ANALYTICAL QUALITY BY DESIGN APPROACH IN RP-HPLC METHOD DEVELOPMENT FOR THE ASSAY OF TERIFLUNOMIDE IN BIOLOGICAL SAMPLES

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Abstract: A reversed-phase high-performance liquid chromatography (RP-HPLC) method has been developed using an ODSC18 column for the analysis of teriflunomide (TRF), in accordance with the regulatory requirement of Analytical Quality by Design (AQbD) in the development of robust analytics. Following risk analysis, a number of critical method variables (CMV) were used as inputs, including the solvent modifier (A), flow rate (B), injection volume (C), column tem (D), and buffer strength (E), while the corresponding Flow rate (F1), injection volume (F2), and buffer strength (F3) of TRF were used as method responses that are likely to have an impact on method performance. The analytical target profile (ATP) was established in accordance with ICH and USP standards. The Central Composite Design (CCD) and Fractional-Factorial Design(FFD) was used as a tool in the investigation of scientific understanding between input variables (A-E) and method responses (F1-F3). The method operable design region (MODR) was arrived at by subsequent analysis of systematic simulation followed by 3D-Contour plots. There were Five candidate methods were selected within MODR and verified experimentally. The r^2 value was 0.997 (TRF) indicating the consistency of the model and reliability of the method in the region. Among the methods, a method Solvent modifier-ACN:0.1AA(A), the Flow rate of 0.75 µM/ml (B), Injection volume of 15 µL/ml (C) Column temp of 27°C (D) and Buffer strength of 50 µM (E) was validated as per ICH Q2R1 guidelines. A conventional RP- HPLC method was developed by trial-and-error method and was compared with the AQbD method. The result indicated the AQbD method was relatively more robust with CMV range ofACN-0.1AA (A), 0.5-1.0(B), 10-20 (C), 25-30(D)and 50-30(E) whilst the conventional HPLC method failed for robustness.

Keywords: AQbD, Teriflunomide, RP-HPLC, Method Development.

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INTRODUCTION

Chemically, teriflunomide is (Z) -2-Cyano-3-hydroxy-but-2-enoic acid-(4-trifluoromethylphenyl) -amide, with a relative molecular mass of 270.2 g mole-1 and the formula C12H9F3N2O2 (Figure 1). Teriflunomide is a powder that is white to almost white, odourless, and non-hygroscopic. Due to its poor solubility and high permeability, it comes under the biopharmaceutical categorization system's class 2 of compounds. [1-2] Teriflunomide has a pH-dependent solubility and a weak acidic pKa of 3.1 at ambient temperature. Teriflunomide becomes much more soluble in aqueous buffers at a rate of 0.02 g mL-1 at pH 1.2 to 8 mg mL-1 at pH 7.6. [3] Teriflunomide inhibits dihydroorotate dehydrogenase, a mitochondrial enzyme involved in de novo pyrimidine production, and is an immunomodulatory drug having anti-inflammatory properties. [3-6]. Teriflunomide is not listed as official medicine in the USP, BP, JP, and Ph. Eur pharmacopoeias. Teriflunomide has been the topic of a broad range of analytical methods that have been reported in the literature, including quantification of Teriflunomide in pharmaceutical dosage forms such as nanoparticles, solids, capsules, and SLNs by HPLC and LC-MS. [6-7] A validated ultra-performance liquid chromatography (UPLC) technique based on the QbD approach is needed to enable the UPLC method for quantifying teriflunomide in API in human plasma, however none have been reported in studies of the effects of biological substances. The UPLC method provides quicker product development for the biological samples, which accelerates the analysis. With sub-2 particle size columns compared to 5 particle sizes, a UPLC method typically yields a 3X higher efficiency and a 9X improvement in throughput with no loss in resolution. The main goal of the suggested research development is to produce a TRF blood plasma UPLC technique employing a QbD methodology for a quick estimate of assay content and in-vivo pharmacokinetics, drug release in the presence of blood samples, and method validation in compliance with ICH criteria. Shorter chromatographic run times result in faster, more inexpensive, more solvent-efficient analysis, which improves the use of biological material in everyday analysis.

