VITAMIN DERIVATIVES, INC.

June 14, 2011

Division of Animal Feeds (HFV-224)
Office of Surveillance and Compliance
Center for Veterinary Medicine
7519 Standish Place
Rockville, MD 20855

To: Division of Animal Feeds, Office of Surveillance and Compliance

The purpose of this letter is to notify you that Vitamin Derivatives, Inc. has determined that the use of Alfacalcidol is GRAS for use as a dietary source of vitamin D for broilers.

Please find enclosed the following in triplicate:

1) Our claim
2) The GRAS monograph
3) A signed copy of the Expert Opinion Letter
4) Resumes for the members of the Expert Panel

One copy of the cited references is also included.

We hope you find this submission complete. Please do not hesitate to contact us if you have any further questions.

Regards,

Dr. Hardy M. Edwards, III

President, Vitamin Derivatives, Inc.

Cc: Dr. Sharon Benz

Dr. Timothy Schell

625 Lem Edwards Road • Winterville, Georgia • 30683-2221 • U.S.A. • Ph: 706.742.2658 • Fax: 706.742.2659

GRAS Exception Claim

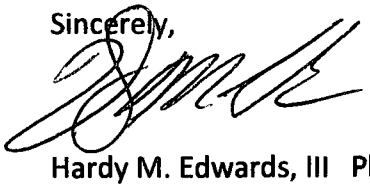
Alfacalcidol (1-alpha-hydroxycholecalciferol) is exempt from premarket approval requirements of the act for use as a dietary source of vitamin D in broiler feed because Vitamin Derivatives, Inc. has determined that such use is GRAS for use as a dietary source of vitamin D in broilers up to 5µg/kg in the finished feed.

This GRAS exception claim is being made by Vitamin Derivatives, Inc., 625 Lem Edwards Road, Winterville, GA 30683.

The basis for this determination has been through scientific procedures.

The data and information that are the basis for our GRAS determination are available for the FDA's review and copying at the address stated above.

Sincerely,



6/14/2011

Hardy M. Edwards, III Ph.D. Date

President, Vitamin Derivatives, Inc.

Expert Opinion Letter

Evaluation of the Generally Recognized as Safe (GRAS) Status of Alfacalcidol

Intended For Use as a Dietary Source of Vitamin D in Broiler Feed

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The undersigned, recognized Experts (hereinafter "Experts"), qualified by scientific training and relevant International experience to evaluate the safety of feed and feed ingredients, was requested by Vitamin Derivatives, Inc. to assess the safety of the use of Alfacalcidol, also known as AlphaD$_3$, 1alpha-hydroxycholecalfiferiol and 1α-OH-Vitamin D$_3$, - Alfacalcidol is a vitamin D$_3$ derivative that has been shown to exhibit significant vitamin D activity in broilers.

Vitamin Derivatives, Inc., hereafter referred to as VDI, wishes to establish by scientific procedures that the use of Alfacalcidol, may be affirmed to be Generally Recognized as Safe (GRAS) as a dietary source of vitamin D in broiler feed. GRAS is proposed for use at levels providing an upper limit of 5 µg per kilogram of finished broiler feed.

A GRAS determination for the AlphaD$_3$ product was based on the weight of the information provided in a comprehensive report prepared by VDI, to assist the Experts. This report was a compilation of documentation supporting the safety of AlphaD$_3$ under the intended conditions of use. The Experts independently and critically evaluated the report and other materials deemed appropriate.

The following summary basis for the discussion of GRAS status was evaluated by the Experts:

- The starting material for AlphaD$_3$, namely Vitamin D$_3$ (cholecalciferol), possesses a long history of safe use as a dietary supplement for both humans and animals;
- The manufacturing process for AlphaD3 is well controlled and consistently produces high product quality;
- The chemical composition of AlphaD$_3$ is well characterized. Product specifications have been provided as well as evidence supporting reproducibility;
- AlphaD$_3$ has been used by humans around the world for decades, particularly by renal failure patients and those suffering from osteoporosis;
- A multitude of published safety data provided the basis for this global acceptance and use of AlphaD$_3$ in humans;
- A recently published toxicity trial featuring AlphaD$_3$ indicated that for broilers fed AlphaD$_3$ up to 15ug/kg (3x of recommended level) of diet, weight gain and tissue damage of organs are not affected;
- Tissue analysis of broilers fed AlphaD$_3$ up to processing represented an absence of AlphaD$_3$ in the meat at the ng/gram level;
- AlphaD$_3$ is currently fed to broilers throughout Latin America and Asia with no reports of adverse events.

Alfacalcidol is a potent analog of vitamin D_3. Available scientific data indicate that the pathways of vitamin D metabolism in chickens and other vertebrate animals, including humans, are similar.

Vitamin Derivatives, Inc. and their bulk chemical supplier (b) (4) (b) (4) prepare Alfacalcidol by an efficient scalable six-step synthesis. Vitamin D_3 is treated with sulfur dioxide to produce two cyclic adducts which are protected via a silicon-protecting group. These protected adducts then undergo sulfur dioxide removal and rearrangement to a single silicon- protected 5,6-*trans*-vitamin D_3. Allylic oxidation then affords the corresponding 1α-hydroxylated derivative, which is then de-protected to yield crystalline 1α-OH-5,6-*trans*-vitamin D_3. Final photochemical isomerization cleanly produces Alfacalcidol (1α-OH-Vitamin D_3) which undergoes final purification via polish filtration and direct crystallization.

Vitamin Derivatives, Inc. plans to market Alfacalcidol as an alternative source of vitamin D activity in broiler feed. The proposed use level for Alfacalcidol in broiler feed would be up to 5μg/kg of feed, a level that corresponds to 40-50μg/kg of vitamin D_3 using the Association of Official Agricultural Chemists International (AOAC International) potency assay.

The experts agreed that decades of Alfacalcidol use by humans around the world and the current widespread use of Alfacalcidol throughout Latin America and Asia in broiler feed is supporting evidence of safety.

Alfacalcidol has been used in humans for the treatment of osteoporosis, chronic renal failure, cancer, hyperparathyroidism and osteomalacia. Alfacalcidol has been prescribed for indefinite periods of time at daily intake levels ranging from 0.25 to 1.00μg/d. These dosages are 29,412 to 117,648 times the estimated usual intake of adults consuming chicken fed Alfacalcidol at 5μg/kg of finished feed.

Based on extrapolation from feed consumption by broiler chickens and from human consumption of chicken, the Expert Panel estimated that mean usual per capita intakes of Alfacalcidol from chickens raised on Alfacalcidol would be 8.5pg/d for adults and 4.25pg/d for children. At the 90^{th} percentile for chicken consumption, usual intakes would be about 17.0 and 8.5pg/d for adults and children respectively.

The study conducted by Covance Laboratories and included in the comprehensive report described how Alfacalcidol residue levels were determined in processed chicken meat. These tests confirmed the calculated estimates as Alfacalcidol were not detected at the ng/level in broilers when fed up to processing.

Based on current available information, even though Alfacalcidol is more potent than vitamin D_3, subsequent human exposure to Alfacalcidol from consumption of chicken meat from broilers fed Alfacalcidol is sufficiently low that it is considered to be of little practical significance.

A number of studies conducted by the D.H. Baker group, University of Illinois at Urbana-Champaign and by the H.M. Edwards, Jr. group, University of Georgia established that when Alfacalcidol is fed at 5μg/kg of feed, no observable adverse affects on growth and final weight of broilers were observed during typical 6-week (42 day) production periods. These studies establish that slower growing chickens, such as New Hampshire x Colombians, can tolerate up to 40μg/kg without seeing an effect on growth performance. In modern fast growth strains, such as Cobb broilers or Ross broilers, there is an indication, though not statistically significant, that 15μg/kg can begin to impact performance.

The Experts felt the recently published Alfacalcidol toxicity trial in broilers by G. M. Pesti's group at the University of Georgia was *pivotal* with regards to establishing the safety of Alfacalcidol use in broilers. At 3x the recommended level in the feed, neither weight gain nor tissue damage was significantly affected.

The Experts recognize that the level of addition of Alfacalcidol to broiler feed is based on the current industry practice of adding vitamin D_3 to broiler feed. In due course further studies on bird performance and product quality may be conducted to determine if further improvements to feeding level and manufacturing can be attained.

After a critical independent evaluation of the available safety information, the undersigned Experts conferred and concluded that Alfacalcidol may be Generally Recognized as Safe (GRAS) by scientific procedures providing an upper limit of intake in broiler feed of 5μg/kg.

John J. Partridge, Ph.D., MRSC Date 09 June 2011

J. J. Partridge Consulting, Inc.

P. O. Box 16832

Chapel Hill, North Carolina 27516-6832

H.L. Shivaprasad, M.S., DVSc, Ph.D. Date 6/8/11

Professor of Clinical Diagnostic Pathology (UC Davis School of Veterinary Medicine)

California Animal Health and Food Safety Laboratory System

CAHFS – Fresno Lab

18830 Road 112

Tulare, CA 93274

Douglas Zaviezo, Ph.D. Date 6/13/2011

Technical Director – Nutrition

Citrex Special Nutrients, Inc.

2766 SW Douglas Road

Miami, FL 33133.

Expert Panel Member

Resumes

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GRAS
Monograph
For
Alfacalcidol

Intended For Use As a Dietary Source of Vitamin D in Broiler Feed

TABLE OF CONTENTS

CHEMICAL IDENTITY:

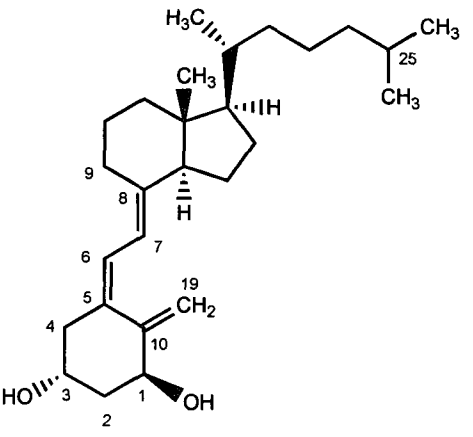
The term Vitamin D is used for a range of closely related compounds which possess the property of preventing or curing vitamin D deficiency. These include 1α-hydroxycholecalciferol (alfacalcidol, 1α-OH-vitamin D_3), calcifediol (25-OH-vitamin D_3) (Amoco, 1994), calcitriol [1α,25-$(OH)_2$-Vitamin D_3], cholecalciferol (Vitamin D_3), dihydrotachysterol (DHT_3) as well as 1α-OH-Vitamin D_2, 25-OH-Vitamin D_2, 1α,25-$(OH)_2$-Vitamin D_2 and ergocalciferol (Vitamin D_2).

