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Determination of dimethylamine in biological samples by high-performance liquid chromatography

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Abstract

A reversed-phase HPLC method for the quantification of dimethylamine in serum and urine is presented. Dimethylamine (DMA) is converted into a stable fluorescent product by precolumn derivatization with fluorenylmethylchloroformate. The DMA derivative is resolved from derivatives of other amines and amino acids by gradient elution with a total run-time of 15 min. The lower limit of determination in biological samples is 0.1 μmol/l. Recoveries from spiked serum samples were 99–107%. Within- and between-run precision were better than 6%. Concentrations of DMA in serum from normal human subjects ($n=8$) and from continuous ambulatory peritoneal dialysis patients ($n=15$) were 3.3 ± 1.5 and 29.1 ± 12.1 μmol/l, respectively.

Keywords: Bacterial overgrowth; Renal failure; Dimethylamine

1. Introduction

Dimethylamine (DMA) is the most abundant low-molecular-mass aliphatic amine in human urine and is also present in other body fluids, including blood and gastric fluid [1,2]. By endogenous nitrosation DMA may be converted into the carcinogenic N-nitrosodimethylamine [2,3].

Three sources of DMA have been identified: diet, bacterial synthesis and endogenous synthesis [4,5]. Significant levels of DMA, trimethylamine and trimethylamine N-oxide (TMAO) are present in fish and it has been observed that fish consumption leads to increased DMA excretion in humans [6]. It has been shown that orally administered TMAO can be converted to DMA, probably by gut bacteria [1]. Choline, a quaternary amine present in the normal diet, can also be converted to DMA by intestinal bacteria [7]. However, Zeisel et al. reported that rats fed a diet devoid of choline as well as germ-free rats excreted the same amount of DMA as control animals [4]. These experiments show that DMA is probably also synthesized by endogenous pathways. Ogawa et al. have shown that rat kidney contains an enzyme that converts dimethylarginine, a post-translationally modified amino acid, into citrulline and DMA [8].

In chronic renal failure (CRF) patients, elevated levels of DMA in blood occur [9–11]. In part, these high levels may be explained by impaired renal excretion. Another factor that may contribute to elevated DMA levels is increased intestinal synthesis by bacteria. Bacterial overgrowth of the small intestine in CRF patients has been reported. It may be

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caused by reduced peristalsis and impaired immunological defense mechanisms [12,13]. Simenhoff et al. have demonstrated that treatment of uraemic patients with antibiotics markedly reduced serum DMA levels [10]. These investigators have also shown that altering the small bowel flora of CRF patients by administration of *Lactobacillus acidophilus* was effective in lowering serum DMA levels [11]. As we are currently investigating the relation between bacterial overgrowth and elevated serum DMA levels in CRF patients, we needed a reliable method to determine DMA in serum, urine and continuous ambulatory peritoneal dialysis (CAPD) effluent.

Various procedures for the analysis of DMA have been described. Nuclear magnetic resonance spectroscopy has been used to identify DMA in plasma and urine samples [14,15]. However, most analytical methods are based on chromatographic separation. Most frequently, aliphatic amines are analyzed by gas chromatography (GC), either by direct injection [16–18] or by head-space sampling after alkalinization of the samples [4,19–22]. Adsorption of free amines, resulting in severe peak tailing, memory effects and low reproducibility has been a serious limitation in GC analysis of these compounds. These problems may be circumvented by derivatization prior to GC. DaCosta et al. [23] analyzed the tosylamide derivative of DMA. Derivatization with isobutylchloroformate was used for the GC analysis of amines in urine by Lundh and Åkesson [24].

Precolumn derivatization has also been applied for analysis of aliphatic amines by HPLC. Lin and Lai [25] developed an HPLC method based on reversed-phase separation of dabsyl chloride derivatives, with UV detection at 425 nm. Elskamp and Schultz [26] used 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD chloride) for derivatization in combination with either UV or fluorescence detection. Baba et al. [27,28] analyzed aliphatic amines, including DMA, in serum and faecal samples as their 2,4-dinitrophenyl derivatives.

