CARBADOX

First draft prepared by

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ADDENDUM

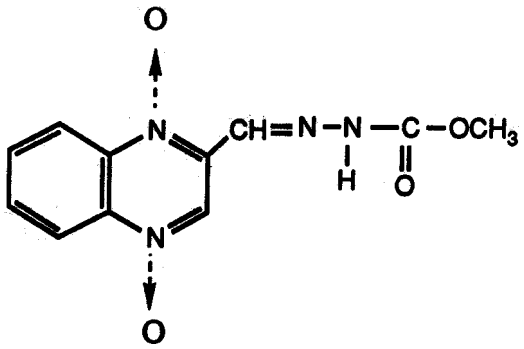
To the carbadox monograph prepared by the 36th meeting of the Committee and published in the FAO Food and Nutrition Paper 41/3, Rome 1991

IDENTITY

Chemical name: Methyl-3-(2-quinoxanyl-methylene) carbazate-N1,N4-dioxide.

Common trade names: Mecadox; Fortigro;GS-6244; Nutriton; Getroxel.

Structural formula:



Molecular formula: $C_{11}H_{10}N_4O_4$

Molecular weight: 262.2

INTRODUCTION

Carbadox was first reviewed by the Committee at its thirty-sixth meeting in 1990 (WHO 1990). In reaching its decision on MRLs for carbadox, the Committee took the following factors into consideration:

- Because of the genotoxic and carcinogenic nature of carbadox and its metabolite desoxy-carbadox, the Committee was not able to establish an ADI.
- Carbadox and desoxy-carbadox can only be detected (<5µg/kg) in tissues for the first 72 hours after treatment, their levels at 28 days withdrawal are negligible.
- More than 90% of total residues in tissues were bound residues and unextractable at 28 days withdrawal.
- With current analytical procedures, quinoxaline-2-carboxylic acid (QCA) is the only carbadox metabolite that can be identified in liver from pigs treated according to good practice in the use of veterinary drugs.
- Bound residues in swine liver at 28 days after treatment would not represent a risk for consumers.
- A 28 day withdrawal time was suitable for residues not to present any risk to consumers.
- Quinoxaline-2-carboxylic acid extracted by alkaline hydrolysis was less than 30 µg/kg after 28 days withdrawal.

- Practical analytical methods are available for measuring quinoxaline-2-carboxylic acid to 30 µg/kg in liver and to 5 µg/kg in muscle.

On the basis of data from studies on the toxicity of quinoxaline-2-carboxylic acid, and on the metabolism and depletion of carbadox, and the nature of the compounds released from the bound residues, the Committee concluded that residues resulting from the use of carbadox in pigs were acceptable and recommended MRLs of 30 µg/kg in liver and 5 µg/kg in muscle of pigs, based on the levels of, and expressed as, quinoxaline-2-carboxylic acid.

The 13th Session of the Codex Committee on Residues of Veterinary Drugs in Foods (CCRVDF) requested that carbadox be given priority for review by the Joint FAO/WHO Expert Committee on Food Additives, as it could be predicted from data on residues that carbadox and desoxycarbadox might be present in tissues from pigs that had not been withdrawn from treatment before slaughter. The Joint Secretariat to JECFA requested requested the following information:

- - All relevant toxicology and residue data, including analytical methods for detecting the parent drug and metabolites in tissues of pigs that have been generated sine the previous evaluation by JECFA.

RESIDUES IN FOOD AND THEIR EVALUATION

Conditions of use

General

Carbadox (GS-6244) is an antimicrobial drug used in the feed of swine for growth promotion, improved feed efficiency, increased rate of weight gain, and to control swine dysentery and bacterial swine enteritis. The commercial product is intended for use in starter and/or grower rations but not in finisher rations.

Dosage

It is usually administered orally in finished feed at 55 mg/kg (50g/ton). (FDA, 1998)

Previous studies of the metabolism of carbadox

The metabolism of carbadox has been studied in rats, monkeys and pigs using [^{14}C] carbadox. labelled in either the *phenyl* ring or the *carbonyl* group of the side-chain. The metabolism of carbadox was characterized by the rapid reduction of the N-oxide groups to give desoxycarbadox, the cleavage of the methyl carbazate side-chain to give the carboxaldehyde and the corresponding carboxylic acid, and the liberation of respired CO_2. The detectable residues in tissues, up to 24 hours after drug withdrawal, were carbadox, desoxycarbadox, quinoxaline-1,4-di-N-oxide-2-carboxaldehyde, and quinoxaline-2-carboxylic acid (QCA*)*. QCA was the only residue in liver detected 24 hours or longer after dose. The metabolism of ring labelled carbadox is summarized in Figure 1 (Pfizer, 1989a).

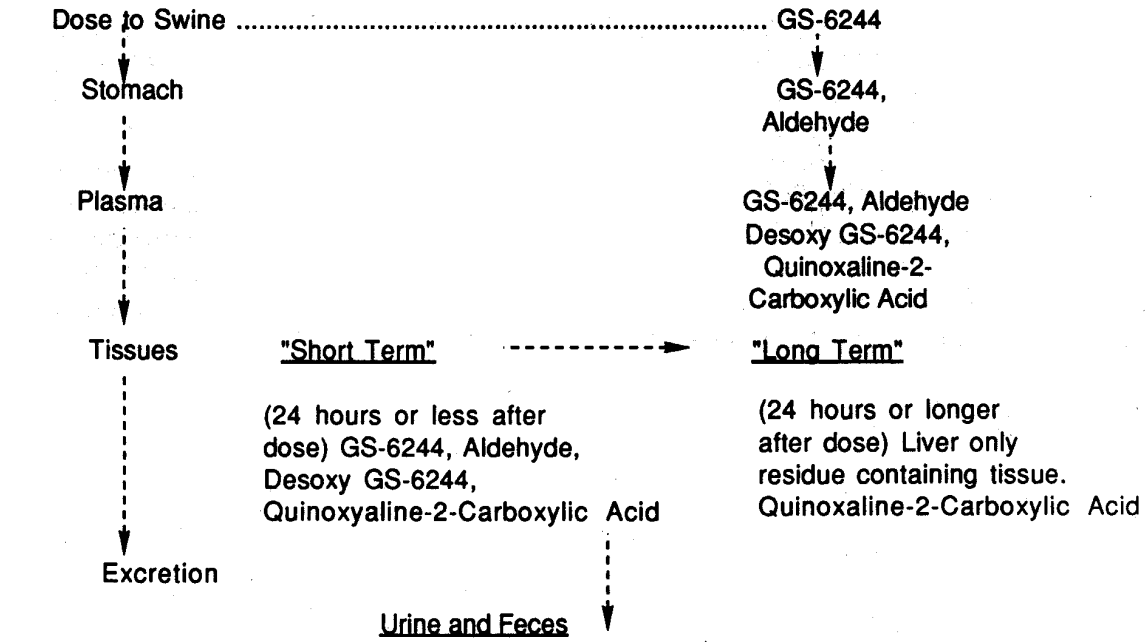
Studies with *carbonyl* labelled carbadox have demonstrated that methylcarbazate is generated. Most of the methylcarbazate is enzymatically hydrolyzed to yield CO_2. Radioactivity in liver decreased with a half-life of two days, and five days after dosing corresponded to 0.12 mg/kg methylcarbazate equivalent that was shown to consist in part of amino acids which were labeled by incorporation of $^{14}CO_2$. The enzymatic hydrolysis of methylcarbazate implies but does not prove the formation of hydrazine. Studies with appropriately labeled drug can measured the residues in tissues arising from methylcarbazate or from quinoxaline derivatives, but no radiotracer method can demonstrate the absence of hydrazine. However, hydrazine was a minor metabolite and would be expected to be present only for a short time before undergoing further metabolism since several enzymatic processes are known to destroy hydrazine. In plasma, free hydrazine was not detected by an assay with a limit of detection of 0.1 mg/kg. The metabolism of carbonyl labeled carbadox is summarized in Figure 2 (Pfizer, 1989a).

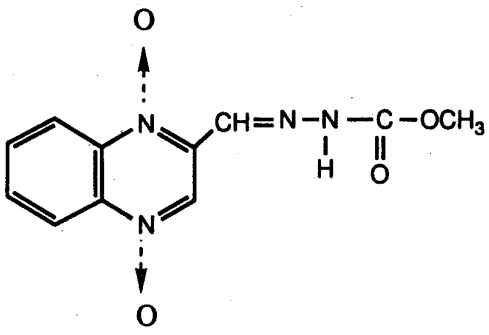
Peak radioactivity concentrations in plasma were observed at approximately 3 hours after dosing, indicating good oral absorption. Carbadox, its aldehyde, desoxy-carbadox and QCA were present in plasma within hours after drug administration, but had disappeared 24 hours later.

Two thirds of the dose was rapidly eliminated with the urine within 24 hours, the remaining with the feces. The major urinary metabolite was shown to be the QCA, which was also excreted in conjugated form. No N-oxides were found in urine. Feces contained some QCA and no unchanged carbadox.

