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Original Article

VALIDATED REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR THE ESTIMATION OF TETRABENAZINE IN SELF-NANO EMULSIFYING DRUG DELIVERY SYSTEMS

SHASHI, NARENDRA KUMAR PANDEY*, BIMLESH KUMAR, SACHIN KUMAR SINGH, DILEEP SINGH BAGHEL, KALVATALA SUDHAKAR, SAURABH SINGH

School of Pharmaceutical Sciences, Lovely Professional University, Punjab-144411, India
***Corresponding author: Narendra Kumar Pandey; *Email: herenarendra4u@gmail.com**

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ABSTRACT

Objective: Self-Nano-Emulsifying Drug Delivery System (SNEDDS) of tetrabenazine (TBZ) was analysed using reverse-phase high-performance liquid chromatography.

Methods: Optimized chromatographic condition was consisted of Acetonitrile (ACN) and 0.1% v/v formic acid in the ratio of 90:10 as a mobile phase in isocratic mode at 25±1 °C. In this C-18 (250 mm×4.6 mm, 5 µm) column was used and absorbance was recorded at 283 nm.

Results: The compound was eluted at a flow rate of 1.0 ml/min and retention time (RT) was observed as 4.34±0.03 min. TBZ showed linearity over 2-10 µg/ml conc. and the value of regression was obtained as 0.9992. The developed method was found precise due to Percentage Relative Standard Deviation (%RSD) was less than 2 %. On the other hand, 0.31 and 0.96 were investigated value for Limit of Detection (LOD) and Limit of Quantification (LOQ), respectively.

Conclusion: The method adopted was found to be robust and can be apply for the determination of drug in different oil, surfactants and co-surfactants for the calculation of drug loading of pharmaceutical product formulation.

Keywords: Tetrabenazine, HPLC, Analytical method development, SNEDDS

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INTRODUCTION

TBZ, a BCS class IV drug, is used to treat chorea due to Huntington's disease and symptomatic treatment of hyperkinetic movement disorders. The exact mechanism of action of TBZ is unknown. It is believed to inhibit Vesicular Monoamine Transporter type 2 (VMAT-2) and it causes a depletion of neuroactive peptides like serotonin, dopamine, norepinephrine [1]. TBZ is a derivative of hexahydro-dimethoxy-benzoquinolizine, and chemically is 9,10-dimethoxy-3-isobutyl-1,3,4,6,7,11b-hexahydro-2*H*-pyrido[2,1-a] isoquinoline-2-one [2] (fig. 1).

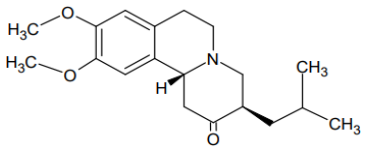

Fig. 1: Structure of TBZ

TBZ depletes monoamines from nerve terminals reversibly. It reduces uptake of monoamines by inhibiting VMAT2, within synaptic vesicles of monoamine pool [3]. It is practically insoluble and undergoes first-pass metabolism [4]. To overcome these problems TBZ loaded self-nano-emulsifying drug delivery system was formulated. Lipid-based formulation approaches have significantly improved the solubility of lipophillic drugs, hence increasing oral bioavailability [5]. SNEDDS are the isotropic mixture of oil, surfactant and co-surfactant, which immediately form oil in water nano-emulsions of 20-200 nm, when diluted in water under disturbance [6]. It requires less amount of drug as compared to other conventional forms. In the Gastro-Intestinal Tract (GIT), because of its self-emulsification process, the drug is present in nano globule form, which enhances its dissolution by providing a large area and provides stability to the drug [7]. It offers stable thermodynamic formulation, improving lymphatic transport and avoiding first-pass metabolism [8].

As previously reported methods of reverse phase high-performance liquid chromatography are available, Reza Mehvar *et al.*, 1986 developed a method in rat and human plasma with water, acetonitrile, acetic acid and triethyl amine as mobile phase in the ratio of 65:33:2.0:0.15 with 0.6 ml/min flow rate and 10 min retention time [9]. As four mobile phase was used in reported method made it complicated and time-consuming. Derangula *et al.*, 2012 reported liquid chromatography-tandem mass spectrometric in human plasma with acetonitrile and 5 mmol ammonium acetate as mobile phase in the ratio of 60:40 with 0.8 ml/min with 2.5 min retention time [1]; this method is not reproducible. To overcome these problems, there is need to develop accurate, precise, simple RP-HPLC method which is less time-consuming and affordable for TBZ.

