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Discussion

Quantification of arginine and its mono- and dimethylated analogs NMMA, ADMA and SDMA in biological fluids by LC–MS/MS: Is LC superfluous?

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1. Introduction

L-Arginine is the substrate of nitric oxide synthase (NOS) which converts this semi-essential amino acid into nitric oxide (NO) and L-citrulline. As endogenous inhibitors of NOS [1], its L-*guanidino*-mono- and -dimethylated L-arginine analogs (Fig. 1), i.e., L-N^G-monomethyl-arginine (NMMA), L-N^G,N^G-dimethyl-arginine (ADMA, asymmetric dimethyl-arginine) and L-N^G,N'^G-dimethyl-arginine (SDMA, symmetric dimethyl-arginine), are of particular analytical, pharmacological and clinical importance (reviewed in Refs. [1–7]). Mass spectrometry (MS) based methods, i.e., GC–MS, GC–MS/MS, LC–MS and LC–MS/MS, for the quantitative determination of L-arginine, NMMA, ADMA and SDMA using commercially available and/or newly synthesized stable-isotope labeled analogs have been developed, thoroughly validated and published in recent years (reviewed in Refs. [2–7]). In all of the reported LC–MS/MS methods for these substances, HPLC ensured reliable quantification. In these methods, HPLC is not required for LC separation of individual analytes, but mainly to minimize matrix effects, notably ion suppression. Despite the need for LC in LC–MS/MS methods, accurate quantification of L-arginine, LNMA, ADMA and SDMA in plasma and urine can be carried out in less than 2 min, using specific collision-induced dissociation (CID) of both native and derivatized analytes [8].

Recently, Weaving et al. [9] reported the synthesis of d_2-SDMA, d_2-ADMA and d_2-NMMA and their use as internal standards (IS) for the simultaneous quantification of the endogenous counterparts in human plasma and urine by MS/MS without preceding HPLC separation, i.e., by directly injecting the eluate of SPE extracts into the mass spectrometer. L-Arginine was quantified by the same method using a commercially available d_7-L-arginine as the IS [9]. The authors concluded that their method "requires neither sample derivatization nor the need for chromatographic separation of analytes, shows good precision and accuracy and is suited for both research purposes and implementation in the busy, routine clinical laboratory" [9]. In our opinion, this study [9] suffers from many analytical and non-analytical shortcomings. In the present article, we discuss the article by Weaving et al. [9] from the analytical point of view, especially focusing on the importance of the HPLC step in quantitative analysis of L-arginine and its methylated derivates in biological samples and on issues closely related to the LC–MS/MS technique including matrix effects.

2. Method validation, matrix effects, comparability

2.1. Method validation

Weaving et al. [9] reported validation data for all investigated analytes in plasma and urine. But, validation, notably accuracy, was

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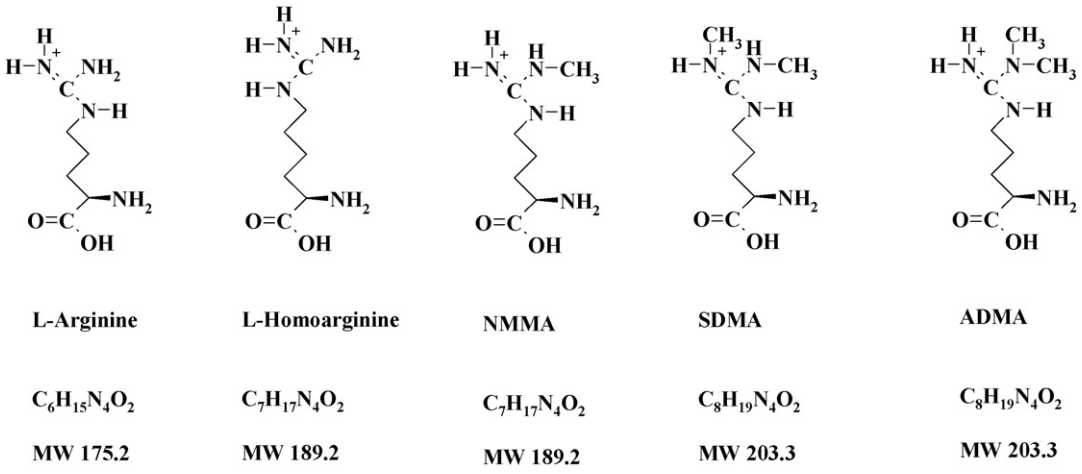

