TECHNICAL NOTE

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Determination of Amphetamine by HPLC After Acetylation

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ABSTRACT: An analytical procedure has been developed for the HPLC determination of amphetamine by off-line pre-column derivatization. The proposed procedure consists of sample preparation by acetylation of amphetamine with acetic anhydride and a subsequent reversed-phase HPLC separation on an octadecyl silica stationary phase with salt-free mobile phase (tetrahydrofuran, acetonitrile, 0.1% triethylamine in water, 15:15:70 v/v) applying UV-detection. The applicability of the elaborated procedure is demonstrated with results obtained by analysis of real samples seized in the Hungarian black market.

KEYWORDS: forensic science, amphetamine, acetylation, derivatization, determination, high-performance liquid chromatography, substance abuse

The number of illicit amphetamine seizures in Hungary has dramatically increased during the past few years. Almost all of the seized preparations contained the amphetamine synthesized by the Leuckart method, and were diluted with sugars and caffeine. In most of the cases, the quantitative determination of amphetamine was requested. The application of gas chromatography in the analysis of amphetamine has gained much popularity among the forensic scientists because of the well-known advantages of this technique (1–3). Capillary electrophoresis with its high resolving power and versatility has also been applied for the analysis of amphetamine and its derivatives (4,5).

Although amphetamine is a volatile compound for the quantitative determination, HPLC is widely used. Due to the basicity of the compound, in order to obtain symmetric peaks, ion-pairing or ion suppression techniques are often used. The latter demands a rather alkaline mobile phase that can damage the majority of commercially available silica-based chromatographic stationary phases. Polymer (6) and alumina (7) stationary phases with alkaline mobile phases are suitable for HPLC separation of amphetamines, but the separation efficiency of these phases is not as good as that of the silica-based phases. Moreover the mechanical stability of the polymer phases is not satisfactory for operating at high pressure. The separation of amphetamines in a reverse phase HPLC mode with mobile phases containing an acidic buffer was also performed (8,9). Longo et al. (10) reported the separation of amphetamines, ephedrine and caffeine as protonated forms, on a silica-based C18 stationary phase in which residual silanols were electrostatically shielded. The authors applied gradient elution with a mobile phase consisting of acetonitrile and 0.02 M phosphate buffer with pH = 3.8. The amphetamine could be detected at 220 nm (10). Moderately alkaline aqueous solutions as mobile phase components were also applied for reversed phase HPLC of amphetamines (11,12). Jane (13) described the application of a silica column with an aqueous methanolic solvent containing ammonium hydroxide and/or ammonium nitrate, for the analysis of amphetamines and other basic drugs. Enantiomers of amphetamines were resolved on chiral stationary phases by using mobile phases with neutral buffers as well as acidic buffers (14,15). Achari et al. (16) applied normal phase HPLC for separation of amphetamine and other drugs. The application of reversed phase ion-pair chromatography with a mixture of acetonitrile and 0.05 M phosphate buffer containing 0.005 M octansulfonic acid sodium salt at pH = 3.5 assures the reliable determination of amphetamine as well as other nitrogen-containing controlled substances (17,18). However, for routine analysis this system is not advantageous because of the buffer content of the mobile phase. The equilibration of the chromatographic phases prior to the analysis is an inevitable "waste of time" operation that increases the specific time of analysis as well as to flush the system salt-free after finishing the daily work in order to avoid the blocking of capillary tubes with the precipitated salt. The overnight running of the mobile phase is not economical as far as depleting the solvent delivery system and the solvent consumption are concerned. To overcome the disadvantages mentioned above, the application of the reversed phase HPLC system with salt-free mobile phase seems to be an alternative solution. The determination of amphetamine by HPLC with UV-detection has not gained much popularity due to the low absorbance of the compound (19). Although the compound has significantly higher response in the low wavelength range (about 210 nm) in an acidic solution than in the higher range, this merit can not always be utilized in HPLC. The mobile phase needed for an appropriate separation is not necessarily transparent in the low wavelength range. For example, tetrahydrofuran is not transparent below 212 nm, and its transparency reaches 50% at 245 nm which does not allow appropriate sensitivity for detection in the low wavelength range. In fact, the selectivity of the UV-detection is better in the high wavelength range than in the low one. Improvements in the detection limits are possible by derivatization. For the derivatization, 9-fluorenylmethyl chloroformate (19),

