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Prevalence of zeranol, taleranol and *Fusarium* spp. toxins in urine: implications for the control of zeranol abuse in the European Union

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There is currently little information concerning the prevalence of zeranol and taleranol in animal urine following metabolism of the naturally occurring Fusarium *spp. toxins. An epidemiological study is described which involves four European Union control laboratories in which 8008 urine samples were screened for the presence of zeranol using a time-resolved fluoroimmunoassay (TR-FIA). Of these samples, 93.6% screened negative for zeranol. All samples testing positive for zeranol were then analysed with a confirmatory method. Based on the confirmatory results, the TR-FIA-positive samples were then categorized as false-positive, true-positive or 'equivocal' (zeranol/taleranol and the* Fusarium *spp. toxins detected). The true-positive samples represented only 0.05% of the total number of samples (n = 4). After statistical analysis, 170 of 174 equivocal samples proved to belong to a 'normal' population in which the amount of zeranol/taleranol could be related to the total amount of* Fusarium *spp. toxins through a linear regression with a 99% prediction interval. This suggested that the presence of zeranol in these samples might be due to in vivo metabolism of the* Fusarium *spp. toxins. The presence of zeranol in the four remaining 'outliers' might be attributable to zeranol abuse rather than to natural contamination. The results are of interest for control laboratories as they might provide an analytical tool to help distinguish between abuse and natural contamination in zeranol testing.*

Keywords: zeranol, zearalenol, *Fusarium*, epidemiology, analytical criteria

Introduction

Zeranol (α-zearalanol) is a non-steroidal oestrogenic growth promoter which increases live-weight gain in food animals. Its administration has been banned within the European Union (EU) (Council Directive 1996a) and Member States are required to monitor food-producing animals for possible abuse (Council Directive 1996b). Zeranol is a semi-synthetic product derived from the naturally occurring mycotoxin zearalenone and it was recently shown in reports from New Zealand (Erasmuson *et al.* 1994, Miles *et al.* 1996) and Northern Ireland (Kennedy *et al.* 1995, 1998) that it might occur naturally in urine and bile from sheep and in cattle following metabolism of the mycotoxins zearalenone and α-zearalenol, which can contaminate animal feedstuffs. Thus, the finding of zeranol in an animal might, on its own, be insufficient proof that malicious abuse of zeranol has occurred. It is therefore necessary to establish quantitative criteria to distinguish zeranol abuse from environmental contamination with *Fusarium* spp. toxins.

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The European Commission (EC) funded a shared-cost research and development project entitled 'Natural Zeranol' (FAIR5-CT97-3443) that aimed to improve reagents and procedures for the implementation of Community policy on the prohibition of the use of zeranol as an anabolic agent in food animals. Most EU control laboratories use commercially available immunoassay kits to screen for the presence of zeranol. However, all these zeranol test kits exhibit cross-reactivity, to a greater or lesser extent, with the *Fusarium* spp. toxins (Cooper *et al.* 2003). As part of this project, a sensitive time-resolved fluoroimmunoassay (TR-FIA) method was developed and validated for the screening of zeranol itself without interference of the *Fusarium* spp. toxins (Tuomola *et al.* 2002, Cooper *et al.* 2002). Furthermore, as part of this project, two different confirmatory methods for zeranol and the *Fusarium* spp. toxins, one based on gas chromatography-mass spectrometry (GC-MS) (Blokland *et al.* 2004) and another one based on liquid chromatography coupled with tandem mass spectrometry detection (LC-MS-MS) (Launay *et al.* 2004) were developed according to EU guidelines (Commission Decision 2002).

However, the development of these screening and confirmatory methods is insufficient to distinguish zeranol abuse from environmental contamination with *Fusarium* spp. toxins. Therefore, the 'Natural Zeranol' project not only aimed to develop screening and confirmatory methods, but also tried to correlate mycotoxin levels with levels of zeranol and taleranol in order to determine tentative criteria to distinguish between zeranol abuse and natural contamination with *Fusarium* spp. toxins. Kennedy *et al.* (1998) had already suggested that the simultaneous determination of zeranol, taleranol and the *Fusarium* spp. toxins might provide such criteria.

