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Stability Indicating RP-HPLC Method for Estimation of Impurities of Vitamin D_3 Analogue and Corticosteroid Used as Antipsoriatic Drugs. An Attempt to Characterize Pre-Calcipotriene

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Abstract

A single RP-HPLC method is developed for estimation of isomeric impurities of vitamin D_3 analogue-Calcipotriol/Calcipotriene (Calci) and impurities of Betamethasone dipropionate (BD). The developed method is capable of separating impurities of Calci and BD, specifically pre-Calcipotriene (Pre-Calci) from other known and unknown impurities. Pre-Calci is isolated and is characterised using few analytical techniques. These impurities are separated using a RP-C_{18} 150 × 4.6 mm, 2.7 µm column maintained at 50˚C. The mobile phase consisted of mixture of water, methanol, acetonitrile and tetrahydrofuran eluted in gradient mode. Detection was done at 264 nm and 240 nm for Calci and BD impurities respectively. The method can be used for determining quality of Calci and BD drugs and ointment based drug products. It is stability indicating related substance method for both the drugs and drug products.

Keywords

Calcipotriene, Pre-Calcipotriene, HPLC, Photo Isomers, Ointment

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1. Introduction

Psoriasis, a chronic inflammatory dermatosis characterized by scaling, infiltration, and erythema, affects approximately 2% of the worldwide population [1]. It is well-known that vitamin D_3 analogues, and Calci, are useful for the treatment of hyper proliferative skin diseases, such as psoriasis vulgaris [2]-[5]. Calci is an analogue of vitamin D used for the treatment of chronic plaque psoriasis and has minimal effects on calcium metabolism. The therapeutic efficacy of Calci has been demonstrated in numerous clinical studies [6]. BD belongs to the class of adrenocortical steroid and used as topicalcorticosteroid in treatment of different skin disorders. BD-Corticosteroid has broad mechanism of action, and inhabits the synthesis of many inflammatory cytokines, production of prostaglandins and nitric oxides, and the expression of adhesion molecules. BD is believed to have anti-inflammatory and anti-pruritic actions that are characteristic of corticosteroids. Several studies have shown that combined therapy with products containing these two active substances is more effective than mono therapy, the additive clinical effects and reduced skin irritationmay be achieved [7]-[10].

The highlights of public assessment reports and defences from product approval package for the drug products of Calci and Calci + BD are as follows.

- Three isomers are mentioned pre-Calci, trans-Calci, and 24-R-Calci. They are also degradation products or related substances [11].
- The release and shelf-life requirements/limits are not identical. Related substances/degradation products and assay are different. Under all three conditions in all batches tested a decrease in assay and an increase in both individual and total related substances/degradation products can be seen [11].
- 5% Calci stability overage is added in manufacturing [12].
- The Calcirelated substances should contain the limits and test for 5, 6 trans Calci [13].

In literature separate HPLC methods are available to estimate impurities of Calci and BD. Also, these methods are limited to estimate only Imp B, Imp C, and Imp D of Calci. However information is not available for quantification of pre-Calci in drug and drug products. Therefore the aim of the current study is to understand formation of pre-Calci, isolate and characterise it, extract both the drugs and impurities from drug product and determine quantitatively related substances of Calci, BD in bulk and combined drug products by single RP-HPLC method.

1.1. Calcipotriol Monohydrate

Calci monohydrate with molecule formula -$C_{27}H_{40}O_3 \cdot H_2O$, IUPAC name (1R,3S,5E)-5-{2-[(1R,3aS,4Z,7aR)-1-[(2R,3E)-5-cyclopropyl-5-hydroxypent-3-en-2-yl]-7a-methyl-octahydro-1H-inden-4-ylidene]ethylidene}-4-methylidenecyclohexane-1,3-diol. H_2O and molecular weight is 430.63. Chemical structure is shown in **Figure 1**. Calci is a white to off-white crystalline powder; the substance is practically insoluble in water, freely soluble in ethanol slightly soluble in methalene chloride. A reversible isomerisation to pre-Calci takes place in solution depending on temperature and time. The activity is due to both. It is sensitive to light [14]. Pre-Calci as an isomer formed in the drug product by a reversible transition, by a slow process which starts when Calci is dissolved and continues until equilibrium is reached. It is influenced by temperature. Therefore, pre-Calci is not a true impurity, and the activity of Calci is due to both substances. So, assay is calculated as the sum of the assays of the isomers [12].

