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Determination of cloxacillin in milk and blood of dairy cows by high-performance liquid chromatography

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Abstract

A rapid and sensitive high-performance liquid chromatographic method is described for the determination of cloxacillin residues in milk and serum. Only a clean-up step after deproteinization is necessary before the analysis. The chromatographic system involves the use of a C_{18} column and ultraviolet absorbance detection at 225 nm. The mobile phase was acetonitrile–0.02 *M* KH_2PO_4 (21:79) at pH 5. Recoveries for cloxacillin were 83.5 and 75.7% in serum and milk, respectively. Detection limits (10 ng/ml in milk and 50 ng/ml in serum) were below the stipulated European Union maximum residue limits for cloxacillin. Thus, the described method showed the same accuracy, precision and sensitivity as the microbiological assays but without interferences caused by other drugs commonly used in therapy. Analysis of different blood and milk samples obtained at different times from dairy cows treated with an intramammary dose of cloxacillin benzatine showed undetectable cloxacillin levels both in milk and blood samples. © 1997 Elsevier Science B.V.

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1. Introduction

Cloxacillin is a β-lactam antibiotic used in veterinary practice because of its activity against Gram positive and Gram negative bacteria. The drug is used in the treatment or prevention of bovine staphylococcal mastitis and is also effective in the treatment of keratoconjunctivitis in cattle produced by *Mycobacterium bovis* [1].

In order to ensure human food safety, the European Union (EU) has established maximum residue limits (MRLs) for veterinary drug residues in food. For this reason, it is important to develop analytical methods to confirm the presence of drugs at the MRL level.

Cloxacillin has been detected in milk using thin-layer chromatography (TLC) [2], high-performance liquid chromatography (HPLC) with ultraviolet (UV) or fluorescence detection [3–7], gas chromatography (GC) or electrospray [8–10] and microbiological assays [11,12]. Microbiological assays are extremely sensitive to β-lactam antibiotics, detecting less than 10 ppb in milk, although they sometimes give false positive results and a simple and confirmatory procedure of comparable sensitivity was not available. The aim of the present study was to develop a method to determine and quantify cloxacillin in serum and milk of dairy cows.

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2. Experimental

2.1. Chemicals

Cloxacillin sodium and cloxacillin benzatine were provided by J. Uriach Labs. (Barcelona, Spain). Oxacillin was obtained from Sigma (St.Louis, MO, USA). HPLC reagent grade acetonitrile, dichloromethane and trichloromethane were purchased from Riedel-de Haën (Seelze, Germany). The other reagents were of analytical-reagent grade. Water was double distilled. Standard solutions of 100 μg/ml and 10 μg/ml of cloxacillin and oxacillin were prepared in 0.02 *M* KH_2PO_4.

2.2. Chromatographic system

The HPLC system consisted of a Rheodyne injector, a Perkin-Elmer Model 250 binary LC pump and a Perkin-Elmer Model 75 detector (Norwalk, CT, USA). Separation was achieved on a C_{18} reversed-phase column (15 mm×3.9 mm I.D., Nova Pack,4 μm, 60 Å, Waters, Milford, MA, USA). The mobile phase was acetonitrile–0.02 *M* KH_2PO_4, (21:79, v/v) pH 5 and the flow-rate was 1.2 ml/min. The chromatogram was monitored at a wavelength of 225 nm. Data processing was handled by a Waters 746 integrator.

2.3. Animals

A single dose of 500 mg/quarter of cloxacillin benzatine was administered by the intramammary route to six dairy cows, in dry period and in the seventh month of pregnancy. Blood samples were collected at 0, 5, 10, 20 and 30 days after drug administration, as well as at the moment of delivery. Then, they were centrifuged at 1932 *g* for 20 min to collect the serum. Milk samples were collected before drying and during milking on the 1st, 3rd, 6th, 9th and 12th day after delivery. Milk from the first jet after delivery was also collected. All the samples were frozen at −80°C until processing.

In order to know the stability of cloxacillin, samples of blood and milk were spiked with 200 ng/ml of cloxacillin and stored at −80°C. Then, samples were processed at different times (1, 4, 8, 15 and 21 days post-freezing) to quantify the amount of cloxacillin.

