Validated Reverse Phase HPLC Method for the Determination of Irinotecan in Pharmaceutical Dosage Forms

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Received 30 August 2006; Accepted 15 October 2006

Abstract: A simple and rapid reverse phase high performance liquid chromatography (RP-HPLC) method was developed and validated for quantitative determination of irinotecan in bulk drug samples and formulations. Irinotecan was analyzed by using reverse phase cyano column (4.6 mm x 25 cm, 5 microns), with mobile phase consisting of phosphate buffer: acetonitrile (75:25 v/v), pH adjusted to 2.5 with phosphoric acid. The flow rate was set 0.8 ml/min and the analysis was performed at wavelength 225 nm using Photo Diode Array (PDA) detector at ambient temperature. The method was validated and stability studies were conducted under different conditions. The retention time for irinotecan was around 5.82 minutes. The calibration curves were linear ($r \geq 0.9998$) over a concentration range from 20.0 to 80.0 µg/ml. Limit of detection (LOD) and Limit of quantitation (LOQ) were 8 ng/ml and 24 ng/ml respectively. The developed method was successfully applied to estimate the amount of irinotecan in injection formulations.

Keywords: RP-HPLC, PDA, Irinotecan
Introduction

Irinotecan (IRT) is an antineoplastic agent primarily used in the treatment of metastatic colorectal cancer. It is chemically (S)-10-[4-(-piperidino)piperidinocarbonyl oxyyl]-4,7-diethyl - 4-hydroxy -1H-pyran [3,4;6,7] indolizin [1,2-b]diethyl-3,14[4H,12H]-dionemono hydrochloride trihydrate (Fig.1). The drug is official in Martindale, The Extra Pharmacopoeia. It is a semisynthetic, water-soluble derivative of camptothecin, which is a cytotoxic alkaloid extracted from plants such as Camptotheca acuminata. Irinotecan and its active metabolite, SN-38, inhibit the action of topoisomerase I, an enzyme that produces reversible single-strand breaks in DNA during DNA replication. These single-strand breaks relieve torsional strain and allow DNA replication to proceed. Irinotecan and SN-38 bind to the topoisomerase I-DNA complex and prevent relegation of the DNA strand, resulting in double-strand DNA breakage and cell death. Irinotecan is cell cycle phase-specific (S-phase).

![Figure 1. Chemical Structure of Irinotecan](image)

Irinotecan is chemically (S)-10-[4-(-piperidino)piperidinocarbonyl oxyyl]-4,7-diethyl-4-hydroxy-1H-pyran[3,4;6,7]indolizin[1,2-b]diethyl-3,14[4H,12H]-dione.

Few HPLC methods for quantitative determination of irinotecan were reported in literature. Majority of these HPLC methods were applied in the determination of irinotecan and it’s metabolites in biological fluids and are mainly useful for therapeutic monitoring of irinotecan. No validated HPLC methods for quantitative determination of irinotecan in bulk drug samples and formulations were reported till date. In order to minimize batch-to-batch variation there is an immense need for developing a rapid, sensitive and validated analytical method for day-to-day analysis of the drug in pharmaceutical dosage forms.

Experimental

Chemicals and reagents

Irinotecan bulk drug and formulations (Irinogen) were kind gifts from BGSS Pharma, Argentina. Acetonitrile (HPLC grade) was obtained from Merck, Mumbai. All other chemicals were of analytical grade and were purchased from Qualigens, Mumbai. HPLC grade deionised water (Nanopure Diamond Barnstead Thermolyne, USA) was used throughout the analysis.

Instrumentation

The HPLC system consisted of a Waters Alliance (Waters Corporation, MA, USA) equipped with a Waters 2695 solvent delivery module in a quaternary gradient mode and a Waters
Validated Reverse Phase HPLC Method

2669 PDA detector. Data acquisition was performed by the Millennium 32® bit software operated on a Pentium® IV microprocessor. Analysis was carried out at 225 nm with a reversed phase Cyano column, (GROM-SIL-120, 250x4.0 mm, 5 µm) at ambient temperature. The mobile phase consisted of phosphate buffer: acetonitrile (75:25 v/v) and pH adjusted to 2.5 with phosphoric acid that was set at a flow rate of 0.8 ml/min. The mobile phase was degassed and filtered through 0.2 µm membrane filter before pumping into HPLC system.

