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Basic extraction studies of benzylpenicillin and its determination by liquid chromatography with pre-column derivatisation

B. WIESE* and K. MARTIN

The Swedish University of Agricultural Sciences, Faculty of Veterinary Medicine, Department of Pharmacology and Toxicology, Uppsala Biomedical Centre, Box 573, S-751 23 Uppsala, Sweden

Abstract: In order to develop a sensitive and precise chemical bioanalysis of benzylpenicillin in biological fluids, basic studies on benzylpenicillin are presented. These studies include pH dependent stability, extraction in aqueous ethylacetate systems in different pH and buffer compositions. A pre-column derivatisation LC method for the detection of benzylpenicillin also is described. The derivatisation of the sample is performed by the β-lactam ring specific formation of the mercuric(II)mercaptide of the penicillenic acid in the presence of imidazole. The UV spectral properties of the derivative are utilised for detection. The chromatographic conditions are optimised with reference to the pH, the methanol, the imidazole and the mercury(II)chloride content of the mobile phase as well as the column temperature.

Keywords: *Benzylpenicillin; liquid–liquid extraction; reversed-phase liquid chromatography; pre-column derivatisation; trace amounts.*

Introduction

The numerous published works dealing with the determination of penicillins reflect the interest and the difficulties in this field. Microbiological [1, 2] and chemical [3, 4] methods have been developed in parallel during the past decades. By the introduction of modern LC [5, 6, 7], it has been possible to avoid the drawbacks of doubtful results caused by active metabolites or anti-bacterial endo- or exogenic substances that may interfere in microbiological methods. Many of the LC methods for penicillin determinations are developed for high concentrations in pure solutions [8, 9]. Methods for the determination of penicillins in biological fluids also have been published [10, 11]. The most sensitive detection methods available today are those based on microbiological measurements. Since the limit of detection for these methods is a few ng ml^{-1} milk [12], a chemical determination method for closer studies in the low penicillin concentration range should be as sensitive as the microbiological ones or, preferably, more sensitive. As the requirements on lower detection limits increase, the number of suitable published chemical methods decreases. Only one method describing the detection of an injected

*To whom correspondence should be addressed. Present address: Apoteksbolaget AB, Central Laboratory, Dept. Biomedicin, S-105 14 Stockholm, Sweden.

amount of 1 ng penicillin-V by a post-column derivatisation LC method seems to be available [13].

In the work presented here basic studies on benzylpenicillin (Pc-G) are performed. These studies include liquid–liquid distribution, stability, UV absorption and chromatographic behaviour. A new LC method for the detection of low sample concentrations of Pc-G is presented. The method involves the formation of the penicillenic acid mercury(II)mercaptide (Pc-GAMM) derivative of Pc-G as a pre-column derivatisation LC method.

The methods should have a very high sensitivity in order to study the relevance of the microbiological determination methods at trace levels of the drug. To make pharmacokinetic calculations, the analytical results must be precise and they should reflect the true concentration of Pc-G in the biological matrix. In order to apply the methods to confirm the positive Pc-G tests obtained by other techniques in food control, it is furthermore essential that the LC-methods yield a positive identification.

According to the above mentioned demands of the bioanalytical LC-methods, the basic studies in this work are rather extensive. The work together with additional optimisation studies of the chromatographic part of the method [14], stress the reliability of the final bioanalytical methods [15, 16].

Experimental

Apparatus

The liquid chromatograph consisted of a Shimadzu Model LC-5A pump, a Model SPD.2A ultraviolet detector and a Model C-R3A integrator (Shimadzu Corporation, Tokyo, Japan). The injector was a Rheodyne 7125 (Berkely, CA, USA) equipped with a 2.0 ml loop. The pH was measured by a Beckman 3500 Digital pH meter (Irvine, CA, USA) and the UV spectra were scanned by a Shimadzu UV-210A spectrophotometer. A Maximator pump, MSF 72 (Schmidt, Kranz and Co., Sorge/Südharz, FRG) was used for column packing.

Chemicals

LiChrosorb RP-18, 5 μm (E. Merck, Darmstadt, FRG) was used as chromatographic support. [Phenyl-4(*n*)-^{3}H]benzylpenicillin was purchased from Amersham International plc (Amersham, Bucks, UK) and Pc-G was kindly supplied by Novo Industri AS (Copenhagen, Denmark). All other chemicals were of analytical grade from E. Merck.

