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Short communication

Quantification of L-arginine, asymmetric dimethylarginine and symmetric dimethylarginine in human plasma: A step improvement in precision by stable isotope dilution mass spectrometry

Jens Martens-Lobenhoffer*, Stefanie M. Bode-Böger

Institute of Clinical Pharmacology, Otto-von-Guericke University, Magdeburg, Germany

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ABSTRACT

The amino acid L-arginine and its metabolites ADMA and SDMA are important markers for a range of diseases in humans. Increased levels of ADMA and SDMA in plasma point to endothelial dysfunction, hypertension, renal impairment and other pathological states. We present here a method to quantify L-arginine, ADMA and SDMA in human plasma, which is suitable to support clinical research in this field. Sample preparation consisted only of protein precipitation and the analytes were separated using a silica based HILIC column. The analytes were detected by ESI MS/MS, providing high selectivity and sensitivity. The calibration functions were linear in the ranges of 7.5–150 μmol/l for L-arginine, 0.15–3 μmol/l for ADMA and 0.2–4 μmol/l for SDMA. These ranges cover the concentrations encountered in healthy and pathological human plasma. The method employs $^{13}C_6$-arginine, D_7-ADMA and, for the first time in LC–MS/MS, D_6-SDMA as internal standards for L-arginine, ADMA and SDMA. Therefore, matrix independency and a high intra-day precision of 0.82% for L-arginine, 2.12% for ADMA and 2.83% for SDMA, were achieved at basal plasma concentrations. The respective inter-day precision values were 4.01% for L-arginine, 3.77% for ADMA and 3.86% for SDMA.

1. Introduction

The amino acid L-arginine (ARG) and its metabolites asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) are involved in many physiological processes in living cells. Of special interest is the transformation of ARG to the cell signaling substance nitric oxide (NO) and citrulline. In neuronal tissue, NO is involved in the processes of learning and memory [1] and in the vascular endothelium it is essential for the regulation of vascular tone and endothelial function [2]. The enzymatic synthesis of NO from ARG is competitively inhibited by ADMA. Thus, elevated levels of ADMA, or even more pronounced, reduced ratios of ARG and ADMA [3], in plasma are considered as prognostic markers for various vascular diseases, such as hypertension, diabetes mellitus, hypercholesterolemia, atherosclerosis and congestive heart failure [4]. SDMA, on the other hand, is not directly involved in the NO synthesis, but can serve as an early marker for impaired glomerular filtration rate (GFR) and the onset of kidney disease, which may have advantages in comparison to the established GFR markers creatinine and cystatin C [5,6]. For both ADMA and SDMA together, elevated levels in plasma can be predictive for adverse outcome of stroke in humans [7].

To facilitate scientific research in these fields, precise, fast, and easy to handle analytical methods for the quantification of ARG, ADMA and SDMA are of constant need. HPLC coupled to tandem mass spectrometry (LC–MS/MS) can be considered as the gold standard for this task because of its unmatched selectivity and sensitivity [8]. However, a very high degree in precision and accuracy in the quantification of ADMA and SDMA is needed to achieve relevant results from clinical studies [9]. In this regard, LC–MS/MS is less favorable because of the susceptibility of current atmospheric pressure ionization sources for matrix effects, which can introduce severe quantification errors if no measures are taken to control them [10]. The most convenient way to overcome the detrimental effects of the matrix on the quantification results is the introduction of stable isotope labeled analogs as I.S.(s) for every analyte to be quantified [8]. Unfortunately, even in recent publications [11–13], no dedicated stable isotope labeled I.S. for SDMA was used. Since in these publications the focus was more on speed than on precision, quite unsatisfying results regarding intra-day and inter-day precisions for ADMA and especially for SDMA were reported.

In this work, we report a fast, easy, precise and accurate method for the quantification of ARG, ADMA and SDMA in human plasma by LC–MS/MS, utilizing the commercially available I.S.(s)

* Corresponding author at: Institut für Klinische Pharmakologie, Leipziger Str. 44, 39120 Magdeburg, Germany. Tel.: +49 391 6713067; fax: +49 391 6713062.
E-mail address: jens.martens-lobenhoffer@med.ovgu.de (J. Martens-Lobenhoffer).

