

## Development and Validation of a HPLC Method for Determination of Pefloxacin in Tablet and Human Plasma

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### Abstract

#### Objective(s)

Developing and validating a simple, efficient, reproducible and economic reversed phase high performance liquid chromatographic (RP-HPLC) method for the quantitative determination of pefloxacin in bulk material, tablets and in human plasma.

#### Materials and Methods

A shim-pack CLC-ODS column and a mobile phase constituting acetonitrile: 0.025 M phosphoric acid solution (13:87 v/v, pH 2.9 adjusted with KOH) were used. The flow rate was 1 ml/min and the analyses performed using ultraviolet (UV) detector at a wavelength of 275 nm using acetaminophen as an internal standard.

#### Results

The developed method showed good resolution between pefloxacin and acetaminophen. It was selective to pefloxacin and able to resolve the drug peak from internal standard and from formulation excipients. The percentage of coefficient variation (CV) of the retention times and peak areas of pefloxacin from the six consecutive injections were 0.566% and 0.989%, respectively. The results showed that the peak area responses are linear within the concentration range of 0.125 µg/ml-12 µg/ml ( $R^2= 0.9987$ ). The limits of detection (LOD) and limits of quantitation (LOQ) for pefloxacin were 0.03125 µg/ml and 0.125 µg/ml. The intra-day and inter-day variation, RSD were 0.376-0.9056 and 0.739-0.853 respectively; also, inter-day variation with relative standard deviation (RSD) were 0.1465-0.821 in plasma. The accuracy results of 70%, 100%, and 130% drugs were 100.72%, 100.34%, and 100.09%, respectively.

#### Conclusion

The method is linear, quantitative, reproducible and could be used as a more convenient, efficient and economical method for the trace analysis of drug in biological fluids, in raw material and tablets.

**Keywords:** Antibiotics, Fluoroquinolone, High Performance Liquid Chromatography, Pefloxacin, Quantitative analysis, Validation studies

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## Introduction

Pefloxacin (Figure 1) is a fluorinated quinolone with an extended antimicrobial spectrum against the majority of Gram-negative microbes and staphylococci including methicillin resistant strains (1, 2). Furthermore, pefloxacin possesses some favorable pharmacokinetic properties, including complete absorption after oral administration, long half-life (10-12 hr) permitting infrequent dosage and rapid penetration into the intracellular and extracellular spaces. It is metabolized in the liver to an N-oxide derivative and to a microbiologically active desmethyl-pefloxacin (norfloxacin), which are excreted in the urine (3, 4). Clinical studies have shown that pefloxacin is highly effective in a wide-range of serious infections (5, 6).

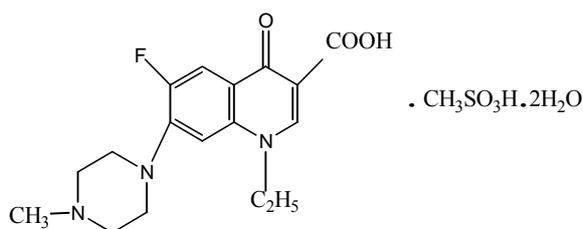


Figure 1. Pefloxacin

Suitable and sensitive analytical methods for determination of drug and their metabolites are essential for successful evaluation of clinical pharmacological, pharmacokinetic, bioavailability, and bioequivalence (7).

Several analytical methods for quantitative determination of pefloxacin in pharmaceutical formulations and in biological fluids are described in the literature, such as Ni *et al* (8). Basavaiah *et al* (9) and Mostafa *et al* (10) studies, which used spectrophotometric method for the determination of pefloxacin in pharmaceutical preparations and human plasma samples. Sun *et al* (11), Fierens *et al* (12) and Flurer (13) used capillary electrophoresis method for the estimation of pefloxacin. Several other studies validated HPLC method for quantitative determination of antibiotics in pharmaceutical preparations (14-19). Beltagi (20) developed a fully validated square wave cathodic adsorptive

stripping voltammetric procedure for the determination of the pefloxacin drug in bulk form, tablets and human serum. Wang *et al* (21) determined the fluorinated quinolone drugs by thin-layer chromatography /fluorescence densitometry. Fratini and Schapoval (22) as well as Jelikić-Stankov *et al* (23) used UV spectrophotometer for the determination of fluoroquinolone. Belal *et al* used titrimetry method for the analysis of pefloxacin (24). Most of the reported methods involve troublesome mobile phase (buffers) and difficult detection methods such as fluorescence or mass detectors. The aim of this study was to develop a simple, economical method for the analysis of pefloxacin.

