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Journal of Chromatography B, 848 (2007) 245–250
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Liquid chromatographic determination of irbesartan in human plasma

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Received 15 June 2006; accepted 20 October 2006
Available online 21 November 2006

Abstract

A simple and sensitive method was developed for determination of irbesartan by liquid chromatography with fluorescence detection. Irbesartan and losartan (I.S.) in human plasma were extracted using diethyl ether:dichloromethane (7:3, v/v) followed by back extraction with 0.05 M sodium hydroxide. Neutralized samples were analyzed using 0.01 M potassium dihydrogen phosphate buffer (containing 0.07% triethylamine as peak modifier, pH was adjusted with orthophosphoric acid to pH 3.0) and acetonitrile (66:34, v/v). Chromatographic separation was achieved on an ODS-C-18 column (100 mm × 4.6 mm i.d., particle size 5 μm) using isocratic elution (at flow rate 1.25 ml/min). The peak was detected using a fluorescence detector set at Ex 259 nm and Em 385 nm, and the total time for a chromatographic separation was ∼13 min. The validated quantitation ranges of this method were 15–4000 ng/ml with coefficients of variation between 0.75 and 12.53%. Mean recoveries were 73.3–77.1% with coefficients of variation of 3.7–6.3%. The between- and within-batch precision were 0.4–2.2% and 0.9–6.2%, respectively. The between- and within-batch relative errors (bias) were (−5.5) to 0.9% and (−0.6) to 6.9%, respectively. Stability of irbesartan in plasma was >89%, with no evidence of degradation during sample processing and 60 days storage in a deep freezer at −70 °C. This validated method is sensitive and simple with between-batch precision of <3% and can be used for pharmacokinetic studies.

Keywords: Irbesartan; Fluorescence detection; Liquid–liquid extraction

1. Introduction

Hypertension is the most prevalent cardiovascular disease in the developed as well as developing countries, affecting as many as one quarter of the adult population. Furthermore, hypertension is an independent risk factor for cardiovascular disease and is associated with an increased incidence of stroke and coronary heart disease. Although there have been many advances in the treatment over the past several decades, less than 25% of all hypertensive patients have their blood pressure adequately controlled with available therapies.

Angiotensin II antagonists are the major development in hypertension management in over a decade. Their excellent lower side effect profile and specificity in the action provide good condition for patient compliance as well as effectiveness. Therefore, these drugs are used as first-line treatment for essential hypertension.

Irbesartan is a non-peptide compound, chemically described as a 2-butyl-3-[*p*-(*o*-1H-tetrazol-5-ylphenyl)benzyl]-1,3-diazaspiro[4.4]non-1-en-4-one (Fig. 1). It is an orally active specific angiotensin II receptor antagonist used, as a hypotensive agent does not require biotransformation into an active form. The oral absorption of irbesartan is rapid and complete with an average absolute bioavailability of 60–80%. Following an oral administration of irbesartan in therapeutic dose (75–300 mg), peak plasma concentration of irbesartan is attained at 1.5–2 h after dosing. Irbesartan exhibits linear pharmacokinetics over the therapeutic dose range [1–2]. On the basis of the reported literature, the peak plasma concentration following administration of 150 mg dose of irbesartan is 1.50 ± 0.29 μg/ml [3–4]. The plasma concentration of irbesartan following sub-therapeutic dose (2 × 25 mg capsules) after 24 h was 45 ng/ml [5].

Although a few HPLC methods with Diode array [6], UV [3,7] and fluorescence detection utilizing solid phase extraction (SPE) or biocompatible in-tube solid phase micro-extraction

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doi:10.1016/j.jchromb.2006.10.044

