Asian Journal of Chemistry; Vol. 37, No. 8 (2025), 1949-1958

ASIAN JOURNAL OF CHEMISTRY

https://doi.org/10.14233/ajchem.2025.33582

A Highly Sensitive Green Analytical UHPLC-MS/MS Method for the Quantification of Trace Level of N-Nitroso Meglumine Impurity in Tafamidis Meglumine Applying the Quality by Design

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Received: 6 March 2025; *Accepted*: 7 July 2025; *Published online*: 31 July 2025; AJC-22073

Tafamidis meglumine is utilized for managing transthyretin amyloidosis (ATTR amyloidosis), a condition arising from improperly folded transthyretin proteins that impact the cardiac and neurological systems. A byproduct identified as N-nitroso megulamine (NNM), classified as a nitroso amine, may exist throughout the manufacturing procedure. To meet the requirements of green analytical principles, NNM quantification was achieved *via* UHPLC utilizing an Luna column (4.6 mm × 150 mm ID, 3.0 µm particle size) at a flow rate of 0.8 mL/min in isocratic mode. The central composite design experimental paradigm was adopted to improve the analytical parameters using design expert statistical software, with statistical significance quantified at *p*-values < 0.05%. The elution methodology included the ionization process utilizing heated ESI in positive ion mode, with identification and quantification executed in MRM mode, transitioning at *m/z* 223.20 to 59.10. A high degree of linearity, indicated by $r^2 > 0.990$, was established from the quantification limit (0.25 µg/mL) to 150% (1.893 µg/mL) of NNM. The validation procedure was carried out in accordance with ICH Q2 guidelines and addressed the parameters of system suitability, accuracy, linearity, detection limit, quantitation limit, specificity and precision. Recovery rates are varied between 99% and 128%. Green assessment tools such as Analytical Eco-Scale, GAPI and AGREE endorse the sustainability of the proposed method.

Keywords: Tafamidis meglumine, N-Nitro megulamine, AQbD, Green analytical chemistry, UHPLC-MS/MS.

INTRODUCTION

Tafamidis and meglumine are primarily recommended for transthyretin amyloidosis (ATTR), including both wild-type and inherited ATTR cardiomyopathy, as well as familial amyloid polyneuropathy (FAP). Tafamidis (Fig. 1a) exerts its action by binding with thyroxine (T4) binding sites of transthyretin (TTR). Tafamidis is an organic compound with the molecular formula of $C_{14}H_9Cl_2NO_3$, while meglumine (Fig. 1b), on the other hand, has the molecular formula of $C_7H_{17}NO_5$. The approximate molecular weight is approximately 441.23 Da. The IUPAC nomenclature of tafamidis meglumine is 2-[3,5-dichloro-4-(2-(3,5-dichlorophenyl)hydrazineylidene)methyl-phenoxy]-N-methyl-3-phenylpropanamide meglumine. It is soluble in DMSO but slightly soluble in water [1,2]. This type of sugar is practically the most widespread and is found in the form of chemical crystals in powdered form.

Usually, tafamidis meglumine is available in the form of a solid dosage form (tablets) and it is administered once a day, either 20 mg or 60 mg [3,4]. Tafamidis meglumine, when given orally, is readily absorbed into the body system. The time taken for the drug to reach the maximum plasma concentration (T_{max}) is 2 to 4 h after administration. It is mostly involved in the pathways of liver through glucuronidation [5,6]. N-nitroso meglumine is characterized by the presence of a nitroso group that is directly bonded to meglumine (Fig. 1c). Meglumine, on its part, has the molecular formula $C_7H_{17}NO_5$, while N-nitroso meglumine contains a nitroso group (–NO). It is therefore concerned with the health implications of nitroso compounds and the meglumine nitroso if not well handled.

N-Nitroso amines are a group of chemical compounds containing nitroso amines known to be carcinogenic and have been studied in various animal species with evidence of possible human carcinogenicity [7]. International regulatory agencies,

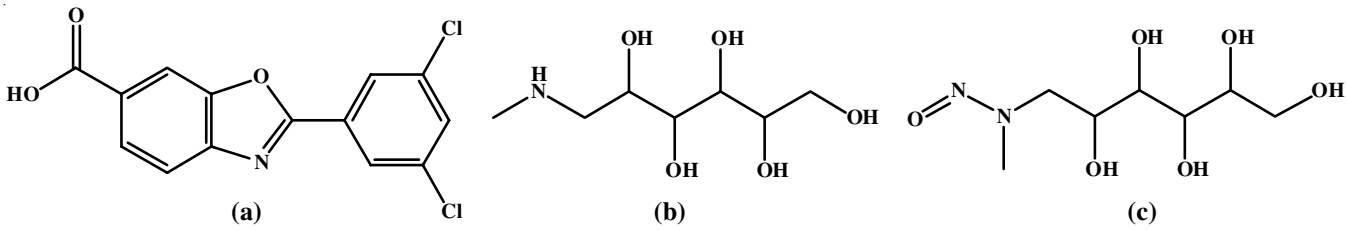

Fig. 1. Chemical structures of tafamidis (a) and meglumine (b) and N-nitroso meglumine (NNM) (c)

including the USFDA, EMA and the WHO, have taken steps regarding this issue. Many drugs have been withdrawn as soon as nitrosamine contaminants are identified. Policies have been developed to demarcate acceptable limits and the procedures for identifying nitrosamines in drugs. New controls, or increased controls for manufacturers to evaluate their processes and risks pertaining to nitrosamine, have been established [8,9]. Sophisticated methods of analysis involving GC-MS, LC-MS, or any other species of chromatography are used for screening and quantitative determines of nitrosamines [10,11].

The good agricultural practices (GAP) and quality by design (QbD) are the two methodologies that help understand the factors, which can further improve the innovation and validation of analytical methods. Green analytical chemistry (GAC) aims to minimize the grievances of analyzed sample analysis, such as the wastage of toxic reagents, excessive generation of wastes and high consumption of energy in analytical processes. Some of the aspects are the elimination of hazardous reagents and wastes, energy, use of renewable resources, design for degradation and safety in analysis [12,13]. Overall, implementing GAP and QbD in method development enable the attainment of sustainable method development, improvement in efficiencies and the minimization of waste, the establishment of sound and reliable methodologies and covering life cycle management strategies [14-16].

Reports have been made about finding N-nitroso meglumine impurity in tafamidis meglumine. Long-term exposure to higher-than-normal levels is known to increase the risk of cancer development and nitrosamine impurities are known to be probable human carcinogens. To ensure safety, it is crucial that the levels of such impurities in pharmaceutical products, including tafamidis meglumine, be monitored carefully. From the literature review, no published literature has been available on the method for identification and quantification of N-nitroso megulamine (NNM) in tafamidis meglumine. The aims of this work were to develop and validate the highly sensitive UHPLC-MS/MS method for the quantification of NNM at an acceptable intake of 1500 ng/day in tafamidis meglumine API as per the reported limit by European Medicines Agency (EMA) and Health Canada [17-20].

