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Determination of opiates and cocaine in urine by high pH mobile phase reversed phase UPLC–MS/MS

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ABSTRACT

A fast and selective ultra performance liquid chromatography-tandem mass spectrometry (UPLC–MS/MS) method for the determination of opiates (morphine, codeine, 6-monoacetylmorphine (6-MAM), pholcodine, oxycodone, ethylmorphine), cocaine and benzoylecgonine in urine has been developed and validated. Sample preparation was performed by solid phase extraction (SPE) on a mixed mode cation exchange (MCX) cartridge. For optimized chromatographic performance with repeatable retention times, narrow and symmetrical peaks, and focusing of all analytes at the column inlet at gradient start, a basic mobile phase consisting of 5 mM ammonium bicarbonate, pH 10.2, and methanol (MeOH) was chosen. Positive electrospray ionization (ESI^+) MS/MS detection was performed with a minimum of two multiple reaction monitoring (MRM) transitions for each analyte. Deuterium labelled-internal standards were used for six of the analytes. Between-assay retention time repeatabilities ($n = 10$ series, 225 injections in total) had relative standard deviation (RSD) values within 0.1–0.6%. Limit of detection (LOD) and limit of quantification (LOQ) values were in the range 0.003–0.05 μM (0.001–0.02 μg/mL) and 0.01–0.16 μM (0.003–0.06 μg/mL), respectively. The RSD values of the between-assay repeatabilities of concentrations were ≤10% at five concentration levels for all analytes, except for pholcodine. Specificity was investigated by determination of the retention times of 96 drugs and internal standards in total. Co-eluting compounds were in all cases separated by the MS/MS detection. No or only minor matrix effects were observed. Total run time, including injection and equilibration time was 5.7 min. The method has been routinely used at the Norwegian Institute of Public Health (NIPH) since August 2007 for qualitative detection of opiates, cocaine and benzoylecgonine in more than 2000 urine samples with two replicates of each sample.

1. Introduction

Opiates are drugs naturally found in the opium poppy, *Papaver Somniferum*, or synthesized from the natural opiates (semi synthetic opiates) [1]. Opiates act on the central nervous system producing analgesia, euphoria, sedation, respiratory depression and cough suppression. Morphine and its analogues are metabolized in the human body by O-dealkylation and/or de-esterification and conjugation with glucuronic acid [2]. Morphine is excreted in urine mainly as morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) and to a lesser extent as free morphine and other metabolites. Heroin is rapidly metabolized in the human body to the active and specific heroin metabolite 6-MAM and further to morphine and conjugated morphine.

Cocaine, an alkaloid obtained from the plant *Erythroxylum coca*, is a stimulant of the central nervous system and an appetite suppressant that is subject to abuse. Cocaine is rapidly metabolized in the human body to the inactive benzoylecgonine, the main metabolite in both blood and urine [2]. Other metabolites are ecgonine methyl ester, ecgonine and norcocaine. To a lesser extent, free cocaine is excreted in the urine.

LC–MS/MS is often used to identify and quantitate drugs in human biological matrices due to the high selectivity and sensitivity, and because there is no need for derivatization of the analytes, which is often necessary for gas chromatography-mass spectrometry (GC–MS) analyses. ESI^+ is the most used ionization principle. Different sample preparation procedures for opiates, cocaine and/or benzoylecgonine in urine for LC–MS and LC–MS/MS analyses are described in the literature. Gustavsson et al. described a LC–MS/MS method with only dilution of the urine specimen before LC–MS/MS analysis [3], while Hegstad et al. have described a method using filtration and dilution of the urine before LC–MS/MS analysis [4]. To reduce the possibility of ion suppression in the ESI source caused by matrix components, a more thorough sample preparation of biological species has been recommended [5–7]. In the former method used for determination of opiates and cocaine at NIPH, the urine

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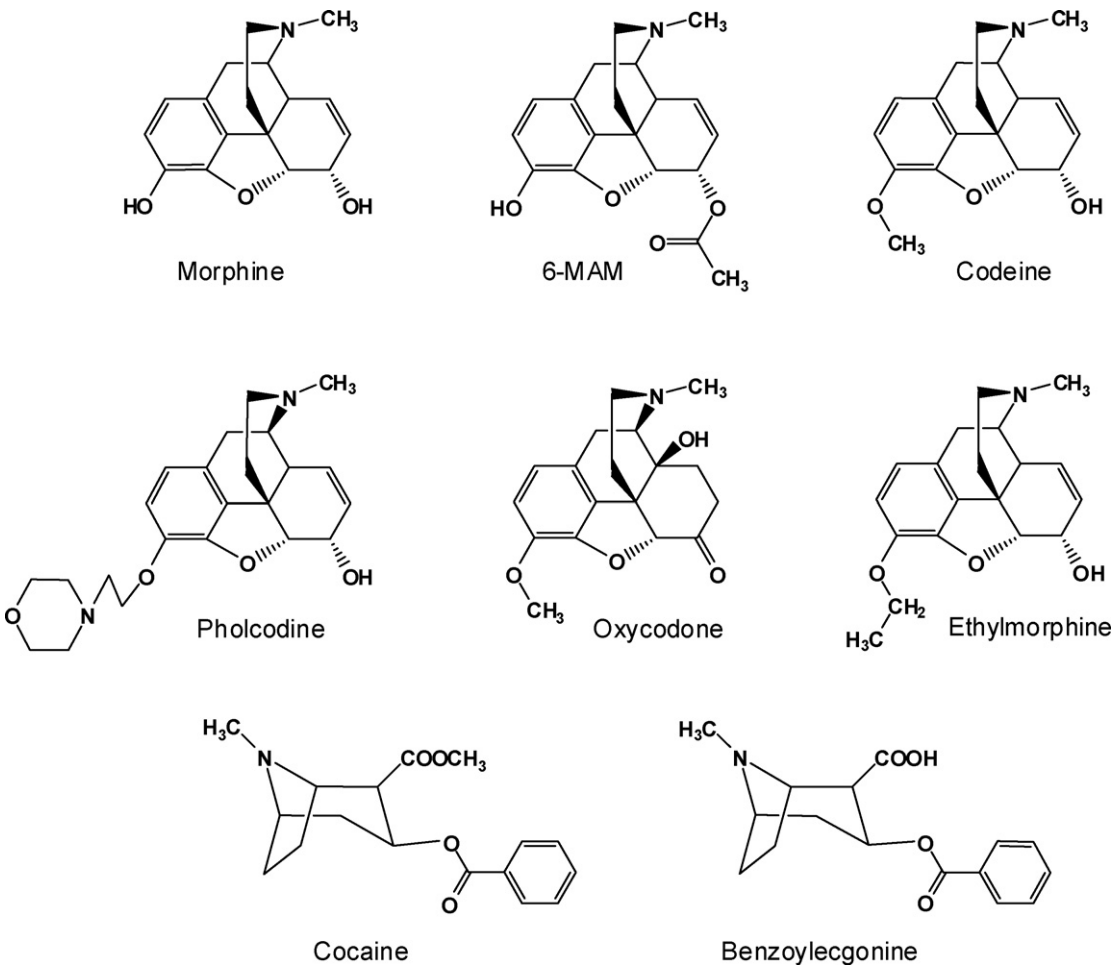


Fig. 1. Molecular structures of the selected opiates, cocaine and benzoylecgonine.

samples were prepared by mixed mode cation exchange SPE and derivatization before GC–MS analysis. The same SPE was used in the presented developed UPLC–MS/MS method. Analytes investigated in this study were morphine, 6-MAM, codeine, pholcodine, oxycodone, ethylmorphine, cocaine and benzoylecgonine. Pholcodine is a drug with two basic amino groups while the remaining analytes all have one basic amino group. Fig. 1 shows the molecular structures of the analytes.

