Published in final edited form as:

Clin Biochem. 2017 November ; 50(16-17): 947–955. doi:10.1016/j.clinbiochem.2017.06.003.

NMR Quantification of Trimethylamine-N-oxide in Human Serum and Plasma in the Clinical Laboratory Setting

Erwin Garcia[†], Justyna Wolak-Dinsmore[†], Zeneng Wang[§], Xinmin S. Li[§], Dennis W. Bennett[‡], Margery A. Connelly[†], James D. Otvos[†], Stanley L. Hazen^{§,‡}, and Elias J. Jeyarajah^{†,*}

[†]LipoScience, Laboratory Corporation of America[®] Holdings, Raleigh, NC

[§]Department of Cellular and Molecular Medicine, Cleveland Clinic, Cleveland, OH

[‡]Department of Cardiovascular Medicine, Cleveland Clinic, Cleveland, OH

[‡]Department of Chemistry and Biochemistry, University of Wisconsin, Milwaukee, WI

Abstract

Background and objectives—Trimethylamine-N-oxide (TMAO) produced by gut microbiota metabolism of dietary choline and carnitine has been shown to be associated with increased risk of cardiovascular disease (CVD) and to provide incremental clinical prognostic utility beyond traditional risk factors for assessing a patient's CVD risk. The aim of this study was to develop an automated nuclear magnetic resonance (NMR) spectroscopy assay for quantification of TMAO concentration in serum and plasma using a high-throughput NMR clinical analyzer.

Methods—Key steps in assay development included: (i) shifting the TMAO analyte peak to a less crowded region of the spectrum with a pH buffer/reagent, (ii) attenuating the broad protein background signal in the spectrum and (iii) using a non-negative least squares algorithm for peak deconvolution. Assay performance was evaluated according to Clinical and Laboratory Standards Institute guidelines. A method comparison study was performed to compare TMAO concentrations quantified by NMR and mass spectrometry (MS).

Results—The within-run and within-lab imprecision ranged from 4.3 to 14.5%. Under the acquisition method employed, the NMR assay had a limit of blank, detection and quantitation of 1.6, 3.0 and 3.3 μ M, respectively. Linearity was demonstrated within the reportable range of 3.3 to

*Corresponding author at: LipoScience, Laboratory Corporation of America[®] Holdings, 2500 Sumner Blvd, Raleigh, NC 27616. Phone 919-256-1131; Fax 919-256-1039; jeyarae@labcorp.com (Elias J. Jeyarajah).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of Interest Disclosure

Drs. Garcia, Wolak-Dinsmore, Connelly, Otvos and Jeyarajah are current employees of Laboratory Corporation of America Holdings (LabCorp). Dr. Bennett is a consultant for LabCorp. Drs. Hazen and Wang report being listed as co-inventors on pending and issued patents held by the Cleveland Clinic relating to cardiovascular diagnostics and therapeutics. Dr. Hazen reports having been paid as a consultant for: Esperion, and Proctor & Gamble. Dr. Hazen reports having received research funds from LipoScience, Pfizer Inc, Proctor & Gamble, and Takeda.

3,000 μM . TMAO measurements using the NMR assay, which involves minimal sample preparation, compared well with values obtained with the MS-based assay ($R^2 = 0.98$).

Conclusions—The NMR based assay provides a simple and accurate measurement of circulating TMAO levels amenable to the high-throughput demands of the clinical chemistry laboratory. Moreover, assay performance enables the levels of TMAO to be quantified in serum or plasma at clinically actionable concentrations for the assessment of cardiovascular disease risks and individualized dietary monitoring.

Keywords

Trimethylamine-N-oxide; Nuclear magnetic resonance spectroscopy; Cardiovascular disease; TMAO

Introduction

Trimethylamine-N-Oxide (TMAO), a metabolite produced from trimethylamine (TMA) containing nutrients abundant in a Western diet, is increasingly recognized as a clinically important metabolite [1–5]. TMAO is produced by a metaorganismal pathway involving an initial rate-limiting gut microbe-dependent step forming TMA, followed by the action of host hepatic flavin monooxygenase 3 (FMO3)[6]. Dietary forms of choline and carnitine are the primary nutrient precursors for generation of TMAO, and circulating levels of both TMAO and these precursors have been shown to be associated with incident cardiovascular disease (CVD) risks [1–12]. Recent reports also linked this proatherogenic metabolite to other diseases such as colorectal cancer [13], obesity [14], fatty liver [15, 16], heart failure [17, 18] and chronic kidney disease [19, 20]. Thus, a simple, accurate and high-throughput method to measure levels of circulating TMAO in routine clinical testing is warranted.

Several mass spectrometry (MS) based methodologies that quantify TMAO have been reported [21–24]. While sensitive and rapid, they require use of a synthetic stable isotope labeled internal standard, uniquely trained personnel, and specialized and expensive equipment not always available in the clinical diagnostic laboratory. The addition of an automated, high-throughput nuclear magnetic resonance (NMR) analyzer to the clinical diagnostic lab has recently become a reality [25, 26]. Here, we present a simple, automated NMR spectroscopy-based assay for quantification of TMAO. Although NMR is a less sensitive analytical technique than MS, sample processing is minimal, and because of the abundance of TMAO in circulation, the method described here can reliably measure TMAO concentrations at clinically actionable concentrations.

Materials and methods

Reagents

TMAO, glucose and sodium phosphate (Na_2HPO_4) were purchased from Sigma Aldrich (St. Louis, MO). Citrate was purchased from Fisher Scientific (Pittsburg, PA). The diluent buffer is composed of an aqueous solution of citrate and Na_2HPO_4 . Serum and plasma specimens from volunteer donors were identified at both LipoScience (now LabCorp, Raleigh, NC) and the Cleveland Clinic in accordance with The Code of Ethics of the World Medical

Association (Declaration of Helsinki) and cleared by the Institutional Review Boards of both institutions. All donors gave informed consent.

