American Journal of Analytical Chemistry, 2011, 2, 303-313
doi:10.4236/ajac.2011.23038 Published Online July 2011 (http://www.scirp.org/journal/ajac)

Determination of Asymmetric Dimethylarginine and Symmetric Dimethylarginine in Biological Samples of Mice Using LC/MS/MS

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Received March 7, 2011; *revised April* 2, 2011; *accepted April* 30, 2011

Abstract

Herein, we present a novel method of asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) determination within biological samples using protein precipitation and LC/MS/MS. Chromatographic separation of ADMA and SDMA was successfully performed using a silica column with optimized elution, or mobile phase, of 10 mM ammonium acetate buffer H_2O/methanol/acetonitrile (20/35/45, v/v) at pH 4. The calibration ranges were 0.50 - 50.0 μg·mL^{-1}, and good linearities were obtained for all compounds (r > 0.99). The intra- and inter-assay accuracies with recoveries and precisions at three concentration levels (*i.e.* 1.00, 5.00 and 25.0 μg·mL^{-1}) were better than 86.9% and 7.36%, respectively. The analytical performance of the method was evaluated by determination of compounds in plasma, urine and tissues from male BALBc/J mice. For the first time, we were able to characterize the distribution of ADMA, SDMA and ADMA/SDMA in plasma, urine, brain, heart, kidneys, liver, lungs, pancreas and spleen. Additionally, we demonstrated that the ADMA/SDMA ratio in the brain was approximately 10-fold lower than all the other biological samples. Only 10 μL of plasma, 1 μL of urine and about 25 mg of tissues were required. These results suggest that the developed methodology was useful in ADMA and SDMA determination within biological samples.

Keywords: Asymmetric Dimethylarginine, Symmetric Dimethylarginine, Creatinine, Arginine, Tissue, Liquid Chromatography/Tandem Mass Spectrometry.

1. Introduction

Asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) are methylated by protein arginine (Arg) methyltransferases (PRMTs) from Arg and metabolized by dimethylarginine dimethylaminohydrolase (DDAH) (**Figure 1**) [1,2]. ADMA and SDMA are endogenous uremic toxins that are associated with chronic kidney disease (CKD) and renal inflammation [3-6]. In fact, Toyohara, *et al.* reported that ADMA was a biomarker for CKD patients [7]. In previous studies, the biological reactions associated with these conditions have been primarily attributed to ADMA, whereas the role of SDMA has been overlooked. However, it has recently been reported that a ratio of Arg, ADMA and SDMA is important in the pathophysiological analysis of cardiovascular diseases, reduced renal functions and other diseases [6,8,9]. Therefore, a methodology for detecting both ADMA and SDMA is warranted. The analytical methods of ADMA and SDMA have been developed using several techniques. The enzyme-linked immunosorbent assay (ELISA) method has been shown to detect cross-reactivity with SDMA [10], albeit with low sensitivity. Given that ADMA and SDMA are structural isomers and the molecular weight is identical at 202.1, chromatographic separation using high performance liquid chromatography (HPLC) with ultra violet (UV), radioimmunoassay and fluorescence (FL) detection was

shown to be necessary [11-15]. The first HPLC method for dimethylarginine detection was reported in 1999 [12]. In 2000, an HPLC method with FL detection was developed, which had high sensitivity, reliability, and good separation of ADMA and SDMA [13,15]. Unfortunately, these HPLC methods are very time consuming. Since 2000, ADMA and SDMA have also been detected by liquid chromatography/mass spectrometry (LC/MS) and liquid chromatography/tandem mass spectrometry (LC/MS/MS) [16-21]. Although detection of a selective product ion with MS/MS fragmentation can be difficult, Bishop, *et al.* and Zotti, *et al.* have been reported that the selective product ions for ADMA and SDMA are at *m/z* 203→46 and 203→172, respectively [17-19]. Additionally, though these methods are more sensitive, ADMA and SDMA have the different pattern of dissociation using various MS system. Di Gangi, *et al*. have developed a reliable method using ultra per-formance LC/MS /MS that is simple and has a short analytical time [21]. This method is sensitive, however preparation for derivatization is time consuming, and ADMA and SDMA have not been separated successfully. The use of capillary electrophoresis (CE) for ADMA and SDMA analysis has also been reported. In fact, recent reports describe using CE/MS/MS and CE/UV for determination of ADMA and SDMA [22,23]. Due to the high number of theoretical plates, these methods have good separation of ADMA and SDMA, high sensitivity and reliability. However, CE is only appropriate for determining ADMA and SDMA in plasma (*i.e.* it is not applicable in tissues or any biological samples). Additionally, homoarginine is not a suitable internal standard (IS) because it is detected in biological samples. Thus, a simple, un-deriva- tive, highly sensitive and reliable method using LC/MS /MS for ADMA and SDMA determination, which can also separate isomers and determine ADMA and SDMA in biological samples, is warranted. The purpose of the present study is to: 1) develop a simple, sensitive and reliable method for ADMA, SDMA, Arg and creatinine (Cr) determination in plasma, urine and tissues, and 2) to determine the distribution of ADMA and SDMA in biological samples of mice. Herein, we have described the chromatographic separation of ADMA and SDMA following HPLC optimization.

