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Short communication

An improved method for the simultaneous determination of losartan and its major metabolite, EXP3174, in human plasma and urine by high-performance liquid chromatography with fluorescence detection

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1. Introduction

Losartan potassium, 2-butyl-4-chloro-1-[*p*-(*O*-1*H*-tetrazol-5-ylphenyl)benzyl]imidazole-5-methanol monopotassium salt (Fig. 1), is a highly selective AT_1-subtype, non-peptide, orally active, angiotensin II (AII) receptor antagonist indicated for the treatment of hypertension [1-4]. An AII receptor antagonist would be expected to provide clinical efficacy while maintaining an excellent tolerability profile when compared with other antihypertensive agents. Characterization of losartan's activity in animals revealed that the 5-carboxylic acid of losartan, EXP3174 (Fig. 1), is an active metabolite which contributes to the overall in vivo activity of losartan in animals [5] and humans [6]. However, losartan is not a prodrug since losartan itself is also a potent AII antagonist.

The pharmacokinetics of losartan in man have been described [7] utilizing a previously published assay method for both losartan and EXP3174 in plasma and urine based on HPLC and UV detection [8]. Recently, in anticipation of needs to support lower dosage strengths of losartan for other indications, development work was initiated to improve the sensitivity of the method. We now report a modified HPLC assay utilizing fluorescence detection for the simultaneous quantitation of losartan and EXP3174 in human plasma and urine. Modifications to the sample preparation procedure of the previously reported HPLC-UV method improved the extraction recovery. The utilization of fluorescence detection improved the detection limit and the selectivity for both losartan and EXP3174.

The structures of losartan, EXP3174 and the internal standard (L-158 854) used in the assay are shown in Fig. 1.

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2. Experimental

2.1. Chemicals

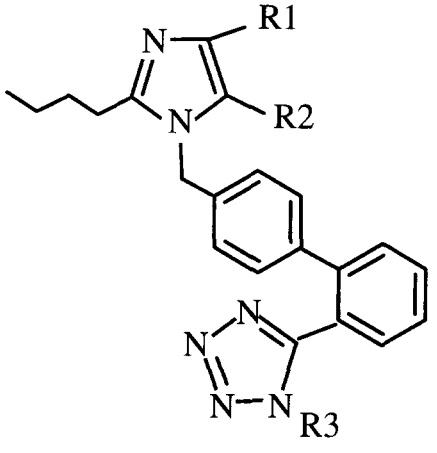
Losartan, EXP3174 and the internal standard (L-158 854) were obtained from Merck Research Laboratories (Rahway, NJ, USA). HPLC grade methyl *t*-butyl ether and isopropanol were purchased from Burdick and Jackson (Muskegon, MI, USA). HPLC grade acetonitrile, hexane, methanol, 85% phosphoric acid, sodium citrate, citric acid and sodium hydroxide were obtained from Fisher Scientific (Fair Lawn, NJ, USA).

2.2. Instrumentation and chromatographic conditions

The liquid chromatographic system was comprised of a Perkin-Elmer Series 410 pump (Norwalk, CT, USA) and a Perkin-Elmer ISS-100 or ISS-200 autosampler (Norwalk, CT, USA) connected to a Perkin-Elmer LC-240 fluorescence detector (Beaconsfield, UK). A Jones Chromatography column block heater (Wales, UK) was used to control the temperature of the column. Separation was achieved on a 5 μm Ultremex CN (250 × 4.6 mm i.d.) from Phenomenex (Torrance, CA, USA). The chromatographic data were analyzed using PE Nelson Systems Turbochrom chromatography software (Cupertino, CA, USA). For plasma analysis, the isocratic mobile phase consisted of phosphoric acid (pH 2.3; 0.015 M)-acetonitrile (75:25, v/v). For urine analysis, the isocratic mobile phase consisted of phosphoric acid (pH 2.3; 0.015 M)-acetonitrile (79:21, v/v). In both cases, sodium hydroxide (2.0 N) was used to adjust the pH of the phosphoric acid. The flow rate was set to 1.25 ml min^{-1} for both plasma and urine analysis. Column temperature was maintained at 35°C. The column effluent was monitored by fluorescence at excitation and emission wavelength of 250 and 370 nm, respectively.

