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Original Article

Stability-Indicating HPLC Method for Quantification of Isotretinoin Impurities in Complex Semi-Solid Dosage Forms

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ABSTRACT

Background: Isotretinoin, a retinoid derivative of vitamin A, is widely used for the treatment of severe recalcitrant acne, reduces sebum production, suppresses sebaceous gland activity, and exhibits potent anti-inflammatory properties and certain dermatological disorders that do not respond to conventional therapy. **Materials and Methods:** Evaluated a new method using a Supelco Ascentis Express C18 column (150 mm × 4.6 mm, 2.7 µm). Gradient elution was employed with mobile phase A (0.1 mL formic acid in 1000 mL water) and mobile phase B (0.1 mL formic acid in 1000 mL of 60:40 methanol: acetonitrile). **Results and Discussion:** A robust and stability-indicating Reverse-Phase High-Performance Liquid Chromatographic (RP-HPLC) method was developed and validated for the quantitative determination of specified impurities in isotretinoin-containing semi-solid formulations. The method exhibited excellent sensitivity, with quantitation limits of 0.03%, 0.04%, 0.02%, and 0.02% for epoxy impurity, 11,13-di-cis impurity, tretinoin impurity, and isotretinoin, respectively. Validation parameters, including accuracy (85-110% recovery), precision, linearity, and robustness, met ICH guidelines. **Conclusion:** The method efficiently resolves isotretinoin from its known impurities, including epoxy impurity, 11,13-di-cis impurity, and tretinoin impurity, forced degradation studies confirmed the specificity and stability-indicating nature of the method. This method is highly suitable for routine quality control of isotretinoin formulations.

Keywords: HPLC, Impurity Profiling, Isotretinoin, Semi-solid Dosage, Stability-indicating Method, Validation.

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INTRODUCTION

Isotretinoin, a retinoid derivative of vitamin A, is widely used for the treatment of severe recalcitrant acne and certain dermatological disorders that do not respond to conventional therapy (Zaenglein *et al.*, 2016). It effectively reduces sebum production, suppresses sebaceous gland activity, and exhibits potent anti-inflammatory properties, thereby targeting multiple pathways involved in acne pathogenesis (Dreno *et al.*, 2018). Despite its therapeutic benefits, isotretinoin is associated with significant adverse effects, including teratogenicity, mucocutaneous dryness, and hepatotoxicity (Hoofnagle *et al.*, 2019). As a result, stringent quality control measures are necessary to ensure the safety, efficacy, and purity of isotretinoin formulations, particularly in complex semi-solid dosage forms such as gels and creams.

The pharmaceutical stability of isotretinoin is a major concern due to its susceptibility to oxidative, thermal, and photodegradation (Liu *et al.*, 2007). Degradation leads to the formation of specified impurities, including epoxy isotretinoin, 11,13-di-cis isotretinoin, and tretinoin, which may compromise drug efficacy and pose potential toxicity risks (Lima *et al.*, 2005). Regulatory authorities such as the International Council for Harmonisation (ICH) and the United States Pharmacopeia (USP) mandate the identification, quantification, and control of impurities in pharmaceutical formulations to maintain drug quality and safety (ICH, 2006a, 2006b; USP 46-NF 41, 2023). However, existing analytical methods for isotretinoin impurity profiling lack specificity, sensitivity, and stability-indicating capabilities, necessitating the development of an advanced chromatographic technique (Bhatti *et al.*, 2019).

High-Performance Liquid Chromatography (HPLC) remains the gold standard for impurity profiling due to its precision, reproducibility, and ability to separate complex mixtures (Snyder *et al.*, 2010). Various HPLC methods have been reported for the quantification of isotretinoin and its related impurities in different dosage forms, including capsules, topical gels, and suspensions (Snyder *et al.*, 2013; Tashtoush *et al.*, 2007; Patel *et al.*, 2011).

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However, most of these methods focus on a single impurity, such as tretinoin (Layegh *et al.*, 2013), and do not comprehensively assess multiple degradation products in a semi-solid formulation matrix (Gandhimathi *et al.*, 2024). Additionally, many conventional methods fail to meet stability-indicating criteria, making them unsuitable for routine quality control in pharmaceutical industries.

