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Original Article

# LC-MS/MS ASSAY FOR IRBESARTAN IN HUMAN PLASMA USING SOLID PHASE EXTRACTION TECHNIQUE: A PHARMACOKINETIC STUDY

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**ABSTRACT**

**Objective:** The objective of this research was to develop a novel liquid chromatography/tandem mass spectrometry (LC-MS/MS) method for the determination of irbesartan in human plasma.

**Methods:** An analytical method based on liquid chromatography/tandem mass spectrometry (LC-MS/MS) has been developed and validated for the quantitative determination of irbesartan in human plasma. The method utilizes irbesartan d4 as internal standard (IS). After solid phase extraction (SPE), analyte and the IS were chromato graphed on a $C_{18}$ columns using a isocratic mobile phase composed of methanol–0.2% formic acid (85:15, v/v) pumped at a flow rate of 0.70 mL/min.

**Results:** Precision and accuracy of the method was determined using five analytical batches in the concentration range of 50.0–9982 ng/ml. All the validation experiments were carried out as per the US FDA guidelines and results met the acceptance criteria.

**Conclusion:** The proposed LC–MS/MS assay method is simple, rapid and enough sensitive for the determination of irbesartan in human plasma. A chromatographic run time set at 2.0 min, thus can analyze more than 300 samples in a day. Also, the proposed method was found to be applicable to clinical studies.

**Keywords:** Irbesartan, Solid–phase extraction (SPE), LC-MS/MS, Method validation, Pharmacokinetics.

## INTRODUCTION

Angiotensin II is the primary vasoactive hormone in the renin-angiotensin system. Its effects include vasoconstriction and the stimulation of aldosterone secretion by the adrenal cortex. Irbesartan antagonizes angiotensin II by blocking AT1 receptors. The drug used mainly for the treatment of hypertension. Irbesartan is an orally active agent, with bioavailability of 60-80%. Following oral administration, peak plasma concentrations are attained at 1.5-2 hours after dosing. The drug has the terminal elimination half-life of 11-15 hours [1, 2].

Literatures survey reveals, many analytical method based on liquid chromatography/tandem mass spectrometry (LC-MS/MS) [3-7], ultra-performance liquid chromatography-tandem mass spectrometry method (UPLC-MS/MS) [8, 9] and mass spectrometry with time-of-flight detection (MALDI-TOF MS) [10, 11] have been reported for the determination of irbesartan in a variety of biological samples. MALDI-TOF MS is a high throughput technique widely used in high molecular weight compound (such as proteins or polymers) analysis, but rarely used in low molecular weight compound analysis. Similarly, UPLC-MS/MS is an ultra-fast techniques possesses advantage of high sensitivity and high sample throughput over conventional LC-MS systems [12, 13]. Of all the methods reported above, only LC-MS/MS are comparable with the present work. Tutunji *et al.,* 2010 [3] reported a method for the simultaneous determination of irbesartan and hydrochlorothiazide in 250 µl of human plasma. Similarly, Prasad *et al.,* 2009 [4] published a method for the simultaneous determination of irbesartan and losartan in rat plasma. Both the methods employs protein precipitation (PP) technique for sample preparation with the chromatographic run time of>4 min, which may not be suitable for routine drug analysis. PP is most likely to cause ion suppression and not efficient to remove the endogenous compounds such as lipids, phospholipids and fatty acids completely. Another method reported by Ganesan *et al.,* 2010 [5] for the determination of irbesartan in 100 µl of human plasma, this method employs liquid–liquid (L–L) extract, evaporation, drying and reconstitution for sample preparation. Ferreirós *et al.,* 2007 [6] reported a LC-MS/MS method for the quantitation of angiotensin II receptor antagonists (ARA-II) in human plasma. This method is not specific for the irbesartan and it may create conflicts in the final results due to improper characterization of selectivity. A promising method was reported by Tiwari *et al.,* 2013 [7] for the determination in irbesartan in human and dog plasma samples with the linearity range of 12.1 to 6018.7 ng/ml using candesartan as internal standard. However, this method is also having the limitation in chromatographic run time>3 min, which may not be suitable for the high through put bio analysis of irbesartan. The analytical method should gratify the scientists in terms of simplicity, sensitivity, runtime, sample volume, time consumption and efficient extraction procedure.

