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Gas chromatographic assay for N,N-dimethylglycine in urine

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ABSTRACT

A gas chromatographic method for the determination of N,N-dimethylglycine in urine has been developed. After clean-up by cation-exchange, N,N-dimethylglycine was derivatized with ethanol and hydrochloric acid to form the corresponding ethyl ester. After evaporation of solvent, N,N-dimethylglycine ethyl ester was extracted into methylene chloride and chromatographed on a gas chromatograph equipped with a packed column containing 10% Carbowax 20 M. The detection limit of the method is 0.01 m*M* N,N-dimethylglycine in urine. This method has been used to detect N,N-dimethylglycine in urine from healthy subjects as well as in urine from patients with metabolic disorders. These findings were verified by gas chromatography–mass spectrometry.

INTRODUCTION

N,N-Dimethylglycine (DMG) is an intermediate in the one-carbon cycle metabolism of betaine [1–6]. In metabolic disorders involving malfunction of enzymes responsible for the conversion of DMG into sarcosine (N-methylglycine) and finally into glycine, elevated levels of DMG may be expected. In a patient suffering from hypersarcosinaemia, Porter *et al.* [7] detected 0.65 m*M* DMG in the urine. DMG was reported to be present in human plasma in small amounts but not in human urine from healthy subjects. Patients suffering from homocystinuria due to 5,10-methylenetetrahydrofolate reductase (EC 1.1.1.68) defiency may be treated with betaine. Betaine is converted into DMG by betaine-homocysteine methyltransferase (EC 2.1.1.5) [6]. Thus in patients treated with betaine, elevated levels of DMG may also be expected.

Few methods for the determination of DMG have been described. Separation by cation-exchange high-performance liquid chromatography and detection by means of the enzyme dimethylglycine dehydrogenase (EC 1.5.99.2) [7] or by UV detection in the range 190–200 nm has been used [8]. A method based on ion-pair chromatography of the methyl ester of DMG on a styrene–divinylbenzene copolymer used a conductivity detector for detection [4]. An isotachophoretic method involving the use of a potential gradient detector has been reported [10].

This paper describes a gas chromatographic (GC) method for the determination of DMG in human urine. Prior to its introduction into the chromatographic

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system, the sample is cleaned up by cation exchange and DMG is derivatized to form N,N-dimethylglycine ethyl ester (DMGE). We also report the amount of DMG found in urine from patients suffering from sarcosinaemia or hyperglycinaemia and in urine from patients suffering from homocystinuria treated with betaine. DMG was found in urine from healthy volunteers in low concentrations.

EXPERIMENTAL

Reagents

The following reagents were used: DMG puriss. from Fluka (Buchs, Switzerland), DMGE from Sigma (St. Louis, MO, U.S.A.), acetyl chloride from Fluka and Dowex 50W-X8, 50–100 mesh from Fluka. N,N-Dimethylaminopropionic acid (DAP) was kindly provided by Hans Bundgaard, The Royal Danish School of Pharmacy, and 6 *M* hydrochloric acid reagent (HCl-R) was obtained by adding 20 ml of acetyl chloride dropwise to 30 ml of 99.9% ethanol.

Chromatography

A Hewlett Packard 5730 A gas chromatograph (Waldbronn, F.R.G.) equipped with a flame ionization detector and connected to an Omniscribe recorder was used. The 2 m x 2 mm I.D. steel column was packed with 10% Carbowax 20 M on Chromosorb W-HP, 100–120 mesh, treated with 2% potassium hydroxide. The injection port, the oven and the flame ionization detector were operated at 150, 140 and 200°C, respectively. Nitrogen was used as the carrier gas at the flow-rate of 25 ml/min.

Formation of DMGE and N,N-dimethylaminopropionic acid ethyl ester (DAPE)

A 50-μl volume of a 2.04 m*M* solution of DMG in 99.9% ethanol was mixed with 50 μl of HCl-R and heated at different temperatures for 30 min in order to test the influence of temperature on the amount of DMGE formed. The formation of the derivatives as a function of the time was tested by heating the mixtures at 70°C as described above. The evaporated reaction mixtures were extracted as described under *Sample preparation*. To examine the evaporation procedure, a known amount of DMGE was dissolved in the reaction mixture, which was evaporated by rotor-vaporization or by a nitrogen stream. The linearity of the procedure was tested in the DMG concentration range from 0.012 to 0.234 m*M*. The standard solutions were 0.01 *M* with respect to hydrogen chloride and 0.012 m*M* with respect to the internal standard DAP. The samples were treated as described under *Sample preparation*.

