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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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A B S T R A C T

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.

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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.

Table 1
Analyte-specific mass spectrometric parameters.

Analyte	Mass transition		Focusing potential (V)	Collision energy (V)	Internal standard
	Q1 (m/z)	Q3 (m/z)			
ADMA	259	214	250	24	D_7-ADMA
	259	70	260	46	D_7-ADMA
SDMA	259	228	280	22	D_7-ADMA
	259	70	260	46	D_7-ADMA
MMA	245	70	260	46	D_7-ADMA
L-Arginine	231	70	270	34	$^{13}C_6$-L-arginine
L-Homoarginine	245	84	260	46	D_4-L-homoarginine
D_7-ADMA	266	77	260	46	–
$^{13}C_6$-L-arginine	237	74	270	34	–
D_4-L-homoarginine	249	88	260	46	–
D_3-ADMA	262	214	250	24	D_7-ADMA
D_6-ADMA	265	214	250	24	D_7-ADMA
D_3-SDMA	262	228	280	22	D_7-ADMA
	262	231	280	22	D_7-ADMA
D_6-SDMA	265	231	280	22	D_7-ADMA
D_3-MMA	248	70	260	46	D_7-ADMA

2.3.2. Rat tissue samples

Frozen rat tissue samples from aorta, kidney medulla, kidney cortex and liver from an ongoing study were weighed before analysis, and per gram tissue 2 mL 0.6 M perchloric acid was added. The samples were homogenized on ice using an Omni-2000 homogenizer (Omni international, Waterbury, CT, USA), and subsequently centrifuged at 1915 × g and 4 °C. The supernatant was transferred to a clean tube and centrifuged for 5 min at 20,160 × g and 4 °C. The resulting supernatant was used for further analysis of the methylated arginines, using both the HPLC-fluorescence method [25,26] and the current LC–MS/MS method.

2.3.3. Human umbilical vein endothelial cells (HUVEC)

For determination of matrix effects, HUVEC were isolated, cultured, and harvested as described previously [29]. HUVEC-pellets were lysed on ice in 500 μL 100 mM NaH_2PO_4 pH 7.3, using an ultrasonic probe (Bandalin Sonopuls mini 20 with MS 1.5 titanium microtip) for 3 × 10 s at ~0.250 kJ (= 90% of maximum power). Proteins were precipitated by adding 200 μL lysate to 200 μL 1.2 M perchloric acid, and after centrifugation (10 min; 4 °C; 20,160 × g) the supernatant was used.

2.4. Sample pretreatment

2.4.1. Sample cleanup

To 200 μL plasma or perchloric acid supernatant, 50 μL of each internal standard solution (D_7-ADMA, D_4-L-homoarginine, and $^{13}C_6$-L-arginine) and 800 μL 0.25 M Na_2HPO_4 were added. The cationic amino acids were extracted with 1-mL (30 mg) Oasis MCX solid phase extraction (SPE) cartridges (Waters, Milford, MA, USA) as described previously [25,26]. After SPE, the samples were collected in glass vials and the solvent was evaporated at 60 °C under N_2.

For the construction of standard curves, calibration samples were prepared by mixing 200 μL of the calibration standards prepared in water with 50 μL of each internal standard solution, and drying under N_2 at 60 °C. The calibration samples were not subjected to SPE, but directly derivatized, since the ratio between analyte and internal standard did not change upon SPE (data not shown).

Additionally, it was determined whether SPE was necessary or if protein precipitation alone would be sufficient. To this end, one aliquot of the plasma pool was subjected to SPE, whereas from a second aliquot proteins were precipitated by adding 200 μL acetonitrile to 200 μL plasma. The samples were thoroughly mixed and after centrifugation (10 min; 4 °C; 20,160 × g), 200 μL of the supernatant was dried under N_2 at 60 °C.

2.4.2. Sample derivatization

The analytes were measured as their butyl-ester derivatives to increase their retention under reversed-phase conditions and allow chromatographic separation of ADMA and SDMA. The derivatization reaction was based on the method described by Schwedhelm et al. [30] and was performed by adding 100 μL of 1.25 M HCl in 1-butanol to the dried samples. The capped vials were mixed thoroughly and heated to 70 °C for 30 min, after which the caps were removed and the samples were dried under N_2 at room temperature. Before analysis the samples were reconstituted in 150 μL mobile phase (described in Section 2.5).

2.5. Instrumentation and settings

Analysis was performed by injection of a 10 μL sample onto a 3.9 mm × 100 mm XTerra MS C18 column with 3.5 μm particles (Waters, Milford, MA, USA) using a Perkin Elmer Series 200 HPLC system (Perkin Elmer Inc., Shelton, CT, USA), comprising pump, degasser, cooled autosampler (4 °C), and column oven (20 °C). Chromatographic separation of the analytes was achieved with isocratic elution at a flow rate of 0.8 mL/min, using 600 mg/L ammonium formate in water–acetonitrile (95.5:4.5, v/v) containing 0.1 vol% formic acid as mobile phase. Using a switching valve (VICI International AG, Schenkon, Switzerland) the first 2 min of the flow after injection were directed to the waste. Before entering the mass spectrometer the flow was split 1:4. Analytes were detected using electrospray ionization (ESI) in positive mode on an API3000 triple quadrupole mass spectrometer (AB Sciex Technologies, Toronto, Canada), with the nebuliser gas at 9 L/min, the collision activated dissociation gas at 4 L/min, and the curtain gas at 10 L/min. For all gasses nitrogen was used. The ion spray voltage was set at 2 kV and the source temperature at 500 °C. The declustering potential (DP) was 40 V, the entrance potential (EP) 10 V, and the collision cell exit potential (CXP) 12.5 V. For all the analytes the dwell time was 150 ms and Q1 and Q3 were operating at unit mass resolution. Multiple reaction monitoring in positive mode was used for analyte quantification, and the analyte-specific mass transitions and analyte dependent parameters are listed in Table 1. In Fig. 1 the structures of all analytes and internal standards are depicted together with the fragments formed after collision. Data was collected and analyzed using Analyst 1.4.2 software (AB Sciex Technologies, Toronto, Canada).

