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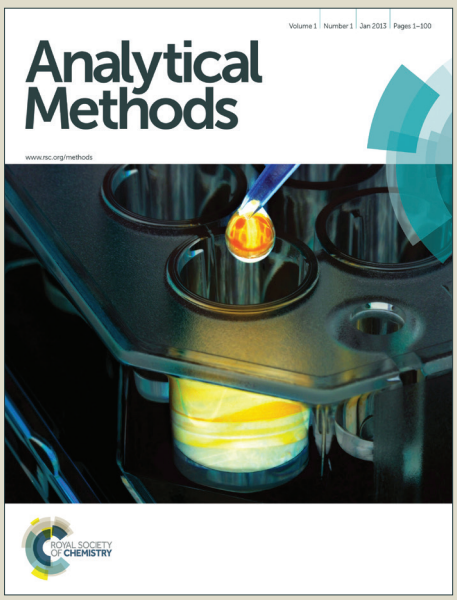

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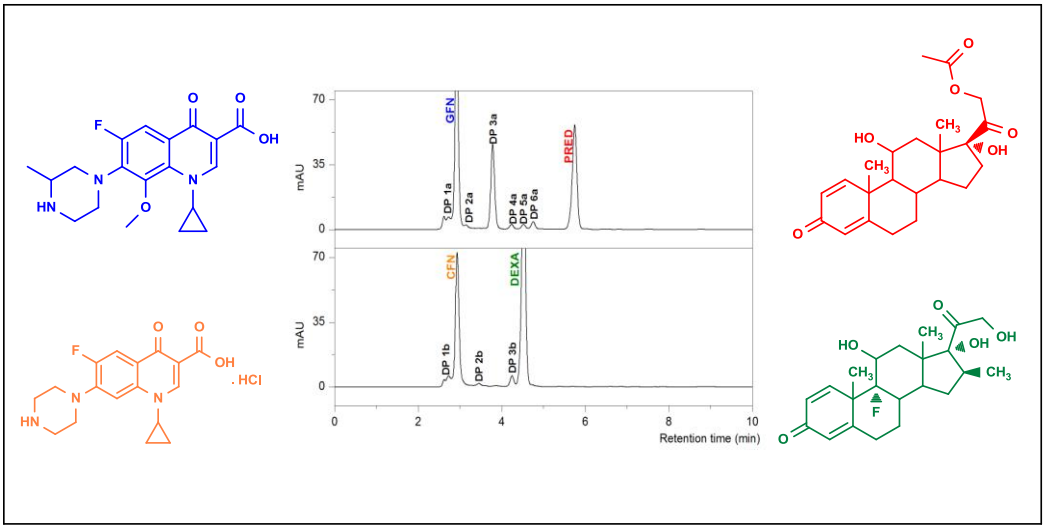

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ARTICLE TYPE

Stability-indicating HPLC-DAD method for the simultaneous determination of fluoroquinolones and corticosteroids in ophthalmic formulations

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The aim of this study was to develop and validate a stability-indicating assay method for simultaneous determination of gatifloxacin and prednisolone acetate, or of ciprofloxacin hydrochloride and dexamethasone in combination and in the presence of degradation products. Reverse-phase high-performance liquid chromatography was used. All analyses were carried out on a Kinetex C18 column and acetronitrile:water (50:50 v/v, pH 3.0) mobile phase with 0.30 mL min^{-1} flow rate. Efficient chromatographic separation of these drugs and their forced degradation products was achieved in less than 6 min and with a peak purity match factor higher than 950. The method showed linearity in the concentration range of 1.2 to 9.6 μg mL^{-1} for gatifloxacin (r=0.9995), 2.0 to 16.0 μg mL^{-1} for prednisolone acetate (r=0.9997), 2.5 to 25.0 μg mL^{-1} for both ciprofloxacin hydrochloride (r=0.9993) and dexamethasone (r=0.9998), precision (relative standard deviation lower than 2%), accuracy (mean recovery 100 ± 2%), and robustness, according to ICH and AOAC guidelines. This method is able to determine simultaneous ophthalmic combinations of these drugs and to separate the drug peaks from their forced degradation product. Additionally, its optimized chromatographic conditions can contribute to minimize organic solvent waste.

Introduction

Fluoroquinolones are an important group of broad-spectrum synthetic antibacterial agents used for the treatment of different bacterial infections.[1] Two drugs of this group are widely used: ciprofloxacin hydrochloride (CFN; Fig. 1) (second-generation) is chemically known as 1-cyclopropil-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazynyl)-3-quinolinecarboxylic acid[2] and gatifloxacin (GFN; Fig. 1) (fourth generation) is chemically known as 1-cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-7-(3-methy-1-piperazinyl)-4-oxo-3-quinolinecarboxylic acid.[2] CFN and GFN have been widely used in the prophylaxis and treatment of ocular infections.[3-10]

Dexamethasone (DEXA; Fig. 1) and prednisolone acetate (PRED; Fig. 1) are the most effective members of the corticosteroid group used for treatment of ocular inflammatory diseases.[11,12] They are chemically known as 16α-methyl-9α – fluoro-1,4-pregnadiene-11β,17α,21-triol-3,20-dione and 1,4-pregnadiene-11β,17α,21-triol-3,20-dione acetate, respectively.[2]

