Validation of Analytical Method of Irbesartan Plasma

in Vitro by High Performance Liquid

Chromatography-Fluorescence

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Abstract: Irbesartan is an antihypertensive drug whose concentration in blood is very small so it requires a sensitive method of analysis, selective and valid for analysis. In this study, it is carried out optimization of analytical conditions and validation for the analysis of irbesartan in plasma. Chromatography was performed on a C18 column (250 × 4.6 mm, 5 μm) under isocratic elution with acetonitrile-0.1% formic acid (46:54 v/v), pH 3.75. Detection was made at excitation 250 nm and emission 370 nm and analyses were run at a flow-rate of 1.0 mL/min at a temperature of 40 ºC. Losartan potassium was used as internal standard. Plasma extraction was done by deproteinization with acetonitrile, mixed with vortex for 30 seconds, then centrifuged it at 10,000 rpm for 10 min. In plasma validation, the recovery was 96.22%, and the lower limit of quantification (LLOQ) in plasma was 2 ng/mL. The method also fulfill the criteria for accuracy and precision intra and inter day by normal values (%Diff) not exceed ± 15%. On the stability study, irbesartan in plasma temperature –20 ºC has been stable for 28 days.

Key words: HPLC, fluorescence, irbesartan, validation, human plasma.

1. Introduction

Hypertension is a common health problem in developed countries and developing countries. The cause of hypertension is diverse due to genetic factors, lifestyle, and stress. Uncontrolled hypertension can lead to various diseases such as stroke, heart failure, diabetic nephropathy, myocardial infarction, kidney failure and even death. According to the guideline of the National Joint Committee, the management of patients with hypertension should be precise and fast, with drugs given singly or in combination therapy drugs [1]. Irbesartan is a class of hypertension drugs called angiotensin II receptor blockers that work on the renin-angiotensin-aldosterone system. Besides useful for lowering blood pressure, drugs known as angiotensin II receptor blockers have a protective effect on the kidneys especially in diabetic patients [2].

Irbesartan is a non-peptide compounds, with the chemical name 2-butyl-3-[[29-(1H-tetrazole-5-yl) [1,19-biphenyl]-4yl]methyl]-1,3-diazaspiro [4,4]non-1-en-4-one. Irbesartan is a hypotensive agent that does not require biotransformation to become active form [3]. Peroral drug absorption is rapid, bioavaibilitasnya about 60-80% and 90% protein bound. At therapeutic doses of irbesartan (75-300 mg), maximum concentration in plasma will be obtained about 1.5-2 hours after dosing [4]. The maximum concentration in plasma after administration of a dose of 150 mg irbesartan is about 1.5 ± 0.29 μg/mL [5].

Numerous HPLC methods for the quantification of irbesartan in biological samples have been reported. These methods involved the use of protein precipitation, liquid-liquid extraction and solid-phase extraction coupled with fluorescence detection or ultraviolet chromatography or high performance liquid
Validation of Analytical Method of Irbesartan Plasma in Vitro by High Performance Liquid Chromatography-Fluorescence tandem mass spectrometry method (HPLC/MSMS) [6-8].

In bioequivalence studies, the proposed method should be simple and able to process hundreds of samples in a limited time. This paper describes a simple, rapid, precise, and accurate HPLC method for determining irbesartan in human plasma in vitro.

2. Materials and Methods

2.1 Chemicals and Reagents

Irbesartan (99.7% on assay) were obtained from Hetero Labs Limited. Losartan Potassium (99.6% on assay) were obtained Ipca Labs Limited. Acetonitrile and methanol were HPLC-grade and were purchased from Merck. The other chemicals and reagents were analytical grade. Human plasma was provided by Indonesian blood bank (Palang Merah Indonesia).

2.2 Chromatographic Conditions

The HPLC system (Shimadzu, Japan) used consisted of a model LC-10AD pump, a fixed manual injection loop of 20 μL, and a model RF-10AXL Fluorescence detector; data acquisition was performed with the SCL-10A processor. The analytical column employed was a Kromasil C18 column (250 × 4.6 mm, i.d., 5 μm).

The mobile phase consisted of acetonitrile-0.1% formic acid (46:54 v/v). The mobile phase was adjusted to pH 3.75 ± 0.01 with 1 N NaOH or dilute phosphate acid (85% v/v), filtered through a 0.45 μm cellulose membrane filter (Whatman) and degassed before use (Elmasonic S60H). The detection wavelength was set at excitation 250 nm and emission 370 nm. Chromatography separation was performed at temperature 40 °C and flow rate was maintained at 1 mL/min.

