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# Development and Validation of a Stability-Indicating RP-HPLC Method for Simultaneous Assay of Betamethasone Dipropionate, Chlorocresol, and for the Estimation of Betamethasone Dipropionate Related Compounds in a Pharmaceutical Cream and Ointment

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## Abstract

**A new stability-indicating reversed-phase HPLC (RP-HPLC) method has been developed and validated for simultaneous assay of betamethasone dipropionate (BD) and chlorocresol and also for the estimation of BD related compounds in a pharmaceutical cream matrix. In addition, this newly developed RP-HPLC method was also demonstrated as suitable for a pharmaceutical ointment product that does not contain chlorocresol. The RP-HPLC method uses a Waters SymmetryShield RP18 analytical column (150 x 4.6 mm). Water (mobile phase A) and acetonitrile (mobile phase B) were used in the gradient elution with a flow rate of 1.5 mL/min and detection wavelength at 240 nm. A Waters XBridge Shield RP18 analytical column (150 x 4.6 mm) was identified as an alternate column. The limit of detection (LOD) and the limit of quantitation (LOQ) are 0.02 µg/mL and 0.05 µg/mL, respectively. The precision of the method for BD is less than 0.3% RSD, and the accuracy of BD ranged from 99.5% to 102.6%. The stability-indicating capability of this method has been demonstrated by analyzing aged samples of the product. This RP-HPLC method was successfully validated per ICH guidelines and proved to be suitable for routine quality control use.**

## Introduction

Pharmaceutical creams and ointments containing betamethasone dipropionate (BD) (9-Fluoro-11β,17,21-trihydroxy-16β-methylpregna-1,4-diene-3,20-dione-17,21-dipropionate, structure in Figure 1) are widely used as an anti-inflammatory agent to relieve a wide variety of skin conditions (e.g., Dermatitis, Psoriasis) (1,2). To perform batch release testing and to conduct stability studies for cream and ointment pharmaceutical products, a stability-indicating analytical method is required to separate the active pharmaceutical ingredient (API) peak from the peaks of all potential degradation products, process related impurities, potential packaging leachables, excipients, and also separate these compounds from each other. Frequently, antimicrobial agents (e.g., chlorocresol) are also included in the topical formulations (3) and need to be monitored at the product release and over the product shelf-life.

The development of a stability-indicating method for steroid containing drug products such as BD is challenging due to the numerous, structurally, similar compounds (Figure 1) that must be separated and monitored throughout the shelf-life of the product (4,5). Several of the compounds shown in Figure 1 are primary degradation products including compounds 1, 2, 4, 5, 6, and 7; the other compounds in Figure 1 are potential degradation products or process related impurities (compounds 6, 7, 8, 9, and 10). The packaging components used for pharmaceutical creams and ointments (e.g., aluminum lined tube with Epoxy Resin and high-density polyethylene or low-density polyethylene closures) and labels (label inks and glue) may contain certain potential leachables (e.g. benzyl alcohol, benzaldehyde, benzophenone, and 2-hydroxy-4-methoxybenzophenone), which can be observed in the cream or ointment products. All potential leachables in the drug product must be separated from BD and its related impurities/degradation products, as well as also from chlorocresol.

The current compendial (USP and Ph.Eur.) monograph methods for BD related compounds (11,12) are not capable of separating BD from all the known related compounds/degradation products. A literature search revealed no known analytical method that can assay both BD and the antimicrobial agent chlorocresol, and estimate all BD related compounds and potential packaging leachables by one method. One reported method (13) is capable of separating BD from several degradants (compounds 1, 4, and 7) including clotrimazole, imidazole, and o-chlorophenyldiphenylmethanol. However, this method cannot separate other related compounds of BD (listed in Figure 1). Another HPLC method is capable of separating BD

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and the related compounds, but due to the product formulation differences (i.e. lotion vs. cream and ointment) a new HPLC method was required (14). Analysis of chlorocresol in the drug product is typically performed by a second method using gas chromatography.

