

# Spectroscopic analysis of bosentan in biological samples after a liquid-liquid microextraction

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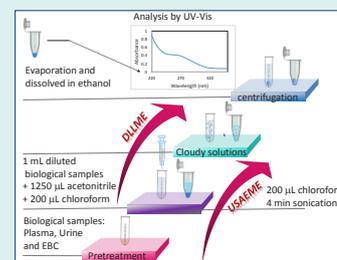
## Abstract

**Introduction:** Microextraction processes with UV-Vis measurement have been developed and validated for analysis of bosentan in biological samples.

**Methods:** In this work, liquid-liquid microextraction procedures (DLLME & USAEME) were employed for cleanup, pre-concentration, and determination of bosentan in biological samples by UV-Vis spectroscopy at 270 nm. The method was validated and applied to the determination of bosentan in spiked serum, exhaled breath condensate and urine samples.

**Results:** Various experimental factors including type of extraction and dispersive solvents and their volumes, pH, sonication time and centrifuging time were investigated. Under the optimum conditions, the method was linear in the range of 1.0–5.0  $\mu\text{g}\cdot\text{mL}^{-1}$ , with coefficient of determination ( $R^2$ ) of > 0.998. The limit of detection (LOD) was 0.07  $\text{mg}\cdot\text{L}^{-1}$ . Recovery of the target analyte in biological samples was 106.2%. The method could be easily applied for higher concentration of bosentan and needs more improvement for application in the pharmacokinetic investigations where more sensitive methods are required.

**Conclusion:** A simple, low cost, precise and accurate spectrophotometric analysis of bosentan in biological samples after liquid-liquid microextraction were developed and validated for routine analyses.



## Introduction

Bosentan or (4-tert-butyl-N-[6-(2-hydroxy ethoxy)-5-(2-methoxy phenoxy)-2-(pyrimidin-2-yl) pyrimidin-4-yl] benzene-1-sulphonamide) monohydrate with molecular formula of  $\text{C}_{27}\text{H}_{29}\text{N}_5\text{O}_6\text{S}\cdot\text{H}_2\text{O}$  is used to treat hypertension and chronic heart failure. It is a competitive antagonist of endothelin-1 acting on both endothelin A and endothelin B receptors of the smooth muscles of pulmonary blood vessels. The maximum serum concentration of bosentan ranged from 959-1,709  $\text{ng}\cdot\text{mL}^{-1}$  and the average time to reach the maximum concentration varied from 1 to 4 h. Less than 3% of bosentan is excreted in urine. Liver metabolism of bosentan produces three metabolites, namely, Ro 48-5033, Ro 47-8634, Ro 64-1056 (see Fig. 1 for struc-

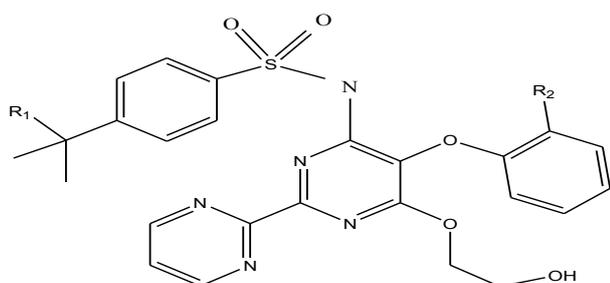
tures). Ro 48-5033 is a pharmacologically active metabolite and may contribute 10% to 20% to the total activity of the parent compound.<sup>1</sup>

An attempt was made to detect bosentan in biological samples by a combination of a micro-volume UV-Vis spectrophotometry with miniaturized approach of dispersive liquid-liquid microextraction (DLLME) and to compare its performance with that of ultrasonic assisted emulsification microextraction (USAEME) to improve selectivity and sensitivity. Pre-concentration step is necessary for analysis of low concentrations in complex matrices. Classical liquid-liquid extraction (LLE) technique was the most commonly used procedure in the past years, but needed high labor intensity, large volumes of organ-

