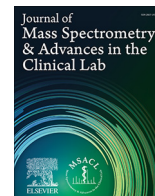




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Research Article

Developing a robust, fast and reliable measurement method for the analysis of methylarginine derivatives and related metabolites



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ABSTRACT

Background: Nitric oxide (NO) plays an important role in endothelial homeostasis. Asymmetric dimethyl arginine (ADMA), L-N monomethyl arginine (L-NMMA) and symmetric dimethyl arginine (SDMA), which are derivatives of methylarginine, directly or indirectly reduce NO production. Therefore, these metabolites are an important risk factor for various diseases, including cardiovascular diseases. Numerous methods have been developed for the measurement of methylarginine derivatives, but various difficulties have been encountered. This study aimed to develop a reliable, fast and cost-effective method for the analysis and measurement of methylarginine derivatives (ADMA, SDMA, L-NMMA) and related metabolites (arginine, citrulline, homoarginine, ornithine), and to validate this method according to Clinical and Laboratory Standards Institute (CLSI) protocols.

Methods: For the analysis of ADMA, SDMA, L-NMMA, arginine, homoarginine, citrulline, ornithine, 200 Å µl of serum were precipitated with methanol, and subsequently derivatized with a butanol solution containing 5% acetyl chloride. Butyl derivatives were separated using a C18 reverse phase column with a 5 min run time. Detection of analytes was achieved by utilising the specific fragmentation patterns identified through tandem mass spectrometry.

Results: The method was linear for ADMA, SDMA, L-NMMA, ornithine, arginine, homoarginine and citrulline in the ranges of 0.023–6.0, 0.021–5.5, 0.019–5.0, 0.015–250, 0.015–250, 0.019–5 and 0.015–250 µM, respectively. The inter-assay CV% values for all analytes was less than 9.8%.

Conclusions: Data obtained from method validation studies shows that the developed method is highly sensitive, precise and accurate. Short analysis time, cost-effectiveness, and multiplexed analysis of these metabolites, with the same pretreatment steps, are the main advantages of the method.

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1. Introduction

Nitric oxide (NO) plays an important role in maintaining homeostasis. NO is synthesized from L-arginine and O₂ by endothelial, neuronal and inducible nitric oxide synthase (NOS) enzymes

Abbreviations: ADMA, asymmetric dimethyl arginine; CE, capillary electrophoresis; CE, collision energy; CLSI, The Clinical & Laboratory Standards Institute; CXP, collision cell exit potential; DDAH, dimethylaminohydrolase; DP, declustering potential; EP, entrance potential; FDA, Food and Drug Administration; GC-MS, gas chromatography–mass spectrometry; HPLC, high performance liquid chromatography; LC-MS, liquid chromatography–mass spectrometry; LC-MS/MS, liquid chromatography tandem-mass spectrometry; L-NMMA, L-N monomethyl arginine; MRM, multiple reaction monitoring; NO, nitric oxide; NOS, nitric oxide synthase; PRMTs, protein arginine methyltransferases; SDMA, symmetric dimethyl arginine.

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[1,2]. Endothelium-derived NO plays an extremely important role in vascular homeostasis by modulating vasodilation, regulating local cell growth and protecting vessels from the damaging effects of circulating cells and platelets [3]. However, increased levels of methylated arginine derivatives, such as asymmetric dimethyl arginine (ADMA), L-N monomethyl arginine (L-NMMA) and symmetric dimethyl arginine (SDMA), reduce NO synthesis directly or indirectly. ADMA and L-NMMA are structurally similar to arginine and are endogenous, competitive inhibitors of the NOS enzyme [4,5]. SDMA indirectly reduces NO levels by inhibiting the uptake of arginine into cells [6]. ADMA, SDMA and L-NMMA are produced during the post-translational methylation of arginine residues in proteins with the enzymes methyl transferases (PRMTs) and released into the cytosol by proteolysis [7]. PRMT-1 plays a role in the formation of L-NMMA and ADMA, while PRMT-2 is responsible for the synthesis of SDMA and L-NMMA

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[8]. Approximately 0.4 μM of ADMA are produced daily in a healthy individual, while approximately 80% is metabolised by the enzyme dimethylaminohydrolase (DDAH) [9]. The plasma concentrations of SDMA, L-NMMA and arginine are approximately 0.5, 0.104 and 65 μM , respectively, in healthy individuals [10]. ADMA is metabolised to citrulline and dimethylamine via DDAH, while L-NMMA is metabolised to citrulline and monomethylamine [11]. ADMA and L-NMMA are mainly eliminated by DDAH, with limited renal excretion. Conversely, while SDMA is not degraded by DDAH, renal excretion is its primary route of elimination [12]. In endothelial cell culture, rat experimental models, and studies in humans, an increase in PRMT activity or a decrease in DDAH activity has been shown to lead to an increase in ADMA levels in cases such as oxidative stress [13]. Homoarginine is a non-essential, non-proteinogenic amino acid synthesized from arginine through the arginine:glycine amidinotransferase (AGAT) enzyme. Elevated homoarginine may compete with arginine and block the production of NO due to structural similarity between these two substrates [14]. Numerous studies have been conducted to evaluate the correlation of methylarginine derivatives, and related metabolites, with cardiovascular diseases. As a result of the studies, it was revealed that increased serum ADMA, SDMA, L-NMMA, citrulline and ornithine levels, and decreased serum homoarginine, arginine levels comprise a risk factor for cardiovascular diseases [15]. Since the kidneys are one of the means of elimination of methylarginines, levels of plasma methylarginine derivatives, especially SDMA are increased in patients with renal failure [16]. In addition, there are studies showing that plasma methylarginine levels are associated with obesity, metabolic syndrome [17], thyroid diseases [18], and neurodegenerative diseases [19]. However, NOS activity is also affected by the concentration of extracellular arginine. As arginine levels can also modulate the inhibitory effect of ADMA with this mechanism, the plasma arginine/ADMA ratio is thought to be a better risk indicator for pathway-related cardiovascular, renal, and neurodegenerative diseases [20]. Therefore, various high performance liquid chromatography (HPLC) [21], capillary electrophoresis (CE) [22], gas chromatography–mass spectrometry (GC–MS) [23], liquid chromatography–mass spectrometry (LC–MS) [24], and liquid chromatography tandem–mass spectrometry (LC–MS/MS) [25] methods have been developed for the measurement of methylarginine derivatives and related metabolites in serum, plasma, urine, and tissue samples [26–28]. However, various difficulties have been encountered when measuring the derivatives of methylarginine. Due to the polarity of these compounds, their retention in conventional reverse phase columns is poor. Generally, low concentrations and differing orders of magnitude of analyte concentrations increase the need for sensitive measurement methods. The fact that derivatives of methylarginine have similar structural and physicochemical properties makes their chromatographic separation difficult. For instance, ADMA and SDMA are enantiomers of each other and are difficult to separate. Therefore, providing chromatographic separation of these compounds requires long run times and complex gradients. In addition, these metabolites are thermally unstable and not volatile [10]. Although ELISA methods allow a large number of samples to be run in a short time, the risk of cross reactivity is high and ADMA values measured by ELISA are approximately 20–50% higher than those measured by chromatographic methods [29]. GC–MS methods provide a good separation between ADMA and SDMA, but require various extraction and derivatization steps, with pretreatment steps taking approximately 2.5–3 h for an average of 10 samples [10,23,26]. HPLC and LC–MS/MS methods were the most widely used methods for these analytes. HPLC–UV based methods were undesirable due to lack of sensitivity and selectivity [30]. HPLC methods primarily measured fluorescent detectors. However, since these compounds do not have chromophores, a fluorescent