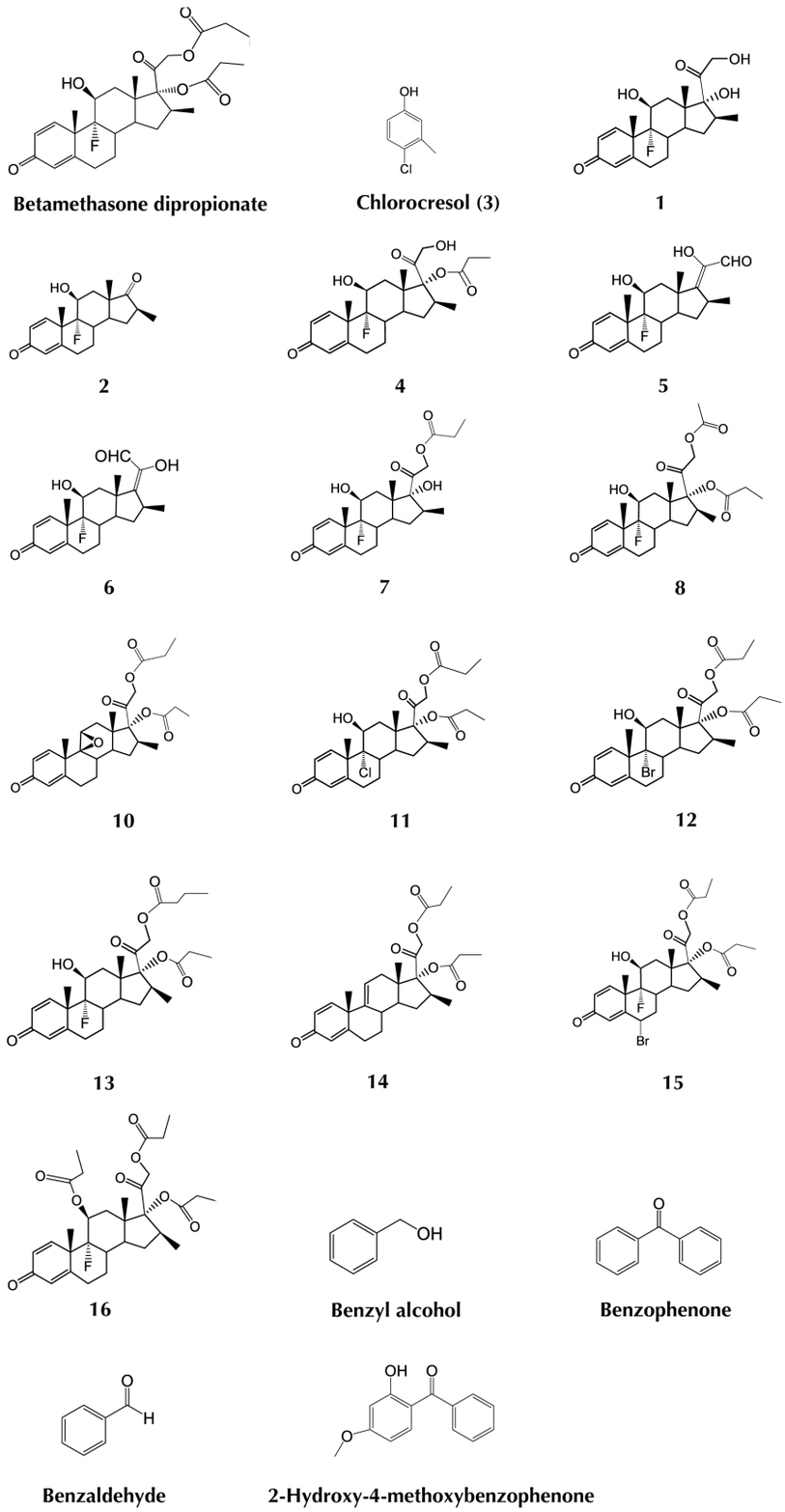


**Figure 1.** Molecular structures of betamethasone dipropionate (9), chlorocresol, all betamethasone dipropionate related compounds, and known leachables. Compound numbers are assigned based on elution order.

In this paper, we describe a reversed-phase high-performance liquid chromatography (RP-HPLC) method for simultaneous assay of BD and chlorocresol as well as the estimation of the BD related compounds and potential leachables. This new HPLC method was successfully validated per ICH guidelines and proved to be suitable for routine quality control use. This method was also demonstrated to be stability-indicating as it can separate all degradation peaks that are present in aged batches of the cream and ointment products. To the best of our knowledge, this is the first method that can separate and accurately quantitate BD, chlorocresol, all known BD degradants and/or related impurities, and potential packaging leachables from a cream and ointment matrix.

## Experimental

### Column

The validation of the final method was performed using a Waters SymmetryShield RP18 column (150 × 4.6 mm, 3.5 µm particle size) as the primary column. The Waters XBridge Shield RP18 column (150 × 4.6 mm, 3.5 µm particle size) was also validated as a true alternate column in case the primary column is no longer commercially available during the lifecycle of this method. Details of all the columns used during method development activities are outlined in Table I.

### Chromatography

HPLC analysis was performed on either an Agilent Technologies 1100 Series HPLC System (Santa Clara, CA), a Hitachi LaChrom Elite HPLC System (San Jose, CA), or a Waters 2695 Alliance Series HPLC System (Milford, MA). All HPLC systems were equipped with a temperature controlled column compartment and an online solvent degasser. The Agilent HPLC systems were also equipped with an LC Spiderling column switching system (Chiralizer Services, L.L.C., Newtown, PA) and ChromSword method development software (Merck KGaA, Darmstadt, Germany) for chromatographic simulations. Data acquisition, analysis, and

