Final Report on Study 275.30

Study title: Provide data on various arsenic species present in broilers treated with roxarsone: Comparison with untreated birds.

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INTRODUCTION

CVM has been questioned about the safety of edible tissues from chickens treated with organic arsenicals, particularly roxarsone. The questions have arisen from (1) recent research indicating uncertainty about the identity and composition of incurred residues in edible tissues of chickens after roxarsone treatment (especially whether inorganic arsenic (iAs) increases in tissues of treated birds) and (2) law suits in the Midwest that contend arsenic in litter has caused ill effects in humans.

This research study was intended to compare arsenic speciation data in birds treated with roxarsone for six (6) weeks, and sacrificed at 0, 3 and 5 days of withdrawal, with that from control chicken tissues (muscle, liver, kidney, blood, bile, gizzard and crop and their contents, and excreta). This research study involved (a) raising of chickens from day-old hatchlings, (b) dosing chickens in treatment group with roxarsone at the maximum intended dose, and (c) analysis of edible tissues (liver and muscle) and other samples from control and treated birds for total arsenic and various arsenic species, so a comparison of arsenic speciation profiles can be made.

The characterization and comparison of the arsenic profiles in tissues of control and treated chickens will allow CVM/FDA to make some conclusions about future regulatory activities for the organic arsenicals. CVM has determined that a safe level

of inorganic arsenic is << 1ppb (So = 0.039 ppt; Sm (muscle, liver) = 0.2 ppt and 0.6 ppt, respectively) (Summary of iAs Carcinogenic RA_Tong20100427.doc). Therefore any new animal drug that contributes to the overall inorganic arsenic burden is of potential concern.

It should be noted that the above calculation is based on the premise that inorganic arsenical species are the primary carcinogenic agent(s). There is a growing body of evidence that implicates methylated As (III) species as being considerably more toxic than the corresponding inorganic As species because they actually are intermediates generated in the pathway for formation of methylated As (V) metabolites that are excreted by humans and other species (Del Razo et al., Coppin et al., and Chen et al.). How does one explain chronic arsenic exposure on hypomethylation of DNA and its potential epigenetic effects (Klingerman and Tennant, Styblo et al., Ren et al., Chen et al., Reichard and Puga)?

There is essentially no data available on the metabolic fate of the organic arsenicals, e.g. roxarsone, used in the production of food animals, i.e., chickens and swine. Could these molecules serve as substrates for the reductases involved in the metabolism of inorganic arsenicals? If reduced to As (III) moieties, can these organic arsenicals undergo subsequent methylations? Are these drugs possible substrates for the glutathione transferase that is implicated in the metabolism of inorganic arsenicals in other species? With no answers to these questions readily available, efforts to

separate arsenic species present in edible tissues should also include identification of as many of the separated arsenic-containing species as possible.

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DESCRIPTION OF MATERIALS AND METHODS

Test and Control Articles

Test Article: The test article is 3-Nitro 20, Roxarsone Type A Medicated Article, NADA 7-891 (Alpharma Inc., Bridgewater, NJ). The active pharmaceutical ingredient, roxarsone, has been characterized by Alpharma. It is supplied in 50 lb bags. The bags were stored in 30 gal drums and labeled according to LAB-031. Refer to the study specific procedure, Preparation of Type A Medicated Feed - Roxarsone in Poultry Feed, for specifics regarding preparation of the medicated feed.

CAS Number: 121-19-7

Lot Number(s): AB770783

Expiration Date: December 2010

Source of Supply: Alpharma Inc. through a local distributor, IVESCO.

Storage Conditions: The test article was stored at refrigerator temperature (~4C) in Feed Bldg., D2.

Composition: 20% Roxarsone + 80% inert ingredient comprised of calcium carbonate, mineral oil and rice hulls (information from MSDS document).

Control Article: The control article is unmedicated feed which was obtained from a commercial vendor, Southern States Inc., SSC-25-235007 ALL GRAIN POULTRY STARTER, Lot #'s G9222 and G9272 supplied in 50# bags. Feed was transferred from the 50# bags to temporary storage bins that were labeled according to SOP# LAB-050. The bins were stored on a pallet in an adjacent room in the building housing the chickens.

Rationale For Dose Selection: The test article was mixed with control feed according to labeled directions so as to provide exposure to the maximum approved level of roxarsone for chickens, i.e., 50ppm or 0.005%.

Dosing Procedure: The test article prepared in control feed according to label directions was administered in feed; feed troughs were topped-off or refilled daily.

Stability Testing: The test article is an approved animal drug and as such there was no need for stability testing of the dosage form. Initially one batch of the medicated feed was prepared on 9/22/2009. Afterwards feed was prepared in two 50lb

batches when the feed bins had approximately one day's ration remaining: 9/24/2009, 10/07/2009, 10/15/2009, and 10/20/2009. Medicated feed batches were transferred to temporary storage bins that were labeled according to SOP# LAB-050. The new batches were "topped-off" with any remaining feed from the previous batch; this insured that the older batch was used up. The feed bins were stored on a pallet in an adjacent room in the building housing the chickens. Stability testing of the medicated feed batches was not done performed due to the lack of an available method during the animal phase of the study.

Animals:

Species:	*Gallus gallus*
Type:	Broiler
Body weigh/age:	1-day old
Number:	102, Animals identified by wing band
Source:	Through USDA - BARC, from Moyers Chicks, Inc. 266 E. Paletown Rd., Quakertown PA 18951-2831; 215-536-3155.

The study protocol for the animal treatment phase was reviewed and approved by the FDA/CVM Office of Research's Institutional Animal Care and Use Committee. The study was conducted in the CVM Office of Research AAALAC approved facility under the auspices of the Good Laboratory Practices (21 CFR part 58).

