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High-throughput liquid chromatographic-tandem mass spectrometric determination of arginine and dimethylated arginine derivatives in human and mouse plasma☆

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Abstract

The balance between nitric oxide (NO) and vasoconstrictors like endothelin is essential for vascular tone and endothelial function. L-Arginine is converted to NO and L-citrulline by NO synthase (NOS). Asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) are endogenous inhibitors of NO formation. ADMA is degraded by dimethylamino dimethylhydrolases (DDAHs), while SDMA is exclusively eliminated by the kidney. In the present article we report a LC-tandem MS method for the simultaneous determination of arginine, ADMA, and SDMA in plasma. This method is designed for high sample throughput of only 20-μl aliquots of human or mouse plasma. The analysis time is reduced to 1.6 min by LC-tandem MS electrospray ionisation (ESI) in the positive mode. The mean plasma levels of L-arginine, ADMA, and SDMA were 74 ± 19 (SD), 0.46 ± 0.09, and 0.37 ± 0.07 μM in healthy humans ($n = 85$), respectively, and 44 ± 14, 0.72 ± 0.23, and 0.19 ± 0.06 μM in C57BL/6 mice. Also, the molar ratios of arginine to ADMA were different in man and mice, i.e. 166 ± 50 and 85 ± 22, respectively.

Keywords: Arginine; Asymmetric dimethylarginine (ADMA); Nitric oxide; LC-tandem MS; Stable isotopes; Symmetric dimethylarginine (SDMA)

1. Introduction

Methylation of arginine residues is a key regulatory process in protein function [1,2]. Protein methyl transferases (PRMTs) transfer methyl groups to each arginine residue to be methylated [1–3]. Two groups of PRMTs are known, PRMT I methylates arginine residues at the same nitrogen of the guanidine group to yield asymmetric dimethylarginine (ADMA). PRMT II methylates arginine residues at two different nitrogens of the guanidine group to yield symmetric dimethylarginine (SDMA) [3]. Subsequently, regular hydrolysis of proteins involves release of the dimethylated arginines ADMA and SDMA. Once released, ADMA inhibits all three isoforms of nitric oxide synthase (NOS) [4,5]. SDMA has been reported to be a weak inhibitor of isolated neuronal NOS [6]. To control for ADMA levels, two dimethylarginine dimethylhydrolases (DDAHs), i.e. DDAH I and II, hydrolyse ADMA to citrulline and dimethylamine [5,7–9]. SDMA is not known to be metabolized by enzyme activity, but seems to be exclusively eliminated by the kidney [10]. Interestingly, even though enzymatically degraded, ADMA plasma levels in humans are of the same order of magnitude as of SDMA [11–13]. However, in other species, the molar ratio of ADMA to SDMA may be different.

Analysis of ADMA and SDMA has been challenging since about 15 years. Analytical methods for the measurement of methylated arginine derivatives have been reviewed elsewhere [14–17]. Mass spectrometric (MS) assays for the quantitative determination of ADMA involve gas chromatography (GC) and liquid chromatography (LC) [13,18–22]. We have previously reported a simple and rapid LC-tandem MS-based method for the quantitative determination of ADMA, SDMA, and arginine in human plasma [13]. This method has the major advantage over

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other LC-MS-based methods in that it distinguishes dimethylated arginine derivatives by specific daughter ions. We have further improved this method to allow for batch-wise sample preparation, introduced internal standard pre-coated 96-well filtration plates, and substantially reduced sample analysis time operating chromatography on a microbore guard column packed with teicoplanin covalently bonded to 5-μm spherical silica. The glycopeptide antibiotic teicoplanin is a large hydrophilic molecule that is able to form several interactions with other compounds. All these refinements, streamlined the analytical determination of ADMA, SDMA, and arginine, and made the method suitable for expeditious clinical and basic science application.

2. Experimental

2.1. Chemical and materials

L-Arginine hydrochloride reference standard was purchased from US Pharmacy (Rockvill, MD, USA). ADMA dihydrochloride (purity > 99%) was from Sigma–Aldrich (Steinheim, Germany) and SDMA dihydrochloride (purity > 99%) from Calbiochem (Schwalbach, Germany). Aqueous stock solutions of L-arginine, ADMA, and SDMA were made by weighing authentic material supplied by the manufacturer. L-[$^2H_7$]-Arginine hydrochloride (L-[2,3,3,4,4,5,5-$^2H_7$]-arginine, 98 at% ^2H) and L-[*guanidino*-$^{15}N_2$]-arginine hydrochloride (99 atom% ^{15}N) were purchased from Euriso-top (Saint-Aubin, France). [$^2H_6$]-ADMA ([3,3,4,4,5,5-$^2H_6$]-ADMA, 98 at% ^2H) and [$^2H_6$]-[$^{13}C_5$]-ADMA ([1,1,1,1′,1′,1′-$^2H_6$]-dimethylamino-[1,2,3,4,5-$^{13}C_5$]-arginine, 98 at% ^2H and 99 at% ^{13}C) were synthesised as described elsewhere [12,16]. 96-Well 0.20-μm Multiscreen HTS microfiltration plates were purchased from Millipore (Molsheim, France), u-shaped 96-well polypropylene plates were from Greiner bio-one (Frickenhausen, Germany), skirted multiply-PCR 96-well polypropylene plates were obtained from Sarstedt (Nümbrecht, Germany), desiccant deshydratant was from Oker Chemie (Goslar, Germany), and aluminium seal were from Sarstedt. All other chemicals were purchased from Sigma–Aldrich.