Reversed phase (RP) LC–MS and LC–MS/MS determinations of basic drugs are usually performed with an acidic mobile phase. A reason for this is that silica based columns, which are most widely used, usually degrade at high pH. Another reason is that acidic mobile phases are thought to increase the ESI^+ sensitivity because of the increased ionization of bases at low pH [8,9]. Several LC–MS/MS methods for determination of opiates, cocaine and/or benzoylecgonine in human biological samples with acidic mobile phases have been published, e.g. in oral fluid [10], in plasma [11–14], in serum [11], in whole blood [11,15,16], in hair [17], in meconium [11] and urine [3,4,11,12,16,18–21]. However, chromatographic performance of basic compounds is usually improved with high pH mobile phases as less silanol interactions, increased retention and increased loadability are achieved [22,23]. The Waters Aquity UPLC BEH columns are compatible with both low pH and high pH mobile phases. LC–MS/MS analysis using high pH mobile phases is not common. However, Wood et al. describes a LC–MS/MS method for quantification of multiple illicit drugs, including morphine, codeine, 6-MAM, cocaine and benzoylecgonine, in oral fluid by LC–MS/MS using a pH 10 mobile phase [24]. Kasprzyk-Hordern et al. have described an UPLC–ESI^+-MS/MS-method for the determination of basic/neutral drugs, including codeine, cocaine and benzoylecgonine, in surface water, and examined both acidic and basic mobile phases [9]. Recently, Lurie and Toske have investigated both acidic and basic mobile phases for UPLC–MS/MS analysis of heroin and heroin impurities, including morphine and codeine [25].

The aim of the present study was to develop a fast and selective UPLC–MS/MS method for the determination of the same analytes determined by the former GC–MS method, morphine, 6-MAM, codeine, pholcodine, oxycodone, ethylmorphine, cocaine and benzoylecgonine in urine. The UPLC–MS/MS method is used for qualitative detection in the sense that results are reported as being positive or negative. The concentrations of the opiates in samples with low analyte concentrations (within the linear ranges) are, in addition, used at NIPH to interpret and report what type of opiate(s) that probably has (have) been taken. This procedure is most commonly used for samples containing morphine, since this drug maybe detected both after intake of morphine and as a metabolite of codeine, ethylmorphine, pholcodine and heroin [2]. The previously used GC–MS method was used in the same manner. The UPLC–MS/MS method was developed to save time and to avoid using the toxic derivatization reagents used for the former GC–MS method used at NIPH. The pressure maximum in UPLC instrumentation, up to 1000 bar, makes it possible to use columns with smaller particles and hence do faster and/or more efficient chromatographic separations compared to commercial high performance liquid chromatography (HPLC) systems that normally have a pressure maximum of 300–400 bar [26–28]. To obtain high

Table 1
Analyte and internal standard transition ions and associated mass spectrometric parameters (cone voltage, collision energy and dwell time).

Compound	Time window	Transition ion	Cone voltage (V)	Collision energy (V)	Dwell time (ms)	Internal standard
Morphine	1.0–1.9 min	286.3 > 201.1[a]	45	25	20	Morphine-D_6
		286.3 > 209.1	45	25	20	
		286.3 > 185.1	45	30	20	
6-MAM	1.9–2.7 min	328.2 > 211.1[a]	45	25	20	6-MAM-D_6
		328.2 > 193.1	45	30	20	
		328.2 > 268.2	45	25	20	
Codeine	1.9–2.7 min	300.3 > 215.1[a]	45	25	20	Codeine-D_6
		300.3 > 225.1	45	25	20	
Ethylmorphine	2.7–3.5 min	314.3 > 229.2[a]	40	25	20	Codeine-D_6
		314.3 > 257.1	40	25	20	
Pholcodine	2.7–3.5 min	399.4 > 381.3[a]	45	25	20	Codeine-D_6
		399.4 > 100.1	45	30	20	
Oxycodone	2.7–3.5 min	316.3 > 298.2[a]	25	25	20	Oxycodone-D_6
		316.3 > 241.2	25	30	20	
Benzoylecgonine	1.0–1.9 min	290.3 > 168.1[a]	30	20	20	Benzoylecgonine-D_8
		290.3 > 150.1	30	25	20	
Cocaine	3.5–4.6 min	304.3 > 182.1[a]	35	25	20	Cocaine-D_3
		304.3 > 150.1	35	20	20	
Morphine-D_6	1.0–1.9 min	292.3 > 201.1[a]	45	25	20	
		292.3 > 209.1	45	25	20	
6-MAM-D_6	1.9–2.7 min	334.2 > 211.1[a]	45	25	20	
		334.2 > 271.2	45	25	20	
Codeine-D_6	1.9–2.7 min	306.3 > 218.1[a]	45	25	20	
		306.3 > 228.1	45	25	20	
Oxycodone-D_6	2.7–3.5 min	322.3 > 304.2[a]	25	25	20	
		322.3 > 247.2	25	30	20	
Benzoylecgonine-D_8	1.0–1.9 min	298.3 > 171.1[a]	30	20	20	
		298.3 > 153.1	30	25	20	
Cocaine-D_3	3.5–4.6 min	307.3 > 185.1[a]	35	25	20	
		307.3 > 150.1	35	20	20	

[a] MRM ions used for quantifications.

chromatographic efficiency, conditions providing focusing of the analytes (no/low elution) at gradient start were wanted. This paper presents a validated UPLC–MS/MS method for the determination of opiates, cocaine and benzoylecgonine in urine. All analytes were almost completely focused at the Aquity BEH C_{18} column at gradient start with a high pH mobile phase. To the best of our knowledge, no UPLC–MS/MS method for the determination of all these opiates and/or cocaine and benzoylecgonine in urine with a high pH mobile phase has been described in the literature.

2. Experimental

2.1. Reagents and standards

2-Propanol, acetic acid, ammonia (25%), ammonium acetate, dichloromethane, disodium hydrogenphosphate, hydrochloric acid, potassium dihydrogenphosphate were purchased from Merck (Darmstadt, Germany). Acetonitrile (ACN) and MeOH were purchased from LabScan (Dublin, Ireland). Ammonium bicarbonate was purchased from Sigma–Aldrich (UK), ammonium formate was purchased from BDH (Pole, England) and formic acid was obtained from BDH Prolabo (Briare, France). Morphine, codeine, pholcodine and ethylmorphine were purchased from Norsk Medisinal Depot (Oslo, Norway). 6-MAM and oxycodone was purchased from Lipomed (Arlesheim, Switzerland). Benzoylecgonine and cocaine were purchased from Sigma-Aldrich (St. Lois, MO, USA). Morphine-D_6, 6-MAM-D_6, codeine-D_6, oxycodone-D_6, benzoylecgonine-D_6 and cocaine-D_3 were purchased from Cerilliant (Round Rock, TX, USA). Type 1 water (18.2 MΩ) was obtained from an in house Milli-Q Biocel from Millipore with an Ultrapore Quantum Organex cartridge.

2.2. Sample specimen

Urine samples received for analyses at NIPH are from different case categories such as forensic autopsies, medical cases, suspected drug abuse by the police or prison inmates, social medicine and workplace drug testing. Preservative-free urine containers were purchased from Sterilin (Staffordshire, UK) and Greiner Bio-One (Kremsmûnster, Austria). Urine samples screened positive for opiates and/or cocaine by an immunological screening (EMIT) method were analysed by the developed UPLC–MS/MS method.

