A RP-HPLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF DILTIAZEM AND QUINOLONES IN BULK, FORMULATIONS AND HUMAN SERUM

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ABSTRACT

High Performance Liquid Chromatographic method was developed and applied for the simultaneous determination of diltiazem and fluoroquinolones in bulk, pharmaceutical formulations and human serum in presence of caffeine as internal standard. A Hiber®, RP-18 column was used with mobile phase consisting of acetonitrile:methanol:water (30:20:50 v/v, pH 3.6) and quantitative evaluation was performed at 230 nm. Suitability of this method for the quantitative determination of the drugs was proved by validation in accordance with the requirements laid down by International Conference on Harmonization (ICH) guidelines.The method was selective, precise, accurate and can be used for routine analysis of pharmaceutical preparations, quality control and clinical laboratories.

Keywords: Diltiazem, floroquinolones, method validation, HPLC determination.

INTRODUCTION

Diltiazem is a benzothiazepine calcium channel blocker with peripheral and coronary vasodilator properties. It lowers the blood pressure and has effect on cardiac conduction. It is administered orally in the treatment of angina pectoris and hypertension and may be given intravenously in the treatment of arterial fibrillation or flutter and paroxysmal supraventricular tachycardia[1]. Flurorquinolones are a class of important synthetic antibiotics, which are active against both Gram (+) and Gram (-) bacteria through inhibition of their DNA gyrase, also they have some activity against myobacteria, mycoplasmas and rickettsias[2].

It has been concluded in one of the studies that calcium channel blocker produced synergistic antimicrobial effect due to its ability to convert cryptic forms of chronic bacterial infections to a non-cryptic form. This synergistic effect was found to be present when they are co-administrated with antibiotics in appropriate doses[3]. Ethan and Cammb[4] demonstrated that patients who are susceptible to cardiac arrhythmias because of underlying heart diseases, metabolic derangement and medications that prolong the QT interval, has raised the issue of the cardiac safety of the newer fluoroquinolones. Hence due to safety issues including cardiotoxic effects various fluroroquinolones have been removed from the market. Macrolides, ketolides and fluoroquinolones as well as other classes of antimicrobial agents have been associated with prolongation of cardiac repolarisation. This effect is most notable with erythromycin, clarithromycin, gatifloxacin, moxifloxacin, levofloxacin and telithromycin[5]. It was also concluded by Mary *et.al.*,[6] that xenobiotics metabolism was inhibited by diltiazem. In another study it was demonstrated that diltiazem may induce P. aeruginosa biofilms, resistance to the first-line fluoroquinolones. In this study, it was demonstrate that for P. aeruginosa biofilms which are resistance to the first-line fluoroquinolones[7]. It was also established that no augmentation of fluoroquinolone activity in the time-kill curves occured for both diltiazem and verapamil. They had no effects on levofloxacin killing curves against SA 1199 or 1199B[8].

Several investigations have been performed for the determination of diltiazem in pharmaceutical formulations[9-13] and in plasma or serum[14-16] by spectrophotometry and high performance liquid chromatography with ultraviolet detection. More over, these methods only focused on separation of individual drug. However, several analytical procedures have also been described for simultaneous separation and enantio separation of diltiazem, its analogs, possible degradation products and metabolites using high performance liquid chromatography [10,17-19]. Similarly several chromatographic methods have been reported for the separation [20,21] and simultaneous determination of two or more fluoroquinolones in human plasma[22-28]. Over the last two decades, there has been growing concern about probable drug interaction between two different categories of co administered drugs which may result in the loss of therapeutic effects of drug. In order to carry out these drug interactions we need an authentic HPLC method. However, in literature no HPLC method for separation and simultaneous determination of diltiazem and floroquinolones has been reported. In present paper a universal HPLC method for the consecutive determination of these drugs in bulk material, pharmaceutical formulations and human serum has been described and validated.

