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Determination of arginine and methylated arginines in human plasma by liquid chromatography–tandem mass spectrometry

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Abstract

Nitric oxide (NO) is synthesized from L-arginine (ARG) catalyzed by the enzyme nitric oxide synthase (NOS) and is important in the regulation of vascular tone, neurotransmission and host defense. *N*,*N*-Dimethyl L-arginine (asymmetric dimethylarginine, ADMA) and *N*-monomethyl L-arginine (MMA) are endogenous inhibitors of NOS. *N*,*N′*-Dimethyl L-arginine (symmetric dimethylarginine, SDMA), the inactive enantiomer of ADMA is also known to be present endogenously. A simple, sensitive and fast LC–MS–MS method was developed to extract and quantitate ADMA, SDMA, MMA and ARG from human plasma. $^{13}C_6$-ARG was used as the internal standard for the assay. Protein precipitation using acetonitrile gave good recoveries of all the compounds from plasma. The compounds were separated by HPLC in less than 15 min using a silica column. The limits of detection for this method were found to be approximately 1 ng/ml for ARG, ADMA and SDMA and 2.5 ng/ml for MMA. The total LC–MS–MS analysis time is less than 15 min making this the fastest and most specific method reported to date. The use of an isocratic liquid chromatographic separation makes this method optimal for high sample throughput. The inter- and intra-day precision (% RSD) and accuracy (% error) for this assay were less than 15%. The average concentrations of ARG, ADMA, SDMA and MMA in plasma from 20 human subjects were found to be 10.9±4.1 μg/ml, 25.1±9.4 ng/ml, 33.2±13.1 ng/ml and 19.6±3.8 ng/ml, respectively.

Keywords: Arginine; Methylarginines

1. Introduction

Nitric oxide (NO) is a cell-to-cell mediator involved in the regulation of vascular tone, host defense, platelet aggregation and neurotransmission. In the cardiovascular system, endothelial release of NO acts to regulate blood flow and blood pressure through the vascular smooth muscle. NO induces the lysis of aggregated platelets, and inhibits platelet aggregation [1,2]. In 1988, Palmer and co-workers [3,4] first reported that nitric oxide along with citrulline is synthesized under physiological conditions by a constitutive Ca^{2+}-dependent enzyme, nitric oxide synthase (NOS) from endogenous or dietary supplemental L-arginine (ARG) exclusively in response to receptor stimulation. This biosynthesis can be inhibited by some analogues of arginine including *N*-monomethyl-L-arginine (MMA) and *N*,*N*-dimethyl L-arginine (asymmetric dimethylarginine, ADMA) which has potent pressor/vasocon-

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strictor effects. *N*,*N*′-Dimethyl L-arginine (symmetric dimethylarginine, SDMA), the inactive isomer of ADMA is known to be present endogenously as well. Hence, the quantification of these intermediates in the nitric oxide pathway in human plasma and tissue including vascular and placental tissue is of great clinical importance [5–19].

The separation and detection of ADMA, SDMA and ARG have been reported using high-performance liquid chromatography (HPLC) [20–24] and capillary zone electrophoresis (CZE) [25]. However, all the HPLC methods reported have used pre-column derivatization using *o*-phthalaldehyde (OPA) to increase the detectability and retention of these compounds on reversed-phase columns. Complex gradient systems were required and the elution times were as long as 70 min not including the equilibration time between runs. Although the run time of the one CZE method is short (approximately 8 min), the sample preparation is tedious [25]. In this paper, we report a simple, fast and sensitive method, which can be used to quantitate ARG, ADMA, SDMA and MMA, simultaneously without interference from other endogenous compounds such as amino acids or their derivatives.

2. Experimental

2.1. Chemicals

N,*N*-Dimethyl L-arginine (asymmetric dimethylarginine, ADMA), L-arginine (ARG) and *N*,*N*′-dimethyl L-arginine (symmetric dimethylarginine, SDMA) were purchased from Sigma (St. Louis, MO, USA). *N*-Monomethyl L-arginine (MMA) was purchased from Research Biochemicals International (Natick, MA, USA). $^{13}C_6$-Arginine (internal standard, I.S.) was obtained from Cambridge Isotope Labs. (Andover, MA, USA). The structures of these compounds are shown in Fig. 1. Formic acid (88%), methanol and acetonitrile were HPLC grade and were obtained from J.T. Baker (Phillipsburg, NJ, USA). Ammonium formate (97+%, Aldrich, Milwaukee, WI, USA) was used without further purification. Deionized water was obtained from a Continental system (Natick, MA, USA).

