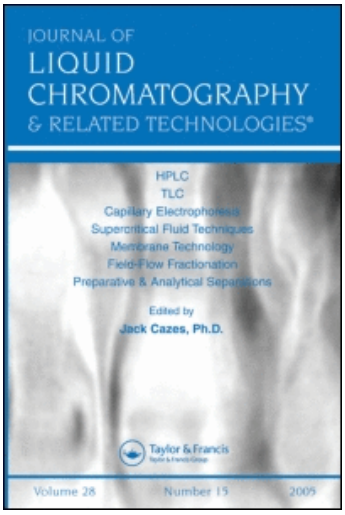

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Determination of Losartan in Human Plasma by Liquid Chromatography Electrospray Ionization Mass Spectrometry (LC-ESI-MS): Application to Bioequivalence Study

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Abstract: A sensitive, simple, rapid, and specific liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) method was developed and validated for the identification and quantification of losartan in a small volume of human plasma. Losartan and I.S. were successfully separated on a CN column with a mobile phase of acetonitrile-0.2% formic acid solution (68:32, v/v). Detection was performed on a single quadrupole mass spectrometer by a selected ion monitoring (SIM) mode via electrospray ionization (ESI) source. The ESI source was set at positive ionization mode. The ion signal of m/z 422.79 and 194.81 were measured for losartan and I.S., respectively. The limit of detection (LOD) was 0.5 ng/mL (signal-to-noise ratio of 10.03) using only 200 μL of human plasma samples. The calibration curve was an excellent linear fit over the range of concentrations 1.0–1000 ng/mL ($R^2 = 0.9987$) of losartan in human plasma. Consequently, all of our results fulfilled the common standard criteria of bioequivalence, 0.80 to 1.25 by the Korean and US Food and Drug Administration. In addition to the confidence intervals (C.I.) 90% of the pharmaceutical parameters, a two-way ANOVA showed no significant difference

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between the two formulations. This method was successfully applied to bioequivalence study of two brands of losartan potassium tablet (100 mg) formulations after a single oral administration.

Keywords: Bioequivalence, LC-ESI-MS, Losartan

INTRODUCTION

Losartan (2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[(2′-(1*H*-tetrazol-5-yl) biphenyl-4-yl)methyl]imidazole, potassium salt) is an orally active selective angiotensin II receptor antagonist employed to treat hypertension high blood pressure.[1] Losartan, which is in the pharmaceutical market in the form of tablets, has been demonstrated to be superior to previous peptide receptor antagonists and angiotensin converting enzyme (ACE) inhibitors due to its enhanced specificity, selectivity, and tolerability.[1,2] Losartan also shows a gradual and long lasting effect, becoming an alternative to hypertension treatment. Losartan potassium is a light yellowish solid with molecular weight, 461; melting point, 183.5–184.5°C; soluble in water (3.3 mg L^{-1} at pH 7.8); pK_a value, 4.9.[3]

Several methods have been reported for the quantitative determination of losartan potassium in tablets. These methods employ techniques such as high performance liquid chromatography (HPLC), super fluid chromatography (SFC), capillary electrophoresis (CE), and high performance thin layer chromatography (HPTLC).[4] Among them, the HPLC method has been the commonly used technique for the determination of losartan in biological fluids as well as in formulations. Reported HPLC methods were mostly based on UV detection[5–8] and fluorescence detection.[9,10] However, these methods have some analytical disadvantages including low sensitivity, need for a large volume of plasma due to inherent sensitivity, complicated preparation method, and long run time for analysis. In order to completely evaluate the pharmacokinetics of losartan in human plasma to support a bioequivalence study, it is necessary to develop and validate a rapid and reliable assay with sensitivity, selectivity, accuracy, and precision. Since a potentially large number of samples are used in bioequivalence studies, an ideal method should have simple preparation, fast on-column separation, and sensitive and specific detection. Liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) is an analytical tool which meets most of these needs. Although LC-MS/MS MS methods have been reported for the determination of losartan, it is too expensive to use for the routine measurements in the preclinical laboratory.[11,12]

The aim of this study was to develop a highly specific LC-ESI-MS method, which is as sensitive as LC-MS/MS for the determi-

nation of losartan in human plasma. In this study, losartan was only analyzed because the analysis of metabolites is not required in bioequivalence studies. This analytical method was applied to determine the level of losartan in plasma following a single dose in human healthy volunteers.

