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De novo synthesis of trideuteromethyl esters of amino acids for use in GC–MS and GC-tandem MS exemplified for ADMA in human plasma and urine: Standardization, validation, comparison and proof of evidence for their aptitude as internal standards☆

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ABSTRACT

Asymmetric dimethylarginine (ADMA, N^G,N^G-dimethyl-L-arginine) is an endogenous inhibitor of nitric oxide (NO) synthesis, a potential risk factor for cardiovascular diseases and a powerful biochemical parameter in clinical studies. In our previous work we have reported on a GC-tandem MS method for the accurate and precise quantification of ADMA in biological fluids using de novo synthesized [$^2H_3$]-methyl ester ADMA (d_3Me-ADMA) as internal standard (IS). This method provides basal ADMA concentrations in biological fluids that agree with those obtained by other groups using other validated methods for ADMA. Unanimously, de novo synthesized stable-isotope labeled analogues are considered not ideal IS, because they must be prepared in a matrix different from the biological sample. Recently, [2,3,3,4,4,5,5-$^2H_7$]-ADMA (d_7-ADMA) has become commercially available and we took this opportunity to test the reliability of the de novo synthesized d_3Me-ADMA as an IS for ADMA in GC-tandem MS. In this article, we report on the re-validation of the previously reported GC-tandem MS method for ADMA in human plasma and urine using d_7-ADMA as IS, and on comparative quantitative analyses of ADMA by GC-tandem MS using d_7-ADMA and d_3Me-ADMA. After thorough standardization of d_7-ADMA and methods validation, we obtained by GC-tandem MS very similar ADMA concentrations in plasma and urine using d_7-ADMA and d_3Me-ADMA. The present study gives a proof of evidence for the aptitude of $^2H_3$-ADMA as IS in GC-tandem MS and suggests that de novo synthesis of stable-isotope labeled alkyl esters of amino acids and amino acid derivates may be a generally applicable method in mass spectrometry-based methods for amino acids. This approach is especially useful for amino acids for which no stable-isotope labeled analogues are commercially available.

1. Introduction

N^G,N^G-Dimethyl-L-arginine (asymmetric dimethylarginine, ADMA; Fig. 1) is an endogenous inhibitor of nitric oxide (NO) synthesis from L-arginine [1]. ADMA has been suggested as a potent risk factor for the development of NO-associated endothelial dysfunction in cardiovascular diseases as well as in chronic renal failure [2–4]. Increase in the extent of inhibition of NO synthesis in vivo due to elevated circulating ADMA concentrations in NO-associated diseases could be the underlying mechanism in these diseases. The interest in this potentially fundamental role of ADMA in basic and clinical research led to the development of numerous analytical methods for the quantitative determination of ADMA in biological systems, notably plasma, serum and urine (reviewed in Refs. [5–10]). Most of these analytical methods revealed comparable ADMA concentrations, for example, of the order of 400–500 nM in plasma and serum of healthy humans [8,10].

In our previous work we showed that GC-tandem MS allows for highly accurate and sensitive quantitative determination of ADMA in human plasma and urine [11,12]. Because no stable-isotope labeled ADMA analogues were commercially available at that time, we tried to overcome this difficulty by using in these GC-tandem MS methods de novo synthesized [$^2H_3$]-methyl ester ADMA (d_3Me-d_0-ADMA; Fig. 1) as internal standard (IS). This approach involves separate *O*-methylation of the carboxylic group of endogenous ADMA by using HCl in CH_3OH and of the carboxylic group of synthetic ADMA by using HCl in CD_3OD (Fig. 2A). After methy-

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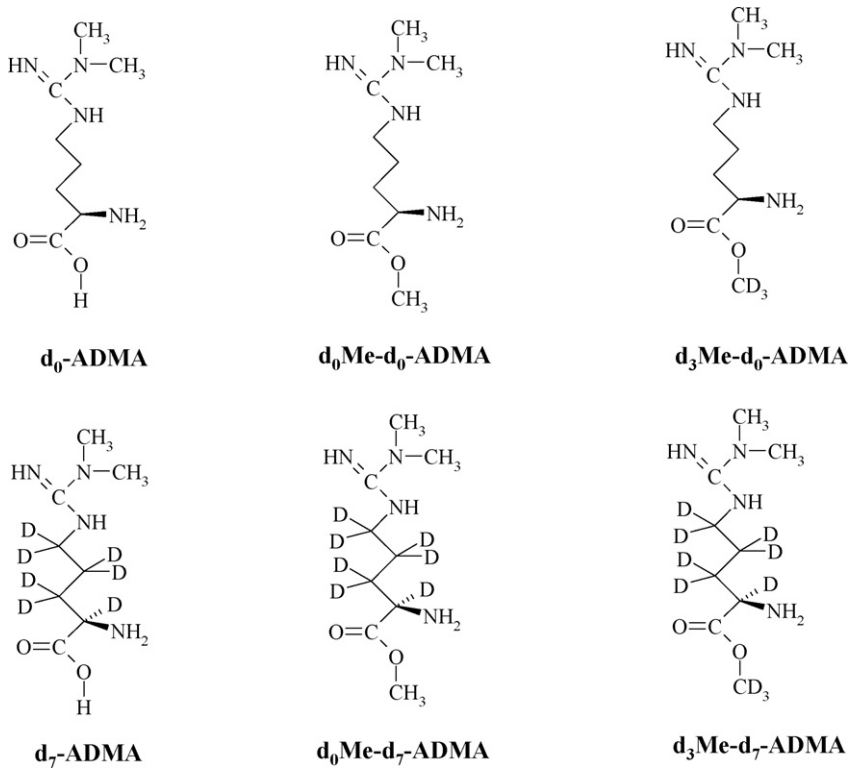

Fig. 1. Chemical structures of unlabeled and deuterium-labeled analogues of asymmetric dimethylarginine (ADMA) as free acids and as label-free or labeled methyl esters.

lation the samples containing the methyl esters of endogenous ADMA (d_0Me-d_0-ADMA) and d_3Me-d_0-ADMA are combined to a single matrix for subsequent derivatization with pentafluoropropionic anhydride (PFPA; Fig. 2A). We have shown that de novo synthesized d_3Me-d_0-ADMA is suitable as an IS for the quantitative determination of ADMA in plasma and urine by GC-tandem MS [11,12]. This method provides ADMA concentrations in human plasma and urine which are in accordance with generally accepted ADMA concentrations (discussed in Refs. [5–10]). Because of the lack of a commercially available stable-isotope labeled analogue of ADMA, we have been not able to give unequivocal proof that the de novo synthesized d_3Me-ADMA is as good as synthetic stable-isotope labeled ADMA analogues in quantitative GC-tandem MS analyses. In 2007, a deuterium-labeled ADMA, i.e., [2,3,3,4,4,5,5-$^2H_7$]-ADMA (d_7-ADMA, Fig. 1), has become commercially available (http://www.eurisotop.de). d_7-ADMA has a well-founded poten-

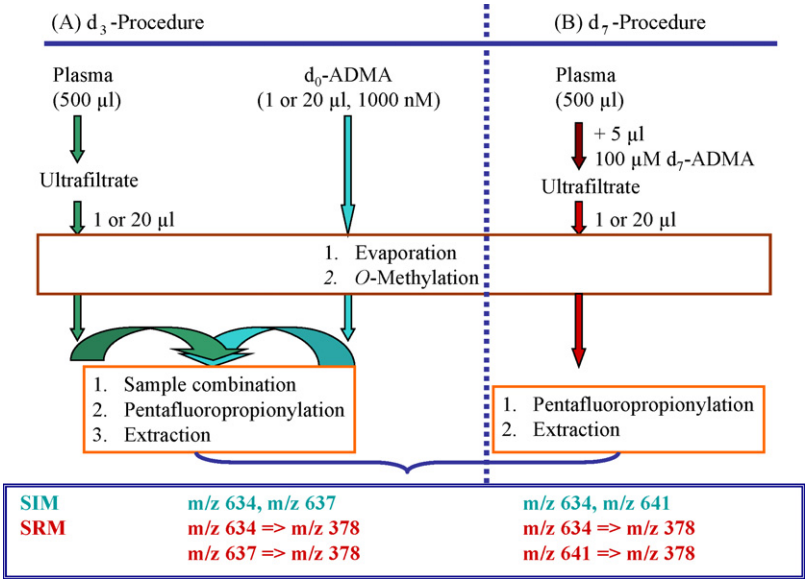

Fig. 2. Simplified schematic of the method for the GC-tandem MS measurement of ADMA in plasma samples involving use of de novo synthesized trideuteromethyl ester as internal standard (A) or the commercially available d_7-ADMA (B). In the d_3-procedure, ADMA present in the plasma sample is methylated in methanol separately from the synthetic ADMA standard which is methylated in deuterium-labeled methanol. After this step, the samples are combined and subjected to a common derivatization step with pentafluoropropionic anhydride in ethyl acetate [11]. In the d_7-procedure, the internal standard d_7-ADMA is added directly to the plasma sample. In the case of urine, the procedures are the same except for the ultrafiltration step which is leaved out. SIM, selected-ion monitoring; SRM, selected-reaction monitoring.

tial to be excellently suited as IS for ADMA in our GC-tandem MS method, analogous to home-made stable-isotope labeled ADMA analogues the utility of which in GC–MS and LC–MS has recently been reported by two other groups [13,14].

