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Review

Chromatographic-mass spectrometric methods for the quantification of L-arginine and its methylated metabolites in biological fluids☆

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Abstract

L-Arginine (Arg) and its methylated metabolites play a major role in the synthesis of the cell signaling molecule nitric oxide (NO). Arg serves as a substrate for the enzyme NO synthase (NOS), which produces NO, whereas monomethylarginine (L-NMMA) and asymmetric dimethylarginine (ADMA) act as competitive inhibitors of NOS. Symmetric dimethylarginine (SDMA) has virtually no inhibitory effect on NOS activity, but shares the pathway for cell entry and transport with Arg and ADMA. Accurate and reliable quantification of these substances in various biological fluids is essential for scientific research in this field. In this review, chromatographic-mass spectrometric methods for Arg and its methylated metabolites ADMA and SDMA are discussed. Mass spectrometric detection provides an intrinsic higher selectivity than detection by means of UV absorbance or fluorescence. Taking advantage of the high selectivity, approaches involving mass spectrometric detection require less laborious sample preparation and produce reliable results. A consensus emerges that the concentration values in plasma of young healthy volunteers are about 65 μM for Arg, 0.4 μM for ADMA and 0.5 μM for SDMA.
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Keywords: Reviews; Arginine; Dimethylarginine; Mass spectrometry; HPLC; GC

Contents

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1. Introduction

1.1. Role of L-arginine and its methylated metabolites in the regulation of nitric oxide production

One class of metabolites of the amino acid L-arginine (Arg) stems from the methylation of its guanidino-nitrogen (N^G) atoms forming N^G-monomethyl-L-arginine (L-NMMA), N^G,N^G-dimethyl-L-arginine (asymmetric dimethylarginine, ADMA) or N^G,N'^G-dimethyl-L-arginine (symmetric dimethylarginine, SDMA) (Fig. 1) [1]. These metabolites derive from the post-translationally methylation of arginine residues in proteins by a group of enzymes termed protein methyltransferases (PRMT). Such methylation reactions play major roles in signal transduction, nuclear transport, or direct modulation of nucleic acid interactions [2]. PRMT-I is responsible for the asymmetric methylation of Arg moieties in the polypeptide chain resulting in the formation of ADMA, whereas PRMT-II forms SDMA. L-NMMA is formed by both isozymes, probably as an intermediate product on the way to the formation of ADMA or SDMA, respectively [3]. PRMT-I appears to be more active than PRMT-II since ADMA residues in the polypeptide chain are about 4 to 5 times more prominent than SDMA residues. The exact ratios between ADMA and SDMA are dependent on the cell type examined [4]. These methylated derivatives of Arg are liberated into the cytoplasm in the course of regular protein breakdown and turnover.

In another metabolic pathway, Arg serves as the substrate for an enzyme family termed nitric oxide synthase (NOS) which cleavages Arg into citrulline and nitric oxide (NO) [5]. The structural analogues of Arg, L-NMMA and ADMA, competitively inhibit these enzymes, whereas SDMA has virtually no such effect [6–8]. On the other hand, SDMA competes with ADMA and Arg for cell entry and re-absorption in the kidney and it must therefore be assumed that SDMA may influence indirectly NOS-catalyzed production of NO [9].

In various tissues NO exerts specific actions, and the production rate of NO is therefore a crucial factor for the physiological function of the respective tissue. For example, in neuronal tissues, NO is involved in the processes of learning and memory [10], and in the vascular endothelium NO is essential for the regulation of vascular tone and endothelial function [11]. NO production rate seems to be regulated by the concentration ratio of Arg and ADMA, and, to a much lesser extend, of Arg to L-NMMA. The minor role of L-NMMA stems from the fact that its concentration is about one order of magnitude lower than that of ADMA. Both ADMA and L-NMMA are eliminated chiefly by the action of the enzyme family dimethylarginine dimethylaminohydrolase (DDAH) [12], which hydrolyzes ADMA and L-NMMA to L-citrulline and dimethylamine or methylamine, respectively. Thus, the distribution of DDAH in NO-generating tissues and its specificity towards ADMA and L-NMMA support the idea that regulation of ADMA levels by DDAH might in turn regulate NOS activity [3]. Impaired activity of DDAH leads to elevated ADMA levels which have been identified as a marker for a range of diseases [13]. The activity of DDAH is largely influenced for example by the presence of reactive oxygen species (ROS) and homocysteine, which is a cardiovascular risk factor by itself [14,15]. Renal excretion is presumably of minor importance for ADMA and L-NMMA, but is, on the other hand, the only known elimination route for SDMA, since DDAH has no activity towards SDMA [16]. Because of this exclusively renal elimination and its largely constant production rate, SDMA has been identified as a new and superior marker for the onset and progression of renal insufficiency [17].

The quantitative determination of Arg, ADMA and SDMA in various biological fluids is of crucial importance to scientific progress in the field of cellular NO production and to investigate the clinical implications of disorders related to NO.

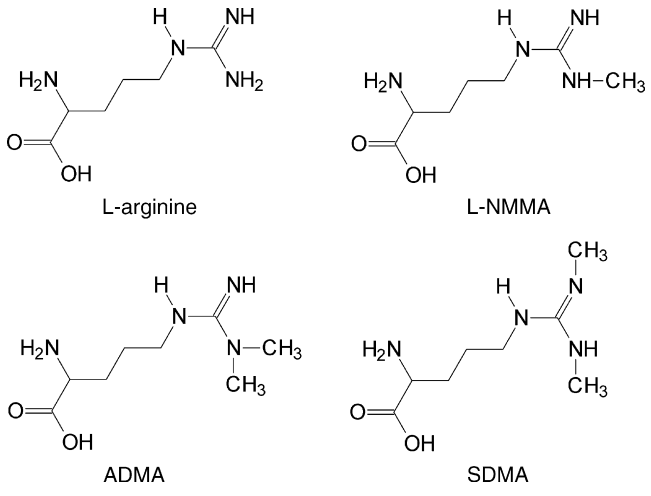

Fig. 1. Molecular structures of Arg, L-MMNA, ADMA and SDMA.

1.2. Methodological considerations regarding the quantification of L-arginine and its methylated metabolites

The quantification of Arg and its methylated metabolites in biological fluids is associated with numerous analytical difficulties. In plasma, most physiological amino acids are present in concentrations of the same order of magnitude as Arg, i.e. they are of the order of 50 μM. Typically, plasma concentrations of ADMA and SDMA are two orders of magnitude lower, with the concentration of L-NMMA being found to be another order of magnitude lower. This condition require assays of high specificity and sensitivity. Arg, ADMA, SDMA and L-NMMA are lacking of chromophores, so that no specific identification of these substances by UV absorbance detection is possible. Furthermore, as amino acids are polar, thermally labile and non-volatile compounds, their analysis without derivatization is difficult by means of reversed phase HPLC and impossible by GC. Likewise, effective separation and detection in CE methods requires derivatization of the analytes [18,19]. Nevertheless, because all physiological amino acids result in similar derivatives, the problem of unspecific detection persists, if the analytes are monitored by detectors of limited selectivity such as UV absorbance and fluorescence detectors. On the other hand, use of mass spectrometers add additional

dimensions of selectivity because analytes are identified due their characteristic molecular mass-to-charge (*m*/*z*) ratio, and, if applicable, by their fragmentation pattern, in addition to their characteristic retention times in HPLC or GC. Therefore, the selectivity of mass spectrometry-based approaches is considerably higher in comparison to conventional detectors used in HPLC and GC, and quantification errors due to interferences are minimized.

