ELSEVIER

Journal of Chromatography B, 702 (1997) 149–155

JOURNAL OF CHROMATOGRAPHY B

High-performance liquid chromatographic assay for the quantitation of irbesartan (SR 47436/BMS-186295) in human plasma and urine

Shu-Ying Chang[a], Daisy B. Whigan[a,*], Nimish N. Vachharajani[b], Rajesh Patel[a]

[a]*Department of Metabolism and Pharmacokinetics, Bristol-Myers Squibb Pharmaceutical Research Institute, One Squibb Drive, P.O. Box 191, New Brunswick, NJ 08903-0191, USA*

[b]*Princeton University, P.O. Box 4000, Princeton, NJ 08543-4000, USA*

Received 26 March 1997; received in revised form 17 July 1997; accepted 17 July 1997

Abstract

A selective, accurate, precise and reproducible high-performance liquid chromatographic assay was developed for the quantitation of irbesartan, an angiotensin II antagonist, in human plasma and urine samples. The method involved solid-phase extraction of irbesartan and internal standard (I.S.) using a 100-mg Isolute CN cartridge. A portion of the eluate was injected onto an ODS analytical column connected to a fluorescence detector that was set at an excitation wavelength of 250 nm and an emission wavelength of 371 nm. The mobile phase consisted of 50% acetonitrile and a 50% weak phosphate–triethylamine solution, pH 3.5, at a flow-rate of 0.8 ml/min. The assay was linear from 1 to 1000 ng/ml with both plasma and urine. In either matrix, the lower limit of quantitation was 1 ng/ml. The analyses of quality control samples indicated that the nominal values could be predicted with an accuracy >95%. The inter- and intra-day coefficients of variation for the analyses in both matrices were <8%. Irbesartan was stable in both human plasma and urine for at least seven months at −20°C. The application of the assay to a pharmacokinetic study is described. © 1997 Elsevier Science B.V.

Keywords: Irbesartan; SR 47436; BMS-186295; Angiotensin II antagonist

1. Introduction

The renin–angiotensin system (RAS) plays a central role in the regulation of blood pressure and in fluid and electrolyte balance. The RAS is an enzyme cascade that leads to the production of the active hormone, angiotensin II (AII). AII causes vasoconstriction by activating the AII–AT1 receptor, and blockade of this receptor will therefore inhibit vasoconstriction [1].

Recent years have seen the development of a new class of antihypertensive agents that act as specific antagonists of the AII receptor. One such agent is irbesartan, which is a potent, long-acting, non-peptide AII receptor antagonist with high specificity for the AT1 subtype [2]. Irbesartan, 2-butyl-3-[(2′-(1H-tetrazole-5-yl)biphenyl-4-yl)methyl] - 1, 3 - diazaspiro [4,4]non-1-en-4-one, is also known as BMS-186295 and SR 47436. It was discovered by Sanofi Recherche and is being developed jointly by Bristol-Myers Squibb (BMS) and Sanofi-Winthrop. The primary indication will be hypertension. On the basis

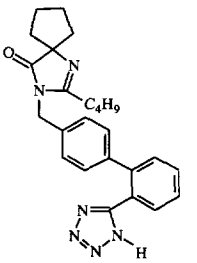
*Corresponding author.

PII S0378-4347(97)00387-3

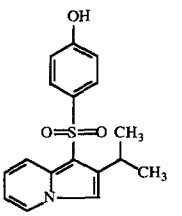
of its mechanism of action, irbesartan has the potential to offer advantages in safety and tolerability over earlier classes of drugs for the treatment of hypertension, diabetic nephropathy and heart failure [3–5].

Irbesartan has a dose-related effect on plasma renin and AII at doses ranging from 5 to 100 mg [6]. Clinical studies in hypertensive subjects have demonstrated that irbesartan safely and effectively lowers blood pressure (BP) with once-daily administration [7,8].

