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Direct analysis of un-derivatized asymmetric dimethylarginine (ADMA) and L-arginine from plasma using mixed-mode ion-exchange liquid chromatography–tandem mass spectrometry

Michael J. Bishop*, Brian Crow, Dean Norton, Ekaterina Paliakov, Joe George, J.A. Bralley

Metametrix Clinical Laboratory, 3425 Corporate Way, Duluth, GA 30096, United States

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Abstract

A high-throughput analytical method was developed for the measurement of asymmetric dimethylarginine (ADMA) and L-arginine (ARG) from plasma using LC/MS/MS. The sample preparation was simple and only required microfiltration prior to analysis. ADMA and ARG were assayed using mixed-mode ion-exchange chromatography which allowed for the retention of the un-derivatized compounds. The need for chromatographic separation of ADMA from symmetric dimethylarginine (SDMA) was avoided by using an ADMA specific product ion. As a result, the analytical method only required a total run time of 2 min. The method was validated by linearity, with $r^2 \geq 0.995$ for both compounds, and accuracy, with no more than 7% deviation from the theoretical value. The estimated limit of detection and limit of quantification were suitable for clinical evaluations. The mean values of plasma ADMA and ARG taken from healthy volunteers ($n = 15$) were 0.66 ± 0.12 and 87 ± 35 μM, respectively; the mean molar ratio of ARG to ADMA was 142 ± 81.

Keywords: Asymmetric dimethylarginine; ADMA; L-arginine; ARG; Plasma; LC/MS/MS; Ion-exchange

1. Introduction

The endothelial derived relaxing factor nitric oxide (NO) has been identified to participate in the regulation of vascular tone, neurotransmission, blood flow and blood pressure [1–3]. Endothelial nitric oxide synthase (eNOS) catalyzes the production of NO from the terminal guanidino nitrogen atoms of L-arginine (ARG) [4]. This enzymatic pathway can be competitively inhibited by methylated arginine metabolites, most notably asymmetric dimethylarginine (ADMA) [5–9]. Elevated levels of plasma ADMA have been associated with atherosclerotic disease, stroke, heart disease, and hypertension [10–22]. Additionally, patients who received intravenous doses of ADMA were shown to have adverse cardiac effects [23].

There are a number of publications that describe analytical methods for the measurement of ADMA and ARG from plasma [24–26]. Analytical methods for the rapid quantification of ARG, along with a number of other essential amino acids, are quite common and have been described in several publications [27,28]. There are fewer publications regarding the measurement of ADMA, however more methods are likely to emerge as research continues to reveal its importance as a biomarker for cardiovascular disease. The most difficult aspect of ADMA measurement is the presence of a similar isobaric biomarker, symmetric dimethylarginine (SDMA), which is another metabolite of arginine methylation. SDMA, like ADMA, is a naturally occurring compound found in plasma. SDMA is similar to ADMA in nearly all chemical properties and only differs in structure by the placement of a methyl group. In order for there to be an accurate method for the quantification of ADMA, both compounds must be separated analytically. The most common analytical approach for this separation requires derivatization prior to chromatographic separation [29–32]. However, the sample preparation needed for derivatization is often very time

* Corresponding author. Tel.: +1 800 221 4640; fax: +1 770 441 2237.
E-mail address: mbishop@metametrix.com (M.J. Bishop).

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Table 1
LC instrument method for the extended chromatographic run

Time (min)	% MPA	% MPB	Flow (mL/min)	Curve	Comments
0.00	100.0	0.0	0.300	1	Initial conditions
1.00	100.0	0.0	0.300	1	1.00 min hold at 100% MPA
5.00	0.0	100.0	0.300	10	% MPA concave down, % MPB concave up
10.00	0.0	100.0	0.300	1	5.00 min hold at 100% MPB
10.01	100.0	0.0	0.300	1	Return to initial conditions
15.00	100.0	0.0	0.300	1	Total runtime equals 15 min

consuming and the derivatized products are not always stable [26]. Similarly, the chromatographic separation of ADMA and SDMA can require extended runtimes which can decrease efficiency [24].

