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Optimization of Morphine and Codeine hydrolysis in urine by experimental design for GC-MS quantification purpose

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1. Introduction

Morphine and Codeine are potent narcotic analgesic substances of natural origin and used in some medical treatments at different levels. Because of its severe side effects and drug addiction effect, Morphine is a controlled substance and for athletes, in particular, its use is prohibited by the World Anti-Doping Agency (WADA) code. A Morphine urinary concentration superior to 1 µg/ml is declared as an adverse analytical finding by the accredited laboratories [1]. Nevertheless, the presence of Morphine in urine may be due to the administration of Codeine, which is a permitted substance for therapeutic purpose. For this reason, both Morphine and Codeine exact concentrations as well as the Codeine/Morphine ratio have to be correctly established [2,3].

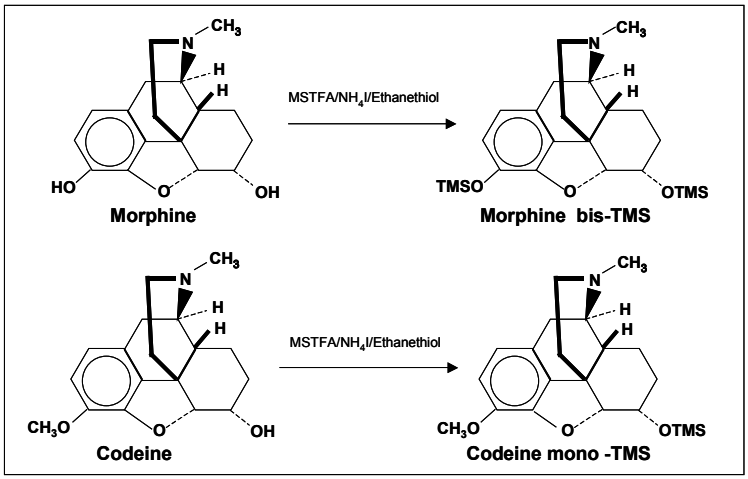

***Figure 1**: Structure of the investigated compounds*

The 1 µg/ml threshold limit is based on the sum of the glucuronide conjugate and free Morphine concentrations. For GC-MS quantification purpose of Morphine and Codeine, the critical step during extraction is the hydrolysis of glucuronide forms. Indeed, the large

standard deviations obtained from proficiency tests on Morphine during 2004 attest, in particular, of the difficulty to assess good hydrolysis conditions.

Consequently, in this study, an experimental design was carried out in order to find the optimal conditions for the hydrolysis of Morphine and Codeine glucuronide forms and to evaluate the robustness of the method. The optimized method was validated and the exactitude of the dosage was evaluated with a certified urine.

2. Experimental

2.1. Standards and sample solutions

Morphine-3-β-D-glucuronide, Codeine-6-β-D-glucuronide and Codeine-(N-methyl-d_3) were purchased by Lipomed AG (Switzerland). Certified urine (UTAK, N° 98815, Lot 5773) with Morphine glucuronide was obtained from InGen (France).

2.2. Urine extraction

2 ml of urine is added with 100 µg/ml of Codeine-D_3 and hydrolysed with concentrated HCl (optimal conditions assessed in this study). Hydrolysed urine is then washed with diethyl ether, basified with carbonate buffer and extracted with diethyl ether/isopropanol. The residue is finally derivatized with 50 µl MSTFA/NH_4I/Ethanethiol in order to obtain the TMS forms of Morphine and Codeine.

2.3. Instrumentation and analytical method

Analyses were performed by GC-MS with SIM (quantification) and SCAN (identification) modes on a Hewlett-Packard 6890 gas chromatograph (HP Analytical Division, Waldbronn, Germany) and coupled with a HP 5973 mass selective detector (MSD). GC separation was achieved on a ZB-5 (5% phenyl-95% dimethyl-polysiloxane (Phenomenex, St. Torrance, CA, USA) column (30 m x 0.25 mm I.D., 0.25 µm film thickness). Temperature programming: 90°C for 0.5 min, ramped at 20°C/min to 320°C and held for 3 min. Injections of 1 µl-samples were made at 270°C in the splitless mode.

2.4. Experimental design

In this study, a central composite design was implemented in order to determine the optimal conditions for Morphine and Codeine hydrolysis. Three experimental parameters (variables) were investigated: HCl volume (between 150 and 1800 µl), the temperature (from 65 to 130 °C) and time of hydrolysis (from 10 up to 110 min). The selected experimental design led to the implementation of 20 experiments which were randomly performed.

