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SENSITIVE ANALYSIS OF THE MYCOTOXIN ZEARALENONE AND ITS METABOLITES IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY[a]

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SUMMARY

A method is described for the analysis of zearalenone and its metabolites, α- and β-zearalenol, in small volumes (0 5-2 0 ml) of biological fluids including milk, blood, plasma, urine and bile, using high-performance liquid chromatography with fluorescence detection Isolation of the toxins from biological fluids was achieved using a series of pH-controlled solvent extractions Detection limits for zearalenone and α-zearalenol were 1 ng/ml, and for β-zearalenol ca 5 ng/ml, both at a signal-to-noise ratio of 3 In bile, however, the detection was ca five times less sensitive owing to interfering substances Recoveries at low ng/ml concentrations were highest from urine (87-94%) and plasma (85-93%), slightly lower from whole blood (78-88%) and milk (75-84%), and lowest from bile (66-77%)

INTRODUCTION

Zearalenone (ZEN) (Fig 1) is a potent estrogenic metabolite produced by several *Fusarium* species and found to be a common and widespread contaminant in cereal grains and animal feedstuffs [1-4]. Various health problems associated with this mycotoxin have been well documented in domestic animals, including decreased feed intake, infertility, vaginal prolapse and enlargement of the uterus and mammary glands [5-12]. Furthermore, ZEN has been reported to be potentially carcinogenic [13] It has also been suggested that ZEN was a contributing factor in cases of precocious sexual development of human infants in Puerto Rico [14] Consequently, there is increasing concern about the potential risk of ZEN to humans, not only from direct exposure to

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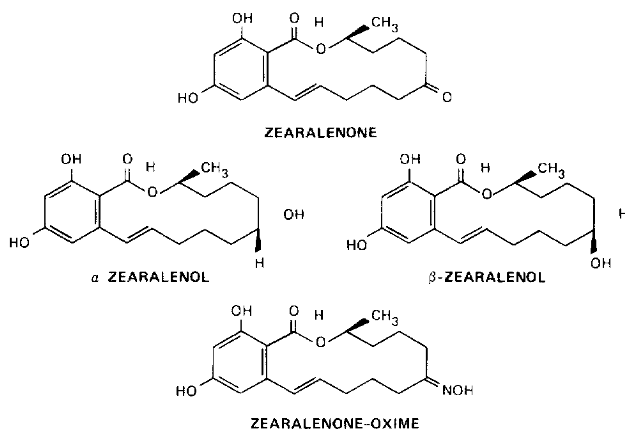

Fig 1 Chemical structures of the compounds of interest

contaminated cereal products, but also through foods from farm animals exposed to ZEN-contaminated feeds [5,15,16]. But while concern over its frequent occurrence has prompted numerous methods for the quantitation of ZEN in cereal grains, relatively few studies have examined the biological fate of this toxin in livestock, and even fewer attempts to detect ZEN-derived residues in animal products have been reported [17–20] In one such study, Mirocha et al [19] did find moderately high levels of ZEN and its metabolites in the milk from a cow fed 25 mg of ZEN per day for a 7-day period; residual concentrations measured on the seventh day amounted to 1359 ng/ml (total metabolites)

To confirm animal exposure to ZEN, or to monitor toxin levels during toxicological experimentation, it is desirable to have a rapid, selective, and sensitive analytical procedure for the simultaneous quantitation of trace concentrations of ZEN and its metabolites, α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL) in various biological fluids Although methods for the determination of ZEN in urine and blood/plasma are available [16,21–23], the procedures may not be directly applicable to more complex samples, such as milk or bile In the few instances where milk has been analysed, procedures were hampered by the necessity for large sample sizes (10–500 ml) requiring lengthy workups, or reduced recoveries (56–71%) of the toxin [11,17–19,24]

