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Simultaneous detection of arginine, asymmetric dimethylarginine, symmetric dimethylarginine and citrulline in human plasma and urine applying liquid chromatography–mass spectrometry with very straightforward sample preparation

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Abstract

Nitric oxide (NO) is synthesized by NO synthase from L-arginine, which can be competitively blocked by endogenous inhibitors such as asymmetric dimethylarginine (ADMA), but not by symmetric dimethylarginine (SDMA). ADMA is degraded by dimethylarginine dimethylaminohydrolase (DDAH) to dimethylamine and citrulline. A growing number of published clinical studies documented a strong correlation between increased ADMA blood levels and cardiovascular morbidity and mortality. We present here a highly sensitive method for the determination of this compounds in plasma and urine by means of HPLC–MS. The sample preparation is very simple and comprises only protein precipitation and concentration in the case of plasma samples and dilution in the case of urine. The samples are derivatized automatically with orthophthaldialdehyde and 2-mercaptoethanol, are separated on a 250 mm × 4 mm RP18 column by gradient elution with formate buffer/methanol and are detected by ESI-MS. The calibration functions are linear and cover the range from normal to pathologic concentration values of the analytes. The intra-day relative standard deviation (R.S.D.) of the assay for ADMA in plasma is 7.5% and the corresponding inter-day R.S.D. is 5.7%. In urine, these values for ADMA are 3.8 and 6.4%, respectively. All other analytes in plasma as well as in urine exhibit intra-day R.S.D. below 8%. The corresponding inter-day R.S.D. are all below 13%.
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1. Introduction

The amino acid arginine (ARG) is metabolized in vivo posttranslationally in proteins by a class of enzymes which are known as protein-arginine methyltransferases [1]. The main products of this methylation reactions are N^{G},N^{G}-dimethylarginine (asymmetrical dimethylarginine, ADMA) and N^{G},$N^{G'}$-dimethylarginine (symmetrical dimethylarginine, SDMA). These methylated species are liberated in the course of protein breakdown and turnover. Both ADMA and SDMA are eliminated by the kidney [2], and additionally, ADMA is converted by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) into citrulline (CIT) and dimethylamine [3]. Nitric oxide (NO) inhibits key processes of atherosclerosis, such as monocyte adhesion, platelet aggregation, and vascular smooth muscle cell proliferation [4]. NO is synthesized by stereospecific oxidation of the terminal guanidino nitrogen of L-arginine by NO synthase (NOS), which can be competitively blocked with guanidino-substituted analogs of L-arginine, such as ADMA. SDMA is inactive with respect to NOS but shares with ADMA and ARG the pathway for cell entry and may therefore indirectly influence the NO production rate [5]. In clinical studies, a strong correlation between increased ADMA blood levels and impaired endothelial-dependent vasodilatation, and cardiovascular morbidity and mortality has been documented in different populations [6–10]. In this context, it is highly desirable to measure the concentrations of ARG and its metabolites in human biological fluids with a highly specific and fast method, which allows a high sample throughput.

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There is a range of methods available for the determination of this compounds. Most of them employ derivatization with orthophthaldialdehyde (OPA), separation with reversed phase chromatography and fluorescence detection [11–14]. These methods require tedious sample preparation with cation-exchange solid-phase extraction. A fully automated version of such a procedure including sample preparation is presented by Dobashi et al. [15], employing a very complicated system with several pumps and switching valves, which is not easily set up in all laboratories. An HPLC method comprising OPA derivatization and fluorescence detection without sample pretreatments other than protein precipitation is presented by Chen et al. [16], but presumably they have not achieved separation of ADMA from SDMA. Caused by the unspecific fluorescence detection, all of these methods have some difficulties with interferences from endogenous substances. Another approach is described by Caussé et al. [17]. They used fluorescein isothiocyanate as fluorescence labeling reagent and separated the analytes by electrophoresis. This method has some drawbacks in terms of quite long derivatization reaction times and difficulties to achieve a single derivatization product for each compound. Quantification of ARG, ADMA and SDMA utilizing HPLC–MS has first been reported by Vishwanathan et al. [18]. They separate the analytes in their underivatized state on a normal phase column and detect them in the multiple reaction monitoring mode of the mass spectrometer. However, in spite of the selective detector, a laborious and expensive sample clean-up with cation-exchange solid-phase extraction has to take place.

We present here a new method for the quantification of ARG, ADMA, SDMA and CIT in biological fluids such as plasma, serum or urine. After minimal sample preparation (only protein precipitation and evaporation in serum or plasma and dilution in urine samples), we use the well known and robust OPA reagent derivatization, but instead of the unspecific fluorescence detection we measure the analytes by electrospray mass spectrometry. This approach combines the two advantages of superior chromatographic properties of the OPA derivatives and the selectivity of a mass spectrometric detection to a very simple and rugged analytical assay.

2. Experimental

2.1. Instrumentation

The HPLC part of the analytical system consists of an Agilent 1100 system (Waldbronn, Germany) comprising a degasser, a binary pump, an autosampler and a thermostatted column compartment, controlled by an Agilent 1100 control module. The chromatographic separation of the analytes take place in a Merck Superspher 100 RP18 250 mm × 4 mm column. The analytical column is protected by a Phenomenex SecurityGuard (Aschaffenburg, Germany) system equipped with a C18 4 mm × 3 mm filter insert. The analytes are detected by a ThermoFinnigan LCQ ion trap mass spectrometer (San Jose, USA) equipped with an ESI ion source. Data were collected and analyzed by the ThermoFinnigan Xcalibur software package, revision 1.1.

