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# A study of impurity profiling via method development and force degradation in hydrocortisone butyrate at low concentration

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## Abstract

**Background** Since long, triamcinolone and fluocinolone, fluorinated derivatives, have been used for treating various types of dermatitis. Rosacea and perioral dermatitis are the most common side effects observed with prolonged use of fluorinated derivatives. Recent studies have shown that these adverse effects are more severe at low doses of fluorinated derivatives compared to low doses of non-fluorinated derivatives. Therefore, this study focused on impurity profiling through force degradation studies of hydrocortisone butyrate in a 0.1% lotion.

**Results** A precise and robust method with low concentration was established for the estimation of hydrocortisone butyrate in bulk and formulations was done using the RP-HPLC technique. The mobile phase consisted of a combination of acetonitrile and purified water (30:70% v/v) and acetonitrile and purified water (95:5% v/v), with detection at a wavelength of 254 nm and a total run time of 20 min. The method demonstrated linearity and accuracy within the concentration range of 0.1–250 µg/mL and 50–125 µg/mL, respectively, with an $r^2$ value of 0.999. Stress stability studies were conducted on hydrocortisone butyrate, revealing 11% degradation in alkaline conditions and 18% degradation in photolytic conditions.

**Conclusion** The established method can be commercially used as it exhibits excellent linearity. Impurities were identified by injecting the reference standard, and their retentions were confirmed. The identified impurities included hydrocortisone, hydrocortisone-21-butyrate, hydrocortisone 3-methyl enol ether 17-butyrate, and hydrocortisone 17, 21-methylorthobutyrate, with retention times of 2.89, 9.14, 13.70, and 16.25 min, respectively. This precise method can be utilized in commercial applications for the accurate identification of hydrocortisone butyrate at low concentrations.

**Keywords** Impurity profiling, Stability-indicating RP-HPLC, Hydrocortisone butyrate, Forced degradation

## Background

Hydrocortisone 17-butyrate (HCB-17) belongs to the class of 21-hydroxysteroids, carrying a hydroxyl group (-OH) at the C-21 of the steroid backbone [1]. It is a novel non-fluorinated topical corticosteroid that has been approved to treat severe skin diseases such as psoriasis and eczema (Fig. 1) [2]. In a double-blind study, a paired comparison was conducted between 0.1% HCB-17 and triamcinolone acetonide, betamethasone 17-valerate (0.1%), and fluocinolone acetonide (0.025%) in patients suffering from these diseases [3–5]. The study found that 0.1% HCB-17 was safer and more effective compared to 1% hydrocortisone (HB) when applied to individuals experiencing atrophy on rosacea caused by prolonged use of other topical corticosteroids, particularly fluorinated ones [6, 7]. This may be attributed to the absence of HCB (0.1%) interfering with the positive effects of

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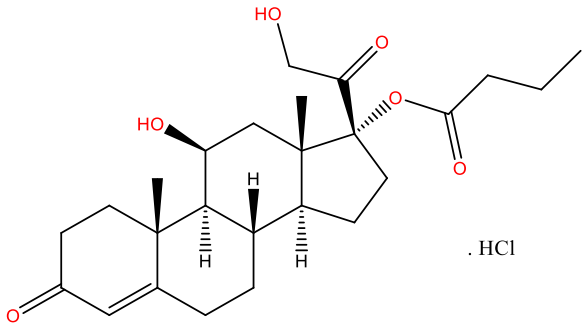



**Fig. 1** Chemical structure of Hydrocortisone butyrate

systemic tetracycline or the elimination of telangiectasia. However, the occurrence of significant rebound eruption in approximately 10% of patients highlights the need for caution when using HCB-17 for prolonged periods.

The findings suggest that HCB-17 may be less likely to cause skin atrophy and adrenal suppression than other potent topical corticosteroids. However, the trials conducted to date have been too brief to draw definitive conclusions due to the lack of infant studies and limited knowledge about possible long-term effects [8–10]. Several analytical methods, such as high-performance liquid chromatography (HPLC) and ultraviolet (UV) spectroscopy, have been reported for the analysis of the drug, but they tend to be expensive [11–15]. Although a few reliable and cost-effective HPLC methods are available [16–18], they do not specifically address the potential presence of impurities in bulk or pharmaceutical formulations of HCB-17. Monitoring and quantification of impurities are of paramount importance to ensure the potency and efficacy of drugs [19]. Therefore, the aim of the current experiment is to develop an accurate, specific, and cost-effective method for the estimation of HCB and related substances using the lowest possible concentration.

## Methods

### Instrumentation

Agilent 1260 Infinity II with a DAD detector and quaternary pump was the tool utilized for development and validation enabled with Agilent's Openlab EzChrom software. The Aczet analytical balance, Digital Systronic pH meter, Labman ultrasonicator, and Millipore vacuum filter pump (XI 5522050) were used for the method development with a nylon filter (for filtration) of Merck Millipore (0.22-µm).

