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Simultaneous analysis of Thebaine, 6-MAM and six abused opiates in postmortem fluids and tissues using Zymark® automated solid-phase extraction and gas chromatography–mass spectrometry

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Abstract

Opiates are some of the most widely prescribed drugs in America and are often abused. Demonstrating the presence or absence of opiate compounds in postmortem fluids and/or tissues derived from fatal civil aviation accidents can have serious legal consequences and may help determine the cause of impairment and/or death. However, the consumption of poppy seed products can result in a positive opiate drug test. We have developed a simple method for the simultaneous determination of eight opiate compounds from one extraction. These compounds are hydrocodone, dihydrocodeine, codeine, oxycodone, hydromorphone, 6-monoacetylmorphine, morphine, and thebaine. The inclusion of thebaine is notable as it is an indicator of poppy seed consumption and may help explain morphine/codeine positives in cases where no opiate use was indicated. This method incorporates a Zymark® RapidTrace™ automated solid-phase extraction system, gas chromatography/mass spectrometry, and trimethyl silane (TMS) and oxime-TMS derivatives. The limits of detection ranged from 0.78 to 12.5 ng/mL. The linear dynamic range for most analytes was 6.25–1600 ng/mL. The extraction efficiencies ranged from 70 to 103%. We applied this method to eight separate aviation fatalities where opiate compounds had previously been detected.
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Keywords: Opiate; Thebaine; Postmortem; Zymark®; Gas chromatography

1. Introduction

Opiates constitute a highly addictive group of commonly prescribed drugs [1,2]. The most commonly prescribed opiate-type compounds are hydrocodone, dihydrocodeine, codeine, hydromorphone, oxycodone, and morphine [3]. These compounds may have severe side effects, including, but not limited to, drowsiness, dizziness, hypotension, unconsciousness, or mental clouding, which can lead to significant impairment [1]. According to the Drug Enforcement Agency, these compounds are some of the most highly abused prescription drugs in America. Therefore, a procedure that allows for the rapid and accurate determination of opiate compounds is a necessity for the field of forensic toxicology.

Thebaine is a naturally occurring opiate that is introduced into the body, along with morphine and codeine, following the consumption of poppy seeds [4–8]. Scientific literature has clearly demonstrated the distinct possibility of a morphine and/or codeine positive due to poppy seed consumption [4–8]. The potential legal consequences of an opiate positive necessitates that laboratories, both drug testing and forensic, differentiate between an opiate positive due to morphine or codeine use and an opiate positive due to poppy seed consumption. In a case where morphine and/or codeine have been detected, a subsequent thebaine positive would indicate poppy seed consumption and could possibly rule out opiate use.

Heroin (diacetylmorphine) abuse is common in the United States. Heroin is quickly metabolized to 6-monoacetylmorphine (6-MAM), which is then metabolized to morphine. 6-MAM has not been detected following the use of morphine or other opiate compounds or the ingestion

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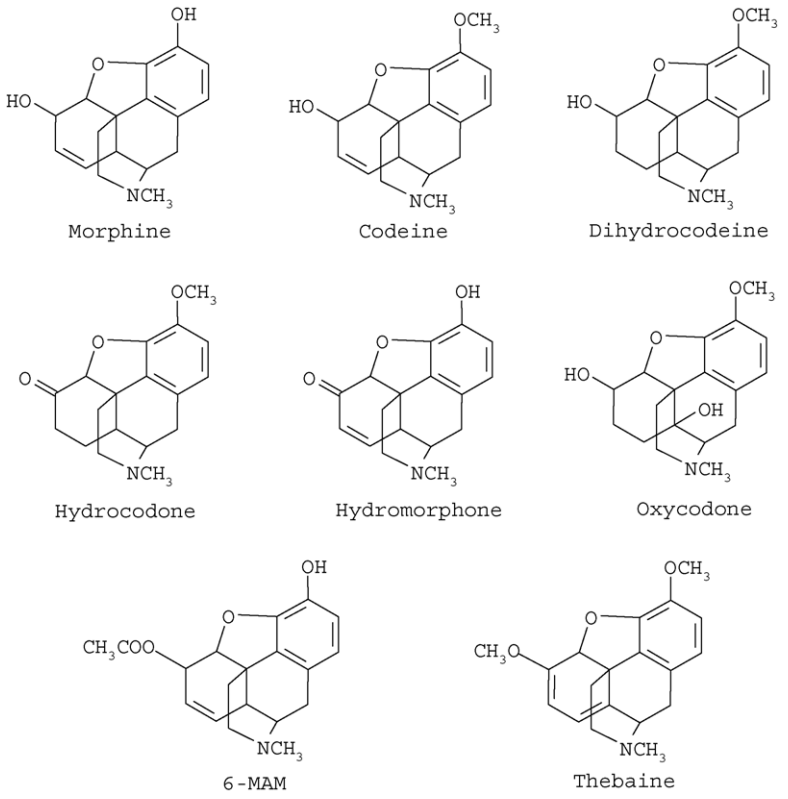

Fig. 1. Chemical structures of eight opiate compounds.

of poppy seeds and is only present following heroin use [5]. Therefore, 6-MAM is considered a heroin-specific catabolite and is legally defensible evidence of heroin use.

