RESEARCH PAPER

Sustainable bioanalytical method for metformin and sitagliptin by using internal standard : Integrating quality by design, micro extraction and green assessment via reverse phase high performance liquid chromatography and application to real patient samples.

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ABSTRACT

Background: To develop robust, ecofriendly and regulatory complaint methods by the integration of green assessment principles with Analytical Quality by Design(AQBD). In routine bioanalytical and pharmaceutical quality control of Metformin Hydrochloride (MET) and Sitagliptin (SIT) employ chromatographic methods that are solvent intensive and environmentally hazardous. These combination of drugs are commonly prescribed for the treatment of type 2 diabetes mellitus.

Objective: This research planned to advance a sustainable Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) method for the synchronous quantification of MET and SIT using Gliclazide (GLI) as an internal standard, incorporating microextraction techniques and comprehensive green assessment tools.

Methods: By risk assessment critical method parameters and attributes were determined and enhanced using Design of Experiments (DOE) with a Central Composite Design Expert 13. Chromatographic separation was performed an a inertsil ODS C18 column (250 mm×4.6 mm, 5µm) with a mobile phase of 6mM potassium dihydrogen ortho phosphate buffer(pH 3.0, adjusted with orthophosphoric acid) and Methanol(83:17%v/v) at 0.8ml/min and detection wavelength at 235nm. Sample preparation employed ultrasound assisted microextraction to minimize solvent usage. The procedure was authenticated in accordance with ICH Q2(R1) guidelines.

Results: The enhanced procedure was sensitive, specific, precise, accurate and robust for synchronous estimation of MET and SIT in plasma. Comparative evaluation using AGREE, MoGAPI, BAGI and RAPI confirmed superior environmental and analytical performance over reported methods.

Conclusion: This authenticated AQBD based RP HPLC method with green sample preparation offers an eco-efficient, regulatory- complaint platform for MET-SIT analysis, supporting pharmaceutical quality control, clinical research and future pharmacokinetic and therapeutic applications.

Keywords: Sustainable bioanalysis, Metformin, Sitagliptin, Gliclazide, Analytical Quality by Design, Microextraction, Green Assessment and High-Performance Liquid Chromatography.

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INTRODUCTION

In high stress life, diabetes mellitus has become a common disease among people. MET stands as the preferred option for treating diabetes mellitus(1). Additionally, various combinations of MET with SIT are common for diabetes management. In the market , numerous pharmaceutical industries are manufacturing this combination of drugs in response to popular demand. MET is a biguanide antihyperglycemic drug and exercise to control glycemic levels in type 2 diabetes. SIT increases the body's production of chemicals that cause the pancreas to release more insulin (2-3). The chemical structures of MET, SIT are depicted in Fig.1.

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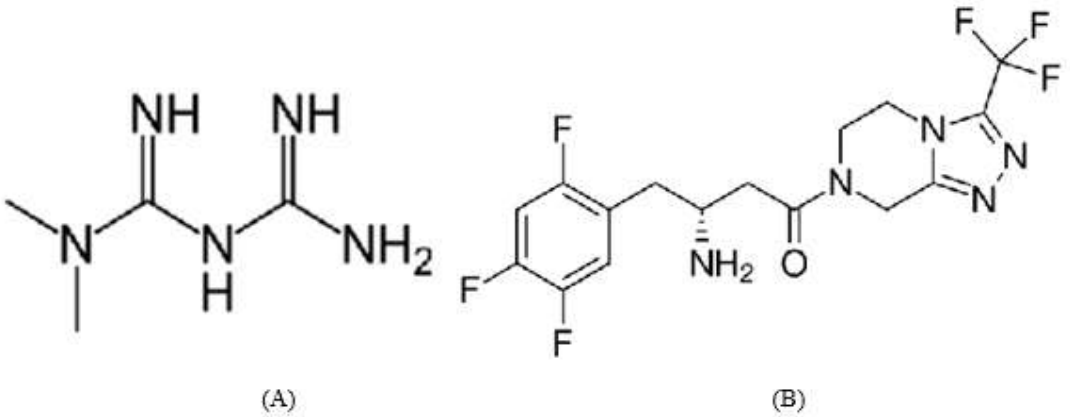

Fig. 1. Chemical structures of anti-diabetic drugs A. Metformin hydrochloride and B. Sitagliptin.

To date, no reported RP-HPLC Method was found employing GLI as an internal standard under these chromatographic conditions after an extensive literature survey(4-5). The enhancement of this RP-HPLC procedure follows the AQBD in accordance with ICH Q14 guideline, planning to reduce organic waste generation and ensure regulatory compliance(6-7). Analytical quality risk assessment using Taguchi design in Design Expert 13 software (trial version) was performed to identity Critical Procedure Parameters (CPPs) and Critical Method Performance Attributes (CMPAs). DOE based response surface modeling (RSM) using Central Composite Design in Design-Expert 13 software (trial version) optimized the RP-HPLC method(8).

In pharmaceutical enhancement, the analysis of drugs within biological matrices is crucial. Liquid-Liquid extraction and Solid-phase extraction are traditional extraction techniques and time-consuming techniques. Usually, involving the use of organic solvents, generates significant amounts of waste that can be hazardous to human health and the environment (9). To avoid these drawbacks, green non-conventional extraction methods, such as Microwave-Assisted Extraction (MAE), Matrix Solid-Phase Dispersion (MSPD) and Ultrasound Assisted Extraction (UAE), are constantly used as pre concentration methods(10-11). Within these techniques, a simple and efficient sample extraction method is Ultrasound-Assisted Extraction (UAE) technique. By increasing the interaction between matrix and solvents, this technique accelerates the mass transfer of analytes. Compared with traditional and other conventional extraction methods, UAE has shown several benefits, including time saving, lower consumption of organic solvents and higher extraction efficiency (11-12). In Green analytical methods, the selection of a proper solvent has become a crucial task not only for effective extraction but also for development (13). To align with green chemistry principles, a novel type of green solvent, known as a Deep Eutectic Solvent (DES), has emerged. DESs are commonly synthesized which are economical, biodegradable and non-toxic (14-15). These components are mixed to form a eutectic mixture with a melting point lower than that of each element due to the generation of hydrogen bonds and Van der Waals interactions. Nevertheless, there are limited studies related to extraction optimization using UAE and DES as a solution for the extraction of drugs from plasma samples(16-17). Its greenness was evaluated using established tools such as the Analytical GREEness metric (AGREE), Modified Green Analytical Procedure Index (MoGAPI), Blue Applicability Grade Index (BAGI) and Red Analytical Performance Index (RAPI) and Red Green Blue color codes (RGB) (18-21).

MATERIALS AND METHODS

Chemicals and reagents

Reference standards of MET, SIT and GLI as an internal standard were obtained as gift samples from kshetra Analyticals (Hyderabad, India). Analytical grade reagent Methanol and orthophosphoric acid were procured from thermo fischer scientific (Mumbai, india). Potassium dihydrogen phosphate (KH2PO4) was obtained from SD Fine Chem Ltd.(Mumbai, India). Double distilled water was prepared using a Milli Q purification system (Sigma-aldrich, bangaleru, india). Filtration was carried out using 0.45µm membrane filters and 0.22µm syringe filters (Thermo fischer scientific) and Human plasma.

