ABSTRACT

Objective: To study the in vivo behaviour and irritant properties of different ocular bio-adhesive inserts of ciprofloxacin hydrochloride (CFX-HCl) prepared using a spray dried (SD2) matrix system consisting of xanthan gum, carbopol, and propylene glycol.

Methods: CFX-HCl in aqueous humor samples was analysed using HPLC method. Applying a mobile phase of 0.01M sodium acetate: methanol (70:30 v/v) with pH around 3.00, and using Purosphere star 100RP-18 column (125 mm × 4.6 mm × 5 µm). The in vivo behaviour and irritant properties of ocular inserts were studied on rabbits. Twelve rabbits were used for the study and were divided into four groups. After placing the insert in the eye 100 µl of the aqueous humor was withdrawn at different time intervals in order to measure the concentration profile of CFX-HCl. The tested formulae R, F1, F2, and F3 were all containing 6.25 mg of CFX-HCl.

Results: The method was well validated according to linearity, recovery, and precision. Where the calibration curve was linear over a concentration range of (2.500–7.826) µg/ml, with an average recovery of 99.76%. The presence of the matrix system enhances the absorption of CFX-HCl and sustains its release up to four days leading to increasing its bioavailability. Also, the ocular inserts of F2 and F3 have better biocompatibility compared with R and F1.

Conclusion: The analysis method was found sensitive, accurate and precise and could be used to assess the in vivo behaviour of CFX-HCl. The ratio of the free drug to the matrix system controlled the rate of drug release.

Keywords: Ciprofloxacin-HCl, Formulation, In vivo study, Ocular inserts, Pharmacokinetic analysis, Sustained release drug delivery.
Validation of the in vivo precision, accuracy, and specificity.

Performance expressed as analytical parameters such as linearity, this method was validated through the evaluation of its expression, precision, accuracy, and specificity.

Validation of the in vivo HPLC analytical method

This method was validated through the evaluation of its performance expressed as analytical parameters such as linearity, precision, accuracy, and specificity.

Chromatographic conditions

The HPLC analysis of CFX-HCl was performed at room temperature using 0.01M sodium acetate: methanol (70:30 v/v) as a mobile phase. Its pH was adjusted to be in the vicinity of 3.00 using glacial acetic acid. The mobile phase was always clarified by filtration through a nylon filter paper, with pore size equal to 0.45 µm, and degassed through a sonicator, then pumped at a flow rate of 1 ml/min on Purosphere star 100RP-18 column (125 mm x 4.6 mm x 5 µm). The peak response was monitored at a wavelength of 280 nm. A sample 40 µl was injected into HPLC system, and the data was acquired using Thermo Quest software.

Linearity

The linearity of the proposed method was established from the calibration curve at several concentration levels (2.50-7.83) µg/ml. Calibration curve was constructed for CFX-HCl in aqueous humor by plotting their response area against their respective concentration, the coefficient of the linear regression equation and the correlation coefficient (R²) were calculated using the linear least squares regression analysis [14,15].

Specificity

In order to confirm that there is no interference between the endogenous components of the aqueous humor and CFX-HCl peaks a blank aqueous humor sample was analysed and compared with aqueous humor containing CFX-HCl. Also, the specificity and selectivity of the analytical method were investigated by confirming the complete separation and resolution of CFX-HCl peak.

Precision

Method precision was determined in terms of repeatability (i.e., analysis repeatability). In order to determine the repeatability six samples of both standard solution and aqueous humor standard of the concentration 4.444 µg/ml CFX-HCl were prepared individually, then each sample was injected into the HPLC system. Repeatability of the areas was determined and expressed as mean±standard deviation (SDEV) and percent relative standard deviation (%RSD) calculated from the obtained data as a precision of the method.

Accuracy

The accuracy of the method was determined in terms of percent recovery. Spiked aqueous humor samples were prepared and extracted to get three concentration levels of 50%, 100% and 200% of the labeled content of CFX-HCl. Another set of standard solutions at the same concentration levels was also prepared. The samples were injected into the HPLC system. The percent recovery was calculated according to the following equation:

\[
\text{% Recovery} = \frac{[A] \times 100}{[B]} \]

Where [A] is the net peak area of the drug in aqueous humor sample, [B] is the peak area of the drug in standard solution.

