Journal of Chromatography, 573 (1992) 295–301
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6126

Simultaneous determination of a novel angiotensin II receptor blocking agent, losartan, and its metabolite in human plasma and urine by high-performance liquid chromatography

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(First received June 4th, 1991; revised manuscript received August 20th, 1991)

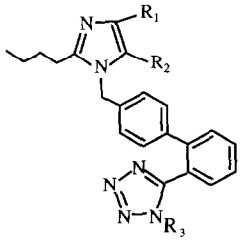
ABSTRACT

A sensitive and selective high-performance liquid chromatographic method for the simultaneous determination of a new angiotensin II receptor blocking agent, losartan (DuP 753, MK-954, I), and its active metabolite, EXP3174 (II), in human plasma or urine is described. The two analytes and internal standard are extracted from plasma and urine at pH 2.5 by liquid–liquid extraction and analyzed on a cyano column with ultraviolet detection at 254 nm. The mobile phase is composed of acetonitrile and phosphate buffer at pH 2.5. The limit of quantification for both compounds in plasma is 5 ng/ml. The limit in urine is 20 and 10 ng/ml for I and II, respectively. The assay described has been successfully applied to samples from pharmacokinetic studies.

INTRODUCTION

The potassium salt of 2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[(2′-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole (losartan, DuP 753, MK-954, I) is a novel chemical entity currently under development jointly by DuPont and Merck. Compound I has been shown to be a potent, orally active and highly specific angiotensin II receptor antagonist and antihypertensive agent [1–4]. In contrast to angiotensin-converting enzyme inhibitors, compound I has been demonstrated to be highly specific for the renin-angiotensin system and does not potentiate the effects of bradykinin [2]. An active carboxylic acid metabolite, EXP3174 (L-158,641, II), has been identified in rat plasma [5] and may contribute to the pharmacological effects produced *in vivo* after administration of I [6]. Thus far, the pharmacokinetics of I in rat and dog have been reported [5,7]. In order to investigate their pharmacokinetic properties in man, a high-performance liquid chromatographic (HPLC) assay was developed and validated for the simultaneous quantification of I and II in human plasma and urine.

The structures of I, II and the internal standard (L-158,854, III) used in the assay are shown in Fig. 1.



	R_1	R_2	R_3
I	Cl	CH_2OH	K
II	Cl	COOH	H
III	2-Chlorophenyl	COOH	H

Fig. 1. Structures of losartan (DuP 753, I), EXP3174 (II) and internal standard L-158,854 (III).

EXPERIMENTAL

Materials

Compounds I, II and the internal standard were obtained from Merck Sharp & Dohme Research Laboratories (Rahway, NJ, USA). HPLC-grade methyl *tert.*-butyl ether (MTBE) and isopropanol were purchased from Burdick and Jackson (Muskegon, MI, USA). HPLC-grade acetonitrile, hexane, methanol and 85% phosphoric acid and reagent-grade sodium phosphate monobasic and sodium hydroxide were obtained from Fisher Scientific (Fair Lawn, NJ, USA).

Equipment

The HPLC system consisted of a Series 410 pump and an ISS-100 autosampler, both from Perkin-Elmer (Norwalk, CT, USA), connected to an Applied Biosystems 783 UV detector (Foster City, CA, USA). The chromatographic data were analyzed using Turbochrom 2700 software (PE Nelson Systems, Cupertino, CA, USA). An Ultremex CN column (250 mm × 4.6 mm I.D., 5 μm) from Phenomenex (Torrance, CA, USA) was used for the HPLC separations.

Chromatographic conditions

The flow-rate was set to 1.0 ml/min and effluent was monitored at 254 nm. The mobile phase composition was 23% acetonitrile and 77% phosphate buffer (100 ml of 0.2 *M* NaH_2PO_4 + 2 ml of 85% phosphoric acid in 2 l of water; pH adjusted to 2.5 with 2 *M* NaOH). All analyses were performed at ambient temperature.

