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Comparison of two methods for dimethylarginines quantification

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ABSTRACT

Objectives: Both dimethylarginines are widely bound to chronic kidney disease (CKD). This study was focused to validate published LC-MS/MS method and compared the measured data with an immunoassay.

Design and methods: The analysis was performed on a Dionex UltiMate 3000 UHPLC-Standard (Thermo Fisher Scientific, Waltham, Massachusetts, USA) with an amaZon SL ion trap (Bruker, Billerica, Massachusetts, USA). Comparison was evaluated by using Passing Bablok regression and Bland Altman plot. Healthy volunteers (n = 40) were used for validation and as control group to patients group (n = 40) with different stages of CKD.

Results: The results in healthy controls determined by the LC-MS/MS (ELISA) method were 0.52 ± 0.0892 with 95 % CI: 0.49–0.55 (0.61 ± 0.1213 with 95 % CI: 0.57–0.64) μmol/L for AD MA and 0.56 ± 0.0810 with 95 % CI: 0.53–0.58 (0.62 ± 0.0752 with 95 % CI: 0.57–0.65) μmol/L for SDMA. In the same way, the patient group values determined by the LC-MS/MS (ELISA) method were 0.82 ± 0.1604 with 95 % CI: 0.75–0.88 (1.06 ± 0.3002 with 95 % CI: 0.94–1.19) μmol/L and 2.14 ± 0.8778 with 95 % CI: 1.47–2.58 (1.65 ± 0.5160 with 95 % CI: 1.40–1.98) μmol/L for ADMA and SDMA, respectively. The correlation between the methods, expressed as the Spearman correlation coefficient (R), was 0.858 (0.8059) for ADMA ($p < 0.0001$) and 0.895 (0.9607) for SDMA ($p < 0.0001$).

Conclusions: ADMA levels determined by the immunoassay were almost 30 % overestimated, in contrast to SDMA levels, which were 3 % underestimated. According to our findings, a better correlation could be obtained by simple sample dilution.

1. Introduction

Asymmetric and symmetric dimethylarginine (ADMA and SDMA), also called “toxic methylarginines”, are non-proteinogenic amino acids formed in proteins by post translation modifications (PTMs) [1]. ADMA, an essential molecule for vascular homeostasis due to its inhibitory effect on the enzyme nitric oxide synthase (NOS), is related to one carbon metabolism [2]. Diseases linked with hyperhomocysteinemia and the overproduction of reactive oxygen species (ROS) are responsible for reducing ADMA degradation because of their inhibitory effect on dimethylarginine dimethylaminohydrolase (DDAH) [3]. Meanwhile, SDMA is an early and sensitive marker of incipient renal injury. Almost 100 % of its daily production is eliminated from the organism by the kidneys. Both

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methylderivatives are competitive inhibitors of L-arginine transport into the cells and activate NFκB [4]. Therefore, dimethylarginines are mainly associated with cardiovascular disease and chronic kidney disease (CKD) [4–10].

The most common method for the quantification of dimethylarginines in human serum or plasma is liquid chromatography with mass spectrometry, with derivatization or without it [11–21]. The second choice involves immunoassays (enzyme-linked immunosorbent assay; ELISA) [22–25]. Separation techniques are well known for having less interference and other benefits compared to immunoassays. Comparison of these two types of methods has shown different correlation results, and only one study focused on both dimethylderivatives; thus, the correlation for SDMA is not well known yet [13,26–28]. Methods like high pressure liquid chromatography with fluorescent detection (HPLC FLD), gas chromatography with tandem mass spectrometry (GC-MS/MS), and capillary electrophoresis with laser induced fluorescence (CZ-LIF) or with ultraviolet detection (CZ-UV) are less frequent [29–35].

The aim of this study was to validate the published ultrahigh pressure liquid chromatography method with tandem mass spectrometry (UHLPC-MS/MS) for ADMA and SDMA quantification in human plasma [13]. The results obtained by the mass spectrometry technique were then compared with the ELISA method.