2.2. Sample preparation

For protein precipitation, 96-well 0.20-μm microfiltration plates were used. Each well of the mircofiltation plate was pre-coated with 800 pmol of L-[$^2H_7$]-arginine and 40 pmol of [$^2H_6$]-ADMA solved in 20 μl of acetone–water (50:50, v/v), corresponding to a concentration of 40 and 2 μM, respectively. [$^2H_6$]-ADMA was used as internal standard for ADMA and SDMA. Pre-coated 96-well filtration plates were dried under air and stored with desiccant deshydratant at room temperature until use. Before sample preparation the 96-well microfiltration plate had to be placed on top of u-shaped 96-well polypropylene plate or skirted multiply-PCR 96-well polypropylene plate. 96-Well microfiltration plates were pre-loaded with 100 μl of methanol in each well. Subsequently, to each well a 20-μl sample aliquot was added. Proteins were precipitated quantitatively by shaking the microfiltration plates on top of the u-shaped or multiply-PCR polypropylene plates for 5 min using an orbital shaker (Heidolph, Schwabach, Germany). To separate analytes from precipitated proteins, the microfiltration plates on top of the polypropylene plates were centrifuged for 5 min at 4000 rpm (Eppendorf, Hamburg, Germany). After centrifugation, the two plates were separated and the eluates were dried by heating at 85 °C for 10 min. Compounds were derivatized, reconstituted, and analysed as described below. The calibrators were treated exactly the same as plasma samples.

2.3. Derivatization

All compounds were analysed as their butyl ester derivatives. Derivatization was performed in 96-well polypropylene plates, u-shaped or multiply-PCR type. In brief, after addition of 100 μl of 1 M HCl in 1-butanol, plates were sealed with aluminium foil (Sarstedt) and u-shaped 96-well polypropylene plates were heated at 65 °C on a 96-well aluminium block (Novodirect, Kehl, Germany) for 15 min. Alternatively, skirted multiply-PCR 96-well polypropylene plates were heated on a 96-well PCR machine (Eppendorf) also at 65 °C for 15 min. For evaporation, aluminium seal was removed and open plates were heated at 85 °C for 30 min. After evaporation of derivatization reagent, samples were reconstituted in 100 μl of acetonitrile–water (60:40, v/v) containing 0.1 wt% ammonium formate. The pH was adjusted to 4 with formic acid. Afterwards, polypropylene plates were transferred to a Prostar Autosampler (Varian, Palo Alto, CA, USA), and 10-μl aliquots were injected onto the chromatographic column from each sample.

2.4. Liquid chromatography-tandem mass spectrometry

Analyses were performed on a Varian 1200L Triple Quadrupole MS equipped with two Varian ProStar model 210 HPLC pumps. Separation of analytes from major matrix components was achieved with a Chirobiotic T, 20 mm × 1.0 mm i.d., microbore guard column packed with teicoplanin covalently bonded to 5-μm spherical silica (Astec, Whippany, NJ, USA). Acetonitrile–water (60:40, v/v, pH 4) containing 0.1 wt% ammonium formate, served as isocratic eluent at 28 °C, with a flow rate of 0.2 ml/min. Nitrogen was used as the nebulizing

Table 1
m/z values of parent ([M + H]$^+$) and product ions, and collision energy (CE) values used in quantitative analyses of L-arginine, ADMA, and SDMA in human and mouse plasma as butyl ester derivatives

Analyte	Parent ion [M + H]$^+$	CE (eV)	Product ion
L-arginine	231	−22	70
L-[$^2H_7$]-arginine	238	−22	77
L-[$^{15}N_2$]-arginine	233	−22	70
ADMA	259	−16	214
[$^2H_6$]-ADMA	265	−16	220
[$^2H_6$]-[$^{13}C_5$]-ADMA	270	−16	219
SDMA	259	−14	228

