Journal of Pharmaceutical & Biomedical Analysis
Vol. 11, No. 10, pp. 993–997, 1993
Printed in Great Britain

0731–7085/93 $6.00 + 0.00

High-performance liquid chromatographic determination of nalidixic acid in rat serum, brain and cerebrospinal fluid

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Abstract: A high-performance liquid chromatographic method for the determination of nalidixic acid (NA) in rat serum, brain and cerebrospinal fluid (CSF) was developed. NA in rat serum and brain homogenate was extracted and injected onto a reversed-phase column. CSF was directly analysed without extraction procedure. The limits of detection were 0.05 μg ml^{-1} for serum, 0.07 μg g^{-1} for brain and 0.02 μg ml^{-1} for CSF, respectively. Calibration curves were linear over the concentration ranges 0.1–50 μg ml^{-1} for serum, 0.12–9 μg g^{-1} for brain and 0.05–10 μg ml^{-1} for CSF, respectively. The reproducibility of NA assay in rat biological media ranged from 1 to 4% relative standard deviations (RSD). The recoveries of NA added to serum and brain were higher than 96% with an RSD of less than 4%. The present method was found to be applicable to pharmacokinetic study of NA in rat serum, brain and CSF.

Keywords: *HPLC; nalidixic acid; serum; brain; cerebrospinal fluid; rat.*

Introduction

Nalidixic acid,1-ethyl-1,4-dihydro-7-methyl-4-oxo-1,8-naphthyridine-3-carboxylic acid (NA) was the first and prototypic drug of the quinolone antimicrobial agents, which was released in 1962 [1, 2] and is still used for urinary tract infections [3]. Many other drugs including new quinolones with chemical structures related to NA have been developed in the last decade. The incidence of side effects by the quinolones was relatively low, but some of them were known to exhibit neurotoxic side effects, such as convulsions [4]. Our recent study reported that lipophilicity may be one of the determinant factors in the permeability of some of the new quinolones from blood into cerebrospinal fluid (CSF) [5]. NA had relatively higher lipophilicity than the new quinolones [6] and convulsions after overdose of NA have been also reported [4]. Therefore, it is important to clarify the kinetics of NA in the CNS. The metabolism of NA has been widely studied and it has been reported that the biotransformation of NA was extensive [3]. However, NA had a higher lipophilicity than its metabolites, hence it seemed reasonable to suppose that the parent compound plays an important role in the entry into the CNS and the serious neurotoxicity of this quinolone. Various HPLC methods have been reported for the determination of NA in biological samples including serum [7–12]. However, none of these methods included the analysis of NA in brain and CSF.

The purpose of this work was to develop a new HPLC assay procedure for the determination of NA in rat brain and CSF, and to apply the method to the pharmacokinetic study of the quinolone. Furthermore, by using this method, we were able to determine NA as both total and unbound forms in serum for the analysis of pharmacokinetic data in the CNS.

Experimental

Apparatus

A Shimadzu LC-10AD high-performance liquid chromatograph (Kyoto, Japan) was used, equipped with a Shimadzu SPD-10A UV spectrophotometric detector operating at 255 nm, and a reversed-phase column (150 mm × 4.6 mm i.d.) packed with Wakosil 5C18, particle size 5 μm (Wako Pure Chemical Industries, Osaka, Japan).

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Chemicals

NA and pipemidic acid (internal standard) of analytical grade were purchased from Nakarai Tesque (Kyoto, Japan) and Sigma (St Louis, MO, USA), respectively. Methanol, acetonitrile, distilled water and sodium lauryl sulphate were of liquid chromatographic grade. All other reagents were of analytical grade.