Current developments in LC/MS/MS have allowed for the separation of ADMA and SDMA by mass spectral analysis [33–36]. One such method requires the derivatization of ADMA and SDMA to produce compounds that form distinct product ions [36]. The advantage of this method is that ADMA and SDMA can be separated spectrally rather than chromatographically. Therefore, shorter chromatographic runs are required for the analysis of ADMA. However, the reduction in chromatographic runtime is somewhat offset by the increased time required for sample preparation. Other publications have achieved spectral separation of ADMA and SDMA without the need for derivatization, reducing the time needed for sample preparation. Although both ADMA and SDMA form the same precursor ion at *m/z* 203.2, differences in the product ion formation have been used to distinguish the compounds. A selective product ion for ADMA has been reported at *m/z* 46 while SDMA produces a selective product ion at *m/z* 172; the use of these specific ions makes the accurate determination of ADMA and SDMA possible without the need for chromatographic separation [33–35]. Although this technique allows for selective spectral determination of ADMA, few analytical methods have been developed using LC/MS/MS that are suitable for high-throughput analysis.

In this report, we describe a simple analytical method for the rapid quantification of ADMA and ARG from plasma. Using the selective ADMA product ion, the need for chromatographic separation from SDMA is avoided. This technique has been adapted here for the development of an accurate analytical method for rapid analysis of ADMA without a lengthy chromatographic separation. Chromatographic peaks were concentrated on instrument using a mixed-mode ion exchange analytical column. The analytical method requires little sample preparation, using centrifuge micro-filtration for sample clean-up with a chromatographic run time of only 2 min. The method was validated by linearity and accuracy experiments with an established limit of quantification that is suitable for clinical measurement of patient samples. The method is currently the most efficient analytical technique for the screening of ADMA and ARG from plasma.

2. Experimental

2.1. Instrumentation and reagents

All chromatography was performed using a Waters (Milford, MA, USA) 2695 high-performance liquid chromatograph. A Waters Quattro-micro tandem mass spectrometer was used for analyte detection. All samples were analyzed using an electrospray ionization source in positive ion mode. HPLC grade acetonitrile was purchased from VWR (VWR International, North America). Trifluoroacetic (TFA) was purchased from Sigma (St. Louis, MO USA). The standards, L-arginine and asymmetric dimethylarginine were also purchased from Sigma. The internal standards, L-lysine, 4, 4, 5, 5,-d_4 (D4LYS) and L-arginine-guanido-$^{15}N_2$ (N15ARG) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Microcon centrifugal filters, ultracel YM-3 (3 kDa cut-off), were purchased from Millipore (Bedford, MA, USA).

2.2. Chromatographic conditions

Two chromatographic separations were performed in the development of this assay. The first extended chromatographic assessment was used to determine the presence of interferences for ADMA, ARG, D4LYS, and N15ARG from plasma. The second short chromatographic assay was developed for

Table 2
LC instrument method for the short chromatographic run

Time (min)	% MPA	% MPB	Flow (mL/min)	Curve	Comments
0.00	100.0	0.0	0.300	1	Initial conditions
0.50	0.0	100.0	0.600	10	% MPA concave down, % MPB concave up
1.00	0.0	100.0	0.800	10	0.50 min hold at 100% MPB
1.01	100.0	0.0	1.000	1	Return to initial conditions
2.00	100.0	0.0	1.000	1	Total runtime equals 2 min

Table 3
MRM transitions and detection settings for ADMA, ARG, D4LYS, and N15ARG

Analytes and I.S.	Precursor ion [M + H]	Product ion	Cone potential (V)	Collision energy (eV)
ADMA	203.2	46.0	25	15
ARG	175.2	70.0	25	20
D4LYS	151.1	87.9	20	15
N15ARG	177.2	70.1	25	20