Fig. 1. Chemical structures, formulas and molecular weights of the protonated molecules of L-arginine, L-homoarginine, L-N^G-monomethyl-arginine (NMMA), L-N^G,N'^G-dimethyl-arginine (SDMA, symmetric dimethyl-arginine) and L-N^G,N^G-dimethyl-arginine (ADMA, asymmetric dimethyl-arginine).

inadequate because it was performed for only two analyte concentrations which are moreover too high and irrelevant on the basis of our current knowledge of the physiology and pathology of the L-arginine/NO pathway [2–7].

The biological variation of ADMA plasma concentrations in healthy humans is remarkably small with a RSD of 12% (n = 726) [10]. Most diseases are associated with very low increases in circulating ADMA concentration which are of the order of about 15% [4,7]. Also, equally minimal changes in ADMA plasma concentrations occur upon pharmacological intervention and/or change in disease state [4,7]. Analytical methods devoted to the quantitative determination of ADMA need to be adequately validated in a relevant concentration range, for instance for added concentrations in the range of 0–1000 nM of ADMA in plasma of healthy humans, as has been reported by us in this journal for ADMA using LC–MS/MS [8] and GC–MS/MS [11,12]. Same considerations equally apply to the linearity of the method, which has been tested for a very high concentration range, with 5 μM and 10 μM being the lowest concentrations for ADMA/SDMA and NMMA, respectively [9]. Consequently, on the basis of the validation data reported by Weaving et al. [9], the accuracy of their method for ADMA and related compounds has not been demonstrated for relevant concentrations, especially for ADMA, SDMA and NMMA in human plasma. In consideration of the relatively poor precision of the method for very high added concentrations of the analytes, for instance of a RSD of 23% for 20 μM of arginine added to plasma and urine [9], there are reasonable doubts about the validity of this HPLC step-sparing MS/MS method for L-arginine, ADMA, SDMA and NMMA.

Satisfactory characterization, stability of the label during chromatography as well as mass spectrometry, standardization of commercially available as well as of self-synthesized stable-isotope labeled analogs of analytes, and final added concentration of the IS in the matrices being analyzed is essential and crucial for reliable quantitative [12–14] and qualitative analyses (see below). In Ref. [9] such important information about the newly synthesized d_2-ADMA, d_2-SDMA and d_2-NMMA is not reported, neither on "cross-talk" and "cross-contamination" investigations between analytes and stable-isotope labeled internal standards.

2.2. Matrix effects

Omission of the HPLC step in LC–MS/MS has been shown to be sufficient for semi-quantitative methods such as in newborn screening for inherited metabolic diseases [15]. However, in that application a specific derivatization and specially designed neutral loss scans had to be performed to get reliable results without HPLC separation. The well-known and important phenomena in LC–MS/MS, i.e., ion enhancement and ion suppression, have been discussed by the authors in general terms but they did not provide any data [9]. Various protocols have been suggested and routinely applied for the evaluation of matrix effects including ion enhancement and suppression [16–18]. However, Weaving et al. did not provide data regarding potential matrix effects in their method [9].

