Chapter 2

High performance liquid chromatographic methods for the assay of drugs
High performance liquid chromatographic method for
determination of deflazacort in pharmaceutical formulations and
human plasma samples

ABSTRACT

A simple high performance liquid chromatographic method for the
determination of an anti-inflammatory drug, deflazacort (DLZ) in
pharmaceutical formulations and human plasma samples has been developed
and validated. Piroxicam (PRX) was employed as an internal standard. The
assay of the drug was performed on a CLC C_{18} (5 \mu, 25 \text{ cm} \times 4.6 \text{ mm i.d}) with
UV detection at 200 nm. The mobile phase consisted of acetonitrile-water
mixture in the ratio of 90:10 and a flow rate of 1 ml/min was maintained. The
standard curve was linear over the concentration range of 0.5-16.8 \mu g/ml
\left(r^2 = 0.9944\right). Analytical parameters have been evaluated. The precision for
within-day and between-day assay as expressed by relative standard deviation
was found to be less than 0.5 %. The method has been applied successfully for
the determination of DLZ in spiked human plasma samples and pharmaceutical
formulations.

The results of this work have been accepted for publication in *Journal of
Liquid Chromatography and Related Technologies* (Taylor and Francis).
**General drug profile**

<table>
<thead>
<tr>
<th>Commercial name</th>
<th>Deflazacort</th>
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<tr>
<td>Chemical name</td>
<td>11 beta,21-dihydroxy-2'-methyl-5' beta H-pregna-1,4-dieno[17,16-d] oxazole-3,20-dione-21-acetate</td>
</tr>
<tr>
<td>Molecular formula</td>
<td>C_{25}H_{31}O_{6}N</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>441.53</td>
</tr>
<tr>
<td>Description</td>
<td>White crystalline powder</td>
</tr>
<tr>
<td>Solubility</td>
<td>Soluble in water, methanol, benzene etc.</td>
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<td>Category</td>
<td>Anti-inflammatory</td>
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</table>

**INTRODUCTION**

DLZ is a synthetic corticosteroid and is used to decrease inflammation. It works within cells and prevents the release of certain chemicals that are important in the immune system. These chemicals are normally involved in producing immune and allergic responses resulting in inflammation. By decreasing the release of these chemicals in a particular area, inflammation is reduced. This helps to control a wide number of disease states, which are characterized by excessive inflammation. These include severe allergic reactions, inflammation of the lungs in asthma and inflammation of the joints in arthritis. The anti-inflammatory potency of DLZ in comparison with prednisolone has been found to be 0.82 in rheumatoid arthritis and other
diseases [1]. DLZ is also known to decrease the number of white blood cells circulating in the blood. It is used in the treatment of certain types of leukaemia, where there is abnormally large number of certain white blood cells in the tissues or blood. In clinical trials, DLZ has been observed to influence calcium balance and carbohydrate metabolism to lesser degrees than prednisolone [2].

Simple HPLC methods have attracted a considerable attention in quality control of drugs and quantitative determination of pharmaceutical and biological samples due to their selectivity, sensitivity and overall versatility. Critical literature survey indicated that no HPLC method has been reported for the determination of DLZ in pharmaceutical formulations. Only a few HPLC methods with different detection systems [3-9] have been reported for its assay in biological samples. Flow injection analysis with UV detection for the determination of DLZ is also available in literature [10]. These methods require solid-phase extraction or expensive equipments, which are not economically feasible for routine use in pharmaceutical industries where numerous samples have to be analyzed. In the present work, we report a simple and sensitive HPLC method for quantitative determination of DLZ in pharmaceutical and biological samples. In the proposed HPLC method, there is no need to extract DLZ from the excipients matrix of pharmaceutical dosage forms and endogenous substances in serum samples, thereby decreasing the error in quantification.
EXPERIMENTAL

Drug solutions

DLZ (100 mg) and PRX (100 mg) were accurately weighed separately into different 100 ml calibrated flasks and dissolved in the mobile phase and diluted to volume with the same.

Standard working solutions

Standard working solutions were prepared individually in mobile phase. Working solutions of each of 250 µg/ml of DLZ and PRX were prepared separately in the mobile phase. Studies on the stability of analytes in standard working solutions showed that there were no decomposition products.

Plasma samples

Human blood samples were collected in dry and evacuated tubes (which contained saline and sodium citrate solution) from different healthy volunteers, after an overnight fast, before breakfast. The samples were handled at room temperature and were centrifuged for 10 min at 1500 rpm for the separation of plasma within 60 min of collection. The plasma samples were spiked with DLZ and internal standard and were extracted with ether. The ethereal layer was evaporated to dryness on a water bath under gentle stream of nitrogen gas at 40 °C. The residue was dissolved in the mobile phase, stored at -20 °C until analysis and 20 µl solution was injected on to the column.

Pharmaceutical preparation

Twenty tablets of DLZ were finely powdered. An amount equivalent to 25 mg of the drug was weighed accurately and transferred into a 100 ml
beaker. Using a mechanical stirrer, the powder was completely disintegrated in mobile phase for DLZ. The solution was filtered and the filtrate was made up to 100 ml with the mobile phase. Suitable amount of the aliquot was taken for assay studies.

**Operating conditions**

Column : CLC C\textsubscript{18} column (5μ, 25 cm x 4.6 mm i.d.) with CLC ODS (4 cm x 4.6 mm, i.d.) as a guard column to protect analytical column.

