RESEARCH PAPER

RP-HPLC Method Development for Estimation of Flutamide loaded Cubosomes: Application of Analytical Quality by Design approach

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ABSTRACT

This study involves RP-HPLC method development for Flutamide, a drug used to treat hirsutism using Analytical Quality by Design (AQbD). AQbD provides a structured, risk-based framework to design reliable analytical methods that ensure pharmaceutical quality control. A Box-Behnken Design was employed to conduct 15 experimental runs, incorporating three independent variables: organic phase concentration (%), flow rate (mL/min), and column temperature (°C). The dependent variables included retention time, tailing factor, and theoretical plates of flutamide. The mobile phase consisted of 90:10 acetonitrile with 0.05% formic acid in water, with flow rate of 1 mL/min analyzed using a Phenomenex Luna C18 column (250 × 4.6 mm, 5 µm). Stability was evaluated under stress conditions through forced degradation testing. The method exhibited outstanding precision and accuracy, with a retention time of 4.36 minutes. Linearity was observed within 2–12 µg/mL (R^2 = 0.999), with detection limit of 0.477 µg/mL and quantitation limit of 1.447 µg/mL. Method validation using ICH Q2 (R2) guidelines confirmed recoveries of 97.03–99.71% in flutamide loaded cubosomes.

Keywords: Analytical Quality by Design, Box-Behnken Design, Flutamide, Hirsutism, RP-HPLC.

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1. Introduction: -

Hirsutism represents a widespread medical condition among females, making it a significant clinical symptom of polycystic ovarian syndrome (PCOS) [1]. Flutamide (FLT**) (Fig. 1)** (2-methyl-N-[4-nitro-3-(trifluoromethyl)phenyl] propenamide) belongs to the trifluoromethyl benzene category. This medication works by blocking specific receptors in areas like the skin, prostate, testes, and hair follicles, which is how it achieves its effects [2,3].

Chromatographic procedures serve as the primary tool for assessing the quantity and identification of substances. Stability measurements coupled with pharmaceutical mixture analysis and drug detection in bodily fluids all utilize this technique [4]. A review of the literature found that multiple analytical strategies exist for FLT determination based on spectrophotometry, GC electron capture detection, HPLC, and voltammetry. A limited set of analyses determined the stability of FLT [5].

Sara Esmaeilzadeh *et.al*. worked together on a reverse-phase HPLC method to measure Flutamide concentration for these protein binding investigations. Ultrafiltration techniques measured protein binding while extracting FLT from samples through a straightforward diethyl ether extraction process that maintained affordability. Acetanilide operated as the reference standard within the HPLC/UV test methodology. For chromatographic separation, the system used an acidic potassium dihydrogen phosphate buffer at pH 3.2 as its mobile phase in conjunction with an acetonitrile-methanol mixture and ran through a reversed-phase C8 column. The determined quantification limit reached 62.5 ng/ml for

Flutamide, and accuracy ratings (86.7–98.5%) combined with precision ratings (0.2–5.3%) met established restrictions. All technique parameters met ICH standards while operating efficiently and exhibiting high sensitivity along with reliability as a method [6]. Vidya Sabale *et.al.* performed the analysis by the High-performance liquid chromatography technique, which is used to measure Flutamide in rat blood. This method is both precise and cost-effective, offering reliable results without incurring high costs. Plasma extraction of Flutamide used methanol as the solvent, while acetanilide acted as an internal standard. The Cromasil C18 column performed chromatography analysis at 227 nm using a 0.8 ml/min methanol-water mobile phase flow rate. The technique achieved accuracy levels of 97–101% with precision below 5% as well as linearity between 100–1000 ng/ml, which had an R^2 value of 0.9947. The methodology determined minimum quantifiable levels at 7.66 ng/ml while detection capability reached 2.52 ng/ml. There was a 96–100% recovery. Researchers tested commercial Flutamide formulations using this methodology to understand their pharmacokinetic behavior [6].

The objective of the current investigation was focused on developing a chromatographic analysis method that demonstrated both simplicity and accuracy in detecting Flutamide in pure form and pharmaceutical products using DoE principles. Research shows that previous analytical methods, despite being commonly used, require complex equipment and long durations.