2.3. Sample preparation

Frozen plasma samples, stored at −20°C, were thawed and allowed to reach room temperature. An 1 ml aliquot of plasma was placed into a polypropylene tube and 125 μl of 1.0 M phosphoric acid were added to each plasma sample to adjust the pH to approximately 2.5. Internal standard solution, 100 μl, (1.0 μg ml^{-1}) were added to each sample and the analytes were extracted with 10 ml of methyl-*t*-butyl ether (MTBE) by shaking for 20 min at 60 rpm. The samples were centrifuged for 5 min at 2060 × *g*. The aqueous layer was frozen in a dry ice/acetone bath and the organic solvent, containing the analytes, was decanted to clean polypropylene tubes containing 200 μl of 0.05 M NaOH and shaken for 15 min at 60 rpm. The samples were once again centrifuged at 2060 × *g* for 5 min. The aqueous layer was separated by freezing as above, the MTBE layer was discarded and the residual MTBE was removed by nitrogen evaporation. The NaOH layer was acidified with 75 μl of pH 4.3, 0.5 M citrate buffer and vortex-mixed. The aqueous fraction was washed by



	R1	R2	R3
Losartan potassium	Cl	CH_2OH	K
EXP3174	Cl	COOH	H
L-158,854	2-Chlorophenyl	COOH	H

Fig. 1. Structures of losartan potassium, EXP3174 and internal standard (L-158 854).

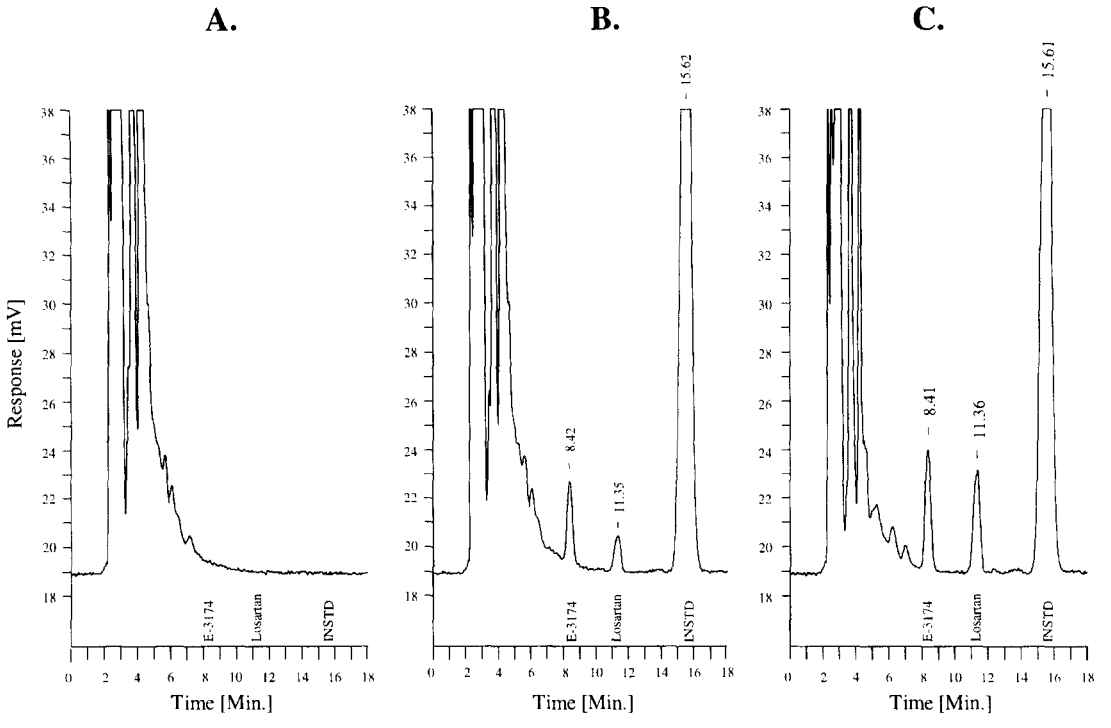


Fig. 2. Representative chromatograms of losartan, EXP3174 (E-3174) and internal standard (INSTD) in human plasma. (A) Control plasma (1 ml); (B) control plasma (1 ml) spiked with 5.0 ng ml^{-1} of losartan and EXP3174 and 100 ng ml^{-1} of INSTD. (C) 2-h Sample (1 ml) from a subject after administration of 12.5 mg oral dose of losartan (concentrations = 15.12 ng ml^{-1} losartan and 7.45 ng ml^{-1} EXP3174).

adding 6 ml of hexane and vortex-mixing for 1 min. 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 of the analytes in the mobile phase, 10 μl of isopropanol were added to the aqueous layer. A 75 μl aliquot out of a total 285 μl was then injected onto the HPLC for analysis.

