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Screening of Vitamin D activity (VDA) of *Solanum glaucophyllum* leaves measured by radioimmunoassay (RIA)☆

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Abstract

The ingestion of *Solanum glaucophyllum* (*SG*) causes a calcinosis of cattle named Enteque Seco (ES). The toxic principle is the 1,25-$(OH)_2D_3$, mainly conjugated as glycoside. This study aims to validate a simple novel method of evaluation of the VDA of *SG* leaves. Aqueous extracts of *SG* were purified using C_{18} minicolumns and assayed by RIA with an antibody raised in rabbits by injection of the acid—C22, 1α-(OH)Vitamin D_3. Data were expresed as glycoside equivalent to 1,25-$(OH)_2D_3$ in ng/g of dry leaves. We compared this data with 1,25-$(OH)_2D_3$ levels measured, in the same samples, by liquid chromatography (HPLC) after enzyme cleavage. This procedure involved the incubation of *SG* leaves with rumen fluid, followed by C_{18}-OH solid phase extraction. The 1,25-$(OH)_2D_3$ fraction was run by HPLC and detection was achieved using a photodiode array detector. Data were expressed as micrograms of 1,25-$(OH)_2D_3$/g dry leaves. A significant regression of 1,25-$(OH)_2D_3$ levels (*Y*) as a function of glycoside RIA 1,25-$(OH)_2D_3$ equivalents (*X*) was found: $Y = 12.02 + 0.35X$ [$R = 0.81$; $P = 0{,}0002$; $N = 15$], allowing us to conclude that this novel assay could be used to estimate the amount of this active principle contained in *SG* leaves.

Keywords: Radioimmunoassay; *Solanum glaucophyllum*; Hypervitaminosis D; 1,25-$(OH)_2D_3$-glycoside

1. Introduction

The ingestion of *Solanum glaucophyllum* (*SG*), growing wild in South America and India [1], causes a calcinosis of cattle named Enteque Seco (ES) in Argentina and Uruguay, and "Espichamento" in Brazil [2]. The active principle associated with the pathological signs of this disease is the 1,25-$(OH)_2D_3$, either free (aglycone) or conjugated with carbohydrates as 1,25-$(OH)_2D_3$-glycoside [3]. There are only a few plant species (6 up to now) that cause calcinosis in animals, and *SG* came to be 100-fold more active than the *Cestrum diurnum* [4]. Economic relevance of *SG* could be seen as being the causal factor of toxicosis of grazing cattle (negative value) and also as a valuable source of Vitamin D_3 active metabolites (positive value). Up to now, a lot of scientific work has been published about pathology and biochemistry of this calcinotic disease in experimental animals, conversely, only a few publications have shown quantitative studies about the Vitamin D synthetic capability of the plant [5,6].

Concerning ES we can say that a great variation of its incidence has been registered among and within farms in diferent years, making it very dificult to evaluate risks in enzootic areas. One of the factors to be considered would be the variations of plant toxicity under different vegetative states and locations. On the other hand, some pharmacological applications of *SG* in human and veterinary medicine as well as in animal husbandry, have been assayed. The main indications known in human medicine comprised chronic renal failure, hypoparathyroidism, and bone disorders. In veterinary medicine the uses have been: the prevention of milk fever, pseudo-vitamin D deficiency of pigs and acidosis in chicks [7]. In animal husbandry, the assay of the effect of *SG* leaves powder on egg shell quality in laying hens was the first

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application [8,9]. Recently, *SG* has been added to the feed of chicks and cows to improve phosphorus utilization, and so, to reduce environmental phosphorus contamination due to animal husbandry [10]. Another promissing application in study is the supplementation of finishing cattle with *SG* to improve meat tenderness [11]. Moreover, *SG* dry leaves powder have been used in homeopathy and in mesotheraphy by french physicians to amelliorate certain pathological calcifications in joints and internal organs [12].

In order to evaluate *SG* toxicity an *in vivo* model has been developed, based on the subcutaneous administration to adult female rabbits of aqueous extracts (AE) of SG dry leaves for a week, measuring body weight during the trial and the calcium content in soft tissues at the end of it [13]. An *in vitro* method based on a radioimmunoassay (RIA) was also developed to estimate the Vitamin D activity (VDA) on glycoside fraction of AE of *SG*, based on the affinity of Vitamin D active glycosides with an antibody raised against 1α-(OH)Vitamin D_3 [14]. Both, toxicity and VDA, showed variation among *SG* field samples. High and significant correlation between the results obtained *in vivo* and *in vitro* was encountered, enabling the use of this RIA assay to study *SG* toxicity [13]. Nevertheless, a quantitative approach is necessary in order to know the actual content of the main active metabolite of Vitamin D_3. One approach may be to cleave the glycosides to yield free 1,25-$(OH)_2D_3$. Rumen fluid is known to be very efficient at cleaving Vitamin D glycosides [15]. In preliminary test we have determined the amount of *SG* leaves powder to be incubated, the incubation conditions, and the method of analysis of this metabolite. We have also determined that incubation of *SG* with deactivated (autoclaved) rumen fluid does not yield detectable 1,25-$(OH)_2D_2$ or 1,25-$(OH)_2D_3$ (data not published).

