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Short communication

Improved method for simultaneous determination of L-arginine and its mono- and dimethylated metabolites in biological samples by high-performance liquid chromatography

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Abstract

An improved method has been developed for the determination of L-arginine and its methylated metabolites, N^G-monomethyl-L-arginine (L-NMMA), N^G,N^G-dimethyl-L-arginine (asymmetric DMA, ADMA) and N^G,$N^{G\prime}$-dimethyl-L-arginine (symmetric DMA, SDMA) in biological samples. Extraction of these compounds with a strong cation-exchange resin AG50W-X8 with L-homoarginine (2-amino-6-guanidinohexanoic acid) as an internal standard gave a recovery of more than 70% except for SDMA from plasma samples. After extracted samples were converted to fluorescent derivatives with *o*-phthalaldehyde (OPA) in an alkaline medium, the following high-performance liquid chromatographic separation with a ODS column (wide-pore size, 300 Å) was successfully performed with an isocratic mobile phase system. The method permits quantitative determination of L-arginine and its methylated metabolites at concentrations as low as 4 μ*M* and 0.18 μ*M*, respectively. Using this method, the levels of L-arginine, L-NMMA, ADMA and SDMA in human plasma, urine and rat tissue were determined.

Keywords: Arginine; Methylarginines

1. Introduction

Nitric oxide (NO) which plays an important role in the regulation of vascular tone, neurotransmission and host defense [1] is synthesized from L-arginine by NO synthase (NOS, EC 1.14.13.39) [2]. Because L-arginine metabolites, N^G,N^G-dimethyl-L-arginine (ADMA) and N^G-methyl-L-arginine (L-NMMA), are capable of inhibiting cellular L-arginine uptake and/or NO synthase activity competitively [3,4], the low availability of cellular L-arginine and/or accumulation of endogenous inhibitors would lead to decreased NO production. Decreased L-arginine/ADMA ratio in blood is thought to be, in part, correlated with hypercholesterolemia [5], congestive heart failure [6,7], arterial occlusive disease [8], chronic renal failure [9] and hypertension [10]. In contrast, exogenous administration of L-arginine was

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shown to restore the physiological activity of NO in experimental hypercholesterolemia [5,11]. For these reasons, methods for determination of L-arginine and its methylated metabolites, such as derivatization with *o*-phthalaldehyde (OPA) or phenylisothiocyante (PTIC) and separation of the corresponding derivatives by high-performance liquid chromatography (HPLC) have been developed [5,7,9,12–17]. However, the published methods have some limitations: although extraction of the biological samples was done with solid-phase extraction (SPE) cartridges containing cation exchangers, such as Bond Elut CBA and/or Bond Elute SCX materials, the use of a suitable internal standard for quantification was not reported for most of the used procedures. Some attempts only focused upon separation of L-arginine, ADMA and N^G,$N^{G\prime}$-dimethyl-L-arginine (SDMA), a biologically inactive stereoisomer of ADMA [3], but not L-NMMA [5,9,12,13,15]. Meyer et al. [14] showed the simultaneous separation of L-arginine, ADMA and L-NMMA in human serum whereas no information about SDMA was reported. Therefore, an accurate and reproducible procedure is required to perform simultaneous determination of L-arginine and its mono- and dimethylated metabolites. This paper describes an improved method for extraction of L-arginine, L-NMMA, ADMA and SDMA from biological fluids and simultaneous separation of these compounds.

2. Experimental

2.1. Chemicals

L-Arginine, ADMA, SDMA, L-homoarginine (2-amino-6-guanidinohexanoic acid), L-NMMA and OPA were purchased from Sigma (St. Louis, MO, USA). The cation exchanger resin AG50W-X8 (100–200 mesh, hydrogen form) was obtained from Bio-Rad Labs. (Richmond, CA, USA). All other reagents and solvents were of analytical or HPLC grade.