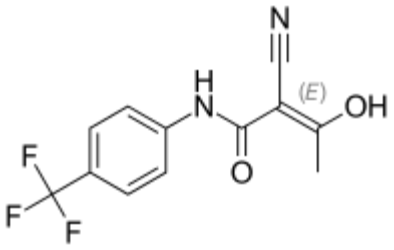

Figure 1: Molecular Structure of Teriflunomide

EXPERIMENTAL

Instruments: The RP-HPLC Waters system, which includes the Waters Pump Control Module-II, Waters 515 Solvent Delivery System (pump), Waters Rheodyne-injector (20 loops), Waters 2489 UV-Visible detector, and Waters Corporation's Empower2 software as a data processor, was used to accomplish chromatographic separation. Sun Fire C18 (4.6 x 150 mm) analytical column, 3.5 m. The mobile phase was degassed using a aultra-sonic cleaner by spinco tech Pvt ltd after being passed through a 0.45 m membrane filter. Chemicals and reagents: Gemcitabine hydrochloride was acquired from a local pharmacy in its GEMzar sterile for injection label and received as a gift sample from the pharmaceutical firm. We utilised analytical grade O-phosphoric acid (SD fine chemicals), acetonitrile HPLC grade (MerckIndia), methanol HPLC grade (MerckIndia), HPLC water (MerckIndia), and methanol.

Chromatographic condition

optimization of chromatographic conditions was done by performing different trails by taking different mobile phases and varying their compositions flow rates, finally, an optimised chromatogram was obtained. The system suitability parameters were presented in Table 1.

Table 1: Chromatographic conditions

Parameters	Method
Stationary phase (column)	Inertsil-ODSC_{18} (250 x 4.6 mm, 5 µ)
Mobile phase	Methanol: Acetonitrile(70:30)
Flowrate(ml/min)	1.0 ml/min
Runtime(minutes)	6 min
Column temperature(°C)	Ambient
Volume of injection loop(µl)	20
Detection wavelength(nm)	247 nm
Drug RT (min)	2.922 min

Preparation of mobile phase

Ortho-phosphoric acid (0.1 per cent) was generated by placing 0.1ml of analytical-grade OPA in a 100ml volumetric flask and adding HPLC-grade water to the mixture to get it up to the proper volume. A membrane filter (Millipore nylon disc filter 0.45 micron) was used in conjunction with a vacuum filter to separate the mobile phase, acetonitrile, and water (0.1 per cent) ortho-phosphoric acid. Before usage, this filtered mobile phase underwent a 15-minute sonication in an ultrasonic water bath.

Defining the QTMP and CAAs

Numerous important analytical attributes (CAAs), including peak area, retention time, and peak tailing, were established to maintain accuracy in the method development process and to generate reasonable findings with little waste utilisation of chemicals and solvents. This in turn aids in lowering the overall cost of the analytical approach.

Factor Screening Studies

To select the most important factors, factor screening tests were carried out using fractional-factorial design (FFD). Table 2 provides a summary of the design matrix, including the buffer strength, organic modifier, flow rate, injection volume, column temperature, and coded levels for each factor listed below the table. According to the traditional FFD design matrix, eight tests were run with the design produced by the Design Expert programme to see how they affected the CAAs. The linear polynomial model was additionally employed to objectively evaluate the influence of variables on each of the CAAs.

Table 2: FFD design matrix for screening of various method variables at their respective low and high levels

Number of Runs	Solvent Modifier (A)	Flow rate (B)	Inj. vol. (C)	Column Temp (^{0}C)(D)	Buffer strength (E)
1	-1	1	-1	1	-1
2	-1	1	1	1	1
3	-1	-1	1	-1	1
4	1	-1	-1	-1	1
5	1	1	-1	1	-1
6	1	-1	1	1	-1
7	-1	1	1	1	1
8	-1	1	-1	1	-1
9	1	-1	1	-1	-1
10	1	-1	1	-1	1
Levels of factor		Levels			

	Low (-)	High (+)
solvent modifier (A)	Acetonitrile	0.1 acetic acid
Flow rate (µM/ml)	0.5	1.0
Column temperature (C°)	25	30
Injection volume (µL/ml)	10	20
Buffer strength (µM)	50	30

Analytical Method Development Using Box-Behnken Design

The most crucial method parameters (CMPs) are anticipated to have a significant impact based on the information acquired by the RAS and FFD design for screening, according to literature reports on optimization studies. At different stages, A3 x 3 Box-Behnken designs (BBD) were employed to optimise the CMP technique (Table 3). To obtain the effects of all the technique CAAs for all the experimental runs, a standard stock was made during the development of the AQbD-method, and after numerous dilutions, a concentration of 10 g/mL was chosen(8-9).

