





## Article

# Photodegradation Assessment of Calcipotriol in the Presence of UV Absorbers by UHPLC/MS<sup>E</sup>

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## Abstract

Calcipotriol, a synthetic vitamin D<sub>3</sub> analogue widely used in psoriasis treatment, requires a detailed stability assessment due to its topical application and potential exposure to UV radiation. As a drug applied directly to the skin, calcipotriol is particularly susceptible to photodegradation, which may affect its therapeutic efficacy and safety profile. The present study focuses on the analysis of calcipotriol photostability. An advanced UHPLC/MS<sup>E</sup> method was employed for the precise determination of calcipotriol and its degradation products. Particular attention was given to the effects of commonly used organic UV filters—approved for use in cosmetic products in both Europe and the USA (benzophenone-3, dioxybenzone, meradimate, sulisobenzon, homosalate, and avobenzone)—on the stability of calcipotriol. Unexpected degradation of calcipotriol was observed in the presence of sulisobenzon. Importantly, this effect was consistently detected in methanolic solution and in the pharmaceutical formulation containing calcipotriol and betamethasone, which is particularly significant from a practical perspective. This finding underscores the necessity of evaluating photostability under real-life conditions, as cosmetic ingredients, when co-applied with topical drugs on the skin, may substantially influence the stability profile of the pharmaceutical active ingredient. The research resulted in the first-time characterization of four degradation products of calcipotriol. The degradation process was found to primarily affect the *E*-4-cyclopropyl-4-hydroxy-1-methylbut-2-en-1-yl moiety, causing its isomerization to the *Z* isomer and the formation of diastereomers with either the *R* or *S* configuration. Computational analyses using the OSIRIS Property Explorer indicated that none of the five degradation products exhibit a toxicity effect, whereas molecular docking studies suggested possible binding of two of the five degradation products of calcipotriol with the VDR.

**Keywords:** calcipotriol calcipotriene; drugs photostability; UV absorbers; vitamin D analogs; psoriasis



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## 1. Introduction

Calcipotriol (Calcipotriene) is a vitamin D analogue used topically for psoriasis treatment. Psoriasis is a chronic disease characterized by autoimmune and genetic origins, resulting in excessive proliferation of keratinocytes and impaired differentiation [1]. In psoriasis, increased T lymphocyte activity and elevated levels of IL-23 and IL-17 cause inflammation and epidermal hyperplasia [2]. Subclinical inflammation can persist even

after skin lesions have healed completely, potentially leading to exacerbations of the disease [3]. Psoriasis affects around 2% of the global population and primarily presents as chronic plaque psoriasis in 90% of cases [1]. Typically, the disease manifests with mild symptoms and localized skin lesions, making topical therapy the preferred treatment option [4]. Psoriasis is an incurable condition, and treatment focuses on managing symptoms and improving quality of life [5]. In addition to skin symptoms, psoriasis can cause thick, ridged, or pitted nails. Severe forms of psoriasis, such as generalized pustular psoriasis, may also lead to systemic symptoms like fever, malaise, and fatigue. In addition to cutaneous manifestations, psoriasis is associated with several comorbidities, including psoriatic arthritis (joint pain, stiffness, and swelling), cardiovascular disease, and metabolic syndrome. Early diagnosis and effective management are crucial to reduce disease burden and improve patient outcomes. Psoriasis significantly reduces patients' quality of life, as its unpleasant symptoms and widespread social stigma often lead to emotional distress and feelings of marginalization [6,7]. For mild to moderate conditions, first-line treatment includes topical corticosteroids, vitamin D analogues, and retinoids. For patients with severe conditions, topical medications are used alongside systemic therapy options such as methotrexate, cyclosporine, and dimethyl fumarate [2].

Corticosteroids are the most common topical treatment for psoriasis; however, the prolonged use of corticosteroids may cause side effects, including corticosteroid-induced skin atrophy, telangiectasia, and tachyphylaxis. The therapeutic efficacy of corticosteroids can be enhanced by the combination with a vitamin D analogue [2,5]. Medicinal products typically contain calcipotriol (50 µg/g) and betamethasone dipropionate (0.5 mg/g), which act synergistically. Calcipotriol induces keratinocyte differentiation and inhibits their proliferation. Betamethasone dipropionate exhibits local anti-inflammatory, antipruritic, immunosuppressive, and vasoconstrictive properties [2]. In randomized, double-blind clinical trials conducted by Douglas et al., a combination of these two substances was shown to be superior to monotherapy with either betamethasone dipropionate or calcipotriol in treating psoriasis vulgaris, demonstrating a faster onset of action [8]. Furthermore, calcipotriol counteracts skin atrophy induced by betamethasone dipropionate [9]. Topical medications containing calcipotriol and betamethasone dipropionate are available in the European Union as gel, ointment, and foam formulations. Among these, the foam formulation is considered particularly innovative due to its enhanced efficacy, improved skin penetration, and greater patient acceptability compared to traditional ointment and gel forms. After applying the foam, the propellants evaporate, leaving a stable, supersaturated solution of calcipotriol and betamethasone without forming crystals [10,11].

