

Original Article

## Accumulation of trimethylamine and trimethylamine-*N*-oxide in end-stage renal disease patients undergoing haemodialysis

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### Abstract

**Background.** Trimethylamine (TMA) is a short-chain tertiary aliphatic amine that is derived from the diet either directly from the consumption of foods high in TMA or by the intake of food high in precursors to TMA, such as trimethylamine-*N*-oxide (TMNO), choline and L-carnitine. The clinical significance of TMA may be related to its potential to contribute to neurological toxicity and 'uraemic breath' in patients with end-stage renal disease (ESRD).

**Methods.** Concentrations of TMA and TMNO in plasma from 10 healthy adults (not on haemodialysis) and 10 adults with ESRD undergoing haemodialysis (pre- and post-dialysis) were determined by gas chromatography–mass spectrometry.

**Results.** The concentrations of TMA and TMNO in pre-dialysis plasma ( $1.39 \pm 0.483$  and  $99.9 \pm 31.9 \mu\text{M}$ , respectively) were significantly ( $P < 0.05$ ) higher than the corresponding levels in healthy subjects ( $0.418 \pm 0.124$  and  $37.8 \pm 20.4 \mu\text{M}$ , respectively). However, there were no significant differences between post-dialysis and healthy subject plasma concentrations. In the ESRD patients, there was a significant ( $P < 0.05$ ) reduction in plasma TMA (from  $1.39 \pm 0.483$  to  $0.484 \pm 0.164 \mu\text{M}$ ) and TMNO (from  $99.9 \pm 31.9$  to  $41.3 \pm 18.8 \mu\text{M}$ ) during a single haemodialysis session.

**Conclusions.** TMA and TMNO accumulate between haemodialysis sessions in ESRD patients, but are efficiently removed during a single haemodialysis session.

**Keywords:** end-stage renal disease; gas chromatography; trimethylamine; trimethylamine-*N*-oxide

### Introduction

Trimethylamine (TMA) is a volatile short-chain aliphatic amine that has the characteristic odour of rotting fish. TMA may be derived from the diet either directly by the consumption of foods high in TMA, such as fish, or by the enterobacterial generation of TMA from food rich in trimethylamine-*N*-oxide (TMNO), choline or L-carnitine [1]. TMA is efficiently absorbed from the gastrointestinal tract [2] and subsequently metabolized by the flavin-containing monooxygenase (EC 1.14.13.8) isoform 3 enzyme (FMO3) in the liver to form TMNO [3]. Individuals with decreased activity of FMO3 develop trimethylaminuria, a condition characterized by increased excretion of TMA, body malodour resembling rotting fish and distressing psychosocial reactions [4].

Patients with end-stage renal disease (ESRD) have qualitatively different as well as higher bacterial populations in the small intestine compared with individuals with normal renal function [5], resulting in greater generation of the short-chain aliphatic methylamines from dietary precursors [6]. Furthermore, as these amines are normally removed from the body by renal excretion, further accumulation may occur in ESRD depending on the efficiency of their removal by haemodialysis. TMA and TMNO are of clinical importance because of their possible contribution to neurotoxicity and 'uraemic breath' in patients with ESRD [7].

In this study, solid-phase microextraction (SPME) and gas chromatography–mass spectrometry (GC–MS) were used to compare plasma levels of TMA and TMNO (pre- and post-dialysis) in patients with ESRD undergoing haemodialysis to those in healthy subjects. A recent report [8] identified a significant analytical problem that can be encountered during the measurement of TMA in human plasma. Thus, conversion of endogenous amines to TMA during sample preparation may have led to overestimation by previous researchers. This is the first time that a validated method has been

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used to measure accurately the extent of accumulation of TMA and TMNO in patients with ESRD.