EXPERIMENTAL

Analytically pure N-nitroso meglumine (99.84%) and tafamidis meglumine (99.89%) were procured from Sigma-Aldrich, USA while analytical grade formic acid, ethanol and ammonium formate was acquired from Thermo Scientific, India. Milli-Q Water was sourced from Supor™ Prime from Cytiva Pvt. Ltd., USA.

Analytical instruments, columns and software: This study was conducted using Waters UHPLC-MS/MS 2695 instrument, featuring column heater, an auto-injector, binary pumps and a sample cooler. For managing the mass spectrometry data, the software MassLynx was employed. The analysis was performed using a Luna Omega column with (4.6 mm × 150 mm ID) 3 µm; a pH meter from Thermo-Scientific was used to measure the buffer pH. The weighing process involved standard and sample measurements with an analytical balance from Radwag and a Micro Balance from Sartorius. A Milli-Q water from Supor Prime was installed. The sample and standard were subjected to sonication using an PCI Analytics, India. Moreover, a pipette was utilized alongside the micropipette, a product from Eppendorf.

Preparation of mobile phase and diluent: The optimized mobile phase consisted as 10 mM ammonium formate buffer (pH 6.0 adjusted with the formic acid) and ethanol in the ratio of 20:80 v/v. Filtered the mobile phase through vaccum filtration through 0.22 µ PVDF filters. The optimized diluent consists of 10 mM ammonium formate buffer and ethanol in the ratio of 20:80 v/v.

Preparation of analytical solutions

Preparation of standard solution: The standard solution for N-nitroso meglumine (NNM) was prepared with respect to the concentration of 0.015 µg/mL in diluent.

Preparation of sample solution: To prepare the sample solution, weighed and transferred the sample equivalent to 10 mg tafamidis meglumine into a 15 mL polypropylene tube, added 2 mL of water and turn to sonicate the sample until it dissolves completely and then added 8 mL of ethanol. Vortex and mixed well, 0 22 µm pore size PVDF syringe filter was used to filter the sample and collected the filtrate in the lower receiving tube to make the final concentration of 1000 ppm.

Analytical method validation: The optimized UPLC-MS/MS method was validated by assessing various validation parameters like system suitability or system precision, specificity, DL, QL, linearity, accuracy and method precision according to ICH Q2 (R1) guidelines.

System suitability or system precision: The optimized UPLC-MS/MS was validated for system by injecting the six replicate injections of standard solution in accordance with the analytical procedure. The RSD for the NNM impurity ion counts of the standard solution should not exceed 15.0%.

Specificity: The optimized UPLC-MS/MS was validated with the specificity by injecting the blank (diluent), standard solution, sample and NNM impurity-spiked sample prepared at 100% specifications limit. It is pertinent to identify there should not be any interference observed from the blank and sample at the elution pertaining to the target NNM impurity peak.

Detection limit (DL) and quantification limit (QL) level: In this optimized method, the DL and QL was calculated by visual examination. Prepared the DL and QL level correspond to 10% (concentration of 0.0015 ppm) and 20% (concentration of 0.003 ppm) of the standard concentration. The precision for QL-level standard solution has been injected into the instrument for six replicates at that level. The peaks of interest should be detectable at the concentration that aligns with the DL level. The RSD for the ion counts of the NNM impurity peak derived from the six replicate injections conducted at the QL level precision should not more than 15.0%.

Linearity: The linearity was determined for the proposed method by injecting the NNM impurity at six different concentrations of QL, 50%, 80%, 100%, 120% and 150% as per the pre-scribed standard concentrations of 0.003 ppm, 0.0075 ppm, 0.012 ppm, 0.015 ppm, 0.018 ppm and 0.0225 ppm into the LC-MS/MS. The determination coefficient (r^2) for the NNM impurity must not below 0.98.

Method precision: The optimized UHPLC-MS/MS was validated with the precision method by injecting the six individual sample preparations spiked with NNM impurity at the standard concentration (100% level at 0.015 ppm). The %RSD for the NNM impurity levels across the six spiked sample preparations should not more than 15.0.

Accuracy: The accuracy of method was determined for the proposed method by injecting a sample with NNM impurity spiked at different concentrations of QL (0.003 ppm), 100% (0.015 ppm) and 150% (0.018 ppm). It is recommended that the recovery rate for NNM impurity at every concentration should be between 70.0% and 140.0% and the RSD for percentage recovery at each level should not exceed 15.0%.

Preparation of spiked sample solution: The spiked sample solutions were prepared in triplicate for QL. 100% and 150% level and subsequently injected into the LC-MS. Approximately 10.0 mg of the sample was accurately weighed and placed into each 15 mL polypropylene tube. Then, 2.0 mL of water was added, followed by thorough vortexing and sonication until the sample fully dissolved, after which added 0.20 mL for QL, 1.0 mL for 100% and 1.5 mL for 150% of standard solution and makeup to the volume to 15 mL of ethanol. This mixture was vortexed and mixed thoroughly. The resulting sample was filtered using a 0.22 µm PVDF syringe filter and subsequently injected into the LC-MS. The % recovery NNM impurity at QL, 100% and 150% was calculated and the %RSD at each level were determined.

Quality by design (QbD) principle in to assess the robustness of the optimized method: The design expert (DE) software has been used extensively to study the robustness of the proposed and optmized the UHPLC-MS/MS using QbD principles. In this research, the optimization of essential method parameters like column temperature, buffer pH and flow rate is done with the help of a design expert to design accurate and precise methods. These procedures are subjected to validation with respect to parameters such as linearity, accuracy and precision to determine their fitness for deployment in quality control (QC) activities. In addition, the software simplifies method development and improvement of method variables, definition of realistic design spaces and compliance with validation guidelines provided by ICH Q2 (R1) [21].

