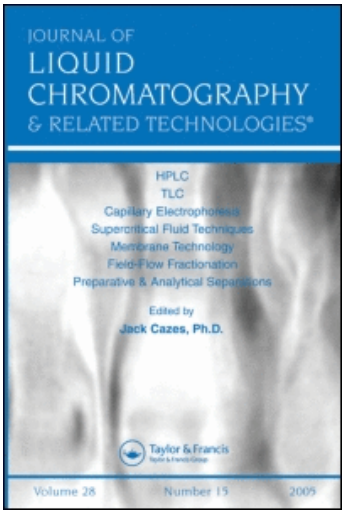

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Application of Solid-Phase Extraction of Fluoroquinolone Derivatives from a Biological Matrix to Their Biodetermination by Liquid Chromatography

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Application of Solid-Phase Extraction of Fluoroquinolone Derivatives from a Biological Matrix to Their Biodetermination by Liquid Chromatography

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ABSTRACT

A solid-phase extraction (SPE) procedure was developed for extraction of relatively hydrophilic amphoteric drugs from plasma coupled with their determination by high-performance liquid chromatography (HPLC). The extraction columns filled with 500 mg of reversed phase octadecyl-bonded silica phase were conditioned with methanol, water, and ammonium acetate solution. After sample application, the sorbent was washed with the same solution. The analytes were then desorbed with the methanol–acetate buffer (pH 2.8) mixture. A composition, volume, and flow-rate of the eluting solvent as SPE parameters were optimised. A method for the determination of fleroxacin and sparfloxacin in plasma using the optimal SPE conditions was validated. The absolute recovery of fluoroquinolones mentioned above was ca. 96% and 92%, respectively.

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Key Words: Fleroxacin; Sparfloxacin; SPE-procedure; Plasma; HPLC analysis.

INTRODUCTION

The high-performance liquid chromatography (HPLC) analysis of drugs in biological material usually requires a sample pre-treatment procedure to release the analyte from a conjugate in a matrix (proteins, endogenous compounds). In current bioanalytical methods, solid-phase extraction (SPE) is increasingly applied for sample preparation instead of classical techniques such as liquid–liquid extraction. Besides, the SPE technique can be fully automated by using the automatic sample preparation with extraction cartridges system (ASPEC), coupled directly with HPLC. This procedure is particularly recommended when the number of samples is large.

High performance liquid chromatography is the predominant method allowing the determination in biological fluids of fluoroquinolone derivatives chosen in this assay (Fig. 1).[1–12]

In bioanalytical procedures mentioned above, the samples were extracted with use of organic solvents, such as dichloromethane–isopropanol containing sodium dodecyl sulfate,[1] dichloromethane–isopropanol,[2] or acetonitrile.[3–5] Kamberi[6] and Vangani et al.[7] were able to determine sparfloxacin (SPA) after precipitating plasma proteins with perchloric acid or acetonitrile–70% perchloric acid, respectively. The SPE was applied by Hobara et al.[8] for a sample cleanup in order to determine fleroxacin (FLE) in rat plasma and to evaluate the pharmacokinetic parameters. Investigators also applied direct determination of FLE[9,10] as the method allowing reducing the time of analysis. In order to minimise hydrolysis during sample preparation, a multidimensional HPLC method with direct injection of plasma was used for the analysis of the desacetylcefotaxime–FLE drug.[11] In most HPLC methods fluorescence detection was preferred to UV detection.[1–4,8,10,12]

The aim of the present work has been to study the best conditions for the development of SPE of FLE and SPA from plasma, coupled with their bioanalysis. The simple method, based on isocratic reversed-phase HPLC assay with photodiode-array detection, was validated with respect to recovery, linearity, precision, and detectability.

EXPERIMENTAL

Apparatus and Conditions

A high-performance liquid chromatograph equipped with the following components is recommended: a 170 Diode Array Detector (DAD), a

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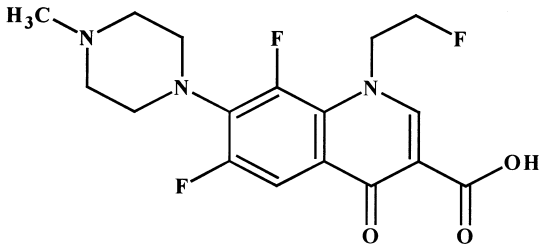
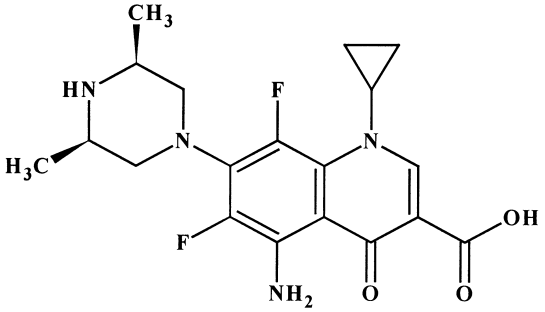