## Subjects and methods

### Patients

The Royal Adelaide Hospital and University of South Australia Human Research Ethics Committees gave approval for the study. Prior to the collection of blood, subject consent was obtained and exclusion criteria included any person who was on, or had taken, oral antibiotics or L-carnitine in the last month. The subjects were also asked to avoid eating any food containing fish (rich in TMA) for 48 h prior to blood collection. Basic demographic data, including age, gender and time on haemodialysis, was obtained. A sample of blood (5 ml) was collected from 10 healthy subjects and pre- and post-haemodialysis from 10 patients with ESRD who were undergoing haemodialysis (thrice weekly) at the Renal Unit, Royal Adelaide Hospital. Blood was collected into a vacuette (Greiner Bio-one; Kremsmunster, Austria) and immediately placed on ice prior to centrifugation (3000 g) at 4°C for 10 min.

### GC–MS conditions

A Varian Star 3400 CX gas chromatograph (CA, USA) fitted with a SPB-1 Sulfur (30 m × 0.32 mm × 4.0 µm film thickness) fused silica capillary column (Supelco, PA, USA) and Varian Saturn 2000 mass spectrometer were used. The GC–MS conditions were splitless injection, injector temperature 250°C and column temperature 120°C isothermal, with helium used as the carrier gas at a flow of 60 ml/min. Mass spectra were collected every 0.25 s using electron impact ionization with automatic gain control. The *m/z* 58 and 66 ions were used to monitor for unlabelled and labelled TMA, respectively. A carboxen-polydimethylsiloxane (75 µm) SPME fibre (Supelco, PA, USA) and narrow bore injector liner were used for analysis (Supelco, PA, USA) and the SPME fibre was conditioned according to the manufacturer's instructions.

### Sample analysis

The plasma samples were analysed for TMA and TMNO using SPME and GC–MS based on the methods described previously [8,9]. Briefly, for the analysis of TMA, plasma (400 µl) was acidified with hydrochloric acid (600 µl, 0.01 M) followed by the addition of milli-RQ water (800 µl) and deuterated TMA (200 µl) as internal standard (14.7 µM free base equivalent, D<sub>9</sub>-DCl salt; Cambridge Isotope Laboratories, MA, USA). On the day of analysis, samples (unknown plasma sample treated as above, acidified calibration standards and acidified quality control samples) were placed on ice and 50 µl potassium hydroxide (5 M) was added to liberate unionized TMA. The samples were subsequently heated to 50°C for 5 min, followed by insertion of the SPME fibre into the headspace for a further 5 min and then insertion of the fibre into the GC–MS.

For the analysis of TMNO, hydrochloric acid (300 µl, 0.01 M) was added to plasma (50 µl) followed by the addition of titanium (III) sulphate in dilute sulphuric acid (10 µl, 45% w/v; Aldrich Chemical Company, WI, USA).

This solution was vortex mixed for 30 s and then left at room temperature for 15 min to allow reduction of TMNO to TMA. Deuterated TMA as internal standard (100 µl, 14.7 µM free base equivalent) and milli-RQ water (550 µl) were added followed by potassium hydroxide (100 µl, 5 M) and the vial sealed immediately. The calibration standards and quality control samples were reduced in an identical manner to the unknown plasma sample. The samples were then analysed for TMA using the method above. Calibration curves were constructed in the range of 0.169–33.8 µM for the TMA assay and 6.66–266 µM additional TMNO in plasma for the TMNO assay. Peak areas for TMA and TMNO were corrected for the response from the basal endogenous level of TMA (or TMNO) prior to determination of unknown plasma samples.

Determining the inter- and intra-day precision and accuracy of each method validated the plasma assays for TMA and TMNO [10]. Briefly, inter-day precision and accuracy for both assays was elucidated by duplicate analysis at each respective quality control concentration on two separate days. Intra-day precision and accuracy was determined by analysis of five samples of each quality control on the same day. The inter-day precision and accuracy of the TMA and TMNO assays was within 15% whilst the intra-day precision and accuracy was within 25% of the nominal concentration. These results were within reasonable limits for the measurement of endogenous compounds [10].

### Calculations

Statistical analysis was performed using the Student's *t*-test to compare differences in the concentrations of TMA and TMNO in plasma pre- and post-haemodialysis (paired *t*-test) and also between healthy subjects and the ESRD group (unpaired *t*-test), with *P* < 0.05 taken to represent significance.

