Journal of Pharmaceutical and Biomedical Analysis 96 (2014) 111–117

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Development and validation of a novel stability-indicating HPLC method for the simultaneous assay of betamethasone-17-valerate, fusidic acid, potassium sorbate, methylparaben and propylparaben in a topical cream preparation

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ARTICLE INFO

Article history:
Received 18 November 2013
Received in revised form 27 February 2014
Accepted 4 March 2014
Available online 25 March 2014

Keywords:
HPLC
Fusidic acid
Betamethasone-17-valerate
Preservatives
Development
Validation

ABSTRACT

A novel stability-indicating reversed phase high performance liquid chromatographic (RP-HPLC) method for the simultaneous assay of betamethasone-17-valerate, fusidic acid and potassium sorbate as well as methyl- and propylparaben in a topical cream preparation has been developed. A 100 mm × 3.0 mm ID. Ascentis Express C18 column maintained at 30 °C and UV detection at 240 nm were used. A gradient programme was employed at a flow-rate of 0.75 ml/min. Mobile phase A comprised of an 83:17 (v/v) mixture of acetonitrile and methanol and mobile phase B of a 10 g/l solution of 85% phosphoric acid in purified water. The method has been validated according to current International Conference on Harmonisation (ICH) guidelines and applied during formulation development and stability studies. The procedure has been shown to be stability-indicating for the topical cream.

1. Introduction

Inflamed skin conditions, such as eczema and dermatitis, can be further complicated by bacterial infections. For this reason, a topical cream preparation containing the active pharmaceutical ingredients (APIs) betamethasone-17-valerate and fusidic acid as well as the preservative compounds potassium sorbate, methylparaben and propylparaben (Fig. 1) has been developed.

Betamethasone-17-valerate (BV) is a potent corticosteroid with anti-inflammatory properties applied topically for the treatment of a variety of skin conditions, including eczema, atopic dermatitis and psoriasis [1] where it helps to relieve associated symptoms such as oedema and itching. Fusidic acid (FA) is a bacteriostatic antibiotic with steroid structure first isolated from the fermentation broth of *Fusidium coccineum* by Godtfredsen et al. at the laboratories of Leo Pharmaceutical Products in 1962 [2]. It is used for the treatment of Gram-positive bacterial infections, particularly those caused by *Staphylococcus* species. It has regained popularity in recent years due to its effectiveness in the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) [3,4].

Potassium sorbate (PS) and both methyl- and propylparaben (MP, PP) are antimicrobial substances, found in many pharmaceutical and cosmetic preparations, which are effective against fungi and Gram-positive bacteria [5]. Their function is to ensure that microbiological purity is maintained throughout the shelf-life of the product.

According to current guidelines [6,7], APIs and preservatives in finished pharmaceutical products must be quantified as a general quality control requirement during both release and shelf-life testing. This is in order to demonstrate an acceptable level of quality throughout the life-cycle of the pharmaceutical product. Initially, the stability of trial batches must be determined during the formulation development phase, necessitating the employment of a stability-indicating analytical procedure. In the past, it was common in analytical laboratories to have several individual HPLC procedures for the separate analysis of APIs and preservatives in the same finished formulation. In recent times, however, there appears to be a trend towards developing a single procedure for the analysis of all components in a single run. Examples include Shaikh et al. [8], who developed a procedure for the simultaneous quantitation of chlorocresol, mometasone furoate and fusidic acid in a topical

