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A VALIDATED BIOANALYTICAL METHOD FOR QUANTIFICATION OF TOLVAPTAN BY RPHPLC-UV

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Abstract
Tolvaptan is a critical therapeutic agent for the treatment of various medical conditions, including hyponatremia and polycystic kidney disease. Developing a sensitive and reliable bioanalytical method for the quantification of Tolvaptan is essential to ensure its quality and efficacy in pharmaceutical formulations. In this study, a novel bioanalytical method for the estimation of Tolvaptan was developed and validated. The method was carried out with an Agilent HPLC with a UV detector. Column Agilent Eclipse XDB C-8 column was used at a flow rate of 1.0 mL/min. Detection was carried out at 253nm. The mobile phase consisting of a mixture of Methanol: 0.05 M Phosphate buffer (pH 5) (70:30 v/v) respectively. Developed method demonstrated excellent linearity over a wide concentration range (0.1 to 5µg/ml) and exhibited precision and accuracy within acceptable limits. The lower limit of quantification (0.5µg/ml) was, indicating the method's sensitivity. This validation will serve as a crucial foundation for its application in pivotal and crucial studies related to bioavailability, bioequivalence, pharmacokinetics, and toxic kinetics.

Keyword: Bioanalytical Method Development and Validation, Tolvaptan, Mobile Phase, HPLC, Column selection

Introduction:

The primary objective of developing bioanalytical methods is to precisely outline the method's design, operational parameters, limitations, and appropriateness for its designated purpose. Additionally, the optimization of the method for validation is a crucial aspect. A bioanalytical method encompasses a series of steps involved in gathering, processing, storing, and analyzing a biological matrix (such as blood, plasma, serum, or urine) to detect a chemical compound. Bioanalytical Method Validation is the systematic process employed to establish the suitability of a quantitative analytical method for biochemical applications. The application of bioanalytical method validation in the quantitative assessment of drugs and their metabolites in biological fluids significantly contributes to the assessment and interpretation of data from bioavailability, bioequivalence, pharmacokinetic, and toxicokinetic studies. These studies typically form a vital part of regulatory filings.[1,2]

Tolvaptan is chemically known as 4-Amino-5-chloro-2,3-dihydro-N-[1-(3-methoxypropyl)-4-piperidinyl]-7-benzofurancarboxamide Butanedioic Acid is a selective vasopressin V2 receptor antagonist (fig. 1).[3]

Tolvaptan serves as a non-peptide antagonist targeting the vasopressin V2 receptor and is primarily prescribed for addressing euvolemic or hypervolemic hyponatremia. Its mechanism of action involves enhancing free water clearance and ameliorating serum sodium levels. Given its therapeutic significance, the development of a sensitive and precise analytical method is imperative for determining Tolvaptan concentrations in pharmaceutical formulations. This medication finds application in the management of hypernatremia (low blood sodium) in individuals with heart failure or syndrome of inappropriate antidiuretic hormone (SIADH). Additionally, Tolvaptan is employed to decelerate the decline of kidney function in adults who face a heightened risk of rapidly progressing autosomal dominant polycystic kidney disease (ADPKD). Tolvaptan binds to V2 receptor with 1.8 times greater affinity than ADH. The drug is highly plasma protein bound (99%). About 40% of tolvaptan is bioavailable and the terminal half-life is about 12 h.[4,5,6,7,8]

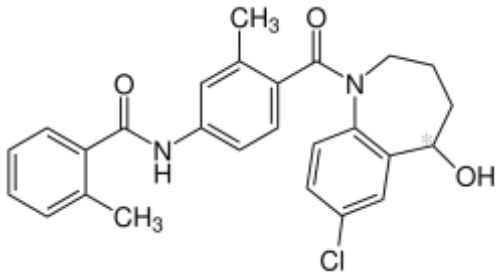

Figure 1: Structure of Tolvaptan

Literature survey reveals few UV spectrophotometric[9,10,11,12,13], RPHPLC[13,14,15,16,17], LC/MS-MS[18,19,20], UPLC[21] and HPTLC[22] methods for Tolvapatan estimation in bulk and pharmaceutical dosage form. Based on the literature review, stability-indicating RP-HPLC and UV-Visible method for the estimation of Tolvaptan was not found. Hence, it was felt that there is a need for a new bioanalytical analytical method.

