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Analysis of morphine, morphine-3-glucuronide, morphine-6-glucuronide, codeine and codeine-6-glucuronide using HPLC

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Abstract: In interpretive forensic and clinical toxicology, there is a great need for the identification and detection of opiate drugs and their various glucuronide metabolites. Glucuronides are usually determined by cleavage of the glucuronide bond with an enzyme (e.g., β-glucuronidase) or acid hydrolysis to yield the parent compound, which is subsequently detected. For Gas chromatography-mass spectrometry (GC-MS) analysis this may be via derivatization to a more volatile or stable form. Direct detection of the glucuronide conjugates overcomes the critical limitations of these approaches.

In this conduct, a rapid and selective reversed-phase high-performance liquid chromatographic (HPLC) assay with gradient elution and ultraviolet detection for morphine, morphine-3-glucuronide, morphine-6-glucuronide, codeine, codeine-6-glucuronide and hydrocodone was developed. The separation was performed on a gemini C18 (Octadecyl carbon chain) analytical column (150 × 2.0 mm, 5 μm) and detected by an ultraviolet (UV) detector at 210 nm. The mobile phase consisted of an acetonitrile–phosphate buffer (0.0125 M, pH 7.5) with elution in the gradient mode ranging from 7.5–60% acetonitrile over 25 minutes (min) with a 0.2 mL/min flow rate. The calibration curves were linear (R2 range 0.997–0.999) in the concentration range 0.025–2 μg/mL for all analytes, using hydrocodone as the internal standard.

Therefore, this conduct aims to develop an efficient method of separation of morphine, codeine and glucuronides by HPLC-UV and to evaluate the use of SPME LC tips for the extraction of morphine, codeine and glucuronides from urine samples.

Keywords: morphine, codeine, glucuronide, toxicology, forensic, HPLC, GC-MS.

تحليل المورفين، المورفين -3 غلوكورونيد، المورفين -6-غلوكورونيد، الكوداين والكوداين -6-الغلوكورونيد باستخدام جهاز الفصل السائل عالي الكفاءة

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الملخص: في علم السموم الشرعي والتفسير السريري، هناك حاجة كبيرة لتحديد واكتشاف العقاقير الأفيونية ومستقلبات الجلوكورونيد المختلفة. عادة ما يتم تحديد الجلوكورونيدات عن طريق انشقاق رابطة الجلوكورونيد مع إنزيم (على سبيل المثال، جلوكورونيداز) أو التحلل المائي الحمضي لإنتاج المركب الأصلي، الذي يتم اكتشافه لاحقًا. بالنسبة لتحليل الطيف الكتلي للغاز GC-MS، قد يكون هذا عن طريق الاشتقاق إلى شكل أكثر تقلبًا أو ثباتًا. الكشف المباشر عن اتحادات الجلوكورونيد يتغلب على القيود الحرجة لهذه الطرق.

في هذا البحث، اختبار كروماتوجرافي السائل عالي الأداء وعكسي الطور عكسي انتقائي مع شطف التدرج والكشف عن الأشعة فوق البنفسجية للمورفين، المورفين 3-الجلوكورونيد، المورفين 6-الجلوكورونيد، الكوكايين، الكوداين -6-الجلوكورونيد والهيدروكودون تم تطويره. تم إجراء الفصل على عمود تحليلي C18 (سلسلة الكربون من 150) (Octadecyl ، 2.0 مم، 5 ميكرون) واكتشفه كاشف الأشعة فوق البنفسجية (UV) عند 210 نانومتر. يتكون الطور المتحرك من مخزن مؤقت من الأسيتون تريل - الفوسفات (0.0125 ، درجة الحموضة 7.5) مع شطف في وضع التدرج تتراوح من 7.5 إلى 60% من الأسيتون تريل على مدى 25 دقيقة (دقيقة) مع معدل تدفق 0.2 مل / دقيقة. كانت منحنيات المعايرة خطية (نطاق R2 من 0.997 إلى 0.999) في نطاق التركيز 0.025-2 ميكروغرام / مل لكل التحليلات، باستخدام الهيدروكودون كمعيار داخلي. لذلك يهدف هذا السلوك إلى تطوير طريقة فعالة لفصل المورفين والكودين والغلوكورونيدات بواسطة HPLC-UV وتقييم استخدام نصائح SPME LCلاستخراج المورفين والكودين والجلوكورونيدات من عينات البول.

الكلمات المفتاحية: مورفين، كودين،جلوكورونايد، السموم، الطب الشرعي.

1- Introduction

In interpretive forensic and clinical toxicology, there is great need for the identification and detection of drugs and their various metabolites. Being able to provide an estimated drug to metabolite ratio gives an idea of the time, dose and the method through which the drug was administered(Kaushik, Levine, & LaCourse, 2006). Determination of metabolite of the different opioids has assisted greatly in the identification of the parent opioid from the human biological fluids. In addition, the identification process of metabolites can assist in determining the period since the drug was used, whether the drug was administered recently in high dosage or it has been administered over a longer period to treat the chronic pain (Chong, Ho, Leung, Lau, & Woo, 2018).

Opium is obtained from the opium-poppy, Papaver somniferum, and is extracted as a sap from the plant (Klimas & Mikus, 2014) . In ancient times, poppy plants were used as an analgesic when conducting surgery and as a form of painkiller(Klimas & Mikus, 2014). Friedrich Sertürner was able to isolate some of the elements of opium in the 1806, and due to its effect of euphoria one of them was named name morphium, which originated from the Greek god of dreams, Morpheus.3 The name was later changed to 'morphine' by Gay-Lussac(Klimas & Mikus, 2014). Opium has other alkaloids that are of clinical use majorly as pain relivers, namely codeine, noscapine and papaverine, which is used to treat

condition caused by muscle spasm, but the most used opiate in the world is morphine (Oliveira et al., 2014).

In recent decades, there have been constant discoveries of different synthetic opiates drugs known as opioids such as Dilaudid, Demerol, Vicodin, Methadone and fentanyl(Jafari-Nodoushan, Barzin, & Mobedi, 2016). The various uses of morphine especially in hospitals are to assist in pain management for patients battling with cancer or other chronic pain. This means that there has been an increase in the use of opiates in the world (Klimas & Mikus, 2014). Questions have arisen about the use of morphine in the long-term for example for dying patients, as its effectiveness cannot be quantified. There has also been an increase in the misuse of medical opioids as many people have become addicted to them (Jafari-Nodoushan et al., 2016). The side-effects such as intense relaxation or loss of analgesic potency, are recorded after the use of opioid analgesics to manage chronic pain have a tendency for addiction (Sloan & Hamann, 2006)(Eckart et al., 2015).

Due to short specific methods and techniques for the identification and detection of drugs of abuse and their various metabolites, there is a need to improve and evaluate different laboratory methods which can help estimate drugs and metabolite ratio. In addition, raise specificity of detection of drugs of abuse in laboratory by altering some methods and techniques.

1.1 Morphine

In 1986, morphine was accepted by the World Health Organisation to be used as a treatment for severe pain, if given to patients in a regulated manner. As shown in Figure 1, morphine is a phenanthrene alkaloid consisting of five rings. The morphine molecule is made active by the C3 and C6 groups combined with the amino group. These features also make the structure rather rigid (Klimas & Mikus, 2014). Morphine a amphoteric (as will be explained in section 1.4) has a pK_a value of 7.9 and 76% of its molecules are ionised at physiological pH, Morphine is relatively soluble in water, while being poorly soluble in lipids because of the C3 and C6 hydrophilic OH groups that are present(Klimas & Mikus, 2014).

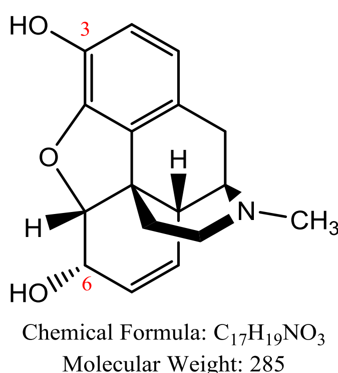


Figure (1) Chemical structure of morphine with highlighted C3 and C6(Skrabalova, Drastichova, & Novotny, 2013).

Morphine can be administrated orally, intramuscular (IM), intravenously (IV) or through a suppository. The injection is provided in different concentrations depending on the requirements of the patient, and choosing the right dosage is important as errors in dosing can result in addiction due to overdose or death("MS Contin, Astramorph (morphine) dosing, indications, interactions, adverse effects, and more," n.d.). 0.1 to 0.2 mg per kilogram in every 4 hours is the starting dose for IV dosage in adults, and 10 mg in every four hours in IM injections ("MS Contin, Astramorph (morphine) dosing, indications, interactions, adverse effects, and more," n.d.).The patient's severity of pain is considered here, their response, and prior treatment using analgesic measures. This is crucial to avoid abuse or addiction to the drugs. During treatment, patients are continuously re-evaluated to assess probable incidence of adverse reactions, and the ability of these drugs to maintain pain control ("MS Contin, Astramorph (morphine) dosing, indications, interactions, adverse effects, and more," n.d.).

UDP-glucuronosyltransferase 2B7 (UGT2B7) in the human body assists morphine to metabolize especially in the liver producing morphine-3-glucuronide (M3G; 45–55%) and morphine-6-glucuronide (M6G; 10–15%), which are the two most important metabolites (Figure 2) (Oliveira et al., 2014). This happens through the process of *glucuronidation* of the C3 phenolic group and C6 hydroxyl group (Oliveira et al., 2014). M3G formation is higher than M6G as more UGT isoforms are involved in M3G formation, such as UGT 1A3, 1A6, 1A9, 1A8 and 1A10 (Figure 2) (Oliveira et al., 2014). M3G has no opioid action as compared to M6G, which has opioid action with the same effects as morphine (Oliveira et al., 2014). Although M3G has no opioid action, it displays some of the side-effects that may be felt following the use of morphine, such as neurotoxicity, allodynia, decreasing morphine analgesia and an antagonistic effect (Oliveira et al., 2014).The ratio of morphine to glucuronide concentration plus the concentration ratio of both major glucuronides allows easy identification of patients who metabolise relatively more M3G than is common, and those who produce relatively more M6G than is common (Khabbazi, Nassar, Goumon, & Parat, 2016). Pharmacogenomics of M3G and M6G varies significantly depending on metabolism of morphine (Khabbazi et al., 2016). As individuals have varying rates of metabolizing morphine due to genetic composition and health, pharmacogenetic rates of these elements also vary (Williams, Patel, & Howard, 2002).There have been various metabolites formations that have been found in the human body that may explain the different variations of pain relief among users (Xu et al., 2018). It therefore means that, there is need for proper quantification of morphine simultaneously with its glucuronide metabolites and more focus on the calculation of ratios, to better understand morphine's side-effects and efficacy(Oliveira et al., 2014).

