

A simple chromatographic method for determining norfloxacin and enoxacin in pharmacokinetic study assessing CYP1A2 inhibition

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ABSTRACT: We developed a simple assay method for the determination of serum and urine norfloxacin and enoxacin using reversed-phase high-performance liquid chromatography and perchloric acid precipitation for sample pre-treatment. Optimized conditions can permit detection of norfloxacin and enoxacin in the same chromatogram, so either compound can be used as an internal standard for another determinant. Supernatants of the precipitated samples were analyzed by the octadecylsilyl silica-gel column under ambient temperature and an ultraviolet wavelength of 272 nm. A mobile phase solvent consisting of 20 mM sodium dihydrogenphosphate (pH 3.0) and acetonitrile (85:15, v/v) was pumped at a flow rate of 1.0 mL/min. The calibration curves for norfloxacin and enoxacin at a concentration of 62.5–1000 ng/mL for serum and 250–4000 ng/mL for urine were linear ($r > 0.9997$). The recoveries of norfloxacin and enoxacin from serum and urine were >94% with the coefficient of variations (CV) <5%. The CVs for intra- and inter-day assay of norfloxacin and enoxacin were <4.2 and <5.5%, respectively. This method can be applied to the pharmacokinetic study of norfloxacin and enoxacin after repeated administration to assess changes in CYP1A2 activity in healthy subjects. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: HPLC; norfloxacin; enoxacin; blood concentration; CYP1A2

Introduction

Norfloxacin and enoxacin are new quinolone antimicrobial agents for the treatment of infections such as urinary tract infections (including cystitis) and sexually transmitted diseases (Swanson *et al.*, 1983; Goldstein, 1987; Siboulet *et al.*, 1988; Well *et al.*, 1998). They are the second generation of quinolones that possess a fluorine atom at the C-6 position and a piperazine at the C-7 position (Fig. 1). These provide broad spectrum cover against Gram-positive and Gram-negative microorganisms (Ball, 2000). These chemical modifications are also associated with inhibitory activity for cytochrome P450 (CYP) 1A2 (Wijnands *et al.*, 1986; Fuhr *et al.*, 1992; Mizuki *et al.*, 1996). Therefore, concomitant use with CYP1A2 substrates (caffeine, theophylline and tizanidine) should involve consideration of possible drug interactions (Davis *et al.*, 1995; Mizuki *et al.*, 1996; Granfors *et al.*, 2004). The blood concentrations of norfloxacin and enoxacin that lead to CYP1A2 inhibition have not been well elucidated.

Determination of the blood concentrations of norfloxacin and enoxacin is usually carried out by high-performance liquid chromatography (HPLC) equipped with ultraviolet (UV) or fluorescence detector. Sample pre-treatments have been conducted by liquid–liquid extraction with dichloromethane or chloroform (Boppana and Swanson, 1982; Groeneveld and Brouwers, 1986; Wise *et al.*, 1986; Hussain *et al.*, 1995; Wallis *et al.*, 1995), C₁₈ cartridge column extraction (Carlucci, 1998) and protein precipitation with acetonitrile, methanol, trichloroacetic acid or perchloric acid (Morton *et al.*, 1986; Griggs and Wise, 1989;

Mascher and Kikuta, 1998; Samanidou *et al.*, 2003; Galaon *et al.*, 2007).

We developed a simple HPLC method for the determination of norfloxacin and enoxacin under identical analytical conditions. The method employed perchloric acid precipitation for sample pre-treatment, isocratic elution on reversed-phase mode and UV detection. It can be applied to the pharmacokinetic study of norfloxacin and enoxacin to assess changes in CYP1A2 activity in healthy subjects.

Experimental

Chemicals and Standard Solutions

Norfloxacin [1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline carboxylic acid] and enoxacin [1-ethyl-6-fluoro-1,4-dihydro-4-

