



Short communication

Liquid chromatographic determination and depletion profile of oxytetracycline in milk after repeated intramuscular administration in sheep

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ABSTRACT

A simple, rapid and specific ion-pair liquid chromatographic method for the routine determination of the marker residue of oxytetracycline in sheep milk, at levels as low as 20 µg/kg, has been developed. Milk samples were acidified and extracted with acetonitrile. The extracts were purified by treatment with ammonium sulphate and concentrated into diluted phosphoric acid. Separation was carried out isocratically on a Nucleosil C₁₈ column using a mobile phase that contained both positively and negatively charged pairing ions. The in-house validated method gave overall recoveries and overall relative standard deviations better than 86% and 4.6%, respectively. The method was successfully applied to study the depletion of oxytetracycline in sheep milk and to estimate the withdrawal period after intramuscular administration of a commercial oxytetracycline formulation.

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1. Introduction

Oxytetracycline (OTC) is licensed for use in a variety of food-producing animals including cattle, sheep, goats, pigs, poultry and fish. The use of OTC in lactating animals may lead to the occurrence of residues in their milk posing a health hazard to the consumer [1]. Moreover, the presence of OTC residues in milk could create critical problems in the production of fermented products such as yoghurt and cheese, since they may cause partial or complete growth inhibition of the starter cultures used for fermentation. The European Medicines Agency (EMA) of the European Union (EU) has established a maximum residue limit (MRL) of 100 µg/kg for the marker residue of OTC in milk [2], which comprises the sum of OTC and 4-epi-oxytetracycline (4-epiOTC).

Since OTC is widely used in lactating animals, milk residue depletion and estimation of withdrawal period of this compound plays an important role to guarantee the safety of milk. A thorough literature survey shows that only limited residue studies concerning milk have been published [3–6], but information on the depletion of OTC marker residue in sheep milk and estimation of withdrawal period after intramuscular injection does not currently exist. For this application, the EU demands a fully validated

method, in compliance with the Commission Decision 2002/657/EC [7], for the quantitative determination of OTC marker residue in milk.

Many liquid chromatographic (LC) methods for analysing tetracyclines in foods of animal origin have been reported over the past twenty years and they have been reviewed recently [8–11]. Several of these methods have been proposed for the determination of tetracyclines in milk [3,12–19], but only the method developed by De Ruyck and De Ridder [19] is able to individually quantify OTC and 4-epiOTC in milk, meeting the EU legislation. Nevertheless, the latter method requires expensive equipment, such as LC–MS/MS, which although valuable for confirmatory purposes, cannot be used in a routine basis to detect OTC residues in unknown milk samples due to the high cost of the analysis.

The objective of this study was twofold; firstly to develop and in-house validate according to EU legislation [7], a fit-for-purpose LC method for the routine determination of OTC and 4-epiOTC in sheep milk, and secondly to apply this new method to study the depletion profile of these compounds in milk. The former objective was effectively realised in view of using simple LC–UV/visible equipment to develop a simple, rapid, specific and cost-effective LC method based on a recently published LC method [20] concerning the determination of OTC residues in animal tissues, with some modifications. The latter objective was accomplished via a large scale experiment with 20 healthy sheep intramuscularly injected with a conventional OTC formulation.

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2. Experimental

2.1. Reagents and standards

The LC-grade acetonitrile and methanol, and reagent grade dichloromethane, phosphoric acid, sulphuric acid, ammonium sulphate, disodium hydrogen phosphate, octanesulphonate sodium, tetrabutylammonium hydrogen sulphate and ethylenediaminetetraacetic acid (EDTA) tetrasodium salt (dihydrate) were from Merck (Darmstadt, Germany). Reference standards of OTC (96.2%) and 4-epiOTC (98.4%) were kindly donated by Pfizer Hellas S.A. (Athens, Greece).

Stock standard solutions were prepared in individual 25-mL volumetric flasks by dissolving ca. 10 mg of each reference standard and diluting to volume with methanol. Mixed standard intermediate solution, containing the two analytes, was prepared by combining appropriate aliquots of each stock solution into a 25-mL volumetric flask and diluting to volume with methanol. Mixed standard working solutions in the range of 50–800 ng/mL were prepared by appropriate dilutions of the mixed standard intermediate solution with 0.1 M phosphoric acid. Stock and mixed standard intermediate solutions were stored at -28°C and used for 1 month. Mixed standard working solutions were stored at 4°C and used for 3 days.

2.2. Animal trial

Twenty, 3-year-old, healthy sheep of a local Greek breed (Chios) were intramuscularly injected in the shoulder/neck region with a conventional OTC formulation at a dose of 10 mg OTC per kg body weight once daily for 5 consecutive days. The study was performed in accordance with all EU recommendations [21] regarding animal welfare.

