A simple, rapid, accurate and precise high performance liquid chromatography (HPLC) method for simultaneous analysis of Paclitaxel and Topotecan was developed. Different analytical parameters, such as linearity, accuracy, precision, specificity with intentional degradation, limit of detection and limit of quantification (LOQ), were determined according to the ICH guidelines. Acetonitrile–water (70:30, 0.1% trifluoroacetic acid) was run on a Phenomenex Luna C-18(2) column in isocratic mode at a flow rate of 1.2 mL/min for simultaneous analysis of the two drugs using a UV detector set at 227 nm. The proposed method showed a retention time ($R_t$) of 14.56 min for Topotecan and 23.81 min for Paclitaxel with a continuous run up to 30 min. The linearity of the calibration curves for each analyte in the desired concentration range was found to be good ($r^2 > 0.9995$). The recovery ranged from 97.9 to 101% for each drug with a relative standard deviation (%RSD) of <2%. Peaks corresponding to each of the drugs exhibited positive values for the minimum peak purity index over the entire range of integrated chromatographic peak indicating high purity of the peaks. Stability analysis revealed that the drugs remained stable for sufficient time. Thus, the developed method was found to be robust and it can be employed to quantify Paclitaxel and Topotecan in commercial sample and rat blood/serum.

Introduction

A combination of Paclitaxel and Topotecan is preferred to single-drug therapy for the treatment of cancer, as it reduces the tumor regression as well as surpresses drug resistance. Paclitaxel and Topotecan have few overlapping toxicities and different mechanisms of action (1–6).

Paclitaxel isolated from Taxus brevifolia, the Western Yew tree, is a hydrophobic drug with a molecular weight of 853.92 g/mol (Figure 1). It is a BCS class IV drug having extremely low solubility (i.e., 4 μg/mL) (7). Paclitaxel is an FDA-approved drug for the treatment of breast and ovarian cancers as well as lumps and AIDS-related Kaposi’s sarcoma (8). Topotecan, a camptothecin analog, on the contrary, is a hydrophilic drug with a molecular weight of 421.451 g/mol having solubility in water up to 1 mg/mL (Figure 2). Topotecan is an inhibitor of DNA topoisomerase I (9–11). It is most active against ovarian and small-cell lung cancer (SCLC), but it is also used to treat hematological malignancies, particularly chronic myelomonocytic leukemia and myelodysplastic syndrome (10, 12, 13). This combination is active against recurrent ovarian carcinoma (14–16), breast cancer (17), cervical tumors (2, 18–20) and SCLC (3).

Different methods have been explored and employed for the determination of Paclitaxel, viz. capillary electrophoresis (21), LC-MS (5) and high performance liquid chromatography (HPLC) (6, 22). Similarly, for Topotecan, the study of lactone ring hydrolysis (23–25) and its estimation using HPLC (26, 27), HPLC with a fluorescence detector (28) and ELISA (29) have been reported in the literature. There are a number of analytical methods discussed in the literature to estimate Paclitaxel and Topotecan alone but no simple and accurate method has been developed so far for the estimation of these drugs in combination. In the present study, a simple, accurate and precise HPLC method was developed for simultaneous estimation of Paclitaxel and Topotecan. Analytical methods have been developed and validated as per the ICH guidelines for analysis of both the drugs in combination.

Experimental

Chemical and reagents

Topotecan and Paclitaxel were generously provided as a gift sample by Fresenius Kabi Oncology Ltd, Haryana, India. All solvents were of the HPLC grade and were filtered and degassed before use.

Preparation of standard solutions

Standard solution containing a combination of Paclitaxel (50 μg/mL) and Topotecan (50 μg/mL) was prepared in acetonitrile (ACN) and stored in an amber-colored flask at 4–6°C. Appropriate dilutions of the standard solution were made in ACN to produce solutions in the range 0.025–2 μg/mL. Samples for the determination of recovery, precision and accuracy were also prepared by spiking control in appropriate concentrations (i.e., 1, 10 and 50 μg/mL) and stored at −20°C.

Apparatus and chromatographic conditions

The analytical technique was developed using Shimadzu (Kyoto, Japan) HPLC equipment, Model-SPD-M20A, fitted with a Phenomenex Luna C-18(2) column (4.6 × 250 mm, dp = 5 μm; Hyderabad, India). The mobile phase consisted of a mixture of ACN and purified water (W) containing 0.1% trifluoroacetic acid (TFA) in the ratio 70:30. The pH of the mobile phase was adjusted to 5 with acetic acid (30), filtered through a 0.22-μm nylon filter and degassed using ultrasonic bath sonicator for 60 min before running the experiment. All experiments conducted on the HPLC were carried out in isocratic mode. Injection volume was 20 μL with a flow rate of 1 mL/min. The
column temperature was maintained at 25°C and elution was monitored at 227 nm using a Photo diode array detector. All chromatographic data were acquired and processed with the Lab Solutions software (31–39).