Various pharmaceutical combinations of fluoroquinolones and topical corticosteroids have been proposed in recent years, such as the combination of GFN and PRED or CFN and DEXA.[13-15] Clinical studies have demonstrated the safety and efficacy of these combinations in the treatment and prophylaxis of ocular infections.[16, 17]

Several analytical methods have been described for the individual estimation of CFN or GFN in pharmaceutical formulations by high-pressure liquid chromatography (HPLC),[18-21] UV spectrophotometry[22-30] and capillary zone electrophoresis.[31] Few UV spectrophotometric and HPLC methods were found for the individual determination of PRED or DEXA in pharmaceutical dosage forms.[32-36]

A literature survey related a capillary zone electrophoresis[37] and HPLC[38] methods for the simultaneous determination of CFN and GFN and others fluoroquinolones in tablets. The binary mixtures of CFN or GFN with other drugs have been determined by HPLC[39-41] and UV spectrophotometry.[42-45] On the other hand, few analytical methods based on chromatographic[46-49] and spectrophotometric[50-51] techniques were reported for the simultaneous determination of CFN or GFN in fixed dose combination with corticosteroid drugs selected for our study. Furthermore, the United States Pharmacopoeia describes a HPLC method for simultaneous determination of CFN and DEXA in otic suspensions.[52] However, most of these methods use buffered mobile phases,[46-47,52] high flow rate (about 1.0 mL min^{-1})[46-48,52]

and complicated mathematical model.[50-51]

Therefore, an extensive review of the literature revealed no analytical method is reported in the official compendiums for the simultaneous determination of GFN and PRED or CFN and DEXA in ophthalmic formulations (eye drops). In addition, no stability-indicating HPLC method for simultaneous determination of both pharmaceutical combinations was found in the literature.

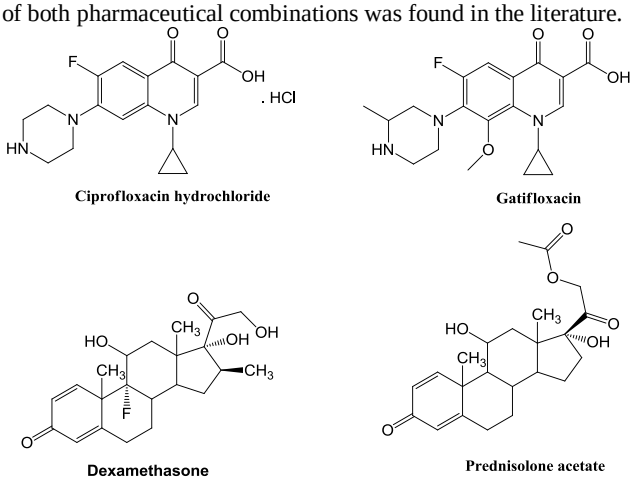

Fig. 1 Chemical structure of the drugs studied.

Experimental

Chemicals and reagents

The GFN and PRED reference standards (assigned purity 100.0%) were kindly donated by Allergan, Inc. (São Paulo, Brazil). Reference standards of CFN and DEXA with stated purity of 98.0 and 100%, respectively, were obtained from EMS Sigma Pharma pharmaceutical company (São Paulo, Brazil). The ophthalmic suspension Zypred® (sample A) containing 3 mg mL^{-1} of GFN and 10 mg mL^{-1} of PRED (declared content) was also donated by Allergan, Inc. The ophthalmic suspension Cilodex® (sample B), which is stated to contain 3 mg mL^{-1} of CFN and 1 mg mL^{-1} of DEXA, was obtained from a local pharmacy. All reference substances, as well as the ophthalmic formulations, were kept protected from light throughout the study. The HPLC grade acetonitrile (Vetec, Brazil), analytical grade phosphoric acid (Synth, Brazil) and fresh ultrapure water from a Milli-Q® Plus system were used in all analyses.

Analytical procedure

HPLC analyses were performed in a system consisting of a Dionex® Ultimate 3000 (Thermo Fisher Scientific, USA), equipped with an Ultimate 3000 RS Variable Wavelength photodiode array detector and Ultimate 3000 pump. The system was connected to a microcomputer with software Chromeleon® 7.1 - Chromatography Data System.

The experimental conditions were optimized at room temperature (24 ± 2 ºC) on a Kinetex C18 column (100 Å, 150×2.1 mm, particle size 2.6 µm) manufactured by Phenomenex Inc., USA. All separations were obtained in isocratic mode using a mobile phase consisting of acetonitrile and water (pH 3.0) in the ratio of 50:50 v/v, respectively. The pH of water was adjusted to 3.0 using phosphoric acid. The flow rate of the mobile phase was 0.30 mL min^{-1} and the sample injection volume was 20 µL. The photodiode array detector was set at 240 nm (DEXA and PRED), 280 nm (CFN), and 290 nm (GFN).

Preparation of standard stock solutions

The stock solutions of reference standards were prepared in order to optimize the experimental procedure and decrease the chance of errors. Therefore, 20 mg of each reference standard (GFN, PRED, CFN, and DEXA) was transferred to 200 mL volumetric flasks in combined form i) GFN plus PRED or ii) CFN plus DEXA. The volumes were completed with acetonitrile to reach 100 µg mL^{-1} of each reference standard. These solutions were used to determine the linearity and accuracy of the proposed method and in the forced degradation studies.