In the present work, we tested the suitability of d_7-ADMA as IS for the quantitative determination of ADMA in plasma and urine of humans by GC-tandem MS [11,12] and we re-validated this method for ADMA in these matrices using d_7-ADMA as IS. Our results suggest that de novo synthesis of stable-isotope labeled alkyl esters of amino acids could be a generally applicable method to prepare useful IS in MS-based methods especially in cases of commercial unavailability of stable-isotope labeled analogues of amino acids and their derivates.

2. Experimental

2.1. Materials and chemicals

Asymmetric dimethylarginine (N^G,N^G-dimethyl-L-arginine; ADMA) dihydrochloride, was purchased from Sigma (Deisenhofen, Germany). Pentafluoropropionic anhydride (PFPA) was obtained from Pierce (Rockford, IL, USA). Tetradeuterated methanol (CD_3OD, 98% at ^2H) was supplied by Aldrich (Steinheim, Germany). [2,3,3,4,4,5,5-$^2H_7$]-ADMA (d_7-ADMA, declared as 99.3% at ^2H by ^1H NMR and each 98% for chiral and chemical purity by HPLC), provided as monohydrate monohydrochloride salt, was purchased from EURISOTOP (Saarbrücken, Germany). The whole powder (declared as 5 mg) was diluted in 190.1 μl of distilled water in the original flask resulting in a nominal concentration of 100 mM of d_7-ADMA. From this stock solution dilutions of 1000 and 100 μM in distilled water were prepared in two steps; stock solution and proportioned dilutions were stored at −20 °C. All other chemicals were obtained from Merck (Darmstadt, Germany). Vivaspin 2 Hydrosart cartridges (cut-off, 10 kDa) were supplied by Sartorius (Göttingen, Germany).

2.2. Procedures for the quantification of ADMA in human plasma and urine

2.2.1. Biological samples—recovery of blood, plasma and ultrafiltrate

Blood (5–7 ml) was drawn from antecubital veins of healthy volunteers using syringes containing EDTA and put immediately on ice. Blood samples were centrifuged at 1500 × *g* and 2 °C for 15 min. The plasma generated was analyzed immediately or stored at −78 °C until further analysis. Routinely, ultrafiltrates (approx. 0.2 ml) from 0.5-ml aliquots of plasma samples were obtained by centrifugation (8000 × *g*, 20 °C, 20 min) using the Vivaspin cartridges. Plasma ultrafiltrate (1 μl or 20 μl) was used for the quantification of plasma ADMA. Urine from healthy volunteers was obtained from spontaneous micturition. Urine samples were analyzed immediately or stored at −20 °C until analysis.

2.2.2. Derivatization procedures

Fig. 2 shows schematically the procedures used for the quantitative determination of ADMA in human plasma by using de novo synthesized d_3Me-d_0-ADMA (i.e., referred to as *d_3-procedure*) or d_7-ADMA (i.e., referred to as *d_7-procedure*).

2.2.2.1. Derivatization procedures involving de novo synthesized d_3Me-d_0-ADMA. Aliquots (1 μl or 20 μl) of plasma ultrafiltrate or urine and of aqueous solutions of ADMA (1 μM for plasma; 20 μM for urine) were evaporated to dryness by means of a nitrogen stream (Fig. 2A). The residues of plasma ultrafiltrate and urine samples were treated with 100-μl aliquots of a 2 M HCl solution in CH_3OH; the residue of the standard ADMA solution was treated with a 100-μl aliquot of a 2 M HCl solution in CD_3OD. Amino acids were methylated by heating the samples separately for 60 min at 80 °C. After cooling to room temperature, the methanolic solution of plasma ultrafiltrate or urine sample was combined with the corresponding sample of methanolic solution of synthetic ADMA. To ensure quantitative transfer, each residue was taken up with a 100-μl aliquot of CH_3OH; solvents and reagents of the combined solutions were evaporated to dryness by a stream of nitrogen gas. The residue was then treated with a 100-μl aliquot of a solution of PFPA in ethyl acetate (1:4, v/v) and the sample was heated for 30 min at 65 °C. After cooling to room temperature, the sample was evaporated to dryness, the residue was reconstituted with a 200-μl aliquot of 0.4 M borate buffer, pH 8.5, and the sample was vortexed immediately for 60 s with toluene (200-μl aliquots for plasma, 1000-μl aliquots for urine samples). Aliquots (100 and 800 μl, respectively) of the upper organic layer were taken and transferred into glass vials, from which 1-μl aliquots were analyzed by GC–MS in the selected-ion monitoring (SIM) mode or by GC-tandem MS in the selected-reaction monitoring (SRM) mode (Fig. 2) as described below.

2.2.2.2. Derivatization procedure involving d_7-ADMA. Routinely, plasma aliquots (0.5 ml) were spiked with 5-μl aliquots of a 100-μM dilution of d_7-ADMA resulting in a final added concentration of 1 μM with respect to the plasma volume (Fig. 2B). Plasma aliquots (0.5 ml) were then ultrafiltered as described above, and 20-μl or 1-μl aliquots of the ultrafiltrate were subjected to further treatment and derivatization as described above except that *O*-methylation is carried out in unlabeled methanol.

2.3. Standardization of d_7-ADMA

d_7-ADMA was standardized by GC–MS and GC-tandem MS using commercially available unlabeled ADMA (d_0-ADMA) as follows. Solutions of d_0-ADMA and d_7-ADMA in distilled water (each 100 μM nominal) were used to prepare mixtures containing fixed nominal concentrations of unlabeled and labeled ADMA at 1.0 μM and variable nominal concentrations of 0.0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1,3 and 2.0 μM) as specified below. A set of three experiments was performed. In experiment (a), the concentration of d_7-ADMA was kept fixed at 1.0 μM, whereas the concentration of d_0-ADMA varied. In experiment (b), the concentration of (de novo synthesized) d_3Me-d_0-ADMA was kept fixed at 1.0 μM, whereas the concentration of d_0-ADMA varied. In experiment (c), the concentration of d_0-ADMA was kept fixed at 1.0 μM, and the concentration of d_7-ADMA varied. From all of these solutions each of the three 1-μl aliquots were derivatized. GC–MS analyses were performed by SIM of the precursor ions at *m*/*z* 634 for d_0-ADMA (i.e., d_0Me-d_0-ADMA), *m*/*z* 637 for d_3Me-d_0-ADMA, and *m*/*z* 641 for d_0Me-d_7-ADMA (see Section 3). GC-tandem MS analyses were performed by SRM of the common product ion at *m*/*z* 378 which was generated from the precursor ions at *m*/*z* 634 for d_0Me-d_0-ADMA, *m*/*z* 637 for d_3Me-d_0-ADMA, and *m*/*z* 641 for d_0Me-d_7-ADMA (see Section 3). The peak area ratios (*R*) of d_0Me-d_0-ADMA/d_0Me-d_7-ADMA ($R_{0/7}$), d_0Me-d_0-ADMA/d_3Me-d_0-ADMA ($R_{0/3}$), and d_0Me-d_7-ADMA/d_0Me-d_0-ADMA ($R_{7/0}$) were determined in experiments (a), (b) and (c), respectively, and plotted versus the varying concentration of d_0-ADMA or d_7-ADMA as applicable. From linear regression analyses between the respective peak area ratio (*y*) and the varied concentration of d_0-ADMA or d_7-ADMA (*x*) the regression equations ($y = a + bx$) were obtained.

In our previous work [11] we found that the yield of the methylation of ADMA and L-arginine in 2 M HCl in methanol is of the order of 90%. In the present work the HCl-catalyzed methylation of ADMA was investigated by GC-tandem MS for mixtures of d_0-ADMA and d_7-ADMA as follows. Ten 20-μl aliquots of 1-μM aqueous solutions

Table 1
Summary of ions [*m/z* (intensity, %)] in the GC-ECNICI-MS mass spectra of the methyl ester tri(*N*-pentafluoropropionyl) derivatives of unlabeled and deuterium-labeled ADMA analogues.

d_0Me-d_0-ADMA	d_3Me-d_0-ADMA	d_0Me-d_7-ADMA	d_3Me-d_7-ADMA	Ion assignment
233 (17)	236 (12)	234 (7)	237 (7)	[PFP-N = C-H/D-$COOCH_3/CD_3$]$^-$ [a]
443 (5)	446 (5)	450 (3)	453 (3)	not assigned
525 (6)	528 (5)	530 (2)	533 (2)	not assigned
565 (4)	568 (4)	572 (2)	575 (2)	not assigned
N.A.	636 (2)	640 (9)	643 (8)	$[M–DF]^-$
634 (100)	637 (100)	641 (100)	644 (100)	$[M–HF]^-$
654 (3)	657 (3)	661 (3)	664 (2)	$[M]^-$

[a] PFP, pentafluoropropionyl. N.A., not applicable.

of d_0-ADMA and d_7-ADMA (i.e., nominal 20 pmol each) were combined. After evaporation to dryness, methylation was performed in 2 M HCl in CH_3OH ($n = 5$) or CD_3OD ($n = 5$). Samples were then paired, evaporated to dryness and acylated with PFPA as described above. GC-tandem MS analysis of all ADMA derivatives was performed by SRM of the product ion at *m/z* 378 which was produced from *m/z* 634 of d_0Me-d_0-ADMA, *m/z* 637 of d_3Me-d_0-ADMA, *m/z* 641 of d_0Me-d_7-ADMA and *m/z* 644 of d_3Me-d_7-ADMA.

