A VALIDATED STABILITY-INDICATING NORMAL PHASE HPLC METHOD FOR THE DETERMINATION OF NAPROXCINOD ALONG WITH ITS CHIRAL AND ACHIRAL IMPURITIES.

Shiva Raj¹, K. Siva Kumari²*, and A. Narasimha Rao²

¹Department of Chemistry, Osmania University, Hyderabad, AP, India-500 007.
²Research and development, Active Pharmaceutical Ingredients, DR. Reddy’s Laboratories, Hyderabad - 500072, India

*E-mail: sivakumarik@drreddys.com

ABSTRACT
A novel stability-indicating normal phase liquid chromatographic (NP-LC) method was developed for the determination of purity of naproxcinod in the presence of its impurities and degradation products. This method is capable of separating all the related substances of naproxcinod along with its chiral and achiral impurities. This method can be also be used for the estimation of assay of naproxcinod. The method was developed using chiralpak IB (250mm×4.6mm, 5µm) column with immobilized stationary phase. n-Hexane, 2-propanol, acetonitrile and triflouro acetic acid in 97.5:2.5:0.2:0.2 (v/v/v/v) ratio was used as a mobile phase. The eluted compounds were monitored at 210 nm. naproxcinod was subjected to the stress conditions of oxidative, acid, base, hydrolytic, thermal and photolytic degradation. The degradation products were well resolved from main peak and its impurities, proving the stability-indicating power of the method. The developed method was validated as per International Conference on Harmonization (ICH) guidelines with respect to specificity, limit of detection, limit of quantification, precision, linearity, accuracy, robustness and system suitability.

Key words: Naproxcinod, Development, Normal phase liquid chromatography, Stability-indicating, Chiral and achiral impurities.

INTRODUCTION
Naproxcinod (nitro naproxen) is a non-steroidal anti-inflammatory drug (NSAID). It is derived from naproxen but substituted with a nitroxybutyl ester to allow it to also act as a nitric oxide (NO) donor. This second mechanism of action makes naproxcinod the first of a new class of drugs, the cyclooxygenase inhibiting nitric oxide donators (CINODs), that are hoped to produce similar analgesic efficacy to traditional NSAIDs, but with less gastrointestinal and cardiovascular side effects.

Naproxcinoid is metabolized to naproxen and a nitric oxide donating moiety. NO has various cardiovascular effects, including vasodilatory and platelet-inhibitory actions as well as the inhibition of vascular smooth muscle proliferation that serves to maintain normal vascular tone. Naproxcinod led to significant improvements in pain and functioning among patients with hip osteoarthritis and was not associated with serious gastrointestinal or cardiovascular adverse events, a multicenter trial found¹.

High performance liquid chromatography (HPLC) is playing a more and more important role for the resolution of drugs enantiomers in the field of pharmaceutical industry². However, the development of the methods for the quantitative analysis of chiral compounds along with other process related substances is extremely challenging³.

In the literature there were limited LC methods have been reported for determination of Naproxcinod in pharmaceutical preparations. The chiral method⁴ reported describes the separation of the two enantiomers of Naproxcinod by Chiral Normal-Phase Liquid Chromatography, but it was out of scope because it was not stability indicative and not determining the other related impurities.
Hence a rapid simple reproducible normal phase stability-indicating HPLC method was developed and validated for the quantitative determination of Naproxcinod along with its chiral and achiral impurities in bulk drugs. So far to our knowledge there was no stability indicating method has been reported using normal phase chromatography for Naproxcinod.

**EXPERIMENTAL**

**Materials and Reagents**

Standards of naproxcinod and its seven impurities namely impurity 1 (99.5%), impurity 2 (98.0%), impurity 3 (99.5%), impurity 4 (99.5%), impurity 5 (99.0%), impurity 6 (98.0%) and impurity 7 (98.5%) were supplied by Dr. Reddy's Laboratories Limited, Hyderabad, India. The HPLC grade n-hexane, 2-propanol, acetonitrile and analytical grade trifluoro acetic acid were purchased from Merck, Darmstadt, Germany.

**Equipment**

The Waters HPLC system (Waters, Milford, USA) used consists of a pump, auto sampler and a PDA detector. The output signal was monitored and processed using empower-2 software. 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).

**Chromatographic conditions**

The method was developed using Chiralpak-IB (250mm×4.6mm, 5µm) column. n-hexane, 2-propanol, acetonitrile and trifluoro acetic acid in 97.5: 2.5: 0.2: 0.2 (v/v/v/v) ratio was used as mobile phase. The mobile phase was filtered through a nylon membrane (pore size0.45µm) filter. The flow rate of the mobile phase was 1.8 ml/min. The column temperature was maintained at 35°C and the wavelength was monitored at 210 nm. The injection volume was 10 µL.

