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Note

Procedure for the determination of amines in small urine samples

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Aliphatic amines are common components of biological systems and their accurate measurement is often a requirement for characterizing biological processes and for clinical purposes. For example, elevated urinary levels of aliphatic diamines have been associated with certain carcimonas [1].

Analytical methods for the determination of amines present in urine must accommodate their highly polar nature, water solubility and a large number of interfering compounds. Previous determinations of amines in biofluids have utilized direct analysis and derivatization and measurement after clean-up of the sample.

Direct methods lower analysis time and reduce systematic errors. Methylamine, dimethylamine and trimethylamine were determined in rat urine using headspace sampling followed by gas chromatography (GC) with nitrogen-specific detection [2]. Although sensitivity was adequate for the μg/ml levels usually observed, ammonia could interfere with the methylamine measurement. Another method used GC of an ultrafiltrate of human serum [3]. This approach utilized a special packed column and ammonia-spiked carrier gas but was unable to measure methylamine in the presence of equal amounts of dimethylamine.

In the majority of reports, the amines have been isolated from the biofluid, derivatized with dinitrofluorobenzene, and the derivatives have been separated by paper [4] or thin-layer chromatography [5–7]. While useful, these methods are time-consuming and interferences can arise from the reaction product of dinitrofluorobenzene and ammonia present in urine. Other promising instrumental techniques for measuring amines using electrochemical detection [8] or gas chromatographic–mass spectrometric (GC–MS) [9] methods have not been applied to urine or other biological materials.

A procedure has been developed in this laboratory to determine trace levels of

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primary aliphatic amines present in small volumes of urine. It includes a simple bonded porous silica column clean-up and direct reaction of the amines in the cleaned-up eluate with pentafluorobenzaldehyde (PFB) to form imine derivatives. The entire procedure, from sample clean-up to imine measurement, can be completed in less than 1 h. GC–MS is used to ensure that all imine isomers will be measured separately, with no interferences.

EXPERIMENTAL

Reagents

The hydrochlorides of methyl-, ethyl- and *n*-propylamine and putrescine and cadaverine were obtained from Fluka (Hauppage, NY, U.S.A.), PFB from PCR (Gainesville, FL, U.S.A.), methyl-[$^2H_3$]amine hydrochloride from MSD Isotopes (St. Louis, MO, U.S.A.), organic-free water from J.T. Baker (Phillipsburg, NJ, U.S.A.) and HPLC-grade acetonitrile from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

Standard solutions of amines in water at the 1 mg/ml level were freshly prepared each week. The derivatizing solution, consisting of 10 mg/ml PFB in acetonitrile, was stored in a freezer to retard decomposition.

Methodology

A 0.5-ml sample of undiluted urine was spiked with 5 μg of methyl-[$^2H_3$]amine internal standard, mixed, and passed through a 1-ml cartridge containing 100 mg of octadecyl (C_{18}) bonded porous silica (J.T. Baker) that had been previously conditioned for reversed-phase chromatography by passing 1 ml methanol followed by 1 ml water through the cartridge. This step removed non-polar interferences present in the urine by adsorption onto the C_{18} bonded silica.

The urine effluent containing the polar constituents was combined with 200 μl of 0.1 *M* borate wash of the cartridge and this mixture was transferred to a 5-ml Reacti-vial (Pierce, Rockford, IL, U.S.A.). Acetonitrile (0.5 ml) containing 5 mg PFB was added, the mixture heated to 80 °C and held at that temperature for 30 min. This step converted the primary amines present in the eluate to PFB-imines [9].

After the reaction mixture had cooled to ambient, 0.5 ml of water were added to the vial and the imine derivatives were adsorbed from the reaction mixture by passing this solution through a second C_{18} cartridge that had been conditioned as before. The cartridge was washed with 1 ml water to remove any remaining water-soluble interferences and dried by aspirating room air through the cartridge for 5 min. The imine derivatives were eluted with 200 μl of ethyl acetate. This convenient isolation procedure using C_{18} bonded silica was developed to obtain an extract of the imine derivatives free from biological materials which could be deleterious to analytical instruments.

