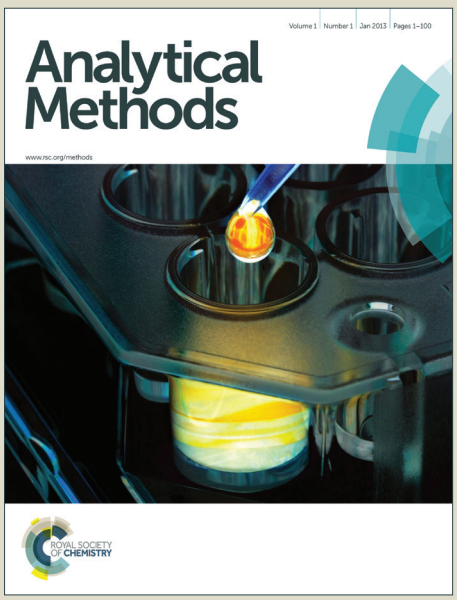
Analytical Methods

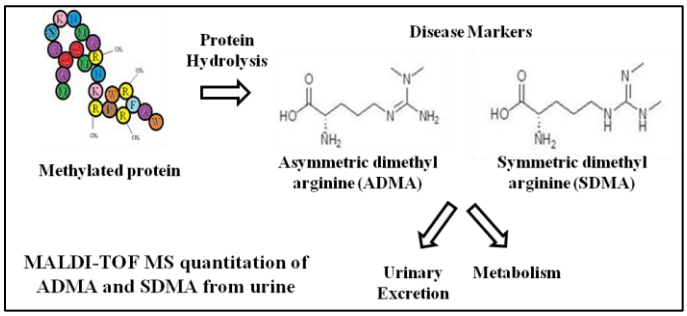
Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods

Graphical and textual abstract



Free dimethylarginines, ADMA and SDMA are released from methylated proteins following protein hydrolysis and serve as disease markers. These are either metabolized or cleared via renal excretion. MALDI-TOF MS quantitation of the free dimethylarginines endogenously present in urine is demonstrated in this work.

Matrix-assisted laser desorption/ionization mass spectrometry analysis of dimethyl arginine isomers from urine[$]

Nivedita Bhattacharya,[†#] Ajeet Singh,[†#§] Avinash Ghanate,[†#] Gayatri Phadke,[†] Dharmesh Parmar,[†#] Deepika Dhaware,[†#] Trayambak Basak,[‡#] Shantanu Sengupta[‡#] and Venkateswarlu Panchagnula[*†#§]

[†]CSIR - National Chemical Laboratory, Dr. Homi Bhabha Rd, Pune – 411 008 India

[‡] CSIR - Institute of Genomics and Integrative Biology, Mathura Road, New Delhi – 110 020 India

[#]Academy of Scientific and Innovative Research, 2 Rafi Marg, Anusandhan Bhavan, New Delhi – 110 001 India

[§] Center for Applications of Mass Spectrometry, 100 NCL Innovation Park, Dr. Homi Bhabha Rd, Pune – 411 008 India

*Corresponding author: Phone: +91 20 2590 2194; Fax: +91 20 2590 2621;

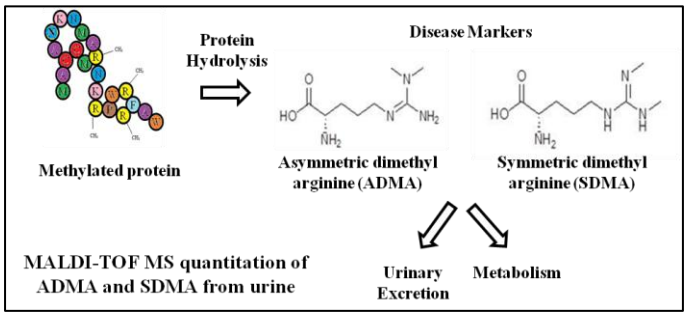
Email address: v.panchagnula@ncl.res.in

[$] Electronic supplementary information available

Abstract

Isomeric asymmetric and symmetric dimethyl arginine (ADMA and SDMA respectively) residues are excreted in urine and are putative markers of cardiovascular and chronic kidney diseases. In this work, we demonstrate simultaneous and quantitative detection of endogeneous ADMA and SDMA from urine samples of healthy subjects using MALDI-TOF MS without any chromatographic separation. The DMA isomers yielded $[M+H]^+$ ions along with their product ions formed due to MALDI in-source fragmentation. The precursor ions were validated using MALDI-TOF MS/MS as well as direct injection ESI-Q-TOF MS/MS. ADMA and SDMA generated unique product ions at ~*m/z* 46 and ~*m/z* 172 respectively in the MS-mode itself. These were advantageously used for full scan-mode absolute quantification of the isomeric metabolites. The *m/z* observed for all the ions was within 10 ppm mass accuracy. The calibration method was established by generating internal standard normalized peak area-based concentration response curves using synthetic standards. Good linearities ($R^2 > 0.95$) with acceptable intra-assay, inter-assay variation (within 15% RSD) and excellent recoveries were observed for quality control samples. Finally, endogeneous concentrations of the metabolites were determined in urine from healthy subjects (*n*=11). ADMA and SDMA were found to be in the range of 1.6-8 µM and 2.9-9.1 µM in urine and were in agreement with previously reported physiological levels.

Graphical and textual abstract



Free dimethylarginines, ADMA and SDMA are released from methylated proteins following protein hydrolysis and serve as disease markers. These are either metabolized or cleared via renal excretion. MALDI-TOF MS quantitation of the free dimethylarginines endogenously present in urine is demonstrated in this work.

