

Determination of asymmetric dimethylarginine (ADMA) using a novel ELISA assay

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Abstract

Asymmetric dimethylarginine (ADMA) is an endogenous competitive inhibitor of nitric oxide synthase (NOS). Elevated ADMA plasma levels have been reported in connection with diseases associated with an impaired endothelial L-arginine-NO pathway and endothelial dysfunction, such as atherosclerosis, hypercholesterolemia, chronic heart failure, diabetes mellitus, and hypertension. NO production by NOS is decreased due to elevated ADMA levels. In fact, there is increasing interest in determination of ADMA levels in samples of various origins. The aim of this work was to develop a precise and easy immunoassay in contrast to the existing methods, such as HPLC, liquid chromatography-mass spectrometry (LC-MS) and gas chromatography (GC)-MS. We determined cross-reactivity in our immunoassay of 1.2% for symmetric dimethylarginine and <0.02% for L-arginine. The limit of quantitation was 0.05 $\mu\text{mol/l}$. We found good correlation of the values measured when we compared our assay with LC-tandem MS ($n=29$; $r=0.984$; $p<0.0001$). We determined ADMA levels in human serum and plasma, mouse and rat plasma, and cell culture supernatant. For human plasma we found a mean of 0.65 $\mu\text{mol/l}$ in healthy subjects. In the plasma of mice and rats we found mean concentrations of 1.05 and 1.09 $\mu\text{mol/l}$, respectively.

Keywords: dimethylarginine; endothelial dysfunction; inhibitor; NO pathway; risk factor.

Introduction

Asymmetric dimethylarginine (ADMA) and its regioisomer, symmetric dimethylarginine (SDMA), are

generated by the degradation of methylated proteins due to protein arginine methyltransferase 1 (PRMT 1) activity (1) and subsequent physiological protein turnover. ADMA, but not SDMA, is an endogenous inhibitor of endothelial nitric oxide synthase (eNOS) (2). ADMA is eliminated from the body either by renal excretion or by degradation to dimethylamine and citrulline by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) (3). ADMA inhibits eNOS by competitive displacement of the physiological substrate, L-arginine, from the enzyme (4, 5). The inhibition leads to decreased NO production in the endothelium of vessel walls. Thus, when ADMA levels are elevated, endothelial dysfunction may result (5).

Elevated plasma levels of ADMA were first reported in patients with renal failure (2), a disease that is closely linked to endothelial dysfunction and is associated with extraordinarily high cardiovascular mortality. To date, elevated ADMA levels have been detected in a large number of diseases associated with an impaired endothelial L-arginine-NO pathway, such as atherosclerosis, hypercholesterolemia, chronic heart failure, type 2 diabetes mellitus, stroke, hyperhomocysteinemia, and hypertension [for a review see ref. (6)]. Moreover, ADMA has recently been shown to be a risk factor for cardiovascular disease (7, 8). Likewise, by experimental administration of ADMA to healthy human subjects, symptoms of endothelial dysfunction, such as impaired renal perfusion, reduced cardiac output, and elevated blood pressure, can be evoked (9, 10).

Taking into account the emerging role of ADMA as a risk factor in cardiovascular disease, there is increasing interest in measuring ADMA levels in human subjects, animals, and cell culture. So far, quantification of ADMA by high-performance liquid chromatography (HPLC) analysis has been the most widely applied method. HPLC analysis is performed by extraction of samples with cation-exchange columns followed by o-phthalaldehyde (OPA) derivatization and reversed-phase HPLC with fluorescence detection (11). This method has been modified by several groups with respect to the extraction procedure (12), the derivatization reagent (13), or the HPLC column used (14). Distinct from HPLC with fluorescence detection, different analytical strategies were applied, among them capillary electrophoresis (15), liquid chromatography-mass spectrometry (LC-MS) (16, 17), and gas chromatography-mass spectrometry (GC-MS) (18, 19). All of these methods are laborious and not available in many laboratories. This led us to adopt a more rapid and ubiquitously feasible method for the analysis of ADMA. We report the development and validation of an enzyme-linked immunosorbent assay (ELISA) for the determination of ADMA concen-

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trations in human subjects, animals, and cell culture supernatants. The analytical performance of the newly developed ELISA kit was assessed by evaluating its specificity, analytical sensitivity, precision, accuracy (recovery, linearity), and comparability with LC-MS and GC-MS.