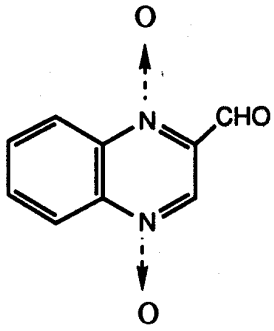
Figure 1

Summary of Ring Labeled Carbadox (GS-6244) Metabolism in Swine

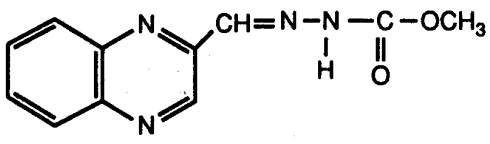




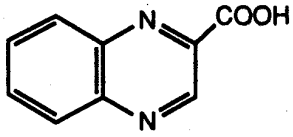
GS-6244



GS-6244 Aldehyde
(Quinoxaline-1, 4-di-N-Oxide-2-Carboxaldehyde)



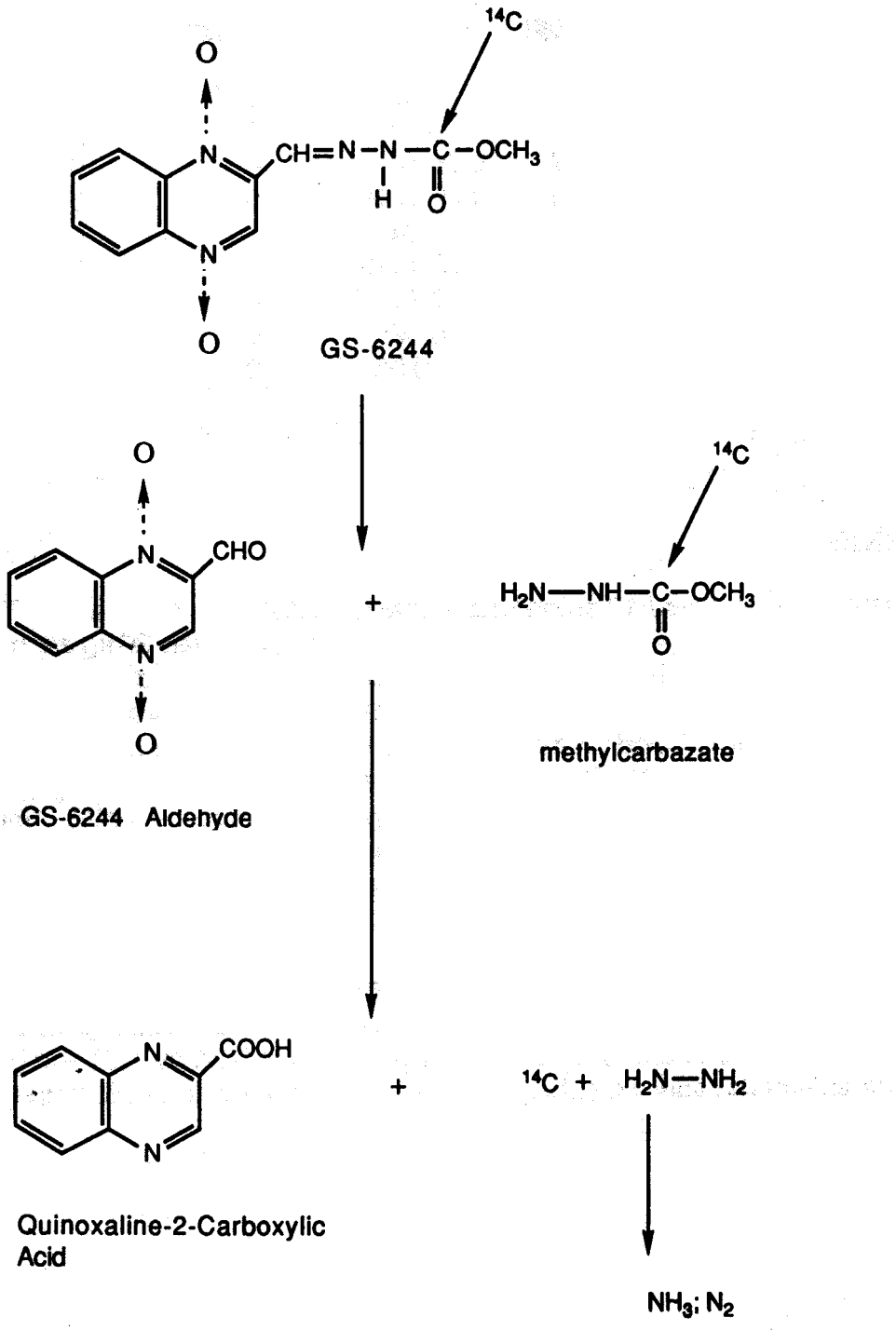
Desoxy GS-6244
(Methyl 3-(2-Quinoxalinylmethylene Carbazate)



Quinoxaline-2-Carboxylic Acid

Figure 2

Summary of ^{14}C-Carbonyl Labeled Carbadox Metabolism in Swine



Residue data

A depletion study of carbadox and desoxycarbadox (MacIntosh et al., 1985) was made in young pigs fed with carbadox-containing rations (55 mg/kg) for one week using a liquid chromatographic method (LOD 2 µg/kg). The presence of carbadox and desoxycarbadox was reported in swine tissues until 72 hours post dose using a single animal at each time point. Results are shown in table 1.

Table 1. Residues (µg/kg) of carbadox and desoxycarbadox in tissues of treated pigs (55 mg/kg, 7 days)

Withdrawal period	Carbadox			Desoxycarbadox		
	Liver	Muscle	Kidney	Liver	Muscle	Kidney
24 hours	<2	19	<2	125	17	186
48 hours	<2	<2	<2	17	9	34
72 hours	<2	<2	<2	<2	<2	<2

This work predicted the possibility of the presence of carbadox and desoxycarbadox in tissues from a pig treated with carbadox that had not been withdrawn.

A pivotal study (Pfizer, 1989c) was conducted in which 10 swine were given unrestricted access to feed containing 55 mg/kg [^{14}C] carbadox (uniformly labelled in the phenyl ring, specific activity of 8.4 µCi/g) for 5 consecutive days and killed at 30, 45 and 70 days, resp., after treatment. Concentration of total residues was measured in tissues (LOD = 1µg/kg).

Table 2. Concentration (µg/kg of carbadox equivalents) of total residues in tissues of treated pigs (55 mg/kg, 5 days)[a]

Withdrawal time (days)	Liver	Kidney	Muscle	Fat
30	74 (50-117)	15 (10-21)	5 (3-6)	2 (1-3)
45	20 (17-21)	5 (4-6)	3 (2-4)	1
70	13 (13-14)	4 (3-4)	2 (2-3)	<1

[a] Numbers in parentheses refer to the range of values obtained.

In another study (Pfizer, 1989d), which was similarly designed, however, in which feed consumption was lower, the tissues were assayed for extractable and bound radioactivity, following sequential extraction with methanol, acetone and n-hexane. The results showed that more than 90% of the total residues in tissues at 30 and 45 days withdrawal time were non-extractable.

The concentration of carbadox and desoxycarbadox in the tissues declined rapidly, and were less than 5µg/kg after 3 days. Extremely low levels of unidentified metabolites remained in the liver at withdrawal periods longer than 7 days. These residues were partially released and converted to quinoxaline-2-carboxylic acid by alkaline digestion of the liver.

The liver tissue was assayed for QCA (as measured by methyl quinoxaline-2-carboxylate) by a method involving alkaline digestion, thin-layer chromatography, gas-1iquid chromatography, and reverse-isotope dilution. The concentration of the residue was 18.9 µg/kg at 30 days withdrawal time, decreasing to 5.5 µg/kg at 45 days, and 1.3 µg/kg at 70 days, representing 24.4 %, 27.5 % and 9.9 % of the total residues respectively.

A study aimed at characterizing the bound residue was conducted using [^{14}C]carbadox (labelled in the phenyl ring). Liver samples obtained after one week following withdrawal of the medicated feed were extracted with enzymes under acidic, neutral, and alkaline conditions. However, no more than 19% of the radioactivity was extractable and no major metabolites could be identified.

Bioavailability studies of the bound residues by *in vivo* methods were not considered feasible because of the low level of residues in the liver tissues at withdrawal periods of four weeks.

Eighteen swine with an average body weight of 25 pounds were fed a ration containing carbadox at 55 mg/kg (50 g/ton) continuously for 47 days until they reached 103 pounds body weight. At this point (zero withdrawal) three swine were sacrificed followed by an additional three swine on each of the days 7, 14, 21, 28, and 35 days of drug withdrawal. The method had a limit of quantification of 30 µg/kg. Residues were below the MRL in liver at 28 days withdrawal. In muscle, they were below the LOQ at all times analyzed. Results are summarized in table 3.