**Solution state stability testing**
Stability testing was carried out to evaluate the stability and extent of degradation of the stock solution containing both the drugs in ACN. Fresh stock solution containing Topotecan (50 mg/mL) and Paclitaxel (50 mg/mL) was prepared and then working solutions at three concentration levels were made from this standard solution and kept at 4–6°C. Sampling was done at regular time intervals for a period of 7 days in triplicate. Each sample was run in HPLC after filtering through a 0.22-μm filter. The peak areas of the individual drugs were compared at different time points to determine the stability as a function of time.

**Peak purity assessment**
Peak purity was assessed using the class VP software for Shimadzu HPLC system based on the degree of similarity of UV spectra across the peak in the range 190–800 nm. Peak purity evaluation was performed with the objective of obtaining additional supportive information during the selection of appropriate analytical conditions that allowed specific determination of both Topotecan and Paclitaxel. The peak was classified as pure if the peak purity index was greater than the single point threshold resulting in a positive value of the minimum peak purity index.

**Validation of the analytical method**
The developed method was validated as per the ICH guidelines for linearity, accuracy and precision and specificity (40, 41). Limit of detection (LOD) and limit of quantification (LOQ) were determined using the serial dilution method.

**Linearity**
The linearity of the method used for Topotecan and Paclitaxel analysis was evaluated from the standard curve of detector response (peak area) against analyte concentration. The concentration range was selected on the basis of anticipated drug concentration in the release study samples and 8-point calibration curves were generated on 3 consecutive days with standard working solutions of their combination. The solutions were injected in triplicate into the HPLC column. The linearity of the analytical procedure was evaluated by plotting detector response (the peak area) against analyte concentration. Linear regression analysis was carried out to calculate the slope, intercept and linear correlation coefficient ($r^2$).

**Accuracy and precision**
Accuracy and precision of the analytical method was determined by analyzing quality control (QC) samples at three different concentrations within the calibration range in triplicate ($n = 3$). QC standards were prepared in the same media and were independent of those used for the preparation of calibration curves. The precision (%RSD) of the analytical procedure was evaluated by determining the intra- and inter-day coefficients of variation and reported as %RSD for a statistically significant number of replicate measurements. The intra-day precision of the selected method was estimated by analyzing samples in the same way as for the intra-day precision assay and was repeated for 3 consecutive days.

**Specificity**
Specificity is the ability of the analytical method to measure accurately and specifically the analyte of interest in the presence of other components that might be expected to be present in the sample. Specificity of the analytical method was evaluated for both the drugs in combination. Assessments were based on quantification limits and forced degradation studies.

**Quantification limits**
LOD and LOQ decide about the sensitivity of the method. LOD is the lowest detectable concentration of the analyte, whereas LOQ is the lowest amount of the analyte in a sample, which could be quantitatively determined with suitable precision and accuracy. LOQ was assessed by the standard deviation of the response and the slope method. Slope $S$ was calculated from the calibration curve of the analyte and the standard deviation was
estimated by running five blank samples while LOD was taken as the one-third of LOQ for their simultaneous analysis, LOQ and LOD were estimated by the serial dilution method.

**Forced degradation studies**

Forced degradation studies were also carried out for Pac-Top to provide an indication of the stability indicating property and specificity of the proposed method. Intentional degradation was attempted to stress conditions, that is, acid hydrolysis (0.5 N HCl/60°C/1 h), base hydrolysis (0.5 N NaOH/60°C/1 h), oxidation (3% H2O2/60°C/1 h), thermal (60°C/48 h) and photo stability (UV 254 nm/48 h). The peak purity test was carried out for Pac-Top peaks by using the PDA detector in stress samples.

**Application of the method**

Estimation of Paclitaxel–Topotecan (20:1, w/w) in serum from rats (n = 3) was performed. Blood free from drugs was collected from rats, after a 30-min coagulation period at room temperature, and the serum was separated out by centrifugation at 1145 × g for 5 min. Pac-Top in DMSO (0.5 mg/mL) was diluted 10 times with serum to yield serum samples (n = 3) at a concentration of 500 ng/mL. Serum was equilibrated at 37°C for 20 min; 500 μL of cold methanol (−20°C) was added to 500 μL of the serum sample in a micro tube. The mixture was vortex-mixed for 30 s. After centrifugation at 5500 × g for 10 min, the supernatant was separated and filtered. Filtrate was injected into the HPLC column immediately.

