*Journal of Chromatography*, 579 (1992) 285–289
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6446

# Determination of sparfloxacin in serum and urine by high-performance liquid chromatography

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(First received February 24th, 1992; revised manuscript received May 15th, 1992)

## ABSTRACT

A specific and sensitive analytical method for the determination of sparfloxacin in serum and urine is described. Serum proteins are removed by precipitation with acetonitrile after the addition of ofloxacin as an internal standard. The supernatant solvent is evaporated in a vacuum concentrator and the dry residue is redissolved in the mobile phase. Separation is performed on a cation-exchange column (Nucleosil 100 5SA, 125 × 4.0 mm I.D., 5 μm particle size) protected by a guard column (Perisorb RP-18, 30 × 4.0 mm I.D., 30–40 μm particle diameter). The mobile phase consisted of 750 ml of acetonitrile and 250 ml of 100 mmol/l phosphoric acid (v/v) to which sodium hydroxide had been added. The final concentration of sodium was 23 mmol/l and the pH was 3.82. Sparfloxacin and ofloxacin were determined by spectrofluorimetry (excitation wavelength 295 nm; emission wavelength 525 nm). The flow-rate was 1.5 ml/min and the retention times were 4.7 (sparfloxacin) and 8.0 (ofloxacin) min. Validation of the method yielded the following results for serum: detection limit 0.05 mg/l; precision between series 10.4–3.6%; recovery 99.5–100.0%; comparison with a microbiological assay $c$(bioassay) = 1.035$c$(HPLC) − 0.06. The test organism was *Bacillus subtilis* ATCC 6633. For urine the results were: detection limit 0.5 mg/l; precision between series 7.8–5.0%; recovery 97.0–97.8%; method comparison $c$(bioassay) = 1.092$c$(HPLC) − 1.09. No interferences were observed in human volunteers. The method can also be applied to stool samples.

## INTRODUCTION

Sparfloxacin (AT4140; RP64206) is a quinolone antimicrobial agent [5-amino-1-cyclopropyl-6,8-difluoro-1,4-dihydro-7-(*cis*-3,5-dimethylpiperazinyl)-4-oxoquinoline-3-carboxylic acid] (Fig. 1). The substance offers high *in vitro* activity against a broad range of pathogenic microorganisms, especially an increased activity against gram-positive organisms compared with other quinolones. So far only two high-performance liquid chromatography (HPLC) methods have been described in confidential manufacturer protocols (Dainippon Pharmaceutical Co. and

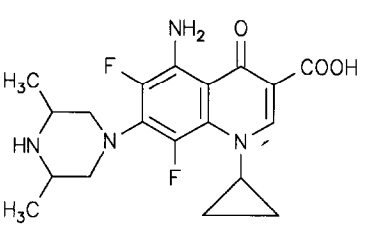


Fig. 1. Chemical structure of sparfloxacin.

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0378-4347/92/$05.00 

Rhone D.P.C. Europe), which differ from the presented method in sample pre-treatment, type of chromatography and method of detection. In this paper we describe a specific and accurate HPLC method for the determination of sparfloxacin in serum and urine.

## EXPERIMENTAL

### *Materials*

Sparfloxacin (RP46206), lot No. 89006, purity 99.8%, was kindly donated by Rhone D.P.C. Europe (Antony Cedex, France). Ofloxacin, lot No. C259/0794, was a generous gift from Hoechst (Frankfurt/Main, Germany). Acetonitrile, concentrated phosphoric acid and sodium hydroxide solution (Titrisol) were purchased from E. Merck (Darmstadt, Germany). Drug-free human serum was a generous gift of Boehringer Mannheim (Mannheim, Germany). Tridistilled water was used throughout all experiments.

### *Solutions*

The following solutions were used: 2 mol/l sodium hydroxide solution; 0.1 mol/l sodium hydroxide solution; and 0.1 mol/l phosphoric acid solution, pH 2.92. A 6.74-ml volume of concentrated phosphoric acid was mixed with 40 ml of 2 mol/l sodium hydroxide and diluted with water to 990 ml. The solution was adjusted to pH 2.92 with approximately 0.4 ml of concentrated phosphoric acid and diluted with water to 1 l.

### *Mobile phase*

Acetonitrile (750 ml) was mixed with 250 ml of 0.1 mol/l phosphoric acid solution, diluted with water to 1 l and adjusted to pH 3.82 with approximately 0.3 ml of concentrated phosphoric acid. The final sodium concentration was 23 mmol/l. The mobile phase was filtered through a 0.45-$\mu$m filter (type HVLP, Millipore, Eschborn, Germany).