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$^{13}C_6$-arginine for ARG, D_7-ADMA for ADMA and, for the first time in LC–MS/MS, D_6-SDMA for SDMA. Applying this isotope dilution technique for all analytes led to non-matrix-dependent, precise and robust quantification results. Sample preparation required only protein precipitation and separation of the analytes was achieved on a silica column by the HILIC separation process.

2. Experimental

2.1. Chemicals

ARG, ADMA (as hydrochloride salts) and SDMA (as di(*p*-hydroxyazobenzene-*p*′-sulfonate) salt) were purchased from Sigma–Aldrich (Munich, Germany). The stable isotope labeled I.S.(s) $^{13}C_6$-arginine hydrochloride (isotopic purity 99%) and D_7-ADMA hydrochloride (isotopic purity 98%) were obtained from Cambridge Isotope Laboratories (Andover, MA, USA) whereas D_6-SDMA (isotopic purity 99%) was purchased from Toronto Research Chemicals (New York, ON, Canada). All other chemicals were of analytical grade or better.

2.2. Plasma samples

Blood samples of about 5 ml from 10 apparently healthy volunteers (7 male, 3 female, age 23–55 years, median 39 years, weight 56–135 kg, median 83.5 kg) were drawn into EDTA plasma sampling tubes (BD Vacutainer Systems, Heidelberg, Germany). All participants gave written informed consent for this study. Plasma was obtained by centrifugation at 2400 × *g* for 10 min. Plasma samples were stored at −80 °C until analysis.

2.3. Analytical equipment

The HPLC part of the analytical apparatus consisted of an Agilent 1100 system (Waldbronn, Germany) comprising a binary pump, an autosampler and a thermostated column compartment. The chromatographic separation took place on a Atlantis™ HILIC silica column with 5 μm particle size and with the dimensions 150 mm × 2.1 mm (Waters, Eschborn, Germany), protected by a SecurityGuard system (Phenomenex, Aschaffenburg, Germany) equipped with a 4 mm × 2 mm silica filter insert. The analytes were detected by a Thermo Fisher Scientific (Waltham, MA, USA) TSQ Discovery Max triple quadrupole mass spectrometer, equipped with an electrospray ionization (ESI) ion source. System control and data handling were carried out by the Thermo Electron Xcalibur software, version 1.2.

2.4. Calibration and quality control samples

A calibration stock solution was prepared in water with the concentrations of 1500 μmol/l for ARG, 30 μmol/l for ADMA and 40 μmol/l for SDMA. From this stock solution, aqueous and human plasma calibration samples in 7 concentration levels ranging between 7.5 and 150 μmol/l for ARG, 0.15 and 3 μmol/l for ADMA and 0.2 and 4 μmol/l for SDMA, were prepared. Quality control samples were prepared from pooled human plasma containing the endogenous concentrations of ARG, ADMA and SDMA (low level). By spiking with the calibration stock solution two further levels (medium and high) from the same plasma pool with added concentrations of 30 μmol/l and 150 μmol/l for ARG, 0.6 μmol/l and 3 μmol/l for ADMA and 0.8 μmol/l and 4 μmol/l for SDMA were prepared.

2.5. Sample preparation

50 μl of calibration samples, quality control samples or unknown samples was mixed with 10 μl of the I.S. solution (containing 462 μmol/l $^{13}C_6$-arginine, 3.49 μmol/l D_7-ADMA and 4.81 μmol/l D_6-SDMA in water). Proteins in the samples were precipitated by adding 60 μl buffer solution (1 ml formic acid and 1 g ammonium formate in 200 ml water, pH 3.5) and 375 μl acetonitrile. After mixing on a vortex mixer, the precipitated proteins were separated by centrifugation at 10,000 × *g* for 5 min. From the clear supernatant, about 100 μl was transferred into autosampler vials with microliter inserts.

