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LC separation of calcipotriol from its photodegradation products and protection possibilities using adjuvants

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Abstract

Mobile phase optimization and reversed-phase column characteristics were used to separate photodegradation products from the parent compound, 24-cyclopropyl-9-,10-secochola-5,7,10(19),22-tetraene-1α,3β,24-triol (calcipotriol). Separation between calcipotriol and its degradation products was obtained with an acetonitrile/water (53:47, v/v) mobile phase on a C_{18} Hypersil ODS column (250 mm length, 4.6 mm id, 5 μm particle size) and a flow rate of 1 ml/min. Using this system, the influence of commonly used solvents in dermatology on degradation was studied. The addition of a UV filter in two concentrations was also evaluated for its possible protective effect to light exposure. Propylene glycol and polyethylene glycol 400 decreased the speed of degradation. The sunscreen 2-hydroxy-4-methoxybenzophenone affords a protection proportional to the filter concentration used in the study

Keywords: Calcipotriol; Photodegradation; Photostability; Sunscreen

1. Introduction

Calcipotriol (Fig. 1) is the first representative in its class of compounds to undergo an intensive preclinical and clinical investigation for the treatment of psoriasis [1], and is considered one of the more important recently developed drugs [2]. It is a synthetically prepared vitamin D_3 analogue used for the treatment of psoriasis, a disease characterized by hyperproliferation and incomplete differentiation of the epidermis [3] and whose influence on calcium and bone metabolism is far less than that of the natural hormone, calcitriol [1,4]. Various in vivo and in vitro metabolic studies have been reported on calcipotriol [5,6]. However, no data are available on its degradation under the influence of light where it was mentioned to de-

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grade very fast when exposed [7]. Being an analogue of the light-sensitive vitamin D_3, the loss of potency or the speed at which calcipotriol degrades under the influence of light was studied.

To provoke photodegradation, calcipotriol was exposed to an artificial light source. A xenon lamp was used for daylight simulation because its spectral distribution is resembling solar distribution [8]. However, in order to determine the presence of degradation products, exposure studies have to be followed by an assay of calcipotriol in which it is separated (e.g. chromatographically) from these degradation products. Since the main goal of any separation method is focused towards selection of optimum conditions [9,10] that will give a satisfying separation of all components in a single run, first the analysis time of the method was optimized by determining an appropriate mobile phase solvent strength. By optimizing the mobile phase selectivity and changing the column characteristics, the speed and sensitivity of the analysis may be enhanced [10].

In view of a resulting loss of drug potency as the undesired outcome of photoinstability [11], light protection studies for calcipotriol were explored. Since there are various solvents used in the study of vitamin D_3 formulations [12–14], four solvents were selected and studied for their possible protective influence on the degradation of calcipotriol as a function of exposure to an artificial light source. Also, a light absorber in the form of a sunscreen was examined. The results of this work will mainly be beneficial to studies related to stability testing.

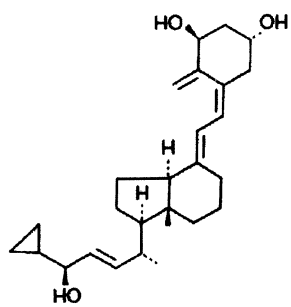

Fig. 1. Structural formula of calcipotriol.

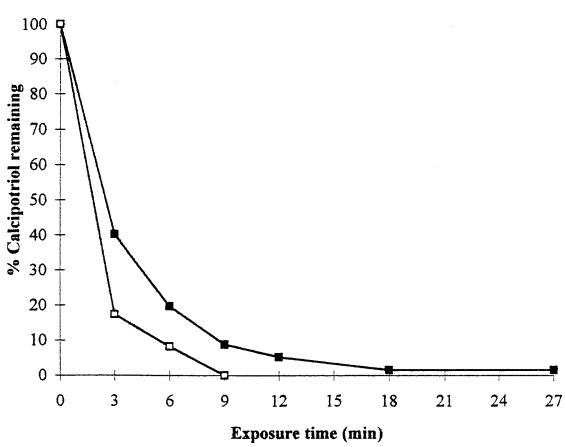

Fig. 2. Degradation profile of calcipotriol (18.64 μg/ml) using two electrical currents: □, 26 A; ■, 13 A. Chromatographic conditions: LiChrosphere C_{18} (5 μm, 125 mm × 4 mm i.d.), flow rate of 1 min/ml, methanol/water (80:20, v/v).

