Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis
33 (2003) 73–84
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Simultaneous determination of losartan and EXP3174 in human plasma and urine utilizing liquid chromatography/tandem mass spectrometry

Michelle Polinko *, Kerry Riffel, Hengchang Song, Man-Wai Lo

Department of Drug Metabolism, Merck Research Laboratories, WP 75A-303, West Point, PA 19486, USA

Received 24 June 2002; accepted 29 January 2003

Abstract

Losartan is an orally active angiotensin II receptor antagonist indicated for the treatment of hypertension. EXP3174 is an active metabolite, which contributes to the overall activity of losartan. Analytical methods for the simultaneous determination of losartan and its active metabolite EXP3174 in human plasma and urine with limited plasma sample size have been developed and validated to support a pediatric clinical program. In both methods, analytes are extracted from the matrixes by liquid–liquid extraction and separated using reverse phase high-performance liquid chromatography (HPLC). A tandem mass spectrometer (MS/MS) with a Turbo ionspray (TIS) interface in multiple-reaction-monitoring (MRM) mode is used for detection of the analytes in both methods. The plasma method has a lower limit of quantitation (LOQ) of 1 ng/ml with a linearity range of 1–500 ng/ml for losartan and EXP3174 using 100 μl of plasma. For the urine method, the LOQ for both losartan and EXP3174 is 2 ng/ml using 0.5 ml of urine, and the linearity range for both analytes is 2–1000 ng/ml. Validation procedures have proven that both methods are robust, accurate, and reproducible. Both methods have been used to assay clinical samples and provided satisfactory results.

Keywords: Losartan; EXP3174; LC–MS/MS; Human plasma and urine; Pediatric

1. Introduction

Losartan (**I**, 2-*n*-butyl-4-chloro-1-[*p*-(*o*-1*H*-tetrazol-5-ylphenyl)benzyl]-imidazole-5methanol monopotassium salt) is a highly selective, orally active, non-peptide angiotensin II receptor antagonist indicated for the treatment of hypertension. It has a more potent active metabolite EXP3174 (**II**, 2-*n*-butyl-4-chloro-1-[2′-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole-5-carboxyl acid) [1–5].

Several analytical methods have been published which determine the concentration of **I** and **II** in human biological fluids. Methods of detection varied from ultraviolet detection [6–9] to later methods that utilize fluorescence detection which greatly improved the sensitivity and selectivity of **I** and **II** [10–13]. The liquid–liquid extraction and fluorescence detection method reported by Lo et

* Corresponding author. Tel.: +1-215-652-8556; fax: +1-215-652-4524.
E-mail address: michelle_polinko@merck.com (M. Polinko).

0731-7085/03/$ - see front matter
doi:10.1016/S0731-7085(03)00348-0

al. achieved the sensitivity needed to analyze lower dosage clinical samples when extracting from 1 ml of plasma and 0.5 ml of urine [10]. (Plasma LOQ at 1 ng/ml for **I**, 0.5 ng/ml for **II**). The large sample volume needed to achieve this sensitivity limits the use of the method.

A plasma method that uses solid-phase extraction (SPE) and negative ionization LC–MS/MS for detection has also been reported [14]. The LC–MS/MS method reaches the same LOQ (1 ng/ml) while extracting from a smaller sample volume of 0.4 ml of plasma. The automated SPE decreases sample preparation time. A disadvantage of the method is lower recoveries are reported as compared with previous methods. The investigators attribute lower recoveries to suppression by the ion source; however, matrix effect was not formally evaluated. Matrix effects are common when using an MS ionspray interface and have been discussed in the literature [15–17]. When analyzing biological samples by MS, endogenous species may be present which co-elute with analytes and effect ionization efficiency at the MS interface. This can compromise reproducibility and accuracy of the method. Therefore, the matrix effect on **I** and **II** using a MS Turbo ionspray (TIS) interface should be evaluated. The method reported here minimizes changes in efficiency of ionization of the analytes by optimizing extraction and chromatographic conditions.

The previous methods referenced were not suitable for analysis of plasma samples with limited volume needed for pediatric clinical samples. Many pediatric clinical samples received were in quantities less than 0.4 ml. Therefore, in this paper, a new method which utilized liquid–liquid extraction for sample preparation and positive ionization LC–MS/MS for detection is described for the simultaneous determination of **I** and **II** in human plasma with sample size of 0.1 ml. Also, the first method to determine **I** and **II** in human urine samples utilizing LC–MS/MS detection is reported. These methods do not compromise the high sensitivity and wide linear range, which are needed to generate high quality data for pharmacokinetics analysis. Clinical data demonstrates the application of these methods. The data establishes long-term stability of samples stored at −20 °C as well as the robustness and reproducibility of the methods.

2. Experimental

2.1. Material

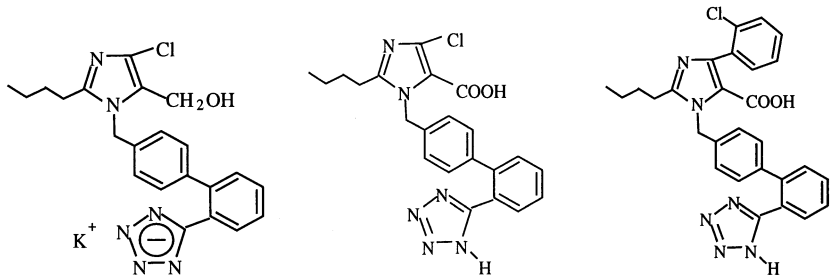
Compound **I** (potassium salt), **II** (hydrochloride salt), and the internal standard (**III**, 2-*n*-butyl-4-(2-chlorophenyl)-1-[2′-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole-5-carboxyl acid) (Fig. 1) were obtained from Merck Research Laboratories (Rahway, NJ). HPLC grade methyl-*t*-butyl ether (MTBE) (HPLC grade) was obtained from Burdick & Jackson (Muskegon, MI). Control human plasma was purchased from Sera-Tec Biologicals (New Brunswick, NJ). Formic acid (99%) was purchased from Sigma (St. Louis, MO). Other chemicals and solvents were from Fisher Scientific (Fair Lawn, NJ).