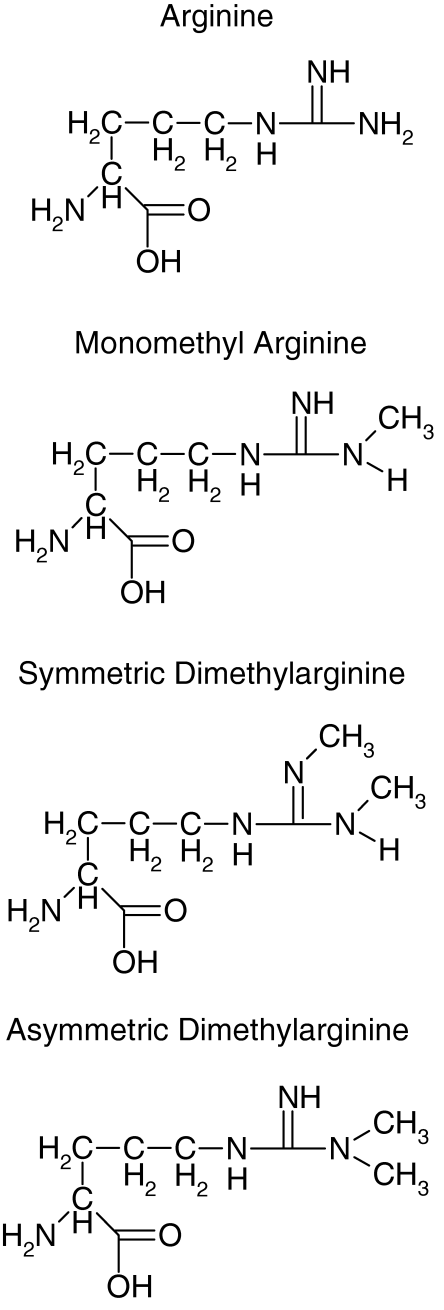

Fig. 1. The chemical structures of L-arginine (ARG), monomethyl L-arginine (MMA), asymmetric dimethyl L-arginine (ADMA) and symmetrical dimethyl L-arginine (SDMA).

2.2. Instrumentation

HPLC separations were achieved on a Hewlett-Packard Model 1100 system equipped with a quaternary pump and an autosampler (Palo Alto, CA, USA). The column utilized was a Brownlee Spheri-5 Silica (100×4.6 mm I.D., 5 μm particle size, Perkin-Elmer, Norwalk, CT, USA). An RP-C_2 1 mm guard column containing C_2 stationary phase was used (Perkin-Elmer). A flow-rate of 300 μl/min was used. Injections (30 μl) of the reconstituted extracts were injected onto the HPLC system for analysis. The mobile phase consisted of 0.1% formic acid containing 10 m*M* ammonium formate in water–0.1% formic acid containing 10 m*M* ammonium formate (95:5, v/v) in methanol–acetonitrile (1:1). The column was heated to 37°C.

Mass spectrometric experiments were performed using a Micromass Quattro II (Beverly, MA, USA) triple quadrupole mass spectrometer interfaced to the HP1100 HPLC system equipped with an electrospray ionization (ESI) probe using nitrogen as the sheath gas. MS control and spectral processing was carried out using MassLynx software, version 2.22 (Micromass). The positively charged molecule of each analyte was selected by mass and focused into the collision cell containing argon gas (99.999% purity) maintained at a pressure of approx. $1.3 \cdot 10^{-3}$ Torr (1 Torr=133.322 Pa). The precursor and collision-induced fragment ions were monitored by a post collision quadrupole analyzer. The source temperature was set at 150°C and the needle and cone voltages were optimized at 3500 V and 35 V, respectively and at 15–20 eV collision energy and a 100 ms dwell time. All measurements were made at unit mass resolution.