Table 1
Major product ions (intensity >5%) obtained from the collision-induced dissociation of the $[M+H]^+$ ions of the unlabeled and labeled underivatized amino acids investigated in the present study.[a]

Analyte	$[M+H]^+$ (m/z)	Product ions (m/z)						
	A	B	C	D	E	F	G	H
L-Arginine	175	–	158	116	60	–	70	
L-Homoarginine	189	–	144	130	60	–	84	–
NMMA	189	–	144	116	74	57	70	–
d_6-NMMA	**195**[*b]	–	**150**[*]	**122**[*]	74	–	**76**[*]	–
d_3-NMMA	**197**[*]	–	**151**[*]	**121**[*]	**77**[*]	**60**[*]	**74**[*]	–
ADMA	203	–	158	116	88	71	70	46
d_6-ADMA	**209**[*]	–	**164**[*]	116	**94**[*]	**77**[*]	70	**52**[*]
d_7-ADMA	**210**[*]	–	**165**[*]	**123**[*]	88	71	**77**[*]	46
SDMA	203	172	158	116	88	71	70	–

Sources and abbreviations for isotopic labeled substances: [2,2′,3,3′,4,4′-$^2H_6$]NMMA (d_6-NMMA), [N^G-[$^2H_3$]methyl-1,2,3,4,5-$^{13}C_5$]ADMA (d_3-NMMA) and [*guanidino*-$^2H_6$]ADMA (d_6-ADMA) were synthesized as described elsewhere [13,14] using stable-isotope labeled precursors from Cambridge Isotope Laboratories (Andover, MA, USA). [2,3,3′,4,4′,5,5′-$^2H_7$]ADMA (d_7-ADMA) was purchased from Cambridge Isotope Laboratories.

[a] Analyses were performed on LC–MS/MS instruments as described elsewhere [8].

[b] Asterisks indicate ions with ^2H and/or ^{13}C label.

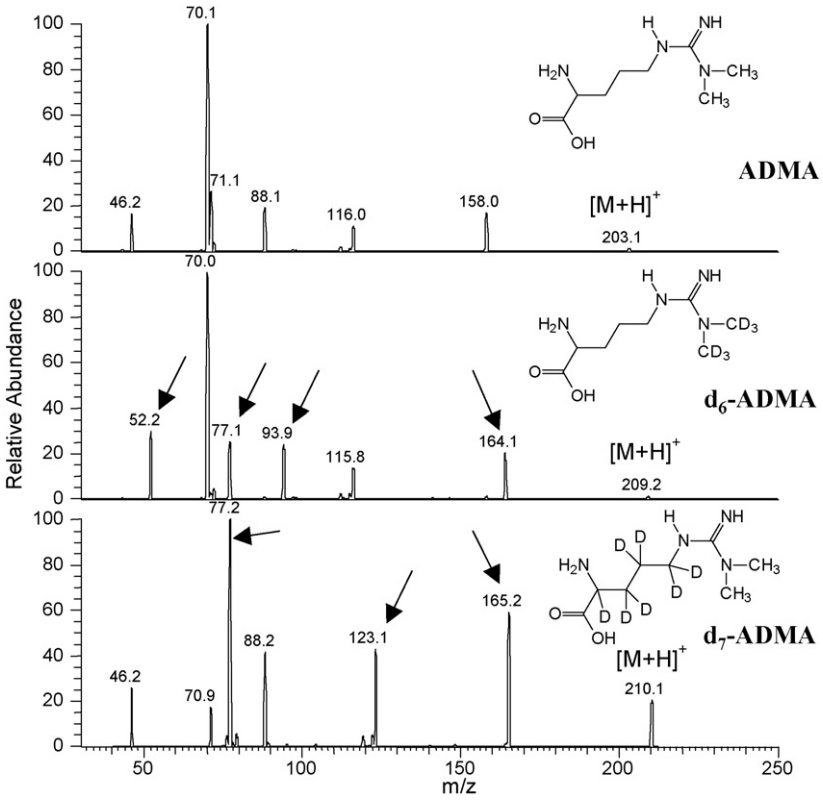

Fig. 2. Product ion mass spectra generated from underivatized ADMA species, i.e., unlabeled ADMA (upper panel), d_6-ADMA (middle panel) and d_7-ADMA (lower panel). The respective molecular ions $[M+H]^+$ were subjected to collision-induced dissociation under identical conditions. Deuterium-containing product ions are indicated by arrows. d_6-ADMA, [N^G-dimethyl-$^2H_6$]ADMA was synthesized according to [14]. d_7-ADMA ([2,3,3′,4,4′,5,5′-$^2H_7$]ADMA) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA).

