Metabolites of Oxytetracycline, Tetracycline, and Chlortetracycline and Their Distribution in Egg White, Egg Yolk, and Hen Plasma

Georg Zurhelle,† Michael Petz,*,† Erika Mueller-Seitz,† and Eike Siewert‡

Department of Food Chemistry, University of Wuppertal, Gauss-Strasse 20, D-42097 Wuppertal, Germany, and Federal Institute for Health Protection of Consumers and Veterinary Medicine, Diedersdorfer Weg 1, D-12277 Berlin, Germany

4-Epioxytetracycline and *N*-demethyloxytetracycline, as metabolites of oxytetracycline (OTC), 4-epitetracycline and *N*-demethyltetracycline, as metabolites of tetracycline (TC), and 4-epichlortetracycline, isochlortetracycline (ICTC), 4-epi-ICTC, and *N*-demethyl-ICTC, as metabolites of chlortetracycline (CTC), were detected in egg yolk and plasma obtained from feeding studies with either OTC, TC, or CTC. In egg white, only OTC, TC with its 4-epimer, and ICTC with its 4-epimer were detected in substantial concentrations. The ratios of epimerization and N-demethylation in the eggs did not change during the medication period. The samples were analyzed by an automated HPLC system (ASTED) with UV, fluorescence, or MS-MS detection.

Keywords: *Oxytetracycline; tetracycline; chlortetracycline; isochlortetracycline; egg white, yolk; microdialysis*

INTRODUCTION

The European Union (EU) (Regulation 281/96) has set maximum residue limits (MRL) of 200 μg/kg for each tetracycline [TC; oxytetracycline (OTC) or chlortetracycline (CTC)] as the sum of parent drug and its 4-epimer in whole eggs. These levels have to be controlled by national authorities within the EU (Regulation 675/92). To validate and verify analytical methods when applied for food inspection, the use of reference material is strictly recommended by the EU (Commission Decision 256/93). However, no reference materials for tetracyclines are available. In an attempt to produce such a reference material, the first dose of medication was chosen to obtain residue levels in whole eggs close to the MRL. Analyzing the eggs from these feeding experiments disclosed the unexpected presence of several metabolites of the tetracyclines formed in vivo (Zurhelle et al., 1999).

Data on the metabolism and distribution of tetracyclines in eggs are summarized by the WHO (1996) and by Roudaut et al. (1987, 1989). The results given there for residues in eggs were obtained by microbiological assays and provide no information about metabolic processes. Nelis and De Leenheer (1982) described the in vivo formation of the 9-hydroxy derivative and the *N*-demethyl derivative of minocycline in humans, and Boecker (1983) described the in vivo formation of the *N*-demethyl derivative of doxycycline in humans, mice, and rats. ICTC was found as a metabolite of CTC in meat of calves by Kuehne (1993). A recent study by Kennedy et al. (1998) using HPLC disclosed 4-epi-ICTC and ICTC as being the principal metabolites of CTC in whole egg. In another recent HPLC study on OTC residues by De Ruyck et al. (1999), only data for the parent compound in whole egg were reported. The distribution of several veterinary drugs (including tetracyclines) between egg white and yolk was reviewed by Kan and Petz (2000). Here, the occurrence and distribution of parent tetracyclines and their metabolites in egg white and yolk are reported including data on hen plasma.

MATERIALS AND METHODS

Chemicals. The parent drugs used in the feeding studies and as standards for the analytical procedure were obtained from Sigma (Deisenhofen, Germany); isochlortetracycline (ICTC) and the 4-epimers of TC, OTC, and CTC as hydrochlorides were obtained from Acros Chimica (Fisher Scientific).

Feeding Study. A total of 60 White Leghorn (Lohmann White SL) and 6 Red Leghorn hens were used in this study. The hens had ad libitum access to water and feed. No additive, for example, citric acid, was used to improve absorption of the tetracycline antibiotics. A control group of 10 hens were fed with unmedicated feed.

Sample Preparation. Egg white was separated from egg yolk directly after the egg shell had been broken. A syringe was used to obtain pure egg yolk material. Prelaid eggs/ovules were obtained from hens slaughtered at the end of the medication period as well as plasma, which was kept frozen at −20 °C until analysis.