2.3. Preparation of solutions and samples

Each assay contained calibrants, control samples and blank samples in addition to the samples, all utilizing 0.50 mL urine. Stock solutions of each analyte were prepared in MeOH in glass volumetric flasks. Working solutions were made in type 1 water by appropriate dilution of the stock solutions. The following was used to convert from μg/mL to μM: ((μg/mL)/molecular mass) × 1000. Two working solutions were made with morphine, codeine, oxycodone, pholcodine, ethylmorphine and benzoylecgonine at 2 and 20 μM concentrations, respectively. Two calibrant working solutions were made with 6-MAM and cocaine at 1 and 20 μM concentrations, respectively. Separate working solutions were made because of potential stability problems of 6-MAM and cocaine. Calibrants with morphine concentrations in the range 0.10–3 μM

(0.029–0.86 μg/mL), 6-MAM concentrations in the range 0.05–3 μM (0.016–0.98 μg/mL), codeine concentrations in the range 0.10–3 μM (0.030–0.90 μg/mL), oxycodone concentrations in the range 0.10 to 6 μM (0.032–1.9 μg/mL), pholcodine concentrations in the range 0.10–6 μM (0.040–2.4 μg/mL), ethylmorphine concentrations in the range 0.10–6 μM (0.031–1.9 μg/mL), benzoylecgonine concentrations in the range 0.10–6 μM (0.029–1.7 μg/mL), cocaine concentrations in the range 0.05–6 μM (0.015–1.8 μg/mL), all in blank urine, were prepared by appropriate dilution of the working solutions. Control working solutions were made in the same way as the calibrants by appropriate dilutions of the stock solutions. Each assay contains three control samples with analyte concentrations: 0.10, 0.40 and 1.0 μM (morphine: 0.029, 0.11 and 0.29 μg/mL, 6-MAM: 0.033, 0.13 and 0.33 μg/mL, codeine: 0.030, 0.12 and 0.30 μg/mL, oxycodone: 0.032, 0.13 and 0.32 μg/mL, ethylmorphine 0.031, 0.13 and 0.31 μg/mL, pholcodine: 0.040, 0.16 and 0.40 μg/mL, benzoylecgonine: 0.029, 0.12 and 0.29 μg/mL, cocaine: 0.030, 0.12 and 0.30 μg/mL). An internal standard working solution containing morphine-D_6, codeine-D_6, oxycodone-D_6 and benzoylecgonine-D_8 and a working solution containing 6-MAM-D_6 and cocaine-D_3, were made in type 1 water. Internal standard concentrations in all samples in each assay were 1 μM (0.3 μg/mL). Sample preparation were performed by adding 0.050 mL of both internal standard working solutions and 0.5 mL 0.67 M Sørensens phosphate buffer (pH 7.4) to each sample (calibrants, control samples, blank samples and urine samples), before extraction on Oasis MCX SPE cartridges (60 cc, 30 mg) from Waters (Wexford, Ireland). The cartridges were conditioned by 2 mL MeOH and 2 mL type 1 water before the urine samples were loaded onto the cartridges. The cartridges were washed with 2 mL type 1 water, 1 mL 0.1 M HCl, and finally 2 mL MeOH before dried under vacuum for at least 2 min before elution with 2 mL dichloromethane/2-propanol/ammonia (80/20/2) into 5 mL glass tubes. The samples were dried at 50 °C with nitrogen gas, reconstituted in 0.400 mL of MeOH/type 1 water (5/95) and transferred to autosampler vial. One microliter of the extracted sample was analysed by UPLC–MS/MS. Validation samples were prepared in the same way as the calibrants.

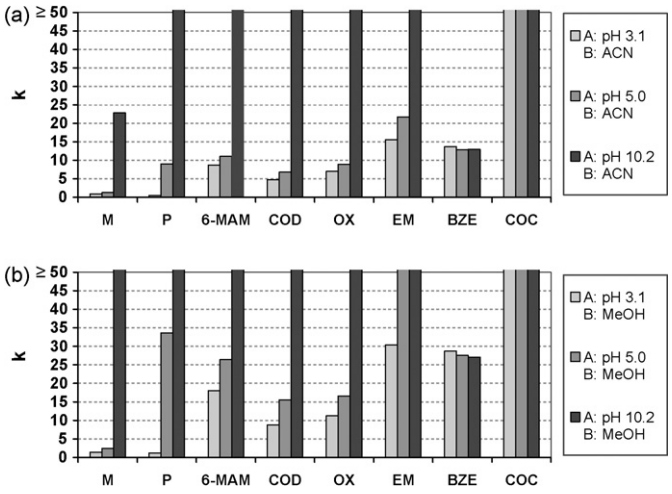


Fig. 2. *k* values obtained with 5% ACN (a) and 5% MeOH (b) in mobile phases with buffer pH 3.1, 5.0 or 10.2 on an Aquity BEH C18 column. *k* values ≥ 50 were in most cases much higher than 50. Abbreviations; M: morphine, COD: codeine, OX: oxycodone, EM: ethylmorphine, BZE: benzoylecgonine, COC: cocaine.

2.4. Instrumentation

An Acquity UPLC with a sample manager and a binary solvent manager, coupled to a Quattro Premiere Xe tandem mass spectrometer from Waters (Milford, MA, USA) was used. Chromatographic separation was performed at 60 °C on an Acquity UPLC BEH C_{18} column (2.1 mm ID × 50 mm, 1.7 μm particles) from Waters (Wexford, Ireland). A column in-line filter was used in front of the column. The mobile phase for the validated method consisted of 5 mM ammonium bicarbonate buffer, pH 10.2 (solvent A) and MeOH (solvent B). Mobile phase flow rate was 0.400 mL/min. Gradient profile (% B) was: 5% B in 0.0–0.15 min, 5–30% B in 0.15–0.30 min, 30–50% B in 0.30–2.70 min, 50–90% B in 2.70–3.80 min, 90% B in 3.80–4.20 min, 90–5% B in 4.20–4.50 min, 5% B in 4.50–5.00 min. The total post-injection equilibration time was 1.2 min, including 0.7 min injection

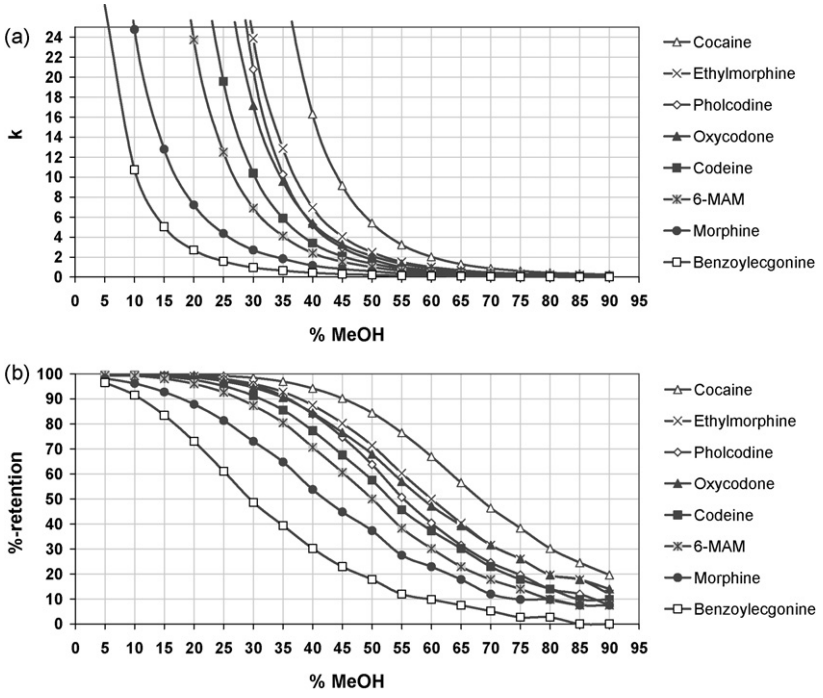


Fig. 3. *k* values (a) and % retention (b) on an Aquity BEH C_{18} column obtained with different amounts MeOH in the mobile phase. 5 mM ammonium bicarbonate buffer (pH 10.2) was used as the aqueous solvent.

time and 0.5 min at the end of the gradient. ESI-MS/MS-detection was performed in multiple reaction monitoring (MRM) mode. Desolvation gas temperature was optimized to 500 °C. Desolvation gas flow was 900 mL/h. Capillary voltage and capillary angle button adjustments were optimized to 1 kV and 5.5, respectively. Table 1 shows the analyte and internal standard transition ions and associated mass spectrometric parameters (cone voltage, collision energy and dwell time).