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Compound	R <sub>1</sub>	R <sub>2</sub>
Bosentan	CH <sub>3</sub>	OCH <sub>3</sub>
Metabolite I (Ro 48-5033)	CH <sub>2</sub> OH	OCH <sub>3</sub>
Metabolite II (Ro 47-8634)	CH <sub>3</sub>	OH
Metabolite III (Ro 64-1056)	CH <sub>2</sub> OH	OH

**Fig. 1.** Structure of bosentan and its metabolites.

ic solvents and is a time consuming procedure, which brings great threat to the environment. New miniaturized methodologies<sup>2</sup> such as DLLME,<sup>3</sup> single-drop microextraction,<sup>4,5</sup> solvent bar microextraction,<sup>6</sup> hollow fiber liquid-phase microextraction,<sup>7</sup> and cloud point extraction<sup>8,9</sup> are employed as alternative methods. DLLME is employed in this work as a powerful microextraction technique demonstrated by Rezaee and co-workers in 2006 for the first time.<sup>3</sup> In their developed method, the analyte is extracted and concentrated into a small volume of the extraction solvent. In this procedure, an appropriate mixture of extractant and dispersant were injected into the aqueous sample by a syringe which forms a cloudy solution and increase contact between phases to extract the sample. The cloudy solution would then be centrifuged and the fine droplets are sedimented at the bottom of the conical test tube. Determination of analytes in the remaining phase can be performed by instrumental techniques.<sup>10</sup> Simplicity of the operation, rapidity, low sample volume, low cost, high recovery and high enhancement factor are some advantages of DLLME.<sup>11</sup> In addition to preconcentration, DLLME could clean up the sample which makes it more reliable for spectrophotometric or spectrofluorimetric analysis of drugs in complex matrices such as plasma.<sup>12</sup> Coupling a miniaturized pretreatment technique with microvolume UV-Vis spectrophotometer<sup>13</sup> may provide a possibility to overcome the low sensitivity of method and expand its fields of applications. In the present work, a microvolume UV-Vis spectrophotometry followed by DLLME for determination of bosentan in biological samples is presented.

## Materials and methods

### Reagents

Bosentan monohydrate was a gift from Danesh Pharmaceutical Company, acetonitrile (ACN), ethanol, methanol, acetone, tetrachloroethylene and chloroform (CHCl<sub>3</sub>) were obtained from Scharlau (Barcelona, Spain). Sodium hydroxide, carbon tetrachloride (CCl<sub>4</sub>), dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>) were purchased from Merck (Darmstadt, Germany). Zinc sulfate was purchased from Ajax Chem-

icals (Auburn, NSW, Australia). All reagents and solvents were of analytical grades. De-ionized (DI) water (Shahid Ghazi Pharmaceutical Company, Tabriz, Iran) was used. Bosentan stock solution was prepared by dissolving the appropriate amount in ethanol to obtain 100 mg.L<sup>-1</sup>. The working solutions were prepared by dilution of stock solutions with ethanol. Drug-free QC plasma samples were provided by Iranian Blood Transfusion Research Center (Tabriz, Iran) and frozen in polypropylene microtubes at -20 °C. Also bosentan spiked plasma samples were freshly prepared. EBC samples were obtained from healthy volunteers (who have not used any drug within 3 months) using a lab-made setup.<sup>14</sup> Drug free urine samples were collected from healthy volunteer.

### Instrumentation

All spectral studies were carried out by PG Instruments T70/T70+ (Leicestershire, England) spectrophotometer using 0.05 mL micro cell (Spectral Cell Inc. Oreland, PA). A vortex from Labtron Company (Tehran, Iran) was used in sample preparation. Sigma centrifuge (Osterode, Germany) was used in protein precipitation step and Hettich centrifuge (Tuttlingen, Germany) was used for sedimentation of the extraction solvent in sample preparation. The pH adjustments were made by a Meterohm<sup>®</sup> pH meter (Herisau, Switzerland). Alex machine (Istanbul, Turkey) was used for ultrasonic performance.

