Quality consideration for the validation of urine tma and tmao measurement by nuclear magnetic resonance spectroscopy in fish odor syndrome

Charlotte Veyrat-Durebex, Isabelle Benz-De-Bretagne, Victoria Clavier, Clement Bruno, Christian R. Andres, Catherine Antar, Benjamin Hennart, Francois Maillot, Lydie Nadal-Desbarats, Helene Blasco

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Veyrat-Durebex Charlotte[1,2], Benz-de Bretagne Isabelle[1,2], Clavier Victoria[1], Bruno Clément[1,2], Andres Christian R[1,2], Antar Catherine[1,2], Hennart Benjamin[3], Maillot François[4], Nadal-Desbarats Lydie[2], Blasco Hélène[1,2]

1-Laboratoire de biochimie et biologie moléculaire, CHRU de Tours, Tours
2-Unité INSERM U 1253, Université de Tours, Tours
3-Service de toxicologie et génopathies, CHU de Lille
4-Service de Médecine Interne, CHRU de Tours, Tours

Corresponding author

Veyrat-Durebex Charlotte

c.veyratdurebex@chu-tours.fr

Blasco Hélène

Helene.blasco@univ-tours.fr

Laboratoire de Biochimie et Biologie Moléculaire, Hôpital Bretonneau, 2 bd Tonnellé, 37044 - Tours cedex 1, France. Fax: (33) 0 2 47 47 86 13

CHRU de Tours, Inserm U1253 "Imaging and Brain", Team Neurogenomics and Neuronal physiopathology ,University of Tours, Faculty of Medicine, 10 Bd Tonnellé, 37032 Tours Cedex 1, France

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Abstract

Objectives

Trimethylaminuria, also known as Fish Odor Syndrome (FOS), is a condition characterized by the presence of high concentrations of trimethylamine (TMA) in urine, sweat and expired air of affected patients. Diagnosis of this benign but unpleasant disease is mainly based on clinical presentation and assessment of TMA and its metabolite, TMAO (trimethylamine-N-oxide), concentrations in urine of patients.

Material and methods

We here described the validation of an analytical method for measurement of TMA and TMAO in urine using nuclear magnetic resonance (NMR) according to the specifications of the ISO 15189 norm. We used a fast validation protocol, based exactitude profile method, enabling to determine accuracy, intra and inter-day precision from a limited number of samples.

Results

The linearity was established from 2.5 to 100 mg/L for TMA measurement and from 10 to 1000 mg/L for TMAO measurement, with good analytical performances i.e. accuracy, intra and inter-day precision. We also report a case diagnose for FOS from this method.

Conclusions

This method validation ensures the robustness of NMR in routine use for diagnosis of trimethylaminuria, as part of the reference center for inherited metabolic diseases at the Tours hospital.

Keywords:

Fish odor syndrome, trimethylamine, TMA, trimethylaminuria, H^1NMR nuclear magnetic resonance

Introduction

Trimethylaminuria, also known as Fish Odor Syndrome (FOS) [1], is a rare condition [2, 3] characterized by the presence of trimethylamine (TMA), whose odor is described as rotten fish, in the urine, sweat and expired air. This autosomal recessive benign metabolic disease is due to mutations in *FMO3* gene (primary trimethylaminuria) [4], leading to a defect of Flavin Mono-Oxygenase 3, which normally oxidizes TMA into non-odorous trimethylamine-N-oxide (TMAO). Consequences of FOS are quite minor for health (such as hypertension, affective disorder, xenobiotic metabolism) but are major for social interaction [5]. Secondary, trimethylaminuria has also been described involving the putative following factors: dietary, gut metabolism, hormonal and enzyme expression [5]. The large majority of cases with primary *FMO3* deficiency will emerge in early childhood and an early and accurate diagnosis is essential for appropriate genetic counselling and long-term management. The diagnosis is based on clinical presentation and urine measurement of TMA and TMAO. Despite there is no guidelines about the adequate excretion of these products, an oxidizing ratio based on the formula TMAO/(TMAO+TMA) (expressed in %) < 80% is often observed in affected patients [5, 6]. Furthermore, a threshold for the detection of symptoms may be a urine TMA concentration superior to 18–20 µmol/mmol creatinine [7]. Chalmers *et al*. also described normal ranges in urine for healthy controls: TMA/creatinine < 10, TMAO from 50 to 1000 µmol/mmol of creatinine and TMA/TMAO ratio < 0.1 [8]. Genetic testing is finally recommended for certitude diagnosis of primary trimethylaminuria.

