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Identification of Vitamin D_3 Hydroxylated Metabolites in *Solanum glaucophyllum* Leaves: Towards Its Biosynthetic Pathway Elucidation †

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Abstract: *Solanum glaucophyllum* is a species of the Solanaceae family, which causes enzootic calcinosis by vitamin D_3 intoxication in breeding cattle grazing in Argentina. Inspired on thrusting forward the study of the biosynthetic pathway of vitamin D_3 in *S. glaucophyllum*, the aim of the present work is to provide knowledge of vitamin D_3 toxicity related to concentration in the mentioned species located throughout the Río Salado basin. We present the development of a reverse phase high performance liquid chromatography (RP-HPLC) method for the analysis of vitamin D_3 and its hydroxylated metabolites. An exhaustive optimization of RP-HPLC method allowed us to perform calcitriol (1α,25-$(OH)_2D_3$) and calcidiol (25-OHD_3) identification, both metabolites obtained by chemical hydrolysis of *S. glaucophyllum* leaves starting off its hydrophilic extract. These results will allow the design of a monitoring scheme for *S. glaucophyllum*, achieving a more selective control of this weed to avoid cattle declining in the Río Salado basin.

Keywords: *Solanum glaucophyllum*; vitamina D_3; HPLC

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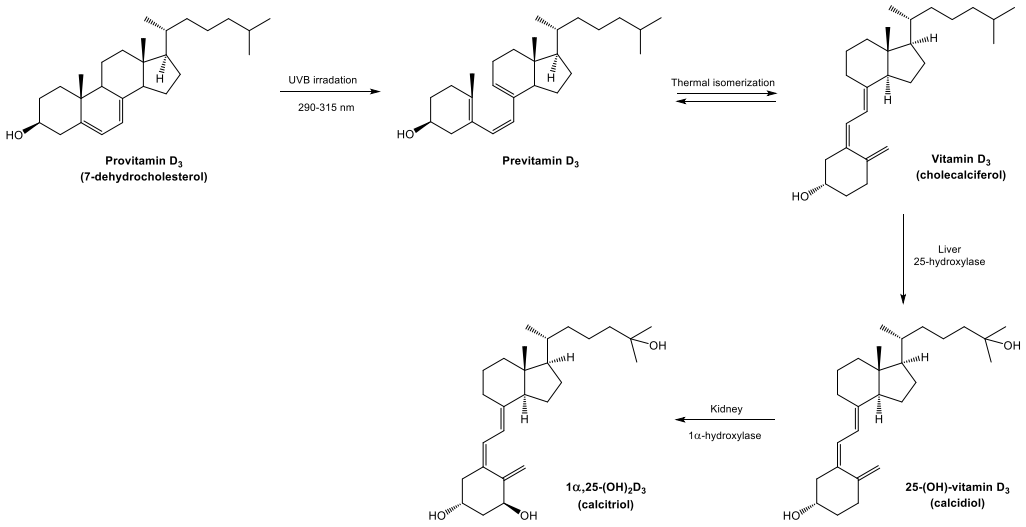
1. Introduction

Solanum glaucophyllum belongs to the Solanaceae family widely distributed in flooded areas of natural grasslands in the northeast of Argentina. Since 1960, its consumption by cattle has been associated with vitamin D_3 intoxication known as enzootic calcinosis. This particular disease is related to alteration in the absorption of vitamin D_3, which produces calcium depositions in muscle tissues and joints of cattle. It is a progressive disease with signs such as stiffness, painful gait, and loss of body condition, being responsible for significant mortality in affected ruminants, thus nowadays produces important economic losses for Argentine livestock [1,2].

Concerning bibliography background, vitamin D_3 is known to be present in species belonging to the Solanaceae family. The principal hydroxylated metabolite found is 1α,25-$(OH)_2D_3$, known as calcitriol, mainly conjugated with carbohydrates as 1α,25-$(OH)_2D_3$-glycoside [3]. The biologically most active metabolite calcitriol, associated with pathological signs of the mentioned disease, has been identified in *S. glaucophyllum* from the enzymatic hydrolysis of the water: ethanol extract obtained from the leaves of this species [4]. In cattle, calcitriol is released throughout enzymatic hydrolysis of 1α,25-$(OH)_2D_3$-glycoside at the ruminal level, after *S. glaucophyllum* leaves ingestion [1].