### Samples

For DLLME under optimum conditions an appropriate volume of water sample was placed in a glass tube with conic bottom and spiked at the range 1.0-5.0 µg.mL<sup>-1</sup> of bosentan. A mixture of 1250 µL of acetonitrile (as disperser solvent) and 200 µL CHCl<sub>3</sub> (as extraction solvent) was injected into a sample solution by using 2.0 mL syringe rapidly, so that a cloudy mixture was formed. The cloudy solution was centrifuged for 5 min at 5000 rpm. Accordingly, fine droplets of extraction phase were sedimented. The sedimented phase at the bottom of conical test tube was entirely transferred into a microtube. The extract was dried under nitrogen stream. The residue was dissolved in 75 µL ethanol and was conveyed to a microvolume UV-Vis spectrophotometer to measure its absorbance at 270 nm.

To compare the extraction of sample in organic solvent we also studied and optimized USAEME method. One milliliter of sample was transferred in to a microtube. Under optimized conditions, 200 µL chloroform was also added to the tube as an organic extractant phase then sonicated for 5 min. Finally, tubes were centrifuged for 4 min at 5000 rpm. The sedimented phase at the bottom of tube after drying was dissolved in ethanol and its absorbance was read by UV-Vis spectrophotometer at 270 nm.

### Biological samples

Analytical procedure for plasma samples were done on 1 mL pretreatment spiked plasma sample (for DLLME diluted up to 7.5 mL in a conic test tube) and extracted

under optimum condition. For pretreatment step, each precipitant agent with different volume ratio was added, to the samples. Our finding demonstrated the best results with  $\text{ZnSO}_4$  (1 M): ACN (10:90 v/v) as precipitant in the volume ratio of 0.5:1 (precipitant/plasma). One milliliter of EBC spiked samples (for DLLME diluted up to 7.5 mL in a conic test tube) were conveyed into a conic tube without any pretreatment. One milliliter of spiked urine samples were prepared daily by dilution of the stock solution. Calibration standards were prepared ( $1.0\text{--}5.0\ \mu\text{g}\cdot\text{mL}^{-1}$ ) for plasma, EBC and urine samples, respectively, and for bosentan using DLLME and USAEME method. Stability assays were prepared by three different concentrations of analyte spiked in EBC, urine, and plasma. The mean of three repetitions were used for calibration curve and other purposes.

## Results

DLLME and USAEME microextraction methods were developed for preconcentration and detection of bosentan. In order to find the appropriate conditions for DLLME and USAEME, different experimental parameters were investigated using a bosentan standard solution in water sample. All validation tests for determination of bosentan in biological samples under optimum conditions were reported according to FDA guidelines.

### Wavelength selection

In order to perform quantitative analysis, the maximum absorption wavelength should have been established. Thus the sample solution containing various concentrations of bosentan dissolved in ethanol was examined according to the DLLME procedure and spectra of sedimented phase were scanned in the range of 190–350 nm, in this work 270 nm was selected for quantitative analysis of bosentan.

### Effect of type and volume of disperser solvent

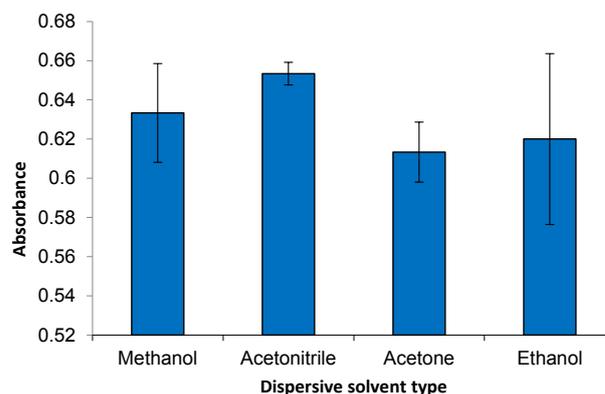
In DLLME, a suitable disperser solvent has to be miscible with both aqueous and organic phases and form a cloudy state that increase contact area between two phases. Dispersive solvents such as menthol, acetonitrile, ethanol and acetone, were examined. Since a higher absorbance signal was obtained using acetonitrile (Fig. 2), it was selected as dispersive solvent for following study. Then the volume of dispersive solvent was investigated at 250, 500, 750, 1000, 1250, and 1500  $\mu\text{L}$ . The obtained results showed the higher response for 1250  $\mu\text{L}$  acetonitrile (Fig. 3).

