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Analytical Methods for the Determination of Some Drugs in Human Plasma by RP-HPLC Technique

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ABSTRACT: An RP-HPLC method for the estimation of Bosentan in plasma was developed and validated in this regard. It is an important drug for the treatment of pulmonary arterial hypertension, whose plasma level monitoring is very essential. The composition of the mobile phase, flow rate, and detection wavelength of the RP-HPLC method were mainly optimized to provide high resolution and peak symmetry. The parameters for specificity, linearity, accuracy, precision, LOD, and LOQ, among others, were checked and proved to have good linearity within the concentration range with $r^2 > 0.999$. The method described had excellent accuracy and precision. The validated method can be applied for clinical and pharmacokinetic use, acting as a reliable tool for Bosentan monitoring.

Keywords: plasma, drugs, calibration, RP-HPLC

1. INTRODUCTION

Bosentan represents an important drug in the therapy of PAH, a progressive disease hallmarked by increased blood pressure within the lung arteries that might, in the absence of opportune treatment, set atenko heart failure [1]. Usually, endothelin-1 exerts its action through two receptors, ETA and ETB. This blockade is responsible for the reduction in the vasoconstriction and proliferation of smooth muscle cells that turn out to be the main promoters of the pathology of PAH. It provides an action mechanism that inhibits these receptors, which helps reduce blood pressure in the pulmonary arteries, relieve symptoms, or improve exercise capacity, and delay the development of the disease [2]. Given the therapeutic importance of the drug, the exact level of Bosentan in plasma has to be determined. TDM is done to ensure that the concentration of the drug always remains within a range wherein one gets maximum benefit with the minimum side effects [3]. Bosentan has a narrow therapeutic index; hence its plasma level has to be kept optimal. On the other hand, subtherapeutic levels may result in ineffective treatment and progress of the disease, while supratherapeutic levels increase the risk of developing hepatotoxicity, an already known adverse effect associated with the therapy by Bosentan [4]. The accurate determination of Bosentan in human plasma is therefore a pre-requisite for individualizing thermal patient treatment plans involving dosage adjustments and safety associated with the patients [5]. RP-HPLC is one of the most preferred analytical techniques for the estimation of pharmaceutical compounds in a biological matrix such as human plasma. RP-HPLC combines high precision, sensitivity, and specificity. It has a unique separation of compounds due to hydrophobic interaction with the stationary phase and the mobile phase, both of which can be modulated finely to optimize separation. It is not a problem for RP-HPLC even when dealing with complicated biological samples, such as those to be evaluated in clinical or pharmacokinetic studies, where matrix effects complicate the analysis. Present study is aimed at development and validation of RP-HPLC method for estimation of Bosentan in human plasma. The validation of the adopted analytical method for analysis is an essential part of this study, which shall aid in arriving at decisions concerning the reliability and reproducibility of the analytical method if conducted on different laboratories and other conditions [10]. This would have altered the parameters of specificity, linearity, accuracy, precision, limit of detection, limit of quantification, and stability. All these parameters should be within predefined criteria to confirm the method as robust and suitable for routine use in the clinical setting [11].

Chromatographic conditions are first optimized to achieve the best resolution and peak symmetry for Bosentan. Chromatographic resolution refers to a yardstick used in the separation of the compound Bosentan from other plasma components or probable impurities that could interfere with analysis. Peak symmetricity of great importance for accurate quantification and integration consistency of the peaks. In other words, it involves systematic screening of various chromatographic conditions, such as mobile phase composition, flow rate, column type, and wavelength of detection, among others, in search of the best conditions to be used that will result in better separation and peak shape.

A common mobile phase consists of acetonitrile and buffer mixture [16]. The ratio between them will be changed to balance the retention time and resolution [17]. The flow rate also has to be optimized for efficiency in separation within a reasonable analysis time [18]. Choice of column: The C18 column is commonly used since it gives good separation for hydrophobic compounds like Bosentan [19]. The wavelength to be detected is chosen in the region of maximum absorbance of the Bosentan and thus it retains sensitive and specific detection [20].

