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Short communication

Sensitive high-performance liquid chromatographic assay for norfloxacin utilizing fluorescence detection

Mohammed S. Hussain*, Vern Chukwumaeze-Obiajunwa, Ronald G. Micetich

Department of Drug Metabolism and Pharmacokinetics, SynPhar Laboratories Inc., 24 Taiho Alberta Center, 4290-91A Street, Edmonton, Alb. T6E 5V2, Canada

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Abstract

A rapid, sensitive and reproducible reversed-phase high-performance liquid chromatographic assay was developed for the determination of norfloxacin. Following protein precipitation with 10% trichloroacetic acid, norfloxacin and the internal standard enoxacin were extracted from plasma with chloroform, dried and reconstituted in the mobile phase. The chromatographic separation of norfloxacin and the internal standard enoxacin was achieved on a C_8 column with fluorescence detection set at 280 and 418 nm for excitation and emission, respectively. The peaks with a resolution factor greater than 1.5 were free from interferences. Excellent linearity ($r^2 \geq 0.998$) was observed over the concentration range 0.025–5.0 μg/ml in plasma. The inter-assay variability was 13.6% or less at all concentrations examined. The suitability of the assay for pharmacokinetic studies was determined by measuring norfloxacin concentration in rat plasma after administration of a single intravenous 10 mg/kg dose.

1. Introduction

Norfloxacin, [1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid, NFX] is a fluoroquinolone antibiotic, which exhibits activity against a broad spectrum of both Gram-positive and Gram-negative bacteria and is highly effective in the treatment of a wide variety of infectious diseases [1–6]. The zwitterionic structure of NFX (Fig. 1) results in rapid dissolution, absorption and tissue penetration. It has been reported that clearance of NFX involves both glomerular filtration and renal tubular secretion and as such, patients with severe renal failure have a prolonged half-life of the drug [7] which requires dosage adjustments [8]. Therefore, a sensitive analytical technique is needed to monitor NFX plasma concentrations in those patients.

This paper describes a simple, rapid and sensitive reversed-phase high-performance liquid chromatographic (HPLC) assay suitable for the determination of NFX in mammalian plasma.

* Corresponding author.

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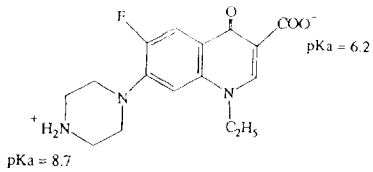

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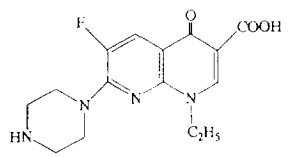

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Fig. 1. Zwitterionic structures of (A) norfloxacin, and (B) enoxacin.

2. Experimental

2.1. Chemicals

Norfloxacin and the internal standard (I.S.) enoxacin were purchased from Sigma (St. Louis, MO, USA). Acetonitrile, chloroform, methanol and sodium hydroxide (BDH, Toronto, Ont., Canada) were all analytical grade. Water was HPLC grade (BDH), and trifluoroacetic acid was analytical grade (Fisher Scientific, Fair Lawn, NJ, USA).

2.2. Chromatography

Samples were mixed with a Vortex Genie 2 mixer (Fisher Scientific) and centrifuged with an Eppendorf centrifuge Model 5415C (Brinkmann Instruments, Westbury, NY, USA). A Savant Speedvac concentrator–evaporator (Savant Instruments, Farmingdale, NY, USA) was used to evaporate the solvents. The HPLC system consisted of a Varian Model 2510 pump (dual), 2584 static mixer, 9100 autosampler and a DS 654 data system (Varian, Sunnyvale, CA, USA). The detector was a Waters 470 scanning fluorescence detector (Waters, Mississauga, Ont., Canada) which was set at 280 and 418 nm for excitation and emission, respectively. Analytical separation of the drug and the I.S. was achieved on a 80×4.6 mm I.D., 5 μm, C_8 column (Zorbax, Brockville, Ont., Canada). The mobile phase was methanol–0.01% trifluoroacetic acid (25:75, v/v) which was pumped at a flow-rate of 1.2 ml/min. Sample preparation and analysis were performed at ambient temperature.

