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Determination of N^G,N^G-dimethyl-L-arginine in rat plasma and dimethylarginine dimethylaminohydrolase activity in rat kidney using a monolithic silica column

Satoko Nonaka [1], Makoto Tsunoda [*,1], Chiaki Aoyama, Takashi Funatsu

Graduate School of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

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Abstract

A fast, simple and sensitive column-switching high-performance liquid chromatography (HPLC)-fluorescence detection method was developed on a monolithic silica column for the determination of N^G,N^G-dimethyl-L-arginine (ADMA), which is an endogenous nitric oxide synthase inhibitor. After fluorescence derivatization of plasma samples or homogenized tissues with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), the samples were injected into the HPLC system. The NBD-derivatized ADMA was trapped on a cation-exchange column and separated within 15 min on a monolithic silica column. The detection limit for ADMA was 36 nM (250 fmol per injection) when the signal-to-noise ratio was 3. A good linearity for calibration curve for ADMA was observed within the range of 140 nM (1.0 pmol per injection) – 140 μM (1.0 nmol per injection) using N^G-monomethyl-L-arginine (L-NMMA) as an internal standard. The proposed method was used for the quantitative determination of ADMA in rat plasma. The concentrations of ADMA in rat plasma were 0.82 ± 0.05 μM ($n = 4$). Furthermore, the method developed was applied to determine dimethylarginine dimethylaminohydrolase (DDAH) enzyme activity in rat kidney, which was assayed by measuring the amount of ADMA metabolized by the enzyme.
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Keywords: N^G,N^G-Dimethyl-L-arginine (ADMA); Dimethylarginine dimethylaminohydrolase (DDAH); Monolithic silica column; 4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F); Fluorescence; Column-switching

1. Introduction

Nitric oxide (NO), synthesized from L-arginine by NO synthase (NOS), is an important regulator of vascular tone, suppression of vascular smooth muscle proliferation, and inhibition of platelet adhesion [1–3]. The synthesis of NO is inhibited by endogenous L-arginine analogues, N^G,N^G-dimethyl-L-arginine (ADMA) [4]. Elevation of ADMA level in plasma has been associated with renal failure [5], hypertension [6], hypercholesterolemia [7], hyperhomocystemia [8], diabetes mellitus [9], and several other diseases.

ADMA is metabolized by dimethylarginine dimethylaminohydrolase (DDAH) [10], which specially hydrolyzes ADMA to citrulline and dimethylamine. There have been reports that a reduced activity of DDAH might be responsible for elevated ADMA concentrations [11,12]. In consequence, the ADMA–DDAH system might be involved in the regulation of endogenous NO synthesis.

To elucidate the arginine–NO pathway and ADMA–DDAH pathway, determination methods of ADMA in biological samples such as plasma or urine should be developed. Several methods using high-performance liquid chromatography (HPLC) [13–17], capillary electrophoresis [18,19], and liquid chromatography–mass spectrometry (LC–MS) [20,21] have been reported. Among them, there are some reports about pre-column derivatization with *o*-phthalaldehyde (OPA) followed by the separation of methylated arginines from rat plasma sample, and fluorescence detection [15–17]. However, these techniques have some undesirable characteristics, such as instability of fluorescence of OPA derivatives and a complicated pre-treatment process.

To resolve the limitations mentioned above, the determination method for three methylated arginines, N^G-monomethyl-L-

* Corresponding author. Tel: +81 3 5841 4761; fax: +81 3 5802 3339.
E-mail address: makotot@mol.f.u-tokyo.ac.jp (M. Tsunoda).
[1] These authors contributed equally to this work.

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arginine (L-NMMA), ADMA, and N^G,$N^{G\prime}$-dimethyl-L-arginine (SDMA) in rat plasma has been developed [22]. In this method, 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was used as fluorescence derivatizing reagent, the detection limit of which was better than the previous method [15–17]. Further, we developed a column-switching HPLC system to remove tedious pre-treatment processes [23]. After the direct derivatization of methylated arginines in plasma sample with NBD-F, the derivatized methylated arginines were on-line extracted with a cation-exchange column, followed by separation on a conventional particulate silica column. However, the method took about 42 min for each analysis cycle.