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http://dx.doi.org/10.1016/j.jpba.2014.03.005
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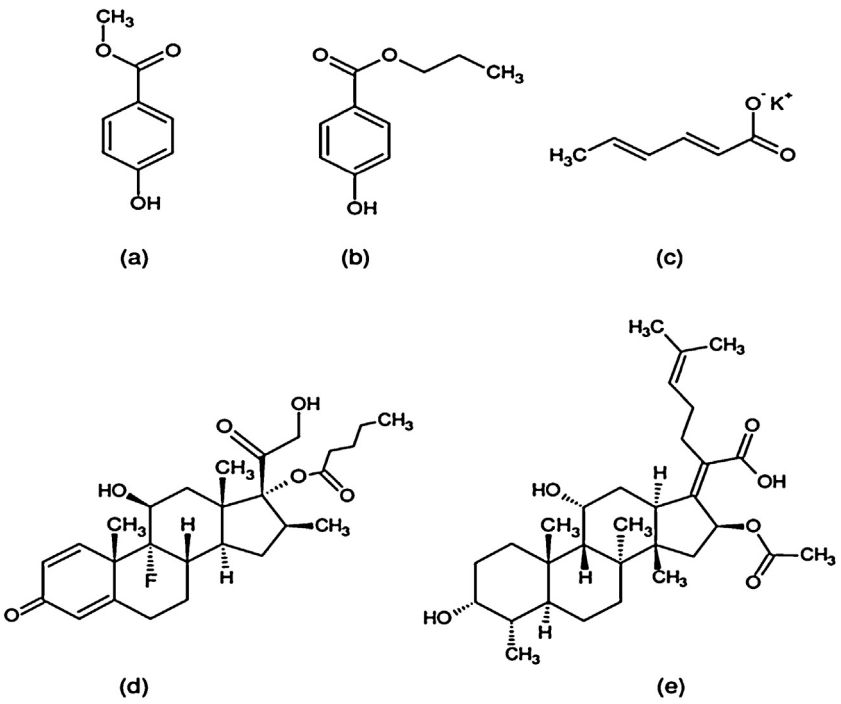

Fig. 1. Chemical structures: MP (a), PP (b), PS (c), BV (d) and FA (e).

cream with a run time of 8 min, and Goswami et al. [9], who developed a procedure for the analysis of halometasone, fusidic acid, methylparaben and propylparaben in a single run. This trend has most likely been driven by advances in analytical technology, such as UPLC and more efficient, more selective analytical columns, as well as environmental awareness issues and cost efficiency.

From the outset the aim of the current analytical development was to employ a single method for the analysis of all compounds. However, none of the analytical methods reported in the literature are suitable for the simultaneous analysis of the 5 components of interest in a single chromatographic run. The majority of reported methods are only suitable for the analysis of a maximum of 1 or 2 of the compounds of interest [10,11] and are often based on poorly selective procedures such as UV–vis spectrophotometry [12] or atomic absorption spectrometry [13] which can no longer be considered state-of-the-art and/or stability-indicating. Consequently, it was necessary to develop a novel procedure for the analysis of all five components. The simultaneous analysis with the aid of a single HPLC run would allow for a much more cost-effective and less time-consuming analysis of cream samples.

In this paper the development of a novel stability-indicating RP-HPLC method for the simultaneous assay of fusidic acid, betamethasone-17-valerate and potassium sorbate, as well as methyl- and propylparaben in a topical cream preparation is reported. The method has been validated according to current International Conference on Harmonisation (ICH) guidelines and applied to development formulations and finished product samples.

2. Materials and methods

2.1. Reagents

Fusidic acid hemihydrate and betamethasone-17-valerate were purchased from OJSC Biosintez (Penza, Russia) and Crystal Pharma (Valladolid, Spain), respectively. Potassium sorbate was obtained from Merck (Darmstadt, Germany) and both methyl- and propylparaben were purchased from Clariant (Pontypridd, UK). All compounds were of Ph. Eur. or cosmetic grade.

Gradient grade methanol and acetonitrile were purchased from VWR International GmbH (Darmstadt, Germany). Purified water was obtained from the in-house purification system at mibe GmbH Arzneimittel (Brehna, Germany). Phosphoric acid (85%, analysis grade) was purchased from Merck (Darmstadt, Germany). All cream samples were provided by mibe GmbH Arzneimittel.

