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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 that causes enzootic calcinosis by vitamin D_3 intoxication in breeding cattle grazing in Argentina. Inspired by 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 the RP-HPLC method allowed us to perform calcitriol (1α,25-$(OH)_2D_3$) and calcidiol (25-OHD_3) identification, both metabolites obtained by the chemical hydrolysis of *S. glaucophyllum* leaves starting from its hydrophilic extract. These results allow the design of a monitoring scheme for *S. glaucophyllum*, achieving a more selective control of this weed to avoid cattle decline in the Río Salado basin.

Keywords: *Solanum glaucophyllum*; vitamin D_3; HPLC

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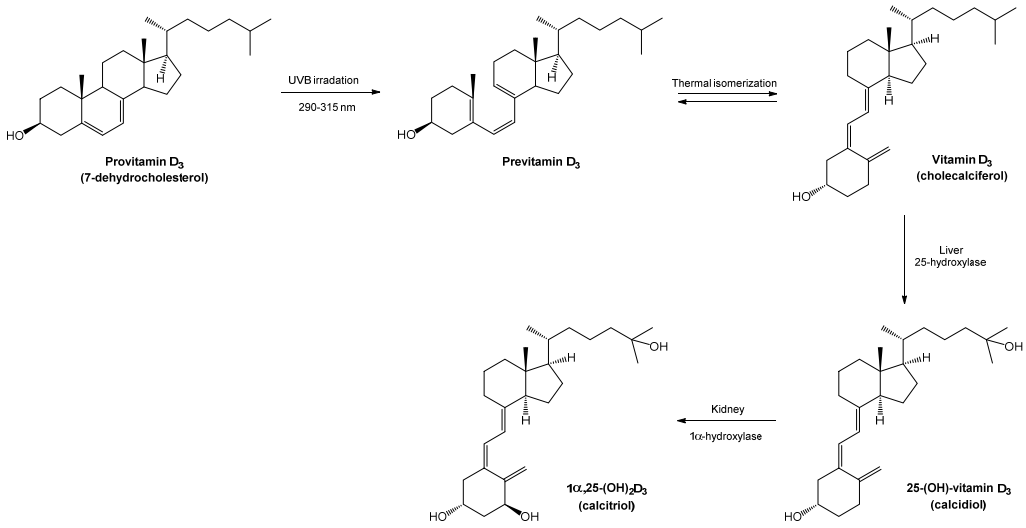
1. Introduction

Solanum glaucophyllum belongs to the Solanaceae family and is widely distributed in the 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 the muscle tissues and joints of cattle. It is a progressive disease with signs such as stiffness, painful gait, and loss of body condition, and it is responsible for significant mortality in affected ruminants that nowadays produces important economic losses for Argentine livestock [1,2].

Concerning bibliographic 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, and is mainly conjugated with carbohydrates as 1α,25-$(OH)_2D_3$-glycoside [3]. The biologically most active metabolite, calcitriol, is associated with pathological signs of the mentioned disease and 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 through the enzymatic hydrolysis of 1α,25-$(OH)_2D_3$-glycoside at the ruminal level after *S. glaucophyllum* leaf 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 the 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 enzymatically hydroxylated at C-25 by the 25-hydroxylase enzyme, producing 25-OHD_3 (calcidiol). The 25-OHD_3 is hydroxylated for a second time 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 by 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 and quantitative analyses 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 validating the optimal starting amount of *S. glaucophyllum* leaves to be hydrolyzed, the chemical hydrolysis conditions, and the method of analysis of the main metabolites in the plant leaf materials.

2. Materials and Methods

2.1. General

The solvents used for extraction and chromatography were previously distilled. The HPLC analysis was performed using isopropanol (HPLC gradient grade for liquid chromatography, LiChrosolv), acetonitrile (HPLC gradient grade for liquid chromatography, LiChrosolv), and ultrapurified Milli-Q water (Millipore, Billerica, MA, USA). All the 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 with thin-layer chromatography (TLC) on silica gel plates (60F-254) and visualized under UV light or by using a *p*-anisaldehyde solution (5 mL *p*-anisaldehyde, 5 mL H_2SO_4 concentered, 1mL acetic acid, and 90 mL ethanol).

The HPLC analysis was conducted on an 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 with an Agilent Cary 60 UV–Vis spectrophotometer. Stock calcitriol and calcidiol standard mixtures were generated using a molar extinction coefficient of 18,300 AU M^{-1} L^{-1} at 265 nm in HPLC-grade isopropanol. The prepared stock standard mixtures were perfused with nitrogen and stored at −20 °C.

2.2. Plant Material

Solanum glaucophyllum (Solanaceae) plant specimens were collected at Dolores in the Buenos Aires province of Argentina from the La Quebrada location (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 separated, and the leaves were spread on the ground indoors to dry superficially, separate from the rest of the soil and foreign bodies. After 24 h, the samples were placed in an oven at 36 °C until reaching a constant weight; this 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), 2 N HCl solution (140 mL) was added. The mixture was stirred for 13 h at 85 °C and for 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 by their stain color on the plate.

