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Determination of dimethylated arginines in human plasma by high-performance liquid chromatography

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Abstract

Using high-performance liquid chromatography (HPLC) with multigradient elution, N^G,N^G-dimethyl-L-arginine (asymmetric-DMA, ADMA) and N^G,N'^G-dimethyl-L-arginine (symmetric-DMA, SDMA) can be separated from human plasma samples. The dimethylarginine compounds in plasma, after extraction with a cation-exchange column, are converted to fluorescent derivatives with *o*-phthaldialdehyde (OPA) in an alkaline medium and the derivatives are separated simultaneously within 50 min on a reversed-phase column (Ultracarb 3 ODS(20)). The recoveries of ADMA and SDMA are over 80% and the method permits quantitative determination of dimethylated arginines at concentrations as low as 0.1 μmol/l in human plasma.

Keywords: Dimethylarginine; Asymmetric dimethylarginine

1. Introduction

Endothelium releases several vasoactive agents that interact with smooth muscle cells to regulate vascular tone, thus contributing to the control of blood pressure [1]. One such agent is the labile humoral factor endothelial derived relaxing factor (EDRF) [2], now identified as nitric oxide (NO) [3] and shown to be derived from the terminal guanidino nitrogen atoms of L-arginine [4]. This NO mediated vasorelaxation has been extensively studied and subsequently many inhibitors of the arginine–NO pathway have been characterized. A common feature of these compounds is a modification of the guanidinic group of L-arginine [5] and it has been shown recently that the endogenously produced N^G,N^G-dimethyl-L-arginine (asymmetric-DMA, ADMA) has potent inhibitory effects on NO synthesis [6]. ADMA and the biologically inactive enantiomer N^G,N'^G-dimethyl-L-arginine (symmetric-DMA, SDMA) (Fig. 1), are known to occur in several tissue proteins, methylated post-translationally, and are released in body fluids after in vivo protein degradation. ADMA has recently been shown to accumulate in plasma from patients with renal failure [6] and the resulting inhibition of NO production could explain, at least in part, the hypertension that occurs in this condition.

Currently available analytical methods for the determination of methylated amino acids, including HPLC, cannot easily differentiate the two enantiomers of dimethylarginine. The purpose of the present study was to establish a simple and rapid HPLC

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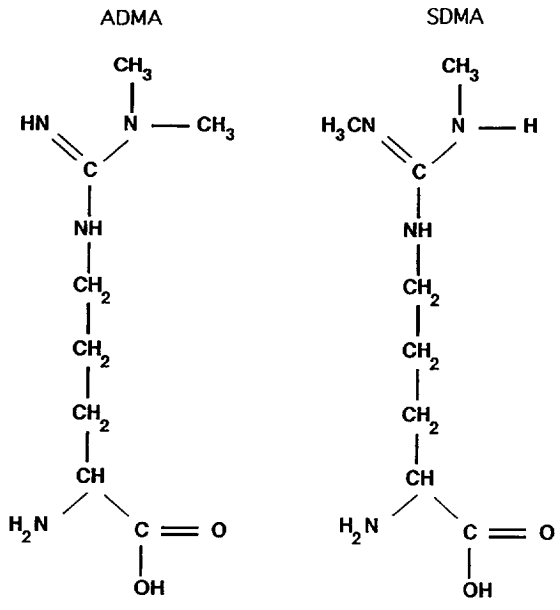

Fig. 1. Chemical structures of ADMA and SDMA.

method utilizing the OPA pre-column fluorescence derivatization technique [7] for analysis of ADMA.

2. Experimental

2.1. Instrumentation

The chromatographic system consisted of an HP-1050 series liquid chromatograph with a HPLC gradient delivery pump, a HP-1050 programmable variable volume autoinjector with a cooling device, a HP 35900 A/D-converter and a HPLC2D ChemStation data system. These components were supplied by (Hewlett-Packard, Waldbronn, Germany). A Waters 474 scanning fluorescence detector (Millipore, Milford, MA, USA) with a 338 nm bandpass excitation filter, a 425 nm longpass emission filter and a flow cell of 5 μl was used for detection. Separation of ADMA and SDMA was performed on a 3 μm Ultracarb 3 ODS(20) (150×4.6 mm O.D.) column (Phenomenex, Torrance, CA, USA).

