CHAPTER VIII
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Development and validation of a HPLC method for the assay of pentoxifylline in pharmaceutical formulations and spiked human plasma

Abstract

A reverse phase high performance liquid chromatographic method for the determination of pentoxifylline (PTF) in pharmaceutical formulations and human plasma samples has been described in the present chapter. The assay of the drug was performed on a CLC C_{18} (5 \mu m, 25 cm x 4.6 mm i.d) with UV detection at 205 nm using piroxicam (PRX) as an internal standard. The mobile phase consisted of acetonitrile-water mixture in the ratio of 90:10 (v/v) at a flow rate of 1 ml/min. The standard curve was linear over the range of 0.3-20 \mu g/ml (R^2 = 0.9984). Analytical parameters have been evaluated. Low values of within-day and between-day assay revealed the reproducibility of the results. The proposed method is simple and selective and could be applicable for routine analysis of PTF in human plasma samples and pharmaceutical formulations.

The results of this chapter have been communicated for publication.
GENERAL DRUG PROFILE

Pentoxifylline (PTF)

Chemical name: 3,7-dimethyl-1-(5-oxohexyl)purine-2,6-dione

Structure:

Molecular formula: $C_{13}H_{18}N_4O_3$

Molecular weight: 278.31

Description: White needle crystal

Solubility: Soluble in water and ethanol and sparingly soluble in toluene

Category: Peripheral vasodilator
INTRODUCTION

Pentoxifylline (Trental®), a methylxanthane derivative, widely used as a vasodilator in the treatment of peripheral vascular disorders as it decreases blood viscosity by increasing red cell deformability [1-6]. In particular, it has been reported to decrease pulmonary injury and associated pulmonary neutrophil and protein sequestration in dog and guinea pig models of sepsis with endotoxin or *Escherichia coli* as the septic insult [7,8]. PTF is officially listed in the British Pharmacopoeia addendum as oxypentifylline [9].

Burrows [10] has described the simultaneous determination of oxpentifylline and three metabolites in plasma by capillary gas chromatography equipped with a Model 18789A nitrogen-specific detector and a Model 18740B capillary inlet system. A HPLC method was described by Jia et al [11] for the assay of PTF in human plasma with UV detection at 273 nm. The system consisted of an internal standard, chloramphenicol and the mobile phase comprised of 0.02 M phosphoric acid (pH 4), methanol and tetrahydrofuran (55:45:1, v/v) with a flow-rate of 1.4 ml/min. Mancinelli et al [12] have reported the use of a solid-phase extraction procedure in the sample preparation. The detection limit reported was considerably high.

Several analytical methods including TLC [13], high-performance liquid-chromatography [11,12,14-20], gas-chromatography [21,22,10], fluorescence polarized immunoassay [23-25] and visible spectrophotometry [26,27] has been reported for the assay of PTF. Thorough literature survey revealed that no attempt has been made to develop a HPLC method for the determination of
PTF employing acetonitrile and water as the mobile phase. All the reported HPLC methods are critical of the pH of the mobile phase used.

In view of the above, the investigator has developed a simple and reliable RP-HPLC (coupled with UV detection) method for the determination of PTF in pharmaceutical formulations and spiked human plasma samples.

**EXPERIMENTAL**

**Stock solutions**

1) Standard solution: Pure PTF solution (500 µg/ml) was prepared in acetonitrile.

2) Internal standard solution: A stock solution of PRX (500 µg/ml) prepared in acetonitrile was used in the study.

**Standard working solutions**

Working solutions of pure PTF and PRX (100 µg/ml) were prepared separately by appropriate dilution with the mobile phase. Stock solution of PTF was further diluted as and when required. Studies on the stability of analytes in standard working solutions showed no difference in peak areas and also there were no decomposition products observed in the chromatogram.

**Sample preparation**

a) Blood Plasma

Human blood samples were obtained from healthy volunteers in dry and evacuated tubes (containing saline and sodium citrate solution). The samples were centrifuged for 10 min at 1500 rpm for the separation of plasma at 28 °C.
within 1 h of collection. The samples were then transferred to polypropylene tubes and stored at -20 °C until analysis. A 20 μl solution was injected on to the column during analysis.

b) Pharmaceutical formulation

Ten tablets of PTF were finely powdered and an amount equivalent to 10 mg of the drug was weighed accurately and transferred into a 100 ml beaker. The powder was completely disintegrated in mobile phase by continuous stirring with a glass rod. The solution was filtered through 0.45 μ Millipore membrane filter and the filtrate was made up to 50 ml with the mobile phase. Suitable amount was taken for analysis.