derivatization pretreatment was performed. Derivatization increased the detectability of the methylarginine derivatives in the fluorescent detector, as well as the column retention of these analytes. However, time spent on pretreatment, and the costs associated with derivatization, have increased [10]. Generally, derivatization with orthophthalaldehyde (OPA) was performed using HPLC methods. Although derivatization with OPA is generally performed in a short time and at room temperature, it has been observed that the derivatives formed quickly decompose (decreasing approximately 5–10% in 24 h and 35% in 72 h). Due to instability problems of OPA derivatives, alternate derivatization reagents, such as naphthalene-2,3-dicarboxaldehyde (NDA), o-phenylenediamine, AccQ-Fluor™ (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate), 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), ninhydrin, and phenylisothiocyanate, have been used. Derivatives with NDA are more stable than OPA, but require longer incubation times (approximately 20 min) [21]. Derivatization with NBD-F increased both the stability of the derivatives (stable at room temperature for approximately 3 days) and the sensitivity of the analysis [31]. Derivatives formed with AccQ-Fluor™ are stable for up to one week, but require longer elution times (approximately 50 min) [32]. In HPLC methods, only chromatographic separation of ADMA and SDMA has been achieved [33]. Hence, for the accurate and reliable analysis of methylarginine derivatives, as with many analytes, there is a need to develop new LC–MS/MS measurement methods with high accuracy, reproducibility and precision based on specific fragmentation patterns rather than chromatographic separation. Our aim in this study was to develop a multiplexed, rapid, cost-effective, practical, and reliable method for the measurement of methylarginine derivatives.

2. Materials and methods

2.1. Study design

2.1.1. Chemicals

ADMA (CAS Number 220805-22-1), SDMA (CAS Number: 1266235-58-8), L-NMMA (CAS Number: 53308-83-1), arginine (CAS Number: 202468-25-5), ornithine (CAS Number: 3184-13-2), citrulline (CAS Number: 372-75-8), homoarginine (CAS Number: 1483-01-8), methanol (CAS Number: 67-56-1), HPLC grade water (CAS Number: 7732-18-5), n-butanol (CAS Number: 71-36-3), acetyl chloride (CAS Number: 75-36-5), formic acid (CAS Number: 64-18-6), bovine serum albumin (CAS Number 9048-46-8), potassium chloride (CAS Number: 7447-40-7), sodium chloride (CAS Number: 7647-14-5), di-sodium hydrogen phosphate (CAS Number: 7558-79-4), potassium dihydrogen phosphate (CAS Number: 7778-77-0) were obtained from Sigma Aldrich (St. Louis, MO, USA). d_7 -ADMA (Catalog No: DLM-7476-5) was obtained from Cambridge Isotope Laboratories.

2.1.2. Sample preparation

Serum ADMA, SDMA, L-NMMA, arginine, ornithine, homoarginine and citrulline levels were measured by modifying the method developed by Gangi *et al* [26]. Briefly, 200 μL of serum sample was transferred into eppendorf tubes and 100 μL of ADMA internal standard (d_7 -ADMA in MeOH) was added. To precipitate proteins, 1000 μL of methanol was added, followed by 30 s of vortexing, and centrifugation at 13000 rpm for 10 min. The supernatants were poured into glass tubes then evaporated under nitrogen gas at 60 °C. 200 μL of a freshly prepared butanol solution, including 5% (v/v) acetyl chloride was added for derivatization. The tubes were sealed and incubated for 30 min at 60 °C. After incubation, the solvents of the mixtures were evaporated again with nitrogen gas. The residues were dissolved in 200 μL of water–methanol

(90:10, v/v%) mixture including 0.1% (v/v) formic acid then 40 µL was injected into the LC-MS/MS system.

2.1.3. Instrumentation and LC-MS/MS method

The Shimadzu HPLC system (Kyoto, Japan) consisted of a pump (LC-20 AD), an automatic sampler (SIL-20 AC HT), and a unit for online degassing (DGU-20A3). Mass spectrometric analyses were performed using an API 3200 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex) equipped with an electrospray ion source (ESI) operating in positive mode. Chromatographic separation was performed using a Phenomenex C18 HPLC column (50 mm × 4.6 mm, part no: 00B-4041-E0). The mobile phases A and B consisted of 0.1% formic acid in water, and 0.1% formic acid in methanol, respectively. The percentage of mobile phase B was programmed as follows: 0.1 min, 15%; 1.0 min, 25%; 2.0 min, 100%; 2.10 min, 15%; 4.90 min, 15%. The total run time was 5 min. Different product-ions for the identification of L-NMMA, arginine, ornithine, citrulline, homoarginine, especially ADMA and SDMA were investigated via the infusion of pure standards of these molecules. The transitions monitored for each analyte were (precursor ion / product fragment ion; *m/z*): ADMA-259.3/214, 259.3/158.0, 259.3/70.0, 259.3/116.0; SDMA-259.3/228.0, 259.3/88.0, 259.3/70.0, 259.3/116.0; citrulline-232.3/113.0, 232.3/159.1, 232.3/70.0; arginine-231.3/70.0, 231.3/172.0, 231.3/116.0; homoarginine- 245.2/84.2, 245.2/211.0, 245.2/130.0, 245.2/186.0; ornithine- 189.0/70.0, 189.0/116.1; L-NMMA-245.3/70.2. These values were entered into the multiple reaction monitoring (MRM) table, and their intensities were tracked by analyzing both calibration solutions and patient samples. Among the ion transitions, those with the highest intensity, selectivity and separation were selected as quantifier ions, while the ion transitions with the second highest intensity were selected as qualifier ions and added to Table 1. The qualifier ions used for ADMA, SDMA, arginine, ornithine, citrulline and homoarginine were 259.3/158.0, 259.3/88.0, 231.3/116.0, 189.0/116.1, 232.3/159.1, 245.2/186.0, respectively. LC-MS/MS method optimization parameters of ADMA, SDMA, L-NMMA, ornithine, citrulline, arginine, homoarginine and internal standard (d7-ADMA) are shown in Table 1.

For DP and CE parameters, the first value written in Table 1 belongs to the quantifier ions, while the second value belongs to the qualifier ions.

Ionspray voltage, source temperature, curtain, ion source (GS1) and ion source (GS2) gas values were adjusted to 5000 V, 350 °C, 20, 40, 60 psi, respectively.

2.1.4. Method validation

The LC-MS/MS method is generally validated according to the Clinical and Laboratory Standards Institute (CLSI) C62-A: Liquid Chromatography–Mass Spectrometry Methods [34]. Linearity, pre-

cision, recovery, matrix effects, stability, carry-over, selectivity and specificity parameters were evaluated in this study.