2.3 Standard Solutions and Quality Control Samples

Primary stock solutions of irbesartan (1 mg/mL) and losartan potassium (1 mg/mL) were prepared in methanol. Then, diluted with methanol to obtain a certain concentration.

Human plasma calibration standards of irbesartan were prepared by spiking an appropriate amount of the working standard solutions into drug-free human plasma. The concentration of irbesartan in calibration curve was 2.05 ng/mL, 5.12 ng/mL, 10.24 ng/mL, 51.20 ng/mL, 256 ng/mL, 512 ng/mL, 1,024 ng/mL, and 5,120 ng/mL. Quality control (QC) samples were prepared at three concentrations that were low (6.14 ng/mL), medium (2,048 ng/mL), and high (4,096 ng/mL).

2.4 Sample Preparation

0.25 mL of plasma containing certain concentrations of irbesartan were added 25.0 μL of the internal standard working solution (6 μg/mL). Three part of acetonitril (750.0 μL) was added to precipitate protein in plasma, vortex-mixed for 30 sec and centrifuged at 10,000 rpm for 10 min. A 20 μL aliquot of the supernatant was injected into the HPLC system.

2.5 Validation of This Method

The validation parameters obtained were specificity, linearity, sensitivity, accuracy, precision, recovery and stability. The method was validated according to USFDA guidance for bioanalytical method validation [9]. Six randomly selected blank plasma samples [9] were processed by a similar extraction procedure and analyzed to determine the extent to which endogenous plasma components may contribute to interference at the retention time of irbesartan, and losartan potassium.

The calibration curves were constructed each day before the analysis of the samples by plotting the peak-area ratio versus the drug concentrations. The lower limit of quantification (LLOQ) was defined as the lowest concentration with a coefficient of variation (CV) of less than 20% and accuracy of 80-120%. The accuracy and precision of the method were assessed intra-day and inter-day during 5 days by determining QC plasma samples at three concentrations which
were low (6.14 ng/mL), medium (2,048 ng/mL), and high (4,096 ng/mL) concentrations, accompanying by a standard calibration curve on each analytical run.

The recovery of irbesartan was evaluated by comparing measured concentration obtained from peak areas of pre-treated quality control plasma samples \( (n = 5) \) with mean measured concentration of those spiked-after extraction samples at the same nominal concentrations. Stability quality control plasma samples were conducted at low and high concentrations and were subjected to short-term (6 h and 24 h) incubation at room temperature, three freeze/thaw cycles, and storage for 28 days \((-20 ^\circ C)\). The stability of primary stock solutions were also being conducted for 25 days \((5 ^\circ C)\).

3. Results

3.1 Specificity

The current method showed excellent chromatographic specificity with no endogenous plasma interference at the retention times of irbesartan and losartan potassium as internal standard. Chromatograms obtained from human blank plasma and human blank plasma spiked with irbesartan \((0.5 \ \mu g/mL)\) and losartan potassium \((20 \ \mu g/mL)\) are shown in Figs. 1A and 1B, respectively. Irbesartan and losartan potassium were well resolved with respective retention times of 6.2 min and 10.4 min.

3.2 Calibration Curve and Limit of Quantification

The calibration curves were linear over the concentration range of 2.05-5,120 ng/mL with a correlation coefficient of 0.9999. The correlation coefficient from replicate calibration curves on different days was more than 0.9995. The lower limit of quantification with a coefficient of variation of less than 20% was 2 ng/mL.

3.3 Precision and Accuracy

The coefficient variation values of both inter- and intraday analysis for 5 days at three concentrations which each concentration is conducted at 5 replicates were less than 6.37% whereas the %Diff were less than 13.43%. The inter- and intra-day precision and accuracy values of the assay method are presented in Table 1.

3.4 Recovery

The mean extraction recoveries of irbesartan at three concentrations \((\text{low, mid, high})\) were 86.23%-113.45%, 85.37%-99.31%, and 85.73%-103.44%, respectively.

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**Fig. 1** Representative chromatogram of human blank plasma (A), human blank plasma spiked with irbesartan (1) and losartan potassium (2) (B).
3.5 Stability

Treated plasma samples were found to be stable at least 24 h when the samples were kept at room temperature (%Diff < 15%). The concentrations of irbesartan in plasma which underwent three freeze-thaw cycles or storage at –20 ºC for 28 days were found to be stable with % differentiation less than 15%. The stability data of irbesartan stored under various conditions and subjected to freeze-thaw cycles are shown in Table 2. The primary stock solutions were also found to be stable for 25 days when were kept at 5 ºC. The stability data of irbesartan stored under various conditions and subjected to freeze-thaw cycles are shown in Table 2.

