Development and validation of reverse phase HPLC method for determination of angiotensin receptor blocking agent irbesartan in plasma

Zeb-un-Nisa[1], Syed Imran Ali[1], Mehwish Rizvi[2], Maqsood Ahmed Khan[1], Rafi Akhter Sultan[3], Rasheeda Fatima[1], Najma Shaheen[3], Farya Zafar[3], Sadia Suri Kashif[1] and Najeeb Khatian[1]

[1]Faculty of Pharmacy, Ziauddin University, Karachi, Pakistan
[2]Dow College of Pharmacy, Dow University of Health Sciences, Karachi, Pakistan
[3]Faculty of Pharmacy and Pharmaceutical Sciences, University of Karachi, Pakistan

Abstract: A sensitive, reproducible and modest analytical procedure was developed and validated for evaluation of irbesartan in human plasma. LLE (Liquid-Liquid extraction) of the drug was carried out with acetonitrile (1:1 v/v). Chromatographic separation of irbesartan was conducted by the help of 4.0mm × 25cm column having L1 packing from plasma and mobile phase utilizing HPLC. The mobile phase comprise of phosphate buffer and acetonitrile in a ratio of 67:33 v/v. The flow rate was set at 1ml/minute and the detector at a wavelength of 220 nm. The resolution of irbesartan was well performed from plasma components. This method was validated and demonstrated linearity with a concentration range of 0.1to 6µg/ml of irbesartan in plasma. Intra-day, inter-day accuracy was found 89.33% to 96.37% while intra-day, inter-day precision was found within the limit of 0.02 and 2.15 respectively. The mean recovery of irbesartan was 97.28%. The efficacy of extraction was proved by above-mentioned results. In plasma, the 0.05 and 0.1µg/ml dilutions were exhibited as the LOD and LOQ of irbesartan. Stability studies disclosed that irbesartan showed stability at -20^{o}C storage.

Keywords: Irbesartan, liquid–liquid extraction, human plasma, HPLC, validation.

INTRODUCTION

Irbesartan (IRB) chemically termed as 2-butyl-3-[[4-[2-(2H-tetrazol-5-yl)phenyl]phenyl]methyl]-1,3-diazaspiro [4,4]non-1-en-4-oneutilized in hypertension. IRB is a non-tetrazole derivative peptide that selectively blocks the binding of angiotensin II to the AT1 receptor, it is also an angiotensin II receptor antagonist. The antagonism of angiotensin-II receptors gives an elevation of renin levels in plasma and angiotensin-II levels, plasma aldosterone concentration declines (Waniand Zargar, 2015). The binding of angiotensin II to the AT1 receptor, results in vasodilation and therefore decrease the effects of aldosterone.

In literature, various analytical procedures were found for the estimation of Irbesartan in biological fluids including liquid chromatography (Rao and Rao, 2016; Tiwari *et al*., 2013; Bae *et al*., 2009; Ferreiros *et al*., 2007), capillary electrophoresis (CE) (Vujic *et al*., 2012) and spectrophotometry (Russo *et al*., 2008). The basic method for estimation of irbesartan in human blood plasma is High-performance liquid chromatography (Anderson *et al*., 2017). A literature survey showed that there are variations in the limit of determination and quantitation (1-10µg/mL) according to various researchers (Rao and Rao, 2016; Caudron *et al*., 2004).

*Corresponding author: e-mail: syedimranali2009@gmail.com

Different procedures were adopted for extraction and preparation of samples in biological fluids. Literature showed various methods including solid-phase extraction (SPE), solid-phase microextraction (SPME), liquid-liquid extraction (LLE), and precipitation of protein (Bae *et al*., 2009; Kristoffersen *et al*., 2007; Erk, 2003). SPE is the principal method for biological samples, however, it is complex and requires more time. SPME is an ideal technique for preparation of small amount of samples, but it is not commonly used for samples of human plasma. However, although LLE is a time-consuming procedure although it is more simple and economical.