Recently, monolithic silica columns have attracted attention as an alternative to conventional particulate silica columns [24,25]. Monolithic silica columns possess a unique biporous structure. This type of column has greater permeability and can therefore be employed with high flow-rates without loss of performance or limitations due to increased pressure. Monolithic silica columns, therefore, achieve faster separation than conventional particulate columns. Although high throughput analyses of drugs using monolithic silica columns have been reported [26–28], applications of monolithic silica columns to endogenous compounds in bio-samples have been limited.

In this study, we adopted monolithic silica columns instead of conventional particulate columns for the rapid determination of ADMA in rat plasma. Furthermore, we applied the method developed to the assay of DDAH activity, which was calculated by measuring the amount of ADMA metabolized by the enzyme.

2. Experimental

2.1. Materials

ADMA and L-NMMA were obtained from Sigma (St. Louis, MO, USA). A stock standard solution was stored in 10 mM HCl at 4 °C. The chemical structures of these compounds are shown in Fig. 1. NBD-F and boric acid were purchased from Wako Pure Chemicals (Osaka, Japan). Acetonitrile of HPLC grade was obtained from Kanto Chemical (Tokyo, Japan). Water was used after purification by Milli RO–Milli Q reagent system (Nihon Millipore, Tokyo, Japan).

2.2. Rat plasma and kidney sample preparation

Male Sprague–Dawley (SD) rats (8-weeks old) were purchased from Charles River Japan Inc. (Kanagawa, Japan). The rats received human care in compliance with the National Institute of Health guideline. The rats were anesthetized with pentobarbital. Blood was quickly taken from the abdominal aorta; then the kidney was immediately removed and chilled on ice. Blood was centrifuged at 800 × g for 10 min at 4 °C. The kidney was homogenized with four volumes of 50 mM sodium phosphate buffer (pH 7.5) containing 0.5 mM 1,4-dithiothreitol on ice. The homogenates were centrifuged at 9100 × g for 40 min at 4 °C, and the supernatant was stored for DDAH activity assay. Each sample was frozen at −80 °C until the assay.

2.3. Fluorescence derivatization procedure

Fluorescence derivatization procedure was based on our previous method [23].

One hundred microlitres of 200 mM borate buffer (pH 9.0), 15 μl of 20 μM L-NMMA solution as internal standard and 30 μl of 120 mM NBD-F in acetonitrile were added to 30 μl of plasma sample. Then, the reaction was carried out at 60 °C for 4 min, and stopped when 40 μl of 6% acetic acid (v/v) was added. After the filtration using Cosmospin filter H (polytetrafluoroethylene membranes filter, 0.45 μm) (Nacalai Tesque, Kyoto, Japan), 50 μl of supernatant fluid of derivatized sample was injected into the column-switching HPLC system using a monolithic silica column.

2.4. HPLC conditions for the determination of ADMA

Determination of ADMA derivatized with NBD-F was performed by the column-switching HPLC system (Fig. 2). The system consisted of two pumps, L-7100 (Hitachi, Tokyo, Japan) and 880-PU (Jasco, Tokyo, Japan), column oven (30 °C) (CO 631A, GL Sciences Inc., Tokyo, Japan), switching valve (HV-992-01, Jasco) and fluorescence detector (FP-920S, Jasco). The pre-column used was CAPCELLPAK MF-SCX cartridge (4.0 mm × 10 mm I.D., SHISEIDO, Tokyo, Japan). The pre-treatment buffer used was 50 mM sodium phosphate buffer (pH 3.2), and the flow rate was 2.0 ml/min. The column utilized for the separation was Chromolith™ Performance RP-18e (100 mm × 4.6 mm I.D., Merck, Darmstadt, Germany) protected by Chromolith™ Guard Cartridge RP-18e (10 mm × 4.6 mm I.D.). For the mobile phase, a mixture of a 50 mM sodium phosphate buffer (pH 3.2)–acetonitrile (96:4, v/v) was delivered in isocratic mode at 6.0 ml/min. The wavelengths of the fluorescence detector were set at 470 and 530 nm for excitation and emission, respectively.