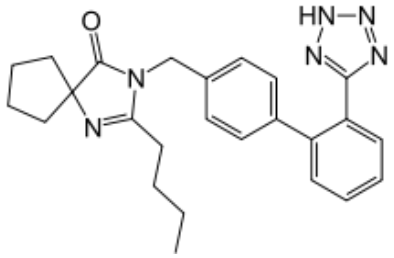
The present paper describes a simple, selective and sensitive liquid chromatography–electro spray ionization tandem mass spectrometry (LC–MS/MS) assay method for the determination of irbesartan in human plasma using irbesartan d4, as an internal standard (IS). Use of isotope labeled compounds as internal standards minimizes matrix effect related problems and variability in recovery between analyte and the IS. This method requires only 50 µl human plasma for solid–phase extraction (SPE) technique without drying, evaporation and reconstitution steps, and minimum usage of organic solvents and demonstrates excellent performance in terms of ruggedness with a sample cut off time 2.0 min.

## MATERIALS AND METHODS

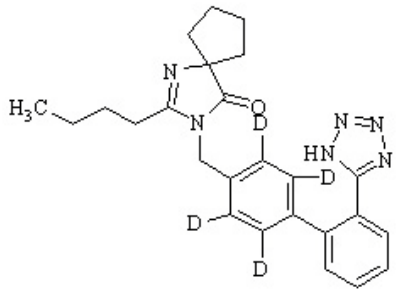
### Standards and chemicals

The reference sample of irbesartan (99.44%) was obtained from Hetero Drugs Limited (Hyderabad, India), while irbesartan d4 (97.90%) was from Clearsynth Ltd., (Mumbai, India). Their structures are presented in fig. 1. Water used for the LC–MS/MS analysis was prepared by using Milli Q water purification system procured from Millipore (Bangalore, India). HPLC grade methanol was purchased from J. T. Baker (Phillipsburg, USA). Analytical grade

formic acid and ammonium acetate was purchased from Merck Ltd (Mumbai, India). The control $K_2$ human plasma sample was procured from Deccan's Pathological Lab's (Hyderabad, India).



**Irbesartan**



**Irbesartan d4 (IS)**

**Fig. 1: Chemical structures of irbesartan and irbesartan d4 (IS)**

**LC–MS/MS instrument and conditions**

An HPLC system (Shimadzu, Kyoto, Japan) equipped with a Hypurity Advance (50 mm × 4.6 mm, 5 µm) (Make: Thermo scientific), a binary LC–20AD prominence pump, an auto sampler (SIL–HTc) and a solvent degasser (DGU–20A3) was used for the study. An aliquot of 15 µl of the processed sample was injected in to the liquid chromatographic system. An isocratic mobile phase composed of a mixture of 0.2% formic acid and methanol (15:85, v/v) was used to separate the analyte from the endogenous components and pumped at a flow rate of 0.70 mL/min (with splitter) into the electro spray ionization chamber of the mass spectrometer. Quantification was achieved with MS–MS detection in positive ion mode for the analyte and the IS using an AB Sciex API–3000 mass spectrometer (Foster City, CA, USA) equipped with a Turboion spray ™ interface at 500 °C. The ion spray voltage was set at 5000 V.

The source parameters viz. the nebulizer gas (GS1), curtain gas and collision gas were set at 5, 15, and 7 psi, respectively. The compound parameters viz. the declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP) were 22, 35, 10, 12 V for irbesartan and for the IS. Detection of the ions was carried out in the multiple–reaction monitoring mode (MRM), by monitoring the transition pairs of *m/z* 429.5 precursor ion to the *m/z* 207.0 for irbesartan and *m/z* 433.5 precursor ion to the *m/z* 211.0 production for the IS. Quadrupoles (Q1 and Q3) were set on unit resolution. Dwell time was set at 200 ms. The analysis data obtained were processed by Analyst software™ (version 1.4.2).

**Preparation of calibration curve standards and quality control samples in human plasma**

Two standard stock solutions of irbesartan were prepared separately in HPLC grade methanol (1 mg/ml) for the preparation of calibration curve standards and quality control samples, respectively. Further dilutions of analyte were prepared in a mixture of methanol and water (50:50, v/v; diluent). A 1 mg/ml of irbesartan d4 stock solution was prepared by dissolving the compound in HPLC grade methanol. The working concentration of irbesartan d4 (25.0 ng/ml) was prepared from the above stock solution using the diluent.

