Research Article

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Development and validation of a stability indicating UPLC-DAD method coupled with MS-TQD for ramipril and thymoquinone in bioactive SNEDDS with *in silico* toxicity analysis of ramipril degradation products

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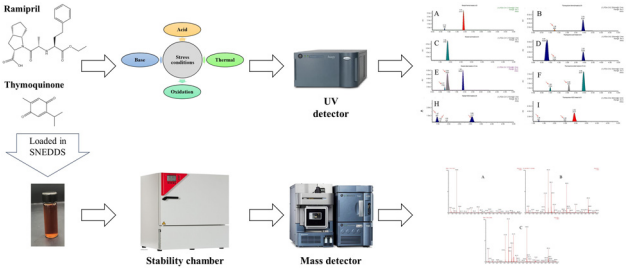
Abstract: The identification of degradation products of therapeutic molecules in pharmaceutical formulations has gained significant attention due to their potential impact on patient safety. Ramipril (RP), an antihypertensive agent, was incorporated into a self-nanoemulsifying drug delivery system (SNEDDS), which greatly enhanced its bioavailability. However, none of the previous studies have investigated the toxicological effects of these degradation products that may form during storage. Moreover, a bioactive SNEDDS containing black cumin oil (BCO) and its bioactive ingredient, thymoquinone (TQ), was used to further enhance the therapeutic activity of RP. To assess the stability of the proposed formulation, a validated ultrahigh-performance liquid chromatography (UPLC) method was developed to simultaneously measure the concentrations of RP and TQ. The formulation was subjected to accelerated stress conditions to facilitate drug degradation. The resulting degradation products were analyzed using mass spectroscopy (MS) to determine their molecular mass, and their chemical structures were *in silico* predicted using Zeneth Nexus software, while their toxicity was assessed using *in silico* Derek Nexus software. RP and TQ, along with their degradation products, were separated using an HSS T3 column at a flow rate of 0.25 mL/min. The detection wavelengths for RP and TQ were 210 and 254 nm, respectively. The developed UPLC method exhibited acceptable linearity for both RP and TQ, with correlation coefficient (r^2) values exceeding 0.9995 and 0.9998, respectively. The method provided accurate, precise, and high-resolution analysis of both drugs and their degradation products within a short run time of less than 3.2 min. The toxicity and mutagenicity of two alkaline degradation products of RP were predicted using *in silico* software Derek Nexus (version 6.3). Several toxicity endpoints, including chromosomal damage, skin sensitization, and hepatotoxicity, were predicted. Overall, the developed method can be used to evaluate the stability and integrity of RP and TQ during the development of the proposed antihypertensive formulation.



Graphical abstract

Keywords: bioactive SNEDDS, ramipril, forced stress degradation, stability-indicating method, TQD-MS

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1 Introduction

The low solubility of therapeutic drug molecules poses a challenge to their bioavailability [1]. Extensive research has been conducted to develop drug delivery systems that can significantly enhance the solubility [2,3]. However, some drug molecules face stability issues due to incompatibility

with excipients in the formulation [4,5]. Therefore, it is essential to identify and examine the toxicological degradation products formed within these drug delivery systems to assess their impact on patient safety [6].

Ramipril (RP, Figure 1a) is a commonly used antihypertensive agent for hypertension treatment [7]. It exerts its pharmacological effects by inhibiting angiotensin-converting enzyme (ACE) [8]. Additionally, RP has shown protective effects on mortality following a heart attack [9]. However, its limited solubility leads to poor dissolution and reduced bioavailability [10]. To address this issue, various drug delivery systems have been employed, including the self-nanoemulsifying drug delivery system (SNEDDS), which has been extensively used to improve the dissolution rate and bioavailability of RP [10–15]. More recently, a bioactive SNEDDS formulation has been developed to enhance the therapeutic activity of loaded drug molecules and improve the bioavailability. This is achieved through the incorporation of oil-containing phytochemicals with nutraceutical properties [16,17].

Nigella sativa has a long history of use in various cultures for treating different diseases, including hypertension [18,19]. Recent clinical studies have demonstrated that the intake of *N. sativa* significantly lowers blood pressure levels compared to control groups [20–22]. Hence, black cumin oil (BCO), an extract of *N. sativa*, can be used to enhance the bioavailability and therapeutic activity of RP. This is due to the presence of a well-known bioactive compound called thymoquinone (TQ, Figure 1b) [23], which exerts antihypertensive effects by reducing ACE production [24,25]. The combination of RP and TQ can potentially offer synergistic antihypertensive benefits.

From a pharmaceutical perspective, BCO can be incorporated into SNEDDS formulations as a bioactive ingredient to improve the bioavailability of naturally occurring TQ, as well as the solubility and bioavailability of RP [10,26]. However, there is a need to develop an analytical method for quantifying the loaded drugs in the prepared SNEDDS formulation during its shelf-life and during its *in vitro* pharmaceutical characterization.

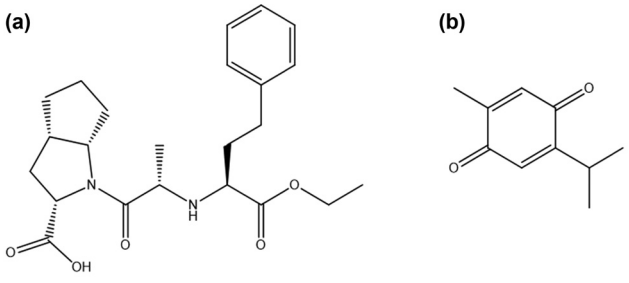


Figure 1: Chemical structures of (a) RP and (b) TQ.

Furthermore, it is crucial to evaluate the potential toxicity of the detected degradation products [27]. Therefore, *in silico* studies were performed using computational methods, specifically Derek Nexus (version 6.3), to assess the potential toxicity and mutagenicity of the proposed compound. Derek Nexus (version 6.3) software is a specialized software specially adopted to assess various toxicity endpoints, such as carcinogenicity, genotoxicity, mutagenicity, teratogenicity, skin irritation, allergic reactions, and impact on fertility. The results from this study could be used as part of an ICH M7 workflow [28,29].

The main objective of this study was to develop a stability-indicating method for analyzing RP and TQ in the proposed formulation. The developed method was validated for linearity, specificity, accuracy, and precision. It was also tested for its ability to separate degradation products under different stress conditions. The established method was then employed as an analytical tool for quantifying the drugs in the proposed pharmaceutical formulation. Additionally, mass spectroscopy (MS) was utilized to identify the exact degradation products in the formulation. The degradation products' chemical structures were further confirmed by *in silico* Zeneth Nexus software. Zeneth software uses a knowledge-based approach to forecast potential pathways for degradation and byproducts under various stress scenarios. Finally, the *in silico* prediction of toxicity for these degradation products was investigated using the Derek Nexus software.

