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Simultaneous determination of asymmetric and symmetric dimethylarginine, L-monomethylarginine, L-arginine, and L-homoarginine in biological samples using stable isotope dilution liquid chromatography tandem mass spectrometry

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ABSTRACT

Production of the endogenous vasodilator nitric oxide (NO) from L-arginine by NO synthase is modulated by L-homoarginine, L-monomethylargine (MMA), asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA). Here we report on a stable isotope dilution liquid chromatography tandem mass spectrometry (LC–MS/MS) method for simultaneous determination of these metabolites in plasma, cells and tissues. After addition of the internal standards (D_7-ADMA, D_4-L-homoarginine and $^{13}C_6$-L-arginine), analytes were extracted from the samples using Waters Oasis MCX solid phase extraction cartridges. Butylated analytes were separated isocratically on a Waters XTerra MS C18 column (3.5 μm, 3.9 mm × 100 mm) using 600 mg/L ammonium formate in water - acetonitrile (95.5:4.5, v/v) containing 0.1 vol% formic acid, and subsequently measured on an AB Sciex API 3000 triple quadrupole mass spectrometer. Multiple reaction monitoring in positive mode was used for analyte quantification. Validation was performed in plasma. Calibration lines were linear ($r^2 \geq 0.9979$) and lower limits of quantification in plasma were 0.4 nM for ADMA and SDMA and 0.8 nM for the other analytes. Accuracy (% bias) was <3% except for MMA (<7%), intra-assay precision (expressed as CV) was <3.5%, inter-assay precision <9.6%, and recovery 92.9–103.2% for all analytes. The method showed good correlation ($r^2 \geq 0.9125$) with our previously validated HPLC-fluorescence method for measurement in plasma, and was implemented with good performance for measurement of tissue samples. Application of the method revealed the remarkably fast (i.e. within 60 min) appearance in plasma of stable isotope-labeled ADMA, SDMA, and MMA during infusion of D_3-methyl-1-^{13}C-methionine in healthy volunteers.

1. Introduction

Asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) are formed through the methylation of L-arginine residues in proteins by two separate classes of enzymes called protein arginine methyltranferases (PRMT) [1], thereby expanding the functional repertoire of these proteins [2]. In the formation of both ADMA and SDMA, L-monomethylarginine (MMA) is formed as an intermediate product [1]. After degradation of the methylated proteins, ADMA, SDMA and MMA are released into their free form. ADMA and MMA are endogenous inhibitors of nitric oxide synthase (NOS) [3–5], the enzyme responsible for the conversion of L-arginine into nitric oxide (NO), the most potent vasodilator in the human body [6]. SDMA is generally considered not to inhibit NOS. However, Tsikas et al. [7] showed that SDMA possesses a weak inhibitory potency towards neuronal NOS. Additionally, SDMA may limit NO production by competing with L-arginine for cellular uptake. For the clearance of ADMA and MMA, cells contain the enzyme dimethylarginine dimethylaminohydrolase (DDAH), which hydrolyzes ADMA to dimethylamine and L-citrulline, and MMA to methylamine and L-citrulline [4,8]. Cationic amino acid transporters in the plasma membrane facilitate the transport of cytosolic ADMA, SDMA, and MMA to the circulation [9,10], from where they are either taken up by other cells or tissues, or excreted by the kidneys [11].

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L-Homoarginine is a structural analog of L-arginine that may also act as a substrate for NOS. However, compared to arginine, the K_m value of homoarginine is much higher, reflecting a lower catalytic efficiency of NOS using homoarginine as substrate [12]. Therefore, production of NO may be reduced at a high L-homoarginine/L-arginine ratio. In its relation to cardiovascular disease it remains unclear whether high or low L-homoarginine levels are beneficial. Recently, low levels of L-homoarginine in plasma have been associated with cardiovascular mortality and stroke in a large cohort study [13,14]. In contrast, numerous studies have revealed an association between high, rather than low, plasma levels of ADMA and cardiovascular disease [15,16]. Likewise, high plasma levels of SDMA have been associated with increased cardiovascular and all-cause mortality [17,18], and may also reflect renal dysfunction [19]. Notably, formation of ADMA, SDMA and MMA, as well as inhibition of NOS, occur inside cells, and therefore, for the prediction of cardiovascular disease, intracellular levels of ADMA, SDMA, MMA, L-arginine, and L-homoarginine may be more relevant than their plasma levels [20].

Investigation of the intracellular metabolism of these compounds and the relation between their intracellular and circulatory concentration, requires a precise and sensitive method that can handle a variety of sample matrices. Liquid chromatography tandem mass spectrometry (LC–MS/MS) is used increasingly for the simultaneous determination of L-arginine, ADMA, and SDMA in biological samples [21,22]. For L-homoarginine a LC–MS/MS method has recently been described as well [23]. Di Gangi first published an ultra-HPLC–MS/MS-method for the combined determination in plasma and urine of all L-arginine analogs that may have an impact on NOS metabolism [24]. With our current HPLC-fluorescence method [25,26] we are able to measure L-homoarginine in combination with L-arginine, ADMA and SDMA, but MMA cannot be quantified since it is used as the internal standard. Furthermore, the HPLC-fluorescence method is very precise and accurate for the analysis of plasma, urine and culture media, but lacks the sensitivity and specificity required for reliable quantification in small tissue samples. Additionally, the fluorescence method is not suitable for the determination of stable isotope-labeled forms of ADMA, SDMA and MMA in tracer studies, which can be very useful in determining the key processes in formation and degradation of these compounds. Therefore, the aim of the present study was to develop and validate a highly precise and sensitive stable isotope dilution LC–MS/MS method for the combined determination of ADMA, SDMA, MMA, L-arginine, and L-homoarginine, in biological samples.

