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A Liquid Chromatography Tandem Mass Spectrometry based method for the Simultaneous Determination of Irbesartan and Hydrochlorothiazide in Human Plasma

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Abstract:
The aim of current study was to develop and validate a rapid, explicit and vigorous assay based on solid phase extraction and liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI MS-MS) for the simultaneous quantitative analysis of Irbesartan and Hydrochlorothiazide in human plasma using Losartan and Hydroflumethiazide as internal standards (IS). The precursor to product ion transitions of m/z 427.3/ 193.0 and m/z 295.8/ 205.1 were used to measure the Irbesartan and Hydrochlorothiazide respectively. The method was validated over a concentration range of 99.9 to 6274.0 ng mL^{-1} for Irbesartan and 3.18 to 500.45 ng mL^{-1} for Hydrochlorothiazide. The method was validated over the parameters like selectivity, matrix effect, sensitivity, linearity, precision and accuracy various stabilities (bench top stability, standard stock solution stability, stock dilution stability, auto sampler stability, freeze thaw stability, long term stability), effect of potentially interfering drugs, dilution integrity, recovery and reinjection reproducibility. The mean % recovery of Irbesartan, Hydrochlorothiazide, Losartan and Hydroflumethiazide were 89.03 %, 83.15 %, 88.89 % and 84.89 % with a precision of 9.39 %, 2.79 %, 4.36 % and 2.12 % respectively. The RSD % of intra-day and inter-day assay was ≤15%. The application of this assay was demonstrated in a bioequivalence study after an oral administration of a tablet containing higher dose of Hydrochlorothiazide and Irbesartan in healthy volunteers.

Keywords: Irbesartan, Hydrochlorothiazide, electrospray ionization, tandem mass spectrometry, human plasma, bioequivalence study.

Introduction

Hydrochlorothiazide, 6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide- 1,1-dioxide is a diuretic of benzothiadiazine class, extremely useful in the treatment of edema, hypertension and hypercalciuria(1,2). Hydrochlorothiazideis an angiotensin II receptor antagonist (3). Irbesartan is a non-peptide compound, chemically described as 2-butyl-3-(p-(o-1H-tetrazol-5-ylphenyl)benzyl)-1,3-diazaspiro(4.4)non-1-en-4-one(1). Irbesartan is a potent and selective angiotensin II receptor antagonist indicated for the treatment of patients with hypertension, nephropathy and type 2 diabetes mellitus (4,5).

Market survey revealed that, new drugs and their combination with another drugs are being introduced in market as they have more patient compliance than a single drug. Combinations of two or more drugs in the pharmaceutical dosage forms are very much useful in Multiple therapies (6). Combination therapy of irbesartan and hydrochlorothiazide can decrease side effects and improve compliance with reduce cost. The various fixed dose combination were approved for Hydrochlorothiazide and irbesartan for 12.5 mg /150 mg, 12.5 mg /300 mg and 25 mg /300 mg

Full Length Original Research Paper

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respectively, for patients with hypertension who do not respond properly to monotherapy of either drug. The US FDA accept waiver request of in-vivo testing with proportional similarity of the formulations across all strength in all other lower dose combination if bioequivalence of higher strength are acceptable [7]. Hence, method development has challenge for their analysis with the help of number of analytical techniques that are available for the estimation of the individual drugs and their combination.

As per literature survey, several analytical methods have already been developed for the determination of Hydrochlorothiazide and Irbesartan either individually or in combination with other drugs in plasma including liquid chromatography tandem mass spectrometry (LC–MS– MS) and HPLC-UV [8-11]. There are various methods reported for Irbesartan and Hydrochlorothiazide in liquid chromatography tandem mass spectrometry (LC–MS– MS), which has time consuming, low sensitivity, low recovery and improper linearity range, which are not sufficient for analysis higher strength combination of Irbesartan and Hydrochlorothiazide [8-11]. The purpose of the current study was to develop and validate a sensitive, robust and a rapid LC–ESI-MS/MS method for simultaneous determination of Hydrochlorothiazide and Irbesartan in human plasma over a wide range, which could make it applicable for use in a bioequivalence study of all strength.

EXPERIMENTAL

Materials and Method

Irbesartan (99.6% w/w on as is basis), Losartan Potassium (99.0% w/w on as is basis) and Hydrochlorothiazide (99.4% w/w on as is basis) were obtained from Clearsynth Labs Ltd, India. Hydroflumethiazide (97% w/w on as is basis) was obtained from Sigma-Aldrich, India. HPLC-grade acetonitrile and methanol were purchased from SD Fine Chem. Ltd. (Mumbai, India). Formic acid were purchased from Merck (Fluka Chemie, GmbH, Germany). Milli-Q water (8.2m Ω(milliohm) and TOC ≤ 50 ppb (parts per billion)) obtained from Milli-Q system (Millipore SAS, Molsheim, France). Hypersil Gold, 5 µm (50*4.6 mm) HPLC column was purchased from Thermo Scientific, USA. All other reagents and chemicals used for these studies were HPLC grade unless specified. The potentially interfering drugs used during the course of validation were obtained from Toronto Research Chemicals Inc. (Acetaminophen), Merck Ltd (Caffeine), Clearsynth Labs (P) Ltd (Acetyl Salicylic Acid) and Vivan Life Sciences Pvt. Limited (Ibuprofen). The HLB cartridges (30 mg/1cc) used for the extraction of analytes along with internal standards were procured from Waters Corporation, Milford, MA, USA.

Instrumentation

LC MS-MS analysis was performed using API 4000 triple quadrupole instrument (Applied Biosystems MDS SCIEX, Toronto, Canada) coupled with Shimadzu HPLC system (Shimadzu SIL HTC, USA) in multiple reaction monitoring (MRM) mode. A turbo electrospray interface in positive ionization mode was used for ionization. Data processing was performed on Analyst software version 1.4.2 (Applied Biosystems MDS SCIEX, Toronto, Canada).