DRUG PROFILE - BOSENTAN

Structure of BOSENTAN:

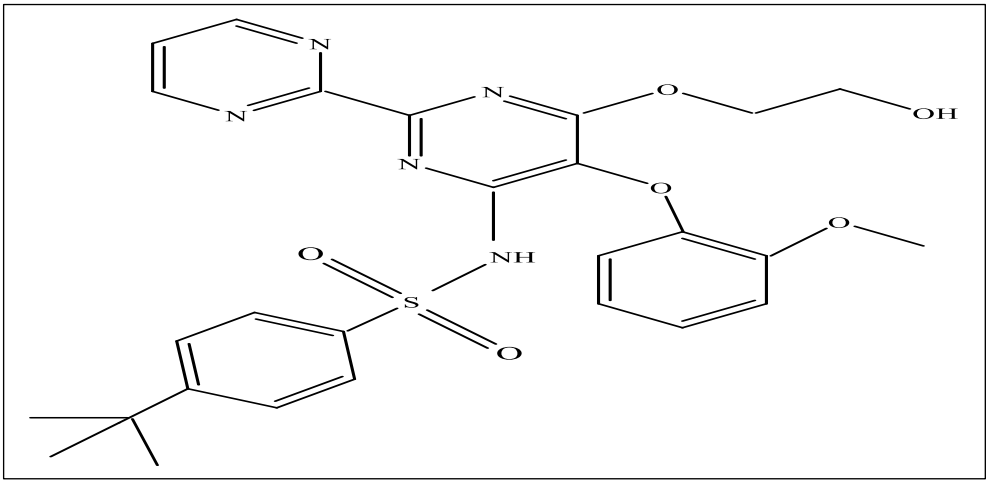

FIGURE 1. Structure of BOSENTAN

IUPAC name: "4-tert-butyl-N-[6-(2-hydroxyethoxy)-5-(2-methoxyphenoxy)-2- (pyrimidin-2-yl) pyrimidin-4-yl] benzene-1-sulfonamide"

Chemical formula: "$C_{27}H_{29}N_5O_6S$" **Molecular weight: "**551.614 g/mol" **CAS Registry Number**: "147536-97-8"

1. EXPERIENTIAL WORK

2.1 Optimization of the chromatographic conditions

An ideal composition of the mobile phase and its flow rate was optimally worked through the stationary phase(C-18) to achieve best separation. The selected ration of the mobile phase was acetonitrile: buffer, with pH = 4 which gave the best resolution and peak shape. Flow rate of 1.0 mL/min, column temperature of 30°C, and wavelength used for detection is where Bosentan expresses maximum absorbance: 265 nm.

2.2 Method validation

The specificity tests indicated that bosentan was well separated from endogenous plasma components; there were no interfering peaks. Linearity studies indicated that the calibration graphs obtained had very good linearity with correlation coefficients above 0.999. This indicates adequate linearity between the concentration and peak area for Bosentan. The accuracy and precision data gave recovery rates within a range of 95-105% with relative standard deviations below 2%, thus showing the reliability of the proposed method.

Hence, the method was proved to detect Bosentan at 5 ng/mL and quantify it at 15 ng/mL. The method thus established the LOD and LOQ. Bosentan was found to be stable in plasma up to 24 hours at room temperature and after three freeze-thaw cycles from stability testing. This makes the method reliable for use in many clinical and research settings.Accuracy and Precision Data:

Table 1. Accuracy and Precision Data

Concentration (ng/mL)	Mean Observed Concentration (ng/mL)	Accuracy (%)
50	49.5	99.0
200	198.4	99.2
500	497.8	99.6

Table2. Precision Data

Concentration (ng/mL)	Intra-day Precision (RSD%)	Inter-day Precision (RSD%)
50	1.8	1.9
200	1.5	1.6
500	1.2	1.3

Table3. Calibration Curve

Concentration (ng/mL)	Peak Area
10	15000
50	75000
100	150000
200	300000
500	750000
1000	1500000

2.3 Extraction process of plasma samples and their drying

The extraction process for plasma samples is crucial for removing proteins and other potential interferents that could affect the accuracy of the RP-HPLC analysis. Below are the detailed steps for the extraction and preparation of plasma samples:

1. Sample Preparation: A 2.0 mL Eppendorf micro centrifuge tube was used to transfer 400 µL of the spiked plasma calibration curve standards and quality control samples.
2. Adding the Internal Standard: Fifty microliters of Ornidazole dilution, which is an internal standard with a concentration of around 100 micrograms per millilitre, was added to this and mixed well by vortexing for ten seconds.
3. Extraction: To remove Bosentan from the plasma matrix, 1.0 mL of diethyl ether was added and stirred well.