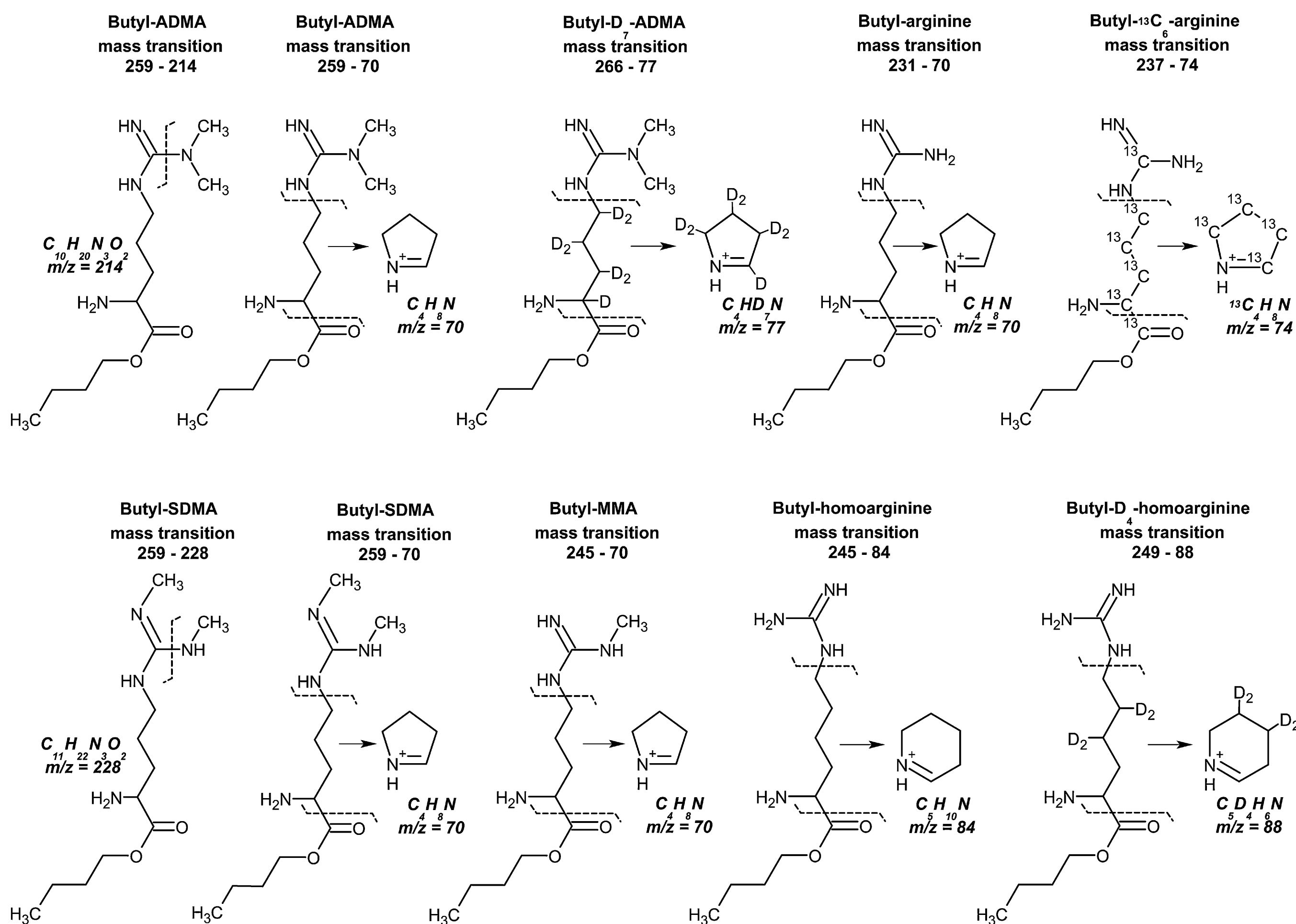

Fig. 1. Structures and proposed fragmentation of the butylated forms of ADMA, SDMA, MMA, L-arginine and L-homoarginine, and the internal standards D_7-ADMA, $^{13}C_6$-L-arginine, and D_4-L-homoarginine. Mass transitions are based on the protonated forms of the analytes and their fragments. ADMA and SDMA produce a unique fragment (m/z = 214 for ADMA, and m/z = 228 for SDMA), but they also share a common fragment (m/z = 70), which has a higher intensity signal. For the measurement of the common fragment chromatographic separation is essential.

2.6. Evaluation of matrix effects

Because no analyte-free matrix is available, the matrix effects were evaluated by post-column infusion of the internal standards (20 μM $^{13}C_6$-L-arginine, 0.2 μM D_7-ADMA, and 0.2 μM D_4-L-homoarginine). During infusion of the internal standards at 1 μL/min, internal standard-free matrices were injected onto the column to reveal potential ion-suppression. For this purpose, we used undiluted, 10-fold diluted, and 100-fold diluted plasma (Section 2.4), a tissue sample from rat liver (Section 2.3.2), and a HUVEC sample (Section 2.3.3), that were subjected to sample cleanup and derivatization before injection.

