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Short Communication

High-performance liquid chromatographic determination of angiotensin II receptor antagonists in human plasma and urine

I. DuP 532 (L-694,492)

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ABSTRACT

A sensitive reversed-phase high-performance liquid chromatographic method with ultraviolet detection was developed for the analysis of a new angiotensin II receptor antagonist, DuP 532 (L-694,492), in human plasma and urine. The analyte and internal standard are extracted from plasma and urine at a pH between 3.3 to 3.6 by liquid–liquid extraction and analyzed on a C_6 column with ultraviolet detection at 254 nm. The mobile phase is composed of acetonitrile and phosphate buffer at pH 2.5. The limits of quantification are 6 and 7.5 ng/ml for plasma and urine, respectively.

INTRODUCTION

Non-peptide angiotensin II receptor antagonists are a new class of novel agents under development for treatment of hypertension [1]. Losartan, the potassium salt of 2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[(2′-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole, the leading compound in the class, is currently undergoing clinical trials. Part of the antihypertensive effect of losartan is attributed to the active carboxylic acid metabolite EXP3174 (III) [2,3]. Research efforts are continuing to discover new analogues of losartan, the activities of which do not require *in vivo* generation of active metabolites. DuP 532 (L-694,492, I, 2-propyl-4-pentafluoroethyl-1-[(2′-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole-5-carboxylic acid) was discovered as an active AII receptor antagonist in *in vitro* AII binding assays and in animal models [4]. In order to investigate the pharmacokinetic properties of I in humans, a high-performance liquid chromatographic (HPLC) assay was developed and validated for the quantification of I in human plasma and urine. The structures of I and the internal standard (II, XA276, 2-butyl-4-pentafluoroethyl-

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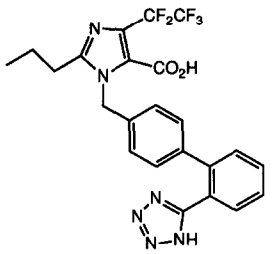
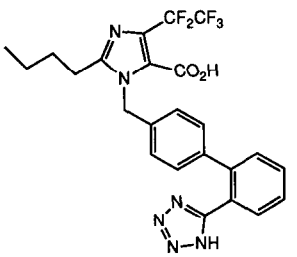


L-694,492



XA-276

Fig. 1. Structures of DuP 532 (L-694,492,I) and internal standard XA-276 (II).

1-[(2'-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]-imidazole-5-carboxylic acid) are shown in Fig. 1.

EXPERIMENTAL

Materials

Compounds I and II were obtained from the DuPont Merck Pharmaceutical Company (Wilmington, DE, 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, sodium hydroxide, citric acid and sodium citrate were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Disposable glass, screw-capped 150 × 16 mm culture tubes and disposable glass, screw-capped 15-ml conical tubes (Baxter, McGraw Park, IL, USA) were used. Heparinized human plasma from Biological Specialty (Lansdale, PA, USA) was used to prepare standard curves.

Equipment

A multi-tube vortexer (VWR, Bridgeport, NJ, USA) was used for mixing during sample work-up. The HPLC system consisted of a Hewlett-Packard 1090L pump equipped with an autosampler 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). A Chromegabond C_6 column (100 mm × 4.6 mm I.D., 3 μm particle size) from ES Industries (Marlton, NJ, USA) was used for the HPLC separations.

Chromatographic conditions

Compound I was detected by ultraviolet detection at 254 nm. The mobile phase consisted of 35% acetonitrile and 65% phosphate buffer. The phosphate buffer was prepared by diluting 80 ml of 1 *M* sodium dihydrogenphosphate and 8 ml of 85% phosphoric acid to 8 l. The final pH was adjusted to 2.5 with 1 *M* sodium hydroxide. The flow-rate was 1 ml/min and the column was kept at ambient temperature for plasma and at 40°C for urine.

