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Simultaneous Analysis of Amphetamine, Methamphetamine, and 3,4-Methylenedioxymethamphetamine (MDMA) in Urine Samples by Solid-Phase Extraction, Derivatization, and Gas Chromatography/Mass Spectrometry

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ABSTRACT: A rapid and effective solid-phase extraction procedure using Bond Elute Certify™ bonded silica sorbent cartridges was adopted to extract amphetamine, methamphetamine, and 3,4-methylenedioxymethamphetamine (MDMA or Ecstasy) from urine samples. The extract was derivatized with trichloroacetic anhydride prior to gas chromatography/mass spectrometry (GC/MS) analysis with selected ion monitoring of the following ions: 190, 91, 188; 204, 91, 202; 162, 135, 202; 194, 123; and 211, 209 for the derivatized amphetamine, methamphetamine, MDMA, d_5-amphetamine, and d_9-methamphetamine, respectively. The first of the ions listed for each compound was used for quantitation. The compound d_5-amphetamine was used as the internal standard for amphetamine, and d_9-methamphetamine was used for methamphetamine and MDMA. Results showed a higher than 65% recovery and a reproducibility with less than a 5% coefficient of variation. When a sample size of 2 mL was used, the lowest detectable concentration was about 50 ng/mL, and a near-perfect fit can be obtained (within the 250 to 4000-ng/mL concentration range studied) using a second-order polynomial model.

KEYWORDS: toxicology, amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA), solid-phase extraction, gas chromatography/mass spectrometry (GC/MS), urine

With the development of various sorbent materials, solid-phase extraction has become an effective approach to isolation and concentration of analyte in biological samples. The solid-phase extraction approach offers the following advantages over the conventional liquid-liquid procedures: (1) there is less usage of organic solvents; (2) it has a shorter sample preparation time; and (3) it is easier to incorporate into the automatic operation process.

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The main thrusts of this study are the integration and improvement of the use of solid-phase extraction [*1,2*], trichloroacetyl derivatization [*3*], and d_5-amphetamine/d_9-methamphetamine internal standards [*4*] for simultaneous gas chromatography/mass spectrometry (GC/MS) analysis of amphetamine (AMP), methamphetamine (METH), and 3,4-methylenedioxymethamphetamine (MDMA) in urine samples. While the monitoring of AMP and METH are required by the mandatory guidelines [*5*] established by the U.S. Department of Health and Human Services, MDMA, also known as Ecstasy, has been reported to cause serious abuse consequences [*6,7*] and its testing is required by some agencies, for example, the U.S. Coast Guard.

The reproducibility, linearity, recovery, and sensitivity established by this study have proven to be effective for routine and high-volume testing of these drugs of abuse.

Materials and Methods

Reagents and Controls

Amphetamine, METH, and MDMA were purchased from Sigma (St. Louis, Missouri). The internal standard 1-phenyl-2-aminopropane-1,2,3,3,3-d_5 (d_5-AMP) and 1-phenyl-2-(methyl-d_3-amino)propane-1,1,2,3,3,3-d_6 (d_9-METH) was obtained from Radian (Austin, Texas) and MSD Isotopes (Dorval, Quebec, Canada), respectively. The derivatizing reagent trichloroacetic anhydride and the 4-methylaminopyridine catalyst were obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin).

A 0.1*M* phosphate buffer was prepared by dissolving 13.61 g of potassium dibasic phosphate in 900 mL of distilled water and adjusting the pH to 6.0 with 1.0*M* potassium hydroxide. Carbonate buffer (pH 9.5) was prepared by dissolving 16 g of sodium carbonate (Na_2CO_3) and 18 g of sodium bicarbonate ($NaHCO_3$) in distilled water and diluting the solution to a final volume of 250 mL.

Standards and controls for urine analysis were prepared in-house using stocks containing 0.1 mg/mL of AMP, METH, and MDMA. The 500-ng/mL solution was used as the one-point calibration standard. Internal standards, d_5-AMP and d_9-METH, were prepared by diluting the 0.1-mg/mL solutions obtained from the suppliers to produce an 8-μg/mL working solution.