In addition, ratios can be used in the interpretation of toxicology results from deaths that occur due to intoxication with either morphine or heroin as they are both extracted from the poppy plant (Bosch, Sánchez, Rojas, & Ojeda, 2007).Heroin (Diacetylmorphine) is a semi-synthetic drug that has become the world's most abused opioid; it is derived from morphine. Heroin is rapidly metabolised to 6-

aceylmorphine (6-AM) in whole blood by deacetylate. Further metabolism of 6-AM to morphine occurs in the liver within 6-25 min of 6-AM. Moreover, codeine can be detected in a small number of heroin cases due to the impurity of the heroin and the fact that it has not been directly metabolised (Ellis, McGwin, Davis, & Dye, 2016).

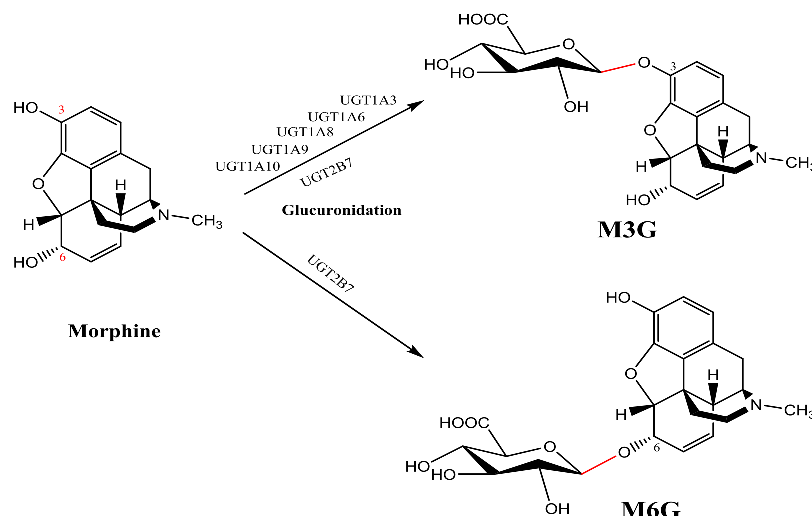


Figure (2) This figure shows the morphine metabolism pathway and glucuronide bonds are highlighted (Chen, Zhao, & Zhong, 2003).

There have been a number of analytical methodologies for the quantification of morphine alone or morphine metabolites in range of biological matrices (Tang, Bada, Ng, & Leggas, 2019). The use of an enzyme to cleave glucuronide bond can enable the parent drug to be detected. This method was, until recently, the most favoured when it came to the identification process of metabolites. With this method, however there are several limitations. Firstly, competitive inhibition of the enzyme in the enzymatic hydrolysis of the drug is one such limitation (Tang et al., 2019). Secondly, there are some conjugates that are not easy to cleave due to the specific nature of their structure such as the different glucuronides formed in each opiate. These methods require almost perfect conditions of the pH, temperature, and incubation time during sample preparations which is time-consuming, and the glucuronides of each of the different drugs require unique methods of analysis (Tang et al., 2019). Many of the processes that are used to quantify morphine are based on liquid chromatography (LC) coupled with different detectors, since there are no specific methods of directly quantifying M3G and M6G metabolites by Gas Chromatography-Mass Spectrometer (GC-MS). GC-MS has been successful for analysing the total and free morphine (as will be explained in section 1.4.1) concentration after sample pre-treatment by hydrolysis (Oliveira et al., 2014). The total morphine is the sum of "free morphine" and morphine released after the hydrolysis of M3G and M6G. The use of LC coupled to mass spectrometry (MS), electrochemical (ECD), fluorescence or UV has been shown to be the best method of identifying the specific metabolites of morphine. Therefore, HPLC-UV was the main focus of this conduct, although MS has a higher sensitivity and specificity than Diode Array Detector (DAD), UV, fluorescence or ECD methods. LC-MS has some limitations when being

used to analyse glucuronides. For example, breakdown of glucuronides inside the spectrometer may give an elevated parent drug concentration.

1.2 Codeine

Codeine is widely used in the world as an analgesic. There is some similarity between the codeine and morphine structures. The codeine structure has an additional methyl group on the C3 hydroxyl group of morphine (changing it from an –OH to a -OCH_3) (Figure 3). Codeine is found alongside morphine in opium, meaning that it can also be extracted (Armstrong & Cozza, 2003). Codeine is a rather weak analgesic due to the weak affinity it has towards the opioid μ-receptors approximately 300 times smaller than morphine. Opioid receptors are inhibitory opioid containing G-protein –coupled receptors. These opioids take the place of ligands in these elements, and are found primarily in the spinal cord, the brain and digestive tract (Cregg, Russo, Gubbay, Branford, & Sato, 2013). Codeine is mainly metabolised through conjugation to form codeine-6-glucuronide and demethylation to form morphine (Frost et al., 2015).

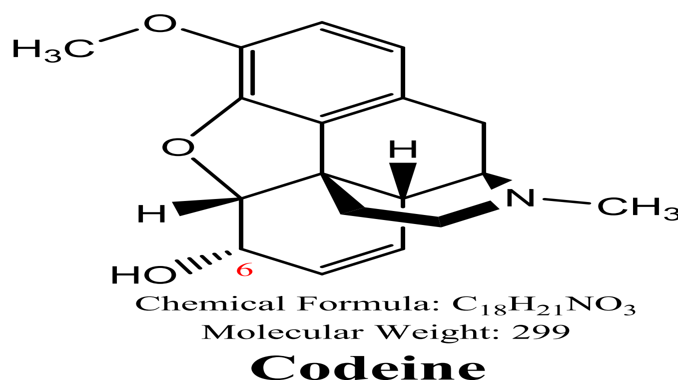


Figure (3) Chemical structure of codeine with highlighted C6 (Carlin, Dean, Bookham, & Perry, 2017).

There is compelling evidence from research such as receptor-affinity studies and clinical analysis, showing that codeine's analgesic effects are mostly due to its metabolism to morphine via the polymorphic cytochrome P450 isoenzyme 2D6 (Frost et al., 2015).

The main pathway that codeine takes is outlined in Figure 4. A big portion of codeine is glucuronidated to form the metabolite codeine-6-glucuronide (C6G), while another small portion is demethylated to form norcodeine *via* the cytochrome P450 isoenzyme 3A4. Norcodeine undergoes further reaction by being glucuronidated to form norcodeine-6-glucuronide (N6G), while the minor part that remains un-glucuronidated, is further demethylated to form normorphine. When codeine is ingested, 0 to 15% of it is O-demethylated to form morphine with the aid of CYP2D6 and further goes on to be glucuronidated to form an inactive metabolite form of morphine; M3G (which is 60% of the morphine

that is formed) and the active metabolite of morphine; M6G (which is 5-10% of the morphine formed) (Frost et al., 2015).

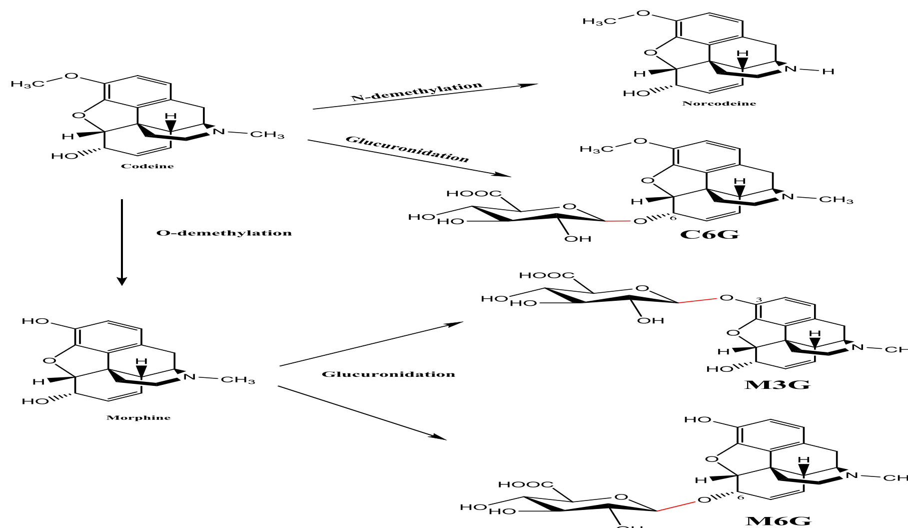


Figure (4)This figure shows the codeine metabolism pathway and glucuronide bonds are highlighted ("Codeine—An overview | ScienceDirect Topics," n.d.).

Further studies have suggested that codeine, C6G and norcodeine are part of the putative mediators of codeine toxicity (Frost et al., 2015). Existing literature on this suggests that codeine and its metabolites may exhibit postmortem redistribution (PMR), but the published data are inconsistent and quite limited (Frost et al., 2015)(Maskell, Albeishy, De Paoli, Wilson, & Seetohul, 2016). PMR means that a change occurred to the drug concentration in the body after death. PMR of opiates might be caused by the bacterial conversion of opiate-conjugates to parent drugs, instability or diffusion processes along concentration gradients (Tolliver, Hearn, & Furton, 2010).

1.3 Opiate analysis

Normally, patient specimens are tested by either quick screening methods such as immunoassay, or more complex technique such as GC-MS, HPLC-UV or LC-MS (Fernández, Morales, Vázquez, Bermejo, & Tabernero, 2006). The analysis of morphine and its metabolites has been done successfully through immunoassay methods (Netriova et al., 2006). This method is highly sensitive, but is not able to distinguish the individual opiates from their respective glucuronides, which may cross-react (Netriova et al., 2006). On the other hand, chromatography systems using either gas or liquid, can be used separate and identify the different analytes, and are therefore of great help for analysis (Bosch et al., 2007). There has not been a reported case of a successful separation of the glucuronides using GC, although GC-MS is routinely used in the analysis of morphine (Sloan & Hamann, 2006).

An LC method coupled with fluorescence detection (FLD) or electrochemical detection (ED) has similar sensitivity to GC-MS with the advantage of including the glucuronides (Bosch et al., 2007). However, there is a clear difference when it comes to the specificity of GC-MS methods (Bosch et al.,

2007). The preparation of samples for HPLC is a very important step. Extraction procedures such as solid-phase extraction (SPE), solid-phase microextraction (SPME) and liquid-liquid extraction (LLE) have been used to separate the metabolites. Differences in the Pk_a values for morphine, M3G and M6G are the main difficulty in the development of extraction methods (Netriova et al., 2006). Morphine contains a phenolic hydroxyl group at C3 with a pK_a of 9–10, a tertiary amine group with a pK_a of 7.9, and an allyl hydroxyl group at C6 (Netriova et al., 2006). The glucuronides contain a carboxylic group with a pK_a ranging from 3 to 4 (Netriova et al., 2006). M3G, due to the glucuronidation, has no phenolic hydroxyl group (Sartori, Lewis, Breaud, & Clarke, 2015).