Table 3. QCA levels (µg/kg) in tissues of swine following 47 days of feeding with carbadox (55 mg/kg)

Withdrawal time (days)	Liver	Kidney	Muscle
0	345 (293-400)	210 (142-299)	<LOQ
7	168 (153-186)	<LOQ	<LOQ
14	82.6 (70-102)	<LOQ	<LOQ
21	45.6 (40-49)	<LOQ	<LOQ
28	<LOQ	<LOQ	<LOQ
35	<LOQ	<LOQ	<LOQ

Numbers in parentheses refer to the range of values obtained.

New information about residues of carbadox

Two new studies were provided, supplying new information on depletion of residues of carbadox from pig liver and muscle. In one, QCA residues were measured in the liver of pigs fed medicated feed containing carbadox in combination with oxytetracycline, after a withdrawal period of up to 42 days. In the other study, QCA, carbadox and desoxycarbadox residues were measured during the first 15 days after administration of medicated feed containing 55 mg/kg. This study provided detailed information on depletion of the carcinogenic residues.

Study carried out to determine a withdrawal time on the basis of the MRL for QCA: *Depletion of QCA residue in liver by growing swine after consumption of carbadox and oxytetracycline in combination*

The combination of carbadox and oxytetracyc1ine is indicated for increased rate of weight gain, improved feed efficiency, treatment of bacterial enteritis caused by *Escherichia coli* and *Salmonella cholerae suis* sensitive to oxytetracyc1ine and treatment of bacterial pneumonia caused by *Pasteurella multocida* sensitive to oxytetracyc1ine. In order to gain regulatory approval to use both products in a single feed, it was necessary to demonstrate that oxytetracycline use would not increase the tissue concentration of the carbadox marker residue above its tolerance limit.

Pigs were fed a diet of 27.5 mg/kg (25g/ton) carbadox in combination with 880 mg/kg oxytetracycline (Heird and Spires, 2002). Carbadox was incorporated into the feed of the other seven pens for 28 days and oxytetracycline was added in combination with carbadox for the last 14 days before the withdrawal period. Animals used in the study had an average body weight of approximately 63 pounds when they first began receiving a diet containing carbadox. The average body weight was 119 pounds when withdrawal began and increased to 203 pounds at the time of slaughter of the last group of animals.

Five animals were sacrificed at each of 7 different time points. The livers were analyzed for QCA (Lynch and Bartolucci, 1982) in order to propose a regulatory withdrawal time on the basis of the MRL recommended by the 36th JECFA. All results were corrected for recoveries. Results are presented in Table 4.

Table 4 . QCA depletion (µg/kg) in liver of swine treated with feed containing 27.5 mg/kg of carbadox

Withdrawal time (days)	No. Observations*	Mean	SD
0	5	133	74
7	5	41	9.3
14	5	28.7	11.8
21	5	6.7	3.1
28	4	3.1	0.095
35	1	2.3**	-
42	2	2.15	0.071

* QCA was below the LOD (2 µg/kg) in one of five animals sacrificed at 28 days, in four of the five animals sacrificed at 35 days, and in three of the five animals sacrificed at 42 days after withdrawal of carbadox. LOQ = 5 µg/kg.

** This value constitutes the detectable observation in only one of the five animals

Based on these results, the QCA depletion curve was fitted using data for groups of pigs slaughtered after 7, 14, 21 and 28 days of withdrawal. Data from 35 and 42 days after last dose were not used in the statistical analysis. A linear model of ln [QCA] vs. time was highly significant ($P < 0.0001$) and the lack of fit test departure from linearity was non-significant ($P = 0.0553$).

The statistical methods used were in agreement with methods proposed by the Center for Veterinary Medicine Guideline (FDA, 1994). Based upon the fitted linear model, and using a statistical tolerance limit for the 99 th percentile of the population with 95 % confidence, a withdrawal period of 22 days was calculated to ensure that the liver QCA concentration, calculated as 13.1µg/kg was below the 30 µg/kg tolerance limit

Short term to describe the depletion of residues during the first 15 days after withdrawal of the drug

A short-term withdrawal residue study was conducted using pigs (13 barrow and 13 gilts) with a range of body weights of 100-125 pounds. The animals were fed a diet containing the maximum approved concentration of 50 g/ton of feed (55mg/kg) for 14 days. Three animals were sacrificed at each of the following withdrawal times: 0, 3, 6, 9, 12, 24 hours, 2, 4, 7, 10, and 15 days. The tissues collected were: muscle, liver, kidney, skin, and fat. Medicated diets were prepared by mixing carbadox into the basal diet and carbadox concentrations were analyzed by validated HPLC methods to ensure adequate homogeneity.

Carbadox, desoxycarbadox and QCA were determined directly in untreated samples of the tissues, in whole tissue samples after incubation with USP simulated gastric fluid (pepsin), in whole tissue samples after incubation with USP simulated intestinal fluid (pancreatin), and in the supernatant of samples after treatment with simulated digestive fluids. QCA was determined by the regulatory GC-ECD method. Residues of carbadox and desoxycarbadox were determined quantitatively by liquid chromatography with tandem mass spectroscopy (LC/ACPI-MS/MS) after extraction with acetonitrile. After enzymatic treatment of the samples, residues of carbadox, desoxycarbadox and QCA were determined by LC/ACPI-MS/MS after extraction with ethyl acetate. The reported LOQs were 50 ng/kg for carbadox, 30 ng/kg for desoxycarbadox and 15 µg/kg for QCA.

The results of this residue depletion study are shown in figures 5 and 6 and in tables 5, 6, 7 and 8. QCA was the main residue in liver followed by desoxycarbadox. Pretreatment of the samples with digestive enzymes increased significantly the amounts of residues found in the tissue. In particular the levels of carcinogenic desoxycarbadox increased by a factor of over 4 when the samples were treated with pancreatin prior to extraction and analysis.

QCA was not detected in muscle, except in two samples taken at early withdrawal times. There was a steep decrease in the concentrations of carbadox and desoxycarbadox in muscle during the first few hours following withdrawal of the medicated feed. The only residue remaining quantifiable until the end of the study was desoxycarbadox.

QCA was not detected or quantified in skin or fat. In skin tissue carbadox residue values are higher than in liver or muscle, being desoxy-carbadox values lower than in those tissues. Levels of carcinogenic residues were increased by pretreatment of samples with enzymes in some cases, but only one sample per time point was analyzed. Carbadox

Figure 5: Depletion of residues of carbadox in liver of swine treated with carbadox at 55 mg/kg for 14 days

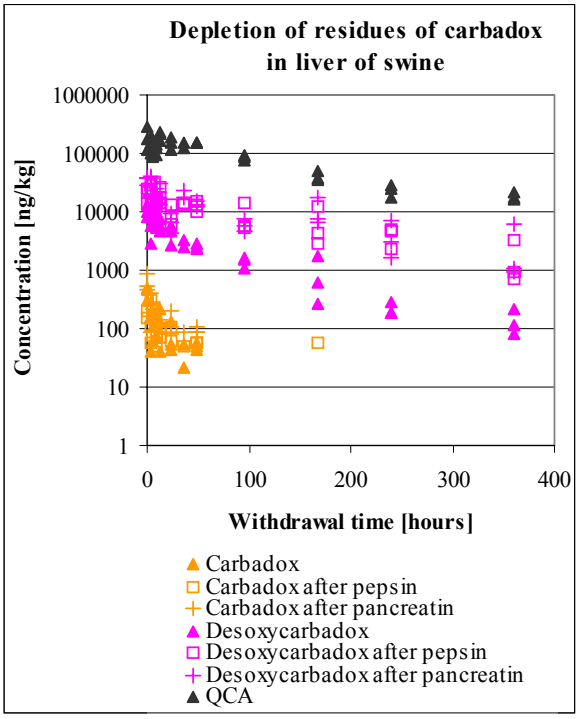
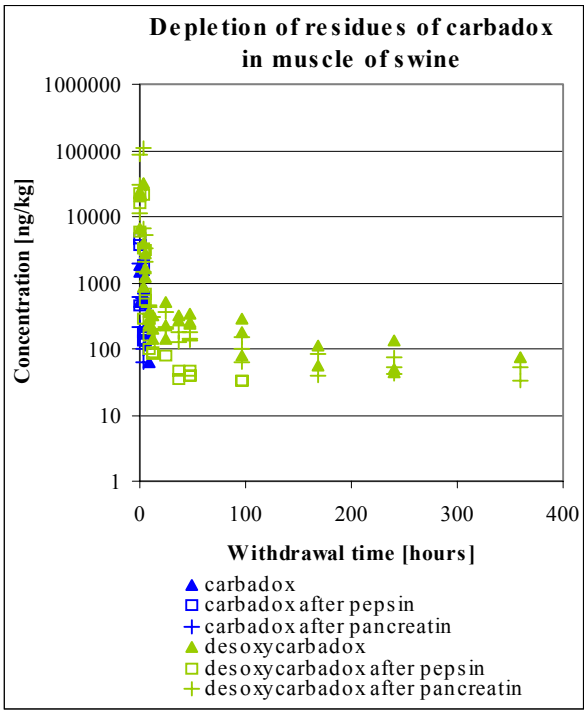


Figure 6: Depletion of residues of carbadox in muscle of swine treated with carbadox at 55 mg/kg for 14 days



was not detectable at 10 days withdrawal in all samples and desoxycarbadox was the only residue quantifiable at 15 days withdrawal.

