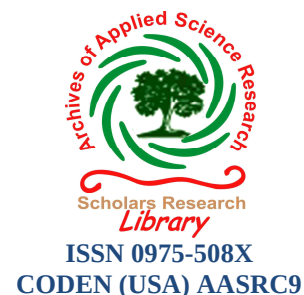




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Development and validation of a stability-indicating NP-HPLC method for simultaneous determination of betamethasone dipropionate and calcipotriene in topical dosage form

Chinmoy Roy^{*1,2}, Jitamanyu Chakrabarty² and Rammohan Rao Ratti¹

¹Analytical Research and Development, Integrated Product Development, Dr. Reddy's Laboratories Ltd., Bachupally, Hyderabad, Andhra Pradesh, India

²Department of Chemistry, National Institute & Technology, Durgapur, West Bengal, India

ABSTRACT

A simple precise NP-HPLC method development and validation for simultaneous determination of Betamethasone Dipropionate and Calcipotriene in topical formulation. Chromatographic separation of drug was achieved on Inertsil Silica 100A^o (250×4.6mm, 3μ) column with mobile phase A n-Hexane, tetrahydrofuran and Ethanol in the ratio 95:5:2 v/v/v, respectively as mobile phase A and mobile phase B as n-Hexane, tetrahydrofuran, Ethanol and Isopropylalcohol in the ratio 65:8:2:25 v/v/v/v, respectively in gradient mode at flow rate of 1.2mL/min. Analytes were detected at 264nm. Linear response ($r > 0.999$) were observed over a range of 1.171 μg/mL to 32.9 μg/mL and 0.017 μg/mL to 3.1 μg/mL for Betamethasone Dipropionate and Calcipotriene respectively. Stability indicating capability of developed method is established by analysing forced degradation samples in which spectral purity of Betamethasone Dipropionate and Calcipotriene along with separation of degradation products from analytes peak. The method was validated as per ICH guideline for specificity, linearity, accuracy, precision, LOD, LOQ, robustness, solution stability and suitable for the quantitative determination of Betamethasone Dipropionate and Calcipotriene in topical dosage form and also for quality control in bulk manufacturing.

Keywords: Stability indicating • HPLC • Normal phase • Assay • Betamethasone Dipropionate and Calcipotriene

INTRODUCTION

Psoriasis is a chronic, inflammatory skin disease affecting 1–3% of the world's population ^[1]. Despite decades of active research, there is currently no cure and different treatment strategies focus on relieving the symptoms and restricting its severity.

Topical corticosteroids and the vitamin D analogue, Calcipotriene, have become mainstays in psoriatic treatment ^[2], each having different mechanisms of action ^[3,4]. Several studies have shown that combined therapy with products containing these two active substances is more effective than mono therapy, and that additive clinical effects and reduced skin irritation may be achieved ^[5-8]. However, current corticosteroid products cannot be applied together with a Calcipotriene product as the drugs are incompatible and will degrade when mixed ^[9,10]. Consequently, patients have been applying one product in the morning and the other in the evening. To explore the effect of simultaneous treatment and to improve patient compliance alleviating the inconvenience of separate applications, it is desirable to have a single product in which both substances are stable and can be applied simultaneously. Due to differences in

physicochemical properties and stabilities it has not previously been possible to successfully combine the two drug substances in a single formulation^[11-13].