Standard and Quality Control Sample Preparation

Primary stock solution of Irbesartan and Hydrochlorothiazide, for preparation of calibration standard and quality control (QC) samples were

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prepared separately. The primary stock solution of Irbesartan (1 mg mL^{-1}), Hydrochlorothiazide(1 mg mL^{-1}), Losartan (1 mg mL^{-1}) and Hydroflumethiazide (1 mg mL^{-1}) were prepared in methanol. The stock solution of internal standards were diluted to concentration of (approximately 50000.0 ng mL^{-1} and 1000.00 ng mL^{-1} for Losartan and Hydroflumethiazide respectively) with diluent solution (Methanol: Milli-Q/HPLC Grade Water:: 50:50, v/v). Aqueous dilutions for spiking were prepared by serially diluting the primary stock solution of Irbesartan and Hydrochlorothiazide with diluent solution (Methanol: Milli-Q/HPLC Grade Water: 50:50, v/v). Spiking of aqueous dilutions in human plasma was done to give eight-point calibration curve, (99.9 to 6274.0 ng mL^{-1}) for Irbesartan and (3.18 ng mL^{-1} to 500.45 ng mL^{-1}) for Hydrochlorothiazide. In a similar way spiking of aqueous quality control dilutions were done in human plasma to prepare the quality control samples consisting of Irbesartan concentrations of 99.9 ng mL^{-1} (LLOQ QC), 263.6 ng mL^{-1} (LQC), 3194.8 ng mL^{-1} (MQC) and 4557.4 ng mL^{-1} (HQC) and for Hydrochlorothiazide 3.18 ng mL^{-1} (LLOQ QC), 8.14 ng mL^{-1} (LQC), 246.65 ng mL^{-1} (MQC) and 362.72 ng mL^{-1} (HQC). Primary stock solutions were kept at 2-12°C when not in use. Spiked calibration standards and QC samples were stored below –50°C.

Sample Preparation

Sample preparation involved aliquoting of 300 µl of plasma into ria vials and addition of 25 µl internal standard dilution (containing Losartan approximately 50000 ng/mL and Hydroflumethiazide approximately 1000 ng/ml), followed by vortexing for a 1 minute. The samples were loaded on conditioned HLB cartridge (30 mg/1cc) with 1.0 ml methanol followed by 1.0 ml of Milli-Q water. This was followed by washing the cartridges with 1 ml washing solution (Methanol: Milli-Q Water :: 05:95, v/v). The cartridges were than dried for approximately 2 minutes. The samples were eluted from dried cartridges 1.0 ml of Methanol into elution tubes. The elute was evaporated to dryness at 50 °C & at constant pressure in nitrogen evaporator followed by reconstitution of the dried samples in 300 µl of mobile phase, Vortex and transfer to HPLC vials for analysis.

Chromatographic and Mass Spectrometric Conditions

The analytes were chromatographically separated using reversed-phase high-performance liquid chromatography (HPLC) with isocratic elution. The mobile phase consisted of Methanol:Buffer Solution (0.1% Formic Acid in Milli-Q Water) :: 90:10 v/v)), which was used at a flow rate of 0.6 mL min-1. The chromatographic separation was performed using Hypersil Gold 50*4.6, 5µm column. For all analyses 10 µL of extracted sample was injected. The total run time of 2.5 minute was found suitable for retaining and separating the analytes from each other and associated interference. The mass spectrometer was operated in the electro spray ionization mode with negative ion detection to monitor the ions with m/z 427.3/ 193.0 for Irbesartan, m/z 295.8/ 205.1 for Hydrochlorothiazide, m/z 421.1/ 126.7 for Losartan and m/z 329.8/238.8 Hydroflumethiazide Fig.1. The source parameters for Irbesartan, Hydrochlorothiaizde, Losartan and Hydroflumethiazide optimized were Curtain gas (CUR): 8 psi, ion spray voltage (IS): -3000 V, source temperature (TEM): 450°C, collision gas (CAD): 6 psi, nebulizer gas (NEB): 12 psi, while the declustering potential (DP), focusing potential

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(FP), collision energy (CE) and cell exit potential (CXP) applied were -80, -123, -18 and -12 V for the Irbesartan and Losartan . The declustering potential (DP), focusing potential (FP), collision energy (CE) and cell exit potential (CXP) applied were -48, -139, -35 and -13 V for the Hydrochlorothiazide and Hydroflumethiazide.

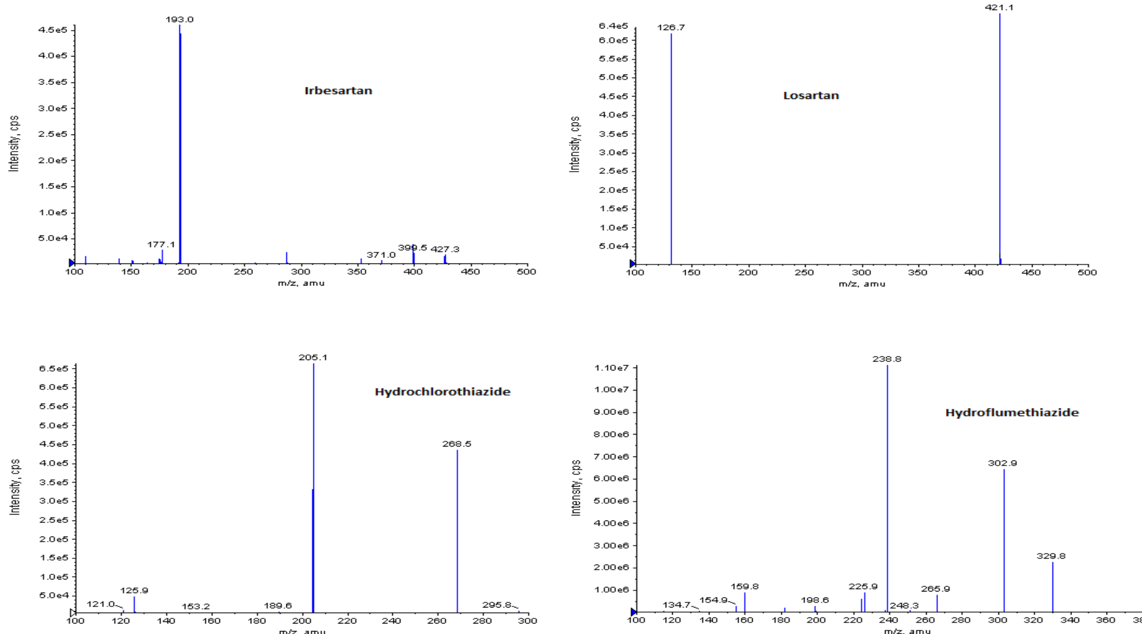

Fig. 1: Product ion spectra of Irbesartan, Hydrochlorothiazide, Losartan and Hydroflumethiazide

Data processing and Regression

The MRM chromatographic peaks were integrated using Analyst software version 1.4.2 after which peak area ratios of Irbesartan to Losartan and Hydrochlorothiazide to Hydroflumethiazide were plotted versus concentration and a linear curve fit, weighted by $1/x^2$ (where x = concentration) was used to produce the regression line.

