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Capillary electrophoretic and micellar electrokinetic separations of asymmetric dimethyl-L-arginine and structurally related amino acids: Quantitation in human plasma

We report the development of efficient electrophoretic methods for the separation and quantification of L-arginine and six naturally occurring derivatives that are structurally and functionally related. Capillary electrophoresis (CE) employing a concentrated borate buffer at pH 9.4 achieves the separation of mixtures containing dimethyl-L-arginine, N^G-monomethyl-L-arginine, L-arginine, L-homoarginine, L-ornithine, and L-citrulline as 4-fluoro-7-nitrobenzofurazan derivatives. In addition, the separation of the isomeric dimethyl-L-arginine derivatives (symmetric and asymmetric) is attained with baseline resolution by micellar electrokinetic chromatography (MEKC) when a high concentration of deoxycholic acid is added as a surfactant to the same running buffer. The influence of buffer type, concentration, and pH on the separation was studied to optimize separation conditions. The limit of quantitation (LOQ) for asymmetric dimethyl-L-arginine in aqueous solution was determined to be 20 μM using UV absorption in a CE separation and 0.1 μM using laser induced fluorescence (LIF) detection in an MEKC separation. This newly developed method was successfully applied for the quantitation of asymmetric dimethyl-L-arginine and L-arginine in human plasma samples at levels that might be used as a clinical diagnostic for cardiovascular disease (0.125 μM LOQ).

Key Words: Capillary electrophoresis; Micellar electrokinetic chromatography; Dimethyl-L-arginine; Amino acids

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1 Introduction

N^G-monomethyl-L-arginine (NMMA) as well as asymmetric N^G, N^G-dimethyl-L-arginine (ADMA) and symmetric N^G, N'^G-dimethyl-L-arginine (SDMA) are naturally occurring amino acids that circulate in blood plasma and are excreted in urine. They are formed from hydrolysis of proteins containing methylated L-arginine residues. L-Arginine and free L-methylarginines are detectable in cell cytosol, plasma, tissues, and urine. L-arginine is metabolized by nitric oxide synthase to nitric oxide (NO) [1], ADMA (but not SDMA) acts as an endogenous competitive inhibitor of this reaction. The inhibition by ADMA of NO synthesis has pathophysiological significance. Defects in NO generation have been associated with the pathogenesis of diseases such as arteriosclerosis, hypertension, and endothelial dysfunctions [2–6]. In the cardiovascular system NO is known for being the most potent vasodilator, and decreased NO biosynthesis has the potential to increase blood pressure, enhance platelet aggregation and leucocyte adhesion, increase vascular smooth muscle growth, alter mitochondrial oxygen consumption, and accelerate the development of arteriosclerotic lesions [1, 7].

Increased concentrations of plasma ADMA have been observed in renal failure, hypertension, hyperhomocysteinemia, hyperglycemia, hypercholesterolemia, and arteriosclerosis, each of which conditions are associated with impaired NO dependent vascular relaxation.

For a thorough analysis of the effects of methylated-L-arginine NO synthase inhibitors it is of central importance to develop a method that allows for the simultaneous determination of minute amounts of ADMA, SDMA, and L-arginine as well as the enzymatic degradation product L-citrulline and the precursor for the biosynthesis of L-arginine and ADMA, L-ornithine. Plasma concentrations for ADMA and SDMA are expected to be between 0.1–3.0 μM and those for L-arginine 20–150 μM. Several reports have previously described the separation of ADMA from some of the structurally related compounds using HPLC [4, 8–10], CE [11], LC-MS [12, 13], and GC-

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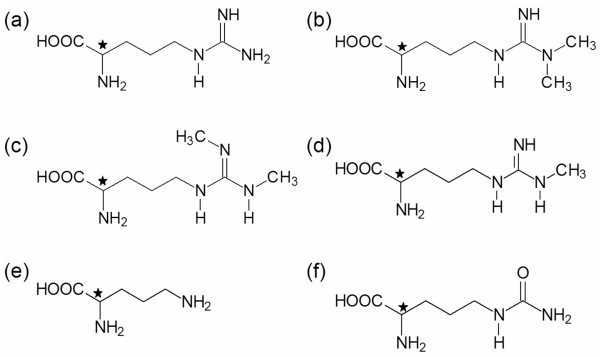

Figure 1. Chemical structures of (a) arginine, (b) asymmetric dimethyl-L-arginine (ADMA), (c) symmetric dimethyl-L-arginine (SDMA), (d) N^G-methyl-L-arginine (NMMA), (e) L-ornithine, and (f) L-citrulline. Stars mark the locations of chiral centers.