This paper describes conditions for the rapid quantitative analysis of low ng/ml levels of ZEN and its metabolites (α-ZEL, β-ZEL) in various biological fluids (milk, urine, bile, blood and plasma), using small sample volumes (0 5–2 0 ml) All samples were obtained from swine, except milk which was from dairy cows

EXPERIMENTAL

Instrumentation

A Spectra-Physics Model SP8000 B-03 high-performance liquid chromatographic (HPLC) system (Spectra-Physics, San Jose, CA, U S A) equipped

with a temperature-programmed oven (30°C) and a Model 8780×R automated sample injector was used A Spectroflow Model 980 fluorescence detector (Kratos, Westwood, NJ, U S A.), excitation wavelength 238 nm and 418 nm cut-off emission filter, range 0.03 μA, attached to an SP4290 computing integrator, was used for detection. All analyses were performed using a stainless-steel 25 cm×4.6 mm I D column packed with reversed-phase RP-18, 5 μm particle size, OD-5A Spheri-5 (Brownlee Labs., Santa Clara, CA, U.S.A) and a Spherisorb S10-ODS (10 μm particle size) pre-column insert (Phase Sep, Norwalk, CT, U.S.A.) The HPLC mobile phase consisted of a water–methanol–acetonitrile mixture (5 4 2, v/v/v), pumped at a flow-rate of 1 2 ml/min Methanol and acetonitrile were HPLC grade, distilled and deionized water (Barnstead's NANOpure Ultrapure Water System, Sybron Corp , Boston, MA, U.S A.) was passed through a Millipore HA 0 45-mm filter prior to use.

Chemicals

Zearalenone, α- and β-zearalenol, and zearalenone oxime (ZOX) were a generous gift from International Minerals & Chemical (IMC) Corp (Terre Haute, IN, U.S.A) β-Glucuronidase (Type IX) was obtained from Sigma (St Louis, MO, U S A)

Biological samples

Milk samples were obtained daily from the 06 00 h milking of dairy cows at the research farm Blood and plasma were obtained freshly as needed from swine by venipuncture. Bile and urine were frozen control samples collected from recent unrelated pharmacokinetic studies involving the catheterization (gall bladder, urinary bladder) of pigs

Hydrolytic treatment of samples

For enzymic hydrolysis of glucuronide conjugates prior to analysis, biological samples (2 0 ml of milk, urine, blood, plasma, or 0 5 ml of bile) were adjusted to pH 5 6–6 6 with 2 0 ml of 0.2 *M* phosphate buffer (pH 5 0), and incubated for 16 h at 37°C after the addition of β-glucuronidase (5000 U/sample) Similar hydrolytic conditions have been reported elsewhere [11,23,25]

Extraction and clean-up procedure

In order that all samples be handled in the same way, prior to the analysis 2.0 ml of 0 2 *M* phosphate buffer (pH 5.0) was also added to the samples not treated with β-glucuronidase

The samples were adjusted to pH 12–14 by the addition of 1 0 ml of 1 0 *M* NaOH, at which time ZOX (60 ng/ml of sample) was added as internal standard Samples were then washed twice with 5 0 ml of 10% (v/v) 2-propanol in diethyl ether using repeated gentle inversion of the mixture for 10–15 s Following centrifugation (2000 *g* for 5 min), the upper organic layer was discarded

each tıme To the remaınıng aqueous layer were added three drops of phenol red ındıcator, and the mıxture was neutralızed to pH 6 8–7 0 by the dropwıse addıtıon of 0 7 *M* acetıc acıd (wıth mıxıng) untıl the pınk color dısappeared. The toxıns of ınterest were then extracted from solutıon usıng two 5 0-ml volumes of 2-propanol–dıethyl ether (10 90, v/v) Samples were mıxed vıgorously (vortex, 30 s) at thıs step Solvent layers were separated by centrıfugatıon (2000 *g* for 5 mın), and the upper organıc phase was saved The combıned organıc extracts (ca 9 ml) were then washed once wıth 2 ml of dıstılled water (vortexed for 20 s and centrıfuged at 2000 *g* for 5 mın) After separatıon the ether layer was evaporated to dryness under nıtrogen usıng slıght heat (40°C), and the fınal resıdue was stored at −10°C untıl analysıs

HPLC analysıs

Resıdues were redıssolved ın 0 3 ml of the HPLC mobıle phase, and a 100-μl alıquot (equıvalent to 167–667 mg sample extract) was ınjected ınto the chromatograph.

