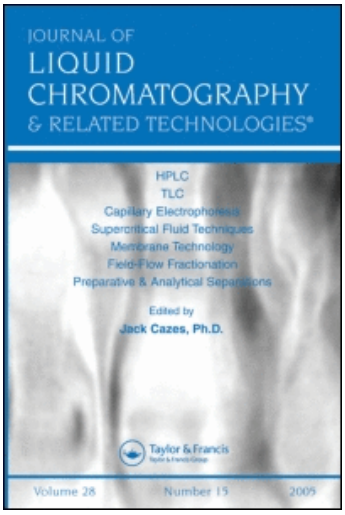

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Determination of N^G-Monomethyl-L-arginine in Human and Dog Serum Using Pre-Column o-Phthaldialdehyde Derivatization and High Performance Liquid Chromatography

A. M. Alak[a]; S. Moy[a]
[a] Fujisawa Research Institute of America, Inc. Northwestern University/Evanston Research Park, Evanston, Illinois

To cite this Article Alak, A. M. and Moy, S.(1997) 'Determination of N^G-Monomethyl-L-arginine in Human and Dog Serum Using Pre-Column o-Phthaldialdehyde Derivatization and High Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 20: 12, 1839 — 1848
To link to this Article: DOI: 10.1080/10826079708005546
URL: http://dx.doi.org/10.1080/10826079708005546

J. LIQ. CHROM. & REL. TECHNOL., 20(12), 1839–1848 (1997)

DETERMINATION OF N^G-MONOMETHYL-L-ARGININE IN HUMAN AND DOG SERUM USING PRE-COLUMN O-PHTHALDIALDEHYDE DERIVATIZATION AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

A. M. Alak, S. Moy

Fujisawa Research Institute of America, Inc.
Northwestern University/Evanston Research Park
1801 Maple Avenue
Evanston, Illinois 60201

ABSTRACT

N^G-monomethyl-L-arginine (NMA) is a selective inhibitor of the enzyme nitric oxide synthase (NOS), which converts arginine to nitric oxide and citrulline. NMA may prove useful in the treatment of autoimmune and inflammatory disorders, as well as in preventing the cardiovascular effects associated with endotoxins and cytokines. An automated high performance liquid chromatographic method for determination of NMA in human serum samples was developed using pre-column derivitization with o-phthaldialdehyde and mercaptoethanol. NMA was extracted from serum using 5-sulfosalicylic acid and methanol, then injected into the HPLC system for on-line derivitization. A two mobile phase gradient (A: 70:30 methanol-water containing 0.01M K_2HPO_4, pH 7.9; B: water containing 0.01M K_2HPO_4, pH 6.9) was employed with 70:30 (A:B) for 15 min, 10:90 through 21 min, and 70:30 through 25 min. A Beckman ODS 3 μm 75 x 3.6 mm column was used with a

Hitachi L-6200A pump set at a flow rate of 1.8 mL/min and fluorescence detection at 340 nm excitation and 450 nm emission. The sensitivity of the assay was <50 ng/mL of plasma and the assay was linear over the range 0.05-500 μg/mL. The method was sensitive, reproducible, and specific for NMA.

INTRODUCTION

N^G-monomethyl-L-arginine (NMA) is a selective inhibitor of the enzyme nitric oxide synthase (NOS), which converts arginine to nitric oxide (NO) and citrulline.[1,2] NO formation is involved in the cardiovascular effects associated with endotoxins and cytokines.[3,4] Septic shock, caused by microbial endotoxins, is characterized by cardiovascular collapse and multiple metabolic derangements. A number of cytokines appear to be involved as intermediates in these endotoxin effects.[3] NO formation appears to be involved in the mechanism, as NO is a potent hypotensive, i.e., an endothelium-derived relaxing factor.[3]

NMA has been studied in experimental animals and humans to determine whether it can be used to prevent the hypotensive effects of cytokines and endotoxins.[3,5-7] NMA, administered to patients with septic shock, largely refractory to epinephrine administration, increased blood pressure.[7] Similarly, NMA increased blood pressure in renal cell carcinoma patients treated with the cytokine interleukin-2. Interleukin-2 treatment, which is among the only effective treatments for this form of carcinoma,[8] is limited by hypotensive effects,[9] which NMA may reverse.[3] Thus, NMA and other NOS inhibitors may have a role in the treatment of autoimmune and inflammatory disorders, as well as in preventing the cardiovascular effects associated with endotoxins and cytokines.