1.3.1 Total morphine

Total morphine is essentially the sum of free morphine and the morphine that is released after the hydrolysis of M3G and M6G. Total morphine also includes several other minor metabolites in minimal quantities (Al-Asmari & Anderson, 2007). These metabolites include, morphine-2,6-diglucuronide, morphine-3-etheral sulphate, normorphine, and normorphine-6-glucuronide. Analysing total morphine using the hydrolysis of M3G and M6G and using these values in calculations increases the uncertainty (Al-Asmari & Anderson, 2007) (Frost et al., 2015).

1.3.2 HPLC-UV-DAD

HPLC-UV is an abbreviation for high performance liquid chromatography coupled with an ultra violet detector while HPLC also known as high pressure liquid chromatography. HPLC is one of the major techniques that is used in separation. In the 1960's HPLC was known as high pressure liquid chromatography and by the end of 1970's it had transformed and improvements were made on the instrumentation and the column material and is now known as high performance liquid chromatography(Netriova et al., 2006).

As shown in Figure 5, HPLC is a technique of separation that utilises a small volume of liquid sample that is injected by a sample injector into the HPLC system, and poured into a tube (column) that is filled with tiny particles (2 to 5 µm in diameter) called the *stationary phase.* Separation starts as the small volume of samples moves down the packed column under the flow of another liquid (the *mobile phase*), which is forced down the tube under high pressure. Individual components of any sample are separated from each other via the column packing, which has various physical and or chemical properties that interact with the analytes (Kaushik et al., 2006). The separated molecules are monitored as they leave the column using a detector (a flow-through device), which measures their respective amounts (Kaushik et al., 2006). Figure 3 shows the processes that are involved in HPLC.

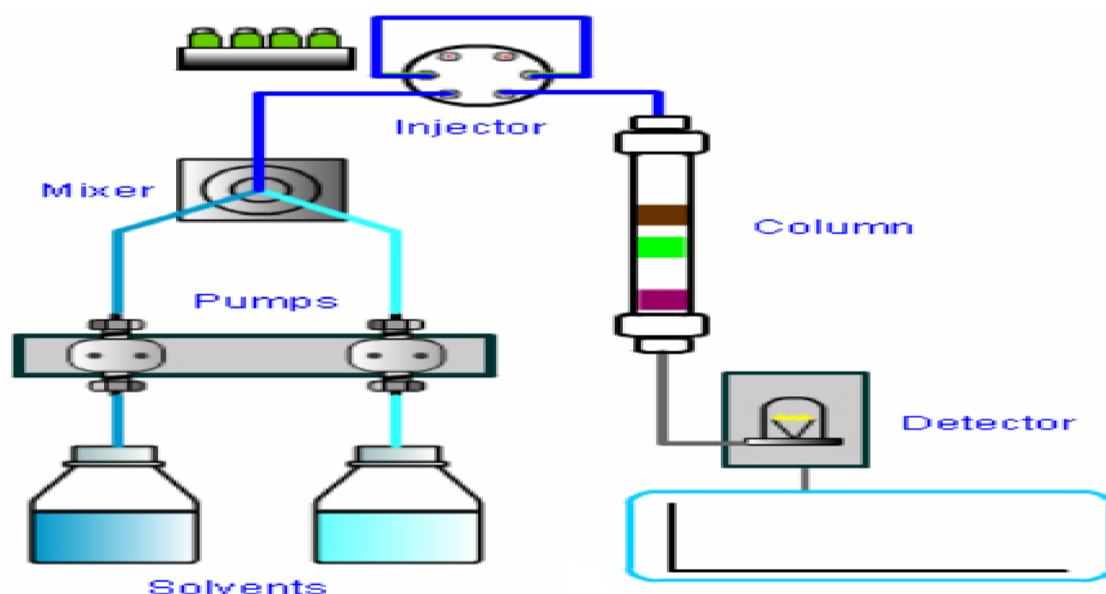


Figure (5) HPLC schematic (Dong, 2006).

Detectors that are commonly used in HPLC are normally based on the ability of analyte molecules to absorb UV light. The popularity of these detectors is because they are cheap, robust, have rela)ively low detection limits and are easy to use. UV detectors are commonly of; single wavelength, multiple wavelength or diode array configurations. DAD can perform qualitative analysis (identification) of the light absorbance intensity through the use of the UV spectra (Dong & Zhang, 2014).

As shown in Figure 6, the (UV) detectors that are commonly used in HPLC measure how monochromatic light of a certain wavelength (normally between 190 nm [UV] and 400 nm [blue light]) is absorbed. This is compared with the intensity of absorption of a reference beam and indicates the concentration of the analyte in the eluent passing through a flow cell (David, Galaon, & Aboul-Enein, 2014).

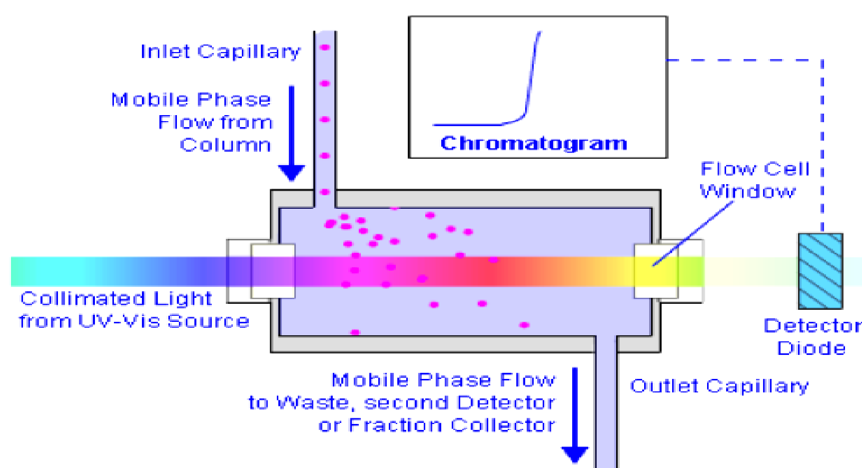


Figure (6) UV Detector for HPLC (Steed, 2007).

DADs, as shown in Figure 7 below, radiate a beam of light that is focussed through the flow cell and then a mechanically controlled or fixed width slit (David et al., 2014). The radiated light is dispersed through a holographic grating, which breaks the white light into its respective wavelengths to be detected by the photodiode array (David et al., 2014).[26] Every photodiode in the system receives a narrow wavelength of the light spectrum and may be used at any one point in the process. Differentiating the wavelengths during the chromatography provides for easy identification of the signals that are deconvoluted hence showing the specific wavelengths during the separation process. This provides the chromatography process with the ability to conduct targeted analysis, simultaneous quantitative analysis, and component identification of different signals that are of specific wavelengths (David et al., 2014).

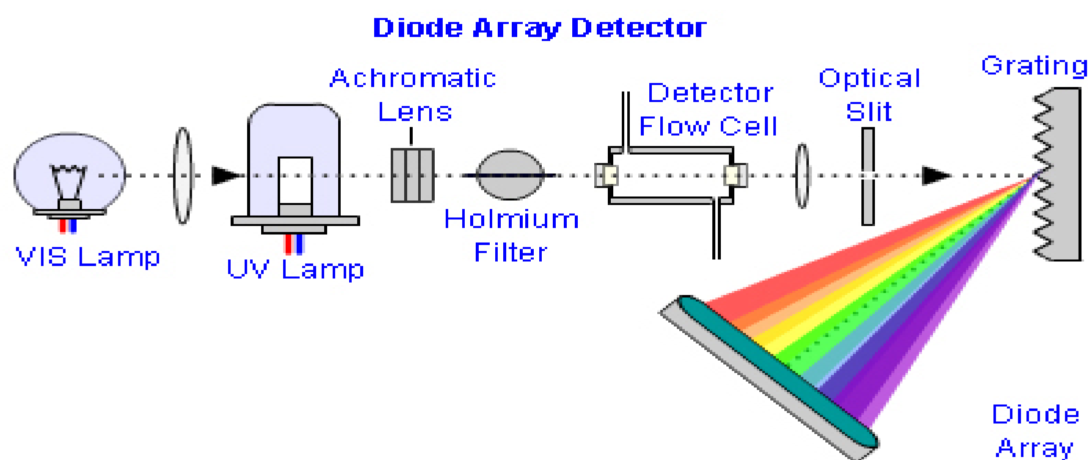


Figure (7) Diode Array Detector (Steed, 2007).

When analysing morphine and glucuronides simultaneously, the preferred choice is HPLC *reverse phase* coupled with a detection method (Netriova et al., 2006). *Reverse phase* HPLC, is normally characterised by mobile phase being more polar than the stationary phase, which may be non-polar (Dong & Zhang, 2014). Common reverse phase stationary phases are hydrophobic and chemically bonded to the surface of a silica support particle. Work by Svensson has become the benchmark for many clinical and pharmacokinetic studies (Svensson, Yue, & Säwe, 1995). Svensson noted that the HPLC-UV method that can be simultaneously conducted with electrochemical detection to determine morphine, M6G and M3G in body fluids, with UV detection at 210 nm (Santos et al., 2008).

1.3.3 SPME

Traditional methods that are used, such as LLE and SPE are tedious, time-consuming and depending on the method, require some dissolving of the sample and evaporating of solvents (Jurado, Giménez, Soriano, Menéndez, & Repetto, 2000). In recent years, SPME has been a welcomed process of extracting the analytes as they are directly absorbed to a fused-silica fibre directly from the sample (Jurado et al., 2000). The fused-silica fibres are normally coated with the relevant stationary phase. The absorption occurs when the fibre is dipped into the sample matrix. Extraction is based on whether the analytes will be

portioned between the sample matrix and the stationary fibre (Jurado et al., 2000). SPME has two main methods of extraction; first, *direct immersion* (DI) of the SPME fibre into the liquid sample matrix, simply termed DI-SPME, and the second, *head-space* (HS) extraction in which the liquid sample matrix is heated in a vial to volatilise the analytes and the fibre is placed just above the sample matrix (Kole, Venkatesh, Kotecha, & Sheshala, 2011). The fibre is placed on the inside of the injection port of the GC, where it is heated and the analytes are thermally desorbed(Jurado et al., 2000) .For LC, desorption of SPME fibres should be done in an appropriate volume of solvent or mobile phase, which can then be injected onto the LC system (Kole et al., 2011). Over the years, many studies have investigated SPME, and the technique has been widely used (Jurado et al., 2000).