2.4. Validation experiments

Accuracy (recovery) and precision (RSD) of the method were determined using freshly obtained and pooled plasma and urine samples from spontaneous micturition from a healthy volunteer. Intra- and inter-assay precision was determined by working up in replicate unspiked and spiked pooled plasma on five consecutive days, and of unspiked and spiked pooled urine samples on one day. For simplicity, the validation and comparison experiments are described in detail in the Results section.

2.5. GC-tandem MS determination of basal ADMA concentrations in human plasma and urine—comparison between d_3-Me-d_0-ADMA and d_7-ADMA

In these comparative studies, plasma and urine samples from previously reported clinical studies (see Ref. [9]) were used. The nominal concentrations of d_3Me-d_0-ADMA and d_7-ADMA corresponded to 1 μM in plasma and 20 μM in urine each.

2.6. GC–MS and GC-tandem MS conditions

GC–MS and GC-tandem MS conditions were essentially the same as described previously [11]. Analyses in the electron-capture negative-ion chemical ionization (ECNICI) mode were performed on a triple-stage quadrupole mass spectrometer ThermoQuest TSQ 7000 (Finnigan MAT, San Jose, CA) directly interfaced with a Trace 2000 series gas chromatograph equipped with an autosampler AS 2000 (CE Instruments, Austin, TX). The gas chromatograph was equipped with a 30-m long fused-silica capillary column Optima 17 (0.25 mm I.D., 0.25 μm film thickness) from Macherey-Nagel (Düren, Germany). The following oven temperature program was used with helium (at a constant flow rate of 1 ml/min) as the carrier gas: 1 min at 90 °C, then increased to 225 and 320 °C at a rate of 15 and 30 °C/min, respectively. Interface, injector and ion-source were kept at 280, 280 and 180 °C, respectively. Electron energy and electron current was set to 200 eV and 300 μA, respectively. Methane (530 Pa) and argon (0.13 Pa collision pressure) were used as reagent and collision gases, respectively. Routinely, collision energy and electron multiplier voltage were set to 18 eV and 1.6 kV, respectively. Aliquots (1 μl from toluene extracts) were injected in the splitless mode by means of an autosampler. In quantitative analyses the dwell time was 50 ms for each ion both in GC–MS and in GC-tandem MS.

3. Results

3.1. GC–MS and GC-tandem MS mass spectra of unlabeled and deuterium-labeled ADMA derivatives

The most intense ions found in the GC–MS mass spectra of the methyl ester tri(*N*-pentafluoropropionyl), i.e., $MePFP_3$, derivatives of unlabeled and deuterium-labeled ADMA analogues are summarized in Table 1. The molecular anions of the $MePFP_3$ derivatives $[M]^-$ were present in the mass spectra at very low intensity. In the ECNICI mass spectra of the ADMA derivatives analyzed the most intense ions were due to loss of HF (20 Da), i.e., $[M–HF]^-$, from the molecules of the $MePFP_3$ derivatives. In the mass spectrum of the $MePFP_3$ derivative of d_7-ADMA we also observed a considerably less intense anion at *m/z* 640 most likely due to loss of DF, i.e., $[M–DF]^-$ (Fig. 3A). Interestingly, an anion $[M–DF]^-$ was absent in the mass spectrum of the $MePFP_3$ derivative of d_3Me-d_0-ADMA, suggesting that the deuterium atom that leaves the molecule during ionization does not come from the trideuteromethyl ester group.

Collision-induced dissociation (CID) of the parent anions $[M–HF]^-$ resulted in the generation of numerous product ions (Table 2). The most intense product ions from the $MePFP_3$ derivatives of unlabeled and deuterium-labeled ADMA analogues were found at *m/z* 378 and *m/z* 360 or *m/z* 361; these ions carry no deuterium atoms (*m/z* 378 and *m/z* 360) or one deuterium atom (*m/z* 361). Obviously, these product ions contain the dimethyl amino group and should therefore be characteristic for ADMA. The product ion mass spectrum of the $MePFP_3$ derivative of d_0Me-d_7-ADMA is shown in Fig. 3B. The product ion with *m/z* 378 has been previously used by us in quantitative GC-tandem MS analyses of ADMA [11,12] and was also chosen for quantitative analyses (SRM mode) in the present study.

3.2. Standardization of d_7-ADMA by GC–MS, GC-tandem MS and HPLC

Injection of 100 fmol of the $MePFP_3$ derivative of d_7-ADMA and SRM of *m/z* 378 produced from *m/z* 634 and *m/z* 641 revealed a peak area ratio of 0.00082 (RSD, 9.3%; $n = 3$) suggesting that the commercially available d_7-ADMA we used in this study does not contain analytically relevant amounts of unlabeled ADMA (i.e., d_0-ADMA).

The results from the GC–MS and GC-tandem MS standardization experiments are summarized in Table 3 and are shown in Fig. 4. The precision (RSD, %) of the measurements ranged between 2% and 17%. In all cases, linear relationships ($r \geq 0.9993$) were observed. Almost identical results were obtained by GC–MS and GC-tandem MS only in experiment (b), i.e., when analyzing d_0-ADMA and d_3-ADMA in their own mixtures. By contrast, in experiments (a) and (c), in which d_7-ADMA was used, clearly different values for the slopes of the regression straight lines were obtained by GC–MS and GC-tandem MS (Table 3, Fig. 4). Interestingly, the reciprocals of the slope values of experiment (a), i.e., 1/0.819 = 1.221 in GC–MS and 1/1.323 = 0.756

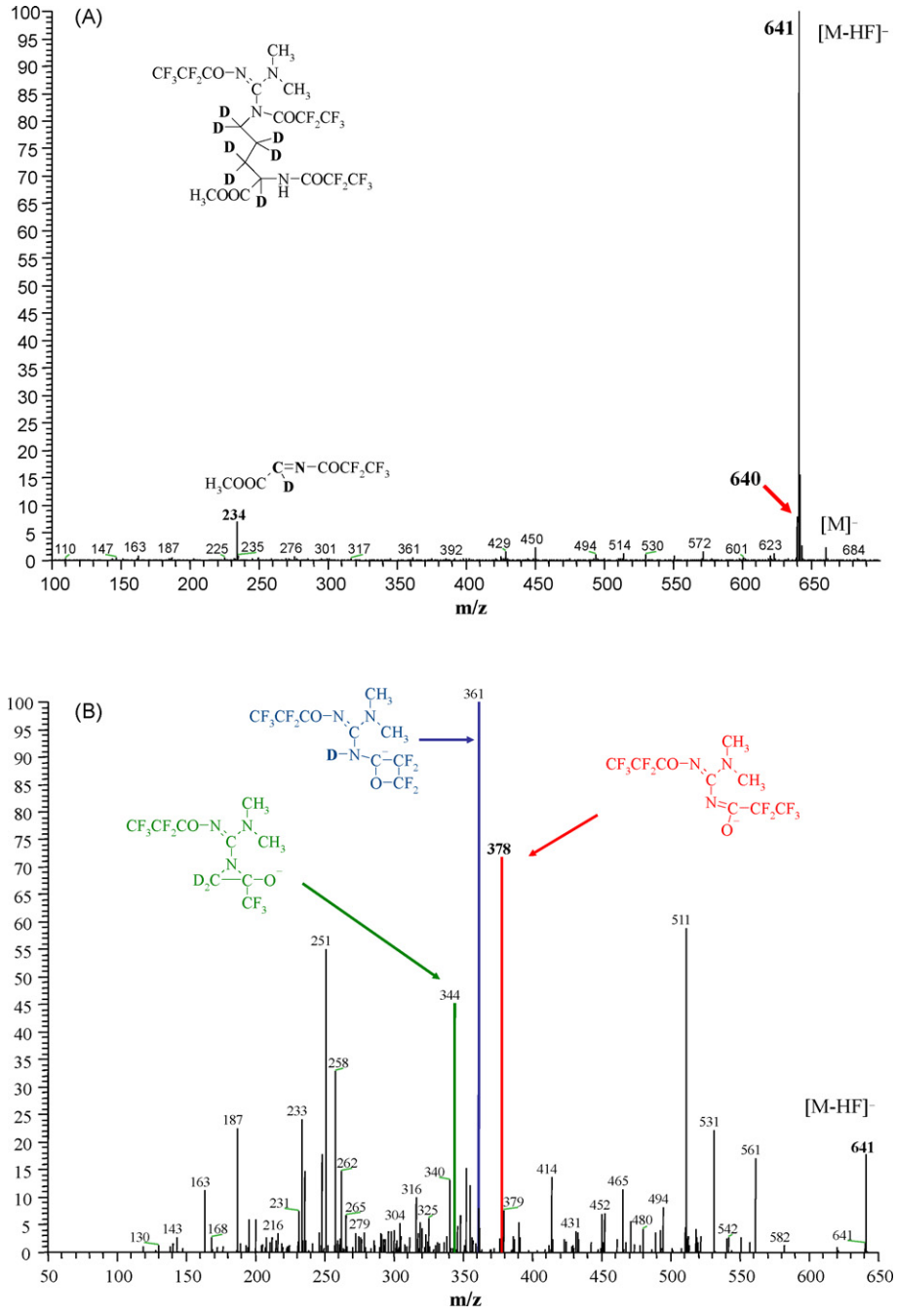

Fig. 3. GC–MS (A) and GC-tandem MS (B) mass spectra in the ECNICI mode of the methyl ester tri(*N*-pentafluoropropionyl) derivative of d_7-ADMA. The parent ion at *m/z* 641 ($[M–HF]^-$ was subjected to collision-induced dissociation (CID) with argon at a collision energy of 18 eV. Insertions in (B) show the putative structures of the most intense product ions.

in GC-tandem MS, are almost identical with the respective slope values observed in experiment (c), i.e., 1.252 and 0.748, respectively. Also, both in GC–MS and in GC-tandem MS analyses, the slope values obtained in experiment (a) are almost identical with the respective slope values from experiment (c). Thus, GC–MS and GC-tandem MS analyses yielded diametrically opposed results: GC–MS suggests that the concentration of d_7-ADMA in the dilution used in these experiments is greater than that of d_0-ADMA, whereas GC-tandem MS suggests the contrary.