Instrumentation

Chromatographic analysis was performed using a Shimadzu prominence-LC. 2030C 3D Plus HPLC system (Shimadzu, Japan) with a quaternary pump and autosampler, and data acquisition was done using LabSolution software. Separation was achieved on a Inertsil ODS C18 column (250mm × 4.6 mm, 5.0 µm). An ultrasonic bath sonicator (Bio Technics India, Mumbai) was used for mobile phase degassing and sample extraction. A deluxe pH meter(Lab India, Thane) was used for pH measurements, and sample weights were measured with an electromagnetic balance (Shimadzu/Shinko Denshi, Japan).

Software and analytical tools

Method optimization was performed using Design-experiment 13 (Star-Ease Inc., Minmeapolis, USA). The greenness of the developed method was evaluated using

the AGREE calculator (https://www.agree.com), complex GAPI (https://www.complexgapi.com), RGB (https://www.rgb.com), MoGAPI (https://www.mdpi.com) and BAGI (https://www.bagi.com).

Preparation of working standard solution

A 1.0 ml of respective stock solution (each stock containing 1000µg/ml of MET, SIT and GLI) into a 10 ml volumetric flask and made up with mobile phase. A concentration of 100.0µg/ml of MET, SIT and GLI working standard solutions was prepared.

Preparation of Hydrophobic Deep Eutectic Solvent (HDES)

A Hydrophobic deep eutectic solvent (HDES) was prepared by mixing tetrabutylammonium chloride(TBAC) and 1-dodecanol in a 2:1 molar ratio. A clear and homogenous liquid was formed by gently heat to 70±5°C under continuous stirring then cool to room temperature and stored in a sealed container until further use.

Optimization of extraction method

Extraction parameters influencing recovery of MET and SIT from human plasma samples were systematically optimized. Key variables included pH extraction solvent volume and sonication time are the key variables. By using a Design of Experiments (DOE) optimization was performed to achieve maximum extraction efficiency while minimizing solvent use.

Preliminary experimentation and failure mode effect analysis

Initial trails were conducted to identify critical method risk parameters affecting chromatographic performance. Several solvent systems and mobile phase modifiers were evaluated with potassium dihydrogen ortho phosphate and methanol identified as optional for sharp, well resolved peaks. Based on spectral analysis 235nm was selected as a UV detection wavelength. Through a brainstorming session more than thirty potential method risk parameters were identified and categorized under methanol, material, machine, manpower, mother nature and measurement domains. These were systematically mapped in an ishikawa (fishbone) diagram (Fig. 2) to support failure mode effect analysis (FMEA) and guide subsequent risk mitigation strategies during method development.

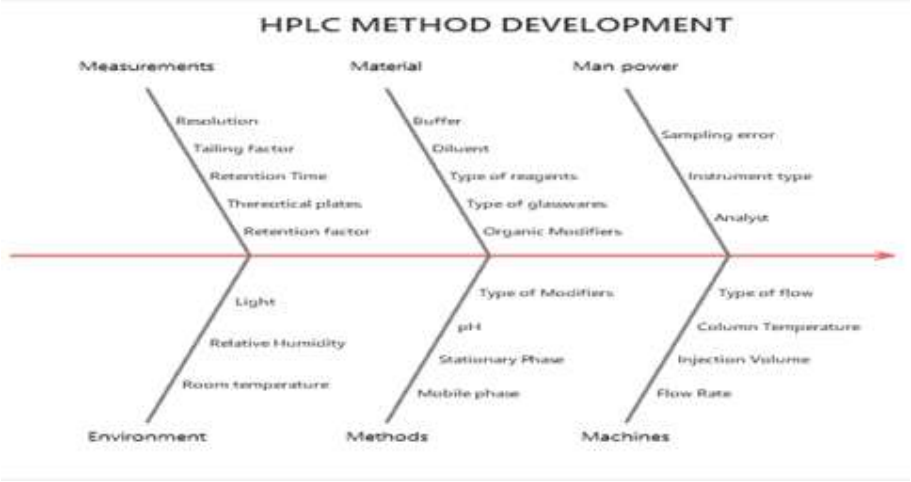

Fig 2 . Ishikawa diagram for method risk factors identification

Failure-mode effect analysis (FMEA)

In fig 3 FMEA was analyzed by a graph risk priority number versus failure words was composed. In the development of RP-HPLC method, each identified failure mode was analysed for its occurrence, severity and detectability. By multiplying score of occurrence, severity and detectability to each failure mode a RPN number has been allotted. By observing resolution and compactness of spots from very low (02) to very high effect (10) the score of occurrence and severity was allotted. By the ability of detection of failure mode very certain (02) to very uncertain (10) the score of detectability was given.

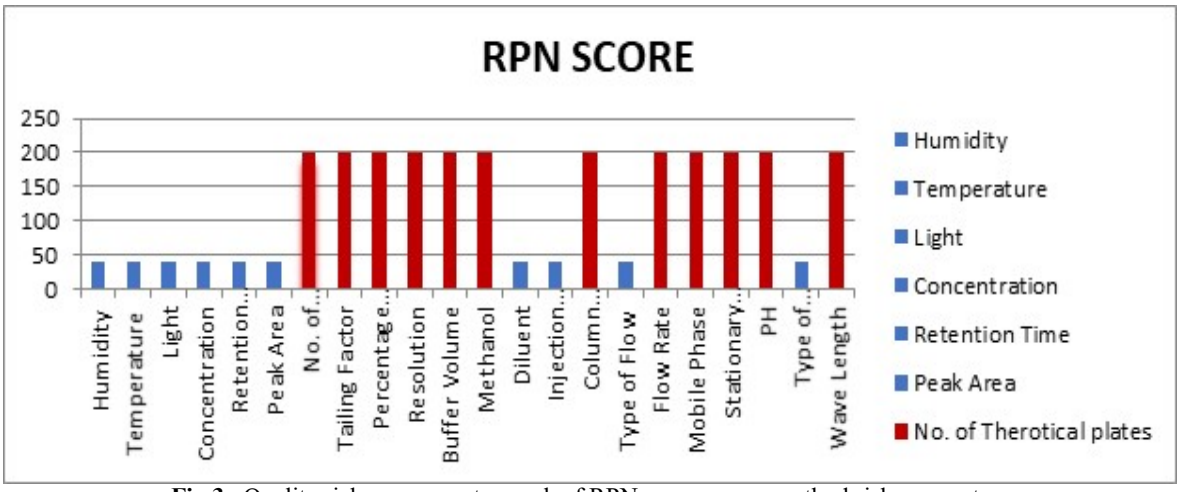

Fig 3 . Quality risk assessment– graph of RPN score versus method risk parameters

Screening of potential failure modes and CQAs by DOE based Taguchi design

The development of an analytical method twelve method risk parameters were found to be critical. All identified critical method risk parameters were screened by DOE based Taguchi design for their primary effect on the resolution of peaks of selected drugs. All twelve method risk parameters were entered at low (-1) to high (+1) levels in design expert software (version 13) upon selecting critical responses. In the laboratory , eight experimental runs were performed by suggested DOE software, responses were measured and entered against the respective experimental run and analysed for effect on responses. Statistical analysis of the main effect on responses was done by ANOVA and a pareto chart (Fig. 4).

