

Analytical Quality by Design-Assisted UPLC–PDA Method for Determination of Navepegritide in Rat Plasma: Validation, Sustainability Assessment, and Pharmacokinetic Application

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Abstract

A rapid, robust, and sustainable bioanalytical method was developed for the determination of navepegritide in rat plasma using an analytical quality by design (AQbD)-assisted ultra-performance liquid chromatography with photodiode array detection (UPLC–PDA). A three-factor, three-level Box–Behnken design was employed to systematically evaluate the effects of acetonitrile content, mobile-phase pH, and flow rate on critical chromatographic attributes, including retention time, tailing factor, and theoretical plate count of navepegritide and vosoritide, which was used as the internal standard. Numerical optimization based on response-surface methodology identified 0.1% (v/v) formic acid in water (50:50, v/v), pH 3.0, and a flow rate of 0.5 mL min⁻¹ as the optimum chromatographic conditions. Separation was achieved on an ACQUITY UPLC BEH Phenyl C18 column (2.1 × 100 mm, 1.7 μm) with detection at 272.4 nm and a total run time of 5 min. Plasma samples were processed using a simple protein-precipitation procedure. The method demonstrated satisfactory selectivity and linearity over 300–12,000 ng mL⁻¹, with a coefficient of determination (R²) of 0.99994. The limit of detection and lower limit of quantification were 100 and 300 ng mL⁻¹, respectively. Matrix-factor values ranged from 97.01 to 97.18%, with variability below 2%. Accuracy ranged from 96.31 to 98.60%, while intra- and inter-day precision ranged from 0.16 to 2.06%. Carryover remained below 20% of the lower limit of quantification response. Navepegritide demonstrated acceptable stability under bench-top, freeze–thaw, processed-extract, autosampler, short-term frozen, and long-term frozen conditions. The method was successfully applied to a single-dose pharmacokinetic study in rats, where a C_{max} of 5896.13 ng mL⁻¹ was attained at 1.5 h, with reported AUC_{0–t} and AUC_{0–∞} values of 39,779 ng·h mL⁻¹ and a terminal half-life of 20 h. Multicriteria sustainability assessment using AGREE, AGREEprep, Analytical Eco-Scale, CaFRI, CACI, ComplexGAPI, ComplexMOGAPI, and BAGI provided a multidimensional evaluation of the environmental and practical characteristics of the procedure. The developed AQbD-assisted UPLC–PDA method therefore provides a rapid, validated, and practically applicable approach for quantification of navepegritide in rat plasma and its use in preclinical pharmacokinetic investigations.

Keywords: Navepegritide; Rat plasma; UPLC–PDA; Analytical Quality by Design; Bioanalytical method validation; Pharmacokinetics.

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INTRODUCTION

Navepegritide is a therapeutic peptide under investigation for the management of growth-related disorders and represents an emerging pharmacological approach in the regulation of skeletal growth [1, 2]. As a biologically active peptide, its pharmacological activity is closely associated with systemic exposure, absorption characteristics, distribution, and elimination. Consequently, reliable characterization of the plasma concentration–time profile of navepegritide is essential during preclinical drug

development for pharmacokinetic evaluation, dose selection, exposure–response assessment, and translational investigations [3]. Quantitative determination of peptide therapeutics in biological matrices, however, is analytically challenging because of their relatively complex molecular characteristics, susceptibility to degradation, low concentrations in plasma, and potential interference from endogenous matrix components. These considerations necessitate the development of a selective, sensitive, precise, and reproducible bioanalytical procedure capable of

accurately quantifying navepegritide in biological matrices [4].

Ultra-performance liquid chromatography (UPLC) provides several advantages for bioanalytical applications, including enhanced chromatographic efficiency, improved resolution, shorter analysis times, and reduced mobile-phase consumption compared with conventional high-performance liquid chromatography [5]. These characteristics are particularly advantageous for peptide analysis, where adequate separation from endogenous plasma components and potential degradation products is essential for reliable quantification [6]. In the present study, UPLC with photodiode-array detection was investigated for the determination of navepegritide in rat plasma.

Vosoritide was employed as the internal standard to compensate for variations associated with sample preparation, injection, and instrumental response. The suitability of vosoritide as the internal standard was established experimentally based on its chromatographic behavior, adequate resolution from navepegritide and endogenous plasma constituents, consistent detector response, and satisfactory performance during the validation experiments [7]. The selection of an appropriate internal standard is particularly important in plasma-based quantitative analysis because it contributes to improved reproducibility and minimizes the influence of analytical variability throughout the sample-processing and chromatographic procedures.

Conventional analytical method development frequently depends on sequential experimentation and empirical adjustment of chromatographic variables. Such approaches may require extensive experimentation and provide limited understanding of the interaction between method parameters. Analytical Quality by Design (AQbD) offers a systematic, science-driven alternative in which method development begins with the definition of an Analytical Target Profile (ATP), followed by identification of critical method attributes (CMAs), critical method parameters (CMPs), risk assessment, and statistically guided optimization using Design of Experiments (DoE) [8]. Implementation of AQbD facilitates identification of influential chromatographic variables, evaluation of their individual and interactive effects, and establishment of an experimentally supported region in which the method can consistently achieve its intended analytical performance. This risk-based strategy can therefore enhance method robustness, reproducibility, and lifecycle understanding while reducing dependence on empirical trial-and-error optimization [9].

Along with analytical performance, the environmental impact of bioanalytical procedures has become an

increasingly important consideration in contemporary pharmaceutical analysis. Green Analytical Chemistry (GAC) emphasizes the reduction of hazardous reagents, solvent consumption, analytical waste, energy requirements, and unnecessary sample-processing steps while maintaining the required analytical quality [10]. UPLC can contribute to improved analytical sustainability through reduced solvent consumption and shorter chromatographic analysis times; however, the overall environmental profile of a bioanalytical method also depends on sample preparation, solvent selection, waste generation, and operational requirements. Therefore, the developed procedure was evaluated using complementary GAC assessment approaches, including the Analytical GREENness metric (AGREE), AGREEprep, Analytical Eco-Scale, ComplexGAPI, ComplexMOGAPI, Carbon Footprint Reduction Index (CaFRI), Click Analytical Chemistry Index (CACI), and Blue Applicability Grade Index (BAGI). The combined application of these tools enabled assessment of the environmental sustainability as well as the practical applicability of the proposed analytical procedure rather than relying on a single greenness metric [11].

A comprehensive literature evaluation indicated a limited availability of validated chromatographic procedures specifically addressing the quantitative determination of navepegritide in rat plasma. The absence of a sufficiently characterized bioanalytical procedure may restrict reliable preclinical pharmacokinetic investigations and subsequent exposure assessment of this emerging therapeutic peptide. Therefore, the present study was undertaken to develop and validate a rapid, selective, precise, and robust AQbD-assisted UPLC method for the quantitative estimation of navepegritide in rat plasma using vosoritide as the internal standard. The developed method was systematically optimized through a risk-based AQbD strategy and subsequently validated for its intended bioanalytical application. Furthermore, the environmental sustainability and practical applicability of the method were comprehensively investigated using multiple GAC assessment tools. The validated procedure was subsequently applied to rat plasma samples obtained from a pharmacokinetic study, demonstrating its suitability for quantitative exposure assessment of navepegritide in a preclinical setting.

MATERIALS AND METHODS

Chemicals and reagents

Navepegritide and vosoritide reference standards were obtained from Glenmark Pharmaceuticals Ltd., Hyderabad, India. HPLC/UPLC-grade acetonitrile and methanol, along with formic acid and triethylamine, were purchased from Merck Life Science Pvt. Ltd.,

Mumbai, India. Ultrapure water was produced using a Milli-Q water purification system (Millipore, Bedford, MA, USA) and had a resistivity of 18.2 M Ω ·cm at 25 °C with a total organic carbon (TOC) content of <10 ppb. Unless otherwise specified, all solvents and reagents used for chromatographic method development, sample preparation, method optimization, validation, and pharmacokinetic analysis were of analytical reagent or UPLC grade. All materials were used as received without further purification.

Preparation of the mobile phase

The mobile phase consisted of 0.1% (v/v) formic acid in ultrapure water and UPLC-grade acetonitrile in a 50:50 (v/v) ratio. The aqueous and organic components were combined under continuous stirring to ensure uniform mixing. The pH of the aqueous phase was adjusted to 2.8–3.2 using triethylamine to obtain suitable chromatographic retention and peak characteristics for navepegritide and vosoritide. The prepared mobile phase was filtered through a 0.22 μ m PTFE membrane filter and degassed by ultrasonication for 10 min before use. The freshly prepared, filtered, and degassed mobile phase was used throughout method development, AQbD optimization, method validation, and analysis of rat plasma samples to maintain consistent chromatographic performance and reproducibility.

Preparation of navepegritide standard solution

An accurately weighed quantity (5 mg) of navepegritide reference standard was transferred to a 10 mL volumetric flask and dissolved using the selected diluent. The solution was made up to volume with the same diluent to obtain the primary stock solution. A 4.8 mL aliquot of this solution was subsequently transferred to a 10 mL volumetric flask and diluted to volume with the diluent. From the resulting solution, 1 mL was transferred to a 10 mL volumetric flask and diluted to volume to obtain the working standard solution. The prepared solution was used for calibration-standard and quality-control sample preparation.

