

Diagnosis of suspected trimethylaminuria by NMR spectroscopy

Priscilla Podadera^a, Jose A.G. Arêas^b, Ursula M. Lanfer-Marquez^{a,*}

^a*Departamento de Alimentos e Nutrição Experimental, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, Av. Prof. Lineu Prestes, 580, CEP 05508-900-São Paulo, São Paulo, Brazil*

^b*Departamento de Nutrição, Faculdade de Saúde Pública, Universidade de São Paulo, São Paulo, Brazil*

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Abstract

Background: Trimethylamine (TMA) is a volatile substance produced in the gut, absorbed into the blood and further metabolized by healthy individuals into trimethylamine-*N*-oxide (TMAO) by TMA-oxidase and then excreted in urine. Patients suffering from trimethylaminuria (TMAU) show an impaired enzymatic oxidation of TMA, excreting this amine in breath, urine and other body secretions which confers an unpleasant body odor.

Methods: We diagnosed a Brazilian adult male patient suspected of trimethylaminuria with a burden of choline bitartrate by monitoring the urinary excretion of TMA and TMAO by proton nuclear magnetic resonance spectroscopy (¹H-NMR).

Results: The patient's urinalyses showed an augmented TMA (12.64±0.95 mg/l) and TMAO (88.42±0.82 mg/l) excretion 6 h after the overload test representing an oxidation capacity of 84.6%, consistent with a heterozygosis condition. Diets containing tuna fish or eggs resulted in an excretion of TMA and TMAO similar to that of the control diet. Only the diet based on dogfish, rich in TMAO, enhanced the excretion of TMA and TMAO reaching 24.65 and 1055.55 mg/l, respectively, in the 0–24 h urine sample.

Conclusions: It was concluded first, that the patient was not able to metabolize the dietary overload of TMA and second, that more studies are needed to substantiate foods that should be avoided, especially regarding fish, due to their high TMA precursor contents.

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1. Introduction

Trimethylaminuria (TMAU), also called fish odor syndrome, is characterized by an offensive body odor

due to an excessive excretion of trimethylamine (TMA) in breath, urine, sweat, saliva and other body secretions [1]. The underlying problem is an impaired hepatic dysfunction of the flavin containing mono-oxygenase (FMO3) system to oxidize TMA to the stable, nonvolatile, odorless trimethylamine *N*-oxide (TMAO). This can be inherited or caused by hormonal and other enzyme activity depressing

* Corresponding author. Tel.: +55 11 3091 3684; fax: +55 11 3815 4410.

E-mail address: lanferum@usp.br (U.M. Lanfer-Marquez).

conditions. Another type of TMAU seems to arise from enhanced liberation of TMA originated from a substrate overload of the enzyme [2–5].

There are not many reports about TMAU worldwide (about 200 cases). This number is likely underestimated. It has been impossible to access precisely true incidence and prevalence due to the unawareness to recognize the condition through clinical symptoms [5]. Another hindrance to the TMAU diagnosis is the lack of clinical laboratories to perform the biochemical analysis of TMA and TMAO on a routine basis. Evidence supports the view that this disease might be much more common than it had been recognized before as new cases are being identified continuously as a consequence of intense systematical investigations [5–9].

Unfortunately, there are no official methods available for analysis of TMA in urine. Although GC has been mostly used for analysis of TMA and TMAO, it has been found not completely satisfactory. GC technique does not allow concurrent analysis of TMA and TMAO in the same sample [10]. The amount of TMAO is quantified indirectly measuring the increase in TMA after TMAO chemical reduction [10–12].

An alternative approach to analyze these metabolites is proton nuclear magnetic resonance spectroscopy ($^1\text{H-NMR}$), a simple high-resolution technique that permits a rapid evaluation of all the H-containing substances in urine. However, the proton NMR technique is relatively insensitive when compared to most other spectroscopic and chromatographic techniques, and it represents the major limitation on the range of its application [13]. Nevertheless, $^1\text{H-NMR}$ has the advantage of permitting the detection of TMA and TMAO in a single run, with little pretreatment of the urine sample [14].