### Effect of type and volume of extractant

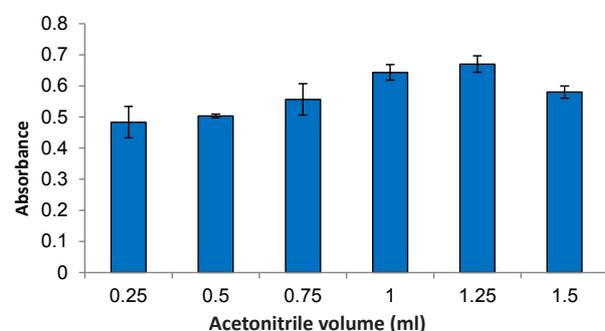
A number of extraction solvents such as  $\text{CHCl}_3$ ,  $\text{CCl}_4$ , and  $\text{CH}_2\text{Cl}_2$  were used for both DLLME and USAEME. Then optimized type of extraction solvent and its volume (as results shown in Figs. 4 and 5), 200  $\mu\text{L}$   $\text{CHCl}_3$  was reasonable for DLLME and USAEME.

### Optimization of sample volume

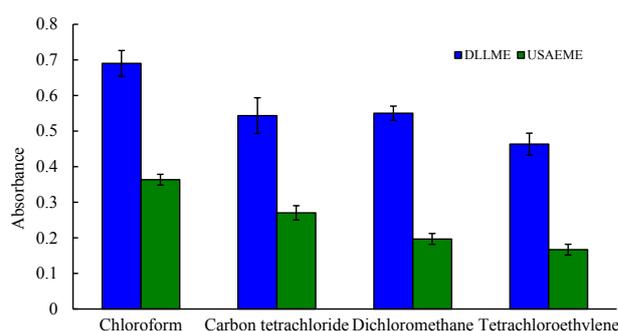
Sample volume effect was studied at four levels 2.5–10 mL containing  $2.0\ \mu\text{g}\cdot\text{mL}^{-1}$  of bosentan. The results (Fig. 6) re-



**Fig. 2.** Effect of disperser kind on the extraction efficiency. Extraction conditions: extraction solvent, chloroform (200  $\mu\text{L}$ ); disperser solvent volume: 1 mL; sample volume, 10 mL; analyte concentration,  $2.0\ \mu\text{g}\cdot\text{mL}^{-1}$  of bosentan; extraction time,  $\sim 0$  min; centrifuging time, 5 min and centrifuging speed, 5000 rpm. The bars indicate the standard deviations (N=3).



**Fig. 3.** Effect of disperser volume on the extraction efficiency. Extraction conditions: extraction solvent, chloroform (200  $\mu\text{L}$ ); disperser solvent: acetonitrile; sample volume, 10 mL; analyte concentration,  $2\ \mu\text{g}\cdot\text{mL}^{-1}$  of bosentan; pH=7.0; extraction time,  $\sim 0$  min; centrifuging time, 5 min and centrifuging speed, 5000 rpm. The bars indicate the standard deviations (N=3).



**Fig. 4.** Effect of extraction solvent kind on the extraction efficiency. Extraction conditions: extraction solvent (200  $\mu\text{L}$ ), dichloromethane, tetrachloroethylene, chloroform, carbon tetrachloride; disperser solvent: Ethanol (1000  $\mu\text{L}$ ); sample volume, 10 mL; analyte concentration,  $2\ \mu\text{g}\cdot\text{mL}^{-1}$  of bosentan; pH=7.0; extraction time,  $\sim 0$  min; centrifuging time, 5 min and centrifuging speed, 5000 rpm. The bars indicate the standard deviations (N=3).

vealed that the analytical signal was increased by addition of volume, then decreased by further addition. Hence,

7.5 mL was used as the optimum sample volume in the DLLME procedure.

