Section A-Research paper

Bioanalytical reverse phase high performance liquid chromatography method development and validation for the estimation of Tetrabenazine in rat plasma

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Abstract

Tetrabenazine (TBZ) in rat plasma can be estimated using a reverse-phase high-performance liquid chromatography (RP-HPLC) approach employing benzoquinolizine as an internal reference. ICH M10 criteria were used to validate the created approach. Acetonitrile (ACN) and 0.1% formic acid were used in a ratio of 90:10 v/v for the separation, with a flow rate of 1 mL/min and a detection wavelength of 283 nm. Benzoquinolizine and TBZ had retention times (RT) of 3.665 and 5.064 minutes, respectively. The developed method's regression coefficient (r2) of 0.996 revealed that it is linear in the 50–250 ng/mL range. Indicating their accuracy and precision, the TBZ recovery (%) was found to be over 92% and their relative standard deviation (%) was determined to be less than 2%. The plasma concentration of TBZ had a limit of detection (LOD) and limit of quantitation (LOQ) of 18.19 ng/mL and 55.14 ng/mL, respectively. To ensure the stability of the medication in plasma,

investigations on short-term stability and freeze-thaw cycles were also carried out. The pharmacokinetics of drug were investigated using the recognised methodology.

Keyword: Tetrabenazine, internal standard, plasma, stability, validation, accuracy

Introduction

Tetrabenazine was first introduce as antipsychotic drug in 1960's but didn't give beneficial effects (Hayden et al., 2009). It was taken off the market in 1966 because it was ineffective as a strong neuroleptic (Guay, 2010). It gained FDA approval in 2008 for the treatment of chorea associated with HD and other hyperkinetic movement disorders (Kaur et al., 2016) (Chen et al., 2012) (Yao et al., 2011) (Hussar, 2008). It's chemical name is *cis* rac-1,3,4,6,7,11b-hexahydro-9,10-dimethoxy-3-(2-methylpropyl)-2H-benzo[a] quinolizin-2-one (Guay, 2010) (Figure 1). It is a benzoquinolizine pharmacophore that is a reversible depletor of monoamines (dopamine, norepinephrine, serotonin, histamine) from nerve terminals (Hussar, 2008) (Derangula et al., 2013). It is a selective, reversible, high affinity inhibitor of VMAT2 inhibition of VMAT2 by TBZ reduces the uptake of monoamines into presynaptic vesicles and thereby depletes concentrations of monoamines, particularly dopamine, as observed in preclinical and human post-mortem studies (Scott, 2011). Adverse events associated with TBZ use are related to the agent's effects within the central nervous system, namely sedation, fatigue, insomnia, depression, somnolence and akathisia (Setter et al., 2009) (Yero & Rey, 2008).

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 0.6mL/min flow rate and 10 min retention time (Mehvar et al., 1986). 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

5mM ammonium acetate as mobile phase in the ratio of 60:40 with 0.8mL/min with 2.5 min retention time (Derangula et al., 2013), 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.

A simple, sensitive, accurate, and precise bioanalytical method has been developed for the estimation of TBZ in rat plasma. The method was validated in terms of accuracy, precision, sensitivity and specificity and found acceptable. The developed method was successfully used to perform the pharmacokinetic analysis of TBZ in plasma.

2. Materials and Methods

2.1 Materials

TBZ was kindly gifted from Synnat Pharma Pvt. Ltd., India. Formic acid purchased from LOBA CHEMIE Pvt. Ltd., Mumbai, India. Acetonitrile was procured from Merck, Mumbai, India. All other chemicals and reagents used were of analytical grade and HPLC grade. The experiment was conducted with triple-distilled water. A mobile phase delivery pump (LC-20 AD; Prominence, Shimadzu, Japan), a photodiode arrays detector (PDA) (SPDM20A; Shimadzu, Japan), a 20 L loop (Rheodyne) for injecting sample, and a C-18 reverse phase column (Nucleodur C18, 250 mm 4.6 mm i.d., 5 Macherey Nagel) were used to separate the components in the high-performance liquid chromatograph. LC solution software was used to operate the HPLC equipment.

