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Short communication

Simultaneous determination of irbesartan and hydrochlorothiazide in human plasma using HPLC coupled with tandem mass spectrometry: Application to bioequivalence studies

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ABSTRACT

A sensitive, specific and selective liquid chromatography/tandem mass spectrometric method has been developed and validated for the simultaneous determination of irbesartan and hydrochlorothiazide in human plasma. Plasma samples were prepared using protein precipitation with acetonitrile, the two analytes and the internal standard losartan were separated on a reverse phase C_{18} column (50 mm × 4 mm, 3 μm) using water with 2.5% formic acid, methanol and acetonitrile (40:45:15, v/v/v (%)) as a mobile phase (flow rate of 0.70 mL/min). Irbesartan and hydrochlorothiazide were ionized using ESI source in negative ion mode, prior to detection by multiple reaction monitoring (MRM) mode while monitoring at the following transitions: m/z 296 → 269 and m/z 296 → 205 for hydrochlorothiazide, 427 → 175 for irbesartan. Linearity was demonstrated over the concentration range 0.06–6.00 μg/mL for irbesartan and 1.00–112.00 ng/mL for hydrochlorothiazide. The developed and validated method was successfully applied to a bioequivalence study of irbesartan (300 mg) with hydrochlorothiazide (12.5 mg) tablet in healthy volunteers (N = 36).

1. Introduction

Hydrochlorothiazide (HCTZ), 6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide, is an effective thiazide diuretic, which has been used alone, or in combination with other antihypertensive drugs including β-blockers, ACE inhibitors, or angiotensin II receptor blockers for the treatment of hypertension and congestive cardiac failure [1,2]. Irbesartan, 2-butyl-3-[[2′-(tetrazol-5-yl)biphenyl-4-yl]-methyl]-1,3-diazaspiro[4.4]non-1-en-4-one, is a potent and selective angiotensin II subtype 1 receptor antagonist indicated for use in patients with hypertension, in addition to those with type 2 diabetes mellitus and nephropathy [3].

A combination dosage form of irbesartan and HCTZ is indicated in the treatment of edema and hypertension. Clinical studies of irbesartan/HCTZ suggest that this combination is clinically effective with a favorable safety profile [4,5]. Irbesartan/HCTZ provided consistent blood pressure lowering and tolerability regardless of age, obesity, and prevalence type 2 diabetes and greater efficacy in patients with high cardiovascular risk [6].

Several chromatographic methods have been reported for the analysis of HCTZ in human plasma individually or in combination, such methods have included: HPLC coupled with UV [7–9] or diode array [10,11], electrochemical detection [12], LC–MS [13,14] or with tandem LC–MS/MS [15–19]. A number of HPLC methods have been reported for the determination of irbesartan in human plasma including; HPLC coupled with UV [20], fluorescence [21,22], or with tandem LC–MS/MS detection [23]. Although the simultaneous determination of irbesartan and HCTZ has previously been suggested by other workers, who employed HPLC coupled with UV detection [24,25], such methods suffered from lack of sensitivity, demonstrated by the lower limits of quantitation (LLOQ) e.g. 75 ng/mL for irbesartan, and 6 ng/mL for HCTZ. However, the analytical requirement for the application on bioequivalence, bioavailability and pharmacokinetic studies necessitate the development of more sensitive method. This is generally achieved if the

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AUC_{0-t} was at least 80% of the $AUC_{0-\infty}$, consequently for a reliable estimate of terminal half-life, at least three to four concentrations should be accurately measured to properly estimate the terminal log linear phase [26]. This requirement has not yet been met in the current analytical literature to be fit for this purpose.

The present paper reports a sensitive tandem LC–MS/MS method for the simultaneous determination of irbesartan and HCTZ in human plasma which demonstrated a LLOQ of 60 ng/mL for irbesartan and 1.00 ng/mL for HCTZ. The developed and validated method has been successfully applied to a bioequivalence study; after dosing with irbesartan (300 mg) and HCTZ (12.5 mg) tablets to healthy volunteers ($N = 36$) in the fasted state.

2. Experimental

2.1. Chemicals and reagents

Working standards of irbesartan (99.2%) and hydrochlorothiazide (101.4%) were kindly provided by Jamjoom Pharmaceuticals (Jeddah, Saudi Arabia). Losartan potassium (99.1%) used as an internal standard (IS) was kindly provided by National Pharmaceutical Industries (NPI, Sultanate of Oman). HPLC gradient grade acetonitrile, methanol and water, in addition to analytical grade formic acid were purchased from Merck (Darmstadt, Germany). All reagents were used without further purification. Blank human plasma was obtained from the blood bank (Amman, Jordan) and stored at −30 °C prior to use.