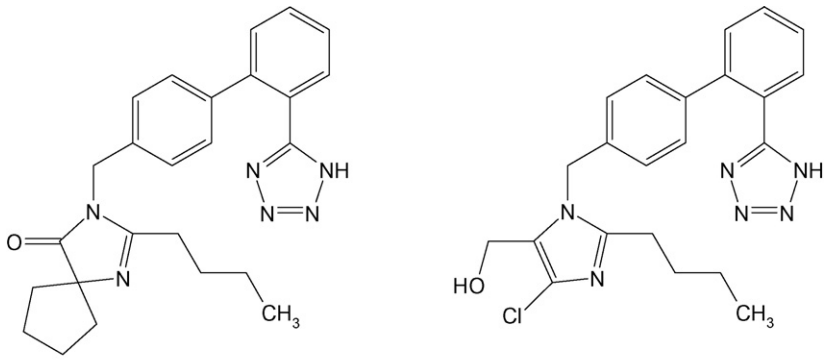

Fig. 1. Chemical structure of irbesartan and losartan (I.S.).

(SPME) for the determination of irbesartan have been previously reported [8–10], literature survey revealed that there is a high variation in the limit of quantitation (1 ng/ml to 10 μg/ml) as reported by different authors [6–10]. The quantitation ranges were reported as 1–1000 ng/ml [8], 5–2000 ng/ml [10], 20–1000 ng/ml [6], 50–4000 ng/ml [9] and 10–60 μg/ml [7]. For routine clinical analysis, a high-throughput analysis with expensive SPE is always not advantageous as such equipment and techniques are not available in most of the laboratories. Therefore, new methods with both simple and sensitive determination of irbesartan are required. This paper describes a simple, economical and sensitive HPLC method with fluorescence detection for the determination of irbesartan in human plasma using a liquid–liquid extraction.

2. Experimental

2.1. Chemicals

Irbesartan and losartan drug substances (internal standard, I.S., Fig. 1) were obtained from Sun Pharmaceutical Industries Ltd., Mumbai, India. HPLC-grade water (Lichrosolve water), methanol, diethyl ether and dichloromethane were from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

2.2. Drug solutions

Stock solutions of irbesartan and losartan (I.S.) for generating standard curves were prepared by dissolving an appropriate amount of each compound in methanol to yield concentration of 1.0 mg/ml. Working standard solutions of irbesartan were obtained by further diluting the stock standard solution with methanol:water (7:3, v/v).

2.3. Chromatography

For chromatographic analysis, Merck-Hitachi (Lachrome®) HPLC equipped with quaternary gradient pump (Lachrome® 7100), Perkin-Elmer fluorescence detector (model-LC 240), an autosampler (L7200) with a rheodyne injector (Rheodyne 7125) holding 100 μl loop was used. The signals were acquired and analyzed using Windows 2000 based, D700 HSM chromatography data station software. The separation of the compounds was made on an ODS-Hypersil-C-18 Column (100 mm × 4.6 mm, 5 μm, Thermo Electron Corporation, USA) at temperature 20 ± 1 °C. The mobile phase used in this study was 0.01 M (1.36 g/l) Potassium dihydrogen phosphate buffer (containing 0.06% triethylamine as peak modifier, pH 3.0 adjusted with orthophosphoric acid) and acetonitrile (66:34, v/v). All analyses were performed under isocratic condition at a flow rate of 1.25 ml/min. The excitation and emission wavelength were set at 259 and 385 nm (response 5, factor 1024). Mobile phase was filtered through 0.45 μm Millipore filter before use and degassed in an ultrasonic bath. The pH meter used was Orion Research (model 611, Orion Research incorporation, USA).

2.4. Sample processing

One millilitre volume of plasma was transferred to a 15 ml glass test tube and the 50 μl of internal standard working solution (60 μg/ml) was spiked. Solution was vortexed and acidified with 150 μl of 1 M-orthophosphoric acid. Then 6 ml aliquot of extraction solvent diethylether:dichloromethane (7:3, v/v) was added using Ceramus® Classic Bottle top Dispenser (Hirsch Mann Laborgerate GmbH, Eberstadt, Germany). The sample was vortexed for 5 min using Vibrax Vortexer (Model VX-Z VXR Basic, IKAWerke GmbH & Co. Staufen, Germany) and centrifuged for 2 min at 1000 × *g*. The organic layer was transferred to a 10 ml clean test tube containing 200 μl of 0.05 M sodium hydroxide (pH > 11). The analytes were back extracted into this aqueous layer by 1 min vortex mixing and centrifugation for 5 min at 1000 × *g*. This aqueous layer was frozen at −30 °C using deep freezer (Platinum 500 V, Angelantoni Industrie S.p.a, Italy) for 10 min. The organic layer was discarded and the aqueous layer thawed and acidified with 100 μl of 0.2 M orthophosphoric acid. Aliquot of 50 μl was injected into HPLC.