Identification and quantitation of opiates in postmortem fluids and tissues are important aspects of forensic toxicology and may provide crucial information in determining the cause of impairment and/or death. This report describes a rapid, automated procedure for the single-step extraction and simultaneous determination of hydrocodone, dihydrocodeine, codeine, oxycodone, hydromorphone, 6-MAM, morphine, and thebaine in postmortem fluids and tissues using a Zymark® RapidTrace™ automated solid-phase extraction (SPE) system and gas chromatography with mass spectrometry (GC/MS). Chemical structure for these eight opiates can be seen in Fig. 1. While published methods exist for the identification and quantitation of various opiate compounds, including thebaine [2,9–15], none offer the sensitivity of this method, combined with the simultaneous extraction and analysis of the eight opiate compounds in postmortem fluids and tissues.

2. Materials and methods

2.1. Chemicals and reagents

All aqueous solutions were prepared using double deionized water (DDW), which was obtained using a Milli-QT_{plus} Ultra-Pure Reagent Water System (Millipore®, Continental Water Systems, El Paso, TX). All chemicals described below were purchased in the highest possible purity and used without any further purification. Hydrocodone, dihydrocodeine, codeine, morphine, hydromorphone, oxycodone, and 6-MAM were purchased from Cerilliant (Cerilliant Corp., Round Rock, TX) as methanolic standards at a concentration of 1.00 mg/mL in sealed glass ampules. Thebaine was purchased from Research Biomedical International (RBI Inc., Natick, MA). Hydrocodone-d_3, dihydrocodeine-d_6, codeine-d_3, morphine-d_6, hydromorphone-d_3, oxycodone-d_3, and 6-monoacetylmorphine-d_6 were purchased from Cerilliant as methanolic standards at a concentration of 0.100 mg/mL in sealed glass ampules. The derivatization reagent, BSTFA with 1% TMCS, was obtained from Pierce (Pierce Inc., Rockford, IL). Sodium acetate, hydroxylamine, potassium phosphate, glacial acetic acid, and β-glucuronidase were purchased from Sigma (Sigma Chemical Co., St. Louis, MO). Methanol, acetonitrile, and ammonium hydroxide were purchased from Fisher (Fisher Scientific, Pittsburgh, PA). Ethyl acetate was purchased from Varian (Varian Inc., Palo Alto, CA).

2.2. Gas chromatographic/mass spectroscopic conditions

All analyses were performed using a bench-top gas chromatograph/mass spectrometer (GC/MS), which consisted of

a Hewlett Packard (HP) 6890 series GC, interfaced with a HP 5973 quadrupole MS (Agilent, Palo Alto, CA). The GC/MS was operated with a transfer line temperature of 280 °C and a source temperature of 250 °C. The MS was tuned on a daily basis using perfluorotributylamine. The electron multiplier voltage was set at 106 eV above the tune voltage. Chromatographic separation was achieved using a HP-ULTRA-1 crosslinked 100% methyl siloxane capillary column (12 m × 0.2 mm i.d., 0.33 μm film thickness). Helium was employed as the carrier gas and used at a flow rate of 1.0 mL/min. A HP 6890 autosampler was used to inject 1 μL of extract into the GC/MS. The GC was equipped with a split/splitless injection port operated at 250 °C in the splitless mode with the purge time of 0.5 min. The oven temperature profile was established as follows: 160–195 °C at 35 °C/min, 195–230 °C at 5 °C/min, 230–290 °C at 40 °C/min and a final hold time of 2.5 min resulting in a total run time of 11 min. Initially, neat standards of each compound (1 μL of a 100 ng/μL solution) were injected individually and analyzed using the full scan mode of the GC/MS, which scanned from 50 to 600 AMU. Quantitation and qualifier ions for each analyte were then selected based on both abundance and mass-to-charge ratio (*m*/*z*). To increase reproducibility and reduce interference, high mass ions were selected when possible. The ions chosen for each respective analyte can be seen in Table 1. Upon selection of unique ions, the MS was run in selected ion monitoring (SIM) mode with a dwell time of 30 ms for each recorded ion.