Two (2) day-old chicks were randomly assigned to one of two test groups: control or roxarsone-treatment. Birds were housed in individual brooder cages; three brooder units (Petersime Model 25D24RE; each holding 24 individual cages) were used for the roxarsone treatment group and two units were used for the control group. The birds were randomly assigned to individual brooder cages as described in the study protocol and each bird was wing-banded. Wing banding was delayed until the 12th day because of purchasing delays encountered at end of fiscal year. Room lighting was adjusted to maintain lights on for 23hr and lights off for 1hr. All the brooders have built-in lights that are either on or off; there are no timers. The lights were kept on at all times because the heaters are tied into the same power supply. The area where the heat source is located does not get direct light and provided a darker environment for the birds if they chose to make use of it. The feed and water trays are located in the lighted portion of the brooder cages. The room temperature was maintained at approximately 80-85°F for the first month and then decreased to 70-75°F. The brooder temperatures were maintained at 90-95°F for the first week and decreased by 5°F weekly thereafter to about 80°F. Feed and water were available *ad libitum*.

Initially, sixteen (16) birds each were assigned to the three roxarsone test groups (16 x 3 = 48), and eight (8) birds each to the control groups (8 x 3 = 24); total minimum number of birds needed = 72. Additional birds were assigned as extras to each of the

groups to account for mortality and to provide additional test samples if needed. Eight birds from the extras (four (4) controls and four (4) from the roxarsone test group) plus the two extra chicks (total = ten chicks) were sacrificed on the day after arrival as an animal health quality control (QC) check. No adverse observations were noted during necropsy of these QC birds.

Diet. All chicks were fed Southern States All Grain Poultry Starter *ad libitum* as the basal diet throughout the course of the study. Feed troughs were refilled as needed. Water was available *ad libitum* and the water troughs were topped off or refilled daily. In an effort to eliminate environment factors such as feed and water as possible sources of arsenic exposure triplicate samples of the initial lot # of the control feed and duplicate samples of building water were analyzed by a GLP-compliant contract laboratory (Test America) for total arsenic using inductively coupled plasma mass spectrometry (ICP-MS). The levels of total arsenic that were measured (feed = 0.53 ppm and water = 2.5 ppb) were within the approved tolerances for feed (50 ppm) and the EPA's tolerance for municipal water (10 ppb). It should be noted that the minimal level of quantitation for inorganic arsenic in feed and water were 0.5 mg/kg and 2.5 µg/L, respectively. Therefore results were essentially zero for practical purposes.

Treatment. Chicks were fed either the control diet or the roxarsone-containing test diet for six weeks. The roxarsone-treatment group received the control diet supplemented with 50 ppm of roxarsone from 3-Nitro® 20 Type A Medicated Article (Alpharma, lot #

AB770783; expiration date – 12/2010). After six weeks, the diet of the roxarsone treatment group was switched to the control diet. Birds from both test groups were terminated by CO_2 asphyxiation on days 0, 3, and 5 following the change to control diet. On each of these post-treatment days, eight (8) birds from the control group and sixteen (16) birds from the roxarsone treatment group were terminated. All remaining "extra" birds were terminated along with the day 5 animals. Selected tissues (blood, bile, liver, kidney, muscle, crop and/or gizzard contents, and excreta) were collected from each animal when sacrificed. Excreta were collected from the trays under each of the individual pens. On the day before the specified withdrawal days, the excreta collection trays were cleaned and lined with fresh paper to collect excreta from the birds on the same day they were terminated.

Sample Collection and Storage:

On scheduled withdrawal termination days, birds were removed from their individual brooder cages and put into a chamber filled with CO_2 until all movements ceased and the birds were unresponsive. Birds were removed from the chamber and decapitated. The bird's neck was placed in a fifty-mL screw cap falcon tube and the bird was held upside down to facilitate bleeding and blood collection. The tube was sealed and the volume of blood recorded; the tube was put on ice. The crop contents were then expressed into a sample tube and the weight of the contents recorded. The majority of the breast muscle was removed, weighed and transferred to the dissecting station for removal of triplicate 0.5g aliquots. The abdominal cavity was

opened and the gizzard contents transferred to a sample collection tube and the weight of the contents recorded. The bile duct of the livers was cannulated with a 20g needle and any bile present was aspirated with a 5mL syringe and the volume recorded. The bile was transferred to a centrifuge tube and put on ice. Afterwards, the liver was removed, weighed and transferred to the dissecting station for removal of triplicate 0.5g aliquots. Similarly, the left and right kidneys were individually removed, weighed and divided into multiple 0.5g aliquots if there were sufficient amounts of tissue; otherwise it was left intact. Tissue samples from individual birds on each collection date were refrigerated until all scheduled necropsies and tissue collections were completed. Necropsies were started at 7am and completed by noon on days 0 and 3; on day 5 the process was extended by an hour or two because of the increased number of samples. When sample collections were completed they were transferred to labeled storage boxes and stored at -80C until removed for analyses. Because the analytical method was being developed concurrently, tissues from some of the "extra" birds from the control and treatment groups were used to provide tissues for method development. In this case, three birds each from the control and treated group were sacrificed on week 4 and one each from the two test groups on week 5. In addition, two (2) birds from the extras in the roxarsone treatment group were sacrificed on day 2 of withdrawal to provide training for a new staff fellow.

Analytical Procedure:

The analytical procedures are described in detail in the DRC Methods, "SOP for ICP-totals 8-31-10.doc" and "SOP for Speciation Method101107.doc". They are further elaborated upon in the Analysts' Report, entitled: "Analysts Report-liver feed premix Final.doc (May need to include amended title?)". For total arsenic analysis, 0.5 – 1g tissue is digested with 10g (7mL) concentrated (70%) nitric acid. After rinsing, etc. the sample volume is ~50 mL and is then analyzed by ICP-MS. For arsenic speciation, 0.5g tissue is extracted with 3mL of an aqueous solution of tetramethylammonium hydroxide (TMAH), diluted with 6.5mL water, filtered thru 30K Centri-prep tubes to remove proteins and other macromolecules, and analyzed by ion chromatography-inductively coupled plasma-mass spectrometry (IC-ICP-MS). The MS is set to detect As ions (*m/z* 75). Peak identification is by retention time matching with external standards, with standard addition used when necessary. Quantification is by comparison to an external calibration curve.