In consideration of these unexpected results we analyzed in triplicate native, non-derivatized d_0-ADMA and d_7-ADMA (from their nominal 10-μM solutions) by cation-pairing reverse-phase HPLC using 1-octansulfonic acid (10 mM) as the ion-pairing agent and UV absorbance detection at 205 nm as described previously for *S*-nitrosothiols [15]. The ratio of the areas of the HPLC peaks of d_0-ADMA and d_7-ADMA in these solutions was calculated to be 0.846 ± 0.008 ($n = 4$). This finding is in accordance with the result obtained from experiments (a) and (c) using GC–MS and suggests that either (1) the concentration of d_0-ADMA in the 10-μM dilution is not 10 μM as originally assumed (i.e., nominal concentration) but 8.46 μM, or alternatively, (2) the concentration of d_0-ADMA in its 10-μM dilution is indeed 10 μM but that of d_7-ADMA is 11.8 μM instead of 10 μM as originally assumed, i.e., on the basis of the declaration of the supplier.

In an additional experiment, mixtures containing nominally equal amounts of d_0-ADMA and d_7-ADMA (i.e., 20 pmol each) were

Table 2
Summary of the major[a] product ions (m/z) and their intensity (%) produced by collision-induced dissociation of the parent ions $[M–HF]^-$ of the methyl ester tri(*N*-pentafluoropropionyl) derivatives of unlabeled and deuterium-labeled ADMA analogues.

ADMA analogue	$[M–HF]^-$											
d_0Me-d_0-ADMA	634	559	527	505	378	360	349	342	259	251	248	232
Intensity (%)	10	15	28	30	100	65	15	60	15	36	15	34
No. of D atoms	0	0	0	0	0	0	0	0	0	0	0	0
d_3Me-d_0-ADMA	637	559	530	508	378	360	358; 352	342	262	254	250	232
Intensity (%)	6	16	45	20	75	100	20; 22	76	20	28	17	36
No. of D atoms	3	0	3	3	0	0	0	0	3	3	2	0
d_0Me-d_7-ADMA	641	561	531	511	378	361	354; 351	344	262	258	251	233
Intensity (%)	18	18	22	60	72	100	15; 17	44	15	34	55	23
No. of D atoms	7	2	4	6	0	1	5; 2	2	3	7	3	1
d_3Me-d_7-ADMA	644	561	534	514	378	361	355	344	265; 261	254	251	233
Intensity (%)	12	15	36	80	97	100	46	45	46; 42	86	40	36
No. of D atoms	10	2	7	9	0	1	6	2	6; 2	3	3	1

[a] Product ions with intensity of ≥15% within the m/z range of 200–650 were considered.

methylated in HCl-containing CH_3OH or CD_3OD, acylated by PFPA and analyzed by GC-tandem MS. The results from the analysis of these samples are shown in Fig. 5. These data indicate that the methylation of the carboxylic group of d_0-ADMA and d_7-ADMA with CD_3OD or CH_3OH proceeds with very similar yields under the derivatization conditions used. Furthermore, these results are similar to those obtained from GC-tandem MS analyses reported above and suggest that the concentration of d_0-ADMA or d_7-ADMA differs from the nominal concentration of 10 μM. From the data of Fig. 5, i.e., from the mean of the reciprocal of the peak area ratios, it results that the concentrations of d_0-ADMA and d_7-ADMA could be 10 and 8.06 μM, or 12.4 and 10 μM, respectively.

3.3. Comparison between d_7-ADMA and d_3-ADMA in the quantitative GC-tandem MS analysis of ADMA in human plasma and urine

In a comparative study, ADMA was quantitated in parallel by GC-tandem MS in plasma and urine samples from 18 subjects using d_7-ADMA and d_3-ADMA as internal standards. Aliquots of the same plasma and urine samples had been analyzed before for ADMA by GC-tandem MS using d_3-ADMA as internal standard as described [11,12]. The previously obtained data and the results generated in the present study are summarized in Table 4.

Paired *t* test analysis of the ADMA concentrations measured in plasma and urine using d_7-ADMA or d_3-ADMA as IS revealed statistically highly significantly ($P \ll 0.01$) different concentrations in the samples analyzed. Interestingly, there was no statistical difference between the ADMA concentrations measured in these samples in the present study and those measured for the first time in May/June 2007 (*P* value was 0.17 for plasma and 0.2 for urine samples) indicating the reliability of the method involving use of d_3-ADMA as IS. The ratio of the ADMA concentrations measured using d_7-ADMA and d_3-ADMA (i.e., d_7/d_3 ratio, $R_{7/3}$) was not statistically different for the plasma and urine samples ($P = 0.2$). The ADMA concentrations measured by using d_7-ADMA (y) and those measured by using d_3-ADMA (x) correlated closely both for plasma ($y = -55 + 1.34x$, $r = 0.9475$) and for urine ($y = -5 + 1.31x$, $r = 0.9941$), with the slope values of the regression equations being almost identical. These findings are comparable to those obtained using d_7-ADMA and GC-tandem MS (see Table 3 and Fig. 5).

We may reasonably assume that the amount/concentration of the reference compound d_0-ADMA, i.e., the dihydrochloride salt of N^G,N^G-dimethyl-L-arginine, which we are using since several years in the de novo synthesis of d_3-ADMA and in quality control [9], is true. On the basis of this assumption and the findings described above, we have to conclude that the concentration of the stock solution and the dilutions of the commercially available d_7-ADMA, i.e., the monohydrate monohydrochloride salt of [2,3,3,4,4,5,5-$^2H_7$]-ADMA, is about 20% higher than assumed by us. This assumption may explain the results obtained from GC–MS analysis, but not those generated by GC-tandem MS analysis. Formation of ions due to loss of HF, i.e., m/z 641, as well as due to loss of DF, i.e., m/z 640, from the $MePFP_3$ derivative of d_7-ADMA suggests that these anions have different chemical structures (Fig. 6). For instance, the ion m/z 641 with structure (A′) could be formed from the loss of the H atom of the α-amido group of the $MePFP_3$ of d_7-ADMA, while the ion at m/z 640 with structure (B′) could be formed from the loss of the D atom at the C-atom on position 2 of the $MePFP_3$ of d_7-ADMA (Fig. 6). In the case of d_0-ADMA we would also obtain two differ-

Table 3
Linear regression equations and correlation coefficients obtained from the standardization of d_7-ADMA by GC–MS and GC-tandem MS using d_0-ADMA or d_3-ADMA as the calibrator.

GC–MS (SIM)			GC-tandem MS (SRM)		
$\frac{m/z\,634}{m/z\,641}$ $R_{0/7}$	$\frac{m/z\,634}{m/z\,637}$ $R_{0/3}$	$\frac{m/z\,641}{m/z\,634}$ $R_{7/0}$	$\frac{m/z\,634 \rightarrow 378}{m/z\,641 \rightarrow 378}$ $R_{0/7}$	$\frac{m/z\,634 \rightarrow 378}{m/z\,637 \rightarrow 378}$ $R_{0/3}$	$\frac{m/z\,641 \rightarrow 378}{m/z\,634 \rightarrow 378}$ $R_{7/0}$
Experiment[a]					
(a) $y = 0.024 + 0.819x$, $r = 0.9995$	N.A.	N.A.	$y = 0.05 + 1.323x$, $r = 0.9993$	N.A.	N.A.
(b) N.A.	$y = 0.023 + 1.005x$, $r = 0.9996$	N.A.	N.A.	$y = 0.019 + 0.998x$, $r = 0.9996$	N.A.
(c) N.A.	N.A.	$y = 0.001 + 1.252x$, $r = 0.9999$	N.A.	N.A.	$y = 0.008 + 0.748x$, $r = 0.9998$

[a] The nominal concentrations used were: In experiment (a): d_0-ADMA, 0–2 μM; d_7-ADMA, 1 μM; In experiment (b): d_0-ADMA, 0–2 μM; d_3-ADMA, 1 μM; In experiment (c): d_7-ADMA, 0–2 μM; d_3-ADMA, 1 μM. N.A., not applicable.