Materials and methods

Chemicals

All fine chemicals were purchased from Sigma (Munich, Germany) or Merck (Darmstadt, Germany).

Reagents

Microtiter plates (96-well) comprised of microtiter strips, eight wells each (Maxisorb; Nunc, Roskilde, Denmark) were coated with ADMA bound to albumin. Standards A–F are ADMA solutions of the following concentrations: 0, 0.1, 0.3, 0.6, 1.0, and 5.0 $\mu\text{mol/l}$, respectively. Control solutions 1 and 2 have ADMA concentrations of 0.25–0.45 $\mu\text{mol/l}$ and 0.6–1.0 $\mu\text{mol/l}$, respectively. An acylation buffer (1 mol/l Tris–HCl buffer, pH 9.1) is provided, together with a lyophilized adjustment buffer (to be dissolved in water) and a lyophilized acylation reagent (N-hydroxysuccinimido carbonic acid to be dissolved in 1.5 ml of dimethylformamide). Rabbit anti-N-acyl-ADMA antiserum is used to detect acyl-ADMA, and the enzyme conjugate is an anti-rabbit-immunoglobulin G (IgG) peroxidase. To generate the immunogene for immunizing the rabbits, ADMA was bound to succinic acid, and subsequently bound to bovine serum albumin (BSA) by the carbodiimide method.

A wash buffer concentrate is diluted 1:10 prior to use. The substrate for the enzyme conjugate is tetramethylbenzidine (TMB). The color reaction is stopped with a stopping solution of 0.3 M sulfuric acid.

All kit reagents are stable for at least 6 months when stored at 2–8°C. The reagents prepared are stable for up to 24 h at 2–8°C.

Samples

Plasma and serum samples were obtained from healthy human subjects. The cell culture supernatants (Dulbecco's modified Eagle medium +5% fetal calf serum) were taken from experiments in our laboratories with human coronary arterial endothelial cells (HCAEC). Rat and mouse plasma samples were from healthy animals from our laboratories.

Quality control sera

Control samples were prepared by adding different stock solutions of known ADMA concentrations to individual plasma samples from normal volunteers or animals. The controls were aliquoted and stored at –80°C.

ELISA kit

Handling of samples Serum or plasma (obtained using EDTA vials) can be used in this ELISA kit. Hemolyzed or lipemic blood samples should not be used in this assay. Samples can be stored for up to 24 h at 2–8°C. Samples not used on the same day can be stored at –20°C for 12 months. Repeated freezing and thawing should be avoided.

Pre-treatment of samples (acylation) Standards A–F (20 μl), control solutions 1 or 2 (20 μl), and 20 μl of sample are pipetted into the wells of a 96-well reaction plate. Acylation buffer (25 μl) and adjustment buffer (25 μl) are added to each well. The reaction plate is shaken for 10 s. Freshly prepared acylation reagent (25 μl) is added and shaken immediately. The reaction plate is incubated for 30 min at room temperature on a horizontal shaker (Titramax 101; Heidolph, Schwabach, Germany). Additional adjustment buffer (1.5 ml) is mixed with water (9 ml) and 100 μl of the mixture is added to each well. The reaction plate is again incubated for 45 min at room temperature. After this step a gel-like clot may occur, but this does not affect the performance of the assay. An aliquot (50 μl) of the prepared standards, controls and samples are used in the ELISA.