**Table I. HPLC Column Screening Study Summary Using Initial HPLC Conditions Developed Using ChromSword**

| Column (Supplier) | Column Description (%Carbon) | Comments: Suitable for further evaluation (yes/no) |
|---|---|---|
| Ace 3 C18 (Mac-Mod Analytical) | 4.6 x 150 mm; 3 µm particles (15.5%) | inadequate separation of compound 2 and chlorocresol and also of late-eluting compounds (no) |
| Prontosil C18-EPS (Mac-Mod Analytical) | 4.6 x 150 mm; 3 µm particles (18%) | inadequate separation of late-eluting compounds (no) |
| Kromasil C18 (Alltech) | 4.6 x 150 mm; 5 µm particles (19%) | no separation of compound 2 and chlorocresol (no) |
| Capcell-Pak C18 UG (Phenomenex) | 4.6 x 150 mm; 3 µm particles (15%) | inadequate separation of all compounds (no) |
| Cosmosil C18-AR (Phenomenex) | 4.6 x 150 mm; 5 µm particles (16%) | inadequate separation of compound 2 and chlorocresol and of late-eluting compounds (no) |
| Develosil RP-Aqueous (Phenomenex) | 4.6 x 150 mm; 3 µm particles (18%) | peaks elute slower than on other columns (no) |
| Synergi MAX-RP (Phenomenex) | 4.6 x 150 mm; 4 µm particles (17%) | inadequate separation of compound 5 and 7 and of late-eluting compounds (no) |
| Synergi POLAR-RP (Phenomenex) | 4.6 x 150 mm; 4 µm particles (11%) | inadequate separation of compound 5 and 7 and also of late-eluting compounds (no) |
| Ultracarb ODS (20) (Phenomenex) | 4.6 x 150 mm; 3 µm particles (22%) | inadequate separation of late-eluting compounds (no) |
| Atlantis dC18 (Waters) | 4.6 x 150 mm; 3 µm particles (12%) | no separation of compound 15 and 16 (no) |
| Nova-Pak C18 (Waters) | 4.6 x 150 mm; 4 µm particles (7%) | no separation of compound 15 and 16 (no) |
| Symmetry C18 (Waters) | 4.6 x 150 mm; 3.5 µm particles (19%) | inadequate separation of compound 2 and chlorocresol, and compound 10 and 11 (no) |
| SymmetryShield RP8 (Waters) | 4.6 x 150 mm; 3.5 µm particles (15%) | chlorocresol and compound 4 co-elute, slight co-elution of compounds 5 and 6, and compounds 15 and 16 (no) |
| SymmetryShield RP18 (Waters) | 4.6 x 150 mm; 3.5 µm particles (17%) | separation of all compounds (recommended for further evaluation) |
| µBondapak C18 (Waters) | 4.6 x 150 mm; 10 µm particles (9.8%) | inadequate separation of all compounds(no) |
| XTerra RP18 (Waters) | 4.6 x 150 mm; 3.5 µm particles (15%) | separation of all compounds (recommended for further evaluation) |
| XTerra MS C18 (Waters) | 4.6 x 150 mm; 3.5 µm particles (15.5%) | no separation of compounds 10 and 11, and compounds 15 and 16 (no) |
| XBridge C18 (Waters) | 4.6 x 150 mm; 3.5 µm particles (12%) | inadequate separation of compound 2 and chlorocresol, compound 10 and 11, and compounds 15 and 16 (no) |
| XBridge Shield RP18 (Waters) | 4.6 x 150 mm; 3.5 µm particles (17.5%) | inadequate separation between compounds 15 and 16 (no) |
| Hydrosphere C18 (YMC) | 4.6 x 150 mm; 3 µm particles (12%) | peaks elute slower, no separation of compounds 10 and 11 (no) |
| ODS-AQ (YMC) | 4.6 x 150 mm; 3 µm particles (14%) | inadequate separation of late-eluting compounds (no) |
| Pack Pro C18 (YMC) | 4.6 x 150 mm; 3 µm particles (16%) | inadequate separation of late-eluting compounds (no) |

reporting were performed using ChemStation (Agilent), EZChrom Elite (Hitachi) and Millennium[32] (Waters) chromatographic software. The final details of the chromatographic conditions used in the validation studies are summarized in Table II.

### Chromatographic performance

Retention time ($t_R$) and resolution factor (Rs) calculations were performed by ChemStation software on the Agilent HPLC systems, EZChrom Elite software on the Hitachi HPLC systems and Millennium[32] software by Waters HPLC systems.

### Solvents and chemicals

BD, chlorocresol, and all BD-related compounds were provided by the Global Quality Services–Analytical Sciences group in Schering-Plough (Merck & Co., Inc. Union, NJ). All HPLC-grade solvents were used as received from Fisher Scientific (Fisher Scientific International Inc., Liberty Lane, Hampton, NH). Water (18.2 MΩ·cm) was used as obtained from a Milli-Q system (Millipore, Billerica, MA).

### Solution preparation

Solutions of BD and chlorocresol were prepared in acidified methanol–water (80:20 v/v) where the methanol was acidified with 0.1% glacial acetic acid for the cream. Solutions of BD were prepared in acidified methanol–water (90:10 v/v) where the methanol was acidified with 0.1% glacial acetic acid for the ointment. The analytical concentration of BD was approximately 0.1 mg/mL, and the analytical concentration of chlorocresol (cream studies only) was ~ 0.165 mg/mL. The standard solutions were used as external standards to quantitate the sample solutions. All solutions and samples were protected from light (15).

### Extraction of cream and ointment samples

Extraction of cream sample was conducted by using 4 g of the cream product with 20 mL of 0.1% glacial acetic acid in methanol in a 50 mL glass centrifuge tube. The sample mixture was heated at 75°C for 10 min and mixed (e.g., via vortex) occasionally during the heating process. After heating, the sample was allowed to cool for ~ 1 min before removing the cap of the centrifuge tube. Two mLs of water were added into the centrifuge tube, the tube was recapped, chilled in an ice bath for 20 minutes, and centrifuged at 3000 RPM for 10 min. After centrifugation, the liquid layer, which contained the analytes of interest (e.g., BD, chlorocresol and the BD related compounds),

resided on top of the solid excipients. An aliquot of the liquid layer was filtered using a 0.45-µm PTFE syringe filter and placed in an HPLC vial for analysis.

Extraction of ointment sample was conducted by using 1.6 g of the ointment product with 10 mL of diluent (0.1% glacial acetic acid in 90:10 methanol–water) in a 50-mL glass centrifuge tube. The sample solution was heated at 70°C for 8 min and mixed (e.g., via vortex) occasionally during the heating process. After heating, the sample was centrifuged at 2500 RPM for 10 min. After centrifugation, the liquid layer, which contained the analytes of interest (e.g., BD and the BD related compounds), resided on top of the solid excipients. An aliquot of the liquid layer was filtered using a 0.1 µm PTFE syringe filter and placed in an HPLC vial for analysis.