3. Results and discussion

3.1. Method development

An UPLC–MS/MS method was explored to save time and to avoid using the toxic derivatization reagents used by the former GC–MS method used at NIPH. Sample preparation procedures were similar as the former GC–MS method with mixed mode cation exchange SPE. Deuterium-labelled internal standards were used for six of the eight analytes to compensate for variable extraction yields and to reduce possible effects of ion suppression/enhancement in the MS source. Codeine-D_6 was chosen as the internal standard for both ethylmorphine and pholcodine due to structure similarities.

Efficient chromatographic separation with narrow and symmetrical peaks is important to obtain a selective LC–MS/MS method and to reduce the possibility of ion suppression and/or ion enhancement in the MS source [5,29]. Focusing of the analytes on the column inlet at gradient start minimizes the effect of pre-column peak broadening and gives a better control of the chromatographic separation. In LC–MS and LC–MS/MS with ESI low amounts of organic solvent in the mobile phase is known to give poor spray quality and low sensitivity [30,31]. Hence, conditions providing increased retention of the most polar compounds are favourable. To minimize the LC–MS/MS instrument operation time and to ease the operator job, short analysis times are desirable as well.

3.1.1. Focusing of analytes at column inlet

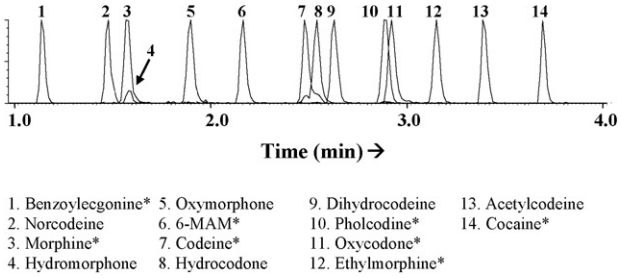
When a compound is completely focused at the column (no elution), the retention time, t_R, and the retention factor, k, increases toward infinity. To investigate if the analytes were focused at the column inlet with 5% B in the mobile phase, a gradient with a 60 min isocratic delay at 5% B was used. After 60 min the amount of B was rapidly increased to 90% and kept at this concentration for 0.7 min to elute all analytes, before it was decreased to 5% B again. Three buffers, 5 mM ammonium formate (pH 3.1), 5 mM ammonium acetate (pH 5.0) and 5 mM ammonium bicarbonate (pH 10.2) were examined as the aqueous part of the mobile phase. ACN and MeOH were examined as organic modifiers. The void time, t_0, was determined to be 0.37 min. Analytes not eluting within the first 60 min were assigned with a retention time of 60 min giving a maximum k value of 161. Fig. 2 shows k values obtained on an Aquity BEH C_{18} column at a column temperature of 60 °C.

Fig. 2 shows that the retention of opiates was much higher when the 5 mM ammonium bicarbonate buffer (pH 10.2) was used as the aqueous part of the mobile phase. Benzoylecgonine, with both an acidic and a basic functional group, had similar retention factors no matter what the pH was in the aqueous part of the mobile phase. Cocaine was highly retained with all mobile phases examined. ACN as the organic modifier (Fig. 2a) gave, as expected from the solvent strengths based on Snyders selectivity triangle [32], less retention than MeOH (Fig. 2b).

Table 2
ESI$^+$ sensitivity (high pH vs low pH mobile phase).[a].

Analyte	Concentration		t_R (min)		Peak area ratio
	μM	μg/mL	pH 10.2	pH 3.1	pH 10.2 vs pH 3.1
Morphine	0.40	0.11	2.08	1.02	~4:1
6-MAM	0.10	0.033	2.36	1.49	~3:1
Codeine	0.40	0.12	2.48	1.38	~3:1

[a] Gradient profile used: in 0.00–0.15 min: 3% B, in 0.15–3.15 min: 3–90% B, in 3.15–4.00 min:90% B, in 4.00–4.30 min: 90–3% B, in 4.30–5.00 min: 3% Six replicate injections of a standard sample was analysed by UPLC–MS/MS both with the high pH and the low pH mobile phase.



1. Benzoylecgonine* 5. Oxymorphone 9. Dihydrocodeine 13. Acetylcodeine
2. Norcodeine 6. 6-MAM* 10. Pholcodine* 14. Cocaine*
3. Morphine* 7. Codeine* 11. Oxycodone*
4. Hydromorphone 8. Hydrocodone 12. Ethylmorphine*

Fig. 4. Chromatographic separation of opiates, cocaine and benzoylecgonine on an Aquity BEH C_{18} column (2.1 × 50 mm) at a column temperature of 60°C and a flow rate of 0.400 mL/min (2.5 μM standard solution). Gradient profile was: 5% B in 0.0–0.15 min, 5–30% B in 0.15–0.30 min, 30–50% B in 0.30–2.70 min, 50–90% B in 2.70–3.80 min, 90% B in 3.80–4.20 min, 90–5% B in 4.20–4.50 min, 5% B. * analytes qualitative determined in the developed UPLC–MS/MS method.

3.1.2. ESI$^+$ sensitivity of basic compounds in RP LC–MS(/MS)

Acidic mobile phases have been thought to be necessary in RP LC–MS and LC–MS/MS to obtain high ESI$^+$ sensitivity of basic compounds. However, basic mobile phases may also give high ESI$^+$ sensitivity. Zhou and Cook have suggested two gas phase reaction mechanisms that explain how NH_4^+ ions present in a high pH mobile phase may improve the ESI$^+$ sensitivity of basic compounds [33]. Recently, Lurie and Toske have investigated both acidic and basic mobile phases for UPLC–MS/MS analysis of heroin, morphine, codeine and several heroin impurities not investigated in this study [25]. They report approximately a 10 fold increase of the sensitivity when a low pH mobile phase was used compared to a high pH mobile phase. In the present study, the peak intensities of morphine, codeine and 6-MAM obtained by using 5 mM ammonium bicarbonate buffer (pH 10.2) was compared to the intensities

Table 3
Retention time repeatabilities.

Compound	Within-assay repeatability (n = 10)		Between-assay repeatability (n = 10)[a]	
	t_R (min)	RSD (%)	t_R (min)	RSD (%)
Benzoylecgonine-D_8	1.127 ± 0.007	0.26	1.128 ± 0.010	0.37
Morphine-D_6	1.539 ± 0.002	0.13	1.548 ± 0.019	0.56
6-MAM-D_6	2.131 ± 0.005	0.06	2.136 ± 0.021	0.40
Codeine-D_6	2.435 ± 0.005	0.03	2.436 ± 0.025	0.43
Oxycodone-D_6	2.875 ± 0.005	0.05	2.873 ± 0.028	0.41
Cocaine-D_3	3.681 ± 0.003	0.02	3.680 ± 0.011	0.11

[a] In total, 225 injections in 10 assays performed over a time period of a month.

Table 4
Accuracy and precision.