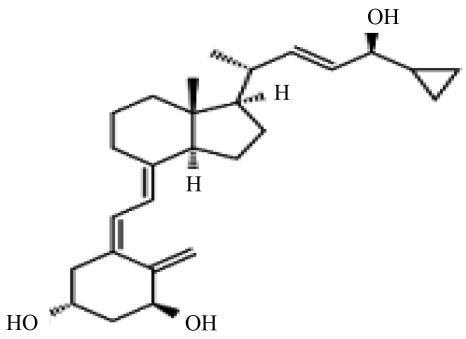


Figure 1. Calcipotriol.

1.2. Betamethasone Dipropionate

Betamethasone dipropionate is chemically 9-fluoro 11*β*,17, 21-trihydroxy-16*β*-methylpregna-1,4-diene-3,20-dione 17,21-dipropionate and it belongs to the class III of adrenocortical steroid. Chemical structure is shown in **Figure 2**. BD id used as topical corticosteroid in treatment of different skin disorders. The possible known impurities in drug and drug products are Imp-A: Betamethasone 17-propionate; Imp-B: Betamethasone 21-propionate; Imp-C: Betamethasone 17-propionate, 21-acetate.

2. Experimental

2.1. Materials

Calcipotriol Monohydrate (Formasa, Taiwan); Betamethasone dipropionate, its impurities (Crystal Pharma, India); Calci impurity mixture (EP Reference standards, EU); Ortho phosphoric acid (OPA) (AR grade, Merck, India); methanol (MeOH), acetonitrile (ACN) (HPLC grade, Merck, India); Tetrahydrofuran (THF) (Merck, India) were used in the present study. High pure water is from Mill-Q water purification system from Millipore.

2.2. Instrumentation

Agilent 1100 series HPLC system equipped with quaternary pump with an online degasser, auto sampler, thermostatted column compartment and variable wavelength detector. Waters alliance HPLC system equipped with 2695 separations module and 2489 UV/VIS detector or 2998 Photodiode array detector. Waters Empower 2 software is used for data acquisition, data processing.

2.3. Preparation of Solutions

2.3.1. Standard Preparation

About 15 mg of pure BD is dissolved in 200 ml of diluent. 15 mg of pure Calci is dissolved in 100 ml of diluent. Transferred 0.5 ml of BD standard stock solution, 0.1 ml of Calci standard stock solution into 100 ml volumetric flask diluted to 100 ml with diluent and mixed. The diluted standard is about 0.75 µg/ml and 0.075 µg/ml of BD and Calci respectively.

2.3.2. Sample Preparation

About 2500 mg of ointment sample containing 1.25 mg of BD and 0.125 mg of Calci is transferred into a 100 mL amber colour volumetric flask, to it 10 mL of n-Hexane is added and sonicated for 15 min for complete dispersion, to this 5 mL of Diluent is added and mixed on a vortex mixture for 5 min. Transferred the mixture to 50 mL centrifuge tube, centrifuge at 5000 rpm for 5 min. Clear lower layer was injected for analysis. Samples are prepared fresh ever time.

2.3.3. Preparation of Pre-Calci Reference Solution

Pre-Calci is prepared by placing 2 mg of Calci in a vile and dissolved in 200 µl of solution made from 1 ml triethylamine and 9 ml of chloroform. Closed the vile and kept it in a water bath at 60˚C for 2 hours.

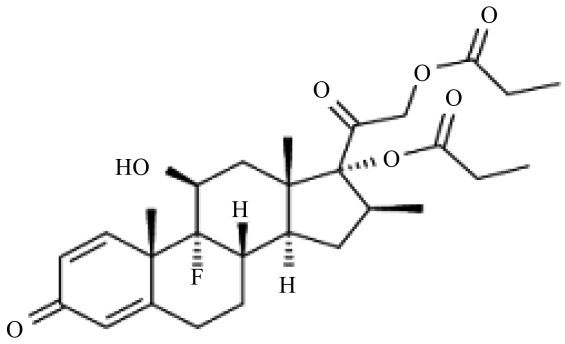


Figure 2. Betamethasone dipropionate.