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: 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 : 5 min

Wavelength : 205 nm
Procedures

Establishment of calibration curve

Working standard solutions of PTF (0.3-20 μg/ml) containing 6 μg/ml PRX were prepared in the mobile phase. Triplicate 20 μl solutions were injected for each working solution. A typical chromatogram obtained is shown in Fig. 1. The values of peak area ratio of standard to internal standard were plotted against the concentration of drug to obtain the calibration graph (Fig. 2). The results were subjected to regression analysis.

Analysis of plasma samples

The plasma samples were spiked with PTF and a fixed amount of PRX (6 μg/ml) and the tube was briefly shaken. Then the mixture was vortex mixed 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 suitable amount of mobile phase and 20 μl solution was injected on to the column and the chromatogram was recorded (Fig. 3).

Analysis of dosage forms

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

The mobile phase was selected 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 acetonitrile-water combination. Hence, the effects of various acetonitrile-water mixtures were investigated to select suitable chromatographic conditions. It was noticed that the acetonitrile-water mixture in the ratio of 90:10 yielded good analytical conditions.

Flow rate

The effect of flow rate was studied by varying the flow rate of the mobile phase from 0.25 to 1.75 ml/min. Lower flow rates led to increase in resolution time and high flow rates led to considerable increase in pressure. However, a flow rate of 1.0 ml/min gave an optimal signal to noise ratio with a reasonable separation time.

Internal standard

The selection of PRX as an internal standard was based on proper resolution, less time for analysis and good chromatographic behavior.

Order of elution

It was observed that the PRX was eluted first followed by PTF.
**Retention times**

The retention times of PRX and PTF were noticed to be 0.76 and 1.98 min, respectively (n=9).

**Selection of wavelength**

Effect of wavelength on the response factor and on the peak resolution was examined over the wavelength range of 180-280 nm. Ideal chromatographic conditions were obtained at the wavelength of 205 nm.

**Linearity of detector response**

Appropriate amounts of aliquots of standard solutions were mixed with 6 µg/ml of PRX and diluted to 5 ml with mobile phase. Then 20 µl solution was injected into the column and chromatogram was noted down. The values of peak area ratio of PTF to PRX were plotted against the concentrations of PTF to construct the calibration graph (Fig. 2). The values of correlation coefficient, slope, intercept and % RSD are given in Table 1. Good linearity was noticed in the range of 0.3-20 µg/ml with $R^2 = 0.9984$.

**Limit of detection and limit of quantification**

The LOD and LOQ [11] values were found to be 0.083 and 0.277 µg/ml, respectively (Table 1).

**Suitability of the method**

The suitability of the method was checked by determining the chromatographic parameters viz., resolution, selectivity and peak asymmetry and the results are shown in Table 2. The calculated values of resolution
(more than 1.5), selectivity (more than 1) and peak asymmetry (less than 2) revealed ideal chromatographic conditions for quantification of PTF.

**Intra- and inter-day assay**

Within- and between-day assay precision and accuracy were evaluated by determining different amounts of PTF within the linear range. Within and between-day assay relative standard deviation values were observed to be less than 1.5% (Table 3), while within- and between-day assay errors (% bias) were found to be less than 2.7% (Table 3). These values revealed that the proposed method was precise and accurate.

**Specificity of the method**

The specificity of the proposed method was evident from the fact that the drugs such as methdilazine hydrochloride, isothipendyl hydrochloride, ceterizine hydrochloride, metaprolol succinate, nimesulide, gabapentin, ampicillin, cloxacillin, cefadroxil and diclofenacsodium did not show interference in the determination of PTF.

**Applications**

**Analysis of plasma samples**

The proposed method was extended for the analysis of PTF in spiked plasma samples. The results of precision for three different concentrations of PTF in plasma are summarized in Table 4. The largest value of % bias was observed to be 2.5. Low values of relative standard deviation and % bias indicated high precision and accuracy of the proposed method.
**Analysis of pharmaceutical preparation**

The proposed method was successfully applied to the assay of tablet preparations containing PTF and the results are shown in Table 5. The low values of relative standard deviation indicated high precision of the method.