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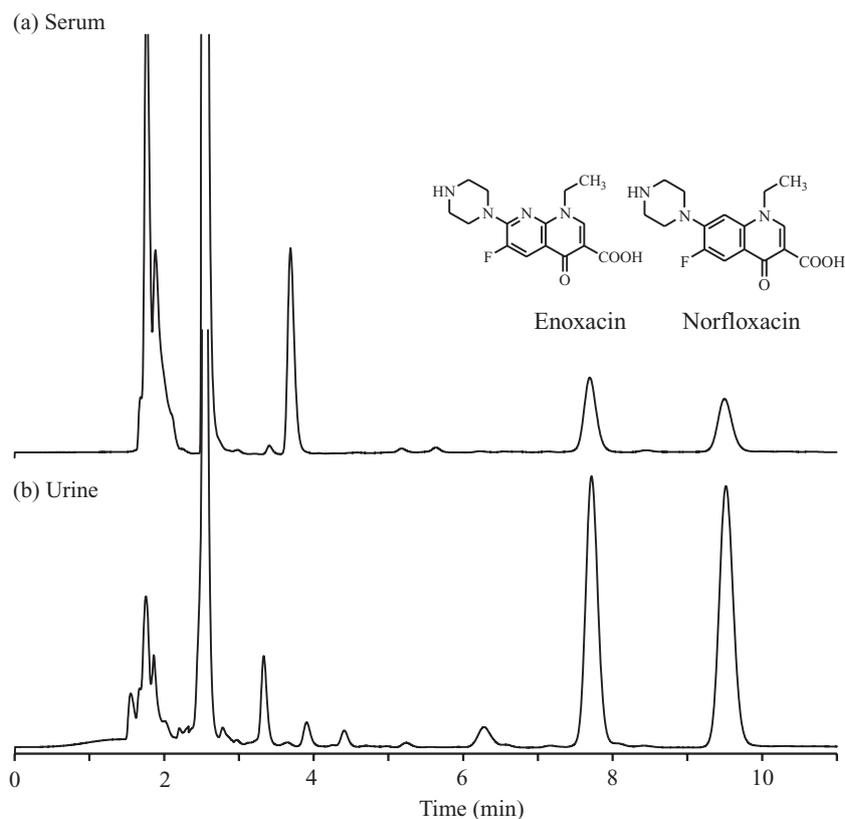


Figure 1. Typical chromatograms for the determination of enoxacin and norfloxacin in serum (a) and urine (b). Blood sample was collected at 3 h after administration of enoxacin (100 mg) on day 3. Urine sample was collected for 9 h after administration of enoxacin (100 mg) on day 3. Serum and urinary enoxacin concentrations were 0.97 and 2.6 $\mu\text{g/mL}$, respectively.

oxo-7-(1-piperazinyl)-1,8-naphthyridine-3-carboxylic acid] were purchased from Sigma–Aldrich (Tokyo, Japan). Acetonitrile (Wako Pure Chemical Industries Ltd, Osaka, Japan) was of HPLC grade. All other chemicals purchased from Wako Pure Chemical Industries Ltd were of analytical reagent grade.

Control samples of serum and urine were prepared using an alternative human serum (Twin-consera L, Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) and human urine, respectively. Standard solutions for norfloxacin and enoxacin of serum and urine were prepared at concentrations of 62.5–1000 and 250–4000 ng/mL. These solutions were stored at -20°C until use. Norfloxacin and enoxacin can be simultaneously detected under the present conditions, so each drug can be used as an internal standard (IS) for measuring the other one. IS solutions dissolved in water were prepared at concentrations of 20 $\mu\text{g/mL}$ for serum and 80 $\mu\text{g/mL}$ for urine.

HPLC Apparatus and Analytical Conditions

The HPLC system used in this study consisted of a pump (CCPM, Tosoh, Tokyo, Japan), a pump controller (Px-8020, Tosoh), a degasser (SD-8020, Tosoh) and a UV detector (UV-8010, Tosoh). An octadecylsilyl silica-gel (ODS) column (TSK-gel, 4.6 i.d. \times 150 mm, Tosoh), used as the analytical column, was maintained at ambient temperature. The detection wavelength was 272 nm. The mobile phase solvent consisting of 20 mM sodium dihydrogenphosphate (pH 3.0) and acetonitrile (85:15, v/v) was pumped at a flow rate of 1.0 mL/min.

Assay Procedure

An aliquot of serum or urine samples (300 μL) spiked with 10 μL of IS solutions was treated with 300 μL of 7% perchloric acid to precipitate

proteins. After vortex mixing for 10 s, samples were centrifuged at 18,500g for 2 min. One hundred microliters of supernatant was injected into the HPLC system. Urine samples were diluted 50-fold with water. Further dilution (2-fold) of the urine samples was conducted when the concentration exceeded the range of calibration curves in the 50-fold dilution.

Pharmacokinetic Study and CYP1A2 Inhibition

Three healthy male subjects (non-smokers) received norfloxacin and enoxacin to investigate the pharmacokinetic parameters and change in CYP1A2 activity. Subjects received 100 mg of norfloxacin three times (08:00, 12:00 and 20:00) on day 1 and twice (08:00 and 12:00) on day 2. After the washout period (2 weeks), they received 100 mg of enoxacin three times (08:00, 12:00 and 20:00) on days 1 and 2, and once (08:00) on day 3. Blood drawing was conducted 1.5, 2, 3, 4 and 6 h after administration of each drug at 08:00 on day 2 for norfloxacin and on day 3 for enoxacin. Urine samples were collected between 08:00 and 17:00 (9 h after administration) on the same day as blood drawing. Alcohol and drinks containing caffeine were not permitted on study days. The protocol was approved by the Ethical Committee of University of Tsukuba.

