The volume in each test tube was adjusted to 10 ml with distilled water. The absorbance of the solutions was measured at 253 nm against distilled water as a blank and a calibration curve was constructed. Similarly, absorbance of sample solution was measured and the amount of acyclovir was determined by referring to the calibration curve.

Recovery studies were carried out by adding a known quantity of pure drug to the pre-analyzed formulation and the proposed method was followed. From the amount of drug found, percentage recovery was calculated.

The proposed method of determination of acyclovir showed molar absorptivity of $1.3733 \times 10^4$ l/mol×cm and Sandell's sensitivity of $0.01642$ µg/cm$^2$/0.001-absorbance unit.

Linear regression of absorbance on concentration gave the equation $y=0.0609x+0.003$ with a correlation coefficient of 0.9997. Relative standard deviation of 0.00134 was observed for analysis of five replicate samples, indicating precision and reproducibility.

Acyclovir exhibits its maximum absorption at 253 nm and obeys Beer's law 2-20 µg/ml. The results of analysis and recovery studies are presented in Table 1. The percentage recovery value 98-100% indicates that there is no interference from the excipients present in the formulation. The developed method was found to be sensitive, accurate, precise and reproducible and can be used for the routine quality control analysis of acyclovir in bulk drug and formulations.

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**A New Liquid-Liquid Extraction Method for Determination of Montelukast in Small Volume Human Plasma Samples Using HPLC with Fluorescence Detector**

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Montelukast is a potent orally active cysteinyl leukotriene receptor antagonist that significantly improves parameters of asthmatics. A new liquid-liquid extraction-based reverse phase liquid chromatography method has been developed and subsequently validated for the determination of montelukast in human plasma. The separation was achieved with C$_8$ column ($150 \times 4.6$ mm, 5 micron) and a mobile phase comprising of a mixture of 10 mM ammonium acetate buffer (pH 3.0) and acetonitrile in a ratio of 35:65 v/v. Montelukast was extracted from human plasma using a liquid-liquid extraction technique with tert-butylmethyl ether. The limit of detection and lowest limit of quantification were 5 and 10 ng/ml respectively. This method was found to be linear over the range of 10 to 1000 ng/ml with a recovery of 53 to 62%. Intraday and interday precision (% CV) was <15% and accuracy ranged from 96.23 to 108.39%. Stability studies showed that montelukast in human plasma is stable during the short-term period of sample preparation and analysis. This method can be used with small volume sample during pharmacokinetic studies.

Montelukast ([R-(E)]-[1-[[1-3-[2-(7-chloro-2-quinolinyl)ethyl]phenyl]-3-[2-(1-hydroxy-1-methylethyl)phenyl]propyl][thio]methyl]cyclo propane acetic acid, fig. 1) is a selective and orally active cysteinyl leukotriene (CysLT$_1$) receptor antagonist. It is used for prophylaxis and chronic treatment of asthma$^{1-3}$. Few liquid chromatographic methods with UV detection$^4$, fluorescence detection$^5$, stereoselective determination of S-enantiomer of montelukast on column switching technique with fluorescence detection$^6$; and 96 well-based extraction methods$^7,8$ are reported for the assay of montelukast in human plasma. The reported methods are based on either protein precipitation techniques, which are preliminary in nature and nonspecific$^9$ or solid phase extraction technique. Emphasis has been laid on the reduction of blood sample volume during analysis and hence less amount of sample withdrawal is required during kinetics-related studies. We report a new liquid-liquid extraction based simple, economic and sensitive HPLC method with fluorescence detection to determine montelukast in human plasma with small plasma sample volume.

Montelukast sodium (Morepen Laboratories Ltd., New Delhi). Blank human plasma (Prathma Blood Bank, Ahmedabad). Quinine sulphate (internal standard), ammonium acetate and sodium bicarbonate (S. D. Fine Chem. Limited, Mumbai). All chromatography solvents were of HPLC grade.

The HPLC system consisted of isocratic pump (Jasco PU-980), autosampler (Jasco AS-950-10) and fluorescence detector (Jasco FP-920). Chromatographic separation was achieved by using Kromasil RP-8, $150 \times 4.6$ mm, 5 micron column (Flexit Jour, Pune). A mixture of 10 mM ammonium acetate buffer (pH 3.0) and acetonitrile (35:65, v/v) was used as mobile phase.

The mixture consisting of 10 mM ammonium acetate buffer (pH 3.0) and acetonitrile (35:65, v/v) was found to be an appropriate mobile phase, allowing adequate separation of drug and the internal standard using a C$_8$ column at a flow rate of 1.0 ml/min. The fluorescence detection was done at excitation and emission wavelengths of 350 and 400 nm respectively. The detector was set on programmed mode, which showed no interference from the endogenous polar impurities (Table 1).

Stock solutions of drug and internal standard (100 µg/ml) were prepared in methanol and stored at 4°C. The working standards of 1 µg/ml and 100 ng/ml were prepared and used within 2 w from the date of preparation.