3. Results

3.1. Method Development

3.1.1. Selection of Diluent

Diluentselection is found to be key factor in extraction of selected drugs from ointment base. It is obvious that ointment base is soluble only in non-polar solvents like n-Hexane and THF, whereas drugs are soluble in polar solvents. The present drug product contains about 96% to 97% w/w of wax based ointment and 0.005% w/w of Calci and 0.0643% w/w of BD. It is difficult to extract all related compounds of drugs at such low concentration. Diluent is finalized by checking recovery of many solvent systems. Highest recovery is found with solvent mixture containing ACN, water at 95:5 v/v. few solvent systems recovery results are presented in **Table 1**.

3.1.2. Chromatographic Conditions

The critical separation of all analytes was achieved using a mobile phase consisting of Component A: Water: MeOH:THF—70:25:5 (v/v/v); Component B: ACN:Water:THF—90:5:5 (v/v/v). Separation was achieved on a Supelco Acentis Express RP-C_{18} 150 × 4.6 mm, 2.7 µm at 50˚C. The injection volume was 20 µL, Diluent (D) was ACN:water—95:5 (v/v). The gradient programme is as follows with Mobile phase component % and Flow (F) mL/min Gradient: Time(T) (min) = 0.1, F = 1.0, A = 98%; T = 2, F = 1.0, A = 98%; T = 15, F = 1.0, A = 70%; T = 28, F = 1.0, A = 70%; T = 30, F = 1.0, A = 72%; T = 5, F = 1.0, A = 72%; T = 55, F = 2.0, A = 5%; T = 62, F = 2.0, A = 5%; T = 65, F = 1.0, A = 92%; T = 70, F = 1.0, A = 92%. Detection is done at 240 nm for BD and BD related compounds, at 264 nm for Calci and Calci-related compounds.

3.2. Method Validation

System suitability solution is prepared by diluting 1 ml of pre-Calci reference solution to 100 ml with diluent and mixed. USP Resolution between Pre-Calci and Calci shall be about 4.0 (**Figure 3**).

Table 1. Solvent selection—recovery of BD and Calci.

Diluent	% Recovery	
	BD	Calcipotriene
Methanol	70.7	75.1
Acetonitrile	93.8	93.6
Acetonitrile:Methanol (50:50 v/v)	89.0	90.6
Acetonitrile:Water (90:10 v/v)	99.9	99.8
Acetonitrile:Water (95:5 v/v)	100.5	99.8

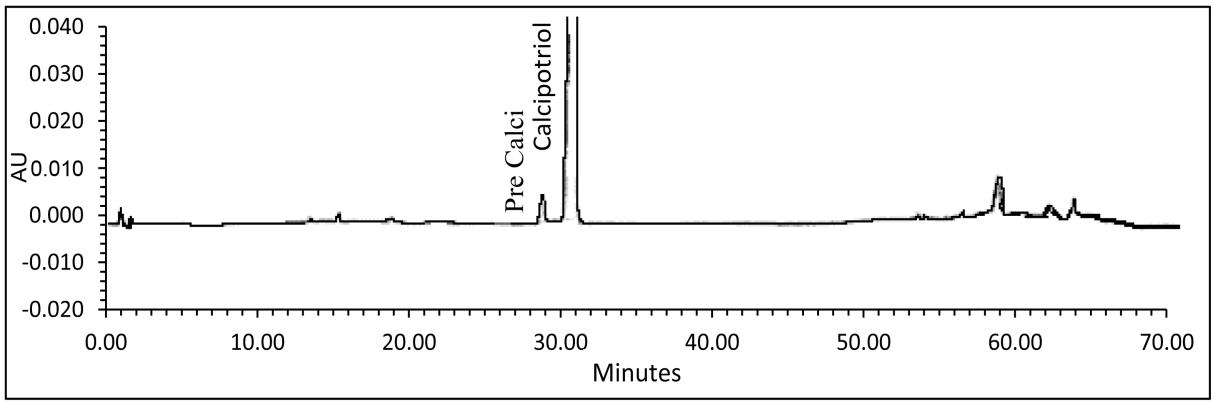


Figure 3. System suitability chromatogram.