The urine samples were prepared using a modified version of the preceding plasma assay. Frozen urine samples were thawed and brought to room temperature. Urine, 0.5 ml, was acidified with 750 μl of a pH 5.0, 1.0 M citrate buffer and extracted with 10 ml of MTBE:hexane (4:1, v/v). The analytes were back-extracted from the organic layer into 0.05 M NaOH as above and the residual MTBE:hexane was removed. The NaOH layer was adjusted to an acidic pH with 75 μl of 0.2 M phosphoric acid. This aqueous fraction was washed with hexane as above and the residual hexane was removed. To improve solubility of the analytes in the mobile phase, 50 μl of isopropanol were added to the aqueous layer. A 65 μl aliquot out of a total 325 μl was then injected onto the HPLC for analysis.

2.4. Quantitation

Calibration standards of losartan and EXP3174 were prepared by spiking appropriate volumes of the losartan/EXP3174 working standard solutions into 1.0 ml of control human plasma or 0.5 ml of control human urine followed by the addition of the internal standard. Calibration curves were constructed from the extracted calibration stan-

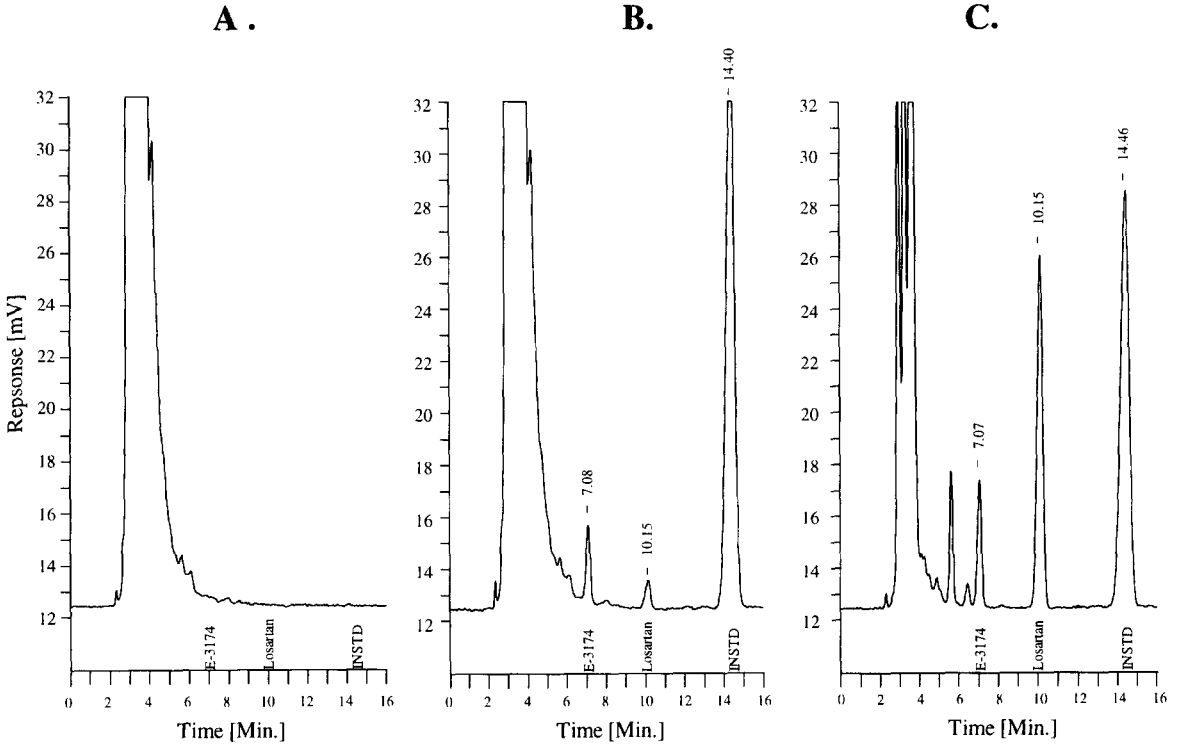


Fig. 3. Representative chromatograms of losartan, EXP3174 (E-3174) and internal standard (INSTD) in human urine. (A) Control urine (0.5 ml); (B) control urine (0.5 ml) spiked with 10 ng ml^{-1} losartan, EXP3174 and 200 ng ml^{-1} of INSTD. (C) 0–3 h Sample (0.5 ml) from a subject after administration of 12.5 mg oral dose of Losartan (concentrations = 174.7 ng ml^{-1} losartan and 21.6 ng ml^{-1} EXP3174).

dards as linear least-squares fitted lines of peak height ratios (losartan or EXP3174 height/internal stardard height) versus standard concentrations. Reciprocal weighting was applied to the concentrations.

3. Results and discussion

The extraction procedures described in the previously published method [8] were modified to improve the extraction recovery of the analytes. Improvements in the extraction recoveries contributed to the improved sensitivity of the assay. The recovery was determined by comparing peak areas from extracted standards ($n = 4–5$ at each concentration measured) with those of unextracted standards. The recovery of both losartan and EXP3174 was greater than 90% (coefficient of variation $< 7\%$), in both plasma and urine, at each concentration measured, across the range of the calibration curves. Solid-phase extraction was briefly explored for sample clean-up but liquid–liquid extraction was found to be a superior approach. Additionally, the pH 5.0, citrate buffer proved to be more effective at controlling the pH of the clinical urine samples than simple acidification.