Preparation of vosoritide internal standard solution

A 5 mg quantity of vosoritide reference standard was accurately weighed and transferred to a 10 mL volumetric flask. The standard was dissolved and diluted to volume with the selected diluent. Subsequently, 2.4 mL of the resulting solution was transferred to a 10 mL volumetric flask and diluted to volume. An aliquot of 1 mL of this solution was further transferred to a 10 mL volumetric flask and diluted to volume with the diluent to obtain the working internal-standard solution at a final concentration of 12,000 ng/mL. The solution was used for spiking plasma samples during sample preparation.

Preparation of calibration and spiked plasma samples

Calibration standards of navepegritide were prepared over the concentration range of 300–12,000 ng/mL by appropriate dilution of the working standard solution with the selected diluent. For preparation of spiked plasma samples, 200 μ L of rat plasma was transferred to a centrifuge tube, followed by the appropriate volume of navepegritide standard solution, 500 μ L of vosoritide internal-standard solution, and 300 μ L of acetonitrile. The volume was adjusted with the selected diluent where required to obtain the desired final calibration concentration. The samples were thoroughly mixed and subjected to centrifugation at 4000 rpm for 20 min. The resulting supernatant was collected, appropriately filtered, transferred to an autosampler vial, and injected into the UPLC system.

Plasma sample extraction procedure

Rat plasma samples were processed using a protein-precipitation approach. Briefly, 200 μ L of rat plasma was transferred into a labelled centrifuge tube. 500 μ L of diluent was added and the mixture was thoroughly homogenized. Subsequently, 300 μ L of methanol was added as the protein-precipitating solvent, and the sample was mixed using a vortex mixer to facilitate protein precipitation and analyte extraction. The samples were then centrifuged at 4000 rpm for 20 min. Following centrifugation, the clear supernatant was carefully separated without disturbing the precipitated protein pellet, transferred to an appropriate LC vial, and subjected to UPLC analysis.

UPLC instrumentation and optimized chromatographic conditions

Chromatographic analysis was performed using an ACQUITY™ UPLC system (Waters Corporation, Milford, MA, USA) equipped with a Binary Solvent Manager (BSM), Sample Manager–Flow Through Needle (SM-FTN), and photodiode-array (PDA) detector. Instrument control, chromatographic data acquisition, peak integration, and quantitative calculations were performed using Empower™ 3 chromatography software (Waters Corporation). Separation of navepegritide and vosoritide (internal standard) was achieved on an ACQUITY UPLC BEH Phenyl C18 column (2.1 $\times$ 100 mm, 1.7 μ m; Waters Corporation). The column was selected following chromatographic screening and AQbD-based optimization based on its favorable separation efficiency, peak symmetry, retention characteristics, and reproducibility. The optimized mobile phase consisted of 0.1% (v/v) formic acid in ultrapure water and acetonitrile (50:50, v/v), with the aqueous phase adjusted to pH 2.8–3.2 using triethylamine. Chromatographic separation was performed under isocratic elution at a flow rate of 0.5 mL min^{–1}. The PDA detector was set at 272.4 nm, which was

established as the common isosbestic wavelength for navepegritide and vosoritide based on their PDA spectral profiles. This wavelength enabled simultaneous monitoring of both compounds with consistent detector response. The injection volume was 5 μL , and the total chromatographic run time was 5 min.

Analytical Quality by Design strategy

Response surface optimization using Box–Behnken experimental design

Following preliminary chromatographic screening and risk assessment, the Analytical Quality by Design (AQbD) approach was implemented to systematically optimize the chromatographic conditions for the determination of navepegritide in rat plasma using vosoritide as the internal standard. A Box–Behnken Design (BBD) was selected as the response surface experimental design and generated using Design-Expert® software. The design enabled simultaneous investigation of the linear, quadratic, and interaction effects of the selected chromatographic variables on method performance while minimizing the number of experimental runs compared with conventional full-factorial experimentation. The reduced experimental requirement also contributed to lower solvent consumption, sample utilization, and instrument operating time, supporting the sustainability objectives of the analytical procedure [12, 13].

Three critical method parameters (CMPs) identified from the preliminary risk assessment were selected for experimental optimization: acetonitrile content in the mobile phase (45–55%, v/v), mobile-phase pH (2.8–3.2), and flow rate (0.4–0.6 mL min^{-1}). Each factor was investigated at three levels, coded as -1 , 0 , and $+1$, representing the low, central, and high experimental settings, respectively. The corresponding experimental combinations were generated according to the BBD matrix, and the chromatographic experiments were conducted in a randomized sequence to minimize the influence of systematic variation and experimental bias. The chromatographic responses selected for optimization comprised retention time (RT), tailing factor (TF), and theoretical plate number (TP) for navepegritide and vosoritide. These responses were selected as critical analytical performance characteristics because they collectively provide information regarding chromatographic retention, peak symmetry, and column efficiency, which are essential for reliable quantitative determination in the presence of the rat plasma matrix. The experimental response data were subjected to response surface analysis, and suitable second-order polynomial models were fitted to describe the relationship between the CMPs and the selected chromatographic responses [14].

The statistical significance and adequacy of the fitted models were assessed using analysis of variance (ANOVA). Model terms, regression coefficients, lack-of-fit, coefficient of determination, adjusted and predicted determination coefficients, and diagnostic plots were evaluated to establish the reliability of the generated models. Three-dimensional response surface and two-dimensional contour plots were subsequently constructed to visualize the individual and interactive effects of the CMPs on chromatographic performance. Numerical optimization using the desirability-function approach was performed by simultaneously imposing the predefined analytical requirements for RT, TF, and TP of navepegritide and vosoritide. The optimized chromatographic condition predicted by the statistical model was subsequently subjected to experimental verification by performing confirmatory runs under the predicted optimum conditions. The agreement between the predicted and experimentally observed responses was used to demonstrate the suitability of the optimized chromatographic conditions before proceeding to bioanalytical method validation [15].

Biological matrix and plasma collection

Rat plasma was used as the biological matrix for UPLC method development, validation, and pharmacokinetic application. Plasma samples were obtained from healthy adult male Wistar rats weighing 200–250 g supplied by the Central Animal Facility, Department of Pharmacy, Chaitanya (Deemed to be University), Hyderabad, India. All animal-related procedures were reviewed and approved by the Institutional Animal Ethics Committee (IAEC) under approval number 268/Ro/Fe/W/2004/CPCSEA and were conducted in accordance with the applicable guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. Prior to experimentation, the animals were acclimatized under controlled laboratory conditions at 25 ± 1 °C temperature and $55 \pm 5\%$ relative humidity, with a 12 h light/12 h dark cycle. Animals were maintained in polypropylene cages with appropriate bedding and had free access to standard laboratory feed and purified water. Blood samples were collected using standardized procedures and transferred immediately into K_2EDTA -containing tubes to prevent coagulation. The collected blood was centrifuged under refrigerated conditions to facilitate plasma separation. The resulting plasma was carefully transferred into labelled polypropylene microcentrifuge tubes and stored at -80 °C until analysis [16].

Drug-free rat plasma was used for preparation of calibration standards and quality-control (QC) samples and for assessment of selectivity, matrix effect, extraction recovery, carryover, and stability

during method validation. The use of the same biological matrix for method development and validation ensured that the analytical procedure was evaluated under conditions representative of its intended application for the quantitative determination of navepegritide in preclinical pharmacokinetic studies [17].

Bioanalytical method validation

The optimized AQB–assisted UPLC–PDA method was validated to establish its reliability and suitability for the quantitative determination of navepegritide in rat plasma. The validation procedure was conducted in accordance with the principles and recommendations outlined in the ICH M10 guideline for bioanalytical method validation and the US Food and Drug Administration (FDA) Bioanalytical Method Validation Guidance [18–20].

System performance verification

System performance was verified before the analysis of calibration standards, QC samples, and pharmacokinetic specimens to confirm the consistency and suitability of the UPLC–PDA system under the optimized chromatographic conditions. Six successive injections of navepegritide at the mid-quality control (MQC) concentration of 6000 ng/mL, containing vosoritide as the internal standard, were analyzed. The system was evaluated with respect to RT, peak-area response, TP count, TF, and chromatographic resolution. The repeatability of the chromatographic response was assessed using the percentage relative standard deviation (%RSD) of replicate injections. The chromatographic system was considered suitable for subsequent analysis when the measured system-suitability parameters complied with the predefined acceptance criteria and demonstrated acceptable injection-to-injection reproducibility.

Selectivity

The selectivity of the developed UPLC–PDA method was assessed using blank rat plasma samples obtained from six independent animals. Chromatograms of individual blank plasma samples were examined and compared with those obtained from corresponding plasma samples spiked with navepegritide and vosoritide (internal standard). The chromatograms were evaluated for the presence of endogenous or other extraneous responses at the respective RTs of navepegritide and the internal standard. Selectivity was considered satisfactory when no significant endogenous interference was observed at the retention positions of either compound that could adversely affect their identification or quantitative determination. Representative chromatograms of blank plasma, blank plasma spiked with navepegritide and vosoritide, and a representative incurred plasma sample were used to demonstrate the selectivity of the method.

