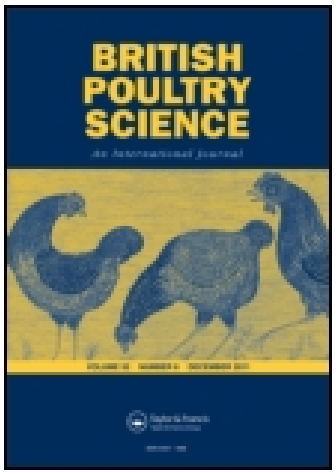

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H. Bachmann[a], S. Autzen[a], U. Frey[b], U. Wehr[c], W. Rambeck[d], H. McCormack[e] & C.C. Whitehead[e]

[a] Herbonis AG, Augst, Switzerland

[b] HES-SO Valais, Institute of Life Technologies, Sion, Switzerland

[c] Bavarian Health and Food Safety Authority, Bavaria, Germany

[d] Institute for Physiology, Physiological Chemistry and Animal Nutrition, Ludwig-Maximilian-University, Munich, Germany

[e] Roslin Institute (Edinburgh), Midlothian, UK

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The efficacy of a standardised product from dried leaves of *Solanum glaucophyllum* as source of 1,25-dihydroxycholecalciferol for poultry

H. BACHMANN, S. AUTZEN, U. FREY[1], U. WEHR[2], W. RAMBECK[3], H. McCORMACK[4] AND C.C. WHITEHEAD[4]

Herbonis AG, Augst, Switzerland, [1]HES-SO Valais, Institute of Life Technologies, Sion, Switzerland, [2]Bavarian Health and Food Safety Authority, Bavaria, Germany, [3]Institute for Physiology, Physiological Chemistry and Animal Nutrition, Ludwig-Maximilian-University, Munich, Germany, and [4]Roslin Institute (Edinburgh), Midlothian, UK

Abstract 1. Chemical characterisation of an extract of *Solanum glaucophyllum* (*SG*) leaves affirmed the predominant presence of 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$) glycosides. The compound 1-(ß-D-glucopyranosyl)-1α,25-dihydroxycholecalciferol was isolated for the first time from a natural source.
2. Vitamin D activity of the extract was confirmed by the calcaemic properties shown in a quail eggshell bioassay. The results suggested a $1,25(OH)_2D_3$ bioavailability of approximately 15%.
3. A broiler feeding experiment replicated in time was carried out with 6 treatments. A basic control diet containing 25 µg cholecalciferol/kg was supplemented with 2.5 and 5 µg free $1,25(OH)_2D_3$/kg, with a product based on dried *SG* leaves (Panbonis) providing 10 µg of $1,25(OH)_2D_3$-glycosides/kg, with two concentrations of an *SG* extract providing 8.8 and 37.8 µg of $1,25(OH)_2D_3$-glycosides/kg.
4. Tibia breaking strength and stiffness were numerically greater in all treatment groups with free 1,25 $(OH)_2D_3$ and with *SG* products compared to controls, though the overall treatment effects only had probabilities in the range of $P = 0.07$ to $P = 0.1$. Values for both characteristics increased progressively, with additions of synthetic $1,25(OH)_2D_3$; values with the dried *SG* product were similar to those with 5 µg synthetic $1,25(OH)_2D_3$/kg.
5. Plasma calcium was mildly elevated ($P < 0.05$) in treatment groups. The *SG* extract treatment containing 37.8 µg $1,25(OH)_2D_3$/kg gave the highest plasma calcium concentration and lowest bodyweight, signs of marginal hypervitaminosis D. Plasma $1,25(OH)_2D_3$ concentrations were in the normal range for all treatments.
6. Tibial dyschondroplasia occurred in only one replicate. The incidences were 31% in controls but considerably lower or zero with all other treatments.
7. Bioavailability of $1,25(OH)_2D_3$ in the *SG* product seemed to be higher in broiler chickens than in Japanese quails.
8. It is concluded that the inclusion of the dried *SG* product as a source of vitamin D_3 in broiler diets at a dietary concentration of 1 g/kg, providing 10 µg $1,25(OH)_2D_3$/kg, is safe and efficacious.

INTRODUCTION

Vitamin D is an essential nutrient with a major role in the regulation of a number of genes, many of which are involved in calcium absorption and transport and cell development. Vitamin D is normally provided in poultry diets as a supplement of cholecalciferol, but this has to be hydroxylated first in the liver to give 25-hydroxycholecalciferol [$25(OH)D_3$] and subsequently in the kidney to give 1,25-dihydroxycholecalciferol [$1,25(OH)_2D_3$]. This last compound is the main metabolically active form of vitamin D and binds to the vitamin D receptor (VDR) which in turn binds to the vitamin D response element in genes. The importance of $1,25(OH)_2D_3$ in calcium homeostasis is

Correspondence to: H. Bachmann, Herbonis AG, Rheinstrasse 28, CH-4302 Augst, Switzerland. E-mail: h.bachmann@herbonis.com
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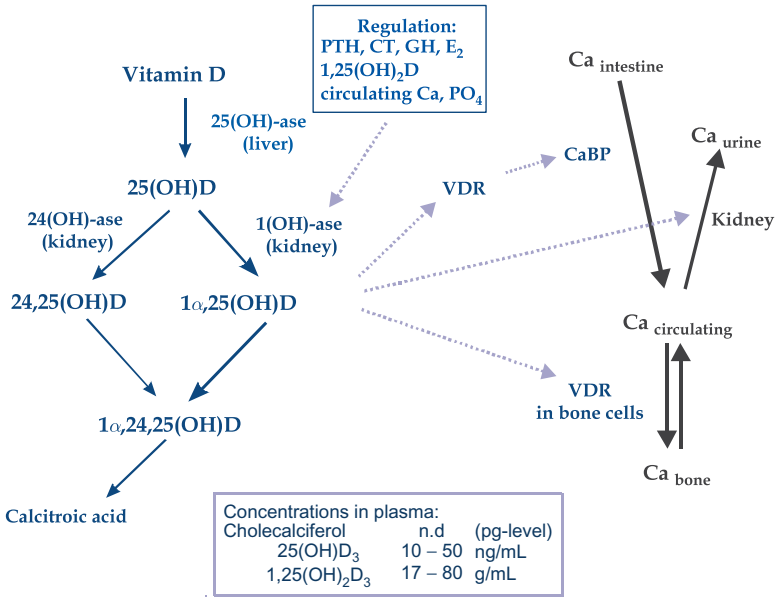