2. Material and methods

2.1. Chemicals

- Standards (N^G,N^G-dimethyl-L-arginine dihydrochloride ≥98 %; N^G,$N^{G'}$-dimethyl-L-arginine di(p-hydroxyazobenzene-p'-sulfonate) ≥ 99 %) were purchased from Merck KGaA (Darmstadt, Germany).
- The internal standard for ADMA (N^G,N^G-dimethyl-d_6-arginine dihydrochloride 98 atom % D, 95 % (CP)) was purchased from Merck KGaA (Darmstadt, Germany). The standard for SDMA (N^G,$N^{G'}$-dimethyl-L-arginine-d_6 98 atom % D; 95 % (CP)) was obtained from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA).
- Solvents (methanol LiChrosolv®; acetonitrile LiChrosolv®) and chemicals (acetic acid LiChropur®; ammonium acetate LiChropur®; 1 M HCl-1-butanol LiChropur®) were purchased from Merck KGaA (Darmstadt, Germany).

2.2. Biological samples

The control group used for validation of the UHPLC-MS/MS method consisted of 40 healthy volunteers: men (n = 20) and women (n = 20), both with an age range between 40 and 64 years (average and SD were 49 ± 7 and 48 ± 7 years, respectively). Patients with different stages of CKD (n = 40) were used for method comparison: men (n = 21) with an age range of 55–86 years (66 ± 10 years) and women (n = 19) with an age range of 34–84 years (64 ± 14 years). Biological samples were centrifuged at 2000 g at 4 °C for 5 min, and their plasma aliquots were stored at −80 °C. Plasma and serum were collected from 27 patients in the control group to evaluate the matrix effect of different samples. ADMA was determined in 79 subjects only, because of one patient's low sample volume.

2.3. UHPLC-MS/MS

The analysis was performed on a Dionex UltiMate 3000 UHPLC – Standard (Thermo Fisher Scientific, Waltham, Massachusetts, USA) with an amaZon SL ion trap (Bruker, Billerica, Massachusetts, USA) and Genius NM32LA nitrogen generator (Peak Scientific Instruments, Inchinnan, Great Britain). The method was created in HyStar (ver. 3.2, © Bruker Daltonik GmBH). The chromatographic part of the method was controlled by Chromeleon (ver. 6.8, SR12, © 1994–2013 Dionex Corporation, Part of Thermo Fisher Scientific) and the ion trap was controlled by TrapControl (ver. 7.2, © 1998–2013 Bruker Daltonik GmBH). DataAnalysis (ver. 4.2, © 1993–2013 Bruker Daltonik GmBH) and QuantAnalysis (ver. 2.2, © 1999–2013 Bruker Daltonik GmBH) were used for the evaluation of measured data.

2.4. ELISA methods for quantification of ADMA and SDMA

Both immunoassays for ADMA and SDMA quantification are based on the same principle: competitive ELISA where acylation of free ADMA or SDMA in plasma or serum samples is necessary before measurement. The acylated products of our compounds compete with solid phase-bound ADMA (or SDMA) for a fixed number of rabbit anti-ADMA (or anti-SDMA) antiserum binding sites. When the system is in equilibrium, free antigen and free antigen-antiserum complexes are removed by washing. The antibody bound to the solid phase ADMA or SDMA is detected by anti-rabbit/peroxidase. The substrate TMB/peroxidase reaction is monitored at 450 nm. The amount of antibody bound to the solid phase ADMA or SDMA is inversely proportional to the ADMA or SDMA concentration in the sample.

The sample preparation and measurement was the same as in the instructions for use of each kit. The following ELISA kits and quality controls for the quantification of ADMA and SDMA were purchased from DLD Diagnostika GmbH (Hamburg, Germany):

- Enzyme immunoassay for the quantitative determination of endogenous asymmetric dimethylarginine (ADMA) in serum or plasma (REF EA201/96) with ADMA high sensitive ELISA quality controls (REF EA29/96) at two concentration levels (0.41 and 0.80 μmol/L);
- Enzyme immunoassay for the quantitative determination of endogenous symmetric dimethylarginine (SDMA) in serum or plasma (REF EA203/96) with SDMA ELISA quality controls (REF EA203/96) at two concentration levels (0.55 and 0.95 μmol/L).

