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Determination of trimethylamine N-oxide level and its metabolic precursors in biological material

The level of trimethylamine N-oxide, exceeding the threshold indices, is a precursor to a number of diseases, leading to disability and death. In this context the definition of titers and the normalization of its levels in the body is one of the stages of preventive medicine. This review presents the methods for determining the levels of TMAO and its metabolic precursors in the biological material. The world practice mainly use the high performance liquid chromatography for TMAO quantitative determination in biological material, the detection is performed using tandem MS/MS spectroscopy, and in some cases nuclear magnetic resonance spectroscopy. The time-consuming sample preparation and complex combinations of the composition of the mobile phase are applied for effective separation and receiving of reliable results. Nevertheless, the problem of the quantitative and qualitative determination of TMAO and his predecessors not only hasn't lost the relevance, but has acquired the new horizons to improve this analysis in view of recent events in the scientific world.

Key words: TMAO, choline and metabolites, atherosclerosis, trimethylaminuria, GC-MS/MS, NMR-spectroscopy, HPLC-MS/MS, ion chromatography, analysis technique.

Trimethylamine N-oxide (TMAO) is the end product of metabolism of phospha- tidyl- choline, entering the organism with food of animal origin (such as red meat, egg yolk, seafood), which under the influence of the intestinal microbiota is metabolized to trimethylamine (TMA), and it enters the liver with a current blood, where is oxidized to its final form TMAO with the help of an enzyme of flavinmonooxygenase (FMO3) genus (Fig. 1) [1, 2]. Initially, in clinical practice, the levels of TMA and TMAO in blood and urine were considered as a diagnostic character of genetically caused disease Trimethylaminuria (insufficient synthesizing of FMO3 enzyme in liver) as well as in the diagnosis of renal disease. From the perspective of environmental protection, the levels of TMAO and other methylamines are important as a component of organic nitrogen spray in the areas with increased levels of these substances that affect the climate and human health as a whole [3]. Also TMAO can be regarded as a new, relatively modifiable risk factor for cardiovascular [1, 2, 4], it is especially important that this group of diseases is the leader for morbidity and mortality worldwide on a global healthcare [5]. According to recent reports, the control of TMAO level allows to monitor the risk of atherosclerosis development and its complications, as well as to evaluate the effectiveness of treatment and prevention, which can be one of the components of personalized medicine. All of the above leads to the necessity of the development and improvement of methods for determining the levels of TMAO in the biological material.

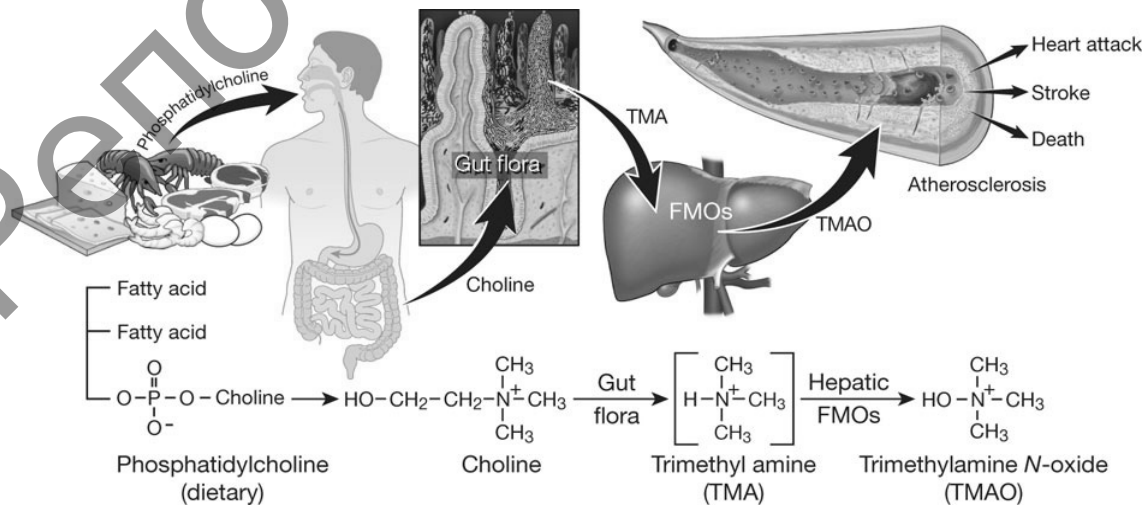

Figure 1. TMAO generation in human organism [1, 2]