Figure 1. *Metabolic activation of vitamin D_3 (cholecalciferol) to the active metabolite and its degradation. Broken lines indicate effects of a compound on metabolic processes in different organs. (Modified after Norman et al., 1982). Abbreviations: VDR: Vitamin D receptor, CaBP: Calcium-binding protein, PTH: Parathormone, CT: Calcitonin, GH: Growth hormone, E2: Estrogen, Ca: Calcium; PO_4: Phosphate.*

shown in Figure 1, where the metabolite interacts with other hormones and factors to regulate calcium absorption from the intestine, absorption or deposition in bone and excretion in kidneys (Norman *et al.*, 1982).

The dietary requirement of broilers for vitamin D_3 quoted by NRC (1994) is 5 µg/kg (200 IU/kg). However, the requirement has been shown to be increased by suboptimal concentrations or ratios of calcium and phosphorus (Waldroup *et al.*, 1965) and the standard commercial practice is to feed very much higher concentrations, often up to the legal limit in many countries of 125 µg/kg (5000 IU/kg). These higher concentrations are needed to optimise bone development but even then they may not be sufficient because some recent research has suggested that concentrations up to 250 µg/kg (10000IU/kg) may be needed to minimise the occurrence of tibial dyschondroplasia (TD) under certain conditions (Whitehead *et al.*, 2004).

TD has been a long-standing problem in broiler production. It is characterised by an impairment of the normal progression of chondrocyte differentiation in the growth plate of long bones, most frequently in the proximal tibia, that results in the formation of a plug of prehypertrophic chondrocytes (Hargest *et al.*, 1985; Loveridge *et al.*, 1992). This abnormality has been shown to be painful in itself (Danbury *et al.*, 2000) and can lead to distortion of tibial growth and consequent lameness. The condition involves both genetic and nutritional factors. Genetic involvement was first established by Leach and Nesheim (1965). The lesion can be visualised with x-rays and selection against TD using this technology has been used to breed lines of birds with lower susceptibility to TD (Sorensen and Su, 2001). However, the technology is more effective in detecting larger TD lesions, so application in commercial breeding programmes has not eliminated susceptibility to TD from current broiler production. There is thus continued interest in nutritional approaches to the problem.

Nutritional factors influencing TD have been reviewed by Whitehead (1997). Low dietary calcium-to-phosphorus ratio can increase the incidence of TD (Edwards and Veltmann, 1983). However, vitamin D and its metabolites have been found to be much more effective in minimising TD. As indicated above, feeding high concentrations of vitamin D (above legal limits) has been shown to help minimise the occurrence of TD (Whitehead *et al.*, 2004). More effective nutritional strategies involve feeding vitamin D metabolites. Dietary supplementation with 1-hydroxycholecalciferol or 25(OH)D_3 can be effective (Rennie and Whitehead, 1996) though relatively high concentrations (70 µg/kg and above) of the latter metabolite may be needed. The most effective metabolite has been found to be 1,25 $(OH)_2D_3$. This effect was first reported by Edwards (1989) and has subsequently been confirmed by many other studies (Edwards, 1990; Edwards *et al.*, 1992; Rennie *et al.*, 1993, 1995; Elliot and Edwards, 1997; Carlos and Edwards, 1998). Dietary concentrations in the range of 2.5 to 10 µg/kg have been found to prevent TD completely, though there can be toxicity problems resulting from hypercalcaemia at the higher end of this range, if the diets also contain higher calcium concentrations (Rennie *et al.*, 1995). The

mechanism of action of vitamin D in preventing TD has not yet been fully established. However, $1,25(OH)_2D_3$ is known to regulate a number of genes involved in cell differentiation, and there have been several studies investigating effects on growth plate chondrocytes (Farquharson *et al.*, 1993, 1996; Loveridge *et al.*, 1993).

Dietary supplementation with synthetic $1,25(OH)_2D_3$ is not practical commercially because of the high cost of chemical synthesis. However, the metabolite occurs naturally in some plants, particularly in *Solanum glaucophyllum* (*SG*, formerly named *Solanum malacoxylon*). This perennial shrub is a native of partly flooded low-lands of Argentina, Brazil and Paraguay, and has come to attention as a result of the toxic symptoms seen when cattle ingested too much of these plants. The symptoms of cattle affected by "*enteque seco*" coincide with hypervitaminosis D (Worker and Carrillo, 1967). Calcaemic properties of *SG* have also been demonstrated in poultry. Feeding dried *SG* plant material to laying hens has been reported to lead to thicker eggshells (Morris, 1977; Azcona *et al.*, 1982). Such observations led to the identification of $1,25(OH)_2D_3$ as the active principle in the plant (Napoli *et al.*, 1977; Boland *et al.*, 2003; De Boland *et al.*, 1978). The metabolite has been shown to be present as a glycoside linked to a variable number of sugar units (Vidal *et al.*, 1985). Since this plant contains substantial amounts of $1,25(OH)_2D_3$ as the main vitamin D-active component, its therapeutic use in human and veterinary medicine has been suggested but has not been achieved with a standardised product until recently.