2.3. Sample selection and storage

A search of the Civil Aerospace Medical Institute (CAMI) database identified eight fatalities from separate civil aviation accidents from the previous 3 years that were reported positive for opiates and also had a majority of the desired biological tissues and fluids (blood, urine, liver, kidney, and muscle) available for analysis. In all cases, blood was stored at −20 °C in tubes containing 1.00% (w/v) sodium fluoride/potassium oxalate until analysis. All other specimens were stored without preservation at −20 °C until analysis. Blood opiate concentrations determined in this study agreed well with those previously determined by our laboratory via another analytical method. All opiate concentrations found were within 10% of the value originally determined, verifying that no deterioration in opiate concentration had occurred.

Table 1
Ions employed and retention times expected for the GC/MS-SIM analysis of eight opiate compounds and their internal standards

Compound	Ions (*m*/*z*)[a]	Retention time
d_6-Dihydrocodeine	**379**, 318, 364	5.99
Dihydrocodeine	**373**, 315, 358	6.01
Thebaine	**383**, 353, 368	6.68
d_3-Codeine	**374**, 375, 346	6.78
Codeine	**371**, 372, 343	6.79
d_6-Morphine	**432**, 417, 404	7.65
Morphine	**429**, 430, 401	7.67
d_3-Hydrocodone	**389**, 300, 374	7.93
Hydrocodone	**386**, 297, 371	7.94
d_6-6-MAM	**405**, 406, 390	8.06
6-MAM	**399**, 400, 340	8.08
d_3-Hydromorphone	**358**, 477, 432	8.31
Hydromorphone	**355**, 444, 429	8.32
d_3-Oxycodone	**477**, 388, 462	8.74
Oxycodone	**474**, 385, 459	8.75

[a] Ions in bold used for quantitation.

2.4. Calibrator and control preparation

Calibration curves for all opiates except thebaine were prepared by serial dilution utilizing bovine whole blood as the diluent. Calibrators were prepared from one set of original stock standard solutions, while controls were prepared in a similar manner as calibrators, using bovine whole blood as the diluent, but from a second set of original stock solutions. Thebaine calibrators and controls were prepared using certified-negative human urine obtained from UTAK Laboratories Inc. (Valencia, CA) as the diluent. Calibration curves were prepared at concentrations ranging from 0.78 to 3200 ng/mL. A minimum of seven calibrators were used to construct each calibration curve. Controls were prepared at concentrations of 100 and 400 ng/mL in pools large enough to provide replicate samples for the entire study. These concentrations were chosen to reflect typical values seen in our laboratory. The internal standard solution, containing d_3-hydrocodone, d_6-dihydrocodeine, d_3-codeine, d_6-morphine, d_3-hydromorphone, d_3-oxycodone, and d_6-6-monoacetylmorphine, was prepared at a concentration of 400 ng/mL in DDW by dilution from the stock standard of each compound.

In human-derived specimens, codeine and morphine are predominately found as glucuronide derivatives. Therefore, we initially hydrolyzed each postmortem urine specimen using the enzyme β-glucuronidase. A solution of β-glucuronidase was prepared by adding 2.5 mL of pH 5.00, 0.10 mM sodium acetate buffer to 250,000 units of the solid enzyme, followed by mixing. This yielded a final concentration of 100,000 units/mL. This solution was stored in a freezer at −20 °C and discarded after storage of a maximum of 30 days. However, it typically was used entirely within 7 days following preparation.

Quantitation was achieved via an internal standard calibration procedure. Response factors for each compound were determined for every sample analyzed. The response factor was calculated by dividing the area of the analyte peak by the area of the internal standard peak. Calibration curves were derived by plotting a linear regression of the analyte/internal standard response factor versus the analyte concentration for each respective calibrator. These calibration curves were then used to determine the concentrations of each opiate compound in the prepared controls and biological specimens.

2.5. Sample preparation and extraction procedure

Postmortem fluid and tissue specimens, calibrators, and controls were extracted in the following manner. Tissue specimens were homogenized using a PRO250 post-mounted homogenizer (Pro Scientific, Oxford, CT). The generator used with this homogenizer was 30 mm in diameter and set to rotate at 22,000 rpm. Tissues were homogenized following a 1:2 dilution with 1.00% NaF in DDW. Three milliliters aliquots of specimen fluids, calibrators and controls, and 3.00 g aliquots of tissue homogenate were transferred to individual 16 mm × 150 mm screw-top tubes. To each specimen, calibrator, and control, 1.00 mL of the internal standard mixture (400 ng) was added. Samples were vortexed briefly and allowed to stand at room temperature for 10 min. One hundred μL of stock β-glucuronidase solution (10,000 units), followed by 2.00 mL of 0.10 M pH 5.00 sodium acetate buffer, was added to each urine sample. The urine samples were then vortexed briefly and incubated at 70 °C for 3 h to facilitate hydrolysis of all glucuronide conjugates. In our initial investigations complete hydrolysis of both codeine and morphine–glucuronide conjugates was achieved after incubation with β-glucuronidase for 2.5 h at 70 °C. However, an incubation time of 3 h was chosen to ensure that specimens with elevated opiate concentrations were also completely hydrolyzed. Following hydrolysis, samples were allowed to cool to room temperature. Nine milliliters of acetonitrile was added to all samples except urine. The samples were then mixed on a rotary extractor that was set to rotate at 15 rpm for 20 min. Following rotation, the samples were centrifuged at 820 × *g* for 5 min. The supernatant was then transferred to clean 16 mm × 100 mm culture tubes. To each sample, including urine, was added 2.00 mL of pH 6.00 phosphate buffer and 0.50 mL of 10% hydroxylamine, which was prepared fresh daily. The samples were covered with parafilm and incubated for 1 h at 60 °C in a water bath.