Calibration model and linearity

Calibration standards of navepegritide were prepared in blank rat plasma over the concentration range of 300–12,000 ng mL⁻¹ and processed using the optimized sample-preparation procedure. The calibration curve was generated by plotting the peak-area ratio of navepegritide to vosoritide (internal standard) against the corresponding nominal plasma concentration of navepegritide. The calibration data were subjected to linear regression using the experimentally selected regression model and weighting scheme to account for variance across the calibration range. The suitability of the calibration model was assessed from the regression parameters, residual distribution, and back-calculated concentrations of the calibration standards. The calibration curve was considered acceptable when the back-calculated concentrations satisfied the predefined bioanalytical acceptance criteria, with the exception of standards permitted to be excluded according to the applicable validation guidelines.

Method sensitivity

The sensitivity of the developed UPLC–PDA method was investigated by determining the limit of detection (LOD) and lower limit of quantification (LLOQ) for navepegritide. The LOD was estimated as the lowest concentration that produced a clearly distinguishable analytical response from the blank matrix, corresponding approximately to a signal-to-noise ratio (S/N) of 3:1. The LLOQ was established as the lowest concentration that could be quantified with acceptable accuracy and precision, with an approximate S/N ratio of 10:1. The experimentally established LLOQ was included as the lowest concentration of the validated calibration range, provided that it fulfilled the predefined bioanalytical acceptance criteria.

Matrix effect

The potential influence of endogenous rat plasma constituents on the chromatographic response of navepegritide was investigated using plasma samples obtained from independent matrix sources. Matrix effects were evaluated at the low-quality control (LQC) and high-quality control (HQC) concentrations by comparing the responses obtained from post-extraction spiked plasma samples with those of corresponding navepegritide solutions prepared in the mobile phase at equivalent concentrations. The assessment was performed using the analyte response and, where applicable, the corresponding internal-standard response to determine any matrix-related alteration in analytical response. The matrix effect was considered acceptable when the calculated response factors demonstrated consistent behavior across the different plasma sources and did not indicate substantial enhancement or suppression of the quantitative response.

Accuracy and precision

The accuracy and precision of the optimized UPLC–PDA method were assessed using navepegritide-spiked rat plasma QC samples at four concentration levels, namely LLQC (300 ng mL⁻¹), LQC (900 ng mL⁻¹), MQC (6000 ng mL⁻¹), and HQC (9000 ng mL⁻¹). Intra-day accuracy and precision were determined by analyzing replicate preparations of each QC level within the same analytical run, while inter-day performance was evaluated by repeating the analysis over three consecutive days under the established chromatographic conditions. Accuracy was expressed as the percentage difference between the measured and nominal concentrations, whereas precision was represented as the percentage relative standard deviation (%RSD) of the measured concentrations. The results were evaluated against the bioanalytical acceptance criteria specified in ICH M10 to establish the reliability and reproducibility of the method across the investigated concentration range.

Carryover assessment

Carryover was investigated by injecting a blank rat plasma sample immediately after the highest calibration standard (12,000 ng mL⁻¹) to determine whether residual analyte or internal-standard responses persisted in subsequent injections. The resulting blank chromatogram was examined at the established RTs of navepegritide and vosoritide. Carryover was considered acceptable when the response in the post-highest-standard blank remained within the acceptance limits specified in ICH M10, thereby confirming the absence of a significant residual response that could affect the accuracy of subsequent sample measurements.

Stability evaluation

The stability of navepegritide in rat plasma was investigated under conditions representative of the anticipated sample collection, handling, processing, storage, and chromatographic analysis. Stability experiments were conducted using plasma QC samples at LQC, MQC, and HQC concentrations. The evaluated conditions included bench-top stability for 8 h at room temperature, processed-sample stability of dry and wet extracts for 24 h, autosampler stability of processed samples for 24 h at 10 °C, three consecutive freeze–thaw cycles with freezing at –70 °C, short-term frozen stability for 2 days at –80 °C, and long-term frozen stability for 30 days at –80 °C. After completion of each stability condition, the samples were analyzed and the measured concentrations were compared with those obtained from freshly prepared QC samples. Navepegritide was considered stable when the observed concentrations remained within the acceptance criteria specified in the ICH M10 Bioanalytical Method Validation Guideline. The stability assessment established the suitability of the

validated method for reliable quantification of navepegritide throughout routine plasma sample handling, processing, storage, and analysis [21].

Application to pharmacokinetic study

The utility of the validated AQbD-assisted UPLC–PDA method was demonstrated through its application to a preclinical pharmacokinetic study of navepegritide in healthy adult male Wistar rats. The animal study was conducted after obtaining approval from the IAEC and in accordance with the applicable guidelines of the CPCSEA, Government of India. A single oral dose of navepegritide, prepared as a suspension in 0.5% methylcellulose, was administered to the experimental animals. Blood samples were collected at predetermined intervals of 0 (pre-dose), 0.5, 1, 1.5, 2, 5, 10, 15, 20, and 25 h following drug administration to characterize the temporal disposition of navepegritide in plasma [22].

Approximately 300 µL of blood was withdrawn at each sampling point into K₂EDTA-containing tubes and immediately processed under refrigerated conditions for plasma separation. The separated plasma was transferred into labelled polypropylene tubes and maintained at –80 ± 10 °C until bioanalytical analysis. Before chromatographic determination, the plasma samples were subjected to the optimized extraction procedure established during method development. Calibration standards and QC samples covering the validated analytical range were included in each analytical batch together with the pharmacokinetic samples to verify the suitability and consistency of the analytical run. Navepegritide concentrations in the plasma samples were quantified using the validated UPLC–PDA procedure, with vosoritide serving as the internal standard. The measured concentration data were plotted against the corresponding sampling times to obtain the plasma concentration–time profile. Pharmacokinetic evaluation was performed by non-compartmental analysis using Phoenix® WinNonlin® software (Version 5.2, Certara, Princeton, NJ, USA). The pharmacokinetic parameters, including maximum plasma concentration (C_{max}), time to reach maximum concentration (T_{max}), area under the plasma concentration–time curve from zero to the last measurable concentration (AUC_{0–t}), area under the curve extrapolated to infinity (AUC_{0–∞}), terminal elimination half-life (t_{1/2}), and apparent clearance (CL/F), were calculated according to standard non-compartmental procedures. The inclusion of calibration standards and QC samples in each analytical batch, together with appropriate sample stability controls, ensured the reliability of the measured plasma concentrations and supported the suitability of the validated method for

pharmacokinetic characterization of navepegritide [23].

Multicriteria sustainability assessment of the developed UPLC method

The sustainability of the developed AQbD-assisted UPLC–PDA method for navepegritide determination in rat plasma was examined using a combination of complementary assessment tools representing different dimensions of GAC. Because no single metric adequately captures the environmental, sample-preparation, resource, and practical aspects of an analytical procedure, a multicriteria evaluation strategy was adopted. The assessment considered the complete analytical workflow, including solvent and reagent consumption, sample preparation, chromatographic analysis, energy requirements, waste generation, and practical applicability.

The overall environmental performance of the method was initially assessed using the AGREE, which evaluates the analytical procedure against the twelve principles of GAC and provides a normalized score on a scale of 0–1 [24]. The sustainability of the plasma sample-preparation procedure was assessed separately using AGREEprep, which places specific emphasis on the characteristics and quantities of reagents, sample handling, energy consumption, waste generation, and other operations associated with sample pretreatment [25]. The Analytical Eco-Scale was additionally applied to quantify the environmental burden of the procedure through penalty points associated with factors such as reagent hazards, energy consumption, occupational considerations, and waste generation [26].

The carbon-related implications of the analytical procedure were further examined using the CaFRI, with consideration of the solvent consumption, chromatographic analysis time, and operational features of the developed method [27]. The CACI was also employed to provide a broader assessment of the analytical workflow by considering factors related to analytical efficiency, simplicity, and sustainability [28]. A process-oriented visualization of the environmental profile was obtained using ComplexGAPI and ComplexMOGAPI. These tools enabled assessment of multiple stages of the analytical workflow, extending from sample collection and preparation to chromatographic determination, reagent and solvent use, instrumentation, and waste generation. Their graphical outputs were used to identify the major contributors to the environmental burden of the developed procedure rather than relying on a single numerical greenness value [29, 30].

In addition to environmental sustainability, the BAGI was used to evaluate the practical applicability of the developed method. BAGI provides information complementary to conventional greenness metrics by

considering characteristics relevant to routine analytical implementation, including operational requirements, analytical performance, simplicity, and applicability. Thus, the BAGI assessment was interpreted as an indicator of the method's practical suitability rather than as a direct measure of environmental greenness [31]. Collectively, the results obtained from AGREE, AGREEprep, Analytical Eco-Scale, CaFRI, CACI, ComplexGAPI, ComplexMOGAPI, and BAGI provided a multidimensional assessment of the developed UPLC–PDA procedure. The combined evaluation allowed the environmental and practical characteristics of the method to be considered alongside its established analytical performance, supporting a more comprehensive appraisal of its suitability for routine determination of navepegritide in rat plasma and its application in pharmacokinetic investigations.