As a crucial part of the drug development process, a rapid, sensitive and selective assay is required to measure drug concentrations in plasma and urine samples from clinical pharmacokinetic studies. There is no methodology reported in the literature to measure irbesartan in body fluid samples. We report an accurate and sensitive validated high-performance liquid chromatographic (HPLC) assay with fluorescence detection for the determination of irbesartan in human plasma and urine samples.



Irbesartan (BMS-186295)



Internal standard (BMS-190462)

Fig. 1. Chemical structures of irbesartan (I) and the internal standard (II).

2. Experimental

2.1. Chemicals and reagents

Irbesartan and internal standard (BMS-190462) were obtained from Sanofi Recherche (Montpellier, France). The chemical structures of irbesartan and internal standard (I.S.) are provided in Fig. 1. HPLC-grade acetonitrile, HPLC-grade methanol and HPLC-grade phosphoric acid (85–88%, approximately 14.7 *M*) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). A dilute phosphoric acid solution, prepared by diluting 1 ml of the commercially obtained phosphoric acid to 100 ml with water, was used in the preparation of samples. Hexane was purchased from Burdick and Jackson (Muskegon, MI, USA). Triethylamine (TEA) was obtained from Sigma (St. Louis, MO, USA). Water was double-distilled and filtered using a Milli-Q-System from Millipore (Milford, MA, USA). Control human plasma was obtained from Biological Specialty (Colmar, PA, USA). All other reagents were of analytical grade and were used without further purification.

2.2. Apparatus and HPLC conditions

The HPLC system consisted of the following components: a Perkin-Elmer/ABI Spectroflow 400 pump and Perkin-Elmer ISS-100 autosampler equipped with a 20-μl loop (Perkin-Elmer, Norwalk, CT, USA), a model F-2000 or F-1080 Hitachi fluorescence detector (Hitachi Instruments, Danbury, CT, USA). The analytical column was a YMC-ODS-AQ, 150×4.6 mm I.D., 5 μm packing, supplied by YMC (Wilmington, NC, USA). Isocratic separation was achieved using a mobile phase consisting of an aqueous phosphoric acid–TEA solution and acetonitrile (50:50, v/v), which was maintained at a flow-rate of 0.8 ml/min. The aqueous phosphoric acid–TEA solution was prepared by adding 1.0 ml of TEA to 1 l of water, then adjusting the pH to 3.5 with commercial phosphoric acid (85–88%, approximately 14.7 *M*). A fluorescence detector set at an excitation wavelength of 250 nm, with the emission wavelength at 371 nm, was used to detect the analytes.

2.3. Extraction cartridges

Isolute Cyano (CN) solid-phase cartridges from Jones Chromatography (Lakewood, CO, USA) were used for sample extraction. Each cartridge contained 100 mg of sorbent with a total reservoir of 3 ml. The cartridges were processed on a Varian Vac-Elut SPS-24 processing station connected to an Analytichem vacuum manifold (Analytichem, Harbor City, CA, USA).

2.4. Standard solutions

Stock solutions of irbesartan and I.S. (250 μg/ml) were prepared in methanol. The stock solutions were stored at 5°C and were found to be stable for at least six months. A secondary I.S. solution, prepared weekly at 1000 ng/ml in a dilute phosphoric acid solution, was used to spike the samples prior to extraction.

2.5. Standard curve and quality control samples

Blank plasma or urine samples were spiked with 1–1000 ng/ml of irbesartan. These spiked samples were used to construct a standard curve. Quality control (QC) samples were prepared in plasma or urine to contain irbesartan concentrations within the standard curve range. These QC samples were used to determine the acceptability of an analytical run. In addition, a QC sample containing an irbesartan concentration greater than 1000 ng/ml was prepared in blank matrix to serve as a dilution QC sample. This dilution QC sample was assayed together with samples that have drug concentrations above the upper limit of quantitation. The dilution QC and study samples were diluted with blank matrix prior to sample extraction.

