Oxacillin residues in milk after drying off with Stapenor® Retard TS†

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Milk samples from 28 cows were analysed for residues of oxacillin after drying off with Stapenor® Retard TS (oxacillin). Analysis was performed with an automated HPLC system consisting of an on-line solid-phase extraction and photochemical post-column derivatization with UV-detection at 300 nm. Although the time interval between treatment and parturition was less than the demanded 55 days, the maximum residue limit of 30 μg kg^{-1} was only exceeded in one case, in which the withdrawal time was 28 days.

Introduction

Mastitis is considered to be the most costly disease[1] affecting dairy cattle worldwide. Dry cow therapy, a part of the mastitis control program for nearly 25 years, is the intramammary use of antibiotics after the last milking of the lactation period. To guarantee an optimal quantity of milk in the next lactation, dairy cows should be provided with a dry period of about 45–55 days prior to the next expected calving. Because susceptibility to mastitis increases acutely during the first two weeks after drying off, antibiotics are generally applied to each quarter of the udder as a prophylactic treatment in order to avoid intramammary problems during the next lactation phase. The penicillinase resistant isoxazolyl penicillins, including oxacillin as used here, are among the most common antibiotics for mastitis therapy. Residues can occur following use of the drug, and concentrations can be higher if the drug is not used according to the producer's recommendations or when cows have a shorter than normal dry period. In the EU, a maximum residue limit[2] (MRL) of 30 μg kg^{-1} has been established for each isoxazolyl penicillin in milk.

The aim of this study was to evaluate the residues of oxacillin in colostral milk of cows dried off with Stapenor® Retard TS (oxacillin) in order to check if any risk for the consumer could occur when applying this drug. For analysis, an automated rapid HPLC method,[3] consisting of an on-line coupled solid-phase extraction (SPE) and post-column photochemical derivatization with UV-detection at 300 nm was used. To check the reliability of the method, two ways of sample preparation, a rapid aqueous, and a time-consuming acetonitrile extraction, were tested.

Experimental

Application and sample collection

28 pregnant cows, selected at random out of a larger herd, were dried off with Stapenor® Retard TS (oxacillin), a trademark of Bayer Vital GmbH (Leverkusen, Germany). The herd was kept under field situation at a dairy unit in northeastern Germany. For drying off each quarter of the mammary gland was treated with a single injection of Stapenor® Retard TS, consisting of 1000 mg oxacillin, according to the product instructions. Within the selected cows, the expected time interval between drying off and parturition was shorter than 52 days. During the dry period no other drugs were applied to the cows. The withdrawal time for milk is 5 days after the start of lactation if drying off takes place 55 days before parturition, or otherwise 60 days after the date of drying off.

Milk samples were gathered under field conditions twice a day for 5 consecutive days, starting on the first day of lactation, and stored at −20 °C until determination. As newborn calves were allowed to drink colostrum from their mothers, milk samples from the first day were only available from 14 cows. Sample collection from the remaining cows started at day 2 (5 cows), day 3 (4 cows), day 4 (3 cows) or day 5 and 6 (one animal each), respectively.

Methodology

Sample preparation

Aqueous extraction[3]

After addition of 1.25 ml acetonitrile as releasing solvent to 20 g of milk, the sample was ultrasonicated for 10 min and then centrifuged at 4 °C for 25 min at 2000*g* for defatting. The fat layer was removed with a spatula, and the skimmed milk was ultrafiltered (30 000 Da) using a stirred cell. A portion of the filtrate (2 ml) was taken for HPLC determination with on-line SPE. For method validation six different raw milk samples were spiked in triplicate with oxacillin at concentration levels of 15, 30 and 60 μg kg^{-1}. Analysis was performed on 6 different days within three weeks, thus 18 single values were obtained for each fortification level.

Acetonitrile extraction[4]

After acidification samples of 25 g of milk were extracted with 90 ml acetonitrile. The aqueous layer was extracted by adding 50 ml dichloromethane and discarded. The organic layer was

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dried with anhydrous sodium sulfate and rotary-evaporated at 30 °C until 0.5 ml were left. The residue was suspended in 3 × 3 ml petroleum ether and oxacillin was extracted with 3 × 2 ml phosphate buffer of pH 7. The solution was diluted with water to a volume of 10 ml and 1 ml was taken for HPLC analysis with on-line SPE.

