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Research article

Qualitative and quantitative assessment of related substances in the clobetasol propionate in topical cream dosage form by RP-HPLC

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ABSTRACT

The identification and control of impurities in drug substance and drug product is considered as very critical factor by regulatory agency for safe and effective use of Drug product for its intended use. A specific and sensitive Reverse Phase High Performance Liquid Chromatography (RP-HPLC) method is developed and validated for the estimation of 11 specified impurities in Topical Clobetasol Propionate Cream. The chromatographic parameters for develop method are C18 column, proportion of Water: Methanol: Acetonitrile: Trifluoro acetic acid as mobile phase at flow rate of 1.2 ml/min and UV detection at 241 nm. The method is validated for parameters as specified in ICH guideline. Developed method is validated for range 0.05–0.75 µg/mL for which Linearity, accuracy and precision was proven with acceptable limits. Method is sensitive as established Limit of Detection (LOD), and Limit of Quantitation (LOQ) are 0.02% and 0.06% respectively.

Keywords: Clobetasol propionate, RP-HPLC, Topical dosage form, Mobile phase, Stationary phase.

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INTRODUCTION

Chronic and painful characteristic with incurable nature of disease makes psoriasis as one of the most sever disease effecting patient quality of life negatively. [1]. Psoriasis is serious global problem due to rate of disease ranges from 0.09 to 11.4% globally. In age group of 50-60 Years [2]. Other factors associated with psoriasis such as social discrimination has significant impact on mental health of the patient. [3].

Psoriasis involves immune stimulation of epidermal keratinocytes but largely cause remains unclear. Other factor that contribute as probable cause are family history, smoking and stress. Commonly some genes and human leukocyte antigens (Cw6, B13, B17) are associated with psoriasis. An environmental trigger is thought to evoke an inflammatory response and subsequent hyper proliferation of keratinocytes. [4,5]. Psoriasis treatment mainly involve three categories of treatment like – topical therapy (e.g., Steroid, Vitamins etc.), Phototherapy (UV light) and Systemic Medication therapy (e.g., methotrexate, cyclosporine, immunomodulatory agents etc.). [6-9]. Topical Therapy is mainly used for Anti-Inflammatory effect and mainly divided in to 5 major categories. The clobetasol propionate cream is one of the most commonly used tropical therapies for treatment of Psoriasis. Clobetasol propionate is the 17-O-propionate ester of clobetasol. Clobetasol Propionate mechanism of action involves initiation of phospholipase A2 inhibitory proteins that controls release of the inflammatory precursor arachidonic acid. [10].

Clobetasol propionate belongs to class of topical glucocortico steroids used for treatment of psoriasis. It is available in crystalline form as white to off white powder. Solubility profile indicates that clobetasol propionate is practically insoluble in water, freely soluble in acetone and in dichloromethane, and sparingly soluble in ethanol. [11]. Various dosage forms such as cream, gel, lotion, spray is availed in market for Clobetasol propionate [12]. The drug substance Clobetasol propionate is official in IP (Indian pharmacopeia), USP (United States pharmacopeia), EP (European pharmacopeia) and BP (British Pharmacopeia). There is total 13 prominent related substances where identified which have possibility to be present in drug substance and formulation. As per the EP monograph the 7 related substances specified namely: A, B, C, D, E, L, M. The Related substances L and M having the unknown structure hence no reference standard was available. [13,14]. Further There are 6 specified and identified detectable related substances namely: F, G, H, I, J, K, which have possibility to be present in drug substance depends on the synthesis and formulation technique used. They are limited by the general acceptance criterion for other/unspecified

impurities and/or by the general monograph substances for pharmaceutical use. It is therefore not necessary to identify these impurities for demonstration of compliance. [15].

As per the ICH guideline and FDA guidance for industry all drug substances and drug products are subject to control of impurities. A threshold-based approach described in the ICH Q3A and Q3B guidelines may be used for the control of impurities in drug substances or drug products generated during the manufacturing process or storage period. The precise control of impurities assures the efficacy and stability of the drug product for its intended purpose. Further the any drug substances and drug products to be marketed in the United States must incorporate the precise analytical procedure with scientifically decided limits for achieving approval by the FDA either via New Drug Applications (NDAs) or Abbreviated New Drug Applications (ANDAs) or through the FDA over-the-counter (OTC) monograph system. [16-18].

