

METHOD DEVELOPMENT AND VALIDATION FOR THE QUANTITATIVE DETERMINATION OF TAFAMIDIS IN HUMAN PLASMA USING LIQUID CHROMATOGRAPHY–TANDEM MASS SPECTROMETRY

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Article Received: 12 August 2026 | Article Revised: 02 September 2026 | Article Accepted: 22 September 2026

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DOI: <https://doi.org/10.5281/zenodo.23052949>

How to cite this Article: Srinivasa Reddy, Nirmala Nayak, Licto Thomas, Imran Ahmed M, Arindam Mukhopadhyay, Saral Thangam (2026) METHOD DEVELOPMENT AND VALIDATION FOR THE QUANTITATIVE DETERMINATION OF TAFAMIDIS IN HUMAN PLASMA USING LIQUID CHROMATOGRAPHY–TANDEM MASS SPECTROMETRY. World Journal of Pharmaceutical Science and Research, 5(10), 18-30.



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ABSTRACT

Tafamidis is a selective transthyretin (TTR) stabilizer indicated for the treatment of transthyretin amyloidosis in adult patients presenting with early-stage symptomatic polyneuropathy. In the present study, a rapid, sensitive, and reliable liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed and validated for the quantification of tafamidis in human plasma. Plasma samples were processed using a liquid–liquid extraction procedure employing tert-butyl methyl ether. Chromatographic separation was achieved on a reversed-phase C18 column using a mobile phase comprising 5mM Ammonium acetate in 0.025% Formic acid and organic mixture (Acetonitrile: Methanol:: 75:25 v/v) at a flow rate of 1.2 mL/min. Detection was performed using multiple reaction monitoring (MRM) in negative electrospray ionization mode, monitoring the transitions m/z 305.4 $\rightarrow$ 261.4 for tafamidis and m/z 312.0 $\rightarrow$ 267.7 for the 13C6 containing internal standard. The method demonstrated a lower limit of quantification (LLOQ) of 10 ng/mL and exhibited linearity over the concentration range of 10–10,000 ng/mL with a correlation coefficient (R^2) greater than 0.99. Validation parameters, including selectivity, precision, accuracy, matrix effect, extraction recovery, and stability, complied with established regulatory acceptance criteria. The validated method was successfully applied to the pharmacokinetic evaluation of tafamidis in human subjects.

KEYWORDS: Tafamidis, metabolite, LCMS/MS, method development, method validation.

INTRODUCTION

Amyloidosis is a rare and progressive disorder characterized by the misfolding of proteins into insoluble amyloid fibrils, which aggregate and deposit extracellularly within tissues and organs. These deposits can affect multiple organs, including the heart, kidneys, liver, spleen, peripheral nervous system, and gastrointestinal tract, leading to progressive organ dysfunction. Amyloidosis is classified according to the nature of the precursor protein involved in fibril formation. Among the various forms, immunoglobulin light-chain amyloidosis (AL) and transthyretin amyloidosis (ATTR) represent the two principal subtypes associated with cardiac involvement.

Immunoglobulin light-chain amyloidosis arises from the abnormal production of monoclonal light-chain fragments by plasma cells, resulting in protein misfolding and direct myocardial toxicity. In contrast, ATTR develops due to destabilization and dissociation of transthyretin (TTR), a transport protein responsible for carrying thyroxine and retinol-binding protein in the circulation and cerebrospinal fluid. Together, AL and ATTR account for approximately 95% of cardiac amyloidosis cases.

Transthyretin is predominantly synthesized in the liver, contributing approximately 90% of circulating TTR, while the remaining fraction is produced by the choroid plexus and retinal pigment epithelium. Under physiological conditions, TTR exists as a homotetramer. Destabilization of this tetrameric structure promotes dissociation into monomers, which subsequently misfold and aggregate into amyloid fibrils. Deposition of these fibrils within the extracellular matrix of various tissues leads to progressive impairment of organ function.