2 Materials and methods

2.1 Materials

RP was purchased from Jai Radhe Sales (Ahmedabad, India). TQ was obtained from Sigma-Aldrich (St. Louis, MO, USA). Hydrogenated castor oil (HCO-30) was acquired from Nicole Chemical Co. (Tokyo, Japan). Transcutol P (TC-P) was obtained from Gattefossé (Lyon, France).

2.2 Preparation of BCO

The procedure for obtaining BCO was described in a previous publication [13]. In summary, black cumin seeds (BCS) were collected from the central part of Bangladesh. The collected seeds were cleaned using fresh water and then dried in open air with exposure to sunlight to remove all moisture. The dried seeds were then cold-pressed to

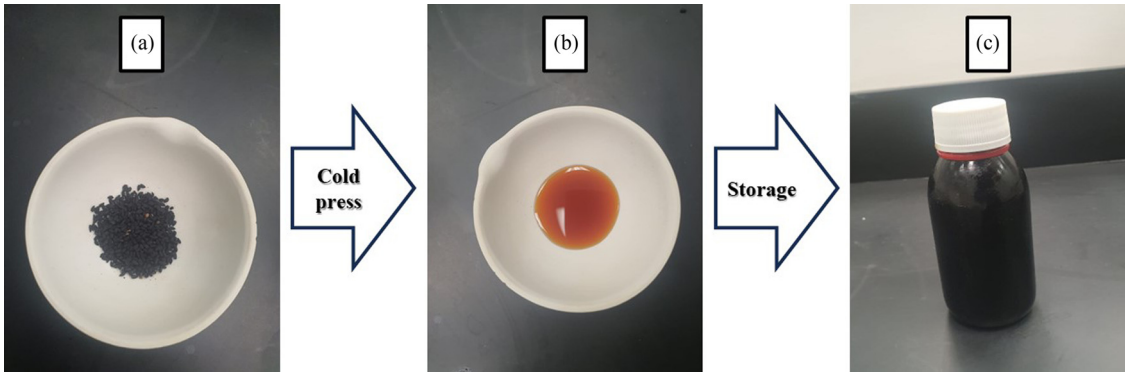


Figure 2: Preparation of BCO: (a) BCS, (b) BCO obtained from compressed BCS, and (c) BCO stored in an amber glass bottle to protect it from light.

extract the oil. The obtained oil was stored in an amber glass bottle with a screw cap, as shown in Figure 2.

2.3 Preparation of BCO-based SNEDDS formulation

The SNEDDS formulation was prepared following the procedure described in the literature [30]. In a 4 mL glass vial, HCO-30, TC, and BCO were accurately weighed in a ratio of 40:27.75:32.25 to create the RP-free bioactive SNEDDS. Afterward, 10 mg of RP was added to 990 mg of the RP-free SNEDDS and subjected to vortexing and sonication to ensure complete solubilization of the drug. The drug content, specifically RP and TQ, was determined using the developed ultrahigh-performance liquid chromatography (UPLC) method to quantify the initial concentrations accurately.

2.4 Preparation of mobile phase (solution A)

To prepare the mobile phase, 1 g of sodium lauryl sulfate was dissolved in a 1 L volumetric flask and then MQ water was added to prepare a 0.1% w/v solution. The pH of the solution was adjusted to 2.4 ± 0.1 using phosphoric acid. The solution was filtered through a 0.45 µm Millipore membrane filter. Acetonitrile (ACN) was then added to the filtered solution in a ratio of 55:45 v/v. The resulting mixture was further adjusted to a pH of 2.75 ± 0.1 using phosphoric acid, and this mixture was named Solution A.

2.5 Instrumentation and chromatographic conditions

The analysis of RP and TQ in the standard solutions, quality control samples, and bioactive SNEDDS formulations was carried out using the Waters Acquity UPLC system (Milford, MA, USA). The UPLC system consists of a binary solvent pump manager connected to a column chamber with controlled temperature. The samples were injected through an Acquity automatic Sample Manager and passed through a connected column. The drug concentrations were analyzed using the equipped Acquity photodiode array (PDA) detector. The mobile phase used for elution was prepared by combining Solution A and ACN in a ratio of 67:33. Separation was performed on an Acquity UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 µm). The flow rate of the mobile phase was maintained at 0.25 mL/min. A sample volume of 2 µL was injected, and the absorbance of RP and TQ was detected at 210 and 254 nm, respectively.

2.6 Preparation of standard stock solution, calibration, and quality control samples

To prepare stock solutions of both drugs, 5 mg of each drug was dissolved in separate 10 mL volumetric flasks using ACN. This resulted in stock solutions with a concentration of 500 µg/mL for each drug. From the prepared stock solutions, serial dilutions were performed by diluting the stock solution with ACN to obtain the desired concentration range. For RP, the concentrations ranged from 1.5 to 50 µg/mL, and for TQ the concentrations ranged from 0.5 to 50 µg/mL.

2.7 Validation studies

2.7.1 Linearity and calibration

To construct the calibration curves, dilutions of RP and TQ with varying concentrations were prepared. These freshly prepared standard solutions were then injected in six

replicates, and the resulting peak areas were recorded. The peak area is a measure of the response obtained from the UV absorbance of the compounds. Using the MassLynx software (Waters Corporation, Milford, MA, USA), the concentration of RP or TQ was plotted against the corresponding measured peak area. The linear regression equations were then calculated to determine the relationship between the drug concentration and absorbance. This included calculating parameters such as the slope, intercept, and correlation coefficient (r^2) of the calibration curves [31].

2.7.2 Specificity

The specificity of the developed analytical method demonstrates its capability to accurately determine the drug concentration even in the presence of other components. This encompasses assessing any potential interference, such as the matrix effect, between the retention time of the drug peak and other components. Matrix components were injected to evaluate method specificity, and the retention times of detected peaks were compared with those of the matrix spiked with RP and TQ. This analysis ensured that any potential interference was properly identified and avoided during drug concentration determination [32].

