ABSTRACT
A simple, isocratic and sensitive high performance liquid chromatographic method was developed for the estimation of Ketoprofen during transdermal diffusion studies across porcine ear epithelium. A reversed-phase (C18) column was used with UV detector at 260 nm. Selected mobile phase contained a mixture of Acetonitrile and double distilled water (40:60 v/v), pH adjusted to 3 using 1% ortho-phosphoric acid solution. Calibration curve showed good linearity over the concentration range of 5-50 µg/ml in ear epidermis permeates of pH 7.4 phosphate buffer (Blank permeates). The applicability of the method was demonstrated by the analysis of porcine ear epithelium permeated samples of Ketoprofen in a diffusion study. The steady state permeability Flux (J) of Ketoprofen through ear epidermis using 5mg and 10mg of Ketoprofen solution in donor cell of diffusion study was found to be 0.0002 µg/sqcm/hr and 0.0006 µg/sqcm/hr respectively.

KEYWORDS: RP-HPLC, Flux, Diffusion study, Ketoprofen.

INTRODUCTION
The aim of the study was to develop a simple, isocratic and sensitive HPLC method for the analysis of Ketoprofen during transdermal permeation studies. Ketoprofen, (RS)-2-(3-benzoylphenyl)-propionic acid (chemical formula C16H14O3) is one of the propionic acid class, a nonsteroidal anti-inflammatory drug (NSAID)\(^1\)\(^2\) has been extensively utilized for treatment of rheumatism.\(^3\) It is associated with oral side effects including gastrointestinal irritation when administered by oral route. The adverse effects may worsen to renal and cardiovascular problems ultimately leading to mortality when used chronically especially in case of geriatric population. The promising method to diminish its adverse effects is to
deliver the drug via skin. Therefore, an eventual need has emerged to develop a transdermal dosage form of Ketoprofen to minimize the oral side-effects and to provide relatively consistent drug levels for prolonged periods. The major problem associated with transdermal drug delivery is barrier properties of stratum corneum which is considered one of the most impermeable epithelia of the human body to exogenous substances. These permeation problems can be minimized by use of chemical permeation enhancers.

Figure 1: Chemical structure of Ketoprofen.

The present study is planned to estimate the Ketoprofen during transdermal permeability study.

MATERIALS AND METHODS
Ketoprofen was purchased from BMR Pharma and Chemicals, Hyderabad. HPLC grade solvents were purchased from Ranbaxy Fine Chemicals Ltd., New Delhi. Phosphoric acid was purchased from Karnataka Fine Chem. Triple distilled water was prepared in laboratory. Porcine ear tissue obtained from local slaughter house.

Chromatographic conditions
The instrument used for the HPLC analysis was Shimadzu LC equipped with LC 20 AT prominence liquid chromatograph pump, and a Shimadzu SPD-20A UV prominence UV/Vis detector. An Inertsil ODS-3V (4.6 mm ×250mm; 5 μm particle size) column was employed during the analysis. An isocratic method was used with a mobile phase containing a mixture of Acetonitrile (ACN) and triple distilled water (40:60v/v). The pH of the used triple distilled water was adjusted to pH 3 with 1% ortho-phosphoric acid solution. Flow rate of the mobile phase was 1.0 ml per minute. The mobile phase was vaccum-filtered through 0.45 μm Millipore membrane and degassed by ultra-sonicaton.
The injection volume was 20 μL. After equilibration with the solvent to obtain a stable baseline, aliquots of calibration solutions containing Ketoprofen and internal standard were injected.

The total run time was 10 minutes. The absorbance of the eluent was monitored at 260nm with a detection sensitivity of 0.250 aufs. All the analysis was performed at room temperature.

**Preparation of Calibration Standards**

**Preparation of Blank Permeates**

Porcine ear tissue was obtained from a local slaughter house immediately after pigs were slaughtered. Ear tissue of freshly slaughtered pigs was immediately transferred to our laboratory. The dorsal hair was removed with a clipper and full thickness skin was surgically removed with the help of micro- dissecting scissor and blunt forceps. Then the ear skin was dipped into the hot water (60ºC) and then fat adhering to epidermis was carefully removed to get epidermis without any damage to stratum corneum. Washed with water and used in permeation studies. Then the permeation studies were initiated within 1hr of isolating ear epithelium. The tissue permeates of the pH 7.4 Phosphate buffer (blank permeates) was prepared by placing the porcine ear epidermis in Franz diffusion cell between donor and receptors and clamped with the help of rubber bands and pH 7.4 Phosphate buffer was added in the donor (5 ml) as well as receptor compartment (35 ml) of the diffusion cells and taking care to avoid the entry of air bubbles. Magnetic bead was added in the receptor to maintain stirring conditions. The entire set up was placed over magnetic stirrer and temperature was maintained at 37ºC by placing the diffusion cell in a water bath for 24 hrs at 37ºC. The Permeates collected after 24 hrs were used as diluent for the preparation of calibration standards. This will eliminate any interference of the components which were eluted during diffusions study from the porcine ear epidermis.

**Preparation of Calibration Standards for HPLC Estimation**

In this method, Internal Standard was also added in a constant amount to calibration samples which will correct for the loss of analyte during sample preparation and sample inlet. Propyphenazone was selected as Internal Standard. Standard stock solution of Ketoprofen and Propyphenazone as Internal Standard (1mg/ml) were prepared in methanol such that each ml of solution will have Ketoprofen and Internal standard equivalent to 1000 μg.
A secondary stock solutions (100 μg/ml) were prepared by dilution of the primary stock solution with pH 7.4 Phosphate buffer for both Ketoprofen and internal standard solutions. From secondary stock solutions aliquots (0.5, 1, 2, 3, 4, 5 ml) of Ketoprofen and 1 ml of internal standard were further diluted to 10 ml with blank permeates to obtain six calibration standards (5, 10, 20, 30, 40 and 50 μg/ml) of Ketoprofen and 10 μg/ml of internal standard using 10 ml volumetric flask. The prepared calibration standards were analyzed after filtering through 0.45 μm Millipore membrane filter.