2.2. Chemicals

ARG and ADMA (as their hydrochloride salts), SDMA (as its di(*p*-hydroxyazobenzol-*p*′-sulfonate salt) and CIT (as pure substance) were obtained from Sigma (Steinheim, Germany). The purity of all substances is higher than 98%. The I.S. $^{13}C_6$-arginine and homoarginine (as their hydrochloride salts, purity 98%) were purchased form Cambridge Isotope Laboratories (Andover, USA) and Aldrich (Steinheim, Germany, purity 97%), respectively. The chemicals for the derivatization reaction were orthophthaldialdehyde and 2-mercaptoethanol, both obtained from Sigma. Ultra pure water was produced in our laboratory by a Barnstead EASYpure UV system (Werner, Leverkusen, Germany). All other chemicals were obtained in analytical grade or better.

2.3. Sample collection

Blood samples of about 5 ml were drawn into sampling tubes (BD Vacutainer Systems, Plymouth, UK) containing either citrate, EDTA or no additives. Blood cells were separated by centrifugation at 2400 × *g* for 10 min and the resulting plasma or serum, respectively, was stored at −80 °C until analysis. Urine samples were stored in plastic containers at −80 °C until analysis.

2.4. Calibration samples

For calibration purposes in serum or plasma, a stock solution of 150 μmol ARG, 50 μmol CIT, 8 μmol ADMA and 6 μmol SDMA in 100 ml water was prepared. Water, serum or plasma from healthy volunteers was spiked with appropriate volumes of this solution to yield samples with added concentrations of ARG from 7.5 to 150 μM, ADMA from 0.4 to 8 μM, SDMA from 0.3 to 6 μM and CIT from 2.5 to 50 μM. This covers the concentration ranges of normal and pathologic samples.

For urine calibration, an analogous stock solution containing 50 μmol ARG, 20 μmol CIT, 50 μmol ADMA and 50 μmol SDMA in water was prepared. Water or urine from healthy volunteers was spiked with appropriate volumes of this solution to yield calibration samples with added concentrations in the range of 2.5–50 μM for ARG, ADMA and SDMA, and 0.5–20 μM for CIT. This covers the concentration ranges of most urine samples, but since concentrations of the analytes vary much more in urine then in serum or plasma, very concentrated urines which exceed the calibration range have to be diluted appropriately with water and measured once again.

2.5. Quality control samples

Quality control samples were prepared from serum and urine from healthy volunteers in two concentration levels. The low level exhibits the concentrations found in pooled human plasma or urine, respectively, representing normal values. The high level results from the low level samples spiked with calibration solution to yield concentrations analog to the highest calibration level. Details are summarized in Tables 2 and 3.

2.6. Sample preparation

From urine samples, 250 μl are mixed with 50 μl I.S. solution (185 μM $^{13}C_6$-arginine and 900 μM homoarginine in water) and 500 μl of 200 mM boric acid buffer pH 9.5. These samples are forwarded to the autosampler of the HPLC system, where the automatic derivatization take place directly before injection.

From plasma or serum samples, 250 μl are mixed with 50 μl I.S. solution (185 μM $^{13}C_6$-arginine and 900 μM homoarginine in water) and 500 μl acetonitrile is added to precipitate proteins. After centrifugation at 3000 × *g* for 5 min the clear supernatant is evaporated in vacuum and the residue is redissolved with 70 μl water. This solution is transferred to the autosampler of the HPLC system, where the automatic derivatization takes place directly before injection.

2.7. Derivatization reaction

The derivatization reagent consists of 50 mg OPA, 53 μl 2-mercaptoethanol, 1 ml methanol and 9 ml of 200 mM boric acid, buffer pH 9.5. This solution is useable for 3 days when stored in a refrigerator at 4 °C.

The autosampler of the HPLC system automatically mixes 50 μl of the sample solution and 25 μl of the derivatization reagent in a vial, let it react for 1 min and injects 50 μl of this mixture.

2.8. Chromatographic conditions and MS detector settings

For both the serum/plasma and the urine assay, the same chromatographic and mass spectrometric parameters are used. The chromatographic separation of the analytes is accomplished by gradient elution. Solvent A consists of 1 ml

