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SIMULTANEOUS DETERMINATION OF THE CATIONIC AND ANIONIC PARTS IN REPOSITORY PENICILLINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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SUMMARY

High-performance liquid chromatographic separations of repository penicillins (hardly soluble salts of organic bases with penicillins) described previously permit only the determination of the penicillin part; the identification or quantitation of the cationic part was possible only by additional analysis. The optimization of the pH value of the mobile phase succeeded in giving a simultaneous determination of the cationic and anionic parts under isocratic reversed-phase conditions.

The elution characteristics of the basic components benzathine, procaine and clemizole depend strongly on the pH of the mobile phase. In mixtures of phosphate buffer and methanol the retention time increases rapidly with increasing pH, whereas the retention time of the penicillin part is only slightly influenced. For the analysis of benzathine penicillins a pH of 5.0–5.5 can be recommended, for procaine penicillin G pH 7.3–7.5 and for clemizole penicillin G pH 2.5–3.0. For all components linear calibration graphs were obtained over the concentration range 0.1–1 mg/ml (injection volume 20 μl). The relative standard deviation, depending on the component and separation system, was 0.5–2%. The separations can be performed within a few minutes at 50°C.

INTRODUCTION

Different methods for the analysis of repository penicillins are described in the various pharmacopoeias. The most important chemical procedure for the quantitative determination of penicillin is still iodimetric titration, which can be traced back to Alcino in 1946[1]. The iodimetric titration is included in the Code of Federal Register (FDA) for all penicillins. A few years ago Karlsberg and Forsman described mercury titration[2], which is included in the draft of the next edition of the European Pharmacopoeia for some penicillins. The cationic component in repository penicillins (benzathine, procaine and clemizole) can be determined be separate chemical analysis. Thus the British Pharmacopoeia describes a titrimetric assay after extraction for

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benzathine[3] as well as for procaine[4]. For procaine different colorimetric methods are also possible[5–7].

Separations of repository penicillins can be performed by thin-layer chromatography (TLC). Fooks and Mattok[8] succeeded in separating free procaine, penicillin G and procaine penicillin G on silica gel plates. The separation system is particularly suitable for tests for identity and purity. Wilson *et al.*[9] described an analogous test method for benzathine penicillin G. As has been demonstrated recently by Nachtmann and co-workers, the high-performance liquid chromatographic (HPLC) determination of penicillin G and V on reversed phases is superior to chemical methods with regard to selectivity and thus to accuracy. The selectivity of HPLC can be explained by the fact that all by-products and degradation products of penicillin are separated[10–12]. For this reason it was obvious to apply HPLC to the analysis of repository penicillins. As the basic cationic part of these compounds also shows UV absorption, it should be possible under appropriate conditions to determine the cationic and anionic component at the same time. The analysis of oral benzathine penicillin V suspensions by HPLC was described by Lebelle *et al.*[13]. According to this method, only the penicillin component can be determined. Tsuji *et al.*[14] succeeded in carrying out a simultaneous determination of procaine and penicillin G. Problems occurred, however, with regard to the reproducibility of separation.

This paper describes the simultaneous determination of the basic and acidic component in benzathine, procaine and clemizole salts of penicillin G and V.

EXPERIMENTAL

The penicillin salts tested, benzathine benzylpenicillin (DBED-G), benzathine phenoxymethylpenicillin (DBED-V), procaine benzylpenicillin (Proc-G) and clemizole benzylpenicillin (Clemizole-G), were manufactured by Biochemie GmbH (Kundl, Austria). All solvents used were of analytical-reagent grade (E. Merck, Darmstadt, G.F.R.). LiChrosorb RP-8 (Merck) was used as the stationary phase for the HPLC separations. This material, particle size 10 μm, was packed in stainless-steel columns (250 × 3.2 mm I.D.) using a slurry technique[15].

