OXYTETRACYCLINE

First draft prepared by

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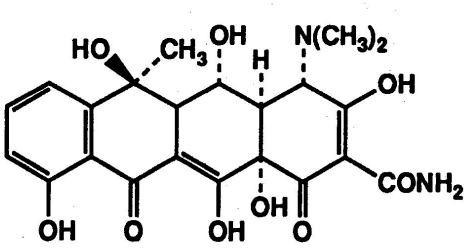
ADDENDUM

To the monograph and its addenda prepared by the 45th, 47th and 50th meeting of JECFA and published in FAO Food and Nutrition Papers 41/8, 41/9 and 41/11, respectively

IDENTITY

Chemical names: 4-(Dimethylamino)-1, 4, 4a, 5, 5a, 6, 11,12a-octahydro-3, 5, 6, 10, 12, 12a - hexahydroxy - 6 -methyl- 1, 11-dioxo-2-naphthacenecarboxamide

Structural formula:



Molecular formula: Oxytetracycline: $C_{22}H_{24}N_2O_9$

Molecular weight: Oxytetracycline: 460.44

INTRODUCTION

At the 50th meeting of JECFA, an ADI of 0-30 μg/kg bw for tetracycline, oxytetracycline and chlortetracycline (group ADI) was established.

At the 54th meeting of the Committee a temporary maximum residue limit (MRL) of 200 μg/kg in fish muscle was recommended which should apply only to oxytetracycline. However, in order to establish final MRLs for this compound the following information in fish were required for evaluation in 2002:

1. Results of a residue-depletion study
2. Validation of the analytical method for fish

Condition of use

General

Oxytetracycline hydrochloride is a broad spectrum antibacterial agent which is widely used to control in fish bacterial diseases including furunculosis (caused by susceptible *Aeromonas salmonicida*) in *Salmonids* (salmon and trout) and Bacterial Haemorrhagic Septicaemia, (caused by susceptible *Aeromonas hydrophila, A. sobia,* and *Pseudomonas* species) in *Catfish* and *Salmonids.*

Dosage

The typical dosage is 75 mg/kg body weight daily for 4 to10 successive days in fish. (Bishop, 1998)

RESIDUES IN FISH AND THEIR EVALUATION

Residue depletion studies with unlabelled compound

The usual form of oxytetracycline used in medicated fish feed is the mono-alkyl trimethyl ammonium salt, normally expressed as oxytetracycline HCl equivalent. However, in the summaries of studies, values have been converted to the oxytetracycline base (OTC equiv).

Residue depletion in northern pike

A pilot study was conducted in northern pike (average weight of 52.7 grams) fed with mono-alkyl trimethyl ammonium oxytetracycline top-coated feed equivalent to 103 mg OTC equiv/kg of bodyweight daily based on the actual 45% feed consumption of 229 mg OTC equiv/kg/day for 10 days (Bernardy et al 2000a). Five fish on day one and four fish on days 2, 4 and 8 post last treatment were collected indiscriminately. The mean temperature was 13.8 ±0.1°C. Samples were taken up to 8 days after treatment. Even at the last sampling time the concentration in the northern pike fillet (skin off) was twofold above the JECFA temporary MRL of 200 µg/kg. An elimination half life of 3.3 days was reported using a mono compartmental model.

Residue depletion in juvenile Coho salmon

In a GLP residue depletion study juvenile Coho salmon, weighing 13-62 g, were assigned to a mass treatment in an experimental 0.69 x 2.44 x 24.4 m concrete raceway. Commercial oxytetracycline medicated feed, containing 7.9 mg OTC equiv/g of feed was given at a dose of 79 mg of OTC equiv/kg /kg/day (offered 50% in the morning and 50% in the afternoon) for 10 consecutive days (Meinertz, 1999; Meinertz et al., 2001).

After cessation of the treatment on the last day, the depletion phase started. Ten fish were collected in 2 groups of five at day 1, 4, 8, 14 and 19 of the depletion phase. The skin-on fillet was stored at -10 °C prior to arrival at the laboratory. Fillets were homogenised to a fine powder using dry ice, which subsequently sublimated from the tissue, the homogenised fillets were stored at -70 °C for maximum 13 weeks prior to analysis.

Subsequently all fillet samples were analysed by an HPLC method (Meinertz et al, 1998) with a LOD of 5.2 µg/kg and LOQ of 18 µg/kg. The method mean accuracy (recovery) ranged from 80% to 105% in Coho salmon skin-on fillet seems to be acceptable. The method mean precision reported to be less than 10%.