Extraction studies

The partitioning of Pc-G was investigated by adding the drug to aqueous buffers to a concentration of 100 μg ml^{-1}. The samples were then extracted with ethyl acetate at equal phase volumes for 1 min on a shaker at room temperature. After centrifugation the concentrations of Pc-G in respective phase were determined by injection of 10 μl directly onto chromatographic system B.

The pH value of the aqueous phase varied from 3.0 to 7.0 with buffers made from phosphoric acid, disodium hydrogen phosphate and sodium dihydrogen phosphate, $\mu = 0.1$. In the pH range 7.7–9.0 different disodium hydrogen phosphate concentrations or Tris(hydroxymethyl)aminomethanehydrochloric acid buffers were used. Sodium bromide was added to the aqueous phase in order to study the sodium ion-pair formation.

Stability of Pc-G

The pH dependent degradation of Pc-G was followed by adding Pc-G to aqueous buffers, pH 2.2–9.0, to a concentration of 100 μg Pc-G ml^{-1}. After suitable times aliquots of 10 μl were taken out and mixed with 200 μl phosphate buffer, pH 6.6, and stored at +4°C until analysed within a few hours by the chromatographic system A.

UV absorption spectra for Pc-G and Pc-GAMM

UV spectra from 190 to 300 nm for Pc-G were obtained in phosphate buffers, pH 3.4, and 6.6, $\mu = 0.1$ with 20% v/v acetonitrile. UV spectrum for Pc-GAMM was scanned from 190 to 400 nm. A derivative, prepared from 10 μg of Pc-G dissolved in 150 μl of 0.1 M Tris-HCl buffer, pH 9.0, was made according to the derivatisation procedure described later. One 150 μl blank sample was treated in the same way. The sample and the blank were then diluted with the mobile phase used in the bioanalysis and UV spectra were obtained. The molar absorptivity, ε, was calculated by Lambert–Beer's law.

Chromatographic studies

Preparation of the mobile phase. A stock solution of imidazole in water (20% w/v), with the pH adjusted to 6.6. by phosphoric acid, was mixed with appropriate amounts of methanol, water and 0.05 M mercury(II)chloride. The eluent was degassed in an ultrasonic bath under vacuum before being used.

One pre-column (4.0 cm × 4 mm i.d.) and one analytical column (15 cm × 4 mm i.d.) were used in series. They were made of stainless steel and equipped with modified Swagelok^T connections and filters of 2 μm porosity, 100 μm thick. The columns were packed by a modified balanced density technique [17] involving the following steps. The support was suspended in chloroform (0.1 g ml^{-1}). The packing reservoir was filled with the slurry and a layer of acetone (about 70 ml) and connected to the packing pump. A 200 ml volume of methanol was used as driving liquid at a pressure of 39 MPa. Before the column was disconnected, the reservoir was emptied and filled with water. About 30 ml of water was then pumped through the column, which was then connected to the LC pump. This last step prevents the packing material from sinking at the top of the column when going directly from methanol in the packing pump to water in the LC pump. The columns were equilibrated with 200 ml of mobile phase when they were newly packed. Both columns were heated to different temperatures up to 50°C with a water jacket connected to a thermostat bath. The void volume of the columns (V_m) was measured by a 20 μl injection of water. Symbols and equations for chromatographic parameters were used according to Kirkland *et al.* [18].

When the LC system was left overnight or longer, the system was washed with about 30 ml phosphate buffer, pH 3.0, $\mu = 0.1$:methanol (55:45 v/v). Equilibration of the column after this washing was done within an hour with the respective mobile phases.

Two other chromatographic systems were used for direct detection of Pc-G at 200 nm: phosphate buffer, pH 6.6, $\mu = 0.1$:acetonitrile (85:15 v/v) (system A) and phosphate buffer, pH 6.6, $\mu = 0.1$:methanol (60:40 v/v) (system B). All other conditions were as described above.

Derivatisation

Benzylpenicillenic acid mercury(II)mercaptide (Pc-GAMM) was formed by 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. From this reagent 30 μl was added to 200 μl

of an aqueous solution of Pc-G. The reaction was performed in 1.5 ml polypropylene tubes with caps (Eppendorf tubes). After mixing, the sample was left in a water bath at 60°C for 20 min. An aliquot or all of the sample was then injected onto the column.