Introduction

Dimethylarginines (DMA) are formed by methylation of arginine residues in proteins by enzymatic action of the protein arginine methyl transferases (PRMT). Depending on the class of PRMTs involved, asymmetric and symmetric dimethylarginines (ADMA and SDMA respectively) are generated.[1, 2] These isomeric residues are hydrolyzed from the methylated proteins and circulate freely in blood. Several underlying molecular mechanisms further aid metabolism and urinary elimination of ADMA and SDMA from the body.[3, 4] ADMA is an inhibitor of nitric oxide synthase (NOS). Several clinical studies have directly linked elevated levels of ADMA to endothelial dysfunction and cardiovascular disease (CVD) in general,[5-8] which is possibly the largest cause of death worldwide. It is also known to influence progression of chronic kidney disorders (CKD)[9] and serves as a possible link between CVD and CKD.[10] Independently, elevated SDMA level has been reported to serve as a better marker than creatinine for decreased glomerular filtration rate (GFR) and is indicative of atypical renal function in early stages.[11] Increased ADMA catabolism index (ADMA/SDMA) has also been reported to be an indicator of sepsis in critically ill patients.[12]

Simultaneous screening of SDMA and ADMA is challenging due to their isomeric nature, low abundance and short half lives in blood plasma.[13] Several methods are currently in use for detecting ADMA and SDMA from biological fluids, but come with limitations.[14] Immunoassay-based methods used for the detection of ADMA cannot estimate SDMA from a sample containing both.[15] The DMA isomers can also be analyzed using HPLC based on retention times alone, and LC-ESI-MS that use precursor to product ion reaction monitoring schemes.[8, 16-20] These approaches invariably involve a chromatographic dimension that limits the throughput of the analysis. Although sensitive detection is achieved with unit-resolution triple quadrupole analyzers typically used for quantitation, the analysis might also be prone to contributions from interfering ions arising from a complex biological matrix.[21, 22] A

significant need for comprehensive, high throughput, and efficient analytical methods continues to exist for translating preliminary clinical findings on DMAs as metabolite disease markers into diagnostics, and for further enabling studies exploring therapeutic routes to treatment.[23]

MALDI-TOF MS offers significant advantages over other currently practiced analytical methods used in metabolite detection. Most importantly, it provides a high throughput that can be crucial to handling large sample sizes. MALDI platforms are simpler to handle and maintain. They are also robust, have a greater tolerance to impurities and involve minimal-to-no sample preparation. Several reports have demonstrated that the limitations associated with matrix interference and desorption ionization processes can be overcome for targeted analysis.[24-35] Tandem MS capabilities have become routine adding significantly to accurate detection. Quantitation using high resolution TOF analyzers offers precise detection based on accurate mass.[21, 36] MALDI MS detection of DMAs has been reported earlier. However, they have either been studied from peptides[37] or as free DMAs derivatized with 6-aminoquinolyl carbamoyl (AccQ) tags.[38]

In this work, we present a quantitative MALDI-TOF MS method for the determination of the endogeneous DMA isomers from urine of healthy human subjects. This method differentiates and quantifies ADMA and SDMA on the basis of unique fragment or product ions arising due to in-source decay (ISD) in MALDI-TOF MS. DMAs were first discovered in urine[39] and furthermore, several reports implied urinary DMAs for causing various diseases.[40-42] To our knowledge, simultaneous MALDI-TOF MS quantitation of free underivatized DMAs from biological fluids has not yet been reported.

Experimental

Reagents and Materials Ultrapure 2,5-dihydroxybenzoic acid (2,5-DHB), NG,NG'-dimethyl L-arginine di(p-hydroxyazobenzene-p'-sulphonate) salt (SDMA), NG,NG-dimethyl arginine hydrochloride (ADMA), potassium chloride, sodium chloride, urea, citric acid, potassium phosphate, creatinine, sodium hydroxide, sodium bicarbonate, LC-MS grade acetonitrile, methanol and trifluoroacetic acid (TFA) were purchased from Sigma Aldrich. Melamine (2,4,6-triamino-1,3,5-triazine) and ascorbic acid was purchased from Loba Chemie (India). Sulfuric acid was purchased from Merck. Deionised water with specific resistivity 18.2 MΩ cm^{-1} was collected from SG ultrapure water unit (Germany).

MALDI-TOF MS analysis and sample preparation Qualitative and quantitative analysis was performed on Waters Synapt HDMS equipped with a MALDI ionization source, and a hybrid quadrupole-orthogonal acceleration (oa) TOF analyzer. The instrument was operated in reflectron 'V' positive ion mode. The pressures at source and TOF were maintained at 8.5 e^{-2} mbar and 7.4 e^{-7} mbar. The TOF and reflectron voltages were maintained at 9.1 and 2.1 kV respectively. The detector voltage was set at 1750 V after detector sensitivity test. Detector sensitivity was optimized by standardizing the ion counts of standard Glu-1-fibrinopeptide B (Waters) as recommended by the manufacturer. Temperature of the room was maintained at 22°C. Quadrupole filters were set to pass ions between 20 - 600 *m/z*. All mass spectra were acquired with optimized laser energy (solid state laser, 355 nm). Laser has following specifications: repetition rate - 200 Hz, average power – 20 mW, pulse width – 3 ns, pulse energy – 100 µJ. Prior to acquisition, the instrument was calibrated with PEG (mixture of PEG 200, 600 and 1000) to obtain rms mass accuracy within 5 ppm. Before starting any analysis, routine calibration check was performed. For sample spotting, standard 96-well stainless steel MALDI target plates were used after rigorous cleaning as per manufacturers' instructions. A calibration in MS/MS mode was also performed to achieve

exact masses of all fragmented ions. To avoid interferences, the precursor ion selection window was adjusted to select precursor ion within a Dalton. The quadrupole was set according to precursor mass to achieve maximum sensitivity and nitrogen gas was used in the collision cell. Following instrumental parameters were used during the analysis: laser energy – 300 units, trap collision energy – 6 eV, transfer collision energy – 4 eV. Direct injection ESI-MS analysis was carried out on Agilent 6540 Ultramass high definition accurate mass Q-ToF instrument was used for all direct injection electrospray related analysis. 2 µL of sample was injected directly into the source. The *m/z* range selected for ESI-MS analysis was *m/z* 40-210. Following instrumental parameters were used during analysis: gas temperature – 350 ° C, gas flow – 5 L/min, fragmentor voltage - 180 V.