2.5. Bioanalytical method validation

2.5.1. Standard and quality control samples

Working solution of irbesartan for the calibration (0.30, 0.60, 1.20, 2.50, 10, 20, 40 and 80 μg/ml) and quality control sample

(0.30, 0.90, 44 and 72 μg/ml) was prepared separately. Working solution for calibration and quality control (10, 20, 40, 44, 72 and 80 μg/ml) was prepared by diluting the 1000 μg/ml stock solution (0.20, 0.40, 0.80, 0.88, 1.44 and 1.60 ml) to 20 ml in volumetric flask using methanol:water (7:3, v/v), while other solutions (0.30, 0.60, 0.90, 1.20 and 2.50 μg/ml) were prepared by diluting the intermediate 40 μg/ml working solution (0.15, 0.30, 0.45, 0.60 and 1.25 ml) to 20 ml. The internal standard working solution 60 μg/ml was prepared by diluting stock solution with diluent. Fifty microlitres of working standard solution was added to 950 μl of drug free plasma to obtain irbesartan concentration of 15, 30, 60, 125, 500, 1000, 2000, and 4000 ng/ml. Similarly, the quality control samples of irbesartan as a single batch [of concentration 15 (LLOQ), 45 (low), 2200 (medium) and 3600 ng/ml (high)] were prepared by spiking the 5% working solution to the 95% of pooled blank plasma. The quality control samples were divided into aliquot in test tube and stored in the deep freezer at −70 °C until analysis.

A calibration curve was made from a blank sample (a plasma sample processed without an I.S.), a zero sample (a plasma processed with I.S.) and eight non-zero samples covering the total range (15–4000 ng/ml), including lower limit of quantification (LLOQ). Such calibration curves were generated on 6 consecutive days. Linearity was assessed by a weighted ($1/x^2$) least squares regression analysis. The acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value except LLOQ, which was set at 20%.

2.5.2. Recovery

Recovery of irbesartan was evaluated by comparing the mean peak areas of six extracted low, medium and high quality control samples with the mean peak areas of six neat reference solutions (unprocessed) containing the same amount of the test compound.

2.5.3. Specificity and selectivity

To evaluate the specificity of the method, drug free plasma sample was carried through the assay procedure and the retention times of the endogenous compounds in the plasma were compared with those of irbesartan or losartan (internal standard). Specificity of the method was assessed to test the matrix influence between different plasma samples. Interference from other or over the counter (OTC) medication was also investigated. The tested compounds were aspirin, ibuprofen, metronidazole, paracetamol, caffeine and valsartan.

2.5.4. Sensitivity

The limit of detection was defined, as analyte responses are at least five times the response compared to blank responses. The lowest standard on the calibration curve was defined as the limit of quantification as an analyte peak was identifiable, discrete and reproducible with a precision of less than or equal to 20% and accuracy of 80–120%.

2.5.5. Accuracy and precision

Within-batch accuracy and precision evaluation were performed by repeated analysis of etoricoxib in human plasma. The batch consists of a calibration standards, six replicates of LLOQ, low, medium and high quality control samples, while between-batch accuracy and precision were assessed by analysis of similar sequence of samples on three separate occasions. The overall precision of the method was expressed as relative standard deviation and the accuracy of the method was expressed in terms of relative error (bias).