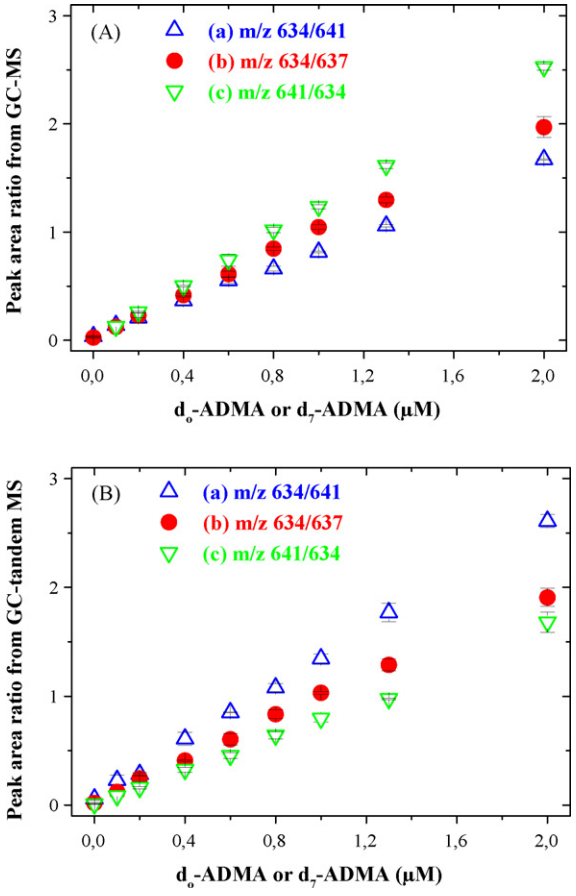

Fig. 4. Plotting of the peak area ratio measured by GC–MS (A) and by GC-tandem MS (B) versus the varied concentration of d_0-ADMA or d_7-ADMA in the standardization experiments using d_0-ADMA or d_3-ADMA as the calibrator each at a nominal concentration of 1 μM. Data are shown as mean ± SD from triplicate analyses. For more details see the text.

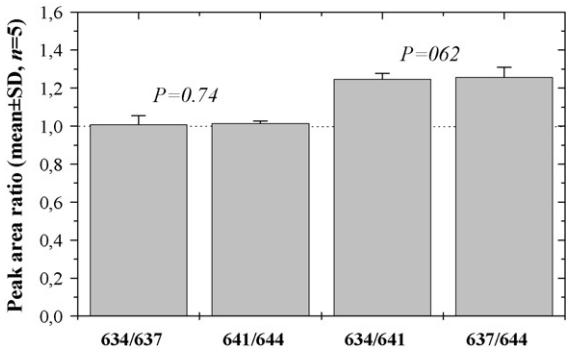

Fig. 5. Mixtures of nominally equal amounts (20 pmol) of d_0-ADMA and d_7-ADMA were methylated separately in CH_3OH or CD_3OD, combined, acylated by PFPA and analyzed by GC-tandem MS. Explanation of the labeling for the peak area (PA) ratios: 634/637 means PA from SRM of *m/z* 378 from *m/z* 634 and *m/z* 637 for d_0Me-d_0-ADMA and d_3Me-d_0-ADMA, respectively. 641/644 means PA from SRM of *m/z* 378 from *m/z* 641 and *m/z* 644 for d_0Me-d_7-ADMA and d_3Me-d_7-ADMA, respectively. 634/641 means PA from SRM of *m/z* 378 from *m/z* 634 and 641 for d_0Me-d_0-ADMA and d_0Me-d_7-ADMA, respectively. 637/644 means PA from SRM of *m/z* 378 of *m/z* 637 and *m/z* 644 for d_3Me-d_0-ADMA and d_3Me-d_7-ADMA, respectively.

ent ions $[M–HF]^-$ but they would have the same *m/z* value, i.e., *m/z* 634 with the structure (A) and structure (B) (Fig. 6). Because of the different structures of these anions it is possible that, on a molar basis, the intensity of *m/z* 640 differs considerably from that of *m/z* 641, e.g., is greater than suggested by Fig. 3A. Thus, the ion *m/z* 640 with the structure (B′) would not be considered in quantitative analyses by GC-tandem MS. Moreover, the corresponding ion *m/z* 634 of unlabeled ADMA with the structure (B) would contribute to the product ion *m/z* 378 that is also produced from the parent ion *m/z* 634 of unlabeled ADMA with the structure (A). Using the data of Table 3, Figs. 4 and 5, it is calculated that a fraction of about 33% of d_7-ADMA does not contribute to *m/z* 378 [e.g., A′ + B′ = 120; 378A/378A′ = 100/(120 − B′) = 1.25, with A′ contributing to *m/z* 378 and with B′ non-contributing to *m/z* 378]. For instance, for a final corrected d_7-ADMA concentration of 1200 nM in plasma and 24 μM in urine, the relevant "true" d_7-ADMA concentrations in the matrices in quantitative analyses by GC-tandem MS would be 800 nM

Table 4
ADMA concentrations in plasma (nM) and urine (μM) of healthy and ill subjects measured by GC-tandem MS using d_7-ADMA or d_3-ADMA as internal standards.

No.	Plasma[a]				Urine[a]			
	d_7	d_3	$d_3$2007[b]	$R_{7/3}$[c]	d_7	d_3	$d_3$2007[b]	$R_{7/3}$[c]
1	614	449	470	1.37	111	93	95	1.20
2	774	643	650	1.21	125	94	103	1.33
3	598	504	500	1.19	65.4	53.1	61.2	1.23
4	563	462	470	1.22	52.9	46.7	52.8	1.13
5	766	591	580	1.30	68.4	58	55.4	1.18
6	576	467	450	1.23	64.3	51.2	52.4	1.26
7	697	544	550	1.28	76.2	58.6	61.1	1.30
8	610	506	490	1.21	54.7	44.1	45.3	1.24
9	613	514	510	1.19	55.3	47.2	39.1	1.17
10	533	433	450	1.23	51.0	42.1	46.5	1.21
11	611	488	530	1.25	78.9	67.8	71.3	1.16
12	483	423	400	1.14	83.9	70.7	72.8	1.19
13	650	502	430	1.30	49.2	41.4	39.9	1.19
14	484	412	400	1.18	48.3	43.2	44.1	1.12
15	456	414	400	1.10	7.58	6.6	6.88	1.15
16	572	466	450	1.23	27.4	23.7	24	1.16
17	546	452	420	1.21	140	108	124	1.30
18	563	438	410	1.29	51	44.2	41.6	1.15
Mean	595	484	476	1.23	67.2	55.2	57.6	1.20
SD	87	62	69	0.06	32.5	24.7	28.1	0.06

[a] The nominal (uncorrected) concentration of d_7-ADMA and d_3-ADMA was each 1 μM in plasma and each 20 μM in urine samples.
[b] The samples had been analyzed for the first time in May/June 2007 using d_3-ADMA as internal standard as described previously [11,12].
[c] $R_{7/3}$ is the ratio of the ADMA concentrations measured by using d_7-ADMA and d_3-ADMA as internal standards.

