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Short communication

Determination of norfloxacin in rat liver perfusate using capillary electrophoresis with laser-induced fluorescence detection

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Abstract

A capillary zone electrophoresis method has been developed for the direct determination of norfloxacin in the physiological perfusate of isolated rat liver. Norfloxacin and the internal standard triamterene were detected using laser-induced fluorescence (LIF) detection with the excitation and emission wavelength of 325 and 435 nm, respectively. The background electrolyte (BGE) was 50 mM phosphate buffer (pH 4.6). The effect of pH and concentration of BGE on the electrophoretic migration and fluorescence response of analytes were examined. Calibration curves were linear over a wide range of 0.01–100 μg/mL. The limit of quantitation was 0.01 μg/mL. The intra- and inter-day relative standard deviation was 3.7%, or less, and the accuracy was 93.2% of the nominal concentration. No endogenous substances were found to interfere. The method was used to characterize the steady-state and transient pharmacokinetics of norfloxacin in the rat liver.

Keywords: Capillary electrophoresis; Hepatic disposition; Laser-induced fluorescence; Norfloxacin; Perfused rat liver

1. Introduction

Norfloxacin is an orally absorbed antimicrobial agent widely used in the treatment of complicated urinary tract infections [1,2]. Following oral administration, norfloxacin is primarily eliminated unchanged by the kidney, thus producing high urinary concentration. The renal clearance of norfloxacin is about 2–3-folds higher than the glomerular filtration rate, indicating that active tubular secretion is involved in its urinary excretion. The metabolic biotransformation of norfloxacin is low and mainly occurs in the liver [1]. Norfloxacin decreases CYP3A and CYP1A activity significantly by competitive inhibition, therefore, it can cause drug interactions when concomitant with medicines that are substrates of these metabolizing enzymes [3,4]. The biliary concentrations of norfloxacin are 3–7 times higher than the concurrent plasma concentrations, suggesting a transporter-mediated excretion [1].

Recently, norfloxacin was shown to be a substrate of the breast cancer resistance protein, a drug efflux transporter that is found in a variety of tissues including intestine, liver and kidney [5]. Norfloxacin displayed nonlinear elimination kinetics in rats [4,6]. Although the nonlinearity may be attributed to a saturation of metabolism or/and secretion, the underlying mechanism remains unknown. Therefore, investigation on the role of transporters and metabolizing enzymes in the pharmacokinetics and drug–drug interactions of norfloxacin is of great interest.

To study the pharmacokinetics of norfloxacin, a suitable sensitive method that allows an accurate measurement of low concentration level is needed. Numerous analytical techniques have been developed for the quantification of norfloxacin. The HPLC methods for the determination of norfloxacin in biological fluids have been reviewed [7,8]. Capillary electrophoresis (CE) techniques have been applied to separate quinolone antibiotics over the past decade, and its applications for the determination of norfloxacin in pharmaceutical dosage forms [9,10], plasma [11], blood [12] and urine [13] have been published recently. It

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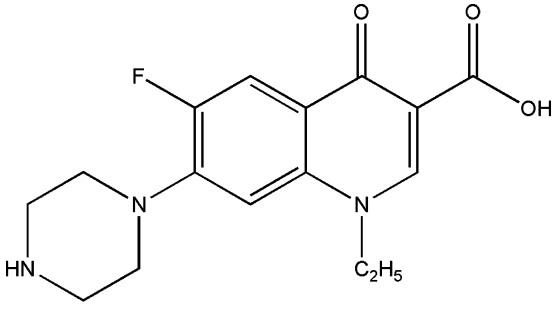
is interesting to note that quantification of norfloxacin in HPLC and CE methods is dominated by UV detection, even though norfloxacin possesses native fluorescence.

CE–laser-induced fluorescence (LIF) methods have been reported to determine ciprofloxacin, enrofloxacin, moxifloxacin, ofloxacin and their metabolites in human plasma and urine [14–17]. Nonetheless, to our knowledge no CE–LIF method for determining norfloxacin in biological samples has been developed to date. In this work, we present the development, validation and application of a simple and sensitive CE–LIF method to quantify norfloxacin concentration in the Kreb's solution as physiological perfusate for kinetic studies of norfloxacin in isolated perfused rat liver. The isolated perfused rat liver is a good model for examining the role of transporters and metabolizing enzymes on drug disposition [18,19].

