The European Agency for the Evaluation of Medicinal Products
Veterinary Medicines and Inspections

EMEA/MRL/769/00-FINAL
January 2001

COMMITTEE FOR VETERINARY MEDICINAL PRODUCTS

COUMAFOS

SUMMARY REPORT (2)

1. Coumafos [O-(3-chloro-4-methyl-2-oxo-2H-1-benzopyrane-7-yl) O,O-diethyl phosphorothioate, [O'O-Diethyl-O''(3-chlor-4-methyl-7-cumarinyl) thiophosphate] (CAS No 56-72-4) is an organophosphorus compound intended to control *Varroa* and *Braula* infections in honey bees. The recommended application rate is two treatments of 32 mg coumafos/hive in aqueous solution, given 7 days apart.

   Coumafos also used as insecticide on crops and has been evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 1990.

   Currently, coumafos is included in Annex III of Council Regulation (EEC) No 2377/90 as follows:

Pharmacologically active substance(s)	Marker residue	Animal species	MRLs	Target tissues	Other provisions
Coumafos	Coumafos	Bees	100 µg/kg	Honey	Provisional MRL expires on 1.7.2001

   Additional data on the validatin of the analytical method were provided in response to the list of questions, further to the establishment of provisional MRLs for coumafos.

2. Like other organophosphorus compounds, the main pharmacodynamic activity of coumafos is cholinesterase inhibition. A lower threshold for cholinesterase inhibition in female rats, as compared to males, was consistently observed in oral and dermal studies in rats. No adverse effects on haematological and urine parameters and on electrolyte excretion were induced in rats by a single oral dose of up to 10 mg/kg bw.

3. Investigations on the absorption, excretion, distribution and metabolism of coumafos have been done in four mammalian species: rats, rabbits, cattle and goats. These studies were performed with coumafos radiolabelled with either ^{32}P or ^{14}C.

   Absorption of coumafos was low after dermal application by spraying or as a pour-on to cattle and goats, with a substantial proportion of the applied dose remaining on the skin at the site of application (cattle: more than 60% of dose after 2 days, goats: 41 to 49% of dose after 7 days). Approximately 4% and 6% of the dose was recovered in the excreta of cattle and goats, respectively. The major radiolabelled metabolite in cattle urine was the hydrolysis product chlorferron (3-chloro-7-hydroxy-4-methyl-2H-1-benzopyran-2-one), which constituted about 80% of the extracted radioactivity. In goat urine, chlorferron and highly polar material were the major metabolites.

   In rats, after single or multiple oral doses of 1 mg/kg absorption of coumafos was rapid and extensive. Peak plasma level of radioactivity occurred 30 minutes after treatment. Absorption of coumafos after a single oral dose of 15 mg/kg bw was only about half that observed with the dose of 1 mg/kg bw.

7 Westferry Circus, Canary Wharf, London, E14 4HB, UK
Tel. (44-20) 74 18 84 00 Fax (44-20) 74 18 84 47
E-mail: mail@emea.eu.int www.emea.eu.int

Elimination of radioactivity was rapid; the elimination half-life in plasma was 2 to 3 hours for oral and intravenous administration and more than 80% of the dose was excreted during the first 24 hours after treatment. Urine was the main route of excretion after a dose of 1 mg/kg bw. Chlorferron was the major radioactive component (more than 75% of extracted radioactivity), with coumafos and coroxon (phosphoric acid 3-chloro-4-methyl-2-oxo-2H-1-benzopyran-7-yl diethyl ester) (being present only in trace). Chlorferron and coumafos were the main radiolabelled components in faecal extract.

4. In rats, the oral LD_{50} of coumafos administered in polyethylene glycol was 113 mg/kg bw in males and 28 mg/kg bw in females.

5. In a 8-week dietary study mice were exposed to coumafos at concentrations of 0, 20, 60, 120 and 180 mg/kg feed, equivalent at least to 0, 2, 6, 12 and 18 mg/kg bw. Plasma and erythrocyte cholinesterases were inhibited at all dose levels. Significant inhibition of brain acetylcholinesterase was present in females at 12 mg/kg bw and above. Transient inhibition was observed at 6 mg/kg bw and above in males killed after 3 weeks of treatment but not in those sacrificed at the end of the experiment. The NOAEL for brain acetylcholinesterase inhibition was 2 mg/kg bw.

   In a 13-week dietary study rats were exposed to coumafos at concentrations of 0, 2, 5 and 10 mg/kg feed, equivalent at least to 0, 0.1, 0.25 and 0.5 mg/kg bw, respectively. A dose-related inhibition of erythrocyte cholinesterases was observed in both sexes at all dose levels. Plasma cholinesterase inhibition was observed in both sexes at 5 mg/kg feed and above. No significant brain cholinesterase inhibition was present. The NOAEL for brain acetylcholinesterase inhibition was higher than 0.5 mg/kg bw.