Green analytical principles (GAP): GAP tools like AGREE, GAPI and eco-scale are essential for assessing the environmental impacts linked to analytical methodologies. These tools play a crucial role in assessing the ecological impacts of approaches employed within the pharmaceutical sector. The advancement of concepts under green analytical chemistry (GAC) has, consequently, led to the development of various tools utilized for evaluating the environmental repercussions of newly devised analytical techniques. The GAPI, AGREE and analytical eco-scale have emerged as widely recognized metrics. These indices incorporate a performance spectrum represented by red, yellow and green indicators to assess the environmental implications of different methodologies, which are scientifically validated and extensively implemented. When comparing the proposed methodologies with the standards set forth by analytical eco-scale, green analytical procedure index (GAPI) and analytical greeness (AGREE), it is noteworthy that the GAPI visualization indicates two critical areas in red, specifically off-line sampling and transportation; these areas correspond to zone 3 in the context of the AGREE visualization. The proposed methodology exclusively utilizes water and ethanol, thereby avoiding the use of organic solvents. The AGREE visualization reflects a comprehensive assessment denoting high ecological compatibility and minimal effects associated with the proposed approaches. The analytical eco-scale serves as a partial quantitative evaluation of analytical methods to greenness. The analytical eco-scale specifically designed for ranking the environmental impact of methodologies; it allocates penalty points based on various parameters, including consumption of energy, different kinds of solvents used, sample processing volume and the generation of analytical solvent waste. The analytical eco-scale score was calculated by subtracting the penalty points from 100, where the analytical score considered as excellent if it exceeding 75 points, it is acceptable if the scores between 75 and 50 and considered it is inadequacy practice if the score is below 50. In conjunction with the analytical eco-scale, GAPI is recognized as a significant instrument for evaluating the eco-friendliness of a methodology, concentrating on six essential factors: the sampling technique employed, the method of sample selection, the sample preparation process, the reagents and solvents utilized, the instruments applied and the types of quantification or qualification methods employed. The adoption of a colour scheme consisting of red, yellow and green shades is utilized to enhance the pictographic representation [22-27].

RESULTS AND DISCUSSION

Optimized LC-MS conditions: The liquid chromatography parameters were optimized to enhance the sensitivity of

the compound under study. This optimization aims to detect the NNM impurity at a lower concentration below the acceptable limit. The sensitivity of the analytes was observed in various solvents like methanol, ethanol and acetonitrile, along with buffer solvents like trifluoroacetic acid (TFA), ammonium acetate and ammonium formate. The best sensitivity was observed using ethanol and ammonium formate (pH 6.0) as mobile phase. The column selection was also assessed to get the proper peak shape and retention of NNM impurity. Columns like Waters Symmetry C_{18} columns, Zorbax C_{18} column and Luna Omega columns were used to assess the selected criteria. The analysis utilizing a Luna® Omega 4.6 mm × 150 mm ID column provided the best sensitivity. The desired sensitivity levels of the method (0.003 ppm for QL of pure NNM in diluent) were attained through separation at a flow rate of 0.8 mL/min with elution of ammonium formate at pH 6.0 and ethanol.

The optimized mobile phase buffer consisting of 10 mM ammonium formate and the pH adjusted to 6.0 with formic acid and the organic constitutes ethanol, functioning in isocratic mode with a 20:80 v/v ratio. The retention and selectivity assessments were carried out on a Luna Omega column with 150 mm × 4.6 mm and a particle size of 3 µm with 0.8 mL/min as mobile phase flow rate and 40 ºC as column temperature and the sample temperature is at 15 ºC. The analytes were characterized utilizing a mass spectrometer; the injection volume was established at 50µl and the overall duration of the analysis was 15 min.

Experimental mass spectrometry procedures were conducted employing a Waters UHPLC-MS/MS apparatus connected to a Q-TOF utilizing electrospray ionization (ESI) as the ionization source operating in negative scan mode. MassLynx software was utilized for data acquisition employing the MRM approach. The operational parameters at the mass source included an interface temperature of 200 ºC, an interface voltage of -1.00 kV, 3.00 L/min is a nebulizing gas flow rate, 220 ºC is a desolvation line temperature, a heat block temperature, 10 L/min a drying gas flow rate, parent ionization occurring at 232 *m/z*, product ionization occurring at 59.10 *m/z*, a dwell time of 100 msec, a collision energy fixed at 22.0 and a total run time of 4.80 min. Nitrogen was utilized as the drying gas (350 ºC, 10 L min^{-1}) and the nebulizing gas (45 psi). High-purity ultrahigh nitrogen was employed as the collision gas. The MRM transition occurred from *m/z* 223.20 to 59.10.

AQbD optimization using surface central composite: For the optimized UHPLC-MS/MS method, the AQbD investigation was carried out for robustness study, the three critical analytical attributes (CQA) are defined *viz*. (i) the pH of the mobile phase buffers was chosen deliberately from the range of 4.0 to 8.0, ensuring meticulous consideration; (ii) Further, the column temperature was maintained accurately in the range of 30 to 50 ºC, since too high or too low temperature may affect the separation of the analytes; (iii) the mobile phase flow rate ranges from 0.6 to 1.0 mL/min.

As mentioned above, the following responses were identified, for example, (i) the retention time of NNM and (ii) the peak asymmetry factor for NNM.

Interestingly, the changes made in the mobile phase buffer pH, column temperature and flow rate, which were implemented during the experiment, were found to have a negligible effect on the identified critical analytical attributes (CQA). The experimental design was also orthogonal with three factors and consisted of the three centre point designs with no blocks. Subsequently, a total of 17 experimental trials were conducted and documented in Table-1 with as much precision as possible.

The investigation was conducted in all 17 trials and the collected data were subjected to thorough analysis. From the analytical results using ANOVA test as shown in Table-2, the P value is significantly below 0.05 for the variables under study, including NNM retention time and NNM peak asymmetry. Such significance further affirms the validity of the model in response to both the variables under analysis. All-factor composite response surface plots including 3D Cube figures, 2D contour figures and 3D contour figures for the first and second

TABLE-1
DESIGN OF EXPERIMENTS (DoEs) TRAILS AND UHPLC-MS RUNS WITH RESULTS

Std	Run	Factor 1	Factor 2	Factor 3	Response 1	Response 2
		A: Buffer pH	B: Flow fate (min)	C: Column temperature (ºC)	NNM retention time (min)	NNM peak asymmetry
16	1	6.0	0.8	40	3.82	1.07
3	2	4.0	1	30	3.45	1.22
4	3	8.0	1	30	3.59	1.22
1	4	4.0	0.6	30	4.25	1.22
10	5	9.36359	0.8	40	3.96	1.11
12	6	6.0	1.13636	40	3.26	1.11
2	7	8.0	0.6	30	4.32	1.22
14	8	6.0	0.8	56.8179	3.91	1.06
7	9	4.0	1	50	3.49	1.01
13	10	6.0	0.8	23.1821	3.85	1.31
9	11	2.63641	0.8	40	3.85	1.06
8	12	8.0	1	50	3.56	1.09
17	13	6.0	0.8	40	3.85	1.06
15	14	6.0	0.8	40	3.87	1.04
5	15	4.0	0.6	50	4.21	1.05
6	16	8.0	0.6	50	4.35	1.10
11	17	6.0	0.463641	40	4.51	1.11

TABLE-2
ANOVA TABLE FOR RESPONSE FACTOR 1 AND RESPONSE FACTOR 2

Response	Source	Sum of squares	df	Mean square	Adjusted R^2	Predicted R^2	F-value	p-value	
NNM retention time	Linear *vs.* mean	1.98	3	0.6588	0.9917	0.9896	641.23	< 0.0001	Suggested
NNM peak asymmetry	Quadratic *vs.* 2FI	0.0253	3	0.0084	0.9891	0.9788	111.18	< 0.0001	Suggested

responses are illustrated in Figs. 2 and 3. From the sources of variation analysis, it is established that in the case of Response 1 (NNM retention time), it is sensitive to change in the flow rate while for Response 2 (NNM peak asymmetry), the changes that will have an impact are those facilitated by the column oven temperature. The tests for goodness of fit known as Fit Statistics show that there was a substantial variability between the Respond 1's Predicted R^2 of (0.9896 and Adjusted R^2 of (0.9917) and Respond 2's Predicted R^2 of (0.9788) and Adjusted R^2 of (0.9891). This existing model provides a valuable tool for exploring the design space.

