ELSEVIER

Journal of Chromatography B, 660 (1994) 170–175

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Determination of N^G-nitro-L-arginine and N^G-nitro-L-arginine methyl ester in plasma by high-performance liquid chromatography

M.J. Whiting[a,*], A.J. Rutten[b], P. Williams[a], A.D. Bersten[b]

[a]*Department of Biochemistry and Chemical Pathology, Flinders Medical Centre, Bedford Park, Adelaide, South Australia 5042, Australia*

[b]*Department of Anaesthesia and Intensive Care, Flinders Medical Centre, Bedford Park, Adelaide, South Australia 5042, Australia*

First received 27 January 1994; revised manuscript received 10 May 1994

Abstract

An HPLC method has been developed for the measurement of the nitric oxide synthase inhibitors, N^G-nitro-L-arginine (L-NOLA) and N^G-nitro-L-arginine methyl ester (L-NAME), in sheep plasma. Using an ion-exchange HPLC column (JWAS 150, 100 × 3.9 mm I.D., Millipore-Waters, Australia) and post-column ninhydrin detection, L-NOLA was separated from valine and other plasma amino acids. When added to sheep plasma, good recovery (mean 102%) and precision (mean coefficient of variation 2.7%) in the measurement of L-NOLA was obtained over the range 2–50 mg/l. L-NAME was unstable in sheep plasma at 37°C, and was converted to L-NOLA with a half-life of 250 min. This method will permit pharmacokinetic parameters to be determined for these potential drugs, and will allow plasma drug concentrations to be correlated with the pharmacodynamic effects of these compounds.

1. Introduction

Nitric oxide is synthesised from the amino acid L-arginine [1]. There are two enzymes known to catalyse this process. A constitutive nitric oxide synthase, which is calcium dependent, is present in several cell types and acts as a widespread mediator of cell–cell and intracellular communication [2]. A second type of nitric oxide synthase is induced after exposure to endotoxin or cytokines, and this enzyme has been implicated in the pathogenesis of septic shock [3].

Certain guanidino-substituted analogues of L-arginine can inhibit nitric oxide synthesis in a stereospecific competitive manner [4,5]. These compounds have potential as new drugs for the reversal of the hypotension associated with severe septic shock [6,7]. Although numerous studies have been carried out to investigate the role of nitric oxide synthase inhibitors in affecting physiological responses in a variety of experimental systems, little attention has been directed to determining pharmacokinetic parameters associated with the administration of these compounds to animals or man. This is partly because assays for the measurement of these compounds have not been described in detail. The aim of this work was to develop a high-

* Corresponding author.

0378-4347/94/$07.00
SSDI 0378-4347(94)00257-6

performance liquid chromatographic (HPLC) method for the measurement of arginine analogues, particularly N^G-nitro-L-arginine (L-NOLA) and N^G-nitro-L-arginine methyl ester (L-NAME) in sheep plasma. An experimental model of septic shock is available in conscious sheep [8] for studies of the haemodynamic effects of L-NOLA and L-NAME.

2. Experimental

2.1. Chemicals

The arginine analogues L-NOLA, L-NAME hydrochloride and L-N^G-monomethyl arginine (L-NMMA) were all purchased from Sigma (St. Louis, MO, USA), as were ninhydrin, hydrindantin, amino acid standards and the internal standard, norleucine. All other chemicals and organic solvents were of HPLC or analytical reagent grade. Mobile phases and ninhydrin solution for HPLC were prepared, filtered through 0.45 μm filters, sparged and stored under nitrogen.

2.2. Preparation of standard solutions

A stock standard solution of L-NOLA and L-NAME was prepared by dissolving both arginine analogues in 6% (w/v) sulphosalicylic acid (SSA) to give final concentrations of 2.56 mg/ml. This solution was diluted in SSA to produce a series of stock solutions covering the concentration range 40 to 2,560 mg/l. A stock solution of the internal standard norleucine was prepared by dissolving 5.7 mg of norleucine in 100 ml of 6% SSA.