DATA CALCULATIONS/MANIPULATIONS

Because of the requirement to use ICP-MS software to evaluate individual data files or analytical sequences and then the subsequent export of this data to Excel spreadsheets for further analyses, one cannot briefly provide a simple explanation of the steps involved in data manipulations/calculations from raw chromatographic data to final summary files. Refer to the Analysts' Report for a listing of data files used to generate summary Excel files and tables. An analysis of liver and muscle tissues for total arsenic content (Table 2 of Analysts' Report) were summarized in the Excel file, "Total iAs_20101209.xls". Subsequently, results of re-analyses of

liver samples for total As, “All liver totals results.xls”, after QA audit were submitted for statistical analysis. The source file, “Chicken liver speciation summary-rev 20110114.xls”, from the As speciation summary tables (Tables 4a & 4b in the Analysts’ Report which originated from the MS Excel spreadsheet, Liver speciation survey-rev20110114.xls. The spreadsheet was renamed to “Liver arsenic speciation.xls”.) was rearranged with separate tabs used to contain data from the control and treated groups, respectively. Within each separate tab, results columns were rearranged to group similar and/or related compounds together (see results section text). Additional columns were inserted so as to calculate the sums of these related groups to facilitate data evaluation. Portion of revised spreadsheet to be used for Table 4 of this report.

STATISTICAL METHODS

Refer to the Statistician’s Reports, “Total As Analyses for JK.doc” and “Non-iAs Analyses for JK.doc”, and “Inorganic Arsenic in Poultry.pdf” and “Final Report for Total As20101212VL.doc”, wherein statistical analyses were conducted using the statistical program “R 2.9. In the “Total As Analyses” report, values that were less than the lowest calibration standard were censored and in both the “Non-iAs Analyses” and “Inorganic As Analyses” reports all values < LLOQ were “censored”. There were no censored data in the report entitled: “Final Report for Total As20101212VL.doc”. Copies of all these reports are attached to this document.

RESULTS and DISCUSSION

Animal Phase

The animal phase of the study concluded without any major incidents. Although we expected a 10-15% mortality rate, only two (2) birds from the roxarsone treatment group died and this occurred during the first two weeks of the study. The use of brooders to house chicks individually was probably a major contributing factor for the low mortality rate observed in this study. In addition, use of the brooders enabled us to randomly assign birds to pens and then to randomly assign birds to various withdrawal/sampling dates. The inclusion of extra birds also enabled us to use a sufficient number of birds for QC testing, intermediate sources of materials for method development, and as potential substitutes in the event of premature mortality of test and/or control animals. Having extra birds also allowed us to have a higher number of test animals for the 5 day withdrawal time point.

The environmental controls for the study room maintained the room at an elevated ambient temperature of ~85°F when the chicks first arrived and then it was decreased to ~81°F from the 2^{nd} - 4^{th} weeks. Data from the following two weeks (22 October until 4 November) were lost because of computer user error by the facility contractor. These environmental facility controls together with the heater controls provided adequate environmental conditions for the birds to grow and thrive. By the end of the experiment, the birds were actually somewhat cramped in their individual brooder cages. Though cramped, they were not hindered in their mobility or in their access to feed and water. This was especially evident when it was time to remove birds for euthanasia and sample collection. Similarly, there were three instances noted in the animal care

logs that birds were “out of pens” (#65 on 10/22/09; unidentified bird on 10/31/09, and #4 on 11/1/09).

So as to eliminate the potential that feed and/or water would be contaminated with high levels of arsenic, samples of water and control feed were submitted to a GLP compliant testing company (Test America Inc.) for analysis of total arsenic using a standard ICP-MS procedure. Only the first (lot# G9222) of the two lots of feed used were analyzed for arsenic contamination. The second lot (G9272) arrived in the middle of the animal phase because it was apparent that we would not have enough feed to finish the study. Because of this, we did not submit samples from this lot for testing. The data obtained from Test America was provided in their report entitled, “J51633-1 Std_Tal_L2 Final Report_jck.pdf”. (Feed = 0.53 ppm (n=3) and water = 2.5 ppb (n=2) where n= number of samples submitted); as per their report each sample was analyzed in duplicate. Based on the levels of arsenic in the feed (0.53ppm), I contacted Southern States to express my concern. I was told that since the level of arsenic was less than the AAFCO established tolerance for feed, 30-50 ppm, there was nothing they could or would do. I was further informed that the facility that produces the chick starter ration does not prepare any medicated feeds, and therefore it is unlikely that the feed was contaminated by any arsenic-containing active pharmaceutical ingredient. The values reported for total arsenic in feed were barely above the lower quantitative limit of 0.5 mg arsenic/kg feed and the value for water was less than the lower limit of 10 µg arsenic/L. During the animal phase, the OR/CFSAN analyst team requested samples of feed used in the study for their use as part of the method development procedure. Samples of control feed and one batch of the medicated feed which had been

prepared previously were provided to and analyzed by the OR/CFSAN analyst team; the results will be discussed under the analytical phase section of this report.

Following replacement of the roxarsone-medicated feed with control feed, birds in both the control (n=8) and roxarsone-treated (n=16) groups on days 0 and 3, and all the remaining control and roxarsone-treated birds on day 5 were randomly sacrificed. The sequence for animal sacrifice was to do all the control birds first and then the birds in the roxarsone-treated group. The processing of birds on specified sample collection days was done in "assembly-line" fashion with one person (DF) designated to document results. At one point during sample processing on day 5, connectivity with the network drive was lost and access to the previously printed necropsy forms were lost and could not be printed for manual completion during the necropsy/sample collection procedure. Therefore minimal data regarding the remaining animals that were processed were entered manually on the back-side of a hard-copy of the necropsy data form. Since a copy of the Excel spreadsheet used to record tissue weights was stored on the laptop, this data file was restored and used to tabulate remaining data. Even though we had collected tissue weights from all the birds terminated under the study, this data was collected to facilitate keeping a tissue inventory as opposed to using the data to determine if there were "production" effects noted between the test groups. In addition, the tissue weight data would have been meaningless without body weights which were not collected.

