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Original Article

Optimization and validation of HPLC methods for *in vitro* and *ex vivo* analyses of bosentan monohydrate in FDA-recommended and biorelevant media

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ABSTRACT

Background and Aims: Bosentan (BOS) is an endothelin receptor antagonist indicated for the treatment of pulmonary arterial hypertension. It is a BCS Class II drug. This study aimed to apply and method validation the new HPLC methods for FDA-recommended and biorelevant media. These methods were used to assess the quantification of BOS in *in vitro* and *ex vivo* studies on lipid-based drug delivery systems (BOS-loaded self-nanoemulsifying drug delivery systems (SNEDDS), and solid self-nanoemulsifying drug delivery systems (S-SNEDDS)) compared to commercial products (Tracleer®). *In vitro* studies include assessments of content uniformity and dissolution in FDA-recommended and biorelevant media. The stability of S-SNEDDS tablets was evaluated in an FDA-recommended medium. The *ex vivo* study assessed the permeability of BOS-loaded SNEDDS, S-SNEDDS tablets, and reference tablets in biorelevant media.

Methods: HPLC was operated using buffer solution: acetonitrile (45:55) with a flow rate of 1.5 mL/min at 220 nm. The injection volume was set at 100 µL. Separation was carried out using a Waters XSelect® HSS C18 column (250x4.6 mm, 5µm) at 25°C.

Results: HPLC methods were validated using ICH Q2(R2) and FDA guidelines. Retention times were found to be between 4.7 and 5.5 in different media. The validated methods were proved to be sensitive, simple, reproducible, rapid, and precise for determining BOS in pharmaceutical formulations and dosage forms.

Conclusion: These new HPLC methods were successfully applied and validated for FDA-recommended and biorelevant media *in in vitro, ex vivo*, and quality control tests of BOS-loaded SNEDDS, S-SNEDDS tablets, and reference tablets.

Keywords: Bosentan monohydrate, HPLC, FDA-recommended and biorelevant media, Pharmaceutical dosage forms, Pharmaceutical formulations

INTRODUCTION

Bosentan monohydrate (BOS) is the first endothelin receptor antagonist to be approved for the treatment of pulmonary arterial hypertension (PAH) treatment (McLaughlin et al., 2005). Oral bosentan (Tracleer®) was approved for the treatment of pulmonary hypertension by the Food and Drug Administration (FDA) on November 20, 2001, and by the European Medicines Agency (EMA) on May 15, 2002. By blocking endothelin receptors, it acts as a vasodilator and neurohormonal blocker, improving left ventricular performance, reducing cardiac vascularization, and improving survival (Ioselevich, Nogid, & Rozenfeld, 2001).

BOS (Ro 47-0203), a substituted pyrimidine derivative without chiral centers, was developed by Hoffman-La-Roche (Basel, Switzerland) in 1994 (Ono & Matsumori, 2002; FDA, 2003). It is a solid, yellowish-white powder. It is very stable in the solid state, non-hygroscopic, does not show polymorphism, and is not affected by light. Slightly soluble in water (1.0 mg/100 mL). It has low solubility in low pH aqueous solutions (e.g., at pH 1.1 and 4.0: 0.1 mg/100 mL, at pH 5.0: 0.2 mg/100 mL) and at pH 7.5, its solubility is 43 mg/100 mL (FDA, 2003). BOS is slightly soluble in hexane, isopropanol, and methanol, soluble in ethyl acetate and ethanol, and freely soluble in dichloromethane and acetone (EMA, 2005). Other physicochemical properties are given in Table 1.

Ultraviolet (UV) spectrophotometric, high-performance thin-layer chromatography (HPTLC), and tandem mass spectrometry (LC-MS/MS) methods have been investigated in different studies for the analysis of BOS from bulk samples, tablet

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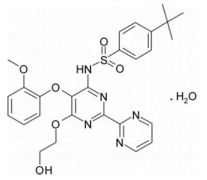
Table 1. Chemical structure and other physicochemical properties of BOS

Chemical structure and chemical name	Molecular formula	Molecular weight (g/mol)	Melting point	log P	pKa	log D
4-tert-butyl-N-[6-(2-hydroxyethoxy)-5-(2 methoxyphenoxy)-2-(pyrimidin-2-yl) pyrimidin-4-yl]benzene-1-sulfonamide monohydrate* 	$C_{27}H_{29}N_5O_6S•H_2O$	569.64**	115°C***	3.1**** (pH 4)	5.5*****	1.3**** (pH 7.4)