2.2. Instrumentation

Two LC–MS/MS systems were used in this research. The LC–MS/MS system for the plasma method consists of a PE Sciex (Thornhill, Ontario, Canada) API 3000 with a TIS interface, a Perkin–Elmer (Norwalk, CT) Series 200 Autosampler, a pair of Perkin–Elmer Series Micro 200 pumps, and a Jones Chromatography (Lakewood, CO) 5795 Column Heater. The data were processed using PE Sciex MACQUAN software (Version 1.5) on a MacIntosh PowerPC G3 computer. The LC–MS/MS system for the urine method consists of a PE Sciex API 365 with a TIS interface, a Perkin–Elmer Series 200 Autosampler, a Perkin–Elmer Series 200 quaternary pump, and a Jones Chromatography 5795 Column Heater. The data were processed using PE Sciex MACQUAN software (Version 1.4) on a MacIntosh PowerPC 9500 computer.

2.3. Standard solutions

Concentrations of **I** are expressed as losartan potassium; **II** concentrations are expressed as the free acid.



I *II* *III*

Fig. 1. Chemical structure of **I**–**III**.

2.3.1. Plasma method

Primary stock solutions of **I** and **II** were prepared separately at 0.2 mg/ml in methanol/water (50:50, v/v). Primary stock solution was further diluted with methanol/water (50:50, v/v) to give a series of working standards with concentrations of both **I** and **II** at 1, 2, 10, 20, 100, 250 and 500 ng/ml. Internal standard (**III**) working solution was prepared at 5 ng/ml in methanol/water (50:50, v/v). All the standard solutions were stored at room temperature. Plasma standards were prepared by adding 0.1 ml of each working standard to 0.1 ml of human control plasma. The resulting plasma standard concentrations ranged from 1 to 500 ng/ml.

Quality control (QC) stock solutions of **I** and **II** were prepared separately at 0.2 mg/ml in methanol/water (50:50, v/v). An appropriate amount of diluted solutions from stock was placed into a 25 ml volumetric flask and then filled with human control plasma to make concentration levels of 1.5, 40 and 400 ng/ml, representing low, medium, and high QCs, respectively. The QC samples were aliquoted in 0.5 ml portions into 2 ml polypropylene centrifuge tubes and stored at $-20\,^{\circ}C$ until assayed.

2.3.2. Urine method

Primary stock solutions of **I** and **II** were prepared separately at 0.5 mg/ml in methanol/water (50:50, v/v). These solutions were further diluted with methanol/water (50/50, v/v) to give a series of working standards with concentrations of both **I** and **II** at 10, 25, 50, 250, 500, 2500 and 5000 ng/ml. Internal standard (**III**) working solution was prepared at 250 ng/ml in methanol/water (50:50, v/v). All the standard solutions were stored at room temperature. Urine standards were prepared by adding 0.1 ml of each working standard to 0.5 ml of human control urine. The resulting plasma standard concentrations ranged from 2 to 1000 ng/ml.

QC stock solutions of **I** and **II** were prepared separately at 0.5 mg/ml in methanol/water (50:50, v/v). An appropriate amount of diluted solutions from stock was placed into a 50 ml volumetric flask and then filled with human control urine to make concentration levels of 6, 120 and 900 ng/ml, representing low, medium, and high QCs, respectively. The QC samples were aliquotted in 2 ml portions into 5 ml polypropylene round bottom tubes and stored at $-20\,^{\circ}C$ until assayed.

2.4. Sample preparation

2.4.1. Plasma method

Human plasma (0.1 ml) was treated with 0.25 ml of 0.5% formic acid, 0.1 ml methanol/water (50:50, v/v), and 0.05 ml of 5 ng/ml internal standard was added. The analytes were extracted using 2 ml of MTBE. Samples were vortexed and centrifuged for 5 min at $\sim$3000 rpm. After freezing the aqueous phase, the organic layer (MTBE) was transferred to a clean polypropylene tube. The MTBE was evaporated under nitrogen to dryness. Samples were reconstituted in 0.2 ml of isopropanol (IPA):0.2% formic acid (25:75, v/v) and vortexed. After transferring to 200 μl autosampler vials, 5–20 μl of sample was injected for LC–MS/MS analysis.

2.4.2. Urine method

Human urine (0.5 ml) was treated with 0.5 ml of 0.5% formic acid, 0.1 ml methanol:water (50:50, v/v), and 0.1 ml of 250 ng/ml internal standard was added. The analytes were extracted using 5 ml of MTBE. Samples were vortexed and centrifuged for 5 min at ~3000 rpm. After freezing the aqueous phase, the organic layer (MTBE) was transferred to a clean polypropylene tube. The MTBE was evaporated under nitrogen to dryness. Samples were reconstituted in 0.2 ml of IPA:0.2% formic acid (25:75, v/v) and vortexed. After transferring to 200 μl autosampler vials, 5–30 μl of sample was injected for LC–MS/MS analysis.