In addition, vitamin D_3 in *S. glaucophyllum* showed a similar to vertebrate's photodependent process of synthesis, but there is few researching regarding quantitative studies about vitamin D_3 biosynthetic pathway of the plant [5]. In vertebrates, vitamin D_3 is synthesized upon UVB irradiation. The UVB irradiation of provitamin D_3 (7-dehydrocholesterol) in the skin breaks the B-ring to form previtamin D_3, which rearranges itself in vitamin D_3 (cholecalciferol). Vitamin D_3 is transported to the liver where it is enzymatic hydroxylated at C-25 by 25-hydroxylase enzyme, producing 25-OHD_3 (calcidiol). The 25-OHD_3 is second time hydroxylated at C-1 in the kidneys to the active metabolite 1α,25-$(OH)_2D_3$ (calcitriol) (Scheme 1).



Scheme 1. Biosynthetic pathway of vitamin D_3 in vertebrates [6].

Inspired on thrusting forward the study of the biosynthetic pathway of vitamin D_3 in *S. glaucophyllum*, the aim of the present work is to provide knowledge of vitamin D_3 toxicity related to concentration in the mentioned species located throughout the Río Salado basin. High performance liquid chromatography (HPLC) allows qualitative / quantitative analysis in order to know the presence and content of related compounds in *S. glaucophyllum* with highly accurate and sensitive results. Herein we present the development of a reverse phase high performance liquid chromatography (RP-HPLC) method for the determination of vitamin D_3 and its hydroxylated metabolites. So far, the present work contributes to validate the optimal starting amount of *S. glaucophyllum* leaves to be hydrolyzed, the chemical hydrolysis conditions, and the method of analysis of main metabolites in the plant material leaves.

2. Materials and Methods

2.1. General

Solvents used for extraction and chromatography were previously distilled. HPLC analysis was performed using isopropanol (HPLC gradient grade for liquid chromatography, LiChrosolv); acetonitrile (HPLC gradient grade for liquid chromatography, LiChrosolv) and ultra purified Milli-Q water (Millipore, Billerica, MA, USA). All solvents were degassed by simultaneous sonication and filtration through 0.2 µm PTFE membranes prior to use. Calcitriol (1α,25-$(OH)_2D_3$) and calcidiol (25-OHD_3) analytical standards were obtained from Sigma-Aldrich (St. Louis, MO, USA). For column chromatography, neutral aluminum oxide Fluka Typ 507C (100–125 mesh) was used. The chromatographies were monitored by thin-layer chromatography (TLC) on silica gel plates (60F-254), visualized under UV light and/or using a *p*-anisaldehyde solution (5 mL *p*-anisaldehyde, 5 mL H_2SO_4 concentered, 1mL acetic acid and 90 mL ethanol).

HPLC analysis was conducted on a LS-MS-Thermo Scientific -UltiMate 3000-MSQ PLUS HPLC system equipped with an Agilent Zorbax SB-Aq stable bond analytical C18 reverse phase column and a fixed wavelength UV detector. Calcitriol (1α,25-$(OH)_2D_3$) and calcidiol (25-OHD_3) were monitored at an absorbance of 265 nm. UV spectra for maximal wavelength standards determination were recorded in an Agilent Cary 60 UV–Vis spectrophotometer. Stock calcitriol and calcidiol standards mixture were generated using a molar extinction coefficient of 18,300 AU $M^{-1}L^{-1}$ at 265 nm in isopropanol HPLC grade. Prepared stock standards mixture were perfused with nitrogen and stored at −20 °C.

2.2. Plant Material

Solanum glaucophyllum (Solanaceae) plant specimens were collected in Dolores in Buenos Aires province of Argentina, in the location La Quebrada (36°17′21.99″ South latitude and 57°36′14.76″ West longitude), in april 2021.

2.3. Harvest and Extraction

The plant material was harvested by hand, preferably at noon to avoid dew and excessive humidity. The stems were separate and the leaves were spread on the ground, indoors to dry superficially and separate the rest of the soil and foreign bodies. After 24 h the sample was placed in an oven at 36 °C until reaching a constant weight, it took approximately 3 days.

Finely ground dry leaves of *S. glaucophyllum* (163 g) were extracted with a solution (1250 L) of water: ethanol (80:20) at 40 °C for 8 h. The extract was concentrated under reduced pressure, giving 36.7 g (3.7%).