* Kaur et al., 2013
** Cohen, Chahine, Hui & Mukherji, 2004
*** Jadhav & Pore, 2017
**** EMA, 2005
*****Roux, Breu, Ertel & Clozel, 1999

dosage forms, pharmaceutical formulations, and in vivo samples (Atila et al., 2014; Marolia, Shah, Bodiwala, Prajapati, & Jariwala, 2015). For BOS, UV spectrophotometry (Das, Narendra, Kumar, & Annapurna, 2010; Kumar, Kumar, & Sankar, 2011; Kumar, Sreenivas, Samal, Dey, & Priyanka, 2011; Narendra, Deepika, & Annapurna, 2012), HPLC (Jadhav et al., 2011; Lavudu, Rani, Chander, & Sekaran, 2013; Jatczak et al., 2016), and LC-MS/MS (Qiu, Zhao, Wang, Xu, & Xu, 2014) methods have been reported in the literature. However, most analytical studies use mass spectrometry and HPLC with UV detection as the methods used for the separation of BOS, its degradation products, and its metabolites in human plasma (Parekh, Shah, Sanyal, Yadav, & Shrivastav, 2012). The HPLC method was optimized using the USP Pending Monograph Version 1-Bosentan (USP, 2012). Since the desired results could not be obtained when using the monograph method, the method was adapted again by changing the column temperature (25°C) and mobile phase ratio (Acetonitrile: Buffer (55:45) in the current monograph method.

The *in vitro* media used in these studies vary, however there are not many studies on the formulation of BOS. The first source for dissolution studies is the FDA dissolution database. Dissolution study evaluations need to be conducted based on the information provided. However, there is no study in the literature on the specific quantitative analysis of BOS in biorelevant media, nor is there any application and method validation for this database. In our previous publications, the data regarding the application and method performed using HPLC were not discussed in detail. This publication is important in terms of providing guidance to people who want to perform their work in different media and drug content analyses, especially in formulation studies on BOS.

This study aimed to apply method validation specific, accurate, sensitive, and rapid HPLC method procedures for the determination of BOS based on the ICH Q2(R2) and FDA guidelines for the validation of analytical methods guidelines (ICH Q2(R2), 2023; FDA, 2001). FDA-recommended dissolution medium (1% SLS in distilled water) and biorelevant media (Fed State Simulating Intestinal Fluid (FeSSIF and its second version FeSSIF-V2) and Fasted State Simulating Intestinal Fluid (FaSSIF and its second version FaSSIF-V2)) were used as dissolution media for in vitro studies. In this respect, the present work is innovative because an HPLC method has not been developed for these media. These methods performed BOS analysis on pharmaceutical dosage form (SNEDDS, S-SNEDDS tablet formulations) samples (Table 2). All analyses were performed and compared with reference tablets (Yılmaz Usta, Timur, & Teksin, 2022; Yılmaz Usta, Olgac, Timur, & Teksin, 2023).

MATERIALS AND METHODS

Chemicals and reagents

Bosentan monohydrate was kindly supplied by Abdi İbrahim (Türkiye). Sodium lauryl sulfate, acetonitrile, and triethylamine were purchased from Merck (Germany). Phosphoric acid and methanol were obtained from Sigma-Aldrich (France). Biorelevant powder was purchased from Biorelevant.com (UK). All the reagents and chemicals were HPLC grade. The reference product is a Tracleer® 125 mg film-coated tablet (Johnson & Johnson, Switzerland) (Expiration date 10/20, Lot number IW067A0401).

HPLC method development and validation for BOS in in vitro and ex vivo samples

Instrumentation

The chromatographic system consisted of an Agilent Technologies Infinity Series 1220 LC (Germany) equipped with a UV diode array detector. XSelect® HSS C18, 5µm, 250 x 4.6 mm (Waters, Ireland) was used for chromatographic separation. The column was 25°C. Separation was carried out with the mo-

Table 2. HPLC methods

	HPLC				
	In vitro Dissolution studies		*Ex vivo* Permeability studies		Drug content analysis
	FDA-recommended medium	Biorelevant media	FDA-recommended medium	Biorelevant media	FDA-recommended medium
S	✓	✓	✓	✓	
S-ST	✓	✓	✓	✓	✓
RT	✓	✓	✓	✓	✓

S: SNEDDS, **S-ST:** S-SNEDDS tablet, **RT:** Reference tablet, **FDA-recommended medium:** 1% SLS in distilled water, **Biorelevant media:** FaSSIF, FeSSIF, FaSSIF-V2, and FeSSIF-V2
SLS: Sodium Lauryl Sulfate, **FaSSIF:** Fasted State Simulating Intestinal Fluid and its second version FaSSIF-V2, **FeSSIF:** Fed State Simulating Intestinal Fluid and its second version FeSSIF-V2

bile phase consisting of a buffer solution (1 mL of triethylamine added to 1 L of distilled water and the solution adjusted to a pH of 2.5 with phosphoric acid) and acetonitrile (45:55) at a flow rate of 1.5 mL min^{-1}. The Millipore 0.45 µm nylon filter was used for the filtration of the mobile phase and was degassed by sonication. The injection volume was set at 100 µL. The wavelength was adjusted to 220 nm. Chromatographic data were obtained from the peak area, which was automatically integrated using Agilent ChemStation software.