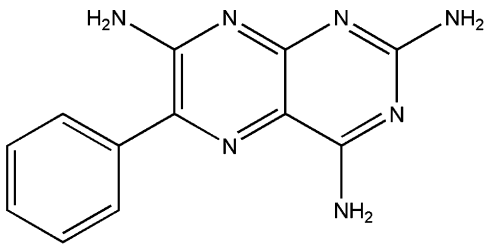
2. Experimental

2.1. Chemicals and reagents

Norfloxacin (Lot MN28005, 96.94%) was obtained from Taiwan Biotech Co. Ltd. (Taoyuan, Taiwan). Triamterene (Lot 36H1206, >99%) (Fig. 1) was purchased from Sigma (St. Louis, MO, USA). Sodium dihydrogen phosphate and disodium hydrogen phosphate were obtained from Kanto Chemical (Tokyo, Japan). Acetic acid, sodium acetate and sodium hydroxide were obtained from Mallinckrodt Baker (Xalostoc, Mexico). Orthophosphoric acid and dimethyl sulfoxide (DMSO) was from Riedel-de Haën (Sealze, Germany). All chemicals were analytical grade reagents and were used without further purification. The water used was Milli-Q Reagent Water (Millipore, Bedford, MA, USA).



Norfloxacin



Triamterene

Fig. 1. Structures of norfloxacin and internal standard triamterene.

2.2. Instrumentation

The CE instrument used was a Beckman P/ACE MDQ system (Beckman Coulter, Fullerton, CA, USA) equipped with a laser-induced fluorescence detector assembly. The 325 nm line of a HeCd-laser with a specific power of 20 mW/mm^2 and an LC500-120RC power supply (Omnichrome, Series 56, model no. 3056R-M-A03, Carlsbad, CA, USA) was used for fluorescence excitation. Fluorescence detection was performed at an emission wavelength of 435 nm. Data collection and processing were performed using 32 Karat^{TM} software 4.0 (Beckman Instruments, Fullerton, CA, USA). The pH was measured with a Suntex SP-701 pH meter (Taipei, Taiwan).

2.3. Capillary electrophoretic conditions

The BGE used in this study was 50 mM phosphate buffer (pH 4.6). An untreated fused-silica capillary with a total length of 60 cm (effective length 50 cm) and an id of 75 μm was employed for separation (Beckman Coulter, Fullerton, CA, USA). The capillary temperature was maintained at 20 °C and the separation voltage was 28 kV. The samples were introduced into the anodic end of the capillary by pressure injection with 0.5 psi for 10 s. At the beginning of a series, the capillary was rinsed with 0.1 M NaOH for 10 min, Milli-Q water for 10 min and with BGE for 15 min. Rinsing procedures were always performed at a pressure of 20 psi. After each run, the capillary was washed with 0.1 M NaOH for 2 min and Milli-Q water for 2 min before re-equilibrating with running buffer for 2 min. At the end of a day the capillary was always rinsed with 0.1 M NaOH and Milli-Q water for 4 min each, and was dried with air for 5 min.

2.4. Standards and controls

Stock solution of norfloxacin (1 mg/mL in 50 mM acetate buffer, pH 5) and the working solution of internal standard triamterene (2 μg/mL in 0.1% DMSO) were prepared monthly and kept tightly sealed at −20 °C. The stock solution of norfloxacin was diluted with water to give the calibration standards at concentrations of 0.01, 0.1, 1, 10, and 100 μg/mL norfloxacin. The quality controls were prepared independently at concentrations of 0.01, 1 and 100 μg/mL prior to the start of sample collection and stored at −20 °C until used.

2.5. Samples preparation

The perfusate samples (100 μL) were diluted 1:2 with Milli-Q water and added with 30 μL of triamterene working solution. The solution was vortex mixed and passed through a 0.22 μm membrane filter before injecting into the capillary.