EXPERIMENTAL

Chemicals and Materials

Losartan potassium and butylparaben as an internal standard (I.S.) were purchased from IPCA (Mumbai, India) and San Fu Chemical Co. Ltd. (Taipei, Taiwan), respectively. HPLC grade acetonitrile was purchased from J.T. Baker (Phillipsburg, NJ, USA) and formic acid was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Water was purified by a Milli-Q system (Millipore Corp., Bedford, MA, USA). All other chemicals were analytical grade and used without further purification.

Instruments and Operating Conditions

The LC-ESI-MS analysis was performed using a waters Micromass ZQ 2000 spectrometer, coupled to a Waters 2690 separations module with an autosampler and MassLynx 4.0 data processor (Milford, MA, USA).

The mass spectrometer was operated in positive ionization mode with selected ion monitoring (SIM). The nebulizer gas was supplied from a high purity nitrogen source. The source and desolvation temperature were set at 150°C and 200°C, respectively; and the voltages of the capillary and cone were 3.2 kV and 20 V, respectively. The gas flow of desolvation and cone were set at 300 L/hr and 50 L/hr, respectively. The chromatographic separation was performed on Phenomenex Luna CN column (150 × 3.0 mm i.d., 5.0 μm particles size) at 40°C. The mobile phase, consisting of ACN – 0.2% formic acid solution (68:32, v/v), was delivered at a flow late of 0.6 mL/min. The $[M + H]^+$, m/z 422.79 for losartan and $[M + H]^+$, m/z 194.81 for I.S., were selected as detecting ions, respectively.

Preparation of Standard Solutions

A stock solution of losartan (100 μg/mL) and I.S. (1.0 mg/mL) were prepared by dissolving in methanol and then storing frozen. Standard

solutions of losartan in human plasma for the calibration curve were prepared by spiking the appropriate volumes of diluted stock solutions, giving trial concentrations of 1, 5, 100, 200, 500, and 1000 ng/mL.

Preparation of Plasma Samples

Each 200 μL of human plasma samples was precipitated with 600 μL absolute ethanol in microcentrifuge tubes after addition of 20 μL I.S. solution (100 μg/mL). The samples were vortexed for 10 min and the mixtures were centrifuged at 10000 × g for 15 min at 4°C. Clear supernatants were then transferred to test tubes and dried under a steam of nitrogen gas at 60°C. Then, 200 μL of mobile phase was added to dissolve the residue, and 10 μL of aliquot was automatically injected into the HPLC-ESI-MS system for analysis.

Validation of Assay Method

The chromatographic method was validated based on five repetitions for five different days to determine specificity, linearity, precision, and accuracy of the HPLC-ESI-MS method.

Specificity

Specificity was assessed by the examination of peak interference from endogenous substances. Drug free human plasma was tested for interference using the proposed preparation procedures and chromatographic/spectroscopic conditions, and the chromatograms were compared with those obtained from plasma sample spiked with I.S. and losartan.

Linearity

The linearity was determined by the calibration curve at six concentrations of losartan: 1, 5, 100, 200, 500, and 1000 ng/mL in plasma samples. The ratio of losartan peak area to I.S. peak area was plotted vs. losartan concentration in ng/mL and the calibration curve was obtained by linear regression.

Sensitivity

The limit of detection (LOD) was defined as the lowest amount of analyte in the plasma samples with the peak of at least five times signal-to-noise ratio but not necessarily quantified. The limit of quantification (LOQ)

was defined as the lowest concentration at which the precision was lower than 20%, the accuracy was within 80–120%, and the signal-to-noise ratio was higher than 10.

Precision and Accuracy

The intra- and inter-day precisions expressed as coefficients of variation (CV%) and inter-day accuracies, expressed as a percentage of the measured concentration to the theoretical concentration, were determined by the analysis of plasma samples spiked at 1, 5, 500, and 1000 ng/mL. The intra-day precisions were determined by analyzing five replicates on the same day. The inter-day precisions and accuracies were determined by analyzing five calibration curves on five different days.

Recovery

The absolute recoveries of losartan from human plasma were performed at low, medium, and high concentration in three replicates (1, 500, and 1000 ng/mL). This was established by comparing absolute responses of losartan and I.S. in human plasma with those in mobile solutions, which represent 100% recoveries.

Bioequivalence Study

The validated method was applied to evaluate the bioequivalence of losartan after an oral dose of 100 mg losartan. Two brands of 100 mg losartan tablets, Ozaltan® (Lot number: LOSA 100-BE-001, Hanmi Pharmaceutical Co. Ltd, Korea; test formulation) and a commercially available reference formulation (Lot number: 05135, Merck Inc., USA), were used in this study.