### *Calibrators and control serum samples*

Serum calibrators and serum samples for assessment of accuracy were prepared by spiking human drug-free serum. Sparfloxacin was dissolved in 1 mmol/l sodium hydroxide solution and appropriately diluted with 0.1 mol/l phosphoric acid solution. Serum calibrators were prepared by mixing four volumes of serum and one volume of dilute sparfloxacin solution. Calibrators for the assay of urine and stools were dilutions of sparfloxacin in 0.1 mol/l phosphoric acid solution. For the control of precision, assayed samples (serum, urine or stool extracts) from volunteers were pooled. Control samples were stored at $-20$ or $-80$°C.

### *Internal standard solution (0.05 mg/l ofloxacin)*

A stock solution of 100 mg/l ofloxacin was prepared by dissolving 25 mg of ofloxacin in 250 ml of water and 5 $\mu$l of concentrated phosphoric acid. Dissolution was enhanced by ultrasound if necessary. The stock solution was further diluted with water (1:200, v/v) and stored in brown Eppendorf vessels at $-20$°C (concentration 0.5 mg/l). For daily use this solution was diluted 1:10 (v/v). All solutions of sparfloxacin and ofloxacin were protected against light during manipulations.

### *Samples and storage*

Serum, urine and stools were stored at $-80$°C immediately after collection. Care was taken against too much exposure to light.

### *Instrumentation*

The mobile phase delivery system (Model LC 2/2, Perkin-Elmer, Überlingen, Germany) was set at a flow-rate of 1.5 ml/min yielding a pressure of 10 MPa. The autoinjector (Model ISS-101, Perkin-Elmer) used a needle wash solution of acetonitrile–water (1:1). The injection volume was 25 $\mu$l for serum and stools and 50 $\mu$l for urine. The sample tray was kept at 20°C. A guard column filled with Perisorb RP-18 (E. Merck; 30 × 4.0 mm I.D.; particle size 30–40 $\mu$m) protected the main column. Separation was performed on a column of Nucleosil 100 SA (Macherey–Nagel, Düren, Germany; 125 × 4.0 mm I.D.; particle size 5 $\mu$m; temperature 20–22°C). A fluorescence detector (Model RF 535, Shimadzu, Duisburg, Germany) was used for sample detection with the

following settings: excitation, 295 nm; emission, 525 nm; high sensitivity; reponse factor slow. An integrator (Model 3390A, Hewlett-Packard, Bad Homburg, Germany) was used for data collection. Calculation of concentrations was performed by means of peak areas for urine and stools and by peak area ratios for serum. The retention time of sparfloxacin was 4.7 min and of ofloxacin 8.0 min.

*Sample preparation*

*Serum.* To 0.50 ml of serum, serum blank or serum calibrator 0.10 ml of internal standard solution and 1.00 ml of acetonitrile were added in a 2-ml Eppendorf vessel. The mixture was agitated for 30 s with a mechanical shaker and centrifuged for 5 min at 10 000 *g*. A 1.50-ml volume of clear supernatant was transferred into a glass tube (75 × 12 mm). The liquid phase was evaported at 40°C in a vacuum concentrator (CON-1000 vacuum concentrator, Fröbel, Lindau, Germany). The run time was 120 min. The solid residue was redissolved in 0.3 ml of mobile phase. The final solution was transferred into the autosampler vessels and injected.

*Urine.* One aliquot of urine or urine calibrator was diluted with four aliquots of phosphoric acid solution, 0.1 mol/, pH 2.92. The diluted solution was transferred into the autosampler vials and injected.

*Stool.* A 0.1-ml volume of urine calibrator was diluted with 9.0 ml of mobile phase. Weighted samples of 100 mg of stools were suspended in 9.0 ml of mobile phase in a 15-ml glass tube. The suspension was shaken for 15 min and thereafter centrifuged for 10 min at 1500 *g*. The clear supernatant was decanted into another glass tube. The residue was resuspended in 9.0 ml of mobile phase, shaken, centrifuged and decanted as before. The extraction of the stool sample was repeated once more. Each extract was transferred into a separate autosampler vessel and injected. For the calculation of stool concentrations the sum of all three extracts was used. The specific density of the stool was assumed to be nearly equal to 1.00 kg/l.