Sample processing

Blood was collected from volunteers following Institutional Review Board approved protocols in K₂EDTA tubes or serum tubes after overnight fast or non-fasting. Plasma or serum was separated following manufacturer specified centrifugation guidelines, and refrigerated up to five days before use, or frozen at -80°C for later use. Fresh or frozen and thawed plasma and serum samples were prepared for NMR analysis by mixing 3:1 (v/v) sample and citrate buffer (pH 4.4) resulting in a target pH of 5.3, either manually or by automated on-board mixing on the Vantera[®] Clinical Analyzer (LipoScience, now LabCorp, Raleigh, NC). During automated sample processing, the Vantera fluidics system drew 240 μL of serum and 80 μL of citrate buffer and, after mixing, introduced the mixture into the detection cell. The Vantera Clinical Analyzer is a fully automated high-throughput NMR platform equipped with a 400 MHz (9.4 T) Agilent spectrometer, a 4 millimeter indirect detection probe and a fixed flow cell that was equilibrated at 47°C via a variable temperature control module (Agilent Technologies, Santa Clara, CA).

NMR data acquisition

One-dimensional (1D) proton (^1H) Carr-Purcell-Meiboom-Gill (CPMG) spectra were recorded. The H₂O resonance was attenuated using water suppression enhanced through T₁ effects (WET) gradient sequence applied for 80.4 milliseconds (ms) [27]. The total spinlock time following the 90° radio frequency pulse was 100 ms. Total acquisition time for each spectrum was 5.5 min (spectral width = 4496.4 Hz, steady state scans = 4, relaxation delay between scans = 5 sec, direct acquisition time = 1.2 sec, number of scans = 48). After the NMR spectrum was acquired, the sample was replaced in the flow cell by the next sample in the queue in less than 30 sec. While the sample was being replaced, spectral processing and result generation was being performed. In total, sample testing and result generation takes approximately 6 min.

Each spectrum was zero-filled to 64K data points and an exponential apodization function (line-broadening = 0.1 Hz) was applied prior to Fourier transformation [28]. Following Fourier transformation, the frequency-domain signal was corrected for phase, tilt and DC offsets, passed through an automated quality check using citrate signal intensity and line width, and the TMAO peak on the spectrum was quantified using mathematical lineshape analysis and deconvolution as described below.

TMAO quantification by peak deconvolution

The TMAO peak was quantified by generating an algorithm that resolved the peak into its potential components. A combination of Lorentzian and Gaussian lineshapes was used to mathematically model the TMAO peak at 3.29–3.30 ppm. A quadratic function and an experimental component, constructed from the spectrum of serum that was devoid of small molecule metabolites, were incorporated into the deconvolution algorithm in order to adequately model the baseline and account for the residual broad signal. The lineshape deconvolution was achieved by a non-negative least squares fitting algorithm [29]. The fit to

the experimental TMAO signal yields amplitude coefficients of the mathematical component shapes used for modeling the signal that have a linear relationship to the analyte concentration [30]. The amplitude coefficients corresponding to the fitted TMAO peak were transformed to concentration units (μM) by using an empirically determined conversion factor. The conversion factor was obtained by calculating the TMAO signal from spectra collected from dialyzed serum spiked with known TMAO concentrations and relating the peak signal areas to the expected concentrations.

Assay performance testing

Sensitivity—The limits of blank (LOB), detection (LOD) and quantitation (LOQ) were determined according to Clinical and Laboratory Standards Institute (CLSI) guidelines [31]. For LOB, 5 dialyzed serum pools devoid of TMAO were used as blank samples and analyzed in quadruplicate per day for 3 days. LOB was calculated as the mean + 1.645*standard deviation of these measurements. On the other hand, serum pools were used for LOD and LOQ. To generate the pools, 329 clinical specimens were analyzed for TMAO and combined based on their natural TMAO content. Five pools with low TMAO content were analyzed in quadruplicate for 3 days and the LOD was calculated as LOB + 1.6525*pooled standard deviation of the 5 individual pools. In order to cover points in a broad concentration range, 5 (out of 18) pools were spiked with the analyte. For each pool, quadruplicate analysis was performed each day for 3 days. Mean concentrations and coefficients of variation (CVs) were calculated for each pool. A power function was fitted to the plot of CV versus mean concentration and the LOQ was estimated at the concentration where the CV was 20%.

Imprecision—Serum pools, targeting low (7.7 μM) and high (20.0 μM) concentrations within the biological range of TMAO, were used to determine within-run and within-laboratory imprecision and repeatability per CLSI guidelines [32]. Within-run imprecision was assessed by analyzing the two serum pools on a single day with 20 replicates for each level. For the within-lab imprecision, the same two pools were analyzed over 10 days wherein 2 replicates were measured for three separate runs per day, while repeatability comprised analysis of duplicates from one run per day for 10 days.

Linearity—Linearity was evaluated by using serum pools with TMAO levels spanning the biological range per CLSI guidelines [33]. Dialyzed serum was used to achieve the low level source pool while the other pools were spiked with the analyte. The resulting TMAO values (mean of 6 measurements) for the 4 source pools were: <1 μM (low), 40.0 μM (medium), 115.9 μM (high) and 5851.1 μM (very high). The 18 pools used to evaluate linearity were generated by serially mixing the four source pools and were analyzed in quadruplicates. Linear regression was performed (Analyse-it v3.90.1, Analyse-it Software, Ltd., Leeds, UK) on the measured (mean of the 4 measurements) and expected TMAO concentrations.