This article has three parts. First, we describe the characterisation of cholecalciferol derivatives in a product derived from dried leaves of *SG* (available as Panbonis) using chemical techniques. Second, we establish the vitamin D activity by the calcemic effects of the product using a Japanese quail eggshell test. This bioassay assesses the vitamin D activity in unknown preparations based on the recovery in eggshell production following a period of depletion of vitamin D. The procedure has been described in detail by Rambeck and Zucker (1985). The test correlates highly with other more demanding tests such the rat line test (AOAC, 1936) or the prophylactic growth assay in chickens (Rambeck *et al.*, 1984). Finally, we demonstrate the effectiveness of the product in optimising bone properties in broilers.

MATERIALS AND METHODS

Chemicals and reagents

Chemicals and solvents are purchased, if not otherwise stated, from Sigma-Aldrich, CH-9471 Buchs, Switzerland and were of the highest purity for analytic work and adequate purity for preparative procedures. 1,25-Dihydroxyvitamin D_3 was purchased from Cerbios-Pharma SA, CH-6917 Barbengo/Lugano, Switzerland.

Preparation of *Solanum glaucophyllum* product and extract

SG was harvested in Buenos Aires Province, Argentina as wild plants and as cultivated plants in Switzerland and elsewhere. Air flow-dried (max. 45°C) leaves were ground in a centrifugal mill (ZM-100 Retsch, www.retsch.de). The dried powder was mixed with wheat middlings to give a product (Panbonis) of an adjusted content of $1,25(OH)_2D_3$ with 10 µg/g analytically determined $1,25(OH)_2D_3$.

For extract preparation, dry *SG* leaves were extracted with 6 volumes (m/v) water/ethanol 8 + 2 at 40°C for 8 h. The extract was filtered through a 0.2 µm filter and evaporated in a rotary evaporator (Büchi, Uster Switzerland) to a concentration of 30% and kept at −20°C for further processing or for lyophilisation.

Purification and characterisation of the *Solanum glaucophyllum* extract

A purification step was performed by size exclusion chromatography on Sephadex G10 (Amersham Biosciences) with water as solvent according to the suppliers protocol. Fractions (1% of the column volume) were collected and total $1,25(OH)_2D_3$ was measured separately after enzymatic glycolysis with a glycosidase kit (Glycoprep, BMA Biomedicals, CH-4302 Augst, Switzerland).

Isolation of active vitamin D metabolites was carried out in the aqueous extract. After standing for 24 h at room temperature, the solution was applied to mass exclusion chromatography on Sephadex G25, followed by low-pressure chromatography on a polystyrene column (Amberlite XAD-1180, Sigma-Aldrich, Buchs Switzerland) and by two preparative HPLC runs on RP18 phase. The isolated component was subjected to analytical methods such as MS, NMR, UV/Vis spectroscopy.

Analyses

$1,25(OH)_2D_3$: Plant material was extracted by an automated solvent extraction system (Dionex ASE-200, www.dionex.com) with 65% ethanol (v/v) and subsequently analysed for $1,25(OH)_2D_3$ by means of an ELISA (Immundiagnostik, Bensheim Germany, www.immundiagnostik.com) according to the suppliers protocol, but without the separation of $25(OH)D_3$. Total $1,25(OH)_2D_3$ was measured after enzymatic glycolysis with the glycosidase kit and subsequent application to the ELISA.

Blood plasma $1,25(OH)_2D_3$ was measured in pools of 1 ml samples with the same ELISA, according to the supplier's protocol using an appropriate dilution with phosphate-buffered saline.

Total carbohydrates were measured with a phenol sulphuric acid method after hydrolysis (Mecozzi *et al.*, 2002) and reducing sugars by a method for reducing sugars in plant tissues (Prado *et al.*, 1998; Chaplin, 2001). Amino acids and peptides were determined by the Kjeldahl nitrogen procedure for proteins. Characterisation of the *SG* leaf material was done by proximate analysis.

High performance thin layer chromatography (HPTLC) was carried out by means of a Linomat 5 (all reagents and equipment from Camag, Muttenz Switzerland, www.camag.com) and 10×10 cm silica plates (Merck, Germany, www.merck-chemicals.com) using cyclohexane/tertiary-butyl methyl ether/methanol (6 + 4 + 1, v/v/v) or tertiary-butyl methyl ether/methanol/water (7 + 2 + 1, v/v/v) as mobile phases. Detection for solanine, chaconine and tomatine and for aglycones solasodine and solanidine after acid hydrolysis (2 h in 2N HCl at 95 °C) was performed with Carr-Price and Dragendorff reagents for alkaloids and plant sterols, with diphenylamine reagent for carbohydrates under visible and UV light. Quantitative analysis for the glycoalkaloids α-chaconine and α-solanine was performed at UFAG-Laboratorien, Sursee, Switzerland www.ufag-laboratorien.ch) with a HPLC/MS-MS method.

Blood calcium, phosphate and alkaline phosphatase were measured with assay kits from Roche Diagnostics (www.roche-diagnostics.com; o-cresolphthalein method for calcium #11730240; ammonium phosphomolybdate method for phosphate #11730347 and p-nitrophenyl phosphate for the alkaline phosphatase #2173107). All methods were adapted to a multititer plate reader (Molecular Devices SpectraMax 190, www.moleculardevices.com) using the same volume proportions and measured at two wavelengths (570 minus 700 nm).

Determination of vitamin D activity by means of the Japanese quail eggshell bioassay

Japanese quail hens (*Coturnix japonica*) caged individually were selected for a laying performance of >80% during a 10-d period. The birds were then given a diet deficient in vitamin D, but containing all other nutrients (see Table 1 and Rambeck and Zucker, 1985).