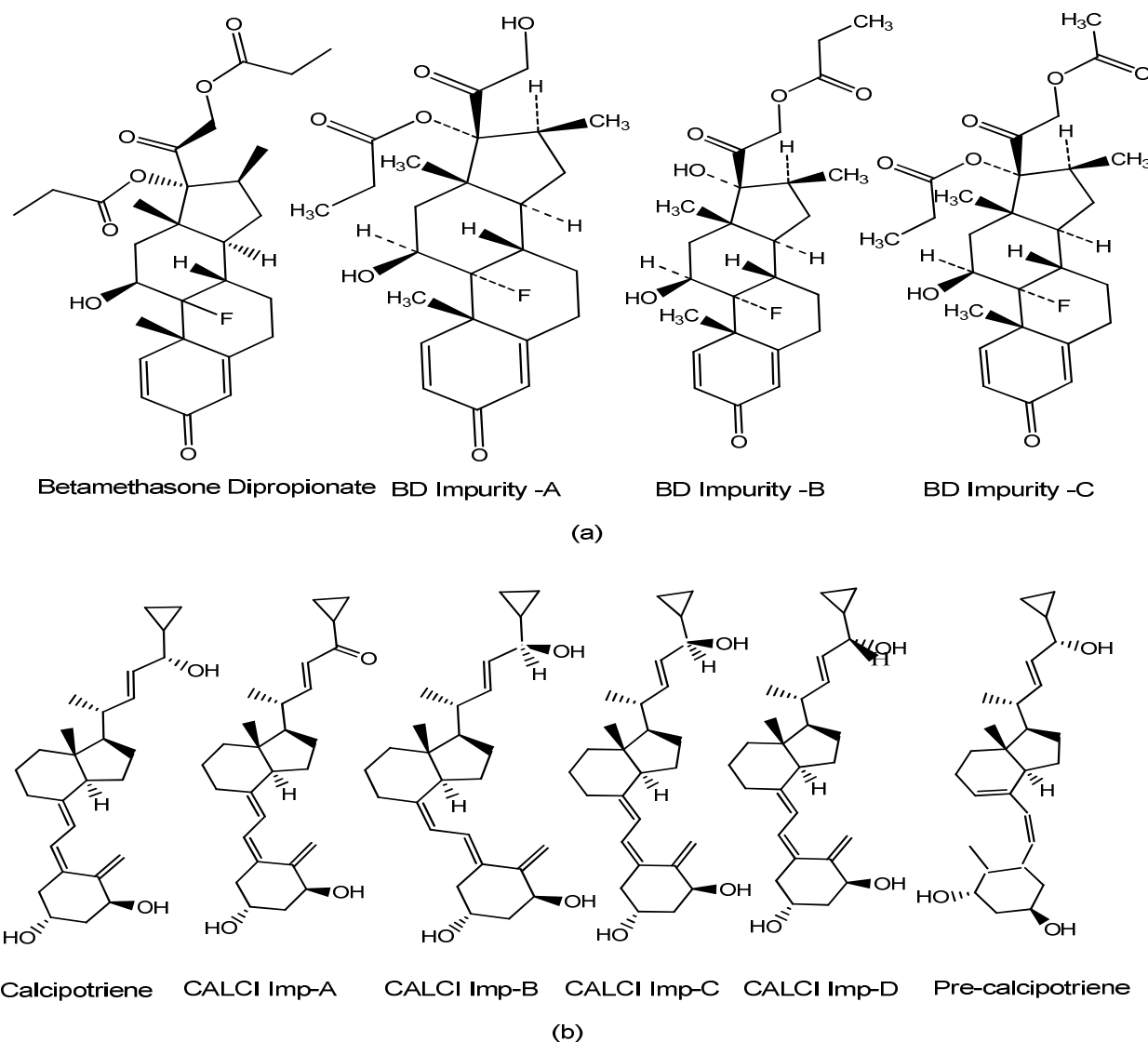


Fig. 1. Chemical Structure of (a) Betamethasone Dipropionate and its three impurities (b) Calcipotriene and its five impurities

Calcipotriene, a synthetic analog of 1,25-dihydroxy vitamin D₃, binds to the vitamin D receptor and act as a heterodimer with the retinoid X receptor (RXR) Vitamin D receptors are present on keratinocytes and lymphocytes. 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 of adrenocortical steroid and used as topical corticosteroid in treatment of different skin disorders^[14].

A detailed literature survey for Betamethasone Dipropionate and Calcipotriene revealed that determination of individual compound or combination with other drugs have been reported by HPLC^[14-24], LCMS^[25], GCMS^[26] and spectrophotometer^[27, 28]. To the best of our knowledge, there is no stability-indicating LC method reported for the simultaneous determination Betamethasone Dipropionate (BD) and Calcipotriene (CALCI) in topical formulation. Therefore, attempts were made in this study to develop a fast, sensitive, selective and stability-indicating normal phase high-performance liquid chromatography (HPLC) method for the simultaneous determination Betamethasone Dipropionate and Calcipotriene in topical formulation. The proposed method is able to separate Betamethasone Dipropionate and Calcipotriene with each other and from its impurities, degradation products and placebo components. The developed LC method was validated with respect to specificity, linearity, limit of detection and quantification, precision, accuracy and robustness. Force degradation studies were performed on the placebo and drug product. Developed method separates all degradation products from Betamethasone Dipropionate and Calcipotriene and it exhibits stability indicating nature.