Animals and drug treatments

Male Wistar rats, 10 weeks old, cannulated into the right jugular vein, were used. A bolus dose of 10 mg kg^{-1} of NA was administered via the cannula to the rats. At designated times after the drug injection, each rat was lightly anaesthetized with ether. The CSF specimen was obtained by cisternal puncture and the blood sample was withdrawn through the cannula. The serum was immediately separated by centrifugation with the serum separator, Fibrichin (Takazono Sangyo, Osaka, Japan) and a portion of the serum was ultrafiltered by using MPS-3 (Amicon, Danvers, MA, USA). Immediately after CSF and blood collection, the rat was sacrificed by microwave irradiation focused on its head for 0.8–1.0 s using a microwave applicator (Toshiba Model TMW-6402A, Tokyo, Japan). The whole brain was readily excised and weighed after careful removal of the dural and subarachnoidal vessels. Drug-free (i.e. blank) blood serum, CSF and brain tissue were also obtained from the control rats which were injected with solvent alone. The biological samples were kept frozen (−20°C) until analysis and NA in these biological samples were completely stable for 1 month.

Standard solutions

The stock solution of NA (100 μg ml^{-1}) or the internal standard (pipemidic acid, 30 μg ml^{-1}) was prepared by dissolving each drug first in a small volume (less than 1.0 ml for 10 mg of any drug) of 0.1 M sodium hydroxide and by diluting the solution with the distilled water. This solution was stored at below 15°C protected from light and was completely stable for 1 month. Working standard solution and internal standard solution were prepared by diluting the stock solution with distilled water.

Analytical procedures

To 50 μl of rat serum were added 0.5 ml of phosphate buffer consisting of 0.2 M disodium hydrogenphosphate 12-water adjusted to pH 7.0 with 0.2 M potassium dihydrogenphosphate and 100 μl of the internal standard solution (10 μg ml^{-1} of pipemidic acid). This mixture was extracted with 3.0 ml of methylene chloride–2-propanol (9:1, v/v) in a 10 ml glass tube, which was shaken vigorously for 10 min. After centrifugation at 3500 rpm for 5 min, 2.0 ml of the organic phase was transferred to another tube and evaporated to dryness under a gentle stream of dry nitrogen at 40°C. The residue was reconstituted in 200 μl of methanol–0.05 M sodium hydroxide (3:2, v/v) by vortexing. A 10 μl aliquot was injected into the chromatograph.

Each whole brain was homogenized in two volumes (v/w) of ice-cold normal saline using a Teflon homogenizer. To a portion (0.5 ml) of the homogenate, 0.5 ml of 0.2 M phosphate buffer (the same as described in the serum extraction procedure), 100 μl of the internal standard solution (3 μg ml^{-1} of pipemidic acid) and 3 ml of diethyl ether were added. The solution was shaken for 5 min and centrifuged at 3000 rpm for 5 min at 4°C. The ether phase was discarded. To the aqueous phase, 1 g of ammonium sulphate and 5 ml of a mixture of organic solvents (methylene chloride–2-propanol, 9:1, v/v) were added. The resultant mixture was shaken for 20 min and centrifuged at 3500 rpm for 5 min at room temperature. An aliquot (4 ml) of the organic phase was evaporated to dryness under a gentle stream of dry nitrogen at 40°C. The residue was reconstituted with 0.5 ml of a mixture of methanol–0.05 M sodium hydroxide (3:2, v/v) under vigorous shaking for 15 min and filtered through a membrane filter (0.45 μm, Chromatodisc® 4A, Kuraboh, Osaka, Japan). A 10 μl aliquot of the filtrate was used for injection onto the HPLC column.

To 30 μl of CSF, 30 μl of 0.05 M sodium hydroxide containing 3 μg ml^{-1} of pipemidic acid was added and mixed. A 10 μl aliquot of the mixure was directly applied onto the column.

Each injected solution was completely stable at room temperature within a day and all procedures of the analysis were performed within 24 h.

HPLC conditions

The mobile phase was methanol–acetonitrile–0.015 M potassium dihydrogenphosphate (3:2:5, v/v/v) containing 2 mM sodium

lauryl sulphate adjusted to pH* 2.5 with phosphoric acid. The flow rate was 0.8 ml min^{-1} and separation was performed at 40°C. The chromatographic data were calculated with a Shimadzu C-R4A data module.