2.2. Instrumentation

The LC–MS/MS system consisted of a high performance liquid chromatograph (Agilent 1200 Series, Agilent Technologies, Waldbronn, Germany) coupled with an Applied Biosystems, Sciex a triple quadrupole mass spectrometer (API 5000, MDS Sciex, Ontario, Canada), equipped with an electrospray ionization (ESI). Data acquisition and processing were controlled by Applied Biosystems/MDS SCIEX Analyst software (Version 1.4.2.).

2.3. LC–MS/MS chromatographic conditions

Chromatographic separation was performed using a Purosphere® STAR C_{18} column (50 mm × 4 mm, 3 μm, Merck, Germany) thermostated at 45 °C. The mobile phase was water with 2.5% formic acid, methanol and acetonitrile (40:45:15, v/v/v (%)). The separation was performed under isocratic conditions with a constant flow rate of 0.7 mL/min, the injection volume was 10 μL.

LC–MS/MS experimental conditions utilized the multiple reaction monitoring (MRM), detection of irbesartan, HCTZ and internal standard (losartan) was performed in the negative ESI mode for their respective $[M-H]^-$ ions. Instrument settings of the MS/MS are summarized in Table 1.

2.4. Standard solutions and calibrators

Stock standard solutions of irbesartan (800 μg/mL) and HCTZ (400 μg/mL) were freshly prepared in acetonitrile. A series of working standard solutions were diluted in acetonitrile to produce eight standard solutions ranging 6.0–600 μg/mL for irbesartan, and 100–11200 ng/mL for HCTZ respectively. Matrix based calibrators were prepared by spiking 100 μL of each standard solution to a final volume of 10.00 mL plasma. Quality control samples were prepared at 0.18, 3.00, and 4.80 μg/mL for irbesartan and 3.00, 55.00 and 88.00 ng/mL for HCTZ. A working standard solution containing 120.00 ng/mL of the IS dissolved in acetonitrile was prepared. All plasma solutions were stored at −86 °C until assay.

2.5. Extraction of analytes from plasma

To a plasma aliquot (250 μL) in an Eppendorf tube, a 0.50 mL volume acetonitrile containing the IS (120.00 ng/mL) was added. The mixture was vortexed (30 s) and centrifuged (4000 rpm for 5 min), 250 μL aliquot of the supernatant was transferred to a 10 mL glass tube. The mixture then evaporated to dryness, under a stream of nitrogen in a block heater (40 °C). The residue was reconstituted with 1.00 mL of water; the mixture was then vortexed (30 s), and a 10 μL aliquot of the solution was injected into the LC–MS/MS system.

2.6. Method validation

The developed method was validated with respect to the following validation parameters: selectivity, linearity and linear working range, limits of detection and quantitation, sensitivity, recovery, accuracy, precision, and stability [27].

2.7. Application

The validated bioanalytical method was successfully applied to evaluate the bioequivalence of two tablet formulations of irbesartan/HCTZ in healthy volunteers: irbesartan/HCTZ tablets of Jamjoom Pharmaceuticals, Saudi Arabia (test product) was compared with CoAprovel™ of Sanofi Winthrop Industries-1, rue de la Vierge-33440 Ambares, France (reference product). The study compared equal doses of (300 mg/12.5 mg tablet) of each product administered to healthy participants under fasting condition. Thirty-six healthy adult male volunteers, age range of 18–45, were included in the study. The volunteers were selected after assessment of their health status, physical examination, ECG, in addition to hematology, biochemistry, electrolytes, and urinalysis testing. The volunteers were free from cardiac, hepatic, renal, pulmonary, gastrointestinal, neurological and hematological diseases. The clinical protocol was approved by Pharmaquestjo Ethics Committee, and the volunteers were given written informed consent.