2.3. Standard solutions

A stock solution of 1 mg/ml NFX was made in 0.01 *M* NaOH (solution 1). This stock solution was further diluted serially with HPLC grade water to generate a set of four working standard solutions (1, 5, 10, and 50 μg/ml). The I.S. solution was made in 0.01 *M* NaOH, and then diluted with water to give a final concentration of 0.1 mg/ml (solution 2). Another stock solution of NFX (to determine the extraction yield) was prepared in chloroform to give a final concentration of 1 mg/ml (solution 3). Solution 3 was further diluted serially with chloroform to generate another set of three working standard solutions (1, 5, and 50 μg/ml). These solutions were stored at 4°C until just prior to use.

2.4. Sample preparation

Drug free rat plasma samples (0.15 ml) were spiked with appropriate NFX working standard solutions to give final concentrations of 0.025, 0.05, 0.1, 0.25, 0.5, 1.0, 2.5 and 5.0 μg/ml NFX. To this was added 10 μl (1.0 μg) of I.S. solution (solution 2). The plasma was deproteinized with 75 μl of 10% trichloroacetic acid and extracted with 0.6 ml of chloroform, mixed on a vortex-mixer for 5 min and centrifuged at 13 800 *g* for 10 min. The organic layer (0.5 ml) was transferred to clean tubes and evaporated to dryness using a Savant SpeedVac concentrator–evaporator. The resulting residues were separately reconstituted in 150 μl of mobile phase and a 50-μl aliquot of each of these solution was employed for HPLC analysis.

2.5. Extraction yield

Appropriate chloroform working standard solutions of NFX were evaporated to dryness and then reconstituted in 0.15 ml of plasma to give concentrations of 0.025, 0.50, or 5.0 μg/ml (three sets of each). The NFX was extracted and evaporated to dryness as previously described. To compare these samples with those that were not extracted, another set of samples containing the above concentrations was prepared without the addition of plasma and subsequent extraction procedure. Peak areas of extracted NFX versus unextracted equivalent NFX concentrations were compared under identical HPLC conditions. The extraction yield of the I.S. was determined by a similar method.

2.6. Treatment of data

Concentrations of NFX in plasma were calculated from the standard curves constructed by plotting peak-area ratios of NFX to the internal standard against the spiked concentration of NFX. A freshly prepared set of calibration samples was run daily, along with drug free plasma extracts. Results are presented as mean ± standard deviation (S.D.).

2.7. Accuracy and precision

The inter-assay reproducibility was evaluated by preparing three sets of calibration curves on three separate days. The accuracy of the method was determined by recovery measurements and the precision was determined by calculating the inter-assay coefficient of variation (C.V.).

2.8. Applicability to pharmacokinetic studies

To test the suitability of the assay for pharmacokinetic studies, a single bolus intravenous dose of 10 mg/kg of NFX was administered to male Sprague–Dawley rats ($n = 6$, body-weight 250–300 g). Serial blood samples (0.25 ml) were collected at 0, 0.25, 0.5, 1, 2, 4, 6 and 8 h via a catheter implanted in the right jugular vein. Plasma (0.15 ml) was immediately separated by centrifugation at 4000 *g* for 5 min, and samples were stored at −20°C until analysis.

3. Results and discussions

The reported HPLC methods for determination of NFX in biological fluids [9–15] either used extensive sample preparation steps [9–11] or did not include an internal standard [9,10,12,14,15]. The mobile phase used ranged from phosphate buffer and acetonitrile [13] to a complex mixture of acetonitrile–methanol–water–phosphoric acid and ion-pairing reagent [12]. The present method is not entirely different from those described earlier, but combines a simple mobile-phase composition, a simple extraction step and a smaller volume of plasma (0.15 ml). The total analysis time was less than 8 min. Thus, the method could be utilized efficiently to process a large number of samples like those collected during pharmacokinetic studies. Although a bioassay [16] is quite sensitive, the antibacterial nature of several NFX metabolites rendered it unsuitable for use in pharmacokinetic studies [11]. Since the 4-quinolone structure in both NFX and the I.S. molecules shows fluorescence, we decided to use a fluorescence detector instead of a UV detector because of its high sensitivity and specificity. Enoxacin was used as the I.S. because of its structural similarity and favorable fluorescence and chromatographic properties. Most of the reported methods used acetonitrile as one of the constituents of the mobile phase. Interestingly, our laboratory found that addition of acetonitrile to an aqueous solution of NFX resulted in turbidity. Therefore, methanol was used instead of acetonitrile to avoid such precipitation in the analytical column.

