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Determination of Losartan and Losartan Carboxylic acid in Human Plasma by New HPLC Method with Fluorescence Detection for Pharmacokinetics Studies

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Abstract:

Losartan is highly effective blood pressure lowering agent who directly works by selectively blocking angiotensin-I (AT-I) receptor. It is primarily metabolized in active form, losartan carboxylic acid. A sensitive and selective HPLC method was developed for determination of losartan and losartan carboxylic acid in human plasma was developed and validated. The drug and its metabolite were extracted from plasma by liquid-liquid extraction with t-Methyl butyl ether.

Valsartan was used as internal standard. A Waters Associates Inc. (Milford, MA, USA) Liquid chromatography was used to complete this study. The analytical apparatus consisted of a 600 quaternary pump and a fluorescence detector. Samples were applied to a repacked 5 µm, 250 x 4.6 mm CN column using auto sampler. Flow rate of 1.25 ml/min was found to give adequate resolution. Separations were performed at 35ºC and monitored at an excitation wavelength of 250nm and an emission wavelength of 370nm. Mobile phase was prepared by mixing 0.015M phosphoric acid (pH 2.3): acetonitrile in a ratio of 72:28. Linearity was established for the range of concentrations 2–300 ng/ml and 3–375 ng/ml for losartan and metabolite respectively. The lower limit of quantitation (LLOQ) was identifiable and reproducible at 2 ng/ml with a precision of 3.28% and at 3 ng/ml with a precision of 2.59% for the drug and metabolite respectively.

The inter batch, between days precision at 3 ng/ml level was found to be 8.07% for losartan. The inter batch, between days precision at 5 ng/ml level was found to be 4% for the metabolite. The precision and accuracy were established at low, medium and high concentration levels. The results were within the accepted limits. The analysis method was found to be sensitive, accurate, and precise for the quantification of losartan and its metabolite in human plasma. It was applied successfully, for pharmacokinetics studies.

Key words: *Losartan, HPLC, Human plasma, Losartan carboxylic acid.*

تحديد عقار اللوسارتان وحامض كاربوكسيلك اللوسارتان في البلازما البشرية بواسطة طريقة جديدة باستخدام جهاز تحليل كروماتوجرافي عالي الكفاءة مع كشف فلورنس للحركة الدوائية.

أحمد عباس حسين

فرع الصيدلانيات، كلية الصيدلة، جامعة بغداد

الخلاصة:

اللوسارتان عقار عالي الفعالية لخفض ضغط الدم الذي يعمل مباشرة من خلال منع انتقائي لمستقبلات أنجيوتنسين. يتم استقلابه أساسا على شكل نشط الى حامض كاربوكسيلك اللوسارتان. وقد تم تطوير طريقة تحليل كروماتاجرافي عالي الكفاءة، حساسة وانتقائية لتحديد اللوسارتان وحامض اللوسارتان كاربوكسيلك في البلازما البشرية وتم التحقق من صحتها.

تم استخلاص العقار ومستقلبه من البلازما عن طريق استخراج السائل السائل مع تي الميثيل بوتيل الأيثر، وقد استخدم فالسارتان كمعيار داخلي.

أجريت عملية الفصل في درجة حرارة 35 مئوية ورصدها على الطول الموجي 250 نانوميتر إثارة والطول الموجي انبعاث 370 نانوميتر. تم إعداد الطور المتحرك عن طريق خلط 0.015 مولاري حامض الفوسفوريك (الرقم الهيدروجيني 2.3): أسيتونتريل في نسبة 72:28. الرسم الخطي لمجموعة من التراكيز 2-300 نانوغرام/مل و3-375 نانوغرام/مل للوسارتان والمستقلب على التوالي. كان الحد الأدنى لتحديد الكميات هي 2 نانوغرام/مل مع دقة 3.28٪ وفي

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3 نانوغرام/مل مع دقة من 2.59٪ للعقار والمستقلب على التوالي. درجة الدقة لايام بين الوجبات في 3 مستوى نانوغرام/مل لتكون 8.07٪ للوسارتان، ودرجة الدقة لايام بين الوجباتفي مستوى 5 نانوغرام/مل أن يكون 4٪ للمستقلب. أشئت الدقة والانضباط في مستويات تركيز منخفضة ومتوسطة وعالية. كانت النتائج ضمن الحدود المقبولة.
لقد وجد ان الطريقة المستخدمة لتحليل حساسة ودقيقة، ودقيقة لتقدير حجم اللوسارتان ومستقلبه في البلازما البشرية وتم تطبيقها بنجاح لدراسة حركية العقار.

الكلمات المفتاحية: اللوسارتان، جهاز تحليل كروماتاجرافي عالي الكفاءة، البلازما البشرية، حامض كاربوكسيلك اللوزارتان

Introduction:

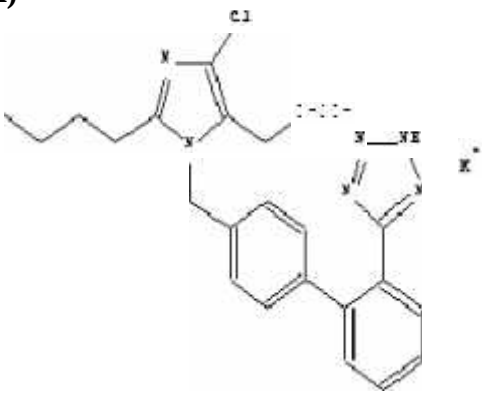
Losartan potassium, 2-butyl-4-chloro-1-[[2'- (1*H*-tetrazol-5-yl) [1, 1'-biphenyl]-4-yl] methyl]-1*H*imidazole- 5-methanol monopotassium salt (figure 1), is the first member of a new class of nonpeptide angiotensin II receptor antagonist [1]. Approximately 14% of an oral dose of losartan is converted to the 5-carboxylic acid metabolite EXP 3174 (figure-1), which is more potent than losartan as an AT1-receptor antagonist. The metabolism of losartan to EXP 3174 and to inactive metabolites is mediated by CYP2C9 and CYP3A4. Peak plasma levels of losartan and EXP 3174 occur 1–3 hours after oral administration, respectively, and the plasma half-lives are 2.5 and 9 hours, respectively [2].

