High Throughput Extraction of Opiates from Urine and Analysis by GC/MS or LC/MS/MS)

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Objective

The prescription of opiate drugs is common for the treatment of chronic pain. Due to the addictive nature of these drugs, many opiates are regulated under Schedule I and routinely tested as part of pre-employment screening or workplace performance monitoring. The confirmation of these analytes by GC/MS can be complicated by two factors, 1) removal of endogenous compounds from urine that can co-elute with target analytes and 2) the presence of non-target opiates which have similar mass ions. The objective of this work was to develop a high throughput assay for opiates in urine utilizing solid phase extraction (SPE) for sample clean up and analysis by either GC/MS or LC/MS/MS.

Method

Urine samples were spiked at 2,000 ng/mL with morphine, codeine, and other commonly encountered opiate related interferences. A 2 mL aliquot of urine was then spiked with internal standards and 400 µL concentrated HCl. Hydrolysis was performed by heating the sample at 90 °C for 40 minutes in a heating block, or in an autoclave for 15 minutes on a liquid cycle. After samples were cooled, they were centrifuged for 10 minutes at 2000 rpm and the pellet was discarded. The hydrolyzed samples were then buffered and extracted using a 30 mg/3 mL SPE tube containing a polymeric cationic exchange sorbent under the conditions specified in Table 1. Wash conditions were optimized to remove polar cationic contaminants that interfered with the GC/MS analysis.

Separation by GC/MS of morphine and codeine from other opiate related inferences was evaluated using two different derivatization procedures. Samples were simultaneously analyzed by LC/MS/MS on an Applied Biosystems® API 3000™ without the need for derivatization.

Table 1: SPE Protocol

	Strata™-X-C 30 mg/3 mL SPE Tubes (Part Number: 8B-S029-TBJ)
	Condition: - 1 mL of Methanol - 1 mL of Water
	Load: - To 1 mL of Urine, add 1 mL of 100 mM Sodium Acetate Buffer at pH 5.8-5.9
	Wash: - 1 mL of 100 mM Sodium Acetate Buffer at pH 5.8-5.9 - 1 mL of Methanol
	Dry: - 5 minutes at 10 inches of Mercury
	Elute: - 1 mL 7:2:1 Ethyl Acetate / Isopropanol / Ammonium Hydroxide - Evaporate to dry at < 40 °C.
	Derivatization: - Reconstitute sample in 50 µL of Pyridine and 50 µL of Propionic Anhydride, vortex - React mixture at 60 °C for 30 minutes - Allow sample to cool and then dry - Reconstitute in 50 µL of Ethyl Acetate

Objective

Due to the increase in sensitivity provided by newer MS instrumentation, especially LC/MS/MS, the volume of urine needed for extraction in order to meet SAMHSA detection limits can be reduced. We were able to reduce the volume to 1 mL of urine, which was then diluted 1:1 with a buffer solution to a total volume of 2 mL. This volume allowed us to use a new optimized SPE format with 30 mg of polymeric cation exchange sorbent packed into a 3 mL tube. Polymeric sorbents offer several important advantages over silica based sorbents, including improvements in tube to tube reproducibility due to metal content in silica, the reduced susceptibility to de-conditioning if the sorbent bed dries out between processing steps, and the improved flow characteristics.

By using a 30 mg bed mass, we were able to significantly reduce the solvent volume required for conditioning, wash, and elution steps. This reduced solvent volume saves both time and money significantly for subsequent blow down steps prior to derivatization. The recoveries for morphine and codeine were 97 % and 98 % respectively, with %CVs < 4 % for replicate injections over three different concentrations, indicating that the sorbent mass was sufficient to reproducibly perform the extraction.

When developing the method, several different wash and elution options were explored that provided visually different results. The opiate species are cationic and were retained via ion-exchange mechanisms on the Strata™-X-C sorbent. Washing with acidified water solutions helps to remove polar acidic and neutral components. In this case, the use of buffer significantly reduced the presence of a yellowish impurity (**Figure 1**). However, even the use of a buffer solution was not sufficient to completely remove this impurity. Additional experiments suggested that this impurity was insoluble in strong organic solutions. By changing the elution solvent system from basified methanol to a mixture of 7:2:1 ethyl acetate / isopropanol / ammonium hydroxide, we were able to almost completely remove this impurity from the final extracts.