2.3. Separation

The separation of ARG, ADMA, SDMA and MMA was explored using various reversed-phase HPLC columns including C_{18}, C_8, C_6, C_2 and silica columns. The components were not retained on the C_{18}, C_8 and C_6 columns. The four components were not well resolved on the C_2 column after optimizing separation conditions such as mobile phase buffer concentration, pH value and organic modifier percentage and type. The separation of the positional isomers, ADMA and SDMA were difficult and since the MS–MS spectra of the two were similar, chromatographic separation was essential. Separation was achieved on a Brownlee Spheri-5 Silica column 100×4.6 mm, 5 μm particle size (Perkin-Elmer). Of the optimizable parameters, the pH value of the buffer and organic modifier percentage had the greatest effect on the peak shape and separation. The components were best separated using a mobile phase of 0.1% formic acid with 10 m*M* ammonium formate in water–0.1% formic acid with 10 m*M* ammonium formate (95:5) in acetonitrile–methanol (1:1, v/v).

2.4. Extraction procedure

The recoveries of ARG, ADMA, SDMA and MMA were explored using various solid-phase extraction cartridges. Each cartridge was first conditioned with 1 ml methanol and 1 ml deionized water. Then 1 ml of the standard solution (1 μg/ml) was loaded and drawn through the cartridge with vacuum (~5 in. Hg; 1 in. Hg=388.638 Pa). The cartridge was then washed with 1 ml deionized water. The analytes and the internal standard were eluted with 2 ml 5% (v/v) ammonia in methanol solution into a clean culture tube. The eluent was then dried under vacuum (SC110A SpeedVac Plus and RVT400 Refrigerated Vapor Trap; Savant, Farmingdale, NY, USA). The residue was reconstituted in 100 μl of mobile phase for LC–MS–MS analysis. Strong-cation exchange (SCX) and CBA cartridges have previously been used for the extraction of ADMA and ARG [20,23]. In this study, four different cartridges were tested: SCX (Ansys, Irvine, CA, USA), CBA (Varian, Harbor City, CA, USA), Oasis HLB (Waters, Milford, MA, USA) and Extract Clean Silica (Alltech, Deerfield, IL, USA) cartridges. The recoveries were less than 50% for the analytes of interest with these cartridges and hence, protein precipitation was performed using 5% trichloroacetic acid (TCA) and acetonitrile. Protein precipitation using acetonitrile gave good recovery for all the components and was cleaner and provided interference free chromatograms. Hence, acetonitrile was used as the protein precipitating reagent.

2.5. Generation of calibration curves

Seven-point calibration curves were generated for ADMA, SDMA, ARG and MMA. Stock solutions were prepared at a concentration of 100 μg/ml for ADMA, SDMA and MMA and at 1000 μg/ml for ARG in deionized water. An intermediate stock containing 1000 ng/ml of MMA, SDMA and ADMA and 100 μg/ml of ARG was prepared in deionized water. This intermediate stock was diluted in water to give concentrations of 20, 50, 100, 200, 400 and 600 ng/ml for ADMA, SDMA and MMA and 2000, 5000, 10 000, 20 000, 40 000 and 60 000 ng/ml for ARG. Quality control (QC) samples (for accuracy and precision) were prepared at a concentration of 60 ng/ml and 500 ng/ml for ADMA, SDMA and MMA and at 6000 ng/ml and 50 000 ng/ml for ARG. The internal standard ($^{13}C_6$-ARG) was prepared at a concentration of 25 000 ng/ml in water. A 500-μl volume of the standard solution was added to 500 μl of blank plasma and 100 μl of the internal standard solution was added. The mixture was vortex-mixed for 30 s in a rotary mixer. Then, 3 ml of acetonitrile was added as a protein precipitating reagent. The tubes were vortex-mixed and then centrifuged at 3000 rpm for 15 min using a Jouan CR4-22 (Winchester, VA, USA) centrifuge. The solutions were filtered through a 0.2-μm nylon syringe filter (13 mm; Alltech) and dried under vacuum. The residue was reconstituted in 100 μl of the mobile phase, microcentrifuged at 12 000 rpm for 5 min and stored at 4°C until assay. The calibration curves were plotted using the ratio of peak areas of ADMA, SDMA, MMA or ARG to internal standard ($^{13}C_6$-ARG) versus concentrations. The correlation coefficients were greater than 0.99 for all analytes.