Mobile phase : acetonitrile : water (90:10, v/v)

Flow rate : 1.0 ml/min

Internal standard : PRX

Temperature : Ambient

Mode : Reverse phase

Membrane : 0.45 μ Millipore

Run time : 4 min
RECOMMENDED PROCEDURES

Chromatographic conditions

HPLC analysis was performed by isocratic elution with a flow rate of 1.0 ml/min. The mobile phase consisting of acetonitrile and water (90:10, v/v) was used throughout. All solvents were filtered through a 0.45 µMillipore membranes filter before use and degassed in an ultrasonic bath. Volumes of 20 µl each of pharmaceutical or plasma samples were injected into the column. Effective quantification was achieved by measuring at 200 nm with UV detection. Chromatographic run time of 4 min was maintained throughout. Throughout the study, the suitability of the chromatographic system was monitored by calculating the capacity factor (k'), the resolution (R), the selectivity (α) and the peak asymmetry (T).

The chromatographic peaks observed were of good shape and completely resolved from one another. The chromatogram of extracted plasma samples did not show any co-eluting interference peak with the analyte or internal standard.

Establishment of calibration

Working standard solutions of DLZ (0.5-16.8 µg/ml) containing fixed concentration of internal standard (2 µg/ml) was prepared in the mobile phase. Triplicate 20 µl injections were made for each standard solution to check the reproducibility of the detector response at each concentration level. The peak area ratio of standard to internal standard was plotted against the concentration of the drug to obtain the calibration graph. The results were subjected to
regression analysis. Typical chromatogram obtained for pure drug sample is shown in Fig. 1.

**Analysis of plasma samples**

The plasma sample obtained as described in plasma sample preparation (page number 47.) was taken and 20 µl solution was injected into the chromatographic column and the chromatogram was recorded (Fig. 2). At the experimental chromatographic conditions, well resolved peaks for standard and internal standards were observed. The retention times of PRX and DLZ were observed to be at 0.75 and 2.00 min, respectively.

**Analysis of tablet**

Appropriate amount of the drug solution obtained by following the procedure described for analysis of pharmaceutical preparations was taken and chromatogram was recorded. The chromatogram at 200 nm showed a complete resolution of all the peaks.

**RESULTS AND DISCUSSION**

**Method development**

**Mobile phase**

The mobile phase was chosen after several trials with methanol, acetonitrile, isopropyl alcohol, triethylamine, water and various buffer solutions in various proportions and at different pH values. A mobile phase consisting of acetonitrile and water (90:10, v/v) was selected to achieve maximum separation and sensitivity.
Flow rate

Flow rate of the mobile phase plays an important role in chromatographic determination of pharmaceutical drugs. The effects of flow rates in the range of 0.5 to 1.5 ml/min were examined in the determination of DLZ. With increase in flow rate, the analyte was eluted quickly and the two peaks (DLZ and PRX) were not resolved properly. When flow rate was decreased, the peaks obtained were broad and required more time for elution. However, with a flow rate of 1.0 ml/min, an optimal signal to noise ratio with a reasonable separation time was noticed. Hence, a flow rate of 1.0 ml/min was maintained for ideal chromatographic conditions.

Internal standard

Various compounds viz., fluoxetine, parecoxib, nimesulide, amoxycillin, cefadroxil, tenofovir and PRX were examined as internal standards. Proper resolution, less time for analysis and good chromatographic behavior (ideal retention time) was observed with PRX with a overall concentration of 2 µg/ml. Hence, PRX was employed as an internal standard in the present study.

Order of elution

It was noticed that the internal standard, PRX was eluted first followed by the analyte, DLZ.
Retention times

The reproducibility of the retention times of PRX and DLZ were calculated based on the average of nine determinations. The retention times of PRX and DLZ were observed to be 0.75 and 2.00 min, respectively. Thus, the total time of analysis was observed to be less than 4 min. These facilitated the analysis of DLZ in short time.

Wavelength

The solution containing DLZ and PRX exhibited maximum absorption at 200 nm and hence, this wavelength was chosen for analysis.

Linearity and regression analysis

The peak area ratio of DLZ to PRX was plotted against the concentration of DLZ to obtain the calibration graph (Fig. 3). The calibration curve was observed to be linear in the concentration range 0.5-16.8 μg/ml with \( r^2 = 0.9944 \). The calibration curve was represented by, \( y = bx + c \), where \( y \) represents the ratio of DLZ peak area to PRX peak area and \( x \) represents the concentration of DLZ.

Limits of detection and quantification

Limit of detection (LOD) was established at a signal-to-noise ratio (S/N) of 3 while limit of quantification (LOQ) was established at a signal-to-noise ratio (S/N) of 10. The limit of detection was calculated to be 0.144 μg/ml while the limit of quantification was found to be 0.432 μg/ml.
Suitability of the method

The chromatographic parameters such as resolution, selectivity and peak asymmetry were evaluated. The calculated resolution values $R$ between each peak-pair were found to be 1.60 and 1.56 while the selectivity values ($\alpha$) were observed to be 2.92 and 2.89 for pure sample and plasma sample, respectively. The capacity factors ($k^1$) were found to be 7.33 and 21.44 for pure samples of DLZ and PRX, while those for plasma samples were observed to be 7.64 and 22.12 for DLZ and PRX, respectively. The peak asymmetry ($T$) values were noticed to be 1.2 and 1.3 for DLZ and PRX, respectively. The above system performance parameters shown in Table 1 indicated that the proposed method is suitable for the assay of DLZ.

