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Determination of benzylpenicillin in milk at the pg 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 in milk at very low levels by a pre-column derivatisation LC-method is described. Nine millilitres of milk were precipitated and extracted. The sample was concentrated 40 times and the recovery of benzylpenicillin was about 90%. The derivatisation of benzylpenicillin was made by the β-lactam ring specific formation of the mercuric mercaptide of the penicillenic acid in the presence of imidazole. A technique called digital subtraction chromatography (DSCh), which uses the built-in background compensation programme of the chromatographic data processor, was applied to the chromatographic step of the analysis. The blank sample required for this technique was obtained by the β-lactam ring specific hydrolysis of benzylpenicillin by penicillinase. By this technique it was possible to reduce interfering background peaks on the chromatograms and to increase the specificity of the method. Standard curves were made in the range 0.2–1000 ng benzylpenicillin ml^{-1} milk. The relative standard deviation (RSD) at 1 ng ml^{-1} milk, corresponding to an injection of 3.9 ng benzylpenicillin-sodium converted to the mercuric mercaptide of the penicillenic acid, was ±7.8% and RSD at 10 ng ml^{-1} milk was ±1.6%.

Keywords: *Benzylpenicillin; milk; trace amounts; reversed-phase liquid chromatography; pre-column derivatisation; digital subtraction chromatography.*

Introduction

The dominating methods for the bioanalysis of penicillins are based on microbiological techniques. The principle of using the inhibition of bacterial growth, caused by the penicillins, as a detection tool is as old as penicillin itself [1]. It is a carefully studied technique, standardised [2] and yet still under development. The relative simplicity of performing large numbers of determinations from different body fluids and tissues with a high sensitivity has made the technique popular in the food industry. In the dairy

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industry for example, many processes like cheese and yoghurt production are carried out by microorganisms, and the presence of an anti-bacterial substance may ruin large amounts of milk. The results obtained from the penicillin analyses do, therefore, fit such industrial processes, irrespective of any anti-bacterial substance originating from penicillin or not. A draw-back of the microbiological bioanalysis of penicillin is that active metabolites or anti-bacterial endo- or exogenic substances may give rise to false positive results for the analysis. This sometimes causes differences of opinion about the origin of an anti-bacterial substance between the dairy and the farmer, and furthermore has public health implications. It has been pointed out that milk samples from cows with elevated somatic cell count can show positive microbiological test results for penicillin after a break of 10 days in the milking of the infected udder. These false positive results also can arise after wrong treatment of the milk prior to the tests caused, for example, by preservatives and the use of penicillinase as an identification step for penicillinase sensitive penicillins [3, 4]. Many of these non-specific positive results correspond to a penicillin concentration of less than 10 ng ml^{-1} milk (0.006 U ml^{-1}).

A detection method for penicillin in milk based on the LC-technique would solve the problems with interfering substances, as LC is much more selective than the microbiological assay. Further studies in the low concentration range would then be possible. As no LC-method sensitive enough was available a bioanalytical LC-method for the detection of benzylpenicillin (Pc-G) in milk has been developed. Benzylpenicillin is the most common penicillin for the treatment of bovine mastitis in Sweden. The method was based on extraction studies of Pc-G presented in an earlier paper [5], where also an LC-method for the detection of Pc-G as the mercuric mercaptide of benzylpenicillenic acid (Pc-GAMM) was reported. In another work [6], the possibility of reducing disturbing chromatographic peaks by a technique called digital subtraction chromatography (DSCh) was described. By using the basic results of these two works [5, 6] a very sensitive and selective pre-column derivatisation LC-method for the determination of Pc-G in milk has been developed.

Experimental

Apparatus

Most of the instruments were manufactured by the Shimadzu Co., Tokyo, Japan. The pump was a Model LC-5A connected to a variable wavelength UV-detector Model SPD-2A, the detector signal was processed by an integrating chromatographic data processor C-R3A. The injector was a Rheodyne Model 7125 (Berkely, CA, USA) equipped with a 2.0 ml loop. The pH was measured with a Beckman 3500 Digital pH meter (Irvine, CA, USA). A Maximator pump, MSF 72, from Schmidt, Kranz and Co. (Sorg/Südharz, FRG) was used for the column packing.

