Development and Validation of a New Stability-Indicating RP-UPLC Method for the Quantitative Determination of Bromfenac Sodium and Its Impurities in an Ophthalmic Dosage Form

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Abstract

A new rapid stability-indicating reversed-phase UPLC method was developed and validated for the determination of Bromfenac sodium and its impurities in Bromfenac ophthalmic solution. During literature search, only a few publications were found about Bromfenac sodium. There is no official monograph in the pharmacopoeias about Bromfenac sodium. Chromatographic separation has been achieved on a polar-embedded Waters Acquity BEH Shield RP18 (100 mm × 2.1 mm, 1.7 μm) column under gradient elution by using a binary mixture of potassium dihydrogen phosphate (0.01 M, pH 3.3) and acetonitrile (ACN) at a flow rate of 0.5 mL/min. Chromatogram was monitored at 265 nm using a photodiode array detector (PDA). The drug and its related impurities are eluted within 13 min. Resolution of Bromfenac sodium and all eight potential impurities have been achieved greater than 4.0 for all pairs of compounds. To prove the stability-indicating power of the method, the drug was subjected to hydrolytic (acid, alkaline and water), oxidative, photolytic and thermal stress, and the major degradation products were identified based on LC–MS analysis. The developed method was validated as per ICH guidelines with respect to specificity, linearity, limit of detection, limit of quantification, precision, accuracy and robustness.

Introduction

Bromfenac sodium sesquihydrate (BRO) chemically described as sodium 2-amino-3(4-bromobenzoyl)phenylacetate sesquihydrate (Figure 1A). Its empirical formula is C15H11BrNNaO3·1½H2O, and the molecular weight is 383.17 g/mol. The drug was approved by United States Food and Drug Administration (USFDA) and marketed under the trade name of Xibrom (Bromfenac ophthalmic solution, 0.09% w/v). Bromfenac ophthalmic solution is a sterile, topical, nonsteroidal anti-inflammatory drug for ophthalmic use (1, 2).

An extensive literature search revealed only few analytical techniques for determining BRO individually based on UV spectrophotometry (3, 4) and high-performance liquid chromatography (HPLC) (5–9). All of the listed techniques are applied to assay of BRO. Jia-yi et al. (10) reported RP-HPLC determination of content and related substances of Bromfenac sodium. According to our findings, none of the currently available analytical methods are stability indicating with respect to quantification of all known and degradation impurities. In the present work, in addition to reported impurities, other possible-related substances and degradation products of BRO were considered for method development by UPLC. The literature survey reveals that no reference exists for the quantitative determination of impurities by a stability-indicating UPLC method. No official United States Pharmacopeia Convention (USP) and European Pharmacopoeia (EP) monographs currently exist for BRO. It is therefore felt necessary to develop an accurate, rapid, selective and sensitive stability-indicating LC method for the determination of BRO and its...
Figure 1. Chemical structure and name of BRO and impurities. (A) BRO, (B) Imp-1, (C) Imp-2, (D) Imp-3, (E) Imp-4, (F) Imp-5, (G) Imp-6, (H) Imp-7 and (I) Imp-8.
related compounds. For this study, we intend to opt for a faster chromatographic technique UPLC to assess whether UPLC can result in shorter analysis times without compromising on the resolution and sensitivity. Hence, a stability-indicating RP-UPLC method was developed for the quantitative determination of BRO and its eight impurities with a shorter run time (18 min) using a simple and cost-effective mobile phase. This method was successfully validated according to the ICH guidelines (11). The developed method is also suitable for the determination of BRO assay.

**Experimental**

**Materials and reagents**

BRO active pharmaceutical ingredient (API), BRO ophthalmic solution drug product and placebo and standards of impurities were supplied by Dr. Reddy’s Laboratories Ltd., Hyderabad, India. HPLC-grade methanol (MeOH), acetonitrile (ACN), analytical grade potassium dihydrogen phosphate (KH₂PO₄), ammonium acetate (CH₃COONH₄), orthophosphoric acid (H₃PO₄), hydrochloric acid (HCl), sodium hydroxide (NaOH) and hydrogen peroxide (H₂O₂) were purchased from Merck, Darmstadt, Germany. Deionized water was prepared using a Milli-Q plus water purification system from Millipore (Bedford, MA, USA).

