1. INTRODUCTION

Benzalkonium chloride, also known as alklydimethylbenzlammonium chloride (ADBAC) [Fig.1.] is a typical preservative used in the ophthalmic formulation. It is a mixture of alkylbenzylidimethyl ammonium chlorides of various even numbered alkyl chain lengths [1]. The greatest biocidal activity is associated with the C12-C16 alkyl derivatives. Several studies have identified allergic reactions to benzalkonium chloride by some individuals [2-8] but several studies have cast doubt on its reputation for other negative health effects [9-10]. It is still widely used in eye washes, nasal sprays, hand and face washes, mouthwashes, spermicidal creams, and in various other cleaners, sanitizers, and disinfectants. Hence it is necessary to estimate the BKC content in the ophthalmic solutions.

The objective of the present work is to determine the content of total benzalkonium chloride in Ketorolac tromethamine ophthalmic solution. Thorough literature search indicates that, no single method is reported for the estimation of BKC in Ketorolac ophthalmic solutions. However some method for the estimation of BKC by HPLC [11], LC-MS [12], MS-MS [13] and other chromatographic methods for the determination of BKC were reported [14-15]. Danijela[1] developed a method for the estimation of BKC in nasal drops by HPLC, not suitable for the estimation in Ketorolac ophthalmic solution. Hence it is necessary to develop a stability indicating method for the estimation of BKC in Ketorolac tromethamine ophthalmic solutions; results reported corresponding to the individual homologs present to give the total benzalkonium chloride.

Ketorolac tromethamine ophthalmic solution 0.4% is a member of the pyrrolo-pyrole group of nonsteroidal anti-inflammatory drugs (NSAIDs) for ophthalmic use. This is available in the market with the brand name of ACULAR™, marketed by Akorn, Inc. The composition contains ketorolac tromethamine 0.4%, benzalkonium chloride 0.006%, sodium chloride, edetate disodium 0.015%, octoxynol 40, purified water, and hydrochloric acid and/or sodium hydroxide to adjust the pH. BKC was used as a preservative in the ophthalmic solution.

2. EXPERIMENTAL

2.1. Chemicals and reagents

BKC standard was purchased from USP and Ketorolac tromethamine ophthalmic solution 0.4% samples were supplied by Dr. Reddy’s Laboratories Limited, IPDO, Hyderabad, India. HPLC grade Acetonitrile, Methanol, and analytical grade Potassium Dihydrogen Phosphate, Triethyl amine, Orthophosphoric acid and Acetoneitrile were purchased from Merck. Water used was obtained by using Millipore MilliQ Plus water purification system.
2.2. Equipment
Acquity UPLC system (Waters, Milford, USA) consists of a binary solvent manager, a sample manager and a PDA detector. Empower2 software was used to monitor the output signal. Cintex digital water bath was used for hydrolysis studies. Photo stability studies were carried out in a photo stability chamber (Sanyo, Leicestershire, UK). Thermal stability studies were performed in a dry air oven (Cintex, Mumbai, India).

2.3. Chromatographic Conditions
The chromatographic column used was Acquity BEH C8, 50 x 2.1 mm, 1.7 µm particle size, and separation was achieved on isocratic method. Mobile phase contains mixture of 0.05M phosphate buffer (added 1mL of Triethyl amine and adjusted the pH to 4.5 with Orthophosphoric acid), acetonitrile in the ratio of 35:65 (v/v). The flow rate, injection volume was 0.5 mL/min and 5 µL. The column temperature was ambient and the peaks were monitored at 208 nm. Mobile phase was used as a diluent for standard and sample preparations.

2.4. Preparation of standard solution
Weigh accurately and transfer about 500 mg of Benzalkonium Chloride working standard into a 100 mL volumetric flask, add about 70 mL of diluent sonicate to dissolve completely, dilute to volume with Diluent and mix. Pipette 3 mL of the above Stock solution into a 50 mL volumetric flask and dilute to volume with diluent and mix. Pipette 4 mL of the above Standard Stock solution into a 50 mL volumetric flask and dilute to volume with diluent and mix.