the rapid determination of ADMA and ARG. Each chromatographic method was performed using a SIELC Technologies (Prospect Heights, IL, USA) Primesep 100 guard column, 10 mm × 2.1 mm, 5 μm maintained at 40 °C throughout the experiment. Two mobile phases were used for elution consisting of 0.01% TFA in de-ionized water (MPA) and 0.25% TFA in acetonitrile (MPB). For the extended chromatographic run, 10 μL of the sample was injected on column with initial mobile phase conditions at 100% MPA and a flow rate of 0.3 mL/min. The initial mobile phase conditions were held constant for the first minute (0–1). A concave gradient was employed following the 1 min hold (1–5 min) to 100% MPB. These conditions were held for 5 min (5–10 min) following the gradient. Finally, the mobile phase composition was returned to the initial conditions after 10 min (10.01–15) resulting in a total run time equal to 15 min (Table 1). The gradient employed in this method utilized a preset instrument gradient, Waters #10, to perform a concave gradient from initial conditions to the final elution conditions (1–5 min). The gradient changes were concave up for MPB; however, the MPA concentration changes from 100 to 0% were concave down. All other gradient changes were made immediately, Waters #1, and do not follow a gradient profile.

The short chromatographic separation was performed using the same chromatographic conditions listed above. The gradient elution profile was adjusted to increase method efficiency. Flow rate adjustments were made throughout the chromatographic separation following a linear profile. 10 μL of the sample was injected on column with initial mobile phase conditions at 100% MPA and a flow rate of 0.3 mL/min. A concave gradient was employed over the first 0.5 min (0–0.5 min) to 100% MPB; the flow rate was increased to 0.6 mL/min. Following the gradient, the mobile phase conditions were held for 0.5 min (0.5–1 min) and the flow was increased to 0.8 mL/min. Finally, the mobile phase composition was returned to the initial conditions after 1 min (1.01–2) and the flow rate was increased to 1.0 mL/min for the remainder of the chromatographic run (Table 2). The total run time was equal to 2 min. The gradient employed in this method utilized a preset instrument gradient, Waters #10, to perform a concave gradient from initial conditions to the final elution conditions (0–0.5 min). The gradient changes were concave up for MPB; however, the MPA concentration changes from 100 to 0% were concave down. The flow rate was increased throughout the chromatographic run in order to increase efficiency and to reduce the time needed for column re-equilibration. The overall elution profile results in a change in mobile phase composition increasing the organic modifier and flow rate to clear the column of any residual compounds.

2.3. Mass spectrometer conditions

Electrospray positive ionization mode was used for the detection of all compounds throughout each experiment. The desolvation gas was set to 800 L/h, while the cone gas was not used. Capillary voltage was maintained at 2.5 kV, with source and desolvation temperatures at 150 and 350 °C, respectively. Each MRM was collected at unit mass resolution with a dwell time of 0.1 s. The cone and collision settings were established individually for each compound for multiple reaction monitoring (MRM) detection. The conditions for detection of all analytes were obtained by direct infusion of a standard solution in line with the HPLC at initial mobile phase conditions. The MRM transitions and appropriate detection settings are presented in Table 3.

2.4. Standard and working solutions

Working stock solutions were prepared for ADMA, ARG, and the internal standard as follows: Working stock solution A (StkA) was made by the addition of 0.0202 g of ADMA to a 200 mL volumetric flask and brought to volume with de-ionized water. Working stock solution B (StkB) was prepared in the same manner by the addition of 0.0211 g of ARG to a 200 mL volumetric flask and brought to volume with de-ionized water. The internal standard solution was prepared by the addition of 0.0015 g of D4LYS and 0.0044 g of N15ARG to a 100 mL volumetric flask and brought to volume with de-ionized water.

2.5. Calibration standards

Five levels of calibration were prepared for ADMA and ARG. The highest calibration level was prepared by the addition of 40 μL of StkA and 6 mL of StkB to a 10 mL volumetric flask and brought to volume with de-ionized water. Calibration standards were prepared by serial dilution of the highest calibration level for the desired calibration range. Five levels of calibration were used for quantification, including a blank, prepared at concentrations of 0.00, 0.25, 0.50, 1.00, 1.50, and 2.00 μM for ADMA and 0.00, 37.5, 75, 150, 225, and 300 μM for ARG.