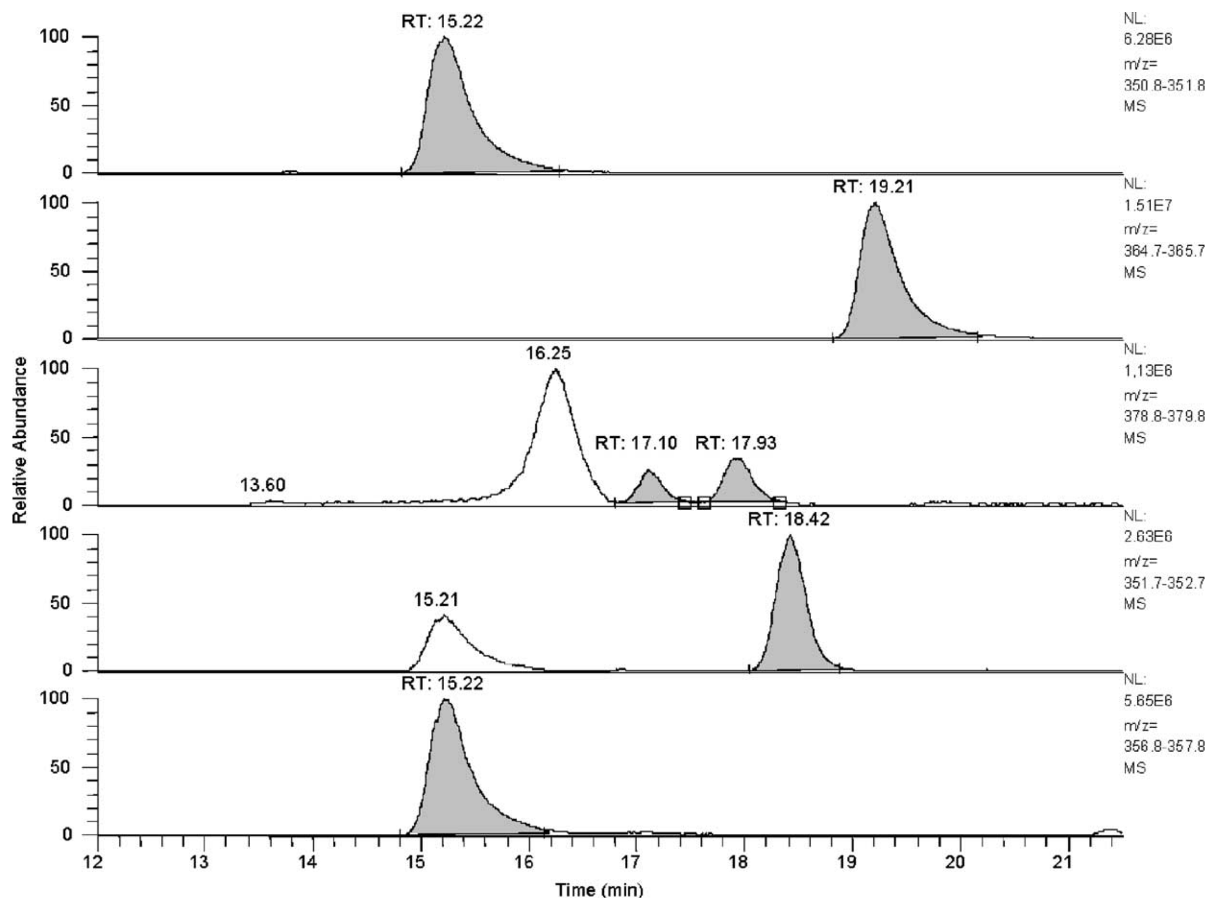

Fig. 1. Chromatogram of a serum sample from a healthy volunteer. The ion traces (*m*/*z*) depicted are 351.3 for ARG, 357.2 for $^{13}C_6$-arginine, 379.2 for ADMA and SDMA, 352.2 for CIT and 365.1 for homoarginine. The concentrations in this sample are 117.9 μM ARG, 0.549 μM ADMA, 0.882 μM SDMA and 37.5 μM CIT.

formic acid (0.1%) and 1 g ammonium formate in 1 l water, resulting in a pH of 3.5. Solvent B is pure methanol. The gradient ratio of solvent B starts with 30% and rises to 50% in 20.5 min. To elute strongly retained substances, the ratio of solvent B is set from 20.6 min to 85%. The chromatographic run ends at 27 min and an additional post-run time of 5 min to reequilibrate the column is necessary. The flow rate is 0.7 ml/min, but is lowered from 12.5 to 20.5 min to 0.5 ml/min to better comply with the needs of the ESI ion source. The column temperature is set to 40 °C. Under these conditions, the retention times for the derivatives of ARG (and also its I.S. $^{13}C_6$-arginine), ADMA, SDMA, CIT and the second I.S. homoarginine are 15.2, 17.2, 18.1, 18.6 and 19.1 min, respectively.

The settings of the ESI ion source are as follows: the capillary temperature is set to 240 °C, the capillary voltage is set to 10 V and an ion-spray voltage of 3 kV is applied. The sheath gas and the auxiliary gas flow-rates are set to 80 and 10 units (about 2.2 and 3 l/min), respectively. A divert valve directs the HPLC flow from 12.6 to 21.5 min of the chromatographic run to the ion source, otherwise to the waste container. During the first 12 min, it is possible to save gas by turning the sheath gas and the auxiliary gas to lower values. The ESI source is working in the "positive mode", producing positive charged ions in the form of [H^+] adduct ions. These ions exhibit mass/charge (*m/z*) ratios of 351.3 for ARG, 357.2 for $^{13}C_6$-arginine, 379.2 for ADMA and SDMA, 352.2 for CIT and 365.1 for homoarginine, respectively. These are the expected *m/z* values for the isoindole derivatives formed from amino acids and OPA reagent [19]. The mass spectrometer is taking single MS full scans in the mass range from *m/z* 349 to 381. The ion traces corresponding to the analytes are extracted from these full scans for subsequent identification and quantification.

3. Results and discussion

3.1. Sample preparation, chromatography and MS detector settings

A very attractive feature of the method described here is the minimal sample preparation. Since there is no extraction at all, no extraction losses occur and this part of the method is therefore very robust and inexpensive since no costly cation-exchange solid-phase extraction cartridges with their difficult extraction behavior have to be used. Serum or plasma samples only have to be concentrated after protein precipitation. This step is quite time consuming, but it can be accomplished unattended. In the case of urine,