2.1.5. Preparation of calibration and working solutions for validation

The calibrators and working solutions used in method development and validation studies were prepared using a surrogate matrix [35]. Different surrogate matrices were investigated for the analysis of methylarginine derivatives by LC-MS/MS. In this context, phosphate-buffered saline solution (0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4 at 25 °C) containing 1% bovine serum albumin (BSA) and various solvents, such as water and methanol, were tested. Following this investigation, phosphate-buffered saline solution containing 1% BSA was selected as the surrogate matrix, as it bears the closest resemblance to serum [36]. Phosphate buffered saline solution containing 1% BSA has a high similarity to the biological matrix since it has a similar protein content and ionic strength as human serum. The calibration and working solutions used in all validation studies were prepared by spiking analytes into the phosphate-buffered saline solution containing 1% BSA solution. All working and calibration solutions were freshly prepared on the day of analysis and stored at +4 °C. To assess the equivalence between the phosphate buffered saline solution containing 1% BSA solution selected as the surrogate matrix and human serum, low, medium and high level analytes were spiked into the phosphate buffered saline solution containing 1% BSA solution for each analyte. For comparison, a serum pool (blank serum sample) was prepared using at least 6 different individuals for each analyte and the metabolite levels of this pool were established. The low, medium and high level analytes were spiked into this serum pool for each analyte in accordance with the levels in phosphate buffered saline solution containing 1% BSA. Equivalence between surrogate matrix and serum was calculated with the following formula [37]:

$$\text{Equivalency (\%)} = \left(\frac{\text{Response serum spike} - \text{Response serum blank}}{\text{Response surrogate spike}} \right) \times 100$$

Response serumspike is the peak area obtained from analyte-spiked sample in human serum. Response serum blank is the peak area obtained from analyte in blank human serum, and response surrogate spike is the peak area obtained from analyte-spiked sample in surrogate matrix. It was found that the equivalence ranged from 91% to 103% for all analytes. Therefore, the matrix effect of phosphate buffered saline solution containing 1% BSA and serum was considered comparable. Although the matrix effect is slightly higher in the measurements performed on the patient samples, the difference in the results obtained with BSA was considered acceptable.

Table 1

Method optimization parameters for ADMA and related metabolites by LC-MS/MS. EP, entrance potential; DP, declustering potential; CE, collision energy; CXP, collision cell exit potential.

Analytes	Precursor ion (<i>m/z</i>)	Quantifier Product ion (<i>m/z</i>)	Qualifier Product ion (<i>m/z</i>)	DP	EP	CE	CXP
ADMA	259.3	214	158.0	40/40	7.5	24/30	4
SDMA	259.3	228	88.0	40/40	7.5	24/36	4
L-NMMA	245.3	70.2	40	40	7.5	24	4
Arginine	231.3	70	116.0	40/45	7.5	24/30	4
Homoarginine	245.2	84.2	186.0	40/45	7.5	24/20	4
Ornithine	189	70	116.1	55/60	7.5	20/20	4
Citrulline	232.3	113	159.1	40/42	7.5	24/20	4
d7-ADMA	266.61	221	40	40	10	24	4

Phosphate buffered saline solution containing BSA, and stock solutions, were prepared separately by dissolving the accurately weighed compounds in prepared solution. Stock solutions were obtained at final concentrations of 48, 40, 44, 40 μM for ADMA, L-NMMA, SDMA, and homoarginine, respectively, and 1000 μM for arginine, citrulline, ornithine, respectively. Calibration solutions of ADMA were prepared at 6, 3, 1.5, 0.75, 0.375, 0.187, 0.093, 0.046, 0.023 μM by serial dilution of the stock solution with phosphate buffered solution containing BSA. Calibration solutions of L-NMMA and homoarginine were prepared at 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, 0.019 μM by serial dilution of the stock solution with phosphate buffered solution containing BSA. Calibration solutions of SDMA were prepared at 5.5, 2.75, 1.375, 0.687, 0.343, 0.171, 0.085, 0.042, 0.021 μM by serial dilution of the stock solution with phosphate buffered solution containing BSA. Calibration solutions of arginine, ornithine and citrulline were prepared at 250, 125, 62.5, 31.25, 15.62, 7.81, 3.91, 1.95, 0.975, 0.488, 0.244, 0.122, 0.061, 0.030, 0.015 μM by serial dilution of the stock solution with phosphate buffered solution containing BSA. IS (d7-ADMA) was also prepared in methanol at concentration of 5 μM. Solutions were stored under refrigeration (2–8 °C) when not in use.

2.1.6. Statistical analysis

Statistical evaluation was performed by SPSS statistical software package version 21.0, MedCalc statistical software 19.2.1 version, EP Evaluator Release 8 version (Data Innovations, South Burlington, VT) and Excel (2010), p < 0.05 was considered as a statistically significant difference.

3. Results

3.1. Method validation

3.1.1. Linearity

The linearity study was performed according to CLSI EP06-A protocol [34]. Calibration solutions for ADMA, SDMA, L-NMMA, arginine, ornithine, citrulline and homoarginine used in the linearity study were prepared as specified in section 2.1.5. Calibration curves were obtained by plotting the ratios of analyte/internal standard peak areas versus nominal analyte concentration. The results were evaluated by linear regression analysis. The correlation coefficients of the ADMA, SDMA, L-NMMA, ornithine, arginine, homoarginine and citrulline calibration curves were found as 0.9935, 0.9996, 0.9917, 0.9913, 0.9931, 0.9982 and 0.9975, respectively. The mass spectrometric method was linear at the 0.023–6.0, 0.021–5.5, 0.019–5.0, 0.030–250, 0.030–250, 0.019–5.0 and 0.030–

250 μM for ADMA, SDMA, L-NMMA, ornithine, arginine, homoarginine and citrulline, respectively.

3.1.2. LOD (Limit of detection) and LLOQ (Lower limit of quantitation)

There are different methods for determining LLOQ. The first approach is to determine the lowest analyte concentration that can be measured with acceptable precision and accuracy [38]. The Food and Drug Administration (FDA) guideline recommends the use of data from ≥ five replicates in of a spiked sample from at least three different runs. The imprecision should be ≤ ±20% and a deviation in accuracy ≤ ±20% [39]. In order to determine the LLOQ values, intra- and inter-assay precision and accuracy were calculated by running a total of 20 samples in five different runs across five days. The accuracy study was performed to determine LLOQ values by running 20 replicates of a pretreated low-level analyte spiked phosphate-buffered saline solution containing 1% BSA solution and the % bias was calculated using the following formula:

$$\text{Bias\%} = \left(\frac{\text{measured value} - \text{expected value}}{\text{expected value}} \right) \times 100$$

LLOQ values were determined to be 0.093, 0.042, 0.039, 0.061, 0.030, 0.078, 0.061 μM for ADMA, SDMA, L-NMMA, ornithine, arginine, homoarginine and citrulline, respectively. The intra and inter-day precision ranged between 2.8% and 9.7% for all analytes at LLOQ concentrations. The accuracy ranged between 88% and 112% for all analytes at LLOQ concentrations. The limit of blank (LOB) levels were calculated with the formula LOB = meanblank + 1.65SDblank after 20 replicates of analyte free phosphate-buffered saline solution containing 1% BSA solution. LOD values were calculated with the formula LOD = LOB + 1.65SDlow concentration sample following 20 replicates of the low level analyte spiked phosphate-buffered saline solution containing 1% BSA [40]. Accordingly, LOD values of ADMA, SDMA, L-NMMA, ornithine, arginine, homoarginine and citrulline were 0.036, 0.028, 0.021, 0.035, 0.018, 0.038, 0.029 μM, respectively.