MS [14]. The most commonly used methods are based on reversed phase HPLC separation and rely on direct UV detection. Necessary pre-column derivatization methods, often employing *o*-phthalaldehyde (OPA) and thiol, require complicated and rather time-consuming derivatization procedures. The extraction and derivatization steps necessary for HPLC detection are generally highly labor-intensive and make the procedure more susceptible to human errors. To date, complete baseline separation of all four basic amino acids, L-arginine and the mono- and dimethylated derivatives as well as the educt and product of biosynthesis, L-ornithine and L-citrulline, was not achieved by a single method.

The present study focuses on the CE and MEKC separations of native ADMA from its isomer SDMA and L-arginine, NMMA, L-citrulline, and L-ornithine. **Figure 1** presents the chemical structures of these compounds. We have explored the influence of various buffers in different concentrations on the separation characteristics. We have found it advantageous to derivatize the amino acids with 4-fluoro-7-nitrobenzofurazan (NBD-F) and use laser-induced fluorescence (LIF) detection. Using this procedure with L-homoarginine as an internal standard, the limit of quantification for ADMA in aqueous solution was found to be 0.1 μM, which corresponds to 20 fg of sample for a 5-s injection.

2 Experimental methods

2.1 Instrumentation

All electrophoretic separations were performed with a Beckman P/ACE 5000 (Beckman Instruments, Fullerton, CA) equipped with either a UV absorbance or an LIF detector. Fused-silica capillaries (36-cm total length, 20-, 30-, 40-, and 50-μm inside diameter, 365-μm outside diameter) were purchased from Polymicro Technologies (Phoenix, AZ). The capillaries were maintained at 20°C. Absorbance detection was performed at 200 or 254 nm; LIF detection of the derivatized amino acids was accomplished using a 3-mW argon-ion laser (λ_{ex} 488 nm/λ_{em} 520 nm). Injections were done at a pressure of 0.5 psi and the injection times varied from 2 to 5 s. All analyses were carried out in a 193 mM borate buffer solution ($Na_2B_4O_7 \cdot 10\ H_2O$) at a pH of 9.3 (saturated at 23°C). For the MEKC experiments 100 mM sodium deoxycholate was added as surfactant to the borate buffer. Data analysis was performed with GRAMS/32 software version 4.02 (Galactic Industries Corp., Salem, NH, USA). All electropherograms presented here were scaled using GRAMS/32. Peaks were assigned by injecting a single component and comparing the migration or retention times as well as by coinjection of one of the components with the mixture.

2.2 Chemicals

Unless stated, all reagents were purchased from Sigma-Aldrich (Milwaukee, WI) in the highest grade available. Anhydrous acetonitrile was stored over molecular sieves (3 Å) for at least 24 hours prior to use. NBD-F (4-fluoro-7-nitrobenzofurazan) was obtained from Fluka (Buchs, Switzerland). SDMA (N^G, N'^G-dimethylarginine di(*p*-hydroxyazobenzene-*p'*-sulfonate)) was purchased from ALEXIS Corporation (San Diego, CA, USA). High purity water (18.2 MΩ) obtained from a Millipore-Q System (Millipore, Marlborough, MA, USA) was used to prepare the borate buffer solution.

2.3 Sample preparation

All amino acid samples were prepared as 5 mM stock solutions in water and diluted with water to the desired concentration before use. NBD-F was diluted to 6.8 mM concentration (5 mg/mL) in anhydrous acetonitrile and stored for a maximum of 3 weeks. All samples as well as the NBD-F solutions were stored at −4°C and heated to room temperature prior to use. A borate buffer at a concentration of 193 mM was used for all derivatization procedures. Derivatized samples were used for a maximum of 8 hours. Pre-column derivatization was performed by mixing the amino acid sample with a greater than 1000-fold molar excess of borate buffer and a greater than 200-fold molar excess of NBD-F solution according to the reaction scheme given in **Figure 2**. To insure complete derivatization, the sample was heated for 6 minutes at 60°C. Although UV detection of the separated derivatized components showed that derivatization was complete even without heating, quantification became significantly more reproducible for very low concentrations when the heating step was included.

Pooled human plasma samples from the Stanford Blood Bank were treated by adding 60 μL of 30% 5-sulfosalicylic acid to 300 μL of human plasma to precipitate the protein.

