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High performance thin-layer chromatographic method for the determination of sparfloxacin in human plasma and its use in pharmacokinetic studies

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Abstract

A rapid and sensitive high-performance thin-layer chromatographic (HPTLC) method has been developed for the measurement of sparfloxacin in human plasma and its use for pharmacokinetic study has been evaluated. Detection and quantitation were performed without using an internal standard. A single stage extraction procedure was followed for extracting sparfloxacin from plasma and a known amount of the extract was spotted on precoated silica gel 60 F254 plates using a Camag Linomat IV autosampler. Sparfloxacin was quantified using a Camag TLC Scanner 3. The recovery study of authentic analytes added to plasma at 0.1 to 0.8 μg ml^{-1} was $94.9 \pm 0.98\%$ and the lowest amount of sparfloxacin that could be detected was 50 ng ml^{-1} plasma. The method provides a direct estimate of the amount of sparfloxacin present in plasma. The method was used for the determination of plasma levels as well as pharmacokinetic parameters of sparfloxacin after oral administration of two marketed preparations to healthy volunteers.

Keywords: Sparfloxacin; High-performance thin-layer chromatography; Pharmacokinetic

1. Introduction

Sparfloxacin (5-amino-1-cyclopropyl-6,8-difluoro 1,4-dihydro-7-(*cis*-3,5-dimethyl-1-piperazinyl)-4-oxoquinoline-3-carboxylic acid; AT 4140, RP 64206) (Fig. 1) [1], is a new fluoroquinolone with a broad antibacterial activity against gram-positive and gram-negative bacteria, glucose nonfermenters, anaerobes and species of *Mycoplasma*, *Chlamydia* and *Mycobacterium* spp. [2–5]. Its pharmacokinetics in animals are characterized by a high degree of tissue distribution, with the exception of the central nervous system and adipose tissue and a long elimination half life ($t1/2$). In all species tested, including man, the plasma protein binding (mainly to albumin) is weak (45–46%) and only one inactive metabolite, the acylglucuronide of sparfloxacin, has been found [6,7].

Two assay systems have been established for the routine determination of sparfloxacin concentrations in various biological fluids: a microbio-

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logical agar well method and a high performance liquid chromatographic (HPLC) method. A microbiological method of sparfloxacin determination has been reported using Molten assay agar with *E. coli* as an indicator organism [8]. The HPLC method reported earlier required a semi-automated solid phase extraction from plasma with the use of internal standard and showed a recovery of $83.3 \pm 2.3\%$ of sparfloxacin which could be improved [9,10]. Moreover the method also seems to be time consuming and expensive.

This paper describes a reliable and selective high-performance thin-layer chromatographic (HPTLC) method which enables the determination of plasma concentration of sparfloxacin when the drug was given orally in dose of 200 mg to healthy volunteers. The method was also used to obtain comparative pharmacokinetic information about the drug in healthy volunteers.

2. Experimental

2.1. Chemicals

A reference standard of sparfloxacin was obtained from Hetero Drugs, Hyderabad, India. Two marketed formulations (A and B) of 200 mg sparfloxacin tablets were used for comparative pharmacokinetic studies. Acetate buffer (pH 3.5) and dichloromethane (analytical grade) were used for extractions. Chloroform, methanol and formic acid (analytical grade) and water were used for developing TLC plates (Silica gel 60 F_{254}, Art 5554, DC-Alufolien, Kieselgel 60 F_{254}, Merck, Darmstadt, Germany).

2.2. Preparation of standards

A stock solution of sparfloxacin was prepared in methanol at a concentration of 0.1 mg ml^{-1}. Standard solutions were obtained by diluting the stock solutions to concentrations ranging from 1 to 25 μg ml^{-1}.

2.3. Preparation of plasma samples

In a 15-ml graduated glass centrifuge tube, sparfloxacin working standard (20 μg ml^{-1}) was added in volumes of 0, 5, 10, 20, 25 and 40 μl to 1 ml of drug-free plasma to provide calibration standards of 0 (no sparfloxacin added), 100, 200, 400, 500 and 800 ng. Each test sample was mixed with 400 μl of acetate buffer (pH 3.5) by vigorous vortex mixing for 2 min and was extracted with 2×3 ml of dichloromethane on a vortex-mixer for 2 min and centrifuged at $700 \times g$ for 10 min. The combined dichloromethane extract was evaporated to dryness at 45°C in a water bath. Unknown plasma samples were prepared in an identical manner except for the addition of sparfloxacin.