The most commonly used methods for the qualitative and quantitative determination of related substances in any pharmaceutical dosage forms are HPLC, UPLC and LCMS. During the literature search it was observed that few analytical methods have been developed for the estimation of clobetasol propionate drug substance alone and combination by Thin Layer Chromatography (TLC), Densitometry, HPLC LC-MS UV Spectroscopy and High-performance Thin Layer Chromatography (HPTLC). [19].

Based on literature search it was observed that analytical method was developed for quantification of related substance in ketoconazole and Clobetasol Propionate Cream by HPLC. [20]. This method separates only impurity-C and impurity-J with a very high LOQ of 0.5%.

Recently an analytical method for Identification and estimation of related substances in tropical preparation of Clobetasol propionate and vitamin D3 analogue successfully determined five related substances of clobetasol propionate by using rapid Ultra high-performance liquid chromatography (UHPLC). This method complies the acceptance criteria for the regulatory submission data for the potential and most commonly observed impurities of Clobetasol propionate [21].

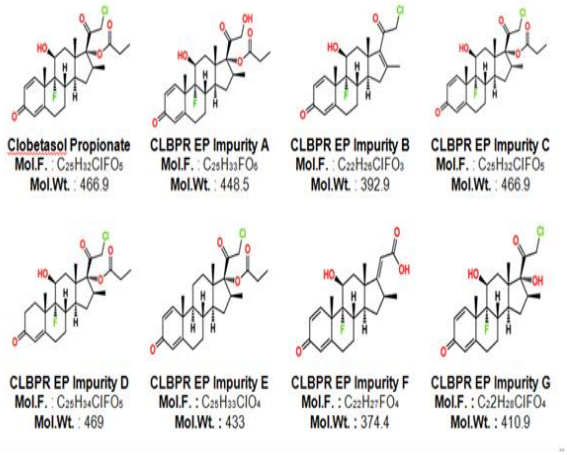
Thus, it was potential requirement to develop RP-HPLC method for the estimation of all related substances specified in European Pharmacopeias (EP) for clobetasol propionate in Tropical dosage form. Method was developed and validated as per ICH Q2 (R1) guideline. Validation of analytical method provide sufficient supportive data that method is Specific, Sensitive, Linear, Precise and accurate for its intended use [22].

MATERIAL AND METHOD

Chemicals and reagents

HPLC grade methanol, acetonitrile, trifluoracetic acid were procured from Merck, Mumbai, India. Samples of Clobetasol propionate (CLBPR) and all its impurities were received as gift sample from SynZeal Research Pvt Ltd. Ahmedabad, Gujrat, India. HPLC grade water was obtained using Milli-Q water purification.

Figure 1: Structures of impurities of Clobetasol propionate. (Received from SynZeal Research Pvt. Ltd. Ahmedabad, Gujarat, India)



Instrumentation and software

Method development and validation studies were performed on Waters Alliance e2695 HPLC system equipped with 2489 UV/visible detector with dual wavelength and 2998 Photodiode Array (PDA) Detector with auto sampler and auto injector unit with sample loop volume of 100µL. The chromatographic data were acquired, monitored, and processed using Empower 3 software from Waters Corporation.

Chromatographic conditions

The stationary phase, Zorbax SB-C18, 250x4.6mm column with 5µ µm partial size was used in the analytical method development and validation activity. The column oven temperature and sample cooler temperature is set at 50°C and 25°C respectively. The injection volume of sample and standard was 100 µl. The mobile phase composed of water, acetonitrile and methanol in ratio of 80:15:5% v/v and add 2mL of trifluoroacetic acid in line A while water, acetonitrile and methanol in ratio of 15:80:5% v/v and add 2mL of trifluoroacetic acid in line B. The flow rate of the mobile phase is set at 1.2 ml minute. The gradient program time is set as per below

Time (Mins)	Mobile Phase-A	Mobile Phase-B
0	80	20
15	65	35
53	35	65
58	5	95
80	5	95
82	80	20
90	80	20

Water: Acetonitrile in proportion of 65: 35 %v/v is used as a diluent. Desired Extraction is achieved using heating, sonication, centrifugation and filtration technique. Detection wavelength is 240 nm.