To the best of the present authors' knowledge, there is little information concerning the prevalence of the interference caused by *Fusarium* spp. toxins in zeranol testing. The present paper reports the results gathered during a major survey conducted in four EU Member States and an epidemiological study conducted as part of the 'Natural Zeranol' project. Four different laboratories (National Agricultural Research Foundation (NAGREF), Thessaloniki, Greece; Laboratorio Nacional de Investigacao Veterinaria (LNIV), Lisbon, Portugal; The National Food Administration (NFA), Uppsala, Sweden; and Department of Veterinary Science, Queen's University Belfast (QUB), Belfast, UK) were involved in this study. Each laboratory collected approximately 2000 urine samples and analysed them using the zeranol-specific TR-FIA mentioned above. All samples testing positive for zeranol were then sent to European Union Community Reference Laboratory, National Institute of Public Health and Environment (RIVM), Bilthoven, the Netherlands, or QUB and analysed for zeranol, taleranol and the *Fusarium* spp. toxins with one of the two confirmatory methods mentioned above. The data generated by the survey were used to investigate a possible correlation between the amount of zeranol and the concentrations of the *Fusarium* spp. toxins in bovine urine and determine tentative criteria whereby zeranol abuse and natural contamination with *Fusarium* spp. toxins may be distinguished.

Materials and methods

Materials and reagents

All solvents were of high-performance liquid chromatography (HPLC) grade and other chemicals were of analytical reagent grade. All chemicals, unless otherwise stated, were supplied by Sigma Co. (Poole, UK). Distilled or de-ionized water was used throughout this study. For TR-FIA measurements, DELFIA Wash solution and DELFIA Enhancement solution were obtained from Perkin-Elmer Life Sciences (Wallac, Turku, Finland). The TR-FIA plates were purchased from InnoTrac Diagnostics Oy (Turku, Finland). For GC-MS and LC-MS-MS measurements, deuterium-labelled internal standards, d_4-zeranol, d_4-taleranol, d_4-α-zearalenol and d_4-β-zearalenol, were obtained from RIVM.

Urine sample collection

A total of 8008 urine samples were collected during this study (NAGREF, 2024; NFA, 1996; LNIV, 2001; QUB, 1987). Most samples were of bovine origin (7852). However, some samples were also of ovine (NAGREF, 68), porcine (NAGREF, 78) and caprine (NAGREF, 10) origin. The samples were taken randomly throughout the country/region concerned and were collected evenly throughout a calendar year. All samples were stored frozen at −18°C in the dark.

Screening method

All the samples collected were screened for zeranol in the laboratory of the country of origin. Immunoaffinity columns (IAC) were used for the extraction of zeranol from urine samples. To ensure homogeneity of the results, the IAC were prepared in the UK laboratory according to the method described by Tuomola *et al.* (2002) and were dispatched to the three other partners. A single set of 20 IAC was sent to each participating laboratory. One set was used for the analysis of all samples in each laboratory. The sample preparation and TR-FIA protocol used were the same in the four countries and have already been described by Tuomola *et al.* (2002) and Cooper *et al.* (2002). For the same reasons as for the IAC columns, the standards used for the TR-FIA were prepared in the UK laboratory and subsequently sent to the other participants. In the four participating laboratories, the europium time-resolved fluorescence was measured using a Victor™ 1420 multilabel counter with Wallac 1420 workstation software and MultiCalc online data reduction software supplied by Perkin Elmer Life Sciences.

Confirmatory methods

All samples testing positive for zeranol with the screening method were then referred to RIVM or QUB for confirmatory analysis. The samples were analysed for zeranol (and its epimer taleranol) and the *Fusarium* spp. toxins (α-zearalenol, β-zearalenol and zearalenone) using a GC-MS method (RIVM) or an LC-MS-MS method (QUB). These two methods are being published elsewhere (Blokland *et al.* 2004, Launay *et al.* 2004). The sample preparation was similar for both methods. Briefly, the urine sample (5 ml) was buffered with acetate buffer (pH 5.2) and deconjugated with β-glucuronidase from *Helix pomatia* at 37°C for 2 h. After liquid/liquid extraction, the extract was cleaned-up using successively C18 and amino solid-phase extraction columns (Varian, Harbor City, CA, USA).

For GC-MS measurement, the extract was then derivatized with heptafluorobutyric acid anhydride (Pierce, Rockford, IL, USA). The analysis was performed on a Hewlett Packard 5890 series II gas chromatograph equipped with a Hewlett Packard 7673 autosampler and a 5989A Mass Spectrometer (type Engine). The column used was a fused silica capillary column HP-1 (12.5 m × 0.20 mm × 0.33 μm). The detection limit for all compounds was 0.5 ng ml^{-1}.