**Results**

**Chromatographic separation**

The Topotecan showed a retention time of 14.3 min, which was well separated from the peak of Paclitaxel with a retention time of 23.9 min. The isocratic mode was employed for the elution of these drugs. Nevertheless, the drugs got eluted within 24 min; the run was further continued for 6 min to ensure the complete removal of traces of drugs from the column and to re-equilibrate the system to initial conditions. Figure 3 illustrates the complete chromatogram generated over 30 min, which shows the peaks of both the drugs. Figure 4 shows the graph of standard curves of Paclitaxel–Topotecan obtained in the range 0.025–2 μg/mL.

**Stability of stock solutions**

Table I represents stability data of the stock solution containing both the drugs. Chromatograms obtained by running three concentrations on the 3rd and the 7th days from the preparation of stock solution have been compared with those obtained initially. Values given under day 3 and day 7 denote the peak area ± SD (%RSD) calculated with respect to the average peak area of the respective concentrations as obtained initially.

**Ruggedness/ robustness testing**

The ruggedness/robustness of the method was checked after deliberately altering the following parameters: composition of the mobile phase, mobile phase flow rate, injection volume, column temperature and detector wavelength (Table II). The parameters of chromatographic separation [retention time, relative retention time (RRT), resolution and number of plates] were not much different on varying the operational parameters.

**Validation of the method**

Validation parameters have been highlighted in Table III for their simultaneous analysis. Purity of the peaks corresponding to the drugs in the method was also established as an additional proof of specificity (Figure 5). Each standard curve was generated in triplicate on 3 consecutive days distributed evenly across the linearity range. Values are reported as mean ± SD of three calibration curves. Accuracy and precision data showed that the recoveries ranged from 99 to 101% for Topotecan as well as for Paclitaxel. Both intra- and inter-day precision (%RSD) of QC standards were less than 2% over the selected range for both the drugs (Table IV).
Accuracy and precision were determined with QC samples. Triplicate samples were analyzed on 3 consecutive days. For intra-day determinations, three standard curves were prepared on the same day. For inter-day determinations, three standard curves were generated on three consecutive days. Accuracy is represented by percent recovery (mean ± SD) and precision by percent RSD.

Specificity evaluation was carried out by analyzing Paclitaxel and Topotecan in combination. Recovery of both drugs from solutions prepared in ACN–W with 0.1% TFA was accessed at three concentration levels in triplicate (Table V). Results of intentional degradation have been summarized in Table VI.

Discussions

Chromatographic separation

Different mobile phase solvents have been reported in literature at various compositions for the separation of Paclitaxel and Topotecan (31–39). Here, Paclitaxel and Topotecan were separated on the ODS column using the mobile phase consisting of ACN:water containing 0.1% TFA in the ratio 70:30. The isocratic mode was employed for the elution of these drugs. Nevertheless, the drugs got eluted within 24 min; the run was further continued for 6 min to ensure the complete removal of traces of drugs from the column and to re-equilibrate the system to initial conditions.

Stability of stock solutions

The stock solution of the combination was found to be stable for 1 week as percent recovery was within statistical limits. Further,
Figure 5. Peak purity curves of: (a) Paclitaxel in simultaneous analysis and (b) Topotecan in simultaneous analysis. Each of the peaks has a positive value of the minimum peak purity index confirming the purity of the peaks and the specificity of the method developed.

### Table IV
Accuracy and Precision Studies for Simultaneous Analysis of Paclitaxel–Topotecan

<table>
<thead>
<tr>
<th>Drug concentration (ng/mL)</th>
<th>Inter-day</th>
<th>Intra-day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>750</td>
<td>650</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precision (%RSD)</td>
<td>1.37 ± 0.41</td>
<td>1.46 ± 0.21</td>
</tr>
<tr>
<td>Accuracy (%recovery)</td>
<td>99.98 ± 1.27</td>
<td>99.42 ± 1.56</td>
</tr>
<tr>
<td>Topotecan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precision (%RSD)</td>
<td>1.18 ± 0.48</td>
<td>0.95 ± 0.71</td>
</tr>
<tr>
<td>Accuracy (%recovery)</td>
<td>100.17 ± 1.18</td>
<td>100.01 ± 0.15</td>
</tr>
</tbody>
</table>
no appreciable change was observed in the measured concentration of the drugs during the period. Recovery and %RSD are seen to be within statistical limits. Hence, the solutions remain stable over a period of 7 days at 4–6°C.