ELISA test procedure Aliquots (50 μl) of the pre-treated standards, controls and samples are pipetted into the wells of the microtiter plate and antiserum solution (50 μl) is added to each well. The microtiter plate is shaken for a short time on a horizontal shaker and the plate is covered with adhesive foil. The plate is incubated for 15–20 h at 2–8°C. After incubation, the solution from each well is removed and the wells are washed with wash buffer (250 μl) four times. Subsequently, enzyme conjugate solution (100 μl) is added to each well and the microtiter plate is incubated for 1 h at room temperature on a horizontal shaker. Then the wells are again washed four times with the wash buffer. After washing, substrate solution (100 μl) is pipetted into the wells and the plate is incubated for 20–30 min on a horizontal shaker. The reaction is stopped with stopping solution and the optical density is read at 450 nm (reference wavelength 570–650 nm) using a microtitre plate reader (Sunrise; Tecan, Crailsheim, Germany) within 1 h.

Comparison methods

The performance of the ELISA assay was checked against two recently developed candidate reference methods. ADMA in human serum samples and cell culture supernatants was determined by ELISA compared to GC-MS. The GC-MS method has been previously described (19).

The determination of ADMA in human serum samples by LC-tandem MS was carried out in the Medical Laboratory Bremen (MLHB, Germany), a commercial medical laboratory. In brief, this analysis comprises reverse-phase HPLC separation followed by selected ion monitoring with an API 4000 (Applied Biosystems, Foster City, CA, USA) triple-quadrupole mass spectrometer (method to be published; H. Kirchherr and W.N. Kühn-Velten, personal communication).

For comparison of the ADMA concentrations determined in human plasma and serum samples by ELISA, the ADMA results in both sample types drawn from one subject were compared by linear regression.

Statistical methods

All data are given as mean \pm SD. The method comparison between ELISA and GC-MS or LC-tandem MS methods was performed by linear regression. Statistical significance was accepted for $p < 0.05$.

Results

Specificity of the kit

We evaluated the specificity of the anti-ADMA antiserum used in our ELISA by determination of the

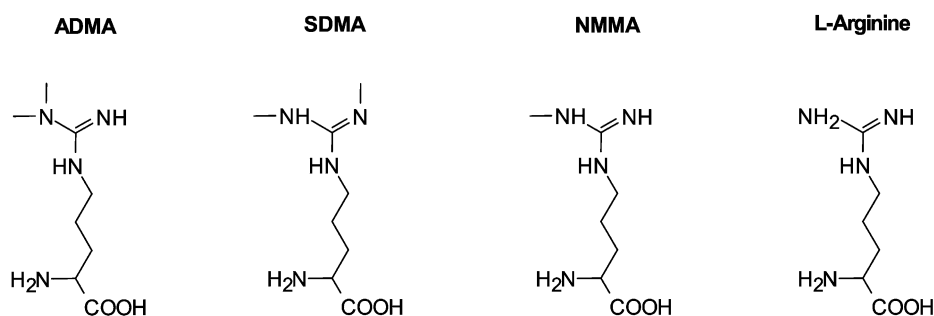


Figure 1 Chemical structures of ADMA and potentially cross-reactive substances.

Table 1 Specificity of the ADMA ELISA.

Substance	ED ₅₀ , ng/ml	Cross reactivity, %
ADMA	126	100
L-Arginine	660000	<0.02%
L-NMMA	12200	1.0
SDMA	10500	1.2

ED₅₀ is the concentration at half the maximum absorption of a concentration-OD curve.

Table 2 Intra-assay variation of the ADMA ELISA.

Sample	ADMA, $\mu\text{mol/l}$		CV, %
	Mean	SD	
1	0.81	0.06	7.5
2	1.76	0.08	4.5

Table 3 Inter-assay variation of the ADMA ELISA.

Sample	ADMA, $\mu\text{mol/l}$		CV, %
	Mean	SD	
1	0.63	0.07	10.3
2	1.01	0.10	9.8
3	1.38	0.13	9.4
4	2.26	0.19	8.3

Table 4 Analytical recovery of ADMA from human serum.