2.2. Instrumentation

Shimadzu Prominence HPLC Systems (Shimadzu, Japan) were used for method development and validation. The HPLC systems were equipped with a binary pump (LC-20AD), a temperature-controlled auto-sampler (SIL-20AC$_{HT}$), a temperature-controlled column compartment (CTO-20AC) as well as an on-line degasser (DGU-20A$_5$) and a photo-diode-array detector (SPD-M20A). The software used was SHIMADZU LC solution version 1.24 SP1.

2.3. Chromatographic conditions and sample preparation

The HPLC column used was the Ascentis Express (Fused-Core®), C18, 100 × 3.0 mm ID, with 2.7 μm particles (Supelco®, Bellefonte, USA) and a suitable guard-column. Mobile phase A comprised of an 83:17 (v/v) mixture of acetonitrile and methanol and mobile phase B of a 10 g/l mixture of phosphoric acid (85%) in purified water. A gradient elution programme was employed (Table 1) with a mobile phase flow-rate of 0.75 ml/min. The column-oven and auto-sampler temperatures were set at 30 °C and 25 °C, respectively. The detection wavelength was 240 nm and an injection volume of 10 μl was used.

Samples were prepared by weighing approximately 1.00 g of cream into a 50.0 ml volumetric flask, adding 30 ml of acetonitrile

Table 1
Gradient elution programme.

Time (min)	%A	%B
START	18	82
16	48	52
25	90	10
27	18	82
35	18	82

and suspending by shaking in a water-bath at 65 °C. Thorough mixing was ensured with the help of a vortexing machine. The resulting suspension was shaken on a flat-bed shaker at approximately 200 shakes per minute and allowed to cool to room temperature before being made up to volume with acetonitrile. After 20 min in a freezer (−20 °C) a portion of the cold suspension was centrifuged at 3000 rpm for 5 min. The supernatant was removed and allowed to warm to room temperature.

A portion of the supernatant was diluted 1–2 (v/v), e.g. 10.0–20.0 ml with purified water. Finally, the solution was filtered through a 0.45 μm regenerated cellulose filter (Macherey-Nagel, Germany) into a suitable HPLC vial. The assay concentrations of the standards were 200.0 and 12.14 μg/ml for FA and BV and 20.0, 8.0 and 1.6 μg/ml for PS, MP and PP, respectively. These concentrations correspond to 100% of the label claim of each component.

3. Results and discussion

3.1. Method development – optimisation of the gradient programme, HPLC column, flow-rate and injection volume

Fusidic acid hemihydrate (see Fig. 1) has acidic character due to the presence of a single carboxyl group with a pK_a of 5.3 [2]. To achieve an acceptable retention of this compound on a C18 column, it needs to remain in its protonated form in solution, so that it can interact with the stationary phase. Its retention is therefore affected by the pH of the mobile phase. Betamethasone-17-valerate as well as methyl- and propylparaben are, on the other hand, neutral compounds. The retention of these compounds should, therefore, remain unaffected by the pH of the mobile phase. Since potassium sorbate is the potassium salt of sorbic acid, it will be present in its ionised form in solution. The retention time of this ionised form will be influenced by the pH of the mobile phase. The compound will only be sufficiently retained on the HPLC column in its protonated form, i.e. in the sorbic acid form which has a pK_a value of 4.8 [14]. On consideration of the above points, an acidic mobile phase containing 10 g/l phosphoric acid (85%) and having a pH of approximately 1.6 was chosen as mobile phase B. Mobile phase A comprised of an 83:17 (v/v) mixture of acetonitrile and methanol.

For the initial experiments a Nucleosil C18 100-5, 250 mm × 4.6 mm ID column with 5 μm particles was chosen. The oven-temperature, detector wavelength and injection volume used were 25 °C, 240 nm and 50 μl, respectively. The initial conditions of the gradient programme were 40% mobile phase A and 60% mobile phase B held for 10 min, after which the % of A was increased to 100% over the next 30 min and held at this composition for a further 10 min. After returning to the start conditions over a period of 5 min the system was equilibrated for a further 5 min giving a total run time of 60 min. The flow rate was 1.0 ml/min.

