ANALYSIS OF OPIATES AND THEIR GLUCURONIDE METABOLITES IN BIOLOGICAL FLUIDS USING MIXED-MODE SOLID PHASE EXTRACTION AND ULTRAPERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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OBJECTIVE

Develop a method to simultaneously extract and quantify morphine and five of its metabolites from plasma.

INTRODUCTION

Morphine is the most commonly used opiate analgesic for the treatment of severe pain [1]. In humans, the major metabolites of morphine are morphine-3β-glucuronide (M3G) and morphine-6β-glucuronide (M6G) [2]. Both of these metabolites are pharmacologically active, but have opposite effects. The effects of M6G have been reported to be equal to or greater than morphine [3], while M3G counteracts morphine's analgesic activity. It is also possible for morphine to form N-oxide, hydroxyl, and acetyl metabolites, the last of which is structurally similar to heroin. Quantitation of all metabolites in biological fluids is critical for applications such as drug metabolism, metabolite profiling, substance abuse, and veterinary medicine. Therefore, it is desirable to simultaneously analyze morphine and all of its metabolites in one analysis.

Both the extraction and analysis of morphine and its related metabolites present some challenges using LC-MS and SPE. First, these compounds are extremely polar, and are poorly retained on traditional reversed-phase C_{18} columns. Furthermore, adequate LC separation of all desired analytes in a short analysis time is difficult. Second, the presence of slightly different functional groups between the metabolites may cause different retention behaviors on mixed-mode SPE sorbents, which can result in lower recoveries for some or all of the analytes. In addition, some morphine metabolites are not stable at the high pH extreme that is employed for elution in traditional mixed-mode SPE protocols. Finally, a fast-scanning MS instrument is required to properly sample each of the peaks (typical peak width of 2-3 seconds) eluting from the UPLC column in order to obtain accurate and precise quantitation.

This work describes a method for the simultaneous extraction and analysis of morphine and five of its related metabolites from plasma using mixed-mode SPE in the µElution format and UPLC-MS/MS. The SPE protocol employs an elution into a neutralizing solvent to maintain analyte stability prior to UPLC-MS/MS analysis [4]. Separation of all six analytes was achieved in 3 minutes at pH ~ 2.7 using a column designed to retain polar analytes under high aqueous conditions. The SPE recovery and repeatability of the method were determined, as well as the linearity and lower limit of quantitation (LLOQ) for each analyte.

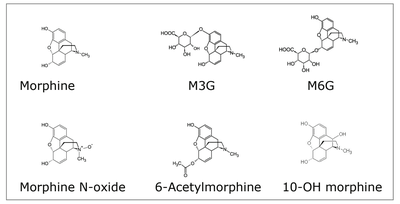


Figure 1. Structures of morphine and five related metabolites.

UPLC-MS/MS METHOD

UPLC® conditions

System:	Waters ACQUITY UPLC® System
Column:	**ACQUITY UPLC® HSS T3 Column 2.1 x 50 mm, 1.8 µm**
Column Temp:	45 °C
Sample Temp:	15 °C
Flow Rate:	0.5 mL/min
Mobile Phase A:	0.1% HCOOH in H_2O
Mobile Phase B:	Methanol
Gradient:	2-10% B in 1 min, then to 98% B in 0.1 min, hold for 0.9 min, reset (3 min total cycle time).
Injection volume:	15 µL
Injection mode:	Partial loop with needle overfill (PLNO; 20 µL loop size)
Weak wash solvent:	95/5 (H_2O/Methanol)
Strong wash solvent:	95/5 (ACN/H_2O)

MS conditions

MS system:	Waters ACQUITY® TQD
Ionization mode:	Electrospray positive
Capillary voltage:	1.0 kV
Dwell time:	10 ms
MRM transitions:	See Table 1

Compound	Reaction (m/z)	Cone Voltage (V)	Collision Energy (eV)
M3G, M6G	462.0>285.9	30.0	35.0
M3G-D3, M6G-D3	465.2>289.0	45.0	36.0
6-Acetylmorphine	328.0>164.9	50.0	35.0
6-Acetylmorphine-D3	331.1>165.0	45.0	50.0
Morphine N-oxide	302.0>161.9	45.0	30.0
10-hydroxymorphine	302.0>57.9	50.0	25.0
Morphine	286.0>200.9	50.0	25.0
Morphine-D3	289.1>201.1	45.0	28.0

Table 1. MS/MS conditions for 6 analytes and 4 deuterated internal standards.

EXAMPLE SEPARATION

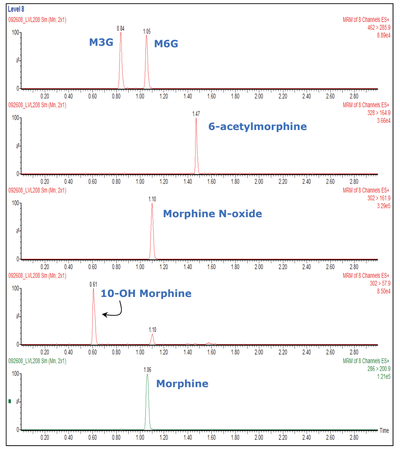
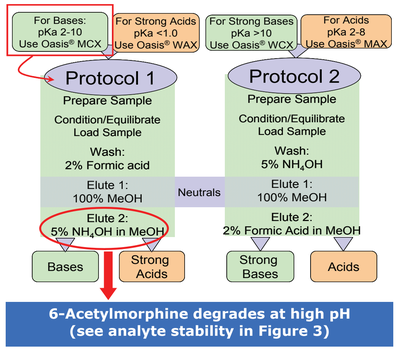


Figure 2. UPLC-MS/MS chromatogram of a plasma sample spiked with 25 ng/mL of each analyte prior to extraction.