The aim of the performed study was to develop and validate a simple, precise, sensitive and RP-HPLC procedure to quantify the drug during pre-formulation and formulation studies.

MATERIALS AND METHODS

Materials

TBZ was gifted from Synnat Pharma Pvt. Ltd., India. Olive Oil, Cotton seed oil, Peanut oil, Paraffin oil, and Eucalyptus oil were purchased from CDH Pvt. Ltd, India. Capryol 90, Capmul MCM, Isopropyl myristate, Capryol PGMC, Labrafil M 1944 CS, Labrasol, Labrafac WL 1349, Labrafac PG, Peceol, Lauroglycol 90, Lauroglycol FCC, Transcutol P, Transcutol HP, Plurol Oleique, and Maisine CC were procured as a gift sample from Gattefosse, Mumbai, India. Tween 20, Tween 60, Tween 80, Span 80, PEG 400, and acetonitrile HPLC grade were purchased from Merck, Mumbai, India. Formic acid, Orthophosphoric acid, and Triethylamine were recieved from LOBA CHEMIE Pvt. Ltd., Mumbai, India.

Formulation development

In a glass tube, 100 µl** of capmule PGMC, 600 µl** of tween 20 and 300 µl** of transcutol P were added and vortexed for 5 min. Then, 12.5

mg of TBZ was added to the formulation and vortex until a monophasic system is formed. Add this mixture to 500 ml water at room temp. while stirring [10]. Prepared SNEDDS were stored at room temperature for further characterization. Formulations were characterized for globule size, zeta potential, and Poly-Dispersity Index (PDI).

Analytical method development

Chromatographic conditions and equipment's

HPLC analysis was carried out using HPLC (instrument from Shimadzu Japan) equipped with a pumping system of LC-20 AD series, a PDA detector (SPDM20A; Shimadzu, Japan), and manual Rheodyne injector (20 µl** loop size). LC Solutions software was used for data processing and interpretation. Sonicator was employed to degas the mobile phase. Calibrated *p*H meter was used to measure the *p*H of prepared formic acid. For estimation of the drug, the stationary phase used was C-18 reversed-phase column (C18, 250 mm×4.6 mm, 5 µm), and the various mobile phases used for the developing method were ACN,-5 mmol ammonium acetate; ACN-0.1% glacial acetic acid; ACN-0.1% ortho-phosphoric acid and ACN-0.1% formic acid by varying their pH and mobile phase ratio. Amongst these, the selected mobile phase consists of a mixture of formic acid with pH 3.2 and ACN (10:90 v/v). The flow rate was fixed to 1 ml/min. The column temperature was ambient. The detection wavelength of the eluent drug was 283 nm.

Preparation of formic acid pH 3.2

In 100 ml volumetric flask formic acid (100 µl**) was taken and filled up to 100 ml using triple distilled water. The pH of this solution was adjusted to 3.2 using triethyl amine. 0.45 µm syringe filter was used to filter the solution and sonicated to remove the air bubbles.

Preparation of mobile phase

The mobile phase was prepared by mixing 90 parts of ACN and 10 parts of formic acid with a *p*H of 3.2. Using 0.45 µm syringe filter, mobile phase was filtered and was ultrasonicated to degas the mobile phase.

Preparation of standard stock solution

Accurately weighed TBZ (10 mg) was dissolved in the mobile phase in a 10 ml volumetric flask and filled with the mobile phase. It gave a stock solution of 1000 µg/ml. Serial dilutions were performed by taking 1 ml of the above solution and making it up to 10 ml resulting in a solution of 100 µg/ml, which on further dilution yield a solution of 10 µg/ml. From the prepared stock solution, serial dilutions were performed to get final concentrations of 2, 4, 6, and 8 µg/ml [11].

Method validation

The developed method was validated as per the ICH Q2 (R1) for linearity, accuracy, precision, robustness, and specificity [12].

System suitability

To determine the system suitability, peak purity index, tailing factor, and Height Equivalent to Theoretical Plate (HETP) [13] were calculated by injecting blank, followed by six replicates of system suitability sample i. e. 10µg/ml TBZ onto the HPLC system [14].