Animals

Six female Sprague Dawley rats were obtained for this study from the Panjab University's Central Animal Home in Chandigarh, India. Rats ranged in age from 11 to 12 weeks and weight from 200 to 250 g. The rats were housed at 25±2°C, 55±10% relative humidity, and 12:12 light:dark cycled husk-lined polypropylene cages. The animals were provided with unlimited access to water as well as a standard pellet diet The study procedure was authorized by the Lovely Professional University School of Pharmaceutical Sciences' Institutional Animal Ethics Committee (Protocol no: LPU/IAEC/2022/05).

Methods

Chromatographic Conditions

HPLC system (Shimadzu LC-20AD, Japan) carry photodiode array detector (SPD-M20A) and a Rheodyne injector (7725i) with 20 µL loop was used for injecting samples. A C18 reverse-phase column (Nucleodur C18, 250 mm x 4.6 mm i.d., 5μ, Macherey Nagel) was used for the elution of plasma components. LC solution software was used for operating the entire system. For drug quantification in rat plasma a bioanalytical method was developed utilizing ICH M10 standards. The mobile phase consisting acetonitrile and 0.1% formic acid was used in the ratio of 90:10 v/v. The flow rate was kept 1 mL/min for develop the chromatogram. 283 nm wavelength was selected to detect the drug. TBZ was analyzed using various mobile phase compositions, such as acetonitrile-0.1% orthophosphoric acid, acetonitrile-water, and 0.1% glacial acetic acid –acetonitrile in different ratio and different pH.

Collection of blood and extraction of plasma

The blood was collected using the orbital sinus technique with the help of a capillary tube. In this rat is handled with thumb and forefinger to stretch the area around eyes. Clean the eyes with the help of cotton and water. Using a capillary tube and radioimmunoassay (RIA) vials containing ethylene diamine tetra acetic acid (EDTA) crystals, blood sample was collected from rats through the retro-orbital puncture. Once, the sinus is punctured, blood comes out via capillary and is collected in EDTA (ethylene diamine tetraacetic acid) vials. The EDTA tubes were centrifuged at 2000 rpm for 15 min. The clear supernatant was withdrawn with a micro pippete and stored for processing in a deep freezer at -20 °C.

Preparation of blank plasma

An adequate amount of plasma (1mL) was taken in eppendorf followed by the addition of 2 mL of ACN. The mixture was vortexed for about 5 min to precipitate the plasma proteins. The clear supernatant was collected in separate eppendorf and was further centrifuged at 2000*g* for 15 min. After this, the obtained supernatant was transferred in a 100 ml volumetric flask and the volume was made up to 100 mL.

Preparation of standard stock solutions

To obtain a solution with a concentration of 100 mg/mL, 10 mg of TBZ was dissolved in 10 mL of acetonitrile in a 100 mL volumetric flask (Solution A). Acetonitrile up to 100 mL was added to a second volumetric flask to dilute Solution A to a concentration of 10 mg/mL. (Solution B). By combining 10 mL aliquots of solution B with 100 mL of acetonitrile to achieve a concentration of 1.0 mg/mL, a further TBZ dilution was produced (Solution C). 20 mL of liquid from solution C was dissolved in a 100 mL volumetric flask, and the volume was increased by 100 mL of acetonitrile to reach a concentration of 200 ng/mL. (Solution D). In a 100 mL volumetric flask, 10 mg of benzoquinolizine were dissolved in a tiny amount of acetonitrile, and the volume was subsequently raised to 100 mL using acetonitrile to get a final concentration of 100 mg/mL. (Solution E).

Preparation of internal standard (IS)

Benzoquinolizine 10 mg/mL was used as the IS for dilution preparation. A quantity of 10 mg was weighed and added to a volumetric flask (100 mL) containing 20 mL of ACN. The solution was sonicated for 10 minutes. The final volume was adjusted to 100 mL using ACN to produce a stock solution with a concentration of 100 µg/mL.

Specificity study

TBZ and blank plasma samples were injected on HPLC using mobile phase acetonitrile: 0.1% formic acid (90:10 v/v) to validate method specificity. These were analysed at 283 nm to identify any interference between the drugs and plasma peaks.

