An Automated Solid Phase Extraction Method for Thebaine, 6-Acetylmorphine and Other Opiates in Urine

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INTRODUCTION

The confirmation and identification of opiates in biological matrices often may lead to more questions than answers. The presence of morphine in urine may be indicative of pain therapy, heroin abuse or poppy seed ingestion. Several studies have proposed using 6-acetylmorphine as the primary marker for heroin abuse (1, 2). Heroin is metabolized to 6-acetylmorphine within minutes after the drug enters the body; this is either conjugated or further metabolized to morphine (1-4).

Thebaine is a marker for poppy seed ingestion. Poppy seeds contain varying concentrations of morphine, codeine, thebaine and other compounds. Thebaine undergoes considerable degradation with exposure to heat, especially in the acidic conditions used in derivatization procedures with BSTFA (5). Laboratories at UCT have developed a method to simultaneously detect and quantify 6-acetylmorphine, thebaine and 10 other opiates in urine using automated solid phase extraction. This procedure utilizes enzyme hydrolysis, solid phase extraction with a Zymark Rapid Trace system and derivatization followed by GC/MS analysis.

MATERIALS AND METHODS

Reagents

Certified ACS grade reagents were purchased from numerous suppliers such as Fisher Scientific (Pittsburgh, PA) and JT Baker (Phillipsburg, NJ). Propionic anhydride was purchased from Aldrich (Milwaukee, WI). Distilled water was prepared using a Millipore purification system. Pyridine was purchased from Regis Technologies (Morton Grove, IL). ß-glucuronidase from Limpets was purchased from Sigma Chemical (St. Louis, MO).

Standards

Thebaine was purchased from Sigma Chemical (St. Louis, MO). Naloxone was purchased from Aldrich (Milwaukee, WI). All other drug standards were purchased as solutions from Cerilliant Corp (Austin, TX). Both the working standard solution of the multiple drugs and the internal standard solution were prepared to 10 mg/mL.

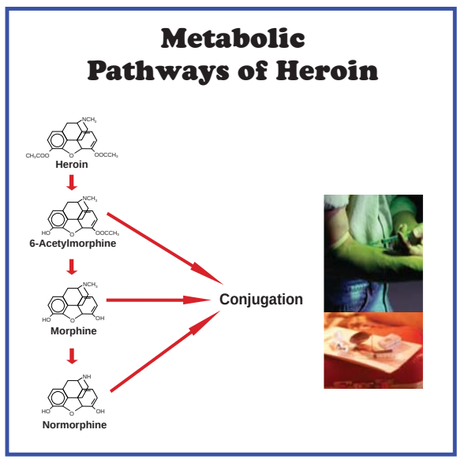
Sorbent

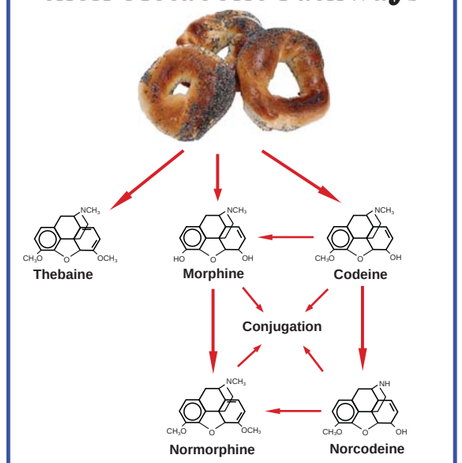
The extraction columns were **CLEAN SCREEN® CSDAU203** containing 200 mg of sorbent in a 3 mL column and were made and prepared by UCT, Inc.

Instrumentation

A Zymark Rapid Trace™ SPE Workstation was used to perform the solid phase extractions. The GC/MS system consisted of a Hewlett Packard 5971A Mass Selective Detector, 7673 Autosampler and a 5890 Gas Chromatograph fitted with a 30 m, 0.25 mm i.d., 0.25 mm film thickness Rtx-5 capillary column.

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Rapid Trace™ is a trademark of Zymark, Corp. (Hopkinton, MA)
Rtx-5® is a trademark of Restek, Corp. (Bellefonte, PA)





SAMPLE PREPARATION

Deuterated internal standard (morphine-d3) solution was added to 13 x 100 mm screw-top culture tubes followed by 4 mL of urine, 3 mL of 100 mM phosphate buffer (pH 6.0) and 12,500 units of ß-glucuronidase from Limpets. The appropriate amount of working standard solution was added to the calibrator sample tube. An appropriate volume of methanol should be added to the tubes to keep a constant volume for all tube to be loaded into the Rapid Trace. The solution was vortexed and heated at 60ºC for three hours. The tubes were then removed from the heat and allowed to cool. The samples were transferred to 13 x 100 mm disposable borosilicate culture tubes and placed in the shuttle of the Zymark Rapid Trace.

