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Quantification of ADMA: analytical approaches

Edzard Schwedhelm

Abstract: Methylated L-arginine analogs are involved in nitric oxide synthase activity regulation. Methods based on high-performance liquid chromatography with fluorescence, capillary electrophoresis, or ion exchange chromatography with absorbance detection were first applied for the quantitative determination of N-monomethyl-L-arginine (NMMA), asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) in human blood and urine. These assays revealed elevated circulating levels of ADMA in various diseases and gave accumulating evidence of the usefulness of ADMA as a cardiovascular risk factor. However, the methods used are hampered by the fact that NMMA, ADMA and SDMA can be distinguished from L-arginine only by means of chromatographic separation. This has promoted the development of alternatives that involve mass spectrometry (MS) technology. Today, various MS-based approaches such as liquid chromatography (LC)-MS, LC-MS/MS, gas chromatography (GC)-MS, and GC-MS/MS are available. L-arginine and its analogs have been subjected to LC-MS analysis with and without further derivatization to their *o*-phthaldialdehyde derivatives. For these methods, labelled L-arginine was used as the internal standard. The first MS-based method that distinguishes NMMA, ADMA, SDMA and L-arginine by mass-to-charge (*m/z*)-ratio has been reported by Tsikas et al. This GC-MS approach has been further improved by Albsmeier et al by introducing labelled ADMA as an internal standard. As an alternative to existing methods, a commercially available ELISA kit has recently been developed and validated.

Key words: asymmetric dimethylarginine; endothelial dysfunction; ELISA; gas chromatography-mass spectrometry; high-performance liquid chromatography; liquid chromatography-mass spectrometry; methylated L-arginine; nitric oxide; nitric oxide synthase

Introduction

Qualification and quantification issues had been addressed several years before Vallance and co-workers introduced asymmetric dimethylarginine (N^G , N^G -dimethylarginine, ADMA) as an endogenous inhibitor of nitric oxide synthase (NOS).¹ Methylation of L-arginine residues of proteins is a well known feature of protein function and metabolism.² Monomethylation, dimethylation and trimethylation of arginine as well as of lysine are observed. Information on methylation status of proteins is obtained after (total) hydrolysis or digestion. The techniques applied for the analysis of protein methylation are ion chromatography and more recently liquid

chromatography-mass spectrometry (LC-MS).^{3–7} Even though the methylation of arginine residues of proteins seems to be ubiquitous, the degree of arginine methylation is organ specific. The extent of methylation yielding dimethylarginine residues is elevated in brain, liver, lung, spleen, kidney and small intestine.⁴ The ratio of ADMA over symmetric dimethylarginine (N^G , N'^G -dimethylarginine, SDMA) is notably increased in heart tissue (a 32-fold excess has been found).⁴ The functional consequence of such observations is currently subject to investigation. While measurement of ADMA and SDMA allows us to distinguish between the two isomers, no information is provided about which protein is methylated and at which position. Electrospray ionization (ESI) tandem mass spectrometry (tandem MS) has recently revealed that dimethylammonium (mass-to-charge ratio (*m/z*) 46) is a specific marker ion (as well as a neutral loss of 45 Da) for ADMA in methylated proteins.^{5–7} These mass spectrometric techniques now enable us to have a substantial insight into the diverse functions of protein arginine methylation, which has been

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speculated to parallel the role of phosphorylation in modification of protein function.²

High-performance liquid chromatography

The major advantage of high-performance liquid chromatography (HPLC) over ion chromatography is a reduction of analysis time from several hours to 30–60 minutes. A sensitive HPLC method for the determination of methylated arginines has been introduced by several groups using *o*-phthaldialdehyde (OPA) for precolumn derivatization and subsequent fluorescence (FL) detection.^{8–10} FL detection is carried out at excitation (λ^{ex}) and emission (λ^{em}) wavelengths of 340 and 455 nm, respectively. The crucial step of the HPLC analysis of methylated arginines is the chromatographic separation of ADMA from SDMA and to resolve both from matrix components.¹¹ Baseline separation of OPA derivatives of ADMA and SDMA can be achieved by means of analytical columns packed with octadecylsilica or phenyl silica, namely, reversed phase (RP) conditions. The analytical column should be at least 150 × 4.6 mm. It should also be protected by a guard column packed with the same material. It is interesting that OPA derivatives of ADMA and SDMA elute on a phenylsilica column in the opposite order to octadecylsilica. To resolve analytes from matrix prior to HPLC-FL analysis, appropriate sample pretreatment has to be performed. This includes: (1) protein precipitation and (2) solid phase extraction (SPE) of biological samples. Protein precipitation can be performed with any convenient organic solvent. The organic layer has to be evaporated prior to SPE. Alternatively, protein precipitation can be accomplished by the addition of 5-sulfosalicylic acid¹⁰ or proteins can be separated by ultrafiltration. In some cases it might be necessary to extract lipids from samples with trifluoroethane before they are subjected to SPE.⁸ However, in some cases it is neither necessary to extract lipids nor to precipitate proteins if SPE is applied. Thus, if not present in large amounts, proteins will be separated during the SPE procedure. To avoid clogging of the columns used for SPE, samples containing proteins should be diluted 1 + 1 with deionized water. Several types of columns have been applied in SPE of ADMA; they are silica based or equipped with a polymeric stationary phase. A major advantage of polymeric material is that column preconditioning becomes unnecessary. Stationary phases are modified with weak carboxylic acid (CBA) or strong cation exchange (SCX) resins.^{8,9,12} The advantage of SCX columns is better retention of the analytes. Columns with a bed of 50–100 mg can be used. In contrast, 500 mg of bed material are recommended for CBA columns. Elution of basic amino acids such as ADMA, SDMA and L-arginine from CBA columns is obtained with 1 M formic acid.