Analyte	Within-assay repeatability ($n = 10$)				Between-assay accuracy and repeatability ($n = 10$)				
	Theoretical concentration		Found mean (μM)	RSD (%)	Theoretical concentration		Found mean (μM)	Accuracy (%)	RSD (%)
	μM	μg/mL			μM	μg/mL			
Morphine	0.050	0.014	0.059	7.0	0.050	0.014	0.051	10	4.4
	0.101	0.029	0.105	5.9	0.100	0.029	0.106	5.6	6.4
	0.402	0.115	0.402	2.9	0.401	0.114	0.407	1.5	7.8
	1.006	0.287	1.031	3.2	1.002	0.286	0.981	−2.1	5.5
	4.023	1.148	4.329	2.9	4.006	1.143	3.613	−9.8	4.3
6-MAM	0.025	0.008	0.025	10	0.025	0.008	0.025	1.6	8.1
	0.050	0.016	0.053	3.6	0.100	0.033	0.103	2.9	7.1
	0.201	0.066	0.202	2.8	0.401	0.131	0.401	−0.1	5.6
	1.006	0.329	1.027	1.9	1.003	0.329	1.002	−0.1	5.1
	4.037	1.322	3.678	2.0	4.014	1.314	3.611	−10	5.3
Codeine	0.050	0.015	0.053	8.6	0.050	0.015	0.051	1.8	10
	0.101	0.030	0.112	5.4	0.101	0.030	0.104	2.5	3.8
	0.404	0.121	0.410	3.3	0.404	0.121	0.396	−1.9	4.5
	1.011	0.303	1.023	3.3	1.009	0.302	0.971	−3.8	4.2
	4.019	1.203	4.144	3.6	4.006	1.199	3.407	−15	4.9
Oxycodone	0.025	0.008	0.027	7.8	0.025	0.008	0.025	0.0	10
	0.100	0.032	0.106	2.3	0.101	0.032	0.098	−3.4	3.7
	0.400	0.126	0.410	2.0	0.406	0.128	0.381	−6.1	3.3
	1.000	0.315	1.059	1.5	1.015	0.320	0.963	−5.1	3.3
	4.044	1.275	4.097	3.7	4.059	1.280	3.671	−9.6	4.1
Ethylmorphine	0.050	0.016	0.055	9.1	0.050	0.016	0.051	2.8	10
	0.101	0.032	0.106	5.7	0.100	0.031	0.110	9.8	9.5
	0.403	0.126	0.396	2.5	0.402	0.126	0.423	5.4	6.1
	1.007	0.316	1.005	2.5	1.005	0.315	1.067	6.5	6.9
	4.078	1.278	3.918	3.5	4.019	1.260	3.730	−7.2	4.9
Pholcodine	0.050*	0.020*	0.019	15	0.050*	0.020*	0.035	−30	33
	0.101*	0.040*	0.085	8.9	0.101*	0.040*	0.088	−12	16
	0.403	0.161	0.363	2.1	0.405	0.162	0.365	−9.0	13
	1.008	0.402	0.974	3.4	1.013	0.404	0.970	−3.3	10
	4.053	1.615	4.171	5.8	4.053	1.615	3.768	−7.0	9.4
Benzoylecgonine	0.025	0.007	0.026	1.8	0.025	0.007	0.024	−3.2	7.7
	0.100	0.029	0.106	2.5	0.101	0.029	0.109	8.2	3.8
	0.401	0.116	0.403	2.0	0.404	0.117	0.417	3.3	2.6
	1.002	0.290	1.028	1.5	1.011	0.293	1.034	2.5	3.9
	4.014	1.161	3.612	1.5	4.044	1.170	3.962	−2.0	4.6
Cocaine	0.025	0.008	0.025	3.8	0.025	0.008	0.025	0.0	4.6
	0.050	0.015	0.054	2.5	0.102	0.031	0.107	5.7	4.9
	0.200	0.061	0.207	1.7	0.406	0.123	0.403	−0.7	2.9
	1.001	0.304	1.079	2.0	1.015	0.308	1.045	3.0	2.8
	4.061	1.232	3.574	2.2	4.061	1.232	3.928	−3.3	3.3

* Concentration below LOQ.

obtained by using 5 mM ammonium formate buffer (pH 3.1). Chromatographic separations were performed on an Aquity BEH C_{18} column with a flow rate of 0.400 mL/min, a column temperature of 60 °C and with MeOH as the organic modifier. Table 2 shows the peak intensities observed.

Table 2 shows increased peak area values for morphine, 6-MAM and codeine with the high pH mobile phase. Signal/noise values of morphine were approximately 2–3 times higher when the high pH mobile phase was used (data not shown). No major signal/noise differences between using the high pH and the low pH mobile phase were observed for 6-MAM and codeine, but large variations of the background noise of the replicates made it difficult to determine the difference. Both morphine, 6-MAM and codeine had shorter retention times with the acidic mobile phase. Poor ESI with low contents of organic modifier may explain why morphine, that eluted first, had lower sensitivity when the acidic mobile phase was used. A similar experiment with LC–MS/MS analysis of the morphine metabolites M3G and M6G also were performed. Increased signal/noise values, narrower peaks and less tailing were observed when the high pH mobile phase was used (data not shown).

3.1.3. *Retention with a high pH mobile phase*

Analyte retention on the Aquity BEH C_{18} column with the mobile phase consisting of 5 mM ammonium bicarbonate buffer (pH 10.2) and MeOH, with varying amounts of MeOH was investigated. Both k and %-retention, the latter defined in Eq. (1), was determined.

$$\%\ \text{Retention} = 100 - \left(\frac{t_0}{t_R}\right) 100 \quad (1)$$

To determine k and % retention, gradients starting with an isocratic elution with various amounts of MeOH were used. After 60 min the amount of B was in all cases rapidly increased to 90% and kept at this concentration for 0.7 min to elute all analytes, before it was decreased to the same amount used in the isocratic part of the gradient. Analytes not eluting within the first 60 min were assigned a retention time of 60 min. Fig. 3 shows k values and % retention values obtained on an Aquity BEH C_{18} column at a column temperature of 60 °C at different amounts MeOH in the mobile phase.

Fig. 3 shows that when the mobile phase contains less than approximately 5% MeOH all analytes are almost completely focused at the column inlet.

3.1.4. Chromatographic separation

Different gradient profiles were investigated to obtain the best compromise between separation of analytes, focusing of the analytes at gradient start and short analysis time. Six additional opiates that may be present in the urine samples, norcodeine, hydromorphone, oxymorphone, hydrocodone, dihydrocodeine and acetylcodeine were included in this experiment to avoid or minimize co-elution and interferences with the validated six opiates. Fig. 4 shows chromatographic separation obtained with the optimized gradient profile.

Fig. 4 shows symmetrical and narrow peaks, with peak widths of 4–6 s, for all analytes. The gradient starting with an isocratic elution at 5% MeOH for the first 0.15 min was chosen to focus all analytes. Co-eluting analytes were separated by the MS/MS detection (see Section 3.2), where two or more MRM transition ions were chosen for each analyte and each internal standard for improved selectivity.

3.2. Method validation

As the aqueous part of the mobile phase, 5 mM ammonium bicarbonate buffer (pH 10.2) was chosen because of the increased retention and the ability to focus the analytes at the column inlet at gradient start. MeOH was chosen as the organic modifier because of increased retention of the analytes even though the lower viscosity of ACN gives a lower back pressure and may improve chromatographic performance. Stability problems of 6-MAM and cocaine in high pH sample solvents are reported [24]. Therefore, MeOH/type 1 water, with the same amount MeOH as at gradient start, 5%, was used as the sample solvent. Recoveries were not investigated in this study, but recoveries of approximately 90–95% were found when the GC–MS method was developed 5–6 years ago using the same SPE (data not shown).

3.2.1. Retention time repeatability

Retention time repeatabilities were investigated for the deuterium labelled internal standards as these are present in all samples. Table 3 shows the retention time repeatabilities obtained.

3.2.2. Accuracy and precision

Accuracies were determined as the average differences in percent between found and theoretical concentration of validation samples at five different concentrations. Within-assay precisions were determined as RSD values of found concentrations of 10 replicate analyses of validation samples. Between-assay precisions were determined as RSD values of found concentrations of validation samples analysed on 10 successive assays. Table 4 shows the between-assay accuracies and the within-assay and between-assay precisions.

Table 4 shows that between-assay accuracy was within ±15% for morphine, 6-MAM, codeine, oxycodone, ethylmorphine, benzoylecgonine and cocaine at all five concentration levels investigated. Accuracy of pholcodine at a concentration of 0.050 μM was ±30% but this concentration is below the LOQ. A possible explanation for the low value is loss of pholcodine due to adsorption to glass walls. The within-assay and between-assay repeatabilities were ≤10% for all analytes at the five investigated concentration levels, except for pholcodine.

