Comparison of Fluorometric Procedures for Assay of Amphetamine

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Abstract □ Five methods for the fluorometric determination of amphetamine were examined. The acetylacetone–formaldehyde procedure for colorimetric analysis of amphetamine was modified, and its accuracy and precision compared favorably with those obtained with a fluorescamine procedure. Only these two methods were suitable for the trace analysis of amphetamine. The formaldehyde–sulfuric acid method is less sensitive and slightly inferior in precision to these two procedures. The native fluorescence method is simple but far less sensitive, and the 2,5-hexanedione method was unsuitable for the quantitative analysis of amphetamine.

Keyphrases □ Amphetamine—comparison of five fluorometric methods □ Fluorometry—determination, amphetamine, comparison of five methods

The continued use of amphetamine and its derivatives as drugs of abuse stimulates continued interest in the evaluation of selective and sensitive methods for the detection and determination of these compounds. In particular, there is interest in the fluorometric determination of amphetamines because of its great sensitivity.

Chemically, amphetamine is a primary alkyl amine having weak native fluorescence. Therefore, most fluorometric procedures for its determination depend upon reactions involving the primary aliphatic amino group. Since many methods of this type have recently appeared, it was decided to undertake the present comparison and, where appropriate, modification of the available methods. This information should be useful in selecting the fluorometric procedure most suited for a particular situation.

EXPERIMENTAL

Fluorescence spectra (uncorrected) were taken on a spectrophotofluorometer[1] equipped with a 150-w xenon source, R-106 photomultiplier, strip chart recorder[2], and 10-mm quartz cells. The instrument was operated in the ratio recording mode, and the slit settings allowed for 6- (excitation) and 10- (emission) nm bandpass.

Dextroamphetamine sulfate[3], sodium amobarbital[4], reagent grade sulfuric acid[5], boric acid[5], hydrochloric acid[5], acetic acid[5], sodium acetate[5], sodium chloride[5], sodium hydroxide[5], reagent grade 2,5-hexanedione[6], acetylacetone[6], *n*-pentanol[6], 40% formaldehyde[6], spectroquality acetone[6], spectroquality ethanol[6], spectroquality chloroform[6], spectroquality dioxane[6], spectroquality acetonitrile[6], and fluorescamine[7] were used without further purification.

The pH 9 borate buffer was prepared by titrating 0.2 *M* boric acid with 0.1 *M* sodium hydroxide. Solutions of required concentration were prepared by dilution with distilled deionized water.

Procedure I—This method is based on the native fluorescence of amphetamine. Amphetamine shows native fluorescence in 0.1 *N* sulfuric acid at the wavelength combination of 260 (excitation) and 282 (emission) nm (1). This observation was confirmed in the present experiments, although the intensity was found to be very weak. Amphetamine did not show fluorescence in other solvents such as distilled water, 0.1 *M* sodium hydroxide, and ethanol.

Procedure II—A procedure of Wachsmuth and Koeckhoven (2) for the colorimetric analysis of amphetamine was used. Amphetamine reacts with 2,5-hexanedione in acetic acid to form 1-(1′-phenylisopropyl)-2,5-dimethylpyrrole, which gives a green fluorescence under UV radiation. The aim was to make this method more sensitive by using the fluorescence rather than absorption. To a 0.5-ml aliquot of amphetamine sulfate in 1 *N* acetic acid, 0.2 ml of 2,5-hexanedione and 5.0 ml of pentanol were added. The mixture was heated in a stoppered tube on a boiling water bath for 50 min. After cooling to room temperature, its fluorescence was observed. The wavelength combination was found to be 400/490 nm (excitation/fluorescence).

Procedure III—A colorimetric procedure of Wachsmuth and Koeckhoven (3) was modified. In this procedure, amphetamine reacts with acetylacetone and formaldehyde to give a colored 1,4-dihydrolutidine derivative. This compound was also found to be fluorescent. A mixture of 2.0 ml of amine solution in 0.1 *N* sulfuric acid, 4.0 ml 50% sodium acetate, 0.25 ml acetic acid, 0.15 ml acetylacetone, and 0.25 ml 35% formaldehyde was heated at 60° for 20 min with occasional stirring. After cooling, it was quantitatively transferred to a 10-ml volumetric flask and diluted to volume with 50% ethanol and its fluorescence was observed. A linear relationship between relative fluorescence intensity (395/475 nm) and concentration was obtained for amphetamine sulfate concentrations below 10 ppm (3.7×10^{-5} *M*). The lower limit of detection (that concentration of amphetamine sulfate giving a signal equal to twice that of the solvent blank) of amphetamine sulfate by this method was found to be 2.3×10^{-9} *M*.

Procedure IV—A general fluorometric procedure for assaying primary amines using fluorescamine reagent was reported (4, 5). This procedure was applied to amphetamine determination without modification. The excitation and emission maxima of the fluorophore lie at 395 and 475 nm, respectively. A 0.2-ml sample aliquot was buffered to pH 9 with 3.0 ml of sodium borate buffer. To this, 1.0 ml of a solution of fluorescamine in acetone (24 mg/100 ml) was added. Rapid addition and constant stirring of the solution are essential for optimal results. The mixture was diluted to 10.0 ml with borate buffer. The calibration curve drawn at 395/475 nm was linear below 10 ppm amphetamine sulfate (3.7×10^{-5} *M*). The lower limit of detection of amphetamine sulfate was 5.1×10^{-11} *M*, employing derivatization with fluorescamine.