The method is validated as per ICH guidelines Q2 (R1) for pure compounds of Calci and BD. Method is found accurate and precise. Specificity is proved by performing force degradation. Acidic, basic, oxidative, humidity, photolytic and thermal stressed samples are analysed. And is found, all known impurity peaks of Calci and BD are pure as they pass the peak purity. LoD and LoQ of BD found to be 0.003 µg/mL and 0.008 µg/mL respectively. LoD and LoQ of Calci found to be 0.002 µg/mL and 0.006 µg/mL respectively. Linearity is established from LoQ to 1.5 µg/mL for BD and 0.15 µg/mL for Calci, which is 0.6% of Sample concentration. Robustness and intermediate precession are within acceptance limits.

3.3. Isolation

3.3.1. Chiral Separation

To isolate Pre-Calci solid compound, preparative chiral chromatography is chosen. Concentrated solution (about 1 mg/ml) of Calci is prepared with ACN as solvent, the solution is kept in water bath at 80˚C for 5 hours, and few microliter solution is injected on to analytical chiral column to ensure formation of pre-Calci. It is found Calci and Pre-Calci are present in the ratio 70:30 percent composition of compounds (mass ratio). 20 ml of isomers mixture is run through preparative chiralpack (Daicel) column, fractions of Pre-Calci are collected. The colour of Pre-Calci solution is observed to be light yellow. The pooled fractions are immediately evaporated under vacuum. Few mg of solid Pre-Calci is formed as residue. Chromatographic purity of isolated solid Pre-Calci is determines as about 96% pure.

3.4. Characterization of Pre-Calci

3.4.1. Liquid Chromatography-EP Method vs Developed Method

Europe Pharmacopeia (EP) Chemical Reference Standard (EP-CRS) is available from EP which contains mixture of Calci, Calci Impurity-C, Calci Impurity-B and Calci Impurity-D along with respective chromatogram. It is confirmed that the EP method is not capable of separating Pre-Calci from Calci. When EP-CRS mixture is analysed by developed method Pre-Calci is perfectly separated. Pre-Calci peak is confirmed by spiking isolated compound on to EP-CRS solution. Pre-Calci peak is also confirmed by spiking "Pre-Calci reference solution" prepared as per EP on to EP-CRS (**Figure 4**).

3.4.2. UV Scans

All separated peaks are scanned by Ultra violet light, from 400 nm to 200 nm on HPLC-DAD detector. absorption maxima (λ_{max}) of Pre-Calci is found to be at 260 nm, The peak is confirmed as Pre-Calci related Substances of Calci as λ_{max} of Calci and its related compounds range from 264 nm to 274 nm. The lowering of λ_{max} for Pre-Calci is because of extended conjunction is part of ring (**Figure 5**).

3.4.3. NMR

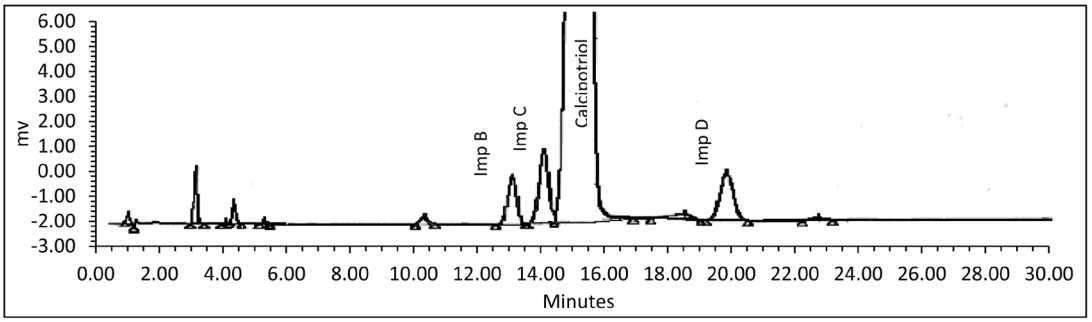
^{1}H NMR spectrum is obtained by subjecting isolated Pre-Calci and pure Calci, dissolved in $CDCl_3$ and analysed on a Bruker 400 MHz NMR spectrometer. In pre-Calci spectrum, the chemical shifts of 1.25 corresponding to ^{1}H at carbon position 19 ($-CH_3$) and chemical shift of 3.55 corresponding to ^{1}H at Carbon position 1 (-CH-). When compared to spectrum of Calci (**Figure 6**) the changes in chemical shifts are in support of available Pre-Calci structure (**Figure 7**).