BIOANALYTICAL METHOD VALIDATION

As a part of method validations following parameters were evaluated:

Selectivity

Selectivity is the ability of the analytical method to differentiate and quantify the analytes in the presence of other expected components in the sample. Selectivity was performed by evaluating at least six different blank matrices for interference at the retention time of analytes and internal standards.

Matrix Effect

Matrix effect was evaluated to asses any undesirable effect from the matrix that can bring about ion suppression/enhancement, decease/increase in sensitivity, increased baseline, imprecision of results, retention time drift and chromatographic peak tailing. Matrix effect was estimated quantitatively through calculation of matrix factor, which is the ratio of peak response in the presence of matrix ions to the peak response in the absence of matrix ions. Matrix effect can further be evaluated from matrix factor as follows.

% Matrix Effect = 1- mean of the matrix factor for Analyte/IS ×100

Sensitivity

Sensitivity of the method was determined by the estimation of the lowest concentration that can

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be measured with an acceptable limit of accuracy and precision. For the estimation of sensitivity six lower limit of quantitation (LLOQ) samples were processed and analysed against a calibration curve and accuracy and precision were determined.

Goodness of Fit

The data of three precision & accuracy batches were used for the estimation for goodness of fit. The back-calculated concentrations of Calibration Curve standards using 1/x and $1/x^2$ weighing were considered for finding the best fit for regression

Linearity

A regression equation generated after processing the three precision and accuracy batches with the best-fit weighing factor was used to found out the linearity of the method. A correlation coefficient (r^2) was used as a benchmark to prove the linearity of the calibration curve.

Precision and Accuracy

Precision and accuracy for Irbesartan and Hydrochlorothiazide was assessed by analyzing three batches comprising of standard blank (blank without IS), standard zero (blank with IS), calibration standards and six replicates of quality control samples (LOQQC, LQC, MQC and HQC) including both intra and inter day runs. The precision of the assay was measured by the calculation of percentage co-efficient of variation over the concentration range of LOQQC, LQC, MQC and HQC samples that were run within a day (intraday) or on different days (inter-day). The accuracy was expressed in percentage and it was calculated as the ratio of the calculated mean values of the LLOQ QC, LQC, MQC and HQC samples to their respective nominal values.

Stock Solution Stability in refrigerator

The stock solution stability was evaluated at 2-12°C for analytes (Irbesartan and Hydrochlorothiazide) and internal standards (Losartan and Hydroflumethiazide) by the preparation of two aqueous mixtures one from the stability standard stock solutions (kept in the refrigerator at 2-12°C) and the other from fresh standard stock solution (comparison stocks). An analysis of six replicates of aqueous mixture samples from stability stock and comparison stock was carried out to evaluate the stability. The percentage change of the mean response of the stability to the comparison stock aqueous mixtures gives an estimate of the stability.

Stock Dilution Stability at Room Temperature

Stock dilution stability was evaluated at room temperature for analytes (Irbesartan and Hydrochlorothiazide) and internal standards (Losartan and Hydroflumethiazide) by the preparation of two aqueous mixtures one from the stability stock dilution solution (kept in room temperature) and the other from fresh standard stock solution (comparison stock). An analysis of six replicates of aqueous mixture samples from stability stock and comparison stock were carried out to evaluate the stability. The percentage change of the mean response of the stability to the comparison stock aqueous mixtures gives an estimate of the stability.

Auto sampler stability

To assess the auto sampler stability of Irbesartan and Hydrochlorothiazide in processed samples, six quality control samples (at LQC and HQC level) were processed and stored into the auto sampler at 5 °C for the stability period of 27 hours. These pre-processed samples were than quantified against freshly spiked calibration curve standards.

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Bench-top stability

The stability of analytes in human plasma stored at room temperature (bench-top stability) was determined by kept six quality control samples (at LQC and HQC level) idle for 6 hours on bench at room temperature then processing bench top stability quality control samples and quantifying them against the freshly spiked calibration curve standards.

Freeze-thaw stability

The freeze-thaw stability was conducted by analyzing the six quality control samples (at LQC and HQC level) that had been frozen and thawed three times. The processed freeze-thaw quality control samples were quantified against a freshly spiked calibration Curve.

Long-term stability

The long-term stability was conducted by analyzing low and high quality control samples stored below -50°C and -20°C for 100 days with freshly prepared calibration standards.

Recovery

Recovery of Irbesartan and Hydrochlorothiazide from the extraction procedure was determined by preparing aqueous recovery comparison samples at LQC, MQC and HQC levels (representing 100 % extraction). Aqueous recovery comparison samples (LQC, MQC and HQC) were prepared by using 30 µL aqueous dilution of each Irbesartan and Hydrochlorothiazide of respective quality control, 250 µL of internal standard dilution (containing Losartan approximately 50000 ng/mL and Hydroflumethiazide approximately 1000 ng/ml) and 2720 µL of mobile phase. The following recovery dilution represents 100% extraction. The aqueous samples (LQC, MQC and HQC) of Irbesartan and Hydrochlorothiazide were compared against 6 sets of processed plasma samples of LQC, MQC and HQC. Recovery of internal standards was also compared at LQC, MQC and HQC level. The recovery was calculated in the following way

$$\%\ \text{Recovery} = \frac{\text{Mean Peak Area Response of Extracted sample}}{\text{Mean Peak Area Response of Unextracted sample}} \times 100$$

Effect of potentially interfering drug

Effect of potentially interfering drug (PID) was evaluated to demonstrate the effect of potential drugs that could affect the analysis (by creating any interference at the retention levels of analytes and internal standards) because of their administration to the study volunteers during the clinical phase of the study. Potentially co-administered drugs prepared at therapeutic concentrations are tested to verify their interfering potential at the RT of an analytes and the internal standards. PID MQC samples were prepared by the spiking of 2 % of the respective PIDs at their expected C_{max} concentration and 1 % of each analyte middle quality control level concentration (MQC) in blank matrix. These samples were analysed against a calibration curve. The effect of potentially interfering drugs (PID) (Ibuprofen, Caffeine, Acetaminophen and Acetyl salicylic acid) on Irbesartan and Hydrochlorothiazideanalys was performed.