RESULTS AND DISCUSSION

Analytical Quality by Design strategy

Response surface optimization of critical chromatographic parameters

Following preliminary chromatographic investigations, acetonitrile content (A), mobile-phase flow rate (B), and mobile-phase pH (C) were identified as the principal CMPs influencing the chromatographic behavior of navepegritide and vosoritide. These parameters were subsequently optimized simultaneously using a three-factor, three-level BBD within the predefined experimental domain. The RSM provided an efficient means of investigating the individual, quadratic, and combined effects of the CMPs while minimizing the number of experimental runs required to characterize the chromatographic system.

The BBD experiments were generated using Design-Expert® software, and the resulting experimental runs together with the observed chromatographic responses are presented in **Table 1**. The selected CMAs comprised RT, TF, and TP for both navepegritide and vosoritide. These responses were selected to collectively describe chromatographic retention, peak symmetry, and column efficiency, which are directly relevant to the reproducible quantitative determination of the analyte and internal standard in rat plasma. The experimental response data were subjected to quadratic response-surface modeling to establish quantitative relationships between the three CMPs and the selected CMAs. The resulting second-order polynomial equations (**Eqs. 1–6**) describe the response behavior across the investigated design space. The statistical significance and adequacy of each model were examined by ANOVA, together with evaluation of R^2 , adjusted R^2 , predicted R^2 , and lack-of-fit. The corresponding statistical parameters and ANOVA results are provided in **Tables 2–7**.

Collectively, these statistical evaluations were used to determine whether the developed models adequately represented the experimental chromatographic system and could therefore be used for response prediction within the studied factor ranges. The regression coefficients obtained from the quadratic models provided quantitative insight into the contribution of the selected CMPs to chromatographic performance. The sign of a coefficient indicated the direction of the corresponding factor effect, whereas the magnitude reflected its relative influence on the predicted response. The linear, quadratic, and interaction terms were therefore considered collectively rather than interpreting the individual chromatographic variables independently.

RT(Navepegritide)

$$= +1.76 - 0.1843 * A - 0.0553 * B - 0.0030 * C + 0.0130 * AB - 0.0040 * AC + 0.0105 * BC + 0.0169 * A^2 - 0.0031 * B^2 + 0.0084 * C^2 \dots \dots \dots \text{Eqn. 1.}$$

TF(Navepegritide)

$$= +1.05 + 0.1587 * A + 0.0587 * B + 0.0050 * C + 0.0175 * AB + 0.0000 * AC + 0.0050 * BC + 0.0196 * A^2 + 0.0046 * B^2 - 0.0029 * C^2 \dots \dots \dots \text{Eqn. 2.}$$

TP(Navepegritide)

$$= +7558.00 - 2.50 * A + 11.87 * B + 2.87 * C - 70.25 * AB + 8.25 * AC - 30.50 * BC - 229.00 * A^2 - 54.75 * B^2 - 34.25 * C^2 \dots \dots \dots \text{Eqn. 3.}$$

RT(Vosoritide)

$$= +3.47 - 0.1618 * A - 0.0481 * B + 0.0044 * C + 0.0045 * AB - 0.0025 * AC + 0.0007 * BC + 0.0120 * A^2 - 0.0012 * B^2 - 0.0077 * C^2 \dots \dots \dots \text{Eqn. 4.}$$

TF(Vosoritide)

$$= +0.9400 + 0.1812 * A + 0.0662 * B + 0.0000 * C + 0.0125 * AB - 0.0050 * AC + 0.0000 * BC + 0.0288 * A^2 + 0.0188 * B^2 + 0.0212 * C^2 \dots \dots \dots \text{Eqn. 5.}$$

TP(Vosoritide)

$$= +9049.33 + 46.00 * A - 14.88 * B + 9.62 * C - 46.00 * AB + 6.00 * AC - 29.25 * BC - 231.04 * A^2 - 64.29 * B^2 - 62.29 * C^2$$

Table 1. Box–Behnken experimental design matrix presenting the coded and actual levels of acetonitrile content, mobile-phase pH, and flow rate, along with the experimentally determined RT, TF, and TP for navepegritide and vosoritide.

Fa	F	F	Res	Res	Res	Re	Re	Re
ct	a	a	pon	pon	pon	sp	sp	sp
c	c	c				on	on	on

	or	t	t	se	se	se	se	se	se
	l	o	o	1	2	3	4	5	6
		r	r						
		2	3						
R	A:	B	C	RT	TF	TP	R	TF	TP
u	O	:	:	(Na	(Na	(Na	T	(V	(V
n	rg	F	p	vep	vep	vep	(V	os	os
	an	l	H	egr	egr	egr	os	ori	ori
	ic	o		itid	itid	itid	ori	tid	tid
	P	w		e)	e)	e)	tid	e)	e)
	ha	r					e)		
	se	a							
		t							
		e							
	%	m		Mi			Mi		
		l/		n			n		
		m							
		i							
		n							
1	55	0	3	1.6	1.2	730	3.	1.	88
		.	.	02	2	1	32	16	12
		5	2				4		
2	50	0	2	1.7	1.1	750	3.	1.	89
		.	.	01	2	1	41	07	36
		6	8						
3	50	0	3	1.7	1.0	752	3.	0.	90
		.	.	75	1	4	45	95	38
		5					2		
4	50	0	2	1.8	0.9	741	3.	0.	88
		.	.	58	6	2	52	87	75
		4	8				4		
5	55	0	2	1.5	1.2	729	3.	1.	87
		.	.	97	3	8	30	19	96
		5	8				1		
6	45	0	3	1.9	0.9	727	3.	0.	87
		.	.	85	1	5	65	8	04
		5	2				4		
7	45	0	3	1.9	0.9	736	3.	0.	87
		.	.	01	3	4	59	83	25
		6					8		
8	45	0	3	2.0	0.9	720	3.	0.	86
		.	.	12		4	68	79	95
		4					7		
9	50	0	3	1.7	1.0	756	3.	0.	90
		.	.	98	9	9	46	96	12
		5					8		
1	55	0	3	1.5	1.2	720	3.	1.	87
0		.	.	65	9	4	28	21	21
		6					5		
1	50	0	3	1.6	1.1	746	3.	1.	89
1		.	.	97	6	5	40	09	12
		6	2				1		
1	45	0	2	1.9	0.9	730	3.	0.	87
2		.	.	64	2	5	62	81	12
		5	8				1		

1	50	0	3	1.8	0.9	749	3.	0.	89
3		.	.	12	8	8	51	89	68
		4	2				2		
1	50	0	3	1.7	1.0	758	3.	0.	90
4		.		12	6	1	49	91	98
		5					2		
1	55	0	3	1.6	1.1	732	3.	1.	88
5		.		24	9	5	35	12	75
		4					6		

Table 2. ANOVA results for the quadratic response surface model illustrating the effects of acetonitrile content, flow rate, and mobile-phase pH of the retention time of navepegritide (Y_1).

Source	Sum of Squares	df	Mean Square	F-value	p-value	significant
Model	0.2986	9	0.0332	27.32	0.0010	significant
A-Organic Phase	0.2716	1	0.2716	223.65	<0.0001	
B-Flow rate	0.0244	1	0.0244	20.11	0.0065	
C-pH	0.0001	1	0.0001	0.0593	0.8173	
AB	0.0007	1	0.0007	0.5567	0.4892	
AC	0.0001	1	0.0001	0.0527	0.8275	
BC	0.0004	1	0.0004	0.3632	0.5730	
A ²	0.0011	1	0.0011	0.8701	0.3937	
B ²	0.0000	1	0.0000	0.0289	0.8717	
C ²	0.0003	1	0.0003	0.2154	0.6621	
Residual	0.0061	5	0.0012			
Lack of Fit	0.0021	3	0.0007	0.3543	0.7956	not significant
Pure Error	0.0040	2	0.0020			
Cor Total	0.3047	14				

Table 3. ANOVA results for the quadratic response surface model illustrating the effects of acetonitrile

content, flow rate, and mobile-phase pH of the tailing factor of navepegritide (Y_2).

Source	Sum of Squares	df	Mean Square	F-value	p-value	significant
Model	0.2323	9	0.0258	13.32	0.0054	significant
A-Organic Phase	0.2016	1	0.2016	104.01	0.0002	
B-Flow rate	0.0276	1	0.0276	14.25	0.0130	
C-pH	0.0002	1	0.0002	0.1032	0.7610	
AB	0.0012	1	0.0012	0.6320	0.4627	
AC	0.0000	1	0.0000	0.0000	1.0000	
BC	0.0001	1	0.0001	0.0516	0.8293	
A ²	0.0014	1	0.0014	0.7305	0.4317	
B ²	0.0001	1	0.0001	0.0400	0.8493	
C ²	0.0000	1	0.0000	0.0162	0.9037	
Residual	0.0097	5	0.0019			
Lack of Fit	0.0064	3	0.0021	1.31	0.4602	not significant
Pure Error	0.0033	2	0.0016			
Cor Total	0.2420	14				

Table 4. ANOVA results for the quadratic response surface model illustrating the effects of acetonitrile content, flow rate, and mobile-phase pH of the theoretical plates of navepegritide (Y_3).

