Journal of Chromatography B, 878 (2010) 2959–2966

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Hydrophilic interaction liquid chromatography and accurate mass measurement for quantification and confirmation of morphine, codeine and their glucuronide conjugates in human urine

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ARTICLE INFO

Article history:
Received 17 May 2010
Accepted 30 August 2010
Available online 9 September 2010

Keywords:
Glucuronide conjugate
HILIC–TOFMS
Quantification
Confirmation
Morphine
Codeine

ABSTRACT

A hydrophilic interaction liquid chromatography–time-of-flight mass spectrometry (HILIC–TOFMS) method for the quantification and confirmation of morphine (M), codeine (C), morphine-3-glucuronide (M3G), morphine-6-glucuronide (M6G) and codeine-6-glucuronide (C6G) is presented. The method was validated in terms of specificity, selectivity, extraction recovery, accuracy, repeatability, linearity and matrix effect. After a straightforward sample preparation by solid phase extraction (SPE) the compounds were analyzed directly without the need for hydrolysis, solvent transfer, evaporation or reconstitution. The HILIC technique provided good chromatographic separation which was critical for isomers M3G and M6G. The analytes were detected after electrospray ionization (ESI) in positive mode with mass accuracies below 2 mDa using a 5-mDa window. A measurement range of 50–5000 ng/ml was applied for calibration using deuterated analogs as internal standards. The precision of the method was 5.7% and 10.2% (RSD) within and between days, respectively. The applicability of the method was demonstrated with authentic urine samples known to contain codeine and/or morphine and their intact glucuronide conjugates. Identification of the analytes was based on in-source collision induced dissociation (ISCID), applying three diagnostic ions with accurate mass.

1. Introduction

Morphine (M) and codeine (C) are two major biologically active opiates found in opium, and they act through μ-receptors in the central nervous system. Morphine is widely used to relieve severe or agonizing pain whereas codeine is used as a milder analgesic and antitussive. In the body, codeine is conjugated by UDP-glucuronosyltransferases to codeine-6-glucuronide (C6G), but can also be considered as a prodrug, since the hepatic cytocrome P450 2D6 enzyme metabolizes codeine to morphine [1]. Morphine is further conjugated to morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G), the latter representing the active metabolite with an analgesic activity equal to the parent drug [2] (Fig. 1). In humans, M3G is the main metabolite of morphine excreted in urine [3].

In human doping control and toxicology, there is a great practical interest in analyzing morphine and codeine simultaneously with their metabolites. The World Anti-Doping Agency (WADA) has banned the in-competition use of morphine [4] and set a threshold value of 1000 ng/ml for total morphine [5]. Since codeine is not included in the prohibited list by WADA, it is important to determine whether a positive morphine finding is a result of codeine metabolism. In toxicology, simultaneous determination of morphine and its glucuronides bears relevance to the detection of drugs of abuse such as heroin. Due to the fast metabolism of heroin through 6-monoacetylmorphine and morphine, high concentrations of M3G in urine suggest heroin abuse and can be detected for days after the intake [6,7].

Glucuronide conjugates are very polar and non-volatile compounds and they are often cleaved to their free forms by enzymatic or acid hydrolysis for the analysis of total concentrations. However, it has been recurrently reported that the optimization of hydrolysis conditions is difficult and the obtained recoveries and hydrolysis rates vary according to the type of enzyme, temperature, incubation time and nature of the conjugated analyte [8–14].

The liquid chromatography–mass spectrometry (LC–MS) applied to the analysis of intact morphine and codeine glucuronide conjugates enables a simpler sample preparation without the need

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doi:10.1016/j.jchromb.2010.08.047

