Application of Chromatographic Response Function in Development of Stability Indicating HPLC Method for Determination of Benoxinate Hydrochloride and Fluorescein Sodium Mixture Using Factorial Design

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ABSTRACT

A simple and rapid stability-indicating RP-HPLC method was developed and validated for the quantitative determination of benoxinate hydrochloride and fluorescein sodium binary mixture. Both drugs were subjected to different stress conditions including hydrolysis under acidic and alkaline conditions, and oxidation by hydrogen peroxide. The optimization of forced degradation conditions was done by the application of experimental factorial design, which helped to enrich levels of degradation products. Different chromatographic response functions were tried to find out the best function that reflects the overall quality of the chromatogram. NCRI was found to be the optimum function, so it was selected as a response to be optimized in factorial design that was implemented to find the optimum chromatographic conditions. The chromatographic conditions obtained from factorial design led to the use of mobile phase consisting of a mixture of 40% acetonitrile and 60% 50 mM potassium dihydrogen phosphate buffer containing 0.01% triethylamine (pH adjusted to 5.0) at a flow rate of 1.5 mL/min and column temperature kept at 40°C. Inertsil ODS-3(250 mm x 4.6 mm, 5μm) column was used as a stationary phase and the detection was performed at 220 nm using a PDA detector. The HPLC method was successfully applied to the determination of benoxinate hydrochloride and fluorescein sodium in a synthetic mixture, and the percent recovery ± standard deviation (SD) was 100.56 ± 0.59 and 98.36 ± 0.29 for benoxinate hydrochloride and fluorescein sodium respectively. The method was found to be simple and rapid with less trial and error experiments by applying factorial design.

Keywords: Chromatographic response function, Factorial design, Benoxinate hydrochloride, Fluorescein sodium, Stability indicating.

1. INTRODUCTION

Looking for the separation strategy in liquid chromatography, an analyst can develop many different approaches, but the aim is always the same; to obtain the best possible chromatogram. When it comes to the complex mixtures, a set of chromatograms which is obtained by changing the chromatographic conditions is very complicated to be evaluated since it is usually very difficult to have all perfect performances (overall resolution, minimum analysis time, uniformed separation, etc.) at the same time. Modifying the experimental conditions so that the better resolution is accomplished, the elution time is often prolonged, and vice versa. Additionally, slowing down the elution of a mixture of
structurally similar compounds can provide better separation, but on the other hand, it will lead to peak deformation. The analyst is then facing the problem what is the prior criterion based on which the final optimum conditions should be selected.1,2

Objective functions are functions designed to give a single response value that reflects the overall quality of a chromatogram. This enables ranking the chromatograms according to these numerical values and helps in the optimization process.

Objective functions that consider both the time and the resolution have two competing aims; to maximize resolution and to minimize run time. The relative weighting of these two parameters in the function is an important factor in considering an objective function’s suitability. Many objective functions have been suggested to be used in the optimization of separation processes.3-5

Berridge proposed the chromatographic response function (CRF) and used it for the optimization of reversed-phase HPLC separations using the modified simplex algorithm.6 Morris et al. recommended the chromatographic exponential function (CEF) for use in optimization strategies and applied this function to optimize the capillary gas chromatographic separation of phenols.7

The chromatographic resolution statistic (CRS) was developed by Schlabach and Excoffier.8 Olsson and Kaufmann used the CRS in a factorial design for optimizing gas-liquid chromatography conditions.9 Duarte improved the CRF (DCEF) to overcome the problem of resolution term “R” in the objective functions because it can only be successfully applied for Gaussian-shaped peaks.10 He applied this function in size exclusion chromatography study of a variety of different organic compounds. The main problem with DCEF is that the resolution and time term is not adequately weighted, so Jancic- Stojanović suggested a new CRF (NCCEF) and applied it to LC separation of raloxifene and its impurities.2

 Forced degradation studies are carried out to produce the potential degradation products which are important for the development of the analytical method to be specific for the API without interference from the degradation products resulting from different stress processes.11 Several approaches are reported to conduct forced degradation studies.12-15 The target percent degradation of a drug is 5-20%. However, stress conditions may be optimized to enrich the degradation products within this range. A full factorial design is a type of experimental design in which all possible combinations of all levels of factors are investigated.16 This helps to arrive at a combination of stress degradation conditions that enrich and maximize the drug’s degradation. Factorial Design was also implemented for the optimization of the chromatographic response function for the separation of the drugs and their degradation products. The literature review reveals the utilization of the Design of Experiment for optimization of forced degradation conditions.17-22 Besides, the use of the Design of Experiment technique has been recently adopted for the optimization of chromatographic conditions to develop stability-indicating methods for different drugs.23-28