For quantitation experiments, an automated batch mode with optimized instrumental parameters was used. The term 'fragment ion(s)' has been used interchangeably to describe the MS mode 'product ion(s)' formed from the in source decay of the $[M+H]^+$ ion in the text.

The method for quantitative analysis from urine was adapted from previously reported methods wherein the calibrations from synthetic metabolite standards were used to determine unknown concentrations of metabolites in urine.[43-46] 100 µM stock solutions of ADMA and SDMA were prepared in acetonitrile: 0.1% TFA (1:1, v/v) and stored at -20°C. 100 µL aliquots were dispensed in separate tubes. A maximum of three freeze thaw cycles were allowed. Dilutions series for the standard calibrator solutions with final concentrations of 0.6, 0.8, 1, 1.5, 2, 2.5, 5, 7.5 and 10 µM, and quality control (QC) samples (1.25, 4 and 8 µM) were prepared separately from the stock solutions and used for quantitation. Due to absence of an endogenous analyte free matrix (in this case urine), synthetic urine was used to generate calibration curves closely resembling urine. Synthetic urine was prepared by mixing the reagents as mentioned in a published protocol and stirring the mixture for 1 hour. [47] Synthetic urine was spiked with the calibrator and QC solutions in equal amounts such that the final

concentration range is similar to the standard calibrator range. In addition, a higher concentration range of analytes were prepared having a final concentration range of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µM along with three different QC samples (25, 55 and 75 µM) in solvent system.

2,5-DHB (20 mg/mL) was prepared in acetonitrile: 0.1%TFA (1:1, v/v) and used as MALDI matrix. 1µL of matrix was spotted on individual wells of MALDI target plate and dried in air. Melamine was used as an internal standard and the final concentration was optimized at 5.3 µM. Analyte samples premixed with internal standard were spotted on to the matrix layer. Absolute quantitation using a chemically dissimilar synthetic internal standard made comparison across spectra possible by removing systematic errors. This is also possibly a viable alternative to using isotopic labels that are costly and often not easily available.

Urine samples Urine samples were collected from eleven healthy volunteers with the prior approval of ethics committee of the Institute of Genomics and Integrative Biology (Institutional Human Ethics Committee, IHEC). Informed consent was obtained from the volunteers prior to collection. Collected urine was mixed with equal volume of methanol to remove any residual proteins and centrifuged at 13,200 rpm for 15 minutes at 4°C. The obtained supernatant was premixed with a non endogenous internal standard (melamine) for reference in absolute quantitation and spotted with 2, 5-DHB for subsequent MALDI-TOF MS analysis. A pooled urine sample from eleven healthy volunteers was prepared. The pooled sample was spiked with the standard QC samples (1.25 and 4 µM) and processed in similar way as the individual urine samples.

Data analysis Qualitative data analysis was performed with Masslynx 4.0 (Waters) and mMass.[48] Absolute and relative data analysis for quantitation was carried out using a homebuilt software tool 'MQ'. MQ was built by understanding the need for a high-throughput tool complementing MALDI-TOF MS with at par efficiencies. It offers a user-

friendly interface for both targeted and non-targeted analysis of MS data in high-throughput manner regardless of the instrumental source, with a spectrum of parameters to choose from. Before data analysis, the raw data (instrumental files) were first converted to mzXML files using massWolf (command line binary made available by SPCTools). The mzXML files were then converted to ascii format using a small module 'mzXML2txt' developed in-house. The time required in this conversion is roughly 15 minutes for a batch of 100 files. In MQ, an alternative peak intensity field is also present. There are two aspects that the algorithm separately caters to. Firstly, peaks are generated from the raw mass spectral data for which the polynomial fitting is used. Quantitation is done separately with either the whole peak or only a portion of the whole peak depending on the resolution of the data. For the peak generation, a set of points defined by a mass extraction window (MEW) flanking the analyte peak were log transformed and a second order polynomial was fit to these log transformed spectral points in order to capture the Gaussian characteristics. This will address any discrepancies introduced by skewed or irregular peak shapes. For quantitation, estimations are usually robust provided a narrow enough MEW is chosen along with high resolution m/z data (as is the case in the present work). This method might introduce errors in peak area estimation when used with low resolution m/z data or when a large MEW is used.

For analysis with Synapt data, a ppm window of 50 was provided. Relative quantitation returns normalized values of analytes with internal standard. Software generated regression coefficients based on unweighted univariate linear regression fitting method and the calibration curves were plotted for absolute quantitation. The time required for a batch analysis required 20 minutes from generating calibration curve to estimating unknowns. Relative quantitation was done with normalization of the analyte peaks of interest with internal standard within chosen ppm error (50 ppm). For more information about the software and link for downloading the utility, please visit http://www.ldi-ms.com.

Results and Discussion

Fig. 1(A) depicts the MALDI-TOF MS representative spectra from the pooled urine sample with methanol analyzed directly. In this spectra, $[M+H]^+$ peak for the isomeric ADMA and SDMA was observed at *m/z* 203.1499 (within 10 ppm) along with the other ensuing product ions in MALDI MS mode. The protonated adduct ions ($[M+H]^+$) and the corresponding *m/z* values observed in the MS mode were consistent with previously published literature for the analytes.[37, 49] These results were also consistent with data from synthetic standards (Supplementary figure 1(A)). Importantly, none of the known peaks from 2,5-DHB interfered with the analyte peaks of interest either in the standards or the urine sample (Fig. 1(A) and Supplementary figure 1(A)). The structures of the DMA isomers, along with their unique product ions are illustrated in supplementary figure 2. Peaks at *m/z* 70.0652, 88.0763, 158.1255 formed due to in source dissociation of the $[M+H]^+$ ion and are common to both were observed in the spectra in the MS mode. Significantly, the unique product ion peaks at *m/z* 46.0648 and 172.1082 (mass accuracies within 10 ppm) corresponding to ADMA and SDMA respectively were also observed in the MS mode itself. These unique fragment ions provide an independent way for confirming the specific DMA isomers in a mixture, which is not possible based on the precursor ion alone.