All the samples and reagents were kept at +10°C in the autoinjector whereas the column was kept at room temperature.

2.2. Chemicals and reagents

The following compounds were used; crystalline salts of N^G,N^G-dimethyl-L-arginine (asymmetric-DMA, ADMA), N^G,N'^G-dimethyl-L-arginine (symmetric-DMA, SDMA), Brij 35 (30%), *o*-phthaldialdehyde (OPA), 2-mercaptoethanol, trichloroacetic acid (TCA) and triethylamine (Sigma, St. Louis, MO, USA); boric acid, monosodium dihydrogen phosphate, disodium monohydrogen phosphate, methanol (HPLC LiChrosolv, gradient quality) and acetonitrile (HPLC LiChrosolv) all obtained from Merck (Darmstadt, Germany); potassium hydroxide (EKA Bohus, Sweden); tetrahydrofuran (THF) (HPLC-grade) (Fisons, Loughborough, UK); nitrogen (Plus) and helium (Plus 4.6) (AGA, Gothenburg, Sweden).

Isolute-SCX (100 mg) columns were obtained from IST (Mid-Glamorgan, UK). Distilled water used for the preparation of buffers and standards was deionized with Milli-Q purification system and syringefilters Millex-GV were obtained from Millipore (Milford, MA, USA).

2.3. Biological samples and sample preparation

The study was approved by the local ethics committee and all subjects gave their written informed consent. Venous blood from 10 healthy volunteers (4 men, age 40±2 years and 6 women, age 40±2 years (means±S.E.M.)) and 10 patients with end stage renal failure (5 men, age 47±3 years and 5 women, age 45±4 years (means±S.E.M.)) was collected into heparinised tubes, immediately cold centrifuged (+4°C) at 2700 *g* for 20 min to separate plasma. All plasma samples were kept at −70°C if not analysed immediately.

Individual 5 mmol/l standard stock solutions of ADMA and SDMA were prepared in distilled water. A standard mixture was diluted first to 500 μmol/l and then to 0.05, 0.1, 0.5, 1.0, 2.5 and 7.5 μmol/l to construct a calibration curve.

Sodium phosphate buffer (0.5 mol/l, pH 8) was prepared by gradual additions of monosodium dihydrogen phosphate (0.5 mol/l) to disodium monohydrogen phosphate (0.5 mol/l) and diluted to a concentration of 0.15 mol/l with distilled water.

ADMA and SDMA were extracted from the

plasma samples with a cation-exchange column, Isolute-SCX (100 mg). All extraction procedures were conducted at room temperature. The column was activated with 1 ml methanol, conditioned with 2 ml TCA (2%) and loaded with 1.5 ml plasma or standard solutions. The column was subsequently rinsed with 1 ml TCA (2%), 3 ml phosphate buffer (pH 8) and 1 ml methanol. ADMA and SDMA were eluted in 2 ml methanol containing 30% distilled water and 2% fresh triethylamine prepared daily. The eluent was then evaporated to dryness at +60°C under nitrogen. The dried extract was redissolved in 100 μl distilled water and passed through a 0.22 μm filter (Millex-GV).

2.4. Chromatographic conditions

Sodium phosphate buffer (0.05 mol/l, pH 7.1) was prepared by gradual additions of monosodium dihydrogen phosphate (0.05 mol/l) to disodium monohydrogen phosphate (0.05 mol/l) and diluted to a concentration of 0.015 mol/l with distilled water. The buffer was filtered through a 0.45 μm filter membrane.

Mobile phase A consisted of 0.015 mol/l phosphate buffer–methanol–tetrahydrofuran (99:0.5:0.5, v/v) and mobile phase B consisted of 0.015 mol/l phosphate buffer–methanol–acetonitrile (40:45:15, v/v). These solutions were degassed continuously with helium during the analysis. The tetrahydrofuran in mobile phase A was of great importance for the success of the separation and it had to be a fresh solution in order to work properly.