Transthyretin amyloid cardiomyopathy (ATTR-CM) is a debilitating and progressive condition characterized by amyloid deposition within the myocardium, frequently accompanied by involvement of the peripheral and autonomic nervous systems. Clinically, ATTR-CM commonly manifests as progressive heart failure with both right- and left-sided symptoms and is often complicated by cardiac arrhythmias, particularly atrial fibrillation, resulting from conduction abnormalities.^[1-7]

Tafamidis, a non-steroidal benzoxazole derivative, functions as a selective kinetic stabilizer of transthyretin. It inhibits the rate-limiting step of TTR amyloidogenesis by selectively binding with negative cooperativity to one of the two thyroxine-binding sites present in the native TTR tetramer. Occupation of a single binding site is sufficient to stabilize the weaker dimer-dimer interface, thereby preventing tetramer dissociation under both physiological and denaturing conditions. Through stabilization of the TTR tetramer, tafamidis reduces the formation of amyloidogenic intermediates and limits amyloid fibril deposition. Clinical evidence from the Transthyretin Amyloid Cardiomyopathy Clinical Trial (ATTR-ACT) demonstrated that tafamidis significantly reduced cardiovascular-related hospitalizations and all-cause mortality in patients with ATTR-CM.^[8,9]

Although several studies have investigated the pharmacological and clinical aspects of tafamidis, reports describing validated bioanalytical methods for its quantification remain limited. Few high-performance liquid chromatography (HPLC) methods have been reported for the determination of tafamidis in pharmaceutical formulations, while only a single LC-MS/MS method has been described for its quantification in rat plasma.^[10,11] One HPLC-UV method is available for determination of tafamidis in patients; however, its applicability is limited by a relatively narrow calibration range.^[12] Another LC-MS/MS method described briefly about its quantification in human plasma, although the method did not adequately address the potential interconversion between tafamidis and its major metabolite,

tafamidis acyl glucuronide.^[13] Therefore, the present study aimed to develop and validate a rapid, sensitive, and robust LC–MS/MS method for the determination of tafamidis in human plasma in accordance with current regulatory guidelines.^{14, 15} Emphasis was placed particularly on minimizing interconversion between tafamidis and tafamidis acyl glucuronide during sample collection, processing, and analysis. To the best of our knowledge, this study represents the first fully validated LC–MS/MS method for the quantification of tafamidis in human plasma that effectively addresses metabolite back-conversion, thereby supporting its application in pharmacokinetic, bioavailability, and bioequivalence studies.

MATERIALS AND METHODS

Materials

Tafamidis and the isotopically labelled internal standard, Tafamidis-13C6, were procured from BioOrganics, India. All solvents, reagents, and chemicals used throughout the study were of analytical or HPLC grade and were obtained from local commercial suppliers. Ultrapure water generated using a Milli-Q purification system was employed for all analytical procedures.

Methods

Liquid Chromatography–Tandem Mass Spectrometry Conditions

Quantitative analysis of tafamidis was performed using an Agilent 1200 UPLC system (Agilent Technologies, Santa Clara, CA, USA) coupled to an AB SCIEX API 4000 triple quadrupole mass spectrometer (Foster City, CA, USA). The chromatographic system consisted of an autosampler, binary pump, system controller, and column oven.

Separation was achieved on a BDS Hypersil C18 analytical column (4.6 × 100 mm, 5 µm particle size). The autosampler tray and column temperatures were maintained at 10°C and 50°C, respectively. An injection volume of 10 µL was employed.

The mobile phase consisted of 5 mM ammonium acetate containing 0.025% formic acid and an organic solvent mixture in a ratio of 30:70 (v/v). The organic component comprised of acetonitrile and methanol in a ratio of 75:25 (v/v). Isocratic elution was performed at a flow rate of 1.2 mL/min using a flow splitter, resulting in a total analytical run time of 4 min.

Mass spectrometric detection was conducted in negative electrospray ionization mode. Optimized source parameters included an ion source temperature of 450°C, nebulizer gas pressure of 45 psi, and dwell time of 400 ms. Quantification was performed using MRM transitions of m/z 305.9 → 261.8 for tafamidis and m/z 312.0 → 267.7 for Tafamidis-13C6. For tafamidis acyl glucuronide metabolite monitoring, the transition m/z 482.0 → 261.8 was employed. Collision energy of –22 V was applied for both tafamidis and internal standard.

Sample Preparation

Plasma samples were prepared using a liquid–liquid extraction procedure. Briefly, 100 µL of human plasma was transferred into labelled reaction vials, followed by the addition of 50 µL of internal standard solution, except in blank samples. Subsequently, 200 µL of 100 mM sodium dihydrogen orthophosphate dihydrate solution was added, and the mixture was vortex-mixed thoroughly.