2.7. Analytical method validation

Validation was done according to the FDA-guidelines for bioanalytical method validation [31]. The tested validation parameters were linearity, intra-assay and inter-assay accuracy and precision, matrix effects, recovery, and re-injection stability. Additionally, as suggested by Araujo [32], accuracy was tested by the comparison with a reference method, for which purpose our previously described HPLC-fluorescence method was used [25,26].

For the determination of the linear range, three series of seven calibration samples were independently measured. Samples contained 10, 20, 40, 80, 120, 200, and 500 μM L-arginine and 0.1, 0.2, 0.4, 0.8, 1.2, 2.0, and 5.0 μM ADMA, SDMA, MMA, and L-homoarginine. Calibration curves were fitted by least-squares linear regression with weighing factor 1/concentration^2.

Intra-assay accuracy and precision were tested using a plasma pool spiked with the QC-solutions (Section 2.2.3). At each concentration level, 15 spiked plasma aliquots, as well as 15 spike-free aliquots, were measured within one analytical run. The accuracy (expressed as % bias) was determined by comparing the mean concentration of the spiked plasma to the expected value (mean plasma concentration + concentration of the spike), whereas the variation of the measured aliquots (CV%) indicated analytical precision. For inter-assay precision aliquots spiked at each concentration level were prepared and measured on 15 separate days.

The recovery, after SPE and derivatization, was determined using a plasma pool spiked with the QC-solutions (Section 2.2.3). At each concentration level, 15 spiked plasma aliquots, as well as 15 spike-free aliquots, were measured within one analytical run. The recovery (%) was calculated from the difference between the mean concentration of spiked plasma and the mean concentration of the spike-free plasma, divided by the concentration of the added spike. We have previously reported data on recovery of analytes after the SPE-procedure, determined using our HPLC-fluorescence method [25]. To test linearity, precision, recovery and accuracy at low concentrations (e.g. for the measurements in cells and tissues), the calibration samples and the spiked and non-spiked plasma

samples were reanalyzed after 10-fold and 100-fold dilution. Finally, re-injection stability was determined by re-injecting the samples from the intra-assay accuracy and precision determination after storage for 2 weeks at 4 °C.

3. Results and discussion

3.1. Chromatography

In Fig. 2A the chromatograms of a calibration sample (0.2 μM L-arginine, and 2 nM ADMA, SDMA, MMA, and L-homoarginine) are shown and in Fig. 2B typical chromatograms of a 10-fold diluted plasma sample. The analytes were measured as their butyl-ester derivatives, which enabled complete chromatographic separation of ADMA and SDMA. This allows the measurement of the most intense common mass transition of ADMA and SDMA (259→70), in addition to the measurement of their unique mass transitions (259→214 and 259→228, respectively) that have a lower intensity. The additional sensitivity may be useful when the concentrations are near the detection limit or when only small quantities of sample are available. To test if the common transition yields equal precision and accuracy, it was validated together with the unique transitions of ADMA and SDMA (Section 3.3).

In biological samples, the channel for L-homoarginine (mass transition = 245→84) showed an additional peak at 3.2 min (Fig. 2B). After injecting solutions of Nε,Nε,Nε-trimethyllysine, Nα-acetyllysine, or Nε-acetyllysine, which may also result in the mass transition 245→84, it was found that Nε,Nε,Nε-trimethyllysine was responsible for the peak at 3.2 min.

3.2. Evaluation of matrix effects

Post-column infusion of the internal standards D_7-ADMA and D_4-homoarginine did not reveal notable interference (<1%) from ion-suppression upon injection of the different matrices (plasma, tissue, or cell lysate, subjected to sample cleanup and derivatization). Only when during infusion of $^{13}C_6$-L-arginine undiluted plasma was injected, ion-suppression was observed at the retention time of L-arginine (48.6%). Upon injection of 10-fold diluted plasma the ion-suppression was reduced (11.1%), and injection of 100-fold diluted plasma virtually eliminated ion-suppression (<1%). Injection of cell or tissue samples did not lead to interference at the retention time of L-arginine. The ion-suppression was most likely caused by competition for ionization between L-arginine and its internal standard in the ESI-source. When L-arginine standards in water (7.2 μM, 26.4 μM and 45.6 μM) were injected, ion-suppression increased in parallel (23.4%, 43.8%, and 51.6%, respectively), in support of this notion. Although the stable isotope-labeled internal standard of L-arginine is affected to the same extent, and should compensate for the reduced ionization at high L-arginine concentrations, for plasma samples it is preferable to use at least a 10-fold dilution.

When sample cleanup of a plasma pool was performed by protein precipitation instead of SPE (see Section 2.4.1), no matrix effects were observed within the runtime of 10 min. However, after 10 min, interferences were observed in the subsequent injections. These interferences should not affect analytes that have their own stable isotope-labeled internal standard. Quantification of MMA and SDMA, however, is done using D_7-ADMA and might suffer from these interferences. To overcome this, interferences from the late-eluting compounds could be removed with a steep gradient after elution of the last analyte of interest, i.e. SDMA. However, this would require re-equilibration of the column before each new injection, thereby increasing analysis time. Therefore, SPE is preferred over protein precipitation for sample cleanup.