2.5. LC–MS/MS conditions

The separation of analytes was performed on a Keystone Valuepak Cyano column (50 × 2.1 mm, 3 μm) from Thermo Hypersil-Keystone (Bellfonte, PA) with a Keystone BetaBasic CN, 20 × 2 mm guard column. Mobile phase consists of acetonitrile:0.2% formic acid (55:45, v/v) with a flow rate of 0.2 ml/min. HPLC column temperature was fixed at 30 °C to avoid retention time change with fluctuation in room temperature.

The analytes are detected by a tandem mass spectrometer (MS/MS) with a TIS interface in positive ionization mode. The positive product ion scan spectra for the protonated molecule $[M+H]^+$ of **I** at $m/z = 423$, **II** at $m/z = 437$, and **III** at $m/z = 513$ are shown in Fig. 2. The precursor → product ion combinations are monitored in multiple-reaction-monitoring (MRM) mode. Based on the ionization of these compounds, the channels used for the quantitative determination of these compounds were m/z 423 → m/z 207 (**I**) and m/z 437 → m/z 235 (**II**). The internal standard (**III**) was monitored at m/z 513 → m/z 207.

2.6. Quantification

A standard curve was constructed daily. The concentrations of **I** and **II** were calculated from the linear least-squares fitted line of peak area ratios of **I** or **II** to the internal standard **III** versus the analyte concentration with reciprocal weighing on the concentration ($1/x$). The standard samples were assayed along with QC and unknown samples.

3. Results and discussion

3.1. Sensitivity and linearity

For the plasma method, the limit of quantitation (LOQ) is 1 ng/ml for both **I** and **II** using 0.1 ml of human plasma. The linear dynamic range is from 1 to 500 ng/ml. For the urine method, LOQ is 2 ng/ml using 0.5 ml of urine. The linearity range is 2–1000 ng/ml. The LOQ and linear range of both methods were chosen based on the range of concentrations of **I** and **II** that would be expected in pediatric clinical samples assuming similar exposure as in adults. Although LOQ is 1 ng/ml in plasma, the sensitivity is achieved using a limited sample volume of 0.1 ml plasma. The extracts are diluted in the reconstitution step by 2-fold and 2.5–10.0% of final volume was typically injected onto LC–MS/MS for analysis. This indicates that a more sensitive linear range could be achieved if needed in future studies.

For all completed experiments to this point, the correlation coefficient (r) for the calibration curves was greater than 0.99. Representative calibration curve parameters for plasma and urine methods from intraday standard curve replicates are footnoted in Table 1.

3.2. Accuracy and precision

3.2.1. Plasma method

Intraday accuracy and precision of the methods was determined by analyzing five replicates of calibration standards at all concentrations. The precision (%CV, $n = 5$) at LOQ (1 ng/ml) was 2.0% for **I** and 7.7% for **II** with an accuracy (percentage of nominal value) of 90.0% for **I** and 90.0% for **II**. For all concentration levels in the standard curve, the precision varied from 0.8 to 5.5% for **I** and 2.1 to 7.7% for **II**, and the accuracy ranged from 90.0 to 105.0% for **I** and 90.0 to 107.0% for **II**. The data is summarized in Table 1.

Precision and accuracy for intraday QC's were determined by analyzing samples at low, medium,

