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Reversed-phase high-performance liquid chromatography method for the analysis of nitro-arginine in rat plasma and urine

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Abstract

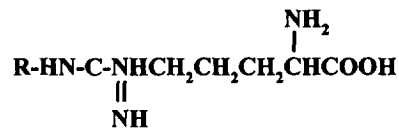
Nitro-L-arginine (L-NNA) is an inhibitor of the enzyme nitric oxide synthase (NOS). We developed a simple, sensitive and reproducible reversed-phase high-performance liquid chromatographic method for detection of nitro-arginine (L- and D-enantiomer) in rat plasma and urine. Samples were treated with perchloric acid, neutralized and eluted through a C_8 reversed-phase column with a mobile phase of 18.5 m*M* heptanesulfonic acid–10% methanol in water, using theophylline as an internal standard. Plasma recovery for both isomers was complete, and the sensitivity limit was 0.5 μg/ml. This method may be used for disposition studies of L-NNA in small animals.

Keywords: Nitroarginine

1. Introduction

Nitric oxide (NO), produced from the endogenous amino acid L-arginine via the enzyme nitric oxide synthase (NOS), is an important mediator involved in the regulation of vascular tone both in animals and humans [1,2]. In addition, NO is believed to be an important mediator in the hypotension developed during septic shock which is nonresponsive to the conventional vasoconstrictor therapy [3,4]. Competitive inhibition of NO production has been shown to cause an increase in blood pressure [2,5]. Nitro-L-arginine (L-NNA), a nitro derivative of the amino acid L-arginine (Fig. 1), is a potent inhibitor [6,7] of NOS, and has been employed in the management of septic shock in humans [8]. The inhibition of NOS by the arginine derivatives has been shown to be stereospecific [9]. In contrast to the L-isomer, the D-enantiomer (D-NNA) has been reported to be ineffective in inhibiting the enzyme NOS both in vitro and in vivo [10,11].

The in vivo pharmacokinetics and pharmacodynamics of NOS inhibitors are not yet well character-



A: R=H, L-arginine

B: R=NO_2, Nitro -L- Arginine

Fig. 1. Chemical structure of the endogenous amino acid L-arginine (A), and the nitro derivative of L-arginine, nitro-L-arginine (B).

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ized. This deficiency may well be due to the lack of bioanalytical methods suitable for use in small experimental animals. An analytical method for detection of L-NNA in sheep plasma has been described [12], but this procedure is quite complex in that it requires gradient elution at elevated temperatures, post-column derivatization and a relatively large sample volume (500 μl). The sensitivity achieved is 2 μg/ml and the run time is about 30 min.

We have developed an alternate method which provides improvements over this existing procedure, allowing for isocratic elution at ambient temperatures, eliminating the need for post-column derivatization and improving the sensitivity of assay to 0.5 μg/ml while permitting the use of a smaller sample size of 100 μl. Application of this analytical method for the pharmacokinetic examination of L- and D-nitro-arginine in rats is demonstrated.

2. Experimental

2.1. Chemicals and reagents

L-NNA, theophylline (anhydrous crystals), heptanesulfonic acid (sodium salt) were purchased from Sigma (St. Louis, MO, USA). D-NNA was obtained from Calbiochem-Novabiochem (La Jolla, CA, USA). All solvents used were of HPLC grade.

2.2. Aqueous standard curves

Stock solutions (877 μg/ml) of L-NNA and D-NNA in double-distilled water were prepared in disposable polystyrene tubes (Becton Dickinson, Lincoln Park, NJ, USA). Working standards (6.6–658 μg/ml) were made by serial dilutions of the stock solution. Stock solutions and the working standards were prepared on a monthly basis and stored at 4°C. Aqueous calibration standards were prepared daily from the working standards by adding 10 μl of each standard and 10 μl of a solution of the internal standard (theophylline, 0.18 μg/ml) into polypropylene microcentrifuge tubes (Brinkmann Instruments, Westbury, NY, USA) containing 107.5 μl double-distilled water to give the final concentrations of 52.0, 34.4, 17.2, 8.6, 1.7 and 0.5 μg/ml. The ratio of the peak area of the L-NNA or the D-isomer to that of the internal standard was plotted vs. the concentration of the isomers to produce the calibration curves. The slope and intercept were obtained by least square linear regression.