Apparatus

For the chromatographic separations an Orlita AE 10-4.4 pump and an Altex 110 A pump in combination with a Rheodyne 7120 injection valve (20 μl) were used. The detection was carried out in the UV region (215–220 nm) with a Schoeffel SF 770 or Perkin-Elmer LC 55 spectrophotometer. The separation column was placed in a thermostated water-bath. All determinations were carried out under isocratic conditions. A Hewlett-Packard 3353 data system was used for integration of the peak areas.

Preparation of samples

The dissolved penicillin salts can be injected into the column without any further preparation. A solution of 1 mg of sample per millilitre of solvent proved suitable for all salts tested. The solvent was composed of phosphate buffer (pH 7.0) (1/15 *M*)–methanol (3:7).

RESULTS AND DISCUSSION

The low solubility of the repository penicillins examined causes some problems. Methanol is certainly an excellent solvent, but it causes rapid degradation of penicillin and the corresponding alkyl-α-D-penicilloic acid is produced[8]. Our own experiments confirmed the instability of penicillin G and V in methanol.

The reaction can be slowed down by adding water to methanol. A compromise could be attained with the mixture of phosphate buffer (pH = 7.0) (1/15 *M*)–methanol (3:7); 1 mg of the salts examined can be dissolved in 1 ml of this mixture without difficulty.

In order to achieve a simultaneous rapid determination of the cationic (basic) and the anionic (acidic) components of repository penicillins it is necessary to find separation conditions that guarantee optimal resolution of the components. The reversed-phase systems hitherto described for penicillins, mostly utilizing pH values of 6.0–7.0[10–12], do not permit such a separation. Elution of the cationic components of the salts cannot be achieved. For this reason, experiments were carried out in order to reach the desired objective by varying the pH of the mobile phase.

In order to facilitate routine analyses the same type of column (250 × 3.2 mm I.D.) was always used. As earlier experiments carried out by Nachtmann and coworkers have shown, LiChrosorb RP-8 is an excellent stationary phase for the separation of penicillins[10,11] and it is advantageous to carry out the separation at high temperatures. An increase in temperature from 20 to 50°C will shorten the analysis time and reduce the back-pressure of the column if the flow-rate is kept constant. At the same time, the plate number of the column is slightly increased. Therefore, all tests described in this paper were carried out at 50°C. For quantitative determinations an external standard was used. Reproducible elution times for all penicillin salts examined were obtained only after conditioning of the column with the sample. For this purpose it was sufficient to inject the sample 5–10 times prior to analysis. Experiments with different batches of the LiChrosorb RP-8 stationary phase showed differences in the elution characteristics described in below by up to one pH unit.

Benzathine penicillins

The most important types are benzathine benzylpenicillin (DBED-G) and benzathine phenoxymethylpenicillin (DBED-V). In penicillin V *p*-hydroxypenicillin V is always present as a byproduct. The elution behaviour of these penicillin salts was examined on a C-8 column (Table I). With constant polarity of the mobile phase the elution behaviour of benzathine depends strongly on pH. In the interesting range from pH 4.0 to 5.5 the elution time and the asymmetry of the peak of benzathine increase with increasing pH. By adding 0.1 % of triethylamine to the mobile phase the peak symmetry could be improved considerable but the retention times remained unchanged.

Within the range examined the elution time of penicillin G depends only slightly on pH. The elution characteristics of penicillin V, on the other hand, differ from those of benzathine. The separation selectivity in the range examined is hardly influenced by a change in the ratio of phosphate buffer to methanol. For the determination of DBED-G the separation system at pH 4.0 could be regarded as most favourable with respect to the time of analysis and separation. Under these con-

TABLE I

ELUTION BEHAVIOUR OF BENZATHINE PENICILLINS

Stationary phase: LiChrosorb RP-8, 10 μm. Mobile phase: phosphate buffer (1/15 *M*)–methanol. Flow-rate: 2.0 ml/min. Temperature: 50°C. Wavelength: 215 nm.