The resolution between the peaks of potassium sorbate and methylparaben proved to be suboptimal during the initial tests. The run time was also longer than desired for an RP-HPLC assay method. The gradient programme, oven-temperature and flow-rate were optimised to reduce the run time. However, the optimised method resulted in a co-elution of the potassium sorbate and methylparaben peaks. It was found that increasing the percentage of aqueous phase at the beginning of the gradient programme to 82% (v/v) enabled a complete baseline separation of both peaks.

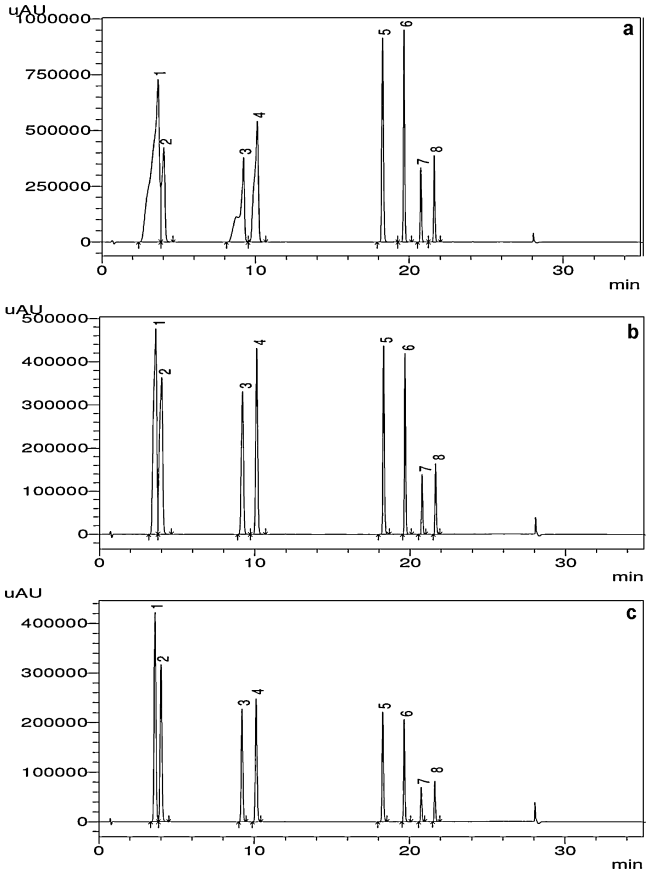

Fig. 2. Optimisation of the injection volume using a mixed standard solution: (a) 50 μl, (b) 20 μl and (c) 10 μl. Chromatographic conditions as described in Table 1. Peak assignment: PS (1), MP (2), betamethasone (3), PP (4), BV (5), betamethasone-21-valerate (6), 3-didehydrofusidic acid (7) and FA (8).

In gradient separations the column chemistry often plays a less significant role than the changing chemistry of the mobile phase, as compared to isocratic separations. Consequently, it is generally possible in gradient separations to use shorter columns than for isocratic separations because the theoretical plate count becomes much less critical. In order to save mobile phase a shorter Ascentis Express 100 mm × 3.0 mm ID column was chosen in combination with a mobile phase flow-rate of 0.75 ml/min. On injecting 50 μl of a mixed standard solution, poor peak shape for the early eluting compounds was observed due to overloading of the alternative HPLC column, which had a smaller inner diameter than the initial column. On reducing the injection volume from 50 μl to 20 μl and then to 10 μl a significant improvement of peak shape was observed (Fig. 2).

The optimised gradient programme, stationary phase dimensions, flow rate and injection volume allowed for a baseline separation of all components (including the main degradation products of the APIs, betamethasone, betamethasone-21-valerate, 3-didehydrofusidic acid, 16-epideacetylfusidic acid and 16-epideacetylfusidic acid-21,16-lactone) within 27 min, with a further 8 min equilibration time giving a total run time of 35 min.

