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Journal of Chromatography B, 741 (2000) 77–83

JOURNAL OF CHROMATOGRAPHY B

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Determination of asymmetrical dimethylarginine by capillary electrophoresis–laser-induced fluorescence

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Abstract

Asymmetric dimethyl-L-arginine (ADMA) is a naturally occurring analogue of L-arginine (L-Arg), the substrate of nitric oxide synthase (NOS). ADMA is a potent endogenous inhibitor of NOS and accumulates in the plasma of patients with renal failure, with peripheral arterial occlusive disease or with clinically asymptomatic hypercholesterolemia. We measured circulating concentrations of L-arginine, symmetric and asymmetric dimethylarginine (SDMA and ADMA, respectively) in human serum. We developed a new method for the rapid determination of these molecules using capillary electrophoresis and laser-induced fluorescence (CE–LIF). All methylated arginines were labeled with fluorescein isothiocyanate (FITC) prior to analysis. Under the capillary electrophoresis (CE) conditions used, methylated arginine derivatives were well separated, with a migration time of around 10 min. These migration times were smaller than the ones of other amino acids which do not have the same charge at pH 10. Consequently, such basic amino acids were well separated from most of the other amines or amino acids. Moreover, CE allowed one to separate all the analogues of fluorescein thiocarbamyl–arginine. The results indicated that CE–LIF is useful as a selective, rapid, cheap and sensitive tool for the determination of methylated arginine products. This new technology might appreciate the endogenous substrate for NO synthase and facilitate the knowledge of the physiological and pathophysiological regulation of NO synthesis.

Keywords: Dimethylarginine; Capillary electrophoresis; Fluoresceine isothiocyanate

1. Introduction

Asymmetric dimethyl-L-arginine (ADMA) is a naturally occurring analogue of L-arginine (L-Arg), the substrate of nitric oxide synthase (NOS) [1]. Recent data suggest that ADMA is produced in several tissues and may be an endogenous inhibitor of NOS. ADMA is found elevated in the plasma of patients with renal failure [2–4], with peripheral arterial occlusive disease [5], and also during pregnancy [6].

Plasma ADMA concentration increases about two-fold in hyper-cholesterolemia, but is unaffected by dietary L-arginine [7]. Recently, it has been reported that plasma concentrations of dimethylarginine were increased in hypercholesterolemic rabbits [8,9] and

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0378-4347/00/$ – see front matter
PII: S0378-4347(00)00034-7

in clinically asymptomatic hypercholesterolemic adults [10].

Analytical methods for ADMA determination usually include ion-exchange chromatography [2] or high-performance liquid chromatography (HPLC) methods that require long and tedious sample preparations in which the sample is loaded onto an ion-exchange column and eluted with different solutions and then concentrated to dryness before injection into the HPLC column [1,2]. Recently, Chen et al. have directly separated ADMA from deproteinized human plasma using *o*-phthaldialdehyde (OPA)–mercaptoethanol as a fluorogenic reagent by reversed-phase HPLC [6].

Our study describes a new method for the rapid determination of methylated arginines, labeled with fluoresceine isothiocyanate (FITC), using capillary electrophoresis and laser-induced fluorescence (CE–LIF).

We evaluated the L-Arg/ADMA ratio in patients with chronic renal failure in hemodialysed treatment, with or without significant coronary stenosis complications.

2. Experimental

2.1. Chemicals

MMA (N^G-monomethyl-L-arginine); L-arginine, L-homoarginine, FITC (isomer I) and all buffer products (boric acid, sodium carbonate and sodium hydrogencarbonate) were obtained from Sigma (St. Louis, MO, USA). ADMA, N^G,N'^G-dimethyl-L-arginine; SDMA, N^G,N^G-dimethyl-L-arginine were obtained from Coger (Paris France)

2.2. Instrumentation

A Zeta automatic CE instrument (Picometrics, Ramonville, France) equipped with a modular LIF detector and a 20 mW argon laser (wavelength 488 nm) was used. The detector was equipped with the 488 nm filter set (Picometrics), including a 488 nm dichroic mirror, two 488 nm notch filters, a 515 nm high pass filter, and a 1.5 mm spatial filter.