Another potential problem in the accurate quantification of Arg and its methylated metabolites is the availability of a suitable internal standard. Most of the conventional HPLC methods use homoarginine as an internal standard, which is, however, also an endogenous substance [20,21]. This may lead to systematic errors in the quantification, since the total concentration of the internal standard, i.e. endogenous plus added, is unknown and variable within individual samples. Similar problems may arise, when L-NMMA instead of homoarginine is used as internal standard [20]. In both cases, this problem can be partly overcome by using these substances at very high concentrations, resulting in an unfavorable concentration ratio of the internal standard and the analytes, and in consequence to increased imprecision and inaccuracy. A general problem of using homologues as internal standards, i.e. substances which are chemically different from the analytes, is the potentially different behavior in the sample preparation procedure. Such differences can be matrix-dependent and may lead to systematic errors in quantification. The only way to completely overcome these problems is the use of stable-isotope labeled analogues as internal standards and mass spectrometers which can discern between the endogenous analytes and their stable-isotope labeled counterparts.

Calibration of assays for endogenous substances such as Arg and its methylated metabolites is also associated with analytical problems and pitfalls. No biological matrix without an endogenous content of the analytes is available. When calibration in a matrix such as plasma is done by adding up the analyte in increasing concentrations, the resulting calibration curve will not go through the origin, but it will intercept the *y*-axis at a value corresponding to the endogenous concentration of the analyte in the matrix. Therefore, the true analyte concentration in the matrix is within the range of the lowest level of the calibration curve, where the analytical imprecision is in general most prominent. On the other hand, when the calibration curve is prepared in aqueous phase free of analyte, a possible matrix effect of the biological fluid will not be taken into account at all [22]. Non-consideration of matrix effects will most probably lead to inaccurate results in conventional methods as well as in approaches based on mass spectrometry. Especially in LC–MS interfaced with atmospheric pressure ionization (API) ion sources matrix effects may be very prone [23,24] (see Section 2.2.1 for a detailed discussion). Again, the problems associated with the construction of calibration curves can be overcome by the use of stable-isotope labeled analogues as internal standards in approaches combining chromatographic separation by HPLC or GC with a mass spectrometric detection, i.e. LC–MS or GC–MS.

2. Chromatographic-mass spectrometric approaches for the quantification of Arg and its methylated metabolites

2.1. Gas chromatographic-mass spectrometric methods

As polar amino acids, Arg and its methylated metabolites are unsuitable for GC unless they are derivatized on all polar centers of the molecules. A procedure for the quantitative determination of ADMA and Arg in biological fluids like human plasma and cell culture supernatant utilizing such a derivatization for subsequent GC–MS–MS analysis has been reported by Tsikas et al. [25]. The sample cleanup step in this method was ultrafiltration of plasma samples to remove proteins and subsequent evaporation of the biological liquid. Derivatization of the acid- and amino-functions of the amino acids took place in a first step by esterification with acidic methanol and in a second step by the conversion of the amino moieties into pentafluoropropionic acid amides with pentafluoropropionic anhydride (PFPA). After such a derivatization, Arg and ADMA were suitable for GC separation, resulting in sharp and symmetric peak shapes on a medium polar Optima-17 capillary column (Macherey Nagel, Germany). In the negative-ion chemical ionization (NICI) GC–MS–MS chromatograms no interfering peak were observed. Since SDMA reacted in the derivatization procedure to a product very different from ADMA, the two structural isomers were separated both by chromatographic and by and mass spectroscopic means. A typical chromatogram obtained from human plasma is depicted in Fig. 2. Internal standard for the quantitative determination of Arg was commercially available [$^{15}N_2$]-Arg. As no stable-isotope labeled I.S. for ADMA was available commercially, ADMA-[D_3]-methylester was synthesized by the reaction of ADMA with CD_3OD, analogously to the first derivatization step of the sample preparation procedure. To avoid scrambling of labeled and unlabeled methyl esters, the synthesis of the I.S. had to be performed separately from the first derivatization step of the samples. Thus, the first step in the sample derivatization was performed without an I.S. for ADMA. Although this is not ideal, Tsikas et al. [25] proved that the esterification step in the derivatization procedure was stable and reproducible, independently of the matrix of the sample. Therefore, the accuracy and precision of the ADMA determination was not impaired. Furthermore, Tsikas et al. [25] proposed the method of the in-line synthesis of a stable-isotope labeled I.S. as suitable for any other amino acid in the sample.

Another procedure for the determination of Arg and ADMA in human plasma and cell culture supernatant has been published by Albsmeier et al. [26]. Sample cleanup consisted of protein precipitation with acetone for plasma and solid phase extraction with carboxy acid ion exchange columns for cell culture supernatant. A two-step derivatization with acidic methanol and PFPA was performed prior to GC separation, similar to the procedure described by Tsikas et al. [25]. After separation on an Optima-17 capillary column (Macherey-Nagel, Germany), the analytes were detected by single stage NICI-MS. Typical chromatograms obtained from cell culture supernatant and human plasma are depicted in Fig. 3. No interferences from endogenous substances were observed in the chromatograms. ADMA

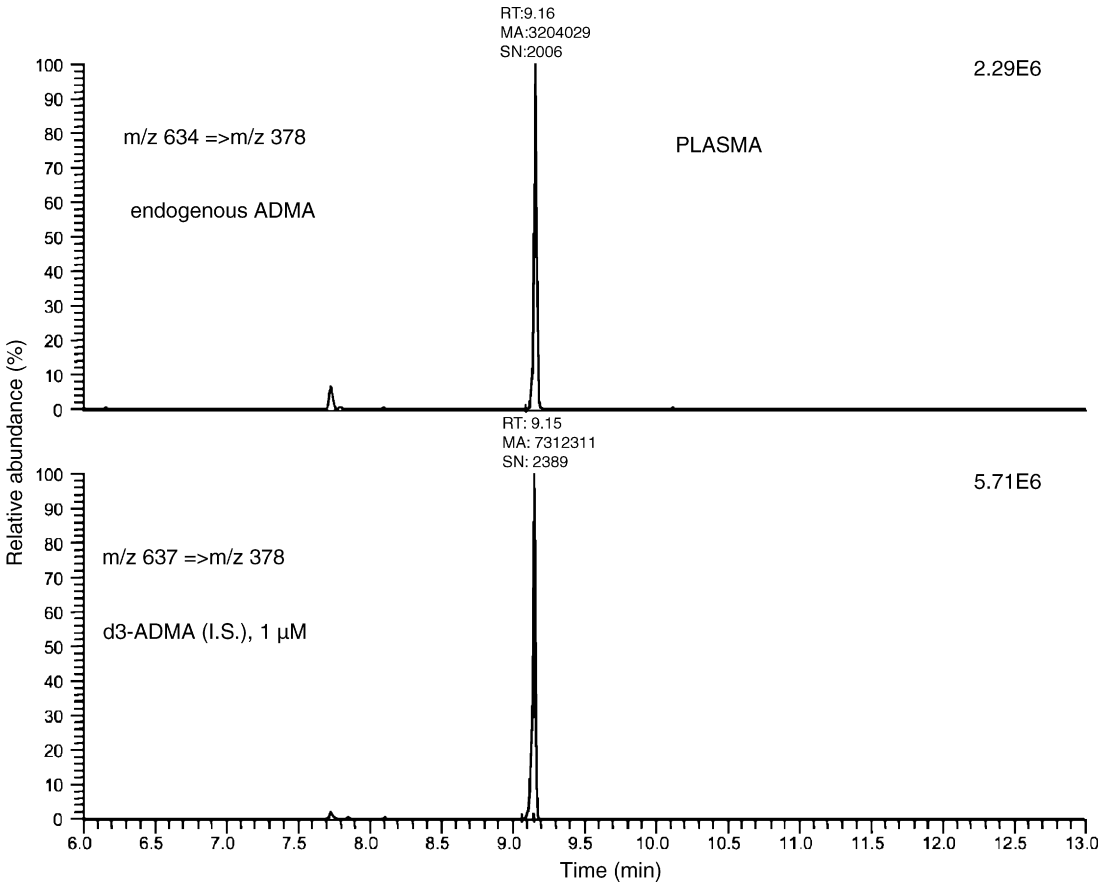

Fig. 2. Typical chromatogram obtained from human plasma by the GC–MS–MS method published by Tsikas et al. [25]. Reprinted from Journal of Chromatography B 798, D. Tsikas, B. Schubert, F.M. Gutzki, J. Sandmann, J.C. Frölich, Quantitative determination of circulating and urinary asymmetric dimethylarginine (ADMA) in humans by gas chromatography–tandem mass spectrometry as methyl ester tri(*N*-pentafluoropropionyl) derivative, 87–99, 2003, with permission form Elsevier.

