Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1066 (2005) 41–45
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

High-performance liquid chromatographic assay of N^G-monomethyl-L-arginine, N^G,N^G-dimethyl-L-arginine, and N^G,$N^{G\prime}$-dimethyl-L-arginine using 4-fluoro-7-nitro-2,1,3-benzoxadiazole as a fluorescent reagent

Satoko Nonaka[a,1], Makoto Tsunoda[a,*,1], Kazuhiro Imai[b], Takashi Funatsu[a]

[a] *Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*
[b] *Research Institute of Pharmaceutical Sciences, Musashino University, 1-1-20 Shinmachi, Nishitokyo-shi, Tokyo 202-8585, Japan*

Received 2 November 2004; received in revised form 11 January 2005; accepted 21 January 2005

Abstract

N^G-Monomethyl-L-arginine (L-NMMA), N^G,N^G-dimethyl-L-arginine (ADMA), and N^G,$N^{G\prime}$-dimethyl-L-arginine (SDMA) are emerging cardiovascular risk factors. A high-performance liquid chromatographic method with fluorescence detection for the simultaneous determination of L-NMMA, ADMA and SDMA is described. The assay employed 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) as a fluorescent derivatization reagent. After solid phase extraction with cation-exchange column, the methylated arginines were converted to fluorescent derivatives with NBD-F, and the derivatives were separated within 32 min on a reversed-phase column. N^{ω}-Propyl-L-arginine was used as an internal standard. Extrapolated detection limits were 12 nM (12 fmol per injection) for L-NMMA and 20 nM (20 fmol per injection) for ADMA and SDMA, respectively, with a signal-to-noise ratio of 3. The calibration curves for L-NMMA, ADMA and SDMA were linear within the range of 50–5000 fmol. The method was applied to the quantitative determination of L-NMMA, ADMA and SDMA in 200 μl of rat plasma. The concentrations of L-NMMA, ADMA and SDMA in rat plasma were 0.16 ± 0.03, 0.80 ± 0.25 and 0.40 ± 0.21 μM, respectively ($n = 5$).

Keywords: N^G,N^G-Dimethyl-L-arginine (ADMA); Nitric oxide synthase; 4-Fluoro-7-nitro-2,1,3-benzoxadiazole; Fluorescence

1. Introduction

Nitric oxide (NO), synthesized from L-arginine by nitric oxide synthase (NOS), plays important roles in the regulation of blood flow and blood pressure, inhibition of platelet aggregation, and neurotransmission [1]. N^G-Monomethyl-L-arginine (L-NMMA), N^G,N^G-dimethyl-L-arginine (ADMA), and N^G,$N^{G\prime}$-dimethyl-L-arginine (SDMA) (Fig. 1) are formed from the degradation of methylated proteins [2]. Asymmetrical methylarginines, L-NMMA and ADMA, presented in plasma are inhibitors for NOS [3,4]. Though SDMA has no biological activity like L-NMMA and ADMA, it can interrupt the transportation of cationic amino acids such as L-arginine into cell [5]. Thus, high concentrations of SDMA in plasma may reduce NO production [6].

Increased concentration of methylated arginines in plasma is associated with hypertension [7], renal failure [8], hypercholesterolemia [9], and diabetes mellitus [10]. Furthermore, previous data on the impairment of arginine metabolism or pools in spontaneously hypertensive rats prompted us to investigate the involvement of the methylated arginines in the hypertensive rats [11,12]. Therefore, measurements of the concentration of these methylated arginines are important for the study of the arginine–NO system and endogenous inhibitors for NOS. However, a reliable and highly sensitive analytical method is lacking.

