Journal of Chromatography, 617 (1993) 191–196
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6924

Gas chromatographic determination of primary and secondary low-molecular-mass aliphatic amines in urine using derivatization with isobutyl chloroformate

Thomas Lundh* and Bengt Åkesson

Department of Occupational and Environmental Medicine, University Hospital, S-221 85 Lund (Sweden)

(First received November 27th, 1992; revised manuscript received May 24th, 1993)

ABSTRACT

A simple routine method for the gas chromatographic determination of methylamine, dimethylamine, ethylamine and methylethylamine in urine is presented. The method is based on a two-phase derivatization procedure with isobutyl chloroformate as reagent. The reaction is quantitative in 10 min. We found no artifact formation of either choline or trimethylamine (dietary amine compounds) or of dimethylethylamine or triethylamine (catalyst amines in the industrial setting). The chromatographic behaviour of the amine carbamates was excellent. The recoveries of methylamine, dimethylamine, ethylamine and methylethylamine in spiked urine samples were 82, 89, 100 and 96%, respectively, and the precision (the relative standard deviation) was 3.6, 1.8, 3.3 and 2.0%, respectively. The method was linear for the studied amine carbamates up to 250 mg/l. The endogenous amine concentrations in urine samples from ten normal subjects were: methylamine, 0.9 mg/l (mean; range 0.3–1.5); dimethylamine, 14.7 mg/l (mean; range 4.6–27.6); ethylamine, 0.8 mg/l (mean; range 0.2–2.3); methylethylamine, less than 0.02 mg/l.

INTRODUCTION

There is an endogenous formation of amines in the body. Humans excrete aliphatic and heterocyclic amines, such as methyl- and ethylamines, in the urine, the predominant one being dimethylamine (DMA). DMA, a secondary amine, is also the most studied amine in this group. The reports of the concentrations of endogenous amines in human urine are limited: methylamine (MA), 0.53–4.8 mg/l [1–3]; DMA 5.8–39 mg/l [1–3]; ethylamine (EA), *ca.* 1 mg/l [2]. No data have been reported for methylethylamine (MEA). Secondary amines may be converted into nitrosoamine, and DMA may be an endogenous precursor of the potential carcinogenic compound nitrosodimethylamine. The low-molecular-mass aliphatic amines excreted in urine mostly originate from conversion of trialkylammonium-containing molecules in the diet, *e.g.* choline [4] and trimethylamine N-oxide in marine fish [5,6].

In general, low-molecular-mass aliphatic amines are analysed by gas chromatography (GC). The samples may be analysed after addition of alkali, either by direct injection [7] or by head-space analysis technique [1], or they may be extracted into an organic solvent before analysis [8]. GC analysis of amines is often problematic, owing to their high polarity and hydrogen-bonding tendency, resulting in tailing peaks and memory effects [9]. However, primary and secondary amines may be derivatized into analytically more suitable molecules. Several methods have been published in which chloroformate has been used to convert amines into carbamates, which exhibit good chromatographic properties [10–12].

* Corresponding author.

0378-4347/93/$06.00

In studies of the metabolic pattern or the pharmacokinetic data of amines it is important to have well evaluated analytical methods. We report here a simple routine method for the determination of primary and secondary low-molecular-mass aliphatic amines in human urine, based on reaction with isobutyl chloroformate in a two-phase derivatization system.

EXPERIMENTAL

Apparatus

A Varian 3400 gas chromatograph (Varian, Palo Alto, CA, USA) with a Varian 8100 autosampler was employed. For the analytical procedure, the injection ports were kept at 220°C. A 10 m × 0.53 mm I.D. fused-silica wide-bore column with a chemically bonded stationary phase, CP-Sil 8 CB, with a film thickness of 5.0 μm (Chrompack, Middelburg, Netherlands), was used. Carrier gas and make-up gas were nitrogen, at flow-rates of 5 and 25 ml/min, respectively. The column temperature was initially 90°C, and then programmed to 220°C at 10°C/min. The amine derivatives were detected with a Varian thermospecific detector (Varian TSD; 250°C with flow-rates for hydrogen and air of 4.2 and 180 ml/min, respectively; bead heating current, 3.03 A; bias voltage, −4.2 V) and the peaks were evaluated with a Maxima 820 chromatography workstation (Millipore, Milford, MA, USA). A Savant AS290 automatic Speed-Vac concentrator (Savant, Farmingdale, NY, USA) was used for evaporation of toluene from the sample solution containing the standards of carbamate derivatives.

