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Sparfloxacin determination in bulk materials, pharmaceutical formulations and human serum by reverse phase HPLC method

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A simple reverse phase high-performance liquid chromatography (HPLC) method was developed and validated for the quantitative determination of sparfloxacin (SPFX) in the bulk material, pharmaceutical formulation and human serum. Purospher Start C_{18} (25 cm × 4.6 mm, 5 µm) and Discovery C_{18} (25 cm × 4.6 mm, 5 µm) columns were used. The mobile phase, methanol, water and acetonitril (60:30:10 v/v/v pH 2.70 adjusted by phosphoric acid), was delivered at a flow rate of 1.0 mL min^{-1}, eluent was monitored by ultra-violet (UV) detector at 290 nm. Gemifloxacin (GFX) was used as an internal standard. The proposed method is specific, accurate (98.11 to 102.83%), precise (intra and inter-day variations were 0.108 to 0.712% and 0.013 to 0.575%) and linearity was within the desired ranges of 2.5 to 100 µg mL^{-1} concentration having r^2 > 0.998. All the results were correlated through analysis of variance (ANOVA) and Student's *t*-test. The limit of detection (LOD) and limit of quantification (LOQ) were 0.0009 to 0.0064 and 0.0028-0.0196 µg mL^{-1}, respectively. This method is not only applicable to routine analysis of SPFX in bulk and pharmaceutical formulations but as well as on human serum samples.

Key words: Sparfloxacin, reverse phase high-performance liquid chromatography (RP-HPLC), serum, analysis of variance (ANOVA), student's *t*-test.

INTRODUCTION

The fluroquinolones are advanced class of synthetic antibiotics having a fluorine atom attached to the central ring system, typically at the 6-position or C-7 position (Nelson et al., 2007; Ivanovo and Budanov, 2006). These agents have broad antibacterial activity for the treatment of a wide variety of infectious diseases (Goodman et al., 2001). Sparfloxacin (SPFX) (Figure 1) is an orally active synthetic broad spectrum third generation quinolone, characterized by good to excellent activity against Gram positive cocci (notably *Streptococcus pneumoniae*) and anaerobes and atypical pathogens. It is also moderately active against some (*Bacteroides fragilis* group) L. monocytogenes resistant (Francis et al., 1997; Andersson and MacGowan, 2003; Barrett et al., 1991; Crumplin, 1988; Sultana et al., 2010).

Literature survey revealed that few analytical methods have been developed for estimation of SPFX in bulk and pharmaceutical dosage form. Akram M. El-Didamony developed fluorescence probe enhanced spectrofluorimetric method for the determination of sparfloxacin in tablets and biological fluids and spectrophotometric determination method of sparfloxacin in pharmaceutical

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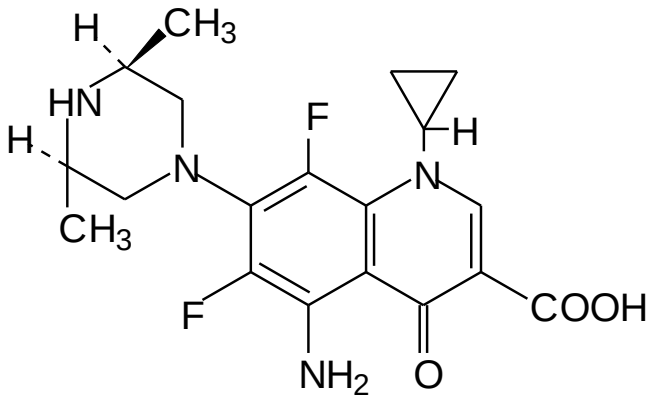

Figure 1. Structure of sparfloxacin.

preparations by ternary complex formation with Pd (II) and eosin (El-Didamony, 2007, 2010). CHO Hea-Young and colleagues developed an high-performance liquid chromatography (HPLC) method for quantitation of SPFX in human serum (Hea-Young et al., 2006) while Argekar and Shah, (1999), Marona and Schapoval, (1999) and Nurun Nahar Rahman and Ahmad, (2007) developed methods for marketed products and stability testing. Another HPLC method was reported for simultaneous determination of sparfloxacin, gatifloxacin and moxifloxacin using levofloxacin as internal standard by Srinivas et al. (2008). Degradation products studies by HPLC have been carried out by Marona et al.**,** (1999).

