HPLC and Densitometric TLC Methods for Simultaneous Determination of Pazufloxacin with Some Co-administered Drugs in Human Plasma

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Abstract

Two chromatographic methods have been developed for determination of pazufloxacin in human plasma with two co-administered drugs, cefoperazone and sulbactam sodium. First method depends on reverse phase high performance liquid chromatography. The plasma sample was extracted using mixture of ethyl acetate and 15% perchloric acid. The method was linear over the concentration range 0.5 to 20 µg/ml, 1 to 30 µg/ml and 1 to 25 µg/ml of pazufloxacin mesylate, cefoperazone and sulbactam sodium, respectively.

The mobile phase used consist of methanol, 0.01 M sodium acetate buffer (pH adjusted to 3 using trifluoroacetic acid) in a ratio 20:80 and 0.5% TEA and flow rate 1 ml/min in isocratic mode and UV-detection at wavelength 240 nm. Second method depends on densitometric thin layer chromatography. The method was linear over concentration range 1 to 20 µg/ml, 6 to 24 µg/ml and 4 to 20 µg/ml of pazufloxacin mesylate, cefoperazone and sulbactam sodium, respectively. The mobile phase used consists of chloroform, methanol and ammonia in the ratio of (11:6:2, v/v/v). Densitometric analysis was carried out at wavelength 240 nm. The stability of pazufloxacin mesylate and the co-administered drugs in plasma was confirmed during three freeze–thaw cycles (−20°C).

Keywords: Pazufloxacin; Co-administered; Plasma; HPLC; TLC

Introduction

Pazufloxacin(-)-(S)-10-(1-aminocyclopropyl)-9-fluoro-3-methyl-7-oxo-2,3-dihydro-7Hpyrido[2,3-de][1,4]benzoxazine-6-carboxylicacid monomethanesulfonate is a fluoroquinolone synthesized by Toyama Chemical Co. Ltd (Tokyo, Japan). This drug has good *in vitro* and *in vivo* activity against a broad range of bacteria, especially Gram-negative bacteria [1,2]. Clinical trials showed its intravenous injection formula was effective in treating respiratory infections and the drug has been approved and is available in Japan [3]. The literature survey revealed that analytical methods reported for the estimation of pazufloxacin mesylate in human plasma include chemiluminescence flow injection [4] and HPLC methods [5-7]. No method has been reported for determination of pazufloxacin mesylate in human plasma with co-administered drugs by HPLC and densitometric TLC. It was reported that the antibacterial activity of pazufloxacin mesylate was enhanced significantly in presence of cefoperazone and sulbactam sodium. Their antibacterial action *in vitro* was characterized by synergism and additive action instead of antagonistic effect [8,9]. The proposed research work describes the estimation of pazufloxacin mesylate with cefoperazone and sulbactam sodium in human plasma by RP-HPLC and TLC using moxifloxacin as an internal standards (Figure 1).
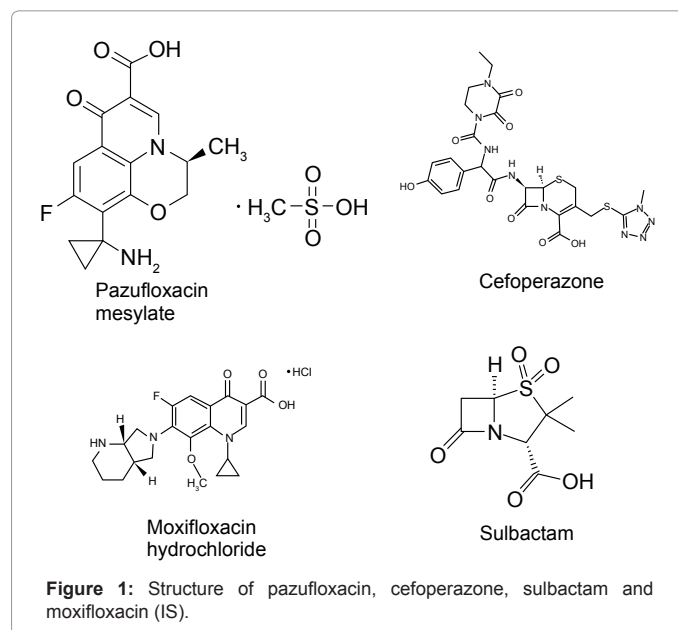
Materials and Methods

Instrumentation

HPLC Knauer instrument (Germany) equipped with K-501 pump, Knauer injector and UV-detector K-2501. Data acquisition was performed on Eurochrom 2000 software. The analytical column employed was X-terra LC-18-DB, (25 cm×4.6 mm×5 µm). The working temperature was 25°C.

DESAGA CD 60 HPTLC densitometer connected to IBM compatible computer fitted with Proquant evaluation software for Windows. (Sarstedt-Gruppe, Germany) with precoated silica gel Plate 60F254 (20 cm×20 cm) 250 µm thicknesses (E. Merck, Darmstadt, Germany) was used as stationary phase. Sample application was done by using DESAGA AS30 HPTLC Applicator. (Sarstedt-Gruppe, Germany).

Figure 1: Structure of pazufloxacin, cefoperazone, sulbactam and moxifloxacin (IS).

Linear ascending development was carried out in 25 cm×25 cm glass chamber. Evaluation of chromatogram was done by using peak areas. Rotatory vacuum evaporator, Buchi Rotavaper R-3000 (Germany).