(A) fleroxacin



(B) sparfloxacin

Figure 1. Chemical structure of 6,8-difluoro-1-(2-fluoroethyl)-1,4-dihydro-7-(4-methyl-1-piperazinyl)-4-oxoquinoline-3-carboxylic acid (A), and of 5-amino-1-cyclopropyl-6, 8-difluoro-1,4-dihydro-7-(*cis*-3,5-dimethylpiperazinyl)-4-oxoquinoline-3-carboxylic acid (B).

Rheodyne L.P valve with 20-μL loop, two Model 306 piston pumps, a Model 811C dynamic mixer, a Model 805 manometric module, a Model 864 degasser (all from Gilson Company, France).

The HPLC system was interfaced to an IBM PC compatible computer using a Uni Point^{TM} Software version 2.1 (Gilson, France). A Manu-CART system contained a LiChroCART analytical column (250 nm $\times$ 4 nm, I.D.) packed with Purospher RP-18 (particle size 5 μm) from Merck was used.

The mobile phases were methanol–5 mmol L^{-1} tetrabutylammonium hydroxide (TBA-OH), pH 2.97 (15 : 85, v/v) and methanol with 25% of tetrahydrofuran (THF)–50 mmol L^{-1} ammonium phosphate $(NH_4)_2HPO_4$, pH 2.95 (27 : 73, v/v) for FLE and SPA, respectively. The pH of eluents was brought to appropriate value with 85% orthophosphoric acid. The mobile

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phases were delivered at a flow-rate of 1.0 mL min^{-1}. The detection was carried out at the analytical wavelengths of 300 nm (SPA) and 285 nm (FLE).

The BAKER spe-12G Glass Column Processor (7018-94) (J. T. Baker) capable of processing up to 12 extraction columns simultaneously was applied. BAKERBOND spe™ (3-mL capacity) SPE columns, packed with 500 mg reversed phase octadecylsilane (C_{18}) bonded to silica gel, from J. T. Baker were used. Extraction columns loaded with plasma samples were used only once, whereas, they were used several times by application of standard solutions of the analytes. The eluting solvent was a mixture consisted of methanol and 10 mmol L^{-1} acetate buffer (8 : 2, v/v). The pH of this mixture was adjusted to 2.8 with concentrated acetic acid.

Chemicals and Reagents

Sparfloxacin (SPA), Fleroxacin (FLE) and norfloxacin (NOR) standard substances were kindly supplied by pharmaceutical companies like Rhône-Poulenc Rorer (Antony Cedex, France), F. Hoffmann-La Roche LTD (Basle, Switzerland), and KRKA (Novo mesto, Slovenia), respectively. Enrofloxacin (ENR) pure substance was obtained from Institute of Drugs, Poland.

Methanol, acetonitrile, THF (Merck, Darmstadt, Germany), and 0.4 mol L^{-1} TBA-OH (Baker, USA) were of HPLC grade. All other reagents were of analytical grade. The water used throughout all experiments was freshly distilled.

The phosphate buffer (pH > 5) was prepared by mixing appropriate volumes of potassium dihydrogenphosphate and disodium phosphate solutions (0.067 mol L^{-1}) (according to Michealis). Phosphate buffer (pH < 5) was prepared by dissolving 9.078 g of potassium dihydrogenphosphate in 1.0 L of water and an aliquot of 100.0 mL, adjusted to required pH with 85% orthophosphoric acid. The acetate buffer (10 mmol L^{-1}) was obtained by dissolving 0.77 g of ammonium acetate in 1.0 L of water and adjusted to pH 2.5 with concentrated acetic acid.

Standard Solutions Preparation

Stock standard solutions of FLE and SPA were prepared in a mixture of methanol–50 mmol L^{-1} phosphoric acid (10 : 90, v/v) at a concentration of 100 μg mL^{-1} (solution A) and 10.0 μg mL^{-1} (solution B). Solution B was obtained by dilution of solution A with the same mixture of solvents.

Stock standard solutions of NOR (500.0 μg mL^{-1}) and ENR (100.0 μg mL^{-1}) as internal standards for FLE and SPA determination,

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respectively, were prepared in a mixture of methanol–50 mmol L^{-1} phosphoric acid (10 : 90, v/v).

