A Rapid Screening Method for Trimethylamine N- Oxide in Human Blood Serum as Potential Indicator for Prediction of Heart Attack and Stroke Risks

Nikhil Ramachandran

ABSTRACT

Introduction: Trimethylamine –N- oxide (TMAO) has been of tremendous clinical interest as it is a biomarker for predicting future heart attack and stroke risks. Red meat, eggs, dairy products and salt water fish are the potential sources of TMAO. Elevated levels of TMAO were associated with the high risk of mortality. This indicates that consumption of diet with high *carnitine* or rich *cholin* could potentially lead to an increased CVD through generation of TMAO. Hence TMAO levels in blood plasma could be used as a biomarker for predicting future heart and stroke risks. Aim: TMAO monitoring is clinically challenging and instrumentation methods available are extremely complicated requiring expensive instrumentation and expertise. This has led to the need of simple and reliable analytical methods. The aim of present study is to develop a new method for the spectrophotometric determination of trimethylamine –N-oxide (TMAO).

Material and Methods: In this proposed method TMAO is enzymatically converted to formaldehyde and is then reacted with Chromotropic acid in acid medium to form a coloured complex.

Results: The complex has an absorption maximum of 535nm a molar absorptivity of 1.48 X 10^4 l $mol^{-1}cm^{-1}$. The colored complex in acidic pH obeys Beer's law in the range of 0.2–2.2 µM. The lower limit of determination is 0.02 µM. The method has been applied for the determination of TMAO in blood plasma, urine, and water. The method is reproducible, selective and sensitive and is free from interferences,

Key Words: Trimethylamine-N-Oxide; Spectrophotometry; Biological Samples, Blood, Urine

INTRODUCTION

Trimethyl amine N- oxide (TMAO) is an organic compound in the class of amine oxide with a formula $(CH_3)_3NO$. It is a product of oxidation of trimethylamine (TMA) and generated in human body by the gut microbial metabolism. It is commonly found in a variety of marine organisms and is known to protect them against the adverse effects of temperature salinity and hydrostatic pressure. TMAO is metabolized into small methylated amines such as tri, di and monomethylamines.[1] Even though TMAO is known for a long time it has become a subject of extensive research only after the recent reports on its direct correlation with cardiovascular diseases (CVD) in humans. In aquatic animals especially the organisms where urea functions as an osmolyte and buoyancy factor, TMAO has shown to restore their natural structure. In some cases TMAO stabilizes folded state of proteins. It is said that amphiphilic structural arrangement of TMAO allows it to form hydrogen bonds with water and preferentially exclude or interact with certain protein functional groups.[2]

TMAO is formed from Trimethylamine which is generated in the gut by the action of biome on dietary cholin and lecithin. Red meat, eggs, dairy products and salt water fish are the potential sources of TMAO. TMA is oxygenated to form TMAO and is finally excreted through the kidney. [3] In some individuals TMA is excreted rather than TMAO which is known as fish order syndrome. [4]

The role of TMAO in CVD is disputed.[5] Presence of elevated levels of TMAO in plasma has adverse effects on human health. In a study by Dr. Tang etal, TMAO levels were increased in patients with stable heart failure and its elevated levels were associated with the high risk of mortality. This indicates that consumption of diet with high *carnitine* or rich *cholin* could potentially lead to an increased CVD through generation of TMAO. Hence TMAO levels in blood plasma could be used as a biomarker for predicting future heart and stroke risks. There are references which indicate the role of TMAO in renal dysfunctions as well. Though the safe level of TMAO is still under investigations, the interquartile range in blood plasma is 2.25-5.79 µM with median concentration about 3.45µM. The plasma TMAO levels show wide intra-individual variations and a large number of factors such as diet, enzymes, bones and kidney functions that determine its level. Literature also suggests certain contradictory findings on these studies including the correlation between TMAO and death. One interesting fact is that many marine organisms have developed an adaptive mechanism to TMAO where as humans haven't.[6]

Considering these facts it can be said that it is important to have routine check of the levels of TMAO in blood plasma. Thus, the development of a rapid and accurate method for the quantification of circulating levels of TMAO is of clinical interest. Several methods for quantifying and

IB II Canadian International School, Bangalore 560064, India

Corresponding author: Nikhil Ramachandran, IB II Canadian International School, Bangalore 560064, India

How to cite this article: Ramachandran N. A rapid screening method for trimethylamine n- oxide in human blood serum as potential indicator for prediction of heart attack and stroke risks. International Journal of Contemporary Medical Research 2020;7(11):K4-K7.