Chemicals

MA, dimethylethylamine (DMEA), triethylamine (TEA) and isobutyl chloroformate were obtained from Janssen (Geel, Belgium), DMA, EA and trimethylamine (TMA) from Sigma (St. Louis, MO, USA) and MEA and dibutylamine (DBA) from Fluka (Buchs, Switzerland). Toluene and methanol were from Labscan (Dublin, Eire) and sodium hydroxide, hydrochloric acid and disodium hydrogenphosphate dodecahydrate from Merck (Darmstadt, Germany).

Preparation of standard derivatives

The isobutyl carbamates of MA, EA, DMA, MEA and DBA (for internal standard) were synthesized by adding 10 ml of MA, EA, DMA, MEA or DBA to 200 ml of toluene and 50 ml of 12 *M* NaOH. Isobutyl chloroformate (10 ml) was added dropwise under magnetic stirring, and the mixture was continuously stirred for 1 h. The organic layer was separated and treated with 50 ml of alkaline methanol (methanol saturated with NaOH) and stirred for 10 min, then 100 ml of 5 *M* NaOH were added and the mixture was stirred for another 10 min. The toluene solution was washed twice with 100 ml of 1 *M* HCl to remove unreacted amine, and the toluene was then evaporated from the carbamates in a Speed-Vac system.

Urine samples

Urine samples were collected in polyethylene bottles, acidified with concentrated HCl (37%; 2 ml per 100 ml of urine) and stored at 4°C until analysis. The stability of amines in urine samples under acidic conditions has been reported elsewhere [8].

Analytical procedure

In a 10-ml test-tube, 2 ml of urine were added to 2 ml of toluene containing the isobutyl chloroformate derivative of DBA as internal standard and 20 μl of isobutyl chloroformate. The mixture was made alkaline with 2 ml of phosphate buffer (0.5 *M*, pH 12.0), after shaking for 10 min and centrifuging (1500 *g*, 10 min), the toluene layer was transferred to a second test-tube, and 1 ml of alkaline methanol was added. The tube was shaken for 5 min, then 3 ml of 5 *M* NaOH were added. The mixture was shaken for a further 5 min, then 1 μl of the toluene layer was injected into the gas chromatograph. Aliquots were analysed with and without addition of the investigated amines. The concentrations of the amines in the samples were calculated according to the following formula: $C_0 = C_\Delta[a_0 / (a_\Delta - a_0)]$, where C_0 = concentration of the amines in the sample, a_0 = peak-area ratio of the sample, C_Δ = concentration of the added amines in the sample and a_Δ = peak-area ratio of the sample with addition of amines.

RESULTS AND DISCUSSION

Standard derivatives

The identities of the standards and internal standard were confirmed by GC–mass spectrometry (MS), and the purity was tested by GC using thermospray detection (TSD) and flame ionization detection. The purity was found to be better than 98%. The standards were stable for more than four weeks without any noticeable degradation when stored in a refrigerator.

Analytical procedure

We found that with a phosphate buffer (pH 12) the reaction was quantitative in 10 min. Other studies show that addition of ammonia may have a catalytic effect on the derivatization of 1,6-hexamethylenediamine [12] or piperazine [13] and increase the recovery. We found that ammonia did not enhance the recoveries of the amines that we studied. On the contrary, the addition of ammonia caused an additional peak in the chromatogram.

Because of the high pK_a values of the investigated amines, a strongly alkaline solution may be preferable for the extraction of the amines into the toluene layer, the medium for the derivatization. However, there is a potential risk of hydrolysing other nitrogen-containing compounds in biological samples that may contribute to the original amine content in the sample.

Artifact formation. Additions of 250 mg/l choline, 100 mg/l TMA, 100 mg/l DMEA or 100 mg/l TEA were used to test for artifact formation. No formation of the conceivable carbamates was detected.