These solutions, stored in a refrigerator at 4°C, were stable for at least one month.

Working standard solutions of FLE (0.25 μg mL^{-1}–8.0 μg mL^{-1}, each containing 5.0 μg mL^{-1} of NOR) and of SPA (0.5 μg mL^{-1}–4.0 μg mL^{-1}, each containing 2.0 μg mL^{-1} of ENR) were obtained by diluting the stock standard solutions with the same mixture solvents in order to measure the recoveries of the determined analytes.

Plasma Samples Preparation

To obtain working solutions for spiking the plasma samples, 1.0 mL of stock standard solutions of FLE, SPA, and I.S. were transferred to volumetric flasks (10.0 mL) and filled up to the mark with methanol.

Increasing volumes of methanolic working solutions corresponding to 0.25, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0 μg of FLE or 0.25, 0.5, 0.75, 1.0, 1.5, 2.0 μg of SPA were transferred to a series of centrifuge test tubes, each containing 0.5 mL of the blood plasma. Next, an aliquot of 0.1 mL of stock standard solution of NOR (5.0 μg) or ENR (1.0 μg) as I.S was added to the samples with FLE or SPA, respectively. The contents of test tubes were filled with methanol up to a volume of 3 mL and the obtained mixtures were centrifuged for 10 min at 3000 *g*.

Sample Extraction

At the beginning of the SPE procedures, the Bakerbond RP-18e extraction columns were located in the holder of the vacuum chamber. The sorbent was conditioned by passing through the columns 1.0 mL of methanol twice, followed by 1.0 mL of distilled water, and 1.0 mL of ammonium acetate (10 mmol L^{-1}). During the next step, a volume of 1.5 mL of the supernatant was loaded onto the corresponding extraction column.

The columns were washed with 1.0 mL of distilled water and 1.0 mL of ammonium acetate (10 mmol L^{-1}). The analyte was eluted from the sorbent at the pressure of 400 mba with five portions of 0.5 mL of a mixture, which consisted of methanol and acetate buffer (10 mmol L^{-1}) (8 : 2, v/v) adjusted to pH of 2.8 with acetic acid. Extracts were evaporated to dryness at 40°C under a stream of nitrogen. The residues of FLE and SPA samples were reconstituted in 0.5 mL and 0.25 mL of methanol–50 mmol L^{-1} phosphoric acid mixture (10 : 90, v/v), respectively. The obtained solutions were injected into the chromatographic system.

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RESULTS AND DISCUSSION

The basic aim of our experiments was the selection of the optimal conditions for SPE with respect to analyte recovery. This analysis was performed on Bakerbond extraction columns containing octadecylsilica sorbent using standard solutions of the analytes instead of spiked plasma samples in order to limit the consumption of these columns. Recovered analytes from plasma in the optimal SPE conditions was confirmed with the spiked plasma samples.

As SPE parameters, a composition, volume, and flow-rate of the eluting solvent was examined.

Some of the extraction scheme and obtained recovery of the analytes are presented in Table 1. In regard to the highest recovery, methanol containing 20% of acetate buffer at pH 2.8 was chosen as the eluting solvent. The addition of acetate buffer and acidic acid (pH 2.8) to methanol was necessary to obtain the satisfactory recovery of the analytes.

The conditioning of the extraction columns was made in two steps. In the first step, the solvation of the sorbent was carried out by use of methanol as an

Table 1. The recoveries of fluoroquinolone drugs obtained by using different eluting solvents.

	Recovery (%)[a]	
Elution solvent (4×0.5 mL)	Fleroxacin	Sparfloxacin
1. Acetone/chloroform (1 : 1)	Lower than 30	
2. Acetonitrile/ethyl acetate (2 : 1)	Lower than 30	
3. Methanol/acetonitrile (2 : 1)	59	60
4. Methanol/DMF (8 : 2)	62	59
5. Methanol	42	35
6. Methanol/water (8 : 2)	45	41
7. 1% Methanolic HCl	48	45
8. 0.5% Methanolic $HClO_4$	72	80
9. 0.5% Methanolic H_3PO_4	54	48
10. Methanol/50 mM phosphate buffer, pH 2.8 (8 : 2)	90	85
11. 10% Methanolic CH_3COOH	82	75
12. Methanol/water/ CH_3COOH (5 : 3 : 2)	85	83
13. Methanol/10 mM acetate buffer, pH 2.8 (8 : 2)	98	95

Note: Conditioning: 2×1 mL methanol and 1 mL water, washing: 1 mL water.
[a]Concentration: 5 μg mL^{-1}.

