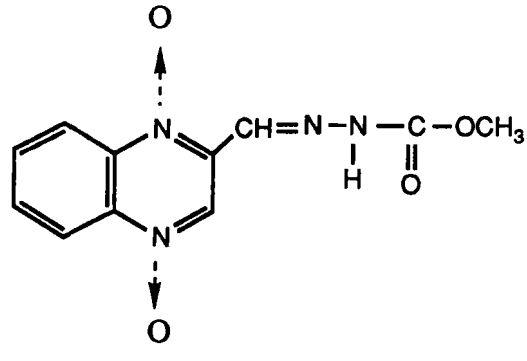
CARBADOX

IDENTITY

Chemical Name: Methyl-3-(2-quinoxalinyl-methylene)carbazate-N1,N4-dioxide

Synonyms: Mecadox; Fortigro; GS-6244; Nutriton; Getroxel

Structural formula:



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

Molecular weight: 262.2

RESIDUES IN FOOD AND THEIR EVALUATION

CONDITIONS OF USE

Carbadox is an antimicrobial drug and is currently used in the feed of swine for growth promotion, improved feed efficiency, increased rate of weight gain, and control of swine dysentery and bacterial swine enteritis. The product is sold for use in starter and/or grower rations but not in finisher rations. In most areas of the world it is fed to pigs at 50 ppm in the feed and may be used in animals up to four months of age, with a four week withdrawal period before slaughter for human consumption. In the U. S. it is approved for use at 55 ppm in pigs up to 35 kg body weight with a 70 day withdrawal period to accord with U. S. practices.

METABOLISM STUDIES

Comparative Metabolism Studies in Swine, Monkeys, and Rats

One pig was administered 3.5 mg/kg 14C-carbadox by stomach tube after 3 weeks of stress with cold drug (50 g/ton feed), while rats(6) were given 5 mg/kg by stomach tube, and one monkey was given 5 mg/kg in a capsule. The specific activity of the drug was 1.97 μCi/mg. Urine and feces were collected and assayed for total radioactivity. The urinary metabolites of carbadox were compared qualitatively by TLC and radiography. The pattern of excretion of radioactivity is shown in Table I for the three species.

Table I. Excretion pattern of radioactivity, expressed as percent of dose, found in swine, monkey and rats after ingestion of 14C-labeled carbadox.

Animal	Dose	Urine	Feces
		0-72 hrs.	0-72 hrs.
Swine	3.5 mg/kg	74.1	16.5
Monkey	5 mg/kg	61.3	7.5
Rats(6)	5 mg/kg	54.0	N.A.

Of the 15 metabolites found in the pig urine, 13 were found in either the rat or monkey urine. One of the metabolites not found in the monkey or rat is the glycine conjugate of quinoxaline-2-carboxylic acid; the other represents only a few percent of the total urinary radioactivity. (von Wittenau, 1978).