### Calculation of percent label claim of BD and chlorocresol and estimation of BD related compounds in cream and ointment samples

The cream sample solutions were bracketed between two standard solutions. For the cream samples, the percent label claim is defined as:

$$\%\ \text{Label Claim} = \frac{P_{Sample}}{\text{Average } RF_{std}} \times \frac{V_{Sample}}{W_{Sample}} \times \frac{100}{0.64} \qquad \text{Eq. 1}$$

where $P_{Sample}$ is the peak area of BD or chlorocresol in the sample chromatogram; $W_{Sample}$ (g) is the weight of the cream or ointment sample; Label Claim (mg/g) for the cream and ointment product is 0.64 for BD and 1.00 for chlorocresol in the cream product; and Average $RF_{Std}$ is the average response factor of BD or chlorocresol in the adjacent bracketing standard chromatograms. The $V_{Sample}$ is the total sample volume. Because the cream contains ~ 70% water, the water content inherent to the composition of the cream needed to be accounted for mathematically in the final, total sample volume [i.e., $V_{Sample}$ = 22.0 + (0.7 * $W_{Sample}$)]. This adaptation of the "$V_{Sample}$" for the cream is not required for the ointment because the ointment formulation does not contain water. The estimation of the BD related compounds is calculated using:

$$\%\ \text{Deg. Products} = \frac{P_{Sample} - i}{\text{Average } RF_{std}} \times \frac{1}{RRF_i} \times \frac{V_{Sample}}{W_{Sample}} \times \frac{100}{0.64} \qquad \text{Eq. 2}$$

where $P_{Sample}$ is the peak area of the related compound. RRF is the ratio of the response factor of each BD related compound relative to the response factor of BD. Again, for the cream, $V_{Sample}$ needs to account for the amount of water present in the formulation.

**Table II. HPLC Parameters and Mobile Phase Gradient Table for HPLC Analysis for Method Validation Studies***

| Time (min) | Flow rate (mL/min) | 100% water (%) | 100% acetonitrile (%) | Gradient curve |
|---|---|---|---|---|
| 0.0 | 1.5 | 74 | 26 | Linear |
| 20.0 | 1.5 | 54 | 46 | Linear |
| 45.0 | 1.5 | 54 | 46 | Linear |
| Column wash and equilibrate to original conditions | | | | |

* Column temperature 35°C; Detection wavelength 240 nm; Injection volume 25 µL.

## Results and Discussion

### Selection of HPLC columns and optimization of mobile phase gradient by method development software system

The method development approach that was used for this method has been previously used by our laboratory (16). Several key chromatographic parameters are evaluated in parallel, including different columns (with various stationary phases), different mobile phase conditions, optimal detection UV wavelength, and column temperatures. This was accomplished using a specificity mixture containing BD, chlorocresol, known related compounds, and potential packaging leachables (at ~1% level) with the aid of ChromSword (which is an artificial intelligence chromatographic method development tool), and an LC Spiderling (which is an automated 9-port column switching system) (16).

Using the techniques and approach previously described (16), different HPLC columns were evaluated capturing several stationary phase functionalities (C18, cyano, amino, phenyl) using various mobile phases and the different organic modifiers for the separation. Methanol, acetonitrile, and isopropanol were screened individually or in combination as potential mobile phases. Mobile phase pH and ionic strength were not evaluated during method development because there are no easily-ionizable functional groups on BD, chlorocresol and all of the BD related compounds (Figure 1).

The best selectivity was observed on a C18 based stationary phases with acetonitrile as the primary organic modifier. Using a preliminary gradient mobile phase system consisting of mobile phase A (water) and mobile phase B (acetonitrile:isoproanol, 17:1, v/v), twenty-one C18 and one C8 HPLC columns were screened using the ChromSword system. The results of column screening studies are presented in Table I. The three critical pairs that were used to determine the best selectivity of the screened columns are; (*i*) compound 2 and chlorocresol pair, (*ii*) the compound 10 and 11, and (*iii*) compound 15 and 16. Based on the results of the column screening studies (Table I), the SymmetryShield RP18 and the Waters XTerra RP18 columns provided the best overall separation of all analytes including the late eluting peaks. Hence, these two HPLC columns were used for further method development work including the optimization of the chromatographic parameters. Additional studies using ChromSword and the SymmetryShield RP18 HPLC column resulted in further modification and simplification of the mobile phase gradient. During method development work, it was observed that the elution order of the peaks drastically changed with minor variations of the method's mobile phase conditions. Generally, the gradient conditions that were favorable for the separation of the peaks eluting before BD were not favorable for the separation of the late eluting peaks. Thus, the optimum mobile phase conditions were a combination of a linear gradient followed by isocratic conditions (Table II).

### Optimization/selection of column temperature, flow rate and UV wavelength

The flow rate of 1.5 mL/min was chosen as optimal to aid in the reduction of the overall run time with an acceptable column back pressure. The column temperature of 35°C was chosen

because all the components in the sample solution were adequately separated, and the column-back pressure was also decreased. An elevated controlled column temperature eliminates/minimizes effects of fluctuating laboratory room temperature on the day-to-day performance of the HPLC method in the QC laboratory. In determining the detection wavelength for the analytical method, several challenges were encountered. The λmax of BD and all the BD related compounds is near 240 nm, except for compound 10, whose λmax is at 248 nm, but the chlorocresol λmax is significantly different at 227 nm (Figure 2). In addition, the chlorocresol concentration is significantly higher than the BD concentration in the cream formulation. Monitoring the cream at λmax of 227 nm, resulted in the chlorocresol peak tailing due to the high concentration of chlorocresol in the product and as a result decreased resolution between chlorocresol and compound 4. Then, the detection wavelength at 240 nm was evaluated for the cream samples. At 240 nm, the chlorocresol peak was reproducible, with minimal peak tailing, and enhanced resolution between chlorocresol and compound 4 (a key degradant of BD). Therefore, the final detection wavelength was set at 240 nm.