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effective solvating agent. Next, the excess of methanol was removed with water and then with ammonium acetate solution (10 mmol L^{-1}, pH 6.8). Spiked plasma samples were loaded on the extraction columns at zero flow in order to adsorb the analytes on the solid phase. Matrix components were washed from the sorbent with ammonium acetate solution (good clean-up efficiency). The clean chromatograms, without interference from plasma components, were obtained. The drugs adsorbent on the sorbent were eluted with the optimal eluting solvent (System 13, Table 1), at the flow-rate of 2 mL min^{-1} that was reached under the pressure of 400 mba. The use of a too low and a high flow during the elution of plasma samples caused a noticeable decrease of the recovery, because only part of the analyte was extracted from the solid phase. In further experiments, the influence of a volume of eluting solvent on an extraction of analytes was evaluated. Finally, a volume of 2.5 mL divided into five parts of the eluting mixture was applied for the elution of FLE or SPA from the extraction columns.

In our work, the photodiode-array detector was used for the selection of the optimum wavelength, the evaluation of the peak purity, and quantification. The analysed fluoroquinolones demonstrate relatively significant absorption properties. Following oral dosing within the recommended therapeutic range, the C_{max} values for plasma are linearly related to increasing doses of the examined quinolones. A maximal plasma concentration of 2.33–7.0 μg mL^{-1} or 0.7–1.97 μg mL^{-1} was monitored after a single oral administration of 200–800 mg dose of FLE or SPA, respectively.[13] The analytical wavelengths of 285 nm and 300 nm were chosen for bioanalysis of FLE and SPA, respectively. In HPLC assay, the internal standard technique was applied using the structure analogues of the investigated drugs as internal standards (NOR and ENR for FLE and SPA determination, respectively). All analyses were performed on highly purified silica gel with a low content of metal impurities used as packing material for the chromatographic column (Purospher ODS).

The mixtures of methanol–5 mmol L^{-1} TBA-OH at pH 2.97 (15 : 85, v/v) and methanol with 25% of THF–50 mmol L^{-1} ammonium phosphate, at pH 2.95 (27 : 73, v/v) were selected as optimal mobile phases for the analysis of FLE and SPA, respectively. The tailing effect of peak analytes was significantly reduced by addition of TBA-OH or THF to the mobile phases mentioned above. Under the described chromatographic condition, the mean retention times (min) (±SD, $n = 5$) were 5.82 ± 0.21 and 9.32 ± 0.44 for FLE and NOR, or 6.42 ± 0.10 and 4.86 ± 0.05 for SPA and ENR. The separation factors between FLE and NOR, or between SPA and ENR, were 7.0 and 3.5, respectively. Tailing factor and peak asymmetry characterising of the analyte peak were 1.30, 1.48 for FLE and 1.31, 1.56 for SPA.

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Selectivity

No endogenous sources of interference were observed at the retention time of the analytes obtained with blank plasma and with spiked plasma containing 2.0 μg mL^{-1} of FLE or SPA as demonstrated by chromatograms presented in Fig. 2 or Fig. 3, respectively. The retention of the analytes was sufficiently high to avoid interference with the front peak. Capacity factors of analysed drugs were higher than 2.2.

Linearity

The linearity was tested at concentrations of 0.25, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0 μg mL^{-1} for FLE and of 0.5, 1.0, 1.5, 2.0, 3.0, 4.0 μg mL^{-1} for SPA. Samples were processed several times ($n = 5$) using the SPE-HPLC procedure

