HPLC- fluorescence determination of valsartan in human volunteers and its application in bioequivalence study of two valsartan tablets

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Abstract: A simple and rapid high-performance liquid chromatographic method with fluorescence detection for the estimation of valsartan in human plasma was developed and validated. Lozartan was used as internal standard. Valsartan and losartan were isolated from plasma by non-extractive procedure; simple protein precipitation with acetonitrile. Separations were performed in low pressure isocratic mode on Zorbax Extend-C18 (4.6 x 150 mm) column, using a mobile phase consisting of phosphate buffer - acetonitrile mixture in the ratio of (50:50 v/v) pH 3.0 was adjusted by orthophosphoric acid) at a flow rate of 1 mL min-111. The detection of valsartan and losartan was carried out at 230 nm (for excitation) and 370 nm (for emission) 253 nm (for excitation) and 374 nm (for emission). The response was linear over a range of 0.2-12 µg ml^{-1}. The limit of detection was 0.2 µg ml^{-1}. The same method was used for the bioequivalence study of two valsartan tablets in 12 healthy, human, Egyptian, male volunteers.
[Ola M. Abdallah and Khalid Abu Zeid. **HPLC- fluorescence determination of valsartan in human volunteers and its application in bioequivalence study of two valsartan tablets.** *Life Sci J* 2013;10(2):583-590]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 85

Key words: Valsrtan; Pharmacokinetic; HPLC; Bioequivalence

1. Introduction

Valsartan *(S*-3-methyl-2- [*N*-({4-[2-(2*H*-1,2,3,4-tetrazol-5-yl) phenyl] phenyl} methyl) pentanamido] butanoic acid (Figure 1), is an angiotensin II receptor antagonist used in the management of hypertension [1].

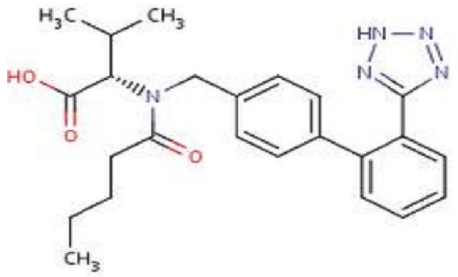

Figure 1: Chemical structure of valsartan

It improves symptoms and quality of life in patients with chronic heart failure. Valsartan treatment had no demonstrable negative effects on growth and development and is used safely as an antihypertensive agent in children less than 6 years old [2].

Valsartan is rapidly absorbed following oral 24 administration. It is quickly eliminated from the body and no significant metabolism takes place. Absolute bioavailability of valsartan is about 25% (range 10%-35%). its peak plasma concentration is reached 2 to 4 hours after dosing, with an average elimination half-life of about 6 hours and AUC and Cmax values of valsartan increase approximately linearly with increasing dose over the clinical dosing range. Valsartan does not accumulate appreciably in plasma following repeated administration [3,4].

Several HPLC methods for the determination of valsartan in human plasma were reported in the literature. Most of these methods employ a liquid extraction procedure with protein precipitation (mostly with methanol), reversed- phase C18 column separation followed by fluorescence [5-11], or ultraviolet detection [12, 13]. Likewise, our proposed method uses liquid extraction and protein precipitation using acetonitrile followed by fluorescence detection. The present method provides results comparable to those obtained using flurometric detection and superior to those utilizing UV detection. However, our method possesses a number of advantages. With the chromatographic conditions described, it was possible to completely resolve valsartan and the internal standard (losartan) peaks within 5 minutes without the additional time required for the back –extraction step used by other methods [5]. The proposed method allows accurate quantification of valsartan concentrations that are expected after regular dose administration. Overall, our method provides a rapid, sensitive, and economically-convenient procedure for the analysis

of valsartan in human plasma samples encountered in pharmacokinetics studies. The same method has also been utilized for the bioequivalence study of valsartan formulations.

2. Material and Methods

Volunteers and clinical protocol:

The study protocol and the informed consent forms were approved by the Ethical Committee of Bioequivalence Center- MSA University (6^{th} October, Egypt). The whole study, which meets the requirements of the declarations of Helsinki, was conducted in accordance with the current Good Clinical Practice (GPC), International Conference Harmonization (ICH) as well as Good Laboratory Practice (GLP) Guidelines [14- 15].Twelve adult male volunteers, non smokers, aged between 18–28 years, weighing between 67and 83 Kg with a mean value of 76.25 ± 5.64Kg, were chosen to participate in the present study. The volunteers were not on concomitant medications and they were free from significant cardiac, hepatic, renal, pulmonary, gastrointestinal, or hematological disease as determined within four weeks prior to the beginning of the study by way of medical histories and physical examinations.