The mean water temperature was about 6 °C and individual data ranged from 4.13 to 8.45 °C. Analysis of non medicated feed showed a low contamination with oxytetracycline about 0.8% of medicated feed and ranged from 0.02 to 1.2%. The effect of such a low contamination was concluded to be non significant.

Oxytetracycline concentrations were fell from 205-1969 µg/kg in day one to <18-58 µg/kg in day 19 with very high CV ranged from 30 to 89%. The terminal half-life of oxytetracycline in Coho salmon given the medicated feed was 4.9 days. Evidence of proper fish feeding during the study was available.

Residue depletion in walleye

Four-hundred-sixty-one walleye, weighing 59.1 ±20 g on average, were used in a GLP study in which fish received mono-alkyl trimethyl ammonium oxytetracycline top-coated feed equivalent to 2.0 mg of OTC equiv/g of feed for 10 days at about 82.5 mg OTC equiv/kg of bodyweight daily for 10 successive days (Bernardy et al 2000c).

Fish were sampled on day 1, 2, 3, 7, 9, 11 and 14 after the last treatment. The mean temperature was 17.5 ±1 °C. Later on, fillet samples were analysed by an HPLC method (Meinertz et al, 1998) with a LOD of 6.5 µg/kg and LOQ of 24.0 µg/kg. The method mean accuracy (recovery) ranged from 81% to 100.5% in walleye fillet seems to be acceptable. The method mean precision ranged from 3.4 to 11.7%.

The mean oxytetracycline fillet residue (skin on) on day 1 fell from 721.2 µg/kg (±33.9%) to 301.4 µg/kg (±31%) in day 14 with high CV ranged from 30.1 to 41.3%. Therefore even at the last sampling time the mean concentration in the walleye fillet remained 1.5 times above the proposed temporary MRL of 200 µg/kg.

An elimination half life of more than 10 days (10.5) was reported using a mono compartmental model. Evidence of support of proper fish feeding was obtained during the experiment.

Residue Depletion in Northern Pike

Two-hundred-seventy-one northern pike (about 9 months old, weighing 114 and119 g on average in each group) were divided to two groups (137 and 134) in two separate tanks in a GLP study (Bernardy et al 2000b).

Fish in group one offered mono-alkyl trimethyl ammonium oxytetracycline top-coated trout feed (about 65.8 mg OTC equiv /kg/day, 2.7 mg of OTC equiv/ g of trout feed) at a rate of 2.4% of the total fish body weight for ten days. In the second group, mono-alkyl trimethyl ammonium oxytetracycline top-coated slow sinking walleye feed (about 87 mg OTC equiv /kg/day, 3.3 mg of OTC equiv/g of slow-sinking walleye feed) was fed at a rate of 2.6% of the total fish body weight for ten days. Fish samples were collected from both groups on days 11, 12, 14, 16, 18 and 20 of study. The mean temperature was 13.8 ±0.1°C. The skin-off fillet samples were analysed by HPLC with a LOD of 6.5 µg/kg and LOQ of 24.0 µg/kg (Bernardy et al 2000a).

The mean oxytetracycline fillet residue (skin off) on days 11 fell from 203 (±21%) to 68 (±25%) µg/kg in day 20 with CV ranged from 21 to 32% in group which received medicated trout feed (about 70.9 mg/kg/day of equivalent oxytetracycline HCl). In the second group receiving medicated slow sinking walleye feed (about 94.2 mg/kg/day of equivalent oxytetracycline HCl), the mean oxytetracycline fillet residue on days 11 fell from 314 (±29%) to 125 (±27%) µg/kg in day 20 with CV ranged from 25 to 33%.

For the elimination half life values of 5.9 and 6.7 days were reported using a mono compartmental model in group one receiving medicated trout feed and in the second group which received medicated slow sinking walleye feed, respectively.

METHOD OF ANALYSIS FOR RESIDUE IN FISH

An HPLC method was validated for the analysis of oxytetracycline in fillets from six species of fish (Stehly and Meinertz, 1997; Meinertz et al, 1998). Those species were: Walleye (*Stizostedion vitreum*), Channel Catfish (*Ictalurus punctatus*), Striped bass (*Morone saxatilis*), White Sturgeon (*Acipenser transmontanus*), Atlantic Salmon (*Salmo salar*), Rainbow Trout (*Oncorhynchus mykiss*).

The procedure, which employs EDTA and trichloroacetic acid during extraction and phenyl solid phase extraction boosted by oxalic acid, was applied for fillets from these six fish species. There was no claim of compliance with GLP.