Swine

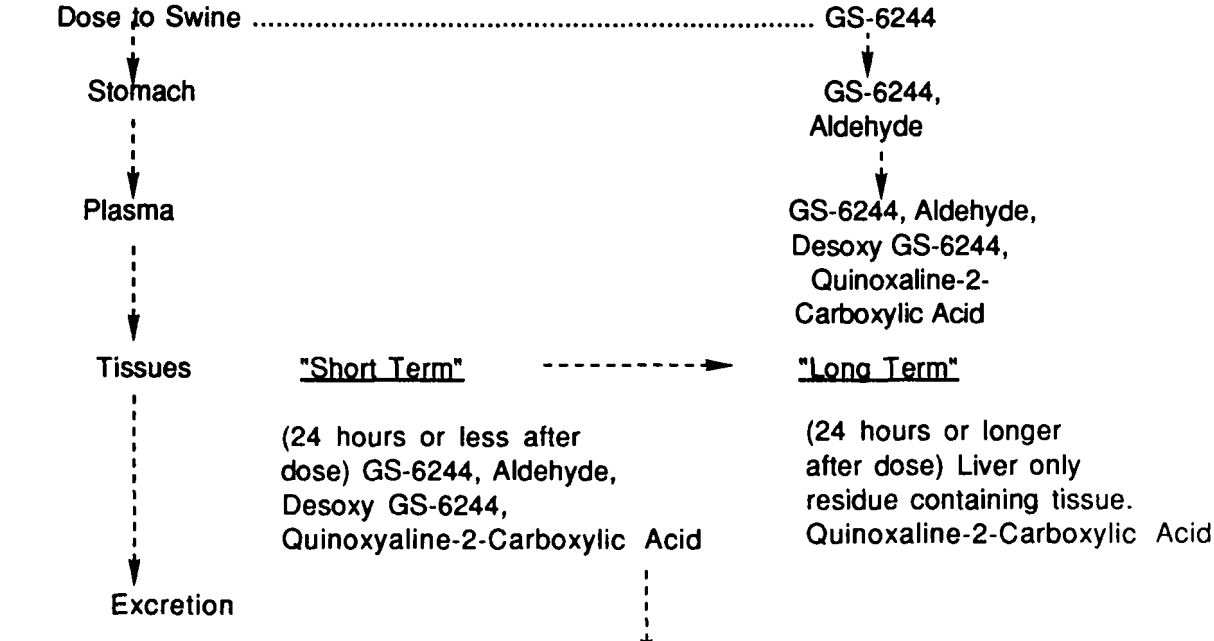
Metabolism studies of carbadox in swine have been performed using both ring labeled and carbonyl labeled 14C-carbadox. Using uniformly ring labeled carbadox, swine of mixed sex approximately seven weeks old were stressed with 50 g/ton cold drug prior to the administration of a single radioactive dose.

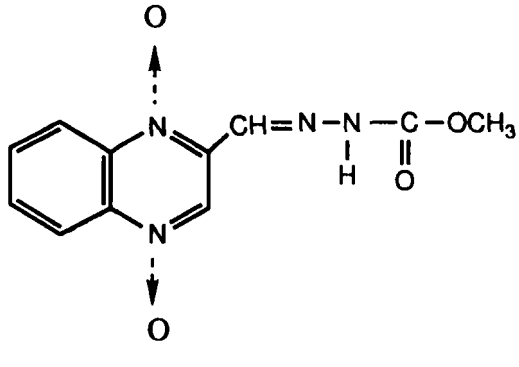
Peak radioactivity concentrations in plasma were observed at approximately 3 hours after dosing, indicating good oral absorption. About two thirds of the dose was eliminated with the urine, the remaining with the feces. The excretion was rapid, more then 95% of urinary radioactivity being excreted within 24 hours.

Very small quantities of Carbadox (GS-6244) and the corresponding aldehyde were found in the stomach 5 hours after ingestion of the drug. Both of these compounds as well as desoxy-Carbadox and quinoxaline-2-carboxylic acid (QCA) were shown to be present in plasma within hours after drug administration, but all four had disappeared within 24 hours. The major urinary metabolite was shown to be quinoxaline-2-carboxylic acid, which was also excreted in conjugated form. No N-oxides were found in urine. Feces contained some quinoxaline-2-carboxylic acid and no unchanged Carbadox. The metabolism of ring labeled carbadox is summarized in Figure 1. (Pfizer, 1989a).

