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Journal of Chromatography B, 688 (1997) 101–106

JOURNAL OF CHROMATOGRAPHY B

Determination of angiotensin II receptor antagonist (E4177) in human plasma and urine by high-performance liquid chromatography

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Received 22 March 1996; revised 11 June 1996; accepted 11 June 1996

Abstract

A sensitive reversed-phase high-performance liquid chromatographic (HPLC) method with fluorescence detection was developed for the analysis of a new angiotensin II receptor antagonist, E4177, in human plasma and urine. The analyte and internal standard (I.S.) are extracted from acidified plasma and urine by liquid–liquid extraction and then refined by solid-phase extraction. The extraction recovery was greater than 90%. E4177 and I.S. were separated from endogenous components in plasma and urine on a C_{18} column using a mobile phase of acetonitrile–water–85% phosphoric acid (27.3:72.0:0.7, v/v). The eluent was monitored by fluorescence with excitation and emission set at 280 and 380 nm, respectively. The assay was linear from 2.5 to 1000 ng/ml of plasma and from 5 to 1500 ng/ml of urine. The limit of quantification was 2.5 and 5 ng/ml for plasma and urine, respectively. Inter- and intra-day coefficients of variation for the plasma and urine ranged from 0.6 to 4.7%. E4177 was stable in plasma and urine for at least 9 months during storage at −20°C, respectively. The method was successfully applied to the determination of E4177 in plasma and urine for a pharmacokinetic study.

Keywords: E4177; Angiotensin II receptor antagonist

1. Introduction

The establishment of angiotensin converting enzyme inhibitors as an effective regimen for hypertension has confirmed that the renin–angiotensin system (RAS) plays an important role in blood pressure regulation [1]. As angiotensin II (A II) is the primary effector molecule of the RAS, much research has been directed at antagonizing its effects at its receptors. Although peptide analogs, such as Saralasin [2] are known to be potent receptor antagonists, their clinical use is limited due to short duration of action, poor oral bioavailability, and partial agonistic activity. E4177, 3-{(2-carboxybiphen-4-yl)methyl}-2-cyclopropyl-7-methyl-3H-imidazo[4,5-b]pyridine, an A II receptor antagonist, has been shown to lower blood pressure in conscious renal hypertensive rats and furosemide-treated dogs [3,4].

Previously reported methods of other A II antagonists used UV detection at 254 nm. The detection sensitivity was reported to be 5~6 ng/ml using 1 ml of human plasma [5,6]. This paper describes a

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PII S0378-4347(96)00272-1

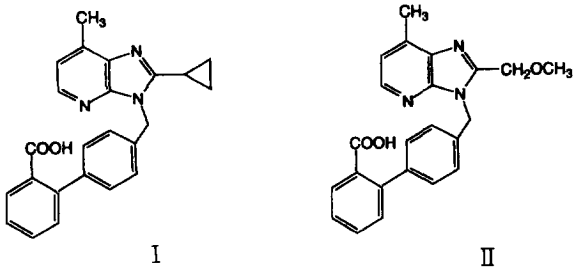

Fig. 1. Chemical structures of E4177 (I) and internal standard (II).

HPLC–fluorescence detection method for the analysis of E4177 in human plasma and urine. The limits of quantification were 2.5 ng/ml for plasma and 5 ng/ml for urine, using 1 ml of human plasma and urine.

To determine the pharmacokinetic properties of E4177 in humans, a HPLC assay was developed and validated for quantification in human plasma and urine. Fig. 1 shows the structure of E4177 and the I.S.

2. Experimental

2.1. Chemicals and reagents

E4177 and I.S. were synthesized at the Eisai Company, Tsukuba Research, Japan. HPLC-grade acetonitrile and methanol were purchased from Kokusan Kagaku (Tokyo, Japan). All other chemicals were of reagent grade. The solid-phase extraction column used propylsulfonic acid (PRS), 10 ml LRC (Varian, Harbor City, CA, USA).

2.2. Instrumentation and operating conditions

The HPLC system consisted of a LC-6A pump (Shimadzu, Kyoto, Japan), a WISP-710B autosampler (Waters, Milford, MA, USA), a reversed-phase column (YMC C_{18} AM-312, 150×6 mm I.D., 5 μm, Yamamura Kagaku, Kyoto, Japan), with temperature maintained at 35°C, and a F-1000 fluorescence detector (Hitachi, Tokyo, Japan) set at 280 nm excitation and 380 nm emission, which was connected to a C-R4AX (Shimadzu, Kyoto, Japan) integrator for the collection data. The mobile phase was acetonitrile–water–85% phosphoric acid (27.3:72.0:0.7, v/v). The flow-rate was set at 1.8 ml/min.