Sample preparation

A 100-μl volume of 1 *M* hydrochloric acid and 100 μl of 12 m*M* DAP were added to 5.0 ml of urine. Distilled water was added to a total volume of 10.0 ml, and the mixture was centrifuged at 2000 *g* for 10 min. A 1000-μl aliquot of the

supernatant was transferred into a 70 mm x 4 mm I.D. cation-exchange column packed with Dowex 50W-X8. Then the column was washed with 3.0 ml of distilled water. DMG was eluted from the column with 3.0 ml of 6 *M* ammonia followed by 20 ml of water. The ion-exchange column was regenerated with 3 ml of 4 *M* hydrochloric acid followed by 7 ml of distilled water. The collected fraction was rotor-evaporated for 15 min at ambient temperature to remove the ammonia. Before freeze-drying, 100 μl of 4 *M* hydrochloric acid were added. The freeze-dried sample was suspended in 1500 μl of HCl-R using an ultrasonic bath for 15 min. The suspension was transferred to a 3.0-ml V-micro vial. The flask used for freeze-drying was washed with 1000 μl of 99.9% ethanol, and the ethanol was transferred to the vial. The reaction vial was placed on a heating block at 75–77°C for 60 min. Subsequently the solvent was evaporated using a nitrogen flow at 70°C for 45 min. After 5 min at ambient temperature, 250 μl of methylene chloride followed by 750 μl of 2 *M* sodium hydroxide were added, and the vial was shaken immediately for 1 min. The upper layer was removed, and 1 μl of the methylene chloride layer was analysed immediately.

Recovery studies

The recovery of DMG from urine and from standard solutions was compared with that of non-treated standard solutions of DMGE in methylene chloride. A standard of DAPE was not available, thus the DMG/DAP ratio was compared with a standard solution of DMG and DAP in 99.9% ethanol treated as described under *Sample preparation*.

Verification of peak identity using GC–MS

The peak identity was verified by injecting standard solutions and urine samples treated as described under *Sample preparation* onto a 30 m x 0.25 mm I.D. fused-silica J & W (Folsom, CA, U.S.A.) DB-5 column with a film thickness of 0.25 μm and coupled to a Finnigan MAT Model 4515 B mass spectrometer (Bremen, F.R.G.) operated in the electron ionization mode at 70 eV. The GC oven temperature was programmed as follows: 0–2 min, 35°C; 2.0–10.0 min, linearly increasing temperature to 100°C; 10–15 min, linearly increasing temperature to 200°C. Mass spectra of corresponding peaks in standards and samples were compared.

RESULTS AND DISCUSSION

Formation of DMGE and DAPE

The amount of DMGE formed increased as the temperature of the reaction mixture increased (Fig. 1). After reaction for 60 min at 75°C no further increase in the amount of DMGE was observed (Fig. 2).

Evaporation by means of the rotor–evaporator showed a recovery of 65 ± 10%, and evaporation using a nitrogen stream a recovery of 78 ± 4%. The

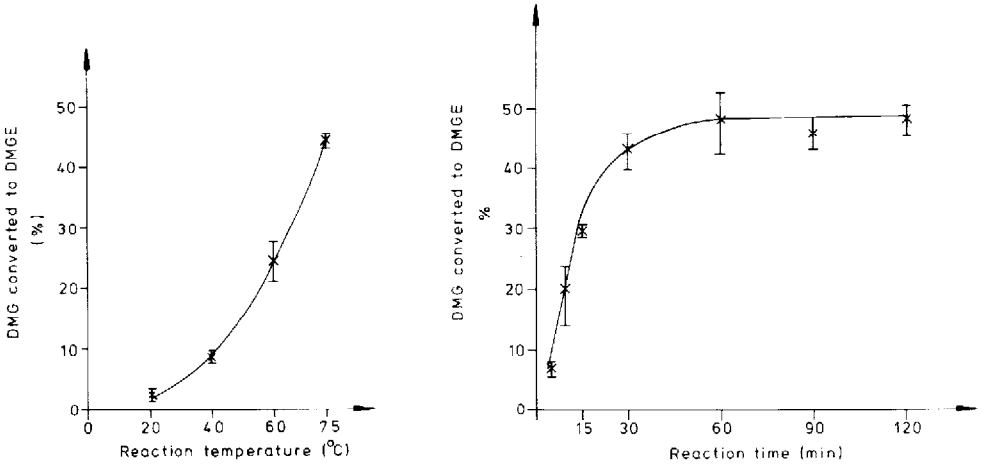

Fig. 1. Formation of DMGE as a function of the reaction temperature. The reaction time was 30 min.

