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Note

Separation of penicillin and cephalosporin diastereoisomers by reversed-phase high-performance liquid chromatography

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Penicillins and cephalosporins are produced by the reaction of the appropriate acylating agent with 6-aminopenicillanic acid, 7-aminodeacetoxycephalosporanic or 7-aminocephalosporanic acid, which then form the characteristic side-chain of each penicillin or cephalosporin. When the acylating agent is a mixture of optical isomers, two different diastereosiomeric penicillins or cephalosporins are obtained. The biological properties of the penicillin and cephalosporin epimers are very different, *e.g.*, D(—)-α-aminobenzylpenicillin (ampicillin), D(—)-α-aminobenzyldeacetoxycephalosporin (cephalexin) and D(—)-α-aminobenzylcephalosporin (cephaloglycin) are far more active than their corresponding L-isomers. With α-phenoxyethylpenicillin, the D-isomer is more active than the L-isomer, but when the two isomers are mixed a synergistic effect occurs and the biological activity of the mixture is similar to that of the more active isomer.

In recent years, much work has been published on the high-performance liquid chromatographic (HPLC) separation of cephalosporins and penicillins and their decomposition products[1]. Special mention should be made of reversed-phase HPLC[2–4], excellent separations having been obtained thereby. However, the literature has not revealed any work on the separation of the diastereoisomers of penicillins or cephalosporins.

On the other hand, separations of racemic mixtures of amino acids by gas chromatography have been described, but not a method suitable for the resolution of racemic mixtures by HPLC. However, the separation by HPLC of the mixture of L- and D-amino acids as diastereoisomeric derivatives using Micropak Si-5 and in isooctane 1.5% isopropanol as the mobile phase has been described[5].

This paper describes the development of a method for separating diastereoisomers of penicillins and cephalosporins by reversed-phase HPLC.

MATERIAL AND METHODS

Reagents

Sodium ampicillin, potassium phenoxyethylpenicillin and cephalexin were supplied by Antibióticos, S.A. (Madrid, Spain).

L(+)-α-Aminophenylacetyl chloride hydrochloride was obtained by the

method of Hardcastle *et al.*[6] for the D-isomer. From this compound L-ampicillin and L-cephalexin were prepared according to published procedures[7,8].

The diastereoisomers of the α-phenoxyethylpenicillin were prepared according to techniques described by Perron *et al.*[9].

To prepare the antibiotic solutions, distilled water was used with the soluble penicillins and cephalosporins and 0.5 *M* sodium hydrogen carbonate solution with the insoluble compounds.

Methanol, orthophosphoric acid, monosodium phosphate, disodium phosphate and all other reagents were of maximum purity and supplied by Merck (Darmstadt, G.F.R.).

Apparatus

All chromatograms were obtained using a Waters M-6000 pump equipped with a Type U6K injector (Waters Assoc.), an Altex 254-nm UV detector and a Hewlett-Packard recorder. The samples were injected with 10-μl syringes (C-160 series, Precision Sampling Co., Baton Rouge, La., U.S.A.). All separations were carried out at room temperature.

The column of μBondapack C_{18} was obtained pre-packed from Waters Assoc.

Chromatographic procedure

The mobile phase consisted of mixtures of appropriate buffers and methanol in various proportions. The buffers used were 0.1 *M* phosphates.

In all instances an injection volume of 10 μl, a flow-rate of 1.5 ml/min and a sensitivity of 0.04 a.u.f.s. were used. All separations were carried out at room temperature.

Determination in serum

A 5-ml volume of rabbit serum was mixed with 1 ml of antibiotic solution sufficiently concentrated to give the required final concentration, then 4 ml of a 7.5% aqueous solution of trichloroacetic acid was added and the mixture was stirred and kept in ice for 15 min. It was then centrifuged at 400 *g* for 10 min. The supernatant was removed and kept at 0° until an aliquot of 90 μl was taken for injection into the chromatograph.

RESULTS

Although penicillins and cephalosporins are ionic compounds that can be separated by ion-exchange chromatography[1], better results are obtained with reversed-phase chromatography[2–4]. We already know that the retention time of penicillins and deacetoxycephalosporins in reversed-phase chromatography depends on the amount of methanol in the mobile phase, as well as on its salt concentration[10]. Variations of the retention times of ampicillin diastereoisomers with pH and for different concentrations of methanol in the mobile phase are shown in Fig. 1. It can be seen that the curves are concave with minima at pH 4–5. As the methanol content of the mobile phase is increased, the curves become flatter and the influence of pH decreases.