Chemicals

Pazufloxacin mesylate (Thonson technology limited, Shanghai, China), cefoperazone and sulbactam sodium (The Nile company for pharmaceuticals and chemical industries, Cairo, Egypt), moxifloxacin hydrochloride (Indo Gulf, India) were received having 99.20%, 99.20%, 99.60 and 99.65% purity respectively. The HPLC grade methanol, acetonitrile, ortho phosphoric acid, chloroform, dichloromethane, ammonia and water were purchased from (Sigma Gmbh, Germany). Analytical reagent grade dipotassium hydrogen phosphate and ammonium acetate were used. Freshly isolated human plasma from collected blood used for research work was supplied by Vacsera, Cairo, Egypt.

Preparation of stock solutions and working standard solutions

Standard solutions preparation was conducted at room temperature. The solutions were protected from light with aluminum foil wrapping and stored at −20°C.

Stock solutions 1.00 mg/ml each of pazufloxacin mesylate, cefoperazone sodium, sulbactam sodium and moxifloxacin hydrochloride were prepared in methanol.

The first working standard solutions of 0.1 mg/ml of pazufloxacin mesylate, cefoperazone sodium, sulbactam sodium and moxifloxacin hydrochloride were prepared by further dilution of stock solutions with mobile phase for HPLC. Second working standard solutions (0.2 mg/ml) of pazufloxacin mesylate, cefoperazone sodium and sulbactam sodium were prepared by further dilution of stock solutions with mobile phase for HPLC and methanol for HPTLC.

Preparation of plasma samples

For HPLC, in a stoppered centrifuge tube, an aliquot quantity of 500 μl plasma was spiked with 50 μl moxifloxacin (internal standard 100 μg/ml) and 450 μl mixture of pazufloxacin, cefoperazone and sulbactam sodium working solutions.

Different aliquots of pazufloxacin were added to provide concentrations of (0.5, 1, 2.5, 5, 10, 15, 20 μg/ml). Different aliquots of cefoperazone sodium were added to provide concentrations of (1, 2, 4, 8, 10, 15, 30 μg/ml). Different aliquots of sulbactam sodium were added to provide concentrations of (1, 2, 5, 10, 15, 20, 25 μg/ml).

The quality control samples (QCs) were prepared in plasma concentration range (1.5, 10, 15 μg/ml), (3, 10, 25 μg/ml) and (3, 10, 20 μg/ml) for pazufloxacin, cefoperazone and sulbactam sodium, respectively. Protein precipitation and extraction were carried out by using 1 ml ethyl acetate and 50 μl 15% perchloric acid. The samples were sonicated for 10 minutes followed by vortex mixing for 5 minutes and centrifugation at 5000 rpm for 15 minutes. The organic layer was transferred to another centrifuge tube and evaporated to dryness at 40°C under vacuum. The residue was reconstituted in 0.25 mls mobile phase and 20 μl injected into HPLC system.

For densitometric TLC, in a stoppered centrifuge tube, an aliquot quantity of 500 μl plasma was spiked with 50 μl moxifloxacin (internal standard 100 μg/ml) and 450 μl mixture of pazufloxacin, cefoperazone and sulbactam sodium. Different aliquots of pazufloxacin were added to provide concentrations of (1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 μg/ml). Different aliquots of cefoperazone sodium were added to provide

concentrations of (6, 8, 10, 12, 14, 16, 18, 20, 22, 24 μg/ml). Different aliquots of sulbactam sodium were added to provide concentrations of (4, 6, 8, 10, 12, 14, 16, 18, 20 μg/ml). The quality control samples (QCs) were prepared in plasma concentration range (3, 12, 18 μg/ml), (18, 20, 23 μg/ml) and (12, 16, 19 μg/ml) for pazufloxacin, cefoperazone and sulbactam sodium, respectively. Protein precipitation and extraction were carried out as previously mentioned in HPLC method. The residue was reconstituted in 0.1 mls methanol and 10 μl were applied to TLC plates.

Chromatographic conditions

For HPLC, the mobile phase used was mixture of methanol, 0.01 M sodium acetate buffer (pH adjusted to 3 using trifluoroacetic acid) in a ratio 20:80 and 0.5% TEA. The mobile phase was freshly prepared and filtered by vacuum filtration through 0.45 μm filter and degassed by ultrasound sonication (Crest Ultrasonics, New York) for 50 minutes just prior to use. The samples were also filtered using 0.45 μm syringe filters (Gelman, Sigma-aldrich). The analysis was done under isocratic conditions at a flow rate 1 ml.min⁻¹ and at room temperature using UV detector at 240 nm.

For densitometric TLC, the mobile phase was selected as mixture of of chloroform, methanol and ammonia in the ratio of (11:6:2 v/v/v) for the development of plates. The densitometric scanning was performed at 240 nm. The samples were also filtered using 0.45 μm filters. Analysis was performed on precoated 20×20 cm silica gel 60 F_{254} aluminium sheets (E.Merck). The plates were pre-washed with methanol and activated at 60°C for 5 min prior to chromatography. Samples were applied to the plates using a DESAGA AS30 Applicator (Germany). Spots were applied 1.5 cm apart from each other and 2 cm from the bottom edge. The chromatographic chamber was pre-saturated with the mobile phase for 45 min the developing distance on TLC-plate was 180 mm.

Method validation

The described methods were validated in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), recovery, selectivity, stability, precision and accuracy according to FDA guidelines regarding standard bioanalytical method validation recommendation [10].