Preparation of quality control standards

Lower Quantified Concentration (LQC), Medium Quantified Concentration (MQC), and Higher Quantified Concentration (HQC) of the calibration curve was resulted on three different level of quality standard [15]. Consequently 6, 4.8 and 7.2 µg/ml was considered for MQC, LQC and HQC, respectively as 6 µg/ml was the centre value of calibration curve.

Linearity and range

The range of an analytical method is the gap between the sample lowest and the highest concentrations of sample for which the analytical procedure has a satisfactory level of precision. Linearity was evaluated by analyzing a series of various concentrations of TBZ. Five concentrations (2, 4, 6, 8, 10 µg/ml) of TBZ were injected six times each, and the regression equation was noted.

Accuracy

The quality and applicability of the developed method were checked by performing the recovery analysis of TBZ at three level i. e., LQC, MQC and HQC of the medium concentration which was 6µg/ml. Standard solutions (LQC, MQC and HQC) were injected six times, and the response mean values were recorded [16]. The percentage recovery was calculated from the following formula [17].

Percentage recovery can be calculated as actual conc. Recovered divided by theoretical conc. and obtained value will be multiplied by 100.

Precision studies

Precision studies were performed in two parts: repeatability and intermediate precision. In repeatability, standard solutions were injected six times each on the same day under the same conditions (intra-day). For the intermediate precision, an inter-day study was carried out by injecting six times of standard solution for three consecutive days and for the inter-analyst study, three different analysts of the same laboratory injected six times of standard solution, which were prepared by other analysts by following the identical conditions of experiment. The mean of responses was noted, and the %RSD was calculated.

Robustness

Robustness of the proposed procedure is to estimate of its value to remain unaltered by modest but considered changes in chromatographic settings, which was investigated by testing the influence of small alterations in terms of variation in the mobile phase such as in *p*H (3.0±0.2), the ratio of mobile phase ACN: Formic acid (88:12, 90:10, and 92:08), and flow rate (1.0±0.2 ml/min). Medium concentration of 6 µg/ml was injected for six times and the effect on the recovery, peak area, and retention time was noted.

Estimation of LOD and LOQ

LOD and LOQ can be calculated by three methods, i. e., visual evaluation, S/N ratio approach and standard deviation of the response and slope. The LOD and the LOQ were determined by the standard deviation of the response and slope method using the following formula [18].

$$LOD = \frac{3.3\,\sigma}{S} \ldots\ldots (1)$$

$$\mathrm{LOQ} = \frac{10\,\sigma}{\mathrm{S}} \ldots\ldots (2)$$

Where S is the slope of the calibration curve and sigma (σ) is the Standard Deviation (SD) of slope.

Application of HPLC method in solubility and drug loading estimation

Determination of drug solubility

For the development of SNEDDS formulation, estimation of TBZ solubility is required. The solubility of different components of SNEDDS, such as surfactant, oil, and co-surfactant, was checked by the HPLC method [19, 20]. The solubility studies of TBZ were carried out in oils (eucalyptus, olive, cotton seed, capmul MCM, peanut, paraffin, caproyl 90, isopropyl myristate and caproyl PGMC), surfactants (tween 20, tween 60, tween 80, span 80, labrafil M 1944 CS, labrasol, labrafac WL 1349, labrafac PG and peceol) and co-surfactants (transcutol P, PEG 400, lauroglycol 90, lauroglycol FCC, transcutol HP, plurol oleique and maisin CC). In 1 ml of co-surfctant, oil, surfactant sufficient amount of 10 mg of drug was which further undergoes vortexing (cyclo mixer REMI, India). The vials were sealed and left for 72 h, with intermittent shaking every hour for 8 h [7, 21]. For thr confirmation of removal of undissolved drug the sample were subjected to centrifugation (REMI CM-12 PLUS, India) at 10000g for 20 min. The supernatants were collected and diluted with methanol, ethanol, chloroform, and hexane to determine the drug quantity [22].