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

0021-9673/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2005.01.052

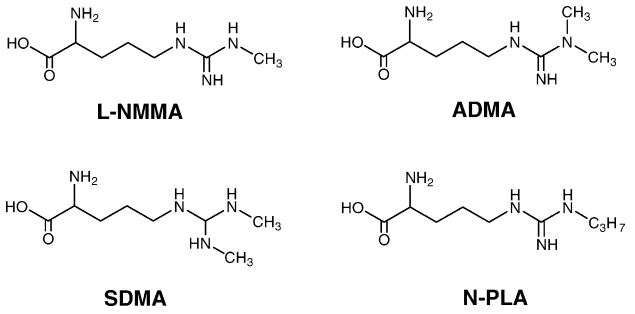

Fig. 1. Chemical structures of methylated arginines (L-NMMA, ADMA, SDMA and N-PLA).

Analytical methods for the determination of methylated arginines include thin layer chromatography [13], electrophoresis [14], ion exchange chromatography [15,16], and monoclonal antibody assay [17]. Recently, liquid chromatography–mass spectrometry (LC–MS) [18,19] and capillary electrophoresis with laser-induced fluorescence (CE-LIF) detection [20] have been the most sensitive determination methods. However, these methods are not ideal for routine clinical purposes because the procedures are time-consuming and the instrumentation is not always available in a routine clinical laboratory.

Reversed-phase high-performance liquid chromatography (HPLC) has also been employed for the sensitive analysis of methylated arginines. These methods include fluorescence detection, and *o*-phthalaldehyde (OPA) was the most commonly used fluorescent derivatization reagent [21–25]. However, OPA derivatives are unstable. Besides the loss of fluorescence during HPLC analysis, methylated arginines were not well resolved under certain chromatographic conditions [21–23,25]. Furthermore, because the concentration of L-NMMA is lower than that of ADMA and SDMA, fluorescence intensities of OPA derivatives were not efficient to determine three methylated arginines simultaneously. 4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was developed as a fluorescent derivatization reagent for amino acid [26]. This reagent has much higher sensitivity and stability than OPA. The purpose of our study was the development of a new method for the simultaneous and sensitive analysis of three methylated arginines using NBD-F as a fluorescent derivatizing reagent.

2. Experimental

2.1. Materials

L-NMMA, ADMA and SDMA were obtained from Sigma (St. Louis, MO, USA). A stock standard solution was stored in 10 mM HCl at 4 °C. N^{ω}-Propyl-L-arginine (N-PLA, Fig. 1) was obtained from Calbiochem (San Diego, CA, USA). NBD-F and boric acid were purchased from Wako (Osaka, Japan). Acetonitrile and methanol (HPLC grade) were obtained from Kanto Kagaku (Tokyo, Japan). Oasis MCX cation-exchange SPE columns (1 ml) were supplied by Waters (Milford, MA, USA). Water was used after purification by a Milli-Q reagent system (Nihon Millipore, Tokyo, Japan).

2.2. Biological samples

Male Sprague–Dawley (SD) rats (8 weeks old) were purchased from Charles River Japan (Kanagawa, Japan). The blood samples were collected into heparinized polyethylene tubes, and were immediately centrifuged at $1710 \times g$ for 10 min at 4 °C. The plasma fraction was collected and stored at −80 °C until the analysis.

2.3. Extraction procedure

L-NMMA, ADMA and SDMA were extracted from plasma samples with Oasis MCX cation-exchange SPE columns at room temperature. The SPE columns were used without preconditioning, and all washing and elution steps were performed by vacuum suction. A Vac-Elut sample preparation manifold, with a capacity of 10 columns (Varian, Harbor City, CA, USA) was used for the SPE procedure. Oil-sealed rotary vacuum pumps GLD-131 C (Ulvac Kiko, Yokohama, Japan) and centrifugal concentrator VC-36N (Taitec, Saitama, Japan) were used for evaporation procedure.

Before analysis, 200 μl of plasma or standard solution was mixed with 100 μl of N-PLA solution and then diluted by adding 700 μl of 50 mM borate buffer (pH 9.0). The solution was applied on the column. The columns were consecutively washed with 1.0 ml of 50 mM borate buffer (pH 9.0), 3.0 ml of water and 1.0 ml of methanol. Methylated arginines were eluted into 2.0 ml tubes with 1.0 ml of concentrated ammonia–water–methanol (10:40:50, v/v/v). The solvent was then evaporated to dryness at 60 °C. The dried extract was dissolved in 100 μl of water and used for the derivatization.