Reagent residues. In accordance with other studies, chloroformate reagent residues in the toluene intended for GC analyses caused adverse effects on the analytical column and influenced the detector sensitivity [12]. The elimination of the reagent residue by evaporation, reported in other studies [12,13], could not be performed because of the volatility of the investigated derivatives. Instead, alkaline methanol was used to eliminate the residue amounts of reagent in the toluene. The volume of the toluene layer was restored by the addition of alkaline aqueous solution. The same technique, with minor modifications, for the elimination of reagent residues in the analysis of plasma samples has been reported by Hartvig *et al.* [14].

The absence of reagent residues in the final toluene layer was confirmed by using this toluene for a second derivatization step. Toluene treated with alkaline methanol was not able to cause any further derivatization, but the untreated toluene could quantitatively derivatize a second sample.

Analysis

Chromatography. The chromatographic behaviour of the amine carbamates was excellent, and there was no sign of decomposition in the chromatographic system. The chromatograms of a urine sample, spiked and non-spiked with the investigated amines, are shown in Fig. 1. Our derivatization method transforms the polar amines into much less polar derivatives. This makes it possible to use a wide range of analytical columns for the separation.

Calculations. The urine samples contained endogenous MA, EA and DMA. Thus, for the calculations of derivatization efficiency, recovery, linearity and precision, the urine samples were analysed before and after the amine addition. For the calculations of the extraction yield, the urine samples were analysed before and after the amine carbamate addition.

Derivatization efficiency. Urine samples collected from ten subjects were spiked with MA, EA, DMA and MEA. The derivatization efficiency, amine carbamate formation, was evaluated by comparing the amounts of amine carbamate formed from the added amines with amine carbamate standards added to the toluene layer prior to derivatization (Table I). The two primary amines had a lower derivatization efficiency compared with the secondary amines. An increase in the reaction time from 10 to 60 min did not increase the derivatization efficiency of the primary amines.

Extraction yield. The partition of the amine carbamates in the two-phase (toluene–buffer) system is favourable. Studies of amine carbamate