2.4. Treatment of blood and milk samples

A volume of 2.5 ml of blood serum and 5 ml of milk, containing 250 ng of oxacillin as internal standard, were adjusted to pH 6.3 with 0.1 *M* HCl and deproteinized with 10 ml of acetonitrile. After centrifugation at 1932 *g* for 20 min, the aqueous phase was transferred to a tube and then extracted with 2×5 ml of dichloromethane by gentle blending for 20 min. Tubes were centrifuged at 1932 *g* for 20 min to allow phase separation. Aqueous phase was discarded and the organic phase was evaporated until dryness using a rotary evaporator. For milk samples, extraction was achieved with 2×5 ml of chloroform. Blood and milk extracts were redissolved with 200 μl of mobile phase and 100 μl of each sample solution were injected in the chromatographic system. Blank blood and milk samples were prepared using the same procedure except that no oxacillin was added.

2.5. Recovery

Drug-free samples were spiked with standard cloxacillin in the range 25–250 ng/ml for milk and 50–500 ng/ml for blood and processed as described (Section 2.4). After extraction, oxacillin (internal standard) was added for analysis. A standard series of cloxacillin containing internal standard was also prepared with the same concentration range and was directly analyzed by HPLC without extraction (external samples). Extraction efficiency was determined by comparison of HPLC results of internal and external samples.

2.6. Calibration

A standard series in the range 25–500 ng/ml of cloxacillin in drug-free milk samples and 50–1000 ng/ml in drug-free blood samples were prepared and processed as described in Section 2.4. Method linearity, precision and accuracy, quantification limit and specificity were calculated ($n=5$). The limit of quantification (LOQ) was determined by studying

the accuracy and precision from samples containing 5, 10, 25, 50 and 100 ng/ml of cloxacillin. The limit of quantification represents the minimum concentration with an accuracy and precision within the established range.

2.7. Stability

In order to confirm the stability of fortified samples of milk and serum, 25, 100 and 500 ng/ml of cloxacillin were added to samples of both matrices. Samples were then frozen at −80°C and analyzed after 1, 4, 8, 15 and 21 days, evaluating the amounts of cloxacillin present.

3. Results and discussion

Figs. 1 and 2 show the chromatograms from blood and milk blank samples and samples spiked with cloxacillin and oxacillin.

The retention times were 5.6 min for cloxacillin and 3.8 min for oxacillin. Cloxacillin and oxacillin peaks showed a good resolution and no interferences, reflecting the high specificity and sensibility of the described method. Other described methods [6,10] use a high volume of sample to increase the sensibility, thereby necessitating the inclusion of more extraction steps. In our method, 2.5 ml of serum and 5 ml of milk were the most suitable volumes to obtain good sensibility, recovery and specificity.

