# Comparison of asymmetric dimethylarginine detection – HPLC and ELISA methods (technical brief)

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**SUMMARY**

*Objective:* Asymmetric dimethylarginine (ADMA) is often discussed in connection with hyperhomocysteinemia and its toxic effect on vessel wall. ADMA concentration is usually measured by HPLC after previous derivatization. Recently, ELISA methods for ADMA determination were introduced and ELISA kits are commercially available. The aim of the study was to compare HPLC and ELISA methods for ADMA determination.

*Settings:* Institute of Clinical Biochemistry and Hematology, Departments of Internal Medicine, Charles University Hospital in Pilsen, Czech Republic.

*Material and Methods:* For HPLC determination we used equipments from Thermo separation product (Florida, USA). After solid-phase extraction on polymer cation-exchange column and the following derivatization with o-phthaldialdehyde the samples were separated using C18 column (mobile phase: 8.7% acetonitrile, 50 mmol/l phosphate buffer, pH 6.5) and a fluorescence detector. $N^G$-monomethyl-L-arginine was used as an internal standard. ADMA® ELISA kit, based on a competitive principle, was obtained from DLD Diagnostika, Hamburg, Germany. ADMA was measured in EDTA plasma of 40 healthy blood donors and 40 hemodialysis patients with hyperhomocysteinemia.

*Conclusion:* In spite of different principles both methods showed a very good correlation (r = 0.944, P < 0.0001). ELISA method reproducibility, calculated from 40 duplicate measurements of hyperhomocysteinemic samples and expressed as a coefficient of variation, was 4.75%. These results show that time consuming HPLC method of ADMA determination can be replaced by ELISA which gives comparable results and has an excellent reproducibility.

*Key words:* ADMA, HPLC, ELISA, hemodialysis.

The adverse effect of hyperhomocysteinemia on a vascular wall can be partially explained by an increasing plasma concentration of asymmetric dimethylarginine (ADMA), a potent inhibitor of nitric oxide synthase. Two L-arginine derivatives: $N^G$-monomethyl-L-arginine (L-NMMA) and $N^G$, $N^G$-dimethyl-L-arginine (asymmetric dimethylarginine – ADMA) competitively block the nitric oxide synthase (NOS) active site and then selectively decrease NO synthesis. As the ADMA blood concentration is about ten-fold higher than that of L-NMMA, it is considered to be the predominant endogenous NOS inhibitor [1, 2].

Patients with end-stage renal disease face a particularly high risk of a cardiovascular disease and possible cardiovascular mortality. Part of their increased risk is due to a higher prevalence of the established risk factors, such as arterial hypertension, diabetes mellitus, lipid metabolism disorders and anemia. Inflammatory processes, high sympathetic activity, and the accumulation of an endogenous inhibitor of NOS – asymmetric dimethylarginine (ADMA), have recently emerged as the cardiovascular risk factors of paramount importance [3]. The time consuming and complicated chromatographic method (HPLC with previous derivatization) is needed for the measurement of ADMA levels. The methods of determining ADMA concentration in plasma applying different detection techniques have already been published [4–8] and only recently has an immunochemical method taken place based on ELISA technique [9].

We collected 80 EDTA plasma samples (40 healthy blood donors and 40 hemodialysis patients) and then compared the results of ADMA concentrations, obtained by HPLC and ELISA methods. The sampling procedure was standardized, plasma samples were collected in EDTA tubes and centrifuged at 3600 g for 5 minutes within 30 minutes; plasma was separated and stored frozen at -70 °C until analysis.

For the HPLC method, we used equipment from Thermo separation product (Miami, Florida, USA). After the solid-phase extraction on the polymer cation-exchange column (OASIS MCX, Waters, Milford, MA, USA) and the following derivatization with o-phthaldialdehyde, ADMA and its stereoisomer, symmetric dimethylarginine (SDMA), were separated simultaneously within 30 min using C18 column (Waters, Milford, MA, USA; mobile phase 8.7% acetonitrile, 50 mmol/l phosphate buffer, pH 6.5) and a fluorescence detector (excitation 340 nm, emission 450 nm). $N^G$-monomethyl-L-arginine was used as an internal standard. This technique is similar to the method described by Teerling et al. [9] with minor modifications. Using a 0.2 ml sample volume, linear calibration was obtained and the signals of stable derivates (ADMA and SDMA) were near the baseline resolution. Although we did not use derivatization just before analysis, our recovery of ADMA was good (92%); also the reproducibility (5.2 %) and the detection limit (below 0.13 µmol/l) were similar to those described by Valtonen et al. [11].