MALDI-TOF MS/MS results of the endogenous isomers from a representative urine sample are shown in Table 1. These are in agreement with previously reported results in published literature,[37, 49] and were also validated individually with the synthetic standards (Supplementary figure 3(A-B)). Mass accuracies (MA) of all the product ions were within 10 ppm. Furthermore, pseudo-MS^3 was performed on the unique fragment ion of SDMA at *m/z* 172.1087 as shown in Supplementary figure 3(C). The resulting product ions correspond to those observed for SDMA precursor ion as well. The tandem MS data thus conclusively ascertains the identities of the metabolite precursor ion detected at *m/z* 203 while the unique

fragment ions at *m/z* 172 and 46 confirm the presence of both the isomers endogenously in the urine samples. Urine samples were also subjected to ESI-Q-TOF MS analysis to qualitatively verify the MALDI-TOF MS data. The resulting MS and MS/MS spectra resulting from CID fragmentation of the precursor ion show the presence of both the metabolites in urine validating the MALDI-TOF results (Supplementary fig. 4).

Limit of detection (LOD) and quantitation (LOQ) were estimated from the standards for all the ions observed in MS mode. S/N ratio was considered as 5 and 10 for LOD and LOQ estimation respectively. A previously reported method was used for the estimation of spectral baseline and noise. [50] Briefly, spectral intensity was plotted as a function of the frequency of occurrence of the specific intensity or below in the proximity of the peak of interest. FWHM for the derivative of this plot was defined as noise while the maximum value or 'highest point density' was taken as the baseline. [50] The baseline value was subtracted from the analyte peak intensity for the S/N estimation. The LOD and LOQ values outlined in Table 2 were averaged for multiple replicates (~18). Unique fragment ion for ADMA at *m/z* 46.0657 showed LOD and LOQ at 0.8 and 1 µM respectively from the solvent system. In synthetic urine, the LOD and LOQ were slightly higher at 1 and 1.25 µM respectively. The unique fragment ion of SDMA at 172.1097 exhibited 0.1 and 0.2 µM as LOD and LOQ, respectively from the solvent system while the LOD/LOQ values in synthetic urine were 0.25 and 0.37 µM. These results were reproducibly observed indicating that the MS mode unique fragment ions are indeed useful for unambiguous detection at trace levels occurring in physiologically relevant concentrations.

Concentration response curves generated from standards of SDMA and ADMA were used for the determination of the metabolite levels from the urine samples for absolute quantitation. Peak areas of the resolved unique fragment ion peaks within a specified MEW were considered for generating the calibration curves as well as determining the levels of ADMA

and SDMA in the urine samples. Fig. 1(B-D) shows the zoomed insets with highlighted peak areas in gray used for quantitation from urine samples. Supplementary figure 1 (B-D) shows the corresponding data from standards. Peak shapes of the ions in context were comparable for standards and urine. Potentially interfering peaks from urine were observed within close proximity. These were nevertheless well resolved from the analyte peaks of interest (Supplementary figure 5(A-B)). As the full scan spectrum contains information for all the product ions as well as the precursor ion, calibration curves for all these ions, common and unique, to ADMA and SDMA were generated (see also Supplementary table 1). Concentration ranges were selected above the LOQ values. The calibration curves were obtained in separate sets over a period of three days and the data generated is summarized in Table 2 for the unique fragment ions. Excellent linearity with correlation coefficient $R^2 > 0.9$ was obtained with reproducible slopes and intercepts for the calibrants both in solvent system and synthetic urine. QC samples yielded recoveries in the range of 84 – 114 % from both the solvent and the synthetic urine systems.(Table 3). Reproducibility of the method was further tested for inter and intra assay variability (Table 3). The %RSD was well within or close to 15% in all these cases with the exception of lowest concentrations of ADMA and SDMA from synthetic urine. Calibration curves in the higher concentration ranges were also estimated along with recoveries as well as inter and intra-assay variations (Supplementary Table 2). The results demonstrate acceptable linearity, QC recoveries and precision. Thus, absolute quantitation was found to be analytically robust exhibiting the required precision and accuracy in both the pure solvent system as well as the synthetic urine matrix (negative control).

Pooled urine sample serves as a positive biological control. Validation using standard addition of known amount of analyte in urine for use as QC samples allows estimation of variations induced by the biological matrix and ion suppression effects, if any.

Concentrations of ADMA and SDMA in pooled urine samples from 11 subjects were first estimated. To this, known amounts of ADMA and SDMA at two concentrations levels were added and used as QC samples. Calibrations from analytes in both solvent system as well as synthetic urine were used to estimate the QC sample recoveries, intra-assay and inter-assay variation. The results obtained across three days are outlined in Table 4. Recoveries in the range of 84 – 133% were obtained. Inter and intra-assay variation were mostly within 15% RSD. These results indicate acceptable accuracy and precision of the method establishing the validity of the method.

Supplementary Table 3 further elaborates the analysis conditions as suggested for high resolution mass spectral quantitation in a recently published article.[22] The data is reported for three different sets analyzed on different days. The mean mass accuracy (MA) for all the *m/z* peaks under consideration was within 10 ppm. Mass accuracy precision (MAP) defined as the mean of standard deviation of MA in different sets was within 6 ppm for all the ions. Mass accuracy variability (MAV), a measure of the instrumental variation over several days estimated by repeating the manufacturer's calibration procedure, was found to be within 5 ppm. Mass accuracy acceptance criterion (MA-AC) defines the acceptable upper and lower limits for MA and was estimated as reported in previous literature.[22] MA-$AC_{standard}$ is based on the MAV and is thus, instrument dependent whereas, MA-$AC_{intraassay}$ relies on the MAP and is analysis specific. For all the peaks of interest, MA-$AC_{standard}$ was in the range of 4-14 ppm whereas the MA-$AC_{intraassay}$ was observed to be above 20 ppm for *m/z* 172.1081, within 20 ppm for *m/z* 46.0651. MEW for quantitation can be categorized into narrow and broad depending upon the MA.[22] Standard MEW is dependent on the MAV, narrowest MEW on the MAP and the broadest MEW on the maximum resolution offered by the instrument. $MEW_{standard}$ for all the peaks were within 15 ppm; the $MEW_{narrowest}$ for *m/z* 46.0651 and 172.1081 was found to be 19.9 and 31.8 ppm respectively. The $MEW_{broadest}$ for all the peaks