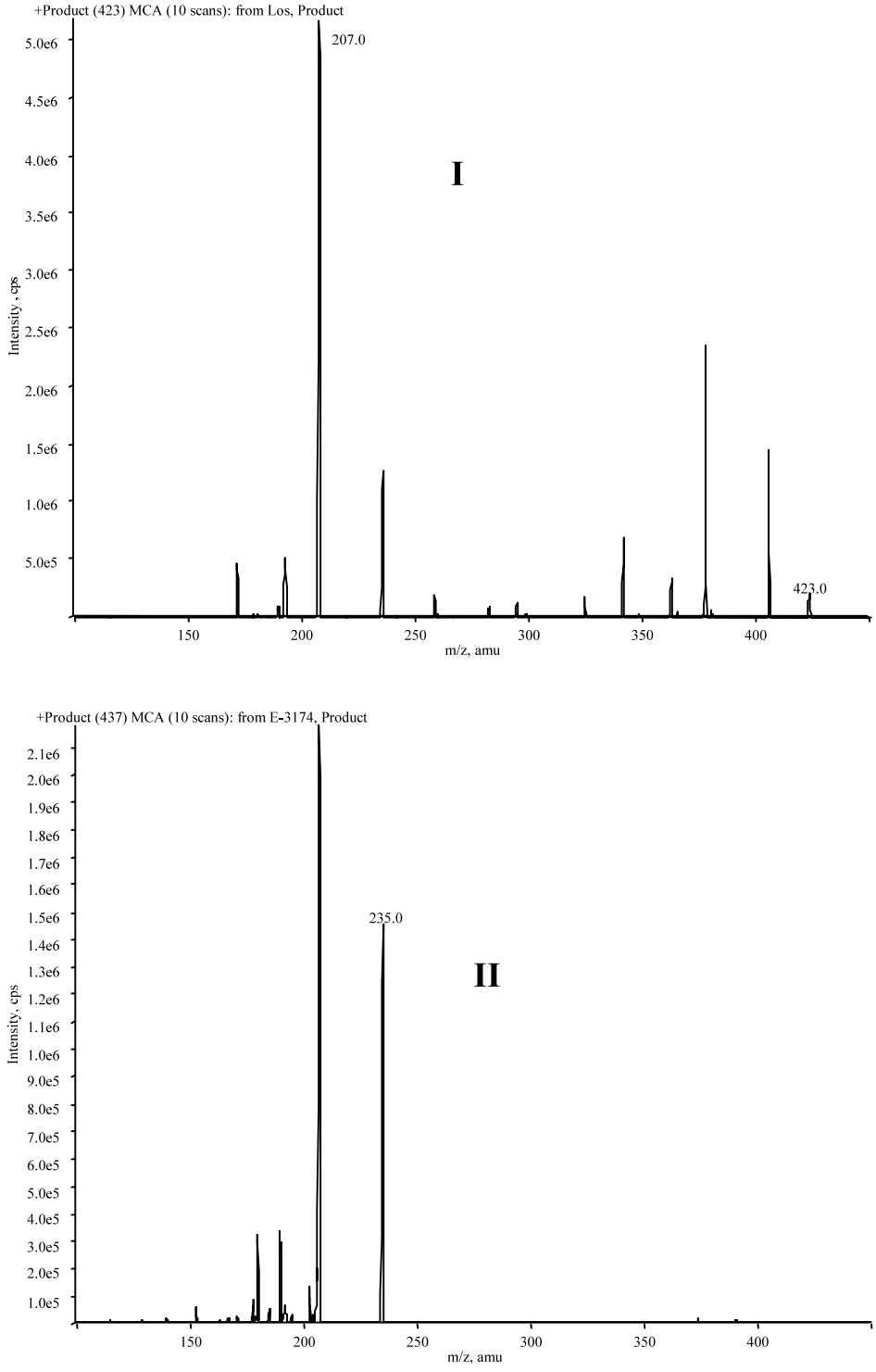


Fig. 2. Product ion scan spectra for **I** (MW = 423), **II** (MW = 437), and **III** (MW = 513).

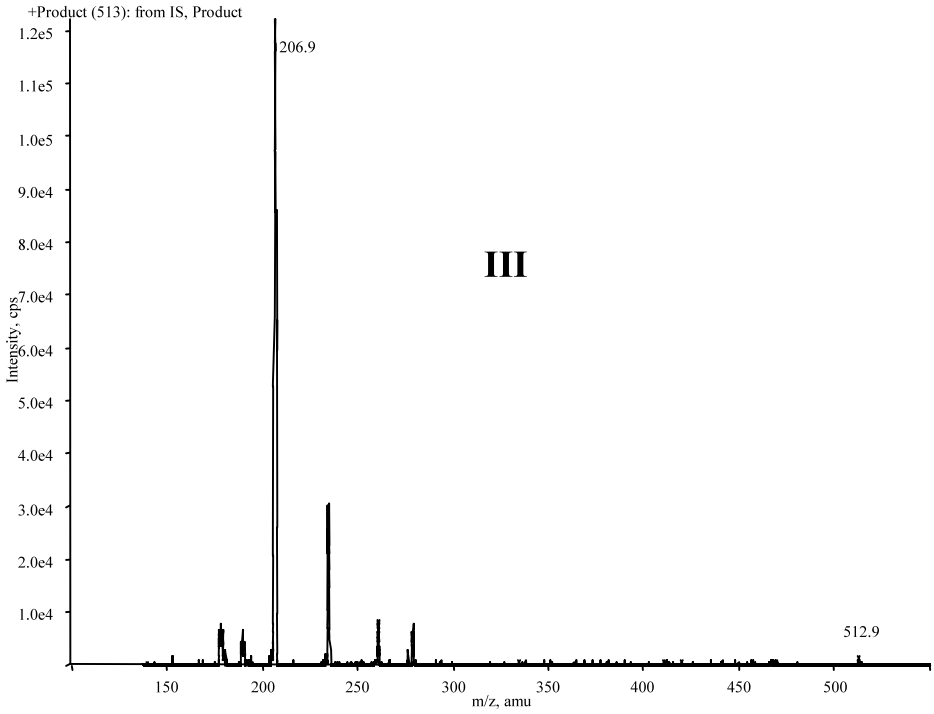


Fig. 2 (*Continued*)

and high concentrations. Table 2 summarizes the means, precision, and accuracy. The precision (%CV) was $\leq 3.8\%$ for **I** and 7.8% for **II** with accuracy range (percentage of nominal value) of 100.0–102.3% for **I** and 93.2–96.5% for **II** over three QC concentrations.

3.2.2. Urine method

Intraday accuracy and precision of the methods was determined by analyzing five replicates of calibration standards at all concentrations. The precision (%CV, $n = 5$) at LOQ (2 ng/ml) was 5.5% for **I** and 5.7% for **II** with an accuracy (percentage of nominal value) of 87.0% for **I** and 88.0% for **II**. For all concentration levels in the standard curve, the precision varied from 1.7 to 6.7% for **I** and 1.2 to 8.8% for **II**, and the accuracy ranged from 87.0 to 108.9% for **I** and 88.0 to 109.2% for **II**. The data is summarized in Table 1.

The precision and accuracy of intraday QC's were determined by analyzing QC samples at low, medium, and high concentrations. Table 2 summarizes the means, precision, and accuracy. The precision (%CV) was $\leq 4.7\%$ for **I** and 9.3% for **II** with accuracy range (percentage of nominal value) of 91.4–103.9% for **I** and 90.0–96.3% for **II** over three QC concentrations.

3.3. Specificity and matrix effect

Human control plasma and urine from five different sources were extracted with and without internal standard to assess the specificity of the method. The conditions specified in the methods were found to be selective for **I**, **II**, and **III**. Interferences from any endogenous compounds in control plasma or urine were negligible at the retention times of **I**, **II**, and **III**. Results for plasma and urine are demonstrated in representative chromatograms for blank control samples (A), control spiked with internal standard (B), and control spiked with analytes (C) in Figs. 3 and 4.

