

Toxicokinetics of cotyledoside following intravenous administration to sheep

C J Botha^a, T Rundberget^b, G E Swan^a, M S G Mülders^a and A Flåøyen^b

ABSTRACT

Cotyledoside, a bufadienolide cardiac glycoside, was administered intravenously to sheep in 2 studies. In experiment 1, sheep ($n = 4$) received 0.0135 mg/kg daily on 5 consecutive days and in the 2nd experiment, sheep ($n = 4$) received 0.027 mg/kg as a single dose. Jugular blood was collected at different time intervals and kinetic parameters were determined. The data fitted a 1-compartmental model. In both experiments a short half-life ($t_{1/2}$) and mean residence time (MRT), a relative small volume of distribution ($V_{d_{ss}}$) and rapid clearance were calculated. In the 1st experiment, $t_{1/2}$ and MRT increased significantly ($P < 0.007$) from Day (D) 0 to D 4. It is suggested that the rapid decline in plasma cotyledoside concentrations in sheep denotes rapid distribution of cotyledoside to the tissues or extracellular spaces and possible accumulation at the biophase.

Key words: cardiac glycoside, cotyledoside, kinetics, krimpsiekte, sheep.

Botha C J, Rundberget T, Swan G E, Mülders M S G, Flåøyen A **Toxicokinetics of cotyledoside following intravenous administration to sheep.** *Journal of the South African Veterinary Association* (2003) 74(1): 7–10 (En.). Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, 0110 South Africa.

INTRODUCTION

Krimpsiekte, a chronic form of cardiac glycoside poisoning, manifests as a paretic syndrome and occurs predominantly in small stock^{5,6}. The disease develops following ingestion of certain members of the Crassulaceae (*Cotyledon*, *Tylecodon* and *Kalanchoe* species). Krimpsiekte is generally believed to be caused by cumulative bufadienolides, with unique neurotoxic properties, encountered in these genera^{5,7}. Naudé and Schultz⁸ coined the term 'cumulative bufadienolides' following the successful demonstration of a cumulative effect with cotyledoside isolated from *Tylecodon wallichii*. These authors determined a subcutaneous LD₅₀ of 0.116 mg/kg cotyledoside in guinea pigs and then injected others subcutaneously with 25 % and 50 % of the subcutaneous LD₅₀ per day until they died. No clinical signs appeared before the LD₅₀ was reached and marked nervous signs occurred when 5 × 25 % or 3 × 50 % the LD₅₀ were administered⁸.

Repeated intravenous cotyledoside administration at 0.01–0.015 mg/kg body mass to sheep induced krimpsiekte,

characterised by tremors, paresis and recumbency³. At this dose no meaningful electrocardiograph abnormalities occurred, indicating that cotyledoside at low doses does not overtly affect the electrical activity of the heart³. Naudé and Schultz⁸ also induced krimpsiekte in sheep with repeated doses of 0.01 mg/kg cotyledoside at intervals of 24 hours or more. Clinical signs observed included weakness, reluctance to stand, unsteadiness, tremors and paresis of the hindquarter muscles, paresis of the neck, arching of the back and standing with the feet close together. Respiratory function was affected in all 3 cases studied, with ruminal stasis and concomitant loss of appetite occurring in one and a transient change in heart function in another.

The acute and subacute toxicity of cotyledoside to sheep was also determined⁸. At 0.05 mg/kg intravenously, 1 of 2 sheep died of typical cardiac glycoside poisoning. The surviving animal exhibited jerky, abdominal respiration and cyanosis. Two sheep that received a single intravenous dose of 0.025 mg/kg developed rumen stasis and polypnoea and both survived.