The plasma clearances of losartan and EXP 3174 (600 and 50 mL/min, respectively) are due to renal clearance (75 and 25 mL/min, respectively) and hepatic clearance (metabolism and biliary excretion). The plasma clearance of losartan and EXP 3174 is affected by hepatic but not renal insufficiency. Losartan should be administered orally once or twice daily for a total daily dose of 25–100 m [3].

It reduces effectively hypertension by suppressing the effects of angiotensin II at its receptors, thereby blocking the renin-angiotensin system [4]. Losartan has been demonstrated to be superior to previous peptide receptor antagonists and angiotensin converting enzyme (ACE) inhibitors because of its enhanced specificity, selectivity, and tolerability[5]. Currently, losartan potassium is marketed alone or combined with hydrochlorothiazide.

Only a few chromatographic methods for the determination of losartan in nanograms /ml plasma levels have been described in the literatures [6-10]. The aim of the present study was to investigate and validate a reversed-phase HPLC method for losartan and its active metabolite determination in human plasma using valsartan (figure 1) as internal phase. This method was to have a limit of reliable quantification of at least 1ng/ml, in order to support clinical studies employing a single dose of 50 mg losartan. At the same time, it was expected that this method would be efficient in analyzing large number of plasma samples supporting pharmacokinetics (e.g. bioavailability / bioequivalence) studies.

(A)



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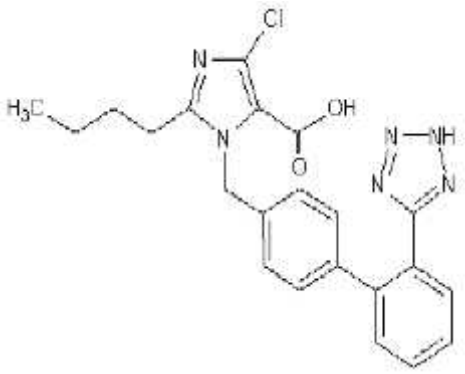

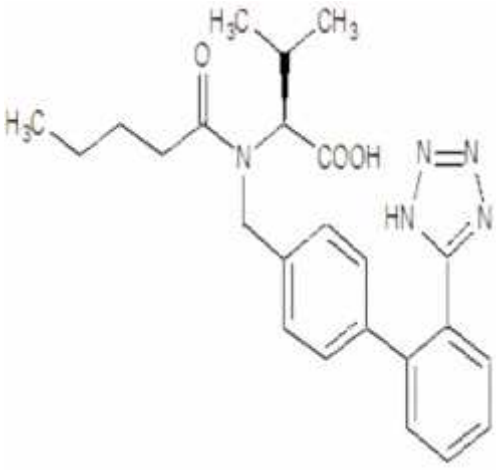

(B) **(C)**

Figure- 1: (A) Losartan, **(B)** Metabolite (Losartan carboxylic acid), **(C)** Valsartan

Materials and method:

Chemicals and reagents

The following chemicals and reagents were used: Losartan, losartan carboxylic acid and valsartan (Sigma, Germany) Acetonitrile HPLC grade (Scharlau chemie, Spain). *ortho*-Phosphoric acid 85% (Panreac, Spain). *n*-Hexane (Scharlau chemie, Spain). *t*-Methyl butyl ether (Acros, Belgium). Sodium hydroxide (BDH laboratories, England). Ammonium dihydrogen phosphate (Scharlau chemie, Spain). Triethylamine (Sigma, USA). Citric acid (BDH laboratories, England).

Instrumentation:

The following instruments and devices were used: HPLC-UV detector V 7057-3" (Knauer, Germany). Waters 600 quaternary pump (Milford, USA). Waters 717 autosampler (Milford, USA). Waters 4872 fluorescence detector (Milford, USA). Waters in-line degasser (Milford, USA). Eppendorf centrifuge 5804 (Germany). IKA-WERKE vortex shaker (Germany). GFL shaker (Germany).

Stock solution preparation:

All stock solutions were prepared employing acetonitrile: methanol: 0.01M ammonium dihydrogen phosphate (30:10:60) mixture as a solvent.

Losartan stock solution:

20.0 mg of losartan potassium were weighed (using losartan potassium standard powder) and dissolved in 10 ml of the solvent to produce a concentration of 2.0 mg/ml of losartan potassium.

Metabolite stock solution:

20.0 mg of metabolite were weighed (using losartan carboxylic acid "*EXP 3174*" standard powder) and dissolved in 10 ml of the solvent to produce a concentration of 2.0 mg/ml.

Internal standard stock solution:

5.0 mg of valsartan were dissolved in 100 ml of the same solvent to produce a concentration of 50 µg/ml.

Standard calibration curves preparation:

Calibration curves were prepared in mobile phase as well as in spiked plasma samples to cover linearity in the range of concentrations of 2–300ng/ml and 3–375ng/ml for losartan and its metabolite, respectively.