Source	Sum of Squares	df	Mean Square	F-value	p-value	significant
Model	2.245E+05	9	24939.76	41.51	0.0004	significant
A-Organic	50.00	1	50.00	0.0832	0.7845	

Phase						
B-Flow rate	1128.12	1	1128.12	1.88	0.2289	
C-pH	66.13	1	66.13	0.1101	0.7535	
AB	19740.25	1	19740.25	32.86	0.0023	
AC	272.25	1	272.25	0.4532	0.5307	
BC	3721.00	1	3721.00	6.19	0.0552	
A ²	1.936E+05	1	1.936E+05	322.31	<0.0001	
B ²	11067.92	1	11067.92	18.42	0.0078	
C ²	4331.31	1	4331.31	7.21	0.0436	
Residual	3003.75	5	600.75			
Lack of Fit	1197.75	3	399.25	0.4421	0.7482	not significant
Pure Error	1806.00	2	903.00			
Cor Total	2.275E+05	1				

Table 5. ANOVA results for the quadratic response surface model illustrating the effects of acetonitrile content, flow rate, and mobile-phase pH of the retention time of vosoritide (Y₄).

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.2289	9	0.0254	61.01	0.0001	significant
A-Organic Phase	0.2093	1	0.2093	502.07	<0.0001	
B-Flow rate	0.0185	1	0.0185	44.44	0.0011	
C-pH	0.0002	1	0.0002	0.3673	0.5709	
AB	0.0001	1	0.0001	0.1943	0.6778	
AC	0.0000	1	0.0000	0.0600	0.8163	

BC	2.250E-06	1	2.250E-06	0.0054	0.9443	
A ²	0.0005	1	0.0005	1.2885	0.3085	
B ²	5.391E-06	1	5.391E-06	0.0129	0.9139	
C ²	0.0002	1	0.0002	0.5263	0.5007	
Residual	0.0021	5	0.0004			
Lack of Fit	0.0013	3	0.0004	1.0523	0.5223	not significant
Pure Error	0.0008	2	0.0004			
Cor Total	0.2310	1				

Table 6. ANOVA results for the quadratic response surface model illustrating the effects of acetonitrile content, flow rate, and mobile-phase pH of the tailing factor of vosoritide (Y₅).

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.3039	9	0.0338	14.78	0.0042	significant
A-Organic Phase	0.2628	1	0.2628	115.02	0.0001	
B-Flow rate	0.0351	1	0.0351	15.37	0.0112	
C-pH	0.0000	1	0.0000	0.0000	1.0000	
AB	0.0006	1	0.0006	0.2735	0.6233	
AC	0.0001	1	0.0001	0.0438	0.8426	
BC	0.0000	1	0.0000	0.0000	1.0000	
A ²	0.0031	1	0.0031	1.3431	0.3000	
B ²	0.0013	1	0.0013	0.5681	0.4850	
C ²	0.0017	1	0.0017	0.7297	0.4320	
Residual	0.0114	5	0.0023			

Lack of Fit	0.0100	3	0.0033	4.77	0.1781	not significant
Pure Error	0.0014	2	0.0007			
Cor Total	0.3153	14				

Table 7. ANOVA results for the quadratic response surface model illustrating the effects of acetonitrile content, flow rate, and mobile-phase pH of the theoretical plates of vosoritide (Y₆).

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	2.429E+05	9	26993.30	20.87	0.0019	significant
A-Organic Phase	16928.00	1	16928.00	13.09	0.00153	
B-Flow rate	1770.13	1	1770.13	1.37	0.2948	
C-pH	741.13	1	741.13	0.5729	0.4832	
AB	8464.00	1	8464.00	6.54	0.0508	
AC	144.00	1	144.00	0.113	0.7522	
BC	3422.25	1	3422.25	2.65	0.1648	
A ²	1.971E+05	1	1.971E+05	152.36	<0.0001	
B ²	15261.85	1	15261.85	11.80	0.0185	
C ²	14327.08	1	14327.08	11.08	0.0208	
Residual	6467.92	5	1293.58			
Lack of Fit	2577.25	3	859.08	0.4416	0.7485	not significant
Pure Error	3890.67	2	1945.33			
Cor Total	2.494E+05	14				

Model validation and response surface interpretation

The adequacy and predictive performance of the BBD models were examined through a combination of

statistical diagnostics and graphical assessments. These evaluations were undertaken to establish the reliability of the fitted quadratic models and to understand how the selected CMPs influenced the chromatographic responses. The collective diagnostic results supported the suitability of the developed models for describing the chromatographic system and for subsequent optimization of the UPLC method. The fitted models were initially evaluated using residual diagnostic plots, including normal probability plots and plots of residuals against predicted responses. As shown in **Fig. 1**, the residuals were distributed randomly around the zero line, with no apparent systematic pattern, curvature, or progressive change in dispersion. The absence of pronounced clustering or influential observations indicated that the assumptions underlying the quadratic regression models were reasonably satisfied. Moreover, the close distribution of the experimental observations around the predicted values indicated satisfactory agreement between the measured and model-estimated responses across the investigated design space. These diagnostic findings provided evidence that the developed regression models were suitable for describing the observed chromatographic behavior.

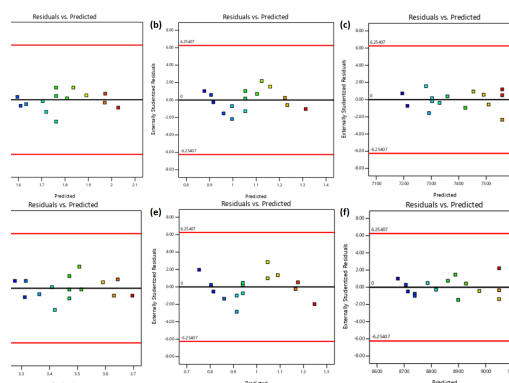


Fig. 1. Residual diagnostic plots for assessment of the adequacy of the developed quadratic response surface models: (a) RT, (b) TF, and (c) TP for navepegritide, and (d) RT, (e) TF, and (f) TP for vosoritide. The random distribution of residuals around the zero reference line, without evident systematic trends or substantial deviations, supports the adequacy of the fitted models and their suitability for predicting chromatographic responses within the investigated design space.

The relative influence of the individual CMPs was further examined using perturbation plots, in which the effect of each factor was assessed while the other variables were maintained at their respective center-point values (**Fig. 2**). The resulting profiles demonstrated that the magnitude and direction of the chromatographic responses varied according to the factor under consideration. Acetonitrile content

produced the most pronounced changes in chromatographic behavior, particularly with respect to analyte retention and detector response. Changes in flow rate also produced substantial variations in chromatographic performance because modification of the mobile-phase velocity alters the residence time of the analytes within the stationary phase and consequently affects retention and separation efficiency. The effect of mobile-phase pH was comparatively moderate but remained important for controlling peak characteristics and chromatographic selectivity. The different slopes observed in the perturbation profiles reflected the relative sensitivity of the individual responses to changes in the corresponding CMPs.

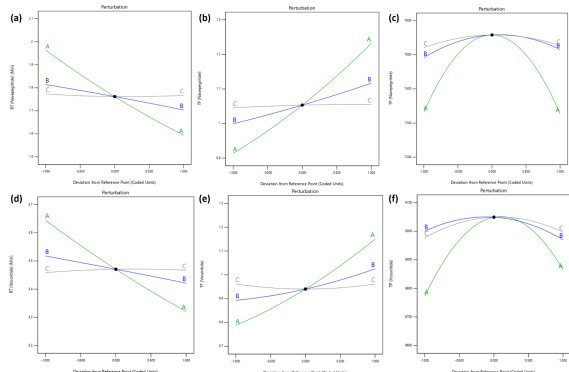


Fig. 2. Perturbation profiles depicting the relative sensitivity of the chromatographic responses to individual Critical Method Parameters: (a) RT, TF, and TP count for navepegritide, and (b) RT, TF, and TP count for vosoritide. Each profile illustrates the effect of varying one factor from its center-point condition while the remaining variables are held constant, enabling comparative assessment of their influence on the predicted analytical responses. The combined and interactive effects of the CMPs were subsequently visualized using three-dimensional response-surface plots. These plots provided a graphical representation of the response behavior within the experimental domain and assisted in identifying the region in which the predefined chromatographic criteria could be simultaneously satisfied. For RT, the response surfaces (Fig. 3) indicated that increasing the acetonitrile proportion and increasing the flow rate progressively decreased the retention of both navepegritide and vosoritide. The reduction in retention with increasing organic solvent content is consistent with reversed-phase chromatographic behavior, as an increase in the proportion of the stronger organic component decreases the interaction of the analytes with the hydrophobic stationary phase and promotes earlier elution. Similarly, a higher flow rate decreases the residence time of the analytes within the column, resulting in shorter retention and reduced analysis

time. Within the investigated design region, these changes could be accommodated while maintaining suitable chromatographic performance. The comparatively smaller effect of pH provided an additional means of fine-tuning the chromatographic environment and analyte–stationary-phase interactions.

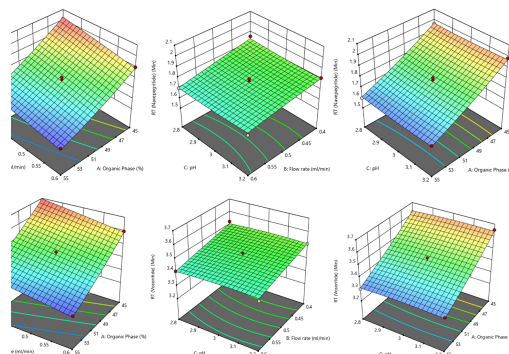


Fig. 3. Three-dimensional response surface plots illustrating the combined effects of acetonitrile content, flow rate, and mobile-phase pH on the RT of (a) navepegritide and (b) vosoritide.

