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Short communication

Sensitive assay of trimethylamine *N*-oxide in liver microsomes by headspace gas chromatography with flame thermionic detection

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Abstract

To compare the trimethylamine *N*-oxygenase activity of liver microsomes from house musk shrew (*Suncus murinus*) and rat, a sensitive method for the quantitation of trimethylamine (TMA) *N*-oxide was developed using gas chromatography with flame thermionic detection. The limit of quantification was 0.5 μ*M* and the calibration curve was linear at least up to 5 μ*M* in incubations containing liver microsomal preparations from *Suncus*. The intra-day RSD values ranged from 10.4 to 12.8 at 0.5 μ*M* and from 3.5 to 6.7 at 5 μ*M*. The inter-day RSD values were 11.6 and 6.5 at 0.5 and 5 μ*M*, respectively. This method provides a sensitive assay for TMA *N*-oxygenase activity in liver microsomes. Using this method we found that *Suncus* was capable of *N*-oxidizing trimethylamine at a very slow rate.

Keywords: Trimethyl-*N*-oxide

1. Introduction

Trimethylamine (TMA) is a strong nucleophilic tertiary alkylamine. It is volatile and therefore emits a ''fishy'' odor. TMA is normally oxidized by hepatic flavin-containing monooxygenase (FMO) and excreted into the urine as TMA *N*-oxide (TMAO), a non-volatile metabolite. Trimethylaminuria is an autosomal recessive human disorder as an inherited polymorphism. Individuals diagnosed as having trimethylaminuria excrete relatively large amounts of TMA in their urine, sweat and breath, resulting in a fishy odor of the body [1–3]. Recently, Cashman et al. [4] reported that decreased human flavin-containing monooxygenase 3 (FMO3) contributed to the abnormal metabolism of TMA in trimethylaminuria patients.

We have studied the expression of hepatic FMOs in mammals to elucidate the physiological significance of FMO [5,6]. In a previous study, we found that the levels of mRNAs for FMOs were extremely low in the house musk shrew (*Suncus murinus*) [7]. *Suncus* is classified as being in the family Soricidae of the order of Insectivola and believed to be phylogenetically closer to primates than rodents [8].

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To determine whether or not this animal species can be a model for trimethylaminuria, it is necessary to compare the TMA *N*-oxygenase activity of *Suncus* to that of other experimental animals, such as the rat, using liver microsomes.

Various analytical techniques have been employed for the determination of TMA *N*-oxide in a biological fluid. These techniques include radioisotope analysis [9], gas chromatography (GC) [10–19] or GC–mass spectrometry (MS) [20]. However, these methods possess insufficient sensitivity or complicated analytical procedure to allow monitoring of the formation of TMA *N*-oxide by *Suncus* liver microsomes.

In the present study, we developed a sensitive headspace GC assay suitable for studies of in vitro TMA *N*-oxygenation with a lower limit of quantification compared to the reported methods using nitrogen and phosphorus-specific flame thermionic detection (FTD).

2. Experimental

2.1. Chemicals and reagents

TMA hydrochloride was obtained from Wako Pure Chemicals (Tokyo, Japan). TMA *N*-oxide dihydrate was obtained from Aldrich (St. Louis, MO, USA). All other chemicals were of the highest grade commercially available.

2.2. Animals

Male *Suncus* (six weeks old) were obtained from Clea Japan (Tokyo, Japan). Male Sprague–Dawley rats (seven weeks old) were obtained from Japan SLC (Shizuoka, Japan). Animals were maintained at a constant temperature of 23±1°C under controlled lighting conditions (lights on from 7.00 to 19.00). They received a commercial chow (Clea Japan) and tap water ad libitum.

2.3. GC instrumentation

GC-17A series gas chromatographs (Shimadzu, Kyoto, Japan) equipped with a FTD system were used for analysis. The column (2 m×3.2 mm I.D.) was packed with 4% (w/w) Carbowax 20M–0.8% (w/w) KOH on Carbopack B (60–80 mesh) (Supelco, Bellefonte, PA, USA). To precondition the column, 10-μl portions of 1% (v/v) ammonium hydroxide solution were injected 20 times over a period of 30 min at 220°C. This treatment converted potassium carbonate in the packing to potassium hydroxide thereby minimizing the adsorption of trimethylamine to the column and preventing tailing of the peak. The operating temperatures of the column, injection port and detector unit were 70°C isothermal, 150°C and 200°C, respectively. The carrier gas was helium at a flow-rate of 20 ml/min.

2.4. Preparation of liver microsomes

Liver microsomes were prepared by sequential centrifugation of the homogenates as described elsewhere [21]. Briefly, the animals were stunned by a blow on the head and decapitated. The livers were removed immediately, washed with 1.15% (w/v) KCl solution, minced and homogenized with three volumes of the same solution. The homogenates were centrifuged at 9000 *g* for 20 min. The supernatant fraction was recentrifuged at 105 000 *g* for 60 min. The microsomes obtained as a pellet were suspended in ice-cold distilled water at a concentration of approximately 10 mg protein/ml and stored at −80°C until use. The protein concentration was determined by the method of Lowry et al. [22] using bovine serum albumin as a standard.