Aim of the study

The aim of the present study was to establish an efficient, reliable, accurate and sensitive method for the separation and quantitative determination of both the drugs simultaneously. Simultaneous determination of both drugs is desirable as this would allow more efficient generation of clinical data and could be performed at more modest cost than separate assays. The applicability of the proposed method was demonstrated later for *in-vitro* interaction studies of diltiazem with floroquinolones.

METHOD

Instrumentation

A Shimadzu HPLC system equipped with LC- 10 AT VP pump and SPD-10 A VP UV–VIS detector was utilized. Chromatographic system was integrated via Shimadzu model CBM-102 to P-IV computer loaded with Shimadzu CLASS-VP software (Version 5.03) for data acquisition and mathematical calculations. Rheodyne manual injector fitted with a 20 µL loop, a Hiber®, Pre-Packed Column RT 250-4.6 Purospher® STAR RP-18 endcapped (5 µm) and DGU-14 AM on-line degasser. In addition, Mattler Toledo electronic balance, microliter syringe and micropore filtration assembly were used in this study. Hilton Pharma (Pvt.) Ltd

Material and reagents

Diltiazem, was kind gift from Hilton Pharma (Private) Limited, caffeine and formulations of diltiazem (Dilzem 30 mg), ciprofloxacin (Axcin 250 mg), levofloxacin (Dynaquin 250 mg), ofloxacin (Oflobid 200 mg) and norfloxacin (Floxacin 400mg) of Park Davis (Pvt.) Ltd, Novartis (Pvt.) Ltd, Barrett Hodgson Pakistan, and Hilton Pharma (Pvt.) Ltd. respectively were obtained from retail pharmacies. Each product was labelled and had an expiry of not less than 365 days at the time of study. All reagents used were of HPLC grade. Acetonitrile, methanol and phosphoric acid 85% (Merk, Germany) and HPLC grade deionized filtered water were used to prepare the mobile phase. Stock solutions of diltiazem and floroquinolones were prepared in the mobile phase. Fresh working solutions were prepared daily. All solutions were filtered (0.45 µm) and degassed using sonicator.

Preparation of solutions

Standard solutions of diltiazem, quinolones and caffeine were prepared by dissolving appropriate amounts of each in mobile phase acetonitrile: methanol: water (30:20:50, v/v) to obtain final drug concentrations of 50 $\mu g mL^{-1}$. For the calibration standards, seven calibrators of each drug were prepared by making serial dilutions from stock solutions. For the assay preparation, the content of 20 tablets were powdered, weighed portion of the powder equivalent to the suitable amount of drug (according to the labeled claimed) was transferred into a 50 mL volumetric flask. The drug was fully dissolved in mobile phase and then diluted with this solvent up to the mark, seven dilutions of each drug were prepared, portion of this solution was filtered through a disposable 0.45 µm filter and then injected.

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Serum drug analysis

Blood samples were collected from healthy volunteers and after cogulation centrifuged at 3000 rpm for 10 minutes. The supernatant (serum) obtained was stored at –20 °C. After thawing, serum was deprotinated by acetonitrile and spiked daily with working solutions to produce desired concentrations of diltiazem and quinolones. 20 µL volume of each sample was injected and chromatographed under above conditions.

Chromatographic conditions

The chromatographic analysis was performed at ambient temperature with isocratic elution. The mobile phase consisted of acetonitrile:methanol:water (30:20:50 v/v) with pH adjusted to 3.6 with phosphoric acid (85%). The pump was initially set at a flow rate of 1.0 $mLmin^{-1}$ and then switched into 1.5 $mLmin^{-1}$, sample volume of 20 µL was injected in triplicate onto the HPLC column and elute was monitored at 230 nm.