Precision

Within- and between-day assay precision was evaluated by determining different concentrations of pure DLZ on the same day and different days. The obtained RSD values for within- and between-day assay are shown in Table 2. The low RSD values indicated that the proposed HPLC method was precise.

Accuracy

A standard working solution containing DLZ (10 $\mu$g/ml) and PRX (2 $\mu$g/ml) was prepared. This standard solution was injected nine times as a test sample. From the respective peak area counts, the concentrations of the DLZ and PRX were calculated using the detector responses. The accuracy, defined in terms of % bias values, was evaluated. The corresponding values are listed in Table 2.
Selectivity

Specificity of the proposed method was confirmed by carrying out the analysis in presence of different drugs such as ampicillin, cloxacillin, paracetamol and ibuprofen. These drugs did not interfere in the determination as evident from the fact that the interfering peaks were not noticed at the retention times of either DLZ or PRX.

Applications

Analysis of plasma samples

The proposed method was applied for the determination of DLZ in plasma samples. The results obtained in intra-day and inter-day assay of plasma samples are summarized in Table 3. The largest value of % bias was -1.80 for intra-day assay while it was 1.00 for inter-day assay. Low values of % RSD indicated high precision of the proposed method.

Analysis of pharmaceutical preparation

The proposed method was successfully applied for the analysis of DLZ in Calcort tablet and the results are shown in Table 4. The low values of relative standard deviation indicated high precision of the method. The % recovery of the drug was observed to be satisfactory.
CONCLUSIONS

In conclusion, a simple, accurate and sensitive reversed phase HPLC method using UV detection has been described for the determination of DLZ in formulations and plasma samples. The method described is rapid since preparation of plasma samples prior to chromatography is relatively simple and the total chromatographic run time is 4 min. The method is capable of determining DLZ accurately down to 0.5 μg/ml which is enough for analysis of DLZ up to 36 h after single dose administration in human plasma with high degree of reproducibility.
REFERENCES


Fig. 1. Chromatogram of pure DLZ (10 μg/ml) solution containing PRX (2 μg/ml).
Fig. 2. Chromatogram of plasma sample containing DLZ (10 µg/ml) and PRX (2 µg/ml).

Fig. 3. Calibration graph for DLZ.
Table 1. Chromatographic separation characteristics of DLZ (10 μg/ml) and PRX (2 μg/ml) analyzed under best conditions.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pure sample</th>
<th>Plasma sample</th>
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<tbody>
<tr>
<td></td>
<td>DLZ</td>
<td>PRX</td>
</tr>
<tr>
<td>Number of theoretical plates</td>
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<td>88.87</td>
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<tr>
<td>Retention time ((t_R)) in min</td>
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<tr>
<td>Capacity factor ((k'))</td>
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<td>Selectivity factor ((\alpha))</td>
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<td>2.89</td>
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<tr>
<td>Resolution ((R))</td>
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<td>1.56</td>
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<tr>
<td>Peak asymmetry ((T))</td>
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<td>1.3</td>
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<tr>
<td>Height equivalent to theoretical plate ((H)) in mm</td>
<td>0.16</td>
<td>0.22</td>
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Table 2. Precision and accuracy data for pure DLZ.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Conc. added, μg/ml</th>
<th>Intra-day assay ((n = 9)) Concentration found, μg/ml</th>
<th>% RSD</th>
<th>% Bias</th>
<th>Inter-day assay ((n = 9)) Concentration found, μg/ml</th>
<th>% RSD</th>
<th>% Bias</th>
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<tbody>
<tr>
<td>DLZ</td>
<td>5</td>
<td>4.96</td>
<td>0.28</td>
<td>-0.80</td>
<td>5.03</td>
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<td></td>
<td>10</td>
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<td>0.31</td>
<td>0.90</td>
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<td></td>
<td>15</td>
<td>14.81</td>
<td>0.45</td>
<td>-1.26</td>
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<td>0.35</td>
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Table 3. Intra-day (1 representative day) and inter-day assay precision and
accuracy for DLZ in human plasma samples.

<table>
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<th>Drug</th>
<th>Plasma Conc. µg/ml</th>
<th>Intra-day assay (n = 9)</th>
<th>Inter-day assay (n = 9)</th>
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<td></td>
<td>Concentration found, µg/ml</td>
<td>% RSD</td>
<td>% Bias</td>
</tr>
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<td>DLZ</td>
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<td>4.91</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>14.84</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>24.68</td>
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Table 4. Analysis of pharmaceutical formulations and recovery studies.

