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Analytical quality by design and greenness assessment–based RP-HPLC method development for stability-indicating assay of Tafamidis

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A novel Analytical Quality by Design (AQbD)-based stability indicating assay method using reverse-phase high-performance liquid chromatography (RP-HPLC**)** integrated with green analytical assessment was developed and validated for the Tafamidis in bulk drug. Critical analytical parameters, including flow rate, mobile phase composition, and pH, were systematically optimized using custom design in Design of Experiments (DoE)**.** The optimized chromatographic conditions provided efficient separation with a retention time of 2.23 min. The method exhibited excellent linearity over the concentration range of 50–150 µg/mL (R^2 = 1.00), with limits of detection and quantification of 0.47 µg/mL and 1.59 µg/mL, respectively as per ICH Q2 R1 guidelines. The forced degradation study performed in typical stress conditions. Under acid and thermal, degradation were high impact of 10.4% and 8.9%, respectively, within acceptable range. Greenness assessment using AGREE and MoGAPI yielded scores of 0.82 and 84**,** indicating excellent environmental sustainability**.** The developed AQbD-based RP-HPLC method offers a rapid and robust analytical platform that is environmentally sustainable, highly reliable, and stability indicating, making it ideally suited for routine quality control of Tafamidis active pharmaceutical ingredient (API) and extending its applicability to finished pharmaceutical formulations in compliance with regulatory standards.

Keywords: AGREE tool, Critical method parameter, Custom design, Greenness, MoGAPI, Method Validation, Risk assessment

Introduction

Tafamidis marketed as Vyndaqel® and Vyndamax, is a breakthrough oral medication received FDA approval in May 2019 for treatment of transthyretin amyloidosis (ATTR amyloidosis) with cardiomyopathy (ATTR-CM) or polyneuropathy (ATTR-PN). Evidence from clinical trials, when compared with placebo demonstrates that by stabilizing transthyretin (TTR) protein it improved tolerance, lowers the risk of heart failure hospitalization and contributes to progression in neurological outcomes as well as overall quality of life in patients[1,2]. Analytical method development, mandated by FDA and EMA, ensures drug batches remain safe, pure, and effective. Techniques such as HPLC, UV-visible, and mass spectrometry are optimized to identify substances, separate impurities, and establish fingerprints. Validation under ICH Q2(R2) defines performance characteristics, making these methods the scientific and legal foundation for market approval[3,4].

High-Performance Liquid Chromatography (HPLC) employed analytical techniques shows high sensitivity, selectivity, precision, and reproducibility[5]**.** Although few RP-HPLC methods have been reported for the estimation of Tafamidis, most are based on conventional trial-and-error optimization and lack integration of Analytical Quality by Design (AQbD) principles or systematic green analytical chemistry assessment. Furthermore, absence in Indian Pharmacopoeial monograph, highlighting the unmet need for a robust, stability-indicating, and environmentally sustainable analytical method. Therefore, the present study aimed to implicate environmentally sustainable metrics based stability-indicating RP-HPLC assay method for Tafamidis API in bulk using an AQbD approach.

Experimental Section

Materials and methods

H_2PO_4, KH_2PO_4, HCl, NaOH, H_2O_2, and methanol were acquired from Thermo Fisher Scientific India Pvt. Ltd. Mumbai, India. Tafamidis was sourced from Rainbow laboratories, Hyderabad, India. The study utilized UV/visible detector (Model No. 2489) equipment and a Waters Alliance HPLC (2695) system using Empower 3 for developing and

validating HPLC methods. Custom Design was utilized with the JMP 18 program to enhance the approach's performance by selecting components and evaluating responses.

Preparation of solutions

Phosphate (KH_2PO_4- 0.1M) buffer preparation: An accurately weighed 1.70 g of KH_2PO_4 was transferred into a 250 mL volumetric flask, dissolved in water and the volume was adjusted up to the mark. The resulting solution was filtered through a 0.45 µm membrane filter and subsequently sonicated for 10 min[7].

