A Simple RP-HPLC Method for the Simultaneous Quantitation of Chlorocresol, Mometasone Furoate, and Fusidic Acid in Creams

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Abstract

A simple, specific, and precise high-performance liquid chromatographic method is developed and validated for the simultaneous determination of chlorocresol (CC), mometasone furoate (MF), and fusidic acid (FA) in a cream formulation. The isocratic mobile phase consists of 1.5% w/v aqueous ammonium acetate buffer–acetonitrile, 55:45 (v/v) of pH 3.8. The column contains octylsilyl chemically bonded to porous silica particle (Symmetry C_8, 150 × 3.9 mm, 5 µm). The detection is carried out using variable wavelength UV–vis detector set at 240 nm. The solutions are chromatographed at a steady flow rate of 1.0 mL/min. The current method separates CC, MF, and FA in less than 8 min with good resolution and peak shapes, minimal tailing, and with retention factors between approximately 1 and 5. Linearity range and percent recoveries for CC, MF, and FA are 10–30, 10–30, and 200–600 µg/mL; and 100.31%, 100.38%, and 100.34%, respectively. The method is validated according to International Conference on Harmonization guidelines and proven to be suitable for stability testing, content uniformity testing, and quality control of these compounds in pharmaceutical preparations.

Introduction

Chlorocresol (CC) is a bactericide, closely related to carbolic acid. It is used as a preservative in many pharmaceutical formulations such as creams and ointments. Preservatives and preservative systems are crucial parts of any cream or ointment formulations which contain them. Other preservatives such as propylparaben and methylparaben have been used for years in cream and ointment formulations. CC is especially used as a preservative for steroid creams. The formulator must be fully aware of the normal procedure for preservative systems in a suitable product which need to be estimated to establish their effectiveness throughout the shelf-life of the product. Knowing the actual concentration of preservative(s) in different formulations is vital in establishing the shelf-life of a product. Apart from this, regulatory agencies often ask for the analytical test result of preservative(s) (1).

Mometasone furoate (MF) is a topical corticosteroid; it has anti-inflammatory, anti-pruritic, and vasoconstrictive properties. Corticosteroids act by the induction of phospholipase A2 inhibitory proteins, collectively called lipocortins. It is postulated that these proteins control the biosynthesis of potent mediators of inflammation such as prostaglandins and leukotrienes by inhibiting the release of their common precursor arachidonic acid (2,3).

For treatment of primary and secondary skin infections caused by sensitive strains of *S. aureus*, Streptococcus species, and *C. minutissimum*, fusidic acid (FA) is generally used. Secondary skin infections are those inflicted by wounds and burns (4–6).

United States Pharmacopoeia 2006 (USP 2006) has described a procedure for the assay of raw material and MF cream by high-performance liquid chromatography (HPLC) using a mixture of methanol and water (65:35 v/v) as mobile phase at a flow rate of 1.7 mL/min, and a stainless steel column (4.6 × 250 mm) containing L7 packing, with the detector wavelength set at 254 nm (7).

The determination of FA raw material, cream, and eye drops has also been described in the British Pharmacopoeia 2005 (BP 2005) by HPLC using a mixture of methanol, 0.05M orthophosphoric acid, and acetonitrile (10:40:50 v/v) as mobile phase at a flow rate of 2.5 mL/min, and a stainless steel column (4.0 × 125 mm) containing lichrospher 100 RP-18 packing, with the detector wavelength set at 235 nm (8). Both of the current procedures (USP, BP) were primarily applied to determine their suitability for use in the simultaneous and individual determination of CC, MF, FA, and 3-ketofusidic acid (FA impurity) using the appropriate column and mobile phase. But the use of the USP and BP mobile phases at flow rates of 1.7 and 2.5 mL/min resulted in high pressure (approximately 3500–4000 psi). FA eluted at approximately 40 min with a ghost peak by applying the method described under the USP monograph "Mometasone furoate cream". However, the BP method has further been tried for the simultaneous determination of CC, MF, and FA. This resulted in lesser elution times for MF and CC at 1.12 and 2.75 min, respectively. The literature survey shows that several methods like HPLC and spectrophotometry have been reported for the determination of MF and FA (9–15). A spectrophotometric method has also been reported for the individual determination of CC in dosage forms (16–19). These methods (USP, BP, and reported) are not suitable for stability analysis of MF or FA cream and ointments, due to the presence of CC as preservative. These methods may not be appropriate for the simultaneous