2.3. Sample collection

Blood was collected into a dry Vacutainer Tube (Becton Dickinson). Five normal patients and 16 hemodialysed patients (HD, Department of Cardiology and Nephrology, University hospital Rangueil, Toulouse, France) with pathological exercise testing and coronary stenosis (detected by coronarography), were taken (before hemodialysis) and separated into two groups: (i) HD patients ($n=8$) with significant coronary lesions (stenosis$\geq$70%), (ii) HD patients without significant (stenosis$<$70%) coronary disease ($n=8$). Serum was obtained immediately after centrifugation of the blood at 2000 *g* for 5 min. Patients sera were stored at −20°C until analysis.

2.4. Derivatization

The derivatization procedure was performed as previously described [11]. All methylated arginines and L-homoarginine were dissolved in carbonate buffer (0.3 *M*, pH 9.5) to give a concentration of 10 m*M* and stored at −20°C. These stock solutions were further diluted with carbonate buffer to various concentrations. We prepared a standard serum calibration curve: (i) by mixing ADMA, L-Arg (10 μl) at final concentrations: of 10, 5, 2.5, 1.25, 0.625, 0.3125 μ*M* for ADMA and of 100, 50, 25, 12.5, 6.25, 3.125 μ*M* for L-Arg; (ii) by adding 90 μl of deproteinised serum pool (sulfosalicylic acid pretreatment).

A 100-μl aliquot of each sample was mixed with 10 μl of distilled water, 50 μl carbonate buffer (0.3 *M*, pH 9.5) and alkalinized with 10 μl of sodium hydroxide (5 *M*). After vortex mixing, 50 μl of FITC solution was added. The solution of FITC isomer I (fluorescein-5-isothiocyanate) was prepared by dissolving 1 mg of FITC in 1 ml acetone. The reaction was performed in the dark for 1 night at ambient temperature.

2.5. Capillary electrophoresis

The capillary equilibration time was realized by a 2 min wash with NaOH (1 *M*), 2 min with 0.1 *M* NaOH, 2 min with water and then 2 min with migration buffer. The analytes were separated on a 85 cm (effective length 50 cm)×50 μm I.D. fused-

silica capillary (Polymicro Technology, Phoenix, AZ, USA). The derivatized sample (1000-fold diluted) was injected automatically into the Zeta CE instrument. Samples (15 nl) were injected by hydrodynamic injection for 1 s.

Separation conditions were 50 m*M* boric acid and 20 m*M* 3-(cyclohexylamino)1-propanesulfonic acid (CAPS) adjusted to pH 9.5, 10 or 11.5. The pH is adjusted using a 10 *M* sodium hydroxide solution. The methylated arginines had a migration time of about 10 min with a separation voltage +30 kV, producing a current of 45 μA (pH 9.5) or 62 μA (pH 11.5) All the methylated arginines and L-homoarginine were baseline resolved at pH 10 and 20 kV (55 μA).

The peak of each methylated arginine was identified by spiking the diluted plasma with known quantities of the corresponding fluorescein thiocarbamyl (FTC) methylated arginine. It was quantified using the linear calibration curve based on the ratio of ADMA/LH, L-Arg/LH peak area, respectively. The peak areas were used for quantitation.

2.6. Statistical method

Results are expressed as means±standard errors of the mean (S.E.M.). Statistical significance was calculated using an unpaired Kruskall–Wallis *H* test.

3. Results and discussion

3.1. Identification of the two isomers ADMA, SDMA

Fig. 1 shows the electropherogram of the FITC-derivatized dimethylarginines. The two isomers were separated with a good resolution at pH 9.5 in 7.5 min. Under experimental conditions of CE–LIF previously reported [11–13], basic amino acids had a shorter migration time and also were identified easily and quickly. These amino acids labeled with FITC have three negative charges due to the fluoresceine nucleus and the carboxylate function of the arginine, and one positive charge due to the guanidinium function (pK_a>12). The total charge is −2. In consequence, these basic amino acids are eluted

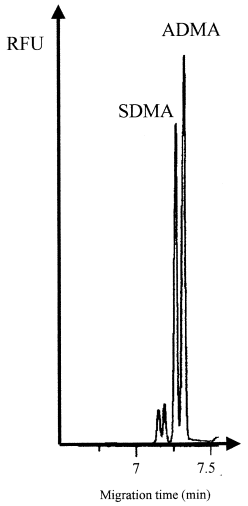

Fig. 1. Electropherogram of standard SDMA and ADMA amino acids. The amino acids are labeled with FITC at $0.5 \cdot 10^{-4}$ *M* and diluted 1000× in water. Migration conditions: boric acid 50 m*M*, CAPS 20 m*M* at pH 9.5, +30 kV (45 μA).

before the others, which have a charge of −3 for neutral amino acids and −4 for acidic amino acids.