Table 3: Design matrix as per the BBD optimization of the HPLC method of TRF

Number of Runs	**Factor 1**	**Factor 2**		**Factor 3**
	Flow rate	**Injection volume**		**Buffer strength**
1	-1	0		1
2	0	1		1
3	-1	-1		0
4	-1	1		0
5	0	0		0
6	0	1		-1
7	1	1		0
8	0	-1		1
9	0	1		1
10	-1	-1		0
Levels of factor		Levels		
		Low (-)	Medium (0)	High (+)
Flow rate (µM/ml)		0.5	0.75	1.0
Injection volume (µL/ml)		7.5	15	20
Buffer strength (µM)		30	50	60

Optimization Data Analysis and Validation of Experimental Design

Multiple linear regression analysis (MLRA) was used to analyse the optimization data with the aid of Design Expert® ver. 13.0.1 software to fit the experimental data to the second-order quadratic polynomial model and calculate the significant effects as well as the effects of interactions. In the end, analysis of several metrics, including P-value, coefficient of correlation (R), predicted error sum of squares (PRESS), and lack of fit, proved the model's suitability. The model coefficients that were statistically significant with a P- values that were less than 0.05 were considered. Additionally, 2D-contour and 3D-response surface plots were created using RSM to explore any potential interactions between the parameters as well as discover any possible links between the variables and their responses. Finally, using a numerical optimization methodology and the desirability function method, the best solution was discovered by "trading off" significant analytical properties in accordance with the selection criteria. Design space for graphical optimization was also found to locate the optimal solution. The experimental design strategy was validated using chromatographic trials or checkpoint runs for the CMPs of the selected chromatographic technique from the knowledge space domain.

Preparation of standard stock and standards solution

To create a stock solution, 10 mg of teriflunomide hydrochloride that had been precisely measured and placed in a 100 ml volumetric flask were first dissolved in a tiny amount of methanol before being subjected to a 10-minute sonication process. a 100mcg/ml concentration was attained.

Preparation of Calibration curve standards

By adding 1–5 ml of standard stock solution to a transferred 10-ml volumetric flask, the calibration curve was created in the concentration range of 10–50 g/ml, and the final volume was adjusted with the mobile phase. The end products were then filtered using 0.45 m membrane filter paper, and the filtrate was used for analysis.

Bio-analytical Method Development

Based on the optimal chromatographic solutions obtained from the selected experimental design, the bioanalytical method development was carried out in Human plasma. The patient's samples were used with the requisite approved protocol. JSSCP/IRB/06/2020-21. The human plasma was collected as per the standard protocol in the heparinized tubes and stored at -20ºC until further use.

Samples Preparation and Extraction Method

A stock of TRF and human plasma, each containing 250 L, were combined before being diluted with acetonitrile to a level of 2 mL and held for two minutes. After samples were held in

a vortex for 60 seconds, they were centrifuged for 15 minutes at a force of 8,050 g. The drug-containing supernatant fraction was isolated, dried, and then reconstituted using 1 mL mobile phase. The samples underwent chromatographic analysis after being filtered with a 0.22 m filter.