In this study, new bio-compatible SPME LC tips developed by Sigma-Aldrich are evaluated for the extraction of morphine, codeine and their glucuronides from urine.

1.4 Aims and objectives

1.4.1 Aims

- To develop an efficient method of separation of morphine, codeine and glucuronides by HPLC-UV.
- To evaluate the use of SPME LC tips for the extraction of morphine, codeine and glucuronides from urine samples.

1.4.2 Objectives

- To investigate the effects of mobile phase composition and gradient to separate the compounds in a single run.
- To determine the linearity of the final method for each drug and glucuronide using an appropriate internal standard. To investigate three different fibre coatings of SPME LC Tips for the extraction of the compounds.

2. Method

1.1 Chemicals and equipment

1.1.1 Chemicals and reagents

BDH Chemicals, sodium dihydrogen orthophosphate 1-hydrate $NaH_2PO_4{\cdot}H_2O$ (analytical reagent) Lot No. A629321 602; BDH Chemicals, disodium hydrogen orthophosphate anhydrous Na_2HPO_4 (analytical reagent) Lot No. F1437681 641; acetonitrile (ACN), batch No. 15C091031 (HPLC grade); methanol (MeOH), batch No. 16Z1753 (HPLC grade); and dichloromethane (DCM), batch No. 14E230500 (HPLC grade) were obtained from VWR chemicals. Ammonium hydroxide solution 20–30% NH_3 (ACS reagent) was obtained from Sigma-Aldrich, and isopropyl alcohol (IPA) (biotechnology grade)

was obtained from Amresco. Blank urine samples were provided by department and were confirmed negative for the targeted analytes.

1.1.2 Drug and metabolite standards

Cerilliant, 1.0 mg/mL morphine M-005 Lot No. FE08141515 expires Nov. 2020; Cerilliant, 1.0 mg/mL codeine C-006 Lot No. FE11021502 expires Dec. 2020; Cerilliant, 100 μg/mL morphine-6β-D-glucuronide M-096 Lot No. FE12121401 expires Jan. 2018; Cerilliant, 100 μg/mL morphine-3β-D-glucuronide M-018 Lot No. FE06011605 expires Aug. 2021; Cerilliant, 1.0 mg/mL hydrocodone H-003 Lot No. FE09091505 expires Sep. 2020; Cerilliant, 100 μg/mL codeine-6β-D-glucuronide C-087 Lot No. FE04221401 expires Apr. 2018. MOR, COD and hydrocodone (HDC) were purchased as solutions in methanol while M6G, M3G and codeine-6-glucuronide (C6G) were purchased as solutions in a solution of water: methanol 1:1.

1.1.3 Equipment and materials

Table 1 shows the equipment and materials that have been used in this conduct.

Table (1) Equipment and materials used in experiments

Equipment and materials	Uses	Supplier
Pipettes 2-20 microliter (μL), 200 μL and 1000 μL	Measuring accurate volumes	Gilson and Eppendorf
Volumetric flasks	Standard preparation	-
Graduated cylinders	Solution preparation	-
Beakers	Mixing solution	-
Bottles	Reservoir solution	
Analytical balance	Measuring accurate masses of different chemical powders	Mettler Toledo XS1003S
pH Meter	Checking the pH of different solutions and buffers	Jenway, 3510 pH Meter
Magnetic stir plate and bars	Mixing solutions	VWR
Vortex	Mixing samples	Fischer Scientific
Sample concentrator/evaporator	Sample evaporation	Thermo Scientific
Orbital shaker	Sample agitation	IKA Vibrax VXR
Water purification system	Providing deionised water	Merck
3.5 mL and 7 mL glass vials	Conditioning SPME LC Tips and for sample preparation	Samco
1.2 mL low-adsorption vials	Sample extraction with SPME LC Tips	Supleco
0.3 mL low-adsorption vials	Sample desorption of SPME LC Tips	Supleco
HPLC vials 2 mL	Place inside the instrument for injection	Agilent
HPLC vial inserts 250 μL	Place inside HPLC vials for low volume	Sigma-Aldrich
SPME LC Tips, C18, PDMS/DVB and C18-SCX Silica (Mixed Mode)	Extraction of non-volatile analytes	Sigma–Aldrich

1.2 Solution preparation

1.2.1 Preparation of working solutions

Two working mixture solutions were prepared at concentrations of 10 µg/mL (working solution A) and 1 µg/mL (working solution B) containing five drugs: MOR, M3G, M6G, COD and C6G. Working solution A (10 µg/mL) was prepared by adding 50 µL of MOR (1 mg/mL), 50 µL of COD (1 mg/mL), 500 µL of M3G (100 µg/mL), 500 µL of M6G (100 µg/mL) and 500 µL of C6G (100 µg/mL) to a 5 mL volumetric flask and adding methanol up to the required mark. Working solution B (1 µg/mL) was prepared by adding 1 mL of working solution A to a 10 mL volumetric flask and using methanol to make it up to the mark. Internal standard (ISTD) working solution (hydrocodone) was prepared at a concentration of 10 µg/mL by adding 100 µL of HDC (1 mg/mL) to a 10 mL volumetric flask and again adding methanol to make it up to the required mark.

1.2.2 Preparation of phosphate buffer (0.0125 M, pH 7.5)

0.386 g of $NaH_2PO_4 \cdot H_2O$ and 1.363 g of Na_2HPO_4 were dissolved in 1000 mL deionised water. The solution was mixed and the pH adjusted to 7.5 ± 0.1 with 100 mM dibasic sodium phosphate to raise the pH or 100 mM monobasic sodium phosphate to lower the pH.

1.2.3 Preparation of desorption solution dichloromethane /isopropyl alcohol /ammonium hydroxide (78:20:2)

A fresh preparation was prepared by adding 40 µL of concentrated ammonium to 400 µL of IPA; this was mixed and then 1560 µL of DCM was added and mixed again.

1.3 Preparation of calibration curve standards

Eight calibration standard solutions were prepared in mobile phase (phosphate buffer 0.0123 M, pH 7.5 + 7.5% acetonitrile) by combining certain volumes of the working solution and the ISTD working solution in a HPLC vial to reach the target concentration, as shown in Table 2.

Table (2) Preparation of calibrators

Calibrator No.	Volume added (µL)				Final concentration (µg/mL)
	Working solution A	Working solution B	ISTD working solution	Mobile phase	
Cal 1	—	2.5	10	87.5	0.025
Cal 2	—	5	10	85	0.05
Cal 3	—	10	10	80	0.1
Cal 4	—	25	10	75	0.25
Cal 5	5	—	10	85	0.5 µg/mL
Cal 6	10	—	10	80	1 µg/mL
Cal 7	20	—	10	70	2 µg/mL

The quantitative determination of MOR, COD and their glucuronides was performed on the basis of peak areas using HDC as ISTD at a concentration of 1 µg/mL. The calibration data graph was recorded by measuring the response of aqueous mixtures of the pure standards at known concentrations. Microsoft Excel was used to draw the calibration curves.

1.4 Chromatographic conditions

The analysis was carried out using an Agilent 1200 HPLC series system consisting of a quaternary pump (G1311A) with four solvent lines, a degassing unit, auto sampler ALS (G1329A), column oven and UV-DAD (G1315D) detector. The opiates were separated with a Gemini column C18 110A 5 µm particle size made by Phenomenex with size 150 × 2 mm. The column oven temperature was set at 25°C. The DAD spectra allowed the wavelength range from 193 to 400 nm to be used for peak identification and purity. The UV signal was set at a wavelength of 210 nm for the detection and quantitation of opiates. The flow rate was 0.2 mL/min with a 20 µL injection volume and the injection sample loop size was 100 µL. The injection volume of 20 µL was chosen on the basis of ≤ 10% of flow rate 0.2 mL/min while the flow rate was chosen based on the 2mm diameter of the column. Peak integration and data acquisition were achieved using the 3D system ChemStation for LC software (Revision B.04.02).

The mobile phase was Solvent A (phosphate buffer (0.0125 M, pH 7.5)) – Solvent B (acetonitrile) and it was eluted in the gradient mode with a constant flow rate of 0.2 mL/min as illustrated in Table 3. The overall analysis run time was 45 min.

Table (3) Composition of mobile phase; Solvent A: phosphate buffer (pH 7.5); Solvent B: acetonitrile.

Time (min)	Solvent A (%)	Solvent B (%)
0	92.5	7.5
2	92.5	7.5
25	35	65
25.1	92.5	7.5
40	92.5	7.5

2.1 These chromatographic conditions were chosen due to method development, as will be explained later in section 3.

1.5 Extraction procedure

The extraction of urine samples was performed on three different fibre coatings of SPME LC Tips (C18, PDMS/DVB and C18-SCX). Two concentrations of drugs (2 µg/mL and 1 µg/mL) were spiked in the urine to extract. These two concentrations were used for extraction due their high response in the chromatogram and to try to initially detect any response from the extraction before checking the

sensitivity. Three different pH values (pH 3, pH 7.5 and pH 11) were used in this procedure to evaluate the extraction of MOR, COD and their glucuronides from urine. The fibres were first conditioned for 30 min; a solution of 50% methanol: water was placed in a 3.5 mL glass vial with enough volume to cover the fibre. A conditioning step was employed to solvate the fibres by the solvent to wet the functional group and make the fibre ready for extraction. The solvated fibres took on a dark-grey appearance, which according to the SPME tips data sheet meant that the fibres were conditioned and ready for extraction.[31] Buffered spiked urine samples were prepared in 1.2 mL low-adsorption vials. The tips were then transferred from the conditioning solution to the 1.2 mL vial to be sure that the tips did not dry out during the transfer (which would be shown by fibre colour changing from grey to white). Sample extractions were performed by exposing samples to the fibres using an agitation speed at 1000 revolutions per minute (rpm) in an orbital shaker for 60 min. After the extraction was complete, the LC tips were transferred to 0.3 mL low-adsorption vials containing 140 µL desorption solution (DCM/IPA/NH_4OH) for the desorption step. Desorption was performed with an agitation speed of 1000 rpm for 20 min. The total volume was evaporated under nitrogen to dryness at room temperature, and reconstituted in 100 µL of mobile phase. The vial cap was changed before the solution was injected into the HPLC system.