2.3. Standard and quality control samples

Stock solutions of E4177 and I.S. were prepared in methanol and stored at 4°C. The plasma standard of E4177 ranged from 2.5 to 1000 ng/ml in plasma and from 5 to 1500 ng/ml in urine. The quality control (QC) samples were 10, 200 and 800 ng/ml in plasma and urine. Both matrix samples were stored frozen at −20°C until the day of analysis.

2.4. Sample preparation

Frozen plasma and urine samples were thawed at room temperature and vortex-mixed for 5 s. A 1-ml volume of 0.1 *M* hydrochloric acid was added to 1 ml volume of plasma or urine in a 15×100 mm glass test tube. After the addition of 100 μl of I.S. (corresponding to 1000 ng/ml), 1 ml of saturated sodium chloride was added.

The mixture was extracted with 3 ml of diethyl ether and shaken for 10 min. After centrifugation for 5 min at 2270 *g*, the upper organic layer was transferred to another glass tube. This was repeated with an additional 3 ml of diethyl ether added to the aqueous fraction. The combined extracted organic layer was aspirated through the solid-phase extraction column at an applied vacuum of approximately 17 kPa. The column was then washed twice with 2 ml methanol. After collection of the wash fluid, tubes (10-ml glass conical tubes) were placed under the tip of the columns, and 2 ml of 0.2 *M* ammonia in methanol was added. After about 30 s, the 0.2 *M* ammonia in methanol was aspirated through the column and into the collection tubes using a vacuum. This was repeated with an additional 2 ml of 0.2 *M* ammonia in methanol. The extracted solvent was evaporated to dryness at 40°C under N_2. The residue was reconstituted in 200 μl of mobile phase, and 50 μl was injected.

2.5. Quantification

Calibration standard curves were calculated by analyzing E4177 in both the matrices and plotting

peak-height ratio of E4177 to the I.S. versus standard concentration. The line of regression was determined by the method of least squares with a weighting factor of 1/concentration^2 [7]. QC samples prepared at three concentrations of plasma and urine were used to assess assay accuracy and precision. The QC sample concentrations corresponded to lower, mid-point and upper part of the calibration curve range.

3. Results and discussion

3.1. Sample preparation

Liquid–liquid extraction of E4177 and I.S. from both matrices was insufficient as the analytes still interfered with endogenous substances. Therefore, both the liquid–liquid extraction and solid-phase extraction column (PRS) method were necessary for refining plasma and urine. By this sample preparation, sufficient resolution of E4177 and I.S. from endogenous substances in human plasma and urine was obtained by the same method (Fig. 2).

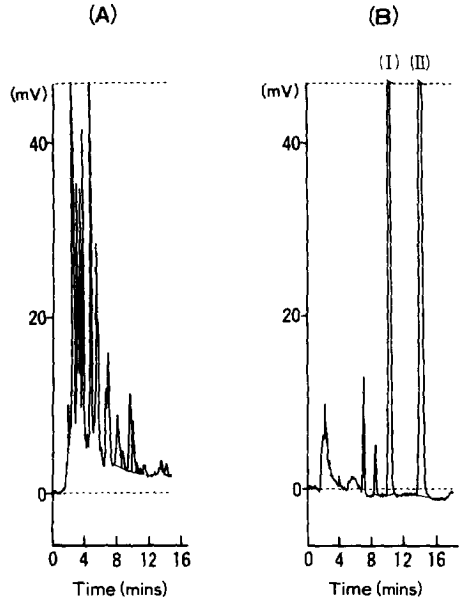

Fig. 2. Chromatograms of the refining procedure on solid-phase extraction from human urine liquid–liquid extraction. (A) Chromatogram of the washing solution with methanol. (B) Extract with 0.2 *M* ammonia and methanol. The arrows indicate the positions of E4177 (I) and I.S. (II).

3.2. Selectivity

E4177 has maxima of UV absorption at 250 and 280 nm, and excitation of fluorescence at 280 nm and emission at 380 nm at acidic pH. HPLC detection of UV and fluorescence were similar regarding peak height. However, use of the fluorescence detector had the advantage of less interference by endogenous substances in chromatograms.

Fig. 3 shows the separation and quantitation of E4177 and I.S. in human plasma and urine. In the chromatograms, obtained from 1.0 ml of drug free plasma and urine, no additional peaks interfering with the determination of drug and I.S. are present (Fig. 3A,C). Fig. 3(B,D) shows chromatograms obtained after extraction of plasma and urine from healthy volunteers containing E4177. The E4177, its main metabolite and the I.S. are well separated with retention times of ca. 12, 8 and 17 min, respectively.

3.3. Precision and accuracy

For validation the report of the conference 'Analytical Method Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies', held in 1990, was used as a guideline [8].