2. Experimental

2.1. Chemicals

An ADMA standard was obtained from Sigma-Aldrich (St. Louis, MO). SDMA and Cr standards were obtained from Wako Pure Chemical Industries (Tokyo, Japan). Arg and L-arginine-$^{13}C_6$ hydrochloride (Arg-$^{13}C_6$) were obtained from Tokyo Chemical Industry (Tokyo, Japan) and Cambridge Isotope Laboratories (Andover, MA), respectively. Creatinine-d_3 (methyl-d_3) (Cr-d_3) was obtained from Toronto Research Chemicals (North York, Ontario, Canada). Methanol (MeOH) and acetonitrile (CH_3CN) of LC/MS grade were obtained from Kanto Chemical (Tokyo, Japan). Ammonium acetate (CH_3COONH_4), acetic acid (CH_3COOH) and formic acid (HCOOH) of LC/MS grade were obtained from Wako Pure Chemical Industries. Ultrapure-grade water was prepared with Purelab Ultra from Organo (Tokyo, Japan).

2.2. Mass spectrometry system and conditions

The MS system was a Thermo Fisher Scientific TSQ Quantum Ultra triple quadrupole mass spectrometer equipped with a heated electrospray ionization (HESI) source. The operating conditions were optimized for each compound by continuously infusing standard solutions dissolved in water (10.0 $\mu g \cdot mL^{-1}$) at a rate of 5 $\mu L \cdot min^{-1}$. Our final analytical conditions for MS are summarized in **Table 1**. HESI was performed in a positive ion mode (pos) for ADMA, SDMA, Arg, Cr, IS1 and IS2. Samples were analyzed using the selected reaction monitoring (SRM) mode, and employing the transition of the $(M + H)^+$ precursor ions to their product ions. The MS/MS transitions were determined in the full scan mode (*m/z* 30 - 250). For the MS/MS analysis, the optimized tube lens offsets and collision energies for collision-induced dissociation (CID) of ADMA, SDMA, Arg, Cr, IS1 and IS2 are summarized in **Table 1**. The pos HESI spray voltages were 1,500 V, the heated capillary temperature was 380˚C, the sheath gas pressure was 65 psi, the auxiliary gas setting was 20 psi and the heated vaporizer temperature was 380˚C. Both the sheath gas and auxiliary gas were nitrogen gas. The collision gas was argon at a pressure of 1.2 mTorr. The LC/MS/MS

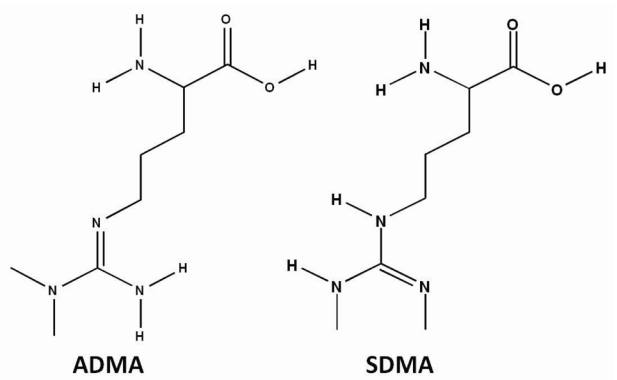

Figure 1. Chemical structure of asymmetric dimethyl arginine (ADMA) and symmetric dimethyl arginine (SDMA).

Table 1. Analytical conditions of HPLC and MS systems for determining ADMA, SDMA, Arg and Cr.

HPLC system	NANOSPACE SI-2 (Shiseido)
Analytical column	Mightysil Si 60 (250 × 3 mm I.D., 5 μm particle size)
Guard column	CAPCELLPAK C18 MGII (10 × 2 mm I.D., 3 μm particle size)
Mobile phase	10 mM CH_3COONH_4-H_2O/MeOH/CH_3CN=20/35/45 (pH = 4)
Flow rate	400 μL·min^{-1}
Oven temperature	40˚C
Divert valve	0 - 4 min: waste, 4 - 14.5 min: detector, 14.5 - 15 min: waste
MS system	TSQ quantum ultra (Thermo Fisher Scientific)
Ionization	HESI (+)
Spray voltage	1500 V
Vaporizer temperature	380˚C
Sheath gas pressure	65 psi
Auxiliary gas pressure	20 psi
Capillary temperature	380˚C
Collision gas pressure	1.2 mTorr
Tube lens offset	ADMA, SDMA: 64, Arg: 54, Cr: 55, IS1: 54 and IS2: 55
Collision energy	ADMA, SDMA: 29 eV (m/z 203.1 > 70.1), Arg: 24 eV (*m/z* 175.1 > 70.1), Cr: 18 eV (*m/z* 114.1 > 44.3), IS1: 24 eV (*m/z* 181.1 > 74.1), IS2: 18 eV (*m/z* 117.1 > 47.3), IS1: Arg-$^{13}C_6$, IS2: Cr-d_3

system was controlled by the Xcalibur software (Thermo Fisher Scientific, San Jose, CA) and data were collected with the same software.