Ethics

Before initiating our trial, the study protocol was approved by the KFDA (Korea Food and Drug Administration). After approval, 24 volunteers were recruited, informed about the test, and asked to sign a consent form. The trial followed the Helsinki Declaration and guideline for the bioequivalence test of the KFDA.[13]

Subjects

Twenty four healthy male volunteers were selected for this study after clinically assessing their health statuses evaluation (physical examination,

electro cardiograph) and hematology, biochemistry, electrolytes, and urinalysis testing. Subjects with a history of drug allergies or idiosyncrasies, renal or hepatic impairment, or drug or alcohol abuse were excluded. Subjects who had used medications of any kind within 2 weeks of the start or during the study were also excluded. The volunteers had the following clinical characteristics (expressed as means ± S.D. (range)): age, 26.0 ± 2.0 years (23–30); height, 176.4 ± 3.7 cm (172–185); body weight, 69.0 ± 7.9 kg (58–86).

Drug Administration

Subjects were advised not to take any medication for 2 weeks before the study and were requested to fast for at least 10 hr overnight the day before each treatment. The study was based on a single dose, randomized, two treatment, two period crossover design. During phase 1 period, after an overnight fast, a catheter was introduced in forearm vein and a predosing blood sample was collected. Twelve volunteers were administered a single dose of test tablets and the other 12 volunteers were administered a single dose of reference tablets. The drug was administered with 240 mL of water and the volunteers were then fasted for 4 hr. A standard lunch and an evening meal were provided at 4 and 8 hr after drug administration in each treatment. After a washout period of 2 weeks, the study was repeated in the same manner (phase 2) to complete the crossover design.

Blood Sampling

Heparinized blood samples (10 mL) were collected from the forearm vein according to the time schedule, which included a blank before drug administration and then at 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10, and 12 hr post dosing. The blood samples were centrifuged at $3000 \times g$ for 10 min. Following centrifugation, plasma samples were stored at $-70^{\circ}C$ until the analysis was performed.

Pharmacokinetic Data

Pharmacokinetic analysis was performed by the BA Calc 2002® program (Seoul National University, Korea), a pharmacokinetic data analysis program. The following pharmacokinetic parameters were assessed for the period of 0–12 hr: the area under the plasma concentration time curves from time zero to the last measurable losartan sample time (AUC_{0-12hr}), the area under the plasma concentration time curves from time zero to infinity ($AUC_{0-\infty}$), the maximum plasma concentration (C_{max}), and the

time to reach maximum plasma concentration (T_{max}) were obtained directly from each volunteer's plasma concentration time curve data by visual inspection. The elimination rate constant (K_e) was obtained as the slope of the linear regression of the log transformed concentration time curve data in the terminal phase. The half-life ($T_{1/2}$) was calculated from 1n2 divided by K_e.

Statistical Analysis

For the purpose of bioequivalence analysis a two way ANOVA was performed with the K-BE Test 2002® program (Seoul National University, Korea) at a significant level of 0.05. Utilizing the program, variances related to sequence, subject, period, and formulation were analyzed. Bioequivalence of the test treatment to the reference treatment was assessed on the basis of the confidence intervals (C.I.) for the "test/reference" mean ratios of these raw variables in relation to the bioequivalence range of 80–125% for the raw data.

RESULTS AND DISCUSSION

Selectivity and Specificity

High selectivity was found for the determination of drugs in plasma samples. According to the mass scan spectrum, m/z 422.79 produced by the quasimolecule ion $[M+H]^+$of losartan and m/z 194.81 produced by the quasimolecule ion $[M+H]^+$ of I.S. were selected for monitoring. The selected ion monitoring (SIM) (+) chromatograms of blank plasma and spiked plasma samples (1 ng/mL) are shown in Figure 1. The chromatographic run time was 10 min for the plasma sample analysis and the retention times of losartan and I.S. were 3.76 min and 3.51 min, min, respectively. There was no interference peak for endogenous components at the retention time of the analytes. No ion suppression effects were observed under the developed sample preparation and chromatographic conditions.

Linearity

The calibration curve was linear in the validated range. The mean equation of the calibration curve, indicating six points, was $y = 0.0063 \times -0.0050$ with the correlation coefficient as $r^2 = 0.9987$, where y represents the peak area ratio of losartan and I.S., and $\times$ represents the losartan concentrations in ng/mL.