2.3. Milk sampling

The animals were milked twice daily (12 h intervals) and milk samples were retained at each milking from day 0 until day 8 after the last intramuscular administration of OTC. One hundred (100) milliliter of milk were sampled after mixing the total quantity of milk collected from each animal and stored at approximately -80°C , pending analysis. Control milk samples were obtained from every sheep just before the first intramuscular administration of OTC.

2.4. Sample extraction and clean-up

Milk (3.00 g) was weighed in a 15-mL graduated centrifuge tube and acidified at pH 2.5–3.0 with 0.4 M sulphuric acid. After addition of acetonitrile to a final volume of 10 mL, the tube was vortex mixed and centrifuged for 1 min at $2000 \times g$. A 6-mL aliquot of the supernatant liquid was pipetted into another tube, where 2 mL of saturated ammonium sulphate solution were also added. Following vortex mixing and centrifugation for 1 min at $2000 \times g$, the top layer separated was transferred into a new centrifuge tube and after addition of 0.5 mL of 0.1 M phosphoric acid and 5 mL of dichloromethane the tube was vortex mixed and centrifuged for 1 min at $2000 \times g$. The supernatant aqueous layer was pipetted into a 1-mL graduated tube and the volume was adjusted with 0.1 M phosphoric acid. A 100- μL aliquot was used for LC.

2.5. Liquid chromatographic analysis

LC analyses were carried out on a modular LC system (Gilson Medical Electronics, Villiers-le-Bel, France) consisting of a model

305 piston pump, a model 805 manometer, a model TC 831 column oven, and a Model 119 variable wavelength UV–vis detector. The detector was linked to a BD 111 Kipp & Zonen (Delft, Holland) pen recorder. Injections were made through a 7125 Rheodyne valve (Rheodyne Inc., Cotati, CA, USA) equipped with 100 μL loop. Chromatographic separation was performed on a Nucleosil 100-5 C₁₈, 5 μm stationary phase in Macherey-Nagel (Germany) analytical (250 mm \times 4.6 mm id) and guard (10 mm \times 4.6 mm id) columns.

Analyses of extracted milk samples and standard solutions were conducted using an acetonitrile–0.01 M disodium hydrogen phosphate (20:80, v/v) mobile phase that contained 5 mM octanesulphonate sodium salt, 3 mM tetrabutylammonium hydrogen sulphate and 0.01% tetrasodium EDTA, and adjusted at pH 3.8 with concentrated phosphoric acid. The mobile phase was delivered in the system at 30°C and a flow rate of 1 mL/min. Detection was made at 370 nm, and recordings were made at a chart speed of 5 mm/min and a detector sensitivity of 0.01 a.u.f.s.

2.6. Determination

Calibration curves were generated by analyzing mixed standard working solutions, plotting the recorded peak heights versus the corresponding mass of the analytes injected, and computing slope, intercept and least squared fit of standard curves. Slope and intercept data of calibration curves were used to compute the mass of the analytes in the 100 μL injected extracts. The concentration (C) of each analyte in milk samples (ng/g) was calculated using the equation $C = 5.56 \times M \times V$, where M is the mass (ng) of the analyte determined in the injected extract (100 μL), and V is the dilution factor, if any, applied.

2.7. Method validation

The validation was performed according to Commission Decision 2002/657/EC [7] using sheep milk as matrix. Essential parameters in validating an analytical procedure, such as specificity, linearity, decision limit, detection capability, sensitivity, accuracy, precision, stability of analytes and applicability, were evaluated.

2.8. Estimation of withdrawal period

The withdrawal period was estimated by applying the recommended by the EMEA [22] Time-To-Safe-Concentration (TTSC) method, using the software WTM1.4 that is downloadable from the EMEA Web site [23].

3. Results and discussion

3.1. Sample extraction and clean-up

The extraction of OTC residues from milk can present a number of problems, mainly its high propensity to bind with proteins to form conjugates that are difficult to extract from milk. Use of an acidic solvent is of great assistance in overcoming this difficulty [12,14,15,17,19], but low recovery has been usually reported. To overcome this problem, extraction of milk samples with 2 volumes of acetonitrile was used, and this resulted in protein precipitation but extraction of OTC and 4-epiOTC was not quantitative unless milk samples had been acidified at pH values lower than 3 [20]. This could be possibly due to incomplete dissociation of non-covalently bound OTC and 4-epiOTC from milk proteins at higher pH values.