were observed to be within 115 ppm and was calculated taking into account maximum possible resolution of the instrument used as 10,000 FWHM. The MEW used for peak area-based quantitation was optimally chosen to be 50 ppm. These results specify the selection as well as the reproducibility of the MA parameters and overall robustness of the high resolution quantitation.

ADMA and SDMA in the urine samples were estimated based on the unique fragment ions with calibrations described above. Fig. 2 presents a box-whisker plot showing five-point graphical distribution of ADMA and SDMA in urine from eleven healthy subjects based on the calibration curves obtained with standards. The observed minimum values for ADMA and SDMA are in the order of 1.6 and 2.9 μM respectively. The observed maximum values for ADMA and SDMA are approximately 8.0 and 9.1 μM respectively. 1^{st}, 3^{rd} quartiles and median are significant as the area between them determines the spread of data across subjects. For ADMA, the observed data (box bordered by 1^{st} and 3^{rd} quartile) spans across a narrow range of 2-3.5 μM whereas, for SDMA the range is spread across 3.5-6 μM. Most importantly, these results indicate that the observed values in urine are similar to those previously reported in literature for normal subjects[39, 51, 52] and conclusively demonstrate the ability of MALDI-TOF MS for quantitation of the DMA isomers from urine samples.

Conclusions

Urine as a biological matrix offers non-invasive sampling and ample sample volume. It also provides a metabolic snapshot of important organs and has the scope for prognostic follow-up.[52] The quantitative MALDI-TOF MS method described herein used minimal urine sample, almost no sample pre-processing, involved no chromatographic separation and offered a high throughput analysis. Furthermore, both the DMA isomers were detected and quantified on a

single analytical platform and in a single analysis. In source fragmentation in MALDI-TOF MS is generally considered a limitation for quantitation. This work illustrates the applicability of a quantitative method that advantageously uses such product ion peak areas. It presents advancement over currently used methods for metabolite analysis with comparable analytical precision and accuracy.[8, 17, 20] In urine, ADMA and SDMA are present in the low micromolar range[39, 51, 52] and are known to increase in diseased conditions and drug abuse.[40-42, 53] The endogenously detected amounts from urine of healthy human subjects in this study are in agreement with physiologically known levels highlighting the potential applicability of the method in clinical analysis. In cases of senile patients or infants, this method can be first used for screening and quantitation of these metabolites and subsequent invasive sampling in other biological fluids.

Acknowledgements

This research was funded by CSIR, India through NCL-IGIB joint research initiative, TFYP CMET project CSC0110 and BIRAC, India. VP gratefully acknowledges the financial support from Department of Biotechnology (India) young investigator award (RGYI grant 102/IFD/SAN/PR-2062/2009-2010) and Ramalingaswamy fellowship along with the NCL intramural grants for the research infrastructure.

List of tables

Table 1. Product ions from collision induced dissociation (CID) of precursor ion from urine samples [37, 49]

	Product ions	***m/z***
ADMA	$[M+H-COOH]^+$	158.1300
	$[(CH_3NH)_2C=NH_2]^+$	88.0841
	$[CH_2CH_2CH_2CH=NH]^+$	70.0638
	$[(CH_3)_2NH_2]^+$	46.0630
SDMA	$[M+H-CH_3NH_2]^+$	172.1108
	$[M+H-COOH]^+$	158.1300
	$[M+H-(CH_3NH)_2C=NH_2]^+$	116.0869
	$[(CH_3NH)_2C=NH_2]^+$	88.0841
	$[(CH_3)N=C=NH(CH_3)]^+$	70.0638

Table 2. Calibration statistics of ADMA (*m/z* 46.0670) and SDMA (*m/z* 172.1090) unique fragment ions analyzed using MALDI-TOF MS for a period of three days (n=6 for each calibrator)

Analyte	Calibration matrix and range (in µM)	Detection limits† (in µM)	Equation; R^2 (for three different days)
ADMA	Solvent system* 1-10	LOD – 0.80 LOQ – 1.00	y=0.003x+0.002; 0.99 y=0.003x+0.004; 0.95 y=0.004x+0.002; 0.92
	Synthetic Urine 1.5-10	LOD – 1.00 LOQ – 1.25	y=0.005x+0.008; 0.93 y=0.002x+0.003; 0.98 y=0.004x+0.002; 0.99
SDMA	Solvent system* 0.6-10	LOD – 0.10 LOQ – 0.20	y=0.321x+0.301;0.98 y=0.375x+0.336; 0.94 y=0.343x+0.321;0.92
	Synthetic Urine 0.6-10	LOD – 0.10 LOQ – 0.25	y=0.561x-0.136; 0.91 y=0.193x+0.334; 0.96 y=0.422x+0.679;0.99

Note: * Acetonitrile : 0.1% TFA in Water (50:50); † n= 18

Table 3. Recoveries, intra-assay and inter-assay variation for unique fragment ions of ADMA (*m/z* 46.0670) and SDMA (*m/z* 172.1090) in QC samples across three different days (n=6 on each occasion)

Analyte	Calibration matrix and range (in µM)	QC samples (µM)	% Recovery	Intra-assay variation (%RSD)	Inter-assay variation (%RSD)
ADMA	Solvent system* 1-10	1.25	109	14.2	9.0
		4	99	12.2	13.1
		8	84	13.1	12.0
	Synthetic urine 1.5-10	1.25	99	12.1	19.2
		4	114	7.4	14.8
		8	84	10.3	10.4
SDMA	Solvent system* 0.6-10	1.25	107	10.6	4.8
		4	103	11.8	5.9
		8	88	10.8	6.8
	Synthetic urine 0.6-10	1.25	96	6.3	19.2
		4	96	9.8	13.6
		8	85	8.9	1.5

Note: * Acetonitrile : 0.1% TFA in Water (50:50)

Table 4. Recoveries, intra-assay and inter-assay variation for unique fragment ions of ADMA (*m/z* 46.0670) and SDMA (*m/z* 172.1090) from pooled urine samples with known amounts of the analytes added and used as QC samples. Inter-assay variation was spread across three different days (n=4 (minimum) on each occasion).