Gas Chromatography/Mass Spectrometry Procedure

A HP 5890 gas chromatograph interfaced with a 5970 mass selective detector (MSD) was used for this study. The gas chromatograph was equipped with a 15-m, 0.25-mm internal diameter, 0.25-μm film thickness, J & W DB-5 (5% phenyl polysiloxane phase) capillary column purchased from J & W Scientific (Folsom, California). Helium was used as the carrier gas, with a flow rate of approximately 1.0 mL/min and a split ratio of 10:1. The injector and GC/MS interface temperature were maintained at 270°C. The injector port has a capillary split injector packed with OV-101 (80/100 mesh). The oven temperature was programed as follows: held at the initial temperature of 180°C for 1 min, increased at a rate of 15°C/min to 250°C, and held at the final temperature for 1 min. The MSD was used in the selective ion monitoring (SIM) mode, with the monitoring of the following ions: *190*, 188, 91 (AMP); *204*, 202, 91 (METH); *162*, 135, 202 (MDMA); *194*, 123 (d_5-AMP); and *211*, 209 (d_9-METH). The first ion listed for each compound was used for the quantitation purpose.

Extraction and Derivatization Procedure

Two-millilitre sample aliquots were pipetted into 50-mL plastic conical-bottom tubes, to which were added 0.25 mL of each internal standard (d_5-AMP and d_9-METH) and 2

mL of 0.1*M* phosphate buffer. The tubes were capped and vortexed for 15 s. The pH of the mixture was adjusted with either 0.1*N* sodium hydroxide (NaOH) or 0.1*N* hydrochloric acid (HCl) to 5 to 7 (if needed). The extraction columns were then assembled in the Vac Elute extraction manifold.

The solid-phase extraction protocol, as shown in Fig. 1, consists of four basic stages: conditioning, loading, rinsing, and eluting. The Bond Elute Certify™ columns obtained from Analytichem International (Harbor City, California) were conditioned sequentially with 2-mL aliquots of methanol and 0.1*M* phosphate buffer (pH 6.0). The urine samples, which were prepared as described above, were then introduced into the appropriately labeled columns. Samples were drawn through the columns under low vacuum, which took at least 2 min for the sample to pass through. The columns were then rinsed with 1 mL of 1.0*M* acetic acid, then dried under full vacuum (15 in. Hg) for 5 min. The columns were rinsed again with 6 mL of methanol under reduced vacuum and dried under full vacuum again for 2 min.

The analytes were eluted with 2% ammonium hydroxide solution in ethyl acetate (prepared fresh daily) into a 15-mL round-bottom glass tube gravimetrically. The eluate was evaporated to dryness under a slow stream of nitrogen at a temperature below 40°C in a water bath. To each sample was added 1 mL of normal NaOH and 2.5 mL of 1-