In recent studies no other neurotoxic substance could be isolated from *T. wallichii* and *T. ventricosus* and it was concluded that the toxic principles contained by these 2 *Tylecodon* species are indeed

bufadienolide cardiac glycosides^{2,3}. The cumulative effect, however, has been questioned as *T. ventricosus* also induced krimpsiekte in sheep following a single, relatively large dose². The intention of the 1st experiment was to demonstrate accumulation of cotyledoside in the plasma of sheep following daily intravenous injections and to determine certain kinetic variables. An additional aim was to assess the recovery of cotyledoside from spiked whole blood. The objective of the 2nd experiment was to determine the same kinetic parameters following a single intravenous administration of cotyledoside to sheep, although at a higher dose, and to refine the initial blood collection points.

MATERIALS AND METHODS

Animals

Four Döhne Merino ewes, approximately 6 months of age and weighing 32–34 kg, were purchased for the 1st experiment and 4 Mutton Merino wethers of similar age and weighing 36–43 kg were obtained for the 2nd experiment. The sheep were identified with ear-tags and housed individually in sheep pens on concrete floors at the Onderstepoort Veterinary Academic Research Unit. They were fed milled lucerne hay and a maize-based concentrate (Epol Ewe and Lamb pellets). The animals had free access to drinking water. During a 2-week adaptation period, clinical examinations were performed. Following induction of krimpsiekte in the 1st experiment, clinical examinations were performed daily and supportive treatment was instituted.

Spiking of whole blood and plasma samples

The possible adherence of cotyledoside to the erythrocyte membrane or absorption into the red blood cells were assessed by spiking whole blood and plasma. Blood was collected in 10 ml heparinised tubes from 1 of the sheep and the haematocrit determined. The heparinised blood samples remained in a water bath at 37 °C until being spiked. Following centrifugation of some of the collection tubes (15 000 rpm for 15 min) plasma

^aDepartment of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, 0110 South Africa.

^bNational Veterinary Institute, Box 8156 Dep., Oslo, 0033 Norway.

*Author for correspondence. E-mail: cbotha@op.up.ac.za
Received: July 2002. Accepted: November 2002.

was collected and spiked to contain 10, 20, 40, 80 and 160 ng cotyledoside/ml before being returned to the water bath. Whole blood was pooled and aliquots were spiked to contain a similar range of cotyledoside concentrations in the plasma, calculated according to the following formula:

$$y \text{ ng/ml plasma } \times (1 - \text{haematocrit}[\%]) = z \text{ ng/ml whole blood.}$$

The spiked blood samples were thoroughly mixed on a rolling mixer (Coulter) and were incubated at 37 °C in a water bath for 60 min. After incubation the blood samples were centrifuged and the plasma collected. The plasma samples were kept frozen at -20 °C until extraction prior to analysis.

Cotyledoside administration

On the day preceding the 1st experiment the sheep were weighed, after having been deprived of food for 12 hours, and dosed according to this weight. A cotyledoside injectable solution (0.025 % m/v) was prepared aseptically by dissolving crystalline cotyledoside in a small volume of warm ethanol before adding the necessary volume of normal saline (auto-sterile 0.9 % NaCl, Renalcare Services). Cotyledoside was administered intravenously at a daily dose of 0.0135 mg/kg³ for 5 consecutive treatments (as clinical signs indicative of krimpsiekte developed on Day (D) 4). The sheep received the cotyledoside injection between 08:00 and 09:00.

For the 2nd experiment a 0.05 % m/v cotyledoside solution was prepared in a similar manner. The sheep each received a single intravenous administration of 0.027 mg cotyledoside/kg (double the previous dose).

Collection of plasma samples

In the 1st experiment blood samples were collected in heparinised vacuum tubes (10 ml) from the jugular vein on D 0 at 0, 2, 7, 15, 30 and 60 minutes, 2, 3, 6, 9, 12, 18 and 24 hours, and once a day before cotyledoside administration on D 1, 2, 3 and 4. On D 4, blood samples were collected at the same intervals as indicated for D 0 with additional samples collected at 36 and 48 hours and on D 7, 9, 11, 14, 18 and 25. In the 2nd study, blood samples (10 ml heparinised tubes) were collected from the jugular vein shortly before and at 2, 5, 9, 15, 20 and 25 minutes after cotyledoside administration. The blood samples were centrifuged within an hour of collection and 5 ml of plasma was collected and stored at -20 °C.