Determination of drug loading in SNEDDS

The SNEDDS were formulated by adding 12.5 mg TBZ in 1 ml mixture of selected surfactant, oil, and co-surfactant. These formulations were diluted with triple distilled water up to 500 ml on a magnetic stirrer at 700-800 rpm at room temperature. The HPLC

method was used to evaluate drug loading in SNEDDS formulation. Using syringe filter formulation sample was filtered [13] and injected into the HPLC system to analyze the TBZ peaks. Percent drug loading was calculated using the following equation [23]:

$$\% \text{ Drug Loading} = \frac{\text{Amount of drug in SNEDDS}}{\text{Amount of formulation components added}} \times 100 \ldots. (3)$$

RESULTS AND DISCUSSION

Formulation

Formulation was evaluated for globule size, PDI, zeta potential and were found to be 68.73±2.79 nm, 0.451±0.08 and-20.2±1.79 mV (fig. 2), respectively. Size of the formulation was showing nano-range, PDI was showing good uniformity and homogeneity between the particles, zeta potential was also showing stability of bilayer.

Selection of mobile phase for TBZ estimation

Several mobile phase compositions in different ratios and pH have been used. Firstly, using ACN: 5 mmol ammonium acetate [1] peaks appeared with splitting and noise (fig. 4A). Secondly, trial with ACN: 0.1% glacial acetic acid was used as a mobile phase for estimating TBZ, but shouldering was observed (fig. 4B). Thirdly, upon using ACN: 0.1% ortho-phosphoric acid as a mobile phase, there was no sharp peak; instead, two peaks with shouldering were observed (fig. 4C). The reported methods have RT between 6.5-10 min [1, 9], and 0.1% formic acid and ACN have RT 4.34±0.03 (fig. 4D). Finally, using ACN: 0.1% formic acid pH 3.2 of ratio 90:10 as mobile phase better results in terms of resolution, sharpness of peaks was observed. Hence, this mobile phase combination was selected for validation. When a blank of 5 mmol ammonium acetate and ACN was injected, there was no peak which interfered with the TBZ peak RT. In addition, the same was observed with 0.1% formic acid and ACN (fig. 3).

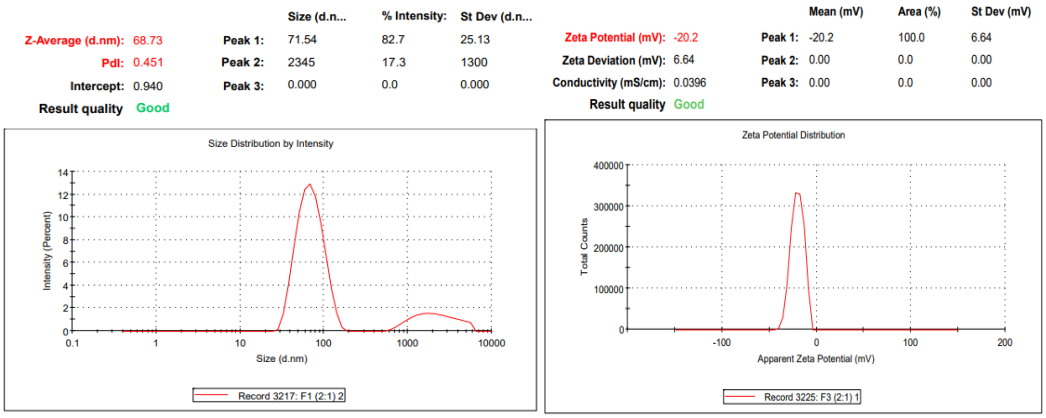

Fig. 2: Globule size, PDI (2A), Zeta potential (2B)