   In a 2-year dietary study rats were exposed to coumafos at concentrations of 0, 1, 5 and 25 mg/kg feed, equivalent at least to 0, 0.05, 0.25 and 1.25 mg/kg bw, respectively. Reduced weight gain was observed in females at 1.25 mg/kg bw. In the same group inhibition of erythrocyte and of plasma cholinesterases was observed in both sexes, whereas brain cholinesterases were unaffected. The NOEL was 0.25 mg/kg bw.

6. In a 1-year dietary study beagle dogs were exposed to coumafos at concentrations of 0, 1, 30 and 90 mg/kg feed, equivalent to approximately 0, 0.025, 0.75 and 2.25 mg/kg bw, respectively. A dose related inhibition of erythrocyte and plasma cholinesterases was observed in animals of both sexes of all treated groups. Significant or slight inhibition of brain cholinesterases was present at the top dose level in females and males, respectively. The NOAEL was 0.75 mg/kg bw for brain acetylcholinesterase inhibition, and 0.025 mg/kg bw for erythrocyte cholinesterase inhibition.

7. In a 2-generation study rats were exposed to coumafos at dietary concentrations of 0, 1, 5 and 25 mg/kg feed, equivalent at least to 0.05, 0.25 and 1.25 mg/kg bw, respectively. No effects were observed on fertility. However, a reduction in the proportion of male live born pups was observed at 1.25 mg/kg bw in F1 and F2 litters; no effect on sex ratio was observed at 0.25 mg/kg bw. Brain cholinesterase activity was significantly reduced in the adult P0 and P1 females at 1.25 mg/kg bw. Plasma and erythrocyte cholinesterases were significantly inhibited in adults of both sexes at 0.25 mg/kg bw and above and in pups at 1.25 mg/kg bw. The NOAEL for this study was 0.25 mg/kg bw/day, based on the brain cholinesterase inhibition.

8. Coumafos elicited signs of apparent maternal toxicity but did not induce teratogenicity or embryotoxicity in rats and rabbits treated orally during the period of organogenesis at doses up to 25 mg/kg bw and 18 mg/kg bw, respectively. However, the proportion of male foetuses was consistently reduced at dose levels of 5 mg/kg bw and above in the rat and at the top dose level of 18 mg/kg bw in the rabbit. No effect were observed at 1.0 mg/kg bw in the rat and 2.0 mg/kg bw in the rabbit.

9. Coumafos did not show any mutagenic or genotoxic potential in a number of *in vitro* tests, with and without metabolic activation, including *Salmonella typhimurium* and *Escherichia c*oli assays, DNA damage in *Escherichia coli*, forward mutation assay on mouse lymphoma cells, chromosome aberrations and sister chromatid exchange on Chinese hamster ovary (CHO) cells, unscheduled DNA synthesis in primary rat hepatocytes. Negative results were obtained also in two oral micronucleus assays in mice. In an *in vitro* gene mutation assay (HPRT locus) in Chinese hamster ovary cells, mutation was produced when a metabolic activation system was present, but there was no mutagenicity in the absence of metabolic activation. In an analogous assay on V79 cells, negative results were obtained. On the basis of available data it was concluded that coumafos is not a genotoxic compound.

10. In a pre-GLP carcinogenicity assay, F-344 rats were exposed to coumafos at concentrations of 0, 10 and 20 mg/kg feed, equivalent at least to 0, 0.5 and 1.0 mg/kg bw. No increase of neoplasm incidence was seen in males: incidence of animals with malignancies was 28%, 24% and 32% at 0, 0.5 and 1.0 mg/kg bw, respectively. In females a not significant but dose-related increase of animals with malignancies was observed: incidence of animals with malignancies was 12%, 20% and 26% at 0, 0.5 and 1.0 mg/kg bw, respectively. The increased incidence of malignancies in females was mostly due to leukaemia (9%, 15% and 22% at 0, 0.5 and 1 mg/kg bw, respectively). However, as ageing F344 rats are known to have a naturally high and variable prevalence of leukaemia, the finding of slightly increased incidence of leukaemia in this study is of little relevance to the evaluation of the carcinogenic risk to human consumers of residues of coumafos in foods. The reliability of these results is also limited by the poor study design (only two treatment dose levels and too few animals in the control groups: 25 per sex).

    In the 2-year dietary chronic toxicity-carcinogenicity study bor:WISW rats, a strain with a low background prevalence of leukaemia, were exposed to coumafos at concentrations of 0, 1, 5 and 25 mg/kg feed, equivalent at least to 0, 0.05, 0.25 and 1.25 mg/kg bw. No carcinogenic effects were observed up to the dose level of 1.25 mg/kg bw. The study was guideline-conform and performed according to GLP. It can be concluded that coumafos is not carcinogenic in rats.