Fig. 2. Formation of DMGE at 75°C as a function of the reaction time.

evaporation process using the nitrogen stream was studied (Fig. 3) and it is clear that it is necessary to control carefully the time of evaporation. It was not possible to obtain data on the recovery of DMG in the interval of 0–30 min as the extraction mixture emulsified in the presence of ethanol. The low recovery obtained after 30 min of evaporation is probably due to the fact that not all solvent had evaporated after 30 min. The ratio of the peak heights of DMGE and DAPE remained constant, the coefficient of variation (C.V.) being 4.7% in the interval from 50 to 70 min of reaction time.

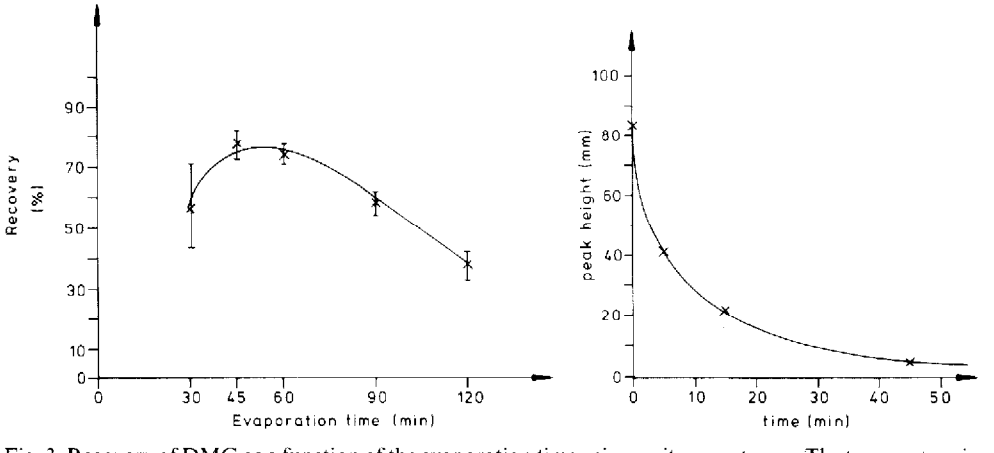

Fig. 3. Recovery of DMG as a function of the evaporation time using a nitrogen stream. The temperature is 75°C ($n=4$).

Fig. 4. Degradation of DMGE in 2 M sodium hydroxide as a function of time at 23°C.

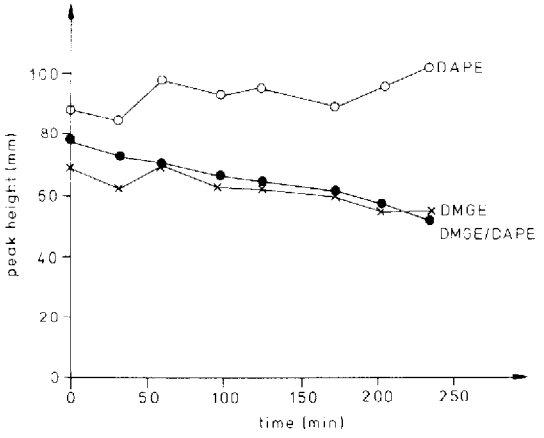

Fig. 5. Stability of DMGE (○) and DAPE (●) and the ratio of DMGE/DAPE (×) after extraction into methylene chloride. (The peak-height ratio shown has been multiplied by 100.)

TABLE I

RECOVERIES OF DMG AND DAP FROM URINE

Each value represents the mean recoveries of DMG and DAP from urine ± C.V. based on four determinations; N.D., not determined.

Medium	Recovery of DMG relative to untreated DMGE in CH_2Cl_2 (%)	DMG/DAP ratio relative to the ratio of a standard solution in 99.9% ethanol (%)	Recovery of DMGE after extraction with 2 *M* NaOH (%)
Spiked human urine	56 ± 22	86 ± 5	N.D.
Standard solution of DMG and DAP in 0.01 *M* HCl, ion-exchanged and freeze-dried before derivatization	72 ± 3	90 ± 2	N.D.
Standard solution of DMG and DAP in 0.01 *M* HCl, freeze-dried before derivatization	36 ± 11	36 ± 3	N.D.
Standard solution of DMG and DAP in 99.9% ethanol	48 ± 18	100 ± 7	N.D.
Standard solution of DMGE in CH_2Cl_2	100 ± 2	N.D.	92 ± 3

Stability studies

DMGE is hydrolysed rapidly in 2 *M* sodium hydroxide (Fig. 4). Thus it is crucial, when DMGE and DAPE were extracted into the methylene chloride phase, to add methylene chloride before adding 2 *M* sodium hydroxide. The DMGE/DAPE ratio after extracting into methylene chloride is shown as a function of time in Fig. 5. The peak-height ratio of DMGE/DAPE decreased as a function of time owing to the instability of DMGE. This makes it necessary to control carefully the time during derivatization as well as the time until injection of the samples onto the column.

