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DEVELOPMENT AND VALIDATION OF A BIOANALYTICAL HPLC METHOD FOR ESTIMATION OF IRBESARTAN IN RAT PLASMA: APPLICATION TO PHARMACOKINETIC STUDIES

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ABSTRACT

A simple, specific, sensitive and rapid reverse phase high performance liquid chromatographic (HPLC) method for the determination of irbesartan (IRB) in small volumes of rat plasma was developed and validated. Biological sample preparation involved simple extraction with organic solvent, followed by dilution with mobile phase to eliminate any chromatographic solvent effects.IRB wasquantitated on a C8 column (4.6 mm i.d. × 300 mm length), using a mobile phase composed of methanol: acetonitrile (70:30 %v/v) which was delivered at a flow rate of 1.0 mL/min. The methodwas proven to be linear over a plasma concentration range of 10 to 1000ng/mL with a mean correlation coefficient of 0.9913. The intra-day and inter-day precision (coefficient of variation) were in the range of 1.9% to 4.3% and 2.7%to 5.4%, respectively. The intra-day accuracy (relative error) were in the range of 2.2% to 5.1%and the inter-day accuracywere in the range of 3.4% to 5.8%. The mean recovery of IRB from rat plasma was found to be 96.93%. The limit of detection (LOD) and the limit of quantification (LOQ) of the developed method were determined to be 1 ng/mL and 10 ng/mL, respectively. Thedeveloped method was successfully applied to quantitatively assess the pharmacokinetics of IRB in wisterrats followinga single oral dose (6.75 mg/kg). The developed method was established as a rapid analytical tool in a pharmacokinetic study as it required short retention time, high precision, sensitivity and small volumes of plasma for analysis.

Key words: *Bioanalytical method, Irbesartan, HPLC, Pharmacokinetics, Rat plasma.*

INTRODUCTION

Irbesartan (IRB) is 2-butyl-3-[(21-(1H-tetrazol-5-yl)biphenyl-4-yl) methyl]-1,3-diazas piro {4,4} non-1-en-4-one (Figure 1), is a synthetic analogue of angiotensin II receptor blocker, utilized for the treatment of hypertension. IRB is a poorly water-soluble drug which displays a dissolution rate-limited absorption pattern in humans and animals. Hence, it can be used as a model drug to assess the influence of various physicochemical, physiological, and dosage form factors on the absorption kinetics and bioavailability of hydrophobic drugs[1-6].A variety of methods have been developed for determination of IRB individually or with combination with some other antihypertensive agents in biological samples[7, 8]. This includes high performance thin layer chromatography (HPTLC) [9], HPLC coupled with mass spectrometric detection (HPLC–MS/MS) [10], spectroscopic and spectrofluorimetric detection [11]. In general, spectroscopicmethod lack sensitivity and cannot distinguish degradation products from the parent compound. Although HPLC–MS/MS and HPTLC methods provide excellent sensitivity, they are not available in all laboratories because of their special requirements and economicconsiderations. Similarly, spectrofluorimetric method utilized either a column switching system or an expensive solid phase extraction cartridges. With respect to these, all reported methods for IRB have various limitations; time-consuming sample clean-up, laborious extraction steps and long chromatographic elution time. Moreover, all these methods have utilized human plasma for the study. As far as our knowledge is concerned there is no method reported till date for the estimation of IRB in rat plasma. Thus the present study was undertaken to develop and validate a simple, sensitive, accurate, precise and reproducible bioanalytical HPLC method for estimation of IRB in rat plasma.

EXPERIMENTAL DETAIL

Chemicals and Reagents

IRB was gifted form Torrent Research Center, Gandhinagar, India. Acetonitrile (HPLC grade), methanol (HPLC grade) and potassium dihydrogen phosphatewere purchased from Merck Chemicals, Mumbai, India. Double deionized water was utilized for entire study.

Animals

Male wister rats with an average weight of 200 ± 20 gm and age ~ 10 weeks (on the day of study) were probed in order to investigate pharmacokinetic behavior of IRB. The study was approved by Institutional Ethics Committee of Department of Pharmaceutical Sciences, Saurashtra University, Rajkot, Gujarat, India (CPCSEA No. SU/DPS/IAEC/1003, Dated: 11/02/2010) and their guidelines were followed throughout the study. All the rats were acclimatized at a temperature 20 ± 2 °C and relative humidity of 45 ± 15%, with a 12 h light/dark cycle over a period of 5 days prior to dose administration. During this acclimatization period, the animals were carefully observed to ensure their good health and suitability for inclusion in the study. For all rats a standard laboratory diet (PranavAgromart Ltd, Baroda, India) and domestic mains tap water were available ad libitum. The animals were disconnected from diet at least 12 h before dosing. During study periods, rats were housed singly in polypropylene and stainless steel cages [12, 13].