Sample preparation

Frozen plasma samples were thawed and brought to room temperature. A 1-ml aliquot of plasma was acidified to pH 2.5 with 125 μl of 1.0 *M* phosphoric acid. After adding 100 μl of internal standard, the analytes were extracted with 10 ml of MTBE by shaking for 20 min at 60 rpm. After centrifugation for 5 min at 2060 *g*, the aqueous layer was frozen in a dry ice–acetone bath and the organic solvent was transferred to clean tubes. The analytes were then back-extracted into 200 μl of 0.05 *M* NaOH (shaken 15 min at 60 rpm). The samples were centrifuged again, as above, and the aqueous layer was separated by freezing. The MTBE was discarded and the NaOH layer was acidified with 75 μl of 0.2 *M* phosphoric acid. Hexane (6 ml) was then added followed by vortex-mixing for 2 min to wash the aqueous fraction. After centrifuging the samples and freezing the aqueous layer, the hexane was discarded and the residual hexane was removed by nitrogen evaporation. To improve the solubility at low pH, 75 μl of isopropanol were added to the aqueous layer. A 110-μl aliquot out of a total of 350 μl was then injected onto the LC system for analysis.

The same assay was used for the analysis of urine with slight modifications. Urine (0.5 ml) diluted with 0.5 ml of water was acidified with 50 μl of 1.0 *M* phosphoric acid and was extracted with 10 ml of MTBE–hexane (4:1, v/v). All other aspects of the procedure were identical to the plasma assay except the injection volume (65 μl for the urine assay).

Quantification

Calibration standards were prepared by adding known amounts of I and II (5–1000 ng) and 100 ng of internal standard to 1 ml of control human plasma or 0.5 ml of control human urine. The sample extraction and HPLC analysis were carried out as described above. Concentrations of I and II were calculated from the linear least-squares fitted line of peak-height ratios of I or II to the internal standard *versus* standard concentrations, with reciprocal weighting on the concentrations. The linear range of the standard curve was from 5 to 1000 ng/ml in plasma for both analytes, 10 to 2000 ng/ml in urine for II and 20 to 2000 ng/ml in urine for I. The limit of quantification was defined as the lowest concentration with a coefficient of variation (C.V.) of $\leq 10\%$.

RESULTS AND DISCUSSION

Sample preparation

Since the analytes are weak acids (the pK_a of the acidic nitrogen in the tetrazole ring is 5.6 and 5.4 for I and II, respectively; the pK_a for the carboxy group in II is 4.2), they could be extracted

from an aqueous medium at low pH into an organic solvent. Even though solvents more polar than MTBE were somewhat more efficient in extracting the analytes from plasma, MTBE was chosen because it gave a cleaner extract. To concentrate and further purify the extract, the analytes were then back-extracted into base. It was found that recovery could not be improved by adding isopropanol to the MTBE because the extraction efficiencies into base for all three compounds were then reduced. After the back-extraction, the extract still contained interfering compounds. The final hexane wash at low pH further selectively removed the chromatographic interferences. We found that washing at basic pH did not provide sufficient clean-up regardless of the wash solvent used, while washing at acidic pH with solvents more polar than hexane removed the analytes of interest along with the interferences. As a final step, the addition of a small amount of isopropanol was necessary to improve the solubility of the analytes at low pH. The removal of the residual hexane was also important to maintain consistent solubility. The isolation method using liquid–liquid extraction was found to be more selective and reproducible for the extraction of both analytes than solid-phase extraction.

Column selection

During initial method development, use of a C_8 column resulted in many late-eluting peaks which significantly lengthened the analysis time. After screening many HPLC columns, the Ultremex CN column was chosen because it eliminated the late-eluting peaks. In general, most CN columns were more effective in producing a cleaner background as compared to C_8 and C_{18} columns. The Ultremex CN provided the cleanest blank along with good separation of the peaks of interest. Since the CN columns from most manufacturers vary from batch to batch, it was necessary to check columns from different batches to maintain consistent chromatography.

Internal standard selection

Many analogues of I and II were screened to serve as internal standard. III was chosen as internal standard because it gave acceptable precision and accuracy data for both I and II, even though III is structurally more similar to II. Chromatographically, III separates favorably from both I and II.