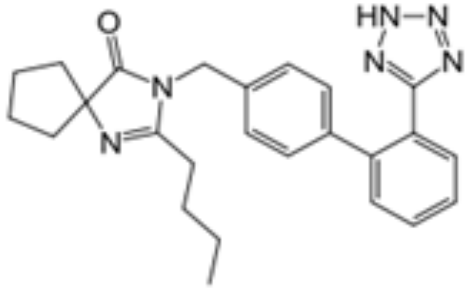

Fig. 1: Irbesartan

The current research work represents an HPLC method for estimation of IRB in biological samples with more accuracy, precision and sensitivity. In the current method, the extraction process is very much simple and less time-consuming.

MATERIALS AND METHODS

Chemicals

Irbesartan was gifted by Aventis Pharma (Pvt.) Limited, Karachi, Pakistan, Phosphoric acid, Triethylamine, Acetonitrile, Methanol (HPLC grade) from Merck (Darmstadt, Germany).

Instrumentation

High-performance liquid chromatographic system including Flow Pump LC-10AT VP, UV/VIS-Detector SPD-10AV VP, Communication Bus Module CBM 102 and Class-GC10 Software (Shimadzu, Kyoto, Japan), Sonicator (Nickel electro, Oldmixon Crescent, England) Syringe micro litre (Hamilton, Reno, Nevada), Analytical balance (Mettler-Toledo, Bekasi, Indonesia), Swinney membrane filter (Millipore, Billerica, USA), Spherisorb C18-column, 5µm, 250mm x 4.6mm (Waters, Milford, USA), Centrifugator (Biocompare, San Francisco , USA).

Chromatographic conditions

Mobile phase comprised of mixed phosphate buffer pH 3.2 and Acetonitrile (67:33 v/v). HPLC was equipped with 4.6mm × 25cm column having L1 packing with a guard-column of C18 packing. The flow rate was set at 1.0mL per minute used while detector adjusted at 220 nm.

Standard solutions

In methanol, the Standard Stock solution of Irbesartan in the concentration of 100µg/ml was prepared which was then further diluted and prepared working standard solution of 10µg/ml. Samples for calibration curve (0.1, 0.2, 0.4, 0.8, 1.5, 3.0 & 6.0µg/ml) as well as quality control (QC) samples (0.3, 2.5 & 5.0µg/ml) were prepared in blank plasma by serially diluting working standard.

Plasma protein precipitation for HPLC analysis

Acetonitrile was utilized for the extraction of the drug from plasma, for that purpose 1ml of acetonitrile incorporated into 1ml of spiked plasma and then vortex for a minute. Finally, centrifuge the above mixture for 5 minutes at 15,000rpm. The supernatant liquid was separate out into a new tube which was filtered through a 0.45µm membrane filter.

Method validation

The Developed analytical procedure was evaluated on various parameters including selectivity, specificity, linearity, precision, accuracy, recovery, sensitivity, stability, robustness and ruggedness as per requirements of FDA (Food and Administration, 2001).

Selectivity & specificity

Selectivity & Specificity were estimated by examining six different samples of blank plasma which obtained from dissimilar batches through multiple blood bank centers. Interfering of the endogenous components and metabolites was observed in blank plasma.

Linearity

For calibration curve spiked plasma samples were prepared by serial dilution in concentration of 0.1µg/ml to 6µg/ml. Linear regression was determine by plotting calibration curve in between plasma drug concentration and the peak area obtained. In addition to three consecutive days, linearity was measured at the beginning of each day on which analysis was performed and individually calibration curve was back calculated to determine actual concentration.

Precision & accuracy

Inter-day, intra-day accuracy and precision were estimated by replicate evaluation of quality control samples on three consecutive days. Compared to the theoretical concentration, accuracy was determined as a percent of the calculated concentration whereas precision was evaluated and quantified as % RSD.

Absolute & relative recovery

QC samples were compared with the samples of the drug in the mobile phase to determine absolute recovery. Furthermore, the relative recovery was evaluated by co-relate the observed concentration with that of theoretical concentration.

Sensitivity

The least possible concentration of drug which can be quantified in plasma with acceptable precision, accuracy & variability was evaluated to determine the sensitivity of the method. For that purpose, spiked plasma samples were analyzed up to the lower concentration of 0.01µg/ml.