Calibration samples were prepared by spiking 950 µl of control $K_2$ EDTA human plasma with the 50 µl working standard solution of the analyte as a bulk, to obtain riluzole concentration levels of 50.0, 100, 200, 500, 1000, 2000, 4001, 5989, 7986 and 9982 ng/ml as a single batch at each concentration. Similarly, quality control (QC) samples were also prepared as a bulk based on an independent weighing of standard drug, at concentrations of 50.3 (lower limit of quantitation quality control, LLOQ QC), 144 (low quality control, LQC), 1105 (medium quality control, MQC1), 5022 (MQC2) and 8968 ng/ml (high quality control, HQC) as a single batch at each concentration.

**Sample processing procedure**

A 50 µl aliquot of human plasma sample was mixed with 10 µL of the internal standard working solution (25.0 ng/ml of irbesartan d4). To this, 100 µL of 2% formic acid was added. The extraction cartridges (Strata-X, 33 µm polymeric sorbent) were conditioned with 1.0 mL of methanol followed by 1.0 mL of water and 1.0 mL of 2% formic acid solution. Then, the entire sample mixture was loaded on to a cartridge and elute with a gentle stream of nitrogen. The extraction cartridge was washed with 1.0 mL of 100 mM ammonium acetate followed by 1.0 mL of water. Analyte and the IS were eluted with 0.50 mL of mobile phase. Aliquot of 15 µL of the extract was injected into the LC-MS/MS system.

**Method validation parameters**

A complete and through, method validation was carried out as per recent US FDA guidelines [14]. The parameters determined were carryover test, matrix effect, selectivity, sensitivity, linearity, precision and accuracy, dilution integrity, recovery, and various stabilities.

**Pharmacokinetic study protocol design**

The proposed method was successfully applied to a pharmacokinetic study in South Indian male subjects ($n$ = 7). The subjects with body body–mass index (BMI) of ≥18.5 kg/m$^2$ and ≤24.9 kg/m$^2$, with body weight not less than 50 kg were selected for the study. All the subjects selected are in the age group of 20-40 years and were fasten for a minimum of 12 prior to drug formulation administration. The protocol approved by the Ethics Committee and subjects provided with informed consent. A 300 mg tablet of irbesartan was administered orally with 200 mL of water. Blood samples were collected at predose and 0.33, 0.67, 1, 1.33, 1.67, 2, 2.33, 2.67, 3, 3.33, 3.67, 4, 4.33, 4.67, 5, 5.5, 6, 8, 10, 12, 16, 24 and 36 h of post dose in K2 EDTA vacutainer (5 mL) collection tubes (BD, Franklin, NJ, USA). The plasma was collected by centrifugation of blood samples at 3200 rpm for 10 min and stored at–70±10 °C till their use. Win Nonlin software (Version 5.2) was used to calculate the pharmaco kinetic parameters of irbesartan by employing non–compartmental model were employed for the preset study.

**RESULTS AND DISCUSSION**

**Mass spectrometry**

Method development starts with the setting of the mass spectrometric parameters. A 100 ng/ml solution of irbesartan and the IS were injected in to the ESI source of the mass spectrometer using infusion pump. Analytes were tuned in positive and negative ionization modes, but the highest intense signals and stable response was obtained in the positive mode. Data acquisition was performed in the multiple reaction monitoring (MRM) modes to obtain better selectivity from the endogenous components. The source dependent parameters such as curtain gas, collision gas, nebulizer gas and auxillary gas were suitably altered to get satisfactory and reproducible response. Likewise, compound dependent parameters namely collision energy, declustering potential and collision cell exit potential were altered to get most intense and consistent product ion Q3 MS spectra of analyte and the IS. Finally, the most sensitive mass transition was observed from m/z 429.5 to 207.0 for irbesartan and from m/z 433.50 to 211.0 for the IS. The dwell time for each transition was set at 200 ms.