The separation of ADMA and SDMA was performed with a multigradient programme by using 100% mobile phase A from start, followed by linear increases in mobile phase B to 10% at 1 min, 25% at 7 min, 40% at 40 min, 63% at 55 min, 100% at 60 min. 100% B was kept for 10 min, followed by a linear decrease in B to 0% at 70 min. An equilibrium time of 8 min at 100% A was maintained prior to the next injection. The analysis was carried out at a flow-rate of 0.8 ml/min.

2.5. Derivatization procedure

The derivatization reagent was prepared by dissolving 5.0 mg of anhydrous *o*-phthaldialdehyde in 100 μl methanol. To this solution 900 μl of 0.4 mol/l borate buffer (adjusted to pH 10.4 with 1 mol/l potassium hydroxide containing 0.6% of 30% Brij 35) was added. This was followed by addition of 5 μl of 2-mercaptoethanol. The mixture was kept at +4°C for 24 h before use. It remained stable for one week, provided that after every three days 1 μl of 2-mercaptoethanol was added.

The derivatization of OPA with amino acids was performed in the autoinjector by automatically mixing 10 μl OPA with 10 μl standard or sample for 1 min in the needle before injecting the mixture into the analytical column. It is important to standardize this procedure, with regard to time, in order to avoid unreliable results in recording the fluorescent intensity of the OPA-amino acid adduct.

2.6. Statistical method

Results are expressed as means±S.E.M. and compared by use of Student's *t*-test.

3. Results and discussion

Currently available analytical methods for the determination of methylated amino acids include paper chromatography [8] and electrophoresis [9], thin-layer chromatography and electrophoresis [10] and ion-exchange chromatography [11–17]. Recently, high-performance liquid chromatography has also been employed for the quantitative and qualitative analysis of dimethylarginines in protein [18] and in plasma [6,19], but the two enantiomers of dimethyl-L-arginine have not been well resolved by these methods. The chromatographic conditions recommended is one of the results of the present investigations for the complete separation of ADMA and SDMA. Furthermore, this improved HPLC method, is more sensitive and faster than other methods published.

Fig. 2a shows a typical chromatogram obtained with plasma from a healthy subject. The OPA derivatives corresponding to dimethylarginines in the sample are separated within 50 min on the Ultracarb 3 ODS(20) column, with gradient elution under HPLC conditions described in Section 2.4.