Publications on this topic are heterogeneous. Based on the scarcity of this disease and its none life-threatening characteristics, there is actually no consensus for diagnosis protocol, including no recommendations for the choice of the analytical method, and no analytical validation protocol. The most appropriate and described analytical method used to measure TMA and TMAO concentrations is nuclear magnetic resonance (NMR) [8]. As French laboratories have to follow an established process to validate all analytical methods according the ISO 15189 norm, we validated the NMR technique by the exactitude method profile, to measure urine concentrations of TMA and TMAO. We finally report the case of a patient diagnosed for FOS from urine analysis by this validated NMR method.

Material and Methods

Samples preparation

Calibration curves and quality controls (QC) were prepared from trimethylamine (TMA), trimethylamine *N*-oxide (TMAO), creatinine, and deuterium oxide (D_2O) purchased from Sigma-Aldrich (Saint Louis, MO). The concentrations for calibration were the followings 2.5-5-10-25-50-100 mg/L and 10-20-50-100-250-500-1000 mg/L for TMA and TMAO measurements, respectively. Two levels of quality controls were prepared in water: low level QC1 containing 5 mg/L of TMA and 100 mg/L of TMAO, and high level QC2 containing 50 mg/L of TMA and 500 mg/L of TMAO. Calibration samples and QC were prepared by adding 50 µL of each standard (TMA and TMAO), 50 µL of creatinine solution (final concentration 15 mmol/L) for normalization to creatinine concentration. Fifty microliters deuterium oxide (D_2O) and 10 µL of trimethylsilylpropionoic acid (TSP) (153 µM final concentration) were then added to each sample for NRM analyze.

Patients' samples were prepared with the same procedure, by adding 50 µL of 0.2 M potassium phosphate buffer in D_2O (pH = 7.4) and 10 µL TSP to 150 µL of urine samples as TMAO chemical shift is dependent on pH value and TMA signal splits when pH is acidic [9, 10]. The patient presented to validate our technique was followed in the reference center for inherited metabolic diseases at the Tours hospital. He was managed as usually in clinical practice and provided the informed consent for genetic testing.

NMR assay

^{1}H-NMR (proton nuclear magnetic resonance) spectra were obtained with a Bruker DRX-600 AVANCE-III HD spectrometer (Bruker SADIS, Wissembourg, France). A "noesypr-1d" pulse sequence was used to acquire 64 scans on a time domain of 64K data points with a spectral width of 7500Hz. After Fourier transformation, baseline and phase correction were applied. Spectral intensities were scaled to the reference region (TSP). Assignments were done using databases as HMDB (http://www.hmdb.ca), ChenomX NMR suite 8.1 evaluation edition (ChenomX Inc, Edmonton, Canada) and in house database of standard chemical.

Method validation

Validation was conducted according to the International Council for Harmonisation (ICH) guidelines [11], and ISO15189 recommendations [12]. Intra- and inter-days precisions, accuracy, and measurement range were assessed by exactitude profile method *via* Neolicy® software. The statistical method used in this software has been previously described [13] and this software has been recently used in our lab and approved for method accreditation by The French Committee for Accreditation.

Each sample was injected in triplicate for 3 different days. Accuracy is reported as percent of the theoretical value (% deviation) and precision is reported as coefficient of variation (CV %). Lower and higher limits of quantification were determined as the lowest and highest concentrations studied with a sufficient accuracy and precision (CV and bias <20%).
No official criteria were defined for intra- and inter-days precisions, accuracy for TMA and TMAO, so we based our criteria on the ICH cut-off at 15%.