Bio-Analytical Method Development and Validation

To the current investigation, we have created a quick and accurate RP-HPLC technique for extract sample quantification. Sun Fire C18 (4.6 x 150 mm) column, 3.5 m, with 0.1 per cent O-phosphoric acid in the mobile phase (pH 3.0) A mixture of 70:30 v/v and acetonitrile was employed. Rate of flow: 0.8 g/ml/min. 268nm was the chosen wavelength for detection. TRF in blood plasma may be determined using the established approach, it was discovered. A typical chromatogram for gemcitabine is displayed in the figure: 2 (10-11)**.**

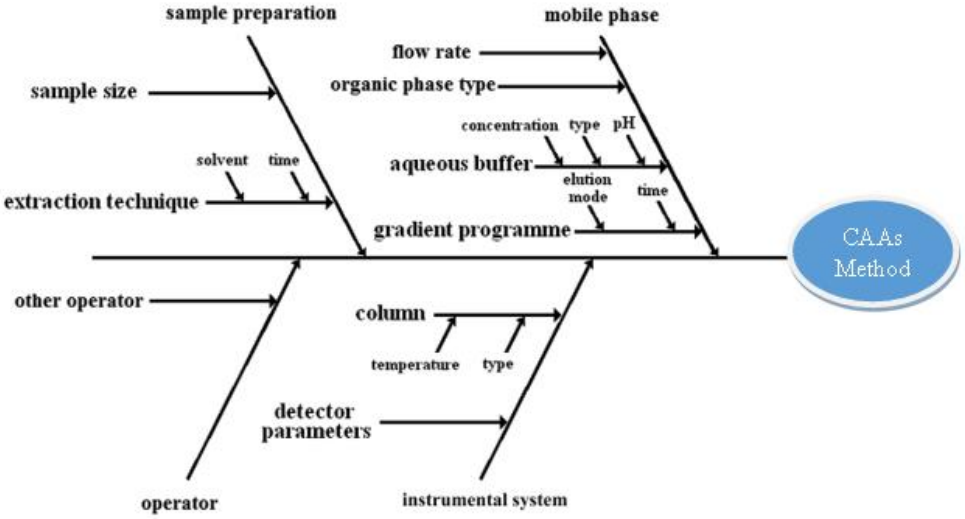

Figure 2: Ishikawa fish-bone diagram depicting the cause-and-effect relationship on the potential CAAs of the analytical method of TRF

VALIDATION

The optimized chromatographic method was completely validated to the procedures in ICH guidelines validation of analytical methods (ICH 2008) (12-17).

Linearity, the limit of detection (LOD), and limit of quantification (LOQ):

The mobile phase was used to dilute the necessary amount of TRF stock solution standard 100 g/ml to create concentrations of 10, 30, 60, 80, and 100 g/ml. This duplicates each concentration that was created and administered to the chromatograph with friendliness. Using weight factor x, the linear least-squares regression model assesses linearity. data analysis using the 2013 version of Microsoft Office Excel. Evaluation was conducted at a significant level of 5%. The correlation coefficient and intercept values are used to evaluate this strategy. The method based on the Intercept's standard deviation and S, the slope, was used to calculate LOD and LOQ.

LOD= 3.3 x σ/(n-1)/slope LOQ= 10 x σ/ (n-1)/slope,…. (1)

Where, σ = Standard Deviation, by these equations LOD was found to be and LOQ was found to be.

Precision

Triple measurements of quality control samples containing 2, 10, 20, 50, and 100 mg/ml of TRF were used to calculate the precision on several days and at various concentrations (2, 4, and 6 h). The intraday precision (RSD) of the approach was established. The intraday precision was calculated using the same concentration levels during the same day at various time intervals, and the intermediate precision was calculated using the RSD of the analysis of the samples prepared with the same concentration but analysed at different times and days at different concentration levels.

Accuracy

The accuracy of the method was determined by the addition of known amounts of TRF (n=3). The recovery studies were performed by standard addition method at (60%, 80%, and 120% concentrations), toa sample solution of known concentration (formulation).these solution was prepared and analysed the total amount recovered was calculated. The mean recovery of the target concentration 100 ± 5% for acceptance.

Specificity

Specificity was determined by forced degradation studies. No interference was observed from the excipients and degradation products. This method was found to be specific.

Stock stability; stability of stock solution was evaluated at two conditions, like room temperature, stored in the refrigerator (2-8 ºc).