Almost all previously reported methods were either for determination of sparfloxacin in dosage form or either in serum plasma but there is no efficient, reliable and precise method for determination of SPFX in bulk, dosage form and human serum together. Here, we report a simple, easy, quick and inexpensive isocratic RP-HPLC method with ultraviolet detection at 290 nm for the determination of SPFX in bulk, dosage form as well as in human serum using gemifloxacin (GFX) as internal standard (IS). Low limit of detection (LOD) and limit of quantification (LOQ) values also merit this method for the determination of sparfloxacin in clinical samples. Moreover, this method was further applied for determination of SPFX in three different marketed formulations.

Aim of the study

The aim of the present study was to establish an efficient, reliable, accurate, sensitive and reproducible method for the quantitative determination of SPFX in bulk, pharmaceutical formulations and human serum samples. As this would allow more efficient generation of quantifiable data and could be performed at more diffident cost.

EXPERIMENTALS

Materials and reagents

Standard bulk drug sample of sparfloxacin were supplied by Abott Pharmaceuticals Pakistan (pvt). Gemifloxacin used as internal standard (IS, Figure 2), was obtained from PharmEvo (Pvt) Ltd, Pakistan; three different formulations of sparfloxacin were used including sparaxin (Abott Pharmaceuticals Pakistan (Pvt)), quspar (100 mg) Schazoo Zaka (Pvt.) Ltd., sparkure (100 mg) Elko organization (Pvt) Ltd. HPLC grade acetonitrile and methanol were obtained from Merck Schuchardt OHG, Darmstadt, Germany.

Softwares

Standard regression curve analysis was performed by use of STATISTICA version 7.0 (USA), without forcing through zero. Linearity graphs were obtained by use of Micro-soft Excel 2007 software. Statistical package for social sciences (SPSS) software version 10.0 (Carry, NC, USA) was used for the calculation of means, standard deviations, homoscedasticity of the calibration plots, analysis of variance (ANOVA) and Student*'s t*-test.

Instrumentation

HPLC system equipped with Shimadzu LC-20 AT VP Pump, SPD-20AV VP Shimadzu UV visible detectors and second HPLC system consisted of an LC-10 AT VP Shimadzu pump, SPD-10AV VP Shimadzu UV visible detector, both connected by CBM-102 communication Bus Module Shimadzu to Intel Pentium 4 machine with Shimadzu CLASS-GC10 software (Version 5.03) and Rheodyne manual injector fitted with a 20 µl loop. Separation was achieved on a Hiber, RT, Purospher STAR C_{18} (25 cm × 4.6 mm, 5 µm) (Merck, Germany) and Discovery C_{18} (25 cm × 4.6 mm, 5 µm) (Supelco, USA). The chromatographic analysis was integrated using a Mobile phase which was sonicated by DGU-14 AM on-line degasser, and filtered through 0.45-micron membrane filter, calibrated Pyrex glassware was used for the solution and mobile phase preparation.

Preparation of solutions and quality control samples

Standard solutions of SPFX and GFX (100 ppm in100 ml) were prepared using mobile phase as solvent. Working solutions were prepared separately by making serial dilutions from the standard solution to obtain concentration between 2.5 to 100 µg mL^{-1} for SPFX and IS (GFX). For quality control (QC) samples, twenty tablets of each formulation were powdered finely and an amount equivalent to 10 mg of SPFX was weighed and then dissolved in the mobile phase. Solutions with high, medium and low concentrations that is; 8, 10 and 12 µg mL^{-1} were prepared, then filtered through a 0.45 µm Millipore filter in order to separate out the insoluble excipients by the same procedure as calibration standards but using different stock solutions. All these solutions and QC samples were stored at 20°C. Once prepared, analyzed daily for inter and intra-day variations of the method, 20 µl of these solutions were injected into LC system and chromatographs were observed.