Analytical Phase

One point that was not addressed in the study protocol is the quantification of total arsenic

residues in the collected samples. Fortunately, our CFSAN collaborators recommended the determination of total arsenic in collected samples as 1) an indication of total exposure and 2) to assess extraction efficiency of any methods that we developed in addition to providing mass-balance data. Data from these and all subsequent analyses were taken from the "Analysts Report-liver feed premix Final20110121.doc".

Midway through the animal phase, a random sample (~50g) from the top layer of the feed bins containing control and roxarsone-medicated feed were provided to the OR/CFSAN analysts for determination of total arsenic content and speciation analysis. They calculated the levels of total arsenic in these feed samples to be 0.156 ppm in the control feed. Although this value was considerably less than that reported by the contract laboratory, the latter's lower level of quantitation was 0.5 mg arsenic/kg feed (500 ppb). They measured 11.3 ppm arsenic in the roxarsone-treated feed which is equivalent to a roxarsone concentration of 39.6 ppm roxarsone. The feed was expected to contain 50 ppm roxarsone. But since this was one small sample out of a 100 lb batch, it is not surprising that results were less than optimal.

The concentration of total arsenic in muscle and liver of chicks are shown in Table 1.

Table 1. Concentration of total arsenic in muscle and liver of untreated control and roxarsone-treated chicks at day 0 and day 5 withdrawal

Sample ID	**Day 0 Control**	**Day 5 Control**	**Day 0 ROX**	**Day 5 ROX**
Muscle	4.9 ± 2.5 ppb	3.1 ± 0.8 ppb	71 ± 20 ppb	28 ± 10 ppb*
Liver	3.4 ± 1.3 ppb	3.3 ± 0.7 ppb	2836 ± 1391 ppb	1062 ± 709 ppb*

*Approved tolerances in muscle and liver are 500 ppb and 2000 ppb, respectively

The tissue residue data indicate that control birds had 3-5 ppb total arsenic in edible tissues regardless of sampling day (0 day vs. 5 day) and/or tissue. These levels of arsenic are consistent with the concentration of inorganic arsenic present in our water supply, 2.5 ppb, but considerably less than the total arsenic measured in the basal feed (150 ppb, 540 ppb). On the other hand, the roxarsone-treated birds had >800-fold more arsenic in the liver at day 0 when compared with control birds; the treated/control ratio of arsenic in muscle at 0 day was ~14-fold. After the 5-day withdrawal, the treated/control ratio in the muscle decreased to ~9-fold. On the other hand, the treated/control ratio in the livers after 5 day withdrawal still showed a 300-fold difference. Although the overall mean concentration of total arsenic in livers of treated birds was less than the 2000 ppb approved tolerance level for uncooked edible tissues, the range of values varied widely, 300 - 2900 ppb.

Results of speciation analyses

FDA and other regulatory agencies consider inorganic forms of arsenic, namely arsenate (As

(V)) and arsenite (As (III)) to be the major species responsible for the carcinogenic activity of this element. However, the regulatory analytical method for total arsenic residues in edible tissues does not address the concern for possible residues of carcinogenic concern in the food. Therefore analysis of the various arsenic species is necessary to determine if dietary supplementation with organic arsenicals will contribute to the carcinogenic burden imposed on individuals consuming edible tissues from chickens.

Because there is a distinct possibility that the inorganic forms of arsenic may be contaminants in the roxarsone pre-mix, the test article, 3-Nitro-20, was analyzed for total arsenic and then for arsenic speciation to assess the distribution of various arsenic species present in the product (see Tables 2 and 3).

Table 2. Arsenic speciation in feed samples and a lot of 3-Nitro-20® (roxarsone) pre-mix.

Sample ID	**Total As**	**As (III) #**	**As (V) #**	**DMA#**	**MMA#**	**3-NH2#**	**N-Ac#**	**ROX#**
Control Feed	156 ppb	24 ppb	32 ppb	trace‡	nd	nd	nd	nd
Medicated Feed	11300 ppb	23 ppb	36 ppb	trace	nd	trace	nd	11300 ppb
Medicated Pre-mix		1000 ppb	12000 ppb			2000 ppb	1600 ppb	3.81%

Abbreviations: As (III) , arsenite; As (V), arsenate; DMA, dimethylarsinic acid; MMA, monomethylarsonic acid; 3-NH2, 3-amino-4-hydroxyphenylarsonic acid; N-Ac, 3-N-acetyl-4-hydroxyphenyarsonic acid ; ROX, roxarsone; nd = not detected. ‡ trace is some quantity between the LOD and the LOQ..

Maximum.API contaminant allowed in finished feed: As (III) < 12.5 µg/kg and As (V) < 25 µg/kg

See calculations below.

Table 3. Initial Speciation Analysis Of Control Feed, Medicated Feed, And Premix.

Concentration is in ppm (mg elemental As per kg) .

