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Determination of L-iminoethyl-L-lysine in serum by liquid chromatography

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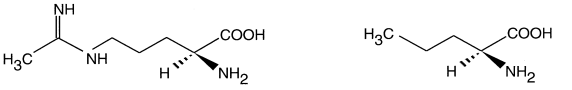
Abstract

A selective and sensitive method is presented for the determination of L-iminoethyl-L-lysine (L-NIL) in rat serum. L-NIL is a selective inhibitor of the inducible nitric oxide synthase. The analytical technique is based upon a two-buffer reversed-phase HPLC system with fluorescence detection of pre-column derivatized amino acid analogue with *o*-phtaldialdehyde. The retention time of L-NIL was 19.4 min. The limit of quantification was 0.5 mg/l. After validation, the method was used to study the pharmacokinetic profile of L-NIL in rats after intravenous as well as oral administration of a single dose.

Keywords: L-Iminoethyl-L-lysine; Amino acids

1. Introduction

Nitric oxide (NO) is an important mediator of inflammation and pain, which is derived from L-arginine by the inducible enzyme nitric oxide synthase (iNOS) [1]. The lysine analogue L-N6-iminoethyllysine (L-NIL) (Fig. 1a) is a selective inhibitor of iNOS used for experimental studies [2,3]. In these investigations L-NIL acts as an antiinflammatory drug in the late phase of inflammation [4,5]. There are no reports of antinociceptive properties of L-NIL, whereas an increase in iNOS was shown in cases of peripheral inflammation in the spinal cord [6]. This remarkable discrepancy between iNOS expression in the spinal cord and the inefficacy of L-NIL in pain related behavior models is reflected in our own results and was the cause to evaluate the pharmacokinetic profile of L-NIL. However, up to now no method has been available for the quantitative measurement of L-NIL.



a) b)

Fig. 1. Chemical structures of L-NIL (a) and L-norvaline (b, internal standard).

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Therefore we developed a selective and sensitive method to determine L-NIL in rat serum using pre-column derivatization with *o*-phtaldialdehyde (OPA) and reversed-phase high-performance liquid chromatography (HPLC) with fluorescence detection.

Several methods to determine levels of amino acids by HPLC have been described in the literature [7,8]. For quantification of L-NIL we first tried to use a method described by Godel et al. [9]. However, the method had to be changed due to an identical retention time of L-NIL and arginine in this system.

2. Experimental

2.1. Equipment

Chromatography was performed with a modular system consisting of a Gynkotek gradient pump (Model 480 with Degasys DG 1310, Germering, Germany), a Jasco FP 1520 fluorescence detector (Gross-Umstadt, Germany), an Abimed 231 XL autoinjector (Langenfeld, Germany), and a column thermostat (Chemdata, Sinsheim, Germany).

2.2. Reagents and solutions

L-NIL was purchased from Alexis Deutschland (Grünberg, Germany). L-Norvaline (Fig. 1b) and amino acid standard solution (AA-S-18, a composition of 2.5 μmol/ml L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-thyrosine and L-valine except L-cysteine at 1.25 μmol/ml) were obtained from Sigma (Deiselhofen, Germany). All other reagents were purchased from Merck (Darmstadt, Germany) in analytical grade. Only acetonitrile and methanol were of HPLC grade.

The internal standard was an aqueous solution of norvaline (1 g/l). For analysis, the amino acid standard was freshly diluted (1:500) in distilled water. The standard solution of L-NIL (1 g/l) was prepared with 0.03 *M* phosphate buffer solution and was stored at −20°C for no longer than 2 months.

The OPA solution was prepared as follows: 5 mg OPA was dissolved in 400 μl of methanol, adding 50 μl of borate buffer (1 mol/l, pH 10.4 adjusted with potassium hydroxide) and 5 μl of 3-mercaptopropionic acid. This reagent was stored at 4°C in the dark and was freshly prepared every week. For analysis, the OPA reagent was diluted (1:2, v/v) with borate buffer and placed in the scheduled vial of the autoinjector.