*Stability of the analyte*

Samples of serum and urine were stable at −20 or −80°C for 6 months if not exposed to daylight or neon light. Calibrator solutions for serum and urine were stable for at least 2 months under the specified conditions. Diluted calibrator solutions were stable at −20°C for at least 5 days. A urine sample exposed to sunlight at 26°C for 24 h lost 14% of the concentration of the drug and yielded abnormal chromatographic peaks. A complete series of extracted serum calibrators was stable at 4°C for 24 h and it was also stable at 20°C for 14 h, which was the longest storage time in the autosampler.

*Comparative microbiological assay*

Serum and urine samples were also analysed by an agar plate diffusion assay [1]. The test organism was *Bacillus subtilis* ATCC 6633. All assays were performed in triplicate.

*Statistical methods*

Results of the microbiological assay and determination by HPLC were evaluated by the method of bivariate regression analysis [2].

## RESULTS

*Method development*

Separations of sparfloxacin from serum, urine and stools of healthy volunteers were achieved by cation-exchange chromatography combined with

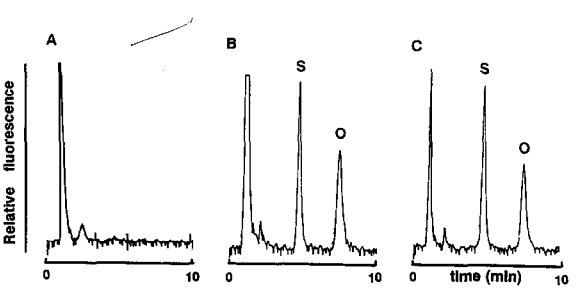


Fig. 2. Chromatograms of (A) blank serum, (B) serum spiked with 1 mg/l sparfloxacin and (C) chromatogram of a volunteer's serum sample containing 1.16 mg/l sparfloxacin 4 h after treatment with 400 mg sparfloxacin by mouth. Peaks: S = sparfloxacin, O = ofloxacin (internal standard).

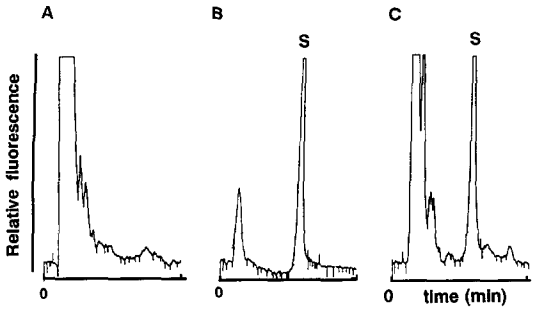


Fig. 3. Chromatograms of (A) blank urine, (B) blank urine spiked with 12.5 mg/l sparfloxacin (S) and (C) a volunteer's urine sample containing 15.0 mg/l sparfloxacin. Collection period 0–6 h after 200 mg of sparfloxacin by mouth.

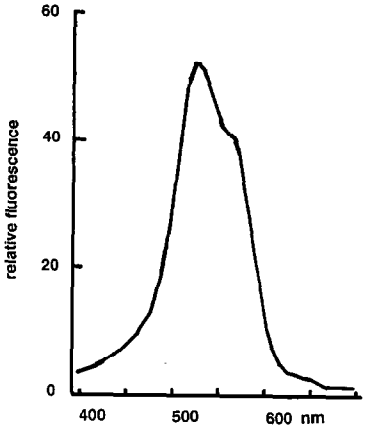


Fig. 5. Uncorrected emission spectrum of 20 mg/l sparfloxacin in mobile phase. Excitation wavelength 290 nm.

reversed-phase chromatography. Typical chromatograms are shown in Figs 2–4. As in classical ion-exchange chromatography the retention time depended on the concentration of the cation in the mobile phase. Furthermore the pH of the sample and mobile phase required careful adjustment. The fluorescence spectrum of sparfloxacin showed a maximum emission at 525 nm when dissolved in the mobile phase (*cf.* Fig. 5).

Absolute recovery of the analyte from serum was 97.8% ($n$ = 6). Extraction rates from the stool samples were 82, 17 and 1%. Thus stool samples should be extracted at least twice.

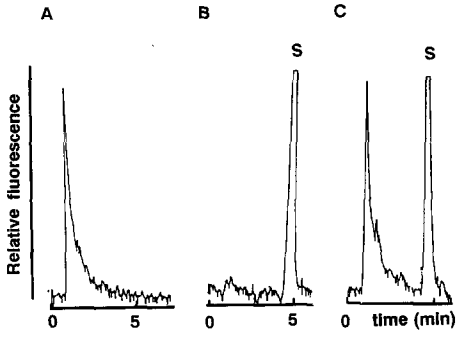


Fig. 4. Chromatograms of (A) a blank stool sample, (B) an aqueous standard containing 5.0 mg/l sparfloxacin (S) and (C) a stool extract containing 3.85 mg/l sparfloxacin. Collection period 0–24 h after 400 mg of sparfloxacin by mouth. Second extract (see under Experimental).