Preparation of stock solution

Stock solutions of BOS with concentrations of 40 µg/mL were prepared by dissolving 2 mg of BOS in 1% SLS and biorelevant media. Biorelevant media were prepared according to the manufacturer's instructions available on biorelevant.com (Biorelevant Media Prep Tool, 2022). These stock solutions were used to prepare working standard solutions with concentrations ranging from 0.0195–10 µg/mL for 1% SLS media, 0.5–20 µg/mL for all biorelevant media by appropriate dilutions. The Sartorius 0.45 µm nylon membrane filter was used for the filtration of all samples. Nylon filter membranes are composed of polyamide polymer filters with different pore sizes, and it is characterized by strong resistance to organic and alkali reagents, large specific surface area, and good permeability (Yue, Zhou, Peng & Zhao, 2022).

Preparation of the calibration curve

Calibration curves were generated using solutions of varying concentrations. The calibration curve was then plotted for 12 concentrations in the range of 0.0195–10 µg/mL for 1% SLS in distilled water, 0.5–20 µg/mL for all biorelevant media.

Method validation

Linearity: To evaluate the linearity parameter, three different stock solutions of BOS in 1% SLS and biorelevant media were prepared. Twelve different concentrations were prepared and injected into HPLC by making appropriate dilutions from these stock solutions. Three analyses were performed at each concentration. Peak areas corresponding to the concentrations were observed. The statistical parameters were calculated.

Precision: The interday precision (reproducibility) was determined on three different days at three different levels (0.625, 5, and 10 µg/mL for 1% SLS), (5, 10, and 16 µg/mL for FaSSIF and FaSSIF-V2), (6, 10, and 16 µg/mL for FeSSIF and FeSSIF-V2), and the intra-day precision (repeatability) study 10 different solutions of the same concentration (0.625 µg/mL for 1% SLS, 10 µg/mL for all biorelevant media) were prepared and analyzed three times in a day. Precision was evaluated using the mean, standard deviation, and relative standard deviation (RSD).

Accuracy (Recovery): Recovery studies were performed on different amounts (80%, 100%, 120%) of bulk BOS samples within the linearity range. The RSD% values were found to be less than 2%, indicating that the method is accurate.

Specificity: The specificity was checked to determine whether the excipients in the formulation showed absorbance at the same wavelength. For this purpose, the method specificity was evaluated by comparing the chromatograms of the BOS, media, and formulation components with those of the blank.

Limit of detection (LOD) and limit of quantification (LOQ): The LOD represents the lowest quantity level of an analyte in the sample. The LOQ represents the lowest quantity level reliably provided for a given signal-to-noise. The standard deviation of the intercepts and mean slope of the calibration curves of BOS were calculated for the LOD and LOQ of the developed method. The results demonstrate the sensitivity of the proposed method. The following equations were used to calculate LOD and LOQ:

$$\text{Detection limit} = 3.3\alpha/S \quad (1)$$

$$\text{Quantification limit} = 10\alpha/S \quad (2)$$

α and S are the response's standard deviation and the calibration curve's mean slope of the calibration curve, respectively.

Stability: The stability of the sample solutions for all media at 4°C, 25°C, and 37°C at 0 and 24 h was investigated. To evaluate the stability 10 µg/mL solutions of BOS were used in all media. Samples were analyzed by HPLC after 0 and 24 h.

Stability studies

The stability of S-SNEDDS tablets loaded with BOS was assessed under three conditions: 4°C, 25±2°C/60±5%, and 40±2°C/75±5%. The BOS quantity was assessed at 0, 1st, 3rd,

6th, and 12th months (Yılmaz Usta et al., 2023). The nine tablets selected at random were powdered, and they (equivalent to 30 mg of BOS) were accurately weighed. A 100 mL volume of 1% SLS in distilled water was added, and the mixture was mixed for 1 h in a magnetic stirrer. The samples were diluted to 20 µg/mL and filtered through a 0.45 µm nylon filter, and HPLC was used for analysis.

Dissolution studies

The *in vitro* dissolution studies were conducted using a USP apparatus II (Agilent 708-DS, USA) at 50 rpm at 37±0.5°C in 900 mL. The BOS-loaded SNEDDS, S-SNEDDS tablets, and reference tablets were placed in 900 mL of biorelevant media and distilled water containing 1% SLS (Yılmaz Usta et al., 2022; Yılmaz Usta et al., 2023). The withdrawn samples of 5 mL were filtered using a 0.45 µm nylon filter, and samples were analyzed at 220 nm by HPLC. The percentages of cumulative amounts were evaluated.