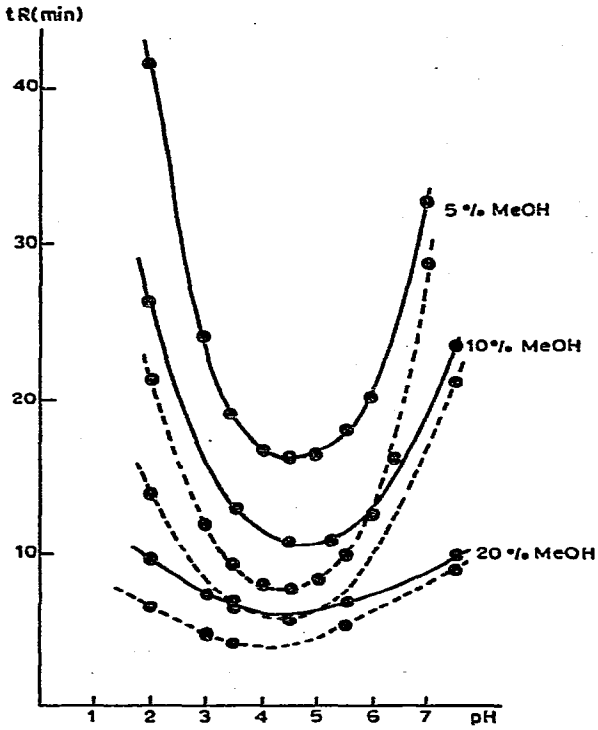

Fig. 1. Variation of retention time with pH and the percentage of methanol in the mobile phase for D-ampicillin (solid lines) and L-ampicillin (broken lines).

We found that the retention times of cephalexin diastereoisomers varied with the pH of the mobile phase in a manner similar to those of ampicillin diastereoisomers, minima also occurring at pH 4–5.

The retention times of the diastereoisomers of α-phenoxyethylpenicillin vary with the pH of the mobile phase in a manner different from those of the diastereoisomers of ampicillin and cephalexin (Fig. 2). It can be seen that the retention times decrease as the pH increases, the slopes of the curves decreasing as the methanol content increases.

Quantitative determination

Apart from an adequate selectivity, quantitative determinations of penicillins and cephalosporins in pharmaceutical raw materials, commercial products and biological materials require low variation coefficients and linearity of the detector response over a wide range. Considering as an example the diastereoisomers of ampicillin and cephalexin, we found that the detector signal in the range 0.1–1 mg/ml was linear, the coefficients of variation being 1.3–1.1% ($n = 9$; without an internal standard). The detection limit was about 0.1 μg/ml.

This method can also be used for determining the diastereoisomers of penicillins and cephalosporins in biological materials. In Fig. 3 typical chromatograms are shown for samples prepared from rabbit serum containing 20 μg/ml of each diastereoisomer of cephalexin.