In Vitro Experiments. Five grams of egg yolk from control hens was adjusted to pH 6 with 15 g of 0.3 mol/L citric acid and spiked with 600 μg/kg of each of the three tetracyclines: OTC, TC, and CTC. Ten grams of egg white was spiked without dilution or pH adjustment. Both samples were incubated at 37 °C for 25 h.

Analytical Procedure (Zurhelle et al., 2000). The samples were analyzed by an automated HPLC system involving microdialysis and a trace enrichment cartridge (ASTED system, Gilson) for the cleanup and analyte enrichment. Tetracyclines and their metabolites with the exception of ICTC and ICTC-derived compounds were detected by UV absorption (360 nm) after chromatography on a reverse-phase C-8 column, whereas the latter were quantified by their fluorescence (350

* Corresponding author (e-mail petz@uni-wuppertal.de; fax ++49-202-439-2784).

† University of Wuppertal.

‡ Federal Institute for Health Protection of Consumers and Veterinary Medicine.

10.1021/jf000141k CCC: $19.00
Published on Web 12/01/2000

Table 1. Residues in Egg Yolk after Feeding Chlortetracycline (1000 mg/kg)[a]

day	total residues	CTC	e-CTC	% epimer[b]	ICTC	e-ICTC	% epimer[b]	N-DM-ICTC	% N-DM-ICTC[c]
1		<LOD[d]	<LOD		<LOD	<LOD		<LOD	
2		<LOD	<LOD		<LOD	<LOD		<LOD	
3	26	<LOQ	<LOD		<LOQ[e]	<LOQ		<LOD	
4	111	27	22.5	45	22.5	29	56	<LOQ	
5	262.5	61	44.5	42	58	74	56	25	9
6	485	116	77	40	109	138	56	45	9
7	547	118	86	42	126	167	57	50	9
8	560	123	99	45	126	162	56	50	9
9	663	161	110	41	143	190	57	59	9
10	673	133	98	42	172	211	55	59	9
11	709	147	115	44	172	214	55	61	9
12	754	159	128	45	178	226	56	63	8
13	735	165	125	43	172	213	55	60	8
14	838	174	135	44	197	260	57	72	9
15	879	178	143	45	15	274	57	74	8
16	936	197	145	42	225	291	56	78	8
17	926	205	140	41	219	285	57	77	8
18	860	161	130	45	214	276	56	79	9
19	605	105	79	43	157	209	57	55	9
20	407	63	47	43	111	150	57	36	9
21	234	35	39	53	62	84	58	14	9
22	120	14	21	60	37	48	57	<LOD	

[a] Medication: days 1−17. Concentrations in μg/kg. [b] % epimer = epimer/Σ (epimer + parent). [c] % N-DM-ICTC = N-DM-ICTC/Σ (ICTC + e-ICTC + CTC + e-CTC + N-DM-ICTC) [d] LOD, limit of detection. [e] LOQ, limit of quantification.

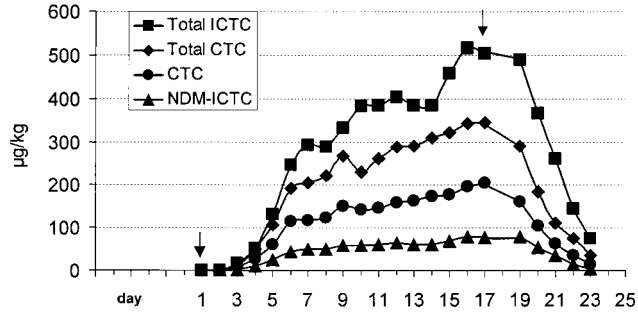

Figure 1. Distribution of CTC and its derivatives in egg yolk after medication with 1000 mg/kg CTC. Day 1 = start of medication; day 17 = end of medication.

nm/420 nm) after adjustment of the HPLC eluate to pH 12.0 or, with less sensitivity, by UV detection (310 nm). Confirmatory analyses were made by LC/MS-MS with electrospray ionization.