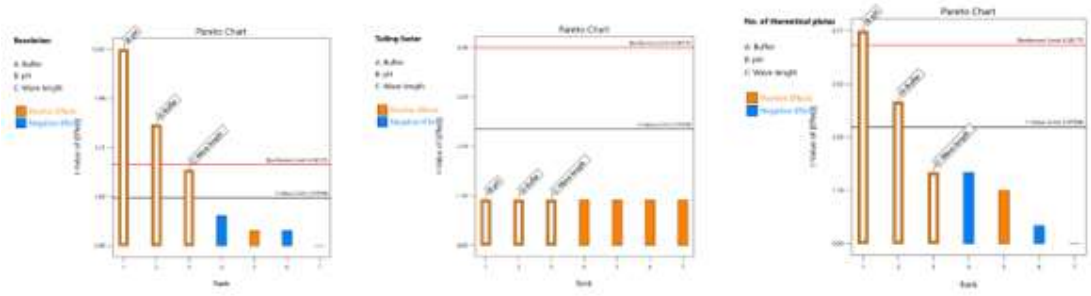

Fig 4. Pareto chart for screening design of resolution, tailing factor and number of theoretical plates.

Response surface analysis modeling and method operable design region identification by central composite design

Critically affecting variables are mobile phase composition, pH and wavelength and critical method attributes are resolution, tailing factor and number of theoretical plates were found by pareto chart analysis and ANOVA by screening design. Central composite design was applied for response surface modelling to study the relationship between critical method variables and attributes by using design of experiment software (Version.13) at three different levels of critical method variables. From the analytical target profile (ATP) of an analytical method for synchronous estimation of MET and SIT in plasma samples method operable design ranges were navigated for operating critical method risk parameters for the desired results out of the 3D contour plots. After screening design, separation and peak shape were found to be critical. Critical method risk parameters were identified and analyzed for their main, interactive and quadratic effect on peak responses by DOE central composite design. All critical method risk parameters were entered at low (-1), medium (0), and high (+1) levels by selecting responses as critical quality attributes in DOE software. In the laboratory seventeen experimental runs were performed and measured by the suggested software responses were entered against the respective experimental run and relationship between critical method variables and attributes were analyzed. The responses were optimized by response surface model.

Control strategy and optimized chromatographic conditions

The development of a robust analytical method for synchronous estimation of MET and SIT in plasma samples, in the Ishikawa diagram, have been identified for framing the control strategy and from the navigation of the method operable design region (MODR), operating ranges for critical method risk parameters are identified. The chromatographic conditions have been selected from the framed control strategy for the development and validation

of an analytical method. The chromatographic separation was achieved with 6Mm of potassium dihydrogen ortho phosphate pH 3 (Adjusted using ortho phosphoric acid) in combination with Methanol (83:17% v/v), ensuring the symmetrical peak shape and minimal baseline noise. A Intersil ODS C18 (250 mm × 4.6 mm, 5.0 µm) assisted efficient resolution with a total run time of 10 min, and detection was monitored at 235nm. The flow rate was maintained at 0.8 ml/min, and the column oven temperature was strictly controlled at 25°C to enhance the separation efficiency.

A 10µl sample injection volume optimized for superior chromatographic performance. Under these conditions, MET and SIT using GLI as an internal standard exhibited a well-defined retention time of 3.36, 5.68 and 9.36 min, confirming the methods reliability and reproducibility.

Sample extraction by UA-DES-DLLME

HDES after thawing to room temperature , 0.05ml of previously frozen plasma samples were separately transferred into tubes. To each sample, a mixture of 200µl tetrabutyl ammonium chloride and 1-dodecanol (2:1) DES and 5µl 0.1N NaOH (pH 12) was added. The tube was sonicated for 15min, a cloudy suspension was formed in the tube, resulting from the dispersion of the DES mixture in the plasma sample. The mixture was centrifuged at 5000rpm for 15 minutes, resulting in phase separation. The dispersive layer was separated by using a 2ml hypodermic syringe and diluted with methanol (1:1,v/v) because of the high viscosity of DES. The solution was then loaded into an auto sampler vial, followed by HPLC analysis.

Method validation

The developed method was validated as per the US-FDA guideline over a linear concentration range of 0.050 to 10,000 µg/ml for MET and 2.00 to 400.00 µg/ml for SIT. The validation parameters, including selectivity and specificity, LOQ, linearity, precision, accuracy, matrix effect, recovery, robustness, stability (bench top, wet extract, freeze-thaw, autosampler, short term, long term, stock solution and refrigerated stock solution stability studies) and central composite design experiment selectivity, were evaluated under the validation section.

Selectivity and specificity

To estimate the selectivity and specificity a ten lots of blank plasma samples were analyzed, out of which six lots free from interference were selected. The potential interfering peak areas for blank samples must be less than 20% of the LLCS peak area of MET and SIT retention.

Limit of Quantification

In a screened plasma lot, six LLOQ standards were prepared and the signal-to –noise ratio (S/N)

was calculated using Shimadzu Lab Solutions software.

Linearity

Calibration standards were developed to get a linearity range of 0.050, 0.101, 0.252, 0.503, 41.006, 2.013, 4.025, 6.008, 8.011 and 10.013µg/ml for MET and 2.010, 4.021, 10.051, 20.103, 40.206, 80.411, 160.822, 240.033, 320.044 and 400.055 for SIT respectively.

Precision and Accuracy

One set contains four different concentrations of quality control standards of LLCS (0.051µg/ml)), MCS (5.038 µg/ml) and HCS (8.53 µg/ml) for MET and LLCS (2.04µg/ml), LCS (6.02µg/ml), MCS (200.78µg/ml) and HCS (340.31µg/ml) for SIT concentration respectively were developed in screened plasma and analyzed each control standards (CS) in six replicates on the same day (Intraday) and five different days (Inter day).

Matrix effect

The matrix effect was determined by comparison with six extracted blank plasma samples in three replicates were spiked with the unextracted concentration of MCS (5.03µg/ml of MET and 200.78µg/ml of SIT) with unextracted standards of the same concentration.

Recovery

The extraction recovery was determined in sextuplicate by comparing the extracted CS with un extracted CS at three different concentrations of LCS (0.151µg/ml), MCS (5.038µg/ml) and HCS (8.539µg/ml) for MET and LCS (6.024µg/ml), MCS (200.788µg/ml) and HCS (340.319µg/ml) for SIT concentrations respectively.