SPE METHOD DEVELOPMENT

OASIS® 2X4 PROTOCOL



ANALYTE STABILITY

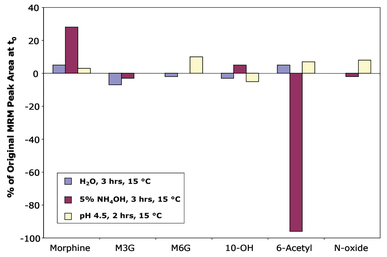


Figure 3. Stability of morphine-related compounds in various solutions at 15 °C. Incubation time is listed on the graph.

FINAL SPE PROCEDURE

Oasis® MCX µElution 96-well plate

1. Spike porcine plasma with the appropriate concentration of each analyte
2. Dilute samples 1:1 with 4% H_3PO_4 in H_2O
3. Condition the wells with 200 µL MeOH
4. Equilibrate with 200 µL water
5. Load 500 µL diluted plasma sample (from #2 above)
6. Wash with 200 µL 2% HCOOH in water
7. Wash with 2x100 µL MeOH
8. **Elute with 2x25 µL of 5% NH_4OH in 90:10 MeOH:H_2O into a collection plate already containing 50 µL of 3% HCOOH in MeOH**
9. Evaporate to dryness at room temperature
10. Reconstitute with 50 µL water

SPE RECOVERY

$$\%\text{Recovery} = \frac{\text{Response}_{\text{pre-extracted spiked sample}}}{\text{Response}_{\text{post-extracted spiked sample}}} \times 100$$

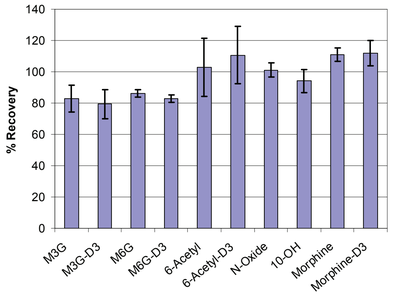


Figure 4. SPE recoveries for each analyte at 25 ng/mL in porcine plasma. The average recovery over a three day period is shown (N=4 on each day).

LINEARITY

Calibration curves for each of the six compounds in porcine plasma were generated at the following concentrations: 0.1, 0.25, 0.5, 1, 5, 10, 25, 50, 100 and 250 ng/mL. The internal standard was spiked to a final concentration of 25 ng/mL for each point. The linear regression was constructed using a weighting of 1/x with the origin excluded. The % deviation for each calibration point was obtained from QuanLynx™ software by comparing the concentration value calculated from the linear regression to the expected value (Table 2).

ANALYTE	Internal Standard	Range (ng/mL)	Linearity (R^2)	% Deviation Range	LLOQ (ng/mL)	S/N @ LLOQ
M3G	M3G-D3	0.25-250	0.997	-11.4 - 8.9	0.25	28
M6G	M6G-D3	0.5-250	0.998	-14.2 - 7.7	0.5	50
6-Acetylmorphine	6-Acetylmorphine-D3	0.25-250	0.997	-10.7 - 16.9	0.25	15
Morphine N-Oxide	Morphine-D3	0.1-250	0.999	-8.4 - 7.5	0.1	81
10-Hydroxymorphine	Morphine-D3	0.25-250	0.999	-3.4 - 6.7	0.25	40
Morphine	Morphine-D3	0.25-250	1.000	-4.5 - 4.6	0.25	21

Table 2. Linearity, % deviation and LLOQ results for morphine and 5 related metabolites.

LLOQ

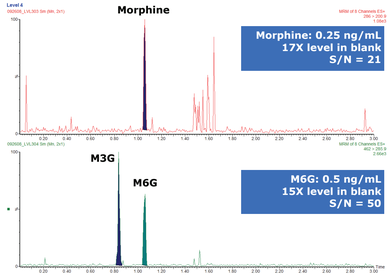


Figure 5. Representative XIC chromatograms at the LLOQ for morphine and M6G in porcine plasma.

CONCLUSIONS

- **A method for the simultaneous extraction and quantitation of morphine and five of its metabolites from plasma was developed using Oasis® MCX µElution SPE and UPLC® -MS/MS.**
- **Total run time for each analysis is 3 minutes, which is suitable for moderate to high throughput bioanalytical applications.**
- **Analyte degradation was minimized by eluting into a neutralizing solvent prior to evaporation.**
- **SPE recovery was between 82.9% and 111.0%, depending on the analyte, and inter-day variation was less than 10% (< 20% for 6-acetylmorphine).**
- **The method was linear over three orders of magnitude with $R^2 \geq 0.997$.**
- **LLOQ values ranged from 0.1 to 0.5 ng/mL with S/N > 15 for all analytes.**

References

1. Y.M. Alnouti, M.K. Shelby, C. Chen, C.D. Klaassen, Curr. Drug Metab. 8 (2007) 79.
2. G. Anderson, L. Christrup, S. Per, J. Pain Symptom. Manage 25 (2003) 74.
3. W.Z. Shou, M. Pelzer, T. Addison, X. Jiang, W. Naidong, J. Pharm. Biomed. Anal. 27 (2002) 143.
4. E.J. Rook, M.J.X. Hillebrand, H. Rosing, J.M. van Ree, J.H. Beijnen, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 824 (2005) 213.

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