Subject's health status was determined following a physical examination, laboratory tests and medical history by a qualified registered MD physician. The physician reviewed all preclinical laboratory tests for each subject. Tests included the following: (i) physical examination: height, weight, blood pressure, heart rate, body temperature and respiratory rate; (ii) blood chemistry: glucose, uric acid, BUN, creatinine AST, ALT, cholesterol and triglycerides; (iii) hematological tests: hemoglobin, hematocrit, ESR, WBCs with differential, RBCs with platelets count and morphology, lymphocytes, MCV, MCH, monocytes, neutrophils, eosinophils, basophils, HIV and Hepatitis B screen; (iv) urine analysis: specific gravity, pH and microscopic examination.These clinical tests were performed in order to determine if they fitted with the participation in the study. Exclusion criteria included extreme weight ranges (overweight or underweight), anemia, liver or renal dysfunction, parasitic and other diseases or conditions that was judged to affect the absorption, distribution and/or elimination of valsartan. All volunteers were given a written informed consent, which explained the nature of the study. All volunteers were interested and willing to participate in the study. The subjects were asked to abstain from taking drugs and alcohol for at least 3 days prior to the study and throughout the study period. On the night before starting the study, the volunteers were instructed to fast for at least 10 hours before drug administration. The study had an open randomized two-period crossover design with a 14-days washout period between doses. The volunteers were arbitrarily divided into two equal groups each of 6 subjects. To the first group the reference formulation was given and to the second group the test formulation was given with a crossover after a washout period of two weeks. On the morning of the experiment, a blood sample was withdrawn from each volunteer to serve as a blank for the drug assay. Each of the 12 volunteers then took one Lasaromep® 81 320 mg per tablet as the test drug, or one Tareg® 82 320 mg per tablet as the reference drug followed by 240 ml of water. Blood samples for plasma drug assay were collected from an indwelling catheter inserted in the antecubital vein of one of the arms. Samples were obtained at 0.0, 0.5, 1.00, 1.50, 2, 2.5,3, 4, 5, 6, 8, 10 and 24.00 hours after drug administration, in heparinized tubes. Plasma was directly separated by centrifugation at 3000 rpm for 10 min, removed out and stored at −20°C until assayed. Four hours after drug administration, the subjects were allowed to eat a standard breakfast. They were then allowed controlled access to water and other non-alcoholic beverages. The volunteers had their second meal (standard lunch) 4 hours later. It should be noted that valsartan is rapidly absorbed from gastrointestinal tract after oral administration and can be administered without regard to food intake. The peak effect of valsartan is evident in 2–4 h; the bioavailability is 25% [16].

Drugs and chemicals

Acetonitrile (ACN) HPLC grade (Fisher Scientific), Dipotassium hydrogen phosphate, K_2HPO_4 (Sigma) and Ultra pure water (Aquatron) was used. Reference Valsartan 99.5% was supplied by Arab Company for Pharmaceuticals & Medicinal Plants (MEPACO). Losartan 99.3 % (internal standard) was purchased from Sigma- Aldrich Co.

Formulations

The following test formulation was employed: lasaromep® 100 film coated tablet (320 mg valsartan/tablet) from MEPACO (batch no. 990210). The reference formulation was Tareg® 102 film coated tablet (320 mg valsartan/tablet) manufactured by Novartis Pharma,

SAE Cairo under license from Novartis Pharma AG., Basle, Switzerland (batch no. Y0004).

Dissolution Study

The test was carried out according to the USP 30 specifications. Six tablets were placed in the vessels of the dissolution apparatus containing 1000 mL of a phosphate buffer with pH 6.8. Rotation was set on 100 rpm using USP apparatus I (Basket type) using Hanson Research SR8 apparatus. Temperature was maintained at 37°C during the time of

dissolution test of 60 min. The tablets were placed in the vessels. Samples (5 ml) were taken at 5, 10, 15, 20, 30, 45, and 60 min. These samples were filtered through a Millipore filter 0.45µm before dilution with phosphate buffer. 20 µL of sample solutions were injected into the liquid chromatograph.

