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Bioanalytical Method Development and Validation of HPLC-VU Method for Quantification of Embelin from Human Plasma

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Embelin is the main bioactive chemical in *E. ribes* berries and possesses a variety of biological properties. Currently, no liquid chromatographic method is available for quantitative estimation of Embelin from human plasma. The purpose of this study was to develop an accurate, precise, and simple reverse-phase high-performance liquid chromatographic method for measuring the amount of Embelin in human plasma. The separation of Embelin was achieved using a Waters C18 (150 × 4.6 mm i.d., 5 µm particle size) column. A mixture of acetonitrile and phosphate buffer whose pH was adjusted to 3.6 in a 20:80 v/v ratio and at a flow rate of 1.4 ml min^{-1} was employed as a mobile phase. The detection was performed at 289 nm. The plasma extraction method was validated for various parameters, including precision, accuracy, and stability. The developed method using human plasma was linear over a range of 13.9-41.65 ng μl^{-1} concentrations with a regression coefficient of 0.984. The accuracy testing revealed the value of the mean percent recovery between 101.54 and 109.15. The mean intra- and inter-day precision of the assay ranged from 105.04 to 91.16% and 0.3628 to 1.4227% RSD, respectively. The extracted samples also showed bench-top and freeze-thaw stability over 72 hours. In human plasma, Embelin was found to be stable. The method's validation parameters satisfied the required criteria for acceptance. From the results, we concluded that the developed method can be used for accurate and precise quantification of Embelin from human plasma.

Keywords: Embelin, RP-HPLC, Bioanalytical method, Human plasma, US-FDA

INTRODUCTION

Embelia Ribes Burm. f., belonging Myrsinaceae family is one of amongst many ancient medicinal plants belonging to India and is commonly known as Vidanga [1]. The fruits of *E. Ribes* contain Vilangin, Christembine, and Embelin (Fig. 1). Embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone) is the main bioactive chemical in *E. Ribes* berries and possesses a variety of biological properties, such as antibacterial, antioxidant, analgesic, anticancer, anti-inflammatory, anthelmintic [2]. In addition, it is used to treat obesity, heart disease, pneumonia, hemorrhoids, mouth ulcers, toothaches, and sore throats [3]. Research on Embelin's ability to fight cancer is extensive. It is a potentially effective chemical for a completely novel class of

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drugs acting against cancer [4], prevents hepatocarcinogenesis [5,6], and also acts as a blood purifier [7]. From the review of the literature, it was found that, apart from UV spectroscopy [8-11], a few other analytical methods *viz*. RP-HPLC [12-17], HPTLC [18-22], Liquid Chromatography-Mass Spectrometry [23-24] and Gas Chromatography-Mass Spectrometry [25] have been published for the determination of Embelin.

The process of identifying and measuring an unidentified compound or a novel compound in a matrix, *viz*., blood and other body fluids, is known as developing the bio-analytical method. The chemical characteristics and concentration of the drug, the matrix, the costs involved, the time taken for the analysis, and whether the estimate is quantitative or qualitative are factors that determine the use of the analytical technique used for quantitation [26,27].

Protein precipitation is routinely employed for the removal of proteins from the sample matrix and to make it interference-free since it is comparatively fast and requires fewer volumes of the organic modifier. Acetonitrile, methanol, acetone, and ethanol are the most common choices for removing plasma proteins and are compatible with HPLC as the mobile phase. A part of the sample matrix is diluted with the precipitating agent, and then centrifugation is carried out. For quantitative analysis, the supernatant is isolated, evaporated to dryness, and reconstituted with a suitable solvent [26,29]. The process of confirming that the analytical technique used for a particular test is appropriate for the quantitation of an analyte is known as bio-analytical method validation [27]. If the results are used to support the registration of a new medicine (NDA or ANDA) or the reformulation of an existing one, then all the bio-analytical procedures need to be verified [30].