## Results

The mean age of the healthy subjects and patients with ESRD included in the study was 32 and 52 years, respectively (Tables 1 and 2). The results revealed a significant difference between the concentrations of TMA in plasma of ESRD patients (pre-dialysis) and healthy subjects ( $1.39 \pm 0.483$  vs  $0.418 \pm 0.124$  µM; *P* < 0.00001) and of TMNO ( $99.9 \pm 31.9$  vs

**Table 1.** Demographic data and plasma TMA and TMNO concentrations in healthy subjects

Subject	Age (years)	Gender	TMA conc. (µM)	TMNO conc. (µM)
1	25	F	0.271	28.9
2	26	M	0.442	21.8
3	33	F	0.320	18.1
4	53	M	0.334	22.5
5	25	M	0.511	18.4
6	32	F	0.360	48.5
7	24	M	0.336	56.5
8	41	M	0.576	32.3
9	28	M	0.652	79.4
10	32	F	0.383	52.1
Mean	32		0.418	37.8
SD	9		0.124	20.4

**Table 2.** Demographic data and characteristics of dialysis for the group of patients with ESRD undergoing haemodialysis

Subject	Gender	Age (years)	Weight (kg)	Time on haemodialysis (years)	Dialyser	Type of dialysis	Membrane	Membrane surface area (m <sup>2</sup> )	Length of single haemodialysis session (h)	Number of dialysis sessions each week	Kt/V	Q <sub>B</sub> urea (ml/min)	Q <sub>D</sub> urea (ml/min)
11	M	57	64	0.7	F8 HPS	Haemodialysis	Polysulphone	1.8	3.5	3	1.39	252	500
12	F	69	101	1	HF 80S	Haemodiafiltration	Polysulphone	1.8	4.5	3	1.67	248	500
13	F	73	51	6.6	F8 HPS	Haemodialysis	Polysulphone	1.8	3.5	3	1.73	252	500
14	M	65	68	2.4	F8 HPS	Haemodialysis	Polysulphone	1.8	4	3	1.62	252	500
15	M	48	150	2.8	F10 HPS	Haemodialysis	Polysulphone	2.2	4.5	3	0.96	259	500
16	F	31	76	0.6	F10 HPS	Haemodialysis	Polysulphone	2.2	4	3	1.32	259	500
17	F	65	77	6.5	F8 HPS	Haemodialysis	Polysulphone	1.8	4.5	3	1.78	248	500
18	M	60	128	0.3	F10 HPS	Haemodialysis	Polysulphone	2.2	4.5	3	1.08	259	500
19	M	66	94	1.2	F10 HPS	Haemodialysis	Polysulphone	2.2	4.5	3	1.16	259	500
20	M	53	87	3.8	HF 80S	Haemodialysis	Polysulphone	1.8	5	3	1.37	248	500
Mean		59	90	2.6		Haemodiafiltration		2.0	4.3	3	1.4	254	500
SD		12	30	2.4				0.2	0.5	0	0.3	4.9	0

37.8 ± 20.4 µM;  $P < 0.0001$ ), suggesting accumulation of these compounds in the ESRD group between haemodialysis sessions (Tables 1 and 3). There was a significant ( $P < 0.001$ ) reduction of ~60% in the concentrations of both compounds following a single haemodialysis session with a post/pre concentration ratio of  $0.397 \pm 0.199$  for TMA and  $0.420 \pm 0.163$  for TMNO. These values were not significantly different ( $P > 0.05$ ) from the corresponding ratio for urea. However, there were no significant differences ( $P > 0.05$ ) in post-dialysis ( $0.484 \pm 0.164$  and  $41.3 \pm 18.8 \mu\text{M}$ ) and healthy subject ( $0.418 \pm 0.124$  and  $37.8 \pm 20.4 \mu\text{M}$ ) plasma concentrations of TMA and TMNO, respectively, reinforcing the efficient removal of these amines by haemodialysis.

