Development and Validation of Bioanalytical Method for Analysis of Simvastatin

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ABSTRACT

This work deals with development of a simple, precise, accurate and economical high performance liquid chromatographic (HPLC) method with ultraviolet (UV) detection for the analysis of antihyperlipidemic drug Simvastatin from human plasma. Nicardipine hydrochloride was employed as the internal standard. Liquid-liquid extraction technique with ethyl acetate solvent was used for preparation of biological samples. Analysis was performed on a HiQSil C18 HS (250X4.6 mm, 5μm) HPLC column using Acetonitrile: 10 mM Ammonium acetate buffer (pH 5.9) (80:20 v/v) as mobile phase at a flow rate of 1 mL/min. Sample injection was done by means of an auto sampler and detection was carried out using UV detector at a wavelength of 238 nm. A good linearity was found in the concentration range of 100-1000 ng/mL. The developed method was validated according to US-FDA and European Medicines Agency (EMA) guidelines for linearity, accuracy, precision and stability. The values obtained were found to be within the prescribed limits. The described method can be applied for pharmacokinetic analysis in real clinical samples.

Keywords: Simvastatin, Human Plasma, HPLC, Bioanalytical Method Validation.


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INTRODUCTION

Simvastatin, \([1S-\{1\alpha,3\alpha,7\beta,8\beta(2S*,4S*), 8\alpha\beta]\}-1,2,3,7,8,8a-Hexahydro-3,7-dimethyl-8-[2(tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl)ethy]-1-naphthalenyl2,2 dimethylbutanoate,\] a cholesterol-lowering agent, used in treatment of hypercholesterolemia. It is also useful in treatment of dyslipidemia and coronary heart disease. It is a potent inhibitor of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase which catalyzes the conversion of HMG-CoA to mevalonate, which is an initial step (rate-limiting step) during the cholesterol biosynthesis and lowers the lipid concentration in body. According to BCS classification system Simvastatin comes under Class II which means it is poorly soluble in water (approx 30 μg/mL) [1] and has Log P value of 4.42 which suggests its high lipophilicity and high permeability. Simvastatin is practically insoluble in water with extremely low intrinsic dissolution rate resulting in poor and variable oral bioavailability (around 5%). It has a short half-life of about 2-3 hrs. It is rapidly absorbed from the GI tract following oral administration, but undergoes extensive first-pass metabolism in the liver and/or GI mucosa by Cytochrome P3A [2].

Literature reports several methods for identification of simvastatin including UV [3], liquid chromatography–UV detection (LC–UV) [4-6], LC with fluorescence detection [7], HPLC–MS/MS [8-10] and Volta metric techniques [11]. The above mentioned reported methods are however time-consuming, required complex sample pretreatment and had long run times of analysis. In this paper, we present a simple, selective and cost effective HPLC method for determination of simvastatin from human plasma using nicardipine hydrochloride as internal standard (IS). The aim of this paper is to give a bioanalytical method with simple sample pretreatment, low run time of analysis per sample and hence less solvent and energy consumption. Structures for both simvastatin and nicardipine hydrochloride are as shown in Figure 1.

![Simvastatin](image1.png)

![Nicardipine Hydrochloride](image2.png)

Figure 1: Structure of Simvastatin and Nicardipine hydrochloride

EXPERIMENTAL

Chemicals

Simvastatin (SIM) and Nicardipine hydrochloride (NIC) were obtained as gift samples from Ranbaxy Laboratories, India and Cipla Ltd., India respectively. Acetonitrile (HPLC grade), ethyl acetate, methanol, ammonium acetate (all of AR grade) were purchased from S. D. Fine Chemicals, (Mumbai, India). Ultrapure water was obtained from PURELAB Flex (ELGA) water purification system. Solvents to be used for HPLC analysis were first filtered through 0.45μm filter and then sonicated to remove entrapped air bubbles on the day of analysis. Drug free human plasma with EDTA as an anticoagulant was supplied by Sir J.J. Mahanagar Blood bank, Byculla, Mumbai, India. Plasma from six sources was obtained which was stored in deep freezer at -20°C until used.

Instrumentation and chromatographic condition

All analyses were performed using Agilent 1200 series HPLC system. System components included a mobile phase degasser unit, a quadratic pump system, an autosampler with a variable injection loop (10μL to 100 μL), temperature controlled column compartment and variable wavelength UV detector (VWD). System control and data acquisition was performed using computer via EZChrom Elite software version 3.2.1.

Chromatographic separation of SIM was achieved using HiQSil C\(_18\)HS (250 X 4.6 mm, 5μm) HPLC column. The mobile phase consisting of Acetonitrile-ammonium acetate buffer (pH 5.9, 10 mM) (80:20 v/v) was pumped at a flow rate of 1 mL/min. The column temperature was kept at room temperature (25°C). The volume of the sample injected for each analysis was 20 μL and 238 nm was used as λ\(_{max}\) for UV detector.