Regarding the quantitative investigations of Embelin following oral delivery in rats, there is only one publication available in the literature. Looking at the broad spectrum of Embelin's pharmacological effects, it was determined that a quick and precise quantitative chromatographic technique was required to quantify Embelin in human plasma. The bio-analysis of pharmaceuticals in plasma can be performed using either RP-HPLC or LC-MS/MS [28]. Thus, the purpose of this work was to develop a simple and accurate bio-analytical method using HPLC for estimating the amount of Embelin in human plasma. It was further validated in accordance with USFDA criteria [30].

METHODS

Materials

Embelin was purchased from Yucca Enterprises, Mumbai, India, and used without further purification. The Pure Lab UHQ water purification unit (ELGA, Mumbai) provided the water for the HPLC. HPLC-grade acetonitrile and methanol were procured from Merck Ltd., Mumbai, India. The Smt. Kashibai Navale General Hospital in Pune, Maharashtra, provided human plasma as a gift. Plasma samples from six different sources were mixed thoroughly to obtain pooled blank plasma. Analytical reagent-grade chemicals were bought from Loba Chemie Pvt. Ltd. in Mumbai, India. Nylon filter papers of 0.45 µm size (Pall India Pvt. Ltd., Mumbai, India) were employed for the filtration of the mobile phase.

Equipment

The drug was separated using chromatography using an LC-2010 CHT (Shimadzu, Japan) that was configured with LC solution software. The HPLC consisted of a pump LC-20 AD with a SIL-20AC HT auto-sampler. The detection was carried out by an SPD20A detector. Plasma samples were prepared and processed using a REMI cyclo-mixer (CM 101) and a REMI refrigerated centrifuge (C24BL). A deep freezer (Panasonic, India) that can maintain a temperature between +10 °C and -22 °C was employed during the stability studies.

Preparation of Mobile Phase

Accurate weight measurements were made for anhydrous disodium hydrogen phosphate (0.900 g) and citric acid monohydrate (1.298 g), which were then dissolved in 1000 milliliters of water. The pH of the resultant solution was adjusted to 3.6 with the help of orthophosphoric acid (OPA). The mobile phase was prepared by mixing phosphate buffer (pH 3.6) and acetonitrile in a 80:20 v/v ratio. Before being used, it was sonicated for ten minutes in an ultrasonicator and filtered using a 0.45 µm membrane filter.

Preparation of Standard Solution and Working Standard Solutions

A 1000 µg ml^{-1} standard stock solution of Embelin was

prepared by dissolving 100 mg of Embelin in 100 ml of methanol using sonication. The standard stock was diluted with methanol to obtain working standard solutions containing the drug at concentrations of 250, 350, 450, 550, 650, and 750 µg ml^{-1}. The quality control (QC) samples were made from working standard solutions in order to get low-quality control (LQC), medium quality control (MQC), and high-quality control (HQC) samples with concentrations of 19.4, 30.5, and 38.88 µg ml^{-1}, respectively.

Extraction Procedure Using Protein Precipitation [20]

After thawing frozen human plasma at room temperature, 400 µl aliquots of the plasma were pipetted into pre-labelled, 10-milliliter centrifugation tubes using a micropipette. Next, 200 µl of each of the working standard solutions of 250, 350, 450, 550, 650, and 750 µg ml^{-1} concentrations was added and vortexed for 30 s. After adding 3 ml of methanol, the tubes' contents were vortexed and agitated for a minute. To achieve phase separation below 10 °C, these solutions were subsequently centrifuged for 15 min at 5000 rpm. Under the stream of nitrogen, the supernatant organic layer was transferred to a different tube, filtered through a 0.22 µ membrane filter, and transferred to HPLC vials for chromatographic analysis.

Method Validation

The developed method was validated in accordance with US-FDA guidance [21] for the industry for the following parameters.