Stability

Spiked plasma quality control samples were placed at different stations to determine the stability of the drug in plasma. For that purpose short term, long term, freeze and thaw drug stability was performed. QC samples were kept at 25 ± 2°C, for a time period estimated for plasma samples examination (approx. 10-12hrs) to evaluate short term stability. Furthermore, QC samples were kept at −20°C for one month to determine long-term stability. In addition to that, 15 QC samples (approx.) were kept for 24hrs at -20°C to determine Freeze and thaw stability.

Robustness & ruggedness

The developed method was also performed in two different laboratories and instruments by different analysts to determine the robustness and ruggedness of the method. During that process, various parameters including flow rate, wavelength, the ratio of mobile phase and temperature were varied to assess their impact on results.

Table 1: System suitability

Parameter	Mean (n=5)	Relative standard deviation (%)	Limit
Retention time	6.0	-	Less than 2
Area	44162.8	0.92	Less than 2
Theoretical Plates	7832	0.97	Less than 2
Tailing Factor	1.39	0.87	Less than 2

Table 2: Linearity

Analyte	Concentration (µg/ml)	*r^2	Equation of regression	y-intercept	**LOD (µg/ml)	***LOQ (µg/ml)
Irbesartan	0.1 – 6.0	0.9995	Y = 24265x – 384.82	384.82	0.01	0.05

*Regression, **Limit of detection, ***Limit of Quantification

Table 3: Accuracy, Precision

Characteristics	Selected concentrations in method validation (µg/ml)			
	0.3	0.6	4	8
	Intra-day			
Mean (n=5)	0.29	0.54	3.81	7.71
% Accuracy	95.33	89.33	95.25	96.37
Standard deviation	0.12	0.14	1.11	1.06
% coefficient of variance	1.9	0.16	0.04	0.02
	Inter-day			
Mean (n=5)	0.26	0.54	3.74	7.41
% Accuracy	87.66	89.16	93.50	92.62
Standard deviation	1.16	0.31	0.22	1.09
% coefficient of variance	0.21	1.68	2.15	0.24

Table 4: Limit of detection

Conc. (µg/ml)	Observed concentration (µg/ml)					Mean	Std. dev.	Coefficient of variance (%)	Accuracy (%)
	Sp-1	Sp-2	Sp-3	Sp-4	Sp-5				
0.1	0.09	0.09	0.09	0.13	0.09	0.98	0.08	1.39	98.0%
0.08	0.07	0.06	0.07	0.06	0.06	0.06	0.01	1.61	77.8%
0.05	0.041	0.030	0.033	0.034	0.028	0.033	0.001	3.03	66.4%
0.01	-	-	-	-	-	-	-	-	-

Table 5: Absolute recovery

S. No.	Conc. (µg/ml)	Peak Area (Mean)		Recovery (%)
		Plasma (n=5)	Mobile Phase (n=5)	
1	0.3	7438.4	7686.7	96.77
2	2.5	59195.2	60539.3	97.78
3	5.0	121460.7	124831.2	97.30
Mean				97.28

Table 6: Relative recovery

Characteristics	0.3µg/ml		2.5µg/ml		5.0µg/ml	
	Conc. obtained	Recovery %	Conc. obtained	Recovery %	Conc. obtained	Recovery %
Mean(n=3)	0.28	93.33	2.46	98.40	4.82	96.40
Std. dev.	0.02	4.51	0.05	2.17	0.04	2.35
%Coefficient of variance	5.13	5.13	3.19	3.19	3.87	3.87

Table 7: Short term stability

Parameter	Low concentration 0.5µg/ml			High concentration 5µg/ml		
Time	0hrs	6hr	12hr	0hrs	6hrs	12hrs
Mean(n=5)	0.509	0.501	0.496	5	4.96	4.87
Std. dev.	0.01	0.017	0.013	0.10	0.12	0.101
%Coefficient of variance	1.96	3.39	2.52	2	2.41	2.07
%Accuracy	100.8	100.2	99.2	100	99.2	97.40

Table 8: Long term stability

Parameter	Low concentration 0.5µg/ml			High concentration 5µg/ml		
	Fresh	2 weeks	3weeks	Fresh	2weeks	3weeks
Mean (n=5)	0.503	0.492	0.469	5.11	4.71	4.76
Std. dev.	0.011	0.012	0.012	0.136	0.008	0.014
%Coefficient of variance	2.18	2.43	2.55	2.66	1.69	0.29
%Accuracy	100.6	98.4	93.8	102.2	94.2	95.2