2.3. Method selectivity

The cation-exchange SPE applied by Weaving et al. [9] for sample preparation can separate basic substances (such as the analytes) from acidic and in part from neutral ones. It can be anticipated that many of the myriads of endogenous substances including their phase I and phase II metabolites may impair the quantification of the target analytes because they are isobaric or may produce isobaric ions.

Weaving et al. [9] have rightly addressed in their article the potential interference of L-homoarginine, which is endogenously present in plasma [19], in the analysis of methylated L-arginine derivates, notably in those HPLC methods that use L-homoarginine as internal standard. However, the authors did not mention that homoarginine, which is isobaric to NMMA ($[M+H]^+$, *m/z* 189) and could theoretically contribute to NMMA, did not interfere with the analysis of NMMA in their method. Indeed, in the product ion mass spectrum of L-homoarginine we did not observe the presence of an intense product ion at *m/z* 70 from *m/z* 189 (intensity <1%) – same with the product ion observed from *m/z* 189 for NMMA (Table 1) – suggesting that endogenous L-homoarginine would not represent a considerable interference in the LC–MS/MS analysis of NMMA. However, this observation does not exclude the existence of other isobaric substances with *m/z* 189, such as *N*-acetyl-lysine – closely structured to homoarginine – and *N*-acetyl-glutamine [20,21], the CID of which could lead to product ions with *m/z* 70. Further isobaric pairs can be found for tryptophan and d_2-ADMA/d_2-SDMA, the IS used by Weaving et al. [9], or for d_7-arginine and tyrosine. Even if no interfering substances have been identified in a particular study, omission of the HPLC step may potently produce erroneous results in quantification. We assume that this could be the case in the study by Weaving et al. [9] for L-arginine, ADMA and LNMA.

2.4. Comparability issues

Agreement of the concentration of a certain endogenous analyte measured with different methods in a particular biological system such as plasma or urine of healthy humans in the basal state is an additional, fairly suitable criterion to value method validity [6]. This criterion has been successfully applied to numerous methods and analytes of the L-arginine/NO pathway [6], including ADMA, SDMA and L-arginine [2–7]. Application of the comparability approach to the concentrations and their variability reported in Ref. [9] reveals considerable disagreement for almost all analytes measured by this group.

The plasma concentrations of 162 ± 76 μM and the variation (RSD, 47%) for L-arginine in nine young healthy male volunteers measured by Weaving et al. [9] are very high and have not been reported in the literature for healthy humans in the basal state, thus far. Most of the reported methods for L-arginine from 16 different groups [3], including LC–MS/MS [8] and GC–MS [11] methods, yielded mean plasma concentrations for L-arginine of about 76 μM, i.e., more than two times lower than those reported by Weaving et al. [9]. This is a disagreement worth mentioning.

At first glance, the mean plasma concentration of 395 nM for ADMA reported by Weaving et al. [9] fits well into those reported by most of the reported MS-based methods (see for instance Refs. [4,7,10]). However, the variation of the mean of the order of 35% (i.e., range 177–627 nM of ADMA) in nine healthy male volunteers in the method of Weaving et al. [9] is considerably higher than that reported by the majority of other groups on a similar number of healthy volunteers [4]. The tracings illustrated in Fig. 5 of the article of Weaving et al. [9], which shows very broad peaks of a width of about 1.5 min at baseline, argue against the accuracy and the

precision of that method. Thus, we have estimated from the peak heights of the tracings of d_2-SDMA and SDMA that the concentration of SDMA in this "typical plasma sample" is about 120 nM. This value is very low and deviates greatly from the SDMA values shown in Table 5 of the article [9].