ADMA, $\mu\text{mol/l}$			Recovery, %
Added	Observed	Calculated	
0	0.59		
0.25	0.85	0.84	101
0.5	0.98	1.09	90
1.0	1.46	1.59	92
1.5	1.86	2.09	89
2.0	2.39	2.59	92
2.5	2.87	3.09	93
3.0	3.37	3.59	94
3.5	3.90	4.09	95
4.0	4.81	4.59	105
Mean \pm SD			94.56 \pm 5.22

cross-reactivity of the substances shown in Figure 1 that may potentially interfere with the assay. The results are shown in Table 1. For SDMA, which has a structure very similar to that of ADMA, cross-reactivity of only 1.2% was detected. For L-arginine, which

is present at up to 100-fold excess in human plasma, cross-reactivity was >0.02%. For L-N^G-monomethyl-arginine (L-NMMA) we found cross-reactivity of 1.0%. The low cross reactivity of the anti-ADMA anti-serum indicates high specificity of the present immunoassay.

ELISA standard curve and analytical sensitivity

A calibration curve was established using ADMA standards from 0 to 5.0 $\mu\text{mol/l}$ with absorbance from 0.3 to 1.8. The analytical sensitivity was calculated as 3 \times SD of the zero standard and was found to be 0.05 $\mu\text{mol/l}$. The range of the ELISA covers the range of normal and elevated ADMA concentrations observed in humans.

Precision

The intra-assay coefficients of variation (CVs) were estimated by repeated measurement ($n=36$) of two control sera, as shown in Table 2. One sample with a lower concentration of ADMA (0.81 $\mu\text{mol/l}$) and one with a higher concentration (1.76 $\mu\text{mol/l}$) were used. The CV was 7.5% for the lower control serum and 4.5% for the higher control serum.

The interassay coefficient of variation was determined by repeated measurement ($n=28$) of four control sera on two consecutive days with concentrations of 0.63, 1.01, 1.38 and 2.26 $\mu\text{mol/l}$, respectively, as shown in Table 3. CVs ranged from 8.3% for the highest control serum to 10.3% for the lowest control serum.

Accuracy

First we estimated the analytical recovery of ADMA in the ELISA at nine different concentrations in human serum, as shown in Table 4. To do this, we added increasing amounts of ADMA to a serum sample. Each serum sample (spiked and non-spiked) was assayed in duplicate. ADMA concentrations were measured, and the percentage recovery was calculated. The mean recovery from all serum samples was 94.6%.

Plasma from mice and rats was handled in an identical manner. The results are shown in Table 5 and Table 6. The mean recovery for mouse plasma was 84.7% and that for rat plasma was 80.7%.

Table 5 Analytical recovery of ADMA from mouse plasma.

ADMA, $\mu\text{mol/l}$			Recovery, %
Added	Observed	Calculated	
0	0.53		
0.25	0.75	0.78	96.2
0.5	0.94	1.03	91.3
1.0	1.25	1.53	81.7
1.5	1.68	2.03	82.8
2.0	2.3	2.52	90.9
2.5	2.3	3.03	75.9
3.0	2.9	3.53	82.2
3.5	3.25	4.02	80.6
4.0	3.65	4.53	80.6
Mean \pm SD			84.69 \pm 6.56

Table 6 Analytical recovery of ADMA from rat plasma.

ADMA, $\mu\text{mol/l}$			Recovery, %
Added	Observed	Calculated	
0	0.79		
0.25	1.0	1.04	96.2
0.5	1.1	1.29	85.3
1.0	1.5	1.79	83.8
1.5	1.75	2.29	76.4
2.0	2.2	2.79	78.9
2.5	2.45	3.29	74.5
3.0	2.75	3.79	72.6
3.5	3.5	4.29	81.6
4.0	3.7	4.79	77.2
Mean \pm SD			80.72 \pm 7.16

Table 7 Linearity on serial dilution of human serum.

Dilution	ADMA, $\mu\text{mol/l}$		Recovery, %
	Observed	Extrapolated	
Original	3.84	3.84	
3:1	2.90	3.87	101
1:1	1.67	3.34	87
1:2	1.22	3.66	95
1:4	0.69	3.45	90
1:9	0.33	3.30	86
1:20	0.16	3.36	88
Mean \pm SD			91.17 \pm 5.78

Samples were spiked with ADMA and then diluted x parts sample plus y parts water. Values were extrapolated from the original undiluted sample.