Benoxyate hydrochloride (BNX HCl), also known as oxybuprocaine HCl is a para-benzoic acid ester that is used as a local anesthetic. Chemically, it is 2-diethyl aminoethyl 4-amino-3-butoxy-benzoate hydrochloride as shown in Figure 1. It is used for topical anesthesia of the eye for the fitting of contact lenses, removal of a foreign body from the cornea, or for minor surgery. Fluorescein sodium (FLO sodium) is disodium 3-oxo-3H-spiro [2-benzofuran-1.9’-xanthene]-3’,6’-bis(olate) as shown in Figure 1. It is a water-soluble fluorescent dye used in permeability and vascular perfusion studies due to its small molecular radius. It is used as a diagnostic aid in corneal injuries and corneal trauma. It has been approved by the FDA for use in externally applied drugs and cosmetics.29 It is also used as a fluorescent tracer to study the blood-brain barrier (BBB) permeability in rodent models.30

Fluorescein sodium and benoxylate hydrochloride ophthalmic solution is indicated for procedures requiring a disclosing agent in combination with an anesthetic agent such as tonometry, gonioscopy, removal of corneal foreign bodies and other short corneal or conjunctival procedures. Different analytical methods have been reported for the determination of BNX HCl31-37 and fluorescein and its derivatives.38-40 However, only one RP-HPLC method is reported in USP for the simultaneous determination of both drugs in ophthalmic solutions. The method involves the use of a mobile phase consisting of sodium 1-pentanesulfonate dissolved in glacial acetic acid, then acetomitrile and triethanolamine are added and pH is adjusted to 3.0 with phosphoric acid. The flow rate is 1.5 mL/min and UV detection is carried out at 254 nm.41 The run time was relatively long (more than 17 minutes), besides, the preparation of the mobile phase is tedious. So, a much more rapid and simple HPLC method is necessary for the assay of BNX HCl and FLO sodium mixture to be used in routine analysis. Moreover, the stability-indicating method is needed for assay of the two drugs in the presence of their degradation products.

Figure 1: Chemical structure of (a) BNX Cl and (b) FLO sodium.

2. METHODS

2.1. Material

BNX HCl was kindly supplied by Egyptian International Pharmaceutical Industries Co. (10th of Ramadan, Egypt) certified to contain 99.8% BNX HCl. FLO sodium was kindly supplied by Sigma Pharmaceutical Industries (Quesna, Egypt).
Sodium Hydroxide analytical grade was purchased from WinLab (UK). The hydrochloric acid analytical grade was purchased from Ridel-de Haën (Germany). Potassium dihydrogen orthophosphate and triethylamine (analytical grade) were purchased from the Oxford laboratory (Mumbai, India). Acetonitrile HPLC grade was purchased from Sigma-Aldrich (USA).

2.2. Instrumentation

A Dionex UltiMate 3000 RS system was used, (Thermo Scientific™, Dionex™, Sunnyvale, CA, USA), equipped with Quaternary RS pump, RS auto-sampler injector, Thermostated RS Column Compartment, and RS Diode array detector (DAD). The instrument was connected to a Dell compatible PC, bundled with Chromelone® 7.1 Chromatography Data System software.

pH measurements were made with HANNA pH 211 Microprocessor pH Meter with a double junction glass electrode. A thermostatically controlled water bath (Messmer, Germany) was used for forced degradation studies. Statistical analysis of data including factorial design was made using Minitab 16 software.

2.3. Chromatographic Conditions

Separation and quantitation were carried out on Inertsil ODS-3(250 mm x 4.6 mm, 5μm) column using of a mixture of 40% ACN and 60% 50 mM potassium dihydrogen phosphate buffer containing 0.01% triethylamine (pH adjusted to 5.0) as mobile phase at a flow rate of 1.5 mL/min. The column temperature was set at 40°C. The detection was performed at 220 nm using a PDA detector.

2.4. Preparation of Stock Standard Solutions

Stock standard solutions of BNX HCl and FLO sodium (1 mg/mL) were prepared in distilled water.