Standard and quality control samples

Primary stock solutions of I and II were prepared by dissolving approximately 10 mg of I into methanol to yield 1 mg/ml. Two separate batches of stock solutions of I were made. After preparing working solutions of I by further dilutions with pH 10 water, one set was used for the preparation of standard curves and the other for the quality controls. The pH 10 solution was prepared by adjusting the pH of water to 10 with 1 *M* sodium hydroxide. The working solution of II was 2.5 μg/ml in pH 10 water. The plasma standard of I ranged from 6 to 1000 ng/ml in plasma and from 7.5 to 750 ng/ml in urine. The quality control samples were 20, 65 and 700 ng/ml in plasma and 22.5, 225 and 525 ng/ml in urine. All quality control samples were stored frozen at −16°C until the day of analysis.

Sample preparation

Control plasma sample was thawed at room temperature and vortex-mixed for 5 s. After adding 50-μl aliquots of the working solution of I and internal standard (corresponding to 125 ng/

ml), a 1-ml volume of plasma was acidified with 0.4 ml of 1 *M* citrate buffer (pH 3.1). The final pH of plasma was 3.3–3.6. The analytes were extracted with 9 ml of MTBE–hexane (50%, v/v) by shaking at 60 rpm for 20 min. After centrifugation for 5 min at 2060 *g*, the aqueous layer was frozen in a dry ice–acetone bath and the top organic layer was transferred to conical tubes. The analytes were then back-extracted into 250 μl of 0.05 *M* NaOH by vortex-mixing for 2 min. The samples were centrifuged as above and the aqueous layer was separated by freezing. The organic layer was discarded and the NaOH layer was thawed to room temperature and acidified to pH 3 with 50 μl of 1 *M* citric buffer (pH 3.1). Hexane (6 ml) was then added followed by vortex-mixing for 2 min to wash the aqueous fraction. After centrifugation, the aqueous layer was frozen and the hexane layer was discarded. The extracts were thawed out at room temperature and the residual hexane was removed by nitrogen evaporation. To improve the solubility at low pH, 100 μl of isopropanol were added to the aqueous layer. A 110-μl aliquot was then injected onto the HPLC column for analysis.

The same assay was used for the analysis of urine with slight modifications. Urine (1 ml) was acidified with 0.2 ml of 0.5 *M* pH 3.0 citric acid buffer. After adding internal standard and MTBE–hexane, the mixture was vortex-mixed for 2 min. The NaOH aqueous layer containing the analytes was acidified with 75 μl of 0.2 *M* phosphoric acid. All other aspects of the procedure were identical to those of the plasma assay.

Quantification

Concentrations of quality control samples were calculated from the linear least-squares fitted line of peak-height ratios of I to the internal standard *versus* standard concentrations, with reciprocal weighting on the concentrations. The limit of quantification of I in plasma or urine was determined during the intra-day validation. It was defined as the lowest concentration of an averaged peak-height ratio ($n = 5$) with a coefficient of variation (C.V.) $\leq 10\%$.

RESULTS AND DISCUSSION

Sample preparation

Since compound I is a weak acid (the pK_a of the acidic nitrogen in the tetrazole ring is 4.65; the pK_a for the carboxy group is 2.55) [5] and it is structurally similar to III, we attempted to extract I using the same procedure as for III by adjusting the pH with phosphoric acid to 2.5 [6]. However, the extraction recovery of I was low at this pH. We explored extraction between pH 1 and 5. The use of pH 3.1 or 3 citrate buffer, at the specified amount, was able to adjust the plasma or urine pH to the optimal range (3.3–3.6). The addition of hexane to MTBE minimized the carry-over of citric acid to the organic phase. The carry-over acidified the NaOH and then lowered the back-extraction yield of the next step. The analytes were back-extracted into NaOH. After the back-extraction, the extract sometimes still contained some interfering compounds. The final hexane wash at pH 3 further selectively removed the chromatographic interferences without removing the compounds of interest. The extraction recoveries of I and II into hexane were very low, especially at acidic pH which prevented the loss of both analytes into hexane. In the final step, the addition of isopropanol was necessary to improve the solubility of the analytes at low pH.

Column selection

During initial method development, an Ultremex CN column was tried on I. The column has been used for the losartan assay [6]. However, we found large inter-column differences in the chromatographic behavior (*e.g.* peak shape) of I on different Ultremex CN columns. We then explored I on short 3-μm C_6 columns and found the 10-cm Chromegabond C_6 column to give the best separation of I from endogenous compounds while also giving the most reproducible peak shapes between columns.