Validation: The optimized UHPLC-MS/MS method was validated following the ICH Q2 (R1) guidelines regarding specificity, system suitability/system precision, method precision, accuracy, linearity, QL and DL.

System precision: The system suitability or system precision was determined by six replicate injections of the N-nitroso meglumine (NNM) standard solution in compliance with USP standards. The %RSD for the six replicate injections pertaining to the NNM ion counts is 2.0 and the average Ion Counts for NNM is 14386.17. which is considerably below the acceptable threshold of 15.0. The suitability criteria for the standard preparation were found to be within acceptable limits.

Specificity: The specificity of the optimized analytical method was determined by injecting the blank (diluent), standard solution and spike sample solution with NNM impurity at the 100% (0.015 ppm) standard concentration. The results also showed that the method had specificity as no interferences were observed at the retention time of NNM. Fig. 4 illustrates the typical ion chromatogram of blank, standard and spiked samples. The retention time of NNM impurity for both the standard and spiked samples are 3.98 and 3.98, respectively.

Linearity: The optimized UHPLC-MS/MS method was assessed with the validation parameter of linearity by injecting NNM standard solutions at QL, 50%, 80%, 100% and 150% of the standard concertation prepared individually. Plotted the calibration curve between peak ion counts against the concentration of NNM. The correlation coefficient for the obtained calibration curve is more than 0.9990, a slope of 11350.5 and a Y-intercept of -43.4868, proving the linearity of method. The corresponding ion count chromatograms characterizing linearity ions are depicted in Fig. 5.

Method precision: The effectiveness of the optimized analytical method validation strategy was assessed through the method precision, where six individual preparations of the NNM impurity spiked sample were examined at the standard

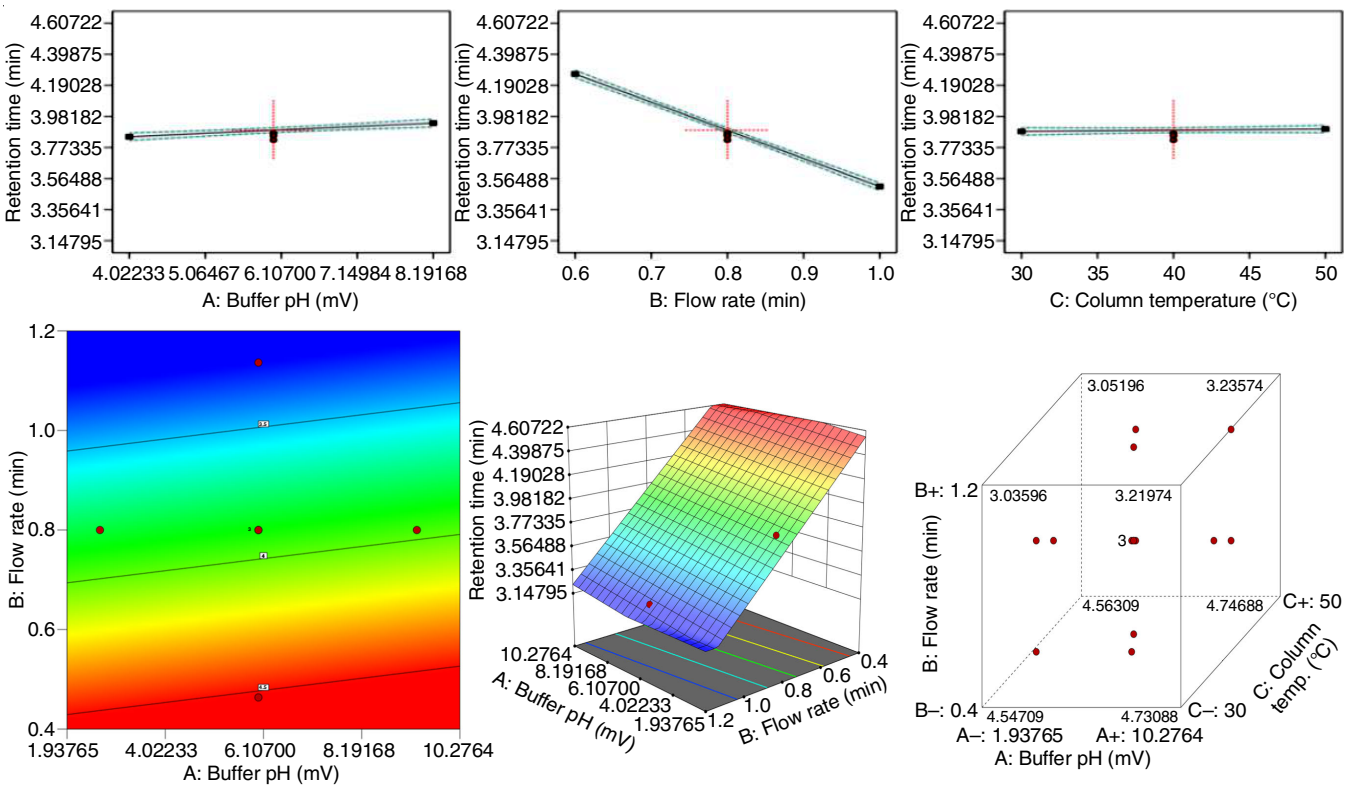

Fig. 2. Evaluation of response factor 1 with all factor coding plot, contour plot, 2D plot, 3D plot and cube plot (NNM retention time)