Sample	DMA	AsIII	MMA	Unk 3.5	Unk 4.5	Unk 5.6	AsV	3-Amino	N-Acetyl	Unk 13	ROX*	Unk 21	Unk 32	Unk 36
Control feed (water extract)	<LLOQ	0.024	<LOD	<LOD	<LOD	<LOD	0.032	<LOD	<LOD	<LOD	0.004	<LOD	<LOD	<LOD
Control feed (TMAH extract)	<LLOQ	<LOD	<LLOQ	<LOD	<LOD	<LOD	0.038	<LOD	<LOD	<LOD	0.015	<LOD	<LOD	<LOD
Medicated feed (water extract)	<LLOQ	0.023	<LOD	<LOD	<LOD	<LOD	0.036	<LLOQ	<LOD	<LLOQ	11.3	0.003	<LOD	0.148
Medicated feed (TMAH extract)	<LLOQ	<LLOQ	<LLOQ	<LOD	<LLOQ	<LOD	0.047	0.010	<LLOQ	<LLOQ	8.00	0.003	0.0462	0.0507
Premix (diluted) (water extract)	<LOD	1.02	<LOD	<LOD	0.442	<LLOQ	12.2	2.03	1.64	0.110	38100	21.9	806	53.3
Premix (diluted) (TMAH extract)	<LOD	0.73	<LOD	<LLOQ	0.446	<LLOQ	12.4	2.07	1.63	0.182	38200	11.7	462	57.5
Premix (direct) (water extract)	<LOD	1.00	<LOD	0.094	0.109	0.178	11.2	1.72	1.45	<LOD	Off scale	10.7	668	39.2
Premix (direct) (TMAH extract)	<LOD	0.77	<LOD	0.074	0.128	0.360	12.5	1.82	1.22	<LOD	Off scale	15.1	419	51.8

*The conversion from ROX concentration as “mg/kg” arsenic to percent roxarsone is: mg/kg /10^6 × 263/75 × 100. Abbreviations: As (III) , arsenite; As (V), arsenate; DMA, dimethylarsinic acid; MMA, monomethylarsonic acid; 3-Amino, 3-amino-4-hydroxyphenylarsonic acid; N-Acetyl, 3-N-acetyl-4-hydroxyphenyarsonic acid ; ROX, roxarsone; LLOQ, lower limit of quantification; LOD, limit of detection; unknowns, (Unk + # (retention time); TMAH, tetramethylammonium hydroxide.

Control feed contained a “trace” amount of DMA, 24 µg/kg As (III), and 32 µg/kg As (V). Whereas the medicated feed, containing 39600 µg/kg total arsenic, contained trace amounts of DMA and 3-NH_2, 23 µg/kg As (III), 36 µg/kg As (V), and 11300 µg/kg ROX. The medicated pre-mix had 1000 µg/kg As (III), 12000 µg/kg As (V), 2000 µg/kg, 3-NH2, 1600 µg/kg N-Ac, and 3.81% ROX, as well as several unknown peaks in the mass chromatogram of arsenic (m/z = 75) (see Table 3). Hence the contribution of the pre-mix to the medicated feed is approximately 3- 4 µg/kg inorganic arsenic (As (III) and As (V)). The mass balance for arsenic was not met for the control feed. Our speciation data for As (III) and As (V) only account for 56 µg/kg inorganic arsenic whereas the total arsenic measured by our analysis was 156 µg/kg, meaning that the speciation only accounted for ~30% of the total.

The criteria for inorganic arsenic species contamination in the finished roxarsone product: As (III) - 0.025% and As (V) - 0.05%, which are based on I_2 potentiometric titrations. Using these values, we calculated what would be the maximal approved levels for inorganic arsenic contaminants in medicated feed containing 3-Nitro 20 Medicated Type A pre-mix:

3-Nitro 20 Medicated Type A pre-mix = 20% Roxarsone API

Medicated Feed = 0.005% Roxarsone (50 mg/kg ROX + 200 mg/kg inert)

From the afore listed Manufacturing Controls, the allowed inorganic arsenic = 0.025% As (III) of API + 0.05% As (V) of API; there is a 2:1 ratio of As (V) /As (III)

= 0.075% As (III + V) in API

= (0.075%/100)*50 mg/kg ROX API in medicated feed

= 0.0375 mg/kg As = 37.5 µg/kg As

= 25 µg/kg As (V) + 12.5 µg/kg As (III) in finished medicated feed

Based on data shown in Table 2, both the control and medicated feeds contain approximately the same amounts of both inorganic arsenic species, namely ~23 µg/kg As (III) and 28% and ~38 µg/kg As (V). These concentrations of inorganic arsenic are approximately 2-fold more As (III) and ~50% more As (V) in both the control and medicated feeds. Since the concentration of inorganic arsenic in control and medicated feeds are essentially equivalent, one cannot conclude that inorganic arsenic species in medicated feed arise from the pre-mix. The pre-mix is comprised of inert ingredients that account for 80% of the total mass. One of these ingredients is rice hulls, which have notoriously high concentrations of arsenic. Rahman *et al*, 2007 reported that the concentration of total arsenic in rice hulls can be 0.7 – 1.6 mg/kg dry weight. However, when this value is incorporated into the feed calculation (rice hulls = 200 mg/kg), the resultant contribution of rice hulls is only 0.23 µg/kg, which is 100-fold less than that of the inorganic arsenic contaminants allowed in the API. Therefore the rice hulls should not contribute to the levels of inorganic arsenic in the API.

Evaluation of Summary Data from Analyst Report and other internal CVM reports

Table 4 of the Analysts' Report provided a summary of all the main arsenic-containing peaks that corresponded to several known arsenic standards. The MS Excel spreadsheet used to generate this table, "Liver speciation summary-rev20110114.xls", was used for further evaluation. Unidentified peaks were also tabulated and listed as "Unk-RT", where RT is the retention time in minutes. The table has been rearranged such that peaks corresponding to the

