Comparison of Four Derivatizing Reagents for 6-Acetylmorphine GC–MS Analysis*

Mark M. Kushnir[1,2], David K. Crockett[1,2], Gordon Nelson[1], and Francis M. Urry[1,2,3]

[1]ARUP Laboratories, Inc., Salt Lake City, Utah 84108; [2]ARUP Institute for Clinical and Experimental Pathology, Salt Lake City, Utah 84108; and [3]Department of Pathology, University of Utah School of Medicine, Salt Lake City, Utah 84112

Abstract

The propionyl, trimethylsilyl, trifluroacetyl, and heptafluoroacyl derivatives of 6-acetylmorphine (6-AM) were evaluated with respect to optimal method performance, derivative stability, and methods characterization for use in gas chromatographic–mass spectrometric (GC–MS) analysis with electron ionization mode and selected ion monitoring. The most common potential interferences and compatibility with other derivatives when used on the same GC–MS were determined for the derivatizing reagents. The propionyl, trimethylsilyl, and trifluroacetyl derivatives produced adequate stability, accuracy, and precision for the method. The 6-AM derivatization with commercially available propionic anhydride generated a relatively small amount of 6-AM-propionyl derivative from the free morphine present in a specimen. The trimethylsilyl derivative obtained by the reaction with MSTFA did not require incubation, was the easiest to prepare, and had the highest potential for use on an automated sample-preparation device. An important advantage of derivatization with MSTFA is elimination of the possibility of heroin decomposition to 6-AM that is due to incubation at elevated temperature.

Introduction

Heroin (3,6-*O*-diacetylmorphine) is a potent narcotic analgesic with a long history of abuse that has resulted in a considerable number of drug-related illnesses, injuries, and deaths. It is metabolized by deacetylation to 6-acetylmorphine (6-AM), which is further metabolized to morphine. Heroin and 6-AM are rapidly eliminated from blood with half-lives of 9 and 38 min, respectively (1,2). Morphine undergoes biotransformation by conjugation to morphine-3-glucuronide and by *N*-demethylation to normorphine. Free and conjugated morphine and 6-AM are the primary heroin metabolites. Heroin is difficult to detect in urine because of rapid and extensive biotransformation and low residual concentration. The identification of heroin use for workplace drug testing starts with immunoassay for opiates followed by confirmatory testing for morphine by gas chromatography–mass spectrometry (GC–MS) operated in the selected ion monitoring (SIM) mode. Correct interpretation of the source of morphine is difficult because morphine in urine can result from heroin, morphine, codeine, or poppy seed intake and may be further complicated when two or more substances containing opiates are consumed. Clinically and forensically relevant concentrations of heroin are usually below the limit of detection of most routine analytical techniques. To provide accurate results for the detection of heroin, a procedure should include such techniques as rapid freezing of the specimen, addition of esterase inhibitors and sodium fluoride, and avoidance of extreme pH conditions during the analysis (3). Because 6-AM can only derive from heroin, it is a specific marker for heroin use. The 6-AM metabolite is generally detectable in urine for less than 24 h after heroin use (4) and exhibits greater stability than heroin, thus requiring fewer precautions during specimen collection and analysis. The new regulations on federal drug testing changed the cutoff concentration of morphine and codeine from 300 ng/mL to 2000 ng/mL with mandatory confirmation of 6-AM for each specimen containing morphine at or greater than 2000 ng/mL (5).

Some methods were developed for simultaneous testing of 6-AM, codeine, and morphine (4,6–9). All of these methods intended to analyze for free morphine concentration, which is not practical for employment drug-testing purposes because total morphine concentration is required. The great disparity between the expected concentrations of 6-AM (approximately 4–100 ng/mL) and total morphine (approximately 800 to above 100,000 ng/mL) requires different instrumental conditions to optimize each compound for accurate qualitative and quantitative analysis. We chose not to include other opiates in the assay because of multiple potential interferences (10,11) and use of acid hydrolysis to convert morphine glucuronide to free morphine. Sample preparation, instrument conditions, and ionization technique are important for reliable performance of a GC–MS procedure for 6-AM. The choice of the derivatizing reagent and derivatization conditions are key factors for the accuracy and precision of a quantitative chromatographic method. Derivatization of the 3-hydroxy group of 6-AM is nec-

*Presented in part at the 49th annual meeting of the American Association for Clinical Chemistry, Atlanta, GA, 1997.

essary to reduce the polarity of the molecule and improve its chromatographic performance. Several derivatization procedures for 6-AM, including propionylation (7,12), silylation (7,13,14), trifluoroacetylation (3,15,16), acetylation with deuterated acetic anhydride (7), and pentafluoropropionylation (9,15), have been described in the literature. Some perfluroacyl derivatives were found to be moisture sensitive and not stable after preparation (9,17), whereas the trifluoroacetyl derivative was suitable for analysis (3,8). Use of the 6-AM propionyl derivative was reported to produce distinctive mass spectra and good chromatographic separation from other related compounds (6,12). Recently, Guillot et al. (6) reported the use of 4-dimethylaminopyridine to perform the propionylation of 6-AM at room temperature to prevent heroin and 6-AM degradation at elevated temperature. No systematic attempt has been published comparing different derivatizing reagents for 6-AM analysis by GC–MS for employment drug-testing application.

The objective of this study was to evaluate four different derivatives of 6-AM with respect to method optimization and characterization, derivative stability, potential interferences, and compatibility with other derivatives when analyzed on the same GC–MS system.