Pharmacokinetic parameters (apparent distribution volume, V/F ; half-life, $t_{1/2}$; apparent total clearance, Cl_{tot}/F) of norfloxacin and enoxacin were simulated by PEDASIM (version 1.0.0.58; Jiho, Tokyo, Japan) based on a one-compartment model. Area under the concentration–time curve (AUC) was calculated using the trapezoid method. Apparent non-renal clearance ($Cl_{\text{non-renal}}/F$) was estimated with Cl_{tot}/F and apparent renal clearance, which was calculated by cumulative urinary excretion ($\text{CUE}_{0-9\text{h}}/\text{AUC}_{0-9\text{h}}$).

CYP1A2 activity was assessed by the paraxanthine–caffeine ratio in the urine collected for the pharmacokinetic study of norfloxacin and enoxa-

Table 1. Intra- and inter-day assay precision for the determination of norfloxacin and enoxacin

Concentration (ng/mL)	Norfloxacin					Enoxacin			
	Intra-day (<i>n</i> = 5)		Inter-day (<i>n</i> = 5)			Intra-day (<i>n</i> = 5)		Inter-day (<i>n</i> = 5)	
	Mean ± SD (ng/mL)	CV (%)	Mean ± SD (ng/mL)	CV (%)	Mean ± SD (ng/mL)	CV (%)	Mean ± SD (ng/mL)	CV (%)	
Serum	62.5	66.1 ± 2.3	3.4	63.8 ± 2.7	4.2	60.0 ± 2.5	4.2	60.8 ± 2.6	4.2
	125.0	130.7 ± 2.8	2.2	126.3 ± 4.5	3.5	122.5 ± 5.1	4.2	123.8 ± 3.4	2.8
	500.0	501.5 ± 4.8	0.9	507.1 ± 7.0	1.4	493.8 ± 9.1	1.8	496.6 ± 12.3	2.5
Urine	500.0	462.9 ± 9.0	1.9	498.3 ± 18.0	3.6	489.3 ± 11.7	2.4	514.8 ± 28.1	5.5
	2000.0	1887.2 ± 68.6	3.6	1930.8 ± 92.7	4.8	2049.9 ± 41.9	2.0	2087.5 ± 101.2	4.8

Table 2. Pharmacokinetic parameters for norfloxacin and enoxacin in three healthy subjects

		Case 1	Case 2	Case 3	Mean ± SD
Age (years)		28	24	24	25.3 ± 2.3
Weight (kg)		52	56	71	59.7 ± 10.0
Norfloxacin	AUC _{0-9h} (µg h/mL)	3.3	3.4	2.5	3.1 ± 0.5
	C _{max} (µg/mL)	0.71	0.84	0.44	0.66 ± 0.20
	CUE _{0-9h} (mg)	57.0	29.6	48.6	45.1 ± 14.0
	CL _{tot} /F (L/h/kg)	0.95	0.94	0.69	0.86 ± 0.15
	CL _{non-renal} /F (L/h/kg)	0.62	0.79	0.42	0.61 ± 0.19
	t _{1/2} (h)	1.6	1.0	4.6	2.4 ± 1.9
Enoxacin	AUC _{0-9h} (µg h/mL)	10.2	4.2	6.7	7.0 ± 3.0
	C _{max} (µg/mL)	1.85	0.98	1.09	1.31 ± 0.47
	CUE _{0-9h} (mg)	63.3	68.5	52.1	61.3 ± 8.4
	CL _{tot} /F (L/h/kg)	0.17	0.39	0.19	0.25 ± 0.12
	CL _{non-renal} /F (L/h/kg)	0.05	0.10	0.08	0.08 ± 0.03
	t _{1/2} (h)	3.0	3.4	5.2	3.9 ± 1.2

cin and the control (without administration of norfloxacin and enoxacin). The paraxanthine–caffeine ratio was examined under a regimen restricting caffeine intake during the study. Urinary levels of paraxanthine (a main metabolite of caffeine) and caffeine were measured by HPLC as previously described with minor modifications (Delbeke and De Backer, 1996; Holland *et al.*, 1998).

