Development and Validation of a Green Stability-Indicating Chromatographic Method for Teneligliptin Hydrobromide Hydrate and Metformin Hydrochloride

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ABSTRACT:

Background: Reverse-phase high-performance liquid chromatography (RP-HPLC) is widely used for simultaneous estimation of pharmaceutical combinations due to its accuracy, precision, and sensitivity. Development of eco-friendly analytical methods using safer solvents is important in green analytical chemistry. Therefore, a simple, cost effective and environmentally benign RP-HPLC method was developed for simultaneous estimation of teneligliptin hydrobromide hydrate (TNLG) and metformin hydrochloride (MET).

Objective: To develop, optimize, and validate a reverse-phase high-performance liquid chromatography (RP-HPLC) method for simultaneous estimation of TNLG and MET using an ethanol–water binary mobile phase in compliance with green analytical chemistry principles and ICH Q2 (R1) guidelines.

Methods: Chromatographic separation was achieved on a Waters Symmetry C18 column (250 × 4.6 mm, 5 µm) with an ethanol–water (30:70, v/v) mobile phase at a flow rate of 1.0 mL/min, UV detection at 254 nm, and an injection volume of 20 µL. Method validation encompassed system suitability, specificity, linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), and robustness. Forced degradation studies were conducted under acid, base, oxidative, thermal, and photolytic conditions.

Results: TNLG and MET eluted at 6.2 ± 0.1 min and 3.5 ± 0.1 min, respectively, with resolution > 2.0. Linearity was established over 10–60 µg/mL for TNLG (R^2 = 0.9998) and 50–300 µg/mL for MET (R^2 = 0.9997). Accuracy (% recovery 98.5–101.5%), precision (%RSD < 2.0%), LOD (TNLG: 0.29 µg/mL; MET: 1.05 µg/mL), and LOQ (TNLG: 0.87 µg/mL; MET: 3.19 µg/mL) complied with ICH acceptance criteria. Stability-indicating capability was confirmed through adequate resolution of degradation products. The AGREE greenness score was 0.78 and GAPI assessment confirmed a predominance of green pictograms.

Conclusion: The developed RP-HPLC method is simple, accurate, precise, robust, stability-indicating, and environmentally benign. Substitution of conventional organic solvents with food-grade ethanol substantially reduces analytical waste toxicity and complies with green analytical chemistry principles. The method is suitable for routine quality control of TNLG–MET fixed-dose combination tablets.

Keywords: RP-HPLC, Green analytical chemistry, Teneligliptin hydrobromide hydrate, Metformin hydrochloride, Stability-indicating method, ICH Q2(R1) validation, Forced degradation

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INTRODUCTION:

Green analytical chemistry (GAC) is a sub-discipline that applies the 12 Principles of Green Chemistry to analytical measurement science. The significance mnemonic to operationalise these principles for analytical laboratories: direct analysis, minimisation of sample preparation, generation of little waste, net reduction of reagent toxicity, information content per measurement, flexibility, in-line monitoring, clean reagents, and economical use of energy.[1]

Conventional HPLC analyses routinely consume 100–1000 mL of acetonitrile or methanol per analytical session, generating toxic waste requiring special disposal. Replacing these solvents with ethanol (food-grade, biodegradable, renewable) dramatically reduces waste toxicity, operator exposure, and disposal costs while maintaining analytical performance.[2]

A green, stability-indicating RP-HPLC method using ethanol–water mobile phase is absent from the literature, creating a gap this study addresses[3].

Drug Profiles:

Teneligliptin Hydrobromide Hydrate (TNLG)

Teneligliptin hydrobromide hydrate is a third-generation, oral, once-daily DPP-4 inhibitor approved in Japan (2012) and India (2015) for the treatment of T2DM. Its molecular formula $C_{22}H_{30}N_6OS \cdot HBr \cdot H_2O$; molecular weight 528.50 g/mol; melting point 246–252°C.[4]

Physicochemical Properties: TNLG is a white to pale-yellow crystalline powder, freely soluble in water and methanol, practically insoluble in chloroform. Its pKa is approximately 7.3. Aqueous solubility exceeds 50 mg/mL at pH 7.0, classifying it as a BCS Class I (high solubility, high permeability) drug [5].

Existing Analytical Methods: Reported methods include UV spectrophotometry at 242 nm, RP-HPLC with acetonitrile–phosphate buffer mobile phases, and LC-MS/MS for pharmacokinetic studies None of the published simultaneous methods employ an ethanol–water green mobile phase system.[6]

Metformin Hydrochloride (MET)

Metformin hydrochloride is the first-line oral antidiabetic agent of the biguanide class recommended by virtually all major diabetes guidelines. Its molecular formula $C_4H_{12}ClN_5$; molecular weight 165.62 g/mol; Melting Point 222-226°C[7].

Physicochemical Properties: MET is a white crystalline powder, very soluble in water (>300 mg/mL at 25°C), practically insoluble in acetone and diethyl ether. Its pKa values are 2.8 and 11.5. High aqueous solubility and complete gastrointestinal absorption place it in BCS Class III (high solubility, low permeability).[8]

MATERIALS AND METHODS:

Chemicals and Reagents

Teneligliptin hydrobromide hydrate working standard (purity: 99.8%) was procured from Aalidhra Pharmachem Pvt. Ltd. (Vadodara, Gujarat, India). Metformin hydrochloride working standard (purity: 99.6%) was obtained from Sohan Healthcare Pvt. Ltd. (Pune, Maharashtra, India). Ethanol (HPLC grade, ≥99.9%) and ultrapure water (18.2 MΩ·cm) were used for mobile phase preparation. Hydrochloric acid (HCl, 35–37% w/v, AR grade), sodium hydroxide (NaOH, AR grade), hydrogen peroxide (H_2O_2, 30% w/v), and all other chemicals were of analytical reagent grade. The commercial tablet formulation Tengli-M 20/500 mg (Mankind Pharma Ltd., India) was purchased from a local pharmacy.