Dilution Integrity

Dilution integrity was estimated by spiking approximately 8.5 times of ULOQ concentration of Irbesartan and Hydrochlorothiazide in the drug free matrix to prepare dilution integrity quality control (DIQC) Sample. Six samples of dilution factor (1/10) were prepared by diluting DIQC sample with blank matrix and back calculation of these QC samples with the appropriate dilution

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factor was carried out against a calibration curve.

Reinjection reproducibility

Reinjection Reproducibility was performed to establish that the reinjection of the samples kept in the auto sampler at controlled temperature has no effect on the result reproducibility. Reinjection Reproducibility was performed by reinjection of a complete precision and accuracy batch after storage in the auto sampler for 26 hours at 5 °C from the last injection of original batch.

Pharmacokinetic study:

A pharmacokinetic study to evaluate the bioequivalence of a test Irbesartan and Hydrochlorothiazide formulation against innovator formulation at 300 mg and 25 mg strength respectively was carried out using the validated method defined in the following manuscript. The study was carried using a open-label, balanced, randomized, two-treatment, two period, two-sequence, single-dose, two-way crossover design. The study was conducted as per the ICH-GCP guidelines after getting approval of the study protocol from the independent ethics committee.

RESULTS AND DISCUSSION

Method Development and Optimization

The scanning and acquisition of the parent and the product ions for Irbesartan, Hydrochlorothiazide, Losartan and Hydroflumethiazide were carried out by continuous infusion of the dilution of analytes and internal standards at appropriate concentration through a pump and sorting out appropriate polarity and ions. Afterwards optimization of mass spectrometric condition for each compound was carried out by continuous infusion and adjustment of the compound dependent parameters as declustering potential (DP), focusing potential (FP) and entrance potential (EP). Parameters such as DP, FP and EP were ramped to provide best signal to noise level for the parent ions. Optimization was carried out for the product ions to trace out the best combination of parameters as collision energy (CE), collision associated dissociation (CAD), cell exit potential (CXP). Afterwards source parameters as curtain gas, nebulizer gas, temperature were optimized by flow injection analysis was using a union in place of column. Then chromatographic conditions were optimized to look for sensitivity, peak shape, separation of peaks and chromatographic run time. The selection of mobile phase was done taking into account symmetric separate peaks with no charge competition in source so that analytes and internal standards response not decrease with huge linear range of analytes. Results derived from several combinations showed that Methanol: Buffer Solution (0.1% Formic Acid in Milli-Q Water) :: 90:10 v/v)) serves the desired purpose with utmost effectiveness. During the early phase of method development attempts were made to choose the right column. Waters Sunfire C18, Phenomenex Gememi C18, YMC pack C8 and Waters X bridge RP18 columns were tested. However, Hypersi; Gold 50*4.6, 5µm column was found to be most suitable for analysis as Irbesartan and Hydrochlorothiazide with retention time were different and shorter run time that further leads to low consumption of mobile phase altogether making the method cost effective. The liquid-liquid extraction method were tested with different solvent alone or in different compositions, but recovery of the

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analytes were low. Hence, solid-phase extraction was tested to improve the recovery with no matrix effect. The use of proper internal standards was done to eliminate the quantitative bias caused by instrumental variation. The deuterated internal standards show charge competition with increase in concentration of analytes in different composition of mobile phase without column, hence Losartan and Hydroflumethiazide were selected which has similar physiochemical properties, ionization condition, appropriate retention time and recovery compared to Irbesartan and Hydrochlorothiazide leading to better tracking of analytes during the course of experiment. The Specificity was carried out in method development in six plasma lots. There was no significant interference observed at the retention time of Irbesartan in presence of ~Cmax concentration of Hydrochlorothiazide and vice- versa.

ASSAY PERFORMANCE

Selectivity and Sensitivity

The selectivity was carried out in six normal plasma lots. No interference of endogenous matrix/impurities was found at the retention time of the analytes and internal standards in normal plasma. Representative chromatograms of extracted blank human plasma (Fig. 2a) and blank human plasma fortified with IS (Fig. 2b), demonstrated the selectivity of the method. Sensitivity was determined by analyzing six replicates of blank human plasma spiked with the analytes at the lowest limit of the quantification (99.9 ng mL-1 for Irbesartan and 3.18 ng mL-1 for Hydrochlorothiazide). The solid phase extraction procedure provide advance sample cleanup and gave very good sensitivity for the analysis of Irbesartan and Hydrochlorothiazide in human plasma The precision and accuracy for Irbesartan at LLOQ was 5.85 % and 105.325 % and for Hydrochlorothiazide 5.24 % and 108.23 % respectively.The representative chromatogram for the LLOQ showing sensitivity was depicted in Fig. 2c.