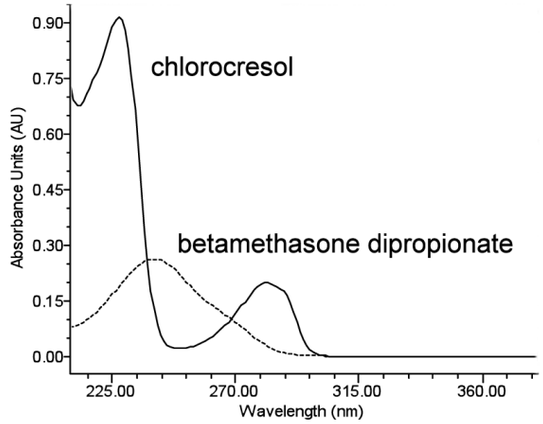


**Figure 2.** Representative spectrum of betamethasone dipropionate and chlorocresol in the cream matrix.

The final optimized method separated all the compounds of interest in 45 min, followed by a column wash and re-equilibration to the initial conditions (Table II). A representative chromatogram is shown in Figure 3 for the cream and ointment matrices. The retention times and relative retention times of the peaks are listed in Table III.

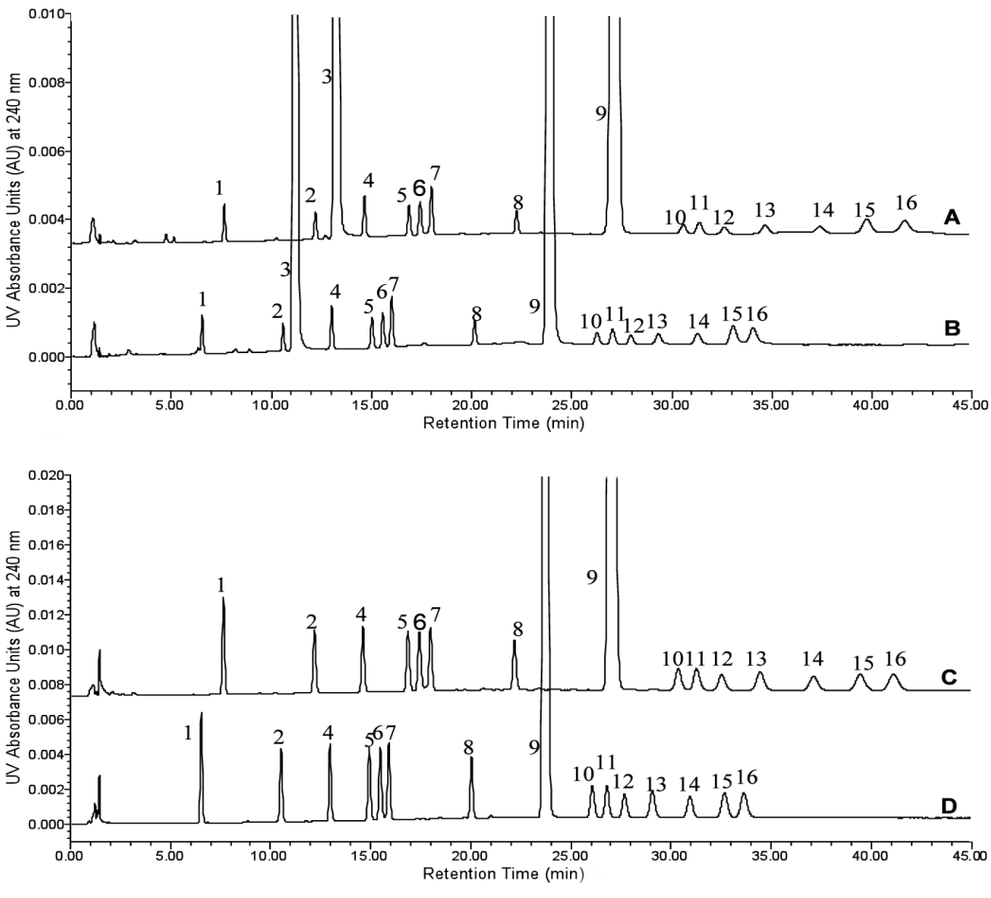


**Figure 3.** Representative chromatograms of a separation of betamethasone dipropionate, chlorocresol, and the betamethasone dipropionate related compounds in the cream matrix on (A) the primary (SymmetryShield RP18) column and (B) the alternate (XBridge Shield RP18) column. Representative chromatograms of a separation of betamethasone dipropionate and related compounds in the ointment matrix on (C) the primary (SymmetryShield RP18) column, and (D) the alternate (XBridge Shield RP18) column. For chromatographic conditions refer to Table II, and for chemical structures refer to Figure 1.

**Sample extraction**

The extraction of pharmaceutical cream and ointments is generally done by (*i*) several heating/cooling cycles in an extraction solvent (13), (*ii*) solid phase extraction (17,18), or (*iii*) liquid–liquid extraction (19–22). In addition, micellar chromatographic approaches have also been used, both alone and in combination with a heating/cooling/centrifugation approach (23, 24). However, each approach has potential challenges in achieving a simple QC-friendly procedure. When either the cream or ointment is heated, the potential exists for degradation of the analytes. When using solid-phase extraction, irreproducible analyte extraction and introduction of cartridge impurities can be introduced into the sample. Liquid–liquid extraction often relies on stronger chemicals or multiple extractions. Micellar preparations can potentially decrease the chromatographic efficiency, including the potential of shortening the column life.