Standard for related substances analysis (0.375ppm)

Weight and transfer about 20 mg of Clobetasol propionate

into 200ml volumetric flask. Add about 150 ml of Acetonitrile and sonicate for 5 minutes to dissolve allow solution to attain room temperature, make volume up to mark with Acetonitrile and mix well. (Stock I). Pipette out and transfer 3ml of above solution (Stock-I) into 200mL volumetric flask, and made volume up to mark with Acetonitrile and mix well. (Stock II), Further pipette out and transfer 5ml of above solution (Stock-II) into 20mL volumetric flask, and made volume up to mark with diluent and mix well.

Extraction procedure for topical formulations samples

Weight and transfer about 3.0 gm of sample into 20ml volumetric flask. Add about 5 ml of acetonitrile, heat at 60°C for 5 minutes in water bath. Vortex the solution for 2minutes and sonicate for 35 minutes with intermittent shaking. Allow solution to attain room temperature, make volume up to mark with diluent and Vortex the solution for 5minutes.Transfer the solution into 50 ml tarson tube and centrifuge the solution at 10,000 RPM for 20 minutes. Filtered the solution from 0.2µ nylon syringe filter and collect the filtrate in HPLC vial after discarding initial 2 ml of filtrate. Inject clear solution in HPLC. Prepared the placebo same as the sample.

METHOD DEVELOPMENT

HPLC has contributed many successes in product development and in quality control for the pharmaceutical industry. The UV detector coupling with HPLC equipment is the most important analytical instrument for preformulation, QC/QA and in-process control in pharmaceutical analysis. HPLC is a rapid, reliable and most widely used analytical tool for related substances evaluation study because of the high-resolution capacity, accuracy, and reproducibility of the equipment. So due to large avaibility and practicality for QC testing the method development for of impurities HPLC instrument was selected.

Selection of stationary phase

Based on several development trials and molecular weight of Clobetasol propionate and its impurities suggests that C_{18} column is optimum for the separation of impurities and Clobetasol propionate. Further extensive trials carried out to optimize the ideal impurities separation on Mobile phase, diluents, temperature and gradient program.

Selection of wavelength

Based upon UV absorption spectra of target compounds and literature survey 240 nm was selected as detection wavelength due to good absorption at this wavelength by all component.

Selection of mobile phase

As the effort is to develop an RS method for cream formulation which involves a high lipid and polymer containing sample matrix the first trial was carried out on gradient combination of water, Acetonitrile and methanol and Formic acid and acetic acid to achieve acidified water portion, mobile phase pH/gradient ratio has significant impact on separation related substance from placebo peaks. When formic acid (0.1%, v/v) and glacial acetic acid (0.1%, v/v) is used as a mobile phase, the separation of component is poor. When and acidified mobile phase with TFA is used optimum separation of all component is observed. Thus, TFA is selected as critical component in Mobile phase. Further Extensive trials were carried out for ideal gradient selection to resolve impurities peaks by keeping acidified water as mobile phase A and acetonitrile as mobile phase B. The use of acetonitrile observed good separation but the closely eluting impurities peaks were observed hence trials were taken by adding methanol in small ratio for two reasons first to achieve the better impurities resolution and to achieve the better impurities peak shapes. The extensive gradient elution program trials were evaluated for achieve the optimum impurities separation profile and finally following mobile phase was finalized.

Mobile Phase A

Water, acetonitrile and methanol in ratio of 800:150:50 and add 2mL of TFA.

Mobile Phase B

Water, acetonitrile and methanol in ratio of 150:800:50 and add 2mL of TFA.

Selection of standard and sample concentration

As per the label claim of clobetsol propionate in cream formulation (0.05%) and limit of clobetasol propionate as per ICH Q3B, impurities in new drug products limit (0.5%) the concentration of Clobetasol propionate in standard prep. was kept 0.375ppm which is 0.5% of sample concentration (75 ppm).

Selection of specification limits and concentration of Known impurities

As per ICH Q3B, impurities in new drug products limit for topical formulation known impurities is (0.5%) the concentration of was known impurities kept 0.5 ppm which is 0.1% of sample concentration (75 ppm).

Selection and rationale for the sample preparation conditions

Some additional precautionary sample preparation and extraction trials were carried out and finalized following steps with rationale as below.