Instrumentation

Chromatographic analysis was performed using a Agilent 1100 Series HPLC System (Waters Corporation, Milford, MA, USA) equipped with a quaternary solvent delivery pump, autosampler, column oven, and a 2998 photodiode array (PDA) detector. Data acquisition and processing were performed using Empower 3 Software (Waters Corporation). Weighing was carried out on an analytical balance (Mettler Toledo XPE205DR, ±0.01 mg). pH measurements used a calibrated pH meter (Eutech Instruments pH 700). Ultrasonic degassing of solvents was performed using a bath-type sonicator (PCi Analytics, Mumbai, 40 kHz). A UV-Visible spectrophotometer (Shimadzu UV-1900, Shimadzu Corporation, Kyoto, Japan) was used for λmax determination.

Preparation of Solutions

Mobile Phase Preparation

The mobile phase consisted of ethanol and ultrapure water in a ratio of 30:70 (v/v). The components were mixed and filtered through a 0.45 µm nylon membrane filter (Millipore) under vacuum before use. The mobile phase was degassed by sonication for 15 minutes. The pH was measured to be 6.8 ± 0.2 without adjustment, which is suitable for both analytes[9].

Standard Stock Solutions

Standard stock solution of TNLG: Accurately weighed 10.0 mg of TNLG working standard was transferred to a 10 mL volumetric flask and dissolved in a minimum volume of mobile phase (ethanol and water), then sonicated for 10 minutes and made up to the mark with mobile phase (ethanol and water) to yield a stock concentration of 1000 µg/mL.

Standard stock solution of MET: Similarly, 50.0 mg of MET working standard was dissolved in mobile phase (ethanol and water) volumetric flask to obtain 1000 µg/mL stock solution[10].

Working Standard Solutions

Working solutions were prepared by appropriate serial dilution of respective stock solutions in mobile phase (ethanol and water) to obtain concentration ranges of 10–60 µg/mL for TNLG and 50–300 µg/mL for MET. Combined working standard solutions at the target concentration (TNLG: 40 µg/mL; MET: 200 µg/mL) were prepared to reflect the therapeutic dose ratio (1:10) present in the FDC tablet[11].

Sample Preparation (Tablet Formulation)

Twenty tablets of Tengli-M 20/500 mg were weighed and finely powdered using a mortar and pestle. A tablet powder equivalent to one tablet was accurately weighed, transferred to a 100 mL volumetric flask, and 60 mL of ethanol and water. The mixture was sonicated for 20 minutes to ensure complete dissolution, then cooled to room temperature and made up to volume with ethanol and water. The solution was filtered through a 0.45 µm nylon membrane filter (Millipore). An appropriate aliquot was diluted to obtain TNLG 40 µg/mL and MET 200 µg/mL in the final solution for injection[12].

Chromatographic Method Development

Initial UV spectroscopic screening was performed to determine the wavelength of maximum absorption (λmax) of TNLG and MET individually, prepared in mobile phase. Both analytes exhibited characteristic

UV absorption at 254 nm, confirming this as the optimal detection wavelength for simultaneous quantification using the PDA detector [13].

Method development was carried out systematically by evaluating the influence of mobile phase composition on chromatographic performance. The primary objective was to achieve adequate baseline resolution (Rs > 2.0), acceptable peak symmetry (tailing factor As = 0.9–1.1), suitable retention times within a 10-minute run, and reliable simultaneous quantification of both TNLG and MET reflecting their 1:10 therapeutic dose ratio present in the Tengli-M 20/500 mg FDC tablet formulation. A Waters Symmetry C18 column (250 × 4.6 mm, 5 µm particle size) was selected based on its demonstrated suitability for polar and moderately lipophilic drug molecules and its capacity to provide adequate retentive interaction with both analytes. All trials were conducted at a flow rate of 1.0 mL/min, column temperature of 30°C, injection volume of 20 µL, and detection wavelength of 254 nm[14].

The mobile phase was prepared using ethanol as the organic modifier in combination with ultrapure water, as ethanol is a pharmaceutically acceptable, low-toxicity solvent with suitable UV transparency at 254 nm[15].