RESULTS AND DISCUSSION

It was already reported by Roudaut et al. (1987, 1989) that for tetracyclines the residue levels in egg yolk are higher than in egg white. They used an unspecific microbiological assay for analysis. A more specific approach was applied here by using HPLC with specific detection methods for investigating the occurrence and distribution of residues.

When spiked material was analyzed as part of the validation procedure for the analytical method, it was shown that the method itself did not lead to the formation of 4-epimers, ICTC, or other possible breakdown products. The limit of detection for epitetracyclines is 10 μg/kg. This means that at least a 5% epimerization at the MRL level (200 μg/kg) can be detected. The same limit of detection holds true for the *N*-demethyl compounds.

The samples with incurred residues from the feeding experiments were analyzed by LC/MS-MS and diode array detection to prove the presence and identity of the tetracyclines and their postulated metabolites. In the chromatograms of the egg or plasma samples of the control group receiving the same feed but without tetracyclines, we observed no metabolites of tetracyclines and no other compounds leading to interfering peaks.

We also checked the stability of the medicated feed that was used in the studies. The epimerization rates of tetracyclines were ≤5%. From these experiments we concluded that any residues besides the parent compounds in egg or plasma samples must have their origin from the in vivo metabolism.

Chlortetracycline. Feeding a diet with 1000 mg/kg CTC led to high residue levels of ICTC besides the parent drug CTC and *N*-demethyl-ICTC in egg yolk (Figure 1 and Table 1). The epimerization rates for ICTC (55−57%) and CTC (41−45%) in yolk were constant during the medication period. At day 17 of the medica-

Table 2. Residues in Egg White after Feeding Chlortetracycline (1000 mg/kg)[a]

day	total residues	ICTC	e-ICTC	% epimer[b]
1		<LOQ	<LOQ	
2	210	165	45	21
3	300	243	57	19
4	267	213	54	20
5	264	209	55	21
6	322	252	70	22
7	321	253	68	21
8	363	286	77	21
9	310	240	70	23
10	382	299	83	22
11	289	222	67	23
12	261	198	63	24
13	241	182	59	24
14	427	339	88	21
15	391	309	82	21
16	309	241	68	22
17	363	281	82	23
18	182	147	35	
19	18	18		
20	19	19		
21	13	13		

[a] Medication: days 1−17. Concentrations in μg/kg. [b] % epimer = epimer/Σ (epimer + parent).

Table 3. Residues in Egg Yolk at Day 17 after Feeding 1300 mg/kg Chlortetracycline to Ten Individual Hens[a]

hen	total residues	CTC	e-CTC	% epimer[b]	ICTC	e-ICTC	% epimer[b]	% total ICTC[c]	N-DM-ICTC	% N-DM-ICTC[d]
C41	1362	241	180	43	360	453	56	66	128	9
C42	4409	940	681	42	1043	1364	57	59	381	9
C43	912	175	139	44	236	287	55	63	75	8
C44	1276	259	211	45	285	408	59	59	113	9
C45	522	89	73	45	138	175	56	66	47	9
C46	1927	407	319	44	444	584	57	59	173	9
C47	769	151	107	41	203	247	55	64	61	8
C48	877	164	124	43	241	272	53	64	76	9
C49	850	181	149	45	201	243	55	57	76	9
C50	1396	252	156	38	356	508	59	68	124	9

[a] Concentrations in μg/kg. [b] % epimer = epimer/Σ (epimer + parent). [c] % total ICTC (ICTC + e-ICTC) = total ICTC/Σ (ICTC + e-ICTC + CTC + e-CTC). [d] % N-DM-ICTC = N-DM-ICTC/Σ (ICTC + e-ICTC + CTC + e-CTC + N-DM-ICTC).

Table 4. Residues in Egg White at Day 17 after Feeding 1300 mg/kg Chlortetracycline to Ten Individual Hens[a]

hen	total residues	ICTC	e-ICTC	% epimer
C41	586	457	129	22
C42	2296	1837	459	20
C43	440	348	92	21
C44	889	667	222	25
C45	307	233	74	24
C46	831	648	183	22
C47	348	275	73	21
C48	472	373	99	21
C49	521	401	120	23
C50	881	687	194	22

[a] Concentrations in μg/kg.

tion (last day) total residues of all CTC compounds were >900 μg/kg, whereas parent CTC just reached 200 μg/kg.