The following analytical characteristics of the ELISA methods are described in the instruction for use for each diagnostic kit: The LODs of the immunoassays are 0.01 μmol/L for ADMA and 0.03 μmol/L for SDMA. ADMA reproducibility was determined for plasma (8.3 %) and serum (7.6 %) samples, and in the range of 6.2–4.9 % for SDMA serum samples only. The average recover values for different ADMA concentration levels were 104 % (rat heparin plasma) and 98 % (rat serum). The recovery of SDMA was 97 % (rat plasma) and 93 % (rat serum). The linearity of the ADMA diagnostic kit was performed with plasma and serum samples, and the linear response range was 0.11–2.55 μmol/L and 0.15–2.72 μmol/L, respectively. For the SDMA immunoassay, the linearity was determined for plasma samples only, within the range of 0.23–1.72 μmol/L. Both kits were tested for cross reactivity. Only the SDMA immunoassay provided information about correlation with the LC-MS/MS method.

3. Experimental

3.1. LC-MS/MS

Separation was performed on a Kinetex 2.6 μm C18 100A HPLC Column 50 x 2.1 mm with a Kinetex UHPLC C18 2.1 mm pre-column. Mobile phase A consisted of methanol and 0.1 % acetic acid, and mobile phase B consisted of 5 mmol/L ammonium acetate and 0.1 % acetic acid (pH = 4.3). Gradient elution was used, with a total run time of 8 min needed for prevention of crossing over and column equilibration. The initial composition of the mobile phases was 10 % A with a linear increase to 100 % A in 3 min, returning back to a composition of 10 % A in 4 min. The flow rate was 0.25 mL/min with 20 μL injection volume. The temperature of the autosampler and column thermostat was 6 °C and 20 °C, respectively. Every measurement of the sample/calibrator/quality control was performed in duplicate and the final result was expressed as an average. Mass spectrometry ionization and fragmentation of the dimethylderivatives and internal standards was optimized on an ion trap mass spectrometer with electrospray ionization (ESI) in a positive mode. The ion source temperature and desolvation temperature were 180 °C. Nitrogen was used as nebulizer (4.0 L/min, 7.3 psi) and desolvation gas. Helium (purity 5.0) was used as the collision gas and set at $3 \bullet 50^{10^{-6}}$ mbar. The capillary voltage was 5000 V. Quantification was performed in the multiple reaction monitoring (MRM) mode (Scan mode: enhanced resolution).

3.2. Stock solutions, calibrators, quality controls, and samples

Stock solutions of ADMA and SDMA (1 mmol/L) were prepared in ultrapure water (Smart2Pure 6 UV/UF system, Thermo Fisher Scientific, Waltham, Massachusetts, USA). Calibration standards were prepared from these solutions by dilution with ultrapure water to give final concentrations of 0.2, 0.4, 0.8, 1.6, 3.2, and 6.4 μmol/L. Stock solutions of the internal standards d_6-ADMA (3.56 mmol/L) and d_6-SDMA (4.80 mmol/L) were prepared in the same way. The concentration of internal standards in each analyzed vial was 0.59 and 0.80 μmol/L, respectively. Quality control (QC) samples were prepared at three concentration levels (low, medium, and high). The low QC was created from the plasma of healthy subjects with concentrations of 0.43 μmol/L ADMA and 0.56 μM SDMA. The medium and high QC samples were prepared from the low QC by spiking with stock solutions, giving final concentrations of 1.23 μmol/L ADMA and 2.06 μmol/L SDMA for the medium QC, and 1.93 μmol/L ADMA and 3.56 μmol/L SDMA for the high QC. Aliquots of stock solutions, working solutions, and QC samples were stored at −80 °C. All were freshly thawed before each sample preparation.

3.3. Sample preparation

The sample preparation described by Boelaert et al. was optimized for our conditions [13]. On the day of preparation, the samples, calibrators, and QC controls were thawed at room temperature (RT), vortex mixed, and centrifuged at 2000 g and 4 °C for 5 min. The first sample preparation step included mixing 160 μL of sample (calibrator or QC sample) and 40 μL of internal standard solution (containing 2.90 μmol/L d_6-ADMA and 4.0 μM d_6-SDMA). Then, 600 μL of acetonitrile was added for protein precipitation and extraction of the analytes. The samples were vortex mixed for 2 min and centrifuged (10,000 g, RT, 10 min). The supernatant was transferred into a new eppendorf tube and then evaporated to dryness under nitrogen (60 °C). The next step was the derivatization with 1 mol/L HCl in butanol, commercial derivatization reagent for creation of butyl-esters. Dried extracts were derivatized by adding 500 μL derivatization reagent, vortex mixed for 2 min, and placed in a water bath at 70 °C for 20 min. After cooling to RT, the extracts were evaporated to dryness under the same conditions as described before. Dried derivatized extracts were reconstituted in 1 mL of the initial mobile phase composition, vortex mixed thoroughly for 10 min, and filtered through nylon syringe filters (0.20 μm) into vials. The samples were measured on the day of preparation.