Preparation of sample stock solutions

The sample solutions were prepared in the same ratio as the labeled amounts in the ophthalmic formulations. An aliquot of 5 mL of sample A (containing 3 mg mL^{-1} of GFN and 10 mg mL^{-1} of PRED) was transferred to a 200 mL volumetric flask. Five milliliters of sample B (containing 3 mg mL^{-1} of CFN and 1 mg mL^{-1} of DEXA) was transferred to a 100 mL volumetric flask. The content of each sample was extracted with 50 mL of acetonitrile under sonication for 15 min. The final volumes were completed with acetonitrile, obtaining a stock sample solution A containing 75 µg mL^{-1} of GFN and 250 µg mL^{-1} of PRED, and a stock sample solution B containing 150 µg mL^{-1} of CFN and 50 µg mL^{-1} of DEXA. These solutions were used to determine the precision and accuracy of the method.

Validation of the HPLC-DAD method

The method was validated based on the ICH and AOAC guidelines, following the validation parameters: specificity, linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ) and robustness.[53,54]

Specificity

The specificity was evaluated through the analysis of a placebo solution as well as by analysis of the drug solutions after forced degradation studies.

Procedure for forced degradation studies

Forced degradation studies were performed according to ICH guidelines.[55,56] Aliquots of each combined standard stock solution were submitted to different stress conditions, and the resulting solutions were analyzed by the proposed chromatographic method via the mean of peak areas (n=3) for each stressed standard solution. The residual amount of active pharmaceutical ingredients (APIs) and the peak purity data were evaluated.

Acidic and basic degradations were performed by transferring 5 mL of each combined standard stock solution to 100 mL volumetric flasks and by adding 5 mL of either 0.5 M hydrochloric acid or 0.5 M sodium hydroxide. The solutions were kept under vigorous mechanical stirring for 4 h at room temperature (24 ± 2ºC). After the stress condition, the solutions were neutralized.

To promote oxidation, a mixture of 5 mL of each combined standard stock solution and 5 mL of 3% hydrogen peroxide solution was placed in 100 mL volumetric flasks and kept at room temperature (24 ± 2º C) for 24 h.

To evaluate the thermal degradation, an aliquot of 15 mL of

each combined standard stock solution was transferred to an erlenmeyer flask and refluxed for 2 h at 80 ºC in the dark. After the stress condition, the solutions were transferred to 200 mL volumetric flasks.

To study the radiation effect, aliquots of 15 mL of each standard stock solution were transferred to 200 mL volumetric flasks. The volumetric flasks were directly exposed to two different stress conditions: i) direct sunlight and ii) cool white fluorescent light (ISO 18909-2006) for 1 h and 48 h, respectively. The photo-degradation steps were evaluated at room temperature (24 ± 2º C).

After the stress conditions, the volumes were completed with the mobile phase and the solutions were filtered before injection in a Millex 0.45 μm filter (Millipore, Bradford, USA).

Linearity

The linearity was determined via calibration curves (peak area versus concentration). For each curve, at least seven concentration levels were used to establish the linear-regression lines by the least-squares method. All chromatographic determinations were performed in triplicate and at room temperature (24 ± 2 ºC). The statistical evaluation was done by analysis of variance (ANOVA).

Precision

The intra-day precision (repeatability) was evaluated by analyzing sample solutions at single concentrations within the linearity range of the proposed method. Aliquots of 5 mL of sample stock solution A and B were transferred, separately, to 100 mL and 50 mL volumetric flasks, respectively. The final volumes were completed with the mobile phase to obtain final concentrations of 3.75 and 12.5 μg mL^{-1} of GFN and PRED or 15 and 5 μg mL^{-1} of CFN and DEXA, respectively. The analyses were performed in six replicates on the same day. To estimate the inter-day precision, the sample solutions were prepared fresh at the same concentration level for each drug, and the responses were determined in six replicates. The procedure was repeated on three consecutive days. The intra- and inter-day precisions are expressed in terms of Relative Standard Deviation (%RSD).

Accuracy

The accuracy was determined by recovery studies, using the standard addition method as recommended by AOAC.[47] Recovery was analyzed by adding known amounts of standard solutions to the sample, followed by analysis using the proposed method. Aliquots of standard and sample stock solutions were transferred to 25 mL volumetric flasks and the final volumes were completed with the mobile phase.

The percentage of recovery (R) was calculated by comparing the theoretical and found concentrations, using the following equation $R = [(C_F - C_U) / C_A] \times 100$; where C_F represents the concentration of analyte measured in the fortified test sample; C_U, the concentration of analyte measured in the unfortified test sample; and C_A, the concentration of the analyte added to the fortified test sample.

Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were calculated based on the standard deviation of the response and the slope of the calibration curves. They were mathematically evaluated by 3.3 σ/s and 10 σ/s equations, respectively, where σ represents the standard deviation of the analytical signal and s is the slope of the corresponding calibration curve.