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

LOD is the lowest concentration of an analyte that can reliably be differentiated from background levels. LOQ of an individual analytical procedure is the lowest amount of analyte that can be quantitatively determined with suitable precision and accuracy. LOD and LOQ were calculated from the standard deviation of the response and the slope of the three linearity curves using the formula 3.3 σ/S for LOD and 10 σ/S for LOQ where σ is the standard deviation of response and S is mean of the slope of three calibration curves [16].

Tailing factor

The tailing factor was estimated by employing the analysis of six standard solutions of the concentration 7.273 µg/ml of CFX-HCl, then was expressed as the mean±standard deviation (SDEV) of the six samples tailing factor.

Materials and Methods

Materials

Ciprofloxacin Hydrochloride (CFX-HCl), and Ciprofloxacin Base (CFX-base) were USP grades obtained from (Biocon, India). Xanthan Gum (XG) and Carbopol 934 (C934) and Propylene Glycol (PG) were obtained from (Shanghai, China), Acetonitrile HPLC grade (Scharlau, Spain), Acetic Acid glacial HPLC grade (Scharlau, Spain), Methanol HPLC grade (Scharlau, Spain), Sodium acetate trihydrate laboratory reagent (Nice chemicals, India). All other excipients and chemicals used were of analytical grade.

Instruments

The High-Pressure Liquid Chromatography (HPLC) Merck Hitachi (Germany), Lachrome A equipment with Interface D-7000, Diode Array L-7455 detector, autosampler L-7200, and L-7150 pump. Transonic Sonicator 460/H, Elma, Germany. Vortex Cyclo mixer, CM 101, Remi equipment. Eppendorf microcentrifuge 5414 was used. Virtex Janke and Kunkel IKA-labor Technik VP2 87-141-09. Hanna pH meter (HBS19N) was used for pH measurements, a 0.45-micrometer membrane filter (Sartorius, Goetting, Germany).

In vivo HPLC analytical method for the determination of CFX-HCl in aqueous humor

The in vivo samples were prepared by protein precipitation method using acetonitrile as a precipitating agent, and the samples were analysed using a validated HPLC method.

Preparation of aqueous humor standards of CFX-HCl

A known amount of CFX-HCl was dissolved in HPLC water to produce a working standard solution of 20 µg/ml, in order to produce CFX-HCl standards in aqueous humor different volumes of the stock solution (20 µg/ml) were added into 70 µl of blank aqueous humor in disposable polypropylene micro centrifuge tubes (1.5 ml, Eppendorf) and vortexed for 15 s, and then 70 µl of acetonitrile was added in order to precipitate proteins, the resulting mixture was mixed using the vortex mixer for 15 s. A centrifugation step was applied for 2 min at 11500 rpm/min in a micro centrifuge (Eppendorf). The aqueous layer was decanted in HPLC glass vial to be ready for injection. The obtained standards were in the concentration range of (1.333-8.333) µg/ml. The same procedure was applied for the preparation of the in vivo samples but without the addition of CFX-HCl.
Stability of samples

Stability studies of aqueous humor spiked with CFX-HCl (2, 5, 8 µg/ml) were carried out over a period of 30 d at -18°C.

In vivo performance of CFX-HCl ocular inserts in an animal model

The aim of this in vivo study was to determine objectively in rabbits the possible irritant properties and the in vivo drug profile of the ocular insert on single administration in the rabbit’s eye [4]. The administration was by placing one insert in the rabbit’s conjunctival cul-de-sac. The in vivo experiment was carried out at the animal house of Jordan University of Science and Technology (JUST). An ophthalmologist attended this study to supervise collecting the aqueous humor samples and monitoring vital signs so as to minimize potential risks.