Development of calibration curve

Aliquots of 0.5, 1.0, 1.5, 2.0, and 2.5 mL of solution D were put into individual 10 mL volumetric flasks and addition of 0.1 mL of plasma was done in above solution. Acetone (1 mL) was added to each sample and thoroughly mixed with a sonicator for 15 minutes to precipitate and denature plasma protein. The entire sample was then centrifuged for 30 minutes at 4 °C using an eppendorf at 10,000 rpm. The supernatant was collected using a micropipette, and the volume was adjusted to 10 mL in a volumetric flask to get TBZ concentrations of 50, 100, 150, 200, and 250 ng/mL and benzoquinolizine concentrations of 10 µg/mL. The final prepared samples were injected into HPLC for evaluation of TBZ and benzoquinolizine.

Validation of the method

The created approach was validated in accordance with the ICH M10 standard (International Conference on Harmonization, 2019). To further evaluate the system's performance, measurements of the tailing factor, theoretical plate, height equal to theoretical plate, detection limit, and quantification limit were checked.

Linearity and range

The concentration was plotted on the X axis, and the mean peak area was plotted on the Y axis, to create the calibration curve. The slope, standard deviation of response (sigma), y-intercept standard deviation of the intercept, and regression coefficient (r^2) were calculated using the calibration data (Hardeep et al., 2022).

Accuracy

To assess the accuracy of the procedure, the absolute recovery of the drug from the quality control samples was assessed. Lower quantified concentration (LQC, 80%), medium quantified concentration (MQC, 100%), and high quantified concentration (HQC, 120%) at the midrange value of 100 ng/mL were the three concentration levels of the procedure that were employed to create the samples. To create these

concentrations, aliquots of solution D in the quantities of 1.2, 1.5, and 1.8 mL were put into separate 10 mL volumetric flasks. Afterwards, 0.1 mL of plasma and 1 mL of solution E were added. The samples were sonicated and centrifuged in an eppendorf at 10,000 rpm for 30 minutes at 4 °C after adding plasma. Then, up to 10mL of ACN were added to make up the volume. These concentrations and benzoquinolizine (10µg/mL) were injected (6 times) in HPLC. The formula shown in the following equation 1 was used to estimate the absolute percentage of drug recovery (Harish et al., 2022):

$$\text{Actual \% recovery} = \text{Actual concentration recovered}/\text{Theoretical concentration}*100 \quad (1)$$

Precision

The new method's precision was assessed using its repeatability and intermediate precision. To assure repeatability, six injections of the LQC, MQC, and HQC samples were made into the same experimental setup on the same day (without the addition of sample solution F). The intermediate accuracy was calculated by calculating LQC, MQC, and HQC samples six times under similar experimental settings but on separate days with different analysts (inter-analyst). Following the collection of the mean data, the percentage relative standard deviation was calculated using the formula shown in the equation 2 below (Cox et al., 2021):

$$\text{\% Relative standard deviation} = \text{Standard deviation of peak area} / \text{Average peak area}*100 \quad (2)$$

System suitability and estimation of LOD and LOQ

Peak purity index, height equivalent to theoretical plate (HETP), theoretical plate, and tailing factor were used to assess system compatibility. The limit of detection (LOD) and limit of quantification (LOQ) were calculated using the standard deviation of response (sigma) and slope of the calibration curve (S). The standard deviation was calculated using the standard deviation of the Y intercepts of the regression line. The following equations 3, 4 were used to calculate the results (D'Cruz et al., 2017) :