For a QC friendly extraction technique, it is desirable that the liquid layer should reside on top of the solid excipients for ease of transfer and sample handling. If the liquid layer is below the solid excipients, then the solid layer needs to be penetrated in order to transfer the liquid layer for analysis. During penetration of the solid layer, solid contaminant can be introduced in the liquid sample, which can cause potential interferences with the analyte peaks.

**Development of extraction procedure for cream sample**

Solid-phase extraction was attempted; however, irreproducible results and on-cartridge degradation were observed over repeated experiments. Thus a simplified heating/cooling/centrifugation pathway was chosen for continued development.

Multiple solvents were tested as the cream product extracting solvent including combinations of water, methanol, ethanol, isopropanol, acetonitrile, and acetone. While methanol, ethanol, isopropanol, and acetone were shown to be approximately equal in terms of extraction recoveries, the addition of water or acetonitrile before the heating step resulted in the reformation of the cream during centrifugation (i.e. no supernatant was formed). However, the addition of a trace amount of water was necessary to ensure the complete precipitation of all excipients upon cooling. If water was not added, the lifetime of the column was severely shortened due to the precipitation of the components from sample solution. The addition of water after the heating step (before cooling) did not result in reformation of the cream during centrifugation. Methanol was chosen as the extraction solvent because the other solvents produced broad/distorted chromatographic peaks. The acidification of the methanol was necessary to slow the degradation from BD to compound 4 in solution. The acidification had no adverse effect on the recovery of any other analytes.

The heating time (10 min) and temperature (75°C) were chosen so as to allow the complete melting of the cream product, resulting in a homogenous mixture during extraction. No evidence of analyte degradation was observed at either the method conditions, at extended extracted time (13 min) or at elevated temperatures (80°C). Glass centrifuge tubes were necessary to minimize the introduction leachable peaks into the sample.

**Development of extraction procedure for ointment sample**

Initially liquid–liquid extraction was examined using iso-octance–methanol–water (30:9:1) for the ointment samples. In a centrifuge tube, 2 g of the ointment product was added. Then, iso-octane–methanol–water (30:9:1) was added, and the sample was vortexed and centrifuged; the lower phase was transferred to a 25-mL volumetric flask. The extraction procedure was repeated, and the lower phase was again transferred to the volu-

**Table III. Retention Times and Relative Retention Times of Betamethasone Dipropionate, Chlorocresol, Betamethasone Dipropionate Related Compounds and Package Leachables in the Cream and Ointment Matrix**

| Compound* | Identity | Retention Time (min) | RRT |
|---|---|---|---|
| Benzyl Alcohol | potential packaging leachable | 3.1 | 0.11 |
| Benzaldehyde | potential packaging leachable | 5.8 | 0.21 |
| 1 | Impurity/Degradant | 7.7 | 0.28 |
| 2 | Impurity/Degradant | 12.2 | 0.45 |
| Chlorocresol (3) | Antimicrobial Preservative | 13.2 | 0.49 |
| 4 | Impurity/Degradant | 14.6 | 0.54 |
| 5 | Degradant | 16.9 | 0.62 |
| 6 | Degradant | 17.4 | 0.64 |
| 7 | Impurity/Degradant | 18.0 | 0.66 |
| Benzophenone | potential packaging leachable | 19.2 | 0.71 |
| 8 | Impurity | 22.3 | 0.82 |
| 2-Hydroxy-4-methoxybenzophenone | potential packaging leachable | 24.2 | 0.89 |
| Betamethasone Dipropionate (9) | Active | 27.1 | 1.00 |
| 10 | Impurity/ Potential Degradant | 30.6 | 1.13 |
| 11 | Impurity | 31.4 | 1.16 |
| 12 | Impurity | 32.6 | 1.20 |
| 13 | Impurity | 34.7 | 1.28 |
| 14 | Impurity | 37.4 | 1.38 |
| 15 | Impurity | 39.7 | 1.46 |
| 16 | Impurity | 41.6 | 1.53 |

* Refer to Figure 1 for chemical structures. Compound numbers were assigned based on elution order.

**Table IV. Summary of Method Linearity Study Results**

| | $r^2$ | | y-intercept | |
|---|---|---|---|---|
| Compound* | Analyst 1 | Analyst 2 | Analyst 1 | Analyst 2 |
| *Cream* | | | | |
| BD† | 0.999991 | 0.999986 | 0.3% | 0.1% |
| Chlorocresol | 0.999643 | 0.999810 | 3.4% | 4.3% |
| 1 | 0.999601 | 0.999949 | 3% | 6% |
| 4 | 0.999827 | 0.999859 | 10% | 5% |
| 7 | 0.999883 | 0.999861 | 20% | –8% |
| 11 | 0.999911 | 0.999919 | 2% | –2% |
| 16 | 0.999953 | 0.999858 | –3% | 3% |
| *Ointment* | | | | |
| BD† | 0.999985 | 0.999989 | 0.1% | 0.1% |
| 1 | 0.999964 | 0.999972 | 6% | 7% |
| 4 | 0.999969 | 0.999976 | 1% | 3% |
| 7 | 0.999940 | 0.999974 | –3% | 0% |
| 11 | 0.999940 | 0.999889 | 13% | 21% |
| 16 | 0.999883 | 0.999979 | –2% | 10% |

* Refer to Figure 1 for chemical structures.
† BD = Betamethasone Dipropionate.

metric flask, and diluted to volume. The BD recoveries ranged from 99–101%. This extraction technique was not pursued further due to several drawbacks: (*i*) the boundary between the liquid phases was difficult to distinguish consistently, making this technique difficult for day-to-day reproducibility, and (*ii*) if some of the non-polar liquid was transferred to the volumetric flask, dilution to volume was difficult because two meniscuses were present.

