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Determination of benzylpenicillin in plasma and lymph at the ng ml^{-1} level by reversed-phase liquid chromatography in combination with digital subtraction chromatography technique

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Abstract: A method for the determination of benzylpenicillin (Pc-G) at very low levels in plasma and lymph is described. Detection at 325 nm of the mercuric mercaptide of benzylpenicillenic acid was made by liquid chromatography via a pre-column derivatization method. By using a digital subtraction chromatography technique, the bioanalytical method could be applied to different kinds of samples whether interfering peaks were present or not. Two different clean-up steps were used for two concentration ranges 0.1–100 μg and 1–1000 ng Pc-G ml^{-1}; namely, precipitation of a 100-μl sample with acetonitrile, or precipitation of a 1- or 2-ml sample followed by concentration via liquid–liquid extraction. The pre-column derivative of Pc-G was achieved using mercury(II)chloride in the presence of imidazole. The blank samples required for the digital subtraction chromatography technique were obtained by penicillinase treatment. Standard curves were made in the two concentration ranges. The relative standard deviation (RSD) at 5 ng Pc-G ml^{-1} plasma was 4.9% and at 5 μg Pc-G ml^{-1} lymph it was 2.8%. The stability of Pc-G, including the problems with non-sterile samples, was studied.

Keywords: *Benzylpenicillin; body fluids; reversed-phase liquid chromatography; pre-column derivatization; trace amounts; digital subtraction chromatography.*

Introduction

Penicillin was introduced in the early 1940s. Its excellent medical properties in combination with a very low toxicity has made the drug very popular. As with most other drugs, which have been used for many years, the dosage regimen for benzylpenicillin (Pc-G) is based upon clinical practice. The interest, however, in trace amounts of antibiotics in products from food producing animals has increased. Pharmacokinetic data from animals, especially in the lower concentration ranges, are therefore important.

The traditional bioanalysis of penicillins is based on microbiological techniques [1, 2]. These techniques are rather easy to perform and have a high sensitivity. The main

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drawback with the microbiological determination methods is the interference from active metabolites or antibacterial substances of endo- or exogenic origin that may give rise to false positive results. Such false results are described for milk samples and could appear in the concentration ranges close to the limit of detection of penicillin for the microbiological methods, >10 ng ml^{-1} (0.006 U ml^{-1}) [3, 4].

A chemical determination method by liquid chromatography (LC) would increase the selectivity. There are several bioanalytical LC-methods for penicillin published [5–7] but none is sufficiently sensitive. In a previous work we presented a method for the determination of Pc-G in milk at the pg ml^{-1} level [8]. The analytical method was based on liquid extraction and chromatography of Pc-G converted to the mercuric mercaptide of benzylpenicillenic acid (Pc-GAMM) [9]. The interference from background peaks was reduced by a digital subtraction chromatography (DSCh) technique [10]. As the same determination technique would be useful for Pc-G in plasma and lymph, the method was applied to these body fluids. In this work, we present a sensitive and selective pre-column derivatization LC-method for the determination of Pc-G in plasma and lymph.

Experimental

Apparatus

Most of the chromatographic instruments were manufactured by the Shimadzu Corp. (Tokyo, Japan). An LC-5A pump was connected to an SPD-2A variable u.v.-detector and a C-R3A chromatographic data processor calculated and printed the detector signal. The injector was a Rheodyne Model 7125 (Berkeley, Calif., USA) equipped with a 2.0 ml sample loop. The LC-columns were packed by a Maximator pump, MSF 72, from Schmidt, Kranz & Co. (Sorge/Südharz, FRG).

Chemicals

LiChrosorb RP-18, 5 μm (E. Merck, Darmstadt, FRG) was used as chromatographic support. Pc-G–potassium was kindly supplied by Novo Industri A/S (Copenhagen, Denmark). Penicillinase was obtained from Løvens Kemiske Fabrik (Ballerup, Denmark). All other chemicals were of analytical grade from E. Merck.