The drug product stability guideline Q1A (R2) issued by the International Conference on Harmonization (ICH) [29] suggests that stress studies should be carried out on a drug to establish its inherent stability characteristics, leading to identification of degradation products and, hence, supporting the suitability of the proposed analytical procedures. It also requires that analytical procedures for testing the stability of samples should be stability-indicating and should be fully validated. Chemical structures of Betamethasone Dipropionate, Calcipotriene and its impurities are presented in Figure 1(a) and (b) respectively.

MATERIALS AND METHODS

Reagents and Chemicals:

BD and CALCI gel, placebo, BD and CALCI working standards and impurities standards of were provided by Dr. Reddy's laboratories Ltd., Hyderabad, India. HPLC grade n-Hexane, Tetrahydrofuran, Ethanol and Isopropylalcohol were used of Rankem, India. 0.22µm PVDF membrane filter was purchased from Millipore, India. 0.2 µm PVDF syringe filter and 0.2 µm Nylon syringe filter was used of Millipore, India. Water for HPLC was generated using Milli-Q Plus water purification system (Millipore, Milford, MA, USA).

BD impurities

Imp-A: Betamethasone 17-propionate;

Imp-B: Betamethasone 21-propionate,

Imp-C: Betamethasone 17-propionate, 21-acetate)

CALCI impurities

Imp-A:(5Z,7E,22E)-24-Cyclopropyl-1 α ,3 β -dihydroxy-9,10-secochola-5,7,10(19),22-Tereaeen-24-one.

Imp-B:(5Z,7E,22E,24r)-24-Cyclopropyl-9,10-secochola-5,7,10(19),22-tereaeen-1 α ,3 β ,24-triol((7Z)-calcipotriol)

Imp-C:(5Z,7E,22E,24S)-24-Cyclopropyl-9,10-secochola-5,7,10(19),22-tereaeen-1 α ,3 β ,24-triol((5E)-calcipotriol).

Imp-D:(5Z,7E,22E,24r)-24-Cyclopropyl-9,10-secochola-5,7,10(19),22-tereaeen-1 α ,3 β ,24-triol(24-epi-calcipotriol)

Pre-calcipotriene:(1R,3S)-5-(2-((1R,3aS,7aR)-1-((2R,5S,E)-5-cyclopropyl-5-hydroxypent-3-en-2-yl)-7a-methyl-2,3,3a,6,7,7a-hexahydro-1H-inden-4-yl)ethyl)-4-methylcyclohexane-1,3-diol compound with ethane (1:2).

Equipment and Instrumentation:

The chromatography analysis was performed using HPLC (Agilent, with Empower2 software) equipped with PDA detector, quaternary solvent manager and auto sampler system. The output signals were monitored and processed using Empower 2 software. Cintex digital water bath was used for hydrolysis studies. Photo-stability studies were carried out in photo-stability chamber (Sanyo, Leicestershire, UK). Thermal stability studies were performed in a dry air oven (Cintex, Mumbai, India).

Chromatographic parameters:

All chromatographic experiments were performed in the gradient mode. Separation was achieved on Inertsil, Silica 100A°, 250X4.6mm, 3µ column as stationary phase by using n-Hexane: Tetrahydrofuran: Ethanol, 95:5:2 v/v/v as mobile phase A and mobile phase B as n-Hexane: Tetrahydrofuran : Ethanol: Isopropylalcohol,65:8:2:25 v/v/v/v keeping gradient programme time (minutes)/mobile phase B(%); 0/7,10/7,12/35,29/35,31/60,37/7. Other parameters such as run time 37 minutes, 1.2 mL/min as flow rate, injection volume of 100µl, column temperature of 25°C were finalized during development. Betamethasone Dipropionate (BD) and Calcipotriene (CALCI) was detected at 264 nm. Mixture of n-Hexane: Tetrahydrofuran (60:40, %v/v) was used as diluents.

Preparation of standard solution:

The stock solutions of Betamethasone Dipropionate (500 µg/mL) and Calcipotriene (50 µg/mL) were prepared by dissolving an appropriate amount of analyte in diluent, separately. Working standard solution was prepared by mixing above stock solutions of Betamethasone Dipropionate and Calcipotriene with final concentration of 20 µg/mL and 2 µg/mL respectively.