Chemicals

As chromatographic support, LiChrosorb RP-18, 5 μm (E. Merck, Darmstadt), was used. Penicillinase was obtained from Løvens Kemiske Fabrik (Ballerup, Denmark). Ampicillin, benzylpenicillin (Pc-G), Pc-G-procaine, procaine-chloride, dihydrostreptomycin sulphate and penicillin-V were kindly supplied by Novo Industri AS (Copenhagen, Denmark). Sulfamethoxazole and trimethoprim were obtained from Sigma (St. Louis, MO, USA). Spiramycin was purchased from Leo Pharmaceutical Ltd. (Copenhagen, Denmark), sulfadimidine from Imperial Chemical Industries Ltd. (UK) and

oxytetracycline from Pfizer Inc. (New York, USA). Tylosin was obtained from Lilly Industries Ltd. (Liverpool, UK). All other chemicals were of analytical grade from E. Merck.

Stability of Pc-G

The stability in milk was studied with fresh chilled and pooled milk delivered from healthy Swedish Red and White, and Black and White cows.

For long term studies at −18°C and −73°C samples of 100 μl at a concentration of 25 μg Pc-G ml^{-1} were used. The proteins in the samples were precipitated with 100 μl of acetonitrile. After centrifugation, 50 μl of the supernatant was diluted with 100 μl of water and 100 μl of this solution was injected onto the LC-column and analysed by the chromatographic system B.

The short-term studies at +4°C and at room temperature were performed with 9 ml samples of 100 ng Pc-G ml^{-1} analysed according to the bioanalysis.

The stability of the penicillenic acid mercuric mercaptide derivative of Pc-G (Pc-GAMM), prepared for injection onto the LC-column according to the analytical procedure, was investigated by preparing three milk samples of 3 μg Pc-G ml^{-1} ready for injection onto the column. The samples were stored at room temperature, +4°C and −18°C, and after suitable intervals 10 μl of the sample was analysed by the chromatographic system used in the bioanalysis.

Extraction studies

The extraction efficiency of Pc-G in the clean up procedure were calculated from partition studies in each step. A 9 ml sample of 27 μg Pc-G ml^{-1} milk was handled according to the bioanalytical procedure and samples of 10 μl from the different liquid phases were analysed by direct injection with chromatographic system B.

Partition of Pc-G between the fat and the aqueous fractions of milk

One millilitre of commercial cream (40% fat) was spiked with 100 μl Pc-G solution. After centrifugation at 18,000 *g* for 5 min, an aliquot of the lower layer (non-fat part) was analysed by chromatographic system B. One millilitre of an aqueous buffer, pH 6.6, was spiked in the same way and the same aliquot was analysed. By comparing the two results the partition to the fat layer could be calculated.

Chromatographic technique

The mobile phase was prepared from stock solutions of imidazole in water (20% w/v) with the pH adjusted to 6.6 by phosphoric acid and 0.05 M mercury(II)chloride in water. Appropriate amounts of water, imidazole, mercury(II)chloride and methanol were mixed in a volumetric flask and the volume was adjusted with water. The final concentrations were methanol 45% (v/v), imidazole 2% (w/v) and mercury(II)chloride 50 μM. The detector wavelength was 325 nm.

In the extraction and some of the stability studies a chromatographic system for direct detection of Pc-G was used: phosphate buffer (pH = 6.6): methanol (60:40 v/v), $\mu = 0.1$ (system B). This system was operated at a detector wavelength of 200 nm. All mobile phases were degassed in an ultrasonic bath under vacuum before use.

One pre-column (40 × 4.0 mm) and one analytical column (150 × 4.0 mm) were used together. They were made of stainless steel equipped with modified Swagelok connectors and filters of 2 μm porosity (100 μm thick). The columns were packed by a

modified balanced density technique [5, 7]. The columns were heated to +50°C by a waterjacket arranged according to [5] and connected to a circulating thermostat bath.