2.5. Preparation of the Binary Mixture

The binary mixture was prepared by accurately transferring 400 μL of the BNX HCL stock standard solution and 250 μL of the FLO sodium stock standard solution into 10 mL volumetric flask, completing to the volume with mobile phase to obtain a solution containing 40 μg/mL BNX HCL and 25 μg/mL FLO sodium.

2.6. Construction Of BNX HCl Calibration Curve

Into a series of 10 mL volumetric flasks, aliquots of BNX HCl stock standard solution (1 mg/mL) were quantitatively transferred; appropriate dilutions were carried out with the mobile phase to yield solutions in the concentration range of 4-80 μg/mL. 20.0 μL portions of each solution were injected in replicates into the chromatographic system.

2.7. Construction of FLO Sodium Calibration Curve

Into a series of 10 mL volumetric flasks, aliquots of FLO sodium stock standard solution (1 mg/mL) were quantitatively transferred; appropriate dilutions were carried out with the mobile phase to yield solutions in the concentration range of 1-50 μg/mL. 20.0 μL portions of each solution were injected in replicates into the chromatographic system.

2.8. Forced Degradation Studies

A mixture of BNX HCl and FLO sodium was subjected to different stress conditions. A full factorial design was implemented to identify optimum degradation conditions.

For acid, alkali, hydrolysis under the neutral condition and oxidative degradation conditions, values of variables like time of exposure, temperature, and strength were chosen to obtain about 20% degradation.

(a) Acid degradation: Acidic degradation solutions were prepared by dissolving 16 mg of BNX HCl and 10 mg of FLO sodium separately in X1 N HCl in a 10 mL volumetric flask and heated under reflux for X2 hours at X3 °C. Two levels were chosen for each of X1, X2, and X3. The high level (+1) for X1, X2, and X3 was 1N, 4 hours, and 100 °C, respectively, and the low level (-1) for X1, X2 and X3 was 0.1 M, 1 hour and 70 °C, respectively.

Aliquots of these solutions (250 μL each) were transferred to 10 mL volumetric flasks, cooled and neutralized with sodium hydroxide, and then completed to volume with the mobile phase.

(b) Alkali degradation: Alkaline degradation solutions were prepared by dissolving 16 mg of BNX HCl and 10 mg of FLO sodium in X1 N NaOH in a 10 mL volumetric flask and heated under reflux for X2 hours at X3 °C. Two levels were chosen for each of X1, X2, and X3. The high level (+1) for X1, X2, and X3 was 0.1 N NaOH, 2 hours, and 40°C, respectively, and the low level (-1) for X1, X2 and X3 was 0.01 N, one hour and 25 °C, respectively.

(c) Oxidative degradation: Oxidative degradation solutions were prepared by dissolving 16 mg of BNX HCl and 10 mg of FLO sodium in 10% hydrogen peroxide in a 10 mL volumetric flask and heated under reflux for X1 hours at X2°C. Two levels were chosen for X1 and X2. The high level (+1) for X1 and X2 was 24 hours and 50°C respectively, and the low level (-1) for X1 and X2 was 4 hours and 30°C respectively.

Since three variables (X1, X2, and X3) were considered at two levels for acid and alkali hydrolysis, a 2^3 full factorial design was applied to set up eight degradation experiments.

Similarly, two variables (X1 and X2) were considered at two levels for oxidative degradation so a 2^2 full factorial design was used to set up four degradation experiments. The experimental matrix for 2^3 and 2^2 full factorial design is shown in Table 1.
2.9. Chromatographic Analysis of Stressed Samples

Each of the stressed samples obtained was cooled, neutralized (for acid and alkaline hydrolysis) and diluted with the mobile phase to get a final concentration of 40 μg/mL BNX HCl and 25 μg/mL FLO sodium, which was chromatographed as previously described. The resulting chromatograms were studied for the appearance of secondary peaks and the % reduction in the area of drug peak concerning standard BNX HCl and FLO sodium solutions. The % reduction in peak area was considered as % degradation. For each stress condition, a blank (control) experiment subjected to the same stress conditions was chromatographed using the proposed HPLC method.