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

Hydrophilic extract pellets obtained from *S. glaucophyllum* leaves were previously purified by chromatography before HPLC analysis. All the 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 autosampler vials with Teflon-coated lids. The HPLC analysis was conducted with an 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. Ultrapurified water was prepared with a 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 of 90:10 acetonitrile:water solution over 18 min. Between each sample injection, an isopropanol blank (HPLC grade) was run. The peak retention times of 1α,25-$(OH)_2D_3$ and 25-OHD_3 analytical standards were employed for the 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]. The exhaustive screening of the RP-HPLC method allowed us to perform both metabolite identifications in the same elution procedure, starting from individual stock calcitriol and calcidiol standards. The HPLC-optimized conditions for the analysis of vitamin D_3 metabolites are 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)
Injection time:	18 min
Flow rate:	0.7 mL/min^{-1}
Column temperature:	25 °C
Wavelength UV detector:	445 nm

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

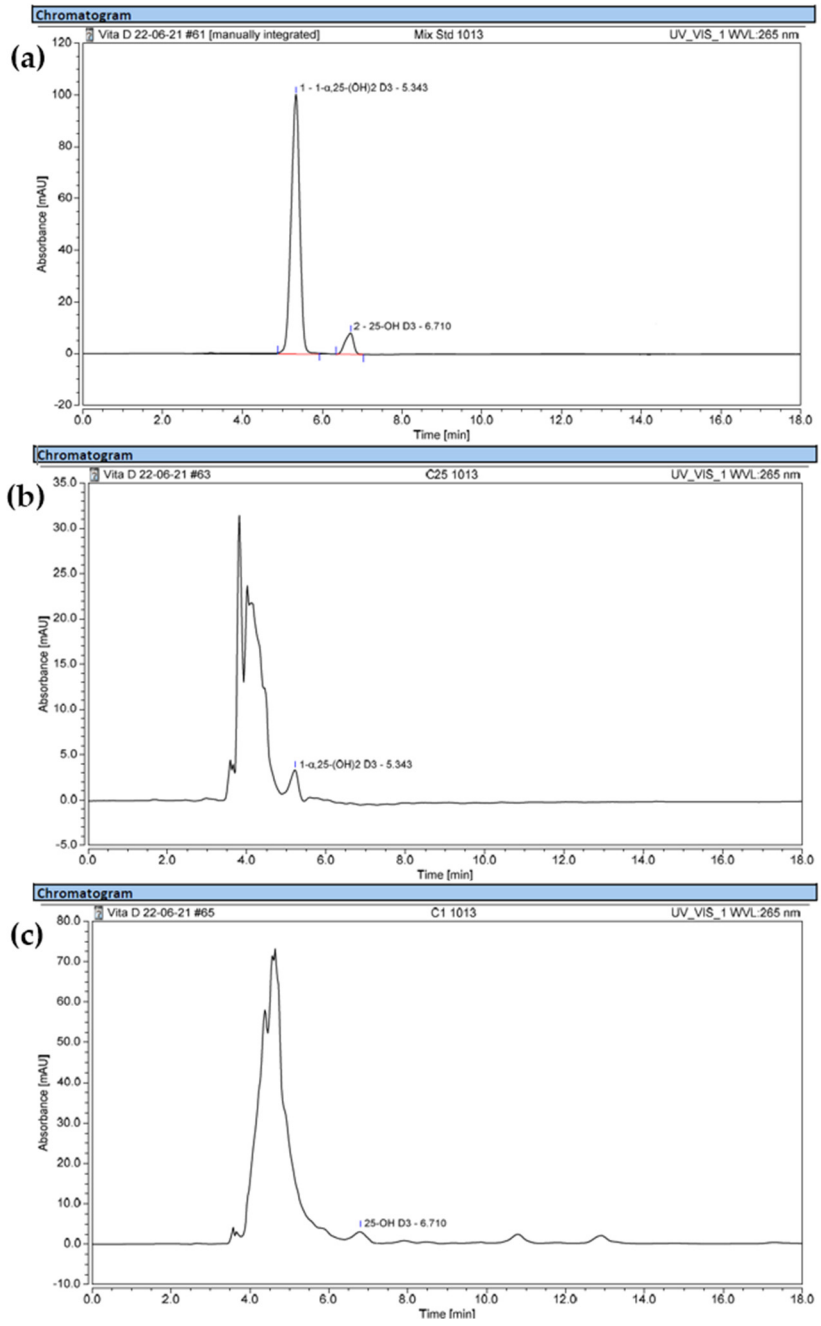


Figure 1. (**a**) HPLC chromatogram of 1α,25-$(OH)_2D_3$ and 25-OHD_3 standards mixtures; (**b**) HPLC chromatogram of purified sample tube 25 showing 1α,25-$(OH)_2D_3$ identification peak; (**c**) HPLC chromatogram of purified sample tube 1 showing 25-OHD_3 identification peak.

4. Conclusions

In order to validate the optimal chemical hydrolysis conditions and the method of analysis of the main vitamin D_3 metabolites in the plant leaf materials of *S. glaucophyllum*, a preliminary quantitative HPLC method was performed. The results obtained here lay the groundwork to improve the process of enzymatic hydrolysis with promising results regarding vitamin D_3 metabolite quantification in the specie. These results improve a smart design management and monitoring scheme for *S. glaucophyllum*, achieving a more selective control of this weed, avoiding cattle decline in the Río Salado basin located in the northeast of Argentina.

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

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