Linearity: The analytical range to be validated was chosen on the basis of the expected plasma concentrations of the studied drugs [5-7,11]. The calibration curve was done for each analyte in the biological sample. The calibration curve should consist of a blank sample (matrix sample processed without internal standard), a zero sample (matrix sample processed with internal standard), and six to eight non-zero samples covering the expected range, including LLOQ that were prepared by adding the required volume of working solution of analyte to blank plasma. The plasma samples were subjected to the sample preparation procedure and injected into the LC.

Plasma calibration curve was prepared by taking area ratio of analyte to internal standard as Y-axis and concentration of analyte (μg/ml) as X-axis.

Accuracy and precision: Accuracy and precision were determined for LQC, MQC and HQC (Low, Middle and High Quality Control) samples with LLOQ. Five replicates of each concentration were analyzed on the same day to determine the within-run accuracy and precision of the method. To confirm the between-run accuracy and precision five replicates of each concentration were analyzed at three separate days.

For HPLC, the used concentrations were (0.5, 1.5, 10 and 15 μg/

ml), (1, 3, 10 and 25 µg/ml), (1, 3, 10 and 20 µg/ml) for pazufloxacin, cefoperazone and sulbactam sodium; respectively.

For TLC, the used concentrations were (1, 3, 12 and 18 µg/ml) equivalent to (0.1, 0.3, 1.2, 1.8 µg/spot), (6, 18, 20 and 23 µg/ml) equivalent to (0.6, 1.8, 2, 2.3 µg/spot) and (4, 12, 16 and 19 µg/ml) equivalent to (0.4, 1.2, 1.6, 1.9 µg/spot) for pazufloxacin, cefoperazone and sulbactam sodium; respectively.

Selectivity: The selectivity of the methods was investigated by analyzing six blank plasma samples. Each blank sample was tested for interference using proposed extraction procedure and the response of the endogenous compounds at the retention times of the studied drugs in plasma samples were compared with the response of LLOQ of the studied drugs.

Recovery: The extraction recovery of analytes was determined by measuring the peak areas of the drugs from the prepared plasma quality control samples. The peak areas of extracted LQC, MQC and HQC were compared to the absolute peak area of the unextracted samples in mobile phase for HPLC and in methanol for TLC containing the same concentration of the drug. To obtain good extraction efficiency the extraction recovery of pazufloxacin and its co-administered cefoperazone and sulbactam sodium was determined using five replicates of each QC samples.

Stability study

Freeze and thaw stability: The stability of pazufloxacin together with co-administered cefoperazone and sulbactam sodium was determined after three freeze and thaw cycles. Five aliquots at each of the LLOQ, low, mid and high quality control concentrations were stored at -20°C for 24 hours and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 24 hours under the same conditions. The freeze–thaw cycle were repeated two more times, and then analyzed.

Short term temperature stability: Five aliquots of each of the LLOQ, low, mid and high quality control concentrations were thawed at room temperature and kept at this temperature for 6 hours and then analyzed.

Long term stability: Long-term stability was determined by storing five aliquots of each of the LLOQ, low, mid and high concentrations of the studied drugs at -20°C for 6 weeks. The concentrations of all the stability samples were compared to the mean values for the standards at the appropriate concentrations from the first day of long-term stability testing.

Stock solutions stability: The stability of stock solutions of each of the studied drugs and the internal standards used were evaluated at room temperature for 10 hours. After completion of the desired storage time, the stability was tested by comparing the instrument response with that of freshly prepared solutions.

Post-preparative stability: The stability of the processed samples was examined by keeping five replicates of the LLOQ, low, mid and high plasma quality control samples at room temperature for approximately 24 hours. The stability was tested by comparing the instrument response with that of freshly prepared samples.

Results and Discussion

HPLC-UV detection and densitometric TLC methods were suggested for the simultaneous determination of each of pazufloxacin with 2 co-administered drugs; cefoperazone and sulbactam sodium.

This work aimed to develop highly selective and sensitive methods with a quantitation limits that cover the expected concentration ranges of all of the studied drugs in human plasma to be able to be used in pharmacological, bioavailability, bioequivalence or other clinical studies to obtain certain pharmacokinetic information.

Extraction procedure optimization

One of the most difficult parts during the method development was to achieve a high and reproducible recovery from the solvent which is used for extraction of the drugs. Different solvents were tried for the extraction of pazufloxacin, cefoperazone and sulbactam sodium from human plasma. First 1 ml n-hexane was used for plasma precipitation. The recovery of all of the three drugs and the internal standard was below 30%. By the use of acetonitrile, the recoveries of pazufloxacin and the internal standard were above 87% but that of the other drugs was below 75%. By the use of ethyl acetate with slight acidification with 15% perchloric acid to increase the precipitation of plasma protein, the recoveries of the all drugs were above 85%. So the ethyl acetate was chosen as the best solvent for extraction.