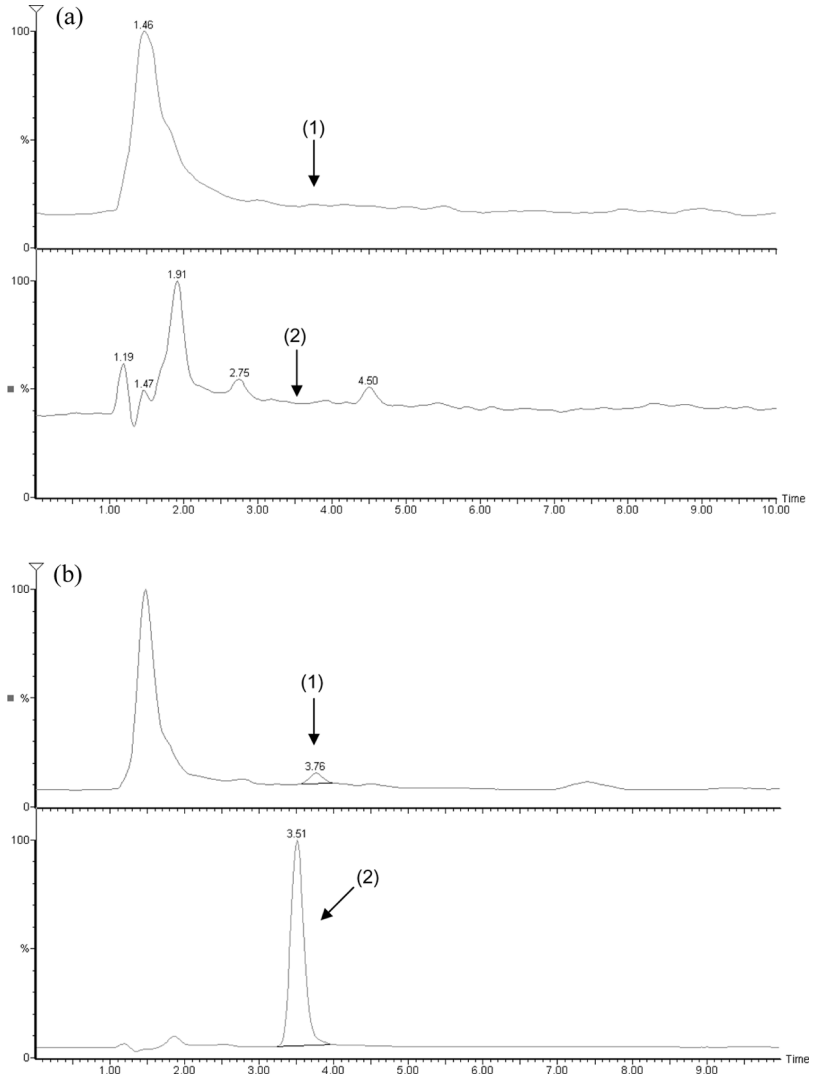

Figure 1. Representative chromatograms of (a) human blank plasma and (b) human blank plasma spiked with 1 ng/mL (LOQ) of losartan and butylparaben as the internal standard (10 μg/mL). Peaks (1) and (2) represent losartan and butylparaben (internal standard), respectively.

Sensitivity

Sensitivity was determined in terms of limit of quantification (LOQ), which was taken as the lowest concentration in the calibration

Table 1. Precisions and accuracies of losartan in human plasma (n = 5)

Losartan concentration (ng/mL)	Precision (CV%)[a]		Accuracy[b]
	Intra-day (n = 5)	Inter-day (n = 5)	
1 (LOQ)	4.79	4.90	99.13 ± 10.69
5	11.36	6.76	89.52 ± 3.36
500	1.35	1.91	105.96 ± 1.66
1000	3.20	2.95	102.07 ± 3.81

[a]Coefficient of variation of peak area ratio of losartan to I.S. (butylparaben).
[b]Data are expressed as [(actual concentration)/(theoretical concentration)] × 100.

range. The LOQ of 1 ng/mL was the same value that was obtained by LC-MS-MS using solid phase extraction.[11] The LOQ was estimated to be 1 ng/mL as shown in Table 1 and Figure 1 (b). The limit of detection (LOD) of the LC-ESI-MS method was approximately 0.5 ng/mL (signal-to-noise ratio of 10.03). In this study, the LC-ESI-MS method, which was not complicated as LC-MS-MS, was used and samples were prepared by simple protein precipitation. Only 200 μL of plasma samples were used in the developed LC-ESI-MS method, whereas 400 μL of plasma samples were used in the LC-MS-MS method.[11] It could be postulated that the developed LC-ESI-MS method was two fold more sensitive than the LC-MS-MS method. The minimum sample requirement and low LOQ value are especially beneficial when analyzing plasma from small animals where only a small volume of blood can usually be collected. This method was sufficiently sensitive for the analysis of losartan in human plasma.