Permeability studies

Franz diffusion cells were used in ex vivo permeability study. Biorelevant media were used as permeation media. The BOS-loaded SNEDDS, S-SNEDDS tablets, and reference tablets were diluted to 1 mL using the appropriate media (1 mL of the samples equivalent to 7.5 mg BOS) (Yılmaz Usta et al., 2022; Yılmaz Usta et al., 2023). SNEDDS was used directly. Tests were carried out at 37°C for 24 h (60, 90, 120, 240, 360, 480, 600, and 1440 min). The samples of 2.5 mL were filtered using a 0.45 µm nylon filter, and samples were analyzed at 220 nm by HPLC. The flux (J) and permeability coefficient (P) values were calculated using Equations 3 and 4 as follows:

$$J = dQ/Adt(g/cm^2min) \quad (3)$$

Q, A, and t stand for substance crossing the goat intestine membrane, the contact area of the membrane, and the time of exposure, respectively.

$$P = J/C_o \text{ (cm/min)} \quad (4)$$

Where C_o is the initial drug concentration in the donor compartment and J is the flux value.

RESULTS AND DISCUSSION

HPLC method development and validation for BOS in *in vitro* and *ex vivo* samples

According to the ICH Q2(R2) guidelines, *in vitro* analysis methods should demonstrate that the relationship between concentration and peak area is linear. For accuracy, recovery, and precision, the RSD% should be below 2%, and it should be proven that it does not peak at the working wavelength for specificity and selectivity. The stability should be confirmed by stability studies at different temperatures (4°C, 25°C, and 37°C). The analysis methods developed to quantify BOS are described below.

Method validation

Linearity: The linearity of the method is the expression that the concentration of the active substance in the prepared sample is directly proportional to the concentration within a specific value range. At least five concentrations should be used to achieve linearity (ICH Q2(R2), 2023). Linearity was evaluated by plotting the concentration (x-axis) against the peak area (y-axis). The calibration curve showed higher regression coefficients (r^2>0.999) for all media. All calibration curves are shown in Figure 1. The other characteristic of the linearity results for BOS is given in Table 3.

Precision: Precision refers to the degree of closeness between successive measurements of a method. Intra-day (repeatability) and interday precision (reproducibility) studies were conducted to determine the precision of the analytical method. The RSD% values were found to be less than 2% (except for the four values in Table 5), which shows the developed method's precision. The results are summarized in Tables 4 and 5.

Accuracy (Recovery): The accuracy of an analysis method is the closeness of the value measured using that method to the known concentration value. The accuracy of the assay method depends on the percent recovery obtained by analyzing samples with known concentrations. The recovery of all media was within the acceptance criteria of 80.0–120.0%. The recoveries (1% SLS in distilled water: 97.7% to 101%, FaSSIF: 95.3% to 103%, FeSSIF: 97.0% to 103, FaSSIF-V2: 92.4% to 101%, FeSSIF-V2: 97.0% to 101%) of BOS were obtained at each concentration. The accuracy results are shown in Table 6.

Specificity: Specificity and selectivity refer to the ability to correctly distinguish the substances to be analyzed in the presence of other substances in the matrix, and the analysis method used should only detect the substance to be analyzed (Thompson, Ellison, & Wood, 2002). The method specificity was verified by comparing the chromatograms of the drug, media, and formulations (without active substance) with those obtained from the blank. There was no peak observed at the retention time of the active substance in the blank formulation samples or the media. Chromatograms are presented in Figures 2 and 3.

LOD and LOQ: The LOD and LOQ were evaluated based on α and S. The results were presented in microgram grades, indicating the method's sensitivity (Table 3).

Stability: The sample solutions of BOS for different media were stable at 4°C, 25°C, and 37°C for 24 h. The stability results are summarized in Table 7.

Stability studies

The HPLC method was used for the stability study (4°C, 25±2ºC/60±5% RH, and 40±2°C/75±5% RH) of BOS-loaded S-SNEDDS tablets. No excipient peaks were observed. The percentage of drug content was within the limits (85%-115%)

