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# Development and Validation of a Reversed-Phase Liquid Chromatographic Method for Analysis of Rofecoxib in Human Plasma

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High performance liquid chromatographic method was developed for the estimation of rofecoxib in human plasma. Varying amounts of rofecoxib (5 to 400 ng) and fixed quantity of nimesulide (500 ng) were added to blank plasma (0.5 mL) and extracted with dichloromethane. The mixture was centrifuged, organic layer was separated, evaporated to dryness under vacuum and reconstituted with 0.1 mL of methanol. Twenty microlitres of this solution was injected into reverse phase C-18 column using a mobile phase consisting of methanol and water (consisting of 0.04% triethylamine and 0.15% glacial acetic acid) in the ratio of 50 : 50 v/v and the eluents were monitored at 272 nm. The method was validated for its linearity, precision and accuracy. The calibration curve was linear in the range of 5 to 400 ng/0.5 mL of plasma. The intra- and inter-day variation was found to be less than 2.3% indicating that the method is highly precise. The mean recovery of rofecoxib from plasma samples containing 50 or 100 ng/mL of the drug was 95.66%. There was no interference of either the drug metabolites or plasma constituents with the proposed HPLC method for the estimation of rofecoxib in human plasma. Due to its simplicity, sensitivity, high precision and accuracy, the proposed HPLC method may be used for biopharmaceutical and pharmacokinetic evaluation of rofecoxib formulations.

**Key Words: Rofecoxib, Estimation, Human plasma, RP-HPLC, Validation..**

## INTRODUCTION

Rofecoxib is used in the treatment of osteoarthritis, relief from acute pain and primary dysmenorrhoea[1]. It is a selective COX-2 inhibitor[2]. Unlike conventional NSAIDs, rofecoxib selectively blocks COX-2 enzyme, which is present in high levels at the site of inflammation. So far, only two analytical methods have been reported for the estimation of rofecoxib in plasma by HPLC[3,4]. The reported methods utilized fluorescence detection with specialized columns and in the latter method run time was more. However, the HPLC methods using the most commonly available columns are preferred. The objective of the present study

was to develop and validate an accurate, sensitive, and simple HPLC method for determination of rofecoxib in human plasma by using RP C-18 column.

## EXPERIMENTAL

Dichloromethane, orthophosporic acid and triethylamine were of analytical grade (Fine Chemicals). The methanol and water used were of HPLC grade (Qualigens). Rofecoxib and nimesulide were gift samples from M/s Torrent Laboratories Ltd, Ahmedabad, India and M/s Dr. Reddy's Laboratories Ltd, Hyderabad, India.

**Instrumentation:** A gradient HPLC (Shimadzu HPLC class VP series) with two LC-10AT VP pumps, variable wavelength programmable UV/Vis detector SPD-10A VP, CTO-10AS VP column oven (Shimadzu), SCL-10A VP system controller (Shimadzu), a disposable guard column LC-18 (Pelliguard™, LC-18, 2 cm, Supelco, Inc., Bellefonte, PA) and RP C-18 column (250 mm × 4.6 mm I.D., particle size 5 μm, Flexit Inc., Pune, India) was used. The HPLC system was equipped with the software "Class-VP series version 5.03 (Shimadzu)".

**HPLC conditions:** Both methanol and water (consisting of 0.04% triethylamine and 0.15% glacial acetic acid) were filtered before use through 0.2 μm membrane filter, and pumped from the solvent reservoir in the ratio of 50 : 50 to the column at a flow rate of 1 mL/min. The column temperature was maintained at 40°C. The volume of each injection was 20 μL. The detector sensitivity was set at 0.0001 a.u.f.s. and eluents were monitored at 272nm.

**Methodology:** Five sets of plasma samples with varying drug concentrations were prepared by spiking drug-free plasma with appropriate volume (100 μL) of a known amount of rofecoxib at a concentration range of 10 to 400 ng/0.5 mL of plasma along with 100 μL of 500 ng/0.5 mL of nimesulide (internal standard) solution.

An aliquot of plasma (0.5 mL) was accurately measured into a 10 mL glass tube with a teflon-lined cap, followed by the addition of 100 μL of 500 ng/mL of nimesulide (internal standard) solution and 5 mL of dichloromethane as the extracting solvent. After mixing for 10 min by means of a vortex mixer, the mixture was centrifuged at 3000 rpm for 10 min. The organic layer was separated and evaporated to dryness under vacuum. The residue was reconstituted with 100 μL of methanol and twenty microlitres of this solution was injected into reversed phase C-18 column. The eluents were monitored at 272 nm. The peak areas of rofecoxib and internal standard were recorded. The ratio of peak area of rofecoxib to that of internal standard (nimesulide) was calculated, and the regression of the peak area ratio over the plasma concentration of rofecoxib was calculated using the least squares method of analysis.