Chromatographic technique

From stock solutions of aqueous 0.05 M mercury(II)chloride and 20% (w/v) of imidazole, with the pH adjusted to 6.6 by phosphoric acid, appropriate amounts were mixed with methanol and water in a volumetric flask. The final concentrations were 2% (w/v) of imidazole and 50 μM of mercury(II)chloride. For the 150 × 4 mm column, 45% (v/v) of methanol was used whilst for the 125 × 4 mm column 4% (v/v) methanol was used. The eluents were degassed under vacuum in an ultrasonic bath before use. The detection wavelength was 325 nm.

Two different column systems were used. For the analysis of the extracted plasma and lymph in the lower concentration range one pre-column (40 × 4 mm) and one analytical column (150 × 4 mm) were used in series. They were made of stainless steel and equipped with modified Swagelok^{T} connectors and filters of 2 μm porosity, 100 μm thick.

The analysis in the higher concentration range, performed by direct injection of precipitated plasma and lymph, were performed on a (125 × 4 mm) Hibar column from E. Merck (Darmstadt, FRG) directly connected to a (4 × 4 mm) disposable cartridge

guard column. The columns were packed by a balanced density technique [11] modified as described previously [9]. The columns and the injector were thermostated to +50°C by a waterjacket arranged according to [9] and connected to a circulating thermostatic bath.

An electric switch synchronized the injector with the integrator. The integrated peak areas were used for the quantitative analyses.

When the LC-system was left overnight or longer, the system was washed with about 30 ml phosphate buffer, pH 3.0/methanol (55:45 v/v), $\mu = 0.1$. Equilibration and warming of the system were done within 1 h of elution of the mobile phase.

As an extra precaution, in order to prevent the deterioration of the column, a filter column filled with about 0.5 cm^3 of the same packing material as the support in the main columns was connected between the pump and the injector.

The extraction studies and the stability studies in lymph with penicillin-V and cloxacillin were performed by direct detection of Pc-G at a detection wavelength of 200 nm and a mobile phase of phosphate buffer, pH 3.4/methanol (55:45 v/v), $\mu = 0.1$. The columns were as described above.

Extraction studies

Plasma (2 ml) and 1 ml of lymph from Swedish Red and White cows were spiked with Pc-G to a concentration of 25 μg ml^{-1}, respectively. The samples were handled according to the bioanalytical procedure. After centrifugation the pellet was dissolved in water. Suitable aliquots from each phase in each step of the extraction scheme, including the dissolved pellet, were analysed by direct injection onto the LC-system for extraction studies described in the preceding section.

Stability studies

Pooled samples of plasma and lymph from Swedish Red and White cows were spiked with Pc-G. Aliquots of 50 μl of the samples were stored at room temperature, −20 and −70°C in 1.5 ml polypropylene tubes with caps. After suitable intervals, the samples were analysed according to the bioanalytical method for high concentration samples (0.1–100 μg Pc-G ml^{-1}).

The degradation of Pc-G in some lymph samples from the pharmacokinetic experiment was studied under special conditions as will be described later.

Clean-up and analytical procedure

1. *Samples in the range 0.1–100 μg Pc-G ml^{-1}*

Extraction. To 100 μl of plasma or lymph, 100 μl of acetonitrile was added for protein precipitation in a 1.5 ml conical polypropylene tube equipped with a cap. After centrifugation at 18,000 ***g*** for 2 min the supernatant was split into two equal volumes by an LC-syringe and transferred into two new 1.5 ml tubes.

Derivatization. To one of the two tubes, 50 μl of a penicillinase solution in water (5000 U ml^{-1}) was added (this formed the blank sample). In order to form the mercuric mercaptide of Pc-GAMM, 50 μl of water and 30 μl of a reagent containing 40% (w/v) of imidazole and 0.1% (w/v) of mercury(II)chloride in water, with the pH adjusted to 6.8 by phosphoric acid, were added to the other tube. After efficient mixing, the two tubes were left for 10 min at room temperature. Then 30 μl of the imidazole-mercury(II) chloride reagent was also added to the penicillinase treated (blank) sample and the two tubes were incubated for 20 min at 60°C. After completion of the reaction, 50 μl of the

penicillinase solution and 50 μl of water were added to the actual sample and 100 μl of water was added to the penicillinase-treated (blank) sample. After a short centrifugation, the samples were ready for injection onto the column.