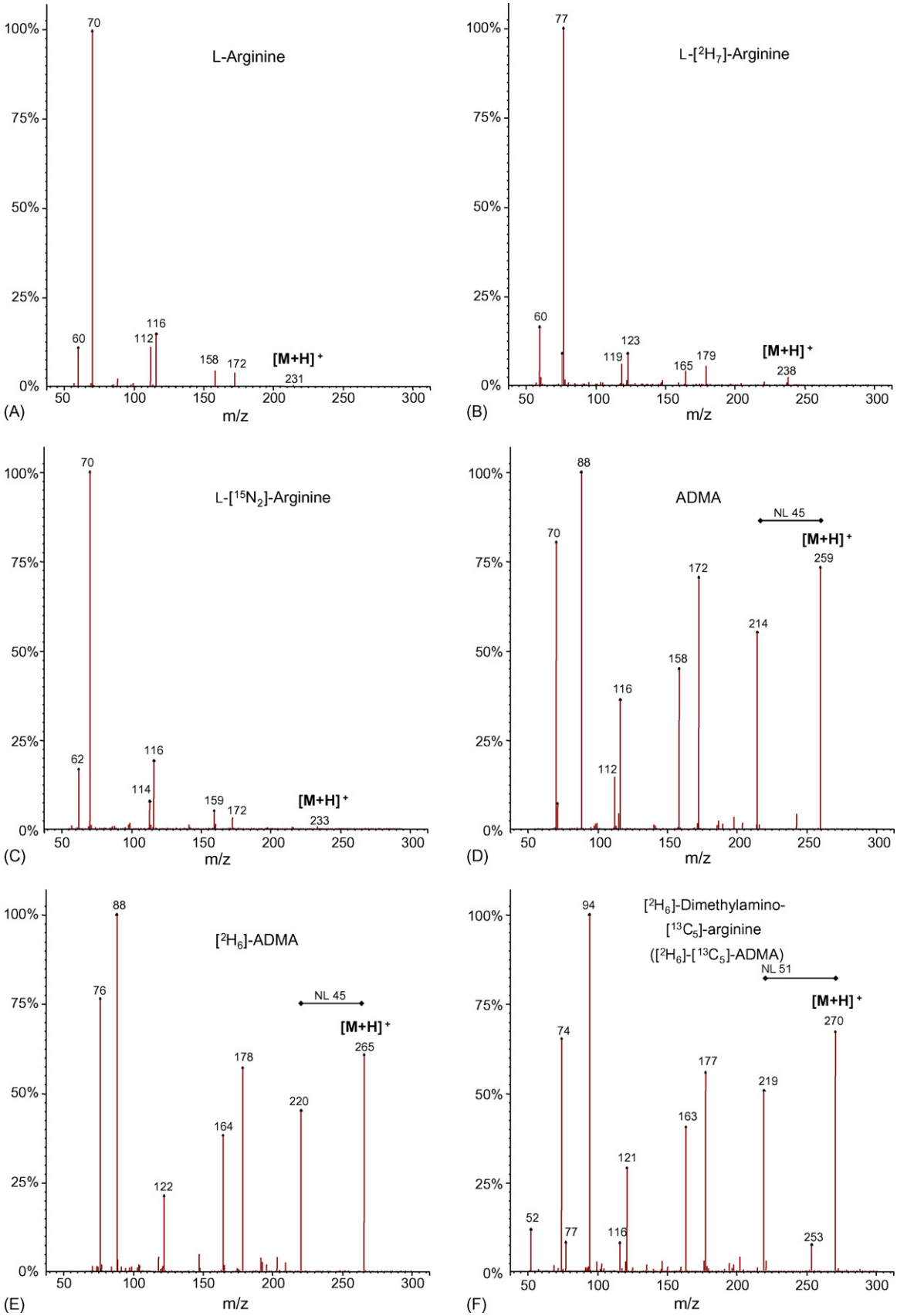

Fig. 1. ESI positive tandem mass spectra of the butyl ester derivatives of L-arginine (A), L-[$^{2}H_{7}$]-arginine (B), L-[$^{15}N_{2}$]-arginine (C), ADMA (D), [3,3,4,4,5,5-$^{2}H_{6}$]-ADMA (E), [1,1,1,1′,1′,1′-$^{2}H_{6}$]-[dimethylamino-1,2,3,4,5-$^{13}C_{5}$]-arginine ([$^{2}H_{6}$]-[$^{13}C_{5}$]-ADMA (F), and SDMA (G). NL: neutral loss. The corresponding parent ions subjected to collision-induced dissociation are indicated by $[M+H]^{+}$.

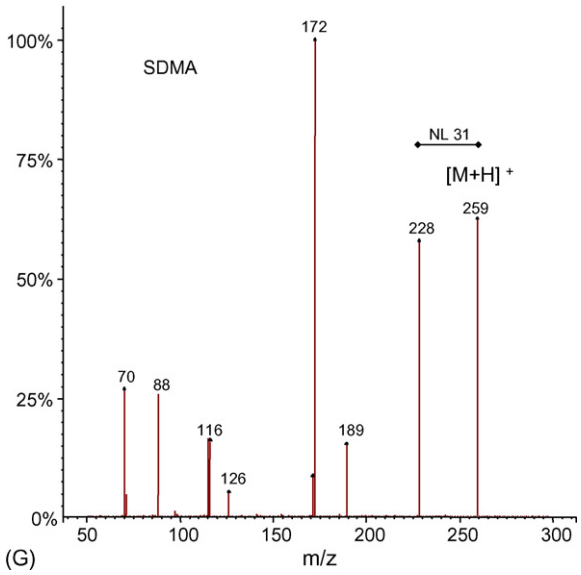

Fig. 1. (*Contuned*).

and drying gas (380 °C) at 90 and 180 l/h, respectively. For positive electrospray ionisation (ESI+) the needle and shield voltage were set at 5600 and 400 V, respectively. The following transitions were used for quantitative analyses in the multiple reaction monitoring (MRM) mode after fragmentation with argon (2 Pa): *m/z* 231 to 70 for L-arginine; *m/z* 238 to 77 [CE −22 eV] for L-[$^2H_7$]-arginine; *m/z* 259 to 214 for ADMA; *m/z* 259 to for SDMA; and *m/z* 265 to 220 for [$^2H_6$]-ADMA (Table 1). Dwell time was set to 190 ms for each ion, inter-scan time was 20 ms in the MRM mode. Thus, a total scan cycle time of 0.97 s resulted.