2. Experimental

2.1. Materials

2.1.1. Photodegradation of calcipotriol

A xenon high pressure lamp XM-450 H/V (ORC Lighting Products, CA) attached to a Carl Zeiss photometer system with a Siemens VX 501 r-5b voltage regulator (Oberkochen, Germany) set at 13 A and 20 V, were used. The lamp is used without filters. The sample holder house with a quartz cuvet of 1-cm pathlength containing the solution is placed at a distance of 28 cm from the xenon lamp. The temperature did not increase more than 3°C during irradiation. For the HPLC experiments with UV detection: a Merck-Hitachi L-6000A Pump (Darmstadt, Germany) with a Perkin–Elmer Elmer LC 90 UV spectrophotometric detector (Connecticut, USA) attached to a Merck-Hitachi D-2500 integrator were used. For the HPLC with diode array detection: Shimadzu LC-10AD pump (Shimadzu, Japan) with a Shimadzu SPD-M10A diode array detector. The loop volume is 20 μl. For solvent degassing, an ultrasonic bath was utilized (Retsch USG, Haan, Germany).

Calcipotriol in isopropanol (1.864 mg/ml) was obtained from Leo Pharmaceuticals (Ballerup,

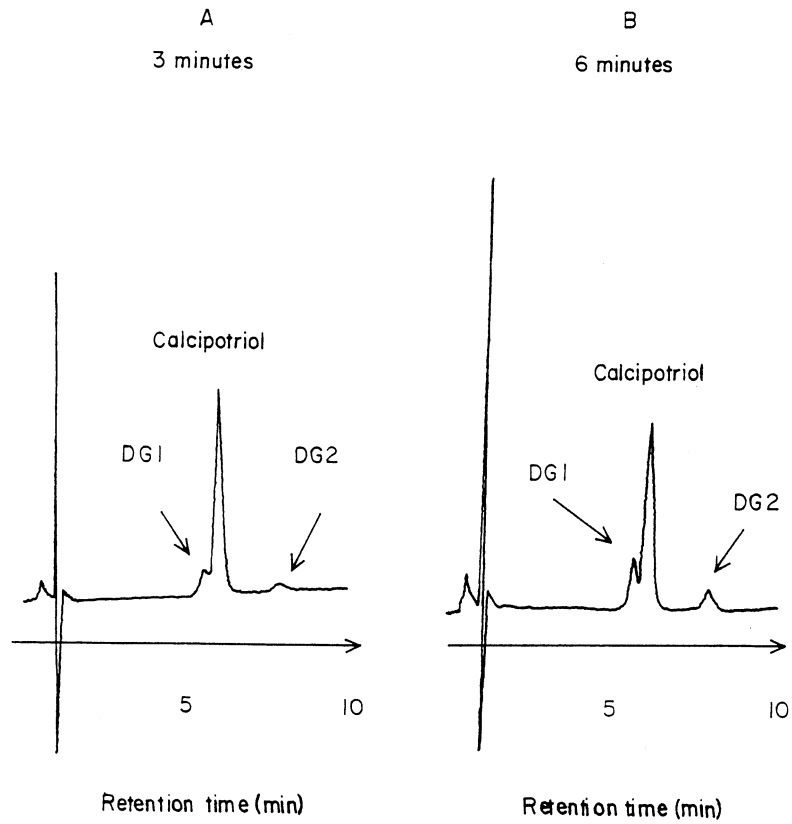

Fig. 3. Two chromatograms from a calcipotriol sample (18.64 μg/ml) exposed to an artificial light source for 3 and 6 min, respectively. Both show the presence of two peaks besides calcipotriol (retention time = 6.02 min). Chromatographic conditions: LiChrosphere C_{18} (5 μm, 125 mm × 4 mm i.d.), flow rate of 1 min/ml, methanol/water (80:20, v/v). DG, degradation products.

Table 1
Resolution data concerning the effect of solvent strength (SST) and organic modifier on the separation of calcipotriol and two unknown degradation products (DG1 and DG2) using a Lichrosphere RP-C_{18}, 125 mm length, 5 μm column

Mobile phase	Composition (v/v)	SST	*k′*			*Rs*	
			1	2	3	R_{12}	R_{23}
MeOH/H_2O	80/20	2.1	4.65	5.08	6.96	0.75	2.26
ACN/H_2O	65/35	2.1	2.44	2.66	2.92	0.77	0.94
MeOH/H_2O	65/35	1.7	40.35	44.42	67.54	1.16	4.51
ACN/H_2O	53/47	1.7	5.02	5.53	6.03	0.94	0.82

MeOH, methanol; ACN, acetonitrile; H_2O, water; SST, solvent strength; *k′*, capacity factor; *Rs*, resolution; R_{12}, resolution DG1–calcipotriol; R_{23}, resolution calcipotriol–DG2.