Table 8 Linearity on serial dilution of mouse plasma.

Dilution	ADMA, $\mu\text{mol/l}$		Recovery, %
	Observed	Extrapolated	
Original	0.97	0.97	
3:1	0.84	1.12	115.5
1:1	0.53	1.06	109.3
1:2	0.46	1.38	142.3
1:4	0.21	1.05	108.2
1:9	0.092	0.92	94.8
Mean \pm SD			114.02 \pm 17.52

Samples were spiked with ADMA and then diluted x parts sample plus y parts water. Values were extrapolated from the original undiluted sample.

To demonstrate the assay accuracy by a different method, linearity studies were carried out. For this purpose we added ADMA to a human serum sample and to mouse and rat plasma samples, respectively. Serial dilutions of these samples were measured in duplicate, and the recovery was calculated. The results are presented in Table 7 for the human sample. Recovery was in the range between 86% and 101%, and the mean recovery was 91.2%. In mouse plasma, we found a mean recovery of 114.0% (Table 8), and in rat plasma, mean recovery was 100.4% (Table 9).

Method comparisons

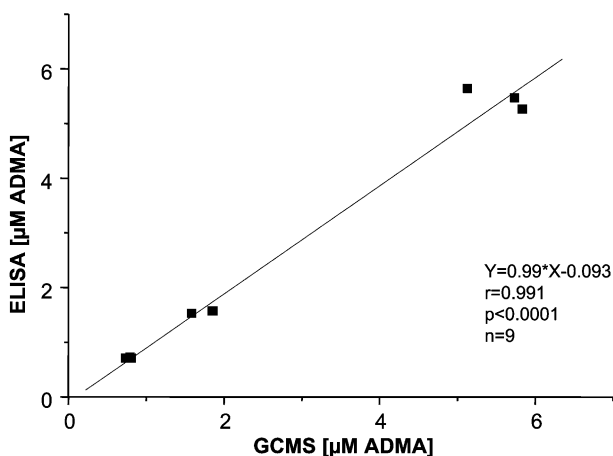
The new ELISA was compared with GC-MS. Nine human serum samples, unmodified and spiked with a wide range of ADMA concentrations, were measured in parallel using GC-MS and ELISA. The results of linear regression analysis showed excellent correlation, with a correlation coefficient $r=0.991$ and $p<0.0001$ (Figure 2).

A total of 29 serum samples with a wide range of ADMA concentrations were measured in parallel using LC-tandem MS and ELISA. The results of linear regression analysis showed comparable correlation, with a correlation coefficient $r=0.984$ and $p<0.0001$ (Figure 3).

Table 9 Linearity on serial dilution of rat plasma.

Dilution	ADMA, $\mu\text{mol/l}$		Recovery, %
	Observed	Extrapolated	
Original	1.15	1.15	
3:1	0.9	1.2	104.3
1:1	0.58	1.16	100.9
1:2	0.42	1.26	109.6
1:4	0.22	1.1	95.7
1:9	0.105	1.05	91.3
Mean \pm SD			100.36 \pm 7.16

Samples were spiked with ADMA and then diluted x parts sample plus y parts water. Values were extrapolated from the original undiluted sample.

**Figure 2** Linear regression analysis of the method comparison for nine human serum samples measured in parallel using GC-MS or ELISA.

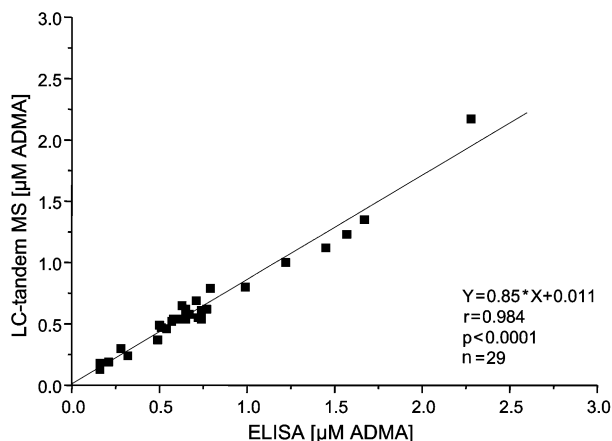


Figure 3 Linear regression analysis of the method comparison for 29 human serum samples measured in parallel by using LC-tandem MS or ELISA.