2.3. Sample preparation and derivatization procedure

An aliquot of the sample (20 μl) and internal standard solution (20 μl) were diluted with distilled water (1:100). A 20-μl volume was transferred to a vial of the autoinjector.

An automated pre-column derivatization procedure was employed using the Abimed autoinjector, equipped with a coldblock (8°C). Two minutes before injection of the sample 40 μl of OPA solution was added. Aliquots (20 μl) of the reaction mixture were injected into the column.

2.4. Chromatography

Mobile phase A consisted of tetrahydrofuran–aqueous buffer solution (2:98, v/v). Mobile phase B consisted of acetonitrile–tetrahydrofuran–aqueous buffer solution (19:7:74, v/v/v). The buffer solution was prepared by dissolving 2.60 g of sodium dihydrogenphosphate dihydrate and 11.92 g disodium hydrogenphosphate dodecahydrate in 1 l distilled water. Fig. 2 shows the gradient program.

The flow-rate of the mobile phase was 1.0 ml/

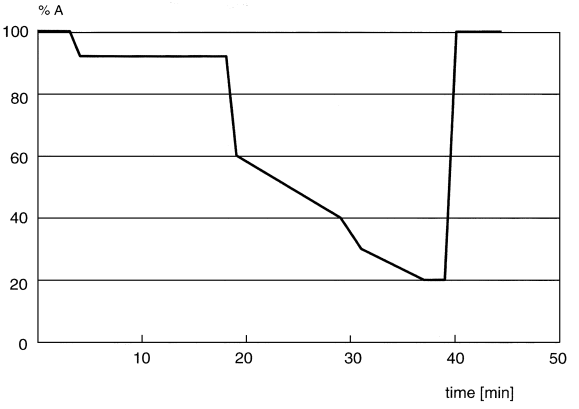

Fig. 2. Gradient program.

min. The stationary phase was Nucleosil C_{18} (120-5) packed in a 250×4 mm I.D. column (Machery–Nagel, Düren, Germany). The column temperature was maintained at 35°C. The fluorescence detector was set at 340 nm for excitation and 455 nm for emission. The peak area ratios of the analyte and the internal standard were determined. For validation of the method as well as quantitative analysis of the substrate the relevant principles [10] were taken into account. The intra-day repeatability of the method was determined by multiple analysis of individual rat serum samples on the same day. Inter-day reproducibility was assessed on 5 different days. The recovery of L-NIL was assessed at 0.5, 5.0 and 40 mg/l by comparing the peak area after pre-column derivatization of rat serum standards with the peak area obtained from injection of the same amount of L-NIL after pre-column derivatization of aqueous standards.

2.5. Collection of serum

A single oral or intravenous (i.v.) dose (D) of L-NIL (8 mg/kg) was administered to six male Sprague–Dawley rats in each case. L-NIL was dissolved in either methylcellulose colloid solution (1%) for oral administration or in physiological sodium chloride solution for i.v. administration. After an overnight fast, blood samples (100 μl) were taken from the retroorbital plexus at 0.5, 1, 2, 3, 4, 6, 7, 8 and 24 h after administration and were transferred to 1.5-ml cups containing 5 μl (100 units) sodium heparinate (Hoffmann-La Roche, Grenzach, Germany) as an anticoagulant. Blood samples were centrifuged immediately and serum probes were stored at −20°C for no longer than 2 months. Furthermore, 24 h after L-NIL administration cerebrospinal fluid (50–100 μl) was collected from the cisterna magna as described elsewhere after terminal heart puncture [11].