Elution of analytes from SCX and polymeric columns is obtained with mixtures of methanol/water/triethylamine or ammonia. Recoveries for L-arginine are crucial and protocols have to be optimized in this respect.^{12,13} All recent protocols for HPLC determination of methylated arginines include the application of an appropriate internal standard. The internal standard has to be added prior to sample preparation (i.e. as the initial step). Several internal standards have been adopted for the simultaneous quantification of ADMA, SDMA and L-arginine: homoarginine,^{14–16} *N*^G-monomethyl-L-arginine (NMMA),^{12,13} and *N*^ω-propyl-L-arginine (N-PLA).¹⁷ A representative chromatogram of a combined standard containing arginine, homoarginine, SDMA and ADMA is depicted in Figure 1. Fluorescence was detected after SPE with CBA cartridges, online OPA derivatization, and separation on a phenyl column (250 × 4.6 mm, Macherey-Nagel, Düren, Germany).¹¹ When applying this method for plasma samples, homoarginine was used as the internal standard. Table 1 summarizes the major characteristics of this and other methods applied. Homoarginine and NMMA, although present in only low quantities in human plasma, may be altered under pathophysiologic conditions such as renal failure.^{19,20} N-PLA is not an endogenous compound. However, since its chromatographic properties are reported to be very similar to SDMA and ADMA, the risk of overlapping signals cannot be excluded.

A major drawback of OPA derivatives is their instability due to rapid decomposition, so on-line derivatization has to be performed. If this is not available, other derivatization agents may be used in combination or instead of OPA. In this regard, the sulfhydryl group used for the reaction with the OPA reagent is of vital importance. The use of 3-mercaptopropionic acid instead of 2-mercaptoethanol for derivatization with the OPA reagent results in more stable derivatives of L-arginine, the internal standard, ADMA and SDMA. After derivatization, the samples have to be stored at 7°C. Peak areas of derivatives were shown to decline by 5–10% within 20–25 hours and by approximately 35% after 72 hours. However, the relative peak area ratios were found unchanged after 72 hours (i.e. 99.4 ± 1.6% of the initial ratio).¹² Further improvement of stability was reported for naphthalene-2,3-dicarboxaldehyde (NDA) derivatives.¹⁷ Owing to the different nature of the NDA derivatives, wavelengths for FL detection have to be adjusted (λ^{ex} = 420 nm and λ^{em} = 483 nm). However, compounds have to be stored at 4°C after derivatization and a linear decline of fluorescence signal by 15.9, 11 and 12.9% for derivatives of arginine, SDMA and ADMA, respectively, was observed after 72 hours.¹⁷ Finally, stable derivatives, even at room temperature, are obtained with the derivatizing agent 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AccQ-Fluor™, Waters, Milford, MA, USA).¹³ FL detection is carried