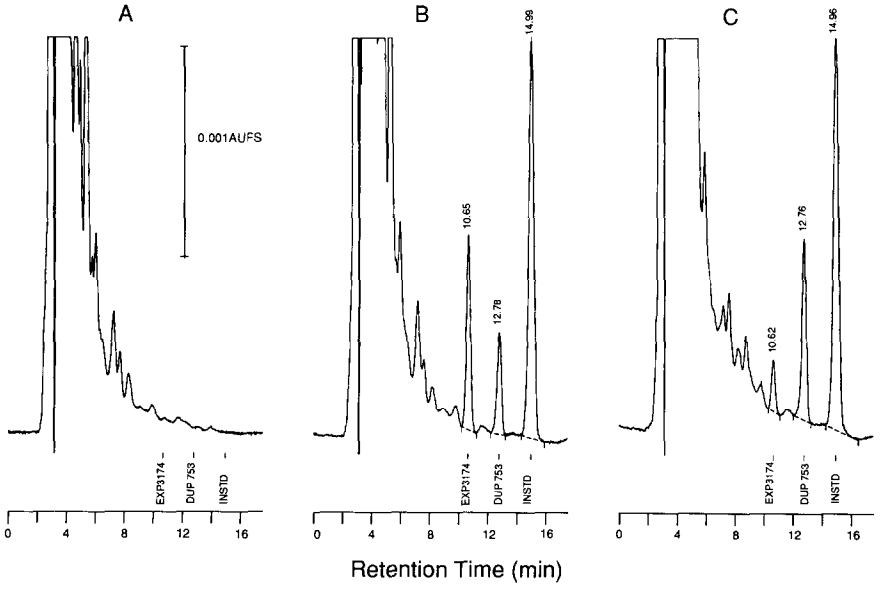


Fig. 2. Representative chromatograms of I (DuP 753), II (EXP3174) and internal standard (INSTD) in plasma. (A) Control plasma; (B) control plasma spiked with 30 ng/ml I and II; (C) 1.5 h sample from a subject given a single 100-mg dose of I (52.6 ng/ml I and 8 ng/ml II).

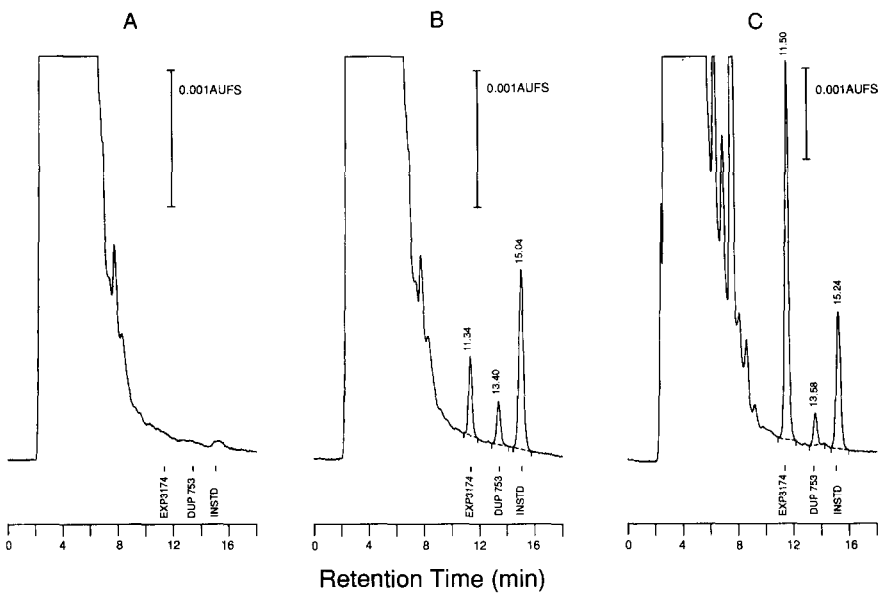


Fig. 3. Representative chromatograms of I (DuP 753) II (EXP3174) and internal standard (INSTD) in urine. (A) Control urine; (B) control urine spiked with 60 ng/ml I and II; (C) 24–36 h urine sample from a subject given a single 100-mg dose of I (58.5 ng/ml I and 368.6 ng/ml II).