For ADMA immunochemical quantification, the ELISA method (kit ADMA® ELISA, DLD Diagnostika GmbH, Hamburg, Germany) and the AUTO-EIA II microplate reader (Labsystems Oy, Espoo, Finland) were used. This competitive method uses the microtiter plate format. ADMA is bound to the solid phase and ADMA in samples is acylated and competes with the bound ADMA for

a fixed number of rabbit anti-ADMA antiserum binding sites. After the equilibrium, free antigen and free antigen-antiserum complexes are removed by washing. The bound antibody is detected by anti-rabbit peroxidase and TMB (3.3',5.5'-tetramethylbenzidine) as a peroxidase substrate. The final product of this reaction is monitored at 450 nm. The amount of antibody is inversely proportional to the ADMA concentration in the sample. Estimate limit of quantification was 0.05 µmol/l; this corresponds to the kit manufacturer's data. The inter-assay CV of ADMA for the kit control 1 (ADMA concentration $0.510 \pm 0.086$ µmol/l) was 14.0 % and for the kit control 2 ($0.892 \pm 0.162$ µmol/l) 18.0 % (N = 5). Both kit controls fell between the target range (control 1: 0.24–0.58 µmol/l, control 2: 0.6–1.0 µmol/l). The repeatability of the ELISA method, expressed as an intra-assay coefficient of variation and calculated from 20 measurements of plasma pool (mean ADMA concentration 1.58 µmol/l), was 11.8%. We also measured in duplicates 40 different plasma samples of hyperhomocysteinemic patients (mean ADMA concentration 1.59 µmol/l, range from 0.82–2.49 µmol/l) and expressed the reproducibility from the differences between the parallel measurements; the variation coefficient was 4.75%.

We compared HPLC and ELISA methods for ADMA determination in a wider range than Valtonen et al. [11]. The ADMA concentration range of our samples was 0.3–3.0 µmol/l. In spite of differing principles, both methods showed a very good correlation (Pearson's correlation coefficient r = 0.944, P < 0.0001), Pasing-Bablok regression model shows a very steep slope according to the wide difference at high concentration levels (Fig. 1). The confidental interval in Bland-Altman (Fig. 2) differential plot leads to hypothesis that at low concentration levels ELISA and HPLC methods are comparable which corresponds to results of Schultze [10] while at higher levels the confidence interval is wider and the comparison is worse. These results show that the time-consuming HPLC method for ADMA determination can be replaced by ELISA which gives comparable results, has good reproducibility and is more suitable for greater series of samples.
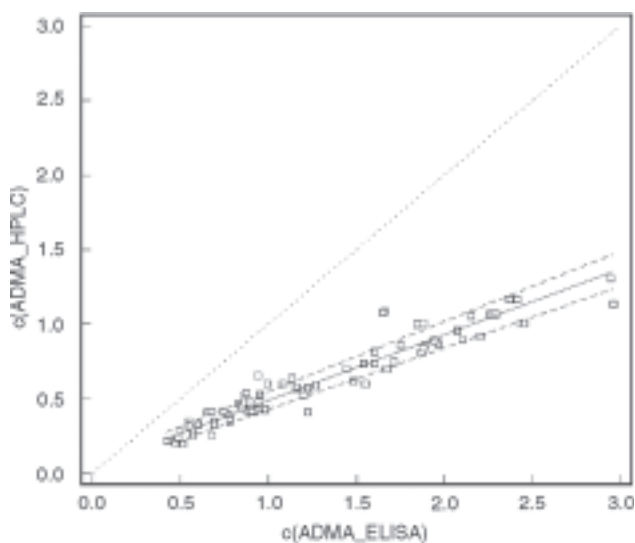


**Fig. 1.** Comparison of HPLC and ELISA methods for ADMA determination (Passing-Bablok regression model)
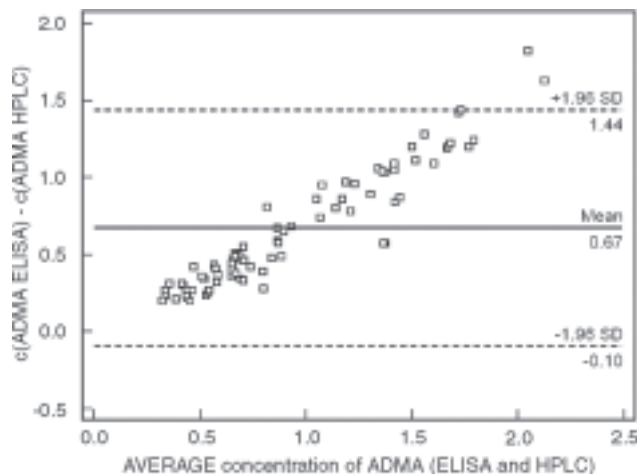


**Fig. 2.** Bland and Altman differential plot (ELISA and HPLC methods), c (concentration)

Conflicting results were published in studies by Schultze et al. [10] and Valtonen et al. [11]. In our study, we confirmed Schultze's results; our correlation coefficient was only a little lower (r = 0.944, P < 0.0001 vs. r = 0.991 obtained by Schultze et al.). Schultze used the same ELISA method but employed a different chromatographic technique (GC-MS) and also the range of our results was different. As Valtonen [11] explained there were some points which can change a correlation curve. Firstly the concentrations in plasma samples were remarkably low in Schultze's paper [10]. On the other hand we measured ADMA concentration in a wider range. Secondly, the kits from the above mentioned papers did not have the same lot number.

According to our results, the ELISA method is less precise but more accessible for laboratories without specific instruments (LC, GC – MS, OASIS columns etc.) and especially for a larger sample series.

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