Quality Control (QC) samples preparation:

Working standard solutions of losartan and metabolite were prepared by serial dilution of stock solution (20µg/ml) using water to attain a concentration of 0.033, 2.2 and 3.3 µg/ml for losartan and 0.055, 2.475 and 3.85 µg/ml for metabolite quality control samples preparation, keeping internal standard at a concentration of 200 ng/ml in each one. All solutions were prepared daily. Quality control samples were prepared by spiking one ml of plasma samples (which was thawed at

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room temperature) with 100µl of the freshly prepared working standard solutions. Accordingly, the plasma samples contain a final concentration equivalent to 3, 200 and 300, respectively of losartan and 5, 225, and 350, respectively of metabolite and 200 ng/ml of valsartan as internal standard.

Methodology:

Chromatographic conditions:

- Column: Zorbax CN 5 µm (250x 4.6 mm) thermo stated at 35°C.
- Mobile phase: 0.015 M phosphoric acid: acetonitrile (72:28, *v/v*).
- Detection: Fluorescence detector λ excitation at 250 nm, λ emission at 370 nm.
- Flow rate: 1.25 ml/min
- Injection volume: 100µl
- Auto sampler temperature: ambient.

Sample preparation:

Samples were double extracted with *t*-methyl butyl ether and hexane. In each time the procedure was the following:

a- Each sample (1 ml plasma) was acidified with 1M phosphoric acid
b- Then plasma samples were extracted using 10 ml *t*-methyl butyl ether.
c- Samples were shaken for 20 minutes and later centrifuged for 5 minutes at 2500 rpm.
d- The upper *t*-methyl butyl ether layer was transferred into another tube containing 200 µl of 0.05M NaOH then shaken again for 15 minutes.
e- The tubes were centrifuged for 5 minutes at 2500 rpm.
f- The aqueous layer was separated by freezing and the *t*-methyl butyl ether layer was discarded.
g- 75µl of 0.5M citrate buffer were added to the aqueous layer and then vortex mixed.
h- The aqueous fraction was washed by adding 6ml hexane and vortex mixing.
i- After centrifuging the samples and freezing the aqueous layer, the hexane was discarded.
j- To improve solubility of the analysts in the mobile phase, 20µl of isopropanol was added to the aqueous layer (theoretically, making the final volume 295 µl).

Samples dilution:

Samples of concentrations higher than upper limit of quantification (ULOQ) of the calibration curve were diluted with the same plasma so to be properly calculated applying the calibration curves ranges. Consequently, 0.3 ml of the plasma sample was diluted with a complementary volume of blank plasma (0.7 ml) and the resultant one ml of the diluted sample was processed.

Method development:

Chromatography was carried out applying the samples to a repacked 5 µm, 250 x 4.6 mm CN Zorbax column using a 96 positions auto sampler. The combination of the mobile phase, prepared by mixing 0.015M phosphoric acid: acetonitrile in a ratio of 72:28, and a flow rate of 1.25 ml/min was found to give adequate resolution. Separations were performed at 35°C with an excitation wavelength of 250 nm and an emission wavelength of 370 nm. The analytical method was validated according to the "FDA Bio analytical Method Validation Guidelines".

The method's sensitivity and specificity was checked by analyzing six different human plasma batches.

For samples of concentrations higher than the ULOQ, justifiable dilution was considered.This necessitated a further validation step. The combination of sample extraction and HPLC was to provide a rapid assay and a valid analytical method free from interfering endogenous plasma components.

Validation protocol:

The method validation was performed using quality control samples (spiked plasma samples with standards of known concentrations). In order to evaluate the integrity and validity of the method the following parameters were evaluated [(11)]:

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Calibration/standard curves:

For the determination of linearity, standard calibration curves of 5 points (non-zero standards) in addition to the blank and zero samples were prepared. Replicate sets of spiked samples were extracted and analyzed on three consecutive days. The calibration curves were evaluated individually by linear regression. Four out of seven none zero standards including LLOQ and ULOQ, should meet the following acceptance criteria:

Not more than 20% deviation at LLOQ.

Not more than 15% deviation for standards above the LLOQ.

Accuracy and Precision:

Intra-day accuracy and precision:

The intra-day precision and accuracy of the assay was measured by analyzing five spiked samples of losartan at three different concentrations; the concentrations were back recalculated by applying the regression equation of the calibration curves. The deviation of the mean from the true value serves as the measure of accuracy.

The precision and accuracy deviation values should be within 15% of the actual values except at LLOQ where it shouldn't deviate by more than 20%. The statistical evaluation includes mean, standard deviation, coefficient of variation, accuracy, and relative error (%).

Intra-day accuracy and precision for losartan:

The intra-day accuracy and precision for losartan was done for three concentration levels namely 3, 200 and 300 ng/ml.

Intra-day accuracy and precision for metabolite:

The intra-day accuracy and precision for metabolite was done for three concentration levels namely 5, 225and 350 ng/ml.

Inter-day accuracy and precision:

The inter-day precision was done at three different concentrations over three days, the concentrations were measured by analyzing forty five samples (five determinations from each concentration per day) and were back recalculated applying the regression equation of the calibration curve.

Inter-day accuracy and precision for losartan:

The inter-day accuracy and precision for losartan was done for three concentration levels namely 3, 200 and 300 ng/ml. At each concentration level, 5 replicates were determined per day.