2.6. Chromatography and detection

From the prepared samples, 10 μl was injected into the HPLC system. The mobile phase consisted of 20% of 0.025% trifluoroacetic acid and 1% propionic acid solution in water (resulting pH = 1.5) and 80% acetonitrile at a flow rate of 0.25 ml/min. The column temperature was set to 30 °C. The column effluent was directed without splitting to the ESI ion source of the mass spectrometer. The capillary temperature was set to 270 °C. Sheath gas, ion sweep gas and auxiliary gas settings were 45, 5 and 15 arbitrary units, respectively. The mass spectrometer was working in the positive mode and the ionization voltage was set to 4500 V. The chromatographic traces of the analytes were recorded in the multiple reaction monitoring mode (MRM), observing the fragment ions m/z 175.2 → 60.1 for ARG, m/z 181.2 → 61.1 for $^{13}C_6$-arginine, m/z 203.1 → 46.1 for ADMA, m/z 210.2 → 46.1 for D_7-ADMA, m/z 203.1 → 172.1 for SDMA and m/z 209.2 → 175.1 for D_6-SDMA. The precursor to product ion fragmentation took place using argon at a pressure of 1.5 mTorr as collision gas. The fragmentation energies were 18 eV, 18 eV, 16 eV, 16 eV, 15 eV and 15 eV, respectively, for the above described fragment ions.

3. Results and discussion

3.1. Sample preparation

A main feature of the current method is the very simple sample preparation. It consisted only of the addition of the I.S., buffering and protein precipitation by the addition of acetonitrile. No cumbersome extraction of the analytes with, for example, solid phase ion exchange columns, was necessary. Thus, the procedure was fast, inexpensive and robust. The utilization of acetonitrile as protein precipitation agent fulfilled the additional purpose of yielding a sample composition very similar to the mobile phase of the HPLC system, thus avoiding peak distortion by injecting samples with a higher elution strength than the mobile phase. The buffering of the samples prior to protein precipitation led to slightly higher and more stable peak intensities in the resulting chromatograms, compared to our previous method without buffering [14]. Other protein precipitation agents, such as methanol with 1% ammonium acetate [12] or methanol:acetonitrile (25:75) [15], were described in the literature. However, the results were comparable with those of our current method in terms of peak shape and absence of interfering peaks.

3.2. Chromatography and detection

The basic amino acids ARG, ADMA and SDMA are very polar and water soluble and therefore very difficult to retain on reversed phase columns without derivatization or ion pairing. Drawbacks of these techniques are that derivatization is laborious and expensive and ion paring is detrimental for the ionization efficiency in the ion source of a mass spectrometer. However, ARG, ADMA and SDMA

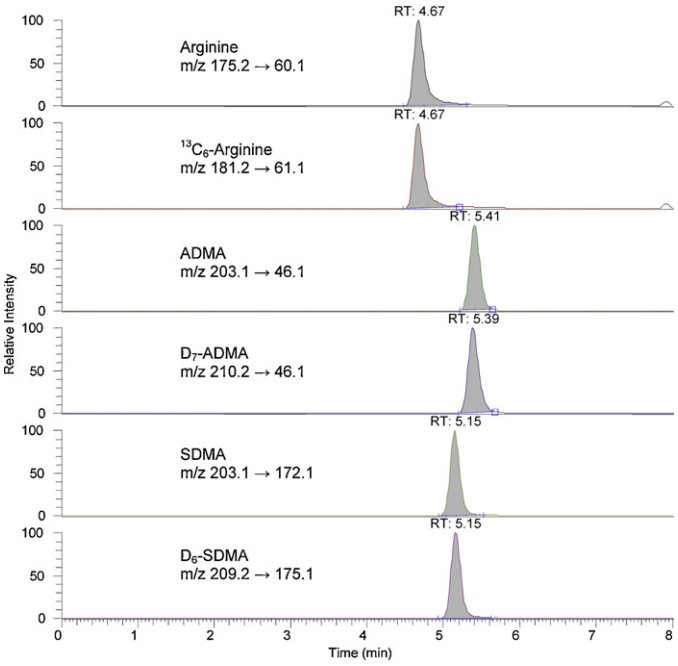

Fig. 1. Typical chromatogram of a human plasma sample from a healthy volunteer. The multiple reaction monitoring ion traces of all analytes are shown. The measured concentrations were 130.3 μmol/l ARG, 0.368 μmol/l ADMA and 0.387 μmol/l SDMA.