#### *Materials and reagents*

The pharmaceutical-grade working standard of hydrocortisone butyrate (purity ≥ 99.80%) was obtained as a gift sample from Aadhaar Life Sciences Pvt. Ltd (Solapur, Maharashtra, India). Impurities, including Hydrocortisone-21-Butyrate (purity = 99.10%) purchased from Sigma Aldrich, India, Hydrocortisone 3-methyl enol ether 17-butyrate (purity = 98.93%) and hydrocortisone 17, 21-methylorthobutyrate (purity = 99.61%) from Sim-Son Pharma Ltd., India. All chemicals, such as HPLC-grade Acetonitrile (ACN), were procured from SD Fine Chemicals, Mumbai, India. Milli-Q water was obtained from Merck, India, for the current study. All weighing was done using calibrated NABL scales, and samples were produced in Class A glassware.

### Chromatographic conditions

The mobile phase consisted of a combination of ACN (30% v/v) and purified water (70% v/v), as well as a mixture of ACN and water (95:5% v/v). A flow rate of 1.2 mL/min was maintained, and detection was performed at 254 nm (Additional file 1: Fig. S7) with an injection volume of 10 µL. The column temperature was kept at 35 °C, allowing a tolerance of ± 2 °C as per robustness guidelines.

### Preparation of mobile phase

*Mobile Phase A** 70% Purified Water: 30% ACN.

Mobile Phase A* was prepared by accurately measuring 700 mL of purified water and 300 mL of ACN, which were then mixed in a suitable container. The resulting mobile phase was filtered through a 0.45 µm nylon membrane and sonicated to degas.

*Mobile phase B* 5% Purified Water: 95% ACN.

Mobile Phase B was prepared by accurately measuring 50 mL of purified water and 950 mL of ACN, which were mixed in a suitable container. The resulting mobile phase was filtered through a 0.45 µm nylon membrane and sonicated to degas.

*Note: If the system suitability requirement is not met, changes to the chromatographic conditions can be made according to the robustness test from method validation. However, only one change can be made at a time.

### Preparation of diluent

Throughout, ACN is used as diluent, pre-filtered through 0.45 µm nylon membrane after a brief sonication.

### Preparation of standard stock solution

#### *Preparation of HCB standard stock solution (1000 µg/mL)*

The drug portion was first dried using standard conditions (78 °C and 3 h) before use. Then, 25 mg of HCB reference standard was weighed and transferred into a 25 mL volumetric flask (VF). The drug was made soluble by adding the necessary amount of diluent and then

sonicated. Once equilibrium was reached, the volume was adjusted to 25 mL using diluent.

#### *Preparation of working standard solution (200 µg/mL)*

The stability of the working solution was observed for 4 days when stored at room temperature. 2.0 mL of stock standard was transferred into a 10 mL volumetric flask (VF). The volume was adjusted using diluent after degassing.

#### *Preparation of HCB lotion formulation for assay (200 µg/mL)*

A sample of 2.0 mL was transferred directly into a 10 mL volumetric flask (VF) using a TC (to contain) pipette. The pipette was rinsed quantitatively using a small quantity of diluent (less than 1 mL). The volume was then adjusted with diluent, and the sample was vortexed for approximately 1 min.

### Forced degradation study

Following the ICH guidelines Q1A(R2) and Q1B [20], degradation studies were conducted on HCB-17. The chromatogram was analyzed based on the drug peak area and the presence of any additional/secondary peaks. Any variations in peak area or the emergence of secondary peaks were considered indicators of degradation.

#### *Photolytic degradation*

An excess quantity of the drug was exposed to UV (254 nm) and white light for a total of 1.2 million lux hours in a petri dish over a 24-h period. Afterwards, a stock solution was prepared by dissolving 10 mg of the drug in a 10 mL volumetric flask. The drug was made soluble by adding the necessary quantity of diluent and then sonicated. Once equilibrium was reached, the volume was adjusted to 10 mL using diluent. Subsequently, the solution was filtered through a 0.45 µm syringe filter before injection.

#### *Thermal degradation*

The thermal property of the drug was determined by placing an excess quantity of the sample in an oven at approximately 80 °C for 4 h. Subsequently, 10 mg of this sample was weighed and transferred into a 10 mL volumetric flask. Around 5 mL of diluent was used to dissolve the stressed solution, and the volume was adjusted to the mark using diluent. Later, the solution was filtered through a 0.45 µm syringe filter before injection.

#### *Acid degradation*

For acid degradation, 10 mg of the drug was accurately weighed and transferred into a 10 mL volumetric flask. To dissolve the drug, 5 mL of diluent was added, and the volume was made up to the mark with diluent. This solution is referred to as the stock solution.

Subsequently, 1 mL of the stock solution was transferred into a 10 mL volumetric flask, and 1 mL of a 200 µL of a 1N hydrochloric acid (HCl) solution was added. The solution was refluxed with diluent at 60 °C for 30 min to induce acid degradation. After refluxing, the solution was neutralized using a 200 µL solution of 1N sodium hydroxide (NaOH) and adjusted to the mark with diluent. Finally, the solution was filtered through a 0.45 µm syringe filter before injection.