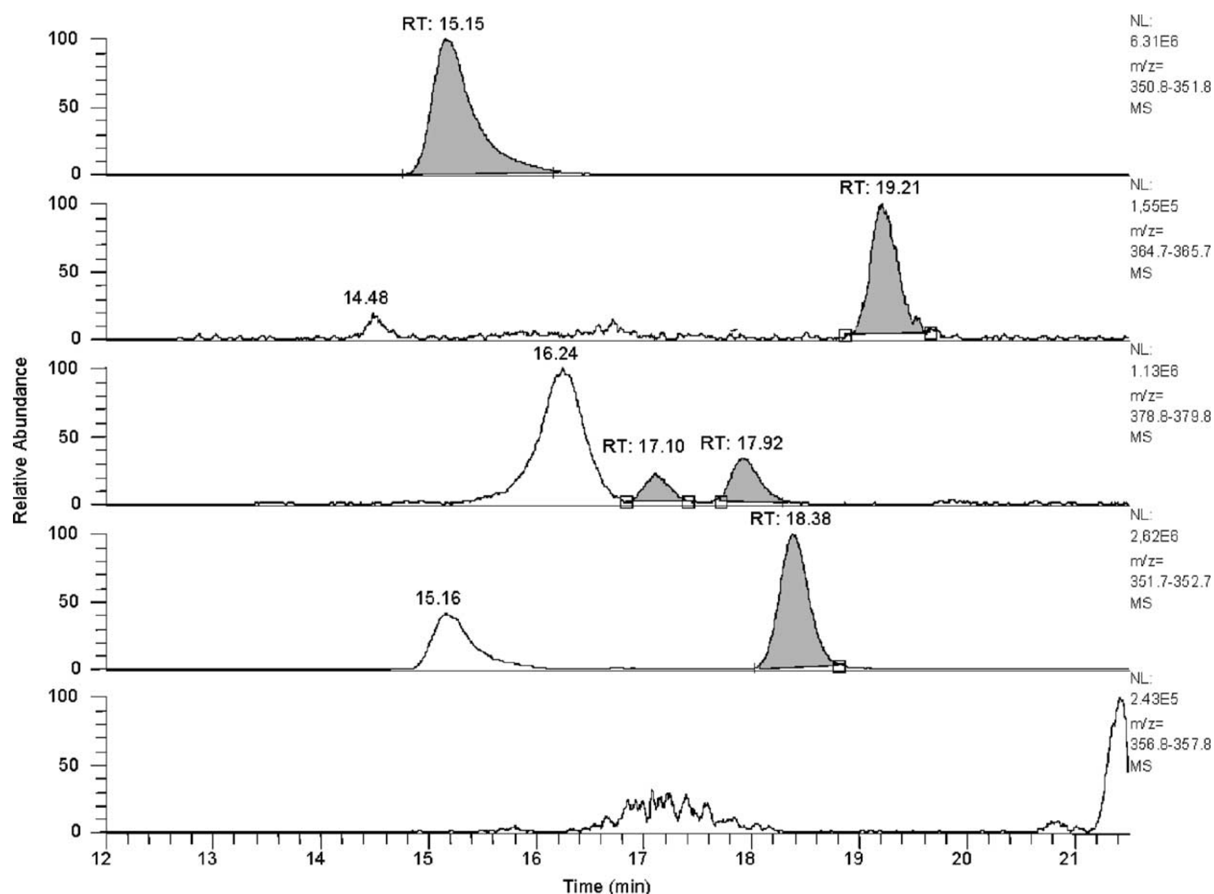

Fig. 2. Chromatogram of the same serum sample as in Fig. 1, but without the addition of internal standards. The ion traces depicted are as in Fig. 1.

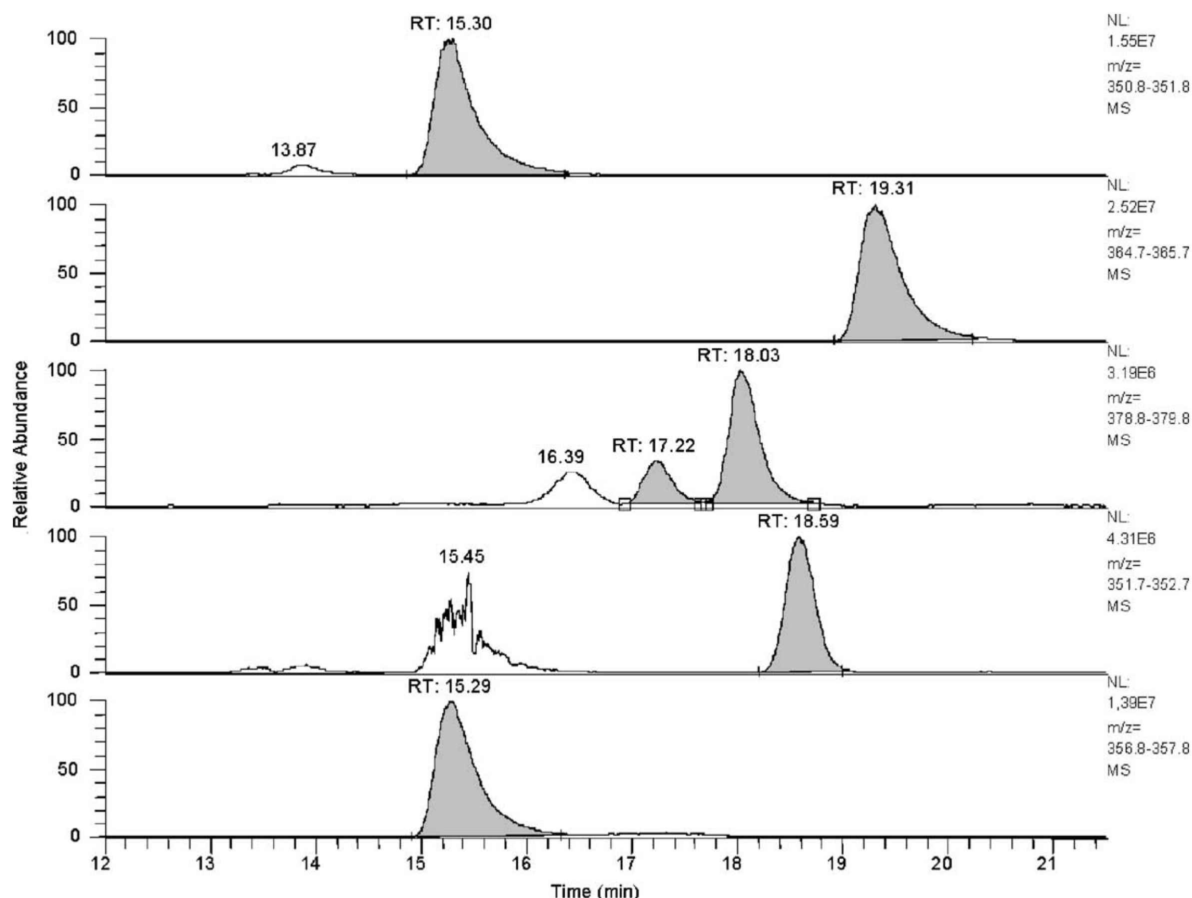

Fig. 3. Chromatogram of a serum sample from a subject with severe renal insufficiency. The ion traces depicted are as in Fig. 1. The concentrations in this sample are 132.4 μM ARG, 1.476 μM ADMA, 4.317 μM SDMA and 33.5 μM CIT.

the only sample pretreatment is that the samples have to be diluted with the buffer used in the derivatization reagent to guarantee for a basic pH.