Two groups of workers have dominated the developments in the total synthesis of the cholecalciferols. Inhoffen's group at Braunschweig reported the first total synthesis of cholecalciferol in 1960 (Inhoffen, 1960), and Lythgoe's group in Leeds reported the total synthesis of precalciferol in 1970 (Dawson, *et al.*, 1970).

The first reporting of the synthetic analogue 1α-OH-Vitamin D_3 was carried out nearly simultaneously by four groups, Barton's (Barton, *et al.*, 1973), Deluca's (Holick, *et al.*, 1973), Lythgoe's (Harrison, *et al.*, 1973) and the Hoffman-LaRoche group (Fuerst, *et al.*, 1973). As the final step in the synthesis of 1α-OH-Vitamin D_3 involves column chromatography or trituration and crystallization, the end result is a high purity compound. 1α-OH-Vitamin D_3 has shown itself to be remarkably heat stable.

The active form of Vitamin D_3, 1α,25-$(OH)_2$-Vitamin D_3, is essential for the proper regulation of calcium and phosphorus homeostasis by enhancing their re-absorption by the proximal tubules of the kidneys and for bone mineralization in animals and man (Norman, *et al.*, 1982; Toffolon, *et al.*, 1975). In poultry, Vitamin D_3 analogs can be up to thirty times more bioactive than Vitamin D_2.

1α-Hydroxycholecalciferol Structure



9,10-secocholesta-5,7,10(19)triene-l,3-diol, (1alpha,3beta,5Z,7E)

$C_{27}H_{44}O_2$

Mol. Wt.: 400.64

PRODUCTION PROCESS: U.S. Serial No. PCT/US2010/040365

a. Synthesis of 1α-OH-Vitamin D_3

Step 1: Preparation of Compound 1

(b) (4)

Step 2: Preparation of Compound 2.

(b) (4)

Step 3: Preparation of Compound 3.

(b) (4)

Step 4: Preparation of Compound 4.

(b) (4)

Step 5: Preparation of Compound 5.

(b) (4)

Step 6: Preparation of 1α-Hydroxy-Vitamin D_3:

(b) (4)

The large scale chemical process involves modifications of the small-scale chemistry described by the Barton group in 1985-1986 (Andrews, *et al.*, 1986).

1α-Hydroxy Vitamin D_3 Process Description

Reaction Scheme:

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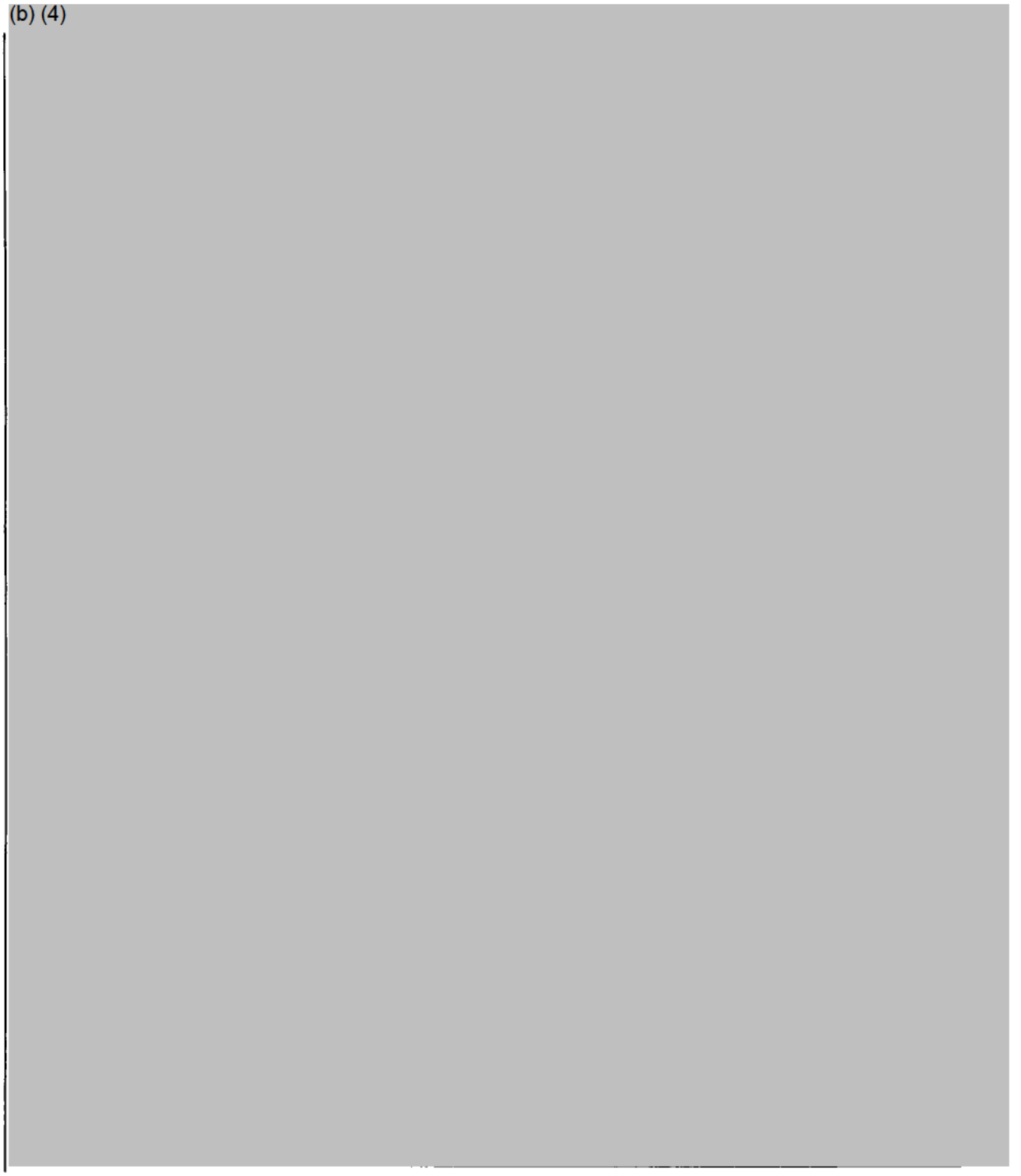

b. Drum-drying of 1α-OH-Vitamin D_3

The drum-dried formulated 1α-OH-Vitamin D_3 is produced batch wise. The ingredient makeup is summarized as follows:

Ingredient	%
Starch	92.21
Peanut Oil	6.00
Sorbitan Monostearate	1.00
FD&C Green #3	0.25
Sorbic Acid	0.20
BHT	0.20
Sodium Benzoate	0.10
1α-OH-Vitamin D_3	0.04
Total	**100.00**

(b) (4)

(b) (4)

OCCURANCE AND USE:

1α-OH-Vitamin D_3 has been synthesized by several research groups (Barton, *et al.* 1973; Holick, *et al.*, 1973; Harrison, *et al.*, 1973; Fuerst, *et al.*,1973). It has a long history of effective and safe use in human medicine in most parts of the world outside the U.S. (Collins and Norman, 2001 and Norman, A.W. *et al* 1994). In Europe, Africa, and the mid-East it is marketed by Leo Pharma as One-Alfa or Lunar and approved for treatment of renal osteodystrophy, postmenopausal osteoporosis, and hypoparathroidism. Teva Pharmaceutical markets the drug in Israel for the same applications as well as hypocalcemia, and Rhone-Poulenc Rorer and Smith Kline & French sell it as Dediol and Diseon respectively. In Japan, both Teijin Ltd and Chugai market it as Onealfa or Alfarol for treatment of osteoporosis and renal problems. Alfacalcidol (1 μg capsules) is listed by the British National Health Service for treating Vitamin D deficiency (NHS Choices Web Site).

1α-OH-Vitamin D_3 has been approved by the European Union for prevention of milk fever in dairy cattle (EMEA, 1998)

In Mexico 1α-OH-Vitamin D_3 is registered for both human and animal use. Since 2007 it has been used extensively throughout the broiler industry in Mexico. 1α-OH-Vitamin D_3 is registered for sale throughout Latin America and is currently being fed to broilers commercially in Brazil, Chile, Colombia, Ecuador, Guatamala, Panama, Peru, Malaysia, Thailand, Phillipines, Vietnam, and South Korea.

The proposed condition of use for 1α-OH-Vitamin D_3 is to provide a dietary source of Vitamin D for broilers at a level of up to 5 μg of 1α-OH-Vitamin D_3/kg of finished feed.

Proposed labeling in the United States for products containing 1α-OH-Vitamin D_3 for the above stated purpose shall read as follows:

Net Weight: 50 Pounds

Product Name: AlphaαD_3 Premix

Purpose: To provide a dietary source of vitamin D in broiler feeds'

Directions for use: Include one-half pound (1/2 lb) of this product in each ton (2000 lbs) of finished feed

Guaranteed Analysis: 348,000 I.C.U. of vitamin D per pound minimum

Feed Ingredients: Ground Limestone, Rice Hulls, Mineral Oil, Vitamin D_3 Supplement

Manufactured for:

Vitamin Derivatives, Inc.
625 Lem Edwards Road
Winterville, GA 30683

ANALYTICAL METHODOLOGY:

Crystalline 1α-OH-Vitamin D3:

Vitamin D determination in feed and food has always been quite challenging from an analytical point of view due to the complex chemistry of this vitamin. AOAC International has validated 11 methods which are *legally defensible* (Blake, C.J. 2005 and Phillips *et al.* 2008).

In a careful review of the literature there are *no* AOAC methods *per se* for analysis of pure D compounds only AOAC methods for the analysis of vitamin D in foods, feed and vitamin mixtures.