Applying medicinal products to the skin for a local effect facilitates safe use of the drug and reduces the risk of adverse effects. The success of local therapy is determined by reaching the necessary concentration of the active pharmaceutical ingredient at the target location. Medications applied topically are exposed to ultraviolet radiation, which can result in photodegradation of the drug. This process can lead to a reduction in the active substance and the generation of by-products with unknown effects [12,13]. Previous research has shown an interaction between calcipotriol and ultraviolet radiation. Lebwohl M. et al. [14] conducted a study in which calcipotriol ointment was applied before and after phototherapy; the ointment was collected and analyzed using reverse-phase high-performance liquid chromatography. UVA exposure led to a decrease in detectable calcipotriol concentrations, indicating that when calcipotriol is used in combination with PUVA, it should be applied after UVA exposure [14]. In research conducted by Jahani M. et al. [15], mometasone furoate, calcipotriol, and their combinations were exposed to various stress conditions. The calcipotriol degradation product, identified as DP4, was formed under conditions of heat exposure, UVA radiation, and oxidation with H<sub>2</sub>O<sub>2</sub>. Based

on UV and LC-MS fragmentation patterns, DP4 was identified as pre-calcipotriol [15]. Bhogadi R. K. et al. [16] developed an RP-HPLC method for the separation of known (Calci Impurity-C, Calci Impurity-B, and Calci Impurity-D) and unknown isomeric impurities of calcipotriol from calcipotriol, pre-calcipotriol, and betamethasone dipropionate. The pre-calcipotriol solution was obtained in a vial by dissolving 2 mg of calcipotriol in 200  $\mu$ L of a solution prepared by combining 1 mL of triethylamine and 9 mL of chloroform, which was closed and kept in a water bath at 60 °C for 2 h. Pre-calcipotriol is an isomer of calcipotriol, formed as a result of a reversible transformation that begins when calcipotriol is prepared in solution and continues until equilibrium is reached. This process also occurs in medicinal products and is affected by temperature. Pre-calcipotriol is not considered an impurity, and the effect of calcipotriol results from both substances. Studies using LC-MS, LC-MS/MS, FT-IR, and NMR confirm the isomeric relationship and structural formula of pre-calcipotriol. It is predicted that calcipotriol undergoes Cis/Trans isomerism at carbon 7, forming Imp-B. This is followed by a rearrangement of double bonds, resulting in pre-calcipotriol [16]. In addition to pre-calcipotriol, other identified isomers of calcipotriol include trans-calcipotriol (5E, 7E), 24R-calcipotriol, and the beta-calcipotriol isomer. These compounds are primarily formed during the synthesis of calcipotriol [16–18]. Vitamin D isomers with (5E, 7E) triene geometry have higher biological activity compared to natural (5Z, 7E) vitamin D compounds [17,19]. The beta-calcipotriol isomer can be utilized as a reference standard for analytical method development or method validation [20].

Photoprotection is recommended by dermatological societies as a key strategy in the prevention of skin cancer. Therefore, a comprehensive understanding of the potential interactions between substances applied simultaneously to the skin, such as UV filters and active pharmaceutical ingredients, is essential to ensure the safe and effective use of topical drug products [12].

Previous studies on tazarotene, bexarotene, terbinafine, and bifonazole have shown that UV filters can significantly impact the stability of drug substances when exposed to UV radiation [21–24]. Building on these findings, the present study aimed to evaluate how commonly used UV filters affect the stability of calcipotriol. To achieve this, a range of filters capable of absorbing UV-A and UV-B radiation was selected for the experiments. The sunscreen agents tested included salicylic acid derivatives such as homosalate (homomenthyl salicylate), which exhibits maximum absorption at 306 nm; dibenzoylmethane derivatives like avobenzone (butyl methoxydibenzoylmethane), serving as UVA I filters with a peak at 357 nm; and benzophenone derivatives including oxybenzone (benzophenone-3, 2-hydroxy-4-methoxybenzophenone) which absorbs in both the UVB (280–315 nm) and short-wavelength UVA (315–355 nm) ranges. Additionally, dioxybenzone (benzophenone-8) and sulisobenzone (benzophenone-4) were included, both characterized by two absorption peaks at 286 nm and 440 nm. Meradimate, a UVA filter exhibiting maximum absorption at 385 nm, was also included in the study [25–27].

The aim of this study was to assess the photostability of calcipotriol under radiation conditions. The UHPLC/MS<sup>E</sup> method used for the determination of calcipotriol was thoroughly validated in accordance with international guidelines, ensuring its accuracy, precision, specificity, and reliability for the assessment of photostability under the tested conditions. The photostability of calcipotriol was tested in the presence of selected chemical UV filters that have been approved for use in cosmetic products in Europe and the United States. Methanol solutions of calcipotriol were tested both with and without the addition of various UV filters, including oxybenzone, dioxybenzone, meradimate, sulisobenzone, avobenzone, and homosalate. Molecular docking simulations were employed to assess the binding potential of the calcipotriol degradation products to the vitamin D receptor (VDR), providing a predictive evaluation of their possible interactions at the molecular level.

## 2. Experimental

### 2.1. Materials and Methods

#### Chemicals and Reagents

Calcipotriol monohydrate (5Z,7E,22E,24S)-24-Cyclopropyl-9,10-secochola-5,7,10(19),22-tetraene-1 $\alpha$ ,3 $\beta$ ,24-triol), pharmaceutical primary standard, European Pharmacopoeia reference standard (Strasbourg Cedex, France), and Calcipotriol TRC (North York, ON, Canada). HPLC-grade methanol, acetonitrile, and formic acid (98%) were purchased from J.T. Baker. Ethanol (99.8%) was obtained from POCH (Gliwice, Poland). Water (quadruple-distilled) with a conductivity of less than 1  $\mu\text{S cm}^{-1}$  was prepared using S2-97A2 distillation apparatus (ChemLand, Stargard Szczecin, Poland). UV absorbers, namely avobenzene (AVB), oxybenzone (Benzophenone-3, BP-3), benzophenone-4 (Sulisobenzene, BP-4), dioxybenzone (Benzophenone-8, BP-8), homosalate, and meradimate, were obtained from Sigma-Aldrich (St. Louis, MO, USA).

