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Food Chemistry 72 (2001) 41–44

Food Chemistry

www.elsevier.com/locate/foodchem

Bound chlortetracycline residues in bones: release under acidic conditions

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Received 29 February 2000; received in revised form 31 May 2000; accepted 31 May 2000

Abstract

Tetracycline residues were frequently found in the bones of slaughtered animals. These bound residues will contaminate products for human consumption, such as mechanically deboned meat. The aim of this study was to examine the behaviour of bound residues of chlortetracycline during in vitro digestion. Dough batches containing poultry meat and 2.5% by weight of bone splinters and bone meal with chlortetracycline residues were incubated with hydrochloric acid and pepsin at 40°C. With the aid of HPLC and agar diffusion tests, evidence was obtained that these bound residues of chlortetracycline could be released during in vitro digestion and become antimicrobially active. Hens fed with chlortetracycline containing meat and bone meal were also able to release bound tetracycline residues.

Keywords: Chlortetracycline; Bound residues; Hens

1. Introduction

The bones of slaughtered animals were found to contain a high incidence of tetracycline residues due to their frequent use in animal husbandry and their pharmacokinetic properties (Kühne, Wegmann, Kobe & Fries, 2000). The tetracyclines in bones are bound to the matrix. Smit, Haagsma and Ruiter (1999) defined bound residues as compounds which are covalently bound to macromolecules and cannot be extracted by aqueous or organic solvents. The possible toxicological significance of these bound residues is still unclear, because the tetracycline-calcium-orthophosphate chelate in bones has been demonstrated to be microbiologically inactive (Forth, Henschler & Rummel, 1987).

Several authors have found that covalently bound residues can be released in vitro by oxidation, by reduction, by the action of proteolytic enzymes, or by acid hydrolysis, depending on the compound under consideration. The release of covalently-bound residues by these methods has been observed for cambendazole (Baer, Jacob & Wolf, 1977), trenbolone (Ryan & Hoffmann, 1978), ronidazole (Wolf et al., 1983) and furazolidone (Hoogenboom, Kammen, van Berghman & Kuiper, 1990). Their in vivo release has also been studied, mainly in rats, using radio-labelled compounds. A recent publication described the release by proteolytic enzymes of tetracycline residues bound to macromolecules in milk and demonstrated the positive effects for microbial detection of tetracyclines in milk serum (Aureli, Ferrini & Mannoni, 1999).

Bound tetracycline residues in bones will contaminate both mechanically deboned meat (Ellerbroek, Kästel, Kolb & Weise, 1998) and meat and bone meal.

The objective of the present study was to investigate whether covalently bound chlortetracycline residues in bones could be released in vitro and in vivo and whether antimicrobial activity could be observed in these released residues. In vitro release was studied under acidic (0.3 M HCl) conditions. To simulate conditions occurring in the stomach, the chlortetracycline chelates were incubated after thorough homogenisation with meat and the addition of pepsin. The in vivo release of chlortetracycline was studied in hens. Analysis was performed by HPLC, followed by UV detection, and several microbiological tests.

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0308-8146/00/$ - see front matter
PII: S0308-8146(00)00198-9

2. Materials and methods

2.1. Design of the experiment

For the in vitro release studies, 11 dough batches were prepared, i.e. 10 batches containing chlortetracyclines and a blank. These batches were used for the release experiments. For the in vitro release of bound chlortetracycline residues, the dough batches were subjected to the digestion treatment described below. They were then centrifuged and both the sediment and supernatant tested for chlortetracycline (parent substance and 4-epimer) and antimicrobial activity using HPLC with UV detection and agar diffusion tests, respectively. For the in vivo release, sterilised bone meals with chlortetracycline residues were fed to hens.

2.2. Preparation of dough batches

Poultry meat and bones were purchased from a local meat plant. After screening for tetracyclines in bones with the screening fluorescence test (Kühne & Ebrecht, 1993), and the confirmation by HPLC with UV and diode array detection of chlortetracycline residues, the fat and meat were thoroughly separated from the bones. Prior to analysis the meat and bones were stored at −20°C.

2.2.1. Muscle

Eleven pectoral muscles from turkey carcasses were carefully deboned, then homogenised using a standard homogeniser (Moulinette, Moulinex) and finally stored in aliquots of 100 g at −20°C prior to analysis.

Each aliquot was analysed for chlortetracycline and 4-epi-chlortetracycline using HPLC. Extraction and clean up were as follows: 20 ml of cold buffer (0.1 m citric acid, Sigma C-0756, and 0.2 M di-sodium hydrogen phosphate; 62:38, v/v, adjusted to pH 4.0) were added to a 5 g sample, homogenised, treated in an ultrasound bath at 50–60 kHz (Bransonic B 221) for 5 min and centrifuged for 5 min at 5000 *g*. Extraction was performed twice. The collected supernatants were transferred to a column filled with a non-ionic polymeric adsorbent (Amberlite XAD-2, Sigma Deisenhofen, art. A-7643). Previously, 6 g of Amberlite XAD-2 had been suspended in 50 ml of methanol (HPLC gradient grade, Roth 7342.1), rinsed in a brown glass column and conditioned with 25 ml hydrochloric acid. After rinsing with 250 ml water, the samples were eluted with 70 ml methanol. The samples were evaporated to 250 µl and resolved with 750 µl of mobile phase for HPLC (0.01 M oxalic acid, Sigma O-0376, and acetonitrile, Roti Solv HPLC, Roth 7330.1; 60:40, v/v).