Table 9: Freeze-thaw stability

Characteristics	0.5µg/ml				5µg/ml			
	Fresh Sample	FT-1	FT-2	FT-3	Fresh Sample	FT-1	FT-2	FT-3
Mean (n=5)	0.494	0.484	0.475	0.478	4.92	4.79	4.92	4.71
Std. dev.	0.004	0.003	0.015	0.007	0.11	0.083	0.025	0.025
%Coefficient of Variance	0.80	0.61	3.15	1.46	2.23	1.73	0.508	0.530
%Accuracy	98.8	96.8	95.0	95.6	98.42	95.8	98.40	94.20

Table 10: Robustness

Parameters	Validated Specs.	Alterations	Peak Area Mean (n=3)	Std. dev.	Coefficient of variance (%)	Recovery (%)
Mobile Phase Composition	67 : 33	0	58998	263.94	0.15	-
		+0.2	59136	251.62	0.18	100.23
		-0.2	58520	260.75	0.16	99.19
Flow Rate	1.0ml/min	0	58998	263.94	0.15	-
		+0.2	58630	271.26	0.13	99.37
		-0.2	59071	268.15	0.12	100.12
Temperature	25°C	0	58998	263.94	0.15	-
		+2.5	58147	254.37	0.18	98.55
		-2.5	58762	261.83	0.17	99.60
Wave Length	220nm	0	58998	263.94	0.15	-
		+0.2	58831	270.78	0.14	99.72
		-0.2	58253	265.59	0.15	98.73

Table 11: Statistical Comparison of Methods

Characteristics	Test Value					
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
Limit of detection	1.005	1	0.498	9.52500	-110.8663	129.9163
Limit of quantification	1.004	1	0.499	26.55000	-309.5291	362.6291

RESULTS

Chromatography

The Chromatographic Peak of Irbesartan in plasma was recorded at 6 minutes (fig. 3) with satisfactory separation and no other peaks found in blank human plasma (fig. 2).

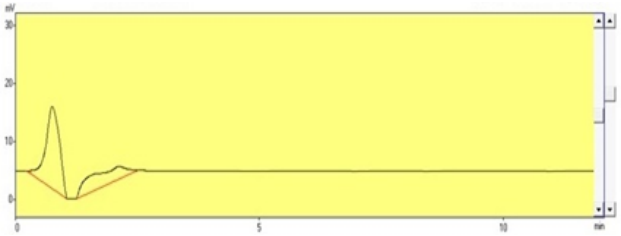

Fig. 2: Representative chromatogram of Blank Plasma

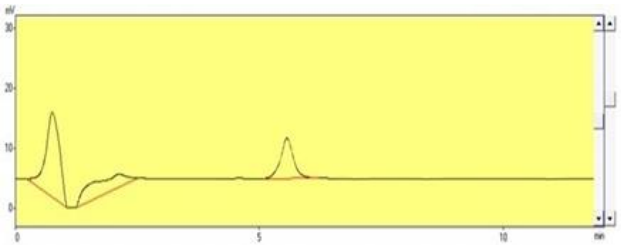

Fig. 3: Representative chromatogram of plasma spiked with Irbesartan

Linearity

Perfect and satisfactory linearity between irbesartan in blank plasma and in mobile phase was proved, the range of concentrations for the establishment of linearity was 0.1µg/ml to 6µg/ml (table 2 & fig. 4).

Accuracy & precision

The intra-day, inter-day accuracy optimized carefully and found perfect, i.e., within the limit of 89.33% and 96.37% respectively. Furthermore the intra-, inter-day precision found 0.02 and 2.15 respectively (table 3).

Limit of detection (LOD) & limit of quantification (LOQ)

Limit of detection and limit of quantification of irbesartan was found 0.05µg/ml and 0.1µg/ml respectively (table 4). The results were comparable with method validation of irbesartan in human plasma on LCMS.

Recovery

The absolute recovery was found 97.28% by comparing peak areas of a spiked plasma sample with the sample of the drug in mobile phase, while relative recovery was found 93.33% to 98.40% (tables 5 & 6).