DOI: http://dx.doi.org/10.21276/ijcmr.2020.7.11.6

determination in biological matrices and environmental samples on titrimetry, gravimetry, Spectrophotometry and hi - phenated techniques including GC MS, LCMS etc has been reported have been reported. [7-8] Stable isotope dilution liquid chromatography, GC-MS methods are being used for biological samples with several limitations.[8] most methods employed require multi step derivatization prior to assay. Some of the methods such as fast atom bombardment mass spectrometry or electrospray quadruple time of flight mass spectrometry and NMR are extremely complex and cannot be conducted without sophisticated instrumentation and analytical expertise. Picric acid methods used in fish and meat industries have several drawbacks due to the explosive nature of reagents and sensitivity. Since most methods do not provide high through put, and most of them are extremely expensive there are limitations in adapting these methods for clinical diagnosis.

AIM

The present study aim to develop, a new rapid screening method and a new spectrophotometric method for an accurate quantitative determination of TMAO for clinical diagnostics. The rapid screening method involves conversion of TMAO into formaldehyde using dimethylase enzyme at room temperature and reacting the resulting aldehyde with chromotropic acid to form a highly selective Xanthylium dye which shows absorption maximum at 535 nm. The dye is formed rapidly in phosphoric acid medium when assisted by micro wave 30 seconds at 1100W microwave and forms the basis for the development spectrophotometric determination of TMAO and development of electronic sensors. The colored complex obeys Beers Law in the range of 0.2 – 2.2 µM. The method has been applied for the determination of TMAO in blood plasma and water. The method is reproducible, selective and free from interferences from creatine, albumin and other common trace elements. The method has been applied for the determination of TMAO in blood serum, and water samples and favorably compared with other methods.

MATERIAL AND METHODS

Apparatus and Reagents: All absorption measurements were conducted on Zeiss Spectrophometer. Calibrated glassware of clinical quality was used for volumetric measurements. All reagents used were of analytical reagents grade obtained from *Sigma Aldrich* otherwise stated.

Chromotropic acid (CA): 0.2% solution was prepared in sulphuric acid.

Dimethylase Enzyme (DMA): 0.001% solution stabilized in phosphate buffer

Trimethylamine N-oxide: 100 µM. Stock solution was prepared in water for standardization from dehydrate standard.

Trichloroacetic acid (TCA) Acetone, demineralized water, Albumin; creatinine, phosphoric acid, sulfamic acid, Zn powder, sulphuric acid, Centrifuge; Syringes, collection tubes grinder, blending jar were used in various stages of experiment. Blood and Urine samples were obtained from National Forensic Laboratory, Bangalore, India and Diagnostic laboratories. The samples were stored at -20 ° C prior to analysis.

Preparation of Calibration curve: To an aliquot of standard solution containing 0.2– 2.5 µM TMAO placed in a 25ml calibrated graduated tube, 2ml of DMA was added and placed on a water bath maintained at 30°C for 10 minutes.1ml of CA reagent was added, shaken well diluted to the mark by adding phosphoric acid through the side of the tube. The tubes were kept in a 1100 w microwave for 30 seconds, and absorbance was measured against distilled water.

TMA in Blood/Plasma: 10ml whole blood samples obtained from a diagnostics laboratory in collection tubes were transferred to a centrifuge tube mixed with TCA acetone mixture (1:1) and centrifuged for 3minutes at 4000RPM. The plasma is further centrifuged for 15 minutes at 2200-2500 RPM. 1 ml of liquid obtained is transferred to the 25ml calibrated graduated tube; 2ml of DMA was added and placed on a water bath maintained at 30°C for 10 minutes.1ml of CA reagent was added, shaken well diluted to the mark by adding phosphoric acid slowly through the side of the tube. The tubes were kept in a microwave for 1 minute and absorbance was measured.

TMAO in Urine: 5ml sample was taken in a centrifuge tube and 2ml TCA –Acetone mixture was added to it. The mixture was centrifuged for 5minutes at 4000RPM and 1ml of the supernatant liquor was analyzed as above.

TMAO in Water: For trace quantities of TMAO in water suitable volume of sample is transferred to graduated tube and analyzed as above. If the concentration is below 0.2ppm, the sample needs to be concentrated using low temperature distillation.