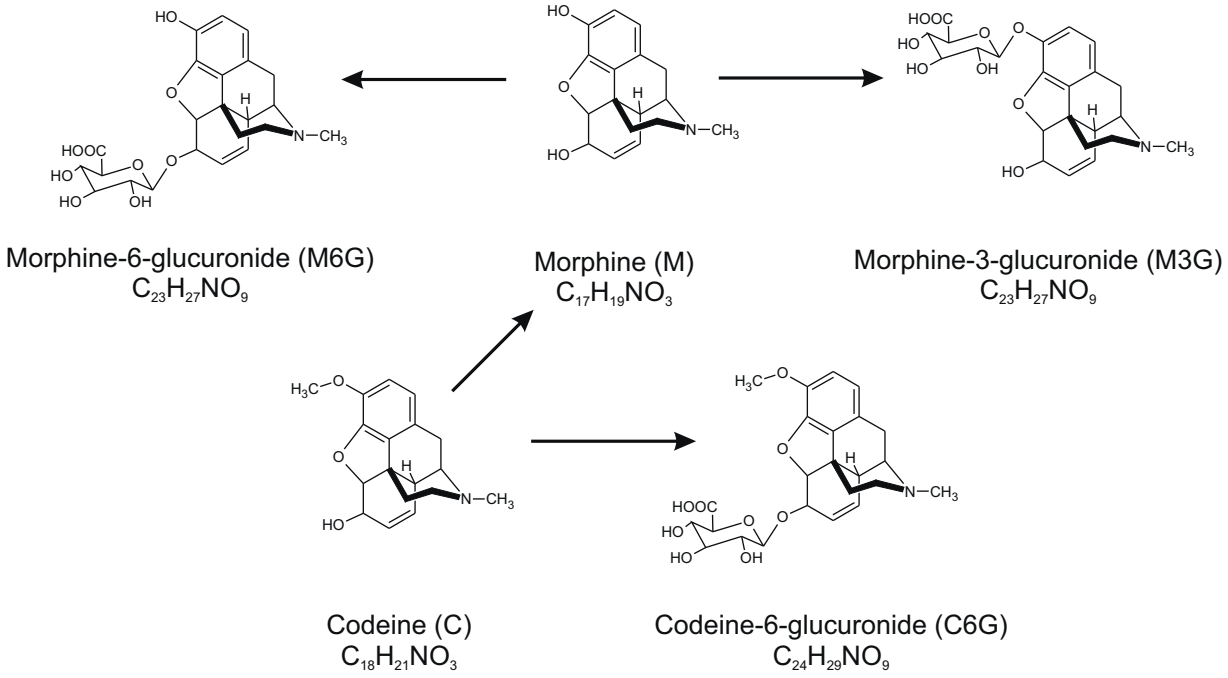

Fig. 1. Structures of morphine, codeine and their glucuronide conjugates.

for time-consuming and laborious hydrolysis and derivatization steps typical for commonly used MS methods with gas chromatography (GC). The applied sample preparation techniques include solid phase extraction (SPE) [15–18], liquid–liquid extraction (LLE) [19] or even direct injection analysis of diluted urine samples [20,21].

LC separation of intact glucuronide conjugates is typically performed with reverse-phase (RP) C18 columns [15–18]. Owing to the hydrophilic nature of the analytes they, however exhibit weak retention and poor resolution in RP-columns. The water content of the mobile phase must thus be rather high to increase the retention, which may suppress ionization in commonly used LC–MS ion sources. To avoid this, normal phase (NP) LC has been applied [22]. However, most NP solvents are not compatible with the electrospray (ESI) interface in MS.

The hydrophilic interaction liquid chromatography (HILIC) technique was introduced some decades ago for the analysis of hydrophilic and polar compounds [23], and the optimization of HILIC parameters was recently discussed elsewhere [24,25]. The compounds are retained on a hydrophilic stationary phase (*e.g.* silica- and polymer-based), and a water miscible, MS compatible mobile phase is used. A few HILIC applications have been published for the analysis of intact morphine glucuronide conjugates in human plasma [26,27], but to our knowledge applications have not been published for urine matrix.

In LC–MS applications, the detection of morphine, codeine and their glucuronide conjugates has been based on low resolution single (MS) or tandem mass spectrometry (MS/MS) techniques, the latter being the technique of choice due to its better selectivity and sensitivity [18–20]. The improved quantitative performance of LC–time-of-flight mass spectrometry (TOFMS) with a wide dynamic range and accurate mass measurement offers competitive quantitative capability with the traditional triple quadrupole set up [28,29], and to our knowledge this technique has not been applied to the studied analytes.

In doping control, the preliminary findings have to be confirmed on the basis of either GC or LC separation and MS detection. WADA has set the identification criteria for different GC– and LC–MS techniques with selected ion and multiple reaction monitoring or full scan modes [30,31]. The criteria consist of retention time (RT), relative ion abundances and minimum number of diagnostic ions. Similarly, the European Union (EU) controls the performance of analytical methods, and for MS detection a system of identification points for different techniques is applied with a minimum requirement of four points for the analysis of abused substances [32]. For example, with high resolution (HR) MS, two points per ion are earned.

In this paper, a straightforward HILIC–TOFMS method for the quantification and confirmation of morphine, codeine and their intact glucuronides is presented. Minimal SPE sample preparation without hydrolysis and solvent transfers is applied to human urine samples. The confirmation of the analytes is based on in-source collision induced dissociation (ISCID), accurate mass and isotope pattern match (SigmaFit). The results of the method validation and authentic samples are provided.

2. Experimental

2.1. Materials

Morphine, M3G, M6G, codeine and their deuterated analogs (purity 99%) were from Cerilliant (Texas, USA), and C6G and its deuterated analog (purity >98%) were purchased from Australian Government National Measurement Institute (Pymble, Australia).

Acetonitrile and methanol were purchased from Labscan (Poch Sa, Swinskiego, Poland), ammonium formate was from Sigma (St. Louis, MO, USA), formic acid of UPLC/MS grade was obtained from LGC Promochem GmbH (Wesel, Germany) and 2-propanol was purchased from Rathburn Chemicals Ltd. (Walkerburn, Scotland). The other solvents and reagents were purchased from Merck (Darmstadt, Germany) and were of high performance (HP)LC or analytical grade. Sep-Pak C18 (50 mg) cartridges by Waters (Milford, MA, USA) were applied for SPE.

Drug-free urine samples used in this study were obtained from healthy volunteers and used either individually or as pooled aliquots.

