



Simple Determination of Bosentan in Plasma Samples by Reversed-Phase High-Performance Liquid Chromatography

Zahra Khalighi ^{1,2}, Hori Ghaneialvar ^{2,3}, Armin Soltani ¹, Ali Khorshidi ⁴, Elahe Karimi ^{2*}, Ardeshir Moayeri ⁵, Naser Abbasi ^{2,6}, Masoumeh Tahmasebi ⁷, Ali Aidy ^{8*}

1. Department of Internal Medicine, School of Medicine, Shahid Mustafa Khomeini Hospital, Ilam University of Medical Sciences, Ilam, Iran

2. Biotechnology and Medicinal Plants Research Center, School of Medicine, Ilam University of Medical Sciences, Ilam, Iran

3. Department of Clinical Biochemistry, School of Medicine, Ilam University of Medical Sciences, Ilam, Iran

4. Department of Epidemiology and Biostatistics, School of Medicine, Ilam University of Medical Science, Ilam, Iran

5. Department of Anatomy, School of Medicine, Ilam University of Medical Sciences, Ilam, Iran

6. Department of Pharmacology, School of Medicine, Ilam University of Medical Sciences, Ilam, Iran

7. Department of Emergency Medicine, School of Medicine, Ilam University of Medical Sciences, Ilam, Iran

8. Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Abstract

Background: In order to measure the plasma levels of Losartan and Bosentan, a sensitive Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) technique was developed.

Methods: To compare bioavailability, the Area Under the Curve (AUC), peak plasma concentration (C_{max}), and time to C_{max} (T_{max}) were employed. The standard curve (150-2400 ng/ml) was linear (R²=0.999), relative errors were between 2.4 to 10.05% and the coefficient of variation (CV%) ranged from 1.52 to 10.88. A single dosage (test and reference) was used for the *in vivo* investigation, which involved 16 healthy individuals.

Results: The AUC₀₋₄₈, AUC₀₋, C_{max}, and T_{max} of the test and reference had no statistically significant differences. The C_{max} and 95% confidence intervals of the ratio of C_{max} of the two formulations were 0.93-0.96 and 97.6-135%, respectively.

Conclusion: Therefore, it was established that generic Bosentan was equivalent to Bosentan from Actelion and that both medications could be regarded as equally effective in clinical settings. The blood level of Bosentan could be measured using this straightforward procedure in all hospital laboratories.

Keywords: Bioequivalence, Bosentan, High performance liquid chromatography, Losartan

To cite this article: Khalighi Z, Ghaneialvar H, Soltani A, Khorshidi A, Karimi E, Moayeri A, *et al.* Simple Determination of Bosentan in Plasma Samples by Reversed-Phase High-Performance Liquid Chromatography. Avicenna J Med Biotech 2024;16(2):104-110.

* Corresponding authors:

Ali Aidy, Ph.D., Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Elahe Karimi, Ph.D., Biotechnology and Medicinal Plants Research Center, School of Medicine, Ilam University of Medical Sciences, Ilam, Iran

Tel: +98 84 3223081

Fax: +98 84 32223081

E-mail:

ilamfarma@gmail.com,

Karimi-e@medilam.ac.ir

Received: 7 Aug 2023

Accepted: 25 Oct 2023

Introduction

Bosentan, whose chemical formula is 4-tert-butyl-N-[6-(2-hydroxy-ethoxy)-5-(2-methoxyphenoxy)-2-pyrimidin-2-ylpyrimidin-4-yl]benzenesulfonamide hydrate (C₂₇ H₂₉ N₅ O₆ S • H₂O), is one of the endothelin receptor antagonists (Figure 1). This drug treats chronic heart failure and Pulmonary Arterial Hypertension (PAH). Endothelin 1 acts on Endothelin A (ETA) and Endothelin B (ETB) receptors in the smooth muscles of pulmonary blood arteries, which this drug antagonizes in a competitive and specific manner, thus inducing significant vasoconstriction ^{1,2}. Bosentan's affinity for

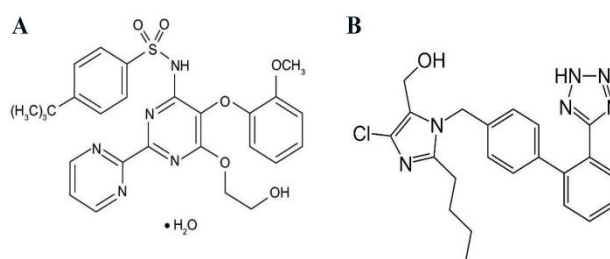


Figure 1. Chemical structure of Bosentan (A) and Losartan (B).

