Detection of Penicillin G and its Benzylpenicilloyl (BPO) - Derivatives in Cow Milk and Serum by means of an ELISA

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ABSTRACT

Pharmacokinetic characteristics of benzylpenicillin and its benzylpenicilloyl (BPO)-derivatives were studied in serum and milk of health cows, using a classical biological assay (Sarcina lutea test) and a competitive ELISA for BPO detection. The plasma level and passage into milk was determined after intramuscular administration of penethamate-hydroiodide and benzylpenicillin-procaine. In serum of cows receiving penethamate-hydroiodide, BPO seemed to persist for a rather long time; the reason for this observation was not clarified. The effect of local (intramammary) application of penicillin G was followed with milk from cows having healthy and mastitic quarters. In all cases, it was found that BPO was not excreted any longer than active penicillin G in milk. In a further survey, 1015 bulk milk samples from two large dairy regions were examined with the ELISA and a biological assay using Bacillus stearothermophilus var. calidolactis. None of the samples showed detectable BPO or antibiotic residues. It is concluded that milk containing inactive penicillin derivatives, like BPO, is not an important source to cause allergies.

In the control of antibiotic residues in food, penicillin poses a special problem since some biologically inactive metabolites, like the benzylpenicilloyl groups (BPO) are known to be allergenic. In this respect, the classical biological assays (9,11) would be insufficient from the allergological point of view (3).

BPO is mainly formed by penicillin reacting with ε-amino groups of lysine in proteins (1,10,16). Reports on allergic reactions, predominantly chronic or recurrent urticaria, highly suspected to be due to penicillin contamination of market milk, are numerous (4,5,10,12,14,16,17,18,20,21). It is widely accepted that up to 10% of a population can be allergic to penicillin (8). Non-detectable BPO residues in food could, therefore, endanger public health. Wal (19) followed the presence of BPO in milk of cattle and serum of pigs, after penicillin administration, by means of a radio immunosorbent assay (RIA). According to his findings, BPO is not excreted longer than active penicillin in milk after intramammary application, but it persists rather long in serum of swine after intramuscular penicillin injection.

Recently we described the use of an enzyme linked immunosorbent assay (ELISA) which detects BPO with a high specificity and a high sensitivity (about 1 ng of BPO hapten/ml) (13). In the present paper we report on use of this ELISA for studying the pharmacokinetics of BPO in dairy cattle after intramuscular administration of penethamate-hydroiodide and benzylpenicillin-procaine, and after intramammary infusion of aqueous penicillin G. The results are compared with those obtained with a bioassay for detection of active penicillin (2). Furthermore, we analysed bulked milk samples with the ELISA and simultaneously with a classical microbiological assay using Bacillus stearothermophilus var. calidolactis for the presence of BPO and active penicillin. The milk samples were randomly collected from two large milk quality control centers. It was of interest to find out whether officially postulated withdrawal times after last penicillin application are sufficient to ensure BPO-free market milk. On the other hand, it was an occasion to observe whether violative actions against the existing legislation are current in the country.

MATERIALS AND METHODS

Kinetic studies

Test animals. Nine healthy Simmental cows with normal milk secretion (California Mastitis Test (CMT) negative) and a daily milk yield of 18.5 to 25 L were used for kinetic studies. Additionally milk of two cows with subclinical mastitis in one quarter each was analysed during and after intramammary treatment. One quarter had a Staphylococcus aureus (non-penicillinase producer), the other one a streptococcal infection. Both affected quarters yielded milk which reacted strongly positive in the CMT.

Milk and serum controls. Before application of antibiotics, samples of serum and milk were taken from all the above-mentioned healthy animals. These samples were used as negative controls in the ELISA and for elaboration of standard curves.

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Treatment schedule and sample collection

Intramuscular (i.m.) application. Three healthy cows received one injection of 10,000 I.U./kg of body weight of benzylpenicillin-diethylaminoethyl ester (penethamate)-hydroiodide (Mamyzin, LEO Pharmaceutical Products, Ballerup, Denmark). Three other cows received a single dose of 15,000 I.U./kg of body weight of an aqueous suspension of benzylpenicillin procaine (Illicocillin® P, Ciba-Geigy, Basel, Switzerland). The medications were injected in the gluteal muscles shortly after milking. Bulked samples from each cow were taken at normal milking times, i.e., about every 12 h. Serum was collected at intervals indicated in Fig. 1 and 2. All samples were kept frozen at -20°C until analysed.

Intramammary applications. Three healthy cows received three times 2 million I.U. of penicillin G (Novo Industrie A/S, Copenhagen) in the left front and right hind quarter at 24-h intervals, immediately after milking. The two cows with a mastitic quarter were treated with the same schedule, including the healthy quarters cross-opposite. All cows were milked with a special quarter milking machine. Quarter milk samples were kept frozen at -20°C until analysed.