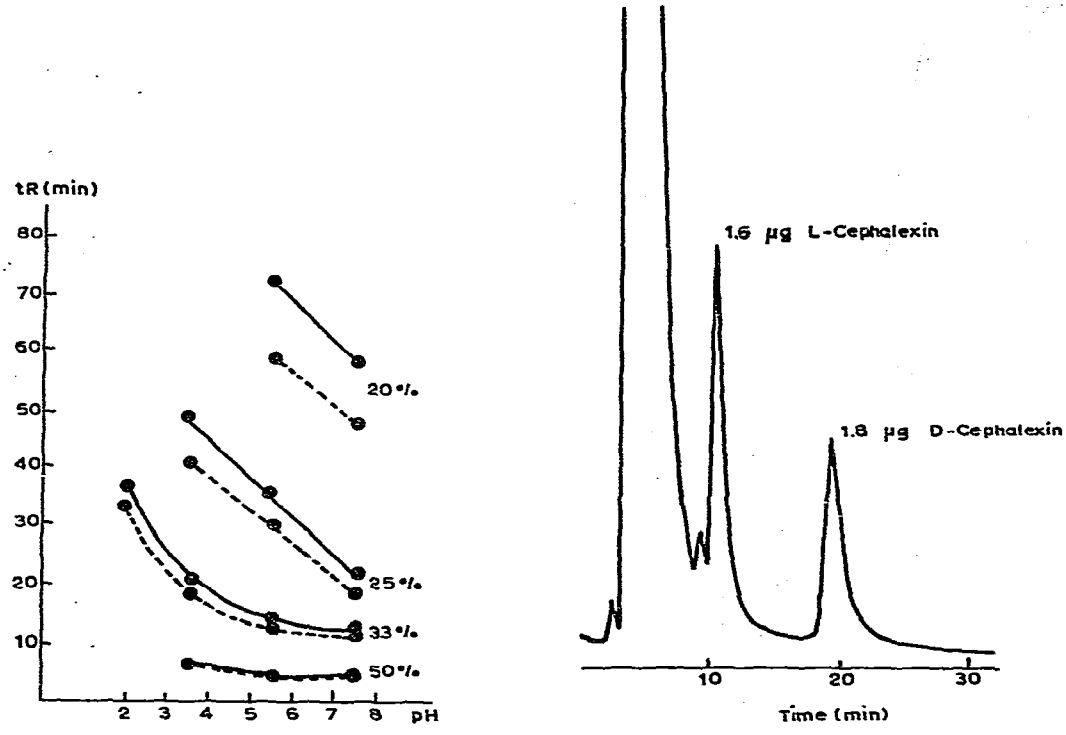

Fig. 2. Variation of retention time with pH and the percentage of methanol in the mobile phase for α-phenoxyethylpenicillin: solid lines, L-form; broken lines, D-form.

Fig. 3. HPLC of cephalexin diastereoisomers in rabbit serum. Column: 30 cm × 4 mm I.D. µBondapak C_{18}. Mobile phase: 0.1 *M* phosphate buffer (pH 3.5) containing 5% of methanol. Flow-rate: 1.5 ml/min. Sensitivity: 0.02 a.u.f.s.

DISCUSSION

Twitchet and Moffat[11] found that, in pharmaceutical products with acidic or basic functional groups, the retention volume was greatly dependent on the pH of the mobile phase. Ampicillin and cephalexin epimers are "amino acids" (p*K* about 2.5 and 7.2 for ampicillins and about 3 and 7.1 for cephalexins) whose isoelectric points coincide approximately with the minimum retention volumes. As the pH values diverge from the isoelectric point, both the contents of the anionic or cationic forms and the retention times increase. Phenoxyethylpenicillin diastereoisomers are only acidic in character, which explains why the graphs of retention times against pH have only a descending branch.

The chromatographic separations of mixtures of penicillin epimers can best be described by the resolution, R_s (Figs. 4 and 5). Fig. 4 shows the relative separations or resolution of a mixture of diastereoisomers of ampicillin with pH for various methanol contents in the mobile phase. A great influence of the pH of the mobile phase on R_s with a methanol content of 10% can be seen. For methanol contents of

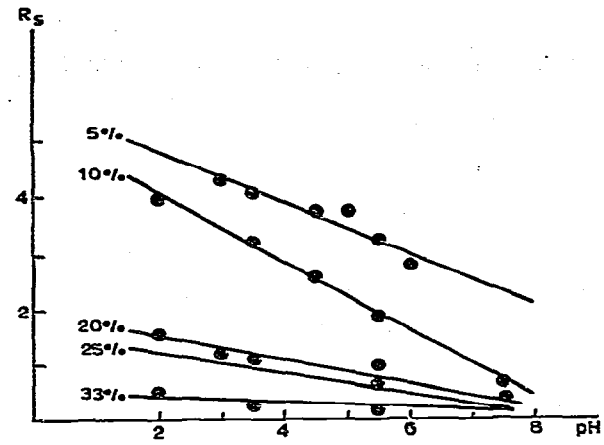

Fig. 4. Variation of the resolution (R_s) of ampicillin diastereoisomers with pH and the percentage of methanol in the mobile phase.

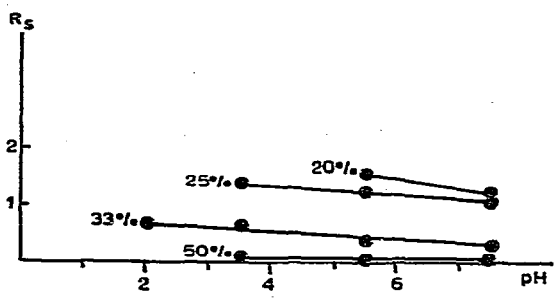

Fig. 5. Variation of the resolution (R_s) of α-phenoxyethylpenicillin diastereoisomers with pH and the percentage of methanol in the mobile phase.

greater than 10%, the influence of the pH is considerably less. The resolution may be expressed by the equation[12]

$$R_s = \frac{1}{4} \cdot \frac{\alpha - 1}{\alpha} \cdot \frac{\bar{k}}{\bar{k} + 1} \sqrt{N}$$

The three parameters in this equation can be considered to be mutually independent and can be evaluated separately in order to establish how each contributes to the variations in R_s. The selectivity coefficient (α) is determined by the distribution coefficient of each epimer. The capacity factor ($\bar{k}$) is determined by the arithmetical mean of the distribution coefficient of each epimer. The efficiency describes the dispersion behaviour of the separation column; an increase in the number of theoretical plates (N) leads only to a non-specific improvement in the resolution.