In egg white of the same eggs only ICTC but no parent CTC or *N*-demethyl-ICTC was detected (Table 2). The constant epimerization rate of ~22% for ICTC in egg white was significantly different from the epimerization rate in yolk.

The influence of the individual hen on the residue concentrations is highlighted by Tables 3 and 4. Total concentrations of all CTC-derived residues for 10 hens obtained after feeding a diet of 1300 mg/kg CTC for 17 days ranked between 522 and 4409 μg/kg in egg yolk and between 348 and 2296 μg/kg in egg white. Despite these differences in the concentration levels, epimerization rate and metabolite pattern were identical.

Oxytetracycline and Tetracycline. As with CTC, the epimerization rate is constant also for OTC and TC, regardless of the individual hen or the concentration of the tetracycline in the feed. OTC was epimerized to 21−24% in egg yolk. In egg white only the parent compound was detected (Table 5). The epimerization rate for TC was 41−50% in egg yolk (Table 6) and 22−26 % in egg white (Table 7). N-Demethylation rates for OTC and TC were 7−8 and 18−21%, respectively, in egg yolk, whereas in egg white no *N*-demethyl products of OTC or TC were detected.

Ovules and Plasma. To investigate potential sites at which metabolism may take place, the hen plasma and pre-laid eggs (ovules) were investigated with the same analytical procedure. In extension of the results of Kennedy et al. (1998) we found that ICTC and its 4-epimer are the principal metabolites also in pre-laid eggs with the same ratio of epimerization in the ovules if compared with the corresponding egg yolk. In plasma samples we found the same metabolites but with lower ratios of epimerization and of demethylation than in the egg yolk samples of the same hen. Residue levels are quite different from hen to hen, and this might be due to the fact that the drug plasma level depends on the laying cyclus of the hen (Harms and Waldroup, 1962).

ICTC concentrations were as high as CTC concentrations. In all future investigations the occurrence of ICTC as a metabolite of CTC should be considered.

N-Demethylation. In the case of N-demethylation we suppose the hen liver as being the place of formation. The monooxygenases of the cytochrome P-450 system might be responsible for this demethylation of the dimethylamino group (Boecker, 1983; Nelis and De Leenheer, 1982). Egg yolk proteins are formed in the liver and are transported via the blood (plasma) into the ovules, whereas egg white proteins are formed in the oviducts (Gilbert, 1971; McIndoe, 1971). Therefore, the residue pattern in egg white is different from the residue pattern in egg yolk. In the chromatograms of the in vitro samples from the experiment described

Table 5. Residues in Egg Yolk and Egg White at Day 14 after Feeding 750 mg/kg Oxytetracycline (Hens A) or 3000 mg/kg Oxytetracycline (Hens D)[a]

	egg yolk						egg white
hen	total residues	OTC	e-OTC	% epimer	N-DM-OTC	% N-DM-OTC	OTC
A1	223	161	44	21	<LOQ		112
A2	216	151	45	23	<LOQ		103
A3	187	142	45	24	<LOD		98
A4	147	106	31	23	<LOD		90
A5	293	210	63	23	<LOQ		180
A6	210	163	47	22	<LOD		120
A7	218	157	46	23	<LOQ		95
D86	829	591	179	23	59	7	387
D87	828	600	165	22	63	8	467
D88	714	496	158	24	60	8	519

[a] Concentrations in μg/kg.

Table 6. Residues in Egg Yolk at Day 14 or 17 (Hens C or B, Respectively) after Feeding 750 mg/kg Tetracycline or at Day 14 after Feeding 3000 mg/kg Tetracycline (Hens F)[a]

hen	total residues	TC	e-TC	% epimer	N-DM-TC	% N-DM-TC
C59	285	121	110	49	54	19
C60	236	101	92	48	43	18
B21	734	306	290	49	138	19
B22	1744	732	672	48	340	19
B23	746	307	303	50	136	18
B24	413	189	148	44	76	18
B25	438	191	164	46	83	19
B26	862	389	309	44	164	19
B27	581	246	226	48	109	19
B28	701	306	255	45	140	20
B29	286	119	106	47	61	21
B30	1721	727	648	47	346	20
F101	2342	996	917	47	429	18
F102	2034	859	818	48	357	18
F103	2337	1115	783	41	439	19

[a] Concentrations in μg/kg.