Sample preparation Step	Rationale
3 times cleaning of glassware with Acetonitrile	To prevent glassware contamination.
Maintaining so nicator temperature below 25°C.	To prevent generation of any unknown impurity.
Sonication for 35 minutes.	To ensure complete extraction impurities from cream base.
Centrifugation at 10,000 RPM for 20 minutes.	To separate clear supernant from cream base to avoid contamination and column care.
Filtration of sample trough 0.22µ Nylon filter	Column care.

Selection of diluent

Significant study has been done for selection of appropriate diluent. Nature of formulation and excipients involve clearly suggest that simple sample preparation technique will not result in to proper extraction of impurities from drug product. Hence, sample extraction technique passes significant role in achieving desired extraction of

impurities from topical dosage form. Based on the different experiments, Acetonitrile is selected as the dispersion solvent and mixture of Acetonitrile and water in the proportion of 65:35 %v/v is selected as a diluent.

Selection of sample extraction procedure

Based different experimental studies sample extraction procedures is finalized as follows, Dispersed the sample matrix in 5 ml of Acetonitrile and heat at 60°C for 5 minutes in water bath this leads most of the excipients matrix melt down into Acetonitrile then vortex and sonicate the solution for 30-35minutes at 25°C water which leads to complete breakdown of cream excipients base, allowed to attain the room temperature and made the volume up to mark with diluents so drug and its impurities goes into diluent. Centrifuged the solution 20 minutes at 10,000 rpm to separate out any excipients residue and filtered from 0.2µ nylon syringe filter and collect the filtrate in HPLC vial after discarding initial 2 ml of filtrate.

Selection of column temperature

Corticosteroids show temperature-dependent separation. Clobetasol propionate also showed temperature dependent separation. Based on experimental studies 50°C column temperature is finalized due to superior peaks separation.

Selection of flow rate

Based on experimental studies flow rate of 1.2 ml/minute is finalized as the flow rate of the method.

Method validation

The method is validated in accordance with ICH Q2 (R1) guideline.

System Precision, System suitability

The system precision was determined by making five replicate injections from a freshly prepared diluted standard solution of (CLB 0.375 ppm) and analyzing for peak area. Further, the single injection of system suitability solution was injected and the resolution between the peaks of Clobetasol propionate and Clobetasol propionate related compound A was determined.

The % RSD of area response of CLBPR peak obtained from replicate standard injections of standard preparation should not be more than 5.0%. The resolution between Clobetasol propionate and Clobetasol rel. compound A should not be less than 1.5 in system suitability solution. The results are summarized in Table 1.

Specificity

This is to be demonstrated by injecting blank, placebo, standard preparation, sample preparation, spiked placebo preparation. The impurities and CLBPR retention time (RT), relative retention time (RRT), and resolution, as is shown in Table 2.0. The purity angle was within the purity threshold limit obtained for the impurities; this confirmed the analyte peak homogeneity. Based on validation studies performed data suggest that method is specific as impurities are separated from each other and there is no interference.

Linearity

As the limit level for each specified were considered as 0.5% level, hence all impurities mixture prepared at limit concentration for linearity determination. The Impurities and CLBPR standard solutions for linearity were prepared at 0.1 to 7.5 µg/ml (0.1% to 150%) of the limit concentration level. The series of linearity solutions for impurities were prepared from working standard solutions at five different concentrations between range of 0.1-0.75 µg/ml. Linearity was established by based on calibration curve. The results are summarized in Table 3.

Limit of detection and limit quantification

The Limit of detection and Limit of quantification were established by residual standard deviation method and by visual evaluation method. The LOD and LOQ are expressed as LOD = 3.3 σ/S and LOQ = 10 σ/S, respectively, where σ = the standard deviation of the response and S=the slope of the calibration curve. The slope S was estimated from the calibration curve of the respective impurity. The estimate of σ was carried out by the residual standard deviation method. The LOD and LOQ estimation by the visual method was done by injecting the diluted working standard solution. The precision was carried out at LOQ level with six injections (n = 6).

Accuracy / Recovery

The accuracy was established by spiking known amounts of impurities in the placebo matrix at three different levels, 50%, 100%, and 150% of specification. The recovery of each impurity was calculated using diluted standard area of Clobetasol propionate and relative response factor of each impurity. The results are summarized in Table 3.

