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Development and validation of a stability-indicating HPLC-UV method for the determination of triamcinolone acetonide and its degradation products in an ointment formulation

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ABSTRACT

A stability indicating high performance liquid chromatography method has been developed for the determination of triamcinolone acetonide (TCA) and its main degradation products in ointment formulations. The method, based on extensive stress testing using metal salts, azobisisobutyronitrile, acid, base and peroxide, showed that TCA undergoes oxidative degradation. All degradation products were identified using HPLC mass spectrometry. Separation and quantification was achieved using an Altima C18 RP18 HP column (250 × 4.6 mm^2, with 5 μm particles) using a mobile phase consisting of acetonitrile and water buffered at pH 7 using 10 mM phosphate buffer. A gradient mode was operated at a flow rate of 1.5 ml/min and detection was at 241 nm. The method showed linearity for TCA and Impurity C in 0.02–125% of the workload, both square roots of the correlation coefficients were larger than 0.9999. Repeatability and intermediate precision were performed by six consecutive injections of both 1.25% and 125% of the work load for both TCA and Impurity C divided equally over two days. RSD were 0.6% and 0.7% for TCA and 0.5% and 0.1% for Impurity C respectively. Accuracy was determined as well, the average recoveries were 99.5% (±0.1%, n = 3) for TCA and 96.9% (±1.3%, n = 3) for impurity C respectively from spiked ointment samples. The robustness was also evaluated by variations of column (old vs new), mobile phase pH and filter retention. The applicability of the method was evaluated by analysis of a commercial ointment formulation. Interestingly, the extensive stress tests were able to predict all degradation products of TCA in a long term stability ointment sample.

1. Introduction

Triamcinolone acetonide (TCA) is a synthetic glucocorticosteroid with immunosuppressive and anti-inflammatory activity. It binds in the target cell to specific cytosolic glucocorticoid receptors and subsequently interacts with glucocorticoid receptor response elements on DNA thereby altering gene expression [1]. TCA has been used for over fifty years and is still frequently prescribed in the treatment of several skin diseases like eczema and psoriasis. It is used in many cream and ointment formulations, including an ointment that is widely used in the Netherlands: TCA ointment FNA (Formulary of Dutch Pharmacists).

Because of the widespread use of TCA in varying matrices several chromatographic methods for the analysis of the compound have been described [2–9]. These methods are suitable for the determination of the TCA content, but not for the quantification of degradation products. The quantification of degradation products is essential in stability research of pharmaceutical products following the ICH Q2 (R1) guideline [10]. Additionally, the degradation of TCA is poorly described in literature. As a consequence, currently no stability indicating method (SIM) for TCA ointment FNA is available.

To develop and validate a method specificity, stressed samples are essential. According to the ICH Q2 (R1) guideline stressed samples should be created using heat, humidity, acid, base, oxidation and light stress [10]. The vagueness of this guideline leads to a

Abbreviations: ACN, acetonitril; AIBN, azobisisobutyronitrile; FNA, formulary of Dutch pharmacists; PG, propyleneglycol; SIM, stability indicating method; TCA, triamcinolone acetonide.

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variety of experience-based approaches which are often not comprehensive in their predictability. The scientific background and practical implementation of adequate stress testing is described extensively elsewhere [11]. Since oxidation is the predominant mechanism of TCA degradation a comprehensive set of oxidative stress testing should be used to attain a more comprehensive prediction of the profile of degradation products [2]. Therefore we incorporated not only a peroxide (e.g. hydrogen peroxide [H_2O_2]), but a radical initiator (e.g. azobisisobutyronitrile [AIBN]) and trace metals (e.g. iron and copper salts) into the set of stress tests [12,13]. All other conditions as mentioned in the ICH-guideline were implemented as well. To show specificity, a four and a half year old ointment sample was used. Degradation product identification was performed in order to assist in method development.

The aim of this study was to develop and validate a HPLC-UV SIM for TCA ointment FNA. In support of this aim TCA degradation products were identified using LC–MS after evaluating the outcome of the set of stress tests.