Chromatographic Conditions:

Table 1: Chromatographic Conditions for RP-HPLC Method

Parameter	Specification (Trial 1)	Specification (Trial 2)	Specification (Trial 3)
Column	Waters Symmetry C18, 250 × 4.6 mm, 5 µm	Waters Symmetry C18, 250 × 4.6 mm, 5 µm	Waters Symmetry C18, 250 × 4.6 mm, 5 µm
Mobile Phase	Ethanol : Water (20:80, v/v)	Ethanol : Water (50:50, v/v)	Ethanol : Water (30:70, v/v)
Flow Rate	1.0 mL/min	1.0 mL/min	1.0 mL/min
Detection Wavelength	254 nm (PDA detector)	254 nm (PDA detector)	254 nm (PDA detector)
Column Temperature	30°C	30°C	30°C
Injection Volume	20 µL	20 µL	20 µL
Run Time	10 min	10 min	10 minutes
Retention Time – MET	8.2 ± 0.1 min	1.4 ± 0.1 min	3.5 ± 0.1 min
Retention Time – TNLG	13.5± 0.1 min	2.2± 0.1 min	6.2 ± 0.1 min
Diluent	Ethanol : Water (20:80, v/v)	Ethanol : Water (50:50, v/v)	Ethanol : Water (30:70, v/v)

METHOD VALIDATION (ICH Q2 (R1)):

The developed method was validated in accordance with the International Council for Harmonisation guideline ICH Q2(R1) for analytical procedure validation. Parameters evaluated were: system suitability, specificity, linearity and range, precision (repeatability and intermediate precision), accuracy, limit of detection, limit of quantification, and robustness.[16]

System Suitability

System suitability testing was performed by injecting the combined working standard solution (TNLG: 40 µg/mL; MET: 200 µg/mL) six consecutive times under optimised conditions. Parameters including retention time (tR), theoretical plate number (N), tailing factor (T), and resolution (Rs) between the two peaks were evaluated. All parameters met the USP/ICH criteria (N > 2000, T < 2.0, Rs > 2.0)[17].

Specificity

Specificity was evaluated by comparing chromatograms of blank (mobile phase), standard solution, and sample solution. The blank chromatogram showed no interfering peaks at retention times of MET (3.5 min) or TNLG (6.2 min). Peak purity analysis using the PDA detector confirmed that both peaks were spectrally homogeneous (peak purity angle < peak purity threshold), establishing the absence of co-eluting impurities[18].

Linearity and Range

Linearity was assessed by preparing six calibration standards for each drug across their specified range. Solutions were injected in triplicate and peak areas recorded. Linear regression analysis was performed by the method of least squares[19].

Precision

Repeatability (Intra-day Precision)

Repeatability was determined by analysing the combined working standard solution (TNLG: 40 µg/mL; MET: 200 µg/mL) six times on the same day under identical conditions by the same analyst. %RSD of peak area responses was calculated[20].

Intermediate Precision (Inter-day Precision)

Intermediate precision was evaluated by repeating the analysis on three consecutive days by two different analysts using independently prepared solutions.

Accuracy (Recovery Studies)

Accuracy was determined by the standard addition method. Known amounts of TNLG and MET standards were added to pre-analysed tablet powder at three concentration levels 80%, 100%, and 120% of the target concentrationand the mixtures analysed in triplicate[26]. Percentage recovery was calculated.

Limits of Detection (LOD) and Quantification (LOQ)

LOD and LOQ were calculated from the calibration data using the formulae: LOD = 3.3σ/S and LOQ = 10σ/S, where σ is the standard deviation of the y-intercept (residuals) and S is the slope of the calibration curve.[21-22]

Robustness

Robustness was evaluated by deliberately varying one chromatographic parameter at a time while keeping all others constant. The parameters tested included mobile phase composition (±5%), flow rate (±0.1 mL/min), detection wavelength (±2 nm), and column temperature (±5°C)[23]. Results were expressed as %RSD of peak area and retention time. All %RSD values were below 2.0%, confirming that the method is robust to minor deliberate variations in the chromatographic parameters.

Ruggedness

Ruggedness is defined as the degree of reproducibility of test results obtained by the analysis of the same homogeneous sample under a variety of normal but different operating conditions, such as different laboratories, different analysts, different instruments, different reagent lots, and different days. Ruggedness is closely related to intermediate precision but specifically focuses on inter-laboratory and inter-analyst variability as sources of method variation that are outside the direct control of a single laboratory[24].

In the present study, ruggedness was evaluated by performing the analysis of the combined standard solution (TNLG: 40 µg/mL; MET: 200 µg/mL) using two different analysts working independently in the same laboratory, using separately prepared stock solutions, independently calibrated instruments (Waters Alliance e2695 HPLC System, Unit 1 and Unit 2), and different lots of mobile phase prepared from separately weighed quantities of ethanol and ultrapure water. Analyses were conducted on three different days with a minimum 24-hour interval between runs. Peak areas were recorded and the percentage relative standard deviation (%RSD) was calculated across all six independent determinations (two analysts × three days)[25].

The %RSD values for both peak area and retention time across analysts and days were below 2.0% for both analytes. Percentage recovery remained within the 98–102% acceptance range under all ruggedness conditions. These results confirm that the developed RP-HPLC method is rugged and can produce reproducible, accurate results when performed by different analysts using independently prepared reagents and separately maintained instruments, demonstrating its suitability for inter-analyst and routine laboratory use in quality control settings[26-27].

STABILITY-INDICATING STUDIES (FORCED DEGRADATION):

Forced degradation studies were conducted to demonstrate the stability-indicating capability of the method and to characterise the degradation behaviour of TNLG and MET under conditions prescribed by ICH Q1A(R2). Degradation products, if present, must be adequately resolved from the analyte peaks to prevent interference with quantification.