Currently available analytical methods for the determination of methylated amino acids include ion-exchange chromatography [14] and HPLC [1,2,4,6], but the two isomers of DMA are not correctly resolved by these two methods. Few studies identified ADMA and SDMA due to very long elution time [1,4], and very poor sensitivity [2]. At this pH, if the migration times were relatively short, we were unable to separate all the different arginine-like molecules. In consequence, other pH and voltages were used in the following study.

In Fig. 1, we noticed some small peaks before the identified standards, these small peaks being related to the standards. Their migration times compared to the major peak ones indicated that they are ''bigger'' species or slightly less negatively charged species, they may be isomers of the major labeled-components. The first small peak derived from the SDMA, the second from ADMA. In contrast with other amino acids previously derivatized with FITC [11,12], we observed an evolution of the electrophoretic profile according to the time. Fig. 2 shows the modification of the electropherogram which was obtained by injecting the same sample at day 1 and day 14. It indicates that the samples were not stable

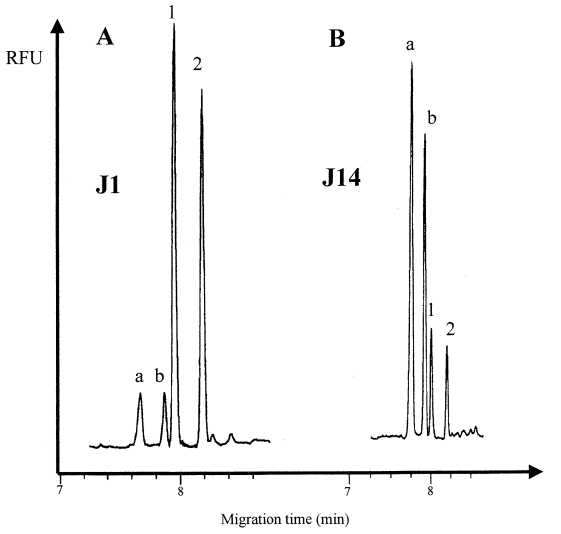

Fig. 2. Electropherogram of standard ADMA and L-arginine (pH 11.5) at different reaction times. (A) Derivatized standard solution after 12 h. (B) Derivatized standard solution after 14 days. Peak (1) and (a): ADMA. Peak (2) and (b): L-arginine.

and that for each standard the smallest peak was converted into a biggest peak and vice versa. Some chemical reactions occurred, modifying the structure of the products and changing the migration profile. The kinetics of these reactions were relatively slow and were determined (Table 1). They were obtained using $\log(1+A)=kt$ and indicated that the reaction was an unimolecular reaction which could be an intramolecular rearrangement. An adjustment of the pH to 2, in a sample of dimethylarginine after derivation was realized, the same behavior was obtained, indicating that there was no fluoresceine thiohydantoine pH-dependent reaction [15]. For these reasons all the samples were analyzed after a 1-night derivatization by FITC. All derivatized samples were stored at −20°C after analysis for possible verification.

Table 1
Kinetic parameters of the curve $\log(1+A)=kt$, where t=time (day), A=surface area of the amino acid peak[a]

Amino acid	Slope	Intercept	r^2
ADMA	−0.0306	0.576	0.86
SDMA	−0.0254	0.516	0.64
L-Arg	−0.0263	0.511	0.81
LH	−0.0293	0.531	0.82

[a] These curves indicate an unimolecular reaction for the transformation of fluoresceine thiocarbamyl arginine-like compounds.