3. Results and discussion

The C18 reversed-phase column with a phosphate buffer (0.0125 M, pH 7.5)– acetonitrile, gradient mode mobile phase showed clear separation of direct injection standards prepared in mobile phase for MOR, M3G, M6G, COD, C6G and HDC. UV data and varying retention times allowed for identification of these products, with M3G having a retention time (RT) of 4.295 min, 11.681 min for M6G, 14.889 min for MOR, 15.353 min for C6G, 19.766 min for COD and 20.369 min for HDC. The ability of the DAD to scan the wavelength range 193–400 nm assisted in identifying the peaks by showing the variation of the absorbance at different wavelengths in a single peak. During method development, many challenges (discussed below) were encountered because of differences in the polarity of the six analytes and the UV detector; complete separation between these analytes was required in order to identify and quantify them. These challenges were optimized to achieve a good chromatogram.

3.1 Solvent selection

Several organic aqueous-based mobile phases were tested. These included phosphate buffer–acetonitrile in combination with methanol in different % compositions, and *o*-phosphoric acid with hexylamine in combination with acetonitrile. These different mobile phases were tested with a Phenomenex Gemini column C18. From these tests, phosphate buffer with methanol or/and acetonitrile was seen to produce the best results in the system (Appendix 1).

Figure 6 shows a chromatogram of the separation of MOR, M3G, M6G and COD after injection of 20 μL of standards which diluted in mobile phase into the HPLC system with a mobile phase composition of phosphate buffer: ACN:MeOH (80:10:10) at wavelength absorbance 210 nm. C6G and HDC were not obtained when analysing this mixture. The remaining compounds were separated, however, M3G (RT at 2.872) is very near the solvent peak (RT at 2.172), which will affect its peak shape. As shown in Figure 8, COD elutes at RT 42.085 min, which means it spent much time inside the column before elution, causing the peak to broaden. This is because it is less polar than morphine and morphine glucuronides and requires more organic solvent to elute at a faster rate. The change in elution mode will be discussed in detail in section 3.2.

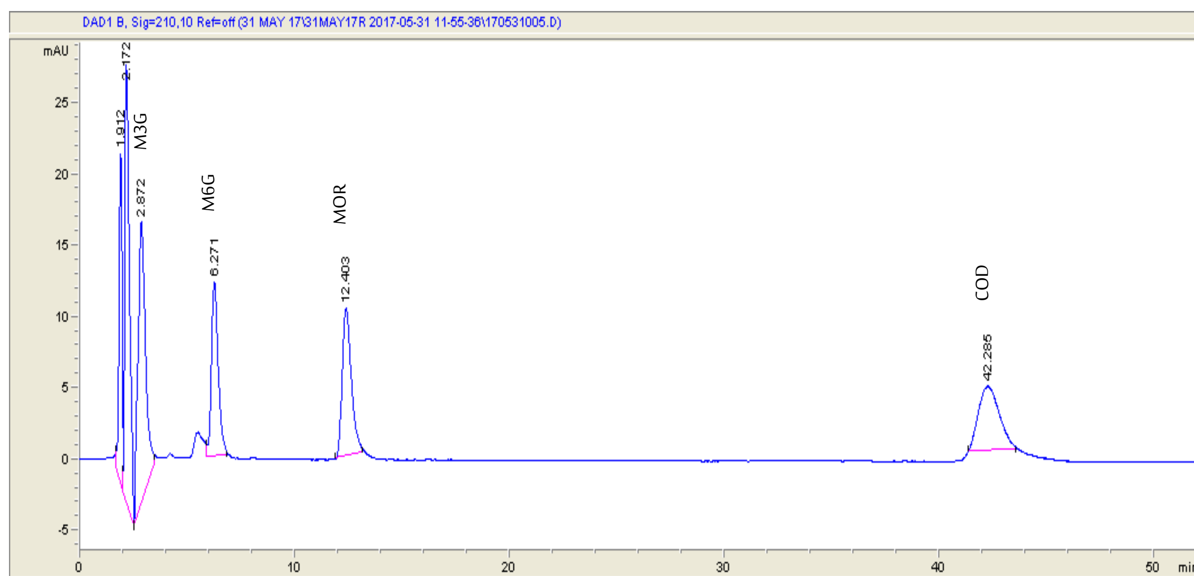
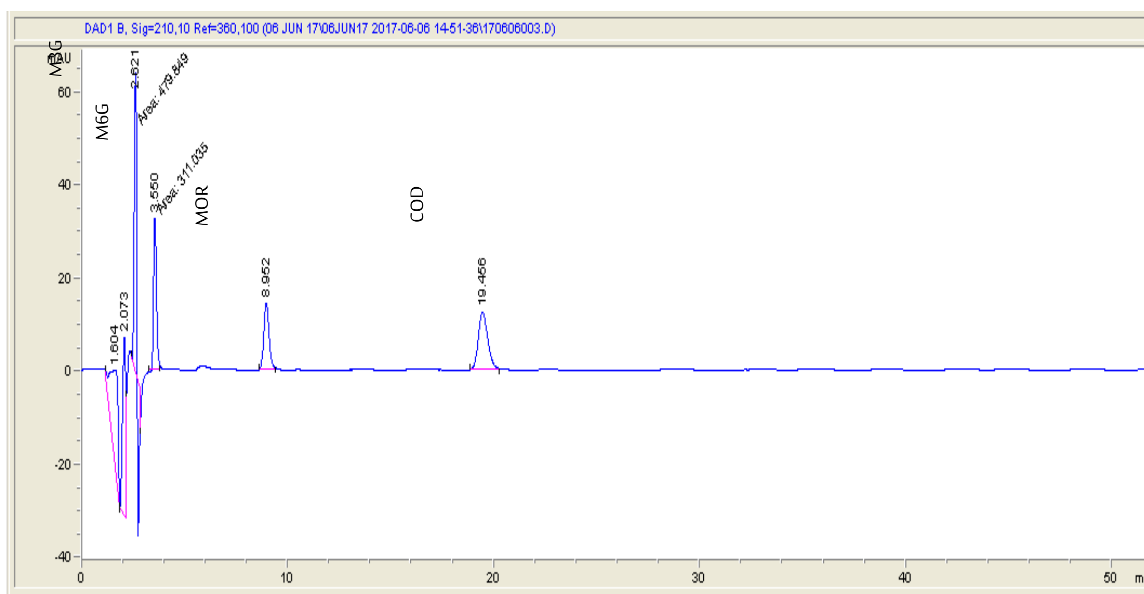
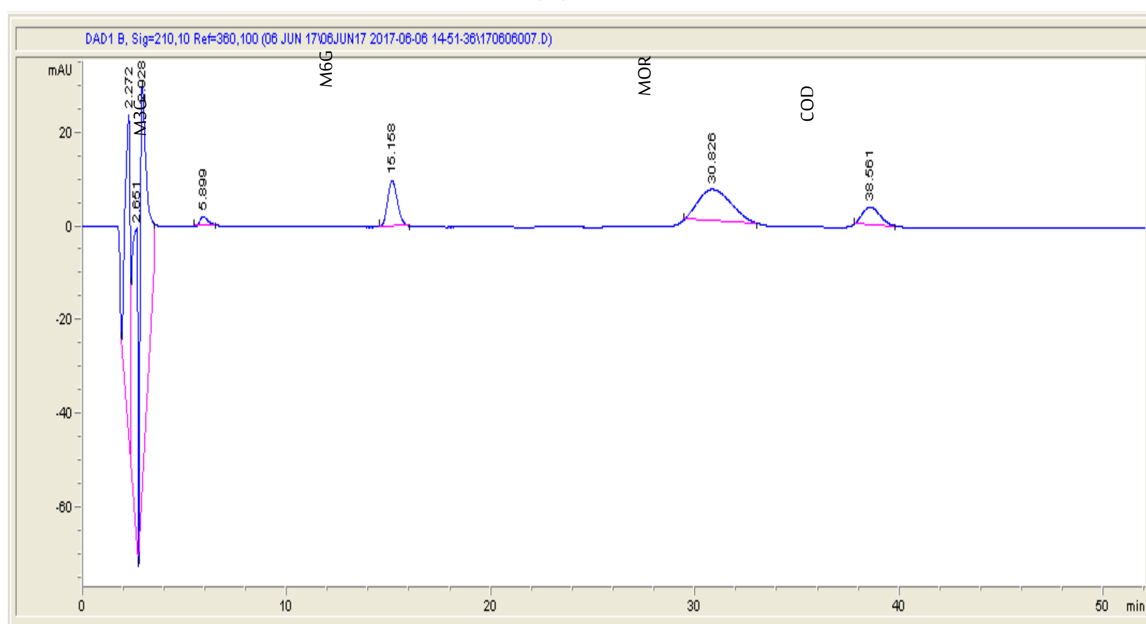


Figure (8) Chromatogram of 1 μg/mL standards with an isocratic elution mobile phase consisting of phosphate buffer:ACN:MeOH (80:10:10) showing full separation between analytes but M3G elutes near to solvent peak, COD shows broadening peak and a long analysis run time.

From the results in Figure 8, it can be seen that there are two problems to solve. M3G elutes near to solvent peak, COD shows broadening peak with a long analysis run time. The first is to delay the elution of M3G and move it away from the solvent peak. We have various parameters that can be manipulated to achieve better separation such as flow rate, column temperature, mobile phase composition and elution mode. However, first we need to modify the mobile phase composition to delay elution of M3G. Different compositions of phosphate buffer, acetonitrile and methanol were tested, but none were effective. Figure 9(A) shows the separation of M6G, MOR and COD using a mobile phase composition of phosphate buffer: ACN (90:10) in less than 20 min. Here, M3G seems to elute very fast, resulting in the interaction of M3G peak with the solvent peak at RT 2.623 min. In contrast, Figure 9(B) shows the separation between M3G, M6G, MOR and COD with poor chromatography and low sensitivity.



(A)



(B)

Figure (9) Chromatogram of 1 µg/mL standards with isocratic elution mobile phases of (A) phosphate buffer:ACN (90:10) and (B) phosphate buffer:MeOH (90:10) (A) shows better separation between analytes but M3G elutes at the same time as the solvent peak while in (B) MOR and COD show an extreme broadening peak and low response for M3G.

Comparison between acetonitrile and methanol indicates that acetonitrile achieves better chromatographic results than methanol. Morphine, codeine and their glucuronide metabolites have absorbance wavelengths of 210 nm, which is a low wavelength absorbance level. As shown in Figure 10(A) and Figure 10(B) the difference between HPLC grade acetonitrile absorbance spectra and HPLC grade methanol absorbance spectra, is that acetonitrile (190 nm) has a lower UV absorbance cut-off wavelength than methanol (205

nm)(Shen, Jin, Xue, Lu, & Dai, 2016) . So, acetonitrile has a lower interference with the analytes compared to methanol, which produces a higher peak for analytes.