Internal standard selection

Since I is structurally similar to III, we screened III as a possible internal standards for I.

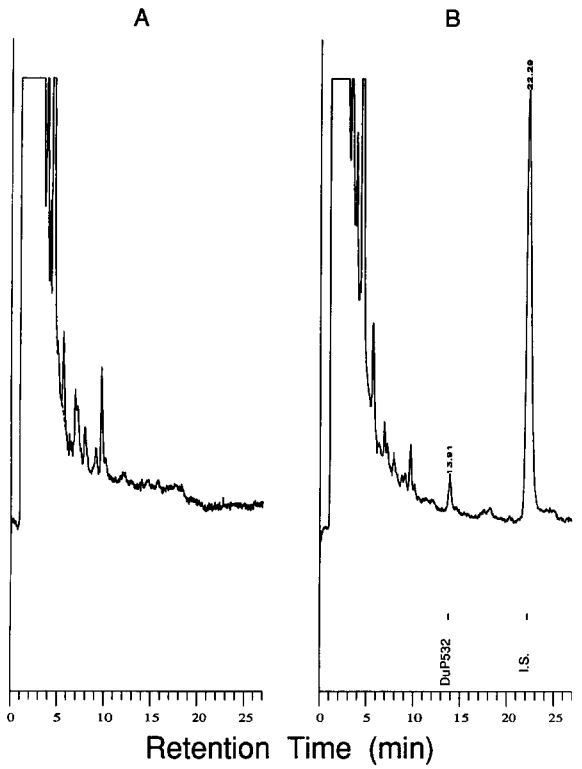


Fig. 2. Representative chromatograms of I (DuP 532) and internal standard (I.S.) in plasma. (A) Control plasma; (B) control plasma spiked with 6 ng/ml I and 125 ng/ml I.S.

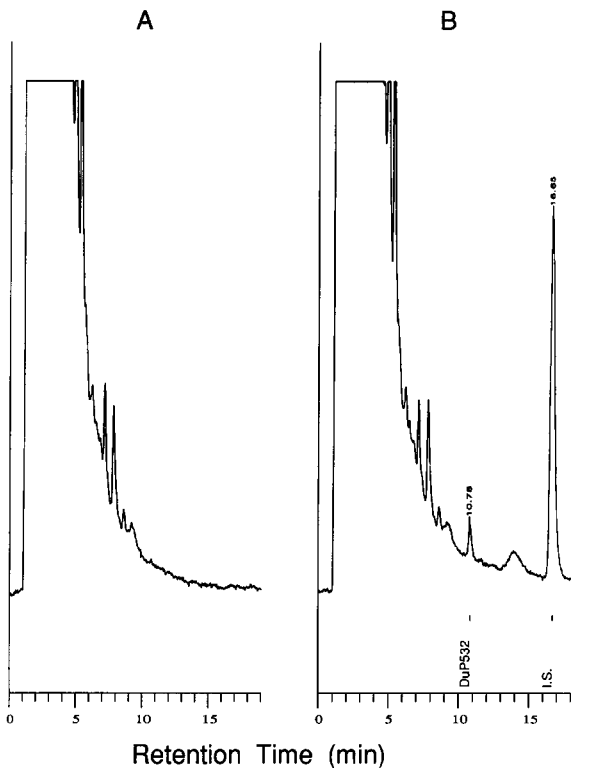


Fig. 3. Representative chromatograms of I (DuP 532) and internal standard (I.S.) in urine. (A) Control urine; (B) control urine spiked with 7.5 ng/ml I and 125 ng/ml I.S.

The extraction behavior of III was different from I between pH 2.5 and 4.5. Other structural analogues of I were evaluated and II was found to be the most suitable with regard to extraction similarity and chromatographic behavior. The similarity of retention times of both extracted and unextracted drugs indicated that they were not altered chemically during the extraction step.