Instrumentation

A Finnigan Model 4000 combination GC–MS system with an INCOS 2300 data system was used for all imine measurements. The GC–MS system was operated

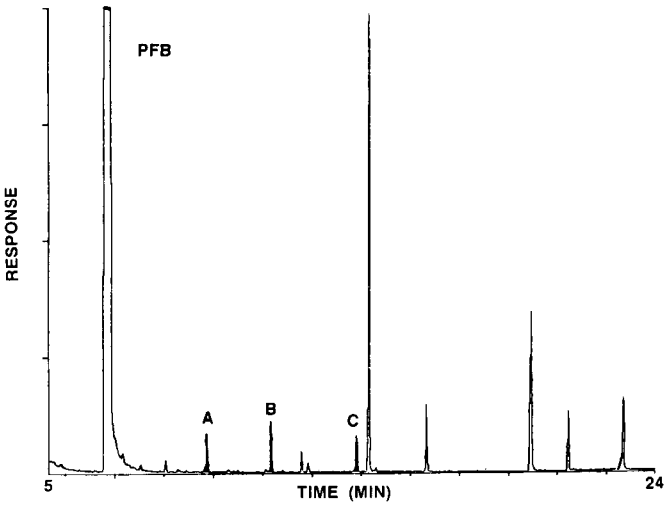

Fig. 1. GC–MS reconstructed-ion chromatogram of an extract from a treated urine sample fortified with 10 μg/ml methyl- (A), ethyl- (B) and *n*-propylamines (C).

in the electron-impact mode using either full-scan (*m/z* 45–400) or multiple-ion detection (*m/z* 208+211). The instrument was fitted with a 30 m×0.25 mm I.D. DB5 (J & W, Rancho Cordova, CA, U.S.A.) fused-silica capillary column which was led directly into the mass spectrometer ion source; helium was the carrier gas at 25 cm/s linear velocity. For each analysis, 1.8 μl of the ethyl acetate eluate were injected splitless at 45 °C, held for 6 min, then programmed to 250 °C at 6 °C/min. A calibration table, generated using water samples containing varying ratios of unlabeled to labeled methylamine, was used for all quantifications.

RESULTS AND DISCUSSION

A typical GC–MS reconstructed-ion chromatogram resulting from application of the method to a sample of urine fortified with three primary alkyl amines is shown in Fig. 1. The responses for each imine derivative are cleanly separated from the unreacted PFB. Common urine constituents such as ammonia, di- and trimethylamine do not interfere since they will not react with PFB.

The clean-up of the urine prior to amine derivatization was essential for removal of the non-polar constituents that cause poor chromatographic separations. Although GC–MS techniques could still be used to measure the PFB-imines, repeated injections of the crude extract caused rapid deterioration of the GC–MS performance. The clean-up step is simple and required little additional time.

Single- and multiple-ion monitoring techniques can be used to increase sensitivity and to distinguish co-eluting isomers. For example, the sensitivity is high because the mass spectra of PFB-imines are dominated by α-cleavage, giving rise to the intense *m/z* 208 base peak. As little as 0.05 μg/ml methylamine in pure water could be detected. Although electron-capture detection would give comparable sensitivity, the excess PFB would cause detector saturation problems thereby

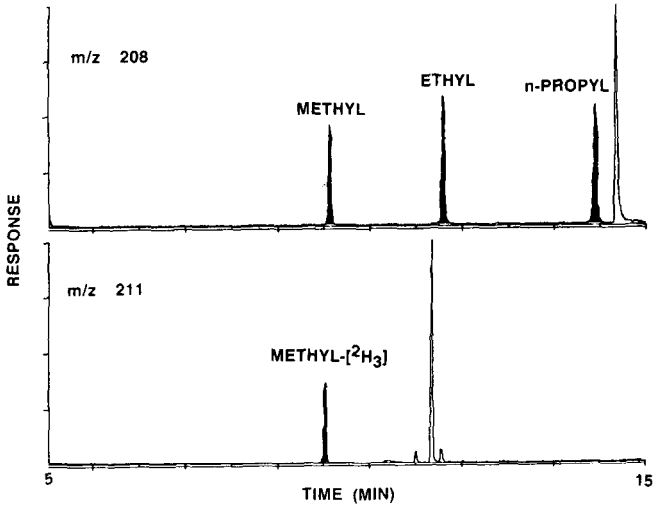

Fig. 2. GC-MS multiple-ion monitoring profile chromatogram of an extract from a treated urine sample fortified with 10 μg/ml [$^2H_3$]methyl-, methyl-, ethyl- and *n*-propylamines.

interfering with PFB-imine measurements. Co-eluting isomers such as *n*-propyl- and isopropylamines can be distinguished because a methyl group in the α-position of the amine alkyl group increases the base peak of the imine derivatives to *m/z* 222.

