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Monitoring of oxytetracycline in ovine milk by high-performance liquid chromatography

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Abstract

A method for 'in vivo' determination of the oxytetracyclin residues in ovine milk at low levels is described. Two groups of Sardinian breed sheep were treated with a dose of oxytetracycline by intramammary infusion and intramuscular administration, respectively. Oxytetracycline residues in extracts obtained from a preliminary cleanup procedure, were detected by an isocratic high-performance liquid chromatography (HPLC) method. Linear calibration plots were obtained over a large concentration range of 1 mg ml^{-1}–10 ng ml^{-1}, with correlation coefficients higher than 0.996. Recoveries between 85.8 and 98.9% were obtained. Limit of detection (LOD) and limit of determination (LOQ) were 5.2 and 17.5 ng ml^{-1}, respectively. This method would be useful for routine monitoring of oxytetracycline residues in ovine dairy milk.

Keywords: Oxytetracycline; In vivo monitoring; Trace amounts; Reversed-phase liquid chromatography; Milk

1. Introduction

Oxytetracycline is frequently used in veterinary practice for treatment of ovine mastitis and microbial infections. This fact has great importance in human pathology, indeed a very modest quantity of this compound in milk might be responsible for toxic effects and allergic reactions [1,2]. Therefore the World Health Organization has recommended a maximum allowable level of 100 ppb [3]. In addition, oxytetracycline residues may cause inhibition of starter cultures in the manufacture of fermented dairy products such as cheese, buttermilk and yogurt [4,5].

Sheep breeding in Sardinia is the most productive income of the local economy; therefore, it is

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of great importance to improve methods of detection of this antibiotic in milk when the dairy sheep are treated. Many methods are available for the determination of oxytetracycline residues in milk. These report bioassays [6] as microbiological tests based on enzyme inhibition [7]. These methods are very sensitive but not specific because they cannot distinguish between different antibiotics. The physicochemical methods are more reliable than the bioassays. The latter are thin layer chromatography [8], electrophoresis [9] and gas–liquid chromatography [10], however most of them cannot be considered sufficiently simple, fast and precise.

Considerable progress has been made recently on the detection of oxytetracycline residues by the use of high-performance liquid chromatography (HPLC). Particularly current HPLC methods have dealt mostly with tetracyclines analysis using reversed-phase columns [11–15]. These methods are much more selective than the microbiological assay and can solve problems with interfering substances such as lysozyme and lactoferrin [7]. With HPLC technique it is possible to reduce interfering background peaks on chromatograms and increase specificity. Indeed oxytetracycline is well isolated from interfering peaks in the blank milk and the peak areas provide good quantitation. Further confirmation of detection of oxytetracycline may be based on the UV spectrum obtained using a diode array detector. Finally with the cleanup procedures all the samples give essentially quantitative recoveries with good accuracy and enough sensitivity.

This paper describes an improved method for the 'in vivo' monitoring of oxytetracycline in milk which combines a cleanup procedure based on the method described by White et al. [11], with a modified HPLC procedure, described by Fletouris et al. [12].

Our data demonstrate the suitability of this method for routine monitoring. The method was applied to determine oxytetracycline residues in milk of two groups of Sardinian breed sheep treated with a recommended dose of oxytetracycline, administered by intramammary infusion (group A) and intramuscular injection (group B), respectively. Concentration/time curves were obtained for 8 days after administration.

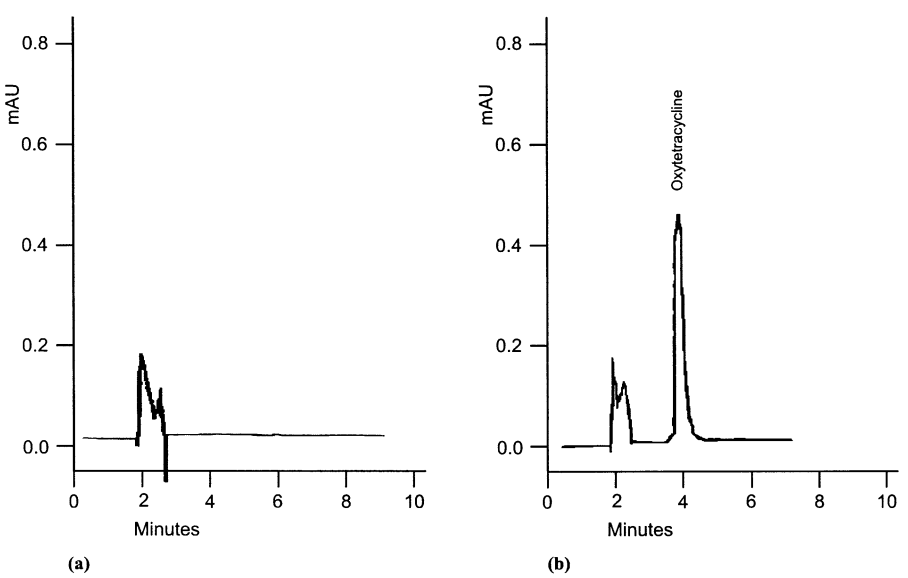

Fig. 1. Chromatograms of extracts obtained from: (a) blank milk sample; and (b) milk sample fortified with oxytetracycline to 0.01 μg ml^{-1}.