Optimization of chromatographic condition

For HPLC, the chromatographic conditions, especially the composition of mobile phase, were optimized to achieve a good resolution and symmetric peak shapes for the analytes and the internal standard, as well as a short analytical time. Initially a mixture of acetonitrile: sodium acetate buffer was used as a mobile phase with a ratio of 30:70 pH=2. The peaks of cefoperazone and sulbactam sodium were overlapped and attached to plasma peak with significant tailing in pazufloxacin peak. Replacement of acetonitrile with methanol resulted in increasing the retention of cefoperazone and sulbactam sodium slightly away from the plasma peak but there was still some overlapping between them. By increasing pH to 3 the retention increased for all of the three drugs with improvement in the resolution of the overlapped peaks. The presence of residual silanol groups is responsible for the tailing that can be generated when amine containing compounds are separated. This interaction occurs because amines are strong silanophiles. Competing molecules such as triethylamine (TEA) interacts with residual silanol groups on the support surface and limits the interaction of basic analytes with these sites thereby decreasing the peak tailing. Triethylamine is commonly used in reversed phase HPLC as a basic mobile phase modifier at concentration 0.1-1% in aqueous phase [12]. With the addition of 0.5% TEA pazufloxacin peak tailing was reduced significantly. The mobile phase used for the simultaneous determination of pazufloxacin and its co-administered cefoperazone and sulbactam sodium was mixture of methanol, 0.01 M sodium acetate buffer (pH adjusted to 3 using trifluoroacetic acid) in a ratio 20:80 and 0.5% TEA. The average retention time (minutes) ± SD, for 6 replicate injections for pazufloxacin, cefoperazone, sulbactam and moxifloxacin, were found to be 6.535 ± 0.05, 3.19 ± 0.07, 4.205 ± 0.05 and 7.486 ± 0.06; respectively.

For densitometric TLC, the proposed TLC method is based on the difference between R_f values of pazufloxacin mesylate and its co-administered drugs which differ in their polarities and consequently in their migration rates on TLC plates. The chromatographic conditions were optimized by spotting the drug with its co-administered drugs on TLC plates and developed in different solvent systems to achieve best separation. Different solvent systems were tried. Initially a system of ethyl acetate and methanol in different ratios were used, but these systems showed poor resolution with excessive tailing in the peaks of cefoperazone and sulbactam which interfered with the peak of

pazufloxacin. Secondly, ethyl acetate was replaced by chloroform which improved the resolution of the separated peaks, but tailing was still observed. By the addition of 2 mls of ammonia, the peaks became sharp and symmetric without tailing. The optimum mobile phase used was was chloroform: methanol: ammonia (11:6:2 v/v/v). The Rf values were 0.31, 0.41, 0.46 and 0.58 for pazufloxacin, cefoperazone, sulbactam and moxifloxacin; respectively.

Method validation

Linearity: For HPLC, the seven point calibration curves were constructed by plotting the peak area ratio of each of pazufloxacin, cefoperazone and sulbactam sodium to moxifloxacin (IS) versus their concentrations in plasma. The mean equations of calibration curves consisting of seven points are $y=0.0817C+0.0061$ for pazufloxacin mesylate with a correlation coefficient 0.9999, $y=0.0828C+0.0028$ for cefoperazone sodium with a correlation coefficient 0.9998 and $y=0.0739C+0.003$ with correlation coefficient 0.9996. Where y represents the ratios of peak area of each drug to that of IS and C represents the plasma concentration of each drug. The values of correlation coefficient confirmed that the calibration curves were linear over the concentration ranges 0.5–20 µg/ml, 1–30 µg/ml and 1–25 µg/ml for pazufloxacin, cefoperazone and sulbactam sodium; respectively.

For densitometric TLC, the calibration curves were constructed by plotting the peak area ratio of each of pazufloxacin, cefoperazone and sulbactam sodium to moxifloxacin (IS) versus their concentrations in plasma. The mean equations of calibration curves consisting of seven points $y=0.122C+0.0438$ for pazufloxacin mesylate with a correlation coefficient of 0.9994, $y=0.0384C+0.0069$ for cefoperazone sodium with correlation coefficient of 0.9990 and $y=0.0782C-0.0253$ for sulbactam sodium with correlation coefficient of 0.9991. Where y represents the ratios of peak area of each drug to that of IS and C represents the plasma concentration of each drug. The calibration curves were linear over the concentration ranges 1–20 µg/ml, 6–24 µg/ml and 4–20 µg/ml for pazufloxacin, cefoperazone and sulbactam sodium; respectively.

Accuracy and precision: The accuracy of the method expressed in term of bias (percentage deviation from true value). The mean value should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%. The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV [10].

Precision of the method was determined by repeatability (intra-day) and intermediate precision (inter-day) and accuracy for set of quality control (QC) sample (low, mid, high) in replicate (n=5). The inter-day and intra-day precision and accuracy for pazufloxacin, cefoperazone and sulbactam sodium were evaluated by assaying the QC samples (low, mid, high) (n=5) in (%CV). In HPLC assay the intra-run precisions were found to be in the ranges of 2.137–5.361%, 0.520–3.316% and 0.643–3.347% and the inter-run precisions were 2.568–6.861%, 0.625–3.708% and 0.743–4.054% for pazufloxacin, cefoperazone and sulbactam sodium, respectively. The accuracy values (%RE) were within the ranges -0.220–3.800%, -0.250–3.500% and -0.700–4.400% for pazufloxacin, cefoperazone and sulbactam sodium, respectively. The above values were within the acceptable range, they show that the HPLC method is accurate and precise.

For densitometric TLC intra-run precisions were found to be in the ranges of 3.090–6.034%, 1.899–5.442% and 2.036–6.650% and the inter-run precisions were 1.346–5.781%, 1.835–5.835% and 2.352–7.900% for pazufloxacin, cefoperazone and sulbactam sodium, respectively. The accuracy values were within the ranges 5.267–11.533%, 2.300–10.883% and -0.069–4.566% for pazufloxacin, cefoperazone and sulbactam sodium, respectively. The low percent coefficient of variation (%CV) and percent relative errors (%RE) were within the acceptable limit. The results of inter-day, intra-day precision and accuracy for pazufloxacin mesylate, cefoperazone and sulbactam sodium are shown in Table 1.