2. Materials and methods

2.1. Chemicals and reagents

FLT was purchased from Dhamtec Pharma and Consultants, Navi Mumbai. We bought concentrated HCL, NaOH, formic acid, methanol, orthophosphoric acid, and HPLC-grade acetonitrile from Hi Media Laboratories Pvt. Limited in Mumbai, India. Every substance utilized in the study, including Poloxamer, Glyceryl monooleate, and Milli-Q, was sourced from the KLE College of Pharmacy located in Belagavi, India.

2.2. Analytical configuration and instrumentation characteristics

Agilent 1220 Infinity II was utilized for analysis, a sophisticated device manufactured in Japan (model LC-20AD). This equipment included a G7111A binary pump for liquid movement and a degasser (G7129A) to eliminate gas bubbles. It was also equipped with an auto-injector (G7115A) for automatically adding samples and a PDA detector to analyze the results. Advanced Open Lab CDS software handled data interpretation alongside data analysis. Advanced Open Lab CDS software operated as the platform for analyzing collected data. A premium Phenomenex Luna C18 analytical column, C-18(2) 100, with inner dimensions of 4.6 mm, length of 250 mm, and particle diameter of 100 Å was used in the research. Mobile phase of acetonitrile and 0.5% formic acid, combined in a 90:10 ratio, was used to analyze Flutamide. The mixture moved at a consistent flow rate of 1 mL/min. A filter made of PVDF material with pores measuring 0.45 µm was used to stop air bubbles from interfering with the analysis. This filter, the Millex HV from Millipore in the USA, uses ultrasonic waves to eliminate gases from the liquid. Throughout the testing, a consistent injection volume of 10 µL was maintained to ensure reliable and uniform results. Such analytical systems implemented detection using a 227 nm wavelength while operating at 35 °C [7,8].

2.3. Working standard solution and stock solution preparation

Stock solution:1 mg/mL solution of FLT was prepared using three different 10 ml volumetric flasks to dissolve 0.1 g of each compound in either acetonitrile for HPLC–DAD, or methanol (for HPTLC) analysis. A working solution of FLT was prepared by transferring stock solutions (1 mg/mL) to 10 mL calibrated flasks. Each flask received acetonitrile until it reached its measurement volume [9].

2.4. RP-HPLC method development with Box-Behnken Design (BBD)

Response surface methodology (RSM) is used in the study along with a thorough HPLC process, taking into account both independent and dependent variables [10,11]. The optimization process made use of a particular design known as BBD, which is present in DoE software version 13.0. BBD yields better results for the responses of independent variables than central composite design (CCD), accomplished with fewer trials and experiments. Three independent variables are the subject of the study: flow rate (X2), organic phase percentage (X1), and the temperature of the column (X3), as shown in Table 1. Corresponding dependent variables are the tailing factor (Y2), RT (Y1), and the quantity of Table 1 describes the theoretical plates (Y3) [12].

2.5. Analytical Method Validation

The HPLC method development followed ICH Q2 guidelines for linearity determination, LOD and LOQ, accuracy assessment, precision evaluation, specificity testing, system quality performance verification, and stability degradation analysis [13,14].

2.5.1. Linearity

The testing of approach linearity involved preparing Flutamide at concentrations from 2 to 12 µg/mL. Five duplicate solutions were created. The plot of peak area

against these concentration levels generated the calibration curve. Testing linearity at the same concentration range occupied three days of measurement [15].

2.5.2. Accuracy

The average percentage recovery from the sample provides the fundamental outcome criteria for experimental recovery tests. The analysis required triplicate measurements of the FLT standard solution at three concentrations of 50%, 100%, and 150%. The analysis of samples resulted in reporting recovery percentages.

2.5.3. Precision

In our regular procedures, we conducted tests to see how accurate the method was on the same day and across different days. Analyses involving duplicate standards along with duplicate sample solutions, produced different results measured on both corresponding and separate days. The calculation of %RSD using intermediate precision and repeatability results showed proper precision levels of the method [16].