Cotyledoside analysis

Plasma proteins were removed with a Sep-Pak C₁₈ Cartridge (Waters) and

Table 1: Kinetic parameters determined in sheep (n = 4) after intravenous administration of 0.0135 mg/kg cotyledoside on Day 0 and after 5 consecutive daily doses (Day 4).

Variable	Day 0 (mean ± SD)	Day 4 (mean ± SD)	P ^a
t _{1/2} (min)	2.5 ± 0.40	4.21 ± 0.37	<0.007
MRT (min)	3.6 ± 0.57	6.07 ± 0.53	<0.007
C _{max} (ng/ml)	12.4 ± 5.09	13.23 ± 4.73	NS
Vd _{ss} (l/kg)	0.72 ± 0.35	0.82 ± 0.29	NS
Clearance (l/kg/min)	0.19 ± 0.07	0.14 ± 0.05	NS

^aProbability level; NS = not significantly different.

cotyledoside eluted with methanol and evaporated to dryness under a stream of nitrogen before transportation to the analytical laboratory. The plasma concentrations of cotyledoside were determined by high performance liquid chromatographic-electrospray mass spectrometry (HPLC-ESMS) analysis. HPLC-ESMS was performed using a 5 micron, 4.6 mm × 150 cm, Waters C-18 symmetry column interfaced to a Finnigan LC-Q electrospray mass spectrometer. Separation was achieved using gradient elution starting with methanol-water (65+35, v/v, both containing 0.1% formic acid), rising to methanol-water (95+5, v/v) over 10 min. The flow rate was 0.7 ml/min. The ESMS was operated in positive ion mode, with selected ion mode (SIM) detection of cotyledoside. An authentic specimen of cotyledoside (MH⁺ = m/z 575), previously isolated from *T. wallichii*³, was used to prepare a standard solution of cotyledoside containing 400 ng/ml. Other reference solutions were prepared by dilution of this solution. Typically, 20 µl injections of reference solutions and plasma were analysed. A calibration curve corresponding to plasma cotyledoside concentrations of 0, 2, 5, 10, 40 and 80 ng/ml was used to determine concentrations. Validation of the LC-MS method revealed that the calibration curve was linear from 2 to 200 ng/ml and the limit of quantification (LOQ) was approximately 0.5 ng cotyledoside/ml when a sample aliquot corresponding to 2 ml plasma was injected.

Kinetic analysis

The kinetic variables were determined with the aid of the software program PC-Nonlin Version 1.1 (Scientific Consultation Inc.).

Statistical analysis

The individual animal data were tabulated and the different kinetic variables were statistically compared with the software program SigmaStat 2.0 (Jandel Corporation, San Rafael). The one-way repeated measures analysis of variance was used to test for significant differences (P < 0.007) between the kinetic

parameters on D 0 and D 4. The data were analysed and ordinary descriptive statistics were determined using the same program.

RESULTS

The kinetic parameters calculated on D 0 and D 4 of the 1st experiment and following a single intravenous administration in the 2nd experiment are tabulated in Tables 1 and 2. The data fitted a 1-compartmental model. In both experiments a short half-life (t_{1/2}) and mean residence time (MRT), a relative small volume of distribution (Vd_{ss}) and rapid clearance were calculated.

Semilogarithmic plots of plasma cotyledoside concentration versus time are presented in Figs 1 and 2. Regression lines fitted to the cotyledoside concentrations detected on D 0 and D 4 gave r² values of 0.98 and 0.93, respectively. Regression analysis of the plasma cotyledoside concentration in the 2nd experiment revealed a r² of 0.98.

Cotyledoside recovery from spiked whole blood and plasma samples was good and ranged from 73 to 118 %. Comparable cotyledoside concentrations were detected following spiking of plasma and whole blood, but the plasma cotyledoside concentration was always 15–33 % higher compared with the spiked whole blood sample throughout the range of concentrations evaluated.