Selectivity: Selectivity was assessed to show that the intended analytes are measured and that their quantitation is not affected by the presence of the biological matrix. For HPLC, (figures 2-5) showed the typical chromatograms of blank plasma and pazufloxacin with its co-administered drugs spiked with plasma. There was no significant interference observed at the retention times of the analytes.

Method	Pazufloxacin mesylate				Cefoperazone sodium					Sulbactam sodium		
	Concentration (µg/ml)	SD (n=5)	CV%	%RE	Concentration	SD (n=5)	CV%	%RE	Concentration	SD (n=5)	CV%	%RE
RP-HPLC												
Intra-day	0.5	0.026	5.361	3.000	1	0.032	3.316	3.500	1	0.032	3.347	4.400
	1.5	0.050	3.358	0.333	3	0.037	1.222	0.433	3	0.040	1.342	0.133
	10	0.211	2.137	1.150	10	0.052	0.520	-0.250	10	0.064	0.643	0.350
	15	0.362	2.411	-0.100	25	0.621	2.572	3.424	20	0.302	1.509	-0.070
Inter-day	0.5	0.033	6.861	3.800	1	0.036	3.708	2.900	1	0.039	4.054	3.800
	1.5	0.050	3.333	1.00	3	0.084	2.801	-0.067	3	0.065	2.165	-0.700
	10	0.422	4.212	-0.220	10	0.062	0.625	0.460	10	0.075	0.743	-0.210
	15	0.382	2.568	0.827	25	0.702	2.806	-0.056	20	0.318	1.580	-0.605
TLC												
Intra-day	1	0.054	6.034	10.500	6	0.306	5.442	6.283	4	0.265	6.650	0.375
	3	0.082	3.090	11.533	18	0.325	1.899	4.933	12	0.401	3.502	4.566
	12	0.526	4.627	5.267	20	0.411	2.103	2.300	16	0.326	2.036	-0.069
	18	0.713	4.203	5.750	23	0.631	2.853	3.826	19	1.020	5.403	0.632
Inter-day	1	0.052	5.781	9.700	6	0.312	5.835	10.883	4	0.302	7.900	4.425
	3	0.081	3.042	11.233	18	0.312	1.835	5.544	12	0.278	2.352	1.483
	12	0.152	1.346	5.875	20	0.562	2.964	5.210	16	0.468	2.938	0.438
	18	0.422	2.585	9.306	23	1.120	5.083	4.204	19	1.114	5.962	1.663

a-CV%: coefficient of variation
b-%RE: percent relative error

Table 1: Accuracy and precision of of pazufloxacin mesylate, cefoperazone and sulbactam sodium in human plasma.
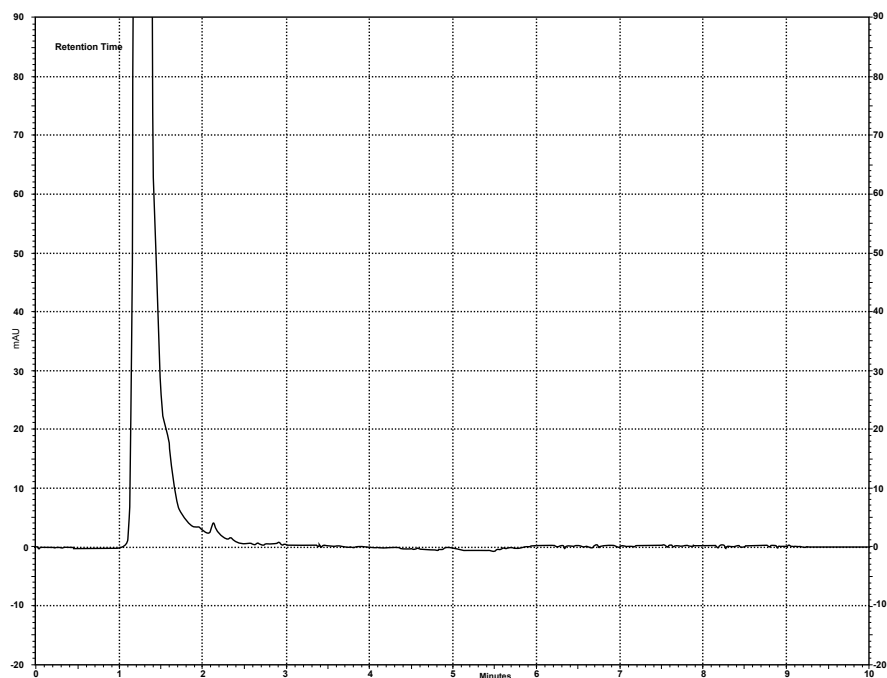
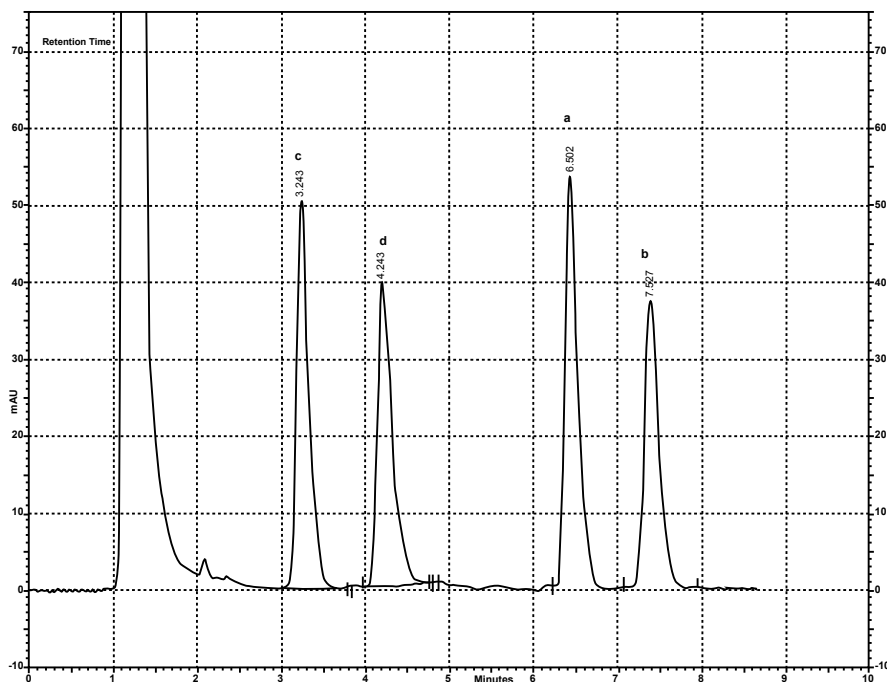**Figure 2:** Chromatogram of blank extracted human plasma sample.