#### Optimization of centrifugation rate and time

In this work, the effects of centrifugation rate and time were examined in the range of 2000-6000 rpm and 2-10 min, respectively. In DLLME, the wide surface area between the extraction solvent and the aqueous phase resulted in more and quick transfer of the analyte from the aqueous phase into the extraction phase. According to the obtained results, 5000 rpm and 4 min were selected as centrifuge rate and time, respectively.

#### Optimization of ultrasonication time for USAEME

Sonication time was examined in the range 1–10 min under constant experimental conditions. The absorbance signal increased with increasing sonication time up to 4 min, remaining constant at longer times. Consequently, 4 min of sonication time was chosen for further experiments.

#### Effect of pH

The extraction of analyte is closely based on pH. Thus, in order to obtain the best absorbance values, the pH were studied in the ranges of 2.0–11.0 adjusted by HCl (1 mol.L<sup>-1</sup>) or NaOH (1 mol.L<sup>-1</sup>). Considering pKa of bosentan (i.e. 5.4) high extraction values is expected in this range. As shown in Fig. 7, the work was done in pH 5.

#### Interferences

To determine bosentan in biological sample, various drugs were investigated as possible interfering agents, and results were shown in Table 1. Acceptable limit was taken < 10%. In this work therapeutic amount of various drugs that were probable to be used simultaneously with bosentan were examined.

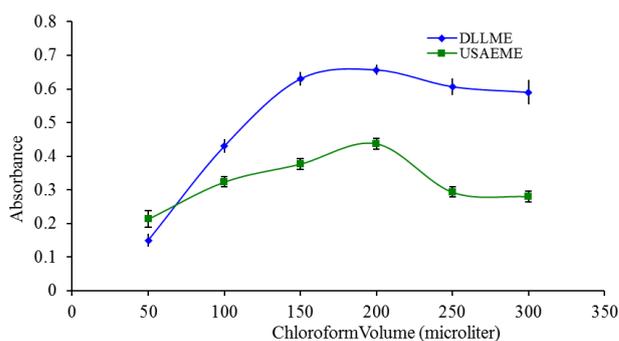
#### Method validation

The developed method was validated according to the FDA guidelines. The calibration, linearity, LOD, LOQ, LLOQ and ULOQ, intra- and inter-day precisions, accuracy, recovery, selectivity (room temperature, refrigerator [4°C and freeze-thaw]) and robustness were tested and the obtained results were summarized in Tables 2-4. As expected, proposed methods had a higher LLOQ in comparison with other reported methods in biological samples, but it cannot ignore its advantages. It can be advised to improve developed methods. The obtained RSDs were less than 5%, while the acceptable RSDs for biological samples according to FDA are less than 20%. The mean of three calibration curves (produced on three different days) was used for the validation studies.

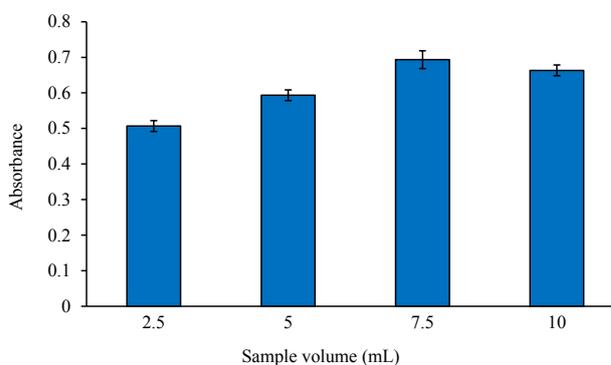
#### Stability

System stability was done to ensure that the system was stable during the time of use. To study stability effect we followed the change of analytes concentration in two ways:

