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Monitoring of benzylpenicillin in ovine milk by HPLC

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Abstract

A method for the determination in vivo of the benzylpenicillin residues in ovine milk at low levels was described. Two groups of Sardinian breed sheep were treated with a dose of penicillin G sodium salt by intramammary infusion and intramuscular administration respectively. The residues were detected by isocratic HPLC method of the extracts obtained from a previous cleanup procedure. Linear calibration plots were obtained over a large concentration range of 1 mg ml^{-1}–10 ng ml^{-1}, with correlation coefficients greater than 0.998. Recoveries between 78.6 and 87.3% were obtained. Limit of detection (LOD) and limit of determination (LOQ) were 2.6 and 8.8 ng ml^{-1} respectively. This method would be useful for routine monitoring of penicillin G residues in ovine dairy milk.

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

1. Introduction

Penicillin G 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 allergic reactions in humans [1,2]. In addition, penicillin is used for starter culture inhibition in the manufacture of fermented dairy products such as cheese, buttermilk and yoghurt [3,4].

Sheep breeding in Sardinia is the most productive income of the local economy; therefore, it is 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 benzylpenicillin residues in milk. These report bioassays as microbiological tests [5], enzyme inhibition [6], immunoassays [7,8] and competitive binding [9,10]. All these methods are very sensitive but not specific because they cannot distinguish between different β-lactam antibiotics. The physicochemical methods are more reliable than the bioassays. The latter are thin layer chromatography [11], electrophoresis [12] and gas–liquid chromatography [13], however most of them cannot be considered sufficiently simple, fast and precise.

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Considerable progress has been made recently on the detection of penicillin G residues by the use of high-performance liquid chromatography (HPLC). Particularly current HPLC methods have dealt mostly with single-component analysis using reversed-phase columns [14,15]. These methods are much more selective than the microbiological assay and can solve problems with interfering substances. With HPLC technique it is possible to reduce interfering background peaks on chromatograms and increase specificity. Indeed penicillin G is well isolated from interfering peaks in the blank milk and the peak areas provide good quantitation. Further confirmation of detection of penicillin G may be based on the UV spectrum obtained using a diode array detector. Finally with the cleanup procedures, even though time consuming, all the samples give essentially quantitative recoveries, and large sample volumes could be avoided leaving, however, an adequate sample volume for injection in order to obtain a good accuracy and enough sensitivity.

This paper describes an improved method for the in vivo monitoring of penicillin G in milk which combines a clean up procedure based on the method described by Moats [15], with a modified HPLC procedure, described by the same author [15].

Our data demonstrate the suitability of this method for routine monitoring. The method was applied to determine benzylpenicillin residues in milk of two groups of Sardinian breed sheep treated with a recommended dose of penicillin G sodium salt, administered by intramammary infusion (group A) and intramuscular injection (group B) respectively. Concentration/time curves were obtained since eighth day from administration.

2. Experimental

2.1. Materials and reagents

Penicillin G sodium salt ($>99\%$) was purchased from Fluka Biochemika. HPLC-grade acetonitrile was purchased from Carlo Erba (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). Phosphate buffer, pH 7, 0.0067 M, was prepared according to the British Pharmacopoeia [16], adjusting the pH with ortho-phosphoric acid when necessary.

2.2. Sample preparation

Primary stock standard solutions of penicillin G (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 in 50 ml conical flask. 20 ml of acetonitrile were slowly added with vigorous swirling. After standing for 10 min the clear supernatant was decanted through a plug of glass wool in the stem of a funnel and collected directly in a 100 ml separator funnel. Methylene chloride (15 ml) was added and, after shaking, the mixture was allowed to separate for 5 min. The lower layer (aqueous phase) was transferred into a 50 ml flask. The remaining organic layer was washed with 3 ml of water. The aqueous phases were mixed and evaporated under reduced pressure in a 40–50°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.