Analyte	Calibration matrix and range (in µM)	QC samples† spiked in pooled urine (µM)	% Recovery	Intra-assay variation (%RSD)	Inter-assay variation (%RSD)
ADMA†	Solvent system* 1-10	1.25	133	7.0	3.6
		4	101	13.6	6.8
	Synthetic urine 1.5-10	1.25	102	7.9	11.4
		4	108	9.1	11.9
SDMA†	Solvent system* 0.6-10	1.25	121	14.5	16.6
		4	84	12.5	14.6
	Synthetic urine 0.6-10	1.25	119	9.5	11.7
		4	99	13.7	12.5

Note: † Values represent only the known amounts added. ADMA and SDMA were determined to be present at 5.8µM and 14.1µM in pooled urine endogenously. Together, the final analyte concentrations fit within the calibration range; * Acetonitrile : 0.1% TFA in Water (50:50)

Figure Captions

Fig. 1. (A) MALDI-TOF MS spectrum of pooled urine sample from 11 healthy human subjects in the range *m/z* 20-210. Annotated peaks represent the detectable ions for ADMA and SDMA along with the melamine peak at *m/z* 127.0729 and creatinine peak at *m/z* 114.0661. (B-D) depicts the zoomed insets with area under curve considered for quantitation highlighted in gray for (B) the protonated adduct ion $[M+H]^+$ of ADMA and SDMA at *m/z* 203.1499, (C) the unique fragment ion at *m/z* 46.0648 for ADMA and (D) the unique fragment at *m/z* 172.1082 for SDMA.

Fig. 2. Box whisker plot representing quartile distribution of ADMA and SDMA in urine of eleven healthy human subjects. ADMA and SDMA are present in the range of 1.6-8 µM and 2.9-9.1 µM respectively. Quartile distribution shows the majority of distribution (area in box bordered by 1^{st} and 3^{rd} quartiles). Majority distribution of the analytes is in the range of 2-3.5 µM and 4-6 µM for ADMA and SDMA respectively.