**Preparation of solutions**

A stock solution of naproxcinod (1.0 mg/ml) was prepared by dissolving appropriate amount of naproxcinod in mobile phase. Working solutions of 1000 and 100µg/ml in mobile phase were prepared from the above stock solution for the related substance determination and assay determination, respectively. A stock solution of impurity (mixture of impurities 1–7) at 0.5 mg/ml was also prepared in mobile phase.

**Analytical Method validation**

The proposed method was validated as per ICH guidelines. The specificity of the developed LC method for naproxcinod was carried out in the presence of its impurities namely imp-1, imp-2, imp-3, imp-4, imp-5, imp-6 and imp-7. Stress studies were performed for naproxcinod to provide an indication of the stability-indicating property and specificity of the proposed method. Intentional degradation was attempted with a stress condition of UV light with not less than 200 watt hours/m² and visible light at illumination of not less than 1.2 million lux hours for 10 days, heat (60°C for 10 days), acid (0.2N HCl for 24 hours), base (0.2N NaOH for 24 hours) and oxidation (3.0% H2O2 for 24 hours) to evaluate the ability of the proposed method to separate naproxcinod from its degradation product. Peak purity test was carried out for the naproxcinod peak by using PDA detector in stress samples. Assay of stressed samples was performed by comparison with qualified reference standard and the mass balance (% assay + %impurities + %degradation products) was calculated.

**Precision**

The precision of the related substances method verified by repeatability and by intermediate precision. Repeatability was checked by injecting six individual preparations of naproxcinod sample spiked with 0.15% of its seven impurities (0.15% of impurities with respect to 1.0 mg/ml of naproxcinod). % RSD of area for each impurity was calculated. The intermediate precision of the method was also evaluated using different analyst and performing the analysis on different days. Precision of
assay method was evaluated by carrying out six independent assays of real sample of naproxcinod at 0.1 mg/ml level against qualified reference standard. The intermediate precision of the assay method was evaluated by different analysts.

**Limit of Detection (LOD) and Limit of Qualification (LOQ)**

LOD and LOQ for impurities 1, 2, 3, 4, 5, 6 and 7 were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations. Precision study was also carried out at LOQ level by injecting six individual preparations of impurities and calculating the % RSD of the area.

**Linearity and Range**

Linearity test solutions for the assay method were prepared from naproxcinod stock solution at five concentration levels from 50 to 150% of assay analyte concentration (50, 75, 100, 125 and 150µg/ml). The peak area versus concentration data was treated by least-squares linear regression analysis. Linearity test solutions for the related substance method were prepared by diluting stock solution to the required concentrations. The solutions were prepared at six concentration levels from LOQ to 150% of the specification level (LOQ, 0.075, 0.15, 0.20, 0.25 and 0.30%).

**Accuracy**

Accuracy of the assay method was evaluated in triplicate using three concentration levels 50, 100 and 150µg/ml on sample. Standard addition and recovery experiments were conducted on sample to determine accuracy of the related substance method. Study was carried out in triplicate using four concentration levels LOQ, 0.5, 1.0 and 1.5µg/ml. The percentages of recoveries for naproxcinod impurities were calculated.

**Robustness**

To determine the robustness of the developed method, experimental conditions were deliberately altered and the resolution between naproxcinod and its impurities and tailing factor for naproxcinod and its impurities were recorded. The flow rate of the mobile phase was 1.8 ml/min, to study the effect of flow rate on the resolution; flow was changed by 0.1 units from 1.7 to 1.9 ml/min. The effect of the column temperature on resolution was studied at 30 and 40 °C instead of 35 °C. The effect of the percent 2-propanol strength on resolution was studied by varying 2-propanol by −5 to +5% while other mobile phase components were held constant.

**RESULTS AND DISCUSSION**

**Method development and Optimization of chromatographic conditions**

The main objective while developing this method was to have a single method for separation of impurities 2, 3, and 4 along with the separation of enantiomer of impurity 5, impurity 6 (naproxen) and enantiomer of naproxcinod (impurity 4) from naproxcinod. As the separation demands the resolution of chiral and non-chiral impurities together, chiral stationary phases based on carbohydrate like cellulose and amylose were chosen as first choice. Initially the separation was attempted in reverse phase. None of the reverse phase-based chiral columns were able to separate all the impurities.

