

Original Research

The measurement of asymmetric dimethylarginine in human plasma by high performance liquid chromatography

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Received: July 22, 2012	Abstract
Accepted: August 13, 2012	Objective: We aimed to establish a high performance liquid chromatography (HPLC) method to measure plasma ADMA levels easily, sensitively and reliably.
Published Online: August 30, 2012	Methods: We used fluorescence detection, 50 mM sodium acetate buffer/ methanol/
DOI: 10.5455/jib.20120813053832	Tetrahydrofuran as a mobile phase (A, 82:17:1; B, 22:77:1, % v, respectively), gradient method, 338 and 425 nm excitation and emission wavelengths, respectively. Samples were
Corresponding Author: Yasemin Gulcan Kurt, Department of Clinical Biochemistry, Gulhane Military Medical Academy, Ankara, Turkey ygkurt@gata.edu.tr	derivatizated with o-Phthaldialdehyde. Results: The recovery was found as 96 to 98,3 %. Intra-assay and inter-assay coefficients of variation (CV) were 2.3% and 2.7%, respectively. Conclusion: These findings suggest that our method is suitable for routine determination of ADMA more reliably.

Key words: ADMA, HPLC, measurement

INTRODUCTION

Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide synthase (NOS) enzyme. Nitric oxide (NO), synthesized from L-arginine by NOS enzyme, contributes to the regulation of blood pressure and to host defense. Nitric oxide is a vasodilator and inhibitor of platelet aggregation, leucocytes migration, cellular adhesion and vascular smooth muscle proliferation (1,2). The main function of NO is to provide the vascular homeostasis. When the NO levels is decreased, endothelial homeostasis is impaired in the direction of vasoconstriction and endothelial dysfunction begins (3).

ADMA is synthesized and released by endothelial cells and present in human plasma in amounts that are sufficient to inhibit NO production [4,5]. Thus, ADMA might be thought as a key molecule that contributes to endothelial dysfunction. Elevated plasma concentrations of ADMA are also present in hypercholesterolemic and hypertensive patients, in patients with chronic heart failure, and in other patient groups at high risk of developing cardiovascular disease.

There are many analytical methods for the determination of ADMA including paper chromatography, thin layer chromatography, electrophoresis, ion-exchange chromatography and monoclonal antibody assay [6-12]. Reserved-phase HPLC has also been employed for the quantitative and qualitative analysis of methylated amino acids in plasma [13-16]. However, because of the complex sample preparation and being too expensive these methods mentioned above are not practical for routine use

In the present study we reported a simple and sensitive method for directly determining ADMA in human plasma with higher precision. Sample pretreatment is not required except to deproteinization with 5-

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sulfosalicylic acid (5-SSA). o-Phthaldialdehyde (OPA) was utilized as a fluorogenic reagent. We prepared a plasma pool from a healthy person and the concentrations of ADMA in plasma were measured with this method (Fig.1). The recovery, intra-assay and inter-assay precision were also investigated in the experiment.

MATERIALS AND METHODS

Chemicals

ADMA, o-Phthaldialdehyde (OPA), mercaptoethanol, acetic acid and boric acid were purchased from Sigma (St. Louis, MO, USA). Tetrahydrofuran (THF), sodium acetate, 5-sulfosalicylic acid (5-SSA) and methanol (HPLC grade) were purchased from Merck (Merck, Germany).

Reagents and Standards

ADMA stock standard solution is prepared with 0.1 M HCl as 0.5 mM concentration and stored at -80°C. Working standard solutions were prepared by diluting the stock solution with 0.1 M HCl between 0,81-100 μ M concentrations. OPA solution is prepared with 500 μ L methanol, 2 mL Borate buffer (0,4 M boric acid, adjusted to pH 10.0 with potassium hydroxide), 30 μ L mercaptoethanol and 10 mg OPA. This solution is stable only two days at 2-8 °C. Sodium acetate buffer is prepared as 50 mM and adjusted to pH: 6.8 with acetic acid.

Chromatographic Conditions

Mobile phases, consisting sodium acetate buffer, methanol and THF (A, 82:17:1; B, 22:77:1, % v, respectively), were degassed ultrasonically and filtered through a 0.2 μ m filter before use. Column temperature adjusted 37°C and flow-rate was 1.0 ml/min during the separation. The mobile phases' gradients are given in Table 1. The wavelengths of fluorescence detector were set at 338 nm for excitation and 425 nm for emission. The areas of peak are used for quantification.