Chromatographic conditions

Agilent 1200 series isocratic quaternary pump HPLC instrument and Agilent autosampler model G 1329A were employed. Separation was accomplished 116 with a Zorbax Extend-C18 (4.6 x 150 mm) and the guard column model Eclipse XDB-C18 (12.5 x 4.6) mm, particle size 5 um. The mobile phase involved a mixture of phosphate Buffer: Acetonitrile in the ratio of (50:50 v/v) (pH adjusted to 3 with orthophosphoric acid), pumped at a flow rate of 1 mL min^{-1}. Agilent Fluorescence detector Model GB was set at at 230 nm for excitation and 370 nm for emission. Quantification of valsartan was obtained by plotting valsartan to internal standard peak area ratios as a function of concentration. Chromatographic peaks were electronically integrated and recorded using Chemstation software.

Extraction of valsartan from Plasma

To 1 ml plasma 100 ul losartan as internal Standard containing 6 µg mL -1125 was added. Samples were extracted using 1 ml acetonitrile, after mixing (30 s), the mixture was centrifuged for 10 minutes at speed of $3.5x10^3$ rpm. 20 ul of the clear supernatant was injected into the liquid chromatograph. Peaks were detected by fluorescence detector (excitation at λ 230 nm and emission at λ 370 nm), were interpreted in the form of reported peak areas. Concentrations of valsartan in unknown samples were calculated with reference to the prepared calibration curve.

Standard and Stock Solutions

Preparation of stock solutions in the mobile phase

Valsartan

A concentrated stock solution was prepared in mobile phase (0.05 M K2HPO4 : ACN in the ratio 50:50 v/v), accurately weighed 100 mg valsartan were dissolved in mobile phase in 100 ml volumetric flask then diluted to volume 136 with the same solvent (solution A). This solution contains 1 mg mL^{-1}. 10 ml from solution A were transferred to 100 ml volumetric flask then diluted to volume by mobile phase (Solution B). This solution contains 100 µg mL^{-1} valsartan.

Losartan

Accurately weighed 100 mg were dissolved in mobile phase in 100 ml volumetric flask (solution C). This solution contains 1 mg mL^{-1}. 3 ml from solution (C) were transferred to 50 ml volumetric flask then diluted to volume by mobile phase (Solution D). This solution contains 60 µg mL^{-1} losartan.

Preparation of calibration standards

Blank plasma samples were spiked with 100 ul freshly prepared standard drug solutions from solution B to contain concentrations of 0.2, 0.4, 0.8, 2, 4 and 8 µg mL^{-1}. Solution A was used to prepare standard drug solutions to contain 10 and 12 µg mL^{-1}.

Preparation of Quality Control (QC) Samples

QC samples were prepared to cover low (0.7 µg mL^{-1}), medium (3 µg mL^{-1}) and high (7 µg mL^{-1}) valsartan concentrations. QC's used in pre and within-study in addition to losartan were employed in order to ensure the accuracy of measurements corresponding to the analyte.

Validation procedures

The validation of this chromatographic analytical method was performed in order to evaluate its linearity, selectivity, stability, precision and accuracy.

Linearity and linear working range

A series of standard plasma solutions, previously spiked with valsartan, were employed as calibrators for constructing calibration growth curves covering the concentrations ranging 0.2-12 µg mL^{-1}. The analytes were extracted using the method described earlier. Linearity was demonstrated both; by calculating the "product moment correlation coefficients" R, and by the visual inspection of individual calibration growth curves. Calibration curves were constructed from the peak area ratio (drug/ internal standard) and the corresponding valsartan concentration in each calibration standard.

Lower limit of quantitation (LLOQ)

LLOQ is defined as the lowest concentration of analyte that can be determined with acceptable precision and accuracy under the stated experimental conditions. It was estimated by analyzing samples with known amounts of valsartan, at progressively lower concentrations, starting at the lower end of the calibration curves. The performance of the assay during the analysis of the study samples was evaluated by the analysis of the quality control (QC) samples.

Recovery

Absolute Recovery

Different concentrations (two QC samples including QCL, and QCH) of valsartan were spiked into plasma and equally into matrix free interference (mobile phase), plasma samples were individually extracted and chromatographed in accordance with **2.5** whereas matrix free QC samples were directly chromatographed.

Relative Recovery

Different concentrations (two QC Samples) of valsartan were spiked into plasma and

extracted in accordance with 2.5. Analysis was repeated on three days to determine the recovered concentrations of valsartan.

Accuracy

Single injections of six individually prepared and extracted plasma samples, Containing 0.7, 3.0 and 7.0 µg mL^{-1} valsartan prepared as described under calibration curve standards were chromatographed and evaluated.