2. Materials and methods

2.1. Chemicals and standard solutions

Trimethylamine hydrochloride ($\text{TMA}\cdot\text{HCl}$), trimethylamine-*N*-oxide dihydrate, ($\text{TMAO}\cdot 2\text{H}_2\text{O}$), deuterium oxide (D_2O), tetramethylsilane (TMS; NMR grade) and carbon tetrachloride were from

Sigma (St. Louis, MO). All other reagents were of analytical grade. Standard solutions of $\text{TMA}\cdot\text{HCl}$ and $\text{TMAO}\cdot 2\text{H}_2\text{O}$ in distilled water were prepared in triplicate. $\text{TMA}\cdot\text{HCl}$ was dried for 2 h at $105\text{ }^\circ\text{C}$ before use, stored in a desiccator until reaching room temperature and then immediately weighed to prepare the standard solutions. The calibration curves of the free base and the oxide ranged 10.5–278.0 mg/l for TMA and 12.0–575.0 mg/l for TMAO, and limits of detection and quantification were calculated according to Winefordner and Long [15].

2.2. $^1\text{H-NMR}$ spectroscopy

^1H -spectra were recorded at $25\text{ }^\circ\text{C}$ on a Bruker DPX 300 spectrometer operating at 300 MHz, according to the methodology described by Maschke et al. [16] with few refinements. A single pulse sequence was used to collect the free induction decays (FIDs), and an exponential apodization function was applied to the FID, with a line broadening of 0.3 Hz. The analytical conditions applied were the following: pulse= 90° , acquisition time=2.65 s, relaxation time=5 s, spectral width=6172.839 Hz and number of scans=32. The water signal was suppressed by applying a secondary irradiation field at the water resonance frequency, and higher sensibility was obtained using an inverse probe.

Five hundred microliters of the standard solutions or urine samples were added to the NMR tubes (507-PP, Ultra Imperial, Wilmad Glass, Buena, NJ, USA) containing 20 μl of D_2O , as an internal lock of the magnetic field. A previously prepared capillary with tetramethylsilane (TMS) was used as reference for chemical shift. The 100- μl capillary was prepared by adding 58 μl of CCl_4 and a few drops of TMS and then sealed with a flame. Peak assignments were made by comparing chemical shifts with those from literature and were confirmed by adding known amounts of TMA and TMAO to the urine samples at 2 concentrations.

TMA and TMAO were quantified by peak integration using the Bruker software WinNMR 1.3 (version 960901.2), and the results were expressed as TMA/TMS or TMAO/TMS ratio. The activity of the enzyme was then calculated as the percentage of total

TMA excreted in form of its oxide as follows (concentration expressed in mmol/l):

$$\% \text{ enzyme activity} = \frac{\text{TMAO} \times 100}{(\text{TMA} + \text{TMAO})}$$

2.3. Case report

A 40-year-old man presenting normal physical and mental development and complaining of episodic fish odor in sweat, urine and breath since adolescence especially after exercises and emotional stress and without any other familiar report of malodor was recruited. The patient had been looking for an explanation of his disorder without success for almost 10 years before he was referred to us. At the age of 37, he had 14.3 mg TMA/l in urine (reference range 0.05–6.80 mg/l). Even so, the case was kept undiagnosed. He was then advised to avoid foods rich in TMA precursor compounds such as fish, eggs, legumes and liver, and eventually, he could get some benefit. Relief of the symptoms was also accomplished by repetitive short-term therapy with antibiotics to reduce the activity of the gut microflora and therefore inhibiting the generation of TMA. The patient collected three random control samples while on a fish-free diet and had not been taking medication 2 weeks before the test period started. During our investigation, he was at the Hospital das Clínicas of the University of São Paulo for a better control of the assays. Hematological and biochemical indices for liver function were normal. This study was previously approved by the Ethic Committee of the Faculty of Pharmaceutical Sciences of the University of São Paulo, and the participant gave his informed written consent.

2.4. Urine samples preparation

For all assays, urine was collected in 1-l plastic bottles and acidified to about pH 1.5 with 10 ml of HCl 6 mol/l, which acts as a preservative and also ensures the conversion of TMA into its water-soluble non-volatile hydrochloride salt. Total volume of urine collected was recorded, and multiple aliquots of 20 ml were stored at -20°C in the dark until analysis. The urine samples had the pH adjusted to 5.8 just before

analysis, following the recommendation of Zuppi et al. [17].