Calibration study. The linearity was evaluated using working solutions of different concentrations of Embelin in plasma (three replicates of each level). These solutions were injected under optimized chromatographic conditions, and peak areas for Embelin were noted at 289 nm. Using basic linear regression between peak areas and concentrations of spiked Embelin over three different days (n = 3), the calibration curve was created. The following formulas were used to compute the limits of detection (LOD), quantification (LOQ), and lower limit of quantification (LLOQ).

LOD = 3.3 X Std. Dev. of y-intercept/Slope

LLOQ = 5 X Std. Dev. of y-intercept/Slope

LOQ = 10 X Std. Dev. of y-intercept/Slope

Where, Std. Dev. = Standard deviation

Selectivity. The selectivity of the method was studied at the lower limit of quantitation (LLOQ) of 13.9 ng $µl^{-1}$ by comparing blank responses of plasma with peak areas produced by the LLOQ samples. It was evaluated to see if the technique could identify drug peaks when plasma was present. It was evaluated to see if the technique could identify drug peaks when plasma was present (Fig. 4).

Accuracy and precision studies. The peak areas of the Embelin for the three QC samples, *viz.*, LQC, MQC, and HQC levels, were determined for six replicates to assess the accuracy of the developed method. By comparing the amount of the drug recovered from plasma supplemented with Embelin with the actual addition, the relative recovery of the drug was ascertained. The findings are determined using the coefficients of variation, standard deviation, and mean percentage recovery. The intraday and interday precision experiments determined the method's precision. On the same day, five replicates of the Low-Quality Control (LQC), Middle-Quality Control (MQC), and High-Quality Control (HQC) levels were used to calculate the method's intra-day precision. After doing three separate analyses on three separate days using three replicates, the method's inter-day precision was examined. The results for accuracy and precision studies are discussed in Tables 2 and 3, respectively. The method is considered precise if the coefficients of variation are within ± 15%.

Stability studies. In order to find any degradation of Embelin in the plasma under the conditions of experiments, stability studies were carried out. Quality control samples produced in a similar manner as calibration standards were used to evaluate the stability of the Embelin. Using two quality control levels, LQC and HQC solution samples at 19.45 ng $µl^{-1}$ and 38.88 ng $µl^{-1}$, respectively, it was determined whether Embelin was stable in the solution and the plasma sample. The LQC and HQC samples solution stability was examined after 6 h of storage at room temperature. To test for freeze-thaw stability, six replicates of the LQC and HQC samples were removed from the deep freezer following a continuous 24-hour freezing period. They were then thawed in a water bath that was maintained at ambient temperature.

After a thorough thaw that lasted at least an hour, the samples were refrozen. This process was run three times, and it was analyzed at the end of the third cycle. Freshly made

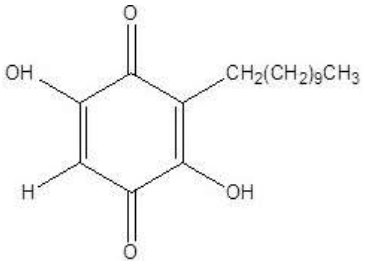

Fig. 1. The chemical structure of Embelin.

sample responses were compared to stable sample responses. Six replicates of LQC and HQC samples of Embelin (19.45 ng μl^{-1} and 38.88 ng μl^{-1}, respectively) were analyzed for bench-top stability at 0 and 6 h at room temperature. The deviation was then computed. To assess the stability over the short term, six injections of LQC and HQC samples were collected during 48 h. The concentration of the zero-day sample was compared with the average concentration.

RESULTS AND DISCUSSIONS

UV- Spectroscopic Studies on Embelin

Embelin was reported to be soluble in methanol; hence, it was selected as a solvent for UV spectroscopic experiments. The UV spectrum scan of the Embelin revealed maximum absorption at 289 nm when scanned against methanol as a blank. Therefore, 289 nm was chosen as the wavelength for further investigations (Fig. 2).