are suitable analytes for the nowadays well established hydrophilic interaction (HILIC) chromatography [16], which is performed on a polar stationary phase with a mobile phase rich in organic modifier. As it turned out, a certain amount of trifluoroacetic acid in the mobile phase was necessary to produce tailing free peaks of the analytes [17]. Since it is well known that trifluoroacetic acid in the mobile phase causes signal suppression in an ESI ion source, a weaker acid (in this case propionic acid) had to be added in excess of the trifluoroacetic acid to alleviate this effect [18]. This remedy, in combination with the high content of acetonitrile in the mobile phase, led to good ionization efficiencies in the ion source and therefore to high intensities of the analyte peaks. The resulting highly acidic mobile phase (pH = 1.5) had no detrimental effect on the column life time, since the silica gel stationary phase without any hydrolysable surface modification is stable at such low pH-values.

As it can be seen in Fig. 1, the analytes showed reasonable retention and sharp and symmetrical peak shapes in the HILIC separation mode. The isocratic mobile phase led to short cycling times of about 8 min. Thus, it may be possible to analyze up to about 180 samples per day. The simple sample clean up procedure had no negative impact on the column life time. The actual column used for this study accumulated over 5000 injections and showed no performance loss so far.

Due to the high intrinsic selectivity of a tandem mass spectrometer, no interferences of endogenous substances were observed (see Fig. 1), despite the unsophisticated sample preparation. Especially the chromatographically difficult to resolve pair ADMA and SDMA was clearly separated by mass spectrometric means by the observation of specific fragment ions for both analytes. The stable isotope labeled I.S.(s) eluted at virtually the same retention times as the respective unlabeled analytes. Owing to the high purity and the 6 or 7 amu higher masses of the I.S.(s), no crosstalk between the analytes and their respective I.S.(s) was observed.

3.3. Calibration and matrix effects

The calibration of endogenous substances like ARG, ADMA and SDMA held special challenges. Since there is no authentic matrix available without these substances, calibration samples were made by adding up the endogenous concentrations of the analytes in plasma calibration samples or the calibration was performed using aqueous samples. However, not taking into account the matrix differences between plasma and water may have contributed to systematical biases in earlier work [19].

In the method described here, such matrix dependencies in the quantification were eliminated by the introduction of stable isotope labeled I.S.(s) for all analytes. The resulting calibration functions from aqueous calibration samples possessed no significant intercepts, whereas the calibration from plasma showed significant intercepts corresponding to the endogenous amounts of the analytes before spiking. However, the slopes for the calibration from aqueous samples and the calibration from plasma were very similar. In the case of ARG, the values were 0.00949 ± 0.00003 and 0.00957 ± 0.00012. The respective values for ADMA were 1.2149 ± 0.0174 and 1.2427 ± 0.0392, and for SDMA 1.0167 ± 0.0124 and 1.0039 ± 0.0134. The differences between the slopes from aqueous calibration samples and from plasma were 0.84% for ARG, 2.24% for ADMA and 1.28% for SDMA. As it was demonstrated by these minor differences, the quantification of the analytes was independent from the substantial differences between the matrix of aqueous and plasma samples. Thus, the calibration functions obtained from aqueous calibration samples were suitable to quantify unknown plasma samples.