We have developed an HPLC method for the analysis of DMA based on reversed-phase separation of the adduct of DMA and 9-fluorenylmethylchloroformate (FMOC-Cl). FMOC-Cl rapidly reacts at room temperature with primary and secondary amines yielding stable products that can be detected fluorometrically with high sensitivity [29–32]. Recently, López et al. [33] reported on an HPLC procedure for the determination of DMA in groundwater, based on precolumn derivatization with FMOC-Cl. In this paper we describe the derivatization and separation conditions and the application of the analytical procedure to the analysis of urine, CAPD effluent and serum samples.

2. Experimental

2.1. Reagents and chemicals

Dimethylamine HCl was obtained from Sigma (St. Louis, MO, USA). 5-Sulfosalicylic acid (SSA), boric acid, potassium dihydrogen phosphate and glycine were supplied by Merck (Amsterdam, Netherlands). FMOC-Cl was from Fluka (Buchs, Switzerland). Gradient-grade acetonitrile was obtained from Biosolve (Barneveld, Netherlands). HPLC-grade water was prepared from demineralized water using a Milli-Q UF Plus water purification system (Millipore, Milford, MA, USA).

2.2. Instrumentation

The HPLC system consisted of a model 616 pump and model 474 fluorescence detector from Waters (Milford, MA, USA) and a model 232-401 autosampler from Gilson (Villiers le Bel, France). Mobile phase was passed through a Degasys model DG2410 inline solvent degasser from Uniflows (Tokyo, Japan). Millennium 2010 software (version 2.10) from Waters was used for instrument control and data acquisition and processing. Analyses were performed on a 10 cm×4.6 mm I.D. Microspher C_{18} column containing 3 μm particles (Chrompack, Middelburg, Netherlands). The analytical column was protected by an 1 cm×2.0 mm I.D. reversed-phase guard column from the same supplier.

2.3. Collection and processing of samples

Blood samples were obtained from 8 healthy volunteers (age 42±14; range 27–65) and 15 CAPD patients (age 55±23; range 22–87). Blood was obtained by venipuncture and allowed to clot at room temperature for 60 min. Serum was prepared by centrifugation (10 min at 3000 *g*) and stored at −20°C prior to analysis. Urine and CAPD effluent samples were also stored at −20°C.

2.4. Derivatization

A stock standard solution of 10 m*M* DMA in water was prepared each month and stored at 4°C. A 100 μ*M* working standard was prepared each day. Samples (urine, serum or CAPD effluent) and standard solutions were deproteinized by mixing a 300-μl volume with 200 μl of a 7.5% (w/v) solution of SSA. After centrifugation (5 min at 3000 *g*, 4°C), 200 μl of the clear supernatant was transferred to a glass tube containing 200 μl of 0.8 *M* borate buffer (pH 9.5). Derivatization was performed by adding 300 μl of a freshly prepared 10 m*M* solution of FMOC-Cl in acetonitrile. After 1 min, excess of reagent was neutralized by addition of 100 μl of a 100 m*M* glycine solution. After centrifugation (5 min at 3000 *g*, 4°C), 400 μl of the supernatant was transferred to a 4 ml glass vial which was sealed with a teflon lined screw-cap. The vials were heated for 10 min at 80°C in a waterbath. After cooling the vials were placed in the autosampler.

2.5. HPLC conditions

50 m*M* potassium dihydrogen phosphate buffer adjusted to pH 6.5 with KOH was used for preparation of the mobile phases. Eluent A consisted of acetonitrile–buffer (50:50, v/v) and eluent B consisted of acetonitrile–buffer (75:25, v/v). The flow-rate was constant at 1.0 ml/min. The column was equilibrated with eluent A. After injection of a 5-μl volume of the sample, a linear gradient was started. Gradient conditions are shown in Table 1. Time between injections was 15 min. After each injection, the sample loading port, needle and injection valve of the autosampler were flushed with 50% acetonitrile, to avoid memory effects. The column effluent was monitored for fluorescence (excitation at 260 nm and emission at 320 nm) and DMA was quantitated on the basis of peak area using external standardization.