known inorganic arsenic moieties, As (III) and As (V) are grouped together with the methylated metabolites, MMA and DMA. The methylated metabolites of the inorganic species were linked with the inorganic species to show that the presence of these metabolites is an indication of the total exposure of the test animals to inorganic arsenic. The presence of these methylated metabolites in edible tissues reflects the probability that increased levels of inorganic arsenic were consumed by the test animals and that we are only measuring the residual compounds not eliminated by normal physiological processes. Depending on the oxidation state(s) of the methylated metabolites, they may in themselves present as toxic metabolite(s) to consumers. At this point we have no idea of the relative chromatographic behavior of the MMA (V) and DMA (V) species vs. the MMA (III) and DMA (III) species – they certainly have different structures with the oxidized forms (+5 valence state) having an additional hydroxyl group. Likewise, roxarsone (ROX) and its metabolites, 3-NH2- and N-Ac-derivatives, were also grouped together. Similarly all the unknowns were grouped together. Extra columns were inserted to reflect what would be considered to be physiologically related groupings, e.g., Total iAs Exposure = (sum of As (III), As (V), DMA, and MMA). As stated above, both of the latter compounds are a direct measurement of exposure to inorganic arsenic and hence should be quantified along with residual amounts of the actual inorganic arsenic species. Similarly, roxarsone and its metabolites should not be segregated from each other and again they represent an overall measurement of exposure to roxarsone and its subsequent metabolism by the chickens treated with this product. There has been some speculation in the literature that these metabolites are potentially more toxic than the parent compound () in the same way the acetylated aromatic amines are more potent carcinogens then their corresponding free amines, e.g., 2-acetaminofluorene vs. 2-

aminofluorene. Since we have no information about the structural identity of any of the unknowns, they should be evaluated as a pooled entity until we know more about these metabolites. Lastly, all of the various subgroups of arsenicals have been summed to generate a column of data that reflects "Total ROX As".

The overall summary table had been separated to address the "Controls" and "Treated" birds in separate tabs; they were subsequently combined. Obviously, data from the controls will not be evaluated for any contributions associated with the organic arsenical moieties even though four birds (3 birds from day 3 and 1 bird from day 5) had peaks with retention times corresponding to that of roxarsone. Surprisingly, four birds from the day 0 group had measurable levels of Unk-4.5 and one bird also had some Unk-3.5. Three birds from Day 3 and five birds from Day 5 also had quantifiable amounts of Unk-4.5. In an effort to ensure that all commercially available arsenic species standards were evaluated, several other arsenic standards were chromatographed under the same conditions used in these analyses: arsenobetaine (RT = 2.8 min), trimethylarsine oxide (RT = 2.8 min), 4-arsanilic acid (4-aminobenzenearsonic acid, RT = 3.6 min), tetramethylarsonium ion (RT = 3.9 min), arsenocholine (RT = 3.92 min), and nitarsone (4-nitrobenzenearsonic acid, RT = 7.6 min). None of these compounds had a retention time that matched any of the unknowns mentioned above, although arsenobetaine and tetramethylarsine oxide both partially co-elute with DMA.

CVM's statistical staff performed an evaluation of results of the "inorganic arsenical residues" (As (III) and As (V), total arsenic in liver tissues, and on each of the main arsenic species that

were consistently observed during IC-ICP-MS speciation analyses. Based on the various LLOQ's for individual analysis data sets, results from all evaluated groups were censored (essentially eliminating a significant number of low values). In some instances, this reduced the statistical evaluation to a comparison of data from the treated groups as a function of withdrawal day. Analysis of the inorganic arsenic species was essentially limited to evaluation of arsenate (As (V)) levels. At Day 0, iAs = 8.13 µg/kg; at day 3, iAs = 1.71 µg/kg, and at day 5, iAs =1.58 µg/kg. Based on the calculated 95% confidence limits and taking into consideration the large number of censored values for Days 3 and 5, there was a significant drop in iAs by Day 3, but there was no difference between Days 3 & 5. Despite the large amount of censored control data, the residual arsenic in treated animals was different than that in controls. Similarly, the total arsenic data in liver tissue at day 5 withdrawal indicate that the treated birds are different than the controls. Not a single control bird had an iAs(V) concentration above the LLOQ over the five day withdrawal period whereas the treated birds displayed a decreasing trend in the proportion of birds with iAs(V) concentrations over the LLOQ (Day 0 = 14/16, Day 3 = 5/16, Day 5 = 9/21). As for the various arsenic species measured by IC-ICP-MS, a conservative value, 2 µg/kg (the higher of two LLOQs) was chosen for censoring data. As a result, all of the values for the control animals were censored. Depending on the arsenic species measured (DMA, MMA, Unk-4.5, and Unk-13), many of the treated animal values were also censored. The treated animals had measurable values for both the 3-NH2 and 3-N-acetyl metabolites. The former declined as the withdrawal days increased and in several instances the values were below the LLOQ (refer to Figs 3&4 of the "Non-iAs Analyses for JK report"). Similar results occurred with the roxarsone peak. Unk-3.5 showed a similar decline related to withdrawal day, but many

of the values were below the LLOQ at days 3 & 5. However, it seems as though the birds may have segregated into two groups with most values at or near the median, but a significant number had values at the 75th quartile and higher. Unk-5.5 and Unk-36 had values that were consistently above the LLOQ and there was minimal decline as a function of withdrawal day (see Fig. 8 & 12 of the "Non-iAs Analyses for JK report"). Unk-21 had a wide range of values that declined over the withdrawal days (Fig. 10 of the "Non-iAs Analyses for JK report"). Unk-32 was not seen in the 0 day group and values were widely separated between those at the LLOQ and those above the 75th quartile; values for this metabolite seemed to increase by the 5 day withdrawal.

Table 3contains results of speciation analyses of control feed, final medicated feeds, and the medicated pre-mix. The control feed contained inorganic As species in addition to some entity that co-eluted with ROX. Water extracts were able to separate As (III) from As (V) whereas TMAH extracts apparently converted As (III) to As (V). On the other hand, medicated feed containing 50 mg/kg roxarsone had measureable levels of the inorganic species and several of the unknowns (Unk-21, Unk-32, and Unk-36). Other unknowns and methylated metabolites were less than the LOD or LLOQ. Except for the methylated metabolites, the unknowns and roxarsone metabolites were quantified in the pre-mix. This may mean that the unknowns seen in the tissues are originating from the various As species in the pre-mix and are then concentrated in the tissues and that we're just seeing their depletion and not necessarily active metabolism.