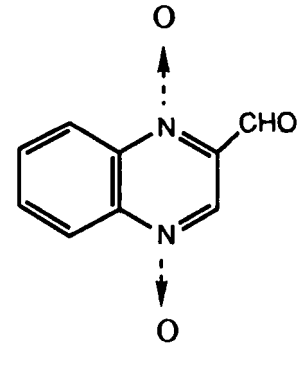
Figure 1

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

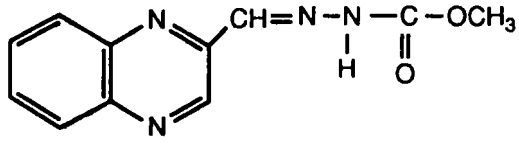




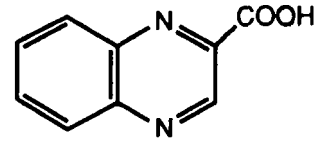
GS-6244



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



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



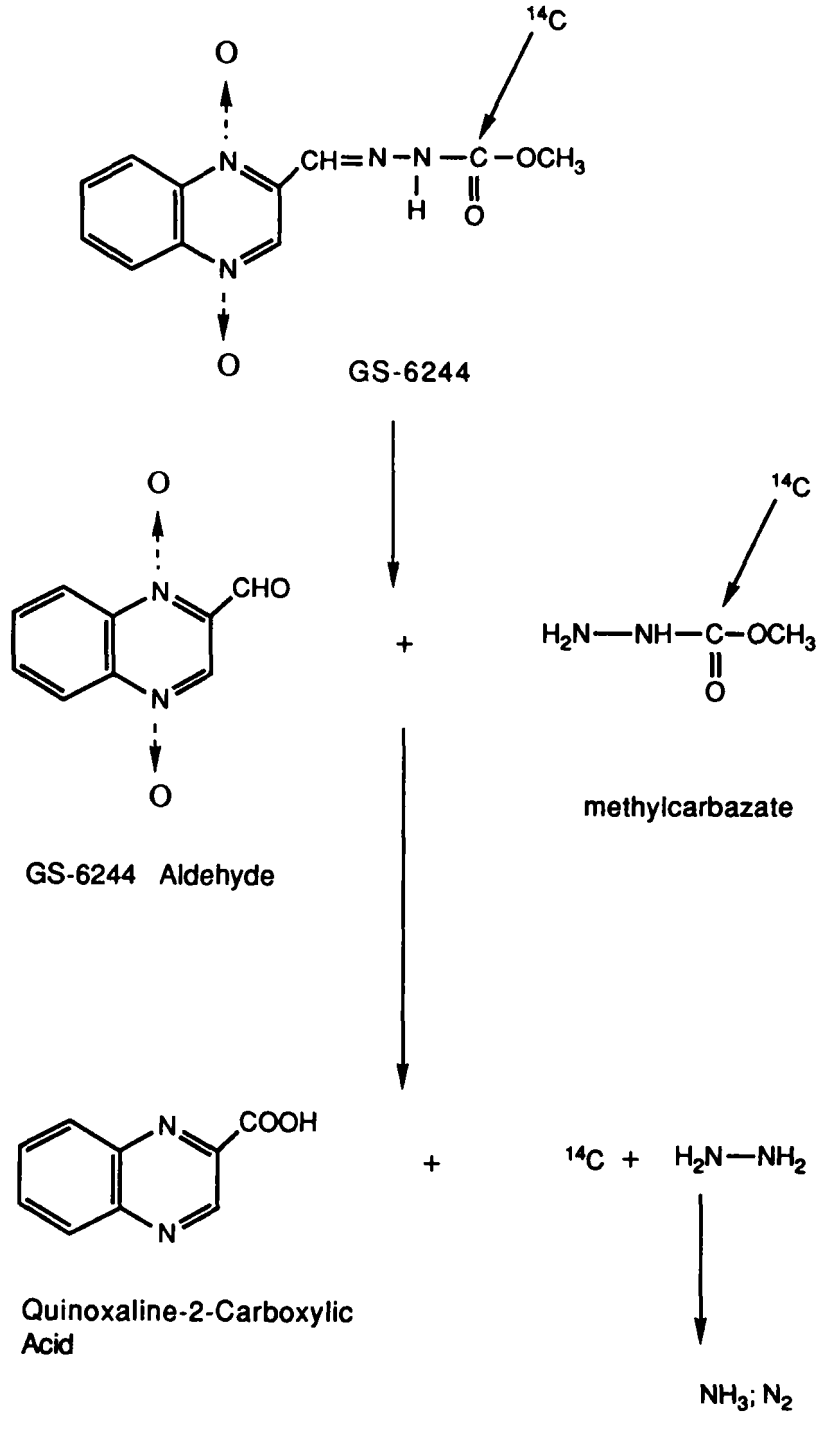
Quinoxaline-2-Carboxylic Acid

Studies with carbonyl labeled carbadox demonstrate that methylcarbazate is generated. Probably as much as 25% is excreted in urine in the form of methylcarbazate derivatives. Most of the methylcarbazate is enzymatically hydrolyzed to yield CO_2, which is exhaled. Radioactivity in liver decreased with a half-life of two days, and five days after dosing corresponded to 0.12 ppm methylcarbazate equivalent, but this radioactivity was shown to consist in part of amino acids which were labeled by incorporation of $14CO_2$.

The enzymatic hydrolysis of methylcarbazate implies but does not prove the formation of hydrazine. While studies with appropriately labeled drug can measure the maximum residues in tissues arising from methylcarbazate or from quinoxaline derivatives and thus can be used to prove their complete absence from tissues, no radiotracer method can demonstrate the absence of hydrazine related residues. Chemical assays and circumstantial evidence do strongly suggest, however, that hydrazine does not form significant tissue residues. In plasma, free hydrazine was not detected by an assay sensitive to 0.1 ppm. This is not unexpected since several enzymatic processes are known to destroy hydrazine. A summary of carbonyl labeled carbadox metabolism is shown in Figure 2. (Pfizer, 1989b).

Figure 2

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



RADIOLABELED RESIDUE DEPLETION STUDIES

The radioactivity remaining in swine tissues after a single oral dose of 3.5 mg/kg of 14C-carbadox is shown in Table II. (Pfizer, 1989a).