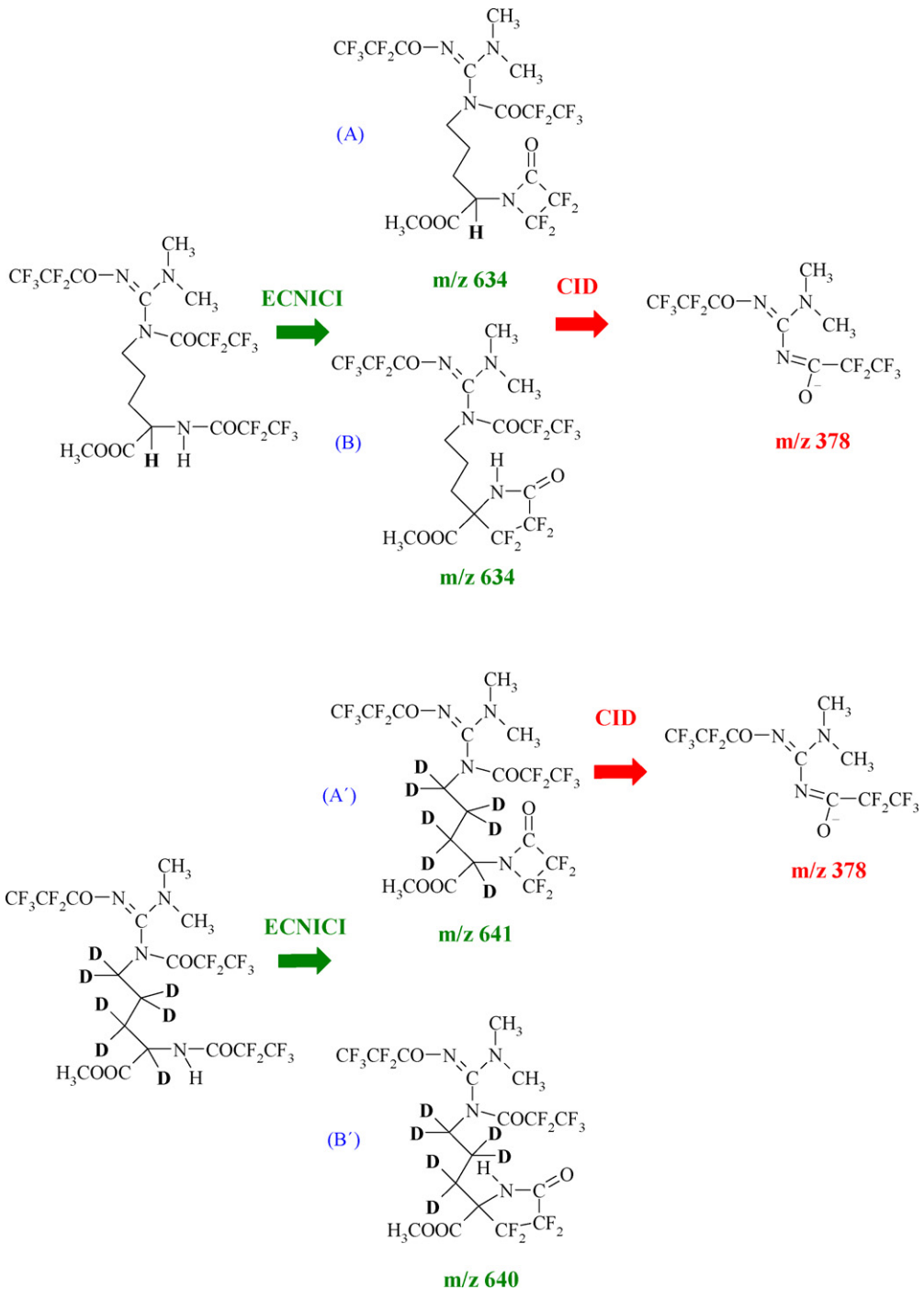

Fig. 6. Proposed chemical structures for the methyl ester tri(*N*-pentafluoropropionyl) (MePFP$_3$) derivatives of d_0-ADMA and d_7-ADMA, of the anions at *m*/*z* 634, *m*/*z* 640, and *m*/*z* 641 formed by ECNICI, and of the product ion at *m*/*z* 378 generated by collision-induced dissociation (CID) of the ions at *m*/*z* 634 for d_0-ADMA and *m*/*z* 641 for d_7-ADMA. ECNICI of d_0-ADMA MePFP$_3$ generates two different ions A and B with the same *m*/*z* value of 634 of which CID leads to the formation of the product ion at *m*/*z* 378. ECNICI of d_7-ADMA MePFP$_3$ also generates two different ions A′ and B′ with different *m*/*z* values, i.e., 641 and 640, of which only the ion *m*/*z* 641 is subjected to CID in the SRM and forms the product ion at *m*/*z* 378.

and 16 μM, respectively. These concentrations were considered in the following experiments on method validation.

3.4. Accuracy, precision, and limit of quantitation of the method

The data on intra- and inter-assay precision and accuracy of the GC-tandem MS method for ADMA in human plasma using d_7-ADMA as internal standard are summarized in Table 5. In the pooled plasma used in these experiments the mean ADMA plasma concentration was measured to be 347 ± 18 nM. The lowest ADMA concentration added to plasma, i.e., 100 nM (29% of the basal ADMA level) was quantitated with high accuracy and precision (RSD, 1.4%), suggesting that even lower concentrations of ADMA externally added to plasma would be quantitated with satisfactory accuracy and precision (Table 5).

The data from the intra-assay precision and accuracy of the method for ADMA in human urine using d_7-ADMA are summarized in Table 6. In the pooled urine sample used for method validation,

Table 5
Intra- and inter-assay accuracy and precision of the GC-tandem MS method for ADMA in human plasma using d_7-ADMA as internal standard.

Day (Ultrafiltrate)	ADMA added (nM)	ADMA measured (nM)[a]	Recovery (%)[b]	Precision (RSD, %)
A (20 μl UF)	0	358 ± 1	N.A.	0.3
	100	457 ± 11	99.0	2.4
	200	545 ± 17	93.5	3.1
	400	757 ± 17	99.8	2.2
	600	955 ± 33	99.5	3.5
	800	1166 ± 49	101.0	4.1
	1000	1326 ± 92	96.8	6.9
	1300	1658 ± 90	100.0	5.4
	2000	2271 ± 55	95.7	2.4
	$y = 358 + 0.98x$, $r = 0.99973$[c]			
A (1 μl UF)	0	362 ± 2	N.A.	0.6
	100	479 ± 5	117.0	1.0
	200	570 ± 4	104.0	0.7
	400	816 ± 10	113.5	1.2
	600	1019 ± 15	109.5	1.5
	800	1232 ± 13	108.8	1.1
	1000	1438 ± 15	107.6	1.0
	1300	1788 ± 2	109.7	0.1
	2000	2483 ± 37	106.1	1.5
	$y = 361 + 1.10x$, $r = 0.99997$[c]			
B (1 μl UF)	0	363 ± 9	N.A.	2.5
	100	437 ± 11	74.0	2.5
	200	536 ± 26	86.5	4.9
	400	767 ± 25	101.0	3.3
	600	941 ± 26	96.3	2.8
	800	1136 ± 3	96.6	0.3
	1000	1314 ± 24	95.1	1.8
	1300	1659 ± 58	99.7	3.5
	2000	2334 ± 54	98.6	2.3
	$y = 354 + 0.977x$, $r = 0.99979$[c]			
C (1 μl UF)	0	317 ± 12	N.A.	3.8
	100	452 ± 10	135.0	2.2
	200	534 ± 32	108.5	6.0
	400	764 ± 22	111.8	2.9
	600	965 ± 14	108.0	1.5
	800	1124 ± 17	100.9	1.5
	1000	1344 ± 15	102.7	1.1
	1300	1686 ± 21	105.3	1.2
	2000	2382 ± 29	103.3	1.2
	$y = 337 + 1.021x$, $r = 0.9994$[c]			
D (1 μl UF)	0	339 ± 28	N.A.	8.3
	100	421 ± 3	82.0	0.7
	200	544 ± 20	102.5	3.7
	400	744 ± 12	101.3	1.6
	600	936 ± 73	99.5	7.8
	800	1119 ± 24	97.5	2.1
	1000	1305 ± 72	96.6	5.5
	1300	1615 ± 86	98.2	5.3
	2000	2386 ± 103	102.4	4.3
	$y = 320 + 1.025x$, $r = 0.99904$[c]			
E (1 μl UF)	0	340 ± 13	N.A.	3.8
	100	449 ± 36	109.0	8.0
	200	559 ± 7	109.5	1.3
	400	794 ± 33	113.5	4.2
	600	1008 ± 44	111.3	4.4
	800	1111 ± 47	96.4	4.2
	1000	1328 ± 19	98.8	1.4
	1300	1686 ± 34	103.5	2.0
	2000	2266 ± 7	96.3	0.3
	$y = 365 + 0.953x$, $r = 0.99977$[c]			

[a] d_7-ADMA was added to plasma samples at a final concentration of 1000 nM, but ADMA concentrations were calculated by using the corrected concentration of 800 nM.
[b] Recovery was calculated as: [(measured − basal)/added] × 100. Plasma ultrafiltrate (UF) volumes of 1 μl or 20 μl were subjected to further analysis.
[c] Between added (y) and measured (x) ADMA concentration. Values are given as mean ± SD, $n = 3$. N.A., not applicable. UF, ultrafiltrate.

endogenous ADMA was measured at about 19 μM. The lowest concentration of ADMA externally added to the urine sample that could be measured with a recovery of 100 ± 20% and a precision (RSD) of ≤20%, was 6 μM (about 31% of the basal level) or 1 μM (about 5% of the basal level) in dependence on the calculation method of recovery (Table 6). Linear regression analysis between found (y) and added (x) ADMA concentrations revealed straight lines ($r > 0.9986$) for plasma (see Table 5) and urine ($y = 19.7 + 1.096x$). These data

Table 6
Intra-assay precision and accuracy (recovery) of the GC-tandem MS method for ADMA in human urine using d_7-ADMA as internal standard.

ADMA added (μM)	ADMA measured (μM)[a]	Precision (RSD, %)	Recovery (%)	
0	19.3 ± 0.21	1.1	N.A.	N.A.
1	20.8 ± 0.19	0.9	150.0[b]	102.5[c]
2	21.9 ± 0.12	0.5	130.0	102.8
3	23.2 ± 0.29	1.3	130.0	104.0
6	25.9 ± 1.41	5.4	110.0	102.4
8	28.7 ± 0.23	0.8	117.5	105.1
10	30.4 ± 0.59	1.9	110.0	103.8
15	36.1 ± 0.24	0.7	112.0	105.2
20	39.4 ± 0.95	2.4	100.5	100.3

[a] d_7-ADMA was added to plasma samples at a nominal final concentration of 20 μM, but ADMA concentrations were calculated by using the corrected concentration of 16 μM.

[b] Recovery was calculated as: [(measured − basal)/added] × 100.

[c] Recovery was calculated as: [measured/(basal + added)] × 100. Aliquots (1 μl) of urine were subjected to further analysis. Values are given as mean ± SD, $n = 5$. N.A., not applicable.

indicate that ADMA can be quantitated by this GC-tandem MS method with high accuracy and precision using the commercially available d_7-ADMA as internal standard even in 1-μl aliquots of plasma ultrafiltrate or of native urine.