3.1.3. Intra- and inter-day precision

The precision study was performed according to the CLSI EP05-A3 protocol [34]. The intra- and inter-assay precision study was performed using a phosphate-buffered saline solution containing 1% BSA prepared by spiking analytes at low and high concentrations, as selected for each analyte throughout the calibration range (Table 2). The intra-and inter-assay precision study was performed separately for each concentration level. EP15 specifies a minimum of 3 replicates per day for a period of 5 days resulting in a total of

Table 2 Precision results of ADMA, SDMA, L-NMMA, arginine, homoarginine, ornithine and citrulline.

Analyte	Added(μM)	Intra-assay			Inter-assay		
		Mean (μM)	SD	CV%	Mean (μM)	SD	CV%
ADMA	1.5	1.481	0.079	5.4	1.479	0.096	6.5
	0.093	0.087	0.006	7.0	0.086	0.004	4.7
SDMA	2.75	2.695	0.029	1.1	2.710	0.173	6.4
	0.17	0.168	0.009	5.6	0.167	0.011	7.1
L-NMMA	2.5	2.499	0.152	6.1	2.496	0.167	6.7
	0.15	0.149	0.01	6.7	0.147	0.013	7.7
Arginine	250	248.18	10.67	4.3	251.57	17.66	7.0
	1.945	1.959	0.095	4.9	1.948	0.128	6.6
Ornithine	250	249.85	15.49	6.2	248.9	17.67	7.1
	15.6	14.96	0.807	5.4	14.90	1.087	7.3
Homoarginine	5	4.096	0.204	5.0	4.087	0.302	7.4
	0.156	0.157	0.008	5.4	0.155	0.008	5.5
Citrulline	250	249.17	10.29	6.9	251.19	19.09	7.6
	1.945	1.968	0.096	4.9	1.917	0.021	1.1

Table 3
Recovery% and matrix effect study results for ADMA and related metabolites.

Analyte	Concentration(μM)	Recovery%	Matrix effect%
ADMA	1.5	106.0	4.0
	0.38	94.1	5.0
	0.093	95.2	6.7
SDMA	2.75	102.9	5.4
	0.68	105.8	3.2
	0.17	94.8	5.6
L-NMMA	2.5	103.4	2.4
	0.62	95.2	1.4
	0.15	95.3	1.3
Arginine	250	101.4	4.9
	31.25	95.7	7.9
	1.945	102.4	8.1
Homoarginine	5	95.8	1.2
	1.25	103.4	3.2
	0.156	99.6	5.0
Ornithine	250	101.3	2.7
	62.5	108.9	4.5
	15.6	104.3	6.2
Citrulline	250	105.3	5.6
	31.25	99.8	3.6
	1.945	95.8	5.7

15 measurements for each inter-assay precision study. In our study, we calculated the inter-assay imprecision based on a total of 20 measurement results by analyzing 4 replicates per day over a 5 day period for each concentration level. Intra-assay imprecision was calculated by analyzing a total of 40 samples, 20 in the morning and 20 in the afternoon at each concentration level, within a day. As a result of the precision study, CV% values were calculated with the following formula:

$$CV\% = \left(\frac{\text{standard deviation}}{\text{mean}} \right) \times 100$$

The results of the intra-day and inter-day precision studies for ADMA, SDMA, L-NMMA, ornithine, arginine, homoarginine and citrulline are shown in Table 2.

3.1.4. Recovery and matrix effects

The recovery study was performed according to the CLSI EP34 protocol [34]. For the recovery experiments, phosphate buffered

saline solution with 1% BSA was spiked with 3 different concentrations of the analytes (Table 3). At each analyte concentration, 2 replicates were analyzed in one analytical run, then recovery was computed as $\text{recovery}\% = \frac{(C2 - C0)}{C1} \times 100$, where C2 is the analyte concentration in the final solution after spiking with known concentration of standard, C0 is the original analyte concentration in the initial solution, and C1 is the added known concentration of standard.

Matrix effects are generally determined by either of two common methods: post-column infusion or post-extraction spiking. We performed the latter method, as previously described by Chambers *et al* [41]. In this method, the low, mid, and high level analyte response in neat solution (such as mobile phase mixture, acetonitrile, methanol) is compared with the response in the analyte spiked matrix at the same levels after pretreatment. Three sets of samples were prepared by spiking low, medium, and high (Table 3) concentrations of all analytes into a mixture of water:methanol (50:50, v/v%) (neat solution). The post-extraction phosphate buffered saline solution containing 1% BSA solution was prepared at the same concentration after following the previously described extraction procedure, but prior to drying the samples down. The matrix effect was calculated using the following formula: $(ME\% = \frac{\text{Mean post-extracted peak area}}{\text{Mean un-extracted peak area}} \times 100)$. Results of recovery and matrix effect studies are shown in Table 3.

3.1.5. Stability

The stability study, performed according to the CLSI EP25-A protocol [34], was carried out with a serum pool prepared from 30 individuals. The effect of freeze-thaw treatment and storage at -20 °C for 45 days was investigated. The study was carried out by measuring the metabolite levels in the serum pool prepared with 30 serum samples on the day of collection and then measuring again after storage at -20 °C on the 15th, 30th, and 45th days. The effect of freeze-thaw was investigated during 4 freeze-thaw cycles following the measurement of metabolite levels on the day of collection. After each analysis, the bias% value was calculated compared to the day of collection (expected value) using the following formula:

$$\text{Bias}\% = \left(\frac{\text{measured value} - \text{expected value}}{\text{expected value}} \right) \times 100$$

Results are presented in Table 4.

Table 4
Stability results of ADMA and related metabolites.

Analyte	Added(μM)	Freeze-thaw stability(bias%)				Frozen (-20 °C) for 45 day (bias%)		
		1	2	3	4	15 days	30 days	45 days
ADMA	1.5	1.9	5	8.13	9.38	2.1	4.3	8.6
	0.093	1.9	6.7	8.1	9.4	2	4.5	8.8
SDMA	2.75	-2.3	3.2	9.4	11.5	3	3.3	10.9
	0.17	-1.2	-2.7	-6.9	-9.9	3.1	4.2	11.1
L-NMMA	2.5	4.8	5.1	9.4	11.7	3.4	4.1	11.8
	0.15	-1.3	-2.6	-5.8	-13	2.1	2.9	10.5
Arginine	250	-1.4	1.1	7.2	8.9	-1.6	-5.8	-10.2
	1.945	-3	-2.4	-1.5	0.6	-4	-6.9	-11.3
Homoarginine	5	-1	-4.5	7.9	8.3	-3	-7.2	-9
	0.156	-2.5	-4.5	-2	8.3	-5	-6.3	-10.1
Ornithine	250	-4.4	-6.2	7.5	10.6	4.5	7.4	10.3
	15.6	-7.9	-8.5	-9.8	-12	7.2	8.1	9.2
Citrulline	250	1.3	4.5	6.2	8.4	2.1	4.8	8.4
	1.945	3.9	6.8	9.9	11.6	3.4	5.2	7.9

Table 5
Evaluation of carryover for ADMA, SDMA, NMMA, Arginine, Homoarginine, Ornithine, Citrulline.