In fat tissue, enzyme treatment of samples increased carbadox and desoxy-carbadox concentrations only in a few samples during the first hours withdrawal time (only one sample analyzed per time point). Carbadox tissue values were very variable with time, being desoxy-carbadox levels lower than those in liver or muscle. Carbadox and desoxycarbadox were not quantifiable between 7 and 10 days withdrawal.

A single sample of kidney at 0, 6, 12, 24, 48 and 96 hours withdrawal time was analyzed to measure carbadox, desoxy-carbadox and QCA. These samples corresponded to the animal liver values that were the highest at each time point. Carbadox and QCA were non quantifiable or were present in very low values after 0 hour withdrawal time. When compared to liver levels, desoxycarbadox values showed a steep decrease during the first hours and are under 424 ng/kg at 96 hours withdrawal time.

Table 5: Carbadox, desoxy-carbadox and QCA in swine liver tissue, fluid extractions and supernatants

Withd. Time	Carbadox (ng/kg)					Desoxy-carbadox (ng/kg)					QCA (µg/kg)
	Tissue	Gastric fluid	Intestinal fluid	Gastric sup.	Intestinal sup.	Tissue	Gastric fluid	Intestinal fluid	Gastric supernatant	Intestinal supernatant	Tissue
0 hours	423 ± 82	173 ±23	621 ±163	150 ±30	590 ±5	10,506±2,151	20,030±4,308	34,666 ±4,437	4,306 ±59	11,800 ±1435	188 ±69
3 hours	119 ±56	153 ±102	244 ±127	99 ±26	305 ±187	5,510 ±2,151	21,500 ±5,880	32,066 ±9,816	3,102 ±1,714	12,106 ±5,256	152 ±75
6 hours	123 ±55	153 ±57	250 ±30	126 ±36	233 ±88	7,260 ±1,069	10,976 ±2,137	11,246 ±4,716	3,550 ±665	11,233 ±974	123 ±32
9 hours	139 ±47	68 ±13	137 ±23	53 (x)	307 ±49	7,750 ±1,805	14,553 ±1,500	19,466 ±3,350	3,586 ±1,052	16,866 ±2,347	119 ±20
12 hours	128 ±71	85 ±12	122 ±14	99 ±22	252 ±56	5,590 ±1,160	19,166 ±5,253	19,700 ±7,239	3,873 ±627	24,666 ±1,915	201 ±30
24 hours	77 ±40	82 ± 22	131 ±55	85 ±13	226 ±62	4,383 ±1,294	9,066 ±2,826	6,846 ±1,997	3,849 ±2,968	21,200 ±978	150 ±26
36 hours*	37 ±15	50 (x)	87 (x)	NQ, ND	154 ±50	2,970 ±420	13,600 ±3,000	20,700 ±2,900	740 ±202	1,705 ±235	141 ±14
48 hours	50 ±7	57.5±0.5*	93 ±8	NQ, ND	168 ±24	2,230 ±256	12,933 ±2,200	13,600 ±1,267	690 ±66	16,966 ±1,144	152 ±2
96 hours	NQ	NQ	NQ	NQ, ND	83 ±14	1,413 ±265	8,293 ±3,900	6,006 ±1,115	1359 ±980	8,156 ±2,642	86.0 ±7.8
7 days	NQ	56 (x)	NQ, ND	NQ, ND	NQ, ND	875 ±628	6,500 ±4,280	10,390 ±4,970	247 ±194	203 ±280	41.0 ±7.2
10 days	NQ	NQ	NQ, ND	NQ, ND	NQ, ND	219 ±48	4,067 ±1,218	3,930 ±2,244	82.0 ±10.2	667 ±198	23.0 ±4.5
15 days	NQ	NQ, ND	NQ, ND	NQ, ND	NQ, ND	138 ±56	1,613 ±1,131	2,693 ±2,387	79.3 ±28.7	210 ±101	18.3±2.0
Controls	NQ, ND	NQ, ND	NQ, ND	NQ, ND	NQ, ND	NQ, ND	NQ, ND	NQ, ND	NQ, ND	NQ, ND	NQ, ND

Data expressed as the mean (n=3 ±SD). (x) only one detectable value ;* only two values
LOQ Carbadox = 50 ng/kg, Desoxycarbadox = 30 ng/kg, QCA = 15 ng/kg. QCA JECFA MRL = 30 µg/kg
ND= non detected, NQ = non quantificable, ND < NQ < LOQ.

Table 6 : Carbadox, desoxy-carbadox and QCA in swine muscle tissue, fluid extractions and supernatants

Withd. Time	Carbadox (ng/kg)					Desoxy-carbadox (ng/kg)					QCA
	Tissue	Gastric fluid	Intestinal fluid	Gastric sup.	Intestinal sup.	Tissue	Gastric fluid	Intestinal fluid	Gastric supernatant	Intestinal supernatan	Tissue
0 hours	1292±585	2961±1818	946 ±767	874±727*	153 ± 70*	16916 ± 7248	14751± 6635	42351±32063	6522 ±298*	21987±14302	18 (x)
3 hours	990 ± 369	732 ± 814	250 ±241	185 ±168	94 ± 40*	11989±13700	8245 ± 9188	38851±49176	3557 ±4471	1592 ± 18510	16 (x)
6 hours	491 ± 228	147 ± 17*	119 (x)	108 (x)	NQ	1881 ± 662	1452 ± 1210	3591 ± 1308	472 ± 132	3376 ± 756	ND
9 hours	64 (x)	NQ, ND	NQ, ND	NQ, ND	NQ	299 ± 55	182 ± 78	445 ± 14	234 ± 38	2283 ± 24	ND
12 hours	ND	NQ, ND	NQ, ND	NQ, ND	NQ	211 ± 72	86.5 ± 2.5	230 ± 47	136 ± 30	141 ± 37	ND
24 hours	NQ, ND	NQ, ND	NQ, ND	NQ, ND	NQ	295 ± 156	77 (x)	258 ± 69	132 ± 50	104 ± 30	ND
36 hours	NQ, ND	ND	NQ, ND	ND	NQ	295 ± 32*	42 ± 6*	152 ± 23*	ND	38 ± 2*	ND
48 hours	ND	ND	NQ, ND	ND	111 (x)	280 ± 44	41 ± 3	153 ± 20	30 (x)	39 ± 6	15 (x)
96 hours	ND	ND	NQ, ND	ND	ND	182 ± 85	33.5 ± 0.5*	103 ± 34	NQ	31 (x)	ND
7 days	ND	ND	NQ, ND	ND	NQ, ND	83 ± 28*	NQ	62.5 ± 22.5*	NQ	NQ	ND
10 days	ND	ND	NQ, ND	ND	ND	76 ± 41	NQ	56.0 ± 13.0	NQ	NQ	ND
15 days	ND	ND	NQ, ND	ND	ND	74 (x)	NQ	43.0 ± 9.0*	NQ	NQ	ND
Controls	NQ, ND	NQ, ND	NQ, ND	ND, NQ	ND, NQ	ND, NQ	ND,NQ	ND,NQ	NQ, ND	NQ, ND	ND,NQ

Data expressed as the mean (n=3 ± SD) . (x) only one detectable value ; * only two values
LOQ Carbadox = 50 ng/kg, Desoxycarbadox = 30 ng/kg, QCA = 15 ng/kg. QCA JECFA MRL = 5 µg/kg
ND= non detected, NQ = non quantificable, ND < NQ < LOQ.

Table 7: Carbadox, Desoxy-carbadox and QCA in Swine Skin Tissue, Fluid Extractions and Supernatants

Withd. Time	Carbadox (ng/kg)					Desoxy-carbadox (ng/kg)					QCA
	Tissue [a]	Gastric fluid [b]	Intestinal fluid [b]	Gastric sup.[b]	Intestinal sup.[b]	Tissue [a]	Gastric fluid [b]	Intestinal fluid [b]	Gastric sup.[b]	Intestinal sup.[b]	Tissue [b]
0 hours	1843 ±1034	1290	382	1520	174	6560 ±2342	143	15300	127	10800	NQ
3 hours	1062 ± 640	647 ± 37	186 ±26	1320±409	303 ±102	6476 ±7594	111 ±58	11765 ±5935	57 ±20	8235 ±4465	NQ
6 hours	782 ± 277	339	150	227	167	1800 ± 652	73	2560	41	933	NQ
9 hours	1167 ± 456	95	853	736	147	1224 ±378	3440	35	43	2270	ND
12 hours	649 ± 277	210	816	756	81	538 ± 83	3420	40	37	2570	ND
24 hours	773 ± 446	172	625	907	166	732 ± 513	5540	107	93	3700	ND
36 hours	585 ± 348*	107	810	690	96	374 ± 11	2900	NQ	43	2160	ND
48 hours	145 ± 38	75	197	185	73	223 ± 101	931	39	42	613	ND
96 hours	98 ± 29*	78	610	67	53	185 ± 86	312	NQ	NQ	222	ND
7 days	NQ,ND	NQ	76	NQ	NQ	83 ± 34	253	NQ	NQ	216	ND
10 days	ND	NQ	NQ	NQ	NQ	77 ± 28	144	NQ	45	108	ND
15 days	NQ,ND	NQ	NQ	ND	NQ	58 ± 8	100	NQ	NQ	94	ND
Controls	NQ, ND	ND	NQ, ND	ND,NQ	ND,NQ	ND,NQ	ND,NQ	ND,NQ	NQ, ND	NQ, ND	ND,NQ

[a] Data expressed as the mean (n=3 ± SD) ; [b]only one data per time point, 36 hours two values.
(x) only one detectable value ; * only two detectable values
LOQ Carbadox = 50 ng/kg, Desoxycarbadox = 30 ng/kg, QCA = 15 µg/kg.
ND= non detected, NQ = non quantificable, ND < NQ < LOQ.