Robustness

The robustness was evaluated by subtle changes of the chromatographic conditions and by the influence of these changes on the peak area, retention time (t_R) and tailing factor (T_f) of the drugs analyzed. The factors (chromatographic conditions) selected to examine the robustness were the flow rate, percentage of acetonitrile and pH of the water in the mobile phase. Each factor was investigated at three levels (- 1, 0, and + 1).[57] Level 0 refers to the normal chromatographic conditions, namely, the conditions employed in the proposed method. From this level, the chromatographic conditions were modified to a higher level (+ 1) or to a lower level (- 1). While one condition was changed, the others remained at level 0. Replicate injections (n = 3) of each sample solution (sample solutions A and B) were performed under these small changes in the chromatographic conditions. The statistical evaluation was done by t-test statistic.

Results and discussion

Method optimization

Choice of stationary phase

Initially, different reverse-phase columns were tested as demonstrated in the study of the influence of different stationary phases on chromatographic parameters (Table 1).

The chromatographic parameters obtained with the Kinetex C18 (column 1) and VertiSep GES C18 (column 2) were within acceptable limits according to ICH and AOAC.[53,54] Only the tailing factor for CFN on column 2 exceeded the acceptable limits, which could be improved with minor changes in the mobile phase. However, column 1 was chosen for the development and validation of the analytical method because it provided excellent values for the chromatographic parameters for all drugs analyzed.

Table 1 Influence of different stationary phases[a] on chromatographic parameters

Drugs	Resolution			Theoretical plates			Tailing factor		
	1	2	3	1	2	3	1	2	3
GFN	13.28	4.87	4.87	4946	3239	1470	1.02	1.43	0.94
PRED				6460	5975	2447	0.99	1.07	1.11
CFN	11.48	5.71	2.66	7419	2867	2599	0.99	3.35	0.83
DEXA				11691	6220	2663	0.97	1.07	1.13

[a] Stationary phases: **1**- Kinetex C18 (Phenomenex, USA); **2**- VertiSep GES C18 (Vertical, USA); **3**- LiChrospher 100RP C8 (Merck, GER). Conditions: mobile phase composed of acetonitrile:water (50:50, v/v), pH 3.0; flow rate: 0.30 mL min^{-1} for column 1; 1.00 mL min^{-1} for column 2; 0.50 mL min^{-1} for column 3; injection volume 20 μL.

The high efficiency of chromatographic separation provided by column 1 is due to the 2.6 μm particle shell technology, which accelerates diffusion and reduces the duration of the mass

transfer.[58,59] Furthermore, the low flow rate (0.30 mL min^{-1}) and the small internal diameter of this column (150 mm × 2.1 mm) required less organic solvent during the chromatographic analysis, minimizing the amount of organic-solvent waste.

Choice of mobile phase

Some mobile phases were tested in a Kinetex C18 column to provide the best chromatographic separation. The influence of the different compositions of the mobile phases on the chromatographic parameters is summarized in Table 2.

Table 2 Influence of different compositions of mobile phases [a] on chromatographic parameters [b] in a Kinetex C18 column

Drugs	Resolution					Theoretical plates					Tailing factor				
	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
GFN	14.80	3.67	2.88	*	*	5248	4306	414	5020	529	1.02	0.65	2.73	1.75	2.12
PRED						7525	10820	9866	*	*	0.99	0.97	0.93	*	*
CFN	11.48	*	1.65	*	*	7396	1532	633	312	7666	0.99	0.93	1.93	3.46	0.91
DEXA						10802	*	1397	*	*	0.98	*	0.99	*	*

[a] Mobile phase compositions: **A-** acetonitrile:water (50:50, v/v), pH 3.0; **B-** acetonitrile:water (50:50, v/v); **C-** acetonitrile:water (75:25, v/v), pH 3.0; **D-** acetonitrile:water (25:75, v/v), pH 3.0; **E-** methanol:water (50:50), pH 3.0. Conditions: flow rate 0.30 mL min^{-1}; injection volume 20 μL.
[b] The asterisk (*) means that the corticosteroid drugs were retained in the column and the chromatographic parameters were not determined.

The acidic mobile phase reduced the retention of fluoroquinolones in the stationary phase. The reported values of pK_a for CFN were 5.76 and 8.78, and for GFN, 5.69 and 8.76.[60] The mobile phase pH influences the acid-base behavior of these drugs. In acidic pH, the amine group (secondary alkylamine) accepts a proton, and in basic pH the carboxyl group (carboxylic acid) donates a proton, showing the amphoteric character of the CFN.[61] Certainly, the same characteristic is attributed to GFN, because it resembles CFN in its chemical structure, except for substitutions of the methoxy group at position 8 and the 3′-methyl group of the piperazinyl ring at position 7 of the quinolone ring.[62] At pH 3.0 the secondary alkylamine groups of these fluoroquinolones remained ionized, leading to lower retention in the reverse stationary phase. The use of a buffered mobile phase is dispensable because the proposed pH value was at least 2 units below the pK_a values of both drugs, providing completely ionized secondary alkylamine groups.[63]

DEXA and PRED have high pK_a values (12.42 and 12.58, respectively)[60] and they are poorly soluble in polar solvents.[2] DEXA and PRED were more retained in the stationary phase when mobile phases composed of a larger amount of water (mobile phase D) were used, and when acetonitrile was substituted for methanol (mobile phase E), as shown in Table 2. The pH adjustment of the mobile phases secured the ionization of the proton acceptor groups. However, pH adjustment alone was not sufficient; it was necessary to optimize the ratio of the organic modifier in the mobile phase. Comparing mobile phases A and D, both composed by acetonitrile and water at pH 3.0, it was clear that reducing the proportion of the organic modifier (acetonitrile) in mobile phase D significantly affected the retention time of the corticosteroid drugs, and therefore the chromatographic parameters could not be determined.