2.6. Sample extraction

The disposable cartridge was conditioned with 2 ml of methanol followed by 2 ml of a dilute phosphoric acid solution. A volume of 1.0 ml of dilute phosphoric acid and 100 μl of I.S. secondary solution were mixed with 250 μl of plasma or urine sample. The mixture was slowly passed through the cartridge. For plasma samples, the cartridge was then washed with 3 ml of a dilute phosphoric acid solution followed by 1 ml of hexane. For urine samples, the cartridge was washed with 4 ml of a dilute phosphoric acid solution. Irbesartan and I.S. were eluted with 1 ml of eluting solution. The eluting solution was a 50:50 (v/v) mixture of methanol and dilute phosphoric acid. After mixing the eluate, a 20-μl volume was injected onto the HPLC system.

2.7. Measurement and calculations

Chromatographic data management was automated using a VAX Multichrom Data Acquisition System (Fisons Instruments, Danvers, MA, USA). Peak height ratios were used in the calculations. The standard curve samples were fit to a linear regression equation, weighting each standard by the reciprocal of its nominal concentration. Irbesartan concentrations in the clinical samples were then determined from the regression equation.

3. Results and discussion

3.1. Method development

Both irbesartan and the I.S. were extracted from plasma and urine samples by solid-phase extraction using a cyano cartridge. For irbesartan, the pK_a corresponding to the loss of the proton from the tetrazole ring is approximately 4.5. It is important to condition the cartridge with dilute phosphoric acid before applying the sample, for quantitative retention of the analytes. For plasma samples, additional washing with hexane removes the lipophilic endogenous substances that might interfere with the chromatographic analysis. For urine samples, this hexane wash is not necessary. Other cartridge sorbents were investigated. C_{18} and C_8 cartridges were more retentive and required a stronger eluting solution for quantitative recovery. In addition, the non-polar cartridges retained more endogenous materials, resulting in more extraneous peaks in the chromatograms. Phenyl cartridges retained the analytes more

strongly than the C_8 or C_{18} cartridges and required either pure methanol or pure acetonitrile for elution. C_2 cartridges gave comparable results for the analytes as the CN cartridges, however, the chromatograms showed more extraneous peaks. All of the results presented in this paper were obtained using cyano cartridges.

The chromatographic system is quite robust. Other ODS columns have been tested, with minimal effect on the resolution of the analytes. Also, in limited trials, a Symmetry C_8 analytical column (Waters, Milford, MA, USA) resolved the analytes from endogenous substances in both plasma and urine. A YMC-ODS-AQ column is recommended because of its demonstrated ruggedness and reproducibility in this assay.

The fluorescence detector used in the assay determined the sensitivity that can be achieved by the assay. Either an Hitachi Model F-2000 or an Hitachi Model F-1080 gave acceptable sensitivities. The use of some detectors made by other manufacturers was not as successful in measuring as low as 1 ng/ml.

The assay has been validated according to the guidelines proposed at the conference on Analytical Methods for Bioavailability, Bioequivalence and Pharmacokinetic Studies [9].

3.2. Selectivity

Sample extraction and chromatographic analysis were developed to produce a selective assay for the analytes. At least ten different lots of commercial plasma and urine donations from ten male volunteers were carefully evaluated for interference in the assay. No interfering components were observed. Typical chromatograms of drug-free plasma and urine samples are shown in Fig. 2.

Studies with ^{14}C-labeled irbesartan showed that more than 80% of the total radioactivity in the plasma was attributed to unchanged irbesartan. About 6% of the plasma radioactivity was attributable to irbesartan glucuronide, and other minor metabolites accounted for most of the remaining radioactivity [10]. All of the metabolites that were isolated and identified were chromatographically resolved from both irbesartan and I.S.