Fig. 7 shows partial GC-tandem MS chromatograms from analyses of ADMA in a human plasma sample analyzed in the comparative study using d_7-ADMA as internal standard before (Fig. 7A) and after spiking with d_0-ADMA. These are typical GC-tandem MS chromatograms that show peaks arising only from endogenous ADMA and the internal standard.

4. Discussion

4.1. Stable-isotope labeled analogues as internal standards in mass spectrometry-based analytical methods

Commercial availability of stable-isotope labeled analogues of natural and synthetic compounds facilitates greatly the accurate quantitative analysis of these compounds in biological fluids by MS-based methods such as GC–MS, LC–MS and their tandem variants. For many compounds, however, stable-isotope labeled analogues are not commercially available. In such cases analysts have to look for alternatives which may include use of homologous compounds or use of stable-isotope labeled analogues of the target analytes to be synthesized in own laboratory. While the first mentioned way is more comfortable, the synthetic way may be by far more challenging and time-consuming but highly recommendable from the analytical point of view.

For ADMA, a biochemical parameter of high relevance in current basic and clinical research [8], no stable-isotope labeled analogues were available in the market until recently. This circumstance and the recognition of the pressing need for a stable-isotope labeled analogue of ADMA have driven investigators to overcome this limitation. So far, two solutions have been proposed: 1) the chemical synthesis of stable-isotope labeled analogues of ADMA as proposed by the groups of Schwedhelm [13] and of Martens-Lobenhoffer [14]; and 2) the chemical de novo synthesis of the trideuteromethyl ester of ADMA (d_3Me-ADMA; Fig. 1) as has been proposed by our group [11]. From the quantitative point of view, synthetic stable-isotope labeled analogues are regarded in general as "ideal" IS because they can be added directly to the biological sample before any other sample treatment. By contrast, de novo synthesis should be, at least in theory, less reliable because this method cannot be performed directly in the biological sample and thus cannot control all changes that undergo endogenous substances or for matrix effects. Nevertheless, on the basis of the agreement on basal concentrations of ADMA in plasma and urine obtained from the use of the "ideal" IS [5,7,10] and from those generated by the use of the putatively less appropriate IS d_3Me-ADMA, one may conclude that the approach based on the use of the de novo synthesized d_3Me-ADMA should be as good as the approaches involving use of synthetic standards. Because of the lack of commercially available stable-isotope analogues, we were not able to prove the aptitude of d_3Me-ADMA as an equally good internal standard for the quantitative determination of ADMA in biological samples. In 2007, [2,3,3,4,4,5,5-$^2H_7$]-ADMA (d_7-ADMA; Fig. 1) has become commercially available, and this enabled us to compare directly and in parallel d_3Me-ADMA with d_7-ADMA and to unequivocally prove the reliability of d_3Me-ADMA as an IS in GC–MS and GC-tandem MS. We assumed that such comparative studies could also provide important information with regard to a more general use of de novo synthesized stable-isotope labeled analogues of amino acids and their derivates in MS-based methods.

4.2. GC–MS and GC-tandem MS characterization of d_7-ADMA

In agreement with our previous study on d_0-ADMA and d_3Me-ADMA [11], we found in the present study that under the derivatization conditions used, i.e., HCl-catalyzed *O*-methylation in CH_3OH or CD_3OD and *N*-perfluoroacylation with pentafluoropropionyl anhydride (PFPA) in ethyl acetate, produces the methyl ester tri(*N*-pentafluoropropionyl) derivatives, i.e., $MePFP_3$ derivatives (see Figs. 1 and 2). The $MePFP_3$ derivatives of unlabeled and labeled ADMA analogues are strong electron capturing, and negative-ion chemical ionization with methane produces mass spectra which contain very few ions (Table 1). In the GC–MS mass spectra of the $MePFP_3$ derivatives we observed the molecular anions $[M]^-$ at very low intensity. The most intense ions were obtained due to neutral loss of HF, i.e., $[M–HF]^-$. Interestingly, we observed anions due to $[M–DF]^-$ exclusively from the $MePFP_3$ derivative of d_7-ADMA suggesting that one deuterium atom of the carbon-chain of d_7-ADMA, most likely the single deuterium atom on carbon-2, leaves the derivative during ionization (Figs. 3A and 6). The formation of $[M–HF]^-$ and $[M–DF]^-$ from d_7-ADMA suggests that these anions have different chemical structures (Fig. 6). Thus, although the intensity of the ion m/z 640 accounts to only about 10% of that of m/z 641, it cannot be excluded that the ion m/z 640 is actually produced to a higher extent than 10%.

Collision-induced dissociation (CID) of the parent ions $[M–HF]^-$ with argon produced many intense product ions which contain varying numbers of D atoms in their molecules (Table 2). The mechanisms by which these product ions were produced are largely unknown and most likely different and complex. In our previous studies we used the product ion m/z 378 for quantitative analyses because of its high intensity [11,12]. This product ion was obtained from all ADMA analogues we analyzed in the present study and does not contain any deuterium atom, suggesting that this mass fragment contains the dimethylamino group and should therefore be characteristic for ADMA (Figs. 3B and 6). Most likely, the product ions at m/z 360 (from d_0-ADMA) and m/z 361 (from d_7-ADMA) do not originate from the consecutive fragmentation of the product ion at m/z 378. This seems also to apply to the product ions m/z 342 and m/z 344. It is therefore likely that at least these product ions are formed independently, i.e., by distinctly different CID mechanisms.

4.3. Standardization of d_7-ADMA

The results of the present study suggest that standardization of commercially available stable-isotope labeled analogues being supplied in mg-amounts, such as d_7-ADMA, are indispensable for accurate quantitative analysis and for reliable comparison of data produced by different groups. Lack of standardization of commer-

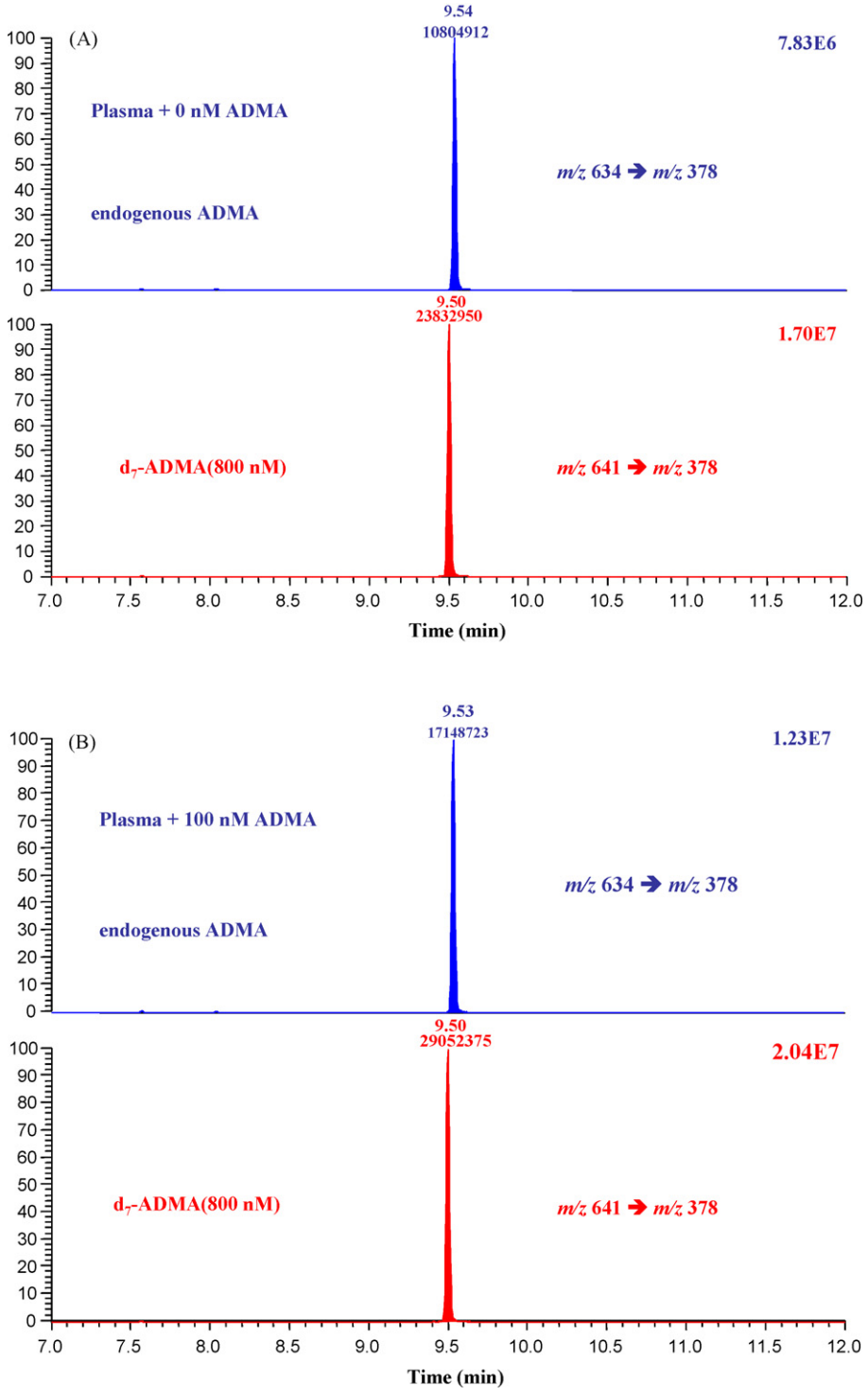

Fig. 7. Partial chromatograms from GC-tandem MS analyses of ADMA in an unspiked human plasma sample (A) and in the same sample spiked with 100 nM of ADMA (B). d_7-ADMA was used as the internal standard at a final nominal concentration of 1000 nM (800 nM after correction; see the text). SRM was performed of the common product ion at *m*/*z* 378 which was produced by CID of the parent ions at *m*/*z* 634 for unlabeled ADMA (upper traces) and *m*/*z* 641 for d_7-ADMA (lower traces). Endogenous ADMA and internal standard were analyzed as their $MePFP_3$ derivatives.