4. Discussion

4.1 Preparation of Plasma Samples

Protein precipitation has the advantages of simplicity and universality, so it was used to prepare the plasma samples [8]. The reason of choosing acetonitrile as the precipitation agent was caused by its ability to precipitate protein, especially when given not less than the volume of blood. It is also usually become the component of mobile phase with the result that the system will be able to accept. Results indicated that direct protein precipitation with acetonitrile was simple and rapid and good separation of the drug and I.S. was achieved using the precipitation method. The centrifugator was used optimally at 10,000 rpm for 10 min. The aim was to obtain the pure supernatant which was ready to be injected.

4.2 Optimization of Mobile Phase

The chromatographic conditions were optimized by injecting analytes with mobile phase containing varying percentages of organic phase and flow rates of mobile phase to achieve good resolution and symmetric peak shapes for irbesartan and losartan potassium, as well as a short retention time. As expected, the retention times increased with decreasing acetonitrile percentage and system flow rates. The chosen mobile phase pH was 3.75 ± 0.01 by giving the most symmetric peak shapes for irbesartan and losartan potassium.

Table 1  Accuracy and precision from the determination of irbesartan in human plasma (n = 25/concentration).

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Mean ± SD (ng/mL)</th>
<th>CV (%)</th>
<th>%Diff</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>5.7972 ± 0.5870</td>
<td>6.85</td>
<td>Min. = –13.77% Max. = 14.67%</td>
</tr>
<tr>
<td>Mid</td>
<td>1957.4585 ± 66.6095</td>
<td>3.41</td>
<td>Min. = –14.63% Max. = 2.81%</td>
</tr>
<tr>
<td>High</td>
<td>3981.4188 ± 0.0358</td>
<td>4.33</td>
<td>Min. = –14.28% Max. = 14.51%</td>
</tr>
</tbody>
</table>

Table 2  Stability data of irbesartan in human plasma.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>CV (%)</th>
<th>%Diff</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short term stability for 24 h in plasma at room temperature</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>7.82</td>
<td>–5.79 - 14.46%</td>
</tr>
<tr>
<td>High</td>
<td>2.82</td>
<td>–9.54 - 8.11%</td>
</tr>
<tr>
<td>Long term storage at –20 ºC for 28 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>2.95</td>
<td>–5.94 - 12.84%</td>
</tr>
<tr>
<td>High</td>
<td>2.20</td>
<td>–13.94 - 10.47%</td>
</tr>
<tr>
<td>Three freeze/thaw cycles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>4.64</td>
<td>–14.46 - 9.02%</td>
</tr>
<tr>
<td>High</td>
<td>6.46</td>
<td>–5.67 - 9.37%</td>
</tr>
</tbody>
</table>
Validation of Analytical Method of Irbesartan Plasma *in Vitro* by High Performance Liquid Chromatography-Fluorescence

Optimal conditions were a mobile phase consisting of acetonitrile: 0.1% formic acid (46:54, v/v) pH 3.75 ± 0.01 arranged by 1 N NaOH and 85% phosphate acid. Under optimum conditions, the chromatographic run time for each sample was completed within 14 min.

4.3 Advantages of the Method

In comparison to previously published HPLC methods for separation and quantitation of irbesartan, the major modifications incorporated into the current method include: simple sample preparation procedures, common and cheap HPLC equipment and mobile phase additives, and a relative short analysis time as well.

Thus the assay is suitable for routine analysis when determining assay on biological samples to perform bioequivalence studies. A simple, rapid, precise, and accurate HPLC method for determining irbesartan in human plasma has been presented. Although lower sensitivity was obtained in comparison to previously published LC methods with mass spectrometry detection, the resulting LLOQ (2 ng/mL) was sufficient for human pharmacokinetic studies.

5. Conclusion

An analytical method developed for irbesartan quantification in plasma samples showed good specificity, sensitivity, linearity, precision, and accuracy over the entire range of clinically significant

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Fig. 2 Chromatogram of irbesartan (1) and losartan potassium (2) in varying percentages of organic phase. The chromatograms representative system in acetonitrile-0.1% formic acid (46:54, v/v) (A); (37:63, v/v) (B); and (40:60, v/v) (C).

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and therapeutically achievable plasma concentrations, thereby enabling its use in bioequivalence trials.

References