From these studies, suitable separation with high resolutions, satisfactory theoretical plates and best peak symmetry were achieved with acetronitrile:water (50:50 v/v, pH 3.0). Under the optimized chromatographic conditions, it was possible to obtain efficient separation of APIs in the ophthalmic formulations. Peak identity was confirmed by the retention time and by the reference spectra match factor. Sharp, symmetrical peaks of GFN, PRED, CFN, and DEXA were obtained at retention times of 2.78, 5.73, 2.75, and 4.50 min, respectively (Fig. 2). All values for the reference spectra match factor were higher than 950, indicating the similarity between the analytes and reference spectra of the library.[64] Furthermore, two unknown related impurities (IMP 1 and IMP 2) were detected in the ophthalmic formulation combining GFN and PRED (Fig. 2a).

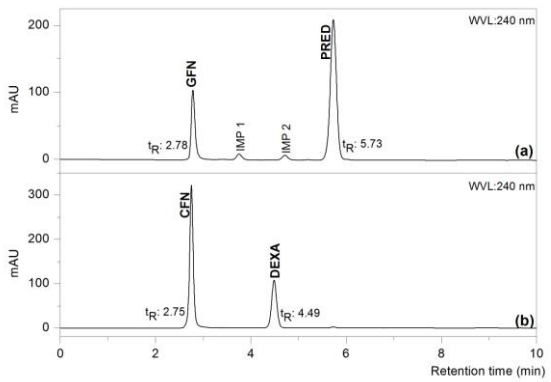

Fig. 2 Chromatograms of (a) gatifloxacin and prednisolone acetate and (b) ciprofloxacin hydrochloride and dexamethasone in pharmaceutical formulation. IMP, unknown related impurity; t_R, retention time. *Conditions*: mobile phase composed of acetonitrile:water (70:30, v/v) pH 3.0; column Kinetex C18; flow rate 0.3 mL min^{-1}; injection volume 20 μL.

Method validation

Specificity

The excipients present in the pharmaceutical dosage forms did not affect the analysis, which proves the specificity of the proposed method.

The specificity of the method was also evaluated by checking the peak purity of all analytes after the forced degradation studies. Chromatographic peak purity data were evaluated from

the spectral analysis report supplied by the photodiode array detection. Peak purity match factor (PPM) values higher than 950 indicate a homogeneous peak.[64] Almost all PPM values for API peaks in chromatograms of stressed standard solutions were higher than 950.The peak purity value was lower than 950 only in oxidative degradation of GFN. The formation of peroxycarboxymidic acid has been observed when acetonitrile is used as a co-solvent in oxidative degradation with hydrogen peroxide. The formation of this secondary reaction product could have affected the GFN peak purity. To resolve these problems, some investigators always perform a parallel oxidative study using azobisisobutyronitrile, which is a less-reactive oxidant and generates degradation products that are more representative of the drugs studied.[65]

All stress conditions were sufficient to degrade the drugs. The fourth-generation fluoroquinolone (GFN) was more stable than the second-generation (CFN). Structural modifications could have caused the difference in stability of these fluoroquinolones. The presence of less substitution in the quinoline nucleus and piperazil group of CFN can increase susceptibility to degradation under different stress conditions.[66] Under acidic and basic hydrolysis (Figs. 3 and 4), CFN was degraded up to 50.45% and 43.75%, respectively, whereas GFN was degraded up to 34.27% and 19.44%, respectively. Under oxidative hydrolysis (Fig. 5), CFN was degraded up to 68.28% and GFN was degraded up to 43.64%. Under thermal degradation (Fig. 6), CFN and GFN were degraded up to 19.74% and 3.94%, respectively. Under photo-degradation, when sunlight was the stressor agent (Fig. 7), CFN was degraded up to 20.27% and GFN was degraded up to 3.13%. However, when white fluorescent light was the stressor agent (Fig. 8), the CFN was degraded less than GFN (4.22% and 7.55%, respectively).

Of the corticosteroid drugs, DEXA was more stable than PRED under the stress conditions evaluated. The corticosteroid monoesters, such as PRED, can be unstable in the presence of acidic, basic and oxidative reagents. The first degradation step is assumed to be hydrolysis of the ester function.[67] Some authors reported that after the ester hydrolysis the free-alcohol function degrades further.[67] Under acidic and basic conditions (Figs. 3 and 4), PRED was degraded up to 52.35% and 95.10%, respectively, whereas DEXA was degraded up to 8.97% and 35.38%, respectively. Under oxidative stress (Fig. 5), PRED was degraded up to 16.56% and DEXA was degraded up to 7.11%. Under thermal stress (Fig. 6), PRED and DEXA were degraded up to 17.19% and 6.93%, respectively. However, in the photolytic degradation the difference in the percentage of degradation of both drugs was smaller. PRED was degraded up to 26.99% and 19.53%, when the stressor was sunlight (Fig. 7) and white fluorescent light (Fig. 8), respectively, while DEXA was degraded up to 21.47% and 13.40% in sunlight and white fluorescent light, respectively.