ETA receptors is somewhat greater than for ETB receptors³. The amount of Bosentan in biological samples has been estimated using methods such as gradient Reverse Phase-High Performance Liquid Chromatography (RP-HPLC), Solid Phase Extraction Liquid Chromatography-tandem Mass Spectrometry (SPE-LC-MS/MS), and UV-visible spectrophotometric methods, which are costly and challenging to implement⁴⁻⁸.

Losartan is one of the less expensive medications frequently used to treat hypertension. Additionally, it can be used with Bosentan to improve PAH⁹. Several methods have been demonstrated for the Losartan potassium determination substance in tablets including Supercritical Fluid Chromatography (SFC), Capillary Electrophoresis (CE)¹⁰, and High-Performance Thin-Layer Chromatography (HPTLC)¹¹. In biological samples, the active component has been determined by HPLC, UV detection¹², fluorescence detection¹³, and liquid chromatography-electrospray ionization tandem mass spectrometry¹⁴.

For the purpose of analysing Bosentan in bodily fluids, many HPLC techniques have been reported¹⁵⁻¹⁷. Bioavailability is the proportion of the drug that reaches the circulation after passing through the barriers to absorption and metabolism¹⁸. Because the characteristics of body structure vary from person to person in terms of absorption and metabolism, it is important to measure the bioavailability of drugs, even for similar medications¹⁹.

The objective of this work was to develop a straightforward, quick, sensitive, economical, specific, and reliable HPLC assay for the quantitation of Bosentan concentrations in plasma. A bioavailability study was carried out using the established methodology. So, a further aim of the present study was to compare the relative bioavailability of generic Bosentan tablet preparation, manufactured by Osve Pharmaceutical Company, an Iranian pharmaceutical company, with that of the Tracleer® (125 mg tablet, Actelion) and quantify Bosentan at the same time with internal standard Losartan.

Materials and Methods

Reagents

Bosentan (125-mg tablets, Batch Num: 019) was purchased from Osve Pharmaceutical Company, Iran, and Tracleer® (Actelion). The standard Bosentan and Losartan and all solvents and chemicals (HPLC GRADE) were purchased from Sigma (Missouri, USA), and Merck (Germany).

Chromatographic conditions

The analysis was performed with HPLC equipped with a PDA detector and a binary dual pump. After selecting the appropriate internal standard and wavelength, the other parameters of the HPLC method were investigated. A German-made C₁₈ column (250×4.6 mm, Knauer, Platin blue, Germany). was used for chromatographic separation. The mobile phase includ-

ed methanol with a pH of 6.4 and 0.1% formic acid. The injection loop capacity was 50 µl, the mobile phase flow rate was 1 ml/min, and the effluent was seen at 270 nm. The drug peak area ratios to the internal standard Losartan measurement were used to accomplish quantification.

Calibration Procedure

To create concentrations of 150, 300, 600, 1200, and 2400 ng/ml, respectively, drug-free plasma (1 ml), 20 µl of internal standard (Losartan, 100 µg/ml), and volumes of 2, 4, 8, 16, and 24 µl of standard Bosentan solution (1 mg/ml, 75 µg) were added to each capped tube. The 100 ml of sample plasma was then mixed with 200 ml of acetonitrile, vortexed for one min with a stirrer, then centrifuged at 12000 rpm for ten min. The mean peak area ratios of the drug were chosen as the device response after isolating and filtering, and 50 µl of the supernatant was then injected into the device (three times). At 150-2400 ng/ml Bosentan, a linear relationship between concentration and method response was seen. The coordinates of the linear regression analysis are as follows:

$$y = 2745.x - 4123$$

$$R^2 = 0.999$$

Precision

Within-day variability: The same drug concentrations used in the standard curve were produced for plasma. On the same day, preparations were made and injections into the HPLC column were made of five samples of each concentration (150, 300, 600, 1200, and 2400 ng/ml, Losartan, 100 µg/ml; respectively) (Table 1A).

Between-day variability

Losartan was synthesised at concentrations of 150, 300, 600, 1200, and 2400 ng/ml, and five replicates of each concentration were monitored daily for four days in a row. Each sample was injected into the HPLC column in a volume of 50 µl. The standard curve equation was then used to compute the equivalent concentration for each sample, and the precision and accuracy of the procedure were determined by the percentage of variation coefficient and error percentage (Table 1B).

Limit of Quantitation (LOQ) and Limit of Detection (LOD)

By comparing the signal-to-noise ratio with known analyte concentrations to the outcomes of the blank sample, LOQ and LOD indices were calculated. The following was found to be the error % and variance coefficient percentage:

- 100 [actual concentration/(actual concentration-calculated concentration)]=error percentage
- 100 (mean/standard deviation)=percentage of variation.