    In a 2-year carcinogenicity assay B6C3F1 mice were exposed to coumafos at concentrations of 0, 10 and 20 mg/kg feed, equivalent at least to 0, 1.0 and 2.0 mg/kg bw. After one year of treatment a compound-related increase of clinical signs was observed in mice of both treated groups, with males showing an excess mortality before termination. A non-significant but dose-related increase of females with malignant tumours was observed at both dose levels: incidence of animals with malignant tumours was 20% in controls, 26% at 1 mg/kg bw and 36% at 2 mg/kg bw. The increased incidence of tumours was mostly due to hepatocellular carcinomas (0%, 8% and 10% at 0, 1 and 2 mg/kg bw, respectively) and to mammary gland carcinomas/adenocarcinomas (0%, 2% and 4% at 0, 1 and 2 mg/kg bw, respectively). The incidence values were higher than background incidence values for each tumour as reported in the scientific literature. Moreover, treated females showed an increased incidence of alveolar/bronchiolar adenomas (8% for both low and high dose groups) as compared to controls (0%); no clear dose-response relationship was evident. Although no increase in tumours was observed in male mice, it could not be excluded that increased mortality in the treated males could have interfered with the ability to detect a rise in the tumour rate.

    More recent studies on mice were not performed.

    On the basis of the data provided, coumafos should be considered as a possible tumourigenic agent in female mice. Since coumafos is non-genotoxic, it is reasonable to assume that a threshold dose exists, below which coumafos causes no tumours. However, in the absence of evidence of a NOEL or a dose-response relationship for the production of lung adenomas, the numerical value of the threshold dose remains unknown.

11. No information was provided on the systemic toxicity of the main coumafos metabolites. Three coumafos metabolites, chlorferron, potasan (O-(4-methyl-2-oxo-2H-1-benzopyran-7-yl) O,O-diethyl phosphorothioate), and the coumafos P-O analogue, were tested in the *Salmonella*-microsomal assay. All three compounds gave negative results.

12. No specific studies were performed on the potential of coumafos for immunotoxicity.

    No alterations of haematological parameters or tissues relevant to immune function were detected in the repeated dose toxicity studies.

13. In a published study coumafos induced reversible neurological signs (impaired gait) in adult hens upon repeated exposure to doses down to 5 mg/kg bw by oral route or 100 mg/kg bw by dermal route. Histological alterations possibly related to delayed neurotoxicity were observed only in hens treated dermally.

    Further studies found equivocal evidence of neuropathy target esterase inhibition in brain and spinal cord of hens, following both oral and dermal exposure to high doses (in the LD_{50} range). No clinical or histological evidence of delayed neurotoxicity was present. Although the studies were not completely adequate, it can be concluded that there are no indications of coumafos inducing delayed neurotoxicity upon oral exposure.

14. Limited studies in humans indicate no inhibition of whole blood cholinesterase following occupational (inhalation) exposure. However, the data are utterly inadequate to derive any conclusion on a dose without effects in humans.

15. In 1968 a temporary ADI of 0 to 0.0005 mg/kg bw was determined by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR), including the parent compound, the O-analogue and the metabolite chlorferron. Since further studies requested were not provided or were unsatisfactory, the temporary ADI was withdrawn in 1980. The compound was reconsidered by the JMPR in 1990. Once more no ADI was allocated due to the lack of studies on developmental and reproductive toxicity as well as of toxicological studies on main metabolites.

16. An ADI of 0.00025 mg/kg bw (i.e. 0.015 mg/person) can be established for coumafos based the lowest NOEL of 0.025 mg/kg bw for erythrocyte cholinesterase inhibition observed in a 1-year feeding study in the most sensitive species dog.

17. The distribution of coumafos within a honeybee colony was studied after application by sprinkling of a commercial product containing 32 mg coumafos/ml. Approximately one-quarter of the total quantity of coumafos applied to a colony was shown to reach the alimentary canal of bees. When bees were fed a syrup containing ^{14}C-labelled coumafos, the total amount of radioactivity recovered in the alimentary canal and haemolymph averaged 68% of the total dose; 73% of the recovered amount was found in the rectum at 24 hours after feeding. The composition of the radioactive fraction was not investigated.

    Transfer of coumafos between bees by the process known as trophallaxis does occur, but it is of minor importance in the distribution of coumafos within a bee colony.

    Coumafos does concentrate in wax. However, the CVMP noted that the regulation of the allowable concentrations of chemicals in bee's wax is a matter for individual Member States and therefore, information on coumafos concentration in wax is considered not directly relevant for the assessment of residues in honey.