Precision

Inter-day precision was evaluated by analyzing plasma samples containing valsartan at two different concentration levels, a low QC sample containing 0.7µg mL^{-1} and a high QC sample containing 7 µg mL^{-1} using six individually prepared replicate determinations on three days. Intra-day analysis was determined upon replicate analysis of 6 check samples at the previously mentioned QC samples.

Selectivity

Selectivity was defined as the ability of the chromatographic method to measure a response from the analyte without influence from the biological matrix. This was accomplished by evaluating injections of mobile phase and 6 individual lots of blank plasma during validation.

Stability

Valsartan stability was studied during sample collection, storage and preparation. All stability investigations were conducted using freshly prepared stock solutions in the mobile phase, as well as in the plasma matrix.

Short-term stability studies

Six aliquots, each of low QC and high QC samples in addition to losartan were thawed once, and were kept unextracted at room temperature before extraction and consequent assay.

Freeze and thaw stability

Analyte stability was determined after three freeze and thaw cycles. Three aliquots of each QCL and QCH sample were stored at –20 °C for 24 hours and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 24 hours under the same conditions. Three freeze and thaw cycles were repeated.

Long-term stability study

Stability of six aliquots, each of low QC and high QC samples in addition to losartan in the plasma matrix under prolonged storage conditions (-20°C), during the study period, was investigated

Pharmacokinetic and statistical analysis

A concentration time curve was plotted and the pharmacokinetic parameters were estimated using non-compartmental methods. The area under the plasma concentration-time curve (AUC0-t) was calculated from the time of administration to the time of last sample by linear trapezoidal rule. The area under the plasma concentration–time curve extrapolated to infinity (AUC0-∞) was calculated according to the following formula:

$$AUC0\text{-}\infty = 219\ AUC0\text{-}t + Clast\ /[Ln2/t½]$$

The ratio AUC0-t/AUC0-∞ as a percent was determined as an indicator for the adequacy of sampling time. Maximum plasma concentration (Cmax) and time to achieve the maximum concentration (tmax) was obtained directly from the concentration time curve. The elimination half-life t1/2 was calculated as t1/2 =Ln (2)/(-b) where b was obtained as the slope of the linear regression of the ln-transformed plasma concentrations versus time in the terminal period of the plasma curve. All the pharmacokinetic and statistical data were calculated using *Kinetica*® 2000 software. For the purpose of bioequivalence analysis, statistical evaluation (ANOVA) was used to assess the effect of formulations, periods, sequences and subjects on AUC0–24, AUC0–∞, and Cmax.

3. Results and Discussion

Results of validation procedures

Under the chromatographic conditions described, valsartan and the internal standard peaks were completely resolved during the run time of the assay with retention times of 2.5 minutes for losartan and 4.0 minutes for valsartan (Figure 2). The broad peak from 1.3–1.7 minutes is related to plasma components that their concentration may be influenced by the presence of valsartan. However, these components do not alter the valsartan quantitation since the peaks of both valsartan and the internal standard are completely resolved.

Calibration curves constructed from the peak area ratio (valsartan/ internal standard) and the corresponding valsartan concentration in each calibration standard were linear from 0.2 to 12 µg mL^{-1}. The mean slope and intercept for the different calibration curves of valsartan in human plasma are presented in Table 1. The correlation coefficient was always greater than 0.999 during the course of the validation.

Table 1: Mean slope and intercept for the different calibration curves of valsartan in human plasma

Calibration Curve Parameter	Mean ± SD
Slope ± SD (Sb)	0.176 ± 0.0045
Intercept ± SD (Sa)	0.031 ± 0.0040
Correlation coefficient	0.999