2.10. Preparation of the Synthetic Mixture

Since the ophthalmic solution containing BNX HCl and FLO sodium mixture is not available in the Egyptian market, the synthetic mixture was prepared to simulate the dosage form. The synthetic mixture was prepared by dissolving accurately weighed 25 mg FLO sodium, 40 mg BNX HCl, 100 mg chlorobutanol, 100 mg boric acid, and 1 gm povidone with distilled water in a 25-mL volumetric flask. An aliquot of 250 μL of this solution was transferred into a 10 mL volumetric flask, completing to the volume with the mobile phase to obtain a solution containing 40 μg/mL BNX HCl and 25 μg/mL FLO sodium. A 20 μL volume of the final solution was injected in triplicate and chromatographed under specified conditions. The concentration of BNX HCl and FLO sodium in assay solutions were calculated from the corresponding calibration curves.

3. RESULTS AND DISCUSSION

3.1. Forced Degradation Studies

FLO sodium was found to be stable in acidic and alkaline hydrolytic conditions as it does not undergo significant degradation while BNX HCl was degraded under acid and alkaline conditions. A full Factorial design was implemented for the optimization of different degradation conditions to obtain about 20% degradation. Three variables were considered at two levels for acid and alkaline hydrolysis, so, a $2^3$ full factorial design was applied to set up eight degradation experiments. Similarly, two variables were considered at two levels for oxidative degradation so a $2^2$ full factorial design was used to set up four degradation experiments. The experimental matrix for $2^3$ and $2^2$ full factorial design is shown in Table 1. (X1, X2, X3, and their levels are described in the experimental section).

Table S1 shows optimum degradation conditions obtained by factorial design and the resulting percent degradation. Supplementary material (Figure S1) shows some of the factorial design results of factors affecting BNX HCl and FLO sodium oxidative degradation such as Pareto chart, contour plot, interaction plot, and response optimizer.

Chromatograms obtained for BNX HCl and FLO sodium under different stress conditions are shown in Figure 2.

Table 1: Experimental design of BNX HCl and FLO sodium degradation in each condition.

<table>
<thead>
<tr>
<th>Expt no</th>
<th>X1</th>
<th>X2</th>
<th>X3</th>
<th>Acid hydrolysis of BNX (%)</th>
<th>Alkaline hydrolysis of BNX (%)</th>
<th>Oxidative degradation of BNX (%)</th>
<th>Oxidative degradation of FLO (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>94.5</td>
<td>80.57</td>
<td>+</td>
<td>50.4</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>40.15</td>
<td>52.43</td>
<td>+</td>
<td>68.5</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>25.5</td>
<td>68.63</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>2.10</td>
<td>14.57</td>
<td>-</td>
<td>14.9</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.62</td>
<td>12.35</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>38.74</td>
<td>40.74</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>10.15</td>
<td>24.43</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>2.97</td>
<td>15.62</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

3.2. Application of Chromatographic Response Function for Development of HPLC Method

FLO sodium was found to be stable in acidic and alkaline hydrolytic conditions as it does not undergo significant degradation while BNX HCl was degraded under acid and alkaline conditions.

For the development of a stability-indicating method for BNX HCL and FLO sodium mixture, forced degradation studies were carried out including acid, alkaline hydrolysis, and oxidative degradation.

FLO sodium was degraded only under oxidative stress conditions. So, an oxidative degraded sample was selected to be used in the optimization of chromatographic conditions. Chromatogram of the oxidative degraded sample contains four main peaks corresponding to BNX HCl, its degradation product, FLO sodium, and its degradation product. To optimize the separation of those four peaks, many responses should be taken into account including resolution between adjacent peak pairs, peak shape, and run time. Instead, objective functions can be used to express the overall quality of the chromatogram in a single numerical value. Three of these objective functions were selected to be used to compare them and find the best function that can be applied in the optimization process. These functions are CRP$^5$, DCRF$^6$, and CRBF$^7$. The effect of pH, the ratio of ACN, and column temperature on the value of different objective functions were studied. The best chromatogram according to each function is shown in Figure 3.
Chromatogram (a) is the best according to CRF while chromatogram (b) is the best according to both $D_{CRF}$ and $N_{CRF}$. However, the run time of chromatogram (a) is relatively long (about 16 minutes). Moreover, the individual resolution between all peaks is not maximized. This means that CRF does not lead to the selection of the optimum chromatographic conditions as it led to the selection of a chromatogram of lower quality.