Among the first researchers, who had synthesized TMAO and spent its qualitative and quantitative determination in 1962, were the scientists from the University of California (USA) J.R. Baker and S. Chaykin. The purpose of their study was to determine the type of liver enzymes and their effect on detoxification (oxidation) of trimethylamine (TMA) to trimethylamine N-oxide (TMAO). The material, in which the level of tertiary amine determines, was the microsomal fraction of pork liver. Preparative liquid chromatography was used, followed by analysis on paper chromatography [6].

In modern time the level of TMAO is usually defined for diagnosis of Trimethylaminuria. The syndrome of disagreeable fish odor (also known as fish odor syndrome or Trimethylaminuria) is the index of metabolic disorder and is characterized by the presence of abnormal amounts of dietary origin tertiary amine, trimethylamine (TMA) in urine, sweat, exhaled air and other secretions. Trimethylamine has a strong smell of rotting fish, and it has on a person, suffering from this disease, a devastating psychological impact on all spheres of life (personal, social, labor). In 2004 Marcus A. Bain et al. [7], based on the earlier experience of their foreign colleagues, who determined the level of TMAO in urine by gas chromatography (GC) [8, 9], high performance liquid chromatography (HPLC) [10], with various detection methods, developed their own methodology using GC. This method using solid phase microextraction (SPME) allowed determining the level of TMAO in human plasma. During the work, it was used gas chromatograph Varian Star 3400 CX with column SPB-1 sulfur (30 m × 0.32 mm, 4.0 μm) and mass-detector Varian Saturn 2000. Evaporator temperature was 250 ºC, the column temperature — 120 ºC, as a carrier gas it was used the isothermal helium with the delivery of 60 ml/min. Carboxy-polydimethylsiloxane fiber (75 μm) was used for SPME. 0.01 M of hydrochloric acid and titanium sulfate (III) dissolved in sulfuric acid (10 μL, 45 % w/v) were poured to plasma. As internal standards the deuterated TMA and TMAOwere used, quantitative calculation was based on the calibration line.

With the aim of the diagnosis of Trimethylaminuria, as well as for studying of the role of betaine and its metabolites in the development of vascular disease, in 2006 the team of scientists from New Zealand conducted the validation of method of 1H NMR-spectroscopy, which, according to their opinion, allows the more efficient identification of methylamine metabolites at the study of their level in urine [11]. Sample preparation was carried out by adding of 1M of hydrochloric acid solution, containing 25 mM of acetonitrile (as an internal standard), into the test material (urine). Also the internal standards of methylamines were used. All spectra were registered and were recorded on a high-resolution investigated liquid phase NMR-spectrometer Varian INOVA 500 at the temperature of 23 ºC in the 5 mm NMR-tubes with deuterated 3 mm lock inserted in them. For measuring it was used 90° RF pulse with duration of 8.1 microseconds. The delay between the pulses was 5 seconds, the detection time 1,982 seconds, the width of the fluctuation band — 8000 Hz. The duration of the analysis was less than 5 minutes. The limit for TMAO detection was 3,37 ppm (15 μM) that, according to the authors' opinion, was above the detection limit using HPLC. The same method, but in more expanded version (with detection of TMAO level not only in the urine and blood), was patented in 2015 by the team of scientists from North Carolina (USA) headed by James D. Otvos [12].

