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Note

L-Carnitine interference in gas chromatographic determination of total trimethylamine in urine: a novel assay method

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In the mammalian gastrointestinal tract, L-carnitine has been reported to be degraded to trimethylamine (TMA) by the endogenous bacterial flora [1,2]. After resorption, practically all the TMA in the liver is converted into the 30-fold less toxic trimethylamine N-oxide (TMA-O) [3,4]. The need for accuracy and sensitivity in determining the very low concentrations at which TMA and TMA-O are present in biological fluids motivated our choice of gas chromatography (GC). TMA and TMA-O may be determined in a sample as total trimethylamine (TTMA) after reduction of TMAO to TMA by the addition of $TiCl_3$.

A number of authors have reported the appearance of "ghost" peaks in the GC determination of TMA [5,6]. This paper presents evidence that this interference is correlated with the presence of carnitine which, therefore, must be separated from TMA. Ion-exchange chromatography [7] is too prolonged a method and does not enable a complete separation of TMA from carnitine. For that reason, an easier and more satisfactory assay method was developed.

EXPERIMENTAL

Gas chromatography

A Varian 3400 gas chromatograph with a flash vapourization injector and a thermoionic specific detector (TSD) were used for the analyses. A flame ionization detector (FID) was used to check the assay.

The column was made of coiled glass (3 m × 6 mm O.D. × 2 mm I.D.) packed with Carbopack B–4% Carbowax 20 M–0.8% KOH (Supelchem, Milan, Italy) [8]; the first 2 cm were packed with 20% lithium hydroxide monohydrate (Fluka, Buchs, Switzerland) on Chromosorb-W-AW-DMCS, 80–100 mesh (Supelchem).

The operating parameters were: column temperature, 95°C isothermal; injector

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port temperature, 150°C; detector temperature, 250°C; helium carrier gas flow-rate, 20 ml/min; hydrogen flow-rate, 5 ml/min (TSD) or 12 ml/min (FID); air flow-rate, 175 ml/min.

In order to avoid the tailing of amine peaks, caused by adsorption on the column, deactivation was necessary. This was accomplished first by adding basic material (KOH) to the packing, secondly by conditioning the column by injecting 1% NH_4OH solution (this treatment converts K_2CO_3 in the packing into KOH), and finally by using an alkaline packing in the first few centimeters of the column length.

The use of the flash vapourization injector allows placing of a glass insert in the injector port to provide easy clean-up and to facilitate removal of non-volatile residues.

Special reagents, standards and apparatus

TMA chloride and TMA-O (Sigma, St. Louis, MO, U.S.A.) powders were dried at 60°C for five days and then stored in a dessicator until ready for use. Aqueous stock solutions used were 12 m*M* TMA-O and 10 m*M* TMA. After dilution with distilled water (at 500–4000 μM concentrations) they could be stored safely at −20°C for several months. A 5 m*M* isopropylamine (internal standard, I.S.) solution (Fluka) was used as a reference standard. Sulphonic acid bonded silica, 1-ml cation-exchange resin columns (LC-SCX) (Supelchem) were used to adsorb TMA. The biological material to be assayed was collected and stored in 0.1 *M* HCl at −20°C.

A solution of 13% $TiCl_3$ in 20% HCl (Fluka) was used to reduce TMA-O to TMA chloride.

To purify the biological sample, the apparatus depicted in Fig. 1 was set up.

Procedure

An aliquot of the urine sample (500 μl) was processed with $TiCl_3$ solution (50

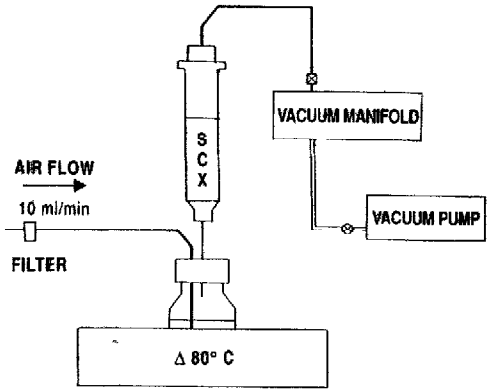

Fig 1. Purification apparatus showing the thermostatic block, the LC-SCX 1-ml column and the vacuum manifold (Supelchem).

μl) for 30 min at room temperature. Excess $TiCl_3$ was eliminated by adding 1.5 *M* $HClO_4$ (1 ml). Elimination of interfering carnitine was accomplished by volatilizing TMA from the alkalinized solution. To that end, 1 ml of perchloric acid supernatant was adjusted to pH 12.2. with 5 *M* KOH, made up to 2.5 ml with distilled water and finally centrifuged at 3470 *g* for 10 min at 6°C. Exactly 1 ml of supernatant was incubated at 80°C for 1 h in a tightly sealed glass ampoule. The volatilized TMA was conveyed to and trapped in an LC-SCX column. The TMA trapped in the column was eluted with 1 *M* $HClO_4$ (1.5 ml); 50 μl of 5 m*M* I.S. and 150 μl of 5 *M* KOH were added to a 0.5-ml aliquot of eluate. After centrifugation, a 0.1-μl aliquot of the supernatant was analysed by GC.

RESULTS AND DISCUSSION

The reliability of the method was clearly established. The correlation of peak-area values resulting from the injection of TMA standard solutions at concentrations of 0.5–4 m*M* was statistically significant ($R = 0.9968$; $P < 1\%$). The linear regression equation was $y = 0.4211x - 0.0302$.

The specificity was evaluated through the volatilization process: TMA was totally separated from the residue and from any interfering substances, and analyses performed with two different types of detector gave similar results (Table I).