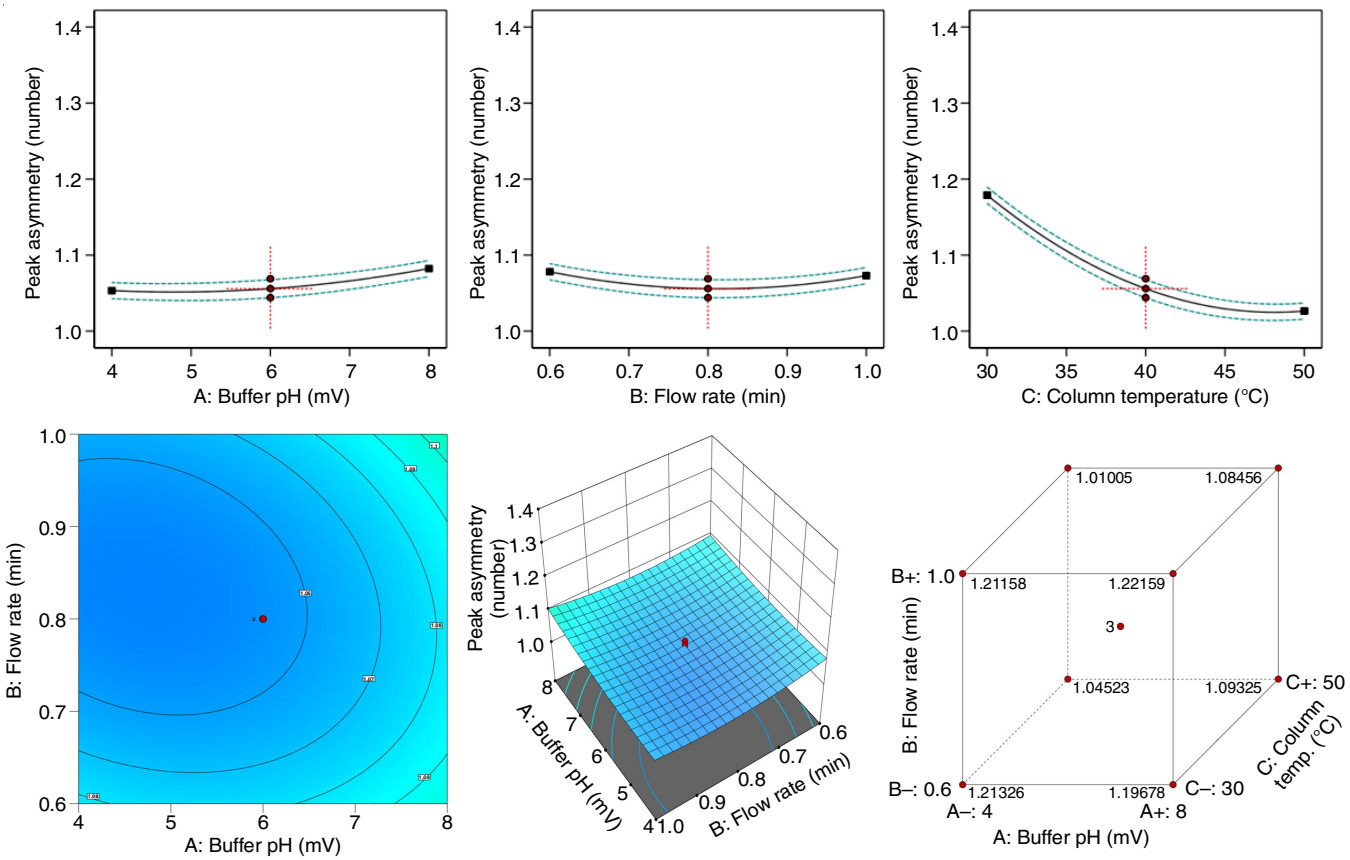

Fig. 3. Evaluation of response factor 2 with all factor coding plot, contour plot, 2D plot, 3D plots and cube plot (NNM asymmetry factor)

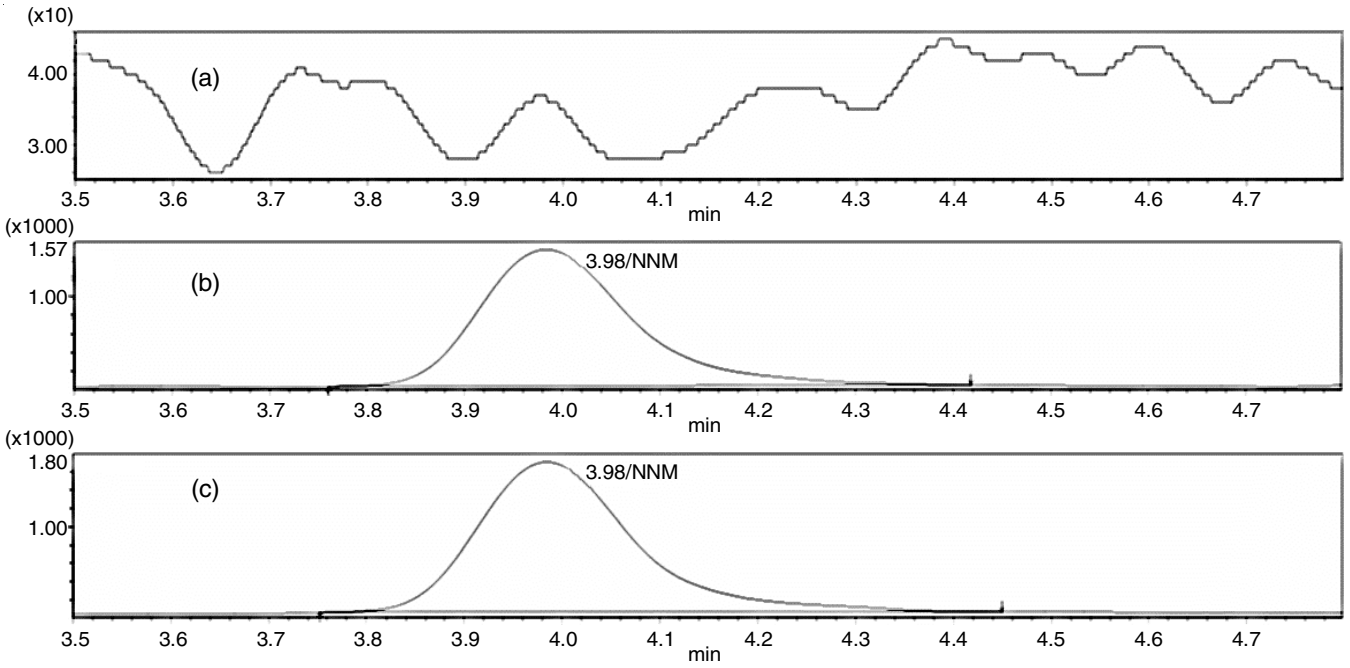

Fig. 4. Typical ion chromatogram of (a) blank, (b) standard and (c) spiked sample

level (0.015 ppm). The RSD values from the six replicate preparations confirm the reliability of method in quantifying NNM using UHPLC-MS/MS is 4.40%, well within the permitted limit of 15.0%.