General procedure for animal’s preparation

The rabbit was chosen as a model for this study because its eye simulates an adult human eye with respect to size, shape, physiology, and composition of tears. Twelve healthy male, New Zealand albino rabbits, weighing between 2.5 and 3.0 kg were accommodated under standard temperature, humidity, and photoperiod light cycles. At the experimenting day, the rabbits were placed in restraining boxes where they could move their eyes and heads freely. They were identified by tattooing their ears. All experiments were carried out following the European Community Council Directives, and the study protocol was approved by the Ethical Committee of the Higher Research Council at the Faculty of Pharmacy, Jordan University of Science and Technology (Irbid, Jordan).

Evaluation of biocompatibility and residence time of inserts in the precorneal area

In this, in vivo study, four formulae were tested. The formulae were chosen according to in vitro dissolution studies carried out previously [13]. Rabbits were divided into four groups, each consisting of 3 rabbits, for each group two trails were carried out, in the first trial one insert was placed in the lower conjunctival sac of the left eye and the right eye was served as a control and vice versa in the second trial. After insertion, the eyelids were closed for 10 seconds in order to prevent the rejection of the insert. The behaviour of inserts after 10, 60, and 180 min from insertion was evaluated by direct visual observation using a skit lamp.

Measurement of CFX-HCl trans-corneal penetration

In order to estimate the amount of CFX-HCl in the aqueous humor of the rabbit eye, around 100 µl of the aqueous humor, was aspirated from the interior chamber at different time intervals using 1 ml insulin syringe fitted with a 26 gauge needle. For the reference formula R the withdrawn samples were at 0, 1, 3, 5, 7, 9, 24 and 32 h, while for F1, F2, and F3 samples were withdrawn at 0, 1, 5, 9, 24, 28, 32, 48, 56, 72, 80, 96, 98, 100, 104 and 120 h. At the end of the experiment, the ocular inserts were removed and the withdrawn samples were stored at -18°C until analysis.

Pharmacokinetic analysis

Ocular inserts were removed from each group at 96 h and the amount of drug remaining in each insert was determined. The area under the concentration in aqueous humor vs. time curve was calculated using the linear trapezoidal rule with extrapolation to infinity, which was done using the commercially available software package TUPPII. The peak aqueous humor concentration of ciprofloxacin (Cmax) and the time to reach Cmax (Tmax) were recorded. Then the statistical analysis of the data was conducted using the software WIN NONLIN (Ver. 3.3).

RESULTS AND DISCUSSION

In vivo HPLC method validation

The analytical method was validated according to the International Council for Harmonization (ICH) guidelines. The method was found accurate, specific, and sensitive for the analysis of CFX-HCl in aqueous humor with complete separation of the drug.

Linearity

The linearity of the method was evaluated from the calibration curve of spiked aqueous humor samples at several concentration levels of CFX-HCl. The peak area of the drug yielded a linear correlation over the concentration range (2.500–7.826) µg/ml. Calibration curve of the spiked aqueous humor standards with the regression equation and their correlation coefficient (R²) are shown in (fig. 2). The results confirmed the linearity of the standard curve over the studied range.

Fig. 2: Calibration curve of CFX-HCl in aqueous humor

Specificity

Representative HPLC chromatograms of blank aqueous humor and aqueous humor spiked with CFX-HCl are shown in (fig. 3) and (fig. 4) respectively, they indicate no any interference from the endogenous components. It is obvious from (fig. 4) that the peak of CFX-HCl was well resolved and completely separated. Therefore, this HPLC method is considered as a sensitive and specific method for CFX-HCl without any interference with the endogenous components might be available in the sample.
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Fig. 3: HPLC chromatogram of blank aqueous humor sample

Fig. 4: HPLC chromatogram of an aqueous humor sample containing CFX-HCl

Precision
The precision representing the repeatability (i.e. analysis repeatability) of standard solutions and spiked aqueous humor standards of CFX-HCl at the concentration of 4.444 µg/ml was studied. The %RSD values were found to be 0.25% and 0.45% for the standard solution and spiked aqueous humor respectively since they are less than 2% then the method is considered precise [3].

Accuracy
The accuracy of the method was determined on the basis of percent recovery at three concentration levels 50%, 100%, and 200% of the labeled content of CFX-HCl. They were found to be 99.43%, 100.09% and 99.76% respectively.