Chromatographic methods were described for isolation of NMA in biological substrates.[10,11] Precolumn derivitization with o-phthaldialdehyde and mercaptans and reverse phase HPLC methods were described for separation of methylated and nonmethylated amino acids, but these methods were not specific for NMA.[12,13]

To date, no sensitive method for the quantitative determination of NMA in human serum samples was available. An automated HPLC method involving precolumn derivatization was developed for determination of NMA in human and canine serum.

MATERIALS

N^G-monomethyl-L-arginine (NMA) standard, 5-sulfosalicylic acid, o-phthaldialdehyde, and 2-mercaptoethanol were purchased from Sigma Chemical Company (St. Louis, MO). Methanol, HPLC Grade, was purchased from Baxter Scientific (Deerfield, IL) and potassium phosphate, dibasic, ACS Grade, from Aldrich Chemical Company (Milwaukee, WI). Drug-free human serum was purchased from Gibco BRL Life Technologies, Inc. (Gaithersburg, MD) and drug-free canine serum from Cocalico Biologicals, Inc. (Reamstown, PA).

The HPLC system consisted of a Hitachi AS 4000 Intelligent Autosampler, a Hitachi L-6200A Intelligent Pump, and a Waters Fluorescence Model 470 detector. All amino acids were separated using a Beckman Ultrasphere C18, 75 x 4.6 mm stainless steel column containing 3 μm ODS packing material.

METHODS

Standards

A stock NMA 1000 μg/mL solution was prepared by weighing 10.0 mg of NMA into a 10 mL volumetric flask and adding methanol to volume. A 100 μg/mL solution was prepared by transferring 1 mL of the first stock solution into a 10 mL volumetric flask and diluting to volume with methanol. Serum standards were prepared by adding stock NMA solutions to 5 mL of human or canine serum to give concentrations of 0.05 to 500 μg/mL.

Serum (standards, quality controls, and samples from dosed subjects) was vortexed vigorously before use. A 50 μL sample was aliquotted into a 1.5 mL polypropylene microcentrifuge tube to which 20 μL of 3% 5-sulfosalicylic acid solution and 250 μL methanol were added. The mixture was vortexed vigorously and then centrifuged at 4000 rpm for 10 min. The resulting supernatant was transferred into 200 μL limited-insert HPLC vials for injection into the system.

The derivitization solution was made by aliquoting 5 mL of o-phthaldialdehyde and 200 μL mercaptoethanol into a 100 mL volumetric flask and diluting to volume with 0.01M dibasic potassium phosphate buffer at pH 9.2. A 100 μL sample of the derivatization solution and a 20 μL sample of

serum supernatant were aspirated and dispensed into a reaction vial. The resulting solution was mixed by repetitive aspiration and dispensing for a reaction time of 3 min. Then 25 μL of the resulting solution was injected into the HPLC system and chromatographed according to the conditions below.

Mobile Phase

Mobile phase A was made of 70% methanol and 30% 0.01M dibasic potassium phosphate buffer, with the pH adjusted to 7.9 with phosphoric acid. Mobile phase B was 0.01M dibasic potassium phosphate buffer, with the pH adjusted to 6.9 with phosphoric acid. The mobile phase was passed through a 0.45 μm nylon membrane filter and degassed prior to use.

HPLC

The flow rate was maintained at 1.8 mL/min and the mobile phase gradient was 70:30 A:B from time 0 through 15 minutes, 10:90 from 16 through 21 minutes, and 70:30 from 22 through 25 minutes. The fluorescence detector was set at 340 nm excitation and 450 nm emission.

The correlation coefficient from least-squares regression calibration curves was constructed by plotting the standard concentration versus the corresponding peak area. The NMA concentrations of quality controls and unknown serum samples were calculated using the regression equation. Intraday accuracy (observed concentration/nominal concentration x 100) and precision (coefficient of variation) were determined by analyzing five samples of three different concentrations on the same day. Interday precision and accuracy were determined by analyzing duplicate samples of three different concentrations over five days.

RESULTS

Under the chromatographic conditions specified, the retention time of NMA in serum was approximately 9 min and there were no interfering peaks. Typical chromatograms in drug-free human serum, spiked human serum (25 μg/mL), and serum obtained from a patient 15 min after intravenous administration of NMA at 4 mg/kg, are shown in Figures 1A, 1B, and 1C, respectively. The same NMA peak is seen in the latter two figures, with no corresponding peak in the untreated sample.