Robustness

Robustness indicates the stability of the developed analytical procedure after the introduction of deliberate variations in the developed method, which can affect its performance, such as accuracy. The flow rate, mobile phase composition and detector wavelength were changed to lower and higher values than the optimized conditions. Changes in the retention time and peak areas were observed and %RSD values were calculated for each factor.

STABILITY STUDIES

Benchtop stability (BTS) (Room Temperature Stability, 24hr)

Six replicates of spiked LCS and HCS (BTS) samples were sit aside at ambient temperature for up to 24hr. samples were processed and compared with newly prepared LCS and HCS (comparison samples).

Freeze and thaw stability (FTS) (after third cycle at -20°C)

Six replicates of low and high concentrations (FTS) samples were frozen at -20°C and subjected to three freeze thaw cycles of 24, 36 and 48h (-20°C to room temperature) and compared with newly prepared LCS and HCS (comparison samples).

Autosampler stability (AS) (2-8°C, 65h)

Six replicates of low and high concentrations (AS) samples were stored in an auto sampler up to 65h at 2-8°C. Stability samples were compared with newly prepared LCS and HCS (comparison samples).

Long term stability (LTS) (-30°C, 45 days)

After completing of the stability period stored at -30°C (45 days), six replicates of LCS and HCS (LTS) samples were compared with newly prepared LCS and HCS (comparison samples).

Clinical samples

Six plasma samples, each with a volume of 0.5ml, were obtained from a diagnostic laboratory (SRK Labs, Guntur, Approval No: SRK/HA/2025-017). These samples were residual specimens from patients diagnosed with type 2 diabetes who were undergoing treatment with MET and SIT. The patients had visited the laboratory for routine diabetic monitoring and biochemical assessments. To ensure confidentially, the samples were anonymized and subsequently stored at -20°C until they were analyzed. The investigators did not perform any direct dosing or blood withdraw. The samples underwent processing and analysis using the optimized SI-DLLME-SFOD coupled HPLC method, with drug concentrations quantified against a calibration curve and results were detailed in table 5.

RESULTS AND DISCUSSION

Analytical quality target profile (ATP) and critical quality attributes(CQAs)

In the present research work a development of a versatile, eco-friendly, and robust chromatographic method for synchronous estimation of MET and SIT in plasma samples with a tailoring factor of 0.9-1.2, a number of theoretical plates more than 2000 and resolution more than 1.5. The analytical target profile (ATP) is the description of quality characteristics of an analytical method with acceptance criteria.

Preliminary trails for failure modes identification

In the present research work, the resolution, the tailing factor and the number of theoretical plates were selected as critical method attributes for the screening of potential method variables. From the results of preliminary trials, potassium dihydrogen ortho phosphate pH 3 and methanol were found to be effective solvents for the separation of MET and SIT. From the results of preliminary experimentation and prior knowledge of chromatography, more than thirty potential risk parameters have been identified and categorized in different class like material, method, machine, measurement , men and mother nature (environment) and shown in Ishikawa (fishbone) diagram for better view of each failure mode for further analysis.

Failure mode effect analysis (FMEA)

By quality risk identification, more than thirty potential risk parameters were identified to be critical for the development of the analytical method as per AT. The screening of all the identified method risk parameters by the design of experiments was a tedious task. Hence, the RPN filtering method was applied for quality risk assessment of identified potential method risk parameters. The listed potential analytical failure modes in the Ishikawa diagram were assessed for their severity, occurrence and detectability for proper peak shape. The RPN scores were calculated for each failure mode and evaluated for their risk in the development of theh RP-HPLC method as per the ICH Q9 guideline. The graph of RPN score versus potential analytical failure mode was plotted to allow a proper review of the criticality of analytical failure modes in RP-HPLC method development.

FMEA was performed by assigning a risk priority number to each failure mode listed in the Ishikawa diagram, based on its occurrence, severity in terms of quality of results and detectability. The occurrence and severity of failure modes were measured by giving a score to each failure mode based on its very low (02), low (04), medium (06), high(08) and very high(10) effect. The score for detectability of risk was given based on its high certainty (02) to low certainty (10). The risk priority number for each method's risk parameter was calculated by multiplying its scores for occurrence (O), severity (S) and detectability (D). All RPN score against the respective method risk parameters (Fig 3) for quality risk assessment. The method risk parameters with an RPN score above 60 were considered critical method risk parameters for method development. Twelve method risk parameters, number of theoretical plates (TP), % Recovery (PR), Resolution, Buffer volume (BV), Methanol, Column, Flow rate (FR), Mobile Phase(MP), Stationary Phase (SP), pH and Wavelength were found critical, having an RPN score of more than 60. Hence, they were selected as critical quality attributes for the development of the chromatographic method as per ATP.

Screening of potential failure modes and CQAs by Taguchi design

Taguchi design was applied for screening of three potential method variables such as MP, WV and pH with two levels and screening of three potential method attributes are resolution, tailing factor and number of theoretical plates using DOE software version 13. Eight experimental runs were suggested in the design matrices. All runs were performed in the laboratory in three replicates, and results were entered against the respective experimental run for analysis by ANOVA. The ANOVA table data and model F values were found at 33.30, 33.30 and 12.96 for resolution, TF and TP respectively. It implies that the model is significant and there is less than a 5% chance that it could occur mainly due to noise. The P-values of MP, Wave length and pH were found to be less than 0.05 in the ANOVA table, which indicated the method risk parameters had a significant main effect on resolution, TF and TP. The P-values of other method risk parameters were found to be above the significant level of 0.05, indicating that none of the method risk parameters are significant for the attributes related to method development. From ANOVA Pareto chart analysis (Fig 4), MP, Wavelength and pH were considered as critical method variables for optimization of chromatographic conditions. From the screening design, Resolution, TF and TP were found significant and were selected as critical analytical attributes.

DOE based response surface analysis by central composite design

Response surface analysis was performed for linking of critical method risk parameters and attributes for optimization of critical method risk parameters. Among all response surface analysis methods, the central composite design was the best response surface design in terms of the minimum experimental runs. Hence, it was selected for response surface analysis. Mobile phase, pH and wavelength were identified as critical method risk parameters that need to be optimized for the development of the analytical method. All three method risk parameters were estimated at three levels in the DOE software by selecting resolution, Tailoring factor and number of Theoretical plates as critical quality attributes for studying their graphical relationship using a DOE based Central Composite Design. Seventeen experimental runs were suggested in design metrices (Table 1). All runs were performed in the laboratory in three replicates and results were entered against the respective experimental run for analysis by ANOVA.

Table 1: Design matrices for central composite design.