During the 1st experiment, 1 of the sheep exhibited clinical signs, typical of krimpsiekte, after 4 daily injections of cotyledoside. The other 3 animals also developed similar clinical signs (tremor, paresis, recumbency, inappetence, weak ruminal movements and/or ruminal

Table 2: Kinetic parameters determined in sheep (n = 4) after intravenous administration of 0.027 mg/kg cotyledoside.

Variable	Mean ± SD
t _{1/2} (min)	3.08 ± 0.59
MRT (min)	4.44 ± 0.85
C _{max} (ng/ml)	33.95 ± 6.0
Vd _{ss} (l/kg)	0.28 ± 0.07
Clearance (l/kg/min)	0.12 ± 0.01

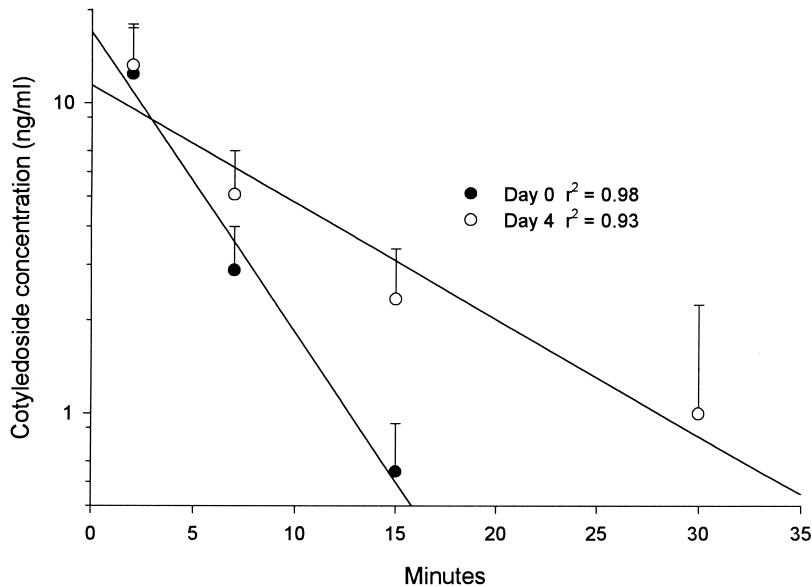


Fig. 1: Plasma cotyledoside concentrations in sheep ($n = 4$) after intravenous administration of 0.0135 mg/kg on Day 0 and following 5 consecutive daily doses (Day 4).

stasis) in varying severity following the 5th consecutive injection. Micturition was noticeable in all sheep. In the 2nd experiment 3 of the 4 sheep developed tachypnoea and forced, abdominal respiration shortly after the single intravenous cotyledoside administration. One of the sheep exhibited inappetence and decreased rumen motility for 2 days following cotyledoside administration.

DISCUSSION

In the 1st experiment plasma cotyledoside concentrations were very low and decreased to below the LOQ soon after 15 min on D 0 (Fig. 1). The same trend occurred on D 4 although the $t_{1/2}$ and MRT increased significantly ($P < 0.007$). In the 2nd experiment, with a 2-fold increase in

dose and more regular blood collecting intervals, the plasma cotyledoside concentration also decreased rapidly and approached the LOQ after 25 min (Fig. 2). The C_{max} increased to a mean concentration of 33.95 ng/ml (range 27.85–42.2) in the 2nd experiment compared to a mean plasma cotyledoside concentration of 12.41 ng/ml (range 7.05–19.3) and 13.23 (range 8.45–17.7) on D 0 and D 4, respectively, in the 1st experiment.