Samples were extracted using a Zymark® RapidTrace™ automated solid phase extraction (SPE) system (Zymark Corp., Hokinton, MA). The SPE cartridges used were 3 mL Varian Bond Elute-Certify I with a 130 mg sorbent bed (Varian Inc., Palo Alto, CA). The RapidTrace™ was programmed with the following parameters: SPE cartridges were conditioned with 2.0 mL of methanol, followed by 2.0 mL of 0.10 M phosphate buffer pH 6.00, both at a flow rate of 8 mL/min. Following conditioning, 6.0 mL of sample was loaded on to each column at a flow rate of 1.5 mL/min. The SPE columns were then rinsed with 2.0 mL of 1.0 M acetic acid at a flow rate of 8 mL/min, dried for 30 s with nitrogen at a pressure of 30 p.s.i., rinsed with 6.0 mL of methanol at a flow rate of 8 mL/min and dried a final time for 30 s at a pressure of 30 p.s.i. The analytes were then eluted with 3.0 mL of 2% ammonium hydroxide in ethyl acetate, which was prepared fresh daily, into 15 mL round-bottom, screw top tubes. To avoid carryover, the RapidTrace™ column plunger was washed with 3.0 mL of elution solvent, and the RapidTrace™ cannula was washed by sequentially passing 6.0 mL of methanol and 6.0 mL of water to waste after completion of each sample extraction.

Each sample eluent was evaporated to dryness in a water bath at 40 °C under a stream of dry nitrogen. Once dry, 25.0 μL ethyl acetate and 25.0 μL BSTFA with 1% TMCS were added to each. The tubes were then capped tightly, vortexed briefly, and incubated in a heating block set to 90 °C for 20 min. Samples were removed from the heating block and allowed to cool to room temperature. The samples were transferred to GC autosampler vials for GC/MS analysis. All specimens were analyzed at one time to avoid inter-assay variations. Specimens with analyte concentrations above the associated calibration curves were diluted by an appropriate factor and re-extracted. When specimen dilution was necessary a control was also diluted by the same factor to ensure dilution accuracy.

2.6. Extraction efficiency/recovery

The method used for the determination of analyte recovery has previously been reported by Johnson et al. [16]. Briefly described, two groups of controls, X and Y, prepared using certified-negative whole blood (urine used for Thebaine), were extracted in the same manner as discussed above. Group X was spiked with a precisely known concentration of each analyte prior to extraction, while group Y was spiked with the same precisely known concentration of each analyte following extraction. Upon analysis, the average response factor obtained from group X was divided by the average response factor obtained from group Y to yield the percent recovery value (100(X/Y) = % recovery) for each of the compounds.

3. Results and discussion

3.1. Method validation

The procedure described herein, which utilizes a Zymark® RapidTrace™ automated SPE system and GC/MS for the detection of opiates and TMS and oxime-TMS opiate derivatives, provides a rapid, reproducible, and sensitive method for the determination of hydrocodone, dihydrocodeine, codeine, oxycodone, hydromorphone, 6-monoacetylmorphine, morphine, and thebaine. Most analyte peaks were completely resolved, with the exception of thebaine and codeine, which had similar retention times. However, each provided ions with unique *m/z*, so no interference was observed. Deuterated hydrocodone, dihydrocodeine, codeine, oxycodone, hydromorphone, 6-monoacetylmorphine, and morphine were used as internal standards for this study. Thebaine had no deuterated analog available, so codeine-d_3 was employed as the internal standard for this analyte. No analyte suffered interference from endogenous/exogenous matrix components. A representative chromatogram demonstrating the separation of the eight analytes is shown in Fig. 2.