METHOD DEVELOPMENT

Experimental design and optimization of isocratic HPLC conditions

In order to select a proper mobile phase for the separation of diltiazem and quinolones isocratic elution was applied. The optimization of the analytical procedure has been carried out by varying the mobile phase composition, flow rate and pH of the mobile phase. Preliminary the mobile phases investigated were methanol and water, found to retain quinolones in the column whereas caffeine and diltiazem separated from each other, therefore we could not use methanol and water as mobile phase. Optimal retention times (quinolones-2.7 minutes, caffeine-3.3 minutes and diltiazem-6.2 minutes) and best resolution were achieved when mobile phase was acetonitrile:methanol:water (30:20:50 v/v) having pH adjusted to 3.6 with phosphoric acid. Mobile phase selection was based on peak parameters (symmetry, tailing), run time, ease of preparation and cost.

VALIDATION PROCEDURE

All validation steps were carried out according to the ICH guidelines[29]. Method validation establishes that the method performance characteristics are suitable for the intended use. Validation entails evaluation of various parameters of the method such as system suitability, selectivity, specificity, linearity (concentration–detector response relationship), accuracy, precision, sensitivity, detection and quantification limit recovery from the matrix[30]. The system suitability was assessed by five replicate analyses of the drug at a concentration of 25 μgmL^{-1}. System suitability of the method was evaluated by analyzing the repeatability, peaks symmetry (symmetry factor), theoretical plates of the column, resolution between the peaks of quinolones, caffeine and diltiazem, mass distribution ratio(capacity factor) and relative retention.

Specificity is the ability of a method to discriminate between the analyte of interest and other components that are present in the sample[31]. The specificity of the method was evaluated to ensure separation of diltiazem and quinolones from the internal standard. For demonstrating the specificity of the method for drug formulation, the drug was spiked and the excipients used in formulation products. The linearity of the method was evaluated at seven different concentrations that ranged from 1.25-25 μgmL^{-1} for diltiazem and quinolones. Here the peak area using absorbance detection was studied for each drug. The accuracy of the method was evaluated from recovery assay which was made on the formulation samples. Thus, known amounts of each drug were prepared in triplicate at three levels (80%, 100% and 120%) and spiked into their corresponding formulation and the average recovery was calculated as the mean value obtained. To test the precision of the method, triplicate determinations (repeatability, intermediate precision, and reproducibility)[31] of each analyte were carried out on two different non-consecutive days. LOD and LOQ were calculated by the equation given in ICH guidelines[29]. Ruggedness of this method was evaluated in two different labs with two different instruments. Lab 1 was in the Research Institute of Pharmaceutical Sciences, Faculty of Pharmacy University of Karachi, while Lab 2 was in the Department of Chemistry, Faculty of Science, and University of Karachi.

RESULTS AND DISCUSSION

The development of HPLC method for the determination of drugs has received considerable attention in recent years because of their importance in routine quality control analysis. HPLC methods generally require complex and expensive equipment, provision for use and disposal of solvents, labor-intensive sample preparation procedure and personal skilled in chromatographic techniques. The goal of this study was to develop a rapid, more accurate, precise reliable least time consuming HPLC method for the simultaneous determination of diltiazem and quinolones in the form of bulk drug samples, its formulations and human serum using the most commonly employed C-18 column with UV detector.