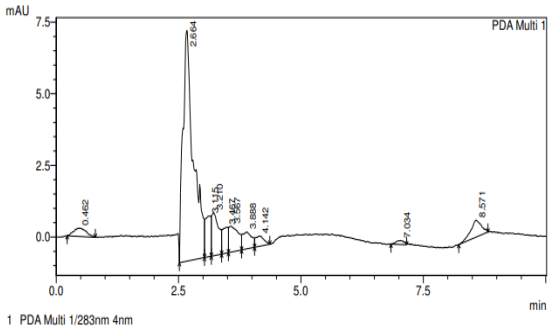

Fig. 3: Chromatogram of blank of ACN and formic acid

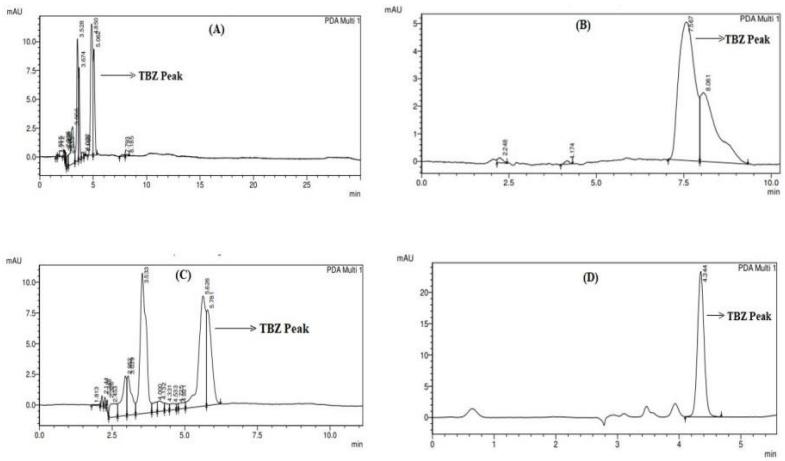

Fig. 4: Chromatogram of TBZ in ACN and 5 mmol ammonium acetate (A), Chromatogram of TBZ in ACN and 0.1% glacial acetic acid (B), Chromatogram of TBZ in ACN and 0.1% ortho-phosphoric acid (C), Optimized chromatogram of TBZ in ACN and 0.1% formic acid (90:10) (D)

Method validation

System suitability

TBZ dilution of 10 µg/ml was injected for system suitability and results were compared with standard and previously reported studies. Tailing factor was found 1.10±0.002 which is less than 2 [1, 9] ensure peak regularity. Theoretical plate was found 6848±43, which is more than the previously conducted studies for quantification of TBZ using HPLC [1, 9] ensure excellent peak efficiency (table 1).

Linearity

The potential of an analytical process to bring out results that are directly proportional to the concentration (quantity) of sample is known as linearity [24]. The linearity of different concentrations of 2, 4, 6, 8, and 10 µg/ml was found. A graph was plotted taking area of peak on y-axis and concentration (µg/ml) along the x-axis (fig. 5). 0.9992 was r^2 value with the regression equation y = 21946x+3403.2.

Table 1: System suitability results for TBZ

Parameters	Value	Limits
HETP	22.01±0.37	Depends upon the theoretical plate
Theoretical plate	6848±43	>2000
Theoretical plate/meter	45652±287	>20000
Tailing factor	1.10±0.002	<2
Peak purity index	0.999	>0.5

*Data are expressed as mean±SD; n=6

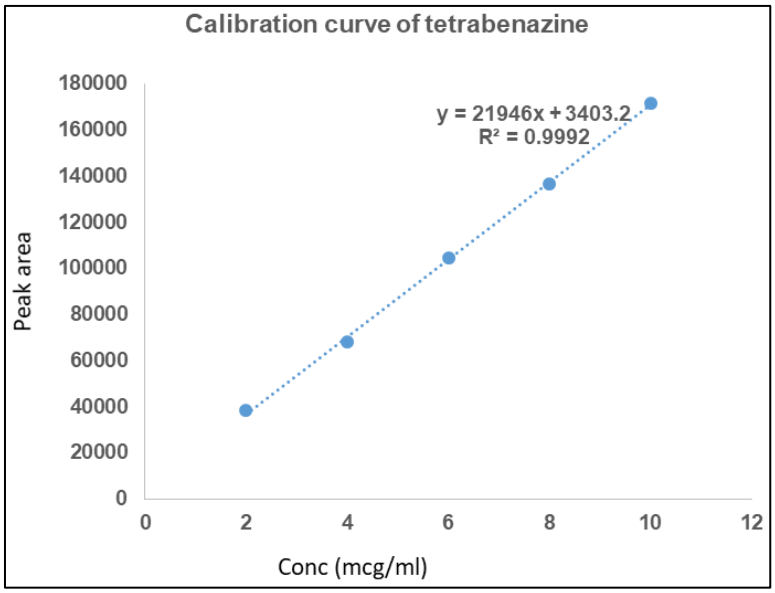

Fig. 5: Calibration curve of TBZ

Accuracy

Accuracy of standard solution was executed by percentage recovery of standard solutions. Percentage recovery was found in between 85.83%-91.38%. The accuracy of a test relates to how closely the results match the true value [25]. The obtained results are depicted in table 2.

Precision

Precision studies have been performed to check whether the method is repeatable. The obtained results are presented in table 3. The observed % RSD for intraday (0.64-1.96%), interday (0.60-1.880%), and interanalyst (0.60-1.91%) which were less than 2% for all the samples, which prove this method was satisfactorily, repeatable and precised [16].