For further clean-up and concentration, solid phase extraction [12,14,15,17–19] is the commonly employed technique in OTC analysis. However, it is well known [9,24] that this technique suffer

from batch-to-batch variability and column clogging, and is invariably time consuming to use, in addition to adding to the cost of the sample preparation.

In this study, in an effort to overcome the aforementioned problems, a simple and cost-effective liquid–liquid partitioning was applied for the clean-up and concentration of OTC and 4-epiOTC from milk. Some purification of the acetonitrile extracts was effectively realized by vortex mixing them with a saturated ammonium sulphate solution. Mixing the acetonitrile phase with dichloromethane and extracting OTC residues with 0.1 M phosphoric acid enabled further purification and concentration.

3.2. Liquid chromatographic analysis

Tetracyclines have a propensity to form chelate complexes with chromatographic packings and to interact with the free silanols present in silica packings [9,11], which significantly contributes to peak tailing and affect quantification. To eliminate peak tailing several attempts were made by adding acids in the mobile phase, but only mobile phases containing oxalic acid (pH 2.0–3.0) produced no tailing peaks of the tetracyclines [8]. These mobile phases, although adequate to separate parent tetracyclines, proved to be inefficient for baseline separation of OTC and 4-epiOTC. To achieve baseline separation of these compounds on reversed phase columns, gradient elution has been used by Cherlet et al. [25] and De Ruyck and De Ridder [19]. Gradient elution has proved to be quite useful for the LC–MS/MS determination of OTC and 4-epiOTC, but for a routine determination an isocratic separation would be most valuable.

In this respect, several mobile phases were previously [20] evaluated for the separation of OTC and 4-epiOTC under isocratic conditions using a Nucleosil 100–5 C₁₈ stationary phase. Best results on separation and resolution could be obtained when the mobile phase had a pH adjusted at 3.8 and contained 3 mM tetrabutylammonium hydrogen sulphate, 5 mM octanesulfonate sodium salt and 0.01% tetrasodium EDTA. Under the optimised conditions OTC eluted at 7.0 min and 4-epiOTC at 8.5 min (Fig. 1).

3.3. Performance of the analytical method

3.3.1. System suitability

The suitability of the LC system was checked with every set of 10 samples by running control and fortified milk extracts, and a complete set of mixed standard working solutions. The LC sys-

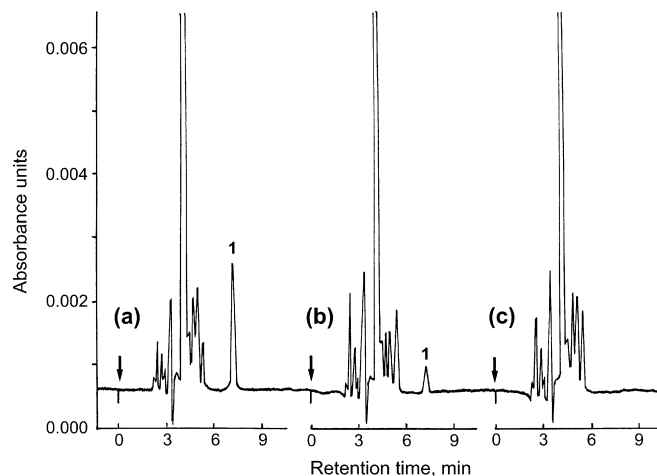


Fig. 2. Chromatograms of incurred milk samples obtained from one sheep at the 4th (a), 7th (b), and 8th (c) milking after the cessation of the intramuscular administration of oxytetracycline at a dose of 10 mg/kg body weight once daily for 5 consecutive days. Peak identification as in Fig. 1.

tem provides baseline resolution and consistent retention times for both OTC and 4-epiOTC. As regards the calibration curves, regression analysis of the data obtained by running a series of working solutions showed that the response for the two analytes was linear in the range examined (4.2–75.0 ng of OTC and 4-epiOTC injected). Calibration curves could be described by the following equations: $y = 0.68 + 1.47x$, $r^2 = 0.9999$, for OTC and $y = -0.21 + 1.69x$, $r^2 = 0.9999$, for 4-epiOTC, where y represents peak height in millimetres and x is the quantity in nanogrammes of each analyte per 100 μ L injected.

3.3.2. Specificity

Chromatograms obtained from 20 different control milk extracts and reagent blanks showed that the peaks attributable to the test compounds were resolved sufficiently from any other peaks from matrix co-extractives to enable reliable quantification (Figs. 1 and 2).