Comparison with stable isotope dilution LC/MS

Serum specimens were obtained from 43 donors. Serum samples from 3 subjects were spiked with 10, 20 and 40 μM TMAO to cover points in the broad range of concentrations encountered clinically. Aliquots were frozen at -80°C until further analysis. Duplicate

samples of frozen serum were analyzed by MS at the Cleveland Clinic (Cleveland, OH) using established methods [21], and in parallel, by NMR at LabCorp as described above. The MS method used stable isotope dilution liquid chromatography tandem mass spectrometry (LC/MS/MS) as previously described [21], and was also independently validated by method of standard additions. The method comparison study was performed in a manner consistent with CLSI guidelines [34]. The correlation between results generated on the two platforms was evaluated using linear regression analysis.

Comparison of TMAO concentrations in serum and plasma

Blood was collected from 20 donors into both *NMR LipoProfile*[®] test serum separator tubes (Lipotube, Greiner Bio-One, Monroe, NC) and K₂EDTA plasma tubes (BD Diagnostics, Durham, NC). Blood in Lipotubes (serum) was allowed to clot at room temperature for 30 min. Blood in K₂EDTA tubes (plasma) was centrifuged within minutes after multiple gentle tube inversion. The tubes were spun at 3,000 RPM for 15 min at room temperature to separate the serum or plasma. For each tube type, 2 donor specimens (out of 20) were spiked with 20 and 40 μ M TMAO to cover points in a broad concentration range. Specimens were aliquoted into 2 mL vials and promptly stored at -80°C until further analysis. TMAO measurements were performed in duplicate for all specimens.

Reference interval

Study was conducted to determine the reference range for TMAO in fasting serum. A total of 153 apparently healthy adult men and women aged 18 to 80 were recruited by LabCorp. This sample group was designated as the normal. Informed consents were obtained from donors. Blood was collected in Lipotubes and processed as described above. TMAO was measured in singlicate. Percentiles were determined (statistical software: JMP version 12.1.0, SAS Institute, Cary, NC) and the reference range was estimated at the 2.5th and 97.5th percentiles.

Specimen stability and number of freeze-thaw cycles

Serum and plasma pools were spiked with TMAO to generate a high level pool. Aliquots of each pool were kept at room temperature, refrigerated (2 to 8°C), frozen (-25 to -10°C) and deep frozen (-80°C). The samples for multiple freeze-thaw cycles were kept at -80°C . Quadruplicate analysis of each aliquot and storage condition was performed on day 0 (as baseline), day 1, day 2, day 3, day 7 and day 15. Stability was assessed by calculating the % bias over time and over multiple freeze-thaw cycles relative to the baseline.

Substance interference testing

A total of 7 endogenous and 13 exogenous substances were tested for potential assay interference consistent with CLSI guidelines [35]. Stock solutions were prepared for each substance in H₂O (20x) or DMSO-d₆ (80x) depending on its solubility. Half of each serum pool was spiked with H₂O or DMSO-d₆ to serve as controls. Two pools containing TMAO near current medical decision points (6 and 10 μ M; as reported by Cleveland HeartLab, Cleveland, OH) were prepared by spiking serum with TMAO stock solution. Analysis was performed using 6 replicates for each pool. The results of the spiked pools were compared to

their corresponding controls by paired difference test [26]. When the difference in results was statistically significant, the difference was checked whether it was considered clinically significant. When interference was observed, the substance was tested at multiple concentrations to estimate the level at which interference exceeded 10%.

Results

Resolution of TMAO peak via pH modification

TMAO has 9 equivalent methyl protons which amplify the intensity of the TMAO ^1H NMR signal. However, a challenge noted early in quantifying the concentration of TMAO in serum by 1D ^1H NMR was that the TMAO methyl singlet is located in a crowded region of the spectrum. Despite using a CPMG sequence that successfully minimized interference by the broad signal of the protein matrix, the ^1H NMR spectrum of serum acquired under normal physiological conditions (pH of 7.4) was not suitable to clearly identify and quantify the TMAO signal. As shown on Figure 1A, the TMAO peak at 3.23 ppm overlaps with the betaine methyl proton peak and is in close proximity to two of the glucose peaks. While a number of endogenous metabolites that contain the same trimethylamine moiety, including choline, betaine and carnitine, have peaks located in the same chemical shift window, TMAO is unique in that it possesses an N-O coordinate bond and is significantly less basic ($\text{pK}_a = 4.56$ [36]), therefore we tested whether we could adjust the pH of the sample to selectively shift the TMAO methyl proton resonance relative to the other trimethylamine containing species. We observed that when a serum sample was subjected to pH titration and analyzed by NMR, the TMAO signal was shifted progressively to the left as the pH of the sample decreased (Figure 1B). While the TMAO methyl proton chemical shift was highly sensitive to pH, the methyl protons of betaine and glucose were far less sensitive and did not shift with changes in pH (Figure 1B). Thus, in order to achieve better resolution of the TMAO peak, we were able to exploit this difference and developed a routine method whereby the sample pH is adjusted to 5.3 with citrate buffer as described under Methods. This pH is lower than when TMAO exists in its almost neutral zwitterion form at pH 6–8 [37] but higher than its pK_a . Thus, the neutral species predominantly exists at pH 5.3. We selected this pH because it moved the TMAO methyl proton peak to a much less crowded region of the ^1H NMR spectrum (between 3.29 and 3.30 ppm). This allowed automated integration of the TMAO peak (described below).

TMAO quantification

The acquired NMR spectra were passed through automated quality check to eliminate pre-analytical errors such as incomplete sample placement and suboptimal magnetic field homogeneity. The TMAO concentrations were quantified by analyzing the TMAO peak at 3.29–3.30 ppm as described in the Methods and illustrated in Figure 2A.