2.4. Chemical Hydrolysis

In a 250 mL two-necked round-bottomed flask, equipped with a condenser loaded with *S. glaucophyllum* extract (0.7 g) was added a 2 N HCl solution (140 mL). The mixture was stirring for 13 h at 85 °C and an additional 18 h at room temperature. Then the reaction mixture was extracted with ethyl acetate (5 × 50 mL). The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure to give 0.126 g of hydrolyzed extract.

2.5. Purification of Hydrolyzed Extract

Chromatography on neutral aluminum oxide (100–125 mesh) of a portion of the hydrolyzed extract (50.0 mg), eluted with dichloromethane/methanol (100:0 to 95:5, step-gradient system) yielded forty tubes. Chromatographic separation was monitored by TLC, using a mixture of dichloromethane: methanol (90:10) as mobile phase. Two major compounds, **1** and **2**, were observed in tubes 1 and 25, respectively, weakly seen for their stain color in the plate.

2.6. Calcitriol (1α,25-$(OH)_2D_3$) and Calcidiol (25-OHD_3) Identification

Hydrophilic extract´s pellets obtained from *S. glaucophyllum* leaves were previously purified by chromatography before HPLC analysis. All tubes containing analytes in purified extract fractions were vacuum dried and dissolved in isopropanol (HPLC grade). The prepared solutions were filtered through a 0.2-µm PTFE syringe filter and placed in brown 2-mL HPLC auto sampler vials with Teflon coated lids. HPLC analysis was conducted on a LS-MS-Thermo Scientific -UltiMate 3000-MSQ PLUS HPLC system equipped with a fixed wavelength UV detector. Calcitriol (1α,25-$(OH)_2D_3$) and calcidiol (25-OHD_3) were monitored at an absorbance of 265 nm. HPLC was performed with a flow rate of 0.7 $mL.min^{-1}$ by binary pumps at 25 °C. The mobile phase consisted of acetonitrile (HPLC gradient grade for liquid chromatography LiChrosolv) and Milli-Q water. Ultra purified water was prepared by Milli-Q Advantage system (Millipore, Billerica, MA, USA), giving a product with a resistivity of ~18.5 MΩ.cm-1. After loading the column with each extract purified fraction dissolved in isopropanol HPLC, the mobile phase was programmed with

an isocratic ratio 90:10 acetonitrile:water solution over 18 min. Between each sample injection an isopropanol blank (HPLC grade) was run. Peaks retention time of 1α,25-$(OH)_2D_3$ and 25-OHD_3 analytical standards were employed for identification of these metabolites in the samples.

3. Results and Discussion

In the present study, *1α,25-$(OH)_2D_3$* and *25-OHD_3* free aglycone metabolites in *S. glaucophyllum* leaves were identified by HPLC analysis [7–10]. An exhaustive screening of RP-HPLC methods allow us to perform both metabolites identification in the same elution procedure, starting from individual stock calcitriol and calcidiol standards. HPLC optimized conditions for analysis of vitamin D_3 metabolites is shown in Table 1.

Table 1. Optimized conditions for chromatographic analysis by HPLC.

Column	**Agilent Zorbax SB-Aq stable bond analytical C18 reverse phase (4,6 mm × 250 mm; 5 μm, Agilent)**
Mobile phase	ACN:H_2O (90:10)
Inyection time:	18 min.
Flow rate:	0,7 $mL.min^{-1}$
Column temperature:	25 °C
Wavelength UV detector:	445 nm

The HPLC chromatogram of *1α,25-$(OH)_2D_3$* and *25-OHD_3* standards mixture are shown in Figure 1a, with peaks retention time of 5.343 min. and 6.710 min., respectively. All purified extracts obtained by chemical hydrolysis of *S. glaucophyllum* leaves starting off its hydrophilic extract were analyzed. *1α,25-$(OH)_2D_3$* and *25-OHD_3* peaks from purified sample tubes 25 and 1, were respectively assigned by comparing their retention time with that of pure standards. Figure 1b shows *1α,25-$(OH)_2D_3$* peak from purified sample tube 25 with a retention time of 5.343 min. Figure 1c shows *25-OHD_3* peak from purified extract tube 1 with a retention time of 6.710 min.