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 performed within 1 h after use with the mobile phase.

An electric starting switch synchronised the injector with the integrator. The peak areas were used for the quantitative calculations.

Clean-up and analytical procedure

Extraction. In Fig. 1 the extraction scheme is shown. Nine millilitres of a well mixed milk sample were poured into a 35 ml test tube (100 × 25 mm). Acetonitrile (18 ml) was added and the mixture was heated under warm tapwater with shaking for 10 s to about 30°C. After centrifugation at 1000 *g* for 30 s the clear supernatant was poured into another tube of the same size. The supernatant was then mixed with 8 ml of a mixture of dichloromethane:petroleum ether (1:1 v/v). 250 µl of 1 M Tris-HCl buffer, pH 9.0 was added and the sample was shaken for 30 s. After a few minutes a two-layer system was established.

The lower layer (aqueous phase) was transferred into a 12 ml test tube (100 × 15 mm) by a pasteur pipette. The remaining organic layer was extracted once more with 1 ml of 0.01 M Tris buffer, pH 9.0, and the aqueous phases were mixed. One millilitre of ethylacetate was added to the aqueous phase and 600 µl of 1 M phosphoric acid also was added. After shaking for 10 s and centrifugation at 2000 *g* for half a minute, the upper

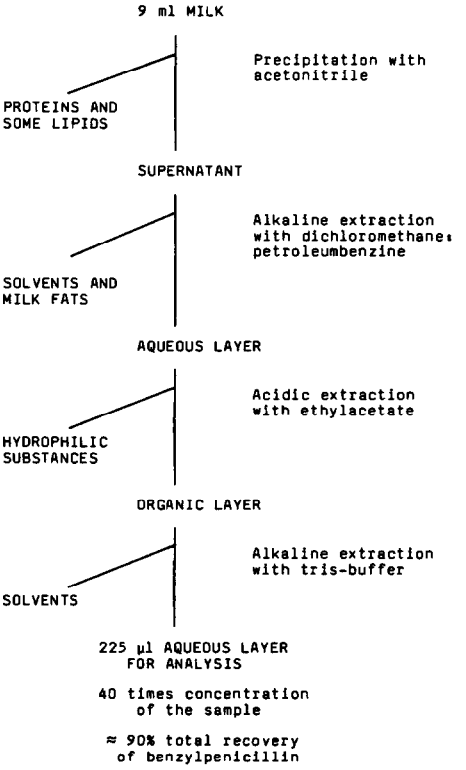

Figure 1
Extraction scheme for benzylpenicillin in milk according to the clean-up procedure.

(organic) layer was transferred by a pasteur pipette into a test tube with conical bottom containing 1 ml of *n*-heptane. The extraction with 1 ml of ethyl-acetate of the acidified aqueous phase was repeated two more times.

The pooled ethyl-acetate phases mixed with *n*-heptane were shaken a few times and centrifuged for a couple of seconds at 2000 *g*. A two-layer system with about 200–300 μl aqueous phase separated the lower (aqueous) layer of which was sucked off with a syringe and discarded.

The remaining organic phase was extracted three times with 75 μl 0.1 M Tris–HCl buffer, pH 9.0 by 10 s shaking and a few seconds centrifugation at 2000 *g*. The aqueous phases were transferred and pooled in a conical 1.5 ml polypropylene Eppendorf test tube with a cap. The tube was flushed with nitrogen for 1 min, then shaken gently and centrifuged for a short time to collect the sample in the bottom of the tube.

Derivatisation

The sample was now divided into equal volumes and transferred to 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, leading 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 added to the penicillinase treated sample. The two samples were then placed in a water bath at 60°C for 20 min. After completion of the reaction, 50 μl of the penicillinase solution also was added to the non-penicillinase treated sample. After a short centrifugation the samples were ready for LC analysis.

Measurements and calculations

The built-in programme 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 storing the chromatograms it was possible to subtract the zero sample from the actual sample and the Pc-GAMM peak integrated. This digital subtraction chromatography technique is described fully in a previous paper [6].