#### 2.2. Standard Solution

A stock solution of calcipotriol was freshly prepared by dissolving 2 mg of the standard in methanol and diluting to 10 mL in a volumetric flask, resulting in a final concentration of 0.2 mg mL<sup>-1</sup>. For method validation, a series of six calibration solutions was prepared in the concentration range of 0.015–0.15 mg mL<sup>-1</sup>. To assess photostability, a methanolic solution of calcipotriol at a concentration of 0.2 mg mL<sup>-1</sup> was prepared.

The photostability of calcipotriol was evaluated using methanolic solutions of various photoprotective agents at a concentration of 1 mg mL<sup>-1</sup>. For this purpose, 10 mg of each compound—oxybenzone, meradimate, sulisobenzene, avobenzene, dioxybenzone, and homosalate—was accurately weighed into separate 10 mL volumetric flasks, and the volume was adjusted to the mark with methanol.

#### 2.3. Photodegradation Studies of Calcipotriol

Calcipotriol was irradiated in methanolic solutions at a concentration of 0.1 mg mL<sup>-1</sup>. For the photodegradation studies, the solutions were placed in quartz Petri dishes (50 mm diameter, Hornik, Poland), which were covered with quartz lids to minimize ethanol evaporation and sealed with parafilm for additional protection.

Photostability testing of calcipotriol under UVA conditions was conducted using a KBF-ICH 240APT.line™ climate chamber (Binder GmbH, Tuttlingen, Germany). During irradiation, samples were positioned 13 cm from the light source. For UVA exposure (maximum wavelength 365 nm), the chamber was maintained at a constant temperature of 25 °C and relative humidity of 60%. The radiation dose was measured with a VLX-3 radiometer (Vilber Lourmat) equipped with a CX-365 sensor, yielding a consistent dose rate of  $1.9434 \cdot 10^{-2} \text{ J cm}^{-2} \text{ min}^{-1}$ . Samples were subjected to UVA irradiation for 60 min.

Photodegradation studies of calcipotriol in methanolic solution were carried out in the presence of organic UV filters. In summary, each 2 mL sample was prepared by mixing 1 mL of calcipotriol stock solution, 0.8 mL of methanol, and 0.2 mL of a selected UV filter solution. Control samples, prepared in the same way but wrapped in aluminum foil to exclude light, served as dark controls. All samples were analyzed using UPLC-MS, and each experimental condition was set up in triplicate.

The photostability of calcipotriol from a foam formulation was evaluated using Enstilar foam (LEO Pharma A/S, Denmark), which contains calcipotriol (50  $\mu\text{g/g}$ ) and betamethasone dipropionate (0.5 mg/g). The samples were prepared by weighing 2 g of foam, adding 10  $\mu\text{L}$  of sulisobenzene solution in propylene glycol, and thoroughly homogenizing the preparation in a mortar. The sulisobenzene solution was prepared by dissolving 10 mg of sulisobenzene in 1 g of propylene glycol. Then, 0.5 g of the mixture was spread evenly

on the PMMA plates (poly(methyl methacrylate) plates). The dark control samples were prepared in the same manner and wrapped in aluminum foil. Following irradiation, the foam was collected from the plates using a spatula, transferred into Eppendorf tubes, and weighed. Subsequently, 1 mL of methanol was added to each tube. Extraction was carried out using a tube shaker (Lab Dancer Vario, IKA) for 20 s, followed by 10 min of ultrasonication in a water bath at 40 °C (Elmasonic S40H, Elma, Wetzikon, Switzerland). After extraction, the solution was filtered through a 0.45 µm pore-size filter before the UPLC-MS<sup>E</sup> analysis.

#### 2.4. UHPLC/MS<sup>E</sup> Analysis

The UHPLC-QTOF system consisted of a Waters Acquity I-Class Plus (Waters Corporation, Milford, MA, USA) coupled to a Waters Synapt XS mass spectrometer (electrospray ionization mode ESI-QTOF). Chromatographic separations were carried out using the Acquity UPLC BEH (bridged ethyl hybrid) C18 column, 2.1 × 100 mm and 1.7 µm particle size, equipped with Acquity UPLC BEH C18 VanGuard pre-column, 2.1 × 5 mm and 1.7 µm particle size. The column was maintained at 40 °C and eluted under gradient conditions using from 95% to 0% eluent A over 10 min at a flow rate of 0.3 mL min<sup>-1</sup>. Eluent A: water/formic acid (0.1%, *v/v*); eluent B: acetonitrile/formic acid (0.1%, *v/v*). Chromatograms were recorded using a Waters eλ PDA detector. Spectra were analyzed in the 200–700 nm range with a 1.2 nm resolution and a sampling rate of 20 points s<sup>-1</sup>.

MS detection settings of Waters Synapt XS mass spectrometer were as follows: source temperature 150 °C, desolvation temperature 250 °C, desolvation gas flow rate 600 L h<sup>-1</sup>, cone gas flow 100 L h<sup>-1</sup>, capillary potential 3.00 kV, and cone potential 30 V. Nitrogen was used for both nebulizing and drying gas. The data were obtained in a resolution MS<sup>E</sup> scan mode (full MS scan with simultaneous data-independent fragmentation), ranging from 50 to 1000 *m/z* at 0.2 s time intervals. Leu-enkephalin was used as a mass reference. Analyses were carried out in positive-ionization mode. Collision activated dissociations (CAD) analyses were carried out with the energy ramp from 10 eV to 60 eV. Argon was used as a collision gas. Data acquisition and analysis software were MassLynx v4.2 and MSe Data Viewer v2.0 (Waters Corporation, Milford, MA, USA).