Automated Solid Phase Extraction

The reagents for extraction consisted of methanol, distilled water, 100 mM phosphate buffer (pH 6.0), 100 mM acetate buffer (pH 4.5) and a fresh solution of ethyl acetate, isopropanol and ammonium hydroxide (84:12:4) as the elution solvent. Table 1 shows the extraction procedure. Each of the lines should be purged with at least 10 mL of the appropriate solvent prior to the extraction procedure.

TABLE 1

Rapid Trace "Create Procedures" Screen for Opiate Analysis

	Step	Source	Output	Vol	mL/min	Liquid Sense
1.	Condition	MeOH	W Org	2,0	12	No
2.	Condition	H2O	W Aqu	2,0	12	No
3.	Condition	PO4	W Aqu	2,0	12	No
4.	Load	Sample	W Bio	4,0	1,5	No
5.	Load	Sample	W Bio	3,7	1,5	No
6.	Rinse	H2O	W Bio	3,0	8	No
7.	Purge-Cannula	H2O	Cannula	5,0	30	No
8.	Rinse	Ac4,5	W Bio	3,0	8	No
9.	Rinse	MeOH	W Org	3,0	8	No
10.	Dry	——	Time =	3,0	min.	No
11.	Purge-Cannula	Elut	Cannula	2,0	30	No
12.	Collect	Elut	Fract1	4,0	2	Yes
13.	Purge-Cannula	MeOH	Cannula	4,0	30	No
14.	Purge-Cannula	H2O	Cannula	5,0	30	No

Derivatization

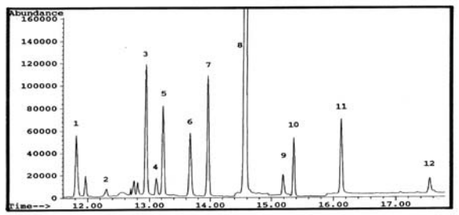
The eluted samples were collected into 12 x 75 mm disposable culture tubes and transferred to 13 x 100 mm screw-top borosilicate culture tubes. The eluate was dried at 40ºC in a TurboVap® LV using a stream of air to aid in evaporation. The tubes were removed and 200 mL of a pyridine:propionic anhydride (1:1) solution was added to each tube. This solution should be prepared fresh daily. The tubes were capped, vortexed and heated for 1 hour at 40ºC. The tubes were removed from the heat and evaporated again at 40ºC. The residue was reconstituted with 50 mL of an ethyl acetate/methanol (70:30) solution, vortexed and transferred to a limited volume insert in an autosampler vial for analysis. TurboVap® is a trademark of Zymark, Corp. (Hopkinton, MA)

GC/MS ANALYSIS

The method used Selected Ion Monitoring (SIM) for three ions of each analyte. The dwell times were set to allow approximately 2.5 cycles per second. The injection port and transfer line were maintained at 250ºC and 280ºC respectively. The initial oven temperature was 100ºC with an isocratic hold for 1 minute following a 2 mL splitless injection. The oven is ramped to 250ºC at 25ºC/minute and a 2.00 minute hold was followed by a ramp to 290ºC at 10ºC/minute and a 0.5 minute hold. The oven was then ramped to 325ºC at 25ºC/minute and held for 3.1 minutes. The total run time was 18.00 minutes. The analytes, ions and relative retention times are listed in Table 2. Figure 1 shows a chromatogram of an extracted 25 ng/mL urine standard.

FIGURE 1

25ng/mL Extracted Standard from Spiked Human Urine



1. Hydrocodone
2. Thebaine
3. Codeine
4. Oxycodone
5. Heroin 10. Naloxone
6. Hydromorphone
7. 6-Acetylmorphine
8 & 9. Morphine and Morphine-d3
11. Nalorphine
12. Norcodeine

Note: Hydrocodone, thebaine and heroin were not derivatized under these conditions. Difficulties were encountered with the peak shape of thebaine. This may be due to our standard being an alkaloid powder, possibly some thermal degradation or the interaction with the injector port. The use of a clean inlet liner and clean column head improved this condition to a degree.

DISCUSSION

The Rapid Trace SPE Workstation performs one extraction in approximately 21 minutes per module using these conditions. It provides reproducible hands-free extractions for the samples and allows the analyst more time to perform other duties. This results in saving a significant amount of money if this assay is performed routinely. Enzyme hydrolysis takes 3 hours, but is gentle enough to leave 6-acetylmorphine and thebaine intact. The use of chemical hydrolysis (acid) will destroy 6-acetylmorphine and thebaine. The extraction procedure gave high recoveries (>85%) for most analytes including thebaine and 6-acetylmorphine. The low recoveries experienced with hydrocodone, naloxone and nalorphine may be due to the age of the standards. Our primary goal for this method was to optimize the recoveries for thebaine, 6-acetylmorphine, codeine and morphine. The determination of synthetic opiates (ie. Oxycodone, hydromorphone, etc) was not optimized in this method. The recovery of oxycodone was very good (96%), however the linearity throughout the range of 12.5 to 1000 ng/mL was not acceptable. This may also be due to the age of the standard. Thebaine did not exhibit linearity to 12.5 ng/mL, however the method was sensitive to detect thebaine at that level. Thebaine does not need to be quantitated; a qualitative determination would be adequate. Derivatization with propionic anhydride provided all analytes with baseline separation. This has often been a problem with many of the opiates (ie. Norcodeine and hydrocodone with codeine using BSTFA). A keto/enol tautomerization occurs in hydrocodone, hydromorphone, oxycodone and oxymorphone. An Oxime is often formed with TMS derivatives to eliminate the enol formation (7). Derivatizing with propionic anhydride at a lower temperature, such as 40ºC, minimizes the enol formation and produced unique ions with high m/z values for each analyte (6).