This method was used for Pc-G concentrations at low levels or only if any interfering peak was >5% of the Pc-G peak, for normal cow milk this was the case in the range <2 ng Pc-G ml^{-1} milk. At higher concentrations the zero sample merely confirmed that the detected peak originated from Pc-G.

Standard curves, recovery and statistics of the method. Standard samples were made by mixing 99 ml of fresh pooled cow's milk with 1 ml freshly prepared Pc-G stock solution to appropriate concentrations. Samples of 9 ml were taken out for analysis with a variable pipette with disposable tip.

Results and Discussion

Extraction according to the clean-up procedure

Milk is a very complex biological matrix that easily tends to form gels in contact with organic solvents. The clean-up extraction of milk for LC-analysis has been performed by

chromatographic purification [8], a combination of organic solvent extractions and chromatographic steps [9] or precipitation of the proteins and organic solvent extractions [10, 11].

Pc-G is an acid with proteolytic behaviour and its partition in different aqueous/organic systems has already been studied under controlled conditions [5]. The results obtained were the basis of the bioanalytical extraction procedure presented here.

The more or less ideal conditions discussed in [5] are not always valid when coming to the milk extractions. Mixtures of organic solvents are used. The milk fat, which varies in composition and concentration, acts as a solvent itself. Different milk samples have different pH, especially those from udders with mastitis, and since for practical reasons each sample cannot be checked in every step of the extraction scheme, the pH and the related distribution ratio for Pc-G (D_{HX} value) may differ. Ethyl-acetate dissolves some water and vice versa, and these equilibria are temperature dependent. The solvents were not equilibrated with the buffers before being used since it was noted that when ethylacetate and a pH 9 buffer were kept together, the alkaline hydrolysis of the ester took place. Within a few hours, the pH of the buffer decreased as acetic acid was formed in the ethylacetate.

All these facts showed the problems to have constant partition of Pc-G in all extraction steps. In order to have as close to a quantitative extraction (>99%) as possible in each step, repeated extractions [12] were used.

The short equilibrium times for the extraction of aqueous-ethyl-acetate systems were investigated in [5] and the same short extraction times were obtained in this bioanalytical study.

Our intention with the presented extraction scheme was to avoid large sample volumes but still have an adequate sample volume left for injection in order to obtain a good accuracy and enough sensitivity.

The double amount of acetonitrile effectively precipitates the proteins in milk [10] and when the sample was heated to ≈30°C the pellet was solid after centrifugation and it was just to turn the tube upside-down to decant the supernatant. Much of the milk fat was also trapped in the pellet. No binding of the drug to the precipitated proteins has been observed.

The addition of dichloromethane–petroleum ether (1:1, v/v) formed a two-layer system with an upper organic layer. The organic layer extracted most of the acetonitrile. The combination of dichloromethane and petroleumether formed a distinct two-layer system and it was easy to suck off the aqueous layer with a pasteur pipette. After adding 250 µl M Tris buffer, pH 9.0 the pH of the aqueous phase was 8.7. Repeated extraction gave >99% of the Pc-G extracted to the aqueous phase.

In the subsequent extraction of the aqueous phase at low pH with ethyl-acetate there was a larger risk of degradation of Pc-G. Experiments with three repeated extractions of Pc-G at pH 2.45 and 2.75 showed that the faster degradation at the lower pH was compensated by the higher D_{HX} value at this pH, and the total recovery for the Pc-G extraction was equal at these two pHs. Not more than 1% was left in the aqueous layer. A degradation of Pc-G after 30 s in pH 2.5 of less than 1% has been observed [13] but the data from the stability studies in [5] showed a somewhat faster degradation. Some of the decrease of the total recovery in the sample extraction must be caused by this degradation.