In the previous LC–MS/MS method published, the investigators attribute lower recoveries to suppression at the ion source; however, the matrix effect was not evaluated [14]. For a method to be

Table 1
Intraday reproducibility for **I** and **II** standards in human plasma and urine

Nominal concentration (ng/ml)	**I**			**II**		
	Calculated[a] concentration (ng/ml)	Precision[b]	Accuracy[c]	Calculated[a] concentration (ng/ml)	Precision[b]	Accuracy[c]
In human plasma						
1	0.9	2.0	90.0	0.9	7.7	90.0
2	2.0	4.0	100.0	1.9	5.5	95.0
10	10.5	3.9	105.0	10.7	4.0	107.0
20	20.7	4.8	103.5	20.8	4.6	104.0
100	104.2	5.5	104.2	104.0	5.2	104.0
250	257.6	3.7	103.0	258.2	3.4	103.3
500	487.2	0.8	97.4	486.6	2.1	97.3
In human urine						
2	1.7	5.5	87.0	1.8	5.7	88.0
5	5.0	6.7	100.0	4.8	8.8	96.8
10	9.9	1.7	99.4	9.9	4.7	99.2
50	54.5	1.7	108.9	54.6	1.2	109.2
100	104.3	3.0	104.3	105.6	5.2	105.6
500	513.9	3.8	102.8	518.9	5.3	103.8
1000	977.7	1.8	97.8	971.5	4.9	97.1

[a] Calculated concentrations are the averages of $n = 5$ standards in daily run. Regression results are calculated using all 35 standards. The plasma calibration equation for **I**: $y = 0.1613x + 0.027$, $r = 0.9992$. **II**: $y = 0.1151x + 0.027$, $r = 0.9991$. The urine calibration equation for **I**: $y = 0.0086x + 0.0048$, $r = 0.9992$. **II**: $y = 0.0041x + 0.0023$, $r = 0.9983$.
[b] Expressed as %CV.
[c] Accuracy = (mean calculated concentration)/(nominal concentration) × 100%.

reproducible and accurate in clinical applications, the changes in the ionization of analytes at the ion source need to be minimized and consistent over varying sources of plasma or urine. Therefore, in this report, the effect on ionization efficiencies for **I** and **II** were determined and minimized through method development. Two groups of samples were prepared by spiking the same amounts of **I** and **II**

Table 2
Intraday reproducibility of human plasma and urine QCs

Nominal concentration (ng/ml)	**I**			**II**		
	Calculated[a] concentration (ng/ml)	Precision[b]	Accuracy[c]	Calculated[a] concentration (ng/ml)	Precision[b]	Accuracy[c]
In human plasma						
400	409.0	3.0	102.3	372.6	3.3	93.2
40	40.8	3.3	102.0	38.6	3.5	96.5
1.5	1.5	3.8	100.0	1.4	7.8	93.3
In human urine						
900	822.3	3.17	91.4	848.0	1.93	94.2
120	124.7	4.01	103.9	115.6	8.91	96.3
6.0	5.9	4.72	98.0	5.4	9.26	90.0

[a] Calculated concentrations are the averages of $n = 5$ individual determinations in the daily run.
[b] Expressed as %CV.
[c] Accuracy = (mean calculated concentration)/(nominal concentration) × 100%.