2.3. Liquid chromatography system and conditions

A NANOSPACE SI-2 LC system comprising an LC pump, auto-sampler, column oven and on-line degasser (Shiseido, Tokyo, Japan) was used. The separations were performed on a Mightysil Si (250 mm × 2 mm I.D., 5 μm particle size) analytical column coupled with a CAPCELL PAK C18 MG II (10 mm × 2 mm I.D., 3 μm particle size) (Shiseido, Tokyo, Japan) guard column maintained at 40˚C (**Table 1**). The effect of the ratio of organic solution in mobile phase on retention was tested by varying the percentage of MeOH and CH_3CN. The percentages of MeOH and CH_3CN were 10, 20, 30, 40, 50, 60, 70 and 80% while keeping the CH_3COONH_4 concentration constant at 10 mM in the mobile phases. The mixture of MeOH and CH_3CN was as follows: 10 mM CH_3COONH_4-H_2O/MeOH/CH_3CN. MeOH and CH_3CN ratios were varied between 10 and 80% while keeping H_2O at 20%. The pH of the mobile phase was between 3.5 and 6.7, and the flow rate was between 300 and 1000 μL·min^{-1}. Retention time (R.T.) and resolution (Rs) values were used to evaluate the retention and separation of ADMA and SDMA, and the R.T. and Rs values of ADMA and SDMA were plotted against the content of MeOH and CH_3CN in the mobile phase. The Rs value was calculated from equation mentioned below:

$$\mathrm{Rs} = \frac{(\text{R. T. of ADMA}) - (\text{R. T. of SDMA})}{0.5 \times (\text{half-width of ADMA}) + (\text{half-width of SDMA})}$$

2.4. Calibration

All peaks were integrated automatically by the Xcalibur software. The ADMA, SDMA and Arg amounts were calculated from the calibration curves using the ratios of their peak areas to that of IS1, and the Cr amounts were calculated from the calibration curve using the ratios to IS2. The range used for the calibration curves of ADMA, SDMA, Arg and Cr was between 0.500 - 50.0 μg·mL^{-1} (*i.e.* 2.47 - 247, 2.47 - 247, 2.87 - 287 and 4.42 - 442 μmol·L^{-1}, respectively).

2.5. Validation of the analytical method

To determine the accuracy and precision, the newly developed method was validated at three concentrations (1.00, 5.00 and 25.0 μg·mL^{-1}) using five samples on three different days. The accuracies and precisions of the method were determined through intra- and inter-day analyses. Accuracy was calculated from the percentage deviation from the mean of the true value, and precision was expressed as the relative error and coefficient of variation (CV, %). The data were validated based on FDA's *Guidance for Industry: Bioanalytical Method Validation* guidelines.

2.6. Animals and collection of biological samples

Male BALBc/J mice ($n = 3$) were kept in a room with a 12-h/12-h light-dark cycle (light cycle from 9:00 to 21:00) at 23˚C - 25˚C and provided water and food *ad libitum*. All procedures used for LC/MS/MS were approved by the committee on the Care and Use of Experimental Animals, Tohoku University, in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health. To demonstrate the biological distribution of ADMA and SDMA, we collected 9 biological samples (plasma, urine and 7 tissues). Mice were first anesthetized with so-