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 214 nm; data acquisition was

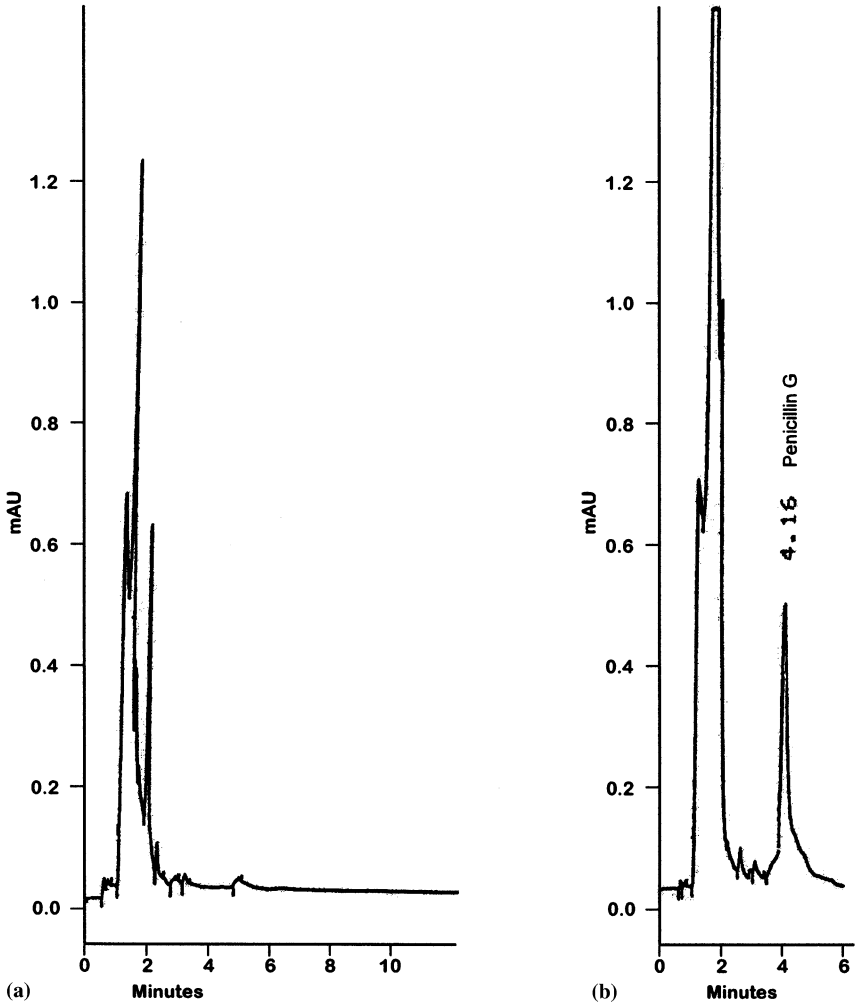

Fig. 1. Chromatograms of extracts obtained from: (a) blank milk sample and (b) milk sample fortified with penicillin G to 0.01 μg ml^{-1}.

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 × 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 7 phosphate buffer–acetonitrile (82:18, v/v) using the HP system; detector sensitivity of $512 \cdot 10^{-4}$ absorbance units and a chart speed of 0.50 cm min^{-1}. Eluted peaks were detected at 214 nm.

2.5. In vivo procedures

The study was conducted for eight 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 24 mg kg^{-1} of penicillin G sodium salt; 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

Table 1
Recovery data of spiked milk samples

Conc. added μg ml^{-1}	Number of replicates	Mean conc. found μg ml^{-1} ± S.D.	Mean rec. %	S.D. %
1000	6	857.6 ± 21.1	85.80	2.45
100	6	78.57 ± 2.92	78.57	3.71
10	6	7.965 ± 0.325	79.65	4.02
1	6	0.851 ± 0.038	85.10	4.70
0.1	6	0.0873 ± 0.0047	87.30	5.36
0.01	6	0.00821 ± 0.00045	82.10	5.48

of the trial and at 12-h intervals during the subsequent seven 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 penicillin G content. Previous studies [11,15] 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 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 214 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: $y = 6.06(\pm 2.55) + 0.2971(\pm 0.0043)x$ and $r = 0.9996$ (range 1000–100 μg ml^{-1}), $y = 0.8861(\pm 0.1824) + 0.3291(\pm 0.0087)x$ and $r = 0.9986$ (range 50–0.5 μg ml^{-1}), $y = 0.0176(\pm 0.0006) + 0.3503(\pm 0.0122)x$ and $r = 0.9982$ (range 0.1–0.01 μg ml^{-1}). Values for the limit of detection (LOD) and limit of determination (LOQ) were 2.6 ng ml^{-1} (0.0026 μg ml^{-1}) and 8.8 ng ml^{-1} (0.0088 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 [17].