Thereafter, 2 mL of tert-butyl methyl ether (TBME) was added to each vial. Samples were mixed using a VibraMax shaker at 2500 rpm for 10 min and centrifuged at 4000 rpm for 5 min at 4°C. Following centrifugation, 1.6 mL of the organic layer was carefully transferred into clean reaction vials and evaporated to dryness under a gentle stream of nitrogen at approximately 40°C.

The dried residue was reconstituted with 1.0 mL of mobile phase and vortex-mixed to ensure complete dissolution. The resulting solutions were transferred into autosampler vials, and 10 µL aliquots were injected into the LC–MS/MS system for analysis.

Preparation of Calibration Standards and Quality Control Samples

Primary stock solutions of tafamidis were prepared in methanol: DMSO (50:50, v/v) at a concentration of 2000 µg/mL, with concentration corrections applied based on purity and accurately weighed amounts. Working solutions ranging from approximately 503.093 to 500,000 ng/mL were prepared by serial dilution of the stock solution using the same diluent.

Calibration standards were prepared by spiking 20 µL of the appropriate working solution into 980 µL of pooled K₂EDTA human plasma obtained from stabilized blood containing 25 mM citrate buffer. To this 100µl of Stabilizing agent (10% stabilizing agent-25mM Citrate buffer) was added. The resulting calibration range covered concentrations from approximately 10.062 to 10,000 ng/mL.

A stock solution of Tafamidis-13C₆ was prepared at 200 µg/mL in methanol: DMSO (50:50, v/v). A working internal standard solution of 4.0 µg/mL was subsequently prepared using methanol water (50:50, v/v).

Similarly, quality control samples were prepared independently by diluting the analyte stock solution to concentrations ranging from approximately 505.613 to 380,000 ng/mL. These solutions were spiked into pooled plasma to obtain QC concentrations ranging from approximately 10.112 to 7600 ng/mL.

All calibration standards and quality control samples were stored at $-70 \pm 10^{\circ}\text{C}$ until analysis. The internal standard solutions were stored at 2-8°C.

Method Validation

Method validation was performed in accordance with the recommendations outlined by the United States Food and Drug Administration (US FDA) and the European Medicines Agency (EMA) for bioanalytical method validation.^{14, 15} The evaluated parameters included selectivity, linearity, precision, accuracy, matrix effect, extraction recovery, and stability. The results of these validation studies are presented in the subsequent sections.

RESULTS AND DISCUSSION

Method Development

Efficient sample preparation is a critical aspect of developing sensitive and selective LC–MS/MS methods, particularly for the quantification of trace concentrations of pharmaceutical compounds in biological matrices. Commonly employed sample preparation techniques include protein precipitation (PPT), liquid–liquid extraction (LLE), and solid-phase extraction (SPE). Although protein precipitation is a simple and rapid approach, it is often associated with matrix-induced ion suppression or enhancement. Solid-phase extraction generally provides cleaner extracts; however, it

is comparatively expensive and labour-intensive. In the present study, liquid–liquid extraction using tert-butyl methyl ether (TBME) was selected because it provided superior extraction efficiency, consistent recovery, and minimal matrix interference.

Tafamidis undergoes biotransformation to form tafamidis acyl glucuronide, a major metabolite reported to represent approximately 22% of the parent drug concentration in urine.^{16, 17} Preliminary investigations revealed that tafamidis acyl glucuronide exhibited limited stability in both whole blood and plasma, resulting in potential back-conversion to the parent compound during sample handling. Further studies demonstrated enhanced metabolite stability in the presence of 25 mM citrate buffer. Consequently, blood samples were stabilized immediately after collection by adding citrate buffer at a blood-to-buffer ratio of 10:1 prior to plasma separation. Plasma samples were subsequently stabilized using the same buffer system to minimize metabolite degradation and prevent elevation of tafamidis concentrations.

Both tafamidis and the isotopically labelled internal standard, Tafamidis-13C6, contain a benzoxazole carboxylic acid moiety. Owing to the acidic nature of this functional group, both compounds readily undergo deprotonation to form $[M-H]^-$ ions. Comparative evaluation of ionization modes revealed that negative electrospray ionization (ESI) produced substantially higher signal intensities than positive ionization and was therefore selected for quantitative analysis.