The lower limits of quantification were defined by the lower ends of the calibration ranges [20]. These values were 7.5 μmol/l for ARG, 0.15 μmol/l for ADMA and 0.2 μmol/l for SDMA. The lower limits of detection at signal to noise ratios of 3 were 0.5 μmol/l for ARG, 0.003 μmol/l for ADMA and 0.005 μmol/l for SDMA. The calibration ranges and the limits of detection and quantification covered the concentrations that were encountered in plasma of healthy and pathological humans [19].

3.4. Precision and accuracy

Pooled quality control plasma samples in 3 concentration levels were utilized for intra-day and inter-day precision testing. In Table 1 the results are summarized. The differences in basal plasma concentrations found in the intra-day and inter-day mean values are due to different plasma pools for the respective quality control sample lots. As it can be seen, all values were well within the limits established by the United States Food and Drug Administration for bioanalytical method validation [20]. Especially the very low relative standard deviations (R.S.D.) of SDMA of about 3% in the intra-day precision testing and about 4% in inter-day testing were strong improvements in comparison to the results of the quite similar, but not using a dedicated stable isotope labeled I.S. for SDMA, method described in [14], where values of less than 7% were reported. Similarly high R.S.D.(s) were reported by El Khoury et al. [12] or even higher values by Di Gangi et al. and Shin et al. [11,13]. The improvement regarding the precision in the current method was attributable to the introduction of the stable isotope labeled I.S. D_6-SDMA for SDMA, which enhanced the performance of the SDMA quantification to the levels also obtained for ADMA and ARG. The low R.S.D.(s) for SDMA, as well as for ARG and ADMA, can be assumed as ideally suited for supporting clinical studies without lowering their statistical power by undue analytical imprecision [9].

Table 1
Precision and accuracy.

Analyte	Level	Spike concentration (μmol/l)	Intra-day precision and accuracy ($n = 10$)			Inter-day precision and accuracy ($n = 5$)		
			Mean (μmol/l)	R.S.D. (%)	Accuracy (%)	Mean (μmol/l)	R.S.D. (%)	Accuracy (%)
L-arginine	Low	0	55.4	0.82	N/A	85.3	4.01	N/A
L-arginine	Medium	30	83.7	1.20	−5.63	117.2	3.46	1.70
L-arginine	High	150	196.6	0.73	−5.87	240.7	3.22	2.31
SDMA	Low	0	0.368	2.83	N/A	1.112	3.86	N/A
SDMA	Medium	0.8	1.163	2.64	2.68	1.920	4.50	0.42
SDMA	High	4	4.338	1.79	−0.76	5.012	3.60	−1.94
ADMA	Low	0	0.344	2.12	N/A	0.652	3.77	N/A
ADMA	Medium	0.6	0.928	3.17	−2.64	1.290	6.09	3.00
ADMA	High	3	3.128	3.75	−7.21	3.758	4.85	2.89

N/A, not applicable.

3.5. Application of the method

To demonstrate the applicability of this method, EDTA-plasma samples from 10 apparently healthy volunteers as described in Section 2.2 were analyzed. We found values of 115.2 ± 18.9 μmol/l ARG, 0.447 ± 0.061 μmol/l ADMA and 0.458 ± 0.121 μmol/l SDMA. These values were in good agreement with the results of much larger general population studies, for example the Hoorn Study, which reported ADMA and SDMA values of 0.497 ± 0.063 μmol/l and 0.526 ± 0.101, respectively [9], or the Framingham Offspring Study with ADMA values of 0.52 ± 0.11 μmol/l [21]. The day to day robustness of the method was proven by the measurement of several hundred samples from larger clinical studies (results are pending publication elsewhere). Regardless of the simple sample preparation procedure, no degradation of the analytical column or the performance of the mass spectrometer was observed over the course of several thousand injections.

4. Conclusion

The method presented here for the determination of ARG, ADMA and SDMA in human plasma is reliable, precise and accurate. The relatively easy, fast and low cost sample preparation procedure makes this approach suitable for large studies. The small required plasma volume of 50 μl enables the measurement even in situations where the total sample size is restricted. If necessary, we believe that the plasma volumes used within the sample preparation procedure could be further downsized without loss in sensitivity because only about 2% of the total prepared sample is injected into the LC–MS/MS system.