Table 8: Carbadox, Desoxy-carbadox and QCA in Swine Fat Tissue, Fluid Extractions and Supernatants

Withd. Time	Carbadox (ng/kg)					Desoxy-carbadox (ng/kg)					QCA
	Tissue [a]	Gastric fluid [b]	Intestinal fluid [b]	Gastric sup.[b]	Intestinal sup.[b]	Tissue [a]	Gastric fluid [b]	Intestinal fluid [b]	Gastric sup.[b]	Intestinal sup.[b]	Tissue [b]
0 hours	444 ± 371	NQ	NQ	86	NQ	7710 ±3790	102	9210	42	6120	ND
3 hours	134 ± 77	NQ	NQ	NQ	61	5935± 6231	83	7780	72	10400	NQ
6 hours	526 (x)	NQ	NQ	NQ	80	1131± 538	313	NQ	NQ	257	ND
9 hours	456 ± 214	NQ	NQ	NQ	NQ	336 ± 81	NQ	110		118	ND
12 hours	323 ± 78	NQ	NQ	66	114	206 ± 32	NQ	70	NQ	71	ND
24 hours	1235 ± 703	NQ	NQ	137	113	159 ± 20	NQ	61	NQ	56	ND
36 hours	609 ± 65	NQ	NQ	NQ	90	93 ± 14	NQ	45	NQ	73	ND
48 hours	663 ± 319	ND	NQ	135	58	106 ± 68	NQ	41	NQ	39	ND
96 hours	353 ± 157	NQ	NQ	NQ	NQ	33 ± 2	NQ	33	NQ	NQ	ND
7 days	NQ	NQ	NQ	NQ	NQ	NQ	NQ	32	NQ	NQ	ND
10 days	NQ,ND	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	ND
15 days	NQ	NQ	NQ	NQ	NQ	NQ	ND	NQ	NQ	NQ	ND
Controls	NQ, ND	ND	NQ, ND	ND,NQ	ND,NQ	ND,NQ	ND,NQ	ND,NQ	NQ, ND	NQ, ND	ND,NQ

[a] Data expressed as the mean (n=3 ± SD) ; [b]only one data per time point, 36 hours two values.
(x) only one detectable value ; * only two detectable values
LOQ Carbadox = 50 ng/kg, Desoxycarbadox = 30 ng/kg, QCA = 15 µg/kg.
ND= non detected, NQ = non quantificable, ND < NQ < LOQ

Other relevant residue studies

US-FDA Supplement Evaluation of Carbadox (NADA 041-061) providing information for establishment of a 42 day slaughter withdrawal period for carbadox in swine tissues.

The Food and Drug Administration of the United States of America has published a residue study on which the legally established withdrawal time for carbadox was based. Thirty-four crossbred pigs (17 gilts and 17 barrows) were given feed containing 55 mg/kgcarbadox. Pigs were killed at 14, 21, 28, 35, 42 and 49 days post dose. Muscle and liver were collected from each animal for QCA residue analysis. All of the tissue samples were analyzed in triplicate. Residues of QCA were determined using the regulatory GC-EC method for QCA (Lynch and Bartolucci, 1982) described above. The method has a limit of quantification of 5 µg/kg. Results are shown in Table 9.

Table 9. Residues of QCA (µg/kg) in tissues of swine fed carbadox at 55 mg/kg for 28 days.

Withdrawal time (days)	No. of Animals	QCA concentration (µg/kg)	
		Liver	Muscle
14	5	51.93 ± 15.14	< LOQ*
21	5	29.09 ± 8.20	< LOQ
28	5	17.72 ± 4.72	< LOQ
35	5	11.23 ± 1.86	< LOQ
42	3	11.16 ± 2.13	< LOQ
49	1	10.90 ± 2.35	< LOQ

* LOQ liver, muscle = 5 µg/kg; LOD liver, muscle = 2 µg/kg

For the purpose of establishing a withdrawal period only the liver residues were used. The withdrawal period was based on a statistical analysis of the depletion data, using an upper tolerance limit containing 99 % of the population with a 95% confidence limit. Using the uncorrected residue data for liver from days 14 to 49, a withdrawal period of 39.34 days was calculated. Based on this data, a 42-day withdrawal time was established.

Comparative Depletion of Residues in liver: estimation of depletion times

The kinetics of the depletion of residues in tissues of treated swine were compared for the above three studies and two additional previous studies mentioned in the report of the 36th meeting of JECFA (Pfizer, 1989 and MacIntosh, 1985).

A number of studies were reviewed and – where possible - quantitatively evaluated using statistical methods, such as linear regression and calculation of statistical tolerance limits and depletion times. The following equation was generally used to describe the depletion of residues:

$$\log_{10} C_t = \log_{10} a + b \times t$$

where C_t is the predicted concentration of the residue at t, a given withdrawal time, a is the concentration extrapolated for zero withdrawal time and b is a rate constant describing the depletion. For all studies and all calculations t was expressed in hours. Upper limits of the 95% or 99% confidence interval for the upper one-sided tolerance limits on the 95th or 99th percentile were calculated for selected relevant studies as a function of the depletion time.

For QCA depletion in liver (Figure 7, table 10) the two newly submitted studies show striking similarities in the kinetic parameters which are reflected in similar calculated depletion times to MRL (11 and 10 days respectivelly), despite a twofold difference in the dose level.

On the other hand, there are significant differences in kinetic parameters between, on the one side, one of the studies evaluated at the 36th Meeting (Pfizer, 1989) and the study published by FDA and, on the other side, the new short term study, although all three studies had been performed at the same dose level. These differences have very significant influence on depletion times calculated from these data sets.

Table 10: Comparison of parameters of four different studies describing the depletion kinetics of QCA in liver of swine.

Study	Cabadox concentration mg/kg /days of feeding	Parameters of linear regression analysis			Depletion time to < MRL (days)	
		a[+]	b [hour^{-1}]	r	Based on averages	Based on tolerance limits
Pfizer 1989	55/47	5.5239	-0.0018	-0.9867	25	*
FDA 1998	55/28	4.8823	-0.0008	-0.9285	21	39.3
Phibro 2002, QCA study	27.5/42	5.1488	-0.0025	-0.9419	11	21.5
Phibro 2002, short term study	55/14	5.2027	-0.0029	-0.9292	10	16.8

[+] a is dimensionless (contents of the residues given in ppt)
* calculation not possible; individual data points are not published.

A comparison was also made between the depletion kinetics determined for desoxy-carbadox in liver and muscle of swine in two studies using the same dose level of 55 mg/kg of parent carbadox (short-term depletion, 14 days and MacIntosh, 1985, 7 days, second study only two data points). While the results obtained for residues in livers were quite similar, there was seemingly a large difference in the results obtained for muscle.

Table 11a summarizes the kinetic characteristics of the short term depletion of the relevant residues of carbadox in liver of swine treated with 55 mg/kg of Carbadox.

The values in Table 11a demonstrate that for the data sets relating to residues of desoxycarbadox the linear regression model fits satisfactorily to the data. This is less true for the carbadox residue data, in particular for the data obtained after treatment of the tissues with pepsin.

Treatment with digestive enzymes increases the recovered concentrations of both, carbadox and desoxy-carbadox. However, the effect is significant only with residues of desoxy-carbadox. Pancreatin is more effective than pepsin.

Figure 7: Comparison of the results of four different QCA depletion studies in liver of swine.

