Natural Zeranol (α-Zearalanol) in the Urine of Pasture-Fed Animals

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Zeranol and its epimer taleranol have been found in the urine of pasture-fed animals. There were similar distributions in samples from slaughterhouses and in samples from an untreated trial group. Amounts ranged up to 1 ng/mL for deer, goats, and lambs, 2.1 ng/mL for sheep, 13 ng/mL for cattle, and 19 ng/mL for horses. The zeranol and taleranol were naturally present and were most likely derived from *Fusarium* species known to be present in New Zealand pasture. It is not known whether zeranol and taleranol were directly produced by *Fusarium* or produced by reduction of the *Fusarium* metabolite zearalenone inside the animals. For horses and cattle, the amounts found were typical of those expected from treatment with RALGRO, a zeranol-containing implant. The levels found imply that xenobiotic anabolic compounds at physiologically effective levels can occur in domestic animals without deliberate treatment.

Keywords: *Zeranol; anabolic; residue; Fusarium; mycotoxin*

INTRODUCTION

Zeranol is an anabolic agent banned in Europe (European Union, 1981). It is produced by Raney nickel reduction of zearalenone, which is an estrogenic toxin produced by the fungus *Fusarium*. The National Chemical Residue Analytical Laboratory of New Zealand is required to analyze samples taken randomly from various species of domestic animals slaughtered for meat export. Provided they are specially tagged, cattle are permitted to be implanted with RALGRO, a silicone implant that contains up to 36 mg of zeranol. As part of regulatory monitoring of use, urine from randomly selected untagged cattle, sheep, deer, goats, and horses was required to be analyzed for a number of anabolic compounds, including zeranol. Zeranol is excreted as the glucuronide, paired with its 7-hydroxy epimer taleranol (β-zearalanol) (Migdalof et al., 1983; Kim et al., 1986). Similarly, any zearalenone ingested is excreted mainly as glucuronides of α-zearalenol and β-zearalenol.

Laboratory-grown *Fusarium* produces zearalenone as a major metabolite but also produces the single-bond analogues zeranol and taleranol. The alkane to alkene ratios vary widely among *Fusarium* isolates (Richardson et al., 1985). Although zeranol has been found as a natural product, any found in animals has been considered to be of industrial origins. The European Union legislation distinguishes between natural and xenobiotic anabolics, with zeranol grouped among the stilbestrols, trenbolone, and other industrial compounds.

The interactions of the six compounds of interest are given in Figure 1. All six compounds can be metabolites of *Fusarium*. Given the ready reduction of the zearalenone double bond by microorganisms (El-Sharkawy and Abul-Hajj, 1988), it is reasonable to postulate that the double bond could be reduced to a single bond inside an animal. For simplicity, reduction of the double bond from zearalenone to zearalanone is not given as it can be achieved by stepwise reactions. The upper (double bond) trio of compounds is in a process of equilibrium as is the lower (single bond) trio. Reduction of the zearalenols to zeranol and taleranol is assumed to be irreversible. An implant of zeranol should not result in the formation of zearalenols. Until now, the presumption has been that zeranol and taleranol when found in urine constituted evidence of anabolic treatment. There are no previous reports that zeranol was found in untreated animals. Previous studies have not simultaneously measured the two metabolite sets in pasture-fed animals. This work reports the discovery of zeranol naturally present in pasture-fed animals at levels comparable to treated animals. The discovery was originally made in routine surveillance of slaugh-

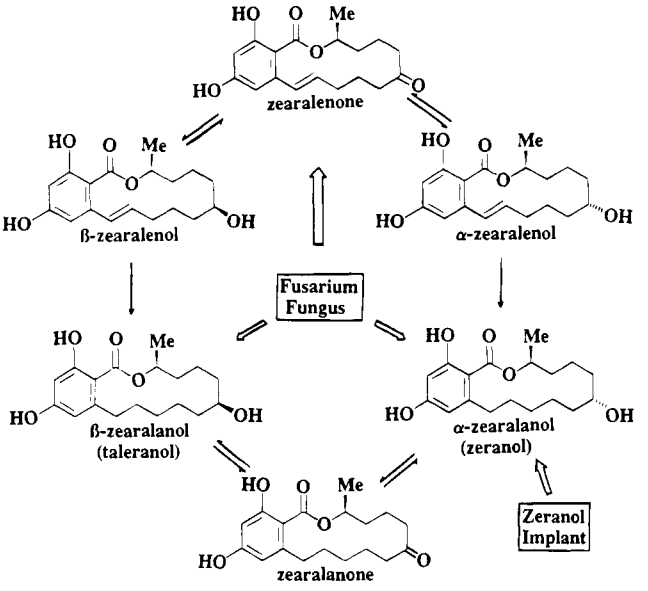

Figure 1. Proposed relationship between the routes that lead to zeranol in the urine of an animal. Zeranol may be directly implanted, ingested as a metabolite of *Fusarium*, or produced in the animal from other metabolites. Only essential conversions are shown. Alcohol–ketone equilibration is rapid compared to the reduction of the double bond.