Denmark); methanol for chromatography was purchased from E. Merck, acetonitrile for HPLC was supplied by Lab-Scan (CRB, Brussels, Belgium); isopropanol p.a. by Merck; propylene glycol BFVI by Lab Fraver (Kontich, Belgium); polyethylene glycol 400 by Lab Flandria (Gent, Belgium); 2-hydroxy-4-methoxybenzophenone by H&R (Holzminden, Germany); water was purified

by Seralpur Pro 90CN (Merck-Belgolabo, Overijse, Belgium). Types of columns used: (i) LiChrosphere C_{18} (5 μm, 125 mm × 4 mm i.d.) from Merck; (ii) Hypersil ODS C_{18} (5 μm, 250 mm × 4.6 mm i.d.) from Alltech (Laarne, Belgium); (iii) Spherisorb ODS-2 C_{18} (3 μm, 150 mm × 4.6 mm i.d.) from Alltech; (iv) Alltima C_8 (5 μm, 250 mm × 4.6 mm i.d.) from Alltech; and (v) LiChrosorb CN (5 μm, 250 mm × 4 mm i.d.) from Merck.

2.2. Methods

Samples were prepared by taking 100 μl of a 1.864 mg/ml calcipotriol solution in isopropanol, diluting with the solvent under study to make a 18.64 μg/ml exposure concentration, placing it in a quartz cuvet (Hellma, Edegem, Belgium) with 1 cm pathlength and exposing to a xenon lamp, as described previously. For samples with UV filter, 7.35 and 4.9 mg/ml stock solutions of 2-hydroxy-4-methoxybenzophenone in methanol were prepared and further diluted with methanol to make exposure concentrations of 7.35 and 4.9 μg/ml. This solution is then used to dilute the 100 μl of calcipotriol to prepare the exposure concentration of calcipotriol described above. Retrieval for all samples was every 30 s during the first 3 minutes and every 3 min thereafter. The solution retrieved is diluted six times with mobile phase for HPLC analysis. Prior to HPLC analysis, the samples were stored in the refrigerator. All samples were prepared under red light at ambient temperature. Samples at all times were kept in amber colored tightly closed bottles, wrapped in aluminium foil and stored at −5°C until use.

2.3. Chromatographic conditions

Methanol and acetonitrile were used as the mobile phase organic modifiers. Each mobile phase was filtered (0.22 μm filter, Millipore, Molsheim, France) and degassed in an ultrasonic bath during at least 5 min. For analysis, the flow rate was set at 1 ml/min. The detection wavelength was set at 263 nm, the wavelength of maximum

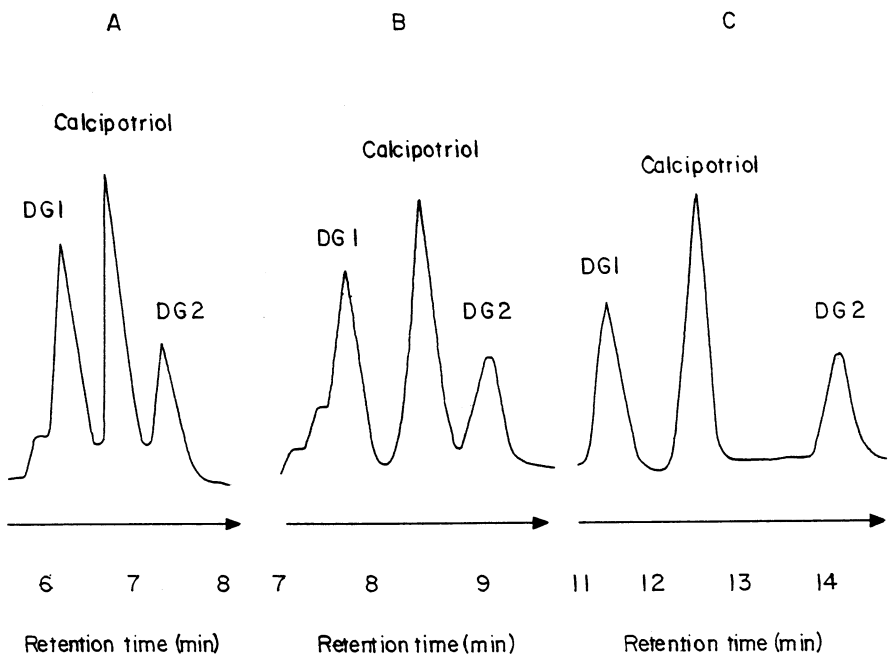

Fig. 4. The effect of the change in RP-C_{18} column characteristics on the separation of components using acetonitrile/water, 53:47 v/v as mobile phase: (A), a Lichrospher 100 prepacked column, 125 mm × 4 mm i.d.; (B), Spherisorb ODS-2 150 mm × 4.6 mm i.d.; (C), Hypersil ODS 250 mm × 4.6 mm i.d..