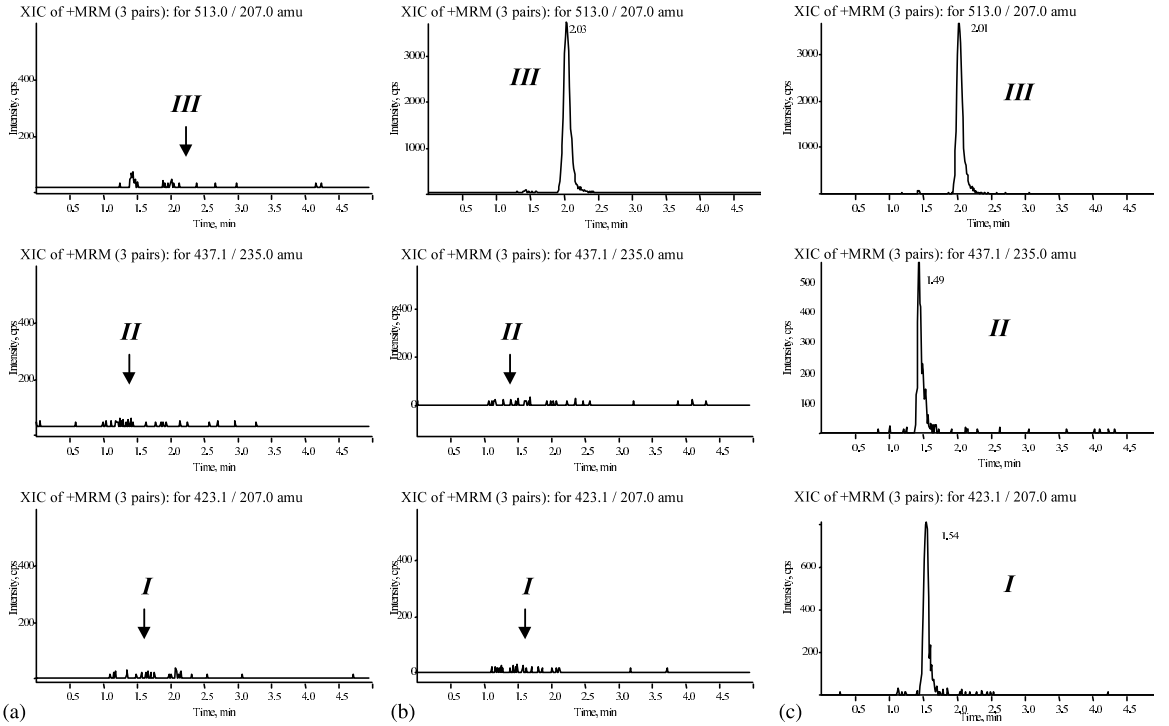


Fig. 3. Representative chromatograms for **I** and **II** in human plasma. (a) Blank control plasma, (b) control plasma spikes with **III** (2.5 ng/ml), and (c) control plasma spiked with **I** and **II** at LOQ (1 ng/ml) and **III** (2.5 ng/ml).

into (1) control plasma or urine extracts and (2) reconstitution solvent. The samples were prepared in replicate ($n = 3$) at three different concentrations of **I** and **II**. The samples were analyzed and chromatographic peak areas of **I** and **II** from these two groups of samples were compared.

For the plasma method, the average matrix effect was −15% for **I** and +11% for **II**. This indicates some suppression of **I** was observed while the ionization efficiency of **II** was enhanced. The analytes were monitored on separate precursor–product ion channels and the specificity of the method including internal standard was established so cross-talk between channels should not be occurring. The increase and decrease of efficiencies reported could be attributed to co-eluting species, which were not removed in extraction. Plasma samples from different sources produced matrix effect at similar levels. The net result was that accurate quantification could still be achieved even with the presence of the minor matrix effect under our experimental conditions.

For the urine method, no significant matrix effect was observed. Average suppression was −1.1% for **I** and −2.4% for **II**.

3.4. Recovery

In the SPE and LC–MS/MS detection method reported [14], recovery was 64.7–80.5% for **I**, 52.2–70.2% for **II**. In our laboratories, a liquid–liquid extraction method had recoveries of greater than 90% for **I** and **II** in plasma and urine [6]. When developing this LC–MS/MS method, the liquid–liquid extraction was modified to (1) extract from smaller sample volume and (2) keep solvents and buffers compatible with MS detector. When optimizing the liquid–liquid extraction, both recovery and matrix effect were evaluated.

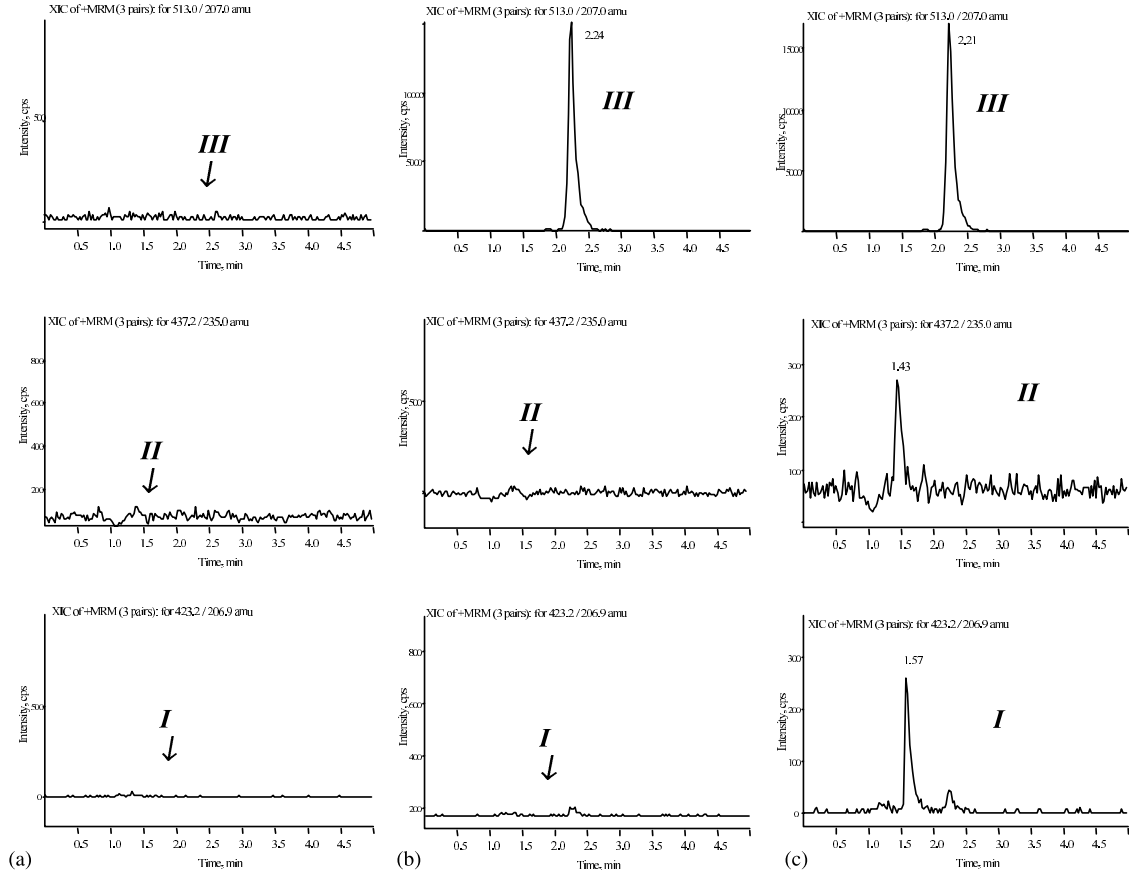


Fig. 4. Representative chromatograms for **I** and **II** in human urine. (a) Blank control urine, (b) control urine spikes with **III** (50 ng/ml), and (c) control urine spiked with **I** and **II** at LOQ (2 ng/ml) and **III** (2.5 ng/ml).