In addition, the sample size required for analysis was small, only 0.15 ml plasma. This sample size was chosen since during pharmacokinetic studies only this amount of plasma could be sampled from each rat at each time point. In spite of this small volume of the plasma, the limit of quantitation (LOQ) was 0.025 μg/ml, which is far below the therapeutic range (1.5–2.0 μg/ml) of the drug [8]. In con-

trast, the reported HPLC methods required 0.5–1 ml plasma for the determination of NFX [9–11,13]. The smaller volume of plasma made the assay suitable for time-course analysis in pharmacokinetic studies in rats or in cases where only small plasma volumes are available. We also achieved good retention and peak shape and resolution without the use of an ion-pairing reagent.

Representative chromatograms of drug-free plasma, plasma spiked with NFX plus internal standard and plasma taken from a rat after a 10 mg/kg intravenous dose are shown in Fig. 2. The HPLC chromatogram of drug-free plasma showed no interfering peaks at the retention times of NFX and the I.S.. The retention times for NFX and the I.S. were 6.9 and 5.8 min, respectively.

The extraction yields of NFX from plasma obtained by comparing extracted versus unextracted samples were 87.5 ± 10.5, 90.5 ± 5.6 and $86.3 \pm 8.5\%$ at concentrations of 0.025, 0.50 and 5.0 μg/ml, respectively. The extraction yields of I.S. were 85.2 ± 4.5 and $82.6 \pm 6.4\%$ at concentrations of 1.0 and 5.0 μg/ml, respectively. The assay was accurate, precise and reproducible (Table 1). Calibration curves were generated by least-squares linear regression analysis. Excellent linearity was observed for all calibration curves over the entire concentration range examined ($r^2 \geq 0.998$). The calibration curves could be typically described by $y = -0.011 + 0.0029x$, where y is the peak-area ratio of the drug to the internal standard and x is the concentration (μg/ml) of NFX in plasma. The error associated with the determinations of accuracy and precision was 13.6% or less, which was well within the range of 20%, an acceptable value as indicated in the conference report on the analytical methods validation [17].

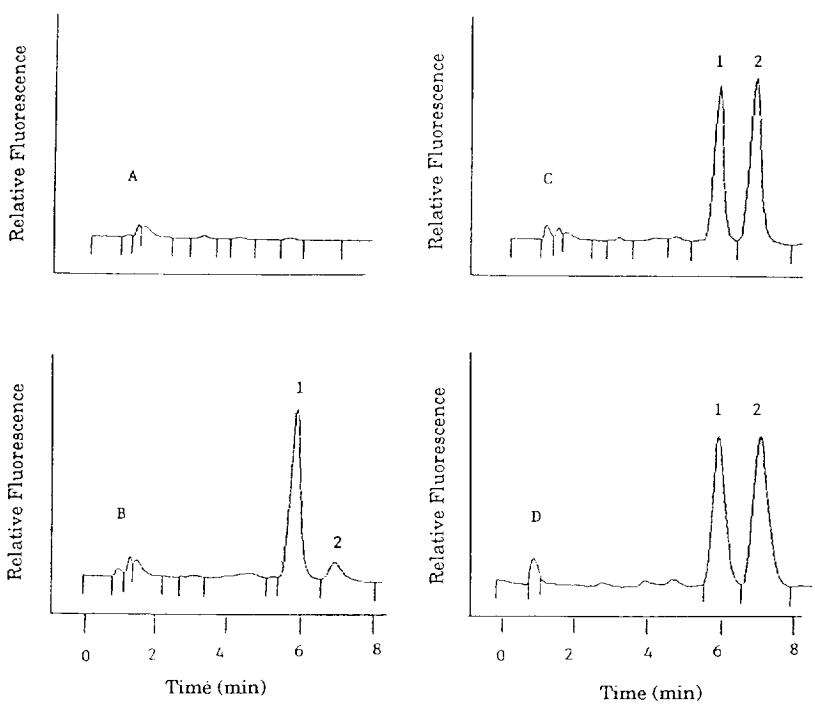

Fig. 2. Chromatograms of (A) drug-free plasma, (B) plasma spiked with 0.025 μg/ml NFX, (C) plasma spiked with 0.50 μg/ml NFX and (D) plasma sample taken 2 h after a single intravenous 10 mg/kg dose of NFX (calculated concentration: 0.54 μg/ml). Peaks: 1 = I.S., 2 = NFX.