2.3. Plasma standard curves

Aliquots of blank rat plasma (100 μl) were placed in the microcentrifuge tubes, and 10 μl of the internal standard (0.18 μg/ml) and 10 μl of each working standards of L- or D-isomers of nitro-arginine were added and vortexed. Plasma proteins were precipitated using 6 μl 70–72% $HClO_4$ (J.T. Baker, Phillipsburg, NJ, USA) and then samples were centrifuged for 2 min at 13 000 *g* (Fisher micro-centrifuge model 325). The supernatant was then transferred and the final pH of the supernatant was readjusted to about 3 by the addition of 1.5 μl 50% NaOH solution (Fisher Scientific Company, Fair Lawn, NJ, USA). The final concentrations obtained were the same as those reported for the aqueous standards.

2.4. Urine standard curves

Rat urine samples (100 μl) were spiked with 10 μl of 0.9 μg/ml internal standard (theophylline) and 10 μl of each aqueous working standard (55–658 μg/ml) of L- or D-isomers of nitro-arginine. Samples were then treated the same way as plasma samples. The final concentrations obtained ranged from 4.3 to 52 μg/ml.

2.5. Apparatus and assay condition

The processed samples were injected onto the HPLC (50 μl for plasma, 10 μl for urine) via a Waters WISP 710 automated injector (Milford, MA, USA). The analysis was carried out with a Beckman Ultrasphere (San Ramon, CA, USA) 5 μm, C_8 reversed-phase column (250×4.6 mm I.D.) which was preceded by a 45×4.6 mm I.D., 5 μm guard column (Beckman, San Ramon, CA, USA). The samples were eluted at room temperature with a

mobile phase consisting of 18.5 m*M* heptanesulfonic acid–10% methanol (J.T. Baker) in double-distilled water. Heptanesulfonic acid was initially dissolved in double-distilled water and then methanol was added and mixed. After addition of methanol, the pH of the mobile phase was adjusted to 2.7 with phosphoric acid (J.T. Baker), as determined by a pH meter, and the mobile phase was filtered and degassed. The mobile phase was pumped with a Waters Model 510 pump at a constant flow-rate of 1.5 ml/min. Detection was accomplished at 280 nm with a Kratos analytical Model 783 spectroflow absorbance detector (Ramsey, NJ, USA). The peak areas were integrated with a Hewlett-Packard (Avondale, PA, USA) Model 3394A integrator. When unknown samples were found to have higher concentration than 52 μg/ml, they were diluted by addition of blank plasma and reassayed.

2.6. Animal studies

Male Sprague–Dawley rats underwent surgery under ether anesthesia for implantation of an indwelling cannula in the right jugular vein. The cannula was threaded to exit at the back of the neck of the animal. One day later, an intravenous bolus dose of 20 mg/kg of either L-NNA or D-NNA in normal saline (1.5 ml injected over 30 s) was given through the jugular cannula, followed by flushing with normal saline (100 μl). Blood samples (400 μl) were taken through the heparinized cannula and the hematocrit was measured. The blood was immediately centrifuged (Fisher micro-centrifuge Model 325) for 2 min at 13 000 *g* and the plasma was stored at −20°C until analysis. Urine was collected throughout the study at 10–12-h intervals. Samples were analyzed by HPLC as described.

2.7. Statistics

The Lagrange polynomial method [13] was used to calculate the area under the plasma concentrations vs. time curve (AUC). Systemic clearance was calculated as dose/AUC. Statistical analyses were performed using Student's *t*-test. Data are expressed as mean±S.D.

3. Results

Spectral analysis revealed that L-NNA absorbs at both 210 and 280 nm with higher absorption at the latter wavelength, which was chosen for detection of both isomers. Typical chromatograms for both blank (A) and spiked plasma and urine (B) containing L-NNA are shown in Fig. 2 and Fig. 3, respectively. The retention times for L-NNA, and internal standard in plasma and urine are presented in Fig. 2 and Fig. 3, respectively. The retention time of D-NNA was similar to that of the L-enantiomer.

Standard curves obtained for both isomers were linear between 0.5 and 52 μg/ml in water and plasma, with correlation coefficients generally >0.995 (see legend to Table 1). Mean recovery for the two isomers in plasma at three different concentrations 34.4, 17.2 and 8.6 were 105±1.8 and 104±1.7 for L-NNA and D-NNA, respectively (n=3 at each concentration).