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determination of CC, MF, and FA together with impurity 3-ketofusidic acid because of interferences with each other. However, as per bibliographical revisions performed, no HPLC analytical method has been reported for the simultaneous determination of these three components in combination, together with 3-ketofusidic acid as impurity.

The present study was aimed at developing a simple, specific, accurate, and precise HPLC method for the simultaneous determination of CC, MF, and FA in commercially available and in-house prepared pharmaceutical formulations, for use in stability studies and quality control applications associated with these ingredients. Molecular structures of the separated compounds are shown in Figure 1. The method can also be used for the determination of the 3-ketofusidic acid impurity in combination with CC, MF, and FA.

The proposed HPLC method is rapid and uses an isocratic mobile phase with low flow rate 1.0 mL/min, instead of 1.7 and 2.5 mL/min as described in USP 2006 and BP 2005. The suitability of the analytical procedure is demonstrated by its stability-indicating ability and optimum chromatographic system suitability parameters used for the determination of these components.

Experimental

Instrumentation

Integrated HPLC systems LC-2010A$_{HT}$ from Shimadzu Corporation (Chromatographic and Spectrophotometric Division, Kyoto, Japan) consisted of a 4-liquid gradient system, high speed auto-sampler, column oven, and UV-vis detector. A Symmetry C_8, 150 × 3.9 mm, 5 µm, stainless steel analytical column from Waters (Milford, MA) was used as stationary phase. Chromatograms were recorded and integrated on a PC installed with Class-VP version 6.13 (Shimadzu) chromatographic software.

Reference substances, reagents, and chemicals

MF was obtained from S.C. Chemicals Ltd. China. FA was obtained from Ercros (Barcelona, Spain), and CC from BDH (Poole, England). Ammonium acetate and orthophosphoric acid, reagent-grade, were purchased from Panreac (Barcelona, Spain). Methanol and acetonitrile (HPLC-grade) were obtained from J.T Baker (Deventer, Holland). Distilled water was obtained from a Milli-Q system (Millipore; Milford, MA). Reference standard MF was obtained from United States Pharmacopoeia Convention (Rockville, MD), while 3-ketofusidic acid and FA reference standards were obtained from European Pharmacopoeia, Council of Europe (Strasbourg, France). The excipients cetostearyl alcohol, cetomacrogal-1000, white petroleum jelly, propylene glycol USP, liquid paraffin, and sodium dihydrogen phosphate were imported from various suppliers. Cream formulations containing CC as preservative, MF, and FA were developed and manufactured in our research and development laboratory and plant.

Chromatographic condition

The isocratic mobile phase consisted of a combination of 1.5% w/v aqueous ammonium acetate buffer and acetonitrile in the ratio 55:45 (v/v), pH 3.8, adjusted with orthophosphoric acid. The mobile phase was filtered and degassed through membrane filter of 0.45 µm porosity under vacuum. A Symmetry C_8, 150 × 3.9 mm, 5 µm, stainless steel analytical column was used as stationary phase. A constant flow rate of l.0 mL/min was employed throughout the analysis. Variable UV-vis detector was set at 240 nm. All pertinent analyses were made at ambient room temperature, and volume of solution injected onto the column was 20 µL.

Extraction solvent

Acetonitrile was found to be the best solvent to extract CC, MF, and FA from cream, because CC, MF, and FA are highly soluble in acetonitrile.

Samples

Test samples were creams prepared in-house with following composition: CC –0.1%, MF –0.1%, and FA –2.0%. Other test samples used were accelerated stability samples with similar composition. Samples were treated according to the test solution preparation.