Table-1.	Gradient	program	for the	separation	of ADMA

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

Equipment

Chromatographic experiments were performed using a Shimadzu (Kyoto, Japan) LC-20A HPLC System, including LC-20AT pump, a SIL-10AD VP auto sampler, a LC-20A degasser, a CTD 10AS VP column oven, a RF 10XL fluorescence detector, a CLASS VP V 6.14 SP1 microprocessor. ADMA separation was performed using a 150 X 4.6 mm C18 ODS-2 reverse phase column (Phenomenex Hypersil, USA) with 5 μ m particle size. The analytical column was protected by ODS-2 cartridge (Phenomenex Hypersil, USA) with 5 μ m particle size.

Sample preparation

Plasma pool obtained from a healthy person and divided aliquot, and all samples stored at -80° C until the study. Frozen samples thawed and 25 mg 5-SSA added to 1 mL sample and then the mixture was left in an ice-bath for 10 min before the study. 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. The derivatization was performed by mixing 10 µL of sample and 100 µL of OPA solution and reacting for 3 min before injecting onto the column. Finally, 10 µL of sample-OPA mixture is injected onto the column.

RESULTS

ADMA standard curve was obtained as shown in Fig. 2. The ADMA standard concentrations were between the range of 0,81-100 µM. ADMA standards' concentrations were correlated with the peak areas that obtained from the chromatograms ($r^2=0.992$, Fig.2). We found that linearity continued even in this wide range. Precision was defined as repeatability (intraassay variation) and reproducibility (inter-assay variation). Intra-assay variation was performed in a plasma sample that obtained a person replicate 10 runs. Inter-assay variation was performed in sample pool analyzed on 10 different days. The coefficient of variation (CV %) values for intra-assay imprecision and inter-assay imprecision were 2.3% and 2.7%, respectively. Inter-assay and intra-assay imprecision values are shown in Table 2. The recovery of ADMA from the plasma sample was determined by spiking known amount of ADMA (2.5-20 µM), and was calculated by comparing the peak response with those obtained from chromatogram. The recovery of ADMA ranged from 96% to 98.3%. Table 3 gives details of the recovery data for the method using a sample.





Figure 1. Chromatogram of a human plasma sample.



Figure 2. The curve of ADMA standards.

Table 2. Intra-assay and Inter-assay imprecision values

Intra-assay imprecision				Inter-assay imprecision			
n	Mean	SD	CV %	n	Mean	SD	CV %
10	1.12	0.03	2.3	10	1.95	0.05	2.7

n: the number of study; SD: standard deviation; CV: coefficient of variation

Test Result (µM)	Spiked Concentration (µM)	Recovery (Mean±SD)(%)	Recovery (Mean±SD)(%)	
0.84 ± 0.02	-	-		
3.24 ± 0.04	2.5	96 ± 1.6		
5.67 ± 0.08	5	96.6 ± 1.6		
10.66 ± 0.02	10	98.2 ± 0.2		
20.5 ± 0.02	20	98.3 ± 0.2		

 Table 3. Mean recovery of ADMA using spiked plasma (n=3)

DISCUSSION

Increased levels of ADMA have recently been indicated as one of the causes of endothelial vasodilator dysfunction, leading to coronary and arterial diseases (17). Therefore, the determination of ADMA is requested for a growing number of clinical studies regarding different conditions of endothelial dysfunction. For this reason a more reliable and manageable method than currently available is needed, especially for large routine analyses of this molecule. Sample preparation, derivatization, chromatographic conditions, reagents and standards of our technique are similar to the HPLC method described by Chen et al (18). Moreover, we would like to highlight some improvements that our method presents.

In our study we used 150 X 4,6 mm C18 ODS-2 reverse phase column (Phenomenex Hypersil, USA) with 5 µm particle size and column temperature was 37°C. Temperature has a broad influence on all chromatographic techniques. Elevated temperatures decrease viscosity and increase solubility. Total analysis time, retention time, peak shape and column efficiency are affected by temperature. The efficiency of a chromatographic column is a measure of the capacity of the column to restrain peak dispersion and thus, provide high resolution. Additionally, temperature control results in improved reproducibility (19). Therefore, to determine the optimum temperature is very important for chromatographic techniques. Chen et al used 27 °C column temperatures for separation of ADMA (18). For optimization of temperature, in our study we used a column oven and column temperature was 37 °C. Intra-assay, inter-assay imprecision values and recovery results of our study are better than previous studies (18,20).

There are many analytical methods for the determination of ADMA. However, because of the complex sample preparation and being too expensive they are not practical for routine use. In this method, derivatization and separation of ADMA is simple, highly reproducible and accurate. We optimized the temperature in this method which can be used for routine determination of ADMA more reliably.

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