Std	Run	Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3
		A:Buffer ml	B:pH	C:Wavelength nm	Resolution	Tailing factor	No. of theoretical plates
6	1	87	2	240	1.7	1.5	2800
9	2	76	3	235	1.4	1.1	2500
5	3	79	2	240	1.3	1.1	2700
13	4	83	3	226.591	1.5	1.3	2500
17	5	83	3	235	1.7	1.5	2700
1	6	79	2	230	1.1	0.9	1800
2	7	87	2	230	1.4	1.2	2700
3	8	79	4	230	1.6	1.4	2800
12	9	83	4.68179	235	2.4	2.2	4400
16	10	83	3	235	1.7	1.5	2700
7	11	79	4	240	2	1.8	3500
15	12	83	3	235	1.7	1.5	2700
8	13	87	4	240	2.3	2.1	4200
14	14	83	3	243.409	2.1	1.9	3100
11	15	83	1.31821	235	1.4	1.2	2600
10	16	89.7272	3	235	2.2	2	4200
4	17	87	4	230	2.2	2	4300

As per the analysis by ANOVA, quadratic model F- values were found at 76.31, 76.33 and 58.67 for resolution, TF and TP respectively, and there is only a 0.01% chance that an F-value this large could occur due to noise. Values of probability less than 0.05 indicate model terms are significant. In this case, the volume of mobile phase and extraction solvent are the main significant effects for responses, while other interactions are not significant for the development of the chromatographic method. The quadratic terms of the volume of mobile phase and extraction solvent were found to the significant ($p < 0.05$), indicating a quadratic relationship between these failure modes and the selected CPAs. The R^2 values , the adjusted R^2 value and the predicted R^2 value for the resolution were found to be 0.9339 and 0.9008 respectively. The R^2 values, the adjusted R^2 value and the predicted R^2 value for the tailing factor were found to be 0.9339 and 0.9004 respectively. The R^2 values, the adjusted R^2 value and the predicted R^2 value for the number of theoretical plates were found to be 0.9701 and 0.9010 respectively. The difference between the adjusted R^2 value and the predicted R^2 value is less than 0.2, which implies that the prediction power of the selected mode was satisfactory. The adequate precision value for the resolution, TF and TP was found to be greater than 4.0, indicating that the model was precise in predicting responses. The 3D contour plots (Fig 5) showed that quadratic models for Resolution, TF and TP were found to be significant for the development of the target RP-HPLC method for simultaneous estimating MET and SIT using GLI as an internal standard in human plasma samples. The 3D surface plots indicate that optimal chromatographic performance is achieved under specific conditions. A resolution of 2.4, T F of 2.2 and a maximum TP 4400 are observed. This synergistic effect significantly enhances extraction efficiency, making it crucial for optimizing bioanalytical HPLC sample preparation to achieve high and consistent recovery rates.

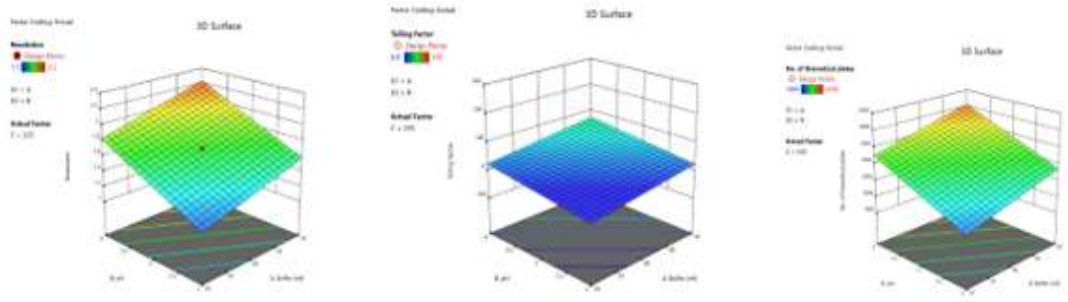

Fig 5. 3D contour plots showing the interaction effects between Buffer and pH on Resolution, Tailing factor and number of theoretical plates.

The responses were optimized by the following model equations

Resolution = + 1.75 Buffer +0.2517 pH +0.3135 wavelength + 0.1471, -10.58298 buffer +0.53919 pH +0.313527 wavelength +0.029422.

Tailing factor =+1.54 Buffer +0.2280 pH +0.3135 wavelength + 0.1471, -11.04439 Buffer +0.056997 Ph +0.313527 wavelength +0.029422.

Number of theoretical plates =+2709.54 Buffer +443.66 pH+573.14 Wavelength +191.04, -1.17391E+05 Buffer +3.05.05768 pH -1689.27624 wavelength +851.25663.

Navigation of MODR

MODR is a term used to represent the design space in the AQBD approach. The quadratic model for resolution , TF and TP was utilized for navigating MODR to optimize CMPs and achieve optimal chromatographic performance (yellow shade in Fig 6). The yellow region indicates conditions where the resolution is 1.5, TF is acceptable (1.2-1.4), and the TP is high (> 2200}. Different theoretical solutions were proposed by the models, and they were tested in the laboratory to confirm the validity of the models and assess the risk migration. The actual values of resolution, TF and TP were found close to predicted values given by software that indicate the risk of the CMPs was reduced at an acceptable level and could be used for framing control strategy for the development of a versatile, economical, eco-friendly and robust chromatographic method for simultaneous estimation of MET and SIT using GLI as an internal standard in biological samples.

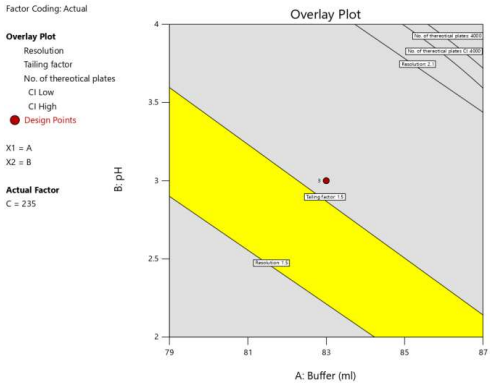

Fig 6. Overlay Plots Showing the Method Operable Design Region (MODR) in Yellow for the combined Influence of buffer and pH on analytical Response Parameters.

Control strategy and model validation

The model validation was performed by measuring the deviation between the predicted values of resolution and tailing factor from actual values and results showed a < 2% relative standard deviation (RSD), indicating that the model was valid for the prediction of responses. From MODR, the operating range of mobile phase composition (6Mm potassium dihydrogen ortho phosphate) was found to be 232 to 240nm. The control strategy was developed for all identified method risk parameters, along with CMPs, to guide the development of the target chromatographic method. From the control strategy perspective, the chromatographic separation of MET and SIT using GLI as an internal standard under study was performed using 6mM potassium dihydrogen ortho phosphate Ph 3 in combination with methanol (83:17%v/v) on a Inertsil ODS column (250mm × 4.6mm, 5.0µm), the retention time of MET and SIT using GLI as an internal standard was found to be 3.363, 5.683 and 9.365 min, and the tailing factor was observed to be within the range of 0.9-1.4, as per the ATP.