$$\mathrm{LOD} = 3.3\sigma/S \quad (3)$$

$$\mathrm{LOQ} = 10\sigma/S \quad (4)$$

Stability study

The stability of plasma samples spiked with TBZ was investigated using three freeze-thaw cycles, with short-term stability at ambient temperature for three hours and long-term stability at -20 °C for three weeks. One RIA vial containing 3 mL of plasma was collected for freeze-thaw stability. The solution was vortexed for five minutes after 10 mg of TBZ (to make a concentration of 1000 mg/mL) was added to this vial. This test tube was kept in a freezer set to a temperature of -20 °C. The frozen sample was removed from the test tube and thawed at room temperature. 1 mL of plasma was extracted (Cycle 1) from the thawed samples, and the remaining 2 mL was kept in the deep freezer for the subsequent cycle. From the extracted plasma (1 mL), the drug was precipitated, and the supernatant was centrifuged. The clear translucent supernatant was collected after centrifugation and diluted with ACN to 100 mL to achieve a concentration of 100 mg/mL. Furthermore, dilutions to produce 120 ng/mL (LQC), 150 ng/mL (MQC), and 180 ng/mL were made (HQC). As in cycle 1 the last frozen plasma sample (2 mL) was removed, allowed to thaw at room temperature, and then 1 mL was extracted (Cycle 2). The remaining 1 mL of frozen plasma was then returned to the freezer. It froze, then was removed and thawed (Cycle 3). In order to prepare LQC, MQC, and HQC samples for Cycles 2 and 3, the procedure was repeated. Each of these solutions received 10 mg/mL of IS addition. All dilutions were made in triplicate, put into HPLC, and had their retention at 283 nm. For each concentration, the mean, standard deviation, and percent RSD were computed. For long-term stability (to reach a concentration of 1000 mg/mL), 1 mL of plasma was added to three RIA vials holding 1 mg of TBZ each. When the mixture had been vortexed for five minutes, all three vials were placed in the freezer at a temperature of -20 °C. The three vials were taken out of the freezer after one, two, and three weeks. The medications were removed from the plasma after each interval, ready for the LQC, MQC, and HQC samples, and then IS (10 mg/mL) was added. Each dilution was created in triplicate and then injected into the HPLC in order to measure the retention time at 283 nm. For each concentration, the mean, standard deviation, and percent RSD were computed.

Statistical analysis

The experimental value are all reported as mean standard deviation (SD). The mean, standard deviation, and percent relative standard deviation were calculated using an MS Excel worksheet. In Graph Pad Prism version 7.0, the calibration curve was developed and the results were compared using Tukey's multiple comparison test. (GraphPad Software Inc., CA, USA). A significant difference in the collected data was shown by a P value less than 0.05.

Results and discussion

Specificity, linearity and range

Developed method was found to be specific for particular drug molecule as blank plasma sample chromatogram, there was no peak present at same retention time and wavelength. On the basis of concentration range (50-250 ng/mL) of plasma based sample the calibration curve was shown to be linear. 0.9964 coefficient of regression was showing linearity. The calibration curve shown in figure 2, and chromatogram of blank plasma and TBZ in rat plasma are given in figure 3.

Accuracy

As per the results, for all levels the value of mean percentage recovery were within 95-105% which is within the standard range. As shown in table 1 the developed method was accurate under experimental condtitions.

Precision

The precision of the developed method was measured by determining the %RSD of the prepared LQC, MQC and HQC samples that were injected in six replicates at intraday, interday, and interanalyst precision under same experimental conditions. Therefore, the %RSD was found to be less than 2, which indicated that the developed method was précised under the test conditions used. The data is presented in Table 2.

Stability study of plasma samples

The stability of the drugs in plasma at three different levels i.e., LQC, MQC and HQC in terms of freeze-thaw, short-term and long-term stability is presented in Table 3, 4 and 5 respectively. The results obtained indicated recovery of the drug within the prescribed limits i.e. more than 90% with %RSD less than 2. This signified the long-term storage of drugs in plasma samples.

LOD and LOQ

The limit of detection (LOD) and limit of quantification (LOQ) were found in rat plasma to be 18.19 ng/mL and 55.14 ng/mL, respectively. These showed that the method was sensitive for detection of the drug at lower concentrations.

System suitability

By using system suitability test chromatographic conditions are examined for further use. For TBZ and Benzoquinolizine tailing factor and theoretical plates is <2 and >2000 respectively which is under acceptable range and showing good efficiency of column throughout the method shown in table 6.

Conclusion

The goal of the research was to create a bioanalytical approach that would be affordable, simple, sensitive, accurate, and precise for measuring tetrabenazine in rat plasma. Tetrabenazine was recovered from plasma samples at a rate of 95–105 percent, which is outstanding. System

suitability experiments showed that the procedure was repeatable and reliable because the RSD of samples with different concentrations utilised for intraday and intermediate precision studies was less than 2%. The method has reduced linearity and range, lower LOQ and LOD values, and dramatically enhances drug recovery from plasma samples when compared to previous techniques for detecting TBZ in biological samples. The created approach can be utilised to research the pharmacokinetics and biodistribution of TBZ or bulk medication in various pharmaceutical formulations.

Conflict of interest

The author declare no conflict of interest.