Table II. Tissue radioactivity (ppm 14C-carbadox equivalents) after a single dose of 3.5 mg/kg 14C-carbadox following three week stress at 50 g/ton. (Pfizer, 1989a)

Days Post Dosing	Animal Number	Liver	Kidney	Muscle	Fat
1	1	1.09	0.39	0(a)	0
3	21	0.54	-	(b)	-
5	10, 13	0.36, 0.44	0	0	0
	14, 15	0.49, 0.48	0	0	0
6	11	0.34	-	-	-
7	2, 3	0.29, 0.26	-	-	-
	12	0.21	-	-	-
14	4	0.096	0	0	0
21	5	<0.03	0	0	0

(a) 0 = 0.1 ppm
(b) Tissue not assayed

Swine

This study involved 10 swine that received radiolabeled carbadox. The animals were crossbred swine (five per sex) and weighed approximately 30 kg each. The ten animals were confined in two pens of five animals each and were given free access to feed containing 55 ppm 14C-labeled carbadox for a period of five consecutive days. After that time they were placed on a basal diet and subsequently were sacrificed in groups at 30 days (3 animals), 45 days (3 animals) and 70 days (4 animals) post dosing. Two nonmedicated swine were killed to supply control tissue samples. Samples of the four principle edible tissues were collected at the time of sacrifice and frozen. The tissues were assayed for total radioactivity and for quinoxaline-2-carboxylic acid where the level of the residue present warranted it.

The 14C-carbadox used in the study was uniformly labeled in the phenyl ring of the heterocycle. The final product was stated to have a specific activity of 8.4 μCi/mg and a radiopurity and a chemical purity of greater than 99% by TLC and HPLC. The report states that the specific activity of the tracer was suitable to allow detection of residues at and above the 1 ppb level.

The tissues were combusted and assayed for total radioactivity. The results are shown in Table III. (Pfizer, 1989c).

Table III. Total radioactivity (ppb 14C-carbadox equivalents) in tissues of swine following five days of feeding 14C-carbadox at 55 ppm.