2.5.4. LOD and LOQ

The sensitivity of analytical techniques relates to LOD and LOQ values. The LOQ stands for the lowest quantifiable quantity of analytes with both precision and accuracy established, while the LOD describes the method's limit to identify the minimum quantifiable amount of analytes. The International Conference of Harmonization guidelines outline three distinct processes. Visual assessment forms the foundation of the first methodology. The data collection based on the curve standard deviation served as the third approach, with signal-to-noise ratio as the second method. The procedure utilized these two formulas to determine LOD and LOQ: LOQ equals 10 SD/S while LOD equals 3.3 SD/S [17, 18].

2.5.5. System Suitability

The system suitability criteria of HPLC method validation ensure both correct operation and precise analysis results from the chromatographic system. The procedure of sample injection occurred six times to confirm the system works correctly while checking retention times, measuring peak areas, theoretical plates, and Flutamide tailing factor [19].

2.5.6. Robustness

Robustness was evaluated to ascertain the method's dependability and resistance to slight changes in the experimental setup. In our study, we assessed resilience by changing the wavelength to 225 and 229 nm. We also adjusted the flow rate to 0.9 and 1.1 ml/min. Additionally, we modified the mobile phase composition to 88:12% and 92:8%. The %RSD of less than 2% indicates that the method performed consistently and reproducibly, even with little adjustments to these parameters. This demonstrates the method's robustness and ability to produce trustworthy results [20,21].

2.5.7. Ruggedness

Ruggedness was done to describe how well the method maintains its performance when experimental conditions undergo minor predetermined alterations. The stability and dependability of an analytical technique can be demonstrated through its ability to function with different analysts and testing laboratories and slightly varying experimental conditions.

2.6. Forced Degradation studies

Forced degradation tests evaluated medication samples' stability levels during stress conditions that included acid and base exposure, oxidation, and photodegradation, together with possible degradation product interference. These evaluations help confirm potential decomposition products while measuring the natural stability features of pharmaceutical active ingredients [22,23].

2.6.1. Acid-base degradation

To experiment, take 2 ml of the Flutamide standard solution and mix it with 2 ml of 0.1 Normal Hydrochloric Acid (HCL) in vials designated for testing acid breakdown. Repeat this by mixing another 2 ml of the Flutamide standard solution with 2 ml of 0.1 Normal Sodium Hydroxide (NaOH) in separate vials for testing base breakdown. Pour these mixtures into a 10 ml measuring flask. Add enough ACN to bring the total volume to 10 ml, ensuring the chemicals are thoroughly combined for the experiment. Two hours of heating occurred for the prepared solutions at 60 to 80°C. The procedure assesses how compounds maintain their integrity in basic environments that could occur in formulation or intestinal processes, while creating acidic scenarios that may arise during stomach passage or manufacturing steps requiring acidic media.

2.6.2. Oxidative degradation

We used a 30% hydrogen peroxide solution for oxidative breakdown. First, take a 10 ml measuring flask. Add 2 ml of a standard solution, which is a liquid with a known composition. Then, add 2 ml of the 30% hydrogen peroxide. This process helps break down substances through a chemical reaction called oxidation. The solution was adjusted to the appropriate level before resting at 60 to 80°C for two hours. This study analyzed how oxidative stress affects the explored material substance while examining its reactions during air interaction and similar environmental exposures [24].

2.6.3. Thermal degradation

The procedure involved filling a volumetric flask of 10 ml with 2 ml of standard Flutamide solution, followed by dilution with ACN up to the mark. The solution

underwent 2 hours of heating at 80°C using a rotatory bath sonicator at a controlled rate.

2.6.4. Photolytic degradation

10 mL clear volumetric flask received 2 mL of standard Flutamide solution under two hours of long-wavelength UV light exposure. The solution received dilution to volume by adding up to 10 mL of ACN following exposure. The chosen volume of solution was moved to the HPLC equipment for evaluation.

2.7. Preparation of Flutamide-Loaded Cubosomes

By the top-down approach, cubosomes were prepared by mixing a blend of stabilizer and lipid in different concentrations [25,26]. The preparation is performed in two steps: the stabilizer and the lipid are melted separately in individual beakers at 60°C, and secondly, the flutamide is added to the molten lipid and then added to a heated stabilizer solution. The mixture above is subjected to the addition of the preheated water at 60 °C dropwise while stirring continuously and homogenizing for 15 minutes under a homogenizer operating at 15,000 rpm . In the second step product mixture is subsequently probe sonicated for five minutes. To avoid exposure to direct sunlight, the resulting cubosome liquid dispersion was kept at ambient temperature in a dark environment. The lipid-based nanocarrier loaded with Flutamide was assessed for particle size, zeta potential, and surface morphological analysis [27].