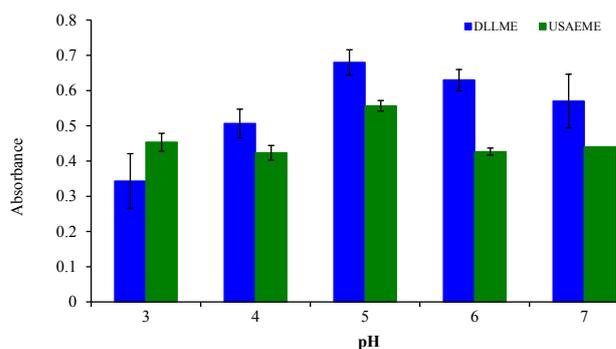
1. After 12 h at 25°C



**Fig. 5.** Effect of extraction solvent volume on the extraction efficiency. Extraction conditions: extraction solvent, chloroform; disperser solvent: Acetonitrile (1000 μL); sample volume, 10 mL; analyte concentration, 2.0 μg.mL<sup>-1</sup> of bosentan; pH=7.0; extraction time, ~0 min; centrifuging time, 5 min and centrifuging speed, 5000 rpm. The bars indicate the standard deviations (N=3).



**Fig. 6.** Effect of sample volume on DLLME. Extraction conditions: extraction solvent, chloroform (200 μL); disperser solvent: Acetonitrile (1250 μL); analyte concentration, 2 μg.mL<sup>-1</sup> of bosentan; pH=7.0; extraction time, ~0 min; centrifuging time, 5 min and centrifuging speed, 5000 rpm. The bars indicate the standard deviations (N=3).



**Fig. 7.** Effect of pH on the extraction efficiency. Extraction conditions: extraction solvent, chloroform (200 μL); disperser solvent: Acetonitrile (1250 μL); sample volume: 7.5 mL; analyte concentration, 2 μg.mL<sup>-1</sup> of bosentan; extraction time, ~0 min; centrifuging speed, 6000 rpm; centrifuging time: 4 min. The bars indicate the standard deviations (N=3).

2. After 3 freeze thaw cycles at 12 h intervals (at 4°C) Results shown in Table 5 revealed no significant changes on stability of analytical results.

#### Robustness

To examine the potential variability such as pH, volume of

extraction solvent and centrifuge time were selected (the range for aforementioned factors were pH=4.5, 5.5, 5.5, extraction solvent's volumes of 180, 200, 220  $\mu\text{L}$  and centrifuge times of 3.5, 4.0, 4.5 min in the experiment). Slight variations are expected when a method is transferred from

**Table 1.** Interference studies in determination of 2  $\mu\text{g}\cdot\text{mL}^{-1}$  of bosentan in aqueous solutions

Interference drug	Concentration spiked ( $\mu\text{g}\cdot\text{mL}^{-1}$ )	$\Delta\text{A}\%$
Carvedilol	0.1	1.6
Furosemide	6	28.9
Verapamil	0.5	0.9
Nifedipine	0.1	1.5
Codeine	0.2	0.5
Losartan	0.6	2.6
Methamphetamine	0.05	3.8
Salicylic acid	250	100
Morphine	0.15	5.5
Nitrocontine	0.01	0.4
Aspirin	100	0.6
Atorvastatin	10	8.7
Propranolol	0.3	3.8

RE: Mean relative error; SD: standard deviation; n.d: not detected.

**Table 2.** Analytical and statistical parameters for the proposed DLLME-UV and USAEME method

Parameter	Level (DLLME)	Level (USAEME)
Linear range ( $\mu\text{g}\cdot\text{mL}^{-1}$ )	1.0-5.0	1.0-4.0
Slope	3.24	5.46
Intercept	-0.018	-0.066
Correlation coefficient	0.998	0.998
Number of data points	6	6
LOD ( $\mu\text{g}\cdot\text{mL}^{-1}$ )	0.7	0.8
LLOQ ( $\mu\text{g}\cdot\text{mL}^{-1}$ )	1.0	1.0
ULOQ ( $\mu\text{g}\cdot\text{mL}^{-1}$ )	5.0	4.0