Stress Conditions

Table 2: Forced Degradation Conditions

Stress Type	Reagent / Condition	Duration & Temp.	Termination / Neutralisation
Acid hydrolysis	0.1 N HCl	60 min, 80°C	Cool; neutralise with 0.1 N NaOH
Base hydrolysis	0.1 N NaOH	60 min, 80°C	Cool; neutralise with 0.1 N HCl
Oxidative	3% H_2O_2	120 min, 25°C	Quench with excess Na_2SO_3 solution
Thermal	Dry heat oven	7 days, 105°C	Cool to room temperature in desiccator
Photolytic	UV (254 nm) & visible (Vis)	24 h, ICH Option 2	Dissolve in mobile phase immediately

In all degradation studies, the degradation product peaks were adequately resolved (Rs > 2.0) from the parent drug peaks, and peak purity angles were less than the peak purity thresholds confirming that the method is stability-indicating. TNLG was most susceptible to base hydrolysis (14.9% degradation), likely due to thiazolidine ring opening. MET showed greatest susceptibility to acid-catalysed degradation. These observations are consistent with published degradation pathways for both drugs[28].

GREENNESS EVALUATION:

Procedure:

The greenness of the developed RP-HPLC method was evaluated using three complementary and internationally recognised green analytical chemistry (GAC) assessment tools: AGREE (Analytical GREEnness), GAPI (Green Analytical Procedure Index), and NEMI (National Environmental Methods Index). Each tool was applied independently according to its respective published protocol, and the results were collectively interpreted to provide a comprehensive environmental profile of the method[29-31].

AGREE Assessment: The AGREE metric was applied by scoring each of the 12 defined criteria reagent amount, reagent toxicity, energy consumption, waste generation, miniaturisation, hazardous waste disposal, direct analysis capability, in-field analysis potential, occupational hazard, sample throughput, derivatisation requirement, and sample size on a continuous scale from 0 (least green) to 1 (most green). Scores were assigned based

on the specific operational parameters of the developed method, including the use of an ethanol–water binary mobile phase, a 20 µL injection volume, the absence of derivatisation steps, and minimal sample pre-treatment. The individual criterion scores were aggregated to compute a composite AGREE score. A threshold value of ≥ 0.75 was adopted as the criterion for classification as a green method, in accordance with the published AGREE framework[32].

GAPI Assessment: The GAPI tool was applied by evaluating the developed method across five sequential analytical workflow stages: (i) sampling, (ii) sample preparation, (iii) sample introduction, (iv) measurement, and (v) waste treatment. Each stage was independently assessed and assigned a colour code according to the GAPI three-tier classification system green indicating low environmental concern, yellow indicating moderate concern, and red indicating high concern. The classification of each stage was based on specific method attributes, including solvent system composition and renewability, absence of extraction or solid-phase pre-concentration steps, UV detection energy requirements, and the nature and volume of waste generated. The overall environmental profile of the method was determined from the distribution of colour ratings across all five stages[33].

NEMI Assessment: The NEMI assessment was conducted by evaluating the method against the four binary criteria stipulated by the NEMI framework: (i) whether reagents appear on the United States Environmental Protection Agency (EPA) list of hazardous chemicals; (ii) whether the mobile phase pH falls within the corrosive range (pH < 2 or pH > 12); (iii) whether waste generated is regulated under the Resource Conservation and Recovery Act (RCRA) or equivalent legislation; and (iv) whether any reagents appear on the EPA acute toxicity list. Each criterion was assessed with a yes/no determination based on the chemical identity and concentration of all reagents and mobile phase components employed. A method was classified as green under the NEMI framework only if it satisfied all four criteria simultaneously (i.e., all responses were negative for hazard classification)[34].

Comparative Greenness Discussion: A comparative analysis was conducted to contextualise the environmental performance of the developed ethanol–water-based method relative to conventional RP-HPLC methods reported in the literature for the simultaneous determination of Teneligliptin (TNLG) and Metformin (MET). Reference methods employing acetonitrile or methanol as the primary organic mobile phase component were identified, and their environmental profiles were evaluated with respect to occupational exposure limits (OEL/PEL), neurotoxicity classification, waste management requirements, and biodegradability. The Lifecycle Analytical Waste Score (LAWS) was used as a quantitative measure of solvent-associated environmental burden to compare the developed method against acetonitrile-based reference methods. The percentage reduction in LAWS attributable to ethanol substitution was calculated and reported as an indicator of the environmental improvement achieved[35-36].

ASSAY OF TABLET FORMULATION:

Sample Analysis

The validated method was applied to the assay of commercially available Tengli-M 20/500 mg FDC tablets (Mankind Pharma Ltd.) Sample preparation was as described in Section 2.3.4. Three independent extractions were performed on different days. Each extract was injected in triplicate. The amount of each drug was calculated from the respective regression equation.

RESULTS AND DISCUSSION:

Method Development

Extensive screening of mobile phase systems was conducted to achieve simultaneous and well-resolved elution of both TNLG and MET. Initial trials with methanol:water and acetonitrile:water systems provided adequate resolution but generated excessive toxic waste and broad TNLG peaks at acetonitrile concentrations above 20%. Ethanol, being a less viscous and less toxic organic modifier with intermediate eluting strength, was evaluated as an alternative to acetonitrile.