Preparation of sample solution:

An accurately weighed sample equivalent to 1.0 mg of BD and 0.1mg of CALCI were taken into 50 mL volumetric flask. About 30 mL of diluent was added to this volumetric flask and sonicated in an ultrasonic bath for 30 min with intermittent shaking. Further diluted up to volume with diluent, mixed well. Centrifuged a portion of solution at 5000 rpm for 15 minutes.

RESULTS AND DISCUSSION

Method development and optimization:

Prime objective of an NP-HPLC method development for determination of BD and CALCI in topical dosage form was: the method should be able to determine assay of drug in single run and should be accurate, reproducible, robust and stability indicating. All degradation product from stress conditions should be well separated from each other and method should be simple to useful in analytical research and quality control laboratory for routine use.

BD and CALCI impurity Spiked sample was used for method development to optimize chromatographic conditions and separation by NP-HPLC. All impurities were spiked in BD and CALCI in such a way to achieve 2 µg/mL for each impurity, 20 µg/mL for BD and 2 µg/mL for CALCI. Furthermore primary developed method was challenged by forced degradation as a pre-validation.

Column selection and mobile phase selection were done simultaneously. A method development was started with waters symmetry C18 150 mm × 3.9 mm, 5 µm column as stationary phase. Mobile phase A was buffer (0.02M phosphate buffer pH 2.5 by orthophosphoric acid) and mobile phase B was 5% tetrahydrofuran in methanol, 50:50 v/v. Flow rate was 1.0 mL/minutes, column temperature 40°C. Co-elution of placebo peak was observed at retention time of CALCI and also late elution of the placebo peak of Tocopherol was observed. Different ratio of mobile phase composition with increase in ratio of tetrahydrofuran in gradient mode was also tried but late elution of placebo peak observed. Further trial was taken with normal phase by using Inertsil Silica 100 A°, 250 × 4.6mm, 3µ column as stationary phase for faster elution of placebo peak. Mobile phase A was n-Hexane, mobile phase B was tetrahydrofuran and mobile phase C was Isopropyl alcohol keeping flow rate 1.0 mL/min, column temperature 25°C and Injection volume 100µL with different gradient programme, placebo peak of Tocopherol was eluted within 5.0 minutes but co-elution of degradation peak of base degradation sample at the retention time of CALCI was observed. Further incorporated ethanol in mobile phase to separate the co-elution of degradation peak from CALCI. Then trial was taken by using n-Hexane, tetrahydrofuran and ethanol in the ratio of 95:5:2 v/v/v, respectively as mobile phase A and mobile phase B as n-Hexane, tetrahydrofuran, Ethanol and isopropylalcohol in the ratio of 65:8:2:25 v/v/v/v, respectively keeping gradient programme time (minutes) / mobile phase B(%); 0/7,10/7,12/35, 29/35, 31/60, 37/7. BD and CALCI peaks are well separated with good resolution and no co-elution of degradation peak. All impurities are well separated in this chromatographic condition and also peak shape of BD and CALCI were found well and obtained chromatogram shown in Figure 2(d).Based on these experiments the final optimized conditions are described below.

With consideration of ointment sample dispersion and to obtain good recovery of sample, using of weak solvents avoided as diluent. Different ratio of n-Hexane and tetrahydrofuran were tried as diluent but recovery problem was observed. With consideration of solubility of two components mixture of n-Hexane, tetrahydrofuran 60:40 %v/v, respectively were used as diluent and satisfactory recovery was achieved.

All chromatographic experiments were performed in the gradient mode. Separation was achieved on Inertsil, Silica 100A°, 250 × 4.6mm, 3µ column as stationary phase by using n-Hexane, tetrahydrofuran and Ethanol in the ratio 95:5:2 v/v/v, respectively as mobile phase A and mobile phase B as n-Hexane, tetrahydrofuran, Ethanol and Isopropylalcohol in the ratio 65:8:2:25 v/v/v/v, respectively. The flow rate was 1.2 mL/min with a gradient programme of (time (min)/ %B); 0/7,10/7,12/35,29/35,31/60,37/7. Other parameters such as run time 37 minutes, injection volume of 100µl, column temperature was maintained at 25°C and detection was monitored at 264 nm. Mixture of n-Hexane and tetrahydrofuran in the ratio 60:40 %v/v, respectively was used as diluents. The stress degraded samples and the solution stability samples were analyzed using a PDA detector covering the range of 200-400nm.