Two kinds of authentic urine samples were used: (1) random patient urine samples in which morphine and/or codeine were confirmed by GC–MS and (2) controlled excretion urine samples that were acquired from three healthy volunteers after an oral administration of codeine (single dose 30 mg *p.o.*). Excretion urine samples were collected over 72 h in 6–10 h fractions. Volumes and specific gravities were determined for each fraction. Protocol of the study was approved by a local ethical committee.

2.2. Sample preparation

A urine sample of 100 μl was centrifuged in an Eppendorf tube with 7125 × *g* (10,000 rpm) for 10 min. The SPE cartridges were conditioned with 1 ml of methanol and 1 ml of water (2 ml/min). The urine samples were applied to SPE along the addition of 100 μl of ISTD solution (500 ng/ml of deuterated analogs of the analytes in water). The cartridges were washed with 1.0 ml water and dried afterwards in full vacuum for 2 min. The analytes were eluted directly into the autosampler vials with 1 ml of 90% acetonitrile in water. A confirmation analysis was performed separately, in which the ISTD solution was substituted with water.

2.3. Liquid chromatography

An Agilent 1200 (Agilent Technologies, Waldbronn, Germany) series rapid resolution LC system with a micro-vacuum degasser, autosampler, binary pump and column oven was used for chromatography. A Zorbax Hilic Plus column 100 mm × 2.1 mm (3.5 μm) from Agilent with in-line frit was used in gradient mode at 25 °C. The mobile phase consisted of 10 mM ammonium formate, pH 6.4 (A) and 10 mM ammonium formate, pH 6.4 in 90% acetonitrile (B). The flow rate was 0.2 ml/min. The gradient started with an isocratic part of 0.5 min with a 100% of mobile phase B. The proportion of A was linearly increased to 45% in 1.5 min, held there for 5.5 min and then decreased back to 0% in 0.5 min. The 100% proportion of B for 3 min was used to equilibrate the column, resulting in an analysis cycle time of 11 min. The injection volume was 3 μl. HyStar version 3.2 by Bruker Daltonics (Bremen, Germany) was used to control the LC instrument.

2.4. Time-of-flight mass spectrometry

The TOF mass spectrometer was a Bruker Daltonics micrOTOF. An orthogonal ESI ion source was applied and ionization was performed in the positive mode. Ionization parameters were optimized with direct injection of the target analytes at 1 μg/ml for each compound by an external syringe (KD Scientific Syringe Pump, MA, USA). The nebulizer pressure was 1.6 bar and dry gas flow (nitrogen) 8.0 l/min. The drying temperature was 200 °C. The applied voltages for capillary, capillary exit and skimmer 1 were 4500, 85.0 and 37.5 V, respectively. The spectral rate was 2 Hz corresponding to 10,000 summation. Mass spectral data were collected within the range of *m*/*z* 50–800 to obtain adequate number of clusters for mass scale calibration. On an average, the resolution for *m*/*z* 430 was 12,500. TOFMS was operated with micrOTOF control version 3.2 (build 23) by Bruker Daltonics. The ISCID was applied to the confirmation of the analytes as a separate analysis. The ionization parameters of ISCID were the same as above, except for capillary exit and skimmer 1 voltages, which were 200.0 and 65.0 V, respectively.

Daily external calibration of TOFMS was performed with sodium formate solution containing 5 mM sodium hydroxide in 2-propanol/0.2% formic acid (1:1, v/v) by syringe injection similarly to our previous study [33].

2.5. Data evaluation

The in-house database for the analytes and their ISTDs were constructed and LC–TOFMS acquisition data were first calibrated by processing with TargetAnalysis (version 1.1, build 192) and DataAnalysis macro (version 3.4) by Bruker Daltonics as described previously [33] with two level rating parameters of 0.15/0.2 min for RT, 5/7 mDa for mass accuracy and 0.03/0.15 for SigmaFit. The values of SigmaFit below 0.050 indicate high probability of correct molecular formula. The quantitative analysis of the samples was performed with a QuantAnalysis software (version 1.8, build 192) by Bruker Daltonics. The calibration curves were generated using peak area ratios of the analyte over the ISTD. The data were fitted to a linear model weighted with 1/× factor applying a 5-mDa window.

2.6. Validation of the method

Validation of the quantitative method consisted of the evaluation of specificity, selectivity, extraction recovery, accuracy, repeatability, linearity and matrix effect. The measurement range was from 50 to 5000 ng/ml, calculated as aglycone concentrations for all analytes. The seven-point calibration curves were obtained with 50, 100, 250, 500, 1000, 2500 and 5000 ng/ml levels as single determinations for each analytical sequence. Three quality control (QC) samples were also applied as single determinations at 100, 1000 and 4000 ng/ml concentrations. Calibration standards and QCs were spiked in pooled drug-free urine and stored at −20 °C.

The specificity of the method was illustrated with six male and female urine samples collected from healthy volunteers and analyzed with and without the addition of ISTD solution. The selectivity of the method was studied with authentic samples containing the following opioids: buprenorphine, dextromethorphan, dextropropoxyphene, methadone, pethidine, oxycodone, oxymorphone, pholcodine and tramadol.

Extraction recovery was evaluated with duplicate drug-free urine samples spiked before and after extraction at a concentration of 500 ng/ml. Calculation was based on peak areas relative to ISTDs.