2.5. Choline loading

The patient fasted overnight and had his early morning first void urine taken, which provided control values. Afterwards, the patient had his normal breakfast and received 3 g of choline bitartrate (50 mg/kg body weight) dissolved in a cup of fruit juice. After the choline load, the 0–6-h urine was collected, and, during that time, he was only given water. Urine was also collected between 6–24 h.

2.6. Ingestion of dietary precursors of trimethylamine

For lunch, the patient was given, every other day, two fried eggs, 300 g of stewed dogfish or 225 g of stewed tuna fish, which were considered the main dietary precursors of TMA on these days. On interval days, the patient was maintained on diets restricted on TMA precursors. The maintenance of 1-day intervals between the ingestion of meals containing TMA precursors was to avoid a fortuitous overlap in TMA or TMAO excretion. The 24-h urine collection started immediately after eating the TMA precursors. Total urine volume was recorded, and results were expressed as metabolite concentration in 0–24-h urine. This assay was followed by another one analyzing the excretion profile after ingestion of a portion of dogfish (350 g). This time, the 0–24 h urine was collected in separate bottles each 2 h.

3. Results and discussion

The methodology of $^1\text{H-NMR}$ for detection and quantification of TMA and TMAO was previously described by Maschke et al. [16]. The limits of detection were 3.03 mg/l for TMA and 3.48 mg/l for TMAO, while the limits of quantification were 10.11 mg TMA/l and 11.59 mg TMAO/l. Regression of the calibration curves were linear over the entire concentration range. Urine samples collected during the pretest period on three separate occasions over a period of several months showed no detectable TMA, while TMAO levels varied widely from 15.3 to 154.0 mg/l (0–24-h urine), the latter being attributed to its

high dependency upon diet. At that time, the patient was on normal diet but without fish and did not complain about malodor.

The interpretation of these first results was indicative of a normal healthy individual. Nevertheless, in view of the clinical symptoms of intermittent appearance of body malodor, a condition of heterozygosity was suggested. The correct diagnosis seemed to be important to start an effective dietary management considering the serious social and behavioral problems associated to the offensive odor.

3.1. Choline loading

Reports have shown that challenge tests by oral administration of a TMA precursor could be useful to identify carriers of occasional sufferers by measuring the *N*-oxidation capacity of the enzyme involved [18,19]. The loading test with 3 g of choline bitartrate revealed an increased amount of TMA as well as of TMAO excreted in the 0–6-h urine sample. The concentration of TMAO was 88.42 ± 0.82 mg/l and of TMA was 12.64 ± 0.95 mg TMA/l. These results revealed that about 84.6% of total TMA (TMA plus TMAO) was excreted as TMAO. The concentration of TMA obtained this time was similar to the level found 4 years earlier when only this amine had been analyzed by a diagnostic center. The analysis of 6–24-h urine was not successful because TMA had been excreted mostly until 6 h after the overload, and the volume of this urine sample was higher, and, eventually, both TMA and TMAO were diluted.

According to Mitchell and Smith [5], the excretion of >10 mg TMA/l, considered the upper limit for normal healthy subjects, should be considered a “larger than normal” loss. Healthy individuals show a capacity of *N*-oxidation in the range of 90% and 99%, heterozygotes between 69% and 99% and

homozygous patients in the range of 11% and 54% [20]. This classification was established by consuming a normal diet (fish-free) and shows an overlap in the range of 90–99% oxidation capacity, which makes it difficult to discern between unaffected individuals and heterozygotes. In these cases, the loading test and the calculation of the amount of total TMA, which is excreted as TMAO, proved helpful for diagnosis. The results obtained evidence of a lower than normal *N*-oxidation capacity of the patient, consistent with a carrier or heterozygote status.

3.2. Excretion of TMA and its oxide after ingestion of dietary precursors

It has been shown that heterozygotes might present different degrees of odor problems. Few carriers can metabolize the daily intake of TMA precursors, while others are more sensitive and unable to oxidize the TMA contents present even in a normal diet [20]. Examining the effects of eggs, dogfish or tuna fish on the urinary excretion of TMA and TMA oxide, it was observed that tuna fish and eggs produced negligible amounts of TMA, whereas the excretion of TMAO was 37.66 ± 5.73 and 24.03 ± 5.46 mg/l (Table 1) in the 0–24-h urine samples, respectively. However, the ingestion of dogfish caused an output of 24.65 ± 2.37 mg TMA/l and 1055.55 ± 45.03 mg TMAO/l.