#### *Alkali degradation*

For alkali degradation, 10 mg of the drug was accurately weighed and transferred into a 10 mL volumetric flask. To dissolve the drug, 5 mL of diluent was added, and the volume was made up to the mark with diluent. This solution is referred to as the stock solution.

Subsequently, 1 mL of the stock solution was transferred into a 10 mL volumetric flask, and 1 mL of a 100 µL of a 1N sodium hydroxide (NaOH) solution was added. The solution was refluxed with diluent at 60 °C for 30 min to induce alkali degradation. After refluxing, the solution was neutralized using 100 µL of a 1N hydrochloric acid (HCl) solution and adjusted to the mark with diluent. Finally, the solution was filtered through a 0.45 µm syringe filter before being ready for injection.

#### *Peroxide degradation*

For peroxide degradation, 10 mg of the drug was accurately weighed and transferred into a 10 mL volumetric flask. To dissolve the drug, 5 mL of diluent was added, and the volume was made up to the mark with diluent. This solution is referred to as the stock solution.

Subsequently, 1 mL of the stock solution was transferred into a 10 mL volumetric flask, and 1 mL of a 3% hydrogen peroxide (H2O2) solution was added. The solution was refluxed with diluent at 60 °C for 30 min to induce oxidative degradation. Afterward, the volume was made up to the mark, and the solution was filtered through a 0.45 µm syringe filter before injection.

### Method validation

The developed method was validated as per ICH Q2 (R1) and USFDA [21] guidelines, considering parameters like Linearity and Range, Specificity (blank, placebo, standard and sample solution), Accuracy, Precision, Robustness, Ruggedness, Limit of Detection (LOD) and Limit of Quantitation (LOQ).

### Solution stability

To know how long the prepared solution is stable, the working standard was analyzed at different time point,

and it was confirmed that it was stable for up to 4 days. This was proved by analyzing the same at 4 different time points. Stability data is shown in (Table 1**).**

The cumulative RSD after 4 days of keeping the VF at room temperature in a dry place away from light was 0.2%. The specification limits are 2% and therefore, the working standard is stable for at least 4 days.

## Results

### Method development and optimization of chromatographic conditions

To achieve satisfactory separation of HC, different combinations of ACN and purified water as mobile phases were tested.

The optimized chromatogram (Fig. 2) and optimized conditions were developed using HPLC system of Agilent 1260 Infinity II embedded with Agilent Zorbax RX—C18 column of 5 microns of 150×4.6 mm size. A gradient programme was run with chromatographic conditions mentioned in Sect. "Chromatographic conditions" using ACN as diluent. The optimized chromatogram was developed at 8.43 min with the run time of 20 min at ambient column temperature. The optimized conditions are summarized in (Table 2).

### System suitability results

Using a series of tests, the suitability and performance of the system were examined. The theoretical plate count, tailing factor, and peak purity are all found to be within the allowed ranges for the ICH guideline system.

### Method validation

#### *Linearity*

Methodological linearity refers to the ability of an analytical method to produce results that are directly proportional to the concentrations of the analyte within a specified range. In this study, five sets of standard solutions were prepared to assess linearity. A calibration curve was constructed by plotting the peak area against the concentration of the standard solutions, and a regression equation was derived using the least-squares method to determine the slope, intercept, and correlation coefficient.

Linearity analysis was conducted across different concentration levels, considering the minimum concentration range of 0.1–250 µg/mL, as shown in (Additional file 1: Table S3). The graph plotted between peak area and concentration demonstrated linearity, as evidenced by the correlation coefficient displayed in (Fig. 3).

**Table 1** Solution stability of hydrocortisone butyrate

| Stability time interval Working Standard | %RSD from five replicates of standard | Tailing (max) | The number of theoretical plates (min) | Total %RSD | Cumulative %RSD of HB |
|---|---|---|---|---|---|
| T-0 | 0.2 | 1.0 | 22,540 | 0.1 | NA |
| 1 day | 0.1 | 1.0 | 22,381 | 0.1 | 0.1 |
| 2 days | 0.1 | 1.0 | 22,505 | 0.2 | 0.1 |
| 4 days | 0.1 | 1.0 | 22,593 | 0.1 | 0.2 |