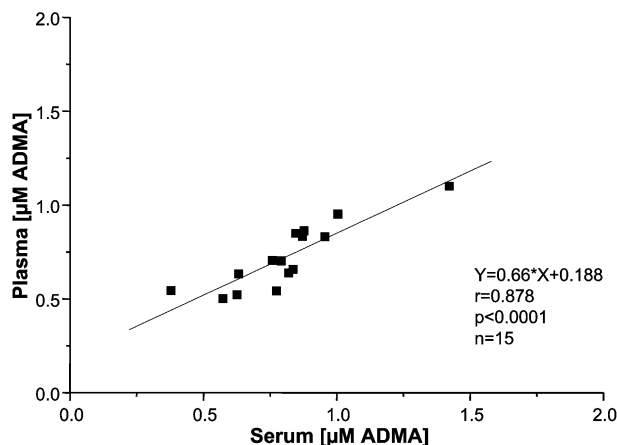


Figure 5 Linear regression analysis of serum and plasma samples from 15 healthy human subjects measured by ELISA in parallel.

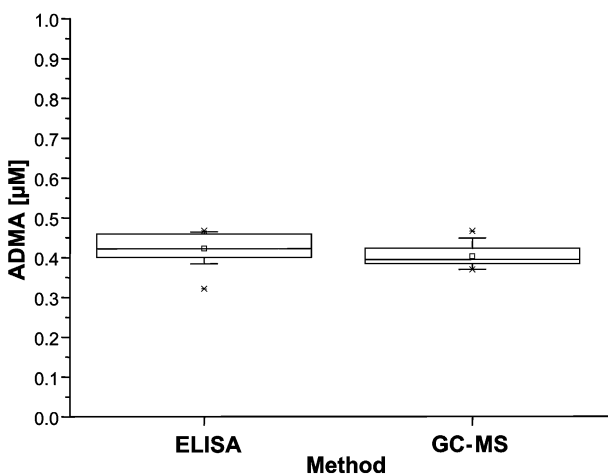


Figure 4 Comparison of ADMA values of 15 cell culture supernatants determined by ELISA and GC-MS.

In addition, we measured 15 cell culture supernatants using ELISA and GC-MS. The results are shown in Figure 4.

Stability of the kit

The stability of the ADMA ELISA kit was investigated by real-time stability studies and under shipping conditions. The kit was found to be stable for at least 1 year when stored at 2–8°C (data not shown).

ADMA determination in plasma and serum

Mean ADMA serum concentration in a group of 10 healthy human subjects was 0.72 ± 0.16 $\mu\text{mol/l}$. Comparison of human plasma and serum samples showed slightly lower ADMA values in human plasma (0.65 ± 0.13 $\mu\text{mol/l}$) compared to serum (Figure 5); however, this difference was not statistically significant.

Mean ADMA concentration in plasma of healthy mice was 1.05 ± 0.17 $\mu\text{mol/l}$. Mice overexpressing the ADMA-metabolizing enzyme, DDAH (20), had signifi-

cantly lower ADMA levels (0.64 ± 0.10 $\mu\text{mol/l}$; $p < 0.05$ vs. control mice; Figure 6). In plasma of healthy rats, mean ADMA concentration of 1.09 ± 0.21 $\mu\text{mol/l}$ was measured. In the conditioned media of cultured human endothelial cells, ADMA levels were determined to be 0.42 ± 0.04 $\mu\text{mol/l}$.