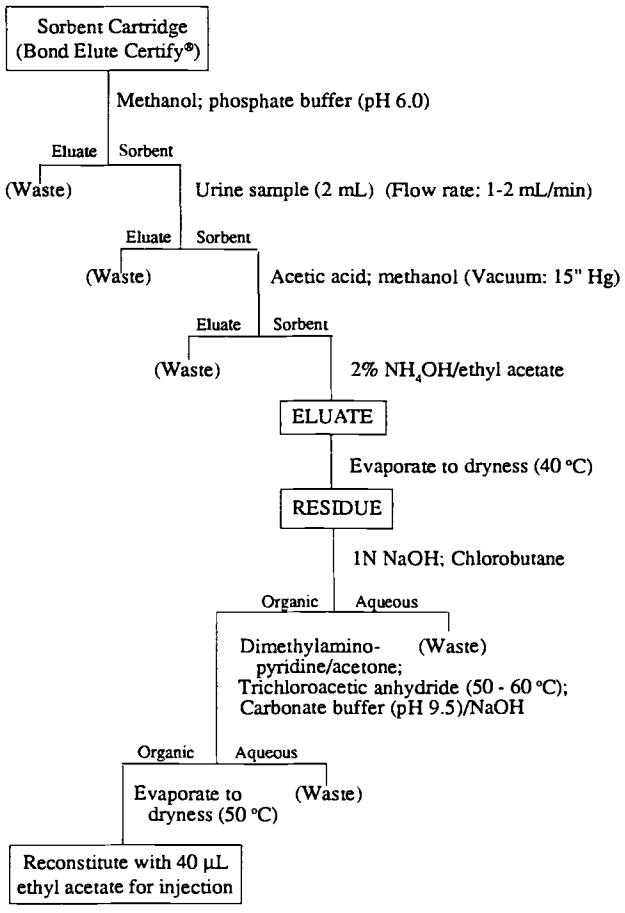

FIG. 1—*Solid-phase extraction and derivatization scheme.*

chlorobutane; then the mixture was vortexed for a minimum of 2 min and centrifuged. The organic layer was transferred to another glass tube after the bottom aqueous layer was frozen in an isopropanol/dry ice bath. The samples were then ready for derivatization.

Derivatization for GC/MS analysis was accomplished by adding 150 μL of 4-dimethylaminopyridine catalyst and 75 μL of trichloroacetic anhydride to the organic phase containing the extracted analytes. The mixture was vortex-mixed for 30 s; 1 mL of H_2O and 0.5 mL of 1.5*M* carbonate buffer (pH 9.5) were added; and the mixture was then vortex-mixed for another 30 s and incubated for 45 to 50 min at 50 to 60°C. After incubation and while warm, the mixture was vortex-mixed vigorously for 1 min. The process was repeated for another minute. (This was necessary to ensure complete destruction of the trichloroacetic anhydride.) The mixture was centrifuged at 1000 to 1500 rpm for a minimum of 5 min. After the lower aqueous phase had been frozen in an isopropanol/dry ice bath, the organic phase was decanted into a 5-mL conical glass tube, and approximately 75% of the organic solvent was evaporated under a slow stream of nitrogen at a temperature of 50 to 60°C. The evaporation was completed at room temperature to minimize evaporative loss. The derivatized specimens were stored in a freezer at −20°C. They were reconstituted with 40 μL of ethyl acetate when GC/MS analysis was performed.

Results and Discussion

The structure and mass spectra of the acylated AMP, METH, and MDMA, and the two deuterated analogues, d_5-AMP and d_9-METH, are shown in Fig. 2. These spectra were used as guides [*8*] for selecting noninterfering high-mass ions for monitoring. Trichloroacetic anhydride [*3*] was used as the derivatization reagent so that the analysis could be performed at a higher temperature to help maintain a cleaner GC/MS system. Since the acylated MDMA has a much longer retention time than AMP and METH, the GC oven temperature was programed so that the three targeted analytes could be chromatographed in a reasonable time period.

Extraction Efficiency

Using the column manufacturer-recommended extraction procedures, followed by the derivatization with trichloroacetic anhydride, does not produce a satisfactory chromatogram (Fig. 3*a*). The addition of a simple cleanup step prior to this derivatization produces a much better chromatographic result, as is shown in Fig. 3*b*.

The extraction recovery was determined from urine standards for the three drugs at the 500-ng/mL concentration level. Triplicate samples were extracted by the solid-phase procedure. A fixed amount of internal standard was added to each of the triplicate extracts and to each of a set of unextracted triplicates containing the same amount of the three analytes. These two triplicate sets were derivatized using the same process. Using d_5-AMP as the internal standard, the percentages of recovery for the extraction process were found to be 66, 81, and 97% for AMP, METH, and MDMA, respectively. The corresponding values were 66, 81, and 95% if d_9-METH was used as the internal standard. The lower recovery obtained for AMP could be due to the evaporative loss in the evaporation step prior to the addition of the internal standard.