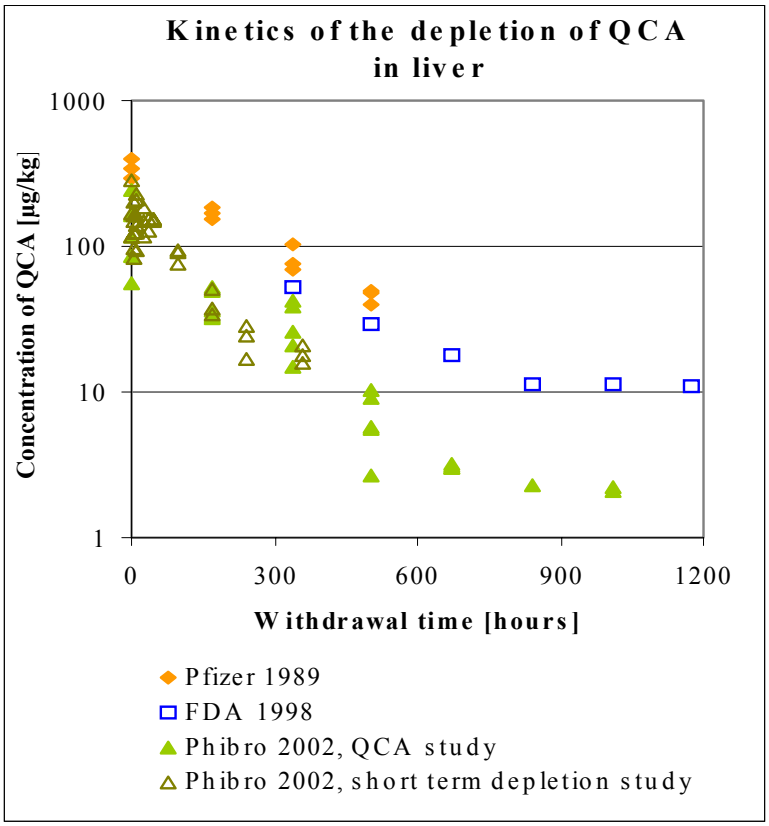


Table 11: Parameters of the kinetics of depletion of residues of carbadox in liver of swine - influence of the treatment of the tissue samples with digestive enzymes:

a) influence on the parameters obtained by linear regression

Residue	Enzymatic treatment	Parameters of the depletion kinetics			
		a[+]	b [hour^{-1}]	r	n
QCA	None	5.2027	-0.0029	-0.9292	35
Carbadox	None	2.2418	-0.0147	-0.6489	23
	Pepsin	2.0182	-0.0026	-0.4156	19
	Pancreatin	2.4231	-0.0120	-0.6519	22
Desoxy-carbadox	None	3.7876	-0.0053	-0.9464	35
	Pepsin	4.2042	-0.0029	-0.8707	35
	Pancreatin	4.2651	-0.0029	-0.7767	35

[+] a is dimensionless (contents of the residues given in ppt)

b) influence of the time required for the residues to deplete to certain limits/concentration

Residue	Regulatory Limits		Depletion times [days after withdrawal of medicated feed] on the basis of:						
	Type of Limit	Numerical value	averages	1-α 95	1-γ 95	1-α 99	1-γ 95	1-α 99	1-γ 99
QCA	MRL	30µg/kg	10.3	15.1		16.8		18.0	

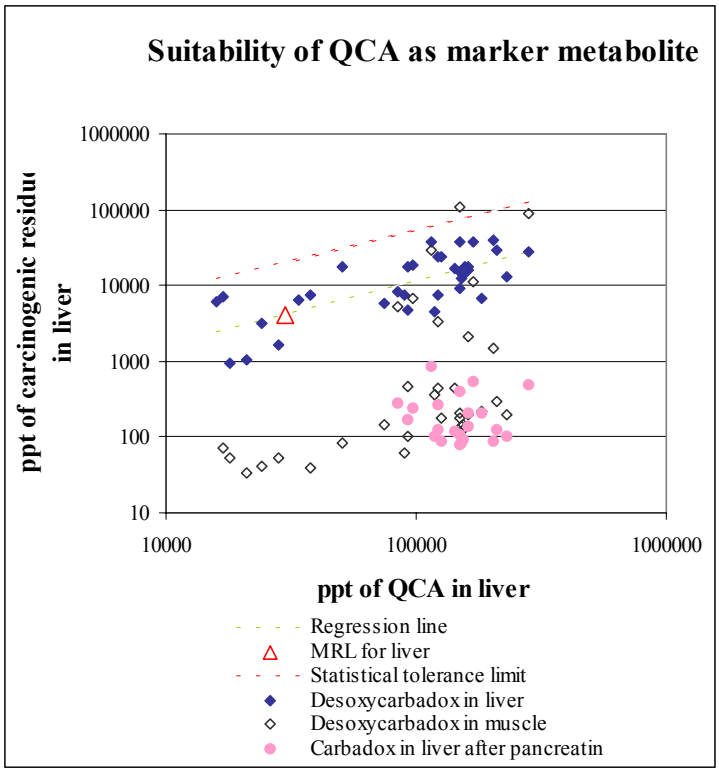
Table 11b provides estimates of required depletion times for QCA. Calculations have been performed on the basis of average concentrations of residues as well as on the basis of (statistical) tolerance limits. Three cases have been considered with respect to the percentiles and upper confidence limits chosen. (95% confidence interval on the 95th percentile, 99% confidence interval on the 95th percentile, 99% confidence interval on the 99th percentile).

Relationship between QCA and carcinogenic residues

The data of the short term depletion study were also used to establish a relationship between the concentrations of the proposed marker metabolite QCA in the target tissue liver and the carcinogenic residues carbadox and desoxy-carbadox in liver and muscle respectively. There is seemingly a linear relationship between the logarithms of the concentrations of QCA and desoxy-carbadox in liver (Figure 8). The relationship between QCA in liver and desoxycarbadox in muscle can only be used over a narrow range of concentrations.

At the MRL for QCA in liver, the average concentrations of the carcinogenic residue desoxy-carbadox in liver estimated by regression analysis were about 4 µg/kg.

Figure 8: Relationship between concentrations of QCA in liver and the concentrations of carcinogenic residues in liver and muscle



METHODS OF ANALYSIS

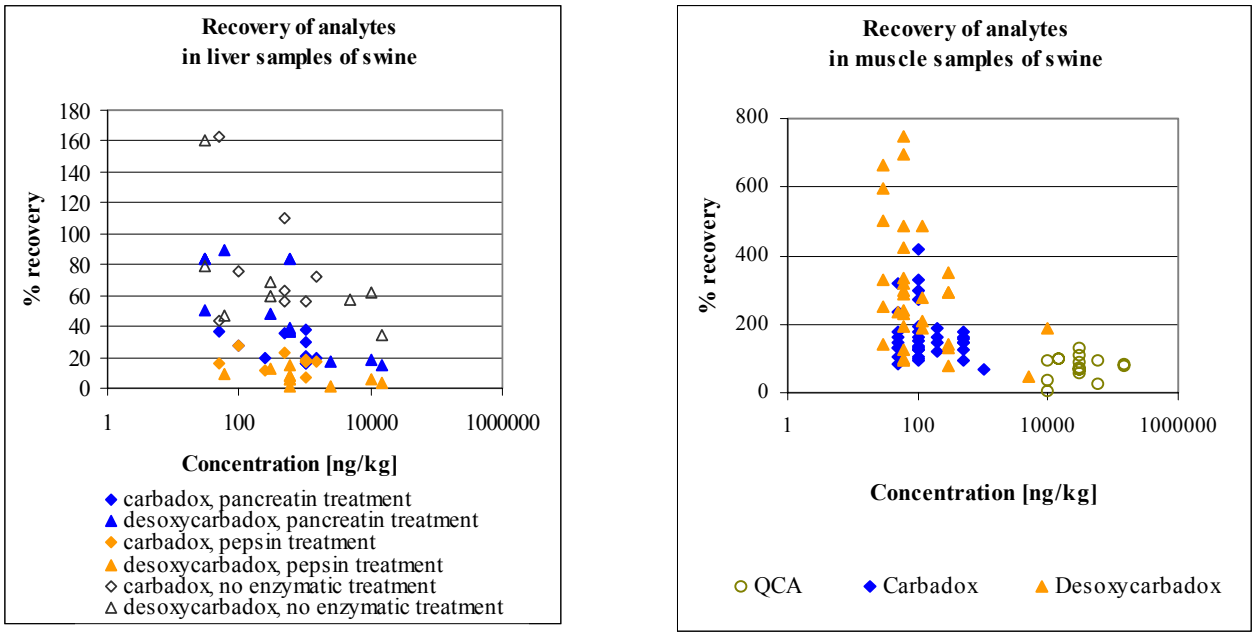
Two methods of determination were used in the three new residue studies considered by the Committee. The recognized regulatory method, based on GC-ECD and accepted by the Committee at its 36th meeting (Lynch and Bartolucci, 1982), was used in two studies to determine residues resulting from treatment of pigs at the dose rate stipulated on the label and at one-half that rate. This method involves alkaline hydrolysis digestion to release bound residues and conversion of any carbadox parent compound or related metabolites present in the tissue to the marker residue, QCA. The principle of the analytical method is as follows: a homogenized tissue sample is hydrolyzed in an alkaline medium, cooled and the hydrolysate acidified. QCA is extracted with ethylacetate. It is then re-extracted from the organic phase using a citric acid buffer. This extract is further cleaned on an ion-exchange column from which QCA is eluted with 14% methanol. After partitioning into chloroform, the solvent is evaporated and the residue is derivatised with n-propanol/sulfuric acid to form the propyl ester. The ester is extracted into toluene and measured using gas chromatography/electron capture detection. Average recoveries were 109.4 % at 10 µg/kg and 80.9% at 50 µg/kg.