2.2. Chromatographic conditions

The chromatographic equipment consisted of a Shimadzu solvent delivery system (a SCL-6A system controller and two LC-6A pump), a Shimadzu Chromatopac C-R1B integrator, and a Shimadzu RF-550 spectrofluorometric detector (Kyoto, Japan). A stainless steel ODS column (250×4.6 mm I.D., 5 μm particles, 300 Å pore diameter) equipped with an ODS guard column (23×4 mm I.D.) from Yamamura Chemical Labs. (Kyoto, Japan) was used. Separation was carried out with 50 m*M* citrate buffer (pH 6.8)–methanol–acetonitrile (920:72:153, v/v/v) at ambient temperature with a flow-rate of 1 ml/min. L-Arginine and its methylated metabolites were measured fluorimetrically at excitation and emission wavelengths of 340 nm and 455 nm, respectively.

2.3. Sample collection

Biological samples, venous blood from seven healthy volunteers (age range 25–35 years, five male and two female subjects) were drawn during fasting for 12 h and collected in heparinised tubes, immediately centrifuged for 20 min at 4°C and 800 *g* for separation of plasma. Morning spot urine samples from nine healthy volunteers (age range 24–35 years, five male and four female subjects) were collected. Wistar male rats (8 weeks old) were sacrificed and the kidney was homogenized in nine volumes of 10 m*M* Tris–HCl buffer (pH 7.5). The homogenates were then centrifuged for 75 min at 90 000 *g* and the supernatant removed and stored at −70°C until use. All the samples were stored frozen at −70°C before use.

2.4. Extraction procedure and derivatization

Human plasma, urine, rat kidney cytosol or standard solutions (0.5 ml each) spiked with 0.5 ml of 4 μ*M* L-homoarginine as an internal standard and 1 ml of 40 m*M* acetate buffer (pH 5.5) was applied to a column packed with AG50W-X8 (1 ml), which had previously been equilibrated with 4 ml of 20 m*M* acetate buffer (pH 5.5). The column was washed with 4 ml of 20 m*M* acetate buffer (pH 5.5), followed by 4 ml of water, 2 ml of 0.05 *M* ammonia, 8 ml of water, and 1 ml of methanol to elute proteins, acidic and neutral amino acids and weakly basic amines from the cartridge. L-Arginine and its methylated metabolites were then eluted with 3 ml of 25% aqueous ammonia–methanol (1:1, v/v). The eluate was evaporated to dryness under nitrogen at

80°C. The residue was dissolved in 0.2 ml of 0.2 *M* borate buffer (pH 8.5). Derivatization of amino acids with OPA was performed by the published methods [13] with minor modifications. Briefly, the OPA reagent was prepared as follows: OPA (5 mg) was dissolved in 100 μl methanol, mixed with 900 μl 0.4 *M* borate buffer (pH 8.5) and 5 μl 2-mercaptoethanol. The OPA reagent should be kept in the dark and freshly prepared every 2 days. The borate buffer solution containing L-arginine and its methylated metabolites was passed through a 0.22-μm filter. An aliquot (45 μl) was mixed with 5 μl of OPA reagent for 30 s exactly at room temperature, and 20 μl of the reaction mixture was immediately injected onto the HPLC system.

2.5. Standard curves and recovery

The concentration ranges for standards were 0.25 to 4 μ*M* for ADMA, SDMA and L-NMMA and 12.5 to 200 μ*M* for L-arginine. All standards were kept refrigerated, and showed no measurable degradation over a period of 10 weeks. The standard curves were calculated by plotting the ratio of areas or heights of L-arginine analogs and L-homoarginine against the concentration of L-arginine and its methylated metabolites. For determination of recovery, 40 nmol of L-arginine and 0.8 nmol of ADMA, SDMA and L-NMMA were added to the biological fluids (0.5 ml each), and then the spiked samples were extracted and determined by the procedure described above.