2.3. Preparation of plasma standards

Sheep plasma was prepared and stored at −15°C after collecting blood into heparinized tubes, mixing and centrifuging at 5 000 *g* for 10 min. Eight standards were prepared at final concentrations of L-NOLA and L-NAME ranging from 0 to 128 mg/l by the addition of 50 μl of stock standard solutions and 450 μl of internal standard in SSA to 500 μl of sheep plasma. After thorough mixing to allow acid precipitation of plasma proteins, solutions were centrifuged at 5 000 *g* for 10 min. The supernatant was adjusted to pH 2.7 with saturated lithium hydroxide prior to filtration through a 0.45 μm pore size microfilter into an autoinjector vial containing a low-volume insert.

2.4. HPLC analysis conditions

Amino acid analysis was performed using equipment from Millipore-Waters Australia (Lane Cove, N.S.W., Australia). Deproteinized plasma samples (100 μl) were injected onto a 100 × 3.9 mm I.D. cation-exchange HPLC column (JWAS 150, 5 μm polystyrene resin, Millipore-Waters Australia), using a Wisp Model 712 autoinjector. A curvilinear buffer pH gradient of constant ionic strength was generated by an M600 solvent delivery system (flow rate 0.4 ml/min) by mixing buffer A (65 mmol/l trilithium citrate, 5% (v/v) methanol, pH 2.72) and buffer B (65 mmol/l lithium borate, 2.7 mmol/l EDTA, pH 10.40). Amino acids eluting from the column were mixed with a ninhydrin solution (0.4 ml/min), containing 110 mmol/l ninhydrin, 2.8 mmol/l hydrindantin, 1 mol/l lithium acetate, pH 5.2 dissolved in 75% dimethylsulfoxide. The mixture was heated to 110°C in a post-column reaction coil before monitoring the absorbance of the eluate at 570 nm using a Model 440 absorbance detector. Chromatographic data were acquired, stored and analysed on personal computer using Waters Maxima 820 software.

2.5. Calibration and calculations

For the calculation of response factors, the ratios of the peak heights of the arginine analogues and that of the internal standard were determined. With final plasma concentrations of L-NOLA and L-NAME of 0, 2, 4, 8, 16, 32, 64, and 128 mg/l, a linear relationship between amino acid concentration and detector response was obtained from unweighted linear regression analysis over the entire range of concentrations used. The equations of the calibration curves and

their correlation coefficients were $y = 0.708x - 2.3$ ($r = 0.995$) for L-NOLA and $y = 0.831x - 1.3$ ($r = 0.999$) for L-NAME, where y is the concentration of amino acid in mg/l and x is the response factor multiplied by the amount of internal standard. The limit of detection was 0.5 mg/l, when calculated as the amino acid concentration that produced a chromatographic peak with a height three times the baseline noise level.

In subsequent experiments to determine the recovery and precision of measurement of L-NOLA and L-NAME in sheep plasma, only three standards containing L-NOLA and L-NAME were used to calibrate the detector response. However, the slope of the standard curve was constant between analytical runs and was 0.692 ± 0.019 (C.V. = 2.7%, $n = 12$) for L-NOLA and 0.835 ± 0.23 (C.V. = 2.8%, $n = 4$) for L-NAME.

3. Results

In order to define their elution profile, a standard mixture of 39 physiological amino acids was separated by ion-exchange HPLC at 43°C, using a curvilinear pH gradient from pH 2.72 to pH 10.40. Under these conditions, acidic amino acids were eluted first from the column, followed by neutral, aromatic and basic amino acids with a total run time of 125 min (see Fig. 1). The arginine analogues L-NOLA, L-NAME and L-NMMA were added to the standard mixture of amino acids and their elution positions are also shown in Fig. 1. L-NOLA eluted with a retention time of 48.5 min, but could not be resolved from valine under these conditions. L-NAME was resolved completely from other amino acids with a retention time of 91.5 min, while L-NMMA co-eluted with arginine with a retention time of 115.6 min and was not further studied.