RESULTS

The absorption spectrum of the conjugated complex is in the range of 392 nm to 620 nm with a maximum at 535 nm (Fig-1). The reagent blank gave negligible absorbance at

Present Method		Reported Method
Blood		
a	6.12 (+/-0.01)	5.88 (+/-0.01)
b	0.52 (+/-0.02)	0.51 (+/-0.3)
c	2.2 (0.01)	2.28(+/-0.4)
d	0	0
Urine		
a	78.8 (+/-0.1)	76.1(+/-0.3)
b	54.0 (+/-0.2)	54.7(+/-0.6)
c	29.6(+/-0.1)	29.1(+/-0.2)
d	5.5(+0.2)	5.2(+/-0.3)
Natural waters		
a	0	0
b	1.8 (+/-0.02)	1.5(+/-0.04)
c	12.6(+/-0.1)	12.0(+/-0.1)
d	47.2(+/-0.2)	48.1(+/-0.3

Table-1: Results of analysis of blood, Urine and water is presented in * µM. The method is compared with reported LCMS method and is comparable, (Mean of three replicate analyses)

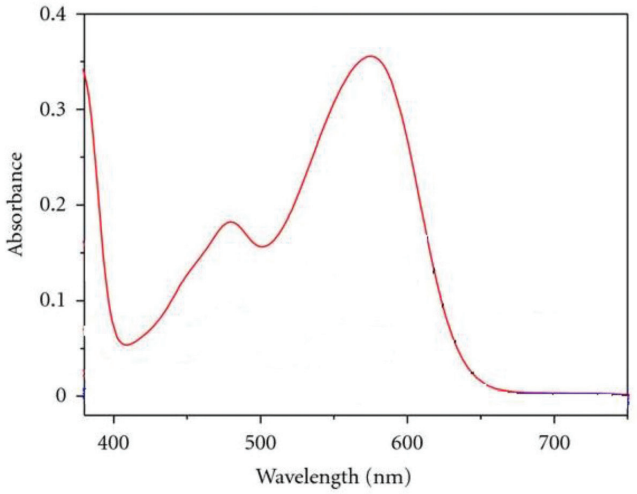

Figure-1: Absorption spectra of the complex formed by the reaction of chromotropic acid and formaldehyde. It shows an absorption maximum of 535nm.

this wavelength. The reagent has negligible absorbance at 392nm. For all further measurements demineralized water was used as reference. The colored complex obeys Beer's law in the range of 0.2– 2.2 µM of TMAO in 25 ml of the final solution in sulfuric acid medium. It was also observed that the sensitivity increases with solvent extraction by three times as reported along with shift in maximum wavelength of absorbance. The lower limit of detection is 0.02 µM. The molar absorptivity of the dye is about 1.48 X 10^4 l $mol^{-1}cm^{-1}$

DISCUSSION

Reproducibility and accuracy of the method was checked by replicate analysis of solutions containing varying concentrations TMAO. The standard deviation and relative standard deviation were 0.001 and +/- 2.1 respectively. Accuracy (%) for 0.2, 0.5, 2.0, 5,100, 200 µM were 98.4, 98.1, 98.6, 99.5, 99.3 respectively. Similarly samples which did not contain any TMAO were spiked with 10 µM of standard and extracted. The percentage recovery of spiked samples was found to be 96.5-98.5%.

Effect of different reagents, Dimethylase enzyme, and chemical matrix were investigated. 2mly of DMA was sufficient for complete enzymatic conversion. For complete color development, 1ml of Chromotropic acid was sufficient. Excess concentration or volume of any of the reagents hardly affected the sensitivity of the method. Excess nitrite/ nitrate if in real samples were removed by adding 1ml of 2% sulfamic acid.

Effect of pH on complete reaction was investigated. Lower acidities reaction was erratic and it was found that for stable reaction and color development, high acidity was required and was maintained with phosphoric acid.

The reaction between TMAO and Chromotropic acid was temperature dependent. It was found that 80°C was most suitable for the complex formation. This was achieved by microwave 1100W for 30 seconds. Higher temperature affected the reproducibility due to evaporation loss. Hence the complete analysis was done at 80°C. It was also observed that 3-4 minutes time is required for optimum color development. Any increased time period has no effect on the reaction. Alternatively the reaction can be conducted in hot water bath with same accuracy.[11]

Effect of temperature on TMAO at different temperatures was studied. The results did not indicate any significance by storage and transport at very low temperatures. We have also observed that 0-4 °C cold storage during shipping is suggested as extreme low temperature increases logistics cost for samples to reach from various places.