The study was an open-labeled, randomized, two-period crossover design, with a 2-week washout interval. The volunteers were hospitalized. After overnight fasting (12 h), the volunteers were orally dosed with a single dose of the assigned tablet with 240 mL of water. Fasting continued 4 h after drug administration. Venous blood samples (8 mL) were collected into labelled EDTA blood tubes, at the following time points: immediately before administration and at 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.25, 2.50, 3.00, 4.00, 5.00, 6.00, 8.00, 10.0, 12.0, 24.0, 36.0, 48.0, 60.0, 72.0, and 96.0 h post-dosing. Blood samples were centrifuged (3000 rpm for 8 min), and the plasma was separated and stored at −86 °C until analysis.

3. Results and discussion

3.1. Chromatographic and mass spectrometric experimental conditions

Method development and optimization was completed by adopting a systematic approach during the optimization process of the experimental conditions. An LC–MS/MS system has been employed for the simultaneous determination of irbesartan and HCTZ in human plasma. The optimized experimental conditions are summarized in Table 1. Detection of both analytes and the internal standard was performed in the negative ionization mode for the corresponding $[M-H]^-$ ions. Product ion mass spectra of the analytes and the IS were recorded, the transition m/z 427 → 175 was chosen for quantitation of irbesartan, whereas the transitions

Table 1
Experimental settings for the tandem mass-spectrometer for the analysis of irbesartan, hydrochlorothiazide and losartan (IS).

Parameter	Value			
Source temperature, °C	600			
Nebulizer gas, psi	60.0			
Turbolon gas, psi	60.0			
Curtain gas, psi	25.0			
Collision gas, psi	12.0			
Ion spray voltage, V	−4500			
Dwell time per transition, ms	100			
Entrance potential, V	−10			
	Irbesartan	Hydrochlorothiazide		Losartan
MRM transition	427 → 175	296 → 269	296 → 205	421 → 179
Collision energy, V	−52	−28	−32	−32
Declustering potential (DP), V	−150	−145	−145	−135
Collision cell exit potential, V	−17	−37	−19	−13

m/*z* 296 → 269, and *m*/*z* 296 → 205 were chosen for the quantitation of HCTZ. The combined areas were utilized for the quantitation of HCTZ to enhance sensitivity. Losartan was monitored at *m*/*z* 179.

As summarized in Section 2.3, the chromatographic conditions were optimized to achieve high resolution, and peak symmetry with a short retention time for both analytes and the internal standard (Fig. 1). Retention times for irbesartan, HCTZ and losartan were at 2.07, 0.72 and 1.98 min, respectively, with a total run time of 4.0 min.

3.2. Selectivity and matrix effects

Six different sources of blank plasma samples were harvested under controlled conditions from volunteers in a fasted state; the samples were extracted and analyzed using the developed method, no interferences were observed at the retention times of irbesartan, HCTZ or the IS. Analytical signals from blank plasma extracts were compared with those obtained from a spiked plasma sample extract at the LLOQs containing both analytes and the IS. This is demonstrated in the representative chro-

