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Determination of trimethylamine and related aliphatic amines in human urine by head-space gas chromatography

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ABSTRACT

A rapid and simple assay procedure employing head-space gas chromatography has been developed for the routine quantification of volatile methylamines and stable trimethylamine N-oxide present in human urine. This assay will enable the rapid screening of patients and aid the diagnosis of fish odour syndrome.

INTRODUCTION

The short-chain aliphatic amines are normal components of human urine which are present in varying amounts dependent upon dietary intake, endogenous production, metabolic capacity and excretory function. Two of these amines are of particular interest because of potential toxicity problems. Dimethylamine may be nitrosated to the reactive dimethylnitrosamine with all its carcinogenic consequencies whereas trimethylamine is associated with the fish odour syndrome.

In healthy individuals the majority of trimethylamine ingested or produced within the body is metabolised and excreted in the urine as its N-oxide, an innocuous non-volatile compound. However, the urine of individuals with fish odour syndrome, a distressing condition with serious psychosocial consequencies, contains large amounts of free trimethylamine which is a pungent volatile substance [1]. The fish odour syndrome is a metabolic disorder in which the enzyme(s) responsible for the N-oxidation of trimethylamine are deficient in their activity. The measurement of both trimethylamine (enzyme substrate) and its N-oxide (enzyme product) in the urine enables an assessment of this enzyme's activity to be made and assists in the diagnosis of fish odour syndrome. Following diagnosis, a patient can be counselled and given practical dietary advice to reduce trimethylamine intake and alleviate the distressing symptoms.

It is now being realised that this condition is more common than previously appreciated and that certain individuals may be carriers of the inherited syndrome, becoming intermittent trimethylaminurics when their already limited enzyme capacities are further compromised [2]. Potential population screening for these problems requires the development of a relatively cheap, reliable, rapid and simple technique for urinary amine quantitation.

Several methods have been described within

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the literature for the measurement of aliphatic amines in biological samples including colorimetric reaction [3–5], paper chromatography [6], thin-layer chromatography [7,8], high-performance liquid chromatography [9,10] and gas chromatography [11–13]. Problems of separation and detection have usually required cumbersome derivatization procedures adding to both time loss and cost. More recently, advantage has been taken of their volatility during sample clean-up but these procedures usually rely on an intermediate step for trapping the volatile amines generated in head-space gas with their subsequent elution before injection ("purge and trap") [14]. In this paper we describe a rapid and simple method whereby the "amine-rich" head-space gas generated above a urine sample is injected directly onto a gas chromatographic column. This technique has been used successfully within our laboratories to analyse over a thousand human urine samples during the past six years.

EXPERIMENTAL

Reagents and chemicals

Methylamine, dimethylamine, trimethylamine, ethylamine, diethylamine and propylamine were purchased as their hydrochloride salts, isopropylamine, *n*-butylamine and isobutylamine as their free bases and trimethylamine N-oxide as its dihydrate (Sigma, Dorset, UK). Methanol, ethanol, acetone, ammonium hydroxide (d_{20}^{20}, 0.880 aq.) and titanous chloride (30%, w/v) were analytical-grade reagents (British Drug House, Merck, Dorset, UK). The powdered hydrochlorides and trimethylamine N-oxide dihydrate were stored in dessicators over silica gel and when required were heated in an oven at 105°C for 2 h prior to use. Isopropylamine, *n*-butylamine and isobutylamine were dried over granular calcium chloride. Aqueous stock solutions (1 mg free base per ml) were found to be stable for several months when stored at −20°C.

Sample collection

Urine samples (0–24 h) were collected from five healthy volunteers under normal dietary conditions, into airtight plastic bottles containing hydrochloric acid (6 *M*; 15 ml) . The total volumes were recorded and aliquots (50 ml) were frozen (−20°C) until analysis. Samples were only thawed once for analysis and then discarded. Multiple analyses required the initial concurrent storage of multiple aliquots.

Sample preparation

Urine (5 ml), spiked with 0.2% (v/v) isopropylamine (30 μl) as internal standard, was placed into a screw-capped glass vial (15 ml) and pelleted potassium hydroxide (3 g) was added before sealing the vial with an airtight PTFE-lined septum cap and leaving on ice to cool. The vial was then heated in an aluminium block at 90°C for 20 min. After this time, but whilst still within the heating block, head-space gas (2 ml) was withdrawn through the septum of the vial with a disposable syringe (Sherwood Medical, Ballymoney, Northern Ireland) and injected immediately and directly onto the analytical column. Only a single injection of gas was made from each vial, the remaining contents then being discarded.

Trimethylamine N-oxide was measured indirectly after its quantitative reduction as an increase in free trimethylamine [15]. Titanous chloride (30%, w/v; 0.2 ml) was added to urine (2 ml) in a screw-capped vial and left for 30 min at room temperature, after which the reduced urine was diluted ten-fold with distilled water and analysed as described above. All analyses were performed in duplicate.