3.3. Method validation

3.3.1. Linearity

The linearity of the method was tested over a calibration range of 10–500 μM for L-arginine and 0.1–5.0 μM for ADMA, SDMA, MMA, and L-homoarginine. For each analyte three independently measured calibration lines were used to determine the linearity. To obtain the calibration lines the peak-area of the analyte divided by the peak-area of the internal standard was plotted on the *y*-axis and the concentration of the standard divided by the concentration of the internal standard on the *x*-axis. Linearity was expressed as mean slope ± SD, mean intercept ± SD, and r^2 (Table 2). ADMA and SDMA have different regression equations for the unique mass transition compared to their common mass transition. This difference is in agreement with the higher intensity of the peaks measured for the common transition. For the 10-fold and 100-fold diluted calibration range, slopes and intercepts were not significantly different from the values reported in Table 2, and for all analytes the calibration curves were also linear ($r^2 > 0.9984$ for 10-fold dilution, and $r^2 > 0.9983$ for 100-fold dilution).

3.3.2. Sensitivity and carry-over

Sensitivity of the method was determined by establishing the lower limit of detection (LOD), defined as signal-to-noise ratio (S/N) ≥ 3, and the lower limit of quantification (LOQ), which was defined as S/N ≥ 10 with an accuracy < 10%. Ideally, LOQ should be determined by analyzing the substances in their matrices. However, since no analyte-free matrix is available, both LOD and LOQ were determined using matrix-free calibration samples prepared in water. For all analytes the LOD was established at 0.2 nM. For ADMA (mass transition 259→70) and SDMA (mass transition 259→70) a LOQ of 0.4 nM was obtained, and for all other analytes, including ADMA (mass transition 259→214) and SDMA (mass transition 259→228), the LOQ was 0.8 nM. For L-arginine the lowest measured concentration was 20 nM, which had a S/N of 559. From this data the LOD was estimated at approximately 0.1 nM and the LOQ at approximately 0.4 nM, which is in the same range as for ADMA and SDMA. Since such low L-arginine concentrations are not anticipated in biological samples, the LOD and LOQ of L-arginine were not experimentally determined.

Carry-over was tested in the two blank injections measured after an injection of the highest standard. For none of the analytes peaks were found at their specific retention times in the two blank injections, indicating that there is no carry-over from previous injections.

3.3.3. Accuracy and precision

Intra-assay accuracy (% bias) and precision (expressed as CV%), and inter-assay precision were determined by using the standard addition method, for which 15 aliquots of the plasma pool were spiked at three concentration levels. In Table 3 data is shown for intra-assay and inter-assay accuracy and precision of undiluted plasma. Intra-assay accuracy at the three concentration levels was < 3% for all analytes except MMA, which was < 7%. Intra-assay precision was ≤3.5% for all analytes, whereas inter-assay precision was <4% for L-homoarginine and ≤9.6% for all other analytes. Both for the 10-fold and 100-fold diluted range in plasma similar results were obtained (data not shown). Additionally, accuracy was tested by the comparison of measurements done on 27 plasma samples using LC–MS/MS with measurements done using our previously validated HPLC-fluorescence method [25,26], which has an inter-assay CV < 4% for all analytes. Mean concentrations of ADMA, SDMA and L-arginine measured with HPLC-fluorescence were in line with previously reported values in healthy subjects measured with this method [25]. Mean concentrations measured with LC–MS/MS were 0.469 ± 0.063 μM for ADMA (259→214),

