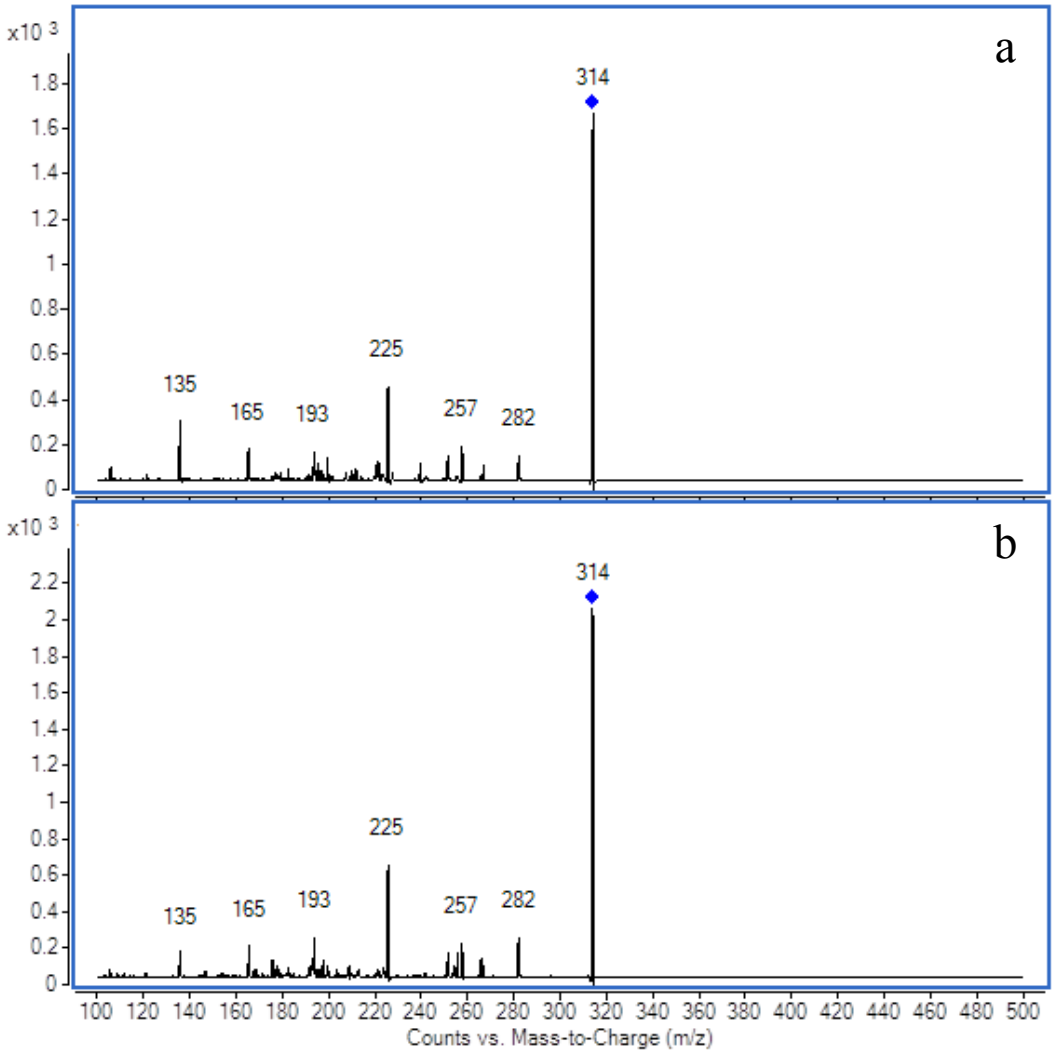


Figure A9: CID spectra of (a) product m/z 314 found in the Swern reaction mixture (CE = 25 eV) and (b) product 314b (CE = 25 eV), indicating that product m/z 314b is a minor product resulting from the Swern conversion of codeine to codeinone.

Table A18: Weighing data for the quantification of 2-nitro-MAM (vial and tube weighed with lids).

1. Empty vial		
	Balance reading (g)	*Total mass (g)*
Z	0.00000	
Empty vial	2.48219	2.48219
Z	0.00000	
Empty vial	2.48221	2.48221
Z	0.00000	
Empty vial	2.48217	2.48217
Z	0.00000	
	Count	*3*
	Average	*2.48219*
	Standard deviation	*0.00002*
2. 2-nitro-MAM solid + vial		
	Balance reading (g)	*Total mass (g)*
Z	0.00000	
2-nitro-MAM solid + vial	2.48391	2.48391
Z	0.00000	
2-nitro-MAM solid + vial	2.48397	2.48400
Z	-0.00003	
2-nitro-MAM solid + vial	2.48399	2.48402
Z	0.00000	
	Count	*3*
	Average	*2.48398*
	Standard deviation	*0.00006*
3. 2-nitro-MAM solid + $CDCl_3$ + vial		
	Balance reading (g)	*Total mass (g)*
Z	0.00000	
2-nitro-MAM + $CDCl_3$ + vial	3.97748	3.97753
Z	-0.00005	
2-nitro-MAM + $CDCl_3$ + vial	3.97751	3.97756
Z	0.00000	
2-nitro-MAM + $CDCl_3$ + vial	3.97757	3.97757
Z	0.00000	
	Count	*3*
	Average	*3.97755*
	Standard deviation	*0.00002*
4. Beaker		
	Balance reading (g)	*Total mass (g)*
Z	0.00000	
Beaker	71.5198	71.51965
Z	0.00015	
Beaker	71.5199	71.51982
Z	-0.00023	
Beaker	71.5199	71.51984
Z	0.00029	