3. Delineation of mechanisms in CID processes

CID processes in GC–MS/MS and LC–MS/MS may be very complex and difficult to interpret. Delineation of fragmentation mechanisms commonly requires use of differently stable-isotope labeled and structurally well-characterized analogs. Proposing fragmentation mechanisms based on theoretical considerations but lacking experimental support is of little help. In our opinion the fragmentation mechanism for ADMA proposed by Weaving et al. [9] is very unlikely. It may reasonably be expected that a fragmentation mechanism would be common for structurally related compounds such as L-arginine, SDMA and NMMA and presumably for many other α-amino acids. Furthermore, the structure for the product ion with *m/z* 46 – most likely $[(CH_3)_2NH_2]^+$ but not $[CH(NH_2)OH]^+$ as proposed in Fig. 3 by Weaving et al. [9] – is very unlikely; presumably such a cation would be very unstable. The authors did not report in their article [9] experimentally generated data that would be supportive of such a fragmentation mechanism and of the proposed structure for the cation with *m/z* 46. Also, the cation $[C_7H_{12}N_3O_2]^+$ (*m/z* 170) shown in Fig. 3 of the article by Weav-

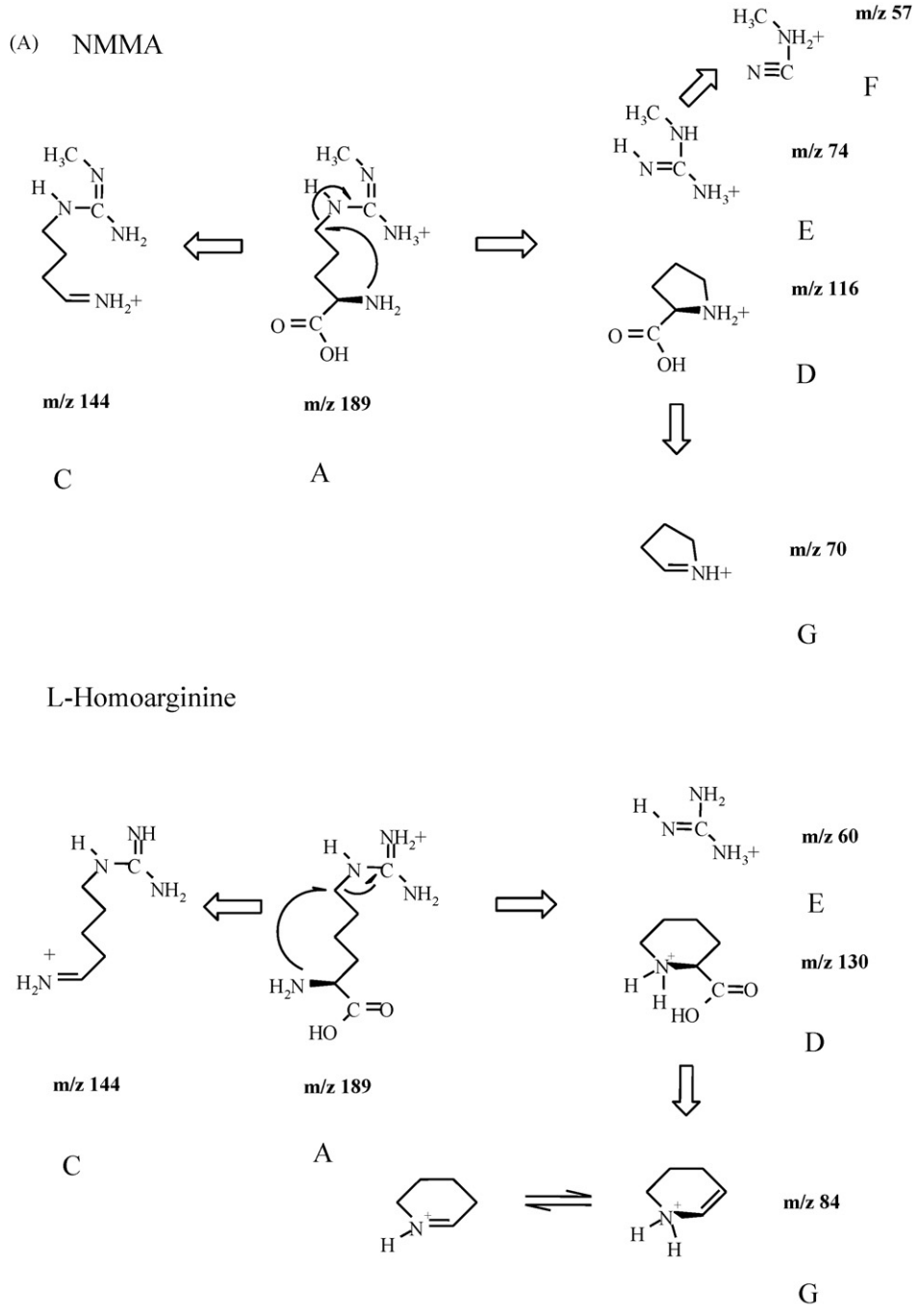

Fig. 3. Proposed mechanisms for the collision-induced dissociation of underivatized NMMA and L-homoarginine (A), and of ADMA and SDMA (B) under LC–MS/MS conditions. See also Table 1.