The same calibration curve concentrations (150, 300, 600, 1200, and 2400 ng/ml, Losartan, 100 µg/ml) were prepared. The samples were dissolved in 100 µl mobile phase before being injected into the HPLC five times with 50 µl each. Free plasma samples-those devoid of medication and internal standards-were then made into a matrix. Then, a drug aqueous solution was

HPLA Assay and Determination of Bosentan

Table 1. Within- and between-days variability for determination of Bosentan in human plasma, a: between days, b: within days

a: within days													
Concentration (ng/ml)		Mean	S.D.	C.V%	Error%								
150		153.6	3.48	2.26	2.4								
300		313.2	6	1.91	4.4								
600		558	8.52	1.52	7								
1200		1113.6	121.2	10.88	7.2								
2400		2158.8	96.12	4.45	10.05								

b: between day													
Day	First day					Second day				Third day			
Conc. (ng/ml)	Mean	S.D.	C.V%	Error%	Mean	S.D.	C.V%	Error%	Mean	S.D.	C.V%	Error%	
150	147.6	9.12	6.18	1.6	153.6	9.12	5.93	2.4	153.6	3.48	2.26	2.4	
300	357.6	12.48	3.49	19.2	346.8	45	12.97	15.6	313.2	6	1.91	4.4	
600	625.2	30	4.79	4.2	592.8	18.4	3.09	1.2	558	8.52	1.52	7	
1200	1213.2	109.44	9.02	1.1	1108.8	135.6	12.22	7.6	1113.6	121.2	10.88	7.2	
2400	2589.6	12.48	0.48	7.9	2589.6	172.8	6.67	7.9	2158.8	96.12	4.45	10.05	

added to each tube, and injections were given at doses of 0.625, 1.25, 1.87, 2.5, 5, 7.5, and 10 $\mu\text{g/ml}$.

In vivo study design: Losartan at five distinct drug concentrations in plasma (150, 300, 600, 1200, and 2400 ng/ml , Losartan, 100 $\mu\text{g/ml}$) were prepared and injected into HPLC. A matrix similarity test was also conducted. The tubes were then filled with a 50 μl aqueous solution of the medication, which was then injected. The below curve area was then read, and the extraction % was computed. The volunteer sample tubes were submerged in a 37°C, 10 min hot water bath. The internal standard solution (50 μl) was added to 0.5 ml of the plasma samples before being centrifuged. Each tube was then filled with 5 ml of dichloromethane, vortexed for 2 min, then centrifuged at 8,000 rpm. Following the aspiration of the aqueous phase and the nitrogen gas flow separation of the organic phase, 200 μl of the mobile phase was added and mixed for 30 s. The prepared sample (50 μl) was injected, and the sample concentration was calculated by comparing the internal standard to the drug's sub-peak level and dividing by the standard curve.

Bioavailability: The human studies ethics committee of the Ilam University of Medical Sciences has given the study approval (Ethical code: IR.MEDILAM.REC. 1398.78). 32 participants were chosen, all of them were between the ages of 20 and 30. The use of different drugs were prohibited for the participants' for two weeks before to and throughout the test.

Numerous clinical and paraclinical assays were performed, including urea, creatinine, alkaline phosphatase, SGOT, SGPT, and FBS. After fasting for the previous night, a blood sample was collected (control at time zero) prior to receiving the single dose of the pill. Next, a four-hour food restriction (save for water) and a 125 mg Bosentan pill were given to each participant.

Blood samples were collected at 0.5, 1.5, 3, 6, 8, 12, 18, 24, and 48 hr after receiving a single dosage of the medication. 32 individuals had their blood tested to determine the drug's maximum plasma concentration (C_{max}) (measurement was done at the C_{max} time).

Analysis of pharmacokinetic: Area Under the Curve (AUC) AUC_{0-12} , peak plasma concentration (C_{max}), $\text{AUC}_{0-\infty}$, time to C_{max} (T_{max}) and $T_{1/2}$ were computed after analysing the volunteer's plasma samples using the standard curve by HPLC using the non-compartmental technique. The slope of the natural logarithm (ln) curve, plotting the drug plasma concentration across time was used to determine the elimination rate constant (K_{el}). By dividing 0.693 by K_{el} , the drug's elimination half-life was estimated. The linear trapezoidal rule was used to calculate the AUC to the last plasma concentration (AUC_{0-t}). The formula for calculating the area under the extrapolated curve to infinity ($\text{AUC}_{0-\infty}$) is $\text{AUC}_{0-t} + C_t/k_{\text{el}}$, where C_t is the last concentration that can be measured. Examination of the individual drug plasma concentration-time profiles led to the determination of C_{max} and T_{max} .