After approximately 8 d, rate of egg-laying dropped below 10%. The birds were then placed in individual cages, 10 birds/treatment. The treatments were allocated to the cages at random and comprised the basal diet (Table 1) supplemented with different doses of the test substances, as shown in Table 2, for 21 d.

Table 1. Composition of the quail diet

Ingredient	g/kg
Maize	434
Soybean meal	390
Calcium carbonate	75
Soy bean oil	40
Soy flour	30
Anilac®	10
Cefkaphos®	10
Sodium chloride	4
Vitamin premix	3
DL-methionine	2.5
Trace element premix	1
Zinc sulphate	0.5

Analyses were (g/kg dry matter): crude protein 264, crude fat 71, crude fibre 39, crude ash 137, N-free extract 489, calcium 28, phosphorus 7.0.

Vitamin and trace element premix (mg/kg dry matter): retinol 4.5, cholecalciferol 33, tocopherol 36, menadion 1.2, thiamine 5.4, riboflavin7.2, pyridoxine 3, cobalamin 0.03, biotin 0.09, folic acid 0.18, iron 62, zinc 80, manganese 81, copper 11, iodide 2.3, cobalt 0.8, selenium 0.9, fluor 4.5, magnesium 60.

Eggs were collected daily, weighed and shell weight was measured after cleaning and drying at 100°C. Eggshell weight per animal and day was calculated for the individual groups between d 5 and 21. Laying performance and feed consumption were monitored weekly. At the end of experiment, the animals were killed and blood samples were taken. Serum calcium, phosphate and alkaline phosphatase were measured individually, whereas $1,25(OH)_2D_3$ was determined in pools of 5 animals. The effects of treatments were analysed by Student's *t*-test. Pairwise comparison was used to determine the difference between treatments (SAS software, SAS Institute Inc., Cary, NC).

Blood parameters

Serum calcium was measured by flame photometry of an aliquot diluted in a 1% lithium chloride solution and acetylene as combustion gas (Photometer ELEX 636, Eppendorf, Hamburg, Germany). For alkaline phosphatase (ALP), serum samples were added to 1 ml substrate solution containing 2.5 mM p-nitrophenyl phosphate (Sigma-Aldrich) in 50 nM sodium hydroxide-glycine buffer of pH 10.0 and containing 0.5 mM $CoCl_2$. The mixture was heated for 2.5 min at 70° C after serum addition and was measured in kinetic mode during the linear phase at 410 nm in a Genesys 10 UV spectrophotometer (Thermo Spectronic). One unit of enzyme activity is defined as the amount of enzyme required to release 1 µmol of p-nitrophenol from pNPP per minute. $1,25(OH)_2D_3$ was determined in serum by means of a competitive Enzyme-Immuno-Assay (EIA), according to the suppliers protocol.

Table 2. Treatments and parameters assessed in the eggshell test with quails

Test compound, µg/kg		Cholecalciferol 2.5	Cholecalciferol 5.0	Cholecalciferol 10.0	1,25(OH)2D3 0.5	1,25(OH)2D3 1.0	SG extract[2] 1.1	SG extract[2] 4.4	SG extract[2] 17.6	SG extract[2] 70.4	SG extract[2] 282.0
Treatment number		1	2	3	4	5	6	7	8	9	10
Eggshell dry weight per animal and day	mg	231	434	738[a]	10	502	17	187	687	802	782
	SD	123	183	169	13	170	23	47	152	71	176
	%[1]	31	59	100	1	68	2	25	93	109	106
Egg weight	g	9.6	10.4	10.9[b]	10.0	10.8	9.8	9.9[b]	11.4	11.5[b]	11.7[b]
	SD	0.76	0.60	0.51	0.00	0.61	2.77	0.83	0.65	0.25	0.27
	%[1]	88	96	100	92	99	90	91	105	106	108
Serum calcium	g/l	215	195	187[a]	259[b]	200	262[b]	233	184	179	282[b]
	SD	70	69	28	86	84	74	92	41	34	109
	%[1]	115	105	100	–	107	140	125	99	96	151
1,25(OH)2D3	pg/ml	21	24	129	4	30	26	58	132	146	–
	%[1]	16	18	100	4	23	20	45	102	113	–
ALP	IU/ml	2314	2371	800	2637	1483	3301	3023	1142	531	609
	SD	620	1011	223	1038	658	251	1261	462	267	234

[1]% compared to control group 3.
[2]Concentrations as analytically determined 1 µg $1,25(OH)_2D_3$ per gram extract.
[a]$P = 0.001$
[b]$P = 0.05$ or
[c]$P = 0.01$ when tested against control group 3.

Evaluation of effectiveness of products from *SG* on performance and bone characteristics in broiler chickens

This experiment was carried out with two replicates separated in time. In each replicate, Ross 308 male broiler chicks were obtained from a commercial hatchery at one d old. The birds were individually wing-banded, weighed and randomly allocated to one of the 6 floor pens, 31 birds per pen, which were deep-littered with wood shavings and fitted with a bell drinker and tube feeder. Dietary treatments were as below:

Treatment	Diet
1	Control (contained 25 µg cholecalciferol/kg)
2	Control + 1,25(OH)2D3 (2.5 µg/kg)
3	Control + 1,25(OH)2D3 (5.0 µg/kg)
4	Control + purified extract [9.5 µg 1,25(OH)2D3/kg]
5	Control + purified extract [37.8 µg 1,25(OH)2D3/kg]
6	Control + dried SG leaves [10 µg 1,25(OH)2D3/kg]

