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Short communication

Determination of N^G,N^G-dimethylarginine in human plasma by high-performance liquid chromatography

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Abstract

N^G,N^G-Dimethylarginine (asymmetric dimethylarginine, ADMA) can be directly separated and measured from deproteinized human plasma using *o*-phthaldialdehyde–mercaptoethanol (OPA reagent) as a fluorogenic reagent by reversed-phase high-performance liquid chromatography. The mean recovery of ADMA was over 96% and the inter- and intra-assay coefficients of variation of amounts were lower than 3.80% and those of retention time were below 0.37% for five runs. The detection limit of the assay is 1 pmol when the signal-to-noise ratio is 3:1. It was observed that the concentration of ADMA was significantly elevated in plasma of patients with pregnancy induced hypertension (PIH) in contrast to healthy pregnant women.

Keywords: Dimethylarginine

1. Introduction

Nitric oxide (NO), synthesized from L-arginine, contributes to the regulation of blood pressure and to host defence. NO synthesis can be inhibited by some analogues of arginine including N^G-monomethyl-L-arginine (L-NMMA) and N^G,N^G-dimethylarginine (asymmetric dimethylarginine, ADMA) which are present in human plasma and urine [1]. ADMA is synthesized and released by endothelia cells in amounts that are sufficient to inhibit NO production [2]. The concentration of dimethylarginine in plasma is ten times greater than that of L-NMMA [1]. Therefore, we measured the concentration of ADMA in plasma in order to determine the possible role of ADMA as an endogenous inhibitor of L-arginine in the NO pathway in patients with pregnancy induced hypertension (PIH).

Analytical methods for the determination of ADMA include paper chromatography [3], thin layer chromatography [4], electrophoresis [5], ion-exchange chromatography [6–9] and monoclonal antibody assay [10]. Reserved-phase HPLC has also been employed for the quantitative and qualitative analysis of methylated amino acids in plasma [11–14]. Complex sample preparation is required for all the methods mentioned above, in order to remove other amino acids which interfere with separation of ADMA. HPLC separation of ADMA from other amino acids in plasma was very difficult because the

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concentration of ADMA in plasma is many times lower than other amino acids, so that the most HPLC methods for determining ADMA in plasma required complicated sample preparation in which the sample was loaded onto an ion-exchange column and eluted twice with different solutions and concentrated to dryness before injection onto the HPLC column [1,11,14].

In present paper we developed a simple, sensitive method for directly determining ADMA in human plasma. Sample pretreatment is not required except to deprotein with solid 5-sulfosalicylic acid (5-SSA). *o*-Phthaldialdehyde (OPA) was utilized as a fluorogenic reagent and the concentrations of ADMA in plasma of patients with pregnancy induced hypertension and in healthy pregnant women were measured with this method. The recovery and reproducibility were also investigated in the experiment.

2. Experimental

2.1. Reagents and standards

Amino acids and ADMA were purchased from Sigma (St. Louis, MO, USA). A stock standard solution (0.5 m*M*) of each compound was prepared in 0.1 *M* HCl and stored in a refrigerator. A working standard solution (50 μ*M*) of each compound was prepared by diluting the stock solution with 0.1 *M* HCl. OPA and 2-mercaptoethanol were purchased from Sigma. Tetrahydrofuran (THF), sodium acetate and acetic acid obtained from Shanghai (Shanghai, China) were analytical-grade reagents. HPLC-grade methanol (Shanghai, China) and Milli-Q quality water were used in the preparation of the mobile phase.

2.2. Equipment

Chromatographic experiments were performed using a Shimadzu (Kyoto, Japan) LC-6A HPLC system, including two LC-6A pumps, an SCL-6A system controller, an SIL-6A autoinjector, a CTO-6A column oven, a RF-530 fluorescence detector, a C-R3A data processor. Separation was performed using a 150×4 mm I.D. Nova-pak C_{18} column with a particle size of 5 μm. The analytical column was protected by a C_{18} guard-pak cartridge (Waters, Millipore, Milford, MA, USA).

2.3. Chromatographic conditions

Mobile phases consisting of 50 m*M* sodium acetate (pH 6.8), methanol and THF (A, 82:17:1; B, 22:77:1) were degassed ultrasonically before use. Each component of the mobile phase was filtered through a 0.2-μm filter. All separations were performed at 27°C and at a flow-rate of 1.0 ml/min. The gradients are given in Table 1. The wavelengths of fluorescence detector were set at 338 nm and 425 nm for excitation and emission, respectively. The areas of peak are used for quantification.