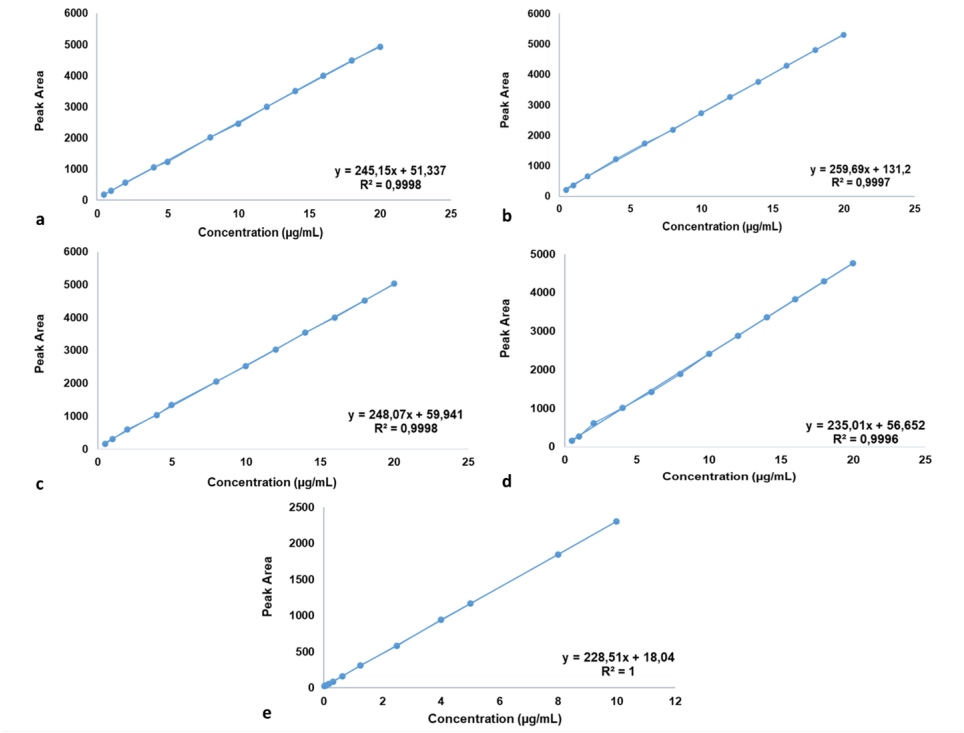


Figure 1. Calibration curves of a) FaSSIF, b) FeSSIF, c) FaSSIF-V2, d) FeSSIF-V2, and e) 1% SLS in distilled water

Table 3. Characteristic properties of the HPLC analysis methods of BOS

	1% SLS	FaSSIF	FeSSIF	FaSSIF-V2	FeSSIF-V2
Linearity range (µg/mL)	0.0195 - 10	5 - 20	5 - 20	5 - 20	5 - 20
Slope*	228.51	245.15	259.69	248.07	235.01
Intercept*	18.04	51.337	131.2	59.941	56.652
Correlation coefficient	1	0.9998	0.9997	0.9998	0.9996
Retention time (min)	5.5	5	4.7	5.1	4.9
LOD (µg/mL)	0.080	0.316	0.265	0.321	0.475
LOQ (µg/mL)	0.245	0.957	0.802	0.973	1.44
Peak height	406	580	627	434	483
Peak width	0.1389	0.1268	0.1238	0.1514	0.1671
Peak area	3652	4709	5173	4289	5258
Peak symmetry	0.971	0.917	0.912	0.846	0.911
Theoretical plate values	25087	24878	23061	18156	13758

*n=3, **SLS:** Sodium Lauryl Sulfate, **FaSSIF:** Fasted State Simulating Intestinal Fluid and its second version FaSSIF-V2, **FeSSIF:** Fed State Simulating Intestinal Fluid and its second version FeSSIF-V2

Table 4. Intra-day precision (repeatability) results of BOS

Injection time of the samples	1% SLS	FaSSIF	FeSSIF	FaSSIF-V2	FeSSIF-V2
1	0.615	10.6	9.71	9.29	11.1
2	0.599	10.7	9.62	9.84	11.0
3	0.617	10.7	9.63	9.43	11.0
4	0.607	10.6	9.67	9.78	11.0
5	0.622	10.7	9.64	9.73	11.1
6	0.621	10.7	9.68	9.97	11.1
7	0.594	10.7	9.67	9.50	11.0
8	0.606	10.8	9.70	9.57	11.0
9	0.591	10.6	9.74	10.1	11.0
10	0.603	10.6	9.67	9.95	11.1
Mean	0.608	10.7	9.67	9.71	11.0
SD	0.011	0.039	0.036	0.254	0.035
RSD%	1.80	0.368	0.375	2.62	0.316

SLS: Sodium Lauryl Sulfate, **FaSSIF:** Fasted State Simulating Intestinal Fluid and its second version FaSSIF-V2, **FeSSIF:** Fed State Simulating Intestinal Fluid and its second version FeSSIF-V2

Table 5. Interday precision (reproducibility) results of BOS

Amount added (µg/mL)	The calculated amount (µg/mL)			Mean	SD	RSD%
	1 day	2 day	3 day			
			1% SLS in distilled water			
0.625	0.639	0.669	0.648	0.655	0.012	1.85
5	5.02	5.13	5.05	5.07	0.059	1.16
10	10.1	9.82	9.83	9.92	0.167	1.68
			FaSSIF			
5	4.82	4.72	4.63	4.72	0.095	2.02
10	9.81	9.67	9.84	9.78	0.075	0.771
16	16.1	15.8	16.2	16.0	0.216	1.35
			FeSSIF			
6	5.91	5.98	5.80	5.90	0.092	1.55
10	9.73	9.87	9.87	9.82	0.080	0.816
16	15.7	15.7	15.9	15.8	0.131	0.830
			FaSSIF-V2			
5	5.15	5.13	4.67	4.98	0.269	5.40
10	10.1	9.99	9.72	9.93	0.196	1.97
16	15.3	15.5	14.0	14.9	0.818	5.47
			FeSSIF-V2			
6	5.96	6.14	5.51	5.87	0.325	5.54
10	10.5	10.5	9.34	10.1	0.686	6.77
16	16.0	15.9	16.1	16.0	0.108	0.674