3.4.4. MS and LC-MS

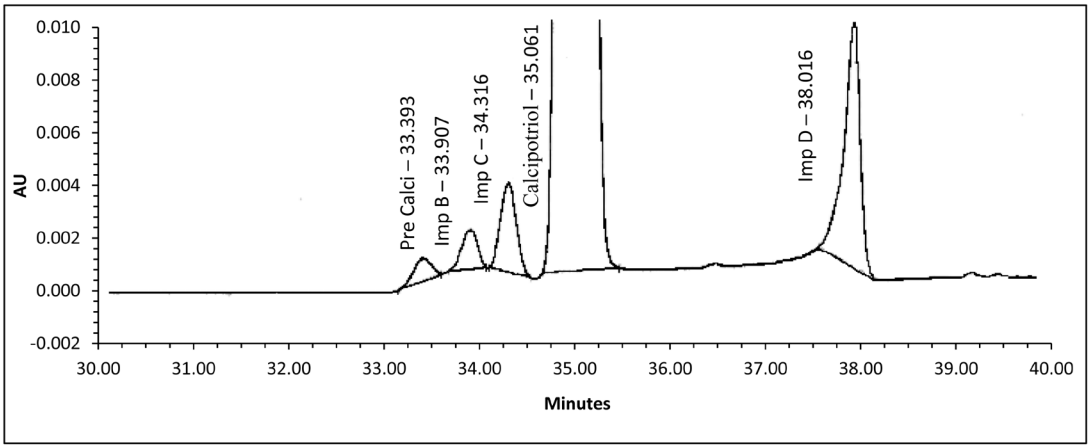
Calci and isolated Pre-Calci are analysed for its mass fragmentation data on a LC-MS and LC-MS/MS. No significant difference in mass fragmentation is observed. Which is in clear support of the fact that Calci and Pre-Calci are isomers.

3.4.5. FT-IR

IR spectrum of isolated pre-Calci and pure Calci are obtained by holding the compounds in a potassium bromide pellet and scanned from 4000 cm^{-1} to 500 cm^{-1} using a Perkin Elmer FTIR spectrometer. A shift in stretching/vibrational wave number (cm^{-1}) corresponds to formation of isomer of Calci, which is supporting its structure. Few wave numbers of Calci and Pre-Calci are compared in **Table 2**.



(a)