2.5. Sample preparation

The activity of TMA *N*-oxygenation was determined by a method described by Gut and Conney [23] with minor modifications. Briefly, a typical reaction mixture consisted of 50 m*M* Tris–HCl buffer (pH 7.4), an NADPH generating system (10 m*M* $MgCl_2$, 10 m*M* glucose-6-phosphate, 0.4 m*M* $NADP^+$ and 0.5 unit of glucose-6-phosphate dehydrogenase) and 0.5 mg protein of microsomes in a final volume of 1 ml. The reaction mixture was preincubated at 37°C for 3 min. Reaction was initiated by the addition of TMA at final concentrations ranging from 0.05 to 8 m*M*. After incubation for 10 min, 25% (w/v) $ZnSO_4$ (0.1 ml) and a

saturated aqueous solution of $Ba(OH)_2$ (0.1 ml) was added to stop the reaction. The mixture was centrifuged at 1300 *g* for 5 min. An aliquot of the clear supernatant (0.5 ml) was placed in a glass vial (27 ml) and made alkaline with 5 *M* NaOH (25 μl) and evaporated to dryness in vacuo at 80°C. The residue was dissolved in 1 *M* HCl (0.4 ml) and 20% (w/v) $TiCl_3$ (0.1 ml). The vial was capped with a PTFE-lined septum, and TMAO contained in the mixture was reduced with $TiCl_3$ at room temperature for 1 h. Before the headspace GC analysis, 10 *M* NaOH (5 ml) was added into the vial, then the vial was immediately capped with a PTFE-lined septum, and kept at 95°C for 30 min. The headspace gas (0.8 ml of headspace gas) was subjected to GC. Only a single injection of gas was performed, then the remaining contents were discarded.

2.6. Preparation of calibration standards

For each run of the assay, accurately weighed 55.6-mg portions of TMAO were dissolved in 50 ml of water. Appropriate dilutions were done with water to obtain standard solutions containing four points in the range of 5–50 m*M* of TMAO. A typical reaction mixture consisted of 50 m*M* Tris–HCl buffer (pH 7.4), an NADPH generating system and 0.5 mg protein of rat liver microsomes. The mixture was incubated at 37°C for 10 min without addition of TMA. After the addition of 25% (w/v) $ZnSO_4$ (0.1 ml) and a saturated aqueous solution of $Ba(OH)_2$ (0.1 ml), calibration standards were prepared in the range of 0.5–5 m*M* by spiking with 0.1 ml each of the standard solution.

3. Results

3.1. Chromatography

In the present study, TMAO was determined as TMA after a chemical reduction. Typical chromatograms for the assay of TMA *N*-oxygenase activity are shown in Fig. 1. The chromatogram from a control incubation contained no peaks co-eluting with TMA.

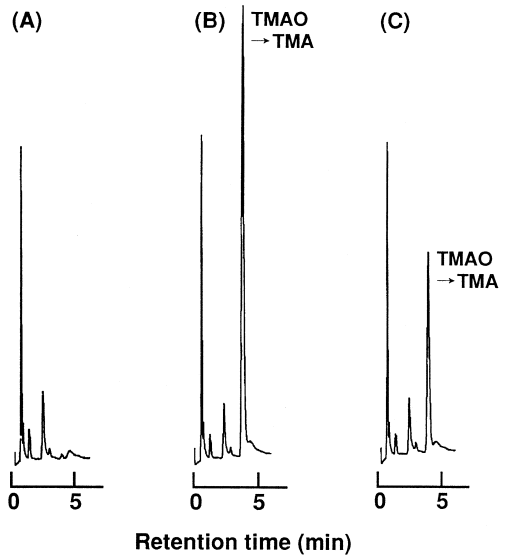

Fig. 1. Representative gas chromatograms for the assay of formation of TMAO from TMA by *Suncus* liver microsomes. The reaction mixture containing 0.5 mg protein of microsomes was incubated without TMA (A), spiked with 20 nmol TMAO (B), or incubated with 100 nmol TMA (C). TMAO was determined indirectly after a quantitative reduction to TMA.

3.2. Quantification

A calibration curve was constructed by plotting the peak-area versus the concentration of TMA in the incubation mixture spiked with TMAO. The correlation coefficient calculated by a linear-square regression analysis was 0.998 over a concentration ranging from 0.5 to 5 μ*M*.