2.4. Instrumentation and chromatoraphic conditions

All residues were redissolved in 100 μl of dichloromethane by vigorous vortex-mixing and 20 μl aliquots of the samples were spotted onto TLC plates with the help of a Camag Linomat IV autosampler. Sparfloxacin (50 ng, 100 ng) reference standards were separately spotted on each TLC plate, as the external standard. The TLC plates were developed (10 cm) in a Camag Twin Trough glass chamber with a solvent system consisting of chloroform:methanol:formic acid:water

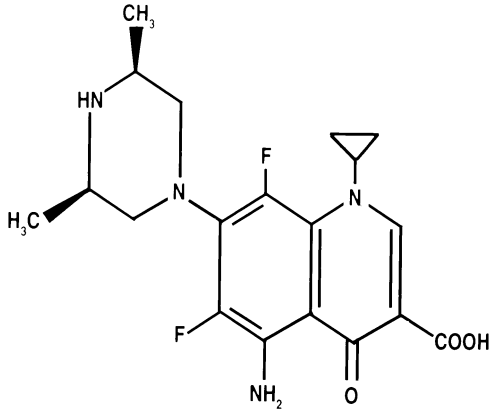

Fig. 1. Chemical structure of sparfloxacin.

(35:4:2:0.25, v/v/v/v), in which the drug had a R_f value of 0.20 ± 0.05 and was separated from other components in plasma. The TLC plates were dried completely, using a hot air drier, after development. Determination of sparfloxacin was done by scanning the fluorescent spots of sparfloxacin (wavelength, 365 nm) on TLC plates with the help of a Camag TLC Scanner 3.

2.5. Quantitation

The quantitation of the chromatograms was performed using the ratio of the peak area of the unknown to that of a standard. A representative standard curve of sparfloxacin was obtained by plotting the area under the peak of sparfloxacin against the concentration over the range of 10–200 ng. The minimum quantifiable concentration of sparfloxacin in human plasma samples was 50 ng ml^{-1}.

2.6. Validation of the method

The recovery of sparfloxacin from plasma was determined by comparing peak areas obtained from plasma to which sparfloxacin (100, 200, 400, 500 and 800 ng) had been added with that of the peak areas obtained from standards. The intra-day precision (random analytical variation) was evaluated by analyzing drug-free plasma samples, to which sparfloxacin had been added at concentrations of 100, 400 and 800 ng, in triplicate. The inter-day precision was determined by analyzing 200, 400 and 500 ng standards simultaneously with the plasma from subjects daily for 5 days. The linearity of the detector response was tested by spotting standards (in triplicate) for each concentration over the range 10–200 ng.

2.7. Pharmacokinetics

Nine healthy male human volunteers were selected for the study after giving written informed consent and having normal biochemical parameters. Subjects were aged 29.77 ± 2.20 years (mean $\pm$ S.D., range 23–42), with a mean body weight of 56.2 ± 2.38 kg (range 44.0–68.0). The study protocol was approved by the Local Ethics Committee of Cadila Pharmaceuticals, Research and Development Unit. Two formulations of sparfloxacin tablets (200 mg) (product A and product B) were selected for the study. One tablet of either brand was administered to each volunteer with 200 ml of water, such that half of the number of volunteers received product A and the rest received product B, in the first part of a single blind cross-over study. The second part of the study was carried out after 7 days. Identical conditions were maintained on both occasions.

Blood samples, obtained from an antecubetal vein prior to dosing and at 1.0, 2,0, 3.0, 4.0, 6.0, 8.0, 12.0 and 24.0 h after dosing, were placed in heparinised tubes. The samples were immediately centrifuged at $1000 \times g$ for 20 min and the plasma samples were separated and frozen at -20°C. until analysis.

Pharmacokinetic parameters were calculated using a model independent method [11]. The peak level (C_{max}) and the time taken to reach peak level (t_{max}) were observed data. The elimination rate constant (Kel) and the terminal elimination half-life ($t1/2$) were estimated by linear regression of the terminal part of the log concentration–time curve. The area under the plasma concentration–time curve (AUC) was determined by the linear trapezoidal rule and extrapolated to infinity ($AUC_{0\rightarrow\infty}$) by dividing the last measurable concentration by the elimination rate constant.

The pharmacokinetic parameters for C_{max}, $AUC_{0\rightarrow\infty}$ and $t1/2$ were compared using analysis of variance and the results are given as the mean value $\pm$ S.E.M. The Wilcoxon test was used to compare t_{max} values. Statistical significance was defined at the $P < 0.05$ level.