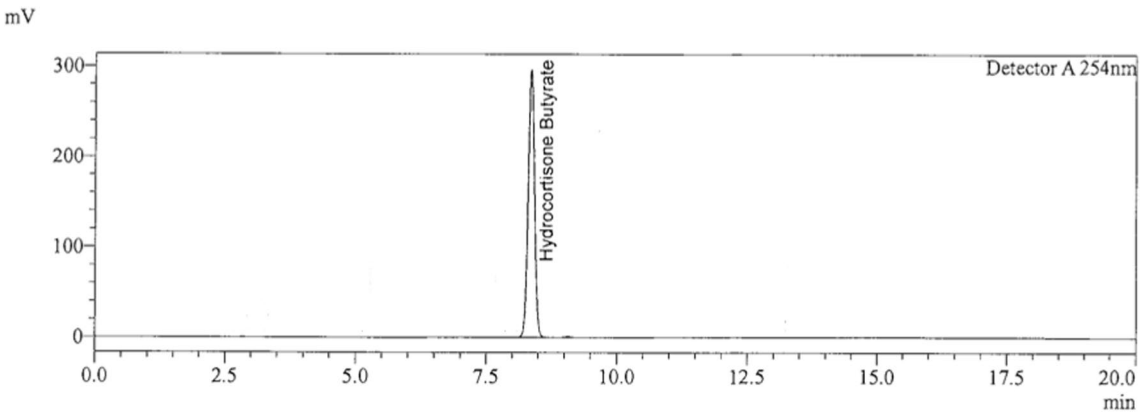



**Fig. 2** Typical chromatogram of HCB with retention time 8.43 min

**Table 2** Showing optimized chromatographic conditions

| Parameter | Condition | | |
|---|---|---|---|
| HPLC instrument | Agilent 1260 Infinity II | | |
| Column | Agilent Zorbax RX—C18 (150×4.6 mm, 5 micron), 80°A | | |
| Wavelength | 254 nm | | |
| Mobile phase | Mobile phase A: 85%<br>Mobile phase B: 15% | | |
| Gradient program | Time minutes | Mobile phase A | Mobile phase B |
| | 0 | 85 | 15 |
| | 12.5 | 50 | 50 |
| | 15.5 | 50 | 50 |
| | 17.5 | 85 | 15 |
| | 20.0 | 85 | 15 |
| Diluent | ACN | | |
| Retention time | 8.43 min | | |
| Run time | 20 min | | |
| Injection volume | 10 µL | | |
| Column oven temperature | 30 $^{0}$C | | |
| Flow rate | 1.2 mL/min | | |
| Column temperature | 35 °C (± 2 °C allowed by Robustness) | | |

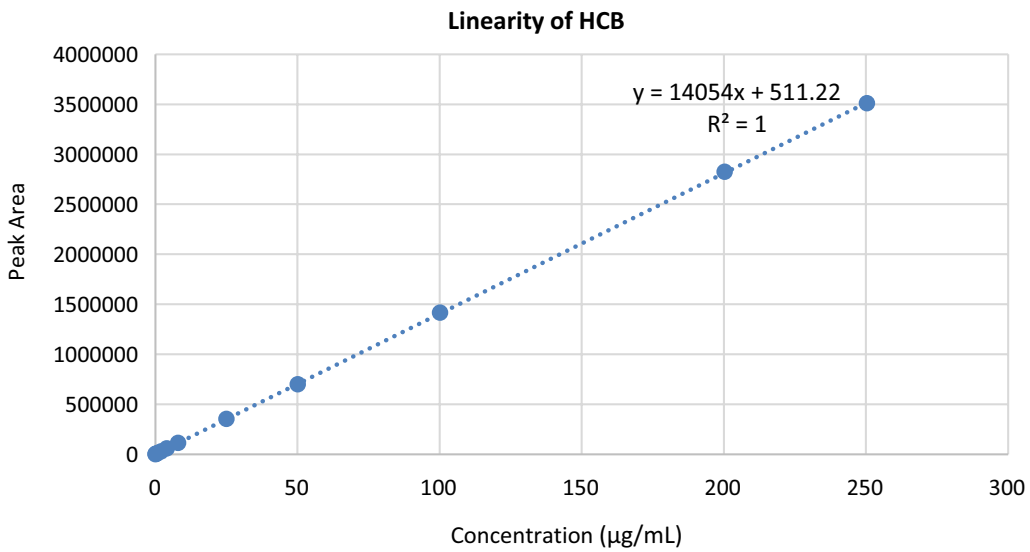


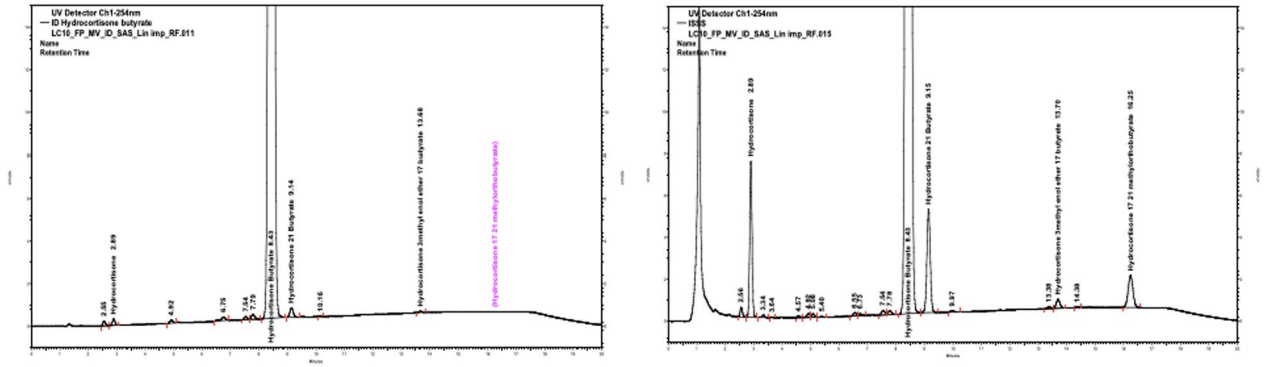

**Fig. 3** Graph showing linearity of HCB

***Range***

The range is derived from linearity, which was evaluated by measuring different concentrations of standard solutions to HCB from range 0.1–250 µg/mL, as shown in (Additional file 1: Table S3).