**Preparation of solutions**

**Preparation of drug stock solution**
The stock solution of Irinotecan was prepared by dissolving accurately weighed quantity of 10 mg of the drug in 10 ml of deionised water (final concentration, 1 mg/ml). From this stock solution, standard solution containing 100 µg/ml irinotecan was prepared by suitably diluting the appropriate volume of stock solution with mobile phase.

**Calibration standards and quality control samples**
Different calibration standards ranging from 20, 30, 40, 60 and 80 µg/ml were prepared by appropriate dilution of standard solution (100 µg/ml) with mobile phase. Three quality control samples at concentrations of 28, 40 and 52 µg/ml representing 70,100 and 130% respectively of assay concentration (40 µg/ml) were prepared from the standard solution. An aliquot of 20 µl of solution was injected into HPLC system.

**Preparation of assay solution**
An accurately measured volume of injection, equivalent to 20 mg of Irinotecan (1 ml), was transferred to a 50-ml volumetric flask, diluted with water to volume and mixed. A volume of 5.0 ml of this solution was pipetted to another 50-ml volumetric flask, diluted to volume with mobile phase and mixed (final Concentration of 0.04 mg/ml) and 20 µl of this solution was injected into the HPLC system.

**Method validation**

**System suitability**
The system suitability was assessed by replicate analysis of six injections of the drug at a concentration of 40 µg/ml. The acceptance criterion was ± 2% for the percent coefficient of variation (%CV) for the peak area and retention times for irinotecan. The number of theoretical plates should not be less than 2500 and the tailing factor should not be more than 2.0.

**Determination of limit of detection & limit of quantitation (Sensitivity)**
A series of dilute solutions were prepared in the range of 0.2%, 0.5% and 1.0% of the assay concentration (40µg /ml) using the standard solutions. Equal volumes (20 µl) of each of the above solutions were injected in 5 times and the areas were calculated due to Irinotecan peak. The standard deviation for the 5 injections for each concentration was calculated. Based on the data obtained, the standard deviation at concentration 0 was calculated and this value was used for the calculation of the Limit of Detection and Limit of Quantitation. The limits of detection (LOD) and quantification (LOQ) were calculated using the following formulae:

\[
\text{LOD} = (3.3 \sigma / S) \quad \text{and} \quad \text{LOQ} = (10 \sigma / S)
\]

Where, \(\sigma\) is the standard deviation of the response and S is the slope of the regression line \(^9,10\).
**Linearity (Calibration curve)**

The calibration curve was constructed with 5 concentrations ranging from 20 to 80 µg/ml. The peak area ratio of the drug was considered for plotting the linearity graph. The linearity was evaluated by linear regression analysis, which was calculated by the least square regression method.

**Accuracy and precision**

Accuracy of the method was carried out by recovery experiments. Quality control sample solutions of 70, 100 and 130% (of 40 µg/ml) containing the excipients used in the formulations were tested and the recovery was calculated in each of the case using the regression line equation developed under the linearity experiment.

Demonstration of precision was done under two categories. The injection reproducibility was assessed by using 6 injections of the standard solution for Irinotecan and the relative standard deviation of the replicate injection was calculated. In addition, to demonstrate method precision, 5 samples from the same batch of formulation were analyzed individually and the assay content (Irinotecan) of each sample was estimated. The average for the 5 determinations was calculated along with the RSD for the replicate determinations.

**Specificity (Forced degradation) and stability**

The specificity of the method was demonstrated through forced degradation studies conducted on the sample using Acid, Alkaline, Oxidative, Reductive and Photolytic degradations. The sample was exposed to these conditions and the main peak was studied for the peak purity (Irinotecan peak), thus indicating that the method effectively separated the degradation products from the Irinotecan active ingredient.

**Acid degradation**

About 20.0 mg of Irinotecan pure drug was accurately weighed and transferred to a 50-ml volumetric flask. About one 1 ml of 1N hydrochloric acid was added. The volumetric flask was placed on a water bath maintained at 60°C for one hour. Then it was cooled, dissolved and volume was made with water.

**Alkaline degradation**

About 20.0 mg of Irinotecan pure drug was accurately weighed and transferred to a 50-ml volumetric flask. 1 ml of 1N sodium hydroxide was added. The volumetric flask was placed on a water bath maintained at 60°C for one hour. Then it was cooled, dissolved and volume was made with water.