Solution preparation

MF and CC standard stock solution

MF and CC standard stock solutions were prepared by transferring 50.0 mg of MF and CC reference standard to a 100-mL volumetric flask. A 70 mL portion of acetonitrile was added, sonicated to dissolve, and cooled to room temperature. The solution was diluted to volume with acetonitrile and mixed. MF and CC standard stock solutions were used to prepare standard solution.

Standard solution

An accurately weighed quantity of 40.0 mg of FA reference standard was transferred to a 100-mL volumetric flask and dissolved by adding 70 mL of acetonitrile. Next, a 4.0 mL portion of MF and CC standard stock solution was added and made up to volume with acetonitrile to obtain a solution containing 0.02 mg of CC, 0.02 mg

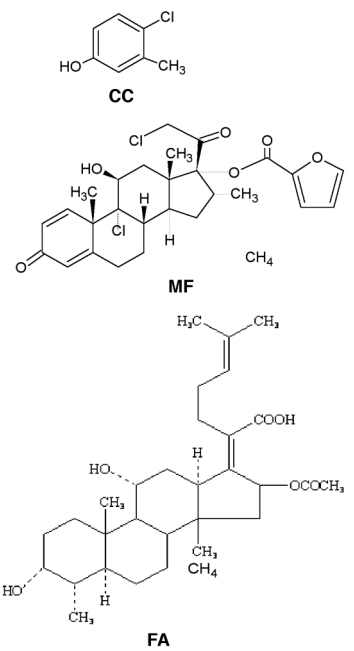

Figure 1. Molecular structures of compounds in this study.

of MF, and 0.4 mg of FA. The solution was mixed and filtered through a 0.45-µm membrane filter, and 20 µL was injected.

System suitability solution

System suitability solution was prepared by transferring a portion of 10 mg of CC, 10 mg of MF, and 5 mg of 3-ketofusidic acid into a 100-mL volumetric flask. The solution was diluted to volume with acetonitrile and mixed. Another 10 mg of FA and 10 mL of the previously mentioned solution were transferred into a 50-mL volumetric flask. The solution was diluted to volume with acetonitrile and mixed. The solution was filtered through a 0.45-µm membrane filter and 20 µL was injected.