The TMAO concentration in human serum is in the low micromolar range and NMR peak intensities can be very low. The acquisition of 48 scans enhances the signal to noise ratio sufficiently for the detection of the TMAO signal. Knowledge of the expected location for the TMAO peak is paramount to quantifying the signal. Small differences in pH of the different specimens result in demonstrable shifts of the TMAO signal peak. The chemical

shift of the much more visible citrate peak (originating from the diluent buffer) is also very pH dependent, whereas the α -anomeric glucose signal at 5.20 ppm is pH invariant. We took advantage of the predictable relationship of relative shifts of the citrate and TMAO signals with respect to the glucose signal, and to accurately predict the TMAO peak location, an empirical function relating the TMAO and citrate offsets from glucose was determined from the plot shown in Figure 2B. Once the TMAO location is determined the deconvolution of the peak takes place as shown in Figure 2A (inset) generating amplitude coefficients from the fit. The amplitude coefficient from the deconvolution is translated to concentration units using a conversion factor (determined only once) obtained using the calibration curve shown in Figure 2C.

Assay performance

The analytical performance of the NMR-based TMAO assay was evaluated for the ability to reliably detect and accurately quantify TMAO in serum. The average value obtained when testing replicate blanks (LOB) was 1.6 μM while the analytical sensitivity or limit of detection (LOD) was 3.0 μM . Testing of 18 serum pools, with TMAO concentrations varying from 1.0 to 100 μM , gave a functional sensitivity or limit of quantitation (LOQ) of 3.3 μM .

The results of precision measurements are summarized in Table 1. The intra-assay (within-run) precision was assessed by analyzing serum pools with two levels of TMAO in one day while the inter-assay (within-lab) variation involved analysis of the same pools in duplicate for three runs each day over 10 days. The repeatability was evaluated by analyzing duplicates of two levels of TMAO per day for 10 days. The low level pool had CVs that ranged from 10.3 to 15.5% while the high level pool had CVs that ranged from 4.3 to 9.8% (Table 1).

To evaluate linearity over the biological range of TMAO, 18 serum pools were analyzed. Regression analysis was performed on the measured and expected TMAO concentrations. Linearity was demonstrated throughout the reportable range of 3.3 to 3000 μM ($R^2 = 1.00$) (Figure 3A).

Method comparison

To compare assay results between the NMR and MS platforms, 52 specimens were analyzed on both platforms. This included serum specimens from 46 individuals, and additional 6 serum samples spiked with TMAO to test a range up to 50 μM . Measurements were performed in duplicate for both methods and the results were averaged. Figure 3B illustrates the plot of TMAO measured by NMR versus MS. Linear regression analysis showed good correlation between the two analytical platforms within the range where 98% of patient TMAO values lie ($R^2 = 0.98$) (Fig. 3B).

Comparison of TMAO in serum and plasma

In this study, measurements were performed in duplicate for added confidence in measuring low TMAO concentrations. Figure 3C compares the NMR-measured TMAO in serum and

plasma specimens obtained from 20 donors. Serum and plasma results correlated well ($R^2 = 0.99$). The plasma TMAO results were on average 6% lower than the serum specimens.

Reference interval determination

The method to measure TMAO in serum by NMR described here was applied to healthy fasting donors ($n = 153$). Population characteristics for this cohort are shown in Table 2. The median age for the population was 43, with 54% being women. The distribution of TMAO concentrations in serum is shown in Table 3. The median TMAO concentration for this relatively small population was $<3.3 \mu\text{M}$ and the 95% reference interval (2.5–97.5 percentile) was <3.3 – $21.1 \mu\text{M}$.

Stability testing

The stability of TMAO as measured on the automated NMR platform was evaluated in serum and plasma samples stored for up to 15 days at room temperature, 4°C , -20°C or -80°C . Measurements were deemed acceptable if they were within 10% of the baseline (day 0) mean TMAO concentration. Results demonstrated that TMAO was stable to day 15 at all 4 temperatures (Table 4). TMAO was also stable for at least 3 freeze-thaw cycles.

Substance interference testing

A total of 7 endogenous and 13 exogenous substances were tested on serum samples with TMAO concentrations near the medical decision points (6 and $10 \mu\text{M}$). The results are shown in Table 5. If a substance elicited interference at the initial test concentration recommended by CLSI guidelines, then the substance was tested at multiple concentrations to estimate the level at which interference exceeded 10% (Table 5).

Discussion

NMR spectroscopy provides simultaneous observation of resonances arising from all the components of a mixture. As such, there is no need to fractionate or physically separate analytes before NMR data acquisition for quantification of metabolites in complex biological samples such as serum or plasma. Unlike MS, NMR has more limited sensitivity, which makes it challenging to measure analytes with concentrations less than several μM . At such levels it is necessary to employ techniques that optimize the ability to quantify analytes such as TMAO at physiological concentrations.

Firstly, complication due to peak overlap between the TMAO and broad resonances from macromolecules such as serum proteins and lipoproteins was minimized by using the CPMG pulse sequence for spectral acquisition. Under these conditions, most of the broad resonances are attenuated during the spinlock time. Secondly, the NMR sensitivity was enhanced by collecting multiple scans and averaging the signals. Thirdly, we employed a pH controlled chemical shift technique that moved the pH-sensitive TMAO signal from a highly overlapped region to a region in the spectrum that does not have any known interferences from naturally occurring metabolites present in serum or plasma. The current TMAO spectral acquisition also allows for the simultaneous measurement of other biogenic amines, namely betaine, carnitine, and choline, which along with TMAO, are also associated with

increased risk of CVD [1, 2, 7, 10, 38, 39]. The chemical shifts for these trimethylamine-containing compounds are sufficiently resolved from other peaks under the current experimental conditions, enabling their simultaneous detection and quantification. While the TMAO peak's sensitivity to pH was utilized to mitigate the resolution issue, it was apparent that slight variations in pH brought about by small differences between samples could move the analyte peak slightly within the preferred location range. On the other hand, there was no difficulty in identifying the citrate peak due to its high concentration. Therefore, we took advantage of the fact that: 1) we could readily identify the citrate peak and 2) the citrate peak is pH sensitive and could aid in automated identification of the location of the TMAO peak once it was pH shifted. In other words, the relative shift of the citrate peak with respect to pH and the location of the pH-insensitive glucose signals allowed for the accurate location of the TMAO peak within a few data points, enabling automated integration and accurate quantification of the intended peak.