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<th>Tablet</th>
<th>Labeled, mg</th>
<th>Found, mg</th>
<th>% RSD</th>
<th>% Recovery</th>
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<td>Calcort</td>
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<td></td>
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<tr>
<td></td>
<td>1</td>
<td>0.991</td>
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<td>99.1</td>
</tr>
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<td></td>
<td>6</td>
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<tr>
<td></td>
<td>30</td>
<td>30.588</td>
<td>0.92</td>
<td>101.96</td>
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Validated HPLC method for the assay of metoprolol succinate in pharmaceutical preparations and spiked plasma samples

Abstract

The objective of the current study was to develop a validated reverse phase high performance liquid chromatographic method for the assay of β-adrenaline receptor blocker, metoprolol succinate (MPS). Separation of drug was successfully achieved on a C18 column utilizing acetonitrile-phosphate buffer as mobile phase in the ratio of 75:25. The detection wavelength was 230 nm. The method was validated and the response was found to be linear in the drug concentration range of 0.4-15.2 μg/ml with correlation coefficient of 0.995. The RSD values for both intra- and inter-day assay were observed to be <0.5 %. The method was applied successfully for the analysis of drug in tablets and also in spiked human plasma samples. Analytical parameters were calculated and the complete statistical evaluation was incorporated.

The results of this chapter have been communicated for publication.
MPS is a cardiovascular beta adrenergic blocker and has been in clinical use for over 30 years. This is one of the most widely prescribed drugs in the world to-day for the treatment of various cardiovascular disorders such as angina pectoris, miocardial infarction, hypertension, cardiac arrhythmia, hyperthyroidism and other related diseases [1]. It is so sensitive that even a small oral dose of the drug gives sufficient blockade. It is official in various pharmacopoeia [2-4]. In view of its biological importance, few analytical methods have been reported for quantitative determination of MPS [5-17], which include gas chromatographic [5,6], high-performance liquid chromatographic [7-10] and spectrophotometric methods [11-17].
The chromatographic separation of a series of P-blockers have been carried out by Svensson et al [9]. Park et al [10] have determined the P-blockers in pharmaceutical preparations and human urine by HPLC with tris(2,2'-bipyridyl)ruthenium(II) electrogenerated chemiluminescence detection. The reported HPLC methods [7-10] suffer from different limitations viz., tedious procedures, complex sample preparation, etc. It was clear from the literature survey that no HPLC method was reported so far for the determination of MPS alone in its pharmaceutical formulations.

Hence, it was thought worthwhile to develop a more precise, accurate, rugged and reliable HPLC method for the determination MPS in its formulations and plasma samples.

**EXPERIMENTAL**

**Stock solutions**

MPS (100 mg) and nimesulide (NIM) (100 mg) were accurately weighed separately into different 100 ml calibrated flasks, dissolved in mobile phase and diluted to the mark with the same. The solutions were diluted as and when required.

**Standard working solutions**

Working standard solutions of each of 250 μg/ml of MPS and NIM were prepared separately in mobile phase. Studies on the stability of analyte in standard working solutions showed that there were no decomposition products.
Preparation of plasma samples

Human blood samples were collected in dry and evacuated tubes (which contained saline and sodium citrate solution) from different healthy volunteers, after an overnight fast, before breakfast. The samples were handled at room temperature and were centrifuged for 10 min at 1500 rpm for the separation of plasma within 60 min of collection. The plasma samples were spiked with MPS and internal standard and were extracted with ether. The ethereal layer was evaporated to dryness on a water bath under gentle stream of nitrogen gas at 40 °C. The residue was dissolved in the mobile phase, stored at -20 °C until analysis and 20 µl solution was injected on to the column.

Pharmaceutical preparation

Twenty tablets of MPS were finely powdered. An amount equivalent to 25 mg of the drug was weighed accurately and transferred into a 100 ml beaker. Using a mechanical stirrer, the powder was completely disintegrated in mobile phase. The solution was filtered and the filtrate was made up to 50 ml with the mobile phase.
Operating conditions

Column : CLC C_{18} column (5 μ, 25 cm x 4.6 mm i.d) with CLC ODS (4 cm x 4.6 mm, i.d.) as a guard column to protect analytical column.

Mobile phase : acetonitrile : phosphate buffer of pH 7 (75:25, v/v)
Flow rate : 1.0 ml/min
Internal standard : NIM
Temperature : Ambient
Mode : Reverse phase
Membrane : 0.45 μ Millipore
Run time : 5 min

PROCEDURE

Chromatographic conditions

HPLC analysis was performed by isocratic elution with a flow rate of 1.0 ml/min. The mobile phase consisting of acetonitrile and phosphate buffer of pH 7 (75:25, v/v) was used throughout. All solvents were filtered through a 0.45 μMillipore membranes filter before use and degassed in an ultrasonic bath. Volumes of 20 μl each, prepared and sample solutions (pharmaceutical or plasma samples) were injected into the column. Measuring at 230 nm effected quantification. The chromatographic run time maintained was 5 min. Throughout the study, the suitability of the chromatographic system was monitored by calculating the capacity factor (k'), the resolution (R), the selectivity (α) and the peak asymmetry (T).
The observed peaks were of good shape and completely resolved from one another. The chromatogram of extracted plasma samples did not show any co-eluting interference peak with the analyte or internal standard.

**Establishment of calibration**

Working standard solutions of MPS (0.1-16 μg/ml) containing fixed concentration of internal standard (10 μg/ml) was prepared in mobile phase. Triplicate 20 μl injections were made for each standard solution to check the reproducibility of the detector response at each concentration level. Chromatograms of solutions (n=9) were recorded. The peak area ratio of standard to internal standard was plotted against the concentration of the drug to obtain the calibration graph. The results were subjected to regression analysis. Typical chromatogram obtained for pure drug sample is shown in Fig. 1.