Precision

The precision of the proposed method was assessed as repeatability (intra-day precision) and intermediate precision (inter-day precision), both expressed as %RSD of % impurity found.

The method precision was evaluated by preparing six replicates of the test sample spiked with each impurity at limit level (Spiked sample preparation) from mixed impurity standard stock solution and analyzing each sample for determination of related substances of each impurity. The RSD of % impurities results of known and unknown impurity each at limit specification concentration should NMT 10.0%.

For intermediate precision six spiked sample preparations at analyzed on different day, different instrument, and using different column. The %Relative standard deviation % of each impurity obtained from six replicates (of intermediate precision) and cumulative RSD of twelve determinations (Six samples from Intermediate and six samples from Method precision) should be NMT 10.0%.

Stability of solutions

The stability of standard solution and spiked sample

solution was evaluated by storing the solutions at 25°C in amber colored volumetric flasks. The solutions were analyzed on different time intervals to evaluate the stability of analytes. The results are summarized in Table 3.

RESULT AND DISCUSSION

Table: 1: System Precision, System suitability

System suitability parameter	Observation	Limit
The resolution between the peaks of Clobetasol propionate and Clobetasol propionate related compound A.	2.5	Not less than 2.0
The % RSD of the area of Clobetasol propionate peak in 5 replicate standard injections.	0.8	Not more than 2.0

Specificity

All impurity peaks were well separated in the spiked sample of API and formulation with a resolution more than 2. In the placebo chromatogram, no coeluting peaks were observed at the RTs of CLBPR and known impurities. Placebo peaks were well separated from the impurity peaks.

Table 2: RT and RRT of Impurities

Name of Impurity	Retention Time (minutes)	Relative retention time
Imp. K	16.11	0.431
Imp. F	18.41	0.492
Imp. A	21.68	0.579
Imp. B	22.58	0.603
Imp. I	24.89	0.665
Imp. G	30.93	0.827
Imp. H	33.26	0.889
Imp. C	34.47	0.921
CLBPR	37.42	1.000
Imp. J	39.50	1.056
Imp. D	41.30	1.104
Imp. E	50.46	1.348

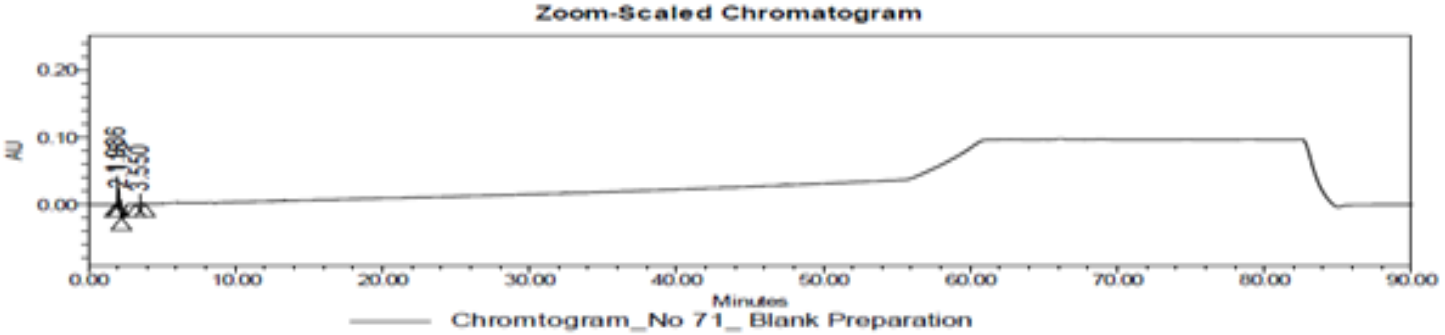
Table 3: Results of LOD, LOQ, precision, linearity, RRF, solution stability, and accuracy