3. Results

3.1. LC–MS and LC-tandem MS of L*-arginine and dimethylated* L*-arginine butyl ester derivatives*

Butylation of amino acids is widely applied in LC–MS analysis of biological samples [24–26]. The parent ions formed from ESI+ of the butyl esters of L-arginine, ADMA, SDMA, and their stable-isotope labelled analogs represent the molecular protonated cations, i.e. $[M+H]^+$ (Table 1). Fragmentation of butylated basic amino acids like citrulline and ornithine is

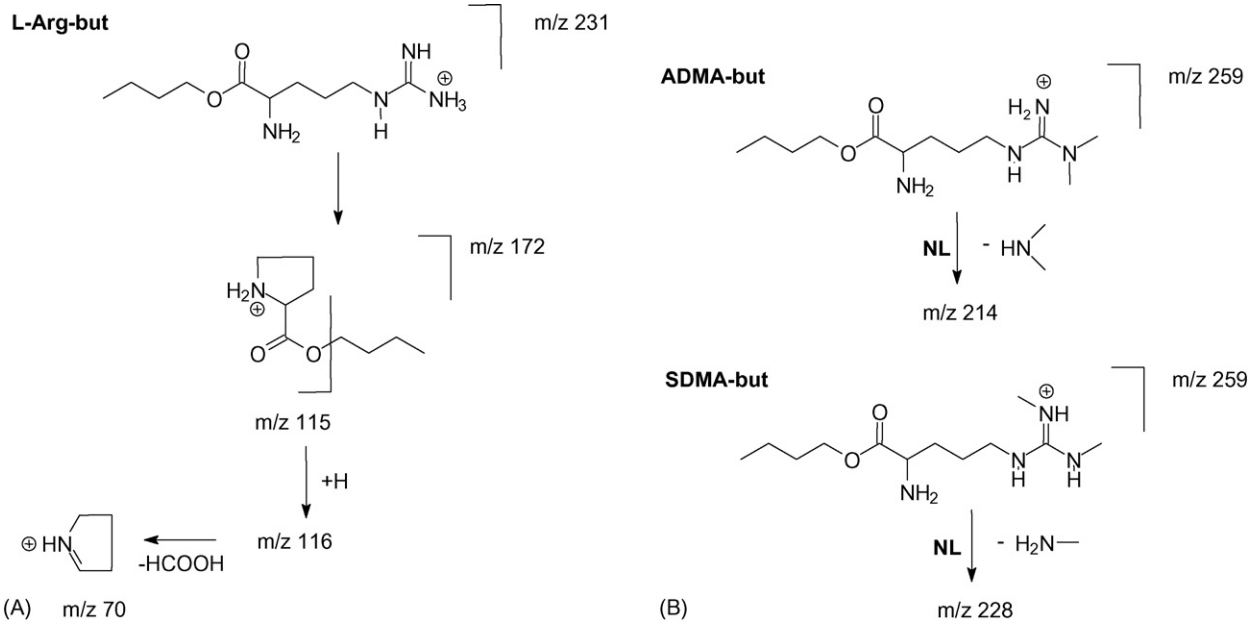

Fig. 2. Proposed mechanisms for the formation of the characteristic product ions of *m/z* 70 for L-arginine (A), of *m/z* 214 for ADMA (B, upper panel), and of *m/z* 228 for SDMA (B, lower panel). The respective parent ions of the butyl ester derivatives of the analytes produced by ESI + were subjected to collision-induced dissociation. L-Arg-but, ADMA-but, and SDMA-but means butyl ester derivatives of L-arginine, ADMA and SDMA, respectively.

characterized by neutral loss (NL) of 119 Da [25]. In contrast, the most intense product ions formed were *m/z* 70 for L-arginine and L-[$^{15}N_2$]-arginine, and *m/z* 77 for L-[$^2H_7$]-arginine (Fig. 1), most likely corresponding to a pyrrolinium ion as a cyclization product (Fig. 2). Also, ADMA and SDMA show a unique fragmentation pattern when analysed as their butyl ester derivatives. Even though ADMA and SDMA exhibit the same protonated molecular ion with *m/z* 259, ADMA fragments by a NL of 45 and SDMA fragments by a NL of 31 (Fig. 1). A NL of 45 was also observed for [3,3,4,4,5,5-$^2H_6$]-ADMA (Fig. 1). ADMA labelled with six deuterium atoms at the methyl moieties of the dimethylamino group, i.e. [1,1,1,1′,1′,1′-$^2H_6$]-[dimethylamino-1,2,3,4,5-$^{13}C_5$]-arginine, shows a NL of 51. These findings suggest that dimethylamine {$(CH_3)_2NH$, 45 Da}; {$(C^2H_3)_2NH$, 51 Da} is cleaved from the molecular ions of unlabelled and labelled ADMA (Fig. 2). Thus, the NL of 31 observed for SDMA is likely to correspond to a monomethylamine (CH_3NH_2, 31 Da) moiety (Fig. 2). It is important to notice that a NL of 31 was not observed from ADMA or [3,3,4,4,5,5-$^2H_6$]-ADMA. The loss of a dimethylamine group is unlikely for SDMA. Indeed, a NL of 45 was absent in the product ion spectrum of SDMA. Esterification of the carboxylic acid function of arginine and dimethylarginines with other alcohols like methanol and ethanol was found to lead to similar fragmentation pattern (data not shown).

3.2. Mode of quantification

Ions used for quantitative analyses in the MRM mode are summarized in Table 1. The retention times of the butyl ester derivatives of L-arginine, L-[$^2H_7$]-arginine, SDMA, ADMA, and [$^2H_6$]-ADMA were 0.837 ± 0.010 (SD) min (RSD 1.2%; $n = 21$), 0.840 ± 0.012 min (1.4%), 0.833 ± 0.015 min (1.8%), 0.840 ± 0.015 min (1.8%), and 0.837 ± 0.014 min (1.4%), respectively. No statistically significant difference in retention time was found for these compounds. A representative partial chromatogram from the simultaneous analysis of L-arginine, L-[$^2H_7$]-arginine, SDMA, ADMA, and [$^2H_6$]-ADMA in human plasma in the MRM mode is depicted in Fig. 3.

