Chapter 5 Method of Analysis
Numerous methods have been reported for the estimation of Diclofenac and Ketorolac in dosage forms and biological fluids. On basis of these methods spectrophotometric methods for estimation of drugs for *in-vitro* studies and HPLC method or *In-vivo* studies was used.

5.1 **SPECTROPHOTOMETRIC METHOD FOR ANALYSIS OF DICLOFENAC DIETHYLAMMONIUM**

Diclofenac reacts with Potassium ferricyanide in alkaline medium to develop orange colored chromophore, the absorbance of which can be measured at 450 nm. 0.5 ml of 1% w/v potassium ferricyanide and 0.2 ml of 6% w/v solution of sodium hydroxide in distilled water were used for color development. All the reagents used were AR grade. The standard calibration curve obtained was plotted absorbance versus concentration and is shown in the Table 5.1. Beers law is obeyed with in a drug concentration of 5 to 25 μg/ml.

5.2 **SPECTROPHOTOMETRIC METHOD FOR ANALYSIS OF KETOROLAC TROMETHAMINE**

Ketorolac tromethamine exhibits absorption maxima at 313 nm in 0.1 N hydrochloric acid. A standard stock solution of concentration 1mg/mL was prepared in methanol and further dilutions were made with 0.1 N hydrochloric acid. All the reagents used were AR grade. The calibration curve was obtained by plotting absorbance versus concentration and is shown in the graph. The linearity experiment shows that Ketorolac tromethamine shows a linear response in the concentration range of 4μg/mL to 16 μg/mL (*Sane et al.*, 1992)
### Table 5-1

**Calibration Curve for Diclofenac Diethylammonium**

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>0.0000</td>
</tr>
<tr>
<td>5.00</td>
<td>0.0865</td>
</tr>
<tr>
<td>10.00</td>
<td>0.1812</td>
</tr>
<tr>
<td>15.00</td>
<td>0.2821</td>
</tr>
<tr>
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<td>0.3731</td>
</tr>
<tr>
<td>25.00</td>
<td>0.4684</td>
</tr>
</tbody>
</table>

CC 0.99983

**Graph:** Calibration curve for Diclofenac Diethylammonium
**TABLE 5-2**

CALIBRATION CURVE FOR KETOROLAC TROMETHAMINE

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Absorbance</th>
</tr>
</thead>
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<td>4.00</td>
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<td>0.6403</td>
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<tr>
<td>CC</td>
<td>0.99619</td>
</tr>
</tbody>
</table>

**CALIBRATION CURVE FOR KETOROLAC TROMETHAMINE**
5.3 HPLC METHOD FOR ANALYSIS OF DICLOFENAC DIETHYL AMMONIUM IN PLASMA SAMPLES.

The plasma samples were analyzed by HPLC. The HPLC system used was Shimadzu LC-10AD equipped with SPD-10A detector and C-R 7A integrator.

Preparation of stock solution

A stock solution of Diclofenac diethyl ammonium was prepared by dissolving 10 mg of Diclofenac diethyl ammonium and 10 mg of Piroxicam (Internal Standard) in 10ml of methanol. The stock solution was diluted appropriately to prepare the standard solution of Diclofenac diethyl ammonium in serum, which was used to prepare the calibration curve. The standard solution (100µg) was injected into the HPLC system to determine the retention time under the chromatographic conditions used in the experiment.

Extraction procedure

The plasma proteins were precipitated with 5 M hydrochloric acid. A pinch of sodium chloride was added into each tube to prevent emulsification during the extraction procedure. The sample was extracted with 5 ml ethylacetate by vortexing the tube for 10 min. The test tube was centrifuged for 10 min. at 2000 rpm. Supernatant was evaporated to dryness under nitrogen on a water bath. The residue was reconstituted with the mobile phase (0.1ml) and analyzed by HPLC method.

All the samples were spiked with the internal standard piroxicam after extraction.

Chromatographic Conditions Diclofenac diethylammonium: (Wong et al.)

Column: ODS [Nova-pak C_{18} (5 µm 25 cm x 4.5 mm i.d)]

Mobile phase: 45% (v/v) Acetonitrile and 55% 0.75 M Sodium acetate buffer at pH 5.0
Flow Rate 1.0 mL / minute

Detection UV at 276 nm

Injection Volume 25 µl

Retention time 5.4 minutes for Diclofenac diethylammonium

7.1 minutes for Piroxicam (Internal Standard)

5.4 HPLC METHOD FOR ANALYSIS OF KETOROLAC TROMETHAMINE IN PLASMA SAMPLES.

The plasma samples were analyzed by HPLC. The HPLC system used was Shimadzu LC-10AD equipped with SPD-10A detector C-R 7A integrator.

Preparation of stock solution

A stock solution of Ketorolac tromethamine was prepared by dissolving 10 mg of drug in 10ml of methanol. The stock solution was diluted appropriately to prepare the standard solution of Ketorolac tromethamine in serum, which were used to construct the calibration curve. A standard solution of Ketorolac tromethamine (100 µg) was injected into the HPLC system to determine the retention time under the chromatographic conditions used in the experiment.

Extraction procedure

The plasma proteins were precipitated by mixing with equal volumes of 0.1 M citrate buffer (pH 3.0). The sample was extracted with 5mL ethylacetate and hexane (30: 70) mixture by vortexing the test tube for 10 min. The test tube was centrifuged for 10 min. at 2000 rpm. Supernatant was evaporated to dryness under nitrogen on a water bath. The residue was reconstituted with 0.1 mL Acetonitrile and water (30:70) and analyzed by HPLC method.
Chromatographic Conditions: Ketorolac tromethamine: (Sane et al., 1992)

**Column**
ODS (Lichrosorb RP-18, 250 mm x 4.5 mm i.d.)

**Mobile phase**
Acetonitrile: Water: Triethylamine

\[
45 : 45 : 0.1
\]

pH adjusted to 3.5 with aqueous phosphoric acid.

**Flow Rate**
1 mL / minute

**Detection**
UV at 243 nm

**Loop Size**
50 µl

**Retention time**
6 minutes