**Instrumentation and chromatographic conditions for UPLC**

Waters Acquity UPLC system equipped with a 2996 photo diode array detector. The output signal was monitored and processed using Empower 2 software (Waters Corporation, Milford, MA, USA). Separation was accomplished on an Acquity UPLC BEH Shield RP-18 column (100 mm, 2.1 mm, and 1.7 μm). Mobile phase A consists of a mixture of 0.01 M KH₂PO₄ buffer (pH 3.3 adjusted with H₂PO₄) and ACN in the ratio of 70:30 (v/v). Mobile phase B consists of a mixture of Milli-Q water and ACN in the ratio of 5:95 (v/v). The mobile phases were filtered through a nylon 0.2-μm membrane filter. The flow rate of the mobile phase was 0.5 mL/min. The UPLC gradient program (time in min/%B) was set as 0.0/15, 3/30, 10/100, 14/100, 14.1/15 and 18.0/15. The column temperature was maintained at 25°C. The detection was monitored at a wavelength of 265 nm. The injection volume was set as 3.0 μL. An equal volume of methanol and water was used as a diluent.

**Preparation of standards solutions and sample preparation**

A stock solution of BRO (450 μg/mL) was prepared by dissolving the drug in diluent. Working standard solutions of 0.45 and 90 μg/mL were prepared from the stock solution for the determination of the related compounds and assay, respectively. The individual stock solutions of BRO (450 μg/mL) were prepared by dissolving BRO (450 μg/mL) in diluent. These solutions were further diluted to 90 μg/mL (5.0–25 μL with diluent) and used for assay.

Similarly, the placebo test preparation was prepared as per the above test procedure for related substances and assay analysis. API test preparation was prepared by dissolving 4.5 mg of BRO to 10 mL with diluent.

**Specificity and mass balance study**

Stress degradation studies were performed according to ICH guidelines Q1A (R2) (12) to demonstrate the stability-indicating nature and specificity of the proposed method. During the process, 5 mL of BRO ophthalmic solution (0.09% w/v) was transferred into a 10-mL volumetric flask and subjected to forced degradation study under acid (0.01 M HCl at 25°C for 1 h), base (0.5 M NaOH at 60°C for 6 h), neutral (water at 60°C for 6 h) and oxidation (5.0% v/v H₂O₂ at 60°C for 5 h) conditions.

The stressed samples of acid and base degradation were neutralized with 0.01 M NaOH and 0.5 M HCl, respectively, and made up to volume with the diluent. BRO ophthalmic solution was placed in a thermally controlled oven at 90°C up to 24 h for thermal stress study. Photolytic degradation was performed by exposing the ophthalmic solution to visible light and UV with minimum exposure of 1.2 million lux-hours and 200 w-h/m², respectively.

The peak purity test was carried out for the BRO peak by using a PDA detector in all stressed samples. The assay of stressed samples was performed (at 90 μg/mL) by comparison with qualified reference standard, and the mass balance (% assay + % impurities + % degradation products) was calculated. The assay was also calculated for the BRO sample by spiking all 13 impurities at the specification level.

**LC-MS-MS conditions**

LC-MS-MS system (Agilent 1200 series liquid chromatography coupled with Applied Bios stems 4000 Q Trap triple quadrupole mass spectrometer with Analyst 1.4 software, MDS SCIEX, USA) was used for the identification of the unknown compounds formed during forced degradation studies. A symmetry shield RP-18 (150 × 4.6 mm, 3.5 μm) column was used as the stationary phase. Ammonium acetate (0.01 M; pH 3.3 adjusted with formic acid) was used as buffer. Buffer and ACN in the ratio of 70:30 (v/v) was used as Mobile phase A. Water and ACN in the ratio of 5:95 (v/v) was used as Mobile phase B. The gradient program (time in min/%B) was set as 0.025, 10/25, 30/50, 50/70, 60/100, 60.1/25 and 65/25. Methanol and water in the ratio of 1:1 (v/v) was used as the diluent. The flow rate was 1.0 mL/min with an injection volume of 20 μL. The analysis was performed in positive electro spray ionization mode. The ion source voltage was 5,000 V. The source temperature was 450°C. GS1 and GS2 are optimized to 30 and 35 psi, respectively. The curtain gas flow was 20 psi. For fragmentation (MS-MS) studies, collision energy and declustering potential were set at 30 eV and 30 V, respectively.