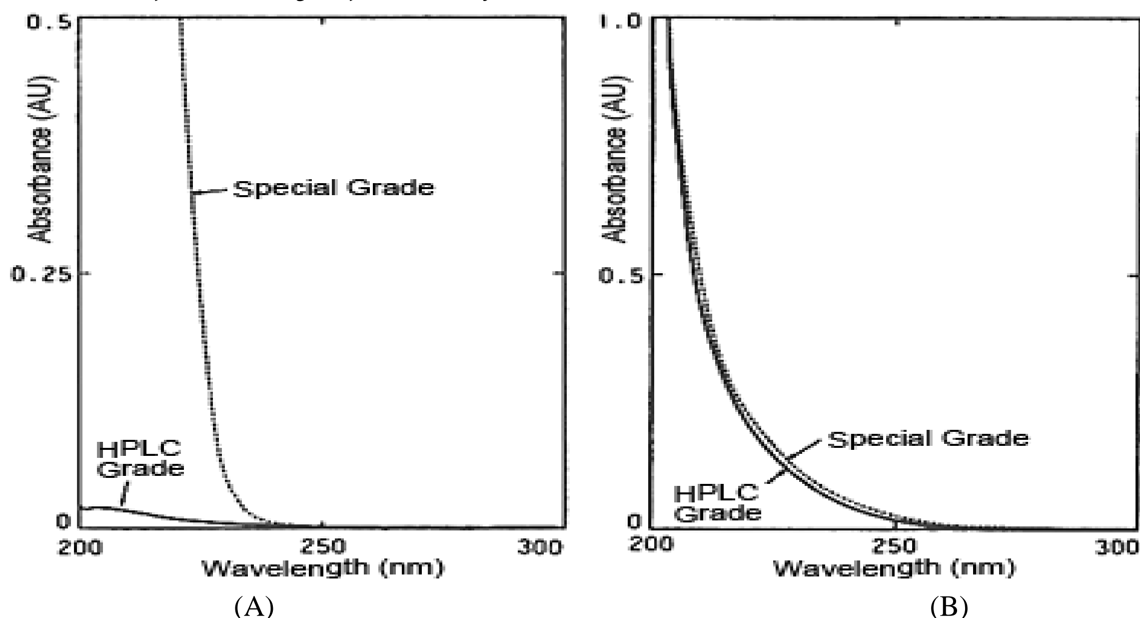


Figure (10) Absorbance spectra for (A) ACN and (B) MeOH show the difference between HPLC grade and special grade within ACN and MeOH and ACN HPLC grade shows the lowest wavelength cut-off.

From Figures 11(A) and (B), it is evident that acetonitrile has a higher elution capacity than methanol, with the retention times of all analytes eluted in Figure 9(A) being less than those in Figure 9(B).

3.2 Elution mode

During method development, many challenges were encountered resulting from the differences in polarity of the six analytes. According to Figure 9, the chromatogram results for MOR, M3G, M6G and COD with isocratic elution and a composition of phosphate buffer: ACN:MeOH (80:10:10), the retention times of all analytes lay between 2.972–42.285 min. A poor chromatogram with a broad peak was obtained for codeine (RT 42.285 min) and a tailing peak was obtained for morphine (RT 12.403 min). Isocratic elution with a different composition ratio of mobile phase showed that the run time was very long and a long gap would be seen between the analytes.

A long analysis time will cause problems in a routine laboratory as they receive many samples and this will cause delays in releasing the results. To optimise the separation of the MOR, M3G, M6G, COD, C6G and the internal standard HDC mixture, a gradient elution could be considered. *Gradient elution* is a variable composition between solvents throughout the run. In gradient elution the ratio of polar to non-polar molecules is changed in the mobile phase, while peak resolution is maintained. This makes it possible for all compounds within a mixture to be eluted. This method is also applicable when the mixture to be separated contains a wide range of compounds with different polarities.[33] Gradient elution is

advantageous because it offers complete separation of the peaks within a short period. Most importantly, the method avoids loss of resolution in early peaks or extreme broadening of later peaks. So gradient elution, as in Figure 11, was set up to separate the compound mixture and the results were very good.

The retention times lay between 3.298–20.423 min for all compounds with an initial composition of phosphate buffer:ACN (90:10) in gradient mode, which is a good run time for separating many compounds. However, the M3G peak still eluted near to the solvent peak in this gradient mode and the initial mobile composition needed to be adjusted to move this peak away from the solvent. To further optimize the chromatographic separation, the initial setup composition of the mobile phase (phosphate buffer:ACN) was changed to (92.5:2.5).

The optimization of chromatographic conditions was found to give better separation with a good retention time for M3G at RT 4.295 min creating good resolution, as shown in Figure 12. Furthermore, the chromatograms for all analytes are narrow in shape and good separation is evident.

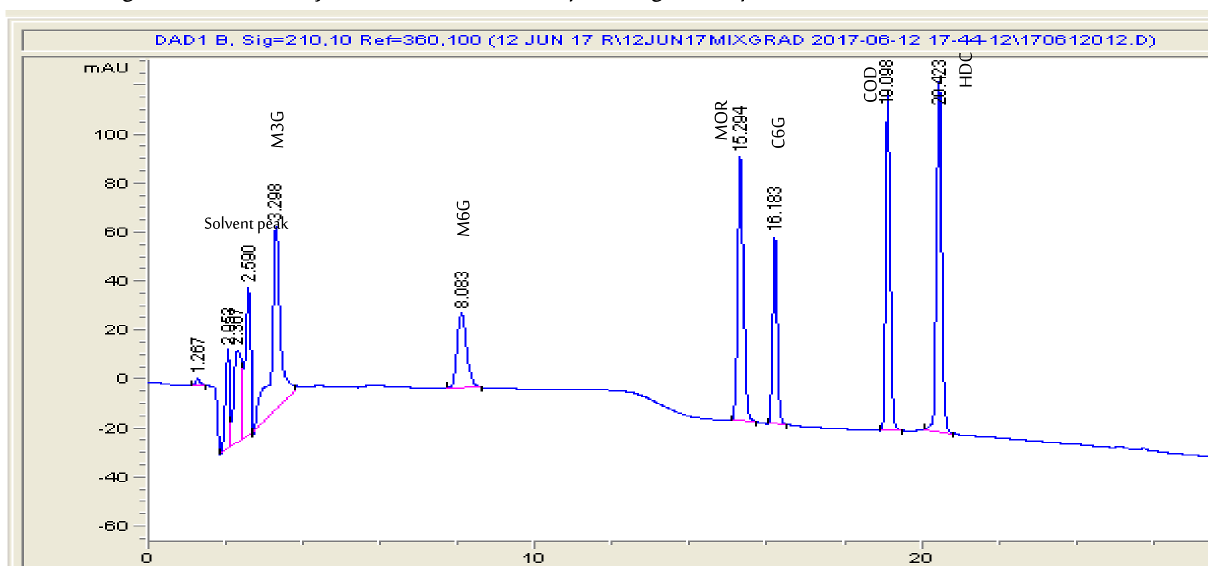


Figure (11) Chromatogram of 2 µg/mL standards with gradient elution and an initial setup composition of phosphate buffer:ACN (90:10). Chromatogram shows M3G peak elute near from solvent peaks.

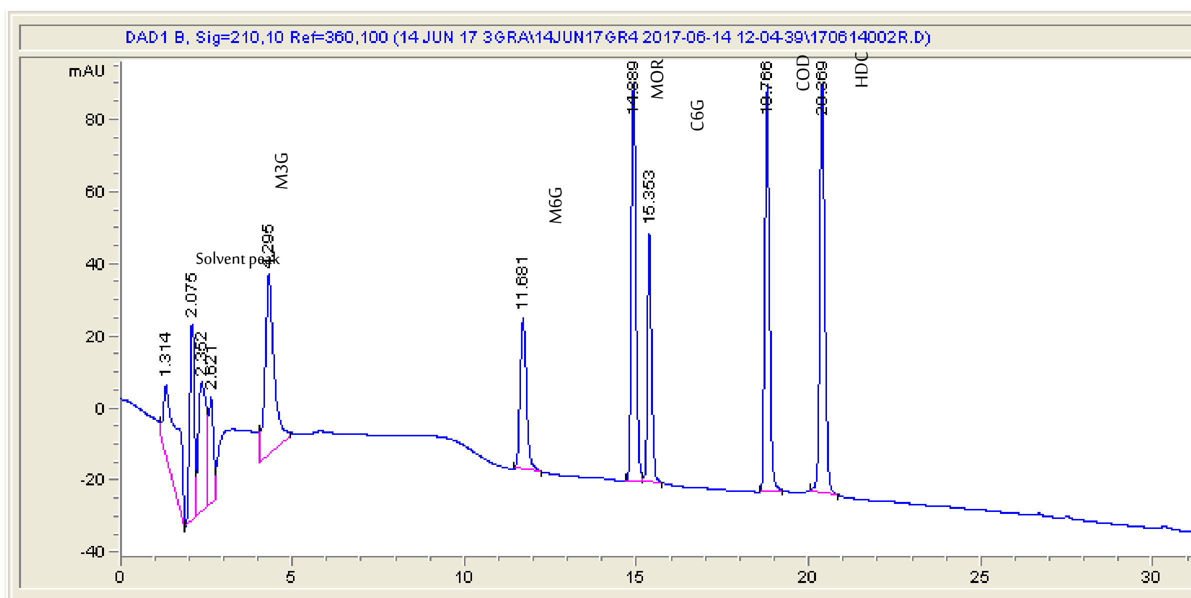


Figure (12) Chromatogram of 2 μg/mL standards with gradient elution and initial setup composition of phosphate buffer:ACN (92.5:2.5) shows better separation between solvent peaks and M3G peak than Figure 9.

3.3 Selectivity and peak identification

Using HPLC-UV-DAD allowed identification of MOR, M3G, M6G, COD, C6G and HDC from their retention times (4.295 min for M3G, 11.681 min for M6G, 14.889 min for MOR, 15.353 min for C6G,19.766 min for COD and 20.369 min for ISTD), their UV absorbance at 210 nm, and their UV spectrum data. As shown in Figure 12, the

separation of compounds was achieved and compared to the individual retention times of each standard (Appendix 2). The UV spectrum allowed the identification of the analytes by their maximum wavelength absorbance, as shown in Figure 13. Furthermore, four different wavelength signals were obtained with each result (wavelengths of 200, 210, 230 and 250 nm) and compared (Figure 13). The result of wavelength 210 nm in Figure 13 shows the best results compared to the other wavelengths.