The optimized mass spectrometric conditions employed multiple reaction monitoring (MRM) transitions of m/z 305.9 $\rightarrow$ 261.8 for tafamidis and m/z 312.0 $\rightarrow$ 267.7 for the internal standard. Chromatographic separation was achieved using a reversed-phase C18 column. Several chromatographic parameters, including buffer composition, pH, and organic solvent composition, were systematically evaluated to optimize analyte response and chromatographic performance. Formic acid, ammonium acetate, and ammonium formate were investigated as mobile-phase modifiers over a pH range of 3–6. In addition, methanol and acetonitrile were evaluated individually and in combination at different proportions.

Among the chromatographic systems evaluated, the mobile phase comprising ammonium acetate buffer and acetonitrile along with methanol provided the most favourable chromatographic conditions, including peak symmetry, retention behaviour, baseline stability, and signal-to-noise ratio. The optimized chromatographic conditions consisted of 5mM ammonium acetate in 0.025% formic acid and organic mixture (acetonitrile: methanol::75:25 v/v) in an isocratic ratio of 30:70 (v/v), delivered at a flow rate of 1.2 mL/min. Under these conditions, tafamidis eluted at a retention time of 2.28min, enabling rapid analysis with excellent reproducibility.

Recovery

Extraction recovery was evaluated by comparing the peak areas obtained from plasma samples spiked with tafamidis prior to extraction ($n = 6$) with those obtained from extracted blank plasma samples subsequently spiked with the analyte and internal standard. The recovery results at low-, medium-, and high-quality control concentrations are summarized in Table I.

Table I: Recovery of tafamidis in spiked human plasma.

LQC			MQC			HQC		
Peak area		Mean percentage recovery	Peak area		Mean percentage recovery	Peak area		Mean percentage recovery
Before extraction	Post-extraction		Before extraction	Post-extraction		Before extraction	Post-extraction	
11453.7	16451.8	69.6	1427080.3	1927323.8	74.0	2889482.7	4097885.3	70.5

The mean extraction recovery of tafamidis across all quality control levels was 71.39%. The observed recovery exceeded the generally accepted minimum criterion of 50% and demonstrated consistent extraction efficiency across the concentration range investigated. These findings confirm that the liquid–liquid extraction procedure using TBME is suitable for the reliable and reproducible extraction of tafamidis and the internal standard from human plasma.

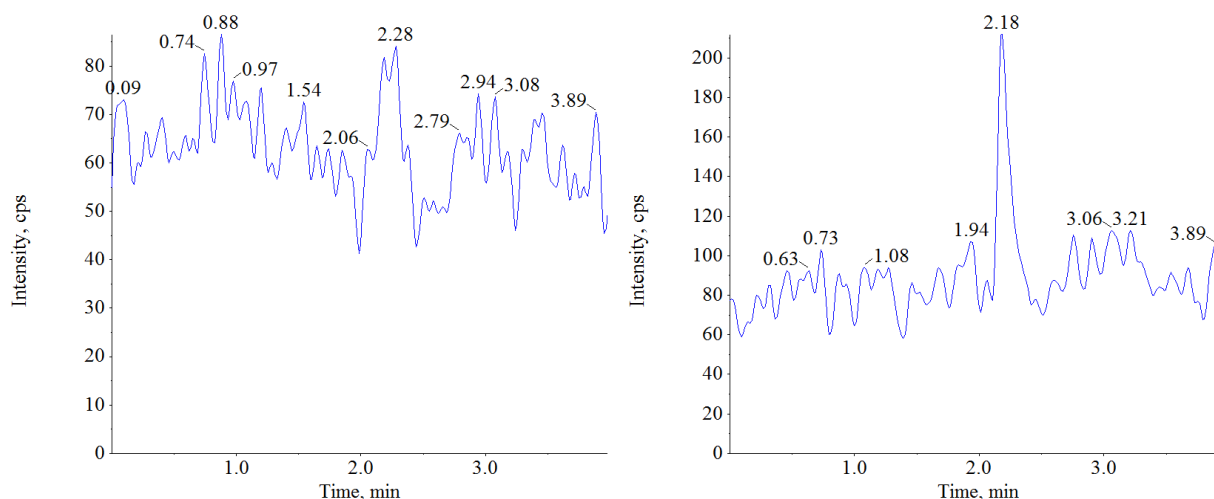
Method Validation

The developed LC–MS/MS method was validated in accordance with current US Food and Drug Administration (FDA) and European Medicines Agency (EMA) bioanalytical method validation guidelines.^[14,15] Validation parameters included selectivity, sensitivity, linearity, accuracy, precision, recovery, matrix effect stability and back conversion experiment.