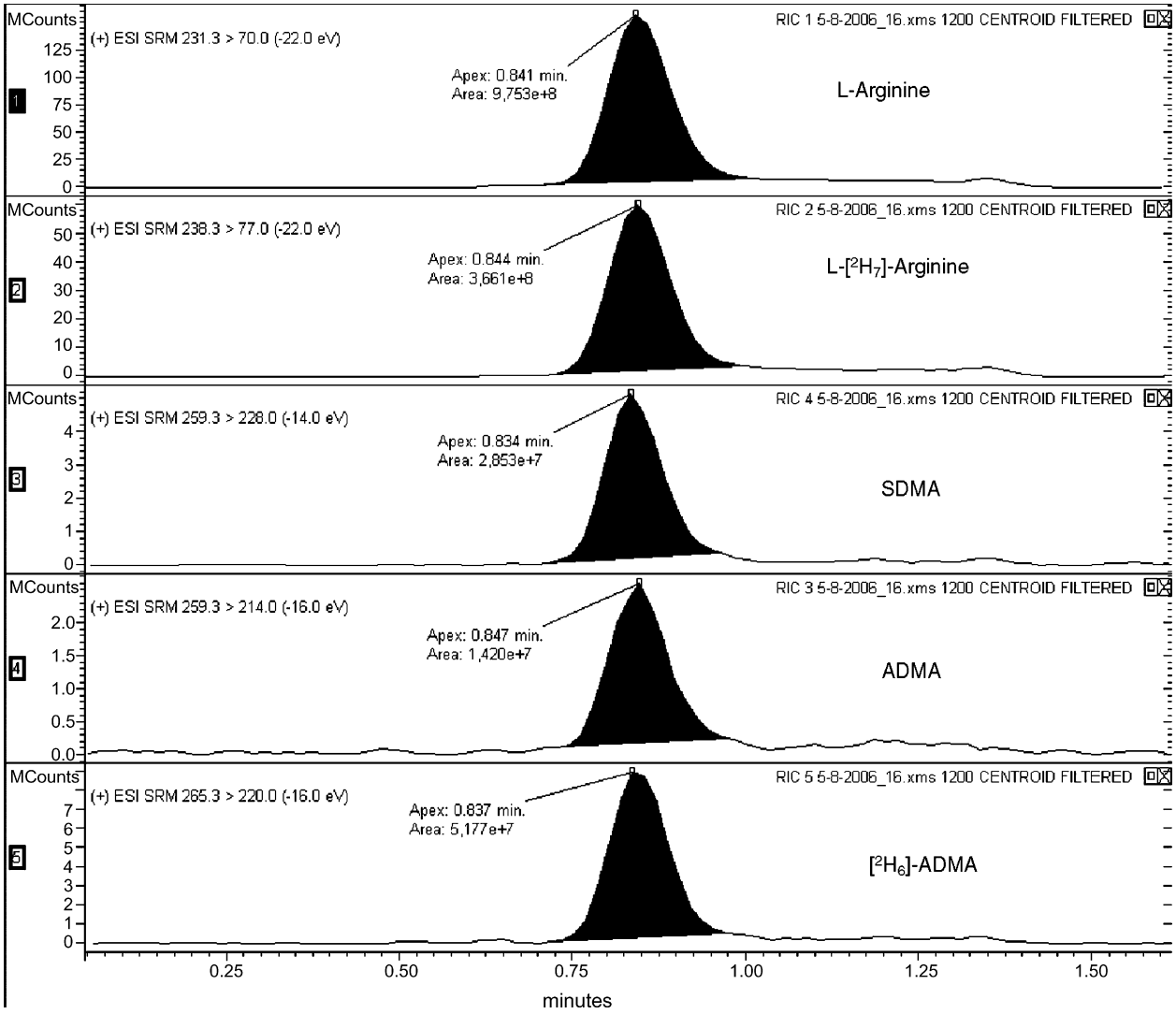

Fig. 3. Partial chromatograms from the LC-tandem MS analysis of L-arginine, L-[$^2H_7$]-arginine (internal standard for L-arginine), SDMA, ADMA and [$^2H_6$]-ADMA (internal standard for ADMA and SDMA) in human plasma as their butyl ester derivatives. Chromatography was performed as described in Section 2. The transitions monitored and the collision energy values used are indicated on the respective tracings and are summarized in Table 1.