Precision and Accuracy

Assessment of the intra- and inter-day variability of the assay was conducted in five different lots of human plasma spiked with losartan over the calibration range of 1–1000 ng/mL. The intra- and inter-day precision and accuracy were determined by analyzing plasma samples

Table 2. Recovery of losartan and butylparaben from human plasma (n = 3)

	Concentration (ng/mL)	Recovery (Mean ± S.D.)
Losartan	1	91.69 ± 1.75
	500	98.57 ± 0.65
	1000	97.8 ± 1.02
Butylparaben	10000	97.38 ± 1.24

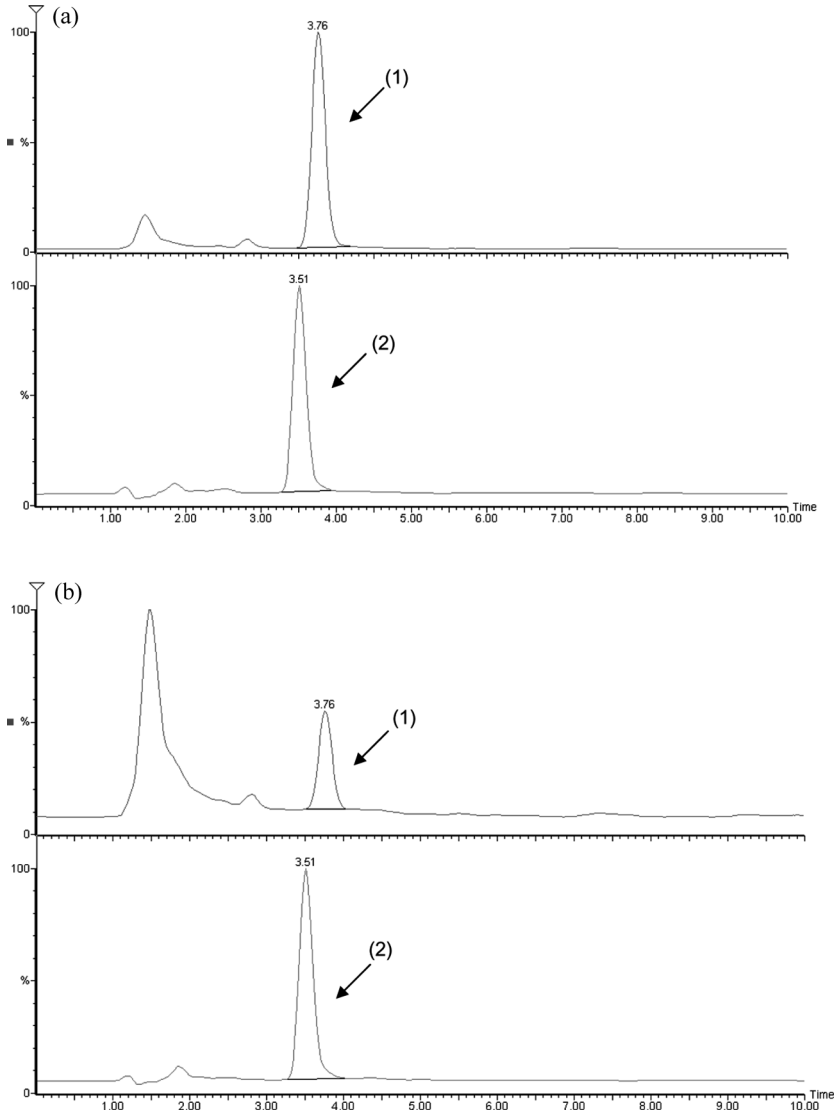

Figure 2. Representative chromatograms of plasma sample after (a) 2 hr and (b) 6 hr following a single oral administration of 100 mg of losartan in tablet form. Peaks (1) and (2) represent losartan and butylparaben (internal standard), respectively.

at the concentrations of 1, 5, 500, and 1000 ng/mL. The results of intra- and inter-day precision and accuracies for losartan in human plasma are presented in Table 1. The R.S.D. of losartan ranged from 1.35 to

11.36% for intra-day and from 1.91 to 6.76% for inter-day, respectively. The accuracy of losartan was expressed as a percentage of the measured concentration to the theoretical concentration ranged from 89.52 to 105.96%.