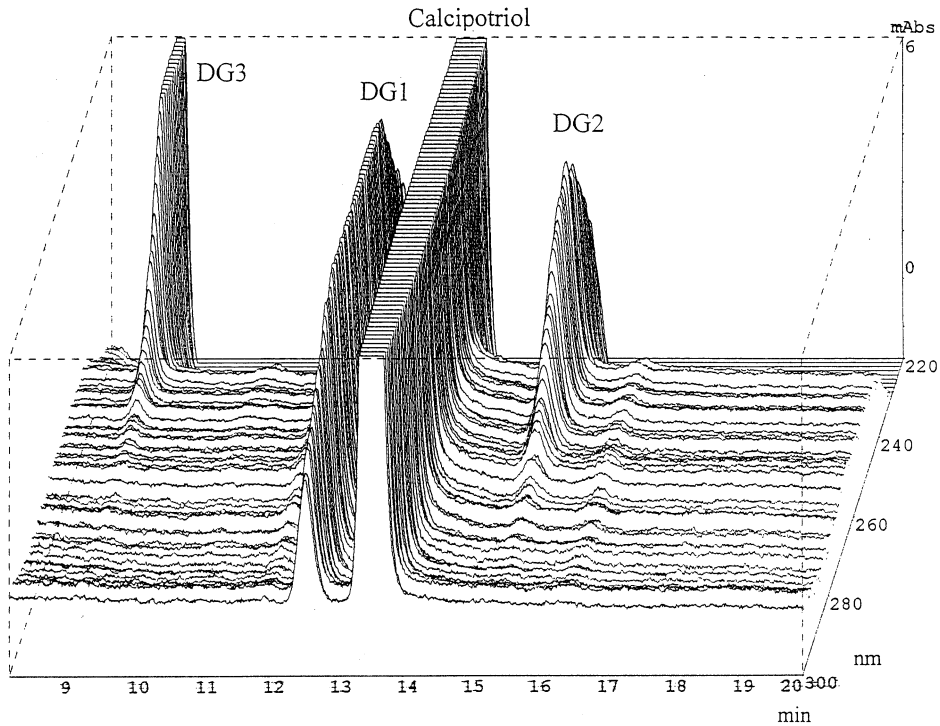

Fig. 5. Diode array chromatograms showing calcipotriol and three degradation products.

absorbance for calcipotriol. Additional detection at 215, 225, 245 and 280 nm was conducted for the photodegradation studies. More details about the mobile phase composition can be found in Section 3.

2.4. Validation of the analysis

The validation of the analysis was done as mentioned before [12]. The robustness was verified by changing the selected mobile phase from 50:50 to 55:45% (v:v) acetonitrile:water.

3. Results and discussion

3.1. Selection of the current of the photometer

It was described [7] that calcipotriol degrades fast under UV light. A xenon lamp was selected to simulate daylight since its spectral distribution is close to solar distribution [8]. The influence of solvent composition on degradation due to irradiation can be proportionally compared. In order to properly track-down the degradation profile of calcipotriol, a search was initiated to determine the amount of electric current that gives an acceptable speed of degradation allowing sufficient time for consecutive retrievals of the exposed sample. Two currents were compared, 26 and 13 A. A methanol/water 80:20 (v:v) mobile phase, a Lichrosphere C_{18} column and a detection wavelength of 263 nm was used to quantify, based on earlier results [12]. At 26 A, the degradation of calcipotriol in methanol was too fast, while it was considerably slower at half the electric current (Fig. 2). The current of 13 A was selected.

Retrieval times were 3 and 6 min after time zero (t_0), the start of exposure. After 6 min, both degradation peaks were clearly detected (Fig. 3). Longer exposures did not show additional degradation peaks at 263 nm. Therefore, 6 min exposure time was selected to optimize HPLC separation.