For LC-MS-MS analysis, the extract was dissolved in water:methanol 50 : 50 (v/v) before injection. Analysis was performed using a Quattro LC (Micromass, Wythenshawe, UK) operated in the negative-ion mode. A Hewlett Packard (Stockport, UK) HPLC system, comprising an 1100 Series binary pump, autosampler and solvent degasser, was coupled via an electrospray interface to the Quattro LC. The LC column used was a Luna 3 μm C_{18} 150 × 4.6 mm (Phenomenex, Macclesfield, UK) and the mobile phase was a mixture of methanol:water (84 : 16 v/v). The detection limit for all compounds was approximately 0.5 ng ml^{-1}. A correlation between the two methods has been described (Launay *et al.* 2004).

Interpretation of results

After confirmatory analysis, screening test positives were then categorized as follows:

- False-positive: *Fusarium* spp. toxins present or absent, zeranol (or taleranol) absent.
- 'Equivocal': *Fusarium* spp. toxins present, zeranol (or taleranol) present.
- True-positive: *Fusarium* spp. toxins absent, zeranol (or taleranol) present.

The data generated by this survey were used to investigate a possible correlation between the amount of zeranol and the concentrations of the *Fusarium* spp. toxins in urine. This was done by statistical analysis of the 'equivocal' screening tests. The aim was to find possible criteria in order to classify equivocal screening test results as either 'positive-zeranol abuse' or 'negative-environmental contamination'.

Results and discussion

Overall screening results

The epidemiological study presented here was carried out as part of the 'Natural Zeranol' project

(FAIR5-CT97-3443) and, therefore, used the TR-FIA assay developed as part of this project. Before commencing this extensive survey, the application of this new assay in different laboratory environments was validated by an interlaboratory ring test in which the four participants in the epidemiological study were involved (Cooper *et al.* 2003). Furthermore, the improved specificity of the zeranol TR-FIA alongside comparable commercially available enzyme immunoassay test kits was demonstrated elsewhere (Cooper *et al.* 2003) and it supported the use of the TR-FIA in order to minimize false-positive screening test results during this study. The overall screening results are shown in table 1. Using the screening test developed within the 'Natural Zeranol' project, the overall incidence of positive samples is fairly low and most samples (93.6%) are negative for zeranol. However, these results do not allow discrimination between natural contamination and zeranol abuse for the samples which screened positive for zeranol. The samples were collected evenly throughout a calendar year in order to assess the impact of seasonality on the number of screening test positive samples. However, this study did not yield any significant results (data not shown) and it was therefore concluded that seasonality was not a factor in the incidence of screening test positive samples.

Confirmatory results

The two confirmatory methods used during this survey were validated as qualitative and quantitative confirmatory methods according to EU guidelines (Commission Decision 2002). Furthermore, 28 bovine urine samples of unknown concentration were selected and analysed using both methods. The results of this study, published elsewhere (Launay *et al.* 2004), showed a very good agreement between the two methods both from a qualitative and a quantitative point of view for all the analytes of interest for this survey, ensuring a good homogeneity of the results of the epidemiological study presented here. Unfortunately, of the 510 samples that screened positive for zeranol, 49 samples could not be analysed with a confirmatory method, either because insufficient material was available for a confirmatory test or because of destruction of the samples following a freezer failure. Thus, 461 screening test positives were analysed with at least one of the two confirmatory methods. After confirmation, as already mentioned above, samples were categorized as true-positive, false-positive or equivocal. The results are shown in table 2. A small majority of screening test positives were categorized as false-positive. However, the proportion of false-positives remains small (3.5%) when compared with the total number of samples screened

Table 1. Summary of the zeranol TR-FIA screening results following analysis of 8008 urine samples.

	Country 1	Country 2	Country 3	Country 4	Total
Samples screened	1987	2001	1996	2024	8008
Samples positive	70	150	94	196*	510
Percentage	3.5	7.5	4.7	9.7	6.4

*Including one porcine and four ovine samples.

Table 2. Summary of the confirmatory results following the mass spectrometric confirmation of 461 samples which screened positive for zeranol using the zeranol TR-FIA.