## Materials and Methods

The HPLC system consisted of a LC-10AT VP Shimadzu liquid chromatograph (Japan) equipped with SPD-100AVP Shimadzu UV-VIS detector. Chromatographic separations were performed on C<sub>18</sub> Shim-Pack CLC-ODS column (6 mm ID×15 cm), attached to a guard column (octa decyl silane guard column), connected to CBM-102 Communications Bus Module Shimadzu (Japan) with PC (P-I I I). In addition, electronic balance, microliter syringe, micropipette and micropore filtration assembly used in this study.

Pefloxacin mesylate and acetaminophen (paracetamol) standard powder were gifted by Aventis Pharma (Pvt) Ltd, Karachi, Pakistan and Zafa Pharmaceutical (Pvt.) Laboratory, Karachi, Pakistan. Eight different brands of pefloxacin mesylate obtained from retailers of Karachi (Pakistan) market. All solvents were of HPLC grade and reagents of analytical grade. Ortho-phosphoric acid (89% (Merck)), acetonitrile (Merck), potassium hydroxide (Merck), ethyl acetate, heparins and the fresh human blood obtained from Baqai Medical University, Karachi, Pakistan and double distilled water used to prepare mobile phase.

### Preparation of stock solution in mobile phase

Stock solution of pefloxacin (400 µg/ml, on dried basis) and acetaminophen (internal standard, 400 µg/ml, on dried basis) were prepared by dissolving in double distilled

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water and a series of dilution for the calibration curves (0.25 µg/ml-12 µg/ml) prepared in mobile phase according to the study design.

### *Preparation of mobile phase*

0.025 M orthophosphoric acid and acetonitrile (87:13 v/v) used as mobile phase whereas, the pH of mobile phase was adjusted to 2.9 with 1 N KOH solution. All solvents and solutions filtered through filtration unit (Millipore, 0.45 µm pore size) and degassed before use. The flow rate maintained at 1 ml/min and volume of injection was 20 µl. Detection performed at a wavelength of 275 nm and analysis was carried out at ambient temperature.

### *Preparation of stock solution in plasma*

Stock solution of pefloxacin (500 µg/ml, on dried basis) and acetaminophen (internal standard, 500 µg/ml, on dried basis) first prepared by dissolving in double distilled water, then it was diluted in fresh plasma to prepare stock solution in plasma (50 µg/ml) and a series of dilution for the calibration curves prepared in plasma according to the study design.

### *Extraction of pefloxacin from plasma*

Extraction carried-out in a ratio of 1:1 with ethyl acetate. Vortex mixing for 1 min and centrifuged for 5 min at 5000 rpm, resulted in deproteinization. Supernatant layer collected and transferred to a 25 ml separate beaker.

This procedure repeated, and organic phase evaporated to dryness at room temperature. The residue reconstituted in 0.5 ml of mobile phase, filtered with 0.45 µm pore size filter paper and 20 µl filtrate was injected into the HPLC column.

### *Validation procedure*

The study was conducted to obtain an affordable and convenient method for HPLC determination of pefloxacin. The experiment carried-out according to the official specifications of United State Pharmacopeias (USP-27), Global Quality Guidelines-2002, International Conference on Harmonization

(ICH-1996) and Centre of Drug Evaluation and Research (CDER-1994) (25-28). The method validated for the parameters like system suitability, specificity, range and linearity, limit of detection, limit of quantification, accuracy, precision, ruggedness and robustness.

The system suitability assessed by six replicate analysis of the drug at a concentration of 20 µg/ml and was used to verify that the resolution and reproducibility of the chromatographic system adequate for the analysis to be done. This method evaluated by analyzing the repeatability, retention time, peak area of pefloxacin, tailing factor, theoretical plates (Tangent) of the column and resolution between the peaks of pefloxacin and acetaminophen (internal standard).

Selectivity was the critical basis for analytical procedure. Chromatographic method determined to ensure separation of pefloxacin from internal standard as well as the separation of active ingredient (pefloxacin) in the presence of excipients used in formulation. Chromatogram was also observed and compared with that of raw material.

A linear relationship evaluated across the range of the analytical procedure. The range of an analytical method is the interval between the upper and lower analytical concentrations of a sample while the method has shown acceptable accuracy, precision and linearity. To evaluate the linearity, LOD and LOQ of the method in reference drug, serial dilutions were made from the standard stock solution in the range of 0.03125-12 µg/ml.

