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

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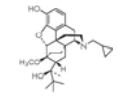
INTRODUCTION

Quantitation of metabolites is crucial for understanding the biotransformation process of any drug. UltraPerformance liquid chromatography (UPLC® technology) provides significant advantages over traditional HPLC with respect to throughput, sensitivity, and resolution. It has also been shown to greatly reduce matrix effects in bioanalytical assays.

Mixed-mode solid phase extraction (SPE) has been shown to be the ideal method for sample preparation for sensitive and robust determination of trace-level components in complex matrices. This is mainly due to the presence of two different retention mechanisms; reversed-phase and ion exchange. Analytes of interest are retained by ion exchange while more hydrophobic interferences that contribute to matrix effects (phospholipids) can be washed from the sample.

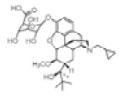
The cumulative benefits of both mixed-mode SPE and UPLC® technology are presented for the analysis of opiates and their glucuronide metabolites in rat plasma. All compounds are extracted in a single experiment, and subsequently analyzed by UPLC®/MS/MS in multiple reaction monitoring (MRM) mode. Total cycle time is 2 minutes, which is suitable for analysis of 500 to 1,000 samples per day. Recovery was greater than 93 % for all analytes and varied less than 6 % between days.

OPIATE STRUCTURES



Buprenorphine
pKa ~ 8.5

Norbuprenorphine



Buprenorphine glucuronide

Norbuprenorphine glucuronide

UPLC®/MS/MS METHOD DEVELOPMENT

Compound	Transition	Cone Voltage (V)	Collision Energy (eV)	Dwell Time (ms)
Buprenorphine	468.2 > 54.9	70	45	15
Norbuprenorphine	414.2 > 101.0	70	40	15
Buprenorphine glucuronide	644.2 > 468.2	70	40	15
Norbuprenorphine glucuronide	590.2 > 414.2	65	38	15

Table 1. MRM transitions for opiate analytes on the TQD mass spectrometer.

Screening Method Protocol

All analyses were performed on an ACQUITY UPLC® system connected in-line to a TQD mass spectrometer (***Electrospray positive mode***).

Column: ACQUITY UPLC® BEH C_{18}, 2.1 x 100 mm, 1.7 µm
Mobile phase A: 0.1% formic acid in H_2O (low pH)
0.1% NH_4OH in H_2O (high pH)
Mobile phase B: 100% Acetonitrile
Gradient: 2-98% B in 5 min, hold at 98% B until 7 min, reset (10 min total cycle time)
Flow rate: 0.5 mL/min
Column temp.: 45 °C
Injection volume: 15 µL (20 µL loop size)
Weak needle wash: 95/5 H_2O/MeOH
Strong needle wash: 95/5 ACN/H_2O

Effect of Mobile Phase pH

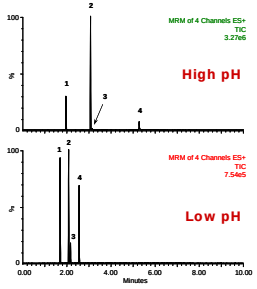


Figure 1. Total ion chromatograms of (1) norbuprenorphine glucuronide, (2) buprenorphine glucuronide, (3) norbuprenorphine, and (4) buprenorphine at high and low pH. Each compound is present at 50 ng/mL in 50/50 MeOH/H2O.

Compound	MS Signal Intensity Low pH	MS Signal Intensity High pH
Buprenorphine	4.51E+05	3.27E+05
Norbuprenorphine	1.39E+05	4.61E+04
Buprenorphine glucuronide	6.41E+05	1.86E+06
Norbuprenorphine glucuronide	6.82E+05	1.53E+06

Table 2. Effect of mobile phase pH on MS signal intensity. Values shown in the table are ion counts for each compound transition.

For the non-glucuronidated compounds, MS signal intensity is up to 3-fold higher at low pH. For the glucuronide metabolites, MS signal intensity is 3-fold higher at high pH. Low pH mobile phases were chosen for the final optimized method due to extremely low MS signals for the non-glucuronidated compounds at high pH.