METHOD DEVELOPMENT

Experimental design and optimization of isocratic HPLC conditions

In the present investigation the best separation of diltiazem from its internal standard and quinolones was achieved using a Hiber®, 250-4.6 STAR RP-18 column which provides efficient and reproducible separation of the components, support with the aim to reduce the retention time (R_t), the retention factor (K') and to improve the resolution (R) of analytes while minimizing solvent usage. Using other type of column under similar experimental condition, the separation lasted about 15 minutes. A mobile phase of acetonitrile:methanol:water (30:20:50 v/v) having pH adjusted with phosphoric acid to 3.6 was provide a reproducible, baseline resolved peak. The lower percentage of acetonitrile in mobile phase results in peak tailing of both components and long analysis duration while higher percentage of acetonitrile in mobile phase results in very little analysis duration. Small changes in pH of the mobile phase had a great influence to the chromatographic behavior of these substances, higher pH of the mobile phase also results in peak tailing and at a lower pH retention time of quinolones and diltiazem was delayed. The criteria to select a given combination of levels were the specificity, peak resolution and omega peak (analysis time). It is obvious from the chromatogram (figure 1 and 3) that quinolones and diltiazem eluted out forming symmetrical peaks and were well separated from the internal standard. The method was found to be rapid as quinolones, caffeine and diltiazem eluted out at 2.7, 3.3 and 6.2 minutes respectively, which is important for routine analysis. More over, all the quinolones eluted at the same retention time i.e. 2.7 minutes but each showed a good resolution from diltiazem and caffeine. In comparison with other published methods for determination of diltiazem and quinolones the advantages of this method are ease of operation, short analysis time (total run time < 10 minutes, which is important for routine analysis), utilization of readily available cost-effective solvents, no matrix interferences, and satisfactory limit of quantification to enable pharmacokinetic studies of diltiazem and quinolones. Reliability, rapidness, simplicity, sensitivity, economical nature, acceptable resolution, good recovery and precision of this method gives it an advantage over the other reported HPLC methods for the determination of diltiazem and quinolones.

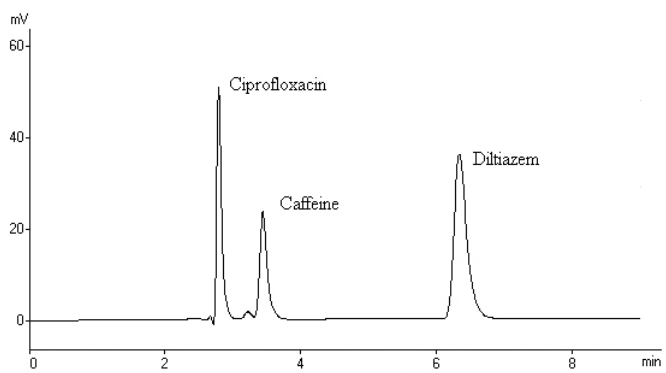

Fig.1: Representative chromatogram showing resolution between ciprofloxacin and diltiazem from the internal standard.

Chromatgraohic condition: Acetonitrile:methanol:water (30:20:50 v/v, pH 3.6)

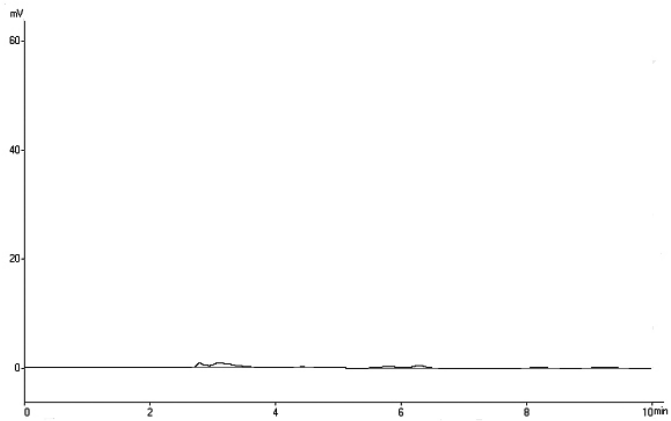

Fig. 2 A typical chromatogram showing blank serum.
Chromatgraohic condition: Acetonitrile:methanol:water (30:20:50 v/v, pH 3.6)

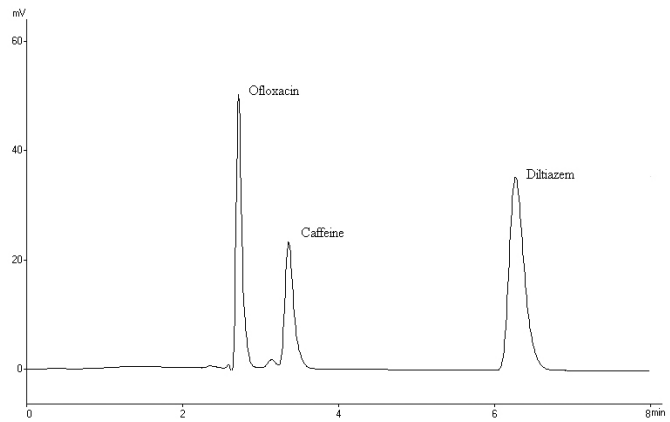

Fig. 3: Representative chromatogram showing resolution between ofloxacin and diltiazem from the caffeine in serum.
Chromatgraohic condition: Acetonitrile:methanol:water (30:20:50 v/v, pH 3.6)