In Figs. 1–3, typical chromatograms of serum from a healthy volunteer, the same serum analyzed without the addition of internal standards and serum from a subject with severe renal insufficiency are depicted. An analogous series of chromatograms from urine are depicted in Figs. 4–6, respectively. From the full-scan MS chromatogram, the specific ion traces of the analytes are extracted. The peaks are well separated, especially the problematic pair ADMA and SDMA show only minimal overlapping. Endogenous substances do not interfere with the analytes. As can be seen in Figs. 2 and 5, no endogenous substances interfere with the peak of the I.S. $^{13}C_6$-arginine. The endogenous amount of the other I.S., homoarginine, is found to be about 1% of the added amount and therefore leads to only minimal systematic deviations. Regarding to the experience of several hundred samples analyzed with this method, the large unidentified peak in front of ADMA and SDMA shows up always in plasma and serum, but it is well enough separated not to compromise the quantification of these compounds.

The MS detector is running in the full-scan single MS mode. This is sufficient in terms of specificity to separate all analytes from interferences, as well in serum and plasma as in urine. A further increase in specificity would be achieved by MS–MS detection. But unfortunately, the fragmentation pattern of the derivatized analytes are very complicated, dispersing the total intensity over many different fragments. Initial experiments with MS–MS detection show that because of this dispersion, the low concentrated substances ADMA and SDMA are beyond the limit of detection in serum and plasma samples. So, MS–MS detection is not applicable with our equipment.

3.2. Calibration and limits of quantification and detection

A common problem in the calibration of assays for endogenous substances is that it is impossible to produce calibration samples which closely resemble a normal biological specimen, but actually do not contain the substance itself. So, a calibration function obtained by spiking a series of normal samples with known amounts of calibration solution did not intercept at zero, but at a value corresponding to the concentration present in the samples before spiking [20]. The calibration function has to be corrected for this additional amount to properly determine the concentrations of unknown samples. Another approach for calibration of

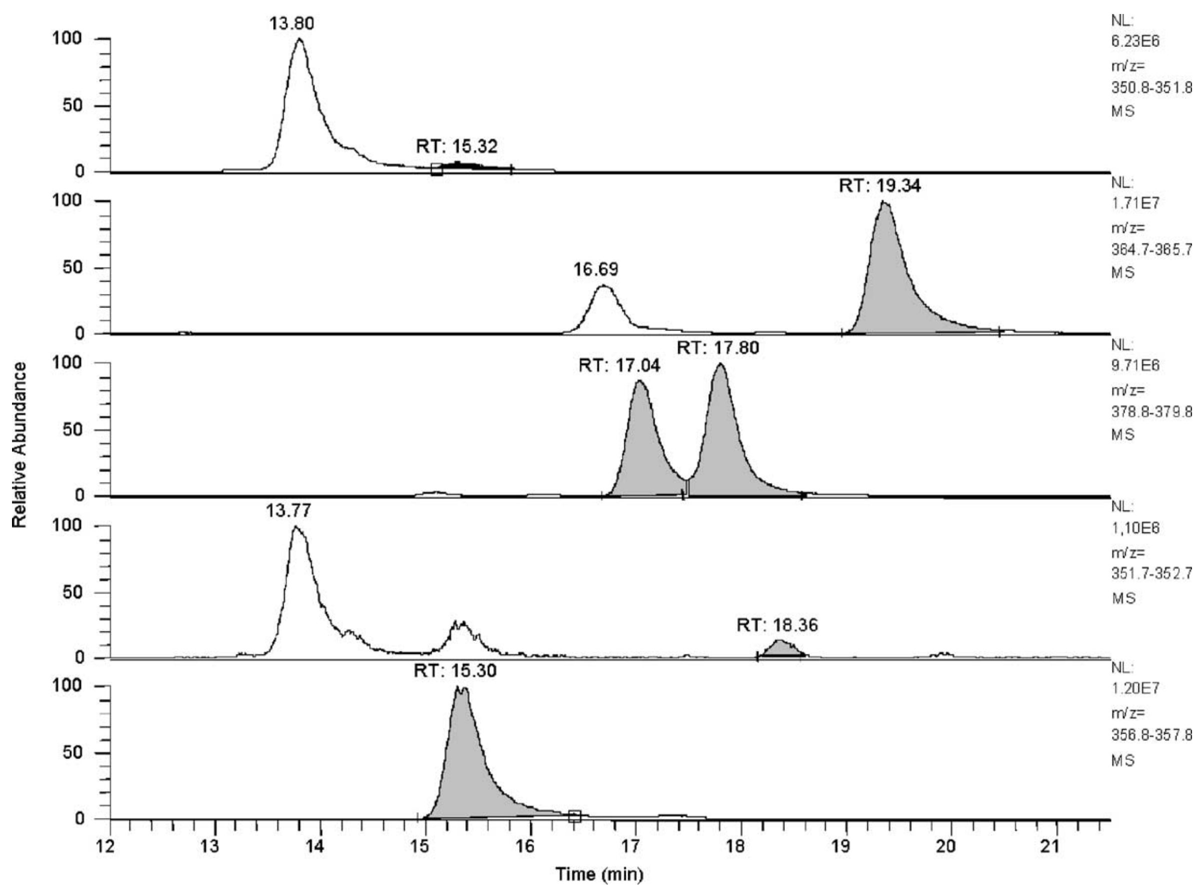

Fig. 4. Chromatogram of a urine sample from a healthy volunteer. The ion traces depicted are as in Fig. 1. The concentrations in this sample are 3.8 μM ARG, 37.4 μM ADMA, 36.9 μM SDMA and 1.8 μM CIT.