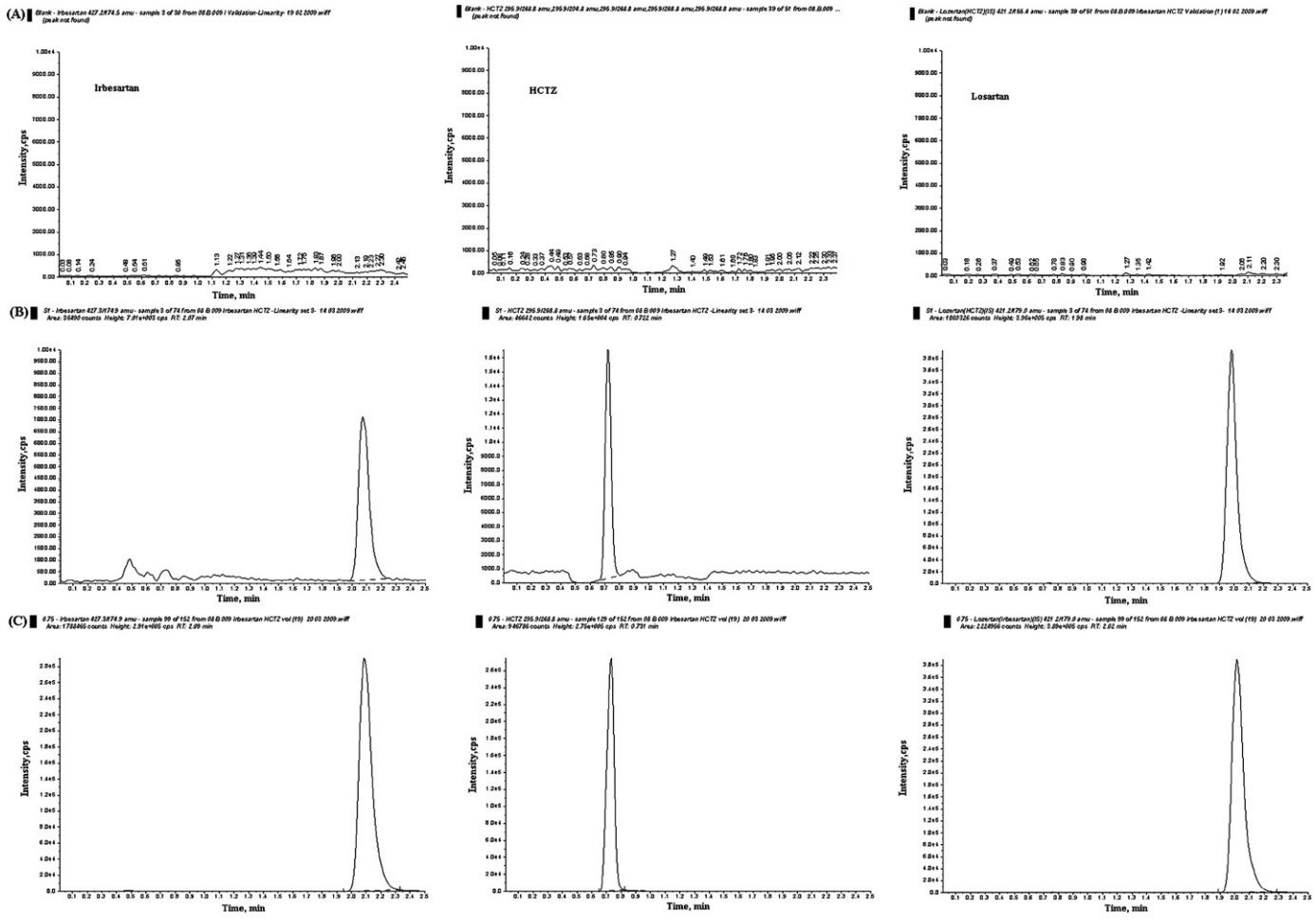

Fig. 1. Mass chromatograms for irbesartan, hydrochlorothiazide and the internal standard losartan from human plasma extractsincluding: (A) blank plasma sample, (B) a blank plasma sample spiked with irbesartan (0.06 μg/mL), hydrochlorothiazide (1.00 ng/mL) and the IS (120 ng/mL), and (C) a volunteer plasma sample; 0.75 h after an oral administration of a tablet containing 300 mg irbesartan and 12.5 mg hydrochlorothiazide in a fasting state.

Table 2
Summary of data pertaining to instrument precision, method, inter-day and intra-day precision and accuracy.

	Irbesartan (μg/mL)			Hydrochlorothiazide (ng/mL)		
Concentration	0.18	3.00	4.80	3.00	55.00	88.00
Instrument precision (CV (%))	3.46	2.21	1.71	2.14	2.99	1.47
Method						
Precision (CV (%))	5.26	0.97	1.74	1.31	0.71	3.50
Accuracy (RE (%))	5.56	3.33	−4.38	2.00	7.78	11.51
Intra-day						
Precision (CV (%))	5.88	2.56	2.13	1.79	2.12	3.20
Accuracy (RE (%))	−5.56	4.33	−2.29	−7.00	−3.22	0.25
Inter-day						
Precision (CV (%))	5.56	1.6	2.37	6.64	4.27	3.66
Accuracy (RE (%))	0.00	4.33	−3.33	0.33	1.40	2.42

CV (%) = SD of measured value/mean measured value × 100. RE (%) = (measured concentration − nominal concentration)/nominal concentration × 100.

matograms of the blank plasma, and those spiked with irbesartan (0.06 μg/mL), HCTZ (1.00 ng/mL) and IS (120.00 ng/mL) (Fig. 1A and B). In addition, chromatograms obtained from test samples after dosing with a tablet containing 300 mg irbesartan and 12.5 mg HCTZ, sampled at 0.75 h after dosing, are illustrated in Fig. 1(C).

Selectivity was also investigated with respect to caffeine and common OTC drugs including: diclofenac, theophylin, and acetaminophen. None of the investigated interferents showed analytical signal(s) at the retention times of irbesartan, HCTZ or the IS.