GC/MS remains the golden standard for SAMHSA opiate testing. The large number of routinely prescribed opiate related compounds that cause positive hits in the ELISA screening assay requires a GC/MS methodology that is able to accurately confirm the presence of morphine or codeine. To improve this separation, the use of a newly developed GC stationary phase specifically designed for the analysis of drugs of abuse was evaluated. To further improve resolution, we investigated the difference in chromatographic performance between BSTFA and propionic acid anhydride derivatized samples.

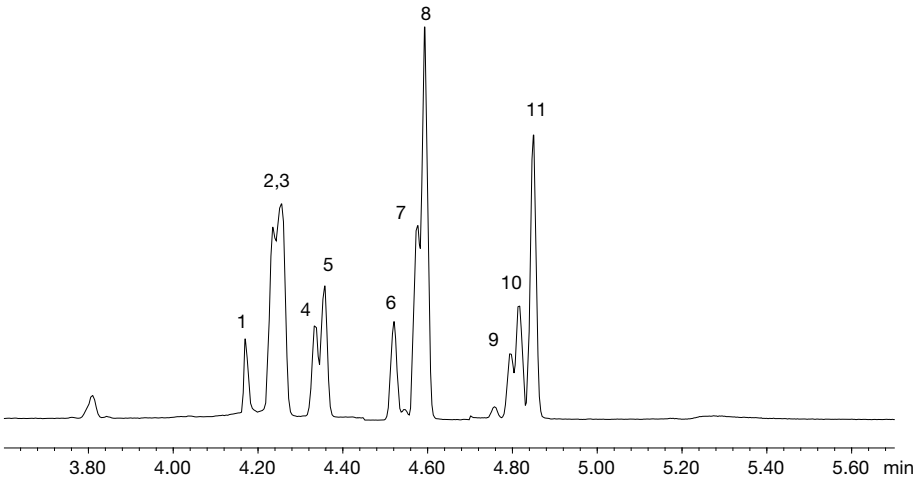
The elution order of morphine and codeine was reversed on the ZB-Drug-1 vs. standard 5 and 5ms phases. BSTFA derivatives were well resolved from all common co-eluting impurities, though problems were observed with norcodeine (**Figure 2**). The use of BSTFA also required the formation of an oxime derivative to prevent multiple reaction products. By forming a propionic acid anhydride derivative, resolution of morphine and codeine from common interferences was greatly improved (**Figure 3**). Furthermore, we found that we were able to eliminate the oximation step, further reducing processing time.

The SPE protocol presented here can also be used to prepare samples for LC/MS/MS. Samples prepared for analysis by LC/MS/MS did not require derivatization (**Figure 4**). LC/MS/MS has been reported to be at least one order of magnitude more sensitive than GC/MS for drugs of abuse. This would suggest that the volume of urine needed for SPE could be further reduced. If volumes of diluted urine could be reduced to about 1.5 mL, it would allow for the use of a 96-well plate SPE format, which can be easily adapted for automation. Automation could be used to streamline workflows and reduce error in sample processing.

Figure 1: Optimization of Wash & Elutions Conditions for SPE

Wash: Buffer Elute: EA/IPA/NH_4OH	Wash: HCl Elute: EA/IPA/NH_4OH	Wash: Buffer Elute: Methanol	Wash: HCl Elute: Methanol

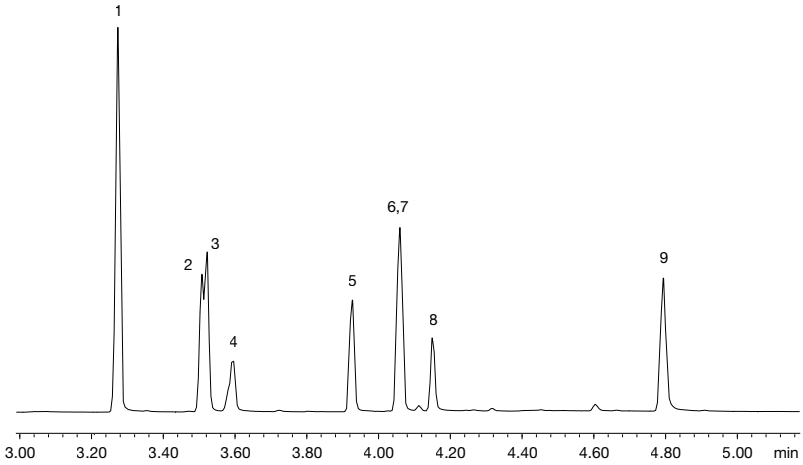
Figure 2: GC/MS Analysis of Samples Derivatized Using BSTFA