endogenous substances is the use of an isotope labeled I.S., which behave (approximately) as the substance itself but can be discerned by a mass spectrometer. In this case, the biological matrix will not affect the measured results and the calibration can be made out of spiked water samples. Unfortunately, in our case only for ARG an isotope labeled I.S. is available. For ADMA, SDMA and CIT the calibration has to be made from serum/plasma or urine in the way described with homoarginine as I.S. Calibration functions obtained in this way from different matrices are summarized in Table 1. As can be seen, for ADMA and SDMA the different matrices have substantial influence on the calibration parameters. CIT also is not as much influenced by the matrix as ADMA or SDMA, presumably because its retention time is very close to the one of its I.S. homoarginine, leading to quite equal matrix effects for both compounds. Thus, ADMA and SDMA and to lesser extend CIT have to be calibrated from authentic matrix samples. In contrast, the calibration parameters of ARG are not very much influenced by the matrix because its isotope labeled I.S. levels out all influences. On the other hand, it can be seen in Table 1 that the serum concentration of ARG is found to be about 60% higher than the plasma concentration. This difference has been observed earlier [14] and seems to relate to the release of ARG from platelets during coagulation. Therefore, while comparing plasma and serum concentrations of ARG attention has to be paid to this effect.

The limits of quantification for this method are half the concentration of the lowest calibration level of the analytes (i.e. 3.75 μM ARG, 0.2 μM ADMA, 0.15 μM SDMA and 1.25 μM CIT). At this concentrations, the R.S.D. of all analytes are below 8%. These concentrations are well below the concentrations expected for samples of normal and pathologic patients.

In Figs. 1 and 4, typical chromatograms are depicted for plasma and urine from healthy volunteers. In plasma as well as in urine, all peaks are well above the limit of detection, which is defined as three times the chromatographic noise. Since samples from pathologic patients usually exhibit higher than normal concentrations of the analytes, the limit of detection is never touched in real samples and therefore is of no concern.

3.3. Inter- and intra-day precision and accuracy

In Tables 2 and 3, the results of the inter- and intra-day precision measurements are summarized. The intra-day precision yields from 10 consecutive measurements of samples representing the highest and lowest calibration concen-

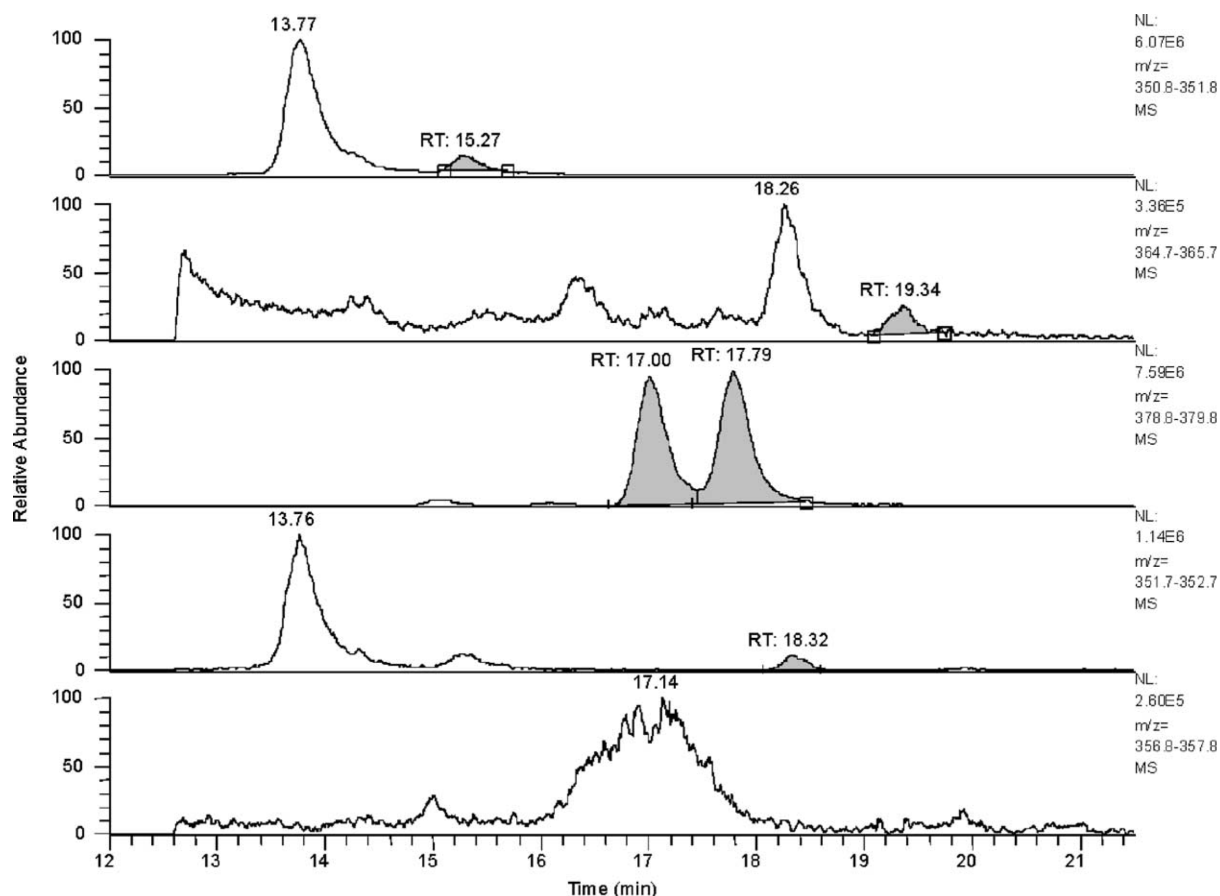

Fig. 5. Chromatogram of the same urine sample as in Fig. 4, but without the addition of internal standards. The ion traces depicted are as in Fig. 1.