### Discussion

Fish are an abundant source of TMNO [1] and both groups of subjects were asked to avoid the consumption of food containing fish for 48 h prior to blood collection. Also, enterobacteria are involved in the conversion of dietary components such as choline and L-carnitine into TMA [11] and, therefore, participants were excluded if they had taken oral antibiotics 1 month prior to the collection of blood. The basis for these restrictions was to minimize the contribution of diet and bacterial generation of TMA on plasma concentrations of TMA and TMNO, thereby focusing more clearly on the factors of ESRD and haemodialysis. The post/pre plasma concentration ratio for TMA and TMNO was similar to that for urea and suggests that there was comparable clearance of these compounds by haemodialysis. The 70% reduction in plasma concentrations of urea following a haemodialysis session suggested adequate and similar haemodialysis efficiency between the patients in this study. Although the mean age of the group of subjects not on haemodialysis (32 years) was much lower than those with ESRD undergoing haemodialysis (59 years), the main purpose of this study was to gain preliminary data on whether there is a significant accumulation of these substances in patients with ESRD, particularly as there may have been analytical shortcomings with previous data [8].

Earlier work by Zeisel *et al.* using GC found the whole blood concentration of TMA to be ~12.5 µM in humans [12] and, by comparison, the results in this study showed mean plasma levels markedly lower ( $0.418 \mu\text{M}$ ) in healthy subjects. Similarly, Simenhoff *et al.* [7] found pre- and post-dialysis TMA serum concentrations in a group of patients with ESRD undergoing haemodialysis of 127 and  $63.4 \mu\text{M}$ , respectively, which are approximately 100 times higher than the concentrations observed in our study. These former values may have overstated the actual TMA concentrations in blood or serum as recent work has indicated that minimizing the amount of potassium hydroxide added to plasma during analytical preparation is crucial for quantifying plasma concentrations of

**Table 3.** Pre- and post-dialysis plasma concentrations of TMA, TMNO and urea in patients with ESRD undergoing haemodialysis

Subject	Pre-dialysis TMA conc. (μM)	Post-dialysis TMA conc. (μM)	Ratio post/pre TMA	Pre-dialysis TMNO conc. (μM)	Post-dialysis TMNO conc. (μM)	Ratio post/pre TMNO	Pre-dialysis urea conc. (mM)	Post-dialysis urea conc. (mM)	Ratio post/pre urea
11	1.43	0.237	0.166	54.5	17.1	0.314	19.7	6.1	0.310
12	2.20	0.308	0.140	80.2	34.1	0.425	19.7	4.9	0.249
13	1.38	0.787	0.570	102	78.2	0.767	23.6	5.4	0.229
14	1.59	0.551	0.347	155	60.0	0.387	20.4	5.4	0.265
15	0.833	0.633	0.760	72.3	42.1	0.582	18.5	8.6	0.465
16	1.27	0.414	0.326	120	57.2	0.477	19.5	6.6	0.338
17	0.702	0.432	0.615	72.2	33.5	0.464	17.9	4.1	0.229
18	1.00	0.442	0.442	132	39.4	0.298	31.1	12.9	0.415
19	1.38	0.420	0.304	124	31.1	0.251	25.03	9.6	0.384
20	2.06	0.617	0.300	87.3	20.7	0.237	20.9	6.8	0.325
Mean	1.39	0.484	0.397	99.9	41.3	0.420	21.6	7.04	0.321
SD	0.483	0.164	0.199	31.9	18.8	0.163	3.98	2.65	0.0811

TMA accurately [8]. Potassium hydroxide is typically added to convert the conjugate acid of TMA (present in plasma) to the volatile TMA, prior to headspace analysis. However, potassium hydroxide catalyses the conversion of quaternary ammonium compounds also found in blood or plasma, for example L-carnitine or choline, to TMA via Hofmann elimination [8]. Therefore, a critical amount of potassium hydroxide is required to allow adequate liberation of TMA for GC-MS analysis without formation of TMA from Hofmann elimination [8]. For instance, the determined concentrations of TMA were 0.576 and 4.23 μM following the addition of 0.25 and 5.35 mmol of potassium hydroxide, respectively, to a plasma sample from a healthy individual [8]. Hence, the addition of even larger amounts of potassium hydroxide, more vigorous conditions (higher temperature), or greater concentrations of potential precursors would be expected to generate an even greater increase in the spurious formation of TMA. The analytical conditions employed by Zeisel *et al.* [12] and Simenhoff *et al.* [7] during sample preparation would have most likely contributed to the formation of TMA through Hofmann elimination [8]. Cashman *et al.* [13] have recently observed that liquid chromatography-MS was insufficiently sensitive to measure plasma concentrations of TMA in humans, which conforms to the low concentrations of the compound. The headspace SPME method described in this current paper allowed for the sensitive analysis of plasma samples for TMA and TMNO whilst ensuring negligible formation of TMA via Hofmann elimination [8].