<u>Unknown metabolite(s)</u>

Although the unidentified peaks are basically unknowns, one can make some inferences about their structure from other published studies. The work of Pizarro, *et al*. (1) provided ample evidence to support the possible presence of arsenobetaine as one of the unknowns after exposure of chickens to sodium arsenate. Fish meal is usually a major protein source used in the preparation of chicken feed and this commodity has elevated levels of arsenobetaine. However, the chromatographic retention time of arsenobetaine did not agree with our results as reflected in Table 5a of the Analysts Report. On the other hand, Falnoga, *et al*. (2) demonstrated that after arsenic trioxide was administered to hens, inorganic arsenic was predominantly bound to a low molecular weight protein (<10,000 daltons) and not to metallothionein as one might have expected.

One significant caveat to the above discussion is that the control feed used in this study is an "all grain" and "complete vegetarian formulated feed" (text from feed tag). As such, one would not expect our diet to contain arsenobetaine and therefore it is highly unlikely that arsenobetaine and/or arsenocholine would be present in our tissue extracts. Chromatography of liver extracts from control birds only showed the presence of DMA and minor amounts of As (III). Other possible arsenic species, including arsenocholine, arsenobetaine, or arsenosugars were not seen. Apparently Falnoga, *et al*. (2) were able to extract all of the liver's arsenic with methanol-water, but only ~66% using Tris buffer to prepare subcellular fractions for protein chromatography. Hughes *et al*. (3) showed that there is a marked difference in the ability of mice to methylate monomethylarsonic acids (MMA) that differ in their valence states: MMA (V) does not undergo further methylation to DMA (V), whereas MMA (III), which is extremely toxic in its own right,

readily undergoes methylation to form DMA (V) (82%) along with small amounts of MMA (V) (6.3%) and DMA (III) (10.9%). Could one or more of the unknowns represent different oxidation states of the known metabolites? This remains to be demonstrated.

Our sample preparation for speciation involved extraction with TMAH and subsequent ultrafiltration through 10K- or 30K-dalton ultrafilters. Considering Falnoga's results, there's a strong possibility that if protein-bound arsenicals arising from roxarsone and/or the inorganics would pass through the ultrafilters, then these might be some of the unknowns that were measured in our sample extracts. Another possibility to consider comes from the work of Kala *et al.* (5) who isolated two arsenic-glutathione complexes in bile that were purportedly formed by the action of the MRP2/cMOAT transporter. Results obtained by the FDA analysts in their method validation experiments (data not included here; refer to the Analysts Report-liver feed premix Final.doc (amended)) indicated that there were differences observed in the amount of arsenic species present in ultrafiltrates obtained using 10K vs. 30K membranes. This is another indication that some of the unknown As peaks are most likely protein- or peptide-bound entities some of which are being sequestered by the ultrafilter membranes. In addition, there could be a significant amount on non-specific binding to the membranes.

The biological fate of roxarsone in chickens has yet to be fully clarified. Can it undergo methylation reactions in the same manner as MMA? Can it participate in arsenolysis reactions with nucleoside phosphates in the same manner that arsenate functions? If so, then this reaction could give rise to arsenosugars. Aschbacher and Feil isolated (4-acetamidophenyl)

dimethylarsine oxide from the urine of pigs treated with arsanilic acid (4-aminophenylarsonic acid) (9), which demonstrates the capacity of animals to methylate and acetylate this organic arsenical. There's no reason to assume that such a reaction sequence would not occur in chickens treated with roxarsone.

Our data demonstrate that the roxarsone premix is contaminated with measurable quantities of inorganic arsenicals and several unknown arsenicals that were detected during speciation analyses using IC-ICP-MS. However, even this observation must be tempered with the fact that the API only represents 20% of the marketed product. The remainder of the product is composed of rice hulls and calcium carbonate. Rice hulls are known for being contaminated with arsenic. In fact, the published values for total arsenic in rice are <0.07 to 3.53 mg/kg and vary by location (6, 7, and 10). However, using the same calculations as performed for the inorganic arsenic species, it is unlikely that the rice hulls are contributing any significant amount of arsenic to the finished medicated diet. One must conclude that some of the unknown arsenicals present in the livers may arise from contaminants that were present in the pre-mix. Whether or not these unknowns can be generated during *in vivo* metabolism remains to be determined.

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PROBLEMS AND DEFICIENCIES

The lack of an analytical method at the start of the animal phase of this study presented considerable challenges. The method was to be developed by DRC staff (HFV-510) in collaboration with CFSAN personnel, but this assignment did not occur until just before initiation of the animal phase of the study. As a consequence, the method was undergoing continual modifications, and actual tissue samples were being used to "test" the method as part of verifying the analytical method. During the method development process, tissues (mostly liver) from the 0 and 5 day withdrawal groups underwent multiple partial thaw and freeze cycles wherein small portions of frozen tissue were removed for analysis. The final method is probably only applicable for use with liver, and possibly kidney tissues, and was not amenable for analysis of muscle. Other tissue-related specimens that were collected from the chickens were not tested because our primary focus was on the need to generate data with edible tissues, i.e., liver and muscle. It is unlikely that this process affected the study results because the samples were being analyzed by those developing/running the method. In several instances, some of the tissues underwent repeated partial thaw/refreeze conditions that may have affected distribution of methylated arsenic and/or inorganic arsenic oxidation states, but should not have any effect on total arsenic in tissues. If analyses of other collected specimens are needed, then they can be processed under a separate study designed for that specific purpose.

Because there were not any methods in place for analysis of either the tissue samples or the medicated feed, it did not seem realistic to collect feed samples for future analyses that may or not be available and performed at some undetermined future date. The feed samples provided to

the analysts were in response to an informal request to provide samples from existing batches of control and medicated feed. As such, the best that could be done was to collect a sample from the top of the feed barrels. Whether or not this minute sample (~50g) was representative of the entire 100 lb batch could not be verified, but since the results (~40 mg/kg) were within 80% of the expected concentration (50 ppm) one can conclude that the feed preparation procedure provided adequate levels of the test article to the test subjects.