Effect of different foreign material and metal ions which are likely to interfere with the procedure were studied. It was observed that many of the foreign ions, proteins like albumin, creatinine etc did not interfere in the procedure as the DMA is a very selective enzyme. Higher concentrations of formaldehyde if present in water was suppressed by zinc sulfuric acid reaction. Since the method is developed specifically for biological samples with no formaldehyde, no interference is observed. Nitrite present was masked with sulfamic acid. Further effect of recovery of TMAO from blood samples were studied by conducting deproteinization and serum separation in single operation. It was found that single step in centrifuge was sufficient. Deproteinization using Methanol is not recommended as oxidation products of methanol may interfere with the absorptivity if large quantities of it are used. To assess the validity of the method, it has been applied for the determination of TMAO in blood samples, and contaminated water. The results of analysis are summarized in Table 1. Multiple replicate analyses were done to ensure the accuracy and reproducibility.

CONCLUSION

The proposed method is compared with two other methods. It is evident from the results described in Table 1 that, this method is rapid, economic, selective, and sensitive and can be replicated in any standard diagnostic laboratories with no sophisticated instrumentation requirements and analytical skills. The lower level of detection is 0.5 µM which is very significant as standard deviation is 0.01. The method can be applied for determination of TMAO in biological and environmental samples with high accuracy.

ACKNOWLEDGEMENT

Authors are grateful to Crescent Polymers Pvt Ltd for providing laboratory facilities. They are also thankful to National Forensic laboratory and Fortis Hospital Bangalore for providing biological samples.

REFERENCES

1. Zeneng Wang, Bruce S Levison, Jennie E Hazen, Lillian Donahue, Xin –Min Li, Stanley L Hazen m, Measurement of Trimethylamine –N-oxide by stable isotope dilution liquid chromatography tandem mass spectrometry, Analytical Biochemistry 455 92014) 35-40.
2. C.A Marmer, L.Choiniere, A Lesimple, Measurement of Urinary Trimethylamine and Trimethylamine oxide by direct infusion electrospray quadruple time of flight

mass spectrometry, Anal Biochem 2010;406:80-82
3. K.A Dacosta, JJ Vrbanc, SH Zeisel, The measurement of dimethylamine N-oxide using capillary gas chromatography-mass spectrometry, Anal. Biochem 1990;187:234-239.
4. M.E. Erupe, A Liberman-Martin, PJ SilvaQG Malloy, N Yonis, DR Cocker KI Puvis Roberts, determination of methylamines and Trimethylamine N Oxide in particulate matter by non-suppressed ion chromatography, Chromatography A 2010;1217:2070-2073.
5. C.A Marmer, L.Choiniere, E.P. Tracy, Measurement of trimethylamine and trimethyl amine Oxide by direct infusion electrospray quadruple time of flight mass spectrometry, Anal Biochem 2010;406:80-82.
6. J.D Bell, J.A Lee, HA Lee, P.J Sadler D.R Wikie,R.H Woodham, Nuclear Magnetic resonance studies of blood plasma and urine from subjects with renal failure. Identification of Trimethylamine –N-oxide, Biochim Biophy. ACTa 1991;1096:101-107.
7. W.G Anderson, P.J Allen, Journal of fish biology 2011;782:667-672
8. S. Veeravalli, I.R Karu Philips, E. A Shepherd, A highly sensitive liquid chromatography electrospray ionization method for quantification of TMAO and creatinine in Mouse urine Methods X 2017;4:310-319
9. J.C Wekell, H.Barnett, New method for analysis of trimethylamine oxide using ferrous sulphate and EDTA J. Food Science 1991;56: 132-138.
10. AD Hatton Gubbsw, Technique for the determination of trimethylamine oxide in natural waters and biological media, Anal.Chem 1999;7: 4886-4891.
11. Paris e. Georgiou et chi Keung (jimmy) ho. The chemistry of the chromotropic acid method for the analysis of formaldehyde, Can. j. chem. 67, 871 (1989).

Source of Support: Nil; **Conflict of Interest:** None

Submitted: 10-09-2020; **Accepted:** 14-10-2020; **Published:** 20-11-2020