3.3.3. Decision limit and detection capability

The decision limit ($CC\alpha$) was determined by analysing 20 control sheep milk samples spiked with OTC and 4-epiOTC at the MRL value of 100 μ g/kg. The $CC\alpha$ values were calculated as the sum of the respective MRL values plus 1.64 times the corresponding standard deviations of the measured concentrations.

The detection capability ($CC\beta$) was determined by analysing 20 control sheep milk samples spiked with OTC and 4-epiOTC at the respective $CC\alpha$. The $CC\beta$ values were calculated as the sum of the respective $CC\alpha$ values plus 1.64 times the corresponding standard deviations of the measured concentrations.

The $CC\alpha$ values calculated for OTC and 4-epiOTC were 106.5 μ g/kg and 107.3 μ g/kg, respectively, whereas the $CC\beta$ values were 114.6 μ g/kg and 115.5 μ g/kg for OTC and 4-epiOTC, respectively.

3.3.4. Sensitivity

The efficiency of the ion-pair chromatographic system coupled with the cleanliness of the extracts, allowed very low limits of detection and quantification to be realized in milk. Analysis results showed that the limits of detection were 8.5 and 8.9 μ g/kg for OTC and 4-epiOTC, respectively, whereas the limit of quantification was 20 μ g/kg for both analytes.

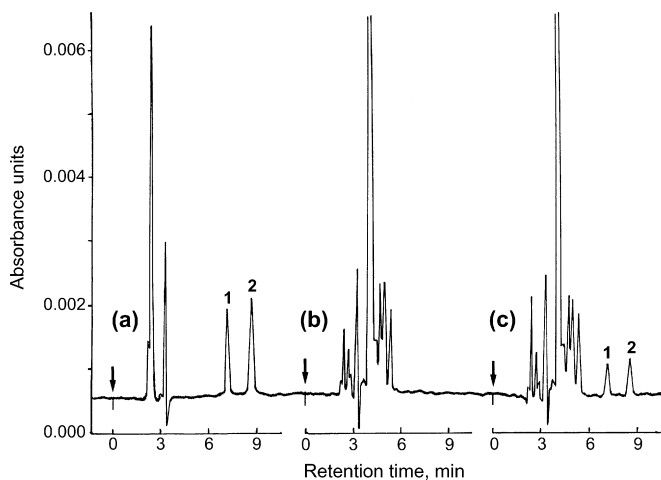


Fig. 1. Typical chromatograms of a mixed standard working solution (a), a control sheep milk sample (b), and a fortified sheep milk sample (c) with 50 μ g/kg oxytetracycline (1) and 4-epi-oxytetracycline (2).

Table 1
Accuracy data for the determination of oxytetracycline marker residue in sheep milk.

Residue	Fortification level (μg/kg)	Mean ^a recovery ± SD (%)	Overall recovery ± SD (%)
OTC	20	102.4 ± 8.3	90.4 ± 6.4
	50	96.2 ± 3.8	
	100	91.5 ± 3.6	
	150	89.7 ± 3.2	
	300	91.3 ± 2.2	
4-epiOTC	20	98.8 ± 8.0	86.7 ± 6.7
	50	88.8 ± 4.1	
	100	85.3 ± 2.6	
	150	84.2 ± 1.7	
	300	87.4 ± 2.0	

^a Mean of six replicates

3.3.5. Accuracy and precision

Overall recoveries were 90.4 ± 6.4% and 86.7 ± 6.7% for OTC and 4-epiOTC, respectively (Table 1), whereas overall intralaboratory repeatability (RSD) for both analytes was better than 5% (Table 2), meeting Commission Decision 2002/657/EC guidelines for accuracy and precision.

3.3.6. Interference test

Several compounds, such as other tetracyclines (tetracycline, 4-epi-tetracycline, chlortetracycline, 4-epi-chlortetracycline, doxycycline), penicillins, sulfonamides, trimethoprim, thiamphenicol, novobiocin, aminoglycosides, thiabendazole, triclabendazole, mebendazole, albendazole and its metabolites, and febendazole and its metabolites, were added to control milk samples at 200 μg/kg, and the samples were submitted to analysis. The results showed that none of the tested compounds interfered with the analysis.

3.3.7. Stability of the analytes

The results of the experiments carried out to investigate the storage stability of the analytes in 0.1 M phosphoric acid indicated

that OTC and 4-epiOTC were stable for at least 6 and 12 days when standard solutions were stored at 4 °C and –28 °C, respectively, but considerable reduction in the concentration of both analytes was observed when standard solutions were stored at 20 °C in the dark. This reduction was more pronounced when the standard solutions were stored in the light.