3.2.3. Matrix effects/ion suppression

Matrix effects (ME) were investigated according to the procedure described by Matuzewski et al. [29]. An exception was that the analytes were spiked directly into the autosampler vials and not into glass tubes and transferred to autosampler vials. The reason for this was to avoid possible adsorption to glass. Two sets of samples were analysed. In set 1, the analytes and internal standards were spiked in autosampler vials containing extracted dried urine samples from eight different sources. In set 2, the analytes and internal standards were spiked into empty autosampler vials. MeOH/type 1 water (10:90) was added to give a sample solvent composition of MeOH/type 1 water (5:95) in both sets 1 and 2. The final sample solvent volume in both sets 1 and 2 were 0.400 mL. ME of each analyte was calculated by Eq. (2).

$$\mathrm{ME} = \left(\frac{\mathrm{PA}_{\mathrm{set\,1}}}{\mathrm{PA}_{\mathrm{set\,2}}}\right) 100 \quad (2)$$

where $\mathrm{PA}_{\mathrm{set\,1}}$ and $\mathrm{PA}_{\mathrm{set\,2}}$ were peak areas from set 1 and set 2.

ME = 100 indicates no matrix effects. ME > 100 indicates possible matrix enhancement, and ME < 100 indicates possible matrix suppression. Table 5 shows the determined ME values.

Table 5 shows that most of the analytes had no or only minor ion suppression. Some ion suppression was observed for 6-MAM and cocaine while their internal standards, 6-MAM-D_6 and cocaine-D_3, did not show any or only minor ion suppression.

Table 5
Matrix effects.

Compound	Concentration		Matrix effect (n = 8)	
	μM	μg/mL	ME	RSD (%)
Morphine	0.2	0.06	94	3.0
	1.0	0.29	95	4.8
6-MAM	0.2	0.07	71	6.5
	1.0	0.33	74	9.0
Codeine	0.2	0.06	93	2.5
	1.0	0.30	95	3.8
Oxycodone	0.2	0.06	94	4.4
	1.0	0.32	94	5.2
Ethylmorphine	0.2	0.06	93	4.8
	1.0	0.31	93	6.6
Pholcodine	0.2	0.08	85	11
	1.0	0.40	91	8.4
Benzoylecgonine	0.2	0.06	89	3.2
	1.0	0.29	91	4.0
Cocaine	0.2	0.06	79	5.4
	1.0	0.30	77	5.5
Morphine-D_6	1.2	0.35	94	4.1
6-MAM-D_6	1.1	0.37	95	5.8
Codeine-D_6	1.1	0.34	95	4.8
Oxycodone-D_6	1.2	0.39	93	5.9
Benzoylecgonine-D_8	1.2	0.36	89	3.7
Cocaine-D_3	1.0	0.31	93	5.7

3.2.4. LOD and LOQ

LOD and LOQ were determined by UPLC–MS/MS analysis of extracted blank urine samples and extracted validation samples with low analyte concentrations from 10 successive assays. The validation samples were prepared in blank urine by appropriate

Table 6
LOD and LOQ and cut-off.[a]

Analyte	LOD		LOQ		Cut-off	
	μM	μg/mL	μM	μg/mL	μM	μg/mL
Morphine	0.009	0.0026	0.028	0.0079	0.10	0.029
6-MAM	0.003	0.0010	0.010	0.0032	0.10	0.033
Codeine	0.007	0.0022	0.024	0.0070	0.20	0.060
Oxycodone	0.005	0.0016	0.016	0.0050	0.20	0.063
Pholcodine	0.05	0.020	0.16	0.064	0.40	0.16
Ethylmorphine	0.006	0.0020	0.021	0.0065	0.20	0.063
Benzoylecgonine	0.003	0.0010	0.010	0.0029	0.20	0.058
Cocaine	0.005	0.0014	0.012	0.0035	0.20	0.061

[a] Cut-off is the detection limit chosen to be used at NIPH per 01.01.2008.

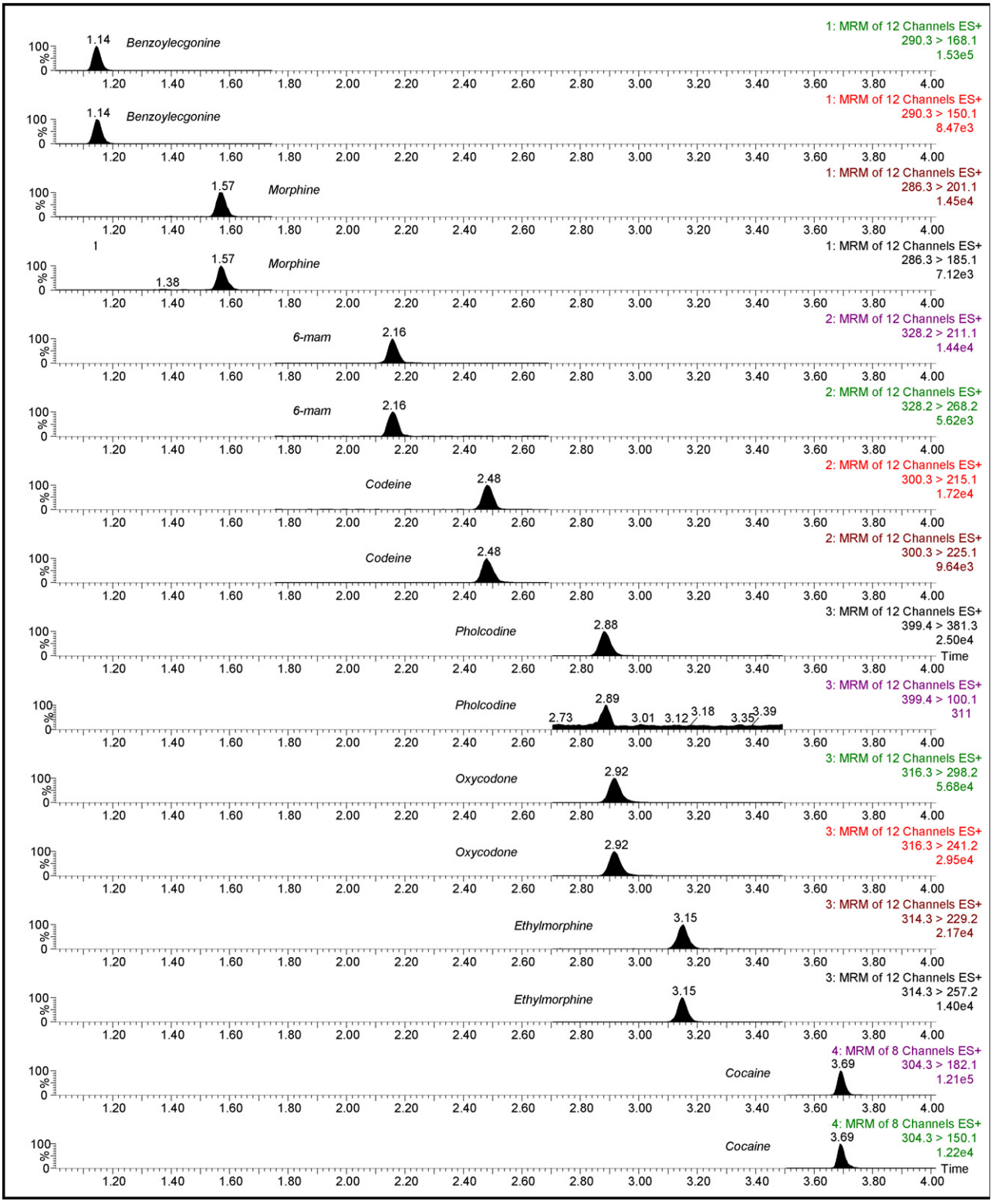


Fig. 5. MRM chromatograms of the selected opiates, cocaine and benzoylecgonine. One microliter of an extracted validation sample with analyte concentrations 0.10 μM (morphine: 0.029 μg/mL, 6-MAM: 0.033 μg/mL, codeine 0.030 μg/mL, oxycodone: 0.032 μg/mL, ethylmorphine 0.031 μg/mL, pholcodine 0.040 μg/mL, benzoylecgonine 0.029 μg/mL, cocaine 0.030 μg/mL) was analysed by UPLC–MS/MS. Conditions as in Fig. 4.

dilutions of the working solutions. In the 10 successive assays blank urine from six different persons was used. The validation samples used to determine LOD and LOQ were prepared with 6-MAM and cocaine concentration of 0.010 μM (0.003 μg/mL), morphine, codeine, oxycodone, ethylmorphine and benzoylecgonine concentration of 0.020 μM (0.006 μg/mL) and pholcodine concentration of 0.10 μM (0.04 μg/mL). LOD and LOQ were determined by Eqs. (3) and (4), respectively.

$$\text{LOD} = \text{mean concentration of blank} + 3 \times \text{SD validation sample} \quad (3)$$

$$\text{LOQ} = \text{mean concentration of blank} + 10 \times \text{SD validation sample} \quad (4)$$

where SD is standard deviation.