Statistical analysis

AUC_{0-t} , $\text{AUC}_{0-\infty}$, and C_{max} were regarded as key variables for bioequivalence study. To evaluate the impact of formulations, times, sequences, and participants on these parameters, a two-way ANOVA for crossover design was employed. For a p-value of 0.05 or below, a difference between two related values was deemed statistically significant. The 90% confidence intervals of the ratio of pharmacokinetic parameters of test to reference products as well as those which were transformed logarithmically were also estimated²⁰. Using SPSS 10, all statistical analyses were carried out.

Results

HPLC assay

Human plasma chromatograms in blank form are shown in figure 2. The Bosentan ratio to Losartan sub-peak level by HPLC was plotted against standard concentrations (150, 300, 600, 1200, and 2400 ng/ml) using the standard curve ($y=2745. x-4123.9$, $R^2=0.9995$). There was no interference at the Bosentan or Losartan retention times. To determine the peak height or peak area of standard curves, two distinct resolved peaks without peak tailing formed after elution. Retention times for Losartan and Bosentan were 4.863 and 2.827 min, respectively at the mobile phase optimum flow rate (1 ml/min). The minimum diagnosable concentration after testing the descending series of the concentration of Bosentan was a concentration with less than 20% error, which was calculated to be 75 ng/ml (Figure 3). Figure 4 shows the concentration of Bosentan in the plasma of a healthy subject after 4.5 hr ingestion of Bosentan tablets.

Within- and between-day variability

The findings of the intra- and inter-day variability are shown in the table 1. Within 2.4 to 10.05% and 1.52 to 10.88, respectively, were the relative errors and CV %. Table 2 displays the findings of solvent-based drug extraction from plasma. More than 93% of the medication is extracted at all concentrations.

In vivo studies

The physical health of the volunteers was assessed throughout the whole trial, and those who had any disorders were disqualified. Figure 5 depicts the mean concentration-time profile following oral administration of Bosentan Tracleer® (Actelion) and (Osve). Ta-

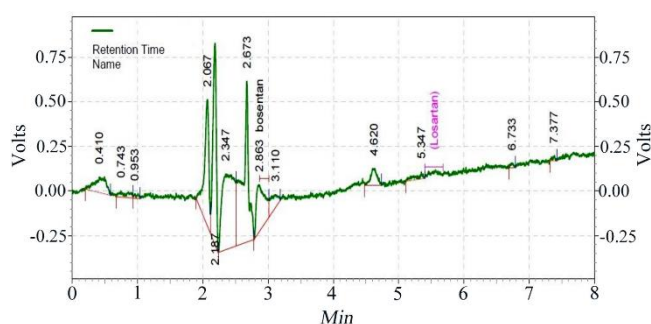


Figure 2. Chromatogram of the blank sample.

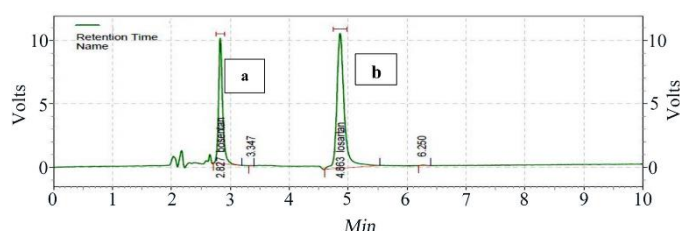


Figure 3. Chromatogram of the standard sample containing Bosentan (a) and internal standard Losartan (b) at a concentration of 100 ng/ml.