2.4. Collection and preparation of samples

Thirty women were accepted for investigation, including ten patients with serious PIH (blood pressure: ≥21.3/14.7 kPa, proteinurine: ++−+++ +), ten patients with moderate PIH (blood pressure: 18.7/13.3 kPa–21.3/14.7 kPa, proteinurine: +) and ten cases of normal pregnancy. Ages of patients with PIH were 23–32 years (27.25±2.77) and 24–32 years (27.00±2.58) for patients undergoing of normal pregnancy. Weights of patients with PIH were 57–65 kg (61.32±5.41) and 56–59 kg (63.25±7.19) for patients undergoing normal pregnancy. The patients with PIH wer treated with magnesium sulphate and methyldopa. The investigation was carried out before parturition and 4–7 days after parturition. Whole blood from patients with PIH and normal pregnant patients was collected into heparinised

Table 1
Gradient programm for the separation of OPA–ADMA and OPA–AAs derivatives

Time (min)	A (%)	B (%)
0	95	5
6	88	12
16	60	40
28	25	75
32	0	100
34	0	100
35	95	5

Solvents: A=50 m*M* sodium acetate pH 6.8–methanol–THF (82:17:1, v/v); B=50 m*M* sodium acetate pH 6.8–methanol–THF (22:77:1, v/v).

tubes (1000 IU/ml) and plasma obtained by centrifugation at 2000 *g* for 10 min. To 1.0 ml plasma, 20 mg of 5-SSA was added [15] and the mixture was left in an ice-bath for 10 min. The precipitated protein was removed by centrifugation at 2000 *g* for 10 min. The supernatant was filtered through a 0.2 μm filter for analysis. For the recovery experiment, 1–20 nmol of ADMA was added to 1.0 ml plasma and deproteinization was performed as described above.

2.5. OPA reagent and ADMA derivatization

OPA (10 mg) was dissolved in 0.5 ml of methanol, and 2 ml of 0.4 *M* borate buffer (0.4 *M* boric acid adjusted to pH 10.0 with potassium hydroxide) and 30 μl of mercaptoethanol were added. This solution is only stable for two days. The derivatization was performed by mixing 10 μl of sample or working standard solution and 100 μl of OPA reagent and reacting for 3 min before autoinjecting onto the column.

3. Results and discussion

3.1. Separation of ADMA from other amino acids in plasma

The concentration of ADMA in plasma is much lower than that of the other amino acids (about 1:50 for arginine) in a healthy person. Good resolution and accurate quantification cannot be reached owing to the interference of other peaks, especially arginine, if the conditions were not appropriate [16]. It is important that mobile phases and gradients were optimal to increase resolution of ADMA. Fig. 1 shows the chromatograms of OPA derivatives of 20 amino acid standards spiked with ADMA (A) and with plasma of a patient with PIH (B). In the present study, the chromatographic conditions were optimized so that the peak of ADMA was eluted at 13.4 min between the peaks of arginine and γ-aminobutyric acid (GABA). There was a duration of 2.3 min from arginine to GABA occurrence, and the peak of ADMA eluting in this period did not suffer from interference by these two peaks and reached baseline resolution. Arginine and citrulline are the most likely interfering compounds as their structures are similar to that of ADMA. Under our experimental chromatographic conditions ADMA and arginine can be baseline resolved while citrulline was co-eluted with Thr before Arg and thus did not interfere