Method Development and Optimization of Chromatographic Conditions

Good system-suitability characteristics were obtained by optimizing the chromatographic conditions. The drug's physicochemical characteristics and polarity were taken into consideration when choosing the mobile phase. Acetonitrile was preferred as an organic modifier over methanol due to its low viscosity, ability to produce sharp peaks and improved column efficiency. The flow rate for which theoretical plates had the maximum resolution between peaks of plasma and Embelin with the optimum run time (15 min) was optimized. Based on the theoretical plate number (greater than 2000) and tailing factor (less than 2), the mobile phase was optimized.

Initially, various trials were conducted to finalize the mobile phase. The retention time of the sample depends on the column characteristics *viz*., the concentration of the bonded phase and the column surface area. Different flow rates (0.6, 0.8, 1, 1.2, and 1.4 ml min^{-1}) were tried. At the end, the mobile phase with a 20:80 v/v ratio of acetonitrile to phosphate buffer and a flow rate of 1.4 ml min^{-1}. was finalized. Embelin's retention period under these conditions was 8.1 ± 0.047 min. The total theoretical plates, which are used to describe typical column efficiency, were found to be 9011 for the peak of Embelin. The tailing factor was found to be less than two. Figures 3 and 4 depict typical chromatograms of blank human plasma and embelin-spiked plasma, respectively.

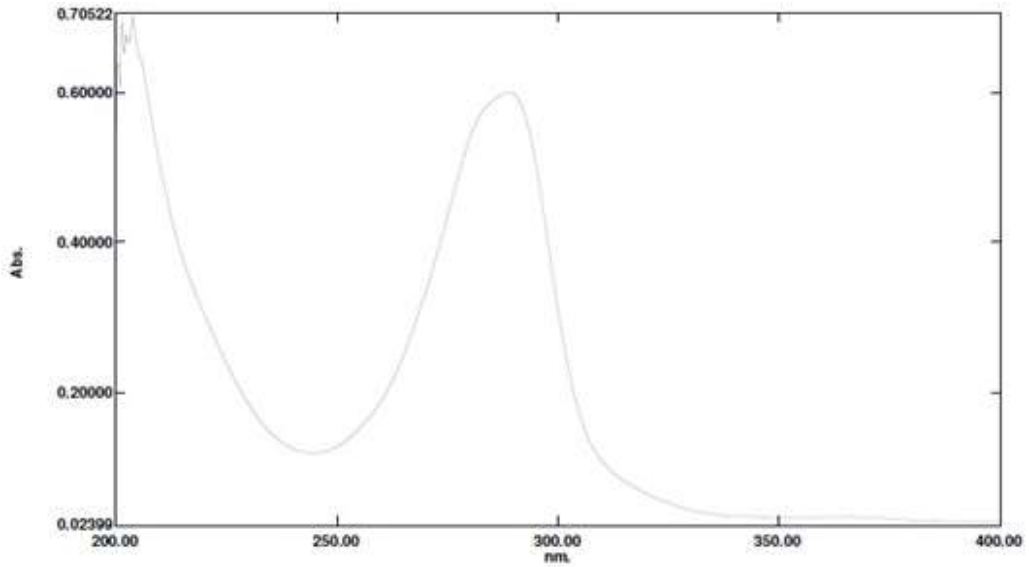

Fig. 2. The UV Spectrum of Embelin in solvent methanol.