Table 1
Recovery data of spiked milk samples

Concn. added μg ml^{-1}	Number of replicates	Mean concn. found μg ml^{-1} ± S.D.	Mean rec.%	S.D.%
1000	6	989.6 ± 23.3	98.96	2.35
100	6	97.47 ± 2.77	97.47	2.84
10	6	9.684 ± 0.371	96.84	3.83
1	6	0.947 ± 0.031	94.70	3.27
0.1	6	0.0891 ± 0.0039	89.10	4.38
0.01	6	0.00858 ± 0.00047	85.80	5.47

Table 2
Precision data for milk samples spiked to 0.1 μg ml^{-1}

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
	0.08347	0.08882	0.08910	0.09318	–	0.08916	0.09005	0.08918
	0.09312	0.08961	0.09203	0.08902	0.09657	0.09013	0.08999	0.08917
	0.08923	0.08941	0.08884	0.09212	–	0.09009	0.08884	0.08231
	0.08911	0.09514	0.08744	0.08717	0.09037	0.08915	0.08437	0.08064
	0.08993	0.09111	0.08975	0.09015	0.9515	0.08691	0.08597	0.09014
	0.08666	0.08457	0.08730	0.08412	0.08270	–	0.09531	0.09015
Mean	0.08859	0.08977	0.08907	0.08930	0.09120	0.08909	0.08909	0.08693
S.D.	0.00325	0.00343	0.00174	0.00332	0.00625	0.00131	0.00381	0.00428
R.S.D.%	3.66	3.82	1.2	3.73	6.86	1.47	4.27	4.92
Recovery%	88.6	89.8	89.1	89.3	91.2	89.1	89.1	86.9

2. Experimental

2.1. Materials and reagents

Oxytetracycline (>98%) was purchased from Fluka Biochemika. HPLC-grade acetonitrile was purchased from Carlo Erba Reagents (Milan, Italy). Deionized and distilled water was purified through a Milli Q system (Millipore). All other reagents were of analytical grade and were purchased from Carlo Erba Reagents (Milan, Italy). Phosphoric acid solution, pH 2.3, 0.02 M, was prepared as described by Fletouris et al. [12].

2.2. Sample preparation

Primary stock standard solutions of oxytetracycline (1000–0.01 μg ml^{-1}) were prepared in water and were stable for 1 month when stored at −25°C. Calibration standard samples were prepared spiking the extracts from blank milk samples with the appropriate volumes of primary stock standard solutions.

For recovery determination six milk samples were spiked by adding the appropriate volume of stock solution to 10 ml of milk, obtaining final concentrations of 1, 0.1, 0.01 mg ml^{-1}, 1, 0.1, 0.01 μg ml^{-1}.

2.3. Extraction and cleanup procedure

A 10-ml volume of milk was measured and poured into a 100-ml conical flask. Two milliliters of 1 N HCl and 48 ml of acetonitrile were slowly added with vigorous swirling. After standing for 5 min the clear supernatant was decanted through a plug of glass wool in the stem of a funnel and collected directly in a 250-ml separatory funnel. Methylene chloride (60 ml) and hexane (120 ml) were added and, after shaking, the mixture was allowed to separate for 5 min. The lower layer (aqueous phase) was transferred into a 100-ml flask. The remaining organic layer was washed with 4 ml of water. The aqueous phases were mixed, neutralized with $NaHCO_3$ 5% solution and

evaporated under reduced pressure in a 50–60°C water bath. Final obtained volumes were: 10 ml for more concentrated sample solutions and 1 ml for more diluted ones. The obtained final solutions were transferred to appropriate autosampler vials.

