Analytical QbD Approach for Development and Validation of RP-High Performance Liquid Chromatography Method for Determination of Tofisopam in Pure Form and Tablets

Megha Kokane¹, Jeeja Pananchery¹*, Monika Jadhav², Ashish Jain³

¹Department of Quality Assurance, Shri. D. D. Vispute College of Pharmacy & Research Center, New Panvel, Navi Mumbai-410206, Maharashtra, India.
²Department of Quality Assurance, C. U. Shah College of Pharmacy, SNDT Women’s University, Sir Vithaldas Thakersay, Santacruz West, Juhu, Mumbai-400049, Maharashtra, India
³Shri. D. D. Vispute College of Pharmacy & Research Center, New Panvel, Navi Mumbai-410206, Maharashtra, India

ABSTRACT
A novel, simple, optimized reversed-phase chromatography method for assay of Tofisopam in pure and tablet form is developed. The experimental trial was by Box Behnken design using the Design Expert® software 10 version. The attributes selected were peak symmetry, number of theoretical, and peak purity. The predicted data satisfied with actual experimental data. The optimized chromatographic conditions required a quaternary pump with a mobile phase of Water: Acetonitrile 25:75 v/v at 1 mL/min, oven temperature at 25°C at 310 nm using C18(250 × 4.6 mm Id, 5μm) column and PDA detector with a run time of 5 min. The method was validated for linearity, precision, accuracy, and specificity. The method produced a linear response over a concentration range of 4–24 ppm with an overall average accuracy of 99.98%. The method was robust, reproducible, and specific with respect to the retention time of tofisopam.

INTRODUCTION
Tofisopam is an antianxiety¹ agent chemically known as 1-(3,4-Dimethoxyphenyl)-5-ethyl-7,8-dimethoxy-4-methyl-5H-2,3-benzodiazepine Fig. 1. It is a white, pale yellowish-white crystalline powder, practically insoluble in water, sparingly soluble in ethanol, freely soluble in methanol, and acetonitrile with melting range 155-159°C.²

There are analytical methods for the estimation of tofisopam with spectrophotometric and spectrofluorimetric³ chromatographic,⁴-⁶ enantiomeric separation,⁷-⁸ gas-liquid chromatography (GLC),⁹ and super-critical chromatography¹⁰ method. All these methods require a large sample volume (1-5 mL) and time-consuming sample clean up procedure. Literature reveals no RP-High

*Corresponding Author: Mrs. Jeeja Pananchery
Address: Department of Quality Assurance, Shri D. D. Vispute College of Pharmacy & Research Center, New Panvel, Navi Mumbai-410206, Maharashtra, India
Email: jeejapananchery@gmail.com

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performance liquid chromatography method developed by quality by design for determination of tofisopam in pure form and tablet. In view of these points, an attempt was made to develop a simple, precise, accurate, and validated method for the determination of tofisopam in pure form and tablet by analytical quality by design approach.

**Materials and Methods**

**Chemicals and Reagents**
The TF was gifted from Ajanta Pharmaceutical, Mumbai India. The commercial dose formulation containing 50 mg TF, Nextril tablet, was procured from the local market. All the reagents and chemicals like acetonitrile, water, methanol were of HPLC grade.

**Instrumentation**
The determination of TF was done by using HPLC (JASCO LC system-1200 series), connected to the autosampler, column oven, and wavelength detector. It consists of a quaternary pump, a Rheodynje injector with a 20 µL loop, and a photodiode array detector. $C_{18} (250 \times 4.6$ mm Id, 5µm) column was used for separation.

**Procedure for Standard Stock Solution**
The standard stock solution was prepared by accurately weighing 100 mg of TF in 100 mL volumetric flask and diluted up to mark with acetonitrile, which gives a concentration of 1000 µg/mL of solution. From the above standard stock solution, an aliquot of 1 mL was transferred into 10 mL volumetric flask, and the volume was made up to the mark with acetonitrile, which gives a concentration of 100 µg/mL of solution (sub-stock solution).