Results and Discussion

Extraction of Pc-G

The instability of Pc-G excluded concentration of the organic phase by evaporation after the organic extraction. In order to obtain a small sample volume with a reduced content of interfering substances, the extraction as well as the back extraction properties of Pc-G were studied and optimised.

Pc-G is a moderately strong acid, pKa = 2.8 [19]. The protolytic properties may be utilised for the extraction procedure of Pc-G from biological fluids. The partitioning of Pc-G in an aqueous organic two phase system depends on the pH of the aqueous phase, the nature of the organic solvent and the presence of counter ions that may lead to ion-pair distribution of Pc-G [20]. Furthermore, the instability of Pc-G at low pH will present restrictions. The distribution of an acid, HX, between an aqueous and an organic phase can be expressed by the distribution ratio, D_{HX}.

$$D_{HX} = \frac{C_{HXorg}}{C_{HXaq}} = \frac{[HX]_{org}}{[HX] + [X^-]} \quad (1)$$

where HX is the acid and X^- the corresponding base. A plot of log D_{HX} versus pH (Fig. 1) should give a straight line with a slope of -1 at pH > pKa + 1 [21]. As can be seen in Fig. 1, D_{HX} increases at pH values >7. This may be explained by the existence of a side-reaction like ion pair distribution of Pc-G to the organic layer. The buffers used in the high pH range were disodium hydrogen phosphate at different molarities. To test the hypothesis of ion-pair extraction, the pH was kept at 8.6 by 0.01 M disodium hydrogen phosphate, where the D_{HX} had a minimum value. The sodium ion concentration was increased to 1 M by addition of sodium bromide. As can be seen in Fig. 1, the value for

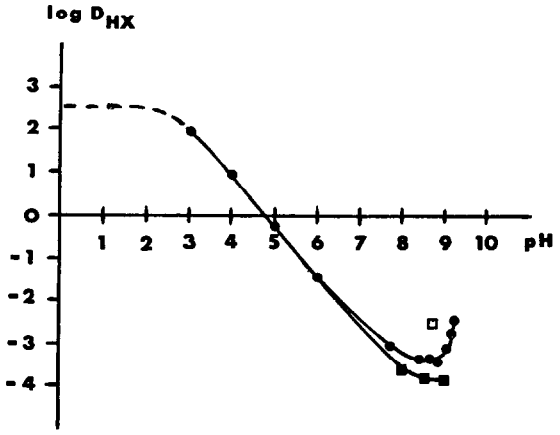

Figure 1
Partition of Pc-G at different pH between different aqueous buffers and ethyl acetate. ● = Sodium phosphate buffers (7.7 < pH < 9.1 = disodium-phosphate: 0.005–0.5 M); ■ = Tris-HCl buffers; □ = 1 M sodium (pH 8.6); --- = calculated value.

D_{HX} increased to almost the same value as for 0.5 M disodium hydrogen phosphate (1 M sodium) at pH 9.2. This evidence indicates the occurrence of ion-pair extraction with the sodium ion as cation.

The last extraction step in a bioanalytical method for Pc-G requires optimal conditions. A good buffer capacity combined with a low D_{HX} value is essential, as this step involves partition of Pc-G to the aqueous phase from an acidic ethyl acetate phase under unfavourable phase volumes. Therefore, instead of different molarities of disodium phosphate, a Tris-HCl buffer (0.1 M) was tested in the higher pH-range, instead of different molarities of disodium phosphate. In Fig. 1 it can be seen that the difference from the ideal straight line for D_{HX} is much less with the Tris buffers than with the sodium phosphate buffers at high pHs.

The main problem with extraction at low pH is the instability of Pc-G. There must be a balance between good extraction efficiency and losses of the substance caused by acidic degradation. The value for D_{HX} should reach a maximum at about pH 2.0 (0.3 log units higher than the D_{HX} value obtained at pH = pKa). As discussed under the stability studies of Pc-G, the drug can be kept only for a few minutes at pH 2.5–3.0, otherwise the degradation will be too great. Only ethyl acetate has been tested as solvent. However, it may be difficult to find a solvent with a better solvation ability for Pc-G than this polar solvent. Experiments to determine the equilibrium time for the extraction of Pc-G at high and low pH values in an aqueous-ethyl acetate two-layer system failed. The extraction was complete after one single stroke of the test tube. Short extraction times therefore could be used.