3.2. Chromatographic conditions

In order to have an appropriate assay of calcipotriol for the stability studies, it is necessary to separate it from all other peaks. Mobile phase optimization following Snyder's solvent triangle [15] was undertaken to locate chromatographic conditions that allow a good separation. In the previous experiments, a reversed phase high performance liquid chromatographic approach with methanol/water, 80:20 v/v mobile phase and a C_{18} column was used. This binary mixture with a solvent strength (SST) of 2.1 was found to separate calcipotriol from calcitriol [12]. However, this mobile phase and a C_{18} LiChrosphere column did not completely separate degradation product DG1 from calcipotriol. The resolution between DGl and calcipotriol is less than one ($Rs = 0.75$) but the individual capacity factors of the four peaks were acceptable (between 1 and 10) namely 3.3, 4.6, 5.1 and 7.0 for DG3, DG1, calcipotriol and DG2, respectively. A second mobile phase was selected by changing the organic modifier in the mobile phase from methanol to a less polar solvent, acetonitrile, at constant SST. There was no good separation and resolutions were all below one (Table 1). A resolution of 1.0 or higher is desired [16] to have an acceptable separation. The SST was then decreased by using a mobile phase with methanol/water (65:35, v/v). The new SST = 1.7 gave a better separation, with $Rs = 1.2$ and 4.5 between DG1 and calcipotriol, and between calcipotriol and DG2, respectively. However, the capacity factors (k') were considered far too large for the three components, namely 40.4, 44.4 and 67.5. The mobile phase with acetonitrile/water (53:47, v/v) having the same SST of 1.7 (Table 1) resulted in nearly separated components (Fig. 4A).

By analyzing the trends of k' and Rs presented in Table 1, further decrease in SST with methanol as the organic modifier will increase k', while with acetonitrile, a decrease in the resolution between calcipotriol and DG2 (R_{23}) will be seen. There-

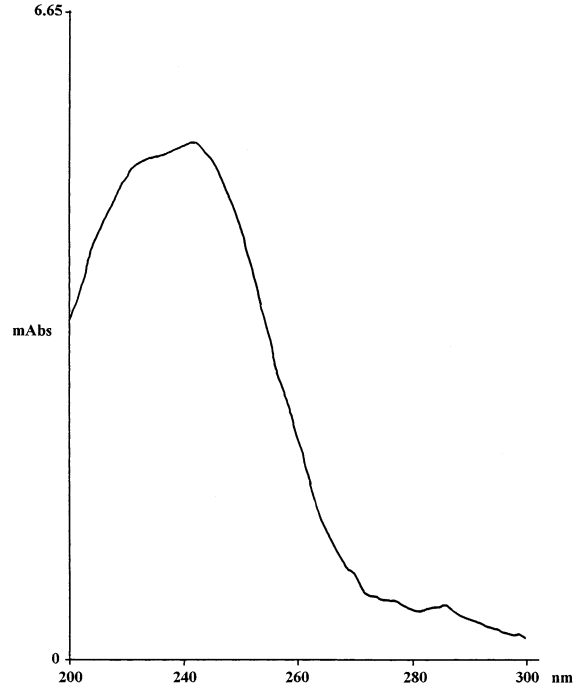

Fig. 7. Diode array spectrum for degradation product DG2.

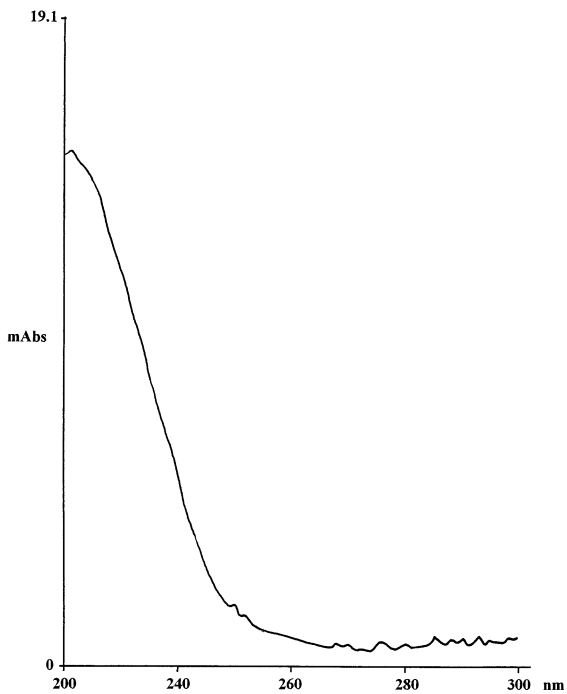

Fig. 6. Diode array spectrum for degradation product DG3.

fore, no further mobile phase optimization was conducted and various columns were tested. So far, the column used in the study was a silica based LiChrosphere RP C_{18} with 5 μm particle size. Considering the fact that a smaller particle size could permit a more adequate separation, 3 μm was used with a slightly longer column of 150 mm, Spherisorb ODS-2. A difference in resolution but not a sufficient improvement was noted (Fig. 4B). Because of a linear relationship between column efficiency and column length [16], the column length was changed to 250 mm, Hypersil ODS. The resulting separation was good (Fig. 4C). It was observed that the drug is always eluting between the DG1 and DG2, denoting that DG1 is somewhat more polar than calcipotriol and DG2. However, for the moment, it is unknown which degradation products are formed. The other tested columns, RP C_8 and a cyanopropyl (CN) one, did not gave good results for a acetonitrile/water, 53:47 v/v as mobile phase. For the RP-C_8, only calcipotriol eluted within the running time of 60 min. For the cyanopropyl (CN) column, no components were eluting within an hour.