2.7.3 Limit of detection (LOD) and lower limit of quantification (LLOQ)

LOD and LLOQ were estimated based on the calculated standard deviation (SD) of responses and slope obtained from the calibration curve using the following equations:

$$\text{LOD} = (3.3 \times \text{SD})/\text{slope},$$

$$\text{LLOQ} = (10 \times \text{SD})/\text{slope}.$$

2.7.4 Accuracy and precision

Accuracy (% recovery) assessed the ability of the developed method to detect the drug concentration close to the predicted value. This was accomplished by comparing the theoretical concentration with the mean concentration of the injected sample, which was determined based on the constructed calibration curve. Precision (relative standard deviation, RSD%) was calculated to evaluate the method's capability to consistently estimate the drug concentration close to its expected value. Typically, this was accomplished by comparing the standard deviation value of multiple injections to the mean of the results. In this study, quality control samples were injected six times over a period of 3 days. The recovery (%) and RSD% for each concentration were calculated to determine the accuracy and precision of the method, respectively.

2.7.5 Stability of the prepared standard solution

In order to assess the stability of the calibration curve under refrigeration conditions, two concentrations were selected for the study. The prepared solutions were stored in a freezer at −20°C, and the drug concentrations were analyzed using the freshly constructed calibration curve on a weekly basis. This allowed for monitoring any potential changes in the accuracy and reliability of the calibration curve over time in the refrigerated storage conditions.

2.8 Stability indicating study

A forced degradation study was conducted to evaluate the ability of the developed method to separate the RP and TQ peaks from any degradation peaks that may arise under various stress conditions. This involved subjecting RP and TQ solutions to oxidative, thermal, acid, and alkaline hydrolysis studies. Following the degradation process, the samples were filtered using a filter syringe and analyzed using the developed UPLC method.

2.9 Application of the developed method

A bioactive SNEDDS formulation, loaded with RP, was prepared to assess the concentrations of RP and TQ after exposure to stability storage conditions [33]. Initially, the concentrations of RP and TQ in the freshly prepared SNEDDS formulation were determined in order to calculate the initial drug concentration. The prepared SNEDDS formulation was then placed in a stability chamber under controlled conditions (40°C and 75% RH) and stored for a period of 2 months. At the end of this incubation period, the SNEDDS formulation from the stability chamber was retrieved, and a chromatogram of the formulation was obtained using the developed UPLC method to evaluate its effectiveness in separating the drugs from any potential degradation products.

2.10 Parameters for the MS scan

The degradation products for the stability sample were determined using a triple quadrupole (TQD) mass spectrometer. TQD was operated in positive electrospray

ionization (ESI+) mode. The TQD was run in the MS scan mode over the range of 50–1,000 Da. The cone voltage was set as 33 V. The capillary voltage, extractor voltage, and RF lens were set at 3.3 kV, 3.0 V, and 0.1 V, respectively. The source temperature and desolvation temperature were set at 150 and 350°C, respectively. Nitrogen was used as the desolvation gas. The rate of desolvation gas flow was 600 L/H. The flow of cone gas was kept at 0.0 mL. The chromatographic conditions regarding the column and mobile phase composition were the same as those of the UV method to track the molecular weight of the compound peak at its exact location. The injection volume was 5 µL.

2.11 *In silico* prediction of the chemical structure and toxicity of the degradation products

A degradation prediction research was conducted using Zeneth software, version 9 (software introduced by Lhasa Limited) in a variety of circumstances. All the three observed DPs were correctly predicted by the adopted Zeneth software.

Lhasa Limited has introduced a software application named Derek Nexus, designed specifically for the assessment of chemical toxicity as well as mutagenicity. This tool employs a knowledge-based approach to generate predictions, allowing users to evaluate potential hazards associated with a chemical by analyzing various endpoints such as mutagenicity, teratogenicity, carcinogenicity, skin irritation, hepatotoxicity, and phototoxicity across different species. The chemical structure under investigation was input, and the prediction process was initiated by executing a setup command in Derek Nexus. Detailed explanations of the predictions can be found in Section 3.

3 Results and discussion

3.1 Optimization of the developed UPLC method

An initial investigation was conducted on various columns and different eluent mobile phases to determine the optimal system components for separating the drugs and potential degradation products. After each optimization, the prepared degradation samples of RP and TQ were injected to assess the separation of the parent drug peak from the degradation products. By employing an isocratic elution method with a mobile phase consisting of solution A and ACN in a ratio of 67:33, and using an HSS T3 column, it was possible to separate both drugs and their degradation peaks. Figure 3 depicts the detected peaks of RP and TQ under the optimized conditions, along with the UV spectrum analysis to identify the maximum wavelength (λ_{max}) using the connected PDA mode. The figure clearly shows that both drugs have distinct λ_{max} values, making it impossible to simultaneously determine RP and TQ at the same λ_{max} unless the method's sensitivity is compromised. As a result, RP and TQ were detected using different UV channels, specifically 210 and 254 nm, respectively, which aligned with previously published data [34,35]. The retention times of RP and TQ were found to be 1.69 and 2.84 min, respectively, with a total run time of 3.2 min.

3.2 Method validation

3.2.1 Selectivity and system suitability

Figure 4a and 4b illustrates the chromatograms of the injected standard solutions of RP and TQ, respectively. In order to examine any potential interference from the matrix before validation, an RP-free bioactive SNEDDS sample (without BCO) was injected [36]. The obtained chromatogram, as shown in Figure 4, verifies the absence of any interference peaks at the retention times of RP and TQ. The analysis of both the UV spectrum and the matrix effect confirms that the detected peaks indeed correspond to the analyzed RP and TQ drugs.

3.2.2 Linearity

The validation parameters for RP and TQ were evaluated in accordance with the ICH guidelines. Standard solutions of RP and TQ were prepared and injected using the previously described UPLC method outlined in Section 3.1. Figure S1 shows the overlay chromatograms obtained from the injected RP and TQ standard solutions. Theoretical drug concentrations were plotted against the corresponding detected peak areas for RP and TQ, as depicted in Figure S1. The resulting calibration curves for RP and TQ demonstrated excellent linearity, as indicated by the coefficient of regression. The calibration curve for RP showed a linear relationship with an r^2 value of 0.9995 within the studied range of 0.5–50 ppm (Figure S2 and Table 1). The equation of the calibration curve, obtained with software, can be expressed as follows: $y = 267.6 \times x + 24.63$. Table 2 presents the back calculation results