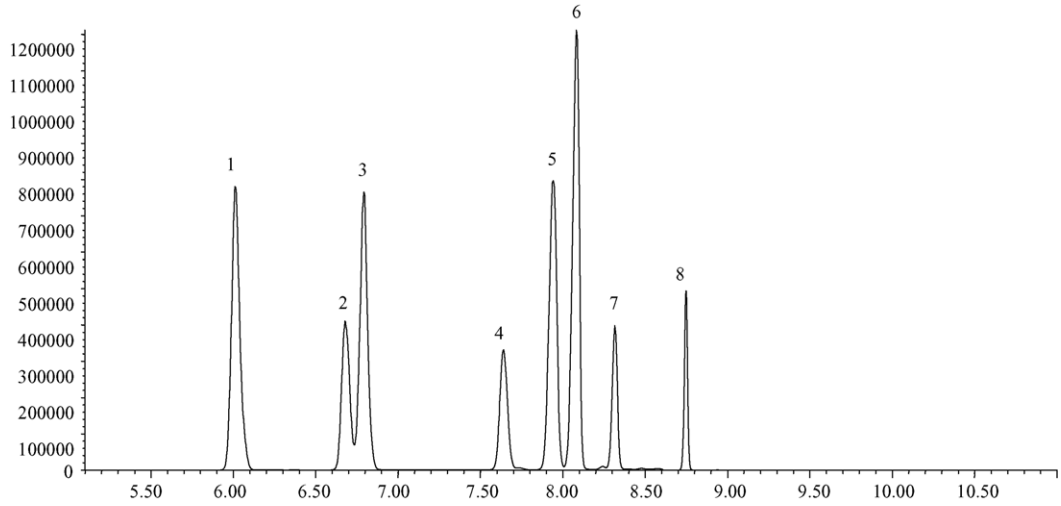

Fig. 2. Representative chromatogram of eight opiate compounds in an extracted whole blood control. Peaks were obtained from a 1 μL injection of a 200 ng/mL whole blood, extracted control. Chromatographic peaks represent ions monitored in SIM mode for each compound as follows: 1, dihydrocodeine; 2, Thebaine; 3, codeine; 4, morphine; 5, hydrocodone; 6, 6-MAM; 7, hydromorphone; and 8, oxycodone.

Numerous derivatizing agents can be employed for the GC/MS analysis of opiates. Propionic anhydride, one such derivatizing reagent, can be used to form propyl-opiate derivatives; however, propionic anhydride is often contaminated with trace amounts of acetaldehyde [17]. Any acetaldehyde present can react with morphine to form 6-MAM, thus falsely suggesting heroin use. Therefore, a trimethyl silane (TMS) derivative is most commonly used for the quantitation of opiate compounds [12,18–21]. However, when forming TMS derivatives of ketone-containing opiates, such as hydrocodone, hydromorphone, and oxycodone, numerous derivatives for each compound can be formed due to keto-enol tautamerization [1,22]. Tautamerization can be avoided by an initial pre-derivatization reaction with hydroxylamine. Hydroxylamine reacts with the ketone moiety of these opiates to form an oxime, which then forms a TMS derivative [22,23].

After evaluating numerous derivatizing agents, we chose to employ TMS and oxime-TMS derivatives due to the efficiency of the derivatizing reaction. We initially investigated the use of a 1% hydroxylamine solution for oxime formation, but this concentration did not produce consistent results across a broad opiate concentration range. It appeared that the 1% solution was not concentrated enough to ensure complete reaction between hydroxylamine and all available ketone moieties. Following further investigation, we found that a 10% hydroxylamine solution produced complete oxime formation for each of the ketone-containing compounds. TMS derivatization following oxime formation makes it possible to simultaneously quantitate both keto and non-keto opiate compounds in one extraction with reproducible results.

Acceptability criteria employed for analyte identification and quantitation were as follows: (1) ion ratios for a given analyte, measured as the peak area of a qualifier ion divided by the peak area of the quantitation ion, were required to be within ±20% of the average of the ion ratios for each respective calibrator used to construct the calibration curve for that analyte; (2) each ion monitored was required to have a minimum signal-to-noise ratio (S/N) of 5; and (3) the analyte was required to have a retention time within ±0.20 min of the average retention time for each respective calibrator used to construct the calibration curve for that analyte. Analytes not meeting these criteria were reported as either negative or inconclusive.

The linear dynamic range (LDR), limit of detection (LOD), limit of quantitation (LOQ) and extraction efficiency (recovery) for each opiate compound, except thebaine, were determined using whole blood as the matrix. Analytical parameters for thebaine were determined in a urine matrix, since we would expect to see thebaine in urine specimens only. The LDR for each analyte is presented in Table 2. In general, LDRs were 6.25–1600 ng/mL. Correlation coefficients for calibration curves used to ascertain that LDRs were all greater than 0.995 when a weighting factor of 1/X was employed. Additionally, Table 2 shows the LOD and LOQ determined for each analyte. The LOD was defined as the lowest analyte concentration detectable that met the above-discussed identification criteria. The LOQ was defined as the lowest analyte concentration detectable that not only met all identification criteria discussed above but also had an experimentally determined concentration within ±20% of its prepared value. The LOD for these opiate related compounds ranged from 0.78 to 12.5 ng/mL. The LOQ for these opiate-related compounds ranged from 0.78 to 25 ng/mL. The LOD and LOQ values determined in this experiment were superior to what had been previously reported [22].