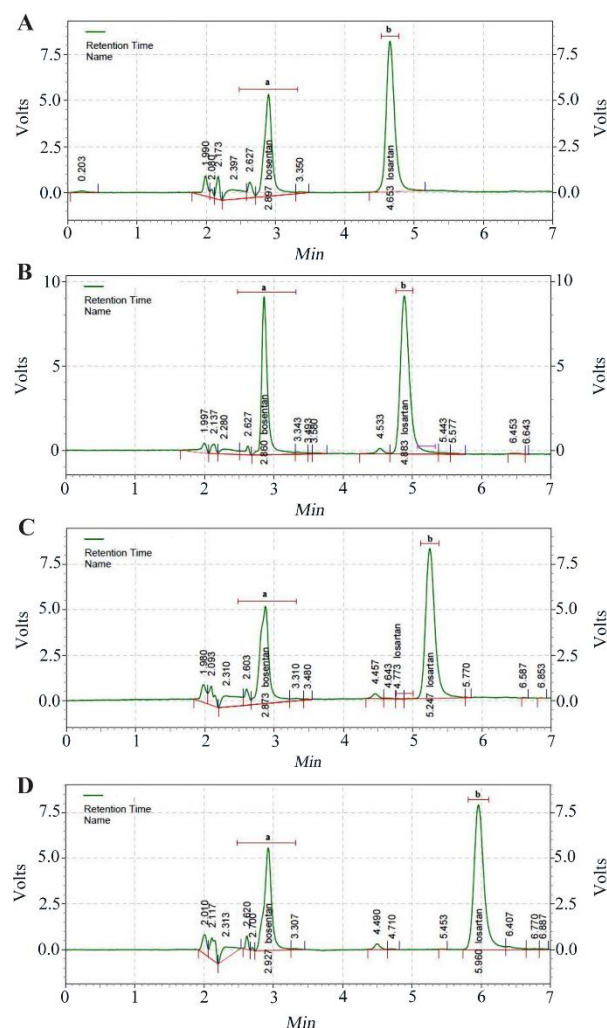


Figure 4. Chromatograms of the volunteers' serum sample analysis (A, B, C, D) containing Bosentan (a) and internal standard Losartan (b) 4.5 hr after ingestion of Bosentan tablet.

Table 2. Bosentan extraction percentage from plasma samples

Concentration (ng/ml)	Extraction average	Standard deviation
150	95.1	5.4
300	93.4	7.1
600	96.5	4.8
1200	93.4	7
2400	95.6	3.8

ble 3 provides a summary of the mean pharmacokinetic parameters for two different brands of Bosentan. The maximum plasma concentration (C_{max}), as determined by the HPLC data, is attained around 3 hr after oral administration. No discernible difference between the two formulations was seen after data log transformation ($p>0.05$). The ratio of C_{max} for the two formulations' 95 percent confidence intervals ranged from 97.6 to 135% (Table 3).

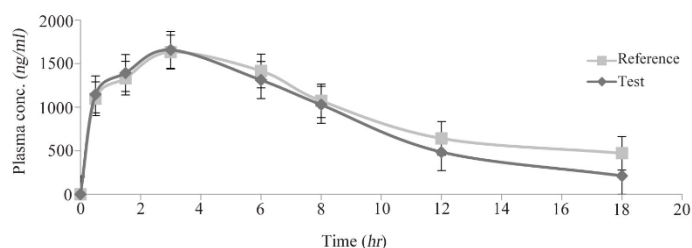


Figure 5. The mean plasma Bosentan levels vs. time profiles following ingestion of a single dose of two 125 mg for a reference (Tracleer®) and test (Osve) products to 32 healthy volunteers. Data are shown as mean±SD.

plasma concentration of Bosentan (150–2400 ng/ml, $R^2=0.9995$), a linear relationship was seen.

In this study the Bosentan detection limit was about 50 ng/ml (signal-to-noise ratio of 3:1) and the quantitation limit with a coefficient of variation of less than 10% was 300 ng/ml using a 0.5 ml plasma sample which was comparable to previously reported methods using differential pulse voltammetry^{32,33}, UHPLC UV method³⁴ or UV-visible spectrophotometer³⁵. Bosentan was detectable in all individuals at the initial sample time (30 min) and five half-lives. An essential aspect of HPLC test is assay accuracy, which is increased

Table 3. Plasma concentration after oral administration of Bosentan and Tracleer® (125 mg)

Parameter	Test (Mean±SD)	Ref (Mean±SD)	Test/Ref (Mean±SD)	CI95% Test/Ref	p-value
C_{max} (μg/ml)	1656.4±120.4	1631.8±105.1	1.01±1.14	0.976-1.35	0.42
Log C_{max}	3.23±2.08	3.21±2.02	1.01±1.02	0.11-0.197	0.59
AUC_{0-48} (μg.h/ml)	22813.04±3435.84	26947.38±3332.76	0.82±1.05	0.88±0.98	0.62
Log AUC_{0-48}	4.36±3.54	4.43±3.52	0.98±1.01	0.02-0.01	0.165
$AUC_{0-\infty}$ (μg.h/ml)	23518.6±3542.1	27780.8±3237.7	0.85±1.09	0.911-1.015	0.632
Log $AUC_{0-\infty}$	4.37±3.55	4.38±3.51	0.99±1.01	0.13-0.11	0.291
T_{max} (hr)	2.81±0.57	2.9±0.82	0.97±0.7	*	*
$T_{1/2}$ (hr)	6.18±0.51	5.82±0.67	1.06±0.07	*	*