2.6. Accuracy spiking standards

The standard solutions used for the accuracy experiment were prepared from StkA and StkB. The highest concentration standard solution was prepared by the addition of 300 μL of StkA and 8 mL of StkB to a 10 mL volumetric flask and brought to vol-

ume with de-ionized water. Spiking standards were prepared by the serial dilution of the highest concentration standard solution. Five levels of spiking standard were prepared at concentrations of 5.0, 7.5, 10.0, 12.5, and 15 μM for ADMA and 133, 200, 267, 333, and 400 μM for ARG.

2.7. Linearity and accuracy

The linearity of the calibration curve was evaluated by linear regression, including the intercept ($y = mx + b$), weighted by $1/x$. Linear curves were comprised of five calibration levels quantified from a standard curve to evaluate reproducibility. Accuracy was evaluated by spike recovery from pooled plasma samples. The baseline plasma level was spiked with five standard solutions of increasing concentration. Spiked samples were prepared by the addition of 10% (v/v) of the specific standard solution to the baseline plasma. The baseline samples were prepared in a similar way i.e. made with 10% de-ionized water in the place of the standard solution. All accuracy samples, including the baseline and spiked samples, were run in duplicate and quantified using a standard curve.

2.8. Limits of detection (LOD) and quantification (LOQ)

The LOD (S/N = 3) and LOQ (S/N = 10) were determined using the regression approach based upon the linear regression of calibration from the established linear range [37]. The sensitivity of the present method was determined from these measurements.

2.9. Sample preparation

Plasma samples were collected in plastic sample tubes and stored at −20 °C. Samples were prepared by the addition of 20 μL of internal standard solution and 60 μL of sample/calibrator to a micro-centrifuge filter. The samples were centrifuged at 13,000 rpm for 10 min. Following centrifugation, 25 μL of filtrate was extracted into Waters total recovery sample vials. The samples were capped and injected on instrument.

2.10. Patient samples

Plasma samples were collected from healthy volunteers ($n = 15$) and were evaluated using the analytical method described here. Plasma samples were collected and stored at −20 °C prior to analysis. All samples were prepared according to the sample preparation method described above. Sample concentrations were quantified using a standard curve.

3. Results and discussion

3.1. Chromatography and MS/MS detection

The analysis of ARG by mass spectrometry has been well established and was developed using common mass spectral techniques [25]. The analysis of ADMA often requires chromatographic separation from SDMA which is another ARG metabolite found in plasma [24–26]. Here, the chromatographic separation of ADMA and SDMA is not required because the compounds can be distinguished by mass spectrometry. As mentioned, the product ions formed from the common precursor ion of *m/z* 203.2 can be used to spectrally distinguish the two compounds [33–35]. Formed by CID of *m/z* 203.2, the selective product ion at *m/z* 46 for ADMA and at *m/z* 172 for SDMA are illustrated in Fig. 1.

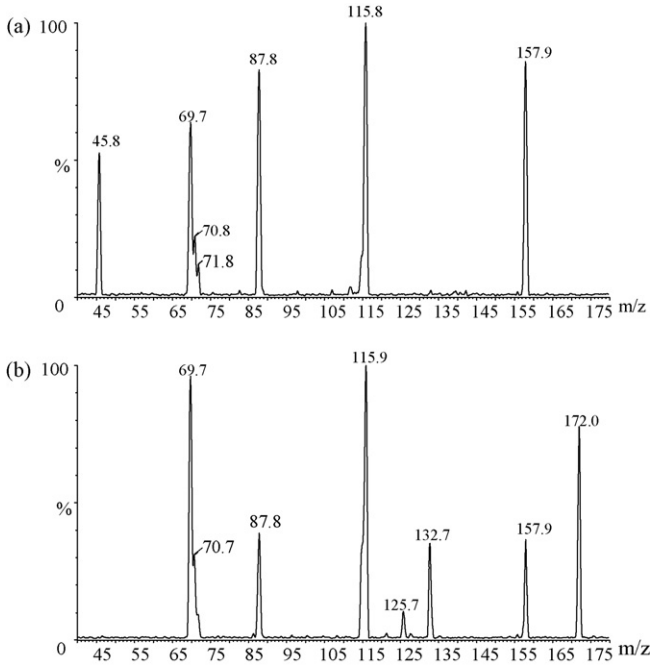

Fig. 1. The mass spectra of daughter scans for *m/z* 203.2, ADMA (a) and SDMA (b).