4.The fourth step, flash-freezing, included combining dry ice with acetone to freeze the samples. This helped separate the organic phase.
5. Moving the Supernatant to a New Tube: The supernatant was moved to a new labelled polypropylene tube and then dried at 40°C under nitrogen.
To prepare the dried residue for analysis, it was mixed with 0.3 mL of mobile phase, vortexed extensively, and then transferred to autosampler vials. The last analysis included injecting a volume of 20 µL.

Representative Chromatograms

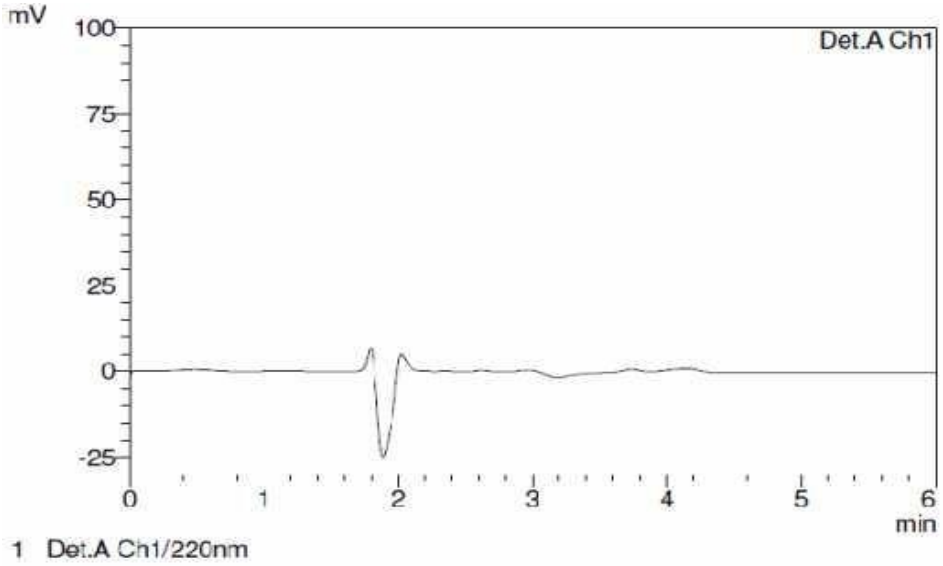

FIGURE 2.- Extract blank plasma

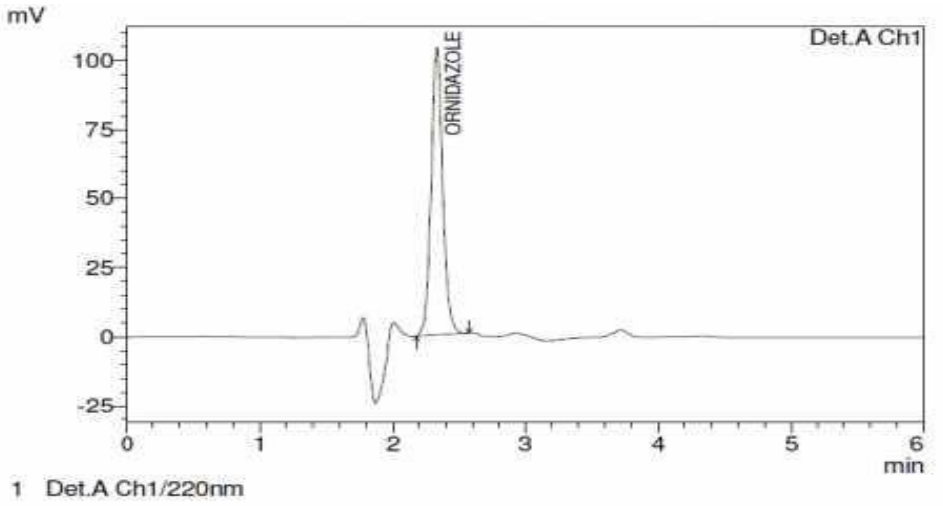

FIGURE 3.- ORNIDAZOLE (IS) spiked

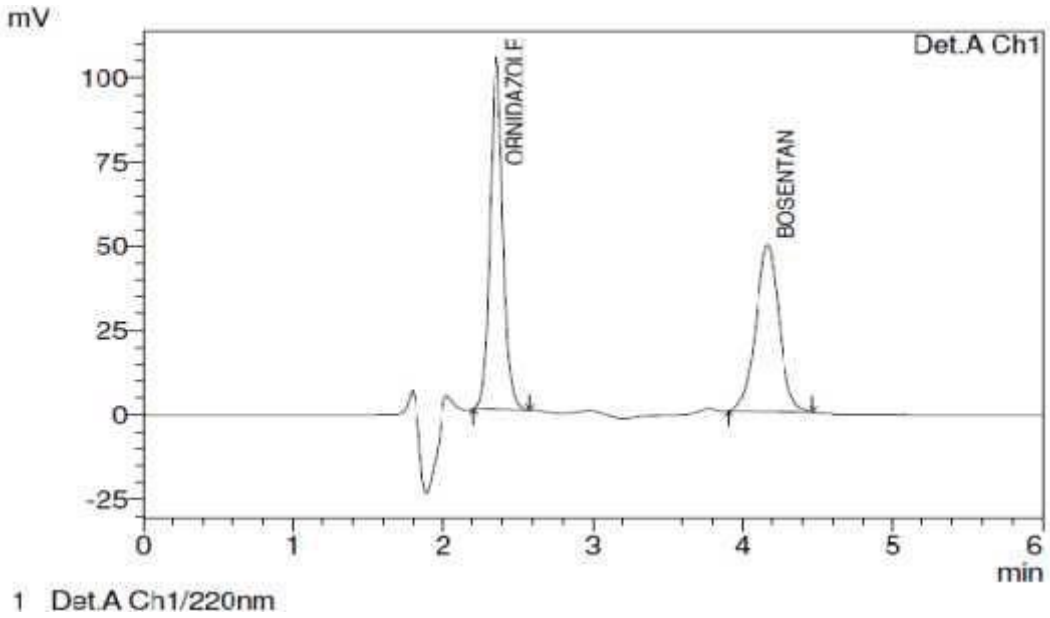

FIGURE 4.- BOSENTAN (drug) and ORNIDAZOLE (IS) extracted from human plasma

3. RESULT AND DISCUSSION

3.1 Screening of plasma lots and specificity

The selectivity of the current approach was assessed by comparing blank K_2EDTA plasma samples collected from various blood donors, which had not been spiked with Bosentan. Upon