Mobile phase	*Retention time (min)*			
	Benzathine	*Penicillin G*	*Penicillin V*	*p-Hydroxypenicillin V*
Buffer* (pH = 5.5)–methanol (1:1)	1.8		1.5	0.9
Buffer (pH = 5.5)–methanol (6:4)	2.8		2.6	1.2
Buffer (pH = 5.5)–methanol (7:3)	5.5	3.7	4.3	1.4
Buffer (pH = 5.0)–methanol (7:3)	4.3	3.8	6.7	2.9
Buffer (pH = 4.5)–methanol (7:3)	2.9	4.0	7.0	1.8
Buffer (pH = 4.0)–methanol (7:3)	1.8	4.1		

* Phosphate buffer (1/15 *M*).

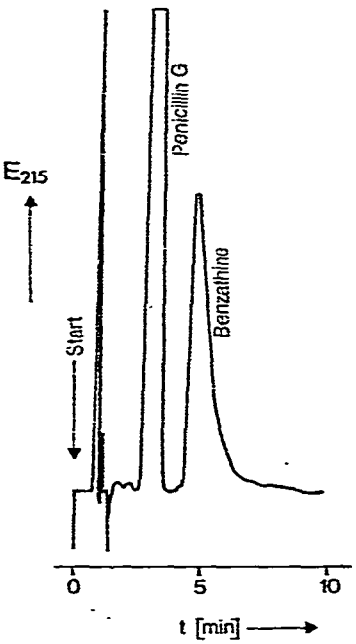


Fig. 1. Determination of benzathine penicillin G by HPLC. Stationary phase: LiChrosorb RP-8, 10 μm. Mobile phase: phosphate buffer (1/15 *M*) + 0.1% triethylamine (pH = 5.5)–methanol (7:3). Flow-rate: 2.4 ml/min. Temperature: 50°C.

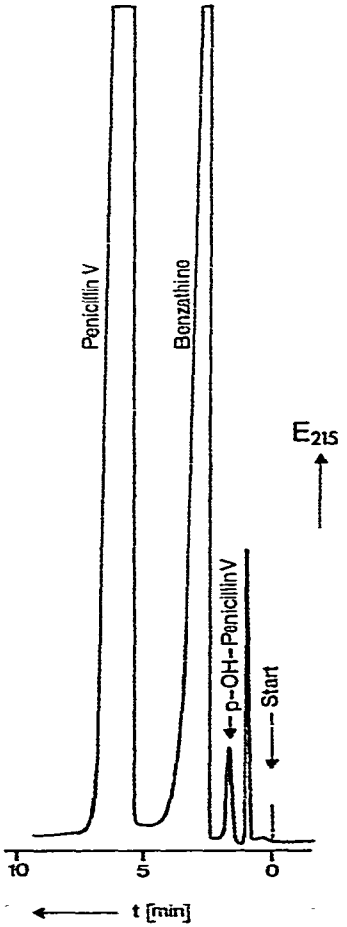


Fig. 2. Determination of benzathine penicillin V by HPLC. Stationary Phase: LiChrosorb RP-8, 10 μm. Mobile phase: phosphate buffer (1/15 *M*) (pH = 4.5)–methanol (7:3). Flow-rate: 1.8 ml/min. Temperature: 50°C.

ditions, however, any degradation products of penicillin present, such as penicilloic acid, are not separated from the benzathine peak. Assuming that such impurities could be present the chromatography has to be carried out at pH 5.5 (Fig. 1). These separation conditions imply an increase in the time of analysis and, in spite of the admixture of triethylamine, asymmetry of the benzathine peak, but, on the other hand, the selectivity of the separation system is improved. All known degradation products of penicillin are eluted before the peak of penicillin G and thus do not interfere in the analysis.

For DBED-V the separation system at pH 4.5 has to be regarded as the most suitable mobile phase. The *p*-hydroxy penicillin V occurring as a small impurity is eluted before the two main components and thus a sensitive detection of this by-product is possible. There is a baseline separation for all three eluted peaks. Fig. 2 shows the corresponding chromatogram obtained for a commercial product of DBED-V. A wavelength of 215 nm was chosen for detection as it represents a favourable compromise for all components of interest. For benzathine as well as for the penicillins linear calibration graphs passing through the origin were found for solutions of 0.1–1.0 mg/ml of sample. The method is sufficiently precise for routine tests. In the above range of concentrations the relative standard deviation ($n = 7$) was 0.8% (conditions as in Fig. 1) and 0.5% (conditions as in Fig. 2) for benzathine, 1.3% for penicillin G and 1.7% for penicillin V.

