Pharmacokinetics of clenbuterol in the ostrich†

P. J. van der Merwe, a S. Toerien a and W. P. Burger b

a Department of Pharmacology, University of the Orange Free State, PO Box 339, 9300 Bloemfontein, South Africa
b Klein Karoo Co-operative Ltd., P.O. Box 241, 6620 Oudtshoorn, South Africa

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The aim of this study was to investigate the pharmacokinetics of clenbuterol in the ostrich as no such data is available. Clenbuterol (2 ng) was given as a single oral dose to nine ostriches. Blood samples were collected over a period of 96 h after administration and urine for a period of 5 d. Plasma and urine samples were frozen at −20 °C pending analysis. Clenbuterol was quantified using a gas chromatograph—mass selective detector. The method for quantification of clenbuterol in plasma was validated by analysing spiked quality control samples at different concentrations. The limit of quantification was determined to be 0.75 ng ml⁻¹ with an absolute recovery of more than 80%. The geometric mean maximum plasma clenbuterol concentration was 4.40 ng ml⁻¹ with 3.0 h as the median time for maximum concentration. The plasma elimination half-life was 19.7 h. The clenbuterol concentration was above 0.75 ng ml⁻¹ in plasma for 48 h and above 1.0 ng ml⁻¹ in urine for 5 d. These data can be useful in residue analysis for clenbuterol in ostriches.

Introduction

β₂-Agonists are used therapeutically in the treatment of broncho-pulmonary disorders in humans and animals. A β₂-agonist, such as clenbuterol, is known to be a growth promoter and a repartitioning agent when used in dosages 5–10 times the agonist, such as clenbuterol, is known to be a growth promoter and a repartitioning agent when used in dosages 5–10 times the recommended therapeutic dose. Clenbuterol is therefore misused in the meat producing industry to increase meat mass and to reduce the fat moiety.

An advantage of β₂-agonists as growth promoting agents is that they are orally active, which allows them to be mixed with animal feed. In food producing animals, the risk posed by residues of growth promoters to the health of the consumer is the major concern. Therefore, European Union (EU) legislation (EU Directive 86/469) prohibits the use of hormonally active anabolic agents such as β₂-agonists. It is therefore prescribed by the EU that monitoring and meat inspection programmes should be carried out to detect the illegal use of anabolic agents. However, before residue analyses can be performed, one should obtain information on the pharmacokinetics of the drugs in the animals of interest.

As the meat of ostriches is consumed by humans and as no information is available on the pharmacokinetics of clenbuterol in the ostrich, it is necessary to obtain such information. The objective of this study was to obtain such information when clenbuterol is given orally to ostriches.

Urine, plasma and tissues, especially livers of slaughtered animals, have been used for the detection of residues of illicitly administered β₂-agonists in animals.2–11 Clenbuterol also accumulates in the pigmented tissues of the eye and in the hair.12–14 For the purpose of this study, only plasma and urine were used, mainly owing to the high cost of killing ostriches to obtain other tissues such as livers and eyes.

Experimental

Standards and chemicals

Clenbuterol hydrochloride, 17α-methyltestosterone, N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) and trimethyl-iodosilane (TMIS) were obtained from Sigma (St. Louis, MO, USA) and 1,4-dithioerythritol from Merck (Darmstadt, Germany). All other solvents, reagents and chemicals were of analytical-reagent grade and obtained from Merck.

Apparatus

A gas chromatograph-mass selective detector (GC-MSD) system [Model 5972 MSD combined with a Model 6890 gas chromatograph (Hewlett-Packard, Avondale, PA, USA)] was used. Injections were made in the splitless mode (time to purge, 0.5 min) on to a 15 m × 0.25 mm id Hewlett-Packard fused silica capillary column (HP-1 MS) with a 0.25 μm film thickness. The column temperature was initially 100 °C and was programmed at 40 °C min⁻¹ to 250 °C, held there for 5 min, and then at 30 °C min⁻¹ to 300 °C and held there for 2 min. Helium was used as the carrier gas at a constant flow rate of 0.9 ml min⁻¹. The injection port and transfer zone temperatures were 250 and 280 °C, respectively, and the ionizing beam was at 70 eV. Data were acquired in the selected ion monitoring (SIM) mode.