Table 1
Accuracy and precision of the method

Norfloxacin concentration (μg/ml)		Accuracy (%)	Precision (C.V., %)
Added	Measured (mean ± S.D.)		
0.025	0.022 ± 0.003	88.0	13.6
0.05	0.048 ± 0.005	96.0	10.4
0.10	0.092 ± 0.009	92.5	9.8
0.25	0.214 ± 0.019	85.5	8.8
0.50	0.429 ± 0.044	85.8	10.2
1.0	0.917 ± 0.083	91.7	9.0
2.5	2.358 ± 0.180	94.3	7.6
5.0	5.007 ± 0.246	100.0	4.9

$n = 9$ (three sets for three days).

In conclusion, the HPLC assay described in this report is simple, sensitive and reproducible, allowing for numerous samples to be processed in a short period of time. Furthermore, the assay is applicable to pharmacokinetic studies of NFX in rats. The plasma concentration–time profile after a bolus intravenous dose of 10 mg/kg NFX to a rat is shown in Fig. 3. Testing of the suitability of the method for analysis of structurally related novel fluoroquinolone antibiotics in plasma is in progress in our laboratory. The method could be successfully applied to monitor NFX levels in humans.

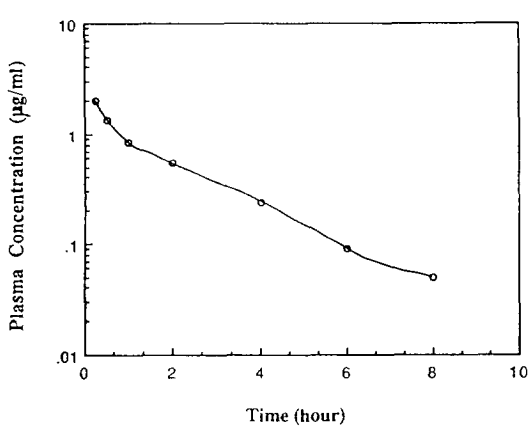

Fig. 3. Plasma concentration–time profile of NFX in a rat following a single intravenous 10 mg/kg dose of NFX.

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References

[1] M.Y. Khan, R.P. Gruninger, S.M. Nelson and R.E. Ckliker, *Antimicrob. Agents Chemother.*, 21 (1982) 848.
[2] H.L. Muytjens, J. van der Ros-van Repe and G. van Veldhnizen, *Antimicrob. Agents Chemother.*, 24 (1983) 302.
[3] Y. Kumamoto and J. Ishigami, *Eur. J. Chemother. Antibiot.*, 3 (1983) 31.
[4] M. Bologna, L. Vaggi, E. Tomei and E. Martini, *Eur. J. Chemother. Antibiot.*, 3 (1983) 41.
[5] A.L. Barry, R.N. Jones, C. Thornsberry, L.W. Ayers, E.H. Gerlach and H.M. Sommers, *Antimicrob. Agents Chemother.*, 25 (1984) 633.
[6] F.W.A. Heesen and H.L. Muytjens, *Antimicrob. Agents Chemother.*, 25 (1984) 123.
[7] D.C. Hooper and J.S. Wolfson, *Antimicrob. Agents Chemother.*, 28 (1985) 716.
[8] G.E. Stein, *Am. J. Med.*, 82 (Suppl. 6B) (1987) 18.
[9] V.K. Boppana and B.N. Swanson, *Antimicrob. Agents Chemother.*, 21 (1982) 808.
[10] C. Forchetti, D. Flammini, G. Carlucci, G. Cavicchio and L. Vaggi, *J. Chromatogr.*, 309 (1984) 177.
[11] L.T. Pauliukonis, D.G. Musson and W.F. Bayne, *J. Pharm. Sci.*, 73 (1984) 99.

[12] W. Schonfeld, J. Knoller, K.D. Bremm, A. Dahlhoff, B. Weber and W. Konig, *Zbl. Bakt. Hyg.*, A261 (1986) 338.
[13] G. Carlucci, P. Mazzeo and G. Palumbo, *Biomed. Chromatogr.*, 7 (1993) 126.
[14] A. Lagana, R. Curini, G. D'Ascenzo, A. Marino and M. Rotatori, *J. Chromatogr.*, 417 (1987) 135.
[15] I. Nilsson-Ehle, *J. Chromatogr.*, 416 (1987) 207.
[16] J. Bland, A. Edison and J. Huber, *Eur. J. Clin. Microbiol.*, 2 (1983) 249.
[17] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowell, K.A. Pittman and S. Spector, *J. Pharm. Sci.*, 81 (1992) 309.