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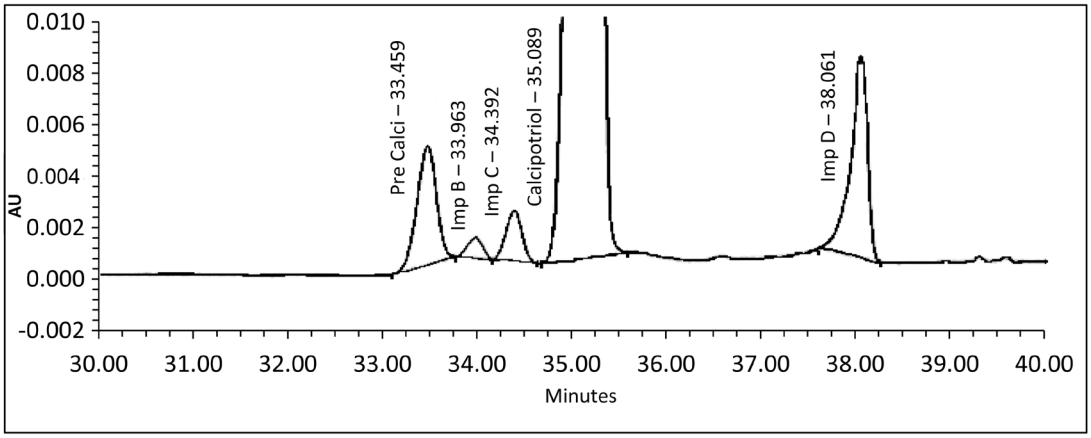
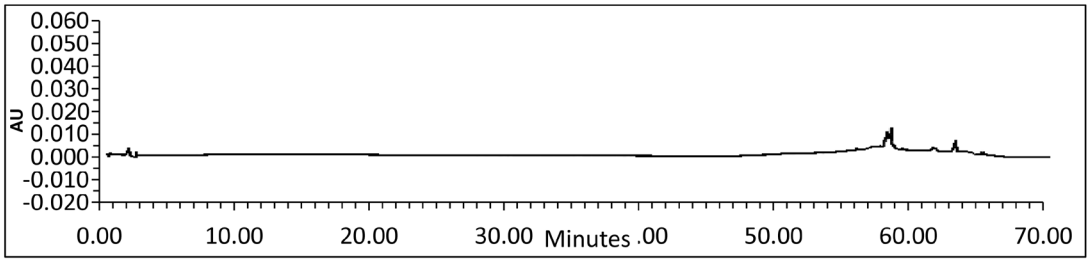


(c)



(d)

Figure 4. Comparison of zoomed chromatograms of (a) EP CRS in EP method; (b) EP CRS in developed method; (c) Isolated pre-Calci spiked on EP CRS in developed method; (d) Blank.

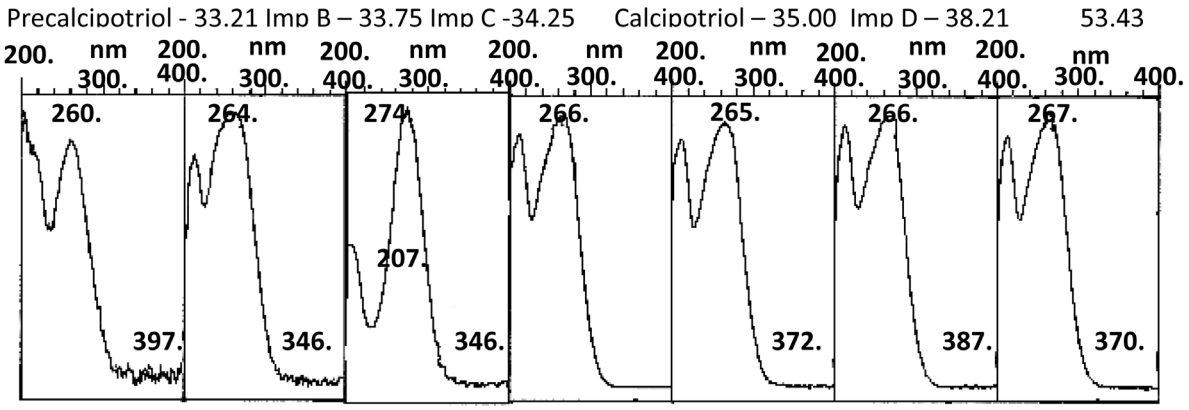


Figure 5. UV scans of Calci and related substance of Calcipotriol.