Collection of urine and plasma samples

Nine ostriches were each given the equivalent of 2 mg of clenbuterol orally. The animals were kept in individual cages with sufficient food and water at the Agricultural Experimental Farm, Oudtshoorn.

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 is to be collected on a daily basis for 5 d. 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 5 d by strapping a bag with a plastic liner at the back of the ostrich. Excreta were recovered every 12 h and, by filtration and centrifugation, relatively faeces free urine was obtained, which was kept frozen at −20 °C pending analysis.
Venous blood samples (20 ml) were collected at 1, 2, 3, 4, 8, 24, 28, 32, 48, 56, 72, 80 and 96 h after clenbuterol administration using 10 ml sterile Vacutainer tubes containing lithium heparin. All the blood samples were collected under the ostrich’s wing. The blood samples were centrifuged and the obtained plasma samples were stored at −20 °C pending analysis.

Preparation of calibration standards

Five calibration standards were prepared by spiking blank ostrich urine or blank ostrich plasma samples with various amounts of clenbuterol to bracket the concentrations of the test samples. The calibration standards covered the range 0–575 ng ml\(^{-1}\) for urine and 0–12 ng ml\(^{-1}\) for plasma.

Extraction of samples

To 5 ml of urine were added 200 µl of K\(_2\)CO\(_3\)–NaHCO\(_3\) solution (20 g of K\(_2\)CO\(_3\) + 20 g of NaHCO\(_3\) + 160 ml of water) and 50 µl of 17α-methyltestosterone (2 mg in 100 ml), as an internal standard. This mixture was extracted with 5 ml of freshly distilled diethyl ether. In the case of plasma samples, 40 µl of K\(_2\)CO\(_3\)–NaHCO\(_3\) solution and 20 µl of 17α-methyltestosterone as an internal standard (both solutions as above) were added to 1 ml of plasma and extracted with 4 ml of freshly distilled diethyl ether. After centrifugation, the organic phase was separated 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, then the trimethylsilyl derivatives were prepared by adding 40 µl of MSTFA–TMIS (1000 + 2 v/v) and 1,4-dithioerythritol (1% m/v) and heating at 60 °C for 20 min. After dilution with 50 µl of toluene, 2 µl of each sample were analysed by GC–MSD. The responses at \(m/z\) 446 and 335 were recorded. Data processing involved integration and the area versus concentration curve was used to quantify the clenbuterol in the test samples.

Validation

The method for quantification of clenbuterol in plasma was validated by analysing quality control samples previously prepared in plasma five times at seven different concentrations, i.e., 16.32, 12.24, 8.16, 4.08, 2.04, 1.02 and 0.68 ng ml\(^{-1}\), to obtain the accuracy and precision of the method. The quality control values were calculated from a standard linear regression curve containing eight different concentrations covering the expected concentration range (0.57–20.07 ng ml\(^{-1}\)). Accuracy was measured as percentage bias and precision as relative standard deviation (RSD).

<table>
<thead>
<tr>
<th>Subject</th>
<th>0 h</th>
<th>1 h</th>
<th>2 h</th>
<th>3 h</th>
<th>4 h</th>
<th>8 h</th>
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* BLQ, below limit of quantification.

Pharmacokinetic variables

Descriptive statistics were applied by using SAS software (V6.12). The various pharmacokinetic variables were calculated using the actual blood sampling intervals (relative to clenbuterol administration).

Maximum concentration (\(C_{\text{max}}\)). \(C_{\text{max}}\) was read directly from the observed concentrations.

Time to maximum concentration (\(T_{\text{max}}\)). \(T_{\text{max}}\) was read directly from the observed concentrations as the blood sampling time corresponding to \(C_{\text{max}}\).