The addition of the stable isotope labeled I.S.(s) $^{13}C_6$-arginine, D_7-ADMA and D_6-SDMA provided for all three analytes quantification results suitable in precision and accuracy for the reasonable support of clinical studies without introducing losses in statistical power [9]. Furthermore, these I.S.(s) make the method completely matrix independent. Thus, it is possible to extend the applicability of the method easily to other biological fluids like urine or cell culture supernatant from humans or from other species like mice or rats. The method is therefore a valuable tool for supporting the scientific research in the field of ARG and its dimethylated metabolites ADMA and SDMA.

Conflict of interest statement

The authors declare to have no conflicts of interests.

References

[1] E. Fedele, M. Raiteri, Prog. Neurobiol. 58 (1999) 89.
[2] J.P. Cooke, V.J. Dzau, Annu. Rev. Med. 48 (1997) 489.
[3] S.M. Bode-Böger, F. Scalera, L.J. Ignarro, Pharmacol. Ther. 114 (2007) 295.
[4] J.P. Cooke, Circulation 109 (2004) 1813.
[5] J.T. Kielstein, S.R. Salpeter, S.M. Bode-Böger, J.P. Cooke, D. Fliser, Nephrol. Dial. Transplant. 21 (2006) 2446.
[6] J.T. Kielstein, H. Veldink, J. Martens-Lobenhoffer, H. Haller, M. Burg, J.M. Lorenzen, R. Lichtinghagen, S.M. Bode-Böger, V. Kliem, Nephrol. Dial. Transplant. 26 (2011) 324.
[7] H. Worthmann, S. Chen, J. Martens-Lobenhoffer, N. Li, M. Deb, A.B. Tryc, A. Goldbecker, Q. Dong, J.T. Kielstein, S.M. Bode-Böger, K. Weissenborn, J. Atheroscler. Thromb. 18 (2011) 753.
[8] J. Martens-Lobenhoffer, S.M. Bode-Böger, J. Chromatogr. B 851 (2007) 30.
[9] T. Teerlink, J. Chromatogr. B 851 (2007) 21.
[10] S. Souverain, S. Rudaz, J.L. Veuthey, J. Chromatogr. A 1058 (2004) 61.
[11] I.M. Di Gangi, L. Chiandetti, A. Gucciardi, V. Moret, M. Naturale, G. Giordano, Anal. Chim. Acta 677 (2010) 140.
[12] J.M. El Khoury, D.R. Bunch, E. Reineks, R. Jackson, R. Steinle, S. Wang, Anal. Bioanal. Chem. 402 (2012) 771.
[13] S. Shin, S.M. Fung, S. Mohan, H.L. Fung, J. Chromatogr. B 879 (2011) 467.
[14] J. Martens-Lobenhoffer, S.M. Bode-Böger, Clin. Chem. 52 (2006) 488.
[15] O. D'Apolito, G. Paglia, F. Tricarico, D. Garofalo, A. Pilotti, O. Lamacchia, M. Cignarelli, G. Corso, Clin. Biochem. 41 (2008) 1391.
[16] Y. Guo, S. Gaiki, J. Chromatogr. A 1074 (2005) 71.
[17] G. Paglia, O. D'Apolito, F. Tricarico, D. Garofalo, G. Corso, J. Sep. Sci. 31 (2008) 2424.
[18] W.Z. Shou, W. Naidong, J. Chromatogr. B 825 (2005) 186.
[19] J. Martens-Lobenhoffer, S.M. Bode-Böger, Eur. J. Clin. Pharmacol. 62 (2006) 61.
[20] U.S. Food and Drug Administration, Guidance for Industry: Bioanalytical Method Validation, FDA, Washington, DC, 2001, http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf.
[21] E. Schwedhelm, V. Xanthakis, R. Maas, L.M. Sullivan, F. Schulze, U. Riederer, R.A. Benndorf, R.H. Böger, R.S. Vasan, Clin. Chem. 55 (2009) 1539.