The method showed acceptable precision (0.9 - 5.8%) and recoveries (83-90%) at a concentration range from 100 µg/kg to 5000 µg/kg. Some individual out of range results were also obtained (walleye, at 100 and 1000 µg/kg with an individual accuracy of 79.3 and 78.4% and at 5000 µg/kg with an individual accuracy of 172.5%; channel catfish, at 100 and 1000 µg/kg with an individual accuracy of 71.6 and 74.0%; striped bass, at 100 and 5000 µg/kg with an individual accuracy of 75.6 and 59.8% and at 10 µg/kg with an individual accuracy 125.8%; white sturgeon, at 10 µg/kg both with an unacceptable mean and an individual accuracy of 59 and 45.4% and at 20 µg/kg with an individual accuracy of 69.8%; rainbow trout, at 100 µg/kg with an individual accuracy of 121.8% and at 1000 µg/kg an individual accuracy 78.5% were obtained which all of them were out of acceptable range at the given concentrations.)

The method precision was further investigated by analysing five endogenously incurred samples in rainbow trout. The concentration was close to the proposed temporary MRLs of 200 µg/kg. The obtained precision was 5.5%. After diluting the homogenate samples with the same amount of fish fillet the obtained precision was 1.6%.

The method's LOD and LOQ ranges were 2-6 µg/kg and 6-22 µg/kg respectively. Oxytetracycline was stable both in working solution and fortified fillet extract at room temperature (21°C) and ambient fluoresce lighting for 14 and 4 days respectively (only changes of 2-3%).

The specificity of the method was demonstrated by lack of detection of any co-eluting compounds in chromatograms of extracts from any species in blank samples. The specificity was further investigated by analyzing some potential interfering chemicals, reported to be used in aquaculture, as working solutions with LC parameters used for oxytetracycline. Interfering characteristic was confirmed by a close elution time (+ 0.5 min) to oxytetracycline one. Of the 19 potential interfering chemical analysed, only sulphadimethoxine sodium salt was confirmed to be an interfering chemical.

Stability of the oxytetracycline in fortified homogenised fillet of at least on species (walleye) at below -70 °C for 12 weeks could not be demonstrated (loss of 37%). Also no data in support of method ruggedness or reproducibility was provided. The results of this study are presented in Table 1.

A bridging study for determination of oxytetracycline in fillets (skin on) of rainbow trout was conducted to evaluate the relationship between the same HPLC method and the official microbial inhibition assay used by FDA, *Bacillus cereus*, ATCC 11778 (Stehly et al., 1999).

The study showed that results from both HPLC and microbiological methods are very close (between 93%and 111% of each other). However this was done at concentrations well above the proposed temporary MRLs of 200 mg/kg (between 300 and 9600 µg/kg). There was no claim of compliance with GLP.

In this study a certified reference standard of oxytetracycline as oxytetracycline dihydrate was used. The intralaboratory validation of the microbiological method at 1000, 2000 and 4000 µg/kg were found to be performed satisfactorily by 2 analysts and over a 3 days period of time. The mean recoveries ranged from 91 to 104%. The obtained precision ranged between 0.31 and 8.35%. A calibration curve with working standards covered oxytetracycline concentrations at 300, 600, 1200, 2400, 4800 and 9600 µg/kg. A linear correlation coefficient (r^2) of at least 0.999 was reported. The limit of detection of the microbiological method ranged from 170 to 470 µg/kg and the limit of quantitation from 290 to 690 µg/kg depending on the analyst, day of analysis and fortification level.

In comparison with the previous HPLC method now oxytetracycline dihydrate was used as certified reference standard instead of oxytetracycline HCl. The recoveries were found to be close. Recoveries in this study ranged from 81.6 to 86.9% using oxytetracycline dihydrate while at the previous study recoveries ranged 83.2 to 98.4% using oxytetracycline HCl.

The results indicated that both HPLC and microbiological methods are very close (between 93 to 111% of each other) of each other. The CV had a smaller range for HPLC (0.36 to 2.14%) than microbiological method (1.48 to 7.35%).