Extraction recoveries for plasma and urine methods were determined by analyzing extracts of standard replicates ($n = 3$) at three different concentrations of **I** and **II**. The extracted samples were compared with neat standard solution spiked in the extracted blank control plasma at the same concentrations. Results were calculated by comparing mean peak areas of the extracted samples with the mean peak areas for the corresponding spike-after-extraction samples. The plasma method had an overall mean recovery of 87.3% for **I**, 104.7% for **II**, and 80.3% for **III**. The urine method had an overall mean recovery of 92.3% for **I**, 91.6% for **II**, and 68.2% for **III**. Precision (%CV) of recovery of **I**–**III** from plasma was $\leq 3.0\%$ and urine was $\leq 8.3\%$. The liquid–liquid extraction from plasma has higher recoveries than the SPE method published. The high recovery contributes to the sensitivity of the method.

3.5. Sample stabilities

Clinical plasma and urine samples were stored at $-20\,^{\circ}C$. Freeze–thaw stability was evaluated by freeze–thawing QC samples for three cycles which consists of thawing the QC samples for 4 h at room temperature and re-freezing at $-20\,^{\circ}C$. In Table 3, accuracy indicates that both **I** and **II** are stable in human plasma and urine after three freeze–thaw cycles.

Stability of analytes in human plasma and urine at room temperature for 24 h was demonstrated by

Table 3
Stability[a] of **I** and **II** QCs in human plasma and urine at varying conditions

	I			**II**		
	High	Med	Low	High	Med	Low
In human plasma						
Nominal concentration (ng/ml)	400	40	1.5	400	40	1.5
Three freeze–thaw cycles ($n=3$)	88.7	90.4	86.7	98.2	93.3	100.0
24 h room temperature ($n=3$)	91.4	94.4	87.6	106.5	105.7	85.7
48 h room temperature autosampler ($n=3$)	90.8	96.0	94.0	96.1	100.3	100.4
In human urine						
Nominal concentration (ng/ml)	900	120	6.0	900	120	6.0
Three freeze–thaw cycles ($n=4$)	105.0	99.3	101.7	97.9	109.0	111.1
24 h room temperature ($n=4$)	108.1	108.5	111.9	108.1	113.6	105.6
24 h room temperature autosampler ($n=3$)	102.9	101.2	96.6	95.5	101.8	103.7

[a] Stability was determined by calculating accuracy of QC values under experimental conditions as compared with nominal QC values.

accuracy in Table 3. Also, stability of analytes in the reconstitution solution on the autosampler at room temperature was demonstrated for 48 h for plasma method and 24 h for urine method. Therefore, reinjection of samples could be done up to 48 h for plasma method and 24 h for urine method from time of extraction.

Finally, long term stability of plasma and urine samples at $-20\,^{\circ}C$ is demonstrated in Table 4. Table 4 summarizes QC interday mean, precision, and accuracy over the clinical studies. For plasma assay, accuracy of plasma QC over three concentrations indicate stability for at least 21 months. For the urine assay, accuracy of urine QC over three concentrations indicate stability for at least 12 months.

3.6. Clinical applications

Both the plasma method and the urine method have been applied successfully to the analysis of samples from pediatric clinical studies, demonstrating the reproducibility and robustness of the method.

Interday precision and accuracy of the plasma method for the clinical studies were determined by analyzing QC samples at low, medium, and high concentrations. The QCs were prepared before the analysis of the study samples and were analyzed in replicate with the daily study samples. Table 4 demonstrates the means, precision, and accuracy for QCs calculated at the completion of two studies, which utilized the current method. The precision for study 1 (%CV, $n=10$ daily runs) was less than 9.6% for **I** and 5.8% for **II** with accuracy range (percentage of nominal value) of 93.5–100.0% for **I** and 89.8–100.0% for **II** over three QC concentrations. The precision for study 2 (%CV, $n=8$ daily runs) was less than 9.0% for **I** and 12.4% for **II** with accuracy range (percentage of nominal value) of 98.3–104.2% for **I** and 86.4–98.3% for **II**.

Interday precision and accuracy of urine method for the clinical studies was determined the same as described for the plasma method. Table 4 demonstrates the means, precision, and accuracy for QCs calculated at the completion of one study, which utilized the urine method. The precision (%CV, $n=7$ daily runs) was less than 8.4% for **I** and 7.1% for **II** with accuracy range (percentage of nominal value) of 92.5–103.8% for **I** and 92.4–103.1% for **II** over three QC concentrations.