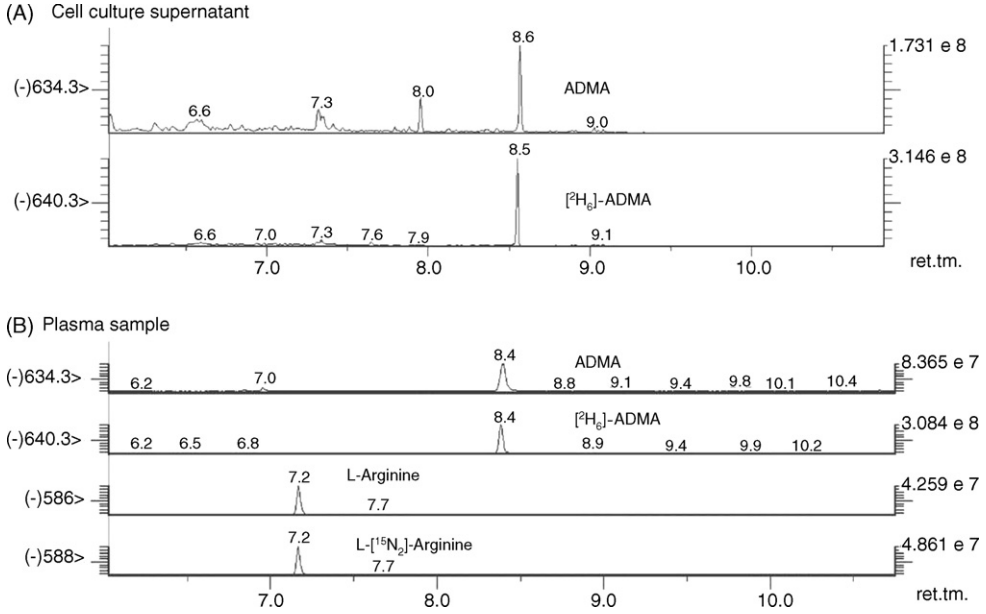

Fig. 3. Chromatograms obtained by the GC–MS method of Albsmeier et al. [26] from cell culture supernatant (A) and human plasma (B). Reprinted from Journal of Chromatography B 809, J. Albsmeier, E. Schwedhelm, F. Schulze, M. Kastner, R.H. Böger, Determination of N^G,N^G-dimethyl-L-arginine, an endogenous NO synthase inhibitor, by gas chromatography–mass spectrometry, 59–65, 2004, with permission from Elsevier.

and SDMA were separated due to the fact that the two substances reacted in the derivatization procedure to different products. The I.S. applied for the quantification of Arg and ADMA were commercially available stable-isotope labeled Arg and in-house synthesized stable-isotope labeled ADMA. The labeled ADMA was synthesized from a [D_6]-ornithine-copper-complex with dimethylamine on activated bromcyan–agarose, following the procedure published by Pundak and Wilchek [27]. Due to the application of stable-isotope labeled analogues for Arg and for ADMA, Albsmeier et al. achieved a stable and accurate method.

2.2. High-performance liquid chromatography-mass spectrometric methods

2.2.1. Matrix effects in liquid chromatography–mass spectrometry

Atmospheric pressure ionization is the currently most used ionization technique for quantitative LC–MS methods. Two general subtypes of this technology exist: electrospray (ESI) and atmospheric pressure chemical ionization (APCI). Both are considered as "soft" ionization procedures and both produce mainly protonated or deprotonated ions without fragmentation [23]. In both ESI and APCI, the ionization rate of the analytes depends strongly on the physicochemical environment in the ion source. ESI is reported to be more susceptible to subtle changes in the characteristics of the LC-effluent than APCI. However, in both methods samples with a complex matrix like biological fluids can cause MS signal suppressions or enhancements, which are termed "matrix effects". It is widely believed that these effects are due to ionization competition between different species eluting from the HPLC column. Matrix effects are generally not reproducible nor repeatable between various samples or even between different injections of the same sample and, thus, can severely compromise quantitative analysis [23]. Therefore, the careful evaluation of matrix effects has to be an integral part of the validation of quantitative methods in LC–MS.

To investigate matrix effects, Souverain et al. [23] proposed a technique in which they infused a solution of the analyte into the LC effluent after injection of samples prepared without the spiking with this analyte. In such an experimental setup, the MS signal of the analyte should be ideally a line at constant intensity while the analyte is infused at a constant rate. Any enhancement or suppression of the analyte signal is therefore due to a matrix effect of the sample. Typical results obtained (according to the sample preparation and the chromatographic method described in [28]) by such experiments are depicted in Fig. 4. The infusion of stable-isotope labeled Arg, which was not present in the prepared samples, resulted in a signal with constant intensity, if a water sample causing no matrix effects was injected. In contrast, the injection of a plasma sample resulted in episodes of strong ionization suppression in the early phases of the chromatogram.

Matrix effects can be minimized by improving the sample preparation to achieve as clean as possible extracts, by optimizing the chromatographic procedure to separate the analytes from the matrix effects, by changing the ionization conditions, or by a combination of the above. However, the most efficient way to circumvent matrix effects is the use of stable-isotope labeled

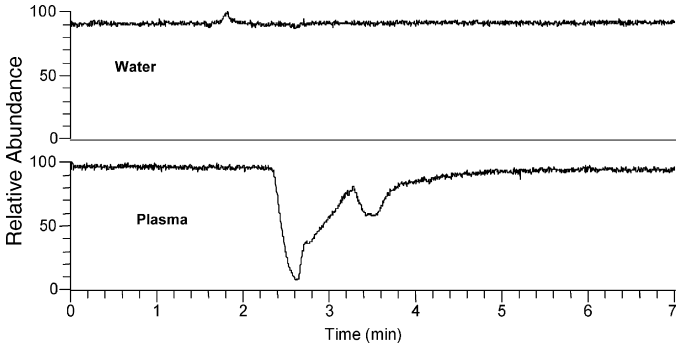

Fig. 4. Evaluation of the matrix effect in plasma. For details see text. Chromatographic setup was according to Martens-Lobenhoffer and Bode-Böger [28]. The observed MS–MS signal of the infused $^{13}C_6$-Arg was m/z 181 → 74.

I.S. [24]. Both the signal intensities of the analyte and its stable-isotope labeled analogue were influenced by any matrix effects in the same way, leaving the ratio between them constant and therefore a reliable quantification can be achieved despite any matrix effects. In Fig. 5, calibration curves of ADMA (obtained with the method described in [28]) are depicted with regard to the I.S. applied (own results, not previously published). As can be seen, the calibration slopes obtained from water and plasma are identical, if [D_6]-ADMA was used as I.S., whereas the slopes differ extremely if instead [$^{13}C_6$]-Arg was applied as I.S. Thus, systematically wrong quantification results may be obtained if no stable-isotope labeled analogue is applied as I.S. and the matrix effect is not adequately considered. This finding underlines the usefulness of stable-isotope labeled I.S. in obtaining reliable results.