To separate L-NOLA from valine and to reduce the analysis time, the HPLC conditions were modified. By increasing the column temperature from 43°C to 52°C, the retention time of L-NOLA was reduced relative to valine, so that these two amino acids were now completely separated. In addition, the total run time was reduced to 80 min by changing the pH gradient and increasing the starting pH from 2.72 (100% buffer A) to 4.64 (75% buffer A, 25% buffer B). The proportion of buffer B was increased linearly to 70% over 35 min, and then to 100% over the next 10 min. Using these conditions, the chromatograms in Fig. 2 show the amino acids present in sheep plasma, both before (A) and after (B) the addition of 44 mg/l of L-NOLA, which eluted with a retention time of 26.9 min.

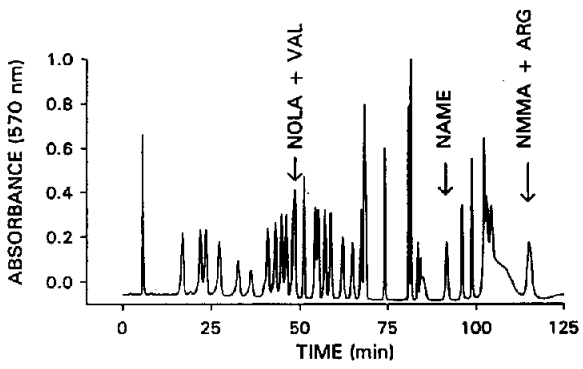

Fig. 1. Chromatogram of physiological amino acids, separated by ion-exchange HPLC at 43°C with a pH gradient and on-line ninhydrin detection. A standard mixture of 39 amino acids was spiked with L-NOLA, L-NAME and L-NMMA to a final concentration of 50 mg/l. Elution positions of the arginine analogues are marked with arrows.

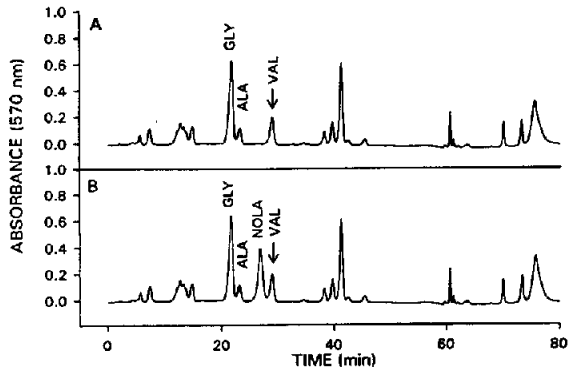

Fig. 2. Chromatography of sheep plasma amino acids, using modified HPLC conditions to separate valine from L-NOLA. Under these conditions, glycine, alanine and valine from normal sheep plasma eluted as shown in A. After the addition of 44 mg/l L-NOLA, the plasma amino acid profile shown in B was obtained. The large peak with retention time of 41.2 min is the internal standard, norleucine.

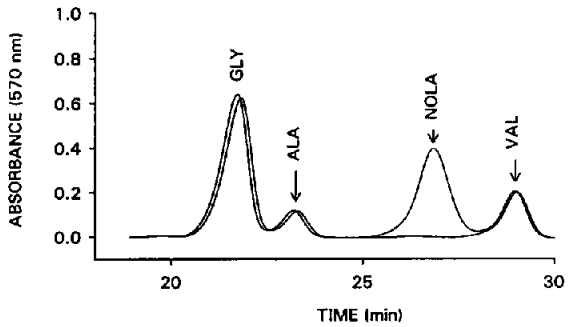

Fig. 3. Overlaid chromatograms of glycine, alanine and valine from sheep plasma, with and without the addition of 44 mg/l L-NOLA. A trace amount of α-amino-isobutyric acid, which eluted slightly before L-NOLA, was present in normal sheep plasma.

Peak retention times correspond to those of glycine (21.8 min), alanine (23.3 min) and valine (29.0 min). In Fig. 3, sheep plasma amino acids eluting between 20 and 30 min are shown in overlaid chromatograms before and after the addition of L-NOLA. Normal sheep plasma contains a low concentration (<1 mg/l) of α-amino-isobutyric acid, which elutes with a retention time of 26.4 min. Although this amino acid is not completely resolved from L-NOLA, its low concentration does not interfere with L-NOLA estimation in plasma at concentrations >1 mg/l.