Robustness

Robustness study was done by changing the pH of the mobile phase (3.0, 3.2 and 3.4), flow rate (0.8, 1.0 and 1.2 ml/min) and the ratio of mobile phase ACN: 0.1% formic acid (88:12, 90:10 and 92:08), respectively. The result of %RSD was in between 1.24-1.67% which were less than 2% (table 4), which showed this method was unaffected by these changes and satisfactory robust. This is the method in which three factors are considered for robustness.

Table 2: Result of accuracy studies

Levels	Concentration of standard solution (µg/ml)	Concentration of drug added (µg/ml)	Recovered concentration (µg/ml)	Recovery (%)	Mean recovery (%)
LQC	4.8	6.0	4.3	89.58	
MQC	6.0	6.0	5.15	85.83	88.93
HQC	7.2	6.0	6.58	91.38	

*Data are expressed as mean±SD; n=6

Table 3: Outcomes of TBZ precision experiment

Parameters	Level	Concentration (µg/ml)	Analytical responses (area), injections 1	2	3	4	5	6	Mean (*N=6)	SD	%RSD
Repeatability (intraday precision)											
	LQC	4.8	99103	98742	98195	99752	98042	98570	98734.00	627.51	0.64
	MQC	6.0	104088	103671	106085	101326	103843	101238	103375.17	1839.80	1.78
	HQC	7.2	148847	147940	145179	146371	153138	145941	147902.70	2895.31	1.96
Interday											
Day 1	LQC	4.8	100800	99300	97149	101644	97810	98708	99235.17	1727.22	1.74
	MQC	6.0	113618	114959	114233	110419	110570	111176	112495.83	2005.40	1.78
	HQC	7.2	160419	160818	159205	158758	159698	157338	159372.67	1251.83	0.79
Day 2	LQC	4.8	102340	103721	102991	103161	102146	102269	102771.33	621.51	0.60
	MQC	6.0	107957	106914	106610	106254	105701	106287	106620.50	769.87	0.72
	HQC	7.2	155035	152976	153634	151066	151668	155023	153233.67	1662.72	1.09
Day 3	LQC	4.8	106031	104058	105570	105581	106324	105939	105583.83	800.29	0.76
	MQC	6.0	137157	136692	137042	137505	138961	139253	137768.33	1072.92	0.78
	HQC	7.2	189687	190240	197962	196294	197082	196424	194614.83	3655.29	1.88
Inter analyst											
Analyst 1	LQC	4.8	97511	99155	98424	99086	98382	98581	98523.16	595.58	0.60
	MQC	6.0	111095	112099	116379	114497	111248	114692	113335.00	2161.41	1.91
	HQC	7.2	152384	156990	150695	153388	156093	153763	153885.50	2332.69	1.52
Analyst 2	LQC	4.8	140015	145172	141923	141502	142676	142410	142283.00	1697.07	1.19
	MQC	6.0	120018	117776	117572	113830	118311	116454	117326.83	2071.83	1.76
	HQC	7.2	160327	157810	162809	160533	161919	158078	160246.00	2004.55	1.25
Analyst 3	LQC	4.8	120202	126452	122477	124958	124815	123884	123798.00	2195.78	1.77
	MQC	6.0	158504	154018	156134	150555	152525	154409	154357.50	2767.10	1.79
	HQC	7.2	196724	199904	200742	197805	194714	200525	198402.30	2408.82	1.21

*Data are expressed as mean±SD; n=6

Table 4: TBZ robustness results

Various parameters	Value	Concentration (µg/ml)	Peak (mean±SD) (*N=6)	Mean of peak area of three value (*N=3)	Retention time (in min) (mean±SD) (*N=6)	Mean of retention times of three values (*N=3)	% Recovery (mean±SD) (*N=3)	Mean of % recoveries of three values (*N=3)
pH	3.0	6.0	112306.70±7105.37	118090.73	4.93±0.01	4.99	105.09±0.97	104.78
	3.2	6.0	114787.50±3189.42	SD=2152.71	5.05±0.77	SD=0.06	103.35±1.02	SD=1.30
	3.4	6.0	119041.00±3597.69	%RSD=1.82	5.01±0.58	%RSD=1.19	105.90±1.23	%RSD=1.24
Flow rate	0.8	6.0	137009.70±3287.00	136192.00	4.42±0.02	4.50	100.00±1.57	100.33
	1.0	6.0	137768.30±2299.50	SD=2107.68	4.50±0.01	SD=0.09	102.00±1.22	SD=1.52
	1.2	6.0	133798.00±2437.00	%RSD=1.54	4.60±0.03	%RSD=2.00	99.00±1.58	%RSD=1.52
Mobile phase ratio (A: B)	88:12	6.0	126103.00±3958.50	124069.30	4.82±0.06	4.94	93.00±1.16	91.33
	90:10	6.0	124168.20±1437.50	SD=2084.75	5.03±0.04	SD=0.06	91.00±1.05	SD=1.52
	92:08	6.0	121937.00±1571.00	%RSD=1.68	4.44±0.09	%RSD=1.12	90.00±1.13	%RSD=1.67