Table 7. Residues in Egg White at Day 14 or 17 (Hens C or B, Respectively) after Feeding 750 mg/kg Tetracycline or at Day 14 after Feeding 3000 mg/kg Tetracycline (Hens F)[a]

hen	total residues	TC	e-TC	% epimer
C59	21	21	<LOD	
C60	17	17	<LOD	
B21	71	55	16	23
B22	173	129	44	25
B23	79	62	17	22
B24	31	31	<LOD	
B25	33	33	<LOD	
B26	89	67	22	25
B27	44	44	<LOD	
B28	70	53	17	23
B29	21	21	<LOD	
B30	185	137	48	26
F101	238	178	60	25
F102	218	165	53	24
F103	254	194	60	24

[a] Concentrations in μg/kg.

below, no signals for the *N*-demethyl derivatives were observed. These metabolites are unquestionably formed in vivo.

Epimerization and Isomerization. The formation of 4-epimers and ICTC has been reported in solutions depending on pH, temperature, and type of buffer (Schwartzman et al., 1979). In an in vitro experiment we spiked egg yolk samples and also egg white samples with the three tetracyclines. The spiked samples were analyzed after incubation at 37 °C for 25 h. This time period was chosen because the egg white formation takes 22−25 h (Ternes et al., 1994). In Table 8 the ratios of epimerization and the formation of ICTC at pH 6.0, 37 °C, and 25 h after spiking with the parent compounds are reported. No epimerization or isomerization occurred in spiked egg yolk or egg white kept at 8 °C and during the automated analytical procedure at ambient temperature.

From the results given in Table 8 we conclude that the formation of ICTC and its 4-epimer and the formation of the 4-epimers of TC in eggs are due to the influence of temperature and time. In the case of OTC probably a temperature >37 °C or longer reaction times are necessary for epimerizsation. In the liver, temperatures of up to 42 °C might be responsible for the 4-epimerization of OTC. These compounds can be considered as in vivo degradation products rather than real metabolites, although they were formed in the hen and not during the analytical process.

Table 8. Epimerization and Isomerization Rates Obtained in Vitro after Spiking Diluted Egg Yolk Samples and Egg White Samples with Tetracycline or Chlortetracycline at pH 6.0 Compared with the in Vivo Results Obtained during Medication

reaction	matrix	% e-OTC	% e-TC	% e-CTC	% e-ICTC	% total ICTC[a]
in vitro, 37 °C, 25 h	yolk	<5	33	28	56	28
	white	<5	25		25	100
in vivo	yolk	21−24	41−50	40−45	55−57	65−80
	white	<5	22−26		19−24	100

[a] % total ICTC (ICTC + e-ICTC) = total ICTC/Σ (ICTC + e-ICTC) + (CTC + e-CTC).

Eisner and Wulf (1963) investigated the influence of temperature on the degradation of CTC in dog urine. After spiking dog urine with CTC, they measured an increasing epimerization of CTC at 37 °C.

Fluorescence of the Egg Shells. With eggs from the medication period we observed a strong fluorescence of the egg shell after excitation with UV light as reported by Lindgren et al. (1970). When the medication was stopped, the egg shells were nonfluorescent (Zurhelle et al., 1999). The intensity was highest after feeding CTC and lower after feeding a diet with identical concentrations of TC or OTC. The egg shells of red layers also showed the fluorescence, but a comparison with a red (brown) egg shell of an unmedicated egg shell is helpful for an inexperienced viewer. The sensitivity of this test could be improved with increasing power of the UV lamp and for red egg shells when the egg was broken and the uncolored inside of the shell was viewed after removal of the inner egg shell skin. From this observation we suggest as a simple screening method checking with a UV lamp whether eggs were laid during a medication period.

LITERATURE CITED

Boecker, R. Analysis and quantitation of a metabolite of doxycycline in mice, rats, and humans by high-performance liquid chromatography. *J. Chromatogr.* **1983**, *274*, 255−262.