3. Results and discussion

Fig. 1 shows a typical chromatogram of authentic L-arginine and its mono- and dimethylated metabolites after derivatization with OPA–2-mercaptoethanol [18]. L-Arginine (t_R=12.5 min), L-NMMA (t_R=16.9 min), ADMA (t_R=21.9 min), SDMA (t_R=22.9 min), and the internal standard L-homoarginine (t_R=19.2 min) were successfully separated in the isocratic mobile phase used. A linear response between the peak area and concentration of L-arginine was obtained over the concentration range 12.5–200 μ*M*, which is expressed by the equation: $y=0.1797x-0.2434$ [*y* is the ratio of L-arginine/L-homoarginine by peak area and *x* is the concentration of L-arginine (μ*M*)] with coefficient of correlation of 0.999. Calibration curves for L-NMMA, ADMA and SDMA were linear over the range 0.25–4 μ*M*, and the linear regressions were expressed as the equations: $y=0.3124x-0.0476$, $y=0.3041x-0.0516$ and $y=0.2407x-0.043$, with correlation coefficients of 0.999, 0.999 and 0.998, respectively [*y* is the ratio of L-NMMA, ADMA or SDMA/L-homoarginine by peak area and *x* is the concentration of them (μ*M*)]. The limit of quantification is 4 μ*M* for L-arginine and 0.18 μ*M* for L-arginine metabolites, respectively. Calibration curves for L-NMMA, ADMA and SDMA were also linear over the range 0.25–4 μ*M* estimated by heights with correlation coefficients >0.992.

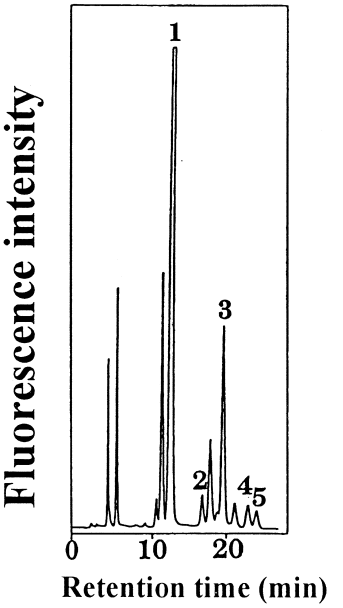

Fig. 1. HPLC chromatogram of a mixture of authentic L-arginine and its methylated metabolites. 1, L-Arginine; 2, L-NMMA; 3, L-homoarginine; 4, ADMA; 5, SDMA. The chromatographic conditions are described in the Experimental section.

The relative instability of OPA amine derivatives, which dependent on the derivatization conditions, often yield falsely low concentrations in particular for samples injected after a long storage time in the autosampler. For this reason, manual injection was selected instead of autosampler in our method. The OPA derivatives under our experimental conditions were found to be stable within 10 min of storage time before injection (data not shown). It has been

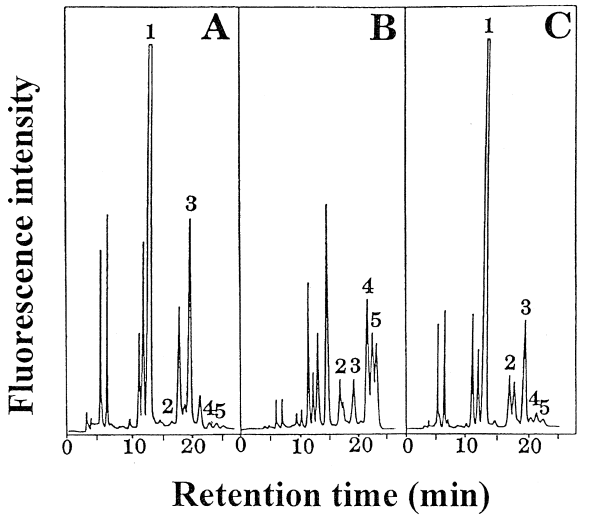

Fig. 2. High-performance liquid chromatograms of human plasma (A), urine (B), and rat kidney cytosol (C) after AG50W-X8 cation-exchange extraction. 1, L-Arginine; 2, L-NMMA; 3, L-homoarginine; 4, ADMA; 5, SDMA. Chromatographic conditions as in Fig. 1.