Discussion

We developed and validated an ELISA assay for the determination of ADMA levels in serum, plasma or other fluids. The ADMA ELISA kit is based on the principles of a competitive immunoassay. The wells of a microtitre plate are coated with ADMA. The acylated ADMA from pre-treated samples and bound ADMA compete for the limited number of binding sites of the antibodies in the antiserum added. After equilibrium has been reached, antigen-antibody complex not bound to the plate is removed. The amount of antibody bound to the microtitre plate is determined by the reaction of tetramethylbenzidine with horseradish peroxidase coupled to the secondary antibody

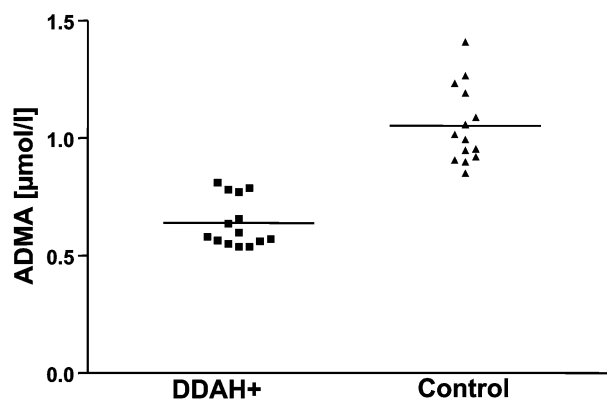


Figure 6 ADMA plasma concentration in DDAH-1 transgenic mice compared to control mice. Each point represents one individual animal; the horizontal lines indicate the mean in each group.

(enzyme conjugate). After the reaction is stopped, the optical density is measured at 450 nm on a microtitre plate reader. The intensity of the color developed is inversely proportional to the amount of ADMA in the sample.

In contrast to the methods available for measuring ADMA, the new ELISA is easy to use and is a high-throughput technique. The combination of the acylation step and the competitive design of the ELISA results in a specific, highly sensitive and non-isotopic immunoassay. The antiserum selected is specific for ADMA and results in negligible cross-reactivity for L-arginine and other endogenous derivatives of L-arginine. The precision of our ELISA has been demonstrated by low intra- and interassay coefficients of variation. ADMA concentrations can be accurately measured across the full range of physiologically relevant concentrations. The values derived from the ELISA correlate well with expected values in recovery tests, and manifest excellent linearity in dilution studies.

The values derived from this ELISA are consistent with those obtained using GC-MS and LC-tandem MS. An interesting finding is the difference in ADMA concentrations found in human serum and plasma. Teerlink and colleagues (21) reported higher L-arginine and SDMA values in human serum compared to plasma for their HPLC method. For ADMA they did not find a statistically significant difference, which might be explained by the low number of samples they compared ($n=10$). Increased release of amino acids from blood cells during clotting might be one possible cause of the higher ADMA, SDMA and L-arginine concentrations in serum compared to plasma. Martens-Lobenhofer and Bode-Böger (17) recently reported a similar difference between serum and plasma ADMA levels. The mechanism behind these observations should be investigated in more detail in the future. Our recovery studies showed that there should be no influence of protein binding on ADMA measurements with our ELISA assay.

Martens-Lobenhofer and Bode-Böger (17) reported a limit of quantitation of 0.2 μM for their LC-MS method, whereas Vishwanathan et al. (16) reached a limit of 0.05 μM . Thus, the sensitivity of our ELISA with a limit of quantitation of 0.05 μM is comparable to the published LC-MS methods.

Although each of the above methods is suitable for the determination of ADMA concentrations in human samples, our ELISA has the advantage of simplicity, in that only standard laboratory equipment and skills are needed. Moreover, the ADMA ELISA is suitable for the determination of ADMA in experimental studies involving rats, mice, or cell culture supernatants with comparable sensitivity and specificity, as demonstrated by our experiments.

In summary, the ELISA kit developed and described here is a highly sensitive and precise high-throughput assay. Accordingly, it should be especially useful for the determination of ADMA levels in human serum and plasma samples in larger clinical studies, and ultimately as a diagnostic test.

Acknowledgements

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