Inter-day accuracy and precision for metabolite:

The inter-day accuracy and precision for metabolite was done for three concentration levels namely 5, 225 and 350 ng/ml. At each concentration level, 5 replicates were determined per day.

Accuracy and precision for quality control samples:

The accuracy and precision for quality control samples were demonstrated by analyzing triplicates of quality control sample at three concentration levels representing the entire range of the standard calibration curve. The analyses were done over a period of two days. The low QC samples were designed to be near the LLOQ, while the mid QC samples were taken near the center. The high QC samples were taken near the ULOQ. Accuracy and precision for losartan QC samples low, mid and high QC samples of losartan were 3, 200 and 300 ng/ml respectively.

Accuracy and precision for metabolite Quality Control samples:

Low, mid and high QC samples of metabolite were 5, 225 and 350 ng/ml respectively.

Recovery:

The absolute peak area (detector response) obtained from the injections of the prepared plasma standards were compared to the absolute peak area (detector response) of an equivalent pure authentic standard, which was prepared to contain a drug concentration assuming 100% recovery. Relative recovery was

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determined by comparing the calculated concentrations of extracted samples to their respective nominal values.

Absolute and relative recovery for losartan:

Absolute recovery was measured by comparing the peak area of extracted samples with peak area of unextracted pure authentic standard solutions at three concentration levels (3, 200 and 300 ng/ml) of losartan. Relative recovery was measured by comparing the back calculated concentrations of extracted samples with their respective nominal values at three concentration levels (3, 200 and 300 ng/ml) of losartan.

Absolute and relative recovery for metabolite:

Absolute recovery was measured by comparing the peak area of extracted samples with peak area of unextracted pure authentic standard solutions at three concentration levels (5, 225 and 350 ng/ml) of the metabolite. Relative recovery was measured by comparing the calculated concentrations of extracted samples with their respective nominal values at three concentration levels (5, 225 and 350 ng/ml) of the metabolite

Absolute analytical recovery for internal standard:

Absolute recovery was measured by comparing the peak area of extracted samples with peak area of unextracted pure authentic standard solution at the concentration level of internal standard to be used (200 ng/ml).

Sensitivity:

The lowest standard concentration in the calibration curve is considered as the lower limit of quantitation (LLOQ), and should meet the following criteria:

A- LLOQ response is five times the response of the blank.

B- LLOQ response is identifiable, discrete and reproducible with precision of 20% and accuracy of 80-120%.

The peak is identifiable, precise and accurate at this concentration.

Specificity:

The method's specificity was determined by screening six different batches of healthy human plasma. The tests were accomplished to ensure absence of interfering endogenous plasma components.

Specificity for losartan:

Specificity of the method was confirmed by the absence of interference peaks at the retention time of losartan.

Specificity for metabolite:

Specificity of the method was confirmed by the absence of interference peaks at the retention time of metabolite.

Application of the method:

The present method was applied to a comparative bioavailability study. The ethics committee on human studies of Baghdad University College of pharmacy approved the study. Ten healthy adult male volunteers aged between 25 to 35 years and weighing from 60 to 80 kg participated in the study.

On the basis of medical history, clinical examinations and laboratory tests including hematology, blood biochemistry and urine analyses, no subject had a history or evidence of hepatic, renal, gastro-intestinal or haematological deviations, or any acute or chronic disease or drug allergy. The subjects were instructed to abstain from taking any medication at least 2 weeks prior to and during the study period. Informed consent was obtained from the subjects after explaining the nature and purpose of the study.

The protocol was the conventional, two-way, crossover study with ten subjects and a one-week washout period. In the first trial period, after an overnight fasting, subjects were given a single oral dose of 50 mg of two marketed formulas tablet (losartan potassium) with 200 ml of water. Approximately 3 ml of blood samples were drawn into heparinized tubes through an indwelling canola before (0 h) and at 0.33, 0.67, 1, 1.33, 1.67, 2, 2.5, 3, 4, 5, 6, 8, 10, 12, 16, 24, 30 & 36 h after dosing.

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The blood samples were centrifuged at 4000 rpm for 10 minutes; plasma was separated and kept frozen at −70°C in coded glass tubes.

Pharmacokinetic Analysis:

The area under the curve to the last measurable concentration (AUC0-t) was estimated by the linear trapezoidal rule and AUC0-∞ also was calculated, where Ct is the last measurable concentration. The peak plasma concentration (Cmax) and corresponding time to peak (t_{max}) were determined by the inspection of the individual drug plasma concentration-time profiles. Also elimination half life ($T_{0.5}$) determined.

Results and discussion:

Standard calibration curves of losartan:

The method exhibits acceptable linear range from 2 to 300 ng/ml. Figure 2 shows a typical calibration plot.

Standard calibration curves of metabolite:

The method exhibits acceptable linear range from 3 to 375 ng/ml. Figure 3 shows a typical calibration plot.

Accuracy and precision Intra-day accuracy and precision:

Intra-day accuracy and precision of losartan:

Intra-day accuracy of the method for losartan ranged from 101.11% to 105.72%, while the intra-day precision ranged from 3.67% to 9.39% at the concentrations of 3, 200 and 300 ng/ml. Data are presented in table-1.

Intra-day accuracy and precision of metabolite:

Intra-day accuracy of the method for the metabolite ranged from 93.7% to 106.6%, while the intra-day precision ranged from 1.5% to 5.1% at the concentrations of 3, 180 and 300 ng/ml. Data are presented in table-2.