The use of authentic methylamine, dimethylamine and trimethylamine separately and together in distilled water and in urine permitted the construction of calibration curves (0.1–150 μg/ml) enabling the quantitation of endogenous urinary amines.

Gas chromatography

A Pye Unicam 4500 series gas chromatograph (Pye Unicam, Cambridge, UK) with a flame ionization detector was used for the analyses. The silanized glass column (170 cm × 4 mm I.D.) was packed with 4% (w/w) Carbowax 20M–0.8% (w/w) potassium hydroxide on Carbopack B graph-

itized carbon support (60–80 mesh) (Supelco, Bellefonte, PA, USA). The column was conditioned by injecting 1% (v/v) aqueous ammonium hydroxide (*ca.* 20 × 10 μl) which converts potassium carbonate in the packing to potassium hydroxide thereby minimizing adsorption of the amines to the column and preventing tailing of the peaks. The operating temperatures of the column, injection port and detector unit were 70°C isothermal, 150°C and 200°C, respectively, with a nitrogen carrier gas flow-rate of 60 ml/min.

RESULTS AND DISCUSSION

The nine volatile compounds examined were clearly resolved and the retention times obtained with this system are given in Table I. Analysis of the normal urine samples showed six or seven peaks (ethanol dependent upon previous alcohol consumption) in addition to the internal standard (Fig. 1). The separation between dimethylamine and its isomer ethylamine became incomplete as the column temperature was raised, giving a single peak at 110°C. The presence of ethylamine in urine was not observed by other workers using a similar system (trimethylchlorosilane–potassium hydroxide column) operating at 160°C, presumably owing to its coelution with dimethylamine [16]. Results obtained for healthy volunteers and fish odour syndrome patients are given in Table II.

The correlation of peak-area ratios (component/internal standard) resulting from the injection of methylamine, dimethylamine and trimethylamine standard solutions were statistically significant for all compounds ($r > 0.99$; $P \ll 1\%$) with the linear calibration range employed spanning 0.1–150 μg/ml. If required, as little as 0.05–0.2 μg/ml could be detected but this was not necessary for routine urine analyses.

The recovery of trimethylamine N-oxide as trimethylamine after its reduction was 98.9 ± 3.4% and those for methylamine, dimethylamine and trimethylamine after processing were 99.3 ± 7.5, 102.0 ± 2.5 and 99.9 ± 4.1%, respectively. Results obtained from several determinations undertaken on multiple aliquots of the same urine sample showed coefficients of variation (C.V.) of 5.0 ± 0.8% for all three amines ($n = 9$). The variability observed over a period of three months between aliquots of the same urine sample analysed at weekly intervals resulted in C.V. values of 5.8% for methylamine, 5.6% for dimethylamine and 7.2% for trimethylamine ($n = 12$).

Potassium hydroxide was initially employed to raise the pH of the urine and ensure that all amines under examination were present as their volatile bases. However, as potassium hydroxide amounts were increased the peak areas obtained for the amines and acetone, a neutral compound, also increased, presumably because the volatility

TABLE I

RETENTION TIMES OBTAINED BY HEAD-SPACE GAS CHROMATOGRAPHY

Compound	Relative molecular mass	Boiling point (°C)	Retention time (min)	Relative retention time
Ammonia	17.03	−33.4	0.52	0.13
Methylamine	31.06	−6.3	0.92	0.23
Dimethylamine	45.08	7.0	1.67	0.42
Ethylamine	45.08	16.6	1.87	0.47
Methanol	32.04	64.7	2.23	0.56
Trimethylamine	59.11	2.9	2.59	0.65
Isopropylamine[a]	59.08	33.5	3.98	1.0
Acetone	58.08	56.5	4.50	1.13
Ethanol	46.07	78.5	4.78	1.20

[a] Internal standard.