The following substances are suitable as external standards: benzathine acetate, sodium penicillin G, potassium penicillin V and sodium *p*-hydroxypenicillin V. The substances used had a purity of greater than 99%.

The method described was used to examine the stoichiometric composition of the above-mentioned penicillins. Table II shows the results for several batches of DBED-G. When considering Table II one has to take into account that theoretically DBED-G is composed of 68.16% of penicillin G, 24.50% of benzathine and 7.34% of water. An experimental determination of the water content was not carried out. The results show that the stoichiometric composition of the batches examined was confirmed in all instances. The differing absolute contents, however, suggest a varying degree of purity of the samples. Analogous results were obtained for DBED-V.

Procaine penicillin G and clemizole penicillin G

Further important salts of penicillin G with depot character are procaine penicillin G and clemizole penicillin G. As a similar dependence of the elution of the cationic component on the pH of the mobile phase was assumed, a pH profile analogous to that for the benzathine salts was investigated. The results are summarized in Table III. The situation is similar to that with the benzathine salts. The elution times for procaine and clemizole increase with increasing pH, whereas the elution time for penicillin G is only slightly changed. Procaine penicillin G can be analysed in both the acidic and neutral pH ranges. For a phosphate buffer–methanol (7:3) mixture at pH 6.0 procaine is eluted before penicillin G, which is in accordance with the results obtained by Tsuji *et al.*[14]. In the alkaline range, however, the order of elution is reversed. If detection of the degradation products of penicillin is desired (stability tests) a pH range of 7.3–7.5 is preferable. Under these conditions the degradation products of penicillin elute before penicillin G and can be identified in the chromatogram. Such a determination is shown in Fig. 3. At a wavelength of 220 nm linear

TABLE II

SIMULTANEOUS DETERMINATION OF THE CATIONIC AND ANIONIC COMPONENTS OF BENZATHINE BENZYLPENICILLIN BY HPLC IN DIFFERENT COMMERCIAL PRODUCTS

Stationary phase: LiChrosorb RP-8, 10 μm. Mobile phase: phosphate buffer (pH = 5.5) (1/15 *M*)–methanol (7:3). Temperature: 50°C. Wavelength, 215 nm.

Product No.	*Content (%)*		*Stoichiometric ratio*
	Penicillin G	*Benzathine*	
1	68.6	24.3	2.03
2	63.9	22.7	2.02
3	69.3	24.1	2.07
4	67.1	24.0	2.01
5	65.5	23.8	1.98
6	69.2	23.6	2.11
7	66.8	23.1	2.08
8	68.9	24.0	2.06
9	62.5	21.7	2.07
10	69.4	23.8	2.10
11	67.9	24.4	2.00
12	66.1	24.4	1.95

calibration graphs were found for both components in the range 0.05–1 mg/ml of sample. At a concentration of 0.5 mg/ml the relative standard deviation ($n = 7$) was 1.2% for penicillin G and 2.0% for procaine. The low precision for the determination of the procaine portion is due to problems of integration caused by peak tailing.

For clemizole penicillin G the elution power of the mobile phase has to be increased by increasing the concentration of the methanol component. Moreover, the analysis can only be carried out in the acidic pH range from 2.5 to 3.0. Owing to the