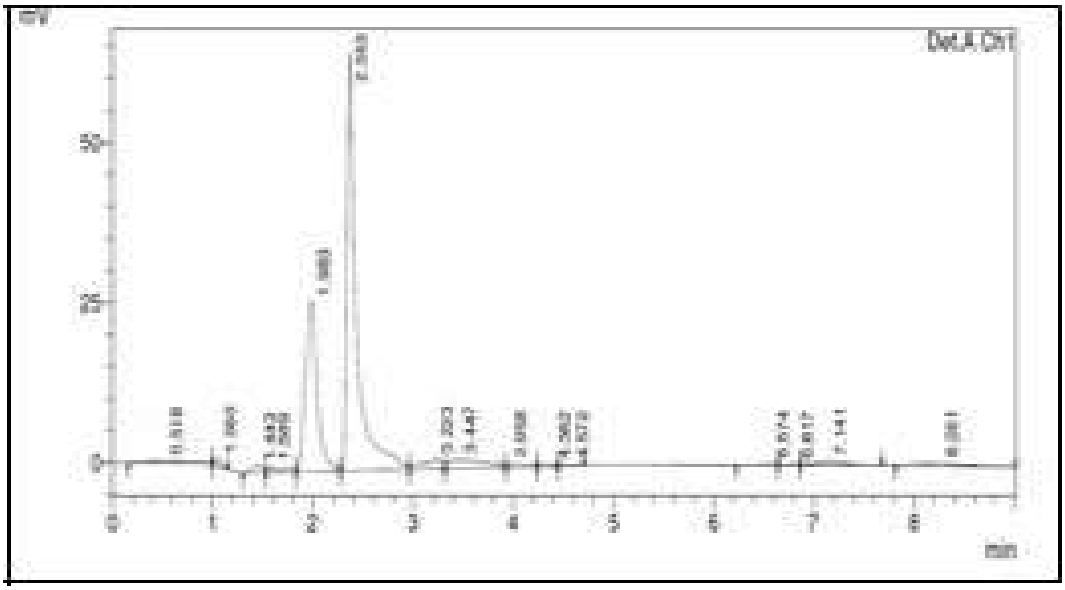

Fig. 3. Chromatogram of blank plasma.

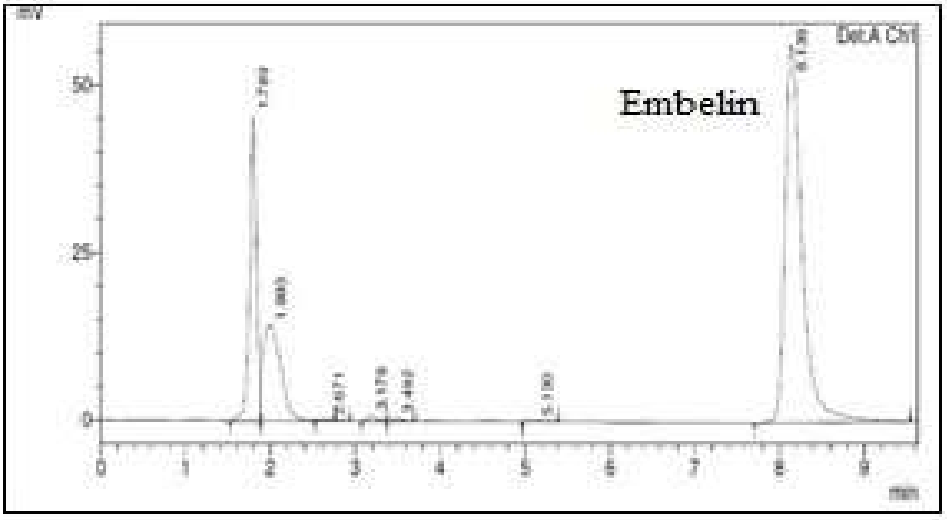

Fig. 4. Chromatogram of plasma spiked with Embelin.