DATA ANALYSIS

Data are reported as mean ± standard deviation (SD) for the replicates. The (RSD) values and accuracy were calculated using Microsoft excel 2003 and graph pad prism-version.9

RESULT AND DISCUSSION

Preliminary Studies

The initial research was done to provide a straightforward LC technique for TRF analysis. As stated in many literature papers, chromatographic separation of TRF was achieved by using complex mobile phase composition, mobile phase flow rate, injection volume, and column oven temperature.

Factor Screening Studies

FFD was used to conduct the study, and the data were accessible as needed using a first-order polynomial equation where 0 represents the intercept and 1 to 7 represents model terms (Eq. 2). The Pareto chart's t-values and Bonferroni limits show that the parameters injection volume and buffer strength significantly affected peak area. However, the responses revealed that factors such as injection volume, flow rate, and buffer strength had a significant impact on retention time and peak tailing. Following the selection of these components as the CMPs, a method optimization study was carried out on them.

$$Y=\beta_0 +\beta_1X_1 + \beta_2X_2 +\beta_3X_3+\beta_4X_4+\beta_6X_6+\beta_7X_7\ldots\ldots\ldots\beta nXn\ldots. \quad (2)$$

Optimization Data Analysis and Response Surface Mapping

The second-order quadratic polynomial model was used to study the main effects and the interaction effects (Table 4) while applying the following model equation mentioned in Eq. (3) for coefficient analysis. (3) Peak area, RT, and peak tailing plots were employed as part of the response surface technique for each of the CAAs (Figure 3A-C) (18-19).

Table 4: REM summarizing risks for HPLC method of TRF

CAAs	Risk estimation matrix (REM)									
	Types of solvent	**Flow rate**	**Flow type**	**Buffer type**	**Buffer strength**	**Injection Volume**	**Column Type**	**Column length**	**Column temperature**	**Sonication time**
Peak area	H	L	H	L	M	H	L	L	L	H
Retention time	H	M	M	L	M	M	L	M	L	H
Tailing factor	H	M	H	M	M	M	L	M	H	M

Parameters

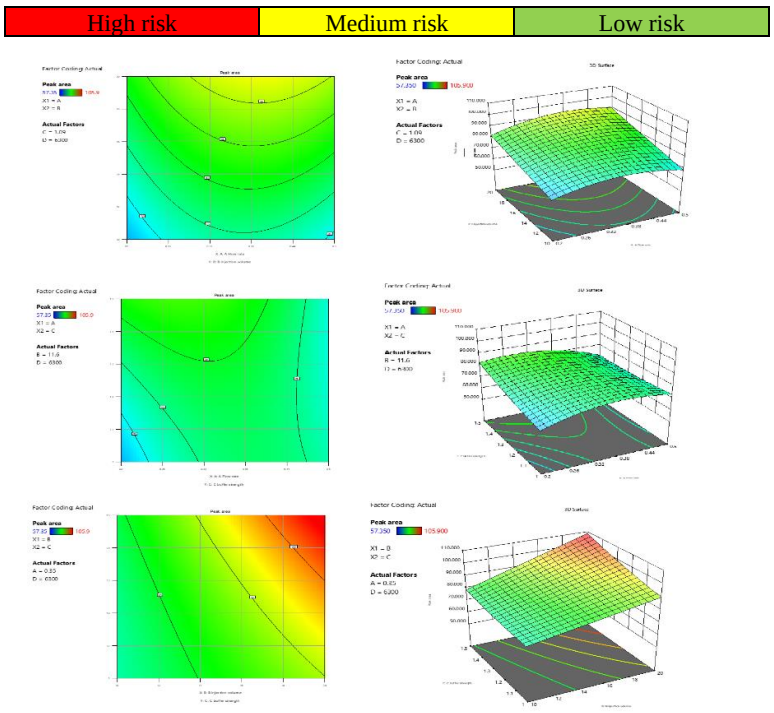

Figure 3A: 3D-response surface plots showing the influence of CMPs, i.e., mobile phase ratio (A), buffer pH (B) and oven temperature (C) on the retention time as the CAA.