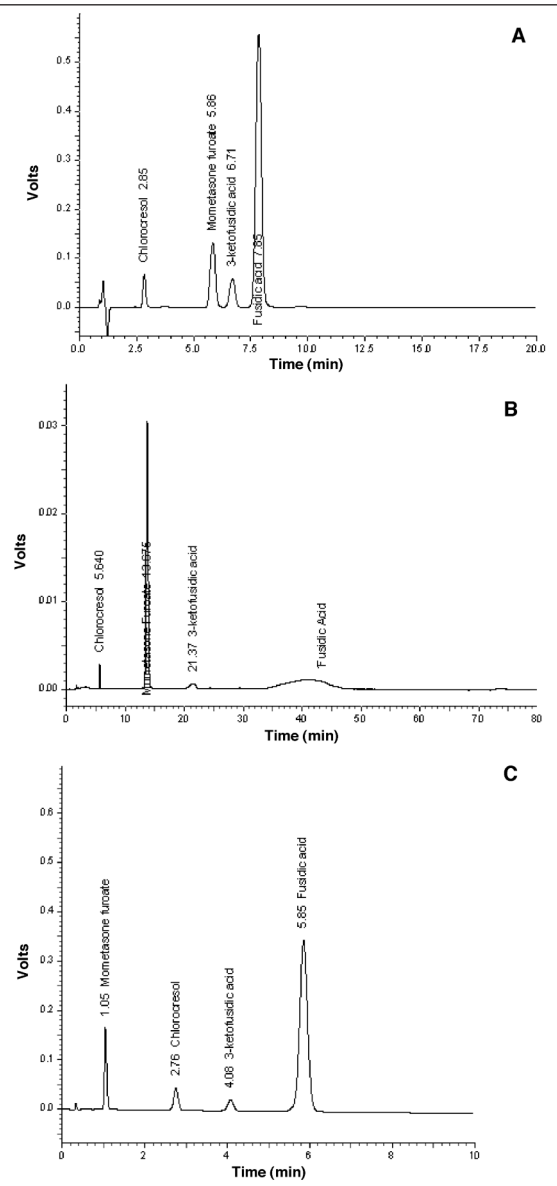

Figure 2. Chromatogram showing CC 0.02 mg/mL, MF 0.02 mg/mL, 3-ketofusidic acid impurity 0.02 mg/mL, and FA 0.4 mg/mL, under the proposed method (A). Chromatogram showing CC 0.02 mg/mL, MF 0.02 mg/mL, 3-ketofusidic acid impurity 0.02 mg/mL, and FA 0.4 mg/mL, under the USP 2006 MF cream method (B). Chromatogram showing CC 0.02 mg/mL, MF 0.02 mg/mL, 3-ketofusidic acid impurity 0.02 mg/mL, and FA 0.4 mg/mL, under the BP 2005 FA cream method (C).

Reference solution

Reference stock solution was prepared by transferring a l0 mg portion of 3-ketofusidic acid into a l00-mL volumetric flask. A 35 mL portion of acetonitrile was added to dissolve, diluted to volume with acetonitrile, and mixed. A 2.0 mL portion of reference stock solution was further transferred to a 50-mL volumetric flask. The solution was diluted to volume with acetonitrile and mixed. This solution contained 4.0 µg/mL of impurity. The solution was filtered through a 0.45-µm membrane filter and 20 µL was injected.

Estimation from formulations

A 1.0 g portion of cream was transferred to a 50-mL volumetric flask, taking care to avoid sticking cream to the walls of the volumetric flask. To this, a 35 mL portion of acetonitrile was added and heated at 80°C for 5 min or until the cream melted completely; in between, the flask was occasionally swirled. The solution was allowed to cool to room temperature. Volume was made up to the mark with acetonitrile and mixed. The solution was centrifuged at 3500 rpm for 8 min; supernatant solution was filtered through a 0.45-µm membrane filter and 20 µL was injected directly onto the column.

Quantitation

Peak areas were recorded for all peaks. Respective peak areas were taken into account to quantitate the label amounts in milligrams per gram of cream by using the following formulas:

$$\text{CC and MF mg/g} = R_u/R_s \times C/100 \times 4/100 \times 50/W \times P/100$$

$$\text{FA} = R_u/R_s \times C/100 \times 50/W \times P/100$$

where R_u is peak area obtained from CC/FA/MF in the investigation solution; R_s are the peak areas obtained from CC/MF/FA in the standard solution; C is the weight, in mg, of respective CC/MF/FA reference standards taken to prepare standard solution; W is the weight, in g, of test sample; and P is the purity of the respective CC/MF/FA reference standards.

Results and Discussion

USP 2006 MF cream assay and BP 2005 FA cream assay method

An assay was made to ascertain the suitability of the chromatographic conditions prescribed in USP 2006 under the monograph "Mometasone furoate cream" for the determination of cream containing CC as a preservative, MF, FA, and 3-ketofusidic acid impurity that may be present in the formulation. The mobile phase at flow rate of 1.7 mL/min was comprised of a mixture of 65 volumes of methanol and 35 volumes of water. Chromatographic procedure was carried out with detector wavelength set at 254 nm and using a stainless steel column (250 × 4.6 mm) containing L7 packing.

The procedure was then applied as described under the monograph "Fusidic acid cream" in BP 2005 to ascertain its suitability for the determination of cream containing the same compounds, excipients, and impurity that may be present in the formulation.