2. Material and methods

2.1. Reagent and chemicals

HPLC grade acetonitrile (ACN), dichloromethane, methanol (MeOH) and hexane were obtained from Avantor Performance Materials (Center Vally, Pennsylvania, USA). Distilled, deionized water was prepared by an Elga Centra R 60/120 system (Woodridge, Illinois, USA). Copper(II) acetate was obtained from Alfa Aesar (Havehill, Massachusetts, USA). Disodium edetate, hydrogen peroxide (H_2O_2), iron(III) chloride ($FeCl_3$), copper(II) chloride ($CuCl_2$), sodium phosphate monobasic (NaH_2PO_4), sodium phosphate dibasic (Na_2HPO_4) and azobisisobutyronitrile (AIBN) were obtained from Merck (Darmstadt, Germany). Propylene glycol (PG) was obtained from Brenntag (Dordrecht, The Netherlands). 1 M hydrogen chloride (HCl) and 0.01 M sodium hydroxide (NaOH) solutions were prepared on site.

TCA ointment FNA consists of 0.1% TCA, 10% PG, 10% lanolin and 79.9% petrolatum.

2.2. LC–MS analysis

MS was conducted on a Micromass Quattro Ultima TQD system equipped with an electrospray ionization (ESI) source (Waters Chromatography, Etten-Leur, The Netherlands). Masses were scanned from m/z 50–1100, gas flow to 530 l/h, gas temperature to 350 °C and voltage 3 kV. Data was analyzed with Masslynx version 4.0 software. The mobile phase components were ACN and water buffered at pH 6.8 using 12 mM ammonium acetate.

To attain insight in the product specific degradation products, a four and a half year old 0.1% TCA ointment FNA sample that was stored throughout its shelf life at room temperature in aluminum tubes was used. The sample was extracted using the sample preparation method provided in Section 2.6 and analyzed using the settings described above. Mass spectra are included in supplementary data.

2.3. HPLC-UV

Chromatography was conducted on a Shimadzu Prominence-i LC-2030C 3D liquid chromatograph with diode array detector (Kyoto, Japan) and an Altima C18 RP18 HP column (250 × 4.6 mm^2, with 5 μm particles) (Mandel Scientific Company, Ontario, Canada). The flow rate was 1.5 ml/min and UV detection was at 241 nm. Mobile phase components were ACN and water buffered at pH 7 using 10 mM phosphate buffer. Injection volume was 20 μl. Chromatograms were obtained and analyzed with Shimadzu LabSolutions software version 5.5.7. A gradient program was run: 0% ACN from start to 12 min, increased to 32% ACN at 12 min, maintained at 32% until 30 min, increased to 70% at 40 min, decreased to 0% at 42 min and maintained at 0% until 47 min.

2.4. Synthesis of Impurity C

The synthesis of Impurity C (compound 2, Fig. 1) was based upon a method described in literature [14]. Impurity C was synthesized by dissolving 600 mg TCA and 31.5 mg copper(II)acetate in 150 ml MeOH. Air was bubbled through the solution for 60 min. The reaction was quenched by adding 20 ml of 2.5 mg/ml disodium edetate aqueous solution. The solution then was concentrated to 30 ml under cold air and was extracted twice with 200 ml dichloromethane. Finally, the dichloromethane was evaporated under cold air to yield Impurity C.

2.5. Stress testing

Stress testing was performed on 0.5% solutions of TCA in PG. These solutions were exposed to the conditions described in Table 1. Conditions were chosen based on a degradation target of 5–20%. HCl and NaOH were used to simulate acid and base catalyzed degradation. AIBN, H_2O_2 and $FeCl_3$ and $CuCl_2$ were used to simulate radical initiator, peroxide and trace metal mediated oxidation respectively. Light stress was omitted because of irrelevancy as TCA is protected against light in the product by its container.

2.6. Sample preparation

Ointment samples were dispersed in hexane and extracted with ACN and water buffered at pH 7 using 10 mM phosphate buffer (1:1). PG solutions were diluted with ACN or ACN-buffer (1:1). Synthesized Impurity C was dissolved in ACN or ACN-buffer (1:1). TCA references were dissolved in ACN-buffer (1:1).