3.2. Standard methylated arginines and arginine

The analysis of standard SDMA, ADMA, MMA, LH and L-Arg by CE–LIF was achieved at pH 10 at a lower voltage (20 kV). A baseline separation of these five amino acids within 13 min is reported Fig. 3. The migration order of these compounds was as follows: first the two dimethylarginines, then the monomethylarginine, L-homoarginine and then arginine. Arginine, homoarginine and methylated arginine have a pK_a superior than pH 12; in consequence they all have the same charge, as explained above. Their migration order is explained by their molecular mass. In this analysis where the normal polarity is used and the positive electrode is at the injection side, the observed velocity v_t of a species is:

$$v_t = v_{eo} - v_{ep} \quad (1)$$

where v_{eo} is the velocity of the electroosmotic flow and v_{ep} is the electrophoretic velocity. The smallest is v_{ep}, the highest is v_t and the shortest is the

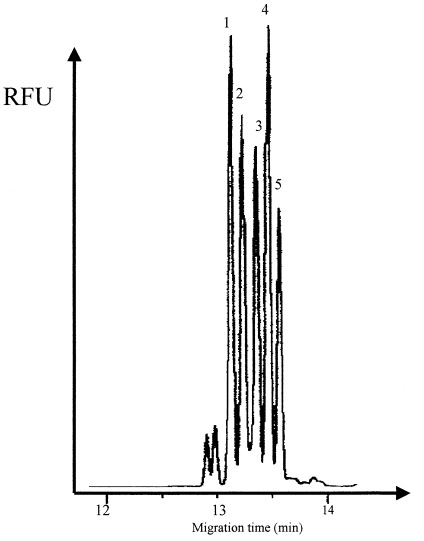

Fig. 3. Electropherogram of standard methylated arginines. (1) SDMA, $6.25\cdot10^{-9}$ *M*; (2) ADMA, $6.25\cdot10^{-9}$ *M*; (3) MMA, $6.25\cdot10^{-9}$ *M*; (4) L-homoarginine (internal standard), $6.25\cdot10^{-9}$ *M*; (5) L-Arg, $5.0\cdot10^{-9}$ *M*. Migration conditions: boric acid 50 m*M*, CAPS 20 m*M* at pH 10, +20 kV (35 μA).

migration time. The dimethylarginines, which have the largest molecular mass, have the lowest mobility and in consequence, have the shortest migration times. On the contrary, arginine, the smallest molecule, has the highest mobility and migrates after all the other arginine-like molecules.

Few studies used an internal standard. Only Vallence et al. [2] added a known amount of homoarginine and no plasma result was shown. In consequence, we used homoarginine in our study, to quantitate ADMA and arginine. On an other hand it is now accepted that ADMA inhibit NO synthesis but SDMA has been shown not to inhibit NO synthesis in vitro and in vivo studies [2], moreover MMA was not previously detected in plasma. So, our aim was to obtain a quicker analysis than previously, where we can only separate ADMA, Arg and homoarginine, and we developed conditions other than the ones described above. The separation of ADMA, L-Arg and LH was run at pH 11.5 and 30 kV and is shown in Fig. 4A.

3.3. Linearity and limit of detection

The limits of detection, defined as a signal-to-noise ratio of 3, for the methylated arginines were lower than 0.05 n*M* injected, that is to say, 0.05 μ*M* in plasma. They were 20–300-fold inferior than the ones mentioned in previous studies [3,7,10].

3.4. Calibration curves

The calibration curves of arginine and each methylated arginine were linear. Table 2 indicates the different equations of calibration. Various arginine calibrators were prepared from standard diluted in carbonate buffer and under the same conditions from a serum pool: respectively, 10, 5, 2.5, 1.25, 0.61, 0.31 μmol/l for ADMA and 100, 50, 25, 12.5, 6.25, 3.12 μmol/l for L-Arg with 10 μmol/l LH (internal standard). The concentrations were calculated according to peak areas. The calibration curves for ADMA or L-Arg quantification were obtained by plotting the peak area ratio of ADMA/LH or L-Arg/LH. The difference of the slopes and intercept between ''standard'' and ''serum'' is relatively important. It is due to the sample treatment where some loss of product can take place.