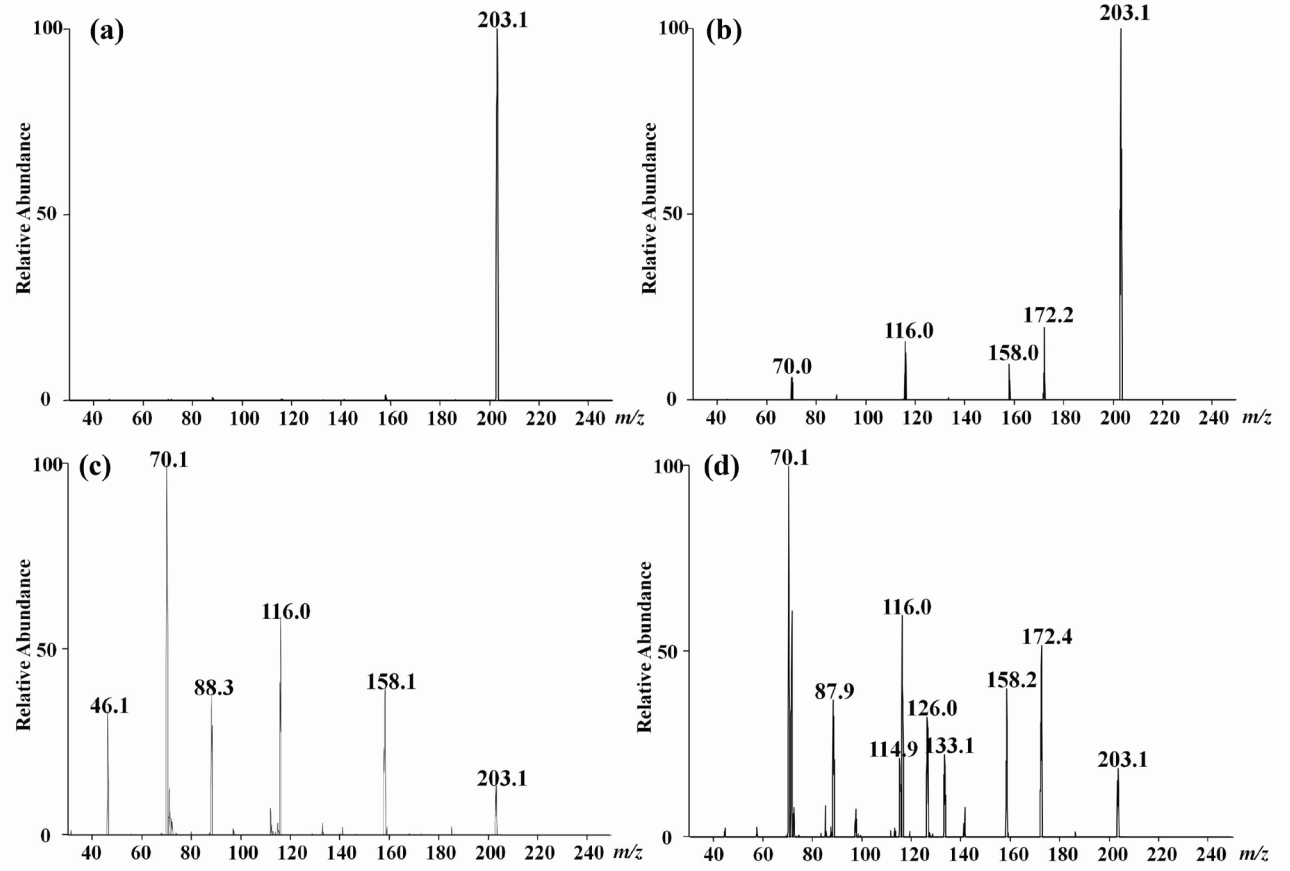

Figure 2. Precursor ion mass spectra of ADMA (a) and SDMA (b), and product ion mass spectra of ADMA (c) and SDMA (d).

dium pentobarbital. Blood was then collected via retro-orbital bleeding, transferred into a 1.5-mL plastic tube with 30 μL of 10 mM ethylenediaminetetraacetic acid, immediately centrifuged at 16,400 × *g* for 10 min, and the supernatant was transferred into a new plastic tube. Mice were then rapidly perfused transcardially with cold saline through the left ventricle and thereby sacrificed. Subsequently, urine and tissues (brain, liver, kidneys, lungs, pancreas, spleen and heart) were quickly removed. All plasma, urine and pulverized tissues were stored at –80˚C.

2.7. Sample preparation of plasma and urine

Sample preparation of plasma and urine for LC/MS/MS was performed as follows. Plasma (10 μL) and urine (1 μL) were transferred into a 1.5-mL plastic tube. Then, 50 μL of internal standard 1 (IS1; Arg-$^{13}C_6$ at 10 μg·mL^{-1}) and IS2 (Cr-d_3 at 1 μg·mL^{-1}), and 250 μL of 0.1% HCOOH/CH_3CN were added. The resulting mixture was homogenized for 30 s in an ultrasonic bath. After centrifugation at 16,400 × *g* for 10 min, the supernatant was transferred into a new plastic tube and evaporated at 60˚C until dry under nitrogen gas stream. The residue was reconstituted in 50 μL of mobile phase, vortexed for 30 s, and passed through a filter (pore size: 0.2 μm, YMC). Subsequently, 1 μL of the filtered solution was injected into the LC/MS/MS system for analysis. The concentrations of the individual compounds were calculated from a regression of the calibration curves. The values were calculated as the mean ± standard deviation (SD). Cr was used as a biomarker to correct for different volumes or urine produced per day [24]. The corrected values of ADMA, SDMA and Arg in urine were calculated by the concentration ratios of the individual compounds (μg·mL^{-1}) to the Cr concentration in urine (μg·mL^{-1}).

2.8. Sample preparation of tissues

For LC/MS/MS analysis, tissue preparation based on previously described methodology [25]. Approximately 25 mg of each tissue was transferred into a 2-mL plastic tube, and 1000 μL of 0.1% HCOOH/CH_3CN, 50 μL of internal standard 1 (IS1; Arg-$^{13}C_6$ at 10 μg·mL^{-1}) and IS2 (Cr-d_3 at 1 μg·mL^{-1}) were added. The resulting mixture was homogenized for 30 s by a sonicator. After centrifu-