In both experiments cotyledoside was rapidly cleared from the plasma following intravenous administration to sheep. Adherence of cotyledoside to erythrocytes after intravenous administration was ruled out as incubation of cotyledoside in whole blood did not result in excessive loss of cotyledoside and the

recovery compared favourably with spiked plasma samples. It is proposed that cotyledoside rapidly distributes from the plasma into the tissues and/or extracellular fluid. HPLC-ESMS analysis indicated no evidence of metabolism. Although limited data points are available, it appears that distribution of cotyledoside from the plasma into the tissues or extracellular fluid is exponential (Figs 1, 2). The discrepancy in the Vd_{ss} ($Vd_{ss} = \text{Dose} \times \text{AUMC}/\text{AUC}$) calculated in experiments 1 and 2 probably reflect differences in sample intervals between the 2 experiments.

In experiment 1 the significant increase in $t_{1/2}$ and MRT from D 0 to D 4 probably reflects saturation at a 'binding site' with less rapid distribution and association of cotyledoside at the 'binding site', resulting in relatively higher plasma concentrations on D 4. A dynamic equilibrium could also have become established where some cotyledoside might redistribute back into the plasma. Another possibility is that cotyledoside, being of high molecular weight, is potentially eliminated by biliary excretion and since this is an active transport process the rate of elimination could be reduced by saturation of the carriers. It is also conceivable that the efficiency of the elimination process is altered due to the toxic effects which were becoming evident by D 4.

Contrary to expectations, the plasma concentrations following daily cotyledoside injections did not increase and remained below the LOQ on each consecutive day prior to cotyledoside administration. The proposed cumulative effect of cotyledoside could not be verified by increased plasma concentrations. However, the effects of cotyledoside were indeed cumulative as clinical signs reminiscent of krimpsiekte developed in all 4 sheep following 5 consecutive daily intravenous injections. This is in agreement with previous studies where a cumulative effect was demonstrated^{3,8}. Cotyledoside is so toxic at minute concentrations that detection of plasma concentrations becomes problematic. In 2 studies designed to determine digoxin pharmacokinetics in adult sheep the therapeutic dose administered intravenously varied from 50–75 $\mu\text{g}/\text{kg}^{1,4}$. In the current study signs of toxicity were induced after a single intravenous injection of 27 μg cotyledoside/kg. This is consistent with the findings reported by Naudé and Schultz⁸ where 2 sheep that received a single intravenous dose of 25 μg cotyledoside/kg developed rumen stasis and polypnoea, but survived.

Distribution from the plasma is so rapid and based on the kinetic parameters it is interpreted as elimination, hence the

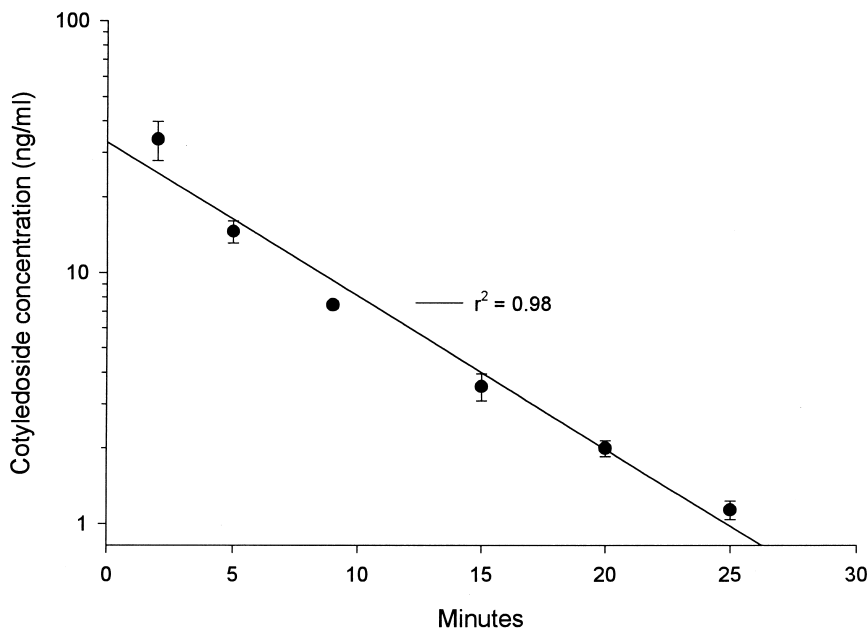


Fig. 2: Plasma cotyledoside concentrations in sheep ($n = 4$) after intravenous administration of 0.027 mg/kg.