**Reductive degradation**

About 20.0 mg of Irinotecan pure drug was accurately weighed and transferred to a 50-ml volumetric flask. Zinc and 1 mL of 1N hydrochloric acid were added. The volumetric flask was placed on a water bath maintained at 60°C for one hour. Then it was cooled, dissolved and volume was made with water.

**Oxidative degradation**

About 20.0 mg of Irinotecan pure drug was accurately weighed and transferred to a 50-ml volumetric flask. 1 ml of hydrogen peroxide (1/20) was added, dissolved and volume was made with water.
Photolytic degradation
About 20.0 mg of Irinotecan pure drug was accurately weighed and transferred to a 50-ml volumetric flask, dissolved and volume was made with water. The solution was exposed to UV light at 254 nm for 8 hours.

In all the forced degradation studies, a volume of 5.0 ml of each of the solutions from the degradation experiments, were transferred to a 50-ml volumetric flask. The volume was made with mobile phase and mixed so as to get a final concentration of 40 µg/ml. Standard solution with final concentration of 40 µg/ml was prepared. And equal volumes of all the above 6 solutions (20 µl) were injected into the chromatograph and the chromatograms were recorded.

Stability
The solution stability was demonstrated by conducting repeated auto-sampler analysis of the standard solution at periodic intervals covering a time period of 24 hours. The areas for each of the experiment were taken and subjected to statistical analysis.

Robustness and ruggedness
The robustness/ruggedness of the method was demonstrated through the study of the following variations.
1. Column to Column Variation
2. Day to Day Variation
3. Analyst to Analyst Variation

The above three parameters Viz., Column, Day and Analyst were studied through a matrix design involving the estimation on two different days using two different columns and 2 different analysts with a total of 4 determinations. Under each of the conditions, samples were analyzed including a duplicate injection for each estimate.

Results and Discussion
Method development and optimization
Irinotecan hydrochloride is freely soluble in water and is ionizable. The drug can be separated on a cyano column as cyano column is slightly polar and it has unique selectivity for polar compounds in both reversed and normal phase modes. Ionizable compounds can best be separated with mobile phases that have a pH value less than or equal to 3.0. The optimization of the method development was done by fixing one variable constant and changing the other variables among mobile phase composition, flow rate and pH of the mobile phase. The pH of the mobile phase was found to be critical. Above the pH of 2.5, the peaks that were obtained were irregular and were asymmetric. Increasing the buffer content enhanced tailing and decreased the plate count. The peak shape and symmetry were found to be good when a mobile phase composition of 75:25 (v/v, buffer of pH 2.5: acetonitrile) was used at a flow rate of 0.8 ml/min at ambient temperature.

Method validation
System suitability
The % CV of peak area and retention time for the drug are within 2% indicating the suitability of the system (Table 1). The efficiency of the column as expressed by number of theoretical plates for the 6 replicate injections was 7734.745 ± 0.013% (mean ± %CV) and the USP tailing factor was 1.38 ± 0.013% (mean ± %CV).
Table 1. System suitability study of Irinotecan

<table>
<thead>
<tr>
<th>Injection</th>
<th>Retention Time (min)</th>
<th>Peak Area</th>
<th>Plate Count</th>
<th>Tailing Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.815</td>
<td>3570143</td>
<td>7621.68</td>
<td>1.40</td>
</tr>
<tr>
<td>2</td>
<td>5.817</td>
<td>3570846</td>
<td>7655.37</td>
<td>1.39</td>
</tr>
<tr>
<td>3</td>
<td>5.818</td>
<td>3573681</td>
<td>7673.83</td>
<td>1.39</td>
</tr>
<tr>
<td>4</td>
<td>5.855</td>
<td>3557208</td>
<td>7873.54</td>
<td>1.38</td>
</tr>
<tr>
<td>5</td>
<td>5.865</td>
<td>3558590</td>
<td>7822.38</td>
<td>1.36</td>
</tr>
<tr>
<td>6</td>
<td>5.875</td>
<td>3559391</td>
<td>7761.67</td>
<td>1.36</td>
</tr>
<tr>
<td>Mean</td>
<td>5.841</td>
<td>3564976.5</td>
<td>7734.75</td>
<td>1.38</td>
</tr>
<tr>
<td>S.D</td>
<td>0.027</td>
<td>7338.213</td>
<td>100.46</td>
<td>0.02</td>
</tr>
<tr>
<td>% CV</td>
<td>0.005</td>
<td>0.002</td>
<td>0.013</td>
<td>0.01</td>
</tr>
</tbody>
</table>

_Determination of limit of detection & limit of quantitation (Sensitivity)_

The results indicated that the method was sensitive enough to detect a concentration of 8 ng/ml and able to quantify at a concentration of above 24 ng/ml.