The peak purity test was carried out for the BRO peak by using a PDA detector in all stressed samples. The assay of stressed samples was performed (at 90 μg/mL) by comparison with a qualified reference standard, and the mass balance (% assay + % impurities + % degradation products) was calculated. The assay was also calculated for the BRO sample by spiking all eight impurities at the specification level.

**Validation parameters**

The proposed method was validated according to the ICH guidelines for its limit of detection (LOD), limit of quantification (LOQ), linearity, precision, accuracy, solution stability and robustness. Moreover,
relative response factors (RRFs) for the impurities of BRO were also determined.

Results

Results of method validation

Specificity

Specificity was demonstrated by spiking the test BRO concentration with 0.15% impurities. An equivalent placebo concentration was prepared (refer preparation of standards solutions and sample preparation section) and injected to evaluate the interference with the analyte peaks. The peaks from the diluent and placebo solution showed no peak interference with analytes; moreover, the adjacent peaks were well separated with Rs > 4.0, indicating the high specificity and selectivity of the method.

Sensitivity

The LOD and LOQ values for all impurities were determined by injecting a series of diluted solutions with known concentration to obtain S/N ratio values of 3 and 10, respectively. For LOQ, the signal-to-noise ratios for all impurities were ranging from 9.6 to 10.5. The precision study was performed at the LOQ level by injecting six individual preparations of BRO and impurities and calculated the % RSD for the areas of each peak. The RSDs were found to be between 2.8 and 4.2%. Accuracy at the LOQ level was verified by injecting three individual preparations of BRO spiked with impurities at the LOQ level. The percent recoveries were calculated for each impurity, and those are ranging from 96.1 to 102.2%. The results were in the range of 0.04–0.07 µg/mL for LOD and 0.11–0.23 µg/mL for LOQ (Table I).

Precision

The repeatability and ruggedness of the method was performed by six individual determinations of BRO ophthalmic solution (450 µg/mL) by spiking with impurities at the specification level. The ruggedness was determined by repeating the same experiment on two different days by different analysts using different equipment. The % RSD was calculated for each impurity (Table I). These results confirmed the high precision.

Linearity

Linearity test solutions were prepared from impurity stock solution at seven different concentration levels ranging from LOQ to 200% of the specification level (i.e., LOQ, 0.27, 0.405, 0.540, 0.675, 0.810, 1.08, and 1.35 µg/mL for Imp-1, Imp-2, Imp-3, Imp-4, Imp-5, Imp-6, Imp-7, Imp-8 and BRO). The calibration curve was drawn by plotting the impurity area versus the concentration. The correlation coefficient obtained was greater than 0.999 for all impurities (Table I).

Accuracy

Accuracy of the method was evaluated by spiking with known amounts of impurities to BRO ophthalmic solution (450 µg/mL) at the level of LOQ, 50, 100 and 150% of specification in triplicate. The percent recoveries were calculated for related substances and those are ranging from 96.6 to 103.4% (Table I). The percent recovery of BRO in the test sample was ranging from 99.2% w/w to 100.8% w/w in its assay method.

Solution stability and mobile phase stability

The solution stability and mobile phase stabilities at a temperature 25°C were evaluated by injecting the test solutions spiked with impurities daily for up to 2 days. The prepared mobile phase was kept constant during the study period. No significant changes in the amounts of impurities were observed during the study. These results confirmed that sample solution and mobile phase were stable up to 2 days at ambient temperature.

Robustness

To determine the robustness of the method, experimental conditions were deliberately altered. The factors chosen for this study, which were the critical sources of variability in the operating procedures such as flow rate (0.5 ± 0.05 mL/min), mobile phase pH (3.3 ± 0.2),

The precision of the assay was evaluated by performing six (n = 6) independent assays of the BRO test sample against the qualified reference standard. The assay results obtained on the two different days (n = 6) were 99.62 ± 0.16 and 99.71 ± 0.35 (mean ± RSD). These results confirmed the high precision.