Calibration graphs

NA was dissolved in drug-free serum to give concentration ranges 0.1–50 μg ml^{-1} (eight points, $n = 5$). The homogenate of drug-free brain was spiked with increasing amounts of NA in the concentration range of 0.12–9 μg g^{-1} wet tissue (six points, $n = 5$). The calibration curve for CSF was prepared by using normal saline spiked with the drug standard solution to make the concentration range of 0.05–10 μg ml^{-1} (eight points, $n = 4$). These samples were then assayed according to the analytical procedures described above. Calibration graphs were constructed by plotting the peak-height ratio of the drug to the internal standard against the concentration.

Reproducibility

Biological samples were taken from the rats at appropriate times after the administration of NA. Ten repeated analyses of serum, brain and CSF samples were carried out.

Accuracy

The accuracy of the present method was evaluated by measuring the recovery of known amounts of NA added to rat serum or brain homogenate. The drug-free serum and brain homogenate were spiked with 3 or 30 μg ml^{-1} and 0.5 or 5 μg g^{-1} of NA, respectively. Recovery was determined by comparing the amount of the drug measured by calculating from an adequate standard with the known added amount.

Results

Typical chromatograms resulting from HPLC analysis of NA in serum, brain and CSF obtained from the rat are shown in Fig. 1. The chromatograms are representative for the analysis of blank samples from the drug-free rats [Fig. 1(A)] or serum, brain and CSF samples taken from the rats after intravenous administration of NA [Fig. 1(B)]. NA and pipemidic acid (internal standard) were eluted at retention times of about 6.7 and 9.3 min, respectively, as well resolved peaks. No interfering endogenous peaks could be detected in any chromatogram of drug-free serum, brain and CSF.

The limits of detection of the assay determined in extracted serum and brain and in CSF (without extraction), defined as a signal-to-noise ratio of 3:1, were 0.05 μg ml^{-1}, 0.07 μg g^{-1} and 0.02 μg ml^{-1}, respectively.

Calibration curves were generated by least-squares linear regression analysis. Satisfactory linearity was observed in the ranges of 0.1–50 μg ml^{-1} for serum, 0.12–9 μg g^{-1} for brain and 0.05–10 μg ml^{-1} for CSF. The regression equations by the method of least-squares were $y = 0.217\ (\pm 0.001)\ x - 0.019\ (\pm 0.011)$, $rI = 0.9999$ for serum; $y = 0.786\ (\pm 0.008)\ x +$