Materials and Methods

Methanol HPLC grade was purchased from S D fine-chem limited, Mumbai and Orthophosphoric acid were bought From Merck Laboratories Pvt. Ltd., Mumbai. HPLC grade water used generated using Lab Link system. Pooled plasma was obtained as gift sample from Sasoon Hospital, Pune.

Preparation of standard stock solution of Tolvaptan

For standard stock solution accurately weighed 10 mg of Tolvaptan transferred to 10 ml volumetric flask and the volume was made up to 10 ml with methanol, to get standard stock solution of Tolvaptan (1000 µg/ml). Further dilutions were made with methanol to produce the stock solutions of 1, 2, 5, 10, 20, 30, 40, 50 µg/ml.

Preparation of Plasma Sample (Quality Control Samples)

Sample preparation consisted of the addition of 0.1 ml of plasma sample in 10 ml test tubes, then 0.1 ml of Std stock solutions was added, then 0.8 ml of methanol was added as precipitating agent to produce the final conc. of 0.1, 0.2, 0.5, 1, 2, 3, 4, 5 µg/ml, then vortex for 3 minutes, centrifugation for 10 minutes at 3000 r.p.m., then the supernatant was injected into HPLC system. The protein precipitation was the preferred choice of separation because of the minimized steps in extraction of drug from matrix.

Selection of Analytical Wavelength

A solution of 10 µg/ml was prepared from standard stock solution of Tolvaptan (1000µg/ml) and scanned over 200-400 nm in UV Spectrophotometer. The maximum absorbance was shown at 253 nm. Hence it was selected as analytical wavelength; UV spectrum is given in Fig. 2.

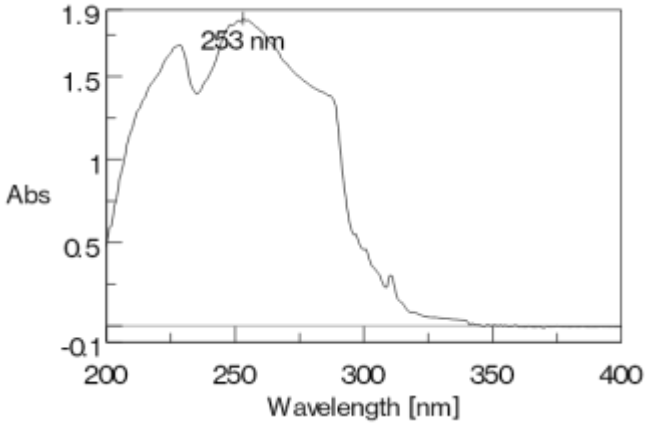

Figure 2: UV-Spectrum of Tolvaptan in methanol (10 µg/ml)

Mobile Phase Optimization

To achieve optimum chromatographic condition various columns and mobile phases were checked using column Agilent Eclipse XDB C-8. The Methanol: 0.05 M Phosphate buffer (pH 5): (80:20 v/v) system was initially tried but did not get a considerable number of theoretical plates as well as peak shape. The ratio changed (70:30 v/v) has resulted in considerable improvement of theoretical plates and appropriate peak shape with appropriate system suitability parameters. The system suitability parameters are given in the Table 1.

Table 1: System Suitability Parameters

Parameter	Obtained values
RT (min)	4.75 ± 0.02
Asymmetry	1.33
Plates (N)	2051

Bioanalytical Method Validation[23,24]

1. Selectivity/Specificity

Selectivity of analytical method is ability of method to differentiate and quantify the drug sample in presence of other interfering substance. The specificity of method is demonstrated by analyzing blank (Mobile Phase), blank plasma, API and spiked plasma (with API), given in fig. 3, 4, 5 and 6 respectively. There was no any interfering peak at the same RT of Tolvaptan.