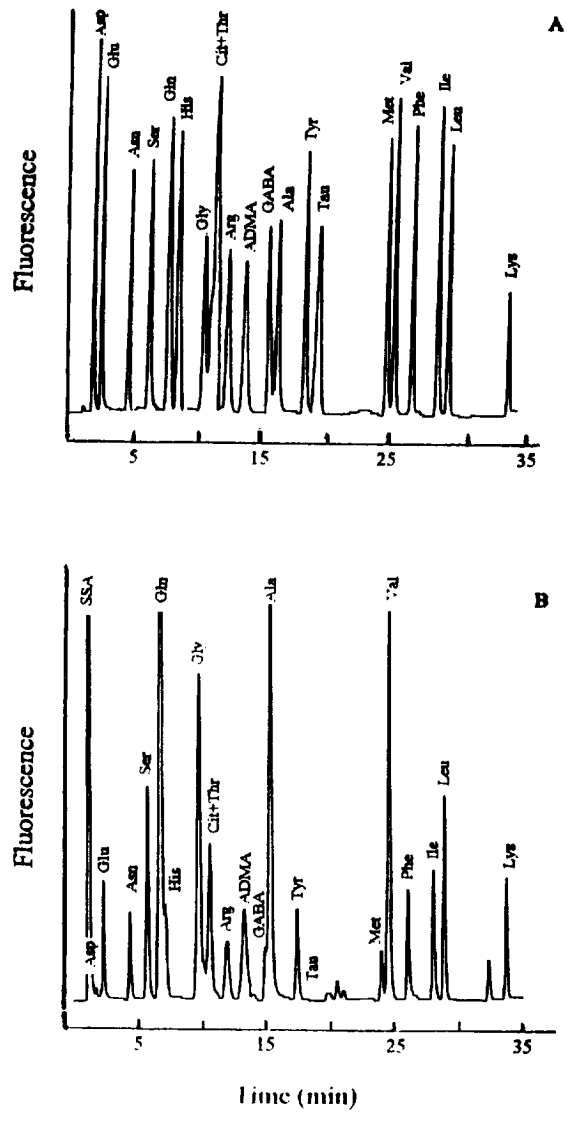

Fig. 1. Typical chromatograms: (A) 25 μmol/l of ADMA and amino acid standard solution, attenuation=3 for all run times. (B) 10 μl of plasma sample of a patient with PIH, attenutaion=8 for 0–13 min, 3 for 13–14 min and 8 for 14–35 min. Column: Nova-pak C_{18} (150×4 mm I.D.); flow-rate: 1.0 ml/min; eluents: sodium acetate–methanol–tetrahydroforan (gradient see Table 1).

with ADMA (Fig. 1A). Other amino acids will not coelute with ADMA due to their different structures.

The proportion of THF in the mobile phases is very important for the separation of ADMA; complete separation cannot be reached between arginine and ADMA when it is less than 1%. When the proportion of THF in the mobile phases is greater than 1%, the retention times of all amino acids varied greatly between runs. Georgi et al. [17] suggested to omit THF during separation of the amino acids, but in our experiment, 1% of THF was added to the mobile phases in order to improve resolution of ADMA. The results show that the ADMA reached baseline resolution and that the retention times of each peak were very stable between runs.

3.2. Recovery and reproducibility of ADMA in human plasma

A standard solution of ADMA was added to 1.0 ml plasma in the range of 1.0–20 nmol and the recovery was determined using the condition described in Section 2.4. Two deproteinization methods [5-SSA and trichloroacetic acid (TCA)] were also compared (Table 2). The results demonstrate that 5-SSA is a deproteinization reagent superior to TCA. The recovery of ADMA ranged from 95.1% to 97.8% for 5-SSA and from 91.9% to 93.7% for TCA. The reproducibility of the method was assessed by calculating the relative standard deviations of the amounts and retention times of ADMA. Table 3 gives details of the precision data for the method using a patient sample pool. The mean inter-assay coefficients of variation (C.V.) ($n=5$) of retention time and amount for OPA–ADMA derivatives are 0.37% and 2.67%, and intra-assay coefficients of variation are 0.34% and 3.73%, respectively. The results showed that the reproducibility and recovery were very satisfactory. We found that the assay is linear up to 1 mmol. The detection limit of the assay is 1 pmol at a signal-to-noise ratio of 3:1.

Table 2
Mean recovery of ADMA using spiked plasma ($n=5$)

Spiked concentration (μM)	Recovery (mean±S.D.) (%)	
	SSA method[a]	TCA method[b]
0.5	95.1±5.2	92.5±6.6
1.0	97.8±4.5	93.7±5.4
5.0	96.3±7.4	91.9±7.2

[a] 5-SSA powder was used a deproteinization reagent.
[b] 5% of TCA was used a deproteinization reagent.

Table 3
Relative standard deviations of the amount and retention time of ADMA in human plasma ($n=5$)

	Retention time		Amount	
	Mean (min)	R.S.D.%	Mean (μM)	R.S.D.(%)
Inter-assay	13.41	0.37	1.85	2.67
Intra-assay	13.45	0.34	1.79	3.73

3.3. Concentration of ADMA in plasma

The concentrations of ADMA in plasma of patients with PIH and those undergoing normal pregnancy are shown in Table 4. The plasma levels of ADMA are significantly elevated in the patients with PIH. This result supported the increasing tendency of plasma ADMA in patients with PIH reported by Fickling et al. [2]; our results showed a higher plasma ADMA level in patients with PIH; however, values in patients undergoing normal pregnancy were very similar. The mechanism of the increase of plasma ADMA in patients with PIH could be due to a renal function disorder whereby elimination of ADMA is decreased or perhaps a decrease in ADMA hydrolysis caused by the inhibition of enzymes by increased levels of calcium in cells [5]. Thorough investigation will be very necessary.

4. Conclusions

Derivatization of ADMA in direct deproteinized plasma with OPA is a simple, highly reproducible,

Table 4
Concentrations of ADMA in plasma of patients with PIH and of normal pregnant women

	n	Concentration (mean±S.D.) (μM)	
		Before parturition	After parturition
Serious PIH	10	3.15±0.55	2.31±0.66
Moderate PIH	10	1.85±0.57	1.55±0.40
Normal pregnancy	10	0.84±0.12	0.83±0.13

accurate procedure. The separation of ADMA derivative is essentially affected by the components of the mobile phase and the gradient program. Optimal chromatographic conditions allowed the ADMA derivative to be baseline resolved from other amino acid derivatives. Complex sample preparation was not required in this method which can be used for routine determination of ADMA in plasma of serum and urine. The run time of each analysis is 36 min.

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