Limit of detection (LOD) and limit of quantitation (LOQ)
LOD value was found to be 0.027 µg/ml, and LOQ was 0.081 µg/ml. These LOD and LOQ values insure that the lowest concentration of CFX-HCl determined in aqueous humor samples can reliably be differentiated from background levels and can be quantitatively determined with suitable precision and accuracy which correlates well with the literature [16].

Tailing factor
The tailing factor was measured and found to be 1.32±0.012 and this is in compliance with the standard range mentioned in the United States Pharmacopeia USP [17].

Stability of samples
Results of the stability study of the spiked aqueous humor samples indicated that the samples were stable for four weeks when stored frozen at -18°C and the degradation occurred was within the recommended limits of biological studies.

Application of the method
The proposed validated HPLC method will be applied for assessing the in vivo behavior and the pharmacokinetics study of CFX-HCl in aqueous humor.

In vivo performance of CFX-HCl ocular inserts
Bioavailability of CFX-HCl in aqueous humor of rabbit’s eye
The bio-adhesive ocular system was studied using four different film coated inserts containing 6.25 mg CFX-HCl, which were given in a single dose and followed up to five days. This drug delivery system could be described as a circular disc consisting of spray-dried CFX-HCl with xanthan gum, cabopol C934, and propylene glycol, and coated with transparent lipophilic rate-controlling film of eudragit RL 100 copolymer [13].

The in vivo behaviour of uncoated inserts was previously studied, but unfortunately, they failed to release all of their drug content inside the rabbit eye since they were transformed into a gel and expelled outside the rabbit eye after 7-8 h from the application.

Based on previous in vitro release study [13], the coated formulae R, F1, F2, and F3 were chosen as models for this in vivo study. Where R is the reference release product, while F1 is representing the slow release behaviour, F2 is the intermediate release behaviour, and F3 is representing the fast release behaviour and they are all containing an amount of 6.25 mg CFX-HCl. The compositions of the studied formulae are shown in table 1. The aqueous humor concentration-time profiles of CFX-HCl release from the different formulae are shown in (fig. 5).
Table 1: The compositions of the studied formulae

<table>
<thead>
<tr>
<th>Formula</th>
<th>Free drug (mg)</th>
<th>SD2 (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>6.25</td>
<td>3.25</td>
</tr>
<tr>
<td>F2</td>
<td>5.00</td>
<td>2.50</td>
</tr>
<tr>
<td>F3</td>
<td>3.00</td>
<td>1.25</td>
</tr>
<tr>
<td>R</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

The pharmacokinetic parameters of the test formulae F1, F2, and F3 are shown in Table 3.

Table 2: Percent of CFX-HCl remaining in coated ocular inserts after removal from rabbits eyes

<table>
<thead>
<tr>
<th>Formula</th>
<th>Tablet 1</th>
<th>Tablet 2</th>
<th>Tablet 3</th>
<th>Tablet 4</th>
<th>Tablet 5</th>
<th>Tablet 6</th>
<th>Mean</th>
<th>SDEV</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>18.98</td>
<td>19.58</td>
<td>22.50</td>
<td>21.36</td>
<td>20.52</td>
<td>21.08</td>
<td>20.67</td>
<td>1.270</td>
</tr>
<tr>
<td>F3</td>
<td>10.22</td>
<td>13.35</td>
<td>12.66</td>
<td>11.10</td>
<td>10.98</td>
<td>11.33</td>
<td>11.61</td>
<td>1.166</td>
</tr>
</tbody>
</table>

The pharmacokinetic parameters of the four CFX-HCl ocular inserts formulae R, F1, F2 and F3 are shown in Table 3.