To prove that the lower recovery for AMP is indeed due to evaporative loss, an additional set of samples was extracted with the addition of internal standards immediately prior to the evaporation step (after all other extraction steps). The apparent percentages of recovery for AMP, METH, and MDMA calculated based on d_5-AMP were 92, 89, and 95%, respectively. The corresponding values were 94, 92, and 99% if d_9-METH was used as the internal standard. It is thus evident that the evaporation step should be conducted at the lowest practical temperature possible.

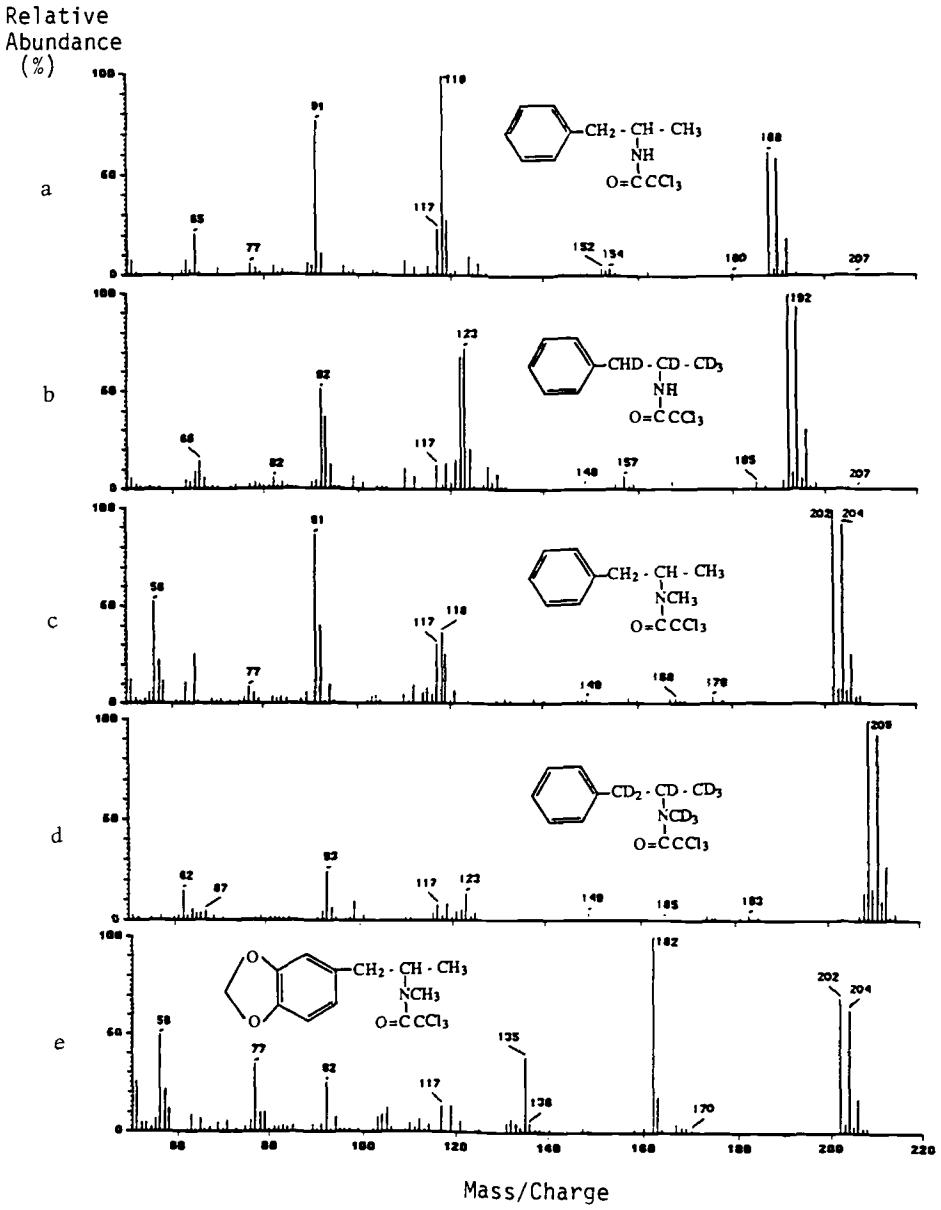

FIG. 2—*Structural information and mass spectra of trichloroacetyl derivatives of amphetamine* (a), *d_5-amphetamine* (b), *methamphetamine* (c), *d_9-methamphetamine* (d), *and 3,4-methylenedioxymethamphetamine* (e).