2.4. Derivatization procedure

First, 105 μl of 100 mM borate buffer (pH 9.0) and 30 μl of 40 mM NBD-F in acetonitrile were added to the 30 μl of sample. Then, the reaction was incubated at 40 °C for 3 min. To stop the reaction, 435 μl of 0.5% acetic acid (v/v) was added. A 10 μl aliquot of sample was injected onto the HPLC.

2.5. Chromatographic conditions

The chromatographic system was composed of Pump L-7100 (Hitachi, Tokyo, Japan), 655A-52 Column Oven (Hitachi) and FP-920S intelligent fluorescence detector (Jasco, Tokyo, Japan). The column used was Unison UK-C_{18} (150 mm × 4.6 mm I.D., Imtakt, Kyoto, Japan).

Mobile phase A consisted of 50 mM sodium phosphate buffer (pH 3.2)–acetonitrile (91:9, v/v), and mobile phase B was acetonitrile. The gradient program was as follows; 0–18 min 100% A, 18–28 min linear change to 70% A,

28–32 min 70% A. The flow rate was set at 0.75 ml/min, and the column oven was maintained at 40 °C. The wavelengths of the fluorescence detector were set at 470 and 530 nm for excitation and emission, respectively.

2.6. Examination of stability of the NBD-F derivatives

Standard solution containing 10 μM L-NMMA, ADMA, SDMA and N-PLA was reacted with NBD-F under the condition expressed above. Stability of the NBD-F derivatives at room temperature was examined for 3 days.

2.7. Validation

Calibration standards for L-NMMA, ADMA and SDMA (1, 2, 5, 10, 20, 50 and 100 μM) were prepared from stock solutions. Additional calibration curves were performed by mixing 200 μl plasma, 100 μl standard solution, 100 μl of 40 μM N-PLA solution and 600 μl of 50 mM borate buffer (pH 9.0). Concentrations of standard samples were 0.5, 1.0, and 2.0 μM. Calibration curves were calculated by plotting the peak area 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 one sample on five different days.

Plasma data were presented as mean ± standard deviation.

3. Results and discussion

3.1. Fluorescent derivatization of methylated arginines with NBD-F

In order to increase the efficiency of derivatization, some parameters such as pH, temperature and reaction time were examined.

It was reported that the optimum pH for the derivatization of amines with NBD-F was above 8 [26]. Therefore, the pH of the derivatization reaction was investigated ranged from 8.0 to 9.0. As shown in Fig. 2, at pH 9.0, the maximum fluorescence intensity was obtained among the pH ranged examined.

Then, optimum temperature was studied, ranged from 40 to 60 °C. Each fluorescence intensity was similar. Because there was less by-products on the chromatogram at 40 °C as compared with 60 °C (data not shown), 40 °C was selected as the optimum temperature. Finally, the optimum fluorescent derivatization condition for 3 min at 40 °C at pH 9.0 was chosen.

3.2. Separation of L-NMMA, ADMA and SDMA

First, the separation of L-NMMA, ADMA and SDMA was investigated under isocratic condition using the mobile phase consisted of 50 mM sodium phosphate buffer (pH 3.2)–acetonitrile (91:9, v/v). However, under the isocratic condition, SDMA was not separated from a by-product. The derivatized samples were then separated on an ODS column using a gradient elution system, and SDMA and the by-product could be separated from each other. The optimum gradient program is described in Section 2.5 above. Fig. 3 illustrates the typical chromatograms of (A) standard solution and (B) rat plasma sample. L-NMMA, ADMA and SDMA were well separated within 32 min.