To further examine matrix effects, the standard curves in spiked human plasma were compared with standards in buffer. The results demonstrated the absence of matrix effects.

3.3. Calibration

Response functions of calibrators were recorded individually for irbesartan and HCTZ. Each was plotted against corresponding concentration level in the dynamic ranges: 0.06–6.00 μg/mL for irbesartan, and 1.00–112.00 ng/mL for HCTZ. Linearity was demonstrated both by visual inspection, and by calculations the correlation coefficient of 0.9977 for irbesartan and 0.9988 for HCTZ.

Using the weighted regression model, with a statistical weight of $1/x^2$, the calibration equations were: $y = 0.2537x + 0.0051$ for irbesartan and $y = 0.0129x + 0.0068$ for HCTZ, where y represents e peak area ratios of the analyte to the IS, and x represents the analyte concentrations.

3.4. Sensitivity and limits of quantitation and detection

The calibration sensitivities were 0.2537 ± 0.0055 and 0.0129 ± 0.0002 for irbesartan and HCTZ, respectively. The LLOQs of irbesartan and HCTZ were found to be 0.06 μg/mL and 1.00 ng/mL, respectively. The limit of detection for irbesartan was 0.01 μg/mL and for HCTZ was 0.51 ng/mL.

3.5. Recovery

Absolute recovery was evaluated by comparing peak areas, obtained from extracted spiked plasma standards with peak areas from standards in the mobile phase. The mean absolute recovery of irbesartan was 48.5, 59.6 and 60.7% for concentration levels of

Table 3
Summary of stability data pertaining to irbesartan and hydrochlorothiazide.

Storage condition	Drug	Nominal concentration[a]	Measured concentration[a]	Recovery (%)[b]
Short term stability (24 h in plasma)	Irbesartan	0.18	0.15	85.55
		4.80	4.87	101.41
	Hydrochlorothiazide	3.00	2.71	90.33
		88.00	81.36	92.45
Autosampler stability after 48 h	Irbesartan	0.18	0.16	88.89
		4.80	4.90	102.08
	Hydrochlorothiazide	3.00	2.87	95.67
		88.00	75.82	86.16
Freeze–thaw cycles ($N = 3$)	Irbesartan	0.18	0.19	105.56
		3.00	2.82	94.00
		4.80	4.58	95.42
	Hydrochlorothiazide	3.00	2.74	91.33
		55.00	51.54	93.71
		88.00	92.37	104.97
Long term stability (135 days, −86 °C)	Irbesartan	0.18	0.19	105.56
		3.00	2.84	94.67
		4.80	4.59	94.67
	Hydrochlorothiazide	3.00	3.20	106.67
		55.00	60.20	109.45
		88.00	98.78	112.25

[a] Irbesartan (μg/mL) and hydrochlorothiazide (ng/mL).
[b] Recovery (%) = measured concentration/nominal concentration × 100.

Table 4
Pharmacokinetic parameters of irbesartan and HCTZ (300 mg/12.5 mg tablet) of the test and reference products administrated to 36 volunteers.

Parameter	Irbesartan		HCTZ	
	Test	Reference	Test	Reference
C_{max}[a]	3.57 ± 1.01	3.42 ± 1.19	69.05 ± 19.09	63.56 ± 17.65
T_{max} (h)	1.65 ± 0.99	1.61 ± 0.81	1.59 ± 0.42	1.88 ± 0.71
$t_{1/2}$ (h)	10.08 ± 3.06	10.32 ± 4.33	9.97 ± 1.80	9.69 ± 1.19
$AUC_{0-96\,h}$[b]	15.56 ± 5.54	15.47 ± 6.73	394.14 ± 81.84	390.77 ± 123.87
$AUC_{0-\infty}$[b]	16.85 ± 5.68	17.11 ± 7.04	422.99 ± 84.19	416.68 ± 123.98

[a] Irbesartan (μg/mL) and hydrochlorothiazide (ng/mL).
[b] Irbesartan (μg h/mL) and hydrochlorothiazide (ng h/mL).

0.18, 3.00 and 4.80 μg/mL. The mean absolute recovery for HCTZ was 28.8, 33.0 and 34.1% for concentration levels of 3.00, 55.00 and 88.00 ng/mL.