**Analysis of plasma samples**

The plasma sample obtained as described in plasma sample preparation was taken and 20 μl solution was injected into the chromatographic column and the chromatogram was recorded (Fig. 2). It was evident that well resolved peaks for MPS and internal standard, NIM were observed. The retention times of NIM and MPS were observed to be at 1.96 and 2.68 min, respectively.

**Analysis of pharmaceutical formulation**

Suitable amounts of aliquots of the drug sample obtained by following the procedure described for pharmaceutical preparation were taken and analyzed. The chromatogram at 230 nm showed a complete resolution of both peaks.
RESULTS AND DISCUSSION

Method development

Selection of mobile phase

The mobile phase was chosen after several trials with methanol, acetonitrile, isopropyl alcohol, triethylamine, water and buffer solutions in various proportions and at different pH values. A mobile phase consisting of acetonitrile and phosphate buffer of pH 7 in the ratio of 75:25 (v/v) was selected to achieve maximum separation and sensitivity.

Selection of internal standard

Several compounds viz., gabapentin, adefovir dipivoxil, tegaserod maleate, lercanidipine, ampicillin, cloxacillin and nimusulide were tested as internal standards. Ideal retention time, proper resolution and less time for analysis was observed with NIM. Hence, NIM was selected as an internal standard.

Effect of flow rate

The effect of flow rate in the range of 0.5 to 1.5 ml/min was examined. A flow rate of 1.0 ml/min gave an optimal signal to noise ratio with a reasonable separation time. With a flow rate of less than or more than 1.0 ml/min yielded asymmetric peaks besides low resolution. Hence, a flow rate of 1.0 ml/min was maintained throughout the study.

Retention times

The retention times of NIM and MPS were observed to be at 1.96 and 2.68 min, respectively. Further, in the present study, the retention times of
NIM and MPS were reproducible. The total time of analysis was observed to be less than 5 min.

**Selection of wavelength**

Effect of wavelength on the response factor and on peak resolution was studied over the wavelength range of 180-280 nm. The solution containing MPS and NIM exhibited maximum absorption at 230 nm. Satisfactory chromatographic conditions were obtained at the wavelength of 230 nm. Therefore, the wavelength of 230 nm was chosen for analysis. The chromatograms obtained at the wavelength other than 230 nm were observed to be asymmetric.

**Order of elution**

It was noticed that NIM eluted first followed by MPS.

**Linearity and regression analysis**

The calibration graph for the proposed method is shown in Fig. 3. The calibration curve was represented by, \( y = bx + c \), where \( y \) refers to the ratio of peak area of MPS to NIM and \( x \) represents the concentration of MPS. Table 1 gives the regression line, correlation coefficient, slope, intercept and % RSD. Excellent linearity was noticed in the range of 0.4-15.2 \( \mu \)g/ml with \( r = 0.995 \).

**Detection and quantification limits**

Limit of detection (LOD) was established at a signal-to-noise ratio (S/N) of 3 while limit of quantification (LOQ) was established at a signal-to-noise ratio (S/N) of 10. The limit of detection was calculated to be 0.130 \( \mu \)g/ml while the limit of quantification was found to be 0.391 \( \mu \)g/ml.
Suitability of the method

The chromatographic parameters such as resolution, selectivity and peak asymmetry were evaluated. The resolution (R) values were found to be >1.5 between each peak-pair while the selectivity values (\(\alpha\)) for pure sample and plasma sample were observed to be >1. The capacity factors (\(k^1\)) were found to be 20.66 and 28.89 for pure sample, while those for plasma samples were observed to be 19.82 and 28.69 for MPS and NIM, respectively. The peak asymmetry (T) values were found to be less than 1.5. The corresponding chromatographic parameters are given in Table 2. These system performance parameters suggested ideal chromatographic conditions for the quantification of MPS.

Precision and accuracy

Within- and between-day assay precision and accuracy of the proposed method were evaluated by determining different concentrations of the drug (n=7). The values of RSD and bias are given in Table 3. The values indicated that the proposed method was precise and accurate.

Specificity

Specificity of the proposed method was confirmed by carrying out the analysis in presence of different drugs such as parecoxib, tenofovir, adefovir dipivoxil, tegaserod maleate, lercanidisine, buzepide, ibuprofen and trazodone hydrochloride. These drugs did not interfere in the determination as evident from the fact that the interfering peaks were not noticed at the retention times of either MPS or NIM.
Analysis of plasma samples

The proposed method was applied to the determination of MPS in plasma samples for validation. The results obtained for intra-day and inter-day assay at three different concentrations in plasma are summarized in Table 4. The largest value of % bias was 1.0 for intra-day assay while it was 2.0 for inter-day assay. Low values of relative standard deviation values for both intra-day and inter-day assay indicated high precision of the proposed method.

Analysis of pharmaceutical preparation

The proposed method was successfully applied to the analysis of MPS in its tablet and the results are shown in Table 5. The low values of relative standard deviation indicated high precision of the method.

CONCLUSIONS

The determination of MPS in its formulations and human plasma using high-performance liquid chromatography with UV detection is developed and validated. The system performance parameters indicated the suitability of the method. All statistical parameters (percentage recoveries, % RSD and % bias) are observed to be within the acceptable limits. Hence, the proposed HPLC method could be adopted for routine quality control for the assay of MPS.
REFERENCES


Fig. 1. Chromatogram of NIM (10 μg/ml) and MPS (10 μg/ml) in pure form.
Fig. 2. Chromatogram of plasma sample containing NIM (10 µg/ml) and MPS (10 µg/ml).