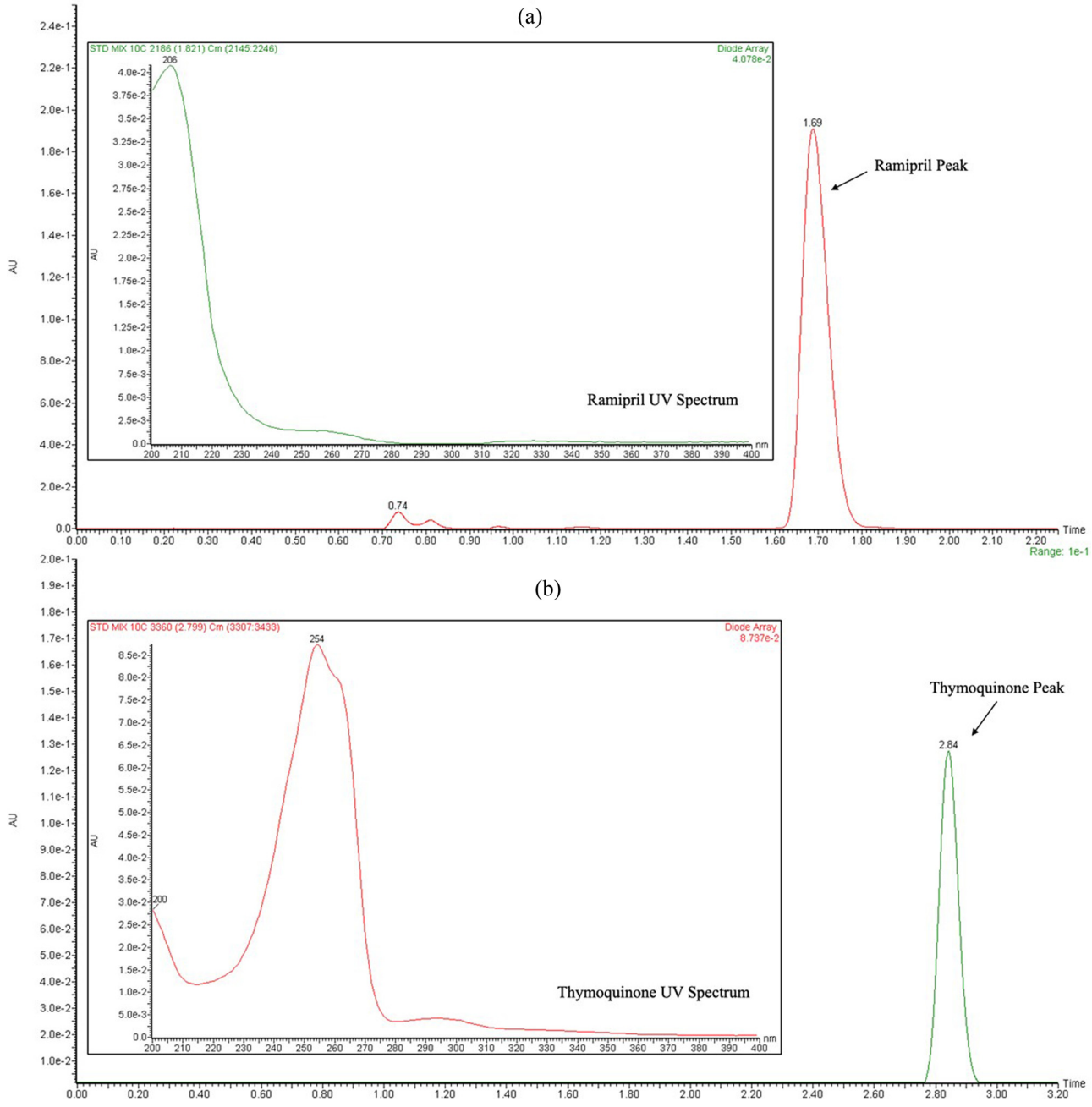


Figure 3: Detected chromatograms of (a) RP standard solution and (b) TQ standard solution along with their corresponding UV spectra.

of the injected replicates. The residual plot in Figure S2 indicates that the collected data were randomly scattered around the zero line, further confirming the linearity of the obtained results.

Regarding TQ, the standard calibration curve was established within the selected range of 0.05–50 ppm (Figure S2). Regression analysis revealed a calculated correlation coefficient (r^2) of 0.9998, indicating excellent linearity across the studied range. The equation of the calibration curve for TQ is represented as $Y = 875.9X - 2.83$. Additionally, Figure S2 illustrates the residual plot, confirming the linearity of the calibration curve by displaying a random distribution of data points around the zero line. Based on these findings, the developed method can be deemed reliable for the simultaneous determination of RP and TQ concentrations.

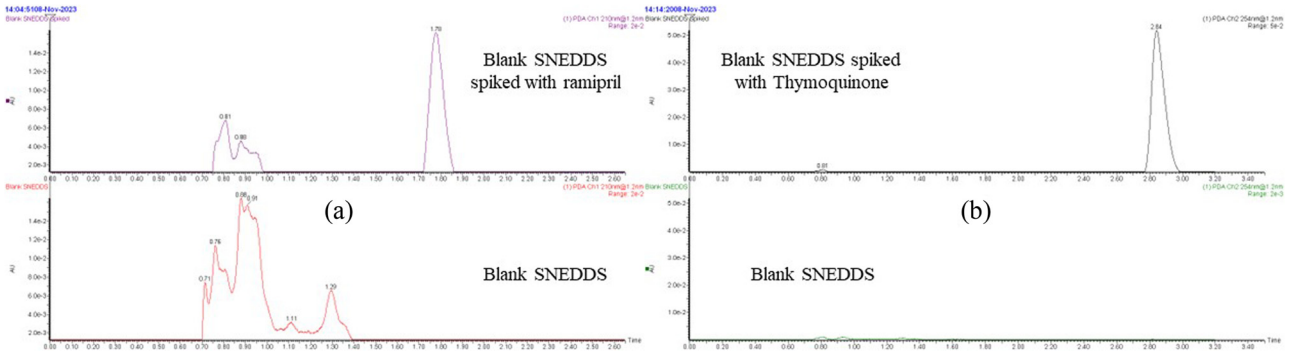


Figure 4: Chromatograms of matrix extraction (RP-free bioactive SNEDDS formulation without BCO) against the counterpart spiked with (a) RP and (b) TQ. The detections were at 210 and 254 nm, respectively.