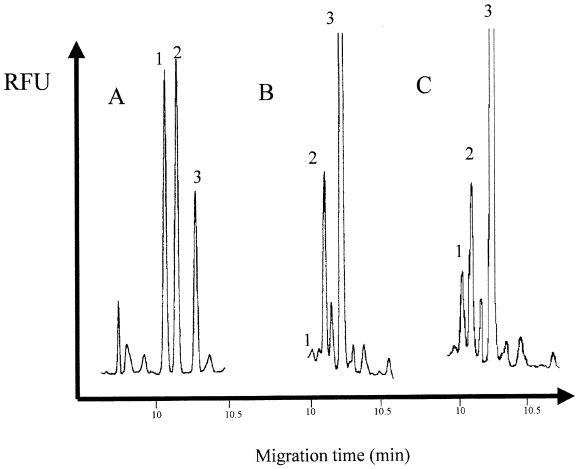

Fig. 4. Electropherograms of serum standards (A), healthy subject serum containing low (B) concentration (0.31 μmol/l) of ADMA and hemodialysed subjects serum containing high (C) concentration (2.26 μmol/l) of ADMA (peak 1), using L-homoarginine (LH, peak 2) as an internal standard and L-arginine (peak 3). Electrophoretic conditions: boric acid 50 m*M*, CAPS 20 m*M*, at pH 11.5, 30 kV (62 μA). Serums were diluted 1000-fold in water prior to the analysis.

3.5. Recovery of ADMA in human serum

Various concentrations of standard solution of ADMA were added to 100 μl of serum (Table 3). We obtained satisfactory results similar to the ones from HPLC methods [4,6,10]. The within-day reproducibility of the ADMA assay was determined with aliquots ($n=6$) prepared independently from the same serum sample, and the between-day reproducibility was calculated from the analysis of the

Table 2
Calibration equation of arginine and ADMA [area = f(concentration)][a]

Amino acid	Slope	Intercept	r^2
Standard ADMA	0.101	−0.022	0.998
Standard L-Arg	0.061	−0.038	0.999
Serum ADMA/LH	0.088	0.0121	0.999
Serum L-Arg/LH	0.061	0.5575	0.991

[a] ''Standard'': prepared directly in buffer. ''Serum'': calibration curves obtained from the standards prepared in serum (after protein precipitation with acid treatment). This treatment could be the reason of the differences between the slopes and intercept values of ''standard'' and ''serum''.

Table 3
Recovery of standard ADMA added to human serum[a]

Patient	Sample	Added	Measured	Recovery (%)
1	0.34	1	1.37	102
		5	5.8	108
2	0.45	0.5	1.04	109
		1	1.58	109
		5	5.53	101

[a] Concentration, μmol/l ($n=5$).

same serum sample that was derivatized each day during three consecutive days. The relative standard deviations which took into account every step (i.e., precipitation, centrifugation, derivatization and injection into the CE–LIF system) were 5.1% and 8.3%, respectively.

3.6. Determination of the L-Arg/ADMA ratio in serum of patients with chronic renal failure during hemodialysis treatment

Fig. 4 shows typical electropherograms obtained with sera from an healthy subject (B) and a hemodialysed patient (C). Some authors previously reported [10] that the study of the L-Arg/ADMA ratio seems to be preferable as compared to the ADMA/SDMA ratio [3,4]. The concentrations of ADMA, L-Arg and the L-Arg/ADMA ratio were determined in sera obtained from hemodialysed patients and are presented in Table 4. As previously described [2], the serum levels of ADMA were found to be significantly elevated ($P<0.01$) in hemodialysed patients. Endogenous ADMA is usually excreted unchanged in urine. In patients with chronic renal failure, who produce little if any urine, serum ADMA concentrations increase in proportion to serum creatinine. These increased serum ADMA concentrations seem sufficient to inhibit NO synthesis [2]. No differences in ADMA concentrations were observed between the two groups of hemodialysed patients having different degrees of coronary stenosis. This may suggest that ADMA (and the alterations in serum L-Arg metabolism) participates early in the formation of atheromatous plaque but probably not to the clinical complications. The increased ADMA concentrations found in patients with coronary lesions, would indicate the participation of ADMA to the development of coronary atherosclerosis, as it has been reported for peripheral arterial occlusive disease [7]. Thorough clinical investigation is required to further elucidate the role of ADMA in patients with chronic renal failure.