Table 1
Gradient program

Time (min)	Flow (ml/min)	Percent A	Percent B
0.0	1.0	100	0
5.0	1.0	0	100
10.0	1.0	0	100
11.0	1.0	100	0
15.0	1.0	100	0

3. Results and discussion

The goal of the present study was to develop a sensitive HPLC method for the determination of DMA in biological fluids.

3.1. Derivatization

We used FMOC-Cl as derivatization reagent, as it reacts equally well with both primary and secondary amines. Derivatization proceeds rapidly at ambient temperature and alkaline pH. The derivatives are very stable and allow fluorometric detection at subpicomol levels [28–31]. A drawback of FMOC-Cl is the fact that both the reagent itself and its hydrolysis product FMOC-OH are intensely fluorescent and may therefore interfere in the chromatographic separation of the amine derivatives. Excess reagent may be removed by extraction with diethylether or pentane but this may result in partial loss of hydrophobic amine derivatives. To avoid this potential problem, we used neutralization of reagent by adding an excess of glycine after derivatization of DMA was complete. In this way the excess FMOC-Cl is converted to the relatively hydrophilic glycine derivative that elutes early in the chromatogram. By this procedure hydrolysis of the reagent is minimized to the extent that the FMOC-OH peak does not interfere with detection of the much smaller peak of the DMA adduct.

3.2. Chromatographic conditions

Using isocratic conditions the DMA peak could only be resolved from the peak of FMOC-OH at a relatively low concentration of acetonitrile in the mobile phase, resulting in an excessively long run-time. We therefore used a simple linear gradient of acetonitrile in buffer, which resulted in a good separation of DMA (eluting at 6.0 min) from FMOC-

OH (eluting at 3.5 min) as shown in Fig. 1A. The large unretained peak corresponds to the FMOC-Cl derivative of glycine, used for neutralization of excess reagent. In addition, SSA used for deproteinization, exhibits fluorescence at the detection wavelengths used and contributes to the unretained peak. As can be seen from Fig. 1A, both chromatograms from a DMA standard and a blank incubation showed a small peak eluting before the DMA peak. Attempts to obtain a complete separation between this blank peak and the DMA peak by manipulating gradient steepness, buffer strength and pH were unsuccessful. Also the use of methanol or acetonitrile–methanol mixtures as modifier did not result in a complete separation of both peaks. During experiments on stability of the FMOC-Cl derivative of DMA, we observed that when derivatized samples were rechromatographed the next day, the blank peak was very much reduced in intensity, whereas the DMA peak remained unchanged. This prompted us to investigate the effect of a heating step after FMOC-Cl derivatization. It appeared that heating the derivatized samples at 80°C for 10 min almost completely abolished the blank peak, without affect-