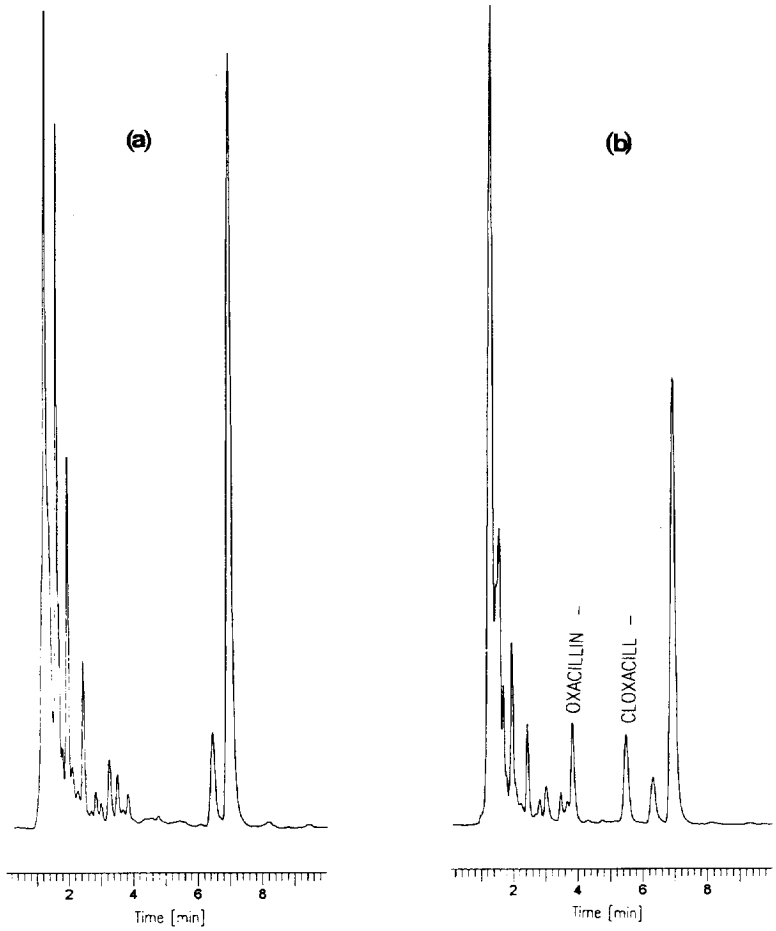

Fig. 1. Chromatograms obtained from a blank (a) and a blood sample spiked with 250 ng of oxacillin and 100 ng/ml of cloxacillin (b).

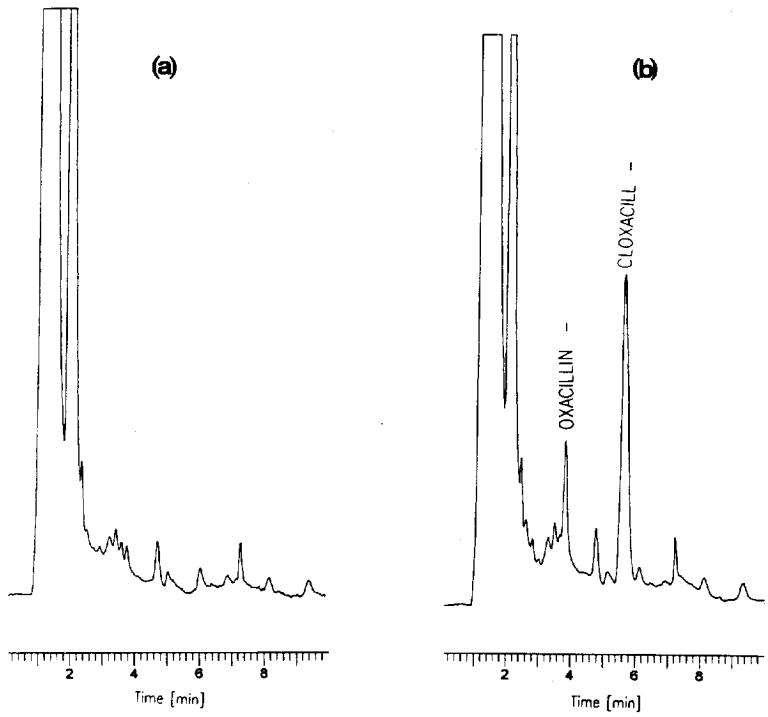

Fig. 2. Chromatograms obtained from a blank (a) and a milk sample spiked with 250 ng of oxacillin and 100 ng/ml of cloxacillin (b).

A pH of 6.4 is necessary for a good recovery of cloxacillin in organic solvents such as methylene chloride. The results obtained show the efficiency of acetonitrile in cloxacillin extraction from milk and serum, although other studies refer different concentrations of acetonitrile in water as the most effective releasing solution for β-lactam antibiotics [7,8]. Furthermore, the simple extraction procedure contributes to obtain a high recovery. The present method improves the sensitivity of screening tests for cloxacillin and presents better separations from interferences. Although peaks were observed in milk samples chromatograms, no additional washing steps were required to clear the evaporated concentrate since no interfering peaks were detected at the same retention time.

3.1. Recovery

Table 1 shows the recovery of cloxacillin obtained from blood and milk samples. In blood and milk samples, recoveries ranged from 79% to 93% and from 75% to 84%, respectively. In milk samples, precision was better at 250 ppb than at lower concentrations. On the other hand, the best precision in blood samples was obtained at 50 ppb and the spiked amount did not influence the recovery. These results show matrix influences in the recovery of cloxacillin due to differences in the drug polarity of milk and blood. The watery blood composition and the physico-chemical properties of cloxacillin may determine the better clean-up of blood samples.