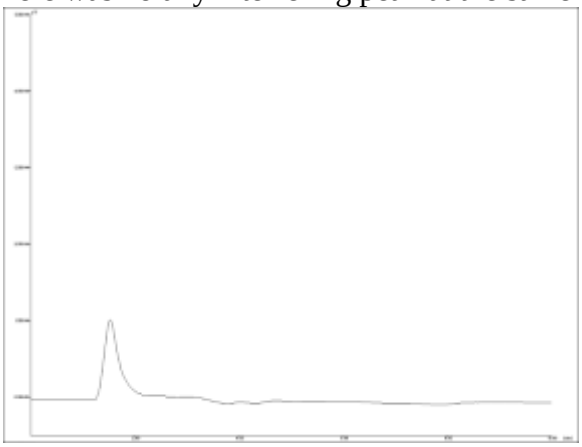

Figure 3: Chromatogram of Blank (MP)

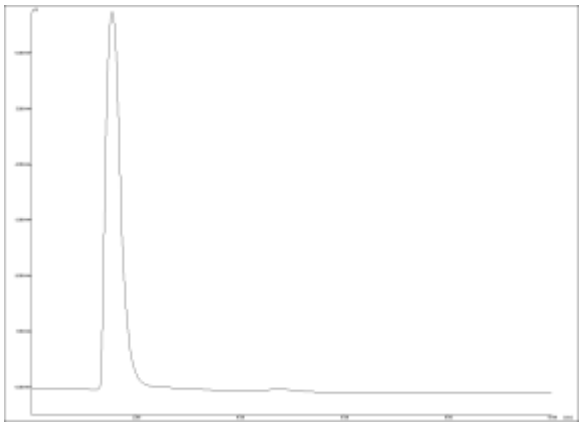

Figure 4: Chromatogram of Blank Plasma

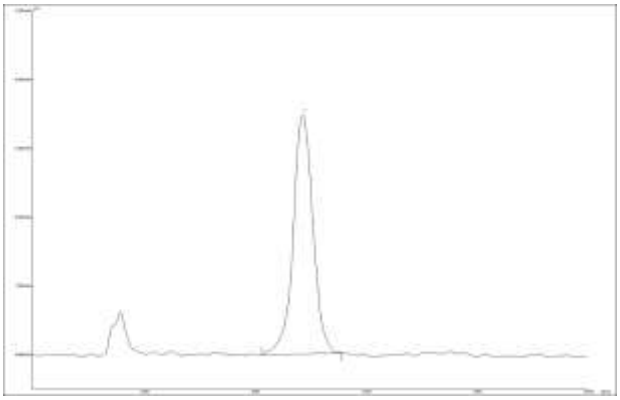

Figure 5: Chromatogram of API – Tolvaptan

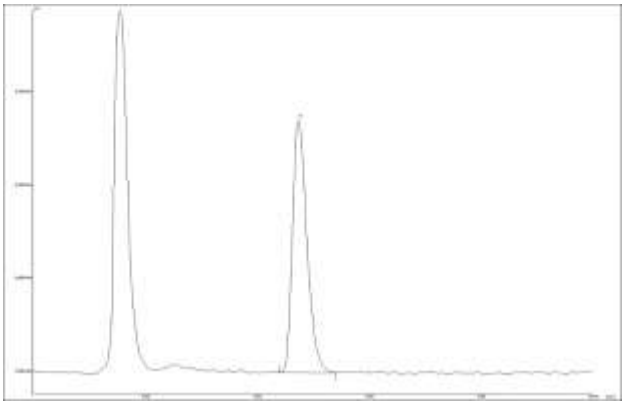

Figure 6: Chromatogram of Spiked Plasma (with API - Tolvaptan)

2. Calibration curve / Linearity

Calibration curve or linearity of method exhibit direct proportionality between detector response and concentration of analyte of interest. Linearity was tested for the range set in concentration of 0.1-5 µg/ml (fig. 7). 6 replicates of QC samples were analysed and peak areas were recorded. The correlation between the known concentration and response was evaluated through a regression analysis of calibration curve constructed using an eight-point (0.1, 0.2, 0.5, 1, 2, 3, 4 and 5 µg/mL) standard calibration curve. Calibration curve was constructed with drug response on Y-axis and concentration on X-axis. The correlation coefficient (R^2) values were calculated (fig. 8).