Days Post Dosing	Animal Number	Liver	Kidney	Muscle	Fat
30	1382	117	12	5	3
	1385	55	10	3	1
	1386	76	18	6	3
	1389	50	21	6	2
		mean = 74			
45	1392	21	6	3	1
	1396	17	5	4	1
	1397	21	4	2	<1
		mean = 20			
70	1381	13	4	3	1
	1387	13	3	2	<1
	1393	14	4	2	<1
		mean = 13			

In an identical study to the above study, except for a decreased level of feed consumption, the tissues were assayed for extractable and bound radioactivity. Ten gram aliquots of the liver, kidney and muscle tissue were extracted in sequence with 100 ml portions of methanol, acetone, and n-hexane. In each case the solvent was added to the tissue in a centrifuge tube, shaken for five minutes, and then centrifuged. The extracted tissue was dried overnight under vacuum and then assayed for total radioactivity. The percentages of radioactivity remaining in the tissue following the solvent extraction averaged 5 to 8% in all three tissues examined. The individual values are shown below in Table IV. (Pfizer, 1989d).

Table IV. Percentages of radioactivity remaining in tissue following extraction sequentially with methanol, acetone, and n-hexane.

Days Post Dosing	Animal Number	Liver	Kidney	Muscle	Fat
30	257	93.0	94.4	98.8	NA
	259	93.3	92.0	92.6	NA
	265	95.0	92.5	94.7	NA
45	260	96.1	100.0	95.7	NA
	264	92.5	92.4	94.7	NA
	271	94.0	91.5	90.0	NA

Liver tissue was assayed for QCA (as measured by methyl quinoxaline-2- carboxylate) by the method based on alkaline digestion, TLC, GLC, and reverse isotope dilution.

Average levels of 18.9 ppb at 30 days, 5.5 ppb at 45 days, and 1.3 ppb at 70 days were reported. The assay results for the individual liver samples are shown below in Table V.

Table V. Methyl quinoxaline-2-carboxylate (expressed as ppb 14C-carbadox equivalents) in tissues of swine.

Days Post Dosing	Animal Number	Liver	Kidney	Muscle	Fat
30	1382	33.8	NA	NA	NA
	1385	11.6	NA	NA	NA
	1386	18.2	NA	NA	NA
	1389	11.9	NA	NA	NA
		mean = 18.9			
45	1392	5.5	NA	NA	NA
	1396	4.4	NA	NA	NA
	1397	6.5	NA	NA	NA
		mean = 5.5			
70	1381	0.8	NA	NA	NA
	1387	1.7	NA	NA	NA
	1393	1.5	NA	NA	NA
		mean = 1.3			

NA = Not assayed

The average percentage of the total residue that represented by QCA is shown below. (Pfizer, 1989c).

Days Post Dosing	Total Residue	QCA	Percent QCA
30	75 ppb	18.9 ppb	24.4
45	20 ppb	5.5 ppb	27.5
70	13 ppb	1.3 ppb	9.9

RESIDUE DEPLETION STUDIES

A liquid chromatographic method was used to monitor a depletion study of carbadox (and its most important metabolite, desoxycarbadox) in young pigs fed carbadox-treated rations for one week. Carbadox was found in blood (20 ppb), blood serum (26 ppb), and muscle tissue 24 h after withdrawal from treated ration; residues were reduced to a trace (<2 ppb) in 48 h, and eliminated by 72 h. Desoxycarbadox, although not detected in blood, was found in muscle (17 ppb) 24 h after withdrawal; it was reduced to 9 ppb at 48 h and to a trace by 72 h. Although no carbadox was detected in liver 24 h after withdrawal,it was reduced to 17 ppb at 48 h and to a trace by 72 h.

Whereas only a trace of carbadox was found in kidney 24 h after withdrawal, 186 ppb desoxycarbadox was found in kidney at 24 h, 34 ppb at 48 h, and a trace at 72 h. No

metabolite of carbadox other than desoxycarbadox was found in extracts of swine tissues during this medicated feed trial, and no metabolite was found in blood extracts by using the established methodology. (MacIntosh et. al., 1985)

Swine on feed fortified with carbadox at use level (55 ppm) for four or twenty-one days showed no unchanged drug levels in tissues (< 5 ppb) or plasma (< 10 ppb) twenty-four hours after drug withdrawal. Desoxycarbadox levels declined below 5 ppb in muscle, kidney and liver by 24, 48 and 72 hours withdrawal, respectively. No (< 10 ppb) desoxycarbadox was found in plasma at 6 and 24 hours withdrawal. QCA persists in liver but is rapidly depleted within twenty-four hours from muscle, kidney and plasma. Approximately 0.03% of the daily dose was excreted in urine and feces as desoxycarbadox. (Pfizer, 1989e).