Mobile phase preparation: The buffer solution was first degassed in an ultrasonicator for 10 min. A mixture was then prepared by combining 125 mL of KH_2PO_4 buffer with 125 mL of methanol in a 1:1 ratio. The prepared mixture was subjected to vacuum filtration and subsequently transferred through a 0.45 µm membrane filter[8].

Diluent preparation: The diluent used was mobile phase.

Formulation of standard stock solutions: In a 25 mL volumetric flask, 10 mg of Tafamidis was precisely weighed, dissolved in 5 mL of methanol and the volume was adjusted using methanol to the proper level. Using HPLC-grade water 1 mL of this solution was diluted to volume in a 10 mL volumetric flask.[9]

Development of AQbD method

AQbD is a methodical, scientific strategy used in the pharmaceutical and other industries to develop and optimize analytical techniques. It emphasizes a proactive and risk-based methodology focusing on understanding and controlling the factors that affect the performance of analytical methods. The important components of AQbD[10] are given below.

ATP (Analytical target profile)

AQbD begins with an analytical target profile (ATP) analogous to QTPP. According to a modern definition, an ATP outlines the intended purpose of a method and serves as a guiding framework for its selection, design and development. In general, the ATP for analytical methods involves (a) identifying the target analytes such as active pharmaceutical ingredient (API) and related impurities, (b) choosing an appropriate analytical technique (e.g., HPLC, GC, HPTLC, ion chromatography) and (c) defining the specific method requirements for assay, impurity profiling or residual solvent determination.

Critical Analytical Attributes (CAA)

CAAs refer to the method parameters that must remain within acceptable limits to fulfill the defined ATP. Typical examples include peak area, retention time, plate count, tailing factor, resolution, and relative retention time.

Critical Method Parameters (CMP)

Critical Method Parameters (CMPs) are controllable analytical variables that directly affect the Critical Analytical Attributes (CAAs) of a method. These parameters such as mobile phase composition, pH, buffer strength, flow rate and injection volume must be carefully optimized and maintained within defined limits to ensure method robustness, reliability and compliance with the desired ATP.

Risk assessment

The process involves three stages: risk identification, risk analysis, and risk evaluation. During risk identification parameters involved in the procedure are outlined depending on their impacton CAAs. From this assessment, a limited number of "critical" CMPs that influence both CAAs and overall method performance are determined.

Design of Experiment

The DoE principles minimize the occurrence of (any) interactions and reduce complexity while allowing for a thorough scientific insight of the many technical aspects and parameters that tend to impact CAAs with minimal testing. Utilizing a variety of factors, DoE can also be utilized to optimize experimental conditions.

Establishment of MODR

These days, computer-aided methods and software are used to optimize settings and the reactions that follow MODR it is also referred as validated acceptable range, is the MODR or ADS (Analytical Design Space) of any analytical method.

Implementation of a control plan and continual improvement

A structured control framework addressing all potential variations is essential to comply with ATP requirements while method transfer and routine application. This can be accomplished by continuously checking system suitability parameters or CAAs. To promote ongoing improvement, a control strategy should be integrated across all critical phases of the method development life cycle.

Optimization of chromatographic conditions

Initial trials were conducted to optimize the final method. Chromatographic separation was attained using a G1 Sciences C8 column, 150×4.6 mm, 3 µm at 25 °C with 0.1 N KH_2PO_4 and methanol were mixed 80:20 (v/v) as mobile phases, which is pumped at a flow rate of 1 mL/min at 250 nm, the UV detector was used[11].

Experimental design

The method was optimized using custom design, considering flow rate, % organic solvent, pH of mobile phase. Different ranges of higher and lower limit was chosen for flow rate: 0.5-1 mL/min and % organic solvent: 20-40 and pH of mobile phase (4.6) was kept constant. JMP 18 was used to generate and analyze a study design consisting of 12 runs (Table 1).

Analytical method validation

Whenever a new or modified analytical method is developed, it must be validated to ensure that it gives consistent and reliable results. This is important so that the method works the same way when used by different people, on similar or different instruments, and in different laboratories. The main goal of method validation is to provide proof that the method does exactly what it is meant to do accurately, reliably and consistently. According to ICH guidelines, the key validation parameters are explained below.