3.2. Method validation

3.2.1. Selectivity

The degradation pathways of betamethasone-17-valerate in topical pharmaceutical preparations have been documented in the literature [15,16]. Betamethasone-17-valerate undergoes an intra-molecular isomerisation under acidic and basic conditions forming the more thermodynamically stable betamethasone-21-valerate. Cleavage of the ester bond results in the alcohol base betamethasone. Kinetic studies regarding these degradation processes indicate that the isomerisation step is dominant over the hydrolysis reaction [17]. Betamethasone-21-valerate should, therefore, be the dominant degradation product of betamethasone-17-valerate in topical preparations at pH values around 5.0. This has been confirmed through purity analysis with a separate analytical procedure.

Data regarding the stability of fusidic acid in finished formulations or details on its potential degradation products do not appear to be available in the literature. However, the current fusidic acid hemihydrate monograph of the European Pharmacopoeia (Ph. Eur.) [18] provides a list of possible impurities of the bulk API. Studies performed by the authors using the above mentioned purity method indicate that the impurities 3-didehydrofusidic acid, 16-epideacetylfusidic acid and 16-epideacetylfusidic acid-21,16-lactone are dominant degradation products of fusidic acid in topical formulations incorporating this API. This data has not yet been published.

Potassium sorbate is known to degrade by oxidation in semi-solid formulations due to the presence of air [19]. This process takes place over a period of months and leads to the formation of carbonyl compounds, such as propenal (acrolein) and malonaldehyde [20,21]. 4-Hydroxybenzoic acid alkyl esters such as methylparaben and propylparaben are generally stable at increased temperature and at pH values below 6. Exposure to pH values at or above 8 results in ester cleavage forming parahydroxy benzoic acid [22]. There are currently no official requirements to detect or quantify degradation products of preservative compounds in pharmaceutical preparations. In general, only preservatives may be employed in drug products that have been shown to be safe and effective and whose degradation products are known to be non-toxic at likely exposure levels. This is in contrast to API compounds whose degradation products must be detected and quantified according to official requirements. Degradation products of the preservative compounds have not been explicitly considered during development and validation of this method, i.e. they have not been identified. The stability-indicating nature of the method has been demonstrated by visual examination of chromatograms from stressed samples (containing all likely degradation products of both the APIs and preservatives), peak purity analysis of the analyte peaks using a diode-array-detector and mass-balance analysis of the APIs from stability samples. This is the standard approach found throughout the literature for assay methods.

Samples which had been stored under accelerated stability conditions (40 °C/75% relative humidity) for 9 months as well as samples stressed under oxidative-, light-stress and increased pH conditions (pH 7) were measured. The oxidative- and light-stress samples were generated by exposing samples of cream to air and daylight for a period of 3 days. The pH stress-test was performed by adjusting the pH of the cream to 7 after manufacture and placing the cream on accelerated stability studies (40 °C/75% RH) for 6 months.

As mentioned earlier, purity studies of the cream formulation with a separate procedure had previously demonstrated that the impurities betamethasone, betamethasone-21-valerate, 16-epideacetylfusidic acid and 16-epideacetylfusidic acid-21, 16-lactone are the dominant degradation products of the APIs. The stressed samples were known to contain all of the above degradation products at concentration levels significantly higher than would be expected over the normal shelf-life of the pharmaceutical product (24 months at 25 °C/60% relative humidity). Under normal conditions the cream is stored in an aluminium tube which is closed with an appropriate cap. This minimises exposure to light and atmospheric oxygen during storage. The stressed samples were therefore considered to be worst case.