	Count	*3*
	Average	*71.51977*
	Standard deviation	*0.00010*
5. Beaker + NMR tube		
	Balance reading (g)	*Total mass (g)*
Z	0.00000	
Beaker + NMR tube	74.1779	74.1779
Z	0.00000	
Beaker + NMR tube	74.1778	74.1778
Z	0.00001	
Beaker + NMR tube	74.1777	74.1777
Z	0.00003	
	Count	*3*
	Average	*74.177779*
	Standard deviation	*0.00011*
6. 2-nitro-MAM solid + $CDCl_3$ + beaker + NMR tube		
	Balance reading (g)	*Total mass (g)*
Z	0.00000	
2-nitro-MAM + $CDCl_3$ + beaker + NMR tube	74.9251	74.9251
Z	0.00000	
2-nitro-MAM + $CDCl_3$ + beaker + NMR tube	74.9251	74.9251
Z	0.00000	
2-nitro-MAM + $CDCl_3$ + beaker + NMR tube	74.9250	74.9250
Z	0.00000	
	Count	*3*
	Average	74.92507
	Standard deviation	*0.00006*
7. 2-nitro-MAM solid + triazine + $CDCl_3$ + beaker + NMR tube		
	Balance reading (g)	*Total mass (g)*
Z	0.00000	
2-nitro-MAM + triazine + $CDCl_3$ + beaker + NMR tube	75.0708	75.07066
Z	0.00014	
2-nitro-MAM + triazine + $CDCl_3$ + beaker + NMR tube	75.0704	75.07038
Z	0.00016	
2-nitro-MAM + triazine + $CDCl_3$ + beaker + NMR tube	75.0702	75.07019
Z	0.00015	
	Count	*3*
	Average	75.07041
	Standard deviation	*0.00024*

Table A19: Weighing data for the quantification of 2-nitro-morphine (vial and tube weighed with lids).

1. Empty vial		
	Balance reading (g)	*Total mass (g)*
Z	0.00000	
Empty vial	2.49637	2.49640
Z	-0.00003	
Empty vial	2.49639	2.49634
Z	-0.00008	
Empty vial	2.49639	2.49638
Z	-0.00009	
	Count	*3*
	Average	2.49637
	Standard deviation	*0.00003*
2. 2-nitro-morphine solid + vial		
	Balance reading (g)	*Total mass (g)*
Z	0.00000	
2-nitro-morphine solid + vial	2.49735	2.49735
Z	0.00000	
2-nitro- morphine solid + vial	2.49733	2.49733
Z	0.00000	
2-nitro- morphine solid + vial	2.49732	2.49734
Z	-0.00002	
	Count	*3*
	Average	*2.49734*
	Standard deviation	*0.00001*
3. 2-nitro-morphine solid + CD_3OD + vial		
	Balance reading (g)	*Total mass (g)*
Z	0.00000	
2-nitro- morphine + $CDCl_3$ + vial	3.38769	3.38769
Z	0.00000	
2-nitro- morphine + $CDCl_3$ + vial	3.38771	3.38771
Z	0.00000	
2-nitro- morphine + $CDCl_3$ + vial	3.38771	3.38771
Z	0.00000	
	Count	*3*
	Average	*3.38770*
	Standard deviation	*0.00001*
4. Beaker		
	Balance reading (g)	*Total mass (g)*
Z	0.00000	
Beaker	71.5204	71.5204
Z	0.00000	
Beaker	71.5204	71.5203