Discussion

Bosentan, a non-peptidic endothelin receptor antagonist, is one of the drugs used to treat hypertension and chronic heart failure²¹. Daily drug usage can lead to drug buildup in the blood, interact with other medicines, and have negative consequences²². Therapeutic Drug Monitoring (TDM), which can change the pharmacokinetics of the drug in clinical settings, is therefore one of the ways to determine the therapeutic and adverse effects of pharmaceuticals²³. Bosentan plasma concentrations have been determined using several techniques, including LC-MS/MS^{4,24-27}. Even though Bosentan plasma determination has been previously reported by HPLC^{28,29}. Although the new HPLC machines have high repeatability injectors, the internal standard technique is utilized to mitigate the error of sample preparation and injection³⁰. However, it is crucial to establish a novel approach in order to more accurately detect Bosentan in plasma. Etodolac has been used as an internal standard to measure Bosentan in plasma in previous studies³¹. One of the most important uses of Bosentan is to control pulmonary blood pressure, and most patients use Losartan to control blood pressure, therefore, in this work Losartan was used as an internal standard¹⁵. Losartan (RT=4.863) and Bosentan (RT=2.827) chromatograms in this investigation lacked any interferences. When the peak area ratios of Bosentan to Losartan were plotted vs. the

by using internal standards. While reliability is the amount of inaccuracy between measurements, repeatability may be thought of separately under identical settings as the repeat estimations were done on a comparable subject³⁶. It is inevitable that there will be some inaccuracy in clinical measures, and the acceptable level will depend on particular circumstances³⁷⁻³⁹. The right statistical methods to determine this kind of repeatability are frequently ineffectual and have the potential to confuse and mislead therapeutic decision-making⁴⁰.

In the current investigation, 32 healthy volunteers who participated in a cross-over trial and administered 125 mg of Bosentan orally had their plasma concentrations measured. The results show that following oral drug administration, C_{max} was attained in 2.8 hr at 1656.4±120.4 (test) and 1631.8±105.1 μg/ml (reference), and no significant difference was seen.

The two oral formulations' C_{max} 95% confidence intervals ratio was 97 to 135%. Consistent with the findings of this investigation, T_{max} and $AUC_{0-\infty}$ correspond to the respective rate and extent of drug absorption, and C_{max} is related to both of these two processes⁴¹. Thus, in order to compare the bioavailability of the two oral final products, all indicated criteria were required between the test and reference AUC_{0-48} , and $AUC_{0-\infty}$ values, there was no statistically significant difference. For the test and form reference, the AUC

extrapolated percent (comparing AUC_{0-48} to $AUC_{0-\infty}$) was 4.37 and 4.38%, respectively. Therefore, the research design was sufficient to assess more than 90% of the AUC utilising the 48-hr sampling period. The AUC_{0-48} and $AUC_{0-\infty}$ for the two products did not differ significantly, indicating that the plasma profiles created by Tracleer® and Bosentan made by Osve Company were similar.

Conclusion

In conclusion, the devised approach was delicate enough to be used to the bioequivalence of Bosentan. According to estimated findings, Tracleer®, a product made by Actelion, and Bosentan tablets made by Osve Company are bioequivalent and both can be thought of as equally effective in clinical settings.

Acknowledgement

This study was supported by the Ilam University of Medical Sciences. The human studies ethics committee of the Ilam University of Medical Sciences has given the study approval (Ethical code: IR.MEDILAM.REC.1398.78).

Conflict of Interest

The authors declare no conflict of interest.

