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UPLC-ESI-MS/MS method for the quantitative measurement of aliphatic diamines, trimethylamine *N*-oxide, and β-methylamino-*L*-alanine in human urine☆

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ABSTRACT

This work describes a quantitative high-throughput analytical method for the simultaneous measurement of small aliphatic nitrogenous biomarkers, i.e., 1,6-hexamethylenediamine (HDA), isophoronediamine (IPDA), β-methylamino-*L*-alanine (BMAA), and trimethylamine *N*-oxide (TMAO), in human urine. Urinary aliphatic diamines, HDA and IPDA, are potential biomarkers of environmental exposure to their corresponding diisocyanates. Urinary BMAA forms as a result of human exposure to blue-green algae contaminated food. And, TMAO is excreted in urine due to the consumption of carnitine- and choline-rich diets. These urinary biomarkers represent classes of small aliphatic nitrogen-containing compounds (N-compounds) that have a high aqueous solubility, low log*P*, and/or high basic pK_a. Because of the highly polar characteristics, analysis of these compounds in complex sample matrices is often challenging. We report on the development of ion-pairing chemistry based ultra-performance liquid chromatography–electrospray ionization–tandem mass spectrometry (UPLC–ESI–MS/MS) method for the simultaneous measurement of these biomarkers in human urine. Chromatographic separation was optimized using heptafluorobutyric acid-(HFBA-) based mobile phase and a reversed-phase C18 column. All four analytes were baseline separated within 2.6 min with an overall run time of 5 min per sample injection. Sample preparation involved 4 h of acid hydrolysis followed by automated solid phase extraction (SPE) performed using strong cation exchange sorbent bed with 7 N ammonia solution in methanol as eluent. Limits of detection ranged from 0.05 ng/mL to 1.60 ng/mL. The inter-day and intra-day accuracy were within 10%, and reproducibility within 15%. The method is accurate, fast, and well-suited for biomonitoring studies within targeted groups, as well as larger population-based studies such as the U. S. National Health and Nutrition Examination Survey (NHANES).

1. Introduction

Aliphatic diamines and other nitrogen-containing compounds (N-compounds) in human urine form as a result of exposure to a broad range of sources and could serve as biomarkers of toxicant exposure. Urinary aliphatic diamines can be formed from exposure to their corresponding diisocyanates, which are used in polyurethane-based paints and coatings [1,2]. These aliphatic diisocyanates may enter the environment through the discharge of industrial waste, application of paint spray, and degradation of consumer products; exposure may subsequently occur through dermal contact, ingestion, and inhalation. The most common aliphatic diisocyanates found in paints and coatings are hexamethylenediisocyanate (HDI) and isophoronediisocyanate (IPDI). Exposed human subjects metabolize these diisocyanates to hexamethylenediamine (HDA) and isophoronediamine (IPDA), respectively. Health effects of diisocyanate exposure include asthma, dermatitis, gastrointestinal irritation, chemical bronchitis, and pneumonitis [3].

Two other health-relevant biomarkers can be analyzed together with HDA and IPDA: β-methylamino-*L*-alanine (BMAA) and trimethylamine N-oxide (TMAO). Exposure to BMAA, a non-proteinogenic amino acid toxin produced by cyanobacteria, occurs through consumption of contaminated seafood and water and is associated with increased risk of neurodegenerative diseases including amyotrophic lateral sclerosis (ALS), Parkinson's disease, and Alzheimer's disease [4–6]. TMAO, a toxicant formed from metabolism of

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phosphatidylcholine- and L-carnitine-rich foods (e.g., dairy, red meat, fish, and certain dietary supplements), is associated with increased risk of cardiovascular disease [7–9]. TMAO affects cholesterol and sterol metabolism and is mechanistically involved in the development of atherosclerosis and cardiovascular disease [8].

Several mass spectrometry-based analytical methods have been developed previously for the measurement of these compounds individually, or simultaneously with a similar class of compounds in urine. HDA and IPDA have been widely analyzed as their perfluoroanhydride derivatives using GC–MS [10–14] and in a few cases using LC-MS [12,15,16]. Samples were acid- or base-hydrolyzed, extracted in a non-aqueous solvent (i.e., liquid-liquid extraction), and reacted with a derivatizing reagent prior to lengthy instrumental analysis (~30 min). Similarly, several GC–MS and LC-MS methods have been reported for BMAA measurements in neurological tissues, cyanobacterial samples, seafood, and staple foods, and in some cases in human and primate urine [4,5,17–23]. However, the chemical specificity has been a concern due to the lack of standard analytical technique for BMAA measurement as well as co-eluting isobaric compounds for GC- and LC-MS method [4,19]. TMAO has been historically measured using GC–MS [24] and very recently using LC-MS [25–28]. However, the methods reported are based on protein precipitation and direct "dilute-and-shoot," which lack proper sample clean-up to avoid potential chemical interferences. We developed a simple, fast, sensitive, rugged, and high-throughput ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method to simultaneously quantify different classes of small aliphatic N-compounds in human urine using ion-pairing chemistry. The analytical challenges regarding the simultaneous measurement of these four analytes using liquid chromatography include their high aqueous solubility, low log*P*, and/or high basic pK_a values. This paper reports the first analytical method that can simultaneously measure all four targeted urinary biomarkers of interest.