Kınetıc study

In a prelımınary pharmacokınetıc study, a dose of 5 mg of zearalenone per kg body weıght (total dose 105 mg) was admınıstered ıntravenously to a pıg prepared surgıcally wıth an ındwelling jugular catheter [26] Concentratıons of ZEN and ıts metabolıtes were analysed ın plasma as outlıned above, and pharmacokınetıc parameters were calculated usıng a two-compartment open model analysıs as suggested by Boxenbaum et al [27]

RESULTS AND DISCUSSION

In the evaluatıon of the practıcalıty of analytıcal technıques for use ın toxıcologıcal research, sımplıcıty, sensıtıvıty and precısıon of the method, as well as ıts applıcabılıty to smaller sample sızes of a varıety of types of bıologıcal fluıd or tıssue are several of the more ımportant consıderatıons

A typıcal chromatogram of a spıked mılk sample ıs presented ın Fıg 2A Under the assay condıtıons used, the followıng retentıon tımes (mın) were obtaıned. 11 4 (β-ZEL), 17 5 (α-ZEL), 19 6 (ZOX), and 22.6 (ZEN) As shown ın Fıg 2B, extracts of control (non-spıked) samples (contaınıng only the ınternal standard, ZOX) produced chromatograms wıthout ınterference by co-extracted endogenous compounds at the retentıon tımes of ınterest It should be noted that, regardless of the fluıd type tested, the chromatograms looked very sımılar ın the regıon of ınterest (10–30 mın) Some samples (ı e bıle, blood) showed greater amounts of materıal beıng eluted earlıer (less than 10 mın), but thıs dıd not ınterfere wıth detectıon of the compounds of ınterest Thıs reflects the selectıvity of the extractıon and clean-up steps, allowıng ZEN and ıts metabolites to be ısolated wıth hıgh quantıtatıon, but removıng any

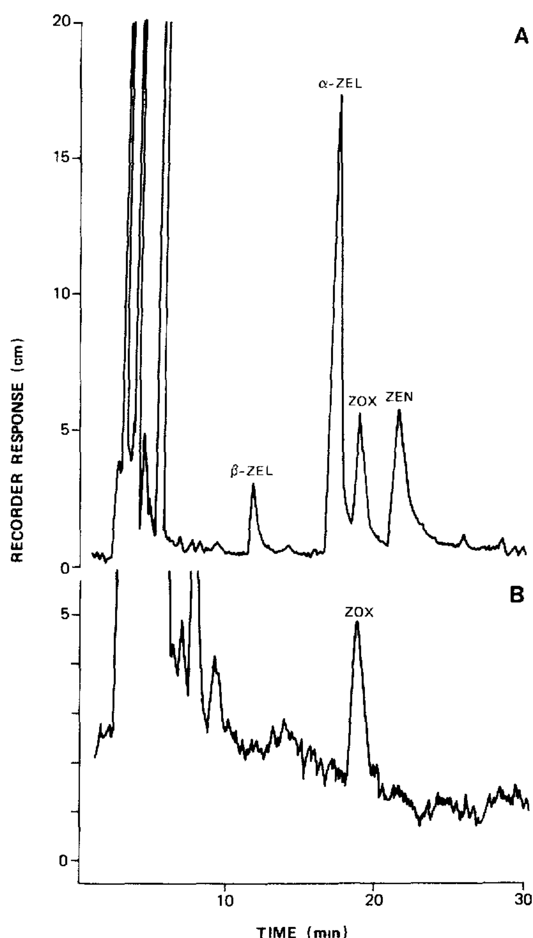

Fig 2 HPLC analysis of (A) ZEN (50 ng/ml), α- and β-ZEL (100 ng/ml each) and ZOX (internal standard, 60 ng/ml) in a spiked milk sample (2 ml) obtained from dairy cow, (B) blank milk sample spiked with only 20 ng/ml ZOX

endogenous interfering components regardless of the sample type In addition, no late-eluting peaks from previous injections were observed to interfere with subsequent chromatograms.