**Precision:** Aliquots of blank plasma (500 μL) were spiked with 100 μL of 500 ng/mL of internal standard and varying amounts of rofecoxib solutions so as to yield concentrations of 10, 50 and 200 ng/0.5 mL. Each plasma sample was extracted as described above and injected into HPLC column (n = 5). Each sample was prepared in triplicate on three consecutive days and injected into HPLC column (n = 5) to observe the precision of the method.

**Accuracy:** The preanalyzed plasma samples containing 100 ng/0.5 mL were added with known quantity of rofecoxib (50 or 100 ng) and subjected to the proposed HPLC method, in triplicate. The difference in the measured concentration and that of the added quantity (50 or 100 ng/0.5 mL) was expressed in terms of per cent recovery.

## RESULTS AND DISCUSSION

The run time of the method was set at 12 min and rofecoxib and nimesulide (internal standard) appeared on the chromatogram in about 7.95 min and 3.6 min respectively (Fig. 1). When the same drug solution was injected 6 times, the retention time of the drug and internal standard were same. This indicates that the proposed HPLC method is rapid, which in turn shows that the method consumes fewer amounts of expensive HPLC solvents. Table-1 shows the mean peak area ratios of rofecoxib solutions for 5 such determinations. When the concentration of rofecoxib and its respective peak area ratios were subjected to regression analysis by least squares method, a high correlation coefficient was observed ($r = 0.9999 \pm 0.078$) in the range of 10 to 400 ng/0.5 mL only. However, the minimum quantifiable concentration was found to be 5 ng/0.5 mL. The regression of rofecoxib concentration over its peak area ratio was found to be $Y = -0.01108 + 1.03363X$ where 'Y' is the peak area ratio and 'X' is the concentration of rofecoxib. This regression equation was used to estimate the amount of rofecoxib either in plasma or in validation study (precision and accuracy).

TABLE- 1
CALIBRATION AND PRECISION OF THE HPLC ASSAY

| Amount of rofecoxib added to 0.5 mL human plasma (ng) | Peak-area ratio* | C.V. (%) |
|---|---|---|
| 0 | 0 | 0 |
| 5 | 0.26400 | 2.58 |
| 10 | 0.58653 | 2.46 |
| 20 | 1.26643 | 1.92 |
| 50 | 2.90408 | 0.95 |
| 100 | 6.00771 | 0.73 |
| 200 | 12.60326 | 0.62 |
| 400 | 25.80539 | 0.51 |

*Mean of five determinations
Regression equation: $Y = -0.01108 + 1.03363X$ ($r = 0.9998$)

The proposed HPLC method was also validated for intra- and inter-day variation. To assess the assay recovery from plasma by extraction procedure, the drug (10, 50, 200 ng/mL) was added to drug free plasma along with 0.5 g/0.5 mL for nimesulide solution (internal standard). The plasma samples were extracted as per the procedure described above, and the resultant filtrate samples were

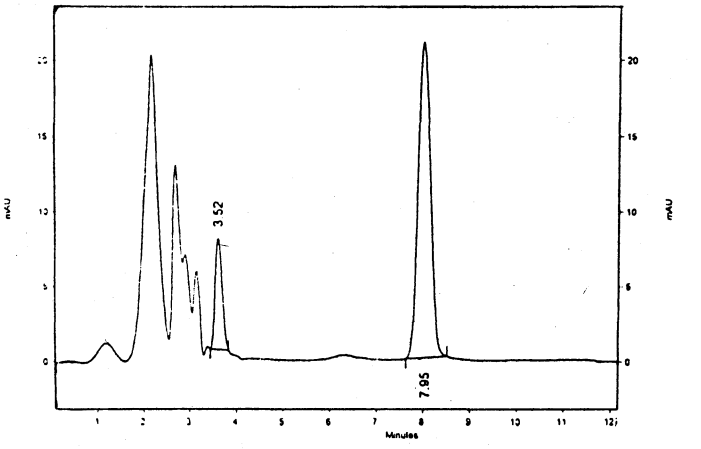


Fig. 1. A typical chromatogram of rofecoxib in human plasma

repeatedly injected on the same day. The coefficient of variation (CV) in the peak area ratio of the drug for five replicates was found to be less than 2%. Also, the inter-day variation (3 days and 5 injections) was found to be less than 2.3%. Thus the results show the proposed method is highly reproducible. when a known amount of drug solution (50 or 100 ng ) was added to plasma containing rofecoxib solution (100 ng/0.5 mL), there was a high recovery (95.66 ± 1.26) of rofecoxib indicating that the proposed HPLC method is highly accurate. The results of the study show that the present HPLC method is simple, rapid, sensitive, precise and accurate for the estimation of rofecoxib in human plasma and could be used for biopharmaceutical and pharmacokinetic evaluation of rofecoxib products.

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