2.7. Method validation

The method was validated according to the ICH Q2 (R1) guideline. Accuracy, precision (including both repeatability and intermediate precision), specificity, linearity, range and detection and quantification limits (LOD and LOQ) were assessed. Appropriate stressed samples were used for the assessment of specificity and resolution. Stressed samples were appropriate if they showed a degradation between 5 and 20%. Compounds were taken into account if they were present in a concentration of ≥1.0%.

2.7.1. Accuracy

For accuracy a freshly prepared ointment matrix was spiked with TCA and Impurity C in concentrations of 100% and 1% of the work load respectively. Recovery was determined on three consecutive runs.

2.7.2. Precision

For precision repeatability and intermediate precision were performed by using six consecutive injections of both 1.25% and 125% of the work load for both TCA and Impurity C in ACN-buffer (1:1) divided equally over two days.

2.7.3. Specificity

The four and a half year old ointment was used to determine the method specificity by determination of the smallest resolution between any two peaks after sample preparation.

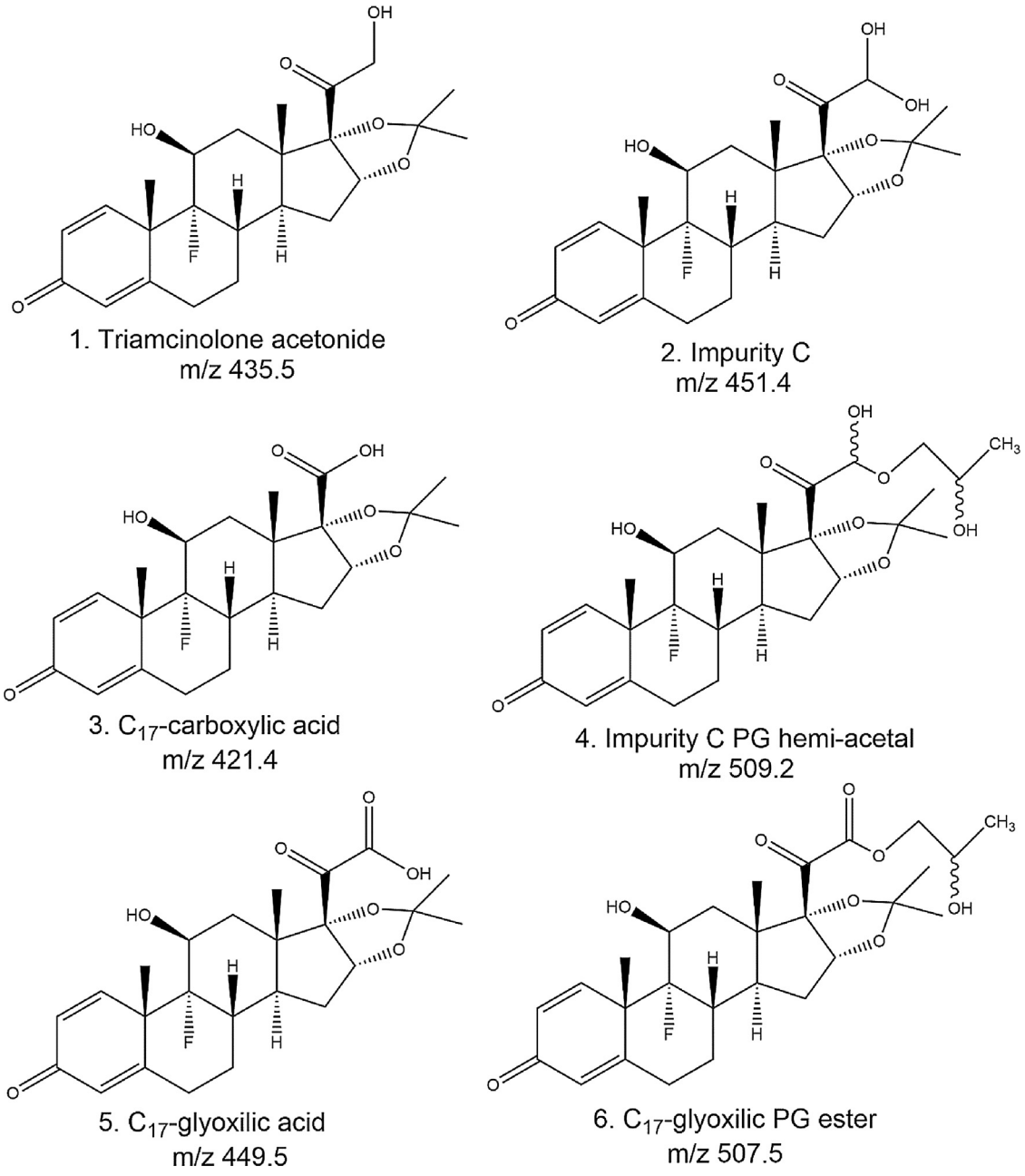

Fig. 1. Molecular structures of triamcinolone acetonide (TCA) and its most predominant degradation products. Structures are based on literature [15,16] in combination with mass determination using HPLC–MS. 1: TCA 2: Impurity C 3: C_{17}-carboxylic acid 4: Impurity C propylene glycol hemi-acetal, 5: C_{17}-glyoxilic acid and 6: C_{17}-glyoxilic acid propyleneglycol ester.

2.7.4. Linearity, range, LOD and LOQ

Linearity for 0.020, 0.1, 0.5, 2.5, 12.5, 62.5 and 125% of the workload was determined for both TCA and Impurity C. The LOD and LOQ were determined by linear regression analysis.

2.7.5. Robustness