Case report

A 45 years-old woman complained of body malodor for at least years. Both negative psychological and social impacts led her to consult a clinician in our reference center of metabolic disorder. This patient had autoimmune hypothyroidism, had neither overweight nor splenomegaly or any other clinical abnormalities. Body malodor was not observed by the clinician. Urine sample was collected after a single choline load in of 520 mg the day before urine collection and the sample was analyzed as previously described. In case of abnormal urine findings, a genetic analysis was planned.

Results

NMR acquisition

The identification of TMA and TMAO was obtained with an excellent resolution enabling the determination of the peak area without any interference (supplementary figure 1). The acquisition time was based on the time sequence used for urine sample quantification (25 min). On the QC, Signal to Noise ratio was superior to limit of quantification (LOQ).

Method validation

As represented on the figure 1, the linearity was established from 2.5 to 100 mg/L (corresponding to 42 à 1692 µmol/L and 2.8 à 112.8 µmol/mmol of urinary creatinine concentration) for TMA measurement and from 10 à 1000 mg/L (corresponding to 133 à 13310 µmol/L and 44 à 887 µmol/mmol of urinary creatinine concentration) for TMAO measurement. The performances of the method were correct with the highest bias at 5.4% and intra- and inter-days variability <15% for both molecules (table 1).

Case report

The patient previously described had a ratio TMAO/(TMA+TMAO) at 21%, a TMA/Cr (µmol/mmol) ratio at 620 (reference ratio < 10), a TMAO/Cr (µmol/mmol) ratio at 167 (reference value 50-1000), and TMA/TMAO ratio at 3.7 (reference ratio <0.1). The majority of these criteria have guided diagnosis toward FOS and it was reinforced by the comparison of these ratio to ratio of other suspected but health patients (not shown). This diagnosis was confirmed by molecular biology, showing that this patient carried two pathogenic variants in the *FMO3* gene, probably responsible for a decreased enzyme activity (c.458C>T, p.Pro153Leu / c.118C>A, p.Leu40Met). Malodorous symptoms were successfully abolished with a low choline diet along with sequential antibiotics as well as riboflavin and folate supplementations.

Discussion

The choice of ^{1}H NMR spectrometry

Despite ^{1}H NMR spectrometry has been described to diagnose metabolic disorders, especially FOS [14], gas chromatography (GC), electrospray ionization tandem mass spectrometry, direct infusion electrospray quadrupole time-of-flight mass spectrometry, and matrix-assisted laser desorption/ ionization time of flight (MALDI-TOF) mass spectrometry have also been reported [5]. NMR has the advantage to be based on a rapid and easy pre-analytical step with buffered D_2O addition without sample denaturation, thus enabling to preserve volatile amine molecules. Conversely, mass spectrometry (MS) require derivatization of samples i.e. longer sample preparation and sample denaturation. The rapid acquisition of the spectrum enables the quantification of the two compounds of interest, with confident quantification based on the integration of the singlets. The low sensitivity of NMR does not preclude this type of technique for the diagnosis of intoxication diseases. To note, MS methods [15] are now currently used in some laboratories for many diagnoses, but there is no advantage of MS for such kinds of diagnosis, and the place of NMR merits to be re-considered for FOS diagnoses and many others [16]. Despite NMR spectrometers are not currently available in most clinical laboratories, a rigorous validation opens the perspective of a larger use of this robust technique usually dedicated to research only.

Validation of urinary measurement of TMA and TMAO

French medical laboratories have to respect some conditions to realize all biological explorations in order to be "accredited" by the French Committee for Accreditation (COFRAC). This accreditation is a guarantee of the quality assessment of biological measurements for prescribers and patients. The process of method validation must follow the ISO 15 189 norm for daily laboratory tests whatever the frequency of these explorations. Thus, we validated the urinary measurement of TMA and TMAO by NMR, in regard to the official recommendations. We based our validation protocol on Neolicy® software enabling a validation with limited number of analyses.

The performances for TMA and TMAO measurement were correct, with high intra- and inter-day precision (CV < 6%) and acceptable accuracy (< 15%). The limits of quantification are suitable for our application with 10.11 mg/L for TMA and 11.59 mg/L for TMAO. Sparse data about analytic performance for urinary TMA and TMAO exploration reported similar findings with NMR (CV between 5 and 10%)[9, 17, 18] or MS [15, 19]. Thus, the validation of our NMR technique is complete and shows that this robust and reliable method fulfills the criteria of the ISO 15189 norm, after the use of exactitude method profile.