The method was used for liver in the one-half dose study and for liver and muscle in the study at the recommended dose rate, with a reported LOQ of 0.005 mg/kg and LOD of 0.002 mg/kg.

A new method based on liquid chromatography-atmospheric pressure chemical ionization tandem mass spectrometry (LC/APCI-MS/MS) was developed for use in a recent study to measure the concentrations of carbadox, desoxycarbadox and QCA directly in tissues (Method EXM-049-037), or in the whole sample or supernatants obtained following treatment with simulated digestive fluids (pepsin or pancreatin) (Method EXM-049-037A). Quantification is based on measurement of a product ion separated in the second stage of MS/MS after fragmentation of a precursor ion formed from the parent molecule in the first stage of MS/MS. This method was developed using liver as this represents the most difficult matrix to be used. Liver was preprocessed by grinding to a powder like consistency in the presence of dry ice. A 1g subsample was extracted with acetonitrile, centrifuged, partitioned in isooctane and evaporated to 2 ml. The final extract was analyzed by LC/MS/MS using a gradient (acetonitrile:water, 14 min) high performance liquid chromatograph system with a phenyl-hexyl column (150 x 4.6 mm x 3µm) and a variable volume injector capable of injecting 25 µl connected with a high sensitivity triple quadrupole Mass Spectrometer via an Atmospheric Pressure Electrospray Ionization Source (700ºC). Precursor to product ion transitions are monitored for each compound and plotted against an external standard curve (carbadox mass transition 263 ➔231, desoxy-carbadox mass transition 231 ➔ 199).(Retention time: carbadox 5.55 min, desoxy-carbadox 6.01 min)

When samples were incubated with digestive fluids the extraction procedure was different. In these cases sodium sulfate was added to the samples and the residues were three times extracted into ethyl acetate. In the case of pancreatin digestion formic acid was added prior to the third extraction with ethyl acetate. The ethyl acetate was evaporated and the residues were reconstituted into acetonitrile. When residues were determined in supernatants of the enzyme digestion the fluids were simply centrifuged and the clear supernatants were directly used for analysis.

Figure 9 (a, b) : Recoveries of analytes in swine tissues.



A linear response was found across the analytical range, with reported LOQs of 50 ng/kg for carbadox, 30 ng/kg for desoxycarbadox and 0.015 mg/kg for QCA. Analyte recoveries were generally variable with the liver samples obtained in this study and decreased to low levels when digestive enzymes were used prior to extraction (see figure 9a). Typically two fortified samples were analyzed under the same conditions with every set of samples in order to estimate recovery. No suitable internal standard was available for recovery correction. When muscle tissues were analyzed, the variability of the analyte recoveries

was even higher – depending on analyte and on sample treatment. The reasons for the unusually high "recoveries" of several hundred percent were not explained in the study. The results are shown in figure 9b.

Analysis of carbadox, desoxycarbadox and QCA after a 4 hour incubation with digestive enzymes showed that carbadox and desoxycarbadox were unstable in the samples treated with pepsin, but pancreatin has little effect on both compounds. In the samples incubated with liver, once again carbadox and desoxycarbadox were unstable in pepsin treated samples, but a decreased was noted for desoxycarbadox in pancreatin treated ones. QCA concentration was unaffected by the enzyme treatment. Results are shown in table 12 and table 13.

Table 12: Carbadox, Desoxycarbadox and QCA in Gastric and Intestinal Fluids: Recovery vs time

	Gastric Fluid: % Recovery			Intestinal Fluid: % Recovery		
Time	**Carbadox**	**Desoxy-C**	**QCA**	**Carbadox**	**Desoxy-C**	**QCA**
0 hours	18.4 ± 12.2	33.6 ± 10.1	98.4 ± 6.7	61.2 ± 5.1	94.1 ± 12.5	31.2 ± 13.7
4 hours	4.34 ± 3.15	0	118 ± 15	86.0 ± 5.9	77.6 ± 6.2	29.1 ± 12.6

Data expressed as the mean (n=12 ± SD)
Most measured values are under the respective LOQ for each compound.

Table 13: Carbadox, Desoxycarbadox and QCA in Gastric and Intestinal Fluids with liver: Recovery vs time

	Gastric Fluid: % Recovery			Intestinal Fluid: % Recovery		
Time	**Carbadox**	**Desoxy-C**	**QCA**	**Carbadox**	**Desoxy-C**	**QCA**
0 hours	39.3 ± 11.1	114 ± 22	42.7 ± 3.3	34.6 ± 6.9	104 ± 33	26.8 ± 7.0
4 hours	13.5 ± 6.8	38.4 ± 33.3	34.2 ± 5.5	4.6 ± 6.9	74.7 ± 10.3	31.9 ± 10.7

Data expressed as the mean (n=12 ± SD)
Most measured values are under the respective LOQ for each compound

APPRAISAL

As no new data on the genotoxic or carcinogenic nature of carbadox and its metabolites had been generated since the previous evaluation, the Committee was again unable to establish an ADI.

New studies were, however, which were provided at the present meeting supplied information on depletion of residues of carbadox in pig liver, muscle, fat and skin. One of the studies, which is ongoing and subject to further evaluation, covers the first 15 days after withdrawal of medicated feed and provides detailed information on depletion of the carcinogenic residues. The results significantly change the information base from that available at the time of the first evaluation.

In reaching its decision on MRLs for carbadox, the Committee at its 36th meeting took various factors into consideration, including the following, which are now fully or partially invalid:

- The Committee concluded that carbadox and desoxycarbadox could be detected in tissues only for the first 72 h after treatment, and their concentrations 28 days after withdrawal are negligible. The new factor is the availability of a new HPLC/MS/MS method with limits of quantification of 50 and 30 ng/kg for carbadox and desoxycarbadox, respectively. With the improved performance of the method, carbadox could be determined quantitatively in liver only up to 48 h, but desoxycarbadox was present in quantifiable concentrations until the end of the study, 15 days after the last administration of medicated feed.
- The Committee at its 36th meeting concluded that more than 90% of the total residues in tissues were bound and could not be extracted 28 days after withdrawal. It agreed that bound residues in pig liver 28 days after treatment would not represent a risk for consumers. With the analytical procedures available at that time, QCA was the only carbadox metabolite that could be identified in liver from pigs treated according to good practice in the use of veterinary drugs. In the new study, the methods included treatment of samples with digestive enzymes (USP systems that mimic gastric and intestinal fluids, respectively). With these techniques, the amounts of desoxycarbadox that could be released from liver tissues were increased by two- to fourfold. The possibility cannot be excluded that, with these techniques, desoxycarbadox could be released at times beyond the 15 days of the duration of the present study.
- The Committee at its 36th meeting also concluded that the amount of QCA extracted by alkaline hydrolysis was less than 30 µg/kg 28 days after withdrawal. Practical analytical methods were available for measuring QCA at concentrations down to 30 µg/kg in liver and 5 µg/kg in muscle. On the basis of studies on the toxicity of QCA and on the metabolism and depletion of carbadox and the nature of the compounds released from bound residues, the Committee concluded that residues resulting from the use of carbadox in pigs were acceptable, provided the concentrations of QCA were below 30 µg/kg in liver and below 5 µg/kg in muscle. The Committee recommended MRLs of 30 µg/kg in liver and 5 µg/kg in muscle of pigs, based on the concentrations of, and expressed as, QCA. While the new studies confirmed the good correlation between the concentrations of QCA and desoxycarbadox in liver and also confirmed the time required to deplete QCA to less than 30 µg/kg, they also showed that desoxycarbadox is still present in liver when the concentrations

of QCA have reached the MRL. Calculation of the relationship between the concentrations of the two metabolites by the Committee by linear regression of the logarithms of the concentrations showed that 30 µg/kg of QCA in liver corresponded to approximately 4 µg/kg of desoxycarbadox. The tolerance limits for the concentration of desoxycarbadox were several times higher owing to the wide variation of the data. Therefore, QCA is not a suitable marker for monitoring carcinogenic metabolites of carbadox in liver in compliance with the MRL recommended by the Committee at its thirty-sixth meeting and QCA does not ensure the absence of carcinogenic residues. QCA is also not a suitable marker for ensuring the absence of carcinogenic residues in muscle.

New studies

Two new studies were provided, supplying new information on depletion of residues of carbadox in pig liver and muscle. In one, QCA residues were measured in the liver of pigs fed medicated feed containing carbadox in combination with oxytetracycline, after a withdrawal period of up to 42 days. In the other study, QCA, carbadox and desoxycarbadox residues were measured during the first 15 days after administration of medicated feed containing 55 mg/kg; this study provided detailed information on depletion of the carcinogenic residues.

The study to determine depletion of QCA residue in pig liver after administration of carbadox and oxytetracycline involved 35 pigs fed a diet containing carbadox at 28 mg/kg (25 g/ton) in combination with 880 mg/kg oxytetracycline. The pigs were treated with carbadox for 28 days and in combination with oxytetracycline for an additional 14 days. Five animals were killed at each of seven times, and their livers were analyzed for QCA by gas chromatography with electron capture detection (GC-EC) (limit of quantification [LOQ], 5 µg/kg). The mean QCA concentrations were 130 µg/kg at 0 days, 41 µg/kg at 7 days, 29 µg/kg at 14 days, 7 µg/kg at 21 days, 3 µg/kg at 28 days, 2 µg/kg (one value) at 35 days and 2 µg/kg at 42 days.