It should be noted that the lower recovery of AMP resulting from the normal operation does not affect the overall qualitative and quantitative aspects of the assay. This is possible because of the relatively high abundance of the ions monitored for AMP and the use of the deuterated analogue as the internal standard.

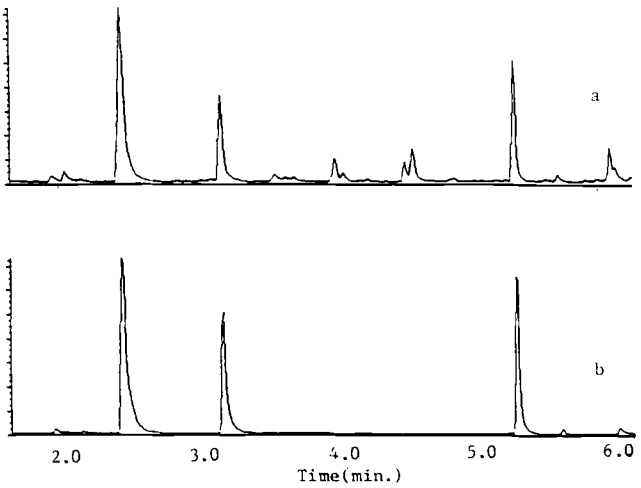

FIG. 3—*Total ion (of the selected ions monitored) chromatograms resulting from the solid-phase extraction without* (a) *and with* (b) *the additional cleanup step.*

Reproducibility

Reproducibilities were studied at two levels—the GC/MS procedure and the entire analytical process. The GC/MS reproducibility study was conducted using a 500 ng/mL control with five injection replicates. The reproducibility for the overall analytical process was examined by testing six replicates of 500-ng/mL controls. Results of these studies, summarized in Table 1, indicate that the overall analytical procedure is highly reproducible, and in the absence of a deuterated analogue, d_0-METH is a suitable internal standard for the quantitation of MDMA.

Calibration Range

Controls with concentrations ranging from 250 to 4000 ng/mL were used for calibration studies. With the same amount of the selected internal standard (d_5-AMP for AMP, d_9-METH for METH and MDMA) used in all samples, the response-concentration relationship is examined by plotting the analyte-to-internal standard quantitation ion intensity ratio (*m/z* 190/194 for AMP, *m/z* 204/211 for METH, and *m/z* 162/211 for MDMA) obtained from these samples. These data were fit with first- and second-order polynomial equations, as shown in Table 2. The resulting standard deviation and mean error data indicate the nonlinear nature of the response-concentration relationship and the suitability of the second-order polynomial model. As an example, the second-order polynomial fit for METH is shown in Fig. 4.

Data shown in Table 3 compare the theoretical value with the observed values obtained using the 500-ng/mL control as the one-point calibrator, and all five controls fit into a second-order polynomial model. With one-point calibration, the observed concentrations for the controls at higher concentration are lower than the expected values, indicating a "curving" phenomenon. This is also evident from the second-order polynomial fit shown in Fig. 4. Without incorporating controls at concentrations lower than 250 ng/mL and comparing the observed with the theoretical values, 250 ng/mL is considered the limit of quantitation for this experimental batch. It is very likely that a lower limit of quantitation can be established if attempted.