screening six separate kinds of blank plasma, it was found that none of them interfered with the analyte or internal standard retention times. Both the standards for the calibration curve and the QC samples used in the validation research were prepared using the same human EDTA plasma lots that were devoid of any contaminants that may interfere.

Table 4. Effect of Interference

AHUMAN PLASMA ID	% INTERFERENCE	
	BOSENTAN	INTERNAL STANDARD
1	3.47	0.25
2	2.77	0.19
3	3.24	0.25
4	2.52	0.34
5	1.46	0.35
6	1.36	0.32
Average	**2.469**	**0.283**

This result shows that all matrices under study meet the selectivity criteria (100%).

3.2 Linearity

Using the technique of least squares, the linearity of the approach was tested using bulk spiked plasma samples. Figure 3.5 shows that the standard curves were linear between 52.52 and 3089.48 ng/mL. R2 is a regression coefficient that falls anywhere between 0.99433 and 0.99750.

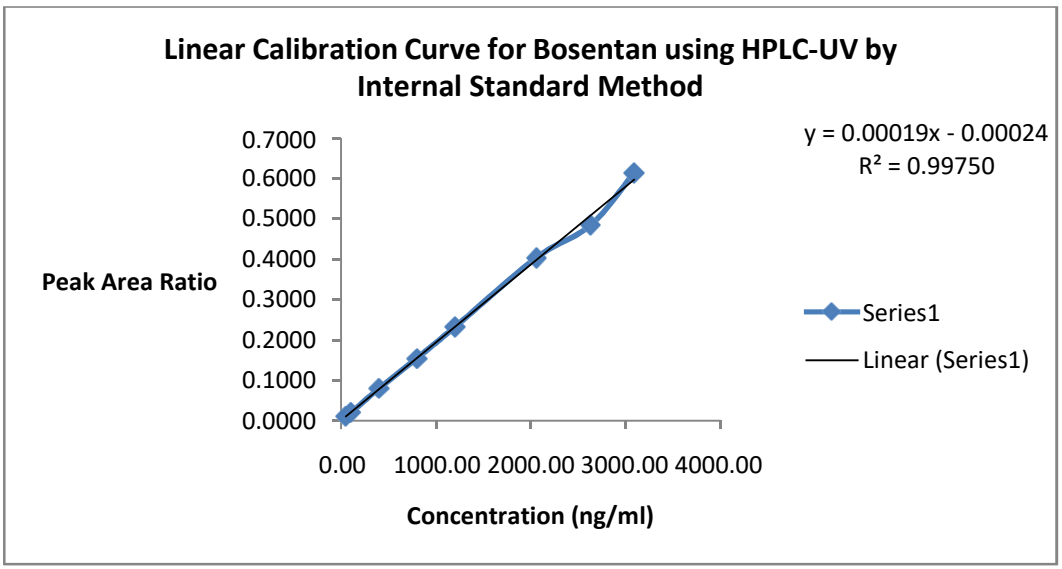

FIGURE 5.- Calibration curve for BOSENTAN (P & A - 01)