Table 1
Calibration functions obtained with different matrices (slope and intercept are values ± S.D.)

Substance	Parameter	Matrix				
		Water	Serum[a]	EDTA[b]	Citrate[c]	Urine
ARG	Slope	0.01010 ± 0.00015	0.00947 ± 0.00036	0.00991 ± 0.00026	0.00909 ± 0.00028	0.01042 ± 0.00025
	Intercept	−0.0185 ± 0.0108	1.50227 ± 0.0265	0.96519 ± 0.0188	0.90143 ± 0.0205	0.09696 ± 0.0060
	Endogenous amount (μM)	0	158.6	97.4	99.2	9.31
ADMA	Slope	0.02098 ± 0.00038	0.01728 ± 0.00023	0.01904 ± 0.00019	0.01817 ± 0.00017	0.01044 ± 0.00035
	Intercept	0.0034 ± 0.0015	0.01194 ± 0.00087	0.01278 ± 0.00074	0.01143 ± 0.00067	0.28133 ± 0.0084
	Endogenous amount (μM)	0	0.691	0.671	0.629	27.0
SDMA	Slope	0.02180 ± 0.00039	0.01839 ± 0.00033	0.02127 ± 0.00020	0.01898 ± 0.00036	0.01243 ± 0.00044
	Intercept	0.0024 ± 0.0011	0.01352 ± 0.00097	0.01374 ± 0.00058	0.01193 ± 0.00105	0.35493 ± 0.0108
	Endogenous amount (μM)	0	0.736	0.646	0.629	28.6
CIT	Slope	0.00403 ± 0.000092	0.00385 ± 0.00007	0.00349 ± 0.00029	0.00357 ± 0.00010	0.00337 ± 0.000039
	Intercept	0.00495 ± 0.0022	0.13665 ± 0.00163	0.13082 ± 0.00535	0.11423 ± 0.00235	0.00607 ± 0.00037
	Endogenous amount (μM)	0	35.5	37.5	32.0	1.80

[a] Serum without additives.
[b] Plasma obtained from sampling tubes containing EDTA.
[c] Plasma obtained from sampling tubes containing citrate.

tration. The inter-day precision date are derived from the measurements of the quality control samples on six sample batches on six different days. As it can be seen in Tables 2 and 3, all parameters fulfill the precision and accuracy requirements for biomedical assays [21].

4. Application of the method

The described method has been used to analyze several hundreds of serum/plasma as well as urine samples. In the case of healthy subjects ($n = 15$), we found concentration