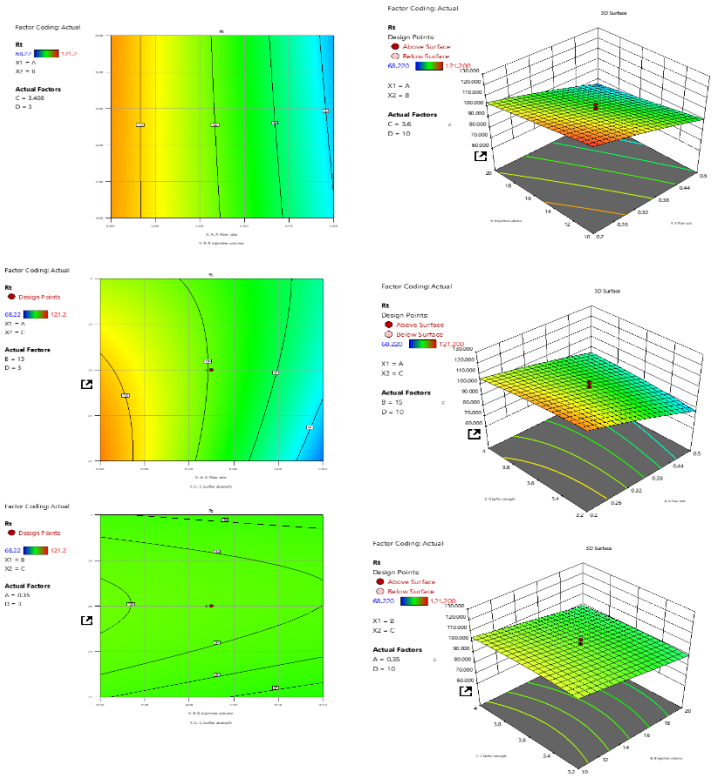

Figure 3B: 3D-response surface plots showing the influence of CMPs, i.e., mobile phase ratio (A), buffer pH (B) and oven temperature (C) on the retention time as the CAA.

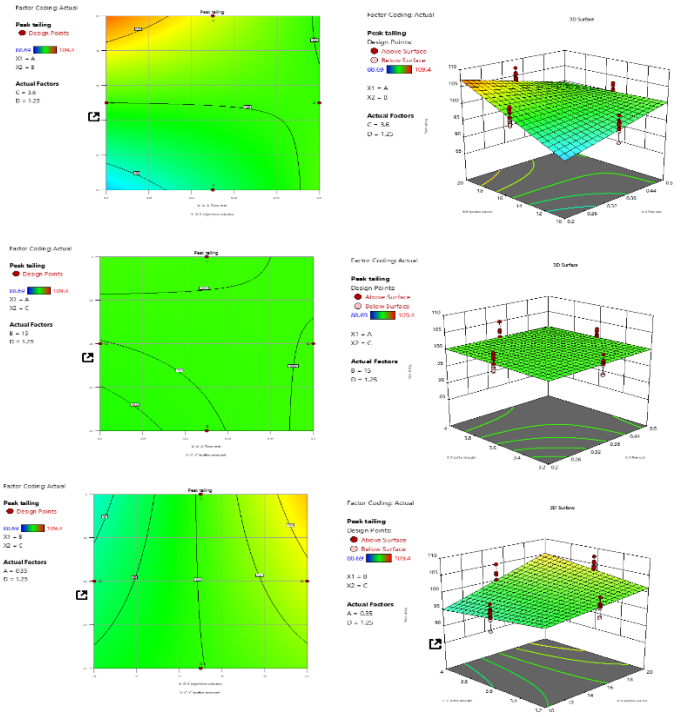

Figure 3C: 3D-response surface plots showing the influence of CMPs, i.e., mobile phase ratio (A), buffer pH (B) and oven temperature (C) on the peak tailing as the CAA

As seen in Figure 4A, the injection volume showed a curved trend whereas the 3D-plot for the peak region revealed a substantially falling trend by flow rate. Contrarily, the peak area of the buffer strength showed a dramatic downward trend.