The control diet was an all-vegetable broiler starter diet based on wheat and soybean meal and formulated to contain 8 g calcium, 6 g available phosphorus and 25 µg cholecalciferol/kg. This diet had the same composition as the imbalanced diet used by Rennie *et al.* (1993) to demonstrate the efficacy of $1,25(OH)_2D_3$ in preventing TD. The relatively low Ca/P ratio was intended to increase TD incidence under laboratory conditions. The remaining experimental diets were prepared by addition of the appropriate supplement (see above) to the control diet. The dried *SG* leaves product contained 10 µg $1,25(OH)_2D_3$/g, so the dietary inclusion level of 1.0 g dried product/kg was calculated to provide a total dietary concentration of 10 µg $1,25(OH)_2D_3$/kg. The purified extracts were prepared separately for the two replicates and, after reanalysis, the amounts used for diet 5 provided 8.8 and 10.1 (mean 9.5) µg of $1,25(OH)_2D_3$/kg for replicates 1 and 2, respectively. For diet 6, the values were 35.2 and 40.4 (mean 37.8) µg of $1,25(OH)_2D_3$/kg, respectively. These differences in values between the replicates were within the 15% error margin involved in the analytical procedure (extraction, hydrolysis and assay).

At 14 d of age, the birds were individually weighed and blood samples (1ml) were taken from the wing vein. Samples were stored immediately on ice and measurement of blood ionised calcium was made within one hour of the samples being taken. The samples were then centrifuged at 700 *g* for 10 min at 4°C, and plasma was stored at −70°C for subsequent measurement of plasma $1,25(OH)_2D_3$. The birds were killed by lethal injection and the growth plate of the left tibia was examined for the presence of lesions. Tissue samples of any abnormal growth plate were taken and fixed in 10% neutral buffered formalin for subsequent histological examination. The right tibia was dissected out and cleaned of any adhering tissue. Bone breaking strengths were determined by three-point destructive bending tests, using a JJ Lloyd LRX50 materials testing machine running the software package Nexygen 2.0 and fitted with a 100 N load cell. The bending jig consisted of two 10 mm diameter steel bar

supports, 30 mm apart at the centre and a 10mm diameter cross head which approached at 30 mm/min. The failure point was set at a load which was 30% of the maximum load. Stiffness was calculated automatically from the load/displacement curve.

Data were analysed by ANOVA using the GENSTAT statistical package (GENSTAT 6.1, Lawes Agricultural Trust, Rothamsted Experimental Station, UK). The nested ANOVA model included replicate, pen and bird in the block statement, giving 5 degrees of freedom for both treatment and residual variation between pens.

RESULTS

Purification and characterisation of the *Solanum glaucophyllum* extract

The vitamin D-active content in collected *SG* was found to vary from 0 to 20 µg $1,25(OH)_2D_3$ (analysed) per g dry leaves, depending on location and time of collection. Under cultivated conditions, a more homogenous content of 16–25 µg/g was found.

Qualitative separation of cholecalciferol derivatives by HPLC/MS yielded after hydrolysis 1,25 $(OH)_2D_3$ and its putative biochemical precursors $25(OH)D_3$, cholecalciferol and 7-dehydrocholesterol. Quantitative estimation indicated that these derivatives were present higher than 90% as $1,25(OH)_2D_3$ in glycosidically bound form and less than 10 % as free $1,25(OH)_2D_3$, $25(OH)D_3$ and cholecalciferol (average of several preparations).

Separation of an aqueous *SG* extract on Sephadex G25 and assay of the individual fractions for $1,25(OH)_2D_3$ after glycolysis showed a molecular size distribution between 700 and 4500 Daltons, suggesting a distribution of bound carbohydrates between 1 and 12 hexose units, with a peak at approximately 1800 Daltons.

Starting from *SG* leaf material containing 17 µg/g analysed $1,25(OH)_2D_3$ after extraction and Sephadex G10 chromatography, the active content could be enhanced to 550 µg/g by further purification of the extract on Amberlite XAD-1180, followed by two preparative runs on HPLC on RP18 phase to yield a light yellow residue of >95% purity (uniformity). The isolated pure compound was characterised by UV analysis, NMR and ESI-TOF as *1-(β-D-glucopyranosyl)-1α,25-dihydroxycholecalciferol, CAS number [89457-77-2]*, whose structure is shown in Figure 2.

Of the typical bulk components, carbohydrates, determined as total sugars after hydrolysis, comprised 85%, amino acids and peptides (8%), inorganic matter (5%), lipids and proteins (less than 2%) of the purified extract. HPTLC chromatography developed with diphenylamine reagent detected glucose, fructose, sucrose and oligosaccharides and polysaccharides (not identified). Due to the extraction conditions, free plant phenols, xanthophylls and chlorophylls and other fat-soluble components are virtually absent. Noteworthy was a high content of quercetin glycosides of 14%, but no free quercetin in the extract.

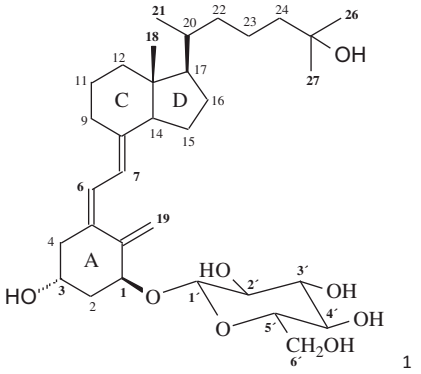

Figure 2. *Structure of the isolated 1,25-dihydroxycholecalciferol-glycoside from Solanum glaucophyllum as 1-(β-D-glucopyranosyl)-1α,25-dihydroxycholecalciferol. CAS number [89457-77-2].*

HPTLC chromatography for alkaloids with Dragendorff and Carr-Price reagent, with and without acid hydrolysis, suggested, in the cultivated plant material, an alkaloid content of <0.1 mg/g. Quantitative analysis for the Solanum-typical glycoalkaloids α-solanine and α-chaconine in the extract was below the detection limit of 0.01 µg/g. By using cultivated plant material, the variability in active components and the bulk composition was less than ±10%.

