Two Efficient Sample Preparations For Opiates Testing in Urine

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Introduction

In 2014, nearly two million Americans either abused or were dependent on prescription opioid pain relievers. 91 Americans die every day from an overdose involving either prescription opioid or heroin. Today's abuse profile combine both clinical and street drugs. Combining efficient hydrolysis based on purified beta-glucuronidases with an automatable clean-up or extraction method enables provides robust and defendable results.

Experiments

Instruments:

All automated extractions were carried out using Orochem's Oroflex Personal Pipettor Robot. All LC-MS/MS methods used a AB Sciex Excion LC system coupled to an API 4500 mass spectrometer with a turbo ionspray ESI source operated in positive ion mode.

Materials:

Panthera Deluxe Polymeric SPE plates, 30 mg/well (Orochem Technologies Inc) and RubyPro Protein Crash plates (Orochem Technologies Inc)) were used for all extractions. Pain management drug standards, their isotope internal standards (IS), codeine-6-β-D- glucuronide, and morphine-3-β-D-glucuronide were purchased from Cerilliant. EBG- β-glucuronidase and BG100 β-glucuronidase were provided by Biotec-La Piedra Biotechnologia SpA(Chile). Mass spec grade methanol, water and formic acid were purchased from Pharmco- Aaper. Ammonium acetate buffer was purchased from Sigma-aldrich. Orochem's EZYPRESS HT 96-well plate positive pressure manifold unit was used for conditioning, washing and processing of SPE plates. Evaporation was carried out using Quikvap 96-well plate evaporator (Orochem Technologies Inc). Human urine was fortified with standards.

Sample Preparation Procedures:

Nine analytes (6-MAM, codeine, morphine, oxymorphone, hydromorphone, oxycodone, noroxycodone, hydrocodone, and horhydrocodone) and their deuterated IS were spiked to blank urine at different concentrations for extraction method validation. Codeine-6-β-D- glucuronide, morphine-3-β-D-glucuronide and 6-MAM were spiked to blank urine at ULOQ level (glucuronides' concentrations are equal to 2000 ng/ml free drug level, 200 ng/ml for 6-MAM) for enzyme hydrolysis recovery test.

I. Protein crash method

1. Add fortified urine (0.1 ml) and 0.2 ml master mix (including ammonium acetate buffer, 66 µL of EBG β-glucuronidase and IS) directly to protein crash plate.
2. Incubate samples for 15 min at 50oC.
3. Add 50 µL methanol. Apply positive pressure and collect the eluate.
4. Add 50 µL water, and inject to UHPLC.

II. Solid phase extraction method

1. Mix fortified urine (0.1 ml) and 0.25 ml master mix (including ammonium acetate buffer, 25 µL of BG100 β-glucuronidase and IS) to 96-well collection plate.
2. Incubate samples for 30min at 68oC.
3. Condition Panthera Deluxe SPE plate with 1 ml of methanol, followed by 1 ml of water.
4. Load hydrolyzed samples.
5. Wash with 1 ml of water, followed by 1ml of 5% methanol
6. Elute with 1 ml of methanol
7. Evaporate at 40oC under nitrogen
8. Reconstitute with 0.3 ml of 5% methanol, and inject to UHPLC.

This method was used to analyze pain management drug panel in urine samples.

III. UHPLC-MS/MS analysis

We used Excion LC (AB Sciex), Gazelle C18 UHPLC column, 1.7 µm, 50 x 2.1 mm (Orochem Technologies Inc.) with a mobile phase gradient of 5-95% acetonitrile with 0.1% formic acid (B) and 0.1% formic acid in 20 mM ammonium buffer (A) and API4500 (AB Sciex) for MRM detection. Total run time was 6 minutes.

Results

I. Separation of opiates in Panthera urine extract:

Several different mobile phases were tested. For opiates, there is no significant difference of peak shapes with or without ammonium buffer; however, ammonium buffer improves benzodiazines peak shapes significantly. As a general method for main drug panel, mobile phase with ammonium buffer is chosen.

No significant fronting or tailing of opiates using Gazelle C18 UHPLC column. Injection solvent strength affects earlier eluate, organic solvent% should be kept at <10%.

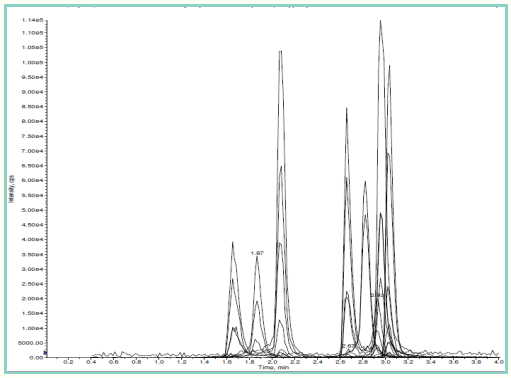

Figure 1. Mass chromatograms of morphine, oxymorphone, hydromorphone, codeine, oxycodone, noroxycodene, hydrocodone, norhydrocode, and 6-MAM (peak from left to right) in fortified urine sample, with Panthera Deluxe SPE process.

II. Solid phase extraction method:

A generic SPE method has been tested for over 37 drugs, it gives over 90% extraction recoveries for most of them.

Analyte	Extraction Recovery %	Matrix Effects %	Analyte	Extraction Recovery %	Matrix Effects %
Hydromorphone	94.1	108.6	Norhydrocodone	95.4	106.5
Morphine	95.8	94.4	Codeine	91.5	114.4
Oxymorphone	93.2	105.1	Hydrocodone	98.8	109.4
6-MAM	94.7	115.3	Noroxycodone	90.9	122.4
Oxycodone	97.6	118.8			

Table 1. Extraction recoveries of opiates using Panthera Deluxe SPE extraction plate

Hydrolysis of codeine-6-β-glucuronide and morphine-3-β-glucuronide using BG100 yield 89.8% and 110.5% recoveries, while labile analyte (6-MAM) gave 100.8% recovery.

III. Protein crash method:

Hydrolysis can be conducted directly on the protein crash filter plate providing a faster, simpler to operate method, at a lower cost. 50 and 100 µl of methanol have been tested, while the difference of organic solvent did not change the extraction recovery, but significantly worsen the peak shapes with higher concentration of methanol. So, 50 µl of methanol is a better choice.

Analyte	Extraction Recovery %	Matrix Effects %	Analyte	Hydrolysis Recovery %
Morphine	62.9	63.7	Morphine-3-β-glucuronide	120.2
Codeine	64.4	104.1	Codeine-6-β-glucuronide	116.6
6-MAM	87.4	96.7	6-MAM	115.9

Conclusion

Two accurate and automated sample prep methods for opiates are tested.

Protein crash filter plate provides on-plate hydrolysis, and it meets the typical screening requirement of PDM and pain management.

Panthera Deluxe SPE method provides excellent recovery, and removes more endogenous in the urine, and increases the lifetime of UHPLC column and mass spectrometer. It is simple and no method development required. For future test, we suggest to use microelute plate, which will reduce the elution solvent amount and evaporation time.

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