Data shown in Table 3 clearly indicate that multiple-point calibration provides more

TABLE 1—*Summary of reproducibility study results.*

Parameter	Internal Standard, d_5-Amphetamine			Internal Standard, d_9-Methamphetamine			Operation
	AMP	METH	MDMA	AMP	METH	MDMA	
$n = 5$							
Mean	508	498	492	508	498	492	GC/MS
Standard deviation	5.7	7.9	23	10	4.8	20	
Coefficient of variation, %	1.1	1.6	4.7	2.0	0.96	4.0	
$n = 6$							
Mean	507	506	545	504	503	541	overall
Standard deviation	5.3	7.5	24	9.7	11	21	
Coefficient of variation, %	1.1	1.5	4.4	1.9	2.1	3.9	

TABLE 2—*First order (linear regression) and second order polynomial fit of the data observed from five controls of known concentrations.*

Drug	Regression Equation[a]	Standard Deviation	Mean Error
AMP	$Y = 0.1179 + 7.6522 \times 10^{-4} X$	0.0567	0.0776
METH	$Y = 0.1575 + 7.6290 \times 10^{-4} X$	0.0812	0.110
MDMA	$Y = 0.0098 + 7.2366 \times 10^{-4} X$	0.0228	0.355
AMP	$Y = 0.0675 + 7.7865 \times 10^{-4} X - 1.2751 \times 10^{-8} X^2$	5.28×10^{-4}	9.82×10^{-4}
METH	$Y = 0.0131 + 1.0200 \times 10^{-3} X - 5.9608 \times 10^{-8} X^2$	4.17×10^{-3}	5.97×10^{-3}
MDMA	$Y = 0.2613 + 7.7865 \times 10^{-4} X - 1.2751 \times 10^{-8} X^2$	1.47×10^{-2}	1.53×10^{-2}

[a] Y = analyte to internal standard quantitation ion ratio; X = concentration of analyte.

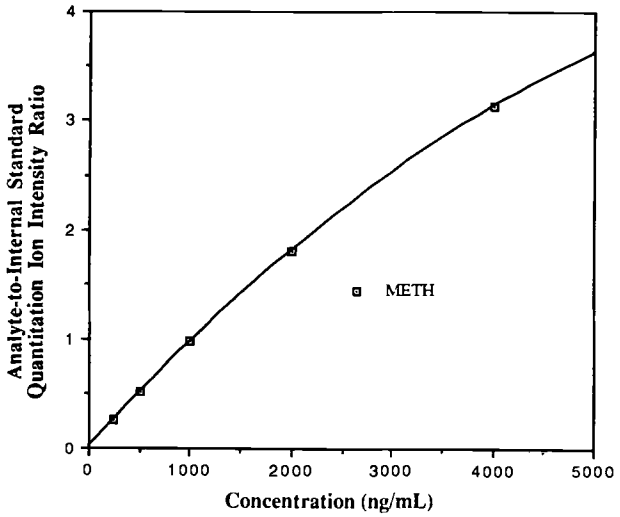

FIG. 4—*Second-order polynomial fit of METH calibration data.*

reliable results on a wider concentration range, especially those at the higher concentration end. However, one-point calibration will provide more accurate results for samples with concentrations in the immediate vicinity of the concentration of the single calibrator. In light of the heavy emphasis on adopting a "cutoff" concentration for reporting a sample as legally (as opposed to scientifically) positive, the merit of using a one-point calibrator at the cutoff concentration should not be overlooked.

Detection Limit

Controls prepared at the concentrations of 100, 50, and 30 ng/mL were used to test the sensitivity of the assay. Using a 2-mL sample size, 40 μL of the final reconstitution volume, and a 2-μL injection volume, the 30 ng/mL control showed relatively low abundance (first row of Fig. 5) for the weakest ions (*m/z* 190, 204, and 135 for AMP, METH, and MDMA, respectively). The corresponding ion abundance for the 50-ng/mL control (second row of Fig. 5) and the 30-ng/mL control with 20-μL reconstitution volume (third row of Fig. 5) are significantly higher. It is thus estimated that the detection limit for the determination of these three drugs is 50 ng/mL following the normal experimental conditions.