Table I gives values calculated for these parameters for ampicillin diastereoisomers at different pH values and concentrations of methanol in the mobile phase. It can be seen that the selectivity term $[(\alpha - 1)/\alpha]$ is maximal for zwitterionic forms, and is greater for cationic forms (low pH values) than for anionic forms (high pH values).

TABLE I

SELECTIVITY TERMS, CAPACITY TERMS AND RESOLUTIONS FOR AMPICILLIN DIASTEREOISOMERS

Percentage values are methanol contents of the mobile phase.

pH	$\frac{\alpha-1}{\alpha}$				$\frac{\bar{k}}{1+\bar{k}}$				R_s			
	5%	10%	20%	25%	5%	10%	20%	25%	5%	10%	20%	25%
2.0	0.51	0.50	0.54	0.39	0.94	0.90	0.53	0.42	4.03	4.0	1.56	1.25
3.0	0.61	—	0.67	—	0.79	—	0.43	—	4.4	—	1.15	—
3.5	0.67	0.68	0.66	0.79	0.71	0.60	0.31	0.28	4.1	3.2	1.17	0.96
4.0	0.72	—	—	—	0.63	—	—	—	4.0	—	—	—
4.5	0.71	0.71	—	—	0.63	0.53	—	—	3.8	2.6	—	—
5.0	0.64	—	—	—	0.67	—	—	—	3.8	—	—	—
5.5	0.56	0.55	0.52	0.50	0.72	0.55	0.37	0.22	3.3	1.9	1.05	0.72
6.0	0.44	—	—	—	0.77	—	—	—	2.7	—	—	—
7.0	0.14	—	—	—	0.89	—	—	—	1.0	—	—	—
7.5	—	0.12	0.14	0.12	—	0.84	0.59	0.40	—	0.72	0.35	0.17

The capacity term $[\bar{k}/(1 + \bar{k})]$, however, is minimal for the zwitterionic form and only slightly higher for cationic than for anionic forms. With 5% of methanol in the mobile phase content, the products of the capacity term and selectivity term are almost constant between pH 2 and 5. The resolution in this pH range is also almost constant. For pH $>$ 5 the decrease in the selectivity term is greater than the increase in the capacity term, and the product of the two decreases, as does the resolution. For each pH level, as the methanol content of the mobile phase is increased, the selectivity coefficient remains almost constant but the capacity factor decreases, thus explaining the decrease in resolution with increase in methanol content of the mobile phase.

With α-phenoxyethylpenicillin isomers (Table II), the selectivity coefficient is lower than for ampicillins and is almost constant, but it decreases as the methanol content is increased. The capacity factor, however, is considerably greater than for ampicillins and decreases slightly as the pH increases. Like the selectivity coefficient,

TABLE II

SELECTIVITY TERMS, CAPACITY TERMS AND RESOLUTIONS FOR α-PHENOXYMETHYLPENICILLIN DIASTEREOISOMERS

Percentage values are methanol contents of the mobile phase.

pH	$\frac{\alpha-1}{\alpha}$			$\frac{k}{1+\kappa}$			R_s		
	20%	25%	33%	20%	25%	33%	20	25%	33%
2.0	—	—	0.12	—	—	0.92	—	—	0.72
3.5	—	0.17	0.13	—	0.91	0.81	—	1.4	0.68
5.5	0.19	0.17	0.12	0.93	0.88	0.73	1.6	1.3	0.48
7.5	0.19	0.17	0.11	0.92	0.81	0.70	1.27	1.2	0.35

the capacity factor decreases as the methanol concentration in the mobile phase is increased, which explains the decrease in resolution as the pH and methanol content increase.

From the above, it can be deduced that with the use of μBondapak C_{18} and the mobile phases described it is possible to separate the diastereoisomers of penicillins and deacetoxycephalosporins in both liquid pharmaceutical and biological forms. For the separation of phenoxyethylpenicillin epimers, the results would probably be improved with the use of a stationary phase, also a reversed phase but with greater selectivity.

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