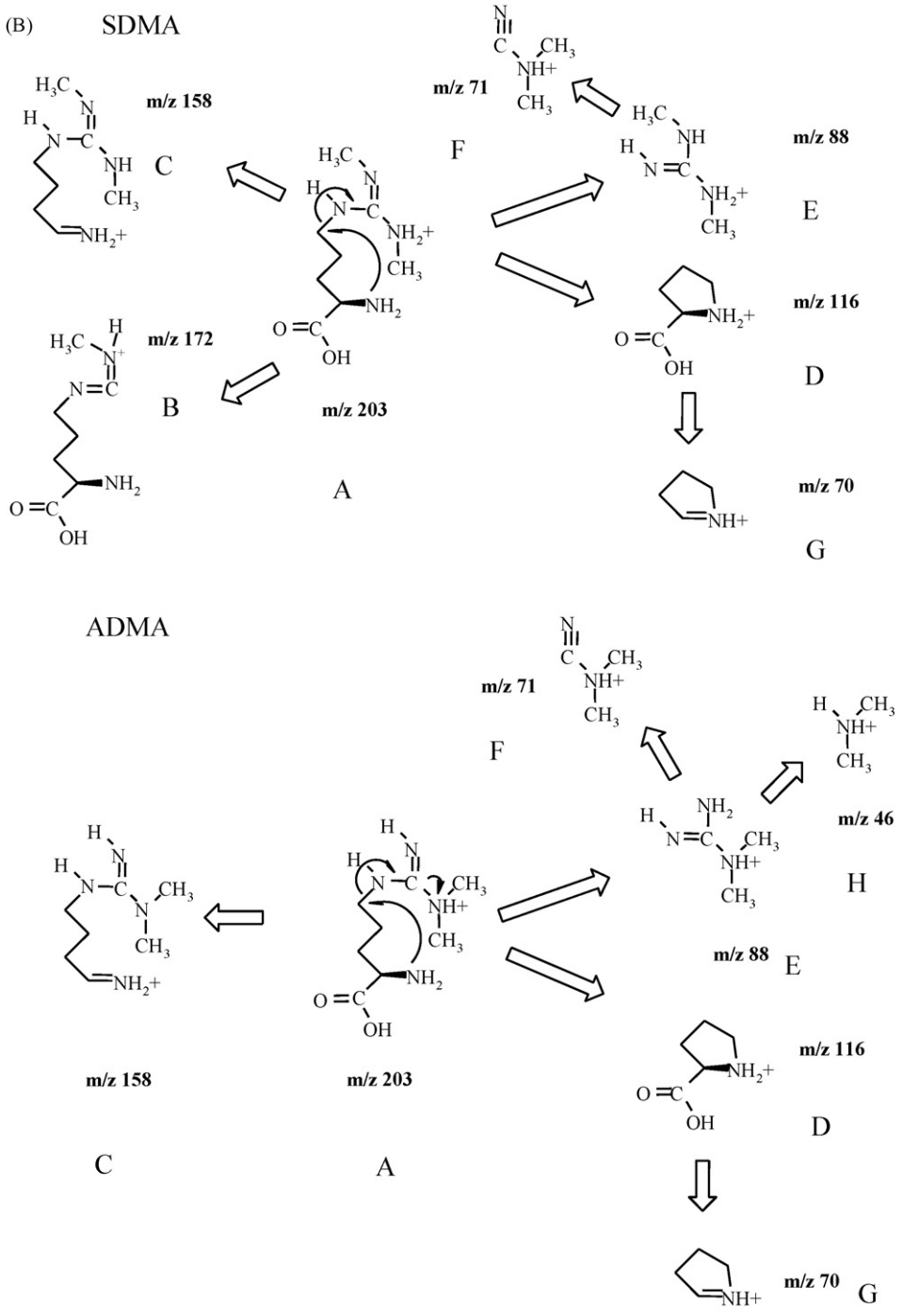

Fig. 3. (*Continued*).

ing et al. [9] contradicts the correct value of *m/z* 172 which has been used to generate the MS/MS spectrum shown in Fig. 2 of the article.

By using differently stable-isotope labeled ADMA analogs, we provide here unequivocal experimental evidence for the formation of the product ion *m/z* 46 and its structural identity as $[(CH_3)_2NH_2]^+$ both for *O*-butylated ADMA [8] and for native underivatized ADMA which contains deuterium in various positions (Fig. 2). The appearance of the ion at *m/z* 46 in the product ion mass spectrum of [2,3,3′,4,4′,5,5′-$^2H_7$]ADMA (d_7-ADMA) and of *m/z* 52 from [guanidine-dimethyl-$^2H_6$]ADMA is an irrefutable proof for the structure $[(CH_3)_2NH_2]^+$ for *m/z* 46 and $[(C_2H_3)_2NH_2]^+$ for *m/z* 52. It is likely that CID of the ions $[M+H]^+$ of ADMA, SDMA, NMMA, L-arginine and L-homoarginine involves a common fragmentation mechanism which is illustrated in Fig. 3.

4. Conclusions

Accurate quantification of ADMA and relatives has been a challenging analytical problem for long time. However, in recent years great progress has been made in this area due to appreciable contribution by many groups from all over the world [7]. Most of the currently available methods, including those being based on GC–MS/MS and LC–MS/MS, are routinely used in basic and clini-

cal research and produce reliable analytical results for ADMA and related compounds within a single run.

Minimization of sample work up in the quantitative analysis of endogenous compounds in biological samples is desirable and very tempting. However, especially in the area of the LC–MS/MS methodology, scientists tend to overestimate the inherent specificity of the MS/MS process. For the great majority of endogenous analytes, abandonment of the