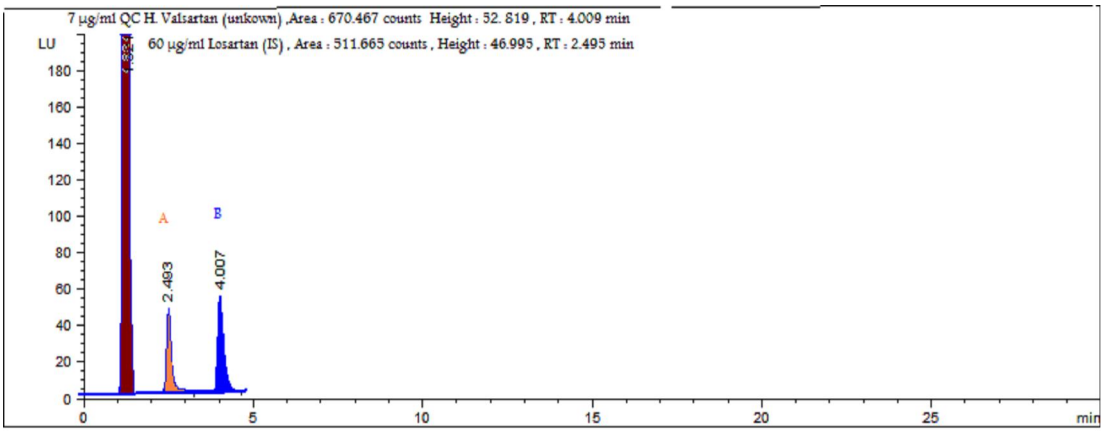

Figure 2: Representative chromatograms for an extract of plasma sample containing 60 µg mL^{-1} Losartan (IS) (A) and Valsartan (7 µg mL^{-1}), High (QC) (B).

The intraday coefficient of variation ranged from 0.90 to 2.90 %, while the inter-day coefficient of variation ranged from 2.97 to 4.24% indicating that the method is precise (Tables 2 and 3). The accuracy of the method was proven as the relative error % obtained was in the range of 1.67 to 7.68 %.

Table 2: Intra-day precision at two different concentrations of quality control samples

Day 1									
Theoretical concentration (µg mL^{-1})	Measured concentrations (µg mL^{-1})						Mean recovered	STDEV (S)	CV (%)
	1	2	3	4	5	6			
0.070	0.558	0.564	0.587	0.587	0.598	0.593	0.581	0.016	2.75
7.000	6.637	6.807	6.864	6.921	6.841	6.847	6.819	0.097	1.42
Day 2									
Theoretical concentration (µg mL^{-1})	Measured concentrations (µg mL^{-1})						Mean recovered	STDEV (S)	CV (%)
0.070	0.621	0.627	0.615	0.615	0.615	0.627	0.619	0.006	0.97
7.000	7.204	7.318	7.375	7.375	7.318	7.375	7.327	0.066	0.90
Day 3									
Theoretical concentration (µg mL^{-1})	Measured concentrations (µg mL^{-1})						Mean recovered	STDEV (S)	CV (%)
0.070	0.621	0.627	0.615	0.615	0.615	0.627	0.619	0.006	0.97
7.000	7.602	7.885	7.488	7.772	7.658	7.658	7.677	0.137	1.78

Table 3: Inter-day precision during the analysis of authentic samples two QC's

	Theoretical concentration (µg mL^{-1})	
	0.070	**7.000**
1	0.615	7.193
2	0.661	7.431
3	0.661	6.977
4	0.615	7.375
5	0.683	7.034
6	0.655	7.431
Mean recovered conc. (µg mL^{-1})	0.648	7.240
STDEV	0.028	0.202
CV%	4.244	2.970

The extraction yield (recovery) was calculated by comparing extracted samples with unextracted samples at two different concentration levels. Absolute recovery of valsartan from plasma was found to be greater than 88%.

The limit of quantification of valsartan in this assay was 0.2 µg mL^{-1}. The retention time of the drug in the standard and the study samples were identical. There were no peaks for endogenous compounds that appeared at the same retention time for valsartan in the chromatograms for six different blank plasma samples.

Evaluation of the effect of short-term storage of extracted plasma samples on the standard curve characteristics and chromatographic behavior of valsartan and internal Standard were also performed. The chromatographic behavior was also unaffected by storage of extracted plasma samples in auto sampler at room temperature for 12 hrs. Freeze-thaw analysis of 3 cycles did not show any major degradation of valsartan. The difference in the drug concentration in all samples in the two analyses was always less than 5 % in each sample.

Dissolution study

The in-vitro release of the valsartan from Lasaromep® was within the acceptable level reported by the profile for valsartan. Based on the value of the similarity factor (*f2*) it can be concluded that the dissolution profiles of the two products are similar as stated by the Center of Drug Evaluation and Research (CDER) at the FDA (*f2* for the two profiles = 78).