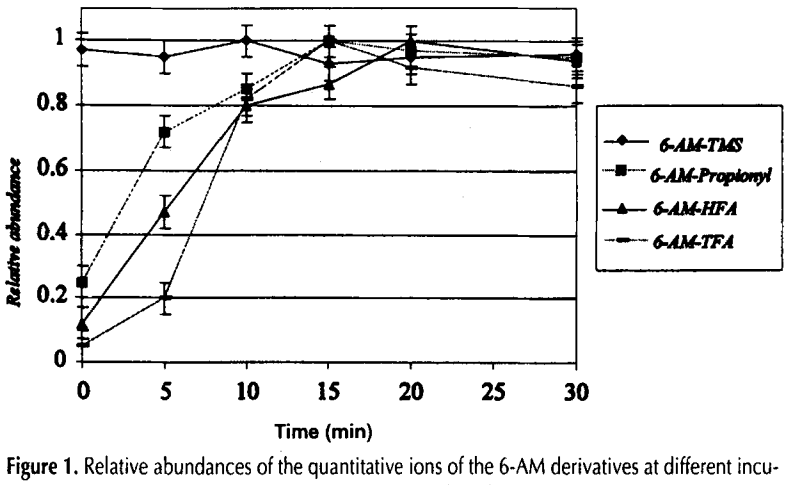

Figure 1. Relative abundances of the quantitative ions of the 6-AM derivatives at different incubation times. Incubation temperatures: TMS, propionyl, and HFA, 90°C and TFA, 120°C.

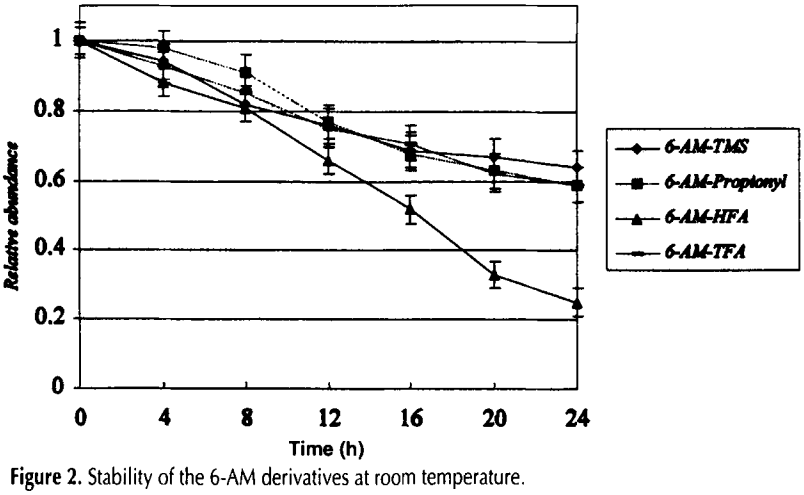

Figure 2. Stability of the 6-AM derivatives at room temperature.

Materials and Methods

Instrumentation

Analysis was performed in the electron ionization (EI) mode using a Hewlett-Packard (HP) 5890 GC with a 5970 mass selective detector (MSD) equipped with a 7673A autosampler and DrugQuant™ software (Palo Alto, CA). The method was also evaluated on a Finnigan Voyager GC–MS equipped with an A200S autosampler, and ToxLab software (ThermoQuest, San Jose, CA). The GCs employed DB-5ms capillary columns (15-m length, 0.25-mm diameter, 0.25-µm film thickness, J&W Scientific, Folsom, CA). Helium was used as the carrier gas, and injection volume was 1 mL.

The Hewlett-Packard GC operating conditions were as follows: splitless injection mode with a purge time of 42 s. Temperature program: 190°C for 1 min, ramped to 285°C at 20°C/min, held 1.2 min, ramped to 310°C at 45°C/min, and held 0.4 min. Injection port temperature was 250°C, and transfer line temperature was 280°C. Carrier gas pressure program: 30 psi held for 0.5 min, ramped to 3.5 psi at 99 psi/min and held 5.5 min, ramped to 30 psi at 50 psi/min, and held 0.7 min.

The Finnigan GC operating conditions were as follows: splitless injection mode with a purge time of 8 s. Temperature program: 160°C for 0.2 min, ramped 70°C/min to 240°C, held 0.5 min, ramped 70°C/min to 300°C, and held 0.5 min. Injection port temperature was 250°C, and transfer line temperature was 300°C. Carrier gas constant flow was 1.5 mL/min.

The GC–MS daily maintenance included replacement of the injection port liner and septum. The instrument was tuned every 24 h with perflurotributylamine. The HP 5970 was operated with the electron multiplier voltage between 200 and 400 mV relative to the tune value. The Finnigan was operated at a detector voltage of 300 mV. All results were obtained on an HP 5970/5890 GC–MS unless otherwise specified.

Reagents, standards, and controls

Heroin, 6-AM and 6-AM-d_6 were purchased from Radian International LLC (Austin, TX). The working 6-AM calibration standard and 6-AM-d_6 internal standard were prepared in acetonitrile at a concentration of 1 ng/mL and stored at 1–6°C. The heroin hydrolysis control was prepared in blank urine pool immediately before the experiment at 400 ng/mL from a freshly opened ampule of heroin standard. Samples containing 6-AM for evaluating method precision and linearity were prepared at 4, 10, 300, and 400 ng/mL in a drug-free urine and stored at 1–6°C. Heptafluorobutyric acid anhydride (HFAA) and *N*-methyl-*bis*-trifluoroacetamide (MBTFA) were purchased from Pierce (Rockford, IL), and *N*-methyl-*N*-(trimethylsilyl)

trifluoroacetamide (MSTFA) was purchased from Campbell Supply (Rockton, IL). Anhydrous pyridine and propionic anhydride were obtained from Aldrich (Milwaukee, WI). All reagents and solvents were of analytical grade.

Sample preparation and calibration

Separation of 6-AM from the matrix was performed on solid-phase extraction (SPE) columns ZSDAU020 (United Chemical Technologies, Inc., Bristol, PA). The calibration standards were prepared at 4, 10, and 40 ng/mL in 5 mL of drug-free urine. To each calibrator, control, and test sample, 100 µL of the working internal standard 6-AM-d_6 was added to a final concentration of 20 ng/mL. A 2-mL aliquot and the same concentrations of the calibration standard and internal standard were used in the assay on the Finnigan GC–MS. The pH was adjusted to 6.0–7.5 with 0.1M K_2HPO_4. The contents of the tubes were transferred onto SPE columns that were previously conditioned with 5 mL methanol, 5 mL distilled water, and 5 mL 0.1M K_2HPO_4 (pH 7.0). The columns were washed by sequential addition of 10 mL deionized water, 5 mL of 0.1M sodium acetate buffer (pH 4.5), 5 mL methanol, and 5 mL methylene chloride/isopropanol (4:1). The columns were dried under a vacuum for 5 min. The analytes were eluted with 3 mL of freshly prepared elution solvent consisting of methylene chloride/isopropanol/ammonium hydroxide (78:20:2). The eluate was immediately dried under a stream of desiccated air at 50–55°C.