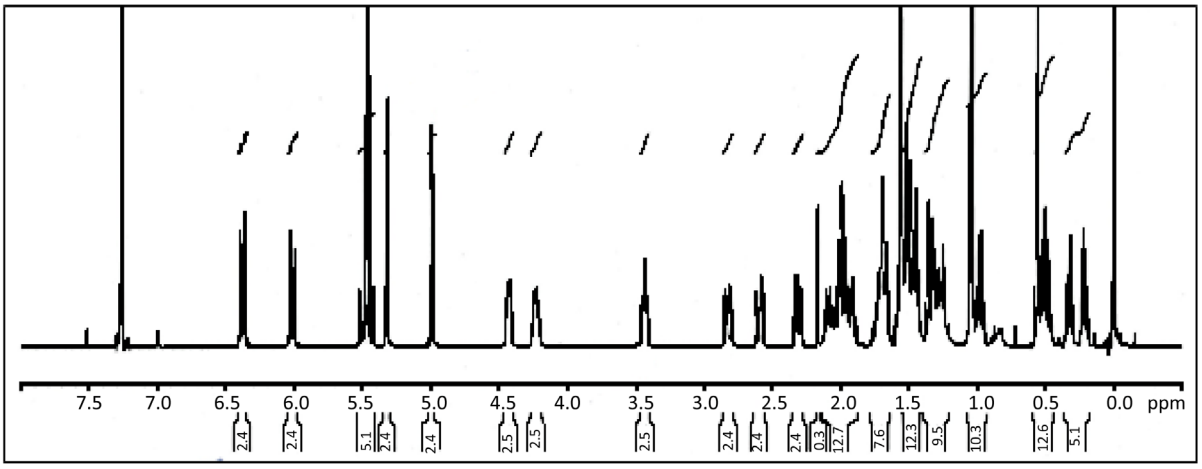


Figure 6. ^{1}H NMR of Calcipotriol.

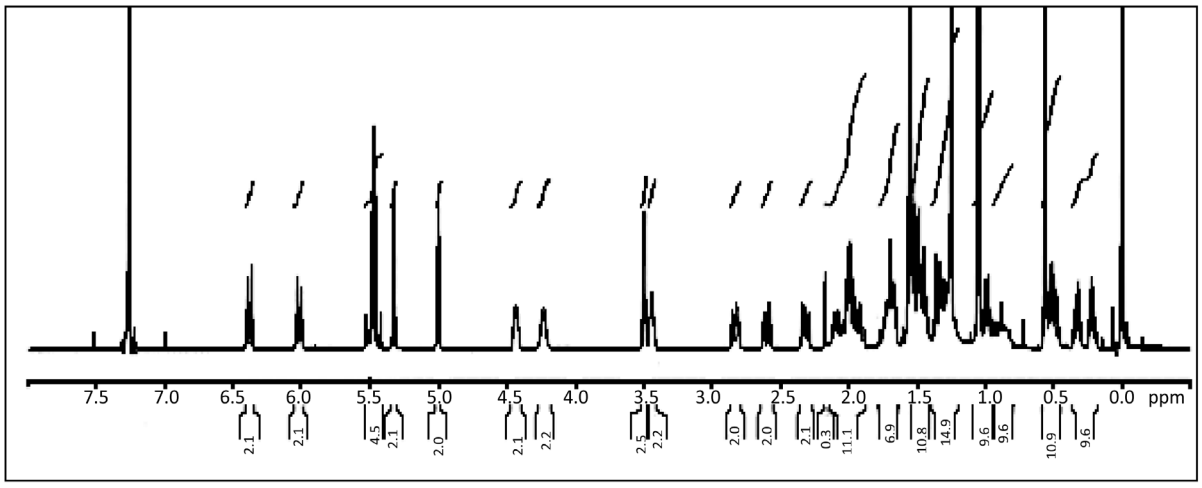


Figure 7. ^{1}H NMR of isolated Pre-Calcipotriol.

4. Discussion

Theoretically it is predicted, Calci undergo Cis/Trans isomerism at carbon 7 forming Imp-B, Followed by rearrangement of double bonds forming Pre-Calci. From different experiments it is noticed that Imp-B levels are

much lower than Pre-Calci, indicating Imp-B is unstable readily converting to Pre-Calci in solution. The equilibrium and rearrangement is shown in **Figure 8**. Structure of pre-Calci is conformed from Product monograph of Dovobet ointment July 2002. The over outcome of current study, chromatographic separation of Calci, isomers of Calci, Impurities of Calci (known and unknown), BD and Impurities of BD (known and unknown) is achieved. **Figure 9** is a representative chromatogram of the proposed HPLC method.

Table 2. Comparison of IR wave numbers of Calci and Pre-Calci.