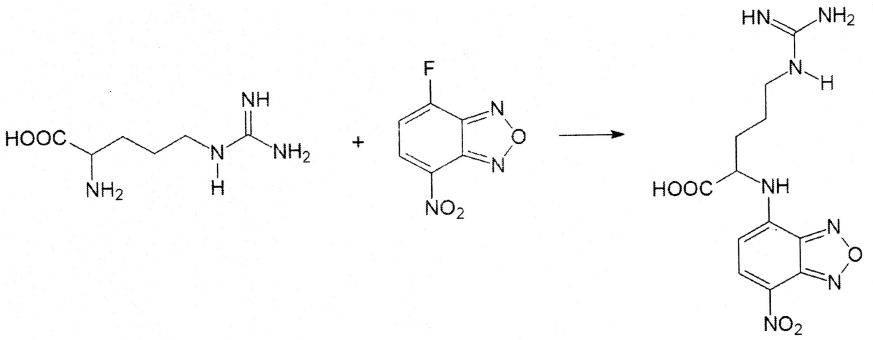

Figure 2. Derivatization of arginine with 4-fluoro-7-nitrobenzofurazan (NBD-F). Typical reaction conditions are 30 μL amino acid solution (c < 10 μM), 10 μL 193 mM borate buffer, and 30 μL 6.8 mM NBD-F solution in anhydrous acetonitrile.

The samples were then vortexed and spun at 13,000 rpm at 4°C for 10 minutes. The supernatant liquid was collected, and 12.5 μL 10 N sodium hydroxide solution was added to adjust the pH to 12.5. One-hundred μL aliquots of these samples were then immediately vacuum dried and stored at −20°C until used. Derivatization was performed immediately before use by adding 30 μL of 6.8 mM NBD-F solution and 10 μL of 193 mM borate buffer pH 9.3, followed by brief vortexing and the heating step described above. For quantitation, 20 μL of the L-homoarginine internal standard was added to the dried plasma sample before derivatization.

2.4 Measurement of ADMA by ELISA

ADMA concentrations were measured using a newly developed, highly sensitive ELISA kit (DLD Diagnostika GmbH, Hamburg, Germany). This technique is based upon the principles of a competitive immunoassay where the acylated ADMA from the pre-treated sample and the bound *N*-acyl-ADMA compete for the limited number of binding sites of the antibodies in the added antiserum. The amount of antibody that is bound to the microtiter plate is determined by the reaction of tetramethylbenzidine with the horseradish peroxidase that is coupled to the secondary antibody. The intensity of the developing color is inversely proportional to the amount of ADMA in the sample and measured by reading the optical density at 450 nm using a microtiter plate reader (Tecan GENios, Salzburg, Austria).

3 Results and discussion

The separation and quantitation of arginine related compounds in body fluids are of central importance because arginine derivatives such as asymmetric dimethyl-L-arginine are known to be important endogenous inhibitors of nitric oxide synthase. Therefore, they could play a major role in assessing the risk for cardiovascular diseases. In what follows, we present a protocol that can be successfully applied to the quantitation of these compounds in human plasma.

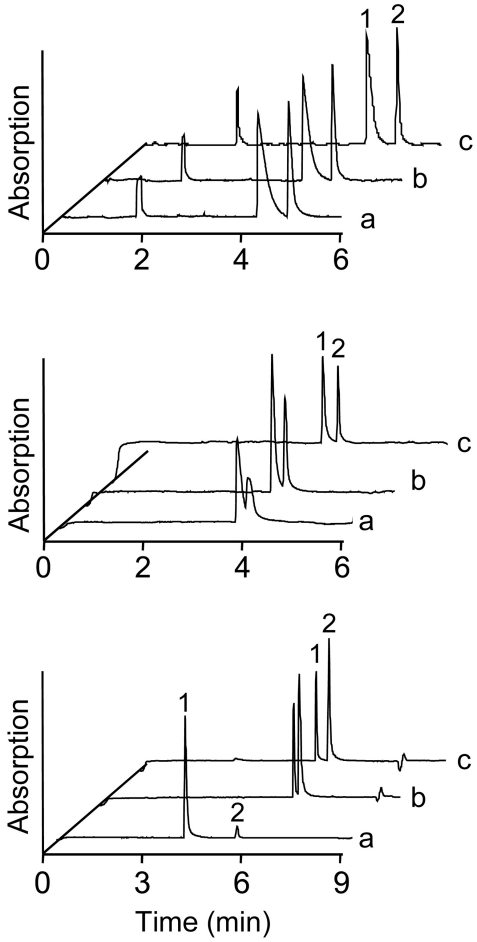

Figure 3. CE separations of an aqueous solution of ADMA (1) and L-arginine (2) with various buffers (top panel: citric acid, pH 1.8; middle panel: ammonium acetate, pH 6.5; bottom panel: phosphate, pH 7.0) at different buffer concentrations: (a) 25 mM; (b) 50 mM; and (c) 100 mM). Separation conditions are 10 kV, 200 nm absorption detection, and 20°C.