Residues of QCA were determined in liver and in muscle by gas chromatography (GC) with electron-capture detection. Results of this experiment are summarized in Table VI. (Lauridsen et. al., 1988).

Table VI. Residues of quinoxaline-2-carboxylic acid in liver and muscle from pigs fed 20 mg carbadox/kg feed from 6-7 kg until 20-27 kg of weight (30-45 days)

		mg/kg pig			
Tissue	Wt. of pig(kg)	Farm 1	Farm 2	Farm 3	Farm 4
Liver	20-27	0.15(0)	0.06(0)	0.14(0)	0.18(0)
Liver	34-37	ND(49)	ND(32)	0.03(18)	0.01(30)
Liver	44-48	ND(49)	ND(32)	ND(36)	ND(57)
Muscle	20-27	ND(0)	ND(0)	ND(0)	ND(0)

ND = not detected (below 0.01 mg/kg). Figures in parentheses are withdrawal times in days.

Eighteen swine averaging 25 pounds body weight were fed a ration containing carbadox at 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 each on days 7, 14, 21, 28 and 35 of drug withdrawal. The method had a limit of determination of 30 ppb. The results are summarized in Table VII. (Pfizer, 1989f)

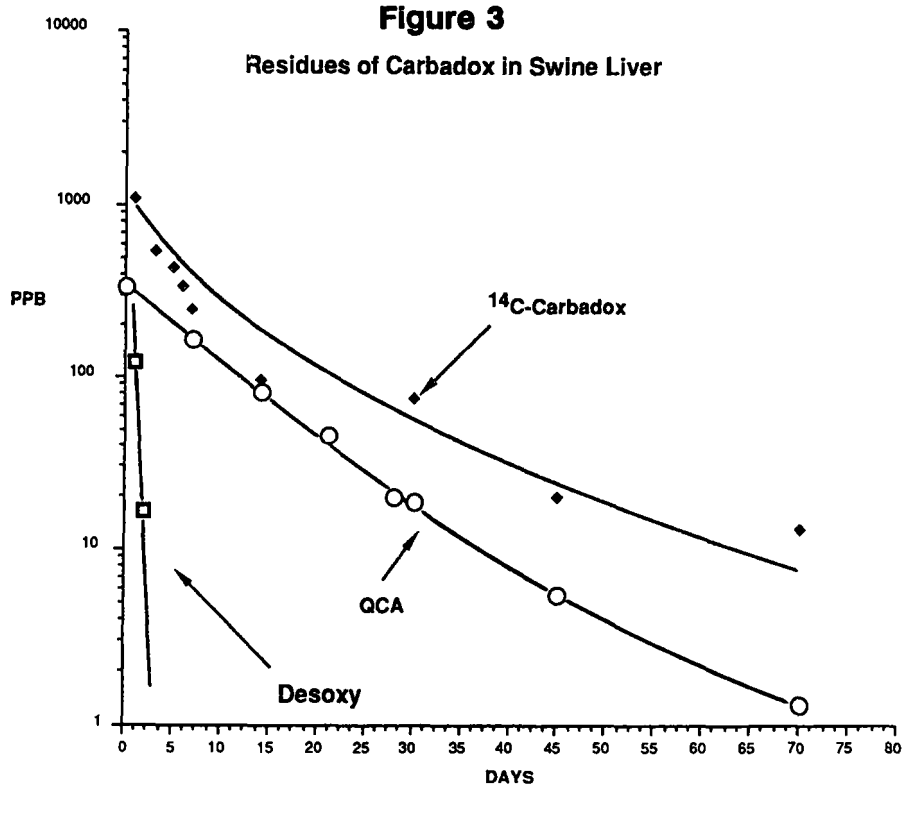
Table VII. QCA levels (ppb) in tissues of swine following 47 days of feeding with Carbadox

Days Post Dosing	Animal Number	Liver	Kidney	Muscle
0	76	400	299	<30
	94	293	191	<30
	109	342	142	<30
7	1	167	<30	<30
	13	186	<30	<30
	48	153	<30	<30
14	38	76	<30	
	66	70	<30	
	100	102	<30	
21	31	48		
	69	40		
	74	49		

(At both 28 and 35 days post dosing, QCA levels in liver for all six animals tested, were less than 30 ppb).