_Linearity_

The calibration curve constructed was evaluated by its correlation coefficient. The peak area of the drug was linear in the range of 20.0 to 80.0 μg/ml. The average areas for each of the concentration obtained were plotted against the concentration of the analyte. The correlation coefficient for the data was calculated as 0.9999 for Irinotecan indicating a strong correlation between the concentration and the area under the curve.

A linear regression graph was drawn between the Concentration of the analyte and the Areas. The regression line observed to be $y = 1E+08x – 277454$ for Irinotecan. Regression analysis was done at confidence level of 0.05 (5%) and the data presented. The data indicated that the difference between the estimated area and the actual area was not significant. These experiments indicated that there was a linear relation between the amount of analyte and the areas within the range studied (50% to 200%). The chromatogram of irinotecan extracted from the formulation and pure irinotecan spiked in the mobile phase can be observed in Fig.2

_Accuracy and precision_

Accuracy of the method was carried out by recovery experiments. Drug standard solutions of 70, 100 and 130% containing the excipients used in the formulations were tested and the recovery was calculated in each of the case using the regression line equation developed under the Linearity experiment. A regression line Graph was drawn using the amount added on the x-axis and the amount found on the y-axis. The slope and intercept were calculated for the regression line (Method of Least Squares), and hypothesis was tested for the correlation between the amount added and amount found, at confidence level of 0.05 (5%), using the t-statistic. The results revealed that there was a strong correlation between the amount added and amount found (Table 2). The results obtained from precision experiments also indicated a good method precision (Table 3).

_Specificity (Forced degradation) and stability_

The specificity of the method was demonstrated through forced degradation studies conducted on the sample using acid, alkaline, oxidative, reductive and photolytic degradations (Table 4). Under each of the conditions, the chromatogram was studied using PDA detector. Using the chromatographic software, the Purity Angle and Purity Threshold were calculated.
Figure 2: Chromatograms of Irinotecan (left to right) extracted from formulation (40 µg/ml) and standard solution (40 µg/ml)

Table 2: Accuracy - Results of recovery experiments

<table>
<thead>
<tr>
<th>Spike level</th>
<th>% Amount added</th>
<th>Amount found</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>0.026</td>
<td>0.02621</td>
<td>100.79</td>
</tr>
<tr>
<td>70</td>
<td>0.026</td>
<td>0.02632</td>
<td>101.22</td>
</tr>
<tr>
<td>70</td>
<td>0.026</td>
<td>0.02636</td>
<td>101.40</td>
</tr>
<tr>
<td>100</td>
<td>0.037</td>
<td>0.03765</td>
<td>101.76</td>
</tr>
<tr>
<td>100</td>
<td>0.037</td>
<td>0.03766</td>
<td>101.78</td>
</tr>
<tr>
<td>100</td>
<td>0.037</td>
<td>0.03767</td>
<td>101.81</td>
</tr>
<tr>
<td>130</td>
<td>0.048</td>
<td>0.04816</td>
<td>100.32</td>
</tr>
<tr>
<td>130</td>
<td>0.048</td>
<td>0.04818</td>
<td>100.37</td>
</tr>
<tr>
<td>130</td>
<td>0.048</td>
<td>0.04818</td>
<td>100.37</td>
</tr>
</tbody>
</table>

Mean 101.09
Standard Deviation 0.0064
% RSD 0.63

Table 3: Precision

<table>
<thead>
<tr>
<th>Precision</th>
<th>Injection Reproducibility</th>
<th>Method Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td>Limit</td>
<td>RSD</td>
<td>Limit</td>
</tr>
<tr>
<td>Irinotecan</td>
<td>2.0%</td>
<td>0.21%</td>
</tr>
</tbody>
</table>

