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Cold-temperature stability of five β-lactam antibiotics in bovine milk and milk extracts prepared for liquid chromatography–electrospray ionization tandem mass spectrometry analysis

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Abstract

The stability of five major β-lactam antibiotics (amoxicillin, ampicillin, cloxacillin, oxacillin, and penicillin G) in fortified milk and in milk extracts prepared for LC-ESIMS/MS analysis was studied at varying cold temperatures (4, −20, and −76 °C). Storage of milk samples at 4 °C resulted in measurable losses of all β-lactams after 6 days (>50% in most cases). Slow degradation of penicillin G, cloxacillin, and oxacillin was observed in milk stored at −20 °C, but no losses were recorded at −76 °C over 4 weeks. All antibiotics showed good stability at all temperature tested in milk extracts prepared for LC-ESIMS/MS analysis. The results of this study emphasize adherence to adequate sample handling and storage protocols as to reflect residue levels at the time of sample submission.

Keywords: Food analysis; Milk; Penicillins; β-Lactam antibiotics; Stability; Method validation

1. Introduction

Antibiotics of the β-lactam group are used intensely in dairy farming, particularly to combat mastitis, a serious disease that inflicts significant economic losses to the world's dairy industry [1]. In fact, more than 179 different antibiotic formulations used for the treatment of mastitis are commercialized and available today either in Switzerland, United Kingdom, France, Italy, or the United States of America. A large number of these drugs contain combinations of different active ingredients, e.g. aminoglycosides, macrolides, or tetracyclines, and more than 75% contain β-lactams that emphasizes their importance in mastitis treatment [1,2].

Several rapid test methods have been developed to ascertain the legislative compliance of raw milk "at farm level" and to ensure that residues do not enter the food chain at concentrations that may pose a health risk to consumers. However, it may be necessary to perform additional confirmatory testing by using alternative analytical techniques (e.g. LC–UV, GC–MS, LC–MS) that cannot be executed "in-the-field". In this case, the samples need to be transported to a testing laboratory and stored until further analysis. Knowledge on the optimal storage conditions is of key importance with regard to the residue stability, and hence of the outcome of the analysis. Therefore, knowledge of analyte stability in the food matrix – besides in reference solution – is one of the performance criteria for analytical methods set in the European Union (EU) commission decision 2002/657/EC [3].

Only a few studies have been performed to date on the stability of β-lactam antibiotics in various food matrices, and most of those reported have been done with animal tissues, such as liver, kidney, and muscle [4–12]. For example, the stability of the antibiotic penicillin G (PEN G) has been studied at storage temperatures of −20 and −76 °C in incurred and spiked bovine tissues and plasma up to time periods of 3 and 6 months [5,6]. Both of these studies reported a loss of PEN G at different rates in the various matrices kept at −20 °C, while the residue levels remained stable at −76 °C. Similarly, ampicillin (AMPI) in porcine muscle tissue stored at −75 °C over a period of 8 months showed no significant difference in

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the residue level [7]. In contrast, the same study shows that the residue level of AMPI started to decline in samples stored at −20 °C after 3 months. O'Brien et al. [8] also reported on the depletion of AMPI in various bovine tissues when stored at 4 and −20 °C, and other studies address the impact of sample preparation (bulk, sliced or ground tissue) before freezing on the depletion rate of β-lactam antibiotics [5–7].

However, there is only limited data available on the stability of β-lactam antibiotics in milk under cold-temperature storage conditions, and one study shows that approx. 60% PEN G – fortified at 0.35 International Units per mL – could be degraded within 48 h at 2 °C [9]. In contrast, Wiese and Martin [10] and Boison et al. [11] reported a constant residue level of PEN G in spiked raw milk (20 and 100 μg/kg) at 4 °C over a period of 6 days. Similarly, raw milk fortified with AMPI (20 μg/kg) and stored at 4 and −70 °C for up to 6 days did not reveal losses of the contaminant [12].

For the confirmation of the occurrence of PEN G, AMOX, AMPI, cloxacillin (CLOX) and oxacillin (OXA) in milk samples, we developed an analytical method encompassing a clean-up based on solid-phase extraction and liquid chromatography coupled with electrospray ionization tandem mass spectrometry (LC-ESIMS/MS) as determinative step [13]. In the context of method validation, we studied the stability of the target analytes in pasteurized milk and the corresponding milk extracts at various cold temperatures over a time period of 28 days, which provides some insight on the optimal storage conditions for the sample transport to a testing laboratory and/or the intermediate period of time pending analysis.