To address these limitations, this study presents a novel, validated, stability-indicating Reverse-Phase HPLC (RP-HPLC) method for the simultaneous quantification of specified impurities in isotretinoin-containing semi-solid dosage forms. The developed method employs a Supelco Ascentis Express C18 column with gradient elution using formic acid-modified methanol and acetonitrile mobile phases. The optimized method ensures baseline separation of isotretinoin and its specified impurities while exhibiting high sensitivity, with quantitation limits as low as 0.02-0.04%. Forced degradation studies, including acid/base hydrolysis, oxidation, thermal stress, and photostability testing, confirm the method's specificity and reliability as per ICH guidelines [ICH Q1A(R2)].

The validated method meets all essential analytical performance parameters, including accuracy (85-110% recovery), precision, linearity, and robustness, making it a powerful tool for impurity profiling in isotretinoin formulations. This study contributes significantly to pharmaceutical quality assurance by providing a reproducible, stability-indicating method that can be implemented in regulatory and industrial settings for routine quality control of isotretinoin-based semi-solid formulations.

In this study, we successfully overcome the difficulties caused by the formulation matrix's complexity by developing a stability-indicating analytical approach for the determination of isotretinoin and its specified impurities in a semi-solid dosage form. We developed an effective chromatographic technique that produced a distinct separation of isotretinoin from its related substances with enhanced sensitivity and repeatability following extensive evaluation with different mobile phase compositions, column chemistries, and gradient programs. The optimized approach showed consistent performance, accurate quantification, and suitability for routine stability tests, in comparison to previous attempts using sodium perchlorate buffers, phosphate buffers, and ammonium salt buffers, which did not provide sufficient resolution. The results achieved in this study provide a validated method for accurately evaluating the quality of semi-solid isotretinoin formulations.

MATERIALS AND METHODS

Materials and Reagents

Isotretinoin working standard and its specified impurities, including epoxy impurity, 11,13-di-cis impurity, and tretinoin impurity, were procured from a certified pharmaceutical supplier. High-Performance Liquid Chromatography (HPLC)-grade methanol, acetonitrile, and formic acid were obtained from Merck (India). Milli-Q water was used for all dilutions, ensuring high purity and minimal contamination. Analytical-grade potassium dihydrogen phosphate, orthophosphoric acid, and dichloromethane were used for buffer preparation and sample extraction. All reagents and solvents were used without further purification.

Instrumentation

A Waters HPLC system equipped with a Photodiode Array (PDA) detector was employed for method development and validation. Chromatographic separation was achieved using a Supelco Ascentis Express C18 column (150 mm × 4.6 mm, 2.7 µm particle size). The mobile phases were degassed using an ultrasonic bath (Sonicator, Branson, USA) and filtered through a 0.45 µm membrane filter before use. Data acquisition and processing were conducted using Waters Empower chromatography software.

Chromatographic Conditions

A gradient elution method was optimized for the quantification of isotretinoin and its impurities. The mobile phase consisted of:

Mobile Phase A: 0.1 mL formic acid in 1000 mL of Milli-Q water.

Mobile Phase B: 0.1 mL formic acid in 1000 mL of a mixture of methanol and acetonitrile (60:40, v/v).

The optimized gradient program was set as follows:

Time (min)	Mobile Phase B (%)
0	50
10	50
45	70
60	70
70	80
80	80
81	50
90	50

The flow rate was maintained at 1.2 mL/min, and the column temperature was set at 40°C. The detection wavelength was fixed at 353 nm, and the injection volume was 10 µL.

Preparation of Standard and Sample Solutions

Accurately weighed 50 mg of isotretinoin working standard was transferred into a 100 mL volumetric flask, dissolved in 5 mL of dichloromethane, and sonicated until complete dissolution. The volume was then adjusted with the diluent (methanol) and mixed thoroughly. A volume of 4.0 mL of the standard stock solution was pipetted into a 50 mL volumetric flask, diluted to volume with methanol, and mixed well. Further dilution was carried out by taking 4.0 mL of the working standard solution into a 100 mL volumetric flask, making up the volume with methanol.