As some of the tissue components will also diffuse into the receptor compartment during the study and which are found to be eluted in a time range of 1–4 minutes, the mobile phase was selected to give a retention time of greater than 6 minutes for the drug.

For every calibration sample ratio of peak areas of Ketoprofen and internal standard were calculated. Standard graph was plotted by taking concentration on X axis and ratio of areas on Y axis. R² and slope were calculated to estimate the linearity.

**Ex-vivo permeation study of Ketoprofen from solution through Porcine ear tissue**

The Franz diffusion cells are thoroughly cleaned with triple distilled water and as previously discussed separated porcine ear epidermis are placed between the donor and receptor compartments of the diffusion cell. The permeation studies were initiated within 1hr of isolating ear epithelium. The pH 7.4 Phosphate buffer was filled into the receptor without any air bubbles. 1mg/ml of pure Ketoprofen solution was prepared by dissolving Ketoprofen in little amount of Ethanol and volume make up with pH 7.4 phosphate buffer. 5 ml of this solution was added into the donor compartment. Then the entire set up was placed over magnetic stirrer and temperature was maintained at 37ºC by placing the diffusion cell in a water bath for 24 hrs at 37º. Then 1ml of sample was withdrawn from the bottom portion of receptor using injection syringe fitted with a catheter. Samples were withdrawn at 1hr, 2 hrs, 4 hrs, 6 hrs, 8 hrs, 10 hrs 12 hrs, 17 hrs and 24 hrs and replaced with 1 ml of pH 7.4 phosphate buffer after every sample withdrawal.

For estimation of Ketoprofen in the permeation, 0.1 ml of permeate was added with 0.1 ml of I.S. solution (100 μg/ml). The volume was made upto 1 ml with mobile phase. This sample was filtered using 0.2 μm Millipore membrane filter before injected into HPLC. Similar study was planned using 5 ml of 2 mg/ml Ketoprofen in ethanol in donor cell.
In both studies, graph is plotted by taking cumulative amount permeated per square on Y axis and time interval on X axis. Flux was calculated from the slope of the straight line of the graph.

RESULTS AND DISCUSSION

Calibration Standard of Ketoprofen by HPLC
The standard graph was found to be linear in the range of 5-50 μg/ml. The $R^2$ and the slope(Y) were found to be 0.9983 and 0.1936 respectively. The values were presented in Table no. 1 and Fig no. 2.

Table 1: Calibration curve of pure drug solution by HPLC.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Concentration(μg/ml)</th>
<th>Ratio (Internal standard / Ketoprofen)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>1.1516</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>2.0205</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
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<td>7.7489</td>
</tr>
<tr>
<td>7</td>
<td>50</td>
<td>9.7895</td>
</tr>
</tbody>
</table>

![Fig. 2: Standard Graph of Ketoprofen in pH 7.4 phosphate buffer by HPLC.](image1)

![Fig. 3: Chromatogram of blank permeates of pig ear epidermis.](image2)
Fig. 4: Chromatogram of Calibration Solution Containing Ketoprofen and IS.

Ex-vivo permeation study of Ketoprofen from solution through Porcine ear tissue
The diffusion study of Pure Ketoprofen drug solution was done by HPLC and the blank peaks due to tissue components showed retention time around 2-3 mins. Ketoprofen’s retention time was observed at 8.4 mins and peaks due to Propyphenazone (I.S) was observed at 6 mins. As some of the tissue components will also diffuse into the receptor compartment during the diffusion study, and many interference with the drug and internal standard peaks. To avoid such interference blank tissue permeates were used in standard graph preparation. The mobile phase was selected to give a retention time of greater than 4 minutes for the drug. The mobile phase and calibration standards were freshly prepared on the day of usage. The model chromatogram of Blank permeates and a calibrated solution containing both drug and IS were shown in the figures 3 and 4.

The values of Diffusion studies were tabulated in table no. 2 and Fig no 5. The flux (J) observed when 5mg and 10mg of Ketoprofen was used in donor was to 0.0002 µg/sqcm/hr and 0.0006 µg/sqcm/hr respectively, which indicates that increasing the drug quantity, in donor has increased permeation.[10,11]

Table 2: Diffusion Study Curve of Pure Ketoprofen Solution by HPLC.

<table>
<thead>
<tr>
<th>Time (Hrs.)</th>
<th>µg of drug permeated per sq.cm.</th>
<th></th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>For 1mg/ml</td>
<td>For 2mg/ml</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.0155</td>
<td>0.0155</td>
<td></td>
</tr>
<tr>
<td>2</td>
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<td>0.0160</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.0159</td>
<td>0.0163</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.0165</td>
<td>0.0172</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.0167</td>
<td>0.0185</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.0172</td>
<td>0.0202</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>0.0189</td>
<td>0.0247</td>
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<tr>
<td>24</td>
<td>0.0205</td>
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CONCLUSION
A simple, isocratic and sensitive HPLC method was developed to estimate the Ketoprofen during Transdermal diffusion study through porcine ear epithelium using for 5mg and 10mg of drug in donor and flux was found to be 0.0002 µg/sqcm/hr and 0.0006 µg/sqcm/hr respectively.

This study can be used to estimate the concentration of Ketoprofen in any transdermal formulation diffusion studies.

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REFERENCES