2. *Samples in the 1-1000 ng ml^{-1} range*

Extraction. (A) Plasma: 2 ml of plasma was divided into two 2.2 ml centrifuge tubes made of propylene with caps. The samples were precipitated with equal volumes of acetonitrile and centrifuged for 2 min at 18,000 ***g***. The supernatants were pooled in a glass test tube and acidified with 140 μl of 1 M phosphoric acid (for cow plasma). The sample was then immediately extracted for 10 s with 0.6 ml of ethylacetate. After centrifugation for 30 s at 2000 ***g***, the organic phase was carefully sucked off with a Pasteur pipette and transferred into a glass tube with a sharp conical bottom. The organic extraction was repeated once more with 1.2 ml of ethylacetate and pooled with the first extract. After addition of 0.8 ml *n*-heptane to the pooled organic layer and a short centrifugation, an acidic aqueous layer was formed at the bottom of the conical tube and was sucked off with an LC-syringe and discarded.

The remaining organic layer was then extracted 3 times with 75 μl 0.1 M Tris–hydrochloric acid buffer pH 9.0, by shaking for 10 s and a short centrifugation at 2000 ***g***. The aqueous phases were pooled in a 1.5 ml polypropylene centrifugal tube and flushed with nitrogen for 1 min. The sample was then ready for derivatization, as described later.

(B) Lymph: the lymph extraction was performed similarly to the plasma extraction with a few differences. 1 ml of lymph was protein precipitated with 1 ml of acetonitrile. After decanting into a glass tube, 100 μl of 1 M phosphoric acid was added to the supernatant (cow lymph). The following steps were identical with the plasma extraction, starting with two organic extractions with 0.6 and 1.2 ml ethyl acetate and ending with a nitrogen flush for 1 min.

Derivatization. The sample was now divided in equal volumes which were transferred to two Eppendorf test tubes. To one of the tubes, 50 μl of a penicillinase solution in water (5000 U ml^{-1}) was added (blank sample). To the other tube, 30 μl of a reagent containing 40% (w/v) of imidazole and 0.1% (w/v) of mercury(II)chloride in water, with the pH adjusted to 6.8 by phosphoric acid, was added, which led to the formation of Pc-GAMM (actual sample). After efficient mixing of the two samples the tubes were left at room temperature. After 10 min, 30 μl of the imidazole-mercury(II)chloride reagent was also added to the penicillinase-treated sample. The two samples were then placed in a water bath at 60°C for 20 min. After reaction, 50 μl of the penicillinase solution was also added to the non-penicillinase-treated sample. After a short centrifugation, the samples were ready for LC-analysis.

Measurements and calculations

The built-in program for background compensation was selected on the chromatographic data processor and the total amount of each of the two split samples were injected and analysed in two consecutive analyses. By this process the two chromatograms were stored and it was possible to subtract the zero sample chromatogram from that of the actual sample, thus enabling the remaining Pc-GAMM peak to be integrated. This DSCh-technique has been described in detail in a previous work [10]. Furthermore, the technique was used for the bioanalysis of Pc-G in milk [8]. Pooled horse plasma was used as a model matrix for complicated chromatograms.

Recovery and standard curves

Plasma and lymph were mixed with 10% (v/v) of Pc-G stock solutions to appropriate concentrations. The samples were taken out by fixed volume pipettes with disposable tips and handled according to the clean-up and analytical procedure.

Pharmacokinetic experiments

Swedish Red and White cows received a single i.v. injection of Pc-G–potassium (10 mg kg^{-1}). Plasma samples were collected i.v. in heparinized vacuum tubes. Lymph was sampled in open heparinized tubes from two permanent catheters draining the two main lymphatic vessels in the left and right udder quarters. Milk samples were also collected by emptying each quarter separately at each sampling time.