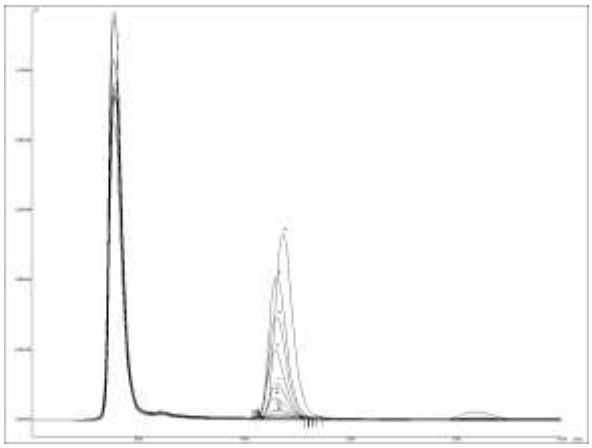

Figure 7: Overlay of Linearity (0.1-5 µg/ml)

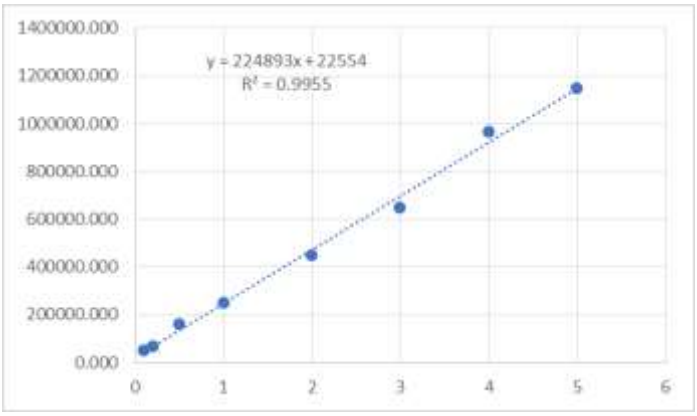

Figure 8: Calibration curve for Tolvaptan in spiked plasma

Table 2: Linearity of Tolvaptan

Conc. (µg/ml).	Area 1	Area 2	Area 3	Area 4	Area 5	Mean	SD	%CV
0.1	58009	48422	42707	46044	46044	48245	5825	12.07
0.2	61000	64863	69561	64632	76590	67329	6003	8.92
0.5	144700	142918	136290	172735	173714	154071	17766	11.53
1	218855	229070	254540	265127	272378	247994	23120	9.32
2	439491	429435	391324	491080	497465	449759	44487	9.89
3	635723	661598	626917	660235	656589	648212	15838	2.44
4	1007637	918371	908008	1032420	969504	967188	54273	5.61
5	1078972	1083275	1082639	1233415	1256415	1146943	89819	7.83

3. Accuracy

Accuracy was estimated by using minimum 5 replicates of 3 concentrations i.e., at LQC (0.5 µg/ml, MQC (2µg/ml), HQC (4µg/ml). The % mean accuracy was determined for all QC samples. Drug area was substituted in regression equation (y=mx+c) to get the concentration of the given sample. The deviation of the average from the theoretical value served as the estimation of accuracy. The accuracy at each concentration level should be within ± 15% of the nominal concentration.

Table 3: Results of Accuracy Studies

Replicates	LQC (0.5 µg/ml)		MQC (2 µg/ml)		HQC (4 µg/ml)	
	Area	Calcu. Conc	Area	Calcu. Conc	Area	Calcu. Conc
1	131527	0.488	436419	1.843	906902	3.932
2	126046	0.464	439699	1.857	917961	3.939
3	128764	0.476	438415	1.852	908372	3.982
4	135942	0.508	424778	1.791	915373	3.939
5	126720	0.467	459279	1.944	922457	4.001
Mean Area	129800		440542		914213	
SD	4042.43		14198.68		6536.57	
%CV	3.11		3.22		0.71	
%Accuracy	**96.98**		**93.05**		**99.28**	

4. Precision

Closeness of the individual measured value of the drug analyte among all aliquots of same volume of the plasma was assessed by injecting six replicates at, LQC, MQC and HQC levels. The precision of the method performed on HPLC-UV was evaluated by determining the %CV of the repeated injections. Intraday precision was evaluated by determining %CV of the response of the repeated injections injected on the same day. On the contrary, Interday precision was calculated after comparison of the measured values of the samples injected on three different days. According to the ICH M10 guideline, the precision (%CV) of the concentrations determined at each level should not exceed ±15%.