3.3. Results of pharmacokinetic study

Overlay graph of mean concentration v/s time curve of the two formulations (Test and Reference) is shown in Figure 3. Descriptive statistics of the major mean pharmacokinetic parameters AUC0–24, AUC0–∞, Cmax, Tmax, Ke and t1/2 for the test and reference formulations are summarized in Tables 4 and 5. The relative bioavailability of valsartan from test tablet (Lazaromep®, 320 mg valsartan, MEPACO) compared to reference tablet (Tareg® , 320 mg valsartan, Novartis) was found to be 95.059% and 97.52% for AUC0–24, AUC0–∞ respectively and the parametric 90% confidence intervals for those pharmacokinetic parameters values were 86.96 - 104.42 for AUC0–24 , 87.9 – 110.27 for AUC0–∞ and 97.4 – 103.84 for Cmax. All values lie entirely within the FDA specified bioequivalent limit (80–125%). ANOVA results showed no significant effects of formulations, subjects or sequence on AUC0–24, AUC0–∞ or Cmax.

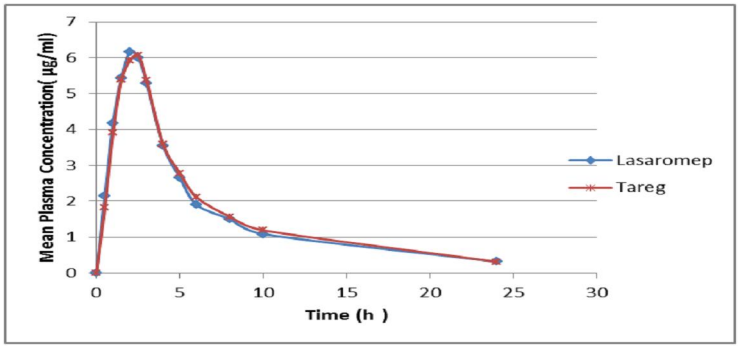

Figure 3: Mean Plasma Concentration Time Curve for Lasaromep and Tareg tablet Products

Table 4: Pharmacokinetic parameters calculated for valsartan after a single oral dose administration of test tablet (320 mg valsartan, MEPACO) to 12 healthy male volunteers.

Pk Parameter	AUC_{0-t}	$AUC_{0-\infty}$	AUC_{0-t} / $AUC_{0-\infty}$	Cmax	tmax	t1/2	Ke
Unit	**µg /ml *hr**	**µg /ml *hr**	**%**	**µg /ml**	**hr**	**hr**	**hr^{-1}**
Mean	37.81	42.55	88.36	6.80	2.25	7.60	0.10
Min	25.55	29.43	76.99	4.26	1.50	4.09	0.06
Max	59.92	62.04	96.58	8.93	3.00	11.45	0.17
SD	9.60	8.77	5.63	1.35	0.50	2.10	0.03
SEM	2.77	2.53	1.62	0.39	0.14	0.61	0.01

Table 5: Pharmacokinetic parameters calculated for valsartan after a single oral dose administration of reference tablet (320 mg valsartan, Novartis) to 12 healthy male volunteers.

Pk Parameter	AUC_{0-t}	$AUC_{0-\infty}$	AUC_{0-t} / $AUC_{0-\infty}$	C_{max}	t_{max}	$t_{1/2}$	K_e
Unit	**µg /ml *hr**	**µg /ml *hr**	**%**	**µg /ml**	**hr**	**hr**	**hr^{-1}**
Mean	39.78	43.63	91.28	6.77	2.25	7.52	0.12
Min	26.01	27.83	76.28	4.62	1.50	1.63	0.04
Max	61.61	63.66	97.99	8.65	3.00	17.90	0.43
SD	9.89	10.43	5.76	1.37	0.54	3.86	0.10
SEM	2.85	3.01	1.66	0.40	0.16	1.11	0.03

4. Conclusion

The validated HPLC method employed here proved to be simple, fast, reliable, selective and sensitive enough to be used in clinical pharmacokinetic studies of valsartan in humans. The statistical analysis of the results of AUC0–24, AUC0–∞ and Cmax using the ANOVA method showed that both the test tablet product (Lazaromep® 320 mg valsartan, MEPACO) and the reference tablet product (Tareg® , 320 mg valsartan, Novartis) are bioequivalent, since they deliver equivalent amounts of valsartan to the systemic circulation at equivalent rates that both AUC0–24h and Cmax ratios are within the 80-125% interval proposed by FDA [17].

Acknowledgment

Special Acknowledgments to Bioequivalence Center, October University of Modern Science and Arts, 6th 289 October City, Egypt, which provided us with the required equipments, chemicals in addition to human volunteers needed to achieve this work.

Authors' Statements

Competing Interests

The authors declare no conflict of interest.

Informed Consent & Ethical Approvals

The institutional and international ethical guides for experiments on human subjects were followed and informed consent was obtained.

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3/12/2013