The robustness was determined using a number of test. Firstly, an old and a new column of the same type were compared for resolution between TCA and Impurity C. Secondly, the influence of mobile phase pH on the resolution between TCA and Impurity C was tested by using a mobile phase at pH 6.5 and 7. Thirdly, the TCA and Impurity C recoveries were determined after filtration by analyzing three injections of 125% of the workload before and after filtration.

3. Results and discussion

3.1. Degradation product identification

An ointment that was stored at room temperature in the drug product container for four and a half years was used to identify the degradation products that may form in the ointment. HPLC–MS analyses were conducted to determine the mass of degradation products. Impurity C (*m*/*z*: 451.4), a C_{17}-carboxylic acid (*m*/*z*: 421.4), a PG hemi-acetal of Impurity C (*m*/*z*: 509.2), a C_{17}-glyoxilic acid (*m*/*z*: 449.5) and a PG ester of the C_{17}-glyoxilic acid (*m*/*z*: 507.5) (Fig. 1; compounds 2, 3, 4, 5 and 6 respectively) were identified as degradation products based on mass. Fig. 1 presents TCA and the five identified degradation products. Comparable degradation products of Impurity C and C_{17}-carboxylic acid are described in lit-

Table 1
Stress testing results. All solutions contained 0.5% triamcinolone acetonide (TCA) in propylene glycol (PG). AIBN: azobisisobutyronitrile.1: TCA; 2: Impurity C; 3: C_{17}-carboxylic acid.

Stress condition				Amount of compound after stress (% of total)				
#	Medium	Temp.	Time	1	2/4	3	5	6
1	TCA ointment	25 °C	4.5 y	76	4.8	9.2	1.2	8.5
2	5 mM HCl	60 °C	7 d	99	–	1.1	–	–
3	0.5 mM NaOH	60 °C	4 d	85	8.1	6.0	–	–
4	11.5 mM AIBN	60 °C	7 d	96	–	–	–	1.8
5	3% H_2O_2	25 °C	7 d	99	–	–	–	–
6	5 mM $FeCl_3$	60 °C	7 d	91	5.5	1.8	1.2	–
7	5 mM $CuCl_2$	60 °C	7 d	98	–	–	–	–