Fluorescence Signal

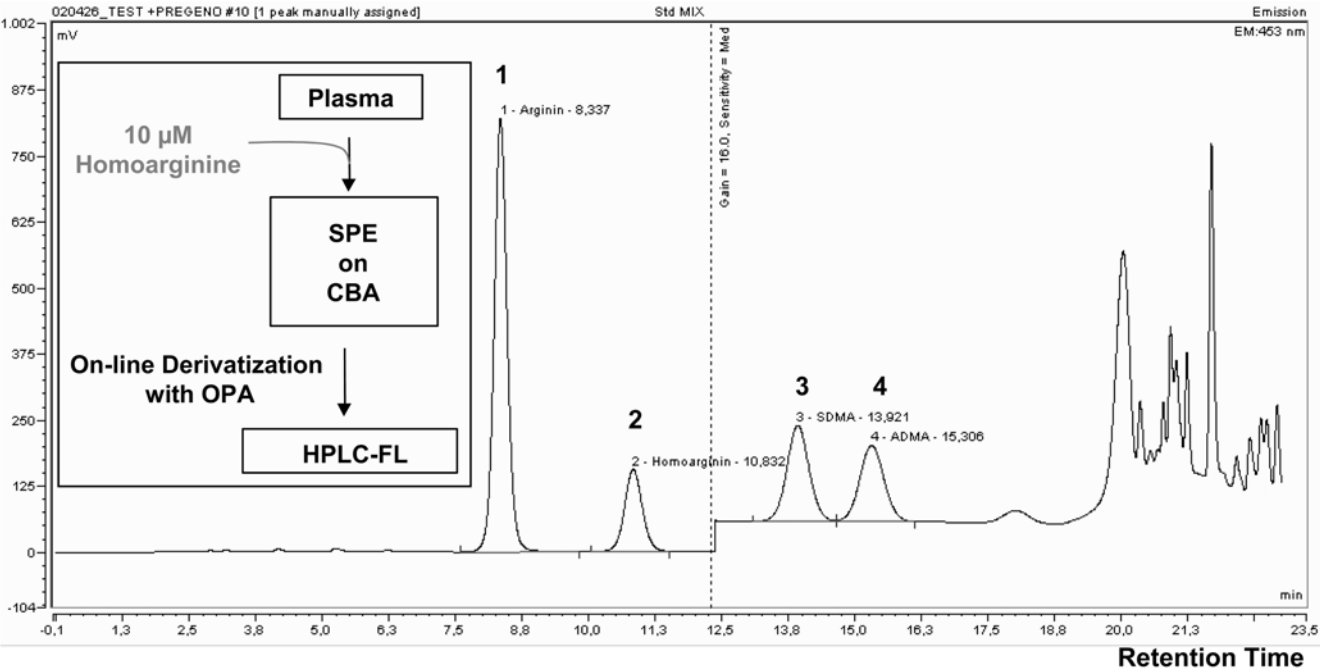


Figure 1 Chromatogram of a combined standard containing 25 μM arginine, 10 μM homoarginine, 1 μM symmetric dimethylarginine (SDMA), and 1 μM asymmetric dimethylarginine (ADMA). Fluorescence was detected after solid phase extraction (SPE) with carboxylic acid (CBA) cartridges, online *o*-phthaldialdehyde (OPA) derivatization, and separation on a phenyl column (250 × 4.6 mm, Macherey-Nagel, Düren, Germany).¹¹ Dashed line indicates gain switch of the fluorescence detector. Peak identification: 1, arginine; 2, homoarginine; 3, SDMA; 4, ADMA.

Table 1 Summary of methods used to detect ADMA in human plasma.

Analysis/detection method	Derivative	Plasma concentration ^a	Detection limit ^a	Reference
<i>HPLC</i>				
Fluorescence	OPA	0.58 ± 0.02 (<i>n</i> = 10)	0.015	Pettersson et al 1997 ⁹
	OPA	0.52 ± 0.21	0.004 ^b	Tsikas et al 1998 ¹¹
	OPA	0.30 ± 0.05 (<i>n</i> = 7)	0.18 ^c	Pi et al 2000 ¹⁵
	OPA	0.42 ± 0.06 (<i>n</i> = 53)	0.01 ^c	Teerlink et al 2002 ¹²
	OPA	0.76 ± 0.12 (<i>n</i> = 35)	0.002	Zhang and Kaye 2004 ¹⁶
	NDA	0.38–1.30 (range, <i>n</i> = 50)	0.01	Marra et al 2003 ¹⁷
	AccQ-Fluor	0.44 ± 0.08 (<i>n</i> = 12)	0.1 ^d	Heresztyn et al 2004 ¹³
<i>Capillary electrophoresis</i>				
Laser-induced fluorescence		0.34 ± 0.02 (serum, <i>n</i> = 5)	0.05 ^e	Caussé et al 2000 ³⁵
<i>Mass spectrometry</i>				
GC-MS	methyl-PFP	0.60 ± 0.08 (<i>n</i> = 10)	2 fmol	Albsmeier et al 2004 ³⁰
GC-MS/MS	methyl-PFP	0.39 ± 0.06 (<i>n</i> = 12)	10 amol	Tsikas et al 2003 ²⁹
LC-MS (ESI)	OPA	0.45 ± 0.13 (<i>n</i> = 15)		Martens-Lobenhoffer et al 2003 ²²
	OPA	0.36 ± 0.07 (<i>n</i> = 47)	0.15 ^c	Martens-Lobenhoffer et al 2004 ²³
LC-MS (APCI)	–	0.48 ± 0.07 (<i>n</i> = 40)	0.01	Huang et al 2004 ²⁵
LC-MS/MS	–	0.12 ± 0.05 (<i>n</i> = 20)	0.005	Vishwanathan et al 2000 ²⁶
	–	0.46 (95% CI, 0.44–0.46, <i>n</i> = 42)	0.017	Kirchherr et al 2005 ¹⁸
	butyl	0.55 ± 0.14 (<i>n</i> = 22)	0.003	Schwedhelm et al 2005 ²⁴
<i>ELISA</i>		0.65 ± 0.13 (<i>n</i> = 10)	0.05 ^e	Schulze et al 2004 ³³