3.6. Precision and accuracy

Ten replicate injections of the same standard mixture in the mobile phase, containing three individual concentrations of both drugs, were utilized to measure instrument precision and accuracy. Six replicate measurements of individual quality control matrix based standards of both analytes were chromatographed to evaluate method precision, as well as intra-day precision and accuracy. Inter-day precision and accuracy were measured over nine days and the results were summarized in Table 2.

3.7. Stability

Stability during sample collection, storage, and processing was investigated. Stability data was evaluated with respect to analytical signals obtained from freshly prepared QC samples.

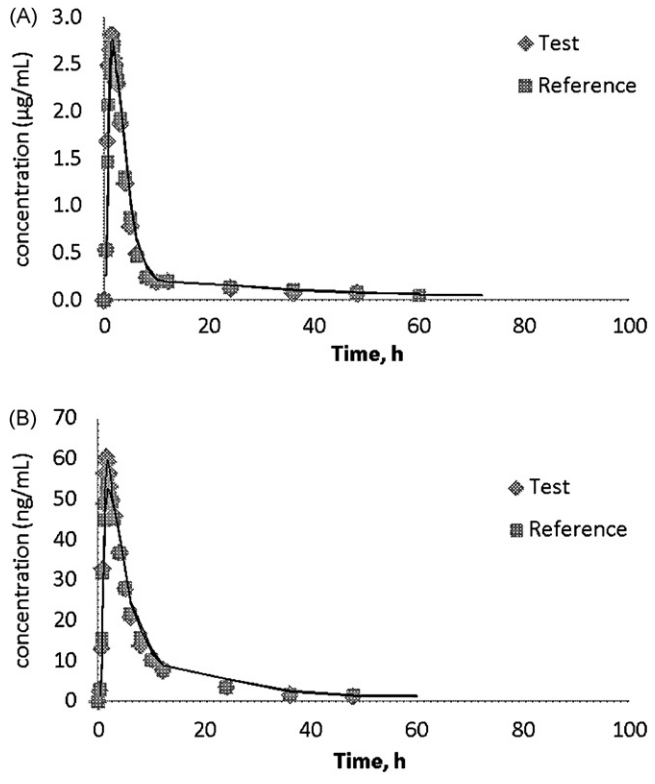

Fig. 2. Mean plasma concentration–time profiles for (A) irbesatan and (B) hydrochlorothiazide after dosing with 300 mg irbesartan and 12.5 mg hydrochlorothiazide of either the test or reference tablet formulations administrated to healthy volunteers ($N = 36$) in the fasting state.

Stability experiments extended throughout the analysis duration until assay of the last harvested sample. For short term stability studies, quality control samples including QC_L and QC_H were thawed and kept un-extracted, at room temperature, for 6, 12 and 24 h. Samples were then extracted and analyzed and signals were compared with freshly prepared samples. Autosampler stability was evaluated over 48 h. Long term matrix based solution stability was investigated under prolonged storage conditions (−86 °C) for the study period. Freeze and thaw stabilities covered three freeze–thaw cycles. The data is summarized in Table 3; these results showed that, there is no significant degradation of either irbesartan or HCTZ was observed under the test conditions.

4. Application

The developed and validated bioanalytical method was successfully applied for the determination of plasma concentrations of both irbesartan and HCTZ in the plasma samples harvested during a bioequivalence study on 36 healthy male volunteers. The mean pharmacokinetic profiles are illustrated in Fig. 2, whereas the pharmacokinetic parameters of C_{max}, T_{max}, $t_{1/2}$, $AUC_{0-96\,h}$, and $AUC_{0-\infty}$ are summarized in Table 4.

5. Conclusion

An accurate and precise LC–MS/MS method for the simultaneous determination of irbesartan and HCTZ in human plasma has been developed and validated. The method demonstrated high selectivity and sensitivity which rendered the method fits for the purpose of its application to measure concentration–time profiles for bioavailability, pharmacokinetic and bioequivalence decision after dosing with two tablet formulations containing 300 mg irbesartan and 12.5 mg HCTZ tablet on healthy volunteers in a fasted state. Calibration graphs were linear in the concentrations ranging 0.06–6.00 μg/mL for irbesartan and 1.00–112.00 ng/mL for HCTZ. Limits of quantitation were 0.06 μg/mL and 1.00 ng/mL for irbesartan and HCTZ, respectively. The method demonstrated high calibration sensitivity (0.2537 and 0.0129 for irbesartan and HCTZ, respectively).

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