Due to the need to improve the diagnosis of Trimethylaminuria, the scientists from many countries introduced new and technically more simplified methods of qualitative and quantitative determination of TMA and TMAO levels. One of these was David W. Johnson, who in 2008 proposed thread-injection electrospray ionization tandem mass-spectrometry for the simultaneous determination of TMA and TMAO in urine for the first time [13].The analysis was performed on the equipment Applied Biosystems/MDS Sciex API 4000. The gas for the collision was nitrogen. A sample (20 μL) was placed through 96-well autosampler Gilson 215, with mobile phase at a rate 150 μL/min (supplied by Agilent 1100 of HPLC-system). The time for the analysis of one sample was 2 minutes. TMAO and TMA marked ^{2}H were used as an internal standard. Sample preparation was carried out in several stages. The internal standards $^{2}N_9TMA$ (1 μL, 1 mg/ml in water) and $^{2}H_9$-TMAO (2 μL, 1 mg/ml in water) dissolved in hydrochloric acid were poured to the test material (urine), then it was added concentrated ammonia solution (1 μL) and ethyl bromoacetate (30 μL, 20 mg/ml in acetonitrile). After 30 minutes, the solution of mobile phase containing acetonitrile/water/formic acid (50:50:0.025) was added.

Subsequently it was conducted 100x dilution of 10 μL of received solution by mobile phase (MP). The following data were obtained for TMA (146,1, 118,1, 400 m/z), 2H9-TMA (155,1, 127,1, 400 m/z), TMAO (76,1, 58,1, 100 m/z) and 2H9-TMAO (85,1, 66,1, 100 m/z). The quantitative calculation was based on the calibration line. Two years later, the team of scientists from Canada used the method of direct input of electro-spray quadrupole time-of-flight mass-spectrometry to determine the TMA and TMAO in urine in order to improve and simplify the given analysis [14]. The difference with the method developed by David

W. Johnson (described above) was in radioactive isotopes, with which the desired material was labeled. Orval A. Mamer and co-workers used the isotope ^{15}N instead the isotope ^{2}H.

In order to protect the human from the use of poor fish and to improve the quality of life of people with Trimethylaminuria the Chinese scientists have developed a method of determining the levels of TMAO, TMA, dimethylamine, formaldehyde in seafood [15]. For this it was used the method of ion chromatography with non-suppressed conductivity. The analysis was performed on the equipment ICS-2000 using theprotective pre-column Dionex Ion-Pac CG17 guard column (50 mm × 4 mm i.d.) and the analytical column DionexIonPac CS17 (250 mm × 4 mm i.d.). The column CS17 has been selected on the basis of benefits at the analysis of organic amines in the method of ion chromatography with not-suppressed conductivity. Mobile phase (MP) consisted of a solution of methanesulfonic acid 3.0 μmol/L. Separation was performed in isocratic mode at 30 ºC temperature of column and MP flow rate 0.8 ml/min. Sample preparation was carried out by homogenization of raw material (finely chopped pieces of fish) in 10 ml of 7.5 % cold trichloroacetic acid (TCA), followed by centrifugation 4000 rpm/min for 15 minutes at 4 ºC. The sediment was subjected to a double back-extractingof 5 ml of 5 % TCA with the repeat centrifugation. All supernatants were mixed with deionized water with subsequent filtration through a fine pore filter paper (20–25μL). The time of TMAO retention was 15,91 min. The quantitative determination was carried out on the calibration line.

The scientists from the University of California (USA) in 2010 at the head of Mark E. Erupe among the first used the above method to determine the level of TMAO (in combination with other amines) in the atmospheric air. This study was conducted for determination of the role of organic nitrogen in the composition of atmospheric aerosols, which plays a significant role in formation of the environment and affecting the climate and human health as a whole [3]. The analysis was performed on the equipment Metrohm 761 Compact IC with protective pre-column Metrosep RP (with steel mesh filter) and analytical column Metrohm-Peak Metrosep C2 (250 mm × 4 mm i.d.). As an eluent was thesolution of 3 mM nitric acid and 3.5 % acetonitrile with mobile phase at a rate of 1 ml/min. The sample was injected manually; the analysis time was 15 minutes. Separation was performed in isocratic mode at a column temperature of 20 ºC. The mix of methylamines (including TMAO) was used as a marker. The samples were taken from the filter of the smoke chamber for sample preparation, then it was conducted the extraction in 10 ml of ultrapure water (Millipore) by ultrasonic dispersion for 30 minutes. The quantitative calculation was based on the calibration line. The lower limit of TMAO detection was 72 μg/L, the retention time — 12 minutes. According to the authors, this method is easy to use and applicable for the determination of the desired substances in low concentrations due to the low detection threshold.