2.2.2. Methods utilizing LC–MS or LC–MS–MS

Vishwanathan et al. reported the first LC–MS–MS method for Arg and its methylated metabolites [29]. After protein precipitation with acetonitrile and solvent evaporation, the underivatized amino acids were separated on a straight phase silica column using a mobile phase consisting of 0.1% formic acid–10 mM ammonium formate in water (solvent A) and acetonitrile–methanol (1:1, v/v, solvent B). The isocratic mobile phase consisted of 95% solvent A and 5% solvent B. The chromatographic run time was about 15 min with complete separation of ADMA and SDMA, but the noise level in the chromatograms was high and the peaks were not always completely separated from endogenous interferences. Especially the peak for L-NMMA was severely distorted by noise and peaks of unidentified endogenous substances (see Fig. 6), leading to doubts about its quantification results. Quantification was carried out by ESI–MS as well as by ESI–MS–MS, with quantification in the MS–MS mode apparently not substantially improving selectivity. No internal standard other then stable-isotope labeled Arg was utilized, leaving the quantification of ADMA, SDMA and L-NMMA prone to errors due to unknown matrix related ionization suppression or enhancement effects. Possibly, the very low concentrations of ADMA and SDMA found in unknown plasma samples by this method can be related to such effects, because the sample preparation of the calibration samples and the unknown samples were performed in a different manner,

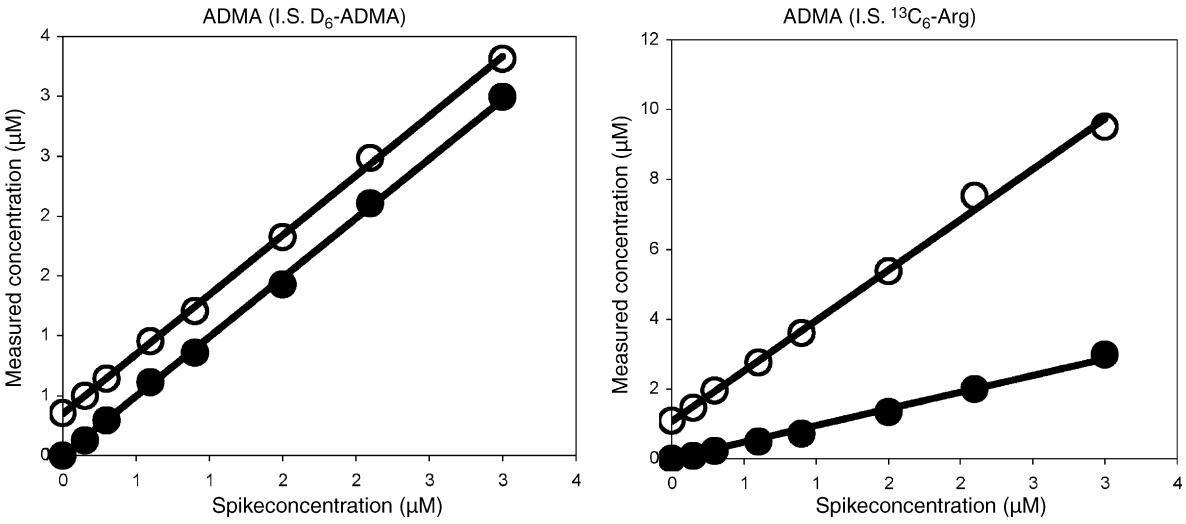

Fig. 5. Calibration curves of ADMA from water (●) and plasma (◯). In the left panel [D_6]-ADMA was used as I.S., in the right panel instead [$^{13}C_6$]-Arg. Results obtained with the method described by Martens-Lobenhoffer and Bode-Böger [28].

leading to different sample constitution with different effects on the ionization process in the mass spectrometer.

The retention of Arg and its methylated metabolites in their underivatized state on a reversed phase C18 column was achieved by Huang et al. from urine [30] as well as from plasma samples [31]. Both methods are quite similar with regard to HPLC separation and MS detection. For plasma, isocratic elution with a mobile phase consisting of water–acetonitrile (90:10, v/v) with 0.5% trifluoroacetic acid (TFA) was applied. The mobile phase for urine samples was water–acetonitrile (95:5, v/v) with 0.4% TFA. It was demonstrated that the amount of the added TFA was critical for the performance of the separation [31]. This is probably due to the ion-pairing abilities of TFA, which make the retention on a C18 column possible. Both described mobile phases lead to estimated chromatographic capacity factors of the analytes in the range of 0.8 to 2.3, but with poor peak shape and unsatisfactory resolution in the case of urine (Fig. 7). Protein precipitation by 5-sulfosalicylic acid was the only sample preparation step in plasma and urine samples. The analytes were detected with APCI–MS in the selected ion monitoring (SIM) mode. The APCI ion source provided satisfactory sensitivity despite the high water content of the HPLC mobile phase and the ionization suppressing properties of TFA. The only internal standard used for all analytes in the two procedures was homoarginine.

As it was demonstrated by Vishwanathan et al. [29] and Huang et al. [30,31], the very polar endogenous amino acids were not easily to retain and separate on reversed phase HPLC columns. As a consequence of these findings, Martens-Lobenhoffer and Bode-Böger [32] developed an assay applicable to human plasma and urine, which derivatized the analytes with *ortho*-phthalaldehyde (OPA) and 2-mercaptoethanol as co-reagent prior to HPLC separation, following similar approaches developed for fluorescence detection [20,33–37]. Unlike HPLC–OPA methods with fluorescence detection, the OPA/2-mercaptoethanol derivatives of Arg, ADMA and SDMA

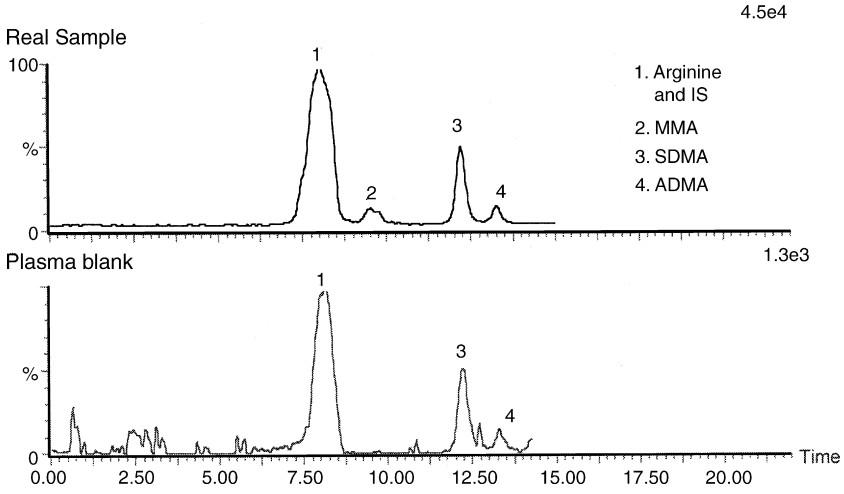

Fig. 6. Typical chromatograms obtained by the method of Vishwanathan et al. [29] from human plasma with LC–MS–MS. Reprinted from Journal of Chromatography B 748, K. Vishwanathan, R.L. Tackett, J.T. Stewart, M.G. Bartlett, Determination of arginine and methylated arginines in human plasma by liquid chromatography–tandem mass spectrometry, 157–166, 2000, with permission from Elsevier.