Foregoing the extraction procedures and moving on to the actual quantification of vitamin D the methodology described in AOAC Method 2002.05 (Blake, C.J. 2005) is excellent for the analysis of pure vitamin D compounds and is essentially the method we have used to quality control our pure compound synthesis of 1α-OH-Vitamin D_3. Our specific conditions are as follows:

HPLC Procedure for the Analysis of 1α-Hydroxyvitamin D_3 (Compounds 3-6)

EQUIPMENT AND MATERIALS:

3.1 Agilent 1100/HPLC system or equivalent
3.2 Column: ES Chromegasphere SI 60, 250x4.6 mm, 5um 60A (Catalog # 155211-SI60)
3.3 HPLC grade Hexane (J.T. Baker or equivalent)
3.4 HPLC grade THF (B&J Brand or equivalent)
3.5 HPLC grade Ethanol (Aldrich or equivalent)

TYPICAL CHROMATOGRAPHIC CONDITIONS

Column: ES Chromegasphere SI 60, 250x4.6mm, 5um 60 A	
Detection:	354 nm
Column Temperature:	35 °C
Injection Volume:	10 µL
Flow Rate:	1.5 mL/min
Mobile Phase:	Hexane(76)/THF(20)/Ethanol(4)
Run Time:	a) Isocratic 12 minutes for Step 4
	b) Isocratic 20 minutes for Step 5, 6

Approximate Retention Times:

Compound	RT(minutes)
3	1.9
4	2.3
5	6.2
6	6.5

SAMPLE PREPARATION:

1.1 Blank (Diluent) Preparation
Hexane(76)/THF(20)/Ethanol(4) (same as mobile phase)
1.2 Purity
Weight and dissolve/dilute about 1mg of the isolated sample to 1ml with Diluent
1.3 Step 4 IPC
Dilute 15 µl to 1.5 ml with Diluent (Adjust concentration as necessary)
1.4 Step 5 and 6
Dilute 50 µl to 2 ml with Diluent (Adjust concentration as necessary)

All vitamin compounds [such as Vitamin D_3, 1α(OH)D_3, 25(OH)D_3 and 1α,25(OH_2)D_3] containing the 5(6), 7(8), 10(19) triene system are in thermal equilibrium with their cognate pre-vitamin structures which possess a 5(10), 6(7), 8(9) triene system. The equilibrium between Vitamin D_3 and pre-Vitamin D_3 is temperature and time dependent; thus, the proportion of pre-Vitamin D_3 present in any preparation can vary depending on the temperature and time of storage (Curtin and Okamura, 1991).

Formal stability studies are ongoing. Pilot stability studies were carried out on product which was spray-dried in January 2006 at an inlet temperature of 220°C and outlet temperature of 95°C (Lot 1002). This lot was packaged in 5 gallon plastic pails with O-ring fitted lids to provide an air-tight seal and warehoused at temperatures ranging from 20-30°C. Over the course of the next year samples of this product were distributed to the University of Georgia, Panama, Mexico, Ecuador, Colombia and Brazil for testing. In all cases full activity in broiler trials was observed. The remainder/majority of this manufacturing lot was shipped to Mexico and consumed in the course of two months (mid-February to mid-April 2007), by a major USA poultry company with satisfactory results.

Certificate of Analysis

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RESEARCH AND DEVELOPMENT CERTIFICATE OF ANALYSIS

Not for Human Use

Project Code: VIT-503

Product Name: 1α-Hydroxyvitamin D_3 (Compound 6)

Lot Number: (b) (4)

Date of Analysis: 11-24-2008

TESTS	SPECIFICATION	RESULTS
Appearance	Report Results	white solid
Identification (NMR)	Conforms to structure	Conforms
HPLC Purity (area %)	≥ 95%	97.4% (Compound 6/Compound 5=98.7/1.3)

"Material for research and development use only. Not manufactured nor tested under cGMP conditions and not suitable for human use."

(b) (4)

12/17/08
Date

12-17-08
Date

1α-OH-Vitamin D_3 Lot History

Lot:	Lot Number:	Weight: (Kg)	Purity: (%)
1	(b) (4)	0.55	96.30
2		0.60	96.00
3		0.64	96.00
4		0.81	97.50
5		0.78	97.60
6		0.76	96.60
7		0.73	96.90
8		0.64	97.60
9		0.74	98.00
10		1.00	95.50
11		1.00	95.80
12		1.10	96.60
13		1.56	98.00
14		1.36	97.80
15		1.68	97.70
16		1.59	97.80
17		1.59	97.40
18		1.63	96.40
19		1.57	98.40
20		1.61	97.90
21		1.49	98.50
22		1.74	96.80
23		1.62	97.30
24		1.54	98.20
25		1.67	97.70
26		1.56	98.10
27		1.61	97.50
28		1.53	97.50
29		1.60	97.60
30		1.57	98.00
31		1.44	97.80
32		1.39	97.40
33		1.45	98.00
34		1.42	97.40
35		1.12	96.30
36		1.31	98.30

b. Drum-dried and Premixed 1α-OH-Vitamin D_3

Once a Vitamin D_3 metabolite has been diluted into a carrier system such that it can satisfactorily be mixed into a Vitamin premix or a finished feed it is very difficult to analyze its activity with conventional bench-top laboratory chemistry (i.e. extraction and HPLC analysis) resulting in higher standard deviations.

The most effective way to determine Vitamin D_3 activity in Poultry Feed Supplements is with the AOAC Official Method 932.16. This assay is comparison, under conditions specified, of efficacy of product under assay with that of USP Cholecalciferol Reference Standard in controlling tibia ash content of growing chicks (AOAC, 1995).

One international chick unit of Vitamin D is equal in biological activity to one unit Vitamin D in USP Vitamin D Reference Standard in this method of assay. Product under assay meets its declared vitamin potency in international chick units of Vitamin D if % ash in H_2O and fat-free bone produced in assay groups by given number of units of Vitamin D is equal to or is greater than % ash produced by same number of units of Vitamin D from USP Reference Standard.

In using the AOAC method Edwards *et al.* (2002), Haussler *et al.* (1973) and Boris *et al.* (1977) determined 1α-OH-Vitamin D_3 was eight, ten and eight times as effective, respectively, as the Cholecalciferol Reference Standard on a weight basis. For a discussion of potencies versus international units, see Norman, 1972.

ESTIMATED EXPOSURE:

United States per capita consumption of chicken in 2004 was 85.4 pounds per person which translated to a boneless, edible weight of 59.2 pounds per person (Buzby, J. C. *et al* 2006). On a daily basis this is 0.16 pounds or 72.57 grams.

1α,25$(OH)_2$-Vitamin D_3 and 1α-OH-vitamin D_3 are approximately 99.9% bound in blood. 1α,25$(OH)_2$-Vitamin D_3 and other Vitamin D metabolites are transported in blood, by an alpha-globulin Vitamin D binding protein. Studies in rats and dogs (Koike *et al.* 1998) have shown that the estimate half-life of either 1α-OH-Vitamin D_3 or 1α,25$(OH)_2$-Vitamin D_3 to be 5 and 8 hours respectively when given orally.

In estimating the exposure of the average consumer of chicken meat to 1α-OH-Vitamin D_3 several factual assumptions can be made.

- Chickens are fasted in the chicken house for approximately 12 hours before they are caught and delivered to the processing plant (another three hours to processing). At processing time only 1/8 of the 1α-OH-Vitamin D_3 consumed in their last meal (assuming they ate immediately before the feeders were raised) would still be circulating based on half-life studies in rats, dogs and humans.

- At 42 days of age chickens eat 0.5 lbs of feed per day. At 5 μg/kg of feed this translates to a daily 1α-OH-Vitamin D_3 consumption, by the chicken, of 1.1 micrograms. Being very

conservative and based on half-life estimates, 0.14 μg (based on total daily intake at 42 days) of 1α-OH-Vitamin D_3 would still be circulating in the chickens bloodstream at the time of catch on the farm.

- Hemoglobin content in mg/gram of chicken muscle after bleed-out is around 0.20 (Griffiths *et al.* 1985)
- Chicken hemoglobin consumption per capita per day is 14.5 mg (72.57 grams times 0.20 mg/gram)

Based on the fact that Vitamin D metabolites are 99.9% bound in blood one can conclude that the main source of exposure to the consumer of 1α-OH-Vitamin D_3 is through his 14.5 mg per day consumption of hemoglobin contained in his 72.6 grams of chicken meat.

It is generally accepted that the total blood volume of a chicken ranges from 8.8 to 10% of the chickens body weight (Autenreid, 2002). The average live processing weight of today's commercial broiler is around 5.25 pounds or 2.38 kilograms. This is 2,380,000 milligrams. Ten percent (blood weight) of this value is 238,000 milligrams which would contain the 0.14 μg of 1α-OH-Vitamin D_3 still circulating at processing. The average consumer would then only take in 0.00006 (14.5 mg/238,000 mg) of this amount or 0.0000085 micrograms (8.5 picograms, 8.5 pg) per day of 1α-OH-Vitamin D_3.

To verify these calculations Vitamin Derivatives, Inc. enlisted the services of Covance Laboratories to assay chickens which had been fed 1α-OH-vitamin D_3 at the 5 μg/kg level in the feed up to sacrifice and compare them to chickens which had had 1α-OH-Vitamin D_3 withdrawn from the feed 14 days prior to sacrifice. Their findings indicated that <2.0 ng/g of 1α-OH-Vitamin D_3 was present in the meat of the birds fed 1α-OH-Vitamin D_3 at the 5 μg/kg level in the feed. The totality of the Covance study is included in this report as follows:

COVANCE
THE DEVELOPMENT SERVICES COMPANY

Analyzing 1ά hydroxy Vitamin D_3 in Whole Chicken Meat utilizing LC-MS/MS

Introduction

Covance received a request to determine the level of 1ά hydroxy-Vitamin D_3 in whole ground chicken meat. The laboratory received 12 whole frozen chickens. Six of the birds were fed 1ά hydroxy-Vitamin D3 up to sacrifice while six birds had the 1ά hydroxy-Vitamin D_3 pulled from their diets 14 days prior to sacrifice. In addition, the laboratory incorporated the analysis of 25 hydroxy Vitamin D_3 during this study.

Method Principle

Samples containing approximately three grams of whole ground chicken meat, that had previously been de-boned and ground under liquid nitrogen, are fortified with internal standard and extracted by sonicating for 30 minutes with 10 mL of 90%ACN/10%H2O. Samples are then diluted to 20 mL with 40%ACN/60%H2O and sonicated for another five minutes. After filtering, 10 mL of the extracts are purified using a pre-condition SPE column. Once eluted off SPE column, the eluent is evaporated under a stream of nitrogen and reconstituted with 0.3 mL of extraction solution for injection on UPLC-MS/MS.

Sample Purification

The SPE column used for this study is an Oasis HBL, 6cc 200mg. The column is conditioned by sequential washings using 6mL of MeOH, 1.0 mL of H2O and 3.0 mL 40%ACN/10%H2O. Once conditioned, 10 mL of the extract is added and the cartridge is washed with 3.0 mL of 40%ACN/10% H2O. The analytes are then eluted with 6 mL of 100%MeOH.. The eluents are evaporated under a stream of nitrogen while being held in a 40° C water bath. Once dry, the samples are then reconstituted with 0.3 mL of 90%ACN/10%H2O. The reconstituted samples are sonicated for10 minutes prior to injection.

Instrument

Analysis was carried out on an API-4000 Q-trap triple quadruple tandem mass spectrometer with Aquity UPLC system (Waters). The column was a Hypersil aQ UPLC column (2.1 x 100 mm, 1.9 µm).