^aConcentrations and detection limits are given in μmol/l unless otherwise indicated; ^bdetermined by our laboratory; ^climit of quantification; ^dlimit of detection in plasma; ^eanalytical sensitivity; APCI, atmospheric-pressure chemical ionisation; CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; ESI, electrospray ionisation; GC, gas chromatography; LC, liquid chromatography; MS, mass spectrometry; NDA, naphthalene-2,3-dicarboxaldehyde; OPA, *o*-phthaldialdehyde; PFP, pentafluoropropionyl.

out at $\lambda^{\text{ex}} = 250$ nm and $\lambda^{\text{em}} = 395$ nm. Derivatives of arginine, NMMA, ADMA and SDMA are stable for at least 7 days at room temperature.

Another method for the analysis of methylated arginines is the combination of on-line extraction, separation with ion-pair chromatography and post-column derivatization of amino acids.²¹ For the ion-pair chromatography, a mixture of the two ion-pair reagents sodium octanoate and cyclohexanecarboxylic acid is used. Even though the column switching protocol is quite complex and three different analytical columns are involved, the analysis of biological samples is completely automated. An equally high degree of automation can be attained by using LC-MS (i.e. liquid chromatography-mass spectrometry).

Liquid chromatography-mass spectrometry

OPA derivatives of L-arginine and its metabolites are also suitable for mass spectrometric analysis with the limitations mentioned above (stability, chromatographic conditions). OPA derivatives of ADMA and SDMA exhibit identical fragmentation patterns under positive ESI (i.e. m/z 379). L-Arginine, L-citrulline and homoarginine isoindole derivatives formed with OPA are discernable owing to different mass-to-charge ratios (i.e. m/z 351, 352 and 365, respectively).^{22,23} The advantage of mass spectrometry is its unique specificity. Thus, the SPE step can be omitted for human plasma and urine. Only a protein precipitation for plasma samples is recommended. To add a further level of specificity, LC-tandem MS is mandatory. OPA derivatives seem to be unsuited because of their complex fragmentation pattern after MS-MS fragmentation.²² Derivatization of the carboxylic acid instead of the primary amino function is a feasible alternative. Derivatization can be carried out with any convenient reagent; for example, esterification or amidation can be performed. Butyl ester derivatives of ADMA and SDMA have the same parent ions (i.e. m/z 259). Nevertheless, butyl ester derivatives of ADMA and SDMA are discernable according to their major daughter ions after MS-MS fragmentation; that is, m/z 214 and 228 for ADMA and SDMA, respectively.²⁴ Thus, any preliminary chromatographic separation (e.g. LC of ADMA and SDMA) can be omitted.

Derivatization of methylated arginines with OPA reagent or 1 M HCl in butanol can be carried out on-line. However, arginine and methylated arginines have also been analysed with LC-MS and LC-tandem MS without derivatization.^{25,26} ADMA and SDMA exhibit identical parent ions after positive atmospheric pressure chemical ionization or ESI (i.e. m/z 203).^{25,26} L-arginine and NMMA are discernable from ADMA and SDMA owing to m/z ratios of 175 and 189 for L-arginine and NMMA, respectively. MS-MS spectra of ADMA and SDMA are very similar for the major

fragments ($\geq 10\%$). Only one major fragment is exclusively formed by SDMA (i.e. m/z 172). This fragment is specific to SDMA since it contains a single methyl group bound to one guanidine nitrogen.²⁶ To overcome this lack of a specific daughter fragment ion for ADMA, SDMA and ADMA have to be separated by chromatography prior to mass spectrometric analysis. In this context a compromise has to be found between sufficient chromatographic separation and short analysis time. Alternatively, a closer look at the minor fragments in the MS-MS spectrum of ADMA seems worth while. Indeed, dimethylammonium (m/z 46) is exclusively formed by ADMA after fragmentation (see introduction). To minimize ion suppression²⁷ at this low m/z ratio, a teicoplanin column can be used.²⁸