2.5.6. Stability

The bench top stability was examined by keeping replicates of the low and high plasma quality control samples at room temperature for approximately 24 h. Freeze–thaw stability of the samples was obtained over three freeze–thaw cycles, by thawing at room temperature for 2–3 h, refrozen for 12–24 h. Autosampler stability of irbesartan was tested by analysis of processed and reconstituted low and high plasma QC samples, which are stored in the autosampler tray for 24 h at 10 ± 1 °C. Stability of irbesartan in human plasma was tested after storage at approximately −70 °C for 60 days. For each concentration and each storage condition, six replicates were analyzed in one analytical batch. The concentration of irbesartan after each storage period was related to the initial concentration as determined for the samples.

2.5.7. Stock solution stability

The working solution (5 μg/ml) of irbesartan was repeatedly ($n = 3$) injected into the chromatograph immediately after preparation (time 0) and at 3, 6, and 9 h after bench top storage at room temperature and 4 °C. This injection protocol was repeated after 1, 3, 6, 8, 15, 27, 45 and 60 days storage of this solution between 4 and 8 °C.

3. Results and discussion

3.1. Chromatography

Fig. 2 shows the representative chromatograms of (A) blank plasma, (B) blank plasma with internal standard, (C) plasma spiked with irbesartan at 15 ng/ml (LLOQ), (D) plasma spiked with irbesartan at 1000 ng/ml, (E) plasma sample from volunteer at zero time and (F) plasma sample from volunteer 12 h after a single dose of 150 mg of irbesartan. The analytes were well separated from co-extracted material under the present chromatographic conditions at retention time of ∼10.0 and ∼5.5 min for irbesartan and losartan, respectively. The peaks were of good shape and completely resolved one from another. The chromatogram of extracted plasma samples did not show any co-eluting interference peak with the analyte or I.S.

There were no interfering peaks present in six different randomly selected samples of drug-free human plasma used for analysis at the retention time of either analyte or I.S. Several compounds (aspirin, ibuprofen, metronidazole, paracetamol, caffeine and valsartan) did not produce any interference with the drug or the internal standard during analysis.