Boecker, R.; Estler, C. J. Quantitative assessment of a newly detected metabolite of doxycycline, in vitro formation and in vivo distribution of N-demethyl-doxycycline in mice. *Drugs Exp. Clin. Res.* **1983**, *9*, 813−819.

De Ruyck, H.; De Ridder, H.; Van Renterghem, R.; Van Wambeke, F. Validation of HPLC method of analysis of tetracycline residues in eggs and broiler meat and its application to a feeding trial. *Food Addit. Contam.* **1999**, *16*, 47−56.

Eisner, H. J.; Wulf, R. J. The metabolic fate of chlortetracycline and some comparisons with other tetracyclines. *J. Pharm. Exp. Ther.* **1963**, *142*, 122−131.

Gilbert, A. B. In *Physiology and Biochemistry of the Domestic Fowl*; Bell, D. J., Freeman, B. M., Eds.; Academic Press: London, U.K., 1971; Vol. 3, pp 1291−1321.

Harms, R. H.; Waldroup, P. W. Relationship of the egg formation cycle to antibiotic blood level in laying hens. *Poult. Sci.* **1962**, *41*, 1932−1936.

Kan, C. A.; Petz, M. Residues of veterinary drugs in eggs and their distribution between yolk and white. *J. Agric. Food Chem.* **2000**, *48*, 6397−6403.

Kennedy, D. G.; McCracken, R. J.; Hewitt, S. A.; McEvoy, J. D. G. Metabolism of chlortetracycline: drug accumulation and extraction in the hen's egg. *Analyst* **1998**, *123*, 2443−2447.

Kuehne, M. Combination of HPLC and agar diffusion test for confirmatory purposes. *Proceedings of EuroResidue II—Conference on Residues of Veterinary Drugs in Food*, Veldhoven, The Netherlands; Utrecht University Press: Utrecht, The Netherlands, 1993.

Lindgren, I.; Nevalainen, T.; Kohonen, J. Tetracyclines and egg shell calcification. *Poult. Sci.* **1970**, *49*, 1357−1358.

McIndoe, W. M. In *Physiology and Biochemistry of the Domestic Fowl*; Bell, D. J., Freeman, B. M., Eds.; Academic Press: London, U.K., 1971; Vol. 3, pp 1209−1220.

Nelis, H. J. C. F.; De Leenheer, A. P. Metabolism of minocycline in humans. *Drug Metab. Dispos.* **1982**, *10*, 142−146.

Roudaut, B.; Moretain, J. P.; Boisseau, J. Excretion of oxytetracycline in eggs after medication of laying hens. *Food Addit. Contam.* **1987**, *4*, 297−307.

Roudaut, B.; Moretain, J. P.; Boisseau, J. Excretion of tetracycline and chlortetracycline in eggs after oral medication of laying hens. *Food Addit. Contam.* **1989**, *6*, 71−78.

Schwartzman, G.; Wayland, L.; Furnkranz, T. A. K.; Selzer, G. Chlortetracyline hydrochloride. In *Analytical Profiles of Drug Substances*; Florey, K., Ed.; Academic Press: New York, 1979; Vol. 8, pp 101−137.

Ternes, W., Acker, L., Scholtyssek, S., Eds. *Ei und Eiprodukte*; Verlag Paul Parey: Berlin, Germany, 1994.

WHO. *Food Additives Series*; WHO: Geneva, Switzerland, 1996; Vol. 36, pp 85−116.

Zurhelle, G.; Petz, M.; Mueller-Seitz, E. Tetracyclines and their metabolites in egg white and egg yolk. *Z. Lebensm. Unters. Forsch. A* **1999**, *208*, 299−300.

Zurhelle, G.; Müller-Seitz E.; Petz, M. Automated residue analysis of tetracyclines and their metabolites in whole egg, egg white, egg yolk and hen's plasma utilizing a modified ASTED-system. *J. Chromatogr. B* **2000**, *739*, 191−203.

Received for review February 1, 2000. Revised manuscript received July 15, 2000. Accepted July 15, 2000.

JF000141K