Accuracy: The optimized analytical method validation was evaluated to assess its accuracy using the standard addition technique. The extraction process was carried out in triplicate at three different levels, namely QL, 100% and 150%; further, the recovery percentages were calculated. The recovery percentages obtained from all the aforementioned samples were compiled and summarized in Table-3. The findings align with the expected pattern of % recoveries, which should fall within

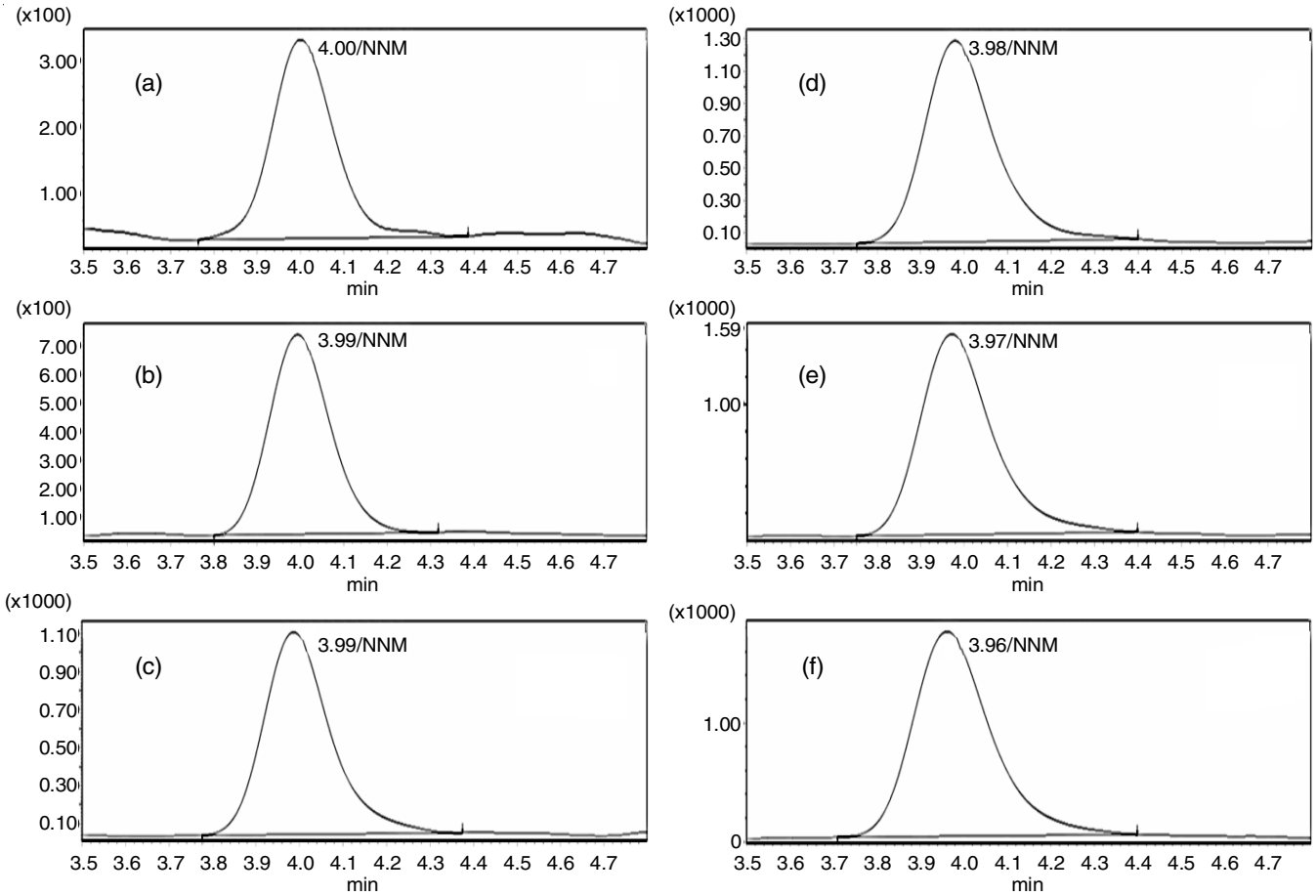

Fig. 5. Typical ion chromatogram of (a) QL level, (b) 50% level, (c) 80% level, (d) 100% level, (e) 120% level and (f) 150% level

TABLE-3
REPRESENTS THE ACCURACY RESULTS FOR NNM IMPURITY

Level & preparations	Impurity added (ppm)	Mean content in as such sample (ppm)	Impurity found in spiked sample (ppm)	Recovery (%)	Mean recovery (%)	RSD (%)
QL-1	0.2511		0.5332	127.36		
QL-2	0.2503	0.2134	0.5462	133.08	127.4	4.4
QL-2	0.2496		0.5173	121.75		
100%-1	1.2393		1.4669	101.15		
100%-2	1.2356	0.2134	1.4979	103.96	105.8	5.4
100%-3	1.2503		1.6166	112.23		
150%-1	1.8607		2.1799	105.69		
150%-2	1.8427	0.2134	2.0479	99.56	101.7	3.4
150%-3	1.8553		2.0669	99.90		

the 70% to 140% range, with the % RSD remaining below 15%. The ion counts chromatograms that demonstrate the accuracy are illustrated in Fig. 6.

DL and QL: The DL and QL denote the minimal quantities of the substance being analyzed. The detection limit (DL) was determined at 10% of the specified threshold, whereas the quantification limit (QL) was established at 20% of the specified threshold. The significant NNM peak is acknowledged at the prescribed concentration for DL, aligning with the defined standards. The % RSD for the ion count associated with NNM impurity is 5.5%, which adheres to the requirement of remaining below 15.0%. Fig. 7 illustrates the representative chromatogram of ion counts at DL and QL levels.

Evaluation of green analytical method: The optimized UHPLC-MS/MS analytical method utilized milli-Q water, ethanol, formic acid and ammonium formate as solvents in the mobile phase, employing ethanol and buffer as diluents instead of toxic chemicals such as methanol or acetonitrile. The column consists of 150 mm × 4.6 mm, 3 µm particle size, with a total runtime of 15 min to analyze two components (one NNM impurity and one tafamidine meglumine), consuming less than 12 mL of ethanol per sample for analysis. For this research, the eco-friendly assessment tools applied to assess environmental impact consisted of GAPI, AGREE and the analytical eco-scale. Table-4 provides a comprehensive overview of the analytical eco-scale results, which evaluates the chemicals or reagents