***Specificity***

The test is considered specific when the analyte can be evaluated without interference from specific components such as impurities, degradants, or excipients. To validate specificity, the chromatograms of




**(a)** **(b)**

**Fig. 4** **a** Chromatogram of hydrocortisone butyrate, **b** Chromatogram of hydrocortisone butyrate spiked with impurities

hydrocortisone butyrate were compared to a blank chromatogram. Related substances (RS) were identified by running individual impurity working standards (as indicated in Additional file 1: Fig. S8 and Fig. 4). The related substances included HC, HCB-21, HC 3-methyl enol ether 17-butyrate, and HC 17, 21-methylorthobutyrate (as listed in Table 3). All the RS are mentioned as references in the USP.

Separate injections of blank diluent and the HCB working standard were performed. It was observed that the retention time of HCB was 3.85 min. Analysis of the chromatogram of the blank diluent confirmed that there was no interference from blank peaks at the retention time of HCB. Related substances were analyzed to identify their peaks. The specificity of the active pharmaceutical ingredient and related substances is summarized below.

### *Accuracy (%Recovery)*

To assess the accuracy of a technique, the degree of correspondence between its test findings and the actual value needs to be examined. In the recovery studies, three different concentration levels were evaluated (as shown in Table 4). For each level, three replicate injections were performed, and the amount of drug present, the percentage of recovery, and the related standard deviation were calculated.

Accuracy was evaluated through triplicate measurements, and it was observed that the method demonstrated accuracy within the ranges of 50%, 100%, and 125%. The relative standard deviations for the accuracy levels of 80%, 100%, and 120% were found to be 0.5%, 0.4%, and 0.4%, respectively. These accuracy assessments determined the method's ability to accurately analyze different concentrations of the drug in solution.

### *Precision*

Analytical precision is assessed by evaluating the agreement between individual test results. Multiple samples from a uniform sample were analyzed to determine repeatability, intra-day, and inter-day variations, which are used to evaluate the precision of the method. This parameter was validated by analyzing samples collected at various times of the day and on different days. Precision was evaluated in terms of instrument precision (the consistency of back-to-back replicate injections of

**Table 3** Specificity of API and its related substances

| **Retention time (major peak)** | | | | | |
|---|---|---|---|---|---|
| **ID** | **HC** | **HCB** | **HCB-21** | **HC-3-methyl enol ether 17-butyrate** | **HCB-17,21-methylorthobutyrate** |
| Diluent | | | | | |
| Working Std -1 | 2.89 min | 8.43 min | 9.14 min | | |
| HCB | 2.89 min | | | | |
| HCB-21 | | | 9.14 min | | |
| HC-3-methyl enol ether 17-butyrate | | | | 13.70 min | |
| HC-17,21-methylorthobutyrate | | | | | 16.25 min |
| All ID Mixture | 2.89 min | 8.43 min | 9.15 min | 13.70 min | 16.25 min |

**Table 4** Result of accuracy showing %RSD

| Spiked amount w.r.t. sample | Prep | Spiked Amount (µg/mL) | Amount Recovered (µg/mL) | % Recovery | Average | %RSD |
|---|---|---|---|---|---|---|
| 50.05 | 1 | 100.104 | 100.800 | 100.7 | 100.3 | 0.5 |
| | 2 | 100.104 | 100.482 | 100.4 | | |
| | 3 | 100.104 | 99.783 | 99.7 | | |
| 100.10 | 1 | 200.208 | 200.708 | 100.2 | 100.2 | 0.4 |
| | 2 | 200.208 | 200.026 | 99.9 | | |
| | 3 | 200.208 | 201.313 | 100.6 | | |
| 125.13 | 1 | 250.260 | 250.291 | 100.0 | 99.7 | 0.4 |
| | 2 | 250.260 | 248.304 | 99.2 | | |
| | 3 | 250.260 | 249.657 | 99.8 | | |

the same concentration), method precision (one analyst injecting 6 different samples with the sample drug concentration and confirming the %RSD), and intermediate precision (a second analyst injecting 6 samples with the same sample drug concentration and confirming the %RSD of the total 12 injections).

The suitability of the HPLC instrument for validation was assessed. Based on the limits specified in (Table 2), the equipment was found to be suitable for conducting the validations. All three types of precision were evaluated after ensuring system suitability, and the reported data in (Table 5**)** indicate that the relative standard deviation for instrument precision, method precision, and intermediate precision were 0.1%, 0.4%, and 0.5%, respectively. The relative standard deviation between method precision and intermediate precision was 0.11%. The %RSD values demonstrated in this method indicate high precision and robustness across different analysts and multiple sample preparations for the same concentration.