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terhouse animals and confirmed in untreated control rams of a university trial flock.

MATERIALS AND METHODS

Reagents. Glucuronidase was from Sigma Chemicals (Helix Pomatia G-0751); zeranol, taleranol, and an internal standard zearalane were gifts from International Minerals Corp., Terre Haute. α-Zearalenol and β-zearalenol were from Sigma Chemicals (Z-0166 and Z-2000), and MSTFA was from Pierce Chemical (48910). Hexane, *tert*-butyl methyl ether (tBME), methanol, and acetic acid were of analytical grade.

Assay Sets. Two sets of urines were simultaneously analyzed for zeranol, taleranol, α-zearalenol, and β-zearalenol. The "regulatory" set comprised 729 urine samples tested between May 1992 and March 1993 for export certification of nontreatment with anabolic substances. Sampling was required to be random throughout the day.

The other set comprised samples from an implant trial at Massey University designed to determine the ability of an analytical laboratory to detect illegitimate anabolic treatment of rams. A test group of 10 rams, born September 1991, were pasture fed over the period May 1992 to March 1993. Urine samples were repeatedly taken, generally mid-morning. Seven rams were not treated with zeranol and were readily distinguished on analysis from three rams treated with RALGRO (36 mg of zeranol). The results for the treated rams will be reported in detail elsewhere. The set of untreated samples reported here constitutes urines from any ram before implants were inserted and from non-zeranol-treated rams thereafter.

Sample Treatment. Urine samples were frozen on collection, shipped to the laboratory by overnight courier, and refrozen until analyzed. Aliquots (5 mL) were analyzed by glucuronidase re-formation of the alcohols, extraction with hexane/tBME (70:30), and then HPLC cleanup of the extracts with programmed fraction collection. A fraction was evaporated under nitrogen, then trimethylsilyl derivatized with MSTFA, and subjected to GC–MS analysis.

Chromatography. The normal phase HPLC separation used a cyanopropyl Whatman PAC column (4.6 mm × 100 mm) in a Varian Vista 5500 HPLC, isocratically pumping hexane/tBME/methanol/acetic acid (695:250:50:5) at 0.9 mL/min. An azo dye (phenylazohomovanillyl alcohol) was used to monitor for drift in retention times. Samples were automatically injected by a Shimadzu SCL-6B\SIL-6B and fractions collected by a Pharmacia FRAC-300.

Residue quantitation used a Hewlett-Packard 5890 gas chromatograph interfaced to a low-resolution mass selective detector (MSD 5970). Output from the GC–MS was downloaded to the statistical package Minitab, via Pascal programs written by one of the authors. Zeranol or taleranol was deemed present in a sample only if a m/z 433 ion peak was found within 2 s of the expected retention time (ca. 20 min) and only if a second confirming peak was present—either the parent ion at m/z 538 or else the m/z 433 ion of the other epimer. The maximum deviation limit of 2 s was also applied to the zearalenols, but the only ion monitored was the parent at m/z 536. A positive urine usually had all four compounds, giving six ion peaks in all, with well-defined time differences.

Figure 2 is a set of GC–MS traces over the GC retention period in which the four compounds elute. The sample was urine from an export certified steer. Zeranol and taleranol were clearly present at the parent mass 538 and fragment mass 433, and the zearalenols were present at the parent mass 536 (and an isotope at 538) and a fragment ion at 433. Sensitivity is excellent even for the peaks below 1 ng/mL, and all peaks are clearly resolved.

The full method, which targets 10 compounds, has been supplied to U.S. Department of Agriculture and to European Union officials and may be requested from the authors. The assay has been designed for regulatory use, emphasizing identification over quantitation. Residue levels of RALGRO zeranol were expected to be about 1 ng/mL in urine after the standard withdrawal period of 65 days (Dixon and Russell, 1983; Dixon et al., 1986). The European Union requires assays to be able to detect down to 2 ng/mL and preferably lower.