**Preparation of Solution and Selection of Wavelength**
From the sub-stock solution (100 µg/mL), aliquot of 1 mL were transferred to 10 mL of volumetric flask and diluted with acetonitrile, to form a solution of 10 µg/mL and it was scanned in the range of 400-200 nm, and the absorbance maximum was noted at 310 nm (Fig. 2).

**Optimization of Mobile Phase**
A variety of solvents with different compositions were screened to find out the ideal mobile phase (Table 1).

**Chromatographic Conditions**
The mobile phase was consists of acetonitrile: water (75:25), and it was filtered, degassed, and sonicated prior to use. A $C_{18} (250 \times 4.6$ mm Id, 5µm) column was used as a stationary phase. The column temperature was kept at 25°C with a thermostatically controlled column oven. The flow rate was maintained at 1.0 mL/min. The detection was carried out at 310 nm wavelength PDA detector, and the injection volume was 20 µL.

**Experimental Design**
The experimental design was constructed using design-expert software version 10 for the study of different variables (oven temperature, percentage of the organic phase, and flow rate) and to verify method performances. The levels of these variables are as given in Table 2. The retention time, peak symmetry, peak purity, and several theoretical plates were used as a response in experimental design as controlling response, which is expected to affect and control method responses. A $3^3$ factorial design consisting of three factors at three levels was considered for the experimental plan. Initially and after confirming that the process is a non-linear, Box-Behnken design was used. The experimental observations along with Design (DOE) plan are shown in Table 3.

**Evaluation of Experimental Results and Selection of Final Method Conditions**
The different method conditions (13 runs) obtained from experimental design was run and evaluated for retention time, peak symmetry, number of theoretical plates, and peak purity. Response surface Box-Behnken statistical screening design was used to optimize and evaluate main effects, interaction effects, and quadratic effects. A 3-factor, 3-level design used is suitable for exploring quadratic response surfaces with Design Expert® (Version 10.0, Stat-Ease Inc).

![Fig. 2: UV spectrum of tofisopam](image_url)

**Table 1: List of mobile phase compositions screened**

| Sr. No. | Mobile phase    | Ratio (v/v) | Remark                       |
|---------|----------------|-------------|------------------------------|
| 1       | Methanol : Water | 90:10       | Peak tailing was observed    |
| 2       | Methanol : Water | 80:20       | Peak fronting                |
| 3       | Methanol : Water | 70:30       | Peak was not proper          |
| 4       | ACN : Water     | 90:10       | Peak was not resolved        |
| 5       | ACN : Water     | 75:25       | Peak was symmetric           |
Method Validation

Method validation is an analytical process by which it is established by laboratory studies that the performance characteristics of the procedure meet the requirement for the intended analytical application.

Linearity

The linearity of TF was assessed in the range of (4–24 µg/mL) in terms of slope, intercept, and correlation coefficient values.

Precision

Precision study was calculated by interday and intraday studies. Both precisions were determined by analyzing 8, 12, 16 µg/mL concentrations daily for 3 days and % RSD were calculated.

Accuracy

Accuracy of the method was confirmed by a recovery study from marketed formulation at 3-level of standard addition. Percentage recovery of TF was found out.

Specificity

Specificity was determined by checking the interference of any possible degradation products. Both the standard and test sample solutions were scanned separately in the range of 200–400 nm.

Ruggedness

It was performed by analyzing three dilutions using different analysts on a different day. The overall % RSD was calculated.

Robustness

The three dilutions were analyzed under different conditions by changing the wavelength and mobile phase composition. The percentage RSD of the assay was calculated.

Stability in Analytical Solution

Prepared sample as per test method and injected in duplicate into HPLC at initial and at different time intervals up to 24 hours. The percentage assay at different time intervals was determined.

Assay

For the assay of TF, twenty tablets were accurately weighed and triturated to make a fine powder. Quantity equivalent to 50 mg was calculated and transferred into 50 mL volumetric flask, diluted with acetonitrile and sonicated for 15 minutes. From the above stock solution, 1 mL was transferred into 10 mL volumetric flask and diluted with acetonitrile, which gives a concentration of 100 µg/mL of solution. From the above solution, an aliquot of 1 mL was transferred into 10 mL of volumetric flask and diluted with acetonitrile, which gives a concentration of 10 µg/mL of solution. The 20 µL of above dilution was injected for HPLC analysis.