The addition of 1 ml of *n*-heptane to the organic layer before the last re-extraction to an aqueous phase will expel the water dissolved in the ethyl-acetate. This water had a low

pH and affected the pH 9.0 buffer negatively at this unfavourable phase volume ratio. Less than 1% of the Pc-G disappeared in this acidic waste. The *n*-heptane also made the organic layer more lipophilic, which led to an increased distribution of Pc-G into the aqueous phase. When an LC syringe was used to collect the aqueous phase >98% of the Pc-G was extracted in this last step.

Derivatisation of Pc-G

Pc-G has its optimal molar absorptivity (ϵ) at a wavelength of 195 nm [5]. The advantages of detecting penicillins as their penicillenic acid derivatives at 325 nm were discussed in a previous work [5]. Also in that work the conditions for the formation of the mercuric mercaptide of the benzylpenicillenic acid (Pc-GAMM) were studied. The final aqueous Pc-G extract of the bioanalytical extraction obtained in this study could be derivatised as it stood since calculated data [14], predicted a good reaction rate in the range pH 6.7–9.0. The nitrogen flush at the end of the extraction guaranteed a pure aqueous solution. The reaction was complete after 15 min and the derivative was stable for 1 h at 60°C [5].

Chromatographic technique

As milk is so variable in its composition and complicated it was understood that a conventional LC-technique could not solve the problems of low level determination of Pc-G.

In spite of the fact that a rather long detection wavelength (325 nm) was used, interfering peaks could not be avoided. A new technique therefore was developed in order to reduce the disturbing peaks, namely digital subtraction chromatography (DSCh). This technique is described and discussed in a previous work [6]. The principle is that a zero sample chromatogram is subtracted from a chromatogram with the searched compound by the chromatographic data processor and a new chromatogram is integrated and printed. This chromatogram presents the compound peak on a straight baseline.

With the milk samples analysed in this study it was not necessary to optimise the chromatographic conditions with reference to the DSCh-technique. If the disturbing peaks are larger, however, it is possible to optimise the conditions as demonstrated in [6].

The chromatographic conditions used in the bioanalysis of Pc-G in milk are based on the studies of the chromatography of Pc-GAMM presented in a previous work [5]. The conditions were chosen to have an optimal performance in order to achieve a visible Pc-GAMM peak at a low sample concentration.

Identification and quantification

In the basic studies of the DSCh-technique [6], the zero sample was represented by a non-spiked sample identical to the spiked sample except for the searched component. In the bioanalysis, which involves a complicated extraction scheme, it was not possible to use a normal zero sample as the identity of each chromatographic peak could not be guaranteed.

Under these circumstances it was better to make an artificial zero sample [6] by a selective withdrawal of the Pc-G peak. This was done by the hydrolysis of the intact β-lactam ring, which is specifically catalysed by penicillinase (β-lactamase).

By splitting the sample at the end of the extraction procedure, two identical portions

were obtained. As the Pc-GAMM was not sensitive to penicillinase and as the degradation product from penicillinase treated Pc-G (benzylpenicilloic acid) was not sensitive to the imidazole–mercury(II)chloride reagent the identity could be kept except for one component, namely the Pc-GAMM.

Less than one part in a thousand of the Pc-G was left after penicillinase treatment for 10 min at room temperature. The penicillinase solution was active for some days when stored at +4°C.

Human milk was chosen as a model for complicated chromatograms where milk from acute mastitis cows was included. Human milk was analysed according to the bioanalytical method with one alteration, in the acidic extraction of Pc-G only 400 µl of 1 M phosphoric acid was added in order not to have a too low pH (Fig. 2). These chromatograms clearly demonstrate that the artificial zero samples obtained from penicillinase treatment were adequate for the DSCh-technique and could be compared with the chromatograms obtained in the basic DSCh-technique studies presented in [6].