2. Experimental

2.1. Chemicals

L-Arginine, ADMA dihydrochloride, SDMA di(p-hydroxyazobenzene-p′-sulfonate) salt, MMA acetate, L-homoarginine hydrochloride, Nε,Nε,Nε-trimethyllysine hydrochloride, Nα-acetyllysine, and Nε-acetyllysine were obtained from Sigma (St. Louis, MO, USA). $^{13}C_6$-L-Arginine ([U-$^{13}C_6$; 99.2%]-L-arginine, 99.2%) and D_7-ADMA ([2,3,3,4,4,5,5-D_7; 98%]-ADMA, 98%) were purchased from Eurisotop (Saint Aubin Cedex, France) and D_4-L-homoarginine ([4,4,5,5-D_4; 98.7%]-L-homoarginine, 98%) from Toronto Research Chemicals (Toronto, Canada). D_3-methyl-1-^{13}C-methionine (95%) was obtained from Isotec (Miamisburg, OH, USA).

Hank's Balanced Salt Solution (HBSS) was obtained from Invitrogen, (Carlsbad, CA, USA). Acetonitrile and formic acid were purchased from VWR prolabo (Amsterdam, The Netherlands), and 1-butanol and 70% perchloric acid from Merck (Darmstadt, Germany). All solvents were of analytical grade. Water was purified with a Milli-Q system from Millipore (Billerica, MA, USA).

2.2. Calibration standards, internal standards, and quality controls

2.2.1. Calibration standards

Because no analyte-free matrix is available, calibration samples were prepared in water.

For each analyte (L-arginine, MMA, ADMA, SDMA and L-homoarginine), a stock solution of 1 mM in water was prepared. From these stock solutions a combined solution of MMA, ADMA, SDMA and L-homoarginine was prepared containing 10 μM of each analyte. This combined solution together with the 1 mM stock solution of L-arginine was used for the preparation of seven calibration standards with concentrations of 0.1, 0.2, 0.4, 0.8, 1.2, 2.0, and 5.0 μM for ADMA, SDMA, MMA, and L-homoarginine and with concentrations of 10, 20, 40, 80, 120, 200, and 500 μM for L-arginine. All stock solutions and 1 mL aliquots of the calibration standards were stored at −20 °C.

2.2.2. Internal standards

For each of the three internal standards, D_7-ADMA, D_4-L-homoarginine, and $^{13}C_6$-L-arginine, stock solutions of 1 mM were prepared in water. The internal standard solutions were diluted with water to 0.2 μM for both D_7-ADMA and D_4-L-homoarginine and to 20 μM for $^{13}C_6$-L-arginine. D_7-ADMA was used for the quantification of ADMA, SDMA and MMA, D_4-L-homoarginine for the quantification of L-homoarginine, and $^{13}C_6$-L-arginine for the quantification of L-arginine (Table 1). The internal standard solutions were stored in aliquots of 1 mL at −20 °C.

2.2.3. Quality controls

For method validation purposes quality control (QC) samples were prepared by spiking a plasma pool. To this end, a separate set of stock solutions of 1 mM for each analyte was made in water. From theses stocks a combined solution was made containing 10 μM MMA, ADMA, SDMA and L-homoarginine, which was used with the L-arginine stock standard of 1 mM to prepare three QC-levels of low middle and high concentration containing 0.55, 0.95 or 2.5 μM ADMA, SDMA, MMA, and L-homoarginine, and 55, 95 or 250 μM L-arginine. The QC solutions were stored in aliquots of 1 mL at −20 °C. Plasma was spiked by drying 200 μL aliquots of the QC solutions under N_2 at 60 °C and reconstituting in 200 μL of a plasma pool (see Section 2.3.1).

2.3. Samples

2.3.1. Plasma samples

For validation of the method a pool of heparin plasma from approximately 100 subjects was prepared. Additionally, plasma was obtained from 27 apparently healthy volunteers after they gave their informed consent, for the comparison between the LC–MS/MS method and our HPLC-fluorescence method [25,26]. To test if the method is sensitive enough for the determination of newly formed D_3-ADMA, D_3-SDMA, D_3-MMA, D_6-ADMA, and D_6-SDMA in plasma during infusion of D_3-methyl-1-^{13}C-methionine, samples of a tracer study were analyzed. The rationale and main results of this study have been previously reported [27,28]. In short, D_3-methyl-1-^{13}C-methionine was given intravenously to both patients with end-stage renal disease and to healthy controls for the determination of methyl-fluxes through the three major pathways of the one-carbon metabolism (transmethylation, remethylation, and transsulfuration). For the current investigation we used samples from the healthy controls that had been stored at −20 °C.