The NMR assay, as developed, offers several advantages over existing methods. While it has comparable throughput (6 min per sample) as some of the MS methods referenced, it involves minimal sample preparation (addition of buffer before placement on the NMR clinical analyzer). Moreover, it does not require separate assay-based calibrations between instruments and avoids the need for costly isotopic dilution in most MS assays. The instruments are calibrated initially using a NMR Reference Standard (15 mM trimethyl acetic acid), and the Reference Standard is run every day to verify instrument performance. In addition, two frozen serum controls with known levels of TMAO are routinely analyzed to ensure assay performance. Despite the fact that NMR is a less sensitive analytical technique than MS, the method described here can reliably measure TMAO concentrations at the medical decision point where TMAO levels indicate a patient may have a higher risk of CVD (typically $>6 \mu\text{M}$).

The assay described herein was optimized with a per sample acquisition time of 5.5 minutes to deliver adequate sensitivity to measure clinically relevant concentrations of TMAO. If higher sensitivity is desired it can be easily achieved by extending the acquisition time or by using a higher magnetic field strength. Alternatively, use of a cryo-probe can improve signal-to-noise ratio significantly. In this setup the radiofrequency (RF) coils and preamplifiers are cooled by liquid nitrogen or liquid helium, which reduces the background noise to yield a two to four fold gain in sensitivity.

Twenty substances were tested for possible interference with TMAO quantification. Thirteen showed either no detected interference or $<10\%$ interference during initial screening at concentrations prescribed by CLSI guidelines. Seven substances were tested at multiple concentrations to determine at which level they produced significant interference. Ibuprofen and urea elicited 10% interference at 28.2 mmol/L and 56.7 mmol/L, respectively. However, these levels are well above the therapeutic and pathological values [33]. Although acetylsalicylic acid showed interference at 0.21 mmol/L, its active metabolite salicylic acid did not show significant interference within the therapeutic concentration (0.72–2.17 mmol/L) [33]. Bilirubin (unconjugated), uric acid and exogenous albumin presented risk for interference. While bilirubin (unconjugated) exhibits an apparent decrease (10%) in measured TMAO at 171 $\mu\text{mol/L}$, uric acid and exogenous albumin presented an apparent

increase in TMAO values within the recommended test concentrations (uric acid: 1,400 $\mu\text{mol/L}$; albumin: 60 mg/mL) [33].

With regard to analyte stability, specimens can be refrigerated or frozen for up to 15 days before testing. This stability was established using pooled serum spiked with additional TMAO ($\sim 60 \mu\text{M}$). The assay stability for specimens with naturally occurring levels of TMAO hasn't been reported and subject of future study. Both plasma and serum can be used for the NMR TMAO assay as reported here. Additionally, there was no significant change in TMAO results for samples that have undergone up to 3 freeze-thaw cycles. This result is consistent with results previously reported for TMAO measured by stable isotope dilution LC/MS/MS showing viability for up to 6 freeze-thaw cycles, and stable across a 10 year period while frozen at -70°C [21]. In the present studies employing the vacutainers described under Methods, TMAO results showed modestly lower levels in plasma compared to serum (approximately 6%). This is consistent with most studies where concentrations of common analytes are lower in plasma than in serum, though no differences were observed between plasma versus serum samples measured by LC/MS/MS in a prior study [21].

Similar to the MS assay, we anticipate that the performance characteristics of this assay will allow it to be clinically useful for assessing patients for CVD risk, as well as identifying those with intestinal dysfunction. For example, in a study that examined the relationship of fasting plasma TMAO levels and incidence of major adverse cardiovascular events (MACE) in 4007 patients undergoing elective coronary angiography, plasma TMAO levels were associated with an increased risk of MACE [hazard ratio for the highest ($>6.2 \mu\text{M}$) compared to the lowest quartile was 2.54; 95% confidence interval, 1.96–3.28; $P<0.001$][3]. Given the LOQ of our NMR-based TMAO assay, individuals at high risk of MACE could be reliably identified, as well as those who are in the 50th percentile (median) or above in a larger population size. The NMR assay described with an LOQ of $3.3 \mu\text{M}$ is well suited to identify patients who are at moderate or high risk of CVD events.

Conclusions

We describe here a simple efficient method to measure circulating TMAO concentrations in serum and plasma by NMR spectroscopy that involves minimal sample preparation. A novel technique that involves the shifting of the TMAO ^1H -NMR signal by controlling the pH thus enabling its quantification was demonstrated. The analytical performance of the assay shows that it can reliably quantify TMAO at levels that are associated with higher CVD risk.

Acknowledgments

Sources of Funding: This research was supported in part by grants from the National Institutes of Health (NIH) and the Office of Dietary Supplements (R01HL103866, R01DK106000, R01HL126827).