Accuracy

The degree to which the measured values resemble the actual or recognized reference values is known as accuracy. In other words, it shows the similarity between the observed result and the actual value. It is also called trueness and is usually assessed by performing at least nine measurements at three different concentration levels within the specified range.

Precision

The degree of agreement (or scatter) between a collection of measurements made from different samplings of a homogenous sample under predetermined conditions is known as the trueness of an analytical procedure. Three levels can be used to assess precision: repeatability (it is the closeness of results over a brief period of time under the same operating conditions), intermediate precision (it refers to the consistency of results within a single laboratory when examined on multiple days, by different analysers, or with different tools) and reproducibility (shows how well data from several labs agree with one another).

Specificity

Specificity for every developmental stage should be demonstrated by the analytical technique. The method should be able to conclusively assess the analyte of interest when all anticipated components—degradants, excipients/sample matrix, and sample blank peaks are present.[12]

Linearity

An analytical procedure is said to be linear if it can yield test findings that, within a given range, are directly proportional to the concentration of an analyte in the sample. A minimum of five concentrations were suggested in order to establish linearity, according to the ICH guidelines. Tafamidis solution in the concentration range of 50 to 150 µg/mL were injected at 250 nm wavelength.

Limit of Detection (LOD)

The least amount of analyte in a sample that can be identified, even though it might not be possible to

Table 1 — Method trail runs by custom design

Sl. No.	Factors			Responses		
	Flow rate	% organic solvent	pH of mobile phase	Retention time	Tailing factor	USP Plate count
1	0.6	40	4.6	3.403	1.42	5698
2	1	20	4.6	2.215	1.17	5911
3	1	40	4.6	2.335	1.41	6154
4	0.6	20	4.6	3.329	1.29	5734
5	1	20	4.6	2.528	1.15	5921
6	0.8	30	4.6	2.485	1.32	5780
7	1	40	4.6	2.465	1.36	6152
8	0.6	40	4.6	3.538	1.47	5598
9	1	20	4.6	2.215	1.17	5911
10	0.6	40	4.6	3.538	1.45	5598
11	1	40	4.6	2.199	1.41	6155
12	0.6	20	4.6	3.326	1.29	5733

measure it precisely, it is known as the detection limit of an analytical procedure. The Limit of Detection (LOD) can be expressed as:

$LOD=3.3\times\sigma/S$

Where σ =standard deviation of the response, S = slope of the calibration curve.

Limit of Quantification (LOQ)

The minimum concentration of an analyte in a sample that can be identified, although not measured accurately, it is referred as the detection limit of analytical method.

$LOQ=10\sigma\times/S$

Where σ = the standard deviation of the response, S = the slope of the calibration curve.

Robustness

The robustness of an analytical method defines its ability to withstand minor but intentional changes in method parameters and shows how reliable it is under typical operating conditions.

Example: influence of changes in a mobile phases pH; composition of the mobile phase; different columns (different lots and/or suppliers); temperature and flow rate[13].

System suitability

System suitability testing of an analytical process is based on the idea that the instrument, analytical process, electronics, and test samples work as a single system that should be examined collectively. The parameters chosen for system suitability depend on the nature of the procedure being validated. In the simplest case, HPLC system suitability is assessed by comparing the chromatogram obtained with that of a standard reference[14].

Forced degradation studies acid stress

5 mL of Tafamidis was diluted to 50 mL, there after put into a 10 mL volumetric flask, and sonicated with 0.1 N HCl for 30 min. The solution was then poured into the HPLC vial and injected into the system, ensuring accurate results.

Base stress

Tafamidis was diluted to 50 mL by pipetting it out of the stock solution in 5 mL increments. After moving 1 mL of the solution to a 10 mL volumetric flask, it was combined with 0.1 N NaOH, adjusted, and sonicated for half an hour. After that, the solution was introduced into an HPLC vial.