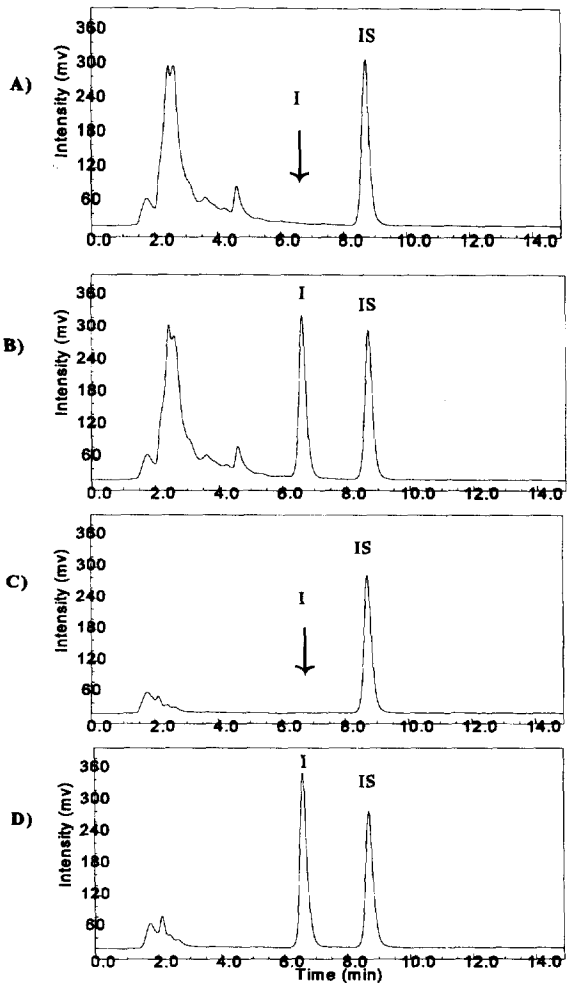


Fig. 2. Typical chromatograms of human plasma and urine samples. (A) Blank plasma spiked with internal standard; (B) clinical plasma sample containing 519 ng/ml of irbesartan; (C) blank urine spiked with internal standard and (D) clinical urine sample containing 495 ng/ml of irbesartan. Peaks: I = irbesartan; IS = internal standard.

3.3. Linearity

Standard curves in plasma or urine were prepared to cover the range of 1–1000 ng/ml. The peak–height ratios of irbesartan to I.S. are directly proportional to the concentrations of irbesartan. Following the recommended procedure, typical linear regression results in the following equation:

Irbesartan concentration (ng/ml) = 0.00196 (ratio) + 0.00489 $R^2 = 0.999$

The lower and upper limits of the standard curve range depend on the detector response. The photomultiplier (PMT) voltage is selected such that at the lower limit of quantitation (LLQ), the *S/N* ratio is about 5:1. Sometimes this setting is too sensitive such that the response at the upper limit of quantitation (ULQ) results in an off-scale peak. When this happens, the injection volume is decreased.

3.4. Lower and upper limits of quantitation

The LLQ is defined as the lowest concentration in the standard curve that back-calculates with adequate precision and accuracy. Evaluation of the standard curves prepared over three days showed that the LLQ is 1 ng/ml for both plasma and urine. At this concentration in both matrices, the accuracy is better than 85% and the coefficient of variation (C.V.) is <15%. In addition, six different lots of matrix were spiked at the LLQ of 1 ng/ml. These LLQ spikes were assayed and the back-calculated results further validated the LLQ of the assay.

The ULQ is the highest concentration in the standard curve that could be analyzed using the chromatographic parameters. Typically, this concentration approached the upper linear limit of the fluorescence detector within a given set of parameters. This concentration can usually be adjusted by controlling the amount of eluate injected. Samples that contain analyte concentrations higher than the ULQ can be assayed after dilution with blank matrix. When this occurs, a dilution QC sample is assayed together with the samples using the same dilution factor.