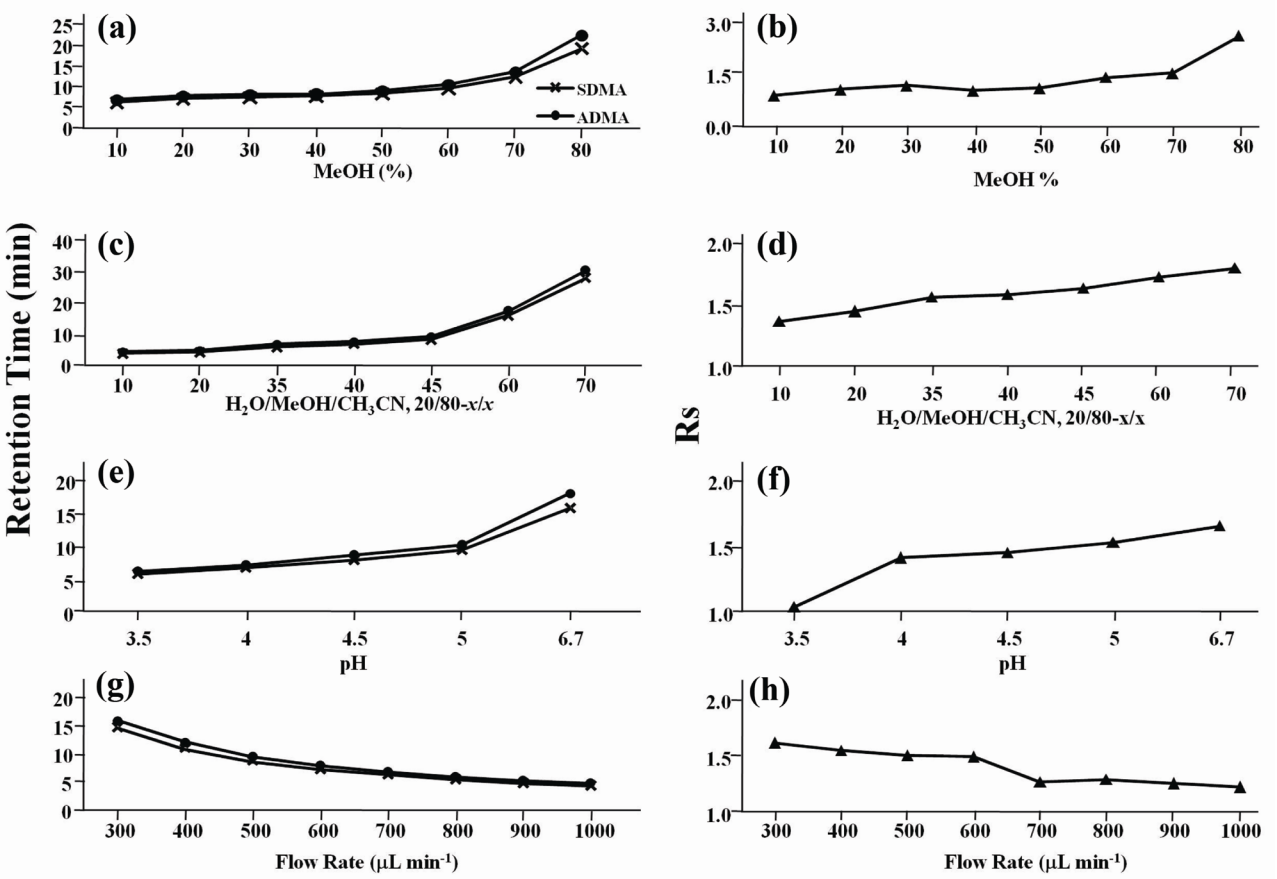

Figure 3. Effect of MeOH percentage in mobile phase (H_2O/MeOH) on retention time (R.T.) and resolution (Rs) (a, b) for ADMA and SDMA. Effect of CH_3CN percentage in mobile phase (H_2O/MeOH/CH_3CN) on R.T. and Rs (c, d). Effect of pH on R.T. and Rs (e, f). Effect of flow rate on R.T. and Rs (g, h).

gation at 16,400 × *g* for 10 min, the supernatant was transferred into a new plastic tube and the preparations were following as described above.

2.9. Statistical analyses

For statistical analysis, we use Microsoft Office Excel 2007 software. The values were calculated as the mean ± SD.

3. Results and discussions

3.1. MS/MS and LC optimization

The ionization of ADMA, SDMA, Arg and Cr was performed in positive ion mode. The optimized HESI of ADMA, SDMA, Arg, Cr, IS1 and IS2 produced abundant $[M + H]^+$ ions at *m/z* 203.1, 203.1, 175.1, 114.1, 181.1 and 117.1, respectively. The conditions for MS/MS detection were optimized for maximum product ion formation through infusion analyses. The precursor and product ion mass spectra of ADMA and SDMA in a product ion scan mode were shown in **Figure 2.** The selective product ion of ADMA and SDMA that has the highest intensity from the precursor ion was the same at *m/z* 203.1→70.1. Martens-Lobenhoffer *et al*. reported the collision induced dissociation (CID) process of ADMA and SDMA [26], and some pervious methods selected different product ion [18,19]. In our MS system, the product ions were as follows: *m/z* 40.1 for ADMA and *m/z* 129.1 for SDMA. However, these ions were not selective and not the highest ions for ADMA and SDMA determination. Consequently, a chromatographic separation of ADMA and SDMA was necessary in our study. Quantification analyses were performed in the SRM mode owing to the high selectivity and sensitivity of SRM data acquisition, in which transitions from the precursor ion into the product ion were monitored at: *m/z* 203.1→70.1 for ADMA and SDMA; *m/z* 175.1→70.1 for Arg; *m/z* 114.1→44.3 for Cr; *m/z* 181.1→74.1 for IS1; and *m/z* 117.1→47.3 for IS2.

The results of retention and chromatographic separation are shown in **Figure 3**. Previously, Paglia, *et al*. described chromatographic separation techniques for

Table 2. Linearity and correlation coefficients of ADMA, SDMA, Arg and Cr.

	Calibration range	Equaition[a]	Correlation coefficient
ADMA	0.50 - 50.0 μg·mL^{-1} (2.86 – 286 μmol·L^{-1})	$y = 0.30x + 0.040$	$r = 0.998$
SDMA	0.50 - 50.0μg·mL^{-1} (4.27 – 427 μmol·L^{-1})	$y = 0.67x + 0.014$	$r = 0.996$
Arg	0.50 - 50.0 μg mL^{-1} (2.87 – 287 μmol·L^{-1})	$y = 0.12x – 0.013$	$r = 0.999$
Cr	0.50 - 50.0 μg·mL^{-1} (4.42 – 442 μmol·L^{-1})	$y = 0.82x + 0.018$	$r = 0.999$

a. x, analyte concentration (μmol·L^{-1}); y, peak area ratio

Table 3. Accuracy of determination method for ADMA, SDMA, Arg and Cr.