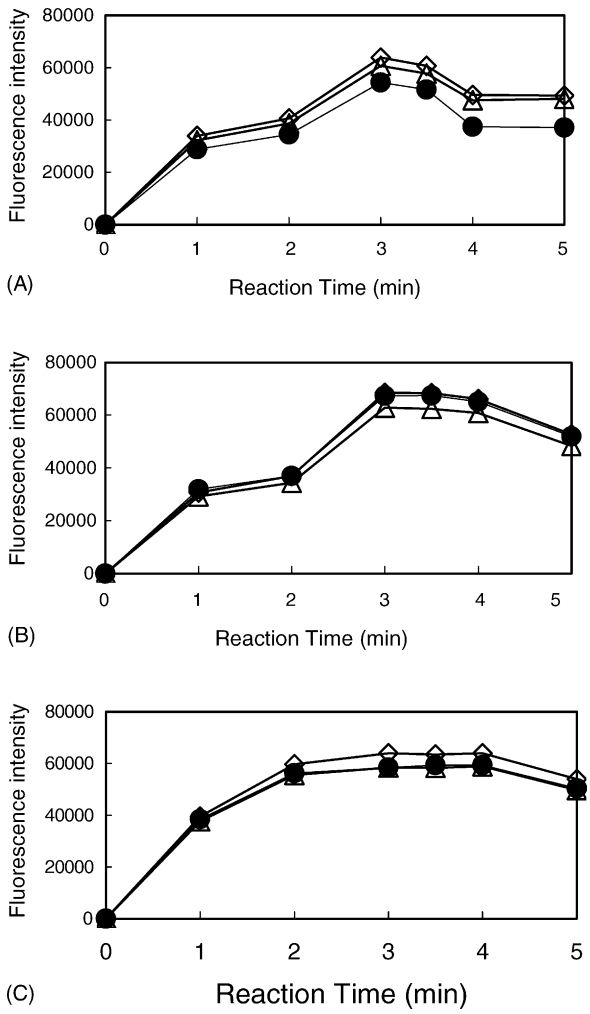

Fig. 2. Effect of pH and reaction time of fluorescent derivatization on the fluorescence intensities of NBD–L-NMMA (◊), NBD–ADMA (△) and NBD–SDMA (●): (A) pH 8.0; (B) pH 8.5; and (C) pH 9.0. The precisions for each point were less than 2%.

3.3. Linearity, detection limit, precision and accuracy

Using the method described here, a validation study on the methylated arginines in rat plasma samples was carried out. Linearity was assessed by adding known amounts of L-NMMA, ADMA and SDMA to rat plasma such that the concentrations ranged from 0.5 to 2 μM. The results were $r^2 > 0.99$ each. Limits of detection (LOD) for the method were

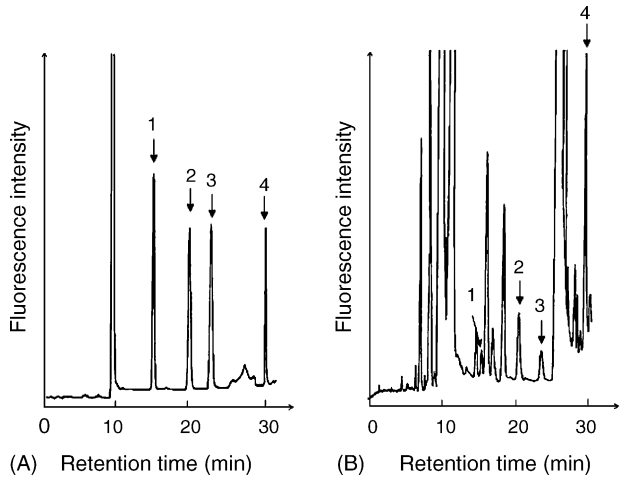

Fig. 3. (A) Chromatogram of a standard mixture containing (1) 10 μM L-NMMA; (2) 10 μM ADMA; (3) 10 μM SDMA; and (4) 5.0 μM N-PLA as internal standard. (B) Chromatogram of rat plasma sample (200 μl) containing (1) 0.17 μM L-NMMA; (2) 0.63 μM ADMA; (3) 0.30 μM SDMA; and (4) 1.5 μM N-PLA as internal standard. A 10 μl aliquot of sample from reaction mixture (600 μl) was injected onto the HPLC. Other HPLC conditions are described in Section 2.