3.3. Precision and accuracy

Precision and accuracy of the assay was determined by the incubation of a mixture containing TMAO at two concentrations (0.5 and 5 μ*M*). The intra-day variation was determined by analyzing six spiked samples at each concentration on the same day. For the determination of inter-day variation, 18 samples were analyzed on three different days. The relative standard deviations (RSDs) ranged from 10.4 to 12.8 at 0.5 μ*M* and from 3.5 to 6.7 at 5 μ*M*. The inter-day RSDs were 11.6 and 6.5 at 0.5 and 5 μ*M*, respectively (Table 1). Addition of internal standard

Table 1
Intra- and inter-day precision and accuracy of TMAO determination

Spiked concentration (μ*M*)	Day	Mean (μ*M*)	RSD (%)	Accuracy (%)
Intra-day (n=6)				
0.5	1	0.465	12.8	−7.01
	2	0.493	11.0	−1.39
	3	0.520	10.4	3.99
5	1	5.03	3.5	0.50
	2	5.18	4.9	3.58
	3	4.68	6.7	−6.39
Inter-day (n=18)				
0.5		0.493	11.6	−1.47
5		4.96	6.5	−0.77

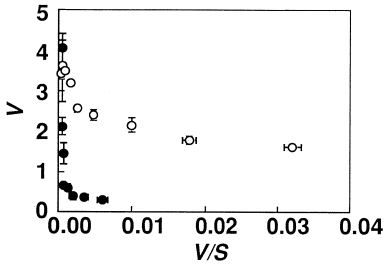

Fig. 2. Eadie–Hofstee plots for the TMA *N*-oxygenase in liver microsomes from *Suncus* and rat. Each point represents the mean±S.E.M. of four animals. Incubation mixture consisted of 50 m*M* Tris–HCl buffer (pH 7.4), the NADPH-generating system and 0.5 mg protein of microsomes as described in detail in Experimental. *V*, Velocity of the TMA *N*-oxygenation (nmol/min/mg protein); *S*, substrate concentration (μ*M*). ●, *Suncus*; ○, rat.

was not required for the established method which gave highly reproducible results.

3.4. Application to in vitro assay

In the present study, the reaction mixture was made alkaline and evaporated to dryness to remove unmetabolized TMA. The chromatogram from the substrate-free reaction mixture spiked with 8 m*M* TMA contained no peaks at the retention time of TMAO, indicating that the residual TMA in the mixture was negligible (data not shown).

The Eadie–Hofstee plots for TMA *N*-oxygenase in liver microsomes from *Suncus* and rat showed a biphasic pattern (Fig. 2). These results suggest that at least two enzymes are involved in the TMA *N*-oxygenation. The enzymes with a high affinity in the TMA *N*-oxygenation in *Suncus* showed a two-fold higher K_m (55 μ*M*) and a four-fold lower V_{max} (0.61 nmol/min/mg protein) compared to those seen in the rat (Table 2). The total CL_i of *Suncus* was calculated to be seven-fold lower than that of the rat.

4. Discussion

Several GC or GC–MS methods for the analysis of TMA or TMAO have been reported. For example, daCosta et al. [20] developed the most sensitive method for the determination of TMAO after chemical reduction using GC–MS. However, complicate procedures were necessary to perform the assay, such as liquid–liquid extraction for precipitation of protein, derivatization of TMA to *N*,*N*-dimethyl-2,2,2-trichloroethyl carbamate. In addition, the GC–

Table 2
Michaelis–Menten kinetic parameters for the TMA *N*-oxygenase in liver microsomes from *Suncus* and rat (each value represents the mean±S.E.M. of four animals)

	Enzyme (1)			Enzyme (2)		
	K_m[a] (1) (μ*M*)	V_{max}[a] (1) (nmol/min/mg protein)	CL_i[a] (1) (μl/min/mg protein)	K_m[a] (2) (μ*M*)	V_{max}[a] (2) (nmol/min/mg protein)	CL_i[a] (2) (μl/min/mg protein)
Suncus	55.4±3.2	0.61±0.08	11.0±0.9	3559±798	3.73±0.59	1.12±0.18
Rat	31.2±3.2	2.49±0.09	81.8±6.4	451±36	1.31±0.04	2.95±0.23

[a] K_m, Michaelis–Menten constant; V_{max}, maximum velocity of TMA *N*-oxygenation; CL_i, intrinsic clearance calculated as V_{max}/K_m.

MS method requires a deuterated internal standard. On the other hand, Zhang et al. [19] reported the method for the determination of TMAO using a headspace GC with flame ionization detection (FID). Although the GC–FID method is suitable for routine use, the lower limit of quantitation about 5 μM was not sensitive enough to monitor the amounts of TMAO formed by *Suncus* liver microsomes. In our in vitro studies of TMA metabolism in *Suncus*, a method more sensitive than the previous method was needed, because the animal species expected to be a poor metabolizer of TMA. Therefore, we developed a sensitive and simple method for the quantitation of TMAO in in vitro study using the headspace GC–FTD. A new assay method developed in the present study showed comparable sensitivity to the GC–MS method of daCosta et al. [20], while the sensitivity was increased about 10-fold over the GC–FID method of Zhang et al. [19]. This was due to the use of FTD instead of FID.

5. Conclusion

A simple, sensitive method has been developed for the analysis of TMAO in liver microsomes and the method has been validated. Using this method, the formation of TMAO from TMA by *Suncus* liver microsomes could be monitored over a wide range of substrate concentrations. Thus, the sensitivity of the new method was adequate for the determination of the kinetic parameters to estimate the TMA *N*-oxygenase activity in *Suncus* liver.

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