Selectivity

Method selectivity was evaluated using eight independent lots of human K₂EDTA plasma, including one lipemic and one hemolyzed plasma lot. Blank plasma samples from each lot were analyzed and compared with corresponding plasma samples spiked at the lower limit of quantification (LLOQ) together with the internal standard.

No significant endogenous interference was observed at the retention times corresponding to tafamidis or the internal standard in any of the plasma lots evaluated. Representative chromatograms of blank plasma and LLOQ samples containing the internal standard are presented in Fig.1 and Fig. 2, respectively. The absence of interfering peaks confirmed the high selectivity of the proposed analytical method for the determination of tafamidis in human plasma.

**Fig. 1: Representative chromatogram of blank plasma for Tafamidis.**

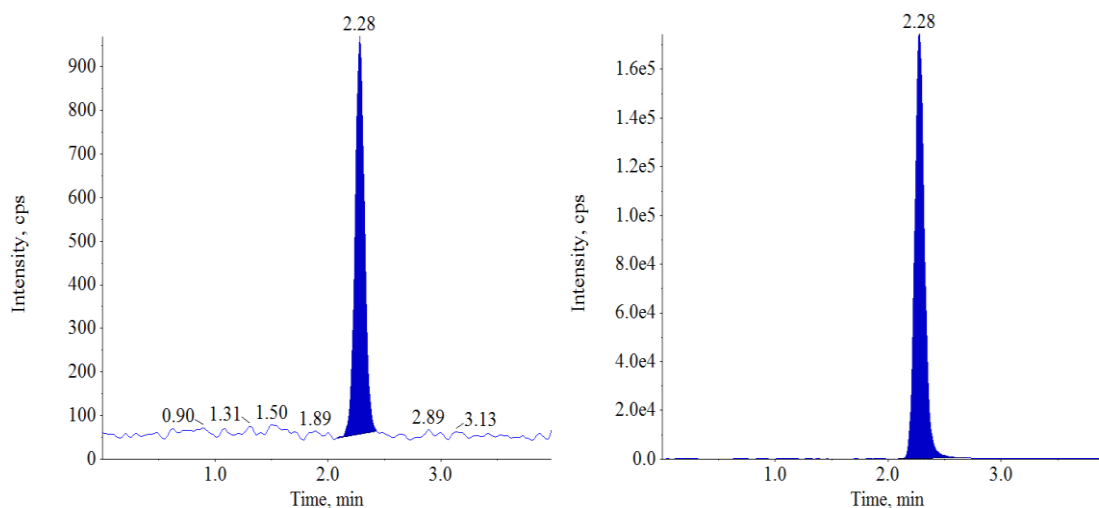


Fig. 2: Representative chromatogram of lower limit of Quantification with IS for Tafamidis.

Linearity and Sensitivity

Calibration curves were established using eight non-zero calibration standards covering a concentration range of 10.058–10,058.241 ng/mL. Linearity was assessed by plotting the analyte-to-internal standard peak area ratio against the nominal concentration of tafamidis and applying weighted linear regression analysis.

Excellent linearity was achieved throughout the validated concentration range, with correlation coefficients (R^2) consistently exceeding 0.99 for all analytical batches. (Fig.3) The calibration data demonstrated reliable quantification across the entire concentration range investigated, confirming the suitability of the method for pharmacokinetic and bioequivalence applications.

The concentrations of the calibration standards were back calculated to assess the accuracy of each calibration point. The accuracy of tafamidis at the calibration points ranged from 98.4% to 102.1%.

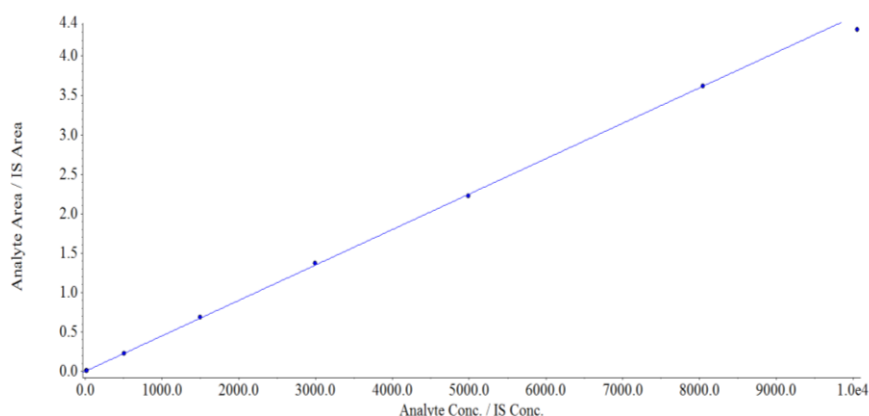


Fig. 3: Representative calibration curve for Tafamidis.