2.5. Preparation of sample solution
Pipette 3 mL (ketorolac tromethamine 0.4%) of the sample into a 10 mL volumetric flask and add 5mL of Diluent sonicate for about 2minutes then dilute to 10 mL with Diluent. Dilute the 5mL of the above solution to 10mL with Diluent. The solution was filtered through a 0.22-µm Nylon membrane filter and injected in UPLC.

2.6. Specificity
Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities [17]. Intentional degradation was attempted by the stress conditions of UV light (1.2 Million Lux hours), heat (105°C for 10 h), acid (0.1N HCL at 60°C for 15h), base (0.1N NaOH at 60°C for 15h), hydrolytic (60°C for 15h), Humidity (25°C/90% RH) and Oxidation (3.0% H2O2 at 60°C for 7h) to evaluate the ability of the proposed method to separate BKJC from the degradation products of Ketorolac tromethamine.

3. RESULTS AND DISCUSSION
3.1. Method development and Optimization
The method was optimized to separate major degradation products formed under varies stress conditions from Ketorolac. The main target of the chromatographic method is to separate closely eluting degradation products at RT’s 0.890 and 1.485 which are eluting very closely to the BKC peaks. The degradation samples were run using different stationary phases like C18, C8 and Mobile phases containing buffers like phosphate and acetate with different pH (2-7) and using organic modifiers like acetonitrile and methanol in the mobile phase. Initial work was done using Inertsil ODS-3 (50mm x 2.1mm, 2 µm) and Zorabx XDB(C18,50mm x 2.1mm,1.8µm) columns using pH 3.0 buffer and methanol as organic modifier, both the columns are not giving satisfactory results, degradant peaks are merging with the BKC peaks. By changing the mobile phase pH and organic modifier separation of degradant peaks was achieved but the tailing factors for the three peaks were greater than 2.0. Acquity BEH C8 column provided an acceptable separation for the three Benzalkonium chloride homolog peaks and degradant peaks, satisfactory system suitability parameters were achieved. It indicated that the elution with pH 4.5 phosphate buffer and acetonitrile in the ratio 35:65; v/v, was successful in separating BKJC peaks and all chromatographic degradation products (Figure 3). The three peaks at RT’s 0.920, 1.585, and 2.728 belong to Homolog C12, C14 and C16 (Fig.2).

Fig. 1. Representation of Benzalkonium chloride

4. Validation of the Method
After satisfactory development of the method it was subjected to method validation as per ICH guidelines [16]. The method was validated to demonstrate that it is suitable for its intended purpose by the standard procedure to evaluate adequate validation characteristics (accuracy, precision, linearity, robustness and stability indicating capability).

4.1. System suitability
A system suitability test of the chromatographic system was performed before each validation run. Five replicate injections of standard preparation was injected and asymmetry, theoretical plates and % RSD of peak area were determined for same. For all system suitability injections, asymmetry was less than 2.0, theoretical plates were greater than 5,000 and % RSD of peak area less than 2.0 was found.

4.2. Precision
The precision of the assay method was evaluated in terms of repeatability by carrying out six independent assays of test sample preparation and the % RSD of assay (intraday) was calculated. Intermediate precision of the method was checked by performing the same procedure on the different day (interday) by another person under the same experimental condition. The RSD values for intra- and inter-day precision were 0.4 and 0.6%, respectively, thereby indicating that the method was sufficiently precise. The results are reported in Table.1.

4.3. Accuracy
The accuracy of an analytical method expresses the nearness between the reference value and found value. The
accuracy of the method was evaluated by spiking the standard solution at three different concentration levels i.e. 50%, 100% and 150% of target test concentration in triplicates and analyzed as per sample procedure. The calculated average percentage recoveries at three concentration levels are ranged from 100-101.2%. Precision was performed by preparing six samples at 50% and 150% spike level concentration. The % RSD values at the two levels 50% and 150% are 0.2 and 1.9 respectively. The results obtained are shown in Table 2.