Results and Discussion

Extraction according to the clean-up procedure

It was assumed that the proteins in lymph were precipitated almost completely by the addition of acetonitrile as was shown for plasma proteins [12]. The extraction schemes for the low concentration samples were based on earlier studies [8, 9], where basic extraction studies and trace analyses of Pc-G in milk were presented.

The extractions were simplified compared to those in the milk analyses [8], as smaller volumes and lower acetonitrile concentrations were used for plasma and lymph. By lowering the pH close to the limit of rapid degradation of Pc-G (pH $\approx$ 2.3), it was possible to achieve a quantitative extraction of the drug in two extractions, due to the good partitioning into the organic phase at this pH [9]. The short extraction times used throughout the method were sufficient [9] and kept the hold-up times in the different medias as short as possible. This is essential because of the instability of Pc-G at high and low pH [14]. The addition of *n*-heptane in the last extraction step separated the acidic water and made the organic phase less polar, which facilitated the extraction of Pc-G to the aqueous phase at the unfavourable phase volume ratios in this step.

The total recoveries of the drug are shown in Table I and were in range 87–91% irrespective of the extraction procedures. Determinations of the precipitated protein pellet dissolved in water showed that about 6% of Pc-G was trapped in these pellets (range: 1–1000 ng ml^{-1}).

Table 1
Extraction of Pc-G from lymph and plasma samples

	RSD (%) $n = 6$	Recovery (%)
Lymph		
10 ng ml^{-1}*	±6.6	86
200 ng ml^{-1}*	±5.3	88
0.2 μg ml^{-1}†	±4.0	90
5 μg ml^{-1}†	±2.8	89
Plasma		
5 ng ml^{-1}*	±4.9	87
100 ng ml^{-1}*	±4.6	87
0.2 μg ml^{-1}†	±4.0	91
5 μg ml^{-1}†	±3.5	89

*According to the method for the low concentration range.
†According to the method for the high concentration range.

Derivatization

The advantages of detecting Pc-G at 325 nm as the Pc-GAMM derivative instead of detection at 195 nm where Pc-G has its u.v.-absorption maximum, were discussed and demonstrated earlier [8, 9]. The samples in the low concentration range were derivatized according to a method for the analysis of Pc-G in milk [8].

For the samples precipitated with acetonitrile only, it was necessary to reduce the acetonitrile concentration by dilution with water prior to the addition of the imidazole-mercury(II)chloride reagent, in order to avoid a two-phase system. The presence of acetonitrile did not affect the reaction rate and the Pc-GAMM formation was completed within 20 min at 60°C.

Identification and quantification

The use of penicillinase as an identification tool for Pc-G is widely used in the microbiological bioassays. It has also been used in LC-bionanalysis of Pc-G [13]. The purpose of the penicillinase addition in this study was to create a blank sample that could be used in the DSCh-technique. The advantage of this technique is that interfering peaks in a chromatogram can be reduced. By using the modern chromatographic data processors it is possible to subtract a blank sample chromatogram from the actual sample chromatogram. The resulting trace, showing the searched compound peak presented on a straight baseline, can then be re-plotted and re-integrated. Accurate and precise chromatographic technique is, however, essential for the success of the operation [10].

The technique not only makes the quantification more universal for body fluids from different individuals or different species, it also yields a more positive identification by means of the β-lactam specific reaction with penicillinase. This is of great importance when the results are required for pharmacokinetic calculations or for confirming trace amounts of Pc-G. In Figs 1A and 1B, spiked horse and cow plasma was analysed. The