**CONCLUSIONS**

A simple, accurate and sensitive reversed phase HPLC method using UV detection has been described for the determination of PTF in plasma and formulations. The analysis of PTF in human plasma with high degree of reproducibility is possible up to 36 h. Simple sample preparation procedure and a short chromatographic time make this method suitable for processing of multiple samples in a limited amount of time.
REFERENCES


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**Fig. 1.** A typical chromatogram showing the separation of PRX and PTF in pure form.
Fig. 2. Calibration graph for PTF.
Fig. 3. A typical chromatogram showing the separation of PRX and PTF in plasma sample.
Table 1. Linearity results, limit of detection and limit of quantification.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>PTF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear dynamic range (µg/ml)</td>
<td>0.3-20</td>
</tr>
<tr>
<td>Regression equation (Y=)</td>
<td></td>
</tr>
<tr>
<td>Slope (b)</td>
<td>0.131</td>
</tr>
<tr>
<td>Intercept (c)</td>
<td>0.0991</td>
</tr>
<tr>
<td>Correlation coefficient (R²)</td>
<td>0.9984</td>
</tr>
<tr>
<td>LOD (µg/ml)</td>
<td>0.083</td>
</tr>
<tr>
<td>LOQ (µg/ml)</td>
<td>0.277</td>
</tr>
<tr>
<td>% RSD</td>
<td>1.88</td>
</tr>
</tbody>
</table>

*Y= bX + c, where X is concentration of drug in µg/ml.
Table 2. System performance parameters of PRX (6 μg/ml) and PTF (10 μg/ml).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Pure sample</th>
<th>Plasma sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PRX</td>
<td>PTF</td>
</tr>
<tr>
<td>Number of theoretical plates</td>
<td>28.48</td>
<td>85.76</td>
</tr>
<tr>
<td>Retention time (t&lt;sub&gt;R&lt;/sub&gt;) in min</td>
<td>0.76</td>
<td>1.98</td>
</tr>
<tr>
<td>Capacity factor (k')</td>
<td>4.84</td>
<td>14.23</td>
</tr>
<tr>
<td>Selectivity factor (α)</td>
<td>2.94</td>
<td>2.96</td>
</tr>
<tr>
<td>Resolution (R)</td>
<td>1.588</td>
<td>1.586</td>
</tr>
<tr>
<td>Peak asymmetry (A&lt;sub&gt;S&lt;/sub&gt;)</td>
<td>1.80</td>
<td>1.40</td>
</tr>
<tr>
<td>Height equivalent to theoretical plate (H) in mm</td>
<td>0.526</td>
<td>0.174</td>
</tr>
</tbody>
</table>

Table 3. Intra-day and inter-day precision and accuracy for the determination of PTF.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration added, µg/ml</th>
<th>Intra-day (n = 9)</th>
<th>Inter-day (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration measured</td>
<td>% RSD  % Bias</td>
<td>Concentration measured</td>
</tr>
<tr>
<td>PTF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.96</td>
<td>1.28  -2.0</td>
<td>1.95</td>
</tr>
<tr>
<td>5</td>
<td>4.91</td>
<td>1.03  -1.8</td>
<td>4.88</td>
</tr>
<tr>
<td>10</td>
<td>9.84</td>
<td>1.42  -1.6</td>
<td>9.74</td>
</tr>
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</table>

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Table 4. Precision and accuracy data for the determination of PTF in human plasma samples.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration added, µg/ml</th>
<th>Measured concentration*, µg/ml</th>
<th>% RSD</th>
<th>% Bias</th>
</tr>
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<tbody>
<tr>
<td>PTF</td>
<td>2</td>
<td>1.95</td>
<td>1.6</td>
<td>-2.5</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4.93</td>
<td>1.9</td>
<td>-1.4</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9.75</td>
<td>2.1</td>
<td>-2.5</td>
</tr>
</tbody>
</table>

*Average of five determinations.

Table 5. Analysis of PTF in pharmaceutical formulations.

<table>
<thead>
<tr>
<th>Tablet&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Labeled, mg/ml</th>
<th>Found*, mg/ml</th>
<th>% RSD</th>
<th>% Recovery</th>
</tr>
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<tbody>
<tr>
<td>Flexital</td>
<td>400</td>
<td>399.6 ± 0.56</td>
<td>1.41</td>
<td>99.86</td>
</tr>
</tbody>
</table>

* Average of five determinations.

<sup>a</sup>Marketed by Sun pharmaceuticals Ltd.