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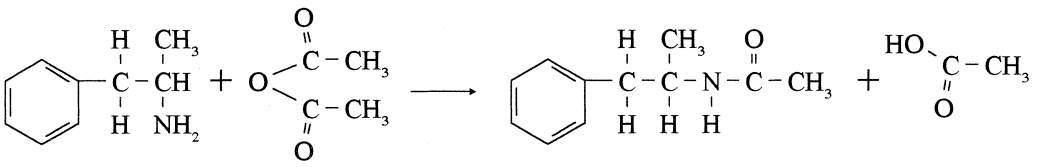

FIG. 1—*Acetylation of amphetamine with acetic anhydride.*

sodium 1,2-naphtoquinone 4-sulfonate (20,21), 3,5-dinitrobenzoyl chloride (22,23), o-phthalaldehyde (24), and 2-naphthoyl chloride (25) were applied as reactants. One of the aims of this study was to develop a simple procedure for the derivatization of amphetamine, and to quantify the derivative formed by reversed phase HPLC using a salt-free mobile phase. For derivatization of amphetamine, the acetylation with acetic anhydride was chosen because the procedure is simple (Fig. 1). Moreover, acetic anhydride is easily obtainable at a reasonable price. The effects of different parameters on the conversion of amphetamine were investigated. Another purpose was to extend the applicability of the HPLC system used in our laboratory for the routine quantification of other controlled substances to the analysis of amphetamine as a derivative. For the reliable determination of MDMA, MDE, cocaine, LSD and heroin in illicit preparations, an HPLC phase system consisting of an ordinary silica-based C18 stationary phase and a mobile phase with components of tetrahydrofuran, acetonitrile and 0.1% triethylamine in water, were introduced with excellent results. The description of results in the analysis of compounds mentioned above appears in a forthcoming paper. It is true that the presence of triethylamine in the mobile phase is not necessary for the analysis of N-acetylamphetamine. The application of the same mobile phase components for all of the target compounds of the analysis is more convenient in the routine work than changing the contents of the mobile phase reservoirs unless it is inevitable.

Experimental

Reagents and Solvents

The acetonitrile and tetrahydrofuran used for HPLC separation were of Lichrosolv grade (Merck, Germany). Triethylamine of synthesis grade (Merck, Germany) and double distilled water were applied. The derivatization agent was acetic anhydride of reagent grade (Reanal, Hungary). The sugars (glucose, fructose, lactose, sucrose) and caffeine were obtained from a pharmacy. The amphetamine was received as the sulfate salt from the Division of Narcotic Drugs United Nations Office in Vienna, Austria. The illicit samples investigated were seized by the Hungarian Police.

Instruments

For GC/MS analysis, a Hewlett-Packard 5890 Series II gas chromatograph connected with a 5989A MS Engine mass spectrometer was used. The HPLC separation was accomplished using a Shimadzu 10A chromatograph system with two LC-10AS pumps, a SIL-10A autosampler and an SPD-M10 diodearray UV-detector. The chromatographic control and data handling was effected by CLASS-LC10 V 1.6 (Shimadzu) software. The derivatization was conducted in an ultrasonic bath of KLN G40/41 (KLN Ultraschall GmbH, Germany).

Derivatization

The powdered samples were weighed into HPLC autosampler vials, and 1 mL of acetonitrile, 40 μL of triethylamine and 10 μL of acetic anhydride were then added to each vial. The presence of triethylamine is important in order to eliminate the disturbing effect of acetic acid that forms during the acetylation (26). The vials were capped and then inserted in an ultrasonic bath. The reaction mixtures were sonicated for 15 min at ambient temperature. The samples with insoluble residues after the derivatization, were filtered. For calibration, different amounts of amphetamine sulfate were derivatized. The conversion of amphetamine and the stability of the N-acetylamphetamine during the storage was checked by GC/MS and/or HPLC analysis of the reaction mixtures.

GC/MS Analysis

The gas chromatographic conditions were as follows: carrier gas, helium; column, HP-5 fused silica capillary (film thickness: 0.25 μm) 15 m × 0.25 mm I.D.; injector temperature, 250°C; oven temperature program, 80°C (2 min), ramped at 20°C/min to 260°C (10 min); MS interface temperature, 280°C; injection volume, 1 μL of reaction mixture.