The response surfaces obtained for TF (Fig. 4) demonstrated that favorable peak symmetry was attained within an intermediate region of the investigated acetonitrile composition and flow-rate ranges. Departures toward extreme levels of these variables produced less favorable peak profiles, indicating the importance of maintaining an appropriate balance between mobile-phase strength and chromatographic flow conditions. Changes in mobile-phase pH further influenced peak symmetry, presumably through modification of the ionization-dependent interactions of the analytes with the stationary phase and residual surface sites. The combined response behavior therefore indicated that controlled adjustment of all three CMPs was necessary to maintain acceptable peak shape for quantitative analysis.

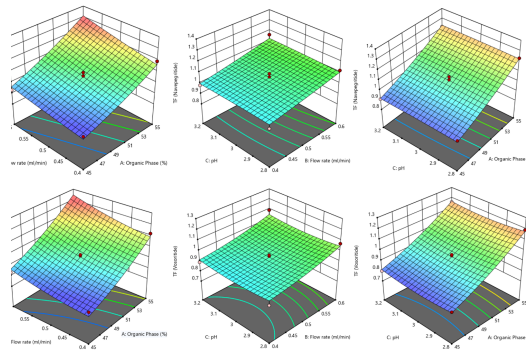


Fig. 4. Three-dimensional response surface plots illustrating the combined effects of acetonitrile content, flow rate, and mobile-phase pH on the TF of (a) navepegritide and (b) vosoritide.

The response surfaces for TP (**Fig. 5**) further demonstrated the dependence of chromatographic efficiency on the combined effects of acetonitrile content and flow rate. Higher plate counts were obtained within an intermediate region of the investigated factor space, suggesting favorable mass-transfer conditions and efficient column utilization under these conditions. Excessive variation in either chromatographic factor resulted in a progressive decline in column efficiency, which may be associated with changes in analyte residence time and mass-transfer characteristics. Although the direct influence of mobile-phase pH on TP was comparatively limited, its interaction with the other CMPs contributed to the overall chromatographic efficiency and helped define the region of acceptable method performance. Taken together, the perturbation and response-surface analyses provided a multidimensional interpretation of the chromatographic system and demonstrated the dependence of RT, TF, and TP on the selected CMPs. The quadratic models enabled identification of an operating region in which the requirements for suitable retention, acceptable peak symmetry, and adequate chromatographic efficiency for navepegritide and vosoritide could be achieved simultaneously.

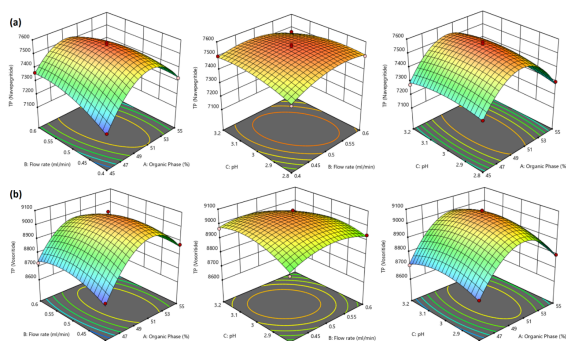


Fig. 5. Three-dimensional response surface plots illustrating the combined effects of acetonitrile content, flow rate, and mobile-phase pH on the TP of (a) navepegritide and (b) vosoritide.

Numerical optimization, design-space definition, and experimental verification

Following establishment of the quadratic response-surface models, numerical optimization was performed using the desirability-function approach in Design-Expert® software. The optimization simultaneously considered the six chromatographic responses corresponding to RT (Y_1 and Y_4), TF (Y_2 and Y_5), and TP count (Y_3 and Y_6) for navepegritide and vosoritide, respectively. The response criteria were defined to obtain an appropriate retention profile and peak symmetry while maximizing chromatographic efficiency. Thus, RT and TF were directed toward lower desirable values within the experimentally acceptable range, whereas TP count was assigned a maximization criterion.

The combination of response constraints generated an overlay contour plot (**Fig. 6**), which enabled simultaneous visualization of the region in which all predefined chromatographic requirements could be satisfied. The common feasible region represented the experimentally supported MODR and provided a multidimensional basis for selecting the final chromatographic operating point rather than relying on optimization of individual variables independently. The desirability function identified 0.1% (v/v) formic acid in water and acetonitrile at 50:50 (v/v), adjusted to pH 3.0, with a flow rate of 0.5 mL min⁻¹ as the optimum chromatographic condition. This combination provided a suitable compromise among retention, peak symmetry, and column efficiency for the determination of navepegritide using vosoritide as the internal standard. The selected conditions were subsequently fixed for method verification and validation.

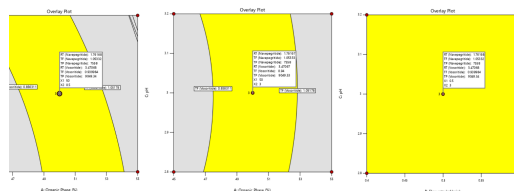


Fig. 6. Overlay contour plots generated using Design-Expert® software depicting the feasible method operable design region (MODR) obtained from simultaneous optimization of the chromatographic responses of navepegritide and vosoritide.

To establish the predictive performance of the optimization models, the numerical solution was subjected to experimental verification under the predicted optimum conditions. Independent replicate chromatographic analyses were performed, and the experimentally obtained values of RT, TF, and TP for navepegritide and vosoritide were compared with the corresponding model-predicted values. The agreement between the predicted and experimentally observed responses, together with the associated prediction errors, confirmed the suitability of the developed response-surface models for optimization within the investigated design space. Under the optimized conditions, navepegritide and vosoritide produced well-defined chromatographic peaks with satisfactory symmetry, reproducible retention behavior, and adequate column efficiency. The representative chromatogram obtained under the optimized UPLC–PDA conditions is presented in **Fig. 7**. The chromatographic profile demonstrated the suitability of the selected conditions for quantitative analysis without evident interference or undesirable baseline disturbances. Overall, the numerical optimization and experimental verification demonstrated that the AQbD strategy successfully translated the statistical design into a controlled and practically applicable

chromatographic procedure, which was subsequently used for validation and quantification of navepegritide in rat plasma.

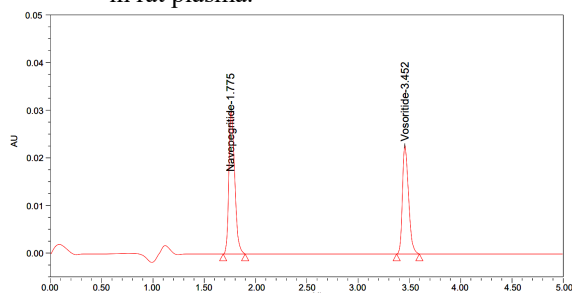


Fig. 7. Representative optimized UPLC–PDA chromatogram demonstrating the separation of navepegritide and vosoritide under the selected chromatographic conditions: 0.1% (v/v) formic acid in water (50:50, v/v), pH 3.0, flow rate 0.5 mL min⁻¹, detection wavelength 272.4 nm, injection volume 5 μ L, and total run time 5 min.

Bioanalytical method validation

The performance of the optimized AQbD-assisted UPLC–PDA method was established in rat plasma according to the principles of the ICH M10 Bioanalytical Method Validation Guideline. The validation results for the developed procedure are summarized in **Table 2**. System suitability was initially examined using six replicate injections of the MQC solution containing 6000 ng mL⁻¹ of navepegritide with vosoritide as the internal standard. The monitored chromatographic parameters, including retention time, peak area, tailing factor, and theoretical plate count, showed variability of less than 2%, demonstrating consistent chromatographic performance and satisfactory injection repeatability under the optimized conditions.

The selectivity of the method was assessed using blank plasma obtained from six individual Wistar rats. Comparison of the blank plasma chromatograms with navepegritide- and vosoritide-spiked plasma samples showed no appreciable endogenous interference at the respective RTs. The fortified samples generated distinct, symmetrical, and well-defined chromatographic peaks. These observations confirmed that endogenous plasma constituents did not interfere with the determination of navepegritide or the internal standard. Representative chromatograms are provided in **Fig. 8**. The calibration characteristics were investigated over the concentration range of 300–12,000 ng mL⁻¹. A progressive increase in the analyte response was observed with increasing navepegritide concentration, demonstrating satisfactory linearity throughout the investigated range. The calibration relationship exhibited an R^2 value of 0.99994, confirming the strong linear association between concentration and the analyte-to-internal-standard

response. The LOD and LLOQ were 100 and 300 ng mL⁻¹, respectively, indicating adequate analytical sensitivity for quantitative determination of navepegritide in rat plasma.