3.1 Separation of underivatized amino acids in aqueous solutions

The separation of ADMA and L-arginine was achieved as native amino acids using several buffer systems covering a wide range of pH. The buffers tested were citric acid with

Table 1. Influence of buffer type and concentration on the CE separation of ADMA and L-arginine. UV detection at 200 nm, 10 kV applied voltage, 20 °C separation temperature.

Buffer type	Concentration [mM]	pH	α	R_S
Citric acid	25	1.8	1.155	2.52
	50		1.163	3.14
	100		1.169	3.96
Citric acid/ammonium acetate	25	5.3	1.049	0.51
	50		1.053	1.12
	100		1.055	1.18
Ammonium acetate	25	6.5	1.088	0.97
	50		1.095	2.27
	100		1.098	4.12
Phosphate	25	7.0	not separated	
	50		1.012	2.02
	100		1.170	5.14
Borate	25	9.3	not separated	
	50		not separated	
	100		not separated	

a pH of 1.8, ammonium acetate/citric acid with a pH of 5.3, ammonium acetate with a pH of 6.5, phosphate buffer with a pH of 7, and borate buffer with a pH of 9.3. We found that the buffer concentration has the largest influence on the resolution R_S. Whereas typical buffer concentrations of 25 or 50 mM gave poor or no resolution or showed strong tailing of the analyte peaks, buffer concentrations of 100 mM or above yielded the best resolution as illustrated in **Figure 3**. The separation factor α on the other hand showed no significant effect upon altering the buffer concentration. For various operating conditions **Table 1** lists the resolution R_S calculated according to

$$R_s = 1.177 \frac{t_R^B - t_R^A}{w_h^A + w_h^B} \quad (1)$$

and the separation factor α calculated according to

$$\alpha = \frac{t_R^B}{t_R^A} \quad (2)$$

In equations (1) and (2), t_R is the migration time of the first (A) and second (B) eluting analyte, and w_h is the peak width at half height of the first (A) and second (B) eluting analyte. Because ADMA and L-arginine were positively charged under all but the most acidic conditions, α in Eq. (2) considers only the total migration times of the analytes and was not corrected by the migration time t_0 of an unretained marker, which would elute after the analytes, and therefore cause α to be smaller than 1 [15].

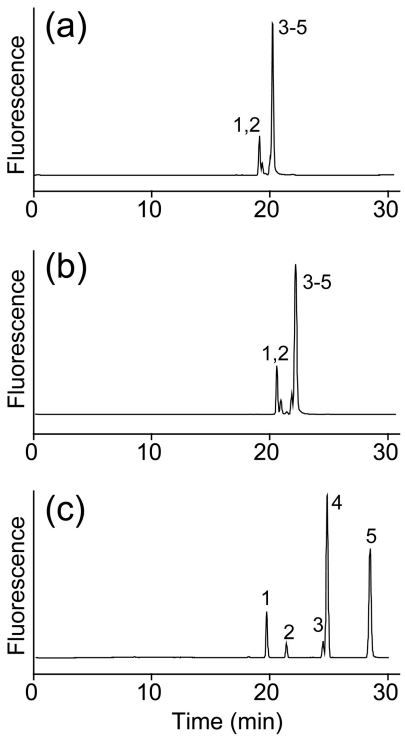

Figure 4. MEKC separation of an aqueous solution of (1) NBD-ADMA, (2) NBD-SDMA, (3) NBD-NMMA, (4) NBD-L-arginine, and (5) NBD-L-homoarginine with 193 mM borate buffer at different deoxycholic acid concentrations (top panel: 10 mM; middle panel: 50 mM, and bottom panel: 100 mM), pH 9.4. Separation conditions are 5-s injection by pressure, 10 kV, excitation wavelength 488 nm, fluorescence detection wavelength 520 nm, and 20°C.