The data of conventional particulate silica column were obtained with the following conditions. The column utilized for separation was Unison UK-C18 (150 mm × 4.6 mm I.D., Imtakt, Kyoto, Japan). The mobile phase consisted of 50 mM sodium

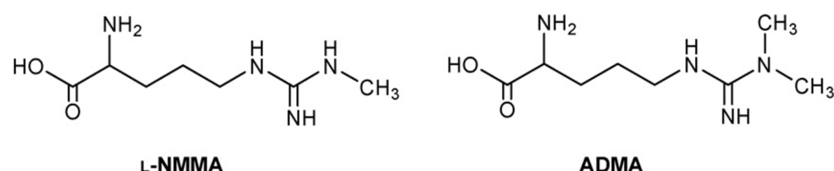

Fig. 1. Chemical structures of L-NMMA and ADMA.

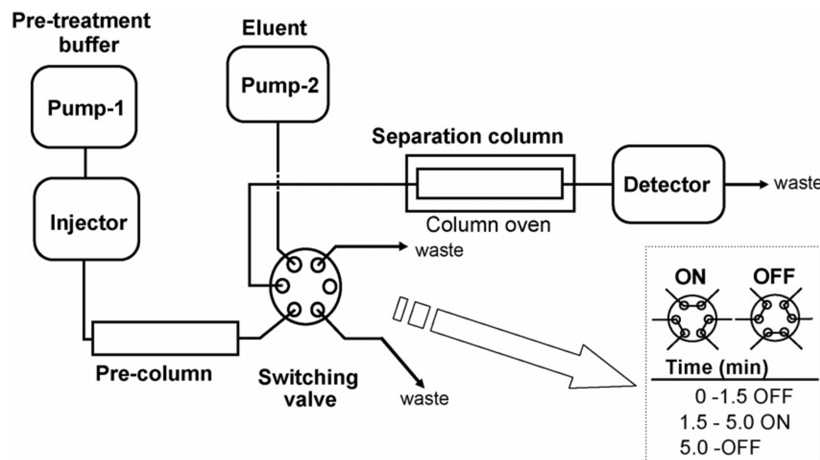

Fig. 2. Block diagram of the column-switching HPLC system for the determination of methylated arginines derivatized with NBD-F. Pre-treatment buffer, 50 mM phosphate buffer (pH 3.2); 2.0 ml/min. Eluent, 50 mM phosphate buffer (pH 3.2)/acetonitrile = 96/4; 6.0 ml/min. Pre-column, CAPCELLPAK MF-SCX cartridge (10 mm × 4.0 mm I.D., SHISEIDO). Separation column, Chromolith™ Performance RP-18e (100 mm × 4.6 mm I.D., Merck) protected by Chromolith™ Guard Cartridge RP-18e (10 mm × 4.6 mm I.D.).

phosphate buffer (pH 3.2)–acetonitrile (91:9, v/v). The flow rate was set at 0.75 ml/min and the column oven was maintained at 40 °C. Other HPLC conditions were the same as mentioned above.

2.5. Validation study

Accuracy was determined by the addition of 0.5, 1.0, 2.0, 4.0, and 8.0 μM each standard sample of ADMA. Calibration curves were calculated by plotting the peak height ratios of analyte over internal standard versus analyte concentration.

The intra-day assay precision was determined by five replicate analyses of the plasma samples on the same day, while the recovery was evaluated simultaneously. The inter-day assay precision was determined by analyzing the above sample that was tested on 5 different days.

Mean concentrations of ADMA in four different rat plasma were calculated. The data were presented as mean ± standard deviation.

2.6. Assay of DDAH activity

Rat kidney samples were divided into two groups, in an ice bath, 30 μl of the rat kidney homogenate and 240 μl of 50 mM sodium phosphate buffer (pH 7.5) were added to 30 μl of 500 μM ADMA. To inactivate DDAH, 30 μl of 30% 5-sulfosalicylic acid dehydrate (SSA) was immediately added to one experimental group, which was then incubated at 37 °C for 20 min. This group provided a baseline of 0% DDAH activity. The other samples were incubated at 37 °C for 20 min before the addition of 30% SSA. They were centrifuged at 9100 × *g* for 10 min at 4 °C. The supernatants were derivatized with NBD-F on the above fluorescent procedure and assayed with the column-switching HPLC system. The difference in ADMA concentration between the two groups was calculated as the metabolic rate. DDAH activity was expressed as decreased ADMA quantum (mol)/min/(mg protein). Protein concentrations of the samples were determined according to Bradford [29].