Procedure for human serum sample

Plasma sample obtained from healthy volunteers were collected and stored at -20°C. Then, 1.0 ml of frozen plasma was mixed with

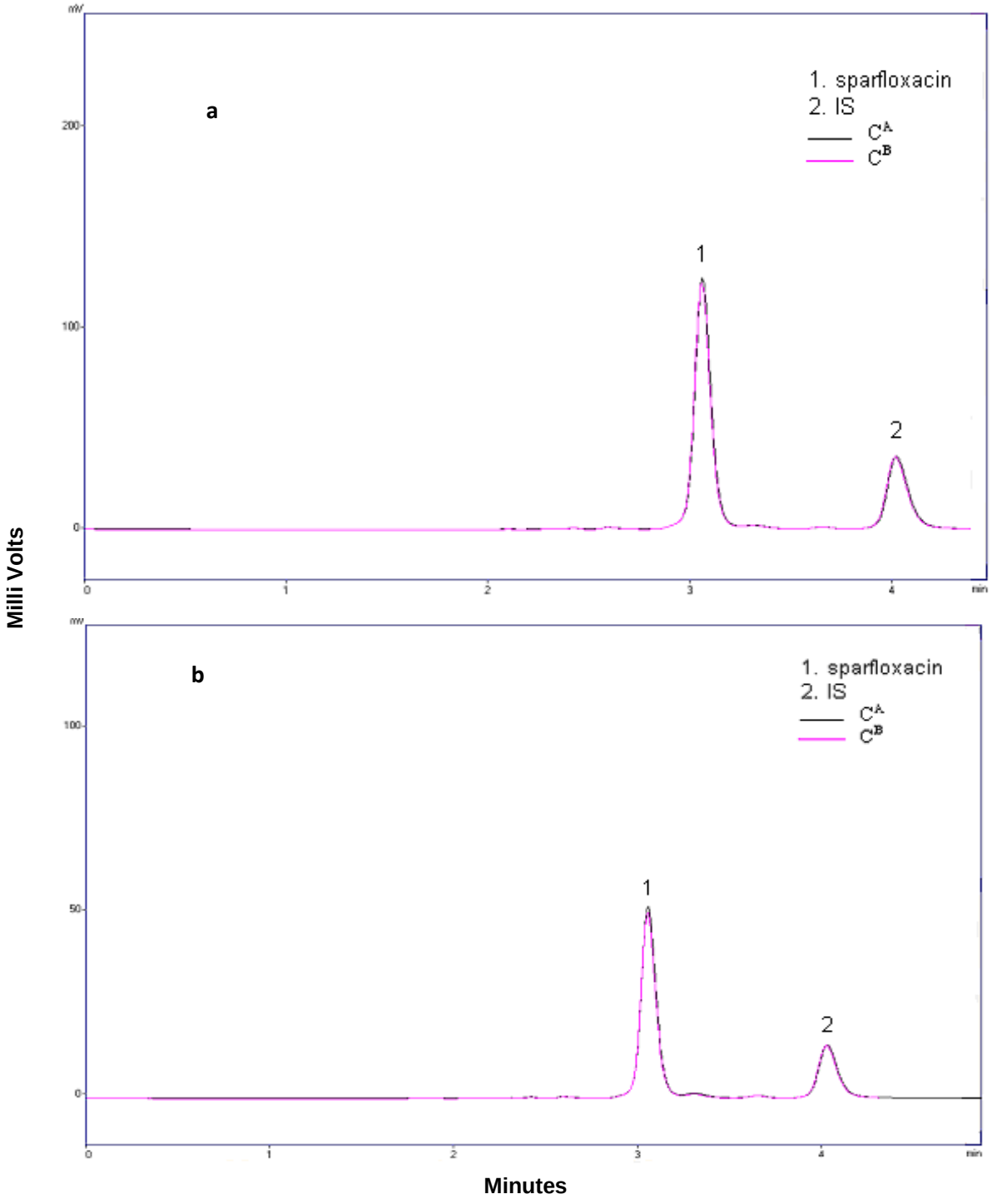

Figure 2. (a) Representative chromatogram of SPFX and IS at 290 nm using System LC 20; (b) representative chromatogram of SPFX and IS at 290 nm using System LC 10.

10 ml of acetonitrile. The mixture was vortexed for one minute and then centrifuged for 10 min at 10,000 rpm and the supernatant was prepared by filtration (0.45 µ pore size membrane filter). An aliquot serum sample was fortified with SPFX to get final concentrations of 2.5 to 100 µg mL^{-1}.

RESULTS AND DISCUSSION

Presented work has been designed to develop a simple, isocratic, precise, accurate and sensitive HPLC method

with UV detection for sparfloxacin determination in bulk, dosage form and in human serum samples. Moreover, the developed method has been applied for quantification of SPFX in its different marketed brands.

