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Optimization and validation of the quantification of morphine from urine by triple quadrupole LC/MS^2

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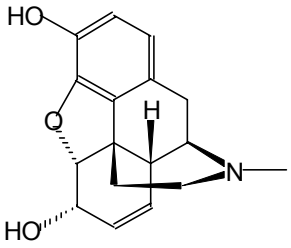
Introduction

According to the WADA rules, morphine at a urinary concentration (free and glucuronide conjugate) greater than 1μg/ml constitutes an adverse analytical finding unless it may have been caused as a result of the administration of a permitted substance such as codeine [1,2].

The methods proposed for the quantification of morphine include GC/MS and LC/MS analysis [3-6]. Morphine analysis by GC/MS requires derivatisation; since the derivatisation step is time consuming and represents an additional source of uncertainty, LC/MS was the technique of choice.



Morphine $C_{17}H_{19}NO_3$, M=285

Materials and methods

Morphine and D3-morphine were purchased from National Measurement Institute – Australian Government, the beta-glucuronidase, type HP-2:, from *Helix Pomatia* from Sigma-Aldrich (USA), the TBME and the acetonitrile from Merck (Germany), and the ultra-pure water was obtained by using the filtration system Simplicity 185, Millipore (Great Britain).

Morphine glucuronides are enzymatically hydrolyzed by β-glucuronidase from *Helix Pomatia*, at pH 5.2 and 55^0C, for 3h. Free morphine is extracted from urine at pH 9.6 with TBME. After centrifugation, the organic layer is transferred into a fresh glass tube and evaporated to dryness. The residue is redissolved in 100μl methanol.

Instrument: triple quadrupole mass spectrometer Agilent 1200/6410 with ESI source.

Table 1. Triple quadrupole LC/MS^2 Agilent 1200/6410 parameters

LC Parameters					**MS Parameters**	
Column	Zorbax XDB-C8 at 30^{0}C (150x4.6mm, part.size 5µm)				Ionization Mode	ESI positive
					Scan Type	MRM
Solvent A	5mM NH_4HCOO, 1‰ formic acid in Millipore ultra-pure water				Dwell time	60ms
Solvent B	5mM NH_4HCOO, 1‰ formic acid in 90% acetonitrile + 10% water				*Source Parameters*	
					ESI Drying Gas	N_2
LC Program	Time (min:sec)	%A	%B	Flow (ml/min)	Gas Temp	350 ^{0}C
					Gas Flow	10 l/min
	0:00	95	5	0.6	ESI Nebulizing Gas	345kPa N_2
	6:00	95	5	0.6	Collision Gas	ultrapure (5.0) N_2
	7:00	30	70	0.6	Capillary voltage	4000V
	12:00	30	70	0.6		
	12:10	95	5	0.6	Delta EMV	300V
	20:00	95	5	0.6		
Injection Volume	1 µl					

Results and discussions

Morphine forms precursor ions by protonation, $[M+H]^+$, m/z 286 for morphine and m/z 289 for ISTD D3-morphine. In figure 1 is shown the product ion mass spectrum generated by the precursor ion for morphine, m/z 286, at 50eV collision energy.

The selected MRM transitions and their relative abundance are shown in table 2. The MRM transition 286>165 of morphine and 289>165 of ISTD D3-morphine are used for quantification.

Table 2. Characteristic parameters for morphine identification

Compound	RT	MRM, collision energy	Relative abundance
Morphine	7.086 min	286>165, 50eV	100%
		286>153, 40eV	78.7%
		286>128, 60eV	78.5%
		286>115, 60eV	53.3%
ISTD D3-Morphine	7.019 min	289>165, 50eV	100%
		289>201, 25eV	81.2%

Assay validation [7]

1. Intermediate precision

The intermediate precision was calculated analyzing 3 series of 6 control samples (different blank urines spiked with 1000ng/ml morphine and 500ng/ml ISTD D3-morphine). The 3 series were extracted at 3 different dates, during 3 weeks, by different analysts and injected once. The intermediate precision was 3.33% (less than 10%).

2. Robustness

There were prepared 5 urine samples spiked with 1000ng/ml morphine and 500ng/ml ISTD D3-morphine. The samples were analyzed one time with the standard set of analytical

parameters and 5 times with one parameter changed: LC column temperature (30 to 25^{0}C), mobile phase composition (5% to 6%B), drying gas temperature (350 to 345^{0}C), drying gas flow (10 to 9L/min), nebulizing gas pressure (50 to 45psi).