HPLC step in LC–MS/MS is not advisable. Preceding sample preparation steps such as SPE and HPLC analysis are necessary for reliable quantitative analysis by MS-based methods of L-arginine and its methylated analogs and numerous other biomarkers. Inadequately and insufficiently validated and tested analytical methods do not form the basis for drawing far-ranging conclusions.

In LC–MS/MS methods, thorough examination of matrix effects and potential interferences in quantitative analyses in accordance with widely accepted literature protocols is indispensable [16–18]. It is obvious that the dispensability of the HPLC step in quantitative LC–MS/MS methods has to be demonstrated experimentally. In our opinion, HPLC is necessary for accurate quantitative determination of L-arginine and its methylated analogs in plasma and urine by LC–MS/MS, because it minimizes matrix-associated effects and eliminates potentially interfering substances. Omission of the HPLC step would result in loss of selectivity and accuracy. Weaving et al. [9] have not provided a solid proof for the analytical reliability of their method for the quantification of L-arginine and its methylated derivates in human plasma and urine.

The problems seen in the LC–MS/MS analysis of L-arginine and its analogs are not unique to these amino acids, but they apply to many other classes of analytes including the large families of the F_2-isoprostanes [22] and the nitrated fatty acids [23]. However, suitable solutions have been provided for these biomarkers too [24,25]. Adequately validated and thoroughly and properly tested analytical methods are the key to successful basic and clinical research, and should be reported in a manner that allows for reliable evaluation of their analytical performance.

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