Since ARG, ADMA, SDMA and MMA are endogenous substances there was no ''real'' blank plasma available. Hence, standard addition method was used. The chromatogram obtained was background subtracted using the blank plasma chromatogram.

2.6. Preparation of plasma samples

Blood was collected into heparinized tubes and centrifuged immediately. The plasma samples were transferred into clean chilled tubes and kept at −20°C until analyzed. The concentrations of ADMA, SDMA, MMA and ARG in 20 patients plasma were determined. A representative LC–MS–MS trace obtained from plasma is shown in Fig. 2. Interference from the background was minimal. Representative LC–MS–MS traces showing blank plasma and a sample from a normotensive volunteer are shown in Fig. 3.

3. Results and discussion

3.1. Development of the LC–MS–MS assay

Due to the low inherent UV absorbance of ADMA, SDMA, MMA and ARG, these compounds are normally converted to their fluorescent derivatives by reaction with OPA. The derivatives are then separated on a C_{18} column using a gradient elution system. Since pre-column derivatization also increases the retention of these compounds in reverse phase systems, the separation can take as long as 70 min. It was difficult to achieve a good separation of underivatized ADMA, SDMA, MMA and ARG on a C_{18} column due to their high polarities and limited retention. Although the analytes could be monitored at 205 nm, sensitivity was quite low. Interference from the biological matrix also made peak identification and integration difficult.

In this study, a simple, fast and sensitive isocratic HPLC method was developed, which utilizes direct injection of the extracted sample, without resorting to derivatization of the analytes, using mass spectrometry (MS) for detection. Acetonitrile was used as the protein precipitating reagent as it gave good, cleaner recoveries and provided interference-free chromatograms. Table 1 illustrates the recovery of the analytes from human plasma.

Two detection options were explored: selected-ion recording (SIR) and multiple-reaction monitoring (MRM) modes. In the SIR mode, m/z 203 (ADMA and SDMA), m/z 175 (ARG), m/z 181 (I.S.) and m/z 189 (MMA) were monitored. These masses represent the protonated molecular ions $[M+H]^+$ for each of the analytes of interest. In the MRM mode, various fragmentation pathways were monitored. The MRM mode has the benefit of increasing the spe-

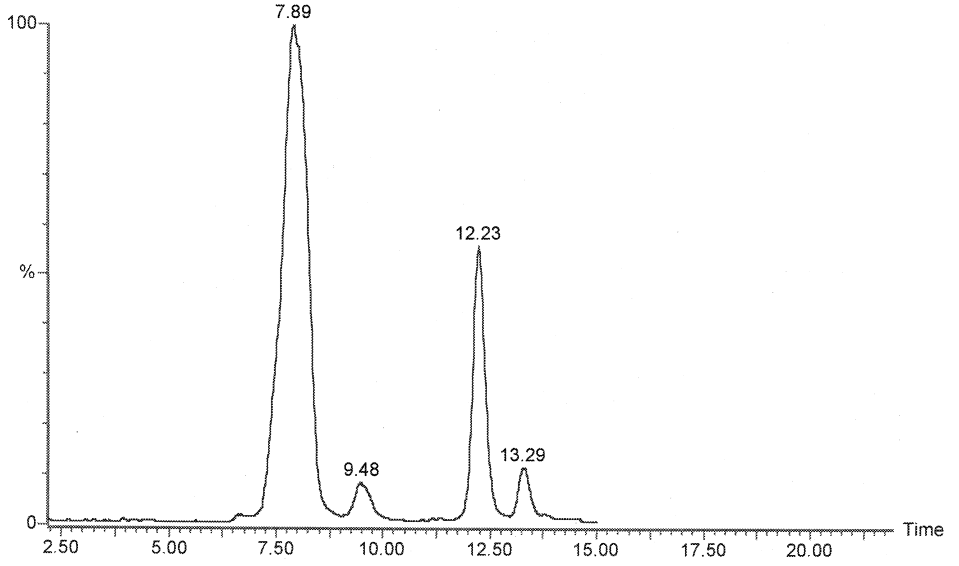

Fig. 2. A representative LC–MS–MS trace of ARG, MMA, ADMA, SDMA and the internal standard $^{13}C_6$-ARG extracted from human plasma. LC was done on a Brownlee silica column (100×4.6 mm, 5 μm) using a mobile phase of 0.1% formic acid with 10 m*M* ammonium formate in water–0.1% formic acid with 10 m*M* ammonium formate (95:5) in methanol–acetonitrile (1:1) at a flow-rate of 300 μl/min. MRM detection was used. ARG elutes at 7.9 min, followed by MMA at 9.5 min, SDMA at 12.4 min at ADMA at 13.3 min. The I.S. ($^{13}C_6$-ARG) elutes along with ARG at 7.9 min.