Figs. 3 and 4 demonstrate that all relevant peaks are baseline separated and there are no obvious interferences. The purity of each of the analyte peaks was evaluated using a photodiode array scan from 200 to 400 nm. Each of the five peaks of interest was found to be pure with this method which demonstrates the stability-indicating properties of the method. The mass-balance values of the APIs for stability samples (see Tables 2 and 3) were calculated by summing the assay values obtained with the present procedure and the content values of relevant impurities obtained with a separate purity method. The mass-balance values lie between 99.4% and 103.8% for stability samples (which had significantly degraded) stored under intermediate (30 °C/65% relative humidity) and accelerated (40 °C/75% RH) conditions. These values are acceptable and further underline the stability-indicating nature of the procedure.

3.2.2. Linearity

The linearity of the detector response for each of the five analytes was checked using an 8-point-calibration over the range 50–150% of the theoretical concentration of each analyte in the sample solution. The correlation coefficients of the best-fitting lines obtained by least-squares regression were all >0.999. The 95% confidence interval of each of the *y*-intercepts contained the value zero, justifying the use of a single-point standard calibration during routine analysis. The residual values demonstrated no particular trend. A summary of the regression data is provided in Table 4.

3.2.3. Precision and accuracy

The repeatability (reproducibility) of the analytical procedure was checked by preparation and measurement of six sample solutions by Analyst A using HPLC Machine A on Day 1. The relative standard deviation (rsd) of the assay values for each of the analytes were below 1.0%. The intermediate precision was determined by preparation and measurement of a further six samples by Analyst B using HPLC Machine B on Day 2. The rsd values of a total of 12 measurements were also <1.0%.

The accuracy of the analytical procedure was determined by preparation and measurement of 9 solutions comprising of placebo which had been spiked with each of the compounds of interest over the range 50–150% of the theoretical sample concentration. The recovery rates for each component lay between 99.1% and 102.4%.

3.2.4. Solution stability and robustness of HPLC parameters

Solution stability was determined by preparation of three standard and three sample solutions (cream) and analyses of these solutions at 3-hourly intervals over a period of 47 h. The solutions were stored at 25 °C and protected from light. No degradation trend was observed for any of the compounds of interest. The content values of relevant degradation products remained unchanged over the measurement period.

In order to test the robustness of the method, the HPLC parameters were deliberately varied from normal conditions as follows: column temperature (±5 °C), flow-rate (±0.2 ml/min), volume of acetonitrile in mobile phase A (±10 ml/l), volume of methanol in mobile phase A (±10 ml/l), detection wavelength (±2 nm) and

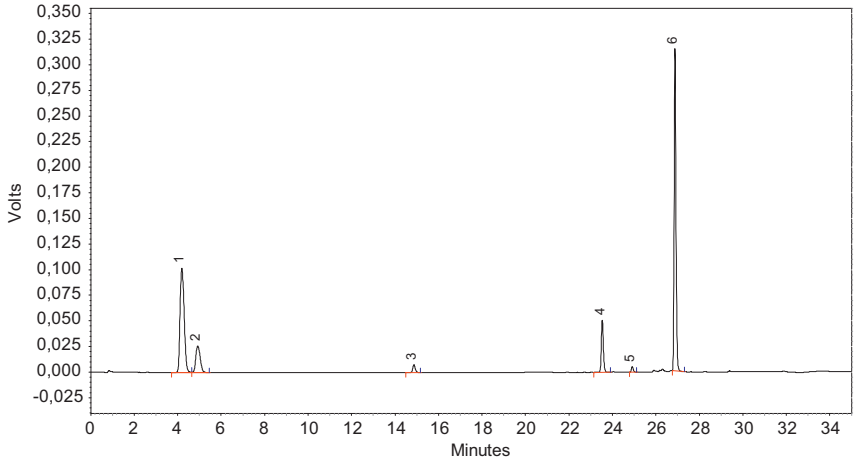

Fig. 3. A chromatogram of topical cream after storage at 40 °C/75% relative humidity for 9 months. The HPLC conditions are the same as those provided in Table 1. Peak assignment: PS (1), MP (2), PP (3), BV (4), degradant betamethasone-21-valerate (5) and FA (6).