The present work was done to validate the simple novel method mentioned above, based on aqueous extraction followed by RIA, to evaluate the total VDA present in *SG*, in order to contribute to the knowledge of the factors that influences the rate of incidence of ES in enzootic areas of the disease. Additionally, this novel method could be used to calculate the amount of *SG* to be included as an additive in animal feeds.

2. Materials and methods

2.1. S. glaucophyllum (SG)

Ground dry leaves of the plant, that had been collected up in Nueve de Julio in Buenos Aires province of Argentina in 1999 and assayed *in vivo* and *in vitro* to evaluate its toxicity [13,14], were used as reference standard (RS). Samples of *SG* leaves and stems were obtained from diferent locations in the Buenos Aires province in Argentina during summer 2003–2004. Stems of *SG* were assayed as a negative control. Samples were treated the same way as RS as was described elsewhere [13,14]. AE of RS and samples were simmultaneously prepared incubating 5 g of finnely ground dry leaves in distilled water, filtering the upernatant through Whatman Number 1 filter discs, and purifing using C_{18} SPE minicolumns. The eluate was run by RIA [14].

2.2. Radioimmunoassay (RIA)

Duplicates of the glycoside fraction obtained from 15 samples (clustered in four groups) were run in four different assays. An antibody to detect 1α,25-$(OH)_2$Vitamin D_3, 1α, 25-(26,27-^{3}H) dihydroxyvitamin D_3 de S.A. = 164 Ci/mmol Dupont as the tracer, and 1α,25-$(OH)_2$Vitamin D_3 as the standard (generously gifted by Dr. M. Uskokovic, Hoffmann-La Roche, NJ, USA), were used. Samples were incubated and assayed on different days, always accompained by an alicuot of RS. Data were expresed as glycoside RIA 1,25-$(OH)_2D_3$ equivalents in ng/g of dry leaves and as % of RS [14].

2.3. Enzyme incubation and chromatography assay (HPLC)

Duplicates of the same samples assayed by RIA were used to quantify the 1,25$(OH)_2D_3$ content by HPLC. The procedure involved the incubation of finely ground (≤1 mm) *SG* leaves with 35 ml rumen fluid at 39 °C for 6 h under anaerobic conditions. The incubation media also contained 500 ng of 1,25-$(OH)_2D_2$ which was added as an internal standard prior to incubation, to account for extraction recovery and also for potential rumen microbial breakdown of the 1,25-$(OH)_2D_3$. Following the incubation period the samples were placed in an ice water bath to stop the reaction. The contents were spun at 1500 × *g* for 15 min and the supernatant removed. Following an additional wash with 10 ml of water the remaining pellet was extracted with 10 ml of methanol. Samples were centrifuged and 2 ml of the supernatants were transferred to 13 mm × 100 mm test tubes. Two milliliters of distilled water and 1 ml of 2% sodium metaperiodate were added and samples allowed stand at room temperature for 15 min. The prepared samples were then applied to C_{18}-OH solid phase extraction cartridges, which were washed successively with 5 ml each of methanol:water (70:30), hexane:methylene chloride (90:10) and hexane:isopropanol (99:1). Finally, the 1,25-$(OH)_2D_3$ fraction was eluted by the addition of 5 ml of hexane:isopropanol (92:8). The 1,25-$(OH)_2D_3$ fraction was dried under vacuum and prepared for HPLC by suspension in 150 μl of mobile phase. The HPLC column used was a Zorbax amino column developed in an isocratic mobile phase of methylene chloride:chloroform:hexane:isopropanol:methanol (70:20:10:0.67:0.33) with a flow rate of 2 ml/min. The 1,25-$(OH)_2D_2$ and 1,25-$(OH)_2D_3$ were detected using a Waters 996 photodiode array detector. Under these conditions 1,25-$(OH)_2D_2$ eluted at ∼34 ml and 1,25-$(OH)_2D_3$ at 46 ml. Typical recovery ranged from 60 to 70%. Quantification of the plant derived 1,25$(OH)_2D_3$ was achieved by relating peak areas of unknowns with those of a standard curve