Table I. Summary of Method Validation for BRO and Its Impurities

<table>
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<tr>
<th>Validation parameter</th>
<th>Imp-1</th>
<th>BRO</th>
<th>Imp-2</th>
<th>Imp-3</th>
<th>Imp-4</th>
<th>Imp-5</th>
<th>Imp-6</th>
<th>Imp-7</th>
<th>Imp-8</th>
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<tr>
<td>RRF</td>
<td>1.36</td>
<td>1.23</td>
<td>1.20</td>
<td>1.05</td>
<td>0.96</td>
<td>2.15</td>
<td>1.42</td>
<td>1.57</td>
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<td>Specifications (%)</td>
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<td>0.15</td>
<td>0.15</td>
<td>0.13</td>
<td>0.13</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
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<tr>
<td>LOQ (µg/mL)</td>
<td>0.05</td>
<td>0.06</td>
<td>0.05</td>
<td>0.06</td>
<td>0.06</td>
<td>0.07</td>
<td>0.04</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>LOQ (µg/mL)</td>
<td>0.15</td>
<td>0.20</td>
<td>0.18</td>
<td>0.19</td>
<td>0.21</td>
<td>0.23</td>
<td>0.11</td>
<td>0.17</td>
<td>0.15</td>
</tr>
<tr>
<td>LOQ accuracy</td>
<td>96.1</td>
<td>97.8</td>
<td>98.5</td>
<td>99.6</td>
<td>100.8</td>
<td>102.2</td>
<td>99.5</td>
<td>98.5</td>
<td>97.8</td>
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<tr>
<td>% RSD (n = 6)</td>
<td>3.2</td>
<td>3.3</td>
<td>4.1</td>
<td>3.8</td>
<td>4.2</td>
<td>3.3</td>
<td>2.8</td>
<td>3.4</td>
<td>3.1</td>
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<tr>
<td>% RSD (n = 6)</td>
<td>2.4</td>
<td>2.5</td>
<td>3.1</td>
<td>2.9</td>
<td>4.7</td>
<td>3.4</td>
<td>3.2</td>
<td>3.0</td>
<td>2.4</td>
</tr>
<tr>
<td>% RSD (n = 6)</td>
<td>2.2</td>
<td>2.3</td>
<td>3.3</td>
<td>3.1</td>
<td>3.5</td>
<td>3.1</td>
<td>3.0</td>
<td>2.5</td>
<td>1.8</td>
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<tr>
<td>Accuracy at 50%</td>
<td>96.6</td>
<td>98.8</td>
<td>99.4</td>
<td>101.1</td>
<td>102.1</td>
<td>103.4</td>
<td>102.1</td>
<td>99.3</td>
<td>99.8</td>
</tr>
<tr>
<td>Accuracy at 100%</td>
<td>97.1</td>
<td>99.1</td>
<td>99.1</td>
<td>100.7</td>
<td>101.5</td>
<td>102.4</td>
<td>101.6</td>
<td>99.1</td>
<td>99.3</td>
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<tr>
<td>Accuracy at 150%</td>
<td>97.3</td>
<td>99.4</td>
<td>99.3</td>
<td>100.3</td>
<td>101.2</td>
<td>101.9</td>
<td>101.1</td>
<td>99.3</td>
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<tr>
<td>Regression equation (y)</td>
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<td>0.9992</td>
<td>0.9994</td>
<td>0.9997</td>
<td>0.9996</td>
<td>0.9998</td>
<td>0.9992</td>
<td>0.9995</td>
<td>0.9992</td>
</tr>
<tr>
<td>Slope</td>
<td>0.12</td>
<td>0.22</td>
<td>0.95</td>
<td>0.18</td>
<td>0.95</td>
<td>0.15</td>
<td>0.41</td>
<td>0.91</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Linearity range is LOQ—200% with respect to 0.15% specification level for BRO and its impurities.

*LOQ precision.

*Repeatability.

*Intermediate precision.
mobile phase composition (mobile phase A: ±5% ACN, mobile phase B: ±5% ACN) and column oven temperature (25 ± 5°C), were identified. Under all deliberate varied chromatographic conditions, the selectivity and the performance of the method were unchanged and proved the robustness of the method.