Recovery of these opiate compounds at both 100 and 400 ng/mL ranged from 70 to 103% (Table 2). Recovery values above 70% are exceptional when considering the sim-

Table 2
LOD, LOQ, LDR and recovery data for eight opiate compounds[a]

Compound	LOD[b] (ng/mL)	LOQ (ng/mL)	LDR (ng/mL)	r^2	Recovery (%) ± S.D.[c]	
					100 ng/mL	400 ng/mL
Morphine	3.12	12.5	12.5–1600	0.998	85 ± 1	76 ± 3
Codeine	1.56	6.25	6.25–1600	0.998	103 ± 9	97 ± 7
Thebaine	6.25	12.5	12.5–1600	0.999	80 ± 6	70 ± 9
Hydrocodone	3.13	6.25	6.25–1600	0.997	94 ± 5	90 ± 1
Dihydrocodeine	1.56	6.25	6.25–3200	0.999	101 ± 5	94 ± 1
Hydromorphone	0.78	1.56	1.56–1600	0.996	97 ± 4	89 ± 5
Oxycodone	6.25	12.5	12.5–800	0.996	92 ± 13	94 ± 9
6-MAM	0.78	1.56	1.56–3200	0.997	97 ± 9	100 ± 2

[a] A blood matrix was used for all opiates in the determination of each analytical parameter above except for Thebaine, for which, a urine matrix was employed.
[b] Concentrations below 0.78 ng/mL were not examined.
[c] $n = 5$ for each recovery group.

plicity of the extraction and the use of whole blood (urine for Thebaine) as the matrix for these experiments.

Carryover from one sample to the next did not occur on either the Zymark® or the GC/MS. Carryover on the Zymark® was investigated by extracting an opiate-negative control following the 3200 ng/mL calibrator. Carryover on the GC/MS was initially investigated and subsequently monitored by the use of ethyl acetate solvent injections. The injection of an ethyl acetate blank following the 3200 ng/mL calibrator showed no carryover contamination. Subsequently, ethyl acetate blanks were utilized between each postmortem specimen throughout the sample sequence to verify that no sample-to-sample contamination occurred.

Intra-day (within day) and inter-day (between days) accuracy and precision were examined for this extraction procedure. Accuracy was measured as the relative error between the experimentally determined and prepared concentrations of a whole blood control. Precision was measured as the relative standard deviation (R.S.D.) of the experimentally determined concentrations of a group of whole blood controls. Pools of controls were created at 100 and 400 ng/mL in volumes large enough to be used for the entire accuracy and precision investigation. 6-MAM was analyzed at concentrations of 10 and 40 ng/mL, as opposed to 100 and 400 ng/mL, due to the expected low concentrations of this analyte in vivo [24]. These controls were stored at 4 °C for the duration of this study. For the intra-day accuracy and precision experiment, a calibration curve was extracted along with five replicates of each control concentration. As shown in Table 3, all analytes at both concentrations yielded relative errors within 15% of the target concentration. Furthermore, all analytes had R.S.D.s within 5%. These results demonstrate the exceptional accuracy and precision of this method.

Table 3
Intra-day and inter-day accuracy and precision data[a]

	Target (ng/mL)	Day 1			Day 4			Day 7		
		Mean (ng/mL)	R.S.D.	%*E*	Mean (ng/mL)	R.S.D.	%*E*	Mean (ng/mL)	R.S.D.	%*E*
Morphine	100	99 ± 2	2	−1	94 ± 3	3	−6	98 ± 1	1	−2
	400	424 ± 1	1	+6	410 ± 14	3	+3	432 ± 3	1	+8
Codeine	100	102 ± 7	7	+2	97 ± 2	2	−3	98 ± 1	1	−2
	400	370 ± 2	2	−8	379 ± 5	1	−5	383 ± 2	0.4	−4
Thebaine	100	87 ± 3	3	−13	90 ± 1	1	−10	92 ± 2	2	−8
	400	345 ± 7	2	−14	354 ± 4	1	−12	355 ± 7	2	−11
Hydrocodone	100	99 ± 4	4	−1	97 ± 4	4	−3	96 ± 7	8	−4
	400	425 ± 12	3.	+6	405 ± 11	3	+1	421 ± 11	3	+5
Dihydrocodeine	100	100 ± 5	5	0	92 ± 1	1	−8	96 ± 1	1	−4
	400	427 ± 2	1	+7	415 ± 6	1	+4	430 ± 3	1	+8
Hydromorphone	100	90 ± 1	1	−10	89 ± 1	1	−11	93 ± 3	1	−7
	400	369 ± 4	0.4	−8	362 ± 5	1	−10	360 ± 2	3	−10
Oxycodone	100	89 ± 3	3	−11	90 ± 4	4	−10	92 ± 5	5	−8
	400	358 ± 1	0.3	−11	360 ± 15	4	−10	357 ± 2	0.1	−11
6-MAM	10	9.9 ± 0.6	6	−1	9.3 ± 0.8	9	−7	9.2 ± 0.8	9	−8
	40	38 ± 1	3	−5	36 ± 1	3	−10	37 ± 2	5	−8