Table 4: Intraday Precision Studies

Concentration Level	Day 1	Day 2	Day 3	Mean	SD	%CV
LQC (0.5 µg/ml)	97527	106046	108765	104113	5863	**5.63**
MQC (2 µg/ml)	376419	339700	388415	368178	25382	**6.89**
HQC(4 µg/ml)	766903	667961	778372	737745	60706	**8.23**

Table 5: Interday Precision Studies

Concentration Level	Day 1	Day 2	Day 3	Mean	SD	%CV
LQC (0.5 µg/ml)	105942	106720	97611	102165	6441	**6.30**
MQC (2 µg/ml)	354778	389279	354778	366278	19919	**5.44**
HQC (4 µg/ml)	864373	822457	847633	844821	21099	**2.50**

5. Recovery

Recovery studies were performed by comparing the chromatographic response for samples after extraction at LQC, MQC and HQC with standard samples in three replicates. Recovery need not be 100 percent, but the extent of the recovery of an analyte should be consistent and reproducible.

Table 6: Results of Recovery Studies

Conc level	Area		%Recovery	%Mean Recovery
	Standard	Spiked plasma		
LQC (0.5µg/ml	159041	144700	90.98	**89.07**
	158449	142918	90.20	

)	158397	136290	86.04	
MQC (2µg/ml)	450348	439491	97.59	**92.15**
	464470	429435	92.46	
	452976	391324	86.39	
HQC (4µg/ml)	1141914	1069504	93.66	**91.48**
	1117867	1032420	92.36	
	1139450	1007637	88.43	

6. Carry Over

Carryover is the impact of the previous injection to the next injection of the analyte. It was determined by injecting blank samples after HQC injection of 4 µg/ml. According to the guidelines, response of samples should be below the LLOQ. Chromatograms obtained are shown in fig. 9, 10.

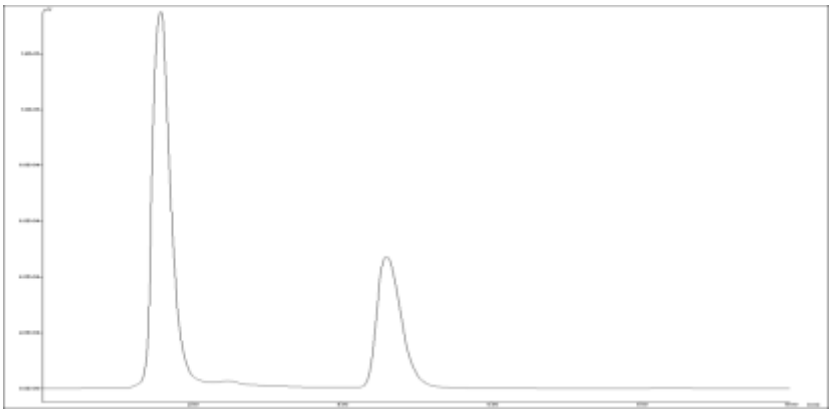

Figure 9: Chromatogram at HQC (4 µg/ml)

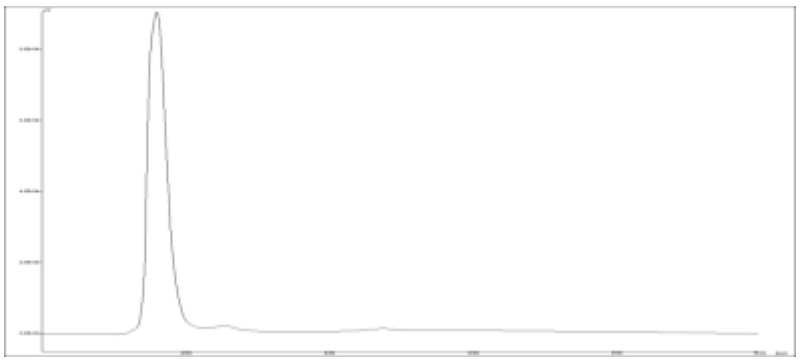

Figure 10: Chromatogram Blank

7. Stability

The purpose of determining stability is to detect any degradation of analyte occurred during entire process of sample collection, storage, extraction, and analysis. It is recommended to determine stability during short term storage, long term storage as well as during freeze thaw cycles. Stability samples should be compared with freshly prepared QC samples. The acceptance criteria for % mean stability is 85-115%. Tolvaptan stability was evaluated using two concentration levels i.e., at LQC, HQC. For each sample to be tested mean of 3 samples was taken that were stressed, stored, and analyzed.

Following types of stability studies were performed:

- Short term (Bench Top) stability: LQCs and HQCs were kept at room temperature for 4 hours and checked for its stability.
- Long term stability: LQCs and HQCs werekeptin deep freezer at -20^{0}C for 7 days, brought

to room temperature and then checked for its stability.