Procedure V—A fluorogenic reaction (6) was used without modification. A 1.0-ml sample aliquot in 0.1 *N* sulfuric acid mixed with 1.0 ml formaldehyde–concentrated sulfuric acid reagent (1:20) and 5.0 ml concentrated sulfuric acid was heated for 15 min at 60°. It was cooled and diluted to 50 ml; then, after 15 min, the intensity of fluorescence was measured at 385/445 nm. The calibration curve constructed at the same wavelength combination was linear below 150 ppm amphetamine sulfate (5.5×10^{-4} *M*). A lower limit of detection of 7.3×10^{-6} *M* amphetamine sulfate was obtained.

Determination of Amphetamine Mixtures—Three synthetic mixtures of dextroamphetamine sulfate and sodium amobarbital were prepared. Each mixture was dissolved in 0.1 *N* sulfuric acid. Amobarbital was removed by extraction with chloroform. Stock

[1] Perkin-Elmer model MPF-2A, Perkin-Elmer Corp., Norwalk, Conn.
[2] Hitachi model QPD-33.
[3] Smith Kline and French, Philadelphia, Pa.
[4] Eli Lilly and Co., Indianapolis, Ind.
[5] Mallinckrodt Chemical Works, St. Louis, Mo.
[6] Matheson, Coleman and Bell Inc., East Rutherford, N.J.
[7] American Instrument Co., Silver Spring, Md.

Table I—Analysis of Amphetamine in Mixtures

Procedure	Mixture[a]	Amphetamine Found[b], mg	Error, %	Standard Deviation[c], mg
III	1	4.85	−3.0	
	2	5.10	+2.0	
	3	5.25	+5.0	±0.11
IV	1	4.80	−4.0	
	2	4.80	−4.0	
	3	4.95	−1.0	±0.13
V	1	4.85	−3.0	±0.18

[a] Each mixture contained 5 mg amphetamine sulfate and 5, 10, and 25 mg amobarbital in Mixtures 1, 2, and 3, respectively. [b] Analysis done in duplicate. [c] Calculated from 10 measurements.

solution (5.0 ml) was pipeted into a 100-ml separator and extracted three times with 20-ml portions of chloroform. The organic phase was discarded and the aqueous phase was diluted with 0.1 *N* sulfuric acid to a region of linear response for the method under consideration. A blank solution was prepared by treating 5.0 ml of 0.1 *N* sulfuric acid in a manner similar to that for the sample. The sample reading was corrected for the blank. Analysis of amphetamine was done using Procedures I–V (Table I).

Determination of Amphetamine in Urine—Known amounts of dextroamphetamine sulfate were added to the normal urine *in vitro* and the samples were kept in a refrigerator. Amphetamine was isolated from urine by extraction of alkaline urine with chloroform and back-extraction with 0.1 *N* sulfuric acid. To the urine sample, 1 *N* sodium hydroxide was added until the pH was about 10. Then 5.0 ml of this was saturated with sodium chloride and extracted with 20 ml and three quantities each of 15 ml of chloroform. Amphetamine was reextracted from the combined chloroform layers into 0.1 *N* sulfuric acid (3 × 10 ml). The acid extract was diluted with 0.1 *N* sulfuric acid to a volume suitable for adequate fluorescence and analyzed by one of the procedures (Table II). A blank was determined in a similar way by treating 5.0 ml of drug-free urine, and the sample reading was corrected for the blank. The use of large quantities of chloroform for extraction minimized emulsion formation.

RESULTS AND DISCUSSION

Procedure I was studied recently in great detail (1) and was not investigated further. This procedure, although simple, is not sensitive. Procedure II gives weak and unstable fluorescence, and the concentration–fluorescence relationship is not linear even at low concentrations. It is also time consuming and was not investigated further. Procedures III and IV are quite sensitive and may be recommended for the trace analysis of amphetamine. Procedure IV is more sensitive, simpler, and less time consuming but the reagent fluorescamine is considerably more expensive than the acetylacetone and formaldehyde used in Procedure III. In Procedure III, it is advisable to take readings after a fixed period of time since the fluorescence intensity slowly increases with time. The intensity is stable for several hours in Procedure IV.

Procedure V is quite satisfactory from the standpoint of stability and reproducibility, but its sensitivity is poor and the precision is slightly inferior to that of the Procedures III and IV. However, it gives higher fluorescence intensity than that obtained from the native fluorescence method. The blank is negligible and the calibration range is linear between 25 and 150 ppm. The fluorescence intensity is stable for a few hours. The fluorophore has two excitation peaks (at 272 and 385 nm), which offer a large wavelength range for excitation. This could be useful in avoiding certain interferences (selective excitation).

Table II—Analysis of Amphetamine in Urine

Procedure	Sample[a]	Amphetamine Sulfate Found[b], ppm	Error, %
III	1	46.5	−7.0
	2	92.0	−8.0
IV	1	46.5	−7.0
	2	90.0	−10.0
V	2	92.5	−7.5

[a] Samples 1 and 2 contained 50 and 100 ppm of amphetamine sulfate, respectively. [b] Analysis done in duplicate.

The presence of twofold excesses of amobarbital does not interfere in these methods. Most amphetamine–amobarbital drug combinations contain more than fivefold amounts of amobarbital, requiring its removal by extraction with chloroform.

About 90% of amphetamine was recovered from urine by the extraction procedure employed. In the present work, amphetamine was added to the urine *in vitro* because of legal complications; however, the situation may be little different when doing analysis of urine from a subject who has received the drug. A few points should be borne in mind. For example, excretion of amphetamine is markedly dependent upon urinary pH, being greatly increased in acid urine (7). After large doses, amphetamine may be detected in urine for several days (7). The presence of *p*-hydroxyamphetamine (a metabolite) will interfere in the methods; however, this metabolite could be separated by TLC (7) prior to the amphetamine determination.

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