Table 4: Specificity Data

<table>
<thead>
<tr>
<th>S.No</th>
<th>Sample Name</th>
<th>Purity Angle</th>
<th>Purity Threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Reductive Degradation</td>
<td>2.154</td>
<td>0.248</td>
</tr>
<tr>
<td>2</td>
<td>Acid Degradation</td>
<td>0.052</td>
<td>0.246</td>
</tr>
<tr>
<td>3</td>
<td>Photo Degradation</td>
<td>0.049</td>
<td>0.249</td>
</tr>
<tr>
<td>4</td>
<td>Oxidation Degradation</td>
<td>0.047</td>
<td>0.244</td>
</tr>
<tr>
<td>5</td>
<td>Alkaline Degradation</td>
<td>0.422</td>
<td>0.268</td>
</tr>
<tr>
<td>6</td>
<td>Irinotecan 100% solution</td>
<td>0.047</td>
<td>0.250</td>
</tr>
</tbody>
</table>
There was a peak at the retention time of Irinotecan in acidic, photolytic and oxidative degradations, while there was some degradation of the Irinotecan under reductive and alkaline conditions. The Irinotecan peak was tested for the peak purity using the chromatographic software. The purity threshold and the purity angle were estimated using the software and compared. In each of the cases, it was observed that the purity threshold was higher than the purity angle indicating that the peak observed was pure except in cases of reductive and alkaline degradations. However, the degradation products in these two conditions did not interfere with the irinotecan peak indicating that the method was specific for the estimation of irinotecan in the product under examination (Fig 3). Stability studies indicated that the samples were stable when kept at bench top for 12 hours (short-term), in auto-sampler for 24 hours. The results of these stability studies were given in Table 5, where the percent change was within the acceptance range of 99-101%.

**Figure 3.** Chromatograms depicting the specificity of Irinotecan

<table>
<thead>
<tr>
<th>Description</th>
<th>Area</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>3,344,820</td>
<td>100.00</td>
</tr>
<tr>
<td>After 6 Hours</td>
<td>3,341,516</td>
<td>99.90</td>
</tr>
<tr>
<td>After 12 Hours</td>
<td>3,346,065</td>
<td>100.04</td>
</tr>
<tr>
<td>After 24 Hours</td>
<td>3,388,471</td>
<td>101.31</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>100.31</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td></td>
<td>0.0067</td>
</tr>
<tr>
<td>% RSD</td>
<td></td>
<td>0.66</td>
</tr>
</tbody>
</table>

**Table 5.** Stability Data for Irinotecan
**Robustness and ruggedness**

The three parameters Column, Day and Analyst were studied through a matrix design involving the estimation on two different days using two different columns with a total of 4 determinations. Under each of the conditions, samples were analyzed including a duplicate injection for each estimate and the assay content of the analyte was estimated. It can be observed from the results that the values are well within acceptance limits of 98-102%, with a RSD of less than 2.0%. Above experiments indicated that the method is rugged and provides consistent and reliable results.

**Application of the method to dosage forms**

The HPLC method developed is sensitive and specific for the quantitative determination of irinotecan. Also the method is validated for different parameters, hence has been applied for the estimation of drug in pharmaceutical dosage forms. Injections (Irinox) from BGSS pharma, Argentina, were evaluated for the amount of irinotecan present in the formulation. Each sample was analyzed in triplicate and the amount of irinotecan in ‘Irinox’ was 101.08%. None of the injection excipients interfered with the analyte peak as seen in the Fig.2.

**Conclusion**

A rapid, specific isocratic HPLC method has been developed for the determination of irinotecan using a PDA detector. The method was validated for accuracy, precision, linearity, specificity & stability, limit of detection & limit of quantitation and robustness & ruggedness. The method uses a simple mobile phase composition, easy to prepare with little or no variation. The rapid run time of 10 min and the relatively low flow rate allows the analysis of large number of samples with less mobile phase that proves to be cost-effective. Efficient UV detection at 225 nm was found to be suitable without any interference from injectable solution excipients or solvents. The calibration curves were linear (r ≥ 0.9998) over a concentration range from 20.0 to 80.0 μg/ml. The relative standard deviation’s (R.S.D.) were <1% and average recovery was above 101.0%. Limit of detection (LOD) and Limit of quantification (LOQ) were 8 ng/ml and 24 ng/ml respectively. Stability experiments indicated that the solutions prepared were stable for a period of 24 hours and the results obtained in this period were reliable. The proposed HPLC method is fast, precise, accurate, sensitive, and efficient and can be used in routine analysis in quality control laboratories.

**References**

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