Accuracy and precision were evaluated as intra- and interday experiments at QC concentration levels. Intraday experiments were performed with six replicates whereas interday measurements were performed in parallel during six different days within 1 month. Intra- and interday repeatability of relative intensities in ISCID measurements at 1000 ng/ml was performed as six replicates.

Stability and repeatability of calibration curves were evaluated with interday repeatability ($n = 6$) data and expressed as linear equation and correlation coefficients.

The matrix effect was evaluated with post-column infusion of the individual analyte (10 μg/ml) to the mobile phase flow from the analytical column. Extracted urine and plain mobile phase samples were injected into the column and the trends in the extracted ion chromatograms (EICs) of the analyte for both samples were compared.

The applicability of the method was demonstrated with two types of authentic samples containing morphine, codeine and their metabolites as described in Section 2.1.

3. Results and discussion

3.1. Sample preparation

The sample pretreatment procedure was straightforward, since there was no need for hydrolysis, and the sole purpose of the applied SPE method was to remove the salts present in the urine samples. Here, due to opposite retention mechanisms of SPE and

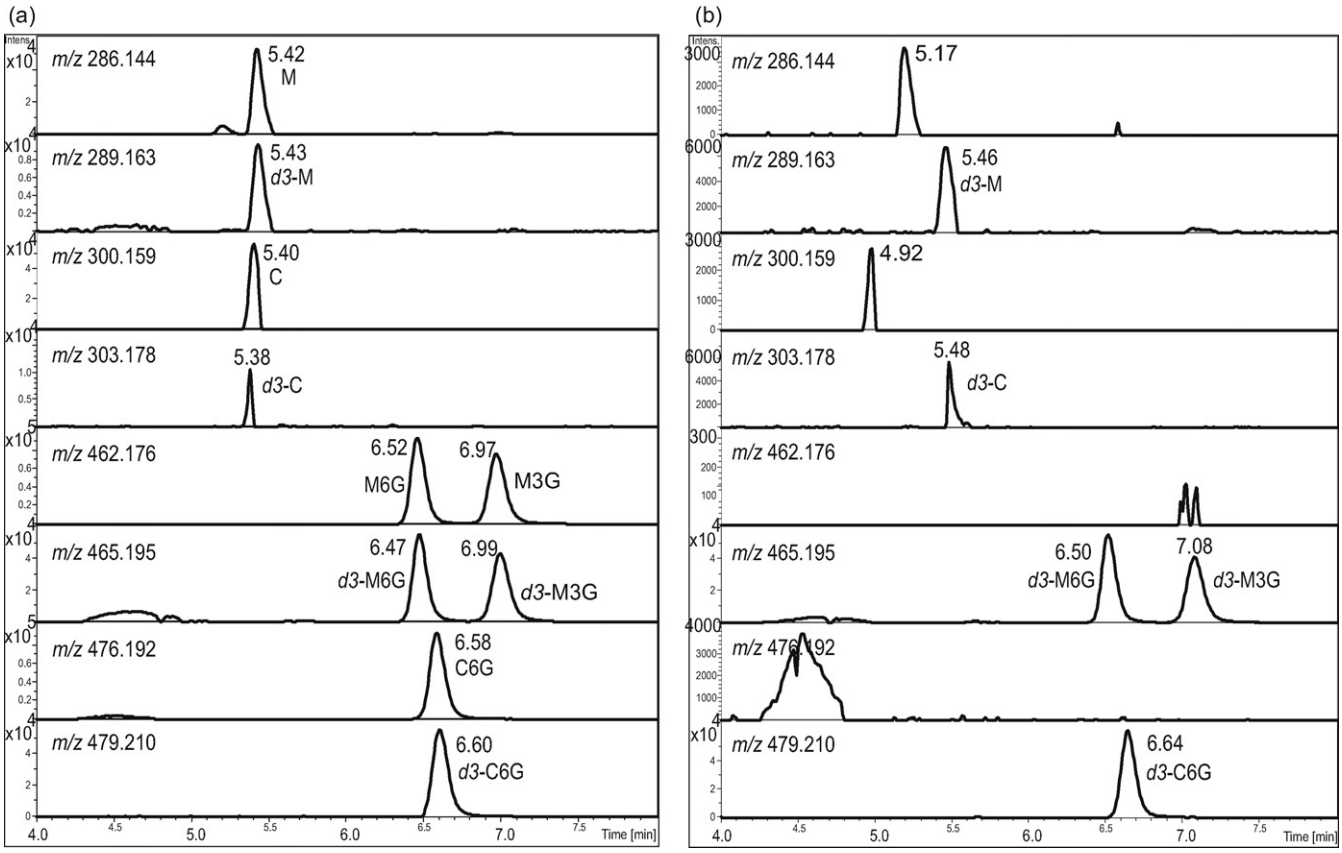

Fig. 2. Extracted ion chromatograms (EICs) of (a) a urine sample spiked at 1000 ng/ml of morphine (M), codeine (C), morphine-6-glucuronide (M6G), morphine-3-glucuronide (M3G) and codeine-6-glucuronide (C6G) and their deuterated analogs (at 500 ng/ml) with a 5-mDa window and (b) a drug-free urine sample showing only the internal standards.