cially available unlabeled synthetic ADMA reference preparations may be a contributor to differing ADMA plasma levels reported in the literature (discussed in Ref. [9]). At present, ADMA is commercially available as the free base, as the dihydrochloride salt and, as in the case of d_7-ADMA, as the monohydrate monohydrochloride salt. By using the dihydrochloride salt of d_0-ADMA – which we are using in our analyses since several years – we found by HPLC analysis of native, non-derivatized ADMA in dilutions that the commercially available preparation must have contained about 20% more d_7-ADMA material than declared by the supplier. This finding collaborates with the observations from the GC–MS analyses of the $MePFP_3$ derivatives in the SIM mode (Fig. 4). In theory, the commercially available d_7-ADMA preparation could contain impurities such as d_6-ADMA. The appearance of the ions *m*/*z* 641 and *m*/*z* 643 in the mass spectra of the $MePFP_3$ derivative of d_7-ADMA (Fig. 3A, Table 1) could originate from d_6-ADMA present as a contamina-

tion of the order about 10%. However, LC–MS analysis of d_7-ADMA did not reveal presence of d_6-ADMA in the d_7-ADMA preparation (personal communication of Dr. Schwedhelm, Hamburg).

Because we wanted to test the applicability of d_7-ADMA as IS in quantitative GC-tandem MS analyses in the SRM mode, we standardized d_7-ADMA by GC-tandem MS using d_0-ADMA as the reference. To our surprise, we found by GC-tandem MS diametrically opposed results as compared to those obtained from HPLC and GC–MS. The most likely explanation for this unexpected finding and contradiction could be that a considerable fraction of the parent ion m/z 641 from the d_7-ADMA $MePFP_3$ derivative does not dissociate to the product ion m/z 378 to the same extent as the m/z 637 from the d_0-ADMA $MePFP_3$ derivative. Under the GC-tandem MS conditions used in the present study, this portion would be relatively constant, i.e., about 33%, suggesting involvement of a specific CID mechanism. Additional investigations would be necessary to delineate the underlying CID mechanism that leads to generation of the product ion m/z 378 using distinctly different labeled ADMA analogues.

We would like to point out that standardization of stable-isotope labeled analogues is of general validity and does not exclusively apply to d_7-ADMA. We have similar experience with many commercially available deuterium-labeled prostaglandins, thromboxane and isoprostanes (unpublished observations). An additional difficulty with such compounds is that they are supplied in solution in μg-amounts that cannot be weighed at all. Such preparations usually require more frequent standardization using the unlabeled compounds which are commercially available at considerably higher and weighable amounts. These considerations do not only apply to commercially available stable-isotope labeled analogues, but they are also valid for "home-made" labeled substances of low molecular weight [16] as well as for macromolecules [17].

4.4. Utility of de novo synthesized trideuteromethyl esters of amino acids and derivates as internal standards in GC–MS and GC-tandem MS

In our previous work [11,12], we have demonstrated the utility of de novo synthesized d_3Me-ADMA for the accurate and precise quantification of ADMA in human plasma and urine samples. The present study gives a direct and solid proof for the aptitude of d_3Me-ADMA as IS for the equally reliable GC–MS and GC-tandem MS quantitative determination of ADMA as $MePFP_3$ derivative. Our study suggests that the de novo synthesis of trideuteromethyl esters of amino acids and their metabolites may be a generally applicable principle for amino acids. This assumption is supported by data observed from the quantitative determination of L-arginine as $MePFP_3$ derivative using [*guanidino*-$^{15}N_2$]-L-arginine as internal standard (data not shown). Obviously, the preparation of trideuteromethyl esters by using hydrochloric acid in CD_3OD proceeds equally effective with synthetic ADMA (as well as with L-arginine, L-ornithine, L-tyrosine and most likely with many other amino acids) as with ADMA from biological matrices such as plasma and urine. In principle, this method is extendable to the isomeric symmetric dimethylarginine (SDMA) [11]. However, $MePFP_3$ derivatives of SDMA seem not to be best suitable for quantitative analyses and would require alternative derivatization reactions for the amino groups (unpublished observations).

We have found that other deuterium-labeled alkyl esters of ADMA such as pentadeutero-ethyl esters and octadeutero-propyl esters are equally suitable as IS in quantitative analyses. However, their synthesis requires use of considerably more expensive deuterium-labeled alcohols as starting materials (e.g., approximate price ratio for CD_3OD: CD_3CD_2OD: $CD_3CD_2CD_2OD$ = 1:2:9) or ^{13}C-labeled alcohols.

4.5. Modes of calculation of accuracy (recovery) values in the quantitative determination of endogenous substances in biological samples

Accuracy describes "The degree of closeness of the determined value to the nominal or known true value under prescribed conditions. This is sometimes termed trueness" [18]. Commonly, accuracy (recovery, %) of an analytical method for a given analyte in a certain matrix is determined by using the general formula (1), where $C_{Measured}$ is the analyte concentration measured in and C_{Added} is the nominal concentration of the analyte added to the sample. However, it should be emphasized that formula (1) is only valid for analytes such as most pharmaceuticals that do not physiologically occur in biological samples or are not present as contaminants therein, i.e., their basal concentration C_{Basal} is zero. If the analyte is physiologically present in the biological sample at a basal concentration C_{Basal}, C_{Basal} must be considered appropriately when accuracy is calculated. In these cases, formula (2) is most frequently used in the literature. Sometimes, however, formula (3) is used for determining methods accuracy for endogenously occurring analytes. The data of Table 6 show that very different and incorrect values for accuracy can be obtained when formula (3) is used instead of formula (2), notably if $C_{Basal} \gg C_{Added}$. These results strongly suggest that formula (2) is best useful for determining true values for accuracy of analytical methods applied to quantitate endogenous compounds in biological samples. In addition, formula (2) is best suited to evaluate the discriminatory power of the analytical method for added concentrations representing a small fraction of the basal analyte concentration.

$$\text{Recovery}\,(\%) = \left[\frac{C_{Measured}}{C_{Added}}\right] \times 100 \quad (1)$$

$$\text{Recovery}\,(\%) = \left[\frac{C_{Measured} - C_{Basal}}{C_{Added}}\right] \times 100 \quad (2)$$

$$\text{Recovery}\,(\%) = \left[\frac{C_{Measured}}{C_{Basal} + C_{Added}}\right] \times 100 \quad (3)$$

5. Conclusions

Commercial availability of stable-isotope labeled analogues is of great help, its freely accessibility to interested investigators facilitates scientific research, and enables a more reliable comparison of analytical results and techniques. However, as this work demonstrates, the use of commercially available stable-isotope labeled compounds as IS in quantitative analyses in biological systems is not automatically safe but it requires adequate standardization. Thus, the results reported here suggest that the commercially available d_7-ADMA preparation, i.e., [2,3,3,4,4,5,5-$^2H_7$]-ADMA·H_2O·HCl (catalog number DLM-7476-0; lot number PR-16061A; 5 mg), tested in this study against accurately weighed ADMA·2HCl reference standard from another commercial source (Sigma), contains about 20% more d_7-ADMA material than declared by the supplier.

The present study provides a solid proof for the utility of the de novo synthesis of trideuteromethyl esters as a useful means for the synthesis of suitable stable-isotope labeled analogues of amino acids for use as IS in quantitative analyses as exemplified here for ADMA in human plasma and urine by using GC-tandem MS. This approach may be of general use for amino acids and amino acid derivates in GC–MS- and LC–MS-based techniques. Thanks the robustness of the HCl-catalyzed methylation of the carboxylic group of ADMA, d_3Me-ADMA is suitable as an IS for the quantitative determination of ADMA in human plasma and urine by GC-tandem MS when analyzed as its $MePFP_3$ derivative. As expected, the commercially available d_7-ADMA was found to be a suited IS for ADMA. By

contrast and to our great surprise, we found that quantification by SRM of the common product ion m/z 378 generated by CID of the parent ion $[M–HF]^-$ of the $MePFP_3$ derivative of d_7-ADMA (i.e., m/z 641) and of unlabeled ADMA (d_0-ADMA, m/z 637) yielded higher ADMA concentrations than expected. We did not yet identify the underlying mechanism, but we were able to determine the extent of the commercially available preparation that does not contribute to the product ion m/z 378. This fraction was highly reproducible and amounted to about 33%. After correction for the falsely too high declared amount of supplied d_7-ADMA material and for the loss of m/z 378 from d_7-ADMA, very similar results were obtained for ADMA in human plasma and urine by using the commercially available d_7-ADMA and the de novo synthesized d_3Me-ADMA as IS.

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