

## Urinary excretion of diethylstilbestrol in the ostrich

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### ABSTRACT

Stilboestrol<sup>®</sup> tablets (20 × 1 mg) were given to 4 ostriches. Urine was collected over a period of 8 days and stored frozen at -20 °C pending analysis. Analyses were performed on a gas chromatograph - mass selective detector for the presence of parent compound and/or metabolites. Diethylstilbestrol and its metabolite, dienestrol, were detected in urine; dienestrol only for 1 day but diethylstilbestrol for 8 days after administration. Residue analysis for the use of diethylstilbestrol as growth promoter can be performed on the urine of ostriches by scanning for parent compound only since it can be detected longer than the metabolite.

**Key words:** diethylstilbestrol, growth promoters, ostrich, residue analysis.

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### INTRODUCTION

In comparison with laboratory animals, knowledge of drug biotransformation in food-producing animals is scarce. However, it is important to obtain a better understanding of the different biotransformation pathways in these species, not only to improve veterinary drug usage but also to guarantee the quality of food of animal origin. In food-producing animals, the risk posed by residues of growth promoters to the health of the consumer is the major concern, and therefore the use of stilbenes and other anabolic agents has been banned by the European Union.

The ostrich is classified as a bird and the meat is consumed by humans. Before residue analysis can be performed one should evaluate the biological matrix of interest. As no information is available on the metabolism and urinary excretion of growth-promoting agents in the ostrich, and since the European Union has strict rules for residue analysis of imported meat, it is necessary to obtain such information.

Several growth stimulants and anabolic agents are available on the South African market. These include zeranol, trenbolone, nandrolone and testosterone. Both

zeranol and trenbolone are designed to be used as implants, while nandrolone and testosterone are produced as intramuscular injectables. Implants are deposited under the skin of the animals, whence they are slowly absorbed into the bloodstream, producing a low concentration over a prolonged period of time. Excretion is slow and prolonged. The oral dosage consists of tablets that are readily absorbed from the gastrointestinal tract. A high concentration over a short period of time is obtained and the drug is more rapidly excreted than occurs in the case of implants. Although diethylstilbestrol (DES) is no longer registered for the South African market, it was available a few years ago as Stilboestrol<sup>®</sup> tablets. However, the European Union still requires that residue analysis should be performed for the stilbenes, *i.e.* DES, dienestrol and hexestrol in urine at a detection limit of 2 ng/ml (EEC letter L/M 18 ANNEX). For this study we have used Stilboestrol<sup>®</sup> tablets registered for oral administration.

The objective of this work was to identify the major metabolites of diethylstilbestrol in the urine of ostriches and to determine which product to detect to prove intake of DES as growth promoter and to determine for how long the major product can be detected.

### MATERIALS AND METHODS

#### Materials

All chemicals were of analytical reagent grade. Stilboestrol<sup>®</sup> tablets were obtained

from Lakato Vet-AG Limited. Sep-Pak C<sub>18</sub> cartridges were obtained from Waters-Millipore, β-glucuronidase-arylsulfatase (*Helix pomatio*) from Boehringer Mannheim, 1,4-dithioerythritol from Merck, and *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA), trimethyliodoslane (TMIS) and 17α-methyltestosterone from Sigma.

#### Apparatus

A gas chromatograph - mass selective detector (GC-MSD) system (Model 5972 MSD combined with a Model 6890 gas chromatograph (Hewlett-Packard)) was used. Injections were made in the splitless mode (time to purge, 0.5 min) onto a 15 m × 0.25 mm i.d. Hewlett-Packard fused silica capillary column (HP-1 MS) with 0.25 μm film thickness. The column temperature was 100 °C, and was programmed to 250 °C at 40 °C/min, hold for 5 min and finally to 300 °C at 30 °C/min and hold for 2 min. Helium was used as a carrier gas with a constant flow of 0.9 ml/min. Injection port and transfer-zone temperatures were 250 °C and 280 °C, respectively, and the ionising beam at 70 eV. Data were acquired in the selected ion monitoring (SIM) mode.

#### Procedures

Four ostriches were each given 20 × 1 mg tablets of Stilboestrol<sup>®</sup> orally. The ostriches, which were between 6 and 8 months of age, were kept in individual cages at the agricultural experimental farm, Oudtshoorn, with sufficient food and water.

Although ostriches are classified as birds, they differ from other birds in that their urine is deposited separately into the urodeum and from there excreted via the same path as the faeces. It is therefore possible to collect urine uncontaminated by faeces, but this is not easy, especially if urine must be collected daily for 8 days. It was therefore decided, for the purpose of this study, to collect the total excreta, since this method is used routinely at the agricultural experimental farm at Oudtshoorn.