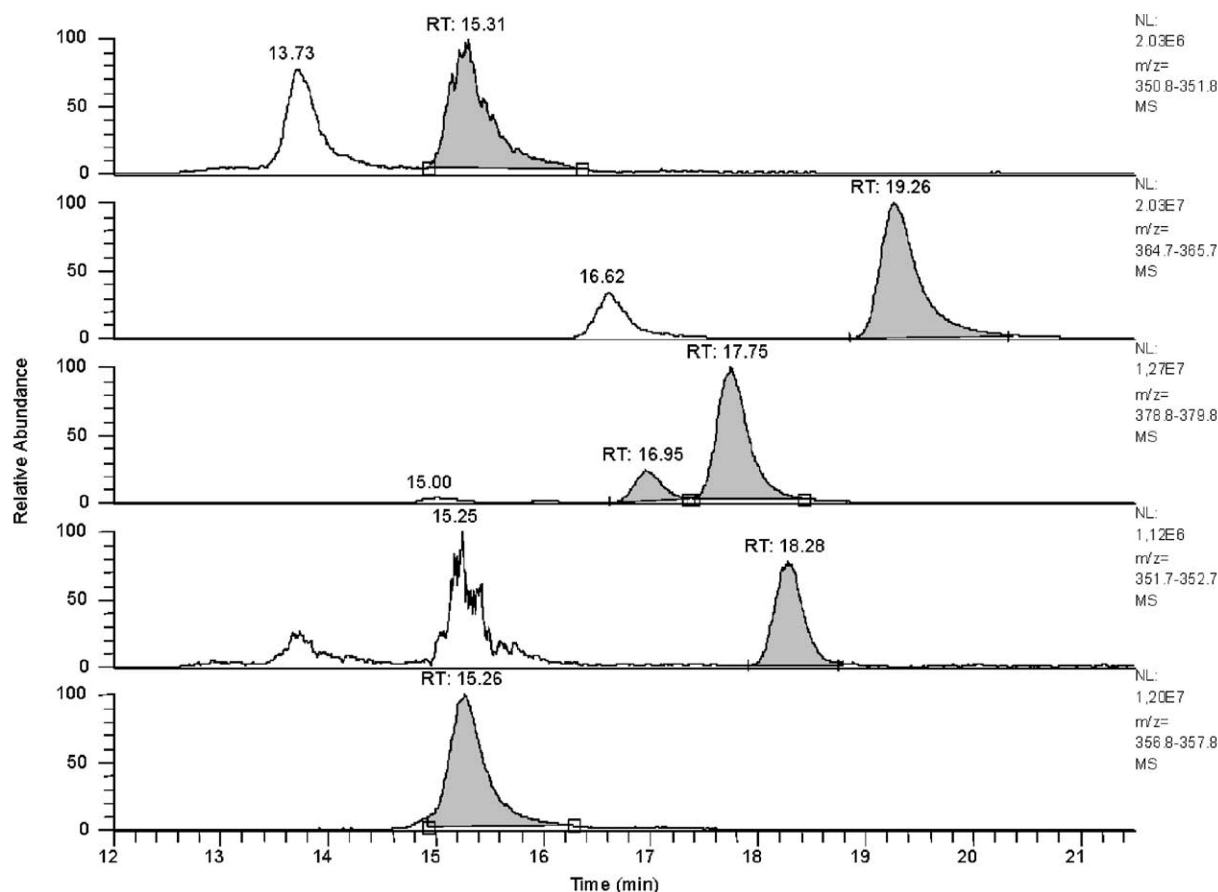

Fig. 6. Chromatogram of a urine sample from a subject with renal insufficiency. The ion traces depicted are as in Fig. 1. The concentrations in this sample are 23.7 μM ARG, 10.2 μM ADMA, 41.6 μM SDMA and 9.3 μM CIT.

values in serum of 119.5 ± 21.6 μM for ARG, 0.453 ± 0.128 μM for ADMA, 0.602 ± 0.168 μM for SDMA and 36.3 ± 10.2 μM for CIT. Urine concentrations from healthy volunteers ($n = 15$) are 11.9 ± 2.4 μM for ARG, 51.7 ± 16.6 μM for ADMA, 50.4 ± 13.0 μM for SDMA and 3.99 ± 4.3 μM for CIT. These values correspond very well with recently published values for healthy subjects and control groups [22,23]. In the case of subjects with severe renal insufficiency ($n = 10$), the serum concentrations are 123 ± 78 μM for ARG, 1.10±0.57 μM for ADMA, 1.53±0.75 μM for SDMA and 38.2 ± 29.7 μM for CIT. The increase of ADMA and SDMA with respect to values of healthy subjects corresponds to previously published data of patients with renal insufficiency [24].

Table 2
Intra-day precision and accuracy (samples prepared according Section 2.6; $n = 10$)

Analyte	Matrix	Low level				High level			
		Spiked concentration (μM)	Found concentration (μM)	R.S.D. (%)	Accuracy (%)	Spiked concentration (μM)	Found concentration (μM)	R.S.D. (%)	Accuracy (%)
ARG	Plasma	0	95.6	3.7	N/A[a]	150	257	5.1	7.6
ADMA	Plasma	0	0.493	7.5	N/A[a]	8	8.65	4.6	1.9
SDMA	Plasma	0	0.497	7.9	N/A[a]	6	6.71	5.3	3.5
CIT	Plasma	0	21.9	5.2	N/A[a]	50	77.9	3.3	12.0
ARG	Urine	0	14.1	8.1	N/A[a]	50	69.5	5.3	8.4
ADMA	Urine	0	36.5	3.8	N/A[a]	50	83.9	2.0	−3.0
SDMA	Urine	0	50.9	3.7	N/A[a]	50	97.7	3.5	3.2
CIT	Urine	0	2.35	6.4	N/A[a]	20	22.9	6.9	2.5

[a] N/A: not applicable because the true concentrations in pooled plasma or urine is unknown.

Table 3
Inter-day precision and accuracy of the quality control samples ($n = 6$)

Analyte	Matrix	Low level				High level			
		Spiked concentration (μM)	Found concentration (μM)	R.S.D. (%)	Accuracy (%)	Spiked concentration (μM)	Found concentration (μM)	R.S.D. (%)	Accuracy (%)
ARG	Plasma	0	35.5	8.7	N/A[a]	150	184	5.8	−1.0
ADMA	Plasma	0	0.420	5.7	N/A[a]	8	8.88	6.9	5.8
SDMA	Plasma	0	0.453	11.8	N/A[a]	6	6.56	5.6	1.8
CIT	Plasma	0	23.0	5.5	N/A[a]	50	73.7	4.8	1.4
ARG	Urine	0	14.1	10.6	N/A[a]	50	68.3	6.4	6.6
ADMA	Urine	0	35.8	6.4	N/A[a]	50	81.5	5.3	−5.0
SDMA	Urine	0	49.7	5.1	N/A[a]	50	105	3.2	−5.0
CIT	Urine	0	3.06	12.4	N/A[a]	20	25.9	10.3	12.3

[a] N/A: not applicable because the true concentrations in pooled plasma or urine is unknown.

5. Conclusion

The described assay for the determination of ARG, ADMA, SDMA and CIT in serum/plasma and urine exhibits some advantages over previously published methods. The derivatization of the analytes with OPA leads to much better chromatographic properties and a better detectability in a mass spectrometer compared to the underivatized state of the analytes. No tedious and expensive sample preparation with cation-exchange solid-phase extraction columns is necessary and therefore a processing step prone to errors can be avoided. Because this extraction step with its imponderabilities is not required, the precision of our assay is considerably better then the one published by Vishwanathan et al. [18] (ARG 3.7% versus 11.3%, ADMA 7.5% versus 8.9%, SDMA 7.9% versus 10.2%). Another benefit of the very simple sample preparation is the possibility to measure CIT in the same run as ARG, ADMA and SDMA. Hence, another biochemical pathway for the elimination of ADMA and therefore the activity of the enzyme DDAH can be monitored without any additional efforts. Furthermore, the method is suitable for serum/plasma samples as well as for urine. The method is selective and sensitive enough to measure samples of healthy as well as pathological subjects. The results generated by this method correspond very well with previously published data. In this way, the reliability of the method is proved independently.

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