3. Results

The peak area was observed to be dependent on the amount of the standard, sparfloxacin and a linear relationship ($r = 0.999$) was found between the peak areas of sparfloxacin at various concentrations over the range 10–200 ng. The solvent system used for development of the plates produced no interference peaks in the area under the curve and all other compounds were distinctly

Table 1
Accuracy and precision of a HPTLC method for the determination of sparfloxacin in plasma

Concentration added (ng ml^{-1})	Concentration detected (mean ± S.D., $n = 5$) (ng ml^{-1})	RSD[a] (%)	Accuracy[b] (%)
100	94.11 ± 1.22	1.30	99.15
200	189.91 ± 2.38	1.26	100.05
400	377.39 ± 4.64	1.23	99.41
500	483.99 ± 5.86	1.21	101.99
800	754.76 ± 9.28	1.23	99.41

[a] Relative standard deviation.
[b] After correction for recovery.

separated. The R_f value of sparfloxacin under the conditions used was found to be 0.20 ± 0.05 and spots were quantified at a wavelength of 365 nm. The accuracy, precision and reliability of the procedure were ascertained by adding known concentrations of drug to drug-free plasma and analyzing five samples of each concentration by the method described for extraction (Table 1). The recovery of sparfloxacin in the extraction procedure from 1 ml of plasma was found to be 94.9 ± 0.98% ($n = 5$). The intra-day and inter-day precisions are given in Table 2.

In order to verify the applicability of this method, the bioequivalence study with two marketed sparfloxacin tablet preparations was conducted in healthy volunteers at a dose of 200 mg. The mean plasma concentration of sparfloxacin at various time points after administration of drug is shown in Fig. 2. The mean maximum concentration (C_{max}) and the mean area under the plasma concentration curve ($AUC_{0\rightarrow\infty}$) for both preparations were comparable and were not significantly different (Table 3). The t_{max} for both preparations ranged between 2 and 6 h. The pharmacokinetic parameters were subject to two-way analysis of variance. The result indicates that the pharmacokinetic profiles of the two formulations are identical and the bioavailability is not significantly different at $P < 0.05$, indicating the bioequivalence of the two products. Considering product A as standard formulation, product B has 105.42% bioavailability. The spot of sparfloxacin in plasma is distinctly separated and, in volunteers, plasma is clearly identified in comparison with blank plasma (Fig. 3). Both the products were analyzed for sparfloxacin content using the proposed technique. The R_f values were found to be the same for tablets and standard sparfloxacin and there

Table 2
Precision data of the HPTLC assay for sparfloxacin

Concentration added (ng)	Peak area[a] (mean ± S.D.)	RSD (%)
Inter-day		
200	5509.93 ± 70.40	1.28
400	11130.00 ± 134.35	1.21
800	21907.75 ± 269.29	1.23
Intra-day		
100	2731.50 ± 35.44	1.30
400	10951.48 ± 138.11	1.26
500	14048.30 ± 169.99	1.21

[a] Calculated for total concentration (integrated value).

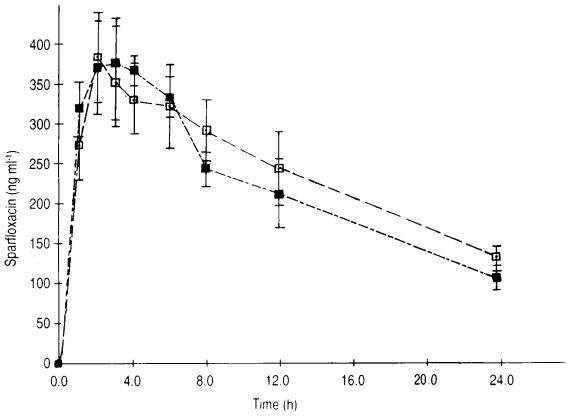

Fig. 2. Plasma concentration after oral administration of 200 mg sparfloxacin as two different formulations (product A, ■; product B, □). Each point represents the mean ± S.E.M. ($n = 9$, cross-over design).

Table 3
Pharmacokinetic parameters of sparfloxacin (200 mg) in human volunteers

Parameter	Product A ($n=9$)	Product B ($n=9$)	P value
C_{max} (ng ml^{-1})	461.3 ± 66.11	489.69 ± 69.93	NS
t_{max} (h)	3.0 ± 0.47	3.33 ± 0.57	NS
$AUC_{0\rightarrow t}$ (ng.h ml^{-1})	5436.02 ± 552.32	5985.39 ± 663.02	NS
$AUC_{0\rightarrow\infty}$ (ng.h ml^{-1})	8538.74 ± 1481.85	9002.12 ± 1091.88	NS
Kel (h^{-1})	0.0522 ± 0.0073	0.0552 ± 0.0065	NS
t1/2B (h)	16.57 ± 4.22	14.31 ± 1.9	NS

Data obtained by cross-over design and presented as mean ± S.E.M.
n, number of volunteers.
NS, not significant ($P<0.05$).

was no interference from the excipients. The contents of sparfloxacin were found to be 98.9 ± 2.4 and 99.2 ± 2.2%, respectively, for products A and B.