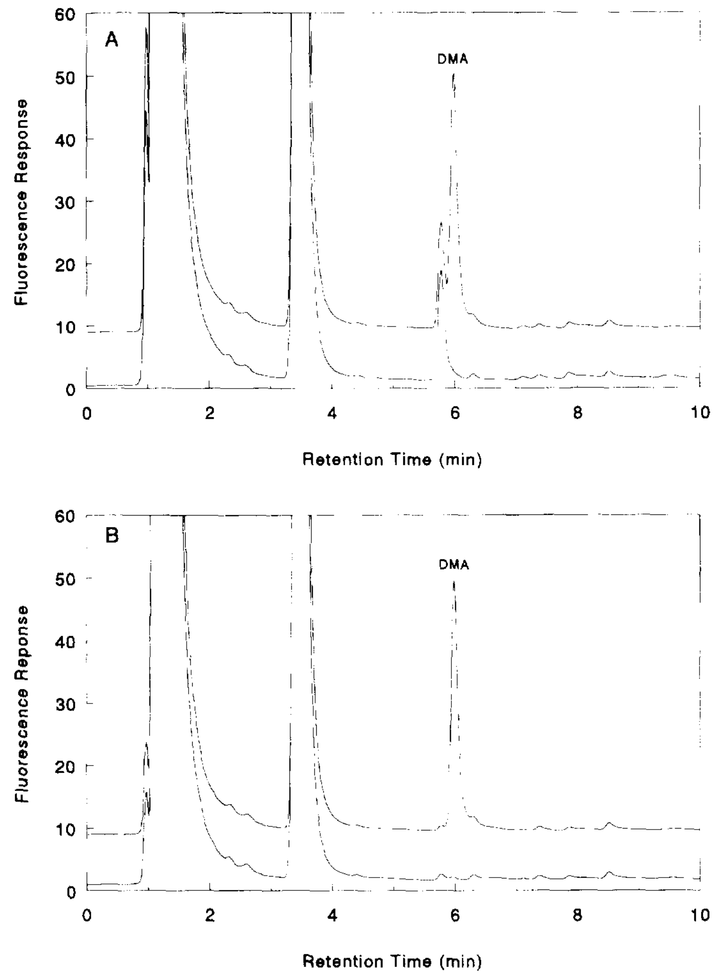

Fig. 1. (A) Chromatogram of a dimethylamine standard (upper trace) and a blank (lower trace). The standard concentration was 48 μmol/l, corresponding to an injected amount of 36 pmol. Derivatization conditions were as described in Section 2.4, with omission of the 80°C heat treatment after derivatization. Chromatographic conditions as described in Section 2.5. (B) Same as (A), after heating at 80°C for 10 min.

ing DMA peak area (Fig. 1B). This heating step was therefore routinely included in the derivatization procedure.

When analyzing biological fluids, the DMA peak must be resolved from the FMOC-Cl derivatives of amino acids that are present in much higher concentrations. We used pH of the mobile phase as the primary tool to obtain adequate resolution. As the DMA adduct is uncharged, its retention time is very insensitive to pH changes. The retention times of amino acid derivatives are strongly pH dependent, due to the presence of an ionizable carboxylate moiety. By systematically varying mobile phase pH we were able to manoeuvre the DMA peak into a region of the chromatogram devoid of amino acid derivatives. Fig. 2A and B show chromatograms of serum samples from a healthy volunteer and a haemodialysis patient, using optimized chromatographic conditions. Although urine contains many more different amines and amino acids than serum, DMA levels in normal urine are approximately two orders of magnitude higher than normal plasma or serum levels. Therefore, conditions that were optimized for serum samples could also be used for the