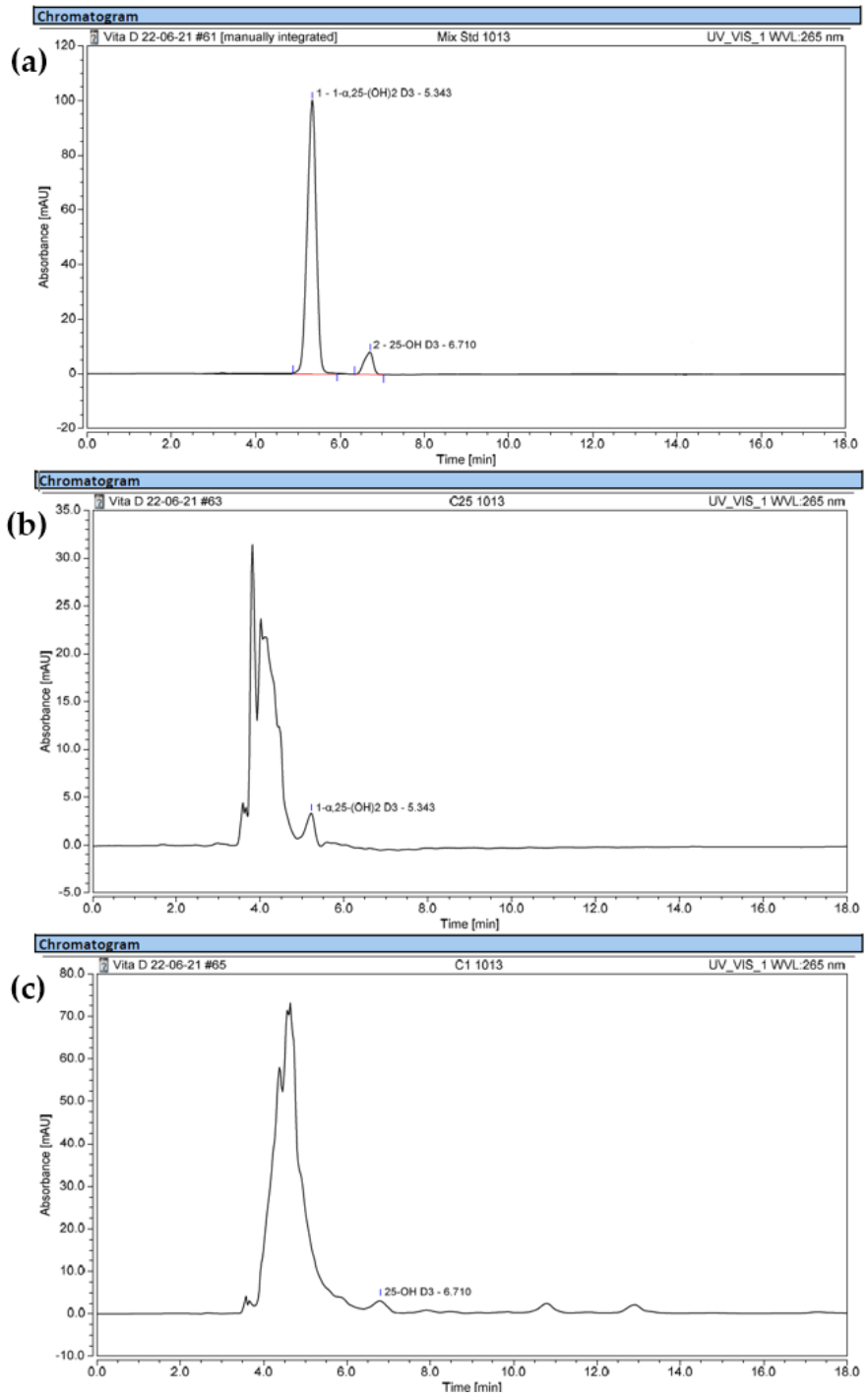


Figure 1. (**a**) HPLC chromatogram of *1α,25-(OH)₂D₃* and *25-OHD₃* standards mixture; (**b**) HPLC chromatogram of purified sample tube 25 showing *1α,25-(OH)₂D₃* identification peak; (**c**) HPLC chromatogram of purified sample tube 1 showing *25-OHD₃* identification peak.

4. Conclusions

In order to validate the optimal chemical hydrolysis conditions and the method of analysis of main vitamin D_3 metabolites in the plant material leaves of *S. glaucophyllum*, a preliminary quantitative HPLC method was performed. The results obtained here lay the groundwork to improve the process from an enzymatic hydrolysis with promising results regarding vitamin D_3 metabolites quantification in the specie. These results will improve a smart design management and monitoring scheme for *S. glaucophyllum*, achieving a

more selective control of this weed, avoiding cattle declining in the Río Salado basin located at the northeast of Argentina.

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Conflicts of Interest: The authors declare no conflict of interest.

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