Solid-phase extraction was explored as a sample extraction technique. However, this process was not optimized as multiple extractions were required.

The final sample extraction method that was explored used a heating/centrifuging technique. Initially, ratios (1:9, 1:7, 1:5, 1:4, and 1:3 of water–methanol) of the extraction solvent were examined, and the BD recovery ranged from 100–102%, and the ratio of water to methanol did not alter the recovery. Water was part of the extraction solvent because it aided in the precipitation of an ointment excipient.

Using a 1:9 ratio of water–methanol as the extraction solvent, the BD recovery remained constant with varying waterbath temperatures (i.e. 60°C, 70°C, and 75°C). Therefore, 70°C was chosen as the heating temperature for the extraction. The ointment samples were extracted at 70°C with varying heating times (8, 12, and 20 min), and the BD recovery was constant for all heating times. Therefore, the final heating time was chosen at 8 min. Finally, acid was added to the extraction solvent to enhance the stability of compound 4. Glass centrifuge tubes were used for the sample extractions as impurity peaks from polypropylene centrifuge tubes were observed to be introduced into the samples.

## Method validation

Two analysts from two different laboratories performed the method validation with respect to the method linearity, accuracy, limit of quantitation (LOQ), limit of detection (LOD), ruggedness, specificity, robustness, and solution stability for both the cream and ointment matrices. Through the method validation, the analytical concentration of BD was ~ 0.1 mg/mL for the cream and the ointment, and the analytical concentration of chlorocresol was ~ 0.165 mg/mL in the cream matrix.

### Method linearity, accuracy, LOQ, LOD, and precision

The BD linearity was analyzed over a range from 0.00005 mg/mL to 0.15 mg/mL, which corresponds to 0.05% to 150% of the analytical concentration. The chlorocresol linearity was analyzed over a range from 0.0825–0.2475 mg/mL, which corresponds to 50% to 150% of the analytical concentration. The linearity of five BD related compounds (compounds 1, 4, and 7 from 0.05 µg/mL to 2 µg/mL; compounds 11 and 16 from 0.1 µg/mL to 2 µg/mL) was analyzed, which corresponds to 0.05% to 2% of the analytical concentration. The tests were performed by spiking a solution of known concentration into a placebo sample.

For analyst 1 and 2, the coefficient of determination ($r^2$) was evaluated for BD, chlorocresol, and for five of the BD related compounds, and the results are summarized in Table IV. The y-intercepts as a percentage of the analytical concentration response for BD and chlorocresol were evaluated, and the y-intercepts as a percentage of the LOQ level response for the BD related compounds were also examined. The results are shown in Table IV.

The data obtained during the linearity studies were used to evaluate the method accuracy for both the cream and ointment matrix (Table V). For BD, percent recoveries from 100% to 104% were observed over the range from 0.0005–0.005 mg/mL and from 99.5% to 102.6% over the range from 0.05–0.15 mg/mL. In the cream matrix, the chlorocresol percent recoveries were 98% to 104% over the range from 0.0825–0.2475 mg/mL. For the BD related compounds, percent recoveries from 93% to 115% were observed over the range from 0.0001–0.0002 mg/mL.

For BD and all BD related compounds except for compounds 11 and 16, the LOQ and LOD were set at 0.05% and 0.03% of the analytical concentration, respectively. For compounds 11 and 16, the LOQ was set at 0.1% and the LOD at 0.05% due to the lower relative response of these late eluting peaks. At these levels, the signal-to-noise ratio (S/N) of the LOQ solutions for the evaluated peaks was ≥ 10, and the S/N of the LOD solutions for the evaluated peaks was ≥ 3.

The method repeatability was evaluated using the recovery results from the low (50%), middle (100%) and high (150%) concentration levels for both BD (cream and ointment) and chlorocresol (cream only). The BD related compounds were evaluated at low (0.2%), middle (0.5%) and high (2.0%) concentration levels (cream and ointment). The results for method repeatability and intermediate precision are summarized in Table V.

**Table V. Summary of Method Recovery, Reproducibility, and Intermediate Precision Results**

| Compound* | Recovery Range (%) | | %RSD Low, Middle, and High Levels ($n$ = 9) | | Absolute Difference in %RSD between Analyst 1 and Analyst 2 |
|---|---|---|---|---|---|
| | Analyst 1†,‡ | Analyst 2†,‡ | Analyst 1 | Analyst 2 | |
| *Cream* | | | | | |
| Betamethasone Dipropionate | 101.5–102.3†; 102–103‡ | 101.6-102.6†; 101–104‡ | 0.3 | 0.3 | 0.0 |
| Chlorocresol | 98–102 | 99–104 | 1.6 | 2.2 | 0.6 |
| 1 | 95–100 | 101–106 | 1.6 | 0.7 | 0.9 |
| 4 | 96–102 | 101–107 | 1.4 | 1.1 | 0.3 |
| 7 | 96–109 | 97–103 | 2.5 | 1.3 | 1.2 |
| 11 | 96–99 | 99–104 | 0.8 | 1.2 | 0.4 |
| 16 | 93–97 | 101–105 | 1.4 | 1.4 | 0.0 |
| *Ointment* | | | | | |
| Betamethasone Dipropionate | 99.5–100.2†; 100–101‡ | 99.7–100.5†; 100–102‡ | 0.2 | 0.3 | 0.1 |
| 1 | 100–110 | 100–105 | 0.8 | 0.8 | 0 |
| 4 | 99–103 | 100–103 | 0.4 | 0.5 | 0 |
| 7 | 98–102 | 99–102 | 1.3 | 0.4 | 1 |
| 11 | 100–109 | 101–115 | 3.3 | 5.4 | 2 |
| 16 | 96–101 | 100–110 | 0.9 | 3.5 | 3 |

* Refer to Figure 1 for chemical structures.
† Recovery Range from 50–150%.
‡ Recovery range from 0.5% to 5%.