Fig. 3. Calibration graph for MPS.
Table 1. Linearity, LOD, LOQ values.

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<th>Parameter</th>
<th>MPS</th>
</tr>
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<tr>
<td>Linear dynamic range (µg/ml)</td>
<td>0.4-15.2</td>
</tr>
<tr>
<td>Regression equation (Y^a)</td>
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<tr>
<td>Slope (b)</td>
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<td>Intercept (a)</td>
<td>0.403</td>
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<tr>
<td>Correlation coefficient (r)</td>
<td>0.995</td>
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<td>LOD (µg/ml)</td>
<td>0.130</td>
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<td>LOQ (µg/ml)</td>
<td>0.391</td>
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<td>% RSD</td>
<td>1.82</td>
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Table 2. Chromatographic separation characteristics of MPS (10 µg/ml) and NIM (10 µg/ml) analyzed under best conditions.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Pure sample</th>
<th>Plasma sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MPS</td>
<td>NIM</td>
</tr>
<tr>
<td>Number of theoretical plates</td>
<td>90.58</td>
<td>91.87</td>
</tr>
<tr>
<td>Retention time (t_R) in min</td>
<td>2.68</td>
<td>1.96</td>
</tr>
<tr>
<td>Capacity factor (k')</td>
<td>20.66</td>
<td>28.89</td>
</tr>
<tr>
<td>Selectivity factor (α)</td>
<td>2.14</td>
<td>2.02</td>
</tr>
<tr>
<td>Resolution (R)</td>
<td>1.62</td>
<td>1.54</td>
</tr>
<tr>
<td>Peak asymmetry (T)</td>
<td>1.33</td>
<td>1.34</td>
</tr>
<tr>
<td>Height equivalent to theoretical plate (H) in mm</td>
<td>0.23</td>
<td>0.28</td>
</tr>
</tbody>
</table>
Table 3. Precision and accuracy data for MPS.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Conc. added, µg/ml</th>
<th>Intra-day assay (n = 7)</th>
<th>Inter-day assay (n = 7)</th>
<th>% RSD</th>
<th>% Bias</th>
<th>% RSD</th>
<th>% Bias</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPS</td>
<td>5</td>
<td>4.98</td>
<td>5.10</td>
<td>0.28</td>
<td>-0.40</td>
<td>0.31</td>
<td>2.0</td>
</tr>
<tr>
<td>MPS</td>
<td>10</td>
<td>10.12</td>
<td>9.92</td>
<td>0.43</td>
<td>1.20</td>
<td>0.39</td>
<td>-0.8</td>
</tr>
<tr>
<td>MPS</td>
<td>15</td>
<td>14.92</td>
<td>15.09</td>
<td>0.37</td>
<td>-0.53</td>
<td>0.42</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Table 4. The results of precision and accuracy for intra-day (1 representative day) and inter-day assay of MPS in human plasma samples.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Plasma Conc. µg/ml</th>
<th>Intra-day assay (n = 9)</th>
<th>Inter-day assay (n = 9)</th>
<th>% RSD</th>
<th>% Bias</th>
<th>RSD (%)</th>
<th>% Bias</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPS</td>
<td>1</td>
<td>1.01</td>
<td>1.02</td>
<td>0.45</td>
<td>1.0</td>
<td>0.47</td>
<td>2.0</td>
</tr>
<tr>
<td>MPS</td>
<td>5</td>
<td>4.98</td>
<td>5.04</td>
<td>0.38</td>
<td>-0.4</td>
<td>0.52</td>
<td>0.8</td>
</tr>
<tr>
<td>MPS</td>
<td>10</td>
<td>10.02</td>
<td>10.18</td>
<td>0.69</td>
<td>0.2</td>
<td>0.60</td>
<td>1.8</td>
</tr>
</tbody>
</table>
Table 5. Analysis of pharmaceutical formulations of MPS.

<table>
<thead>
<tr>
<th>Tablet</th>
<th>Labeled, mg</th>
<th>Found*, mg</th>
<th>% RSD</th>
<th>% Recovery*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seloken-XL&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25</td>
<td>24.92</td>
<td>0.96</td>
<td>99.68</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>49.96</td>
<td>0.98</td>
<td>99.92</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100.11</td>
<td>1.10</td>
<td>100.11</td>
</tr>
<tr>
<td>Metolar&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25</td>
<td>24.95</td>
<td>1.35</td>
<td>99.80</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>49.96</td>
<td>0.89</td>
<td>99.92</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>99.94</td>
<td>1.50</td>
<td>99.94</td>
</tr>
<tr>
<td>Meto-FR&lt;sup&gt;c&lt;/sup&gt;</td>
<td>25</td>
<td>25.19</td>
<td>1.12</td>
<td>100.76</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>49.93</td>
<td>0.97</td>
<td>99.86</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100.09</td>
<td>1.05</td>
<td>100.09</td>
</tr>
</tbody>
</table>

<sup>*</sup>Average of nine determinations.

<sup>a</sup>Astra Zeneca, India, Ltd.

<sup>b</sup>Cipla pharmaceuticals Ltd.