Determination of vitamin D activity by means of the Japanese quail eggshell test

In the quail eggshell test, the onset of egg laying was faster in the groups treated with synthetic 1,25 $(OH)_2D_3$ or *SG* extract than in the groups receiving vitamin D_3 (data not shown). Regain of 50 % laying performance occurred in groups 4–11 as soon as 24 h after the start of treatment, whereas in groups 1–3 receiving cholecalciferol at 72 h of treatment. Repletion with 2.5, 5.0 and 10 µg cholecalciferol/kg diet showed a linear dose–response in the increase of eggshell dry weight, as shown in Table 2. Eggshell weight did not reach the maximum with a dose of 1.0 µg synthetic 1,25 $(OH)_2D_3$. With the *SG* extract, eggshell weight reached a maximum with a dose of 70.4 µg 1,25 $(OH)_2D_3$/kg.

Other characteristics also showed changes. Egg weight was significantly ($P < 0.05$) higher in the *SG* groups (8, 9 and 10), receiving 17.6, 70.4 and 282 µg/kg feed $1,25(OH)_2D_3$ compared to the standard group 3, receiving 10 µg/kg feed

vitamin D_3. Serum calcium was within normal ranges for all the treatments, except at the highest dose of 282 µg of 1,25$(OH)_2D_3$/kg from the *SG* extract, where it was elevated significantly ($P < 0.05$). Serum 1,25$(OH)_2D_3$ concentrations increased at higher concentrations of each of the dietary additives whereas plasma alkaline phosphatase declined.

An evaluation of the eggshell weight per animal and day from data given in Table 2 suggested a half size effect for the standard vitamin D_3 at doses of 4.5 µg cholecalciferol/kg, 0.9 µg synthetic 1,25$(OH)_2D_3$/kg and 7.5 µg 1,25$(OH)_2D_3$/kg for the *SG* extract.

Broiler feeding experiment

The results of the broiler feeding experiment, combining the results from both replicates, are shown in Table 3. Differences in live weight at 14 d were only significant at $P<0.07$ and did not show any consistent numerical differences between the control and the diets containing synthetic or natural 1,25$(OH)_2D_3$. Tibia breaking strength showed a treatment effect significant at $P = 0.10$ with all treatments containing synthetic or natural 1,25$(OH)_2D_3$ showing higher values than the control. Similar responses were seen for tibia stiffness with an overall treatment effect significant at $P = 0.07$. Blood ionised calcium concentrations were significantly ($P < 0.05$) lower with the control than for all other treatments, except that containing 5.0 µg synthetic 1,25$(OH)_2D_3$/kg. Plasma 1,25$(OH)_2D_3$ concentrations were within the normal range for all treatments.

TD incidence in replicate 1 was: control, 31%; treatment 2, 7%; treatment 3, 4%; treatment 4, 0%; treatment 5, 7%; treatment 6, 0%. TD was not seen in the second replicate.

Some analytical data are given in Supplemental Table S1 (available via the online version of this article at: http://dx.doi.org/10.1080/00071668.2013.825692).

DISCUSSION

Characterisation of extracts from the *Solanum glaucophyllum* leaves indicated that more than 90% of the vitamin D active substances were derivatives of 1,25$(OH)_2D_3$ glycosidically-linked to carbohydrate units. Small proportions of 7-dehydrocholesterol, cholecalciferol and 25(OH)D_3 were also identified. These findings are consistent with the theory that the biosynthetic route to 1,25$(OH)_2D_3$ is the same in plants as in animals. For instance, Skliar *et al.* (1992) found a relative distribution of 29% cholecalciferol, 8% 25(OH)D_3, 53% 1,25$(OH)_2D_3$ and 10% 1,24,25-trihydroxycholecalciferol in extracts from *Solanum glaucophyllum (*formerly named *S. malacoxylon*) treated with bovine ruminal fluid. However, it was assumed that the trihydroxy derivative had been the result of the microbial action of the ruminal fluid. A qualitative study on extracts from the *SG* cultures grown in the dark identified the presence of 7-dehydrocholesterol, cholecalciferol, 25(OH)D_3 and 1,25$(OH)_2D_3$ (Curino *et al.*, 1998). These authors concluded that a photolytic step was not necessary for the cleavage of the B sterol ring in the conversion of 7-dehydrocholesterol to cholecalciferol. However, it is our experience that 1,25$(OH)_2D_3$ concentration is low in *SG* plants grown under glass (data not shown). *SG* collected in their natural habitat was analysed to contain between 0 and 20 µg 1,25$(OH)_2D_3$/g dry plant matter, depending on location, year and weather conditions. A cultivated variety showed a more homogenous active content of 17–25 µg/g.

The most common derivatives of 1,25$(OH)_2D_3$ found in the present study on *SG* involved a linkage through the 1-position to a single D-glucose moiety (*1-(β-D-glucopyranosyl)-1α,25-dihydroxycholecalciferol*). The molecular size distribution indicated the presence of other oligo glycosides. The chromatographic elution pattern showed a distribution corresponding to 1 to 12 glycoside units, with a maximum at approximately 4 units.