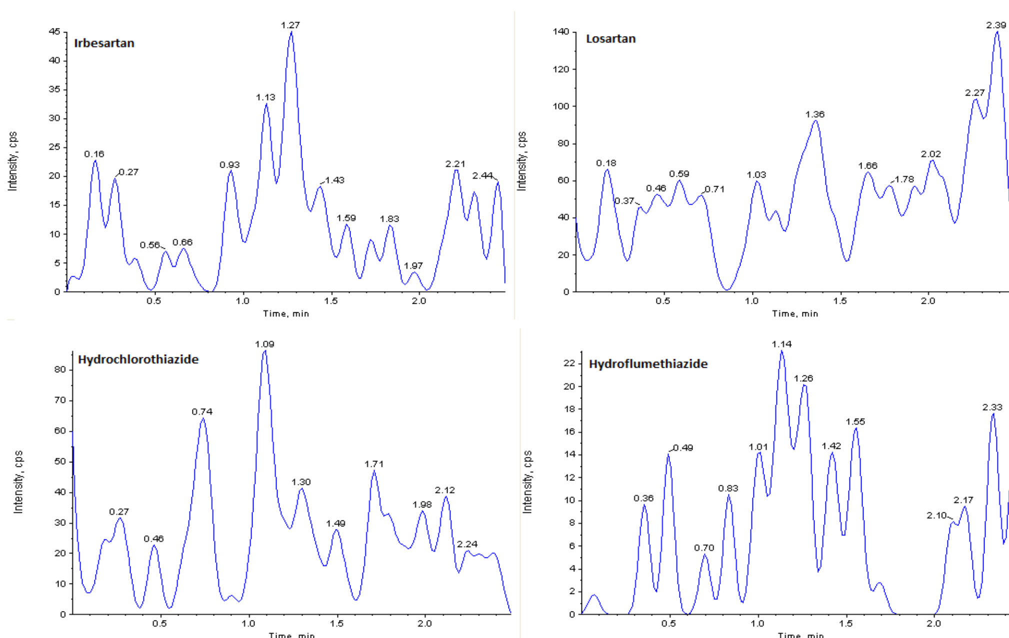

Fig. (2a): Representative Chromatograms of extracted blank human plasma

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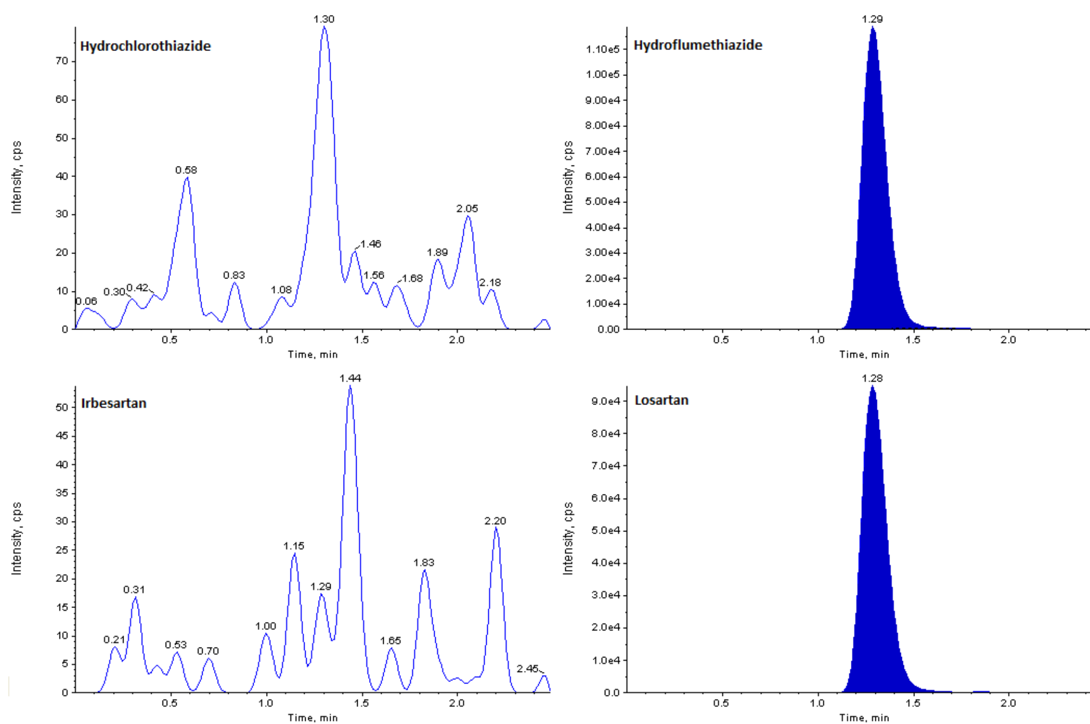

Fig. (2b): Representative Chromatograms of extracted blank human plasma fortified with Internal Standard