Figure 3: Chromatogram of extract from plasma sample spiked with (a) Pazufloxacin, (b) moxifloxacin, (c) cefoperazone sodium and (d) sulbactam sodium.

Recovery: Absolute recovery was calculated by comparing peak areas obtained from freshly prepared sample extracted with unextracted standard solutions of the same concentration. Recovery data was determined in triplicates at three concentrations (low, mid, high) as recommended by the FDA guidelines [10]. The average recovery of pazufloxacin, cefoperazone and sulbactam sodium for RP-HPLC, determined at the three concentrations (low, mid, high concentration) of each were found to be 90.728, 89.408 and 90.493%; respectively.

For HPTLC the average recovery using the three concentrations for pazufloxacin, cefoperazone and sulbactam sodium were found to be 90.557, 91.061 and 91.187% as shown in table 2.

Sensitivity: Sensitivity of the method is defined as the lowest concentration that can be measured with an acceptable limit of accuracy and precision which is lower than 20% [10]. The accuracy and precision at lower limit of quantitation (LLOQ) analyzed by using five
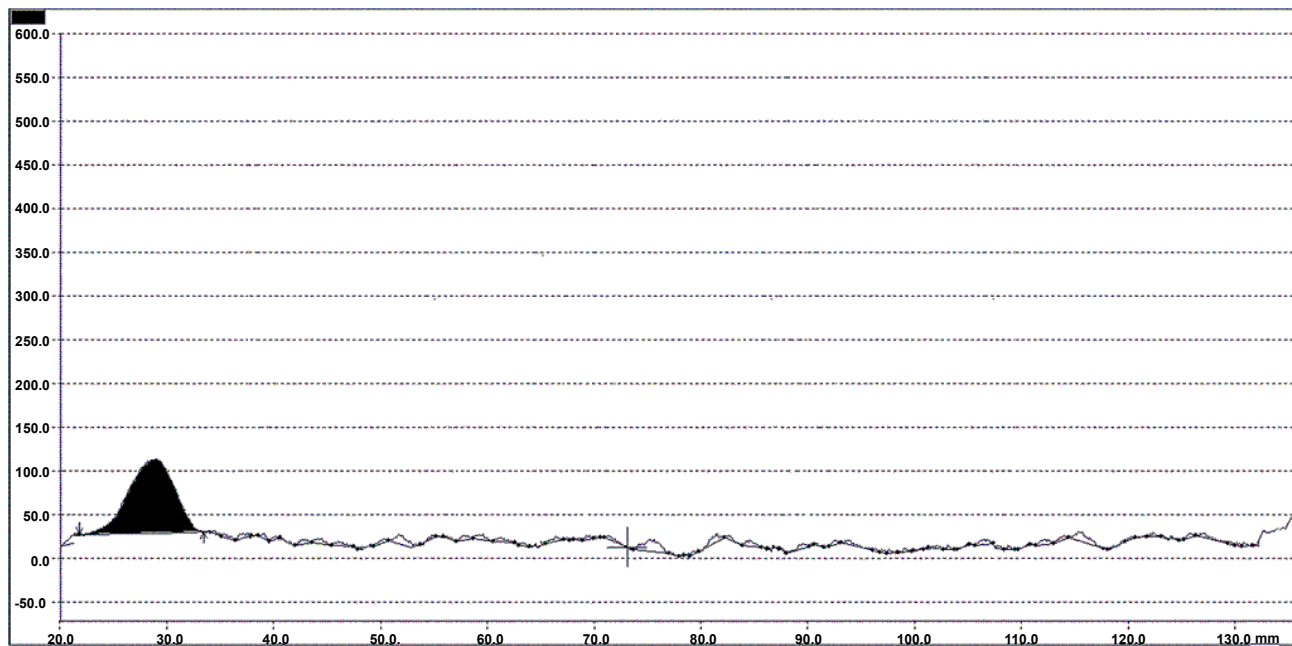
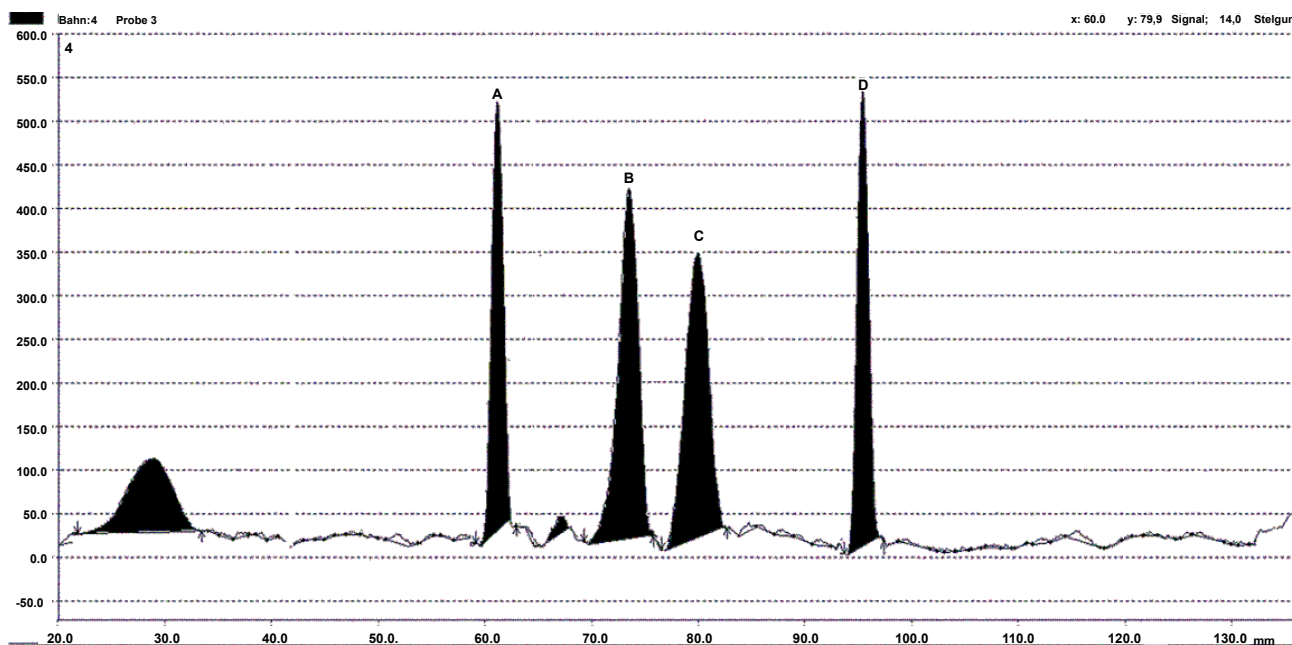**Figure 4:** Densitogram of blank plasma sample.