**Optimization of chromatographic conditions**

Proper selection of mobile phase, flow rate and analytical column resulted improved sensitivity and selectivity of an analytical assay. A variety of volatile buffers like ammonium acetate and ammonium

formate in varying the strengths from 1 mM–20 mM were evaluated for their suitability in combination with the organic solvents such as methanol and acetonitrile as a isocratic mobile phase. But the results obtained were not satisfactory in terms of reproducibility and sensitivity. Hence, the acidic buffers namely formic acid and acetic acid was tried in combination with methanol and acetonitrile using different analytical columns. Finally, an isocratic mobile phase composed of 0.2% formic acid and methanol (15:85, v/v) and Hypurity advance (50 mm × 4.6 mm, 5 µm) column gave good peak shape and response even at lowest concentration level for the analyte and the IS. The retention time was obtained for the analyte and the IS was 1.3 min with the flow rate of 0.70 mL/min. The total chromatographic time was set at 2 min.

For LC–MS analysis, there must be an appropriate extraction technique which can yields maximum recovery with minimal or no matrix effect. Hence, SPE was tried with Starata X polymeric sorbent, Oasis HLB, Bond Elut Plexa and Orpheus C18 extraction cartridges with/without acidic buffer addition to obtain the clean sample and to remove the possible interferences. Additionally, SPE provides clear extracts than the PP and LLE and the influence on sensitivity is significantly less. Of all the above, promising results were obtained with Starata X polymeric sorbent cartridge, which can produce a clean chromatogram for a blank sample and yields the highest recovery for the analyte and the IS from the plasma. Stable labeled isotopes as internal standard are helpful in minimizing the matrix effect and limit the variable recovery between analyte and the IS. Also, these standards will increase the bio analytical assay precision and accuracy. Hence, in the present work irbesartan stable labeled isotope irbesartan–d4 was employed as an internal standard.

**Chromatography and selectivity**

Method selectivity was assessed by analyzing the 8 extracted blank human plasma samples derived from the 8 individual sources. As displayed in the fig. 2A, no momentous direct interference in the blank plasma traces was observed from endogenous substances in drug–free human plasma at the retention time of irbesartan and the IS. Moreover, no significant interference was found from the IS to the MRM channel of the analyte (fig. 2B). Fig. 2C depicts a representative LLOQ sample ion–chromatogram for irbesartan along with the IS (50.0 ng/ml). A typical chromatograms resulting from the analysis of the subject blank plasma sample and 2 h subject plasma sample after the administration of a 300 mg oral single dose of irbesartan is shown in the fig. 3. The sample concentration was determined to be 3460 ng/ml.