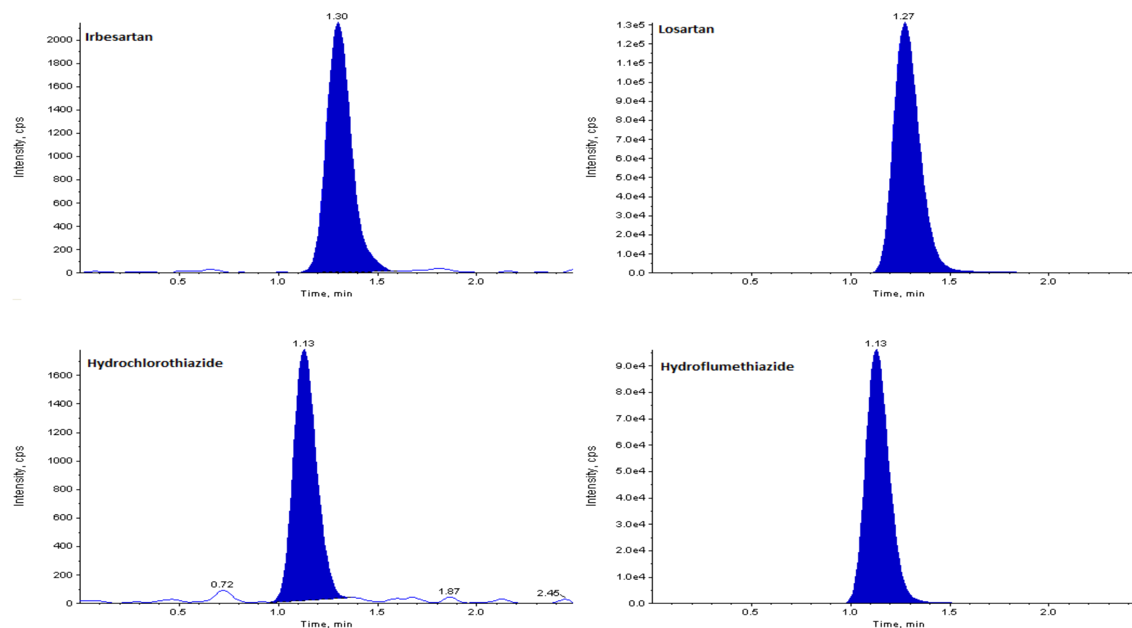

Fig. (2c): Representative Chromatograms of LLOQ

Matrix effect assessment

The matrix effect on the ionization of Irbesartan and Hydrochlorothiazidein the LC/MS-MS method was determined by processing of six different set of plasma lots in duplicate. The neat (non matrix based) samples were prepared using mobile phase, aqueous dilutions of Irbesartan and Hydrochlorothiazide at the LQC and HQC levels and internal standards dilution to give concentration within the level of extracted value. The processed matrix effect blanks samples were reconstituted with neat samples. The matrix effect on the estimation of Irbesartan and Hydrochlorothiazide was determined as the %

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RSD (relative standard deviation) of the matrix factor or the variability of the matrix factor. Matrix factor is estimated by the comparison of the area response of the blank samples with the neat samples. The variability of matrix factor (reported as % CV of matrix factor) was 1.49 % (HQC) and 6.05 % (LQC) for Irbesartan, 2.81 % (HQC) and 3.14 % (LQC) for Hydrochlorothiazide, 1.58 % (HQC) and 5.01 % (LQC) for Losartan and 2.31 % (HQC) and 5.69 % (LQC) for Hydroflumithiazide.

Goodness of Fit and Linearity

The goodness of fit results showed $1/x^2$ to be the best fit for regression. Calibration curves were linear over the concentration range 99.9 to 6274.0 ng mL-1 for Irbesartan and 3.18 to 500.45 ng mL-1 for Hydrochlorothiazide. The precision and accuracy batches evaluated gives a mean linear equation for the calibration curve $y = (0.00009046 \pm 0.00002038) x + (0.002253 \pm 0.0002534)$ for Irbesartan and $y = (0.002303 \pm 0.00007571) x + (0.00237 \pm 0.0008265)$ for Hydrochlorothiazide, where y was the peak area ratio of analyte to the IS and x was the concentration of analyte. The correlation coefficient (r^2) for Irbesartan and Hydrochlorothiazide were above 0.99.

Precision and Accuracy

The linearity, precision and accuracy evaluations were performed on three batches of spiked plasma samples. The intraday precision and accuracy were calculated after repeated analysis in three analytical runs. The intra and inter-batch accuracy was determined by calculating percentage nominal of quality control sample from the theoretical concentration. The within and between-day precision was determined in terms of relative standard deviation (% RSD). Precision of the assay was measured by the percent coefficient of variation over different concentration levels. The acceptance criteria for within and between batch precision were 20% for LLOQ QC and 15% for other non-zero concentrations. The accuracy of the assay was defined, as the ratio of calculated mean values of the quality control sample to their respective nominal values, expressed as percentage and the criteria for accuracy was 100 ± 20% for LOQQC and 100 ± 15% for other concentrations. For Irbesartan intraday experiments, the precision ranged from 0.53 % to 5.56 % and the accuracy ranged from 99.02 % to 103.39 % whereas for the interday experiments, precision ranged from 1.36 % to 10.00 % and the accuracy ranged from 97.62 % to 104.40 % . For Hydrochlorothiazide intraday experiments, the precision ranged from 0.87 % to 11.48 % and the accuracy ranged from 98.11 % to 108.32 % whereas for the interday experiments, precision ranged from 5.60 % to 11.02 % and the accuracy ranged from 96.60 % to 102.24 %. The intra and inter day precision and accuracy data is presented in Table 1(a) and 1(b).

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Table 1(a): Intra- and inter-day precision and accuracy data for Irbesartan

QC	LLOQ QC	LQC	MQC	HQC
	Nominal Concentration			
Intra day	3.18 ng mL^{-1}	8.14 ng mL^{-1}	246.65 ng mL^{-1}	362.72 ng mL^{-1}
Mean	3.120	8.305	255.835	392.888
S.D. (+/-)	0.3583	0.1819	2.2295	3.9792
C.V. (%)	11.48	2.19	0.87	1.01
% Nominal	98.11	102.03	103.72	108.32
N	6	6	6	6
Inter day				
Mean	3.072	8.267	245.191	370.841
S.D. (+/-)	0.3386	0.4822	13.7232	25.4029
C.V. (%)	11.02	5.83	5.60	6.85
% Nominal	96.60	101.56	99.41	102.24
N	18	18	18	18

Table 1(b): Intra- and inter-day precision and accuracy data for Hydrochlorothiazide

QC	LLOQ QC	LQC	MQC	HQC
	Nominal Concentration			
Intra day	**3.18 ng mL^{-1}**	**8.14 ng mL^{-1}**	**246.65 ng mL^{-1}**	**362.72 ng mL^{-1}**
Mean	3.120	8.305	255.835	392.888
S.D. (+/-)	0.3583	0.1819	2.2295	3.9792
C.V. (%)	11.48	2.19	0.87	1.01
% Nominal	98.11	102.03	103.72	108.32
N	6	6	6	6
Inter day				
Mean	3.072	8.267	245.191	370.841
S.D. (+/-)	0.3386	0.4822	13.7232	25.4029
C.V. (%)	11.02	5.83	5.60	6.85
% Nominal	96.60	101.56	99.41	102.24
N	18	18	18	18

Stability study

Stability studies were performed to evaluate the Irbesartan and Hydrochlorothiazide stability in across different parameters. Stock solution of Irbesartan, Hydrochlorothiazide and internal standards (Losartan and Hydroflumithiazide) were found stable in refrigerator at 2-12°C for 7 days with a percentage change of -0.81 % to 0.81 %. Stock dilution of Irbesartan, Hydrochlorothiazide and internal standards (Losartan and Hydroflumithiazide) were stable at room temperature for 22 hours at room temperature with a percentage change of -2.22 % to -0.12 %. Spiked Irbesartan and Hydrochlorothiazide was stable in human plasma (bench top stability) at room temperature for 6 hours. Irbesartan and Hydrochlorothiazide was found stable in the final extract for analysis while placed in the autosampler up to 27 hours. Irbesartan and Hydrochlorothiazide was found to be stable in plasma for at least three freeze and thaw cycles. The Irbesartan and Hydrochlorothiazide spiked plasma samples stored below -50°C and -20°C for long- term stability experiment were found to be stable for 100 days. Stability results for Irbesartan and Hydrochlorothiazide in matrix across different parameters are illustrated in Table 2(a) and 2(b).