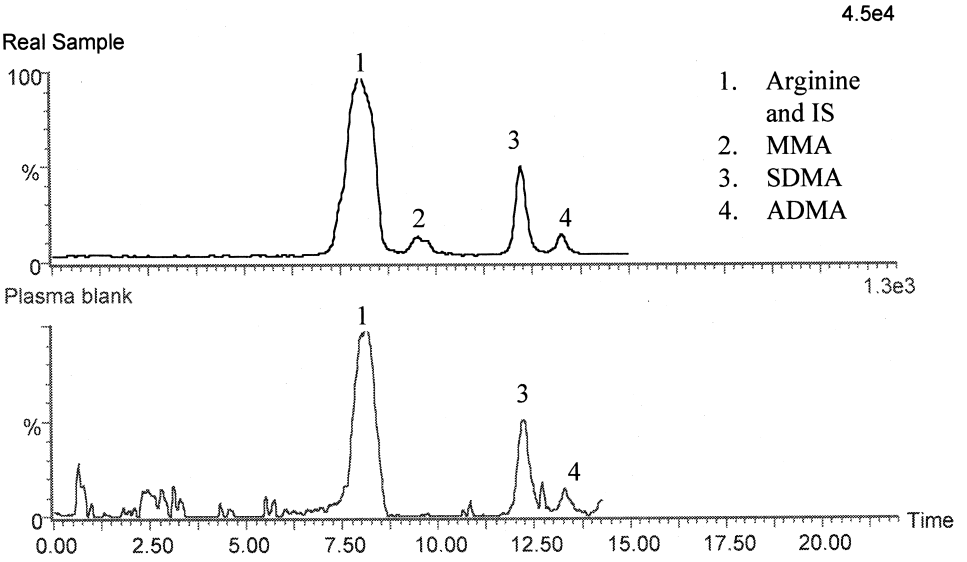

Fig. 3. LC–MS–MS trace of a normotensive sample and the plasma blank. For conditions see Experimental section. LC was done on a Brownlee silica column (100×4.6 mm, 5 μm) using a mobile phase of 0.1% formic acid with 10 m*M* ammonium formate in water–0.1% formic acid with 10 m*M* ammonium formate (95:5) in methanol–acetonitrile (1:1) at a flow-rate of 300 μl/min. MRM detection was used.

Table 1
Percent recovery of compounds from plasma ($n=5$)

Analyte	Recovery (%) (mean±SD)			
	ARG	MMA	SDMA	ADMA
Cal 2 level	83.6±6.9	73.2±5.9	78.2±2.8	95.9±3.2
Cal 5 level	82.1±6.1	72.5±9.0	80.4±8.4	94.1±5.8

cificity of the assay by using the tandem mass spectrometric features of this instrument. For the detection of ARG, the transition from m/z 175→60 was monitored. For ADMA, the transition from m/z 203→158 [loss of $(CH_3)_2NH$] and for SDMA the transition m/z 203→172 was monitored. For MMA, the transition m/z 189→70 was monitored. Both SIR and MRM typically increase the sensitivity of a mass spectrometric method by a factor of 50–100 when compared to full scan modes of operation. In addition, the signal-to-noise ratios are usually better and more accurate peak integration and better reproducibility are obtained.

Both the SIR and MRM methods of analysis were used to determine accuracy, precision and limit of detection (LOD) and have found them to be equivalent. The MRM mode of operation showed improved performance over the SIR mode in cases of high background noises in detecting the analytes. This increased performance is due to the greater selectivity of tandem mass spectrometry. Due to the advantages of the MRM mode of operation, it was used for the determination of ARG and the methylated arginines.

The precision (RSD) of the method was within 12% from run-to-run and day-to-day (Tables 2 and 3). The accuracy of the method was calculated using the equation: accuracy=(measured concentration−spiked concentration)/(spiked concentration)·100. The accuracy of the measured concentrations using the QC samples was better than 11%.