Results and Discussion

The proposed work was planned for the development and evaluation of the RP-HPLC method for the estimation of TF in bulk and pharmaceutical formulation. Accordingly, the initial solvent was selected for TF, and acetonitrile showed good solubility for it. Further, the chromatographic
conditions were analyzed and parameters were finalized, as shown in Table 4. TF showed a sharp peak in mobile phase Acetonitrile: water (75:25) with retention time 4.46 minutes, as shown in Fig. 3.

**QbD Implementation**

Design Expert® (Version 10.0, Stat-Ease Inc) software was used for the statistical analysis of the experimental observations and the analysis was conducted by using Box-Behnken design. The inputs given to software are given in Tables 5 a, b.

Inputs were given to the software and design was run, which provided selected 13 runs to be performed. After completing 13 runs, the results in terms of responses like retention time, peak symmetry, number of theoretical plates and peak purity were entered in software to give a statistical evaluation in terms of degrees of freedom (Tables 6 and 7) along with 3D surface plot of desirability as shown in Figs. 4-8.

**Method Validation**

**Linearity**

The TF showed linearity between concentration range of 4–24 ppm and had a good correlation coefficient of 0.999 with slope 49297 and y-intercept 24739 for the graph of concentration against area Fig. 9.

**Precision**

Results of precision are shown in Table 8, and it shows a standard deviation of less than 2, which indicates that the proposed method is precise.

**Accuracy**

Results of accuracy are shown in Table 9. The percentage recovery was 99.98 and also showed a standard deviation << 2, which states that the method is accurate.

**Specificity**

It was tested for the interference of any degraded substance, and as shown in the chromatogram (Fig. 10),

![Fig. 3: Chromatogram of TF using ACN: Water (75:25)](image)

![Table 4: Chromatographic conditions](image)

![Table 5 (a): Design summary for optimization by using Box-Behnken design](image)

![Table 5 (b): Design summary for optimization by using Box-Behnken design](image)

![Table 6: Evaluation of degrees of freedom](image)
Analytical QbD Approach for Development and Validation of RP-HPLC Method for Determination of Tofisopam in Pure Form...

Table 7: Obtained solutions for optimized condition

| Number | Oven temperature | Organic concentration | Flow rate | NTP | Peak symmetry | Desirability |
|--------|------------------|-----------------------|-----------|-----|---------------|--------------|
| 1      | 25.000           | 24.952                | 1.031     | 6671.988 | 0.998         | 0.9854       |

Table 8: Precision studies

| Concentration ppm | Area (n = 6) | Std Dev | % RSD | Mean RSD |
|-------------------|--------------|---------|-------|----------|
| 8                 | 415585.5     | 495.6518| 0.119266|          |
| 12                | 606419.8     | 1321.209| 0.21787 | 0.121064 |
| 16                | 797341.3     | 207.7592| 0.026056|          |

Table 9: Accuracy studies

| Sample | Formulation (µg/mL) | Pure drug (µg/mL) | % Recovery | Average | % RSD |
|--------|---------------------|-------------------|------------|---------|-------|
| S1: 80%| 10                  | 8                 | 99.28      |         |       |
| S1: 80%| 10                  | 8                 | 99.28      | 100.04  | 0.15  |
| S1: 80%| 10                  | 8                 | 99.28      |         |       |
| S2: 100%| 10                  | 10                | 99.18      |         |       |
| S2: 100%| 10                  | 10                | 99.30      | 99.63   | 0.23  |
| S2: 100%| 10                  | 10                | 99.18      |         |       |
| S3: 120%| 10                  | 12                | 99.49      | 100.26  | 0.25  |
| S3: 120%| 10                  | 12                | 99.49      |         |       |
| Overall average |                  |                   | 99.98     |         |       |
| Overall SD      |                  |                   | 0.31      |         |       |
| Overall % RSD   |                  |                   | 0.31      |         |       |

Fig. 5: Contour plot of peak symmetry

Fig. 6: Contour plot of peak purity

Fig. 7: Contour plot of NTP

Fig. 8: Contour plot of Rt
there is no interference as it showed a clear peak without any another peak.