**Ruggedness/robustness testing**

The ruggedness/robustness testing of the method with deliberate alterations in parameters, viz. composition of the mobile phase, mobile phase flow rate, injection volume, column temperature and detector wavelength, resulted in acceptable recoveries of Paclitaxel and Topotecan, i.e., more than 96%. The parameters of chromatographic separation (retention time, RRT, resolution and number of plates) were not much different on varying the operational parameters.

**Validation of the method**

The method developed for simultaneous analysis of Topotecan and Paclitaxel was validated for analytical performance parameters such as linearity, accuracy, precision, specificity and quantification limits as per the ICH guidelines. Linear regression analysis confirmed that the $r^2$ values for both drugs were found to be >0.9995, confirming the linear relationship between the concentration of the drug and the area under the curve.

The calculated LOD and LOQ concentrations confirmed that the methods were sufficiently sensitive. Specificity evaluation was carried out by analyzing Paclitaxel and Topotecan in combination. It was observed that the peaks of each of the drugs were well separated and not being interfered. It was found that recoveries of both Topotecan and Paclitaxel were within statistical limits under different stress conditions tested.

Further, peaks corresponding to each of the drugs obtained by the proposed method were seen to be pure. Thus, the method is confirmed to be specific to each of the two drugs in combination. Hence, the method can be suitably employed for quantitative analysis of a combination of Paclitaxel and Topotecan in the samples.

### Table V

**Results of Specificity Studies**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Actual concentration (ng/mL)</th>
<th>Calculated concentration (ng/mL)</th>
<th>%Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Topotecan</td>
<td>750</td>
<td>749.62 ± 9.520 (1.27)</td>
<td>99.9499</td>
</tr>
<tr>
<td></td>
<td>550</td>
<td>550.91 ± 5.398 (0.98)</td>
<td>100.166</td>
</tr>
<tr>
<td></td>
<td>350</td>
<td>349.89 ± 0.664 (0.19)</td>
<td>99.96975</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>750</td>
<td>750.98 ± 6.158 (0.82)</td>
<td>100.131</td>
</tr>
<tr>
<td></td>
<td>550</td>
<td>549.02 ± 5.215 (0.95)</td>
<td>99.82239</td>
</tr>
<tr>
<td></td>
<td>350</td>
<td>348.96 ± 5.0599 (1.45)</td>
<td>99.70424</td>
</tr>
</tbody>
</table>

### Table VI

**Analysis of the Forced Degradation Study of Samples**

<table>
<thead>
<tr>
<th>Stress conditions/duration</th>
<th>Degradation ($n=3$, mean ± SD)</th>
<th>Peak purity data*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Paclitaxel</td>
<td>Topotecan</td>
</tr>
<tr>
<td>Acid hydrolysis (0.5 N HCl/60°C/1 h)</td>
<td>4.83 ± 0.19</td>
<td>3.71 ± 0.26</td>
</tr>
<tr>
<td>Base hydrolysis (0.5 N NaOH/60°C/1 h)</td>
<td>5.42 ± 0.37</td>
<td>7.54 ± 0.13</td>
</tr>
<tr>
<td>Oxidation (3% H2O2/60°C/1 h)</td>
<td>8.10 ± 0.17</td>
<td>6.38 ± 0.09</td>
</tr>
<tr>
<td>Thermal (60°C/48 h)</td>
<td>0.46 ± 0.08</td>
<td>1.1 ± 0.53</td>
</tr>
<tr>
<td>Photo (UV 254 nm/48 h)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Peak purity values in the range of 980–1000 indicate a homogeneous peak.

### Conclusion

The HPLC method was developed for simultaneous estimation of Paclitaxel and Topotecan in various samples, viz. commercial sample and rat blood/serum. The developed method is simple, rapid and reliable enough employed for analysis of the two drugs simultaneously using the mobile phase, i.e., ACN and water (with 0.1% TFA). Run time for Topotecan (14.3 min) ensures its rapid estimation without any interference from Paclitaxel. Paclitaxel elutes out in 23.9 min with a total run time of 30 min, which seems to be reasonable with the reported run time. Gradient program has not been designed, since step gradient is known to give sudden shock and stress to the column diminishing its life. Validation report confirms that the method has good linearity, accuracy, precision, adequate specificity and purity, and it can be employed to find out the concentration of Paclitaxel and Topotecan in commercial samples, various dosage forms and rat blood/plasma.

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We thank Dr Dhiraj Khattar, Director, Formulation Development & Delivery Systems, Fresenius Kabi Oncology Limited, Haryana, India, for rendering gift samples of Paclitaxel and Topotecan for analytical work.

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