Abbreviations

1D	one dimensional
CLSI	Clinical and Laboratory Standards Institute

CPMG	Carr-Purcell-Meiboom-Gill
CV	coefficient of variation
CVD	cardiovascular disease
¹H	proton
LOB	limit of blank
LOD	limit of detection
LOQ	limit of quantitation
NMR	Nuclear magnetic resonance
MS	mass spectrometry
TMAO	Trimethylamine-N-oxide

References

1. Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. *Nature*. 2011; 472:57–63. [PubMed: 21475195]
2. Koeth RA, Wang Z, Levison BS, Buffa JA, Org E, Sheehy BT, et al. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. *Nat Med*. 2013; 19(5): 576–85. [PubMed: 23563705]
3. Tang WH, Wang Z, Levison BS, Koeth RA, Britt EB, Fu X, et al. Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. *N Engl J Med*. 2013; 368:1575–84. [PubMed: 23614584]
4. Zhu W, Gregory JC, Org E, Buffa JA, Gupta N, Wang Z, et al. Gut microbial metabolite tmao enhances platelet hyperreactivity and thrombosis risk. *Cell*. 2016; 165:111–24. [PubMed: 26972052]
5. Senthong V, Li XS, Hudec T, Coughlin J, Wu Y, Levison B, et al. Plasma trimethylamine N-oxide, a gut microbe-generated phosphatidylcholine metabolite, is associated with atherosclerotic burden. *J Am Coll Cardiol*. 2016; 67:2620–8. [PubMed: 27256833]
6. Bennett BJ, de Aguiar Vallim TQ, Wang Z, Shih DM, Meng Y, Gregory J, et al. Trimethylamine-N-oxide, a metabolite associated with atherosclerosis, exhibits complex genetic and dietary regulation. *Cell Metab*. 2013; 17:49–60. [PubMed: 23312283]
7. Wang Z, Tang WH, Buffa JA, Fu X, Britt EB, Koeth RA, et al. Prognostic value of choline and betaine depends on intestinal microbiota-generated metabolite trimethylamine-N-oxide. *Eur Heart J*. 2014; 35:904–10. [PubMed: 24497336]
8. Senthong V, Wang Z, Fan Y, Wu Y, Hazen SL, Tang WH. Trimethylamine N-oxide and mortality risk in patients with peripheral artery disease. *J Am Heart Assoc*. 2016; 5(10) pii: e004237.
9. Senthong V, Wang Z, Li XS, Fan Y, Wu Y, Tang WH, et al. Intestinal microbiota-generated metabolite trimethylamine-N-oxide and 5-year mortality risk in stable coronary artery disease: The contributory role of intestinal microbiota in a courage-like patient cohort. *J Am Heart Assoc*. 2016; 5(6) pii: e002816.
10. Tang WH, Wang Z, Li XS, Fan Y, Li DS, Wu Y, et al. Increased trimethylamine N-oxide portends high mortality risk independent of glycemic control in patients with type 2 diabetes mellitus. *Clin Chem*. 2017; 63:297–306. [PubMed: 27864387]
11. Shafi T, Powe NR, Meyer TW, Hwang S, Hai X, Melamed ML, et al. Trimethylamine N-oxide and cardiovascular events in hemodialysis patients. *J Am Soc Nephrol*. 2017; 28:321–31. [PubMed: 27436853]

12. Suzuki T, Heaney LM, Bhandari SS, Jones DJ, Ng LL. Trimethylamine N-oxide and prognosis in acute heart failure. *Heart*. 2016; 102:841–8. [PubMed: 26869641]
13. Bae S, Ulrich CM, Neuhauser ML, Malysheva O, Bailey LB, Xiao L, et al. Plasma choline metabolites and colorectal cancer risk in the women's health initiative observational study. *Cancer Res*. 2014; 74:7442–52. [PubMed: 25336191]
14. Warriar M, Shih DM, Burrows AC, Ferguson D, Gromovsky AD, Brown AL, et al. The tmao-generating enzyme flavin monooxygenase 3 is a central regulator of cholesterol balance. *Cell Rep*. 2015; pii: S2211-1247(14)01065-1. doi: 10.1016/j.celrep.2014.12.036
15. Dumas ME, Kinross J, Nicholson JK. Metabolic phenotyping and systems biology approaches to understanding metabolic syndrome and fatty liver disease. *Gastroenterology*. 2014; 146:46–62. [PubMed: 24211299]
16. Chen YM, Liu Y, Zhou RF, Chen XL, Wang C, Tan XY, et al. Associations of gut-flora-dependent metabolite trimethylamine-N-oxide, betaine and choline with non-alcoholic fatty liver disease in adults. *Sci Rep*. 2016; 6:19076. [PubMed: 26743949]
17. Tang WH, Wang Z, Fan Y, Levison B, Hazen JE, Donahue LM, et al. Prognostic value of elevated levels of intestinal microbe-generated metabolite trimethylamine-N-oxide in patients with heart failure: Refining the gut hypothesis. *J Am Coll Cardiol*. 2014; 64:1908–14. [PubMed: 25444145]
18. Organ CL, Otsuka H, Bhushan S, Wang Z, Bradley J, Trivedi R, et al. Choline diet and its gut microbe-derived metabolite, trimethylamine N-oxide, exacerbate pressure overload-induced heart failure. *Circ Heart Fail*. 2016; 9:e002314. [PubMed: 26699388]
19. Tang WH, Wang Z, Kennedy DJ, Wu Y, Buffa JA, Agatista-Boyle B, et al. Gut microbiota-dependent trimethylamine N-oxide (TMAO) pathway contributes to both development of renal insufficiency and mortality risk in chronic kidney disease. *Circ Res*. 2015; 116:448–55. [PubMed: 25599331]
20. Missailidis C, Hallqvist J, Qureshi AR, Barany P, Heimbürger O, Lindholm B, et al. Serum trimethylamine-N-oxide is strongly related to renal function and predicts outcome in chronic kidney disease. *PLoS One*. 2016; 11:e0141738. [PubMed: 26751065]
21. Wang Z, Levison BS, Hazen JE, Donahue L, Li XM, Hazen SL. Measurement of trimethylamine-N-oxide by stable isotope dilution liquid chromatography tandem mass spectrometry. *Anal Biochem*. 2014; 455C:35–40.
22. Ocque AJ, Stubbs JR, Nolin TD. Development and validation of a simple UHPLC-MS/MS method for the simultaneous determination of trimethylamine N-oxide, choline, and betaine in human plasma and urine. *J Pharm Biomed Anal*. 2015; 109:128–35. [PubMed: 25767908]
23. Liu J, Zhao M, Zhou J, Liu C, Zheng L, Yin Y. Simultaneous targeted analysis of trimethylamine-N-oxide, choline, betaine, and carnitine by high performance liquid chromatography tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2016; 1035:42–8.
24. Heaney LM, Jones DJ, Mbasu RJ, Ng LL, Suzuki T. High mass accuracy assay for trimethylamine N-oxide using stable-isotope dilution with liquid chromatography coupled to orthogonal acceleration time of flight mass spectrometry with multiple reaction monitoring. *Anal Bioanal Chem*. 2016; 408:797–804. [PubMed: 26573169]
25. Matyus SP, Braun PJ, Wolak-Dinsmore J, Jeyarajah EJ, Shalauova I, Xu Y, et al. NMR measurement of LDL particle number using the Vantara Clinical Analyzer. *Clin Biochem*. 2014; 47:203–10. [PubMed: 25079243]
26. Matyus SP, Braun PJ, Wolak-Dinsmore J, Saenger AK, Jeyarajah EJ, Shalauova I, et al. HDL particle number measured on the Vantara, the first clinical NMR analyzer. *Clin Biochem*. 2015; 48:148–55. [PubMed: 25438074]
27. Smallcombe SHPS, Keifer PA. Wet solvent suppression and its applications to LC NMR and high-resolution NMR spectroscopy. *J Magnet Reson*. 1995; A117:295–303.
28. Derome, A. Modern NMR techniques. Pergamon Press; 1988. Chapter 2
29. Lawson, CL., Hanson, RJ. Solving least squares problems. Philadelphia, PA: Society for Industrial and Applied Mathematics; 1974.
30. Jeyarajah EJ, Cromwell WC, Otvos JD. Lipoprotein particle analysis by nuclear magnetic resonance spectroscopy. *Clin Lab Med*. 2006; 26:847–70. [PubMed: 17110242]