TABLE I

TTMA LEVELS IN A RAT URINE SAMPLE AND IN A TMA-O AQUEOUS SOLUTION

	TTMA (mean ± S.D.) (μM)		TTMA (%)
	TSD	FID	
Control urine	2269 ± 176 ($n = 5$)	2374 ± 154 ($n = 5$)	105
TMA-O-enriched urine	3189 ± 112 ($n = 5$)	3283 ± 288 ($n = 5$)	103
TMA-O-aqueous solution	1104 ± 154	1055 ± 143	96

Table II indicates that in non-purified urine samples (analysed without separating TMA from carnitine) TTMA values were 49% higher than in purified samples, and that a further 61% overestimation of TTMA values occurred in non-purified, carnitine-enriched urine from carnitine-treated rats. Then, 5 min after GC injection of a non-purified sample, injection of 1 μl of 1% KOH solution gave rise to a "ghost" peak with the same retention time as TMA. The areas of the ghost peaks occurring after two successive injections of 0.1 μl of 1% KOH were 28 and 23% of the area of the peak of the previously injected non-purified sam-

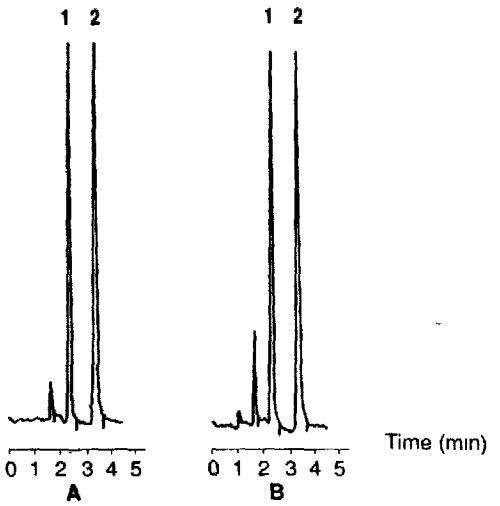

Fig 2. (A) Chromatogram of a 0.1-μl injection of 11.9 pmol TMA standard solution (peak 1) and 35.7 pmol I S. (peak 2); (B) chromatogram of a 0.1-μl injection of a urine sample. Sensitivity, × 12; chart speed, 0 5 cm/min.

ple. As shown in Table II, this phenomenon was particularly apparent after injection of a highly concentrated L-carnitine aqueous solution: the purified solution did not reveal the presence of TMA, whereas the non-purified solution had a TMA concentration of 2096 μM, owing to contamination of the injector with carnitine.

The ghost peaks did not appear after injection of TMA standard solutions [5] or of urine samples processed as described above. The purification method adopted allowed determination of urinary TTMA in those samples were L-carnitine levels were found to be 35 times higher than the basal values (data not shown). The elimination of interfering urinary carnitine was further confirmed by the observation that the concentration of carnitine in the samples remained unchanged before and after purification (Table II).

Fig. 2 shows chromatograms of an aqueous 2000 μM TMA solution (A) and of a urine sample (B) processed according to the method described above. During 4-min GC analyses, TMA (peak 1) and I.S. (peak 2) were eluted with good resolution and separation at the levels of the injected picomolar amounts.

The recovery of TTMA, which was 99% after treatment with $TiCl_3$ (first step), was 83% after evaporation, resin binding and elution. The second stage of the process, therefore, is the most critical one of the analysis.

The reproducibility is demonstrated by the results obtained from several determinations carried out on urine samples and on TMA standard solutions with coefficients of variation (C.V.) of 5.0% (n = 9) and 6.5% (n = 11), respectively (Table III). The variability observed over a period of eight months with measurements carried out twice a week resulted in a C.V. value of 8.0% (Table III).

TABLE II

INTERFERING EFFECT OF L-CARNITINE ON TTMA DETERMINATIONS IN 24-h URINE SAMPLES

Male albino Wistar rats (300 g) were fed a balanced diet and treated orally with 20 mg/kg water (control urine) or with 310 μmol/20 ml/kg L-carnitine (L-carnitine-enriched urine). Samples were analysed before and after purification.

	Non-purified samples			Purified samples		
	TTMA		Total carnitine (μM)	TTMA (μM)	Total carnitine	
	μM	%			μM	%
Control urine	3095 ± 184 (n = 4)	149	207.5 ± 27 0 (n = 12)	2077 ± 123 (n = 4)	203 1 ± 12.7 (n = 2)	98
L-Carnitine-enriched urine	3763 ± 266 (n = 5)	161	521.2 ± 9 19 (n = 2)	2357 ± 174 (n = 5)	547.1 ± 11.5 (n = 2)	105
L-Carnitine aqueous solutions	2096 ± 50·2 (n = 2)		6912 ± 17 0 (n = 2)	0 (n = 2)	6881 ± 18.4 (n = 2)	100

TABLE III

REPRODUCIBILITY AND VARIABILITY OF TTMA DETERMINATIONS IN AQUEOUS SOLUTION AND RAT URINE SAMPLES

Sample	Concentration (mean ± S D.) (μM)	n	C V (%)	Recovery (%)
Reproducibility				
2000 μM TMA	1654 ± 107.6	11	6 5	82.7
Urine	3609 ± 181 8	9	5 0	
Variability over time				
2000 μM TMA after eight months	1617 ± 129.3	12	8 0	81 0
Urine after two months	2551 ± 59.2	4	2.3	

It can be concluded that the GC method described here for the determination of urinary TTMA has good reproducibility, high specificity and sensitivity, and does not suffer from any interference due to the presence of L-carnitine.

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