The following product ions from the precursor ion of 383.2 were monitored: 365.4, 135.1, and 157.2. The results were calculated from the 365.4 product ion while the other two ions were used for confirmation. The internal standard was monitored by the reaction of 386.5 to 368.5. The reaction of 383.2 to 365.4, was found to exhibit the strongest signal with the least interferences and was used for quantitative measurement. Identification of each analyte is confirmed by at least two multiple reaction product ions.

UPLC Conditions

Injection volume: 8 uL

Flow rate: 0.45 mL/min

Mobile phase A: 0.1%FormicAcid/5%ACN/15%MeOH/80%H2O;

Mobile phase B: 0.1%FormicAcid/25%ACN/75%MeOH;

UPLC run time: 13 minutes.

Results and Discussion

Linearity results can be found in table 1. All standard curves demonstrated acceptable linearity. Standards were prepared at five different concentrations; 2, 5, 10, 20 & 50 ng/mL. The internal standard was added to the standards at a concentration of 100 ng/mL.

Limit of quantification (LOQ) and limit of detection (LOD) can be established by several different procedures. For this study, the laboratory utilized a pool of ground chicken meat fortified at a minimal level in the vicinity of the theoretical LOQ. The laboratory analyzed 10 replicates from this pool of fortified chicken meat. The results are expressed as ng/ml of the solution and as ng/gram in the sample. The LOD is established as three times the standard deviation of the 10 replicates. The LOQ is established as 10 times the standard deviation of the 10 replicates. The data can be found in table 2.

Table 3 contains the results from the analysis of the two samples of whole ground chicken meat. Both samples yielded values of <2.0 ng/g for the 1ά hydroxy Vitamin D3 while both samples did yield values for 25 hydroxy Vitamin D3 that were approximately 6.2 ng/g.

Table 4 contains the results from the analysis of both the 1ά hydroxy Vitamin D3 and the 25 hydroxy Vitamin D3. Each standard was prepared and subjected to the entire method procedure. The amount recovered ranged from 101 to 111%.

One major challenge was that no source of an isotope labeled 1ά hydroxy Vitamin D3 was available for use as an internal standard. As a result, the laboratory incorporated an isotope labeled 25 hydroxy Vitamin D3 for use as the internal standard. While the only difference between the two compounds is the position of the OH substitution, the laboratory found that the two reacted differently when subjected to purification and instrumental analysis. Experiments were conducted that involved altering the concentration of ACN in the final extracts. Recoveries of both compounds were optimized when the final concentration of ACN in the extracts was 65%.

To determine accuracy, triplicate preparations of ground chicken meat were fortified at three different levels. These levels, as outlined in the initial proposal were; 2, 5 and 10 times the baseline level. As the laboratory determined the baseline levels in both samples to be < 2.0 ng/g, the subsequent fortification amounts are; 4, 10 and 20 ng for both compounds.

Initial recoveries were in the 80% range when the concentration of ACN was at 55%. These data are not included in the report but will remain a part of the permanent record. The second series of recoveries, which can be found in table 5, were generated with the final concentration of ACN at 75%. These recoveries ranged from a low of 88.5% to a high of 137%. The higher recoveries were found in the samples that were fortified with 20 ng. The third series of recoveries, which can be found in table 6, utilized a final concentration of ACN of 65%. These recoveries are considered acceptable as they range from a low of 92.4% to a high of 104%. The relative standard deviation (RSD) for the nine recoveries on the1ά hydroxy Vitamin D3 recoveries is 4.94% and the RSD for the nine recoveries on the 25 hydroxy Vitamin D3 is 4.82%. Over all statistics for all 18 recoveries for each compound can be found in table 7. Example chromatograms can be found in Appendix 1.

Table 1
Linearity of Calibration Curve

Day	Correlation Coefficient
1	0.9979
2	0.9994
3	0.9986
4	0.9996

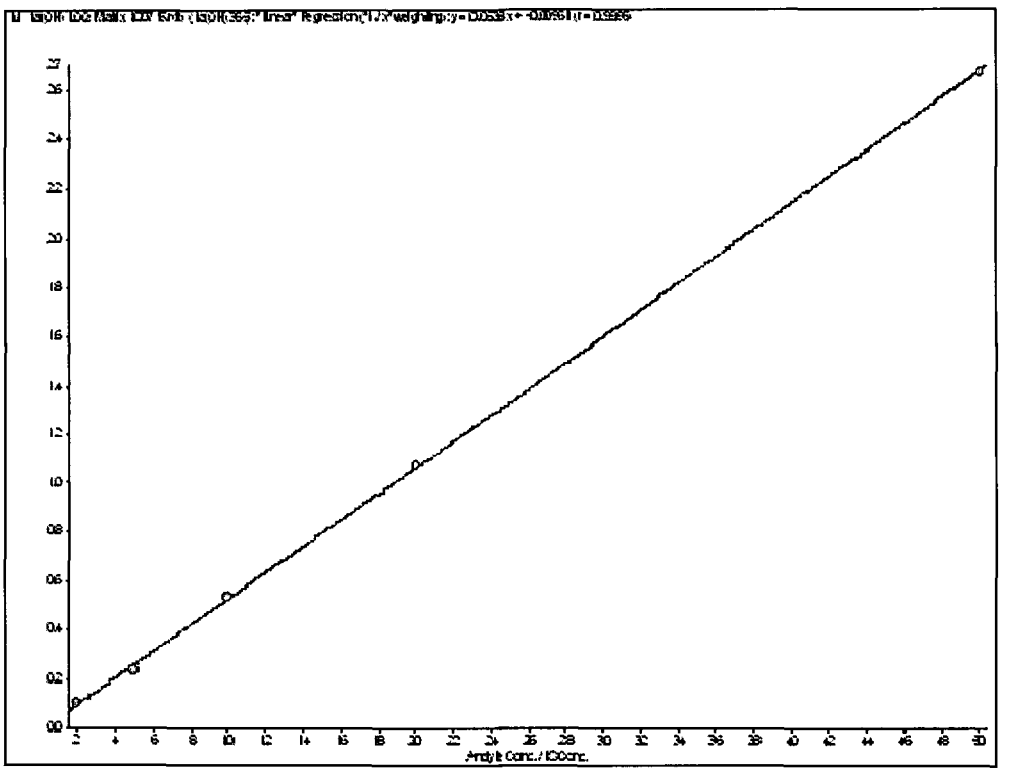

Table 2
LOD/LOQ Data

Replicate	Concentration of Solution (ng/mL)	Actual Concentration (ng/g)
1	16.0	3.20
2	16.6	3.32
3	15.9	3.18
4	17.1	3.42
5	15.9	3.18
6	18.0	3.60
7	15.2	3.04
8	17.4	3.48
9	17.6	3.52
10	16.3	3.26
n	10	10
Mean	16.6	3.32
Standard Deviation	0.897	0.1794
Relative Standard Deviation (%)	5.40	5.40

	Concentration of Solution (ng/mL)	Actual Concentration (ng/g)
Limit Of Detection	2.69	0.54
Limit Of Quantitation	8.97	1.79

Table 3
Results on Whole Ground Chicken Meat (ng/g)

	Pulled 14 Days Prior to Sacrifice		Fed to Sacrifice	
	1 α (OH) Vitamin D3	25 (OH) Vitamin D3	1 α (OH) Vitamin D3	25 (OH) Vitamin D3
Assay 1	< 2.0	13.5	< 2.0	17.8
Assay 2	< 2.0	14.5	< 2.0	20.3

Table 4
Standard Recoveries Without Matrix (%)

	1 α (OH) Vitamin D3	25 (OH) Vitamin D3
Assay 1	105	101
Assay 2	111	107

Table 5
Day 1 Fortified Recoveries With Matrix (%)

Fortification Levels	Replicate	1 α (OH) Vitamin D3	25 (OH) Vitamin D3
4 ng	1	104	101
	2	93.0	88.5
	3	108	107
10 ng	1	111	101
	2	105	99.4
	3	109	98.2
20 ng	1	131	92.5
	2	128	100
	3	137	110

Day 1 Statistics		
Mean (n=9)	114	99.7
Standard Deviation	14.6	6.543
Relative Standard Deviation (%)	12.8	6.56

Table 6
Day 2 Fortified Recoveries With Matrix (%)

Fortification Levels	Replicate	1 α (OH) Vitamin D3	25 (OH) Vitamin D3
4 ng	1	92.5	105
	2	99.5	111
	3	99.0	113
10 ng	1	91.2	98.6
	2	87.6	112
	3	99.0	103
20 ng	1	97.3	105
	2	98.9	103
	3	102	101

Day 2 Statistics		
Mean (n=9)	96.3	106
Standard Deviation	4.759	5.11
Relative Standard Deviation (%)	4.94	4.82

Table 7
Overall Statistics

	1 α (OH) Vitamin D3	25 (OH) Vitamin D3
Mean (n=18)	105	103
Standard Deviation	13.92	6.48
Relative Standard Deviation (%)	13.3	6.29