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Table 2(a): Stability data for Irbesartan in matrix

Sample Concentration ng mL-1	**n=6 Mean ± SD (%)**	**Precision (%)**	**Accuracy (%)**
Bench top stability(6 hrs)			
LQC (263.6)	236.32 ±5.233	2.21	89.64
HQC(4557.4)	4545.07 ± 75.708	1.67	99.72
Autosampler stability (27 hrs)			
LQC (263.6)	231.05 ±4.168	1.80	87.65
HQC(4557.4)	4588.35 ±98.361	2.14	100.68
Three freeze thaw cycles			
LQC (263.6)	243.57 ± 6.519	2.68	92.40
HQC(4557.4)	4814.13 ± 451.174	9.37	105.63
Stability for 100 days			
at ≤ -50⁰ C			
LQC (263.6)	247.95 ±2.464	0.99	94.06
HQC(4557.4)	4519.90 ± 255.105	5.64	99.18
at ≤ -20⁰ C			
LQC (263.6)	246.10 ± 11.392	4.63	93.36
HQC(4557.4)	4486.52 ± 254.514	5.67	98.44

Table 2(b): Stability data for Hydrochlorothiazide in matrix

Sample Concentration ng mL-1	**n=6 Mean ± SD (%)**	**Precision (%)**	**Accuracy (%)**
Bench top stability(6 hrs)			
LQC (8.14)	8.545 ± 0.1668	1.95	104.98
HQC(362.72)	326.068 ± 11.5999	3.56	89.89
Autosampler stability (27 hrs)			
LQC (8.14)	8.047 ± 0.5408	6.72	98.85
HQC(362.72)	325.717 ± 1.8235	0.56	89.80
Three freeze thaw cycles			
LQC (8.14)	8.248 ± 0.7301	8.85	101.33
HQC(362.72)	342.788 ± 37.2500	10.87	94.50
Stability for 100 days			
at ≤ -50⁰ C			
LQC (8.14)	7.368 ± 0.2399	3.26	90.52
HQC(362.72)	327.758 ± 11.9718	3.65	90.36
at ≤ -20⁰ C			
LQC (8.14)	7.525 ± 0.2333	3.10	92.44
HQC(362.72)	329.782 ± 12.3525	3.75	90.92

Dilution Integrity and Reinjection Reproducibility

The precision (% CV) and accuracy (mean % nominal) ranged up to 1.37 % and 101.74 % for Irbesartan and 1.14 % and 103.71 % for Hydrochlorothiazide with a dilution factor of 1/10. The mean % nominal ranged of Irbesartan from 89.93 % (LQC) to 109.54 % (LLOQ QC) with a precision ranging from 0.94 % (MQC) to 2.33 % (LLOQ QC) and for Hydrochlorothiazide from 91.52 % (MQC) to 104.15 % (LLOQ QC) with a precision ranging from 0.56 % (HQC) to 6.65 % (LQC) in reinjection reproducibility. The Irbesartan and Hydrochlorothiazide samples were stable in reinjection reproducibility at 5 °C for 26 hours.

Recovery and effect of potentially interfering drugs

The mean % recovery of Irbesartan, Hydrochlorothiazide, Losartan and Hydroflumethiazide were 89.03 %, 83.15 %, 88.89 % and 84.89 % with a precision of 9.39 %, 2.79 %, 4.36 % and 2.12 % respectively. The back

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calculated concentrations of QC sample spiked with PID were found to be within ± 20 % of the actual concentration of the QC sample for Irbesartan and Hydrochlorothiazide. Hence, the tested PIDs have no effect on the analysis of Irbesartan and Hydrochlorothiazide.

Pharmacokinetic evaluation

A non-compartmental pharmacokinetic model with WinNonlin, version 5.3; Pharsight,USA was used for the analysis of concentration data . Representative mean plasma concentration profile curve of test and reference given in Fig.3a and 3b.

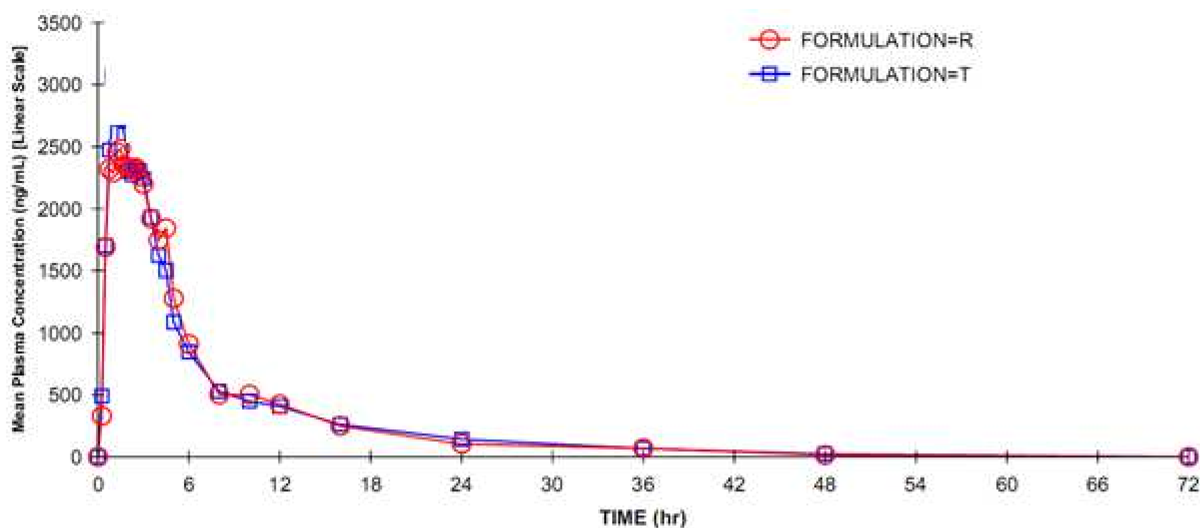

Fig. 3 (a): Mean plasma concentration-time curves for Irbesartan (300 mg) following single-dose administration of test and reference tablets