**Table 3.** Assay of precision and accuracy of QC samples

	Concentration ( $\mu\text{g}\cdot\text{mL}^{-1}$ ; n=3)	Accuracy (RE% $\pm$ SD)	Inter-assay precision (RSD%; n=3)	Intra-assay precision (RSD%; n=3)
Plasma	0	n.d	n.d	n.d
	1.3	1.3 $\pm$ 7.4	1.3 $\pm$ 7.4	1.3 $\pm$ 4.6
	2.0	2.0 $\pm$ 2.0	1.9 $\pm$ 2.0	1.8 $\pm$ 4.8
	3.0	3.0 $\pm$ 3.0	3.4 $\pm$ 3.0	3.4 $\pm$ 2.2
Urine	0	n.d	n.d	n.d
	1.3	1.3 $\pm$ 8.4	1.3 $\pm$ 8.4	1.3 $\pm$ 9.6
	2.0	2.2 $\pm$ 3.0	1.9 $\pm$ 3.0	1.8 $\pm$ 4.8
	3.0	3.2 $\pm$ 3.0	3.4 $\pm$ 3.0	3.4 $\pm$ 3.2
EBC	0	n.d	n.d	n.d
	1.3	1.3 $\pm$ 3.8	1.3 $\pm$ 3.8	1.3 $\pm$ 5.6
	2.0	2.0 $\pm$ 2.4	2.0 $\pm$ 2.4	2.0 $\pm$ 3.1
	3.0	3.0 $\pm$ 2.2	3.0 $\pm$ 2.2	3.1 $\pm$ 4.0

RE: Mean relative error; SD: standard deviation.

one laboratory to another. The results of the study represented no significant changes on the analytical results suggesting the robustness of the method.

## Discussion

In the present work, liquid microextraction methods (DLLME & USAEME) for extraction of bosentan from biological fluids (exhaled breath, plasma and urine) followed by a spectrophotometric determination were proposed. To assess the performance of the proposed methods, the analytical parameters were compared with those of other methods used for the analysis of bosentan. A few methods based on spectrophotometric, LC-MS and HPLC have been reported for determination of bosentan<sup>15-19</sup> which are summarized in Table 6. However all of these methods are not simple, fast and advanced chromatographic methods coupled with tandem MS procedure for the accurate and sensitive determination of bosentan in biological matrices, which are inherently more sensitive and selective than UV-Vis spectrophotometry. The major problems are

**Table 4.** Intraday and interday analytical precision and accuracy for DLLME-UV determination of bosentan in QC sample

	Nominal concentration ( $\mu\text{g}\cdot\text{mL}^{-1}$ ; n=3)	Found concentration ( $\mu\text{g}\cdot\text{mL}^{-1}$ ; $\pm$ SD; n=3)	Relative recovery % ( $\pm$ SD)
Plasma	0	n.d	n.d
	1.3	1.3 $\pm$ 0.01	99.2 $\pm$ 7.4
	2.0	1.9 $\pm$ 0.03	94.0 $\pm$ 2.0
	3.0	3.4 $\pm$ 0.01	101.3 $\pm$ 3.0
Urine	0	n.d	n.d
	1.3	1.4 $\pm$ 0.01	103.8 $\pm$ 5.5
	2.0	2.0 $\pm$ 0.01	99.0 $\pm$ 4.4
	3.0	3.2 $\pm$ 0.02	107.0 $\pm$ 3.8
EBC	0	n.d	n.d
	1.3	1.4 $\pm$ 0.01	96.5 $\pm$ 5.6
	2.0	2.0 $\pm$ 0.01	97.5 $\pm$ 3.1
	3.0	3.2 $\pm$ 0.01	105.3 $\pm$ 4.0