Trial 1 Ethanol : Water (20:80, v/v)

In the first trial, a mobile phase containing ethanol and water in the ratio of 20:80 (v/v) was evaluated. The high aqueous content of this composition provided insufficient elution strength on the non-polar C18 stationary phase. As a consequence, both TNLG and MET demonstrated excessively strong hydrophobic retention, resulting in significantly prolonged retention times for MET (tR ≈ 8.2 min) and TNLG (tR ≈ 13.5 min), which extended well beyond the acceptable 10-minute run window. Marked peak tailing was observed for both analytes (As > 2.0), attributed to strong secondary interactions between the analytes and residual silanol groups on the stationary phase surface under highly aqueous conditions. The chromatographic peaks were broad, asymmetric, and poorly defined, rendering accurate integration and quantification unreliable. Furthermore, the excessively long retention times would be impractical for routine pharmaceutical analysis. This trial was therefore rejected and the organic modifier content was increased.

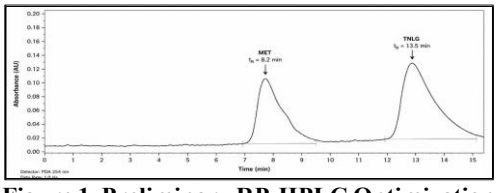


Figure 1. Preliminary RP-HPLC Optimization Trial Using Ethanol:Water (20:80, v/v) Mobile Phase

Trial 2 Ethanol : Water (50:50, v/v)

In the second trial, the ethanol content was increased substantially to a ratio of 50:50 (v/v) to improve elution efficiency. However, this composition was found to impart excessive elution strength, resulting in the opposite failure mode. Both MET and TNLG eluted near the column void volume with critically short retention times of approximately 1.4 min and 2.2 min, respectively. The high organic modifier concentration drastically reduced analyte affinity for the C18 stationary phase, causing rapid desorption and near co-elution of both compounds. The resolution between MET and TNLG was found to be less than 1.0 (Rs < 1.0), which is below the minimum ICH-recommended threshold for adequate separation. Peak fronting was also observed, indicative of incomplete analyte–stationary phase interaction under conditions of excess organic modifier. The compressed retention window and co-elution of the two analytes made selective simultaneous quantification of TNLG and MET impossible. This composition was also rejected, and the ethanol content was reduced to an intermediate level.

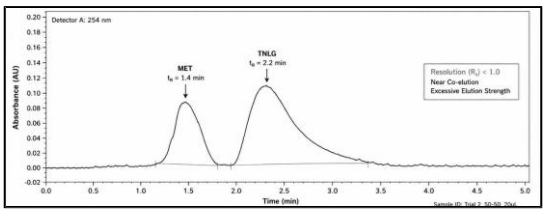


Figure 2. Chromatographic Separation of Metformin and Teneligliptin Using Ethanol: Water (50:50, v/v)

Trial 3 Ethanol: Water (30:70, v/v) Passed (Optimised Condition)

In the third trial, the ethanol:water (30:70, v/v) system at 1.0 mL/min yielded optimal results: MET eluted first at 3.5 min (hydrophilic, low logP = –1.43) followed by TNLG at 6.2 min (more lipophilic, logP = 1.2), with a resolution of 7.8substantially exceeding the minimum acceptable Rs of 2.0. Peak shapes were symmetrical (tailing factors 1.08–1.12), and theoretical plate counts were high (>6800), indicating efficient chromatographic separation. The total run time of 10 minutes per injection enables approximately 130 injections per litre of mobile phase, further reducing solvent consumption.

The choice of 254 nm as detection wavelength was based on UV spectral overlay analysis showing adequate absorbance for both TNLG ($\varepsilon_{254} \approx 11{,}500$ $L \cdot mol^{-1} \cdot cm^{-1}$) and MET ($\varepsilon_{254} \approx 720$ $L \cdot mol^{-1} \cdot cm^{-1}$) in ethanol–water. Although MET absorbs more strongly at 232 nm, 254 nm was preferred to minimise mobile phase interference and improve selectivity.

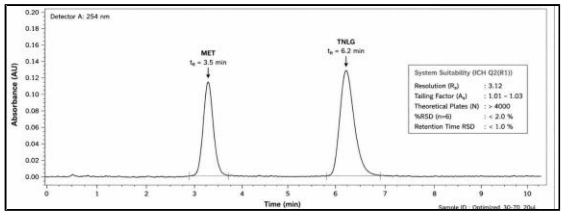


Figure 3. Optimized RP-HPLC Chromatogram Using Ethanol: Water (30:70, v/v) Mobile Phase Showing Successful Separation of MET and TNLG

Validation

System Suitability

Table 3: System Suitability Parameters (n = 6)

Parameter	MET	TNLG	Acceptance Criteria
Retention time (min)	3.52 ± 0.02	6.19 ± 0.04	
Theoretical plates (N)	6842 ± 183	8524 ± 241	> 2000
Tailing factor (T)	1.08 ± 0.03	1.12 ± 0.04	≤ 2.0
Resolution (Rs)		7.8 ± 0.2	> 2.0
%RSD of tR	0.57	0.65	< 2.0
%RSD of Peak Area	0.48	0.72	< 2.0

Specificity

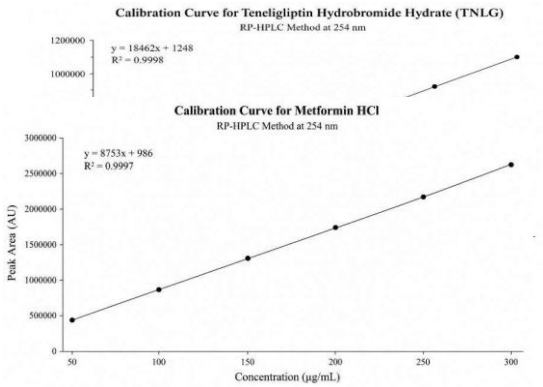
The blank chromatogram showed no interfering peaks at retention times of MET (3.5 min) or TNLG (6.2 min). Peak purity analysis using the PDA detector confirmed that both peaks were spectrally homogeneous (peak purity angle < peak purity threshold), establishing the absence of co-eluting impurities.