Derivatization

The derivatization experiments were carried out in a dry heating block and bead bath (Fisher Scientific, Pittsburgh, PA). The 6-AM-propionyl derivative was prepared by reconstituting the residue with 50 µL pyridine and 50 µL propionic anhydride and incubating 15 min at 90°C. Excess derivatizing reagent was evaporated under a desiccated air stream at 50°C, and the residue was reconstituted with 25 µL acetonitrile. The trifluroacetyl derivative (6-AM-TFA) was prepared by reconstituting the residue with 40 µL MBTFA and incubating at 120°C for 15 min. The trimethylsilyl derivative (6-AM-TMS) was prepared by reconstituting the residue with 25 µL MSTFA and 75 µL ethyl acetate. No incubation was necessary. The heptafluoroacyl derivative (6-AM-HFA) was prepared by the addition of 25 mL HFAA and 100 mL ethyl acetate. The tubes were incubated at 90°C for 15 min, the excess derivatizing reagent was evaporated at room temperature, and the residue was reconstituted with 40 mL of acetonitrile.

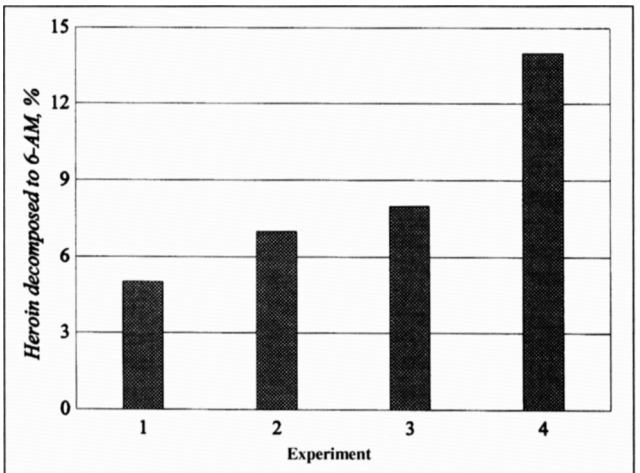

Figure 3. Heroin decomposition to 6-AM during the procedure with MBTFA derivatization. 1, unextracted standard derivatized at 90°C for 15 min; 2, unextracted standard derivatized at 120°C for 15 min; 3, extracted standard derivatized at 120°C for 15 min; 4, the same procedure as 3 but with 60-min delay in evaporation of the elution solvent before derivatization.

Derivatization study

A volume of 2 mL of the calibration standard was transferred into tubes and the acetonitrile was evaporated under a desiccated air stream. The designated amount of derivatizing reagent and solvent (as described in the Derivatization section) were added to the tubes, and the tubes were vortex mixed and placed into the heating block. To stop the reaction, the tubes were immersed in an isopropanol–dry ice bath for 30 s. After derivatization, 20 µL of a derivatized internal standard (IS) was added quantitatively to each tube, and the tubes were vortex mixed for 5 s. Propionyl and heptafluoroacyl derivatives were evaporated at room temperature. The residues were constituted with 50 µL of acetonitrile, transferred into autosampler vials, and injected into the GC–MS. The ratio of the 6-AM peak area to the IS peak area was determined. Each experiment was performed in duplicate.

The IS for the derivatization study was prepared by aliquoting 500 µL of 6-AM-d_6 standard into an empty tube. The solvent was evaporated, appropriate derivatizing reagent was added, and the tubes were incubated in the heating block at 90°C for 15 min.

Artificial 6-AM-propionyl formation from free morphine during propionic anhydride derivatization

An unextracted standard containing 100,000 ng of free morphine and 100 ng of 6-AM-d_6 in methanol was aliquoted to a glass tube. The methanol was evaporated, and the residue was derivatized with propionic anhydride according to the described procedure. The prepared aliquots were analyzed on the Finnigan GC–MS.

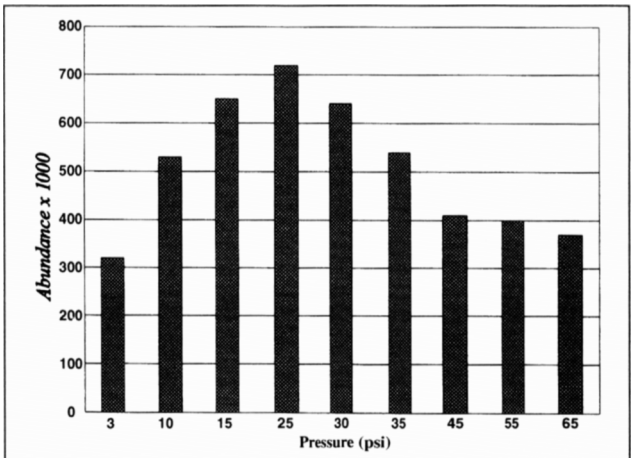

Figure 4. Peak abundance of 6-AM-TFA as a function of the carrier gas injection-port pressure (purge time 42 s, pressure pulse 30 s).

Interference study

The relative retention times for the compounds included in the interference study were determined with derivatized standards of each compound injected separately. Interference of the compounds generating the same ion fragments and similar retention times were evaluated with real and spiked samples.

Compatibility of the derivatives

Mutual interference of the assays using different derivatives was evaluated by assessing long-term performance of the methods on the same instruments. During method development, the 6-AM assays with evaluated derivatizing reagents were used on multiple GC–MS instruments dedicated to assays using TMS, *t*-BDMS, fluroacyl, alkyl derivatives, and assays of underivatized analytes.

