M. Kolmonen[1,2)], A. Leinonen[1)], T. Kuuranne[1)], A. Pelander[2)], I. Ojanperä[2)]

Quantitation of morphine and its glucuronides in human urine by HILIC-TOFMS

[1)] Doping Control Laboratory, United Medix Laboratories Ltd., Helsinki, Finland

[2)] Forensic Toxicology Division, Hjelt Institute, University of Helsinki, Finland

Introduction

Morphine (M) is a widely used opiate in pain treatment. It is metabolized by liver via conjugation to morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G), the latter having an equal analgesic activity to the parent drug [1] (Figure 1). In human, the most abundant urinary metabolite is M3G [2].

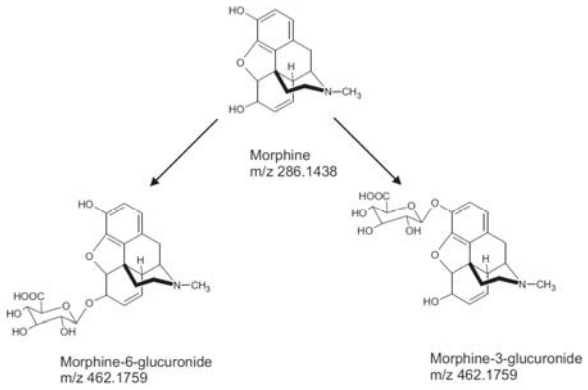

Figure 1 Structures of morphine and its glucuronide conjugates

In human doping control, the World Anti-Doping Agency (WADA) has banned the in-competition use of morphine [3] and set a threshold value of 1000 ng/ml for total morphine [4]. The glucuronide conjugates are very polar and non-volatile compounds and therefore require traditionally an acidic or enzymatic hydrolysis and derivatization before gas chromatographic-mass spectrometric (GC-MS) analysis [5-7]. However, it has been shown that the optimization of the hydrolysis is difficult and several factors *e.g.* temperature, incubation time, type of enzyme affect recoveries [8-13].

Even though liquid chromatography (LC) suits better for polar and non-volatile compounds than GC, the retention of glucuronide conjugates in typical reverse-phase (RP) column is weak and often with poor chromatographic resolution [14, 15]. The water content of the mobile phase must be rather high to increase the retention that may induce ionization problems in common LC-MS ion sources. Hydrophilic interaction liquid chromatography (HILIC) technique was designed for the analysis of compounds having poor retention on RP-columns [16]. The procedures for optimizing HILIC parameters for opioids and their glucuronide conjugates were presented recently [17, 18].

The present study describes a solution for exact and repeatable quantitation of morphine and its glucuronide conjugates relevant in doping control by HILIC-TOFMS without any hydrolysis or loss of chromatographic resolution. The applicability of the method is demonstrated with authentic urine samples containing morphine, codeine and their glucuronides.

Materials and Methods

Morphine, M3G, M6G and their deuterated analogues (purity 99%) were from Cerillant (Texas, USA). 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 solid phase extraction (SPE). Other tested HILIC stationary phases were SeQuant ZIC®-HILIC (Merck KGaA, Darmstadt, Germany), Acquity UPLC® BEH Hilic (Waters, MA, USA) and Kinetex™ HILIC (Phenomenex, USA).

Drug-free urine samples used in this study were obtained from healthy volunteers and used either individually or as pooled aliquots. Excretion urine samples were obtained from a healthy volunteer after a single administration of 30 mg codeine (*p.o*). The samples were collected over 72 hours in 6-10 hour fractions. The protocol of the study was approved by a local ethical committee (Finnish Medicines Agency; obtained statement 20.11.2008).

A urine sample of 100 µl was centrifuged in an Eppendorf tube with 16000*g* (10000 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 with the addition of 100 µl of internal standard (ISTD) solution (500 ng/ml of deuterated analogues in water). The cartridges were washed with 1 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.

An Agilent 1200 (Agilent Technologies,Waldbronn, Germany) series rapid resolution LC system was used for chromatography. A Zorbax Hilic Plus column 100 x 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% 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.