In summary, CE–LIF is useful as a selective, rapid, cheap and sensitive tool for the determination of methylated arginine products. This new technology might appreciate the endogenous substrate for NO synthase and facilitate the knowledge of the physiological and pathophysiological regulation of NO synthesis.

4. Abbreviations

MMA	N^G-Monomethyl-L-arginine
ADMA	Asymmetric N^G,N'^G-Dimethyl-L-arginine
SDMA	Symmetric N^G,N^G-Dimethyl-L-arginine
LH	L-Homoarginine
L-Arg	L-Arginine
FITC	Fluorescein isothiocyanate
CE	Capillary electrophoresis
LIF	Laser-induced fluorescence
NOS	Nitric oxide synthase

Table 4
Concentrations of ADMA in sera of hemodialysed patients with or without significant coronary lesions (coronary stenosis>70%)[a]

	n	L-Arg (μM)	ADMA (μM)	L-Arg/ADMA
Control	5	85±6	0.343±0.022	248±55
Without	8	121±19	0.950±0.217	127±49
With	8	144±26	1.205±0.201	119±41
P		<0.05	<0.01	<0.01

[a] Mean±standard error of mean. P between hemodialysed and control patients were calculated by unpaired Kruskall–Wallis H test.

Acknowledgements

This work was supported by grants from the Conseil Régional Midi-Pyrénées. We are very grateful to Professor M. Galinier and Dr. C. Goudable (Cardiology and Nephrology Departments, respectively, University Hospital Rangueil), who provided us with patient sera.

References

[1] K.S. Park, H.W. Lee, S.Y. Hong, S. Shin, S. Kim, W.K. Paik, J. Chromatogr. 440 (1988) 225–230.
[2] P. Vallance, A. Leone, A. Calver, J. Collier, S. Moncada, Lancet 339 (1992) 572–575.
[3] R.J. MacAllister, M.H. Rambausek, P. Vallance, D. Williams, K.H. Hoffmann, E. Ritz, Nephrol. Dial. Transplant. 11 (1996) 2449–2452.
[4] A. Pettersson, L. Uggla, V. Backman, J. Chromatogr. B 692 (1997) 257–262.
[5] R.H. Böger, S.M. Bode-Böger, W. Thiele, W. Junker, K. Alexander, J.C. Frölich, Circulation 95 (1997) 2068–2074.
[6] B.M. Chen, L.W. Xia, R.Q. Zhao, J. Chromatogr. B 692 (1997) 467–471.
[7] S.M. Bode-Böger, R.H. Böger, S. Kienke, W. Junker, J.C. Frölich, Biochem. Biophys. Res. Commun. 219 (1996) 598–603.
[8] X. Yu, Y. Li, Y. Xiong, Life Sci. 54 (1994) 753–758.
[9] R.H. Böger, S.M. Bode-Böger, A. Mugge, S. Kienke, R. Brandes, A. Dwenger, J.C. Frolich, Atherosclerosis 117 (1995) 273–284.
[10] R.H. Böger, S.M. Bode-Böger, A. Szuba, P.S. Tsao, J.R. Chan, O. Tangphao, T.F. Blaschke, J.P. Cooke, Circulation 98 (1998) 1842–1847.
[11] E. Caussé, R. Terrier, S. Champagne, M. Nertz, P. Valdiguié, R. Salvayre, F. Couderc, J. Chromatogr. A 817 (1998) 181–185.
[12] G. Nouadje, H. Rubie, E. Chatelut, P. Canal, M. Nertz, Ph. Puig, F. Couderc, J. Chromatogr. A 717 (1995) 293–298.
[13] E. Caussé, N. Siri, H. Bellet, S. Champagne, C. Bayle, P. Valdiguié, R. Salvayre, F. Couderc, Clin. Chem. 45 (3) (1999) 412–414.
[14] M. Kimoto, T. Sasakawa, H. Tsuji, S. Miyatake, T. Oka, N. Nio, T. Ogawa, Biochim. Biophys. Acta 1337 (1997) 6–10.
[15] H. Maeda, N. Ishida, H. Kawauchi, K. Tuzimura, J. Biochem. 65 (1969) 777–782.