Sensitivity and Linearity

The developed method demonstrated excellent sensitivity for the quantification of tafamidis in human plasma. The lower limit of quantification (LLOQ) was established at 10.058 ng/mL, with precision and accuracy ranging from

3.44% to 5.76% and 97.90% to 101.02%, respectively. In addition, the limit of detection (LOD) was determined to be 2.514 ng/mL, corresponding to a signal-to-noise ratio greater than 36.113.

The high signal-to-noise ratio observed at the LLOQ indicates the robustness and sensitivity of the analytical method. These findings suggest that further reduction of the quantification limit or the use of smaller plasma volumes may be feasible without compromising analytical performance. Consequently, the method may be particularly advantageous in studies where sample volume is limited, such as paediatric or special patient populations.

Precision and Accuracy

The intra-day and inter-day precision and accuracy of the method were evaluated by analysing six replicates ($n = 6$) of quality control samples at four concentration levels: lower limit of quantification (LLOQ), low-quality control (LQC), medium-quality control (MQC), and high-quality control (HQC). Three precision and accuracy batches were prepared using freshly spiked samples, while an additional three batches were prepared from bulk-spiked plasma and analysed on three separate days. The results are summarized in Table II.

Across all concentration levels, intra- and inter-day precision values were within 10.3%, demonstrating excellent reproducibility of the assay. Accuracy values ranged from 90.88% to 107.07% of nominal concentrations, indicating satisfactory agreement between measured and theoretical values. Accuracy was calculated as $[(\text{mean observed concentration})/(\text{nominal concentration})] \times 100$, while precision was expressed as the coefficient of variation (%CV).

The obtained results complied with regulatory acceptance criteria and confirmed the reliability of the method for routine quantitative analysis of tafamidis in human plasma.

Table II: Accuracy and precision of analysis in the quality-control tafamidis samples.

QC sample & conc (ng/ml).	Freshly spiked				Bulk spiked			
	Intra-day		Inter-day		Intra-day		Inter-day	
	Range of Accuracy (%)	Range of % CV	Average Accuracy (%)	Average % CV	Range of Accuracy (%)	Range of % CV	Average Accuracy (%)	Average % CV
LLOQ (10.18)	92.23 to 96.50	4.55 to 5.79	94.04	5.28	93.17 to 105.42	2.18 to 4.77	100.27	6.41
LQC (27.37)	99.30 to 105.54	1.46 to 3.73	103.20	3.77	99.82 to 102.42	1.34 to 2.69	100.82	2.36
MQC (3564.39)	97.94 to 105.58	1.50 to 2.34	102.07	3.71	100.48 to 102.42	0.39 to 1.14	101.63	1.14
HQC (7748.66)	96.97 to 102.35	0.65 to 1.67	99.12	2.75	97.69 to 99.29	0.70 to 1.38	98.54	1.18

Matrix Factor

Matrix effects were evaluated using plasma samples obtained from eight independent human sources, including one haemolyzed and one lipemic plasma lot. Blank plasma samples were extracted and subsequently spiked with tafamidis and internal standard at LQC and HQC levels to generate post-extraction samples representing the presence of matrix components. Corresponding aqueous samples containing equivalent concentrations of analyte and internal standard were prepared in reagent blank and served as matrix-free controls.

The matrix factor for tafamidis ranged from 93.57% to 104.24% at the LQC level and from 105.38% to 107.85% at the HQC level. The coefficient of variation across all plasma lots remained below 15%, meeting regulatory requirements.

As shown in Table III, the mean matrix factors were 96.5% and 106.3% at LQC and HQC levels, respectively, with corresponding %CV values of 3.9% and 0.9%. These findings indicate that endogenous plasma constituents had minimal influence on ionization efficiency, confirming the absence of significant matrix effects and demonstrating the robustness of the proposed analytical method.