Figure 4B shows the curved relationship between flow rate and retention time, as well as the gradually dropping trend of injection volume and inclining trend of buffer strength. The peak tailing response surface plot shown in Figure 3C revealed a linearly increasing trend for flow rate's influence, while a sharp dip was seen for injection time at all levels. On the peak tiling, the buffer strength, on the other hand, displayed a rising pattern. The repose surface analysis plots generally indicated interaction effects between the CMPs and method CAAs. $Y=\beta_0 +\beta_1X_1 + \beta_2X_2 +\beta_3X^2{}_1+\beta_4X^2{}_1+\beta_6X^2{}_1+\beta_7X^2{}_1\ldots\ldots\beta nX^2{}_1n\ldots.$ (3).

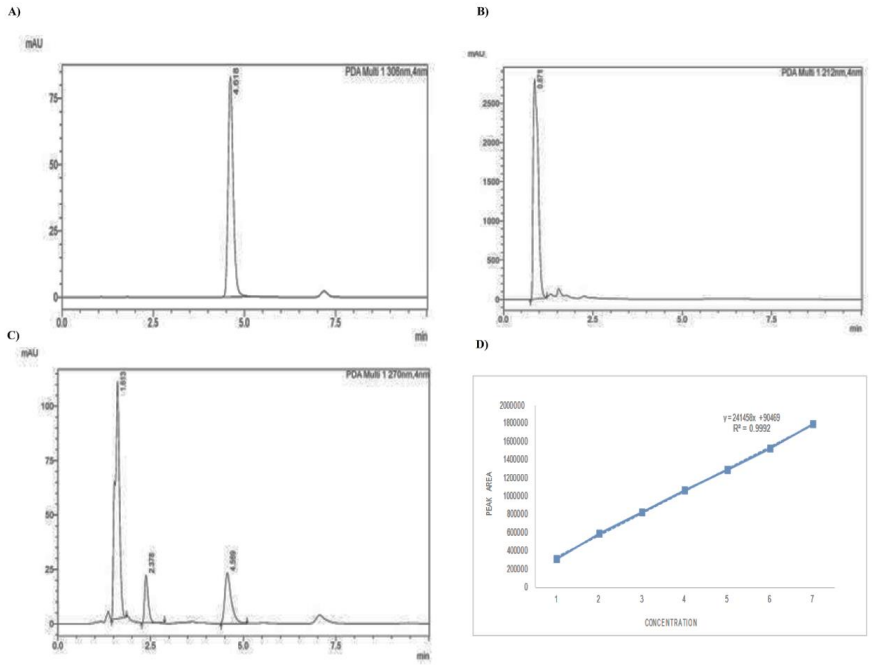

Figure 4: Chromatograms of TRF standard solution at a concentration of 15 µg/mL (A), Blank plasma at LLOQ (B), TRF standard solution spiked with plasma at a concentration of 1000Ng/mL (C), and at Linearity graph of TRF (D)

Search For the Optimum Solution and Validation Studies

In order to attain the intended results, i.e., to get the desirability function close to 1, this was actually achieved by "trading-off" different CAAs through numerical optimization. The design specialist suggested a mobile phase with an ACN:phosphate buffer (0.1 per cent v/v Ortho phosphoric acid) ratio of 30:70 and a flow rate of 0.5 mL/min as the ideal solution. The AQbD method was significantly assisted by the graphical optimization of the optimum process, as shown in Figure 5, which also showed the ideal analytical method specifications within the analytical design space that is coloured yellow. Strong correlation has been seen between the CAAs' predicted and observed values. based on the experimental design methodology's validation, with a percent prediction error (or percent bias) of less than 5%. This suggested that the experimental setup had a strong capacity to anticipate the responses.

Analytical and Bio analytical Method Validation

Linearity: The linearity calculation was kept close to achieve the lowest divergent outcome, with a maximum value of percent bias in limits of 5%. The concentrations employed for the purpose ranged 2 to 50 g/mL, which was further confirmed by the residual plot. Table 5 represents the coefficient of polynomial equations as per the second-order quadratic model for each CAA. Higher values of the coefficient of correlation (0.997, p0.001) were used to validate the analytical method. Also, it was determined that the method's LLOQ was 2 g/mL and its ULOQ was 50 g/mL, confirming that the devised method's working window was accurate.