Inter-day accuracy and precision Inter-day accuracy and precision of losartan:

Inter-day precision of the method for losartan ranged from 3.68% to 9.62%, at the concentrations of 3, 200 and 300 ng/ml. Data are comprehended in table-3.

Inter-day accuracy and precision of metabolite:

Inter-day precision for the metabolite rangedfrom 4% to 4.71% at the concentrations of 5, 225 and 350 ng/ml. Data are comprehended in table-4.

Recovery:Absolute and relative recovery of losartan:

The absolute and relative recovery determined for losartan shown to be consistent, precise and reproducible at the three levels 3, 200 and 300 ng/ml. Data are depicted in tables 5 and 6. **Absolute and relative recovery of metabolite:**

The absolute and relative recovery determined for metabolite shown to be consistent, precise and reproducible at the three levels 5, 225 and 350 ng/ml. Data are depicted in tables 7 and 8.

Absolute analytical recovery of internal standard:

Table-9 depicts the results of absolute analytical recovery of internal standard (valsartan).

Sensitivity:

Lower limit of quantitation of losartan:

The lower limit of quantitation for losartan is considered to be 2 ng/ml, with a precision of 3.28%. Data for losartan LLOQ is presented in table-10.

Lower limit of quantitation of metabolite:

The lower limit of quantitation for metabolite is considered to be 3 ng/ml, with a precision of 2.59%. Data for metabolite LLOQ is presented in table-11.

Specificity:

Specificity was evidenced by the lack of interfering peaks in the chromatograms of plasma samples. Chromatograms represented in figures 4 to 9 illustrate the excellent chromatography and resolution obtained using CN column under the described conditions.

Figure-4 shows an HPLC chromatogram for a blank plasma sample indicating no endogenous peaks at the

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retention times (t_R) of losartan, metabolite or internal standard (Valsartan).

Application of the validated method to routine losartan's analysis:

The data obtained from volunteers' plasma routine analysis are listed in tables 12, 13, 14 and 15. The analyses of plasma samples were accomplished in accordance with the "FDA Bio analytical Method Validation Guidelines" [1]. Good Laboratory Practices and the Bio Center SOPs were followed. The following was strictly adhered to during the samples analysis:

A. Assays of all losartan's plasma samples and its metabolite were completed within the time period for which stability data are available.

B. Calibration curve: A standard curve including blank matrix was generated for each analytical run and was used to determine the sample concentrations in the unknown authentic samples. The same analyst prepared the calibration curves and the samples to be analyzed.

C. Quality Control (QC) Samples: For each run, six QC samples were analyzed, two at each of the low, mid and high concentrations.

As a practice, blank sample (sample at zero time) from the subject for whom unknown samples were analyzed, was included in a run to check selectivity of the method.

QC samples were analyzed together with the unknown authentic samples and were dispersed evenly in a low-high and high-low sequence throughout the batch, in order to detect analytical problems "if any". Criteria of acceptance of QC samples were based on a combined accuracy and precision criteria with an arbitrary range around the "nominal value".

At least four of six QC samples were always within $\pm$ 15% of their respective nominal value; two of the six QC samples (not both at the same concentration) were never outside the $\pm$ 15% respective nominal value.

Conclusions:

The developed method of analysis provided a sensitive and specific assay for losartan and its metabolite (losartan carboxylic acid) in human plasma. It was shown that this method is suitable for the analysis of losartan and its metabolite in the biological samples collected for the bioequivalence study of Cozaar® tablet (losartan) versus of losartan tablet of Indian company.

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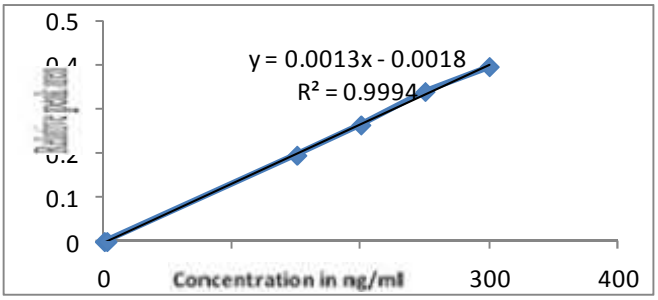

Figure- 2: Standard calibration curve plot of losartan.

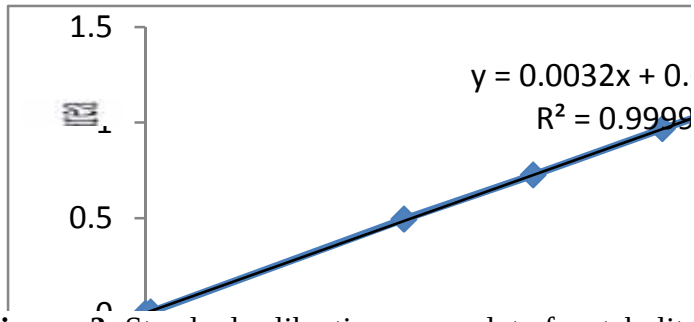

Figure- 3: Standard calibration curve plot of metabolite.

Table-1: Intra-day accuracy, precision and relative error for losartan determination in plasma's spiked samples.

Analyzed on Day	Losartan concentration in human plasma		
	QC sample (3 ng/ml)	QC sample (200ng/ml)	QC sample (300ng/ml)
Day 1	3.477	185.323	316.159
	3.500	218.767	310.880
	2.857	200.672	305.213
	3.056	190.654	295.646
	2.968	203.320	288.754
Mean	**3.1716**	**199.75**	**303.33**
SD	**0.298**	**12.9**	**11.14**
Precision as CV %	**9.39**	**6.46**	**3.67**
Accuracy %	**105.72**	**99.88**	**101.11**

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Table-2: Intra-day accuracy, precision and relative error for metabolite determination in plasma's spiked samples.