Recovery tests were performed using biological samples spiked with known amounts of ZEN and its metabolites The results in Table I show excellent consistency within sample type. Depending on the biological fluid, recoveries between 66% and 94% were obtained, with standard deviations of 0.7–6.2% Recoveries at low ng/ml concentrations were highest from urine (87–94%) and plasma (85–93%), slightly less from whole blood (78–88%) and milk (75–84%), and lowest from bile (66–77%). At a signal-to-noise ratio of 3 (S/N=3),

TABLE I

RECOVERIES OF ZEARALENONE AND METABOLITES FROM VARIOUS SPIKED BIOLOGICAL FLUIDS[a]

Concentration added (ng/ml)	*n*	Recovery (mean ±S D) (%)		
		ZEN	α-ZEL	β-ZEL
Plasma[b]				
500	4	90 9±2 1	92 0±0 8	89 9±2 1
100	6	85 3±2 5	89 4±2 5	92 8±3 4
20	6	87 7±3 0	85 0±1 9	87 3±2 7[c]
Blood[b]				
500	4	84 3±3 3	87 5±1 1	87 8±1 8
100	5	78 2±2 6	86 3±2 2	87 3±0 8
20	6	81 8±1 9	82 9±2 8	83 4±2 1[c]
Urine[b]				
500	4	94 0±2 7	93 6±2 2	92 3±2 1
100	6	92 3±0 7	91 2±2 5	87 9±2 3
20	6	87 3±3 1	89 1±2 3	91 6±1 7[c]
Bile[b]				
500	6	76 7±3 2	71 3±2 7	75 3±1 9
200	6	74 8±2 6	69 4±2 6	67 8±3 0
40	7	71 8±2 4	66 0±2 0	70 1±2 3
Milk[d]				
1000	4	75 2±3 9	75 5±3 9	83 8±2 6
150	6	75 8±1 3	75 8±3 1	83 0±2 2
25	6	81 9±1 9	78 7±2 9	81 8±2 5[e]
3	8	78 1±5 8	77 5±6 2	–[f]

[a]Samples of 2 ml were used except bile, of which 0 5 ml was used Zearalenone oxime (60 ng/ml of sample) was used as internal standard
[b]From pig
[c]Analysis at 30 ng/ml
[d]From dairy cow
[e]Analysis at 16 ng/ml
[f]Not quantitated accurately at this level

the detection limit in most sample types tested was found to be 1 0 ng/ml for ZEN and α-ZEL, and 5 0 ng/ml for β-ZEL. Bile was the exception because greater background interference by endogenous components necessitated that smaller sample volumes (0 5 ml) be used, which reduced the detection sensitivity for ZEN and α-ZEL to 6 ng/ml, and ca 20 ng/ml for β-ZEL

The inclusion of ZOX as an internal standard contributed greatly to the simplicity of quantitative analysis and quality of recorded data Its similarity

to the compounds of interest makes it an ideal standard (Fig 1) Analytical recoveries were calculated by comparing peak-height ratios of the extracted toxins to the internal standard Calibration curves constructed by plotting response ratios were linear for extracted 2 0-ml samples (0 5 ml for bile) spiked with 1–200 ng/ml ZEN and α-ZEL and 3–600 ng/ml β-ZEL (typical linear correlation coefficient >0.99) Although the actual detector response to the toxins was linear over a much greater range, samples containing higher levels of toxins should be prepared with the addition of a greater amount of internal standard in order to maintain the linearity of the calibration ratio