2.6. Pharmacokinetic methods

Serum–concentration time curves after injection of an i.v. dose or an oral dose were evaluated with a non-compartmental analysis using TOPFIT [12]. The apparent half-life, $t_{1/2,\lambda_z}$, was calculated as $\ln(2)/\lambda_z$, where λ_z denotes the time constant of terminal slope. The area under the serum–concentration time curve after injection of an i.v. dose ($AUC_{i.v.}$) and an oral dose (AUC_{or}) was calculated using the linear trapezoidal rule. The extrapolated AUC after the last observed serum concentration was obtained by dividing this serum concentration by λ_z. Subsequently, the percent absolute bioavailability, F, was calculated as

$$F\,(\%) = \frac{AUC_{or} \cdot D_{i.v.}}{AUC_{i.v.} \cdot D_{or}} \cdot 100$$

3. Results

The described method yields meaningful chromatograms for the quantitative analysis of L-NIL within 44 min (Fig. 3). The retention times of L-NIL and internal standard were 19.4 min and 33.5 min, respectively. Without the addition of L-NIL or internal standard the chromatograms contained no relevant spurious signals when analyzing serum probes (Fig. 4) or diluted amino acid standard solution (1:500). Chromatograms obtained from six different rat serum probes showed that the method is adequately specific.

Five separate analytical series analyzed in duplicate were used to verify linearity of the calibration curve for the relevant range of up to 40 mg/l in rat serum. The standard curves required for our purposes were plotted with concentrations of 0, 0.5, 1, 2.5, 5, 10, 20 and 40 mg/l. The correlation coefficients (r^2) were greater than 0.999.

Checks continuing for 2 months demonstrated that the stability of L-NIL in rat serum at three concentrations (0.5, 5, 40 mg/l, $n=5$ in each case) including two freeze–thaw cycles and the standard solution of L-NIL (1 g/l water) stored at −20°C is adequate.

The intra- and inter-day assay precision and accuracy for low, medium and high concentrations of L-NIL in rat serum are summarized in Table 1. The recoveries at concentrations of L-NIL of 0.5, 5 and 40 mg/l were 89.3±2.9, 90.4±2.5 and 94.8±3.0%, respectively. The limit of quantification (LOQ) in serum was 0.5 mg/l.

The validated method was used successfully to study the pharmacokinetic profile of L-NIL after i.v. as well as oral administration of a single dose to six

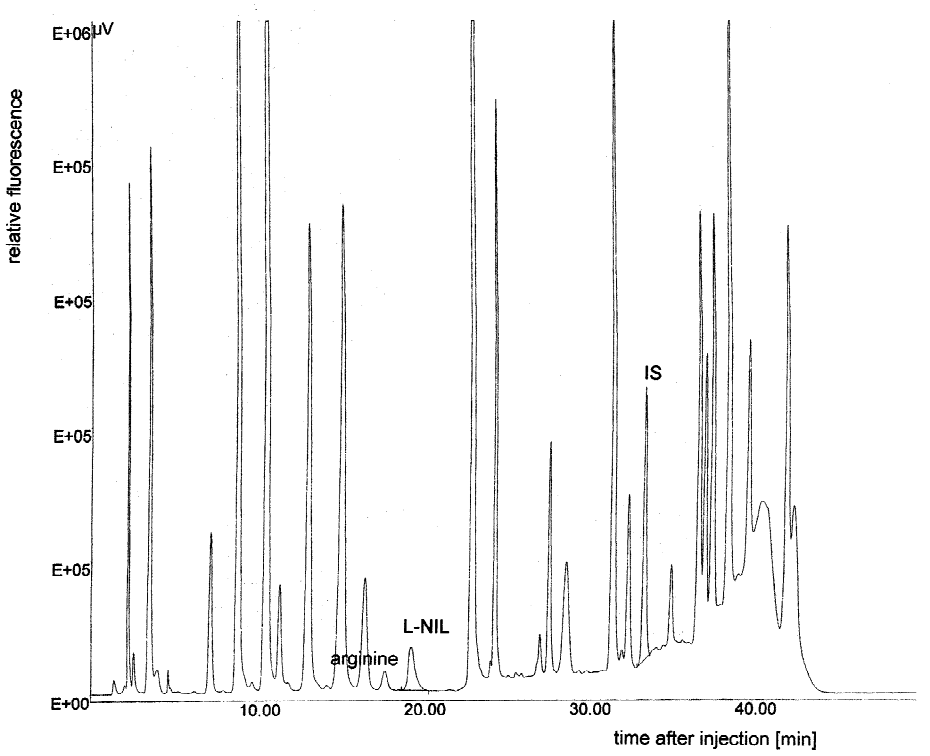

Fig. 3. Chromatogram of L-NIL (8.3 mg/l) and internal standard in rat serum 3 h after administration of 8 mg/kg i.v.