Analyzed on Day	Metabolite concentration in human plasma		
	QC sample (5 ng/ml)	QC sample (225 ng/ml)	QC sample (350 ng/ml)
Day 1	5.346	200.088	345.771
	5.115	223.987	343.591
	5.644	200.429	351.223
	5.445	210.654	338.987
	5.097	218.989	350.993
Mean	**5.33**	**210.83**	**346.11**
SD	**0.23**	**10.76**	**5.18**
Precision as CV %	**4.32**	**5.1**	**1.5**
Accuracy %	**106.6**	**93.7**	**98.89**

Table- 3: Inter-day accuracy, precision and relative error for losartan determination in plasma's spiked samples.

Analyzed on Day	Losartan concentration in human plasma		
	(3 ng/ml)	(200 ng/ml)	(300 ng/ml)
Day1	3.113	210.449	300.009
	3.12	192.683	308.940
	2.768	187.672	315.972
	2.801	203.552	297.646
	2.918	204.314	272.762
Day 2	2.592	194.698	306.313
	2.487	200.395	205.641
	2.801	201.812	313.481
	2.508	190.147	302.110
	3.098	206.395	320.361
Day 3	3.032	201.913	285.742
	2.899	196.341	303.305
	2.998	202.394	321.691
	3.121	205.023	295.486
	2.564	214.540	282.987
Mean	**2.85**	**200.82**	**295.49**
SD	**0.23**	**7.39**	**28.43**
Precision as CV %	**8.07**	**3.68**	**9.62**
Accuracy%	**95**	**100.41**	**98.5**

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Table- 4: Inter-day accuracy, precision and relative error for metabolite determination in plasma's spiked samples.

Analyzed on Day	Metabolite concentration in human plasma		
	(5 ng/ml)	(225 ng/ml)	(350 ng/ml)
Day1	5.258	211.288	345.641
	5.015	223.69	343.591
	5.430	201.319	350.143
	5.159	198.031	339.318
	5.097	218.151	348.883
Day 2	5.324	195.102	370.159
	5.169	210.448	371.575
	4.983	211.210	361.496
	4.998	226.395	367.176
	4.943	225.658	320.517
Day 3	4.546	223.119	335.194
	5.079	214.721	339.845
	4.983	205.487	374.622
	5.059	220.767	364.591
	5.012	209.301	353.946
Mean	**5.07**	**212.98**	**352.45**
SD	**0.2**	**10.04**	**15.61**
Precision as CV %	**4**	**4.71**	**4.43**
Accuracy%	**101.4**	**106.49**	**100.7**

Table- 5: Absolute analytical recovery of losartan.

Concentration ng/ml	HPLC peak area for Losartan					
	Direct inject	In human plasma			Mean	Recovery%
		n#1	n#2	n#3		
3	99298	56079	55231	52696	54668.67	55.06
200	7911307	3906393	4199464	4039016	4048291	51.17
300	10090744	5957818	6402309	6172672	6177599.67	61.22

Table- 6: Relative recovery of losartan.

Conc. (ng/ml)	Recovered Drug from human plasma				Mean	CV %
3	**Measured Conc.**	2.6	3.01	3.3	2.97	11.85
	Relative Recovery%	86.67	103.33	110	100	
200	**Measured Conc.**	190.23	212.34	218.56	207.04	7.19
	Relative Recovery%	95.12	106.17	109.28	103.52	
300	**Measured Conc.**	271.89	301.02	324.67	299.19	8.84
	Relative Recovery%	90.63	100.34	108.22	99.73	

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Table-7: Absolute analytical recovery of metabolite.

Concentration ng/ml	HPLC peak area for metabolite					
	Direct inject	In human plasma			Mean	Recovery%
		n#1	n#2	n#3		
5	526160	236196	264889	207110	236065	44.87
225	1965901	9094808	9239987	8861722	9065505.67	46.11
325	23329350	13319815	11921504	10692564	11977961	51.34

Table-8: Relative recovery of metabolite.

Conc. (ng/ml)	Recovered Drug from human plasma				Mean	CV%
5	**Measured Conc.**	5.54	5.1	4.42	5.02	11.23
	Relative Recovery%	110.8	102	88.4	100.4	
225	**Measured Conc.**	201.56	200.87	241.76	214.73	10.9
	Relative Recovery%	89.58	89.28	107.45	95.44	
350	**Measured Conc.**	377.86	354.04	324.91	352.27	7.53
	Relative Recovery%	107.96	101.15	92.65	100.59	

Table-9: Absolute analytical recovery of internal standard (Valsartan).

Concentration ng/ml	HPLC peak area for Internal Standard (Valsartan)					
	Direct inject	In human plasma			Mean	Recovery %
		n#1	n#2	n#3		
200	20177623	15947167	14277856	15246654	15157225.6	75.12

Table-10: Lower limit of quantization for losartan.

Concentration (ng/ml)	Actual concentration (ng/ml)	Accuracy %	Mean (ng/ml)	C.V%
2	1.89	94.5	1.8	3.28
	1.77	88.5		
	1.81	90.5		
	1.815	90.75		
	1.731	86.55		

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Table- 11: Lower limit of quantitation for the metabolite.