Three different calibrators were used, L-arginine, ADMA, and SDMA, at seven different concentrations ($n = 5$, each): 0, 0.5, 1, 25, 50, 100, and 250 μM of L-arginine, as well as 0, 0.05, 0.1, 0.5, 1, 2, and 4 μM for ADMA and SDMA. The linear regression equations for peak area ratios (LC-tandem MS, y) and ratios of injected unlabelled and labelled calibrators (x) were $y = 1.002x - 0.009$ ($r^2 = 0.999$) for L-arginine, $y = 1.003x + 0.005$ ($r^2 = 0.999$) for ADMA, and $y = 2.002x + 0.01$ ($r^2 = 0.992$) for SDMA. We have previously reported a two-fold greater slope for SDMA in comparison to ADMA in standard curves in solvent [13]. The reason for this observation is a weaker fragmentation of the SDMA butyl ester derivative. However, standard curves were calculated to account for these differences and the slopes of the standard curves were included in all plasma concentration calculations. Plasma concentrations were calculated by using the following Eq. (1):

$$\text{Conc. analyte}[\mu\text{M}] = \frac{\text{Peak area analyte}}{\text{Peak area } IS} \times \frac{\text{Conc. } IS[\mu\text{M}]}{\text{Slope } SC} \quad (1)$$

whereas IS means internal standard, and SC is slope of standard curve.

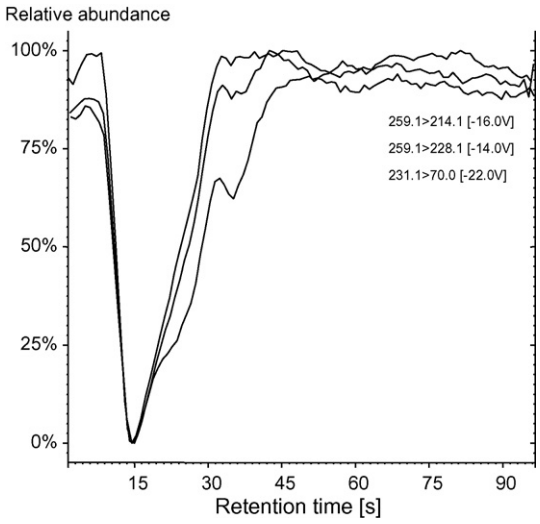

Fig. 4. Combined partial chromatograms from the LC-tandem MS analysis of continuous infusion of L-arginine, SDMA, and ADMA after injection of a 10-μl aliquot of a worked up plasma sample showing ion suppression. Chromatography was performed as described in Section 2. The transitions monitored and the collision energy values used are indicated on the respective tracings and are summarized in Table 1.

Table 2
Intra-assay accuracy and precision of the method for L-arginine, ADMA, and SDMA in human plasma ($n = 5$ for each concentration)

L-Arginine added[a]	L-Arginine measured minus basal level[b]	Accuracy (% bias)	Precision (RSD %)
0	0	N.A.	1.3
0.5	0.57	13	1.3
1	0.99	−0.8	2.5
25	25.6	2.2	2.1
50	50.4	0.7	1.7
100	98.9	−1.1	2.5
250	250.1	0.0	3.6
Mean ± SD		2.4 ± 5.0	2.2 ± 0.8
ADMA added[a]	ADMA measured minus basal level[c]	Accuracy (% bias)	Precision (RSD %)
0	0	N.A.	2.9
0.05	0.053	6.4	3.5
0.1	0.095	−5.3	5.4
0.5	0.49	−1.6	1.9
1	1.03	3.4	2.4
2	2.01	0.6	3.0
4	3.99	−0.3	3.1
Mean ± SD		0.6 ± 3.6	3.2 ± 1.0
SDMA added[a]	SDMA measured minus basal level[d]	Accuracy (% bias)	Precision (RSD %)
0	0	N.A.	1.4.
0.05	0.042	−15.8	2.4
0.1	0.105	4.8	3.1
0.5	0.46	−7.6	4.3
1	1.03	2.9	3.9
2	1.92	−3.8	5.4
4	3.93	−1.7	4.1
Mean ± SD		3.5 ± 6.8	3.5 ± 1.2

N.A., not applicable.
[a] All concentrations are given in μM.
[b] Mean basal level was 43.8 μM.
[c] Mean basal level was 0.66 μM.
[d] Mean basal level was 0.61 μM.

Table 3
Summary of the results from the determination of the LLOQ values of the method for L-[$^{15}N_2$]-arginine and [$^2H_6$]-[$^{13}C_5$]-ADMA in human plasma ($n = 5$ for each concentration)

L-[$^{15}N_2$]-Arginine added[a]	Adjusted L-[$^{15}N_2$]-arginine measured[b]	Accuracy (% bias)	Precision (RSD %)
0.02	0.022	10	10
0.05	0.040	−20	8.5
0.1	0.123	23	11
0.25	0.282	13	5.2
0.5	0.557	11	8.4
1.0	0.992	−0.8	7.1
[$^2H_6$]-[$^{13}C_5$]-ADMA added[a]	[$^2H_6$]-[$^{13}C_5$]-ADMA measured	Accuracy (% bias)	Precision (RSD %)
0.002	0.0023	15	58
0.005	0.0056	12	16
0.01	0.0099	−1.2	11
0.025	0.027	7.8	13
0.05	0.0493	−1.3	8.0
0.1	0.101	1.4	2.0

[a] All concentrations are given in μM.
[b] ^{13}C and ^{18}O of endogenous L-arginine contributed to the chromatographic trace of L-[$^{15}N_2$]-arginine with a signal corresponding to 0.22 μM L-[$^{15}N_2$]-arginine. Measured L-[$^{15}N_2$]-arginine was corrected for this amount.

The lower limit of detection (LLOD, 5 μl injected), defined as a signal-to-noise (S/N) ratio between 3 and 6, was determined to be 5 pg (5.7 nM) for L-arginine (S/N = 3.2) and each 500 fg (0.5 nM) for ADMA (S/N = 3.5) and SDMA (S/N = 5.9), respectively. Ion suppression was checked by infusion experiments [23]. After injection, the matrix from plasma samples suppressed the signal for the first 20 s of chromatography by >90% ($n = 5$). After 45 s the signal was completely restored (Fig. 4).