SLS: Sodium Lauryl Sulfate, **FaSSIF:** Fasted State Simulating Intestinal Fluid and its second version FaSSIF-V2, **FeSSIF:** Fed State Simulating Intestinal Fluid and its second version FeSSIF-V2

Table 6. Accuracy and recovery results of BOS for all media

Level of recovery (%)	Amount added (µg/mL)	The calculated amount (Mean±SD) (µg/mL)	Recovery (%)	RSD (%)
		1% SLS in distilled water		
80	2	1.95 ± 0.04	97.7	1.79
100	2.5	2.52 ± 0.03	101	1.26
120	3	3.04 ± 0.03	101	1.02
		FaSSIF		
80	2	2.06 ± 0.01	103	0.525
100	2.5	2.42 ± 0.02	96.7	0.703
120	3	2.86 ± 0.07	95.3	2.46
		FeSSIF		
80	2	1.95 ± 0.01	97.6	0.496
100	2.5	2.58 ± 0.03	103	1.33
120	3	2.91 ± 0.03	97.0	0.851
		FaSSIF-V2		
80	2	2.01 ± 0.08	100.6	4.09
100	2.5	2.46 ± 0.10	98.2	4.09
120	3	2.77 ± 0.08	92.4	2.99
		FeSSIF-V2		
80	2	1.94 ± 0.03	97.0	1.46
100	2.5	2.45 ± 0.01	97.9	0.266
120	3	3.04 ± 0.21	101	0.214

SLS: Sodium Lauryl Sulfate, **FaSSIF:** Fasted State Simulating Intestinal Fluid and its second version FaSSIF-V2, **FeSSIF:** Fed State Simulating Intestinal Fluid and its second version FeSSIF-V2

except for the 12th month at 40°C (Table 8). In the 12th month at 40°C, this out-of-limit result was related to the forced stability condition.

Dissolution studies

According to the FDA and EMA report, the solubility of BOS is a pH-dependent and poorly soluble drug. Hence, analysis and interpretation of the *in vitro* dissolution data is essential for predicting *in vivo* (FDA, 2003; EMA, 2005). The developed HPLC method can determine data with sufficient precision and accuracy. The percentages of cumulative BOS dissolution are presented in Table 9.

Permeability studies

The analysis method was found satisfactory. The flux and permeability coefficients were calculated and are presented in Table 10. The proposed method can be used to determine BOS.

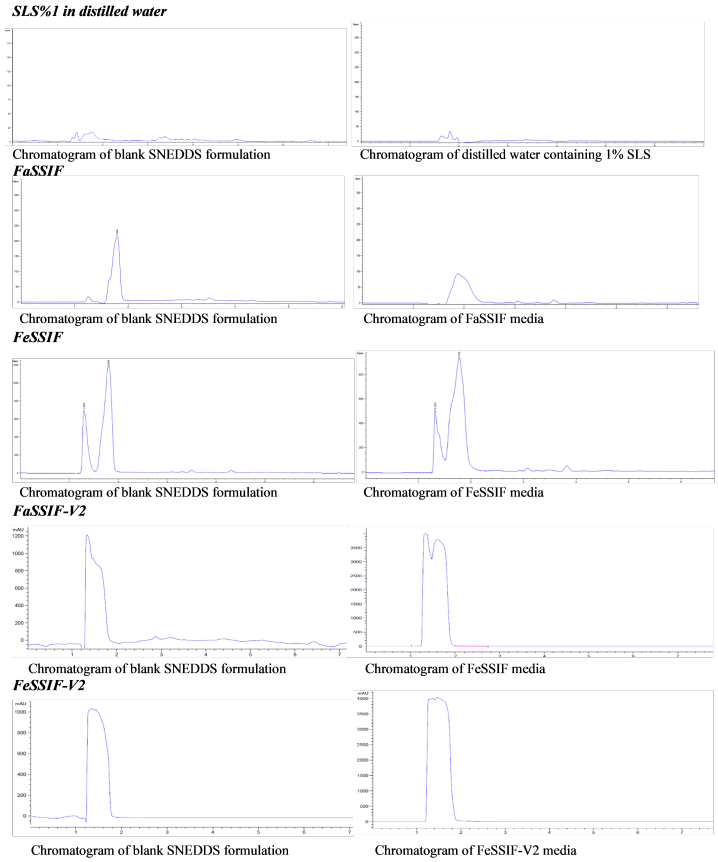


Figure 2. Chromatograms of FaSSIF, FeSSIF, FaSSIF-V2, FeSSIF-V2, and 1% SLS in distilled water and blank SNEDDS formulation in FaSSIF, FeSSIF, FaSSIF-V2, FeSSIF-V2, and 1% SLS in distilled water
SLS: Sodium Lauryl Sulfate, **FaSSIF:** Fasted State Simulating Intestinal Fluid and its second version FaSSIF-V2, **FeSSIF:** Fed State Simulating Intestinal Fluid and its second version FeSSIF-V2