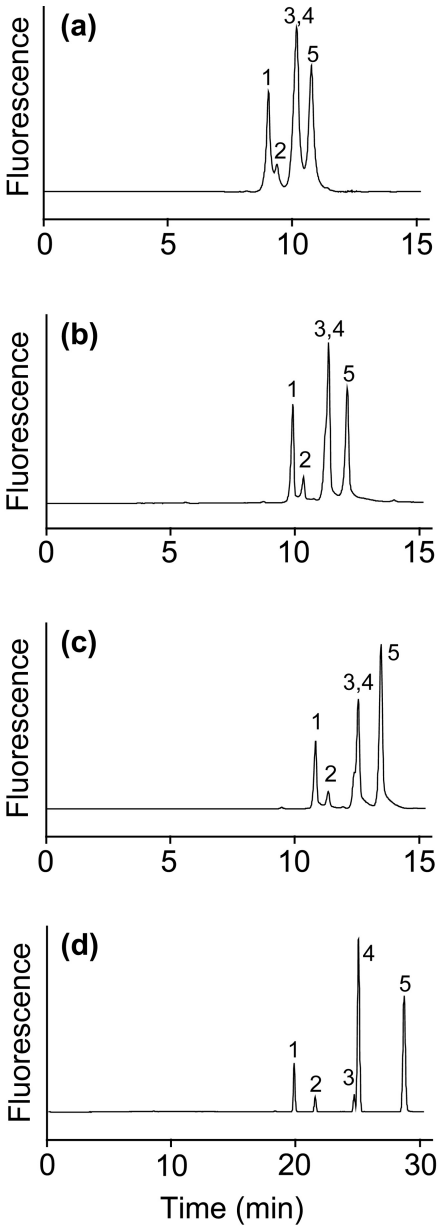

Figure 5. MEKC separation of an aqueous solution of (1) NBD-ADMA, (2) NBD-SDMA, (3) NBD-NMMA, (4) NBD-L-arginine, and (5) NBD-L-homoarginine with 100 mM deoxycholic acid at different borate buffer concentrations: (a) 25 mM; (b) 50 mM; (c) 100 mM; and (d) 193 mM. Separation conditions are the same as in Figure 4.

3.2 Influence of buffer and surfactant concentration on the CE and MEKC separations of NBD derivatized amino acids in aqueous solutions

As demonstrated above for underivatized ADMA and L-arginine, the buffer concentration has a major influence on the separation and peak shape. The same is true for the separation of the NBD-derivatized amino acids. **Figure 4** shows the influence of increasing surfactant concentration while keeping the buffer concentration constant. Increasing the concentration of the deoxycholic acid from 50 to 100 mM dramatically increases peak resolution but also doubles the retention time. A similar effect can be observed for the variation of the buffer concentration. **Figure 5** shows the increase of resolution and the improvement of the peak shape by increasing the buffer concentration from 25 to 193 mM. Comparing the CE and MEKC separations in **Figure 6.a and b** we note that the separation of NBD-NMMA from NBD-L-arginine decreases as the surfactant is added. This behavior is attributed to a difference between electrophoretic and electrokinetic separations. For higher surfactant concentrations the peak resolution improves for all of the analytes but not for NBD-NMMA and NBD-L-arginine.

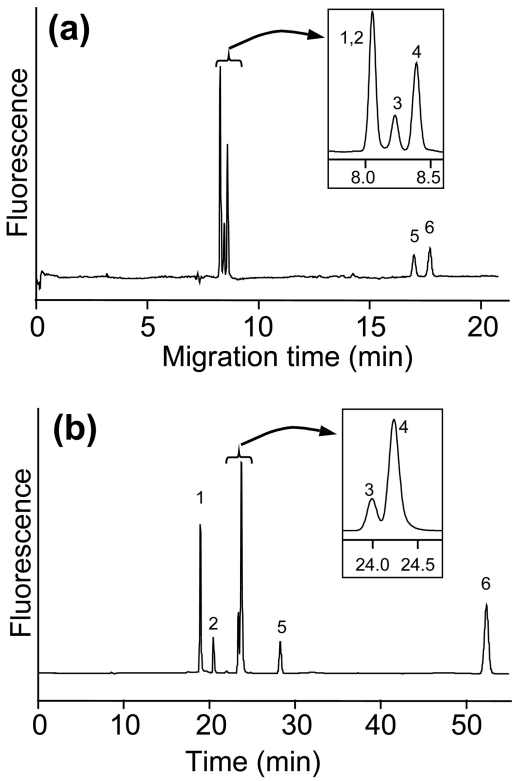

Figure 6. Separation of an aqueous solution of (1) NBD-ADMA, (2) NBD-SDMA, (3) NBD-NMMA, (4) NBD-L-arginine, (5) NBD-L-citrulline, and (6) NBD-L-ornithine carried out using (a) CE and (b) MEKC. In (a) the running buffer is 193 mM borate and in (b) the buffer contains 100 mM sodium deoxycholate. The separation conditions are the same as in Figure 4.