3.5. Extraction recovery

Two sets of standards containing irbesartan and I.S. were prepared for each matrix. One set was prepared in the eluting solution and the other set was prepared in the control human plasma or urine. Plasma and urine standards were processed as previously described while standards prepared in eluting solution were injected onto the column directly. By comparing the slopes of the extracted and non-extracted curves, the recovery of irbesartan was ≈90%, regardless of the matrix. The recovery of the I.S., calculated as the ratio of the average I.S. peak height in the extracted samples versus the average I.S. peak height in the non-extracted samples multiplied by 100, is also ≈90%.

3.6. Accuracy and precision

The accuracy and precision of the assay were determined by assaying QC samples in five replicates on at least three different days. A fourth QC sample (dilution QC) was spiked above the ULQ and diluted with blank matrix prior to extraction, to demonstrate the accuracy and precision of analyses where dilution is necessary. Statistical evaluation of the results established good accuracy and precision of the method. For the four-day analyses of plasma QC samples, the mean accuracy ranged from 95 to 100%. Within- and between-run precision were <8% for all concentration levels (Table 1). For the three-day analyses of urine QC samples, the mean accura-

Table 1
Within- and between-run accuracy and precision for the irbesartan plasma assay

Nominal concentration (ng/ml)	Mean observed concentration (ng/ml)	Deviation (%)	Between-run precision (% R.S.D.)	Within-run precision (% R.S.D.)
5	4.98	−0.40	0.0[b]	4.76
500	477	−4.60	1.59	3.16
850	847	−0.35	7.35	3.11
2000[a]	2004	0.20	4.70	3.28

[a] Dilution quality control sample.
[b] No significant additional variation was observed.

Table 2
Within- and between-run accuracy and precision for the irbesartan urine assay

Nominal concentration (ng/ml)	Mean observed concentration (ng/ml)	Deviation (%)	Between-run precision (% R.S.D.)	Within-run precision (% R.S.D.)
25	23.9	−4.40	0.73	1.78
200	192	−4.00	2.13	1.29
375	384	2.40	2.03	1.83
2000[a]	1919	−4.05	2.50	2.84

[a] Dilution quality control sample.

cy ranged from 95 to 102%, and the within- and between-run precision were <3% for all concentration levels (Table 2).

During a typical week ($n=5$), the slopes of plasma and urine standard curves were quite reproducible with a C.V. of <5%, further supporting the precision of the assay in either matrix.

3.7. Stability

Stability of irbesartan in eluting solution under ambient conditions was assessed by comparing the absolute peak heights of both irbesartan and the I.S. over a 24-h period. All of the results indicated that both irbesartan and I.S. were stable in eluting solution at ambient conditions for at least 24 h.

The stability of irbesartan in plasma and urine was determined by periodic analysis of spiked samples over seven months. The results indicated that no significant degradation occurred over the seven-month period. In addition, select clinical plasma samples, which were assayed repeatedly over seven months, showed good stability of irbesartan at −20°C. Irbesartan was also shown to be stable in plasma and urine under ambient conditions for at least 24 h. Furthermore, irbesartan was stable through at least five freeze–thaw cycles.

3.8. Application

The assay has been successfully used to analyze thousands of plasma and urine samples from Phase I and II clinical studies. Typical chromatograms of plasma and urine samples collected post-dose are shown in Fig. 2. With slight modifications to the sample size assayed, the methodology has been applied to rat, rabbit and monkey plasma samples.

4. Pharmacokinetics of irbesartan

In a Phase I clinical study that was designed to delineate the pharmacokinetics of irbesartan in young subjects, a single 50-mg dose of irbesartan was administered orally to each of twelve male subjects. Serial blood and urine samples were collected over a period of 96 h after dosing. Plasma and urine samples were analyzed using the method described herein. The mean plasma irbesartan concentration–time profile is depicted in Fig. 3. The mean (SD) values for the peak plasma concentration (C_{max}) and the area under the plasma concentration–time curve were 942 (358) ng/ml and 5571 (2165) ng h/ml,