The best quantitative data are obtained with an isotope-labelled internal standard so that similar partitioning, derivatization efficiency and GC retention behavior can be realized. The PFB derivative of methyl-[$^2H_3$]amine can be measured, free of interference from the unlabeled analyte, at *m/z* 211. The simultaneous detection of methylamine and the deuterated internal standard is shown

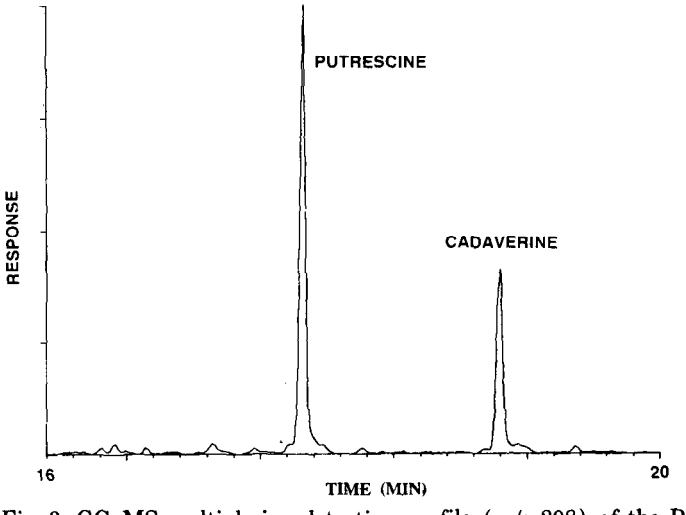

Fig. 3. GC-MS multiple-ion detection profile (*m/z* 208) of the PFB-diimines of putrescine and cadaverine fortified in urine at 10 μg/ml.

in Fig. 2. Response relative to concentration was determined by analyzing pure water containing 10 μg/ml methyl-[$^2H_3$]amine and 50 ng/ml to 10 μg/ml methylamine. A plot of the resulting data showed good linearity and a correlation coefficient of 0.983.

The accuracy of the procedure was tested by spiking a sample of urine from a 38-year-old male with varying amounts of methylamine. The results, listed in Table I, show an average accuracy of 9%.

The reproducibility for lower levels of methylamine was determined by analyzing four aliquots of the unspiked urine sample. The resulting data in show a coefficient of variation of 4% at the low methylamine level of 0.95 μg/ml. Comparable precision was obtained for urine sample volumes as low as 0.1 ml, and the low background levels suggest that amounts as low as 0.1 μg/ml could be determined. The applicability of the method to these small sample sizes may be a necessary feature for some clinical applications and a requirement for potential application to blood and serum studies.

Other primary amines can almost certainly be determined based on interpretation of additional test data. The chromatograms shown in Figs. 1 and 2 illustrate the wide separation between the three amine derivatives, allowing easy measurement. The equivalent responses suggest that the same high sensitivity could also be obtained. Quantification could be achieved by the method of standard additions or by the use of additional labelled standards.

The possible selective and sensitive detection of the aliphatic diamines putrescine and cadaverine is suggested by the data shown in Fig. 3. This chromatogram was obtained from a treated extract of urine spiked with 10 μg of the two diamines. The low background and high sensitivity are typical for primary aliphatic amines analyzed by this method.

This procedure measures the free amine concentration in the sample. To determine total amine content, an initial acid hydrolysis to cleave any amine conjugates would be necessary. The pH of the cleaned-up sample would then have to be adjusted to 10 before derivatization with PFB.

TABLE I

ACCURACY OF THE AMINE DETERMINATION

Urine was spiked with internal standard and analyte, cleaned up, derivatized with PFB, and the isolated imines were measured with GC–MS.

Sample No.	Concentration methylamine★ (μg/ml)	
	Spiked	Found
1	10	9.5
2	10	10.5
3	20	20.4
4	20	19.2
5	30	27.1
6	30	21.2

★Corrected for background level of methylamine.

CONCLUSION

This procedure is straightforward, can be applied to small volumes and is amenable to simultaneous processing of multiple samples. It is not suitable for secondary or tertiary amines.

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