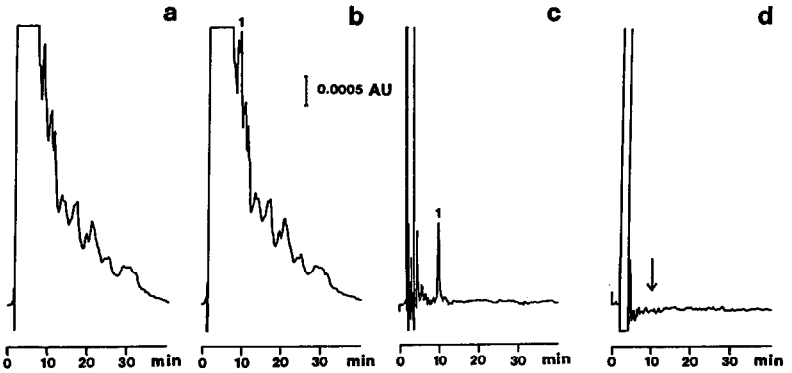

Figure 2
Chromatograms from human milk. Analysis performed according to the bioanalytical method. Samples: 2 ng Pc-G ml^{-1} milk (a, b, c); 0 ng Pc-G ml^{-1} milk (d). (a) Penicillinase treated sample (zero sample); (b) actual sample; (c) interfering peaks removed by the use of the digital subtraction technique, reintegrated and replotted (a minus b), 1 = Pc-GAMM; (d) blank milk performed according to (c). Arrow indicates the retention time of Pc-GAMM.

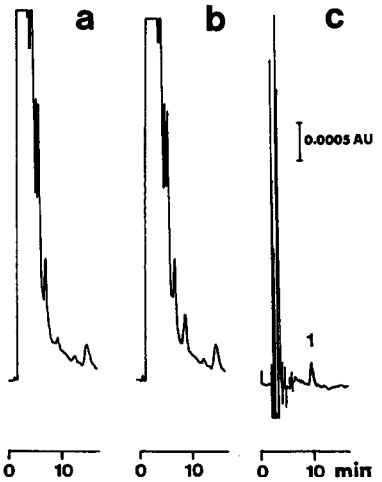

Figure 3
Chromatograms of spiked milk from healthy cows. Analysis performed according to the bioanalytical method. Sample: 0.5 ng Pc-G ml^{-1} milk. (a–c), see Fig. 2(a–c).

In Fig. 3, pooled spiked milk from healthy cows was analysed according to the bioanalysis.

The described method, with a selective transformation of the analysed substance in combination with the DSCh-technique, would be of great advantage in order to simplify and increase the confidence of the identification as well as the accuracy in the chromatography of substances that can be selectively transformed.

Accuracy and standard curves

The reproducibility and recovery of the bioanalytical method for Pc-G in milk are demonstrated in Table 1. A reference factor was calculated from the average of eight 10 ng Pc-G ml^{-1} milk samples, analysed according to the bioanalysis. This reference factor was used by the chromatographic data processor to calculate the concentrations of the samples. Three standard curves for Pc-G in milk were made by plotting the determinations of single injections from each concentration with the added amount on the x-axis and the found amount on the y-axis. The first standard curve was 0.2, 0.4, 0.5, 0.6, 0.8 and 1.0 ng Pc-G ml^{-1} milk, the equation was $y = 1.022x - 0.03$ and $r = 0.9877$. The second was: 2, 5, 10, 20 and 50 ng ml^{-1} with the equation $y = 0.9519x - 0.64$, $r = 0.9974$. The third: 100, 200, 500 and 1000 ng ml^{-1}, with the equation $y = 0.9767x + 2.57$ and $r = 0.9994$.

The partitioning of Pc-G into milk fat

In a previous work [15] the problems with milk samples containing phenylbutazone were discussed. As phenylbutazone was strongly partitioned into the milk fat at neutral pH, the sampling was complicated. The fat content of the milk may increase from 1% in the first amount of milk to 10% in the last amount that leaves the udder during milking [16]. Furthermore the fat separates from the aqueous phase of the milk if not agitated during storage. Depending on the partitioning of a drug into the fat phase of the milk one has to be careful when taking an aliquot of milk in order not to lose or enrich the drug. The partition experiment with 40% cream showed that Pc-G does not enter the milk fat at neutral pH.