Instrumentation

A High Performance Liquid Chromatograph (HPLC) with work station of Shimadzu LC SOLUTION was employed for present investigation. The system consisted Shimadzu UFLC 20-AD as binary solvent delivery system, Shimadzu 7D Rheodyne Injector as loop injector and Photo Diode Array detector as a source of detection.

Chromatographic Conditions

A combination of methanol and acetonitrile (70:30 %v/v) was selected as mobile phase. Samples were separated using Phenomenex Luna® C8 column with a pore size 100 Å, length 300 mm and internal diameter (i.d.) 4.6 mm. The mobile phase was injected to the system using binary pumping mode at a flow rate of 1 mL/min. For all samples, injection volume and run time were fixed as 20 µL and 10 min, respectively.

Preparation of Mobile Phase

For preparing a mobile phase, HPLC grade methanol and acetonitrile were filtered through a 0.2 µm membrane filter and subjected to degassing in an ultrasonic bath (Frontline FS-4, Mumbai, India) for a period of 15 min.

Preparation of Standard Solutions

A primary stock solution (1 mg/mL) was prepared by dissolving 10 mg of IRB in 10 mL of HPLC grade methanol. The stock solution was suitably diluted with HPLC grade methanol to obtain working range of standard solutions. The working standard solutions were prepared in duplicates, out of which one set was used to prepare calibration curve and the other was used to generate quality control (QC) samples. Plasma used in the study was isolated from rat blood by centrifugation at 10000 rpm for a period of 15 min at 4°C, using a centrifuge (Remi Laboratory Instruments, Mumbai, India). The calibration curve samples were prepared by spiking 500 µL of drug free rat plasma with 100 µL of previously diluted working standard solution in order to obtain final concentrations of 10, 25, 50, 75, 100, 250, 500, 750 and 1000 ng/mL. The QC samples [30 ng/mL (LQC), 250 ng/mL (MQC) and 900 ng/mL (HQC)] were prepared by spiking second set of working solutions to the pooled blank plasma. All samples were stored at refrigerated cold conditions (2-8°C) and equilibrated to room temperature prior to use [14, 15].

Sample Preparation

Prior to sample analysis, 100 µL of each solution was extracted using 300 µL of diethylether: dichloromethane (60:40% v/v) for protein precipitation. Further, each of the mixtures was vortexed for a period of 5 min in a vortexer (GeNei, Bangalore, India) with subsequent centrifugation at 10000 rpm, for a period of 10 min at 4 °C using a centrifuge. For each sample, an aliquot of a supernatant was isolated and subjected to dryness. The residue was reconstituted in 100 µL of mobile phase and subsequently centrifuged at 10000 rpm for 10 min at 4 °C in a centrifuge. The supernatant was finally collected and directly injected into the HPLC system. This procedure was followed for all samples of calibration curve and QC [14, 15].

Construction of Calibration Curves

The values of peak areas were plotted against their respective concentrations in order to constructthe calibration curve for IRB. Linear regression analysis was performed for each set of data using Microsoft Excel® version 2010 (Microsoft Corporation, Washington, USA).

Validation Parameters

Linearity and range

The linearity and range of calibration curve was evaluated with ninecalibration standards containing different concentrations of respective drug (10, 25, 50, 75, 100, 250, 500, 750 and 1000 ng/mL). The study was repeated in triplicates to confirm reproducibility of results. The concentrations of the test samples were back-calculated using linear regression analysis [14, 15].

Selectivity

Selectivity of developed method was assessed by comparing chromatograms of three different batches of blank plasma obtained from three individual rats with those of corresponding standard plasma samples [14, 15].

Accuracy and precision

Intraday precision and accuracy of developed method was determined by analyzing six replicates of QC samples at three concentrations in a single sequence. Similarly, for interday precision and accuracy, six replicates QC samples at three concentrations were analyzed on three consecutive days. The accuracy of method was determined by calculating % relative error (%RE) whereas the precision was determined by calculating % relative standard deviation (%RSD) [14, 15].

Robustness

The robustness of developed method was studied by evaluating the effect of small but deliberate variations in chromatographic conditions. The parameters studied were flow rate and mobile phase composition [14, 15].

Recovery (Extraction efficiency)

To investigate extraction efficiency of developed method, a set of samples (n=6) at each QC level were prepared by spiking drug into plasma samples and processed further (pre-extraction). Similarly, a second set of plasma samples were processed first and spiked post-extraction at each QC levels. Extraction recovery for each analyte was determined by calculating the ratios of peak areas of the pre-extraction samples to those of the samples of post-extraction [14, 15].