Electron impact mass spectra were recorded in the m/z range of 40 to 400. The ion source temperature was 200°C.

HPLC Analysis

The chromatographic separation was done in a reverse phase mode by using a mixture of acetonitrile-tetrahydrofuran-0.1% triethylamine in water (15:15:70 v/v) as the mobile phase at a flow rate of 1.5 mL/min, and a 25 cm × 4 mm I.D. column, packed with chemically bonded octadecyl silica (BST Rutin 10 C18, BST, Hungary) as stationary phase. The chromatogram was monitored by UV-detection at wavelengths of 220 nm and 260 nm, respectively. The UV-spectra were recorded from 200 to 300 nm. From the sample solutions, volumes of 10 μL were injected.

Study of Effects of Sugars and Caffeine on Derivatization

In order to investigate how the derivatization might be effected by the most commonly encountered diluents of illicit amphetamine preparations, mixtures of 5 mg amphetamine sulfate, 50 mg sugar, and 10 mg caffeine, respectively, were derivatized. The sugars selected for the experiments were glucose, fructose, lactose and sucrose.

Study of Stability of N-acetylamphetamine

The stability of the N-acetylamphetamine during the storage in a refrigerator at 10°C was investigated both in the unchanged reaction mixture and after dilution of the mixture to double volume with water. In some cases, the addition of water was advantageous

because the sugars and water-soluble additives dissolve and filtration can be avoided. The filtration might cause waste. The N-acetylamphetamine contents of the stored samples were checked by GC/MS every third day for 9 weeks.

Determination of Linear Range and Proportional Error of Procedure

For the determination of the linear range of the procedure, different amounts of amphetamine sulfate, up to 15 mg, were derivatized. In order to investigate the proportional error of the procedure, 50 mg amounts of glucose were spiked with different amounts (1.02, 3.06, 5.10, 7.14 and 10.20 mg) of amphetamine sulfate and derivatized. The same amounts of amphetamine sulfate were derivatized in the absence of glucose.

Determination of Limit of Detection of N-acetylamphetamine by GC/MS and HPLC

Derivatized mixtures with known N-acetylamphetamine contents were diluted and analyzed. The amount of N-acetylamphetamine that resulted in a signal to noise ratio equal to 3, was regarded as the limit of detection.

TABLE 1—*Factors examined in ruggedness tests and their values.*

Factors	Maximum Value	Method Value	Minimum Value
Composition of acetylation solvent, ratio of acetonitrile to triethylamine (v/v)	1010:45	1000:40	990:35
Volume of acetic anhydride	12 µL	10 µL	8 µL
Volume of acetylation solvent	1010 µL	1000 µL	990 µL
Reaction time	18 min	15 min	12 min

Ruggedness Testing

For studying the ruggedness of the derivatization procedure, the Plackett-Burman method (27,28) was applied by using two-level eight experiment designs. Four factors (composition of acetylation solvent, volume of acetylation agent, volume of acetylation solvent and reaction time) were examined in the ruggedness tests. The remaining three factors were assigned to the so-called "dummies" whose effects are usable for the calculation of experimental error. The factors and their levels are described in Table 1. The maximum and minimum values were selected to cover a reasonable range in which the change of method values can be expected owing to the inadequate control of the experimental conditions. In each experiment, 5 mg of amphetamine sulfate was derivatized. The reaction mixtures were analyzed by HPLC. The effects of factors were calculated by using the peak areas of N-acetylamphetamine. The experiments were evaluated graphically by half-normal plotting the effects of factors as applied previously (17).

Results and Discussion

GC/MS Analysis

Total ion chromatograms obtained by the analysis of a typical illicit sample before and after acetylation are shown in Fig. 2, respectively.

The upper chromatogram of Fig. 2 demonstrates the separation of an extract obtained by acetonitrile/triethylamine (100:4 v/v) from an illicit amphetamine sample, where peaks related to amphetamine, caffeine and N,N-di(β-phenilisoprophyl)-amine (DPIA) are present. The lower chromatogram shows the separation of the acetylated extract. The amphetamine could not be detected, and the N-acetylamphetamine formed appears as a peak at a retention time of 6.08 min while the caffeine and DPIA stayed unchanged.