#### 2.5. Method Validation

The purpose of validating the ultra-performance liquid chromatography method combined with tandem mass spectrometry (UPLC-MS/MS) was to ensure its reliability for quantifying calcipotriol, even in the presence of its degradation products. The developed analytical procedure underwent validation following the International Conference on Harmonization (ICH) Q2 (R2) guidelines for analytical method validation [28].

##### 2.5.1. Specificity

The specificity of the method was evaluated by assessing the chromatograms of the mobile phase solution, the chromatograms of the methanolic calcipotriol solution, and the chromatograms after photodegradation in the presence of selected chemical UV filters. The obtained chromatograms were analyzed considering retention time (TR), peak areas, peak shape, and peak separation.

##### 2.5.2. Linearity

The linearity of the method was assessed by analyzing six methanolic calcipotriol solutions in the concentration range of 15–150 µg mL<sup>-1</sup>. Each of the solutions was injected into a column three times in the amounts of 10 µL. Linear regression analysis was conducted using Statistica 13.3 (StatSoft) based on the peak areas and known concentrations of calcipotriol solutions. The slope of the regression line, y-intercept, standard deviation of the

slope and intercept, correlation coefficient,  $R^2$  value, and standard error of residuals for the calibration curve were calculated. The Shapiro–Wilk test was used to determine whether the residuals of the dependent variable have a normal distribution. Furthermore, the homoscedasticity of the random component was evaluated using the Bartlett test, and the presence of autocorrelation in the residuals was examined using the Durbin–Watson test.

#### 2.5.3. Limit of Detection (LOD) and Limit of Quantification (LOQ)

The determination of the limit of detection (LOD) and the limit of quantification (LOQ) was carried out using mathematical equations based on the standard error of estimate (Se) and the slope (a) of the calibration curve. Specifically, LOD was calculated as  $3.3 \times \text{Se}/a$ , while LOQ was determined as  $10 \times \text{Se}/a$ . Additional assessments of precision and accuracy were performed using solutions with concentrations near the established LOD and LOQ to ensure the validity of these calculated limits.

#### 2.5.4. Precision

Method precision was assessed via repeatability studies, where six replicate injections of a methanolic calcipotriol solution at a concentration of  $100 \mu\text{g mL}^{-1}$  (representing 100%) were analyzed within a single day. The coefficient of variation (CV) for the peak area responses was then calculated to evaluate the consistency of the measurements.

#### 2.5.5. Accuracy

The accuracy of the method was evaluated by analyzing methanolic calcipotriol solutions at three different concentration levels:  $75 \mu\text{g mL}^{-1}$ ,  $100 \mu\text{g mL}^{-1}$ , and  $150 \mu\text{g mL}^{-1}$  (corresponding to 75%, 100%, and 150%, respectively). Each concentration was tested in triplicate. The recovery for each sample was determined, and the mean recovery was calculated for each concentration level to assess the method's accuracy.

#### 2.5.6. Robustness

The deliberate small changes of flow rates and column temperatures were made around the optimal values to demonstrate the robustness of the method. For UPLC methods, the mobile phase flow rate was  $0.30 \text{ mL min}^{-1}$ ; to study the effect of the flow rate on resolution, the flow rate was changed to  $0.28$  and  $0.32 \text{ mL min}^{-1}$ . The effect of the column temperature was studied at  $38^\circ\text{C}$  and  $42^\circ\text{C}$  (instead of  $40^\circ\text{C}$ ), and the mobile phase composition was changed  $+2\%$  from the initial composition. In all the deliberately varied chromatographic conditions (flow rate, column temperature, and mobile phase composition), examined compounds were adequately resolved, and the order of elution remained unchanged.

### 2.6. *In Silico* Toxicity Prediction

OSIRIS Property Explorer was used to predict the mutagenicity, tumorigenicity, irritation, and reproductive effects of calcipotriol and its photodegradation products [29].

### 2.7. Molecular Modeling

Molecular docking studies were performed using the Small-Molecule Drug Discovery Suite 2024-4 (Schrödinger Inc., New York, NY, USA) on a workstation operated by a Linux Ubuntu 24.04 LTS system. The ligand molecules were prepared by LigPrep and docked using Glide SP to a VDR model based on the 1S19 (RCSB PDB) experimental structure [30]. The pdb coordinates were processed using the Protein Preparation Workflow and minimized using Prime. Such a conformational model was used for the calcipotriol re-docking (resulting in a reproduction of the experimental binding mode) and the docking of



the degradation products (CP-1—CP-5), with the following re-scoring using the MM-GBSA minimization algorithm.

### 2.8. Statistical Analysis

Statistical analyses were performed using Statistica v. 12 (StatSoft, TIBCO Software Inc., Palo Alto, CA, USA).