Biological diagnosis in practice

Early diagnosis may enable a disease management based on low choline diet, antibiotics to control bacteria in the gut, the use of activated charcoal to sequester TMA, or new other treatments as recently reviewed such as application of the polymersome-based formulation [20]. One of the main difficulties in such diagnosis is the standardization and the robustness of the process to investigate this disease, after taking into account diet, age, and concomitant infection for example.

To avoid false negative, it is advised to ingest sufficient amount of substrate. The most admitted protocol is based on a 300 g marine fish meal followed by the urine collection two to 12 hours after the meal or alternatively on the Nijmegen protocol, with timed urine collection for 6–8 hours post meal [21]. An oral load with choline or TMA (600 mg) may also help in the diagnosis [5] but whatever the protocol used, it could be not informative due to the nature of gut colonization, gut transit time, and alteration in gastric emptying.

The published reports of FOS cases showed variability in ratios findings, compared to the cut-offs determined as the references [6, 22]. Interestingly, few FOS patients (confirmed by genetic exploration) present all pathological ratios previously cited. According to these observations,

and taken into account the progress in molecular biology, normal ratio of TMA and TMAO must not preclude genetic analysis of *FMO3* in case of marked clinical phenotype. In our case, the fish odor was not obvious but all ratios were pathological which led to a genetic exploration confirming the diagnosis. Urinary TMA and TMAO measurement can be done quickly, guiding diagnosis, but diagnosis of certainty or exclusion will be provide by genetic analysis of *FMO3* gene keeping in mind that genetic explorations can be long. Both, biological explorations (urinary TMA and TMAO, and genetic) have to be discussed and performed after deep phenotype determination, case by case.

In conclusion, we validated here an NMR method to quantify urinary TMA and TMAO according to the required criteria to be accredited by COFRAC, thus allowing its routine use for diagnosis of trimethylaminuria. This work opens the opportunity to use the exactitude method profile to validate other NMR applications helpful for metabolic disorders diagnosis.

Declaration of interest

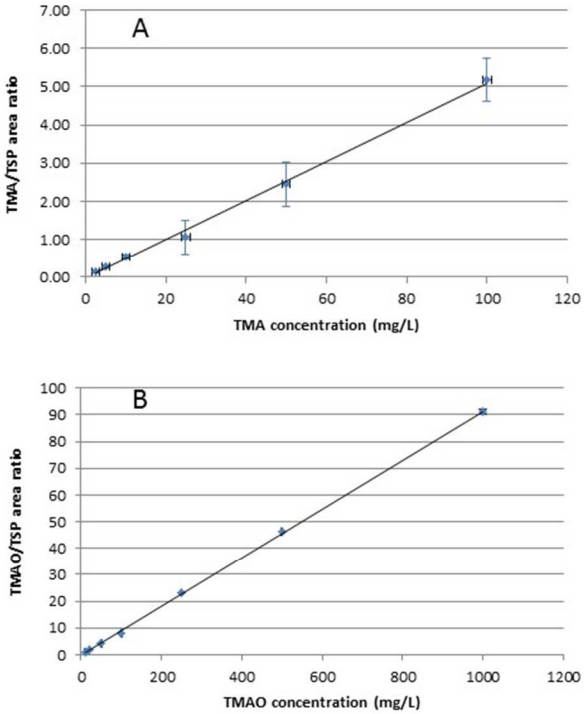
The Authors declare having no competing interests.

Table 1: performances of method validation

	Levels	Accuracy (%)	Intraday precision (%)	Interday precision (%)
TMAO	100 mg/L	-0.46	7.3	14.6
	500 mg/L	-0.12	3.7	5.4
TMA	5 mg/L	5.4	4.9	12.2
	50 mg/L	-1.56	2.1	5.4

Legend of figure

Figure 1: Calibration curve of TMA (A) and TMAO (B) measurement using H^1NMR.
Curves are represented as TMA (or TMAO)/TSP area ratio in function of TMA (or TMAO) concentration in mg/L.



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