3.3. Validation of the method

We validated our LC-tandem MS method by adding different concentrations of L-arginine, ADMA, and SDMA in quintuplicate to 20 μl aliquots of plasma. L-Arginine was added at 0, 0.5, 1, 25, 50, 100, and 250 μM. ADMA and SDMA were added at 0, 0.05, 0.1, 0.5, 1, 2, and 4 μM. Data from the intra-assay validation experiments in human plasma are listed in Table 2. For all analytes and concentrations imprecision (RSD) was below 6%. The observed bias for all added concentrations was not greater than ±8% except for the addition of 0.5 μM L-arginine (13%) and the addition of 0.05 μM SDMA (−16%). Linear regression analysis between measured (y) and added (x) concentrations yielded slopes and y-axis intercepts of 0.999 and 43.8 μM ($r^2 = 0.999$) for L-arginine, of 0.998 and 0.66 μM ($r^2 = 0.999$) for ADMA, and of 0.981 and 0.61 μM ($r^2 = 0.996$) for SDMA. The addition of L-arginine, ADMA, or SDMA did not result in the measurement of higher concentrations of the other analytes.

Since all investigated compounds are endogenously present, we determined the lower limit of quantification (LLOQ) for L-arginine and ADMA by using stable isotope analogs, i.e. L-[$^{15}N_2$]-arginine and [$^2H_6$]-[$^{13}C_5$]-ADMA, respectively. L-[$^{15}N_2$]-Arginine was added to 20 μl aliquots of pooled plasma at 0.02, 0.05, 0.1, 0.25, 0.5, and 1 μM ($n = 5$, for each concentration). [$^2H_6$]-[$^{13}C_5$]-ADMA was added at 0.002, 0.005, 0.01, 0.025, 0.05, and 0.1 μM ($n = 5$, each concentration). Data from the LLOQ determination in human plasma are listed in Table 3. Acceptable accuracy and precision were obtained for 0.25 μM of L-[$^{15}N_2$]-arginine and for 0.005 μM of [$^2H_6$]-[$^{13}C_5$]-ADMA.

We also determined inter-assay accuracy and precision (RSD, $n = 10$ for each concentration) of the method for human plasma samples at three different added concentrations of L-arginine, ADMA, and SDMA. Unspiked plasma samples with measured basal concentrations of 70.9 ± 4.0 μM for L-arginine, 0.37 ± 0.02 μM for ADMA, and 0.32 ± 0.02 μM for SDMA were analysed (first level). Also, L-arginine, ADMA and SDMA were added at 100, 0.5, and 0.5 μM, respectively (second level), and finally L-arginine, ADMA and SDMA were added at 250, 1, and 1 μM, respectively (third level). The data from inter-assay accuracy and precision of the method are summarized in Table 4.

3.4. Basal plasma levels and molar ratios in human and mouse plasma

All samples were analysed batch-wise on 96-well plates. Work-up time for one 96-well plate was 2 h including sample preparation and derivatization. Analysis time for one 96-well plate was 3 h. We measured L-arginine, ADMA and SDMA in plasma samples of healthy human volunteers by our LC-tandem MS method. Fig. 3 shows a typical chromatogram from the simultaneous LC-tandem MS analysis of these analytes in a human plasma sample (20 μl). The mean levels of L-arginine, ADMA, and SDMA in plasma of healthy humans ($n = 85$) were 74 ± 19, 0.46 ± 0.09, and 0.37 ± 0.07 μM, respectively. The mean molar ratio amounted to 166 ± 50 for L-arginine and ADMA, and to 1.24 ± 0.24 for ADMA and SDMA. We also measured arginine and dimethylated arginines in plasma samples of C57BL/6 mice ($n = 30$). Samples were also aliquoted (20 μl) and frozen at −20 °C prior to analysis. The mean levels of L-arginine, ADMA, and SDMA were 44 ± 14, 0.72 ± 0.23, and

Table 4
Inter-assay accuracy and precision of the method for L-arginine, ADMA, and SDMA in human plasma at three different levels ($n = 10$ for each concentration)

L-Arginine added[a]	L-Arginine measured minus basal level[b]	Accuracy (% bias)	Precision (RSD %)
0	0	N.A.	5.6
100	95.8	−4.2	5.3
250	257.3	2.9	3.5
Mean ± SD		−0.6 ± 3.5	4.8 ± 0.9
ADMA added[a]	ADMA measured minus basal level[c]	Accuracy (% bias)	Precision (RSD %)
0	0	N.A.	5.4
0.5	0.51	2.9	4.5
1	0.98	−1.7	3.3
Mean ± SD		0.6 ± 2.3	4.4 ± 0.9
SDMA added[a]	SDMA measured minus basal level[d]	Accuracy (% bias)	Precision (RSD %)
0	0	N.A.	5.2
0.5	0.49	−1.5	4.9
1	0.98	−2.2	4.5
Mean ± SD		−1.8 ± 0.3	4.9 ± 0.3

N.A., not applicable.
[a] All concentrations are given in μM.
[b] Mean basal level was 70.9 μM.
[c] Mean basal level was 0.37 μM.
[d] Mean basal level was 0.32 μM.