Results and Discussion

Method optimization

The derivatization conditions were optimized by evaluating the influence of solvent addition, incubation temperature, and time on derivative recovery (data not shown). The optimal conditions for each derivatizing reagent were determined using experimental design methods (18). The best recovery for the MBTFA derivatization was obtained without solvent addition. Ethyl acetate was found to significantly increase recovery for the HFAA and MSTFA derivatization. Pyridine addition was necessary for propionic anhydride derivatization. For MSTFA derivatization, no difference in the recovery was observed with various incubation times and temperatures. The 6-AM-TMS derivative formation takes place instantaneously in the injection port, and no preliminary incubation is necessary. Maximum recoveries for derivatization with propionic anhydride and HFAA were obtained by incubation at 90°C. The best recovery for MBTFA derivatization was observed with incubation at 120°C. The peak-area ratio relative to the value obtained at the maximum recovery for each of the derivatives is presented in Figure 1. A derivatization time of 15–20 min was optimal for MBTFA, HFAA, and propionic anhydride derivatization.

Stability of the derivatives was evaluated at room temperature in sealed autosampler vials by analyzing the samples in duplicate every 4 h in three seperate runs. It was observed that the 6-AM-TMS derivative was very unstable in polypropylene vials, therefore only vials with glass inserts were used in the assay for all the derivatives. The plot of the peak-area ratio relatiave to the initial value observed immediately after the derivatization is shown in Figure 2. No difference was observed in stability among the propionyl, TMS, and TFA derivatives within 24 h after formation. Decrease of the peak abundance for the derivatives ranged from 40% for the 6-AM-TMS to 48% for the 6-AM-propionyl. Changes in the peak abundance were more rapid for the HFA derivative with losses of 50% of the original abundance within 15 h of preparation. It was observed that the HFA derivative decomposed faster after prolonged exposure to moist air. If no delays that allowed prolonged HFA derivative exposure to moist air occurred, the stability was analogous to the results obtained for the rest of the derivatives. The results for the 6-AM-HFA derivative stability were consistent with the observations of Paul et al. (12).

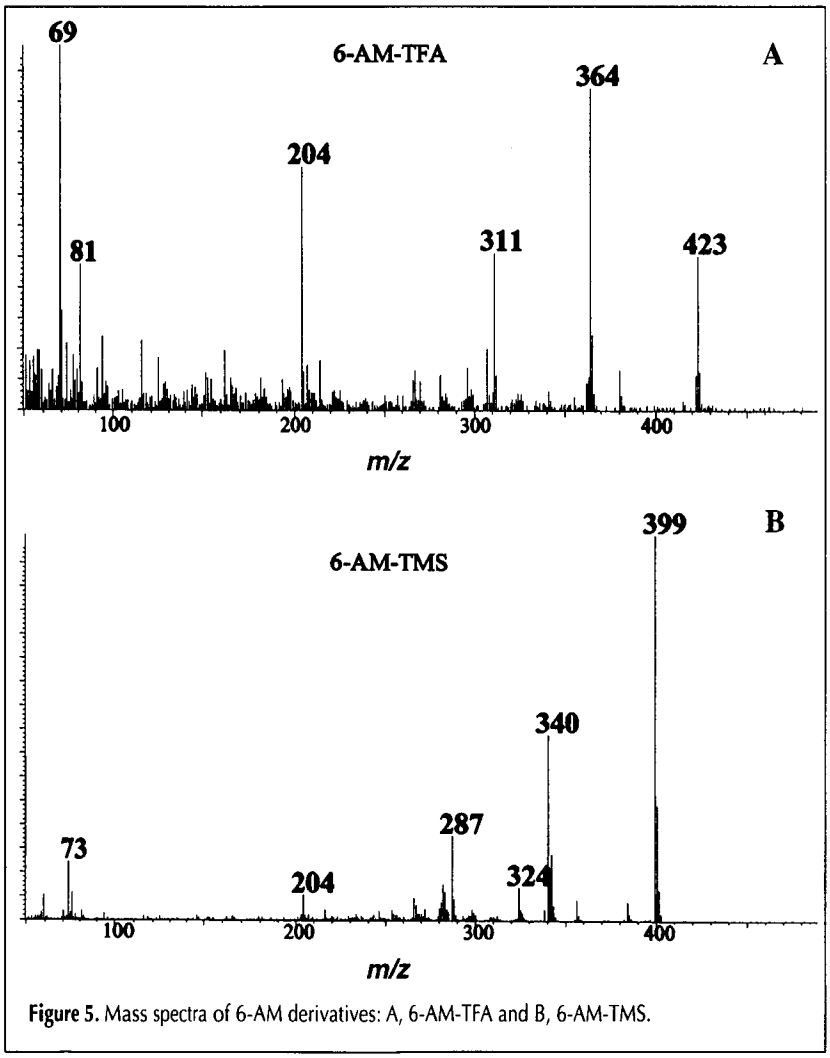

Figure 5. Mass spectra of 6-AM derivatives: A, 6-AM-TFA and B, 6-AM-TMS.

The 3-acetyl group of heroin can easily hydrolyze at extreme pH conditions and exchange during derivatization to form the same derivative as 6-AM, and hydrolysis of both the 3- and 6-acetyl groups would produce the same derivative as morphine (1,2,5). To assess any increase in 6-AM and morphine concentration during the procedure, unextracted standards containing 2000 ng of heroin (equivalent to 400 ng/mL in a 5-mL aliquot) and 100 ng of 6-AM-d_6 were derivatized with MBTFA at 90°C and 120°C for 15 min. To evaluate decomposition during the extraction, urine standards that contained the same amount of heroin and 6-AM-d_6 were extracted. The first

Table I. Retention Times and Ion Fragments of the 6-AM Derivatives

Compound	Retention time* (min)	Ion fragments (*m/z*)
6-AM-TFA	4.40	423†, 364, 311
6-AM-propionyl	6.05	383†, 268, 324
6-AM-TMS	5.22	399†, 340, 287
6-AM-HFA	4.54	523†, 464, 465

* HP 5890/5970 GC–MS system.
† Quantitating ion.