2. Experimental

2.1. Chemicals and material

Amoxicillin (13.7% H_2O), ampicillin (sodium salt), azlocillin (sodium salt), penicillin G (sodium salt) and cloxacillin (sodium monohydrate salt) were purchased from Sigma (Buchs, Switzerland). Oxacillin (sodium monohydrate salt) was obtained from Riedel-de Haën GmbH & Co. (Seelze, Germany). Potassium benzyl(d_7-phenyl)penicillinate (chemical purity >95%, d_7-PEN) was custom synthesized by Toronto Research Chemicals Inc. (North York, ON, Canada). The antibiotics were stored in a dry atmosphere at 4 °C (AMOX, AMPI, CLOX, OXA), at ambient temperature (PEN G, azlocillin) or at −20 °C (d_7-PEN).

Formic acid, *n*-hexane, ethanol, sodium chloride, disodium hydrogen phosphate dihydrate were of p.a.-grade (Merck, Darmstadt, Germany). Water was either purified in-house or purchased from Merck (LiChrosolv). Methanol (LiChrosolv, Merck) and acetonitrile (J.T. Baker, Phillipsburg, NJ, USA) were of HPLC-grade.

Bakerbond C_{18} solid-phase extraction (SPE) cartridges (3 mL, 500 mg) were obtained from J.T. Baker and cut-off filter devices (Microcon-10, nominal molecular weight limit 10,000 Da) from Millipore Corporation (Bedford, MA, USA).

2.2. Milk samples

For the stability tests, full-cream milk (pasteurized) was purchased off-the-shelf and stored at 4 °C for maximum 1 day up to the start of the tests. Milk samples with incurred residues (positive response with the Delvo SP) were obtained from a milk collection center in the United Kingdom. The samples were shipped to the Nestlé Research Center in Switzerland in a frozen state and stored at −30 °C until analysis according to the procedure described in detail elsewhere [13].

2.3. Analytical method

All milk samples were prepared and analyzed according to a procedure described elsewhere in detail [13]. In essence, the procedure entails centrifugation for milk fat removal, analyte extraction by mixing equal aliquots of the defatted milk and 100 mM phosphate buffer (pH 9.2). The subsequent clean-up encompasses a liquid–liquid extraction using *n*-hexane and a solid-phase extraction step (Bakerbond C_{18} cartridge, 50% acetonitrile/50% phosphate buffer, 50 mM, pH 8, as SPE eluent). After volume reduction and pH adjustment to 7, the final extract was passed through a cut-off filter device (10,000 Da). Prior to the LC-ESIMS/MS analysis, azlocillin was added at a final concentration of 63 μg/L.

Measurements were conducted on an Alliance 2690 HPLC coupled to a Quattro LC tandem mass spectrometer (Waters, Rupperswil, Switzerland). A YMC ODS-AQ column (50 mm × 2 mm i.d., particle size 3 μm) was used for analyte separation, running a linear gradient from 100% solvent A (0.1% formic acid solution) to 100% solvent B (water/acetonitrile (35:65, v/v), containing 0.1% formic acid) in 13 min at a flow rate of 0.3 mL/min. The column temperature was set to 35 °C and the injection volume was 10 or 15 μL.

The analytes were recorded by acquiring two or three compound-specific selected reaction monitoring (SRM) traces in positive ESI mode. The SRM trace m/z 462 → 208 of azlocillin was recorded to compensate for potential variations of the injection volume or MS ionization efficiency. The cone voltages and collision energies, which were optimized for each SRM trace, ranged from 18 to 20 V and −10 to −24 eV, respectively.

2.4. Experimental design and practice

The experiments were designed to test the stability of AMOX, AMPI, CLOX, OXA, and PEN G present in pasteurized full-cream milk and milk extracts intended for LC-ESIMS/MS analysis. A portion of pasteurized milk (2 L) was fortified with the five analytes each at a level of 10 μg/kg. Eight aliquots (20 mL) of fortified milk were extracted immediately. Two extracts were subsequently analyzed (control

samples). The remaining six extracts were divided into three aliquots and stored at 4, −20 and −76 °C until analysis.

Eighteen aliquots (40 mL) of the fortified milk were transferred to 45 mL polypropylene tubes and six tubes each were stored at 4, −20 and −76 °C. After a set period of time (1, 2, 3, 7, 14, and 28 days), extracts in duplicate were prepared from one of the milk samples kept at the three various temperatures and analyzed together with the corresponding milk extracts already stored.