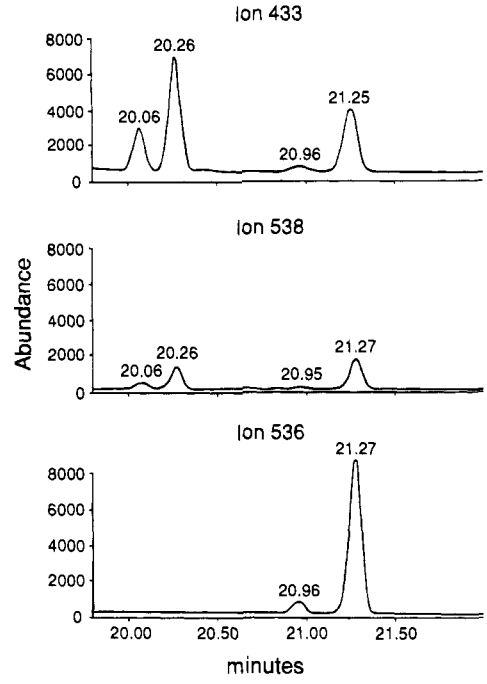

Figure 2. Set of GC–MS chromatographs for an export certified steer. Peaks are 0.51 ng/mL of zeranol at 20.06 min, 0.79 ng/mL of taleranol at 20.26 min, 0.86 ng/mL of α-zearalenol at 20.96 min, and 4.60 ng/mL of β-zearalenol at 21.25 min. Generally, these ions are free of interference, even for urine from pasture-fed animals.

Table 1. Mean and Maximum Quantities of Zearalanols (ZAL) and Zearalenols (ZEL) Found in the Urine from the Survey of Export Animals (Sorted by Percent Positive for ZAL)

			ZAL (ng/mL)		ZEL (ng/mL)	
species	no.	% pos	mean	max	mean	max
goats	27	33	0.08	0.56	3.2	19
deer	41	34	0.07	0.94	2.1	15
lambs	90	42	0.07	0.77	2.5	34
sheep	80	49	0.11	2.1	2.7	86
horses	76	62	0.54	18.8	86.0	2157
cattle	415	68	0.58	12.3	6.7	163

Analytical batches had 25–40 samples and three recoveries ranging from 1.0 to 10 ng/mL. GC–MS response was linear over that range, and linear extrapolation was assumed for all quantitation.

RESULTS

ZAL and ZEL. The equilibration of the epimeric alcohols requires that each trio of single-bond and total double-bond compounds must be treated together. (Bottom trio versus top trio in Figure 1.) To a good approximation, only the four alcohols need be considered. The ketones were found to be a trivial component in urine excretion and were not routinely analyzed. ZAL is defined here as the sum of the epimer pair zeranol and taleranol, and ZEL the sum (α + β) of zearalenol epimers, in units of ng/mL. [Terminology is based on "A" for alkane and "E" for alkene and a terminal "L" for alcohol. Towers (1992) similarly referred to zearalenone as ZEN.]

Regulatory Analysis Set. Of 729 urine samples tested for export certification, 59% were found positive for ZAL and 89% positive for ZEL. Table 1 is a list of the numbers tested for each species, percentage found positive for ZAL, and the mean and maximum ZAL and ZEL found. As many results were near the limit of

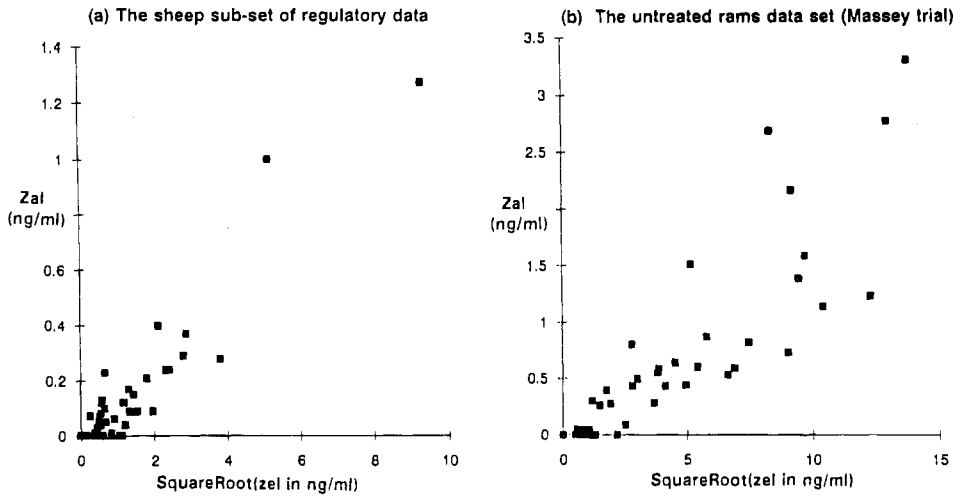

Figure 3. Plot of zearalanols (ZAL) against the square root of the zearalenols (ZEL) for two ovine groups.