Although ADMA can be distinguished from SDMA, prior to the development of a rapid analytical assay a longer chromatographic run is required to determine whether or not interfering compounds are present. The SIELC Primesep 100, a mixed-mode analytical column, was used for all experiments. This column utilizes both reversed phase and ion-exchange for analyte retention. The strong interactions between the charged zwitterionic amino acids and the anionic stationary phase allows for increased retention with shorter columns. The ionic retention also allows for the development of a chromatographic method without the need for sample derivatization, increasing the efficiency of sample preparation. Using a pooled plasma sample, the selectivity of the MRM transitions for ADMA, ARG, D4LYS, and N15ARG was determined using an extended chromatographic method. The MRM transitions for all compounds contained no observable interferences (Fig. 2). Without the presence of matrix interferences a more rapid chromatographic approach was developed. For the purpose of method efficiency, the shortest analytical column available was considered in order to achieve the shortest possible run time. The guard column is used here only to provide minimal retention needed to produce a concentrated sample peak and to reduce band broadening. The chromatographic plots for the rapid assessment of plasma ADMA and ARG, including internal standards, are shown in Fig. 3.

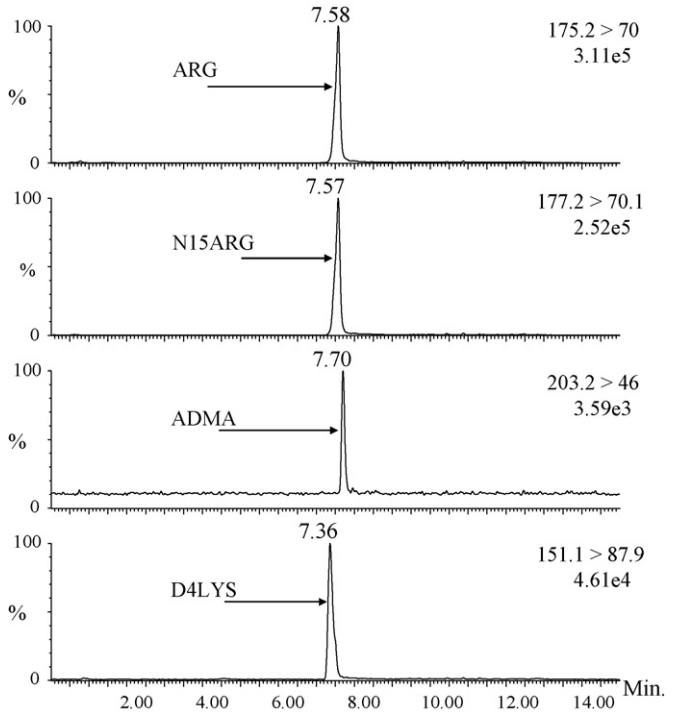

Fig. 2. Chromatogram using the extended analytical run of a plasma sample illustrating the selectivity of the MRM transitions for ARG, N15ARG, ADMA, and D4LYS.