APPENDIX 1: EXAMPLE CHROMATOGRAMS

Figure 1: 50 ppb 1α (OH) Vitamin D3 standard

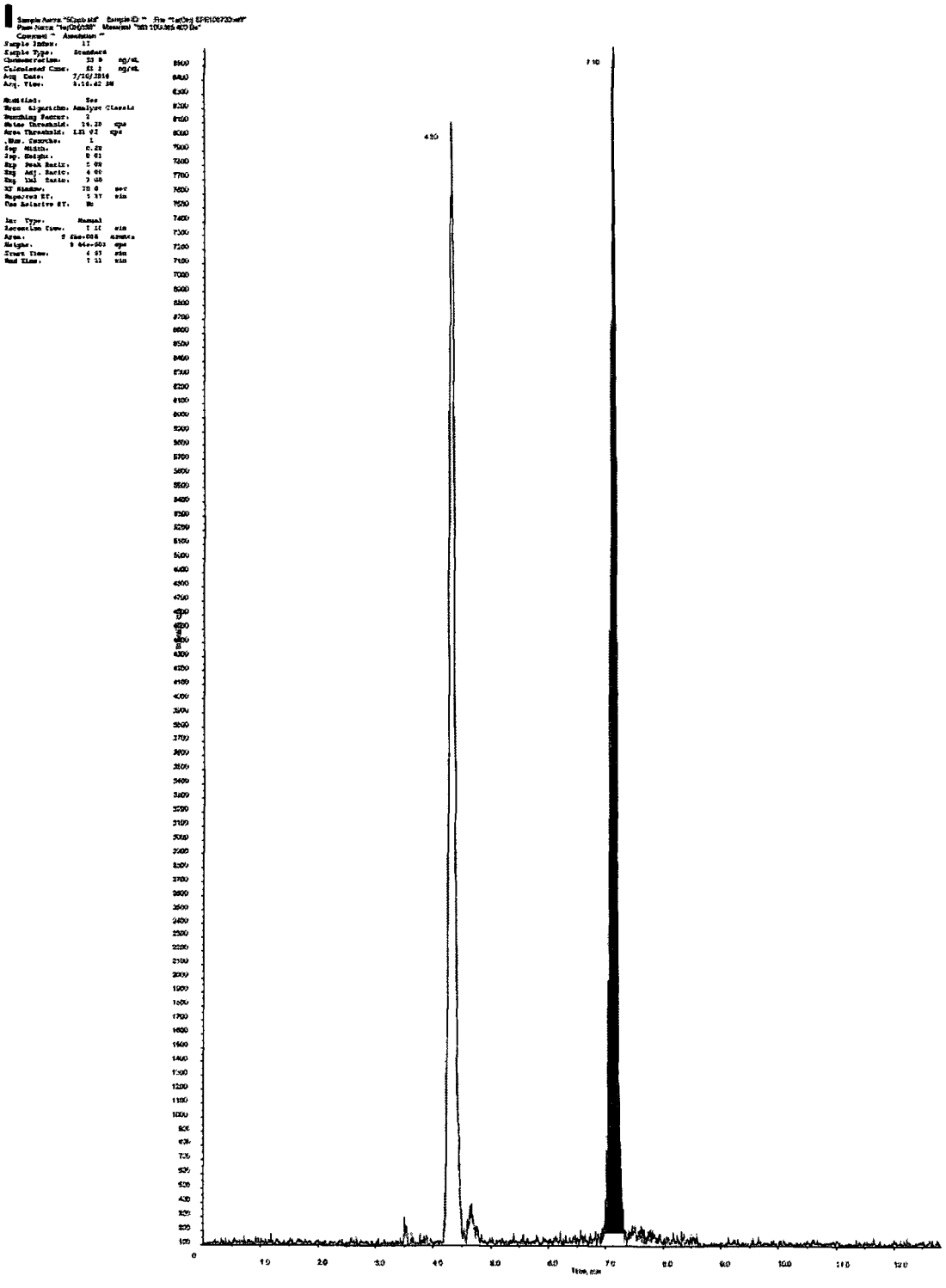

Figure 2: 50 ppb 25 (OH) Vitamin D3 standard

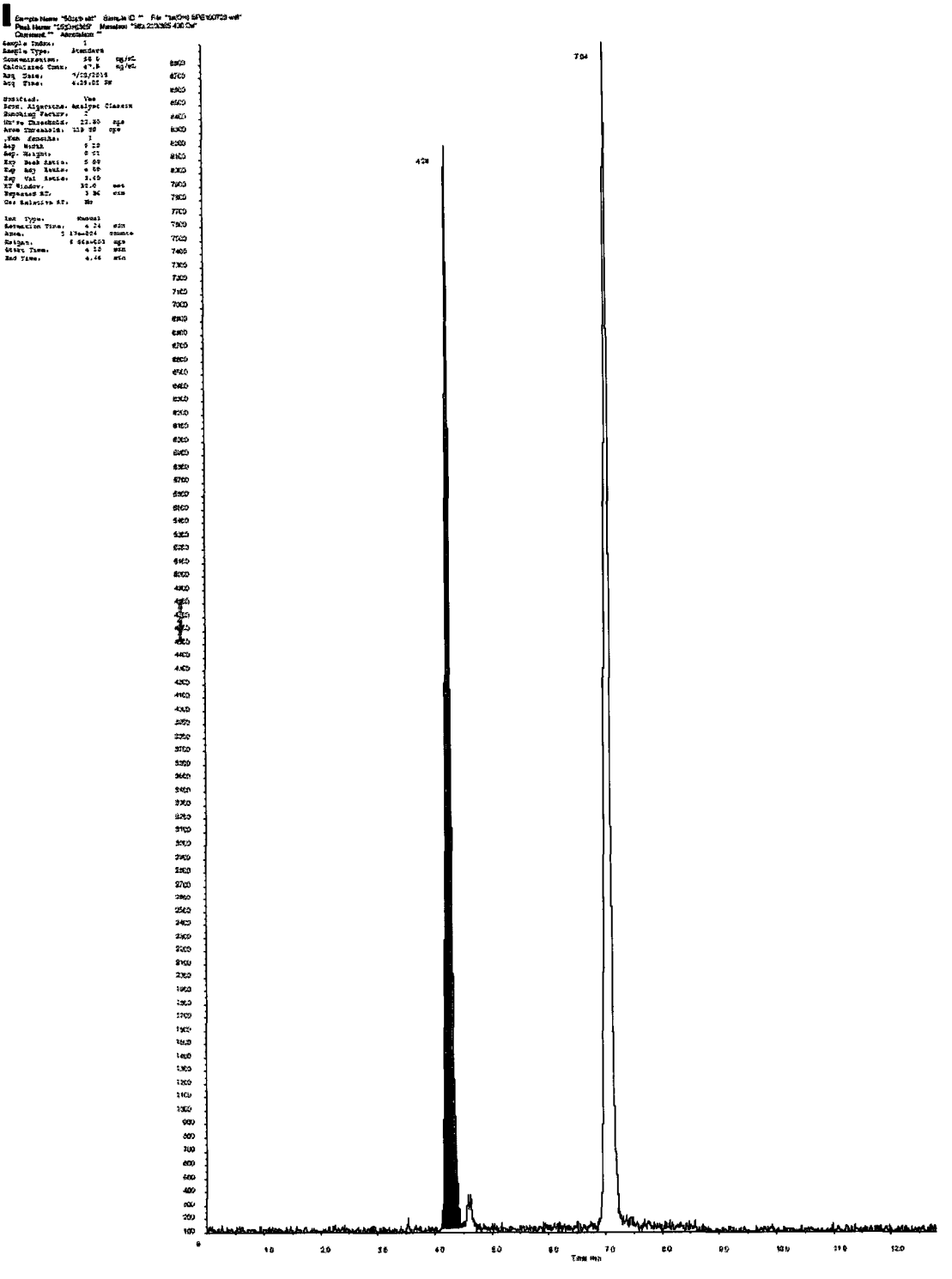

Figure 3: 50 ppb internal standard

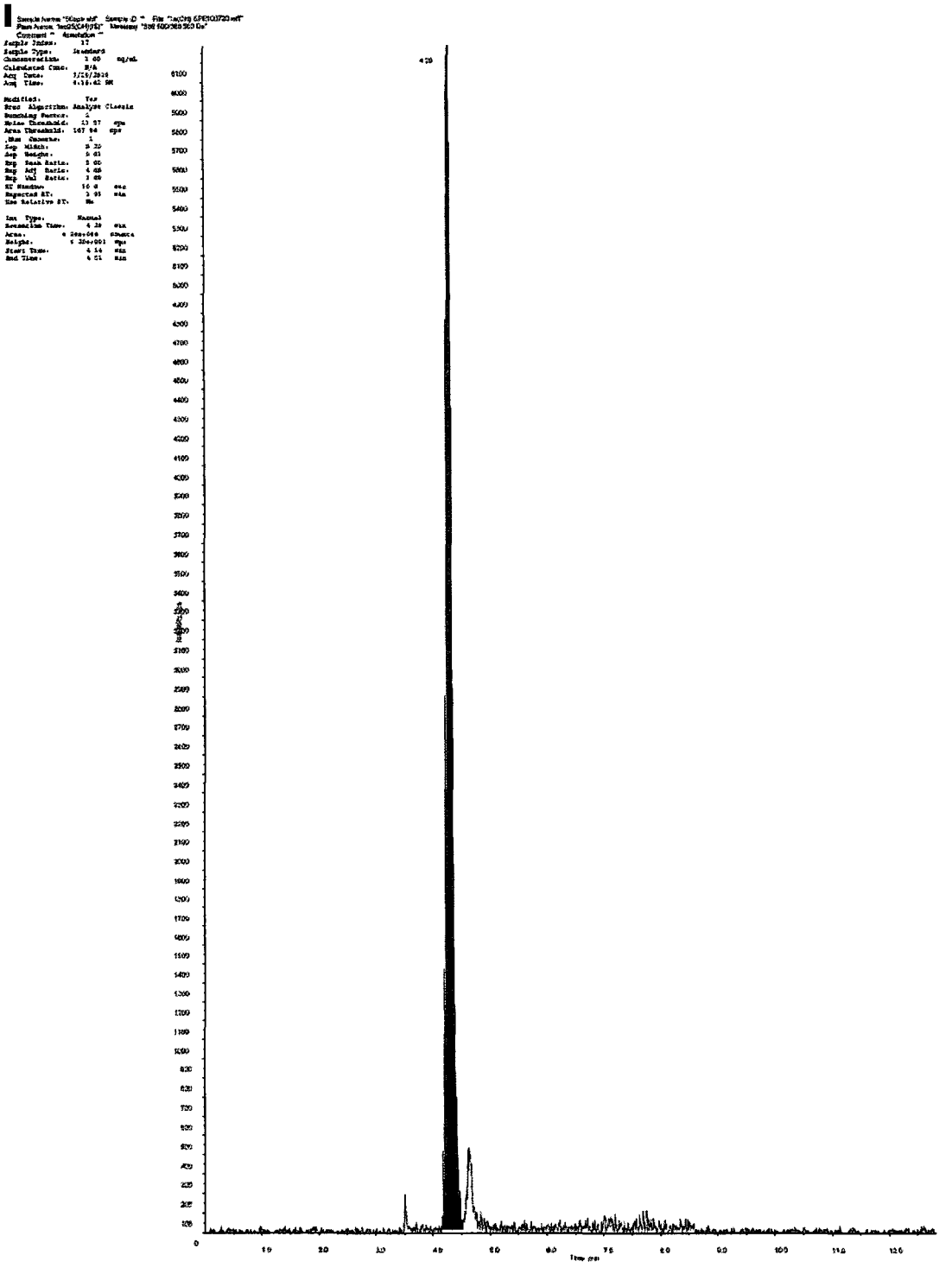

Figure 4: 1α (OH) internal standard with chicken matrix (fed to sacrifice)