Bulk milk

Five hundred and ten milk samples from different producers, all delivered on the same day, were collected in the quality control center of the city of Bern (Milkwirtschaftlicher Kontroll- und Beratungsdienst der Nordwestschweiz, Bern). Five hundred and five samples of 289 deliverers, controlled by the quality control center of the city of Basle (Milkwirtschaftlicher Kontroll- und Beratungsdienst der Nordwestschweiz, Basle) were collected during 14 d. The samples were kept frozen until analysed.

Competitive ELISA for detecting BPO

The competitive test used for the present investigation is described in detail elsewhere (13). Microtiter plates (Flow Laboratories, Virginia, USA) were coated with a rabbit anti-BPO human serum albumin (HSA) antibody (2 ng of protein/ml 0.1 M Na-carbonate buffer pH 9.8). After incubation of 16 h at 22°C in a humid chamber, plates were stored at -70°C. Before use plates were defrosted and then washed by hand (four times) with 0.5% Tween 20, 0.9% NaCl solution. The labeled antigen, BPO-phosphatase, was diluted 1:1500 in a 0.1 M Na-phosphate buffer pH 8.0, and was added in a ratio of 1:9 to milk samples to obtain a final dilution of 1:15 000. The milk samples were analysed after heating for 10 min at 100°C to eliminate unspecific reactions.

Serum samples were tested at a dilution of 1:50 in the phosphate buffer, the final dilution of BPO-phosphatase being 1:10 000 for sera. The test samples in duplicate (milk or serum) mixed with the labeled antigen, were filled into the wells of the coated plates which were incubated at 22°C in a humid chamber for 24 h. After the washing procedure as mentioned above, the substrate nitrophenyl-phosphate (Merck, Darmstadt, West Germany), 0.05 M Na-carbonate buffer pH 9.8 with 1 mM MgCl2 was added. Absorbance was read 1 h after adding the substrate with a multi-scanner Titertek (Flow Laboratories) at a wavelength of 405 nm.

For kinetic studies, individual standard competition curves were established by adding different BPO-eta-aminoacaproic acid (EAC) concentrations to the negative milk and serum controls. Our ELISA, however, also detects active penicillin (about 10 times less sensitively than BPO hapten of BPO-hydroiodide). The absorbance (OD 405) of the individual samples was compared with the OD 405 of the standard curves. Herewith the BPO-hapten equivalent concentration could be calculated with a pocket computer (Texas Instruments SR-51-II). To determine the absorbance limit for finding BPO-contaminated milk samples, we applied the following calculation (7,15): The (x±2s) value was determined by the average absorbance (x) and standard deviation (s) of known BPO-negative milk, taken along on all plates. This value was then adjusted for each plate by multiplication with the factor obtained by the following calculation:

\[ \text{absorbance of BPO negative milk obtained with individual plate} = \frac{x}{(\text{average absorbance obtained with all batch plates})} \times (\text{adjusted } (x±2s)) \]

Samples showing an absorbance below the adjusted (x±2s) value on the individual plate, were considered to be positive. A positive control was taken along on each plate.

Microbiological assays

For detection of active penicillin we used a microbiological cylinder plate bio-assay with Sarcina lutea ATCC 9341 in antibiotic medium No. 1 (Difco, Detroit, Michigan/USA) (2). For screening of antibiotic residues in bulk milk, we used a test on growth inhibition of Bacillus stearothermophilus var. colidolactis in agar (Delvotest® P multi; Gist Brocades, Delft, Netherlands).

RESULTS

Detection of active penicillin G and BPO-hapten in serum after intramuscular injection of penethamate hydroiodide and benzylpenicillin procaine is represented in Fig. 1 and 2, respectively. The long persistence of BPO-hapten in the absence of active penicillin G after penethamate-hydroiodide application is noteworthy (Fig. 1). Serum samples collected before the administration served as a negative control and for establishing standard curves as described in methods. In serum of cows which received benzylpenicillin procaine, BPO-hapten was not detected any longer than active penicillin G (Fig. 2).

The result of active penicillin G and BPO hapten excreted in milk after intramuscular penetration of penethamate hydroiodide injection, and the excretion of active penicillin G in milk after administration of benzylpenicillin procaine is plotted in Fig. 3. The BPO hapten values ranging near the detection limit of our ELISA system are not very accurate. After intramuscular benzylpenicillin procaine administration, no penicillin metabolites could be detected in milk with the ELISA.