3. Results and discussion

3.1. Evaluation of monolithic silica columns

The use of conventional columns containing 3 or 5 μm small silica particles often results in high pressure with high flow rate. A monolithic silica column has a silica skeleton containing mesopores of approximately 13 nm and macropores of approximately 2 μm diameter. Both constitute a three-dimensional network, which contributes to low back pressure. Thus, monolithic silica columns enable high flow rate analysis holding competent chromatographic characteristics [24,25]. To measure the performance of a monolithic silica column under the optimized HPLC condition, column efficiency (number of theoretical plates) and resolution factor (between ADMA and its adjacent peak, homoarginine) were evaluated. These factors were calculated at the flow rates of 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, and 7.0 ml/min. The chromatographic parameters on each flow rate are summarized in Table 1. Column efficiency generally remained the same in a range from 3.0 to 6.0 ml/min (Table 1). In addition, at the flow rate of 6.0 ml/min, retention time was within

Table 1
Comparison of column efficiency (N, number of theoretical plates), resolution factor (R_s, between ADMA and its adjacent peak, homoarginine), retention time (R_t) and back pressure in: (A) monolithic silica column and (B) conventional particulate silica column

Flow rate (ml/min)	N (/m)	R_s	R_t (min)	Pressure (bar)
(A) Monolithic silica column				
1.0	22,988	1.7	40.7	20
2.0	19,764	1.7	20.8	41
3.0	50,867	2.7	15.1	66
4.0	53,444	2.6	11.8	92
5.0	58,875	2.8	9.8	119
6.0	59,404	2.6	8.2	137
7.0	51,658	2.6	7.0	166
(B) Conventional particulate silica column				
0.75	324,900	2.9	22.5	58

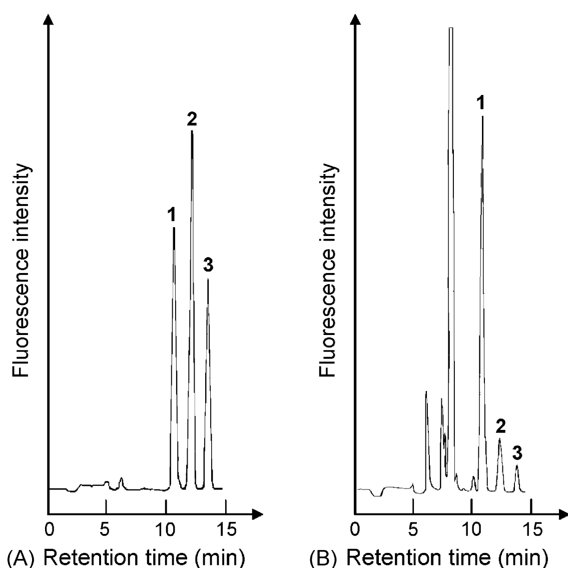

Fig. 3. Chromatogram (A) of standards and (B) obtained from rat plasma sample with the column-switching HPLC system. Peaks: (1) L-NMMA, (2) homoarginine, and (3) ADMA.

10 min holding the appropriate back pressure (137 bar). Hence, 6.0 ml/min was chosen for the optimized flow rate. Although column efficiency of a monolithic silica column was not comparable to that of conventional particulate silica column, the retention time was considerably shortened, and the value of the resolution factor was efficient with the monolithic silica column.

3.2. Chromatograms

In this study, the column-switching HPLC system was employed in the same way as the previous one [23]. After derivatization with NBD-F, biological samples were directly injected into the column-switching HPLC system. NBD-methylated arginines were retained on a cation-exchange column, and were eluted between 1.5 and 5.0 min while other acidic and neutral amino acids were not retained. NBD-methylated arginines were selectively injected into the monolithic silica column by automated valve switching. Fig. 3 shows the chromatograms (A) of standards (each 10 μM) and (B) obtained from rat plasma sample (10 μM L-NMMA as internal standard and 0.78 μM ADMA). These methylated arginines were well separated within 15 min, which was one-third of the time required for the previous method using conventional particulate silica column. We have ascertained that SDMA was eluted after ADMA, which is not shown in the chromatogram. L-NMMA was used as internal standard in this system, because the peak of L-NMMA could not be found in the chromatogram obtained from samples of control rat plasma.