HILIC analytical column, the SPE effluents in 90% acetonitrile could be analyzed directly without any solvent transfer, evaporation or reconstitution, thus lowering the risk of contamination and carry-over. Furthermore, there was no need for adjusting pH, buffering or dilution of the samples since the applied SPE cartridges possessed sufficient retention for the analytes in varying conditions.

The extraction recoveries were within 99–104%. The obtained results were clearly better than in previously published studies where extraction recoveries as low as 45% were reported for glucuronide conjugates [16–18]. The observed ion suppressions for the analytes were: C ±0%, M −40%, M6G −9.4%, C6G ±0%, which was considered acceptable. In the quantification, the ion suppression was compensated by the application of deuterated analogs of the analytes as ISTDs.

3.2. Liquid chromatography

The main objective was to obtain good resolution for M6G and M3G because by being isomers, they cannot be differentiated solely by accurate mass of the protonated molecules. To achieve this goal, different RP and HILIC columns were studied (data not shown), and common C18 phases do not generally offer adequate baseline separation for these positional isomers. The

Table 1
Accuracy and precision of quantitative HILIC–TOFMS method.

	RT (RSD %)	Accuracy and precision								
	Mean min	100 ng/ml			1000 ng/ml			4000 ng/ml		
		Mean conc.	RSD %	Accur. %	Mean conc.	RSD %	Accur. %	Mean conc.	RSD %	Accur. %
Intraday (n = 6)										
C	5.4 (0.09)	nd	nd	nd	1065.0	6.9	106.5	4302.6	6.0	107.6
M	5.5 (0.07)	94.8	9.4	94.8	987.8	4.8	98.8	4150.1	7.0	103.7
M6G	6.5 (0.06)	94.7	10.1	94.7	1046.9	2.9	104.7	4275.1	3.5	106.9
C6G	6.6 (0.06)	90.0	5.5	90.0	883.2	2.4	88.3	3596.8	6.3	89.9
M3G	7.1 (0.08)	103.1	5.9	103.1	1030.3	2.1	103.0	4261.8	6.8	106.5
Interday (n = 6)										
C	5.4 (0.15)	nd	nd	nd	1094.0	9.1	109.4	4617.4	7.9	115.4
M	5.5 (0.15)	97.4	16.3	97.4	939.0	8.3	93.4	3982.5	6.2	99.6
M6G	6.5 (0.06)	107.0	13.8	107.0	1039.5	9.2	104.0	4187.9	8.0	104.7
C6G	6.6 (0.18)	88.9	13.4	88.1	860.5	9.1	93.9	3786.0	6.6	95.4
M3G	7.1 (0.20)	117.6	16.4	117.7	1062.4	8.9	106.2	4302.3	9.3	107.6

Table 2
Protonated molecules and fragment ions applied in quantification and confirmation by the HILIC–TOFMS method.[a]

Compound	[M+H]$^+$	Fragment ions m/z	
C	300.1594 ($C_{18}H_{22}NO_3$)	243.1016 ($C_{15}H_{15}O_3$)	225.0910 ($C_{15}H_{13}O_2$)
M	286.1438 ($C_{17}H_{20}NO_3$)	229.0859 ($C_{14}H_{13}O_3$)	201.0910 ($C_{13}H_{13}O_2$)
M6G	462.1759 ($C_{23}H_{28}NO_9$)	286.1438 ($C_{17}H_{20}NO_3$)	229.0859 ($C_{14}H_{13}O_3$)
C6G	476.1915 ($C_{24}H_{30}NO_9$)	300.1594 ($C_{18}H_{22}NO_3$)	243.1016 ($C_{15}H_{15}O_3$)
M3G	462.1759 ($C_{23}H_{28}NO_9$)	286.1438 ($C_{17}H_{20}NO_3$)	229.0859 ($C_{14}H_{13}O_3$)

[a] The protonated molecules were used in quantification whereas all ions were included in the ISCID confirmation analysis.

best separation was obtained with a non-bonded silica HILIC column at room temperature with mildly acidic buffer (Fig. 2). The parameters affecting HILIC separation such as buffer concentration, pH, temperature and stationary phase configuration were systematically studied (data not shown). Buffer concentration had the biggest effect on the retention of the analytes, and a change in elution order of free morphine and codeine versus their glucuronide conjugates was observed approximately at 8 mM. The applied buffer concentration of 10 mM appeared to be robust for chromatographic separation, and no difference between formate and acetate buffers was observed. Additionally, the concentration was low enough to avoid ESI ion suppression. The ability of HILIC columns to retain polar compounds was corroborated by the elution order of the analytes, *i.e.* more polar glucuronide conjugates eluted last from the column. Codeine and morphine co-eluted as a compromise to achieve better resolution for morphine glucuronides, but owing to their different masses, they could be separated by MS. The applied gradient elution was a simple one, with a total cycle time of 11 min. The LC method proved to be stable with minimal variation in RT of the analytes over time (Table 1), and more than 1000 injections could be carried out without any weakening in the chromatographic performance.