Table 6 shows the LOD, LOQ and cut-off values for the different analytes.

The cut-off values of Table 6 is similar to the values used for the former GC–MS method.

Fig. 5 shows MRM chromatograms of an extracted validation sample with analyte concentrations 0.10 μM for all analytes.

3.2.5. Carry over

Carry over was investigated by UPLC–MS/MS analysis of standard samples containing morphine, 6-MAM, codeine, oxycodone, ethylmorphine, pholcodine, cocaine or benzoylecgonine at a concentration of 250–310 μM (72–102 μg/mL) followed by UPLC–MS/MS analyses of an extracted blank urine sample. % carry over was determined by comparing the found concentrations in the blank samples versus the theoretical concentration in the standard solution. Mean % carry over values ($n \geq 6$) of the six opiates in the first and second blank were 0.006–0.06% and 0.002–0.02%, respectively. The highest % carry over values were observed for morphine. Mean % carry over values ($n \geq 6$) of cocaine and benzoylecgonine in the first and second blank were 0.001–0.04% and 0.000–0.007%, respectively. % carry over seemed to increase with the number of injections on the column. To reduce the possible effect of carry over and for improved quality insurance two replicates of each urine sample from routine cases are analysed by the developed UPLC–MS/MS method.

3.2.6. Specificity

Specificity was investigated by UPLC–MS/MS analysis of different drugs for determination of their retention times and MH^+ ions. Table 7 shows the retention times of different drugs analysed by the gradient profile used for the validated method.

Table 7 shows that some compounds have both similar retention times and the same or almost the same MH^+ ions. Hydromorphone and morphine had similar retention times, the same MH^+ ion and basically the same MRM ions. However, the relative response of the MRM ion 286 > 185 versus 286 > 201 and 286 > 209 made it possible to separate these two compounds by the MS/MS detection. Table 8 shows the relative responses of

Table 7
Retention times and MH^+ ions of different drugs sorted by increasing retention times.

Compound	t_R (min)	MH^+	Compound	t_R (min)	MH^+
Theophylline	0.65	181	Carisoprodol	3.18	261
Paracetamol	0.77	152	Ketamine	3.20	238
Morphine-3-glucuronide	0.90	462	Zolpidem	3.28	308
Normorphine	0.95	272	Alprazolam	3.32	309
Morphine-6-glucuronide	1.04	462	Acetylcodeine	3.39	342
Benzoylecgonine-D8	1.12	298	N-Desmethyldiazepam	3.58	271
Benzoylecgonine	1.14	290	Reboxetine	3.63	314
Phenylpropanolamine	1.23	152	Pethidine	3.63	248
7-Aminonitrazepam	1.28	252	Norbuprenorphine	3.67	414
7-Aminoclonazepam	1.30	286	Cocaine-D_3	3.68	307
Cathine	1.39	152	Diazepam	3.69	285
Lamotrigine	1.45	256	Cocaine	3.69	304
Norcodeine	1.48	286	Midazolam	3.71	326
7-Aminoflunitrazepam	1.53	284	Tramadol	3.74	264
Morphine-D_6	1.54	292	Olanzapine	3.78	313
Morphine	1.57	286	Risperidone	3.78	411
Hydromorphone	1.58	286	Mirtazapine	3.82	266
Ephedrine	1.66	166	Citalopram	3.87	325
2-oxo-3-OH-LSD	1.87	356	Quetiapine	3.89	384
Oxymorphone	1.89	302	Haloperidol	3.93	376
2-oxo-3-OH-LAMPA	1.92	356	Fluvoksamine	3.94	319
Norbuprenorphineglucuronide	2.01	590	Paroxetine	3.94	330
MDA	2.05	180	Clozapine	3.95	327
6-MAM-D_6	2.14	334	Venlafaxine	3.96	278
6-MAM	2.16	328	Buprenorphineglucuronide	3.96	644
Amphetamine	2.20	136	Fentanyl	4.06	337
Moclobemide	2.21	269	Fluoxetine	4.09	310
Ketobemidone	2.37	248	Doxepin	4.13	280
Codeine-D6	2.44	306	Mianserin	4.14	265
Codeine	2.48	300	Orphenadrine	4.15	270
MDMA	2.53	194	Nortriptyline	4.17	264
Hydrocodone	2.54	300	Dextropropoxyphene	4.19	340
Strychnine	2.55	335	Nefasodone	4.21	470
Amisulpride	2.55	370	Perphenazine	4.21	404
Nitrazepam	2.59	282	Promethazine	4.21	285
Zopliclone	2.62	389	Dixyrazine	4.23	428
Dihydrocodeine	2.63	302	Zuclopenthixol	4.25	401
Clonazepam	2.65	316	Methadone	4.26	310
Carbamazepine	2.67	237	Flupenthixol	4.27	435
Metamphetamine	2.74	150	Sertaline	4.31	306
Flunitrazepam	2.81	314	Levomepromazine	4.31	329
Oxycodone-D6	2.88	322	Amitriptyline	4.33	278
Pholcodine	2.88	399	Alimemazine	4.33	299
LSD	2.89	324	Clorpromazine	4.36	319
Oxycodone	2.92	316	Clomipramine	4.39	315
MDEA	3.13	208	Trimipramine	4.41	295
Ethylmorphine	3.15	314	Chlorprothixene	4.42	316
Oxazepam	3.18	287	Buprenorphine	4.56	468

Table 8
Relative MRM ion responses of morphine and hydromorphone.

Morphine		Hydromorphone		*n*	Relative responses (peak heights)			
μM	μg/mL	μM	μg/mL		286 > 201		286 > 185	
					Height	RSD	Height	RSD
0.10	0.035	–	–	3	100	14	48	7.5
0.50	0.18	–	–	3	100	13	42	7.0
5.0	1.8	–	–	3	100	6.9	42	5.6
–	–	0.10	0.035	3	100	17	4900	6.0
–	–	0.50	0.18	3	100	8.1	5800	3.4
–	–	5.0	1.8	3	100	7.2	5200	1.0

the MRM ions 286 > 201 and 286 > 185 of standard solutions containing morphine or hydromorphone at different concentration levels.

Table 8 shows that the relative response of 286 > 185 versus 286 > 201 is approximately 100 times higher for hydromorphone than for morphine.

Table 7 also shows that hydrocodone has similar molecular mass and almost the same retention time as codeine. The difference between the relative response of the MRM ion 300 > 199 versus 300 > 215 and 300 > 225 for codeine and hydrocodone also makes it possible to separate these two compounds by the MS/MS detection (data not shown).