Minimizing Carryover

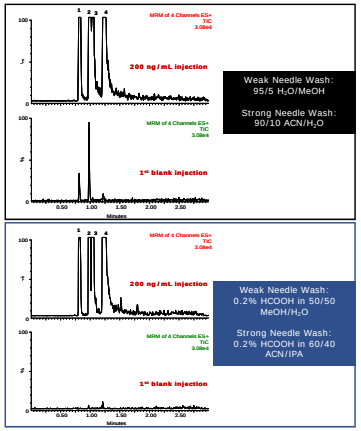
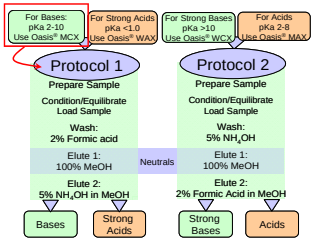


Figure 2. Minimizing carryover using optimized wash solvents. Mobile phase A is 0.1% HCOOH in water. Mobile phase B is ACN. Gradient from 5-95% B in 2 min, hold at 95% B for 0.5 min, reset (3 min total cycle time). ACQUITY UPLC® BEH C_{18} column, 2.1 x 50 mm, 1.7 µm. Column temperature is 30 °C. Injection volume is 5 µL. Peak ID is identical to Figure 1.

OASIS 2X4 PROTOCOL



OPTIMIZED SPE/UPLC®/MS/MS METHOD

Solid Phase Extraction (Oasis® MCX µElution plate)

1. Rat plasma spiked with 20 ng/mL each analyte.
2. Sample pretreated with 1:1 dilution in 4% H_3PO_4 in H_2O.
3. Condition with 200 µL MeOH.
4. Equilibrate with 200 µL H_2O.
5. Load 400 µL sample (200 µL spiked plasma + 200 µL 4% H_3PO_4 in H_2O).
6. Wash with 200 µL 2% formic acid in H_2O
7. Wash with 200 µL MeOH
8. Elute with 2 X 25 µL 5% NH_4OH in MeOH
9. Dilute 1:1 with H_2O and inject 5 µL onto UPLC®/MS/MS

UPLC®/MS/MS

Column: ACQUITY UPLC® BEH C_{18}, 2.1 x 50 mm, 1.7 µm
Mobile phase A: 0.1% formic acid in H_2O
Mobile phase B: 100% Acetonitrile
Gradient: 15-60% B in 1 min, to 95% B at 1.01 min, hold at 95% B until 1.5 min, reset (2 min total cycle time)
Flow rate: 0.5 mL/min
Column temp.: 30 °C
Injection volume: 5 µL (20 µL loop size)
Weak needle wash: 0.2% formic acid in 50/50 MeOH/H_2O
Strong needle wash: 0.2% formic acid in ACN/IPA (60/40)

$$\% \text{ Recovery} = 100 \times \frac{\text{RESPONSE Extracted Sample (with analyte(s))}}{\text{RESPONSE Post-Extracted SPIKED Sample}}$$

OPIATE ANALYSIS IN RAT PLASMA

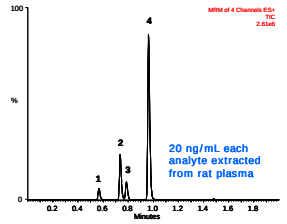


Figure 3. Total ion chromatogram of a rat plasma sample spiked with 20 ng/mL of each opiate. Peaks: (1) norbuprenorphine glucuronide, (2) buprenorphine glucuronide, (3) norbuprenorphine, (4) buprenorphine.

RECOVERY

Day	Bup.	Norbup.	Bup. glucuronide	Norbup. glucuronide
1	97.5	102	91.6	95.1
2	88.3	92.1	98.2	99.7
3	95.4	94.2	92.2	89.9
AVG	93.7	96.1	94.0	94.9
% RSD	5.1	5.4	3.9	5.2

Table 3. % Recovery of opiates from rat plasma over a three day period. N = 4 on each day.

CONCLUSIONS

- A method for extraction and analysis of opiates and their glucuronide metabolites was developed using Oasis® MCX µElution SPE and UPLC®-MS/MS.
- All analytes were stable throughout the extraction and analysis procedure.
- Average recovery for all compounds was greater than 93% over several days, and varied less than 6%.
- The 2 minute UPLC®-MS/MS analysis is suitable for high throughput bioanalysis.

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