One critical point for high recovery of the toxins is careful control of pH during extractions, which is of particular importance when analysing milk The initial addition of 1 *M* NaOH serves several purposes At elevated pH (12–14) levels, milk particulate material is forced into solution, resulting in a clearer, more homogeneous sample Furthermore, the weakly acidic (phenolic) toxins are ionized and remain in the aqueous phase, thus permitting a simple clean-up extraction [two 5 0 ml values of 2-propanol–diethyl ether (10 90, v/v)] This not only prevents interfering compounds from being carried through the ensuing analysis, but also appears to remove those endogenous components that would normally cause emulsification during the solvent extraction of milk at a lower pH (5–7) This emulsion, once formed, cannot be broken completely,

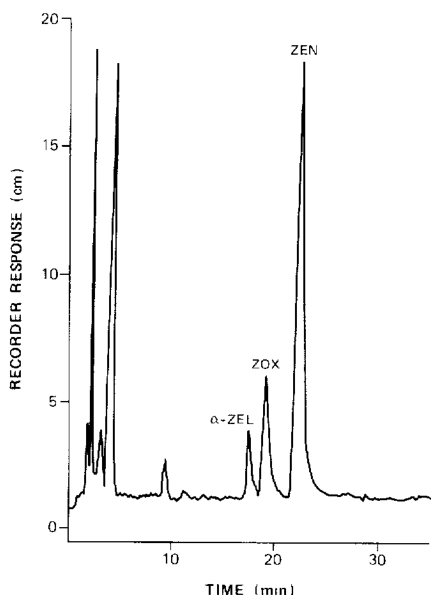

Fig 3 Plasma obtained from a pig 60 min after intravenous dosing (5 mg ZEN per kg) Samples were incubated with β-glucuronidase prior to analysis Calculated concentrations (ng/ml) were based on comparison of untreated and β-glucuronidase-treated samples ZEN, 1425, conjugated ZEN, 705, α-ZEL, 147, conjugated ZEL, 258 ZOX (700 ng/ml) was added as internal standard

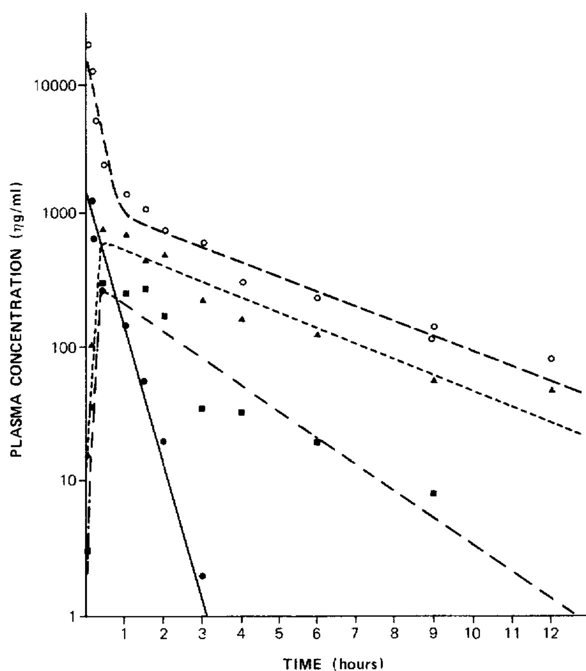

Fig 4 Plasma concentration-time curve of zearalenone and its metabolites following intravenous administration of a single dose of 5 mg/kg to a pig ○ = zearalenone, ● = α-zearalenol, ▲ = conjugated zearalenone, ■ = conjugated α-zearalenol

TABLE II

PRELIMINARY PHARMACOKINETICS PARAMETERS OF ZEARALENONE AND ITS METABOLITES FOLLOWING INTRAVENOUS ADMINISTRATION (5 mg/kg) TO A PIG