The percentage of TMNO present in urine as total TMA (TMA and TMNO) has been used to phenotype individuals for FMO3 activity: individuals with trimethylaminuria have a genetic mutation in the *FMO3* gene, resulting in compromised *N*-oxidation of TMA [14]. In this current study, the group of subjects with ESRD on haemodialysis and those not on haemodialysis had ~99% of summed TMA and TMNO in plasma in the form of TMNO and, therefore, each subject had normal FMO3 activity.

The clinical significance of TMA and TMNO may be related to their potential to form the carcinogen *N*-nitrosodimethylamine and their association with abnormal neurological symptoms observed in patients with ESRD [15]. Other studies have shown higher levels of *N*-nitrosodimethylamine in the gastrointestinal tract of patients with ESRD compared with healthy controls [16] and, therefore, its potential contribution to an increased incidence of cancer in ESRD patients [17] should be carefully examined. *N*-nitrosodimethylamine does not elicit its carcinogenic effects directly, but requires metabolic activation in the liver, particularly by the cytochrome P450 2E1 isoenzyme, to form highly reactive alkylating intermediates that have the ability to methylate target organ DNA [18]. Mildly acidic conditions are conducive to the formation of *N*-nitrosodimethylamine from TMA and TMNO and, therefore, the acidic conditions of the stomach provide the most likely site for its formation

in the body [18]. Whether elevated plasma levels of TMA and TMNO translate to increased levels in gastric fluid is not known. However, compounds with similar structural properties to TMA and TMNO are excreted from blood to gastric fluid [19]. The presence of bacteria or chronic inflammation has also been highlighted in other research as favouring the formation of *N*-nitrosodimethylamine from TMA and TMNO, implying that other sites of the body have the potential to form this carcinogen [18]. In our study, the pre-dialysis plasma concentrations of TMA and TMNO were significantly greater than those in healthy subjects ( $P < 0.00001$  and  $P < 0.0001$ , respectively). There was also a significant reduction ( $P < 0.001$ ) in the pre-dialysis plasma concentrations of these substances following a haemodialysis session. Whether the accumulation of TMA and TMNO in the interdialysis period is associated with adverse effects is not currently known.

The ratio of the plasma concentrations of TMA and TMNO between ESRD patients (pre-dialysis) and healthy subjects ( $C_U/C_N$ ) was found to be 3.3 and 2.6, respectively. Since a large  $C_U/C_N$  ratio increases the probability that a particular compound may show toxicity in the presence of harmful biological effects *in vivo* (reviewed by Vanholder *et al.* [20]), the exact toxicological significance of TMA and TMNO in uraemic syndrome remains controversial; particularly given that in this current study, excessive accumulation did not occur and haemodialysis was sufficient for normalizing the plasma levels of these two compounds. Moreover, the impact of ESRD patients consuming dietary precursors to TMA and TMNO should be explored, because specific foods and dietary supplements could have a large impact on the accumulation of TMA and TMNO.

In conclusion, using a validated analytical method, we have for the first time compared TMA and TMNO levels between healthy adults and ESRD patients stabilized on haemodialysis. The results from this study indicate that there was a significant difference between the pre-dialysis concentrations of TMA and TMNO in the plasma of ESRD patients as compared with individuals who were not on haemodialysis, suggesting accumulation of these compounds between haemodialysis sessions. The ratio of TMA and TMNO in plasma suggested no impairment of TMA *N*-oxidation in this patient group. Future work could compare the plasma levels of these compounds in patients on intermittent dialysis (for example, haemodialysis three times per week) to patients on continuous dialysis (for example, peritoneal dialysis). Further investigations are also required to examine whether accumulation is also evident with other short-chain aliphatic methylamines, such as dimethylamine.

*Conflict of interest statement.* None declared.

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