Table 1: Linear regression analysis of RP and TQ via the developed UPLC method

Parameter	RP	TQ
Linearity range (µg/mL)	1.5–50	0.5–50
Correlation coefficient (r^2)	0.9995	0.9998
LOD (µg/mL)	0.57	0.24
LLOQ (µg/mL)	1.73	0.73

3.2.3 LOD and LLOQ

The LODs for RP and TQ were estimated via the calculated slope of intercept method from the constructed calibration curve and found to be 0.57 and 0.24 µg/mL, respectively. Correspondingly, the calculated LLOQs for both drugs were determined to be 1.73 and 0.73 µg/mL for RP and TQ, respectively. Moreover, the quality control sample prepared to represent LLOQ demonstrated good inter-day accuracy with % recoveries of 96.92 and 102.2 for RP and TQ, respectively. Additionally, the calculated RSD% found to be 1.56 and 6.12 for RP and TQ, respectively, indicates the acceptable precision of the developed UPLC method.

3.2.4 Accuracy and precision

To estimate the accuracy of the developed method, percentage recovery was calculated following the injection of quality control samples. In addition, the precision of the developed UPLC method was assessed to determine its

Table 2: Percent recovery of RP and TQ

Theoretical concentration (µg/mL)	Mean (µg/mL)	Accuracy (%)	Precision (RSD %)
RP			
1.5	1.47	97.78	3.85
2.5	2.47	98.67	2.31
5	4.97	99.33	1.15
7.5	7.63	101.78	1.54
20	20.03	100.17	1.04
50	49.87	99.73	1.27
TQ			
0.5	0.47	94.00	0.24
0.75	0.70	93.33	0.33
1.5	1.43	95.56	3.85
2.5	2.43	97.33	2.31
5	4.93	98.67	2.31
7.5	7.50	100.00	2.31
20	19.97	99.83	0.29
50	50.23	100.47	0.46

Table 3: Intra- and inter-day precision for the analysis of RP and TQ

Quality control sample	Intra-day accuracy and precision		Inter-day accuracy and precision	
	Accuracy (%)	Precision (RSD%)	Accuracy (%)	Precision (RSD%)
RM				
1.5 µg/mL	97.37	0.15	96.26	1.56
4.5 µg/mL	92.67	2.25	91.94	1.94
10.0 µg/mL	92.1	4.65	91.56	3.37
17.5 µg/mL	105.17	3.78	106.36	3.17
40.0 µg/mL	100.9	0.26	100.76	0.36
TQ				
0.5 µg/mL	105	3.82	102.12	6.12
1.5 µg/mL	96.7	4.63	96.66	3.62
10.0 µg/mL	95.17	3.65	94.56	2.75
17.5 µg/mL	104.73	2.4	105.52	2.03
40.0 µg/mL	100.17	0.78	100.34	0.6

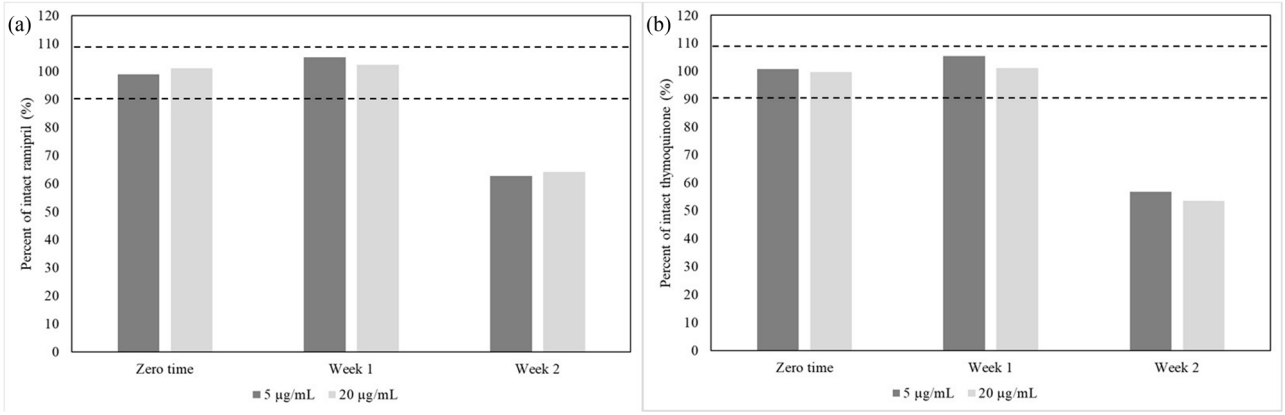


Figure 5: Histograms of back-calculated concentrations for the standard solution of (a) Ramipril and (b) Thymoquinone stored in the refrigerator for 2 weeks.

repeatability. Five quality control samples were prepared for RP, consisting of concentrations of 1.5, 4.5, 10.0, 17.5, and 40.0 µg/mL, representing LLOQ, 3LLOQ, 20% upper limit, 35% upper limit, and 80% upper limit, respectively. Similarly, quality control samples were prepared for TQ with concentrations of 0.5, 1.5, 10.0, 17.5, and 40.0, representing LLOQ, 3LLOQ, 20% upper limit, 35% upper limit, and 80% upper limit, respectively. Table 3 presents the % recovery of these five concentrations with RSD % for intra- and inter-day measurements. The % recovery was found to range from 91.6 to 106.4%, and RSD% was less than 6.2%.

3.2.5 Stability of the calibration curve in a freezer

In order to assess the stability of the prepared calibration curve in a freezer, two concentrations (5 and 20 µg/mL) were chosen. The drug solutions were stored in amber vials with tight screw caps. Figure 5 illustrates the percentage recovery of the injected drug concentrations over a 2-week period. As shown in the figure, the recovered drug concentrations remained within the range of 90–110% after 1 week of storage, affirming the reliability of utilizing standard solutions for daily routine analysis. However, an