TABLE 2

Analyte Names, Ions and Observed Retention Times for Propyl Derivatives

RRT*	Name	Base Ion	2nd Ion	3rd Ion
0,81	Hydrocodone**	299,0	242,0	214.0
0,84	Thebaine**	311,2	296,2	312,2
0,89	Codeine-d3	358,1	285,1	232,1
0,89	Codeine	355,0	282,0	229,0
0,90	Oxycodone	371,3	314,2	298,1
0,91	Heroin**	327,2	369,2	268,2
0,94	Hydromorphone	285,1	341,1	227,8
0,96	6-acetylmorphine	327,2	268,0	383,2
0,99	Oxymorphone	357,3	300,2	413,3
1.00	Morphine-d3	344,3	271,3	400.3
1.00	Morphine	341,2	268,1	397,3
1.04	Naloxone	327,1	383,2	328,2
1.05	Nalorphine	367,2	350,2	294,2
1.11	Norcodeine	223,1	224,1	397,3
1.20	Normorphine	210,1	383,2	236,1

* Relative Retention Times based upon Morphine-d3 ISTD.
** Not derivatized under these condition

Recovery

Five 125 ng/mL standards were prepared by adding the appropriate amount of working drug standard to drug free urine. These standards were extracted using the previously described procedure. Five 125 ng/mL unextracted standards were prepared by adding the appropriate amount of working drug standard to a test tube which was dried at 40ºC and derivatized using the procedure described above. The recovery of each analyte was calculated by comparing areas under the curve of the base ion for the extracted and unextracted standards and was performed in pentuplicate (N=5). The average recovery for each drug was over 85% (range=85.3-103.5%) with the exceptions of hydrocodone (66%), naloxone (50%) and nalorphine (53%). Thebaine averaged 97.5 % recovery and 6-acetylmorphine averaged 100.5% recovery.

Linearity

All of the analytes were linear from 12.5 to 1000 ng/mL with the exceptions of thebaine, oxycodone and naloxone. They were limited to 25 ng/mL to 1000 ng/mL.

Assay Variability

Interday and intraday variability was assessed by extracting the six concentration levels in pentuplicate on three separate days. Tables 3, 4 and 5 reflect the averaged daily data and table 6 shows the averaged data for the three days combined.

Stability

Propyl derivatives have been reported to be stable for over 5 days (6).

Sensitivity

This assay has adequate sensitivity and selectivity for all of the tested analytes. Many of them should be able to be quantitated below 12.5 ng/mL, however in this validation only tested to 12.5 ng/mL. Five milliliters of sample could be used if greater sensitivity is desired.

CONCLUSIONS

The combination of enzyme hydrolysis, automated SPE and derivatization at 40ºC with propionic anhydride proves to be reliable and simple, and is gentle enough for the analysis of thebaine and 6-acetylmorphine. There can be significant cost savings using automated SPE and propionic anhydride. The method is also rugged and reproducible on a day to day basis. The method gave the following advantages:

- Baseline separation for all of the analytes investigated.
- Propyl derivatives gave stable and unique fragmentation patterns that improved upon the detectability and positive identification of similarly structured opiates.
- A single sample preparation (SPE), derivatization and analysis by GC/MS can be performed on all major opiate analytes.

TABLE 3

INTERDAY and INTERDAY VARIABILITY
Results reported as % CV, N=5 for 6 concentrations

	Day 1	Day 2	Day 3	AVERAGE
Hydrocodone	75%	6.0%	9.2%	7.7%
Thebaine	15.5%	7.0%	13.%	12.0%
Codeine	3.9%	4.8%	3.9%	4.2%
Oxycodone	9.3%	7.1%	15.3%	10.5%
Heroin	2.7%	4.1%	4.2%	3.7%
Hydromorphone	7.9%	5.0%	7.7%	6.9%
6-acetylmorphine	2.8%	3.9%	3.8%	3.5%
Morphine	3.1%	3.6%	2.7%	3.1%
Naloxone	12.3%	10.6%	12.4%	11.8%
Nalorphine	7.3%	6.6%	6.7%	6.8%
Norcodeine	3.7%	6.2%	7.0%	5.6%
Normorphine	5.0%	5.0%	6.8%	7.3%



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