Table5: Coefficient of polynomial equations as per the second-order quadratic model for each CAA

Coefficient sequence code	Polynomial coefficients for CAAs		
	Peak area	Retention time	Peak tailing
β1	-7386.23	+0.40	+0.061
β2	+25483.23	-0.038	+0.025
β3	+11320.50	-1.18	+0.035
β4	+14264.50	+0.88	-1.000
β5	+89649.80	+2.15	+1.97
β6	+10855.25	-0.38	-0.21

β7	+15254.47	+1.26	+0.36
β8	+43317.00	+1.96	+0.030
β9	+23893.23	-0.018	+0.039
P-Value	****	****	****
R^2	0.9995	0.9987	0.9974

Note : data represents *>0.1, ***>0.01 and ****>0.001, all data's replicate (n=3).

Accuracy

Detailed information was obtained from a chromatographic assay for several TRF shown in table 6 of QC samples. Based on a few selected concentrations of TRF, the data generated from the samples analysed for evaluating the accuracy of the developed method showed relatively very good percent recovery as compared to earlier reports, i.e., between 99.5% and 101.16%, and percent RSD value within the limit of 2 percent, supporting high degree of accuracy of the TRF analytical method. It was also shown that the information obtained from bioanalytical samples spiked with the aforementioned medicines in human plasma was reliable.

Table 6: Accuracy data of the method for TRF in Human plasma

Fixed concentration	Levels	Concentration (µM/ml)	(%) of recovery	SD	Accuracy (RSD)
TRF(15µM/ml)	LOW:50	5	100.98	15.06 ± 1.04	0.04
	MED:80	10	99.85	20.07 ± 1.08	0.025
	HIGH:120	20	99.97	19.42 ± 0.89	0.04

The observed percentage ranged from 89.8 to 92.22 percent, demonstrating the method employed to measure pharmaceuticals in biological samples had an excellent level of accuracy and reproducibility. The amount of medication recovered from plasma samples has slightly decreased because of interactions with plasma proteins and loss during the liquid-liquid extraction process.

Precision

Results show the analytical information needed to evaluate the method's accuracy; different samples were kept for this evaluation of intra-day and inter-day investigations in table 7. Greater percentages of QCT recovery were seen for LQC (5 g/mL), MQC (10 g/mL), and HQC (15 g/mL), ranging from 94.8 to 97.4 per cent with RSD values under 2 percent. These concentrations were set up so that users could select the TRF standard concentrations. Additionally, Percent recovery in the range of 99.8 to 100.9 percent was found after examination of precision data in bioanalytical samples, demonstrating the established approach's good precision in biological samples. With a percent recovery between 97.5 percent and 98.3 percent, the method for extracting medications from rat plasma showed a slight decline in accuracy values, indicating very little change because of drug loss throughout the extraction process.

Table 7: Precision data of the method for TRF in Human plasma

	Concentration (µM/ml)	(%) of recovery concentration	(%) of recovery	Precision (RSD)
Intra-day	5	5.03±0.63	100.3	0.068
	10	10.01±1.34	100.1	0.015
	20	19.98±1.72	99.98	0.055
Inter-day	5	5.18±0.94	100.18	0.084
	10	10.05±0.78	100.05	0.031
	20	20.03±0.74	100.02	0.062

LOD and LOQ

In comparison to bioanalytical samples, the LOD and LOQ for the QCT were found to be 9.67 ng/mL and 29.3 g/mL, respectively. This verified and offered solid proof of the great degree of sensitivity of the devised analytical approach for quantifying the drug in specimens.

CONCLUSION

For the determination of the TRF in human plasma, a straightforward, affordable, and sensitive bioanalytical method based on the QbD-based approach was devised. The target design space region was delineated around the best chromatographic solution. In terms of linearity, precision, accuracy, and specificity for the medication in analysed samples, method validation produced very positive findings. In conclusion, the QbD-based studies show the analytical method for TRF quantification's tremendous applicability. The established method is now applicable for bio analytical applications during pharmacokinetic and dermatokinetic research thanks to the validation strategy, which also further ensured the method's effectiveness.

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COMPLIANCE AND ETHICAL STANDARD

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Consent For Publication: All authors have approved the manuscript and given consent for publication.

Competing Interest: There is no conflict of interest.

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Authors Contribution: All authors equally contributed.

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