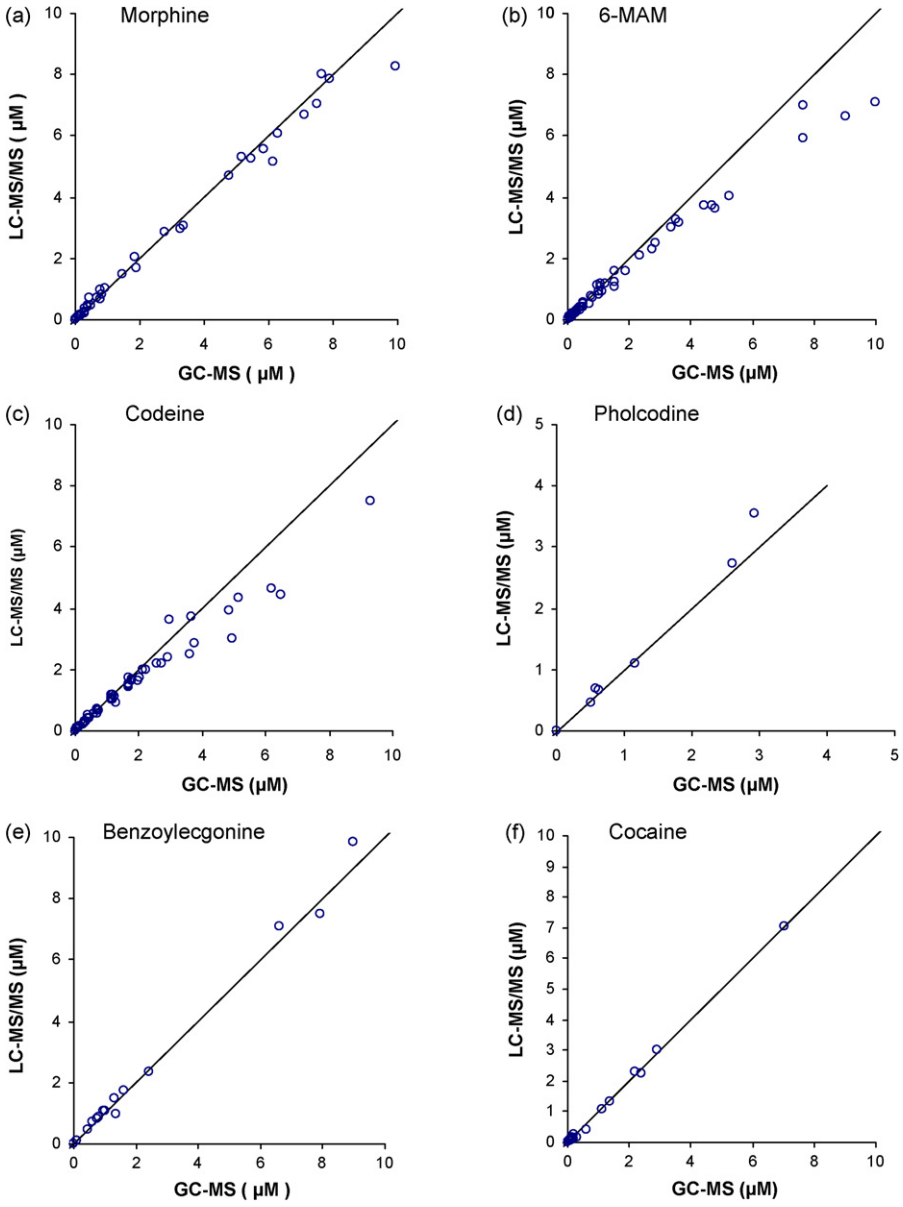


Fig. 6. a–f Comparison of quantification by the former GC–MS-method and the developed UPLC–MS/MS-method. Samples with found morphine concentrations within 0.05–10 μM (0.014–2.9 μg/mL), 6-MAM concentrations within 0.05–10 μM (0.016–3.3 μg/mL), codeine concentrations within 0.05–10 μM (0.015–3.0 μg/mL), pholcodine concentrations within 0.05–5 μM (0.020–2.0 μg/mL), benzoylecgonine concentrations within 0.05–10 μM (0.014–2.9 μg/mL) and cocaine concentrations within 0.05–10 μM (0.015–3.0 μg/mL) by using the UPLC–MS/MS method are shown.

Table 9
Linear ranges.

Analyte	Linear ranges	
	μM	μg/mL
Morphine	0.1–3	0.03–0.9
6-MAM	0.1–5	0.03–1.6
Codeine	0.1–4	0.03–1.2
Oxycodone	0.1–10	0.03–3.2
Pholcodine	0.1–8	0.04–3.2
Ethylmorphine	0.1–8	0.03–2.5
Benzoylecgonine	0.1–10	0.03–2.9
Cocaine	0.1–10	0.03–3.0

3.2.7. Linear ranges

Linear ranges were investigated by UPLC–MS/MS analysis of six extracted validation samples with analyte concentrations from 0.10 to 10 μM (0.03–3 μg/mL of all analytes except pholcodine with analyte concentrations from 0.04 to 4 μg/mL). Deviations more than 10% from linearity indicated that maximum concentration was reached. Table 9 shows the linear ranges obtained.

The calibration curves of oxycodone, benzoylecgonine and cocaine were linear up to the maximum concentration level investigated shown in Table 9. The calibration curves of morphine, 6-MAM, codeine, pholcodine and ethylmorphine were concave above the maximum linear range. Variations in linear ranges have been observed over time, possible caused by sample cone and/or capillary being dirty.

3.2.8. Method comparison

Method comparison was performed by analysing 100 urine samples by the former GC–MS method and by the developed UPLC–MS/MS method. Fig. 6a–f shows the quantification of morphine, 6-MAM, codeine, pholcodine, cocaine and benzoylecgonine. Only linear calibration curves have been used both for the UPLC–MS/MS and the GC–MS method. Upper standards have been deleted from the calibration curves when declining from linearity.

Fig. 6a–f shows good agreements of the calculated concentrations between the two methods. No false positive or negative detections by the UPLC–MS/MS method were observed. Several samples contained morphine, 6-MAM, codeine, benzoylecgonine and/or cocaine with concentrations above the maximum concentrations shown in Fig. 6. Generally for high concentrations the found UPLC–MS/MS concentrations were lower than the found GC–MS concentrations due to shorter linear ranges of the UPLC–MS/MS method (data not shown). However, since the analytical results are reported only as positive and negative and that only low analyte concentrations (within the linear ranges) are used for interpretations at NIPH, it is not important to find the accurate drug concentration(s) of these samples. Only one of the 100 samples analysed contained oxycodone, and only one of the samples contained ethylmorphine, but both analytes were detected by both methods.

3.2.9. Stability

Stability of the analytes in the autosampler vials was investigated by UPLC–MS/MS analyses of six extracted validation samples, four extracted control samples and two extracted blank samples. The samples were analysed twice, once the same day as they were extracted and the next time after one week in the autosampler at 4 °C. Internal standard peak responses from the two UPLC–MS/MS analyses were within ±30% for morphine-D_6, 6-MAM-D_6, oxycodone-D_6. Internal standard peak responses were for the second UPLC–MS/MS analysis in the range ±10% to ±40% for codeine-D_6 and in the range ±30% to ±55% for cocaine-D_6, indicating loss of compound. The quantified values from the two UPLC–MS/MS analyses were found to be within ±20% for all analytes indicating that the internal standards compensated for possible loss of analyte.

4. Application of method

The developed UPLC–MS/MS method has been routinely used at NIPH since August 2007 for qualitative detection of opiates, cocaine and benzoylecgonine in more than 2000 urine samples. Two replicates of each sample have been analysed. Each assay contains calibrants, three quality control samples and two blank urine samples as well as the authentic urine samples. Criteria for acceptable quality control sample performance are that the found concentrations are within theoretical concentrations ±24%. The UPLC–MS/MS method was ISO 17025 (2005) accredited in December 2007.

5. Conclusion

A fast and selective UPLC–MS/MS method for the determination of morphine, 6-MAM, codeine, oxycodone, pholcodine, ethylmorphine, benzoylecgonine and cocaine has been developed and validated. A high pH mobile phase consisting of 5 mM ammonium bicarbonate buffer, pH 10.2, and MeOH provided narrow and symmetrical peaks and repeatable retention times. All analytes were almost completely focused at the column inlet at gradient start (5% MeOH). Within-assay repeatability and between-assay repeatability at five concentration levels showed RSD values ≤10% for all analytes except for pholcodine. Deuterium-labelled internal standards were used for six of the analytes for improved qualitative and quantitative determination and to reduce possible effects of ion suppression. Analysis time, including injection and column equilibration time, was 5.7 min. The method has been routinely used at NIPH since August 2007 for more than 2000 urine samples.

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