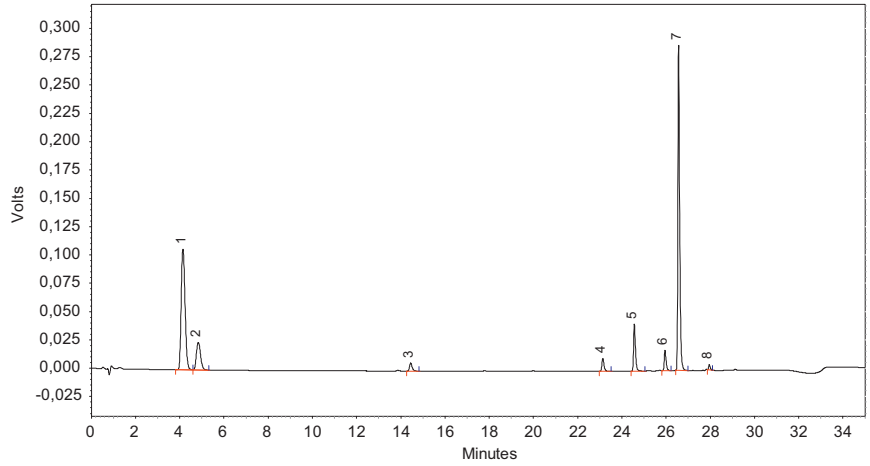

Fig. 4. A chromatogram of a stressed sample (pH 7, 40 °C, 6 months) of topical cream showing relevant degradation products. The HPLC conditions are the same as those provided in Table 1. Peak assignment: PS (1), MP (2), PP (3), BV (4), degradant betamethasone-21-valerate (5), degradant 16-epideacetylfusidic acid (6), FA (7) and degradant 16-epideacetylfusidic acid-21,16-lactone (8). The relative retention times of the 3 degradants are 0.93, 0.98 and 1.05 relative to fusidic acid.

gradient slope (±4%). Some of the conditions tested, e.g. increasing the column-oven temperature by 5 °C lead to a reduced resolution between the potassium sorbate and methylparaben peaks. Consequently, a resolution of at least 1.5 between both of these peaks has been stipulated as a system suitability criterion.

3.3. Analysis of cream samples

During formulation development over 30 trial formulations of topical cream were analysed. Many of the early formulations proved to be highly unstable. With the aid of the developed assay

Table 2
Stability data obtained under intermediate (30 °C/65% relative humidity) ICH stability conditions over a 12-month storage period.

30 °C/65% relative humidity	0 months	3 months	6 months	9 months	12 months	Units
Assay						
Potassium sorbate	100.0	83.2	71.1	60.7	56.4	
Methylparaben	100.0	101.0	96.4	98.9	94.8	% relative to start value
Propylparaben	100.0	103.1	100.0	103.0	98.5	
Betamethasone-17-valerate	100.0	100.0	99.2	101.0	99.2	
Fusidic acid	100.0	101.5	99.0	99.9	99.6	
Degradation products of betamethasone-17-valerate						
Betamethasone-21-valerate	0.87	1.25	1.90	2.69	3.08	
Sum of all degradation products	0.96	1.25	2.00	2.82	3.08	
Mass-balance	101.0	101.3	101.2	103.8	102.3	
Degradation products of fusidic acid						% (n.d. = not detected, n.q. = not quantifiable)
3-Didehydrofusidic acid	0.33	0.36	0.38	0.38	0.40	
16-Epideacetylfusidic acid	n.d.	n.q.	n.q.	0.05	n.q.	
16-Epideacetylfusidic acid-21,16-lactone	n.d.	n.d.	0.06	0.05	0.07	
Sum of all degradation products	0.33	0.36	0.44	0.48	0.47	
Mass-balance	100.3	101.8	99.4	100.4	100.1	

Table 3
Stability data obtained under accelerated (40 °C/75% relative humidity) ICH stability conditions over a 6-month storage period.