3. Results and discussion

3.1. Method Development

Various mobile phases have been tested to develop an HPLC method for flutamide detection. One of the mobile phase formulations incorporates methanol (% v/v): The combination of 60:40 (v/v) 0.05 formic acid to water and methanol to orthophosphoric acid (55:45 v/v), Acetonitrile:0.5 ml Formic Acid in MilliQ Water (60:40 v/v). Selecting the mobile phase for Flutamide followed a balance between assay sensitivity and testing stability period alongside ease of preparation and utilization of recognized solvents. A valid RP-HPLC methodology with selective detection emerged for identifying Flutamide through its reliable sensitivity and specificity analysis. During the retention duration of 4.459 minutes, a mobile phase consisting of ACN and 0.5 Formic acid in Milli-Q water at a percentage ratio of 90:10 produced outstanding peak definition, optimal Flutamide resolution, and normal back pressure at a flow rate of 1 ml/min. The detecting wavelength for Flutamide was set at 227 nm by identifying its highest absorption point. Figure No.3 displays the Flutamide Standard Chromatogram.

3.2. RP-HPLC method development with Box-Behnken Design

Using the BBD, three independent variables were selected from the factor screening tests to be varied at three levels: low (-1) and moderate (0), and high (+1). These were the percentage of the organic phase, the column temperature, and the flow velocity. The response variables chosen were RT (Y1), TF (Y2), and TP (Y3) as dependent factors. In Table 2, the Box-Behnken Design 15 runs design response is shown. Flutamide's retention time (Y1) responses, in minutes, ranged from 3.11 to 5.4 minutes for the minimum and maximum, respectively. The tailing factor (Y2) for Flutamide ranged from 1.32 to 1.79. The theoretical Plate range of Flutamide's (Y3) responses ranged from 5541.23 to 5975.36.

To determine the importance of factors and relationships for response variables, the software conducts experimental results by ANOVA. The presentation of the design results used is the statistics details in (Table 2). Polynomial equations are used to estimate the correlations of factors and response measures. A negative result will show the antagonistic influence on the model, while a positive result will be given to register the synergistic effect, which is indicated in the arrows. All the equations' high observed correlation coefficients guarantee that the chosen responses will provide the optimal values.

The 3D reaction surface plots for Y1 (RT) may be used to show that RT reduces with the rise in the organic phase and improves with the increase in flow rate. As far as the RT is concerned, which decreases as this temperature parameter increases, column temperature has minimal influence (Figure 2a). Similarly, when the concentration of the organic phase increases with the flow rate in Y2, as is evident from (Figure 2b), we agree that there is a direct correlation since the tailing factor concentration increases steadily. For comparison reasons, even when the column temperature changed, the tailing factor did not change. Generally, raising the concentration of the organic phase and flow rate to 90%v/v and 1 mL/min, respectively, has resulted in increased theoretical plates and better separation for Y3 (theoretical plates). The theoretical plate number does decrease for these and higher levels (Figure 2c). Even though a large value of column temperature is given as the default for Y3, the histograms for Y3, which has the lowest sensitivity to column temperature change, reflect a shift in the column temperature. Optimal values for the parameters were determined by using Stat-Ease, Inc.'s Design-Expert software, version 13.0: A 90% v/v organic phase, a 1 mL/min flow rate, and a 30◦C column temperature was used. With a 5% prediction error, these predictions can be considered relatively accurate compared to the experimental results, specifically in Run 5.

3.3. Validation of Analytical Method

The method received approval for regular laboratory use, addressing critical factors such as accuracy, precision, resilience, detection and quantification limits, linearity, and range. Testing the improved HPLC method for Flutamide confirmed it met the standards of the ICH Q2 (R1) Guidelines, demonstrating its ability to produce consistent and accurate results in laboratory settings.