2.6. Calibration and validation

The model for the calibration curve of norfloxacin used the peak area ratio of norfloxacin to triamterene (PAR) and the norfloxacin concentration (C), as given in the following equation: $\ln(\text{PAR}) = \text{Slope} \times \ln C + (y \text{ intercept})$. The slope and

y intercept were determined by a nonlinear least-squares program (*WinNonlin*, Professional Version 2.1, Pharsight Inc., Mountain View, CA, USA), using nominal concentrations and measured PARs from calibration standards. Norfloxacin concentrations were estimated from PARs using the formula: $C = e^{(\ln(PAR) - (y\ intercept))/slope}$. Intra-day precision was evaluated by analyzing the spiked controls six times over one day in random order, while inter-day precision was evaluated from the analysis of each control once on each of six different days. Assay precision (coefficient of variation, C.V.) was assessed by expressing the standard deviation of the measurements as a percentage of the mean value. The accuracy was estimated for each spiked control by comparing the nominal concentration with the assayed concentration. The lower limit of quantitation was the lowest non-zero concentration level, which could be accurately (relative error < 20%) and reproducibly (C.V. < 20%) determined. Assay selectivity was examined in relation to interference from endogenous substances in drug-free outflow perfusate.

2.7. Pharmacokinetic application

Male Sprague-Dawley rats were obtained from the Animal Breeding Center of National Cheng Kung University. The study protocol complied with the Institutional Guidelines on Animal Experimentation of National Cheng Kung University. The *in situ* perfused rat liver preparation was similar to that described in previous studies [18,19]. Norfloxacin (6 mg/L) was infused, at a flow rate of 20 mL/min, into the perfused rat liver for 40 min ($n = 3$) to assess its hepatic extraction at steady-state. The outflow perfusate samples were collected between 5 and 40 min at 5–10 min intervals. To characterize the transient kinetics of norfloxacin in the rat liver, 100 μg of norfloxacin was injected as a bolus dose into the portal vein during a constant perfusion of drug-free perfusate at a flow rate of 20 mL/min ($n = 3$). The total effluent was automatically collected at 1-s intervals, using a motor driven carousel (Pan Chun Scientific Corp., Taiwan) for 2 min, and at 5 and 10 min. The kinetic parameters of norfloxacin were determined by non-compartmental analysis using *WinNonlin*.

3. Results and discussion

3.1. Method development

Separation of quinolone antibiotics in CE has been accomplished mainly by fused silica capillaries with BGE consisting of acetate, borate or phosphate buffers [9–17]. The addition of surfactants [9,11], organic solvents [9,11,12], ion-pairing reagents [11,14–16] and chiral selector [16] improves resolution and peak symmetry. Norfloxacin exhibits strong intrinsic fluorescence with maximal intensity occuring in weakly acidic solutions at pH 3.5–4.0, with excitation maxima at 278 nm and 310–330 nm and an emission maximum at around 440 nm [20–22]. In this work we focused on the determination of norfloxacin, therefore, capillary zone electrophoresis with a simple phosphate buffer solution was employed. And, the excitation and emission wavelength of 325 and 435 nm, respectively, were used for LIF detection of norfloxacin.

The effect of pH (2, 4, 6, 8, 10) of 50 mM phosphate buffer on the fluorescence response and electrophoretic migration of norfloxacin was investigated. It was found that the fluorescence response was maximal at pH 4, and it decreased significantly as the pH increased to basic pH. At pH 10, the response was only 3.9% of that at pH 4. The pH–fluorescence response profile of triamterene showed a similar trend as that of norfloxacin. As the analytes are mainly positively charged at pH 2–6, their migration times decrease significantly with the increase of buffer pH due to the increasing electroosmotic flow. Because triamterene is smaller than norfloxacin, it has a higher electrophoretic mobility and migrates faster than norfloxacin (Fig. 2). For pH above 8, triamterene (pK_a 6.2) is unionized, thus its migration time decreases continuously with the increase of pH. In contrast, norfloxacin is predominantly in anionic form at pH above 8. Its migration time increases with the buffer pH, because the electrophoretic flow is increased and is moving in the opposite direction of the electroosmotic flow (Fig. 2). Based on the above information, a pH of 4.6 was selected to minimize migration times while maintaining adequate resolution and good fluorescence response for the analytes.