Results

Chromatograms and Assay Precision

A typical chromatogram for the determination of norfloxacin and enoxacin is shown in Fig. 1. The peaks representing enoxacin and norfloxacin were observed at the retention times of 7.7 and 9.5 min, respectively. An interfering peak for norfloxacin and enoxacin was not observed on the chromatograms for both serum and urine samples.

The calibration curves for levels of norfloxacin and enoxacin determined at the concentration of 62.5–1000 ng/mL for serum (norfloxacin: $Y = 1.042 \times 10^{-3} X - 0.011$, $r = 0.9997$; enoxacin: $Y = 1.267 \times 10^{-3} X - 0.0021$, $r = 0.9999$) and 250–4000 ng/mL for urine (norfloxacin: $Y = 0.2862 \times 10^{-3} X - 0.0054$, $r = 0.9999$; enoxacin: $Y = 0.3796 \times 10^{-3} X + 0.0086$, $r = 0.9998$) were linear. The detection limits for norfloxacin and enoxacin were 3.0 ng in serum and 12.0 ng in urine as the injected amounts, respectively.

The recoveries of norfloxacin and enoxacin from spiked samples of serum and urine were 94.3–100.8% with coefficient of variation values (CVs) in the range of 0.3–4.2%. The assay precision of norfloxacin and enoxacin was evaluated by intra- and

inter-day validation. The CVs for the intra-day assay of norfloxacin and enoxacin were 0.9–4.2% and for the inter-day assay were 1.4–5.5%, respectively (Table 1).

Pharmacokinetic Study and Urinary Paraxanthine–Caffeine Ratio

We applied the HPLC method to samples obtained from the three healthy subjects (cases 1–3) in the pharmacokinetic study of norfloxacin and enoxacin. The pharmacokinetic parameters were estimated based on simulating serum concentration–time profiles. The calculated pharmacokinetic parameters listed in Table 2 were in accordance with other reports (Lode *et al.*, 1989; Sörgel *et al.*, 1989; Fillastre *et al.*, 1990). The urinary paraxanthine/caffeine ratios in the norfloxacin (22.7 ± 20.8) and enoxacin (8.0 ± 3.7) phase were lower than that in the control phase (70.1 ± 63.1).

Discussion

The procedure employing perchloric acid precipitation for sample pre-treatment and HPLC conditions were identical for norfloxacin and enoxacin assays. This provides a great advantage in clinical application for both drug assays. Among sample pre-treatment procedures such as solid-phase extraction (Carlucci, 1998) and liquid–liquid extraction (Boppana and Swanson, 1982; Groeneveld and Brouwers, 1986; Wise *et al.*, 1986; Hussain *et al.*, 1995; Wallis *et al.*, 1995), perchloric acid precipitation is a simple way to extract norfloxacin and enoxacin. HPLC conditions were

optimized to be convenient with the common ODS column, UV detection and simple mobile-phase solvents. Thus, the method was ultimately optimized in terms of simplicity for norfloxacin and enoxacin assay in the clinical setting.

The present method was sufficiently sensitive and precise for examining the pharmacokinetics of norfloxacin and enoxacin. The recoveries for both drugs from sample specimens (serum and urine) were >94.3% with the CVs being <4.2%. Intra- and inter-day assay precision was sufficient with CVs being <5.5% (Table 1), which were almost identical to those in other studies (Boppana and Swanson, 1982; Groeneveld and Brouwers, 1986).

Norfloxacin and enoxacin are well-known CYP1A2 inhibitors *in vitro* and *in vivo* (Fuhr et al., 1992). This may cause drug interaction with CYP1A2 substrates, such as caffeine, theophylline and tizanidine; blood concentrations were increased during concomitant use with norfloxacin or enoxacin. The blood concentrations of norfloxacin and enoxacin leading to CYP1A2 inhibition have not been elucidated. The urinary paraxanthine-caffeine ratio was used as an index of CYP1A2 activity because the caffeine was predominantly demethylated to paraxanthine via CYP1A2 pathway (Kalow and Tang, 1993). Preliminary results for the change in the urinary paraxanthine-caffeine ratio revealed that the ratios for the enoxacin phase (8.0) and the norfloxacin phase (22.7) were smaller than that for and control phases (70.1). This suggested that both new quinolone antibiotics possessed the inhibitory activity of CYP1A2 and the activity for enoxacin was greater than that of norfloxacin. The difference between the drugs may be due to the higher AUC and C_{max} in enoxacin compared with norfloxacin (Table 2).

In conclusion, the present HPLC assay method for determining serum and urinary levels of norfloxacin and enoxacin can be applied to the pharmacokinetic study of both drugs after repeated administration for assessing the change in CYP1A2 activity *in vivo*.

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