### *Robustness*

Robustness testing is performed to evaluate the method's sensitivity to variations in critical parameters. While equipment calibration is a primary and mandatory step before use, changes were made to the column temperature (as shown in Table 6a) and mobile phase composition (as indicated in Table 6b) to assess the method's robustness. All runs were conducted in triplicate using working standards.

No changes were observed in the theoretical plate, asymmetry, or peak purity as a result of variations in column temperature and mobile phase concentration. However, a slight shift in the retention time of the HCB peak was observed when the organic phase concentration in mobile phase A was decreased, leading to a later elution of the peak. Conversely, an increase in the ACN concentration in mobile phase A resulted in an earlier elution of the peak. This change in retention time can be attributed to the polarity difference between acetonitrile and the drug, which influences the elution behavior.

### *Limit of detection (LOD) and limit of quantitation (LOQ)*

Linearity was assessed for HCB (0.01–0.64 µg/mL; r2 = 0.9998), HC (0.03–1.29 µg/mL; r2 = 0.9995), and HC-21B (0.02–6.16 µg/mL; r2 = 0.9996). LOD and LOQ values were calculated based on linearity and recovery data

**Table 5** Instrument precision, method precision, and intermediate precision and system suitability for HCB

| System suitability—HCB | | | | Peak area | | |
|---|---|---|---|---|---|---|
| Reps | RT | Asymmetry | Theoretical plates | Instrument precision | Method precision | Intermediate precision |
| Rep 1 | 8.43 | 1.00 | 22,389 | 2,837,117 | 2,835,144 | 2,836,541 |
| Rep 2 | 8.43 | 1.00 | 22,381 | 2,838,791 | 2,825,614 | 2,831,645 |
| Rep 3 | 8.43 | 1.00 | 22,501 | 2,835,769 | 2,816,584 | 2,826,254 |
| Rep 4 | 8.43 | 1.00 | 22,388 | 2,835,579 | 2,834,165 | 2,836,154 |
| Rep 5 | 8.43 | 1.00 | 22,415 | 2,841,484 | 2,841,241 | 2,851,245 |
| Rep 6 | 8.43 | 1.00 | 22,428 | 2,837,894 | 2,851,621 | 2,863,251 |
| Average | 3.83 | | | 2,838,848 | 2,834,062 | 2,840,848 |
| %RSD | 0.00 | | | 0.1 | 0.4 | 0.5 |

**Table 6** a Method robustness showing effect of column temperature. b Method robustness showing effect of change of mobile phase

| **Parameter** | | **Column temperature** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | **Decrease** | | | **Normal** | | | **Increase** | | |
| | | **33 °C** | | | **35 °C** | | | **37 °C** | | |
| | | **RT** | **RRT** | **RES** | **RT** | **RRT** | **RES** | **RT** | **RRT** | **RES** |
| *a* | | | | | | | | | | |
| HC | | 2.91 | 0.34 | NA | 2.91 | 0.34 | NA | 2.91 | 0.35 | NA |
| HCB | | 8.55 | 1.00 | 29.7 | 8.48 | 1.00 | 29.7 | 8.41 | 1.00 | 29.5 |
| HCB-21 | | 9.27 | 1.08 | 3.3 | 9.20 | 1.08 | 3.3 | 9.11 | 1.08 | 3.2 |
| HC-3-methyl enol ether butyrate | | 13.81 | 1.62 | 19.0 | 13.75 | 1.62 | 19.6 | 13.67 | 1.63 | 20.3 |
| HC-17,21- butyrate ortho ester | | 16.51 | 1.93 | 9.2 | 16.32 | 1.92 | 9.1 | 16.12 | 1.92 | 9.0 |
| The %RSD for the HCB Area from five replicate injections of standard | | 0.2 | | | 0.1 | | | 0.2 | | |
| Tailing for HCB peak in all standard injections (max) | | 1.0 | | | 1.0 | | | 1.0 | | |
| The number of theoretical plates in all working standard injections for HCB (min) | | 22,298 | | | 22,593 | | | 22,788 | | |
| Total %RSD for the HCB Area | | 0.2 | | | 0.1 | | | 0.2 | | |
| % Assay | HCB (%Assay) | 98.6 | | | 99.9 | | | 98.8 | | |
| % RC | HC | 0.09 | | | 0.08 | | | 0.09 | | |
| | HCB-21 | 0.43 | | | 0.45 | | | 0.39 | | |
| | % Unknown max impurity | 0.05 | | | 0.05 | | | 0.05 | | |
| | % Total Impurities | 0.78 | | | 0.78 | | | 0.71 | | |
| **Parameter** | | **Robustness [change in mobile phase-A composition]** | | | | | | | | |
| | | **Decrease in organic** | | | **Normal organic** | | | **Increase in organic** | | |
| | | **ACN:Water (27:73 v/v)** | | | **ACN:Water (30:70 v/v)** | | | **ACN:Water (33:67 v/v)** | | |
| | | **RT** | **RRT** | **RES** | **RT** | **RRT** | **RES** | **RT** | **RRT** | **RES** |
| *b* | | | | | | | | | | |
| HC | | 3.24 | 0.35 | NA | 2.91 | 0.34 | NA | 2.64 | 0.34 | NA |
| HCB | | 9.28 | 1.00 | 31.2 | 8.48 | 1.00 | 29.7 | 7.66 | 1.00 | 27.4 |
| HCB-21 | | 10.01 | 1.08 | 3.4 | 9.20 | 1.08 | 3.3 | 8.33 | 1.09 | 3.1 |
| HC-3-methyl enol ether butyrate | | 14.56 | 1.57 | 19.9 | 13.75 | 1.62 | 19.6 | 12.83 | 1.67 | 19.2 |
| HC-17,21- butyrate ortho ester | | 17.31 | 1.87 | 9.3 | 16.32 | 1.92 | 9.1 | 15.28 | 1.99 | 8.9 |
| The %RSD for the HCB Area from five replicate injections of standard | | 0.1 | | | 0.1 | | | 0.1 | | |
| Tailing for HCB peak in all standard injections (max) | | 1.0 | | | 1.0 | | | 1.1 | | |
| The number of theoretical plates in all working standard injections for HCB (min) | | 27,299 | | | 22,593 | | | 18,693 | | |
| Total %RSD for the HCB Area | | 0.1 | | | 0.1 | | | 0.1 | | |
| % Assay | HCB (%Assay) | 99.1 | | | 99.9 | | | 98.8 | | |
| % RC | HC | 0.08 | | | 0.08 | | | 0.08 | | |
| | HCB-21 | 0.44 | | | 0.45 | | | 0.43 | | |
| | % Unknown max impurity | 0.05 | | | 0.05 | | | 0.07 | | |
| | % Total impurities | 0.77 | | | 0.78 | | | 0.81 | | |