Internal standards in mass spectrometry

Accurate quantification in mass spectrometry is realized by stable isotope dilution of samples. Compounds labeled with stable isotopes are used as internal standards of arginine and its metabolites and added to crude samples. Labeled compounds show similar chemical properties. Nevertheless, they can be discerned from the analytes because of their higher m/z ratios in mass spectrometry. So far [$^{13}\text{C}_6$]arginine (98% ^{13}C isotopic purity, Cambridge Isotope Laboratories, Andover, MA, USA) and L-[guanidine- $^{15}\text{N}_2$]arginine (98% ^{15}N isotopic purity, Cambridge Isotope Laboratories) have been used as internal standards for the quantification of L-arginine in LC- and gas chromatography (GC)-MS.^{22,23,29,30} A stable isotope labeled internal standard for the quantification of ADMA is not yet commercially available. However, an appropriate internal standard may be synthesized in situ with the samples,²⁹ or easily prepared in the laboratory. Synthesis follows the synthetic route described by Pundak and Wilchek.³¹ L-[3,3,4,4,5,5- $^2\text{H}_6$]Ornithine (>98% ^{15}N isotopic purity, Euriso-top, Saint Aubin, France) or [$^2\text{H}_6$]dimethylamine gas, free base (99% ^2H isotopic purity, Cambridge Isotope Laboratories) were used as labeled educts.^{23,24,30} An appropriate stable isotope-labeled internal standard for SDMA has not yet been synthesized because SDMA is not available via the synthetic route described by Pundak and Wilchek.

Gas chromatography-mass spectrometry

Derivatization is an indispensable prerequisite for the GC-MS analysis of amino acids. Compounds are too polar and not volatile enough in the absence of derivatization. To date, L-arginine, NMMA, SDMA and ADMA have been derivatized with fluorine compounds. This allows highly sensitive analysis with negative-ion chemical ionization (NICI).³²

Thus, analytes are converted to their methyl ester *N*-pentafluoropropionyl amide derivatives and subsequently analyzed in the NICI mode with GC-MS or GC-tandem MS.^{29,30} NMMA and ADMA are analyzed as their tri(*N*-pentafluoropropionyl) amide derivative, L-arginine as its tetra- and SDMA as its di-derivative. Thus, all analytes are discernable owing to different GC retention times and MS ions. In GC-MS the ions m/z 640 and m/z 586 are monitored in the selected ion monitoring mode for ADMA and L-arginine derivatives, respectively. The limit of detection (LOD) was determined to be 2 fmol ADMA with a signal-to-noise ratio of 11.8 ± 1 .³⁰ In GC-tandem MS the transitions m/z 640 to m/z 378 and m/z 586 to m/z 295 are monitored in the selected reaction monitoring mode for ADMA and L-arginine derivatives, respectively. The LOD was determined to be lower in comparison to GC-MS owing to the higher selectivity of tandem MS: 1 fmol of deuterated ADMA was analysed with a signal-to-noise ratio of 343 ± 30 , suggesting a LOD of approximately 10 amol of ADMA.²⁹ With this very sensitive assay it was also possible to generate a daughter ion spectrum from the endogenous compound eluting from the GC

column at the retention time of ADMA. This spectrum was virtually identical with authentic ADMA, irrefutably identifying the endogenous compound as ADMA. Endogenous ADMA was identified in human urine and plasma. Urine samples are derivatized without any preliminary sample purification. Plasma samples are derivatized after ultrafiltration or protein precipitation. A comparison between the urine and plasma samples from 10 individuals measured with GC-MS and GC-tandem MS revealed almost identical values for ADMA. The ratios of values obtained with GC-MS to GC-tandem MS were 1.173 ± 0.097 and 1.022 ± 0.034 for human urine and plasma, respectively.²⁹ To date, a limitation in GC-(tandem) MS analysis of methylarginines is the unavailability of an appropriate internal standard for SDMA.