3.2. MS–MS spectral analysis

The MS–MS spectra of ARG, $^{13}C_6$-ARG, ADMA, SDMA and MMA are tabulated in Table 4. The formation of an intermediate complex as illustrated in Fig. 4 is an important step in the fragmentation of protonated amino acids. The loss of ammonia yields m/z 158. The subsequent loss of CO or loss of COOH from the molecular ion may lead to the formation of m/z 130. This loss of ammonia most likely arises from the most basic site in the molecule, the guanidyl group and not from the alpha amino group: this loss is possible only for ARG and MMA. Similar compounds ADMA and SDMA do not lose ammonia. The loss of COOH has been reported for ^{252}Cf-plasma desorption but in fast atom bombardment (FAB) mass spectra, the loss is known to be exclusively NH_3+CO. The composition and structure of the ion at m/z 130 in the present work is unknown but is believed to be consistent with the

Table 2
Intra-day precision and accuracy for spiked samples

Analyte	Concentration added (ng/ml)	Concentration found[a] (ng/ml)	Precision (RSD, %)	Accuracy (% error)
Arginine	3000	2967.4±335.3	11.3	1.1
	25 000	25 373.4±2333.9	9.2	1.5
Monomethylarginine	30	32.4±3.0	9.2	8.1
	250	253.3±28.2	11.1	1.4
Symmetric dimethylarginine	30	29.7±3.0	10.2	1.1
	250	263.9±14.1	5.3	5.5
Asymmetric dimethylarginine	30	29.8±2.6	8.9	0.8
	250	249.8±24.6	9.9	0.1

[a] Mean±SD ($n=4$).

Table 3
Inter-day precision and accuracy for spiked samples

Analyte	Concentration added (ng/ml)	Concentration found[a] (ng/ml)	Precision (RSD, %)	Accuracy (% error)
Arginine	3000	2794.4±290.6	10.6	6.8
	25 000	26 267.4±1007.1	3.8	5.1
Monomethylarginine	30	33.1±2.2	8.4	10.3
	250	238.2±33.9	14.2	4.7
Symmetric dimethylarginine	30	28.3±3.5	12.3	5.6
	250	254.7±5.7	2.3	1.9
Asymmetric dimethylarginine	30	30.5±3.2	10.5	1.8
	250	229.6±15.8	6.9	8.2

[a] Mean±SD (n=9).

less energetic FAB process [26]. The intermediate complex then dissociates giving rise to peaks at m/z 60 [$C^+(NH_2)_3$] and m/z 116 [loss of $HN{=}C(NH_2)_2$]. The loss of CO and H_2O from m/z 116 leads to m/z 70. These proposed losses are consistent with those observed from the $^{13}C_6$-labeled internal standard.

In the case of MMA, the loss of methylamine leads to m/z 158. The loss of NH_3+CO or COOH results in the formation of m/z 144. An intermediate complex similar to the one formed for ARG dissociates to give m/z 74 and m/z 116. The loss of H_2O and CO from m/z 116 leads to the formation of the stable m/z 70.

For ADMA, the loss of dimethylamine leads to the formation of m/z 158. An intermediate complex is formed similar to that of ARG and MMA, which dissociates to give m/z 88 and m/z 116 (protonated proline). This protonated proline subsequently decomposes by the loss of H_2O and CO to give m/z 70.

SDMA undergoes similar dissociations to ADMA, but gives rise to a unique peak at m/z 172 by the loss of methylamine. The other peaks m/z 158, m/z 116, m/z 70 and m/z 88 are also observed in the collision-induced dissociation (CID) mass spectrum of ADMA. There is also the formation of a peak at m/z 133 which could be $^+NH_3$-$(CH_2)_3$-CH-(NH_2)-COOH. All these fragmentations are similar to those reported earlier by Dookeran et al. [26].