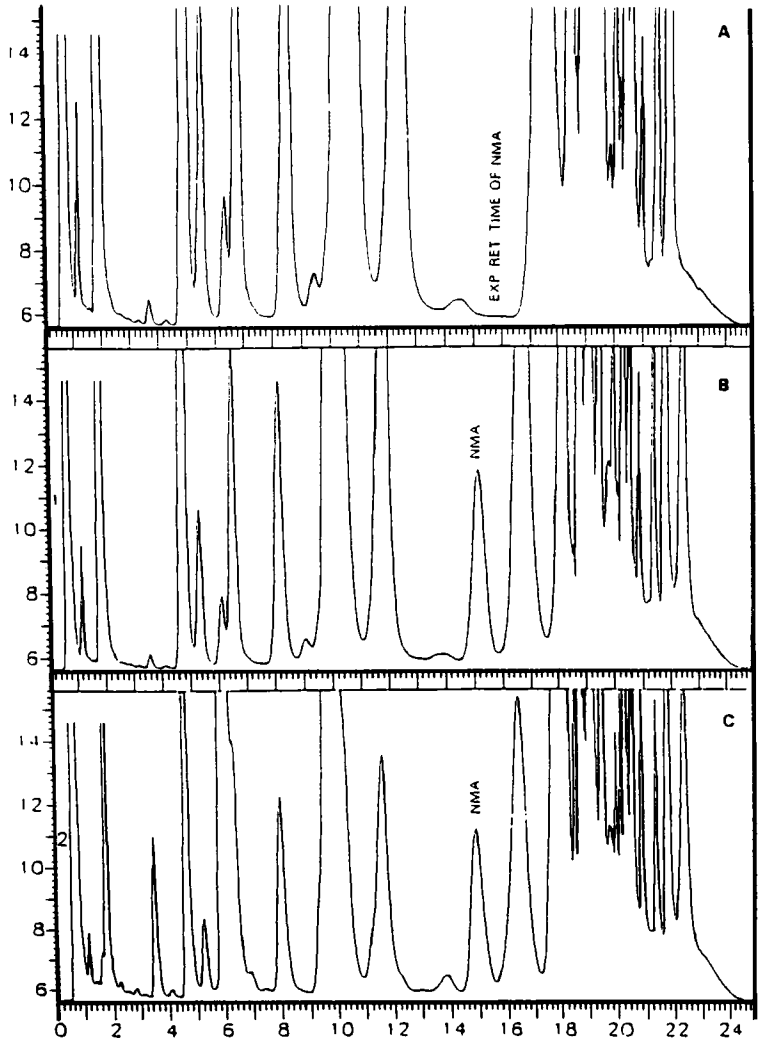

Figure 1. Representative chromatograms in (A) control human serum, (B) human serum spiked with NMA at 25 μg/ml, and (C) human serum obtained 15 min after intravenous administration of 4 mg/kg.

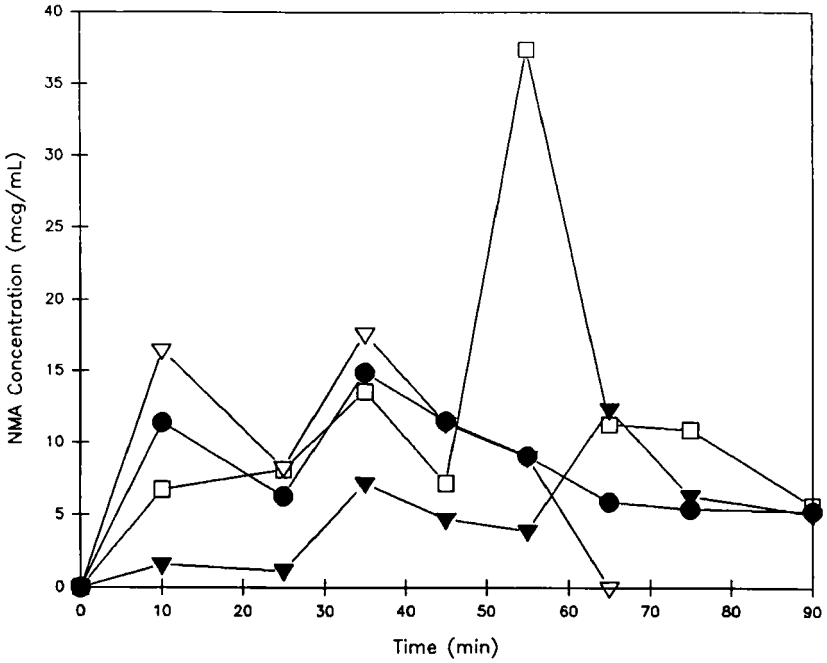

Figure 2. Concentrations of NMA determined over time in human serum samples from four subjects treated intravenously with 4 mg/kg.