Limits of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ of developed method were estimated on the basis of standard deviation and slope of the calibration curve as 3.3 δ/m and 10 δ/m, respectively. Where δ was the regression standard deviation of intercept and m was the slope of calibration curve [14, 15].

Pharmacokinetic Studies

The applicability of the developed HPLC methodfor IRB in rat plasma was demonstratedby the results obtained from pharmacokinetic studiesconducted on male wister rats (n=12). Each rat of was treated with oral dispersions of IRB at a dose of 6.75 mg/kg [16] in a single dose by curved gastric gavage tubes directly into the stomach. The dose volume for all administration was maintained at 5 mL/kg [12, 13]. Serial blood samples (500 µL) were collected from retro-orbital venous plexus with hematocrit over a period 72 h (five biological half-life). Rats were further divided into two subgroup (n=6) for convenient blood sampling over entire study periods as recommended by the experts of IAEC (Institutional Animal Ethics Committee). Blood samples from each group were collected at predetermined time intervals alternatively from each subgroup into heparinized plastic tubes. All these samples of whole blood were kept in refrigerated cold conditions (2-8°C) until separation of plasma. Each sample was processed further by the method

as mentioned under sample preparation and subjected to HPLC analysis for the estimation of drug content by a previously validated bioanalytical method. The pharmacokinetic calculations were performed on the basis of plasma concentration–time data using Kinetica® version 5.1 (Thermo Scientific, USA) pharmacokinetics and pharmacodynamics software [17-19].

RESULTS AND DISCUSSION

Analysis is an important component in formulation and development of any drug molecule. A suitable and validated method has to be available for the analysis of drug(s) in bulk, drug delivery systems, in vitro and in vivo. If such suitable method for specific need is not available then it becomes essential to develop a simple, sensitive, accurate, precise, reproducible method for the estimation of drug samples. The present investigation was aimed to develop and validate bioanalytical HPLC method for estimation of IRB in rat plasma [14, 15, 20-22].

Selection of Chromatographic Conditions

The chromatographic conditions were selected on the basis of backpressure, peak resolutions, peak shapes and day-to-day reproducibility of the retention time (Table 1). For selecting a mobile phase initial trial was taken using methanol and potassium dihydrogen phosphate buffer (pH 3.5) in various phase ratios but the peaks were not of a good shape. Utilization of acetonitrile in place of phosphate buffer improved the peak shapes and hence, 70:30 of methanol: acetonitrile was selected as mobile phase for further trials (Figure 2).

Validation

Linearity and range

The mean regression equation of three standard curves for IRB was y = 562.67x + 34082. Where y presented the peak area of drug and x was the plasma concentration of drug. The precisions (% CV) of the slope and intercept were less than 2% for both drugs which indicated minimum variations [14, 15]. The calibration curve was linear over the studied concentration range (10–1000 ng/mL) with a mean correlation coefficient more than 0.99 (Table 2 and Figure 3).

Selectivity

It is the ability of an analytical method to differentiate and quantify analyte in the presence of other components of the samples. Each blank sample was evaluated for interference with respective drug [14, 15]. The results revealed that the analyte (IRB) was well separated from co-extracted material under the adopted chromatographic conditions. The retention time (Rt) was 6.2 min (Figure 2). In addition to this, the chromatogram of extracted plasma samples did not show any co-eluting interference peak with the analyte which suggested good degree of selectivity for the developed method.

Accuracy and precision

The accuracy of an analytical method describes the closeness of test results to the true concentration of analytes whereas the precision is measure of degree of reproducibility of analytical method [14, 15]. The results of intraday and interday precisions are summarized in Table 3revealed that developed method was accurate and precise for quantification of IRB in plasma samples.

Robustness

The low values of %RSD for each of drug proposed that during all deliberate variations, assay value of test preparation (MQC) was not affected and it was in accordance with that of actual (Table 4). Hence, developed analytical method was considered to be robust [14, 15].

Recovery (Extraction efficiency)

The high value of recoveries for IRB specified that insignificant amounts of drug was lost during plasma protein precipitation step [14, 15]. Lower values of %RSD advocated high degree of extraction efficiency of developed method (Table 5).

Limits of Detection (LOD) and Quantitation (LOQ)

The LOD and LOQ were observed as 1 ng/mL and 10 ng/mL, respectively. These low values were indicative of high sensitivity of developed method.