METHOD VALIDATION

The newly developed method has been validated and holds well for the determination of drug in raw materials, dosage formulations and serum. For validation of analytical methods, the guidelines of the International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use[29] have recommended the accomplishment of system suitability, selectivity, specificity, linearity, accuracy test, precision, sensitivity, limit of detection and quantification of the method.

System suitability testing

Typical system suitability results are summarized in table 1, all the values for the system suitability parameters are within limits[32]. Three clear peaks were observed for the individual drugs, the separation of diltiazem and quinolones obtained by this method is significantly better. The resolution (% RSD) obtained by this method for quinolones, caffeine and diltiazem were ≤ 0.7 for all peaks and tailing was ≤ 1.21.

Selectivity and Specificity

The selectivity and specificity of the method was established through the study of resolution factor of the peak of diltiazem from that of quinolones (table 1). The method demonstrated good resolutions and was found to be free of interference from the excipients used in formulation products and thus, the method is specific for diltiazem and quinolones.

Table 1: System suitability parameters

Parameters	% RSD		
	LEVO*	CAF**	DLZ***
Retention time (R_t in minutes)	1.6687	1.7320	0.9262
Capacity factors (K')	1.6522	1.7210	0.9321
Theoritical plates (N)	0.1726	0.4838	0.5651
Tailing factor (T)	1.0402	0.9065	1.211
Resolution (R)	0.700	0.41	0.1511

*Levofloxacin , **Caffeine, ***Diltiazem

Range and linearity

Table 2 shows the regression statistics of concentration analytical response, the standard deviation of the regression line and the optimum linear range (μgmL^{-1}) for each compound. Calibration curves were constructed in the range of expected concentrations (1.25-25 μgmL^{-1}) and were found to be linear within the quantification ranges for all the assayed drugs using a linear regression. Beer's law is obeyed over this concentration range (figures 4, 5). Accordingly, using intercept, excellent linearity was obtained in all cases with correlation coefficients < 0.999.

Table 2: Regression statistics of the proposed method

Analytes	Goodness-of-fit (R^2)	Std error	Std error of estimate	Intercept	Slope
(Bulk material)					
CIP*	0.99646	3097.22	4912.06	28595.52	5840.65
LVO**	0.99610	5888.62	9339.07	34440.73	10582.36
OFX***	0.99613	7423.46	11773.28	13013.32	13398.99
NOR****	0.99537	1674.69	2655.99	37051.80	2767.26
DLZ*****	0.99801	3996.73	6338.64	7860.351	10071.02
(Serum)					
CIP	0.99559	3414.201	5414.77	27607.73	5766.05
LVO	0.99617	5777.72	9163.20	34499.84	10474.83
OFX	0.99616	7423.64	11773.28	13013.32	13398.99
NOR	0.9955	6272.25	9947.51	13013.32	2754.30
DLZ	0.99788	4103.40	6507.81	7224.40	10020.87