Method Development

The suggested technique has been verified as environmentally friendly, in accordance with ICH guidelines. It utilizes an eco-solvent system, accessible via various tools and software, to estimate the synchronous concentration of MET and SIT in plasma samples. A mobile phase consisting of 6mm potassium dihydrogen ortho phosphate Ph 3 (adjusted using ortho phosphoric acid): Methanol (83:17% v/v), gave the best signal along with a marked improvement in the peak shape and low baseline noise was observed using the Intersil ODS (250mm × 4.6mm, 5.0µm) analytical column with a flow rate of 0.8 mL/min. The column temperature was maintained at a constant temperature of approximately 25°C, and the temperature of the autosampler was maintained at 4°C. The retention time of the analyte (MET, SIT using GLI) was eluted at 3.36, 5.68 and 9.36 min with a 10 min total runtime. Different procedures, including PPT (Protein Precipitation), SPE (Solid- phase Extraction) and LLE (Liquid-Liquid extraction), were optimized. Out of all, it was observed that the LLE followed by ultra wave assisted sonication using a deep eutectic solvent (UA-DES-DLLME) was suitable due to its less matrix effect, sustainability and high recovery (Fig 7).

Optimization of pH

For the RP-HPLC bioanalysis of synchronous estimation of MET and SIT using GLI in plasma, extraction conditions were optimized by adjusting the pH to enhance analyte recovery using a deep eutectic solvent. Maximum recovery was observed at pH 3.0, facilitating efficient extraction into the DES phase.

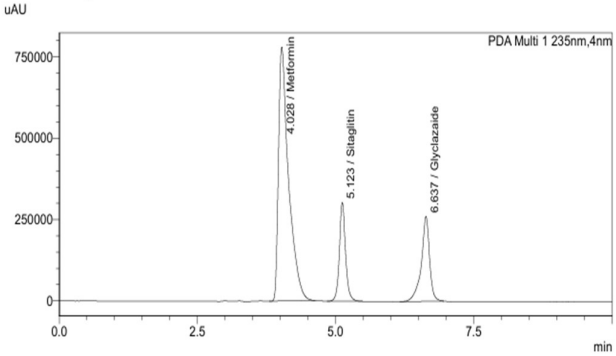

Fig 7. Chromatograms of metformin and sitagliptin using Gliclazide as an internal standard optimisation of hydrophobic deep eutectic solvents.

To identify the most effective hydrophobic deep eutectic solvent (HDES) system for enhanced bioanalytical extraction, a range of HDES combinations were systematically optimized by varying TBAC (Tetrabutylammonium Chloride), TBAB (Tetrabutylammonium Bromide), Choline Chloride(Chcl) as hydrogen bond acceptor (HBA) and 1-Dodecanol, methanol, decanoic acid, octanoic acid as hydrogen bond donor (HBD) along with their molar ratios of 1:1, 1:2 and 2:1. Among the tested combinations, TBAC-1- dodecanol in a 2:1 molar ratio exhibited the highest extraction recovery (97.64%), indicating superior hydrophobic interactions and solubilization efficiency for the target analyte. Other promising combinations included TBAC : Methanol (1:1) with a recovery of 75.2% and TBAB :Methanol(1:2) at 70:30%. In contrast , DESs involving choline showed comparatively lower recovery values, with ChCl : 1-dodecanol (1:2) yielding 55.50%. This optimization study demonstrates that the nature and ratio of HBA and HBD significantly influence extraction efficiency, and TBAC : 1-dodecanol (2:1) was selected for further method development due to its excellent performance.

METHOD VALIDATION

Selectivity and Specificity, Limit of Quantification (LOQ)

No significant response was observed as a retention time of MET and SIT in blank plasma as compared to LLOQ and blank samples. The limit of quantification for this method was proven as the lowest concentration of the calibration curve, which was 0.050 µg/ml for MET and 2.010 for SIT respectively.

Linearity

The developed bioanalytical method demonstrates well-defined calibration ranges for MET (0.050-10.013µg/ml) and SIT (2.010-400.055 µg/ml), using GLI as an internal standard. multiple calibration and QC levels (LLOQ, LQC, MQC, HQC) ensure robust method validation. Linearity assessment showed excellent calibration performance with R^2 >0.998 for both analytes. MET quantification displayed high precision (%CV < 3%) and accuracy near 100%. SIT calibration similarly achieved strong linearity ($R^2 \geq 0.9986$) and precision (%CV < 5%). These results confirm the method's sensitivity, reliability and accuracy. The structured approach supports its application in pharmacokinetic, clinical and bioanalytical studies (Table-1 and 2).

Precision and Accuracy

Precision and Accuracy validation confirmed reliable quantification of MET and SIT across all QC levels, with

mean values near nominal concentration and %CV mostly below 10%. Both within and between batch results MET bioanalytical validation limits for LLOQ, LQC, MQC and HQC demonstrating strong reproducibility, sensitivity and robustness for clinical and pharmacokinetic plasma analysis (Table-1 and 2).

Recovery studies

Recovery studies showed consistent efficiency for MET and SIT across all QC levels, with overall recoveries of 82.31% (CV% = 0.74) and 87.16% (CV% = 0.57) respectively. The internal standard GLI achieved a mean recovery of 78.33% confirming extraction consistency. These results demonstrate efficient , reproducible plasma concentration and support the reliability of the sample preparation procedure for quantitative analysis.

Matrix effect

Matrix effect evaluation for MET showed negligible plasma interference with %nominal values of 101.66% (LQC) and 100.11% (HQC) and low variability (CV% ≤ 2.27%). Sensitivity assessment confirmed suitability, with LLOQ precision at %CV 7.34% and % nominal 103.00%. calibration remained strong (R^2 = 0.9960), demonstrating selectivity, sensitivity and minimal matrix interference, ensuring reliable plasma quantification of MET.

Robustness

The robustness of the method was evaluated by introducing deliberate variations in analytical parameters. The resulting % RSD values were below 5.0%, confirming the methods robustness and reliability under minor procedural deviations (Table 3).

Long term stability studies (30 days)

Long term stability studies at -70°C over 30 days confirmed MET and SIT stability, with calibration curves remaining highly linear (R^2 = 0.9990 for MET; 0.9998 for SIT). QC samples showed % nominal values within 96.40-104.33% (MET) and 97.96-101.78%(SIT), with % stability above 96%. These findings validate the storage protocol and ensure reliable quantification for pharmacokinetic and clinical studies.

Stability (Autosampler stability, bench top stability, wet extract stability, freeze thaw stability, short term stability at -20°C).

Comprehensive stability studies confirmed that MET and SIT remain stable under various storage and handling conditions, including autosampler, bench-top, wet extract and freeze thaw cycles. Both analytes maintained % nominal concentrations of 90 to 104% with low variability and freeze thaw cycling showed minimal impact on integrity. Short term stability at -20°C exceeded 97% for both drugs and calibration curves remained highly linear (R^2 > 0.996). These results validate the method robustness for accurate quantification across diverse laboratory scenarios (Table-1 and 2).