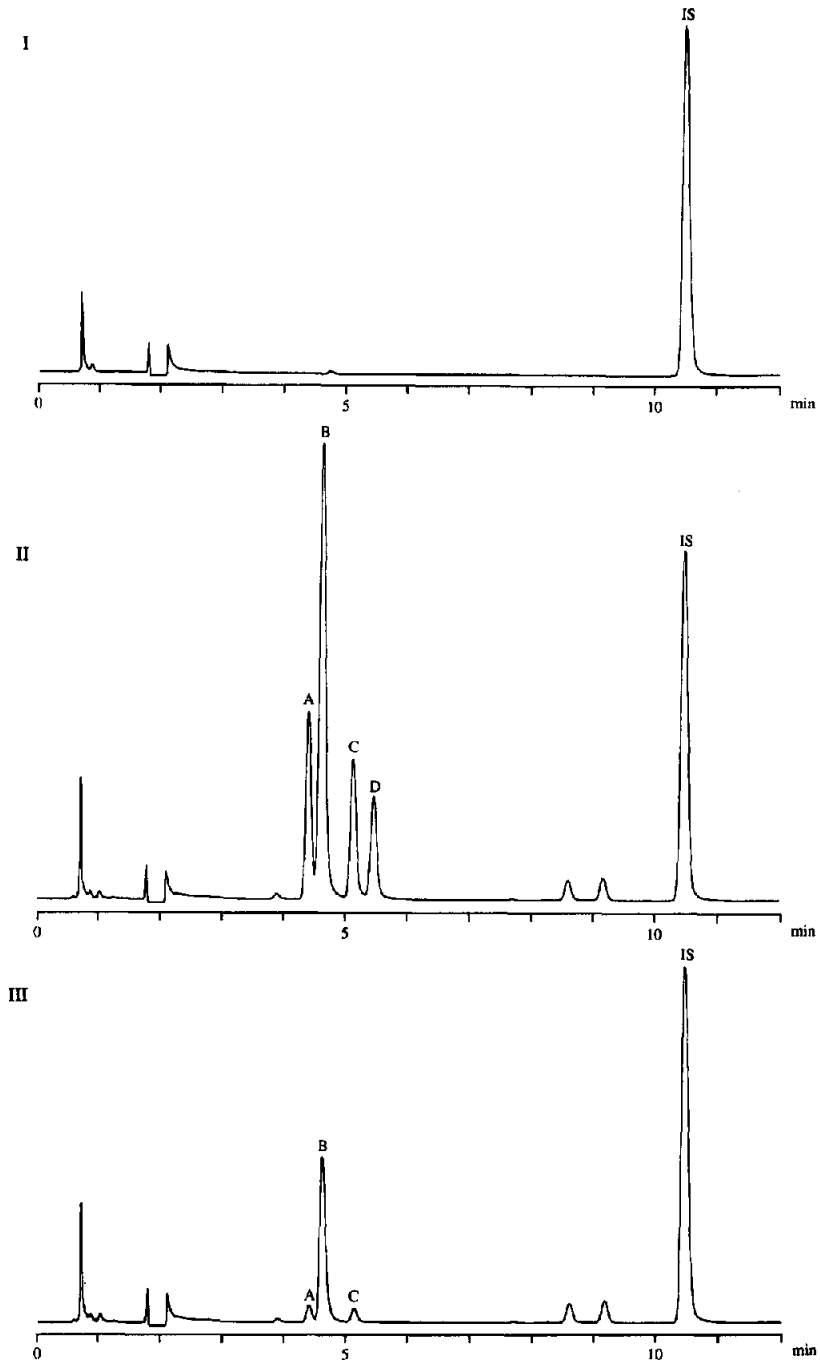

Fig. 1. Chromatograms of (I) a toluene blank in which urine was replaced by 0.1 *M* HCl in the work-up procedure, (II) a urine sample spiked with methylamine (A, 10.4 mg/l), dimethylamine (B, 19.0 mg/l), ethylamine (C, 9.8 mg/l) and methylethylamine (D, 9.1 mg/l), and (III) an unspiked urine sample. The internal standard (IS) is the isobutyl chloroformate derivate of di-*n*-butylamine.

TABLE I

DERIVATIZATION EFFICIENCIES, EXTRACTION YIELDS AND RECOVERIES OF METHYLAMINE, ETHYLAMINE, DIMETHYLAMINE AND METHYLETHYLAMINE IN URINE SAMPLES

Amine	Concentration added (mg/l)	Derivatization efficiency (%)		Extraction yield (%)		Recovery (%)	
		Mean	R.S.D.[a]	Mean	R.S.D.[a]	Mean	R.S.D.[a]
Methylamine	10.4	87	2.3	94	1.7	82	3.6
Ethylamine	9.8	91	2.2	98	1.6	89	1.8
Dimethylamine	19.0	99	2.4	102	1.7	101	3.3
Methylethylamine	9.1	97	1.9	99	1.6	96	2.0

[a] Relative standard deviation; $n = 10$.

standards added to toluene prior to the analytical procedure showed almost complete partition into the toluene layer (Table I). However, the volume of the toluene layer after the addition of alkaline methanol must be restored with an alkaline aqueous solution, because the carbamate derivatives of the primary amines are slightly basic.

Recovery. The recoveries of MA, EA, DMA and MEA were quantitative. The mean recoveries of the amines added to urine samples collected from ten subjects are shown in Table I. The recoveries are expressed as the product of the derivatization efficiency and the extraction yield. The calculations were performed by comparing the amounts of derivatives formed from the added amines with known amounts of the amine carbamate standards.

Linearity. Urine samples spiked with the amines under investigation in the concentration range 0.5–1000 mg/l showed a linearity of the amine carbamates formed from the added amines up to 250 mg/l (MA, $y = 0.082x - 0.051$, $r = 1.00$; DMA, $y = 0.12x - 0.25$, $r = 1.00$; EA, $y = 0.16x - 0.081$, $r = 1.00$; MEA, $y = 0.16x - 0.29$, $r = 1.00$). In practice, dilution of the samples eliminates the upper limit.

Because of the endogenously formed amines, the detection limits for the method were not determined. The minimum detectable amount, for all amines studied, was estimated to be 0.02 mg/l.