The TOF mass spectrometer was a Bruker Daltonics micrOTOF. An orthogonal ESI ion source was applied and ionization was performed in the positive mode. 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. Daily external calibration of TOFMS and data evaluation was performed as described previously [19]. Two level rating parameters of 0.15/0.2 min for RT and 5/7 mDa for mass accuracy were applied. The quantitative analysis of the samples was performed with 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/x factor applying a 5-mDa window.

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 one month. 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 authentic urine samples containing morphine, codeine and their metabolites.

Results and Discussion

The optimization of sample preparation was focused on achieving a straightforward method. By applying an opposite retention mechanism (C18) than in HILIC analytical column, solvent transfer, evaporation and reconstitution could be avoided with extraction recoveries between 99 and 104 %.

Several different HILIC stationary phase configuration were tested, however the most effective separation for glucuronides were achieved with Zorbax Hilic Plus column (Figure 2).

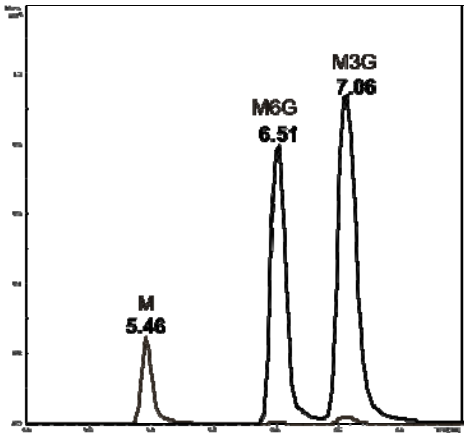

Figure 2 Separation for morphine (M) and morphine-6- and 3-glucuronides (M6G, M3G) standards (1000 ng/ml) in optimized conditions with Agilent Zorbax Hilic Plus column

The results for HILIC optimization are presented in Figure 3. As seen, the most critical factor effecting the separation is buffer concentration even changing the elution order of morphine concentratios above 8 mM.

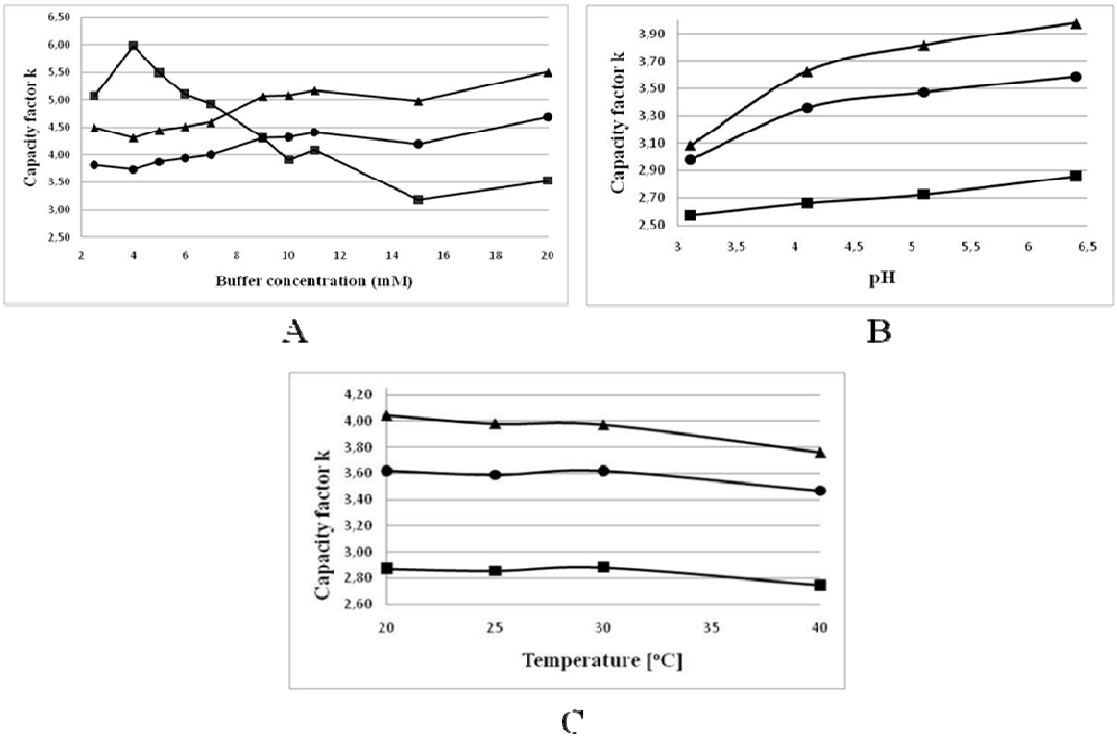

Figure 3 Effect of (A) buffer concentration, (B) pH and (C) temperature on the retention of morphine and its glucuronides in HILIC column (■ morphine, ▲morphine-3-glucuronide, ● morphine-6-glucuronide)