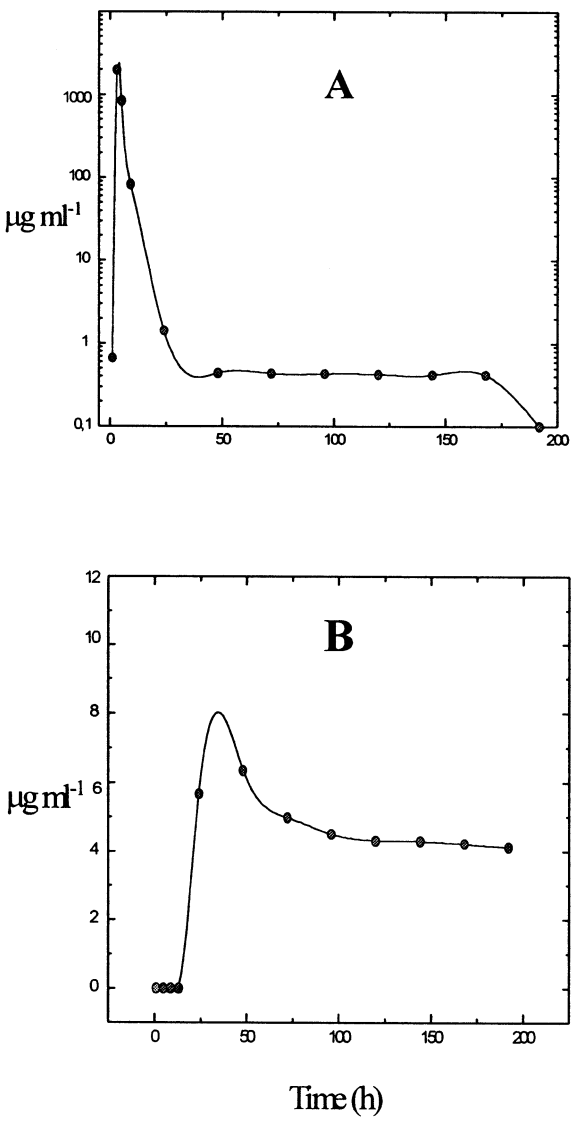

Fig. 2. Concentration/time curves: A: intramammary infusion; and B: intramuscular injection.

2.4. Instrumentation and HPLC conditions

HPLC analyses were carried out on a Hewlett–Packard liquid chromatograph HP 1084-B, variable-volume injection and variable-wavelength UV detector set at 355 nm; data acquisition was controlled by system integrator (HP 79850 B LC terminal). Injections were automatically made on a reversed-phase Supelco Supelcosil LC-18-DB column (5 μm; 4.6 mm × 150 mm). The mobile phase was degassed using helium delivered at a rate of 1 ml min^{-1}. Injection volume was 100 μl for all standards and samples of the final milk extracts. The analyses were carried out at room temperature under isocratic conditions with the pH 2.3 phosphoric acid solution-acetonitrile (76:24, v/v) using the HP system; detector sensitivity of 512×10^{-4} absorbance units and a chart speed of 0.50 cm min^{-1}. Eluted peaks were detected at 355 nm.

2.5. 'In vivo' procedures

The study was conducted for 8 days on two groups of six Sardinian breed sheep (five experimental plus one untreated control per group; middle weight 45 kg). Both groups were treated with a single dose of 20 mg kg^{-1} of oxytetracycline; an intramammary infusion was administered to the first group after the morning milking; the second group was treated by intramuscular administration. Control milk samples, taken from untreated sheep, and all other samples were collected at 6-h intervals during the first day of the trial and at 12-h intervals during the subsequent 7 days. All samples were stored at −25°C until analyzed.

3. Results and discussion

In the present work extracts obtained from milk cleanup were analyzed by HPLC to determine oxytetracycline content. Previous studies [11–13] demonstrated that extraction/deproteinization was simple, rapid and gave essentially quantitative recoveries. Our intention with the previously described cleanup was to avoid the use of large

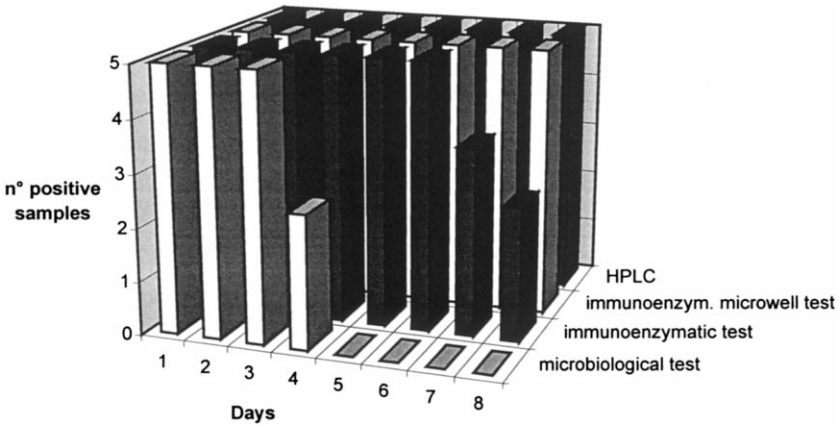

Fig. 3. Qualitative comparison between HPLC method and biological assays [17].

sample volumes keeping an adequate injectable volume without losing accuracy and sensitivity.