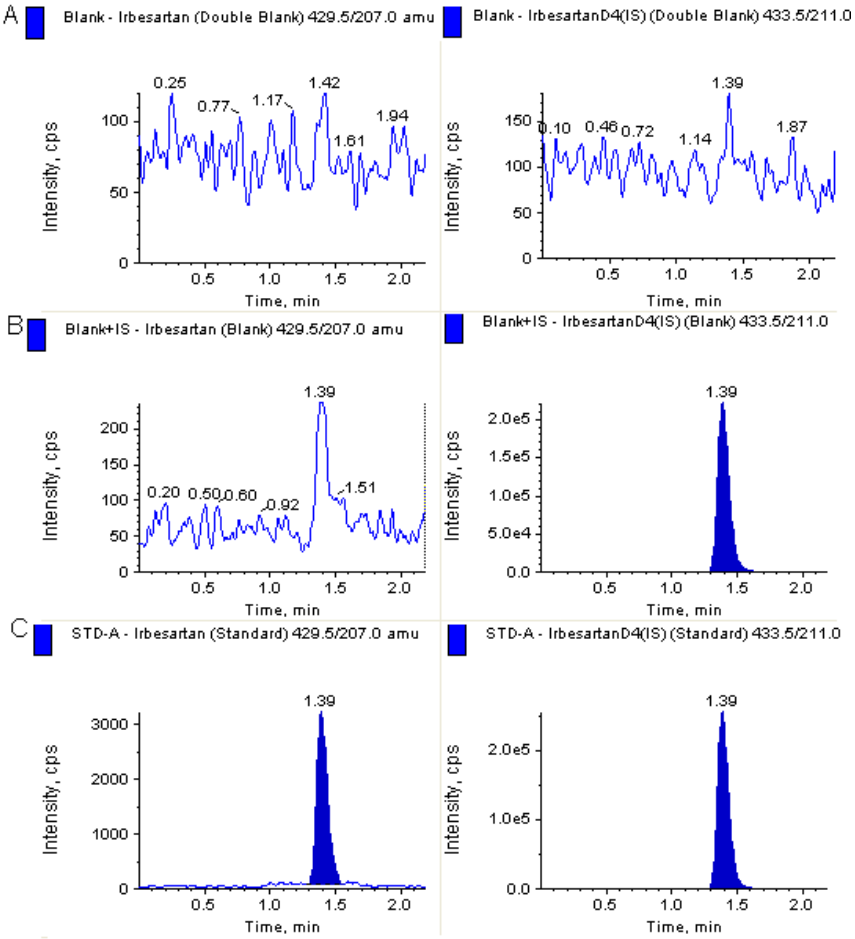



**Fig. 2: A representative MRM channel chromatograms of irbesartan (left panel) and the IS (right panel) in human blank plasma (A), and human plasma spiked with IS (B), a LLOQ sample along with IS**

**Matrix effect**

Matrix effect experiment was conducted using 8 human plasma lots including one hemolytic and lipemic plasma obtained from the 8 different sources. The average matrix factor valve calculate as the response of the post spiked sample/response of neat sample at LQC and HQC level was 1.00 and 1.01, which indicated negligible suppression or enhancement.

**Sensitivity**

Sensitivity of an analytical method can also be called as the lowest limit of reliable quantification (LLOQ) for the analyte. The LLOQ of the proposed method was set at 50.0 ng/ml. At this concentration, the precision and accuracy results were found to be 3.99% and 100%, respectively. The signal–to–noise ratio (S/N) at LLOQ level was ≥10.

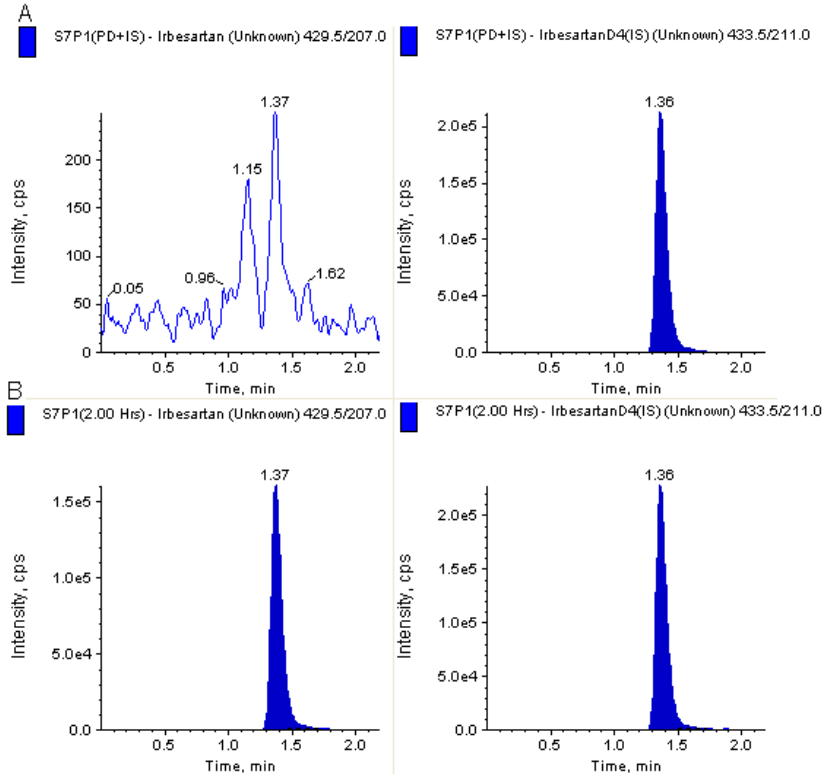



**Fig. 3: A model MRM channel chromatograms resulting from the analysis of the subject blank plasma sample along with the IS (A) and 2.0 h subject plasma sample (B), after the administration of a 300 mg oral single dose of irbesartan tablet**

**Linearity, precision and accuracy**

A total of 5 successful calibration curves were generated during the entire course of validation. Linearity of the method was established in the concentration range of 10.0–10005 ng/ml. A correlation coefficient ($r^2$) of ≥ 0.9936 was obtained for all the runs. Each CC was analyzed individually by least square weighted ($1/x^2$) linear regression as it produces the best fit for the concentration–detector response relationship. The precision and accuracy for calibration standards ranged from 96.0% to 106% and 1.35% to 6.31%, respectively.