Stability studies

Solutions of Pc-G have their maximum stability at a pH of 6.6 [19] with about the same increasing degradation rate at increasing or decreasing pH. Figure 2 shows the stability of Pc-G at pH values suitable for extraction and, as can be seen, the acid extraction is the more gentle.

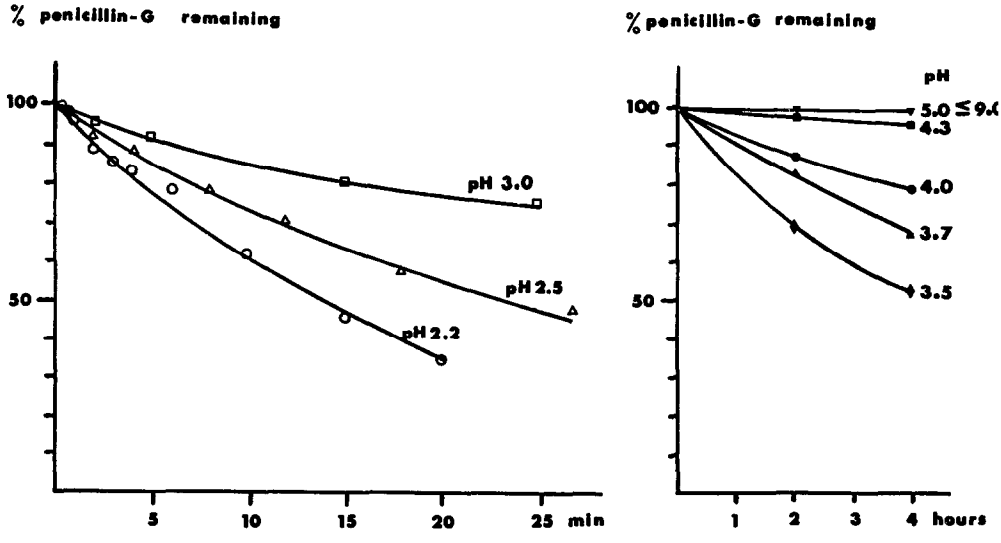

Figure 2
Instability of Pc-G at room temperature in aqueous buffer at different pH. Samples 100 μg Pc-G ml^{-1}.

Derivatisation of Pc-G

The UV-spectrum of Pc-G is shown in Fig. 3a. The transformation of penicillins into the corresponding penicillenic acid in order to detect the drug at wavelengths >300 nm has been used earlier. This is more favourable for bioanalytical purposes as interference from other compounds is minimised. The problem with the fast degradation of the acid has been solved in different ways [22, 23]. By a method developed by Bundgaard *et al.* [4, 24, 25], a stable derivative of penicillenic acid is formed by a β-lactam ring specific reaction. Mercury(II)chloride reacts with many penicillins in neutral solutions to form the mercuric mercaptide of the corresponding penicillenic acids; the reaction is catalysed by imidazole as shown in Fig. 4.

The imidazole is described as a catalyst that migrates from the molecule [25], but as shown in the chromatographic studies, both imidazole and mercury(II)chloride must be present all the time in order to keep the derivative stable.

The molar absorbance (ε) is about 20,000 (1 mol^{-1} cm^{-1}) at 325 nm for Pc-GAMM (see Fig. 3b), compared with an ε of 38,000 (1 mol^{-1} cm^{-1}) at 193 nm for Pc-G at low pH.

The mercuric mercaptide derivative of penicillin has been utilised in a post- as well as in a pre-column derivatisating LC method [10, 26]. The advantage of the post-column derivatisation technique is that it simplifies the chromatographic part of an analysis, as known chromatographic data for the parent compound can be used for the separation