Analytical Parameters and Method Validation

Selectivity studies. The selectivity of a method defines how well it can differentiate and quantify the analyte when there are other components in the sample. The selectivity of the procedure was examined by studying five replicates of plasma samples spiked at LLOQ (13.9 ng μl^{-1}). The actual amount of Embelin present in the spiked samples was determined using the best-fitted regression equation. Embelin showed a mean actual concentration and standard deviation of 13.9080 ± 0.2365 ng μl^{-1}. Table 1 displays the actual concentrations discovered for six replicates, together with the mean real concentration and coefficients of variation. Quality control samples were selected from the range over which the response of the analyte was linear. The LQC sample was selected as the next concentration from the LLOQ. The MQC sample was selected as the middle concentration of the range,

Table 1. Selectivity for Bioanalysis

Replicate No. LLOQ (13.9 ng µl^{-1})	Calculated Conc.
1	13.76
2	14.01
3	13.98
4	13.59
5	14.20
Mean	13.9080
SD	0.2365
%CV	1.70
%Mean	100.052

Table 2. Results of Accuracy Studies

Sr. No.	LQC (19.45 ng µl^{-1})	MQC (30.55 ng µl^{-1})	HQC (38.88 ug µl^{-1})
1	21.43	32.64	40.65
2	20.62	32.47	38.85
3	21.31	30.59	40.62
4	20.75	29.46	40.03
5	22.07	29.98	37.28
Mean*	21.23	31.02	39.48
SD*	0.5818	1.4514	1.4324
% CV*	2.7400	4.677	3.6275
%Mean*	109.15	101.63	101.54

and the HQC sample was selected as the concentration less than the ULOQ (upper limit of quantification).

Linearity and range. For six working standard solutions ranging from 13.9 to 41.65 ng µl^{-1}, Embelin demonstrated an excellent correlation coefficient (r^2 = 0.9844). Three duplicates of each working standard were analyzed, and peak areas were noted. The areas of peaks were plotted against the corresponding concentrations to create the calibration graph. By calculating the coefficient of correlation and standard deviation values, the linearity of calibration graphs and the adherence of the system to Beer's law were validated.

Accuracy and precision studies. The areas of peaks of the Embelin for the three QC samples were determined for six replicates in order to assess the accuracy of the established method. By comparing the amount of the drug recovered from plasma supplemented with Embelin with the actual addition, the relative recovery of the drug was ascertained. The results of recovery studies are expressed as the mean % recovery ± standard deviation. The detailed results for the accuracy studies are given in Table 2. During validation, the percentage coefficients of variation of various quality control samples were used to assess the precision of this approach. For each of the six replicates of the quality control samples, intraday precision was measured at nominal concentrations of 19.45, 30.55, and 38.88 ng µl^{-1} for LQC, MQC, and HQC, respectively. The % coefficients of variation for all the concentration levels were 0.4449, 1.3950, and 1.6824, respectively, which were within the acceptable limits. The reproducibility of the method was evaluated in three replicates on three different days for LQC, MQC, and HQC (19.45, 30.55, and 38.88 ng µl^{-1}). The outcomes are expressed as the mean amount of drug found in plasma at the three concentration levels. For every quality control sample at the LQC, MQC, and HQC levels, the percentage coefficients of variation of the computed concentrations ranged from 0.4449, 1.0684, and 1.7425, respectively, which was found to be within the bounds of acceptance of 15.00%. The detailed results for intraday and interday precision studies are given in Table 3.

LOD and LOQ determination. By injecting different amounts of the standard solution under chromatographic conditions, the LOD and LOQ were ascertained. Signal-to-noise (S/N) ratios of 3:1 and 10 were used to define the LOD and LOQ, respectively. Embelin's LOD and LOQ levels were determined to be 8.083 and 24.494 ng µl^{-1}, respectively.