Name	Method precision (% RSD)	Inter-mediate precision (% RSD)	Linearity (µg/ml)	Correlation coefficient (r^2)	RRF	S/N ratio (LOD)	S/N ratio (LOQ)
Imp. A	4.8	3.2	0.047 - 0.743	0.994	1.000	8.3	21.5
Imp. B	3.0	1.5	0.051 - 0.751	0.995	1.480	7.2	35.4
Imp. C	2.8	5.8	0.043 - 0.743	0.989	1.394	5.8	40.7
Imp. D	4.1	2.8	0.045 - 0.744	0.996	1.429	8.6	31.5
Imp. E	5.6	6.2	0.052 - 0.750	0.998	2.605	5.8	25.8
Imp. F	8.2	4.1	0.047 - 0.721	0.990	1.610	6.5	18.7
Imp. G	6.5	3.7	0.051 - 0.752	0.997	1.325	7.8	22.5
Imp. H	7.8	4.2	0.050 - 0.750	0.986	1.092	11.2	31.5
Imp. I	4.2	4.7	0.048 - 0.748	0.980	1.505	9.8	29.7
Imp. J	5.9	1.7	0.044 - 0.744	0.992	1.040	10.8	30.1
Imp. K	3.7	6.5	0.046 - 0.745	0.989	1.263	12.2	45.2
Limit	**NMT 10.0%**	**NMT 10.0%**	**NA**	**NLT 0.980**	**NA**	NA	NLT10.0

Table 4: Results of solution stability and accuracy

Name	RT (Minutes)	Precision at LOQ (%RSD)	Accuracy at LOQ (%)	Accuracy at 50% level (%)	Accuracy at 100 % level (%)	Accuracy at 150 % level (%)	Solution stability (%Difference from initial)
Imp. A	21.68	3.4	90.4	84.6	92.7	94.9	-1.8
Imp. B	22.58	4.7	88.3	85.2	88.8	91.1	2.1
Imp. C	34.47	2.1	91.7	84.9	96.5	95.2	3.5
Imp. D	41.30	3.4	89.5	98.1	96.1	94.6	2.7
Imp. E	50.46	2.7	88.7	83.8	90.4	94.3	1.5
Imp. F	18.41	2.5	98.4	106.2	99.7	93.7	1.7
Imp. G	30.93	3.2	93.5	115.9	102.2	101.4	2.6
Imp. H	33.26	2.4	91.4	92.8	98.4	96.6	1.5
Imp. I	24.89	2.7	95.1	112.1	109.7	100.8	-1.8
Imp. J	39.50	3.5	95.8	97.5	92.2	91.6	1.2
Imp. K	16.11	4.1	98.7	97.4	91.1	89.2	-1.7

Figure 2: Chromatogram of Blank Preparation



	Name	RT	Area (µV*sec)
1	Blank-1	1.94	147257
2	Blank-1	2.12	67587
3	Blank-1	3.55	17812
Sum			232656