Method optimization and chromatographic conditions

For selection of optimal chromatographic conditions, different C_{18} stationary phases have been tried. Best separation, adequate resolution, short retention time and symmetric peak of SPX and IS were achieved by two difference columns which were C_{18} Hiber RT 250-4.6 Purospher STAR RP-18 (25 cm × 4.6 mm, 5 µm) (Merck, Germany) and Discovery C_{18} (25 cm × 4.6 mm, 5 µm) (Supelco, USA). Appropriate wavelength was investigated for determination of SPFX and IS by scanning solution of both drugs on UV-visible spectrophotometer. It was observed that the maximum absorbance of drug was obtained at 290 nm (Figure 3). Same solution was injected in HPLC at 230, 260 and 290 nm. At 290 nm, SPFX showed maximum absorbance. At 230 nm, both SPFX and GFX (IS) showed absorbance but peaks of GFX was not so prominent. While at 260 nm, GFX showed good absorbance but SPFX absorbance became low. Initially, methanol and water were tried in the ratio of 80:20 (v/v), as a result, SPFX and IS did not separate properly, so the above mobile phase was varied as 70:30 (v/v), both drugs separated but the peak was not symmetrical. For best response, acetonitrile was added to the mobile phase and final mobile phase was composed of methanol/water/ acetonitrile in the ratio of 60:30:10, v/v/v at which both drugs showed good resolution with typical peak nature and symmetry. To select the optimum mobile phase, pH range 2.5 to 4.0 were investigated, excellent performance was achieved at pH 2.75 adjusted with phosphoric acid. Total run time was 7 min; short analysis times are essential for routine analysis.

Effect of pH on mobile phase was also studied in the range of 2.5 to 4.0 adjusted with phosphoric acid. The pH of mobile phase had a little impact on resolution and the best separations were observed at 2.70. Retention time of SPFX was 3.6 ± 0.2 and 6.0 ± 0.2 min for IS, at a flow rate of 1.0 ml min^{-1}. The specificity of the method was established through the study of resolution factor of sparfloxacin peak. Peaks were identified using retention times, after injecting separately.

Method validation

The developed method was validated by various parameters which include system suitability, selectivity, specificity, linearity, accuracy test, precision, robustness, ruggedness, sensitivity, limit of detection and quantification, according to International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use, ICH (2005) and USP (2005).

System suitability

It is an essential component of method validation to make certain that the operational system is running appropriately throughout the analysis (Shabir, 2003; Ermer, 2001; USP, 2007). The system was equilibrated with the initial mobile phase composition, followed by 10 injections of the same standard. These 10 consecutive injections were used to evaluate the system suitability on each day of method validation (Table 1).

Linearity of the method

Linearity is generally reported as the variance of the slope of the regression line and was performed with known concentrations of SPFX, 2.5, 5, 10, 25, 50 and 100 µg mL^{-1}, respectively. Linear least squares regression procedure was used for obtaining calibration curves as given in Table 1. The correlation coefficient (r^2) value is ± 0.998. Homoscedasticity of the calibration plots, tested by Friedman's tests were found to be significantly linear over the tested ranges.

Accuracy of procedure

The accuracy of an analytical procedure measures the closeness of agreement between the values. Recovery tests were performed by adding known amounts of standard solutions to sample followed by analysis using proposed method. Three runs were performed for every concentration and result range was 98.11 to 102.83% indicating its high rank of accuracy (Table 2). The average recovery for each level was calculated as indicated by Association of Official Analytical Chemists International (Somia et al., 2012; AOAC International, 2002).

Intraday and inter-day precision

The precision of the proposed method was investigated with respect to repeatability. Intra-day and inter-day precision were determined by observed responses of freshly prepared solutions after replicate (n = 6) injection of sample solutions (Table 3). The precision of the method was analyzed as relative standard deviation (RSD%) throughout the linear range of concentrations (Shabir, 2003; Ermer, 2001; USP, 2007). All the results