The effect of the plasma matrix on the analytical response was examined at the selected QC concentrations. The calculated matrix-factor values ranged from 97.01 to 97.18%, with variability below 2%. The narrow range of matrix-factor values indicated that endogenous plasma components produced no substantial alteration of the chromatographic response. The consistency observed across the evaluated concentrations further supported the reproducibility of the sample-preparation procedure and the suitability of the developed UPLC–PDA method for analysis of navepegritide in the biological matrix. Accuracy and precision were evaluated at LLQC (300 ng mL⁻¹), LQC (900 ng mL⁻¹), MQC (6000 ng mL⁻¹), and HQC (9000 ng mL⁻¹). The measured accuracy values ranged from 96.31 to 98.60%, while the intra-day and inter-day precision values ranged from 0.16 to 2.06%. The relatively low variability across the investigated QC levels demonstrated good repeatability and intermediate precision, while the accuracy values confirmed close agreement between the measured and nominal concentrations. The results complied with the acceptance requirements of ICH M10, establishing the quantitative reliability of the method over the validated concentration range.

Carryover was investigated by injecting blank plasma immediately after the highest calibration standard. The residual response observed in the subsequent blank injection remained below 20% of the LLQC response for navepegritide, confirming that significant carryover was absent under the optimized chromatographic conditions. This finding is particularly relevant for sequential analysis of biological samples, where residual analyte response could otherwise influence measurements at lower concentrations. Collectively, the validation results demonstrated that the optimized UPLC–PDA method exhibited satisfactory selectivity, linearity, sensitivity, matrix compatibility, accuracy, precision, and carryover control for the determination of navepegritide in rat plasma. The high calibration correlation ($R^2 = 0.99994$), low matrix-related variability, and precision values of 0.16–2.06% demonstrate the reproducibility of the analytical procedure. In combination with the rapid chromatographic separation achieved through the AQbD-based optimization strategy, these characteristics establish the suitability of the developed method for reliable quantification of navepegritide in rat plasma and its subsequent application in pharmacokinetic studies.

Table 2. Linearity, sensitivity, matrix effect, accuracy, and precision of the developed UPLC–PDA method for navepegritide in rat plasma.

Parameter	Sub-parameter	Doxecitine
Linearity	Range (ng mL ⁻¹)	300–12000
	Regression equation	y = 0.000269x + 0.000692
	Correlation coefficient	0.99994
Sensitivity	LOD (ng mL ⁻¹)	100
	LOQ (ng mL ⁻¹)	300
Matrix Effect	HQC (CV / %Accuracy)	0.52 / 97.01
	LQC (CV / %Accuracy)	1.03 / 97.18
Accuracy & Precision (QC levels)	LLQC (300 ng mL ⁻¹) Recovery (%)	96.31
	LLQC Inter-day CV	1.20
	LLQC Intra-day CV	1.18
	LQC (900 ng mL ⁻¹) Recovery (%)	97.40
	LQC Inter-day CV	2.06
	LQC Intra-day CV	1.96
	MQC (6000 ng mL ⁻¹) Recovery (%)	98.70
	MQC Inter-day CV	0.16
	MQC Intra-day CV	0.21

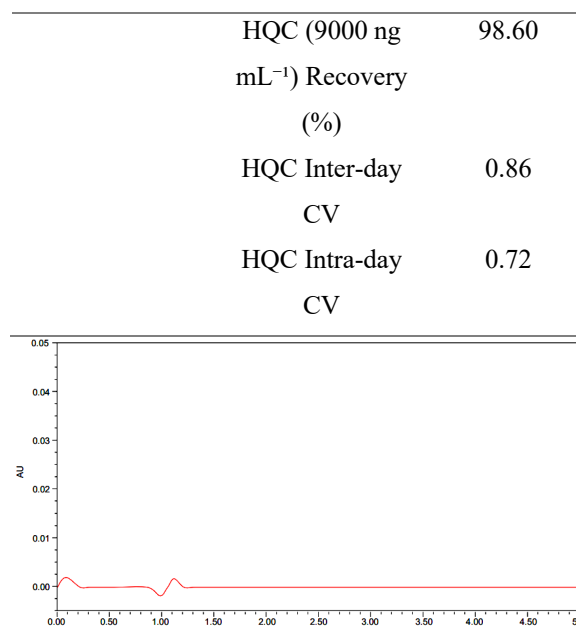


Fig. 8. Representative chromatogram of processed blank rat plasma obtained under the optimized UPLC–PDA conditions, showing the absence of significant endogenous interference at the retention times of navepegritide and vosoritide (internal standard).

Stability performance of navepegritide in rat plasma

The stability of navepegritide in rat plasma was investigated under storage and handling conditions representative of the intended bioanalytical workflow to confirm the integrity of the analyte during sample collection, processing, storage, and chromatographic analysis (**Table 3**). Following 8 h of bench-top exposure at room temperature, navepegritide exhibited recoveries of 97.85–98.62%, with %CV values ranging from 0.64–1.25%, indicating satisfactory short-term stability without appreciable loss of analyte. Similarly, three successive freeze–thaw cycles involving freezing at –70 °C resulted in recoveries of 96.85–99.61%, with %CV values of 0.64–1.92%, demonstrating that repeated freeze–thaw exposure did not substantially compromise the stability of navepegritide.

The stability of processed samples was evaluated using dry and wet extracts maintained for 24 h. Navepegritide retained 96.04–98.24% of the nominal concentration in dry extracts, with %CV values of 0.47–2.45%, whereas the corresponding recoveries for wet extracts ranged from 96.04–99.14%, with %CV values of 1.15–3.82%. These results indicated adequate stability of navepegritide in the processed extracts during the specified storage period. Autosampler stability was subsequently assessed by maintaining processed samples at 10 °C for 24 h. Recoveries of 96.12–98.63% were obtained, with %CV values ranging from 1.15–2.68%, demonstrating

that the processed samples remained sufficiently stable during the evaluated autosampler period.

Short-term frozen storage at -80°C for 2 days resulted in recoveries of 94.72–98.36%, with corresponding %CV values of 1.45–2.96%. Although a modest reduction in recovery was observed at the LQC and MQC levels, the measured concentrations remained within the predefined bioanalytical acceptance criteria. Following long-term frozen storage at -80°C for 30 days, navepegritide showed recoveries ranging from 88.82–95.63%, with %CV values of 2.12–5.45%. The relatively greater reduction in recovery during prolonged storage, particularly at the lower QC concentrations, indicated some decrease in analyte recovery under extended frozen-storage conditions; nevertheless, the results remained within the predefined acceptance criteria for stability assessment. Overall, the stability evaluation demonstrated that navepegritide remained sufficiently stable under bench-top, freeze–thaw, processed dry-extract, processed wet-extract, autosampler, short-term frozen, and long-term frozen conditions investigated in the present study. The observed accuracy and precision across the tested QC levels support the integrity of navepegritide throughout the evaluated sample-handling and storage conditions and further establish the suitability of the developed AQbD-assisted UPLC–PDA method for reliable analysis of rat plasma samples and subsequent pharmacokinetic investigations.

Table 3. Stability assessment of navepegritide in rat plasma under different storage and handling conditions. Accuracy is expressed as the percentage of nominal concentration recovered, and precision is reported as %CV.

Stability condition	QC level	Accuracy (%)	Precision (%CV)
Bench-top (8 h, room temperature)	HQC	98.62	0.64
	MQC	98.41	0.85
	LQC	97.85	1.25
Freeze–thaw (3 cycles, -70°C)	HQC	99.61	0.64
	MQC	98.78	1.74
	LQC	96.85	1.92
Autosampler (24 h, 10°C)	HQC	98.63	1.15
	MQC	97.01	2.32
	LQC	96.12	2.68
Dry extract (24 h)	HQC	98.24	0.47
	MQC	97.61	1.52
	LQC	96.04	2.45

Wet extract (24 h)	HQC	99.14	1.15
	MQC	98.54	2.54
	LQC	96.04	3.82
Short-term frozen (2 days, -80°C)	HQC	98.36	1.45
	MQC	95.14	2.82
	LQC	94.72	2.96
Long-term frozen (30 days, -80°C)	HQC	95.63	2.12
	MQC	89.45	4.24
	LQC	88.82	5.45

Pharmacokinetic application of the validated UPLC–PDA method

The applicability of the validated AQbD-assisted UPLC–PDA method was demonstrated through a single-dose pharmacokinetic investigation of navepegritide in rats. Following oral administration of a single dose of navepegritide, plasma samples were collected at predetermined time intervals of 0.5, 1, 1.5, 2, 5, 10, 15, 20, and 25 h post-dose. A predose plasma sample was also collected to assess potential endogenous interference. The resulting plasma concentration–time profile is presented in **Fig. 9**.