Table III: Matrix effects for analytes in eight different lots of human plasma.

Analyte	LQC				HQC			
	Average Area in absence of matrix	Average area in presence of matrix	Matrix factor for analyte	% CV	Average area in absence of matrix	Average area in presence of matrix	Matrix factor for analyte	% CV
Tafamidis	38096.5	36775.4	96.5	3.9	5257464.3	5590173.6	106.3	0.9

% CV $\leq$ 15 is the acceptable limit.

Dilution Integrity

Dilution integrity was assessed by diluting samples exceeding the upper limit of quantification with blank human plasma at dilution factors of two-fold (2T) and four-fold (4T). Six replicate samples were processed and analyzed together with freshly prepared calibration standards.

The method successfully quantified diluted samples up to a concentration of 15,497.33 ng/mL. Precision values for the 2T and 4T dilutions were 0.63% and 1.96%, respectively, while corresponding accuracy values were 95.55% and 96.28%. These results demonstrate that samples with concentrations above the validated calibration range can be accurately quantified following dilution without compromising analytical performance.

Carryover Assessment

Carryover was evaluated to verify the effectiveness of the needle and injection port washing procedure. The assessment sequence consisted of injections of LLOQ sample, blank plasma, upper limit of quantification (ULOQ) sample, followed by two consecutive blank plasma injections.

No detectable analyte carryover was observed in any of the blank samples following ULOQ injections. These results confirm the adequacy of the washing procedure and indicate that carryover does not affect the accuracy or reliability of the assay.

Stability Studies

Comprehensive stability assessments were performed to evaluate the stability of tafamidis under various storage and processing conditions. Both aqueous and plasma-based stability studies were conducted in accordance with regulatory recommendations.

Stability in Aqueous Solutions

Short-Term Stock Solution Stability (STSS)

Stock solutions of tafamidis and the internal standard were stored at approximately 25°C and evaluated after 24 h. LLOQ and ULOQ samples prepared from the stored stock solutions were compared with freshly prepared reference samples. The observed stability values ranged from 99.40% to 99.97%, demonstrating that the stock solutions remained stable for at least 24 h at room temperature (Table IV).

Long-Term Stock Solution Stability (LTSS)

Long-term stability was evaluated using stock solutions stored at 2–8°C for 34 days. Stability values of 101.18% and 103.38% were obtained for LLOQ and ULOQ concentrations, respectively. These results indicate that tafamidis stock solutions remain stable for at least 34 days under refrigerated storage conditions (Table IV).

Table IV: Stability studies of aqueous stock solutions of Tafamidis.

Stability check	Concentration	Average peak area		% Stability
		Stored solution ^a	Fresh solution ^a	
STSS (24 h)	LLOQ	12189.000	12316.000	99.40
	ULOQ	3444990.000	3461071.833	99.97
LTSS (34 days)	LLOQ	12231.833	12091.833	101.18
	ULOQ	3762655.500	3640561.667	103.38

^an = 6

Stability in Human Plasma

Bench-Top Stability

Bench-top stability was assessed by allowing frozen plasma samples at LQC and HQC concentrations to thaw on wet ice for 6 h. Stability values of 95.91% and 97.83% were obtained for LQC and HQC samples, respectively, indicating that tafamidis remained stable during routine sample handling procedures (Table V).

Freeze–Thaw Stability

Following four freeze–thaw cycles, tafamidis exhibited stability values of 97.69% at LQC and 98.14% at HQC. These results demonstrate that repeated freezing and thawing did not significantly affect analyte stability (Table V).

In-Autosampler Stability

Processed samples stored in the autosampler at 10°C for 50 h remained stable, with stability values of 96.24% and 96.14% at LQC and HQC concentrations, respectively (Table V).

Wet Extract Stability

Wet extract stability was evaluated after storage of extracted samples at 25°C. Stability values of 97.13% and 99.59% were obtained for LQC and HQC samples, respectively, confirming analyte stability throughout the sample processing period (Table V).

Overall, all stability assessments satisfied the predefined acceptance criteria of 85–115%, demonstrating that tafamidis remained stable under all tested storage and handling conditions. The results of the stability evaluations are presented in Table V.

Table V: Stability studies of tafamidis in human plasma.