Eggs had been reported to be potent TMA precursors, but studies suggest that they contain only small amounts of choline in its free form (0.4 mg/100 g) and that the majority is in the bound form, as lecithin, incapable to be converted into TMA [21,22]. Therefore, any increase failure in urinary excretion of TMA or its oxide after the ingestion of egg yolk could be explained. This result is in agreement with a previous report where the ingestion of lecithin did not elicit an augmentation of TMA in urine [22]. Like-

Table 1
Trimethylamine (TMA) and trimethylamine *N*-oxide (TMAO) excretion after ingestion of different diets

Diets	Control	Dogfish	Control	Tuna fish	Eggs
TMA (mg/l)	Traces*	24.65 ± 2.37	10.95 ± 0.31	Traces*	Traces*
TMAO (mg/l)	25.85 ± 2.03	1055.55 ± 45.03	51.80 ± 7.44	37.66 ± 5.73	24.03 ± 5.46
Oxidative ability (%)	–	97.12	78.83	–	–

Analysis in traces.

Oxidative ability: $\text{TMAO} \times 100 / (\text{TMA} + \text{TMAO})$.

* Below quantification limits.

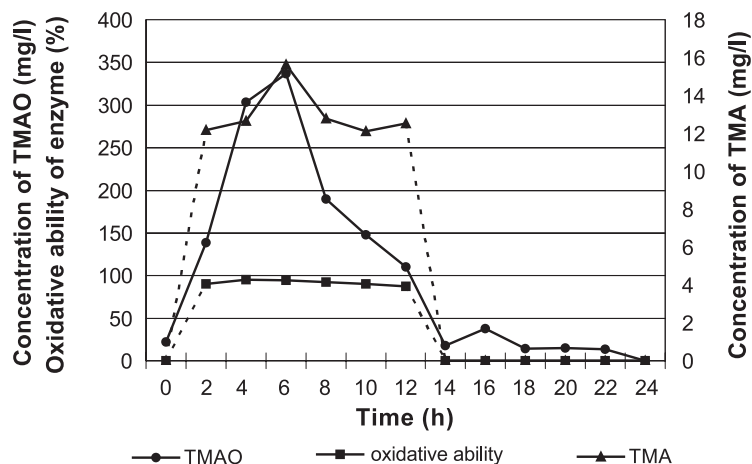


Fig. 1. Urinary excretion of trimethylamine *N*-oxide (TMAO) and trimethylamine (TMA) after ingestion of dogfish, monitored each 2 h over 24 h, and respective oxidative ability of TMA (dotted lines) correspond to the excretion points below quantification limits.

wise, tuna presents choline and TMA oxide in low concentrations, which were not high enough to cause an increase in TMA excretion. Both tuna and egg yolk showed an excretion pattern similar to that obtained after a control diet.

On the other hand, dogfish (*Squalus acanthias*) presents a high content of TMAO by itself (approximately 158 mmol/kg) [23], which undergoes partial reduction (40–60%) in the gut being absorbed and then reoxidized again in the liver [24]. The lack of efficiency of reoxidation by the FMO3 enzyme caused the increase in urinary excretion of TMA.

3.3. TMA excretion profile after eating dogfish

The results of this assay are shown in Fig. 1. After the patient had eaten a single amount of 350-g dogfish, rich in both TMA and TMAO, it was observed that there was an increase in the urinary levels of unoxidized TMA and of TMAO reaching after 6 h, 15.65 mg/l and 336.51 mg/l, respectively. These values were similar to those caused by ingestion of choline bitartrate, meaning that the subject can hardly cope with that burden. Due to the low concentration for evaluation, TMA was quantified only between 2 and 12 h.

The *N*-oxidation ratio as the percentage of total TMA (TMA+TMAO) excreted in the form of TMAO was calculated for each urine sample excreted between 2 and 12 h and showed values varying

between 87% and 95%. These values were slightly higher than those calculated by the choline overload probably due to the high levels of TMAO originally present in that fish, which had been absorbed and excreted directly in urine interfering with the TMAO/total TMA ratio.

More studies are needed to substantiate what fish species can evoke the production of TMA. Actually, the generally accepted statement that fish have to be avoided by heterozygotes should be reviewed, considering that only the elasmobranchs contain high amounts of TMAO [25].

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