Fluorescence detection improved the selectivity and sensitivity of the original UV method, resulting in a cleaner background and higher analyte response. The fluorescence response of losartan and EXP3174 was determined to be highly pH dependent. After exploring the fluorescence response of both losartan and EXP3174 between pH 2 and 7, we determined the optimal pH for

Table 1
Accuracy and precision of plasma standard curves

Nominal concentration (ng ml^{-1})	Losartan			EXP3174		
	Mean found (ng ml^{-1})	%Deviation from nominal	%CV[a]	Mean found (ng ml^{-1})	%Deviation from nominal	%CV[a]
Intra-day reproducibility ($n = 5$)						
0.5	—[b]			0.54	+8.0	9.3
1.0	1.02	+2.0	5.4	1.00	0.0	4.2
2.5	2.53	+1.2	1.7	2.44	−2.4	2.3
5.0	4.91	−1.8	2.0	4.91	−1.8	1.5
25.0	24.81	−0.8	1.1	24.63	−1.5	1.3
50.0	49.71	−0.6	1.4	49.15	−1.7	1.1
250.0	250.53	+0.2	0.5	248.48	−0.6	1.0
500.0	499.99	0.0	0.5	502.86	+0.6	1.3
Linear regression line, 1/x weighting, all data:						
	$y = 0.009621 \cdot x$-0.000101			$y = 0.021908 \cdot x + 0.000513$		
	$r^2 = 1.0000$			$r^2 = 0.9998$		

[a] Peak-height ratios used to calculate %CV for intra-day precision.
[b] Below standard curve, not studied.

analysis to be between pH 2 and 3. Above pH 3, the fluorescence intensity of both compounds decreases rapidly with increasing pH. The fluorescence intensity at pH 2.5, compared with that at pH 7, is 30- and 40-fold higher for losartan and EXP3174, respectively. Additionally, the native fluorescence of EXP3174 is greater than that of losartan. At the pH of the mobile phase (≈ 2.3), the fluorescence response of EXP3174 is approximately twice that of losartan. UV irradiation of the column effluent did not increase the fluorescence response of the analytes.

The cyano column was chosen because it gave very good band spacing of the analytes of interest, provided excellent separation from endogenous peaks in each of the matrices and minimized the incidence of late-eluting peaks. The exact pH of the mobile phase was chosen to optimize the band spacing between the analytes and maximize the response of losartan and EXP3174 without sacrificing the life of the analytical column. Representative chromatograms are shown in Fig. 2 and Fig. 3.