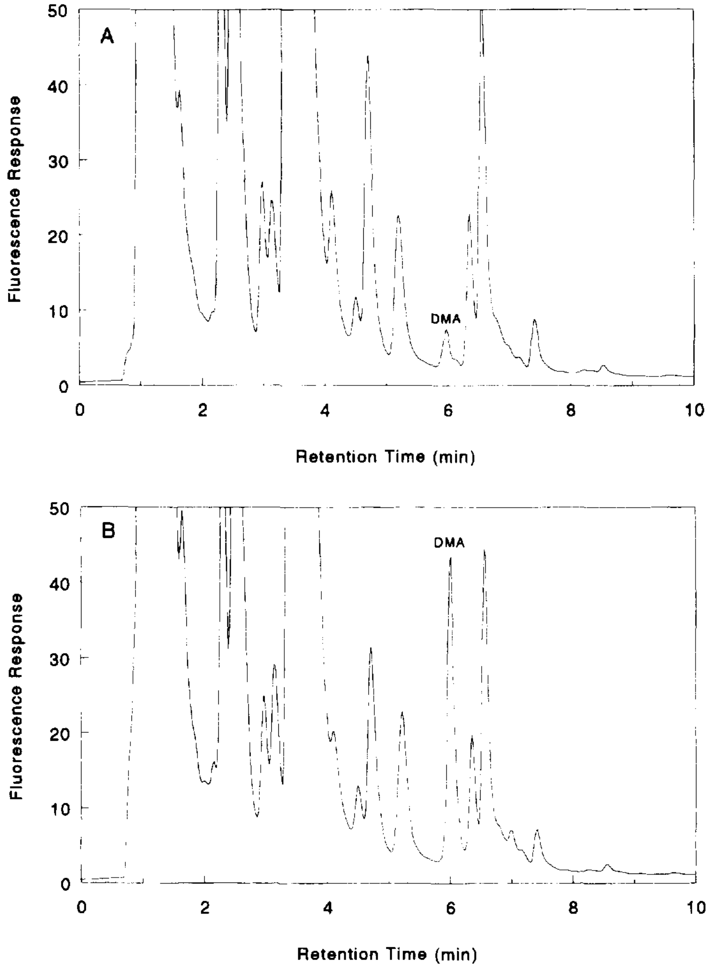

Fig. 2. (A) Chromatogram of a serum sample from a healthy subject (4.7 μmol l^{-1} dimethylamine, corresponding to an injected amount of 3.5 pmol). (B) Chromatogram of a serum sample from a continuous ambulatory peritoneal dialysis patient (49.8 μmol l^{-1} dimethylamine, corresponding to an injected amount of 37.4 pmol). Derivatization and chromatographic conditions as described in Sections 2.4 and 2.5.

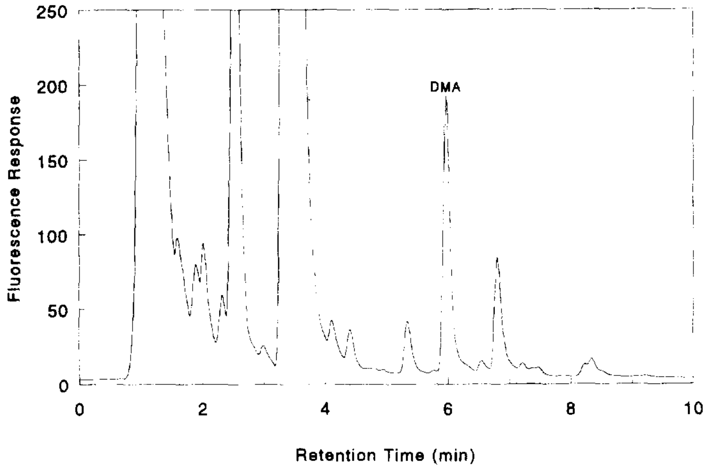

Fig. 3. Chromatogram of a urine sample from a healthy volunteer (237 μmol l^{-1} dimethylamine, corresponding to an injected amount of 178 pmol). Derivatization and chromatographic conditions as described in Sections 2.4 and 2.5.

analysis of urine as shown in Fig. 3. In Fig. 4 a chromatogram of a CAPD effluent is shown.

3.3. Assay performance

Standard curves showed a linear relationship between integrated peak area (y) and concentration (x) in the range 0.001–0.48 mM. (y=880 244x+487; r=0.9996).

Using a detector gain of 100, the detection limit at a S/N ratio of 3 was 0.02 μmol/l, corresponding to 0.015 pmol on column. Due to the presence of very small unidentified peaks eluting close to the DMA peak in chromatograms of derivatized serum samples, the limit of determination is approximately 0.1 μmol/l.

Results on within-run and between-run precision of the determination of DMA in serum are given in Table 2. Within-run coefficients of variation were below 5%. Between-run precision was determined by analyzing a serum pool on 9 occasions over a four-month period. The between-run coefficient of vari-