Standard curve

The standard curve was linear in the range 0.01–0.23 m*M* DMG in urine. The detection limit, determined as a signal-to-noise ratio of 3, was 0.01 m*M* DMG in urine for the method described under *Sample preparation.*

Recovery studies

The results of the recovery studies given in Table I demonstrate that it is necessary to use the internal standard DAP as it compensates a rather important variation in the recoveries of DMG relative to an untreated solution of DMGE in methylene chloride.

Application of the method

Table II shows the amount of DMG found in urine from patients suffering from homocystinuria, hypersarcosinaemia or hyperglycinaemia and from healthy volunteers. Patients suffering from homocystinuria were treated with betaine. Creatinine is an endogenous substance excreted in the urine at a rate close to the glomerular filtration rate. Thus the concentration of creatinine in urine can be used to compensate for variations in the concentration of DMG in urine due to

TABLE II

DMG FOUND IN HUMAN URINE SAMPLES FROM HEALTHY VOLUNTEERS AND FROM PATIENTS SUFFERING FROM HOMOCYSTINURIA, SARCOSINAEMIA AND HYPERGLYCINAEMIA

Urine sample	DMG found (m*M*)	DMG/creatinine (m*M*/m*M*)
Healthy volunteer (A)	0.01	Not determined
Healthy volunteer (B)	0.04	Not determined
Homocystinuria patient (C)	1.96	0.56
Homocystinuria patient (D)	11.4	1.31
Sarcosinaemia patient (E)	0.06	0.01
Hyperglycinaemia patient (F)	0.18	0.18

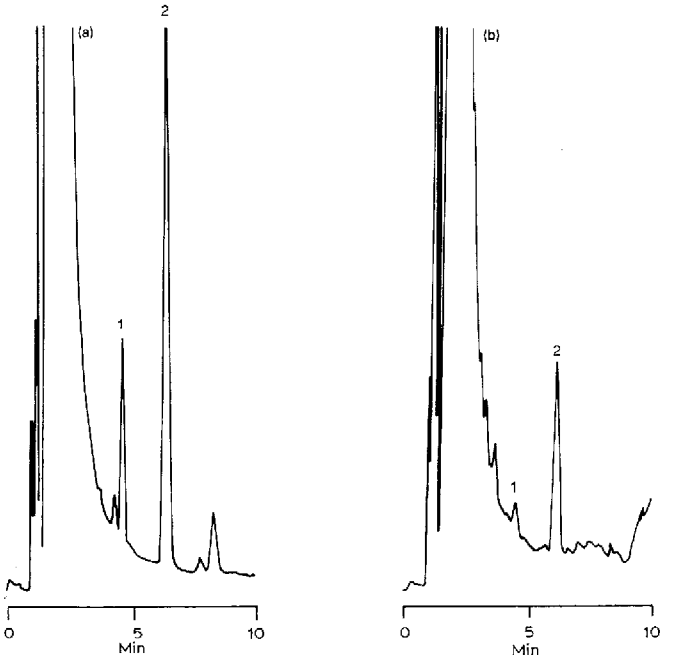

Fig. 6. (a) Chromatogram of a urine sample from a healthy subject; (b) chromatogram of a urine sample from a patient suffering from homocystinuria treated with betaine. Peaks: 1 = DMGE; 2 = DAPE.

variations in diuresis. A representative chromatogram is shown in Fig. 6. The gas chromatographic–mass spectrometric (GC–MS) examination of the urine samples verified the presence of DMG in all samples. We found that DMG was present in human urine from two healthy volunteers in concentrations of 0.04 and 0.01 m*M*, respectively. The concentration of DMG in urine samples from patients suffering from homocystinuria treated with betaine were very high. The concentrations of DMG in urine from patients suffering from hypersarcosinaemia and hyperglycinaemia were slightly elevated.

CONCLUSIONS

The method developed can be used to determine DMG in human urine. The low detection limit in urine (0.01 m*M* DMG) enables the determination of DMG in urine samples from healthy volunteers. In order to obtain valid data it is necessary to control carefully the timing of several steps in the analysis procedure.

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