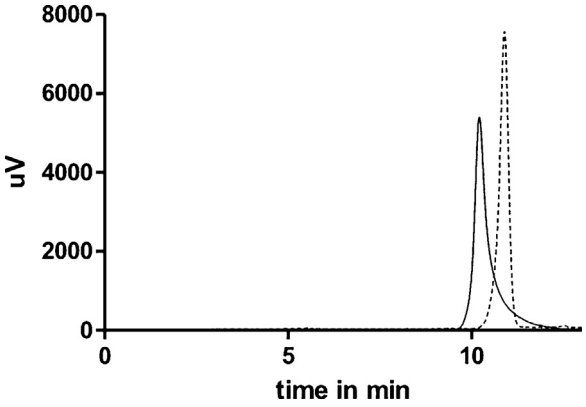

Fig. 2. Chromatograms of synthesized Impurity C dissolved in propylene glycol (PG) and diluted with either acetonitrile (ACN) (black) or ACN-water buffered at pH 7 with 10 mM phosphate buffer (dashed line).

erature as degradation products of TCA and hydrocortisone [2,15]. The PG hemi-acetal is described in literature as a comparable degradation product of triamcinolone acetophenonide in the presence of PG [16]. The C_{17}-glyoxilic acid has been described before for flurandrenolide in a cream formulation only, but not for TCA or other described corticosteroids [2,14,15,17]. For the PG ester of the C_{17}-glyoxilic acid no prior report was found. However it seems logical that such an acid may form an ester when (di-)alcohols such as PG are present. Furthermore, the C_{17}-carboxylic acid and the C_{17}-glyoxilic acid showed a shift in retention time in response to mobile phase pH.

3.2. Optimization of chromatographic conditions

The starting point of the optimization was the TCA related substances method of the European Pharmacopoeia (monograph 0533). To use this method for the TCA ointment FNA an extraction procedure was added to the sample preparation. Extraction of the ointment was performed by dispersing it in hexane followed by extraction using ACN as extraction solvent.

In the Pharmacopoeia method the C_{17}-carboxylic acid (compound 3) showed poor retention and peak symmetry. Therefore this method was adapted and further developed. The retention was improved by changing the gradient program from 32 to 0% ACN at the start of the program. Peak symmetry was improved by buffering the aqueous mobile phase at pH 7 using 10 mM phosphate buffer.

During injection in the HPLC the sample was diluted with water. This led to the conversion of the PG hemi-acetal to impurity C during analysis due to an abundance of water. This is reflected in a broad impurity C peak that impaired proper integration was the result. This issue was resolved by changing the extraction solvent from ACN to ACN-water buffered at pH 7 with 10 mM phosphate buffer (1:1). In this way, the PG hemi-acetal converted to impurity C prior to injection. Fig. 2 shows the chromatograms of synthesized impurity C, dissolved in PG and diluted fifty times with ACN or ACN-water buffered at pH 7 with 10 mM phosphate buffer.

In Fig. 2 it is clearly shown that changing the extraction solvent to ACN-water buffered at pH 7 improves the peak shape. Obviously, in the presence of water a hemi-acetal such as compound 4 converts to its aldehyde hydrate form (Impurity C), this conversion is shown in Fig. 3. Similar equilibria have been described before [18], and have shown to be dependent on both the alcohol and aldehyde. Therefore it is likely that when a different solvent is used in a comparable product this equilibrium differs from what we have seen in this study.

3.3. Stress testing results

Stress tests were conducted in PG as a model for the ointment. Since the solubility of TCA in PG is >100 times higher than its solubility in lanolin and petrolatum it can be assumed that TCA is predominantly present in the PG phase and that degradation of TCA will consequently take place in the PG phase within the ointment. Table 1 presents the results.

Table 1 presents an overview of the outcomes of the stress studies. Firstly, six degradation products were found in the aged TCA ointment. However compound 4 is detected as compound 2 as described in Section 3.2 and thus this degradation product is described as compound 2/4 in Table 1. Secondly, it can be seen that in acidic conditions, in AIBN, peroxide and $CuCl_2$ only minor degradation occurred but in alkaline conditions or when $FeCl_3$ was present degradation was more extensive. Corticosteroids in alkaline conditions have been described before to study degradation patterns [15,19,17]. $FeCl_3$ however is more uncommon to use in corticosteroid degradation studies. Thirdly, by combining conventional stress tests (acid, base and peroxide) with the more unconventional AIBN, $CuCl_2$ and $FeCl_3$ (as recommended in literature [12,13]) all degradation products that formed in the actual drug product were found.