Table I. Comparison of System Suitability Parameters

	CC			MF			FA		
Parameters	A*	B†	C‡	A	B	C	A	B	C
Retention factor	0.90	2.81	0.84	2.90	8.12	0.30	4.23	26.23	2.90
Peak asymmetry	1.03	1.07	1.01	0.95	1.05	1.01	1.00	§	1.06
Resolution (CC-MF-FA)	—	—	—	9.32	19.46	9.81	4.53	§	10.33
Theoretical plates	2504	10237	1559	3156	8228	2490	4679	§	4110

* A: proposed method; † B: USP 2006 method; ‡ C: BP 2005 method; § Ghost peak detected.

Chromatographic procedure was carried out with detector wavelength set at 235 nm and using a stainless steel column (125 × 4.0 mm) containing lichrospher 100 Rp-18 packing. Both of the previously mentioned conditions were followed initially to evaluate CC, MF, FA, and 3-ketofusidic acid impurity, except the use of a Symmetry C_8, 150 × 3.9 mm, 5 µm column (octylsilyl). As per the method described under the USP 2006 monograph, CC and MF compounds eluted at 5.64 and 13.68 min, respectively, and FA eluted at 40 min with a ghost peak. The retention time (RT) was found to be 1.12 min for MF, 2.75 min for CC, and 5.74 min for FA, as per the method described in the BP 2005 monograph. The lesser RT of the MF peak as compared to the proposed method may be due to the increased flow rate of 2.5 mL/min as mentioned in BP 2005; this may still reduce over a period of time with increasing column life, and thus can cause significant concern for the accurate quantitation of this component. Another factor to consider is the FA ghost peak eluted at 40 min in the USP 2006 method. Chromatograms recorded as per the proposed methods (USP 2006 and BP 2005) are shown in Figure 2. A meaningful comparison of system suitability parameters of the proposed USP 2006 and BP 2005 methods is shown in Table I.

Chromatography

A chromatographic system comprised of 0.5% w/v aqueous ammonium acetate and acetonitrile (45:55, v/v), pH 3.0, at constant flow rate of l.0 mL/min as mobile phase, a Symmetry C_8, 150 × 3.9 mm, 5 µm, stainless steel column as stationary phase, and detector wavelength at 240 nm resulted in overlapping of MF and impurity peaks at an RT of 3.4 min. However, the peaks of CC (RT 1.0 min) and FA (RT 10.0 min) eluted with resolution of 8.5. A mobile phase of 1.5% aqueous solution of ammonium acetate and acetonitrile (55:45, v/v, pH 3.8) was manipulated and optimized in isocratic conditions on the previously mentioned column to obtain symmetrical peak shapes and good separation between CC and MF (resolution 9.33) and MF and 3-ketofusidic acid (resolution 4.0); resolution between FA and 3-ketofusidic acid was found to be 3.0, which meets resolution criteria specified in USP 2006. An increase in acetonitrile (65%) and decrease in ammonium acetate (35%) in the mobile phase resulted in a decrease in run time but with less resolution between the peaks. It was noted that a slight decrease in ammonium acetate concentration (< 1.5%) resulted in decreased resolution between CC, MF, FA, and the impurity peak. An increase in ammonium acetate concentration possibly resulted in symmetrical peak shapes with good resolution between all these components. The detector wavelength set at 240 nm allowed sufficient absorption of CC and MF together with FA and impurity. A typical chromatogram of the test solution is shown in Figure 2A.

Method validation

The test method for the simultaneous determination of CC, MF, and FA was validated to include the essential demands of International Conference on Harmonization guidelines (20). Parameters like specificity, linearity, accuracy, precision, range, robustness, and system suitability were examined.

Specificity

No interferences were observed due to the obvious presence of excipients like cetostearyl alcohol, cetomacrogal-1000, white petroleum jelly, propylene glycol, liquid paraffin, sodium dihydrogen phosphate, and 3-ketofusidic acid. The principal impurity 3-ketofusidic acid, which may have been present in the formulation, was separated from main peak of FA with a resolution factor of more than 3.0 (Figure 3).