The depletion of total residues of carbadox and QCA over the withdrawal periods of zero to seventy days is summarized in Figure 3.

Figure 3



METHODS OF RESIDUE ANALYSIS

Identity of Residue at 28 Days Withdrawal

There were a number of experiments directed towards the chemical identification of swine liver radioactivity. The overall objective of these efforts was to disrupt liver in one fashion or another and separate the radioactivity from natural tissue constituents. These experiments were not successful since the objective was not achieved. However, the failure of these efforts to define the nature of the long-term residues can best be explained by the unexpected behavior as to distribution in solvent systems, stability, etc. of quinoxaline-2-carboxylic acid, the compound which subsequently was identified as a major part of liver radioactivity. These experiments are briefly described below.

Liver Enzyme Digestions. Portions of swine liver containing radioactive residues were treated with a number of enzyme preparations in an attempt to disrupt the tissue and free the radioactive moieties for extraction. However, attempts to extract radioactivity with chloroform at acidic, neutral and basic pH's resulted in little recovery of the label. The best results were obtained with extractions of pepsin digests on the acid side which gave approximately 19% recovery of the label. Ion exchange and sephadex column chromatography of pepsin digests yielded no specific separation of label. Generally the radioactivity was spread out with no specific peaks.

Hypothetical -CH = N-N- Tissue Bond. During attempts to elucidate the chemical nature of tissue radioactivity, it was proposed that labeled drug was attached to tissue via the carbazate side chain. If this were the case, semicarbazone cleaving reactions would be expected to regenerate a quinoxaline aldehyde suitable for extraction. Through peracid oxidations, the quinoxalines are oxidized to quinoxaline di-N-oxide. However, the recovery of label was generally unsatisfactory, suggesting that the -CH = N-N- bond did not exist in tissues.

Tissue Extraction Methods. Separation analysis of long-term liver residues revealed the following information: Polar solvent extracts remove negligible liver 14C-residues, whereas dialysis and tissue fractionations indicate the label resides largely with proteinaceous constituents. The soluble drug-related residues distribute indiscriminately with tissue mass upon further characterization, with no indication of a predominate metabolite.

Analytical Methods

Carbadox-related residues in swine liver are assayed by conversion to QCA. The conversion, liberation, and isolation of the residue is accomplished by alkaline hydrolysis of tissue, followed by isolation of the analyte from indigenous hydrolysis products through extraction and chromatographic procedures. Using electron capture gas chromatography (GLC/EC), the limit of determination is 30 ppb. This method has been subjected to interlaboratory validation and is the official method of the U. S. Food and Drug Administration. (Lynch, 1976).

A confirmatory method has been developed to identify QCA in swine liver at 30 ppb. QCA is isolated from liver hydrolysates by solvent extraction and ion-exclusion

chromatography, and a methyl ester derivative is identified by gas liquid chromatography/mass spectrometry with selected ion monitoring. (Lynch et. al., 1982)

A recent publication claims the limit of determination may be extended to 10 ppb utilizing electron capture chromatography, and comparable isolation and purification techniques described by the gas chromatography-mass spectrometry method above. (Lauridsen et. al., 1988).

The limit of determination has been extended to 3 ppb using GLC/MS with ion trap detection and in combination with the use of a stable isotope derivative of the ester of QCA. (Lynch et. al., 1989).