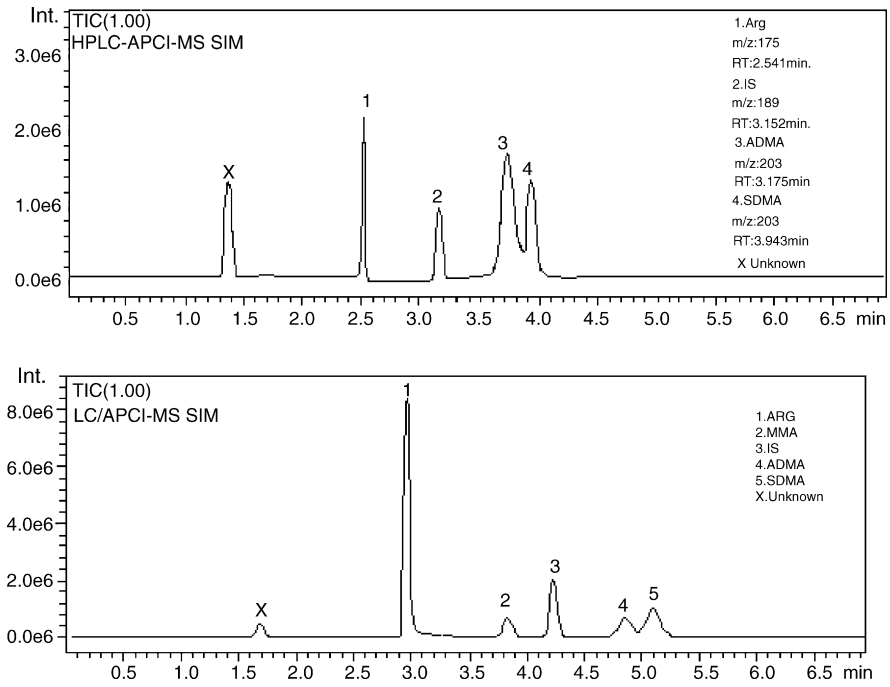

Fig. 7. Typical chromatograms from human urine (top) and plasma (bottom) by the methods described by Huang et al. [30,31]. Reprinted from Analytica Chimica Acta 487, L.F. Huang, F.Q. Guo, Y.Z. Liang, Q.N. Hu, B.M. Cheng, Rapid simultaneous determination of arginine and methylated arginines in human urine by high-performance liquid chromatography–mass spectrometry, 145–153, 2003, with permission from Elsevier and reprinted from Analytical and Bioanalytical Chemistry 380, L.F. Huang, F.Q. Guo, Y.Z. Liang, B.Y. Li, B.M. Cheng, Simultaneous determination of L-arginine and its mono- and dimethylated metabolites in human plasma by high-performance liquid chromatography–mass spectrometry, 643–649, 2004, with permission from Springer.

were analyzed by ESI–MS [32]. Because of the superior selectivity of the mass spectrometric detection, the laborious sample cleanup necessary for the relatively unselective fluorescence detection could be avoided, and sample preparation was reduced to protein precipitation for plasma and dilution for urine samples. In addition to Arg and its methylated metabolites, the amino acid L-citrulline, which is also involved in the Arg metabolic pathway (see Section 1), could be quantified in the same run. This was impossible in HPLC fluorescence assays applying sample preparation with solid phase extraction [20,34–36], in which citrulline was excluded in the sample cleanup processes. HPLC separation was achieved by elution with a formic acid/ammonium formate buffer (solvent A)—methanol (solvent B) gradient on a C18 column. The gradient started at 70% solvent A and 30% solvent B, while solvent B was increased to 50% during the run-time. Since ADMA and SDMA produced OPA derivatives with identical chemical formula, they could not be discerned by LC–MS. Quantification of ADMA and SDMA by this method required their complete chromatographic separation by using quite long gradient run times of 27 min. The internal standards used in this method were commercially available stable-isotope labeled Arg for the quantification of Arg and homoarginine for the quantification of ADMA, SDMA and citrulline. Thus, the quantification of ADMA, SDMA and citrulline was susceptible to matrix related effects on the ionization efficiency of the mass spectrometer and therefore not optimal in terms of reliability, accuracy and precision.

In consequence of the shortcomings of the above discussed LC–MS method [32], Martens-Lobenhoffer et al. [38] developed an advanced method with the main feature of applying in-house produced stable-isotope labeled ADMA in addition to stable-isotope labeled Arg as a second internal standard. The labeled ADMA was synthesized from a [D_6]-dimethylamine with an ornithine-copper-complex on activated bromcyan–agarose, following the procedure published by Pundak and Wilchek [27]. Another improvement of the method regarded the chromatographic separation with better peak shapes due to the addition of trimethylamine to the mobile phases (Fig. 8). Stable-isotope labeled analogues as internal standards both for Arg and for ADMA made the quantification of theses substances independent of the biological matrix and therefore very reliable in terms of accuracy and precision.

Kirchherr and Kühn-Velten [39] reported an LC–MS–MS method for the quantification of ADMA and SDMA in human plasma, utilizing separation of the analytes in their underivatized state on a porous graphitic carbon HPLC column (Hypercarb, Thermo Electron). This material allowed the retention of amino acids due to its unique interaction properties. After protein precipitation by the addition of acetonitrile–methanol (9:1, v/v), the samples were diluted with water and underwent subsequent HPLC chromatography with a water–0.1% TFA (solvent A) and acetonitrile (solvent B), applying a gradient ranging from 5 to 20% solvent B. The addition of TFA to the mobile phase was necessary to achieve complete chromatographic separation of ADMA and SDMA. The analytes were determined by ESI-turbo-ion-spray in the MS–MS mode. Analysis time was short (6 min), chromatography featured sharp and symmetrical peaks and do not show any interferences from endogenous substances. Because ADMA and SDMA could not be completely discriminated by MS–MS in this method, accurate quantification

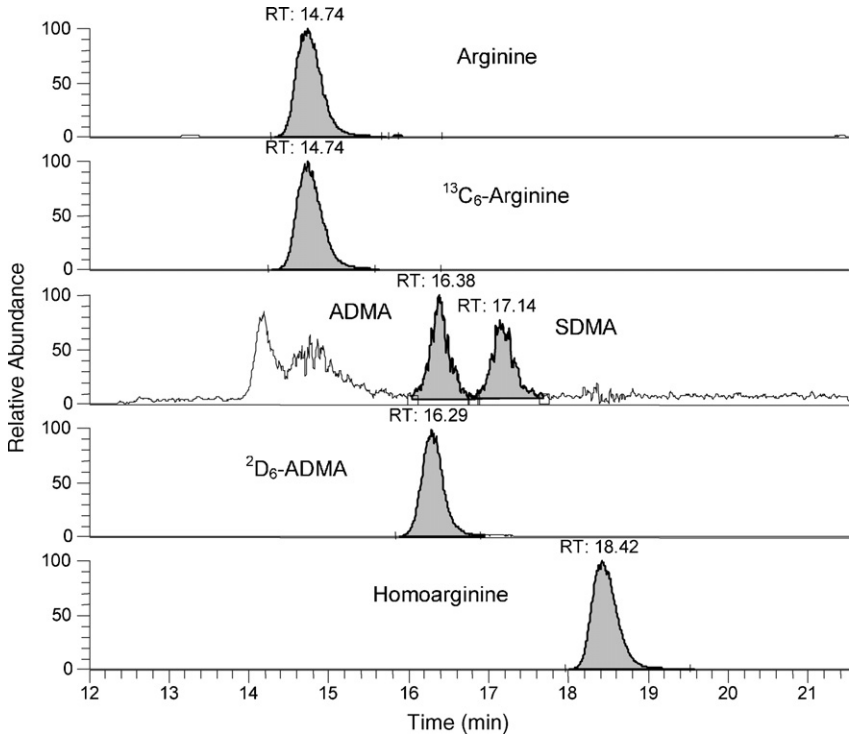

Fig. 8. Typical chromatogram obtained by the method of Martens-Lobenhoffer et al. [38] from human plasma.

required complete chromatographic separation of ADMA from SDMA. Commercially available stable-isotope labeled Arg and stable-isotope labeled leucine were tried as internal standards, with better results provided by the leucine analog. However, the leucine peak in the chromatograms was very distorted and a matrix related ionization suppression or enhancement was obvious by the fact that the calibration slopes of ADMA and SDMA were dependent on the I.S. and the matrix (water or plasma) investigated.

A new type of derivatization to achieve retention on RP-HPLC for Arg and its methylated metabolites and to improve their mass spectrometry behavior was reported by Schwedhelm et al. [40]. They esterified the carboxylic groups of the analytes with 1-butanol in HCl solution after protein precipitation of the biological samples and solvent evaporation. The derivatized samples were separated on a short C18 column using a mobile phase consisting of water–0.1% formic acid (solvent A) and methanol–0.1% formic acid (solvent B), applying a gradient ranging from 98:2 to 50:50 for the ratio of solvent A and solvent B, respectively. The chromatograms showed sharp and symmetrical peaks and analysis time took only 4 min, providing a high sample throughput. Under these conditions, ADMA and SDMA were not completely separated, but these substances were discriminated by MS–MS due to different fragment ions. The observed m/z for the precursor → fragment ion transitions were m/z 259 → 214 and 259 → 228 for the derivatives of ADMA and SDMA, respectively. These fragment ions related to the neutral loss of dimethylamine and methylamine, respectively, both moieties distinctive for their respective parent molecules. The method featured commercially available stable-isotope labeled Arg for Arg and in-house synthesized stable-isotope labeled ADMA (same as described in [26]) as internal standard for ADMA and SDMA. This validated LC–MS–MS method led to accurate and precise results for Arg, ADMA and SDMA in human plasma.