To further validate the accuracy of the purpose assay method regarding recovery from plasma, six different known concentrations of the pefloxacin standard (range from 0.25-12 µg/ml) in mobile phase and plasma were analyzed. Samples for recovery studies (in pharmaceutical dosage form) also prepared by known amount of drug at three concentration levels (in triplicate) i.e. 70%, 100% and 130% levels of the target pefloxacin and were analyzed according to the procedures.

The precision of the method investigated with respect to repeatability (a minimum of 6 determinations at 100% of the test

concentration, system suitability), intermediate precision (intra-day and inter-day variation), and reproducibility (by means of an inter-laboratory trial). To evaluate the ruggedness of the method, the procedure repeated in the laboratory of Baqai Medical University, Karachi. Robustness studies performed on method precision, using a sample concentration of 20 µg/ml by making slight variations in flow rate, concentration of acetonitrile and change in pH of mobile phase.

For bioanalytical studies, the stability of analyte in plasma was evaluated, in order to verify that no degradation has taken place between the time of sample collection and the analyses.

#### **Assay for pefloxacin tablets**

A total of 20 tablets were thoroughly weighed; ground to a fine powder and an amount of powder equivalent to average weight of tablets transformed to a 250 ml volumetric flask. It was dissolved in the mobile phase and shaken for about 10-15 min, then filtered through 0.45 µm filter paper. Filtrated sample solution further diluted in the mobile phase to make the final concentration of working solution equivalent to 20 µg/ml.

## **Results**

In order to validate a simple and efficient

method for the analysis of the drug in pharmaceutical formulations, as well as in plasma, preliminary tests performed with the objective to select adequate and optimum conditions. Parameters, such as detection wavelength, ideal mobile phase and their proportions, optimum pH and concentration of the standard solutions carefully studied. Several eluents tested using different proportions of solvents, such as acetonitrile, methanol, buffers with different pH and water. Present study showed a simple, sensitive, and an easy HPLC method to operate, using UV detection for the determination of pefloxacin in raw material, tablets and in the human plasma.

#### **System suitability**

This test was performed by collection of data from replicated injection of standard or resolution solution (acetaminophen+ pefloxacin) given in the Table 1. The percentage coefficient variation (CV%) of the retention times and of the peak areas of pefloxacin from the six consecutive injections of the resolution solution were 0.566% and 0.989%, respectively. The Mean theoretical plate count, based on USP tangent calculations (30) for pefloxacin peak was 8197.23, and the resolution between pefloxacin and acetaminophen was 21.358.

Table 1. System suitability results.

Injection number	Retention time (min)	Peak area of pefloxacin	Tailing factor	Tangent	Resolution
1	17.66	561362	1.166	8195.3	21.5
2	17.77	566205	1.166	8195.3	21.16
3	17.67	576081	1.148	7948.8	21.5
4	17.73	565825	1.166	8195.3	21.66
5	17.57	562965	1.166	8195.3	21.0
6	17.86	560986	1.186	8453.4	21.33
<b>Mean</b>	17.71	565571	1.166	8197.2	21.36
<b>CV (%)</b>	0.566	0.989	1.031	1.947	1.146

#### **Linearity and range**

Standard calibration curve constructed, using known amounts of pefloxacin in the concentration range of 0.125-12.0 µg/ml including the LOQ range of 0.125 µg/ml with a regression analysis ( $R^2=0.9987$ ). The least square method used to calculate the regression equations. The value of intercept recorded +650.87 and slope 31690 whereas the LOD was 0.03125 µg/ml.

#### **Specificity**

A consequence of this requirement is that the resolution of the analyte from the other components (co-administrated drugs such as pefloxacin and acetaminophen) should be more than 1.5-2.0 (31). Specificity was also determined in the presence of excipients used in the formulation of pefloxacin mesylate tablets 400 mg.

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### Absolute recovery

The accuracy of an analytical method was given by the extent by which the value obtained deviates from the true value. In biological samples, the recovery should be  $\pm 10\%$  and the acceptance criterion for recovery data is 98-102% or 95-105% for drug preparation (31). Thus the mean absolute recovery of the method calculated within the range of 90.92 to 98.59% from plasma (Table 2) and the percentage of CV of this absolute recovery was 1.006% to 6.724%.