The ADMA and SDMA peaks produced by the

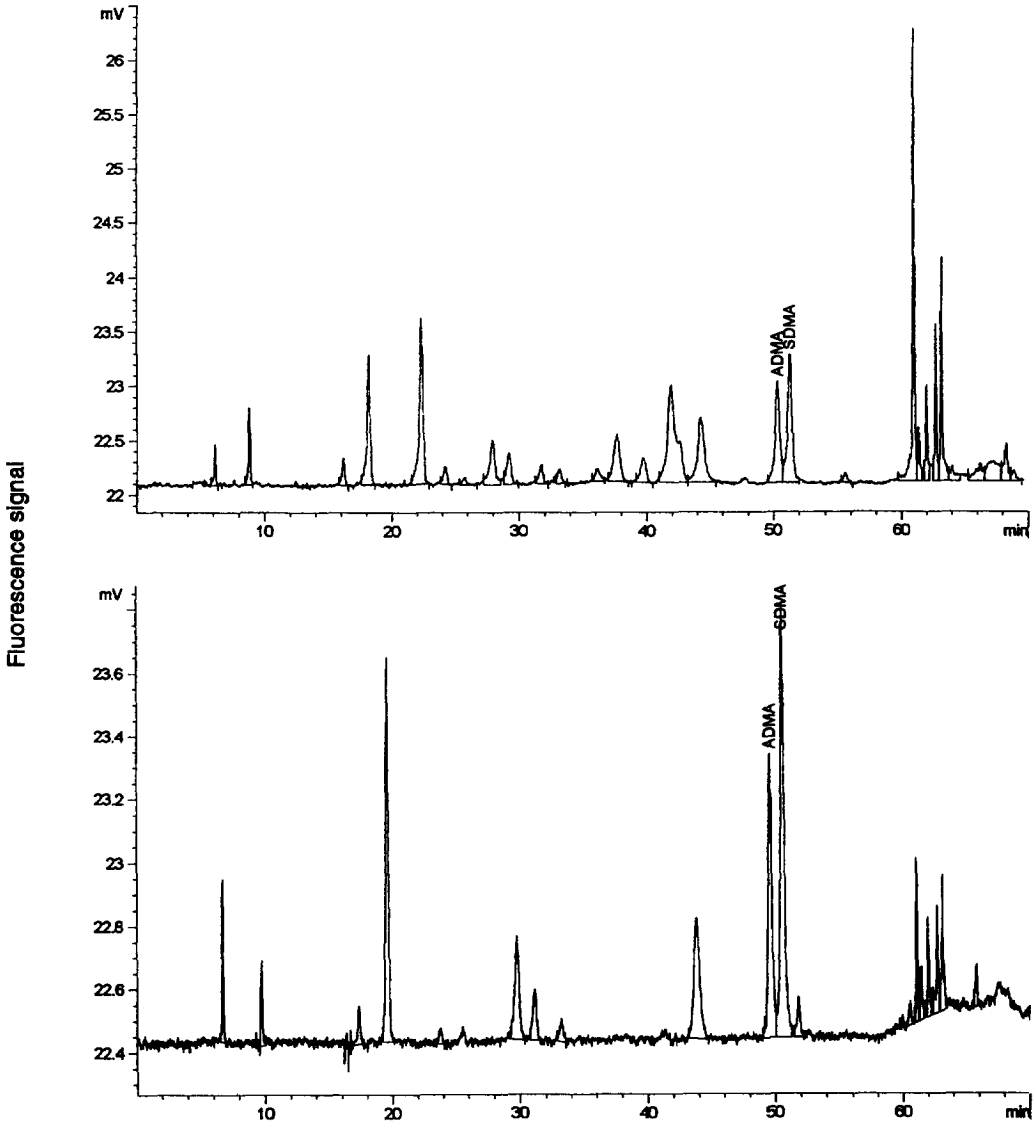

Fig. 2. (a) Typical chromatogram of OPA-derivatized dimethyl-L-arginines in human plasma. (b) HPLC separation of ADMA and SDMA standards (0.5 μmol/l) derivatized with OPA. For chromatographic conditions see Section 2.4.

derivatization with OPA, can be readily identified on the basis of their retention times in comparison with the standard compounds (Fig. 2b). Each peak represents 0.5 μmol/l corresponding to 5 pmol per injection of 10 μl, suitable for this HPLC method.

The limits of detection, defined as a signal-to-noise ratio of 3, for the dimethyl arginines determined by the presented method are 0.015 μmol/l (ADMA) and 0.01 μmol/l (SDMA), respectively.

A modified [6] plasma extraction procedure with a cation-exchange column was used in order to exclude interfering acidic and neutral amino acids, present in the plasma samples. Because of the basic nature of the dimethylarginines, the extraction pro-

Table 1
Recovery of standard methylated amino acids added to human plasma prior to sample preparation

Standard amino acid added	Amount added (μmol/l plasma)	Recovery (%)
ADMA[a]	1.0	85
	5.0	83
SDMA[a]	1.0	82
	5.0	80

[a] ADMA and SDMA represent *N,N*-dimethyl-L-arginine and *N,N'*-dimethyl-L-arginine, respectively.

cedure gave fairly good recoveries (>80%, coefficient of variation 3–11%) regardless of the amount of standard amino acids added (Table 1).

The method of analysis gives reproducible results in terms of retention time and integrated area, with coefficients of variation <0.2% and 5.6% (ADMA), <0.1% and 4.8% (SDMA), respectively, by running 8 replicates from one plasma sample. Furthermore, duplicates from the same plasma sample were assayed on 5 different occasions, giving the intra-assay coefficients of variance for retention time and integrated area, 3.2% and 7.6% (ADMA), 4.4% and 7.1% (SDMA), respectively.

Concentrations of ADMA and SDMA in plasma, relative to a spiked standard (0.05–7.5 μmol/l), from 10 healthy humans were analyzed by the present method. The mean concentrations of ADMA and SDMA were 0.58±0.02 μmol/l and 0.56±0.02 μmol/l, respectively, giving an ADMA–SDMA ratio of 1.04 (range from 0.79 to 1.40), which is in good agreement with the previous studies [6,8].