2.5. Data analysis

The signal areas of the analytes AMOX, AMPI, CLOX, OXA, and PEN G were normalized to the signal area of azlocillin. The response ratios [analyte/azlocillin] obtained on days 1, 2, 3, 7, 14, and 28 were compared to the corresponding response ratio at day 0, using Dunnett's test for comparing *k* means with a control [14]. A least significant difference (LSD) at a 5% confidence level was calculated.

3. Results and discussion

In accordance to procedures outlined in EU commission decision 2002/657/EC [3], the stability of the five β-lactam antibiotics in milk was tested at −20, −76 °C, and additionally, at 4 °C in order to evaluate the analyte stability under refrigerator conditions for a short-term storage of a maximum of 3 days. The same study was also conducted with milk extracts prepared for LC-ESIMS/MS analysis intending to define the optimal storage conditions. In this study, antibiotics were spiked at trace levels, i.e. 10 μg/kg, which are close to the EU Maximum Residue Limits (MRLs) in fresh milk, i.e. 4 μg/kg for PEN G, AMOX and AMPI, and 30 μg/kg for CLOX and OXA [15].

3.1. Stability in milk

All analytes were stable at 4 °C for a maximum of 3 days, but significantly lower concentrations were measured after 7 days. Further loss was recorded over the subsequent 21 days up to complete loss of the antibiotics, i.e. below the detection level of the analytical method, and exemplified for CLOX, OXA, AMOX, and PEN G in Fig. 1. Our observations of the partial depletion of all five analytes spiked at 10 μg/kg in pasteurized milk within 7 days appears to contradict previously published studies with PEN G and AMPI, that have shown stability of individual compounds over the same time period/temperature range [11,12]. However, an earlier report on PEN G stability revealed losses of approx. 60% within 2 days at a storage temperature of 2 °C [9]. Several points must be taken into account when comparing published data. The aforementioned studies were conducted on raw milk, and the contribution of inherent enzyme activity, microbial load and growth, possible presence of somatic cells, etc. all play a role on the rate and pathways of degradation of the individual antibiotics. For this study, we chose to use pasteurized milk, which represents a more "controlled" matrix with regard to microbiological quality, and thus allows a better comparison of the behavior of the individual antibiotics in milk versus the milk extracts.

Along the tests, we also observed a clear decline in pH from 7 to 5–6 and an increase in protein precipitation after the centrifugation step in milk stored at 4 °C. The loss of analyte is probably not correlated to the observed drop in pH, as the more acid-resistant isoxazoyls (CLOX, OXA) were also depleted to more or less the same degree as the more acid-sensitive AMPI, AMOX and PEN G. In addition, PEN G is reported to be rather stable in the pH range of 5.0–8.0 [16].

Raw milk samples that had undergone spoilage (reflected by protein precipitation and pH drop to 4.5–6.4) during intermediate storage showed a reduction of the signal response of the internal standard (d_7-PEN) by 80–90%, compared to a fresh pasteurized full-cream milk sample (control) that was analyzed simultaneously (data not shown). The drastic decline in MS response of d_7-PEN that was added at a final concentration level of 10 μg/kg directly before sample preparation may be due to co-precipitation during centrifugation, i.e. increased non-specific binding to milk proteins or bacterial cells [17]. Therefore, the loss of analyte may be due to microbial and/or enzyme activity [18], or merely linked to lower extraction efficiency in spoiled milk.

The antibiotics AMOX and AMPI retained stability throughout the chosen storage period when kept at −20 °C. In contrast, the levels of PEN G, OXA and CLOX at the same temperature showed minor but significant reduction (10–20%) after only 3 days relative to the control milk sample (Table 1). No losses of the analytes were observed in milk samples stored −76 °C over the entire 4-week period (data not shown). It was rather surprising to detect a slight but significant difference in the stability of the five residues in fortified milk kept at freezing temperature (−20 °C), i.e. reduction in levels of PEN G, OXA, and CLOX, versus good stability of AMOX and AMPI over a 14-day period. Slow degradation of PEN G in raw milk at −18 °C over longer periods of time has been observed elsewhere [9], which highlights the importance of storage under deep-freeze temperatures (e.g. −76 °C) if samples cannot be analyzed within a realistic time frame.

3.2. Stability in milk extracts

The short-term stability of all five β-lactam antibiotics in the milk extracts was estimated from data of multiple injections of 10 milk extracts stored at 5 °C in the autosampler chamber over a time period of >13 h, resulting in an intra-assay precision of 3–6% [13].