Results of forced-degradation studies
Initially, the drug was subjected to 1 M HCl solution at room temperature for 5 h, BRO completely converting into Imp-1 ([M + H]+ 318.2 (RRT ∼0.88)). Hence gradually the concentration of HCl reduced to 0.01 M HCl, where 6.4% degradation was observed in 1 h at room temperature. Under base hydrolysis (0.5 N NaOH at 60°C temperature for 6 h), one low level unknown degradation product (RRT ∼0.45) was formed. In water hydrolysis (water at 60°C for 6 h), 0.6% degradation was observed where Imp-1, Imp-6 and one unknown impurity (RRT ∼1.71, [M + H]+ 302) were formed. When the drug was subjected to peroxide degradation (3% H2O2 at 60°C for 5 h), 1.5% degradation was observed, where Imp-1, Imp-6 and unknown impurity-1 ([M + H]+ 332 (RRT ∼1.13)) and unknown impurity-2 ([M + H]+ 302, RRT ∼1.71) were found as degradants. In the presence of thermal degradation, 0.9% degradation was observed where Imp-1 and Imp-6 were formed. The BRO solution was subjected to photolytic degradation using UV and visible light with exposure of 1.2 million lux-hours and 200 w·h/m2. No considerable degradation (0.5%) was noticed, where low level unknown Imp-1 (RRT ∼1.49) and unknown Imp-2 (RRT ∼1.59) were formed. The above results confirmed that the drug product was very sensitive toward acid hydrolysis and peroxide degradation (Figure 2).

The results from the peak purity assessment revealed that the purity angle was less than the purity threshold in all of the stressed samples, indicating peak homogeneity. The principal peak [M + H]+ value ([M + H]+ 334.2 under all stress conditions supported the identification of BRO. The mass balance (% assay + % impurities + % degradation products) was calculated for all of the stressed samples and found to be close to 99.0% (Table II), representing the stability-indicating nature of the developed UPLC method.

Discussion
Method development and optimization
The main objective of method development was to achieve simple, rapid and efficient separation between BRO and its related compounds. The main difficulty was to obtain sufficient selectivity and resolution in shorter run time with structurally similar impurities.

Due to the slight acidic nature of BRO (pKa 4.29), a low-pH buffer (pH 2.5), a low-pH buffer (pH 2.5; 0.01 M) and Mobile phase B: ACN–water (80:20, v/v) on phenyl, C8 and C18 columns. The C18 column provided the largest number of peaks. Thus, further experiments were carried out using a BEH shield RP-18 column.

Wavelength selection
BRO and related compounds have similar UV absorption spectra, and the absorption maximum observed at 265 nm. A detection wavelength of 265 nm was selected based on the full-range UV spectral data due to its high sensitivity for all related substances and minimal difference in response factors.

Buffer pH and concentration
The effect of the buffer pH on the retention times of BRO and its impurities was studied from pH 2.0 to 7.0 while keeping the other chromatographic parameters unchanged. At pH 4.0, 5.0, 6.0 and 7.0, the early elution of BRO was observed with a broad peak shape. This is due to the presence of a hydrophilic ionizable functional group (–COOH). The retention of other impurities was found to be not
Evaluation of mobile phase and gradient program

BRO impurities have both hydrophilic and hydrophobic attributes. Because the impurities in the mixture have a wide range of polarities, the need for a gradient run was assessed using Mobile phase A: 0.01 M KH₂PO₄ buffer (pH 3.3) and Mobile phase B (ACN–water: 90:10 v/v). The initial gradient run (time in min)/%B: 0/0 and 40/100) provided an estimate of the percentage of organic ratio and approximate retention times for the impurities. The retention times of the first and last impurity peaks were 3 and 34 min, respectively. To minimize the run time with better separations, many attempts were made with different organic solvent compositions in Mobile phases A and B, along with different gradient elutions (Figure 3A). It was found that use of phosphate buffer (pH 3.3), ACN in the ratio of 70:30 (v/v) as mobile phase A; water, ACN in the ratio of 5:95 (v/v) as mobile phase B with gradient elution (time(min)/%B): 0/0.15, 3/30, 10/100, 14/100, 14.1/15 and 18.0/15 enabled separation for all components with good peak shape, resolution and retention (Figure 3A).