Figure 5: Densitogram of extract from plasma sample spiked with (A) Pazufloxacin, (B) moxifloxacin, (C) cefoperazone sodium and (D) sulbactam sodium.

replicate (n=5) of the sample at the LLOQ concentration. The accuracy is determined by %RE at this LLOQ concentration. For HPLC the lower limit of quantitation was found to be 0.5 µg/ml with CV=5.361%, %RE=3.00 for pazufloxacin mesylate. The LLOQ was found to be 1 µg/ml with CV=3.316% and %RE=3.500 for cefoperazone. For sulbactam sodium LLOQ was 1 µg/ml with CV=3.347% and %RE=4.400.

LOD values were found to be 0.165, 0.33 and 0.33 µg/ml for pazufloxacin, cefoperazone and sulbactam sodium, respectively.

For TLC lower limit of quantitations were found to be 1 µg/

ml with CV=6.034% and %RE=10.500, 6 µg/ml with CV=5.442% and %RE=6.283 and 4 µg/ml with CV=6.650% and %RE=0.375 for pazufloxacin, cefoperazone and sulbactam sodium, respectively, which were within the acceptable limit.

LOD values were found to be 0.33, 1.8 and 1.2 µg/ml for pazufloxacin, cefoperazone and sulbactam sodium, respectively.

Stability study: In stock solution stability the studied drugs with their internal standards samples were left at room temperature for 10 h. Comparison of the results with freshly prepared stock solution showed

Method	Pazufloxacin mesylate			Cefoperazone sodium			Sulbactam sodium		
	Concentration	Recovery %	CV%	Concentration	Recovery %	CV%	Concentration	Recovery %	CV%
RP-HPLC	0.5(μg/ml)	89.000	5.393	1	87.400	7.094	1	89.500	8.268
	1.5	91.667	3.782	3	88.600	3.837	3	90.400	4.462
	10	90.620	4.359	10	90.250	5.008	10	91.230	5.601
	15	91.627	5.195	25	91.380	4.469	20	90.840	6.066
TLC	1(μg/ml)	88.500	5.876	6	91.583	6.588	4	88.525	7.117
	3	88.600	3.311	18	90.844	4.373	12	92.100	6.198
	12	92.933	2.260	20	89.420	5.715	16	93.256	6.173
	18	92.194	3.929	23	92.396	5.887	19	90.868	7.095

a-CV%: Coefficient of Variation %

Table 2: Results of recovery of pazufloxacin mesylate, cefoperazone and sulbactam sodium in human plasma.