In contrast to the results obtained with the milk samples, all β-lactams were stable in milk extracts stored at 4 °C over 14 days, i.e. >90% of the initial concentration. A significant loss of around 15% was observed in the extract stored for 4 weeks at refrigerator temperature for all analytes with the ex-

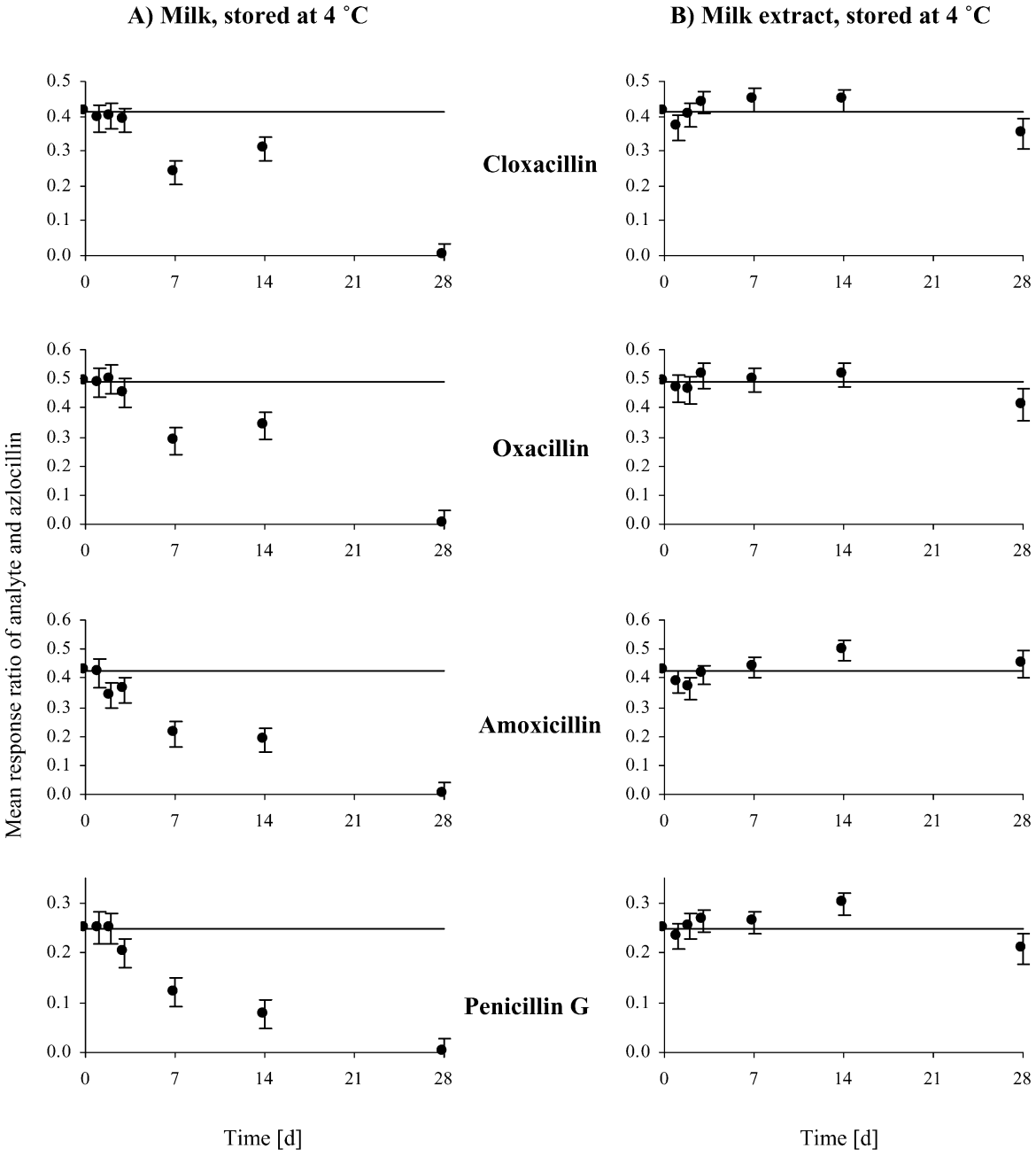

Fig. 1. Mean response ratios [analyte/azlocillin] with least-significant-difference intervals ($p = 0.05$) obtained from (A) fresh milk samples and (B) milk extracts stored at 4 °C (CLOX, OXA, AMOX and PEN G) over 4 weeks. The milk samples were initially fortified at a level of 10 μg/kg. The baseline corresponds to the values obtained from two determinations analyzed at day zero.