3.3.1. Linearity

Tests were conducted using five different concentration levels to study the linearity of the Flutamide analyte. The concentrations of Flutamide were found to range from 2 to 12 micrograms per milliliter (µg/ml). From the calibration curves shown in Figure 4, plotting concentration vs. peak area revealed that the analyte has a good linear correlation. Flutamide demonstrated a good linearity over the established concentration ranges since the correlation coefficient was 0.9992%. This evaluation ensures that the method is more dependable and appropriate to the analytical applications by validating its efficiency in accurately measuring analyte concentrations within the intended range.

3.3.2. Accuracy

The research data on recovery was applied to establish accuracy levels. The laboratory utilized three spiking levels to create corresponding sample solutions, including 50%,100%, and 150%. Data about percentage recovery emerges through the proposed HPLC procedure, which is presented in Table 3. This recovery range of 99.28%, 98.49%, and 101.18% confirms the developed method's accuracy in compliance with ICH Q2 (R2) standards.

3.3.3. Precision

The repeatability RSD% measurement from six repeated tests of 100 µg/ml Flutamide solution produced values lower than 0.082. Table 4 illustrates the assessment of both intraday and interday precisions. The accuracy of the developed approach was confirmed when the RSD% values were maintained below 2.

3.3.4. LOD and LOQ

The Limit of Detection (LOD) is the detection threshold for precise analyte measurement, and the Limit of Quantification (LOQ) is the quantification threshold. As determined by the analytical method, Flutamide's LOD and LOQ values are 0.477 µg/mL and 1.447 µg/mL, respectively. The approach demonstrates suitable application in analytical settings requiring low detection limits because its LOD and LOQ values allow accurate and sensitive Flutamide quantification.

3.3.5. System Suitability

To verify the different parameters, including the retention time, theoretical plates, peak asymmetry, etc., the system suitability test was applied to a representative chromatogram and %RSD of six injections that were replicated. The results are presented in Table 6.

3.3.6. Robustness

Our objective was to assess the process's completeness and its ability to adapt to minor variations in the experimental setup. By significantly adjusting the mobile phase blend, change in wavelength, and flow velocity, we investigated the sturdiness in this work. The RSD% remained below 2%, demonstrating that the methodology continued to produce reliable and replicable results shown in Table 7 despite these little changes. This stability highlights how adaptable the method is in consistently producing outcomes despite slight environmental variations.

3.3.7. Ruggedness

Ruggedness was used to determine how well the strategy performed when used by many analysts while maintaining the same experimental settings. The results in the Table. 8 showed that the %RSD is within the acceptable range, at less than 2%. This indicates that the technique maintained its accuracy and dependability when used by many operators.

3.3.8. Application of method to quantify in formulation

Using the optimum approach, Flutamide in the Flutamide Loaded Cubosomes developed in this study was successfully estimated through the quantitative analysis of data collected along with peak area and sensitivity. The results were satisfactory and optimal with accuracy and percentage coefficients of variation. In further contributing to the development of this field, this paper also provides further research on its applicability in ordinary analysis. The HPLC chromatogram for the synthesized Flutamide-loaded cubosomes is presented in Figure 5.This chromatogram shows the results of an HPLC study conducted at 227.4 nm using a Diode Array Detector (DAD). Different compounds' retention times are displayed on the X-axis (duration in minutes), and absorbance intensity is shown on the Y-axis (mAU). Smaller peaks indicate impurities or other components, while a dominating peak at about 4.5 minutes indicates the main constituent in the sample. The main peak's height and sharpness point to a high concentration of that compound, while the peak's symmetry and width show how well the compound was separated. Smaller peaks that appear before and after the primary peak could indicate degradation products, related compounds, or contaminants.

3.3.9. Forced Degradation studies

The study indicates that the peak levels of the drug and those of its breakdown substances are separate from one another, proving the presented approach's usability. In the acid degradation investigation, Flutamide showed slight degradation peaks of 28.75%

degradation. Similar to this, Flutamide degradation rates were seen in the base degradation investigation, along with the presence of tiny deteriorated peaks. Both peaks were greatly reduced under oxidative stress conditions, and both medications showed minor damaged peaks, according to the oxidative degradation investigation, which also revealed degradation rates of Flutamide. Flutamide revealed a 7% degradation rate under the photolytic degradation study. The drug was barely affected by photolytic degradation conditions, suffering a slight loss in peak height. The dependability and stability of the approach conceived, as revealed in Table 9, were validated by the stability of the peaks under all degradation studies and by the fact that there was no change in the RT of the drug.