Central composite design experiment selectivity

CCDE selectivity testing confirmed high specificity and reproducibility for MET and SIT at the LLOQ, with mean areas of 12,753.0 % (CV 2.87%) for MET and 26,600.5% (CV 3.18%) for SIT. The internal standard showed consistent response with % CV 0.85%. These results demonstrate precise, robust quantification and effective separation from plasma components, supporting the methods suitability for bioanalytical applications.

Table 1. Validation performance characteristics of the developed bioanalytical RP-HPLC method for Metformin quantification in human plasma.

a. Linearity and Accuracy					
Parameter	**Nominal Conc. (µg mL^{-1})**	**Measured Conc. (µg mL^{-1})**	**SD**	***%RSD**	***%Accuracy**
Linearity range	0.050–10.000	0.050–10.013	0.00048-0.20493	0.98–2.67	98.52–103.14
Correlation coefficient (r^2):0.9998					

b. Intra-day and Inter-day Precision & Accuracy						
Spiked Plasma Concentration (µg mL^{-1})	**Within-run (Intra-day)**			**Between-run (Inter-Day)**		
	***Concentration measured (µg mL^{-1}) mean±S.D**	**%RSD**	**%Accuracy**	***Concentration measured (ng mL^{-1}) mean±S.D**	**%RSD**	**%Accuracy**
0.051	0.00548	10.63	101.05	0.00621	11.53	105.56
0.151	0.00507	3.36	99.93	0.00543	3.63	99.06
5.038 8.539	0.04972 0.22732	0.99 2.72	99.52 97.84	0.03166 0.21091	0.63 2.54	99.66 97.26
c. Recovery						

Drug	**Concentration(µg mL^{-1})**	***%Recovery**	**%RSD**
MET	LQS	81.78	2.08
	MQS	82.16	4.50
	HQS	82.97	4.12

Mean		82.31	0.74
d. Matrix factor			
Drug	**Concentration (μg mL^{-1})**	***%Matrix factor**	**%RSD**
MET	LQS	101.66	2.27
	HQS	100.11	1.12
e. Matrix stability			

Spiked Plasma concentration (μg mL^{-1})	**Room temperature Stability (24h)**		**Wet Extract Stability**		**Autosampler stability**		**Freeze and thaw stability 3rd Cycle (48h)**	
	***Concentration measured (μg mL^{-1}) mean±S.D**	***%RSD**	***Concentration measured (μg mL^{-1}) mean±S.D**	***%RSD**	***Concentration measured (μg mL^{-1}) mean±S.D**	***%RSD**	***Concentration measured (μg mL^{-1}) mean±S.D**	***%RSD**
0.151	0.1513±0.00550	3.64	0.1575±3.65	3.65	0.1528±0.00075	4.96	0.1440±0.00540	3.75
8.539	8.5080±0.07830	0.92	8.470±0.06489	0.77	8.4760±0.0774	0.91	8.3797±0.13843	1.65

*Mean of six replicates (n=6)

Table 2. Validation performance characteristics of the developed bioanalytical RP-HPLC method for Sitagliptin quantification in human plasma.

f. Linearity and Accuracy					
Parameter	**Nominal Conc. (μg mL^{-1})**	**Measured Conc. (μg mL^{-1})**	**SD**	***%RSD**	***%Accuracy**
Linearity range	2.000–400.000	2.010–400.055	0.03158-9.53932	1.57–3.03	94.03–103.11
Correlation coefficient (r^2):0.9996					

g. Intra-day and Inter-day Precision & Accuracy						
Spiked Plasma Concentration (μg mL^{-1})	**Within-run (Intra-day)**			**Between-run (Inter-Day)**		
	***Concentration measured (μg mL^{-1}) mean±S.D**	**%RSD**	**%Accuracy**	***Concentration measured (ng mL^{-1}) mean±S.D**	**%RSD**	**%Accuracy**
2.048	0.11515	5.66	99.27	0.12477	6.09	100.10
6.024	0.13611	2.27	99.51	0.06973	1.16	99.91
200.788	1.48760	0.74	99.80	1.36547	0.68	99.75
340.319	15.67158	4.53	101.61	1.20145	0.35	100.02
h. Recovery						

Drug	**Concentration(μg mL^{-1})**	***%Recovery**	**%RSD**
MET	LQS	86.66	1.69
	MQS	87.16	3.80
	HQS	87.66	2.36
Mean		87.16	0.57
i. Matrix factor			

Drug	**Concentration (μg mL^{-1})**	***%Matrix factor**	**%RSD**
MET	LQS	100.30	1.66
	HQS	99.94	0.37
	i. Matrix stability		

Spiked Plasma concentration (μg mL^{-1})	Room temperature Stability (24h)		Wet Extract Stability		Autosampler stability		Freeze and thaw stability 3rd Cycle (48h)	
	*Concentration measured (μg mL^{-1}) mean±S.D	*%RSD	*Concentration measured (μg mL^{-1}) mean±S.D	*%RSD	*Concentration measured (μg mL^{-1}) mean±S.D	*%RSD	*Concentration measured (μg mL^{-1}) mean±S.D	*%RSD
0.151	5.9825±0.21675	3.62	5.7197±0.22735	3.97	5.4223±0.36617	6.75	6.0453±0.10758	1.78
8.539	340.1230±1.85326	0.54	338.8798±2.30708	0.88	340.5262±1.50775	0.44	340.5567±1.19981	0.35

*Mean of six replicates (n=6)

Table 3. Robustness data

Parameter	Variation	Retention Time (min)	%RSD	Peak Area	%RSD	Retention Time (min)	%RSD	Peak Area	%RSD
		Metformin				Sitagliptin			
Flow rate (0.8 ± 0.1 mL/min)	0.7 mL/min	4.93	0.62	2613280	0.53	6.84	0.58	1845621	0.61
	0.9 mL/min	4.89	0.34	2582851	0.29	6.77	0.41	1829348	0.37
Buffer volume (30 ± 2 mL)	28 mL	4.91	0.75	2581439	0.46	6.80	0.69	1834472	0.52
	32 mL	4.93	0.49	2593096	0.27	6.83	0.45	1840189	0.34
Wavelength (235 ± 2 nm)	233 nm	4.88	0.36	2586713	0.41	6.79	0.39	1832284	0.43
	237 nm	4.92	0.61	2579670	0.34	6.82	0.56	1827563	0.38
Column temperature (40 ± 2 °C)	38 °C	4.89	0.76	2598427	0.52	6.81	0.64	1839055	0.47
	42 °C	4.91	0.32	2594319	0.46	6.78	0.35	1836748	0.40

The robustness of the developed RP-HPLC method was evaluated by introducing small deliberate variations in flow rate, buffer volume, detection wavelength, and column temperature. The retention times for Metformin (4.88–4.93 min) and Sitagliptin (6.77–6.84 min) showed minimal variation under all tested conditions. Similarly, peak area responses remained consistent for both analytes. The %RSD values for retention time and peak area were found to be below 1% for both Metformin and Sitagliptin, indicating high precision under varied conditions. Slight changes in flow rate and wavelength resulted in negligible shifts in chromatographic behavior, while buffer volume and column temperature variations did not significantly affect analyte detection. Overall, the results demonstrate that the method is robust, as minor changes in chromatographic parameters did not significantly influence retention time or peak area. This confirms the reliability and suitability of the method for routine simultaneous estimation of Metformin and Sitagliptin.