To prepare quality control (QC) samples, appropriate volumes from working solutions of montelukast were transferred to 50 ml flask and diluted to the mark with blank human plasma and thoroughly mixed. The

**TABLE 1: SUMMARY OF METHOD AND VALIDATION PARAMETERS**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity</td>
<td>10 - 1000 ng/ml</td>
</tr>
<tr>
<td>Correlation coefficient (r)</td>
<td>0.99896</td>
</tr>
<tr>
<td>Recovery</td>
<td>53-62%</td>
</tr>
<tr>
<td>Limit of detection</td>
<td>5 ng/ml</td>
</tr>
<tr>
<td>Lowest limit of quantification</td>
<td>10 ng/ml</td>
</tr>
<tr>
<td>Programme mode</td>
<td></td>
</tr>
<tr>
<td>Excitation</td>
<td>350 nm</td>
</tr>
<tr>
<td>Emission</td>
<td>400 nm</td>
</tr>
<tr>
<td>Attenuation</td>
<td>128 (For internal standard)</td>
</tr>
<tr>
<td></td>
<td>8 (For montelukast)</td>
</tr>
<tr>
<td>Gain</td>
<td>8</td>
</tr>
<tr>
<td>Retention time</td>
<td></td>
</tr>
<tr>
<td>Internal standard</td>
<td>1.9 min</td>
</tr>
<tr>
<td>Montelukast</td>
<td>12.0 min</td>
</tr>
<tr>
<td>Total run</td>
<td>15.0 min</td>
</tr>
</tbody>
</table>

![Fig. 1: Structure of montelukast](image)
concentrations of montelukast were 30, 150 and 800 ng/ml in blank human plasma to represent low, middle and high controls (HQC, MQC and LQC) respectively. The QC samples were stored at -80°C.

Fifty microlitres of internal standard (10 µg/ml of quinine sulphate in methanol) was added to each 200 µl of human plasma samples and vortexed for 10 s. Saturated solution of sodium bicarbonate (50 µl) was added to it, followed by vortexing for 10 s. Ter-butylmethylether (2 ml) was added, followed by vortexing for 2 min and centrifuged at 5000 g for 5 min. Organic layer was transferred to another set of labelled test tubes. Organic layer was evaporated under nitrogen. Residue was dissolved in 150 µl of mobile phase and 100 µl of this sample was injected for analysis.

Calibration curves were prepared by adding known amounts of montelukast (2, 4, 8, 15, 50, 100 and 200 ng) to 200 µl of blank human plasma. An aliquot of 50 µl of the internal standard in methanol (10 µg/ml) was added to each sample. The samples were extracted as described above. The standard curves were constructed by plotting ‘the peak area ratio of montelukast to that of internal standard’ versus ‘their respective concentrations.’ The calibration curves were obtained by least square linear regression analysis. The calibration curves showed linearity over the range of 10-1000 ng/ml for montelukast. The coefficient of correlation was found to be more than 0.99.

For intraday precision and accuracy, six replicates of QC plasma samples of montelukast (6, 30 and 160 ng/200 µl) were extracted as described above and the concentrations were calculated from the standard curve; while for interday precision and accuracy, six replicates of QC plasma samples of montelukast (6, 30 and 160 ng/200 µl) were analysed on three consecutive days, along with the standard calibration curves. Results of intraday, interday precision and accuracy are shown in Table 2.

In order to calculate recovery of the extraction procedure, six QC plasma samples of montelukast (800 and 150 ng/ml) were analysed and the peak area ratios of montelukast to that of internal standard were compared with those of unextracted montelukast in mobile phase (Table 1).

For bench top stability, three replicates of montelukast (HQC, MQC and LQC) were analysed at 0 and 24 h at room temperature; while for autosampler stability, three replicates of montelukast (HQC, MQC and LQC) were analysed at 0 and 24 h at room temperature; and for freeze-thaw stability, three replicates of montelukast (HQC, MQC and LQC) were frozen at -20° and analysed after 1, 2, 3 and 4 freeze-thaw cycles. The stability

### Table 2: Assay Precision and Accuracy of Montelukast

<table>
<thead>
<tr>
<th>Amount added (ng/ml)</th>
<th>N</th>
<th>SD (±)</th>
<th>% CV</th>
<th>Inter day (ng/200 µl)</th>
<th>N</th>
<th>SD (±)</th>
<th>% CV</th>
<th>Accuracy*</th>
</tr>
</thead>
<tbody>
<tr>
<td>800.00</td>
<td>6</td>
<td>0.03</td>
<td>4.37</td>
<td>18</td>
<td>0.19</td>
<td>4.21</td>
<td>108.39</td>
<td></td>
</tr>
<tr>
<td>150.00</td>
<td>6</td>
<td>0.05</td>
<td>6.39</td>
<td>18</td>
<td>0.02</td>
<td>3.03</td>
<td>96.33</td>
<td></td>
</tr>
<tr>
<td>30.00</td>
<td>6</td>
<td>0.01</td>
<td>8.10</td>
<td>18</td>
<td>0.04</td>
<td>15.20</td>
<td>108.33</td>
<td></td>
</tr>
</tbody>
</table>

*Mean of six replicates

For bench top stability, three replicates of montelukast (HQC, MQC and LQC) were analysed at 0 and 24 h at room temperature; while for autosampler stability, three replicates of montelukast (HQC, MQC and LQC) were analysed at 0 and 24 h at room temperature; and for freeze-thaw stability, three replicates of montelukast (HQC, MQC and LQC) were frozen at -20° and analysed after 1, 2, 3 and 4 freeze-thaw cycles. The stability
studies showed that montelukast in human plasma are stable during short-term periods of sample preparation and analysis.

A typical HPLC chromatogram of extracted sample of human plasma spiked with 30 ng/ml of montelukast with internal standard and blank human plasma is illustrated in fig. 2. The retention times of internal standard and montelukast were 1.9 min and 12.0 min respectively with total run time of 20 min.

A new liquid-liquid extraction based method has been developed and subsequently validated for the determination of montelukast in human plasma with small plasma sample volume with fluorescence detection. This method has been found to be specific and cost-effective and can be used for quantitative analysis of montelukast during bioavailability/bioequivalence studies and clinical trials.

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