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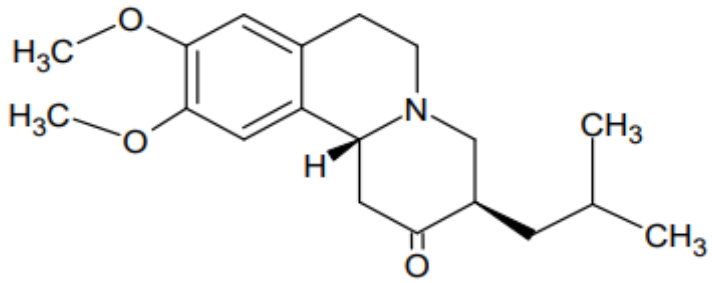

Figure 1: Structure of Tetrabenazine

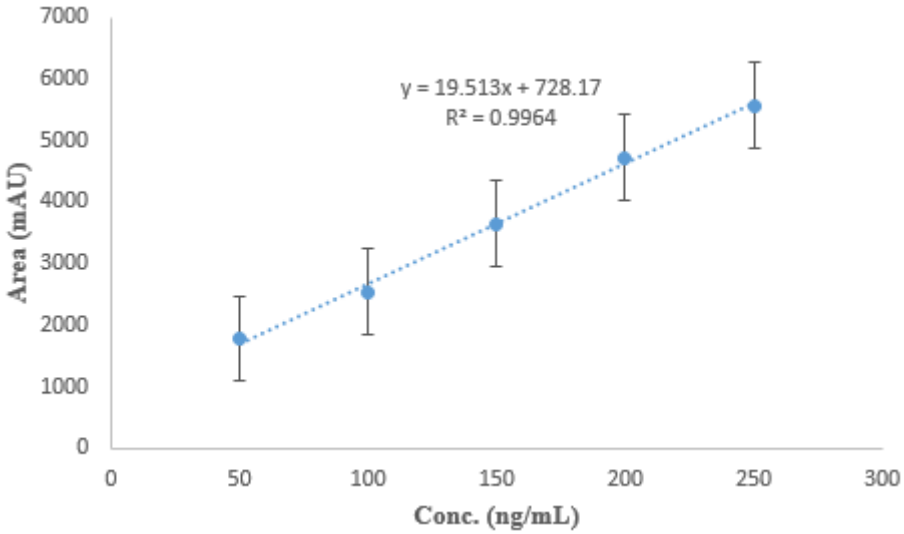

Figure2: Calibration curve of TBZ

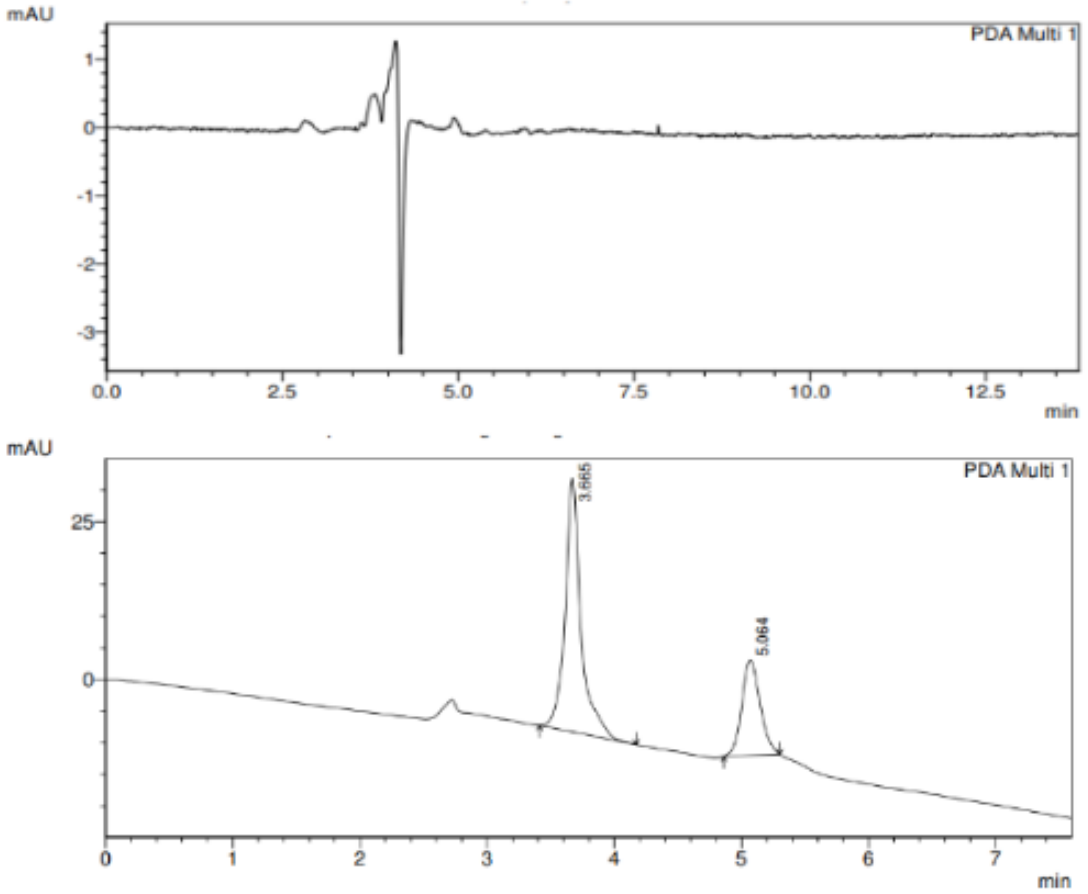

Figure 3: (A) Chromatogram of Blank Plasma, (B) Chromatogram of Benzoquinolizine (RT. 3.665) and TBZ (RT. 5.064) in rat plasma

Table 1: Results of accuracy study

Level	**Concentration of sample solution (ng/mL)**	**Total concentration of solution, actual (ng/mL)**	**Concentration of drug recovered (ng/mL), (n=5)**	**% Recovery**	**Mean % recovery**
LQC	50	120	112.62 ± 1.39	93.85 ± 0.86	99.89 ± 0.64
MQC	50	150	150.54 ± 0.87	100.36 ± 0.59	
HQC	50	180	189.87 ± 0.49	105.48 ± 0.48	

Table 2: Results of precision study

Parameters	Level	Conc. (ng/mL)	Analytical responses						Mean (N=6)	SD	%RSD
			1	2	3	4	5	6			
Repeatability (intraday precision)											
1 hour	LQC	120	2907	2956	2934	2987	2911	2946	2940.16	27.28	0.92
	MQC	150	3683	3694	3675	3629	3687	3672	3673.33	21.12	0.57
	HQC	180	4446	4413	4436	4482	4465	4491	4455.50	26.86	0.60
2 hour	LQC	120	2981	2945	2971	2943	2974	2991	2967.50	17.77	0.59
	MQC	150	3757	3786	3711	3794	3784	3786	3766.67	27.73	0.73
	HQC	180	4501	4587	4468	4472	4485	4531	4507.33	41.31	0.91
3 hour	LQC	120	2878	2876	2883	2914	2897	2905	2892.17	14.20	0.49