Concentration (ng/ml)	Actual concentration (ng/ml)	Accuracy%	Mean (ng/ml)	C.V.%
3	3.1	103.33	3.089	2.59
	3.149	104.96		
	2.95	98.33		
	3.12	104		
	3.129	104.3		

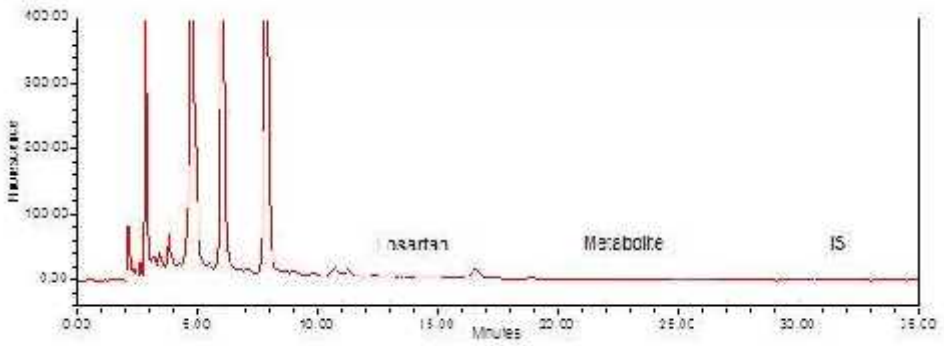

Figure- 4: HPLC chromatogram of a blank human plasma sample.

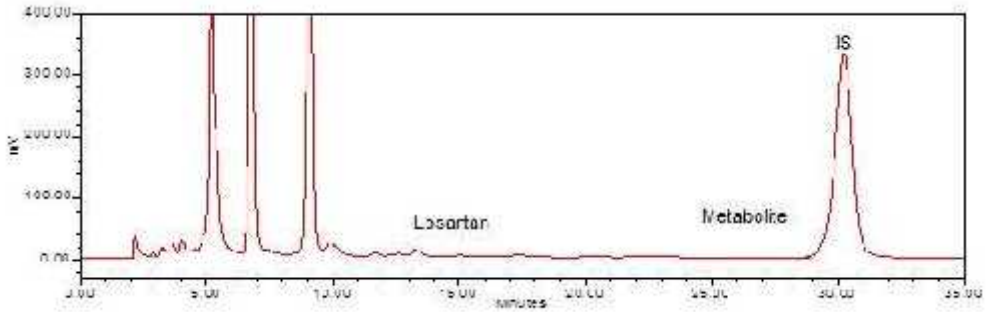

Figure-5: HPLC chromatogram of a zero sample containing 200 ng/ml internal standard (Valsartan).

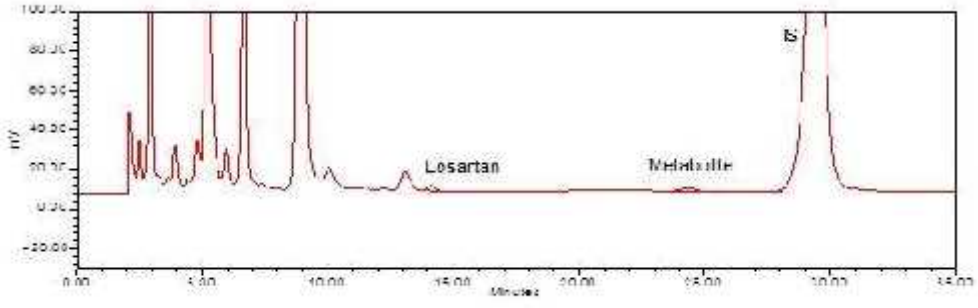

Figure-6: HPLC chromatogram showing human plasma sample containing 2 ng/ml losartan, 3 ng/ml metabolite and 200 ng/ml internal standard (Valsartan).

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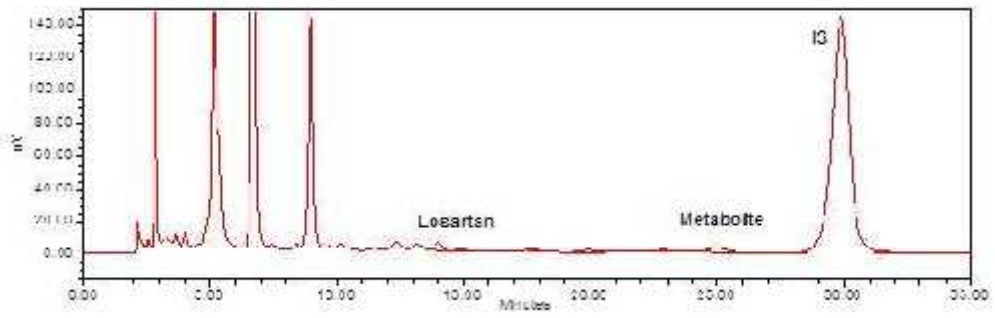

Figure-7: HPLC chromatogram showing human plasma sample containing 3 ng/ml losartan, 5 ng/ml metabolite and 200 ng/ml internal standard (Valsartan).