2.2.2. Chromatography

Separation was performed with a 125 × 4 mm cartridge column (Merck 1.50943-0001) filled with LiChrospher at 0.4 ml min^{-1} at ambient temperature. The HPLC system consisted of a HPLC pump (type 64, Knauer), an injection valve with a 20 µl loop (Knauer), a UV-detector (SPD-10 A, Shimadzu) operated at 370 nm and connected to an integrator (C-R 5 A-chromatopac, Shimadzu), and a diode array detector (SPD-M6A, Shimadzu). For the calculation of results, the external standard method was used. The external standards were chlortetracycline hydrochloride (Sigma C 4881) and 4-epi-chlortetracycline (Acros Chimica 26823-1000). Additionally, the concentrations were corrected for mean percentage recovery, calculated from samples spiked with 1 mg kg^{-1} chlortetracycline. The mean recovery was 55% (S.D. = 5.8; $n = 5$).

2.2.3. Bones

A long bone was taken from the same turkey carcasses that provided the muscles and, after removal of the marrow, sawn into fragments of < 1 mm diameter. Bone fragments and bone meal were also stored at −20°C prior to analysis. The bones were incubated with 1 M hydrochloric acid for 10 h at + 8°C. The sediment was extracted once more. The combined supernatant was filtered und immediately loaded on a previously conditioned XAD-2 Amberlite™ column. The full methodology of the extraction of chlortetracycline and 4-epi-chlortetracycline from bones has been described elsewhere (Kühne et al., 2000).

The bone meal was also tested for *antimicrobial activity*: 1 g of bone meal was suspended in phosphate buffer (pH 5.5) and transferred to excavations on a test plate with standard II agar adjusted to pH 6.0, with 10^4 spores of *Bacillus subtilis* BGA ml^{-1}, and incubated for 18 h at 30°C. 97.5 g of homogenised meat and 2.5 g of bones (50% bone splinters and 50% bone meal) were accurately weighed in a 250 ml beaker and thoroughly mixed.

2.2.4. In vitro release by 0.3 M HCl

A 12.5 g aliquot of the dough was transferred to a clean 100-ml beaker. A 25 ml volume of the digestion solution was added (100 ml water at 40°C, 0.5 g pepsin and 1 ml 25% HCl). The dough was mixed for 30 min on a warm mixer under pH control. After 15 min, pH values reached a steady state of 5.7–6.0. The mixture was centrifuged for 15 min at 5000 *g*. After decantation, the supernatant was directly used for chromatography.

2.2.5. Antimicrobial activity after digestion

The supernatant (see above) was adjusted to pH 7.0 with sodium hydroxide and used for the agar diffusion test. 100 µl of the samples were transferred to previously prepared test plates with *Bacillus subtilis* BGA (see above). In addition, a tetracycline-resistant strain of *Bacillus subtilis* BGA (Merck art. 10649, lot 3244086)

Table 1
Inhibition zones (mm) of chlortetracycline on test plates with standard II agar with *Bacillus subtilis* BGA, with the addition of $MgSO_4$ and with a tetracycline-resistant strain of *Bacillus subtilis* BGA (tc-resist.)

Concentrations (μg ml^{-1})	Standard II	Standard II and $MgSO_4$	Standard II (tc-resist.)
0.01	0	0	0
0.05	5.0	0	0
0.1	8.5	2.0	0
0.2	9.5	4.0	1.0
0.4	10.0	5.5	2.0
0.6	10.5	6.5	3.0
0.8	11.5	7.0	4.5
1.0	13.0	7.5	5.0

and *Bacillus subtilis* BGA with an addition of 10 mg $MgSO_4$ * 7 H_2O ml^{-1} agar (Hamburger, Carleton & Harcourt, 1957) were used for confirmatory purposes. Table 1 gives the inhibition zones obtained from chlortetracycline standard solutions on the different test plates.

2.2.6. In vivo release

Four hens were fed a standard layer diet supplemented with sterilized bone splinters and bone meal containing chlortetracycline concentrations of 35 250 μg kg^{-1} ad libitum for 10 days. Two hens served as controls. They were all humanely killed 1 day later and the *os femoris* was examined for chlortetracycline residues as described above.