Since 2011 the level of TMAO in the human body is given a new meaning. A number of researchers have proven the relationship of TMAO high titers as the end product of metabolism of choline and betaine with an increased risk of developing of cardiovascular diseases, myocardial infarction and stroke [1, 2, 4]. This fact has increased the interest in the improvement of methods for determining the level of these substances in biological samples.

Thus, Zeneng Wang et al., were among the first to carry out this analysis as part of their research work with reference to cardiovascular risk. The liquid chromatography coupled with electrospray ionization spectrometry was used to determine the metabolic profile. Structural identification of targeted analytes was performed using a combination of methods: HPLC/MS/MS, multinuclear NMR-spectroscopy and GC/MS. Sample preparation was carried out by deproteinization of plasma by means of ice-cold methanol. After centrifugation, the supernatant was introduced into Rexchrom Phenyl column (4,6×250 mm, 5 μm) with a flow rate of MP 0.8 ml/min. Gradient elution was used, at first 10 mM ammonium formate was used longer than 0.5 minutes with the transition to 5 μM of ammonium formate, 25 % methanol and 0.1 % formic acid (for 8 minutes), after it was used 100 % methanol and the water for analytes separation [1]. In analysis mode of MS1 positive ions they were obtained the analytes with m/z 76, 104, 118. d9-TMAO was used as the internal standard. The quantitative determination was carried out on the calibration line.

The same group of authors refined the method of determining the TMAO level in the biological material in 2013 [16]. The analysis was conducted using a pump system 4LC-20AD Shimadazu, autosampler SIL-HTC and system switching the valve of double columns connected with the mass-spectrometer API 4000 Q-TRAP. Sample preparation consisted of mixing of 20 μL of the test plasma with 10 μM of internal standard (d9-TMAO) in 80 microliters of methanol with followed centrifugation for 10 minutes at 20000g speed at 4 ºC. The resulting supernatant was injected into the column Luna silica (4,6×250 mm, 5 μm) at a rate of MP 0.8 ml/minute at gradient elution. Discontinuous gradient used for better separation of analytes by mixing a composition of eluent «A» (0.1 % propanoic acid in water) with eluent «B» (0.1 % acetic acid in meth-

anol) in different ratios, ranging from 2 % «B» linearly to 15 % «B» within 11 minutes, then linearly to 100 % «B» for 5 minutes with the followed return to 2 % «B». The desired substance was monitored using electrospray ionization in the mode of positive ion with multiple reaction monitoring (MRM) with typical product-ion transitions m/z 76→58 and 85→66 amu. Retention time was ≈ 9 minutes, the minimum quantification threshold of detection was 0.05 μM, max > 200 μM[16].

A similar method, but with some modifications, was used by Liam M. Heaney et al. in 2015. Differences with the method created by Zeneng Wang were as follows: the composition of MP, as the solvent «A» the mixture of 0.025 % ammonium hydroxide with 0.045 % formic acid (pH 8.1) was used, the solvent «B» was acetonitrile, the characteristics of the used column Acquity UPLC BEH HILIC (130 Å, 2,1 mm × × 100 mm, 1.7 μm). The analysis results were similar to the previous method.