In addition, the comparison of the stability of the combined dosage forms gave two different results. For the GFN and PRED combination, the fluoroquinolone was more stable than the corticosteroid, as observed by Razzaq et al.[68] who evaluated the stability of a combination of moxifloxacin and prednislone in pharmaceutical formulation. However, for the CFN and DEXA combination, the corticosteroid was more stable than the fluoroquinolone.

The largest amount of degradation products (peaks of six degradation products) was generated under acidic and basic conditions for the GFN and PRED combination. The smallest amount of degradation products was obtained under thermal degradation for the CFN and DEXA combination, where no impurity peak was observed.

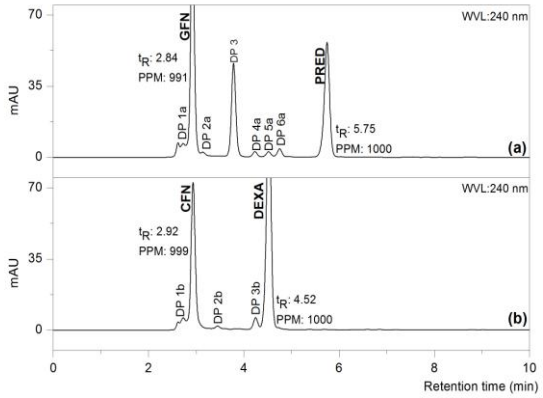

Fig. 3 Chromatograms of (a) gatifloxacin and prednisolone acetate and (b) ciprofloxacin and dexamethasone under acidic hydrolysis. DP, degradation product; PPM, peak purity match factor; t_R, retention time. *Conditions*: mobile phase composed of acetonitrile:water (70:30, v/v) pH 3.0; column Kinetex C18; flow rate 0.3 mL min^{-1}; injection volume 20 μL.

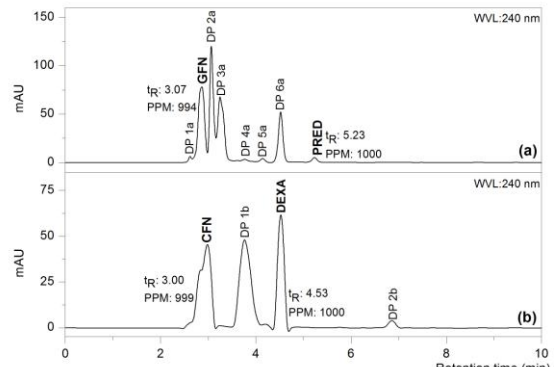

Fig. 4 Chromatograms of (a) gatifloxacin and prednisolone acetate and (b) ciprofloxacin hydrochloride and dexamethasone under basic hydrolysis. DP, degradation product; PPM, peak purity match factor; t_R, retention time. *Conditions*: mobile phase composed of acetonitrile:water (70:30, v/v) pH 3.0; column Kinetex C18; flow rate 0.3 mL min^{-1}; injection volume 20 μL.

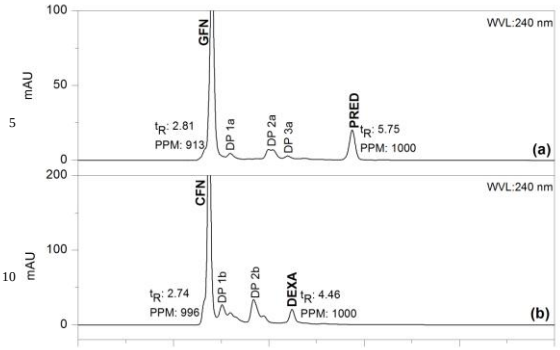

Fig. 5 Chromatograms of (a) gatifloxacin and prednisolone acetate and (b) ciprofloxacin hydrochloride and dexamethasone under oxidative hydrolysis. DP, degradation product; PPM, peak purity match factor; t_R, retention time. *Conditions*: mobile phase composed of acetonitrile:water (70:30, v/v) pH 3.0; column Kinetex C18; flow rate 0.3 mL min^{-1}; injection volume 20 µL.

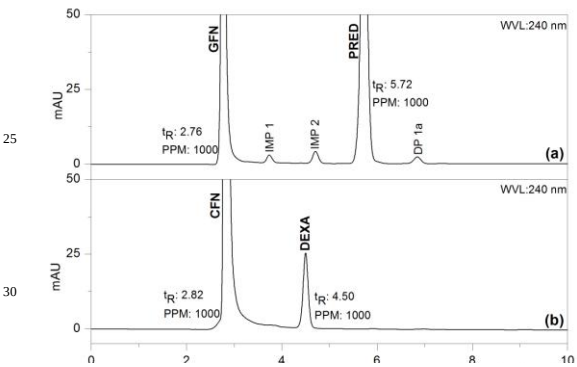

Fig. 6 Chromatograms of (a) gatifloxacin and prednisolone acetate and (b) ciprofloxacin hydrochloride and dexamethasone under thermal degradation. DP, degradation product; IMP, unknown related impurity; PPM, peak purity match factor; t_R, retention time. *Conditions*: mobile phase composed of acetonitrile:water (70:30, v/v) pH 3.0; column Kinetex C18; flow rate 0.3 mL min^{-1}; injection volume 20 µL.