Intra-day accuracy and precision of the standard curves were examined by assaying replicate standard curves in a single run.The close correlation between the nominal and found concentrations of the standards in the calibration curves for intra-day data show that the assay was linear over the concentration range investigated and reproducible. The standard curve intra-day data, including the regression line parameters, are listed in Table 1 and Table 2.

Intra-day and inter-day accuracy and precision of the method were evaluated using quality control (QC) samples which were prepared at low, medium and high concentrations (losartan: 2.5, 40 and 400 ng ml^{-1} in plasma; 16, 160 and 1600 ng ml^{-1} in urine and EXP3174: 1, 40 and 400 ng ml^{-1} in plasma; 8, 160 and 1600 ng ml^{-1} in urine). Intra-day accuracy and precision were determined by assaying replicates of the above QC samples in a single run. The inter-day accuracy and precision were examined by analyzing plasma and urine QC samples daily with clinical study samples over a period of 34 weeks. For both plasma and urine QC samples, the coefficients of variation (CV) were less than 5% for the intra-day and inter-day evaluations. The mean found concentrations did not deviate from the nominal con-

Table 2
Accuracy and precision of urine standard curve

Nominal concentration (ng ml^{-1})	Losartan			EXP3174		
	Mean found (ng ml^{-1})	%Deviation from nominal	%CV[a]	Mean found (ng ml^{-1})	%Deviation from nominal	%CV[a]
Intra-day reproducibility ($n = 5$)						
2.0	—[b]			1.7	−15.0	4.1
5.0	4.4	−12.0	4.2	4.9	−2.0	3.6
10.0	9.7	−3.0	4.5	10.1	+1.0	1.3
50.0	53.0	+6.0	0.8	53.4	+6.8	1.1
100.0	108.4	+8.4	0.7	107.8	+7.8	0.4
500.0	495.5	−0.9	1.2	494.3	−1.1	1.1
1000.0	1020.7	+2.1	0.2	1026.4	+2.6	1.2
2000.0	1973.3	−1.3	0.7	1968.3	−1.6	0.6
Linear regression line, 1/x weighting, all data:						
	$y = 0.008337 \cdot x + 0.004735$			$y = 0.021192 \cdot x + 0.006673$		
	$r^2 = 0.9994$			$r^2 = 0.9993$		

[a] Peak-height ratios used to calculate %CV for intra-day precision.
[b] Below standard curve, not studied.

centrations by more than 8%, for both analytes in either matrix for both intra-day and inter-day evaluations, showing that the assay is sufficiently accurate and precise. The quality control sample intra-day and inter-day data are listed in Table 3 and Table 4.

The linear ranges of the standard curves for the validated assay in plasma were 1–500 ng ml^{-1} for losartan and 0.5–500 ng ml^{-1} for EXP3174. The linear ranges of the standard curves for the validated assay in urine were 5–2000 ng ml^{-1} for losartan and 2–2000 ng ml^{-1} for EXP3174.

The limit of quantitation was defined as the lowest concentration in the standard curve with an intra- day CV $\leq$ 15% and with a mean-fitted value that did not deviate from the nominal value by more than 15%. The limits of quantitation in plasma were 1 ng ml^{-1} and 0.5 ng ml^{-1} for losartan and EXP3174, respectively. The limits of quantitation in urine were 5 and 2 ng ml^{-1} for losartan and EXP3174, respectively. Compared with the previously published method, this represents a five- and ten-fold increase in sensitivity for losartan and EXP3174 in plasma, respectively. For the urine assay, there was a four- and five-fold increase in sensitivity for losartan and EXP3174, respectively.

In order to further evaluate the accuracy of the improved method, human clinical plasma and urine samples that had been analyzed by the previously published method were reanalyzed using the improved method. The new quantitations were compared with the previously determined quantitations as shown in ig. 4. The 1:1 correlation shows that the new quantitations were nearly identical to those of the previous analysis. This demonstrates that the improved method is accurate and specific for both losartan and EXP3174.

The method described has been successfully applied to the quantitation of losartan and EXP3174 in about 1500 plasma and 900 urine samples over a 12-month period. The data from a representative subject given 12.5 mg of losartan orally is shown in Fig. 5.