Another lipophilic component that may be present in milk is the oil based veterinary mastitis products containing salts of Pc-G, as for instance Pc-G-procaine. These sticky depot products are administered by intramammary infusion and may presumably stay intact for some time on the tissue walls. When the udder is milked, these aggregates may be present in the milk sample. Experiments with an oil-based Pc-G-procaine product (Streptocillin™ vet., Novo) added to the 9 ml milk sample, ready for extraction and analysed according to the bioanalysis, resulted in a Pc-G concentration in the milk sample corresponding to the added amount. This showed that the extraction procedure was sufficient to recover Pc-G trapped as a procain salt in fat droplets.

Table 1

Sample concentration (ng Pc-G ml^{-1} milk)	n	Relative standard deviation (%)	Recovery (%)
1	10	±7.8	86
10	8	±1.6	87
100	8	±1.1	91
1000	8	±2.7	91

In conclusion, the udder must be well emptied and the samples must be taken out from well mixed milk in order to have a representative Pc-G concentration for the total milk volume.

Stability studies

Solutions of Pc-G have their maximum stability at a pH of 6.6 [17] with about the same increasing degradation rate at increasing and decreasing pH. The stability of Pc-G in milk is demonstrated in Fig. 4. It is important to chill the milk as soon as possible; at +4°C the milk samples were reasonably stable for some days and then the degradation of Pc-G accelerated. This accelerated degradation also could be seen at room temperature. It may have been caused by microbiological processes in the milk, which lowered the pH or took an active part in the degradation. This may be of importance when incubating milk samples at +37°C, as is done in biological penicillin tests. The frozen samples were much more stable, but at −18°C a slow degradation occurred. The stability at −70°C was much better, and these results corresponded with stability studies for other penicillins in biological fluids [18].

Samples prepared for injection onto the LC-column as the Pc-GAMM derivatives of Pc-G could be stored at room temperature if they were analysed during the same day; 95% of the compound remained after 10 h. Storage at +4°C prolonged the storage time up to 30 h, and at −18°C the prepared sample was stable for at least 1 week.

Limit of detection

An equation giving the relationship between the injected amount on an LC-system (m) and the UV-absorption at peak maximum (A_{max}), the molar absorption (ε), the retention volume for the peak (V_R) and the number of theoretical plates of the column (N), can be written:

$$m = \frac{\frac{C_{max}}{\varepsilon} \cdot \sqrt{2\pi} \cdot V_R}{\sqrt{N}} \quad (1)$$

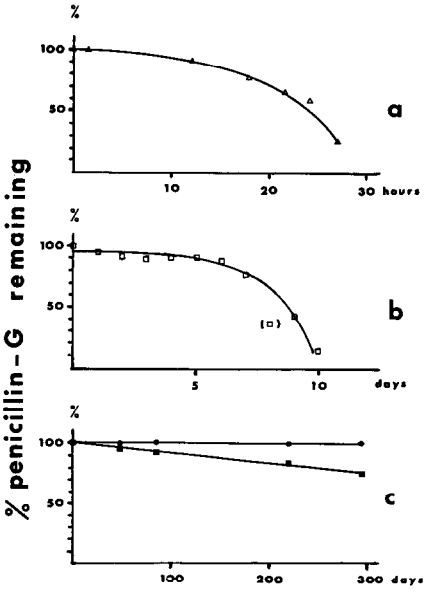

Figure 4
Stability of Pc-G in milk at different temperatures. (a) Room temperature, 100 ng Pc-G ml^{-1}; (b) +4°C, 100 ng Pc-G ml^{-1}; (c) ● = −70°C, ■ = −18°C, 25 µg Pc-G ml^{-1}.

The equation is discussed in a previous work [5], where the nature of the eluted peak was studied.

Equation (1) is useful for the calculation of the limit of detection for the method. By practice, a minimum peak height of three times the noise level is often used. The noise was measured on the printed detector signal and calculated to 0.000037 absorbance units (AU). Three times this value put in equation 1 gives an injected amount of 2.33×10^{-12} mol — the minimum detectability [19] — corresponding to 830 pg of Pc-G-sodium. Since the injected sample in the bioanalysis originated from 4.5 ml milk and the recovery was 86% the limit of detection [19] would be 214 pg Pc-G ml^{-1} milk. A sample of 200 pg Pc-G ml^{-1} milk was analysed, confirming the calculations above (Fig. 5).