Pharmacokinetic Study

The proposed HPLC method was successfullyapplied to monitor quantitatively the time courseof plasma IRB concentrations after oral administration of a single 6.75 mg/kg dose to adult male wister rats.The mean plasma drug concentration-time profileobserved in these pharmacokinetics studies isshown in Figure 4. The values of all major pharmacokinetic parameters like; maximum plasma concentration (C_{max}), time required for maximum plasma concentration (T_{max}), area under curve ($AUC0_{-\infty}$), area under first moment curve ($AUMC0_{-\infty}$), terminal half-life ($t_{1/2}$), mean residence time (MRT) and clearance have been summarized in Table 7.

CONCLUSIONS

A simple, rapid, specific, sensitive and reproducibleHPLC method for the quantitative determinationof IRB in small volumes of rat plasma hasbeen developed and validated. The method issuitable for studying the pharmacokinetics of IRB using the rat asan animal model.

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We would like to thank Torrent Research Center for providing gift sample of irbesartan.

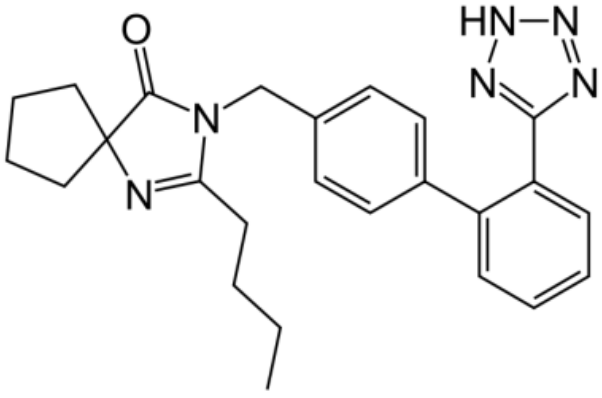

Figure 1. Structure of Irbesartan

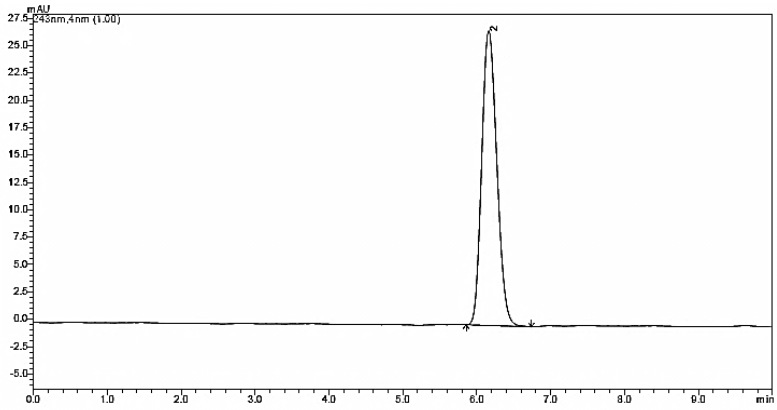

Figure 2 : HPLC Chromatogram of IRB

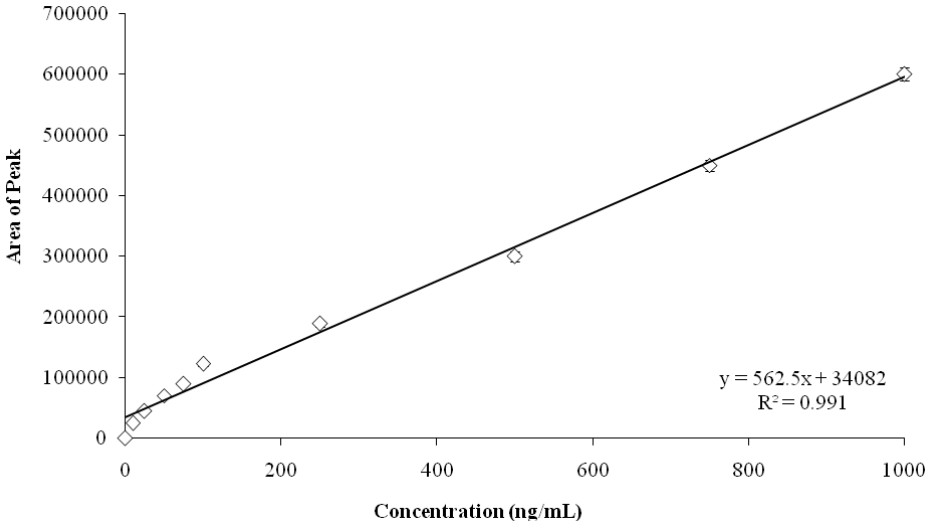

Figure 3 : Calibration curve for plasma samples of IRB

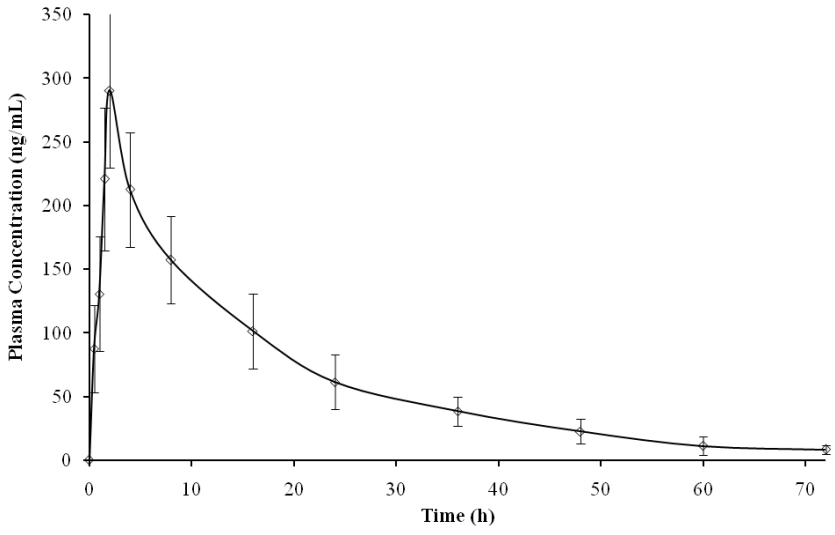

Figure 4 : In vivo pharmacokinetic profiles of IRB, Error bar represents SD (n=6)

TABLES

Table 1 : Chromatographic conditions for bioanalytical method development

Chromatographic Conditions	
Pumping Mode	Binary
Mobile Phase	Methanol and acetonitrile (70:30 %v/v)
Column	Phenomenex Luna® C8 Pore size - 100 Å, Length - 300 mm, i.d. - 4.6 mm