3.3 CE separation of NBD-derivatized amino acids in aqueous solutions

Figure 6.a shows that CE separation of NBD-ADMA, NBD-NMMA, NBD-L-arginine, NBD-L-citrulline, and NBD-L-ornithine is achieved employing 193 mM borate

buffer at pH 9.3 in a 50-μm ID capillary. Using UV absorbance, a limit of quantitation ($S/N > 10:1$) of 20 μM concentration for ADMA was determined, whereas LIF detection had a limit of quantitation of 0.1 μM in a 30-μm ID capillary. In a CE separation under these conditions, NBD-SDMA coelutes with NBD-ADMA, whereas at a lower buffer concentration of 20 mM, NBD-SDMA and NBD-ADMA separate but NBD-NMMA and NBD-L-arginine coelute. Also the separation of NBD-ADMA and NBD-L-arginine deteriorates with decreasing buffer concentration. If NBD-L-homoarginine is added to the sample mixture, it coelutes with L-arginine. The coelution of some of the L-arginine-related basic amino acids could be explained by their very similar size and charge distributions, which lead to nearly identical electrophoretic mobilities of these compounds.

3.4 MEKC separation of NBD-derivatized amino acids in aqueous solutions

Adding deoxycholic acid to the separation buffer as a surfactant allows the separation of NBD-ADMA, NBD-SDMA, NBD-NMMA, NBD-L-arginine, NBD-L-citrulline, NBD-L-ornithine, and NBD-L-homoarginine. The separation is achieved by the additional electrokinetic interaction of the analytes with the micellar pseudo-stationary phase [16]. Figure 6.b presents a typical separation of the NBD-derivatized analytes. Compared to the CE separation, the time required for the MEKC separation is significantly longer because of the additional chromatographic interactions between the pseudo-stationary phase and the analytes. Other surfactants such as cholic acid, taurocholic acid, and sodium dodecylsulfate were tested but did not lead to comparable separations under these conditions.

3.5 Influence of the capillary inner diameter on the resolution and limit of detection of NBD derivatized amino acids in aqueous solutions

Decreasing the capillary inner diameter from 50 μm to 20 μm reduces Joule heating inside the capillary at high buffer concentration conditions, thereby allowing the application of higher voltages while significantly decreasing the resulting current. This leads to a marked improvement in the separation time while resolution R_S and separation factor α stay essentially the same (cf. **Figure 7**). Smaller inner diameters also lead to an increased separation efficiency, which can be observed as an increase of the plate numbers N from up to 400,000 plates per meter for a 50-μm ID capillary to up to 1,300,000 plates per meter for a 20-μm ID capillary. This increase in efficiency more than compensates for the loss in detection path length and leads to an increase in the limit of detection (LOD, $S/N > 3:1$) with decreasing capillary inner diameter. For capillary inner diameters smaller than 20 μm clogging became a problem with plasma samples; therefore, a diameter of 30 μm was chosen.

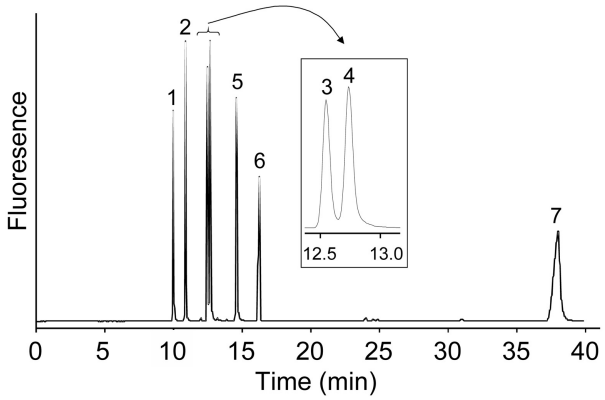

Figure 7. MEKC separation of an aqueous solution of (1) NBD-ADMA, (2) NBD-SDMA, (3) NBD-NMMA, (4) NBD-L-arginine, (5) NBD-L-homoarginine, (6) NBD-L-citrulline, and (7) NBD-L-ornithine in a 30 μm ID capillary. The separation conditions are the same as in Figure 4.