Navepegritide was readily quantifiable in plasma over the investigated sampling period, demonstrating the suitability of the developed method for pharmacokinetic sample analysis. The plasma concentration increased from $1077.77\text{ ng mL}^{-1}$ at 0.5 h to $3295.89\text{ ng mL}^{-1}$ at 1 h, reaching a maximum concentration (C_{max}) of $5896.13\text{ ng mL}^{-1}$ at 1.5 h (T_{max}). Thereafter, the plasma concentration progressively declined to $3450.18\text{ ng mL}^{-1}$ at 2 h, followed by concentrations of 2537.14 , 1894.40 , 1176.34 , and 620.85 ng mL^{-1} at 5, 10, 15, and 20 h, respectively. At 25 h, the navepegritide concentration was reported as 0 ng mL^{-1} , indicating the absence of measurable drug concentration at the final sampling point. Non-compartmental pharmacokinetic analysis was performed using the observed plasma concentration–time data with Phoenix® WinNonlin® Version 5.2. The calculated area under the plasma concentration–time curve from zero to the last sampling time (AUC_{0-t}) was $39,779\text{ ng}\cdot\text{h mL}^{-1}$, while the reported area under the curve extrapolated to infinity ($\text{AUC}_{0-\infty}$) was also $39,779\text{ ng}\cdot\text{h mL}^{-1}$. The maximum plasma concentration was $5896.13\text{ ng mL}^{-1}$, occurring at 1.5 h, indicating rapid attainment of peak systemic exposure following administration. The reported terminal elimination half-life ($t_{1/2}$) was 20 h. The concentration–time profile demonstrated an initial increase in circulating navepegritide concentrations followed by a sustained decline during the subsequent sampling intervals. The inclusion of calibration standards and QC samples with the pharmacokinetic samples ensured appropriate analytical control

throughout the bioanalytical sequence. The plasma samples were processed after addition of the internal standard and analyzed together with QC samples at four concentration levels. The stability criterion specified for the pharmacokinetic samples was a percentage difference not exceeding $\pm 20\%$, supporting the integrity of the analyzed samples under the applied storage and processing conditions. Collectively, the observed plasma concentration–time profile and calculated pharmacokinetic parameters demonstrate the applicability of the validated AQbD-assisted UPLC–PDA method for quantification of navepegritide in rat plasma. The method provided reproducible measurement of navepegritide concentrations across the investigated sampling interval and was therefore suitable for its application in the preclinical pharmacokinetic study.

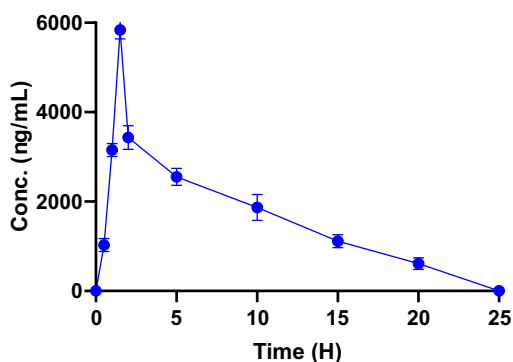


Fig. 9. Plasma concentration–time profile of navepegritide following single-dose administration in rats, showing the observed plasma concentrations over the sampling period of 0.5–25 h.

Multicriteria sustainability assessment of the developed UPLC method

The environmental profile and practical applicability of the developed UPLC–PDA procedure for navepegritide determination in rat plasma were evaluated using complementary GAC and WAC concepts. The complete analytical workflow was assessed using AGREE, AGREEprep, Analytical Eco-Scale, CaFRI, CACI, ComplexGAPI, ComplexMOGAPI, and BAGI, thereby providing a multidimensional evaluation of solvent consumption, sample preparation, waste generation, carbon footprint, operational requirements, analytical efficiency, and practical applicability. The ComplexGAPI pictogram (**Fig. 10a**) provided a visual representation of the environmental characteristics of the complete analytical workflow. The predominance of favorable zones reflected the relatively simple plasma sample-preparation procedure, limited processing steps, absence of derivatization, and straightforward protein-precipitation approach. The less favorable contributions were primarily associated

with the use and disposal of acetonitrile and the energy requirements associated with UPLC operation. The overall profile was supported by the limited sample manipulation and the relatively short 5-min chromatographic run, which reduced solvent consumption and analytical waste generation.

Quantitative evaluation using AGREE (**Fig. 10b**) produced a score of 0.74, indicating favorable conformity with the twelve principles of Green Analytical Chemistry. This favorable score was associated with the streamlined sample-preparation procedure, limited waste generation, relatively low solvent consumption, short chromatographic analysis time, and efficient use of analytical resources. The 5-min run time reduced the volume of mobile phase required per analysis and consequently limited solvent waste generated during routine operation. The AGREEprep score of 0.69 (**Fig. 10c**) further indicated favorable characteristics of the plasma sample-preparation procedure, particularly because of its simple protein-precipitation-based extraction and limited manipulation of the biological matrix. The Analytical Eco-Scale score of 76 classified the procedure as an environmentally acceptable analytical method. The major penalty contributions were associated principally with organic solvent use and instrumental energy consumption, whereas the relatively limited number of reagents and uncomplicated sample-processing procedure helped reduce the overall environmental burden.

The carbon and analytical workflow characteristics of the developed method were further examined using CaFRI and CACI. A CaFRI score of 78 (**Fig. 10d**) indicated a favorable carbon-footprint profile, supported by the simplified plasma-processing procedure, relatively low solvent requirements, and optimized chromatographic conditions. The combination of limited sample manipulation and a 5-min UPLC analysis reduced both solvent and operational requirements, thereby contributing to the observed carbon-footprint profile. The CACI score of 81 (**Fig. 10e**) further indicated a favorable balance among analytical performance, procedural simplicity, and sustainability-related characteristics. The comprehensive ComplexMOGAPI assessment yielded a score of 89 (**Fig. 10f**), indicating a favorable overall environmental profile of the analytical workflow. This assessment further demonstrated that integration of simplified plasma preparation with optimized chromatographic conditions can reduce the environmental burden associated with routine bioanalytical analysis.

The practical applicability of the developed procedure was subsequently evaluated using the BAGI within the WAC framework (**Fig. 10g**). The method achieved a BAGI score of 77.5, indicating favorable practical

applicability with respect to operational simplicity, analytical performance, throughput, and suitability for routine implementation. In contrast to the greenness metrics, BAGI was interpreted primarily as an indicator of practical applicability rather than as a direct measure of environmental performance. Collectively, the results obtained from the complementary sustainability and applicability metrics demonstrate that the developed AQbD-assisted UPLC–PDA method provides a favorable balance between analytical performance, environmental considerations, and practical implementation. The multidimensional profile was supported by the simplified protein-precipitation-based plasma processing, limited procedural manipulation, optimized use of acetonitrile, and short 5-min chromatographic run, which collectively helped reduce solvent consumption, waste generation, and operational requirements. The integration of AQbD-based chromatographic optimization with systematic sustainability assessment therefore provides a comprehensive framework for evaluating the environmental and practical characteristics of the developed method for determination of navepegritide in rat plasma and its application to pharmacokinetic investigations.

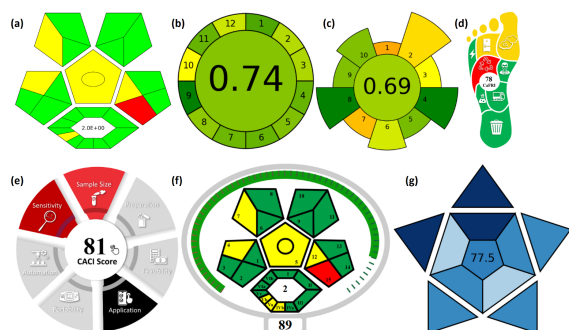


Fig. 10. Integrated assessment of the environmental sustainability and practical applicability of the developed UPLC–PDA method using (a) ComplexGAPI, (b) AGREE, (c) AGREEprep, (d) CaFRI, (e) CACI, (f) ComplexMOGAPI, and (g) BAGI metrics.

CONCLUSION

An AQbD-assisted UPLC–PDA method was successfully developed and validated for the determination of navepegritide in rat plasma. The application of a Box–Behnken experimental design enabled systematic assessment and optimization of the principal chromatographic parameters, namely acetonitrile content, mobile-phase pH, and flow rate, while simultaneously considering retention time, peak symmetry, and column efficiency for navepegritide and vosoritide. The optimized method employed a BEH Phenyl C18 column with a 50:50 (v/v) aqueous formic acid–acetonitrile mobile phase at pH 3.0, a flow

rate of 0.5 mL min^{−1}, and detection at 272.4 nm, providing a total analysis time of 5 min. The validated procedure exhibited satisfactory selectivity, linearity, sensitivity, matrix compatibility, accuracy, precision, carryover control, and stability over the investigated analytical range. The high correlation coefficient ($R^2 = 0.99994$), low matrix-related variability, and acceptable accuracy and precision demonstrate the reliability of the method for quantitative analysis of navepegritide in rat plasma. Its successful application to a single-dose rat pharmacokinetic study further demonstrated its suitability for analysis of biological samples and characterization of the plasma concentration–time profile of navepegritide. Importantly, the method-development strategy integrated AQbD principles with a multicriteria sustainability assessment encompassing environmental, sample-preparation, carbon-footprint, workflow, and practical-applicability dimensions. The favorable profiles obtained from AGREE, AGREEprep, Analytical Eco-Scale, CaFRI, CACI, ComplexGAPI, ComplexMOGAPI, and BAGI indicate that the developed procedure combines analytical performance with consideration of environmental and operational requirements. Overall, the developed method provides a rapid and systematically optimized analytical platform for determination of navepegritide in rat plasma and can support its application in preclinical pharmacokinetic investigations.

DECLARATION OF COMPETING INTEREST

The authors confirm that there are no known competing financial interests or personal relationships that could have influenced the findings presented in this paper.

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DATA AVAILABILITY

Data will be available request from authors.

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