Hydrolysis

By pipetting 5mL of Tafamidis from the stock solution and diluting it to 50 mL, 1 mL of the solution was removed and transferred into a 10 mL volumetric flask. The mixture was then sonicated for half an hour after the volume was adjusted with water. Afterwards, the solution was put into an HPLC vial**.**

Peroxide

5 mL Tafamidis from stock solution was pipetted and diluted to 50 mL, 1 mL of the solution was poured into a 10 mL volumetric flask. 1% H_2O_2 was incorporated, diluted, and sonicated for 30 min. The solution was then injected into an HPLC vial and analyzed using a HPLC.

Heat

A 5 mL portion of Tafamidis stock solution was diluted to 50 mL. From this, 1 mL was transferred into a 10 mL volumetric flask, made up with diluent, and subjected to heating at 60 °C for 30 min. The prepared solution was then placed in an HPLC vial and analyzed.

Sunlight

5 mL Tafamidis was diluted from stock to 50 mL, then 1 mL of solution was added to a 10 mL volumetric flask and left in sunlight for six days and then introduced into a HPLC vial. From the degradation data, it was found that Tafamidis showed degradation in acid (10.4%) and heat (8.9%) stressed condition, but degradation was below 50% of limit and the degraded product did not interfere with the main peak.

Results and Discussion

Analytical techniques indicate product quality and strength throughout its lifetime. HPLC methods primary aim is to isolate and quantify target analyte for contaminants and excipients. JMP 18 software was used for optimization study and statistical analysis to develop the analytical RP- HPLC method for establishing the design space and control strategy. The present method achieved a rapid chromatographic analysis with a retention time of 2.23 min using GL Sciences C18 column and a mobile phase of phosphate buffer and methanol at detection 250 nm**.** (Fig. 1), This composition is cost-effective, non-toxic, and environmentally friendly compared to previously reported methods by Gandhi *et al*., who reported a retention time of approximately 3.3 min**,** using column YMC Triart C18 column with a mobile phase