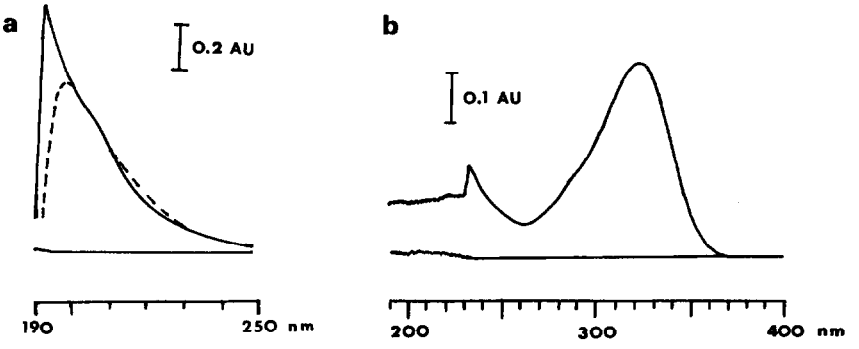

Figure 3
(a) UV spectra for PC-G at different pH. 10 μg Pc-G-sodium/ml phosphate buffer, μ = 0.1:acetonitrile, 80:20 (v/v). pH 3.4 (———) and pH 6.6 (------). (b) UV spectrum for Pc-GAMM. Corresponding to 7.5 μg Pc-G-sodium/ml mobile phase as in Fig. 8.

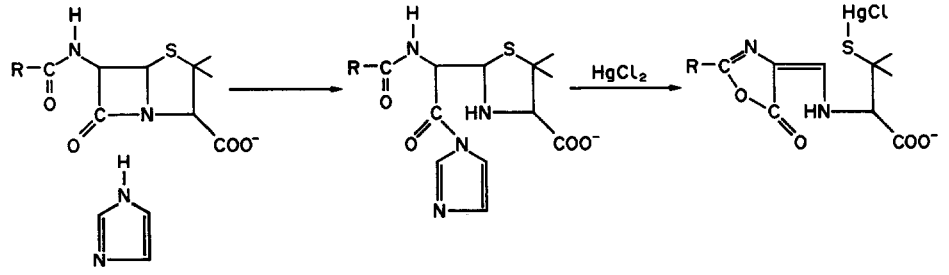

Figure 4
Derivatisation of penicillins. According to Bundgaard and Ilver [4].

step. The total chromatographic system will, however, be more complicated and problems may arise in trace analysis which requires a stable, low-noise base-line at highest sensitivity. These problems were overcome by a pre-column derivatisation LC method. The final temperature and concentrations of imidazole and mercury(II)chloride for the sample derivatisation were with small variations chosen according to [4]. The concentration of imidazole was somewhat higher than in [4], to guarantee a complete reaction within 15 min. Experiments showed a maximum yield of Pc-GAMM after 15 min and the derivative was stable for 1 h at ±60°C which was in agreement with [4].

Chromatographic studies

The good detector response for Pc-G at 193 nm at low pH is described above. As an acid, the Pc-G has a longer retention time in an acidic LC system than at neutral pH [10]. Unfortunately, a lot of endogenous substances in biological fluids, especially in milk, are acids with a similar pH dependence retention mechanism and a good molar absorptivity around 200 nm. By forming a derivative of Pc-G not only a more favourable wavelength can be used for detection, the retention at neutral pH is also increased. The k' for Pc-G is 1.6 compared with 4.1 for Pc-GAMM in the chromatographic system used in the final assay.

Many penicillins are known to form ion-pairs with quaternary ammonium compounds like tetrabutylammonium ion [20] and ion-pairs may also be formed with buffer components on a reversed-phase LC system [10]. Indications of such an ion-pair retention model, caused by the sodium ion in the buffer at neutral pH, is shown in Fig. 5, here the ionic strength of the sodium phosphate buffer was varied at high and low pH. In reversed-phase ion-pair chromatography the k' is increased by increasing counter ion concentration [21]. As can be seen, this occurs at pH 6.6 where Pc-G is protolysed and is able to form an ion-pair with a cation, e.g. sodium. At a pH close to the pKa of Pc-G (2.8) the degree of protolysation of the carboxylic function is very much less than at neutral pH, and here the variation of the k', with varying ionic strength, is very small. Regarding Pc-GAMM, it was not possible to influence the retention at neutral pH by varying the ionic strength or by adding tetrabutylammonium to the mobile phase.