Potential Interference

Since false METH-positive results have been reported by reputed laboratories, the GC/MS characteristics of the following drugs were studied by processing the following drugs individually and together with AMP, METH, and MDMA in the analytical procedure: phentermine, ephedrine, norephedrine, pseudoephedrine, and norpseudoephedrine. The total ion chromatograms resulting from the scan (*m/z* = 51 to 350) and SIM (using ions adopted for monitoring the AMP, d_5-AMP, METH, d_9-METH, and MDMA) modes are shown in Fig. 6*a* and *b*, respectively. As identified in these figures, seven peaks with distinct retention times were identified for this eight-drug mixture. The retention time of phentermine cannot be readily differentiated from that of AMP. However, the mass spectra (Fig. 7 and Fig. 2*a*) of phentermine and AMP are sufficiently different, and the presence of phentermine cannot be misidentified as AMP. It should be noted,

TABLE 3—*Theoretical versus observed values of controls using a one-point calibration curve fit into a second-order polynomial equation.*

Theoretical Concentration, ng/mL	AMP		METH		MDMA	
	1-Point Calibration Observed (% deviation)	2nd-Order Polynomial Observed (% deviation)	1-Point Calibration Observed (% deviation)	2nd-Order Polynomial Observed (% deviation)	1-Point Calibration Observed (% deviation)	2nd-Order Polynomial Observed (% deviation)
500	500 – calibrator	499 (−0.2%)	500 – calibrator	497 (−0.6%)	500 – calibrator	483 (−3.4%)
250	256 (+2.4%)	254 (+1.6%)	258 (+3.2%)	256 (+2.4%)	291 (+16.4%)	250 (0.0%)
1000	966 (−3.4%)	993 (−0.7%)	970 (−3.0%)	993 (−0.7%)	973 (−2.7%)	1034 (+3.4%)
2000	1814 (−9.3%)	2006 (+0.3%)	1792 (−10.4%)	2007 (+0.4%)	1761 (−12.0%)	1980 (−1.0%)
4000	3266 (−18.4%)	3999 (−0.03%)	3111 (−22.2%)	3999 (−0.03%)	3358 (−16.1%)	4003 (+0.08%)