The intra–day precision (%CV) ranged from 0.87 to 4.39% and the accuracy was within 103–109%. For inter–day tests, the precision (%CV) varied from 4.05 to 7.90% and the accuracy was within 98.1–104%. The results for intra–day and inter–day precision and accuracy results in 6 different quality control levels are summarized in table 1.

**Table 1: Precision and accuracy data for irbesartan**

| Quality control | Run | Concentration found | % CV | Accuracy (%) |
|---|---|---|---|---|
| **Intra–day variations (12 replicates at each concentration)** | | | | |
| LLOQ | | 51.7 ± 2.27 | 4.39 | 103 |
| LQC | | 150 ± 2.89 | 1.92 | 105 |
| MQC1 | | 1208 ± 12.6 | 1.04 | 109 |
| MQC2 | | 5391 ± 48.2 | 0.89 | 107 |
| HQC | | 9395 ± 81.8 | 0.87 | 105 |
| **Inter–day variations (30 replicates at each concentration)** | | | | |
| LLOQ | | 49.3 ± 3.89 | 7.90 | 98.1 |
| LQC | | 142 ± 7.69 | 5.42 | 98.8 |
| MQC1 | | 1153 ± 71.1 | 6.17 | 104 |
| MQC2 | | 5165 ± 209 | 4.05 | 103 |
| HQC | | 9003 ± 416 | 4.62 | 100 |

Spiked concentrations of LLOQ, LQC, MQC1, MQC2 and HQC are 50.3, 144, 1105, 5022, and 8968 ng/ml, respectively

**Recovery**

The recovery results obtained with the SPE procedure for the analyte and the IS was good and reproducible. The mean recovery of irbesartan was 84.8±5.55% with the precision (%CV) range of 0.70–1.43% and the recovery of the IS was 81.4%.

**Dilution integrity**

The real time plasma sample concentrations obtained above the upper limit of quantitation (ULOQ) are can be quantified by dilution the samples with the blank matrix. The upper concentration limit of irbesartan can be extended to 16940 ng/ml by using two–and four–fold dilution with screened human blank plasma. The precision (%CV) for dilution integrity of two–and four–fold dilution was found to be 0.87% and 0.95%, whereas the accuracy results were found to be 105% and 88.6%, respectively.

**Stability experiments**

Analyte stability in plasma samples under different conditions such as bench top stability (12 h), repeated freeze–thaw cycles (4 cycles)

and long–term stability at–70 °C for 95 days was evaluated during the method validation. Additionally, processed samples stability (Reinjection stability 75 h, auto sampler stability 48 h and wet extract stability at 2–8 °C 42 h) were also evaluated. The samples were considered stable when the mean % nominal values of the analyte were found to be within ±15% of the predicted concentrations for the analyte at their LQC and HQC levels. The results obtained by the various stability tests were summarized in the table 2. Therefore, the results were found to be within the acceptable limits during the entire validation.

**Table 2: Stability data for irbesartan in plasma (*n*=6)**

| Stability test | QC (spiked concentration (ng/ml) | Concentration found (ng/ml) | %CV | Accuracy/Stability (%) |
|---|---|---|---|---|
| Bench top[a] | 144 | 143± 1.98 | 1.39 | 99.5 |
| | 8968 | 8980 ± 101 | 1.12 | 100 |
| Process[b] | 144 | 143 ± 2.94 | 2.06 | 99.4 |
| | 8968 | 8989 ± 116 | 1.30 | 100 |
| Process[c] | 144 | 141 ± 2.49 | 1.76 | 98.4 |
| | 8968 | 8968 ± 115 | 1.28 | 100 |
| FT[d] | 144 | 142 ± 2.38 | 1.67 | 98.9 |
| | 8968 | 8969 ± 161 | 1.79 | 100 |
| Re–injection[e] | 144 | 147 ± 3.51 | 2.38 | 103 |
| | 8968 | 9555 ± 54.0 | 0.56 | 107 |
| Long–term[f] | 144 | 140 ± 1.68 | 1.20 | 97.2 |
| | 8968 | 9367± 88.9 | 0.95 | 104 |