**Table 5.** Stability study of bosentan in biological samples

Sample	Concentration (µg/ml)	Freeze-thaw stability		Room temperature stability		4 °C	
		Concentration found(µg/ml)	Accuracy (%RE)	Concentration found(µg/ml)	Accuracy (%RE)	Concentration found (µg/ml)	Accuracy (%RE)
Plasma	1.0	1.1	9.0	1.1	5.0	1.1	12.0
	2.0	2.1	4.0	2.1	4.5	2.1	5.5
	3.0	3.1	1.7	3.1	3.4	3.2	5.0
EBC	1.0	1.0	3.0	1.0	4.0	1.1	9.0
	2.0	2.0	1.5	2.1	5.0	2.1	4.0
	3.0	3.0	1.3	3.1	3.5	3.1	4.6
Urine	1.0	1.1	7.0	1.1	5.0	1.1	10.0
	2.0	2.1	4.0	2.1	2.5	2.0	2.0
	3.0	3.1	4.0	3.1	2.7	3.1	3.7

Three freeze-thaw cycles; room temperature 12 h; and 4 °C stability data for studied drugs in urine.

**Table 6.** Comparison of reported methods for analysis of bosentan in biological samples

	LLOQ	Linear range	Sample	Ref
UFLC-MS/MS	0.5 ng.mL <sup>-1</sup>	0.5-2000 ng.mL <sup>-1</sup>	Rat plasma	15
UPLC-MS/MS	5.0 ng.mL <sup>-1</sup>	5-1000 ng.mL <sup>-1</sup>	Human plasma	16
LC-MS/MS	2 ng.mL <sup>-1</sup>	2-1000 ng.mL <sup>-1</sup>	Plasma	17
Narrow bore HPLC-MS-MS	1 ng.mL <sup>-1</sup>	1 to 10 000 ng.mL <sup>-1</sup>	Biological matrices	18
	A 0.5 ng.mL <sup>-1</sup>	0.5-200 ng.mL <sup>-1</sup>	Human plasma	
	B 0.5 ng.mL <sup>-1</sup>	0.5-200 ng.mL <sup>-1</sup>	Human plasma	
LC-MS/MS	C 2 ng.mL <sup>-1</sup>	2-1000 ng.mL <sup>-1</sup>	Human plasma	
	D 2 ng.mL <sup>-1</sup>	2-1000 ng.mL <sup>-1</sup>	Human plasma	19
	E 5 ng.mL <sup>-1</sup>	5-1000 ng.mL <sup>-1</sup>	Plasma, rat, dog	
	F 1 ng.mL <sup>-1</sup>	1-1000 ng.mL <sup>-1</sup>	Plasma, rat	
	G 2 ng.mL <sup>-1</sup>	2-10000 ng.mL <sup>-1</sup>	plasma	

much more cost and not common use in laboratories. So, we proposed DLLME & USAEME microextraction based methods combined with UV-Vis spectrophotometry as a commonly and low cost, simple and fast procedure which are efficiently preferred in routine analysis of bosentan. The main disadvantage of our methods is that they are not as good as those because of larger detection limit. The sensitivity of the methods could be improved by further investigations including employing more efficient extraction systems.

### Conclusion

A rapid, simple and low cost DLLME microextraction process with UV-Vis measurement was developed and validated for analysis of bosentan in biological samples and the results were compared with USAEME method. The suggested method is comparable to other reported techniques. Also an important advantage of this work is UV-Vis detection system which was easy to use and simple. To the best of our knowledge, only few methods are available for determination of bosentan and this method offers a simple way for the determination of bosentan in biological samples. The results demonstrated that the proposed method had good recoveries. Therefore, this technique is feasible for quantitative analysis of bosentan in real sample, and could be used in routine analysis for

most of bioanalytical purposes, however it is not a suitable method for pharmacokinetic studies in which highly selective and sensitive methods should be employed.

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### Ethical issues

Volunteers signed a consent form which was approved by Ethics committee of Tabriz University of Medical Sciences.

### Competing interests

No competing interest to be declared.

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## Research Highlights

### What is current knowledge?

- √ Bosentan is used to treat pulmonary hypertension.
- √ Only few methods are available for determination of bosentan in biological fluids.

### What is new here?

- √ A simple, fast and low cost method for analysis of bosentan in different biological samples (exhaled breath condensate, plasma and urine) is provided.

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