Calci (cm^{-1})	Assignment	Pre-Calci (cm^{-1})
3447.13	O-H Stretch	3426.79
3310.21	O-H Stretch	Absent
2974.44	C-HStretch	Absent
2928.38	C-H Stretch	2945.70
2867.63	C-H Stretch	2881.67
1671.02	C=C	1664.32
1637.27	C=C	1632.45
1444.42	$-CH_3$, > CH_2	1445.13
1060.66	C-O Stretch	1068.74
1018.23	C-O Stretch	1024.09

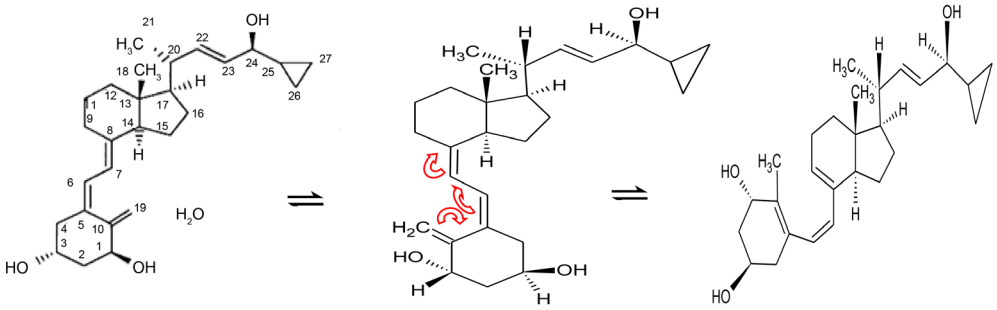


Figure 8. Predicted scheme for Pre-Calci Formation.

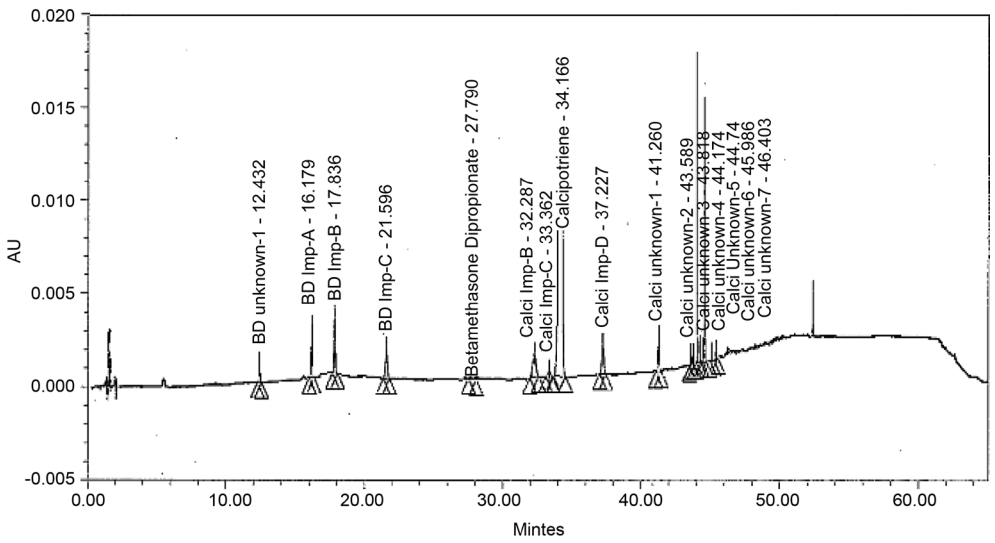


Figure 9. Chromatogram of known and un known impurities of BD and Calci.

5. Conclusion

The separated peak is conformed as Pre-Calci. The developed HPLC method can separate EP-CRS mixture into five peaks—Calci, imp-B, imp-C, imp-D and Pre-Calci; BD, imp-A, imp-B, imp-C of BD from each other. The method is superior to current available methods for BD and Calci. The method can be employed for qualitative and quantitative determination of impurities of Calci and BD drugs and drug products. Many queries and deficiencies with respect to assay, related substances, batch release, shelf life stability, and overages can be addressed by using this method. The developed and validated method shall be a quality control method for release and stability testing.

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