	ADMA	SDMA	NMMA	Arginine	Homoarginine	Ornithine	Citrulline
Mean of low-low results (µM)	0.096	0.19	0.201	2.12	0.21	15.82	2.19
Mean of high-low results (µM)	0.125	0.222	0.205	4.62	0.231	18.62	5.24
SD low-low results (µM)	0.018	0.031	0.042	1.62	0.053	3.9	1.81
Allowable carryover values (µM)	0.054	0.093	0.126	4.86	0.159	11.7	5.43
Carryover (µM)	0.029	0.032	0.004	2.5	0.021	2.8	3.05

3.1.6. Carryover study

The carryover study was performed by analyzing low and high level samples according to the order specified in CLSI EP10-A3 [34]. Low level analyte-containing samples were prepared in phosphate buffered saline solution containing BSA for ADMA, SDMA, L-NMMA, ornithine, arginine, homoarginine and citrulline at 0.093, 0.17, 0.15, 15.6, 1.95, 0.16 and 1.95 µM, respectively. High level analyte containing samples were prepared in phosphate buffered saline solution containing BSA for ADMA, SDMA, L-NMMA, ornithine, arginine, homoarginine and citrulline at 1.5, 2.75, 2.50, 250, 250, 5 and 250 µM, respectively. Pre-treated samples containing high and low concentrations of analyte were placed in a specific order and analyzed by LC-MS/MS. This study was performed separately for each analyte. Eleven copies of the low sample and ten copies of the high sample were run in the following order.

L1-L2-L3-H1-H2-L4-H3-H4-L5-L6-L7-L8-H5-H6-L9-H7-H8-L10-H9-H10-L11.

For each analyte, the measurement results from the LC-MS/MS were entered into the EP Evaluator Release 8 program (Data Innovations, South Burlington, VT) and carry-over values were calcu-

lated. The acceptability criteria for carryover were based on guidelines described in the CLSI protocol EP10-A3. Carryover values were calculated for each analyte using the following formula: Carryover = (mean of high-low results) - (mean of low-low results). Accordingly, the carryover values for ADMA, SDMA, L-NMMA, arginine, homoarginine, ornithine and citrulline were determined to be 0.029, 0.032, 0.004, 2.50, 0.021, 2.80 and 3.05 µM, respectively. Acceptable carryover is <3 × SD low-low results. SD low-low values were calculated as 0.018, 0.031, 0.042, 1.62, 0.053, 3.9 and 1.81 µM for ADMA, SDMA, L-NMMA, arginine, homoarginine, ornithine and citrulline, respectively. Therefore, the carryover values for ADMA, SDMA, L-NMMA, arginine, homoarginine, ornithine and citrulline should be <0.054, 0.093, 0.126, 4.86, 0.159, 11.7 and 5.43 µM, respectively (3 × SD low-low results). The carryover values calculated for all analytes were acceptable. The results of the carryover study are shown in Table 5.

3.1.7. Selectivity and specificity

Selectivity and specificity studies were carried out in accordance with FDA guidelines [39]. The selectivity study was per-

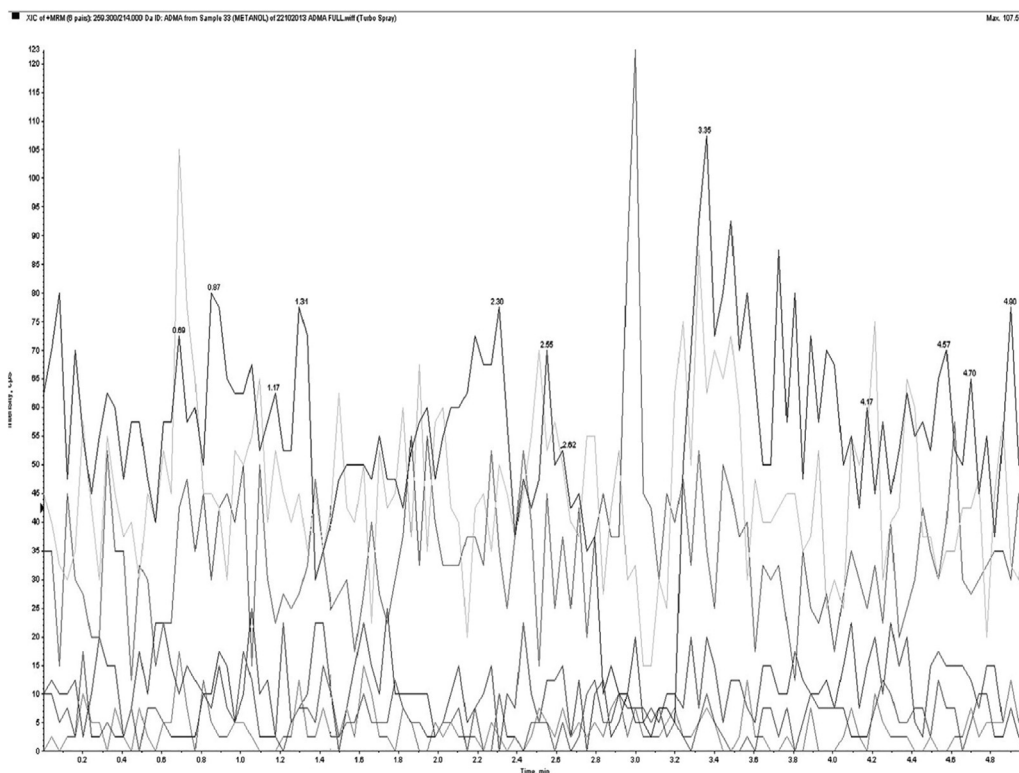


Fig. 1. The chromatogram of the blank sample.