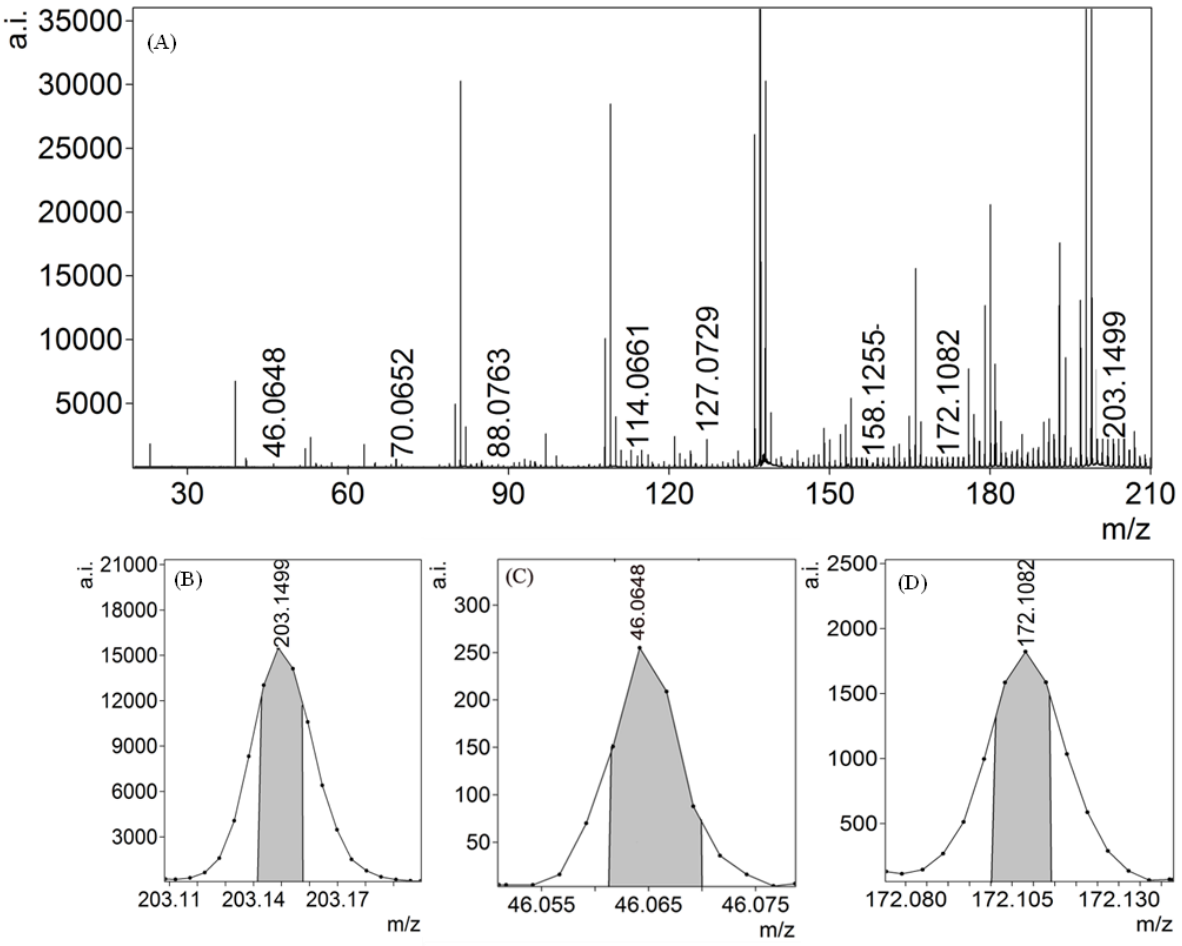


Fig. 1. (A) MALDI-TOF MS spectrum of pooled urine sample from 11 healthy human subjects in the range *m/z* 20-210. Annotated peaks represent the detectable ions for ADMA and SDMA along with the melamine peak at *m/z* 127.0729 and creatinine peak at *m/z* 114.0661. (B-D) depicts the zoomed insets with area under curve considered for quantitation highlighted in gray for (B) the protonated adduct ion $[M+H]^+$ of ADMA and SDMA at *m/z* 203.1499, (C) the unique fragment ion at *m/z* 46.0648 for ADMA and (D) the unique fragment at *m/z* 172.1082 for SDMA.

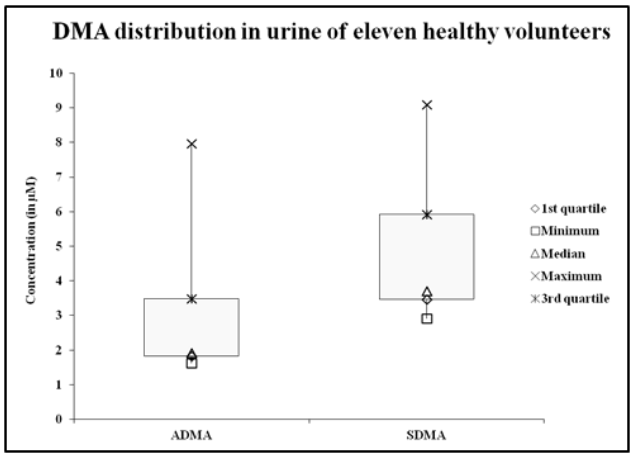


Fig. 2. Box whisker plot representing absolute quartile distribution of ADMA and SDMA in urine of eleven individual healthy human subjects. ADMA and SDMA are present in the range of 1.6-8 µM and 2.9-9.1 µM respectively. Quartile distribution shows the majority of distribution (area in box bordered by 1st and 3rd quartiles). Majority distribution of the analytes is in the range of 2-3.5 µM and 4-6 µM for ADMA and SDMA respectively.

References

1. M. T. Bedford and S. Richard, *Mol. Cell*, 2005, 18, 263-272.
2. M. T. Bedford and S. G. Clarke, *Mol. Cell*, 2009, 33, 1-13.
3. T. Teerlink, Z. M. Luo, F. Palm and C. S. Wilcox, *Pharmacol. Res.*, 2009, 60, 448-460.
4. A. J. Pope, K. Karuppiah and A. J. Cardounel, *Pharmacol. Res.*, 2009, 60, 461-465.
5. A. Meinitzer, U. Seelhorst, B. Wellnitz, G. Halwachs-Baumann, B. O. Boehm, B. R. Winkelmann and W. März, *Clin. Chem. (Washington, DC)*, 2007, 53, 273-283.
6. E. Schwedhelm, V. Xanthakis, R. Maas, L. M. Sullivan, F. Schulze, U. Riederer, R. A. Benndorf, R. H. Böger and R. S. Vasan, *Clin. Chem. (Washington, DC)*, 2009, 55, 1539-1545.
7. K. Sydow, S. P. Fortmann, J. M. Fair, A. Varady, M. A. Hlatky, A. S. Go, C. Iribarren, P. S. Tsao and A. I. for the, *Clin. Chem. (Washington, DC)*, 2009, 56, 111-120.
8. I. M. Di Gangi, L. Chiandetti, A. Gucciardi, V. Moret, M. Naturale and G. Giordano, *Anal. Chim. Acta.*, 2010, 677, 140-148.
9. J. T. Kielstein, R. H. Böger, S. M. Bode-Böger, J. C. Frolich, H. Haller, E. Ritz and D. Fliser, *J. Am. Soc. Nephrol.*, 2002, 13, 170-176.
10. S. Ueda, S. I. Yamagishi, Y. Kaida and S. Okuda, *Nephrology*, 2007, 12, 582-590.
11. J. T. Kielstein, H. Veldink, J. Martens-Lobenhoffer, H. Haller, M. Burg, J. M. Lorenzen, R. Lichtinghagen, S. M. Bode-Böger and V. Kliem, *Nephrol. Dial. Transpl.* , 2011, 26, 324-328.
12. G. Iapichino, M. Umbrello, M. Albicini, P. Spanu, G. Bellani, F. Polli, R. Pavlovic, M. Cugno, I. Fermo and R. Paroni, *Minerva Anestesiol.*, 2010, 76, 325-333.
13. J. T. Kielstein, D. Tsikas and D. Fliser, *Eur. J. Clin. Pharmacol.*, 2006, 62, 39-44.

14. E. Schwedhelm, *Vasc. Med.*, 2005, 10, S89-S95.

15. J. Martens-Lobenhoffer, S. Westphal, F. Awiszus, S. M. Bode-Böger and C. Luley, *Clin. Chem. (Washington, DC)*, 2005, 51, 2188-2189.

16. J. M. El-Khoury, D. R. Bunch, E. Reineks, R. Jackson, R. Steinle and S. H. Wang, *Anal. Bioanal. Chem.*, 2012, 402, 771-779.

17. C. M. Brown, J. O. Becker, P. M. Wise and A. N. Hoofnagle, *Clin. Chem. (Washington, DC)*, 2011, 57, 701-709.

18. H. Kirchherr and W. N. Kühn-Velten, *Clin. Chem. (Washington, DC)*, 2005, 51, 249-252.

19. E. Schwedhelm, J. Tan-Andresen, R. Maas, U. Riederer, F. Schulze and R. H. Böger, *Clin. Chem. (Washington, DC)*, 2005, 51, 1268-1271.