Accuracy also determined by preparing

samples of PEF-c 400 mg (code of finished pack) in triplicate at the 70%, 100%, and 130% levels of the target pefloxacin concentration and analyzed according to the procedure. The percentage recovery ranged from 100.09% to 100.72% of the label claim of pefloxacin mesylate tablets at all three levels of the recovery analysis, and the percentage of CV values for each level, ranged from 0.36% to 0.76%. The overall mean percentage recovery was 100.38 per tablet with an over all CV percentage of 0.199%.

Table 2. Recovery data of standard concentration solution of pefloxacin in mobile phase and in plasma (Expressed in  $\mu\text{g/ml}$  based on calibration curve).

Standard concentration	Amount measured ( $\mu\text{g/ml}$ )		Recovery (%)	CV (%)
	Mobile phase	Plasma		
12 $\mu\text{g/ml}$	12.35	11.914	96.47	2.54
8 $\mu\text{g/ml}$	8.96	8.164	91.116	6.574
4 $\mu\text{g/ml}$	4.329	3.936	90.92	6.724
1 $\mu\text{g/ml}$	1.12	1.075	95.98	2.899
0.5 $\mu\text{g/ml}$	0.538	0.491	91.264	6.46
0.25 $\mu\text{g/ml}$	0.283	0.279	98.59	1.006

### Selectivity

The selectivity of the method determined by comparison of chromatograms obtained from standard concentration of pefloxacin in mobile phase and chromatograms of extracts obtained from plasma (Figure 2A). A good separation between pefloxacin and acetaminophen achieved by use of the chromatographic

conditions (Figure 2B). Retention times were 17.34 and 5.828 min respectively, and changed less than 3% in both intra-day and inter-day analyses. Whereas, the specificity of the method determined by running a blank solution and no interference found with other extraneous components (Figure 2C).

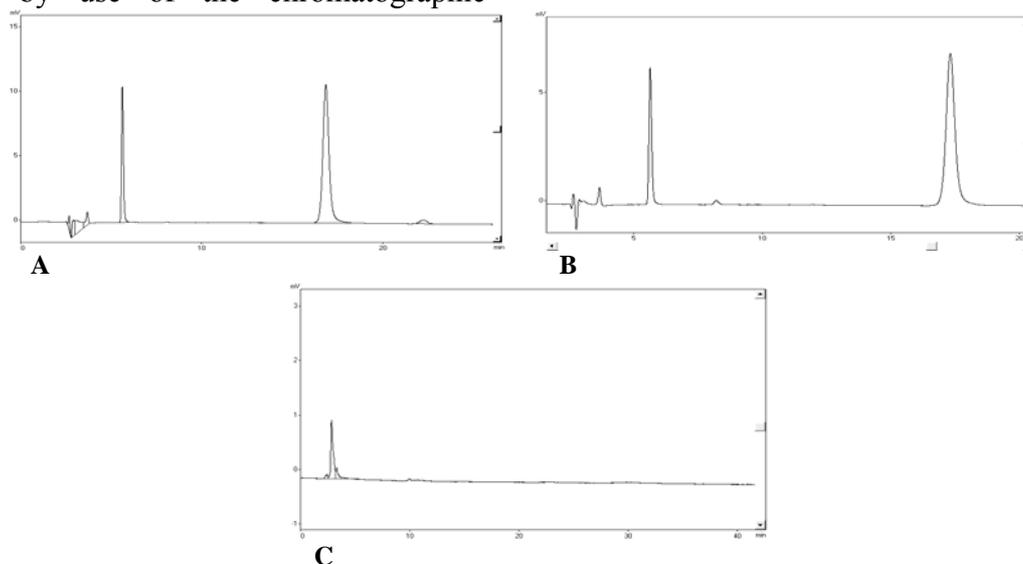


Figure 2. A: Standard concentration of pefloxacin in mobile phase. B: Same standard concentration of pefloxacin in plasma. C: Blank sample of plasma. Chromatograms obtained under the same conditions.

A = Chromatogram of pefloxacin with acetaminophen in mobile phase shows good separation, B = Chromatogram of pefloxacin with acetaminophen in plasma shows good separation, C = Without active ingredient chromatogram shows no additional peak at the retention time.

**Precision and accuracy**

The precision of the method evaluated by determining the intra-day and inter-day CV percentage of the measured concentrations of pefloxacin in the same samples of mobile phase. The reproducibility (intra-day precision) and repeatability of system (inter-

day precision) checked by injecting the different concentrations of standard solution on the same day and different days respectively, which shows in significant variation (Tables 4 and 5).