4. Discussion

The proposed HPTLC method can measure the plasma concentration of sparfloxacin at a dose of 200 mg per oral administration. By this established method, the plasma concentration of sparfloxacin reached a maximum 2–6 h after administration and disappeared after 24 h (Fig. 2). This trend is very much in agreement with the HPLC method reported earlier [10]. It seems that the limit of detection is indeed lower in HPLC analysis (15 ng ml^{-1}), using 1-ethyl 6-chloro 1,4-dihydro 7-(4-methyl 1-piperazinyl)4-oxo-quinoleine 3-carboxylic acid, whereas, in the proposed HPTLC analysis, the limit of detection is 50 ng ml^{-1}, without using any internal standard. However, the recovery and relative standard deviation (RSD) are improved in the proposed HPTLC analysis, compared to the reported HPLC method. The recovery of sparfloxacin by the proposed HPTLC analysis was found to be 94.9 ± 0.98%, compared to 83.3 ± 2.3%, as reported for HPLC analysis.

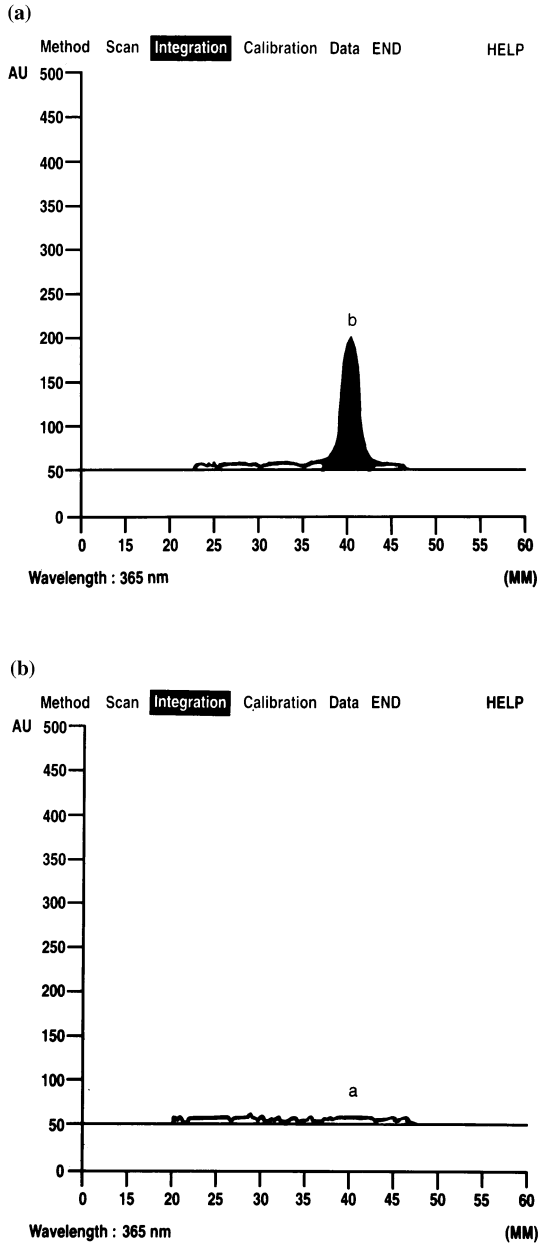

Fig. 3. Chromatograms of: (a) blank plasma (b) plasma samples (collected at 2 h) of healthy volunteer having received sparfloxacin 200 mg orally.

The RSD was found to be much less using the proposed HPTLC analysis ($< 1.30\%$). This method can also be used to accurately determine sparfloxacin in tablets without interference from the excipients.

5. Conclusions

The proposed HPTLC method for the estimation of sparfloxacin in plasma has certain advantages over other reported methods. For example: (1) It gives a clear picture of the total drug present after absorption and thus has direct clinical relevance; (2) It is economical and faster than previously published methods. On a single plate, at least 10–12 samples can be analyzed in 4–5 h; (3) Unlike earlier methods, this method does not require an internal standard and quantification can be done using reference drug as the external standard; (4) The recovery of the drug is improved compared with the HPLC method $94.91 \pm 0.98\%$; (5) The method described is a sensitive and specific assay for sparfloxacin in plasma and is suitable for pharmacokinetic studies after therapeutic doses.

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