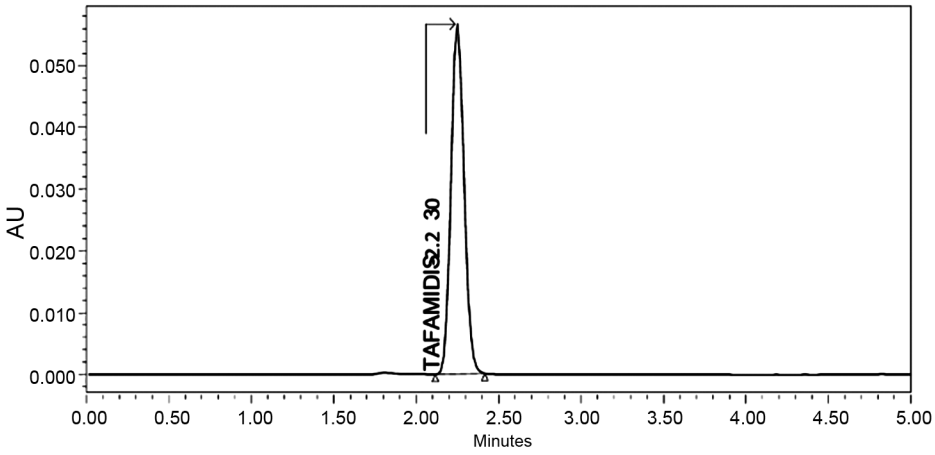

Fig. 1 — Optimal chromatogram

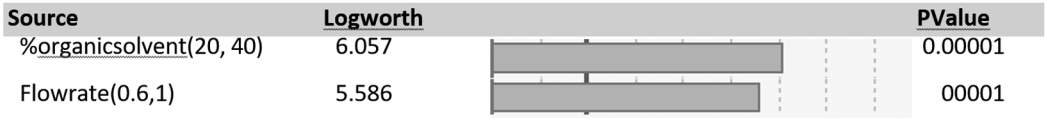

Fig. 2 — Effect summary

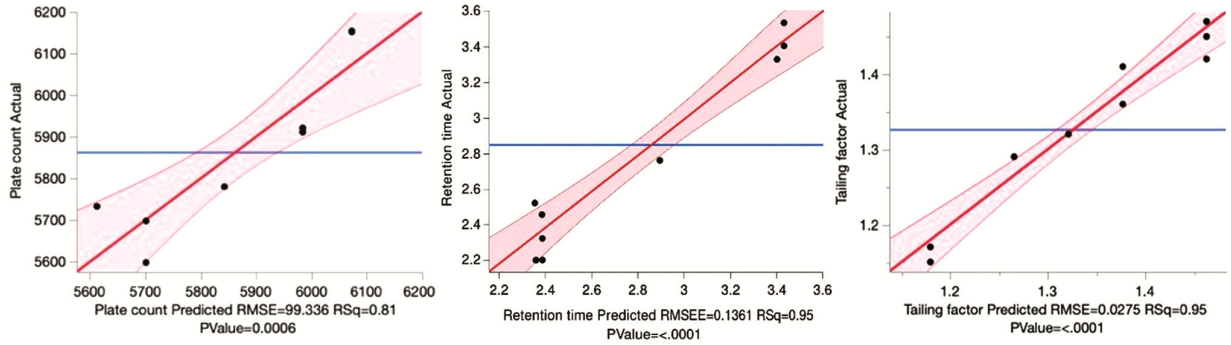

Fig. 3 — Model fit of specific HPLC technique responses

Ammonium Acetate and Acetonitrile at detection 206 nm whereas the QbD-based method developed by Khan *et al*. reported a retention time of about 5.02 min using C18 column with a optimized QbD mobile phase.

Statistical optimization using DOE

Design of experiment by custom design

In Fig. 2, it manifests that method is impacted by factors such as % organic solvent & flow rate. A bar representing the % organic solvent and flow rate crosses the blue vertical line to indicate the significance as shown in Fig. 2.

Model fit of specific HPLC technique responses

The model fit was evaluated using data from all 12 runs, enhancing methods robustness. As shown in Fig. 3, at a significance threshold of $p<0.01$, $R^2 = 0.95$ and $p = < 0.0001$ for response retention time, $R^2=0.95$ and $p=<0.0001$ for response tailing factor, and $R^2=0.81$ and $p=0.0006$ for response plate count, found to be statistically significant. The high R^2 values and low p-values suggest that the model being evaluated is a good fit for the data.

Surface graphs

Retention time: The (3D) surface response plot for retention time was constructed to investigate the influence of flow rate (0.6-1.0 mL/min) and % organic solvent (20-40) on the retention time of the analyte. The surface plane slopes downward in both directions, showing that both factors act synergistically to shorten the retention time (Fig. 4).

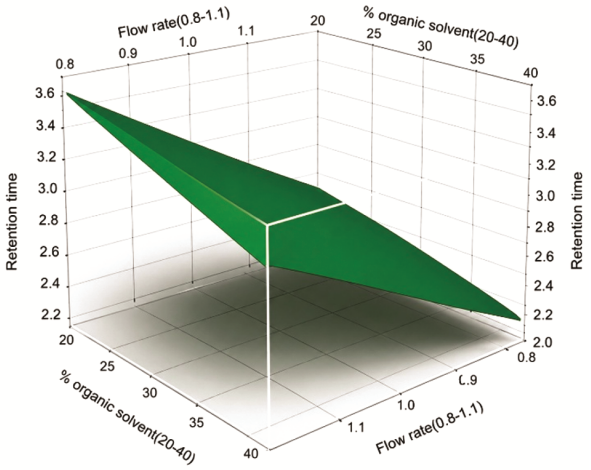

Fig. 4 — Surface graph of Retention time

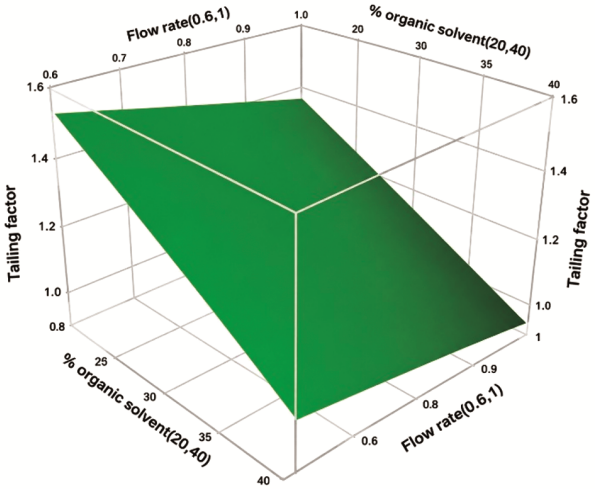

Fig. 5 — Surface graph of tailing factor

Tailing factor: The 3D surface plot for tailing factor represents the interaction between flow rate and % organic solvent on the tailing factor showed a mildly sloped plane. This suggests that peak symmetry remains within permissible range, confirming the reliability of the method (Fig. 5).