31. CLSI document EP17-A: Protocols for determination of limits of detection and limits of quantitation; approved guideline. Wayne, PA USA: Clinical and Laboratory Standards Institute; 2004.
32. CLSI document EP5-A2: Evaluation of precision performance of quantitative measurements methods; approved guideline-second edition. Wayne, PA: Clinical and Laboratory Standards Institute; 2004.
33. CLSI document EP6-A: Evaluation of the linearity of quantitative measurement procedures: A statistical approach. Wayne, PA: Clinical and Laboratory Standards Institute; 2003.
34. CLSI document EP9-A2: Method comparison and bias estimation using patient samples; approved guideline-second edition. Wayne, PA: Clinical and Laboratory Standards Institute; 2002.
35. CLSI document EP7-A2: Interference testing in clinical chemistry; approved guideline second edition. Wayne, PA: Clinical and Laboratory Standards Institute; 2007.
36. Lin TY, Timasheff SN. Why do some organisms use a urea-methylamine mixture as osmolyte? Thermodynamic compensation of urea and trimethylamine N-oxide interactions with protein. *Biochemistry*. 1994; 33:12695–701. [PubMed: 7918496]
37. Singh R, Haque I, Ahmad F. Counteracting osmolyte trimethylamine N-oxide destabilizes proteins at pH below its pKa. Measurements of thermodynamic parameters of proteins in the presence and absence of trimethylamine N-oxide. *J Biol Chem*. 2005; 280:11035–42. [PubMed: 15653673]
38. Lever M, George PM, Slow S, Bellamy D, Young JM, Ho M, et al. Betaine and trimethylamine-N-oxide as predictors of cardiovascular outcomes show different patterns in diabetes mellitus: An observational study. *PLoS One*. 2014; 9:e114969. [PubMed: 25493436]
39. Tang WH, Wang Z, Shrestha K, Borowski AG, Wu Y, Troughton RW, et al. Intestinal microbiota-dependent phosphatidylcholine metabolites, diastolic dysfunction, and adverse clinical outcomes in chronic systolic heart failure. *J Card Fail*. 2015; 21:91–6. [PubMed: 25459686]

Highlights

- An NMR spectroscopic method to measure serum TMAO levels is reported.
- Measurements compare well to established LC/MS/MS based assay.
- The NMR clinical assay is fully automated and offers high throughput.
- Enables further study of TMAO relationships to CVD risk and patient management.

Fig. 1. Spectra collected on a NMR clinical analyzer. **A)** CPMG spectrum of serum containing endogenous TMAO (4 μM) acquired under normal physiological conditions (pH 7.4), **B)** Spectra obtained after adjustment to the indicated pH (TMAO signal marked with a star for emphasis) illustrating the shift in TMAO peak position at varying pH relative to glucose and betaine.

Fig. 2.

TMAO Peak modelling. **A)** The glucose and citrate reference peaks used to locate the TMAO peak. The inset shows the experimental TMAO peak (black) overlaid with a mathematical fit (red) from a composite of Gaussian, Lorentzian and quadratic functions (shown separately in blue). **B)** The pH-dependent linear relationship between TMAO and citrate offsets from glucose, utilized to find the TMAO peak in the spectrum. **C)** Standard curve used to convert amplitude coefficient to concentration unit.

Fig 3.