References

- Rubin LJ, Badesch DB, Barst RJ, Galie N, Black CM, Keogh A, et al. Bosentan therapy for pulmonary arterial hypertension. *N Engl J Med* 2002;346(12):896-903.
- Kiowski W, Sütsch G, Hunziker P, Müller P, Kim J, Oechslin E, et al. Evidence for endothelin-1-mediated vasoconstriction in severe chronic heart failure. *Lancet* (London, England). 1995;346(8977):732-6.
- van Giersbergen PL, Halabi A, Dingemans J. Single- and multiple-dose pharmacokinetics of Bosentan and its interaction with ketoconazole. *Br J Clin Pharmacol* 2002; 53(6):589-95.
- Parekh JM, Shah DK, Sanyal M, Yadav M, Shrivastav PS. Development of an SPE-LC-MS/MS method for simultaneous quantification of Bosentan and its active metabolite hydroxyBosentan in human plasma to support a bioequivalence study. *J Pharm Biomed Anal* 2012;70: 462-70.
- Atila A, Ozturk M, Kadioglu Y, Halici Z, Turkan D, Yayla M, et al. Development and validation of UFLC-MS/MS method for determination of Bosentan in rat plasma. *J Pharm Biomed Anal* 2014;97:33-8.
- Bhavya Sri K, Mounika CH. Development and validation of uv-visible spectrophotometric method for analysis of Bosentan in spiked human plasma. *International Journal of Current Pharmaceutical Research* 2019;11(4):108-10.
- Yokoyama Y, Tomatsuri M, Hayashi H, Hirai K, Ono Y, Yamada Y, et al. Simultaneous microdetermination of Bosentan, ambrisentan, sildenafil, and tadalafil in plasma using liquid chromatography/tandem mass spectrometry for pediatric patients with pulmonary arterial hypertension. *J Pharm Biomed Anal* 2014;89:227-32.
- Qiu X, Zhao J, Wang Z, Xu Z, Xu RA. Simultaneous determination of Bosentan and glimepiride in human plasma by ultra performance liquid chromatography tandem mass spectrometry and its application to a pharmacokinetic study. *J Pharm Biomed Anal* 2014;95:207-12.
- Li H, Zhang S, Tan B, Qiang Y, Li W, Chen S, et al. Investigation of Losartan Potassium as an eco-friendly corrosion inhibitor for copper in 0.5 M H₂SO₄. *Journal of Molecular Liquids* 2020;305(1):112789.
- Qiang Y, Guo L, Li H, Lan X. Fabrication of environmentally friendly Losartan potassium film for corrosion inhibition of mild steel in HCl medium. *Chemical Engineering Journal* 2021;406:126863.
- Zhao Z, Wang Q, Tsai EW, Qin XZ, Ip D. Identification of Losartan degradates in stressed tablets by LC-MS and LC-MS/MS. *J Pharm Biomed Anal* 1999;20(1-2):129-36.
- Soldner A, Spahn-Langguth H, Mutschler E. HPLC assays to simultaneously determine the angiotensin-AT₁ antagonist Losartan as well as its main and active metabolite EXP 3174 in biological material of humans and rats. *J Pharm Biomed Anal* 1998;16(5):863-73.
- Selvadurai M, Meyyanathan SN. Sensitive and accurate estimation of Losartan potassium formulation by high-performance thin-layer chromatography. *Pharm Methods* 2011;2(2):95-8.
- Iwasa T, Takano T, Hara K, Kamei T. Method for the simultaneous determination of Losartan and its major metabolite, EXP-3174, in human plasma by liquid chromatography-electrospray ionization tandem mass spectrometry. *J Chromatogr B Biomed Sci Appl* 1999;734 (2):325-30.
- Anandakumar K, Jambulingam M, Rmaesh J, Subarla SJ, Sangeetha P, Raja M. Development and validation of RP-HPLC method for the dissolution study of bosentan in bulk and in pharmaceutical dosage form. *Current Journal of Applied Science and Technology* 2018;18(2).
- Weber C, Schmitt R, Birnboeck H, Hopfgartner G, van Marle SP, Peeters PAM, et al. Pharmacokinetics and pharmacodynamics of the endothelin-receptor antagonist bosentan in healthy human subjects. *Clin Pharmacol Ther* 1996;60(2):124-37.
- Khan MA, Sinha S, Todkar M, Parashar V, Swamy Reddy K. Development and validation of a stability indicating analytical method for the related substances of Bosentan drug substance by HPLC. *American Journal of Scientific and Industrial Research* 2012;3(2):69-80.
- Seth P. An in-vivo bioequivalence study of a new nifedipine extended release dosage form, 'Opticaps'. *Drug Development and Industrial Pharmacy* 2008;20(9): 1605-12.
- Emami J, Varshosaz J, Falamarzian M, Tahvilian R. High performance liquid chromatographic determination, pharmacokinetic and comparative bioavailability studies of cisapride. *J Pharm Biomed Anal* 2003;33(3):513-20.
- Bhadoriya A, Dasandi B, Parmar D, Shah PA, Shrivastav PS. Quantitation of tadalafil in human plasma using a