	MQC	150	3810	3845	3788	3791	3812	3867	3818.83	28.44	0.74
	HQC	180	4554	4513	4562	4538	4573	4560	4550.00	19.58	0.43
Intermediate precision (interday)											
Day 1	LQC	120	2971	2987	2978	2964	2943	2983	2971.00	14.61	0.49
	MQC	150	3740	3786	3757	3764	3776	3701	3754.00	27.75	0.73
	HQC	180	4424	4491	4398	4437	4401	4389	4423.33	34.36	0.77
Day 2	LQC	120	2914	2962	2933	2951	2978	3001	2956.50	28.46	0.96
	MQC	150	3717	3729	3799	3685	3825	3738	3748.83	48.16	1.28
	HQC	180	4424	4393	4457	4436	4382	4445	4422.83	27.05	0.61
Day 3	LQC	120	2963	2945	2925	2887	2903	2888	2918.50	28.54	0.97
	MQC	150	3724	3699	3785	3755	3759	3801	3753.83	34.45	0.91
	HQC	180	4510	4526	4508	4534	4511	4491	4513.33	13.73	0.30
Intermediate precision (interanalyst)											
Analyst 1	LQC	120	2895	2861	2872	2873	2894	2904	2883.16	15.33	0.53
	MQC	150	3801	3843	3784	3915	3851	3876	3845.00	43.89	1.14
	HQC	180	4454	4487	4435	4398	4482	4435	4448.50	30.41	0.68
Analyst 2	LQC	120	2987	2945	2915	2845	2888	2934	2919.00	44.68	1.53
	MQC	150	3936	3958	3972	3961	3999	3971	3966.16	18.88	0.47
	HQC	180	4501	4523	4572	4531	4511	4584	4537.00	30.65	0.67
Analyst 3	LQC	120	2832	2943	2951	2876	2947	2866	2902.50	46.50	1.60
	MQC	150	3847	3858	3892	3867	3794	3765	3837.16	43.77	1.14
	HQC	180	4605	4598	4572	4592	4584	4575	4587.16	11.86	0.25