**Ruggedness**

It showed an average percentage 99.089 which is within range and standard deviation 0.185 which is less than 2, indicating that the proposed method is satisfying criteria of ruggedness (Table 10).

**Robustness**

The results of robustness studies by varying mobile phase composition and wavelength are shown in (Table 11). RSD less than 2 indicated that the method is robust.

**Solution Stability Studies**

Table 12 shows the results for solution stability, and

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**Table 10: Ruggedness studies**

| Sr.No. | Analyst-1 (%) | Analyst-2 (%) |
|--------|---------------|---------------|
| Sample -1 | 99.40 | 99.09 |
| Sample -2 | 98.48 | 99.54 |
| Sample -3 | 98.71 | 100.68 |
| Sample -4 | 98.69 | 99.09 |
| Sample -5 | 98.61 | 99.31 |
| Sample -6 | 98.38 | 99.09 |
| Average | 98.71 | 99.46 |
| SD | 0.3593 | 0.621 |
| %RSD | 0.363 | 0.624 |
| Overall Average | 98.71 | 99.089 |
| Overall SD | 0.35 | 0.185 |
| Overall %RSD | 0.36 | 0.186 |

**Table 11: Robustness studies**

| Sample ID | Normal condition | Low λmax 309 nm (%) | High λmax 311 nm (%) | Low composition (74:24) (%) | High composition (76:26) (%) |
|-----------|------------------|---------------------|---------------------|-----------------------------|-----------------------------|
| Sample 1  | 99.40            | 98.6                | 98.8                | 100.3                       | 99.4                        |
| Sample 2  | 98.48            | 99.7                | 99.2                | 99.1                        | 99.0                        |
| Sample 3  | 98.71            | 98.5                | 100.9               | 99.7                        | 99.5                        |
| Sample 4  | 98.69            | -                   | -                   | -                           | -                           |
| Sample 5  | 98.61            | -                   | -                   | -                           | -                           |
| Sample 6  | 98.38            | -                   | -                   | -                           | -                           |
| Average   | 98.71            | 98.9                | 99.6                | 99.7                        | 99.3                        |
| SD        | 0.3593           | 0.67                | 1.12                | 0.60                        | 0.26                        |
| % RSD     | 0.363            | 0.67                | 1.12                | 0.60                        | 0.27                        |
| Overall Average | -          | 98.9                | 99.1                | 99.1                        | 99.0                        |
| Overall SD | -               | 0.35                | 0.69                | 0.52                        | 0.28                        |
| Overall % RSD | -         | 0.36                | 0.69                | 0.53                        | 0.28                        |

**Table 12: Solution Stability**

| Time Interval (hrs) | Area | % TF |
|---------------------|------|------|
| Initial             | 514781 | 99.4 |
| 8                   | 510254 | 98.48|
| 16                  | 511395 | 98.71|
| 24                  | 511287 | 98.69|
| Correlation (8)     | 0.98  |      |
| Correlation (16)    | 0.987 |      |
| Correlation (24)    | 0.986 |      |
correlation was found to be less than $2^{[11]}$, indicating that TF is stable in acetonitrile: water (75:25).

**Assay**

Results of assay are shown in Table 13. The percentage of RSD was 0.62 which is within limits.

**Conclusion**

Tofisopam is an antianxiety drug. In this study, the concentration of organic phase, oven temperature, flow rate were considered as a qualitative variable that was controlled with three independent variables peak symmetry, retention time, and NTP. The experimental design suggested the robust MODR region for the TF HPLC method developed. All the validated parameters were within the acceptable criteria of ICH guidelines. Thus a simple, rapid, selective, precise, accurate, and robust HPLC method was developed for the estimation of TF in pure form and its tablet dosage form.

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