Stability test	Samples	Average concentration		% Stability
		Stored sample (ng/ml)	Fresh Sample ^a (ng/ml)	
Bench top (6.0 h)	LQC	27.010	28.567	95.91
	HQC	7391.076	7545.646	97.83
Freeze thaw (4 cycles)	LQC	27.511	28.567	97.69
	HQC	7414.532	7545.646	98.14
In-autosampler (50 h)	LQC	27.102	28.567	96.24
	HQC	7263.348	7545.646	96.14
Wet extract (5 h)	LQC	27.352	28.567	97.13
	HQC	7524.278	7545.646	99.59

^an = 6

Extended Precision and Accuracy

To further demonstrate method reproducibility, an extended precision and accuracy experiment was conducted. One complete calibration curve and 36 sets of quality control samples at LQC, MQC, and HQC levels were processed and analysed within a single analytical run, resulting in a total of 118 samples.

The method demonstrated excellent reproducibility, with precision values of 1.79%, 0.98%, and 0.85% for LQC, MQC, and HQC samples, respectively. Corresponding accuracy values were 99.77%, 100.88%, and 97.27% (Table VI).

These results confirm the robustness of the method for high-throughput bioanalytical applications involving large sample sets.

Table VI: Extended precision and accuracy of tafamidis.

Nominal conc. (ng/mL)	LQC 27.374		MQC 3564.386			HQC 7748.664		
Mean ^a calculated conc. (ng/mL)	Accuracy (%)	% CV	Mean ^a calculated conc. (ng/mL)	Accuracy (%)	% CV	Mean ^a calculated conc. (ng/mL)	Accuracy (%)	% CV
27.312	99.77	1.79	3595.616	100.88	0.98	7536.870	97.27	0.85

^an=36

Metabolite Back-Conversion Assessment

A dedicated back-conversion study was conducted to evaluate potential interconversion between tafamidis and its acyl glucuronide metabolite during sample handling and analysis. Experiments were performed using concentrations corresponding to the reported C_{max} values following administration of 20 mg and 61 mg doses. Samples containing either tafamidis or tafamidis acyl glucuronide were stored at -70°C, thawed under controlled conditions, processed according to the validated extraction procedure, and analyzed together with freshly prepared reference samples. The extent of metabolite-to-parent conversion was assessed by quantifying the tafamidis response observed in metabolite-containing samples. The observed back-conversion remained well below the predefined acceptance criterion, with the mean analyte response in metabolite samples remaining less than 20% of the average LLOQ response. These findings confirm the effectiveness of the citrate-buffer stabilization strategy and demonstrate that the developed method accurately quantifies tafamidis without significant interference arising from metabolite conversion.

CONCLUSION

A rapid, sensitive, and reliable LC-MS/MS method was successfully developed and validated for the quantitative determination of tafamidis in human plasma. The method demonstrated excellent selectivity, linearity, precision, accuracy, extraction recovery, and stability across the validated concentration range of 10–10,000 ng/mL.

The liquid-liquid extraction procedure provided efficient and reproducible analyte recovery with negligible matrix interference. Furthermore, appropriate stabilization strategies were implemented to minimize potential interconversion between tafamidis and its acyl glucuronide metabolite during sample collection and processing. The use of an isotopically labelled internal standard, Tafamidis-13C6, enhanced assay reliability and complied with current regulatory recommendations for bioanalytical method development.

The method achieved a lower limit of quantification of 10 ng/mL using only 100 μ L of human plasma, demonstrating excellent analytical sensitivity. Owing to its high signal-to-noise ratio, the method may be adaptable to applications requiring lower sample volumes or lower quantification limits.

Overall, the validated method is robust, reproducible, and suitable for high-throughput analysis of clinical samples. It can be effectively applied in pharmacokinetic, bioavailability, bioequivalence, and therapeutic drug monitoring studies. The successful application of this method in a human bioequivalence study further confirms its suitability for routine bioanalytical investigations involving tafamidis.

STATEMENT OF ETHICS

Blood was collected from human volunteer as per the protocol approved by Ethics committee.

COMPETING INTERESTS

All authors hereby declare that no competing of interests is associated with the publication of this manuscript.

AUTHORS' CONTRIBUTIONS

All authors have equal contribution in this work.

FUNDING SOURCES

No external funding or other financial support was received for this study.

ACKNOWLEDGEMENT

The authors thank the management of Norwich Clinical Services for providing the opportunity to complete the project.

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