Table 1
Recovery of cloxacillin obtained in blood and milk samples of dairy cows and spiked with different concentrations of the drug

Spiked concentration (ng/ml)	Recovery (%)	
	Blood	Milk
25	–	84±16
50	81±6	75±9
100	79±14	76±5
250	81±10	76±4
500	93±8	–

Data are expressed as mean±S.D. (n=3).

Table 2
Precision and accuracy of cloxacillin determination in milk samples spiked with different concentrations of the drug

Spiked concentration (ng/ml)	Measured concentration (ng/ml)	R.S.D. (%)	Accuracy (%)
25	28±8	28	12
50	55±8	14	10
100	100±9	9	9
250	230±20	8	2
500	490±10	2	2

Data are expressed as mean±S.D. (n=5).

3.2. Calibration

Blood and milk samples spiked with five different concentrations of cloxacillin were analyzed. All analysis were performed in quintuplicate. The peak-area ratios (cloxacillin to oxacillin as internal standard) showed a linear relationship with the concentration over the range 25–500 ng/ml and 50–1000 ng/ml for milk and blood respectively. The equations were $y=11.2x+0.17$ ($r=0.9935$) for blood samples and $y=1.9x+0.15$ ($r=0.9894$) for milk samples, y being the peak-height ratio (cloxacillin to oxacillin) and x the cloxacillin concentration (ng/ml).

Precision of the method can be expressed as inter-day variability in the cloxacillin concentration range for milk samples and blood samples. The accuracy of the method can be measured by the difference between the concentrations observed and those calculated, and be expressed as the relative error. These results are shown in Tables 2 and 3. As expected, precision and accuracy of the method in milk samples improved as the spiked concentration increased. However, precision and accuracy in blood samples were related to the spiked concentration.

The limit of quantification for cloxacillin was 10 ng/ml and 50 ng/ml for milk and blood samples, respectively. In milk, these levels were lower than the MRL fixed by EU (30 ppb). In blood, the levels were below the detection limit. In different studies, the detection limit obtained by liquid chromatography was higher, necessitating other detection systems to confirm low levels of cloxacillin in samples [12–14].

Slow release antibiotic is administered to dairy cows by intramammary route two to three months before delivery (dry period) in order to avoid infection risks. For this reason, it is necessary to guarantee that the milk obtained from treated animals after delivery is free of antibiotic residues. Results showed that the cloxacillin levels in serum were below the detectable levels after five days of administration (<50 ng/ml) and were not detectable in milk samples (<10 ng/ml), including the milk from the first jet after delivery. This means that the total amount of the drug was removed before the cow started next milking. The high detection limit, the good recovery and the clean-up reached with this methodology suggest the reliability of the results and, therefore, milk from treated cows may be consumed without risk for the human health.

These results are in accordance with the properties

Table 3
Precision and accuracy of cloxacillin determination in blood samples spiked with different concentrations of the drug

Spiked concentration (ng/ml)	Measured concentration (ng/ml)	R.S.D. (%)	Accuracy (%)
50	42±2	5	16
100	96±18	19	4
250	270±30	11	8
500	540±50	9	8
1000	990±70	7	2

Data are expressed as mean±S.D. (n=5).

of the drug and the tissue where the drug was placed (mammary gland). It is well known that cloxacillin is a β-lactam antibiotic with a pK_a of 2.7. In the mammary gland (pH around 6.8), this molecule is expected to be present in non-dissociated form. Therefore, the drug would be unable to cross the cellular membrane. The drug would be absorbed slowly and then serum and tissue concentrations would be very low. Accordingly, levels found in mammary gland during drying could be considered as therapeutic, whereas the drug would have practically disappeared at the moment of milking.

3.3. Stability

Cloxacillin is stable throughout the freezing process. No differences were observed between the amount of cloxacillin spiked and that observed in the samples stored during different periods of time. These results enable us to assure that the concentration detected in samples from cows treated with cloxacillin is correct.

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