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Histological Technique for the Identification of Poisoning in Wildlife by the Rodenticide Calciferol

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Anticoagulant rodenticides are widely used to control rats and mice in the United Kingdom. However, as a result of increasing resistance to some compounds in rodent populations, the acute rodenticide, ergocalciferol, has been introduced. In accord with most references in the literature, ergocalciferol will be referred to as calciferol in this paper.

Calciferol is available in commercial formulations which also contain the anticoagulant rodenticide warfarin. Calciferol has a long half-life in mammals (Buckle et al., 1972) and its toxicological action at the recommended bait concentration (0.1%) is to promote the intestinal absorption of calcium and resorption of bone calcium (Buckle et al., 1972). The resultant hypercalcaemia can lead to the calcification of blood vessels (Gillman et al., 1960) and histologically visible deposition of calcium may occur in the cardiac muscle, lungs, gastro intestinal tract and the kidneys (Grant et al., 1963).

During the field assessment of environmental hazard and monitoring studies of the commercial use of pesticides, there is a requirement to identify and confirm the cause of death of any non-target casualties associated with rodenticide treatments. Although calciferol can be chemically determined in rodenticide baits, such analytical detection has not proved possible in animal tissues or stomach contents from field samples. These analytical difficulties probably arise from the rapid conversion of calciferol to metabolities. In view of the existing problems of detection, an attempt has been made to exploit the severe tissue calcium deposition, following calciferol ingestion by evaluating a histochemical method involving the anthraquinone dye, alizarin red S pH 4.1-4.3, which is a specific stain for calcium. The following study was carried out to determine whether the exposure of test rats (*Rattus norvegicus*) and quail (*Coturnix coturnix japonica*) to calciferol could be confirmed by the deposition of calcium in kidney and whether this could be used as a diagnostic technique for application to field samples. Stability of these deposits after death was also investigated.

MATERIALS AND METHODS

Calciferol was obtained as a 2% concentrate in vegetable oil. Test animals were fed normal laboratory diet containing 0.1% by weight calciferol, the recommended field concentration. Before being fed to the test animals, the prepared feed was analysed by the HPLC method of MacNicoll and Simpson (1983) to confirm the initial concentration and purity of calciferol in the feed. Since some degradation may result from exposure to sunlight, the 0.1% calciferol diet was stored in the dark prior to use. Once mixed in bait, the calciferol is stable for periods up to three weeks (MAFF, 1973, 1974; MacNicoll and Simpson, 1983).

Laboratory-reared male albino rats and male and female Japanese quail were given a standard rodenticide toxicity test regime. This involved 2 days feeding *ad libitum* with 0.1% calciferol in diet 41B powder or turkey starter crumbs respectively, followed by normal untreated food. Experimental animals and a control were killed using ether at 0-4 days after removal from the test diet and kidneys were removed for histological examination. Any dead animals were preferentially sampled on each day. A further batch of male rats was also placed on a similar test regime but all rats were killed with ether following removal from the 0.1% calciferol diet (day 0). Rats were stored at room temperature and kidneys from these rats were then sampled 0-7 days after death to verify the stability of kidney calcium deposition since many tissue samples from suspected poisoning incidents in the field are not submitted for examination until several days after death.

Following their removal, rat and quail kidneys were placed in 10% buffered (pH 7.0) formalin and after fixation were routinely processed to paraffin blocks. Tissue sections 6μ thick were stained with a 2% solution of alizarin red S pH 4.1-4.3. The specificity of the stain was also checked by similar staining of reference tissue sections known to contain or to be free from calcium deposits.

RESULTS AND DISCUSSION

The staining results obtained using alizarin red S method are summarised in Table 1. Male rats and quail exhibited a similar pattern of calcium deposits. Female quail showed lower initial deposits of calcium but by day 2 after removal from the treated diet, the calcium deposits were similar to those seen in male rats and quail.

Calcium deposits were detected using the alizarin red S staining method in kidneys of male rats over the period 0-7 days after death indicating the stability of tissue calcium deposits. Kidneys exhibiting severe tissue autolysis by day 7 still provided stainable calcium deposits after fixation and processing.

Table 1. Comparison of staining results using Alizarin Red S pH 4.1-4.3 on rat and quail kidney sections obtained from animals fed 0.1% calciferol bait for 2 days and killed up to 4 days after transfer to untreated bait

	Days after removal from poisoned bait														
	0			1			2			3			4		
Animal No.	1[c]	2	3	4[c]	5	6	7[c]	8	9	10[c]	11	12	13[c]	14	15
Rat ♂	0	+	+	t	+	+[b]	0	+	+[b]	t	+[b]	+[b]	t	+	+
Quail ♂	0	+	+	0	+	+	t	+	+	0	+	+	0	+	+
Quail ♀	+[a]	t	t	0	0	+	0	+	+	0	+	+	0	+	+

0 = no stainable deposits
t = only a few trace deposits per kidney section
+ = large areas of deposition throughout kidney section
a = diseased kidney
b = found dead
c = control sections

The alizarin red S pH 4.1-4.3 histochemical staining method differentiated between kidneys from animals exposed to 0.1% calciferol in their diet and control animals which had no exposure. Some very small foci of calcium deposits were observed in the kidneys of rats and male quail and were scored as trace deposits. These trace calcium deposits were seen mainly in the control rats and would seem to represent background levels associated with spontaneous kidney lesions and disease which occur when rats are not maintained in specific pathogen free conditions. Previous work (unpublished) in this laboratory using kidney tissue obtained from wild rats *(Rattus norvegicus)* of different ages showed that extensive kidney calcium deposits were very uncommon. When they did occur, the calcium deposits did not show any similarity to the calcium deposits observed in calciferol exposed rats. One female

control quail kidney (day 0) gave a positive calcium result and further investigation showed that this was probably due to disease.

The results of this study demonstrate that the alizarin red S pH 4.1-4.3 method can provide a rapid and easily reproducible method for detection extensive calcium deposition in the kidneys of wildlife casualties thought to involve the rodenticide calciferol. It is important that tissue sections are examined from animals of each species that have not been exposed to calciferol in order to provide the necessary background levels of kidney calcium deposits. Various methods of quantifying the observed extensive calcium deposits after exposure to calciferol are currently being investigated.

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REFERENCES

Buckle RM, Gamlen TR, Pullen IM (1972) Vitamin D intoxication treated with porcine calcitonin. Brit Med J 3:205-207

Gillman T, Grant RA, Hathorn M (1960) Histochemical and chemical studies of calciferol induced vascular injuries. Brit J Exp Path 41:1-18

Grant RA, Gillman T, Hathorn M (1963) Prolonged chemical and histochemical changes associated with widespread calcification of soft tissues following brief acute calciferol intoxication. Brit J Exp Path 44:220-232

MacNicoll AD, Simpson WJ (1983) The stability of ergocalciferol in rodenticidal baits. J Hygiene 90:253-258

McGee-Russell SM (1958) Histochemical methods for calcium. J Histochem Cytochem 6:22

MAFF (1973) Technical Circular 30, Ministry of Agriculture, Fisheries and Food, London

MAFF (1974) Technical Circular 31, Ministry of Agriculture, Fisheries and Food, London
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