The effects of concentration of phosphate buffer (10, 20, 30, 40, 50 mM, pH 4.6) on the fluorescence response and migration of norfloxacin and triamterene were examined. When the buffer concentration increased, the fluorescence and migration times of both the analytes increased linearly whereas the resolution between the two analytes improved progressively. Accordingly, the buffer molarity of 50 mM was used.

Most of the CE methods of quinolone antibiotics either used structure-related quinolones as the internal standards or did not have an internal standard. The electrophoretic migration and fluorescence behavior of triamterene were similar to those of norfloxacin in acidic buffer solutions. Both analytes show good fluorescence under acidic conditions and can easily be analyzed with LIF. Therefore, the suitability of triamterene as the internal standard is justified.

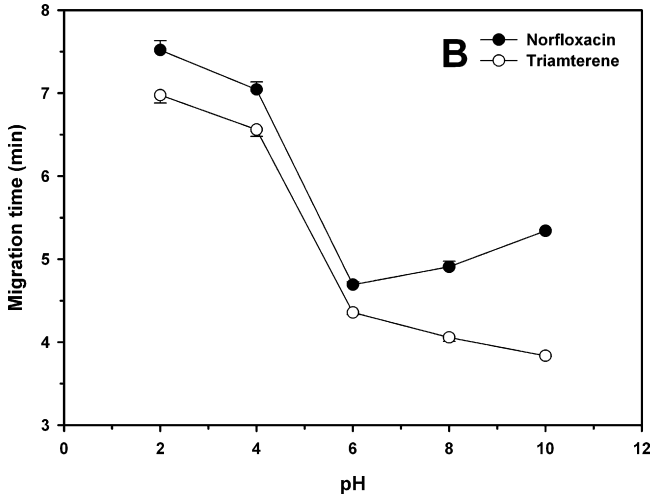


Fig. 2. Effect of pH on the migration time (mean ± SD, $n = 3$) of norfloxacin and triamterene.

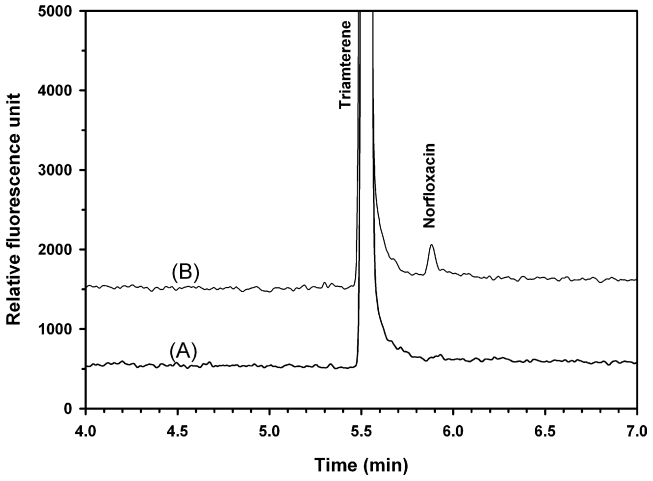


Fig. 3. Electropherograms of blank perfusate (A) and a spiked sample containing 0.01 μg/mL of norfloxacin (B). Electrophoretic conditions: 50 mM phosphate buffer, pH 4.6; capillary, 60 cm × 75 μm; temperature, 20 °C; applied voltage, 28 kV; pressure injection, 10 s at 0.5 psi.

Fig. 3 represents electropherograms of a blank perfusate and spiked sample containing 0.01 μg/mL of norfloxacin. As can be seen in Fig. 3, a good separation of norfloxacin and triamterene was achieved under the electrophoretic conditions specified. The method is specific for norfloxacin. At the corresponding migration times of the analytes, no interfering peaks were observed when blank perfusate samples were analyzed.