Table 2. Number of Animals in the Export Group Found with Specified Ranges of Zearalanols and Zearalenols (Total of 729 Assays)

range (ng/mL)	ZAL	ZEL
0.000–0.001	301	82
0.001–0.25	217	126
0.25–0.50	72	77
0.50–1.0	56	81
1.0–2.0	28	84
2.0–5.0	34	75
5.0–10.0	5	51
10.0–20.0	2	54
20.0–100.0	0	47
above 100.0	0	12
also positive[a]	14	40
% positive	58.7	88.8

[a] "Also positive" refers to assays in which instrument drift resulted in taleranol and β-zearalenol not being measured. The samples were positive but only partly quantitated.

detection, there is some correlation between percent positive and mean ZAL. The larger animals, cattle and horses, had higher ZAL values. Horses were particularly likely to have high ZEL values, whereas cattle had a higher ratio of ZAL to ZEL.

On graphing ZAL versus ZEL, a clear pattern was observable, with low ZEL always implying low ZAL. In only 1% of cases was ZAL greater than ZEL, with a maximum excess of 0.07 ng/mL. But there were differences between the species. Deer and goats both had low ZAL levels with a somewhat scattered plot. Lamb data showed an even stronger scatter plot, some points typical of data for adult sheep and others with very low ZAL to ZEL ratios. The least scatter for a wide range of values was found for adult sheep. Regression gave $\log(\text{ZAL}) = (0.51 \pm 0.07) \times \log(\text{ZEL}) + \text{constant}$. This suggested that ZAL was proportional to the square root of ZEL. Figure 3a is a plot of ZAL versus the square root of ZEL for this set.

Regression of ZAL against the square root of ZEL for each species produced slopes that clustered into two groups. Cattle (steer, bull, dairy, and beef cow) had a slope near 0.5, and all other species had slopes near 0.15. That is, cattle were distinguished by being more likely to excrete a higher ratio of single bond (ZAL) components to double bond (ZEL) components.

Table 2 gives the amounts found summed over all species for a series of ranges. The majority of ZAL levels were below 2 ng/mL (the European assay performance mark). The distribution is skewed, with few high values and many blanks and low positives.

The seasonal data will be reported in detail at a later date, after trace-back of samples to slaughter date. Briefly, samples were analyzed at a constant rate throughout the year, generally within a month of slaughter. The pattern found was a rise in the occurrence of all four compounds in January (summer), and then the majority of samples were positive for all four compounds until levels dropped in August (mid-winter). The levels stayed low from September through December.

Ram Implant Trial. A plot of ZAL versus the square root of ZEL is given in Figure 3b. Regression of the 43 points gave a slope of 0.16 ± 0.01, and the intercept was not significant. This was similar to that found for the regulatory sheep set (0.12 ± 0.01).

The samples taken before August had a mean ZEL value of 35 ng/mL and then values dropped sharply, and those from August to December had a mean of 2.4 ng/mL. Sampling frequency decreased in the final stages of the trial, but the mean value had increased again to 36 ng/mL by the following March (when the trial ended).

DISCUSSION

New Zealand is an isolated country, and the sole source of industrial zeranol is from importation by a single company. Veterinary control of anabolic use is strict. In general, farmers comply with required drug practice, and few positives were expected. When zeranol was first found in untagged randomly sampled animals in August 1991, it was thought to be due to illegal untagged treatment. The levels found were similar to those expected from RALGRO treatment. The presumption of treatment was not substantiated by field officer visits to the farmers, and was furthered weakened by subsequent discoveries of zeranol in sheep and goats, which were uneconomic to implant nor had any been legitimately implanted. Initially, zearalenols were not part of the assay. Given the potential *Fusarium* link, the assay was extended to include the zearalenols, which were immediately found (October 1991). The four alcohol metabolites always occurred as a group. Assay sensitivity was increased and the percentage of positives found exceeded any plausible scenario of misuse. The positive Massey ram samples, where the controls were known not to be treated, finally proved that the observed ZAL had to arise from a natural source.