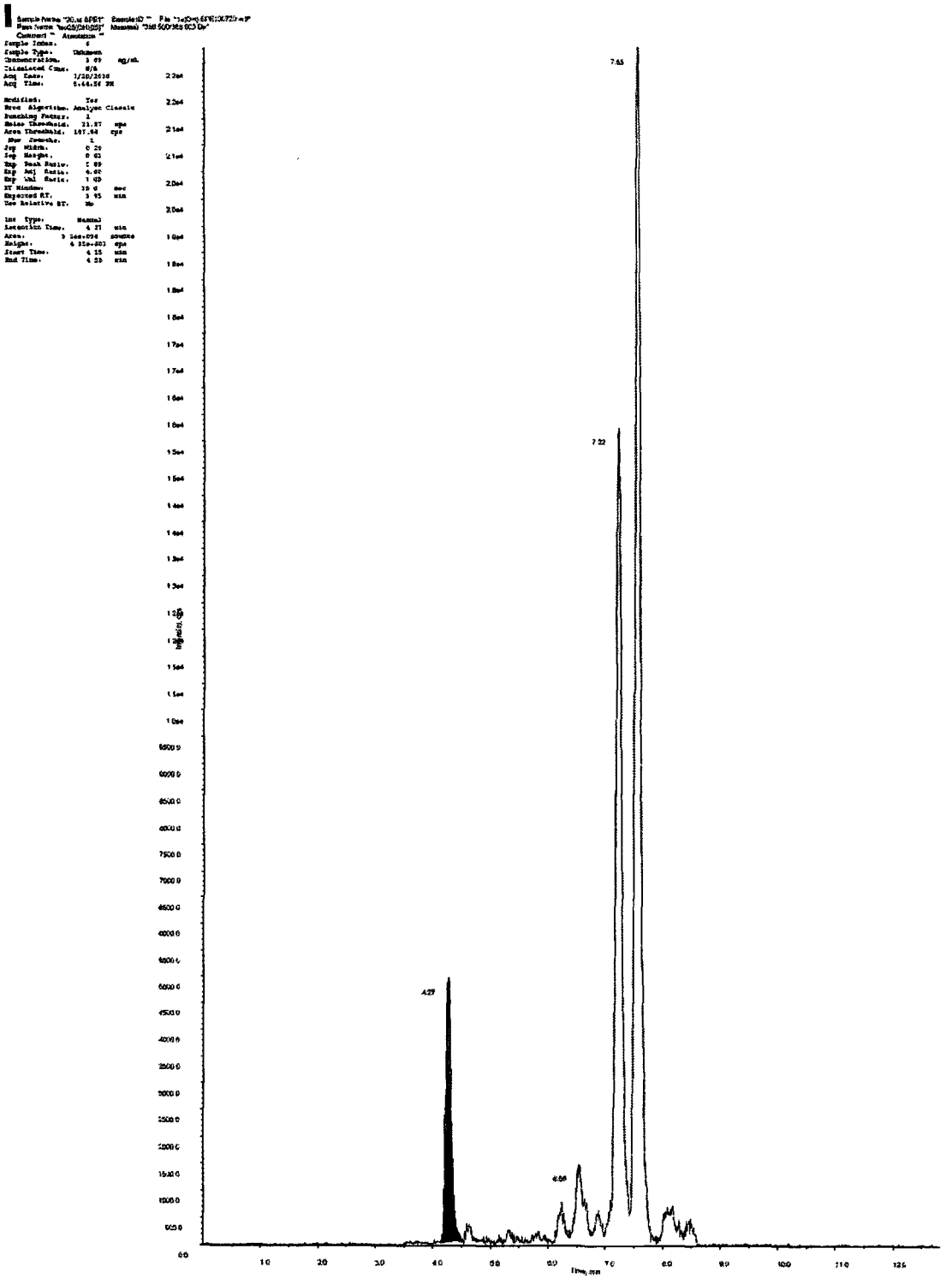

Figure 5: Chicken matrix (fed to sacrifice) 25 (OH) Vitamin D3

Figure 6: 1α (OH) chicken matrix (fed to sacrifice)

SAFETY AND TOXICITY:

For use as a feed additive in broilers, an important segment of the human food supply, it is essential that 1α-OH-Vitamin D_3 be shown to be safe, not only to the target species (broiler chickens) but especially to the end consumers. This section presents a ***"White Paper"*** summary of the published literature on the metabolism, acute and sub-acute toxicity, developmental and reproductive toxicity, and teratogenicity of 1α-OH-Vitamin D_3. It also summarizes its uses in human medicine and the dosage levels found safe in humans.

A. METABOLISM.

The general metabolism of 1α-OH-Vitamin D_3 in chicks and rats has been well-studied, first by Holick (Holick *et al*, 1976a, 1976b). In both species 1α-OH-Vitamin D_3 is rapidly converted to 1α,25-$(OH)_2D_3$; in rats, 92% of the initial injection had disappeared from the intestine in 12h, 98% after 24 hr, and after 96 hours it had completely disappeared from intestine, blood, and bone. In Vitamin D-deficient chickens, 1α-OH-Vitamin D_3 disappeared from blood in 6 hr and from the intestine in 24 hr.

Following the metabolism of tritiated 1α-OH-Vitamin D_3 in male chicks showed that it was hydroxylated at both C-25 and C-24, and deactivated by esterification (Edelstein *et al*, 1978). A Berkeley group (Walters *et al*, 1983) demonstrated that in chicks, 1α-OH-Vitamin D_3 effects its activity at the 1α,25$(OH)_2D_3$ receptors. The C-25 hydroxylation is effected by a cytochrome P-450 enzyme (Guo, 1993). 25-Hydroxylation was demonstrated in both the rat and human liver (Saarem and Pedersen, 1985). The rapid action of pure 1α-OH-Vitamin D_3 on intestinal calcium uptake in the rat, however, suggested that it may not need to be hydroxylated at C-25 in order to be biologically active (Toffolon *et al*, 1975).

The pharmacokinetics of 1α-OH-Vitamin D_3 were investigated in laboratory rats and dogs (Koike *et al*, 1998; Kawase *et al*, 2000).

Intestinal absorption following oral administration of 24(S)-^{3}H-1α-OH-Vitamin D_3 (specific activity of 3.8 Ci/mmol) was found to be 80% and 90% in normal and vitamin D deficient rats, respectively. The maximum plasma concentration of 1α-OH-Vitamin D_3 and 1,25-dihydroxy-vitamin D_3 was measured at 4 and 24 hours, respectively (Koike *et al.*, 1998).

In dogs, a distribution half life of 7 hours was observed following intravenous administration of 0.2 μg/kg bw of ^{3}H-1α-OH-Vitamin D_3 and the maximum plasma concentration of 1,25-dihydroxy-vitamin D_3 was 0.218 pmol/ml at 4 to 6 hours after dosing. Following oral administration of 0.2 μg/kg bw of ^{3}H-1α-OH-Vitamin D_3, plasma levels of 1α-OH-Vitamin D_3 and 1,25-dihydroxy-vitamin D_3 increased immediately with respective half-lives ($t_{1/2\beta}$) of 5 and 8 hours and C_{max} values of 0.265 and 0.328 pmol/ml at 4 hours after dosing (Koike *et al.*, 1998).

Radioactivity following oral administration of 14(*S*)-^{3}H-1α-OH-Vitamin D_3 (specific activity of 3.8 Ci/mmol) was distributed mainly in plasma, liver and small intestinal mucosa in normal and Vitamin D deficient rats. Distribution to the cytosol and nuclear fractions of small intestinal mucosa was also apparent. The amount of radioactivity recovered in the feces over a period of 6 days

corresponded to 39% and 49% of the administered dose after intravenous and oral dosing, respectively. A small percentage of non-volatile metabolites was excreted in urine. It is known that metabolites resulted from metabolism of 1,25-dihydroxy-Vitamin D_3 are either less potent or biologically inactive (Koike *et al.*, 1998).

In humans, peak serum concentration is reached in approximately 12 hours after a single dose, with a duration of up to 48 hours (One-Alpha Product Information, Leo Pharma Inc). The plasma half-life is 3 hours.

B. ACUTE AND SUBACUTE TOXICITY

1. POULTRY

Baker's group at the Univ. of Illinois has published numerous studies on the performance of chicks fed various levels of 1α-OH-Vitamin D_3, not testing for toxicity but nevertheless showing the beneficial effect on weight gain and increased total bone ash. (Biehl *et al*, 1995, 1998; Biehl and Baker, 1997a, 1997b). Extensive but unpublished broiler field trials in which 5 μg of 1α-OH-Vitamin D_3 per kg feed was fed have indicated that weight gain is not affected and mortality of the birds is reduced; the results are summarized below.

FIELD TRIALS	1	2	3	4
		Weight gain (grams)		
Standard Diet	2504	1999	2155	2155
+1α-OH-Vitamin D_3	2526	1994	2114	2275
		Mortality (%)		
Standard diet	5.24	4.25	7.69	5.16
+ 1α-OH-Vitamin D_3	3.11	3.00	2.75	3.60

FIELD TRIAL LOCATIONS:

1. Panama, 2005. Melo, Ross 308 males, 42 days.

2. Mexico, 2006. Nutrix, Ross 308 males, 45 days.

3. Ecuador, 2006. Grasas Unicol, Ross 308 males, 42 days

4. Mexico, 2009. Buenaventura, Cobb 500 males. 42 days

A recent study of the possible toxicity of 1α-OH-Vitamin D_3 in broilers was conducted at the University of Georgia (Pesti and Shivaprasad, 2010). The design of this study was evaluated and critiqued by FDA (IFA 10896). The objective of this study was to evaluate growth and tissue changes when increasing levels of 1α-OH-Vitamin D_3 were added to broiler feed. The measurements taken included body weight gain, livability, feed consumption and efficiency, gross pathology, and histopathology of several tissues. This study was conducted according to FDA Good Laboratory Practice (GLP) as required under 21 CFR Part 58. There were four treatments reflecting 1α-OH-Vitamin D_3 fed at 0, 1x, 3x and 5x of the proposed use level of 5 μg/kg. The performance data are seen in the following table:

Effects of added 1α-OH-Vitamin D_3 on production parameters (42 day)

1α-OH-Vitamin D_3 level (μg/kg)	Body Weight Gain (kg)	Feed Intake (kg)	Feed Conversion Ratio (feed/gain)	Mortality (%)
Pooled data				
0	2.87±0.11	4.64±0.10	1.62±0.04	14.29±4.52
5	2.83±0.10	4.60±0.09	1.63±0.04	8.33±4.67
15	2.56±0.10	4.37±0.15	1.71±0.04	11.43±6.12
25	1.82±0.17	3.64±0.11	2.11±0.25	11.91±4.76
Pr>F	<0.0001	<0.0001	0.0414	0.8717
Males				
0	3.10±0.02	4.80±0.10	1.55±0.03	23.81±2.38
5	3.04±0.03	4.75±0.11	1.56±0.04	16.67±6.30
15	2.69±0.15	4.44±0.28	1.65±0.03	15.71±12.32
25	1.67±0.33	3.73±0.17	2.41±0.46	9.52±6.30
Pr>F	0.0018	0.0102	0.0862	0.6453
Females				
0	2.63±0.05	4.48±0.13	1.70±0.03	4.76±2.38
5	2.61±0.04	4.46±0.10	1.70±0.02	0
15	2.43±0.09	4.30±0.18	1.77±0.06	7.14±4.13
25	1.96±0.15	3.55±0.17	1.82±0.06	14.29±8.25
Pr>F	0.0031	0.0066	0.2712	0.2724

The overall growth rate of the birds in this trial was excellent, with 3.10 kg males and 2.63 kg females at 42 days. Feed efficiency was also excellent, 1.55 and 1.70 feed to gain for males and females respectively. The only disappointing aspect of this trial was the high level of mortality, nearly 24% in the control males and 5% in the control females. The cause of death in these birds was mainly ascites, which is associated with high rates of growth in commercial birds.