Parameter	ZEN	α-ZEL	Conjugated ZEN	Conjugated α-ZEL
α (min^{-1})	4 38 10^{-2}	–	–	–
β (min^{-1})	4 00 10^{-3}	3 37 10^{-2}	4 18 10^{-3}	7 63 10^{-3}
$t_{1/2\alpha}$ (min)	15 8	–	–	–
$t_{1/2\beta}$ (min)	173 3	20 6	165 8	90 8
$AUC_{0\to\infty}$ (ng min ml^{-1})	508 917	29 940	147 274	40 087
Percentage of total[a]	70 1	4 1	20 3	5 5

[a]Percentage based on total plasma area under curve (AUC) for ZEN and its metabolites

and ultimately interferes with the analysis by reducing recoveries and contaminating chromatographic peaks.

Control of pH is also important since the internal standard, zearalenone oxime, is labile under too acidic conditions Therefore, care should be taken to

add the internal standard after the initial adjustment of pH with NaOH, and to prevent the pH from dropping much below the 6 8–7 0 range when neutralizing the sample with the weak acetic acid solution.

This extraction/clean-up procedure has been developed bearing in mind that an additional hydrolysis step may be required prior to analysis Several authors have reported that ZEN and its metabolites undergo considerable biotransformation in various species to the corresponding glucuronide conjugates [19,22,23,25,28,29] Typically, the extent of conjugation is determined by quantitation of the toxins prior to and following hydrolytic treatment with β-glucuronidase. The difference between the amount of free toxin (untreated sample) and the total toxin measured (treated sample) represents the amount of conjugated compound present. This is of particular importance when attempting to determine total residues in an animal-derived product (i e milk), or monitoring the toxin's metabolic fate in animals following exposure Because hydrolysis is carried out in a solution buffered to the pH range 5 5–6 6, it is important that when the pH of the sample is adjusted to 12–14, sufficient NaOH is added to overcome the buffer

Application to experimental samples

A chromatogram resulting from the analysis of a plasma sample obtained from a ZEN-dosed pig is shown in Fig 3. The sample was incubated overnight at 37°C in the presence of β-glucuronidase No interference by endogenous substances could be detected One peak occurring at ca. 9 8 min did appear randomly, but only after hydrolytic treatment and in both control and dosed samples, indicating a contaminant from an external source

Fig 4 shows the plasma concentration–time profile following intravenous dosing of ZEN to a pig Calculated pharmacokinetic parameters are given in Table II The disappearance of free ZEN over the 12-h period monitored is best described as a two-compartment open model. a rapid distribution phase (α), followed by a slower elimination phase (β) These preliminary findings further suggest that elimination of ZEN from plasma is due, in part, to its biotransformation to α-zearalenol, and the corresponding conjugated metabolites which were hydrolysable in the presence of β-glucuronidase Accordingly, the role of metabolism in the toxicokinetics of ZEN should be evaluated further

CONCLUSION

A rapid and sensitive method is described for the quantification of trace levels of zearalenone and its metabolites in a small volume of biological fluid

The method presented here is suitable for blood, plasma, urine, bile and milk Sample preparation is straightforward and recoveries are acceptable and reproducible One main advantage of this procedure compared with other published methods we have tried is that a greater number of samples can be processed during a shorter period In our laboratory we can typically prepare 30–36 samples ready for HPLC analysis per day This compares with 8–12 samples per day for methods starting with larger sample volumes [23,24], in which output is limited by the need to evaporate large solvent volumes, or by having several evaporation steps during the procedure However, with smaller sample sizes it is important that the detectors used for the quantification be sufficiently sensitive

The procedure described here is being utilized currently in a series of in vivo pharmacokinetic studies This is of particular benefit when sample size collection is limited The need for smaller samples is also advantageous in that less stress is placed in the animal In addition there is less chance that the kinetics will be affected by excessive sampling over time (i e. blood collection should be limited to 10% of the total blood volume, which is ca 0.8% body weight)

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