Table 7. Stability results for BOS on all media

	Time (h)	Amount added (µg/mL)	The calculated amount (µg/mL)	Recovery (%)
		1% SLS in distilled water		
Initial		10	9.83	98.3
4ºC	24	10	9.66	96.6
25ºC	24	10	9.26	92.6
37ºC	24	10	9.73	97.3
		FaSSIF		
Initial		10	9.53	95.3
4ºC	24	10	9.28	92.8
25ºC	24	10	9.31	93.1
37ºC	24	10	9.38	93.8
		FeSSIF		
Initial		10	9.69	96.9
4ºC	24	10	9.65	96.5
25ºC	24	10	9.52	95.2
37ºC	24	10	9.59	95.9
		FaSSIF-V2		
Initial		10	10.1	101
4ºC	24	10	8.72	87.2
25ºC	24	10	8.91	89.1
37ºC	24	10	9.51	95.1
		FeSSIF-V2		
Initial		10	9.61	96.1
4ºC	24	10	10.3	103
25ºC	24	10	10.7	107
37ºC	24	10	10.5	105

SLS: Sodium Lauryl Sulfate, **FaSSIF:** Fasted State Simulating Intestinal Fluid and its second version FaSSIF-V2, **FeSSIF:** Fed State Simulating Intestinal Fluid and its second version FeSSIF-V2

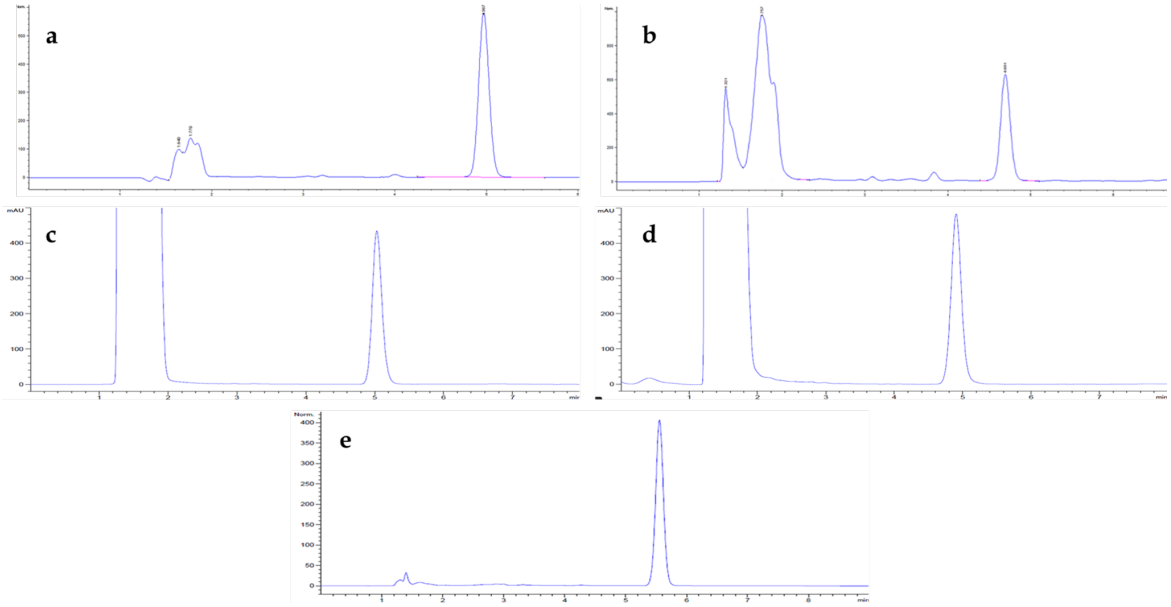


Figure 3. Chromatograms of BOS solutions in a) FaSSIF, b) FeSSIF, c) FaSSIF-V2, d) FeSSIF-V2, and e) 1% SLS in distilled water
FaSSIF: Fasted State Simulating Intestinal Fluid and its second version FaSSIF-V2,**FeSSIF:** Fed State Simulating Intestinal Fluid and its second version FeSSIF-V2, **SLS:** Sodium Lauryl Sulfate

Table 8. Percentage of drug content of BOS for stability conditions

Time (month)	4°C	25±2°C/60±5% RH	40±2°C/75±5% RH
Initial		103%	
1	103%	99.7%	91.9%
3	102%	98.8%	90.9%
6	100%	97.1%	90.9%
12	102%	98.8%	81.4%

n=3

Table 9. Percentages of cumulative dissolution in 1% SLS in distilled water, FaSSIF, FeSSIF, FaSSIF-V2, and FeSSIF-V2 of the reference tablet, BOS-loaded SNEDDS, and BOS-loaded S-SNEDDS tablets