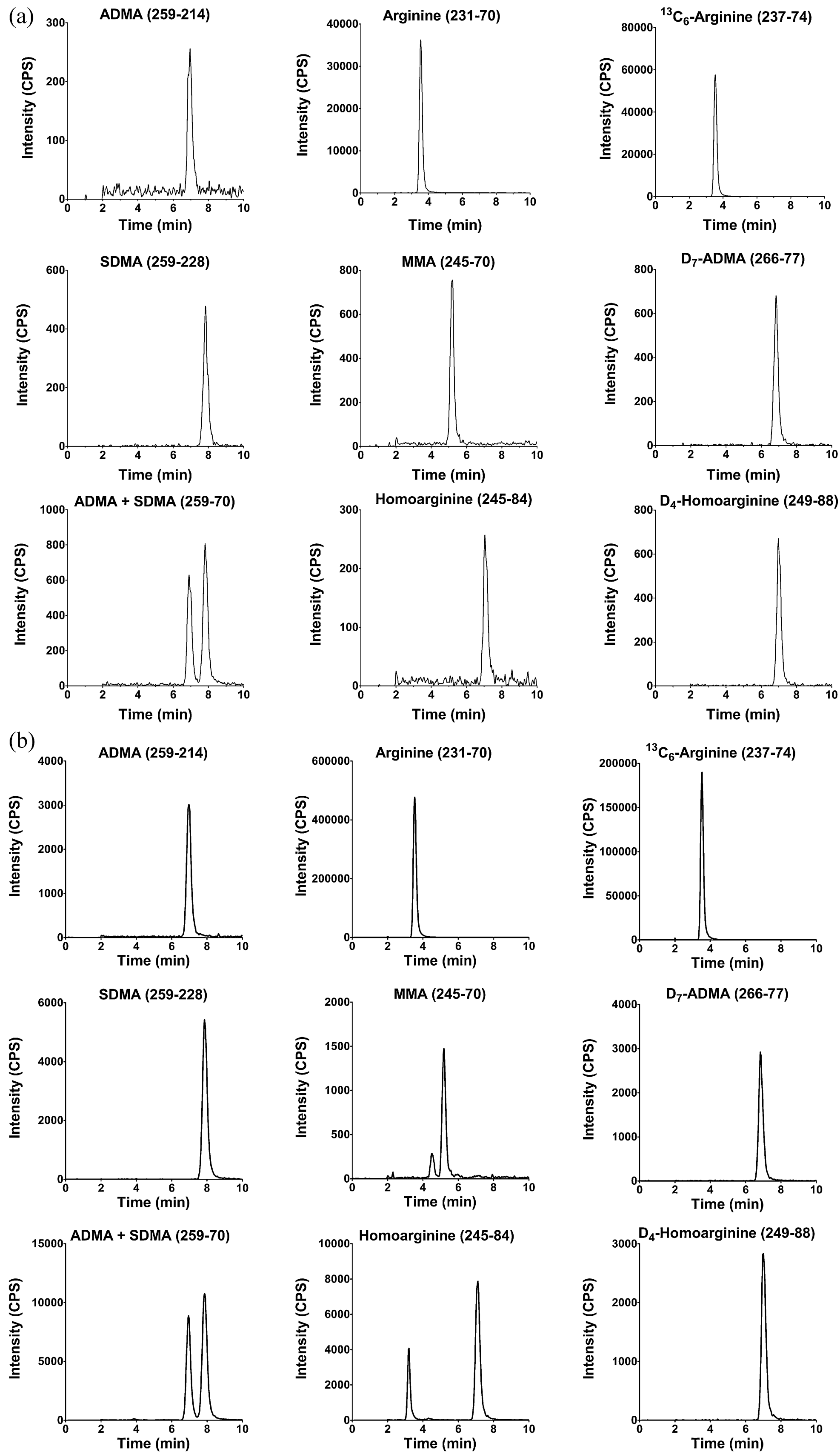

Fig. 2. Typical chromatograms of a standard solution and a plasma sample. The calibration sample (A), taken from the 100-fold diluted calibration range, contained 0.2 μM L-arginine and 2 nM ADMA, SDMA, MMA, and L-homoarginine. The plasma sample (B) is diluted 10-fold.

Table 2
Regression curves for linearity in plasma.

Analyte	Range (μM)	Slope Mean (SD)	Intercept Mean (SD)	Correlation r^2
ADMA (259–214)	0.1–5.0	1.88 (0.082)	−0.0082 (0.010)	0.9989
ADMA (259–70)	0.1–5.0	4.87 (0.215)	−0.0197 (0.020)	0.9991
SDMA (259–228)	0.1–5.0	3.39 (0.111)	−0.0064 (0.011)	0.9991
SDMA (259–70)	0.1–5.0	6.56 (0.275)	−0.0093 (0.019)	0.9991
MMA	0.1–5.0	4.63 (0.318)	0.0120 (0.024)	0.9981
L-Arginine	10–500	0.157 (0.004)	−0.2203 (0.478)	0.9979
L-Homoarginine	0.1–5.0	1.98 (0.090)	−0.0077 (0.012)	0.9985

Data is based on 3 separately measured calibration series.
0.2 μM D_7-ADMA was used for the quantification of ADMA, SDMA and MMA, 0.2 μM D_4-L-homoarginine for the quantification of L-homoarginine, and 20 μM $^{13}C_6$-L-arginine for the quantification of L-arginine.

0.484 ± 0.066 μM for ADMA (259→70), 0.509 ± 0.083 μM for SDMA (259→228), 0.512 ± 0.081 μM for SDMA (259→70), 97.8 ± 18.6 μM for L-arginine, and 2.01 ± 0.647 μM for L-homoarginine. For L-homoarginine and SDMA, these values differed by <2.5% from those measured with HPLC-fluorescence. Although values for ADMA and L-arginine, obtained with LC–MS/MS were slightly higher compared to HPLC-fluorescence (~8% for L-arginine and ADMA 259→214, and ~11% for ADMA 259→70), results obtained with both methods correlated well for all analytes (Fig. 3). Because MMA functions as the internal standard for the HPLC-fluorescence method, measurements could not be compared for this analyte.

3.3.4. Recovery and stability

The recovery of the analytes was determined using a standard addition procedure (Section 2.7). For ADMA and SDMA, recovery ranged from 99.1% to 103.2%, and for the other analytes recovery ranged from 92.9% to 102.3% (Table 3).

Re-injection of the derivatized samples after two weeks of storage at 4 °C showed a deviation of <5% from the first measurement, and did not influence accuracy or precision. Therefore, samples that are stored at 4 °C can be re-injected safely within two weeks after sample preparation.

3.4. Method implementation

3.4.1. Rat tissue samples

L-Arginine, ADMA and SDMA in rat tissue samples were previously determined using our HPLC-fluorescence method. However, in some of these samples ADMA and SDMA peaks suffered from interferences in the matrix around the same retention time (Fig. 4 left panel), which made accurate quantification difficult. Therefore, the tissue samples were remeasured using the newly validated LC–MS/MS method (Fig. 4, right panel). Because of the better selectivity and higher sensitivity the new HPLC–MS/MS method allowed more reliable quantification of ADMA and SDMA in rat tissue homogenates.

3.4.2. Plasma samples from a tracer study

Previously we performed a tracer study [27,28], in which D_3-methyl-1-^{13}C-methionine was given intravenously to patients with

Table 3
Accuracy, intra-assay precision, inter-assay precision, and recovery in plasma.