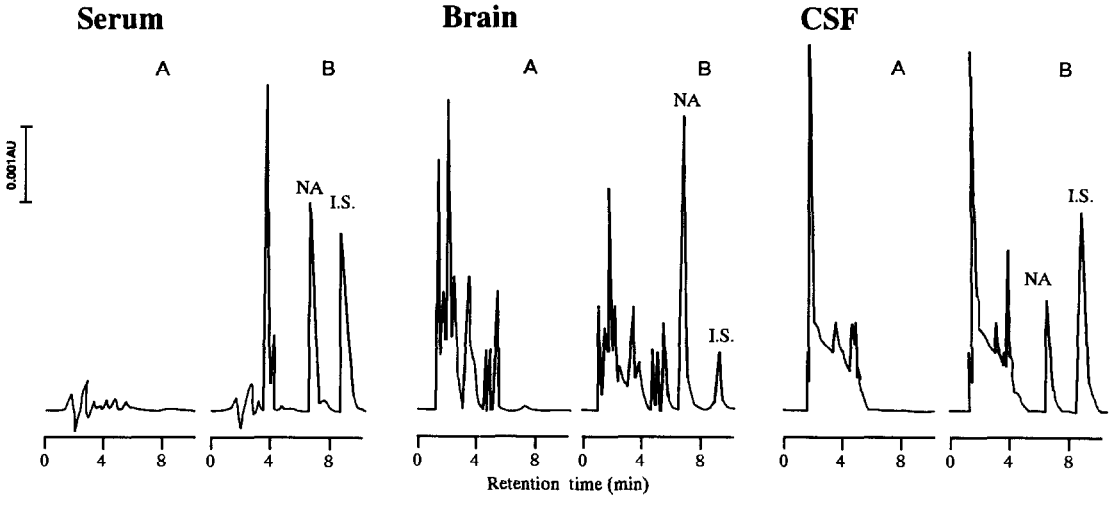

Figure 1
Typical chromatograms of nalidixic acid (NA) obtained from rat serum, brain and CSF. (A) Blank samples; (B) samples obtained from rats 150 min (serum), 10 min (brain), 100 min (CSF) after intravenous administration of 10 mg kg^{-1} of NA, I.S., internal standard (pipemidic acid).

0.006 (±0.038), $rI = 0.9999$ for brain; and $y = 0.674$ (±0.003) $x - 0.001$ (±0.012), $rI = 0.9999$ for CSF, where y is the peak-height ratio of the drug to the internal standard, x is the concentration in serum (μg ml^{-1}), brain (μg g^{-1}) or CSF (μg ml^{-1}). Each value for the regression slope or intercept was expressed as the estimated value with ± SE.

Table 1 shows the precision of this assay method of NA in serum, brain and CSF. The serum and brain assays had relative standard deviations (RSD) below 5% at the concentration tested and assays in CSF had RSDs below 2%.

Table 2 shows the recovery data for NA spiked to drug-free serum and brain tissue homogenate at two different concentrations. The recoveries for NA were 96.2 and 95.7% at the concentrations of 3 and 30 μg ml^{-1} in serum, 103.7 and 104.4% at the concentrations of 0.5 and 5 μg g^{-1} in brain, respectively, with the RSDs less than 4% in all cases.

The present method was then used in the preliminary pharmacokinetic study on NA. Figure 2 shows the concentration versus time data of NA after bolus intravenous administration of 10 mg kg^{-1} of NA to rats. The serum total concentrations of NA showed a mono-exponential decline with time. Serum free concentration of NA was found to decline in proportion to its serum total concentration.

Table 1
Precision of the assay of nalidixic acid (NA) in rat serum, brain and CSF

Sample (unit)	NA level*	RSD (%)
Serum (μg ml^{-1})	4.85 ± 0.04	2.8
	49.7 ± 0.05	3.1
Brain (μg g^{-1})	0.432 ± 0.006	4.1
	5.00 ± 0.03	2.3
CSF (μg ml^{-1})	0.478 ± 0.003	1.8
	5.90 ± 0.02	0.9

RSD, relative standard deviation.
*Mean ± SE of 10 determinations.

NA concentrations in brain and CSF attained the maximum value immediately after the drug injection and then declined with serum free concentration.

Discussion

Several methods have been described for the assay of NA in biological media, i.e. in plasma, urine or fish tissue [7–12]. These methods, however, were lacking in the analysis of NA in brain and CSF. The metabolic biotransformation of NA was extensive [3]. However, the diffusion into CNS for NA would be greater than that for any of its metabolites, presumably because of its greater lipophilicity. Therefore, the present method was intended to investigate CNS levels of the parent compound alone. We have previously described the HPLC methods for determination of a quinolone antibacterial agent, ciprofloxacin (CPFX) in rat brain and CSF using fluorescence detection [13]. However, fluorescence intensity of NA was very low as compared with that of CPFX. Since the fluorometric detection of NA required relatively large amounts of the sample [11] unless some specific derivatization procedure is available, we attempted to utilize the UV detection of NA. There was an inevitable limitation to the smallest sample size for CSF. The proposed method could be adapted for relatively small size of serum, brain and CSF samples such as less than 50 μl, 200 mg and 30 μl, respectively. This method involved the solvent extraction procedure for the serum and brain samples and the direct injection of CSF samples after dilution with an equal volume of the internal standard solution. No appreciable interferences of endogenous substances were detected. Cuisinaud *et al.* described a HPLC method for NA with reversed-phase column and similar mobile phase to our method [9]. In their report, metabolites of NA were eluted earlier than NA