The quantitative method was appropriately validated within a concentration range of 50-5000 ng/ml (calculated as free morphine). Even after a simple sample clean up, urine matrix

did not interfere the detection of the analytes and the ion suppression was -40 % for morphine and approx. -9 % for glucuronide conjugates. The method was also unaffected by other matrix and opiate structured compounds and different storage conditions. Repeatability was measured as six replicates intra- and interday with RSD below 16% (Table 1). The calibration was repeatable with good linear regression (over 0.998) throughout the concentration range (Table 2).

Table 1 The validation results of repeatability

	Intraday			**Interday**		
	Concentration ng/ml Mean % (RSD%), n=6			**Concentration ng/ml Mean % (RSD%), n=6**		
	100	**1000**	**4000**	**100**	**1000**	**4000**
M	98.8 (9.4)	987.8 (4.8)	4150.1 (7.0)	97.4 (16.3)	939.0 (8.3)	3982.5 (6.2)
M6G	94.7 (10.1)	1046.9 (2.9)	4275.1 (3.5)	107.0 (13.8)	1039.5 (9.2)	4187.9 (8.0)
M3G	103.1 (5.9)	1030.3 (2.1)	4261.8 (6.8)	117.6 (16.4)	1062.4 (8.9)	4302.3 (9.3)

Table 2 Statistics of calibration

	Slope			**R^2, 1/x**	
	mean	**RSD%**	**y-intercept**	**mean**	**RSD%**
M	3.1	5.1	0.18	0.998	0.235
M6G	0.8	5.9	0.02	0.998	0.168
M3G	0.8	7.5	0.02	0.999	0.155

n=6

The suitability of the method was demontrated with the excretion urine samples after codeine administration. The chromatographic separation of morphine glucuronides as well as codeine glucuronide was unaffected by the urine matrix and clean extracted ion chromatograms were obtained for the analytes (Figure 4).

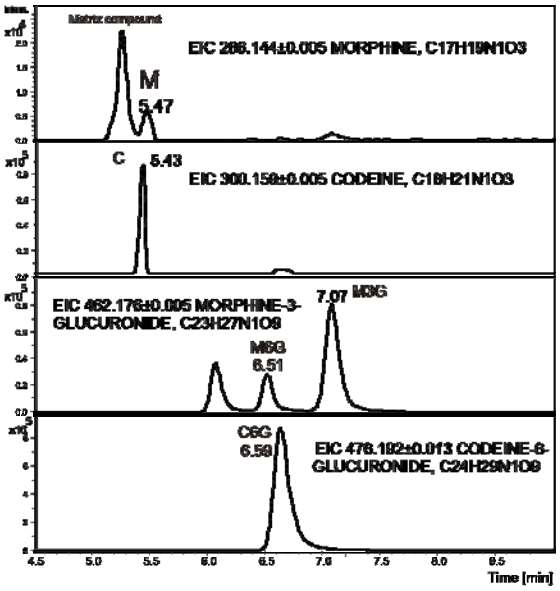

Figure 4 Extracted ion chromatograms (EIC) obtained from an excretion urine sample collected 8 hours after a single oral administration of 30 mg codeine. The measured concentrations were (ng/ml): morphine (M) 129, codeine (C) 1159, morphine-6-glucuronide (M6G) 329, morphine-3-glucuronide (M3G) 1717 and codeine-6-glucuronide (C6G) 23763.

Conclusions

A simple and straightforward sample preparation method was established without need for hydrolysis minimizing contamination and carry over. An adequate resolution for morphine glucuronide conjugates was achieved after thorough optimization of HILIC conditions. Sensitive and accurate quantitation of morphine and its intact glucuronide conjugates in human urine was performed with wide linear range and repeatable calibration demonstrating as well the good suitability of TOFMS for quantitation.

Acknowledgements

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