Table 1. Validation of analytical methods (LC) for residues of oxytetracycline in fish fillet tissues from six species

Parameter		Walleye		Channel catfish		Striped bass		White sturgeon		Atlantic salmon		Rainbow trout	
Matrix		Skin off fillet		Skin off fillet		Skin off fillet		Skin off fillet		Skin on fillet		Skin on fillet	
Accuracy (%)		See recovery		See recovery		Se recovery		See recovery		See recovery		See recovery	
Recovery (%)													
	10 (µg/kg)	80.0*		87.3		92.0		59.0		85.1		98.4**	
	20 (µg/kg)	79.7		87.8		91.3		89.4		92.5		89.1	
	100 (µg/kg)	85.0		89.3		85.6		90.4		87.1		85.9	
	1000 (µg/kg)	86.8		86.0		86.8		89.9		85.0		83.2	
	5000(µg/kg)	86.4		89.8		85.5		87.5		84.6		84.9	
Calibration curve		Triplicate x at least 4 STD		Triplicate x at least 4 STD		Triplicate x at least 4 STD		Triplicate x at least 4 STD		Triplicate x at least 4 STD		Triplicate x at least 4 STD	
Linearity		NR		NR		NR		NR		NR		NR	
Limit of detection (LOD) (µg/kg)		2		3		6		3		4		4	
Limit of Quantitation (LOQ) (µg/kg)		6		9		22		10		13		12	
Precision (% CV)													
	10 (µg/kg)	12*		8.3		17		20		16		8.5**	
	20 (µg/kg)	6.0		4.1		6.2		11		5.0		3.3	
	100 (µg/kg)	4.0		1.5		1.5		2.9		3.1		2.5	
	1000 (µg/kg)	5.0		5.8		1.6		1.9		1.8		2.1	
	5000(µg/kg)	2.5		1.9		0.9		1.2		1.5		2.7	
Reproducibility - Inter-laboratory precision		NI		NI		NI		NI		NI		NI	
Repeatability		See precision		See precision		See precision		See precision		See precision		See precision	
Sensitivity		See LOQ		See LOQ		See LOQ		See LOQ		See LOQ		See LOQ	
Specificity		No endogenous interfering compound and only 1 exogenous out of 19		No endogenous interfering compound and only 1 exogenous out of 19		No endogenous interfering compound and only 1 exogenous out of 19		No endogenous interfering compound and only 1 exogenous out of 19		No endogenous interfering compound and only 1 exogenous out of 19		No endogenous interfering compound and only 1 exogenous out of 19	
Ruggedness		NI		NI		NI		NI		NI		NI	
Analyte Stability		%		%		%		%		%		%	
	In sample matrix (homogenized tissue) at 21°C and for the following days:	< 0.5 x MRL	> 4 x MRL	< 0.5 x MRL	> 4 x MRL	< 0.5 x MRL	> 4 x MRL	< 0.5 x MRL	> 4 x MRL	< 0.5 x MRL	> 4 x MRL	< 0.5 x MRL	> 4 x MRL
	14	- 4.6	- 6.7	MD	- 7.2	+ 3.7	- 0.4	+ 5.0	- 9.2	-0.3	4.0	- 5.0	+ 2.3
	28	- 25	+ 3.8	- 9.3	- 8.0	- 1.5	- 6.6	+ 5.0	-9.6	+ 8.0	+ 3.8	- 8.3	- 9.2
	56	- 36	- 8.0	- 13	- 8.0	+ 0.2	- 9.2	+ 5.0	-13	+ 7.5	+ 4.8	- 4.8	- 2.2
	84	- 37	- 6.4	+ 13	- 1.6	+ 4.2	- 12	+ 5.0	-7.2	+ 0.1	+ 0.3	- 9.7	- 2.5

* Measured at 6 (µg/kg).

** Measured at 12 (µg/kg).

NR = Not reported

NI = Not Investigated

MD = Missing Data

Table 2. Validation of analytical method (LC) for residues of Oxytetracycline in fillets tissue of Coho salmon (*Oncorhynchus kisutch*) and northern pike (*Esox lucius*)