rats in each case. For these measurements, the calibration curves were established daily using seven serum concentrations (0, 0.5, 1, 2.5, 5, 10, 20 and 40 mg/l). Furthermore, 10% of the measured probes were quality control (QC) samples. QC samples were incorporated in duplicate in at least three different concentrations (0.5, 5 and 40 mg/l) into each run. The results of the QC samples provided the basis for accepting or rejecting the run.

Fig. 5 presents a typical example of serum concentration–time profile of L-NIL in a rat following intravenous and oral administration. L-NIL levels were below the LOQ 48 h after administration. The pharmacokinetic data after i.v. and oral application are summarized in Table 2. The median oral bioavailability of L-NIL was estimated to be about 89%. Concentrations in cerebrospinal fluid 24 h after administration were below the LOQ.

Furthermore, the octanol–water partition coefficient ($L = 0.02$, pH 7.4) of L-NIL was evaluated. The protein binding ability of L-NIL in the serum of rats amounted to 45%.

4. Discussion

As L-NIL does not have sufficient UV-absorbing power for the required sensitive determination, the substance has to be derivatized. OPA has been used for the detection of amino acids and various peptides as fluorescent derivatives [7,8].

The OPA derivatives can be generated rapidly and simply by mixing amino acids with the OPA reagent. As the OPA derivatives are labile a pre-column derivatization was performed. In order to achieve optimum derivatization of L-NIL, several conditions, such as reaction temperature, reaction time and stability of L-NIL derivatives in the system were studied. 2-Mercaptopropionic acid was more suitable

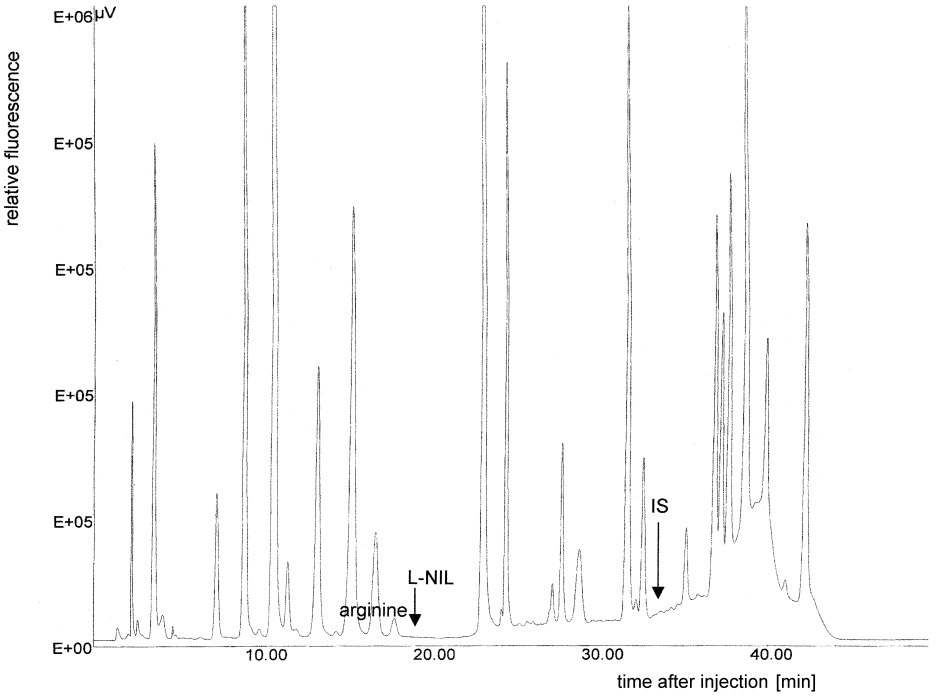

Fig. 4. Chromatogram of rat serum without L-NIL or internal standard.

for the quantitative detection than 2-mercaptoethanol as higher L-NIL peaks were achieved in the chromatograms. Ultracentrifugation of serum probes did not result in any advantages for quantification of the substance.

