DEVELOPMENT AND VALIDATION OF FIRST ORDER DERIVATIVE UV SPECTROPHOTOMETRIC METHOD FOR SIMULTANEOUS ESTIMATION OF FLUOROMETHOLONE ACETATE AND KETOROLAC TROMETHAMINE IN OPHTHALMIC DOSAGE FORM

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ABSTRACT
A simple, accurate and precise First order derivative spectrophotometric method developed for the simultaneous estimation of Fluorometholone acetate (FLU) and Ketorolac tromethamine (KTC) in ophthalmic dosage form. The first derivative values were measured at 296nm for KTC and 226nm for FLU. The linearity range for zero order was carried out by using the concentration range 10-70µg/ml for both the drug. The correlation coefficient of FLU and KTC for zero order was found to be 0.999 and 0.998 respectively. At zero crossing point (ZCP) of KTC (296nm) FLU showed a measurable derivative absorbance whereas at zero crossing point (ZCP) of FLU (226nm) KTC showed an appreciable derivative absorbance value. Precision study showed that % RSD was within range of acceptable limits (<2%). The % recovery for FLU and KTC was found to be within range of 98-102% and 98-101% respectively. The percentage assay was found to be 98.24% and 99.18% for FLU and KTC. The result of analysis has been validated as per ICH Q2 (R1) guideline.

KEY WORDS: Fluorometholone acetate (FLU), Ketorolac tromethamine (KTC), UV Spectrophotometry, First order derivative Spectrophotometry.

INTRODUCTION
Fluorometholone acetate [9-fluoro-11β,17-dihydroxy-6α-methylpregna-1, 4-diene-3, 20-dione, 17-acetate] is a Glucocorticoid, is selective phospholipase A\textsubscript{2} inhibitory proteins antagonist and used in Palpebral and Bulbar conjunctiva, cornea and anterior segment of the globe. Fluorometholone acetate is official in USP (USP, 2004). Ketorolac tromethamine [5-benzoyl-2, 3-dihydro-1H-pyrollidine-1-carboxylic acid with 2- amino-2-(hydroxymethyl) propane – 1, 3- diol] is a Non steroidal anti-inflammatory drug a selective COX-1 and COX-2 antagonist and used as analgesic and anti-inflammatory agent. Ketorolac tromethamine is official in IP and USP (Indian Pharmacopoiea-10).

Both drugs are formulated together in the form of ophthalmic solution for treatment of allergic conjunctivitis and vernal keratoconjunctivitis (Sweetman SC. 2005, Irvine S.R 1953). The chemical structures of both drugs (The Merk index) were shown in figure 1.
From literature survey it reveals that very few methods had been developed for determination of Fluorometholone acetate and Ketorolac tromethamine by spectrophotometry and HPLC either alone or in combination with other drug, but no method has been developed for these in combined dosage form by first order derivative UV spectrophotometry. So the purpose of this work was to develop a simple, accurate and sensitive first order derivative spectrophotometric method for determination of Fluorometholone acetate and Ketorolac tromethamine in ophthalmic dosage form.

MATERIALS AND METHODS

Instrument:
The instrument was double beam UV-visible spectrophotometer (Shimadzu, model 1800, Software: UVProbe 2.31) having two matched quartz cells with 1 cm path length. Sonication of sample solutions was done using ultrasonic cleaner (Sonica 2200MH).

Materials:
Fluorometholone acetate (FLU) drug sample was procured from Syntho Pharmaceuticals Pvt. Ltd. Lucknow (Uttar Pradesh, India) and Ketorolac tromethamine (KTC) drug sample was gifted by Cadila Pharmaceuticals Pvt Ltd. Dholka (Gujarat, India). Eyedrops (Eyetrust, Qualitron Bio Medica Pvt. Ltd. Mumbai, India) was purchased from local market, containing Fluorometholone acetate 0.1% w/v and Ketorolac tromethamine 0.5% w/v per 5 ml Eyedrops. Methanol (95%) was purchase from Astron chemicals Pvt, Ltd. India.

METHODS

Preparation of standard stock solution:
The stock solution having 1000µg/ml concentration of FLU and KTC were prepared separately by dissolving accurately weighed 100mg of both drugs in 100 ml methanol. Further dilutions of standard stock solutions of both drugs were made with methanol to get the working standard stock solutions of 100µg/ml concentration of FLU and KTC.

METHOD DEVELOPMENT (First order derivative)
Selection of scanning range and sampling wavelength:
The standard solutions of FLU and KTC were diluted with methanol individually to get the concentration of 10µg/ml and 50µg/ml respectively and were scanned in UV range 200-400 nm. The λmax of both the drugs were found to be 239nm and 319nm respectively in normal UV spectra shown in figure 2.

Development of first order derivative spectra:
The spectral data was then processed to obtain first order derivative spectrum at wavelength interval of 2nm for the range of 200-400 nm. The λmax of both the drugs were found to be 239nm and 319nm respectively in normal UV spectra shown in figure 2.