3.4. Validation

The validation parameters of the developed method were the limit of detection (LOD), limit of quantification (LOQ), linearity, precision, recovery, freeze-thaw stability, and matrix effects. The LOD was characterized by a calibrator with noise three times higher than blank and a coefficient of variation (CV) lower than 20 % lower than 20 %, measured in triplicate. The LOQ was defined as three times the LOD. The linearity was observed by a calibration curve with ten concentration points. QC samples at three concentration levels were used to determine the precision in a series (each level with n = 10) and between series (each level n = 10 on different days). Relative recoveries (%) were calculated as the ratio of the difference between spiked and unspiked QC samples and the theoretical concentration added. For the freeze thaw stability test, every QC sample level was frozen from one to four times. To evaluate the matrix effects of biological samples, we spiked water and blank plasma to prepare calibrate curves and compared the response from patient's

plasma and serum samples (n = 27) to monitor possible differences in results.

4. Results

Butyl-esters of ADMA and SDMA were separated on a Kinetex 2.6 μm C18 100A HPLC Column 50 x 2.1 mm with a Kinetex UHPLC C18 2.1 mm precolumn. Gradient elution was used, with a total run time of 8 min and an injection volume of 20 μL. Detection was performed at their unique mass transitions (259 → 214 *m/z* for ADMA; 265 → 214 *m/z* for ADMA-IS; 259 → 228 *m/z* for SDMA; and 265 → 231 *m/z* for SDMA-IS). The basic characteristics of the analytes are shown in Table 1 and their chromatograms with mass spectra in Fig. 1. The LOD was 0.01 μmol/L and the LOQ was 0.03 μmol/L for both dimethylderivatives. The linearity results obtained using a 10 point calibration curve (in the range 0.05–10 μmol/L for both) are shown in Table 2. Calibration curves were also prepared in a limited but sufficient range (0.2–6.4 μmol/L) to cover the results expected in the control and patient groups. The average slope, intercept, and coefficient of determination from eight calibration curves are shown in Table 3. Other analytical properties of the method (repeatability, intermediate precision and recovery) are summarized in Table 4. The freeze-thaw stability test was performed for three QC levels, measured in triplicate, with samples being frozen from one to four times. The CVs of each QC level through all frozen cycles were lower than the CV value for precision between series; thus, this test did not impact on the measured analytes.

The CVs of each pair of comparison samples (plasma versus serum) were less than 6.07 % and 6.16 % for ADMA and SDMA, respectively; thus, the difference between plasma and serum is close to the repeatability and intermediate precision values.

Fig. 2 shows the ADMA and SDMA serum concentrations determined by different methods in all samples (Fig. 2A and B) and in separate groups (healthy controls vs. patients with different stages of CKD) determined by LC-MS/MS only (Fig. 2C and D). The results are expressed as medium concentrations ± standard deviation, with a 95 % confidence interval (CI). The ELISA method overestimates the ADMA levels (Fig. 1A) (0.67 ± 0.2020 with 95 % CI: 0.60–0.73 μmol/L *vs*. 0.82 ± 0.3359 with 95 % CI: 0.72–0.91 μmol/l for the LC-MS/MS and ELISA method, respectively), in contrast to the SDMA levels (Fig. 1B) (0.80 ± 1.0215 with 95 % CI: 0.64–1.33 μmol/L *vs*. 0.85 ± 0.6537 with 95 % CI: 0.68–1.24 μmol/L for the LC-MS/MS and ELISA method, respectively). The results in healthy controls determined by the LC-MS/MS (ELISA) method only were 0.52 ± 0.0892 with 95 % CI: 0.49–0.55 (0.61 ± 0.1213 with 95 % CI: 0.57–0.64) μmol/L for ADMA and 0.56 ± 0.0810 with 95 % CI: 0.53–0.58 (0.62 ± 0.0752 with 95 % CI: 0.57–0.65) μmol/L for SDMA (Fig. 1C and D). In the same way, the patient group values determined by the LC-MS/MS (ELISA) method were 0.82 ± 0.1604 with 95 % CI: 0.75–0.88 (1.06 ± 0.3002 with 95 % CI: 0.94–1.19) μmol/L and 2.14 ± 0.8778 with 95 % CI: 1.47–2.58 (1.65 ± 0.5160 with 95 % CI: 1.40–1.98) μmol/L for ADMA and SDMA, respectively (Fig. 1C and D). The correlation between the methods, expressed as the Spearman correlation coefficient (R), was 0.858 (0.8059) for ADMA ($p < 0.0001$) and 0.895 (0.9607) for SDMA ($p < 0.0001$).