*Ciprofloxacin, **Levofloxacin, ***Ofloxacin, ****Norfloxacin, *****Diltiazem

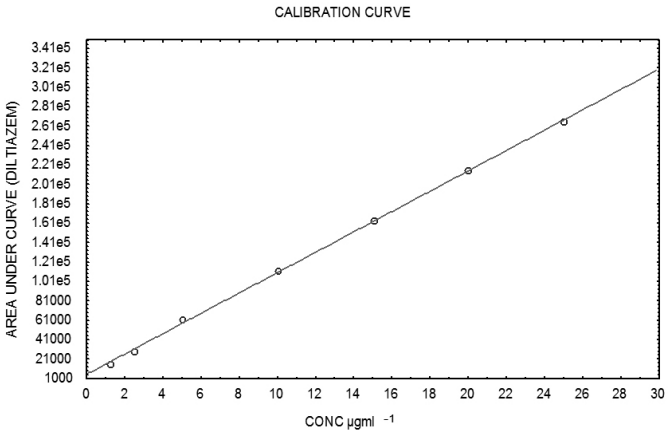

Fig. 4 Calibration curve of diltiazem showing linearity over the concentration range of 1.25-25 µgmL^{-1}.

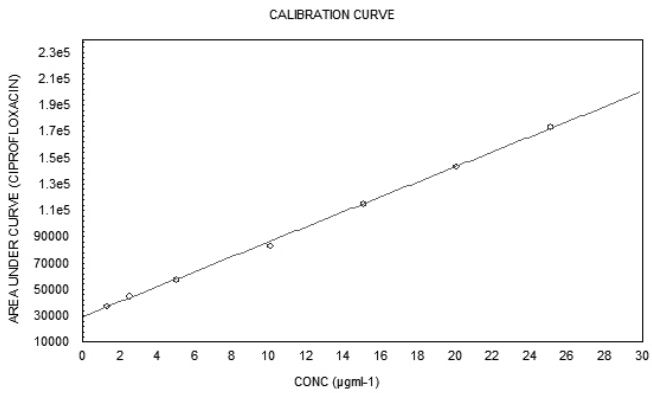

Fig. 5. Calibration curve of ciprofloxacin showing linearity over the concentration range of 1.25-25 µgmL^{-1}.

Accuracy and recovery

Data corresponding to these recovery assays for the studied analytes are presented in table 3**.** The accuracy ranged from 98.9-100.6%, at low, medium and high levels for all investigated analytes. As can be observed, in serum the values ranged within 92.9-101.1%. The data given in table 3 shows that there is no significant difference between the amount of drug spiked in serum and the amount recovered. Thus, serum did not interfere with the estimation.

Table 3: Method accuracy from recovery assays for the studied analytes

Parameters →	% Recovery ←					→ Recovered Concentration(µgmL^{-1}) ←				
Assay (µgmL^{-1})	CIP*	LVO**	OFX***	NOR****	DLZ*****	CIP	LVO	OFX	NOR	DLZ
1.25	97.5	97.4	97.1	99.9	97.8	1.21	1.21	1.21	1.24	1.2
2.5	92.9	97.9	99.4	100.3	96.1	2.32	2.4	2.4	2.5	2.4
5	93.1	98.2	98.3	99.5	101.1	4.65	4.9	4.9	4.9	5.0
10	98.0	99.3	98.5	98.4	98.5	9.80	9.2	9.9	9.8	9.8
15	98.6	99.3	96.4	99.9	98.6	14.7	14.9	14.4	14.9	14.7
20	98.9	99.8	99.8	99.9	99.5	19.7	19.9	19.9	19.9	19.9
25	98.9	99.8	97.4	98.9	99.1	24.7	24.7	24.3	24.7	24.7
Spiking (Bulk material)										
(% level)										
80	100.2	99.9	101.5	99.8	100.4	16.0	15.9	16.2	15.9	16.0
100	100.6	100.1	100.1	100.0	99.9	20.1	20.0	20.0	20.0	19.9
120	100.0	100.4	99.9	98.9	100.0	24.0	24.1	23.9	23.6	24.0
Spiking (Serum)										
(% level)										
80	100.2	99.8	99.9	99.9	100.6	16.0	15.9	15.9	15.9	16.0
100	99.9	100.2	98.7	99.8	100.0	19.9	20.0	19.7	20.0	20.0
120	99.9	100.6	99.7	99.7	99.9	23.9	24.1	23.9	23.9	23.9

*Ciprofloxacin, **Levofloxacin, ***Ofloxacin, ****Norfloxacin, *****Diltiazem

Precision

The intra-and inter-batch precision was evaluated by assaying the samples (table 4). In this assay, the intra-batch precision and the inter-batch precision was ≤ 0.73 in bulk materials and 0.8% or less in human serum. The results demonstrated that the values were within the acceptable range and the method was sufficiently accurate and precise. They also fulfill the AOAC requirements and accordingly were considered suitable for analytical purposes[33].