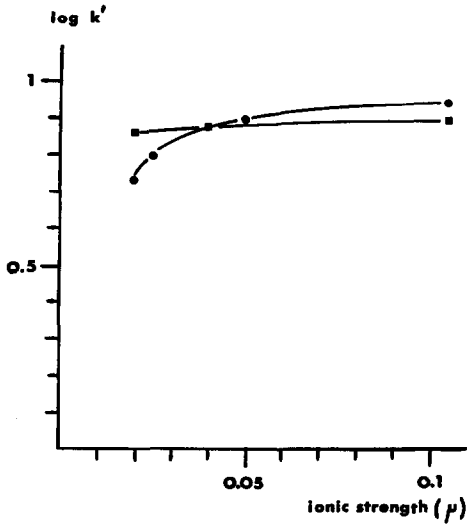

Figure 5
Influence of the ionic strength on the retention of Pc-G at different pH. Column: See Fig. 8. Mobile phase: ■ = phosphate buffer, pH 3.3:acetonitrile, 79:21 (v/v); ● = phosphate buffer, pH 6.6:acetonitrile, 85:15 (v/v). Different ionic strengths.

Furthermore the k' value did not change to any larger extent in the pH range 2.5–7.0. This may be due to an increase in the pKa value.

It was found to be necessary to have imidazole or mercury(II)chloride present in the mobile phase for chromatography of Pc-GAMM. The influence of the imidazole concentration in the mobile phase on the column efficiency is demonstrated in Fig. 6. The high concentration samples are sensitive to variations in the imidazole concentration. The decreasing efficiency of the lower sample concentrations at 4% imidazole in the eluent however may be due to diffusion effects, since the eluent became more viscous, noted by a rising back pressure. The ratio of the imidazole to Pc-GAMM concentrations must be $>10^3$ in order to have a good chromatographic performance. The measured column efficiency is in this study an indication of the stability of the molecule, since the peak became more and more deformed with decreasing imidazole concentration and eventually disappeared.

As expected the k' of Pc-GAMM decreased exponentially from 8.6 to 3.4 when the methanol concentration of the mobile phase was increased from 40 to 60%. A weak influence on the k' of Pc-GAMM, similar to that of an organic modifier, also was observed for the imidazole concentration of the mobile phase. The k' decreased linearly from 3.1 to 2.6 when the imidazole concentration was increased from 1% to 4%.

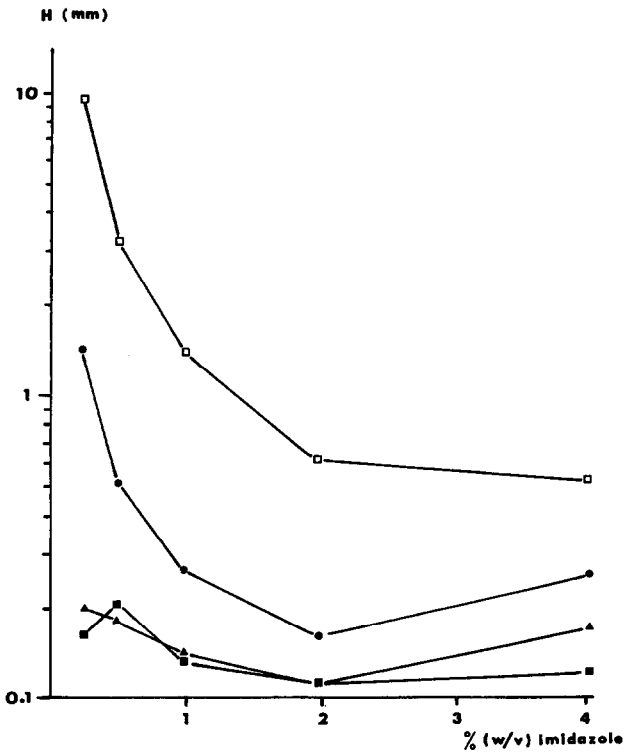

Figure 6
Influence of the imidazole concentration on the column efficiency. Column: see Fig. 8. Mobile phase: 370 μM mercury(II)chloride, pH 6.6, adjusted with phosphoric acid: methanol, 55:45 (v/v), different imidazole concentrations. Samples: injected amounts of Pc-GAMM corresponding to: □ =10 μg Pc-G; ● = 1 μg Pc-G; ▲ = 0.1 μg Pc-G; ■ = 0.01 μg Pc-G.