Recovery

The absolute recoveries of losartan and I.S. were presented in Table 2. The average absolute recovery values of losartan were approximately higher than 98% at low, medium, and high losartan concentrations. The mean recovery for I.S. was 97.38%. The sufficient recoveries were achieved to perform bioavailability studies.

Bioequivalence of Losartan

The developed LC-ESI-MS method was applied to the analysis of losartan in human plasma after the single oral administration of two losartan tablets with the dose of 100 mg in 24 healthy male volunteers. Plasma chromatograms of volunteer administered losartan are illustrated in Figure 2, which represents the typical chromatograms of losartan in human plasma at 2 hr (a) and 6 hr (b) after the administration in 24 healthy male volunteers.

The mean plasma concentration time profiles of losartan after a single oral dose of 100 mg of each tablet formulation are shown in Figure 3. The pharmacokinetic parameters of the two losartan formulations are shown in Table 3. Mean maximum concentrations of the test and reference were found to be 592.11 ± 189.08 ng/mL and 573.20 ± 202.36 ng/mL, respectively. The 90% C.I. of C_{max} was 0.95 to 1.14, which was within the acceptable criterion of 0.8 to 1.25. As for the $AUC_{0-12\,hr}$, test values were 875.51 ± 455.11 ng · hr/mL and the reference values were 806.71 ± 358.09 ng · hr/mL. The 90% C.I. of

Table 3. Pharmacokinetic parameters of losartan in human plasma of 24 healthy volunteers after an oral administration of 100 mg losartan

Parameters	Test	Reference
AUC_{0-12h} (ng · hr/mL)	875.51 ± 455.11	806.71 ± 358.09
$AUC_{0-\infty}$ (ng · hr/mL)	885.77 ± 461.83	820.09 ± 371.09
C_{max} (ng/mL)	592.11 ± 189.08	573.20 ± 202.36
T_{max} (hr)	1.10 ± 0.42	1.05 ± 0.45
K_e (hr^{-1})	0.34 ± 0.12	0.36 ± 0.14
$T_{1/2}$ (hr)	2.36 ± 1.22	2.42 ± 1.23

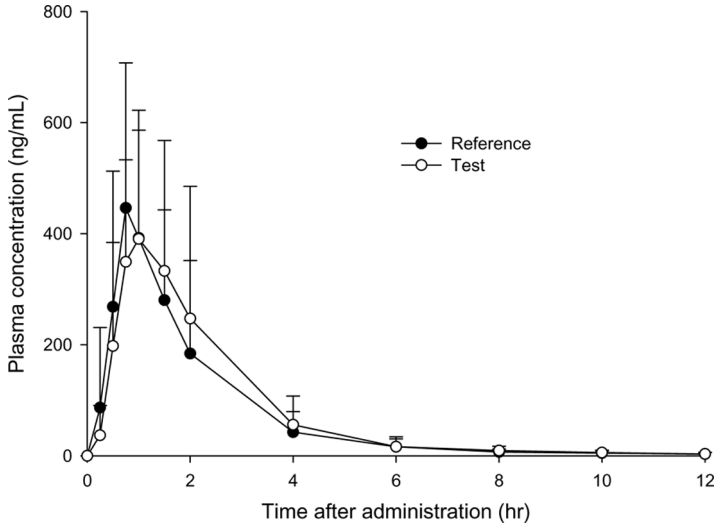

Figure 3. Plasma concentration time profiles of losartan following a single oral administration of 100 mg of test and reference losartan in tablet form in 24 healthy male volunteers. The results represent the mean value (n = 24).

$AUC_{0-12\,hr}$ was 0.96 to 1.15, which was within the acceptable criterion of 0.8 to 1.25 by the Korean and US Food and Drug Administration. From these results, the two losartan tablet formulations are bioequivalent for both the rate and the extent of absorption. Furthermore, it might be suggested that the developed LC-ESI-MS method could be applied to the routine determination of losartan in biological fluid.

CONCLUSION

We have established a sensitive, simple, rapid, and specific analytical method for the determination of losartan in human plasma using the liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) method. This analytical method has been successfully used to provide the bioequivalent study of losartan in human plasma. Moreover, it is suggested that the developed LC-ESI-MS method can be applied to routinely monitor the concentration of losartan.

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