3.3 PRECISION AND ACCURACY

Replicated analyses (n = 6) of Quality-control samples containing varying doses of Bosentan were used to assess accuracy and precision. Table 3.4 displays the outcomes. The findings were all within the permissible range of precision and accuracy, which are 2.59–5.35% and 101.14–104.36%, respectively.

Table 5. Back calculated concentration of Bosentan

ID		Concentration (ng/mL)			
	QC ID	HQC	MQC	LQC	LLOQ QC
PA-01	1	2814.13	1695.94	173.52	55.91
	2	2884.08	1573.70	164.49	59.68
	3	3008.44	1535.30	173.13	58.99
	4	2986.65	1498.98	166.32	56.87
	5	2878.87	1534.46	162.31	54.48
	6	2865.58	1553.76	158.86	55.68
N		**6**	**6**	**6**	**6**
Average		**2906.29**	**1565.36**	**166.44**	**56.94**

Standard Deviation		**75.219**	**68.584**	**5.887**	**2.022**
CV (Precision %)		**2.59**	**4.38**	**3.54**	**3.55**
Nominal Conc.		**2860.63**	**1544.74**	**163.40**	**54.86**
Accuracy (%)		**101.60**	**101.33**	**101.86**	**103.79**
PA02	13	2861.97	1724.77	176.47	56.86
	14	2950.41	1609.90	168.27	61.06
	15	3071.62	1567.54	176.77	60.23
	16	2981.87	1496.58	166.05	56.78
	17	2848.64	1518.35	160.61	53.91
	18	2814.29	1525.95	156.02	54.68
N		**6**	**6**	**6**	**6**
Average		**2921.466**	**1573.848**	**167.365**	**57.254**
Standard Deviation		**97.328**	**84.215**	**8.347**	**2.882**
CV (Precision %)		**3.33**	**5.35**	**4.99**	**5.03**
Nominal Conc.		**2860.63**	**1544.74**	**163.40**	**54.86**
Accuracy (%)		**102.13**	**101.88**	**102.43**	**104.36**
PA 03	19	2820.75	1699.93	173.93	56.05
	20	2936.84	1602.49	167.50	60.78
	21	3029.84	1546.22	174.36	59.41
	22	2967.26	1489.25	165.24	56.50
	23	2839.24	1513.34	160.08	53.73
	24	2808.38	1522.74	155.69	54.57
N		**6**	**6**	**6**	**6**
Average		**2900.386**	**1562.330**	**166.133**	**56.839**
Standard Deviation		**90.671**	**77.608**	**7.443**	**2.746**
CV (Precision %)		**3.13**	**4.97**	**4.48**	**4.83**
Nominal Conc.		**2860.63**	**1544.74**	**163.40**	**54.86**
Accuracy (%)		**101.39**	**101.14**	**101.67**	**103.61**

4. CONCLUSION

The results developed and validated the RP-HPLC method for the estimation of Bosentan in human plasma, thereby proving its robustness and reliability. With mobile phase composition, optimized flow rate, and detection wavelength, this method gives high resolution and peak symmetry. Parameters checked and validated for their conformity to predefined criteria in validation included specificity, linearity, accuracy, precision, LOD, LOQ, and stability. Very fine linearity was obtained with a correlation coefficient above 0.999, which confirms the accuracy and precision of the measurement. At a concentration of 5 ng/mL, determinations and at 15 ng/mL quantification showed good sensitivity for Bosentan. Stability tests demonstrated that Bosentan is stable in plasma under various conditions, adequacy confirmed for routine clinical and pharmacokinetic applications. It supports pharmacokinetic studies that are important in the optimization of Bosentan therapy in patients with pulmonary arterial hypertension and allows for the delivery of reliable data for therapeutic drug monitoring. This validated RP-HPLC method is, therefore, very useful in assuring patient safety and treatment efficacy and may have wider applicability across different laboratories and conditions. Further research in the application of the method in several biological matrices and various patient populations may increase its potential for clinical use.

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