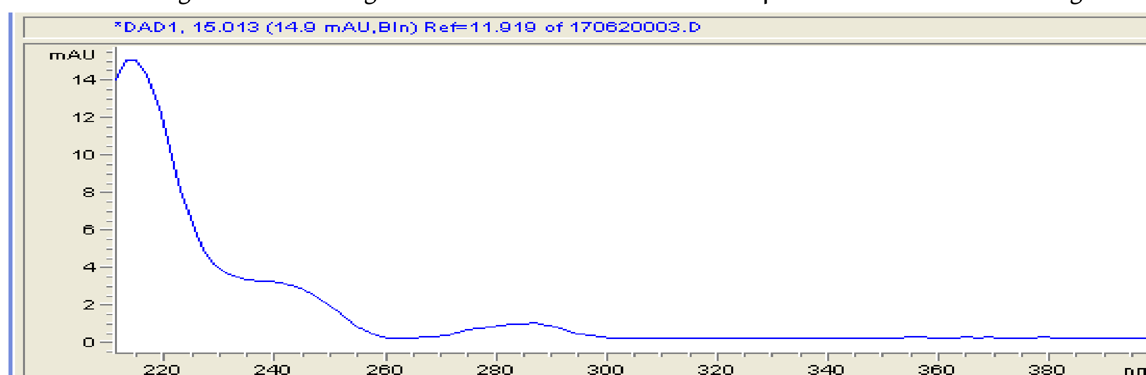


Figure (13) UV Spectrum of morphine from HPLC software shows the UV spectral range for MOR from 200 nm to 400 nm and shows the maximum wavelength absorbance at 210 nm.

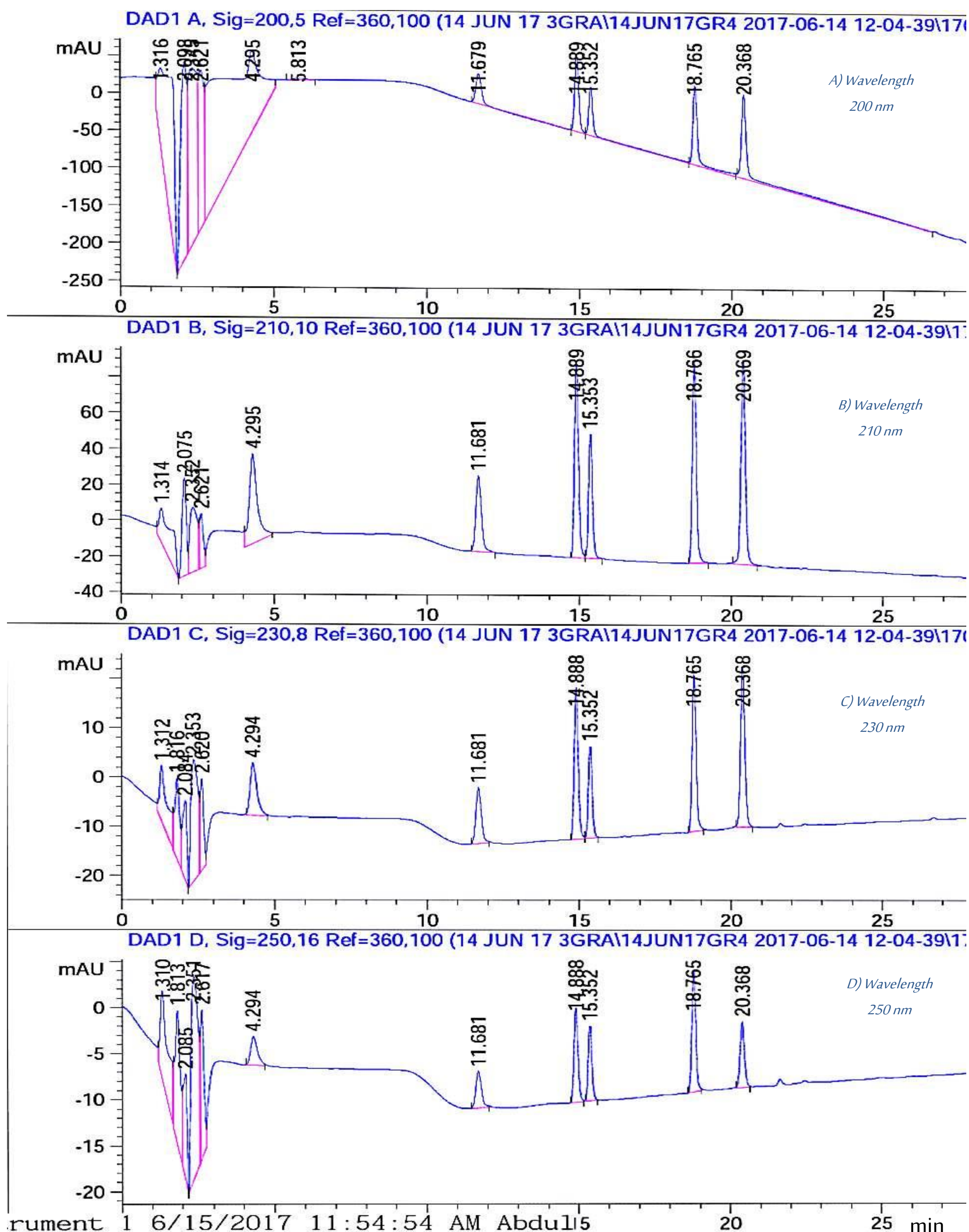
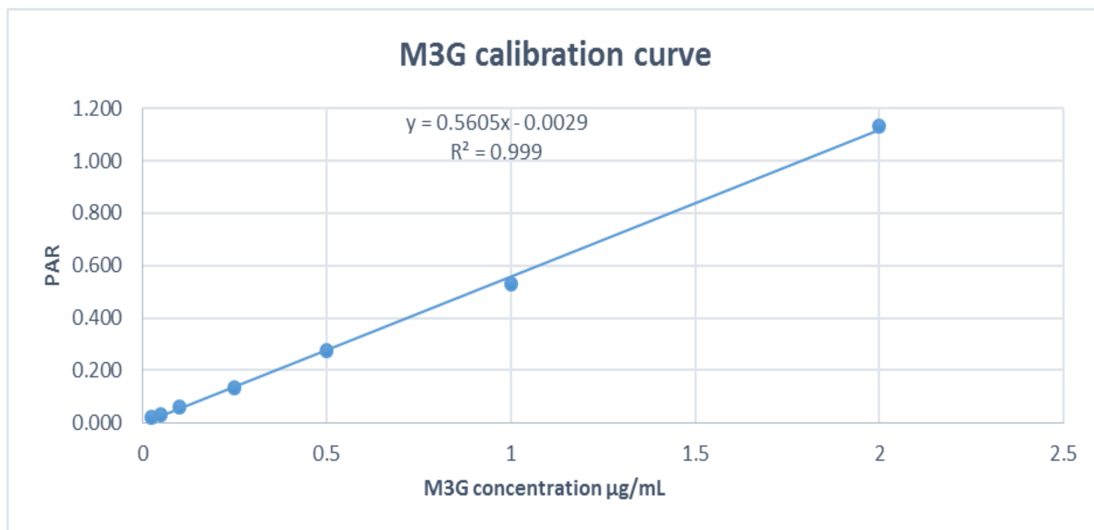


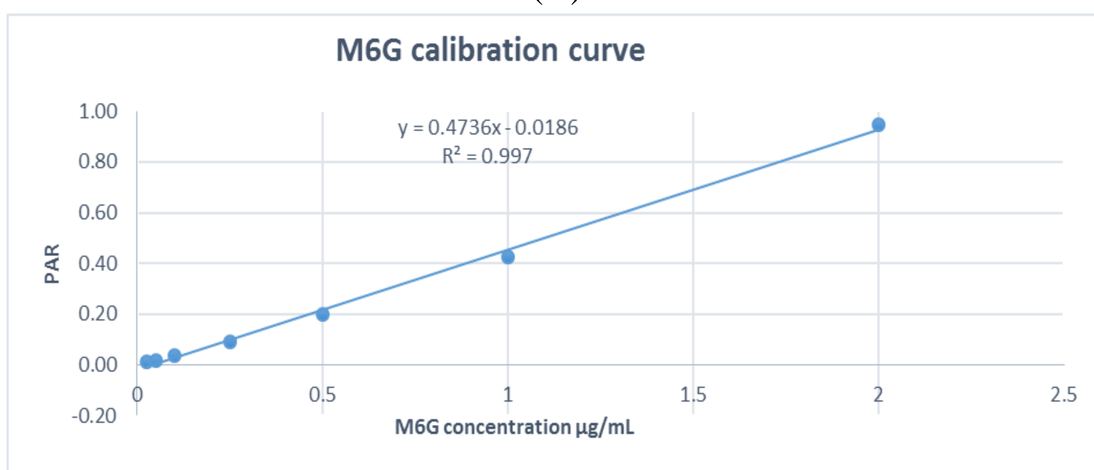
Figure (14) Chromatogram of 2 µg /mL standards shows comparison of signals of four different wavelengths of (A) 200, (B) 210, (C) 230 and (D) 250 nm and it can be observed that signal (B) 210 nm had the highest wavelength absorbance for all opiates.

3.4 Calibration

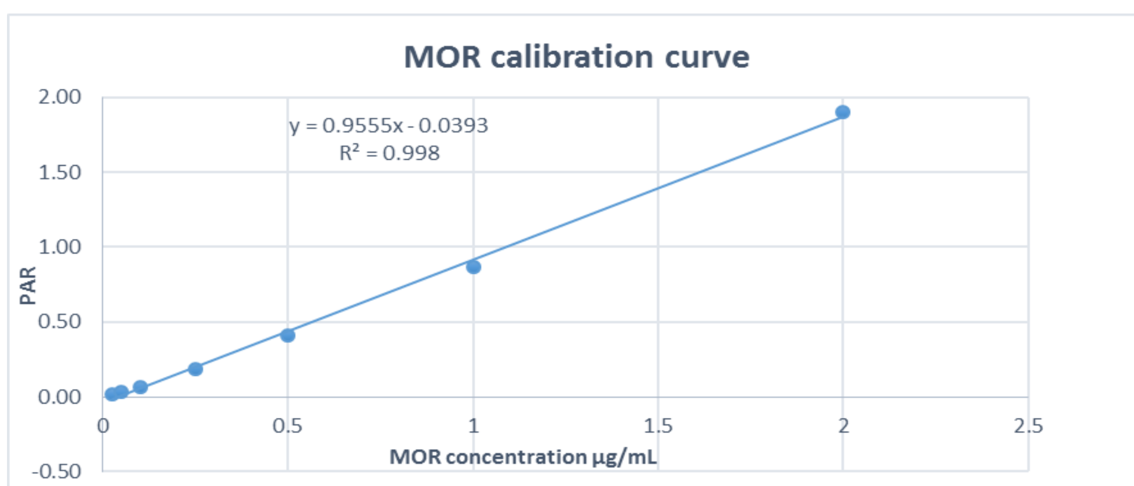
The calibration curves for all drugs were linear in the concentration range 0.025, 0.05, 0.1, 0.25, 0.5, 1.0, 2.0 µg/mL with R^2 = 0.999 for M3G (Figure 14(A)), 0.997 for M6G (Figure 14(B)), 0.998 for MOR (Figure 14(C)), 0.999 for C6G (Figure 14(D)) and 0.999 for COD (Figure 14(E)) (Appendix 3).