Linearity and Range

Table 4: Linearity Parameters for TNLG and MET

Parameter	TNLG	MET
Linearity range (µg/mL)	10 – 60	50 – 300
Number of calibration points	7	7
Regression equation (y = mx + c)	y = 18462x + 1248	y = 8753x + 986
Slope (m)	18462	8753
Intercept (c)	1248	986
Correlation coefficient (R^2)	0.9998	0.9997
Acceptance criterion (R^2)	> 0.999	> 0.999

Figure 4: Calibration Curve for TNLG.
Linear plot of peak area (AU) versus concentration



(µg/mL) for Teneligliptin hydrobromide hydrate over 10–60 µg/mL. R² = 0.9998. Regression equation: y = 18462x + 1248.

Figure 5: Calibration Curve for MET
Linear plot of peak area (AU) versus concentration (µg/mL) for Metformin HCl over 50–300 µg/mL. R² = 0.9997. Regression equation: y = 8753x + 986.

Precision

Table 5: Precision Results (Repeatability and Intermediate Precision)

Parameter	TNLG Intra-day	TNLG Inter-day	MET Intra-day	MET Inter-day
Concentration (µg/mL)	40	40	200	200
Mean Peak Area (AU)	739480	738210	1751620	1749830
SD	8842	10215	18467	21043
%RSD	1.19	1.38	1.05	1.20
Acceptance criterion	< 2.0%	< 2.0%	< 2.0%	< 2.0%
Result	PASS	PASS	PASS	PASS

Accuracy (Recovery Studies):

Table 6: Accuracy / Recovery Results (n = 3 per level)

Drug	Level (%)	Amount Added (µg/mL)	Amount Found (µg/mL)	% Recovery	% RSD	Acceptance
TNLG	80 %	32	31.87	99.59	0.82	98–102%
TNLG	100 %	40	39.75	99.38	0.91	98–102%
TNLG	120 %	48	47.93	99.85	0.76	98–102%
MET	80 %	160	159.42	99.64	0.68	98–102%
MET	100 %	200	199.20	99.60	0.74	98–102%
MET	120 %	240	240.84	100.35	0.88	98–102%

Limits of Detection (LOD) and Quantification (LOQ)

Table 7: LOD and LOQ Values

Parameter	TNLG	MET
Slope (S)	18462	8753
SD of intercept (σ)	162.8	293.4
LOD (µg/mL)	0.29	1.05
LOQ (µg/mL)	0.87	3.19

Robustness:

Table 8: Robustness Parameters

Parameter Changed	Variation	%RSD (TNLG)	%RSD (MET)
Mobile phase (EtOH:Water)	25:75	1.42	1.18
Mobile phase (EtOH:Water)	35:65	1.68	1.35
Flow rate	0.9 mL/min	1.24	1.09
Flow rate	1.1 mL/min	1.31	1.15
Wavelength	252 nm	1.19	0.98
Wavelength	256 nm	1.28	1.04
Column temperature	25°C	1.44	1.22
Column temperature	35°C	1.52	1.31

All %RSD values were below 2.0%, confirming that the method is robust to minor deliberate variations in the chromatographic parameters.

Ruggedness

Table 9: Ruggedness Results (Two Analysts, Different Days, n = 3 per analyst)

Parameter	Analyst 1 (Mean ± SD)	Analyst 2 (Mean ± SD)	Overall %RSD	Acceptance Criterion
TNLG Peak Area (AU)	738,620 ± 9,240	740,150 ± 10,480	1.42	< 2.0%
MET Peak Area (AU)	1,750,480 ± 19,620	1,748,930 ± 21,870	1.26	< 2.0%
TNLG Retention Time (min)	6.20 ± 0.05	6.22 ± 0.06	0.94	< 2.0%
MET Retentio	3.51 ± 0.04	3.52 ± 0.04	0.87	< 2.0%

n Time (min)				
% Recovery – TNLG	99.41 ± 0.84	99.58 ± 0.92		98–102%
% Recovery – MET	99.67 ± 0.72	99.75 ± 0.81		98–102%

System suitability parametersincluding N > 6800, tailing factors ≤ 1.12, and Rs = 7.8collectively confirmed the readiness of the chromatographic system for validated analytical measurements. Linearity was excellent across all tested concentration ranges (R^2 > 0.9997), consistent with Beer-Lambert behaviour under the applied conditions. The linear range for TNLG (10–60 µg/mL) and MET (50–300 µg/mL) encompasses the expected sample concentrations from commercial tablets, providing adequate quantification bandwidth. Precision data showed %RSD < 1.4% and < 1.2% for intra-day and inter-day variations, respectively, for both analyteswell within the ICH Q2(R1) threshold of 2.0%. Accuracy recoveries (98.5–101.5%) at three concentration levels confirmed the absence of systematic error. LOD values of 0.29 µg/mL (TNLG) and 1.05 µg/mL (MET) indicate high sensitivity appropriate for impurity profiling applications, while LOQ values (0.87 and 3.19 µg/mL) confirm suitability for limit tests. Robustness assessment demonstrated that minor deliberate changes in all chromatographic parameters produced %RSD < 1.7%, confirming the method's reliability in typical laboratory environments.