Plate count: The 3D surface plot generated for plate count revealed a gently declining surface as the flow velocity and proportion of organic modifier increases. This suggests that column efficiency is highly sensitive to both flow rate and solvent composition and the best performance can be achieved under milder flow rate and % organic solvent (Fig. 6).

Statistical optimization of selected responses of the HPLC method

Numerical optimization: The prediction profiler reveals a constant relationship between parameters

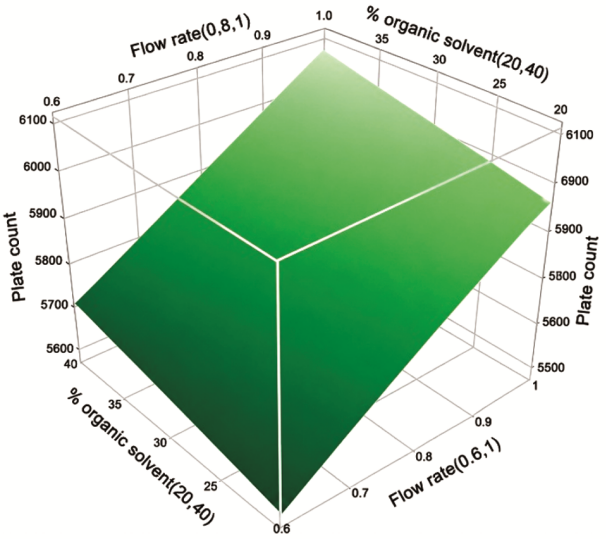

Fig. 6 — Surface Graph of Plate count

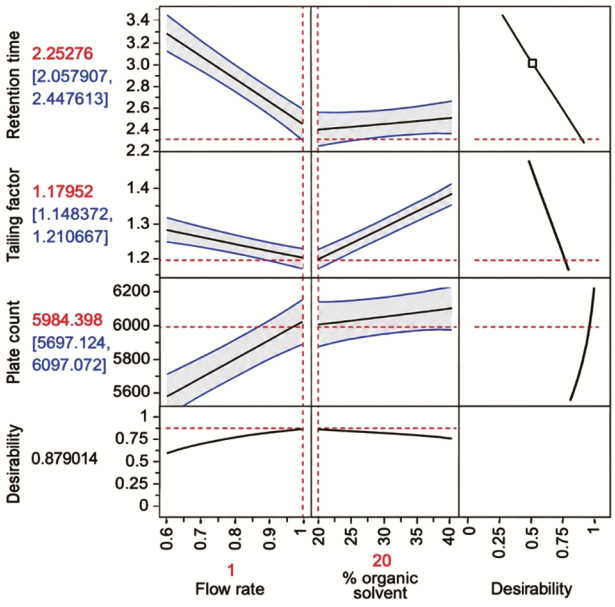

Fig. 7 — Prediction profiler with maximized desirability

and responses, revealing variability in extreme results. Fig. 7 shows a global desirability value of 87.9%, indicating the model's validity for achieving desired goals for all three responses.