	Added per sample (μg·mL^{-1})	Intra-day (n = 5)			Inter-day (n = 3)
		Day 1	Day 2	Day 3	
ADMA	1.00	1.05 ± 0.01	0.95 ± 0.01	0.95 ± 0.01	0.98 ± 0.06
	5.00	5.45 ± 0.05	5.45 ± 0.04	5.15 ± 0.05	5.35 ± 0.17
	25.0	27.0 ± 0.20	25.3 ± 0.20	24.9 ± 0.25	25.7 ± 1.12
SDMA	1.00	1.10 ± 0.01	0.95 ± 0.02	0.95 ± 0.01	1.00 ± 0.09
	5.00	5.65 ± 0.05	5.60 ± 0.05	5.85 ± 0.05	5.70 ± 0.13
	25.0	27.1 ± 0.20	25.5 ± 0.20	25.9 ± 0.25	26.1 ± 0.83
Arg	1.00	0.90 ± 0.49	0.90 ± 0.32	0.90 ± 0.30	0.90 ± 0.01
	5.00	4.75 ± 0.06	4.90 ± 0.09	4.55 ± 0.06	4.73 ± 0.18
	25.0	25.2 ± 0.26	25.1 ± 0.35	24.3 ± 0.48	24.9 ± 0.49
Cr	1.00	1.00 ± 0.04	1.00 ± 0.06	1.05 ± 0.04	1.02 ± 0.03
	5.00	5.05 ± 0.15	5.10 ± 0.14	4.90 ± 0.15	5.02 ± 0.10
	25.0	22.1 ± 0.15	23.3 ± 0.43	23.1 ± 0.60	22.8 ± 0.64

Table 4. Precision of determination method for ADMA, SDMA, Arg and Cr.

	Added per sample (μg·mL^{-1})	Intra-day (n = 5)			Inter-day (n = 3)
		Day 1	Day 2	Day 3	
ADMA	1.00	0.19%	1.02%	0.87%	6.32%
	5.00	0.88%	0.66%	0.86%	2.98%
	25.0	0.69%	0.80%	0.93%	4.20%
SDMA	1.00	0.28%	1.36%	1.11%	7.36%
	5.00	1.00%	0.82%	0.99%	2.86%
	25.0	0.79%	0.86%	1.09%	4.02%
Arg	1.00	5.84%	3.08%	3.30%	1.66%
	5.00	1.22%	1.85%	1.02%	0.43%
	25.0	0.97%	1.35%	1.99%	0.49%
Cr	1.00	3.53%	6.16%	3.52%	1.43%
	5.00	3.40%	2.58%	3.02%	0.43%
	25.0	1.10%	1.86%	2.52%	0.74%

ADMA and SDMA using a silica column [20]. Thus, additional conditions of the mobile phase were tested to obtain better retention and separation of ADMA and SDMA.

Using either MeOH or CH_3CN as the mobile phase did not result in a good separation of ADMA and SDMA.

Table 5. Value of ADMA, SDMA, Arg, Cr and ADMA/SDMA in biological samples (*n = 3*).

Biological samples	ADMA	SDMA	Arg	Cr	ADMA/SDMA
plasma ($\mu g \cdot mL^{-1}$)	0.289 ± 0.031	0.047 ± 0.004	15.4 ± 0.3	0.795 ± 0.060	6.13 ± 0.11
urine ($\mu g \cdot mL^{-1}$)	43.0 ± 8.4	12.4 ± 1.8	27.2 ± 20.7	553 ± 109	3.48 ± 0.63
(mg/mgCr)	0.085 ± 0.012	0.023 ± 0.007	0.046 ± 0.03		
brain ($\mu g \cdot mL^{-1}$)	0.207 ± 0.026	0.113 ± 0.011	39.9 ± 0.4	96.7 ± 2.4	1.86 ± 0.40
heart ($\mu g \cdot mL^{-1}$)	1.17 ± 0.83	0.069 ± 0.047	34.6 ± 0.4	110 ± 30	17.6 ± 0.16
kidney ($\mu g \cdot mL^{-1}$)	10.4 ± 0.3	0.485 ± 0.122	142 ± 9	9.27 ± 1.96	22.3 ± 5.5
liver ($\mu g \cdot mL^{-1}$)	7.50 ± 1.3	0.596 ± 0.224	3.91 ± 1.27	4.04 ± 0.27	13.4 ± 3.8
lung ($\mu g \cdot mL^{-1}$)	0.977 ± 0.256	0.047 ± 0.016	22.6 ± 6.8	4.92 ± 1.56	21.4 ± 2.9
pancreas ($\mu g \cdot mL^{-1}$)	3.14 ± 0.91	0.237 ± 0.088	129 ±116	37.8 ± 6.9	13.6 ± 1.5
spleen ($\mu g \cdot mL^{-1}$)	33.3 ± 4.71	1.30 ± 0.36	168 ± 14	7.48 ± 1.26	26.1 ± 3.4

Retention in MeOH was much shorter than in CH_3CN because of interactions of ADMA and SDMA with the silica column was stronger in MeOH than in CH_3CN. Although the Rs value increased depending on the percentage of MeOH, all of the peaks had poor shapes due to tailing (data not shown). On the other hand, using a mixture of MeOH and CH_3CN rather than MeOH or CH_3CN alone resulted in better separation, retention and peak shapes. In fact, 45% of CH_3CN produced the best retention and Rs value (Rs = 1.62). Because of hydrophilic interactions, the distribution of ADMA and SDMA between the silica column and MeOH was too weak and CH_3CN was too strong. Thus, a mixture of MeOH and CH_3CN results in the best retention and separation of ADMA and SDMA. Furthermore, retention time is important for high-throughput analysis. In 45% of CH_3CN, retention time was significantly increased for both ADMA and SDMA due to the strong hydrophobicity of CH_3CN, which retained the targeted compounds on the silica column.