Table 4: Intra-and inter day, variation /intermediate precision of the method

Conc. (µgmL^{-1}) →	Day 1(% RSD) ←					→ Day 2(% RSD) ←				
	CIP*	LVO**	OFX***	NOR****	DLZ*****	CIP	LVO	OFX	NOR	DLZ
(Bulk material)										
1.25	0.275	0.263	0.156	0.023	0.073	0.717	0.059	0.019	0.035	0.717
2.5	0.582	0.396	0.098	0.009	0.055	0.009	0.008	0.013	0.606	0.009
5	0.018	0.411	0.232	0.033	0.017	0.023	0.014	0.324	0.313	0.023
10	0.128	0.651	0.060	0.493	0.005	0.007	0.460	0.041	0.016	0.007
15	0.050	0.147	0.082	0.076	0.534	0.733	0.203	0.240	0.073	0.733
20	0.261	0.212	0.020	0.023	0.050	0.259	0.027	0.682	0.158	0.259
25	0.244	0.455	0.343	0.216	0.001	0.001	0.019	0.212	0.049	0.001
(Serum)										
1.25	0.073	0.132	0.156	0.106	0.073	0.054	0.105	0.156	0.019	0.442
2.5	0.055	0.185	0.026	0.082	0.055	0.063	0.064	0.026	0.085	0.027
5	0.017	0.800	0.066	0.781	0.017	0.048	0.023	0.066	0.101	0.031
10	0.005	0.651	0.118	0.278	0.005	0.007	0.016	0.118	0.072	0.060
15	0.534	0.097	0.126	0.195	0.534	0.014	0.008	0.126	0.050	0.024
20	0.050	0.002	0.027	0.226	0.050	0.075	0.014	0.027	0.133	0.001
25	0.001	0.315	0.127	0.145	0.001	0.043	0.071	0.127	0.069	0.006

*Ciprofloxacin, **Levofloxacin, ***Ofloxacin, ****Norfloxacin, *****Diltiazem

Ruggedness

The assay results indicated that the method was capable with high precision did not show any notable deviations from acceptable limits.

Limit of detection and quantification

We have evaluated LOD and LOQ as three and ten times, respectively, the ratio between the standard deviation of regression and the slope of the calibration line have been evaluated as depicted in table 5. LOQ generally falls outside the optimum linear ranges for every analyte. However, as mentioned above these ranges should be regarded as limiting optimum values instead of absolute ones. Thus, analyte at concentrations higher than the LOQ can be determined without risks from the calibration graph.

Table 5: Sensitivity (μgmL^{-1}) of the proposed method

Studied analytes	CIP*	LVO**	OFX***	NOR****	DLZ*****
LOD					
Bulk material	0.0219	0.0196	0.0093	0.0147	0.0034
Serum	0.0111	0.0154	0.0164	0.0308	0.0336
LOQ					
Bulk material	0.0665	0.0596	0.0284	0.0446	0.0104
Serum	0.0337	0.0469	0.0499	0.0935	0.0183

*Ciprofloxacin, **Levofloxacin, ***Ofloxacin, ****Norfloxacin, *****Diltiazem

CONCLUSION

The new HPLC method described in this paper provides a generic, simple, universal, convenient and reproducible approach for the simultaneous identification and quantification that can be used to determine diltiazem and any of the four quinolones. In summary, the proposed method can be used for the drug analysis in routine quality control. In addition, this method has the potential application to clinical research of drug combination, multi-drug pharmacokinetics and interactions.

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