20. C. L. Gopu, P. R. Hari, R. George, S. Harikrishnan and K. Sreenivasan, *J. Chromatogr. B, : Anal. Technol. Biomed. Life Sci.* 2013, 939, 32-37.

21. F. Hernandez, J. V. Sancho, M. Ibanez, E. Abad, T. Portoles and L. Mattioli, *Anal. Bioanal. Chem.*, 2012, 403, 1251-1264.

22. B. Rochat, E. Kottelat and J. McMullen, *Bioanalysis*, 2012, 4, 2939-2958.

23. A. Meinitzer, J. T. Kielstein, S. Pilz, C. Drecencehsler, E. Ritz, B. O. Boehm, B. R. Winkelmann and W. März, *Clin. Chem. (Washington, DC)*, 2011, 57, 112-121.

24. P. J. Lee, W. Chen and J. C. Gebler, *Anal. Chem.*, 2004, 76, 4888-4893.

25. L. Sleno and D. A. Volmer, *Rapid. Commun. Mass. Sp.*, 2005, 19, 1928-1936.

26. L. Sleno and D. A. Volmer, *Anal. Chem.*, 2005, 77, 1509-1517.

27. S. Notari, C. Mancone, M. Tripodi, P. Narciso, M. Fasano and P. Ascenzi, *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.*, 2006, 833, 109-116.

28. L. Sleno and D. A. Volmer, *Rapid. Commun. Mass. Sp.*, 2006, 20, 1517-1524.

29. J. A. Campbell, D. S. Wunschel and C. E. Petersen, *Anal. Lett.*, 2007, 40, 3107-3118.

30. P. Kovarik, C. Grivet, E. Bourgogne and G. Hopfgartner, *Rapid. Commun. Mass. Sp.*, 2007, 21, 911-919.

31. S. Notari, C. Mancone, T. Alonzi, M. Tripodi, P. Narciso and P. Ascenzi, *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.*, 2008, 863, 249-257.

32. D. Yukihira, D. Miura, K. Saito, K. Takahashi and H. Wariishi, *Anal. Chem.*, 2010, 82, 4278-4282.

33. D. Miura, Y. Fujimura, H. Tachibana and H. Wariishi, *Anal. Chem.*, 2009, 82, 498-504.

34. M. Persike and M. Karas, *Rapid. Commun. Mass. Sp.*, 2009, 23, 3555-3562.

35. M. Persike, M. Zimmermann, J. Klein and M. Karas, *Anal. Chem.*, 2010, 82, 922-929.

36. J. L. H. Jiwan, P. Wallemacq and M. F. Herent, *Clin. Biochem.*, 2011, 44, 136-147.

37. P. M. Gehrig, P. E. Hunziker, S. Zahariev and S. Pongor, *J. Am. Soc. Mass. Spectrom.*, 2004, 15, 142-149.

38. T. Shirakawa, K. Kako, T. Shimada, Y. Nagashima, A. Nakamura, J. Ishida and A. Fukamizu, *Mol. Med. Report.*, 2011, 4, 615-620.

39. Y. Kakimoto and S. Akazawa, *J. Biol. Chem.*, 1970, 245, 5751-5758.

40. M. F. Lou, *Science*, 1979, 203, 668-670.

41. D. Wang, S. Strandgaard, M. L. Borresen, Z. M. Luo, S. G. Connors, Q. Yan and C. S. Wilcox, *Am. J. Kidney Dis.*, 2008, 51, 184-191.

42. C. Wolf, J. M. Lorenzen, S. Stein, D. Tsikas, S. Stork, F. Weidemann, G. Ertl, S. D. Anker, J. Bauersachs and T. Thum, *Int. J. Cardiol*, 2012, 156, 289-294.

43. M. Davids, E. Swieringa, F. Palm, D. E. C. Smith, Y. M. Smulders, P. G. Scheffer, H. J. Blom and T. Teerlink, *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.*, 2012, 900, 38-47.

44. S. Ertürk, S. M. Cetin and S. Atmaca, *J. Pharmaceut. Biomed.*, 2003, 33, 505-511.

45. K. Shrivas and D. K. Patel, *Spectrochim. Acta A*, 2011, 78, 253-257.

46. M. J. Silva, N. A. Malek, C. C. Hodge, J. A. Reidy, K. Kato, D. B. Barr, L. L. Needham and J. W. Brock, *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.*, 2003, 789, 393-404.

47. S. Y. Lee, E. Son, J. Y. Kang, H. S. Lee, M. K. Shin, H. S. Nam, S. Y. Kim, Y. M. Jang and G. S. Rhee, *B. Kor. Chem. Soc.*, 2013, 34, 1131-1136.

48. M. Strohalm, M. Hassman, B. Kosata and M. Kodicek, *Rapid Commun. Mass Sp.*, 2008, 22, 905-908.

49. S. A. Appolonova, M. A. Dikunets and G. M. Rodchenkov, *Russ. J. Phys. Chem. A*, 2009, 83, 520-529.

50. D. M. Horn, R. A. Zubarev and F. W. McLafferty, *J. Am. Soc. Mass Spectr.*, 2000, 11, 320-332.

51. J. Martens-Lobenhoffer and S. M. Bode-Böger, *Clin. Chem. (Washington, DC)*, 2006, 52, 488-493.

52. S. Bouatra, F. Aziat, R. Mandal, A. C. Guo, M. R. Wilson, C. Knox, T. C. Bjorndahl, R. Krishnamurthy, F. Saleem, P. Liu, Z. T. Dame, J. Poelzer, J. Huynh, F. S. Yallou, N. Psychogios, E. Dong, R. Bogumil, C. Roehring and D. S. Wishart, *Plos One*, 2013, 8, 1-28.

53. S. A. Appolonova, M. A. Dikunets and G. M. Rodchenkov, *Eur. J. Mass Spectrom.*, 2008, 14, 201-209.