Literature survey reveals that the critical chiral separations were achieved by using the columns containing either amylose tris (3,5-dimethylphenylcarbamate) or cellulose tris (3,5-dimethylphenylcarbamate) packing materials. Hence, experiments were carried out in the normal phase using two chiral columns viz. chiralpak AD and chiralpak AD-H, both containing amylose tris (3,5-dimethylphenyl carbamate) packing material. Chiralpak-ADH was found to be showing separation of all impurities in normal phase mode using hexane and 2-propanol in mobile phase but run time exceeding 60 min and peak shapes were not sharp.

Chiralpak–IB immobilised chiral column nearly equivalent to chiralpak AD-H and it has broad range of applications, Chiralpak–IB column was selected for further improvement of resolution of all seven impurities with in short run time of 15 min with good peak shapes. Addition of little amount of acetonitrile and triflouoro acetic acid modifiers improved the peak shape resulting in further increase in resolution of impurities. The mobile phase composition was fixed as. n-Hexane, 2-propanol, acetonitrile
and trifluoro acetic acid in 97.5:2.5:0.0:2.0 (v/v/v/v) ratio with an isocratic elution. The flow rate of the mobile phase was fixed as 1.8 ml/min. Mobile phase was chosen as the diluent, as the blank chromatogram was clean without any interference with analyte peak and the impurity peaks.

Under optimized conditions naproxcinod and its six impurities were well separated with resolution greater than 2.0; typical retention times were approximately 5.2, 2.6, 3.6, 4.2, 4.7, 7.0, 7.7, 8.5 min for naproxcinod, impurity 1, impurity 2, impurity 3, impurity 4, impurity 5, impurity 6 and impurity 7; respectively. System suitability parameters were evaluated for naproxcinod and its seven impurities. Tailing factor for all seven impurities and naproxcinod was found to be less than 1.3. Resolution of naproxcinod and its six potential impurities was greater than 3.0 for all pairs of compounds. Stability study results as per ICH Q1A (R2) for Naproxcinod were given in Table 2.

Results of forced degradation studies

Naproxcinod was found to degrade significantly in acid hydrolysis and in base hydrolysis of impurity 5 and impurity 6 (Fig. 2). This was confirmed by co-injecting impurity 5 and 6 to these degraded samples. naproxcinod was found to be stable under photolytic thermal degradation, and oxidation conditions. Photodiode array detector was employed to check and ensure the homogeneity and purity of naproxcinod peak in all the stressed sample solutions. Assay studies were carried out for stress samples against naproxcinod qualified working standard. The mass balance (% assay + % impurities + %
degradants) results are presented in Table 3. The purity and assay of naproxcinod was unaffected by the presence of its impurities and degradation products and thus confirms the stability-indicating power of the developed method.

![Typical chromatograms of naproxcinod spiked with its impurities and its forced degradation samples.](image)

**Table -1: Regression and precision data**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Naproxcinod</th>
<th>Imp-1</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>
</tr>
</thead>
<tbody>
<tr>
<td>LOD (µg/ml)</td>
<td>0.005</td>
<td>0.009</td>
<td>0.008</td>
<td>0.006</td>
<td>0.006</td>
<td>0.006</td>
<td>0.005</td>
<td>0.008</td>
</tr>
<tr>
<td>LOQ (µg/ml)</td>
<td>0.0018</td>
<td>0.0029</td>
<td>0.0028</td>
<td>0.0022</td>
<td>0.0022</td>
<td>0.0022</td>
<td>0.0018</td>
<td>0.0029</td>
</tr>
<tr>
<td>Slope (b)</td>
<td>48200</td>
<td>45008</td>
<td>39852</td>
<td>32400</td>
<td>35890</td>
<td>32400</td>
<td>42006</td>
<td>45006</td>
</tr>
<tr>
<td>Intercept (a)</td>
<td>-326</td>
<td>572</td>
<td>320</td>
<td>-291.2</td>
<td>278</td>
<td>-180.2</td>
<td>-376</td>
<td>-456</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.9990</td>
<td>0.9998</td>
<td>0.9986</td>
<td>0.9987</td>
<td>0.9992</td>
<td>0.9997</td>
<td>0.9998</td>
<td>0.9999</td>
</tr>
<tr>
<td>Precision (% RSD)#</td>
<td>2.1</td>
<td>2.2</td>
<td>1.8</td>
<td>2.2</td>
<td>2.3</td>
<td>2.1</td>
<td>2.3</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Fig.-2: Typical chromatograms of naproxcinod spiked with its impurities and its forced degradation samples.
### Method validation

#### Precision

The % RSD of assay of naproxcinod during the assay method precision study was 0.55% and the % RSD for the area of impurities 1–6 in related substance method precision study was within 1.5%. The % RSD of the assay results obtained in the intermediate precision study was within 0.5% and the % RSD for the area of impurities 1–7 were well within 2.5%, conforming good precision of the method. The % RSD values are presented in Table 1.