The electron impact (EI) mass spectrum of amphetamine and N-

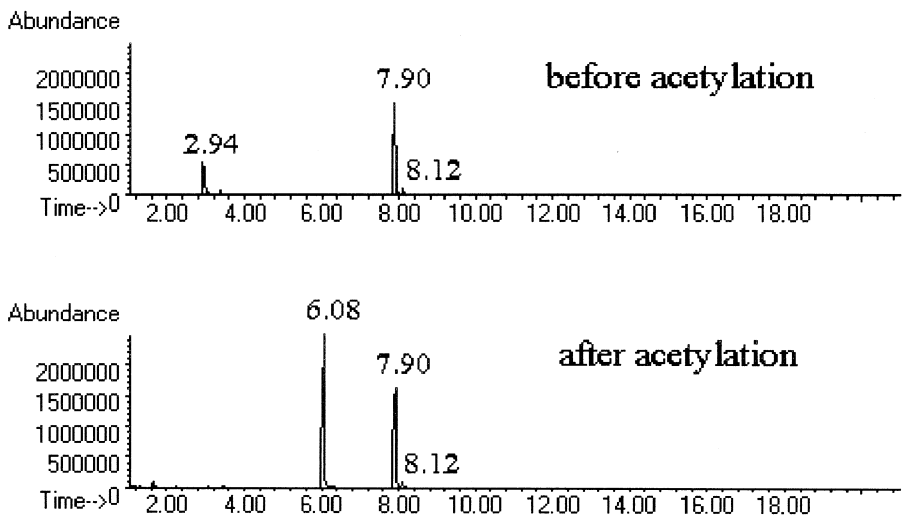

FIG. 2—*Total ion chromatogram of an extract prepared by mixture of acetonitrile-triethylamine (100:4 v/v) from an illicit amphetamine powder (upper chromatogram) and the same sample after acetylation with acetic anhydride (lower chromatogram). Retention times (min): amphetamine—2.94; N-acetylamphetamine—6.08; caffeine—7.90; N,N-di(β-phenilisopropyl)-amine*—8.12. *Characteristic byproduct of the Leuckart-synthesis.*

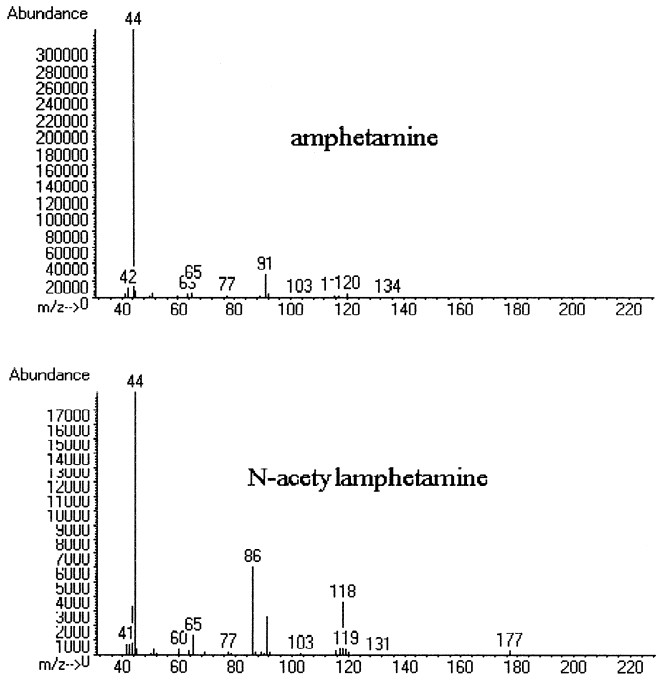

FIG. 3—*EI mass spectrum of amphetamine and N-acetylamphetamine.*

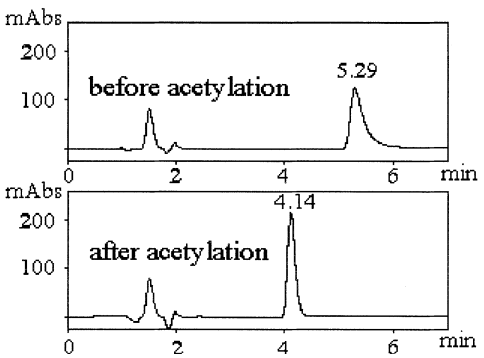

FIG. 4—*Chromatograms obtained by HPLC analysis of an illicit amphetamine sample before and after acetylation, respectively (λ = 260 nm) (for sample preparation and chromatographic conditions see text). Retention times (min): amphetamine—5.29; N-acetylamphetamine—4.14.*

acetylamphetamine are shown in Fig. 3. In the EI mass spectrum of amphetamine, the base peak (m/e: 44) is accompanied by other fragments of very small intensity. The mass spectrum of N-acetylamphetamine next to the base peak (m/e: 44), shows the peak referring to the molecular ion (m/e: 177) as well as some other fragments (m/e: 86, 118) with significantly high intensities.