	Country 1	Country 2	Country 3	Country 4	Total
Samples tested	65	146	94	156	461
False-positive	23 (35.4%)	84 (57.5%)	82 (87.2%)	94* (60.3%)	283* (61.4%)
Equivocal	42 (64.6%)	61 (41.8%)	11 (11.7%)	60 (38.5%)	174 (37.7%)
True-positive	0	1 (0.7%)	1 (1.1%)	2 (1.2%)	4 (0.9%)

*Including one porcine sample

during this study. Furthermore, in a large majority of these samples, no *Fusarium* spp. toxins were detected during confirmatory analysis, suggesting that these false-positive did not arise from *Fusarium* spp. toxins cross-reactivity but rather from matrix interferences. The number of true-positive samples corresponding to the samples for which the detection of zeranol/taleranol unequivocally arose from abuse was very low ($n = 4$, 0.05% of the total number of samples screened) and was in agreement with what was expected by the participants due to their previous experience in zeranol testing as control laboratories. The number of equivocal samples represented 2.2% of the total number of samples screened during the survey. This population of samples is the most interesting as the presence of zeranol (and/or taleranol) in those samples was confirmed along with *Fusarium* spp. toxins. As a consequence, it is currently impossible to determine whether the presence of zeranol (and/or taleranol) arose from abuse or from environmental contamination. Therefore, a possible correlation between the amount of zeranol/taleranol and the concentrations of the *Fusarium* spp. toxins in these equivocal samples was investigated as part of this study in order to propose tentative analytical criteria to distinguish between zeranol abuse and environmental contamination.

Statistical analysis

A linear regression was carried out relating zeranol and taleranol concentrations (response variable) against *Fusarium* spp. toxin concentration (explanatory variable) for the samples belonging to the equivocal population. Both the response and explanatory variables were subject to logarithmic (base 10) transformation. The resulting regression parameters and associated standard errors generated permitted the calculation of a 99% prediction interval for the estimated values of the response variable to any given value of the explanatory variable (McConway *et al.* 1999). This permitted a determination of whether or not the observed value for the response variable fell in this range. To calculate a prediction interval the following equation was used:

$$\alpha + \beta x_0 \pm t(\text{d.f.}) \sqrt{s^2 \left(\frac{(x_0 - \bar{x})^2}{s_{xx}} + \frac{1}{n} + 1 \right)},$$

where n is the number of observations and t(d.f.) is the desired percentage point from the t-distribution. Thus, for a prediction interval of, for example, 99% and an x value of x_0, the above equation will give two values between which we are 99% confident that the true y value should lie for this x value.

To visualize more easily the results of this statistical analysis, the samples belonging to the equivocal population were plotted (figure 1). The $\log_{10}$ of the explanatory variable (*Fusarium* spp. toxins concentration) was plotted on the x-axis and the $\log_{10}$ of the response variable (zeranol and taleranol concentration) on the y-axis. Four samples, all of which came from Country 4 (solid symbols), had a response variable which fell outside the 99% prediction interval (in each case: $p < 0.0001$) for the estimated value of the response variable (given the value of their explanatory variable), and are referred to hereafter as 'outliers'. The remaining 170 samples (open symbols) had a response variable which fell inside the 99% prediction interval for the estimated value of the response variable (given the value of their explanatory variable). To facilitate the discussion, this population will be referred to hereafter as the 'normal population'. The linear regression parameters relating to the normal population, that may be used for determining, with a specified confidence interval, whether or not an individual sample belongs to that normal population, are shown in table 3.

It must be emphasized that the finding of a sample that lies outside the prediction interval does not provide absolute proof that zeranol abuse has occurred. However, it does indicate that the sample belongs, with a measurable degree of certainty, to a non-normal population. Similarly, the finding of a sample that lies within the prediction interval does not constitute proof that zeranol has not been deliberately administered to the animal from which the sample was taken. However, it does indicate that the sample is indistinguishable from that which has been termed here the 'normal population'. Field reports of zeranol abuse in the EU are comparatively rare. In Northern Ireland, for example, zeranol implant pellets have not been found during post mortem meat inspection of suspect animals for more than 10 years. Zeranol is generally not the 'anabolic agent of choice' for farmers wishing illegally to promote the growth of their animals. Drugs such as the β-agonists are much more effective growth promoters and have been much more widely abused in the EU and elsewhere. The balance of probabilities