Apparent terminal half-life (\(t_{\text{1/2}}\)). The terminal half-life of clenbuterol was calculated from the non-linear regression of a single exponential function to the terminal phase of the plasma concentration versus time profile. The regressions were carried using the method of non-linear least squares. The terminal half-life was then calculated as

\[ t_{\text{1/2}} = \ln 2/z = 0.693/z \]

where \(z\) is the terminal rate constant.

Area under the plasma concentration versus time data pairs with extrapolation to infinity [\(\text{AUC}(0–\infty)\)]. \(\text{AUC}(0–t_{\text{last}})\) was extrapolated to infinity by adding \(C(t_{\text{last}})/z\). Thus,

\[ \text{AUC}(0–\infty) = \text{AUC}(0–t_{\text{last}}) + C(t_{\text{last}})/z \]

where \(\text{AUC}(0–\infty)\) is the area under the curve from 0 h to infinity, \(z\) is the terminal rate constant and \(C(t_{\text{last}})\) is the last quantifiable concentration. \(\text{AUC}(0–\infty)\) is characteristic of the extent of absorption of the drug.

Results and discussion

The mean absolute recoveries of clenbuterol determined in triplicate at 2.04, 11.61 and 20.54 ng ml\(^{-1}\) were 86, 87 and 98%, respectively. The limit of quantification (LOQ) was determined from the data obtained for the assayed quality controls during validation, since these data include determinations of the analyte at concentrations close to the limit of detection. The LOQ is defined as that concentration of clenbuterol which can still be determined with acceptable precision (RSD < 20%) and accuracy (bias < 20%). The LOQ was set at 0.75 ng ml\(^{-1}\).

Owing to the problems of obtaining the urine samples from the ostrich, the method for quantification of clenbuterol in urine was not validated fully. The limit of detection (LOD) in urine was determined as 0.7 ng ml\(^{-1}\).

The individual plasma clenbuterol concentrations for each ostrich and the actual blood sampling intervals are given in Table 1. Concentrations below the LOQ are indicated by BLQ.

![Table 1. Plasma clenbuterol concentrations for each ostrich](image-url)
All BLQ values were replaced by half the LOQ value for calculation of the descriptive statistics. The mean plasma clenbuterol concentration versus time profile is presented in Fig. 1. After 56 h the plasma clenbuterol concentration was below 0.75 ng ml\(^{-1}\) (LOQ). The plasma clenbuterol pharmacokinetic variables are given in Table 2.

The results for clenbuterol in urine are shown in Fig. 2. Clenbuterol was rapidly excreted and the concentration in urine was above 1 ng ml\(^{-1}\) for 5 d. One should bear in mind that this is only a concentration in a specific urine sample and it is not possible to extrapolate to the amount excreted or to determine any other pharmacokinetic parameters. Owing to the problem with urine collection, the urine was collected from only four ostriches.

**Conclusion**

The method described is precise, accurate and sensitive enough to be used for residue analysis of ostrich samples for clenbuterol. The data for the concentration of clenbuterol in both the plasma and urine can be useful in the residue analysis for clenbuterol in ostriches.

The authors thank all their co-workers at the Ostrich Research Centre and the Agricultural Experimental Farm at Oudtshoorn who assisted with the blood and urine sampling.

**References**


**Table 2** Pharmacokinetic variables for plasma clenbuterol

<table>
<thead>
<tr>
<th>Variable</th>
<th>Geometric mean ± geometric s (n = 9)</th>
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<tr>
<td>C\textsubscript{max}</td>
<td>4.40 ± 1.38 ng ml(^{-1})</td>
</tr>
<tr>
<td>T\textsubscript{max}</td>
<td>3.0 h (median)</td>
</tr>
<tr>
<td>AUC(0–(\infty))</td>
<td>138.7 ± 1.55 ng h ml(^{-1})</td>
</tr>
<tr>
<td>t(_1/2) (elimination)</td>
<td>19.7 ± 1.37 h</td>
</tr>
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</table>

**Fig. 1** Plasma clenbuterol concentration versus time profile.

**Fig. 2** Urinary excretion of clenbuterol.