Both intra- and inter-day accuracy and precision of the calibration standard curves in plasma and urine were examined. As shown in Tables 1 and 2, the C.V. values were all within 4.7%. Furthermore, the small percentage differences between nominal and found concentrations of the standards showed that the assay was sufficiently accurate (Tables 1 and 2).

Inter-day precision of the method was examined using QC samples. For plasma QC samples analyzed over a 10-day period, C.V. was less than 5.4%. In urine, the C.V. was less than 4.1% for QC samples analyzed over a 9-day period.

3.4. Stability

The stability of E4177 has been studied at concentrations of 20 and 400 ng/ml in human plasma and urine during a period of 9 months at −20°C and after four freeze–thaw cycles. The stability of extracted solution from plasma and urine for the measurement of E4177 was evaluated during storage at room temperature for 48 h in the autosampler.

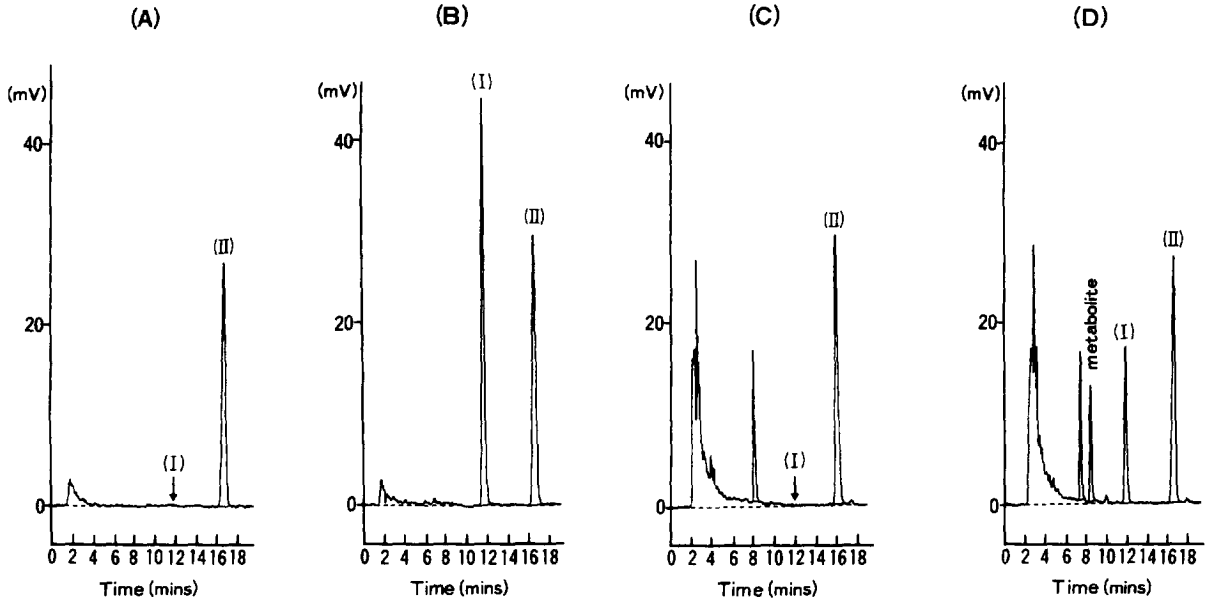

Fig. 3. Typical chromatograms of human plasma and urine samples. (A) Extract of blank plasma with 100 ng/ml I.S. (B) Plasma sample from a healthy volunteer. Concentration is ca. 300 ng/ml of E4177. (C) Extract of blank urine with 100 ng/ml I.S. (D) Urine sample from a healthy volunteer. Concentration is ca. 70 ng/ml of E4177; the retention time of the metabolite of E4177 is 8 min. Peaks: I=E4177; II=I.S.

Table 1
Intra-day reproducibility results of E4177 in human plasma and urine ($n=4$)

Spiked concentration (ng/ml)	Mean found concentration (ng/ml)	Accuracy (%)	C.V. (%)
Plasma			
2.5	2.50	100.0	1.7
5	4.92	98.4	3.6
10	10.28	102.8	3.6
25	25.38	101.5	1.8
50	51.30	102.6	1.1
100	97.80	77.8	2.1
250	246.5	98.6	1.3
1000	986.0	98.6	0.8
Linear regression line, $1/C^2$ weighting all data: $y=0.01027x+0.00365$ ($r=0.99944$)			
Urine			
5	5.06	101.1	2.1
10	9.70	97.0	4.7
25	25.30	101.2	0.8
50	51.45	102.9	1.2
100	98.7	98.7	1.3
250	248.3	99.3	0.7
1000	984.0	98.4	0.9
1500	1522.5	101.5	0.9
Linear regression line, $1/C^2$ weighting all data: $y=0.01049x+0.00173$ ($r=0.99952$)			