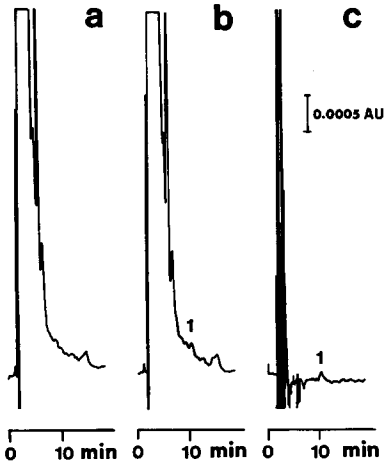

Figure 5
Chromatograms of spiked milk from healthy cows. Analysis performed according to the bioanalytical method. Sample: 200 pg Pc-G ml^{-1} milk. (a–c), see Fig. 2(a–c).

Interfering substances

The following substances were tested for possible interference in the bioanalysis of Pc-G in milk: ampicillin, oxytetracycline, penicillin-V, spiramycin, dihydrostreptomycin, sulphadimidine, sulphamethoxazole, trimethoprim and tylosin. At a concentration of 10 μg ml^{-1} milk only ampicillin and penicillin-V were positive. Ampicillin gave some small peaks in the Pc-G area, none larger than the corresponding peak of 1 ng Pc-G ml^{-1} milk, and some of the peaks were sensitive to penicillinase. Somewhat surprisingly, the penicillenic acid mercury mercaptide of penicillin-V has the same k′ value as Pc-GAMM, though the k′ for penicillin-V is about the double of that of Pc-G at neutral pH in a methanol-phosphate buffer HPLC system [20]. The recovery for penicillin-V in the clean-up procedure is about the same as for Pc-G, but the maximum yield in the derivatisation step in the bioanalysis with the imidazole–mercury(II)chloride reagent was reached after only 10 min, and the degradation of the derivative also was faster at room temperature. The peak area was about 75% of that of Pc-GAMM at maximum yield.

In Sweden, penicillin-V is not registered for use in cows, and will therefore cause no problems, however there are possible ways of distinguishing between the two penicillins. As they are both sensitive to penicillinase, this substance cannot be used, but the difference in acid stability between the two penicillins [17] can be used as an identification tool. By adding 20% (volume) of 1 M phosphoric acid to each of the two split identical samples at the end of the extraction schedule described in the bioanalysis,

the pH will be lowered to about 2.0. After 1 h at room temperature the samples are derivatised as described, but only for 10–15 min. About 10% of the Pc-G and 90% of the penicillin-V will then be present in the samples. Other substances that could be expected to interfere are degradation products of Pc-G. Penicillin solutions are known to give rise to several degradation products after storage at different pHs [21]. If the disappearance of Pc-G was followed by two different methods of analysis it would be possible to see if any of the methods were disturbed by some degradation product when comparing the degradation rate obtained by the two methods. The degradation of Pc-G was followed at pH 2.2 for 20 min and at pH 7.5 for 67 h by both direct detection of Pc-G by the chromatographic system B and detection of the Pc-GAMM derivative according to the bioanalysis. No difference in degradation rate of Pc-G was noted between the two methods of analysis and the rate constants were in agreement with other studies [21].

Conclusions

A bioanalytical method for a selective detection of benzylpenicillin in milk is presented. The sensitivity was about 10 times better than those of microbiological determination methods. The use of the digital subtraction chromatography (DSCh) technique makes it possible to apply the method to different kinds of milk samples, irrespective of interfering peaks. Chromatograms from the analysis of cow's and human milk are shown. The use of penicillinase in the DSCh-technique increases the specificity of qualitative analysis. The method can, therefore, be used for the control of trace amounts of benzylpenicillin in commercial milk.

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