ception of AMOX (Fig. 1). In this context, it has to be noted that the data for milk extracts are based on the analysis of one extract, while the values for the stability in milk represent an average of two independent determinations. Thus, the variation due to sample preparation is not taken into account for the milk extracts, which may explain their apparently enhanced analyte levels. The greater stability of the residues in the extracts versus pasteurized milk at refrigerator temperatures is not surprising. The cell-free extract is comprised of milk co-extractives that potentially show similar chemical behavior as the β-lactams during sample preparation. In addition, fluctuations in pH were avoided in the extracts due to the $H_2PO_4^-/HPO_4^{2-}$ buffering system (pK_{a2} 7.2) of the extraction solvent. Moreover, the analytes were stored in an aqueous environment without any organic solvent and buffered at pH 7, which is close to the pH (6.6) that provides maximum stability of PEN G [19]. In contrast, pasteurized milk represents a complex, non-sterile system where bacterial

Table 1
Stability of CLOX, OXA and AMOX in milk and milk extract samples stored at −20 °C for a maximum of 4 weeks

Time (days)	Cloxacillin		Oxacillin		Amoxicillin	
	Milk[a]	Milk extract[b]	Milk[a]	Milk extract[b]	Milk[a]	Milk extract[b]
0[c]	0.41 (5.5%)	0.41 (5.5%)	0.49 (7.6%)	0.49 (7.6%)	0.42 (8.6%)	0.42 (8.6%)
1	0.39 (5.7%)	0.38 (2.0%)	0.50 (6.0%)	0.45 (3.8%)	0.43 (7.4%)	0.40 (5.0%)
2	0.42 (11.4%)	0.40 (2.0%)	0.50 (13.4%)	0.48 (2.1%)	0.41 (12.4%)	0.35 (2.0%)
3	0.39 (2.5%)	0.42 (4.1%)	0.43 (6.5%)	0.50 (2.0%)	0.41 (4.2%)	0.40 (5.0%)
7	0.32 (4.4%)	0.41 (6.9%)	0.38 (6.4%)	0.49 (2.9%)	0.36 (8.3%)	0.41 (10.1%)
14	0.37 (7.2%)	0.44 (3.9%)	0.43 (8.4%)	0.51 (7.1%)	0.44 (6.8%)	0.47 (7.4%)
28	0.34 (7.2%)	0.36 (4.8%)	0.41 (6.4%)	0.41 (8.1%)	0.44 (6.8%)	0.47 (3.7%)

Entries represent the mean signal response ratio [analyte/azlocillin], relative standard deviation given in the parentheses.
[a] Two determinations, except for the sample analyzed after 3 days, three or four injections.
[b] One determination, three or four injections.
[c] The initial concentration of antibiotic was 10 μg/kg each.

growth and enzymatic metabolism may occur even at freezer temperature of −20 °C.

No significant depletion of all five analytes was observed in the milk extracts stored at freezer temperatures of −20 and −76 °C, except for CLOX, OXA, and PEN G in the extracts kept at −20 °C after a storage of 4 weeks as shown in Table 1 for CLOX and OXA. Hence, milk sample extracts containing trace levels of residues can be stored for extended periods of time at −76 °C pending analysis, without the risk of depletion of the antibiotics.

4. Conclusion

The findings of this study emphasize the importance of analyte stability studies in food matrixes and sample extracts. Testing laboratories should include these parameters in their method validation procedures and procure these data routinely where quantitative methods are being established, and not only restrict investigations to stability of analytes in reference solutions [3].

During transportation and intermediate storage, milk samples suspected to contain antibiotic residues should be kept under freezing conditions or stabilized by adding an appropriate additive. Both measures are aimed at inhibiting analyte degradation due to microbial and/or enzymatic activities and preventing milk spoilage that may affect the efficiency of the extraction procedure, in this case established for the determination of the target compounds by LC-ESIMS/MS.

A testing laboratory should avoid unnecessarily extended storage times of samples, and thus stability data of the analytes at −20 °C up to 28 days seems appropriate. If storage at freezer temperatures is not applicable or milk samples are close to spoilage, a rapid work-up is warranted, since metabolic processes and/or matrix transformation may still progress, albeit slowly, at refrigerator temperatures, while the stability of the target analytes in the milk extracts seems to be guaranteed for at least 14 days under the same storage conditions.

For long-term storage, deep-freezing conditions at −76 °C are required to ensure the original levels of contaminant residues in the milk samples or sample extracts.

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