Fig. 3 shows a comparison of the determination of ADMA and SDMA for both methods by Bland-Altman and Passing-Bablok plots. Fig. 2A shows the Bland-Altman plot with a positive bias (0.19 μmol/L) and an increasing trend of difference at elevated levels, with almost 30 % overestimation. The same trend was confirmed by Passing Bablok regression in Fig. 2B. On the other hand, the ELISA method (Fig. 2C) underestimates the SDMA concentration by about 3 %, only with a negative bias (−0.21 μmol/L), and has the same trend of increasing the difference at higher levels.

5. Discussion

We validate published LC-MS/MS method for the separation of dimethylarginine butyl-esters and compare these results with commercial available immunoassay. All parameters of validation are described in section 2.3.3 Validation. The levels of dimethylarginines observed in the control group, determined by the LC-MS/MS method, are similar to published values [13–15,18,36]. Patients with different stages of CKD had significantly higher concentrations of both SDMA ($p < 0.0001$) and ADMA ($p < 0.0001$) than healthy controls. The elevated level of SDMA in plasma is probably a reflection of its insufficient elimination by renal excretion in CKD patients. In contrast to the symmetric derivative, the main mechanism (>80 %) for ADMA degradation is hydrolysis by DDAH, an enzyme expressed primarily in the kidneys, to citrulline and dimethylamine. In diseases involving the overproduction of DDAH inhibitors (such as ROS or homocysteine), ADMA is also elevated. These trends are also consistent with the experience of other authors [4,7,13,28,37].

Only one study (Boelaert et al., 2016) was focused on a comparison of LC-MS/MS and ELISA methods for the determination of both dimethylderivatives. The authors observed a positive correlation for both dimethylderivatives ($R = 0.78$; $p < 0.0001$ for ADMA and $R = 0.72$; $p < 0.0001$ for SDMA) and an increasing trend of the difference between results at higher values [13]. Their study included CKD patients only, and one internal standard of ADMA was used for both analytes, *i.e.*, for ADMA and SDMA. Our higher correlation of SDMA could be a result of using two unique internal standards (*i.e.,* ADMA and SDMA), in contrast to the mentioned study. The development of an immunoassay for ADMA determination was described by Schulze et al., who compared serum samples of healthy

Table 1
Basic characteristics of ADMA and SDMA.

Analyte	tR (min)	Parent ion (*m/z*)	Fragmentation ion (*m/z*)
ADMA	1.6	259	214
ADMA - IS	1.6	265	214
SDMA	1.8	259	228
SDMA - IS	1.8	265	231