Table 4. Intra-day variation (Expressed in µg/ml based on calibration curve).

Standard concentration	Amount measured (µg/ml)			Mean	Recovery (%)	CV (%)
	8.30 am	1.0 pm	5.0 pm			
12 µg/ml	11.649	11.689	11.691	11.676	97.30	0.204
	11.903	11.853	11.703	11.819	98.497	0.879
Mean	11.776	11.771	11.697	11.748	97.9	0.376
4 µg/ml	4.275	4.307	4.278	4.287	107.175	0.413
	4.306	4.395	4.278	4.326	108.15	1.412
Mean	4.290	4.351	4.278	4.306	107.662	0.905
1 µg/ml	0.923	0.916	0.929	0.923	92.3	0.723
	0.894	0.933	0.903	0.910	91.0	2.231
Mean	0.908	0.924	0.916	0.916	91.65	0.856

Table 5. Inter-day variability (three different concentrations of standard solution injected on different days).

Standard concentration	Amount measured (µg/ml)			Mean	Recovery (%)	CV (%)
	1st day	2nd day	3rd day			
12 µg/ml	11.649	11.949	12.03	11.876	98.97	1.689
	11.903	11.668	11.854	11.808	98.4	1.05
Mean	11.776	11.808	11.941	11.842	98.683	0.739
4 µg/ml	4.275	4.349	4.318	4.314	107.85	0.859
	4.306	4.366	4.281	4.318	107.95	1.012
Mean	4.290	4.358	4.299	4.316	107.9	0.853
1 µg/ml	0.923	0.904	0.939	0.922	92.23	1.887
	0.894	0.932	0.908	0.911	91.15	2.106
Mean	0.908	0.918	0.924	0.917	91.7	0.836

**Stability in matrices**

Analyte stability should be evaluated after three freeze and thaw cycles (32-35). Samples were analyzed on three consecutive days

(inter-day precision, in plasma). The percentages of obtained CV values were lower than 3% (Table 6).

Table 6. Inter-day variability in plasma.

Standard Concentration	Amount measured (µg/ml)			Mean	Recovery (%)	CV (%)
	1st day	2nd day	3rd day			
12 µg/ml	12.453	12.328	12.272	12.351	102.925	0.750
	11.903	11.968	11.854	11.908	99.23	0.480
Mean	12.178	12.148	12.063	12.129	101.08	0.492
4 µg/ml	4.087	3.852	3.998	3.979	99.475	2.981
	4.006	4.126	3.981	4.038	100.95	1.919
Mean	4.046	3.989	3.989	4.008	100.2	0.821
1 µg/ml	0.975	0.987	0.988	0.983	98.3	0.736
	0.994	0.982	0.986	0.987	98.7	0.619
Mean	0.984	0.984	0.987	0.985	98.53	0.146

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### Robustness

Robustness study performed on absolute recovery sample # 2 (100%) by making slight variations in flow rate, amount of acetonitrile, and change in pH, one at a time. Acceptable robustness results obtained in the range of 98-102% (Table 7).

pefloxacin was also rechecked in the laboratory of Baqai Medical University, Karachi, Pakistan in the same concentration range (0.125 µg/ml – 12 µg/ml) as were used in the Department of Pharmaceutics, Faculty of Pharmacy, University of Karachi and the obtained result was  $\leq 1$  regression value.

### Ruggedness

The procedure developed for the analysis of

Table 7. Robustness test.

Parameters	Changes	% Recovery	% of Target
<b>Target conditions</b>		100.34	100.0
<b>Flow rate</b>	0.9 ml/min	99.6	99.26
	1.1ml/min	100.9	100.56
<b>Change in pH</b>	2.7	101.4	101.05
(mobile phase)	3.1	101.8	101.45
<b>Acetonitrile variation</b>	11 %	99.90	99.64
	15 %	100.4	100.06

### Assay of pefloxacin mesylate tablets 400 mg

The developed method applied for the determination of pefloxacin content in marketed formulation (tablets 400 mg). The assay result showed that this method was

sensitive and specific for the quantitative analysis of pefloxacin in raw material and also in dosage form. Acceptable results obtained in the range of 95-105% (Tables 8 and 9).

Table 8. Assay comparisons of 8 different brands of pefloxacin mesylate tablets (400 mg). (Weight of standard drug= 19.3868 mg (dried basis)).