Evaluation of column stationary phase

Five different nonpolar stationary phases with different selectivities and hydrophobicities were selected and screened for separations. The typical retention behaviors of BRO and its impurities at various stationary phases are depicted in Figure 3B. The observations of various column behaviors are summarized.

In the BEH C8 column, close elution for Imp-1 and BRO (Rs ∼1.5) and Imp-2 and Imp-3 (Rs ∼1.8) was noticed. Imp-1 closely eluted with BRO and poor peak shapes were observed with HSS T3 column. The phenyl column yielded good separation for all components except Imp-1, which closely eluted with BRO (Rs ∼1.2). The BEH C18 column gave good separation for all components but close elution for Imp-2 and Imp-3 (Rs ∼1.9). The BEH Shield RP18 column produced good separation for all components. Finally, the polar embedded Waters Acquity BEH Shield RP18 column (100 mm x 2.1 mm, 1.7 µm) was evaluated for separation of all impurities and found to be efficient with good resolution between critical pairs, Imp-1 and BRO (Rs ∼4.8) and Imp-1 and Imp-2 (Rs ∼4.4) (Figure 3B).

Evaluation of diluent and placebo interference

A mixture of Milli-Q water and methanol in the ratio of 1:1 was selected as a diluent. This diluent was found to be more suitable for BRO and its impurities. Placebo interference was also verified and found that no interference was observed at the retention time of BRO and its impurities. The filter (Millipore Nylon membrane, PVDF and GFC) interference was checked and found that no peaks observed at the retention time of BRO and its impurities.

A rapid and efficient separation was achieved on a BEH Shield RP18 column (100 x 2.1 mm, 1.7 µm) using phosphate buffer (pH 3.3), ACN in the ratio of 70:30 (v/v) as Mobile phase A, water, and ACN in the ratio of 5:95 (v/v) as Mobile phase B. The chromatogram was monitored at 265 nm for related compounds of BRO using a gradient program (minor (min)/%B): 0/0.16, 3/30, 10/100, 14/100, 14.1/15 and 18.0/15 with a mobile phase flow rate of 0.6 mL/min and an injection volume of 3 µL. The system suitability parameters were evaluated for BRO and its impurities (Figure 3C). The tailing factor for all impurities and BRO were found to be less than 1.2. The resolution (Rs) between all components is greater than 4.0 (Table III).

Relative response factor

RRFs were established for all known impurities as the ratio of the slope of impurities and the slope of BRO. The slope value obtained with the linear calibration plot was used for the determination of RRF (Table I).

Conclusion

A selective stability-indicating RP-UPLC method has been developed for the quantitative determination of Bromfenac sodium and its impurities in Bromfenac ophthalmic solution. This method is capable of separating all eight impurities with good resolution (Rs >4) within 13 min. This method exhibited excellent performance in terms of sensitivity and speed. Forced degradation studies were conducted, and the major degradants were identified using LC–MS. The developed method was fully validated per the ICH guidelines and found to be specific, precise, accurate and linear. Thus, the method is stability-indicating.
Figure 3. Method development chromatograms. (A) Retention of BRO and its impurities with gradient elusion, (B) selectivity differences of BRO and its impurities by using different stationary phases and (C) typical chromatogram of test sample spiked with all impurities in final chromatographic conditions. This figure is available in black and white in print and in color at JCS online.
and can be used for routine analysis of production samples and to check the stability of samples of BRO ophthalmic solution.

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Conflict of interest statement. The authors declare no conflict of interest.

References


Table III. System Suitability Data

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Impurity name</th>
<th>RT (in min)</th>
<th>Resolution Factor</th>
<th>Tailing factor</th>
<th>Plate count</th>
<th>% RSD</th>
<th>n = 6</th>
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<tbody>
<tr>
<td>1</td>
<td>Imp-1</td>
<td>2.893</td>
<td>1.1</td>
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<td>2</td>
<td>BRO</td>
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<td>20,638</td>
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<td>3</td>
<td>Imp-2</td>
<td>5.901</td>
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<td>Imp-3</td>
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</tbody>
</table>

n, Number of determinations; RSD, relative standard deviation. *BRO diluted standard (0.45 µg/mL).