as presented in Additional file 1: Tables S4 and S5. For LOD determination, peaks were detected for six replicate injections within the specified range mentioned in Additional file 1: Table S4. LOQ was determined based on the average % recovery (90–130%) and % RSD (< 10%) for six replicate injections, as indicated in Additional file 1: Table S5.

Based on the precision and linearity data (Additional file 1: Fig. S9, Tables S4 and S5), the LOD and LOQ for HCB were found to be the same, at 0.025 µg/mL. The

LOD and LOQ values were significantly low, indicating that the method is highly efficient in detecting low drug concentrations. These LOD and LOQ values can be employed during cleaning validation in the industry to determine if the manufactured vessel or equipment is free from API stains. Furthermore, based on the linearity data (Additional file 1: Fig. S9), LOD and LOQ values were determined for hydrocortisone butyrate and its related compounds.

The LOD can be calculated as:

$$\mathrm{LOD} = 3.3 \times (\mathrm{S.D/slope})$$

where, SD = Standard deviation of the y intercepts of five calibration curves, Slope = Slope of five calibration curves.

And the LOQ can be calculated as:

$$\mathrm{LOQ} = 10 \times (\mathrm{S.D/slope})$$

where, SD = Standard deviation of the y intercepts of five calibration curves, Slope = Slope of five calibration curves.

## Discussion

Several analytical methods, including RP-HPLC and GC, GC–MS, have been reported for the estimation of HCB-17, but none of them have been evaluated for impurities. In the current method, HCB was eluted at 8.43 min using a gradient system with a mobile phase combination of ACN:Water (A-30:70% v/v) and ACN:Water (B-95:5% v/v). The method was developed considering the minimum concentration of both the drug sample and mobile phase (organic solvent), resulting in a more sensitive and cost-effective approach. Stability-indicating RP-HPLC methods are responsible for detecting differences between active pharmaceutical ingredients and any degradation products that may form under specific conditions. These methods evaluate the effects of stressors such as pH, temperature, and other conditions to understand the drug stability during storage and analysis. The summarized results can be found in Table 7a.

The stress degradation study demonstrated degradation of up to 18% under photolytic conditions and 11% under basic conditions. Acidic and peroxide conditions resulted in 2% and 1% degradation, respectively, based on the mass balance ratio. No significant degradation peak was observed in the current method during the heat degradation study. Comparison of the %impurity of related substances revealed that the highest impurity (9.64%) was observed in the base sample, while the lowest impurity (0.62%) was found in the peroxide sample, as shown in Table 7b and Fig. 5.