The difficulty in the development of the system was the separation of L-NIL and arginine. Under the shown conditions, the separation is now complete (retention time of L-NIL 19.4 min, of arginine 17.8 min).

However, as it was not necessary to quantify amino acids, the system was only applied to the quantification of the lysine analogue L-NIL.

L-NIL is not a physiological amino acid. Therefore, the substance can be used successfully as an internal standard for the quantification of amino acids.

As for the described application the used serum volume is very little, the method is suitable for pharmacokinetic investigation in rats and other small animals.

However, the L-NIL concentration in cerebrospinal

Table 1
Precision and accuracy for low, medium and high concentrations of L-NIL in rat serum

Added concentration (mg/l)	Intra-day ($n=5$)			Inter-day (5 days, $n=5$ each)		
	Measured concentration (mean±SD, mg/l)	RSD (%)	Accuracy (%)	Measured concentration (mean±SD, mg/l)	RSD (%)	Accuracy (%)
0.5	0.52±0.04	7.7	4.0	0.54±0.07	13.6	7.3
5	4.86±0.66	13.6	−2.8	4.91±0.46	9.4	−1.7
40	39.05±1.23	3.2	−2.4	42.65±2.89	6.8	6.6

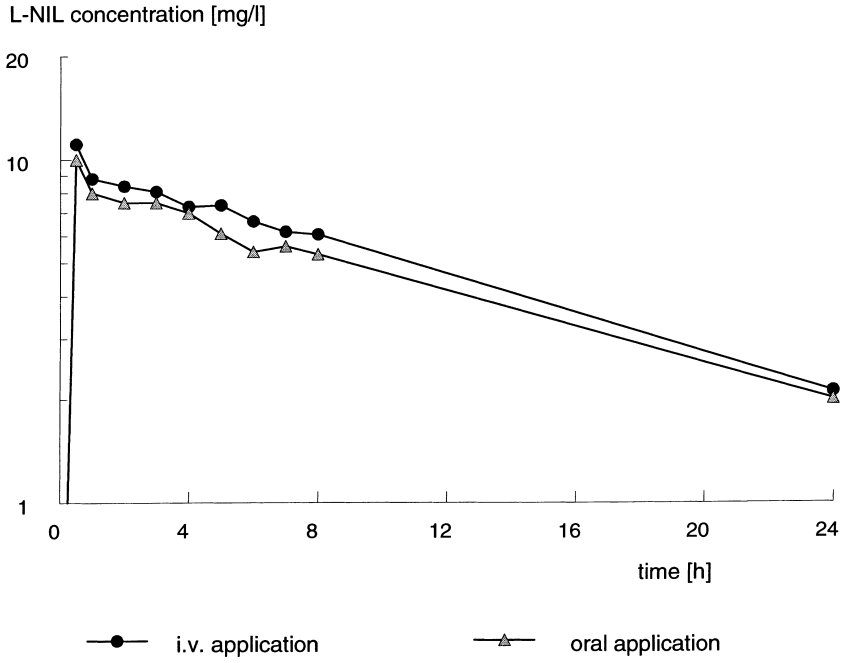

Fig. 5. Serum concentration–time profile of L-NIL following intravenous and oral administration of 8 mg/kg in rat.

Table 2
Pharmacokinetic data after i.v. and oral administration of L-NIL (8 mg/kg) in rat

Administration	AUC (mg min/l)	CL_{TOT} (ml/min/kg)	V_z (l/kg)	$t_{1/2}$ (h)
i.v.	6351±1254	1.2±0.5	1.4±0.7	12.7±2.5
Oral	5680±1978	1.4±0.6	1.7±0.7	13.5±3.1

fluid below the LOQ after i.v. and oral application could explain the inefficacy of peripherally administered L-NIL in pain-related models.

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