Analytical method validation:

After satisfactory development of method it was subjected to method validation as per ICH guideline ^[30]. The method was validated to demonstrate that it is suitable for its intended purpose by the standard procedure. Analytical method validation was carried out by means of system suitability, specificity, accuracy, precision, linearity, LOD, LOQ, robustness and solution stability.

System suitability:

System suitability parameters were measured so as to verify the system, method and column performance. The % RSD (relative standard deviation) of BD and CALCI were count of five replicate injections (standard preparation) was below 0.20%. Low values of % RSD of replicate injections indicate that the system is precise. Results of other system suitability parameters such as theoretical plates, tailing factor are presented in (Table 1).

Table 1: System suitability results (precision, intermediate precision and robustness)

Parameter	Theoretical plates For BD (NLT 10000)	Tailing factor for BD (NMT 2.0)	% RSD* of Standard Area of BD (NMT 2.0)	Theoretical plates For CALCI (NLT 10000)	Tailing factor for CALCI (NMT 2.0)	% RSD* of Standard Area of CALCI (NMT 2.0)
Precision	18000	1.0	0.3	53660	1.0	0.5
Intermediate Precision	17919	1.0	0.2	56270	1.0	0.4
At 1.4 mL/min flow rate	16780	1.0	0.8	59409	1.0	0.8
At 1.0 mL/min flow rate	19034	1.0	0.1	54754	1.0	0.2
At 30°C column temp.	18219	1.0	1.1	58603	1.0	1.2
At 20°C column temp.	17148	1.0	0.4	56553	1.0	0.3
Mobile phase A n-Hexane +2%	17651	1.0	0.1	57449	1.0	0.1
Mobile phase A n-Hexane -2%	20709	1.0	0.2	67771	1.0	0.9
Mobile Phase-B IPA +2%	17734	1.0	1.0	55589	1.0	0.4
Mobile Phase-B IPA -2%	18118	1.0	0.9	66446	1.0	0.6

* Determined on five values. IPA Isopropylalcohol

Specificity:

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities [30]. Forced degradation studies were performed to demonstrate selectivity and stability indicating capability of the proposed NP-HPLC method. Figure 2 shows that there is no interferences at the RT (retention time) of BD and CALCI due to blank, placebo, impurities and degradation products. Significant degradation for CALCI was observed when Sample was subjected to treat with hydrogen peroxide oxidation conditions (3% H₂O₂, 70°C, 10 min). Significant degradation for BD was observed when the drug product was subjected to acid hydrolysis (0.01N HCl, RT, 5 min) and base hydrolysis (0.005N NaOH, RT, 5 min). Also significant degradation for CALCI was observed when the drug product was subjected to photolytic exposed (1.2 million lux hours, 200wh/m² UV light) and thermal exposed (60°C, 2hr). Degradation chromatograms Peroxide, acid hydrolysis, base hydrolysis, photolytic degradation and thermal degradation study are presented in (Figure 3). Peak due to BD and CALCI were investigated for spectral purity in the chromatogram of all exposed samples and found spectrally pure. The purity and assay of BD and CALCI were unaffected by the presence of its impurities and degradation products and thus confirms the stability-indicating power of the developed method. Results from forced degradation study are given in (Table 2).

Table 2: Data of forced degradation study for Betamethasone Dipropionate and Calcipotriene

Stress Conditions	Betamethasone Dipropionate				Calcipotriene			
	PA	PTH	%Deg.	Purity Flag	PA	PTH	%Deg.	Purity Flag
Acidic hydrolysis (0.01N HCl at RT, 5min)	0.116	1.139	5.3%	No	0.369	1.422	4.3%	No
Alkaline hydrolysis (0.005N NaOH at RT, 5min)	0.074	1.067	23.0%	No	0.312	1.293	ND	No
Oxidation (3% H ₂ O ₂ at 70°C, 10min)	0.042	1.051	2.0%	No	0.327	1.346	17.6%	No
Thermal (At 60 °C, 2 hr)	0.044	1.051	2.0%	No	0.408	1.451	9.0%	No
Photolytic (1.2 million lux hr visible light and 200 wh/m ² UV light)	0.044	1.051	2.2%	No	0.503	1.486	3.9%	No

ND No degradation RT Room temperature PA Purity angle PTH Purity Threshold Deg. Degradation