Stability studies. Drug stability in a biological fluid was a function of the storage conditions, the chemical properties of the drug, the matrix, and the container system. Stability procedures should evaluate the stability of the analyte during sample collection and handling after long-term (frozen at the intended storage temperature) and short-term (benchtop, room temperature) storage conditions. The study evaluated the stability of Embelin in plasma samples across two concentration levels, LQC and HQC, and in various stability conditions, such as benchtop and short-term stability studies (-20 °C for 72 h). In order to determine the mean drug

Table 3. Results of Intraday and Interday Precision Studies

Sr. No.	LQC (19.45 ng µl^{-1})	MQC (30.55 ng µl^{-1})	HQC (38.88 ng µl^{-1})
Intraday Precision studies			
1	21.37	33.51	40.72
2	21.41	33.64	40.84
3	21.43	33.29	41.14
4	21.21	33.51	40.86
5	21.37	33.72	40.65
Mean*	21.3580	33.5340	40.8420
SD*	0.0775	0.1459	0.1678
%CV*	0.3628	0.4350	0.4108
%Mean*	109.8	109.75	105.04
Interday Precision studies			
Day 1	18.74	26.91	37.85
Day 2	18.78	27.35	38.04
Day 3	18.62	27.46	39.09
Mean#	18.7133	27.2400	38.3267
SD#	0.0680	0.2376	0.5453
%CV#	0.3634	0.8722	1.4227
%Mean#	96	91.16	98.57

Note: *(n = 5), Note: #(n = 3).

concentration and coefficients of variation, the drug concentration in the stability samples was determined. All stability study findings fall within acceptable limits, as can be seen from the data presented in Table 4. Embelin remained stable in human plasma for at least 72 h, based on the results of freeze-thaw stability testing. According to the findings of studies on short-term stability, for 48 h at -20 °C, the quality control samples were stable. The mean content of Embelin found in the LQC and HQC samples was within the acceptance limit of 85.00-115.00%, which confirmed that Embelin was stable during the extraction process. The findings of stock solution studies additionally demonstrated stability. The detailed results for stability studies are depicted in Table 4.

CONCLUSION

This work describes the development of a bio-analytical method and validation for RP-HPLC-based Embelin quantification in human plasma. The developed method was found to be simple, accurate, precise, and reproducible. The protein precipitation method reported by the authors is less time-consuming and requires a smaller amount of solvent. Acceptable results in accuracy and precision were obtained within range. The stability studies were done at two different levels, *viz*., LQC and HQC, and were found to be within a limit, which confirmed that the Embelin was stable during the extraction process. Further, these studies can be extended to conduct pharmacokinetic studies of Embelin in different biological fluids.

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Table 4. Results of Stability Studies

Stability	Parameter	Concentrations			
		LQC (19.4 ng µl^{-1})		HQC (38.88 ng µl^{-1})	
		Stability	Comparison sample	Stability	Comparison sample
Bench top stability (n = 6)	Mean	19.19	21.2833	36.7598	40.7900
	SD	0.757461	0.1489	1.4578	0.2813
	%CV	2.1487	1.2514	3.9658	0.6897
	%Stability	90.17		90.09	
		LQC (19.4 ng µl^{-1})		HQC (38.88 ng µl^{-1})	
		Stability	Comparison sample	Stability	Comparison sample
Freeze and Thaw stability (n = 6)	Mean	22.1183	21.5333	37.45	40.5917
	SD	0.074	0.2943	0.7536	0.4380
	%CV	0.3351	1.3671	2.011	1.0790
	%Stability	102.71		92.26	
		LQC (19.4 ng µl^{-1})		HQC (38.88 ng µl^{-1})	
		Stability	Comparison sample	Stability	Comparison sample
Short term stability (n = 6)	Mean	22.055	21.5333	36.2033	40.5917
	SD	1.0606	0.2943	1.3782	0.4380
	%CV	4.8089	1.3671	3.8068	1.0790
	% Stability	102.41		89.18	
		LQC (19.4 ng µl^{-1})		HQC (38.8 ng µl^{-1})	
		Stability	Comparison sample	Stability	Comparison sample
Stock solution stability (n = 6)	Mean	22.0167	21.43	44.24	40.81
	SD	1.0382	0.26007	1.05	0.511
	%CV	4.7148	1.2136	2.29	1.25
	%Stability	102.73		108.40	

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