3.3. Validation of the developed method

The calibration curve for ADMA was linear in the range of 140 nM (1 pmol per injection) to 140 μM (1 nmol per injection). The correlation coefficients were higher than 0.999. Detection limit (signal-to-noise ratio = 3) for the ADMA was 36 nM (250 fmol per injection). Compared to our previous data using conventional particulate silica columns, the detection limit was comparatively high. However, this detection limit was enough to determine ADMA in rat plasma as shown in Fig. 3(B).

Table 2
Accuracy data for determination of ADMA expressed as recovery of added analyte from spiked plasma sample

ADMA added, μM ($n = 3$)	Concentration (mean ± S.D., μM)	Precision (R.S.D., %)	Recovery (%)
0	0.78 ± 0.017	2.1	–
0.5	1.2 ± 0.02	2.0	89
1.0	1.7 ± 0.04	2.3	94
2.0	2.6 ± 0.08	3.2	91
4.0	4.7 ± 0.19	4.0	97
8.0	8.7 ± 0.02	0.22	98

Intra-day and inter-day coefficients of variances (CVs) for ADMA were 5.1 and 7.2%, respectively ($n = 5$). The accuracy for ADMA from spiked plasma samples ($n = 3$) is shown in Table 2. The concentrations of ADMA in four different rat plasma were 0.82 ± 0.045 μM. These concentrations are consistent with the previous reports [22,23].

3.4. DDAH activity assay in rat kidney

DDAH activity in rat kidney was calculated by measuring the amount of ADMA metabolized by this enzyme. First, we optimized enzymatic reaction conditions. The optimum pH values (50 mM sodium phosphate buffer) for the enzyme reaction were found to be 7.5 (data not shown). Metabolism of ADMA by DDAH was linear with incubation time up to 30 min and enzymatic activity was linearly related to its volume up to 60 μl.

Fig. 4(A) shows the chromatogram obtained from rat kidney sample without incubation. The chromatogram obtained from rat kidney sample incubated is shown in Fig. 4(B). Comparing these two chromatograms, in the presence of DDAH, the peak of ADMA was decreased due to the enzyme reaction.

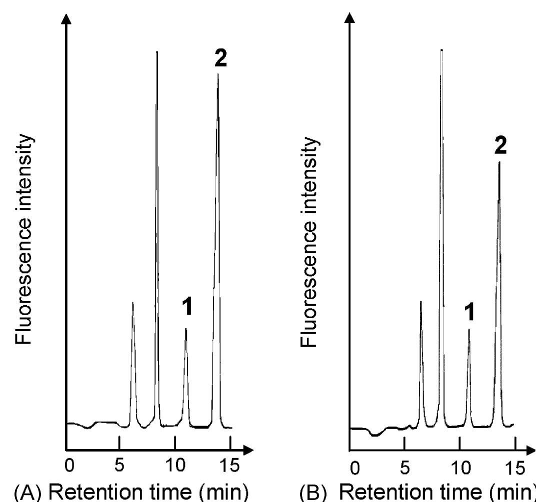

Fig. 4. (A) Chromatogram obtained from rat kidney sample without incubation containing (1) 10 μM L-NMMA as internal standard and (2) 48 μM ADMA. (B) Chromatogram obtained from rat kidney sample after incubation containing (1) 10 μM L-NMMA as internal standard and (2) 34 μM ADMA.

We have ascertained that intra-day and inter-day precision for DDAH activity assay were 6.8 and 10.0%, respectively ($n = 10$). Using the optimized method, mean DDAH activities in three different rat kidneys were determined to be 0.69 ± 0.07 pmol (ADMA)/min/(mg protein) ($n = 3$).

Another major method for DDAH activity assay has been dependent on measuring the formation of L-citrulline [30]. However, DDAH is not the sole source of citrulline, as NOS also produces citrulline. In contrast, our method of directly measuring the amount of ADMA metabolized by enzyme was simple and accurate.

4. Conclusions

A simple and rapid method for the determination of ADMA in rat plasma using a monolithic silica column was developed. Moreover, we succeeded in applying this method to DDAH activity assay. The method should contribute to the elucidation of the mechanism related to the ADMA–DDAH system.

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