Additionally, pH dramatically affected the retention, separation and peak shapes. ADMA and SDMA were strongly retained at pH 5 and over. The Rs value at pH 3.5 was very low (Rs = 1.03) and the peak shapes and intensity were worse at pH 4.5 and over. The flow rate was determined from the Rs value of ADMA and SDMA. The optimal Rs value was 1.5 at a flow rate of 400 $\mu L \cdot min^{-1}$. The best retention, separation and peak shapes were achieved at pH 4 and a flow rate of 400 $\mu L \cdot min^{-1}$. These results indicate that the optimal mobile phase consists of 10 mM CH_3COONH_4 H_2O/MeOH/CH_3CN (20/35/45, v/v) adjusted to pH 4 using CH_3COOH. Separations were performed on a Mightysil Si (250 mm × 2 mm I.D., 5 μm particle size) analytical column coupled with a CAPCELL PAK C18 MG II (10 mm × 2 mm I.D., 3 μm particle size) (Shiseido, Tokyo, Japan) guard column maintained at 40˚C. A valve was used to divert the LC effluent to waste during the first 4 min and last 0.5 min of the chromatographic run. Typical chromatograms of aqueous standard solutions are shown in **Figure 4**. The retention times of ADMA, SDMA, Arg, Cr, IS1 and IS2 were 13.1, 12.0, 9.2, 5.1, 9.2 and 5.1 min, respectively.

3.2. Limit of quantification (LLOQ) and lower limit of detection (LLOD)

The lower limit of quantification (LLOQ) was considered as the lowest concentration that was measurable with a CV of >20 and a signal-to-noise (S/N) ratio of >10. The lower limit of detection (LLOD) was defined as the concentration with an S/N of 3. The analytical procedure was sensitive with LLOQ and LLOD values for ADMA, SDMA, Arg and Cr of 2.47, 2.47, 2.87 and 4.42 $\mu mol \cdot L^{-1}$ and 0.742, 0.742, 0.862 and 1.33 $\mu mol \cdot L^{-1}$, respectively. The LLOD values obtained using our methodology were similar to those previously reported for ADMA and SDMA determination in biological samples [23]. However, the previously reported methodology required 100 μL of plasma, whereas our methodology required only 10 μL of plasma and 1 μL of urine. These results suggest that the sensitivity of our novel methodology was 10-fold greater than the previously reported methodology.

3.3. Linearity

The linearity of the calibration curves was evaluated using seven concentrations (0.250, 0.500, 1.00, 2.50, 5.00, 25.0 and 50.0 $\mu g \cdot mL^{-1}$). The calibration curves of ADMA, SDMA, Arg and Cr were all linear over a range of 0.500 - 50.0 $\mu g \cdot mL^{-1}$ (2.47 - 247, 2.47 - 247, 2.87 - 287 and 4.42 - 442 $\mu mol \cdot L^{-1}$, respectively) (**Table 2**). A linear regression analysis was performed on these portions

of the curves, and it was found that the correlation coefficient was greater than 0.999 for all analytes.

3.4. Accuracy and precision

The results for the accuracy and precision are shown in **Tables 3** and **4**, respectively. The intra- and inter-day accuracies ranged from 86.9% to 112% for the three concentrations used with all of the compounds. The intra- and inter-day precisions ranged from 0.19% to 7.36% for the three concentrations used with all of the compounds. These results indicate that our methodology has good reliability and repeatability. Validation was determined by spiking the standard compounds in plasma. Generally, validation of analytical methods is necessary, since the effects of ion suppression are variable in different types of tissues, and this is achieved by spiking each tissue sample. In our methodology, matrix effects could be avoided by using a silica column and the optimal mobile phase, which allows for chromatographic separation of ADMA and SDMA in several biological samples.

3.5. Tissue distribution of ADMA and SDMA in BALBc/J mice

ADMA, SDMA, Arg and Cr levels determined from various tissue samples of mice are shown in **Table 5**. Although, all compounds were detected, some samples contained analytes at concentration levels outside the linear working range. These samples were adjusted into the working range of the calibration and were reanalyzed. The SRM chromatograms of biological sample are shown in **Figure 5**. ADMA and SDMA levels were high in urine, kidneys, liver, pancreas and spleen. With the exception of low liver levels, Arg had similar urine and tissue levels as ADMA and SDMA. Cr levels were high in plasma, urine, brain, heart and pancreas. In previous studies, Arg/ADMA or ADMA + SDMA/monomethylarginine (MMA) ratios were described to have an important role evaluating disease progression [27]. Therefore, we calculated ADMA/SDMA ratios for tissue comparisons (**Table 5**). ADMA/SDMA ratios in the brain