*Data are expressed as mean±SD; n=6

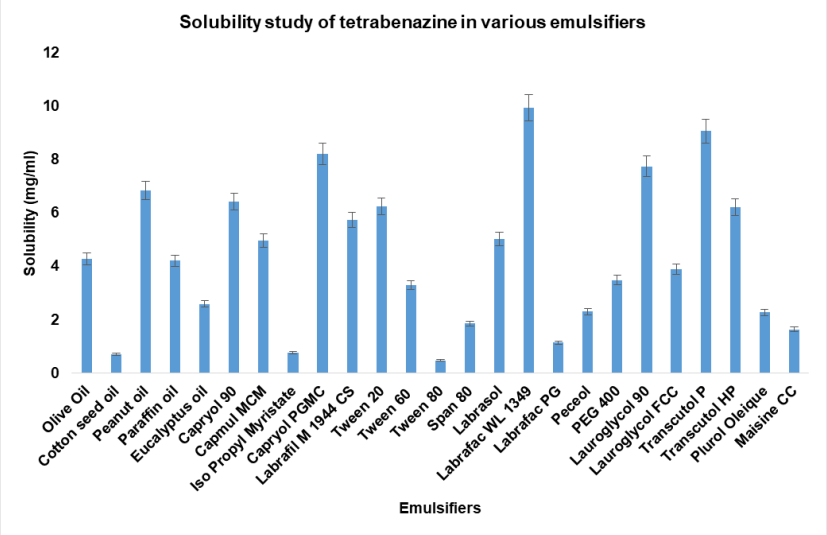

Fig. 6: Solubility of TBZ in oils, surfactants and co-surfactants, *data are expressed as mean±SD; n=3

Estimation of LOD and LOQ

As per ICH guidelines, LOD and LOQ were calculated by the standard deviation of response and slope. The method has very low LOD and LOQ values i. e. 0.31 µg/ml and 0.96 µg/ml, respectively, indicating that the presented method for TBZ estimation has high sensitivity [23].

Application of HPLC method in estimation of drug solubility

Solubility of TBZ

The solubility study of TBZ was done in various oil, surfactants and co-surfactants. The solubility was found highest in Labrafac WL1349, Transcutol P, and Capryol PGMC in surfactant, co-surfactant, and oil respectively (fig. 6). This study helps to select key components for SNEDDS formulations.

Determination of drug loading

The drug loading was found to be 79.2±1.6% in SNEDDS.

CONCLUSION

The developed RP-HPLC method for determining TBZ was reliable, selective and simple, providing adequate precision and accuracy with a lower limit of quantification and detection. The validation studies reported that the developed method was rugged and robust. So this method can be used to estimate the presence of TBZ in various pharmaceutical formulations. Further, the developed method can be used to determine the solubility of TBZ in different oil, surfactant and co-surfactants.

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AUTHORS CONTRIBUTIONS

Conceptualization Shashi (S.), Narendra Kumar Pandey (N. K. P.),; review and editing, S., N. K. P., and Sachin Kumar Singh (S. K. S.),; resources, S., N. K. P., Bimlesh Kumar (B. K.), S. K. S.,; Design, S., N. K. P., Kalvatala Sudhakar (K. S.), Saurabh Singh (S. S.); data collection and/or Processing S., N. K. P., B. K., S. S., K. V.; Analysis and Interpretation, N. K. P., Dileep Singh Baghel (D. S. B.); writing original draft, S., D. S. B., N. K. P.; supervision, N. K. P., S. K. S., B. K.

CONFLICT OF INTERESTS

No conflict of interest was declared by the authors.

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