Selectivity

At the retention time of drug, no other interfering peak was observed in blank plasma and spiked blood plasma as well so that perfect selectivity was claimed (fig. 2).

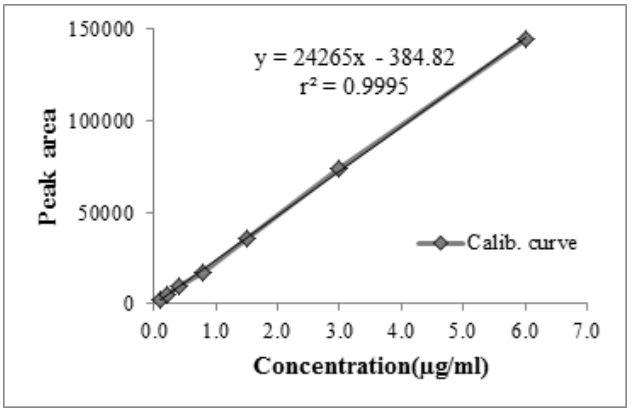

Fig. 4: Calibration curve at various concentration of Irbesartan Standard

Stability

The drug was found stable while analyzing the stability of the drug in plasma at different time intervals and at various temperatures in the short term, long term, and freeze-thaw stability as well, shown in (tables 7 to 9).

Robustness & ruggedness

The developed method was examined for robustness and ruggedness by varying various parameters and also the current method was analyzed by two different analysts in two different laboratories (table 10).

DISCUSSION

The major objective in development of present HPLC method is to access the identification and separation between irbesartan and other components. The method involves Liquid-Liquid extraction with the help and modification of pharmacopoeial method (USP, 2011). This HPLC method possesses numerous advantages, simple requirements, easy process of sample composition, good sensitivity and ease of handling in comparison with other methods including HPLC-tandem mass spectrometry (LC-MS-MS), HPLC with fluorescence detection and HPLC with UV detection. (Wani and Zargar, 2015; Rao and Rao, 2016; Gonzalez *et al* ., 2002; Shakya *et al*., 2007). In current method, preparation of plasma samples was achieved by simple extraction with acetonitrile. It has enhanced the rate of drug recovery.

The method was validated according to FDA guidelines and USP (USP, 2011; Food and Administration, 2001). The method has good selectivity which is elucidative for a description of irbesartan clearance in *in vivo*. The LOD was found 0.05µg/ml whereas LOQ was found 0.1µg/ml. LOD & LOQ Values of current method was compared with methods reported in literature (table 11). Limit of detection and limit of quantification of 1 to 2µg/ml was reported for irbesartan in dosage forms on UV Spectrophotometer (Kumar and Annapurna, 2015). LOD

& LOQ of irbesartan was found in the concentration of 0.019 & 0.053µg/ml in human plasma (Rane *et al*., 2010). In other reported methods, irbesartan was determined in combination with diuretic hydrochlorothiazide and medicinal agents using HPLC, LCMS, and Spectrophotometry (Kristoffersen *et al*., 2007; Gonzalez *et al*., 2002; Shakya *et al*., 2007; Zhang *et al*., 2006). High instrumentation and operations cost of LCMS makes it less favorable for analytical procedures. The current HPLC method seems more suitable for pre, post clinical pharmacokinetic studies and can be successfully used for the assessment of irbesartan in the mobile phase and Plasma in small concentrations. Furthermore, the current method effectively utilize for detection of drug in healthy volunteers in pharmacokinetic study.

CONCLUSION

A precise, rapid and consistent method for the analysis of irbesartan in biological fluids has been examined which met the criteria of validation successfully. The method of bioanalysis depicted its validity for *in vivo* analysis of irbesartan and gives more exact and specific results.

REFERENCES

Anderson JFF, Gerlin MCG, Sversut RA, Oliveira LCS, Singh AK, Amaral MS and Kassab NM (2017). Development and validation of an isocratic HPLC method for simultaneous determination of quaternary mixtures of antihypertensive drugs in pharmaceutical formulations. *Acta Chromatogr.*, **29**(1): 95-110.

Bae SK, Kim MJ, Shim EJ, Cho DY, Shon JH, Liu KH and Shin JG (2009). HPLC determination of irbesartan in human plasma: Its application to pharmacokinetic studies. *Biomed. Chromatogr.*, **23**(6): 568-572.