HPLC Analysis

Typical chromatograms monitored at 260 nm, of a nonacetylated and acetylated amphetamine sample, respectively, are shown in Fig. 4. In chromatograms monitored at 220 nm, the peaks corresponding to amphetamine and N-acetylamphetamine, respectively, appear with higher intensities and the peaks referring to the excess of acetic anhydride and triethylamine in the derivatized sample, are also visible in the retention time range of 1.0 to 1.5 min. The chromatograms detected at 260 nm are much more "clear" than those detected at 220 nm which can be interpreted by the different selectivities obtained at the two wavelengths.

As can be seen in Fig. 4 the amphetamine has an asymmetrical peak with tailing contrary to that of N-acetylamphetamine. The UV-spectra of compounds recorded by the diode array detector is shown in Fig. 5. The two compounds have nearly the same UV-spectrum. The spectra show that the local maximum at 260 nm is more intense for the N-acetylamphetamine than for that of the amphetamine.

Even though the aqueous mobile phase component (0.1% triethylamine in water) itself is rather alkaline (pH> 10), it might have less aggressive effects against the silica when it is mixed with the organic components, because the system has been found to be stable in routine use for several months.

Stability of the N-acetylamphetamine

The derivative was found to be stable at least for 9 weeks at 10°C in the dark even in the presence of water.

Study of Effects of Sugars and Caffeine on Derivatization

A good agreement was found between the peak areas of N-acetylamphetamine when each sugar and caffeine was investigated. Therefore, these frequently occurring components of illicit amphetamine preparations do not disturb the derivatization of amphetamine.

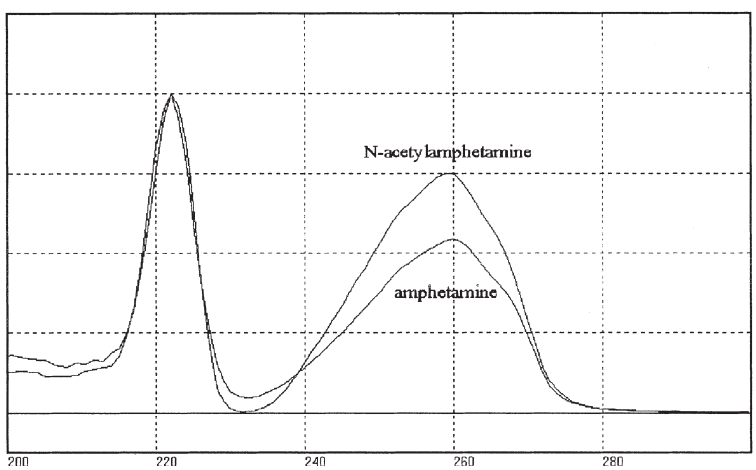

FIG. 5—*UV-spectrum of amphetamine and N-acetylamphetamine (Solvent: HPLC mobile phase; for details see text).*

Linear Range of Procedure

Linear relationship could be established between the peak areas of N-acetylamphetamine (*A*) and amounts of amphetamine sulfate derivatized (*m*) in the range of 0.4 to 11 mg at the conditions of derivatization and HPLC analysis, described in the Experimental section. The calculated correlation equation with the standard deviation of constants and correlation coefficient (*r*) is as follows: $A = (257550 \pm 1948) * m + (66646 \pm 11438)$; $r = 0.999$, where "*A*" is the peak area of N-acetylamphetamine and "*m*" is the amount of amphetamine sulfate derivatized.

Determination of Proportional Error of Procedure

In Fig. 6 the amounts of amphetamine obtained for samples containing sugar (*Y*), are plotted against the amounts of amphetamine obtained for sugar-free samples (*X*). A good linearity can be observed in the plot. The calculated correlation equation with the standard deviation of constants and correlation coefficient (*r*) are as follows: $Y = (1.003 \pm 0.008) * X + (0.054 \pm 0.048)$; $r = 0.999$, where "*Y*" is the amount of amphetamine obtained for sugar-containing samples and "*X*" is the amount of amphetamine obtained for sugar-free samples. According to the results the proportional error of the procedure is not significant in the range studied.