All excreta were collected for a period of 8 days by strapping a bag with a plastic liner at the back of the ostrich. Excreta were recovered every 12 h. Contaminated

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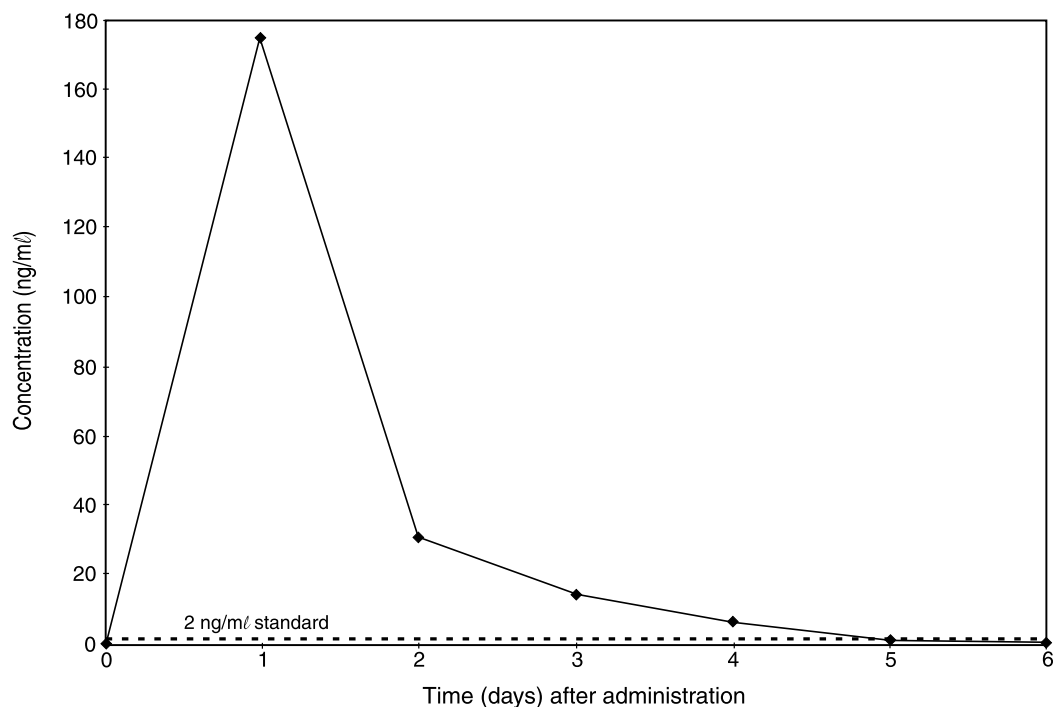


Fig. 1: Excretion of diethylstilbestrol in the urine of ostriches ( $n = 4$ ).

urine was obtained by filtration and centrifugation and stored frozen at  $-20^{\circ}\text{C}$  pending analysis.

#### Extraction of urine samples

A 5 ml volume of the sample was applied to a Sep Pak  $\text{C}_{18}$  cartridge, which was pretreated according to the manufacturer's instructions (5 ml methanol and 5 ml water)<sup>12</sup>. The cartridge was washed with 5 ml water and the analytes eluted from the cartridge with 4 ml methanol. The eluate was evaporated under reduced pressure on a rotavapor.

Glucurono- and sulfo-conjugates of the analytes in urine were hydrolysed enzymatically by dissolution of the dry residue in 1 ml buffer (0.25 mol/l, acetate, pH 5.2) and incubated at  $50^{\circ}\text{C}$  for 3 h with 25  $\mu\text{l}$   $\beta$ -glucuronidase-arylsulfatase.

After addition of 200  $\mu\text{l}$   $\text{K}_2\text{CO}_3$  (0.5 g/l) and 50  $\mu\text{l}$  17 $\alpha$ -methyltestosterone (2 mg/100 ml methanol), the analytes of interest were extracted with 5 ml freshly distilled diethyl ether. The organic phase was transferred and dried under a stream of high-purity nitrogen.

The residue was left in a desiccator for at least 1 h to remove any traces of water,

whereafter the trimethylsilyl derivatives were prepared by adding 40  $\mu\text{l}$  MSTFA-TMIS (1000 + 2 v/v) with 1.4-dithioerythritol (1 % m/v) and heating at  $60^{\circ}\text{C}$  for 15 min.

Toluene (80  $\mu\text{l}$ ) was added and 2  $\mu\text{l}$  of the sample was analysed by gas chromatography - mass spectrometry (GC-MS).

#### RESULTS AND DISCUSSION

When DES is given orally to ostriches, parent compound and the metabolite dienestrol can be detected in the urine.

The analytes (DES and dienestrol) were quantified by measuring the area (A) of the masses  $m/z$  412 and 410 respectively for each analyte, and  $m/z$  446 for the internal standard, by integration.

The area ratios  $A_{412}:A_{446}$  and  $A_{410}:A_{446}$  were used. A calibration curve was constructed by spiking stock solutions of the analyte to blank ostrich urine. The calibration standards covered the range 1.7–500 ng/ml.

The results obtained for the excretion of DES in the urine are given in Fig. 1. DES is rapidly excreted and the concentration is above 2 ng/ml for only 4 days although it can be detected for 8 days. (The EU has

specified a detection level of 2 ng/ml in urine for stilbenes.) One should bear in mind that it is only a concentration of a specific urine sample and owing to the problem of obtaining urine samples from the ostrich it is not possible to extrapolate to amount excreted.

The concentration of dienestrol is just above 2 ng/ml for 1 day only and can be detected for 2 days only.

#### CONCLUSION

Residue analysis for the illegal use of diethylstilbestrol as growth promoter in ostriches can be performed by scanning for parent compound only since it is present in the urine at higher concentrations and can be detected for a longer period than the metabolite.

#### REFERENCES

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