**Table 7** a Forced Degradation results of working standard. b Forced Degradation results of Impurities

| Degradation condition | Sample ID | HCB | | |
|---|---|---|---|---|
| | | % Assay | Peak purity | % Degradation |
| *a* | | | | |
| Control | Working standard | 100.6 | 1.000 | NA |
| Acid degradation | Working standard | 98.9 | 1.000 | 2 |
| Base degradation | Working standard | 89.7 | 1.000 | 11 |
| Peroxide degradation | Working standard | 99.3 | 1.000 | 1 |
| UV (Photolytic) | Working standard | 83.0 | 1.000 | 18 |
| Thermal degradation | Working standard | 100.5 | 1.000 | 0 |

| Sample | % Related compounds | | | | | |
|---|---|---|---|---|---|---|
| | HC | HCB-21 | HC- 17,21-Methyl ortho butyrate | HC 3- methyl enol ether 17-butyrate | % Unknown max | % Total impurities |
| *b* | | | | | | |
| Control | 0.11 | 0.39 | ND | ND | 0.07 | 0.63 |
| Acid | 0.12 | 1.07 | ND | ND | 0.11 | 1.41 |
| Base | 1.21 | 8.23 | ND | ND | 0.09 | 9.64 |
| Peroxide | 0.10 | 0.42 | ND | ND | 0.05 | 0.62 |
| UV | 0.08 | 0.42 | ND | ND | 0.12 | 0.85 |
| Heat | 0.09 | 0.45 | ND | ND | 0.06 | 0.70 |

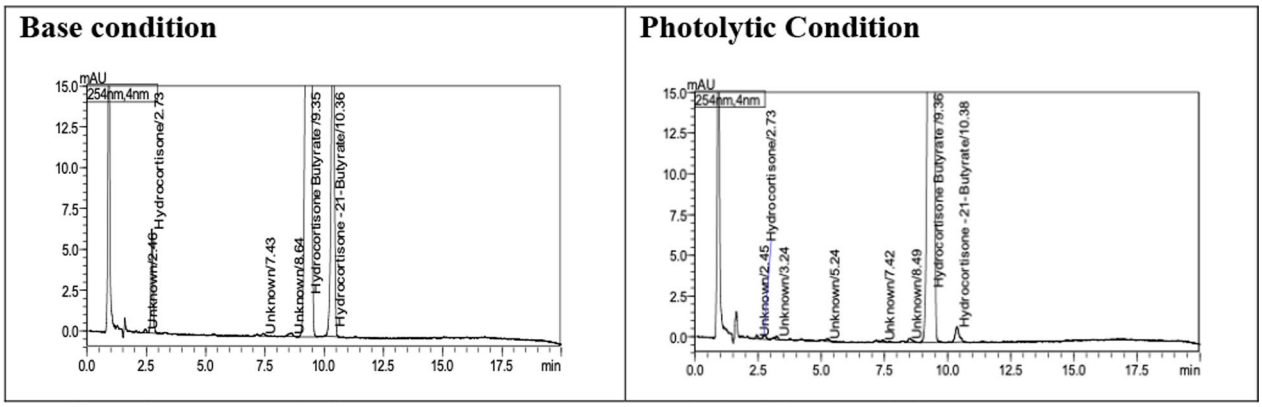



**Fig. 5** Base and photolytic condition chromatograms

### %Assay for hydrocortisone butyrate lotion 0.1%

Based on the validated method, an assay was carried out on hydrocortisone butyrate lotion 0.1%. The assay was found to be at 99.78% for the marketed formulation. The results are summarized in (Table 8).

## Conclusion

Most of the reported methods focused on determining the targeted drug (HCB) without considering its potential impurities. However, it is essential to identify and quantify major impurities like HCB-21 and HC to avoid adverse effects and toxicity associated with HCB. In this research article, a precise and accurate RP-HPLC-based method was developed for the estimation of HCB in bulk drugs and formulations. The method was validated for accuracy, precision, and robustness. Forced degradation studies revealed that HCB is highly susceptible to basic conditions, with more than 5% degradation observed under basic and photolytic conditions. Hence, it is crucial to store the bulk drug and formulation away from light.

**Table 8** Result of assay by proposed method

| Parameters | Results |
|---|---|
| Standard area | 2,837,117 |
| | 2,838,791 |
| | 2,835,769 |
| | 2,835,579 |
| | 2,841,484 |
| | 2,837,894 |
| Mean area | 2,837,117 |
| Sample area | 2,830,875 |
| Amount found | 99.78 mg per gram of lotion |
| % Assay | 99.78 |

### Abbreviations

| | |
|---|---|
| HC | Hydrocortisone |
| HCB | Hydrocortisone butyrate |
| HCB-17 | Hydrocortisone-17-butyrate |
| HCB-21 | Hydrocortisone-21-butyrate |
| RSD | Relative standard deviation |
| ACN | Acetonitrile |
| %RSD | Relative standard deviation |
| LOD | Limit of detection |
| LOQ | Limit of quantitation |

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s43094-023-00558-1.

**Additional file 1**. Supplementary data obtained from trials using different mobile phases, UV spectroscopy, and identification of wavelength, impurity identification, and linearity graph for the estimation of LOD and LOQ are presented as figures, indicated as **Figs. S1, S2, S3, S4, S5, S6, S7, S8 and S9**. Supplementary data on the results of linearity, specificity, and precision are presented in a table, indicated as **Tables S1, S2, S3, S4 and S5**.

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### Author contributions

HG and VB equally contributed for the manuscript.

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### Availability of data and materials

The data for verification is provided with supplementary file and rest of data if required, will be available upon request.

## Declarations

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

The authors declare no competing financial interest.

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