[a]after 12 h at room temperature; [b] after 42 h at 2-8 °C; [c] after 48 h in auto sampler at 10°C; [d] after 4 freeze and thaw cycles; [e] after 75 h of Reinjection; [f] at–70 °C for 95 days

**Application of the proposed method**

The validated method was then successfully applied to quantify irbesartan plasma concentration for a pharma cokinetic study in healthy adult male subjects (*n*=7) under fasting condition. The obtained pharma cokinetic results are summarized in the table 3 and the mean plasma concentration *vs* time profile of irbesartan in health subjects was shown in the fig. 4. The obtained pharmacokinetic results were in close proximity when compared with the earlier reports [7]. An incurred sample reanalysis (ISR) conducted to authenticate the subject sample concentrations. The ISR results obtained were less than 15% difference in concentrations (table 4), shows the proposed method is good and reproducible.

**Table 3: Pharmacokinetic parameters of irbesartan after single oral administration of 300 mg tablet to healthy subjects (*n*=7, mean±SD)**

| Parameter | Estimated value |
|---|---|
| $C_{max}$ (ng/ml) | 3852±492 |
| $t_{max}$ (h) | 2.00±0.47 |
| $AUC_{0-t}$ (ng h/ml) | 15605±4319 |
| $AUC_{0-inf}$ (ng h/ml) | 16281±4214 |
| $t_{1/2}$ (h) | 10.7±4.52 |

**Table 4: Incurred samples re–analysis data of irbesartan.**

| Sample | Initial conc. (ng/ml) | Re–assay conc. (ng/ml) | Difference[a] (%) |
|---|---|---|---|
| 1 | 3595 | 3440 | 4.42 |
| 2 | 170 | 150 | 12.5 |
| 3 | 3914 | 3951 | -0.94 |
| 4 | 157 | 147 | 6.76 |
| 5 | 4219 | 4279 | -1.43 |
| 6 | 186 | 165 | 11.5 |
| 7 | 3990 | 3689 | 7.83 |
| 8 | 163 | 154 | 5.53 |
| 9 | 3141 | 3096 | 1.46 |
| 10 | 278 | 269 | 3.29 |
| 11 | 3399 | 3277 | 3.68 |
| 12 | 156 | 159 | -1.80 |
| 13 | 4348 | 4098 | 5.91 |
| 14 | 254 | 258 | -1.67 |

[a] Expressed as [(initial conc. –re–assay conc.)/average]×100%.

**CONCLUSION**

The LC–MS/MS assay presented in this paper is simple, rapid and enough sensitive for the determination of irbesartan in human plasma suitable for pharma cokinetic or bioavailability/ bioequivalence application in humans. The method was fully validated as per the recent US FDA guidelines. Sample extraction with SPE technique with direct injection devoid drying and reconstitution steps gave good and reproducible recoveries and provides effective sample cleanup. The total run time per analysis of each sample is 2.0 min which allows analysis of more than 300 samples in a single day. The method showed suitability for pharma cokinetic studies in humans. From the results of all the validation parameters, we can conclude that the developed method can be useful for bioavailability and bioequivalence (BA/BE) studies and routine therapeutic drug monitoring with the desired precision and accuracy.

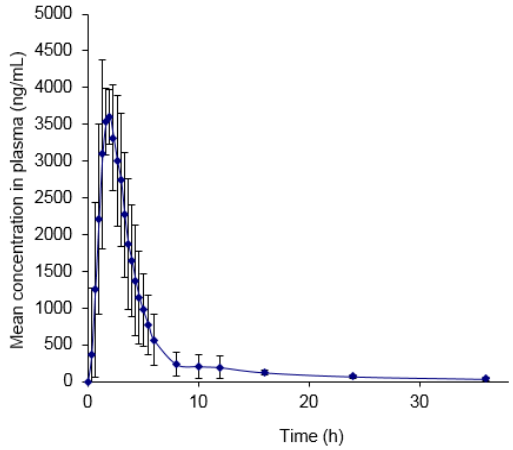



**Fig. 4: Mean plasma concentration–time profile of irbesartan in human plasma following oral administration of irbesartan (300 mg tablet) to healthy volunteers (*n*=7)**

**ACKNOWLEDGEMENT**

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**CONFLICTS OF INTERESTS**

The authors declare no conflict of interest

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