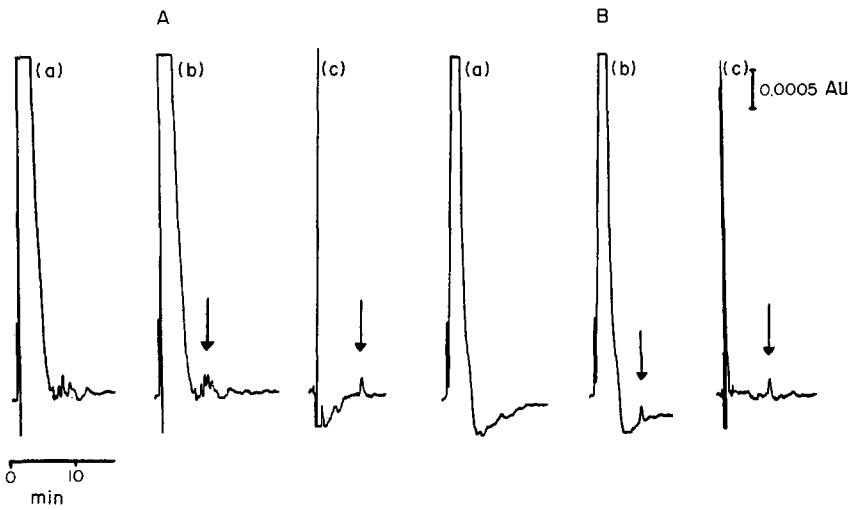

Figure 1
Chromatograms of 30 ng Pc-G–potassium ml^{-1} plasma from horse (A) and cow (B) according to the bioanalytical procedure for the high concentration range. a, Artificial blank sample; b, actual sample; c, resulting chromatogram obtained by the subtraction of b from a, according to the DSCh-technique described in the bioanalytical method. Arrow indicates the mercuric mercaptide of Pc-GAMM.

horse plasma was used as a model for samples giving rise to complicated chromatograms. The disturbance from other peaks in the horse plasma chromatograms was removed by the DSCh technique, as can be seen in the resulting chromatograms. In chromatograms where the disturbing peaks are small compared with the analyte peak, like in the cow plasma sample (Fig. 1B), it was not necessary to use the DSCh technique. The zero sample confirmed the absence of interfering peaks as in conventional LC. The technique was sufficient for different kinds of injected samples, aqueous solutions or as a mixture with organic solvents, which is in agreement with the basic studies of the DSCh technique [10]. In Fig. 2, a lymph sample from a cow is analysed according to the low concentration range procedure.

Stability studies

A Pc-G solution has its maximum stability at a pH of about 6.6 [14]. Among the body fluids, milk is the closest to this optimal pH, but long-time storage of Pc-G solutions in milk required a temperature of −70°C in order to give an acceptable stability [8]. The higher pH values of plasma and lymph made it necessary to freeze the samples within a few hours (Fig. 3). Furthermore, as can be seen in Figs 4a and 4b, the samples have to be

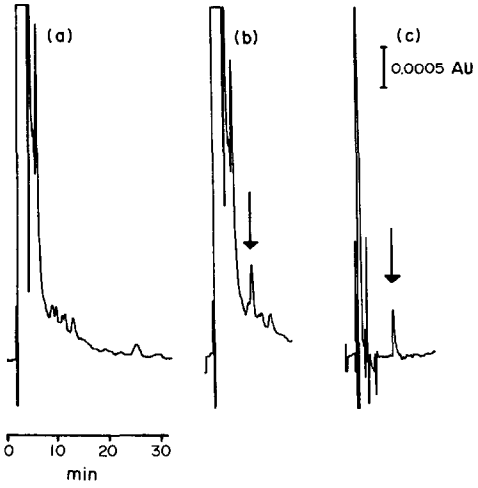

Figure 2
Chromatograms of 5 ng Pc-G–potassium ml^{-1} cow lymph according to the bioanalytical procedure for the low concentration rante. For a, b and c, see Fig. 1. Arrow indicates the mercuric mercaptide of Pc-GAMM.

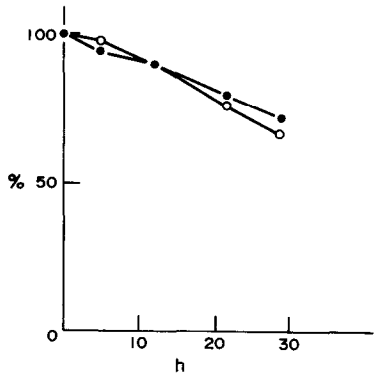

Figure 3
Stability of Pc-G in plasma and lymph from cow at room temperature. Samples: ●, plasma; ○, lymph; 5 μg Pc-G–potassium ml^{-1}.