## 3. Results and Discussion

### 3.1. Method Validation

The developed method was validated in accordance with the guidelines of ICH Q2 (R2), and it is suitable for the determination of calcipotriol and its photodegradation products in the tested methanolic solutions. The validated UPLC-MS/MS method was specific to the tested substance and guaranteed good peak separation both before and after UV irradiation. The proposed method was validated for the determination of calcipotriol in the presence of degradation products and chosen organic UV filters. In order to assess the specificity of the new UHPLC method, the solutions of calcipotriol with chosen UV filters after 1h of UVA irradiation were investigated. Adequate separation was achieved for both the primary compound and its degradation products. Additionally, the UV filters employed did not produce any extraneous signals in the chromatogram that might overlap with the peaks corresponding to the main substance or its degradation products. Table 1 presents the summarized results of the method validation. The linearity of the method was evaluated by examining six methanolic calcipotriol solutions within a concentration range of 15–150  $\mu\text{g mL}^{-1}$ . The linear regression equation was  $y = 83451x - 86683$ . The linear regression analysis showed good linearity of the method across the entire concentration range, with correlation coefficients and determination coefficients ( $R^2$ ) of 0.9990 and 0.9979, respectively. The y-intercept of the regression analysis for calcipotriol did not achieve statistical significance. The Shapiro–Wilk test confirmed that the residuals of the dependent variable are normally distributed, with  $p = 0.15490$ , which is higher than the significance level of  $\alpha = 0.05$ . The Durbin–Watson statistic was utilized to test for the presence of autocorrelation of the random component. The test statistic value,  $d = 1.77$ , exceeds the upper critical value,  $d_U = 1.39$ , at a significance level of  $\alpha = 0.05$  ( $k = 1$ ,  $n = 18$ ), indicating no autocorrelation. Furthermore, Bartlett’s test was employed to verify the homogeneity of variance. The probability  $p = 0.942656$  is greater than the significance  $\alpha = 0.05$ , indicating that the variance of response is homogeneous. The LOD and LOQ values are 7.39  $\mu\text{g mL}^{-1}$  and 50  $\mu\text{g mL}^{-1}$ , respectively. The measurement range of the method is from 50 to 150  $\mu\text{g mL}^{-1}$ . The precision was assessed through repeatability testing, resulting in a coefficient of variation of 2.41%. The accuracy of the method was evaluated using recovery analysis at three concentration levels: 75%, 100%, and 150%. The average percent recovery ranged from 99.17% to 101.55%.

**Table 1.** Method Validation Parameters.

Parameter	Calcipotriol
Retention time (min) <sup>a</sup>	8.04
Limit of detection ( $\mu\text{g mL}^{-1}$ )	7.39
Limit of quantitation ( $\mu\text{g mL}^{-1}$ )	50
Linear range ( $\mu\text{g mL}^{-1}$ )	15–150

Table 1. Cont.

Parameter	Calcipotriol
Regression equation (y): <sup>b</sup>	
Slope ( $a \pm S_a$ )	$83,451 \pm 955$
Intercept ( $b \pm S_b$ )	$-86,683 \pm 79,389$
$t = b/S_b$ <sup>c</sup>	$1.09188 < t_{\alpha,f}$ statistically insignificant
Normality of residuals <sup>d</sup> (Shapiro–Wilk test) <sup>b</sup>	$0.92444$ ( $p = 0.1549$ )
Correlation coefficient	$0.999$
$R^2$ value	$0.9979$
Recovery level 75%	$99.17$
Recovery level 100%	$101.55$
Recovery level 150%	$99.82$
Precision (CV)	$2.41$

<sup>a</sup> Mean SD ( $n = 6$ ). <sup>b</sup> Regression equation  $y = ac + b$ ;  $c$ , concentration of the solution;  $y$ , peak area;  $S_a$ , standard deviation of the slope;  $S_b$ , standard deviation of the intercept. <sup>c</sup>  $t$ , Calculated value of Student's  $t$ -test;  $t_{\alpha,f} = 2.1009$ , critical value of Student's  $t$ -test for degrees of freedom  $f = 18$ . <sup>d</sup> Normal distribution of residuals if  $p > 0.05$ .

### 3.2. UV Irradiation of Calcipotriol

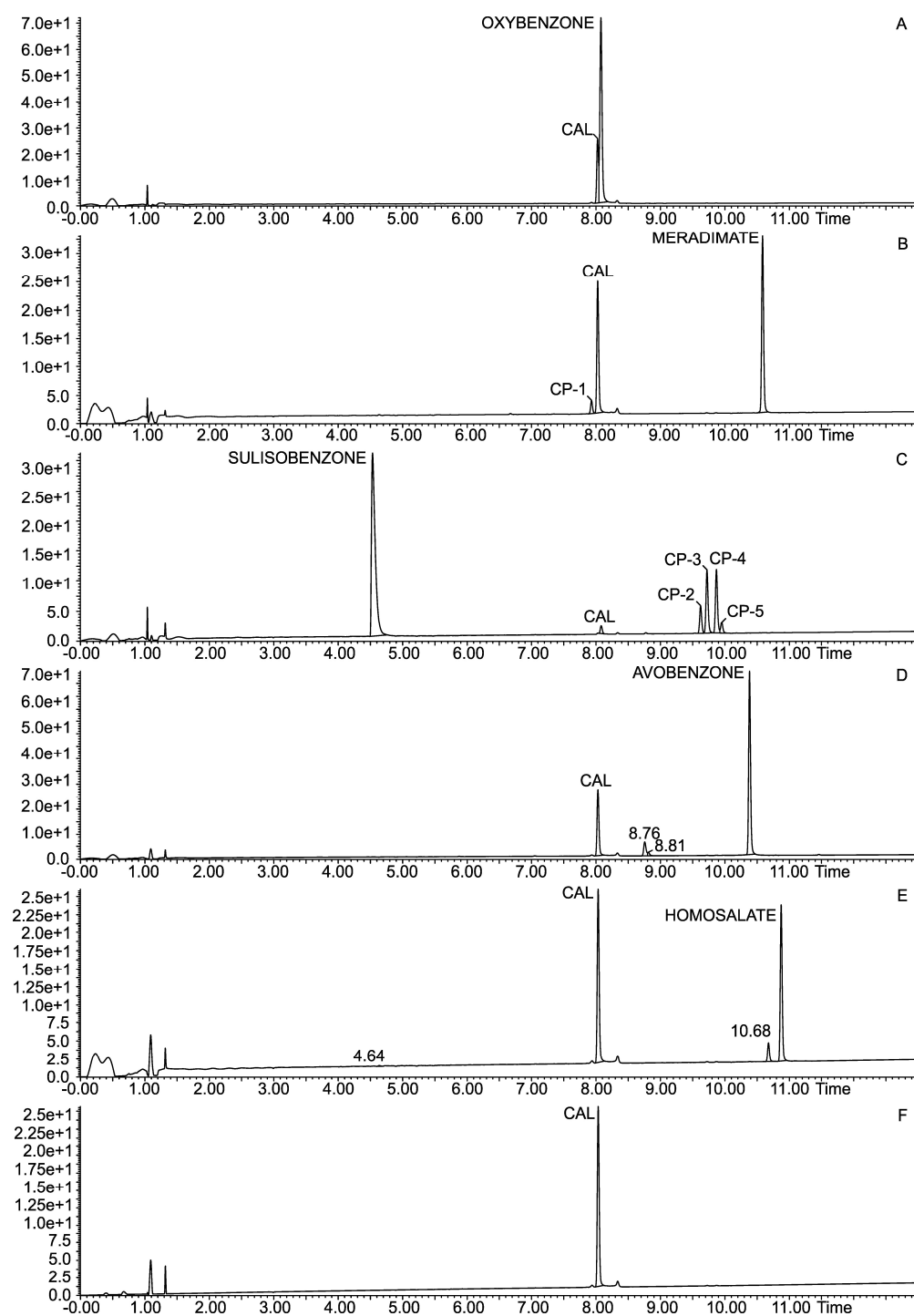
Subsequently, the photostability of calcipotriol under UVA irradiation was evaluated in the presence of the following photoprotective agents: oxybenzone, meradimate, sulisobenzene, avobenzone, dioxybenzone, and homosalate. The photostability testing of calcipotriol was performed in solutions with and without the presence of UV absorbers. The samples were exposed to UVA light for 60 min. The chromatograms of the organic UV filters under study, calcipotriol and its photodegradation products in methanolic solutions after 1 h of irradiation, are presented in Figure 1. The dark control samples were prepared in parallel by shielding them with aluminum foil to prevent light exposure. These samples were then maintained under the same experimental conditions as the irradiated samples for 1 h.