Basal levels of ADMA

An overview of basal plasma levels in humans measured with different assays is given in Table 1. An ELISA for the determination of ADMA has been successfully developed and is now commercially

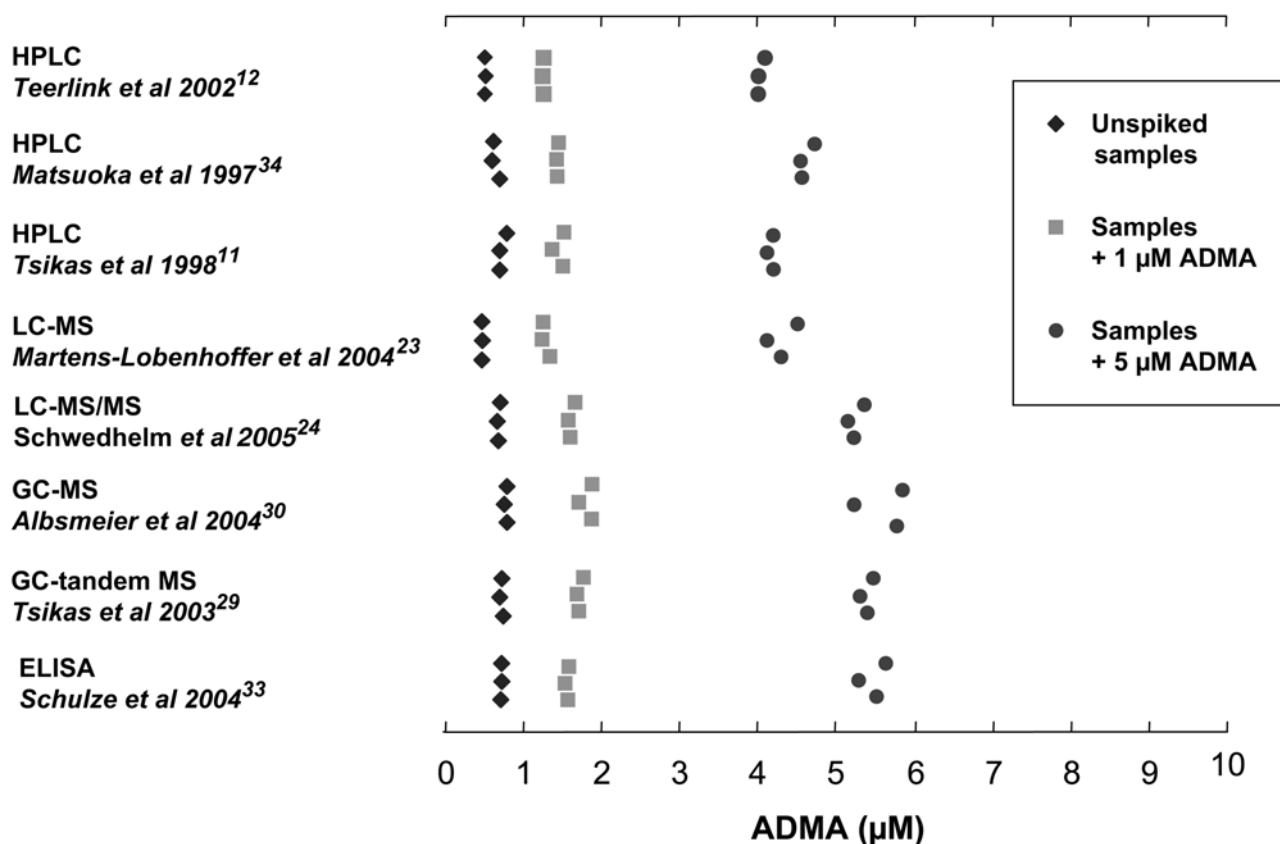


Figure 2 Interlab comparison of analysis of human blood samples for asymmetric dimethylarginine (ADMA). Nine different samples were analysed: three equal basal samples, three equal samples spiked with 1 μ M ADMA, and three equal samples spiked with 5 μ M ADMA. Methods are indicated according to samples analysed in each laboratory. HPLC, high-performance liquid chromatography; LC, liquid chromatography; GC, gas chromatography; MS, mass spectrometry; ELISA, enzyme-linked immunosorbent assay.

available.³³ Basal plasma levels obtained with this assay and with other techniques are quite similar. To obtain an overview of different assays performed in different laboratories, an interlab comparison was carried out. Nine different samples were analysed: three basal samples, three samples spiked with 1 μ M ADMA, and three samples spiked with 5 μ M ADMA. The results are given in Figure 2.

Conclusion

More than ever there is a particular need for reliable methods for the determination of ADMA and other metabolites in the L-arginine pathway. The scientific interest in ADMA and related compounds has increased quickly in the last few years. Thus, availability and rapidity are important for analytical methods in this field. Two different strategies seem to meet these criteria, at least in part that is: ELISA and mass spectrometry. Both these technologies are equal in terms of their rapidity. However, the technology required for ELISA is widely available. Nevertheless, mass spectrometry is unsurpassed in its unique selectivity. By means of mass spectrometry, it was possible to identify unambiguously ADMA on the basis of its distinct mass spectrum. Thus, any other method for the quantification of ADMA should be compared with the gold standard of mass spectrometry. This comparison should be carried out according to the rules of Bland and Altman.³⁶ Nevertheless, in clinical research a single comparison may not be adequate. It is therefore indispensable to have quality controls and interlab comparisons. For the future, the primary analytical challenge in the field of ADMA will be to maintain high standards and continually perform quality control procedures.