In the short-term study to investigate depletion of residues during the first 15 days after withdrawal of the drug, 34 pigs were fed a diet containing the maximum approved concentration of 50 g/t of feed (55 mg/kg) for 14 days. Three animals were killed 3, 6, 9, 12 and 24 h and 2, 4, 7, 10 and 15 days after withdrawal, and the concentrations of carbadox, desoxycarbadox and QCA were determined either directly in tissues, in whole tissue samples after incubation with USP simulated gastric fluid (pepsin) and USP simulated intestinal fluid (pancreatin) or in the supernatant of samples after treatment with simulated digestive fluids. QCA was determined by GC-ECD. Residues of carbadox and desoxycarbadox were determined quantitatively by liquid chromatography with tandem mass spectroscopy (LC-MS/MS) after extraction with acetonitrile. After enzymatic treatment of the samples, residues of carbadox, desoxycarbadox and QCA were determined by LC-MS/MS after extraction with ethyl acetate. The reported LOQs were 50 ng/kg for carbadox, 30 ng/kg for desoxycarbadox and 15 µg/kg for QCA.

In this study, QCA was the main metabolite in liver, followed by desoxycarbadox. Carbadox depleted in liver from 420 ng/kg at 0 h to 50 ng/kg 2 days after withdrawal, desoxycarbadox depleted from 10 500 ± 2200 ng/kg at 0 h to 140 ± 56 ng/kg 15 days after withdrawal, and QCA depleted from 190 000 ± 69 000 ng/kg at 0 h to 18 000 ± 2000 ng/kg 15 days after withdrawal (standard deviations are included to indicate the wide variation of the results).

Pretreatment of the samples with digestive fluids increased the amounts of carcinogenic residues found in all tissues. In liver, the concentration of carbadox increased to 620 ± 160 ng/kg at the time of withdrawal but was not quantifiable 10 days later. The concentration of desoxycarbadox increased by more than fourfold when the samples were treated with intestinal fluid, and large quantities were present 15 days after withdrawal (35 000 ± 4400 ng/kg at 0 h, 3000 ± 2400 ng/kg 15 days after withdrawal).

QCA was detected in muscle tissue only in two samples taken 0 and 3 h after withdrawal. The concentrations of carbadox and desoxycarbadox decreased steeply in all muscle samples during the first few hours after withdrawal of the medicated feed, and carbadox was not detectable 12 h after withdrawal. The only residue that was quantifiable up to the end of the study (15 days) was desoxycarbadox, which was found in very small quantities (74 ng/kg in tissue, 43 ng/kg after intestinal fluid treatment). QCA was not detected in skin or fat. Carbadox was not detectable in skin tissue 10 days after withdrawal, and desoxycarbadox was the only residue quantifiable 15 days after withdrawal, occurring in small quantities (< 100 ng/kg). Neither carbadox nor desoxycarbadox was quantifiable 7 days after withdrawal. One sample of kidney from an animal in which the values in liver were the highest at each time was analyzed 0, 6, 12, 24, 48 and 96 h after withdrawal. QCA was not detected 24 h after withdrawal, and no carbadox was found 48 h after withdrawal. Desoxycarbadox deplete quickly, from 22 000 ng/kg at 0 h to 420 ng/kg 96 h after withdrawal.

In a study on carbadox residues in the USA, 34 crossbred pigs (17 gilts and 17 barrows) were given feed containing carbadox at 55 mg/kg for 28 days. Muscle and liver were collected from each animal for determination of QCA residues by the regulatory GC-EC method (LOQ, 5 µg/kg). The concentration of residue in muscle was below the LOQ. Those of QCA were 52 µg/kg 14 days after withdrawal, 29 µg/kg at 21 days, 18 µg/kg at 28 days, 11 µg/kg at 35 days, 11 µg/kg at 42 days and 11 µg/kg at 49 days. The concentration of QCA was less than 30 µg/kg 28 days after withdrawal.

Methods of analysis

Two methods of determination were used in the three new residue studies considered by the Committee. The recognized regulatory method, based on GC-ECD and accepted by the Committee at its thirty-sixth meeting, was used in two studies to determine residues resulting from treatment of pigs at the dose rate stipulated on the label and at one-half that rate. This method involves alkaline hydrolysis digestion to release bound residues and conversion of any carbadox parent compound or related metabolites present in the tissue to the marker residue, QCA. The method was used for liver in the one-half dose study

and for liver and muscle in the study at the recommended dose rate, with a reported LOQ of 0.005 mg/kg. This analytical method has been routinely used in many regulatory laboratories for over a decade.

A new method based on liquid chromatography-atmospheric pressure chemical ionization tandem mass spectrometry (LC/APCI-MS/MS) was developed for use in a recent study to measure the concentrations of carbadox, desoxycarbadox and QCA. The method had better specificity than the regulatory GC-ECD method. Quantification is based on measurement of a product ion separated in the second stage of MS/MS after fragmentation of a precursor ion formed from the parent molecule in the first stage of MS/MS. A linear response was found across the analytical range, with reported LOQs of 50 ng/kg for carbadox, 30 ng/kg for desoxycarbadox and 0.015 mg/kg for QCA. The reported LOQs may be conservative, as they were the lowest concentrations at which the method was tested to meet the requirements of precision and recovery, but they do not necessarily represent the lowest possible concentrations that might be found experimentally. The method was tested on liver, kidney, muscle, skin and fat, with acceptable recoveries from all tissues except muscle at or above the concentrations noted above. The recovery from muscle was in the range of 400% at concentrations < 1000 ng/kg, suggesting that the external calibration used was unsuitable due to matrix effects. Enhancement due to the matrix may occur in analytical methods for traces based on mass spectral detection. The method offers excellent capability for the determination of trace concentrations of carbadox and desoxycarbadox. It supplements the GC-ECD regulatory method for determination of total residues of toxicological concern and allows determination of the contribution of parent carbadox and dexoxycarbadox to the residues measured as QCA. Additional product ions are available, which, while not used for quantification, may provide additional information for confirmation. Data to support recognition of the method as a confirmatory method were not provided for evaluation.

The LC-MS/MS method was also developed for analysis of supernatants derived from digestion of tissues containing carbadox residues with two enzyme systems considered to be representative of gastrointestinal processes. After a 4-h digestion with the enzymes, both carbadox and desoxycarbadox were found to be unstable when treated with pepsin, but pancreatin had little effect on either compound. Digestion with the addition of liver to the fluid also showed the instability of carbadox and desoxycarbadox with pepsin treatment and a decrease in the concentration of desoxycarbadox after pancreatin treatment. QCA recovery was not affected by either treatment.

Relationship between QCA and carcinogenic residues

The results of the short-term depletion study were also used to establish a relationship between the concentration of the marker metabolite QCA in the target tissue, liver, and of the carcinogenic residues carbadox and desoxycarbadox in liver and muscle, respectively. A good linear relationship was found between the logarithms of the concentrations of QCA and desoxycarbadox in liver, but no such relationship was determined for muscle tissue. For a concentration of QCA in liver of 30 µg/kg, the average concentration of the carcinogenic residue desoxycarbadox in liver was estimated by regression analysis to be about 4 µg/kg.

Conclusions

The new data confirm that carcinogenic residues, in particular desoxycarbadox, are present in edible tissues during the depletion of parent carbadox. The relatively long persistence of the residues was a new finding. The results also show that, after administration of the highest recommended dose of 55 mg/kg in feed, QCA depletes to below the MRL for liver recommended by the Committee at its 36th meeting within a short time (approximately 17 days on the basis of the upper limit of the 95% confidence interval on the 99th percentile).

The experiments conducted with digestive enzymes showed that the true concentrations of the carcinogenic metabolites in tissues cannot yet be estimated with certainty, since an unknown portion of the releasable residue is destroyed during incubation with the enzymes. Therefore, the total residue measured in the supernatant after enzyme digestion and in the remaining tissue represents a lower estimate of the total present in the tissue. The fraction of this residue that could be considered to be bioavailable might be lower, but this value cannot be determined with reasonable certainty

As the Committee was unable to allocate an ADI for carbadox, there is no accepted reference point for comparison with the new data on residues. Therefore, on the basis of the new data, the MRL for QCA recommended by the Committee at its thirty-sixth meeting is not supported for determining residues of carbadox of toxicological concern in liver.

The MRL of 5 µg/kg recommended by the Committee at its thirty-sixth meeting for QCA in muscle is not supported by the new data. Desoxycarbadox was found at all times up to 15 days, but QCA was found in only two samples collected 0 and 3 h after withdrawal. Therefore, the relationship between the concentrations of QCA and desoxycarbadox is not known.

After reviewing the new studies, the Committee could not determine the amounts of residues of carbodox in food that would have no adverse health effects in consumers. The Committee decided to withdraw the MRLs of carbadox recommended by the Committee at its 36th meeting.

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