Aqueous and plasma standards stored at 4°C and −20°C were found to be stable for up to four months with no detectable degradation. Standard curves in urine were linear between 4.3 to 52 μg/ml. The

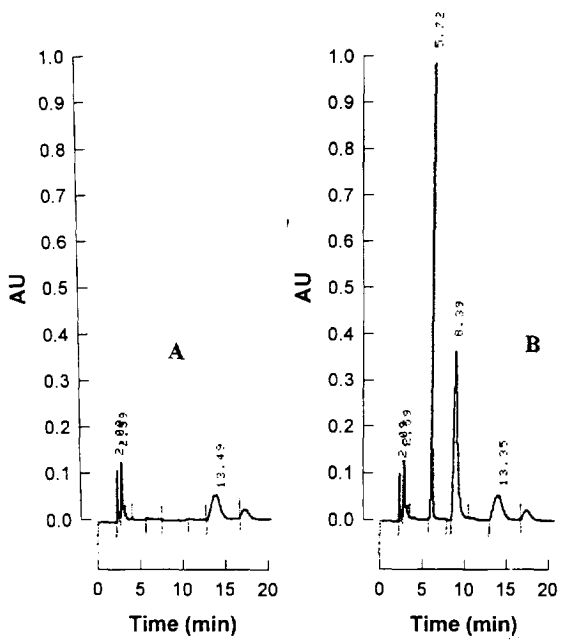

Fig. 2. HPLC chromatogram of blank (A) and spiked (B) rat plasma. The L-NNA and theophylline peaks were observed at 5.7 and 8.3 min, respectively.

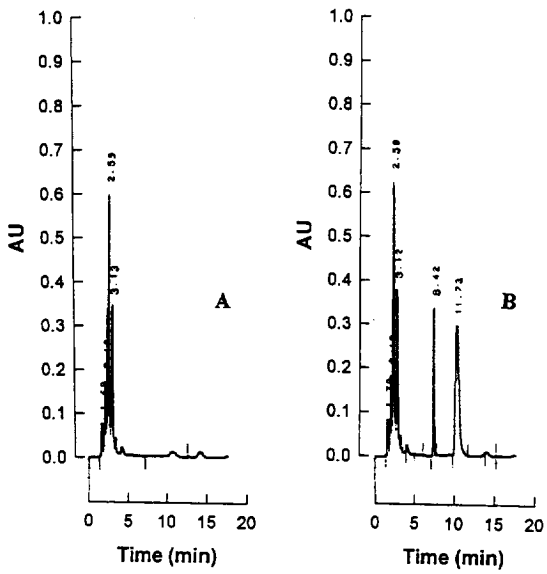

Fig. 3. HPLC chromatogram of blank (A) and spiked (B) rat urine. The L-NNA and theophylline peaks were observed at 8.4 and 11.7 min, respectively.

mean recovery of the two isomers in urine at three different concentrations 34.4, 17.2, 4.3, were 97.8±0.7% and 94.4±3.6% for L- and D-enantiomers, respectively ($n=3$ at each concentration). The intra- and inter-day coefficient of variation for L- and D-enantiomers in plasma and urine are summarized in Table 1 and Table 2, respectively. Spiked urine samples stored at room temperature for up to 24 h were stable with no detectable degradation (data not shown). Under the same analytical conditions, nitro-L-arginine methyl ester (L-NAME), the ester derivative of L-NNA which is often used in pharmacological studies, was detectable at a retention time of 58 min, but considerable on-column degradation to L-NNA was observed.

Fig. 4 shows the pharmacokinetic profile of the of L-NNA and D-NNA in the rat after bolus intravenous dose of 20 mg/kg. The hematocrit values measured in both treatment groups were comparable (L-NNA= 44%±4.36%, $n=3$; D-NNA=50%±3.11%, $n=4$). Peak plasma concentrations up to 100 μg/ml were

Table 1
The intra- and inter-day variability for L- and D-NNA in rat plasma at five different concentrations

Concentration (μg/ml)	Coefficient of variation (%)			
	Intra-day		Inter-day	
	L-NNA	D-NNA	L-NNA	D-NNA
34.4	2.04	6.34	5.93	8.87
17.2	2.91	1.21	4.19	8.13
8.6	3.61	6.17	4.89	4.65
1.7	5.88	5.56	6.25	11.11
0.5	5.97	8.47	12.07	7.01

The regression equations for L- and D-NNA in water ($n=3$) and plasma ($n=3$) were: L-NNA in water, $Y=0.10(\pm0.01)X-0.00(\pm0.05)$, $r=0.999(\pm0.001)$; L-NNA in plasma, $Y=0.09(\pm0.01)X-0.03(\pm0.02)$, $r=0.999(\pm0.001)$; D-NNA in water, $Y=0.09(\pm0.01)X+0.01(\pm0.02)$, $r=0.999(\pm0.001)$; D-NNA in plasma, $Y=0.09(\pm0.01)X+0.1(\pm0.1)$, $r=0.997$ (±0.003).