1α-OH-Vitamin D_3 had practically no effect on growth rate when fed at 5 μg/kg of diet. Growth rates and feed conversion ratios were impaired by 15 μg/kg of 1α-OH-Vitamin D_3, but not enough to be declared significantly different at the 0.05 level of probability. Twenty-five μg/kg of 1α-OH-Vitamin D_3 significantly decreased feed consumption, growth rate and feed conversion ratios.

Growth depression was greater in the males than in the females, resulting in a significant ($P<0.05$) interaction between 1α-OH-Vitamin D_3 and gender for gain and feed efficiency. Again, this was probably related to the high rate of growth in the males and is a quantitative rather than qualitative difference. However, females consumed less feed overall, although more feed per unit of gain. This suggests that there may be a difference in the abilities of males and females to tolerate high levels of 1α-OH-Vitamin D_3.

Despite the response differences due to gender, there was no significant difference for males or females fed 15 μg/kg of 1α-OH-Vitamin D_3. Therefore any qualitative differences appear to be at toxicological and not nutritional levels of intake.

2. DOGS.

Studies have been carried out in Japan (Makita *et al*, 1978a; Izawa *et al*, 1980; Ichikawa *et al*, 2000) to evaluate the safety in beagles. The LD_{50} of 1α-OH-Vitamin D_3 in this species was 500-700 μg/kg orally, 300-400 μg/kg i.v. Hypercalcemia was observed when a dose of 0.08 μg/kg was maintained for 12 months, whereas 0.02 μg/kg over this time period was non-toxic. A dose of 0.10 μg/kg, given three times weekly over 14 weeks, caused no adverse effects.

3. RODENTS.

The oral LD_{50} values for mice and rats for 1α-OH-Vitamin D_3 were found to be 440 to 476 μg/kg and 340 to 720 μg/kg, respectively. The intravenous and subcutaneous LD_{50} values were 56 to 71 μg/kg and 85 to 96 μg/kg for mice and 56 to 101 μg/kg and 62 to 100 μg/kg for rats. (Sjoden *et al*, 1985, report an LD_{50} of 200 μg/kg in the rat; 5 μg/kg/day killed half the rats in four weeks). A level of 50 μg/kg/day induced atrophy of the prostate and testes. Following intravenous dosing of mice there was depression of movement, reduced grooming and squinting at doses of 24 μg/kg bw or more. Necropsy findings included gastrointestinal congestion, hydrothorax, hyperanemia and swelling of the lungs in survivors (Makita *et al.*, 1976, 1977a). In weanling mice, serum calcium is elevated at doses of 2.78-3.45 μg/kg, and doses of 50-250 ng/kg cause severe nephrocalcinosis (Crocker *et al*, 1985).

Administered to uraemic rats, 1α-OH-Vitamin D_3 aggravated the development of aortic lesions (Krog *et al*, 1988).

Various investigators have determined the levels of 1α-OH-Vitamin D_3 feeding at which no adverse affects are observed in rats and mice: 0.02 μg/kg/day for three months (Izawa *et al*, 1978); 0.09 to 0.9 μg daily for six weeks (Lindholm *et al*, 1981); 2.5 μg/kg/day for 30 days (Makita *et al*, 1976); 0.02 μg/kg for 6 months (Makita *et al*, 1977b); 0.08 μg/kg/day for 13 weeks (Nishida *et al*, 1989); 0.5 μg/kg in mice (Makita, 1977b).

4. COWS.

Mullen *et al*, 1979, reported that 1α-OH-Vitamin D_3 caused anorexia in calves when given in large doses (15 μg weekly for four weeks). Postmortem examination revealed macroscopic lesions typical of Vitamin D hypervitaminosis.

Alfacalcidol was approved in 1998 by the European Agency for the Evaluation of Medicinal Products, Veterinary Medicines Evaluation Unit, for prevention of parturient paresis (milk fever) in dairy cows at the end of pregnancy (1-2 doses of 350 μg per animal). It has also been found effective for treatment of "downer cow syndrome" (Barlet and Davicco, 1992).

C. DEVELOPMENTAL AND REPRODUCTIVE TOXICOLOGY; TERATOGENICITY

A fertility study with 1α-OH-Vitamin D_3 was performed with Wistar rats by oral administration doses of 0, 0.02, 0.1, 0.5 and 2.5 μg/kg bw/day to the males for at least 60 days and to females for 14 days prior to mating. Toxicity to the parent rats was evident at doses greater than or equal to 0.5 μg/kg bw/day and adverse effects upon mating performance, resulting in a reduced pregnancy rate, were present at these doses. Mating performance and fertility were unaffected at 0.02 and 0.1 μg/kg bw/day. There were no effects upon the number of *corpora lutea*, implantations and incidence of fetuses with external, visceral or skeletal anomalies. No effects were apparent on the postnatal growth and behavioral development of the offspring. The reproductive capacity and performance of the F1 generation were unaffected by the treatment given to their dams. A NOEL of 0.1 μg/kg bw/day was retained (Makita, *et al.* 1978c).

The potential of 1α-OH-Vitamin D_3 to induce adverse effects when administered from day 17 of pregnancy to day 21 *post partum* was evaluated in pregnant Wistar rats given oral doses of 0, 0.02, 0.1, 0.5 and 2.5 μg/kg bw/day. Administration of 2.5 μg/kg bw/day suppressed weight gain and depressed lactation. Bodyweight gain was slightly reduced at 0.5 μg/kg bw/day. However, treatment at doses up to 2.5 μg/kg bw/day did not adversely affect parturition or the nursing instinct of the dams. A decreased number of pups with reduced bodyweight were reared to weaning in the 2.5 μg/kg bw/day dose group. There was also a delay in the onset of the vaginal opening in the female offspring obtained from dams treated at 2.5 μg/kg bw/day. The reproductive capacity of the F1 generation was unaffected by the treatment given to their dams. A NOEL of 0.5 μg/kg bw/day was retained (Makita, *et al.* 1978b).

Wistar rats were treated with oral doses of 1α-OH-Vitamin D_3 at 0, 0.02, 0.1, 0.5 and 2.5 μg/kg bw/day from days 7 to 17 of pregnancy. The dams treated at 2.5 μg/kg bw/day showed decreased weight gain and food consumption. While a little fetal lethality was observed at this dose, teratogenicity was not recognized except for a small number of fetuses with anomalies considered

spontaneous. Increased intra-uterine death rate and retardation of fetal growth and ossification occurred as secondary consequences to the severe maternal toxic effects at 2.5 µg/kg bw/day. A NOEL for reproductive parameters was identified at 0.1 µg/kg bw/day. (Makita, *et al.* 1978b).

Early studies tested possible toxicity in laying hens from excess feeding of 1α-OH-Vitamin D_3 (Soares *et al*, 1979, 1983; Abdulrahim *et al*, 1979). Soares initially found that hens fed 1α-OH-Vitamin D_3 at a level of 5 µg/kg for 20 weeks had lower hatchability and a higher incidence of embryonic abnormalities. His later paper showed that while feeding levels of 6.8-15 µg caused weight loss, no adverse effects were observed at a level of 5 µg/kg or lower. 1α-OH-Vitamin D_3 did not appear to be transferred into the egg.

The potential teratogenic effect of 1α-OH-Vitamin D_3 was also studied in Himalayan rabbits that received oral doses of 0, 0.02, 0.08, 0.2 and 0.5 µg/kg bw/day. Effects on maternal body weight gain were seen in all dosage groups although there was no clear dosage-dependency when doses between 0.02 and 0.2 µg/kg bw/day were considered. There was an increased fetal resorption rate in the 0.08, 0.2 and 0.5 µg/kg bw/day dose groups although there was a lack of clear dosage dependency. Of 134 fetuses, three malformations occurred in the 0.02-0.20 µg /kg/day group and one grossly malformed fetus in the 0.50 µg /kg/day group. A NOEL for maternal adverse reactions was not defined in this study, however the highest dose that did not adversely affect the outcome of pregnancy was 0.02 µg/kg bw/day (Makita, *et al.* 1977).

D. MUTAGENICITY AND CARCINOGENICITY

The potential of 1α-OH-Vitamin D_3 to induce reverse mutations in *Salmonella typhirium strains* (TA 98, TA 100, TA 1535, TA 1538 and TA 1537) was examined using the spot test and plate incorporation methods devised by Ames. Solutions in DMSO of 0.25 to 250 µg/plate were applied in the spot test and 250 µg/plate was found to represent the limit of solubility in the plate incorporation method. There were no increases in the numbers of revertant colonies either in the presence or absence of metabolic activation (EMEA, 1998).

The potential of 1α-OH-Vitamin D_3 to induce forward mutations at the thymidine kinase locus in cultured mouse lymphoma L5178Y in the absence and in the presence of metabolic activation was also assessed. 1α-OH-Vitamin D_3 did not induce any dose-related or statistically significant increases in the frequency of mutant colonies (EMEA, 1998).

E. 1α-OH-Vitamin D_3 IN HUMAN MEDICINE

1. Renal Osteodystrophy and Hyperparathroidism.

The first application of 1α-OH-Vitamin D_3 in human medicine was its development by Leo Pharma in the early 1970s for treatment of renal osteodystrophy and hyperparathyroidism (Binderup *et al*, 1997). The experience gained from treatment of renal bone diseases in France has been reviewed, and the specific conditions for which it is justified have been described (Fournier *et al*, 1995).

A study of 36 Danish patients (Rix *et al*, 2004) concluded that long-term treatment with alfacalcidol was safe and might be beneficial for the preservation of bone mass in the pre-dialysis stages of chronic renal failure.

A one-year controlled study in Japan (Shimamatsu *et al*, 1981), in which 1α-OH-Vitamin D_3 was given orally to 24 chronic hemodialysis patients, initially at a dose of 2 μg daily but reduced to 1 μg daily for the last nine months, confirmed that serum calcium was increased to the normal level after one month and sustained at this level for one year. No adverse effects were seen apart from three patients with scleral calcification.