<sup>c</sup>Mono Pharmaceuticals Ltd.
High-performance liquid chromatographic determination of parecoxib in bulk and dosage forms

ABSTRACT

A simple and sensitive RP-HPLC method for the determination of parecoxib (PXB) in pharmaceutical formulations has been developed and validated. The mobile phase consisting of methanol-water (90:10 v/v) with a flow rate of 1 ml/min was employed. The linear range of detection was found to be 0.5-14.5 μg/ml ($r = 0.9988$). Intra- and inter-day assay relative standard deviations were observed to be less than 0.5%. The method has been applied successfully for the determination of PXB in pharmaceutical preparations. Analytical parameters were calculated and suitable conclusions were drawn.

The results of this chapter have been communicated for publication.
General drug profile

Commercial name : Parecoxib
Chemical name : N-[4-(5-methyl-3-phenyl-oxazol-4-yl)phenyl]sulfonylpropanamide

Structure :

![Structure Diagram]

Molecular formula : C$_{19}$H$_{18}$N$_{2}$O$_{4}$S
Molecular weight : 370.1
Description : Colorless, practically odorless, amorphous powder
Solubility : Readily soluble in water
Category : Cyclooxygenase Inhibitor, Anti-inflammatory

INTRODUCTION

PXB is a prodrug of valdecoxib, a selective inhibitor of cyclooxygenase 2 (COX 2) administered as an intramuscular or intravenous injection. Traditionally oral NSAIDs such as ibuprofen and diclofenac are used to treat mild to moderate acute pain. However, this may be problematic when patients are unable to take oral medication or are nauseous and vomiting. PXB is an effective analgesic in acute pain.
Critical literature survey revealed that no attempt has been made so far to develop analytical method for the assay of PXB in bulk and pharmaceutical formulations, except the method which was developed by our group [1]. This prompted the investigator to develop a simple HPLC method for the determination of PXB in different samples. The proposed method does not require any internal standard.

**EXPERIMENTAL**

**Stock solution**

PXB (100 mg) was dissolved in mobile phase and diluted to the mark with the same in a 100 ml volumetric flask. The solution was diluted as and when required.

**Standard working solutions**

Working solutions of pure PXB were prepared separately in the mobile phase. Studies on the stability of analytes in working solution showed that there were no decomposition products in the chromatogram and also no difference in area-ratio during analytical procedure and even after storing for several days at 4 °C.

**Pharmaceutical formulations**

An appropriate volume of injection sample containing PXB was diluted with mobile phase and then filtered through 0.45 μ Millipore membrane filter. It was then degassed by keeping it in an ultrasonic bath. The known volumes of this drug solution were used for assay.
Operating conditions

Column : CLC C_{18} column (5 \mu, 25 \text{ cm} \times 4.6 \text{ mm i.d}) with CLC ODS (4 \text{ cm} \times 4.6 \text{ mm, i.d.}) as a guard column to protect analytical column.

Mobile phase : methanol : water (90:10, v/v)

Flow rate : 1.0 ml/min

Temperature : Ambient

Mode : Reverse phase

Membrane : 0.45 \mu Millipore

Run time : 5 min

PROCEDURE

Establishment of calibration curve

Working solutions of pure PXB (0.5-14.5 \mu g/ml) were prepared separately in the mobile phase. Triplicate 20 \mu l injections were injected for each working solution to see the reproducibility of the detector response at each concentration level. A typical chromatogram obtained is shown in Fig. 1. The peak area of PXB versus its concentration was plotted to obtain the calibration graph (Fig. 2). The results were subjected to regression analysis to obtain calibration equation and correlation coefficient.

The suitability of the chromatographic system was monitored by calculating the capacity factor (k'), resolution (R) and peak asymmetry (A_S).
Analysis of pharmaceutical formulations

An aliquot of the drug (obtained by following the procedure described in the sample preparation for pharmaceutical formulation) was taken and analyzed using the same chromatographic conditions.

RESULTS AND DISCUSSION

Method development

Mobile phase

For reverse phases, the retention of organic samples is always high with water as eluent. Increasing the concentration of organic solvents in water can accelerate the elution of organic compounds. The mobile phase was chosen after several trials with methanol, acetonitrile, isopropyl alcohol, triethylamine, water and various buffer solutions of different pH in various proportions. Better results were observed with methanol-water combination and hence, different ratios of acetonitrile to water were tried to select the suitable ratio. It was observed that at higher concentration of water in the mobile phase, the retention time of PXB was noticed to be high and PXB was eluted poorly off the column. The best elution of the drug was achieved with methanol and water combination in the ratio of 90:10.

Flow rate

Flow rate of the mobile phase plays a crucial role in chromatographic determination of pharmaceutical drugs. The effect of flow rate was investigated by varying the flow rate of the mobile phase from 0.25 to
1.75 ml/min. Increased resolution time was observed with lower flow rates of the mobile phase. However, high flow rates led to considerable increase in pressure. A flow rate of 1.0 ml/min exhibited an optimal signal to noise ratio with a reasonable separation time and hence permitted good analytical conditions for the assay of PXB.

**Retention time**

The reproducibility of the retention time of PXB was calculated based on the average of nine determinations. The retention time of PXB was observed to be 1.01 min.

**Wavelength selection**

The solution of PXB exhibited maximum absorption at 200 nm and hence, this wavelength was chosen for analysis.