3. Results

3.1. In vitro experiments

HPLC detected no chlortetracycline in the muscles. Chlortetracycline concentrations of 15 100 to 81 266 μg kg^{-1} were found in bone splinters and bone meal. Antimicrobial activity could not be detected (Table 2). The resulting chlortetracycline residues in the dough batches were calculated and found to be between 378 and 2032 μg kg^{-1}.

The direct examination of supernatants with HPLC showed that the in vitro digestion led to a significant release of chlortetracycline (Table 2). At the same time, some antimicrobial activity could be demonstrated in the microbial assay with *B. subtilis*. This was confirmed as specific activity through the use of both the tetracycline- (tc-)resistant strain and the plate with Mg ions. The sediment showed no detectable antimicrobial activity.

Table 3 shows the results of the recovery calculations for chlortetracycline. On average, 35.3% of the bound chlortetracycline residues from the bone portion were found in the supernatant after the in vitro digestion. The antimicrobial activity was 17.8% of the calculated chlortetracycline concentrations in the dough batches before the in vitro digestion.

Control samples showed neither detectable chlortetracycline concentrations nor any antimicrobial activity.

3.2. In vivo experiments

The bones from hens fed with the control diet did not contain chlortetracycline residues. The bones from four hens fed with the bone meal supplement contained detectable chlortetracycline residues. The mean concentration was 153 μg kg^{-1} with a standard deviation of 180.

4. Discussion

Tetracycline residues in bones were already detected 40 years ago (Milch, Rall & Tobic, 1957). Nevertheless, these residues were found to be of no toxicological significance (Anhalt, 1980). This might be due to the fact that tetracycline residues in bone tissue show no in vitro antimicrobial activity. A few years ago, we looked for a suitable extraction procedure for tetracyclines in bones and found one with hydrochloric acid to be most appropriate (Kühne & Ebrecht, 1993). The high solubility of tetracyclines in acid solution raised the question of whether the digestion of bone particles in weak acid solutions, such as occur in a monogastric's stomach, could break the tetracycline chelates.

The results of our in vitro experiments gave clear evidence that chlortetracycline residues in bone tissue could be released during a simulated digestion with hydrochloric acid and pepsin. The supernatants that were examined by HPLC without any previous extraction

Table 2
Examination of bones, dough batches, supernatants and sediments for chlortetracycline[a,b]

	CTC in meat	CTC in bone (μg kg^{-1})	Antimicrobial activity (AA) in bone	CTC in dough batch (μg kg^{-1} calculated)	CTC in supernatant (μg kg^{-1} dough) HPLC/*AA*	AA in sediment
Mean/S.D.	nd	41 801/26 893	nd	1045/672	341[c]/182 *156/69*	nd

[a] Chlortetracycline concentrations are the sum of chlortetracycline and 4-epi-chlortetracycline.
[b] $n = 10$; nd = not detectable; S.D., standard deviation.
[c] The mean for 4-epi-chlortetracycline was 164 μg kg^{-1}.

Table 3
Recovery of bound CTC as released and antimicobially active CTC after in vitro digestion

	A. Bound CTC	B. Released CTC	C. Antimicrobial active CTC
	CTC in dough (μg CTC kg^{-1} calculated)	CTC in the supernatant (% of A)	CTC with antimicrobial activity in the supernatant (% of B)
Mean/S.D.	1.045/672	35.3/7.9	51.6/18.8

showed 24.6–49.4% of the chlortetracycline concentrations originally bound to the bone matrix. This is much more than Smit et al. (1999) found for suphadimidine. They achieved a release of only 6–14% after an incubation period of 4 h.

The released chlortetracycline residues showed antimicrobial activity on the microbiological plate test with *Bacillus subtilis*. The specifity of this activity was confirmed by the use of both an inactivating $MgSO_4$-additive and a tc-resistant strain of *Bacillus subtilis*.

The mean antimicrobial activity in the supernatant represented only 51.6% of the calculated total chlortetracycline concentrations. This is not surprising, as the total chlortetracycline concentrations consisted of almost equal parts of parent substance and 4-epimer. The antimicrobial activity of 4-epi-chlortetracycline is only 5% compared with the parent substance, but the epimer will be easily transformed into the parent substance when incubated with more alkaline solutions (Hlavka & Boothe, 1985).

5. In vivo experiments

Detectable residues of chlortetracycline were found in the bones of hens after they had been fed contaminated bone meal for only 10 days. Hens were able to release bound chlortetracycline residues and to absorb the substance from the intestine. This provides some evidence that the tetracycline residues found in mechanically separated meat for human consumption (Ellerbroek et al., 1998) may also be released in vivo.

The occurrence of tetracycline residues in food and feed is undesirable because of the possible induction of resistance in pathogenic micro-organisms. The scientifically-based ADI- (acceptable daily intake) levels for tetracyclines and the resulting MRLs (maximum residue levels) for food of animal origin (Commission of the European Community, 1999) must avoid all negative effects on human health. Bound residues of tetracyclines should not be ignored in this continuous evaluation process.

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