Preparation of stock solution and working standard solution

Stock solutions, having concentration of 1000 µg/mL (1.0 mg/mL), each of SIM and NIC were prepared in methanol. The standard solution of SIM was then appropriately diluted with mobile phase to get working standard solutions having concentrations 2,
4, 8, 12, 16 and 20 μg/mL. The standard stock solution of NIC was diluted with mobile phase to yield 20 μg/mL of working standard solution. All the solutions were stored at 2-8°C until use.

Preparation of calibration standards and QC samples

Plasma samples were spiked with working standard solutions to yield calibration curve standards in concentration range of 100-1000 ng/mL. Quality control (QC) standards were prepared in plasma having concentrations of 300 ng/mL (Low Quality Control i.e. LQC), 500 ng/mL (Middle Quality Control i.e. MQC) and 900 ng/mL (High Quality Control i.e. HQC).

Plasma sample preparation

Blank human plasma 200 μL was spiked with 50 μL of appropriate concentration of drug solution and 25 μL IS solution in an eppendorf tube and vortexed for 1 min. Liquid-liquid extraction technique was used to extract the drug from spiked plasma samples. 1 mL of extracting solvent was added and mixed for 1 min on the vortex mixer. The eppendorf tubes were then centrifuged at 5000 rpm for 10 min at 4°C. From the supernatant 800 μL aliquot was transferred to test tubes and evaporated to dryness under a steady stream of nitrogen gas at 50°C for 2 min. The residue thus obtained was reconstituted with 250 μL methanol and injected into HPLC system for chromatographic analysis.

Bioanalytical method validation

Validation of a bioanalytical method is described in various guidelines such as US-FDA [12] and European Medicines Agency (EMEA) guidelines [13]. Developed method was validated for parameters such as selectivity, linearity, accuracy, precision, recovery, carryover and stability.

Selectivity

The selectivity should be performed to show that developed method is able to separate and quantify the drug and IS from interferences present in the sample matrix. It was ensured at the lower limit of quantification (LLOQ) of the drug. The experiment was performed using plasma from six different sources. Absence of interfering components is accepted where the response for interfering substance is less than 20% of LLOQ for the analyte and less than 5% for IS.

Linearity

Standard calibration curves were generated on each validation day. The linearity was evaluated by plotting the peak area ratio of SIM to IS versus concentration of SIM. Regression analysis was performed by ordinary least square linear regression.

Accuracy and precision

Accuracy and precision were studied by analyzing a minimum of three bioanalysed batches over three different days. Each run consisted of one blank sample, one zero sample, one standard curve containing all CC standards along with five replicates of LLOQ, LQC, MQC and HQC samples. According to EMEA guidelines, the percent coefficient of variation (%CV) determined at each concentration level should be within ±15% (±20% for LLOQ) and percent relative error (%RE) should be within ±15% (±20% for LLOQ) of the nominal value.

Recovery

Recovery experiment was performed as per US FDA guidelines by comparing the results for extracted drug samples from plasma at three concentrations (LQC, MQC and HQC) with unextracted standard at same concentration of the drug, representing 100% recovery. Recovery of IS was determined by comparing peak area of extracted zero sample with that of unextracted standard of IS, representing 100% recovery.

Carry-over

Carry-over is the appearance of drug signal in blank sample after the analysis of high concentration sample. It was performed by injecting blank plasma sample immediately after the injection of upper limit of quantification sample (ULOQ) sample of the standard curve and studying the chromatogram of blank sample obtained.

Stability

Stability studies were carried out by analyzing three replicates each of LQC (300 ng/mL) and HQC (900 ng/mL). Freeze and thaw stability for SIM in plasma samples was studied by freezing at -20°C and thawing unassisted at room temperature (3 cycles). Short term stability was assessed by keeping the samples at room temperature for 6 hours prior to analysis. For long term stability, three replicates of LQC and HQC were kept at -20°C in deep freezer for 20 days prior to analysis. The samples obtained were then spiked with IS, subjected to LLE and chromatographic analysis.

RESULTS AND DISCUSSION

Chromatographic behavior of SIM and IS

A trial and error approach was used in the bioanalytical method development. Various mobile phases were tried and chromatographic separation for drug and IS was studied. Chromatographic conditions were first optimized for the standard solutions.
of drug and IS and then applied to the processed plasma samples. Under these conditions, NIC and SIM were separated at retention time of 7.70 min and 9.90 min respectively as shown in Figure 2.

![Figure 2: Chromatogram of SIM and NIC extracted from plasma](image)

Selection of extracting solvent

Liquid–liquid extraction is a traditional but effective method to extract drugs from biological samples. It can be quickly developed and applied to most categories of drug compounds [14]. Different extracting solvents were tried for the liquid-liquid extraction experiments, namely ethyl acetate, acetonitrile, dichloromethane and methanol. Ethyl acetate was selected as extracting solvent depending on the interferences at the retention time of the drug and recoveries after extraction.