An LC–MS–MS method for the separation and quantitative determination of 76 underivatized amino acids and related compounds including Arg and ADMA by reversed phase ion-pairing chromatography was described by Piraud et al. [41]. The ion-pairing agent was tridecafluoroheptanoic acid, and the separation took place on a reversed phase C18 column with an acetonitrile gradient. Sample preparation consisted of protein precipitation for plasma as well as for urine samples. The internal standards for Arg and ADMA were stable-isotope labeled Arg and stable-isotope labeled lysine, respectively. This method was originally designed for the detection of inherited disorders in amino acids, and therefore the quantification of ADMA by this method should be considered rather as a "by-product". Thus, the validation of the method focused on other aspects than the quantification of ADMA. The retention times of Arg and ADMA were very close in this system. Thus, one may suspect that a separation of ADMA and SDMA has not been achieved. Also, the observed fragment ion of ADMA was also present in SDMA, suggesting that ADMA and SDMA cannot be quantified separately by this LC–MS–MS method. Method validation was performed in samples spiked with super-physiologically high concentrations of ADMA, i.e. 200 μM for plasma and 213 μM for urine. The method by Piraud et al. [41] should be regarded as not applicable to the quantitative determination of ADMA in biological fluids in unaltered and altered states of the L-arginine/NO pathway and it is not further discussed in this review.

Recently, Martens-Lobenhoffer et al. reported an LC–MS–MS method for the separation of Arg, ADMA and SDMA by hydrophilic interaction liquid chromatography (HILIC) [28]. In this method, the amino acids were separated in their underivatized state on a straight phase silica column. However, no typical straight phase chromatography mobile phase was used, but a mobile phase consisting of water, acetonitrile, TFA and propionic acid (10:90:0.025:1, v/v). In a HILIC separation, analytes are distributed between the relatively apolar mobile phase and a thin film of water which builds up on the silica surfaces of the stationary phase. HILIC has great advantages in LC–MS analysis of polar substances over reversed phase chromatographic approaches. The most important one is that the mobile phase greatly favors the ionization process in the ESI ion source [42]. The amount of TFA used in the mobile phase of this method was required to provide symmetrical peak shapes, whereas propionic acid was added to compensate for the sensitivity loss caused by the ion suppression effects of TFA in the ESI ion source. The chromatographic run time was 8 min. ADMA and SDMA were not completely separated under these chromatographic conditions, but they were completely discerned by their different fragmentation pattern in the mass spectrometer working in the MS–MS mode. The observed *m/z* values of the fragment ions were *m/z* 203 → 46 for ADMA and *m/z* 203 → 172 for SDMA, respectively. The fragment ion *m/z* 46 was related to the formation of a dimethylammonium ion, a moiety that was found only in ADMA and not in SDMA, whereas the fragment ion *m/z* 172 was related to the neutral loss of methylamine, a moiety that was found only in SDMA and not in ADMA. In observing these fragment ions, no cross-talk was observed between ADMA and SDMA and vice versa. This MS–MS fragmentation pattern of ADMA and SDMA is depicted in Fig. 9. A typical chromatogram obtained from human plasma is depicted in Fig. 10. The sample preparation in this method comprised only protein precipitation by using the HPLC mobile phase. Stable-isotope labeled Arg (commercially available) and ADMA (in-house-made, described in [38]) analogues were used as internal standards. The method was validated for Arg, ADMA and SDMA in human plasma, urine and in cell culture supernatant. Quantification of Arg and ADMA was independent of the matrix, whereas SDMA had to be calibrated in authentic matrix. Precise and accurate quantitative results were obtained for all three analytes.

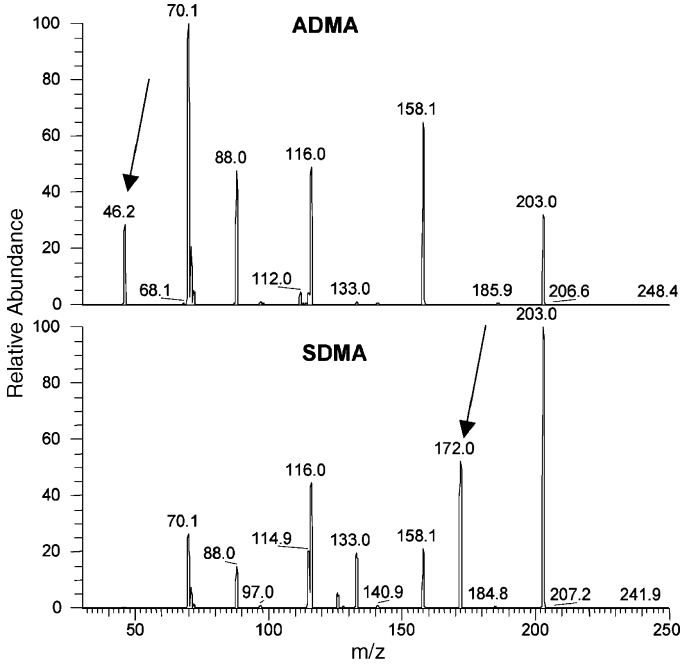

Fig. 9. MS–MS fragmentation pattern for ADMA and SDMA. The distinctive fragment ions *m/z* 46 for ADMA and *m/z* 172 for SDMA are marked.

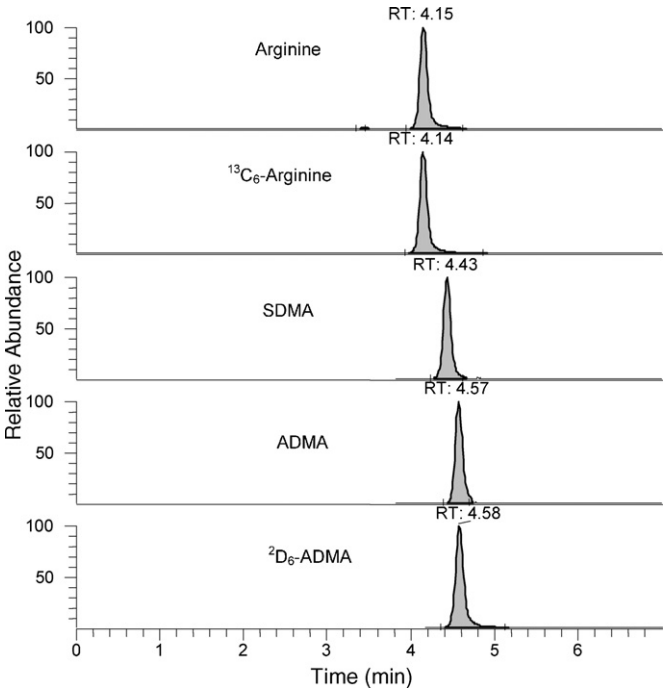

Fig. 10. Typical chromatogram obtained by the method of Martens-Lobenhoffer and Bode-Böger [28] from human plasma.