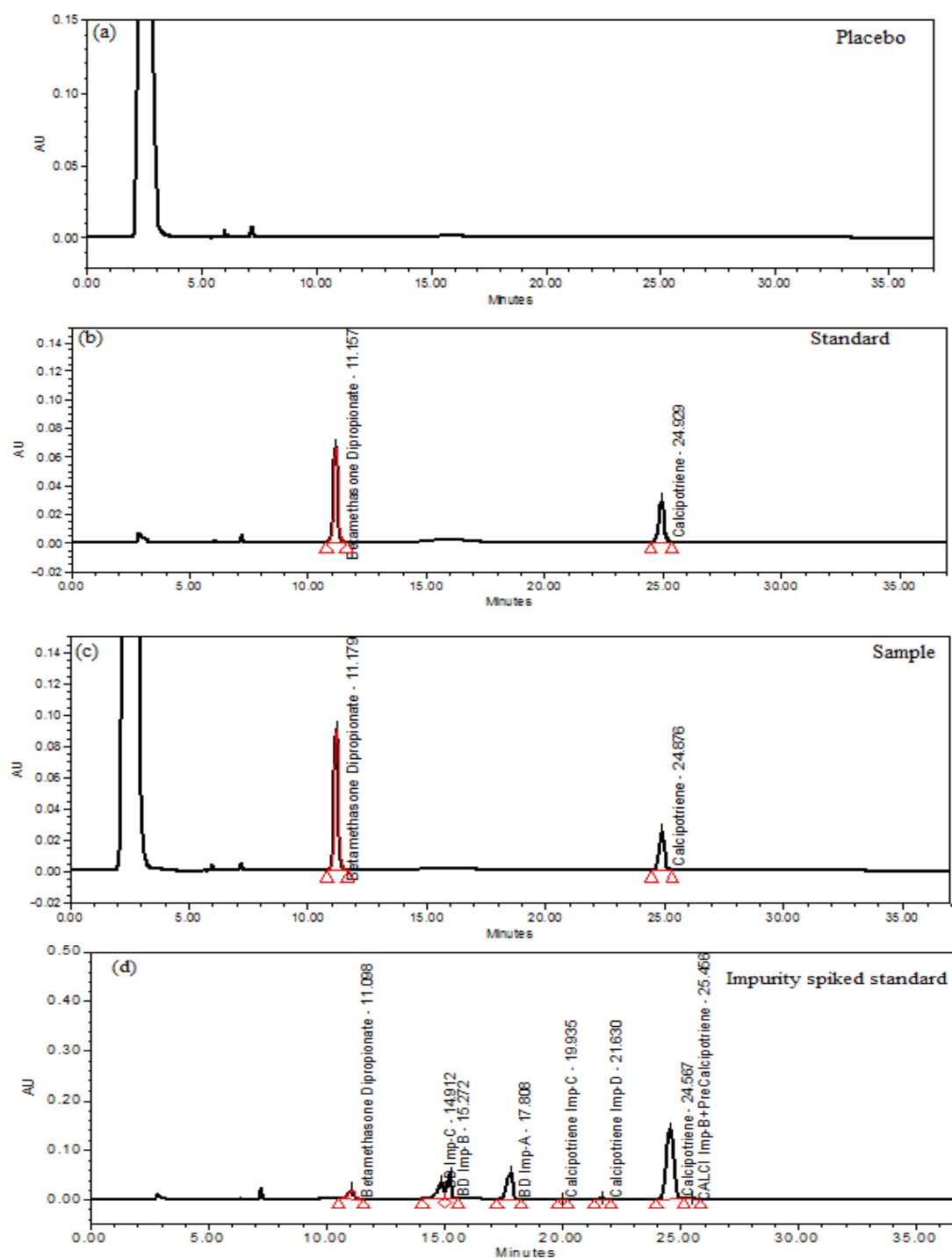


Fig. 2. Typical chromatogram of (a) placebo, (b) standard (c) sample and (d) impurity spiked standard