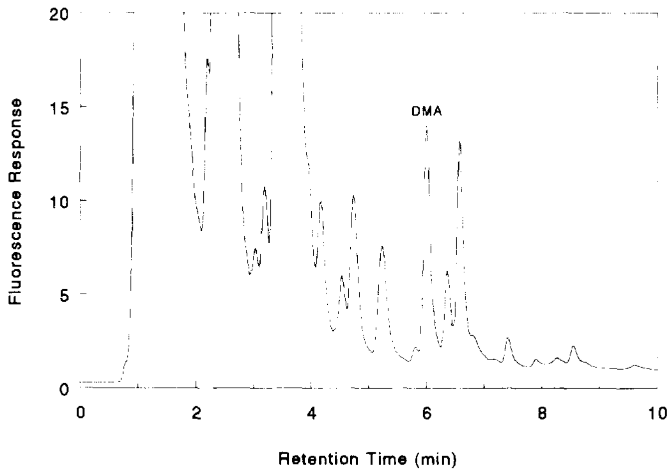

Fig. 4. Chromatogram of dialysis effluent obtained from a continuous ambulatory peritoneal dialysis patient (15.3 μmol l^{-1} dimethylamine, corresponding to an injected amount of 11.5 pmol). Derivatization and chromatographic conditions as described in Sections 2.4 and 2.5.

Table 2
Within-day and between-day precision of the determination of dimethylamine in human serum

	No. tests	Dimethylamine (μmol/l)		
		Mean	S.D.	C.V. (%)
Within-run	9	3.21	0.15	4.6
	12	28.33	0.73	2.6
Between-run	9	33.00	1.72	5.2

Table 3
Recovery of dimethylamine from serum samples spiked at different concentrations

DMA added (μ*M*)	No. tests (*n*)	Recovery±C.V. (%)
7.0	3	106.7±13.7
15.0	6	103.2±10.3
75.0	6	99.7±6.5

ation of 5.2% is acceptable, considering that no internal standard was used. We did not perform experiments to assess the precision of the determination of DMA in urine or CAPD effluent. As DMA levels in these fluids are higher than in normal serum, it can be expected that precision is the same or better as for serum samples.

Accuracy was determined by spiking a number of serum samples ($n=3$–6) at three different levels. The results presented in Table 3 show that recovery was close to 100%.

3.4. Application to biological samples

As shown in Figs. 2–4 the method is suitable for the determination of DMA in serum, urine and CAPD effluent. We measured DMA concentrations in serum from healthy controls and CAPD patients. The results presented in Table 4 show that DMA levels in CAPD patients are approximately 10-fold higher than in normal subjects. The serum DMA concentrations are comparable to the values of 3.2±0.9 μmol l^{-1} for normals and 31.9±7.0 μmol l^{-1} for haemodialysis patients reported by Baba et al. [27].

Table 4
Dimethylamine concentration in serum from normal subjects and continuous ambulatory peritoneal dialysis patients

	n	Mean±S.D. (μmol/l)	Range (μmol/l)
Normal subjects	8	3.3±1.5	1.0–5.5
Dialysis patients	15	29.1±12.1	14.4–49.8

4. Conclusions

A sensitive and reproducible HPLC method has been developed for the quantification of DMA in serum, urine and CAPD effluent samples. Sample pretreatment and derivatization is simple and rapid. Derivatization with FMOC-Cl is complete within 1 min at room temperature. This compares favourably to other derivatization reagents, that require long reaction times or elevated temperatures, e.g. 1 h at room temperature for dabsyl chloride [25], 2.5 h at 60°C for NBD chloride [26] and 40 min at 100°C for 2,4-dinitrobenzenesulfonate [27,28]. In addition, some of these methods require extraction of the reaction product by fluid–fluid extraction [25,27,28], thereby increasing sample work-up time and decreasing precision. In amino acid analysis procedures based on FMOC-Cl derivatization, the excess reagent is usually removed by extraction with an organic solvent [29–32], with the inherent risk of partial loss of the derivatized analytes. López et al. [33] using FMOC-Cl derivatization for the analysis of DMA in groundwater, observed significant loss of derivatized DMA after extraction of excess reagent with hexane. They solved this problem by careful control of the organic content of the reaction mixture. We have used a different approach, by neutralizing excess FMOC-Cl by addition of a large amount of glycine. The resulting glycine adduct is hardly retained during chromatography and does not interfere with the analysis of DMA.

Due to the stability of the FMOC-Cl derivative of DMA, large numbers of samples can be derivatized batchwise, followed by automated HPLC analysis. The method developed is a valuable tool for the study of DMA metabolism in CRF patients and its relation to small bowel bacterial overgrowth.

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