According to ICH Q1B guidelines, “Photostability testing of new active substances and medicinal products”, assessing photostability is an essential component of evaluating the stability of active pharmaceutical ingredients and finished medicinal products [31]. However, the simultaneous application of UV filters and cosmetic products may affect the stability of the active pharmaceutical ingredient (API) under UV radiation exposure [21–24]. Calcipotriol degradation was observed in the applied conditions, only in the samples containing sulisobenzene—the chromatogram reveals four degradation products of calcipotriol CP-2—CP-5 (Figure 1C). In chromatogram D, peaks at retention times of 8.76 and 8.81 correspond to the degradation products of UV filter avobenzone. Likewise, chromatogram E displays a peak at a retention time of 10.68, which is attributed to the UV filter homosalate. Partial E-Z isomerization of calcipotriol was observed in the presence of meradimate (Figure 1B). In dark control samples containing calcipotriol and UV filters, calcipotriol also underwent unexpected degradation in the presence of sulisobenzene, resulting in the formation of degradation products CP-2 to CP-5.

The stability of an active substance in a drug formulation can vary compared to its stability in solution. Therefore, it is essential to conduct stability studies both on active pharmaceutical ingredients and on finished products. In medicinal products, excipients may influence the degradation kinetics of the active substance. In this study, the stability of calcipotriol in a foam formulation was assessed by irradiating samples containing the active compound, both alone and in the presence of the selected chemical UV absorber sulisobenzene. The photodegradation pattern of calcipotriol in the tested foam samples, in the presence of



a UV filter, was analogous to that observed in solutions containing sulisobenzene, with chromatographic peaks corresponding to degradation products CP-2 to CP-5.



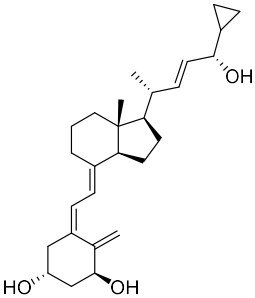
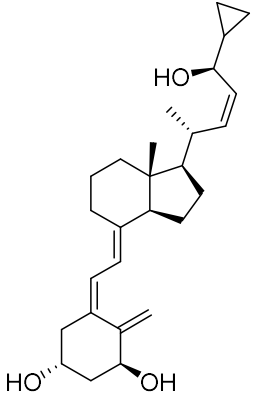
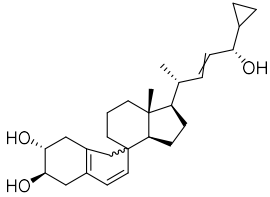
**Figure 1.** UHPLC/MS<sup>E</sup> analysis of photostability of calcipotriol in methanol solution after exposure to UVA irradiation for 1 h: (A) calcipotriol with oxybenzone; (B) calcipotriol with meradimate; (C) calcipotriol with sulisobenzene; (D) calcipotriol with avobenzone; (E) calcipotriol with homosalate, and (F) pure standard substance.