Five capsules were weighed, and their contents were transferred into a 250 mL amber-colored conical flask. A volume of 30 mL of dichloromethane was added, and the mixture was sonicated for 15 min to dissolve the content completely. An additional 100 mL of methanol was added, and sonication was continued for another 15 min. The mixture was cooled to room temperature, further diluted with 120 mL of methanol, and mixed well. The final solution was filtered through a 0.45 µm PVDF filter before injection into the HPLC system. A 25 mL volumetric flask was filled with 3 mL of dichloromethane, and the volume was made up with methanol used as blank.

Method validation

The developed HPLC method was validated according to ICH guidelines Q2(R1) to ensure its suitability for routine impurity profiling. The following validation parameters were assessed: System suitability was evaluated by injecting a standard solution three times under the optimized chromatographic conditions. The acceptance criteria included:

- % Relative Standard Deviation (RSD) of peak areas ≤10%.
- Tailing factor ≤2.0 for the isotretinoin peak.

Specificity was assessed by analyzing blank, placebo, and impurity-spiked solutions. Forced degradation studies were performed to confirm the stability-indicating nature of the method under different stress conditions:

Stress Condition	Treatment
Acid Hydrolysis	1N HCl at room temperature for 1 hr.
Base Hydrolysis	1N NaOH at room temperature for 1 hr.
Oxidation	1% H_2O_2 at room temperature for 30 min.
Thermal Stress	Heated at 80°C for 1 hr.
Water Stress	Stored in water at room temperature for 1 hr.
Photostability	Exposed to 1.2 million lux hours/200 watts/hr.

Chromatograms were analyzed to assess peak purity and degradation product formation.

Linearity was established by preparing solutions at six concentration levels ranging from LOQ (Limit of Quantitation) to 150% of the target concentration. Calibration curves were constructed by plotting actual concentration versus peak area, and the correlation coefficient (R^2) was determined. Precision was evaluated by spiking isotretinoin impurities into the test solution at known concentrations and analyzing six independent preparations. Accuracy was assessed at three levels (LOQ, 100%, and 150%), with recoveries required to be within 85-110%.

LOD and LOQ were determined based on signal-to-noise ratios, where:

LOD was set at a signal-to-noise ratio of 3:1.

LOQ was set at a signal-to-noise ratio of 10:1.

The established LOQs for the impurities were 0.03% for epoxy impurity, 0.04% for 11,13-di-cis impurity, 0.02% for tretinoin impurity, and 0.02% for isotretinoin. Stability studies were performed by storing standard and test solutions at room temperature and analyzing them at 0, 24, and 48 hr. The similarity factor was determined, and results within ±2% of initial values were considered stable. All experiments were performed in triplicate, and statistical analyses were conducted using Microsoft Excel. Mean values, standard deviations, and Relative Standard Deviations (%RSD) were calculated.

RESULTS

The best mobile phase was determined by combining acetonitrile, methanol, and formic acid in a study of various organic modifiers. This approach achieved greater resolution between isotretinoin and its related substances, enhanced peak symmetry, and was compatible with mass spectrometric detection (Gudapati *et al.*, 2024).

The final optimized method employed a Supelco Ascentis Express C18 column (150 mm × 4.6 mm, 2.7 µm), which provided high-efficiency separation with baseline resolution

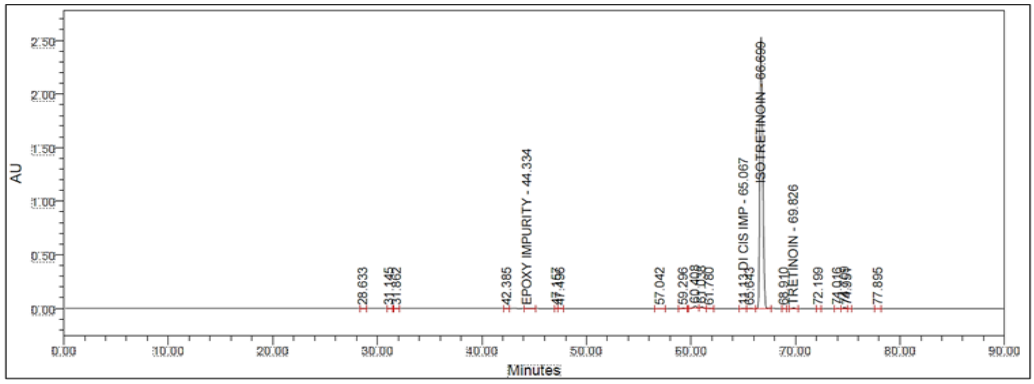

Figure 1: Typical chromatogram of Acid hydrolysis stress sample.

between isotretinoin and its specified impurities-epoxy impurity, 11,13-di-cis impurity, and tretinoin impurity. The gradient elution profile was fine-tuned to minimize peak tailing and co-elution while maintaining a reasonable run time. The optimized conditions resulted in well-defined peaks with a flow rate of 1.2 mL/min, a column temperature of 40°C, and a UV detection wavelength of 353 nm, ensuring high sensitivity.