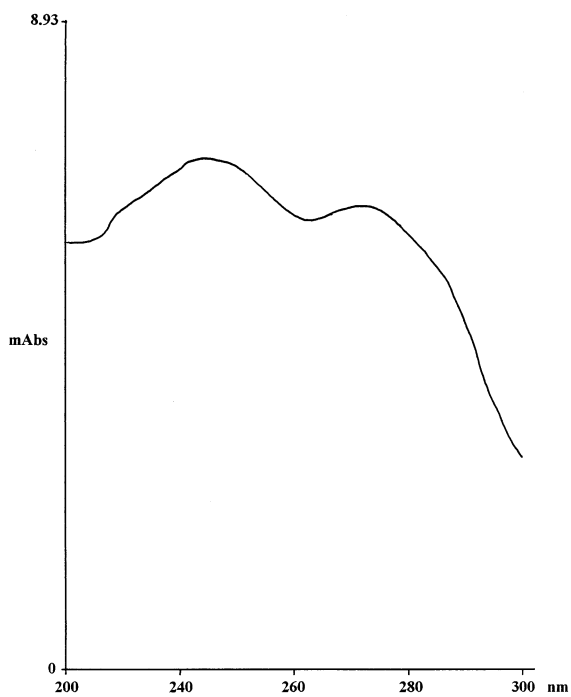

Fig. 8. Diode array spectrum for degradation product DG1.

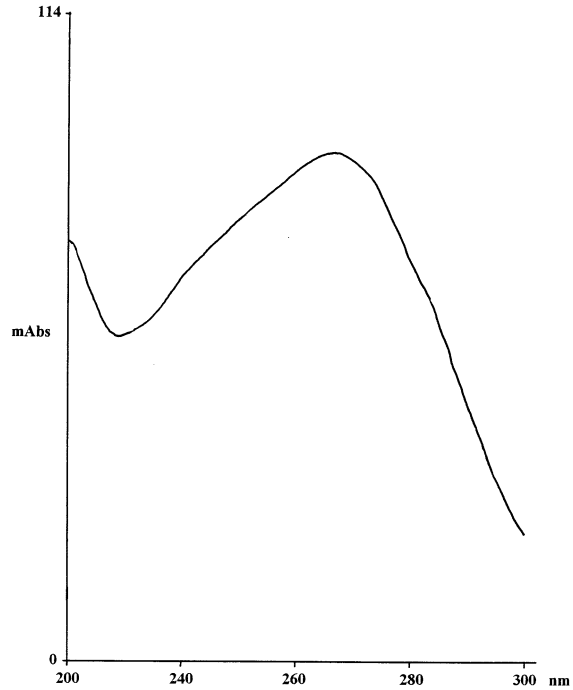

Fig. 9. Diode array spectrum for calcipotriol.

Table 2
Solvents selected for the photoprotective study

Solvent	Structural formula	Use
Methanol	CH_3OH	Solvent in the study of calcipotriol
2-Propanol (isopropyl alcohol)	$CH_3CH(OH)CH_3$	Solvent for calcipotriol supplied from manufacturer
1-,2-Propanediol (propylene glycol)	$CH_3CH(OH)$ $-CH_2OH$	Solvent in pharmaceutical preparations (i.e. topical)
Polyethylene glycol 400	$OH(C_2H_4O)_nH$	Solvent in pharmaceutical preparations (i.e. topical)