The ZAL positives followed the seasonal pattern of zearalenone production by *Fusarium* in pasture (Towers, 1992). The highest levels of zeranol in sheep urine correspond to an excretion rate of about 0.01 mg/day. As pasture intake of zearalenone is of the order of 1 mg/day for a sheep in late summer (in the worst affected areas), the ready explanation is that ZAL is directly or indirectly derived from ingested *Fusarium* metabolites.

Somewhat artificially, the source of ZAL can be postulated as either arising solely from the ingestion of zeranol and taleranol, or else solely from transformation within the animal of a precursor metabolite. Any complete explanation has to account for the three characteristics observed, namely the existence of ZAL in season at about 1 ng/mL levels, the apparent square root dependency of ZAL on ZEL, and the variation in ZAL and ZEL from species to species. At present, both the intrinsic and extrinsic explanations are valid.

Hypothesis 1: Extrinsic Source of ZAL. Zearalanols have been found in laboratory rice cultures of *Fusarium*, where cultures that were weak producers tended to a higher ratio of ZAL to ZEL (Richardson et al., 1985). In particular, ZAL was 17% of ZEL (by weight) for *F. culmorum*, a species prevalent in New Zealand pasture (di Menna et al., 1987, 1991). This is sufficient to explain all the ZAL found. The apparent square root function was unexpected, in that ZAL would have been expected to be a fixed fraction of ZEL. One explanation is that the instances of high ZEL levels are associated not with heavier infestations of *F. culmorum* but with *Fusarium* species that are strong producers of zearalenone. As these happen to be weak ZAL producers, a nonlinear curve results.

The variation in ZEL residues observed between animal species possibly reflects the grazing practices for those species. Sheep, deer, and goats nip grass closely, which results in less grass forming dead litter and possibly a lower zearalenone loading. Cattle pull out longer material, and would consequently have more *Fusarium* metabolites ingested per kilogram of dry matter. This explanation is dependent on dead litter being an enhanced source of zearalenone. di Menna et al. (1991) found green material a poorer source than bulk or dead material, which were similar. There have been no studies on the zeranol and taleranol content of pasture. The rate of ingestion of anabolic compounds is open only to conjecture, and research is needed to establish likely intake levels for various species under typical pasture and weather conditions. Horses were found to be peculiar, having a low ratio of ZAL to ZEL, but produced the highest ZEL levels. Unlike the other species which are rarely other than pasture fed in New Zealand, horses are supplemented with hay and cereals. This may increase the fungal metabolite load.

Hypothesis 2: Intrinsic Source of ZAL. Alternatively, the three characteristics can be ascribed to processes occurring within an animal—digestive or liver reduction of the double bond following ingestion of zearalenone. Studies of microbial transformations of zearalenone have found metabolites with a reduced double bond, and most of those microbes also reduced the carbonyl (El-Sharkawy and Abul-Hajj, 1988). It is quite reasonable to propose that zearalenone is reduced to zeranol and taleranol in the rumen, although reduction in the liver is also a possibility.

The outstanding group are the cattle with a high ZAL to ZEL ratio. Reduction in the rumen explains the presence of ZALs. The monogastric horses can be expected to have a lower ZAL to ZEL ratio than the similarly sized ruminant cattle. The smaller animals with a smaller volume to digestive surface area can be expected to have a faster uptake of low molecular weight substances, which does not allow as much time for the slow ZEL to ZAL reduction. The digestive process argument gives a plausible explanation of interspecies variation.

Within a species, the apparent square root function arises because the enzymic sites are limited, and overloading the capacity allows unchanged zearalenone to be absorbed before reduction can take place. The single variable square root function is an adequate descriptor of the rather scattered data but does fail to properly describe the tendency to a straight line at low zearalenol levels. The true function may be something closer to the Michaelis equation. The total ZAL plus ZEL loading is dependent still on extrinsic factors, which is why the supplemented feed horses have a low ratio but high levels.

Conclusions. Zeranol and taleranol occur naturally, and in the appropriate season are at levels similar to those expected from treating animals. Both the intrinsic or the extrinsic explanations qualitatively describe the results, and further research will be required to define the contributions each make.

Cattle and horses were strong excretors of ZAL. It seems reasonable therefore that the naturally occurring zeranol and taleranol had an anabolic effect on the animals, with the co-ingested zearalenols possibly modifying the effects predicted on the basis of a pure zeranol implant. If the intrinsic model is correct, cattle are efficient converters of zearalenone to zeranol and taleranol, and a combination of supplementary food (as for horses) given to cattle should result in strong xenobiotic anabolic stimulated growth. The observation of "natural zeranol" has more than a regulatory impact. Natural xenobiotic anabolic compounds may be an important component of animal growth.

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