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
	0.07830	0.08344	0.08731	—	0.09159	0.08416	0.08634	0.07696
	0.08881	0.08977	0.08030	0.09015	0.09081	0.08113	0.08615	0.08774
	0.09052	0.09001	—	0.08607	0.08651	0.08509	0.08711	0.09038
	0.08970	0.08711	0.08341	0.09305	0.08643	0.08881	0.08537	0.09333
	0.08621	0.08022	0.08475	0.08225	0.07415	0.09691	0.08763	0.08973
	0.09033	0.09351	0.08503	0.08671	0.08812	0.07998	0.09458	0.08865
Mean	0.08731	0.08734	0.08416	0.08765	0.08627	0.08601	0.08786	0.08780
S.D.	0.00468	0.00484	0.00257	0.00412	0.00631	0.00618	0.00338	0.00564
R.S.D. %	5.36	5.54	3.06	4.70	7.31	7.18	3.84	6.42
Recovery %	87.3	87.3	84.2	87.6	86.3	86	87.9	87.8

The recovery of the method was studied by spiking milk samples at six fortification levels with aqueous penicillin 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 $y = -1.47 + 0.858x$, $(r = 0.9998)$. Therefore, the slope (0.858 ± 0.01) of this regression line could be used as an estimate of overall recovery corresponding to $85.8 \pm 1\%$.

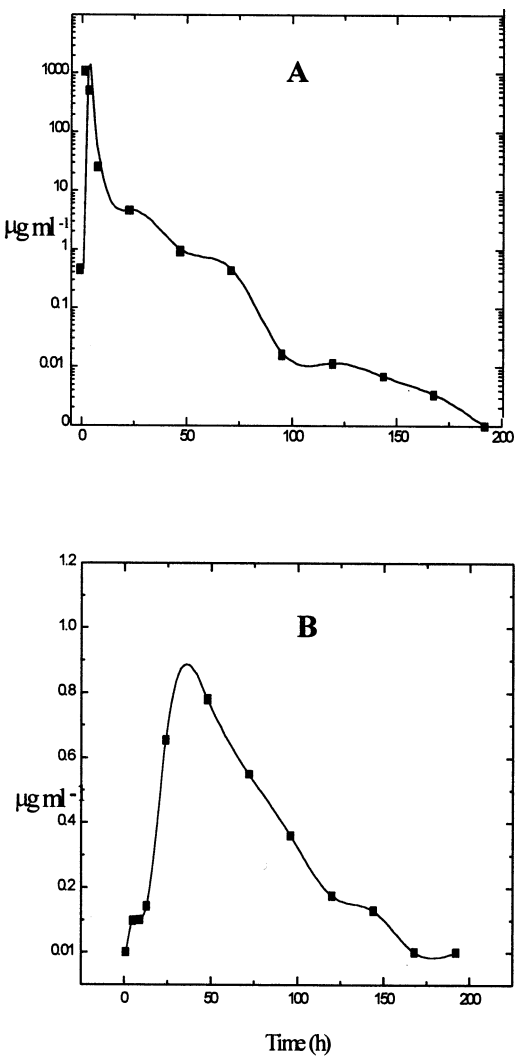

Fig. 2. Concentration/time curves: intramammary infusion A, Intramuscular injection B.

The precision of the method was also studied by assaying, on each of eight different days, several milk samples spiked with penicillin 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 eight days. Results are shown in Table 2; only two 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 penicillin G every 50 sample injections (after washing with the eluent for 20 min) and the mean of the observed retention time was 4.24 min $\pm 2.4\%$ S.D.

The present method provided the necessary mean for the determination of penicillin G residues in real samples (in vivo) studying two groups of Sardinian breed sheep for eight 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 27% of penicillin, while the concentration remained fairly elevated up to the 72 h and persisted in milk for as long as 8 days after treatment.

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 decreased gradually to no detectable levels at 8 days after the injection.

4. Conclusions

Curves concentration/time showed penicillin G 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 around 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 penicillin G in milk and would be useful for

routine monitoring of penicillin G 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 penicillin G residues in milk of treated ovine.

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