Bioanalytical method validation

Selectivity

Six replicates of LLOQ (sample with 100 ng/mL of SIM) and blank plasma from six different sources were studied for any interference at the retention time of drug. Blank plasma did not show any interference at the retention time of drug and IS which can be seen in Figure 3. Hence it was proved that the method was selective for the analysis of drug of choice.

Linearity

A six point calibration curve was constructed by plotting peak area ratios of SIM/NIC versus SIM concentration in the range of 100-1000 ng/mL. Linearity was established by linear regression analysis. The regression equation obtained for standard curve was $y = 0.002 x + 0.095$ with co-efficient of regression ($r^2$) of 0.994.

![Figure 3: Representative chromatogram of blank plasma sample](image)
**Accuracy and precision**

The accuracy and precision within the batch and between the batch were calculated and the results are given in Table 1. Accuracy was determined as percent relative error (% RE) and precision was measured as percent relative standard deviation (% RSD). The within batch RSDs was found to be in range of 3.33-8.32 % at all concentration levels and the between batch RSDs were in range of 2.59-9.75 %. The within batch % RE varied between -8.31 % and +6.22 % at all concentration levels and the between batch % RE values were between -6.01 % and +2.80%. All the results obtained are found within the acceptable limits as per the guidelines.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Concentration</th>
<th>Within batch (n=5)</th>
<th>Between batch (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LLOQ (100 ng/mL)</td>
<td>LQC (300 ng/mL)</td>
<td>MQC (500 ng/mL)</td>
</tr>
<tr>
<td>Mean</td>
<td>100.33</td>
<td>275.06</td>
<td>516.93</td>
</tr>
<tr>
<td>% RSD</td>
<td>8.32</td>
<td>3.33</td>
<td>3.75</td>
</tr>
<tr>
<td>% RE</td>
<td>0.33</td>
<td>-8.31</td>
<td>3.38</td>
</tr>
<tr>
<td>% Nominal</td>
<td>100.33</td>
<td>91.69</td>
<td>103.38</td>
</tr>
</tbody>
</table>

**Table 1: Results for accuracy and precision.**

Recovery of SIM was compared by calculating the peak area of SIM from an extracted plasma sample with that obtained from an unextracted standard at the same concentration for QC samples containing 300 ng/mL, 500 ng/mL and 900 ng/mL of SIM. Recovery of IS was tested at concentration of 500 ng/mL. Results for recovery are given in Table 2.

<table>
<thead>
<tr>
<th>Level</th>
<th>Concentration (ng/mL)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LQC</td>
<td>300</td>
<td>62.06337</td>
</tr>
<tr>
<td>MQC</td>
<td>500</td>
<td>69.43032</td>
</tr>
<tr>
<td>HQC</td>
<td>900</td>
<td>68.16719</td>
</tr>
<tr>
<td>IS</td>
<td>500</td>
<td>52.21146</td>
</tr>
</tbody>
</table>

**Table 2: Results for recovery studies.**

Results showed that no carryover for both drug and IS was found after injecting the ULOQ sample after blank sample.

**Stability**

The results of stability studies are shown in Table 3. From the results it was observed that all the stability results were found to be in acceptable limits for accuracy and precision. No significant degradation observed in the samples stored at different conditions. Hence, it can be concluded that the drug was stable for six hrs at room temperature (short term stability), for three freeze-thaw cycles and for 20 days at -20°C (long term stability).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Short term stability</th>
<th>Freeze-thaw stability</th>
<th>Long-term stability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LQC</td>
<td>HQC</td>
<td>LQC</td>
</tr>
<tr>
<td>LQC</td>
<td>313.01</td>
<td>914.40</td>
<td>305.68</td>
</tr>
<tr>
<td>HQC</td>
<td>288.65</td>
<td>865.24</td>
<td></td>
</tr>
<tr>
<td>Mean % RSD</td>
<td>5.90</td>
<td>2.47</td>
<td>4.46</td>
</tr>
<tr>
<td></td>
<td>8.70</td>
<td>6.76</td>
<td></td>
</tr>
</tbody>
</table>
CONCLUSION

A simple, accurate and precise bioanalytical method based on HPLC-UV detection for quantification of SIM from human plasma was developed and validated as per US FDA and EMEA guidelines. Simple liquid-liquid extraction procedure using ethyl acetate as the extracting solvent was able to extract drug from the biological matrix. Analysis requires short period of time and less solvents. Thus, this method can be used for bioanalysis of real clinical samples.

AUTHOR'S STATEMENT

Competing Interests

The authors declare no conflict of interest.
REFERENCES