Assay	PEF <sub>A</sub>	PEF <sub>B</sub>	PEF <sub>C</sub>	PEF <sub>D</sub>	PEF <sub>E</sub>	PEF <sub>F</sub>	PEF <sub>G</sub>	PEF <sub>H</sub>
<b>1</b>	399.74	403.45	409.70	398.40	403.71	395.91	396.79	408.1
<b>2</b>	396.88	397.65	407.82	406.99	401.57	397.12	407	405.95
<b>SUM</b>	796.62	801.1	817.52	805.4	805.27	793.03	803.79	814.05
<b>Mean</b>	398.31	400.55	408.76	402.699	402.64	396.51	401.9	407.02
<b>±SD<sup>a</sup></b>	2.027	4.10	1.34	6.073	1.52	0.86	7.22	1.52
<b>±SEM<sup>b</sup></b>	1.43	2.90	0.94	4.29	1.07	0.61	5.10	1.08

SD<sup>a</sup> = standard deviation, SEM<sup>b</sup> = standard error of mean

### Discussion

Recently, HPLC has become applicable for the antibiotics analysis, allowing accurate determination of the concentration in relatively small body fluid samples. There is a need to

consider the successive steps for the development of HPLC method. Particularly the problems related to the standardization of sample preparations and to development of mobile phase must be emphasized.

Table 9. Analysis of variance for content assay of 8 different brands of pefloxacin tablets.

Source of Variation	SS	DF	MS	F	P-value	F crit
Between Groups	233.87	7	33.4104	2.28314	0.13509	3.5004
Within Groups	7.0657	8	4.63322			
Total	350.93	1	-			

SS= Sum of squares, DF= degree of freedom, MS=mean square, F= computed f

At the 0.05 level, the means are not significantly different.

F= 0.244006, T – Stat (Paired) = -1.52434, T-Stat = -0.69159 (t-Test: Two-Samples Assuming Equal Variances)

T-Stat = -0.69159 (t-Test: Two-Samples Assuming Unequal Variances).

Compared to the HPLC method mentioned earlier, the studied HPLC method has some advantages. First, the extraction procedure is simple and involves only one step. Other advantages are using a commonly used reversed-phase chromatographic column, simple composition of an isocratic mobile phase and UV absorbance measurement for detection. The method has been proved to be linear, reproducible, quantitative and applicable to human plasma samples. The proposed method in this study was an improved and validated method of Al-Obaidy *et al* (29). The proposed method is simple and does not involve laborious and time-consuming sample preparation.

Although for validation of the present method we used acetaminophen as an internal standard, acetaminophen is not structurally similar to the pefloxacin whereas, according to the ICH the internal standard must be structurally similar to the main compound of interest. The reason for this selection was that the actual study of pharmacokinetics is to estimate the drug interaction of pefloxacin with acetaminophen *in vivo*. Though, the method required for analysis should be the same as that used for validation.

The second important consideration was its retention time, which was not very prompt. The reason behind it, is the *in vivo* study in which the plasma impurities usually appeared at 5-6 min, so it was necessary to keep the actual peak beyond the initial 7-8 min.

This study shows that the plasma samples found to be stable after three weeks (as reported by Abanmi *et al* (36) with no

significant change in concentration when stored at  $-20^{\circ}\text{C}$ .

The present study is a cost effective or economical method, because 10-15 companies in Pakistan are engaged in formulating the pefloxacin tablets. But still they are confused about their analysis is economical (solvent wise), simple (steps wise), and easy to perform.

The range of analysis of Santoro *et al* (14) was 4-24  $\mu\text{g/ml}$  and was only applied on formulation. It can not be used for biological metrics where as, the present study has the analysis range from 0.25-12 $\mu\text{g/ml}$  with  $R^2=0.9987$ , can be used in pharmacokinetics study *in vivo* because the relationship between concentration and area of peaks is also linear in plasma.

Brkich *et al* (38) used fluorescent detector whereas, in the present study, UV-visible detector is simply used which is easily available. We are Pakistan based researchers and most of our Pharmaceuticals have HPLC with UV-visible detector. So, it is the need of our industries to develop such methods that can easily be analyzed by available facilities. Extraction done by acetonitrile in the mentioned method. The area under the peaks obtained by this extraction was markedly lower as compared to the values obtained by aqueous solution (it is mentioned in their paper). So to elucidate the problem, authors used acetonitrile: water ratio, and they also obtained markedly high variation coefficient i.e. 6-8 %. Whereas, in the proposed study, ethyl acetate was used to extract the drug from plasma. This organic solvent could easily be evaporated at room temperature and the

residue can simply be dissolved in mobile phase before injecting it in HPLC and the results seem to be optimal. This change in technique makes the whole method simple, convenient and operator friendly.