3.3. Time-of-flight mass spectrometry

The ions used for quantification and confirmation are listed in Table 2. Since the deuterated analogs used as ISTD produced in ISCID partly the same fragments as the analytes, confirmation analysis was performed in the fourth sample aliquot which was extracted in the same batch without the addition of ISTD. ISCID TOFMS parameters were selected based on direct injection studies measuring the intensity of separate fragments as a function of capillary exit/skimmer 1 voltages (3:1). The protonated molecules of the analytes and two fragments formed the basis of the confirmation analysis. The fragments were selected based on previously published fragmentation patterns for morphinans in ESI [34] and on the results of the present validation. The selected fragments for the aglycone moieties were formed by the loss of an amine ($CH_2CHNHCH_3$, $\Delta m = 57$), water and carbon monoxide [34]. The same fragments could be found from the mass spectra of triple quadrupole MS/MS.

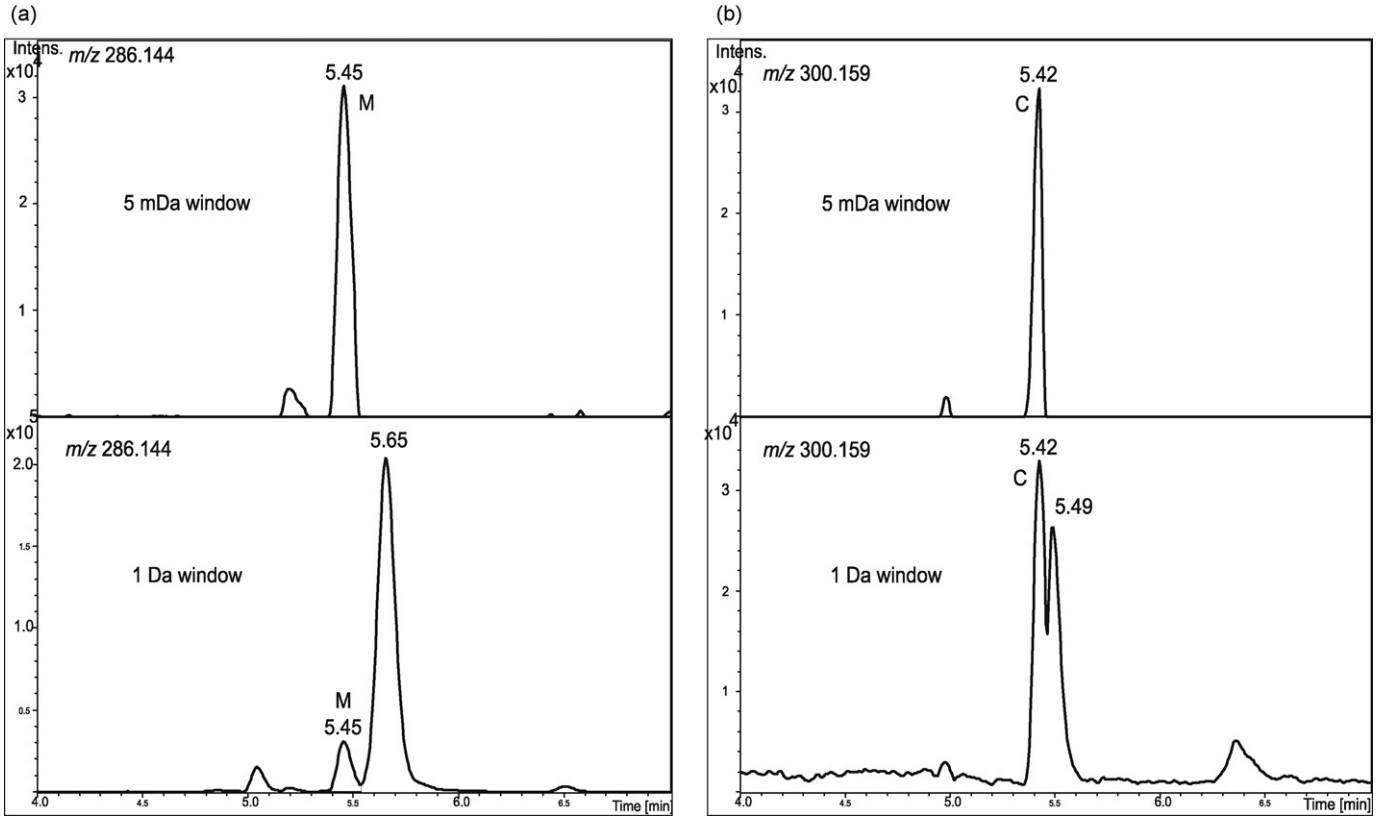

Fig. 3. Extracted ion chromatograms of (a) morphine and (b) codeine with 5-mDa and 1-Da detection windows in a urine sample spiked at 1000 ng/ml.

Table 3
Calibration statistics, $n = 6$.

Compound	Slope Mean (RSD %)	y-Intercept Mean	R^2 Mean (RSD %)
C	1.3 (15.3)	−0.79	0.996 (0.6)
M	3.1 (5.1)	0.18	0.998 (0.2)
M6G	0.8 (5.9)	0.02	0.998 (0.2)
C6G	0.9 (7.7)	0.02	0.996 (0.3)
M3G	0.8 (7.5)	0.02	0.999 (0.2)

3.4. Validation of the method

The validation of the quantitative HILIC–TOFMS method consisted of specificity, selectivity, accuracy, repeatability and linearity.