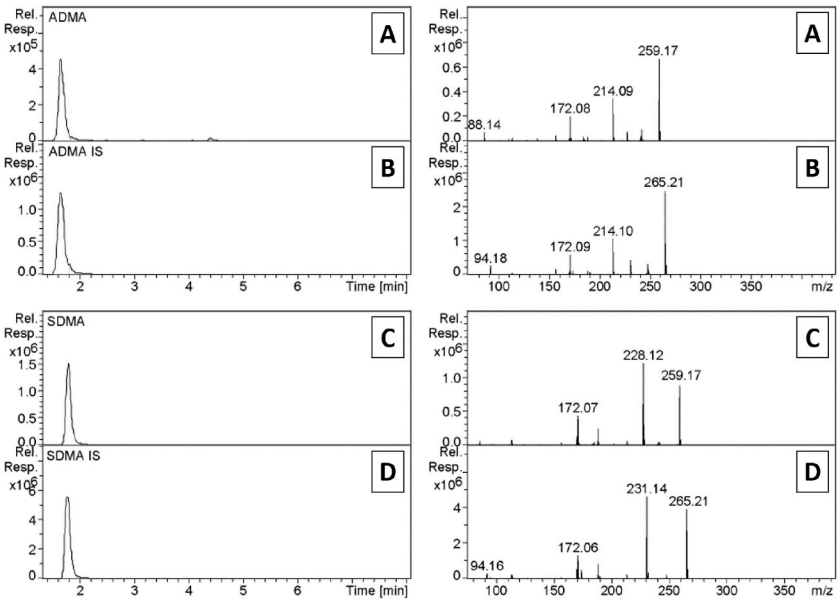

Fig. 1. ADMA (Fig. 1A), ADMA internal standard (Fig. 1B), SDMA (Fig. 1C) and SDMA internal standard (Fig. 1D) chromatograms with their mass spectra.

Table 2
Linearity of the ADMA and SDMA calibration curves.

Analyte	Range (μmol/L)	Slope	Intercept	Coefficient of determination
ADMA	0.05–10.00	0.577494	0.020716	0.999087
SDMA	0.05–10.00	0.504136	−0.048028	0.999423

Table 3
Characteristics of the calibration curves.

Analyte	Slope (±SD)	Intercept (±SD)	Coefficient of determination (±SD)
ADMA	0.4846 (±0.1074)	0.0127 (±0.0428)	0.9994 (±0.0004)
SDMA	0.4555 (±0.0533)	−0.0165 (±0.0278)	0.9996 (±0.0003)

Table 4
Analytical properties of the LC-MS/MS method for ADMA and SDMA measurement.

Analyte	QC level	Repeatability (%)	Intermediate precision (%)	Added concentration (μM)	Recovery (%)
ADMA	LOW	4.73	6.12	–	–
	MEDIUM	4.43	5.07	0.80	100.37
	HIGH	4.80	5.69	1.50	105.56
SDMA	LOW	4.34	6.16	–	–
	MEDIUM	4.38	5.35	1.50	92.38
	HIGH	4.18	3.18	3.00	96.76

individuals ($R^2 = 0.984$, $p < 0.0001$) and presented the importance of different matrices on the ELISA method, which overestimates ADMA concentrations by about 20 % in comparison to the LC-MS/MS method. Their level of correlation could depend on the smaller number of measured samples ($n = 29$) with the average concentration of 0.72 ± 0.16 μmol/L [27]. Martens-Lobenhoffer et al. performed a comparison of human plasma samples for healthy controls, patients with end-stage renal failure, and other diseased individuals. Their findings are in accordance with our results: the ELISA method overestimates the ADMA concentrations determined by LC-MS/MS almost twice and mainly with respect to higher values [28]. Another comparison was performed by Pecchini et al. in patients with CKD only. They observed the same trend of overestimation, expressed mainly in patients with lower values of glomerular filtration rate (and thus higher ADMA concentrations) [26].

According to our findings, the ELISA method overestimates ADMA values by approximately 28 %, which is in agreement with other authors. SDMA had an inverse trend, with a slight underestimation (3 %), in contrast to the published literature which showed a