3. Comparison of chromatographic-mass spectrometric approaches

3.1. Discussion of the analytical characteristics of the methods

In analytically oriented publications, the customarily discussed criteria for analytical performance, i.e. limit of detection (LOD) and limit of quantification (LOQ), are not of very much benefit in the case of the quantification of Arg and its methylated metabolites. Since there is no relevant biological matrix available without an endogenous content of these analytes, LOD and LOQ values are usualy evaluated from non-biological matrix samples, e.g. in aqueous solution. In these cases, the analytical methods always provided plenty of sensitivity reserve. Thus, LOD and LOQ data derived from analyses in aqueous solutions of the analytes have limited significance. Nevertheless, in Table 1 the LODs and LOQs of the here-discussed methods are summarized. The LOQs were assessed by two different methods: either by the definition that the peak to noise ratio has to be 1:10, or

Table 1
Limits of detection (LOD), limits of quantification (LOQ) and intra-day precision (R.S.D.) of MS-based methods in human plasma

First author [ref.]	LOD (μM)			LOQ (μM)			R.S.D. (%)[a]		
	Arg	ADMA	SDMA	Arg	ADMA	SDMA	Arg	ADMA	SDMA
Vishwanathan [29]	0.006	0.005	0.005	5.7	0.05	0.05	11.3	8.9	10.2
Tsikas [25]		0.0001			0.0003[b]			3.2	
Martens-Lobenhoffer [32]				3.75	0.20	0.15	3.7	7.5	7.9
Albsmeier [26]		0.0004			0.05			4.71	
Huang [31]	0.3	0.01	0.01	1.0	0.2	0.2	3.7	4.3	4.4
Martens-Lobenhoffer [38]				7.5	0.15	0.2	1.31	4.65	6.45
Kirchherr [39]		0.17	0.14		0.05	0.05		6.0	6.9
Schwedhelm [40]				1	0.05	0.05	3.3	2.6	2.5
Martens-Lobenhoffer [28]	0.4	0.02	0.01	7.5	0.15	0.20	4.5	5.5	3.9

[a] Intra-day R.S.D. values of the lowest reported concentration (basal or lowest spike-level) of plasma samples.
[b] LOQ defined as peak to noise ratio of 1:10. In all other cases LOQ defined as lower end of calibration range.

with respect to the lower end of the calibration range, whereas the first definition usually results in a lower LOQ.

Of much more interest are the analytical characteristics precision, accuracy and selectivity. The absolute accuracy (i.e. the deviation of a measurement from the "true value") of a method is quite difficult to evaluate and is discussed under the next subheading. The precision values (summarized in Table 1) of the here discussed methods are generally satisfactory, with relative standard deviations (R.S.D.) in plasma samples being less than 8% at the physiological concentration level. The only exception is the method of Vishwanathan et al. [29] with R.S.D. values ranging between 8.9% and 11.3%, depending on the analyte.

The selectivity of the here-discussed mass spectrometric methods is considerably better than in assays depending on conventional detection technologies such as fluorescence or UV absorbance. Despite the use of less sophisticated sample preparation procedures in GC–MS and LC–MS methods, generally these methods provide unequivocal identification and reliable quantification of Arg, ADMA and SDMA in the presence of numerous endogenous substances. A critical point in the HPLC assays remains the separation of ADMA from SDMA. Especially the two procedures of Huang et al. displayed marginal [31] to poor [30] (see Fig. 7) separation between ADMA and SDMA. Schwedhelm et al. [40] and Martens-Lobenhoffer and Bode-Böger [28] overcame the problem of incomplete chromatographic separation by means of the MS–MS technology that enables to distinguish ADMA and SDMA unambiguously by their different fragmentation pattern. In GC–MS methods, the distinction between ADMA and SDMA relies on a different principle, because the two substances are derivatized to different products which can be separated both chromatographically and mass spectrometrically.

3.2. Basal levels determined by the different methods

The most vital performance criterion of a quantitative analytical method is the accuracy of the results produced. Here accuracy is defined as the deviation of the measurements from the "true value", which in principal cannot be known for endogenous substances. In the past, there were very large discrepancies in the reported values for ADMA and SDMA in human plasma of healthy subjects, leading to difficulties in drawing clinical relevant conclusions from study data [22]. In the meantime, this discussion was put essentially to rest [43]. In Table 2, the basal levels for Arg and its methylated metabolites measured in healthy controls by the different mass spectrometry-based analytical methods are summarized. As can be seen, a consensus seems to emerge that the "true" concentration values in plasma of healthy volunteers are about 65 μM for Arg, 0.4 μM for ADMA and 0.5 μM for SDMA. This is in line with values obtained from conventional methods [18–20,34–36]. A more comprehensive comparison can be found in [22]. Remaining differences may be due to the selection of the individuals included in the studies. The only method which produced greatly deviating results of

Table 2
Concentration values of ARG, L-NMMA, ADMA and SDMA measured in human plasma in healthy volunteers by MS-based methods

First author [ref.]	Validated matrix	Arg (μM)	L-NMMA (μM)	ADMA (μM)	SDMA (μM)
Vishwanathan [29]	Plasma	62.6 ± 24.0	0.104 ± 0.020	0.124 ± 0.046	0.164 ± 0.065
Tsikas [25]	Plasma, urine			0.390 ± 0.062	
Martens-Lobenhoffer [32]	Plasma, urine	119.5 ± 21.6[a]		0.453 ± 0.128[a]	0.602 ± 0.168[a]
Albsmeier [26]	Plasma, cell culture supernatant			0.600 ± 0.076	
Huang [31]	Plasma	72.64 ± 17.31	0.35 ± 0.14	0.48 ± 0.07	0.41 ± 0.05
Martens-Lobenhoffer [38]	Plasma	63.9 ± 23.9		0.355 ± 0.066	0.460 ± 0.092
Kirchherr [39]	Plasma			0.455 ± 0.180	0.678 ± 0.037
Schwedhelm [40]	Plasma	65.6 ± 23.4		0.55 ± 0.14	0.69 ± 0.23
Martens-Lobenhoffer [28]	Plasma, urine, cell culture supernatant	60.6 ± 18.3		0.370 ± 0.061	0.449 ± 0.055

[a] Serum values. Please note that Arg serum levels are known to be about 70% higher than Arg plasma values [20].

Table 3
Time requirement for sample preparation and chromatography for Arg and its metabolites in MS-based methods

First author [ref.]	Method, need of derivatization	Estimated sample preparation time (h)	Chromatographic run time (min)
Vishwanathan [29]	LC–MS, no derivatization	0.25	15
Tsikas [25]	GC–MS–MS, derivatization	2.5	11
Martens-Lobenhoffer [32]	LC–MS, derivatization	1	32
Albsmeier [26]	GC–MS, derivatization	3	10.8
Huang [30,31]	LC–MS, no derivatization	0.5	7
Martens-Lobenhoffer [38]	LC–MS, derivatization	1	32
Kirchherr [39]	LC–MS–MS, no derivatization	0.25	6
Schwedhelm [40]	LC–MS–MS, derivatization	1	4
Martens-Lobenhoffer [28]	LC–MS–MS, no derivatization	0.2	8

0.124 μM for ADMA and 0.164 μM for SDMA, respectively, was the one of Vishwanathan et al. [29]. More doubts remain about the concentration levels for L-NMMA in plasma. The only two reported values of 0.104 μM [29] and 0.35 μM [31] for this substance in human plasma by chromatographic-mass spectrometric methods are strongly divergent. Furthermore, values found with HPLC-fluorescence methods of about 0.05 μM [20,44] are much lower than the reported ones here.

In serum samples, the Arg concentration values found are about 70% higher than in corresponding plasma samples [20,32]. This additional Arg may be due to some release during the coagulation process. It is very important to take this effect into account when comparing plasma and serum samples for Arg levels. For ADMA and SDMA no such strong differences between plasma and serum were observed. Only small and usually insignificant dilution effects probably caused by the EDTA solution in the sampling tubes were shown ([20], own unpublished results).

Whereas for human plasma reference values for Arg and its methylated metabolites can be defined, in other matrices such as urine and cell culture supernatants reported concentration values are very sparse. Typical values reported for human urine (all units in μmol/mmol creatinine) were 3.4 for ADMA [25] or 2.92 for ADMA and 3.72 for SDMA [28] or 3.94 for ADMA and 3.70 for SDMA [45], respectively. Because of the shortage in published data, a qualified discussion about the absolute accuracy of the methods in these matrices is not possible at the present time.

3.3. Time requirements to perform the different methods

The requirements in time and laboratory workforce are very different between the chromatographic-mass spectrometric assays reviewed in the present work. In Table 3, the estimated times required for sample preparation and chromatography are summarized. In the case of the two GC methods, a two-step derivatization process with repeated reaction-evaporation cycles has to be performed. The whole sample preparation procedure takes about 2.5–3 h in both methods [25,26]. However, the chromatographic run time is short (i.e. 11 min) in comparison. In contrast, the sample preparation for most of the described LC–MS or LC–MS–MS methods is very easy and fast [28,29,39]. None of the sample preparation procedures utilizes SPE columns, and only the procedure by Schwedhelm et al. [40] requires off-line derivatization. The chromatographic run time of the LC–MS or LC–MS–MS methods is also very short, enabling high throughput analysis [28,31,39,40].