Table 2
Inter-day reproducibility results of E4177 in human plasma and urine

Spiked concentration (ng/ml)	Mean found concentration (ng/ml)	Accuracy (%)	C.V. (%)
Plasma ($n=9$)			
2.5	2.47	98.6	2.8
5	5.09	101.7	4.6
10	10.23	102.3	3.1
25	24.78	99.1	2.5
50	50.50	101.0	3.1
100	101.70	101.7	2.1
250	248.25	99.3	1.9
1000	977.00	97.7	1.7
Linear regression line, $1/C^2$ weighting all data: $y=0.00958x+0.00354$ ($r=0.99944$)			
Urine ($n=4$)			
5	4.86	97.2	0.9
10	10.56	105.6	1.8
25	24.28	97.1	1.2
50	51.45	102.9	2.1
100	108.90	108.9	1.2
250	248.50	99.4	0.6
1000	958.00	95.8	2.5
1500	1449.00	96.6	1.4
Linear regression line, $1/C^2$ weighting all data: $y=0.00898x+0.00429$ ($r=0.99867$)			

Table 3 shows the stability data of the analytes in plasma and urine at −20°C. Table 4 shows the stability data of the analytes in human plasma and urine after four freeze–thaw cycles and in solution extracted from plasma and urine in an autosampler at room temperature. E4177 was stable under these conditions and during this storage period.

Table 3
Stability data in human plasma and urine at −20°C ($n=3$)

Time (months)	Concentration (ng/ml)	Residue (%)	Concentration (ng/ml)	Residue (%)
Plasma				
0	20.91	100.0	427.00	100.0
1	21.86	104.5	442.96	103.7
6	20.39	97.5	422.91	99.0
9	22.19	106.1	450.91	105.6
Urine				
0	20.36	100.0	434.61	100.0
1	21.36	104.9	444.31	101.8
3	20.89	102.6	441.58	101.6
6	21.56	105.9	434.20	99.9
9	22.01	108.1	447.30	102.9

Residue (%) is a percentage of concentration compared with initial analysis.

3.5. Recovery and limit of quantification

The mean absolute recoveries of the analyte from plasma and urine were more than 90% for both E4177 and I.S. The recovery was determined by comparing peak heights from unextracted standards with those of extracted standards, across the range of each standard curve. The limits of quantification were 2.5 ng/ml in plasma and 5 ng/ml in urine.

3.6. Application of method

The method has been used successfully for quantifications in human plasma and urine following 10–240 of oral doses. Fig. 4 shows a plasma concentration–time curve for E4177 in healthy volunteers after oral administration, indicating that the limit of quantification was adequate for the determination of plasma levels after dosing.

Table 4
Stability data after four freeze–thaw cycles and extracted solution from human plasma and urine ($n=3$)

Matrices	Mean concentration (ng/ml)				
	Four freeze–thaw cycles		Extracted solution		
	Initial	After freeze–thaw	Initial	RT[a] 24 h	RT 48 h
Plasma	20.91	19.99	20.91	21.68	19.76
	427.00	320.42	427.00	424.01	418.46
Urine	20.36	21.44	20.36	20.69	20.77
	434.61	454.57	434.61	430.26	435.91

[a] RT=room temperature.

4. Conclusion

In conclusion, a simple, sensitive and validated HPLC assay for the quantitative determination of a new angiotensin II receptor antagonist (E4177) in human plasma and urine is described. The assay methods for both plasma and urine are identical, and liquid–liquid and solid-phase extraction methods can conveniently be used. The recovery of this method is reproducible and greater than 90%. The peak shapes and retention time separations are always reproducible. The method is suitable for the evaluation of the pharmacokinetics of E4177 in humans.

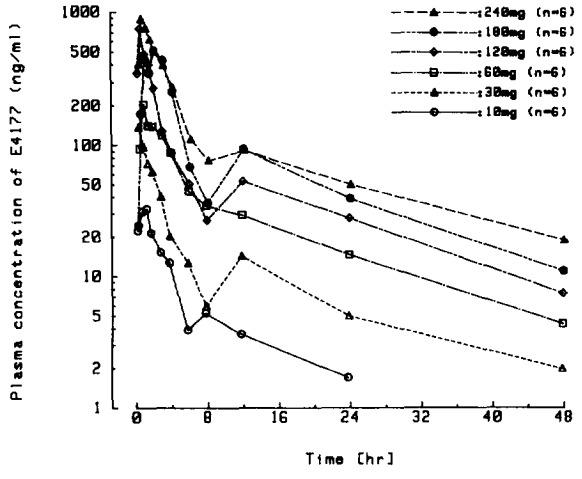

Fig. 4. Mean plasma concentration vs time curve for E4177 after a single oral administration of 10–240 mg.

Acknowledgments

The authors thank Ms. M. Taguchi for her excellent assistance in the preparation of this manuscript.

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