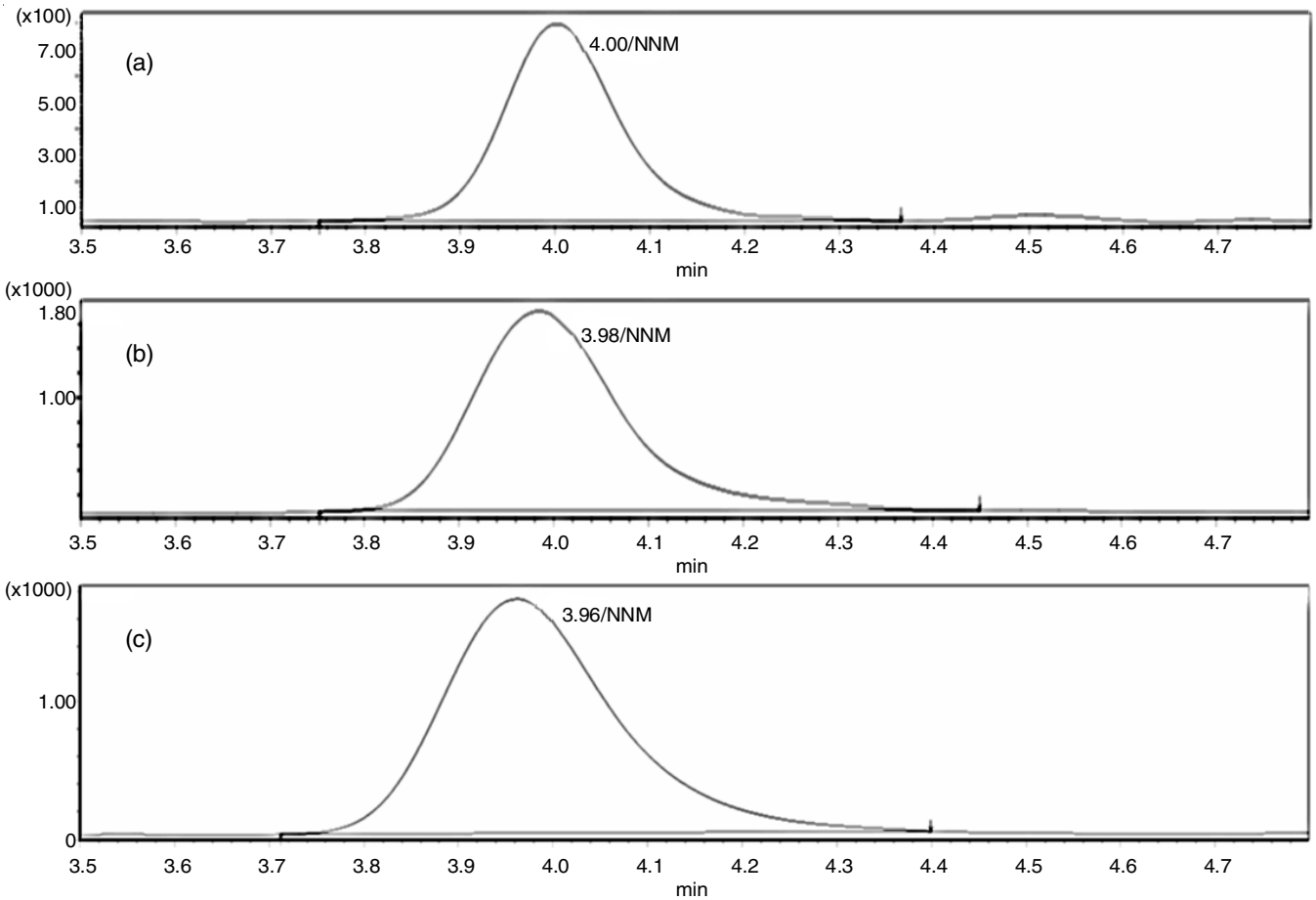

Fig. 6. Typical ion chromatogram of (a) QL level, (b) 100% level, (c) 150% level

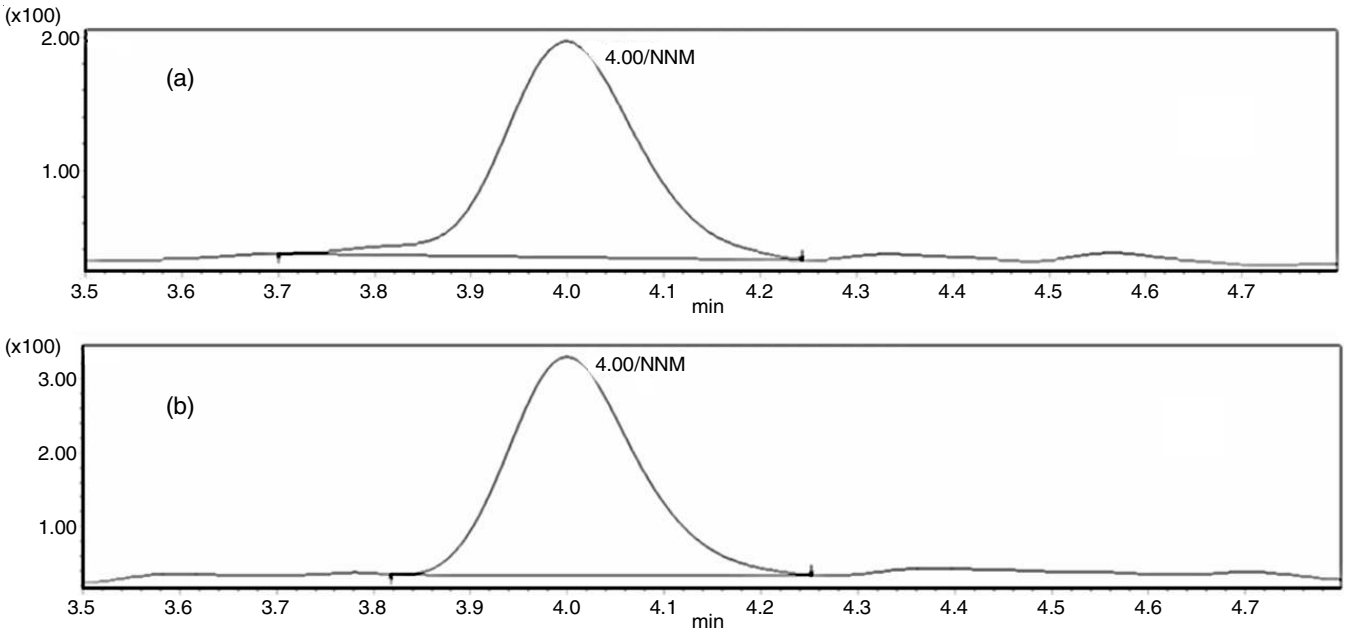

Fig. 7. Typical ion chromatogram of (a) DL level, (b) QL level

employed, the energy consumption of devices, the waste generation and the disposal methods in both the proposed methodology and alternative approaches. The analysis shows that the cumulative penalty for the suggested approach is 12, with the greenness score reaching 88, which is significantly positive.