Linearity

Peak areas versus concentrations in microgram per milliliter were plotted for CC, MF, and FA at the concentration range between 50% and 150% of target level. Five points were taken for each linearity range. CC, MF, and FA showed linearity in the range 10–30, 10–30, and 200–600 µg/mL, respectively. Linear regression equations and correlation coefficient *(r^2)* are provided below:

$Y_{CC} = 28428.3x - 10612.8$ ($r^2 = 0.999$)

$Y_{MF} = 114908.2x - 101758$ ($r^2 = 0.999$)

$Y_{FA} = 469546.2x + 162133.6$ ($r^2 = 0.999$)

Accuracy

Accuracy of the proposed HPLC determination was evaluated from assay result of components. Accuracy was evaluated by performing the assay of samples, and calculated from peak area responses of different samples by component recovery method.

Stock solutions

Stock solution was prepared by dissolving accurately weighed portions of 40 mg of CC, MF, and 800 mg of FA in acetonitrile to produce a 200 mL solution.

Appropriate portions of the stock solution were spiked into blank placebo matrix to provide concentrations of 50%, 75%, 100%, 125%, and 150% of target level. Mean recovery of spiked samples was 100.31% for CC, 100.38% for MF, and 100.34% for FA (Table II).

Precision

Instrumental precision was determined by six replicate determinations of standard solution, and the relative standard deviations (RSD) were 0.21% for CC, 0.12% for MF, and 0.24% for FA.

Method precision or intra-assay precision was performed by preparing six different sample solutions involving different weights. Each solution was injected in triplicate under the same conditions and mean value of peak area response was taken for each solution. Corrections in area were made for each weight taken to prepare six sample solutions, and RSD of peak area response was calculated from the six solutions. RSD were 0.23% for CC, 0.46% for MF, and 0.62% for FA.

Intermediate precision was performed by two different analysts employing different instruments to analyze samples. Standard solution and six different samples at 100% target level were prepared by each analyst. RSD obtained from 12 assay results by two analysts were 0.52% for CC, 0.38% for MF, and 0.50% for FA.

Range

The range of a method is defined as lower and higher concentrations for which the method has adequate accuracy, precision, and linearity. To demonstrate the range, six samples each of low concentration (50% of target level) and high concentration (150% of target level) similar to accuracy samples were prepared by spiking drug substance into blank matrix (placebo). Each sample was analyzed in duplicate. At low concentration, mean recovery of CC, MF, and FA was found to be 99.97%, 100.20%, and 99.99%, respectively. RSD obtained from these determinations were found to be 0.68% for CC, 0.98% for MF, and 0.86% for FA. At high concentration, mean recovery of CC, MF, and FA was found to be 100.20%, 100.10%, and 100.80%, respectively. RSD obtained at higher concentration level were found to be 0.40% for CC, 0.65% for MF, and 0.70% for FA.

Robustness

Robustness of the proposed method was performed by keeping chromatographic conditions constant with the following differences: (*i*) Changing mobile phase composition from buffer (55% v/v)–acetonitrile (45% v/v) to buffer (45% v/v)–acetonitrile (55% v/v); (*ii*) Variation in the mobile phase pH from 3.8 to 4.1; (*iii*) Changing flow rate from 1.0 mL/min to 1.2 mL/min.

Standard solution was injected six times in replicate for each minor change. System suitability parameters like resolution, peak asymmetry, theoretical plates, retention factor, and RSD were recorded for each peak and found to be within acceptable limits of validation criteria.

Six test samples at target concentration levels were prepared and analyzed in duplicate for each change. Recoveries and RSD were calculated for each component during each change and found to be 99.99–100.20% and less than 1.0%, respectively.

System suitability

System suitability tests were performed on chromatograms obtained from system suitability solution to check parameters such as peak areas, column efficiency, peak asymmetry, and resolution between CC, MF, 3-ketofusidic acid, and FA peaks. The %RSD of results obtained from six replicate injections of system suitability solution was not more than 2.0%.