12 fmol for L-NMMA, and 20 fmol for ADMA and SDMA at signal-to-noise ratio of 3 with standard samples, while the LODs for ADMA and SDMA using OPA derivatization and fluorescence detection were reported to be 50–200 fmol [21–23,25].

Intra-day assay CVs ($n = 5$) for L-NMMA, ADMA and SDMA were 2.89, 5.56 and 2.44%, respectively. Inter-day assay CVs ($n = 5$) for L-NMMA, ADMA and SDMA were 9.50, 6.73 and 8.58%, respectively. Data on accuracy, expressed as recovery of analyte from spiked plasma samples ($n = 3$), are depicted in Table 1. These data showed good reproducibility of the present HPLC method.

Table 1
Accuracy and precision data for determination of L-NMMA, ADMA and SDMA

	Concentration (mean ± SD, μM)	Precision (RSD, %)	Recovery (%)
L-NMMA added, μM ($n = 3$)			
0	0.17 ± 0.005	2.89	
0.5	0.68 ± 0.004	0.58	102
1.0	1.2 ± 0.006	0.52	99
2.0	2.2 ± 0.008	0.37	101
ADMA added, μM ($n = 3$)			
0	0.72 ± 0.04	5.56	
0.5	1.2 ± 0.04	3.25	102
1.0	1.7 ± 0.06	3.51	99
2.0	2.8 ± 0.09	3.19	105
SDMA added, μM ($n = 3$)			
0	0.41 ± 0.01	2.44	
0.5	0.90 ± 0.03	3.33	98
1.0	1.4 ± 0.05	3.60	98
2.0	2.4 ± 0.06	2.47	101

3.4. Stability of the NBD-F derivatives

In previous reports [21,23], OPA derivatives were found to be unstable, which may lead to a loss in precision and accuracy of the assay. Marra et al. found that naphthalene–2,3-dicarboxaldhyde (NDA) derivatives were more stable than the OPA derivatives [27]. In this study, we focused on the improvement of the stability of derivatives by using NBD-F as fluorescent derivatizing reagent.

Each fluorescence of the NBD-F derivatives of standards (L-NMMA, ADMA, SDMA and N-PLA) was stable for at least 3 days at room temperature. This data clearly demonstrate that our method has much better stability of fluorescence derivatives over time compared to the previous methods. Thus, a large number of samples could be derivatized in one time, and stored at room temperature for at least 3 days before analysis.

3.5. Concentration of L-NMMA, ADMA and SDMA in rat plasma

The concentrations of L-NMMA, ADMA and SDMA in rat plasma were 0.16 ± 0.03, 0.80 ± 0.25 and 0.40 ± 0.21 μM, respectively ($n = 5$). The mean rat plasma levels of methylated arginines were in agreement with an earlier report (0.18, 0.60 and 0.30 μM for L-NMMA, ADMA and SDMA, respectively) [24].

4. Conclusions

A method for the simultaneous determination of L-NMMA, ADMA and SDMA was developed using NBD-F as a fluorescent derivatization reagent. Fluorescent derivatives of methylated arginines with NBD-F were highly sensitive and stable compared with other fluorescent derivatives. The method may be useful in the investigation of the role of methylated arginines in a large number of pathological conditions.

Acknowledgements

The authors thank Dr. Chang-Kee Lim, MRC Bioanalytical Science Group, for his kind suggestions and valuable discussion. We would like to thank Imtakt for his kind supply of Unison UK-C_{18} column. This research was supported in part by grants (to M.T.) from the Mitsubishi Chemical Corporation Fund and from Takeda Science Foundation.