Fig. 8 provides the visual layout of AGREE and GAPI; the AGREE tool declares the proposed UHPLC-MS/MS indicated a 'greenness', yielding a score of 0.75, which exceeds the minimum passing score of 0.50. Table-5 specifies the applications of the AGREE tool utilized to evaluate the GAP integrated

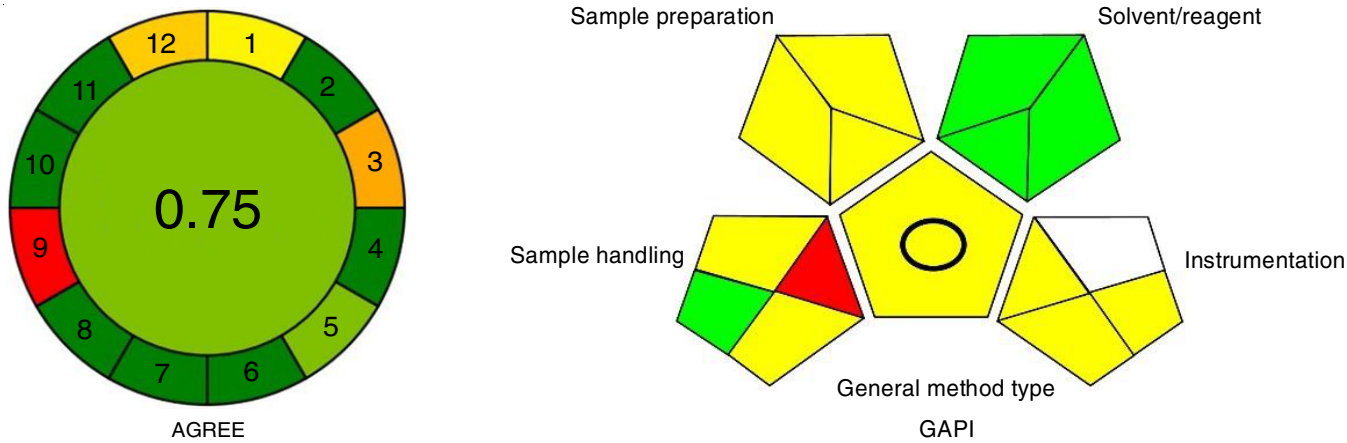

Fig. 8. Pictograms of GAPI and AGREE for the proposed analytical method

TABLE-4
EVALUATING THE PROPOSED GREEN ANALYTICAL METHOD ON UHPLC-MS/MS USING THE ANALYTICAL ECO-SCALE

Name	Penalty points
Chemicals or reagents	
NH_4HCOOH	0
HCOOH	6
CH_3CH_2OH	4
Instruments	
Consumption of energy per sample on UHPLC-MS (1.5 kWh)	2
Waste generation	0
Waste	
Generation of total volume of waste (> 10 mL)	5
Waste treatment	1
Penalty points	12
Total Score (100-Penalty points)	88
Green ness score for the proposed method	Excellent

into the optimized UHPLC-MS/MS method. In Fig. 8, the AGREE visualization of the study emphasizes the sustainability of method as eco-friendly, with the primary concern being the consumption of total energy for UHPLC-MS/MS instrument, highlighted in red colour. Table-6 presents a comprehensive analysis of the GAPI tool's application in assessing the sources of sample, types of method employed, the greenness or toxic usage of reagents and solvents used for sample preparation, the usage of type of instrumentation and number of analytes quantified with the proposed methodology. The GAPI results shows that the methodology is eco-friendly, apart from the sample management component noted in red.

Conclusion

The optimized analytical method for the proposed UHPLC-MS/MS method was validated according to the ICH Q2 (R1) guidelines, the results illustrate that the analytical methodology can effectively quantify N-nitroso meglumine (NNM) at minimum QL level at 0.003 ppm, which is below the acceptable threshold level within the tafamidis meglumine formulation utilizing Luna Omega column with 4.6 mm × 150 mm and 3 µm as stationary phase column. The optimized method was validated with the parameters like the specificity, linearity, accuracy, QL, DL and method. The method is shown to be specific as there is no interference observed at the retention time of the analyte. The correlation coefficient for the calibration

TABLE-5
EVALUATING THE PROPOSED GREEN ANALYTICAL METHOD ON UHPLC-MS/MS USING THE AGREE TOOL

GAC principles	Sample procedure
Type of sample preparation utilized	Off-line analysis
Quantity of Sample Size utilized	1 g
Measurement of samples utilized	At-line
Combining analytical procedures and operations to conserve energy and lowers reagent consumption	3 distinct steps
Selection of automated and compact techniques	Semi-Automatic technique used for the proposed method and it is miniaturized
Utilization of derivatization technique.	No derivatization utilized
Reduce excessive generation of analytical waste	1 g
Analyze number of analytes in single run and samples per hour	2 analytes analyzed per run; 4 samples analyzed per hour
The use of energy should be minimized:	
Technique that requires the highest energy.	UHPLC-MS
Calculate the total energy consumption in kWh.	1.5 kWh
Reagents derived from renewable sources.	All are bio-based reagents used
Toxic substances ought to be removed.	No reagents or solvents are toxic to the environment
Enhance the safety of the operator.	The threats that are not avoided are a. Bio accumulative; b. Highly flammable; c. Explosive

TABLE-6
EVALUATING THE PROPOSED GREEN ANALYTICAL METHOD ON UHPLC-MS/MS USING THE GAPI TOOL

Sample sourcing	
Collection of samples	Off-line
Preservation of samples	Physical or chemical
Transportation of samples	None
Storage of samples	Under normal conditions
Method type and sample preparation	
Extraction utilized	Simple procedure
Extraction scale	Micro-extraction
Toxic solvent or reagent used	Green solvents/reagents used
Additional treatment	No additional treatment
Reagents and solvents	
Reagent/solvent amount	< 10 g used
Adverse health effect	NFPA = 0 to 1.
Adverse safety effect	Highest NFPA flammability = 0 or 1
Instrumentation	
Energy utilized per sample	< =1.5 kWh
Hazardous occupational	Hermetic sealing
Waste generation	1-10 mL (1-10g)
Treatment for waste generation	Degradation, passivation
Quantification/qualification	
Symbol O	The proposed method for the qualification and quantification for NNM impurity in Tafamidis meglumine

curve from QL to 150% is more than 0.98 and the accuracy results were meeting the accepting limits. The AQbD approach was utilized to assess the robustness of the proposed analytical method, employing the ANOVA statistical test, which yielded a p-value < 0.05 which is significant. The green analytical principles assessment study, encompassing AGREE, GAPI and the analytical eco-scale, demonstrates that the proposed method is environmentally sustainable, with analytical eco-scale results surpassing 88. This analytical approach demonstrates significant utility in quality control (QC) laboratories for the detection and quantification of NNM within the tafamidis meglumine formulation.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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