The analysis of the experimental data proved that the minor variation of these parameters has no significant effect on the method performance; and therefore the method is robust.

3. Carryover

Morphine area in the blank urine sample injected after a control sample spiked with 5000ng/ml morphine (5 times the threshold value) represents 0.04% (less than 5%) of morphine area in the spiked sample.

4. Specificity and matrix interferences

Specificity was evaluated by analyzing 6 different blanks of urine and a positive control sample. The positive control sample was blank urine spiked with 1000ng/ml morphine and 500ng/ml ISTD D3-morphine.

No interferences were noticed in the blank urine samples analyzed, at the retention time of morphine and D3-morphine, in the monitored MRM transitions.

5. Limit of quantification and limit of detection

The limit of quantification is 269ng/ml. It was calculated from the calibration curve data.

At a urinary concentration of morphine of 50ng/ml, the signal to noise ratios obtained are significantly higher than 3, for all four characteristic MRM transitions of morphine; the chromatogram is shown in figure 2. Therefore the limit of detection is < 50ng/ml.

6. Linearity

A calibration curve for morphine was generated using blank urine spiked at 500, 750, 1000, 1250, 1500, 1750 and 2000ng/ml.

The calibration curve is linear within the selected range. The calibration equation is $y = 0.0023x + 0.0286$; the linear correlation coefficient for morphine $r^2 = 0.9975$.

7. Uncertainty

The total uncertainty was evaluated by combining the uncertainties introduced by:

- Reference Materials: purity and weighing uncertainty;
- Solutions preparation: volume uncertainty;
- Calibration curve: standard deviation of the analytical result (sx_0);
- Analytic equipment: instrument repeatability;
- Method: method repeatability.

The extended uncertainty, for k=2 and confidence interval 95%, U = 7.76%.

The contribution of different uncertainty sources are shown in figure 4.

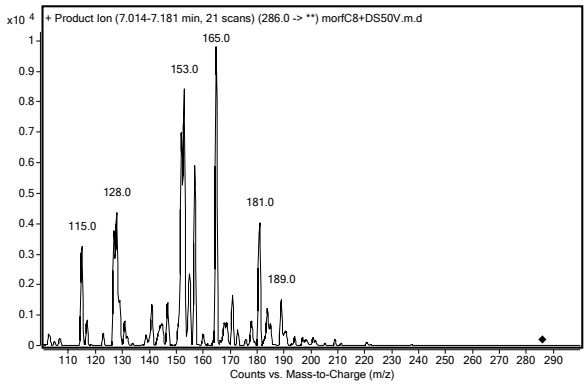


Figure 1. Product Ion mass spectrum of Morphine ($[M+H]^+$=286) using a CE of 50eV.

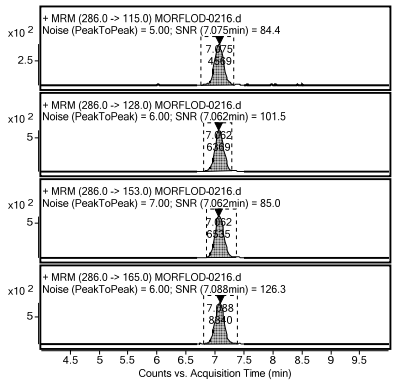


Figure 2. S/N ratios at 50ng/ml.

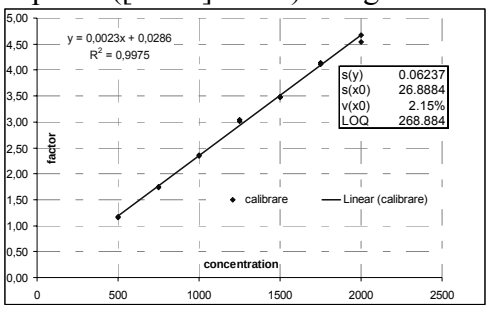


Figure 3. Calibration curve for morphine.

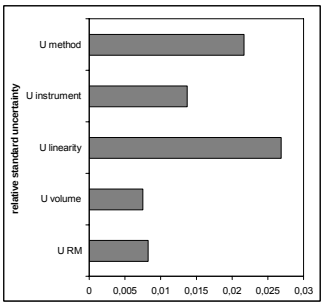


Figure 4. Budget of uncertainty.

Acknowledgements

The authors wish to thank the Romanian National Anti-Doping Agency for support.

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