Development of first order derivative spectra:
The spectral data was then processed to obtain first order derivative spectrum at wavelength interval of 2nm for the range of 200-400 nm. It was observed that FLU shows ZCP at 296nm and KTC shows ZCP at 226nm. At ZCP of FLU (296nm), KTC showed a measurable dA/dλ (Skoog et al. (2000), Beckett et al. (2001)) whereas at ZCP of KTC (226nm), FLU
showed a measurable \(\text{d}A/\text{d}\lambda\). Hence the wavelengths 226nm and 296nm were selected as analytical wavelengths for determination of FLU and KTC first order derivative method respectively shown in figure 2.

**METHOD VALIDATION**

The above proposed method was validated according to ICH Q2 R1 guidelines for validation of analytical procedures (ICH. 2005) in order to determine the linearity, Accuracy, Precision and Assay of marketed formulation.

**Linearity and Range:**

Calibration curve constructed was linear over a selected range of 10-70µg/ml for both drugs. The aliquots of both the drugs used in linearity studies were converted to first derivative spectra and the derivative absorbance at 296nm and 226nm for FLU and KTC were measured respectively. The calibration curve of responses against concentration was plotted was shown in figure 3 and 4. Each concentration was repeated five times. Correlation coefficient and regression line equations for FLU and KTC were calculated and were shown in table no.1.

**Accuracy:**

The accuracy of the developed method was determined by finding out the amount of recovery of Fluorometholone acetate and Ketorolac tromethamine. For the accuracy standard addition method was used where, as known amount of FLU and KTC were added to the known concentration (30µg/ml) of Eyedrop solution. The amount recovered was found by measuring the absorbance of the solution and was expressed as mean recovery of samples with upper and lower limits of percent relatives of standard deviation. Recovery was done at three different levels i.e. 80%, 100% and 120%, within the linearity range of both the drugs.

**Precision**

**Repeatability (n=6):**

For the repeatability study, from the working stock solution of both drugs, aliquot of 4 ml was transferred to a separate 10 ml volumetric flask and diluted up to mark with methanol such that it gives the concentration of 40 µg/ml of FLU and KTC both. The absorbance of the solutions was measured at 226nm and 296nm respectively. The procedure was repeated six times and % RSD was calculated and shown in table no. 3.

**Intraday Precision (n=3):**

From the working stock solution, aliquots of 3 ml, 4 ml and 5 ml were transferred to separate 10 ml volumetric flask and diluted up to the mark with methanol to give concentration of 30, 40 and 50µg/ml for both drugs. The solutions were analysed three times on the same day and % RSD was calculated and shown in table no. 3.

**Interday Precision (n=3):**

From the working stock solution, aliquots of 3 ml, 4 ml and 5 ml were transferred to separate 10 ml volumetric flask and diluted up to the mark with methanol to give concentration of 30, 40 and 50µg/ml for both drugs. The solutions were analysed three times on three different days and % RSD was calculated and were shown in table no. 3.

**Limit of Detection (LOD) and Limit of Quantification (LOQ):**

Limit of detection (LOD) is the minimum concentration of the analyte in the sample which can be analysed by the instrument. Limit of quantification (LOQ) is the minimum concentration of the analyte that can be reliably quantified. The Limit of detection (LOD) and Limit of quantification (LOQ) were measured using following formula. The values of LOD and LOQ for FLU and KTC were shown in table no. 5.

\[
\text{LOD} = 3.3 \times (\text{SD}/\text{Slope})
\]

And \(\text{LOQ} = 10 \times (\text{SD}/\text{Slope})\)

Where, SD = Standard deviation of the Y-intercepts of the 5 calibration curves.

Slope = Mean slope of the 5 calibration curves.

**Assay of ophthalmic formulation:**

Commercially available marketed ophthalmic solution containing both Fluorometholone acetate and Ketorolac tromethamine(Eyetrust)
were used for the study. Eyedrop solution equivalent to 100 mg of Ketorolac tromethamine and 20 mg of Fluorometholone acetate and 80 mg pure Fluorometholone acetate was added by standard addition method transferred in to a 100 ml volumetric flask to bring both drugs in 1:1 ratio and stock solution of this was prepared in methanol, sonicated for 15 min, the volume was adjusted up to the mark with same solvent. Then solution was filtered through whatmman filter paper No. 41. This stock solution contains Ketorolac tromethamine 100µg/ml and Fluorometholone acetate 100µg/ml. Then the appropriate dilution of 35µg/ml was made using methanol as solvent. All the determinations were carried out in triplicate. The absorbance of the prepared solutions was measured at ZCP of FLU and ZCP of KTC and then the concentration of both the drug was calculated using calibration curve equation. The amount of the drug found in dosage form was shown in table no. 4.