Table 3: Short term stability for plasma samples of TBZ

Actual concentration of drug (ng/mL)	**Area 1 (cm^2)**	**Area 2 (cm^2)**	**Area 3 (cm^2)**	**Mean (cm^2)**	**S.D.**	**%RSD**	**Amount of drug recovered in plasma (ng/mL)**	**Recovery (%)**
1 hour								
120LQC	2912	2933	2931	2925.33	11.59	0.39	112.59	93.83
150MQC	3640	3657	3634	3643.66	11.93	0.32	149.41	99.61
180HQC	4475	4462	4438	4454.50	17.13	0.38	191.16	106.20
2 hour								
120LQC	2873	2974	2932	2926.33	50.73	1.73	113.72	94.76
150MQC	3682	3725	3701	3702.67	21.54	0.58	151.97	101.31
180HQC	4412	4546	4483	4480.33	67.03	1.49	191.70	106.50
3hour								
120LQC	2988	2873	2921	2927.33	57.76	1.97	112.70	93.91
150MQC	3759	3683	3625	3722.33	67.20	1.82	151.97	101.15
180HQC	4472	4567	4413	4484.00	77.69	1.73	192.47	106.93

Table 4: Freeze thaw stability for plasma samples of TBZ

Actual concentration of drug (ng/mL)	**Area 1 (cm^2)**	**Area 2 (cm^2)**	**Area 3 (cm^2)**	**Mean (cm^2)**	**S.D.**	**%RSD**	**Amount of drug recovered in plasma (ng/mL)**	**Recovery (%)**
Cycle 1								
120LQC	2871	2959	2867	2899.00	52.00	1.79	111.25	92.70
150MQC	3697	3683	3745	3708.33	32.51	0.87	152.72	101.82
180HQC	4512	4599	4636	4562.33	63.65	1.38	197.51	109.73
Cycle 2								
120LQC	2967	2894	2943	2934.67	37.20	1.26	113.07	94.23
150MQC	3622	3746	3685	3684.33	62.00	1.68	151.49	100.99
180HQC	4559	4467	4585	4537.00	62.00	1.36	195.19	108.44
Cycle 3								
120LQC	3011	3057	2966	3018.00	36.01	1.19	117.34	97.79
150MQC	3758	3699	3705	3720.66	32.47	0.87	153.35	102.23
180HQC	4512	4568	4587	4555.67	38.99	0.85	196.15	108.97

Table 5: Long term stability for plasma samples of TBZ

Actual concentration of drug (ng/mL)	**Area 1 (cm^2)**	**Area 2 (cm^2)**	**Area 3 (cm^2)**	**Mean (cm^2)**	**S.D.**	**%RSD**	**Amount of drug recovered in plasma (ng/mL)**	**Recovery (%)**
1st week								
LQC	2876	2948	2971	2931.67	49.59	1.69	112.57	93.81
MQC	3652	3741	3678	3690.33	45.76	1.24	151.33	100.89
HQC	4564	4644	4538	4582.00	55.24	1.21	196.89	109.38
2nd week								
LQC	2864	2947	2972	2927.67	56.53	1.93	112.37	93.64
MQC	3757	3786	3691	3744.67	48.69	1.30	154.11	102.74
HQC	4587	4614	4549	4583.33	32.65	0.71	196.96	109.42
3rd week								
LQC	2836	2891	2876	2867.67	28.43	0.99	109.30	91.09
MQC	3754	3717	3675	3715.33	39.52	1.06	152.61	101.74
HQC	4587	4637	4696	4638.67	52.51	1.13	199.79	110.99

Table 6: System suitability parameters

Parameters	Value	Limits
HETP	25.86±0.2	Depends on theoretical plate
Tailing Factor	1.17±0.05	<2
Theoretical Plate	7337.33±34.83	>2000