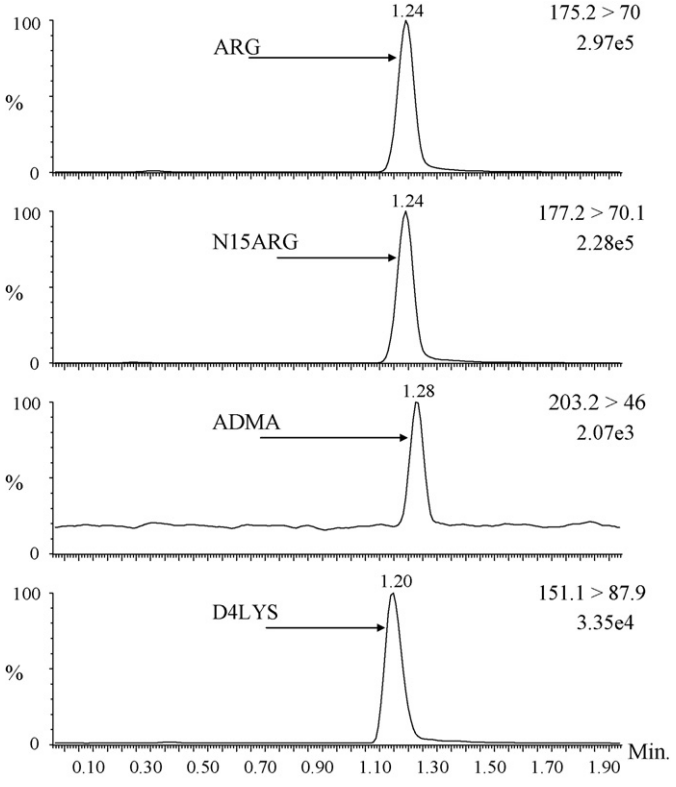

Fig. 3. Chromatogram of a plasma sample illustrating ADMA, ARG, and internal standards.

Table 4
Accuracy results for ADMA and ARG from standard spike levels in plasma

Experimental results: average measured concentration in μM, (% recovery)		
Accuracy sample	ADMA	ARG
Baseline, [ADMA/ARG] μM	0.699, (N/A)	85.8, (N/A)
Spike level-1, [0.50/13.3]	1.19, (98)	99.5, (103)
Spike level-2, [0.75/20.0]	1.40, (93)	106, (100)
Spike level-3, [1.00/26.7]	1.71, (101)	113, (103)
Spike level-4, [1.25/33.3]	1.91, (97)	120, (104)
Spike level-5, [1.50/40.0]	2.31, (107)	126, (100)

3.2. Linearity and accuracy

Linearity was evaluated based on observed percent deviation (%Dev) of five calibrators and a blank calculated from a standard curve. The calibration plots were fit to a linear equation of slope and intercept ($y = mx + b$) weighted by $1/x$. All slopes had r^2 values greater than 0.995. Deviations of the calculated standard values from theoretical values were less than 15% for all analytes. The accuracy of each analyte was evaluated based on the percent recovery for five levels of spiked samples compared with a baseline of pooled plasma. The percent deviation from the theoretical value for the recovered spike was less than 15% for all spikes (Table 4).

3.3. Limits of detection (LOD) and quantification (LOQ)

The LOD (S/N = 3) and LOQ (S/N = 10) were measured for each analyte based upon the linear regression of calibration from the established linear range. The calculated values of LOD and LOQ were 0.06 and 0.20 μM for ADMA, 2.5 and 8.2 μM for ARG, respectively. The measured LOD and LOQ indicate adequate sensitivity for clinical assessments and are consistent with a recent report [33].

3.4. Patient samples

Healthy human plasma samples ($n = 15$) were assayed and quantified from a standard curve. The mean value of plasma ADMA and ARG taken from collected patient samples were 0.66 ± 0.12 and 87 ± 35 μM, respectively. The mean molar ratio of ARG to ADMA was 142 ± 81.

4. Conclusions

Recent developments in tandem MS method development have allowed for selective determination of ADMA from SDMA without chromatographic separation. This technique was utilized here to develop a rapid analytical method for accurate determination of ADMA and ARG in plasma. The method presented here requires only filtration for sample preparation and a short chromatographic analysis of 2 min. Possible interferences from plasma were assessed using an extended chromatographic run; no interfering plasma components were observed. The analytical method was validated by linearity and accuracy experiments with an established LOQ that is suitable for clinical

analyses. Mean values of ADMA and ARG taken from healthy volunteers ($n = 15$) were 0.66 ± 0.12 and 87 ± 35 μM, respectively; the mean molar ratio of ARG to ADMA was 142 ± 81.

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