Figure 3: Chromatogram of Blank Preparation

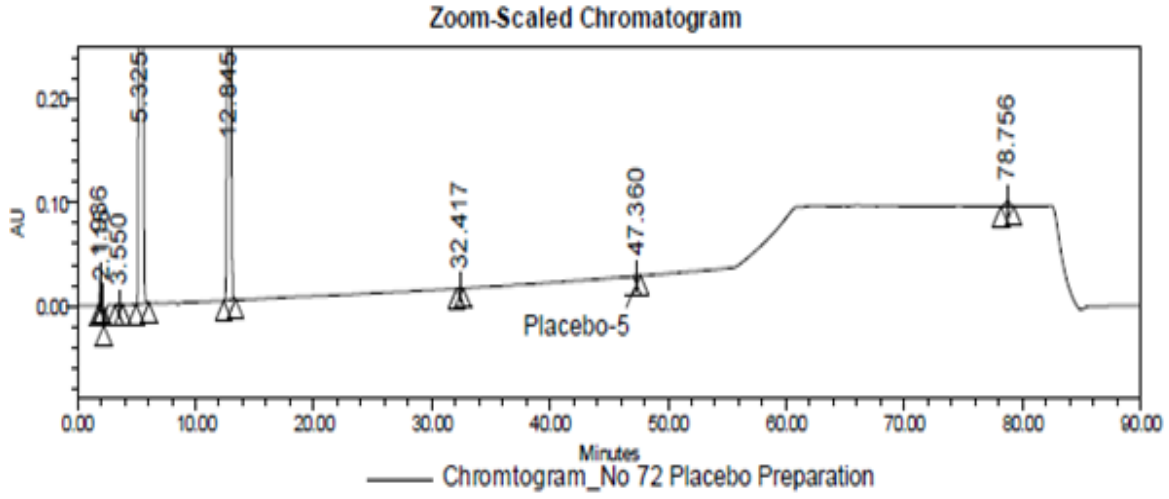
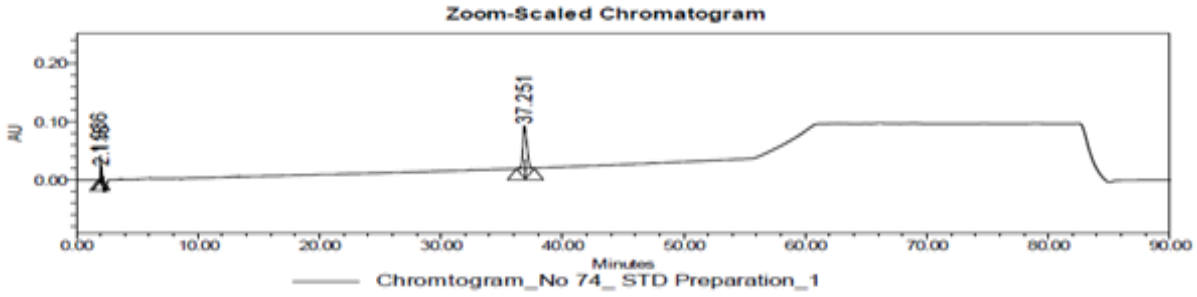
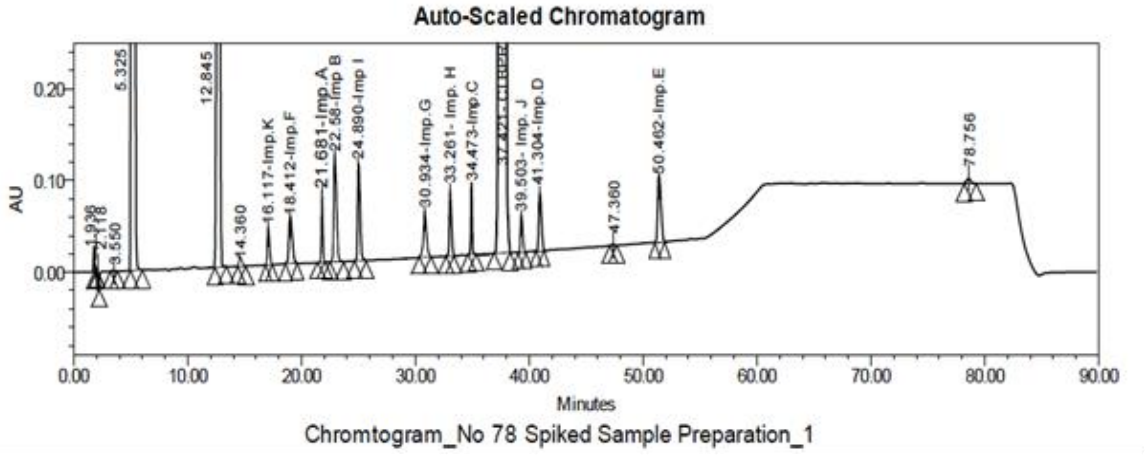


Figure 4: Chromatogram of Placebo Preparation



	Name	RT	Area (µV*sec)
1	Blank-1	1.94	137568
2	Blank-1	2.12	66842
3	CLBPR	37.251	68026
Sum			272436

Figure 5: Chromatogram of standard Preparation



DISCUSSION

Aim of a present work is to develop a Specific, sensitive, precise and accurate RP-HPLC method for its intended use.

Specificity is established as there is good separation between all impurities without any interference. Range is established for concentration level 0.05–0.75 µg/mL. The LOD values around 0.02% and LOQ around 0.06%. Method is precise as %RSD is less than 5%. Accuracy is established between 50-150%.

This is the first reported RP-HPLC method for the determination of total 11 specified related substances as per European pharmacopeia of clobetasol propionate in its cream dosage form. The present study provides new information and supporting data for Qualitative and quantitative assessment of related substances in the Clobetasol Propionate in topical cream dosage form by RP-HPLC.

CONCLUSION

A simple and new stability indicating RP-HPLC method has been developed for the determination of related substances of Clobetasol propionate in cream dosage form. The method was validated (ICH guidelines) by linearity, precision, accuracy and precision and this method is highly helpful for the identification and quantification of impurities and related substances. The proposed method is specific and the system suitability parameters are within acceptable criteria.

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