- sensitive and rapid LC-MS/MS method for a bioequivalence study. *J Pharm Anal* 2018;8(4):271-6.
21. Lausecker B, Hess B, Fischer G, Mueller M, Hopfgartner G. Simultaneous determination of Bosentan and its three major metabolites in various biological matrices and species using narrow bore liquid chromatography with ion spray tandem mass spectrometric detection. *J Chromatogr B Biomed Sci Appl* 2000;749(1):67-83.
 22. Tanaka S, Uchida S, Hakamata A, Miyakawa S, Odagiri K, Inui N, et al. Simultaneous LC-MS analysis of plasma concentrations of sildenafil, tadalafil, bosentan, ambrisentan, and macitentan in patients with pulmonary arterial hypertension. *Die Pharmazie* 2020;75(6):236-9.
 23. Markert C, Schweizer Y, Hellwig R, Wirsching T, Riedel KD, Burhenne J, et al. Clarithromycin substantially increases steady-state Bosentan exposure in healthy volunteers. *Br J Clin Pharmacol* 2014;77(1):141-8.
 24. Al-Ghazawi M, Tutunji M, Aburuz S. Simultaneous determination of sildenafil and N-desmethyl sildenafil in human plasma by high-performance liquid chromatography method using electrochemical detection with application to a pharmacokinetic study. *J Pharm Biomed Anal* 2007;43(2):613-8.
 25. Ma B, Shang X, Zhang Q, Li J, Liu Y, Cao X, et al. Rapid analysis of tadalafil in human blood plasma and seminal plasma by liquid chromatography/tandem mass spectrometry. *J Pharm Biomed Anal* 2013;77:149-57.
 26. Nirogi R, Kandikere V, Komarneni P, Aleti R, Padala N, Kalaikadhiban I. LC-ESI-MS/MS method for quantification of ambrisentan in plasma and application to rat pharmacokinetic study. *Biomed Chromatogr* 2012;26(10):1150-6.
 27. Enderle Y, Witt L, Wilkens H, Grünig E, Haefeli WE, Burhenne J. Simultaneous quantification of endothelin receptor antagonists and phosphodiesterase 5 inhibitors currently used in pulmonary arterial hypertension. *J Pharm Biomed Anal* 2017;143:291-8.
 28. Lavudu P, Rani AP, Chander AP, Bala sekaran C. Determination of bosentan in pharmaceutical dosage forms by high performance liquid chromatography. *International Journal of Drug Delivery* 2013;5(2):146-51.
 29. Selvadurai Muralidharan, Kumar JR. Simple estimation of bosentan in tablet formulation by RP-HPLC. *American Journal of Analytical Chemistry* 2012;3(11): 715-8.
 30. Ohtaka R, Maeda M, Iwagami T, Ueda T, Kimura Y, Imai K, et al. [Precision of internal standard method in HPLC analysis]. *Yakugaku Zasshi* 2003;123(5):349-55. Japanese.
 31. Mannam RaYI. Estimation of bosentan monohydrate in male rabbit plasma by using RP-HPLC method. *Journal of Applied Pharmaceutical Science* 2017.
 32. Atila A, Yilmaz B. Determination of bosentan in pharmaceutical preparations by linear sweep, square wave and differential pulse voltammetry methods. *I ran J Pharm Res* 2015;14(2):443-51.
 33. Sajedi-Amin S, Asadpour-Zeynali K, Khoubnasabjafari M, Rashidi F, Jouyban A. Development and validation of ultrasound assisted and dispersive liquid-liquid microextractions combined with HPLC-UV method for determination of bosentan in human plasma and urine. *Journal of the Brazilian Chemical Society* 2017;28(5):868-77.
 34. Jatczak M, Sidoryk K, Kossykowska M, Luniewski W, Zagrodzka J, Lipiec-Abramska E. Development and validation of a UHPLC UV method for the in-process control of bosentan monohydrate synthesis. *Chromatographia* 2016;79(17):1131-41.
 35. Dey S. Method development and estimation of Bosentan monohydrate in bulk and pharmaceutical dosage forms using UV-Visible Spectrophotometer. *Journal of Pharmacy Research* 2011;4(6):1713-5.
 36. Plana JC, Galderisi M, Barac A, Ewer MS, Ky B, Scherrer-Crosbie M, et al. Expert consensus for multimodality imaging evaluation of adult patients during and after cancer therapy: a report from the American Society of Echocardiography and the European Association of Cardiovascular Imaging. *J Am Soc Echocardiogr* 2014; 27(9):911-39.
 37. Bartlett JW, Frost C. Reliability, repeatability and reproducibility: analysis of measurement errors in continuous variables. *Ultrasound Obstet Gynecol* 2008;31(4):466-75.
 38. Shammass HJ, Hoffer KJ. Repeatability and reproducibility of biometry and keratometry measurements using a noncontact optical low-coherence reflectometer and keratometer. *Am J Ophthalmol* 2012;153(1):55-61.e2.
 39. Galderisi M, Henein M, D'Hooge J, Sicari R, Badano L, Zamorano J, et al. Recommendations of the European Association of Echocardiography How to use echo-Doppler in clinical trials: Different modalities for different purposes. *Eur J Echocardiogr* 2011;12:339-53.
 40. Watson PF, Petrie A. Method agreement analysis: a review of correct methodology. *Theriogenology* 2010;73(9):1167-79.
 41. Karthikeyan K, Mahat MY, Chandrasekaran S, Gopal K, Franklin PX, Sivakumar BJ, et al. Bioanalytical method development, validation and quantification of dorsomorphin in rat plasma by LC-MS/MS. *Biomed Chromatogr* 2013;27(8):1018-26.