Table 2
Accuracy of the assay of nalidixic acid in rat serum and brain

Sample	Added*	Found*†	Recovery†	RSD (%)
Serum	3.0	2.89 ± 0.03	96.2 ± 1.1	2.9
	30.0	28.7 ± 0.4	95.7 ± 1.2	2.7
Brain	0.5	0.519 ± 0.009	103.7 ± 1.8	4.0
	5.0	5.22 ± 0.03	104.4 ± 0.6	1.4

*Serum (μg ml^{-1}), brain (μg g^{-1}).
†Mean ± SE of five or six determinations.

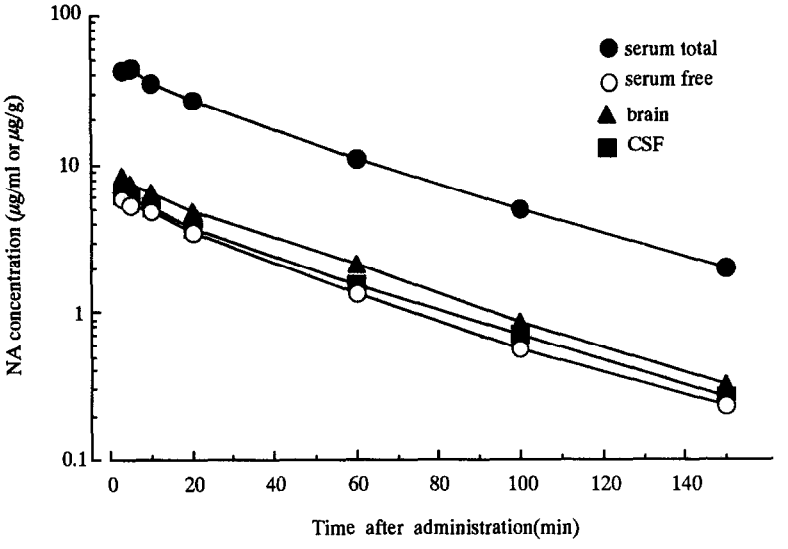

Figure 2
Nalidixic acid (NA) concentrations in serum, brain and CSF after intravenous administration of 10 mg kg^{-1} of NA to rats. Each point represents the mean of five rats.

owing to their higher polarity. As expected, no metabolites were detected in our condition. It was suggested that the present method did not extract metabolites of NA and/or eluted them with front peaks. NA concentration as low as 0.1 μg ml^{-1} in serum, 0.12 μg g^{-1} in brain or 0.05 μg ml^{-1} in CSF could be measured using this method.

Since the present assay method was expected to be applicable to the detailed study of NA disposition in serum, brain and CSF, both reproducibility and recovery tests were examined. The reproducibility of rat biological samples indicated very low relative standard deviations. In the accuracy study, the measured amounts of NA yielded more than 95% of the known amount spiked to the serum sample or the brain tissue homogenate. There was no problem with the recovery of NA in CSF itself. Both reproducibility and recovery in the determination of NA were satisfactory over a wide range of concentration. The present method which the authors developed for the determination of NA was simple, sensitive, precise and accurate enough to utilize for the detailed pharmacokinetic studies of the drug, as shown in Fig. 2.

Conclusions

The HPLC method for the determination of NA in rat serum, brain and CSF has been developed and provided a sufficiently sensitive, accurate and reproducible analytical procedure. It is expected that this method will be used successfully in kinetic studies of NA in the CNS.

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[Received for review 21 December 1992; revised manuscript received 16 February 1993]