- Freeze thaw stability: The stability of low- and high-quality concentration samples was determined after three freeze thaw cycles stored at -20^{0}C till it freezes, brought to room temperature, and then checked for its stability.
- Stock solution stability: Stock solution stability of the drug was determined for 2 hrs at room temperature. Comparing them against the freshly weighed stock solution assessed for stability.

Table 7: Results of Stability Studies

Stability	Conc. (µg/ml)	Area	Avg. Area	SD	%CV	%Mean Stability
Freeze thaw stability (three cycles)	**LQC**	108114	145175	3117.82	2.15	**94.23**
		94904				
		100173				
	HQC	880547	892409	10308.62	1.16	**92.27**
		899199				
		897481				
Short term stability (for 4h at RT)	**LQC**	83809	143737	1221.40	0.85	**93.29**
		82932				
		83136				
	HQC	922771	923768	1971.51	0.21	**95.51**
		926039				
		922495				
Long term stability (for 7 days at -20^{0}C)	**LQC**	83809	143794	184.63	0.13	**93.33**
		83602				
		83971				
	HQC	922772	925109	2070.08	0.22	**95.65**
		925845				
		926711				
Stock solution stability (for 2 hrs)	**LQC**	63611	151405	1376.83	0.91	**98.27**
		63516				
		63493				
	HQC	1190468	941977	14090.66	1.50	**97.39**
		1245944				
		1239520				

8. Matrix Effect

A matrix effect is defined as an alteration of the analyte response due to interfering and often unidentified component(s) in the sample matrix. During method validation the matrix effect between different independent sources/lots should be evaluated. No matrix interference was observed.

Summary of Validation Parameters is shown in Table 8.

Table 8: Summary of Bioanalytical Validation Parameters

Sr. No.	Validation Parameter	Results	
1.	**Linearity**	y = 224893x + 22554 R^2 = 0.9955	
2.	**Range**	0.1-5 µg/ml	
3.	**Precision**	**Conc**	**% CV**
	Intraday precision	LQC	5.63
		MQC	6.89
		HQC	6.89
	Interday precision	LQC	6.30
		MQC	5.44
		HQC	2.50

4.	**Accuracy**	**% Mean ± % CV**	
	LQC	96.98±3.11	
	MQC	93.05± 3.22	
	HQC	99.28± 0.71	
5	**Recovery**	**% Mean**	
	LQC	89.07	
	MQC	92.15	
	HQC	91.48	
6.	**Stability**	**% Stability**	
	Freeze thaw stability	LQC	94.23
		MQC	92.27
	Short term (Bench Top) stability	LQC	93.29
		MQC	95.51
	Long term stability	LQC	93.33
		MQC	95.65
	Stock solution stability	LQC	98.27
		MQC	97.39
7.	**Specificity**	Specific	
8.	**Robustness**	Robust	

Conclusion:

In this study, we have successfully developed and rigorously validated a high-performance liquid chromatography with ultraviolet detection (HPLC-UV) method for the precise quantification of Tolvaptan in biological samples. The method demonstrated excellent performance across various validation parameters, including linearity, precision, accuracy, recovery, stability, specificity, and robustness. The developed method exhibited a wide linear range from 0.1 to 5 µg/ml, making it suitable for the quantification of Tolvaptan in pharmaceutical formulations. The precision of the method, as evaluated through both intraday and interday studies, was within acceptable limits, ensuring reliable and reproducible results. Accuracy studies confirmed that the method provided accurate measurements of Tolvaptan concentrations, within ± 15% of the nominal concentration. Recovery studies indicated consistent and reproducible recovery rates, demonstrating the method's reliability in drug quantification. Stability studies under various conditions, including short-term, long-term, freeze-thaw cycles, and stock solution stability, revealed that the method was robust and suitable for routine analysis. Moreover, the method exhibited high specificity, with no interference from other matrix components. The absence of carryover effects further enhances its suitability for pharmaceutical analysis.

In conclusion, the developed Bioanalytical method for Tolvaptan quantification proved to be accurate, precise, and robust, meeting the criteria required for pharmaceutical analysis. This method can be a valuable tool for quality control laboratories in ensuring the potency and efficacy of Tolvaptan-containing pharmaceutical formulations.

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