18. Fifteen bee populations (5 per dosage) suffering from varroasis were treated topically with two application of coumafos given 7 days apart at the dose of 1.5, 3.0 and 4.5 mg/honeycomb channels (equivalent to 32 mg per colony; recommended dose is 4.0 mg/honeycomb channel). The honey samples were collected 70 to 74 days after the second treatment and analysed by HPLC (limit of detection: 1 µg/kg for coumafos, 2 µg/kg for coroxon and potasan, limit of quantification: 2 µg/kg for coumafos, 25 µg/kg for coroxon and potasan). The coumafos residues ranged from 1.2 to 13.3 µg/kg at the dose of 1.5 mg, from 2.7 to 9.4 µg/kg at the dose of 3.0 mg and from 5.5 to 34.0 µg/kg at the dose of 4.5 mg. The only two metabolites coroxon and potasan analysed were both below the limit of detection in all samples.

Two colonies were treated with 32 mg of coumafos per colony twice. Honey samples were collected 69 days after the second treatment: residues of coumafos (limit of detection: 1 µg/kg, limit of quantification: 2 µg/kg), coroxon and potasan (limit of detection: 2 µg/kg for both, limit of quantification: 25 µg/kg) and chlorferron (limit of detection: 1 µg/kg, limit of quantification: 10 µg/kg) were investigated by HPLC. Residues were below the detection limits for all compounds tested.

Bee colonies infected with *Varroa* were treated twice at a 7-day interval. Six-weeks after the second treatment, honey samples were analysed for coumafos residues by spectrophotometry with TLC (limit of detection: 10 µg/kg, the limit of quantification was not calculated). Colonies treated in spring included 4 colonies treated with two doses of 32 mg and 5 colonies treated with two doses of 16 mg. With the exception of one case with concentrations of 23 µg/kg from the series treated with the lower dose, all the values were below 10 µg/kg. Another seven samples taken from the winter feed had a concentrations between 10 and 99 µg/kg.

Five bee colonies infected with varroasis were treated with medicated sugar solution containing 37.5 mg of coumafos applied to each populated honeycomb passage. The honey samples were taken 196 days after the last treatment, and analysed for coumafos residues by HPLC (limit of detection: 1 µg/kg, limit of quantification: 2 µg/kg). Residue values ranged from 24 to 115 µg/kg.

19. Coumafos residues in five honey samples from 21 colonies that had been treated with 32 mg of coumafos twice were analysed by GC-ECD (electron capture detection, limit of detection: 2 µg/kg, limit of quantification: 3 µg/kg) about 3 months after the second treatment. The average residue concentration in the honey samples was 3 µg/kg.

    Forty-five bee colonies in four different apiaries were treated with coumafos at the recommended dose of 32 mg twice at an interval of one week, in spring. During the summer honey was assayed for coumafos residues by GC method (limit of detection: 2 µg/kg, limit of quantification: 3 µg/kg). The honey samples showed an average concentration of coumafos residues of 2 µg/kg.

    Twenty-one samples of honey were collected from colonies treated with coumafos. A GC method was used to detect coumafos (limit of detection: 0.5 µg/kg). Two honey samples contained detectable amounts of coumafos (1 to 2 and 5 to 6 µg/kg). The concentration of coumafos in the honey remaining in the combs ranged from less than 0.5 µg/kg to 4 µg/kg).

20. Concentrations of the metabolites coroxon, potasan and chlorferron were below the limit of detection of the analytical method in all samples analysed in the residue depletion studies, therefore coumafos can be retained as marker residue. No information on the ratio coumafos to total residues was provided.

21. A validated analytical method for the quantitative determination of residues of coumafos in honey by gas chromatography with an electron capture detector was proposed in ISO 78/2 format with a limit of detection of 2 µg/kg and a limit of quantification of 50.0 µg/kg.

Conclusions and recommendation

Having considered that:

- an ADI of 0.00025 mg/kg bw (i.e. 0.015 mg/person) has been established,
- honey samples collected 70 to 74 days from bee colonies after two treatments with 32 mg of coumafos showed residues of coumafos ranged from 5.5 to 34.0 μg/kg,
- coumafos can be considered as marker residue, since the residue concentrations of the metabolites chlorferron, coroxon and potasan in honey samples tested were below the detection limit of the analytical method,
- a validated routine analytical method for monitoring residues in honey is available;

the Committee for Veterinary Medicinal Products recommends the inclusion of coumafos in Annex I of Council Regulation (EEC) No 2377/90 in accordance with the following table:

Pharmacologically active substance(s)	Marker residue	Animal species	MRLs	Target tissues	Other provisions
Coumafos	Coumafos	Bees	100 μg/kg	Honey	

Based on this MRL value, the daily intake will represent about 13% of the ADI. This leaves an ample margin for intake of residues resulting from pesticidal uses, for which no exact figures were available.