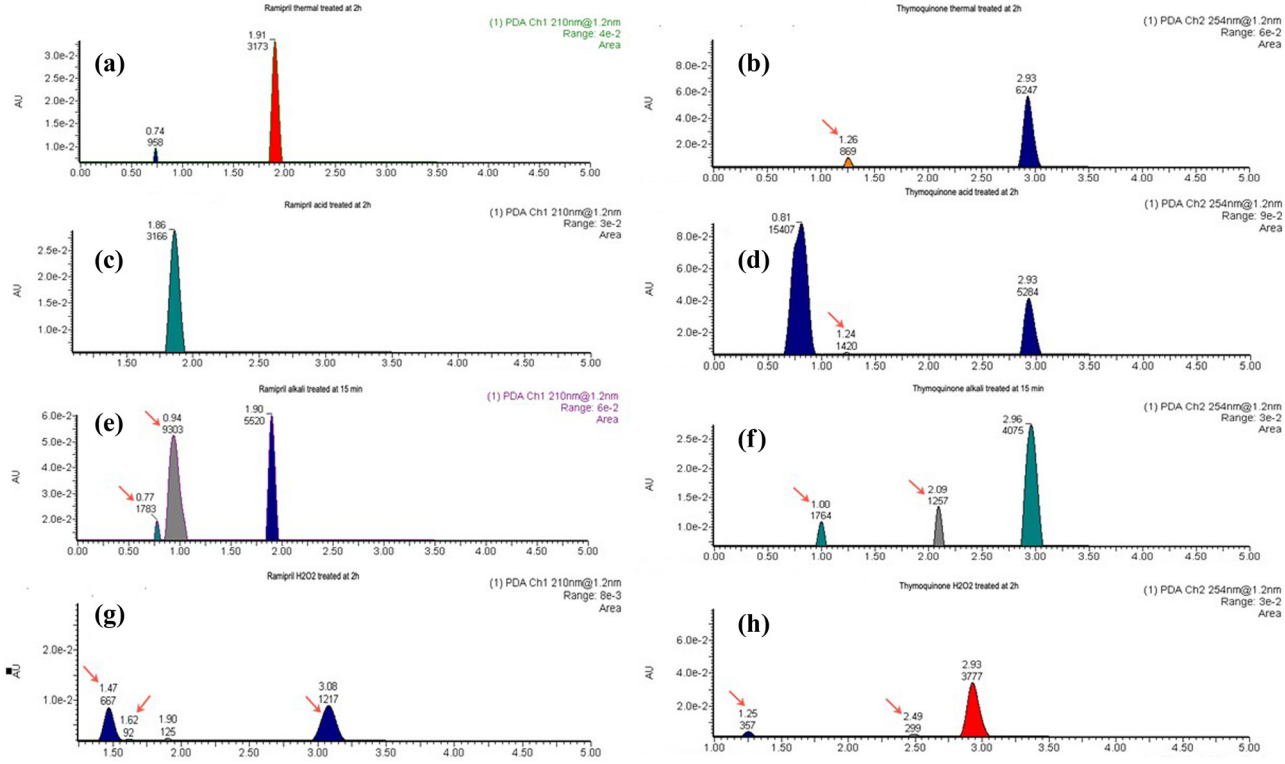


Figure 6: Chromatograms obtained from the forced degradation study, including (a and b) thermally treated, (c and d) acid-treated, (e and f) alkaline-treated, and (g and h) oxidative samples of RP and TQ, respectively.

Table 4: Published stability-indicating methods used quantification of RP and TQ

Drug name	Mobile phase	Stationary phase	Run time (min)	Detection wavelength (nm)	References
RP	ACN + 100 mM sodium perchlorate solution (pH 2.5)	SUPELCOSIL LC-8 column	8	210	[38]
	10 mM ammonium acetate buffer (pH 6) + methanol	cyanopropyl column	15	210	[39]
	ACN + water	C18 column	7	215	[40]
	ACN + 50 mM ammonium acetate	XBridge C18 column	6	240	[41]
	ACN + 4.5 M sodium lauryl sulfate as buffer	Zorbax XDB-C18 column	3	210	[34]
	0.1% Sodium lauryl sulfate + ACN	Acquity UPLC HSS T3 column	3.25	210	Current work
TQ	Water + ACN	HSS-T3 C18 column	3.5	294	[42]
	0.1% Formic acid + methanol	C18 column	12.5	254	[43]
	0.1% Sodium lauryl sulfate + ACN	Acquity UPLC HSS T3 column	3.25	254	Current work

additional week of storage (a total of 2 weeks) led to a significant decrease in drug concentrations. Consequently, it is recommended to prepare a fresh standard solution each week to ensure the accuracy of back-calculated data based on the constructed calibration curve.

3.3 Forced degradation study

To investigate the effects of acid, alkaline, thermal, and oxidative hydrolysis on the prepared stock solutions of RP and TQ, they were incubated with 1 N hydrochloric acid, 1 N sodium hydroxide, pure water, and 6% hydrogen peroxide, respectively. The samples were then heated at 80°C for 2 h to accelerate hydrolysis under the tested conditions. Notably, the alkali-forced degradation study observed complete degradation of RP and TQ. Consequently, the samples were incubated at room temperature for approximately 15 min to detect the original parent peaks along with their corresponding degradation products. Figure 6 displays the chromatograms obtained following RP and TQ hydrolysis studies. Furthermore, the resolution value exceeded 1.5, indicating that both drugs were well separated without any interference [37]. These findings suggest that the developed method can accurately quantify both drugs even in the presence of degradation products. In addition, Table 4 summarizes the previously published stability-indicating methods in terms of the mobile phase used, column type, run time, and detected wavelength. Our method demonstrates a significantly shorter run time of just 3.5 min while effectively detecting both drugs and their degradation products, showcasing its superiority over the previously published methods.

3.4 Pharmaceutical application in characterization of developed SNEDDS formulation

To assess the pharmaceutical characteristics of the prepared RP-loaded bioactive SNEDDS formulation, its mixed components were examined for their ability to spontaneously form a nanoemulsion upon exposure to aqueous media in the gastrointestinal tract (GIT). The formulated mixture was diluted and mixed using a magnetic stirrer for a duration of 5 min. Subsequently, the particle size of the resulting dispersion system was measured using a Zetasizer, as depicted in Figure 7. It was observed that the dispersed system exhibited a nanosize range, with a

			Size (d.n...	% Intensity	Width (d.n...
Z-Average (d.nm):	96.92	Peak 1:	160.5	100.0	146.0
PdI:	0.286	Peak 2:	0.000	0.0	0.000
Intercept:	0.910	Peak 3:	0.000	0.0	0.000
Result quality	Good				

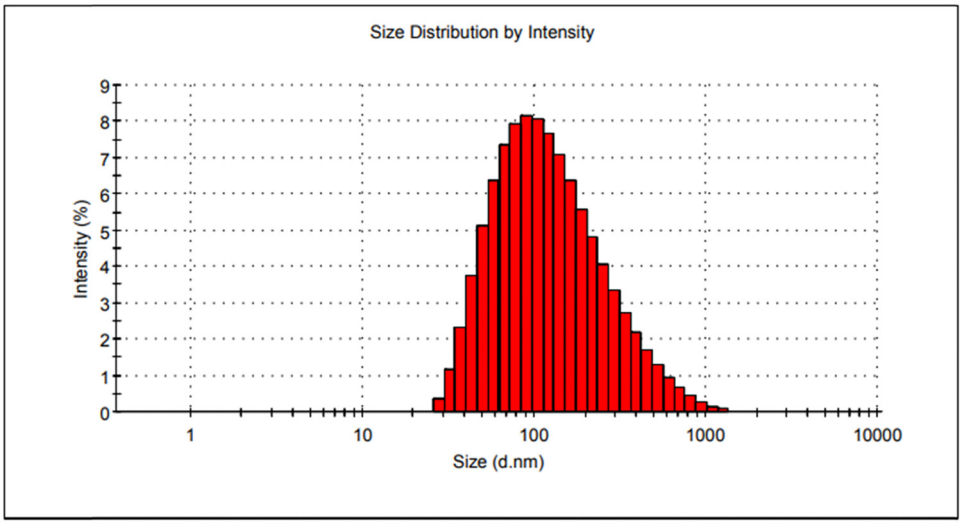


Figure 7: Histogram of particle size distribution of RP-loaded bioactive SNEDDS formulation dispersed in distilled water (1:1,000).