TABLE I

PRECISION AND ACCURACY OF PLASMA STANDARD CURVES

Nominal concentration (ng/ml)	Losartan		II	
	Mean found (ng/ml)	C.V.[a] (%)	Mean found (ng/ml)	C.V.[a] (%)
Intra-day repeatability (n = 5)				
5	5.0	8.3	5.2	6.9
10	10.0	5.6	10.5	3.1
30	29.9	4.6	29.7	6.2
70	71.0	1.5	65.8	5.6
100	98.9	3.1	95.3	2.5
300	300.4	0.7	300.9	3.1
700	699.1	0.9	711.7	2.9
1000	1000.7	1.2	995.8	3.9
Linear regression line, $1/x$ weighting, all data:				
	$y = 0.008128x + 0.009858$		$y = 0.015528x - 0.009077$	
	$r^2 = 0.9998$		$r^2 = 0.9981$	
Inter-day reproducibility (n = 13)[b]				
5	5.2	7.5	5.4	5.0
10	10.0	8.9	10.2	3.0
30	29.8	4.0	29.3	1.3
70	70.4	3.5	69.1	2.6
100	100.1	4.2	96.3	4.5
300	290.6	3.2	289.8	2.4
700	682.7	3.2	685.8	2.5
1000	1026.0	2.6	1029.3	1.9

[a] Peak-height ratios used to calculate C.V. for intra-day repeatability

[b] Thirteen standard curves over a two-month period.

Selectivity

Representative chromatograms are shown in Figs. 2 and 3. No peaks interfered with the detection of I, II or internal standard in plasma or urine. Under the chromatographic conditions described, the retention times of I, II and internal standard were approximately 13, 11 and 15 min, respectively.

Linearity

The small percentage differences between nominal and found concentrations of the standards in the standard curves (Tables I and II) for both intra- and inter-day data showed that the assay was linear over the concentration range investigated. The intra-day regression line parameters are also listed in Tables I and II.

Precision and accuracy

Standard curves. Both intra- and inter-day accuracy and precision of the standard curves were examined. As can be seen in Tables I and II, the C.V. values were all less than 10%. Furthermore, the small percentage differences between nominal and found concentrations of the standards showed that the assay was sufficiently accurate.

Quality control samples. Inter-day precision and accuracy of the method were examined using quality control (QC) samples which were prepared at low and high concentrations (20 and 800 ng/ml for plasma; 40 and 1600 ng/ml for urine) and analyzed daily with clinical study samples. For plasma QC samples analyzed over a seven-week period, C.V.s were less than 10% and mean concentrations found were 92% of nominal or

TABLE II

PRECISION AND ACCURACY OF URINE STANDARD CURVES

Nominal concentration (ng/ml)	Losartan		II	
	Mean found (ng/ml)	C.V.[a] (%)	Mean found (ng/ml)	C.V.[a] (%)
Intra-day repeatability (n = 5)				
10	–[b]		10.7	6.5
20	19.7	3.9	20.1	2.2
60	59.2	5.0	57.9	6.1
140	139.5	4.4	136.6	4.2
200	199.9	4.5	188.7	5.9
600	631.6	5.6	625.9	4.2
1400	1388.9	4.4	1410.3	5.2
2000	1981.3	0.6	1979.8	1.9
Linear regression line, $1/x$ weighting, all data:				
	$y = 0.008413x - 0.000334$		$y = 0.017247x - 0.021402$	
	$r^2 = 0.9984$		$r^2 = 0.9981$	
Inter-day reproducibility (n = 6)[c]				
10	–[b]		10.6	7.0
20	20.8	1.8	20.5	1.8
60	59.7	3.0	59.1	5.1
140	138.2	1.4	136.2	1.6
200	198.7	1.1	193.5	1.8
600	587.1	1.9	587.1	2.2
1400	1399.0	0.5	1397.9	0.7
2000	2016.5	0.7	2025.1	0.3

[a] Peak-height ratios used to calculate C.V. for intra-day repeatability

[b] Below standard curve, not studied.

[c] Six standard curves over a ten-day period.