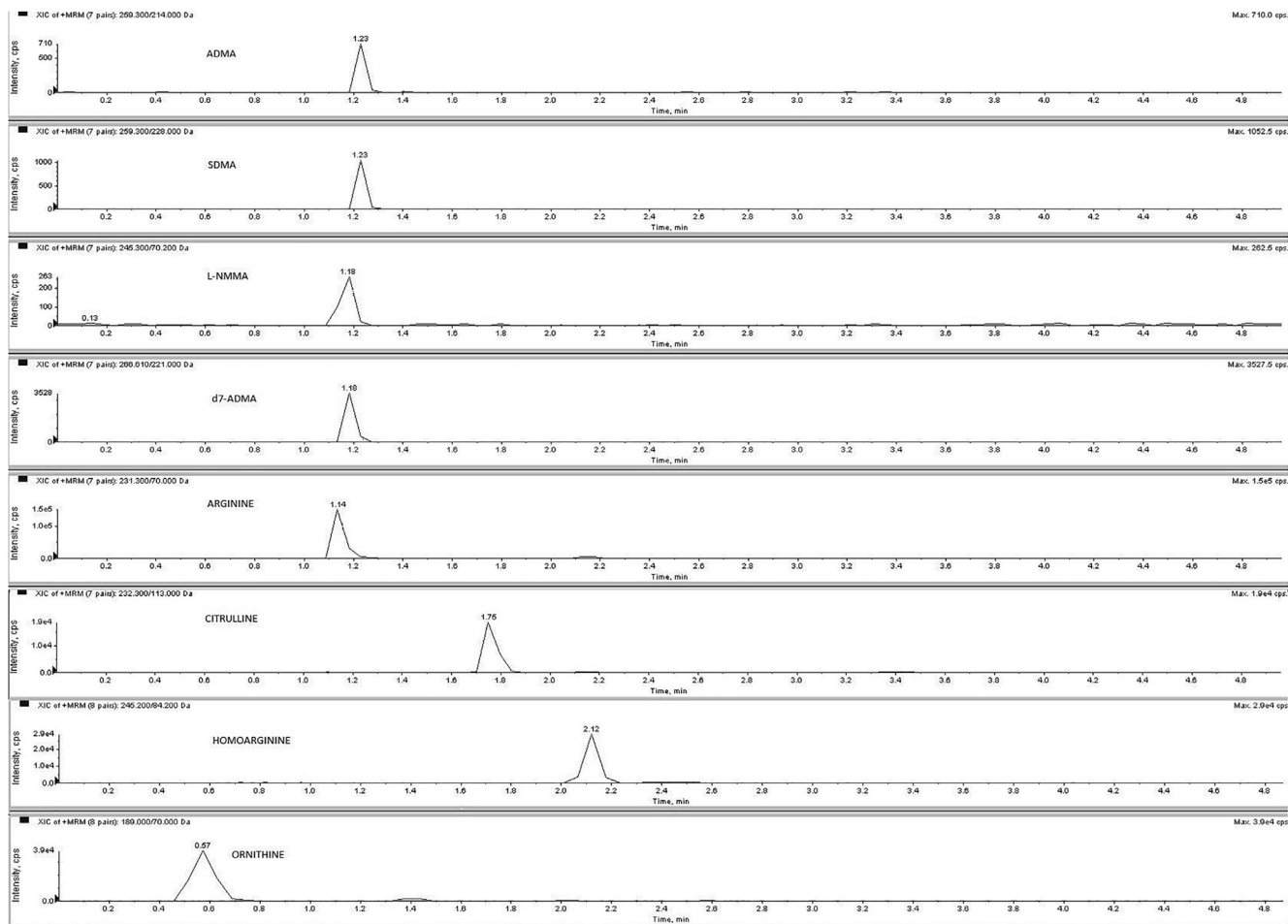


Fig. 2. The chromatogram of ADMA, SDMA, L-NMMA, arginine, homoarginine, ornithine and citrulline of the patient sample. 1, ornithine; 2, L-NMMA; 3, arginine; 4, d7-ADMA, 5, ADMA; 6, SDMA; 7, citrulline; 8, homoarginine.

formed using the analyte-free bovine serum albumin solution used in the preparation of the calibrators [36]. It was found that the chromatogram of bovine serum albumin did not contain an interfering peak at the expected retention time for ADMA, SDMA, L-NMMA, arginine, ornithine, homoarginine, citrulline or the internal standard (d7-ADMA). The response of the d7-ADMA (internal standard) in the blank did not exceed 5% of the d7-ADMA response in the calibrators. ADMA and SDMA (C8H18N4O2, exact mass = 202.142976 u) are isobaric metabolites, therefore, potential interferants for each other. In order to show the specificity of the method for these metabolites, an interference study was carried

out by adding 0%, 25%, 50%, 75% and 100% SDMA solution at a concentration of 5.5 μM to ADMA solutions in 0.093, 0.75 and 1.5 μM bovine serum albumin, and the bias% calculated. Bias values were found to vary between 2.9% and 8.7%. There was no interference between ADMA and SDMA. The chromatogram of the blank sample is shown in Fig. 1.

3.1.8. Application of the method for patient samples

Following the validation study, to demonstrate the applicability of the method, ADMA, SDMA, L-NMMA, arginine, homoarginine, ornithine and citrulline levels were measured in 46 patients who

Table 6

Pre- and post- hemodialysis levels of ADMA, SDMA, L-NMMA, ornithine, arginine, homoarginine, citrulline, creatinine, urea and eGFR. and arginine/ADMA ratio of patients with chronic renal failure.

Metabolite	Pre-hemodialysis	Post-hemodialysis	p
ADMA(μM)	1.06(0.43–3.36)	0.51(0.21–8.97)	p < 0.001
SDMA(μM)	2.51(0.25–9.37)	1.06(0.22–4.76)	p < 0.001
L-NMMA(μM)	0.12(0.02–0.78)	0.037(0.01–1.57)	p < 0.001
Arginine(μM)	94.95(39.50–326.02)	80.95(25.50–312.01)	p = 0.130
Ornithine(μM)	43.21(7.45–220.09)	26.01(4.31–345.02)	p < 0.001
Homoarginine(μM)	3.03(0.63–18.94)	1.27(0.23–10.13)	p < 0.001
Citrulline(μM)	94.85(39.50–326.03)	42.61(17.72–146.20)	p < 0.001
Arginine/ADMA	87.24(34.87–311.11)	171.28(4.71–926.13)	p = 0.001
Creatinine(mg/dL)	7.24(3.21–13.91)	2.37(1.08–6.19)	p < 0.001
Urea(mg/dL)	121.50(47.03–207.04)	29.5(6.01–97.11)	p < 0.001
eGFR(ml/min/1.73 m ²)	7.33(3.35–17.50)	30.19(8.56–72.50)	p < 0.001

Table 7

Correlation and significance values for serum ADMA, SDMA, L-NMMA, arginine, ornithine, citrulline, and homoarginine with creatinine, urea and eGFR. Spearman correlation coefficients were calculated using data obtained from patient samples.

Analyte	Creatinine		Urea		eGFR	
	r	p	r	p	r	p
ADMA	0.451	p < 0.001	0.454	p < 0.001	-0.432	p < 0.001
SDMA	0.471	p < 0.001	0.471	p < 0.001	-0.488	p < 0.001
L-NMMA	0.468	p < 0.001	0.393	p < 0.001	-0.451	p < 0.001
Arginine	0.112	p = 0.301	0.065	p = 0.549	-0.097	p = 0.367
Homoarginine	0.373	p < 0.001	0.346	p = 0.001	-0.381	p < 0.001
Citrulline	0.101	p = 0.347	0.068	p = 0.530	-0.083	p = 0.440
Ornithine	0.204	p = 0.057	0.156	p = 0.147	-0.189	p = 0.078

had undergone hemodialysis for at least 2 months in the dialysis unit of our hospital. Three milliliters of blood were collected from each patient and in serum separator gel tubes and centrifuged at 3500 rpm for 15 min. Separated serum samples were stored at -80 °C until analysis. The study was approved by Selcuk University ethics committee (Number: 2020/137, Date:18/03/2020). The chromatograms of ADMA, SDMA, L-NMMA, arginine, homoarginine, ornithine and citrulline from a representative patient sample are shown in Fig. 2.

ADMA and related metabolites concentrations along with eGFR, creatinine and urea levels of the patients with chronic renal failure are shown in Table 6.

Correlations for serum ADMA, SDMA, L-NMMA, arginine, ornithine, citrulline and homoarginine with creatinine, urea, eGFR are presented in Table 7.