On the other hand, chromatogram (b) represents the best chromatogram regarding run time (11.5 minutes) as well as individual resolution between all peaks. This indicates that both $D_{CRF}$ and $N_{CRF}$ are good objective functions that can be used to reflect the real overall quality of chromatograms. However, $N_{CRF}$ (suggested by Jancic – Stojanovi) was selected to be used in the current study due to some reported drawbacks of $D_{CRF}$.

Besides, $N_{CRF}$ was applied to the chromatographic separation of raloxifene and its impurities which is similar to the current study.

Figure 3: The best chromatogram obtained according to (a) CRF and (b) both $D_{CRF}$ and $N_{CRF}$.

3.3. Optimization of $N_{CRF}$ by Full Factorial Design

For more optimization of the developed HPLC method, the full factorial design was carried out. Three factors were selected for optimization which are: % ACN, pH of the aqueous portion of the mobile phase, and column temperature. The response to be optimized is $N_{CRF}$. A set of eight experiments were performed and the value of $N_{CRF}$ for each experiment was calculated as shown in Table S2. Some of the Pareto charts, contour plots, interaction plot, and response optimizer results are shown in Figure 4. The system suitability criteria of the proposed HPLC method are listed in Table 2.

Table 2: Results of system suitability tests for the HPLC method.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>BNX HCl</th>
<th>FLO sodium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention time ($R_T$) (min)</td>
<td>3.94</td>
<td>7.30</td>
</tr>
<tr>
<td>Theoretical plates (N)</td>
<td>5126</td>
<td>7039</td>
</tr>
<tr>
<td>HETP (cm)</td>
<td>0.0049</td>
<td>0.0035</td>
</tr>
<tr>
<td>Asymmetry factor</td>
<td>1.32</td>
<td>1.19</td>
</tr>
<tr>
<td>Resolution</td>
<td>11.98</td>
<td></td>
</tr>
</tbody>
</table>
Optimum chromatographic conditions obtained from the response optimizer involve the use of 50 mM potassium dihydrogen phosphate buffer containing 0.01% triethylamine (pH adjusted to 5.0) and acetonitrile in a ratio of 60:40, v/v as mobile phase and column temperature is kept at 40°C as shown in Figure 5.

**Figure 5:** HPLC chromatogram of mixture of BNX HCl and FLO sodium oxidative degraded sample. Chromatographic condition: Inertsil C18 (250 - 4.6 mm i.d., 5 lm); flow rate: 1.5mL/min, mobile phase: a mixture of 40% ACN and 60% 50 mM potassium dihydrogen phosphate buffer containing 0.01% triethylamine (pH adjusted to 5.0); detection: 220 nm. Column temperature: 40°C.

### 3.4. Method Validation

The proposed method was validated regarding linearity, accuracy, repeatability, and intermediate precision according to ICH Q2 (R1) recommendations.32

#### 3.4.1. Linearity and range

The linearity of BNX HCl was established by preparing a calibration curve in the range 4-80 μg/mL and 1-50 μg/mL for FLO sodium. Triplicates of each solution were injected and chromatograms were recorded. The mean area under peak was plotted against concentration (μg/mL) to construct the calibration curves. The correlation coefficient and regression equations were determined as shown in Table 3.

**Table 3:** Linearity regression data for BNX HCl and FLO sodium using the proposed HPLC method.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>BNX HCl</th>
<th>FLO sodium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range</td>
<td>4 – 80 μg/mL</td>
<td>1 – 50 μg/mL</td>
</tr>
<tr>
<td>slope</td>
<td>0.2097</td>
<td>1.1270</td>
</tr>
<tr>
<td>SE of slope</td>
<td>0.0011</td>
<td>0.0061</td>
</tr>
<tr>
<td>Intercept</td>
<td>-0.0027</td>
<td>-0.4114</td>
</tr>
<tr>
<td>SE of Intercept</td>
<td>0.0488</td>
<td>0.1707</td>
</tr>
<tr>
<td>Correlation coefficient (r)</td>
<td>0.9998</td>
<td>0.9998</td>
</tr>
<tr>
<td>SE of estimation</td>
<td>0.0851</td>
<td>0.3182</td>
</tr>
</tbody>
</table>

#### 3.4.2. Accuracy

Accuracy of the method was determined by calculating the mean % recovery of triplicate determination for BNX HCl and FLO sodium at three concentrations within the linearity range as shown in Table 4.