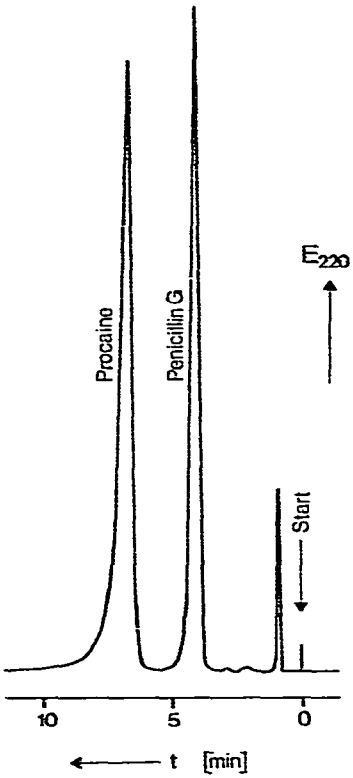
TABLE III

ELUTION BEHAVIOUR OF PROCAINE PENICILLIN G AND CLEMIZOLE PENICILLIN G

Stationary phase: LiChrosorb RP-8, 10 μm. Mobile phase: phosphate buffer (1/15 *M*)–methanol. Flow-rate: 1.8 ml/min. Temperature: 50°C. Wavelength: 220 nm.

Mobile phase	*Retention time (min)*		
	Procaine	*Clemizole*	*Penicillin G*
Buffer* (pH = 4.0)–methanol (7:3)	1.1	17.6	4.7
Buffer (pH = 5.0)–methanol (7:3)	1.3	26.0	4.4
Buffer (pH = 6.0)–methanol (7:3)	1.5		3.0
Buffer (pH = 7.0)–methanol (7:3)	4.2		3.7
Buffer (pH = 7.3)–methanol (7:3)	7.1		4.4
Buffer (pH = 7.5)–methanol (7:3)	8.0		4.0
Buffer (pH = 2.5)–methanol (6:4)		4.7	2.2
Buffer (pH = 3.0)–methanol (6:4)		5.2	1.7

* Phosphate buffer (1/15 *M*).



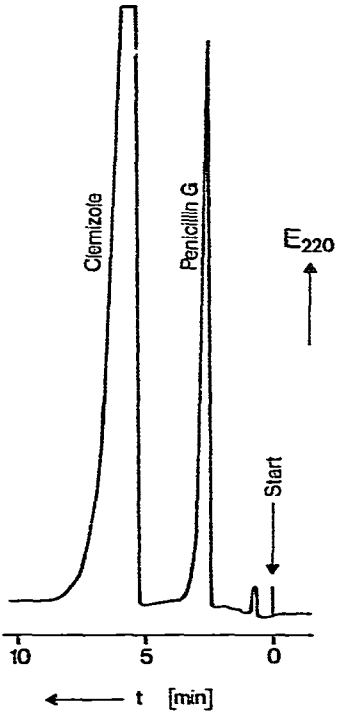


Fig. 3. Determination of procaine penicillin G by HPLC. Stationary phase: LiChrosorb RP-8, 10 μm. Mobile phase: phosphate buffer (1/15 *M*) (pH = 7.3)–methanol (7:3). Flow-rate: 1.9 ml/min. Temperature: 50°C.

Fig. 4. Determination of clemizole penicillin G by HPLC. Stationary phase: LiChrosorb RP-8, 10 μm. Mobile phase: phosphate buffer (1/15 *M*) (pH = 2.5)–methanol (6:4). Flow-rate: 2.1 ml/min. Temperature: 50°C.

rapid chromatographic separation no degratation reaction of penicillin G could be observed. Fig. 4 shows a typical chromatogram for clemizole penicillin G. As with procaine penicillin G, linear calibration graphs were obtained for clemizole and penicillin G at 220 nm in the range of 0.05–1 mg/ml of sample. The relative standard deviation ($n = 7$) was 0.5% for penicillin G and 0.7% for clemizole. The concentration was 0.5 mg/ml. The fact that the values are superior to those of procaine penicillin G can be explained by the more favourable peak symmetry.

CONCLUSIONS

The HPLC methods described permit the simultaneous quantitative determination of the cationic and anionic components of repository penicillins within a few minutes. They are superiour to all hitherto described procedures for this group of substances. Their application is not limited to the quality control of the pure penicillin salts. The analytical characterization of mixtures of repository penicillins with soluble penicillins or mixtures of different repository penicillins is also possible.

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