#### Limit of Detection and Limit of Quantification

The determined limit of detection, limit of quantification and precision at LOQ values for naproxcinod and its five impurities were reported in Table 1.

#### Linearity

Linearity calibration plot for the assay method was obtained over the calibration ranges tested, i.e. 50–150µg/ml and correlation coefficient obtained was greater than 0.999. The result shows that an excellent correlation existed between the peak area and concentration of the analyte. Linear calibration plot for the related substance method was obtained over the calibration ranges tested, i.e. LOQ to 0.30%. The correlation coefficient obtained was greater than 0.999. The above result shows that an excellent correlation existed between the peak area and the concentration of impurities 1–7.
Accuracy
The percentage recovery of naproxcinod was ranged from 98.5 to 100.5%. The percentage recovery of impurities in naproxcinod samples varied from 98.0 to 102.0%.

Table -3: Summary of forced degradation studies.

<table>
<thead>
<tr>
<th>Stress condition</th>
<th>% impurities formed</th>
<th>% Assay</th>
<th>Mass balance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>imp-1 imp-2 imp-3 imp-4 imp-5 imp-6 imp-7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acid hydrolysis</td>
<td>0.05 0.04 ND ND ND 14.6 2.3</td>
<td>82.5</td>
<td>99.5</td>
</tr>
<tr>
<td>Base hydrolysis</td>
<td>0.05 0.04 ND ND ND 1.8 0.8</td>
<td>97.2</td>
<td>99.9</td>
</tr>
<tr>
<td>Neutral hydrolysis</td>
<td>0.04 0.04 ND ND ND ND ND</td>
<td>99.0</td>
<td>99.8</td>
</tr>
<tr>
<td>Oxidative degradation</td>
<td>0.03 0.05 ND ND ND ND ND</td>
<td>99.1</td>
<td>99.9</td>
</tr>
<tr>
<td>Thermal degradation</td>
<td>0.04 0.04 ND ND ND ND ND</td>
<td>99.0</td>
<td>99.8</td>
</tr>
<tr>
<td>Photo degradation</td>
<td>0.05 0.04 ND ND ND ND ND</td>
<td>99.0</td>
<td>99.9</td>
</tr>
<tr>
<td>ND: not detected.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table-4: Peak Purity results of forced degradation samples

<table>
<thead>
<tr>
<th>Degradation Condition</th>
<th>Naproxcinod</th>
<th>imp-6</th>
<th>imp-7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Purity Angle</td>
<td>Purity Threshold</td>
<td>Purity Angle</td>
</tr>
<tr>
<td>Acid hydrolysis</td>
<td>1.800</td>
<td>2.030</td>
<td>4.515</td>
</tr>
<tr>
<td>Base hydrolysis</td>
<td>1.581</td>
<td>2.420</td>
<td>5.045</td>
</tr>
<tr>
<td>Oxidation</td>
<td>1.552</td>
<td>2.506</td>
<td>ND</td>
</tr>
</tbody>
</table>

Robustness
To determine the robustness of the developed method, experimental conditions were deliberately altered and the resolution between Naproxcinol and its impurities and tailing factor for Naproxcinol and its impurities were recorded. The flow rate of the mobile phase was 1.8 ml/min, to study the effect of flow rate on the resolution, flow was changed by 0.1 units from 1.7 to 1.9 ml/min. The effect of the column temperature on resolution was studied at 30° C and 40° C instead of 35° C. The effect of the percent 2-propanol strength on resolution was studied by varying ethanol by −3 to +3% while other mobile phase components were held constant.

Solution stability
The % RSD of the assay of naproxcinol during solution stability experiments were within 1%. No significant changes were observed in the content of six impurities during solution stability experiments. The solution stability experiment data confirms that the sample solutions used during assay and the related substance determination were stable for 48 h.
CONCLUSIONS

The simple isocratic normal phase LC method developed for quantitative analysis of naproxcinod and related substances in samples is precise, accurate, linear, robust and specific. Satisfactory results were obtained from validation of the method. The method is stability-indicating and can be used for routine analysis of production samples and to check the stability of samples of naproxcinod in bulk drugs.

ACKNOWLEDGEMENTS

The authors wish to thank the management of Dr. Reddy’s Laboratories Ltd for supporting this work.

REFERENCES

4. Kai Zhang, Na Xue, Zhifang Yuan, Lin Li, Xiaowei Shi, Liang Cao, Yumin Du, Journal of Chromatographic Science, 49(4), 272

[RJC-822/2011]