Results of validation parameter

Accuracy

The accuracy of Tafamidis was evaluated using the standard addition method at three concentration levels as shown in Table 2. A known quantity of the drug was spiked into the pre- analyzed sample, and the percentage recovery was determined. The results

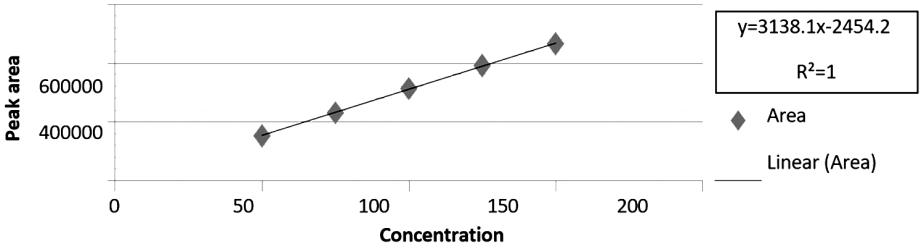

Fig. 8 — Linearity of Tafamidis

Table 2 — Accuracy studies or % recovery studies

Tafamidis Spiked level	Sample Weight	Sample Area	µg/mL added	µg/mL found	% Recovery	Accuracy
50%	175.00	154421	99.000	98.29	99	
50%	175.00	154101	99.000	98.08	99	Mean=99.6
50%	175.00	154120	99.000	98.09	99	
100%	350.00	311424	198.000	198.21	100	
100%	350.00	311451	198.000		100	SD=0.5
100%	350.00	311669	198.000	198.37	100	
150%	525.00	467440	297.000	297.52	100	
150%	525.00	467402	297.000	297.49	100	%RSD= 0.5
150%	525.00	467541	297.000	297.58	100	

Table 3 — Robustness studies by changing composition of mobile phase

Sl. No.	Sample name	Peak name	RT(min)	Area (µV*sec)	USP tailing	USP plate count
1	COMP-1	TAFAMIDIS	1.829	313365	1.14	5991
2	COMP-2	TAFAMIDIS	2.468	449601	1.19	5960

Table 4 — Robustness studies by changing the pH

S. No.	Sample name	Peak name	RT (min)	Area (µV*sec)	USP tailing	USP plate count
1	pH-1	TAFAMIDIS	2.239	313864	1.14	6095
2	pH-2	TAFAMIDIS	2.234	313965	1.18	5993

showed recoveries in the range of 99–100%, which falls within the ICH-accepted limit of 98–102%.

Precision

The % RSD for the precision is 0.1% and was within the acceptable limit of ≤2%.

Linearity

As per the linearity graph as shown in Fig. 8, the method was found to be linear as the R^2 was found to be within the limit of ≥ 0.95.

Specificity

The HPLC method is specific for Tafamidis as there is no interference from blank, placebo or degradation products at the retention time of Tafamidis.

Limit of Detection (LOD) & Limit of Quantification (LOQ)

The lowest amount of sample that can be detected was found to be 0.47 µg/mL and the minimum quantifiable concentration of the sample was determined to be 1.59 µg/mL as per ICH guidelines.

Robustness

Robustness was evaluated by performing the assay under minor variations in flow rate, column temperature, pH, and mobile phase composition. The outcomes met system suitability requirements, confirming that the developed method was robust and exhibited no changes (Tables 3-5).

System suitability

System suitability parameters pass for tailing factor (less than 2.0), plate count (more than 2500) along with acceptable peak area and retention time-2.23 min, % RSD (acceptable limit:≤2%) as specified in Table 6. To check for conditions as essential, for every 6 samples a bracketing standard was kept.

Table 5 — Robustness studies by changing the temperature

S. No.	Sample name	Peak name	RT (min)	Area (µV*sec)	USP tailing	USP plate count
1	TEMP-1	TAFAMIDIS	2.030	349526	1.18	5986
2	TEMP-2	TAFAMIDIS	2.468	449601	1.19	5960

Table 6 — System suitability

SI. No.	Sample name	Peak name	RT (min)	Area (µV*sec)	USP Plate count	USP tailing
1	STD-2	TAFAMIDIS	2.231	312786	5920	1.19
2	STD-2	TAFAMIDIS	2.233	312842	5942	1.18
3	STD-2	TAFAMIDIS	2.231	313344	5838	1.19
4	STD-2	TAFAMIDIS	2.237	313835	5970	1.18
5	STD-2	TAFAMIDIS	2.236	313625	5922	1.18
Mean			2.23	313286.4		
%RSD				0.1		

Table 7 — Forced degradation studies

Stress	Sample weight (mg)	Sample area	% Assay	% Degraded
Acid stress	350	281432	89.56	10.4
Base stress	350	297905	94.80	5.2
Peroxide stress	350	291468	92.75	7.2
Heat stress	350	286013	91.02	8.9
Photo stress	350	299991	95.46	4.5
Hydrolysis stress	350	310713	98.88	1.2

Forced degradation studies

The stability of Tafamidis was tested in extreme environments like heat, light, acidic, basic, peroxide, and water. Temperature and acid stressed conditions led to noticeable degradation, with comparable degradation peaks under the acceptable limits. Details are given in Table 7.