Fig.7 Chromatograms of (a) gatifloxacin and prednisolone acetate and (b) ciprofloxacin hydrochloride and dexamethasone under photo-degradation using sunlight as the stressor. DP, degradation product; IMP, unknown related impurity; PPM, peak purity match factor; t_R, retention time. *Conditions*: mobile phase composed of acetonitrile:water (70:30, v/v) pH 3.0; column Kinetex C18; flow rate 0.3 mL min^{-1}; injection volume 20 µL.

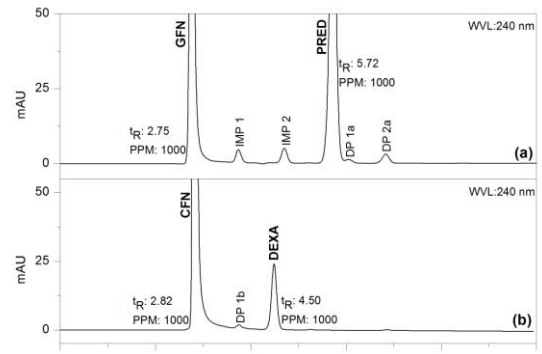

Fig. 8 Chromatograms of (a) gatifloxacin and prednisolone acetate and (b) ciprofloxacin hydrochloride and dexamethasone under photo-degradation using white fluorescent light as the stressor. DP, degradation product; IMP, unknown related impurity; PPM, peak purity match factor; t_R, retention time. *Conditions*: mobile phase composed of acetonitrile:water (70:30, v/v) pH 3.0; column Kinetex C18; flow rate 0.3 mL min^{-1}; injection volume 20 µL.

Linearity

Linear calibration curves were evaluated in the concentration ranges from 1.2 to 9.6 µg mL^{-1} for GFN, 2.0 to 16.0 µg mL^{-1} for PRED, and 2.5 to 25.0 µg mL^{-1} for both CFN and DEXA. The correlation coefficients were 0.9995, 0.9997, 0.9993 and 0.9998 for GFN, PRED, CFN, and DEXA, respectively. The statistical ANOVA evaluation indicated a significant linear regression. These results indicated a linear correlation between peak areas and drug concentrations.

Precision

The precision parameters (%RSD) expressed as repeatability (intra-day) and as intermediate precision (inter-day) are shown in Table 3. For GFN and PRED, the values of %RSD were lower than 0.80% (repeatability) and 1.65% (intermediate precision). For CFN and DEXA, all %RSD values were lower than 0.95%. Therefore, the proposed method has good precision for the simultaneous determination of these drugs.

Table 3 Intra-day and inter-day precision results for the proposed HPLC method

Drugs	Theoretical concentration (µg mL)	Content found ± RSD (%)			
		Intra-day precision [a]			Inter-day precision [b]
		Day 1	Day 2	Day 3	
GFN	3.75	102.40 ± 0.59	100.53 ± 0.63	99.20 ± 0.15	100.71 ± 1.64
PRED	12.50	100.24 ± 0.79	99.36 ± 0.26	99.04 ± 0.23	99.55 ± 0.60
CFN	15.00	98.40 ± 0.40	99.87 ± 0.19	100.07 ± 0.36	99.42 ± 0.91
DEXA	5.00	103.00 ± 0.32	104.00 ± 0.24	103.80 ± 0.16	103.60 ± 0.55

[a] Mean of 6 determinations. [b] Mean of 3 determinations on each of 3 consecutive days.

Accuracy

The mean recovery values (Table 4) were 100.50 ± 1.69%, 99.60 ± 1.60%, 101.05 ± 0.93% and 100.33 ± 0.98%, for GFN, PRED, CFN, and DEXA, respectively. These recovery results were within acceptable limits[54] (100 ± 2%), and they suggest a good accuracy of the proposed method.

Table 4 Recovery results for the proposed HPLC method

Drugs	Fortified theoretical concentration (μg mL^{-1})	Experimental concentration found (μg mL^{-1}) [a]	Recovery (%)	
			Result	Mean ± RSD (%)
GFN	1.20	1.23	101.69	100.50 ± 1.69
	2.40	2.37	98.56	
	3.60	3.64	101.25	
PRED	4.00	4.13	101.44	99.60 ± 1.60
	8.00	7.89	98.64	
	12.00	11.84	98.72	
CFN	3.00	3.04	101.44	101.05 ± 0.93
	6.00	6.10	101.72	
	9.00	8.99	99.98	
DEXA	1.00	1.02	101.23	100.33 ± 0.98
	2.00	2.01	100.48	
	3.00	2.98	99.28	

[a] Mean of 3 determinations.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD values were 0.088, 0.229, 0.038, and 0.016 μg mL^{-1} for GFN, PRED, CFN, and DEXA, respectively (signal to noise ratio of 3:1). The LOQ were 0.265, 0.693, 0.115, and 0.0493 μg mL^{-1} for GFN, PRED, CFN, and DEXA, respectively. These results indicated the sensitivity of the method.