3.3. Limits of detection

The LODs for ADMA using OPA derivatization and fluorescence detection are reported to be about 2 ng/ml from human plasma [20–25]. The method

Table 4
m/z ratios and relative intensities from positive ion electrospray MS–MS spectra

Arginine (ARG) @18 eV	$^{13}C_6$-arginine @15 eV	Monomethyl-arginine @18 eV	Asymmetric dimethylarginine @12 eV	Symmetric dimethylarginine @12 eV
175 (15%)	181 (100%)	189 (30%)	203 (100%)	203 (100%)
158 (10%)	61 (45%)	158 (20%)	158 (30%)	172 (50%)
130 (25%)	121 (40%)	144 (40%)	116 (25%)	158 (20%)
116 (45%)	164 (20%)	116 (75%)	88 (25%)	133 (35%)
70 (100%)	135 (15%)	74 (55%)	70 (10%)	116 (40%)
60 (45%)	74 (20%)	70 (100%)		88 (20%)

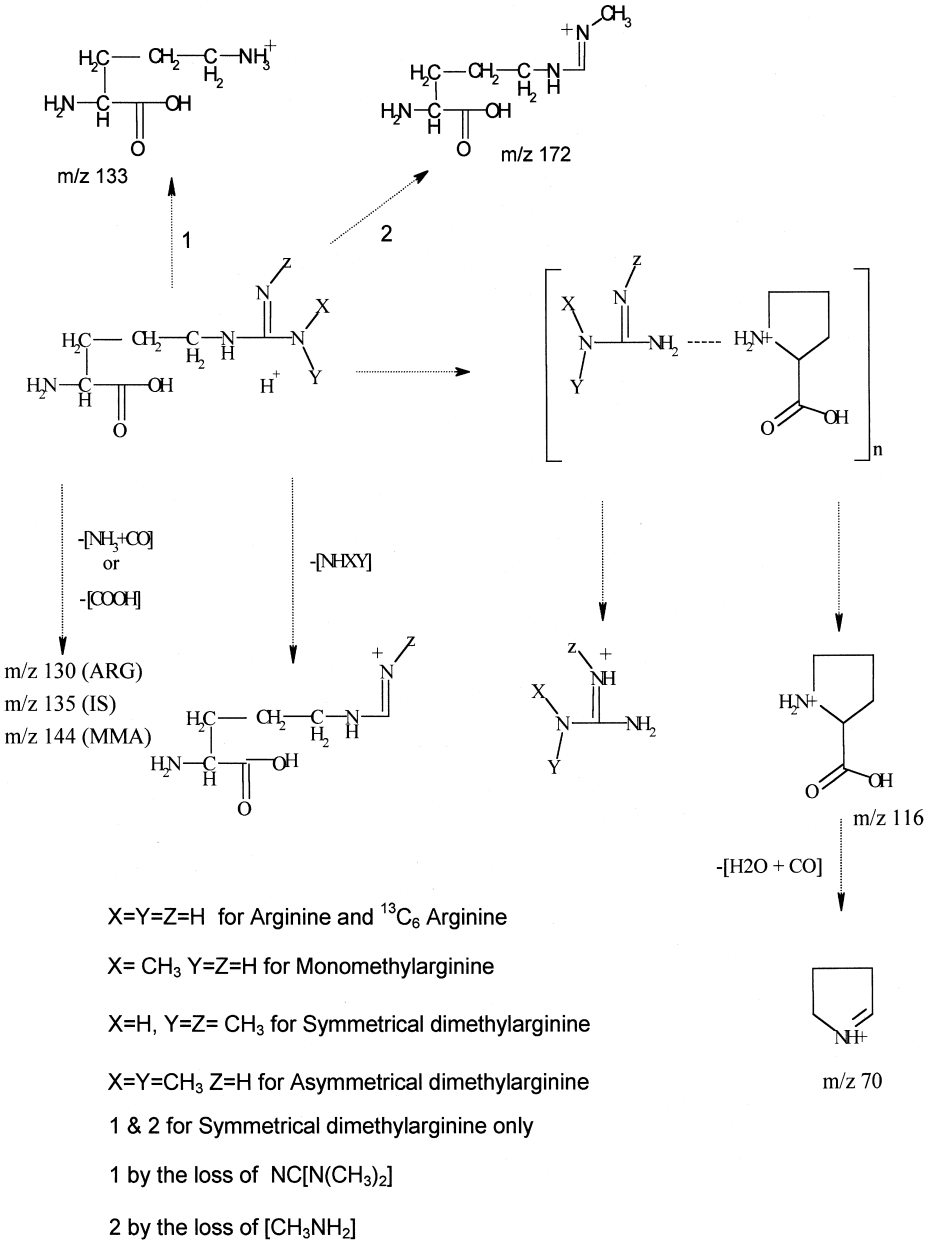

Fig. 4. Fragmentation mechanisms of arginine and the methylated arginines studied.