Z	0.00001	
Beaker	71.5204	71.5203
Z	0.00001	
	Count	*3*
	Average	*71.52034*
	Standard deviation	*0.00006*
5. Beaker + NMR tube		
	Balance reading (g)	*Total mass (g)*
Z	0.00000	
Beaker + NMR tube	73.6878	73.6878
Z	0.00000	
Beaker + NMR tube	73.6877	73.6877
Z	0.00000	
Beaker + NMR tube	73.6875	73.6875
Z	0.00000	
	Count	*3*
	Average	*73.68767*
	Standard deviation	*0.00016*
6. 2-nitro-morphine solid + CD_3OD + beaker + NMR tube		
	Balance reading (g)	*Total mass (g)*
Z	0.00000	
2-nitro-morphine + $CDCl_3$ + beaker + NMR tube	74.1317	74.1317
Z	0.00000	
2-nitro-morphine + $CDCl_3$ + beaker + NMR tube	74.1317	74.1317
Z	0.00000	
2-nitro-morphine + $CDCl_3$ + beaker + NMR tube	74.1317	74.1317
Z	0.00000	
	Count	*3*
	Average	*74.1317*
	Standard deviation	*-*
7. 2-nitro-morphine solid + triazine + CD_3OD + beaker + NMR tube		
	Balance reading (g)	*Total mass (g)*
Z	0.00000	
2-nitro-morphine + triazine + $CDCl_3$ + beaker + NMR tube	74.2226	74.2226
Z	0.00000	
2-nitro-morphine + triazine + $CDCl_3$ + beaker + NMR tube	74.2226	74.2226
Z	0.00000	
2-nitro-morphine + triazine + $CDCl_3$ + beaker + NMR tube	74.2226	74.2226
Z	0.00000	
	Count	*3*
	Average	*74.2226*

	Standard deviation	-

Table A20: Weighing data for triazine in $CDCl_3$ (vial weighed with lid).

1. Empty vial		
	Balance reading (g)	*Total mass (g)*
Z	0.00000	
Empty vial	2.53582	2.53582
Z	0.00000	
Empty vial	2.53587	2.53587
Z	0.00000	
Empty vial	2.53582	2.53582
Z	0.00000	
	Count	*3*
	Average	2.53584
	Standard deviation	*0.00003*
2. Triazine + vial		
	Balance reading (g)	*Total mass (g)*
Z	0.00000	
Triazine + vial	2.54671	2.54678
Z	-0.00007	
Triazine + vial	2.54670	2.54669
Z	-0.00006	
Triazine + vial	2.54671	2.54669
Z	-0.00008	
	Count	*3*
	Average	*2.54672*
	Standard deviation	*0.00005*
3. Triazine + $CDCl_3$ + vial		
	Balance reading (g)	*Total mass (g)*
Z	0.00000	
Triazine + $CDCl_3$ + vial	4.03829	4.03829
Z	0.00000	
Triazine + $CDCl_3$ + vial	4.03839	4.03837
Z	0.00002	
Triazine + $CDCl_3$ + vial	4.03839	4.03837
Z	0.00004	
	Count	*3*
	Average	4.03834
	Standard deviation	*0.00005*

Table A21: Weighing data for triazine in CD_3OD (vial weighed with lid).

1. Empty vial		
	Balance reading (g)	*Total mass (g)*
Z	0.00000	
Empty vial	2.46235	2.46235
Z	0.00000	
Empty vial	2.46237	2.46237
Z	0.00000	
Empty vial	2.46237	2.46234
Z	0.00003	
	Count	*3*
	Average	*2.46235*
	Standard deviation	*0.00002*
2. Triazine + vial		
	Balance reading (g)	*Total mass (g)*
Z	0.00000	
Triazine + vial	2.47307	2.47307
Z	0.00000	
Triazine + vial	2.47296	2.47296
Z	0.00000	
Triazine + vial	2.47303	2.47303
Z	0.00000	
	Count	*3*
	Average	*2.47302*
	Standard deviation	*0.00006*
3. Triazine + CD_3OD + vial		
	Balance reading (g)	*Total mass (g)*
Z	0.00000	
Triazine + CD_3OD + vial	3.35777	3.35777
Z	0.00000	
Triazine + CD_3OD + vial	3.35786	3.35786
Z	0.00000	
Triazine + CD_3OD + vial	3.35787	3.35787
Z	0.00000	
	Count	*3*
	Average	*3.35783*
	Standard deviation	*0.00006*

List of publications related to this research

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S. Luong, A.T. Ung, J. Kalman and S. Fu. Transformation of codeine and codeine-6-glucuronide to opioid analogues by urinary adulteration with pyridinium chlorochromate: potential issue for workplace drug testing. *Rapid Commun. Mass.* sp. **2014,** *28,* 1609-1620.

S. Fu, S. Luong, A. Pham, N. Charlton, U. Kuzhiumparambil. Bioanalysis of urine samples after manipulation by oxidising chemicals: technical considerations. *Bioanalysis.* (accepted for publication on 27th March 2014).

S. Luong and S. Fu. Detection and identification of 2-nitro-morphine and 2-nitro-morphine-6-glucuronide in nitrite adulterated urine specimens containing morphine and its glucuronides. *Drug Test. Anal.* **2014**, *6,* 277-287.

S. Luong, R. Shimmon, J. Hook and S. Fu. 2-Nitro-6-monoacetylmorphine: potential marker for monitoring the presence of 6-monoacetylmorphine in urine adulterated with potassium nitrite. *Anal. Bioanal. Chem.* **2012**, *403,* 2057-2063.

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