The analytical recovery and precision of the measurement of L-NOLA added to sheep plasma at four different concentrations is shown in Table 1. From 24 plasma analyses, the average recovery of L-NOLA, added to plasma at concentrations ranging from 2 to 50 mg/l, was 102% and the average coefficient of variation (C.V.) of measurement was 2.7%. A slight bias was observed in this experiment, in that low concentrations were slightly overestimated while high concentrations were underestimated. The retention time of L-NOLA for these 24 samples was 26.73 ± 0.13 min (C.V. 0.47%), while that of the internal standard norleucine was 41.16 ± 0.08 min (C.V. 0.19%). Retention times were constant between analyses and between analytical runs, provided the pH of the two eluting buffers used to generate the pH gradient was kept constant between preparations.

The analytical recovery and precision of the measurement of L-NAME added to sheep plasma at three different concentrations was also examined, and is shown in Table 2. Unexpected low recoveries of L-NAME were obtained, but it was noted that L-NOLA was also present in plasma samples. The sum of L-NAME and L-NOLA recovered was over 88% of the amount of L-NAME added to the plasma (Table 2). This finding suggested that L-NAME was not stable in sheep plasma. A further experiment in which L-NAME was added to sheep plasma, which was then incubated at 37°C with gentle shaking, confirmed that L-NAME was converted to L-NOLA in vitro. As shown in Fig. 4, the half-life of L-NAME under these conditions was around 250 min. Incubation of L-NAME and L-NOLA in 0.15 *M* NaCl at 37°C did not result in any breakdown of these compounds.

Table 1
Recovery and precision of measurement of NOLA in sheep plasma using ion-exchange HPLC

NOLA added (mg/l)	NOLA measured (mg/l)	C.V. (%)	Recovery (%)
0	0.1 ± 0.1	–	–
2	2.3 ± 0.1	3.5	115 ± 4
10	10.2 ± 0.2	1.7	102 ± 2
20	19.5 ± 0.7	3.5	97 ± 3
50	45.4 ± 1.1	2.3	91 ± 2
Mean		2.7	102

Results are expressed as the mean ± S.D. of 6 determinations.

Table 2
Recovery and precision of measurement of NAME in plasma using ion-exchange HPLC

NAME added (mg/l)	NAME measured (mg/l)	C.V. (%)	Recovery (%)	NOLA measured (mg/l)	Total recovery (%)
0	–	–	–	–	–
2	0.9 ± 0.5	60.9	44 ± 26	1.1 ± 0.3	100 ± 35
10	6.0 ± 0.3	4.4	60 ± 3	3.1 ± 0.4	92 ± 7
50	25.0 ± 0.7	3.0	50 ± 1	18.6 ± 2.8	88 ± 7

Results are expressed as the mean ± S.D. of 6 determinations.

4. Discussion

Various arginine analogues are being used as inhibitors of nitric oxide synthesis to investigate the roles of nitric oxide in different biological systems. In spite of widespread interest in these inhibitors, there are few published methods available to monitor the levels of these compounds in blood or other biological fluids. Sook Park et al. [9] separated several methylated amino acids, including L-NMMA, as their phenyl isothiocyanate derivatives on a 2-column HPLC system with isocratic elution. In studying stereoisomers of dimethylarginine, Vallance et al. [10] used capillary electrophoresis to quantitate these analogues in a urine sample. Krejcy et al. [11] recently described a HPLC assay for L-NAME and L-NOLA in dog plasma, using ultraviolet detection after chromatography on a silica gel column. Other work has described the effects of L-NMMA and L-NOLA on the metabolism of citrulline and arginine in endothelial cells, but the HPLC method described was not used to measure the concentrations of the arginine analogues [12].