Table 1

Intraday and Interday Variability in the NMA Assay

Nominal Concentration (μg/mL)	Intraday		Interday	
	Accuracy	Precision	Accuracy	Precision
1.0	95.4%	1.6%	100.0%	7.1%
10.0	110.1%	14.9%	106.0%	9.1%
100.0	109.9%	5.6%	111.8%	4.7%

In addition, no interfering peaks were seen in any other patient samples examined in this study. Canine serum showed the same pattern, with no interfering peaks and a retention time of approximately 9 min for NMA (data not shown).

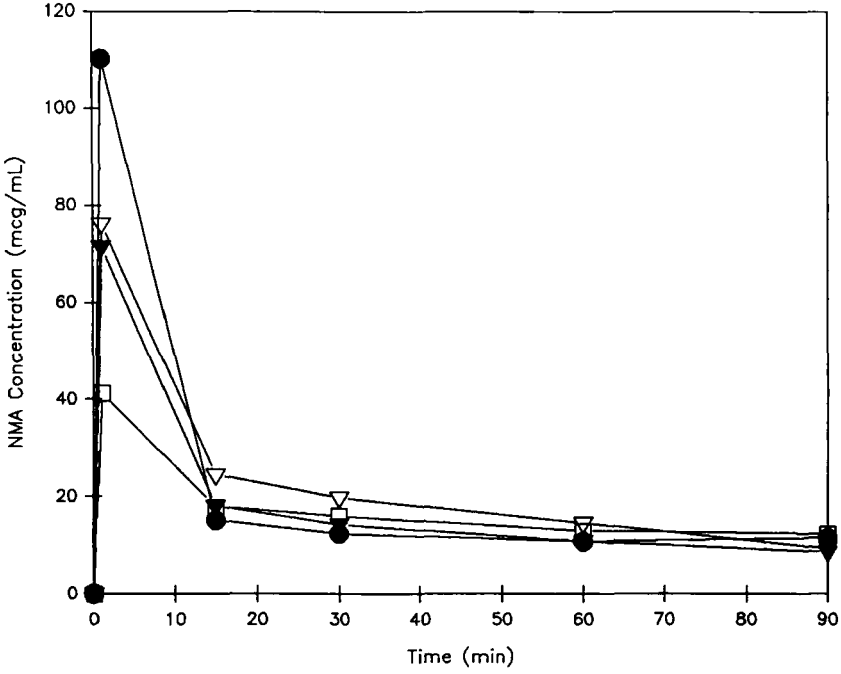

Figure 3. Concentrations of NMA determined over time in canine serum samples from four subjects administered 20 mg/kg by intravenous bolus.

The quantitative NMA concentration range was 0.05-500 μg/mL in serum. The correlation coefficient from least-squares regression calibration curves were typically >0.985. The accuracy and precision of the method were determined for intraday and interday variability (Table 1). The method was used to analyze serum samples from human and canine subjects enrolled in clinical and preclinical studies of NMA. Data from human subjects who received NMA intravenously at 4 mg/kg over 30 min for three doses are shown in Figure 2. Data from canine subjects who were administered a 20 mg/kg intravenous bolus dose of NMA over 30 seconds are shown in Figure 3.

DISCUSSION

Reverse phase HPLC is a powerful method for assaying amino acids in biological fluids. Pre-column derivitization methods were previously developed

for determination of amino acids, with o-phthaldialdehyde offering the highest sensitivity.[12,13] Reaction of amino acids with o-phthaldialdehyde and mercaptans produces thio-substituted isoindoles, which are highly fluorescent products.[12,13]

The adducts formed are unstable, which can limit the reproducibility and quantitative ability of assays based on such derivitization.

In this study, online automated derivitization was used to optimize the derivitization time and enhance the capacity of the method. The method could be used to analyze 25-50 samples in a single run.

The manual serum extraction procedure applied prior to automated HPLC was very quick. The method was sensitive, accurate, and specific for NMA in serum.