Parameter		**Coho salmon**		**Northern pike**	
Matrix		Skin on fillet		Skin off fillet	
Accuracy (%)		See recovery		See recovery	
Recovery (%)*					
	10 (µg/kg)	110		98	
	13 (µg/kg)	99		NS	
	500 (µg/kg)	98.4		95	
	1000 (µg/kg)	94		90	
	2000 (µg/kg)	92		91	
	4000 (µg/kg)	90.8		89	
Calibration curve		NR		NR	
Linearity		r2 = 0.99998 + 0.000062		r2 = 0.99998 + 0.000062	
Limit of detection (LOD) (µg/kg)		3.8		2.8	
Limit of Quantitation (LOQ) (µg/kg)		13		9.5	
Precision (% CV)*					
	10 (µg/kg)	12		9.7	
	13 (µg/kg)	6.7		MD	
	500 (µg/kg)	0.97		1.5	
	1000 (µg/kg)	3.4		2.3	
	2000 (µg/kg)	2.8		2.8	
	4000 (µg/kg)	1.1		7.3	
Reproducibility - Inter-laboratory precision		NI		NI	
Repeatability		See precision		See precision	
Sensitivity		See LOQ		See LOQ	
Specificity		No endogenous interfering compound and no interference from florfenicol		No endogenous interfering compound and no interference from florfenicol	
Ruggedness		NI		NI	
Analyte Stability		%		%	
	In sample matrix (tissue extract) at ambient temperature (<25°C) and ambient fluoresce lighting for these days:	1002 (µg/kg)	2004 (µg/kg)	1000 (µg/kg)	2000 (µg/kg)
	4	0	0	+ 1	+ 1
	8	0	- 1	0	0
	In sample matrix (homogenized tissue) at 21°C and for the following days	1002 (µg/kg)	2004 (µg/kg)	1000 (µg/kg)	2000 (µg/kg)
	14	- 2	- 3	- 2	- 2.5
	28	- 1	0	+ 3	+ 5
	56	- 3	- 3.5	+ 11	+ 6

*****At nominal concentrations of 10, 500, 1000, 2000 and 4000 µg/kg [actually were 10, 13, 512, 1024, 2048 and 4008 µg/kg in Coho salmon and 10,512, 1002, 2004 and 4008 µg/kg in northern pike.]

NR = Not reported

NI = Not Investigated

MD = Missing Data

The same HPLC method was further validated for the quantification of oxytetracycline in fillets tissue of Coho salmon (*Oncorhynchus kisutch*; skin on) and northern pike (*Esox lucius*; skin off) in study complying with GLP (Bernardy et al. 2000a).

A linear correlation coefficient (r^2) of 0.99998 $\pm$ 0.000062 was reported. At concentrations that ranged from 10/13 - 4000 µg/kg, the method showed acceptable recoveries (90-105%) and precision (0.97 - 12%) with LOD and LOQ of 2.8/3.8 µg/kg and 9.5/13 µg/kg, in Coho salmon and northern pike, respectively. In Coho salmon, at 10 µg/kg while considering a CV of 12%, the maximum range of accuracy (123.2%) could slightly go above the maximum acceptable range of 120%.

The method precision was further investigated by analysing replicates of endogenously incurred samples in Coho salmon (n=5) and northern pike (n=8). The mean concentrations were 1240 and 1840 µg/kg after ten days of treatment for two types of fish, respectively. The obtained values for precision were 2.0 and 5.1% for two types of fish, respectively.

The method precision was acceptable in endogenously incurred samples. However, the concentrations used were more than six to nine times higher than the proposed temporary MRL of 200 mg/kg.

Oxytetracycline was stable both in working solution and in fortified fillet extract at room temperature (<25°C) and ambient fluoresce lighting for 4 and 8 days respectively (only 1% changes). Oxytetracycline in fortified homogenized fillet of Coho salmon was stable during 8 weeks of storage below -70°C (the maximum loss of 3.5%). However, surprisingly, in case of northern pike, despite of 2 - 2.4% initial loss, gains of 6 - 11% were observed after 8 weeks of storage. The results of this study are presented in Table 2. No data in support of method ruggedness or reproducibility was provided.

APPRAISAL

The submitted HPLC method was validated in a number of GLP and non-GLP studies for the analysis of oxytetracycline in fillets from eight species of fish.

In the range which covers the proposed maximum residue limit of 200 µg/kg, the method resulted in acceptable precision, accuracy (recovery), LOD and LOQ. However, more data in support of method ruggedness or reproducibility would be desirable.

The obtained data from skin off fillet/s can not be extrapolated to target tissue defined by JECFA (muscle with adhering skin in normal proportions).

With the exception of the first study, all other studies were conducted in comparison with the MRL of 2000 µg/kg which was established recently for the US. Therefore it is necessary to calculate the exact withdrawal time, if applying MRL of 200 µg/kg.

MAXIMUM RESIDUE LIMIT

In recommending MRL, the Committee took into account the following factors:

- An HPLC method validated for the analysis of oxytetracycline residues in fillets from eight species of fish at concentrations including the MRL of 0.2 mg/kg was available.
- Some of the studies of residues were conducted at concentrations that included the temporary MRL of 0.2 mg/kg for muscle and involved analysis of muscle with adhering skin in normal proportions.

The Committee recommended that the temporary MRL of 0.2 mg/kg for fish muscle established at its fifty-fourth meeting be made permanent. This MRL applies only to oxytetracycline.

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