PDI value lower than 0.3, indicating the uniformity and homogeneity of the dispersion system [44,45]. This finding suggests that the prepared formulation has the potential to enhance the bioavailability of both RP and TQ, leading to improved therapeutic outcomes in the treatment of hypertension [46,47].

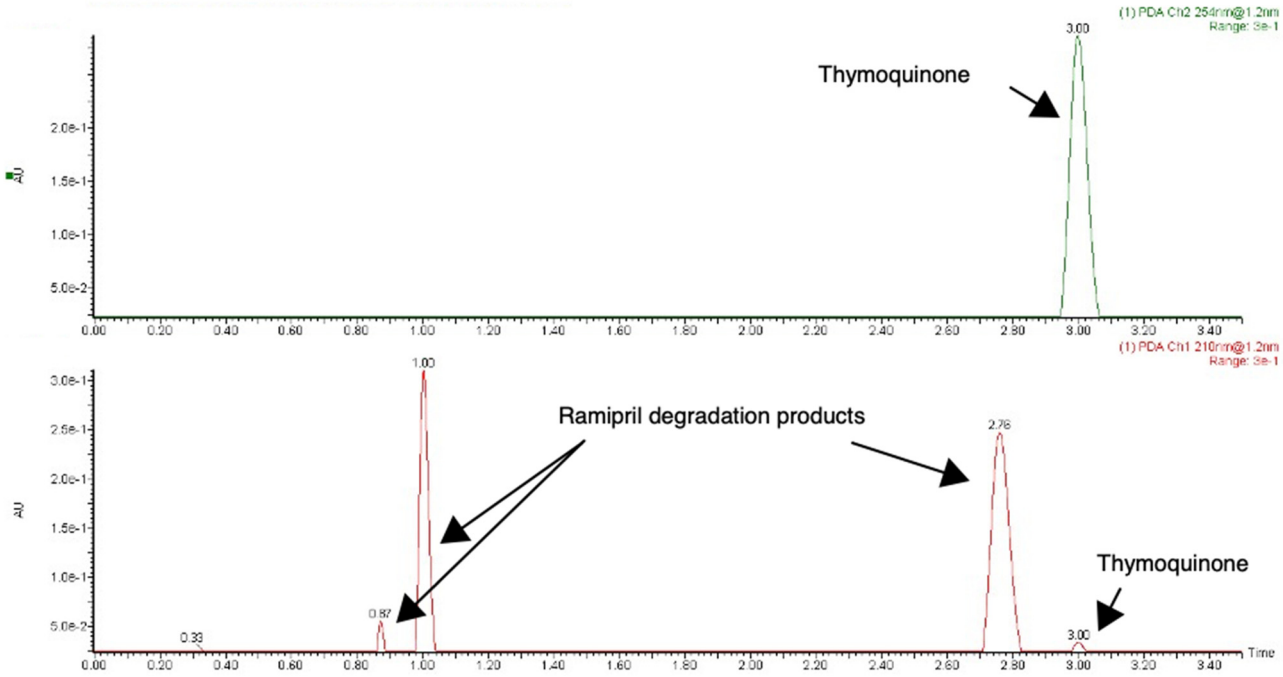


Figure 8: Chromatograms obtained from RP- and TQ-loaded bioactive SNEDDS incubated at 40°C with 75% RH for 2 months.