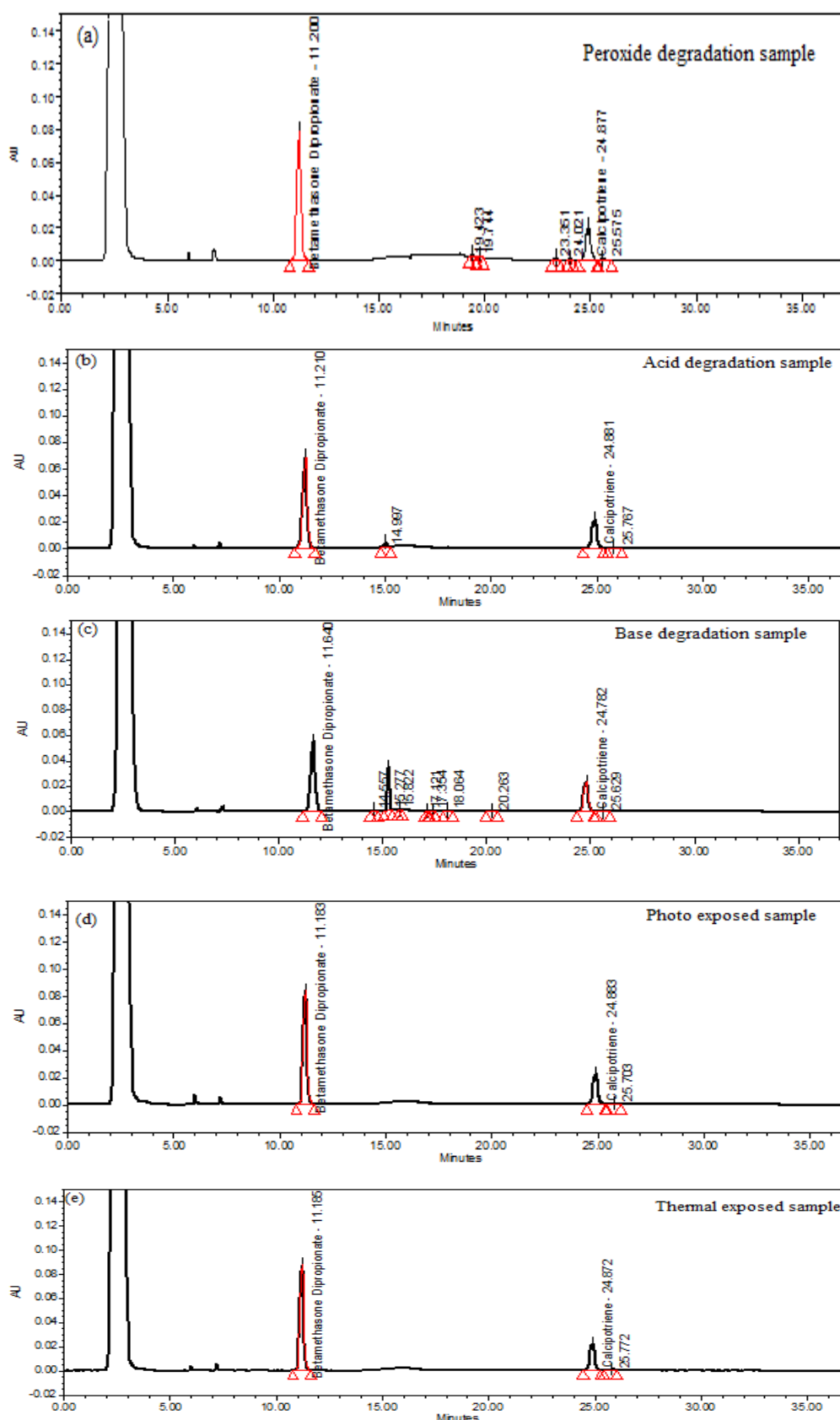


Fig. 3. A typical chromatogram of (a) peroxide oxidation sample, (b) acid hydrolysis sample, (c) base hydrolysis sample, (d) photolytic light exposed sample and (e) thermal exposed sample

Method Precision:

The precision of the assay method was verified by repeatability and by intermediate precision. Precision was investigated using sample preparation procedure for six real samples of ointment samples and analyzing by proposed

method. The average % assay (n=6) of BD and CALCI were 99.8% and 99.1% respectively with RSD of less than 1.0%. Intermediate precision was studied using different column, and performing the analysis on different day. Results are presented in (Table 3) along with intermediate precision data. Low values of % RSD, indicates that the method is precise.