References

[1] S. Moncada, A. Higgs, N. Engl. J. Med. 329 (1993) 2002.
[2] J.R. McDermott, Biochem. J. 154 (1976) 179.
[3] P. Vallance, A. Leone, A. Calver, J. Collier, S. Moncada, J. Cardiovasc. Pharmacol. 20 (1992) 60.

[4] J. Leiper, P. Vallance, Cardiovasc. Res. 43 (1999) 542.
[5] E.I. Closs, F.Z. Basha, A. Habermeier, U. Forstermann, Nitric Oxide-Biol. Chem 1 (1997) 65.
[6] C. Fleck, A. Janz, F. Schweitzer, E. Karge, M. Schwertfeger, G. Stein, Kidney Int. 59 (2001) 14.
[7] H. Matsuoka, S. Itoh, M. Kimoto, K. Kohno, O. Tamai, Y. Wada, H. Yasukawa, G. Iwami, S. Okuda, T. Imaizumi, Hypertension 29 (1997) 242.
[8] C. Fleck, F. Schweitzer, E. Karge, M. Busch, G. Stein, Clin. Chim. Acta 336 (2003) 1.
[9] S.M. Bode-Böger, R.H. Böger, S. Kienke, W. Junker, J.C. Fröhlich, Biochem. Biophys. Res. Commun. 219 (1996) 598.
[10] F. Abbasi, T. Asagmi, J.P. Cooke, C. Lamendola, T. McLaughlin, G.M. Reaven, M. Stuehlinger, P.S. Tsao, Am. J. Cardiol. 88 (2001) 1201.
[11] W.R. Feathers, Q.R. Rogers, R.A. Freedlan, Am. J. Physiol. 224 (1973) 127.
[12] P. Prados, H. Matsunaga, T. Mori, T. Santa, T. Fukushima, H. Homma, C. Kasai, K. Imai, Biomed. Chromatogr. 13 (1999) 27.
[13] E. Tyihak, S. Ferenczi, I. Hazai, S. Zoltan, A. Patthy, J. Chromatogr. 102 (1974) 257.
[14] M. Reporter, J.L. Corbin, Biochem. Biophys. Res. Commun. 43 (1971) 644.
[15] W.K. Paik, S. Kim, Biochem. Biophys. Res. Commun. 27 (1967) 479.
[16] Y. Kakimoto, Biochim. Biophys. Acta 243 (1971) 31.
[17] M. Kimoto, G.S. Whitley, H. Tsuji, T. Ogawa, J. Biochem. 117 (1995) 237.
[18] L.F. Huang, F.Q. Guo, Y.Z. Liang, Q.N. Hu, B.M. Cheng, Anal. Chim. Acta 487 (2003) 145.
[19] J. Martens-Lobenhoffer, S.M. Bode-Böger, J. Chromatogr. B 798 (2003) 231.
[20] E. Caussé, N. Siri, J.F. Arnal, C. Bayle, P. Malatray, P. Valdiguié, R. Salvayre, F. Couderc, J. Chromatogr. B 741 (2000) 77.
[21] A. Petterson, L. Uggla, V. Backman, J. Chromatogr. B 692 (1997) 257.
[22] B. Chen, L. Xia, R. Zhao, J. Chromatogr. B 692 (1997) 467.
[23] T. Teerlink, R.J. Nijveldt, S. de Jong, P.A.M. van Leeuwen, Anal. Biochem. 303 (2002) 131.
[24] Y. Dobashi, T. Santa, K. Nakagomi, K. Imai, Analyst 127 (2002) 54.
[25] J. Pi, Y. Kumagai, G. Sun, N. Shimojo, J. Chromatogr. B 742 (2000) 199.
[26] K. Imai, Y. Watanabe, Anal. Chim. Acta 130 (1981) 377.
[27] M. Marra, A.R. Bonfigli, R. Testa, I. Testa, A. Gambini, G. Coppa, Anal. Biochem. 318 (2003) 13.