[a] $n = 5$ for all measurements. Accuracy was measured as the relative error (%*E*) from the target concentration. Precision was measured as the relative standard deviation (R.S.D.) obtained from five replicate measurements. Blood was used as the matrix for all opiates except Thebaine, for which, urine was employed.

Inter-day accuracy and precision were evaluated by extracting five replicates of each of two control concentrations on days 4 and 7. The quantitative values determined on each of these days were derived from the calibration curves originally prepared on day 1. The results obtained after storage of each control lot at 4 °C for 4 and 7 days can be seen in Table 3. For a majority of these opiate analytes, the concentrations determined on days 4 and 7 showed no significant difference from those obtained on day 1. This agrees well with other published findings [22]. The RSD for each of the opiate compounds was less than 10% on days 4 and 7. Even though no apparent decrease in concentration was observed over the 7-day storage period at 4 °C, as a good laboratory practice and in an effort to maintain a high degree of accuracy, we would recommend (1) preparing new calibration curves at the beginning of each new opiate analysis and (2) prompt toxicological analysis once a postmortem specimen has been thawed.

3.2. Method application: postmortem specimen analysis

In the event of a fatal civil aviation accident, specimens from accident victim(s) are routinely sent to the FAA's Foren-

Table 4
Opiate concentrations (ng/mL or ng/g) found in victims of fatal civil aviation accidents

	Morphine	Codeine	Thebaine[a]	Hydrocodone	Dihydrocodeine	Hydromorphone	Oxycodone
Case #1							
Blood	–	–	–	–	–	–	–
Urine	122	47	22	–	–	–	–
Liver	–	–	–	–	–	–	–
Case #2							
Blood	260	–	–	–	–	–	–
Liver	870	–	–	–	–	–	–
Kidney	1786	–	–	–	–	–	–
Muscle	120	–	–	–	–	–	–
Case #3							
Blood	23	–	–	–	–	–	–
Urine	526	–	–	–	–	–	–
Liver	83	–	–	–	–	–	–
Kidney	121	–	–	–	–	–	–
Muscle	21	–	–	–	–	–	–
Case #4							
Blood	–	13	–	22	270	–	–
Urine	–	154	–	1447	2023	–	–
Liver	–	78	–	142	18,000	–	–
Kidney	–	68	–	122	15,837	–	–
Muscle	–	19	–	42	3264	–	–
Case #5							
Blood	–	–	–	18	13	D	–
Urine	–	–	–	262	78	178	–
Liver	–	–	–	20	D	D	–
Kidney	–	–	–	36	D	D	–
Muscle	–	–	–	D	D	D	–
Case #6							
Blood	–	–	–	102	24	–	–
Liver	–	–	–	447	106	–	–
Kidney	–	–	–	210	39	–	–
Muscle	–	–	–	99	13	–	–
Case #7							
Blood	–	–	–	36	14	–	–
Urine	–	–	–	35	376	–	–
Liver	–	–	–	82	73	–	–
Kidney	–	–	–	125	23	–	–
Muscle	–	–	–	39	7	–	–
Case #8							
Blood	–	–	–	–	–	–	232
Urine	–	–	–	–	–	–	2534
Liver	–	–	–	–	–	–	755
Kidney	–	–	–	–	–	–	298

D, detected but below LOQ; (–), not detected.
[a] Urine was employed as the matrix in the preparation of the Thebaine calibration curve.

sic Toxicology Research Laboratory for toxicological analysis. Postmortem fluid and tissue samples obtained from eight separate civil aviation fatalities that occurred over the past 3 years and had previously been screened positive for opiates by GC/MS were re-examined using this novel method. The fluid and tissue samples selected for analysis were blood, urine, liver, kidney, and skeletal muscle. The eight aviation fatalities chosen for this investigation had a majority, if not all, of the desired specimens available for analysis. The results obtained from this analysis can be seen in Table 4.