This work is cost effective as compared to Groeneveld *et al* (18) in a way that dichloromethane was used for the extraction which dries under nitrogen. Moreover, its mobile phase was comprised of 0.04 M phosphoric acid, tetrabutyl ammonium iodide, and methanol, which is more complex solvent system as compared to the present work. This more complex design increases the chance of error and inter-operator variations.

### Conclusion

Analytical data of the present study can be used to screen the drug potential, that aids in

the development of drug synthesis, support formulation studies, monitor the stability of bulk pharmaceuticals and tests final product for release. Using the proposed method of HPLC, the *in vivo* blood level determination of pefloxacin would be convenient as the limit of detection of pefloxacin in human plasma was as low as 0.0625 µg/ml.

### Acknowledgment

Deep appreciation for the Department of Pharmaceutics, Faculty of Pharmacy, University of Karachi and Institute of Pharmaceutical Sciences, Baqai Medical University, Karachi, Pakistan, for giving us moral and generous help during this research. The authors declare that they have no conflict of interests.

### References

1. Mitsuhashi S. Comparative antibacterial activity of new quinolone-carboxylic acid derivatives. *Rev Infect Dis* 1989; 10:S27-31.
2. Yourassowsky E, Van der Linden MP, Crokaert F, Gluyczynski Y. *In vitro* activity of pefloxacin compared to other antibiotics. *J Antimicrob Chemother* 1986; 17:S19-28.
3. Frydman AM, LeRoux Y, Lefevre MA, Djebbar F, Fourtillan JB, Faillot J. Pharmacokinetics of pefloxacin after repeated intravenous and oral administration (400 mg bid) in young healthy volunteers. *J Antimicrob Chemother* 1986; 17:65-79.
4. Gerding DN, Hitt JA. Tissue penetration of the new quinolones in humans. *Rev Infect Dis* 1989; 11:S1046-1057.
5. Webster A, Gaya H. Quinolones in the treatment of serious infections. *Rev Infect Dis* 1989; 10: S225-230.
6. Lauwers S, Vincken W, Naessens A, Peirard D. Efficacy and safety of pefloxacin in the treatment of severe infections in patients hospitalized in intensive care units. *J Antimicrob Chemother* 1986; 17:111-115.
7. Raghov G, Meyer MC. High-performance liquid chromatographic assay of tolbutamide & carboxytolbutamide in human plasma. *J Pharm Sci* 1981;70:1166-1167.
8. Ni Y, Wang Y, Kokot S. Multicomponent kinetic spectrophotometric determination of pefloxacin and norfloxacin in pharmaceutical preparations and human plasma samples with the aid of chemometrics. *Spectrochim Acta A Mol Biomol Spectrosc* 2008;70:1049-1059.
9. Basavaiah K, Prameela HC, Somashekar BC. Spectrophotometric determination of pefloxacin mesylate in pharmaceuticals. *Acta Pharm* 2007; 57:221-230.
10. Mostafa S, El-Sadek M, Alla EA. Spectrophotometric determination of enrofloxacin and pefloxacin through ion-pair complex formation. *J Pharm Biomed Anal* 2002; 28:173-180.
11. Sun SW, Chen LY. Quantitative determination of gatifloxacin, levofloxacin, lomefloxacin and pefloxacin. *J Chromatogr A* 1997; 766:215-224.
12. Fierens C, Hillaert S, Van den Bossche W. The qualitative and quantitative determination of quinolones of first and second generation by capillary electrophoresis. *J Pharm Biomed Anal* 2000; 22:763-772.
13. Flurer CL. Analysis of antibiotics by capillary electrophoresis. *Electrophoresis* 1997; 18:2427.
14. Santoro MI, Kassab NM, Singh AK, Kedor-Hackmam ER. Quantitative determination of gatifloxacin, levofloxacin, lomefloxacin and pefloxacin fluoroquinolonic antibiotics in pharmaceutical preparations by high-performance liquid chromatography. *J Pharm Biomed Anal* 2006; 40:179-184.
15. Samanidou VF, Demetriou CE, Papadoyannis IN. Development of a validated HPLC method for the determination of four penicillin antibiotics in pharmaceuticals and human biological fluids. *Anal Bioanal Chem* 2003; 375:623-629.
16. Joshi S. Quantitative determination of ciprofloxacin and norfloxacin in pharmaceutical preparations by high performance liquid chromatography. *J Pharm Biomed Anal* 2002; 28:795-809.