3.6 Application of the method for the analysis of human plasma samples

The developed method could be transferred to human plasma samples for the quantification of ADMA and L-arginine using L-homoarginine as an internal standard. L-Homoarginine was chosen because it does not interfere with the separation and is not naturally present in biological samples. **Figure 8** depicts the MEKC separation of a human plasma sample that was spiked with 16 mM each of NBD-ADMA, NBD-L-arginine, and NBD-L-homoarginine. The different peak areas arise from the different amounts of amino acids already present in the plasma sample and the different detector response for each ana-

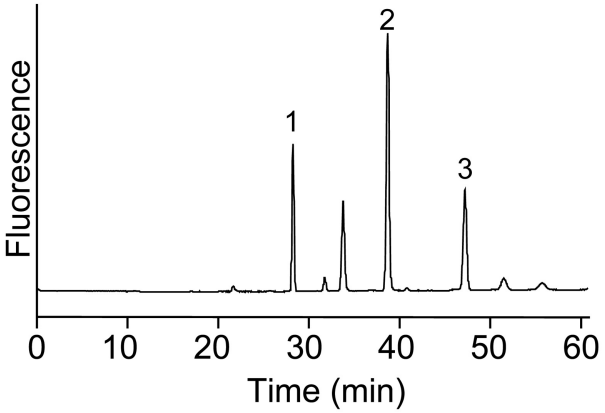

Figure 8. MEKC separation of a human plasma sample spiked with 16 mM solutions of (1) NBD-ADMA, (2) NBD-L-arginine, and (3) NBD-L-homoarginine. The separation conditions are the same as in Figure 4.

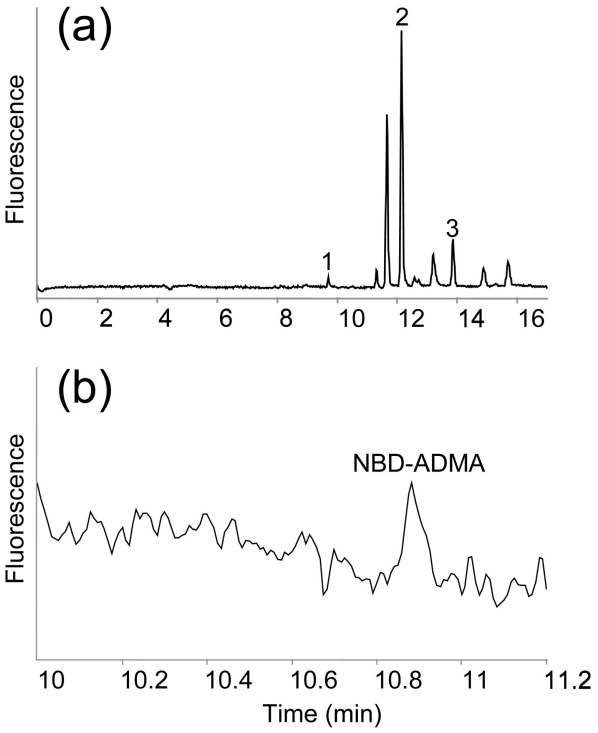

Figure 9. MEKC separations in human plasma of (a) 10 μM NBD-ADMA (1), 100 μM NBD-L-arginine (2), 50 μM NBD-L-homoarginine (3), and (b) 0.125 μM NBD-ADMA with 193 mM borate buffer containing 100 mM deoxycholic acid. Separation conditions are 5-s injection by pressure, 20 kV, excitation wavelength 488 nm, fluorescence detection wavelength 520 nm, and 20°C.

lyte. Even at reduced concentrations, these amino acids were detected in plasma samples. **Figure 9.a** shows the MEKC separation of 10 μM NBD-ADMA, 100 μM NBD-L-arginine, and 50 μM NBD-L-homoarginine. **Figure 9.b** demonstrates that the lowest concentration of NBD-ADMA that can be reliably measured in human plasma is 0.125 μM.