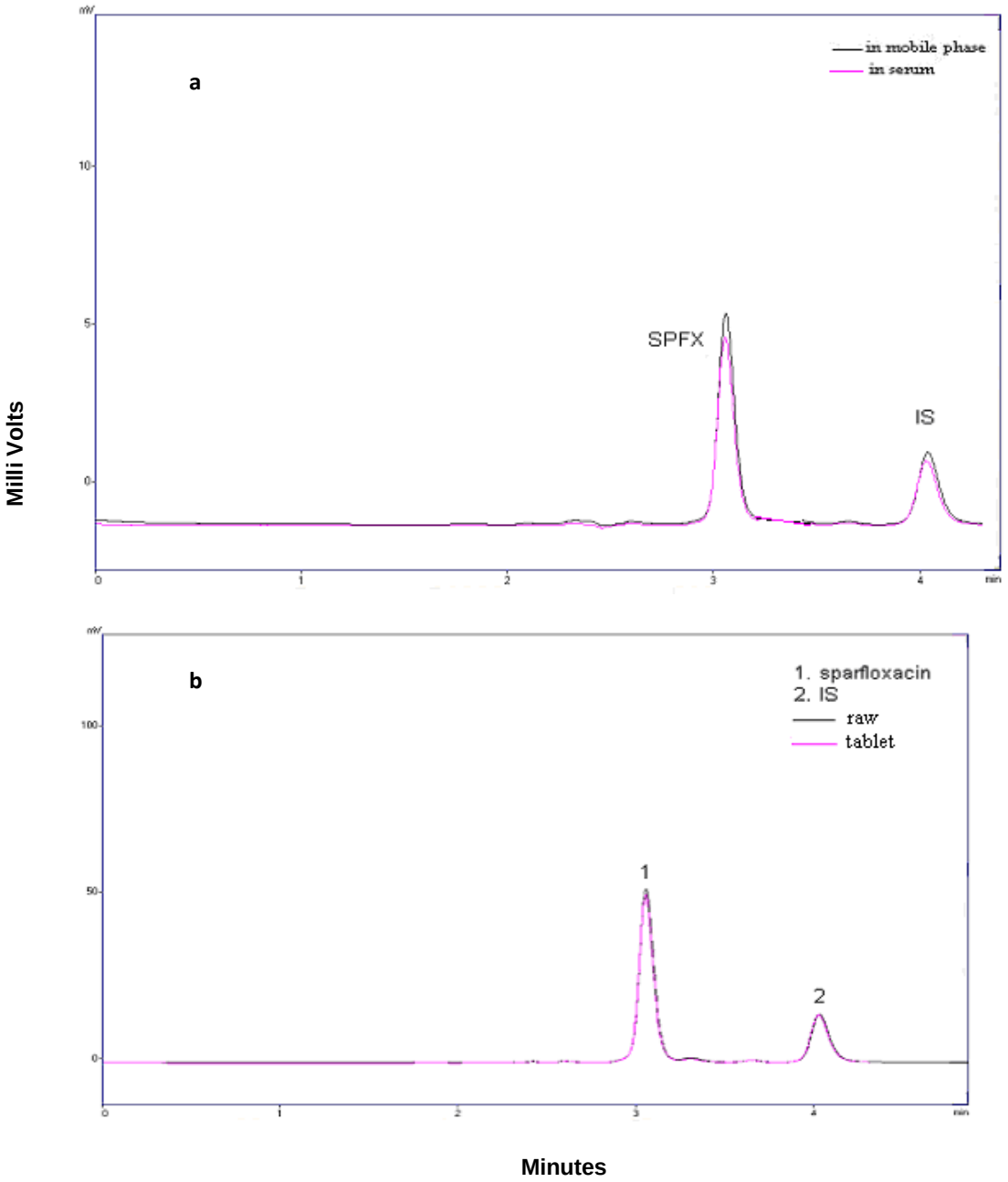

Figure 3. (a) Representative chromatogram of SPFX and IS at 290mm in both mobile phase and human serum; (d) representative chromatogram of SPFX and IS at 290 mm with and without excepients.

were correlated and found non-significant by student*'s t*-tests indicating no remarkable difference in intra and inter day precision.

Analysis of marketed products

Assay was done by taking three different brands of sparfloxacin. The peak of SPFX in marketed tablets was observed at 2.5 min and all the results were found to be in acceptable limits. The percent recovery was in good agreement with the label claims thereby suggesting that there is no interference from any excipients, which are normally present in tablets. The active content of drug in tablets was found to be 100.12% ± 0.994. Results were further verified by statistical evaluation, using one way analysis of variance (ANOVA) and the *F*-ratio at 95% confidence level as shown in Table 4 and found

Table 1. System suitability parameters and regression characters of the proposed method.