Caudron E, Laurent S, Billaud EM and Prognon P (2004). Simultaneous determination of the acid/base antihypertensive drugs celiprolol, bisoprolol and irbesartan in human plasma by liquid chromatography. *J. Chromatogr. B.*, **801**(2): 339-345.

Erk N (2003). Simultaneous determination of irbesartan and hydrochlorothiazide in human plasma by liquid chromatography. *J. Chromatogr. B.*, **784**(1): 195-201.

Ferreiros N, Iriarte G, Alonso RM and Jimenez RM (2007). Development of a solid phase extraction procedure for HPLC-DAD determination of several angiotensin II receptor antagonists in human urine using mixture design. *Talanta*, **73**(4): 748-756.

Food Drug and Administration (2001). *FDA Guidance for Industry: Bioanalytical Method Validation*. US Department of Health and Human Services, Food and Drug Administration, Centre for Drug Evaluation and Research (CDER), Centre for Vetrinary Medicine, Rockville, MD.

Gonzalez L, Lopez JA, Alonso RM and Jimenez RM (2002). Fast screening method for determination of angiotensin II receptor antagonists in plasma by high-performance liquid chromatography with fluorimetric detection. *J. Chromatogr. A.*, **949**(1-2): 49-60.

Kristoffersen L, Oiestad EL, Opdal MS, Krogh M, Lundanes E and Christophersen AS (2007). Simultaneous determination of 6 beta-blockers, 3 calcium-channel antagonists, 4 angiotensin-II antagonists and 1 antiarrhytmic drug in post-mortem whole blood by automated solid phase extraction and liquid chromatography mass spectrometry: method development and robustness testing by experimental design. *J. Chromatogr. B.*, **850**(1-2): 147-160.

Kumar JSP and Annapurna MM (2015). New spectrophotometric methods for the simultaneous determination of irbesartan and hydrochlorothiazide in combined dosage forms. *Pharm. Meth.,* **6**: 120-125.

Rane VP, Patil KR, Sangshetti JN, Yeole RD and Shinde DB (2010). Stability indicating LC method for simultaneous determination of irbesartan and hydrochlorothiazide in pharmaceutical preparations. *J. Chromatogr. Sci.*, **48**(7): 595-600.

Rao KT and Rao LV (2016). Rapid Stability-indicating RRLC Method for Simultaneous Estimation of Irbesartan and its Related Impurities. *Indian J. Pharm. Sci.,* **78**: 252-258.

Russo R, Guillarme D, Nguyen DTT, Bicchi C, Rudaz S, and Veuthey JL (2008). Pharmaceutical applications on columns packed with sub-2 µm particles. *J. Chromatogr. Sci.*, **46**(3): 199-208.

Shakya AK, Al-Hiari YM and Alhamami OM (2007). Liquid chromatographic determination of irbesartan in human plasma. *J. Chromatogr. B.*, **848**(2): 245-250.

Tiwari HK, Monif T, Verma PRP, Reyar S, Khuroo A H., and Mishra S (2013). Quantitative estimation of irbesartan in two different matrices and its application to human and dog bioavailability studies using LC-MS/MS. *Asian J. Pharm. Sci.,* **8**(6): 346-355.

USP 34 NF 29 (2011). The United States pharmacopeia and the National formulary. The United States Pharmacopeial Convention, Rockwille, MD.

Vujic Z, Mulavdic N, Smajic M, Brboric J and Stankovic P (2012). Simultaneous analysis of irbesartan and hydrochlorothiazide: an improved HPLC method with the aid of a chemometric protocol. *Molecules*, **17**(3): 3461-3474.

Wani TA and Zargar S (2015). New ultra-performance liquid chromatography-tandem mass spectrometry method for the determination of irbesartan in human plasma. *J. Food Drug Anal.* **23**: 569-576.

Zhang M, Wei F, Zhang YF, Nie J and Feng YQ (2006). Novel polymer monolith microextraction using a poly (methacrylic acid-ethylene glycol dimethacrylate) monolith and its application to simultaneous analysis of several angiotensin II receptor antagonists in human urine by capillary zone electrophoresis. *J. Chromatogr. A.*, **1102**(1-2): 294-301.