RESULT

Figure 2: Zero order (a) and first order (b) spectra of FLU (10µg/ml) and KTC (50µg/ml) in methanol

Figure 3: Linearity graph for first order derivative of FLU

\[
y = 0.005x + 0.009 \\
R^2 = 0.999
\]
Figure 4: Linearity graph for first order derivative of KTC

Table no. 1: Optical Characteristics

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Fluorometholone acetate</th>
<th>Ketorolac tromethamine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>226 nm</td>
<td>296 nm</td>
</tr>
<tr>
<td>Beer’s law limit (µg/ml)</td>
<td>10 – 70</td>
<td>10 – 70</td>
</tr>
<tr>
<td>Regression equation</td>
<td>Y = 0.005x + 0.009</td>
<td>Y = 0.007x – 0.012</td>
</tr>
<tr>
<td>Slope (m)</td>
<td>0.005</td>
<td>0.007</td>
</tr>
<tr>
<td>Intercept (c)</td>
<td>0.009</td>
<td>0.012</td>
</tr>
<tr>
<td>Correlation coefficient ($R^2$)</td>
<td>0.999</td>
<td>0.998</td>
</tr>
</tbody>
</table>

Table no. 2: Results of Recovery studies

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration of STD drug</th>
<th>Recovery level (%)</th>
<th>Amount of drug added (µg/ml)</th>
<th>Amount of drug recovered (µg/ml)</th>
<th>% Recovery ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLU</td>
<td>30 µg</td>
<td>80</td>
<td>24</td>
<td>53.46</td>
<td>98.80 ± 0.0049</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>30</td>
<td>59.41</td>
<td>99.01± 0.0008</td>
</tr>
<tr>
<td>Drug</td>
<td>Concentration (µg/ml)</td>
<td>Average ABS ± SD</td>
<td>% RSD</td>
<td></td>
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<tr>
<td><strong>REPEATABILITY (n=3)</strong></td>
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<tr>
<td>FLU</td>
<td>40</td>
<td>0.231 ± 0.0033</td>
<td>1.471</td>
<td></td>
<td></td>
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<tr>
<td>KTC</td>
<td>40</td>
<td>0.268 ± 0.0009</td>
<td>0.354</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>INTRA-DAY PRECISION (n=6)</strong></td>
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<tr>
<td>FLU</td>
<td>30</td>
<td>0.184 ± 0.0037</td>
<td>1.801</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>0.233 ± 0.0028</td>
<td>1.211</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.291 ± 0.0023</td>
<td>0.809</td>
<td></td>
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<tr>
<td>KTC</td>
<td>30</td>
<td>0.199 ± 0.0042</td>
<td>1.781</td>
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</tr>
<tr>
<td></td>
<td>40</td>
<td>0.268 ± 0.0012</td>
<td>0.464</td>
<td></td>
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<tr>
<td></td>
<td>50</td>
<td>0.338 ± 0.0017</td>
<td>0.501</td>
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<tr>
<td><strong>INTER-DAY PRECISION (n=6)</strong></td>
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<tr>
<td>30</td>
<td>0.181 ± 0.0009</td>
<td>0.519</td>
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</tbody>
</table>

*SD = standard deviation, *STD = standard deviation
### Table no. 4: Analysis of Ophthalmic formulation

<table>
<thead>
<tr>
<th>Drug</th>
<th>Label claim (mg/ml)</th>
<th>Amount found (mg/ml)</th>
<th>% Drug found ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLU</td>
<td>1</td>
<td>0.982</td>
<td>98.24 ± 0.0009</td>
</tr>
<tr>
<td>KTC</td>
<td>5</td>
<td>4.960</td>
<td>99.18 ± 0.0047</td>
</tr>
</tbody>
</table>

*SD = standard deviation, ABS = Absorbance

### DISCUSSION

The present paper describes the estimation of FLU and KTC in ophthalmic dosage form by First order derivative method. The Beer-Lambert’s concentration range was found to be 10-70µg/ml for both drug FLU and KTC at 226 nm and 296 nm respectively. The correlation coefficient was found to be 0.999 for FLU and 0.998 for KTC (Table no. 1) for proposed method.

Precision was determined by studying repeatability, intraday and interday precision. The standard deviation and Relative standard deviation (%RSD) were calculated for both the drugs. The % RSD for proposed method were found to be not more than 2.0% which indicates good intermediate precision (Table no. 3). The values of LOD and LOQ were 0.818µg/ml and 2.481µg/ml for FLU and 0.584µg/ml and 1.771µg/ml for KTC respectively (Table no. 5). Percentage estimation of FLU and KTC in ophthalmic dosage form was 98.24% and 99.18% by
the proposed method respectively (Table no. 4).

**CONCLUSION**

A simple, accurate and precise UV first order derivative spectrophotometric method has been developed for the estimation of FLU and KTC in combination. It has advantage that it eliminates the spectral interference from one of the two drugs while estimating the other drug by selecting zero crossing point in the derivative spectra of each drug at selected wavelength. The developed method can be successfully applied to analysis of marketed formulation.

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