### 3.3. Identification of Degradation Photoproducts

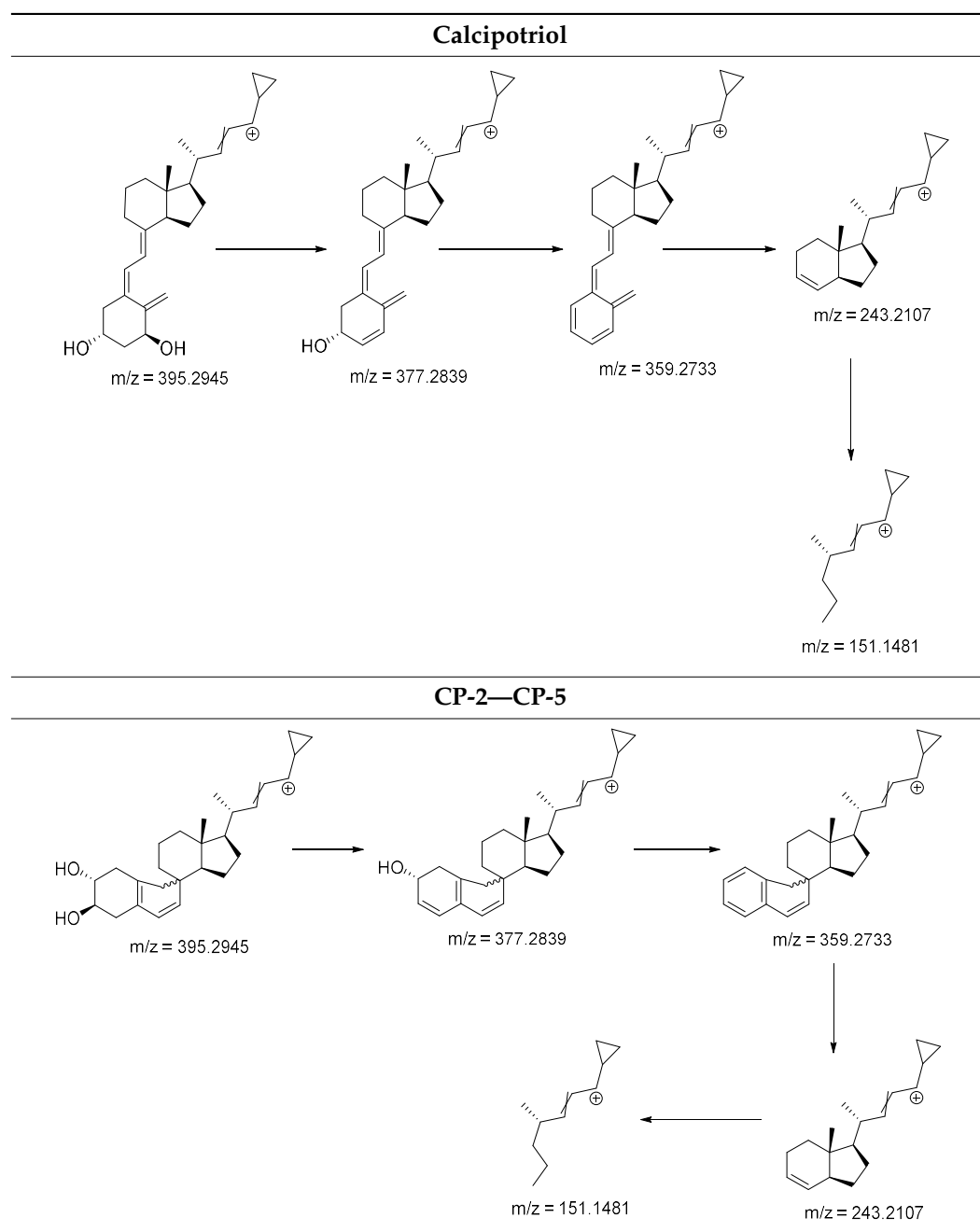
In the present study, the identification of calcipotriol degradation products was accomplished using UHPLC/MS<sup>E</sup> experiments. The proposed structures of the degradation products are shown in Table 2. The proposed fragmentation patterns of calcipotriol and

its degradation products are shown in Table 3. The degradation process was found to affect primarily the E-4-cyclopropyl-4-hydroxy-1-methylbut-2-en-1-yl moiety, causing its isomerization to the Z isomer. Additionally, the products CP-2 to CP-5 are formed by intramolecular [4+2] photoaddition in the hexa-1,3,5-triene fragment of calcipotriol, giving diastereomers with the R or S configuration at the resulting spiro carbon atom. Regarding the activation mechanism of sulisobenzone, upon UV irradiation, it generates an active triplet state on the ketone moiety, which may subsequently activate calcipotriol in a following step. When benzophenone is exposed to ultraviolet light, its photoreactive benzophenone moiety absorbs the energy and undergoes photolysis. Benzophenone absorbs UV photons, which excite the molecule from its ground state to the singlet excited state. The molecule efficiently undergoes intersystem crossing, transitioning from the singlet excited state to the triplet excited state. The resulting triplet-state ketone is highly reactive, exhibiting pronounced biradical character. The triplet-state intermediate is significantly more reactive than the ground state, enabling benzophenone to initiate further chemical transformations [32–34]. To the best of our knowledge, this is the first report describing these photodegradation pathways and the detailed structures of calcipotriol photoproducts.

**Table 2.** Proposed structures of degradation products of calcipotriol.

Compound	RT [min]	[M+H] <sup>+</sup>	Fragmentation Ions	Structure
Calcipotriol	7.93	395.2945 *	151.1481, 243.2107, 359.2733, 377.2839	
CP-1	8.08	395.2945 *	151.1481, 243.2107, 359.2733, 377.2839	
CP-2	9.62	395.2945 *	151.1481, 243.2107, 359.2733, 377.2839	
CP-3	9.72			
CP-4	9.87			
CP-5	9.95			

\* [M-H<sub>2</sub>O]<sup>+</sup>.