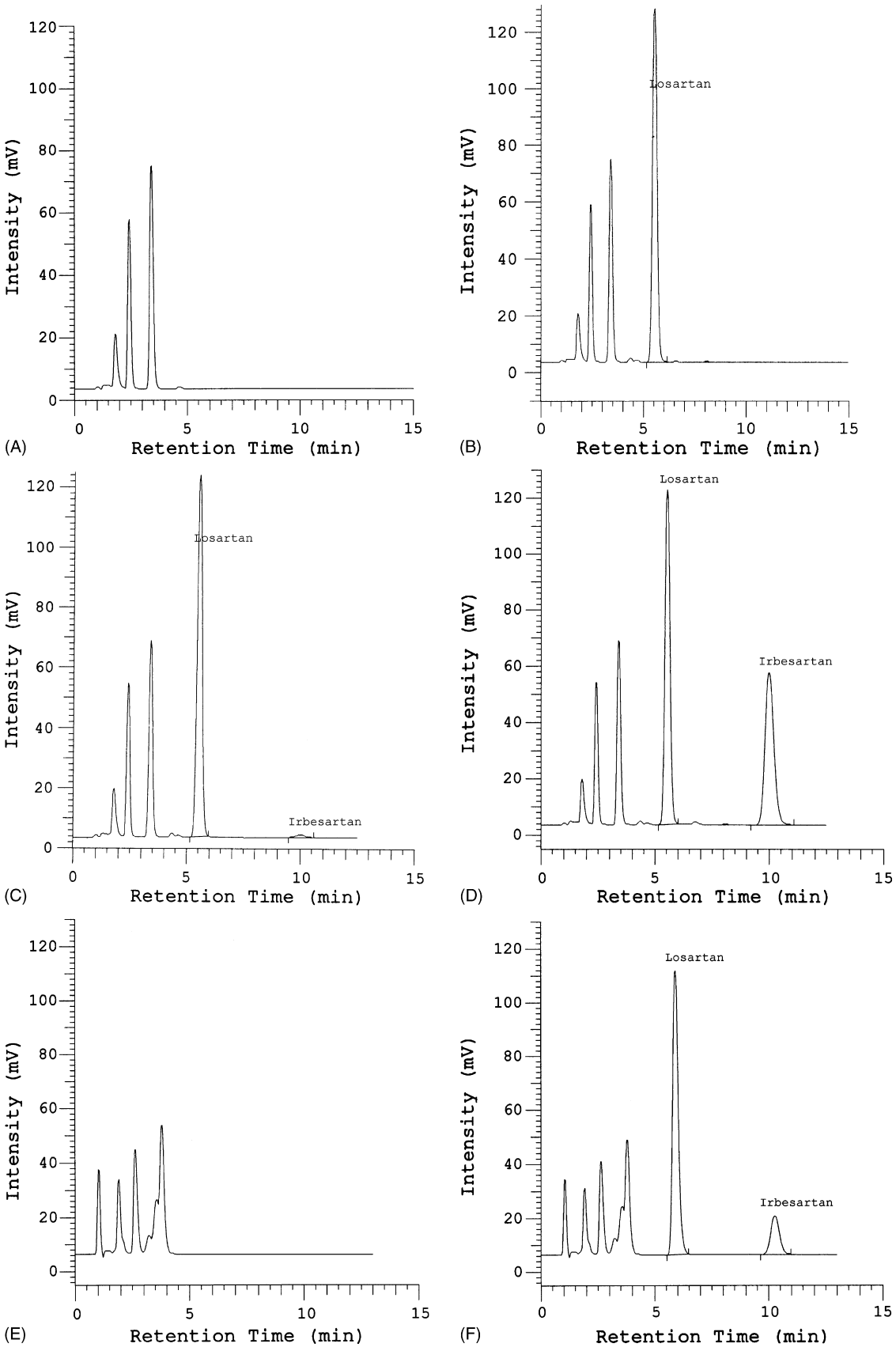

Fig. 2. Chromatograph resulting from (A) analysis of blank human plasma, (B) human plasma spiked with 3000 ng/ml of losartan (I.S.), (C) human plasma spiked with 15 ng/ml of irbesartan and 3000 ng/ml of losartan, (D) human plasma spiked with 1000 ng/ml of irbesartan and 3000 ng/ml of losartan, (E) plasma sample from volunteer at zero time and (F) plasma sample from volunteer 12 h after 150 mg dose of irbesartan (the plasma concentration was determined to be 248.76 ng/ml).

Table 1
Extraction recovery of analyte from plasma ($n=6$)

Analyte	Concentration added (ng/ml)	Recovery (%)	CV (%)
Irbesartan	45	73.3	3.7
	2200	74.6	5.4
	3600	77.1	6.3

3.2. Recovery and linearity

Recovery from plasma was calculated by comparing the peak areas of pure standards prepared in methanol:water (7:3, v/v), and injected directly into the analytical column with those of extracted plasma samples containing the same amount of the test compound ($n=6$ each). Mean recoveries were 73.3–77.1% with coefficients of variation of 3.7–6.3% at three different concentrations ranging for irbesartan (45, 2200 and 3600 ng/ml) (Table 1).

Calibration curves were linear over the concentrations range from 15 to 4000 ng/ml for irbesartan ($r=0.999$ or better) with coefficients of variation between 0.75–12.53%. The best-fit calibration curve were achieved with linear equation $y=mx+c$ with $1/x^2$ weighting factor. The mean linear equation of calibration curve were achieved with linear equation $y=mx+c$ with $1/x^2$ weighting factor. The mean linear equation of calibration curve for the analyte was $y=0.0008184\ (\pm 0.00004488)x - 0.00090328\ (\pm 0.002972)$, where y was the peak area ratio of the analyte to the I.S. and the x was the concentration of the analyte. Precision and relative error of back-calculated concentrations of standard solutions for irbesartan are mentioned in Table 2.

3.3. Sensitivity

The limits of detection and quantification were 2 and 15 ng/ml, respectively. The precision and relative error for LLOQ were 12.5 and 3.10%, respectively.

3.4. Precision and accuracy

Within- and between-day precision and accuracy were evaluated by assaying quality controls with different concentrations of irbesartan. Within- and between-day relative standard deviations (precision, %CV) were less than 6.2 and 2.2%, respectively. Within- and between-day relative errors (bias, %) were less than 6.9 and 0.9%, respectively (Table 3).