reported that endogenous components such as acidic amino acids and amines in biological samples interfere with the determination of L-arginine and its methylated metabolites by HPLC. Although earlier investigations [5,8] targeted at this subject had shown that L-arginine, ADMA and SDMA could be extracted effectively from human plasma by using CBA solid-phase extraction cartridges, there were still interfering peaks in the HPLC chromatogram. The use of a strong cation exchanger AG50W-X8 resin, which has been applied to separate L-citrulline from L-arginine for determining NOS activity [19], was thus attempted in this study. No appreciable components interfering with the separation of L-arginine analogs were detected. Typical chromatograms of human plasma, urine and rat kidney cytosol after AG50W-X8 extraction are shown in Fig. 2. It should be noted that if some unknown peaks with retention times longer than 25 min appear in the HLPC chromatogram, they could be removed by a washing step with 30% (v/v) acetonitrile in water for 10 min before injection of the next sample. Table 1 shows the mean recoveries of L-arginine, L-NMMA, ADMA and SDMA from human plasma, urine and rat kidney cytosol by AG50W-X8 extraction. More than 88% of L-arginine, 75% of L-NMMA, 71% of ADMA and 65% of SDMA were recovered from biological samples with relative standard deviations (RSDs) ranging from 1.3% to 10% for 3–4 determinations.

The present method allows one to measure concentrations of L-arginine, L-NMMA, ADMA and SDMA in human plasma, urine and kidney of rat (Table 2). The mean plasma levels of L-arginine, ADMA and SDMA obtained were in agreement with those reported by other authors [13–15,17]. To our knowledge, only Meyer et al. [14] have reported concentration of L-NMMA (0.1 μM) in human serum. Our data reveal that L-NMMA levels were in a similar range as those of ADMA or SDMA, but the standard derivation of L-NMMA was large, which may be due to large individual variation. Rat kidney contained high levels of L-arginine and L-NMMA and relative low levels of ADMA and SDMA as compared with plasma. The levels of ADMA, SDMA and L-NMMA in urine were relatively high but L-arginine could not be detected because of its lower limit of detection.

In this study, we have developed for the first time

Table 1
Recoveries of L-arginine and its methylated metabolites from an AG50W-X8 cation cartridge

Compound	Plasma		Urine		Kidney	
	Recovery[a] (%)	RSD (%)	Recovery[a] (%)	RSD (%)	Recovery[a] (%)	RSD (%)
L-Arginine	90.7	3.6	88.9	6.9	100.1	7.2
L-NMMA	75.2	10.0	87.7	2.2	96.1	9.9
ADMA	71.3	3.9	75.2	9.2	76.5	3.5
SDMA	65.3	8.2	70.6	6.1	80.5	1.3

[a] Mean of three to four determinations. Recoveries were estimated by adding 40 nmol of L-arginine and 0.8 nmol of ADMA, SDMA and L-NMMA to each sample.

Table 2
Concentrations of L-arginine and its methylated metabolites in the plasma and urine of healthy human subjects and kidney of male Wistar rats[a]

Compound	Biological sample		
	Plasma (n=7) (nmol/ml)	Urine (n=9) (nmol/mg of creatinine)	Kidney (n=5) (nmol/g of tissue)
L-Arginine	60.72±18.96	n.d.	907.3±27.5
L-NMMA	0.31±0.18	105.2±60.2	20.7±6.1
ADMA	0.30±0.05	376.8±73.8	11.2±2.5
SDMA	0.34±0.06	394.7±93.3	6.8±0.6

[a] Samples were treated as described in the Experimental section. Each value is the mean±SD.
n.d., Not detectable because of low limit of detection.

a simple and reliable procedure for the simultaneous and quantitative determination of L-arginine, L-NMMA, ADMA and SDMA in plasma, urine and tissue. Because impairment of systemic NO production from L-arginine by NOS is thought to cause cardiovascular diseases [20], the determination of ratios of L-arginine to endogenous NOS inhibitors, L-NMMA and ADMA, would provide useful information about the role of L-arginine methylation in NO-dependent physiological and/or pathophysiological actions.

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