3.2. Method validation

The developed method was validated with respect to linearity, accuracy, precision, sensitivity and specificity. The calibration curves were linear from 0.01 to 100 μg/mL. The mean (±SD) regression equation for six replicated calibration curves constructed using 100 μL of perfusate samples on different days was: $\ln(\mathrm{PAR}) = (1.031 \pm 0.005) \times \ln C + (0.132 \pm 0.019)$, $r^2 = 0.9998 \pm 0.0001$. Precision and accuracy (0.01–100 μg/mL) were investigated (Table 1), and in all cases the within-day and between-day precision was acceptable at a C.V. of 3.7% or less. In addition, accuracy was within 6.8% when compared with nominal concentrations across this range. From this experiment, the LOQ of the method was determined to be 0.01 μg/mL, with the within-day imprecision and error of 3.1% and −3.3%, and the between-day imprecision and error of 2.0% and −6.5. The S/N ratio at LOQ was about 6 (Fig. 3).

3.3. Method application

The assay was applied to study the hepatic disposition kinetics of norfloxacin in rats. Fig. 4 shows the mean availability versus time profile of norfloxacin during constant infusion of 6 mg/L. The hepatic availability of norfloxacin increased rapidly to 68% 5 min after infusion and its distribution reached equilibrium within 20 min. The steady-state availability was 88 ± 4%, suggesting that hepatic clearance of norfloxacin is relatively low. The outflow concentration–time

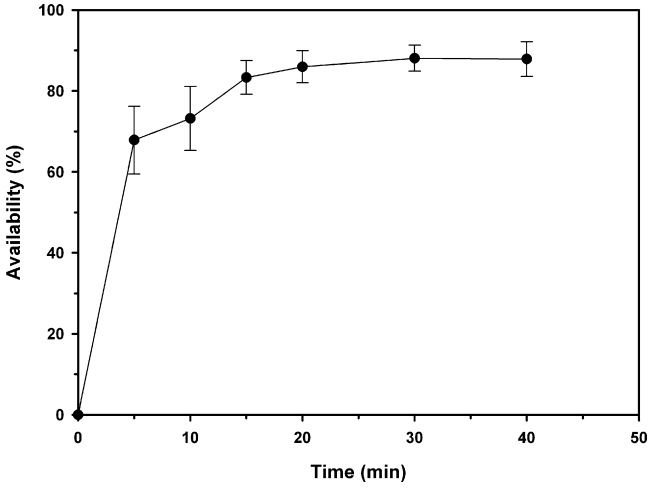


Fig. 4. Steady-state availability of norfloxacin following constant infusion of 6 mg/L in the isolated perfused rat liver.

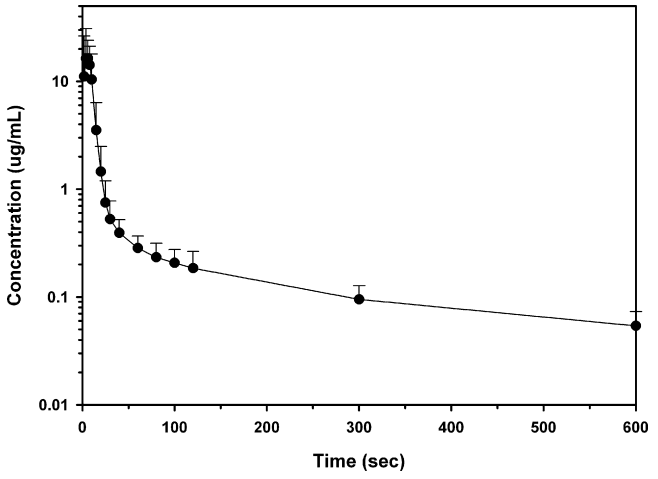


Fig. 5. Outflow concentration–time profile of norfloxacin following a 100 μg bolus in the isolated perfused rat liver.