HPLC analysis

For sample clean-up and analyte enrichment, an HPLC-integrated SPE, based on a restricted access material (alkyl-diol-silica from Merck, Darmstadt, Germany), was used. Separation was performed on a reversed-phase HPLC column, and oxacillin was detected at 300 nm after irradiation with a Hg-lamp (λ_{max} = 254 nm) for 60 s.[3]

Results and discussion

The results of the validation including the aqueous extraction as sample preparation are given in Table 1. The limit of quantification (LOQ) and the limit of detection (LOD) were calculated according to the German National Standard Method DIN 32645.[5] The mean recovery rate obtained for spiked raw milk samples was 75.4% with a coefficient of variation 5.0%. Further details of the method concerning, *e.g.*, the apparatus or validation data, are given by Ibach and Petz.[3] To check the reliability of the rapid aqueous extraction for the analysis of colostral milk samples with regard to extraction efficiency, the samples containing incurred residues of oxacillin were also extracted with acetonitrile. This solvent is known as a releasing agent to cleave adsorptive bonds between penicillins and matrix components[6] and is also used in the German official method for the determination of penicillins by capillary gas chromatography.[7]

Comparison of aqueous and acetonitrile extraction

When acetonitrile was used for the extraction of spiked samples, recoveries >90% for oxacillin were obtained showing the higher efficiency of the organic solvent extraction. At the fortification level of 30 μg kg^{-1} the recovery rate obtained for spiked raw milk samples was 91.1 ± 2.5% (n = 8). But when corrected for recovery the results of both extraction procedures showed good conformity indicating that reliable results for oxacillin in milk could be obtained by using the rapid aqueous extraction, which allows the analysis of up to 30 samples per day. In contrast, using the acetonitrile extraction with its time-consuming evaporation step and liquid–liquid extraction procedures, only 6–8 samples could be prepared for analysis within one working day. Moreover, a large amount of organic solvents was needed for the acetonitrile extraction, whereas for the aqueous sample preparation only 1.25 ml acetonitrile were used. Table 2 presents the oxacillin concentrations of the 13 incurred samples analysed with both methods. Only the highly viscous milk samples, which were collected directly after calving, contained higher concentrations when acetonitrile extraction was used. These samples, which were difficult to ultrafilter because of their consistency, should be extracted with organic solvents.

Table 1 Validation data for oxacillin in spiked raw milk samples: mean recovery rates (n = 18 at each level, n = 3 at 6 different days) with coefficients of variation (day-to-day precision), limit of quantification (LOQ) and limit of detection (LOD)

Fortification level/ μg kg^{-1}	Recovery ± RSD (%)	LOD[a]/ μg kg^{-1}	LOQ[a]/ μg kg^{-1}
15	75.1 ± 5.8		
30	75.0 ± 5.7	3.2	10.2
60	75.9 ± 3.3		

[a] Calculated according to the German National Standards Method DIN 32645.[5]

Oxacillin residues in dried-off cows

The cows examined in this study had a time interval between drying off and parturition of 28 to 52 days, which was much shorter than the withdrawal time. As the depletion of a drug is dependent on the formulation and the concentration applied, studies should be made for each product.

This study was performed in order to evaluate if residues of oxacillin above the MRL could occur after drying off dairy cows with Stapenor® Retard TS. Analysis was performed with the aqueous sample preparation. Fig. 1 shows the typical HPLC chromatograms of a standard solution, a colostral milk blank and a spiked colostral milk sample at a fortification level of 30 μg kg^{-1}.

In most milk samples, the oxacillin concentration was below the limit of quantification or no residues were detectable (Table 3). The milk of five cows contained oxacillin concentrations (mean values) of 11.0–19.6 μg kg^{-1} only during the first two days of lactation. At days three and four after parturition residues of 12.9–18.1 μg kg^{-1} were analysed in the milk of two cows. Only the milk of one cow with a drying-off time of 28 days (half of the demanded withdrawal time) showed oxacillin residues, in three samples above the MRL, during the entire investigation period.