The column efficiency for Pc-GAMM also is dependent upon the mercury(II)chloride concentration in the mobile phase, as is demonstrated in Fig. 7. The mercury(II)chloride concentration in the eluent should be just about twice the molarity of the Pc-GAMM in the injected sample. Obviously the equilibrium of the reaction of Pc-GAMM must be maintained by the continuous presence of both imidazole and mercury(II)chloride.

A mobile phase containing 2% (w/v) of imidazole and 50 μM of mercury(II)chloride was chosen for the quantitative determinations. The decreasing column efficiency for high sample concentrations did not effect the integrated peak areas.

In Fig. 8, typical Pc-GAMM chromatograms are given. As can be seen, it is possible to detect an injected amount of Pc-GAMM corresponding to less than 2 ng of Pc-G.

Column temperature

The advantages of elevated column temperature for the chromatography of penicillin-V have been described earlier [27]. The present experiments show that shorter retention times with maintained column efficiency and decreased back pressure at a given concentration of organic modifier also are possible for Pc-G at elevated column temperatures. However, for acidic eluents, the on-column degradation of Pc-G will be faster at the higher temperatures.

The temperature dependence of the column efficiency for Pc-GAMM is demonstrated in Fig. 9. Notable is the difference between two thermostating designs. The plate height

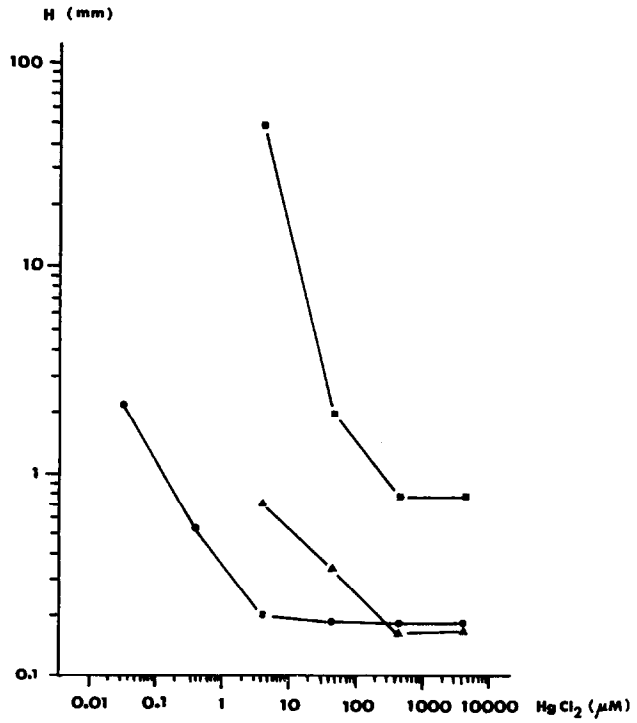

Figure 7
Influence of the mercury(II)chloride concentration on the column efficiency. Column: see Fig. 8. Mobile phase: 2% (w/v) imidazole, pH 6.6, adjusted with phosphoric acid:methanol, 55:45 (v/v), different mercury(II)chloride concentrations. Samples: injected amount of Pc-GAMM corresponding to: ■ = 10 μg Pc-G; ▲ = 1 μg Pc-G; ● = 0.250 μg Pc-G.

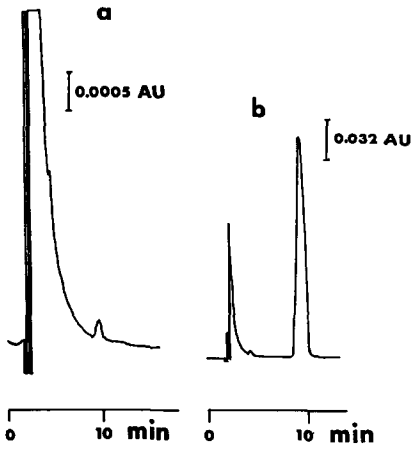

Figure 8
Chromatograms of Pc-GAMM. Samples prepared according to the described derivatisation procedure. The injected amount corresponds to (a) 2 ng and (b) 2000 ng Pc-G-potassium. Column: LiChrosorb RP-18 5 μm, one guard column 40 × 4 mm and one analytical column 150 × 4 mm. Column temperature: 50°C. Mobile phase: water:methanol 55:45 (v/v), 2% imidazole (w/v), pH 6.6 adjusted with phosphoric acid, 50 μM mercury(II)chloride.