**Method specificity**

The method specificity was demonstrated by showing that the method was capable of resolving BD, chlorocresol, and all BD related compounds. For the cream samples, a specificity mixture consisting of BD and chlorocresol at ~ 100% levels and all BD related compounds at ~ 1% levels was analyzed for the cream samples. A specificity mixture for the ointment samples was prepared in the same manner as the cream specificity mixture minus chlorocresol. In addition, five representative cream and ointment samples (two expired and three unexpired) were analyzed.

All BD related compounds were separated from BD and chlorocresol for the cream samples, and all BD related compounds were separated from BD for the ointment samples. The minimum resolution between each related compound was 1.6 between compounds 10 and 11. All other resolutions were 1.8 or greater. In addition, the purity of the BD and chlorocresol peaks in the specificity mixtures, the cream samples, and the ointment samples were evaluated based on a photodiode-array (PDA) scan. In all cases, the purity angle was less than the purity threshold for BD and for chlorocresol, indicating that both peaks are pure.

**Method robustness**

Deliberate variations in HPLC and sample extraction parameters were made to demonstrate the robustness of the method. The HPLC parameter variations studied included column temperature (30°C and 40°C), flow rate (1.3 and 1.7 mL/min), injection volume (20 and 30 µL), gradient slope (10% slower, 10% faster), detection wavelength (238 and 242 nm), HPLC system (Agilent), grade of mobile phase A water (HPLC grade) and column lot (two additional lots). The sample extraction parameters studied included volume of diluent, water bath temperature, centrifuge time, centrifuge speed, heating time, shaking time during heating, volume of filtrate discarded and lot of filter used.

The % label claim of BD varied ± 3% (percent relative difference) from the analytical method determination for both the cream and ointment samples. The % label claim of chlorocresol varied ± 3% (percent relative difference) from the analytical method determination for the cream samples. The estimation of each related compound varied ± 0.1% (percent absolute difference) from the analytical method estimation for both the cream and ointment samples. The resolution between compounds 4 and 7 was consistent for all variations studied. The peak symmetry of the BD peak varied between 1.0 and 1.2 for the cream and ointment samples. The peak symmetry of the chlorocresol peak varied between 1.1 and 1.2 for the cream samples. The S/N ratio of the LOQ level BD peak ranged from 10 to 40 for both the cream and ointment samples.

**Solution Stability**

The extracted cream sample solutions, the extracted ointment sample solutions, and standard solutions were prepared for the solution stability study. Each solution was split into two parts with one stored at room temperature and the other was stored under refrigeration (2–8°C). All solutions were quantitated against fresh standard solutions on the respective day. All solutions were protected from light during the stability period.

Under both room temperature and refrigerated conditions, the percent relative difference of BD was within ± 2% of the initial concentration for both the cream and ointment samples, and the percent relative difference of chlorocresol was within ± 1% of the initial concentration for the cream samples. The percent absolute difference of all quantifiable (at the QL level or above) related compounds was within ± 0.1% of the initial estimation. No additional peaks were observed at any of the time points in comparison to the day 0 analysis. The solutions were considered stable for 7 days at room and under refrigerated temperatures.

**Sample reproducibility**

The reproducibility of the sample extraction for the cream and ointment products was also evaluated. Six samples of an expired cream and ointment batch were extracted and the %RSD's of the results were determined. The %RSD of the %label claim of BD was 0.5% and 0.1% for the cream and ointment samples, respectively. The %RSD of the %label claim of chlorocresol in the cream sample was 0.3%. The %RSD of the estimation of compound 4 was 1.8% and 0.5%, of compound 7 was 0.5% and 0.5%, and of compound 16 was 1.8% and 2.5% for the cream and ointment samples, respectively. All other detected related compounds had peak responses lower than the LOQ for the cream samples. In the ointment samples, two unknown peaks (RT = 13.2 and 29.7) were observed above the LOQ with a %RSD of 0.9% and 3.9%, respectively.

**Alternate column**

The Waters XBridge Shield RP18 analytical column (150 × 4.6 mm, 3.5 µm particle size) was identified as an alternate column to the Waters SymmetryShield RP18 analytical column (150 × 4.6 mm, 3.5 µm particle size). Both columns contain polar embedded functional groups. Although the retention on the XBridge Shield RP18 column was slightly less than on the SymmetryShield RP18 column, the elution profile for all compounds was the same on both columns (Figure 3). Additionally, the response of BD and the quantitated related compounds was shown to be equivalent on both columns for the cream and ointment samples. The response of chlorocresol in the cream samples was shown to be equivalent on both columns.

## Conclusion

A new RP-HPLC method has been developed for simultaneous assay and identification of BD along with the estimation of all BD related compounds in a commercially available cream and ointment based product, and for the assay and identification of chlorocresol in the cream product. The new HPLC method was successfully validated per ICH guidelines and proved to be suitable for routine quality control use. This method was also demonstrated to be stability-indicating as it can separate all the degradation peaks that are present in aged batches of the cream and ointment products. To the best of our knowledge, this is the first method that can separate and accurately quantitate BD, chlorocresol, all known BD degradants and impurities, and potential packaging leachables from cream and ointment products.

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