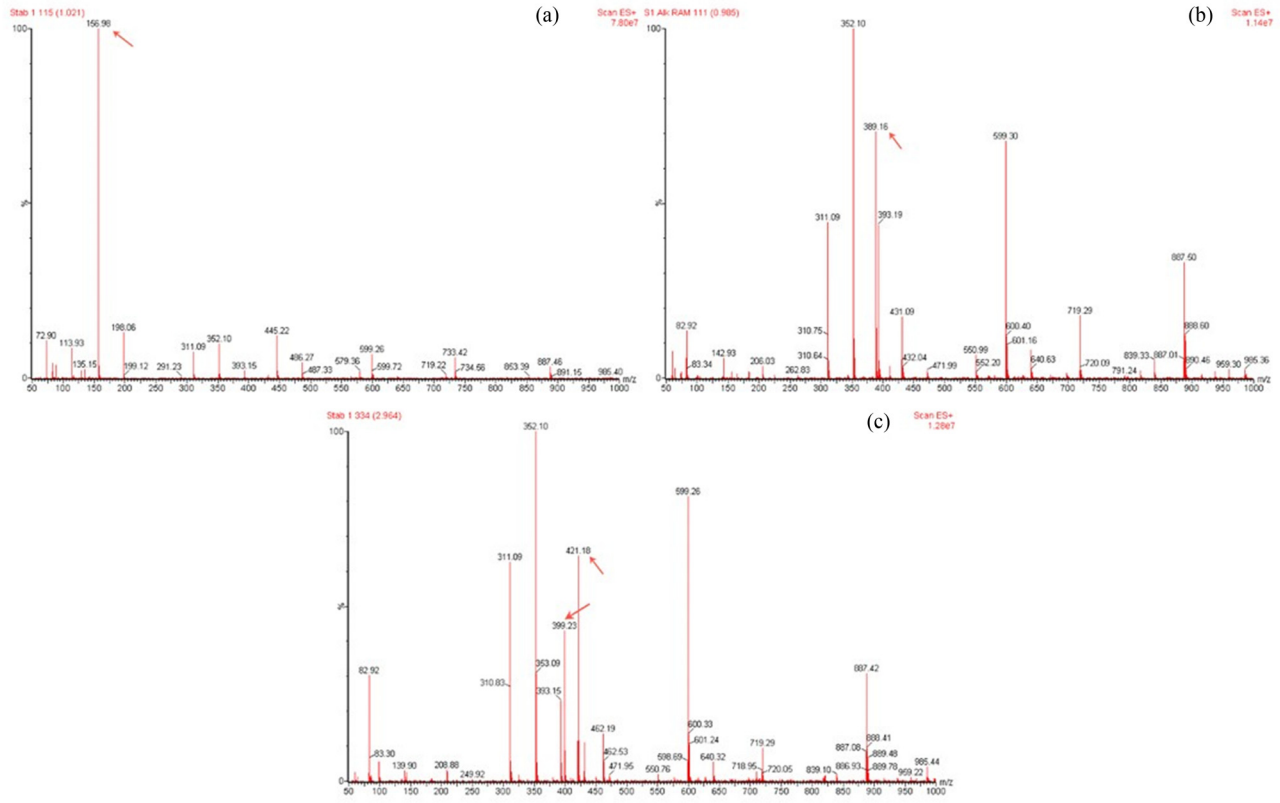


Figure 9: Mass spectra of detected degradation products at (a) 0.87, (b) 1.00, and (c) 2.76 min.

Table 5: Practically observed and theoretically predicted degradation products by Zeneth software for RP

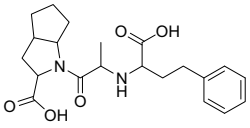
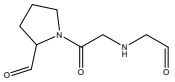
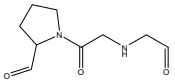
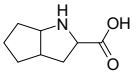
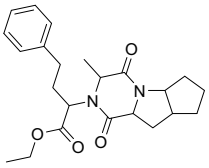
Name	Transformation name	Conditions	Exact mass
D1	Hydrolysis of amide	Water; pH	155.0946
D2	Hydrolysis of alkyl ester	Water; pH	388.1998
D3	Lactamization of the amino acid or derivative	pH, Temperature	398.2206

3.5 Application of UPLC

According to the existing literature, both RP and TQ are known to be sensitive to heat and humidity [48,49]. To assess the stability of the prepared RP-loaded bioactive SNEDDS, an accelerated study was conducted by subjecting it to incubation at 40°C/75%RH, aimed at enhancing the degradation of RP and TQ. Both drugs were extracted from the incubated formulation and subsequently injected to evaluate the capability of the established method to distinguish the parent drug peaks from the resulting degradation products. Figure 8 illustrates the chromatograms of stability samples at the selected channels (210 and 254 nm) used for the detection of RP and TQ, respectively. The figure clearly demonstrates that RP was completely degraded, as no peak was detected at the drug's retention time. On the other hand, TQ's peak was detected in the corresponding channel, with no detected degradation. Therefore, it can be concluded that the developed method successfully separated and quantified RP and TQ even in the presence of degradation products. The detected degradation products were subjected to mass scanning in order to identify their respective molecular weights. Figure 9 displays the molecular weights of the degradation products at 0.87, 1.00, and 2.76 min, which were measured as 156.98, 389.16, and 399.23, respectively. The molecular weight 421.18 can be assumed to be the sodium adduct of 398.22. The molecular weights of the three degradation products were extra confirmed by *in silico* Zeneth software. The Zeneth RP degradation prediction model suggests that the main factors responsible for RP degradation are amide hydrolysis, alkyl ester hydrolysis, and lactamization of the amino acid or derivative.

The results are displayed in Table 5, showing the molecular weights of the degradation products, the stress condition for their formation, and the transformation name.

Table 6: Toxicity and mutagenicity prediction of alkaline degradation products of RP

Derek			
Chemical structure	**Structural alert code**	**Structural alert**	**Endpoints for toxicity**
	562		Teratogenicity
	614		Hepatotoxicity
		No detected toxicity	
		No detected toxicity	

3.6 *In silico* toxicity prediction of the alkaline degradation products of RP

The toxicity and mutagenicity of the alkaline degradation products of RP were estimated using the Derek (version 6.3). Table 6 presents the results of toxicity and mutagenicity studies performed. Predictions were generated by employing various criteria, encompassing humans, monkeys, pigs, dogs, rabbits, guinea hamsters, mice, primates, rats, bacteria, and *Salmonella typhimurium*. Multiple endpoints, including carcinogenicity, chromosomal damage, hepatotoxicity, skin sensitization, teratogenicity, nephrotoxicity, neurotoxicity, phototoxicity, phospholipidosis, and numerous disorders, were calculated as part of the analysis. The following conclusions are here: the first degradation product of PR (retention time = 1.00 min) was anticipated to induce hepatotoxicity and teratogenicity due to *N*-acyl-pyrrolidine, -piperidine, or their analogue. But this group was also found in RP; accordingly, the toxicity of other alkaline degradation products is comparable to the main drug. The other two degradation products (retention times = 0.87 and 2.76 min) showed no toxicity.

4 Conclusions

A validated UPLC method for the simultaneous estimation of RP and TQ and their degradation products has been successfully established. The results obtained from method validation demonstrated good linearity, accuracy, and precision, ensuring the reliability of the developed method. Furthermore, our proposed RP-loaded bioactive SNEDDS formulation displayed the ability to form a nanoemulsion upon dispersion. Standard solutions of RP and TQ were subjected to various stress conditions, including acid, alkaline, thermal, and oxidative hydrolysis, to evaluate the stability and degradation behavior of the drugs. The developed method effectively separated the parent drug peaks from the formed degradation products, enabling accurate quantification. Additionally, the validated method was employed to estimate the levels of both drugs in the formulation after exposure to these stress conditions. Moreover, we employed the Derek software to evaluate the *in silico* toxicity of the alkaline degradation products of RP. The predicted outcomes indicated limited toxicity, notably hepatotoxicity and teratogenicity, associated with the first degradation product. The present study revealed that the developed UPLC method indicated that direct contact between the SNEDDS formulation and RP resulted in the formation of toxicological degradation products during storage. Therefore, an alternative approach should be used to enhance the dissolution and bioavailability of RP with no impact on the safety of treated patients.

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analysis – E.M.E.; investigation – E.M.E., A.Y.S., and A.A.S.; resources – M.K.; data curation – E.M.E.; writing – original draft preparation – E.M.E., A.Y.S., and H.W.D.; writing – review and editing – E.M.E., A.A.S., A.Y.S., and M.A.; visualization – E.M.E. and A.Y.S.; supervision – E.M.E.; project administration – E.M.E.; funding acquisition – E.M.E. All authors have read and agreed to the published version of the manuscript.

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Ethical approval: The conducted research is not related to either human or animal use.

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