3.4. Method validation results

A chromatogram of the four and a half year TCA ointment sample is shown in Fig. 4. In this chromatogram the identity of the peaks is marked with numbers corresponding to the degradation products in Fig. 1.

The method validation results are summarized in Table 2 and further discussed in the following paragraphs.

3.4.1. Accuracy

Recovery of TCA and Impurity C from a freshly prepared ointment matrix was determined three consecutive times. Mean (standard deviation [sd]) recovery from the ointment was 99.5% (0.1%, n = 3) for TCA and 96.9% (1.3%, n = 3) for impurity C respectively.

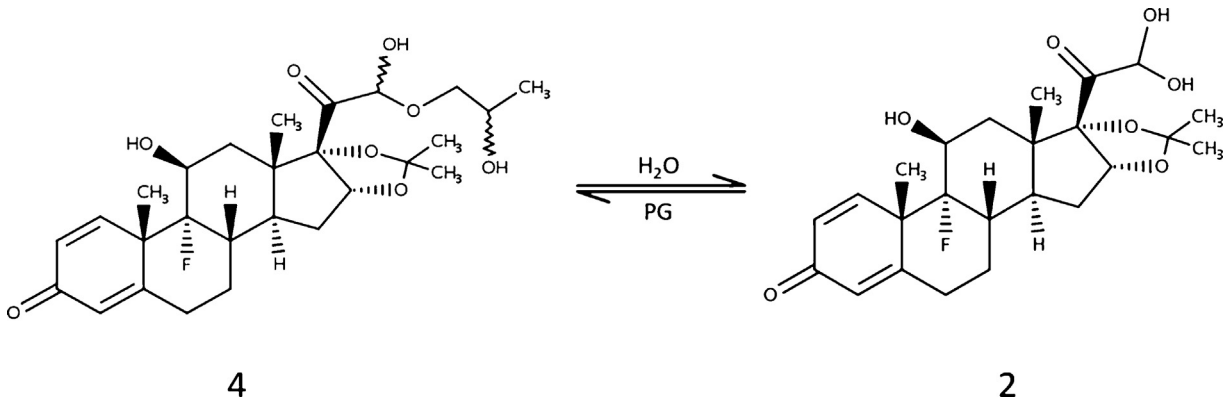

Fig. 3. Equilibrium between Impurity C propylene glycol (PG) hemi-acetal (compound 4) and Impurity C (compound 2).

Table 2
Summary of method validation results, TCA = triamcinolone acetonide Imp. C = Impurity C, if applicable (SD) is presented for n = 3.

	Description of test	Results
Accuracy	Recovery	TCA: 99.5% (0.1); Imp. C: 96.9% (1.3)
Precision	%RSD for 1.25% and 125% of workload	TCA: 0.6% and 0.7%; Imp. C: 0.5% and 0.1%
Specificity	Resolution	>7.1
Quantification limits	LOD	TCA: 0.008%; Imp. C: 0.011%
	LOQ	TCA: 0.025%; Imp. C: 0.032%
Robustness	Resolution old vs new column	6.5 (0.0) (old) vs 7.1 (0.0) (new)
	Resolution mobile phase pH 6.5 vs 7.5	4.5 (0.15) (low pH) vs 6.5 (0.0) (high pH)
	Tailing factor C_{17}-carboxylic acid for pH 6.5 vs pH 7.5	1.45 (0.1) (low pH) vs 5 (0.0) (high pH)
	Recovery after filter retention	TCA: 100% (0.4); Imp. C: 98.9% (0.2)