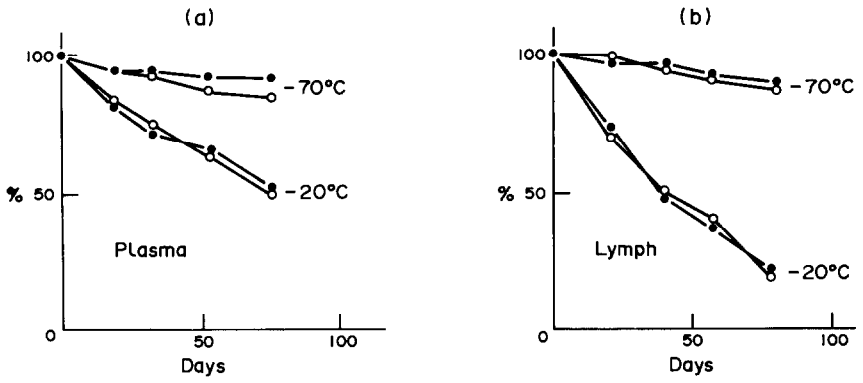

Figure 4
Stability of Pc-G at different concentrations in plasma (a) and lymph (b) from cow at different temperatures below 0°C. Concentrations: ●, 10 μg; ○, 1 μg Pc-G–potassium ml^{-1}.

analysed within a month to guarantee a sample with no more than a small percentage loss of the analyte — though they were stored at −70°C.

During pharmacokinetic studies of Pc-G in the cow, where the lymph concentrations were included, it was noted that some determinations of Pc-G in lymph were remarkably lower than expected. In Fig. 5, it can be seen that at 1, 2.5 and 4 h the concentrations were lower in lymph from the left udder quarters compared to the right quarters. Furthermore, the decrease in the Pc-G concentration from the left quarters did not follow a harmonic curve. When the failing samples were re-analysed, the concentrations had decreased even more. Further experiments to study these phenomena were therefore performed. The stability of the Pc-G present in some of the lymph samples from the left udder quarters in Fig. 5 was followed at room temperature. If the drug was degraded faster in some of the samples, this could explain elimination curves like those in Fig. 5. The results in Fig. 6 were as expected, the degradation rates of Pc-G in the failing samples were increased compared with the other samples. This experiment eliminated the possibilities that a deficient analytical procedure had caused the non-expected values.

In order to find the origin of the decreased stability of Pc-G in these samples, two more penicillins were added to a lymph sample that had shown a fast degradation of Pc-G. The penicillins were phenoxymethylpenicillin (Pc-V), which differs from Pc-G by being much more acid-stable, and cloxacillin, which compared with Pc-G and Pc-V is less sensitive towards degradation by penicillinase.

Three samples of lymph from the left udder quarters, sampling time: 4 h (see Fig. 5), were spiked each with one of the three penicillins to a concentration of 25 μg ml^{-1}. The degradation was followed at room temperature. After 25 h 14% was remaining of Pc-G and 9% of Pc-V, but as much as 84% still remained of cloxacillin. Hence, it followed that the fast degradation of Pc-G in the 4-h sample was not acid catalysed as Pc-V had about the same degradation rate. The relative stability of cloxacillin compared with the other two penicillins indicated, however, a penicillinase-dependent degradation of Pc-G (and Pc-V).

Further experiments showed that it was possible to slow down the degradation of Pc-G in the lymph by the presence of cloxacillin. The results in Fig. 7a indicate a competitive inhibition of the degradation of Pc-G by cloxacillin.

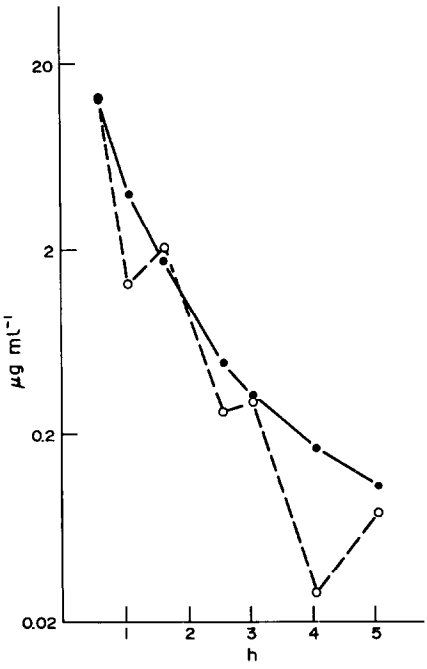

Figure 5
Lymph concentrations of Pc-G from one cow after i.v. injection of Pc-G–potassium. Samples: ●, lymph collected from the right udder quarters; ○, lymph from the left udder quarters (see Material and Methods, pharmacokinetic experiments).