Analyte	Intra-assay[a]					Inter-assay[a]	
	Spike (μM)	Measured (μM)	Accuracy (%)	Precision (%)	Recovery (%)	Measured (μM)	Precision (%)
ADMA (259–214)	0	0.568	NA	2.0	NA	0.566	5.6
	0.492	1.06	−0.1	1.8	99.8	1.08	6.9
	0.849	1.44	1.8	2.5	103.0	1.43	7.8
	2.24	2.79	−0.6	1.7	99.3	2.80	7.5
ADMA (259–70)	0	0.576	NA	1.4	NA	0.569	6.4
	0.492	1.08	0.8	1.8	101.8	1.06	8.0
	0.849	1.44	1.3	2.1	102.3	1.42	9.1
	2.24	2.79	−0.7	1.6	99.1	2.79	8.5
SDMA (259–228)	0	0.657	NA	1.7	NA	0.721	7.9
	0.537	1.19	−0.4	3.0	99.1	1.27	9.4
	0.927	1.61	1.9	1.7	103.2	1.66	9.5
	2.44	3.09	−0.1	2.0	99.9	3.09	6.7
SDMA (259–70)	0	0.662	NA	1.6	NA	0.725	6.9
	0.537	1.20	0.4	2.8	100.9	1.26	9.6
	0.927	1.61	1.5	2.2	102.5	1.65	9.0
	2.44	3.09	−0.2	1.9	99.7	3.10	5.6
MMA	0	0.089	NA	3.5	NA	0.088	8.0
	0.551	0.605	−5.4	2.3	93.7	0.585	9.3
	0.952	0.997	−4.2	2.3	95.5	0.948	9.4
	2.51	2.42	−6.9	1.8	92.9	2.30	8.7
L-Arginine	0	94.4	NA	1.7	NA	120	7.6
	52.8	145	−1.2	1.2	96.6	169	5.7
	91.2	188	1.1	1.7	102.3	211	6.0
	241	326	−2.7	1.1	96.2	351	3.8
L-Homoarginine	0	1.37	NA	1.4	NA	1.35	2.6
	0.557	1.90	−1.4	1.5	95.0	1.88	3.7
	0.962	2.34	0.3	2.6	100.7	2.28	3.7
	2.53	3.84	−1.8	2.0	97.2	3.76	2.4

NA: Not applicable.
[a] Based on 15 samples per concentration level.