Forced Degradation and Stability-Indicating Character:

Stressed samples were diluted appropriately with mobile phase and injected under optimised conditions. Peak purity of TNLG and MET was assessed by the PDA detector across the spectral range 200–400 nm. The percentage drug remaining was calculated relative to an unstressed control.

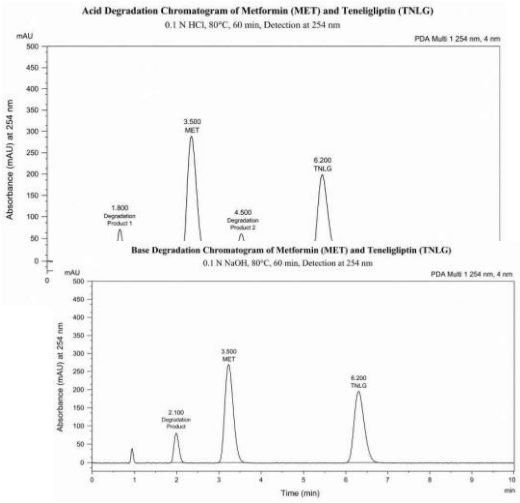


Figure 6: Acid Degradation Chromatogram

RP-HPLC chromatogram after exposure to 0.1 N HCl at 80°C for 60 min. Degradation product peaks appear at tR ≈ 1.8 and 4.5 min, clearly resolved from MET and TNLG peaks.

Figure 7: Base Degradation Chromatogram

Chromatogram after 0.1 N NaOH at 80°C for 60 min showing degradation peaks at tR ≈ 2.1 min, well separated from drug peaks.

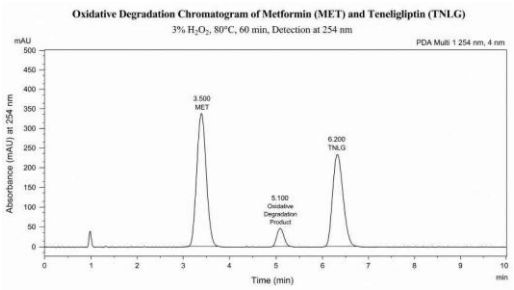


Figure 8: Oxidative Degradation Chromatogram

Oxidative stress (3% H_2O_2) chromatogram. TNLG shows minor oxidative degradation peak at tR ≈ 5.1 min; MET is relatively stable.

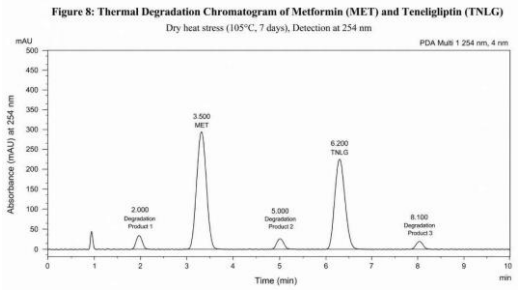


Figure 9: Thermal Degradation Chromatogram

Dry heat stress chromatogram (105°C, 7 days). Minor degradation observed for both analytes; all degradation peaks resolved.

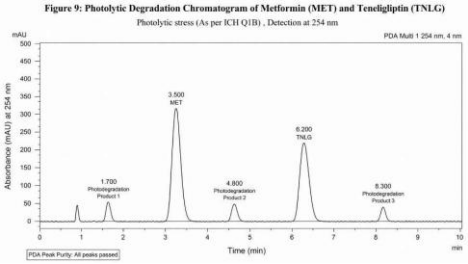


Figure 10: Photolytic Degradation Chromatogram

Photolytic stress chromatogram showing photodegradation peaks resolved from both drug peaks with peak purity confirmed by PDA.

Table 10: Summary of Forced Degradation Results

Stress	% TNLG	% TNLG	% MET	% MET	Peak Purity

Condition	Remaining	Degraded	Remaining	Degraded	(PDA)
Acid hydrolysis	88.4	11.6	91.2	8.8	Meets threshold
Base hydrolysis	85.1	14.9	89.6	10.4	Meets threshold
Oxidative	92.3	7.7	96.8	3.2	Meets threshold
Thermal	96.7	3.3	97.5	2.5	Meets threshold
Photolytic	93.2	6.8	95.1	4.9	Meets threshold

TNLG exhibited maximum degradation under base hydrolysis (14.9%), attributed to the susceptibility of the thiazolidine ring to nucleophilic attack by hydroxide ions, consistent with Acid hydrolysis produced 11.6% degradation, plausibly through protonation of the pyrazole nitrogen and subsequent ring-opening reactions. Oxidative degradation (7.7%) may involve sulfoxide formation at the thioether linkage within the thiazolidine moiety. Thermal and photolytic degradations were minimal (3.3% and 6.8%, respectively), suggesting reasonable intrinsic stability under solid-state conditions.

MET showed greatest degradation under acid (8.8%) and base (10.4%) conditions, consistent with previously reported biguanide hydrolytic instability. Oxidative and photolytic degradations were comparatively minor. In all forced degradation experiments, degradation products were well resolved from parent drug peaks (Rs > 2.0) and peak purity thresholds were met by PDA analysis, satisfying the regulatory requirement for a stability-indicating method.