The effectiveness of the cleanup procedure allowed chromatographic analysis of milk samples under isocratic conditions and the chromatograms recorded at 355 nm were free of interfering extraneous peaks, in the extracts of blank as well as in spiked samples (Fig. 1).

The linearity of calibration curves, obtained spiking the extracts of blank milk samples, was studied over a large concentration range of 1 mg ml^{-1}–10 ng ml^{-1}. The wide range was necessary for the determination of residues in samples obtained after intramammary infusion; indeed the residue quantities were particularly high in the first sampling.

Three standard curves were plotted as the peak-area vs. concentration with six points each. The equation of standard curves were: $y = -51.4(\pm 20.5) + 2.283(\pm 0.034)x$ and $r = 0.9995$ (range 1000–100 μg ml^{-1}), $y = -3.87(\pm 2.09) + 1.918(\pm 0.054)x$ and $r = 0.9984$ (range 50–0.5 μg ml^{-1}), $y = -0.0011(\pm 0.0010) + 0.511(\pm 0.024)x$ and $r = 0.9966$ (range 0.1–0.01 μg ml^{-1}). Values for the limit of detection (LOD) and limit of determination (LOQ) were 5.2 ng ml^{-1} (0.0052 μg ml^{-1}) and 17.5 ng ml^{-1}(0.0175 μg ml^{-1}), respectively in the lowest range. The LOD and the LOQ were defined as the concentrations obtained calculating the standard deviation of the lowest range multiplied by three and ten times, respectively [16].

The recovery of the method was studied by spiking milk samples at six fortification levels with aqueous oxytetracycline standard solutions and analyzing six replicates. The concentration ranged from 1000 to 0.01 μg ml^{-1}. Least-squares and regression analysis of the data presented in Table 1 show that the relationship between 'added' and 'found' was adequately described by the linear regression $y = -0.3327 + 0.9898x$, $(r = 0.9999)$. Therefore, the slope (0.9898 ± 0.007) of this regression line could be used as an estimate of overall recovery corresponding to $98.98 \pm 0.7\%$.

The precision of the method was also studied by assaying, on each of the 8 different days, several milk samples spiked with oxytetracycline at 0.1 μg ml^{-1} (intermediate level). To improve the precision the analyses were carried out by the same operator, using the same material and performing six trials each day during 8 days. Results are shown in Table 2; three of the 48 values were doubtful and were discarded.

Finally the stability of the column was evaluated calculating the retention time of a standard solution of oxytetracycline every 40 sample injections (after washing with the eluent for 20 min) and the mean of the observed retention time was 4.12 min $\pm$ 4.05%S.D.

The present method provided the necessary mean for the determination of oxytetracycline residues in real samples ('in vivo') studying two groups of Sardinian breed sheep for 8 days.

Fig. 2A shows the milk concentration-time curve for intramammary infusion. The highest concentration was observed, as expected considering this administration method, at the second sampling with a recovery of 53.4% of oxytetracycline, while the concentration remained fairly elevated up to the 170 h and decreased to no detectable levels on the eighth day.

Fig. 2B shows the milk concentration-time curve for intramuscular injection. In this case the highest concentration was observed at 48 h, while during the following days the concentration remained quite elevated up to 8 days after the injection.

Fig. 3 shows the qualitative comparison between HPLC method and the biological assays applied to the same Sardinian breed sheep described by De Santis et al. [17]. Only one (TC microwell test) of the two immunoenzymatic tests was comparable to HPLC method, while the biological test was inadequate.

4. Conclusions

Concentration/time curves showed oxytetracycline residues for a long period in both administration ways. Surprisingly high concentrations were obtained also in intramammary infusion where a rather quick clearance was expected [18]. On the other hand intramuscular administration resulted in antibiotic concentration values over the limit of determination still at day 8.

In conclusion the results of the present study show that the proposed cleanup-HPLC method is an efficient and reliable means of quantitating oxytetracycline in milk and would be useful for routine monitoring of oxytetracycline residues. Since 12–14 samples can be easily processed by a single operator in an 8-h working day and only a single cleanup step is used, the method is suitable for detection on large scale of oxytetracycline residues in milk of treated ovine.

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