Determination of 3-ketofusidic acid and other impurities

In the chromatogram obtained with test solution, area due to any secondary peak other than the solvent peak and principal peaks of CC, MF, and FA was less than the area of 3-ketofusidic acid impurity peak obtained with reference solution (less than 0.25%). The sum of all secondary peaks was less than twice the area of impurity peak obtained with reference solution

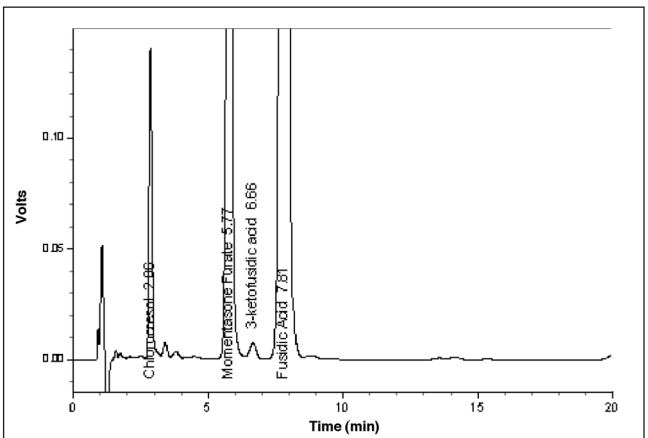

Figure 3. Chromatogram showing separation of 3-ketofusidic acid impurity in the sample matrix under the proposed method.

Table II. Accuracy Data* (Analyte Recovery)

No.	Theoretical amount (mg/mL)	Theoretical (% of target level)	Determined amount (mg/mL)	Determined (% of target level)	Recovered (%)	Bias (%)
CC						
1	0.010	50	0.00998	49.90	99.80	–0.20
2	0.015	75	0.01503	75.15	100.20	+0.20
3	0.020	100	0.02012	100.60	100.60	+0.60
4	0.025	125	0.02491	124.55	99.64	–0.36
5	0.030	150	0.03031	151.55	101.33	+1.33
MF						
1	0.010	50	0.01013	50.65	101.30	+1.30
2	0.015	75	0.01498	74.90	99.86	–0.14
3	0.020	100	0.02008	100.40	100.40	+0.40
4	0.025	125	0.02521	126.05	100.84	+0.84
5	0.030	150	0.02985	149.25	99.50	–0.50
FA						
1	0.20	50	0.20101	50.25	100.50	+0.50
2	0.30	75	0.30033	75.08	100.11	+0.08
3	0.40	100	0.39923	99.80	99.80	–0.20
4	0.50	125	0.50243	125.60	100.48	+0.48
5	0.60	150	0.60501	151.25	100.83	+0.83

* Average of three determinations.

Table III. Long-Term and Accelerated Stability Data

No.	Storage condition	Initial	3 months	6 months
CC				
1	Long-term*	100.30%	100.50%	100.45%
2	Accelerated†	100.30%	100.25%	100.20%
MF				
1	Long term	101.10%	100.80%	100.90%
2	Accelerated	101.10%	100.75%	100.44%
FA				
1	Long term	99.98%	100.00%	100.20%
2	Accelerated	99.98%	99.95%	99.90%

* Long-term = 30°C/65% RH; † Accelerated = 40°C/75% RH.

(less than 0.5%). The detection limit for 3-ketofusidic acid was found to be 0.009 µg/mL.

Stability studies

Stability studies were performed on three batches of in-house prepared formulations. The samples were subjected to long-term stability at 30ºC/65% relative humidity (RH) and accelerated stability at 40ºC/75% RH for a period of 12 months. Samples were withdrawn at regular intervals of 0, 3, 6, and 12 months and analyzed for the stability of the drug in formulation. Stability data of the formulation is shown in Table III.

Conclusion

The proposed HPLC method is rapid, direct, specific, accurate, and precise for the simultaneous determination of CC, MF, and FA from creams and ointments. The method can be used to determine 3-ketofusidic acid and other impurities in creams and ointments containing FA. The described method is suitable for stability studies, routine analysis, and quality control of creams and ointments or other pharmaceutical preparations containing these ingredients, either alone or in combination.

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