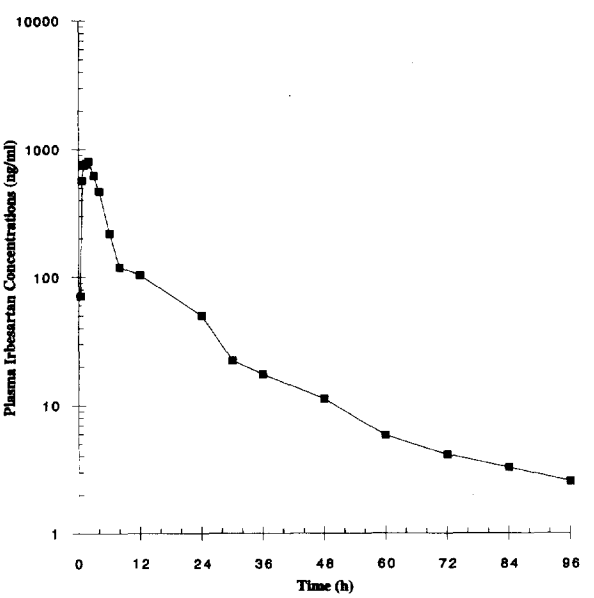


Fig. 3. Mean plasma irbesartan concentration–time profile following a single 50-mg dose of irbesartan to each of twelve healthy male subjects.

respectively [11,12]. The mean terminal elimination half-life was around 20 h. Less than 2% of the administered dose was recovered as unchanged irbesartan from the urine. The renal clearance was about 3 ml/min.

In summary, the HPLC procedure with fluorescence detection for the determination of irbesartan in human plasma or urine samples is sensitive, selective, accurate and precise.

References

[1] M. Ondetti, B. Rubin, D. Cushman, Science 196 (1977) 441.
[2] C. Cazaubon, J. Gougat, F. Bousquet, P. Guiraudou, R. Gayraud, C. Lacour, A. Roccon, G. Galindo, G. Barthelemy, B. Gautret, C. Bernhart, P. Perreaut, J.C. Brelière, G. Le Fur, D. Nisato, J. Pharmacol. Exp. Ther. 265 (1993) 826.
[3] R.T. Eberhardt, R.M. Kevak, P.M. Kang, W.H. Frishman, J. Clin. Pharmacol. 33 (1993) 1023.
[4] S.S. Gottlieb, K. Dickstein, E. Fleck, J. Kostis, T.B. Levine, T. LeJemtel, M. DeKock, Circulation 88 (1993) 1602.
[5] Y. Lacourcière, H. Brunner, R. Irwin, B.E. Karlberg, L.E. Ramsay, D.B. Snavely, T.W. Dobbins, E.P. Faison, E.B. Nelson, J. Hypertens. 12 (1994) 1387.
[6] J. Ribstein, J. Sissmann, A. Picard, M. Bouroudian, A. Mimran, J. Hypertens. 12 (1994) 131.
[7] J. Sissmann, M. Bouroudian, C. Armagnac, Y. Donazollo, M. Latreille, R. Panis, J. Hypertens. 12 (1994) S92.
[8] A.H. Van den Meiracker, P.J. Admiraal, J.A. Janssen, J.M. Kroodsma, W.A. de Ronde, F. Boomsma, J. Sissmann, P.J. Blankestijn, P.G. Mulder, A.J. Man In't Veld, M.A.D.H. Schalekamp, Hypertension 25 (1995) 22.
[9] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, Eur. J. Drug Metab. Pharmacokinet. 16 (1991) 249.
[10] T. Chando, D. Everett, A. Kahle, A. Starrett, N. Vachharajani, W. C. Shyu, K. J. Kripalani and R. H. Barbhaiya, Proceedings of the Seventh North American ISSX Meeting, San Diego, CA, 1996, p. 322.
[11] M. Gibaldi and D. Perrier, Pharmacokinetics, 2nd edition, Marcel Dekker, New York, 1982, pp. 409–417.
[12] S. Riegelman, P. Collier, J. Pharmacokinet. Biopharm. 8 (1980) 509.