4. Conclusion:

This study aimed to determine and measure Flutamide, an antiandrogenic drug commonly utilized in managing hirsutism associated with polycystic ovary syndrome (PCOS), using a simple, fast, and inexpensive high-performance liquid chromatography (HPLC) method. The main intention of the study was to demonstrate that the proposed approach can be applied uniformly to pharmaceutical formulations and bulk drug substances, including cubosomes and other advanced formulations, all while complying with validation requirements established by the International Council for Harmonization (ICH). A retention period of 4.36 min and an adjusted mobile phase of 0.5% formic acid and acetonitrile (90:10, v/v) were used in the developed method on a C18 column to bring about a fast and efficient approach for the analysis. The method validity surveys, such as linearity ($R2 > 0.999$), accuracy (recovery range, 98–102%), and precision (RSD% LPL for intra- and interday reverse correctness), demonstrated the linear predictive equations suitable for quality control of NAT product in finished drugs.The method also displayed good specificity, preventing interference by any excipients or degradation products, but best sensitivity, low LOD, and LOQ values. Studies on forced degradation confirmed the test method confirmed its ability to withstand stress and thus a stability-indicating test. In summary, the analytical method HPLC gives a proven, reliable, and cost-effective simple technique to evaluate with activities of drugs the quality of Flutamide. Its accuracy and sensitivity, and also its durability make it suitable for clinical and research environments, including the development and testing of new formulations for the treatment of androgen-related cysts in women.

Abbreviation

FLT: Flutamide, **ICH:** International Conference of Harmonisation, **QbD:** Quality by Design, **HPLC:** High-Performance Liquid Chromatography, **HPTLC:** High-Performance Thin-Layer Chromatography, **BBD:** Box-Behnken Design, **DAD:** Diode Array Detector, **RSM:** Response Surface Methodology, **LOD:** Limit of Detection, **LOQ:** Limit of Quantification, **ACN:** Acetonitrile, **DoE:** Design of Experiment**, RT:** Retention Time, **TF:** Tailing Factor, **TP:** Theoretical Plate

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Declarations:

Consent for publication

Not applicable.

Availability of data and materials

The authors confirm that the data supporting the findings of this study are available within the article and in the supplementary files provided.

Competing interest/Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethics approval

Not Applicable

Generative AI Declaration

No generative artificial intelligence (AI) tools were used in the conception, design, data collection, analysis, or writing of this study. All content was prepared entirely by the authors.

Author contributions:

Autho1: Supervision, Resources, Project administration, writing- review & editing, Methodology. **Author 2**: Writing – original draft, Investigation, Conceptualization. **Author 3:** Formal analysis, Validation and Writing -review & editing

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Table Captions:

Table 1. Level and Chromatographic Factors for Box-Behnken Design

Table 2. Box-Behnken experimental design implementation and the associated results for the suggested HPLC technique

Table 3. Accuracy performed on developed RP-HPLC Method for Flutamide

Table 4. Intraday and Interday precision studies

Table 5. LOD and LOQ for developed RP-HPLC Method for Flutamide

Table 6. System suitability performed on developed RP-HPLC Method for Flutamide

Table 7. Robustness data for RP-HPLC Method for Flutamide

Table 8. Ruggedness parameters of the developed method

Table 9. Forced Degradation Studies

Figure Captions:

Figure 1. Structure of Flutamide

Figure 2. (a):(Y1) Retention Time, (b) :(Y2) Tailing Factor, (c):(Y3) Theoretical Plate.

Figure 3. Flutamide Standard Chromatogram

Figure 4. Calibration curve of Flutamide

Figure 5: Chromatograms illustrating the forced degradation studies of Flutamide under various stress conditions (a) acidic, (b) basic, (c) oxidative, (d) thermal, and (e) photolytic. The plots demonstrate the formation of degradation products/ peak asymmetry and confirm the stability indicating capability of the developed RP-HPLC method through effective separation of parent compounds from their degradation peaks.