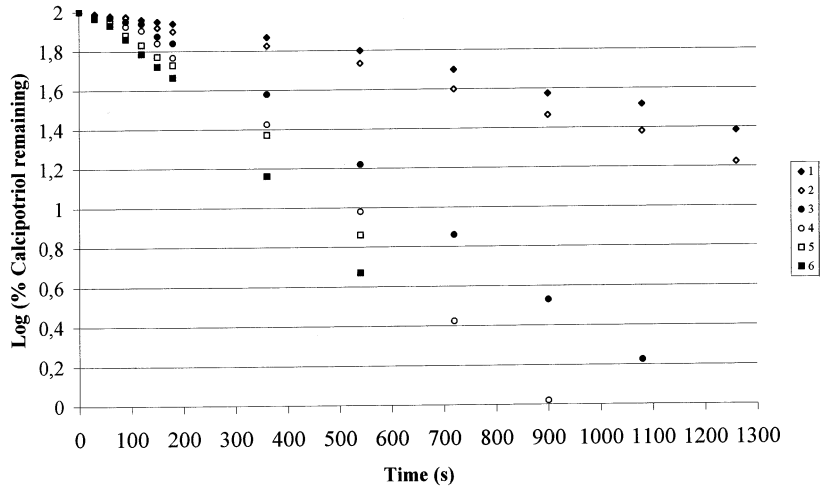

Fig. 10. Log (% calcipotriol remaining) as a function of exposure time. Solvents: ■, methanol; □, isopropanol; ●, propylene glycol; ○, PEG 400; ◆, UV filter 7.35 μg/ml; ◇, UV filter 4.9 μg/ml.

Several wavelengths were then studied for the presence of other degradation products. Since initially no diode array detector was at our disposal, only at a limited number of wavelengths was measured, 215, 225, 245 and 263 nm. A third peak (DG3) was detected at 215–245 nm with retention time of 9.5 min and well separated from the other two degradation products.

To check the peak purity, chromatographic analysis was performed with a photodiode-array detector, which allowed a simultaneous monitoring in the range of wavelengths between 200 and 300 nm. At the same time, in order to know if at a higher concentration (since originally we were limited in the concentration that could be applied) a separation of the three components still can be achieved, a 100 μg/ml calcipotriol in methanol was exposed for 6 min following the procedure stated in Section 2. After exposure, this was diluted with acetonitrile/water, 53:47 v/v (mobile phase) to make 50 μg/ml solution for injection to avoid that the sample solvent would have a too high solvent strength compared to the mobile phase using again the Hypersil ODS C_{18} column. The fourth peak, already earlier noted at 215 nm was clearly detected (Fig. 5).

The individual spectra are shown in Figs. 6–9. We note that DG3 shows no absorption at 263 nm. DG2 shows a maximum at 241 nm, DG1 two maxima, the first at 242 nm, the second at 275 nm. All are different in spectrum from calcipotriol. As a conclusion, we can state that at higher concentrations the calcipotriol peak is still well separated from the three degradation products, enabling stability studies on calcipotriol.

Validation was executed at 263 nm, maximum wavelength of calcipotriol. For the limit of detection, 10 ng/ml was noted. The limit of quantification by injecting six times was considered 20 ng/ml calcipotriol with a 3.5% relative standard deviation. Linearity was observed from 20 to 100 ng/ml with a correlation coefficient of 0.999.

Table 3
Test for differences in protective properties of the solvents at exposure times from t_0 to t_{180} ($t'_{(95\%)}$, critical value; $t_{calc.}$, calculated value)

Solvents	t-value, $t_0 - t_{180}$		
	$t_{calc.}$	$t'_{(95\%)}$	Sig.
Propylene glycol, PEG 400	0.2281	3.4538	NS
PEG 400, isopropanol	0.4063	0.5014	NS
Isopropanol, methanol	0.6886	3.0822	NS
PEG 400, methanol	0.8434	0.753	S
Propyleneglycol, methanol	1.0052	0.5504	S
Propylene glycol, Isopropanol	0.6279	0.3717	S

Changing the mobile phase, composed of acetonitrile:water to 55.45 and to 50:50% (v:v) had no noticeable effect on separation of calcipotriol and its degradation products. It could therefore be concluded that robustness was good.

3.3. Influence of solvents on photodegradation

Photo stability testing of a drug substance involves a study of the degradation rate of the drug in solution when exposed for a period of time to a source of irradiation [8]. Since the influence of solvents on the degradation rate of a given drug is of great importance [17], and since the degradation of calcipotriol is known to be fast [7,12], the following investigations were conducted to determine the possible influences of solvents used in dermatology, on the degradation of calcipotriol. The solvents selected are given in Table 2. For this part of our work, chromatographic detection was fixed at 263 nm, the maximum wavelength of calcipotriol. Since it is customary within the pharmaceutical industry to express drug degradation referring to the time to lose a certain amount [8], the solvents were first evaluated in this way. It was observed (Fig. 10) that calcipotriol dissolved in propylene glycol lost potency the slowest among the solvents studied followed by polyethylene glycol (PEG) 400, while in methanol and isopropanol it was faster. This protective effect of propylene glycol was also detected in a photostability study on nifidipine [11]. As polar solvents tend to accelerate reactions [8], methanol gave the fastest degradation.