Table 1
Within-day and between-day accuracy and precision for the determination of norfloxacin in physiological perfusate

$C_{nominal}$ (μg/mL)	Within-day			Between-day		
	C_{est} (μg/mL)	C.V. (%)	Error (%)	C_{est} (μg/mL)	C.V. (%)	Error (%)
0.01	0.01	3.1	−3.3	0.01	2.0	−6.5
1	1.00	0.6	0.1	1.05	1.6	5.3
100	95.8	2.9	−4.2	93.2	3.7	−6.8

$C_{nominal}$: nominal concentration; $C_{est.}$: estimated concentration; $n = 6$.

profile of norfloxacin following a single bolus dose of 100 μg is illustrated in Fig. 5. Evidently norfloxacin displayed two-compartmental distribution characteristics in the liver, in which transport was limited by a permeability barrier. These results are consistent with the findings in human, and confirm that norfloxacin in not extensively metabolized and its distribution in the rat liver is mediated by transporters. The results also demonstrated that this simple and rapid CE–LIF method is sensitive to follow disposition kinetics of norfloxacin in animal study.

4. Conclusion

In conclusion, a simple, rapid and sensitive capillary zone electrophoresis method with laser-induced fluorescence detection has been developed for the direct determination of norfloxacin in the physiological perfusate of isolated *in situ* rat liver. The method offers low quantitation limit with good accuracy and precision and selectivity for monitoring concentration of norfloxacin. The method was applied successfully to investigate the steady-state and transient pharmacokinetics of norfloxacin in the rat liver.

Acknowledgments

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References

[1] G.E. Stein, Am. J. Med. 82 (Suppl. 6B) (1987) 18.
[2] D. von Rosensteil, D. Adam, Drugs 47 (1994) 872.
[3] R.A. Mclellan, R.K. Drobitch, M. Monshouwer, K.W. Renton, Drug Metab. Dispos. 24 (1996) 1134.
[4] J.D. Davis, L. Aarons, J.B. Houston, Pharm. Res. 12 (1995) 257.
[5] G. Merino, A.I. Álvarez, M.P. Pulido, A.J. Molina, A.H. Schinkel, J.G. Prieto, Drug Metab. Dispos. 34 (2006) 690.
[6] S. Marchand, M. Chenel, I. Lamarche, C. Pariat, W. Couet, J. Pharm. Sci. 92 (2003) 2467.
[7] G. Carlucci, J. Chromatogr. A 812 (1998) 343.
[8] F. Balal, A.A. Al-Majet, A.M. Al-Obaid, Talanta 50 (1999) 765.
[9] S.-W. Sun, A.-C. Wu, J. Liq. Chromatogr. Related Technol. 22 (1999) 281.
[10] C. Fierens, S. Hillaert, W. Van den Bossche, J. Pharm. Biomed. Anal. 22 (2000) 763.
[11] M. Hernández, F. Borrull, M. Calull, J. Chromatogr. B 742 (2000) 255.
[12] M. Ferdig, A. Kaleta, T.D.T. Vo, W. Buchberger, J. Chromatogr. A 1047 (2004) 305.
[13] B. Deng, C. Su, Y. Kang, Anal. Bioanal. Chem. 385 (2006) 1336.
[14] K.-H. Bannefeld, H. Stass, G. Blaschke, J. Chromatogr. B 692 (1997) 453.
[15] J.G. Möller, H. Staß, R. Hening, G. Blascheke, J. Chromatogr. B 716 (1998) 325.
[16] C. Horstkötter, G. Blaschke, J. Chromatogr. B 754 (2001) 169.
[17] E. Horstkötter, D. Jiménez-Lozano, J. Barrón, C. Barbosa, G. Blaschke, Electrophoresis 23 (2003) 3078.
[18] C.-H. Chou, M. Rowland, J. Pharm. Sci. 86 (1997) 1310.
[19] C.-H. Chou, J. Pharm. Pharmacol. 52 (2000) 1011.
[20] P.T. Djurdjevic, M. Jelikic-Stankov, D. Stankov, Anal. Chim. Acta 300 (1995) 253.
[21] A.I. Drakopoulos, P.C. Ioannou, Anal. Chim. Acta 354 (1997) 197.
[22] M. Consuelo Cuquerella, M.A. Miranda, F. Bosca, J. Phys. Chem. A 110 (2006) 2607.