A study of hypoparathyroid patients in Israel, which followed the administration of 1α-OH-Vitamin D_3 for a total of 2,040 patient-months, found that a mean daily dose of 1 μg achieved a stable level of serum and urinary calcium with a week, and concluded that it is a safe and effective drug for long-term therapy of this condition (Halabe *et al*, 1994). Supplemental 1α-OH-Vitamin D_3 (1.5 μg daily) added to methimazole treatment also proved valuable for treating hyperthyroidism in patients with Graves' disease (Kawakami-Tani *et al*, 1997).

Low doses of 1α-OH-Vitamin D_3 (0.04-0.09 μg/kg), if started early, prevent secondary hyperparathyroidism in children on peritoneal dialysis (Saarinen, 2007). A 12-month study of 1,159 German patients with chronic kidney disease, from 241 nephrological centers, showed that mean doses of 0.43 μg/day of 1α-OH-Vitamin D_3 prevented progression or caused regression of secondary hyperparathyroidism (Reichel, 2010).

2. Osteoporosis.

1α-OH-Vitamin D_3 has been in use for more than 30 years in the treatment of osteoporosis (Kubodera, 2009), and studies in France (Pouillies *et al*, 1992), Germany (Ringe, J.D., *et al*, 2005a, 2005b), Italy (Nuti *et al*, 2006), Switzerland (Dambacher *et al*, 1997) and Japan (Shiraki *et al*, 1993, 1996) all have concluded that it is safe to use at doses up to 1.0 μg per day. A three-year study of patients in Germany and Switzerland found only mild side effects at a dose of 1 μg daily (Ringe *et al*, 2005b). In a 1999 summary, Gallagher concluded that the potential for toxicity when using 1α-OH-Vitamin D_3 is minimal when the recommended conventional dose (1 microgram per day) is used (Gallagher, 1999). A recent study (Schacht and Ringe, 2010) of over 2,000 Swiss patients confirmed that daily therapy with 1.0 μg of 1α-OH-Vitamin D_3 increased muscle power and function with an incidence of adverse drug reactions of only 0.52%. In a long-term study of 7000 Japanese patients treated with 1 μg/day of 1α-OH-Vitamin D_3, hypercalcemia occurred in only 4% of the patients (Shiraki *et al*, 1993, 1996). The applications have not been confined to elderly patients; a study in Egypt (El-Husseini *et al*, 2004) confirmed the value of alfacalcidol (0.25 μg daily) in renal transplant patients aged 17 or under. And in a group of premenopausal women, mean age 35, with collagen diseases, combination therapy for two years with thiazide, calcium, and 1α-OH-Vitamin D_3 (0.75 μg) was found effective in preventing glucocorticoid-induced osteoporosis (Yamada, 1989), though long-term administration was discouraged in patients undergoing chronic glucocorticoid therapy.

3. Other.

Several studies have demonstrated that 1α-OH-Vitamin D_3 has serious potential for treating some forms of human cancer. It was shown to be an effective treatment of follicular, small-cleaved cell type of non-Hodgkins lymphoma, with no recorded toxicity in 34 patients at a dose of 1.0 μg daily for 14 months (Raina *et al*, 1991). In combination with vitamin K, it has also been shown to

improve anemia and thrombocytopenia (low platelet count) in leukemia patients with low to intermediate myelodysplastic syndrome (Akiyama *et al*, 2010).

1α-OH-Vitamin D_3, along with insulin, was given to 35 adult patients with latent autoimmune diabetes at a dose of 0.5 μg per day for one year; pancreatic beta-cell function was significantly preserved without any severe side effects (Li *et al*, 2009).

In 140 patients with rheumatoid arthritis, a daily dose of 1-2 μg for 16 weeks prevented reduction in bone density (Yamauchi *et al*, 1989). The higher dose caused a significant increase in serum calcium, and a dose of 1.0 μg/day was judged to be suitable for long-term treatment.

Interestingly, a double-blind, placebo-controlled study in men with impaired glucose tolerance showed a moderate decrease in blood pressure on a daily dose of 0.75 μg of 1α-OH-Vitamin D_3 (Lind *et al*, 1988).

This thirty-year record of use in treatment of a variety of human disorders, with a minimum of serious side effects, argues strongly that the recommended small doses (of the order of 1.0 μg or less) are safe in humans. As Thys-Jacobs *et al* concluded in a 1997 review, 1α-OH-Vitamin D_3 causes toxic effects (primarily hypercalcemia) in human subjects at doses above 1.0 μg/day, but doses of 0.5-1.0 μg/day appear to be safe. For comparison, according to Colston *et al*, 1989, the normal human daily production of $1,25(OH)_2D_3$, the primary metabolite of 1α-OH-Vitamin D_3, is about 1 μg.

SAFETY EVALUATION:

FDA has defined 'safe' as 'a reasonable certainty in the minds of competent scientists that the substance is not harmful under the intended conditions of use' [(21 CFR 170.3(i)].

a. 1α-OH-Vitamin D_3 safety in poultry

The intended use level of 1α-OH-Vitamin D_3 in poultry feed is 5μg/kg. In older slower growing strains of chickens such as New Hampshire x Columbians it is clear that 1α-OH-Vitamin D_3 does not affect growth up to as much as 40μg/kg (Biehl *et al*, 1995).

In modern fast growing strains, 15μg/kg of 1α-OH-Vitamin D_3 seems to be the upper level affecting growth rate. Growth and tibia ash studies indicate that when 1α-OH-Vitamin D_3 is fed at 5μg/kg all goals can be accomplished without affecting bird performance while leaving a performance margin of safety of 3x.

It should be noted that in the University of Georgia trial, mortality was not adversely affected when the birds were fed 1α-OH-Vitamin D_3 at 5x or 25 μg/kg, the highest level required by FDA. This is notable in that the chance of a mixing error of this magnitude is very rare in the poultry industry. Also notable is that the 5x level was fed for the entire 42 day growing period, a nearly inconceivable scenario in the commercial feed industry.

Six studies (Abdulrahim *et al.*, 1979; Atencio *et al.*, 2005; Atencio *et al.*, 2006; Carlos and Edwards, 1998; Frost *et al.*, 1990; Snow *et al.*, 2003) evaluated the performance of breeders and layers when fed the various analogs Vitamin D_3, 25-OH-Vitamin D_3, 1α-OH-Vitamin D_3 and 1α,25-$(OH)_2$-Vitamin D_3. Several of the studies showed improvements, particularly in egg shell weight, when fed 1α-OH-Vitamin D_3. Abdulrahim *et al.*, 1979 determined that when 1α-OH-Vitamin D_3 is provided as the sole Vitamin D supplement in breeder feed, it does not affect hatchability. The Carlos paper indicated that having 1α,25-$(OH)_2$-Vitamin D_3 in the feed prevented a rapid decrease in egg production due to a *Mycoplasma gallisepticum* infection. It should also be noted that feeding 1α,25-$(OH)_2$-Vitamin D_3 to broilers has been shown to ameliorate vitamin A toxicity (Aburto *et al*, 1998).

Several studies (Edwards, 1990; Edwards, 2000; Mitchell *et al.*, 1997; Elliot and Edwards, 1997; Elliot *et al.*, 1995; Rennie and Whitehead, 1996; Stevens and Blair, 1987) evaluated the efficacy of numerous Vitamin D_3 analogs in combating tibial dyschondroplasia in broilers and found both 1α,25-$(OH)_2$-Vitamin D_3 and 1α-OH-Vitamin D_3 to be very effective preventatives.

Numerous industry trials have been conducted with 1-α-(OH)-Vitamin D_3. These trials are summarized on page 17. Notably, mortality invariably decreases while performance is maintained even with lower levels of phosphorus in the feed.

b. 1α-OH-Vitamin D_3 safety in humans

Based on an average daily consumption calculation of 0.0000085 μg (8.5 pg) of 1α-OH-Vitamin D_3 in humans from the remaining hemoglobin in chicken meat (from the calculations on pp 12-13), the safety of this level of consumption can be evaluated. In countries where 1α-OH-Vitamin D_3 is registered and used by the human population, the typical daily intake ranges from 0.25 to 1.00 μg. It is recommended that people taking 1α-OH-Vitamin D_3 on a daily basis should periodically test their blood calcium levels. A danger in taking too much 1α-OH-Vitamin D_3 is hypercalcemia.

Another danger in taking too much Vitamin D is the potential teratogenic effect. 25-OH-Vitamin D_3 and 1-α-(OH)-Vitamin D_3 have both been shown to cause birth defects in rabbits. In the Makita paper (Makita, *et al.* 1977) a dose of 0.02 μg/kg bw/day of 1-α-(OH)-Vitamin D_3 was considered a safe level and thus would not adversely affect the outcome of the pregnancy. For a 110 pound woman 0.02 μg/kg bw/day translates to 1.00 μg/day.

Being very conservative and assuming a level of 1.00 μg has the potential to cause hypercalcemia or a birth defect, we can back calculate how much chicken on a daily basis would need to be consumed to create this scenario. Simply, 1.00 divided by 0.0000085 gives a value of 117,647. Thus a person would need to consume 18,824 pounds (117,647 times 0.16 pounds per day) of chicken in a day to take in 1.00 μg of 1α-OH-Vitamin D_3, an inconceivable amount.

c. 1α-OH-Vitamin D_3 safety to the environment

The discovery in 1993 that 1,25$(OH)_2$-Vitamin D_3 increased natural phytate phosphorus utilization in chicks (Edwards, 1993) led to a flurry of activity in the research area. Biehl *et al.* 1995 showed the same improvement in phytate phosphorus utilization with 1α-OH-Vitamin D_3. In addition he demonstrated that 1α-OH-Vitamin D_3 was additive with phytase. Additional published studies by both the Edwards and Baker laboratories confirmed these early findings (Biehl and Baker, 1997a, 1997b; Biehl *et al.*, 1998; Edwards, 2002; Kasim and Edwards, 1998; Mitchell and Edwards, 1996a, 1996b and Snow *et al.*, 2004).

A synopsis of all of these studies indicates that in the absence of each other, both 1α-OH-Vitamin D_3 and phytase can account for 0.10% available phosphorus in a typical broiler diet. For instance, if a broiler starter diet is formulated to have 0.45% available phosphorus in the diet, the addition of either 1α-OH-Vitamin D_3 or phytase would allow the nutritionist to formulate the diet with just 0.35% available phosphorus having confidence that the additional 0.10% needed for optimal performance would be liberated from inositol phosphate found commonly in corn, soybean meal or other feedstuffs.

The discovery that 1α-OH-Vitamin D_3 and phytase are additive (Biehl *et al*, 1995; Mitchell and Edwards, 1996b), increased further the amount the nutritionist could reduce the available phosphorus by up to 0.15%. The ramification of this finding is highly beneficial to the environment. Using 1α-OH-Vitamin D_3 and phytase together allows for the maximum reduction in phosphorus excretion to the environment amongst all known feed technologies.

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