Later they began to appear the new works confirming the correlation of TMAO high titers with the development of atherosclerosis [17–19], heart failure [20], inflammatory bowel disease (Crohn's disease, ulcerative colitis) [21], the more detailed description of the metabolic pathway of TMAO formation on the basis of nutritional factor [22–24]. All of these moments give scientists new tasks to increase the number of simultaneously defined TMAO metabolites and precursors, to reduce the time of analysis, to increase the sensitivity of the method, including the simplification the methods of analysis and at the same time the optimization of its reliability.

For example, the team of Latvian scientists have developed and conducted the validation of the method for the simultaneous determination of TMAO level in combination with L-carnitine and its biological precursor γ-butyrobetaine (GBB) in human plasma using HPLC/MS/MS [25]. For the sample preparation 900 μL of solution of acetonitrile and methanol (3:1 v/v) containing the tap (200 ng/ml) was poured the 40 μL of plasma. Supernatant was used for analysis after centrifugation (13000g, 10 minutes). The system Acquity HPLC with column Acquity HILIC BEH (2,1×50 mm, 1.7 μm) was used for the separation of analytes. Elution was conducted by the gradient principle from 75 to 55 % acetonitrile in 10 μM of aqueous solution of ammonium acetate (pH4) at a rate of MP 0.25 ml/minute. The analytes were monitored using electrospray ionization in the positive ion mode with multiple reactions monitoring on a triple quadrupole mass-spectrometer. The mass-spectrometer was configured as follows: capillary voltage 3,3 kV, desolvation and source temperature 120 and 350 ºC respectively. Nitrogen flow rate 500 L/h. The product-ion transitions for TMAO amounted m/z 75,8 → 58,3, for L-carnitine — m/z 146,11 → 87,11, for GBB — m/z 175,44 → 86,0. The time TMAO detection was 1,95 min, L-carnitine — 1,85 min, GBB — 2,21 min. The quantitative determination was carried out on the calibration line.

All of the following methods for determining of the level of choline structural metabolites are largely identical. Analysis was performed using HPLC/MS/MS in the positive ion mode. Relative differences were presented in the composition and the speed of mobile phase, in the type of analytical column and the time ofanalytes retention in accordance with applied chromatographic systems.

One of the latest it is possible to mark the work of Xueqing Zhao et al [26], which improved method for determination of choline, betaine, TMA and TMAO to diagnose diseases associated with nutrition, bowel diseases and the risk of life-threatening diseases. Sample preparation was carried out as follows: acidified test material (plasma, urine) was extracted, and then derivatization using tert-butylbromoacetate in acetonitrile and ammonium hydroxide in water was performed. After the centrifugation, the supernatant was transferred to a vial for subsequent analysis. Chromatographic separation was performed on the column Atlantis Silica HILIC (4,6×50 mm, 3μm). Column temperature was 40 ºC, the eluent flow rate 1 ml/minute. The solvent «A» was the composition of acetonitrile and water (1:9) with 10 μM of ammonium formate and 0.125 % formic acid. The solvent «B» consisted of acetonitrile and water (9:1) with 10 μM of ammonium formate and 0.125 % formic acid. The gradient elutionwas used. The analytes and their respective isotopes were monitored by specific ion transitions: 104→45 for choline, 118→59 for betaine, 174→59 for TMA, 76→58 for TMAO. The retention time for TMAO was 2,41min.

Steuer et al. [27] using the same clinical objectives conducted another improvement of the analysis abovelisted substances in plasma, serum and human urine. They proposed the method for rapid and simultaneous determination of compounds of quaternary ammonium of phosphatidylcholine origin such as choline, betaine, O-acetyl-L-carnitine, L-carnitine and TMAO. Plasma, serum, urine were deproteinized with methanol with following centrifugation and the supernatant sampling. The column Phenomenex Luna-HILIC (4,6×150 mm, 3 μm) was used for the separation of substances, the detection and quantitative analysis was performed by LC-MS/MS electrospray ionization in the positive ion mode recording. The temperature of column was 35 ºC, mobile phase flow rate — 0.75 ml/min. The analysis was conducted using a gradient elution. Solvent «A1» con-