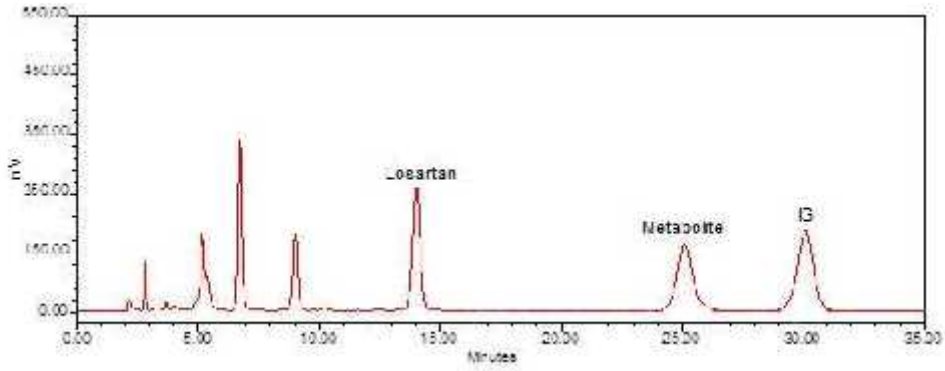

Figure-8: HPLC chromatogram showing human plasma sample containing 200 ng/ml losartan, 225 ng/ml metabolite and 200 ng/ml internal standard (Valsartan).

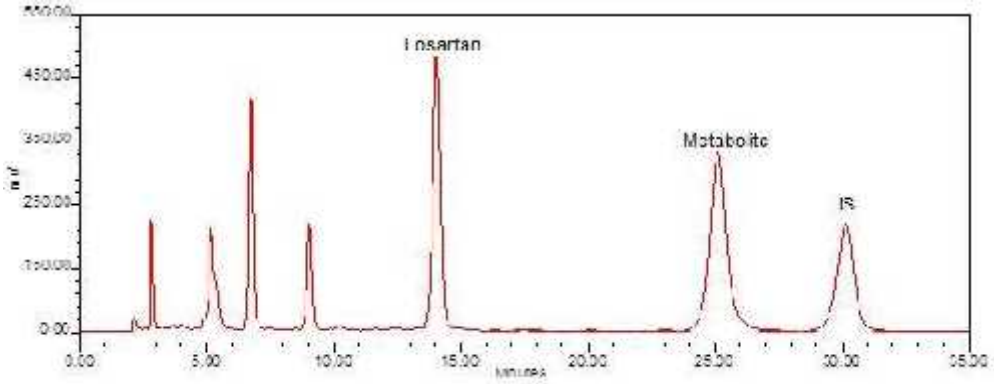

Figure-9: HPLC chromatogram showing human plasma sample containing 300 ng/ml losartan, 350 ng/ml metabolite and 200 ng/ml internal standard (Valsartan).

Table-12: The individual and the descriptive statistics of the pharmacokinetic parameters of losartan following the administration of single dose 50 mg of Cozaar ® tablet (losartan) to 10 healthy adult male volunteers in fasting, randomized two-way crossover design.

Pk Parameter	C_{max} (ng/ml)	T_{max} (hr)	AUC_{0-t} (ng.hr/ml)	$AUC_{t-\infty}$ (ng.hr/ml)	$AUC_{0-\infty}$ (ng.hr/ml)	$T_{0.5}$ (hr)
Arithmetic Mean	215.88	1.68	503.86	13.72	517.57	3.212
± SD	130.95	0.86	209.67	19.25	209.87	1.42
Min	107.8	0.67	167.1	3.6	175.4	1.62
Max	5544.2	3	947.5	67.6	956.1	5.71

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Table-13: The individual and the descriptive statistics of the pharmacokinetic parameters of losartan following the administration of single dose of dose 50 mg of losartan tablet of Indian company 10 healthy adult male volunteers in fasting, randomized two-way crossover design.

Pk Parameter	C_{max} (ng/ml)	T_{max} (hr)	AUC_{0-t} (ng.hr/ml)	$AUC_{t-\infty}$ (ng.hr/ml)	$AUC_{0-\infty}$ (ng.hr/ml)	$T_{0.5}$ (hr)
Arithmetic Mean	202.47	1.36	471.96	11.7	483.66	2.969
± SD	131.98	0.86	162.86	10.53	162.31	1.43
Min	97.1	0.67	228.6	3.7	232.5	1.02
Max	459	3	812.8	35.7	821.6	5.62

Table-14: The individual and the descriptive statistics of the pharmacokinetic parameters of losartan metabolite following the administration of single dose of 50 mg of Cozaar ® tablet (losartan) 10 healthy adult male volunteers in fasting, randomized two-way crossover design.

Pk Parameter	C_{max} (ng/ml)	T_{max} (hr)	AUC_{0-t} (ng.hr/ml)	$AUC_{t-\infty}$ (ng.hr/ml)	$AUC_{0-\infty}$ (ng.hr/ml)	T0.5 (hr)
Arithmetic Mean	301.54	4.3	2272.48	37.19	2309.68	5.983
± SD	159.07	1.39	1064.44	17.22	1080.02	1.12
Min	66.8	2.5	763.6	8.3	771.9	3.34
Max	489.9	6	3783.1	65.7	3848.8	7.14

Table-15: The individual and the descriptive statistics of the pharmacokinetic parameters of losartan metabolite following the administration of single dose of 50 mg losartan tablet of Indian company tablets 10 healthy adult male volunteers in fasting, randomized two-way crossover design.

Pk Parameter	C_{max} (ng/ml)	T_{max} (hr)	AUC_{0-t} (ng.hr/ml)	$AUC_{t-\infty}$ (ng.hr/ml)	$AUC_{0-\infty}$ (ng.hr/ml)	T0.5 (hr)
Arithmetic Mean	276.72	4.95	2165.99	35.77	2201.75	6.024
± SD	155.59	1.64	1085.03	24.88	1093.81	1.04
Min	52	2.5	626.5	8.4	634.9	4.51
Max	550	8	3859.1	76.3	3935.3	7.77