Table 2
The intra- and inter-day variability for L- and D-NNA in rat urine at three different concentrations

Concentration (μg/ml)	Coefficient of variation (%)			
	Intra-day		Inter-day	
	L-NNA	D-NNA	L-NNA	D-NNA
52.0	2.44	8.97	6.67	5.26
34.4	1.69	5.17	1.69	9.43
4.3	2.67	3.90	10.12	12.05

The regression equations for L- and D-NNA in water ($n=3$) and urine ($n=3$) were: L-NNA in water, $Y=0.016(\pm0.003)X+0.00(\pm0.01)$, $r=0.999(\pm0.001)$; L-NNA in urine, $Y=0.017(\pm0.001)X+0.00(\pm0.02)$, $r=0.998(\pm0.003)$; D-NNA in water, $Y=0.018(\pm0.002)X+0.00(\pm0.01)$, $r=0.999(\pm0.001)$; D-NNA in urine, $Y=0.016(\pm0.002)X+0.02(+0.01)$, $r=0.999(\pm0.001)$.

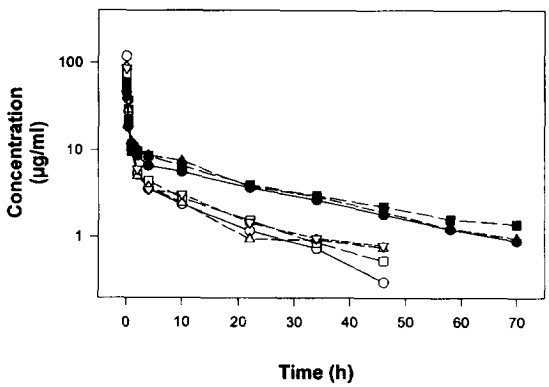

Fig. 4. Semi-logarithmic plot of plasma concentrations of L-NNA (closed symbols, $n=3$), and D-NNA (open symbols, $n=4$) vs. time after i.v. bolus administration (20 mg/kg) in rats.

observed after administration of both isomers. The pharmacokinetics of the two enantiomer of nitro-arginine were shown to be different, with the L-enantiomer having a longer elimination half-life than the D-enantiomer (22.3±2.4 h vs. 14.2±2.2 h, $P=0.006$) and a slower systemic clearance (70.9±8.2 ml/h per kg vs. 170±20 ml/h per kg, $P=0.0004$). No L-NNA was detected in the urine over the study period of 70 h. However, 3.6±1.1% ($n=3$) of the D-enantiomer dose was detected in urine over the first 10 h of dosing.

4. Discussion

We have described a simple, reproducible and sensitive assay for the analysis of nitro-arginine in rat plasma and urine. The assay utilizes a wavelength of 280 nm for detection at which NNA has the highest UV absorptivity. The sensitivity of the assay is adequate for detecting nitro-arginine in the rat plasma over three terminal half lives (70 h) of the inhibitor at the dose administered. The time required for the preparation of the samples is less than 10 min per sample and the plasma volume required is small (100 µl). Although both nitro-arginine and the internal standard eluted within 15 min of column injection, the run time of each chromatogram was close to 30 min due to the presence of late-eluting components at about 27 min post injection (not shown in Fig. 2 and Fig. 3). In this study, plasma standards were used for the pharmacokinetic studies.

The present studies indicate that the pharmacokinetics of L-NNA and D-NNA are substantially different. Thus the lack of activity of D-NNA vs. L-NNA observed in vivo [11] may arise not only from a stereospecific difference in their inhibitory effect on NOS, but also from the faster systemic clearance of the D-isomer as well. Some of the pharmacokinetic properties of L-NNA have been reported elsewhere [14].

In summary, we have developed a simple and specific assay for the determination of nitro-arginine in rat plasma and urine. Several properties of the assay, viz., simplicity, short run time, small volume and short sample preparation time make this method suitable for use in pharmacokinetic studies even in small animals.

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