**Linearity of detector response**

Linearity and range of the method were determined by analyzing different solutions containing 0.2-18 µg/ml of PXB under the chromatographic conditions mentioned above (n = 9). Then 20 µl solution was injected into the column and chromatogram was noted. The peak area of PXB was plotted against its concentration to obtain the calibration graph (Fig. 2). Table 1 gives the regression line, correlation coefficient, slope, intercept and % RSD. Excellent linearity was noticed in the range of 0.5-14.5 µg/ml with $r = 0.9988$. 

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Limits of detection and quantification

The values of LOD and LOQ [2] were evaluated and were observed to be 0.164 μg/ml and 0.492 μg/ml, respectively (Table 1).

Suitability of the method

The suitability of the method was checked by determining the chromatographic parameters viz., resolution, capacity factor and peak asymmetry and the results are shown in Table 2. The observed values of resolution (more than 1.5) and peak asymmetry (less than 2) revealed ideal chromatographic conditions for the quantification of PXB.

Precision

The precision of the method (Intra- and inter-day variations of replicate determinations) was checked by injecting PXB (n = 9) at the LOQ level. The results obtained are recorded in Table 3. These results highlighted the reproducibility of the method.

Accuracy

A standard working solution containing PXB was prepared and the mixture was injected (n = 9) and chromatogram was recorded. From the respective area counts, the concentrations of the PXB were determined using the calibration graph. The values of % bias are recorded in Table 3 and these results revealed accuracy of the proposed method.
Application

Analysis of injection sample

The proposed method was successfully applied to the analysis of PXB in injections and the results are shown in Table 4. The low values of relative standard deviation indicated high precision of the method.

CONCLUSIONS

The proposed HPLC method showed acceptable linearity, precision and accuracy over the concentration range mentioned. The method described is rapid, since the total chromatographic run time is about 5 min. The chromatographic method could be used to analyze a large number of pharmaceutical formulations in quality control laboratories. Hence, the proposed method could be adopted for the assay of PXB in pharmaceutical preparations.

REFERENCES

Fig. 1. A typical chromatogram showing the elution of PXB (8 µg/ml).
Fig. 2. Calibration graph for PXB.
Table 1. Linearity, limit of detection and limit of quantification.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PXB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear dynamic range (µg/ml)</td>
<td>0.5-14.5</td>
</tr>
<tr>
<td>Regression equation ( Y^a )</td>
<td></td>
</tr>
<tr>
<td>Slope (b)</td>
<td>0.2833</td>
</tr>
<tr>
<td>Intercept (c)</td>
<td>0.5930</td>
</tr>
<tr>
<td>Correlation coefficient (r)</td>
<td>0.9988</td>
</tr>
<tr>
<td>LOD (µg/ml)</td>
<td>0.164</td>
</tr>
<tr>
<td>LOQ (µg/ml)</td>
<td>0.492</td>
</tr>
<tr>
<td>% RSD</td>
<td>1.64</td>
</tr>
</tbody>
</table>

\( Y = bX + c \), where X is concentration of drug in µg/ml.

Table 2. System performance parameters.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>PXB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention time ( (t_R) ) in min</td>
<td>1.01</td>
</tr>
<tr>
<td>Capacity factor ( (k') )</td>
<td>10.22</td>
</tr>
<tr>
<td>Resolution (R)</td>
<td>2.02</td>
</tr>
<tr>
<td>Peak asymmetry ( (A_s) )</td>
<td>1.08</td>
</tr>
<tr>
<td>Number of theoretical plates (N)</td>
<td>95.52</td>
</tr>
<tr>
<td>Height equivalent to theoretical plate (H) in mm</td>
<td>0.15</td>
</tr>
</tbody>
</table>
Table 3. Intra-day (1 representative day) and inter-day precision and accuracy for the determination of PXB.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Conc. added, µg/ml</th>
<th>Intra-day (n = 9) Concentration measured</th>
<th>% RSD</th>
<th>% Bias</th>
<th>Inter-day (n = 9) Concentration measured</th>
<th>% RSD</th>
<th>% Bias</th>
</tr>
</thead>
<tbody>
<tr>
<td>PXB</td>
<td>5</td>
<td>4.93</td>
<td>0.94</td>
<td>-1.40</td>
<td>4.96</td>
<td>0.71</td>
<td>-0.80</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9.94</td>
<td>1.05</td>
<td>-0.60</td>
<td>9.96</td>
<td>0.63</td>
<td>-0.40</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>14.92</td>
<td>0.86</td>
<td>-0.53</td>
<td>14.95</td>
<td>1.08</td>
<td>-0.33</td>
</tr>
</tbody>
</table>

Table 4. Analysis of PXB in pharmaceutical formulations.

<table>
<thead>
<tr>
<th>Injection(^a)</th>
<th>Labeled, mg/ml</th>
<th>Found*, mg/ml</th>
<th>% RSD</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vorth-P</td>
<td>40</td>
<td>39.88</td>
<td>1.28</td>
<td>99.73</td>
</tr>
<tr>
<td>Valus-P</td>
<td>40</td>
<td>39.96</td>
<td>0.92</td>
<td>99.91</td>
</tr>
</tbody>
</table>

* Average of five determinations.
\(^a\) Marketed by Glen Copel Marketing.