Table 3. Live weight, tibia breaking strength and stiffness, blood ionised calcium and plasma 1,25-dihydrocholecalciferol [1,25$(OH)_2D_3$] at 14 d in broilers given diets supplemented with different concentrations of 1,25$(OH)_2D_3$, a product based on dried leaves of Solanum glaucophyllum and extracts of the leaves

Treatment	1	2	3	4	5	6	SED	Significance
Live weight (g)	287	297	281	291	274	306	8.6	**
Tibia breaking strength (N)	57.7	63.8	65.7	59.2	60.9	68.1	3.1	*
Tibia stiffness (N/mm)	45.8	50.6	52.9	46.6	46.2	52.5	2.1	**
Blood ionised calcium (mm)	1.21[a]	1.30 [b]	1.26[ab]	1.30[b]	1.33[b]	1.31[b]	0.03	***
Plasma 1,25$(OH)_2D_3$ (pmol/ml)	48.8	50.8	44.0	46.1	47.3	40.6	pooled samples	

Treatments: 1, Control (contained 25 µg cholecalciferol/kg); 2, Control + 1,25(OH)2D3 (2.5µg/kg); 3, Control + 1,25(OH) 2D3 (5.0 µg/kg); 4, Control + purified extract [9.5 µg 1,25(OH) 2D3/kg]; 5, Control + purified extract [37.8 µg 1,25(OH) 2D3/kg]; 6, Control + dried SG leaves [10.0 µg 1,25 (OH) 2D3/kg].

Values are means of 60 observations, 30 from each of the 2 pens, except plasma 1,25$(OH)_2D_3$ which is the mean of 2 samples each pooled from 30 birds/replicate.

Mean values within the same row sharing a common superscript letter are not statistically different at $P < 0.05$.

*$P = 0.10$; **$P < 0.07$; ***$P < 0.05$.

Other derivatives that have been identified include chains of up to 9 glucose units comprising 1, 2 or 4 units of the disaccharide kojibiose (2-O-α-D-glucopyranosyl-D-glucose) and terminally linked to a fructose moiety (Vidal *et al.*, 1985). A further type of derivative, a diglycoside, has been described by Zimmerman *et al.* (2003) in which one glucopyranoside moiety was linked to the 1-position and another to the 3-position of the seco-steroid structure.

The presence of the alkaloid solasodine in seeds and callus cultures of *SG* has been described by Jain and Sahoo (1986). However, the present study suggested that alkaloid comprised only a barely detectable component in leaves and extracts of *SG* from cultivated plants. Semi-quantitative estimation by HPTLC showed for *SG* plants a content of less than 0.1 mg/g of alkaloid-positive spots co-migrating with the *Solanum* species typical glycoalkaloids α-solanine and α-chaconine, as well as the aglycons solanidine and solasodine after acid hydrolysis. There is thus little danger of alkaloid toxicity from *SG* since this concentration is considerably less than that found in potato sprouts, for instance (Friedman, 2006). The other extract constituents are the typical plant components such as carbohydrates, inorganic salts, organic and amino acids, but only traces of fats, proteins and insoluble fibres are found. The hydrophilic extraction method makes it unlikely to enrich lipophilic compounds. A remarkably high content of quercetin glycosides is present in the flavonoid class.

Further characterisation of the *SG* leaves and extract shows aside of the unique $1,25(OH)_2D_3$- and quercetin- glycosides content, no notable differences to other green plants abundantly present in the feed. From our experience and observations from the wild habitat, all effects can be contributed to the vitamin D metabolite content. An eventual combinational effect of the vitamin D and quercetin needs further investigation.

The quail eggshell bioassay confirmed the vitamin D biopotency of the *SG* extract. Activity estimation for the cholecalciferol standards was in good agreement with published values (Rambeck and Zucker, 1985). Serum measurements of $1,25(OH)_2D_3$ showed that cholecalciferol supplementation in groups 1 and 2 was too low to achieve normal concentration of $1,25(OH)_2D_3$ (100–150 pg/ml), whereas in group 3, a concentration of 10 μg/kg gave a normal serum concentration and a near maximal response in eggshell weight. This concentration also gave values for serum calcium, $1,25(OH)_2D_3$ and ALP that could be considered normal. The responses to supplementation with the free $1,25(OH)_2D_3$ suggested that the amounts given were insufficient to achieve optimal responses in eggshell weight or serum $1,25(OH)_2D_3$. However, there was a strong response in eggshell weight to additions of the *SG* extract over the range of supplemental concentrations. Quails given this extract also started laying eggs one to two d earlier than animals given vitamin D_3. This difference can be explained by the time needed for the two enzymatic steps in the endogenous transformation of cholecalciferol to $1,25(OH)_2D_3$ (see Figure 1). Normal values of eggshell weight, serum calcium, $1,25(OH)_2D_3$ and ALP occurred with a measured dietary content of 70.4 μg $1,25(OH)_2D_3$/kg in the *SG* extract. The highest concentration of $1,25(OH)_2D_3$ (282 μg/kg) gave normal shell production, but significantly elevated serum calcium, indicative of a beginning hypercalcaemia. This was not observed to result in calcification of soft tissues by pathological examination.

For free $1,25(OH)_2D_3$, it is known that the safe dose range for broilers is 2.5–10 μg/kg whereas higher doses have resulted in reduced weight gain and clinical hypervitaminosis (Weiser *et al.*, 1990). Even 10 μg/kg can cause hypercalcaemia if given in conjunction with higher dietary concentrations of calcium (Rennie *et al.*, 1995). There is no equivalent information on the toxicity of glycosidically linked $1,25(OH)_2D_3$. However, assuming an approximately similar toxicity for laying quail as for broilers, the apparent tolerance of quail to a concentration of 70.4 μg $1,25(OH)_2D_3$/kg might be considered surprising. This observation can be explained in two ways. One by the comparative responses in eggshell output to free $1,25(OH)_2D_3$ and glycosidically linked $1,25(OH)_2D_3$ in *SG* which indicate a lower bioavailability of glycosidically bound $1,25(OH)_2D_3$ in the quail. Based on the responses in eggshell production in the Japanese quail test, it was estimated that synthetic $1,25(OH)_2D_3$ was 5 times more active than cholecalciferol. The glycosidically linked $1,25(OH)_2D_3$ in the purified extract was 1.7 times less active than cholecalciferol and approximately 7 times less active than synthetic $1,25(OH)_2D_3$. These findings suggest that in quail, the bioavailability of $1,25(OH)_2D_3$ in *SG* is approximately 15%. However, the plateau of eggshell production was achieved in group 8 at a dose of 17.6 μg $1,25(OH)_2D_3$/kg while a subclinical hypercalcaemia occurred only at 282 μg/kg which counts for a safety factor of 16 and clearly higher than for free $1,25(OH)_2D_3$.