4. Discussion

Following the determination that ADMA and related metabolites are risk markers for cardiovascular and renal diseases, many methods have been developed for their measurement. However, difficulties have been encountered when measuring derivatives of methylarginine. With the development of tandem mass spectrometry, sensitive, selective and reliable measurement is possible using specific analyte fragmentation patterns. By focusing on analyte specific m/z ratios, fragmentation-based separation can be achieved where chromatographic separation alone was difficult or impossible. A derivatization step was applied to improve the retention of analytes on a reverse phase column, and increase sensitivity of analysis [10]. Existing mass spectrometric methods commonly perform derivatization using butanol-HCl, while in the method we have developed, butanol-acetyl chloride was used following protein precipitation with methanol [24,48,51]. Here, we have established a method by which arginine, ADMA, SDMA, L-NMMA, homoarginine, ornithine and citrulline can be analyzed in the same chromatogram. For ADMA and SDMA, separation was achieved using a specific fragmentation pattern using ion transitions of 259.3 to 214.0 and 259.3 to 228.0 m/z , respectively. The method was linear at 0.023–6.0, 0.021–5.5, 0.019–5.0, 0.030–250, 0.030–250, 0.019–5.0 and 0.030–250 μM for ADMA, SDMA, L-NMMA, ornithine, arginine, homoarginine and citrulline, respectively. LLOQ values were determined to be 0.093, 0.042, 0.039, 0.061, 0.030, 0.078 and 0.061 μM for ADMA, SDMA, L-NMMA, ornithine, arginine, homoarginine and citrulline, respectively. The inter-assay imprecision was less than 7.8% at concentration levels other than the LLOQ, while they were less than 9.8% at LLOQ levels. The accuracy ranged between 88% and 112% for all analytes at LLOQ concentrations. Recovery% values ranged from 94.1% to

108.9% for all analytes, while the matrix effect% ranged from 1.2% to 8.1%. Our method is advantageous compared to other LC-MS/MS methods. The various mass spectrometric methods reported for methylarginine derivatives were summarized in Table 8.

Our method allows multiplexed analysis of ADMA, L-NMMA, SDMA, arginine, citrulline, ornithine, and homoarginine. Most established LC-MS/MS methods only measure levels of metabolites such as arginine, ADMA and SDMA [24,43,44,48,49,51–53]. A few methods also measure creatinine, L-NMMA, citrulline and/or homoarginine [42,45,46,49]. Other advantages of our method include a short run time and cost-effectiveness. Existing methods report analysis times ranging from 1.6 to 23 min [51,53]. In the method developed by Lobenhoffer et al., which measured ADMA, SDMA, arginine and citrulline, using OPA for derivatization, the analysis time is 21 min [45]. In the method developed by Yi et al., which measured arginine, ADMA, and SDMA, using acetonitrile for precipitation followed by butanol derivatization, the analysis time is 10 min [24]. The analysis time of the method described herein is only 5 min. Our method provides analyte separation based on specific precursor/product ion transitions of metabolites rather than chromatographic separation alone. Furthermore, increasing the total flow rate and using a shorter C18 column resulted in early elution of compounds from the column and reduced retention times.

According to the comparison of our method's LOQ values with previously published methods [44,47,49,50], it was observed that our method had a lower limit of serum ADMA quantitation (0.093 μM). The mean recovery % value of the developed method for all analytes was approximately 100%, while it was observed that the recovery% values showed wide variations in previous methods [50,52,54]. CV% values were reported higher than 10% for methylarginine derivatives in previous methods [46,50,52,54], whereas CV% values were <9.8% in our method. Another advantage of our method was that in contrast to methods in which the endogenous compounds, such as L-NMMA and homoarginine, were used as internal standards [24,55], d_7 -ADMA was used as an internal standard in our method. Moreover, in order to demonstrate the applicability of the method, serum ADMA, SDMA, L-NMMA, arginine, homoarginine, ornithine, citrulline levels were measured in the samples of hemodialysis patients and compared with previous literature data performed in hemodialysis patients. For example, in the study conducted by Wahbi et al., ADMA, SDMA and arginine levels were found to be 0.99, 4.53, 116 μM in pre-hemodialysis patients, and 0.63, 2.95, 104 μM in post-hemodialysis patients, respectively [56]. In the study conducted by Schmidt et al., ADMA, SDMA, arginine and citrulline levels were 4.14 ± 0.78 , 1.41 ± 0.19 , 81 ± 9 , 112 ± 14 μM in pre-hemodialysis patients, and 1.45 ± 0.42 , 0.62 ± 0.10 , 66 ± 11 , 44 ± 3 μM in post-

Table 8

The various methods reported for methylarginine derivatives.

Method	Analyte	Extraction	Derivatization	Device	CV % ranges	LOD/LOQ	Linearity	Column	Sample	Recovery %	Matrix effect%	Time	IS	Ref.	
ESI-LC-MS/MS	ARG, MMA, HARG, ADMA, SDMA, CIT	NA	DEPC	Waters system model Acquity UPLC equipped with a Waters tandem quadrupole mass spectrometer(TQD) (Waters Italia, Milan,Italy)	4.0-5.4	0.01 -0.03 $\mu\text{mol/L}$	0.31-5 $\mu\text{mol/L}$	100mm \times 4.6mm Zorbax Eclipse Plus C18 3.5 $\mu\text{mol/L}$ column	Plasma	92-107	NA	10 min	d_6 -ADMA	Sotgia et al., 2019 [42]	
ESI-LC-MS/MS	ARG, ADMA, SDMA	MeOH,ACN(PP)	NA	Alliance 2695,Waters Co., Milford, MA, USA) HPLC with Triple Quadrupole MassSpectrometer equipped with an electrospray ion source (ESI)operating in positive mode (Micromass Quattromicro, Waters-USA)	2.3-8.4	NA	0.22-1.29 $\mu\text{mol/L}$	Luna silica column, 3 $\mu\text{mol/L}$, 100 \times 2 mm i.d, Phenomenex-USA)	Plasma	96.2-100.5	NA	6 min	$^{13}\text{C}_6$ -L-Arg	D'Apolito et al., 2008 [43]	
ESI-LC-MS/MS	ARG, ADMA, SDMA	ACN, ammonium formate buffer solution (PP)	NA	Agilent 1100 system (Waldbronn,Germany equipped with Thermo Fisher Scientific (Waltham, MA, USA) TSQ Discovery Max triple quadrupole mass spectrometer equipped withan electrospray ionization (ESI) ion source	4.2-11.3	0.003/0.15 $\mu\text{mol/L}$	0.15-3 $\mu\text{mol/L}$	Atlantis HILIC silica column with 5 μm particle size and with the dimensions150 mm \times 2.1 mm (Waters, Eschborn, Germany)	Plasma	NA	NA	8 min	d_7 -ADMA	Martens-Lobenhoffer et al., 2012 [44]	
42	ESI-LC-MS	ARG, ADMA, SDMA, CIT	ACN(PP)	OPA/ME	Agilent 1100 system (Waldbronn, Germany) comprising a ThermoFinnigan LCQ ion trap mass spectrometer (San Jose, USA) withan electrospray ionization (ESI) ion source	2.0-8.1	NA	0.4- 8 $\mu\text{mol/L}$ for serum 2.5-50 $\mu\text{mol/L}$ for urine	Merck Superspher 100 RP18 250mm \times 4mm column	Plasma, Urine	NA	NA	21 min	$^{13}\text{C}_6$ -arginine and HARG	Martens-Lobenhoffer et al., 2003 [45]
ESI-LC-MS/MS	ARG, CIT ADMA, SDMA	ACNcontaining 0.5% acetic acid and 0.025% TFA (PP)	NA	Shimadzu LC-20AD delivery pump, SIL-20AC autosampler and CBM-20A system controller(Shimadzu Scientific Instruments; Columbia, MD) and ABI/Sciex API3000 triple quadrupole massspectrometer (Applied Biosystems, Foster City, CA)	2.09-14.4	NA / 0.025 $\mu\text{mol/L}$	0.025-1.000 $\mu\text{mol/L}$	150mm \times 2.1mm Alltima HP HILIC 3 $\mu\text{mol/L}$ column	Rat plasma, urine, cell lysate	NA	NA	6 min	$^{15}\text{N}_4$ -ARG	Shin et al., 2011 [46]	
ESI-LC-MS/MS	ADMA, HCY	0.1% formic acid in MeOH	NA	Waters Alliance (Waters Co., Milford, MA, USA) system Micromass Quattro micro triple quadrupole mass spectrometer (Micromass UK Limited, Manchester, England)	2.4-4.8	0.01-0.69 $\mu\text{mol/L}$	0,69-131,47 $\mu\text{mol/L}$	Atlantis HILIC silica (100 mm \times 2.1 mm, 5 $\mu\text{mol/L}$, Waters)	Urine	94.9-101.1	NA	4.5 min	cystamine dihydrochloride	Gopu et al., 2011 [47]	
ESI-LC-MS	ADMA, SDMA, ARG	ACN	HCl and n-butanol	Shimadzu LC-10 system (Shimadzu,Kyoto, Japan) with LC/MS-2010 single quadrupolemass spectrometer (Shimadzu, Kyoto, Japan)	3.0-7.8	NA-0.08 $\mu\text{mol/L}$	0.08-5 $\mu\text{mol/L}$	Thermo Hypersil-Keystone Hypurity C18 (150mm 2.1 mm,5 $\mu\text{mol/L}$) analytical column	Plasma	93-101	NA	10 min	HARG	Yi et al., 2011 [24]	