*Method validation*

Concentration *versus* peak area curves were linear in the following range: for serum, 0.1–2.0 mg/l; urine, 1.56–50.0 mg/l; stool, 0.5–20.0 mg/l. Detection limits were calculated from the variation of six calibration graphs: serum, 0.05 mg/l; urine, 0.46 mg/l; stools, 0.14 mg/l.

For the measurement of imprecision pooled samples were used. The results are summarized in Table I. For the three matrices imprecision, ex-

TABLE I

RESULTS OF IMPRECISION

| Matrix | $n$ | Concentration (mg/l) | Coefficient of variation (%) |
|---|---|---|---|
| *Within-series* | | | |
| Serum | 10 | 0.55 | 5.0 |
| | 10 | 1.50 | 2.7 |
| Urine | 10 | 6.00 | 5.8 |
| | 10 | 13.00 | 3.4 |
| | 10 | 20.00 | 4.3 |
| Stool | 10 | 1.02 | 4.6 |
| | 10 | 4.67 | 2.6 |
| | 8 | 9.84 | 2.9 |
| *Between-series* | | | |
| Serum | 15 | 0.30 | 10.4 |
| | 16 | 0.91 | 3.6 |
| Urine | 22 | 6.40 | 7.8 |
| | 22 | 20.60 | 5.0 |
| Stool | 4 | 0.85 | 2.4 |
| | 4 | 4.43 | 4.7 |

TABLE II

ACCURACY RESULTS

| Matrix | $n$ | Spiked value (mg/l) | Recovery (mean ± S.D.) (%) |
|---|---|---|---|
| Serum | 10 | 0.55 | 100.0 ± 5.0 |
| | 8 | 1.00 | 99.5 ± 3.1 |
| | 10 | 1.50 | 99.5 ± 2.7 |
| Urine | 10 | 6.00 | 97.0 ± 3.3 |
| | 10 | 13.00 | 97.8 ± 3.4 |
| Stool | 8 | 10.00 | 98.0 ± 2.9 |

pressed as the coefficient of variation, ranged within series from 2.6 to 5.8% and between series from 2.4 to 10.4%.

Accuracy was determined by spiking blank matrices from healthy volunteers. Results are given in detail in Table II. Mean recovery rates varied from 97.0 to 100.0%.

No endogenous interferences were observed in serum, urine, or stools from healthy volunteers.

Results obtained by HPLC were compared with results obtained by a microbiological assay. Data of bivariate regression analysis are given in Table III. Although there was a small significant difference between the HPLC and microbiological assay results from serum it is not considered relevant. No significant differences between HPLC and microbiological results from urine were observed.

## DISCUSSION

At the time of this practical work on sparfloxacin no published HPLC method was available. Two internal methods developed by the manufacturers differed in sample preparation, the separation principle, detection method and internal standard [3,4]. Precipitation of serum proteins and subsequent removal of the liquid phase by a vacuum concentrator was very efficient with an absolute recovery rate of 98% after a single extraction. Only stool samples required at least two extractions.

TABLE III

METHOD COMPARISON: HPLC ($x$) VERSUS MICROBIOLOGICAL ASSAY ($y$) RESULTS

| Matrix | $n$ | Slope $b$ | Intercept $a$ (mg/l) | $p$ Value |
|---|---|---|---|---|
| Serum | 71 | 1.035 | −0.06 | <0.01 ***[a] |
| Urine | 48 | 1.092 | −1.09 | >0.20 n.s. |

[a] Difference significant.

Sparfloxacin is a polar substance that can be separated from endogenous and exogenous compounds by a cation-exchange column. The benefit of the use of a reversed-phase guard column of low capacity was an empirical finding. Similar conditions of chromatography have been successfully used for the separation of ofloxacin and fleroxacin from their metabolites [5]. Detection by fluorescence proved more specific than by UV absorbance. Ofloxacin was a suitable internal standard although its maximum fluorescence emission differs from sparfloxacin (480 *versus* 525 nm). The main metabolite of sparfloxacin, most likely a glucuronide, does not fluoresce, which is a minor drawback of the method presented. The sensitivity of the fluorescence detector is slightly higher than that of a comparative UV absorbance detector. Further results of validation and method comparison with a microbiological assay are considered acceptable for serum and urine. The method has not been fully evaluated for stool samples. Existing data of validation suggest that it may also be suitable for stools.

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