Application to clinical samples

The HPLC method that was developed and validated in this study proved to be effective to measure MET and SIT concentration in the six patient's samples. The method was able to resolve both analytes without significant interference from endogenous plasma components, confirming, their selectivity in real biological matrices. As shown in table 6, the plasma concentrations of MET ranged from 190 to 980ng/ml. (mean ± SD; 592 ± 267ng/ml), while SIT concentrations ranged from 135 to 995 ng/ml. (mean ± SD; 536 ± 293 ng/ml) across the patient samples. All the concentrations were above the LLOQ and within the calibration range. The concentrations observed in patient plasma samples align with published C_{max} values for 500mg dosing in clinical pharmacokinetic studies. For MET extended release (500 mg twice daily), mean C_{max} was reported as 607ng/ml (increasing to 905ng/ml at 1000mg DR) and SIT, single – dose ascending studies in healthy Caucasians reported C_{max} values as low as 488ng/ml at 500mg, indicating that our linearity window is appropriate for accurate quantification. The successful detection of both drugs in patient using the SI-DLLME-SFOD extraction followed by HPLC demonstrates the practical applicability of the method under real clinical conditions. Importantly, the recovery and sensitivity of the method were sufficient to detect concentrations within the therapeutic window, confirming its suitability for pharmacokinetic monitoring and therapeutic drug monitoring purposes (Table 4).

Table 4. Patient plasma samples concentration levels.

Number of patients	Metformin Conc (µg/ml)			Sitagliptin Conc (µg/ml)		
1	255.13	254.98	255.20	53.16	53.11	53.19
2	790.64	790.52	790.65	103.29	103.26	103.28
3	423.81	423.79	423.82	198.67	198.65	198.66
4	78.32	78.16	78.40	350.22	350.29	350.19
5	290.43	290.40	290.45	107.84	104.88	104.82
6	560.79	560.76	590.82	224.07	224.09	224.09

ASSESSMENT OF GREENNESS FOR DEVELOPED METHOD

Analytical GREEness (AGREE)

The optimized analytical method was assessed using two recognized green analytical chemistry (GAC) metrices: AGREE and MoGAPI AGREE, developed by Pena Pereira et al. evaluates analytical procedures based on the 12 principles of green analytical chemistry. Each principle is represented as a segment in a circular pictogram, where the color gradients from red to green indicate the level of compliance. The overall greenness score ranges from 0 to 1, with values above 0.6 generally considered green. The optimized method achieved a score of 0.85 (Fig. 8), demonstrating a high degree of environmental compatibility.

AGREE prep

This metric evaluates the impact of sample preparation on environmental techniques, representing a significant advancement in analytical chemistry. This tool helps optimize their techniques for ecological sustainability without compromising analytical quality (19). Each of the ten criteria in this assessment system has a value ranging from 0 to 1. The program combined weighted scores to provide an overall rating, which was displayed as a round pictogram and ranged from 0 to 1. Ten weighed trapezoid bars, each representing a different criterion, encircle and display the overall score in this representation. With a score of 0.79, as illustrated in fig 8, the optimized approach proved to be more sustainable.

MoGAPI

The MoGAPI tool introduced by Plotka–Wasylka [20] provided a holistic evaluation of the entire analytical workflow. It uses a five part pictogram with color – coded segments to indicate the environmental and health impact of each procedural step. Green denotes minimal impact , yellow denotes moderate impact, and red denotes high impact. The optimized *method* exhibited six green and only three red segments, reflecting a strong environmental profile. Compared to earlier chromatographic methods, the present HPLC method showed a total MoGAPI score of 78 (Fig. 8).

Blue applicability grade index (BAGI)

The BAGI tool (21) provides a comprehensive assessment of the environmental performance and the overall flexibility of an analytical method. This tool assigns a score between 25 and 100 based on ten key criteria, including technique, reagents, sample preparation and automation. Higher scores indicate better alignment with green and practical analytical principles. The results are displayed using a color-coded asteroid chart, where blue shades reflect increasing levels of conformance. As shown in fig 8. The optimized method scored 75, which confirms the superior greenness of the technique.

Assessment of redness

The red analytical performance index (RAPI) [22] is a novel tool designed to quantitatively and visually assess the analytical performance of various methods. It complements the existing greenness metrics by focusing on validation – related and functional criteria. The RAPI assesses ten key performance criteria, all of which are equally weighed in a five-level scoring system (0, 2.5, 5.0, 7.5 and 10). A total score (0-100) was displayed centrally in the output pictogram to reflect the overall performance of the method. As shown in fig 8, the developed method was evaluated using the RAPI and was found to have a total score of 80.

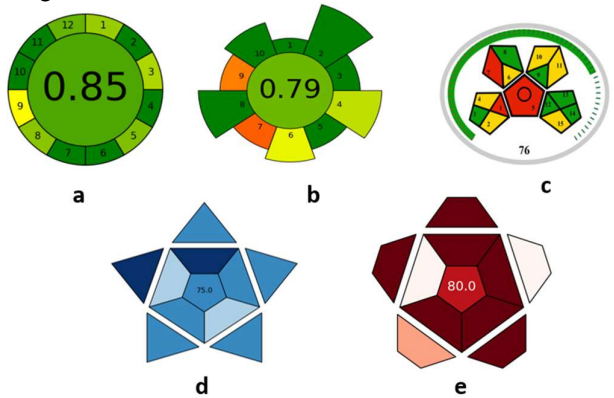

Fig 8. Visual assessment of analytical method greenness using multiple greenness evaluation tools: a) AGREE (b)

AGREE prep c) MoGAPI d) BAGI e) RAPI.

CONCLUSION

A robust and environmentally sustainable bioanalytical method has been successfully developed and validated for the synchronous estimation of MET and SIT using GLI as an internal standard by RP-HPLC. The integration of (QbD) principles facilitated systematic risk assessment and design space optimization , ensuring method robustness and reproducibility. The application of microextraction techniques minimized sample preparation complexity, improved extraction efficiency, and reduced organic solvent usage, thereby aligning with the objectives of green analytical chemistry. Furthermore , a comprehensive green assessment confirmed the method's eco-friendly profile, emphasizing its sustainability. The validated method demonstrates high precision, accuracy, linearity and sensitivity, making it highly suitable for pharmaceutical quality control, bioanalytical studies and regulatory submissions. Overall, this research establishes a scientifically rigorous, cost-effective and environmentally responsible analytical platform for routine analysis. The clinical sample analysis gave an insight into the method's clinical applicability, where the aim was to determine drug exposure in the study population. This method can be extended to pharmacokinetic research, therapeutic monitoring and toxicological studies as per regulatory compliance.

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