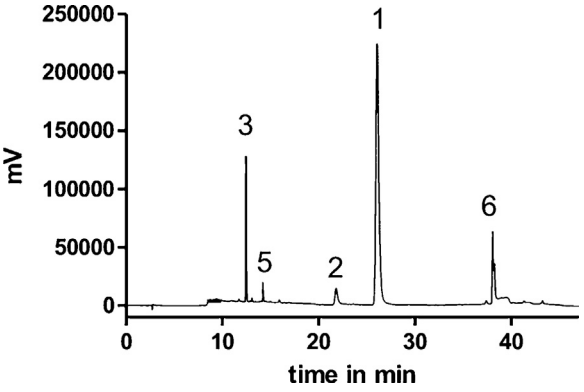

Fig. 4. Chromatogram of a four and a half year sample of 0.1% TCA ointment FNA stored at room temperature throughout shelf life. The peaks are marked with numbers corresponding to the components presented in Fig. 1.

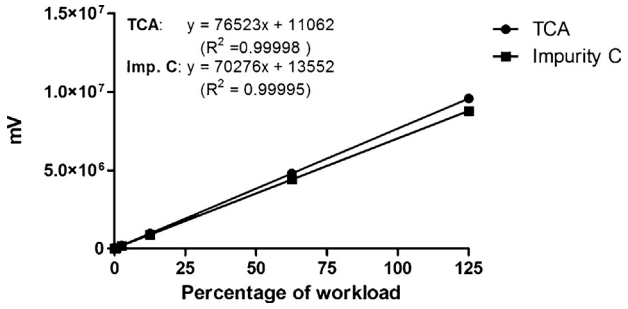

Fig. 5. Triamcinolone acetonide (TCA) and Impurity C (Imp. C) calibration curves for 0.02–125% of the work load.

3.4.2. Precision

RSD for 1.25% and 125% of the workload were 0.6% and 0.7% for TCA and 0.5% and 0.1% for Impurity C respectively.

3.4.3. Specificity

Stressed sample 1 (Table 1) was used during the specificity assessment, because all degradation products were present. Resolution between impurity C and TCA was 7.1. Resolutions amongst other components were greater than 7.1. Matrix components showed no interfering peaks.

3.4.4. Linearity, range, LOD and LOQ

The equation of the calibration curves were y = 76523x + 11062 with R^2 = 0.99998 for TCA and y = 70276x + 13552 with R^2 = 0.99995 for Impurity C. Fig. 5 presents the calibration curves. LOD and LOQ were 0.008% and 0.025% for TCA and 0.011% and 0.032% for Impurity C respectively.

3.4.5. Robustness

Robustness with respect to the column was performed by three consecutive injections of stressed sample 1 (Table 1) on both an old and a new column of the same type. Mean (sd) resolutions between TCA and Impurity C using the old and new column were 6.5 (0.0, n = 3) and 7.1 (0.0, n = 3) respectively.

Robustness with respect to the mobile phase pH was performed by three consecutive injections of stressed sample 1 using mobile phase buffered at pH 6.5 and 7.5. Mean (sd) resolutions between TCA and Impurity C for pH 6.5 and 7.5 were 4.5 (0.15, n = 3) and 6.5 (0.0, n = 3) respectively. Mean (sd) tailing factors of C_{17}-carboxylic acid for pH 6.5 and 7.5 were 1.45 (0.1, n = 3) and 5 (0.0, n = 3) respectively.

Robustness with respect to filter retention was performed by three injections of 125% of the work load using both filtered and unfiltered samples. Mean (sd) recoveries for the filtered and unfiltered were 100% (0.4%, n = 3) for TCA and 98.9% (0.2%, n = 3) for Impurity C respectively.

4. Conclusion

A stability indicating HPLC-UV method was developed, validated and applied to a relevant pharmaceutical ointment, TCA ointment FNA. During the development degradation products were identified and an innovative method to influence a hemi-acetal aldehyde hydrate balance was found in adapting the extraction solvent. Only by using uncommon stress tests (AIBN, $CuCl_2$ and $FeCl_3$) all degradation products that were found in the actual product after long term storage were found. Thereby indicating the importance of comprehensive oxidative stress test designs in pharmaceutical development. The described method can be used in practice for TCA ointment FNA and potentially for other ointments as well.

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