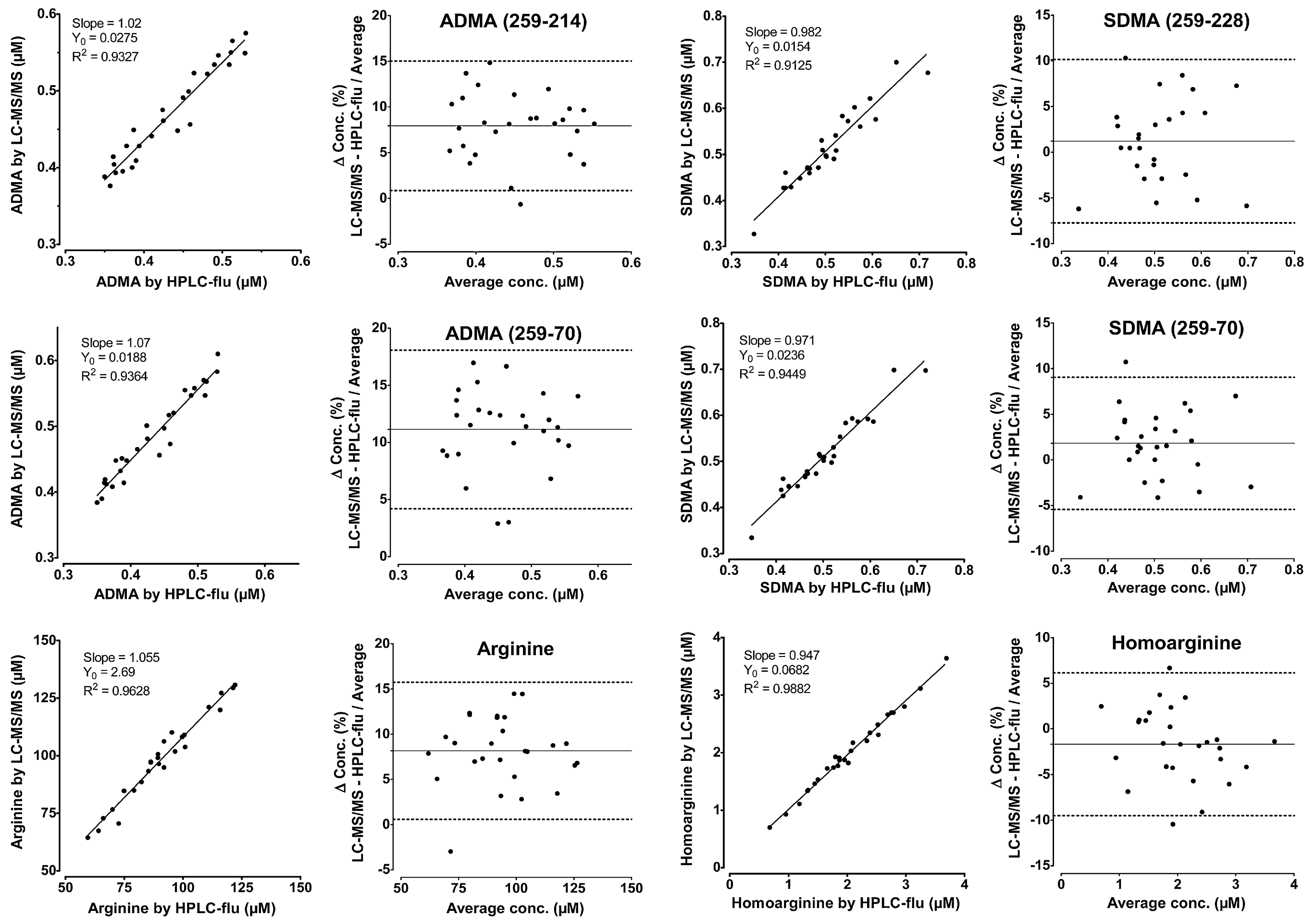

Fig. 3. Comparison of plasma samples measured with HPLC-fluorescence and LC–MS/MS. Scatter plots (left) and Bland-Altman plots (right) for the comparison of concentrations (conc.) of ADMA, SDMA, L-arginine and L-homoarginine measured with HPLC-fluorescence (HPLC-flu) and LC–MS/MS in plasma obtained from 27 healthy volunteers. The methods could not be compared for MMA, since it functions as the internal standard in the HPLC-fluorescence method.

end-stage renal disease and to healthy controls. By measuring the levels of D_3-methyl-labeled S-adenosyl-methionine, S-adenosyl-homocysteine and homocysteine, the methyl-fluxes through the three major pathways of the one-carbon metabolism (transmethylation, remethylation, and transsulfuration) were evaluated. One of the possible fates of the D_3-methyl-group is its PRMT-catalyzed transfer to arginine residues in proteins, resulting in labeled MMA, ADMA, and SDMA, the latter two containing either one or two labeled methyl groups. However, the analysis of these compounds was beyond the scope of the original study. Because plasma samples from this tracer study were still available, we had the opportunity to test the sensitivity of our new method for the determination of D_3-MMA, D_3-ADMA, D_3-SDMA, D_6-ADMA, and D_6-SDMA.

Within 60 min after starting infusion of D_3-methyl-1-^{13}C-methionine, D_3-ADMA, D_3-SDMA, and D_3-MMA in plasma reached levels above the detection limit (Fig. 5). This means that within this time frame, the supplemented D_3-methyl-1-^{13}C-methionine is transported into the cell and converted to S-adenosyl-methionine, from which the methyl group is transferred to an arginine residue of a protein that is subsequently degraded, releasing ADMA, SDMA or MMA into the cytosol, from where they are transported to the plasma via cationic amino acid transporters. It is remarkable how fast these subsequent processes lead to appearance of D_3-methyl-labeled analytes in plasma. However, the possibility that, next to PRMT-catalyzed methylation of protein-incorporated arginine residues, also direct methylation of free L-arginine by other N-methyltransferases occurs, cannot be fully excluded.

The tracer study was originally not undertaken for the determination of methylated arginines, but for the determination of methyl fluxes through the one-carbon metabolism. Therefore, the supplemented amount of D_3-methyl-1-^{13}C-methionine might not have been optimal for the formation of labeled ADMA, SDMA and MMA. Within the 300 min follow-up a steady state was not reached, which is probably the reason that D_6-ADMA and D_6-SDMA in plasma remained undetectable.

3.5. Potential method limitations

SDMA was quantified using D_7-ADMA as internal standard. SDMA has a different retention time than D_7-ADMA and different analyte-specific mass spectrometric parameters, which could potentially lead to differences in ion-suppression. However, no matrix effects were observed during post-column infusion of D_7-ADMA. Furthermore, intra-assay and inter-assay precision for SDMA was similar to that of ADMA, which has its own stable isotope-labeled internal standard. The assay could be further improved by incorporating a commercially available or newly synthesized stable isotope-labeled SDMA as internal standard [33].

Plasma concentrations of ADMA and SDMA exhibit low intra-individual biological variation [34,35], which leads to optimal imprecision goals of $\leq$3% for ADMA and $\leq$ 5% for SDMA [36,37]. In contrast to our current HPLC-fluorescence method, the inter-assay precision of the LC–MS/MS method does not meet this goal.