2.5. Optimisation of hydrolysis parameters

For each experiment, the area ratio between Morphine or Codeine and the internal standard IS (Codeine-d3) was systematically calculated. A mathematical model was obtained which represents the relationship between the responses and the independent variables. The quadratic regressions allowed to determine the optimal conditions by maximizing the ratios between Morphine or Codeine and the internal standard:

	Variable		
Substance	**V_{HCl} (µl)**	**Temperature (°C)**	**Time (min)**
Morphine	820	120	53
Codeine	1280	109	71
Selected conditions	900	120	60

***Table 1**: Optimal conditions for the hydrolysis of Morphine and Codeine*

3. Results and discussion

3.1. Response surfaces and robustness evaluation

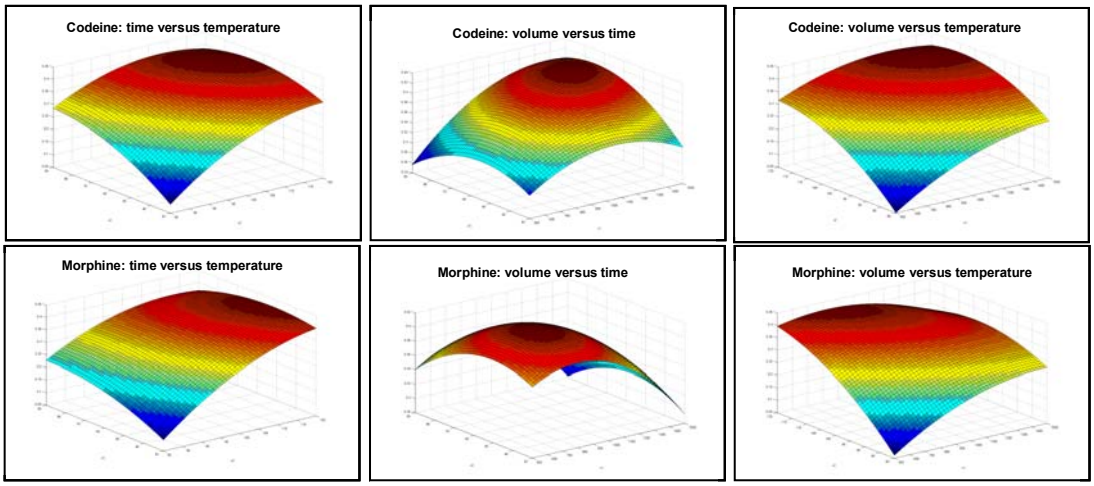

***Figure 2**: Response surfaces for Morphine and Codeine*

It is possible to visualize response surfaces as a three-dimensional plot of two factors, while keeping the other constant at its optimal value, and to determine an optimal zone where the best hydrolysis recovery can be performed for further validation and dosage in urine. The robustness of the method at the optimum can also be evaluated by this way. The optimal conditions selected for the simultaneous hydrolysis of Morphine and Codeine are: 90 µl of HCl at 120 °C during 60 min.

3.2. Method validation

The method was validated at the optimised conditions and good results were obtained in terms of precision, linearity, LOD, LOQ and reproducibility of the extraction. The experimental conditions were then applied for the simultaneous quantification of Morphine and Codeine glucuronide forms in a certified urine in order to assess the accuracy of the method. In this

particular case, it is important to point out the necessity to use the glucuronide forms of Morphine and Codeine for the establishment of the calibration curve. Finally, the total uncertainty of the method has also been evaluated.

Parameter	**Compound**	
Precision	Morphine	Codeine
Intra-day		
migration time	0.02 %	0.01 %
peak area ratio	3.20 %	2.50 %
Inter-day		
migration time	0.04 %	0.02 %
peak area ratio	4.50 %	3.40 %
Linearity (from 0.1 to 5 µg/ml)	**glucuronide form**	**glucuronide form**
(Y = ax + b) **a**	1.2251	1.3507
b	0.1611	0.1003
r^2	0.9903	0.9938
LOD	10 ng/ml	10 ng/ml
LOQ	30 ng/ml	30 ng/ml
Extraction reproducibility	4.8 %	6.6 %
Accuracy (glucuronide forms)		
label claim	2.43 µg/ml	1.50 µg/ml
amount found	2.38 µg/ml	1.51 µg/ml
Recovery	98 %	101 %
Total uncertainty	14.8 %	16.8 %

Table 2*: Validation data for the optimised method*

4. Conclusion

An experimental design was used for the simultaneous optimisation of three experimental parameters for the hydrolysis of morphine and codeine in urine. After evaluation of response surfaces, it was possible to determine the optimal conditions for the hydrolysis (900 µl HCl, 120 °C, 60 min) and to evaluate the robustness of the method. The method was validated and good performances were obtained in particular regarding the accuracy of the method for the dosage of both substances in urine. In this particular case, it is necessary to use the glucuronide forms of Morphine and Codeine for the assessment of the calibration curve.

5. Acknowledgement

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6. References

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