The limit of quantitation (0.05 μg/mL) was sufficient for the determination of serum NMA levels in humans and dogs up to 24 hours after dosing. This method is suitable for use in therapeutic drug monitoring of NMA in clinical trials.

ACKNOWLEDGMENTS

This study was supported by Fujisawa USA, Inc. Patient and canine serum following NMA dosing were obtained from Dr. Robert G. Kilbourn at The University of Texas M.D. Anderson Cancer Center, Houston, TX.

REFERENCES

1. H. Kolb, V. Kolb-Bachofen, "Nitric Oxide: A Pathogenic Factor in Autoimmunity," Immunol. Today, **13**, 157-160 (1992).

2. J. A. Corbett, R. G. Tilton, K. Chang, K. S. Hasan, Y. Ido, J. L. Wang, M. A. Sweetland, J. R. Lancaster Jr., J. R. Williamson, M. L. McDaniel, "Aminoguanidine, A Novel Inhibitor of Nitric Oxide Formation, Prevents Diabetic Vascular Dysfunction," Diabetes, **41**, 552-556 (1992).

3. R. G. Kilbourn, O. W. Griffith, "Overproduction of Nitric Oxide in Cytokine-Mediated and Septic Shock," J. Nat. Cancer Inst., **84**, 827-831 (1992).

4. A. K. Nussler, M. Di Silvo, T. R. Billiar, R. A. Hoffman, D. A. Geller, R. Selby, J. Madariaga, R. L. Simmons, " Stimulation Of Nitric Oxide Synthase Pathway In Human Hepatocytes By Cytokines And Endotoxin," J. Exp. Med., **176**, 261-264 (1992).

5. E. Nava, R. M. J. Palmer, S. Moncada, "Inhibition of Nitric Oxide Synthesis in Septic Shock: How Much Is Beneficial?," Lancet, 338, 1555-1557 (1991).

6. J. Meyer, L. D. Traber, S. Nelson, C. W. Lentz, H. Nakazawa, D. N. Herndon, H. Noda, D. L. Traber, "Reversal of Hyperdynamic Response to Continuous Endotoxin Administration by Inhibition of NO Synthesis," J. Appl. Physiol., **73**, 324-328 (1992).

7. A. Petros, D. Bennett, P. Vallance, "Effects of Nitric Oxide Synthase Inhibitors on Hypotension in Patients with Septic Shock," Lancet, **338**, 157-1558 (1991).

8. S. A. Rosenberg, M. T. Lotze, Y. C. Yang, P. M. Aebersold, W. M. Linehan, C. A. Seipp, D. E. White, "Experience with the Use of High-Dose Interleukin-2 in the Treatment of 652 Cancer Patients," Ann. Surg., **210**, 474-484 (1989).

9. R. Lee, M. Lotze, J. Skibber, E. Tucker, R. Bonow, F. Ognibene, J. Carrasquillo, J. Shelhamer, J. Parillo, S. Rosenberg, "Cardiorespiratory Effects of Immunotherapy with Interleukin-2," J. Clin. Oncol., **7**, 7-20 (1989).

10. T. Nakajima, Y. Matsuoka, Y. Kakimoto, "Isolation and Identification of N^G-Monomethyl,N^G,N^G-Dimethyl- and N^G,N'^G-Dimethylarginine from the Hydrolysate of Proteins of Boving Brain," Biochim. Biophys. Acta, **230**, 212-222 (1971).

11. Y. Kakimoto, S. Akazawa, "Isolation and Identification of N^G,N^G- and N^G,N'^G-Dimethyl-Arginine, N^M-Mono, Di-, and Trimethyllysine, and Glucosylgalactosyl- and Galactosyl-L-Hydroxylysine from Human Urine," J. Biol. Chem., **245**, 5751-5758 (1970).

12. K. S. Park, H.Y. Lee, S.-L. Hong, S. Shin, S. Kim, W. K. Paik, "Determination of Methylated Amino Acids in Human Serum by High Performance Liquid Chromatography," J. Chromatog., **440**, 225-230 (1988).

13. B. N. Jones, S. Pääbo, S. Stein, "Amino Acid Analysis and Enzymatic Sequence Determination of Peptides by an Improved O-Phthaldialdehyde Precolumn Labeling Procedure," J. Liq. Chromatog., **4**, 565-586 (1981).

Received April 28, 1996
Accepted May 14, 1996
Manuscript 4178