	Pazufloxacin			Cefoperazone			Sulbactam sodium		
	Plasma concentration (μg/ml)	Found* (Mean ± SD)	CV%	Plasma concentration (μg/ml)	Found* (Mean ± SD)	CV%	Plasma concentration (μg/ml)	Found* (Mean ± SD)	CV%
Freeze-thaw	0.5	0.489 ± 0.041	8.384	1	0.968 ± 0.0325	3.357	1	0.972 ± 0.036	3.704
	1.5	1.498 ± 0.043	2.884	3	3.026 ± 0.0541	1.788	3	3.112 ± 0.0684	2.198
	10	10.063 ± 0.302	3.001	10	9.962 ± 0.2011	2.019	10	9.985 ± 0.311	3.115
	15	15.121 ± 0.355	2.348	25	30.062 ± 0.506	1.683	20	20.121 ± 0.663	3.295
Short term	0.5	0.482 ± 0.041	8.506	1	0.958 ± 0.041	4.279	1	0.976 ± 0.042	4.303
	1.5	1.502 ± 0.046	3.036	3	2.984 ± 0.0622	2.084	3	3.022 ± 0.0552	1.827
	10	9.996 ± 0.285	2.851	10	10.025 ± 0.2133	2.128	10	10.025 ± 0.338	3.372
	15	14.933 ± 0.341	2.284	25	30.018 ± 0.522	1.739	20	20.021 ± 0.625	3.122
Long term	0.5	0.486 ± 0.038	7.819	1	0.978 ± 0.036	3.681	1	0.952 ± 0.053	5.567
	1.5	1.493 ± 0.043	2.908	3	3.112 ± 0.033	1.067	3	2.966 ± 0.0632	2.131
	10	9.889 ± 0.233	2.356	10	9.912 ± 0.196	1.982	10	9.954 ± 0.328	3.295
	15	15.021 ± 0.411	2.736	25	29.932 ± 0.814	2.719	20	19.912 ± .744	3.736
Post preparative	0.5	0.481 ± 0.036	7.484	1	0.953 ± 0.045	4.722	1	0.974 ± 0.038	3.901
	1.5	1.500 ± 0.051	3.406	3	2.995 ± 0.0415	1.386	3	2.984 ± 0.0521	1.746
	10	9.984 ± 0.195	1.953	10	10.062 ± 0.1652	1.642	10	10.163 ± 0.203	1.997
	15	14.965 ± 0.406	2.713	25	30.052 ± 0.425	1.414	25	20.012 ± 0.741	3.703

a-* Mean of five determinations

Table 3: Stability study of Pazufloxacin, Cefoperazone and Sulbactam sodium in human plasma by the proposed HPLC method.

	Pazufloxacin			Cefoperazone			Sulbactam sodium		
	Plasma concentration (μg/ml)	Found* (Mean ± SD)	CV%	Plasma concentration (μg/ml)	Found* (Mean ± SD)	CV%	Plasma concentration (μg/ml)	Found* (Mean ± SD)	CV%
Freeze-thaw	1	0.932 ± 0.043	4.614	6	5.448 ± 0.365	6.700	4	3.741 ± 0.204	5.453
	3	2.847 ± 0.068	2.388	18	17.996 ± 0.503	2.795	12	11.484 ± 0.336	2.926
	12	11.896 ± 0.332	2.791	20	19.598 ± 0.632	3.225	16	15.658 ± 0.516	3.295
	18	16.984 ± 0.685	4.033	23	21.814 ± 0.762	3.507	19	18.365 ± 0.388	2.113
Short term	1	0.941 ± 0.038	4.038	6	5.865 ± 0.385	6.564	4	3.899 ± 0.196	5.027
	3	2.871 ± 0.071	2.473	18	17.652 ± 0.416	2.357	12	12.006 ± 0.425	3.540
	12	11.445 ± 0.205	1.791	20	19.852 ± 0.811	4.085	16	15.741 ± 0.602	3.824
	18	17.062 ± 0.625	3.663	23	21.745 ± 0.698	3.210	19	19.012 ± 0.374	1.967
Long term	1	0.925 ± 0.051	5.514	6	5.462 ± 0.395	7.232	4	3.602 ± 0.216	5.997
	3	2.762 ± 0.074	2.679	18	17.062 ± 0.541	3.171	12	11.836 ± 0.325	2.746
	12	11.303 ± 0.217	1.920	20	18.326 ± 0.506	2.761	16	15.322 ± 0.425	2.774
	18	17.622 ± 0.387	2.196	23	21.881 ± 0.852	3.894	19	18.385 ± 0.811	4.411
Post preparative	1	0.957 ± 0.045	4.702	6	5.841 ± 0.398	6.814	4	3.902 ± 0.215	5.510
	3	2.911 ± 0.065	2.233	18	17.124 ± 0.526	3.072	12	12.001 ± 0.347	2.891
	12	11.395 ± 0.122	1.071	20	19.629 ± 0.805	4.101	16	15.877 ± 0.285	1.795
	18	17.818 ± 0.528	2.963	23	22.001 ± 1.022	4.645	19	18.625 ± 0.502	2.695

a- * Mean of five determinations

Table 4: Stability study of Pazufloxacin, Cefoperazone and Sulbactam sodium in human plasma by the proposed TLC method.

that there was no significant difference between response of freshly prepared solutions and samples of the studied drugs after 10 h.

Freeze–thaw stability was determined after three freezes–thaw cycles for five replicate of LLOQ, low, mid and high QC samples. The samples were stored at −20°C temperature for 24 h. Then thaw at room temperature. No significant difference between freeze–thaw samples and freshly prepared samples was observed.

The result of stability experiments shows that no significant degradation occurred at ambient temperature for 6 h for short term stability, at -20°C for 6 weeks for long term stability and for the post preparative stability for 24 h after comparing with freshly prepared sample. Results of stability for both RP-HPLC and TLC methods are shown in tables 3 and 4.

Conclusion

The proposed RP-HPLC and Densitometric-TLC methods for the estimation of pazufloxacin mesylate in human plasma are selective and sensitive.

Sensitivity of the method is suitable for handling various plasma levels of the drug. The method is economical and faster than earlier published methods. In future these methods can be used for bioequivalence study.

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