0.19 ± 0.06 μM, respectively. The mean molar ratio amounted to 85 ± 22 for L-arginine and ADMA, and to 4.2 ± 0.7 for ADMA and SDMA.

4. Discussion

Derivatization of arginine, ADMA, and SDMA to their butyl esters and collision-induced dissociation (CID) resulted in the formation of specific product ions for arginine, ADMA, and SDMA (Fig. 1). The observed MS/MS fragmentation of the butyl ester derivatives allows for the simultaneous determination of ADMA, SDMA, and L-arginine in plasma without any chromatographic separation. However, chromatography cannot completely be omitted. Infusion experiments showed considerable ion suppression by plasma matrix components after 15 s of chromatography (Fig. 4). Thus, matrix components have to be eliminated prior to detection of analytes. Elimination of matrix components can be achieved by preceding SPE and/or liquid chromatographic separation on normal or reversed phase material [27]. Chromatography on octadecyl reversed phase material usually involves a solvent gradient to separate arginine and dimethylarginines from polar and non-polar matrix. The advantage of teicoplanin material over reversed phase material is its ability to retain amino acids at higher organic solvent fractions, e.g. 60 vol% acetonitrile [28]. Thus, matrix constituents causing ion suppression elute from the analytical column (20 mm × 0.1 mm i.d.) packed with teicoplanin within the first 45 s, while arginine, ADMA, and SDMA butyl ester derivatives are retained for 0.8 min. After elution of the analytes, the next sample can be injected without any further rinsing and re-equilibration of the teicoplanin column. Thus, in contrast to other LC–MS-based methods [13,20–22], sample run time was reduced to 1.6 min and chromatography of analytes was completed within 1 min in the present LC-tandem MS method.

The herein described method includes derivatization of samples, heating and evaporation of derivatization reagent (Fig. 5). These steps and the preceding protein precipitation step are performed off-line. To reduce laboratory work needed to process

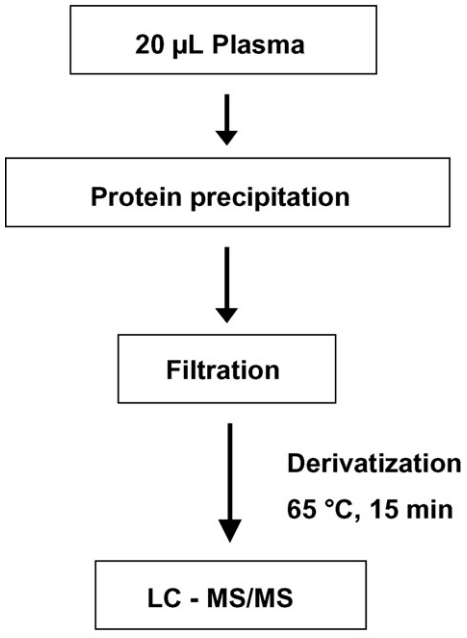

Fig. 5. Summary of the most relevant sample preparation steps of the LC-tandem MS method for the quantitative determination of L-arginine, ADMA, and SDMA in plasma samples.

samples we have introduced a 96-well plate system. The 96-well plate system consists of a filtration plate adapted to the bottom polypropylene plate. The bottom polypropylene plate is used for derivatization process and is directly applied to the autosampler of the LC-tandem MS system. One further advantage of the 96-well plate system is that sample volume is reduced to 20 μl of plasma. This is advantageous for plasma samples from small animals like mice in which plasma volume is limited. To reduce stochastic error, plasma samples have to be pipetted only once to the filtration plate and no subsequent manual transfer is needed. Also, filtration plates were pre-coated in our laboratory with internal standard to guarantee identical conditions for all samples. Several 96-well plates can be performed at the same time further reducing laboratory work and saving time. Thus, in contrast to previous chromatographic methods our present method allows for high-throughput analysis of arginine and dimethylated arginines.

Convenience of handling of the 96-well plate system used in the present study is comparable with a commercially available ELISA kit for ADMA determination [29,30]. The advantage of the present LC-tandem MS method over the ADMA ELISA kit is the simultaneous determination of arginine, ADMA and SDMA. This allows for the calculation of ratios of arginine to ADMA and of ADMA to SDMA. The molar ratio of arginine to ADMA is one determinant of the substrate availability for NOS [4,5]. In a meta-analysis between NO production rate and logarithmic plasma arginine to ADMA concentration ratio a significant correlation was found [4]. We could demonstrate that the arginine to ADMA ratio is considerably different in mice than in man. We found a two-fold higher ratio in man. The reason for this observation is not clear and may be due to species-specific differences. One explanation for this finding may be a species-specific difference in DDAH I and II activity. So far, data on DDAH I and II activity are very limited [7–9]. In a DDAH I transgenic C57BL/6J mice model ADMA plasma levels were 39% lower, i.e. 0.44 μM, in comparison with wild type littermates, i.e. 0.72 μM [31]. Interestingly, in these mice human DDAH I was overexpressed. Thus, the overexpression of the human DDAH I in transgenic mice resulted in ADMA concentrations which were comparable to human plasma levels of ADMA observed in our study, i.e. 0.46 μM.

In conclusion, we combined convenient 96-well sample preparation technique with specific, accurate and sensitive LC-tandem MS to allow for simultaneous high-throughput determination of arginine, SDMA, and ADMA. We have validated our method and applied it for the determination of arginine and dimethylated arginine derivatives in 20-μl aliquots of human or mice plasma.

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