A second argument may be different uptake characteristics in which the cleavage of the glycosidic part performed by intestinal glycosidases modifies the resorption of the free $1,25(OH)_2D_3$ in order to reduce high peak concentrations in circulation. Such a prodrug-like effect would be difficult to prove. However, Londowski *et al.* (1986) has shown in the rat that parenterally applied $1,25(OH)_2D_3$-glycosides are cleaved in blood circulation. In addition, a recent

publication (Bachmann *et al.*, 2013) demonstrated a different plasma profile between free 1,25(OH)$_2$D$_3$ and 1,25(OH)$_2$D$_3$-glycosides in rats.

The broiler trial was intended to show the potency of the *SG* product as a source of vitamin D. The basal diet was not formulated to be wholly adequate for broilers. Instead, the low Ca/P was designed to increase the possibility of TD and the concentration of 25 µg cholecalciferol/kg was meant to give a good level of performance without necessarily maximising bone quality. Although the "official" NRC (1994) requirement for cholecalciferol is 5 µg/kg, it is known that the requirement is increased with imbalanced Ca/P (Waldroup *et al.*, 1965). With normal, adequate diets, a supplemental concentration of cholecalciferol is often 125 µg/kg. However, recent findings of Whitehead *et al.* (2004) have shown that the requirements of modern broilers can be considerably above 125 µg/kg to give optimum bone characteristics.

In the present study, overall treatment effects for bone strength and stiffness were only significant in the range $P = 0.07$ to $P = 0.1$. However, both characteristics showed progressive increases to the addition of 2.5 and 5 µg free 1,25(OH)$_2$D$_3$/kg in the control diet. Addition of the dried *SG* product, providing a dietary content of 10 µg 1,25(OH)$_2$D$_3$/kg, gave similar bone strength and stiffness to the higher free 1,25(OH)$_2$D$_3$ treatment. The *SG* extracts gave poorer bone characteristics than the other treatments containing 1,25(OH)$_2$D$_3$, and it was noticeable that the treatment providing 37.8 µg 1,25(OH)$_2$D$_3$/kg gave weakest growth and had highest plasma ionised calcium concentration than all the other treatments. These observations could perhaps indicate that this concentration of total 1,25(OH)$_2$D$_3$ was on the margin of hypervitaminosis D. Plasma 1,25(OH)$_2$D$_3$ concentrations for all treatments were within a normal range.

A high incidence of TD occurred with the control treatment in the first replicate. Unfortunately, TD did not occur in the second replicate so the TD data are not suitable for statistical analysis. The reason for the non-appearance of TD in the second replicate is unknown, but may be related to genetic differences in the second batch of birds or to differences in husbandry of parents or treatment of eggs during incubation that may have influenced TD in unknown ways. However, in the first replicate, all treatments containing 1,25(OH)$_2$D$_3$ in any form, decreased the incidence to very low or zero. This observation is consistent with the wide body of published information showing the effectiveness of 1,25(OH)2D3 in preventing TD (Edwards, 1989; Edwards *et al.*, 1992; Rennie *et al.*, 1993; 1995; Elliot and Edwards, 1997; Carlos and Edwards, 1998) and suggest the vitamin D substance in *SG* is functioning metabolically in the bird as 1,25(OH)$_2$D$_3$. A metabolic effect attributable to 1,25(OH)$_2$D$_3$ is consistent with other studies showing that phosphorus utilisation in broilers can be improved by feeding 1,25(OH)$_2$D$_3$ (Edwards, 2002) or *SG* (Cheng *et al.*, 2004). This last observation suggests that *SG* may have wider benefits for poultry, allowing lower phosphate feed supplementation and decreasing the phosphorous content of excrements.

The finding that 10 µg of 1,25(OH)$_2$D$_3$/kg provided by the dried *SG* product was comparable to the concentration of 5 µg free 1,25(OH)$_2$D$_3$/kg in enhancing bone characteristics, which suggests that the *SG* product may have provided more available 1,25(OH)$_2$D$_3$ than the 1.5 µg/kg that might be calculated on the basis of the quail data. Furthermore, the observations on the *SG* extracts that the treatment providing 37.8 µg total 1,25(OH)$_2$D$_3$/kg was possibly toxic suggests that this treatment was contributing substantially more than the 5.7 µg available 1,25(OH)$_2$D$_3$/kg that might have been calculated on the basis of the quail data. Bioavailability of glycosidically linked 1,25(OH)$_2$D$_3$ may thus be considerably higher in broilers than in laying quail.

In conclusion, it is apparent that the product obtained from dried leaves of *Solanum glaucophyllum* is a good source of vitamin D$_3$ activity in the form of 1,25(OH)$_2$D$_3$ glycosidically linked to a variable number of hexose units. The 1,25(OH)$_2$D$_3$ is partially available to poultry, as shown by the quail eggshell test and bone and growth responses in broilers, with the probability that bioavailability is higher in broilers than the 15% calculated for quail. Inclusion of the dried *SG* product in a dietary concentration of 1 g/kg, providing 10 µg 1,25(OH)$_2$D$_3$/kg, has thus been shown to be safe and efficacious for broiler feeding. Benefits that might be expected from its use could be improved performance, bone quality and phosphorus utilisation.

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