Linearity and method comparison data for the TMAO assay. **A)** Results of linearity testing for the NMR TMAO assay, **B)** Comparison of TMAO concentrations (graph 0–50 µM, n = 52; inset 0–15 µM, n = 46) determined by NMR and LC/MS. **C)** Comparison of TMAO concentrations obtained from plasma versus serum specimens as described in the Methods section.

Table 1

Within-run and within-laboratory imprecision and repeatability for the NMR TMAO assay.

TMAO (μM)				
		Within-run ^a	Within-lab ^b	Repeatability ^b
Low	Mean	7.7	6.1	6.2
	SD	0.8	0.9	1.0
	%CV	10.3	14.5	15.5
High	Mean	20.0	17.7	17.6
	SD	0.9	1.7	1.6
	%CV	4.3	9.8	9.1

Based on CLSI guidelines using 2 controls that had mean TMAO values near the current medical decision points.

^aBased on 1 run of 20 tests.

^bBased on 3 runs per day in duplicate for 10 days (n = 60 per pool).

Table 2

Population characteristics of healthy controls (n=153).

Clinical characteristic	Values
Age (years)	
Median (Interquartile range)	43 (32–53)
BMI	
Median (Interquartile range)	24.5 (21.8–27.3)
Male (%)	45.8
Race (%)	
African American	7.2
Asian	19
Caucasian	71.2
Other	2.6
Blood pressure >140/90 mmHg (%)	5.2
Indicated tobacco consumption (%)	6.5

Table 3

Distribution of TMAO values for reference range study participants.

Percentile	TMAO (μM)
0	<3.3
2.5	<3.3
10	<3.3
25	<3.3
50	<3.3
75	5.5
90	9.1
97.5	21.1
100	72.2

Table 4
Stability of the TMAO analyte at different storage conditions and multiple freeze-thaw cycles.

Specimen	Day	Room temp		Refrigerated		Frozen		Deep		Freeze/Thaw	
		Mean (µM)	% Bias	Mean (µM)	% Bias	Mean (µM)	% Bias	Mean (µM)	% Bias	Mean (µM)	% Bias
Serum ^a	0	62.0	–	62.0	–	62.0	–	62.0	–	62.0	–
	1	59.9	3.3	56.5	8.8	61.1	1.4	61.7	–0.4	62.0	0.1
	2	62.2	–0.3	61.7	0.4	56.3	9.1	61.9	0.0	60.2	–2.8
	3	59.8	3.5	61.4	0.8	61.5	0.8	61.7	–0.3	60.9	–1.7
	7	62.1	–0.2	57.9	6.6	60.3	2.7	61.8	–0.2	60.2	–2.8
	15	64.6	–4.2	63.1	–1.9	62.5	–0.9	60.5	–2.4	–	–
Plasma ^b	0	64.6	–	64.6	–	64.6	–	64.6	–	64.6	–
	1	62.6	3.1	63.9	1.0	62.8	2.8	58.0	–10.3	65.0	0.7
	2	60.6	6.1	64.2	0.5	63.5	1.6	61.0	–5.5	56.3	–12.8
	3	59.6	7.7	58.8	9.0	50.3	22.1	59.3	–8.2	60.0	–7.0
	7	60.9	5.7	62.1	3.9	61.0	5.6	61.4	–4.9	63.4	–1.8
	15	66.5	–2.9	68.1	–5.5	58.3	9.7	63.3	–2.0	–	–

^a Plain serum tube;

^b EDTA plasma tube; n = 4; % Bias calculated relative to mean of day 0.

Table 5

Summary of interference testing for TMAO measurement by NMR.

Substance	Drug Name	Test Concentration	Concentration Eliciting Interference ^a
Bilirubin, unconjugated	–	0–200 µg/mL (342 µmol/L)	> 100 µg/mL (171 µmol/L)
Bilirubin, conjugated	–	288 µg/mL (342 µmol/L)	–
Creatinine	–	50 µg/mL (442 µmol/L)	–
Hemoglobin	–	2 mg/mL	–
Urea	–	0–2.6 mg/mL (42.9 mmol/L)	> 2.6 mg/mL (42.9 mmol/L)
Uric acid	–	0–235 µg/mL (1.4 mmol/L)	> 140 µg/mL (0.83 mmol/L)
Protein (albumin)	–	0–60 mg/mL	> 0.83 mg/mL
Acetaminophen	Tylenol	200 µg/mL (1324 µmol/L)	–
Acetylsalicylic acid	Aspirin	0–660 µg/mL (3.62 mmol/L)	> 38 µg/mL (0.21 mmol/L)
Atorvastatin	Lipitor	48 µg/mL	–
Enalapril dihydrate	Vasotec	0.33 µg/mL (0.86 µmol/L)	–
Fenofibrate	Tricor	45 µg/mL (125 µmol/L)	–
Hydralazine hydrochloride	Apresoline	180 µg/mL	–
Hydrochlorothiazide	HCT	6.0 µg/mL (20.2 µmol/L)	–
Ibuprofen Sodium salt	Advil	0–560 µg/mL (2425 µmol/L)	> 560 µg/mL (2425 µmol/L)
Metformin Hydrochloride	Glucophage	600 µg/mL	–
Metoprolol tartrate	Lopressor	13 µg/mL (18.7 µmol/L)	–
Naproxen Sodium	Aleve	550 µg/mL (2170 µmol/L)	–
Nifedipine	Adalat	0.4 µg/mL (1156 nmol/L)	–
Salicylic acid	–	0–600 µg/mL (4.34 mmol/L)	> 420 µg/mL (3 mmol/L)

Test concentrations were obtained from CLSI EP7-A2 guidelines Appendix C, where available.

^aConcentration at which substance elicited a >10% change in the TMAO value.