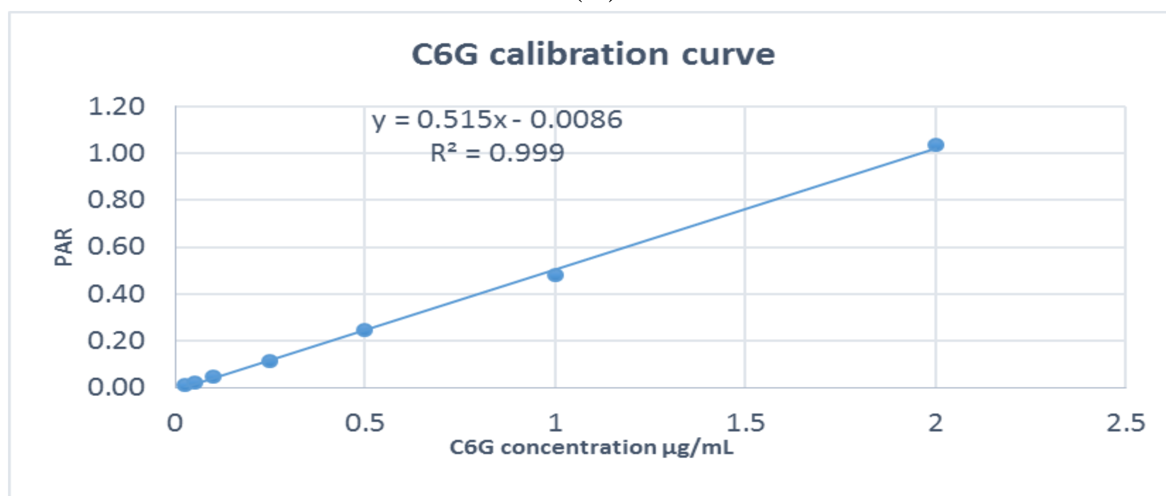
(A)



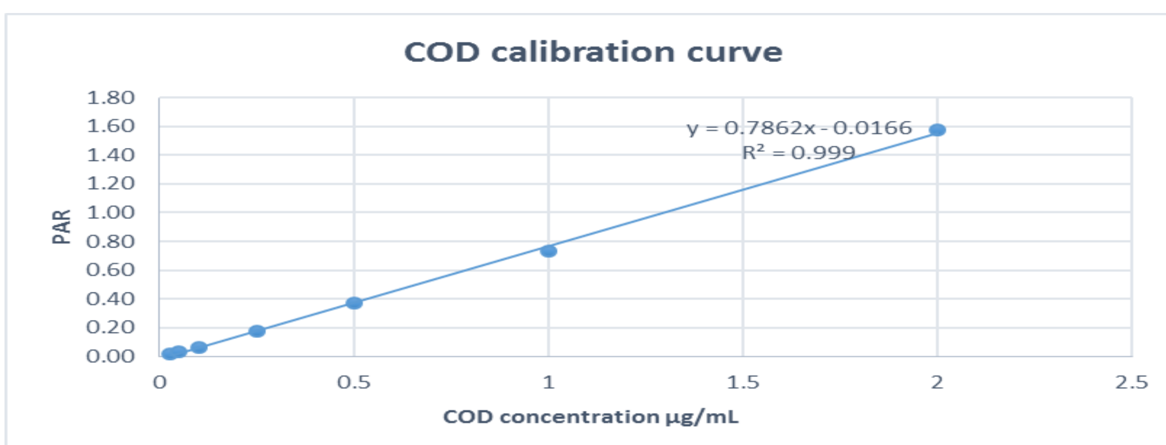
(B)



(C)



(D)



(E)

Figure (15) Un-extracted calibration curves with a good R^2 values for all analytes; drawn by using Excel sheet (A) M3G, (B) M6G, (C) MOR, (D) C6G and (E) COD.

3.5 SPME LC Tips extraction

In this study, new SPME LC tips were used to extract morphine, codeine and their glucuronides from urine with different pH levels, but no drugs could be detected in any of the analysed urine samples. The SPME LC tips are still under evaluation and there are few papers published using this technique. However, (Reyes-Garcés, Bojko, & Pawliszyn, 2014) report that when different coating thin-film SPME (C18, polystyrene-divinylbenzene (PS-DVB) and hydrophilic–lipophilic balance (HLB)) fibers were compared, the absolute recovery between different drugs varied, whereby M6G and MOR exhibited very low recovery when using a C18 coating, while the HLB coating showed better recovery for M6G, MOR and COD (Figure 15) (Reyes-Garcés et al., 2014). Moreover, M6G has a lower recovery, at less than 10%, when using the HLB coating (Reyes-Garcés et al., 2014).

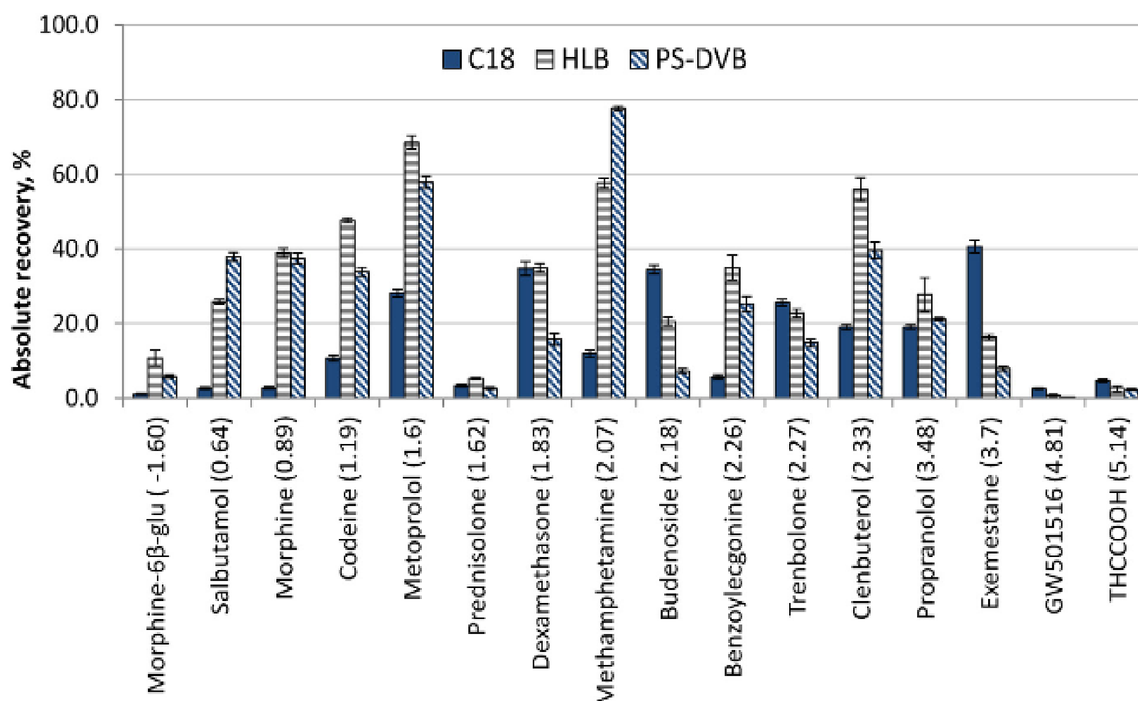


Figure (16) Taken from Reyes-Garcé. Comparison of C18, HLB and PS-DVB coating performance in the extraction of various drugs from plasma spiked at 100 ng/mL and it can be seen that the recovery for M6G is very low.

Unfortunately, because of time limitations, further investigation of the parameters could not be conducted with the SPME LC tips e.g., extraction time, pH, desorption solvent, etc.

3.6 Further work

The HPLC-UV system had low sensitivity – this could be improved with a new UV detector or by combining the UV detector with another detector such as an electrochemical detector to improve the sensitivity. Complete validation of the method and an increase in sensitivity are important before conducting analyses using biological samples, to ensure the method is robust and able to detect low concentrations. The validation will need to examine many parameters such as precision, accuracy, selectivity, linearity and stability. Precision and accuracy will be established using the Lower Limit of Quantification (LLOQ) as well as using low, medium and highly concentrated solutions(Hess, Sydow, Kueting, Kraemer, & Maas, 2018). Further optimization of gradient elution composition may decrease the run time, decrease solvents volumes, and save time.

There is still no published work focusing on direct extraction of morphine and codeine glucuronides using a new technique such as SPME LC tips. Here, research can be conducted on how different parameters can be manipulated to facilitate the extraction of these compounds.

More investigation of the effects of mobile phase composition and gradient to separate the compounds in a single run can assist detect drug of abuse. And also, increase sensitivity of test to determine the linearity of the final method for each drug and glucuronide using an appropriate internal

standard. More studies to investigate three different fibre coatings of SPME LC Tips for the extraction of the compounds are recommended.

4. Conclusion

A rapid and selective reversed-phase high-performance liquid chromatographic assay with gradient elution and ultraviolet detection for morphine, morphine-3-glucuronide, morphine-6-glucuronide, codeine, codeine-6-glucuronide and hydrocodone was developed. The HPLC-UV method has advantage over GC-MS as it does not require hydrolysis of glucuronides for detection. Prior derivatization is also not required for LC systems. However, MS detectors still have a higher sensitivity and selectivity than UV detectors. High-performance liquid chromatography, a reliable method, has been used extensively in laboratories for pharmacokinetic and toxicology studies. The chosen mobile phase showed clear separation of morphine, codeine and their glucuronides with good linearity. Extraction of morphine, codeine and their glucuronides with using SPME LC tips still requires additional research.

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