4.4. Linearity
The calibration curves plotted for BKC was linear over the concentration range of 6-18 µg/ml. Sum of the three Peak areas was plotted against concentrations and linearity regression analysis performed for the resultant curve. The Correlation coefficient value for BKC was 0.999.

4.5. Specificity
The results of stress testing studies indicated a high degree of selectivity of this method for BKC. Photodiode array detection was also used as an evidence of the selectivity of

Table -1: % Assay of six samples

<table>
<thead>
<tr>
<th>Precision set-1</th>
<th>Precision set-2</th>
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<tbody>
<tr>
<td>100.2</td>
<td>100.0</td>
</tr>
<tr>
<td>100.4</td>
<td>101.3</td>
</tr>
<tr>
<td>101.3</td>
<td>Mean: 101.5</td>
</tr>
<tr>
<td>100.3</td>
<td>% RSD: 0.4</td>
</tr>
<tr>
<td>100.1</td>
<td>100.6</td>
</tr>
<tr>
<td>100.6</td>
<td>100.2</td>
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</tbody>
</table>

Table – 2: Results of Accuracy (% recovery)

<table>
<thead>
<tr>
<th>Spiked level</th>
<th>Amount added (µg)</th>
<th>Amount recovered (µg)</th>
<th>% recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>%, n=3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BKC 50</td>
<td>0.12</td>
<td>0.121</td>
<td>101</td>
</tr>
<tr>
<td>100</td>
<td>0.24</td>
<td>0.240</td>
<td>100</td>
</tr>
<tr>
<td>150</td>
<td>0.36</td>
<td>0.364</td>
<td>101.2</td>
</tr>
</tbody>
</table>
the method, and to evaluate the homogeneity of the drug peaks. The degradatnts of Ketorolac during the acid, base, peroxide, heat, humidity and light degradations were well separated from all the three BKC peaks. The peak purities of the three BKC peaks are passed. The peak purities are tabulated in Table-3. Typical chromatograms obtained following the assay of sample and base stressed samples are shown in Fig. 3.

4.6. Solution stability and Mobile phase stability

The stability of standard and sample solution was established by injecting the solutions at the intervals of 1 and 2days, calculated the % assay of aged sample and similarity factor for aged standard against freshly prepared standard solution. The difference in assay from initial to 2 days aged sample was less than 1.0% and the similarity factor for aged standard was 1.0; proving the stability for both standard and sample solutions up to 2days.

Mobile phase stability was established by injecting the freshly prepared standard and sample solution using aged mobile phase at the time intervals of 1 and 2days. The system suitability parameters were passed and the difference in % assay of sample solution from initial to 2days was less than 1.0%, proving the mobile phase was stable up to 2 days.

4.7. Robustness

The robustness is a measure of method capacity to remain unaffected by small, but deliberate changes in chromatographic conditions and this was studied by testing the influence of small changes in pH of buffer (±0.2 units), change in column temperature (20°C and 30°C) and change in flow rate (± 5%). No significant effect was observed on system suitability when small changes were made to chromatographic conditions. Thus proved the robustness of the test method.

5. Conclusion

A simple UPLC method was successfully developed and validated for the determination of BKC in Ketorolac tromethamine ophthalmic solution. The total run time was 3 minutes, with in which the three peaks of BKC and the degradation products of Ketorolac tromethamine were well separated. Method validation results have proved that the method is selective, precise, accurate, robust and stability indicating. This method can be successfully applied for the routine analysis as well as stability studies. Overall the method provides high throughput solution for determination of BKC in Ketorolac ophthalmic solution with excellent selectivity, precision and accuracy.

References


[16] ICH Q2 (R1), Validation of Analytical Procedures: Text and Methodology, 2005

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