Assessment of greenness

The greenness of the method was evaluated using the AGREE tool and MoGAPI index, both provide a comprehensive assessment based on the 12 principles of Green Analytical Chemistry. AGREE is a free software tool which can be downloaded from the link http://mostwiedzy.pl/AGREEprep[6] (version 0.5 beta)[15]. MoGAPI (Modernized Green Analytical procedure Index) is an advanced greenness assessment tool which expands from original GAPI tool which provides more detailed evaluation zones, better colour gradation and enhanced ability to differentiate different methods involved from sample collection to waste disposal (link-bit.ly/MoGAPI) [16].

Assessment of greenness applied on developed RP-HPLC method for Tafamidis. This involves minimal external sample treatment and reduced preparation steps with enhanced eco-friendly nature. The optimized chromatographic run time was 2.230 min with a flow rate of 1.0 mL/min, which is less when compared to other methods thereby reducing solvent and energy consumption. The mobile phase composition consisted of phosphate buffer (KH_2PO_4) and methanol (80:20, v/v), adjusted to pH 4.6, under isocratic conditions. The AGREE tool generated a clock-like pictogram with an overall score of 0.84 as shown in the Fig. 9 while MoGAPI yielded a score of 84 as shown in the Fig. 10 both indicating that the method is highly green and highly acceptable by environment.

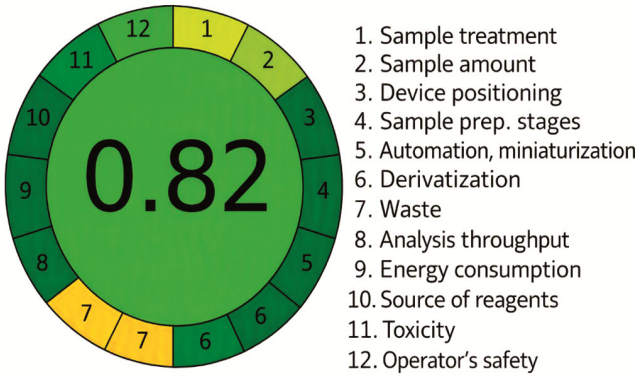

Fig. 9 — Pictogram representation of greenness of the developed method using AGREE

Conclusion

A stability indicating AQbD-based RP-HPLC method for the quantitative estimation of Tafamidis API in bulk was successfully developed and optimized using a C18 column with a flow rate of 1.0 mL/min, providing a short retention time of 2.230 min. Application of AQbD principles enabled systematic control of critical analytical parameters, enhancing method robustness in accordance with ICH Q8 and ICH Q14 guidelines. The developed method satisfied all validation requirements as per ICH Q2 (R1) guidelines, demonstrating acceptable accuracy, precision (%RSD < 2%), linearity, specificity, and robustness, confirming its suitability for routine quality control analysis. Forced degradation studies indicated noticeable degradation under acidic and thermal stress conditions compared to others and under acceptance limit establishing the stability-indicating nature of the method. Greenness evaluation using AGREE and MoGAPI confirmed excellent compliance with green analytical chemistry principles, mainly due to reduced solvent consumption, short run time, and minimal sample preparation. Overall, the developed method is reliable, robust, environmentally sustainable, and regulatory compliant, and is recommended for routine quality control, stability testing, and pharmaceutical quality assurance of Tafamidis API. The study was limited to Tafamidis API in bulk, and evaluation on finished pharmaceutical dosage forms is recommended for broader industrial applicability.

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Conflict of Interest

The authors declare no conflict of interest.

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