TABLE III

INTER-DAY ACCURACY AND PRECISION OF QUALITY CONTROL (QC) SAMPLES

Compound	Low QC				High QC			
	Concentration (ng/ml)		*n*	C.V. (%)	Concentration (ng/ml)		*n*	C.V. (%)
	Nominal	Found			Nominal	Found		
Plasma[a]								
Losartan	20	19.2	12	9.9	800	736.7	12	6.3
II	20	19.6	12	3.3	800	777.8	12	5.7
Urine[b]								
Losartan	40	40.8	5	4.2	1600	1562	5	1.0
II	40	40.3	5	1.5	1600	1628	5	2.0

[a] QCs were run on twelve days over a seven-week period.
[b] QCs were run on five days over an eight-day period.

better. In urine, the C.V.s were less than 5% for QC samples analyzed over an eight-day period and the mean concentrations found were within 2% of nominal (Table III).

Stability

Analysis of plasma stability control samples studied at concentrations of 20 and 800 ng/ml over a period of thirty weeks showed I and II to be stable when stored at −20°C. Mean found concentrations at the end of thirty weeks were 98–102% of nominal. The analytes at concentrations of 40 and 1600 ng/ml were also stable for at least ten weeks in frozen urine. Mean found concentrations at the end of ten weeks were 91–104% of nominal. Long-term stability data are being gathered at regular intervals as part of an ongoing study.

Recovery

The mean absolute recovery of the analytes from plasma was 91 and 85% for I and II, respec-

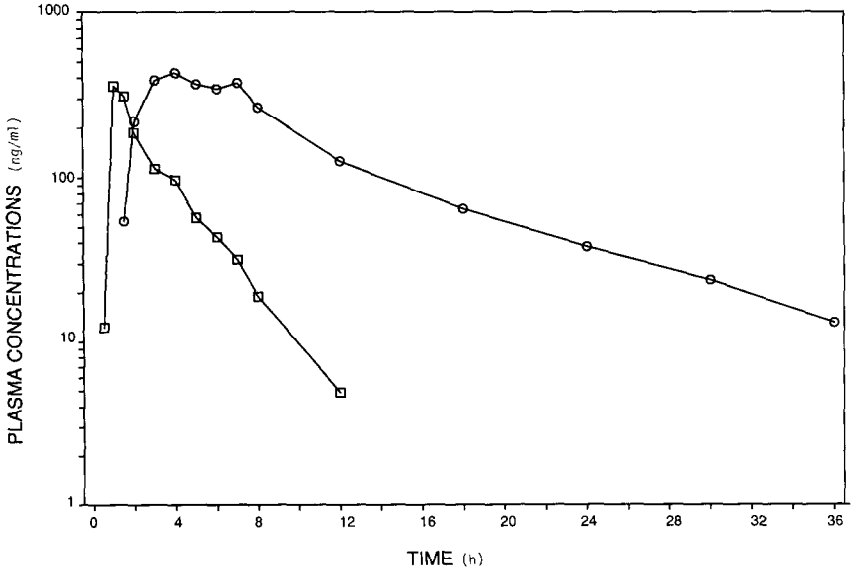


Fig. 4. Plasma concentrations of I (□) and II (○) following a single 100-mg oral dose of I in a healthy subject.

tively. The mean recovery from urine was 98 and 88% for I and II, respectively. The recovery was determined by comparing peak areas from unextracted standards with those of extracted standards, across the range of each standard curve.

Limit of quantification

The limit of quantification (intra-day C.V. $\leq 10\%$) for both compounds in plasma was 5 ng/ml. The limit in urine was 20 ng/ml for I and 10 ng/ml for II.

Analysis of clinical samples

The method described has been successfully applied to the quantification of I and II in about 1500 plasma and 330 urine samples over an eleven month period. The data from a representative subject given 100 mg of I orally is shown in Fig. 4. Urinary excretion was found to be a minor route of elimination for I and II following an oral dose. The amounts of I and II excreted in urine in ten subjects given 100 mg of I were 3.4 and 5.2 mg, respectively.

CONCLUSION

An HPLC assay method is reported for I and II in plasma and urine. The method has been applied to evaluate the pharmacokinetics of I in man.

ACKNOWLEDGEMENTS

We thank Dr. Wallace Ashton, Exploratory Chemistry, MSDRL (Rahway, NJ, USA) for supplying the internal standard L-158,854 and Drs. Linyee Shum and David Christ of Du Pont Merck Company for their helpful suggestions during the early development of the assay.

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