Fig. 5 is a representative plasma concentration–time profile of **I** and **II** in a 4-year old hypertensive patient following seven daily doses of 0.71 mg/kg

Table 4
Interday Reproducibility of Human Plasma and Urine QCs

	Nominal concentration (ng/ml)	I			II		
		Calculated concentration (ng/ml)	Precision[a]	Accuracy[b]	Calculated concentration (ng/ml)	Precision[a]	Accuracy[b]
In human plasma							
Study 1 ($n = 10$ runs)[c]	400	383.7	5.0	95.9	388.0	5.6	97.0
	40	37.4	4.7	93.5	37.9	4.2	94.8
	1.5	1.5	9.6	100.0	1.5	5.8	100.0
Study 2 ($n = 8$ runs)[d]	400	403.7	4.4	100.9	359.4	2.4	89.8
	40	39.3	5.1	98.3	34.6	5.4	86.4
	1.5	1.6	9.0	104.2	1.5	12.4	98.3
In human urine							
Study 2 ($n = 7$ runs)[d]	900	832.3	6.7	92.5	831.3	7.1	92.4
	120	121.4	8.4	101.2	120.7	7.1	100.6
	6.0	6.2	8.3	103.8	6.2	5.8	103.1

[a] Expressed as %CV.
[b] Accuracy = (mean calculated concentration)/(nominal concentration) × 100%.
[c] Daily QC values are the averages of $n = 2$ or 3 individual determinations in the daily run.
[d] Daily QC values are the averages of $n = 2$ individual determinations in the daily run.

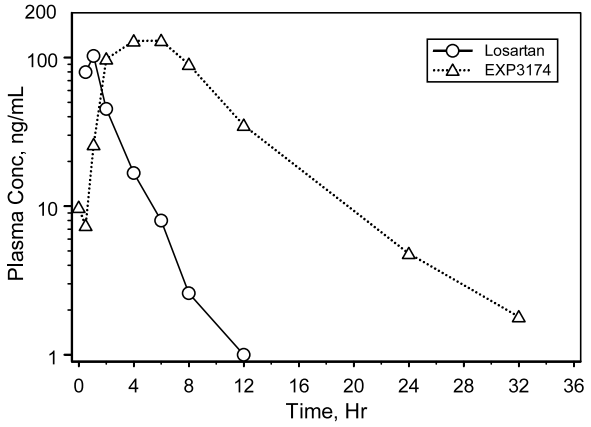


Fig. 5. Plasma concentration–time profile of **I** and **II** on day 7 in a 4-year-old hypertensive patient following seven daily doses of 0.71 mg/kg daily doses of losartan potassium.

of losartan potassium. Urinary recovery on day 7 was 1.3 and 2.72% of the dose as **I** and **II**, respectively.

4. Conclusions

The bioanalytical methods to accurately determine the concentrations of compounds **I** and **II** from human plasma and urine with limited plasma sample size were developed and validated. The plasma method has a LOQ of 1 ng/ml using only 0.1 ml of plasma and a linear range of 1–500 ng/ml for both compounds. The method lowers the volume of plasma needed for extraction by increasing the sensitivity and recovery while minimizing matrix effects. The urine method is the first to be reported using LC–MS/MS detection. The LOQ is 2 ng/ml and linear range is 2–1000 ng/ml for both compounds. The methods have been proven fast, specific, accurate, and sensitive. Both methods have been successfully applied to clinical sample analysis and produced satisfactory results which demonstrates that the methods are reproducible and robust.

References

[1] P.B.M.W.M. Timmermans, P.C. Wong, A.T. Chiu, et al., Pharmacol. Rev. 45 (1993) 205–251.
[2] A.H. Gradman, K.E. Arcuri, A.I. Goldberg, L.S. Ikeda, E.B. Nelson, D.B. Snavely, C.S. Sweet, Hypertension 25 (1995) 1345–1350.
[3] P.C. Wong, W.A. Price, A.T. Chiu, J.V. Duncia, D.J. Carini, R.R. Wexler, A.L. Johnson, P.B.M.W.M. Timmermans, J. Pharmacol. Exp. Ther. 255 (1990) 211–217.
[4] T. Tamaki, A. Nishiyama, S. Kimura, Y. Aki, M. Yoshizumi, H. Houchi, K. Morita, Y. Abe, Cardiovasc. Drug Rev. 15 (1997) 122–136.
[5] M.W. Lo, M.R. Goldberg, J.B. McCrea, H. Lu, C.I. Furtek, T.D. Bjornsson, Clin. Pharmacol. Ther. 58 (1995) 641–649.
[6] C.I. Furtek, M.W. Lo, J. Chromatogr. 573 (1992) 295–301.
[7] H. Lee, H.O. Shim, H.S. Lee, Chromatographia 42 (1996) 39–42.
[8] A. Soldener, H. Spahn-Langguth, E. Mutshler, J. Pharm. Biomed. Anal. 16 (1998) 863–873.
[9] D. Farthing, D. Sica, I. Fakhry, A. Pedro, T.W.B. Gehr, J. Chromatogr. B 704 (1997) 374–378.
[10] M.A. Ritter, C.I. Furtek, M.W. Lo, J. Pharm. Biomed. Anal. 15 (1997) 1021–1029.
[11] E. Cagigal, L. Gonzalez, R.M. Alonso, R.M. Jimenez, Talanta 54 (2001) 1121–1133.
[12] L. Gonzalez, J.A. Lopez, R.M. Alonso, R.M. Jimenez, J. Chromatogr. A 949 (2002) 49–60.
[13] K.M. Williamson, J.H. Patterson, R.H. McQueen, K.F. Adams, Jr, J.A. Pieper, Clin. Pharmacol. Ther. 63 (1998) 316–323.
[14] T. Iwasa, T. Takano, K. Hara, T. Kamei, J. Chromatogr. B 734 (1999) 325–330.
[15] D.L. Buhrman, P.I. Price, P.J. Rudewicz, J. Am. Soc. Mass Spectrom. 7 (1996) 1099–1105.
[16] I. Fu, E.J. Woolf, B.K. Matuszewski, J. Pharma. Biomed. Anal. 18 (1998) 347–357.
[17] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 70 (1998) 882–889.