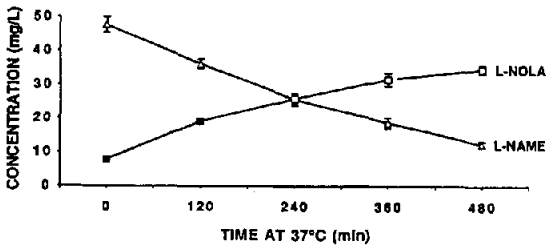

Fig. 4. Conversion of L-NAME to L-NOLA by sheep plasma in vitro. To sheep plasma incubated at 37°C was added 47.5 mg/l L-NAME. Samples were analysed for L-NAME and L-NOLA every two hours.

In the present work, conventional amino acid analysis using cation-exchange chromatography with ninhydrin detection has been used to determine the elution characteristics of L-NOLA, L-NAME and L-NMMA. The addition of a nitro group to the guanidino moiety of arginine changed the charge of this amino acid, so that instead of eluting as a basic amino acid, like arginine or L-NMMA, L-NOLA eluted near valine, a neutral amino acid. To completely separate L-NOLA from valine, which is a major plasma amino acid, an increase in column temperature was required. Acceptable recovery and precision in the measurement of L-NOLA in sheep plasma was then obtained. The availability of a reliable method for L-NOLA estimation should facilitate pharmacokinetic studies of this potential drug, and studies are in progress in our laboratory to examine the disposition and vascular effects of L-NOLA during septic shock in sheep.

Although well resolved from other amino acids, L-NAME determination in sheep plasma was complicated by its unexpected instability and in vitro conversion to L-NOLA. This finding has recently been reported by Krejcy et al. [11], who found the half-life of L-NAME in dog plasma at 37°C was 222 min. Nevertheless, L-NAME could still be a useful compound, since it is likely to be taken up by cells more readily than L-NOLA [11].

Alternative methods of amino acid analysis

involve pre-column derivatization prior to separation by reversed-phase HPLC. To this end, several derivatizing agents are in use, including *o*-phthaldialdehyde, 9-fluorenylmethylchloroformate and phenylisothiocyanate. In preliminary studies, we have found that L-NOLA also can be resolved from other amino acids by reversed-phase HPLC of the phenylisothiocarbamyl-derivatives (unpublished data).

Acknowledgement

We would like to thank Ms. J. McCulloch for typing the manuscript.

References

[1] R.M.J. Palmer, D.S. Ashton and S. Moncada, *Nature*, 333 (1988) 664–666.
[2] S. Moncada, R.M.J. Palmer and E.A. Higgs, *Pharmacol. Rev.*, 43 (1991) 109–142.
[3] P. Vallance and S. Moncada, *New Horizons*, 1 (1993) 77–86.
[4] P.K. Moore, O.A. Al-Swayeh, N.W.S. Chong, R.A. Evans and A. Gibson, *Br. J. Pharmacol.*, 99 (1990) 408–412.
[5] D.D. Rees, R.M.J. Palmer, R. Schulz, H.F. Hodson and S. Moncada, *Br. J. Pharmacol.*, 101 (1990) 746–752.
[6] A. Petros, D. Bennet and P. Vallance, *Lancet*, 338 (1991) 1557–1558.
[7] S. Geroulanos, J. Schilling, M. Cakmakci, H.H. Jung and F. Largiader, *Lancet*, 339 (1992) 435.
[8] D.L. Traber, H. Redl, G. Schlag, D.N. Herndon, R. Kimura, T. Prien and L.D. Traber, *Am. J. Physiol.*, 254 (1988) H833–H839.
[9] K. Sook Park, S. Shin, S. Kim and W.K. Paik, *J. Chromatogr.*, 440 (1988) 225–230.
[10] P. Vallance, A. Leone, A. Calver, J. Collier and S. Moncada, *Br. J. Pharmacol.*, 101 (1990) 746–752.
[11] K. Krejcy, S. Schwarzacher and G. Raberger, *Arch. Pharmacol.*, 347 (1993) 342–345.
[12] M. Hecker, J.A. Mitchell, H.J. Harris, M. Katsura, C. Thiemermann and J.R. Vane, *Biochem. Biophys. Res. Commun.*, 167 (1990) 1037–1043.