Column: Zebron ZB-Drug-1
Dimensions: 10 meters x 0.18 mm x 0.18 µm
Part No.: 7CD-G023-08
Injection: Split 10:1 @ 260 °C, 1 µL
Carrier Gas: Helium @ 0.7 mL/min (constant flow)
Oven Program: 180 °C to 340 °C @ 20 °C/min
Detection: MS @ 320 °C
Sample:

1. Morphine-d3
2. Morphine
3. Norcodeine
4. Codeine-d6
5. Codeine
6. Oxymorphone
7. Hydromorphone-d6
8. Hydromorphone
9. Oxycodone-d6
10. Oxycodone
11. Hydrocodone

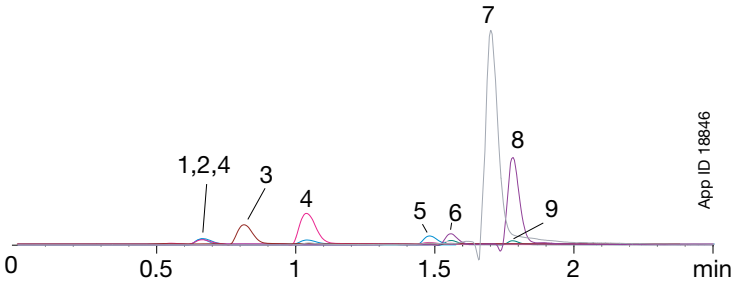
Figure 3: GC/MS Analysis of Samples Derivatized Using Propionic Acid Anhydride



Column: Zebron ZB-Drug-1
Dimensions: 10 meters x 0.18 mm x 0.18 µm
Part No.: 7CD-G023-08
Injection: Split 10:1 @ 260 °C, 1 µL
Carrier Gas: Helium @ 0.7 mL/min (constant flow)
Oven Program: 185 ºC to 320 ºC @ 30 ºC/min for 3 min
Detection: MS @ 320 °C
Sample:

1. Hydrocodone
2. Codeine-d6
3. Codeine
4. Oxycodone
5. Hydromorphone
6. Morphine-d3
7. Morphine
8. Oxymorphone
9. Norcodeine

Figure 4: LC/MS/MS Analysis Without Derivatization



Column: Kinetex 2.6 µm C18 100 Å

Dimensions: 50 x 2.1 mm

Part No.: 00B-4462-AN

Mobile Phase: A: 0.1 % Formic acid in H_2O
B: 0.1 % Formic acid in Acetonitrile

Gradient: 95:5 (A/B) for 0.24 min then linear to 70:30 over 1 min, hold for 1.25 min, then to 5:95 for 0.3 min, then to 95:5 for 1.8 min

Flow Rate: 450 µL/min

Temperature: 25 °C

Detection: Mass Spectrometer (MS)

Sample:

1. Morphine-d3 (IS)
2. Morphine
3. Oxymorphone
4. Hydromorphone
5. Norcodeine
6. Codeine-d6
7. Oxycodone
8. Hydrocodone
9. Codeine

Table 2: Mass Ions for Chromatographic Analysis

	GC/MS – BSTFA	GC/MS – Propyl	LC/MS/MS
Codeine-d6	*377, 349	*361,288,235	MRM 306.4>*152.0
Codeine	*371, 343, 234	*355,282,229	MRM 300.4>*152.0
Hydrocodone	*386, 297, 371	*299,242,214	MRM 300.4>*199.1
Hydromorphone-d6	*361, 450	*291,347	
Hydromorphone	*355, 444, 429	*285,341,228	MRM 286.2>*185.2
Morphine-d3	*432, 417	*344,271,400	MRM 289.2>*152.2
Morphine	*429, 414, 401	*341,268,397	MRM 286.2>*152.2
Norcodiene	*429, 414, 356	*223, 397, 101	MRM 286.1>*152.2
Oxycodone-d6	*480, 465	*377,320	
Oxycodone	*474, 459, 460	*371,314,298	MRM 316.4>*298.5
Oxymorphone	*532, 517, 287	*357,300,413	MRM 302.3>*227.0

Conclusion

A new procedure for the extraction and analysis of opiates from urine was developed that dramatically reduced overall sample processing time and improved chromatographic results by either GC/MS or LC/MS/MS.