Table 1: Results of forced degradation.

Stress Condition	% Degradation	Purity Flag
Control (No Stress)	0.46%	No
Acid Hydrolysis (1N HCl, RT, 1 hr)	2.08%	No
Base Hydrolysis (1N NaOH, RT, 1 hr)	0.50%	No
Peroxide Oxidation (1% H_2O_2, RT, 30 min)	0.53%	No
Thermal Stress (80°C, 1 hr)	0.49%	No
Aqueous Stress (Water, RT, 1 hr)	0.50%	No
Photostability (1.2 million lux hours)	11.46%	No

DISCUSSION

System suitability tests were performed to assess the reproducibility and efficiency of the chromatographic system. Key parameters, including the tailing factor, theoretical plate count, and peak area repeatability, met the acceptance criteria as per ICH guidelines. The tailing factor for the isotretinoin peak was 1.0, indicating good peak symmetry, and the %RSD for peak area in replicate injections was found to be 1.1%, demonstrating excellent precision.

Specificity was evaluated by analyzing blank, placebo, standard, and impurity-spiked solutions. No interference was observed at the retention times of isotretinoin and its impurities, confirming the method's selectivity. Forced degradation studies were conducted under acidic, basic, oxidative, thermal, aqueous, and photolytic conditions to evaluate the stability-indicating capability of the method. The results of forced degradation are summarized in Table 1, demonstrated that isotretinoin exhibited significant degradation under photostability stress (11.46% degradation), confirming its susceptibility to light-induced isomerization (bee *et al.*, 2001). Acid hydrolysis chromatogram shown in Figure 1 resulted in 2.08% degradation, whereas oxidative stress led to minor degradation (0.53%), indicating moderate oxidative stability. No significant degradation was observed under basic,