17. Sanzgiri D, Knaub SR, Riley CM. Quantitative determination of gatifloxacin, levofloxacin, lomefloxacin and pefloxacin fluoroquinolonic antibiotics in pharmaceutical preparations by high-performance liquid chromatography. *Anal Profiles Drug Subst Excipients* 1994; 23:325–369.
18. Groeneveld AJ, Brouwers JR. Quantitative determination of ofloxacin, ciprofloxacin, norfloxacin and pefloxacin in serum by high performance liquid chromatography. *Pharm Weekbl Sci* 1986; 8:79-84.
19. Montay G, Blain Y, Roquet F, Le Hir A. High-performance liquid chromatography of pefloxacin and its main active metabolites in biological fluids. *J Chromatogr* 1983; 272:359-365.
20. Beltagi AM. Determination of the antibiotic drug pefloxacin in bulk form, tablets and human serum using square wave cathodic adsorptive stripping voltammetry. *J Pharm Biomed Anal* 2003; 31:1079-1088.
21. Wang PL, Chen L, Fan YF. Selective separation and simultaneous determination of trace levels of five types of fluorinated quinolone drugs by thin-layer chromatography /fluorescence densitometry. *J AOAC Int* 2001; 84:684-688.
22. Fratini L, Schapoval EES. In sensitive spectrophotometer methods for the determination of amoxicillin, ciprofloxacin. *Int J Pharm* 1996; 127:279–282.
23. Jelikić-Stankov M, Veselinović D, Malesev D, Radović Z. Spectrophotometric determination of pefloxacin in pharmaceutical preparations. *J Pharm Biomed Anal* 1989; 7:1571-1577.
24. Belal F, Al-Majed AA, Al-Obaid AM. Methods of analysis of 4-quinolone antibacterials. *Talanta* 1999; 50:765–786.
25. United States Pharmacopeia. 28th ed. United States Pharmacopeial Convention, Rockville: 2005.
26. Global Quality Guideline, Validation of analytical procedures 2002; Number: G-6.9, Version 1.0.
27. ICH Q2B, Guidance for Industry, Validation of analytical procedures—methodology, 1996.
28. Reviewer guidance validation of chromatographic methods, Center for drug evaluation and research, food and drug administration 1994.
29. AL-Obaidy, Po SS, McKiernan ALWPJ, Glasgow JFT, Millership J. Assay of paracetamol and its metabolites in urine, plasma and saliva of children with chronic liver disease. *J Pharm Biomed Anal* 1995; 13:1033-1039.
30. United States Pharmacopeia, XXV11. Chromatography, 2004.
31. Mohammad Y, Gunawan I. Validation of chromatographic methods of analysis. Profiles of drug substances, excipients, and related methodology, Vol 32, 2005.
32. Garofolo F. Bioanalytical method validation, In: Chan CC, Lam H, Lee YC, Zhang XU, editors. Analytical method. Validation and instrument performance verification. Wiley-Interscience; 2004. p.105-138.
33. Hartmann C, Smeyers-Verbeke J, Massart DL, McDowall RD. Validation of bioanalytical chromatographic methods. *J Pharm Biomed Anal* 1998; 17:193-218.
34. Guidances for Industry, Bioanalytical method validation, US department of health services, food and drug administration, center for drug evaluation and research, center for veterinary medicine.
35. Dadgar D, Burnet PE, Choc MG, Gallicano K, Hooper JW. Application issues in bioanalytical method validation, sample analysis and data reporting. *J Pharm Biomed Anal* 1995; 13:89-97.
36. Abanmi N, Zaghoul I, El Sayed N, Al-Khamis KI. Determination of pefloxacin and its main active metabolite in human serum by high performance liquid chromatography. *Ther Drug Monit* 1996; 18:158-163.
37. Wilson, Gisvold's. Textbook of organic and medicinal and pharmaceutical chemistry. 10th ed. 1998.
38. Brkich GÉ, Arzamastsev AP, Kaz'mina ÉM, Mikhalev AV, HPLC determination of pefloxacin and ciprofloxacin in blood serum, *Pharm Chem J* 1999; 33:219-221.