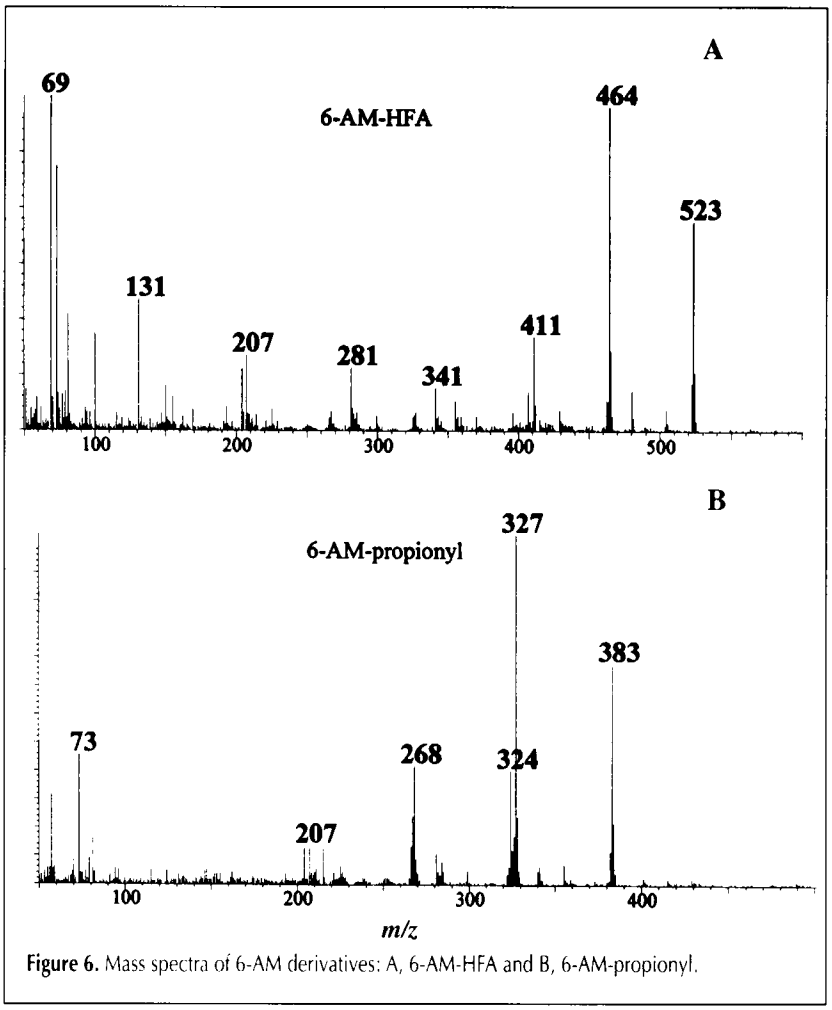

Figure 6. Mass spectra of 6-AM derivatives: A, 6-AM-HFA and B, 6-AM-propionyl.

standard was extracted according to the procedure and the second with the evaporation of the SPE column eluate delayed for 1 h. The extracts were derivatized at 120°C for 15 min. The samples were analyzed for 6-AM and morphine. The results are displayed in Figure 3. The derivatization at 90°C for 15 min converted 5% of heroin to 6-AM. By increasing the derivatization temperature to 120°C, an additional 2% of heroin was converted. Extraction by the employed procedure accounted for approximately 1% of the 6-AM formation. A 6% increase was observed when the eluate from the SPE column was kept for 1 h at room temperature prior to evaporation and derivatization. No morphine was detected in the samples at any of the evaluated conditions. The extent and the final products of heroin decomposition may be different with other sample preparation techniques, derivatizing reagents, and derivatization conditions. An advantage of on-column derivatization with MSTFA is elimination of the possibility of heroin decomposition to 6-AM because of incubation at the elevated temperature.

Propionic anhydride derivatization of 6-AM in the presence of an elevated concentration of free morphine was found to produce artificial 6-AM-propionyl which would falsely increase the 6-AM concentration in the specimen. The extent of this potential increase was evaluated by analyzing standards containing 100,000 ng of morphine and 100 ng of 6-AM-d_6 in triplicate. The derivatization experiments were performed by incubation at 60, 90, and 120°C using two different lots of propionic anhydride. The amount of 6-AM-propionyl was found to be independent of the incubation temperature and was 0.03 to 0.05% of the free morphine content in the specimen. One possible explanation of this phenomenon may be the presence of a small amount of acetic anhydride impurity in the commercially produced propionic anhydride. The problem is aggravated by an unmonitored and variable amount of acetic anhydride impurity in commercially available propionic anhydride.

The low target concentrations of 6-AM in urine require that the sample preparation method produce a clean extract for optimal

Table II. Mean of Within-Run Accuracy* and Imprecision for the 6-AM Derivatives on the HP 5890/5970 GC–MS System

Target concentration (ng/mL)	6-AM-propionyl			6-AM-TFA			6-AM-HFA			6-AM-TMS		
	Mean (ng/mL)	CV (%)	Accuracy (%)	Mean (ng/mL)	CV (%)	Accuracy (%)	Mean (ng/mL)	CV (%)	Accuracy (%)	Mean (ng/mL)	CV (%)	Accuracy (%)
4	3.9	0.50	96.6	4.3	0.70	107.0	3.9	1.10	97.5	3.9	1.17	97.5
10	10.2	0.40	101.8	9.5	0.93	94.9	10.1	0.55	101.2	10.5	1.05	105.0
300	309.6	0.77	103.2	319.2	1.20	106.4	258.0	3.12	86.0	304.5	0.35	101.5

* n = 3 for 6-AM-propionyl and 6-AM-TFA, and n = 2 for 6-AM-HFA and 6-AM-TMS.

sensitivity. In order to obtain a cleaner extract an additional SPE column wash was incorporated in the method (19) prior to elution of the analytes. A significant improvement in extract cleanliness was obtained with the same solvent mix composition as used in the SPE elution solvent methylene chloride/isopropanol (4:1).