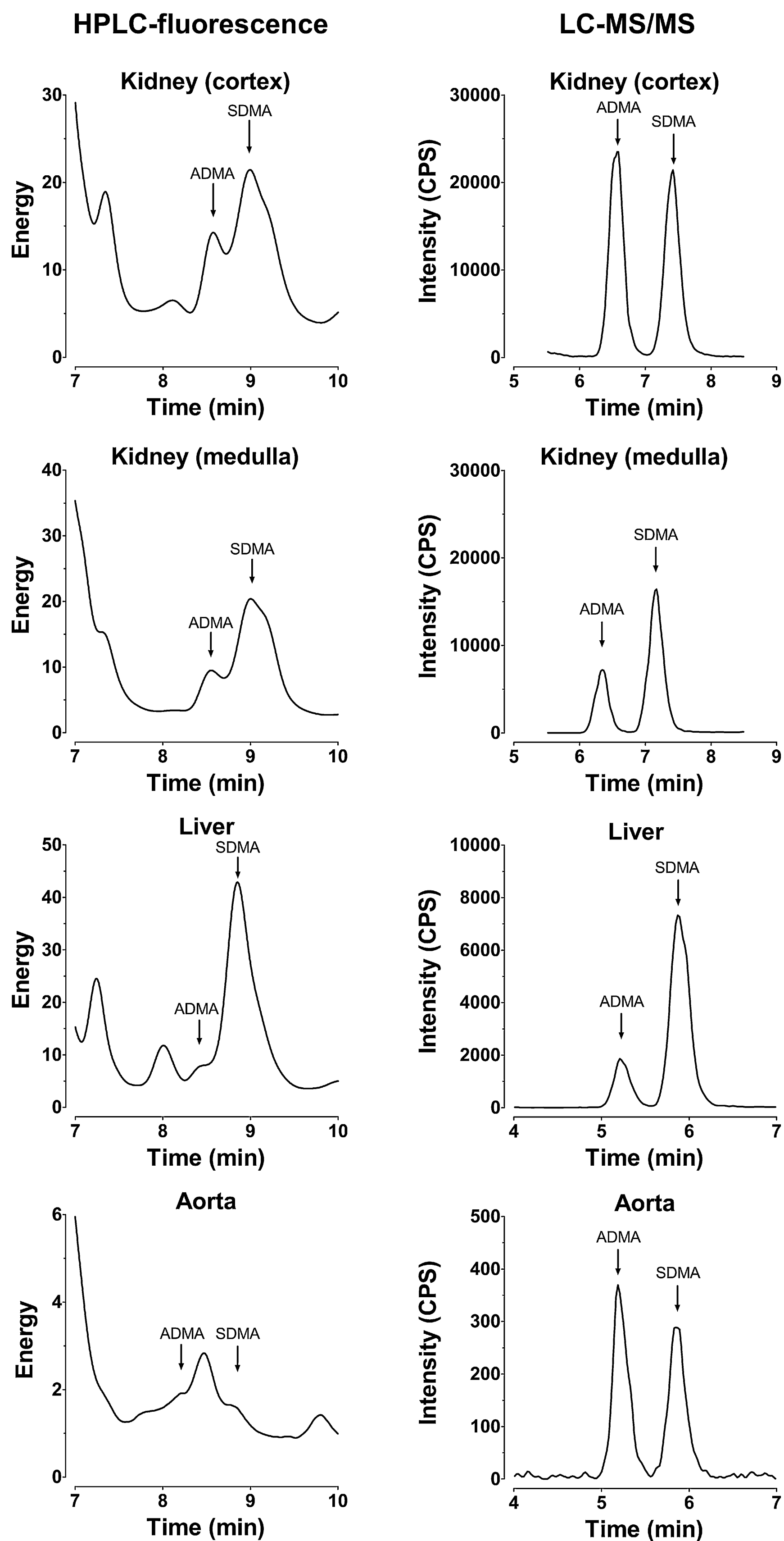

Fig. 4. ADMA and SDMA in rat tissue measured with HPLC-fluorescence and LC–MS/MS. Chromatograms for ADMA and SDMA of rat tissue samples measured using the method with fluorescence detection are depicted on the left and using the newly developed method with mass spectrometry detection (transition 259→70 for ADMA and SDMA) on the right.

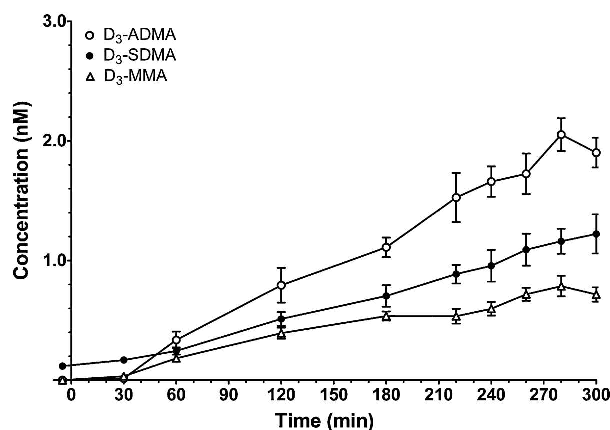

Fig. 5. Appearance of D_3-ADMA, D_3-SDMA, and D_3-MMA in plasma during D_3-methyl-1-^{13}C-methionine infusion in healthy subjects. Within 60 min after start of the infusion, D_3-ADMA, D_3-SDMA, and D_3-MMA in plasma reached detectable levels. D_6-ADMA and D_6-SDMA did not reach detectable levels during the 5-h infusion period.

Therefore, for the measurement of ADMA, SDMA, L-arginine and L-homoarginine in plasma, the HPLC-fluorescence method [25,26] is preferable (CV <3% for ADMA and <4% for SDMA), whereas the LC–MS/MS method is more suitable for quantification of these analytes in small tissue samples and cultured cells, for the quantification of MMA, and in tracer studies.

4. Conclusions

An LC–MS/MS method was developed for the simultaneous determination of ADMA, SDMA, MMA, L-arginine, and L-homoarginine in plasma, cells and tissues. The method was validated by determining linearity, recovery, lower limits of detection and quantification, accuracy, intra-assay precision, inter-assay precision, and re-injection stability. Previously, MS/MS-methods for the determination of L-arginine and its methylated forms [22,38], and for L-homoarginine [23] in plasma and urine have been reported. Only one other group reported on a combined method for the determination of ADMA, SDMA, MMA, L-arginine, L-homoarginine, and L-citrulline in plasma and urine [24]. But like most methods, because concentrations in plasma and urine are well above the detection limits, the method does not make use of chromatographic separation [21,39]. Butylation of the analytes allows chromatographic separation of ADMA and SDMA, which enables the use of their most intense common mass transition. Consequently, the separation of ADMA and SDMA benefits the selectivity and sensitivity for measurements in cells and tissues.

Implementation of the method for analysis of rat tissue samples yielded satisfactory results, demonstrating the suitability of this method for measurements in very small tissue samples. Furthermore, the method is sensitive enough to detect appearance of D_3-ADMA, D_3-SDMA, and D_3-MMA in plasma within 1 h after starting infusion of labeled methionine, demonstrating its potential for studying intracellular metabolism of methylated arginines with stable isotope tracer methodology.

Disclosures

None.

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