The specificity was evaluated with six male and female ($n = 12$) drug-free urine samples. No interfering signals were detected with the m/z of the analytes by applying 5-mDa detection window. The accurate mass measurement with high resolution assured the validity of the quantification, since it was observed that by using unit resolution (*e.g.* in common quadrupole MS), interfering matrix compounds were present with similar m/z values those found for morphine and codeine (Fig. 3). Selectivity of the method for opioids (buprenorphine, dextromethorphan, dextropropoxyphene, methadone, pethidine, oxycodone, oxymorphone, pholcodine and tramadol) was studied with authentic positive urine samples. There was no observed interference from these compounds.

Accuracy and precision of the method are shown in Table 1. The precision of the method was on the average 5.7% and 10.2% (RSD) within and between days, respectively. Accuracies were within ±15%, which is a normal acceptance criteria for bioanalytical methods [35]. Compared with previously published methods with common MS detection [16–18,20], the results obtained here were at least equal to the above values, thus proving the suitability of the HILIC–TOFMS method for quantification.

Statistics of the calibration curves are presented in Table 3. The calibration was stable and repeatable with good linear correlation (over 0.99) for all analytes within the calibration range of 50–5000 ng/ml, suggesting the feasibility of current TOFMS for wide-range quantification. Considering previous publications involving triple quadrupole and ion trap, the applied calibration range was equivalent or even broader for all analytes [16–18,21].

The applicability of the method was demonstrated with two types of authentic samples, patient urine samples previously reported by qualitative GC–MS-method to contain codeine and/or morphine, and excretion urine samples after a single oral dose of 30 mg codeine. The results of the patient urine samples are shown in Table 4. The results indicate that glucuronide conjugates play a major role in the total concentrations of morphine and codeine, and potent hydrolysis for glucuronide conjugates is consequently emphasized in the measurements of total urinary concentrations. The majority of the samples had to be diluted heavily for quantification of M3G and C6G. The total codeine concentrations were higher than total morphine concentrations (except in cases 5, 9 and 10), indicating codeine administration. In the three cases 5, 9 and 10, notably higher total morphine concentrations were measured, suggesting heroin abuse. In addition, for cases 5 and 10, GC–MS reported only morphine, whereas low concentrations of C6G were detected by HILIC–TOFMS. Illicit heroin can contain small amounts of codeine as an impurity, however M3G is the main metabolite excreted in urine [19,36,37]. Morphine and codeine can also be detected in urine after consumption of poppy seeds as stated before [38–40]. Altogether, interpretation of the origin of positive codeine and morphine findings is ambiguous.

An excretion study was performed with three volunteers. After a single oral dose of 30 mg codeine, glucuronide conjugates could be detected even up to 50 h or longer, as reported in earlier studies [6,41,42]. The peak concentrations were reached in the first 6–8 h with the following average values ($n = 3$, ng/ml): C 1349 ± 228, M 101 ± 44, M6G 317 ± 158, C6G 34965 ± 20180 and M3G 1096 ± 570, calculated to correspond aglycone concentrations relative to a specific gravity of 1.020. There were large variations between individuals in the excretion of different metabolites. On the average, the total excretion of codeine was 20.9 ± 1.7 mg from which the majority (89.7%) was in the form of C6G. This is consistent with earlier observations [6,19]. The average proportion of other analytes were: 4.8% M3G, 3.9% C, 1.2% M6G and 0.4% M. The relations of different metabolites were similar between individuals, even though the concentrations and excretion times varied. These metabolite relations were similar to those published after administration of 120 mg of codeine [43]. An example of excretion profile for one female volunteer is presented in Fig. 4.

Confirmation of the analytes was based on ISCID with protonated molecule and two fragment ions. Mass error, SigmaFit and repeatability of ion ratios were studied at the 1000 ng/ml concentration level within and between days (Table 5). The protonated molecule was the most abundant ion for all the other analytes except for M3G, for which it was the aglycone. The average precision (RSD) of the relative intensities was 8.2% and 12.1% for intra- and interday, respectively. Mass accuracy was excellent with an average of 1.05 mDa. The SigmaFit could be used as an additional confirmation parameter for intensive fragments with values lower than 0.05, indicating that the resulted molecular formulae were correct with high probability.

The presented HRMS confirmation method fulfills WADA and EU criteria for identification [30,32]. With three diagnostic ions, the minimum requirement of three diagnostic ions by WADA for LC–MS technique was achieved. For the EU system of identification points, six points were earned exceeding the minimum requirement of four points. Furthermore, extra reliability for confirmation

Table 4
Results of authentic patient urine samples with qualitative GC–MS and quantitative HILIC–TOFMS.[a]

Case	Qualitative GC–MS	Quantitative HILIC–TOFMS (ng/ml)				
		C	C6G	M	M6G	M3G
1	C, M	2853	63,367	242	1084	5800
2	C, M	3022	187,483	400	4086	12,788
3	C, M	2722	52,157	259	1831	6823
4	C, M	867	15,290	–	334	1367
5	M	–	31	–	201	765
6	C, M	1247	94,902	568	2245	6342
7	C, M	208	11,140	113	77	282
8	C, M	4142	44,533	142	640	6262
9	C, M	5399	1554	1191	4453	17,105
10	M	–	104	–	424	1949

[a] Concentrations of glucuronide conjugates are calculated as corresponding aglycone concentrations.