Table 8 (continued)

Method	Analyte	Extraction	Derivatization	Device	CV % ranges	LOD/LOQ	Linearity	Column	Sample	Recovery %	Matrix effect%	Time	IS	Ref.
ESI-LC-MS/MS	ADMA, SDMA, ARG	Acetone	HCl and n-butanol	Varian ProStar HPLC systems with Varian 1200L Triple Quadrupole mass spectrometer	0.6–5.6	3–50 nmol/L	0.025–4 µmol/L	Varian analytical column [50x 2.0 mm (i.d.)] packed with Polaris C18-Ether (3 µmol/L bead size)	Plasma	90.8–105	NA	4 min	d ₆ -ADMA	Schwedhelm et al., 2005 [48]
HESI-LC-MS/MS	ADMA, SDMA, ARG creatinine	0.1% formic acid in ACN	NA	NANOSPACE SI-2 (Shiseido, Tokyo, Japan) HPLC system with Thermo Fisher Scientific TSQ Quantum Ultra triple quadrupole mass spectrometer	0.2–7.2	0.742–2.47 µmol/L	0.50–50.0 µg/mL	MightySil Si 60 (250 × 3 mm I.D., 5 µmol/L particle size)	Plasma, urine, tissue	94.6–100.5	NA	<15%	Arg- ¹³ C ₆ and Cr-d3	Saigusa et al., 2011 [49]
ESI-LC-QTOF-MS	ARG, CIT, ADMA, SDMA	ACN	PFBC	NanoAcquity UPLC system with AXevo G2 Q-TOF MS (Waters)	1.7–14.9	0.03–0.1 µmol/L	0.05–2.5 µmol/L	Acquity HSST3 column (50 × 1.0 mm, 1.75 µmol/L) with 0.22 µmol/L membrane inline filter (Waters)	Serum	88.93–110.9	2.84–14.79	14.5 min	d ₇ -ADMA	Wiśniewski et al., 2017 [50]
ESI-LC-MS/MS	ARG, ADMA, SDMA	MeOH	HCl and n-butanol	Varian 1200L Triple Quadrupole MS equipped with two Varian ProStar model 210 HPLC pumps	1.3–5.4	0.5 nM/NA	0–4 µmol/L	Chirobiotic T, 20mm×1.0mm i.d., microbore guard column packed with teicoplanin covalentlybonded to 5µm spherical silica (Astec, Whippany, NJ, USA)	Plasma	NA	NA	1.6 min	d ₆ -ADMA	Schwedhelm et al., 2007 [51]
ESI-LC-MS/MS	ADMA, SDMA, ARG	1% ammonium acetate in MeOH and 1% formic acid in ACN	NA	Thermo Fisher TSQ Quantum Access with a TLX-4 HPLC system and tandem mass spectrometer	1.4–12.2	NA	0.55–4.43 µmol/L	Polaris Si-A analytical column	Plasma	99–155	NA	5 min	¹³ C-ARG and d ₇ -ADMA	El-Khoury et al., 2012 [52]
ESI-LC-MS/MS	ADMA, SDMA	TCA (PP)/SPE	NDA/ME	Varian 1200L LCMS/MS system (Agilent Technologies, Palo Alto, CA, USA) with a triple quadrupole mass analyzer	2.7–6.8	2.6–8.7 nM	0.05–2.5 µmol/L	Kinetex 75 × 3.0 mm, 2.6 µm C18 column from Phenomenex (Torrance, CA, USA)	Plasma	93.79–105.07	NA	23 min	N-propyl-ARG	Hui et al., 2012 [53]
LC-ESI-QTOF	ARG, ADMA, SDMA, CIT	ACN(PP)	Benzoyl chloride	Nano-Acquity UPLC system equipped with Xevo G2 XS QuadrupoleTOF MS (waters)	1.6–14.5	0.03–0.08 µmol/L	0.05–2.5 µmol/L	Acquity HSST3 column (50 × 1.0 mm, 1.75 µmol/L) from Waters	Serum	86.78–127.82	NA	10 min	d ₇ -ADMA	Fleszar et al., 2018 [54]
ESI-LC-MS/MS	HARG, ARG, ADMA, SDMA	MeOH(PP)	NA	HPLC Agilent 1100 series was equipped with an Agilent 1100 LC/MSD SL quadrupole ion trap	NA	NA- 0.06 µmol/L	0.1–25 mg/L	Supelcosil™ LC-Si 3.3 cm × 4.6 mm i. d., 3 µmol/L particle size	Urine	94.0–98.0	NA	20 min	NMMA	Servillo et al., 2013 [55]

ACN, acetonitrile; ADMA, asymmetric dimethylarginine; APDS, 3-aminopyridyl-N-succinimidyl carbamate; ARG, arginine; CIT, citrulline; DEPC, diethylpyrocarbonate; MMA, monomethyl arginine; HARG, homoarginine, HCY, homocysteine; ME, 2-mercaptoethanol; NA, not available; NDA, naphthalene-2,3-dicarboxaldehyde; OPA, o-phthalaldehyde; PP, protein precipitation; SDMA, symmetric dimethylarginine; SPE, solid-phase extraction; TCA, trichloroacetic acid, TFA, trifluoroacetic acid. LOD, LOQ, linearity values for ADMA and other parameters are described for all analytes listed.

hemodialysis patients, respectively [57]. Analyte levels measured by the current method in hemodialysis patients were found to be consistent with the results reported in previous studies (Table 6).

5. Conclusions

A highly useful method has been developed for the measurement of ADMA and related metabolites. The main advantages of our method are the short run time, high reproducibility, cost-effectivity and most importantly, it allows multiplex analysis with high sensitivity. The measurement results we obtained from dialysis patients also show that the method can be used reliably for patients in the clinic.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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