	15 min	30 min	45 min	60 min	90 min
			1% SLS in distilled water		
Reference tablet	90.2±2.6	96.9±5.4	100±2	101±2	102±1
BOS-loaded SNEDDS	80.2±19.9	103±0	104±0	104±1	104±1
BOS-loaded S-SNEDDS tablet	58.8±4.8	74.7±5.0	81.8±4.6	85.3±4.7	88.3±3.2
			FaSSIF		
Reference tablet	21.9±0.3	27.3±0.8	30.1±0.3	31.8±0.1	32.4±2.9
BOS-loaded SNEDDS	82.5±4.6	94.7±1.0	97.4±0.3	97.1±0.7	97.2±1.2
BOS-loaded S-SNEDDS tablet	44.9±0.9	59.8±0.5	67.1±4.1	72.4±3.1	80.0±4.1
			FeSSIF		
Reference tablet	8.72±0.9	10.1±0.1	10.7±0.9	10.7±0.5	11.0±0.1
BOS-loaded SNEDDS	70.8±6.1	84.6±1.3	86.7±1.4	87.3±2.1	87.7±1.0
BOS-loaded S-SNEDDS tablet	51.3±12.9	61.4±7.4	70.1±8.0	74.0±5.5	82.4±2.3
			FaSSIF-V2		
Reference tablet	14.9±0.4	17.9±0.5	19.7±0.8	20.5±0.2	22.3±0.8
BOS-loaded SNEDDS	81.5±11.3	89.0±2.4	93.1±0.5	94.1±0.4	94.1±0.8
BOS-loaded S-SNEDDS tablet	55.9±11.3	69.8±9.9	72.8±1.9	80.3±9.2	82.4±7.8
			FeSSIF-V2		
Reference tablet	17.0±0.2	18.5±0.5	19.3±1.7	19.3±0.2	20.1±2.6
BOS-loaded SNEDDS	91.5±10.2	93.6±3.3	98.7±2.9	99.5±1.9	99.1±1.9
BOS-loaded S-SNEDDS tablet	58.8±3.8	75.4±1.8	82.1±1.8	85.2±1.1	88.2±1.2

mean ± SD, n=3, **SLS:** Sodium Lauryl Sulfate, **FaSSIF:** Fasted State Simulating Intestinal Fluid and its second version FaSSIF-V2, **FeSSIF:** Fed State Simulating Intestinal Fluid and its second version FeSSIF-V2

Table 10. Flux and permeability coefficient parameters of BOS-loaded SNEDDS, S-SNEDDS tablets, and reference tablets in goat intestine membranes from different biorelevant media

		SNEDDS	S-SNEDDS tablet	Reference tablet
A	**Flux (μg cm^{-2} min^{-1})**	54.4±20.2	44.8±28.0	8.85±1.83
	Permeability coefficient (x 10^{-2} cm min^{-1})	0.181±0.067	0.597±0.374	0.118±0.024
E	**Flux (μg cm^{-2} min^{-1})**	208±26.6	3.2±2.89	2.69±0.317
	Permeability coefficient (x 10^{-2} cm min^{-1})	0.692±0.089	0.042±0.038	0.036±0.004
A-V2	**Flux (μg cm^{-2} min^{-1})**	417±262	24.2±19.2	6.36±0.816
	Permeability coefficient (x 10^{-2}cm min^{-1})	1.39±0.874	0.322±0.255	0.085±0.011
E-V2	**Flux (μg cm^{-2} min^{-1})**	229.±85.0	6.06±3.48	3.36±0.802
	Permeability coefficient (x 10^{-2} cm min^{-1})	0.763±0.283	0.081±0.046	0.045±0.011

mean±SD, n=3, **A:** FaSSIF, **E:** FeSSIF, **A-V2:** FaSSIF-V2, **E-V2:** FeSSIF-V2, **FaSSIF:** Fasted State Simulating Intestinal Fluid and its second version FaSSIF-V2, **FeSSIF:** Fed State Simulating Intestinal Fluid and its second version FeSSIF-V2

CONCLUSION

The validated HPLC methods for the quantitative determination of BOS were applied and method validation was successfully performed for pharmaceutical formulations and pharmaceutical dosage forms samples for FDA-recommended and biorelevant media in *in vitro* and *ex vivo* studies. The HPLC methods validation studies were conducted based on the ICH Q2(R2) guideline. The methods were applied and successfully validated for linearity, accuracy, precision, selectivity, specificity, LOD, LOQ, and repeatability for *in vitro* and *ex vivo* permeability studies. These methods can be used for *in vitro, ex vivo*, and quality control tests of BOS-loaded SNEDDS, S-SNEDDS tablets, and reference tablets.

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