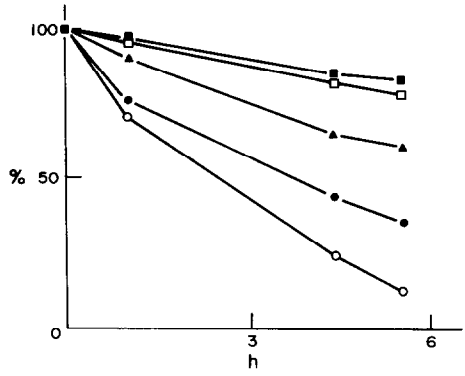

Figure 6
Stability at room temperature of Pc-G in different lymph samples collected in the pharmacokinetic experiment in Fig. 5. Samples: lymph from the left udder quarters, sampling times according to Fig. 5. ■, 1.5 h; □, 0.5 h; ▲, 2.5 h; ●, 4.0 h; ○, 1.0 h. Pc-G concentrations: as presented in the lymph according to Fig. 5.

When the same experiments with Pc-G and cloxacillin were repeated in a pure aqueous buffer (pH 7.4) with penicillinase added to 0.05 U ml^{-1}, the competitive inhibition was about the same as in lymph, as can be seen in Fig. 7b.

Furthermore, it was possible to decrease the degradation rate of Pc-G by high concentrations of the Pc-G itself, probably by a saturation of the binding sites of penicillinase.

The results pointed towards the possibility that the fast degradation of Pc-G in some of the lymph samples was caused by penicillinase activity, probably of microbiological origin. This assumption was supported by a bacterial growth test of some lymph samples. The samples that showed the fastest degradation of Pc-G also showed a rich growth of a

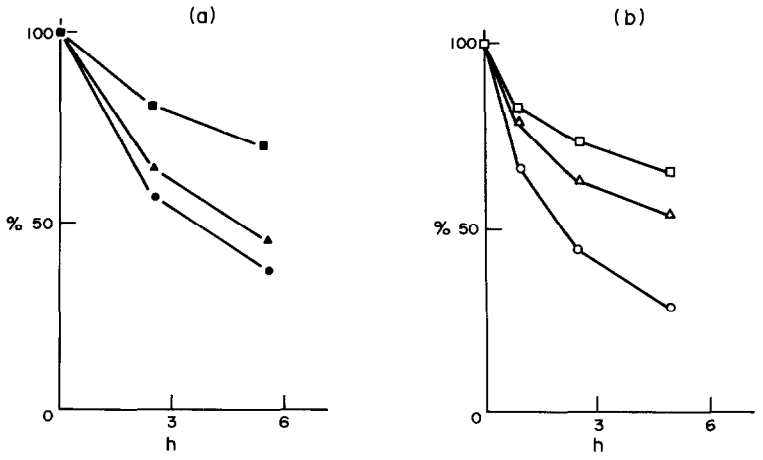

Figure 7
The inhibition effect of cloxacillin on the degradation of Pc-G in different medias at room temperature. Samples: (a) lymph collected at 4 h from the left udder quarters according to Fig. 5. The concentration of Pc-G was adjusted to 2 μg ml^{-1}. Cloxacillin was added to 0 μg ml^{-1} (●), 4 μg ml^{-1} (▲) and 40 μg ml^{-1} (■). (b) Penicillinase in physiological buffer pH 7.4 (0.05 U ml^{-1}). The Pc-G concentration was spiked to 2 μg ml^{-1}. Cloxacillin was added to 0 μg ml^{-1} (○), 20 μg ml^{-1} (△) and 50 μg ml^{-1} (□).

mixed bacterial flora when the lymph was seeded on agar plates, while the stable lymph samples showed a very scarce bacterial growth when incubated on agar plates.