System	Column	t_R	K'	N	T	(R_s)	r^2	SEE	S.E	Y	Regression equation	LOD (µg mL^{-1})	LOQ (µg mL^{-1})
LC 10	C^A	3.057	0.33	6564	1.11	3.27	0.9991	19985.75	11164.32	-46821.5	Y=16203X-46822	0.00648	0.01963
	C^B	3.054	0.46	6594	1.11	3.25	0.9991	14276.13	7974.842	-9623.6	Y=11110X-9623.6	0.00231	0.007
LC 20	C^A	3.051	0.46	6333	1.13	2.99	0.9987	44651.51	24942.95	-54558.5	Y=29925X-54559	0.00094	0.02856
	C^B	3.049	0.26	6374	1.13	3.16	0.9983.	52202.34	29160.95	-44623.5	Y=29715X-44624	0.00093	0.00283

C^A = Purospher STAR, C^B = Discovery, t_R = Retention time, K'= Capacity factors, N = Theoretical plates, T = Tailing factor, R_s = Resolution, SEE = Standard error of estimate, SE = standard error, Y = Intercept, LOD = Limit of detection, LOQ = Limit of quantitation, Conc. 2.5-100 µgmL-1.

Table 2. Accuracy of sparfloxacin.

Column System	Parameter	C^A				C^B			
		Assay (spiking method)		Assay in serum		Assay (spiking method)		Assay in serum	
	Conc. (µg mL^{-1})	Conc. Found	% Recovery	Conc. Found	%Recovery	Conc. Found	%Recovery	Conc. found	% Recovery
LC 10	8	8.148	101.85	8.152	101.9	8.129	101.617	8.124	101.55
	10	10.038	100.387	10.035	100.35	10.029	100.293	10.019	100.19
	12	12.33	102.75	12.35	102.961	12.205	101.711	12.195	101.625
LC 20	8	8.2	102.62	8.16	102	7.956	99.455	8.01	100.125
	10	10.25	102.57	10.19	101.9	10.283	102.833	10.279	102.79
	12	11.77	98.142	11.89	99.08	12.292	102.437	12.293	102.441

non-significant.

Limit of detection and quantitation

The LOD and LOQ of this method were determined from the coefficient of variation of a known concentration of SPFX. The LOD and LOQ for this assay were calculated from three and ten times the noise level of the response (Shabir, 2003; Ermer, 2001; USP, 2007) which are given in Table 1. The LOD and LOQ were 0.0009 to 0.0064 and 0.0028 to 0.0196 µg mL^{-1}, respectively (Table 2).

Specificity and selectivity

The selectivity and specificity of proposed method was evaluated during the entire study through possible interference due to excipients present in the pharmaceutical formulations. The method confirmed good resolutions (Table 1). It was found to be free of interference from the excipients used in pharmaceutical formulation and it indicated the specificity of the system. Specificity was also determined by screening four different samples of controlled human serum which were free from interfering endogenous plasma components.

Robustness

Robustness of the method was assessed by

Table 3. Precision of sparfloxacin.

Column	C^A			C^B		
Systems	Formulation (%RSD)		Serum (%RSD)	Formulation (%RSD)		Serum (%RSD)
	D_1	D_2	D_1	D_1	D_2	D_1
LC 10	0.495	0.575	0.494	0.712	0.799	0.717
	0.226	0.366	0.225	0.277	0.256	0.273
	0.201	0.565	0.201	0.254	0.215	0.251
	0.475	0.577	0.572	0.499	0.497	0.492
	0.366	0.367	0.368	0.156	0.153	0.155
	0.565	0.566	0.569	0.115	0.114	0.113
LC 20	0.383	0.396	0.384	0.400	0.431	0.403
	0.236	0.213	0.239	0.384	0.323	0.387
	0.108	0.109	0.106	0.371	0.377	0.372
	0.296	0.293	0.292	0.231	0.233	0.235
	0.013	0.012	0.013	0.023	0.021	0.024
	0.091	0.094	0.093	0.077	0.074	0.073
t-Test: paired two sample for precision						
Systems	Columns	t stat	P (T<t) two-tail	-	-	-
LC 10	C^A	-2.093	0.091	-	-	-
	C^B	-.197	0.852	-	-	-
LC 20	C^A	0.345	0.744	-	-	-
	C^B	0.363	0.732	-	-	-

Where, C^A = Purospher STAR, C^B = Discovery, D_1 = Intra-day and D_2 = Inter-day variations

deliberate variations made to the method parameters such as composition (± 5) of the mobile phase, pH (± 0.2), flow rate (± 0.2), and detection wavelength (± 30). Therefore, five repeated samples were injected under small variations of each parameter. The method proved to be fairly steady as there is no considerable drift in the factors as given in Table 5.