Table 3
Intra-day accuracy and precision of quality control samples

Compound	Low QC (Concentration (ng ml^{-1}))				Medium QC (Concentration (ng ml^{-1}))				High QC (Concentration (ng ml^{-1}))			
	Nominal	Mean found	%Deviation from nominal	%CV	Nominal	Mean found	%Deviation from nominal	%CV	Nominal	Mean found	%Deviation from nominal	%CV
Plasma[a]												
Losartan	2.50	2.51	+0.4	1.19	40.00	40.53	+1.3	0.77	400.00	408.68	+2.2	0.92
EXP3174	1.00	1.04	+4.0	3.01	40.00	39.96	−0.1	0.87	400.00	404.09	+1.0	0.70
Urine[b]												
Losartan	16.0	15.2	−5.0	2.4	160.0	153.2	−4.3	1.2	1600.0	1551.4	−3.0	0.8
EXP3174	8.0	7.9	−1.3	1.7	160.0	160.4	+0.3	0.6	1600.0	1615.8	+1.0	0.6

[a] $n = 6$.
[b] $n = 6$.

Table 4
Inter-day accuracy and precision of quality control samples

Compound	Low QC (concentration (ng ml^{-1}))				Medium QC (concentration (ng ml^{-1}))				High QC (Concentration (ng ml^{-1}))			
	Nominal	Mean found	%Deviation from nominal	%CV	Nominal	Mean found	%Deviation from nominal	%CV	Nominal	Mean found	%Deviation from nominal	%CV
Plasma[a]												
Losartan	2.50	2.52	+0.7	3.3	40.00	40.48	+1.2	1.6	400.00	409.23	+2.3	1.8
EXP3174	1.00	1.08	+8.0	4.8	40.00	40.81	+2.0	2.9	400.00	412.11	+3.0	3.2
Urine[b]												
Losartan	16.0	15.4	−3.6	3.8	160.0	153.7	−4.0	2.7	1600.0	1570.1	−1.9	3.1
EXP3174	8.0	8.2	+2.5	3.4	160.0	160.4	+0.3	2.2	1600.0	1616.5	+1.0	2.0

[a] $n = 31$ over a period of 34 weeks.
[b] $n = 28$ over a period of 34 weeks.