Table 3: Summary of the mean values of pharmacokinetic parameters of CFX-HCl following ocular administration of the four coated ocular inserts formulae

<table>
<thead>
<tr>
<th>Formula</th>
<th>AUC 0-∞ (µg h/ml)</th>
<th>AUC 0-∞ (µg h/ml)</th>
<th>C max (µg/ml)</th>
<th>t max (h)</th>
<th>t 1/2 (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>68.08 (6.857)</td>
<td>70.59 (6.038)</td>
<td>5.18 (0.557)</td>
<td>7.00 (0.000)</td>
<td>5.51 (1.690)</td>
</tr>
<tr>
<td>F1</td>
<td>192.80 (24.306)</td>
<td>194.69 (25.150)</td>
<td>3.33 (0.322)</td>
<td>24.00 (0.000)</td>
<td>10.49 (1.146)</td>
</tr>
<tr>
<td>F2</td>
<td>190.01 (9.057)</td>
<td>190.30 (8.991)</td>
<td>4.40 (0.536)</td>
<td>24.00 (0.000)</td>
<td>2.78 (0.856)</td>
</tr>
<tr>
<td>F3</td>
<td>122.72 (8.595)</td>
<td>128.93 (10.833)</td>
<td>4.53 (0.357)</td>
<td>9.00 (0.000)</td>
<td>8.88 (2.109)</td>
</tr>
</tbody>
</table>

Table 4: Relative bioavailability in terms of AUC 0-∞, AUC 0-∞, and C max of (F1/R, F2/R and F3/R), following ocular administration of ocular inserts containing 6.25 mg of CFX-HCl

<table>
<thead>
<tr>
<th>AUC 0-∞ ratios</th>
<th>AUC 0-∞ ratios</th>
<th>C max ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1/R</td>
<td>2.861</td>
<td>1.891</td>
</tr>
<tr>
<td>F2/R</td>
<td>1.816</td>
<td>2.777</td>
</tr>
<tr>
<td>F3/R</td>
<td>2.715</td>
<td>1.839</td>
</tr>
<tr>
<td>F1/R</td>
<td>1.839</td>
<td>0.646</td>
</tr>
<tr>
<td>F2/R</td>
<td>0.646</td>
<td>0.855</td>
</tr>
<tr>
<td>F3/R</td>
<td>0.855</td>
<td>0.876</td>
</tr>
<tr>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>0.510</td>
<td>0.344</td>
<td>0.205</td>
</tr>
<tr>
<td>0.456</td>
<td>0.291</td>
<td>0.240</td>
</tr>
<tr>
<td>0.291</td>
<td>0.240</td>
<td>0.084</td>
</tr>
<tr>
<td>0.240</td>
<td>0.084</td>
<td>0.141</td>
</tr>
<tr>
<td>0.084</td>
<td>0.141</td>
<td>0.165</td>
</tr>
</tbody>
</table>

*mean±SDEV, n = 6
It is obvious from the results shown in the table that all bioavailability values (F) of the test formulae are greater than 1, meaning that none of F1, F2, or F3 are bioequivalent to R [18]. Also as the amount of free drug decreases and the amount of SD2 increases, the bioavailability of CFX-HCl increases. The relationship between AUC0-\(\infty\) and the amount of SD2 available in the formula was constructed, and a linear correlation was observed as shown in (fig. 6). The bioavailability of ocular inserts of ciprofloxacin was previously reported by other investigators, where the insert was a combination of ciprofloxacin, methylcellulose, hydroxypropyl methyl cellulose, hydroxypropyl cellulose and Eudragit RS100. The in vivo studies of the ocular inserts showed that ciprofloxacin hydrochloride had a significant effect on the reduction of induced ocular conjunctivitis, where the bacterial load in the treated groups with inserts was reduced by two folds compared to control groups [4].

This relationship supports the conclusion that by increasing the amount of the matrix SD2 in the formula the extent of absorption of ciprofloxacin, methylcellulose, hydroxypropyl methyl cellulose, hydroxypropyl cellulose and Eudragit RS100. The in vivo studies of the ciprofloxacin hydrochloride had a significant effect on the reduction of induced ocular conjunctivitis, where the bacterial load in the treated groups with inserts was reduced by two folds compared to control groups [4].