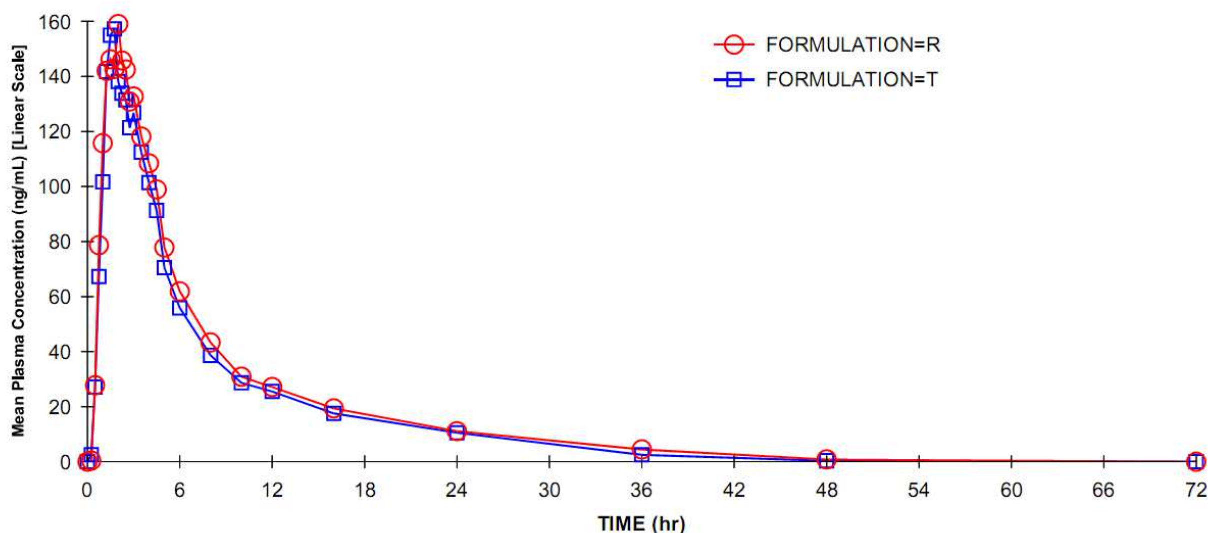

Fig. 3 (b): Mean plasma concentration-time curves for Hydrochlorothiazide (25 mg) following single-dose administration of test and reference tablets

Conclusion

A highly specific, selective, and rapid LC-ESI MS-MS method for the simultaneous determination of Irbesartan and Hydrochlorothiazide in human plasma has been developed and validated with a lower limit of quantification of 99.9 ng mL-1and 3.18 ng mL-1 respectively. The validated method provides an easy way of estimation of Irbesartan and Hydrochlorothiazidein plasma with highly

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efficient sample cleanup associated with good recovery and negligible matrix effect. The validated method has been successfully applied for performing pharmacokinetic evaluation to adjudge the bioequivalence of two formulations of Irbesartan and Hydrochlorothiazidewith 300 mg and 25 mg dose respectively in healthy volunteers.

Conflict of Interest

All the authors of the article declare no conflict of interest

References

1) Sweetman S C (2007) (ed) Martindale. The Complete Drug Reference, 35th ed.
2) The Pharmaceutical Press, London, 2007: p. 1316, 1307.
3) Block J H, Beale J M. (eds). Wilson and Gisvold's Textbook of Organic Medicinal and Pharmaceutical Chemistry, 11th ed. Lippincott Williams & Wilkins, Philadelphia, 2004: p. 610.
4) Mizuno M, Sada T, Ikeda M, Fukuda N, Miyamoto M, Yanagisawa H and Koike H. Pharmacology of CS-866, a novel nonpeptide angiotensin II receptor antagonist. European Journal of Pharmacology 1995; 285: 181-188.
5) Croom, K.F.; Curran, M.P.; Goa, K.L.; Perry, C.M. Drugs. 2004, 64, 999–1028.
6) Huang, Z.H. Chin. J. New Drugs Clin. Rem. 2006, 26, 384–387.
7) US DHHS, FDA and CDER. Guideline for Industry: Co development Two or More New Investigational Drugs for use in Combination. US Department of Health and Human Services, Food and Drug Administration and Center for Drug Evaluation, 2013. Available at: www:fda.gov/ downloads/Drugs /Guidance Complicance Regulatory Information / /Guidances/UCM236669.pdf
8) US DHHS, FDA and CDER. Guideline for Industry: FDA Draft Guidance on Irbesartan and Hydrochlorothiazide. Available at: www.fda.gov/downloads/Drugs //Guidance ComplianceRegulatoryInformation/Guidances/ ucm086212.pdf
9) Czerwińska K, Mazurek AP. Identification and determination of selected angiotensin II receptor antagonist group drugs by HPLC method, Acta Pol. Pharm., 2011, 68 (6), 831-837.
10) Bae SK, Kim MJ, Shim EJ, Cho DY, Shon JH, Liu KH, Kim EY, Shin JG. HPLC determination of irbesartan in human plasma: its application to pharmacokinetic studies. Biomed. Chromatogr., 2009, 23 (6), 568-572.
11) Tutunji LF, Tutunji MF, Alzoubi MI, Khabbas MH, Arida AI. Simultaneous determination of irbesartan and hydrochlorothiazide in human plasma using HPLC coupled with tandem mass spectrometry: Application to bioequivalence studies. Pharm Biomed Anal. 2010; 51:985-990
12) Zhang RR, Chen XH, Li Q, Liu WT, Yang WW, Bi KS, Sun LX. Liquid chromatography coupled with mass spectrometry method for the simultaneous quantification of irbesartan and hydrochlorothiazide in human plasma. Journal of Chinese Pharmaceutical Sci. 2011; 4: 360-368.

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