The sensitivity of the method can be enhanced by increasing specimen aliquot volume and injection volume or decreasing the final reconstituted volume. Another way to improve sensitivity is to use pressure pulse in the GC inlet during the injection (20). The procedure used a high inlet pressure at the time of injection, followed by a rapid reduction of the pressure to a flow rate optimal for the capillary GC separation and MS performance. The 6-AM-TFA quantitative ion-peak abundance as a function of the carrier gas pressure in the injection port is presented in Figure 4. The 6-AM peak abundance doubled when the pressure was increased from 4 to 25 psi. The abundance gradually decreased with a further increase in pressure. Maximum sensitivity for the methods, when analyzed within 4 h after derivative formation with all the other conditions equal, was observed for the 6-AM-TFA and 6-AM-TMS derivatives.

Table III. Between-Run* Accuracy and Imprecision for 6-AM-Propionyl, TFA, and TMS Derivatives

	6-AM-propionyl†		6-AM-TFA†		6-AM-TMS†		6-AM-TMS‡	
Concentration (ng/mL)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
4	3.39	101.1	3.17	109	0.00	92.5	3.00	95.8
10	1.92	101	1.31	97.7	0.00	106.0	2.53	99.3
300	6.3	92.1	1.76	91.7	1.25	101.5	N/A	N/A
400	N/A	N/A	N/A	N/A	N/A	N/A	0.63	94.6

* $n = 3$.
† HP 5890/5970 GC–MS system.
‡ Finnigan GC–MS system.

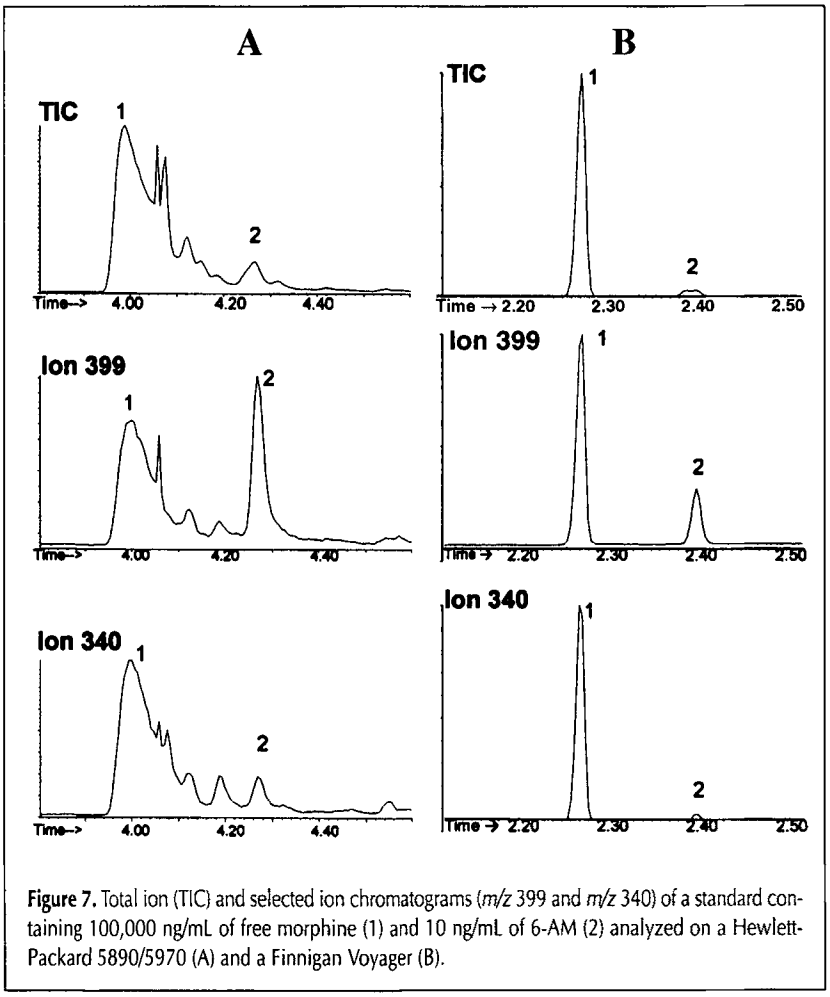

Figure 7. Total ion (TIC) and selected ion chromatograms (*m/z* 399 and *m/z* 340) of a standard containing 100,000 ng/mL of free morphine (1) and 10 ng/mL of 6-AM (2) analyzed on a Hewlett-Packard 5890/5970 (A) and a Finnigan Voyager (B).

Accuracy, precision, and linearity

Mass spectra of the 6-AM derivatives are contained in Figures 5 and 6. The ion mass fragments used are presented in Table I. The abundances of the secondary and tertiary ions for all the derivatives were adequate for GC–MS in the SIM mode. The limit of quantitation was 1 ng/mL for the TFA derivative, and 4 ng/mL for the remaining derivatives with the criterion of maintaining a qualitative ion mass ratio of ± 20% relative to the same ion mass ratio of the 10-ng/mL calibrator. The ion mass ratios at the lower concentration for the propionyl, HFA, and TMS derivatives were affected by cross-contribution of the ion fragments from the 6-AM-d_6 internal standard derivatives. Using an accuracy criterion of within 85–115% of the target concentration, and precision within 10%, the upper limit of linearity for all the methods was 300 ng/mL.

To assess method accuracy and precision, urine samples containing 4, 10, and 300 ng/mL of 6-AM were analyzed in triplicate using the derivatized extracts within 4 h after preparation. The mean results for within-run accuracy and imprecision are presented in Table II. The results were acceptable for all four derivatizing reagents, but some decrease in accuracy was observed for the HFA derivative at 300 ng/mL. Between-run accuracy and imprecision (Table III) for the propionyl, TFA, and TMS derivatives produced acceptable results.

The 6-AM-TMS assay performance was also evaluated on the Finnigan Voyager GC–MS system. The advantages of this instrument compared to earlier generations of GC–MS instruments include the ability of the MSD to work at greater carrier gas flow rates, increased temperature ramp rate, improved scan frequency, and use of a more sensitive photomultiplier. These advancements led to a considerable improvement in assay run time, sensitivity, and increased the linear range. With all other conditions equal the assay on

the Finnigan Voyager performed adequate with 2.5 times smaller sample volume and 5.25 times shorter purge time compared with the assay used on the HP 5890/5970. The improved instrument sensitivity allowed to improve the GC column performance by a decreasing amount of sample injected in the instrument, extending linear range and eliminated detector overloading for morphine. The data for between-run accuracy and imprecision for the 6-AM-TMS assay on GC–MS systems are presented in Table III.