λ Scanning Range	190-800 nm
Flow Rate	1 mL/min
Injection Volume	20 µL
Run Time	10 min

Table 2 : Calibration curves for plasma samples of drugs

Concentration(ng/mL)	*Peak Area*
10	25331.45 ± 800.42
25	45421.63 ± 1014.46
50	70321.27 ± 1134.64
75	89304.65 ± 1645.31
100	122901.23 ± 3111.43
250	189053 .57± 5231.53
500	300530.25 ± 8342.54
750	450331.76 ± 9012.43
1000	600321.37 ± 11123.47

The results are of mean ± SD (n=3)

Table 3 : Intra and Interday accuracy and precision of bioanalytical method

Nominal Concentrations	*Mean Area ± SD (n=6)*	*%RE*		*%RSD*	
		Intraday	*Interday*	*Intraday*	*Interday*
LQC	28.98 ± 1.45	5.1	5.8	4.3	5.4
MQC	252.67 ± 3.12	3.3	4.3	2.5	3.1
HQC	892.91 ± 7.42	2.2	3.4	1.9	2.7

Table 4 : Robustness study of bioanalytical method

Flow Rate (mL/min)	*0.8*	*1.0*	*1.2*
Mean (n=3)	188831	189053	189134
%RSD	3.43	2.34	4.16
Mobile Phase Ratio (%v/v)	***65:35***	***70:30***	***75:25***
Mean (n=3)	188734	189053	188921
%RSD	3.35	1.45	5.32

Table 5 : Recovery study of bioanalytical method

Nominal Concentration	*% Recovery*	*%RSD*
LQC	98.6	3.33
MQC	97.3	2.67
HQC	94.9	4.78

Table 6 : Summary of validation parameters of bioanalytical method

Parameters	*Results*
Linearity Range (ng/mL)	10 - 1000
Correlation Coefficient(r^2)	0.9913
Slope	562.67
Intercept	34082
Limit of Detection (LOD) (ng/mL)	1
Limit of Quantification (LOQ) (ng/mL)	10
Retention Time (R_t) (min)	6.2

Table 7 : Results of in vivo pharmacokinetic study of IRB

Parameters	*Mean ± SD (n=6)*
C_{max}(ng/mL)	290.25 ± 95.1
T_{max}(h)	2.01 ± 0.25
$AUC_{0-\infty}$ (ng·h/mL)	4666.38 ± 680.35
$AUMC_{0-\infty}$ (ng·h^2/mL)	86094.71 ± 937.76
$t_{1/2}$	14.20 ± 1.1
MRT (h)	18.45 ± 1.42
Clearance (mL/h)	0.2893 ± 0.0045

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