reported here is more sensitive. The LOD was explored by reducing the concentration of standard solutions gradually. To avoid interference from previous injections, a pure water blank was injected to ensure that the system was clean prior to the next injection. The LODs for ADMA, SDMA, ARG was found to be 1 ng/ml and 2.5 ng/ml for MMA with a signal-to-noise ratio of 3. The limit of quantitation of this study is 10 ng/ml for ADMA, SDMA and MMA and 1000 ng/ml for ARG.

3.4. Significance of the results

Concentrations of ARG, ADMA, SDMA and MMA in plasma were calculated using the calibration curves. The average concentrations of ARG, ADMA, SDMA and MMA were found to be 10.9±4.1 μg/ml, 25.1±9.4 ng/ml, 33.2±13.1 ng/ml and 19.6±3.8 ng/ml. The concentrations are tabulated in Table 5.

The concentration range reported for ARG varied from 8.7 to 22.0 μg/ml [20,27,28]. The average concentration reported for MMA was 19.0±1.9 ng/ml [20]. The values for total dimethylarginines (ADMA and SDMA) varied from 60.6 to 240 ng/ml [20,21,25]. The values reported here for ARG, ADMA, SDMA and MMA are well within the average values reported earlier. The values reported for ADMA and SDMA are somewhat lower than previously reported values but are consistent with the ratios of ADMA:SDMA which is approximately 0.76 (range 0.60–1.35). The high standard deviations (SDs) associated with the measurement of these compounds indicate the possibility that concentrations of ARG and methylated arginines may be useful as diagnostic markers for the progression of heart diseases. However, a greater sample population with subsequent correlation to patient disease state will be necessary before any detailed clinical studies can be made.

4. Conclusion

The clinical relevance of the L-arginine/nitric oxide pathway in vascular disease has been demonstrated in numerous studies. Alterations in the synthesis of nitric oxide have been demonstrated in the pathophysiology of several cardiovascular diseases including atherosclerosis, hypertension and diabetes. Thus, it is of great interest to determine mechanisms responsible for alterations in the synthesis of nitric oxide. Recently, ADMA has been determined to be an endogenous inhibitor of the enzyme nitric oxide synthase, which is responsible for the synthesis of

Table 5
The concentrations of ARG, ADMA, SDMA and MMA from healthy volunteers

Subject	Concentration of (ng/ml):			
	ARG	ADMA	SDMA	MMA
1	5872.6	40.1	38.5	19.6
2	14 033.4	47.6	32.0	20.2
3	11 074.6	26.7	33.0	20.3
4	5765.4	34.4	39.4	19.1
5	10 990.6	29.1	45.9	21.8
6	19 582.1	27.2	53.3	19.3
7	16 275.7	25.9	46.2	19.9
8	9762.2	37.2	58.3	20.1
9	9281.4	28.5	40.4	20.0
10	9532.8	24.9	42.1	19.4
11	8221.6	17.9	36.8	16.6
12	8395.9	13.4	32.9	17.2
13	8528.9	18.8	33.4	17.3
14	11 339.0	11.6	12.5	18.3
15	13 227.1	25.6	26.6	16.5
16	10 281.0	21.6	23.4	17.0
17	7631.8	15.1	15.8	16.4
18	18 289.4	17.9	13.3	34.3
19	4202.2	17.2	16.3	17.8
20	15 803.8	20.3	22.8	20.8
Mean±SD	10 904.6±4172.6	25.0±9.4	33.1±13.1	19.6±3.8

nitric oxide from ARG. Increases in the generation of ADMA and/or MMA or a decrease in the concentration of ARG might result in a reduction in the synthesis of nitric oxide with a subsequent reduction in vasodilation. By simultaneously determining ARG and ADMA using this method, one can determine whether the cause of decreased vasodilation is due to altered substrate concentration or due to increased production of ADMA and MMA. Moreover, this method may also be useful for evaluating therapeutic regimens, which could influence nitric oxide production by altering the concentrations of ADMA and MMA.

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