Limit of Detection

The limit of detection was 10 μg by HPLC analysis at 260 nm and 5 ng by GC/MS in scan mode, respectively.

Ruggedness Testing

According to the results of graphical evaluation, the effects of the examined factors (Table 1) did not exceed the experimental error of the procedure, which indicates that none of the investigated factors had significant influence on the quantitative determination in the range studied.

Determination of Amphetamine in Illicit Sample

A caffeine-containing illicit amphetamine powder was analyzed by both reverse-phase ion-pair chromatography and the proposed procedure applying acetylation. By the procedure based on reversed phase ion-pair chromatographic separation of a sample prepared by extraction of the powder with acidic aqueous mobile phase, 53.25 ± 1.83% amphetamine base could be determined. The determination applying the proposed method based on the acetylation resulted in 54.53 ± 1.45% amphetamine base. In both experiments seven parallel determinations were run. In performing the appropriate statistical tests, no differences can be established between the results obtained by the two procedures.

Summary

An analytical procedure based on the acetylation of amphetamine and subsequent reversed-phase HPLC separation of the derivative applying a salt-free mobile phase has been described for the determination of amphetamine. The proposed procedure is an alternative to other analytical procedures, and widens the scale of tools applicable in the analysis of amphetamine.

The advantages of the elaborated procedure are as follows: the N-acetylamphetamine can be detected at 260 nm with higher sensitivity than the amphetamine; owing to its basicity, the derivative has significantly better peak shape than the amphetamine has even by using an ordinary, basically nondeactivated silica-based C18 column; the same HPLC system can be used for the determination of amphetamine as one applicable for the determination of other controlled substances such as MDMA, MDE, cocaine, LSD and heroin. The most common additives of illicit amphetamine preparations, including sugars and caffeine, do not affect the quantification of amphetamine as an acetylated derivative. The acetylation of amphetamine can be a useful tool in the identification of the compound by GC/MS. By a complex evaluation of chromatographic and spectral data obtained by the analysis of underivatized and sub-

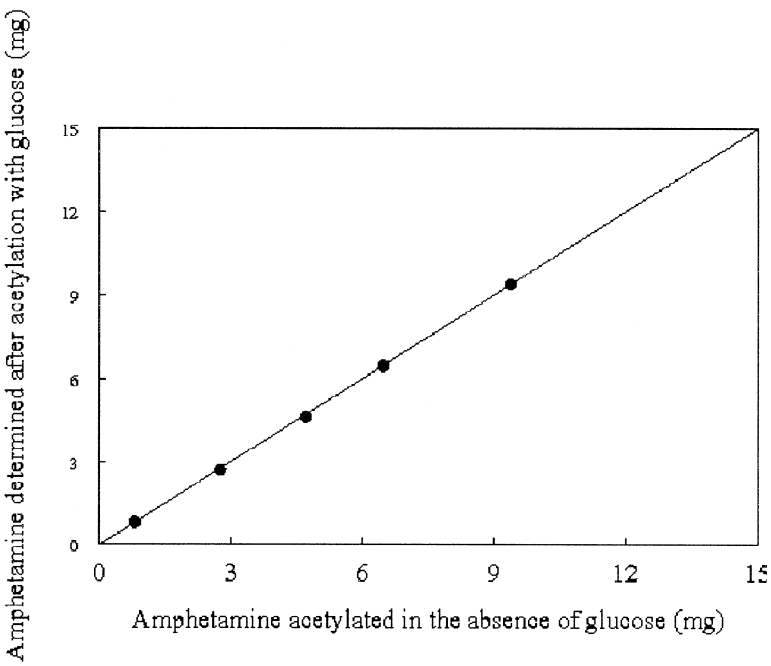

FIG. 6—*Plot for determination of proportional error of procedure.*

sequently derivatized samples, reliable identification is possible even though the amphetamine is present at a low concentration. The proposed procedure has been found to be rugged in a reasonable range with respect to the derivatization conditions evaluated. The procedure was applied with good results in the International Proficiency Testing of National Laboratories for Forensic Drug Analysis, organized by the United Nations Drug Control Programme.

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