The results of in vivo evaluations clearly demonstrated that CFX-HCl releasing insert system produced a significant, aqueous humor concentration throughout the 5-days insertion of one unit. Apparently, the application of the controlled drug insert system significantly minimizes the dose required of CFX-HCl for an effective management of corneal ulceration and conjunctivitis. In other words, the therapeutic efficacy of CFX-HCl in corneal ulceration and conjunctivitis treatment has been improved with the use of CFX-HCl insert system to control its ocular delivery. The above results support the conclusions reported by previous investigations and studies [20,21], that various ophthalmic systems such as inserts, ointment, suspensions, and aqueous gels have been developed in order to increase the residence time of the dose in addition to enhancing the ophthalmic bioavailability. Where the conventional ophthalmic delivery systems always result in poor bioavailability and therapeutic response because of the rapid precorneal elimination of the drug.

Statistical evaluation of the data

The Statistical analysis was performed using WIN NONLIN® (ver 3.3). The Two-way multiple measures (Analysis Of Variance) ANOVA was used to test for potential differences among the four coated ocular formulae F1, F2, F3 and R. Critical levels of significance were set at P<0.05. Also, the results were corrected for pairwise comparisons using the formulae Least-Square-Means Differences Test (LSM). There are significant differences between the in vivo release profiles for the four formulae due to the “Formula” variable (P<0.05) at α = 0.05.

In order to examine the significant differences between different mean values for the pharmacokinetic parameters of the four formulae, the pair-wise comparisons using Least-Significant-Means Difference Test was done. In terms of AUC0-\(\infty\) and AUC0-t, all the formulae are significantly different from each other except F1 and F3 since their P value was>0.05.

The 90% confidence interval (90% CI) for AUC0-\(\infty\), AUC0-t and Cmax measures of relative bioavailability and bioequivalence lied within an acceptance interval of [0.80-1.25] for some formulae. None of the formulae F1, F2 and F3 has been bioequivalent with R in terms of AUC0-\(\infty\), AUC0-t, and Cmax, lower and upper 90% CI are outside the 0.80-1.25 interval. F1 and F2 are also bioequivalent in terms Ln AUC0-\(\infty\), and AUC0-t. Also, F3 and F2 are bioequivalent in terms of LnP Cmax, values 90% CI. This was expected from the in vitro dissolution data [13].

Evaluation of biocompatibility of inserts in the precorneal area

Treatments F1, F2, and F3 were given in comparison with the reference formula R. The eyes were observed for redness, lacrimal secretion, mucoidal discharge, swelling of the eyelid, and response to ocular stimuli on a score basis up to 5 d. It was observed that ocular inserts F1, F2 and F3 have better biocompatibility compared with R and this could be due to the smaller size they have.

Importantly for previous investigators working on developing ocular inserts of different drugs [22], there has been a concern regarding the toxicity, non-biodegradability, and non-biocompatibility of synthetic polymers, so the trend was to use a combination of synthetic and biopolymers with well-known biocompatibility and biodegradability.

CONCLUSION

A rapid and precise RP-HPLC/UV method was used for the determination of CFX-HCl in aqueous humor. The method was validated according to the standard guidelines. The extraction procedure exhibited an excellent recovery of CFX-HCl. The inserts based on F1 and F2 formulae exhibited a profile typical of a controlled release delivery system, so they have a good potential to provide an effective drug concentration over days in the aqueous humor with a reduced number of applications. Fair biocompatibility of the matrix system in the rabbit eye was observed, then the insert formulae F1, F2, and F3 were with better biocompatibility compared with the reference formula R.

In conclusion, the ocular bio-adhesive inserts of CFX-HCl combined with the matrix system SD2 could be considered as a promising biocompatible controlled release dosage form for the treatment of corneal ulceration and conjunctivitis.

ACKNOWLEDGMENT

We would like to thank the Arab Pharmaceutical Manufacturing Co. Ltd. (Al salt, Jordan) for donating the ciprofloxacin hydrochloride (USP grade), xanthan gum, carbopol C934, and Eudragit RL100. This research was supported by grant # 147/2002 from the Jordan University of Science and Technology (Irbid, Jordan).

CONFLICTS OF INTERESTS

Declared none

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How to cite this article