The results obtained with milk samples collected after intramammary infusion of penicillin G are shown in Fig. 4. The ELISA did not detect BPO hapten any longer than the microbiological test reveals penicillin activity. In the same experiment, the quarters receiving no penicillin G also excreted active penicillin and BPO hapten. It is noted furthermore that mastitic quarters excreted penicillin...
Figure 2. Mean serum concentration of penicillin G detected with Sarcina lutea test and BPO-hapten detected with ELISA after intramuscular injection of benzylpenicillin-procaine 15,000 I.U./kg body weight in 3 cows.

Figure 3. Penicillin G concentration determined with Sarcina lutea test in milk of 3 cows after intramuscular injection of penethamate hydroiodide (10,000 I.U./kg body weight) and of 3 cows after intramuscular injection of benzylpenicillin-procaine (15,000 I.U./kg body weight). BPO-hapten concentration determined with ELISA in milk of the 3 cows receiving penethamate hydroiodide.

Figure 4. Mean penicillin G concentration in milk after intramammary infusion of 3 × 2 million I.U. aqueous penicillin G in 8 healthy quarters of 5 cows and 2 mastitic quarters of 2 cows determined with a bioassay (S. lutea test). Mean BPO-hapten concentration (ELISA) in milk from healthy quarters and mastitic quarters. Penicillin G concentration and BPO-hapten concentration in 10 healthy quarters of 5 cows cross opposite to treated quarters.

Table 1. Adjusted absorbance distribution of bulk milk samples as obtained with ELISA.

<table>
<thead>
<tr>
<th>Range</th>
<th>Range OD_{405} nm</th>
<th>Number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-2S</td>
<td>&lt;0,422</td>
<td>0</td>
</tr>
<tr>
<td>X±2S</td>
<td>0,422-0,682</td>
<td>851</td>
</tr>
<tr>
<td>X±1S</td>
<td>0,487-0,617</td>
<td>446</td>
</tr>
<tr>
<td>&gt;X+2S</td>
<td>&gt;0,682</td>
<td>164</td>
</tr>
</tbody>
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DISCUSSION

Use of the competitive ELISA is appropriate to detect the inactive BPO-derivatives of benzylpenicillin. However, the test also detects active penicillin ten times less sensitively than BPO-hapten in monovalent BPO-EAC (13). BPO-hapten in polyvalent BPO-derivatives is detected more sensitively than those in BPO-EAC. We regard a major part of residues expressed in BPO-hapten of BPO-EAC, obtained with the ELISA, to be active penicillin. The high persisting level of BPO-hapten in serum of cows receiving penethamate hydroiodide is difficult to interpret. Penethamate hydroiodide is more immunogenic than penicillin procaine (6). The application could herewith act as a booster for preformed anti-BPO antibodies which theoretically interfere in the competitive ELISA (13). On the other hand, it could be possible that unsplit inactive penethamate hydroiodide may persist at this level for a long time in serum. The presented kinetic results with milk confirm the results from Wal et al. obtained with a RIA (19). We could demonstrate that BPO and other possible metabolites of benzylpenicillin, detectable with serological methods, are not excreted any longer than active penicillin G in milk. This observation points out that existing withdrawal times for milk according to the Swiss milk delivery regulation (3 d after parenteral and 5 d after intramammary treatment) are effective to prevent penicillin residues in market milk for most penicillin preparations used for treatment during lactation.
Furthermore, it can be concluded that market milk presents practically no risks to human health with regard to penicillin allergies. This consideration is enforced by the negative results obtained with the screening of bulk milk from two larger dairy regions. Concerning the results obtained with the competitive ELISA for detection of BPO-groups, a restriction must be made; the detection limit of about 10 ng BPO-hapten/ml milk (X-2s) is slightly above the accepted immunogenic tolerances which lay between 1.2-1.8 ng of penicillin/ml milk (5,11). However, when extrapolating Fig. 3 and 4 (logarithmic linear functions) the BPO-hapten concentrations at the limit of withholding times are quite well within or below the cited immunogenic tolerances. It has also to be considered that in normal milk with a buffered pH of about 6.4, penicillin is very stable, and BPO formation is probably limited (1). The negative results obtained with the biological assay (Delvotest) in bulk milk samples suggest that the extent of contamination of the Swiss market milk with antibiotics, in particular with penicillins, is negligible.

The convincing success of dermatologists in healing chronic urticaria patients also showing adverse reactions to penicillin, after recommending a diet free from milk and its products, can probably not always be explained merely on the basis of penicillin or BPO contamination of milk. It is known that milk, even milk free of antibiotic residues, is one of the most common allergens among food-sensitive patients (17). In our opinion, great prudence has to be observed when incriminating penicillin and its derivatives as the cause of allergic reactions after milk consumption. According to our results, milk is probably not a common penicilloyl carrier.

In conclusion, we are convinced that control measures generally applied for prevention of antibiotic residues in milk are very effective to protect the consumer.

REFERENCES