As far as the stability of the analytes in matrix is concerned, the results indicated that the test compounds were stable during the storage of spiked and incurred milk samples (*n* = 10) at –80 °C for at least 5 months. As regards the stability of the analytes in the final milk extracts (*n* = 10), the results indicated that no significant change in the concentration of OTC and 4-epiOTC, during storage for 3 days at 4 °C in the dark, was observed, but an approximately 8% reduction in the concentration of the analytes was noted after 9 days of storage in the dark.

Moreover, experiments were conducted to evaluate the possible epimerization of OTC to form 4-epiOTC due to sample preparation. The results showed that the 4-epimer was not formed during the extraction/clean-up procedure, which was applied to milk samples that were fortified only with OTC. Furthermore, the possibility of 4-epiOTC to convert back to OTC during analysis of milk samples that were fortified only with 4-epiOTC did not corroborate.

3.4. OTC residues depletion

The results of OTC residues depletion in sheep milk are shown in Table 3, whereas typical chromatograms of incurred sheep milk samples are presented in Fig. 2. The results indicated that none of the 20 sheep treated produced milk with 4-epiOTC concentrations above the limit of quantification of the developed method. This finding is clearly not in accordance with the results of a previously published study [20] where the presence of 4-epiOTC in the tissues of the OTC treated sheep, as a consequence of *in vivo* epimerization, was proved.

As far as the concentration of OTC in sheep milk is concerned, the results presented in Table 3 indicate that OTC concentration

Table 2
Precision data for the determination at three different days of oxytetracycline marker residue in sheep milk fortified with both oxytetracycline and 4-epi-oxytetracycline.

Residue	Fortification level (μg/kg)	Mean ^a concentration found ± SD (μg/kg)			RSD (%)			Overall RSD (%)
		Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	
OTC	50	48.1 ± 1.9	45.4 ± 1.9	47.0 ± 2.2	3.9	4.2	4.8	4.6
	100	91.5 ± 3.6	93.3 ± 2.7	88.9 ± 3.8	4.0	2.9	4.2	
	150	134.6 ± 4.9	131.4 ± 4.9	138.6 ± 5.6	3.6	3.7	4.0	
4-epiOTC	50	44.4 ± 2.1	44.9 ± 1.8	46.3 ± 2.0	4.6	3.9	4.3	4.4
	100	85.3 ± 2.6	91.5 ± 2.5	87.6 ± 3.6	3.0	2.8	4.1	
	150	126.3 ± 2.6	130.7 ± 4.4	133.9 ± 3.5	2.1	3.4	2.6	

^a Mean of six replicates.**Table 3**
Concentration (μg/kg) of oxytetracycline marker residue in the milk of 20 sheep after the intramuscular administration of oxytetracycline at a dose of 10 mg/kg body weight once daily for 5 consecutive days.

Milking post-treatment	OTC concentration found ^a		4-epiOTC concentration found ^a
	Concentration range	Mean concentration ± SD	
1	513.8–1557.3	940.5 ± 284.0	<20.0
2	366.2–1053.5	647.5 ± 218.9	<20.0
3	66.2–369.1	191.8 ± 95.4	<20.0
4	34.4–207.1	96.6 ± 51.4	<20.0
5	<20.0 to 87.6	53.7 ± 18.3 (<i>n</i> = 11) ^b	<20.0
6	<20.0 to 66.0	47.9 ± 13.7 (<i>n</i> = 5) ^b	<20.0
7	<20.0 to 34.6	33.2 ± 1.3 (<i>n</i> = 3) ^b	<20.0
8	<20.0	<20.0	<20.0

^a Values have been corrected for recovery.^b Number of sheep with OTC concentration above the limit of quantification.

dropped below the MRL, five (5) milkings after the last intramuscular administration.

3.4.1. Estimation of withdrawal period

Using the TTSC method recommended by the EMEA [22], a withdrawal period of 6.589 milkings was estimated. Since this point does not make up full milkings, the withdrawal period would have to be rounded up to the next milking [22]. Therefore, a withdrawal period of 7 milkings (84 h) for the conventional OTC formulation was estimated to guarantee consumer safety.

4. Conclusions

The developed method needs very small sample size, offers considerable savings in terms of solvent requirements, costly materials, sample manipulation and time of analysis, and presents satisfactory analytical characteristics with respect to recovery, sensitivity, selectivity, and repeatability. Sample throughput (extraction/clean-up/LC determination) is 16 samples in 4 h by a single analyst. The method utilizes commercially available reagents and equipment and is designed to be performed easily by trained analysts. Owing to these advantages, the method might be considered suitable for routine monitoring as well as for depletion studies of OTC residues in milk. The developed method was successfully applied for studying the depletion of OTC residues in sheep milk.

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