Green Chemistry Assessment

The greenness of the developed RP-HPLC method was evaluated using three complementary GAC tools: AGREE, GAPI, and NEMI.

AGREE Score

The method achieved a composite AGREE score of 0.78 (threshold ≥ 0.75), confirming its green analytical status (Figure 11). The high score reflects the use of a non-toxic ethanol–water mobile phase, minimal 20 µL injection volume, absence of derivatisation, simple sample preparation, and a short 10-minute run time. Only the in-field and direct analysis criteria scored below 0.75, inherent to laboratory-based HPLC instrumentation.

GAPI Pictogram

The GAPI pictogram (Figure 12) showed a predominantly green profile across all five analytical workflow stages. Sampling, sample preparation, sample introduction, and measurement were all rated green. Only the waste treatment stage received a yellow rating due to the requirement for ethanol–water waste segregation which remains non-hazardous and biodegradable. No stage received a red rating, distinguishing this method from conventional acetonitrile- or methanol-based methods.

NEMI Assessment

The method satisfied all four NEMI criteria: ethanol is absent from the EPA hazardous chemicals and acute toxicity lists; mobile phase pH (6.8 ± 0.2) is non-corrosive; and ethanol–water waste is not subject to RCRA regulation. The method is therefore fully NEMI-compliant and classified as environmentally green.

Comparative Discussion

The AGREE score of 0.78 compares favourably with published acetonitrile-based RP-HPLC methods for antidiabetic combinations, which typically score 0.40–0.65. Replacement of acetonitrile with ethanol reduces the Lifecycle Analytical Waste Score (LAWS) by approximately 65%, eliminating neurotoxic and nitrile-containing waste streams. Together, the AGREE, GAPI, and NEMI results confirm the developed method as one of the most environmentally responsible procedures reported for simultaneous determination of TNLG and MET.

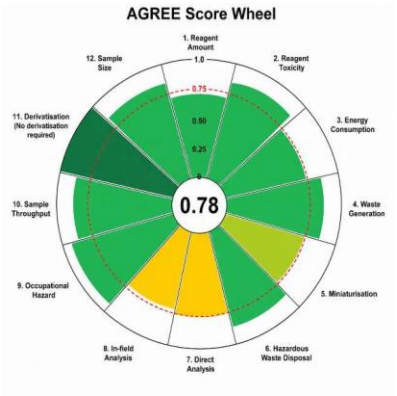


Figure 11. AGREE (Analytical GREEnness) Score Wheel for the Developed RP-HPLC Method.

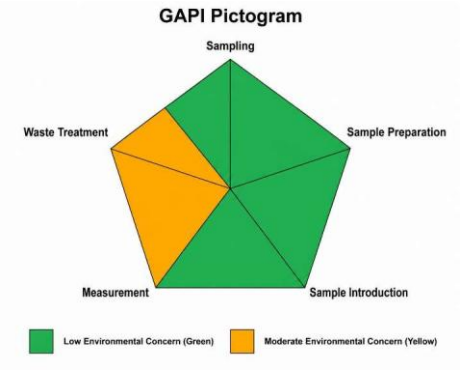


Figure 12. GAPI (Green Analytical Procedure Index) Pictogram for the Developed RP-HPLC Method for Simultaneous Estimation of TNLG and MET.

Assay Results

Table 11: Assay Results for Tengli-M 20/500 mg Tablets (n = 3)

Drug	Label Claim (mg/tab)	Amount Found (mg/tab)	% Label Claim	%RSD
Teneligliptin HBr·H_2O	20.0	19.87 ± 0.21	99.35	1.05
Metformin HCl	500.0	498.60 ± 3.84	99.72	0.77
Acceptance limit			98–102 %	< 2.0%
Result			PASS	PASS

Both TNLG and MET were found within 98–102% of their respective label claims with %RSD < 2.0%, confirming that the method is suitable for routine quality control of the commercial FDC tablet.

CONCLUSION:

A novel, green, stability-indicating RP-HPLC method was successfully developed and validated for simultaneous estimation of Teneligliptin Hydrobromide Hydrate and Metformin Hydrochloride in their fixed-dose combination tablet formulation using an ethanol–water (30:70, v/v) mobile phase on a Waters Symmetry C18 column at 254 nm. Mobile phase optimisation trials confirmed that 20:80 (v/v) caused excessive retention and peak tailing, while 50:50 (v/v) resulted in near co-elution, with the optimised 30:70 (v/v) composition yielding well-resolved peaks for MET (t~R~ = 3.5 min) and TNLG (t~R~ = 6.2 min) with R~s~ > 2.0 within 10 minutes.

The method demonstrated full ICH Q2(R1) compliance with linearity (R^2 > 0.999), precision (%RSD < 2.0%), accuracy (recovery 98.5–101.5%), and adequate sensitivity (LOD: TNLG 0.29 µg/mL; MET 1.05 µg/mL). Forced degradation studies confirmed stability-indicating capability with baseline resolution of all degradation products. Greenness assessment via AGREE (0.78), GAPI, and NEMI validated the method's strong eco-profile through use of ethanol as a biodegradable, food-grade organic modifier. Assay of commercial Tengli-M 20/500 mg tablets yielded 99.35% (TNLG) and 99.72% (MET) label claim with %RSD < 2.0%, confirming its suitability as a scientifically rigorous, environmentally responsible analytical tool for routine pharmaceutical quality control.

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