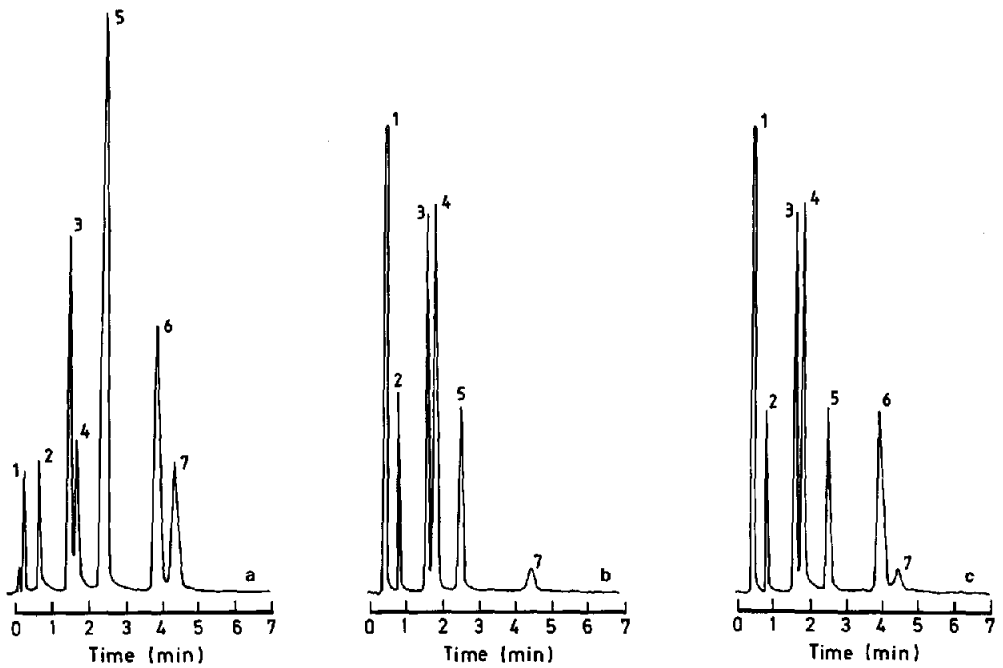

Fig. 1. Head-space gas chromatogram of (a) standard mixture, (b) blank urine and (c) blank urine with internal standard. Peaks: 1 = ammonia; 2 = methylamine; 3 = dimethylamine; 4 = ethylamine; 5 = trimethylamine; 6 = isopropylamine (internal standard); 7 = acetone. Concentrations used for standard mixture trace (a) were 5 μg/ml for peaks 1–5, 12 nl/ml for peak 6 and 4.8 nl/ml for peak 7.

of these compounds was facilitated by the intense ion strength created in solution (Fig. 2). However, the signals obtained from the various compounds did not increase equally, especially that for trimethylamine which effectively levelled out. This was not unexpected given their differing chemical structures and physical properties. In order to standardise the assay at a sufficient sensitivity to measure urinary amines whilst minimizing reagent use, 3 g of potassium hydroxide were routinely employed.

Several effects were noticed when sample heat-

TABLE II

URINARY EXCRETION OF ALIPHATIC AMINES BY HEALTHY SUBJECTS AND PATIENTS WITH FISH ODOUR SYNDROME

Results are expressed as the median values (with ranges) obtained for the two groups of five subjects.

Compound	Urinary excretion (mg per day)			
	Healthy volunteers		Fish odour syndrome patients	
	Median	Range	Median	Range
Methylamine	6.5	4.4–13.6	13.4	5.5–39.5
Dimethylamine	18.6	8.4–27.2	16.6	10.3–27.0
Trimethylamine	0.9	0.2–2.7	12.0	8.7–56.1
Trimethylamine N-oxide	30.7	10.7–60.6	10.1	8.3–54.8

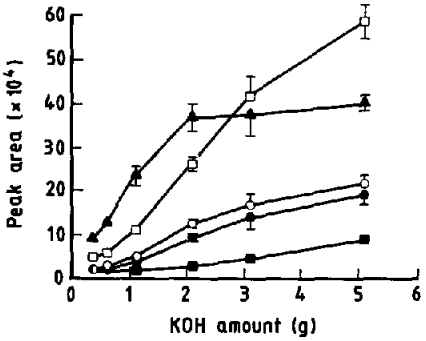

Fig. 2. Effect of potassium hydroxide concentration on amine peak areas. □ = acetone; ▲ = trimethylamine; ○ = isopropylamine; ● = dimethylamine; ■ = methylamine.

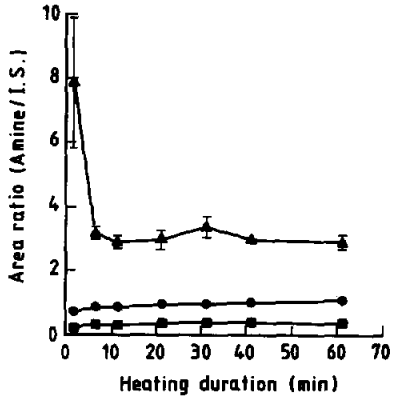

Fig. 3. Effect of heating time on peak-area ratios (amines/internal standard). ▲ = trimethylamine; ● = dimethylamine; ■ = methylamine.

ing times were varied (Fig. 3). Before heating the peak area for trimethylamine was relatively large compared to those for the other amines, leading to an abnormally high ratio when compared to the internal standard. During the 0–15 min period the peak area corresponding to isopropylamine increased rapidly with the ratio value for trimethylamine falling and those for methylamine and dimethylamine rising slightly. After 15 min all the ratios of amines to the internal standard were constant and remained so, with minor fluctuations, for up to 60 min.

The amine content of urine samples stored in a freezer (−20°C and −70°C) did not change significantly over a three-month period. However, those samples stored at room temperature or those which had undergone freeze–thaw cycles showed great variation in change; the amine levels in some samples remained almost constant whereas those in others altered dramatically Maintaining the urine at pH 1 or adding sodium azide (to a final concentration of 0.4%, w/v) at pH 7 minimized these changes, suggesting that they were due, at least in part, to the action of microbes present within the urine samples.

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