Loss of environmental (temperature and humidity) data during the final two weeks of the animal phase was a result of "operator error" on the part of the facilities contractor. However this data was not absolutely necessary and the loss of it during the last two weeks of the animal phase would not be expected to affect the study outcome because the temperature in the specific location was already down to normal room temperature. The data obtained during the early phase of the study confirmed that our room temperature requirements were being met.

Conforming Statement: This study was conducted under the auspices of the GLP's, although certain requirements related to test article sampling and stability testing (LAB-032), equipment qualification and electronic records were not followed: 1) Because of the short duration of the actual animal phase and the lack of an appropriate analytical procedure, a sampling plan and collection of test article mixtures specifically designed for test article stability was not done. 2) Not all the equipment used on this study were "formally" qualified as per installation, operational, or performance criteria, even though they were maintained and calibrated as required by GLP's. Several of the instruments were tested against operational

standards (Agilent ICP-MS and Agilent 1200LC). 3) Electronic records were not always maintained as per 21 CFR Part 11.

DATA ARCHIVAL

All animal care records, assay worksheets, computer-generated reports, spectra, etc. generated under the appropriate analytical SOP's have been identified as raw data. In addition to hard copies of the original data sheets, electronic files generated by computer controlled equipment were transferred to DVD-R media. Interim versions of summary files were used to facilitate the QA audit. Final versions of these data files on DVD media will be submitted along with the final report and its attachments for archiving in the CVM/OR archives, MOD2, Bldg. A, Rm. G606. Tissue samples will be held in storage at -80C in the event additional analyses are performed under different studies.

SUMMARY AND CONCLUSIONS

Control and roxarsone-medicated chicken feed have detectable levels of inorganic arsenic that are higher than the allowed contamination levels of the roxarsone API. In addition the roxarsone medicated premixes contained several unknown arsenical species. Sample preparation extraction procedures used for arsenic speciation analyses, did not provide reproducible recoveries of most arsenic species using incurred samples. Thus, it is difficult to compute mass balance data even though data obtained using spiked samples produce results that approximated near 100%

recoveries. Perhaps this failure to obtain 1:1 correlations with incurred specimens is a result of arsenicals binding to macromolecules *in vivo* that do not pass through the ultrafiltration membranes used in the analyses. The analysts also demonstrated that arsenic species were irreversibly binding to the resin in the chromatographic column. These are just some of several method-related questions that need to be investigated further.

Roxarsone and its metabolites were present in liver tissues from chicks fed roxarsone-medicated feed. The incurred levels of inorganic arsenic species were highly variable in treated chicks but appeared to be significantly greater than that in the untreated control birds. Withdrawal of the medicated feed led to a time-dependent decrease in various arsenic species, many of which were unidentified. Whether or not these unknowns could pose any toxicological risk will be dependent upon their subsequent identification and testing. Due to censoring of inorganic arsenic speciation data that was below the LLOQ, descriptive statistics (Table 2 of Second Revision As Report.doc) and confidence intervals (Table 3 of Second Revision As Report.doc) for the treated birds were estimated using the Kaplan-Meier estimator (Helsel, 2005 and Nelson, 1982). No descriptive statistics for the control birds could be estimated due to the high level of censoring (100%). Despite this, one could conclude that the treated birds were different from the controls at day 5 withdrawal. Total arsenic analysis for both liver and muscle indicated that the mean of measured levels were below the established tolerance values of 2 mg/kg and 0.5 mg/kg. Various arsenic species that were separable by IC analysis of birds from both test groups indicated that several unknowns in the treated birds were significantly above that of the controls and they appeared to follow a depletion pattern that was a function of withdrawal day.

LIST OF ATTACHMENTS

Study Protocol and Amendments:

- Study Protocol (pdf version)
- Study Protocol Amendment #1 (pdf version)
- Study Protocol Amendment #2 (pdf version)
- Study Protocol Amendment #3 (pdf version)
- Study Protocol Amendment #4 (pdf version)

Facilities:

- J51633-1 Std_Tal_L2 Final Report_jck.pdf
- E1-TREND-11-12-2009.xls converted to pdf
- E-1-9-17-09.xls converted to pdf
- E-1 Trend 10-22-09.xls converted to pdf
- E-1-TREND-10-8-09.xls converted to pdf

Test System:

- ChickShippingLabel.pdf
- AnimalHealthStatusVerificationForm_90923.pdf
- Study SOP Deviation Report (pdf version)
- Daily Animal Inspection and Cleaning Records (pdf version)
- Study Protocol Deviation Report (pdf version)
- ContolChickFeed.pdf (pdf version)

- Roxarsone_TestArticleInfo_Annotated.pdf
- Roxarsone_TestArticleInfo&MSDS.pdf
- Preparation of Type A Medicated Feed.pdf
- Necropsy&SampleCollectionProcedure.rtf
- AnimalTerminationList&ScheduleForDays_0_3_5withdrawal.pdf
- DocumentationOfUseOfBirds_jck91110.pdf

Analytical Method and Results:

- Signed Analysts Report with Attachments.pdf (includes Amendment to Report)
- FCC ICP tune SOP.doc (pdf version)
- SOP for ICP-MS analysis for total arsenic.doc (pdf version)
- SOP for Speciation of arsenic compounds related to roxarsone use in chickens.doc (pdf version)
- Chicken liver speciation summary-rev 20110114.xls
- Amendment to Final Report for Study 27530.doc (pdf version)

APPROVALS:

J Hand
Director, Division of Animal Research — 2/14/11 Date

Carol V. Cope
Quality Assurance Officer, CVM — 2/14/11 Date

Director, Office of Research, — 2/18/11 Date

JC Kennedy
Study Director — 18 Feb 2011 Date