Obviously, one has to be very observant when collecting biological samples in open tubes, especially in places like veterinary clinics, where a constant pressure from antibiotics may select penicillinase producing bacterias.

As has been shown, there are possibilities of reducing the degradation of Pc-G by competitive inhibition with, for example, cloxacillin in high concentrations. Another substance which may be useful as a stabilizing agent for Pc-G is clavulanic acid [15, 16], which is used together with Pc-G (Synulox^{T}, Beecham Ltd) as a potent inhibitor of various types of bacterial penicillinase.

Anyhow, the use of such agents should make it necessary to check the formation rate of the blank sample required for the DSCh technique, as this blank was made by the addition of penicillinase.

Standard curves and recovery

Table 1 shows the recovery and the reproducibility of the bioanalytic method. The standard curves were linear in the stated ranges and the correlation coefficients (r) were better than 0.9991 in all cases.

Limit of detection

The limit of detection defined as a peak height ratio of 3 times the baseline noise, was 0.8 ng Pc-G ml^{-1} plasma and 1.6 ng Pc-G ml^{-1} lymph. Regarding lymph, it was not possible to get more than 1 ml at each sampling in the pharmacokinetical experiment.

There were, however, no problems concerning the increase of the sample amounts if the extraction conditions were adapted. Compare the bioanalytical method for Pc-G in milk [8] where a sample of 9 ml milk was extracted and analysed down to 200 pg Pc-G ml^{-1}.

Pharmacokinetical experiments

Figure 8 demonstrates the kinetics of Pc-G in different body fluids from the cow. The plasma and lymph samples were analysed according to the described bioanalytical methods. In the range 100–300 ng Pc-G ml^{-1} two samples of each biological matrix were analysed by the methods for the high as well as the low concentration range. This was done in order to confirm that the two methods yielded corresponding results. The milk samples were analysed according to [8].

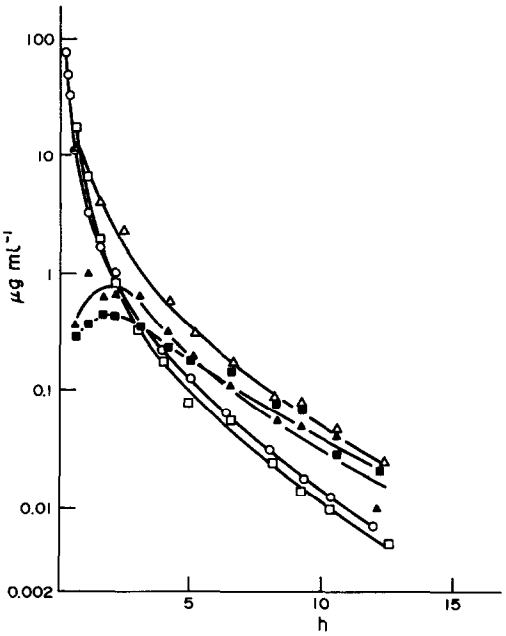

Figure 8
Plasma, lymph and milk concentrations in one cow after i.v. injection of Pc-G–potassium. Plasma and lymph were analysed according to the bioanalytical procedures, milk was analysed according to ref. 8. Samples: ○, plasma; □, lymph from the left udder quarters; △, lymph from the right udder quarters; ■, milk from the hind left udder quarter; ▲, milk from the hind right udder quarter.

Conclusions

A bioanalytical method for a selective detection of Pc-G in plasma and lymph is presented. The sensitivity and accuracy of the method makes it suitable for pharmacokinetic studies at concentration levels under the limit of detection for microbiological methods. The use of the (DSCh) technique makes it possible to apply the method to different kinds of samples, irrespective of interfering peaks. The use of penicillinase for the DSCh-technique increases the specificity, and the method can therefore be adapted to control trace amounts of benzylpenicillin in foods.

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