Precision and accuracy. Urine samples from ten subjects were spiked with MA, DMA, EA and MEA. The within-day precision for the de-

TABLE II

WITHIN-DAY PRECISION FOR THE DETERMINATION OF METHYLAMINE, ETHYLAMINE, DIMETHYLAMINE AND METHYLETHYLAMINE IN URINE SAMPLES

Amine	Concentration added (mg/l)	Concentration found[a] (mg/l)		Precision[b] (%)
		Mean	Range	
Methylamine	10.4	10.3	10.2–10.4	1.0
Ethylamine	9.8	9.6	9.3–9.8	2.1
Dimethylamine	19.0	18.8	18.2–19.3	1.9
Methylethylamine	9.1	8.6	8.3–8.7	1.9

[a] The calculations were performed by use of amine standard addition.

[b] Relative standard deviation, $n = 10$.

TABLE III

CONCENTRATIONS OF ENDOGENOUS METHYLAMINE, ETHYLAMINE, DIMETHYLAMINE AND METHYLETHYLAMINE IN URINE

Amine	Endogenous concentration (mg/l)	
	Mean[a]	Range
Methylamine	0.9	0.3–1.5
Ethylamine	0.8	0.2–2.3
Dimethylamine	14.7	4.6–27.6
Methylethylamine	< 0.02	–

[a] $n = 10$.

termination was good (Table II). Urine samples analysed within a two-week interval showed no significant differences (Students t-test; $p > 0.05$). The calculations were performed by analysis of samples with and without the addition of amine standards.

Endogenous amine concentration. The concentrations of endogenous MA, DMA, EA and MEA in the urine samples from ten normal subjects are shown in Table III. The concentrations are in accordance with data reported in other studies [1–3].

CONCLUSION

A GC method has been developed for the determination of primary and secondary low-molecular-mass aliphatic amines. The two-phase derivatization with isobutyl chloroformate as reagent improved the chromatographic behaviour of the compounds under study, and offers a simple method for the accurate and precise determination of amines in urine. There was no artifact formation originating from the endogenous TMA. Because the carbamate reference standards are not commercially available, we recommend that the evaluation is performed with the addition of amine standards.

ACKNOWLEDGEMENTS

The study was supported by the Swedish Work Environment Fund and the Medical Faculty, Lund University. The technical assistance of Ms. Åsa Amilon is acknowledged.

REFERENCES

1 S. Lowis, M. A. Eastwood and W. G. Brydon, *J. Chromatogr.*, 278 (1983) 139.
2 H. Heberer, *Z. Klin. Med.*, 41 (1986) 143.
3 S. H. Ziesel, K.-A. DaCosta and J. T. LaMont, *Carcinogenesis*, 9 (1988) 179.
4 S. H. Zeisel, J. S. Wishnok and J. K. Blusztajn, *J. Pharmacol. Exp. Ther.*, 225 (1983) 320.
5 D. J. B. Da Ponte, J. P. Roozen and W. Pilnik, *J. Food Technol.*, 21 (1986) 33.
6 S. H. Zeisel and K.-A. DaCosta, *Cancer Res.*, 86 (1986) 6136.
7 G. Audunsson and L. Mathiasson, *J. Chromatogr.*, 315 (1984) 299.
8 T. Lundh, B. Ståhlbom and B. Åkesson, *Int. Arch. Occup. Environ. Health*, 44 (1991) 81.
9 A. Di Corsa and R. Samperi, *Anal. Chem.*, 46 (1974) 977.
10 N. O. Ahnfelth and P. Hartvig, *Acta Pharm. Suec.*, 17 (1980) 307.
11 N. O. Ahnfelth, P. Hartvig and K. E. Karlsson, *Chromatography*, 16 (1982) 60.
12 G. Skarping, M. Dalene, T. Brorsson, J. F. Sandström, C. Sangö and A. Tiljander, *J. Chromatogr.*, 479 (1989) 125.
13 G. Skarping, T. Bellander and L. Mathiasson, *J. Chromatogr.*, 370 (1986) 245.
14 P. Hartvig, W. Hands, J. Vessman and C. M. Svahn, *Anal. Chem.*, 48 (1976) 390.