References

- Vallance P, Leone A, Calver A, Collier J, Moncada S. Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. *Lancet* 1992; **339**: 572–75.
- McBride AE, Silver PA. State of the arg:protein methylation at arginine comes of age. *Cell* 2001; **106**: 5–8.
- Paik WK, Kim S. Omega-N-methylarginine in protein. *J Biol Chem* 1970; **245**: 88–89.
- Nakajima T, Matsuoka Y, Kakimoto Y. Isolation and identification of N^G -monomethyl-, N^G,N^G -dimethyl-, and $N^G,N^{G'}$ -dimethylarginine from the hydrolysate of proteins of bovine brain. *Biochim Biophys Acta* 1971; **230**: 212–22.
- Rappsilber J, Friesen WJ, Paushkin S, Dreyfuss G, Mann M. Detection of arginine dimethylated peptides by parallel precursor ion scanning mass spectrometry in positive ion mode. *Anal Chem* 2003; **75**: 3107–14.
- Brame CJ, Moran MF, McBroom-Cerajewski LD. A mass spectrometry based method for distinguishing between symmetrically and asymmetrically dimethylated arginine residues. *Rapid Commun Mass Spectrom* 2004; **18**: 877–81.
- Gehring PM, Hunziker PE, Zahariev S, Pongor S. Fragmentation pathways of N^G -methylated and unmodified arginine residues in peptides studied by ESI-MS/MS and MALDI-MS. *J Am Soc Mass Spectrom* 2004; **15**: 142–49.
- Bode-Böger SM, Böger RH, Kienke S, Junker W, Frölich JC. Elevated L-arginine/dimethylarginine ratio contributes to enhanced systemic NO production by dietary L-arginine in hypercholesterolemic rabbits. *Biochem Biophys Res Commun* 1996; **219**: 598–603.
- Pettersson A, Ugglä L, Backman V. Determination of dimethylated arginines in human plasma by high-performance liquid chromatography. *J Chromatogr B Biomed Sci Appl* 1997; **692**: 257–62.
- Chen BM, Xia LW, Zhao RQ. Determination of $N(G),N(G)$ -dimethylarginine in human plasma by high-performance liquid chromatography. *J Chromatogr B Biomed Sci Appl* 1997; **692**: 467–71.
- Tsikis D, Junker W, Frölich JC. Determination of dimethylated arginines in human plasma by high-performance liquid chromatography. *J Chromatogr B Biomed Sci Appl* 1998; **705**: 174–76.
- Teerlink T, Nijveldt RJ, de Jong S, van Leeuwen PA. Determination of arginine, asymmetric dimethylarginine, and symmetric dimethylarginine in human plasma and other biological samples by high-performance liquid chromatography. *Anal Biochem* 2002; **303**: 131–37.
- Heresztyn T, Worthley MI, Horowitz JD. Determination of L-arginine and NG , NG - and NG , NG' -dimethyl-L-arginine in plasma by liquid chromatography as AccQ-Fluor fluorescent derivatives. *J Chromatogr B Analyt Technol Biomed Life Sci* 2004; **805**: 325–29.
- Böger RH, Bode-Böger SM, Szuba A et al. Asymmetric dimethylarginine (ADMA): a novel risk factor for endothelial dysfunction: its role in hypercholesterolemia. *Circulation* 1998; **98**: 1842–47.
- Pi J, Kumagai Y, Sun G, Shimojo N. Improved method for simultaneous determination of L-arginine and its mono- and dimethylated metabolites in biological samples by high-performance liquid chromatography. *J Chromatogr B Biomed Sci Appl* 2000; **742**: 199–203.
- Zhang WZ, Kaye DM. Simultaneous determination of arginine and seven metabolites in plasma by reversed-phase liquid chromatography with a time-controlled *ortho*-phthalaldehyde precolumn derivatization. *Anal Biochem* 2004; **326**: 87–92.
- Marra M, Bonfigli AR, Testa R, Testa I, Gambini A, Coppa G. High-performance liquid chromatographic assay of asymmetric dimethylarginine, symmetric dimethylarginine, and arginine in human plasma by derivatization with naphthalene-2,3-dicarboxaldehyde. *Anal Biochem* 2003; **318**: 13–17.
- Kirchherr H, Kühn-Velten WN. HPLC-tandem mass spectrometric method for rapid quantification of dimethylarginines in human plasma. *Clin Chem* 2005; **51**: 249–51.
- Marescau B, Nagels G, Possemiers I et al. Guanidino compounds in serum and urine of nondialyzed patients with chronic renal insufficiency. *Metabolism* 1997; **46**: 1024–31.
- Mendes Ribeiro AC, Roberts NB, Lane C, Yaqoob M, Ellory JC. Accumulation of the endogenous L-arginine analogue NG -monomethyl-L-arginine in human end-stage renal failure patients on regular haemodialysis. *Exp Physiol* 1996; **81**: 475–81.
- Dobashi Y, Santa T, Nakagomi K, Imai K. An automated analyzer for methylated arginines in rat plasma by high-performance liquid chromatography with post-column fluorescence reaction. *Analyst* 2002; **127**: 54–59.