Robustness

The results for robustness (Table 5) indicated that the change in the chromatographic conditions did not significantly modify the peak areas, retention time, and tailing factors of the drugs (GFN, PRED, CFN, and DEXA).

As seen in Table 5, a 1% change in acetonitrile or 3.3% in the pH of the mobile phase slightly affected the peak area of all drugs, without affecting the retention time and tailing factors. Finally, a 10% change in the flow rate affected the peak area and the retention time. However, these effects did not compromise the analyses of the drugs.

Table 5 Robustness evaluation of the proposed HPLC method

Chromatographic changes		Drugs											
		GFN			PRED			CFN			DEXA		
Factor[a]	Level	Area	t_R[b]	T_f[c]	Area	t_R[b]	T_f[c]	Area	t_R[b]	T_f[c]	Area	t_R[b]	T_f[c]
A: % of acetonitrile in mobile phase (v/v)													
49	-1	37.56	2.67	1.07	13.95	5.89	0.96	149.75	2.65	0.96	15.08	4.50	0.95
50	0	37.25	2.67	1.01	14.11	5.71	0.97	149.48	2.64	1.00	14.89	4.42	0.96
51	+1	37.39	2.67	1.04	14.19	5.89	0.96	149.80	2.65	1.00	15.09	4.33	0.96
Mean ± S.D. (n = 3)		37.40 ± 0.15	2.67 ± 0.00	1.04 ± 0.03	14.08 ± 0.12	5.83 ± 0.10	0.96 ± 0.01	149.68 ± 0.17	2.65 ± 0.01	0.99 ± 0.02	15.02 ± 0.11	4.42 ± 0.09	0.96 ± 0.01
B: pH of mobile phase													
2.90	-1	39.85	2.67	1.02	14.07	5.73	0.99	150.22	2.65	0.98	15.12	4.39	0.96
3.00	0	37.25	2.67	1.01	14.11	5.71	0.97	149.48	2.64	1.00	14.89	4.42	0.96
3.10	+1	38.85	2.67	1.04	14.27	5.74	0.97	149.82	2.65	0.97	15.12	4.43	0.96
Mean ± S.D. (n = 3)		38.65 ± 1.31	2.67 ± 0.00	1.02 ± 0.02	14.15 ± 0.11	5.73 ± 0.02	0.98 ± 0.01	149.84 ± 0.37	2.65 ± 0.01	0.98 ± 0.02	15.04 ± 0.13	4.41 ± 0.02	0.96 ± 0.01
C: Flow rate (mL min^{-1})													
0.27	-1	38.45	2.97	1.07	13.63	6.35	0.97	149.18	2.93	1.00	15.24	4.92	0.96
0.30	0	37.25	2.67	1.01	14.11	5.71	0.97	149.48	2.64	1.00	14.89	4.42	0.96
0.33	+1	38.11	2.43	1.08	13.67	5.21	0.97	146.06	2.41	0.98	15.73	4.02	0.95
Mean ± S.D. (n = 3)		37.94 ± 0.62	2.69 ± 0.27	1.05 ± 0.04	13.80 ± 0.26	5.76 ± 0.57	0.97 ± 0.00	148.23 ± 1.91	2.66 ± 0.26	0.99 ± 0.01	15.29 ± 0.42	4.45 ± 0.45	0.96 ± 0.01

[a] Three factors (A, B and C) were slightly changed at three levels (+1, 0, −1); each time a factor was changed from level 0 the other factors remained at level 0. [b] Retention time. [c] Tailing factor.

Application of the method

The proposed HPLC-DAD method is suitable for simultaneous determination of GFN and PRED as well as CFN and DEXA in ophthalmic dosage formulations in routine analysis. For commercial formulations, the results are presented in Table 6.

Table 6 Analysis of commercial formulation using the proposed HPLC method

Commercial formulations[b]	Drugs	Theoretical concentration ($\mu g\ mL^{-1}$)	Content found ± RSD (%) [a]
Sample A	GFN	3.75	100.23 ± 0.74
	PRED	12.50	99.84 ± 0.10
Sample B	CFN	15.00	100.05 ± 0.09
	DEXA	5.00	102.71 ± 0.24

[a] Mean of 3 determinations. [b] Sample A was Zypred® (Allergan Inc., São Paulo, Brazil) containing labeled amounts of 3.0 mg mL^{-1} GFN and 10 mg mL^{-1} PRED. Sample B was Cilodex® (Alcon Ltda, São Paulo, Brazil) containing labeled amounts of 3.0 mg mL^{-1} CFN and 1 mg mL^{-1} DEXA.

Conclusion

A stability-indicating HPLC-DAD method was developed and validated for simultaneous determination of two ophthalmic combinations of a fluoroquinolone and a corticosteroid, *viz.*, gatifloxacin and prednisolone acetate or ciprofloxacin hydrochloride and dexamethasone. This method showed specificity, precision, accuracy, sensitivity, and robustness for the assay of the fluoroquinolone and corticosteroid in ophthalmic formulations. In addition, the method was successfully applied to separate the active pharmaceutical ingredients from their forced degradation products. The results presented suggest that the proposed method can be used in routine quality control analysis. The method's optimized chromatographic conditions can contribute to minimize organic solvent waste.

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