short $t_{1/2}$ and MRT as well as the rapid clearance. The short $t_{1/2}$ most probably reflects distribution, but on the other hand it may represent elimination and thus could be affected by elimination processes. Pharmacokinetic variables have been determined for digoxin, a cardenolide cardiac glycoside. A distribution half-life ($t_{1/2\alpha}$) of 0.72 h and an elimination half-life ($t_{1/2\beta}$) of 15.2 h have been determined in ewes¹. In another study a $t_{1/2\beta}$ of 7.15 h was calculated for sheep⁴. Digoxin is not a cumulative cardiac glycoside and renders itself to conventional pharmacokinetic analysis.

Based on the rapid decline of plasma cotyledoside concentrations and the development of clinical signs after 5 consecutive injections it was concluded that cotyledoside quickly distributes into the tissues and/or extracellular fluid. Thus, it is surmised that cotyledoside binds somewhere to the tissues, most probably at the biophase, where it is retained. Additional experiments to demonstrate the accumulation of cotyledoside in tissues are envisaged.

ACKNOWLEDGEMENTS

Funding received from the South African Veterinary Foundation and the Faculty of Veterinary Science, University of Pretoria, is gratefully acknowledged. The research reported here emanates from Project 36.5.361, approved by the Animal Use and Care and Research Committees of the Faculty of Veterinary Science of the University of Pretoria.

REFERENCES

1. Berman W, Musselman J, Shortencarrier R 1982 The pharmacokinetics of digoxin in newborn and adult sheep. *Journal of Pharmacokinetics and Biopharmaceutics* 10: 173–186
2. Botha C J, Kellerman T S, Schultz R A, Erasmus G L, Vlegaar R, Retief E 1998 Krimpsiekte in a sheep following a single dose of *Tylecodon ventricosus* (Burm. f.) Toelken and the isolation of tyledoside D from this plant species. *Onderstepoort Journal of Veterinary Research* 65: 17–23
3. Botha C J, van der Lugt J J, Erasmus G L, Kellerman T S, Schultz R A, Vlegaar R 1997 Krimpsiekte, associated with thalamic lesions, induced by the neurotoxic cardiac glycoside, cotyledoside, isolated from *Tylecodon wallichii* (Harv.) Toelken subsp.

wallichii. *Onderstepoort Journal of Veterinary Research* 64: 189–194

4. Dix L P, Bai S A, Rogers R A, Anderson D L, Riviere J E 1985 Pharmacokinetics of digoxin in sheep: limitations of the use of biological half-life for interspecies extrapolation. *American Journal of Veterinary Research* 46: 470–472
5. Kellerman T S, Coetzer J A W, Naudé T W 1988 *Plant poisonings and mycotoxicoses of livestock in southern Africa*. Oxford University Press, Cape Town
6. Kellerman T S, Naudé T W, Fourie N 1996 The distribution, diagnoses and estimated economic impact of plant poisonings and mycotoxicoses in South Africa. *Onderstepoort Journal of Veterinary Research* 63: 65–90
7. Naudé T W, Anderson L A P, Schultz R A, Kellerman T S 1992 "Krimpsiekte": a chronic paralytic syndrome of small stock caused by cumulative bufadienolide cardiac glycosides. In James L F, Keeler R F, Bailey E M, Cheeke P R, Hegarty M P (eds) *Poisonous Plants*. Proceedings of the Third International Symposium, Iowa State University Press, Ames: 392–396
8. Naudé T W, Schultz R A 1982 Studies on South African cardiac glycosides II. Observations on the clinical and haemodynamic effects of cotyledoside. *Onderstepoort Journal of Veterinary Research* 49: 247–254