APPRAISAL

Although two of the residues in edible tissue resulting from the use of carbadox in swine feed are suspect carcinogens, carbadox and desoxycarbadox, the conditions of use of the drug and the depletion patterns of the residues lessen the human food safety concerns for these two metabolites. Good animal husbandry practices enable the use of carbadox in swine feed to be followed by a lengthy withdrawal period (28 days). As carbadox and desoxycarbadox can only be detected (<5 ppb) in plasma, urine and tissues for the first 72 hours after the drug is withdrawn, their levels in edible tissues at 28 days withdrawal are negligible.

The residues of carbadox can be monitored for at least 28 days by analyzing for the noncarcinogenic compound, QCA. Furthermore, residues in liver are bound or unextractable after 28 days of withdrawal; QCA is released only with alkaline digestion. Although analytical methods are available to measure QCA at withdrawal periods longer than 28 days, the increased complexity of the methods and the slow decline of the total residues from 30 to 70 days probability does not justify a withdrawal period longer than 28 days.

REFERENCES

Lauridsen, M. G., Lund, C., and Jacobsen, M. (1988). Determination and Depletion of Residues of Carbadox, Tylosin and Virginiamycin in Kidney, Liver, and Muscle of Pigs in Feeding Experiments. J. Assoc. Off. Anal. Chem. 71, 921-925.

Lynch, M.J. (1976). Rapid Determination of Quinoxaline-2-Carboxyl Acid in Swine Liver at 30 ppb by Ion-Exclusion and Electron Capture Gas-Liquid Chromatography. Unpublished report submitted to FAO by Pfizer, Inc. Groton, CN, USA. Method published in USA Federal Register, Vol 37, No 192, 10-3-72; Code of Fedreal Regulations: 21 CFR 556.100.

Lynch, M. J. and Bartolucci, S. R. (1982). Confirmatory Identification of Carbadox-Related Residues in Swine Liver by Gas-Liquid Chromatography/Mass Spectrometry with Selected Ion Monitoring. J. Assoc. Off. Anal. Chem. 65, 66-70.

Lynch, M. J. and Mosher, F. R. (1989). Determination of Carbadox-Related Residues in Swine Liver by Gas-Liquid Chromatography/Mass Spectrometry with Ion Trap Detection. Unpublished report from Pfizer, Inc., Groton, CT, USA. Submitted to FAO by Pfizer, Inc., Groton, CT, USA.

MacIntosh, A. I., Laurlault, G., and Neville, G. A. (1985). Liquid Chromatographic Monitoring of the Depletion of Carbadox and its Metabolite Desoxycarbadox in Swine Tissues. J. Assoc. Off. Anal. Chem. 68, 665-671.

Pfizer (1989a). Carbadox in Pigs Radiotracer Metabolism C14-Phenyl Labeled. Unpublished report. Submitted to FAO by Pfizer, Inc. Groton, CN, USA.

Pfizer (1989b). Carbadox in Pigs Radiotracer Metabolism C14-Carbonyl Labeled. Unpublished report. Submitted to FAO by Pfizer, Inc. Groton, CN, USA.

Pfizer (1989c). A C14-Carbadox Radiotracer Tissue Residue Study in Growing Swine. Unpublished report No. 1525N-60-87-005. Submitted to FAO by Pfizer, Inc. Groton, CN, USA.

Pfizer (1989d). A C14-Carbadox Radiotracer Tissue Residue Study in Growing Swine. Unpublished report No. 1525N-60-87-004. Submitted to FAO by Pfizer, Inc. Groton, CN, USA.

Pfizer (1989e). Tissue Depletion - Carbadox, Desoxycarbadox, QX-2-COOH. Unpublished report. Submitted to FAO by Pfizer, Inc. Groton, CN, USA.

Pfizer (1989f). Tissue Residue Studies with Carbadox in Swine Unpublished report No. 1520A-74-003. Submitted to FAO by Pfizer, Inc. Groton, CN, USA.

von Wittenau, M.S (1978). Comparative Metabolism Studies in Swine, Monkeys and Rats of Carbodox (GS-6244). Unpublished report. Submitted to FAO by Pfizer, Inc. Groton, CN, USA.