3.7 Quantitation of ADMA and L-arginine in plasma samples

Quantitation of NBD-ADMA and NBD-L-arginine in plasma samples was achieved by using NBD-L-homoarginine as an internal standard. First calibration curves were measured to assure a linear detector response in the expected concentration range. The concentration range of interest is 0.1 μM to 10 μM for ADMA and 1 μM to 100 μM for L-arginine. The concentration of the internal standard was kept constant at 16 μM for all measurements. When plotting the peak area ratios of analyte (NBD-ADMA or NBD-L-arginine) to NBD-L-homoarginine against the concentration of analyte, highly linear calibration curves were obtained ($R^2 > 0.9994$). To account for the different detector responses for each of the analytes and the internal standard, correction factors f were determined by measuring the peak area ratios of analyte A_a to the internal standard A_{IS} and multiplying these ratios with the respective concentrations of analyte c_a and the internal standard c_{IS} according to:

$$f = \frac{A_{IS} \cdot c_a}{A_a \cdot c_{IS}} \quad (3)$$

For ADMA a correction factor f_1 of 0.62 was found, and for NBD-L-arginine the correction factor f_2 was 0.87; these factors were independent of the concentration as long as the concentration ratio of analyte to internal standard was kept constant.

The concentration of NBD-ADMA or NBD-L-arginine was then calculated by determining the peak areas of the analytes $A_{a\,pls}$ and the internal standard $A_{IS\,pls}$ in the plasma sample. From the concentration of the internal standard in the plasma sample $c_{IS\,pls}$, which was added prior to the derivatization procedure and the previously determined correction factor f, the concentration of the analytes $c_{a\,pls}$ could be determined according to:

$$c_{a\,pls} = f \cdot \frac{c_{IS\,pls} \cdot A_{a\,pls}}{A_{IS\,pls}} \quad (4)$$

For ADMA a plasma concentration of 0.42 ± 0.02 μM was found and for L-arginine the value was 27.4 ± 3.4 μM. The inter-day precision for 18 consecutive measurements yielded a maximum relative standard deviation of 4.85% for ADMA and 12.31% for L-arginine.

Analysis of the same sample in an ELISA assay for ADMA leads to a plasma concentration of 0.57 ± 0.02 μM with an inter-day relative standard deviation of 3.6%. The use of this method has been recently validated in our laboratory. The analytical recovery of ADMA was 87.5% in human plasma, 84.7% in mice plasma, and 80.7% in rat plasma. Linearity studies showed a mean recovery of 99.3% in human plasma, 122.9% in mice plasma, and 114.1% in rat plasma. The interassay coefficient of variation was 1.5%, as determined by repeated measurements of control human plasma on four consecutive days. Quantitation of L-arginine by ELISA is not available.

Comparison of these results with those obtained by MEKC shows a significant deviation of the two methods of about 25%. Large deviations of different methods are not uncommon, as can be seen from **Table 2**. These deviations can be mainly attributed to losses from purification and derivatization steps that greatly vary from method to method.

4 Conclusions

Capillary-format separation of NBD-derivatized ADMA, SDMA, NMMA, L-arginine, L-homoarginine, L-citrulline,

Table 2. Results reported in literature for ADMA and arginine concentrations in (several) healthy human volunteers.

ADMA [μM]	L-Arginine [μM]	Method	Source	Reference
0.453 ± 0.128	119.5 ± 21.6	LC-MS	serum	13
0.390 ± 0.06	n.d	GC-MS	plasma	14
1.459 ± 1.002	n.d.	HPLC	plasma	10
1.710 ± 0.21	57.1 ± 5.7	HPLC	plasma	4
0.124 ± 0.046	62.57 ± 23.5	LC-MS	plasma	12
0.343 ± 0.022	85 ± 6	CE	serum	11
0.560 ± 0.02	n.d.	HPLC	plasma	9

n.d. = not determined.

and L-ornithine can be achieved using MEKC. We have demonstrated that the buffer and surfactant concentrations have a major influence on the peak shape as well as on the resolution of these amino acids. The strong influences of buffer and surfactant concentrations show that electrophoretic and electrokinetic separation mechanisms are both equally important for the separation. The speed of the separation could be improved by decreasing the inner diameter of the capillary without compromising the resolution of the peaks or the LOD. The low LOD of the amino acids of interest by this method hold promise for its use in routine biomedical applications. The described method is superior to previously reported chromatographic procedures because co-elution of other structurally related amino acids can be excluded, complicated two-dimensional chromatographic systems that are susceptible to failures are avoided, and the time required for sample preparation, derivatization, and analysis is significantly reduced.

Acknowledgements

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