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COMPARISON OF BONDED, POLYMERIC AND SILICA COLUMNS FOR CHROMATOGRAPHY OF SOME PENICILLINS

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SUMMARY

The retention of ampicillin, amoxicillin, and penicillin G was determined on ODS and cyanopropyl (CN)-bonded to silica, on an all-organic polymeric reversed-phase column (styrene–divinylbenzene) and on bare silica. Solvents tested were 0.01 *M* H_3PO_4 (pH 1.6)–acetonitrile and 0.002 *M* KH_2PO_4 (pH 4.6)–acetonitrile in the proportions 100:0 to 10:90. Retention with 0.002 *M* KH_2PO_4 was weaker than with 0.01 *M* H_3PO_4 at all proportions of acetonitrile on all packings. With 0.01 *M* H_3PO_4, retention of penicillins on the polymeric column decreased rapidly from 100 to 50% aqueous phase and remained low to 10% aqueous phase. On the ODS bonded column, retention was similar, except that retention of the aminopenicillins, amoxicillin and ampicillin increased at $<30\%$ aqueous phase. On the CN-bonded column, results were similar to those on the ODS column, except that the aminopencillins were less strongly retained when using 100% 0.01 *M* H_3PO_4. On the bare silica, penicillin G was poorly retained at all proportions of acetonitrile, while retention of aminopenicillins increased rapidly at $<50\%$ 0.01 *M* H_3PO_4. Retention of aminopenicillins at $<50\%$ 0.01 *M* H_3PO_4 was parallel on bonded and bare silica but weaker on the bonded silica. A bonded organic layer weakened but did not prevent binding of amines to silica.

INTRODUCTION

It is well known that interactions with the silica support may affect chromatography of basic compounds on reversed-phase packings in which an organic layer is bonded to silica[1–7]. So-called "end capping" procedures appear to be only partially successful in blocking this effect[1–5].

Binding to silica may cause broadening or distortion of chromatographic peaks of basic compounds[1–7]. As the proportion of water in the solvent is decreased to low levels, interaction with the silica may become the dominant effect on the bonded columns and basic compounds show normal-phase behavior[2–4]. Thus, a plot of retention *vs.* fraction of water on a bonded reversed-phase column typically shows a U-shaped curve with basic compounds[2,4]. Interaction with silica may be detrimental when it causes peak distortion but may also improve separations[1,4,8]. Tylosin was

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separated from interferences in biological materials by normal-phase chromatography at low water concentrations on a bonded reversed-phase column[8].

In a previous study of chromatography of penicillins, it was shown that, on a bonded ODS column, compounds with a basic function give broader chromatographic peaks than those with only carboxylic acid functions. This interference could be overcome by adding a silanol blocking agent to the mobile phase or by using an all-organic polymeric packing[1]. The present study was undertaken to determine the effect of pH and low water concentrations on chromatographic behavior of the penicillins.

EXPERIMENTAL

Chemicals

Acetonitrile and methanol were of HPLC grade from several sources; other chemicals were of reagent grade.

Antibiotic standards

Penicillin G, ampicillin, and amoxicillin were purchased from Sigma (St. Louis, MO, U.S.A.). Stock solutions of 1 mg/ml were prepared weekly in water and stored under refrigeration. Working dilutions of 0.01 mg/ml were prepared in water or in 80–95% acetonitrile, as required.

Apparatus

A Varian Model 5000 liquid chromatograph with a UV-50 variable-wavelength detector set at 220 nm, and a Valco automatic loop injector with a 200-μl loop (Varian, Sunnyvale, CA, U.S.A.) were used.

Chromatographic columns

The analytical columns used were Varian Micropak Si-5, 5-μm silica, 250 × 4.0 mm I.D. and 150 × 4.0 mm I.D., Micropak MCH-5, 5-μm bonded ODS, 150 × 4.0 mm I.D. and Micropak CN-4, 4-μm bonded cyanopropyl, 250 × 4.0 mm I.D. (all from Varian), PLRP-S, 5-μm styrene–divinylbenzene copolymer, 150 × 4.6 mm I.D. (Polymer Labs., Amherst, MA, U.S.A.), and Supelcosil LC-18-DB, 5-μm bonded ODS, 150 × 4.6 mm I.D. (Supelco, Bellefonte, PA, U.S.A.). Respective guard columns used were: for Si-5 none, for MCH-5, a MCH-10 cartridge, for CN-4, a CN-10 cartridge, for PLRP-S none, for Supelcosil LC-18-DB a LC-18-DB guard cartridge.