sisted of 10 μM ammonium acetate in 90 % acetonitrile with water (9:1), the solvent «B1» consisted of 10 μM acetate buffer with pH 4. The second mobile phase consisted of «A2» of 10 μmol of ammonium formate and 90 % acetonitrile and «B2» — 10 μmol of ammonium formate in water at pH 3. Autosampler temperature was set to 10 ºC. Validation method was carried out in accordance with international guidelines regarding selectivity, consistent contamination of samples, the limit of quantitation (LQ), linearity, accuracy, reliability, reproducibility and stability of the treated sample. Ionic analytes transitions made the identical performance to the above methods. The retention time for TMAO was 7.27 min.

Conclusions

Thus, by analyzing all of the above, it is found that the level of trimethylamine N-oxide exceeding the threshold is a precursor of a number of diseases, leading to disability and death, in this context, the definition of titers and the normalization of its levels in the body are one of the stages of preventive medicine. High performance liquid chromatography is mainly used in world practice for quantitative determination of TMAO in biological material. Given the structural features of TMAO the detection is performed using tandem MS/MS-spectroscopy and nuclear magnetic resonance spectroscopy in some cases. It should be noted that such equipment is very expensive, so it is not always available. Taking into account the multicomponent composition of the research material the time-consuming sample preparation and complex combinations of the composition of the mobile phase are used for efficient separation and obtaining of reliable results.

Nevertheless, the problem of the quantitative and qualitative determination of TMAO and his predecessors not only hasn't lost the relevance, but has acquired the new horizons to improve this analysis in view of recent events in the scientific world. Speaking about the evolution of techniques, it is clear that the work aimed at their improvement, due to changes of technical component of analysis, the number of simultaneously defined analytes, reducing time of analysis, and also the improvement of the method of sample preparation and the composition of the mobile phase for the separation.

The development of rational sample preparation and efficient methods of quantitative determination of TMAO in plasma using HPLC-MS/MS for the early diagnosis of diseases of the circulatory system is carried out in the Share laboratory of the Scientific-research Center of Karaganda state medical university.

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А.Б. Марченко, С.А. Ивасенко, А.А.Турмухамбетова

Биологиялық материалда триметиламин оксиді деңгейін және оның метаболитті орынбасушыларын анықтау

Триметиламин оксиді көптеген аурулардың бастамасы болып өлім мен жұмысқа қабілетсіздікке әкеліп соғады. Профилактикалық медицинаның ең маңызды рөлдерінің бірі титрлеу мен ағзадағы қалыпты деңгейін анықтау болып табылады. Берілген шолуда ТМАО деңгейін анықтау әдісі мен биологиялық материалдағы оның метаболитті орынбасушылары көрсетілген. Бүгінде биологиялық материалдағы ТМАО санын анықтауда жоғары дейгейдегі сұйықты хроматография тандемін, яғни спектроскопия MS/MS және кей жағдайларда резонанстық ядролық спектроскопия әдістерін, қолданады. Ұтқыр фазаның күрделі комбинациялық құрамы тиімді бөлініп, сенімді нәтижелер алуға көмектеседі, сол себепті жоғары сұранысқа ие болады.

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Определение уровня окиси триметиламина и его метаболических предшественников в биологическом материале

Окись триметиламина является предшественником многих заболеваний, приводящих к нетрудоспособности и смерти. В этом контексте определение титров и нормализация его уровней в организме являются одной из важных задач профилактической медицины. В настоящем обзоре представлены методики для определения уровней ТМАО и его метаболических предшественников в биологическом материале. Сегодня главным образом используются высокоэффективная жидкостная хроматография для количественного определения ТМАО в биологическом материале, тандемная спектроскопия MS/MS и в некоторых случаях — спектроскопия ядерного магнитного резонанса. Подготовка проб и сложные комбинации состава мобильной фазы применяются для эффективного разделения и получения надежных результатов, благодаря чему являются весьма востребованными.