Ruggedness

The ruggedness of the method was established in two different labs. Lab 1 was Research Institute of Pharmaceutical Sciences, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Karachi while 2nd lab was lab 9, Department of Chemistry, Faculty of science, University of Karachi. Work was carried out on two different instruments; LC 20 and LC 10, using two different columns Purospher STAR C_{18} and Discovery C_{18} for the study on different days by different analysts. All the results were correlated by applying one-way ANOVA and the differences were found non significant. Concluding that the method was capable within acceptable limits in precision, but the peak area was affected with change of wavelength (Table 1).

Application of the proposed method

The proposed method is not only applicable for the routine quality control assay of sparfloxacin in bulk, pharmaceutical dosage form and serum but also for the clinical evaluation, pharmacokinetic, bio-equivalence and interaction studies where low volume of blood or plasma is needed due to the simplicity of the separation procedure, shorter run time (7.0 min) and the cheaper mobile phase. The proposed method also gains its significance, being applicable to the wider range of detection wavelengths.

Conclusion

A simple and reliable HPLC method has been developed successfully for the monitoring of SPFX, in bulk, pharmaceutical dosage formulation and human serum. This method has been established for the first time and was not reported earlier. The above developed method has

Table 4. Application of the proposed method for the determination of marketed products

Brands	Sparaxin™	Sparkure™	Quspar™	
sample	conc. Found (mg)/% recovery	conc. Found (mg)/% recovery	conc. found (mg)/% recovery	
1	99.77	99.65	99.68	
2	99.88	99.95	99.94	
3	99.86	99.82	99.85	
4	99.67	99.86	99.88	
5	99.56	99.36	99.37	
6	99.94	99.77	99.72	
7	100.26	100.18	99.96	
8	100.27	100.57	100.06	
9	100.12	100.07	100.17	
				total
Count	9	9	9	27
Sum	899.33	899.23	898.63	2697.19
Mean	99.925	99.914	99.847	99.895
S.D	0.2495	0.342	0.2355	0.827
Variance	0.0622	0.117	0.0554	0.2962
Result	***SS***	**df**	***MS***	**F**
Between Groups	0.105	3	0.0349	
Within Groups	1.878	32	0.0586	0.596
Total	1.983	35	-	

Where, *label claim is 100 mg.

Table 5. Robustness of the proposed method (n=6).

Parameters	Level	t_R	K'	T	(R_s)
pH of mobile phase					
2.5	-0.2	3.051	0.31	1.11	3.27
2.7	0	3.057	0.33	1.11	3.25
2.9	0.2	3.055	0.33	1.13	3.32
Mean± S.D (n=6)		3.053±0.0028	0.323±0.011	1.116±0.011	3.28±0.036
Flow rate (ml min^{-1})					
0.8	-0.2	3.057	0.26	1.11	3.37
1	0	3.059	0.33	1.13	3.25
1.2	0.2	3.053	0.36	1.13	3.32
Mean± S.D (n=6)		3.055±0.0028	0.316±0.051	1.123±0.011	3.313±0.060
Percentage of water in mobile phase (V/V/V)					
25	-5	3.051	0.27	1.13	3.24
30	0	3.054	0.33	1.11	3.27
35	5	3.057	0.37	1.11	3.32
Mean± SD (n=6)		3.054±0.0042	0.323±0.0503	1.116±0.011	3.27±0.040
Wavelength (nm)					
230	-60	2.49	4.7	1.42	2.39
260	-30	2.51	4.3	1.43	2.36
290	0	3.057	0.33	1.11	3.25
Mean± SD (n=6)		2.685±0.014	2.515±3.090	1.32±0.1819	2.66±0.505

t_R = Retention time, K'= Capacity factors, N = Theoretical plates, T = Tailing factor, R_s = Resolution.

been conducted on three different wavelengths, validated as per ICH guidelines and the results were then correlated. Results indicate that the 290 nm is the wavelength where the area under curve (AUC) of SPFX is maximum, in comparison to 230 and 260 nm, providing the same chromatographic conditions. Therefore, we can report that the best suited wavelength for the determination of SPFX is 290 nm provided the same chromatographic conditions were followed.

The limit of quantification, small sample volume and short chromatographic time of this method are particularly adapted for routine assay. The short analysis time (< 7 min) enables its application in routine and quality control analysis of finished products. The proposed method has been effectively applied to quantities SPFX in three different commercially available brands.

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