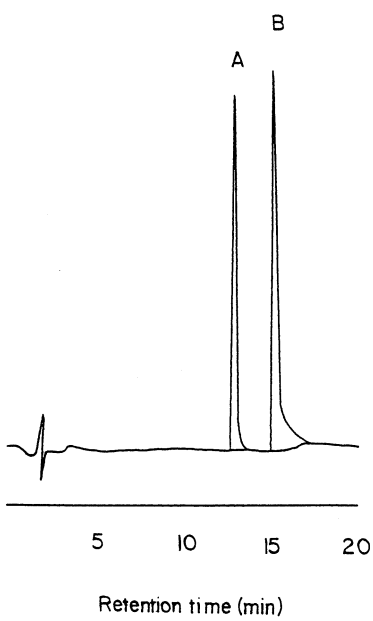

Fig. 11. Chromatogram of calcipotriol (A) and the UV filter (B). Column, Hypersil ODS RP18 (5 μm), 4.6 × 250 mm, mobile phase: acetonitrile/water (53:47, v/v).

The detection of three degradation products, DG1 and DG2 appearing first, and DG3 appearing when DG2 diminishes, suggests a complex degradation pattern. However, when plotting the remaining log concentration of calcipotriol as a function of time (Fig. 10), the results suggest an overall bimodal exponential first-order reaction [17], one until about 180 s of exposure and another until the end of exposure.

To compare the protective effect of the solvent to the degradation of calcipotriol, a statistical method [18] was used where the degradation lines in different solvents were compared by means of a t-test. Since there are two slopes observed in each solvent, the calculations could be applied to each pathway. However due to too few measurements for $t_{180} - t_{\infty}$, only data from $t_0 - t_{180}$ are presented (Table 3). There was no significant difference observed for slopes with propylene glycol and polyethylene glycol, polyethylene glycol and isopropanol, and isopropanol and methanol at a 5% level of significance. Slopes between propylene glycol and methanol, polyethylene glycol and methanol, polyethylene glycol and methanol, and propylene glycol and isopropanol which are further from each other were found to be significant at 5%. The results reported concern t-tests between the different lines. No global conclusion from the results of Table 3 is allowed since that concerns a multiple comparison problem which would require a correction of the applied α-values. The significant differences found between the different solvents, indicate however, that there is a difference in the decrease of calcipotriol or in the protective effects. The results seem to indicate that propylene glycol is more protective than for instance methanol or isopropanol.

3.4. Influence of a sunscreen

With calcipotriol absorbing in the short-wave region of the daylight spectrum, a light absorber in the form of a UV filter was tried. UV filters provide vital protection against damaging effects of UV radiation [19]. Thoma and Klimek described the principle of photoprotection by spectral overlapping [20]. 2-Hydroxy-4-methoxybenzophenone is a broad spectrum UV absorber in the short-wave region and commercially available as a sunscreen [21]. The chromatographic separation between calcipotriol and 2-hydroxy-4-methoxybenzophenone was obtained with the acetonitrile/water (53:47, v/v) mobile phase and the C_{18} Hypersil ODS column, the same chromatographic system that separated calcipotriol from the degradation products as described earlier (Fig. 11). Two concentrations of 2-hydroxy-4-methoxybenzophenone were selected for the exposure study based on its spectrum of absorption and on the principle of spectral overlapping.

HPLC results showed that the amount of calcipotriol remaining as a function of exposure time is higher in the solution with a higher amount of UV filter (7.35 μg/ml) compared to the solution containing a smaller concentration (4.9 μg/ml). The concentration of 2-hydroxy-4-methoxybenzophenone seems to give a proportional protective effect on calcipotriol in the examined range (Fig. 10). From Fig. 10, it can be observed that the UV filter in both concentrations gave a better protective effect for calcipotriol than the various solvents used. After 900 min of irradiation, the protective factor of the highest concentration of the UV filter is ca. 1.5 higher than with the lowest concentration and ca. ten times higher than with propyleneglycol. From the combined results one could expect that the use of the sunscreen with propylene glycol (instead of methanol as was here the case) as solvent would form the most protective combination.

4. Conclusion

The chromatographic system described in this work can mainly be used in the test for the presence of photodegradation products in the raw material and during stability studies for calcipotriol. It was also shown that in the presence of an artificial light source, the degradation of calcipotriol is very fast and three degradation products were found. For the photoprotective study of solvents, propylene glycol affords the slowest degradation to calcipotriol among the solvents tested. In all solvents used, the degradation of calcipotriol undergoes at least two pathways and can be described as a bimodal overall first order reaction. More experiments are necessary to study these degradation products in detail. 2-Hydroxy-4-methoxybenzophenone as an UV filter affords a protection against degradation relative to the filter concentration used. This addition of the UV filter can enhance the shelf life of dermatological calcipotriol preparations.

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