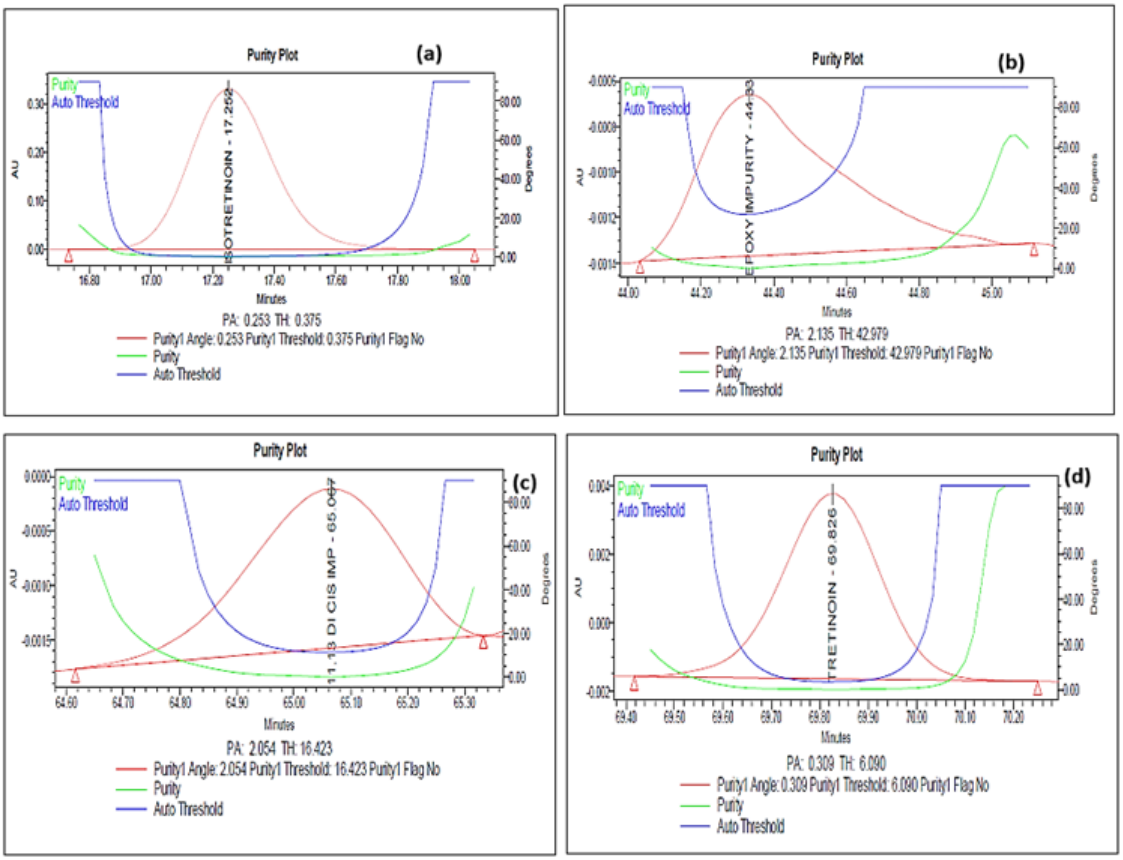

Figure 2: Purity plot for Acid hydrolysis stress sample (a) Isotretinoin (b) Epoxy Impurity (c) 11,13 Di Cis Impurity and (d) Tretinoin.

aqueous, or thermal conditions, confirming isotretinoin's resilience to these environments.

All degradation peaks were well resolved in acid hydrolysis stress as shown in Figure 1, and purity analysis of isotretinoin and its known impurities are shown as purity plots in Figure 2 confirmed the absence of co-eluting peaks, validating the stability-indicating nature of the method.

Linearity was established for isotretinoin and its impurities over the LOQ-150% concentration range. Calibration curves exhibited excellent linearity, with correlation coefficients (R^2) exceeding 0.997 for all analytes shown in Figure 3. The slopes and intercepts were consistent, indicating a robust response across the concentration range.

The Limits of Detection (LOD) and Limits of Quantitation (LOQ) were determined as follows:

Epoxy impurity: LOD=0.01%, LOQ=0.03%

11,13-di-cis impurity: LOD=0.01%, LOQ=0.04%

Tretinoin impurity: LOD=0.01%, LOQ=0.02%

Isotretinoin API: LOD=0.01%, LOQ=0.02%

The LOQ accuracy ranged between 85% and 110%, meeting regulatory acceptance criteria.

The method exhibited high precision, with %RSD values of ≤7.4% for all impurities across six replicate injections. Accuracy studies were performed at LOQ, 100%, and 150% levels, and the recoveries of isotretinoin and its impurities were within 91-105% shown in results of accuracy (Table 2), indicating excellent accuracy and minimal matrix interference.

Solution stability was evaluated for both standard and test preparations over 48 hr. The similarity factor between freshly

Table 2: Results of Accuracy.

Impurity	LOQ Recovery (%)	100% Recovery (%)	150% Recovery (%)
Epoxy impurity	94.0%	91.0%	99.8%
11,13-di-cis impurity	93.6%	101.7%	101.3%
Tretinoin impurity	94.5%	91.7%	91.8%