Endogenous ADMA is assumed to be excreted unchanged in the urine and in patients with chronic renal failure, plasma concentrations of dimethyl-arginines have been shown to be elevated in proportion to serum creatinine [6]. Increased plasma ADMA concentrations (1.25±0.07 μmol/l) in patients with end stage renal failure were also found in the present study (Fig. 3). Interestingly, plasma concentrations of SDMA (3.15±0.20 μmol/l) were significantly ($p<0.01$) higher compared to plasma ADMA concentrations, changing the ADMA–SDMA ratio to 0.4 in this group of patients. This may exploit the fact that SDMA is more susceptible to renal excretory function, while ADMA also undergo metabolic

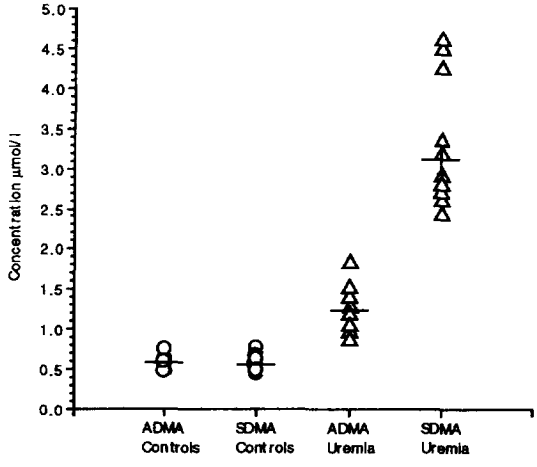

Fig. 3. Individual plasma concentrations of ADMA and SDMA in healthy controls and in patients with end stage renal failure. Compared to healthy controls, $*p<0.01$.

clearance by N^G,N^G-dimethylarginine dimethylaminohydrolase [20–23].

In a separate series of experiments, the stability of dimethylarginines in blood samples was studied. No significant degradation of either ADMA nor SDMA was found in whole blood or plasma during up to 24 h in room temperature compared to blood samples taken on ice and subsequently cold centrifugated (+4°C) and stored at −70°C (Table 2).

Increased plasma ADMA concentrations, either due to increased protein methylation, increased protein degradation and/or decreased renal elimination, may not only be of interest with respect to control of blood pressure, but also with respect to platelet aggregation, white cell function and vascular smooth muscle proliferation, physiological events where the arginine–NO system plays an important regulatory role.

The OPA derivatives of dimethylarginines, obtained by pre-column derivatization, could be used for quantitative measurements of plasma concentrations down to 0.15 μmol/l (ADMA) and 0.10 μmol/l (SDMA).

In addition, the amount of plasma required for the analysis was 3 ml, which could easily be obtained from patients. Hence, the method described can readily be employed for quantitative analysis of ADMA and may be useful for the further under-

Table 2
Viability of ADMA and SDMA in whole blood and plasma at room temperature (25°C)

Time before sample[a] was frozen at −70°C (h)	Amino acid[b]	Concentration when analysed (μmol/l)[c]
Blood		
0	ADMA	0.55±0.02
	SDMA	0.45±0.02
2	ADMA	0.57±0.03
	SDMA	0.42±0.02
8	ADMA	0.61±0.02
	SDMA	0.52±0.02
24	ADMA	0.52±0.02
	SDMA	0.38±0.01
Plasma		
0	ADMA	0.40±0.02
	SDMA	0.31±0.01
2	ADMA	0.42±0.02
	SDMA	0.32±0.02
8	ADMA	0.41±0.01
	SDMA	0.36±0.02
24	ADMA	0.41±0.03
	SDMA	0.31±0.02

[a] Blood and plasma from one healthy subject.
[b] ADMA and SDMA represent *N,N*-dimethyl-L-arginine and *N,N'*-dimethyl-L-arginine, respectively.
[c] Each value represents the mean±S.E.M. of four samples.

standing of the physiological and pathophysiological regulation of NO formation.

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