Operating conditions

Standard solutions of penicillins, containing 2 μg/200 μl, were injected in water or 80–95% acetonitrile, depending on which was judged to have the least eluting power under the conditions used. In case of doubt, injections were made in both solvents. Operation was isocratic with the solvents indicated. Flow-rates were 1 ml/min. Column dead-volume was judged from the shortest time following injection at which a discernible change in the baseline was observed.

RESULTS AND DISCUSSION

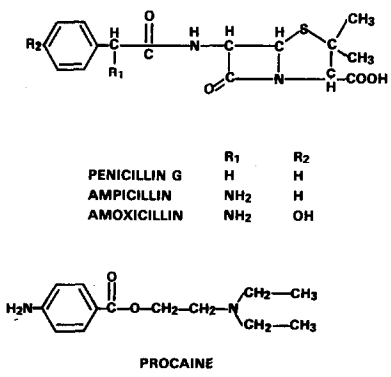
The aminopenicillins, ampicillin, and amoxicillin, are derived from penicillin G by addition of an amino and an amino and a *p*-hydroxy group, respectively, to the side chain (Fig. 1).

Retention on four types of packings was determined to evaluate the relative importance of the bonded organic layer and the silica in binding of these compounds to bonded reversed-phase columns at various proportions of organic solvent and water. The effect of the organic phase alone was evaluated using a styrene–divinylbenzene copolymer packing. Retentions on a more polar cyanopropyl layer, bonded to silica, and on bare silica were also determined. Determinations were made using both 0.01 *M* H_3PO_4 (pH 1.6) and 0.002 *M* KH_2PO_4 (pH 4.6) as the aqueous solvents.

Fig. 2 shows capacity factors of the three penicillins on a bonded ODS column (MCH-5) with 0.01 *M* H_3PO_4 (pH 1.6) at various proportions of acetonitrile. Under these conditions, ionization of the carboxyl groups (pK_a 2.7)[9] is suppressed and the aminopenicillins are present as the amine cation. Penicillin G is readily soluble in organic solvents at this pH[1,9] and, as would be expected, was more strongly retained on the reversed-phase column than the amphoteric compounds. Ampicillin and amoxicillin, although not readily soluble in organic solvents, were retained strongly with 0.01 *M* H_3PO_4. Retentions decreased rapidly with an increasing proportion of acetonitrile. With the aminopenicillins, retentions increased at >70% acetonitrile while retention of penicillin G did not.

Fig. 3 shows the same results on a Supelco LC-18-DB column. This packing was tested because the manufacturer reported that the packing was "specially deactivated" for use with basic drugs[10]. The results show that bonding of the aminopenicillins at low water concentrations was actually somewhat stronger than on the MCH-5 packing. Thus, the "deactivation" process used had little effect on the affinity of the packing for basic compounds.

Fig. 4 shows results with a polymeric (PLRP-S) column, which were similar



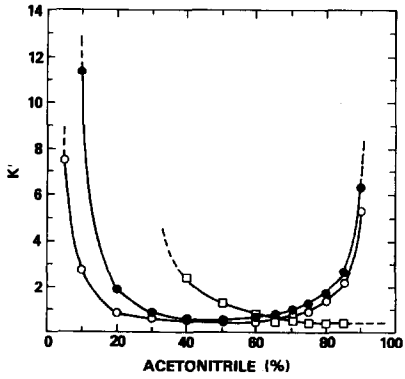


Fig. 1. Chemical structures of compounds studied.

Fig. 2. Effect of the proportion of acetonitrile in the mobile phase (0.01 *M* H_3PO_4) on retention factors of ampicillin (●—●), amoxicillin (○—○) and penicillin G (□—□) on a bonded ODS reversed-phase column (MCH-5).