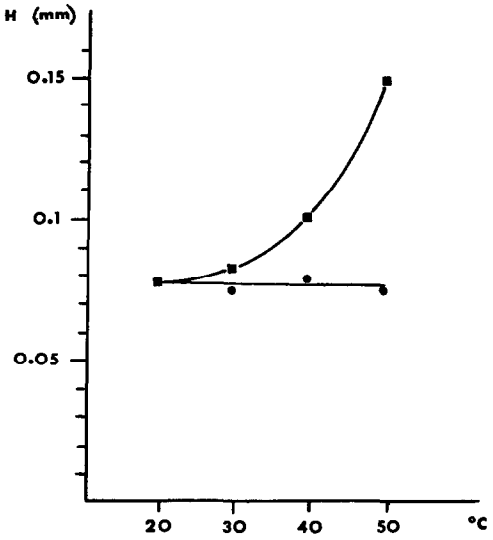

Figure 9
Influence of the column temperature on the column efficiency. Column: See Fig. 8. Mobile phase: See Fig. 5. Sample: injected amount of Pc-GAMM corresponding to 0.02 μg Pc-G. Heating designs: ■ = only the column; ● = the column, inlet capillary and loop thermostated (see text).

increased with elevated temperature when the column including the ¼″ end fittings was thermostated. However, if the capillary and the low dead volume connection between the injector and the column were thermostated by a longer water jacket and parts of the loop and the injector were surrounded by the warm outlet tube from the jacket the plate height was not affected by elevated temperature. Hence, it is necessary to avoid the temperature gradient developed when a colder eluent flow enters a warmer column. The retention times for all retained peaks are reduced by elevated temperature. The k' of Pc-GAMM decreased from 8.2 to 3.4 when the column temperature was increased from 20 to 50°C. In comparison with decreasing the k' by a higher organic modifier content of the mobile phase, the elevated column temperature also decreased the back pressure over the column from 380 to 210 kg cm^{-2} in the range 20–50°C. The peak areas were constant at all temperatures which indicate that no degradation of the sample occurred.

The nature of the eluted peak

The fact that both imidazole and mercury(II)chloride must be present during the whole analysis may be somewhat confusing when considering the reaction scheme (Fig. 4).

By analysing the Pc-GAMM derivative of tritium labelled (phenyl-4[n]-^{3}H)Pc-G according to the described method and then measuring radiometrically the collected peak from the column, it was clear that most of the radioactivity was recovered with the derivative peak. The injection of the same amount of underivatisated [^{3}H]Pc-G in the chromatographic system B showed that 86% of the activity was recovered in the Pc-GAMM peak compared to the mother substance. This shows a high degree of reaction of Pc-GAMM, which is in agreement with other results [4].

Provided that the Pc-GAMM formation is complete it is possible to calculate the molar absorbance, ε, from Fig. 3b by the Lambert–Beer's law. The ε for Pc-GAMM, dissolved in the mobile phase used in the bioanalysis, is then 20,900 (1 mol^{-1} cm^{-1}). In order to check if this absorbance originates from discrete molecules that elute in the chromatographic system used, an equation giving the relationship between the amount of sample injected (m) and the maximum concentration in the eluted peak (C_{max}) for Gaussian peaks in LC chromatograms [21] can be used:

$$m = \frac{C_{max} \times \sqrt{2\pi} \times V_R}{\sqrt{N}}, \tag{2}$$

where V_R is the retention volume for the peak and N is the number of plates of the column.

Combination of equation 2 and the Lamberg–Beer law gives:

$$m = \frac{\frac{A_{max}}{\varepsilon} \sqrt{2\pi} \times V_R}{\sqrt{N}}. \tag{3}$$

Equation 3, applied to a chromatogram of Pc-GAMM, gives an ε for the substance in the eluted peak of 19,700 (1 mol^{-1} cm^{-1}) corresponding rather well to earlier determined molar absorptivity for Pc-GAMM.

Conclusions

The partition of benzylpenicillin in aqueous organic systems at different pH and buffer compositions was studied as well as the stability of the drug at different pH values. Furthermore a pre-column derivatisation method for the detection of benzylpenicillin by reversed-phase LC at a detector wavelength of 325 nm was developed and optimised. The results obtained will be the basis for the development of very sensitive bioanalyses of benzylpenicillin.

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