4. Conclusion

The quantification of Arg, ADMA and SDMA in various biological fluids by chromatographic-mass spectrometric means has reached a high degree of maturity. There is a range of methodologies for the chromatographic separation available to the user: from derivatization and GC separation to different kinds of HPLC separations without derivatization, such as polar interaction on porous graphitic carbon, HILIC or ion-pair chromatography, to standard RP-HPLC after derivatization. Mass spectrometry coupled to chromatography provides high sensitivity and selectivity, making these assays superior to the ones depending on conventional detection technologies such as UV or fluorescence. In applying LC–MS–MS instead of single stage mass spectrometry, an even higher degree of selectivity can be achieved, making a mass spectrometric distinction of the structural isomers ADMA and SDMA possible. The well-known susceptibility of LC–MS systems with API ion sources to matrix effects [23,24] makes the use of stable-isotope labeled Arg and ADMA as internal standards very advisable. Stable-isotope labeled internal standards are compensating for all matrix effects and improve the reliability of the assays largely. For Arg and ADMA, but not for SDMA, stable-isotope labeled analogues are available in the meantime. Short chromatographic run times and quick and easy sample preparation make some of the here discussed assays suitable for cost-effective high-throughput analysis. Because methods based on chromatography coupled to mass spectrometric detection can be regarded as “gold-standard”, especially if stable-isotope labeled analogs are used as I.S., such approaches can help in the establishment of reference values for Arg and its methylated metabolites in normal and pathological conditions. Careful application of such methods can pave the way to more consistent results in clinical investigations.

References

[1] Y. Kakimoto, S. Akazawa, J. Biol. Chem. 245 (1970) 5751.
[2] J.D. Gary, S. Clarke, Prog. Nucleic Acid Res. Mol. Biol. 61 (1998) 65.
[3] J.M. Leiper, P. Vallance, Eur. J. Clin. Pharmacol. 62 (Suppl. 13) (2006) 33.
[4] P. Bulau, D. Zakrzewicz, K. Kitowska, B. Wardega, J. Kreuder, O. Eickelberg, Biotechniques 40 (2006) 305.

[5] S. Moncada, A. Higgs, N. Engl. J. Med. 329 (1993) 2002.
[6] S.M. Bode-Böger, R.H. Böger, S. Kienke, W. Junker, J.C. Frölich, Biochem. Biophys. Res. Commun. 219 (1996) 598.
[7] R.M. Palmer, D.S. Ashton, S. Moncada, Nature 333 (1988) 664.
[8] P. Vallance, A. Leone, A. Calver, J. Collier, S. Moncada, Lancet 339 (1992) 572.
[9] C. Fleck, A. Janz, F. Schweitzer, E. Karge, M. Schwertfeger, G. Stein, Kidney Int. Suppl. 78 (2001) S14.
[10] E. Fedele, M. Raiteri, Prog. Neurobiol. 58 (1999) 89.
[11] J.P. Cooke, V.J. Dzau, Annu. Rev. Med. 48 (1997) 489.
[12] T. Ogawa, M. Kimoto, K. Sasaoka, J. Biol. Chem. 264 (1989) 10205.
[13] J.P. Cooke, Circulation 109 (2004) 1813.
[14] T. Teerlink, Clin. Chem. Lab. Med. 43 (2005) 1130.
[15] T. Teerlink, Vasc. Med. 10 (2005) S73.
[16] R.J. Nijveldt, P.A. van Leeuwen, C. Van Guldener, C.D. Stehouwer, J.A. Rauwerda, T. Teerlink, Nephrol. Dial. Transplant. 17 (2002) 1999.
[17] S.M. Bode-Böger, F. Scalera, J.T. Kielstein, J. Martens-Lobenhoffer, G. Breithardt, M. Fobker, H. Reinecke, J. Am. Soc. Nephrol. 17 (2006) 1128.
[18] E. Causse, N. Siri, J.F. Arnal, C. Bayle, P. Malatray, P. Valdiguie, R. Salvayre, F. Couderc, J. Chromatogr. B 741 (2000) 77.
[19] G. Trapp, K. Sydow, M.T. Dulay, T. Chou, J.P. Cooke, R.N. Zare, J. Sep. Sci. 27 (2004) 1483.
[20] T. Teerlink, R.J. Nijveldt, S. de Jong, P.A.M. van Leeuwen, Anal. Biochem. 303 (2002) 131.
[21] P. Valtonen, J. Karppi, K. Nyyssonen, V.P. Valkonen, T. Halonen, K. Punnonen, J. Chromatogr. B 828 (2005) 97.
[22] J. Martens-Lobenhoffer, S.M. Bode-Böger, Eur. J. Clin. Pharmacol. 62 (2006) 61.
[23] S. Souverain, S. Rudaz, J.L. Veuthey, J. Chromatogr. A 1058 (2004) 61.
[24] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.
[25] D. Tsikas, B. Schubert, F.M. Gutzki, J. Sandmann, J.C. Frölich, J. Chromatogr. B 798 (2003) 87.
[26] J. Albsmeier, E. Schwedhelm, F. Schulze, M. Kastner, R.H. Böger, J. Chromatogr. B 809 (2004) 59.
[27] S. Pundak, M. Wilchek, J. Org. Chem. 46 (1981) 808.
[28] J. Martens-Lobenhoffer, S.M. Bode-Böger, Clin. Chem. 52 (2006) 488.
[29] K. Vishwanathan, R.L. Tackett, J.T. Stewart, M.G. Bartlett, J. Chromatogr. B 748 (2000) 157.
[30] L.F. Huang, F.Q. Guo, Y.Z. Liang, Q.N. Hu, B.M. Cheng, Anal. Chim. Acta 487 (2003) 145.
[31] L.F. Huang, F.Q. Guo, Y.Z. Liang, B.Y. Li, B.M. Cheng, Anal. Bioanal. Chem. 380 (2004) 643.
[32] J. Martens-Lobenhoffer, S.M. Bode-Böger, J. Chromatogr. B 798 (2003) 231.
[33] B.M. Chen, L.W. Xia, R.Q. Zhao, J. Chromatogr. B 692 (1997) 467.
[34] J. Meyer, N. Richter, M. Hecker, Anal. Biochem. 247 (1997) 11.
[35] A. Pettersson, L. Uggla, V. Backman, J. Chromatogr. B 692 (1997) 257.
[36] J. Pi, Y. Kumagai, G. Sun, N. Shimojo, J. Chromatogr. B 742 (2000) 199.
[37] W.Z. Zhang, D.M. Kaye, Anal. Biochem. 326 (2004) 87.
[38] J. Martens-Lobenhoffer, O. Krug, S.M. Bode-Böger, J. Mass Spectrom. 39 (2004) 1287.
[39] H. Kirchherr, W.N. Kühn-Velten, Clin. Chem. 51 (2005) 249.
[40] E. Schwedhelm, J. Tan-Andresen, R. Maas, U. Riederer, F. Schulze, R.H. Böger, Clin. Chem. 51 (2005) 1268.
[41] M. Piraud, C. Vianey-Saban, C. Bourdin, C. Acquaviva-Bourdain, S. Boyer, C. Elfakir, D. Bouchu, Rapid Commun. Mass Spectrom. 19 (2005) 3287.
[42] Y. Guo, S. Gaiki, J. Chromatogr. A 1074 (2005) 71.
[43] E. Schwedhelm, Vasc. Med. 10 (Suppl. 1) (2005) S89.
[44] B. Anderstam, K. Katzarski, J. Bergstrom, J. Am. Soc. Nephrol. 8 (1997) 1437.
[45] M. Al Banchaabouchi, B. Marescau, I. Possemiers, R. D'Hooge, O. Levillain, P.P. De Deyn, Pflügers Arch. 439 (2000) 524.