40 °C/75% relative humidity	0 months	3 months	6 months	Units
Assay				
Potassium sorbate	100.0	90.4	89.3	% relative to start value
Methylparaben	100.0	98.2	97.8	
Propylparaben	100.0	100.0	100.6	
Betamethasone-17-valerate	100.0	95.9	92.7	
Fusidic acid	100.0	100.0	99.0	
Degradation products of betamethasone-17-valerate				
Betamethasone-21-valerate	0.87	5.32	9.60	
Sum of all degradation products	0.96	5.32	9.75	
Mass-balance	101.0	101.2	102.5	
Degradation products of fusidic acid				% (n.d. = not detected, n.q. = not quantifiable)
3-Didehydrofusidic acid	0.33	0.37	0.37	
16-Epideacetylfusidic acid	n.d.	0.07	0.09	
16-Epideacetylfusidic acid-21,16-lactone	n.d.	0.12	0.21	
Sum of all degradation products	0.33	0.56	0.67	
Mass-balance	100.3	100.6	99.7	

Table 4
Regression data from the linearity experiments.

Substance	$y = mx + c$	r	r^2	Residuals	95% confidence interval for y-intercept
Potassium sorbate	$y = 65{,}680x + 13{,}804$	0.9999	0.9999	No trend	$c \pm 16{,}944$
Methylparaben	$y = 47{,}190x + 7311$	0.9992	0.9984	No trend	$c \pm 15{,}460$
Propylparaben	$y = 39{,}561x + 1108$	0.9992	0.9985	No trend	$c \pm 2673$
Betamethasone-17-valerate	$y = 24{,}520x + 2978$	0.9998	0.9997	No trend	$c \pm 5486$
Fusidic acid	$y = 6896x + 20{,}841$	0.9998	0.9996	No trend	$c \pm 28{,}059$

procedure it proved possible to determine that betamethasone-17-valerate was isomerising rapidly to its corresponding 21-valerate ester. This isomerisation process was shown to be dependent on the pH of the aqueous phase of the cream as well as on the storage temperature. Additionally, and quite surprisingly, the isomerisation rate was shown to be significantly influenced by the concentration of the primary emulsifier in the cream formulation. At higher concentrations of emulsifier, e.g. 5%, the isomerisation occurs rapidly with 5% betamethasone-21-valerate being formed at pH 5.0 ± 0.2 after 3 months storage under the conditions 25 °C/60% relative humidity. Reducing the emulsifier concentration leads to a significant reduction of the isomerisation rate. After 3 months storage under the conditions 25 °C/60% relative humidity and at pH 5.0 ± 0.2, only 0.1% betamethasone-21-valerate was observed when the emulsifier concentration was reduced to an appropriate level.

The data also indicated that the preservative compound, potassium sorbate, degrades rapidly (Tables 2 and 3). Degradation of potassium sorbate in semi-solid formulations is common and is not considered critical as long as it can be demonstrated that the quantities present at the end of shelf-life are sufficient to ensure microbiological purity of the preparation. This can be investigated by employing a test for efficacy of microbial preservatives as described in any of the major pharmacopoeias. In contrast to potassium sorbate, the API fusidic acid as well as the preservative compounds methyl- and propylparaben proved to be stable under real-time (25 °C/60% RH), intermediate (30 °C/65% RH) and accelerated (40 °C/75% RH) storage conditions. The results clearly indicate that the method is stability-indicating for the topical cream preparation.

4. Conclusions

A RP-HPLC method was developed to simultaneously determine the content of fusidic acid, betamethasone-17-valerate, potassium sorbate as well as methyl- and propylparaben in a topical cream preparation. The method was validated according to current ICH guidelines and demonstrated to be selective, linear, precise, accurate and robust within the validated range. The method has been shown to be stability-indicating due to its ability to separate the most important degradation products of the APIs and detect changes of content of the active substances and preservatives over the shelf-life of the pharmaceutical product.

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