The psychoactive concentrations of these opiate compounds, as well as their pharmacokinetics are pharmacodynamics, are beyond the scope of this paper. These topics are, however, covered extensively elsewhere [25]. As previously stated, deuterated analogs of hydrocodone, dihydrocodeine, codeine, morphine, hydromorphone, oxycodone, and 6-MAM were used as internal standards in this study. A deuterated analog of thebaine was not available. Therefore, to obtain reliable quantitative results, urine was used as the calibration matrix for thebaine, since the detection of thebaine was expected in urine specimens only. The interpretation of quantitative data resulting from the analysis of specimen types other than that used to create the calibration curve should be closely scrutinized due to variations in extraction efficiencies when deuterated analogs are not available.

Morphine was found in three of the eight cases examined and was the only opiate detected in two of these cases. Morphine concentrations in the various fluids and tissues ranged from 0 to 260 ng/mL, 122 to 526 ng/g, 0 to 870 ng/g, 121 to 1786 ng/g, and 21 to 120 ng/g in blood, urine, liver, kidney, and muscle, respectively. Specimens with analyte concentrations above the associated calibration curves were diluted by an appropriate factor and re-extracted, so that the results fell within the LDR for each analyte. The general trend observed for the highest to lowest concentration of morphine between specimen types analyzed was urine > kidney > liver > blood > muscle.

Codeine was identified in two of the eight cases investigated and had a concentration of 13 ng/mL, 78 ng/g, 68 ng/g, and 19 ng/g in blood, liver, kidney, and muscle, respectively; urine levels ranged from 47 to 154 ng/mL. One of the codeine-positive cases tested positive for morphine and Thebaine, while the other case tested positive for hydrocodone and dihydrocodeine.

Thebaine was identified in one of the cases examined at a concentration of 22 ng/mL in urine. For comparative purposes, morphine and codeine were found in the urine of this case at 122 and 47 ng/mL, respectively. We did not identify any of these analytes in the corresponding blood or liver specimens. No additional specimens were analyzed in this case. An in-depth review of the case history revealed no medically legitimate opiate use. Furthermore, finding a urinary morphine concentration greater than that of codeine and both opiates at low concentration, along with the presence of thebaine, suggests that the morphine and codeine present in this case is a result of poppy seed consumption. It must be stressed, however, that it is possible following poppy seed ingestion to find morphine and codeine in urine without detecting thebaine. Therefore, the absence of thebaine cannot preclude poppy seed consumption as the source of morphine and codeine present in a case.

Hydrocodone and dihydrocodeine were found in four of the eight cases examined. Blood concentrations for hydrocodone and dihydrocodeine ranged from 18 to 102 ng/mL and 13 to 270 ng/mL, respectively. Urine concentrations for hydrocodone and dihydrocodeine ranged from 35 to 1447 ng/mL and 78 to 2023 ng/mL, respectively. Liver concentrations for hydrocodone and dihydrocodeine ranged from 20 to 447 ng/g and <1.5 to 18,000 ng/g, respectively. Kidney concentrations for hydrocodone and dihydrocodeine ranged from 36 to 210 ng/g and <1.5 to 15,837 ng/g, respectively. Muscle concentrations for hydrocodone and dihydrocodeine ranged from <3 to 99 ng/g and <1.5 to 3264 ng/g, respectively. As can be seen from these data, there was no apparent correlation between hydrocodone and dihydrocodeine concentrations within any of the specimen types analyzed. Additionally, no apparent trend was observe d concerning which specimen type contained the highest concentrations of hydrocodone and dihydrocodeine.

Hydromorphone was found in one of the hydrocodone/dihydrocodeine positive cases. Hydromorphone was below the LOQ in all specimens except urine, which was found to be 178 ng/mL. Oxycodone was found in one of the eight cases examined. Oxycodone concentrations in the fluids and tissues investigated were 232 ng/mL, 2534 ng/mL, 755 ng/g, and 298 ng/g in blood, urine, liver, and kidney, respectively. The trend observed for the highest to lowest concentration of oxycodone between specimen types analyzed was urine > liver > kidney > blood.

Each case was analyzed for the presence of 6-MAM; however, none was detected through the course of these experiments. Thus, the lack of 6-MAM was not surprising when considering the incapacitating consequences of heroin use and the resulting inability to operate an aircraft. To further validate this new procedure with regards to 6-MAM, we analyzed past College of American Pathologist Forensic Urine Drug Testing samples known to contain 6-MAM. Our findings were all within 10% of those previously reported.

4. Conclusion

An automated method that is rapid, robust, and sensitive has been developed for the identification and subsequent quantitation of eight opiate compounds in postmortem fluids and tissues. This method offers the ability to simultaneously analyze numerous commonly encountered opiates and thebaine in one simple extraction. Application of this procedure demonstrates the effectiveness of GC/MS for the separation and subsequent detection of opiate and TMS/oxime-TMS derivatized opiate compounds. The relative simplicity of this

procedure and its proven applicability to both postmortem fluids and tissues should make the quantitation of these opiate compounds more readily attainable for the field of forensic toxicology.

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