- 22 Martens-Lobenhoffer J, Bode-Böger SM. Simultaneous detection of arginine, asymmetric dimethylarginine, symmetric dimethylarginine and citrulline in human plasma and urine applying liquid chromatography-mass spectrometry with very straightforward sample preparation. *J Chromatogr B Analyt Technol Biomed Life Sci* 2003; **798**: 231–99.
- 23 Martens-Lobenhoffer J, Krug O, Bode-Böger SM. Determination of arginine and asymmetric dimethylarginine (ADMA) in human plasma by liquid chromatography/mass spectrometry with the isotope dilution technique. *J Mass Spectrom* 2004; **39**: 1287–94.
- 24 Schwedhelm E, Tan-Andresen J, Maas R, Riederer U, Schulze F, Böger RH. LC-tandem MS method for the analysis of asymmetric dimethylarginine (ADMA). *Clin Chem* 2005 (in press).
- 25 Huang LF, Guo FQ, Liang YZ, Li BY, Cheng BM. Simultaneous determination of L-arginine and its mono- and dimethylated metabolites in human plasma by high-performance liquid chromatography-mass spectrometry. *Anal Bioanal Chem* 2004; **380**: 643–49.
- 26 Vishwanathan K, Tackett RL, Stewart JT, Bartlett MG. Determination of arginine and methylated arginines in human plasma by liquid chromatography-tandem mass spectrometry. *J Chromatogr B Biomed Sci Appl* 2000; **748**: 157–66.
- 27 Annesley TM. Ion suppression in mass spectrometry. *Clin Chem* 2003; **49**: 1041–44.
- 28 Dalton RN, Turner C. Measurement of asymmetric and symmetric dimethylarginines using stable isotope dilution electrospray mass spectrometry-mass spectrometry. Poster presented at the 2nd International Symposium on ADMA: 2004 Nov 6; New Orleans, LA.
- 29 Tsikas D, Schubert B, Gutzki FM, Sandmann J, Frölich JC. Quantitative determination of circulating and urinary asymmetric dimethylarginine (ADMA) in humans by gas chromatography-tandem mass spectrometry as methyl ester tri(*N*-pentafluoropropionyl) derivative. *J Chromatogr B Analyt Technol Biomed Life Sci* 2003; **798**: 87–99.
- 30 Albsmeier J, Schwedhelm E, Schulze F, Kastner M, Böger RH. Determination of *NG*, *NG*-dimethyl-L-arginine, an endogenous NO synthase inhibitor, by gas chromatography-mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 2004; **809**: 59–65.
- 31 Pundak S, Wilchek M. Synthesis of guanidine-*N*-alkylarginines by the use of polymeric pseudoureas. *J Org Chem* 1981; **46**: 808–809.
- 32 Tsikas D. Analysis of the L-arginine/nitric oxide (NO) pathway. The unique role of mass spectrometry. *Curr Pharm Anal* 2005; **1**: 15–30.
- 33 Schulze F, Wesemann R, Schwedhelm E et al. Determination of asymmetric dimethylarginine (ADMA) using a novel ELISA assay. *Clin Chem Lab Med* 2004; **42**: 1377–83.
- 34 Matsuoka H, Itoh S, Kimoto M et al. Asymmetrical dimethylarginine, an endogenous nitric oxide synthase inhibitor, in experimental hypertension. *Hypertension* 1997; **29**: 242–47.
- 35 Caussé E, Siri N, Arnal JF. Determination of asymmetric dimethylarginine by capillary electrophoresis-laser-induced fluorescence. *J Chromatogr B Biomed Sci Appl* 2000; **741**: 77–83.
- 36 Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1986; **i**: 307–10.