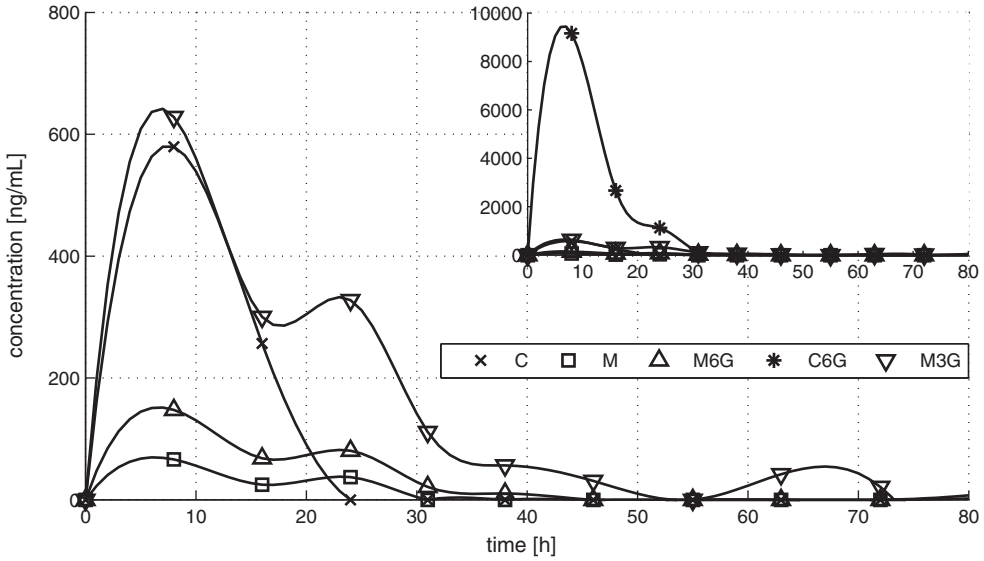

Fig. 4. Adjusted urinary concentrations for a female volunteer as a function of time elapsed after the administration of a single dose of 30 mg codeine. The small picture includes all the analytes. Adjusted concentration is calculated as in Ref. [44]: $c_{adj} = c_{measured} \times (1.020 - 1/(SG - 1))$ in which SG is the measured specific gravity of the urine fraction. C, codeine; M, morphine; M6G, morphine-6-glucuronide; C6G, codeine-6-glucuronide; M3G, morphine-3-glucuronide.

Table 5
Data of confirmation analysis at the 1000 ng/ml concentration level, $n = 6$.

m/z	Relative Intensities (%)		Mass error (mDa)	SigmaFit
	Intraday	Interday	Intraday	Intraday
	Mean (RSD %)	Mean (RSD %)	Mean	Mean
C				
300.1594	100	100	1.15	0.026
243.1016	35 (18)	32 (23)	1.13	0.090
225.0910	41 (9.0)	45 (19)	1.73	0.162
M				
286.1438	100	100	0.98	0.030
229.0859	31 (10)	33 (12)	0.97	0.068
201.0910	35 (7.3)	43 (13)	0.60	0.163
M6G				
462.1759	100	100	1.18	0.006
286.1438	39 (6.0)	38 (8.4)	0.90	0.016
229.0859	4.5 (12)	5.2 (13)	1.15	0.045
C6G				
476.1915	100	100	1.28	0.007
300.1594	25 (3.1)	26 (6.1)	1.08	0.013
243.1016	4.6 (7.2)	4.9 (12)	0.78	0.024
M3G				
462.1759	74 (3.5)	73 (6.8)	1.22	0.005
286.1438	100	100	0.73	0.006
229.0859	6.8 (6.0)	7.5 (7.2)	0.80	0.022

was provided with accurate mass and SigmaFit. For the moment, no exact guidelines exist for accurate mass measurements except for Food and Drug Administration's recommendations for exact mass measurements [45].

4. Conclusions

HILIC–TOFMS proved to be feasible for quantitative analysis of morphine, codeine and their glucuronide conjugates. This is important, as the repeatability and suitability of enzymatic or acidic hydrolysis of glucuronide conjugates has been questioned. A straightforward SPE method without hydrolysis, evaporation or reconstitution was applied to obtain high recovery and to minimize sample contamination and carry-over. Selectivity and baseline separation of glucuronide conjugates was achieved with HILIC. Current TOFMS allowed quantification with a wide linear range and accurate mass. It was possible to identify the analytes from matrix background with a narrow mass window. Confirmation of the analytes was based on the protonated molecule and two diagnostic fragment ions produced with ISCID. With these diagnostic molecules, confirmation and identification criteria of WADA and EU were fulfilled.

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