Interference study

The relative retention times for some compounds that may interfere with the assays are shown in the Table IV. The need for enhanced method sensitivity by increasing sample and injection volume created potential problems because it also concentrated morphine and other compounds coextracted from the specimen aliquot. Although most of the coextracted compounds are transparent in the SIM mode, GC column overloading from high concentrations of these compounds may occur during analysis and interfere with method performance. The potential compounds coeluting with 6-AM were hydromorphone and desipramine for the propionyl derivative, oxycodone and normorphine for the TMS derivatives, and hydromorphone and norcodeine for the HFA derivative. The propionyl derivative of desipramine and prazepam have the major ion fragment 324 in common with 6-AM-propionyl. This could interfere with 6-AM if the peaks are not sufficiently separated. The small distances between the 6-AM, morphine, and hydromorphone peaks were noteworthy because the compounds have the same secondary and tertiary ions in the mass spectra of propionyl, TFA, HFA, and tertiary ion for the TMS derivatives. Oxycodone can potentially interfere with the secondary and tertiary ions for the 6-AM-TMS assay. The presence of morphine and hydromorphone may affect the performance of the method if the peaks are not well separated from 6-AM. For TFA, HFA, and TMS derivatives, morphine elutes before 6-AM, and the derivatives may interfere with 6-AM if the column is overloaded. Because morphine is the major metabolite present after heroin use and has a close retention time to 6-AM for all the derivatives, it is important to have adequate separation of morphine from 6-AM. The advantage of the propionyl derivative is that morphine elutes after 6-AM and does not affect the analysis. Hydromorphone and oxycodone are not present in a specimen after heroin use and would exist only if the donor had also used these drugs. It is therefore important to have morphine, hydromorphone, and oxycodone peaks well separated from 6-AM.

The Finnigan Voyager GC–MS system increased sensitivity and extended the linear range of the detector, allowed the use of a decreased sample aliquot and injection volume. This was very favorable for the assay performance, significantly decreased the potential for column and detector overload, and improved peak separation from the potential interferences. Figure 7 presents chromatograms of a specimen containing 100,000 ng/mL of morphine and 10 ng/mL of 6-AM derivatized with MSTFA analyzed on both HP 5890/5970 and Finnigan Voyager GC–MS systems.

Table IV. Relative Retention Times of Potentially Interfering Compounds with the 6-AM Assays Relative to the Internal Standard 6-AM-d_6

Compound	TFA	Propionyl	TMS	HFA
6-AM-d_6	1.000	1.000	1.000	1.000
6-AM	1.004	1.005	1.004	1.003
Codeine	0.899	0.899	0.909	0.919
Desipramine	N/A	1.002	N/A	N/A
Heroin	1.305	1.210	1.406	1.266
Hydrocodone (underiv.)	1.166	1.154	0.880	1.122
Hydrocodone (mono)*	0.866	1.091	0.921	0.903
Hydromorphone (mono)	0.973	0.975	0.961	0.994
Hydromorphone (bis)†	0.774	0.791	0.942	0.869
Morphine	0.821	1.082	0.948	0.895
Norcodeine (bis)	1.063	1.082	0.956	1.002
Normorphine	0.956	1.082	0.987	0.961
Oxycodone (bis)	1.067	0.909	1.023	0.954
Oxycodone (mono)	1.537	0.851	0.948	0.844
Prazepam	N/A	0.939	N/A	N/A

* The single derivative.
† The double derivative.

Compatibility of the derivatives with other methods

Depending on the nature of the derivatizing reagent and the cleanliness of the extract, the method may negatively affect performance of the GC–MS for other assays. While developing the method, interference of the studied derivatives on other methods analyzed on the same GC–MS system was evaluated. The assays used on the same instruments included underivatized analytes (phencyclidine, meperidine, and methadone metabolite), HFA derivatives (amphetamines and normeperidine), TMS derivatives (9-carboxy-THC, benzoylecgonine, and opiates), *tert*-butyldimethylsilyl (*t*-BDMS) derivatives (benzodiazepines), and alkyl derivatives (barbiturates). Table V presents data for compatibility of the derivatives for long-term use of the methods on the same GC–MS system. The 6-AM TFA and HFA derivatives were compatible with underivatized analytes, alkyl derivatives, and not compatible with HFA, TMS, and *t*-BDMS derivatives of other drugs. The 6-AM-propionyl was compatible with alkyl derivatives, TMS and *t*-BDMS deriva-

Table V. Compatibility of the 6-AM Derivatives with Other Methods on the Same GC–MS

Evaluated derivatives of other drugs	6-AM derivative			
	TFA	HFA	TMS	Propionyl
Underivatized	+*	+	–	–
HFA	–†	–	–	–
Alkyl	+	+	+	+
TMS	–	–	+	+
t-BDMS	–	–	+	+

* + the derivative is compatible.
† – the derivative is not compatible.

tives and not compatible with underivatized analytes, and perfluoroacyl derivatives. The TMS derivative was compatible with TMS, *t*-BDMS, and alkyl derivatives of other drugs. Incompatibility of different methods on the same instrument is related to deterioration of the column after prolonged exposure to various derivatizing reagents and coextracted sample matrix components. Compatibility with different derivatives was improved on the Finnigan Voyager GC–MS system by introducing less sample on the column through using a shorter purge time.