Table 2
Precision and accuracy (relative error) of back-calculated concentration of standard sample for irbesartan in spiked plasma

Spiked concentration (ng/ml)	Concentration found (mean ± S.D., $n=6$) (ng/ml)	Precision (%)	Relative error (bias, %)
15	15.47 ± 1.94	12.53	3.10
30	27.35 ± 1.90	6.94	−8.85
60	58.66 ± 2.18	3.72	−2.23
125	120.57 ± 5.74	4.76	−3.55
500	494.53 ± 23.88	4.83	−1.09
1000	1015.85 ± 41.90	4.12	1.59
2000	1962.29 ± 81.51	4.15	−1.89
4000	4012.17 ± 30.01	0.75	0.30

Accuracy was expected as percent error (relative error) [(measured concentration − spiked concentration)/spiked concentration] × 100 (%), precision was quantitated by calculating within- and between-day %CV values.

3.5. Stability

Stability results are given in Table 4. Twenty-four hour room temperature storage and freeze–thaw cycles for low and high quality controls samples indicated that irbesartan was stable in human plasma under experimental condition. QC samples were stable for at least 60 days, which are frozen at approximately −70 °C. Results from autoinjector stability indicate that the irbesartan is stable when kept in the autosampler for up to 24 h.

3.6. Assay application

The described assay has been successfully employed without any interference to quantitate irbesartan in human plasma samples obtained from a volunteer following the administration of single 150 mg dose of the drug. The representative chromatograms of plasma sample are shown in Fig. 2E and F.

Table 3
Precision and accuracy (relative error) for the determination of analysis in spiked plasma

Spiked concentration (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	Precision (%)	Relative error (bias, %)
Within assay precision ($n=6$)			
15	16.03 ± 0.99	6.2	6.9
45	47.06 ± 1.91	4.1	4.6
2200	2227.50 ± 20.62	0.9	1.3
3600	3577.31 ± 120.41	3.4	−0.6
Between assay precision ($n=3$)			
15	14.18 ± 0.31	2.2	−5.5
45	44.84 ± 0.17	0.4	−0.4
2200	2203.70 ± 25.42	1.2	0.2
3600	3631.79 ± 21.78	0.6	0.9

Table 4
Stability of the irbesartan in samples

Sample concentration (ng/ml)	Concentration found (ng/ml)	Precision (%)	Relative error (%)
Short term stability for 24 h ($n = 6$) in plasma			
45	41.38	3.3	−8.1
3600	3645.51	2.4	1.3
Freeze–thaw stability ($n = 6$)			
45	40.11	4.7	−10.9
3600	3678.50	3.8	2.2
Autosampler stability ($n = 6$)			
45	41.58	7.4	−7.6
3600	3626.35	6.8	0.7
Sixty days stability at −70 °C ($n = 6$)			
45	43.58	6.2	−3.2
3600	3620.67	7.1	0.6

4. Conclusion

The HPLC method described here is simple, sensitive, specific and fully validated as per guidelines. The previously reported methods (SPE and in-tube SPME) for the analysis of irbesartan in plasma were very expensive due to the expensive sample preparation techniques. Besides the SPE [8] and in-tube SPME [10] methods, the present liquid–liquid extraction based isocratic HPLC method with its LLOQ (15 ng/ml) and LOD (2 ng/ml) values is more sensitive and precise than the other previously reported methods [6,7,9]. Good validation data were achieved for linearity, assay precision, accuracy and recovery. This validated method covers the wide range of linearity for irbesartan (15–4000 ng/ml), which is suitable for the estimation of irbesartan at different therapeutic dose levels for pharmacokinetic studies as well as therapeutic drug monitoring. The HPLC procedure described for the determination of irbesartan is applied to pharmacokinetic studies.

Acknowledgements

Authors wish to thank Dean, Faculty of Pharmacy and Medical sciences, Amman University and University of Jordan, Amman and Managing Director, Jordan Center for Pharmaceutical Research, Amman for providing necessary facilities.

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