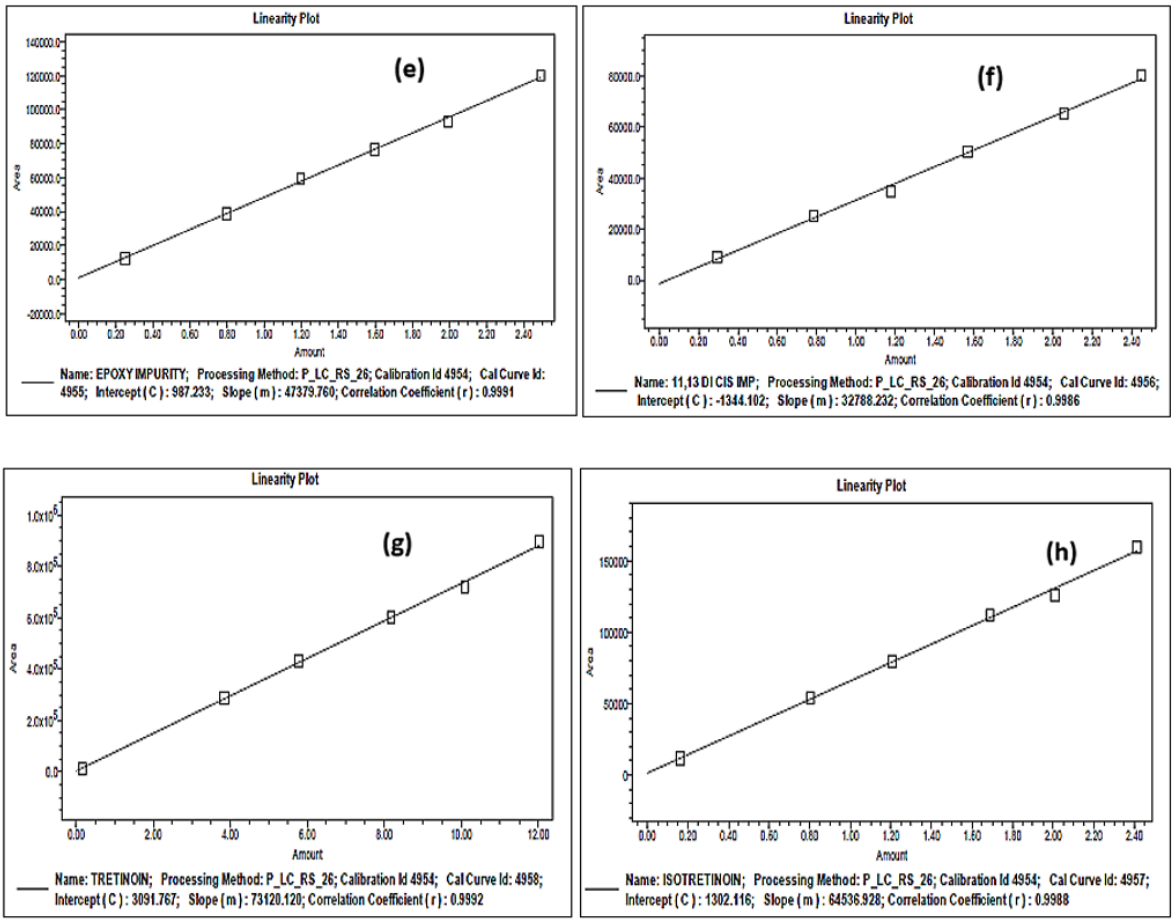

Figure 3: Linearity plot for (e) Epoxy impurity (f) 11, 13-Di cis impurity (g) Tretinoin impurity and (h) Isotretinoin.

prepared and stored solutions was within ±2%, confirming the short-term stability of the method. The test solution remained stable for 24 hr, while the standard solution exhibited stability for 48 hr when stored at room temperature. Robustness was assessed by making deliberate changes to flow rate (±0.2 mL/min), column temperature (±5°C), and mobile phase composition (±5%). No significant variation in retention time, resolution, or peak area was observed, indicating that the method is robust and reliable for routine use. The validated method was successfully applied to analyze commercial isotretinoin semi-solid dosage forms. The impurity levels were found to be well within the acceptable pharmaceutical limits (≤0.1%), confirming the suitability of the method for routine quality control and batch release testing.

CONCLUSION

A robust, precise, and stability-indicating RP-HPLC method was developed and validated for the simultaneous quantification of isotretinoin and its specified impurities in a complex semi-solid dosage matrix. The method demonstrated excellent specificity, precision, accuracy, and robustness, making it suitable for regulatory and industrial applications. It effectively separates degradation products, ensuring compliance with ICH guidelines, and is recommended for routine impurity profiling in pharmaceutical formulations.

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ABBREVIATIONS

RP-HPLC: Reverse Phase High Performance Liquid Chromatography; **ICH:** International Council for Harmonization; **LOQ:** Limit of Quantitation; **LOD:** Limit of Detection; **HCl:** Hydrochloric Acid; **RT:** Room Temperature; **NaOH:** Sodium Hydroxide; **H_2O_2:** Hydrogen Peroxide; **USP:** United States Pharmacopeia; **PDA:** Photodiode Array Detector; **RSD:** Relative Standard Deviation; **UV:** Ultraviolet; **NM:** Nanometre; **mg:** Milligram.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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