The study indicates that nearly all oxacillin residues, found after drying off with Stapenor® Retard TS, were below the MRL

Table 2 Oxacillin concentration (μg kg^{-1}) in incurred milk samples from two different methods of sample preparation (corrected for recovery: 75.4% aqueous extraction and 91.1% acetonitrile extraction, respectively)

Sample	Aqueous extraction	Acetonitrile extraction
1	18.1[a]	22.6[a]
2	13.0	13.3
3	16.5	16.9
4	12.9	9.7
5	32.4[a]	44.3[a]
6	23.1	24.8
7	17.0	17.0
8	15.1	12.7
9	24.0	25.8
10	31.5	29.7
11	11.4	10.5
12	31.4	25.7
13	28.7	26.4

[a] Highly viscous samples.

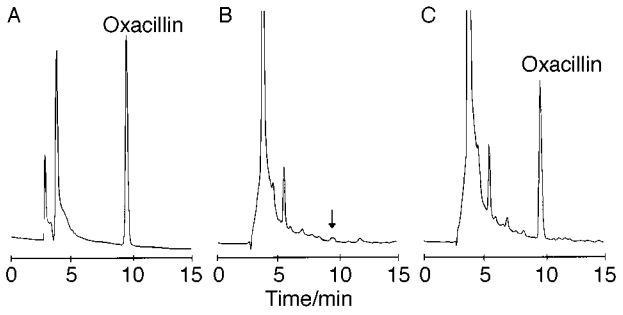

Fig. 1 HPLC chromatograms of: A, oxacillin standard equivalent to 30 μg kg^{-1}; B, colostral milk blank; C, colostral milk spiked with 30 μg kg^{-1} oxacillin.

Table 3 Oxacillin contents (μg kg^{-1}) of the analysed colostral milk samples. The results are corrected for recovery (75.4%)

		Samples from days after parturition											
	Dry Period	1		2		3		4		5		6	
No.	(days)	m[a]	e[a]	m	e	m	e	m	e	m	e	m	e
1	28	32.4	23.1	17.0	—[d]	15.1	24.0	31.5	11.4	31.4	28.7	10.5	—
2	28	<LOD[b]	<LOD										
3	29	<LOQ[c]	<LOQ	13.2	<LOQ	<LOQ							
4	29	18.1	<LOQ	13.1	16.5	<LOQ	12.9	<LOQ	<LOQ				
5	29	—	—	—	—	—	—	—	—	—	—	<LOQ	<LOD
6	30	<LOD	<LOD										
7	31	—	—	—	—	<LOD	<LOD						
8	32	<LOQ	<LOQ										
9	33	<LOD	<LOD										
10	33	<LOD	<LOD										
11	33	—	—	11.0	<LOQ	<LOQ							
12	35	—	—	—	—	<LOD	<LOD						
13	36	—	—	—	—	—	—	<LOD	<LOD				
14	36	—	—	—	—	—	—	18.1	<LOQ	<LOQ			
15	38	<LOD	19.6	<LOQ	<LOQ								
16	38	—	—	<LOD	<LOD								
17	40	<LOD	11.3	<LOQ									
18	42	18.3	16.6	<LOQ	<LOQ								
19	42	—	—	—	—	—	—	—	—	<LOD	<LOD		
20	43	—	—	<LOD	<LOD								
21	43	—	—	—	—	—	—	<LOD	<LOD				
22	44	—	—	—	—	<LOD	<LOD						
23	45	—	—	<LOD	<LOD								
24	45	—	—	<LOD	<LOD								
25	48	<LOQ	<LOD										
26	49	—	<LOD	<LOD									
27	49	—	—	—	—	<LOD	—	<LOD					
28	52	<LOD	<LOD										

[a] Morning/evening milk. [b] Value below limit of detection (<3.2 μg kg^{-1}). [c] Value below limit of quantification (<10.2 μg kg^{-1}). [d] Sample not available.

even when the time between treatment and calving was shorter than the demanded 55 days.

Conclusion

By comparing the aqueous with the organic solvent extraction it could be shown that the recently developed rapid HPLC method, including the aqueous sample preparation, could be used to analyse incurred colostral milk samples. As a measure of consumer protection a residue study was performed, evaluating the oxacillin concentration in colostral milk after drying off cows with Stapenor® Retard TS. No residues above the MRL are to be expected when the drug is applied according to the producer's instructions and when the withdrawal time is respected.

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