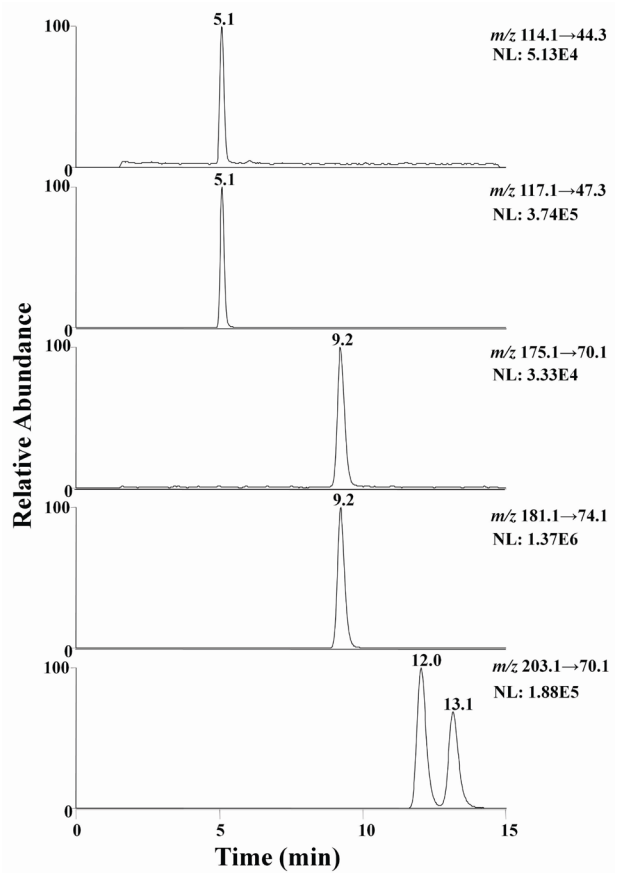

Figure 4. SRM chromatograms of *m/z* 114.1→44.3 for Cr, *m/z* 117.1→47.3 for Cr-d_3, *m/z* 175.1→70.1 for Arg, *m/z* 181.1→74.1 for Arg-$^{13}C_6$ and *m/z* 203.1→70.1 for ADMA and SDMA from standard compounds.

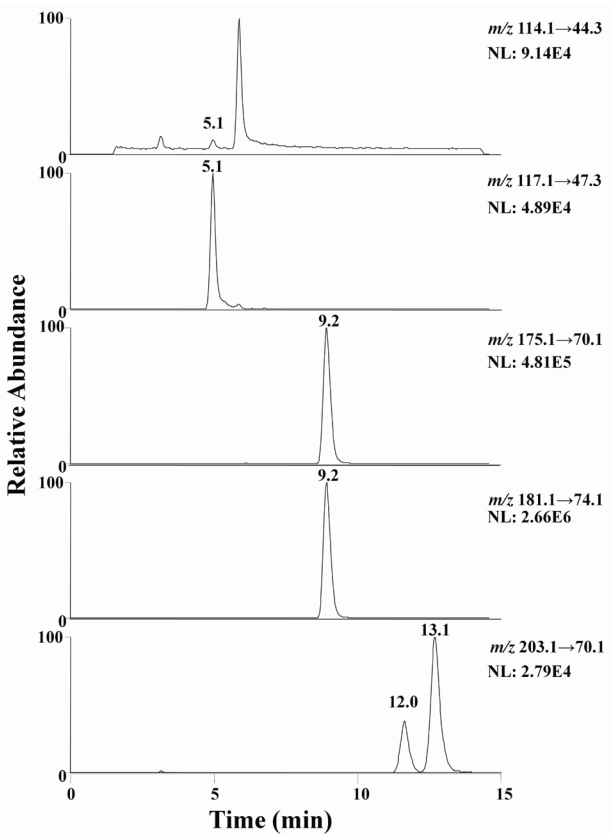

Figure 5. SRM chromatograms of *m/z* 114.1→44.3 for Cr, *m/z* 117.1→47.3 for Cr-d_3, *m/z* 175.1→70.1 for Arg, *m/z* 181.1→74.1 for Arg-$^{13}C_6$ and *m/z* 203.1→70.1 for ADMA and SDMA from a biological sample (plasma).

and plasma were lower than in the other tissues. The present study is the first to demonstrate lower level of ADMA/SDMA in the brain. DDAH is an enzyme that is known to metabolize endogenous nitric oxide synthase (NOS) inhibitors such as MMA and ADMA to citrulline [28]. The gene expression of DDAH I and II has been reported by Tra *et al.*, and DDAH I have been more distributed in the brain than the other organs [29]. In addition, DDAH I and NOS were up-regulated in neurons following nerve injury [30]. These reports indicated that ADMA might be expeditiously metabolized by DDAH I in the brain, and the central nerves system might be protected by the low concentration of ADMA from the affection of NOx via the inhibition with NOS. Although the reason and mechanism for these findings cannot be elucidated in the present study, the data suggests that the brain and plasma may have high levels of SDMA in relation to ADMA. It has been suggested that the relative value is just as important as the absolute value for revealing the mechanisms of ADMA and SDMA. Thus, our novel methodology proved to be useful in detecting ADMA and SDMA in various biological samples with high sensitivity and selectivity.

4. Concluding remarks

We have developed a methodology for ADMA and SDMA determination in biological samples using LC/MS/MS. Furthermore, it was validated that this methodology can determine ADMA and SDMA levels with high sensitivity and reliability. This methodology is the first of its kind that can determine tissue distribution of ADMA and SDMA with good separation. Additionally, this methodology requires small sample sizes, specifically only 10 μL of plasma, 1 μL of urine and about 25 mg of tissues were required in the present study. Furthermore, the ADMA/SDMA ratio was found to be lower in the brain than in any other tissues. Although the mechanism for this finding is unclear, the developed methodology was useful in determining ADMA and SDMA levels in biological samples.

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