Table 3: Method precision results

Peaks	Precision (Day-1)		Intermediate precision (Day-2)	
	% Assay [#]	% RSD*	% Assay [#]	% RSD*
Betamethasone Dipropionate	99.8	0.60	100.0	1.00
Calcipotriene	99.1	0.61	98.9	0.97

Average of six determinations. * Determined on six values

Accuracy:

To confirm the accuracy of the proposed method, recovery experiments were carried out by standard addition technique. Three different levels (50 %, 100 % and 150 %) of standards were added to pre-analyzed placebo samples in triplicate. The percentage recoveries of BD and CALCI at each level and each replicate were determined. The mean of percentage recoveries (n =3) and the % RSD was calculated. The amount recovered was within ± 1 % of amount added, which indicates that the method is accurate and also there is no interference due to excipients present in topical formulation. The results of recoveries for assay are shown in (Table 4).

Table 4: Accuracy results

Spiked level	Betamethasone Dipropionate	Calcipotriene
50% % Recovery [#]	100.0	100.0
% R.S.D.*	0.62	0.0
100% % Recovery [#]	99.8	100.3
% R.S.D.*	0.48	1.52
150% % Recovery [#]	99.3	100.9
% R.S.D.*	0.54	0.34

* Determined on three values.

Mean of three determinations.

Limit of detection (LOD) and quantification (LOQ):

The LOD and LOQ were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations. The limit of detection and limit of quantification values of BD and CALCI are reported in (table 5).

Linearity:

Linearity was demonstrated from LOQ % to 150 % of standard concentration using minimum six calibration levels of test concentration (LOQ-32.9 $\mu\text{g/mL}$ for BD and LOQ-0.017 $\mu\text{g/mL}$ for CALCI), which gave us a good confidence on analytical method with respect to linear range. The response was found linear for all BD and CALCI from LOQ to 150 % of standard concentration and correlation coefficient was also found greater than 0.999. Bias was also found within ± 0.03 . The result of Correlation coefficients, Y-intercept of the calibration curve and % bias at 100% response for BD and CALCI are presented in (Table 5).

Table 5: Evaluation of LOD, LOQ and linearity data

Parameter	Betamethasone Dipropionate	Calcipotriene
LOD ($\mu\text{g/mL}$)	0.056	0.005
LOQ ($\mu\text{g/mL}$)	1.171	0.017
Linearity range ($\mu\text{g/mL}$)	1.17-32.9	0.017-3.1
Correlation coefficient	0.9999	0.9999
Intercept (a)	196.102	-77.706
Slope (b)	40992.207	204034.986
Bias at 100% response	0.022	-0.018

Robustness:

The robustness as a measure of method capacity to remain unaffected by small, but deliberate changes in chromatographic conditions was studied by testing influence of small changes in flow rate ($\pm 0.2\text{mL/min}$), change in column oven temperature ($\pm 5^\circ\text{C}$), change in mobile phase-A composition ($\pm 2\%$ of n-Hexane and Tetrahydrofuran composition) and change in mobile phase-B composition ($\pm 2\%$ of n-Hexane and Isopropylalcohol composition). No significant effect was observed on system suitability parameters such as theoretical plates, tailing factor and % RSD of BD and CALCI, when small but deliberate changes were made to chromatographic conditions. The results are presented in (Table 1) along with system suitability parameters of precision and intermediate precision study. Thus, the method was found to be robust with respect to variability in above conditions.

Stability of analytical solutions:

Stability of sample solution was established by storage of sample solution at ambient temperature for 24 hours. BD and CALCI sample solution was re-analyzed after 12 and 24 hours time intervals and assay was determined and compared against fresh sample. Sample solution did not show any appreciable change in assay value when stored at ambient temperature up to 24 hours, which are presented in (Table 6). The results from solution stability experiments confirmed that sample solution was stable for up to 24 h during assay determination.

Table 6: Solution stability results

	Initial	After 12 hrs.	After 24 hrs.
Betamethasone Dipropionate	99.8	100.1	100.6
Calcipotriene	99.1	99.5	99.7

CONCLUSION

A gradient NP-HPLC method was successfully developed for the determination of BD and CALCI in topical dosage form. The method validation results have proved that the method is selective, precise, accurate, linear, robust, filter compatible and stability indicating. The run time (37.0 min) enables for rapid determination of drug. Moreover, it may be applied for determination of BD and CALCI in the study of content uniformity, tube homogeneity and in-vitro release test profiling of BD and CALCI topical dosage forms, where sample load is higher and high throughput is essential for faster delivery of results.

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