Selectivity

Representative chromatograms are shown in Figs. 2 and 3. No endogenous peaks interfered with the detection of I and II in plasma and urine. Under the chromatographic conditions described, the retention times of I and II were 14 and 22 min, respectively, in plasma and 11 and 17 min, respectively, in urine. The differences in retention times of I and II in plasma and urine samples were due to slight differences in the column temperature, mobile phase pH and age of the column.

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 support the linear relationship between peak-height ratios of I to the internal standard and concentrations of I over the investigated concentration range. To further support the linear relationship, weighted linear regression analyses were done between nominal and fitted concentrations for intra-day plasma and urine standard curves. The 95% confidence limits included 1 and 0 for slope and intercept, respectively. The linearity range of I was 6–1000 ng/ml for plasma and 7.5–750 ng/ml for urine. The intra-day linear regression line parameters are also listed in Table I.

TABLE I

INTRA-DAY REPEATABILITY OF PLASMA AND URINE STANDARD CURVES

Nominal concentration (ng/ml)	Mean found concentration (ng/ml)	C.V.[a] (%)
Plasma		
6	6.6	5.5
15	14.1	5.8
35	37.0	10.0
100	98.4	5.0
300	294.3	1.0
700	704.5	3.7
1000	1003.2	4.6
Linear regression line, $1/x$ weighting, all data: $y = 0.0098x + 0.0029$ ($r^2 = 0.9974$)		
Urine		
7.5	7.6	9.0
22.5	22.1	2.4
52.5	52.5	7.1
75	75.4	3.3
225	226.2	3.5
525	521.0	3.1
750	753.0	3.1
Linear regression line, $1/x$ weighting, all data: $y = 0.0099x + 0.0043$ ($r^2 = 0.9999$)		

[a] Peak-height ratios were used to calculate the coefficients of variation (C.V.) ($n = 5$).

Precision and accuracy

Standard curves. Both intra- and inter-day accuracy and precision of the standard curves were examined. As shown in Tables I and II, the C.V. values were all within 10.3%. 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 was examined using quality control (QC) samples. For plasma QC samples analyzed over a six-day period, C.V.s were less than 3.7% and mean concentrations found were 95.4% of nominal or better. In urine, the C.V.s were less than 8.4% for QC samples analyzed over a three-day period and the mean concentrations found were 105.4% of nominal or better (Table III).

TABLE II

INTER-DAY REPRODUCIBILITY OF PLASMA AND URINE STANDARD CURVES

Nominal concentration (ng/ml)	Mean found concentration (ng/ml)	C.V. (%)
Plasma (n = 4)		
6	6.1	4.4
15	15.8	3.8
35	35.5	3.0
100	95.9	2.2
300	303.9	4.4
600	696.0	1.3
1000	1006.8	1.9
Urine (n = 3)		
7.5	7.9	8.8
22.5	22.4	2.4
52.5	52.6	10.3
75	77.9	1.8
225	232.7	4.7
525	526.0	0.7
700	747.8	2.3

Recovery

The mean absolute recovery of the analytes from plasma and urine was 90% for both I and II. The recovery was determined by comparing

TABLE III

INTER-DAY ACCURACY AND PRECISION OF QUALITY CONTROL SAMPLES

Nominal concentration (ng/ml)	Found concentration (ng/ml)	C.V. (%)
Plasma (n = 6)[a]		
20	19.9	3.7
65	66.8	2.3
700	692.3	2.0
Urine (n = 3)[b]		
22.5	23.2	8.4
225	228.3	5.6
525	553.1	2.2

[a] QCs were run over a six-day period.

[b] QCs were run over a three-day period.

peak heights 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. $\leq 10\%$) was 6 ng/ml in plasma and 7.5 ng/ml in urine.

CONCLUSION

A reversed-phase HPLC assay method is described for the new angiotensin II receptor antagonist I in human plasma and urine. Citric buffer has very good buffer capacity in the pH range 3–5. By using this buffer, the pH vlaues of plasma and urine are able to be adjusted to a very narrow range (3.3–3.6) and the recovery of the described method is consistent, reproducible and greater than 90%. The peak shapes and the column behavior are always reproducible. The method is suitable for the evaluation of pharmacokinetics of DuP 532 (L-694,492) in humans.

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