**Table 4:** Evaluation of accuracy for the determination of BNX HCl and FLO sodium.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Conc. taken (μg/mL)</th>
<th>Conc. found (μg/mL)</th>
<th>% Recovery</th>
<th>Mean recovery ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNX HCl</td>
<td>8</td>
<td>8.02</td>
<td>100.31</td>
<td>101.20 ± 0.79</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>20.37</td>
<td>101.83</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>40.58</td>
<td>101.45</td>
<td></td>
</tr>
<tr>
<td>FLO sodium</td>
<td>5</td>
<td>5.01</td>
<td>100.19</td>
<td>99.55 ± 0.68</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>12.46</td>
<td>99.66</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>24.71</td>
<td>98.83</td>
<td></td>
</tr>
</tbody>
</table>

#### 3.4.3. Precision

Repeatability (intraday precision) was determined by calculating the SD and % RSD for triplicate determinations of three concentrations of BNX HCl and FLO sodium within the linearity range on the same day. Intermediate (interday) precision was calculated by triplicate determinations of BNX HCl and FLO sodium at three concentrations within the range of the linearity on three different days. The % RSD was not more than 2% as shown in Table 5.

#### 3.4.4. Robustness

Deliberate changes in the column temperature (± 2°C), mobile phase composition (± 2%), and pH of an aqueous portion (± 0.1) were made. The %RSD between the area under peak and that obtained under optimized chromatographic conditions were determined. The results are shown in Table S3. The low
value of % RSD of the percentage recoveries indicated the robustness of the method.

**Table 5:** Evaluation of the precision of the proposed HPLC method for the determination of BNX HCl and FLO sodium.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Intraday</th>
<th>Interday</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Conc. taken</td>
<td>Conc. found</td>
<td>%RSD</td>
<td>Conc. taken</td>
<td>Conc. found</td>
</tr>
<tr>
<td></td>
<td>(µg/m L)</td>
<td>(µg/m L)</td>
<td></td>
<td>(µg/m mL)</td>
<td>(µg/m L)</td>
</tr>
<tr>
<td></td>
<td>8.059</td>
<td>8.027</td>
<td>0.44</td>
<td>8</td>
<td>7.97</td>
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<tr>
<td></td>
<td>7.99</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BNX HCI</td>
<td>20</td>
<td>20.31</td>
<td>0.27</td>
<td>20</td>
<td>20.14</td>
</tr>
<tr>
<td></td>
<td>20.37</td>
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</tr>
<tr>
<td></td>
<td>40.51</td>
<td>40.63</td>
<td>0.17</td>
<td>40</td>
<td>39.98</td>
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<tr>
<td></td>
<td>40.60</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>FLO sodium</td>
<td>5</td>
<td>5.03</td>
<td>0.41</td>
<td>5</td>
<td>5.01</td>
</tr>
<tr>
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<td>4.98</td>
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<tr>
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<td></td>
<td>24.70</td>
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</tbody>
</table>

**3.4.5. Limit of detection and limit of quantitation**

The ICH guidelines for calculation of LOD and LOQ were followed. The method was based on the standard deviation of the blank response and the slope of the calibration curve.

The calculated LOD and LOQ for BNX HCl were found to be 0.31 µg/ml and 0.95 µg/ml respectively. For FLO sodium, the calculated LOD and LOQ were found to be 0.1 µg/ml and 0.31 µg/ml respectively.

**3.5. Application to a Pharmaceutical Dosage Form**

The proposed method was successfully applied for the determination of BNX HCl and FLO sodium in the laboratory prepared synthetic mixture. Three replicates were determined. Satisfactory results were obtained for both drugs as shown in Table S4. This confirmed that the excipients did not show any interference.

**4. CONCLUSIONS**

Forced degradation studies of BNX HCl, FLO sodium mixture were carried out and oxidative degradation conditions were optimized using full factorial design. Different objective functions were tried to find out the best function that reflects the overall quality of chromatogram. NCREF was found to be the optimum function, so it was used as the response to be optimized in factorial design that was performed to find the optimum chromatographic conditions. The method is simple, sensitive, and rapid stability-indicating HPLC method for the determination of benoxinate hydrochloride and fluorescein sodium in the presence of their potential degradation products as well as in ophthalmic solution within eight minutes.

**CONFLICTS OF INTEREST**

The authors declare no conflict of interest.

**5. REFERENCES**

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