**Table 3.** Proposed fragmentation patterns for calcipotriol and degradation products.

Previous studies have confirmed that calcipotriol is susceptible to photodegradation, and several chromatographic methods have been developed to separate the parent compound from its photoproducts. Cirunay et al. [35] demonstrated the separation of calcipotriol from its degradation products by HPLC and examined the protective effects of various solvents and UV filters; however, they did not characterize the degradation products themselves. In the experiment, a xenon lamp was employed due to its spectral distribution being similar to that of sunlight. After 3 and 6 min of irradiation, two unknown degradation products (DG1 and DG2) were obtained, as well as DG3, which appears when DG2 diminishes, suggesting a complex degradation pattern [35]. Lebwohl M. et al. also observed a decrease in detectable calcipotriol concentrations in the ointment after exposure to UVA radiation [14]. In research conducted by Jahani M. et al., the degradation product of calcipotriol obtained under the influence of UVA radiation was identified as DP-4, which is pre-calcipotriol, based on UV and LC-MS fragmentation patterns [15].

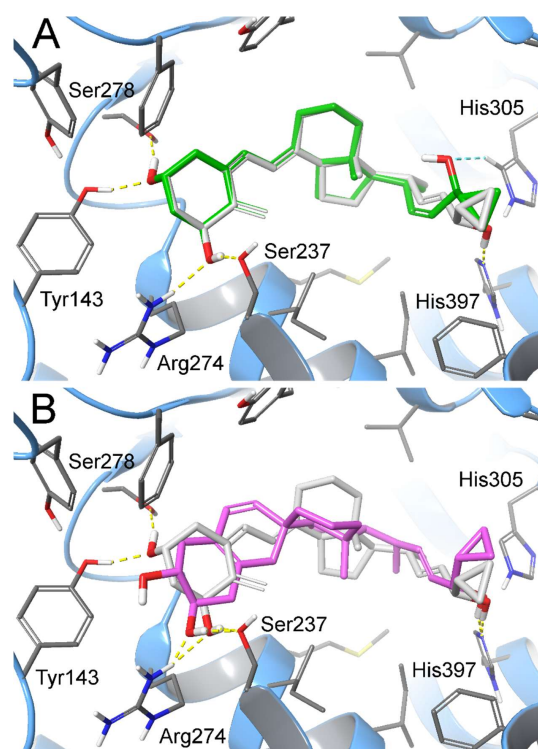
### 3.4. In Silico Toxicity Predictions

The OSIRIS Property Explorer server was used to evaluate the toxicity profiles of calcipotriol and each of its detected degradation products. The analysis revealed that none of the five degradation products exhibited mutagenic, tumorigenic, reproductive, or irritant properties.

### 3.5. Molecular Modeling

Molecular docking studies on a model of the VDR ligand-binding domain resulted in reasonable binding modes for two photodegradation products—CP-1 and one of the CP-2—CP-5 isomers (specifically, the E isomer with R-configuration at the spiro carbon atom, referred to as CP-ER). To some extent, the compounds mimicked the interactions of calcipotriol, suggesting potential biological activity, as reflected by their MM-GBSA score values.

CP-1 interacted in a similar manner to calcipotriol; however, its predicted binding free energy was higher, suggesting weaker pharmacological activity of the Z isomer (−129.5 kcal/mol, compared with −139.4 kcal/mol predicted for calcipotriol). Both molecules formed favorable hydrogen bonds with polar amino acid residues. The 4-methylenecyclohexane-1,3-diol moieties interacted with Ser237 and Arg274, as well as with Ser278 and Tyr143. The differing interactions of the 5-cyclopropyl-5-hydroxypent-3-en-2-yl fragment explain the poorer performance of the Z isomer—instead of forming a hydrogen bond with His397, it formed a weaker aromatic hydrogen bond with His305 (Figure 2A).



**Figure 2.** Predicted binding modes of (A) CP-1 (green) and (B) CP-ER (pink), in comparison with the experimentally resolved calcipotriol (grey) interactions in the VDR ligand-binding domain (LBD). The protein model used for docking was based on PDB entry 1S19—the VDR LBD co-crystallized with calcipotriol [30]. The compounds formed hydrogen bonds (yellow dashed lines) and aromatic hydrogen bonds (cyan dashed lines).

The second degradation product that succeeded in the docking study was CP-ER. Its spiro[indene-4,2'-naphthalene]-6',7'-diol moiety was able to interact solely with Ser237 and Arg274, while the second terminal fragment of the molecule retained interaction with His397, characteristic for calcipotriol (Figure 2B). Nevertheless, the predicted binding free energy for CP-ER was even higher, reaching  $-108.2$  kcal/mol.

#### 4. Conclusions

The newly developed UHPLC/MS<sup>E</sup> method, validated in accordance with ICH Q2(R2) guidelines, demonstrated high specificity, linearity over the range of  $50\text{--}150\text{ }\mu\text{g mL}^{-1}$ , satisfactory precision, and accuracy. Photostability studies indicated that calcipotriol undergoes significant degradation in the presence of the UV filter sulisobenzene, which led to the formation of four main degradation products (CP-2 to CP-5). Exposure to sulisobenzene, a commonly used UV filter, induced degradation of calcipotriol both in methanolic solution and commercial formulation. The identification of the degradation products was achieved using UHPLC/MS<sup>E</sup>, and their structures and fragmentation patterns were proposed for the first time. In silico toxicity assessment using the OSIRIS Property Explorer indicated that neither calcipotriol nor its photodegradation products exhibited mutagenic, tumorigenic, reproductive, or irritant properties. Two out of the five degradation products interacted with the VDR in a manner similar to calcipotriol; however, their predicted binding free energies were higher, indicating a potentially weaker pharmacological effect. These findings expand our understanding of calcipotriol stability, emphasizing the significance of assessing interactions not only with excipients but also with commonly used UV filters. The study demonstrates that real-world conditions—such as the simultaneous use of pharmaceutical and cosmetic products—can markedly affect drug stability, which is crucial for maintaining therapeutic efficacy.

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**Conflicts of Interest:** The authors declare that they have no conflicts of interest.

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