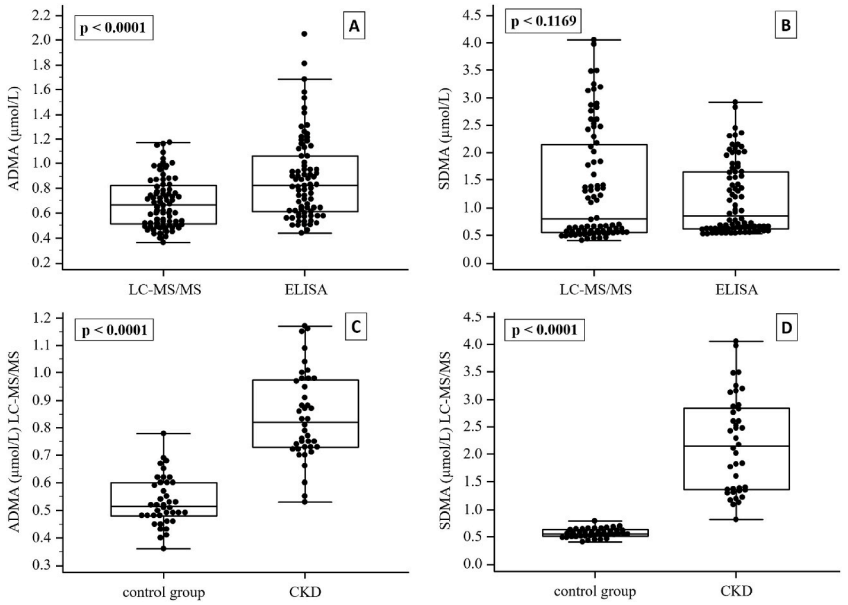

Fig. 2. ADMA (2A) and SDMA (2B) serum concentrations (µmol/L) determined by LC-MS/MS and ELISA method in all samples; ADMA (2C) and SDMA (2D) serum concentrations determined by the LC-MS/MS method only in a control group and in patients with different stages of chronic kidney disease.

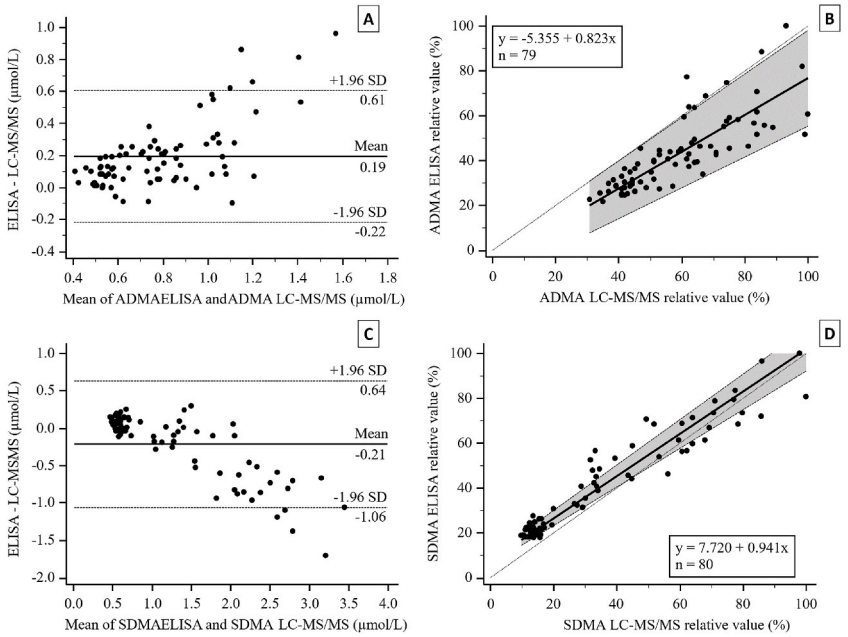

Fig. 3. Bland-Altman and Passing-Bablok plots for ADMA (3A, 3B) and SDMA (3C, 3D) quantification method comparison (LC-MS/MS and ELISA).

slightly inversed trend with no fixed bias [13]. In both cases, the mean difference between methods increases at higher concentrations, thus mainly in the CKD group. The dissimilarity of trends in both dimethylderivatives could depend on different concentration levels in diseased patients. The linearity range in the ADMA diagnostic kit is suitable for the values expected in both healthy individuals and CKD patients. The linear response of the SDMA diagnostic kit was only up to 1.72 µmol/L; thus, two-fold sample dilution was required in patient groups where SDMA levels were significantly higher. In accordance with the SDMA method comparison results, a simple sample dilution could be a possible tool to reduce the matrix effects in biological samples.

6. Conclusion

LC-MS/MS, the reference method, rules out matrix effects, with a guarantee of determining the true ADMA and SDMA values. ADMA levels determined by the immunoassay were almost 30 % overestimated, in contrast to SDMA levels, which were 3 % underestimated. According to our findings, a better correlation could be obtained by simple sample dilution.

Author contribution

Vendula Sudová;, Pavel Prokop, Ladislav Trefil performed the measurements, with data interpretation; Vendula Sudová, Jaroslav Racek: wrote the manuscript; Jaroslav Racek, Daniel Rajdl: proofread the manuscript.

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The manuscript was edited by a native speaker and at the same time an expert in the field of clinical chemistry (Proof Reading company).
The Ethics Committee of the University Hospital of Plzen approved the study and all participants gave their written consent to participate.

Declaration of competing interest

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

Data availability

The data that has been used is confidential.

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