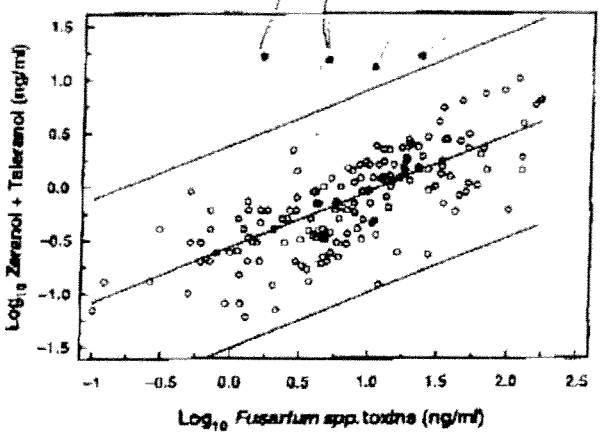

Figure 1. Samples (n = 174) belonging to the 'equivocal' population which contained zeranol and taleranol in addition to the Fusarium *spp. toxins. The samples (n = 4) represented by a solid circle fell outside the 99% confidence interval, i.e. they are 'outliers'. The remaining samples (n = 170) fell*

Table 3. Linear regression parameters relating to the 170 samples belonging to the 'normal population'.

Parameter	Symbol	Value
Mean X	$\bar{x}$	0.8733
Intercept	α	−0.6000
Slope	β	0.5318
Degrees of freedom	d.f.	168
Sum $((X-\text{mean } X)^2)$	s_{xx}	69.6738
$(Y-\text{predicted } Y)^2$/d.f.	s^2	0.0916

suggests, therefore, that the zeranol and taleranol occurring in samples classified as belonging to the normal population is present as a result of metabolism of dietary *Fusarium* spp. toxins.

Unlike the samples belonging to the normal population, the outliers fell outside the 99% prediction interval. Furthermore, as shown in table 4, the concentration of zeranol and taleranol is generally higher than the total concentration of the *Fusarium* spp. toxins. Kennedy *et al.* (1998) have suggested that in contaminated samples, the total concentration of *Fusarium* spp. toxins is much higher than the concentration of zeranol and taleranol. Thus, it is very unlikely that the presence of zeranol and taleranol arises from the metabolism of the *Fusarium* spp. toxins only. Therefore, it is suggested here that the presence of zeranol and taleranol in these four samples did not arise from natural contamination but rather may have arisen from zeranol abuse.

Conclusion

A major survey was carried out as part of the project 'Natural Zeranol' (FAIR5-CT-1997-3443). Four con-

Table 4. Concentrations (ng ml^{-1}) of the different metabolites for the four outliers (Country 4), all of which fell outside the normal population ($p < 0.0001$).

	Zeranol	Taleranol	α-Zearalenol	β-Zearalenol	Zearalenone
Sample 1	3.6	9.3	1.9	4.8	3.6
Sample 2	4.6	10.7	0.9	3.8	0
Sample 3	5.1	11.3	0.8	0.8	0
Sample 4	6.3	10.2	2	16	2.7

trol laboratories from four different EU countries collected each approximately 2000 urine samples and tested them for zeranol using a TR-FIA immunoassay. A total of 93.6% of all samples screened negative for zeranol. All samples found positive were then re-tested using a confirmatory method based either on GC-MS or on LC-MS-MS. After confirmatory analysis, screening test positives were then categorized as false-positive, true-positive or equivocal. A total of 2.2% of the samples surveyed during this study proved to be equivocal and 0.05% of the samples proved to be true-positive. The equivocal samples were then subjected to statistical analysis. In four of the 174 equivocal samples (all of which came from Country 4), the lack of correlation between the concentration of zeranol/taleranol and the total concentration of the *Fusarium* spp. toxins coupled with the fact that zeranol/taleranol was found at relatively high concentrations suggested that the presence of zeranol/taleranol arose from abuse rather than from environmental contamination. Of the remaining 170 samples, termed the 'normal population', the presence of zeranol/taleranol in these samples probably arose from natural contamination rather than from abuse. These results are important for control laboratories as they might provide an analytical tool to help distinguish between abuse and natural contamination in national residue control schemes.

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Log10 Zearenol + Taierenol (ng/ml)
1.5
1.0
0.5
0.0
−0.5
−1.0
−1.5
−1
−0.5
0.0
0.5
1.0
1.5
2.0
2.5
Log10 Fusarium spp. toxins (ng/ml)