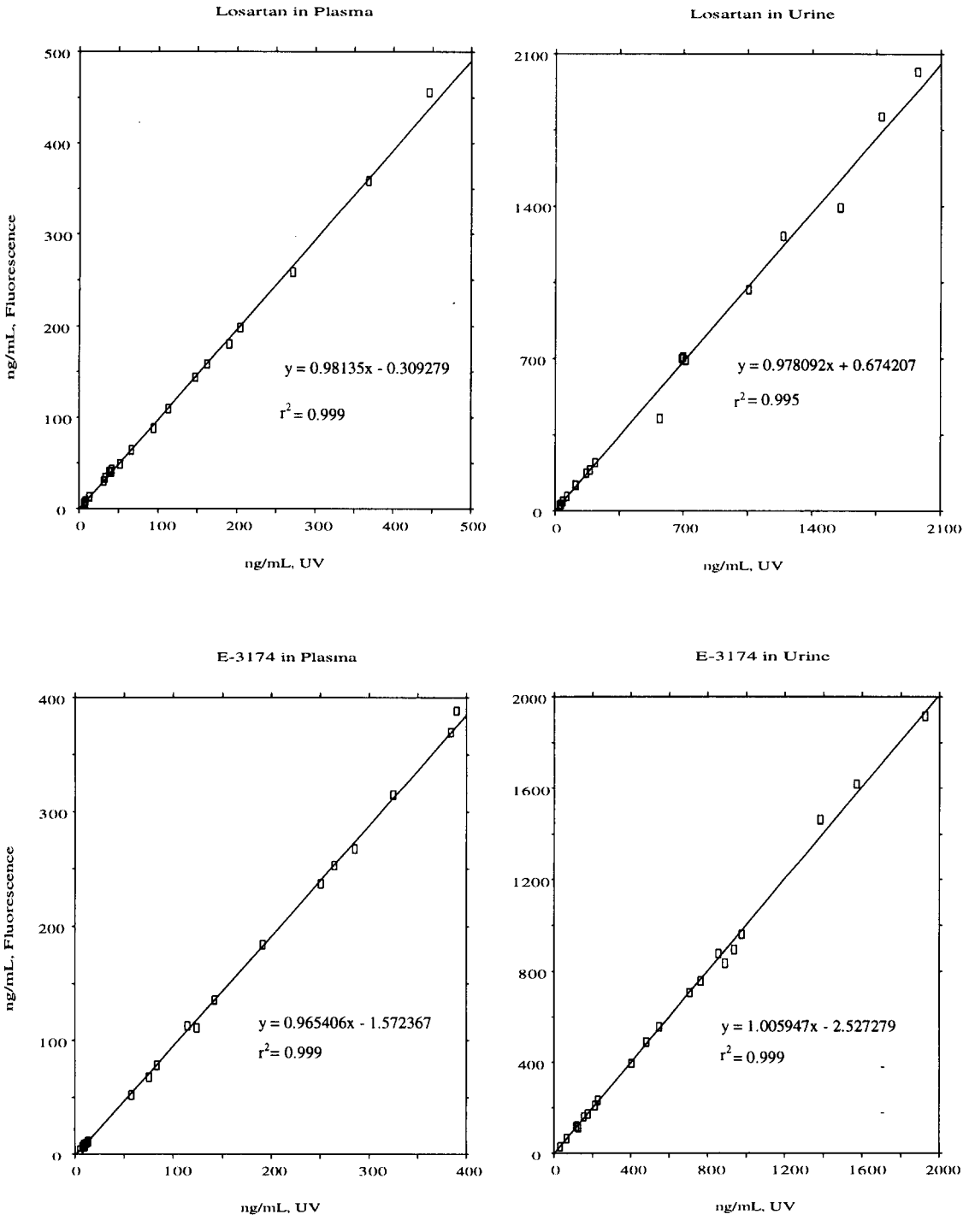


Fig. 4. Concentrations determined by fluorescence method versus UV method.

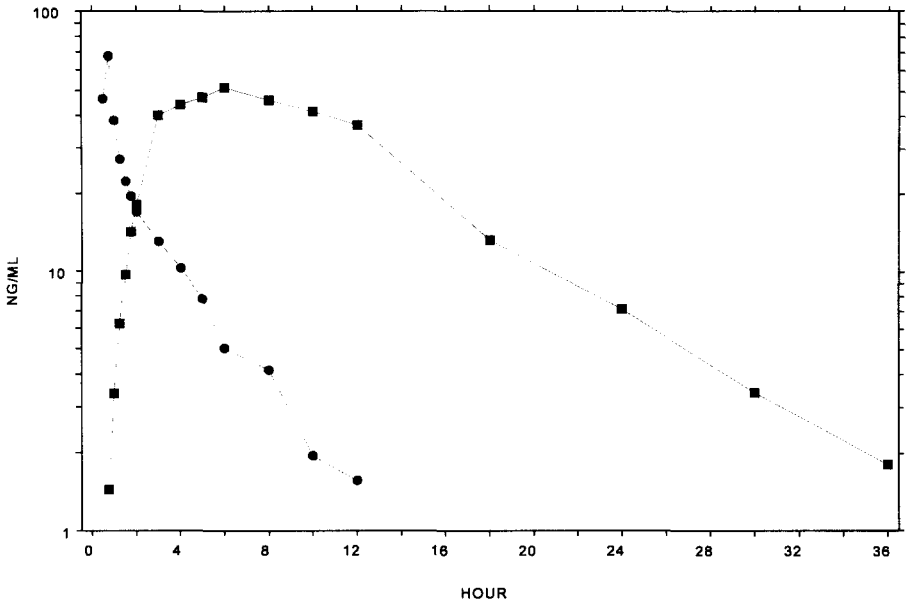


Fig. 5. Plasma concentrations of losartan (●) and EXP3174 (■) following the administration of a single 12.5 mg losartan tablet in a healthy subject.

4. Conclusions

An improved HPLC assay method is reported for losartan and EXP3174 in plasma and urine. The method is selective, reproducible and sensitive. Significantly lower limits of quantitation were achieved in both plasma and urine, compared with the previously published method [8]. It has replaced the HPLC-UV method in evaluating the pharmacokinetics of losartan in humans.

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