2. Experimental

2.1. Materials

HPLC-grade heptafluorobutyric acid (HFBA), LCMS Optima™-grade methanol, AcroSeal® 7 N ammonia in methanol, and synthetic urine were purchased from Fisher Scientific (Suwanee, GA). Reagents 1 N sodium hydroxide (NaOH) and 6 N hydrochloric acid (HCl) were purchased from Sigma-Aldrich (St. Louis, MO). Trifluoroacetic acid (TFA), pentafluoropropionic anhydride (PFPA), and analytical standard-grade HDA, IPDA, BMAA and TMAO were purchased from Sigma-Aldrich (St. Louis, MO). IPDA-[^{13}C, $^{15}N_2$], BMAA-[^{13}C, $^{15}N_2$], and TMAO-[$^{13}C_3$] were purchased from IsoSciences (Kings of Prussia, PA), and HDA-[$^{2}H_{12}$] from Toronto Research Chemicals (Ontario, Canada). Human urine pool was collected anonymously following a protocol approved by the CDC Institutional Review Board.

2.2. Calibration solutions

Individual master stocks of neat standards and internal standards (1 μg/mL) were prepared in methanol/water (50:50 v/v). Master stocks were diluted in water to make a set of working stocks of seven different concentrations. All calibration solutions were prepared by diluting the working stocks by a factor of 10 in 0.1% HFBA (v/v) in water. The concentration range of calibration solutions is shown in Table 6. Internal standard concentrations ranged from 1 to 18 ng/mL and were assumed constant for the quantitation. All stock solutions were stored at −70 °C prior to use.

2.3. Sample preparation

The sample preparation workflow involved acid hydrolysis followed by automated solid phase extraction (SPE). For acid hydrolysis, 1000 μL sample was prepared by mixing 250 μL of urine followed by 100 μL of 6 N HCl (final concentration of acid is 0.6 N), 50 μL of the internal standard mixture, and 600 μL of water. The sample solution was placed in a heating block (VWR, Radnor, PA) set at 80 °C for 4 h. The solution was allowed to cool to room temperature and was subsequently adjusted to pH ~1.0 using 500 μL of 1 N NaOH prior to performing SPE. Strata XC (30 mg, 3 mL) mixed-mode strong cation 96-well plates from Phenomenex (Torrance, CA) were used for sample clean-up. The SPE plates were conditioned using 1 mL of methanol followed by equilibration using 1 mL of HPLC grade water. The hydrolyzed samples were then loaded and washed with 1 mL of 0.1 N HCl acid followed by 2 mL of methanol. Finally, analytes were eluted twice with 500 μL of 7 N NH_3 in methanol solution (i.e., SPE elution solvent). The SPE elution solvent bottle was chilled at 4 °C using Thermal Lab Beads™ and a cooling tray (TecaLAB™, Chicago, IL). SPE was performed using a Biotage® Extrahera™ sample preparation system (Charlotte, NC). Following SPE, eluents were evaporated to dryness in an SPE Dry evaporation system from Biotage® (Charlotte, NC) under nitrogen gas at 60 °C. Prior to UPLC-MS/MS analysis, dried samples were reconstituted in 1 mL of water/methanol (95:5 v/v) with 0.1% of HFBA using an automated vortex mixer for 10 min (VWR, Radnor, PA).

2.4. UPLC-ESI-MS/MS analysis

The analytical run was performed using an Acquity I-Class UPLC system (Waters Corporation, Milford, MA) equipped with 2.1 × 100 mm, 2 μm ACE Excel2 SuperC18 Column (Mac-Mod Analytical, Chadds Ford, PA). The UPLC system was coupled to a 5500 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source (Sciex, Framingham, MA). Chemical separation was performed using the solvent gradient of 0.1% aqueous HFBA (v/v, mobile phase *A*) and 0.1% HFBA in methanol (v/v, mobile phase *B*) as shown in Table 1. Column and sample manager temperatures were set to 50 °C and 10 °C, respectively. Injection volume was 2 μL using the full loop injection mode. The mass spectrometer was operated in positive ion ESI scheduled multiple reaction monitoring (sMRM) mode with a target scan time of 0.3 s for 30 s MRM detection window. Optimized ion source parameters included the following: ESI voltage, 2.5 kV; CAD gas, 7 psi; curtain gas flow, 30 psi; nebulizing gas (GS1) flow, 45 psi; heating gas (GS2) flow, 55 psi; and heater temperature, 650 °C. The chemical structures and mass-to-charge ratios of the precursor ions monitored are shown in Fig. 1. Other compound-dependent parameters are shown in Table 2.

2.5. Data analysis

All LC-MS/MS data were generated in Analyst 1.6.2 (Sciex, Framingham, MA) and processed in MultiQuant 3.0.2 (Sciex, Framingham, MA).

Table 1
Gradient elution table for chromatographic separation. Mobile phases *A* and *B* are 0.1% (v/v) HFBA (aq.) and 0.1% HFBA (v/v) in methanol, respectively.

Time (min)	Flow rate (mL/min)	% Mobile phase B (by volume)
Initial	0.2	5
0.75	0.5	5
1.75	0.5	50
3.25	0.3	95
3.5	0.3	5
5.0	0.2	5