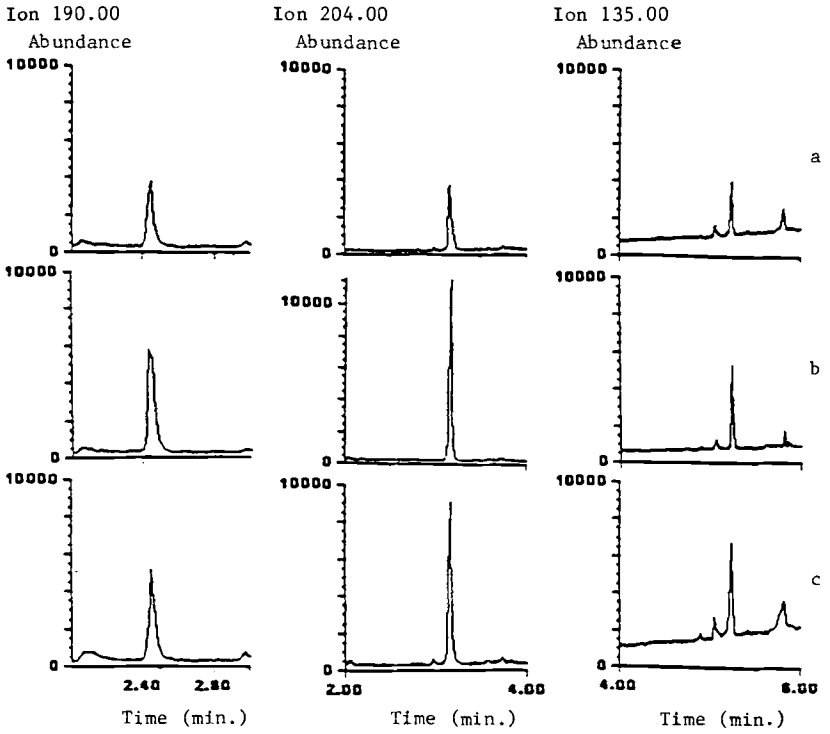

FIG. 5—*Ion chromatograms showing the intensities of the weakest ions* (m/z *190, 204, and 135 for amphetamine, methamphetamine, and methylenedioxymethamphetamine, respectively) obtained from a 30-ng/mL* (a) *and* (c) *and a 50 ng/mL* (b) *control. Chromatograms* (a) *and* (b) *were obtained using a 2-mL sample, 40-μL reconstitution, and 2-μL injection volume, while* (c) *was obtained using a 2-mL sample, 20-μL reconstitution, and 2-μL injection volume.*

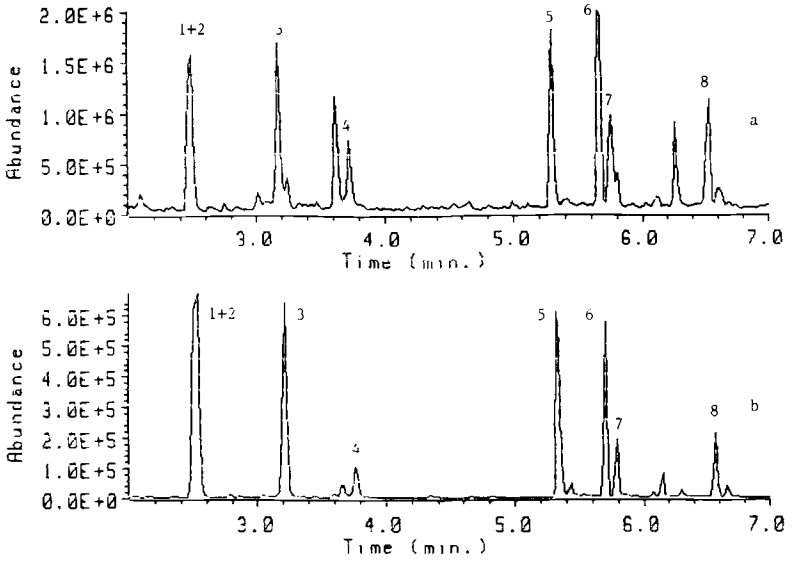

FIG. 6—*Scan* (a) *and SIM* (b) *ion chromatograms of derivatized AMP (1), phentermine (2), METH (3), ephedrine (4), MDMA (5), norpseudoephedrine (6), norephedrine (7), and pseudoephedrine (8).*

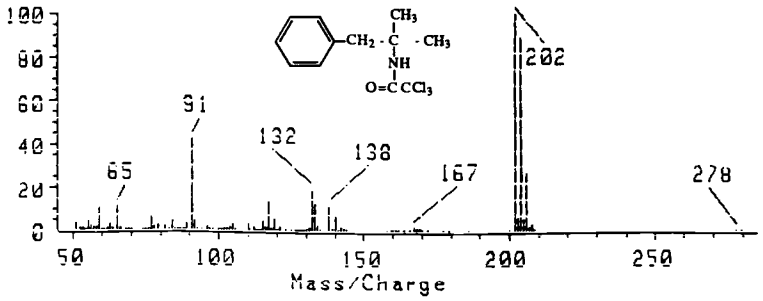

FIG. 7—*Mass spectrum of derivatized phentermine.*

however, that the use of *m/z* 91 should be avoided for the identification of AMP; the presence of phentermine will contribute to the intensity of this ion and result in unacceptable intensity ratios of this ion and other ions monitored for the identification of AMP.

Conclusions

This study showed that with the additional cleanup step introduced, Bond Elute Certify™ is an effective solid-phase medium for extraction of the amphetamine drugs studied. The recoveries are better than 65%, and the reproducibilities show no worse than 5% coefficient of variation when using d_5-AMP as the internal standard for AMP, and d_9-METH for METH and MDMA. The detection limit of 50 ng/mL and a calibration range of 250 to 4000 ng/mL are routinely attainable.

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