Conclusions

No significant difference was observed in the stability of the 6-AM propionyl, TMS, and TFA derivatives within 24 h after formation when stored in glass autosampler vials. The HFA derivative was the least stable and decomposed by 50% within 15 h. The 6-AM-TMS derivative was significantly less stable when stored in polypropylene vials. All the derivatives produced adequate precision for the method. Significant improvement in the cleanliness of the extract was obtained by incorporating an additional wash of the SPE columns with a solvent mix consisting of methylene chloride/isopropanol (4:1). The sensitivity of the 6-AM method can be enhanced by using a pressure pulse during the injection and subsequent carrier gas pressure programming during the GC analysis. The most common potential interferences for the respective assays are hydromorphone (propionyl, TFA, HFA), oxycodone (TMS), and desipramine (propionyl), and it is therefore important to have these peaks well separated from 6-AM. An advantage of the propionyl derivative is that morphine elutes after 6-AM, whereas morphine elutes just before 6-AM for the other derivatives and, if not adequately separated, may affect the analysis. The 6-AM-TMS and 6-AM-propionyl derivatives were the most compatible with other methods when analyzed on the same GC–MS. Of the evaluated derivatives, 6-AM-TMS produced by the reaction with MSTFA was the easiest to prepare, did not require incubation, had sufficient sensitivity, and had the highest potential for use on an automated sample-preparation device. Another important advantage of the on-column derivatization with MSTFA is the elimination of the possibility of heroin decomposition to 6-AM caused by incubation at the elevated temperature.

The 6-AM derivatization with propionic anhydride generated a relatively small amount of 6-AM-propionyl derivative produced from the free morphine present in a specimen. A possible explanation of the phenomenon may be the presence of a small unmonitored and variable amount of acetic anhydride impurity in commercially produced propionic anhydride reagent.

With all the conditions equal, the assay on the new generation of GC–MS instrumentation is significantly more sensitive than on the older HP 5890/5970 GC–MS system. This allows such advantages as using less sample for the analysis, enhanced sensitivity, improved instrument performance, and separation from potentially interfering compounds.

References

1. R.C. Baselt and R.H. Cravey. *Disposition of Toxic Drugs* and *Chemicals in Man*, 4th ed. Chemical Toxicology Institute, Foster City, CA, 1995, p 366.
2. G.R. Nakamura, J.I. Thornton, and T.T. Noguchi. Kinetics of heroin deacetylation in aqueous alkaline solutions and in human serum and whole blood. *J. Chromatogr.* **110:** 81–89 (1975).
3. B.A. Goldberger, W.D. Darwin, T.M. Grant, A.C. Allen, Y.H. Caplan, and E.J. Cone. Measurement of heroin and its metabolites by isotope-dilution electron-impact mass spectrometry. *Clin. Chem.* **39:** 670–675 (1993).
4. E.J. Cone, P. Welch, J.M. Mitchell, and B.D. Paul. Forensic drug testing for opiates: I. detection of 6-acetylmorphine in urine as an indicator of recent heroin exposure: drug and assay considerations and detection times. *J. Anal. Toxicol.* **15:** 1–7 (1991).
5. Changes to the testing cutoff levels for opiates for federal workplace drug testing. *Fed. Regist.* **63:** 483–484 (1998).
6. J.G. Guillot, M. Lefebvre, and J.P. Weber. Determination of heroin, 6-acetylmorphine, and morphine in biological fluids using their propionyl derivatives with ion trap GC–MS. *J. Anal. Toxicol.* **21:** 127–133 (1997).
7. L.J. Bowie and P.B. Kirkpatrick. Simultaneous determination of monoacetylmorphine, morphine, codeine, and other opiates by GC–MS. *J. Anal. Toxicol.* **13:** 326–329 (1989).
8. D.C. Fuller and W.H. Anderson. A simplified procedure for the determination of free codeine, free morphine, and 6-acetylmorphine in urine. *J. Anal. Toxicol.* **16:** 315–318 (1992).
9. J. Fehn and G. Megges. Detection of O^6-monoacetylmorphine in urine samples by GC–MS as evidence of heroin use. *J. Anal. Toxicol.* **9:** 134–138 (1985).
10. J. Fenton, J. Mummert, and M. Childers. Hydromorphone and hydrocodone interference in GC/MS sssay for morphine and codeine. *J. Anal. Toxicol.* **18:** 159–164 (1994).
11. M.M. Kushnir, C. Jones, R. Struempler, and F.M. Urry. Eliminating interferences of related compounds in opiates GC/MS analysis. *Clin. Chem.* **42:** S218 (1996).
12. B.D. Paul, J.M. Mitchell, L.D. Mell, and J. Irving. Gas chromatography/electron impact mass fragmentometric determination of urinary 6-acetylmorphine, a metabolite of heroin. *J. Anal. Toxicol.* **13:** 2–7 (1989).
13. S.J. Mulé and G.A. Casella. Rendering the "poppy-seed defense" defenseless: identification of 6-monoacetylmorphine in urine by gas chromatography/mass spectrometry. *J. Anal. Toxicol.* **14:** 1427–1430 (1988).
14. T. Vu-Duc and A. Vernay. Evaluation of sample treatment procedures for the routine identification and determination at nanogram level of O^6-monoacetylmorphine in urine by capillary GC and dual NPD–FID. *J. High Resol. Chromatogr.* **13:** 162–166 (1990).
15. G.F. Grinstead. A closer look at acetyl and pentafluoropropionyl derivatives for quantitative analysis of morphine and codeine by gas chromatography/mass spectrometry. *J. Anal. Toxicol.* **15:** 293–298 (1991).
16. B.A. Goldberger, E.J. Cone, T.M. Grant, Y.T. Caplan, B.S. Levine, and J.E. Smialek. Disposition of heroin and its metabolites in heroin related deaths. *J. Anal. Toxicol.* **18:** 22–28 (1994).
17. B.D. Paul, L.D. Mell, Jr., J.M. Mitchell, J. Irving, and A.J. Novak. Simultaneous determination of codeine and morphine in urine by capillary gas chromatography and mass spectrometry. *J. Anal. Toxicol.* **9:** 222–226 (1985).
18. R.O. Collier, Jr. and T.J. Hummel. *Experimental Design and Interpretation.* McCutchan Pub. Corp., Berkeley, CA, 1977, pp 337–352.
19. Clean Screen7 extraction columns application manual. Bristol, PA, 1995, p 43.
20. P.L. Wylie, R.J. Phillips, K.J. Klein, M.Q. Thompson, and B.W. Hermann. Improving splitless injection with electronic pressure programming. *J. High Resol. Chromatogr.* **14:** 649–655 (1991).

Manuscript received August 18, 1998;
revision received December 7, 1998.