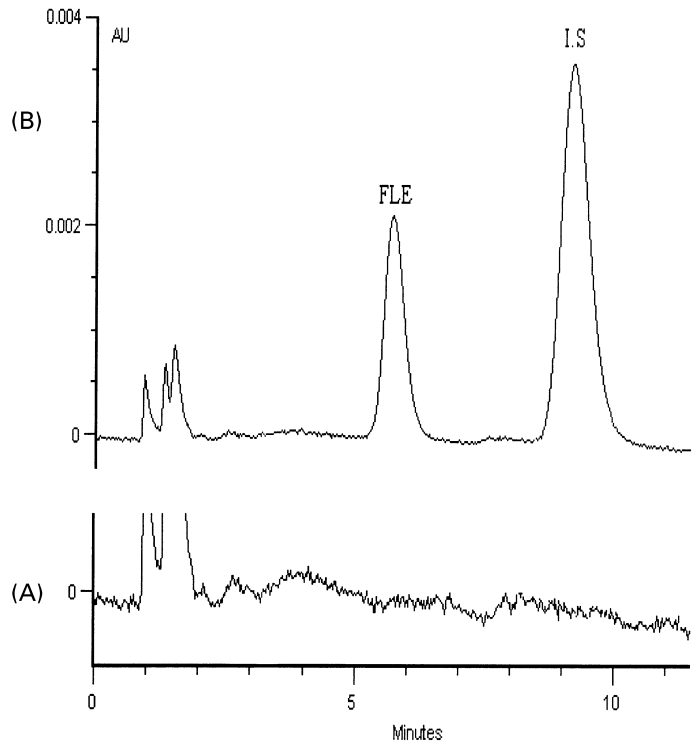

Figure 2. Typical chromatograms of extracts from blank plasma (A) and plasma spiked with 2.0 μg mL^{-1} of FLE (B) obtained by using SPE-HPLC method.

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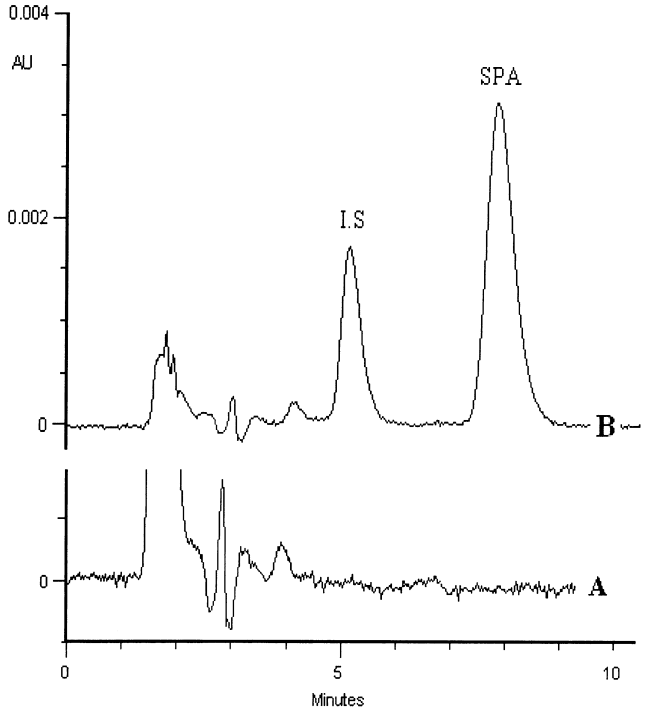

Figure 3. Typical chromatograms of extracts from blank plasma (A) and plasma spiked with 2.0 μg mL^{-1} of SPA (B) obtained by using SPE-HPLC method.

described above. By plotting peak high ratios of the analytes determined to the appropriate internal standard ($h/h' = Y$) against the concentration (x) the regression lines expressed by the following equations was constructed:

$$Y_{\mathrm{FLE}} = 12.5198(\pm 0.224)x + 0.0457(\pm 0.041), \quad r = 0.9994$$
$$Y_{\mathrm{SPA}} = 24.3213(\pm 1.072)x + 0.0527(\pm 0.053), \quad r = 0.9993$$

The linearity of the obtained curves was demonstrated by the good coefficient correlation (r) and was confirmed by using Mandel's fitting test (FLE: TV = 2.4 < F = 7.50, SPA: TV = 1.09 < F = 7.68).

Detectability

Minimum detectable concentration, using a signal to noise ratio of 3 was determined to be 0.82 ng and 1.2 ng injected, corresponding to a concentration

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of 0.041 μg mL^{-1} plasma and of 0.06 μg mL^{-1} plasma for FLE and SPA, respectively.

Recovery

The recovery of analytes from spiked plasma samples was examined at different concentration levels in the range from 0.25 to 8.0 μg mL^{-1} (seven specimens) for FLE, and in the range from 0.5 to 4.0 μg mL^{-1} (six specimens) for SPA. Three replicate measurements were performed at each level. The recovery was calculated by comparing the concentrations measured in plasma samples (x) with those found in the standard solutions (x'): %recovery = $(x/x') \times 100$.

The mean recovery of the extraction was found to be 95.94% for FLE and 91.75% for SPA.

Precision

The precision of the method was evaluated by repeating measurements of series of samples prepared as described above (three times in the same day and one time after three days).

The within-day repeatability, calculated as the relative standard deviation (%RSD), was changed from 0.53 to 3.60% (FLE) and from 0.78 to 4.85% (SPA). Relative standard deviation values expressed as the between day reproducibility were varied from 5.0% to 8.8% for both drugs.

In conclusion, the presented SPE of FLE and SPA from biological matrix is simple in performance and reliable for plasma sample preparation. Solid-phase extraction procedures can be recommended for HPLC bioanalysis of these fluoroquinolones.

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