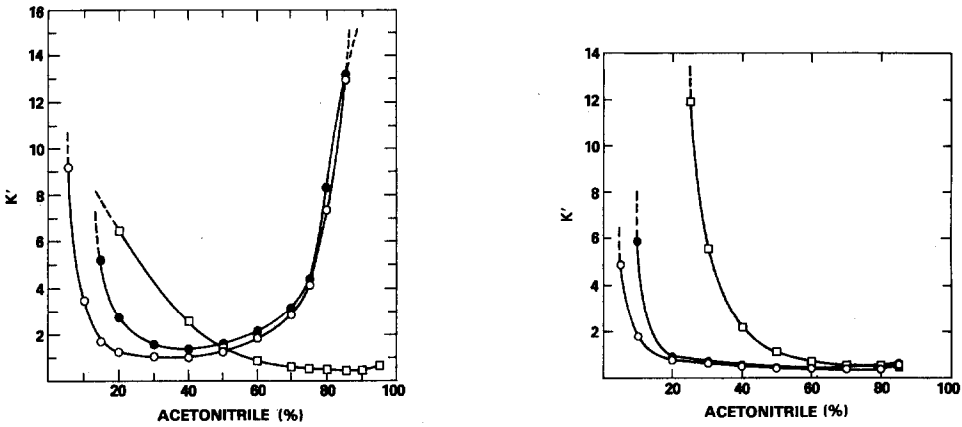


Fig. 3. Effect of the proportion of acetonitrile in the mobile phase (0.01 *M* H_3PO_4, pH 1.6) on retention factors of ampicillin, amoxicillin and penicillin G on a "specially deactivated" bonded ODS reversed-phase column (LC-18-DB). Symbols as in Fig. 2.

Fig. 4. Effect of the proportion of acetonitrile in the mobile phase (0.01 *M* H_3PO_4, pH 1.6) on retention of three penicillins on a polystyrene/divinylbenzene copolymer column (PLRP-S). Symbols as in Fig. 2.

to those on the bonded ODS columns, except that there was no increase in retention of the aminopenicillins at high acetonitrile concentrations.

Fig. 5 shows the results with CN (cyanopropyl) bonded to silica. This packing is considered more polar than bonded ODS. Results were similar to those on bonded ODS, except that reversed-phase binding of the aminopenicillins was weaker at 0–30% acetonitrile.

Fig. 6 shows results on bare silica. Binding of the aminopenicillins increased as acetonitrile concentration increased. The retention curves on the silica and the

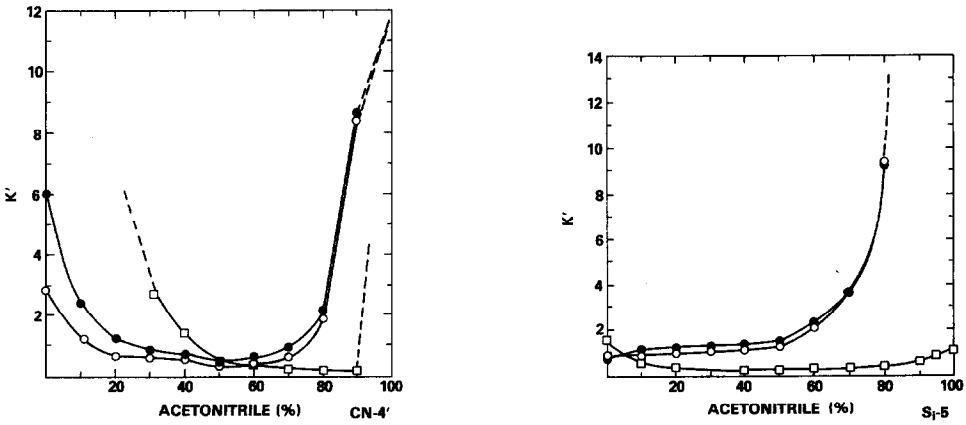
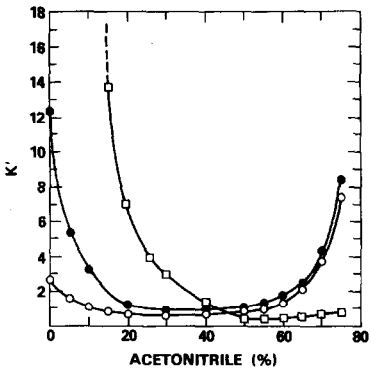


Fig. 5. Effect of the proportion of acetonitrile in the mobile phase (0.01 *M* H_3PO_4, pH 1.6) on retention of three penicillins on a cyanopropyl bonded reversed-phase column (CN-4). Symbols as in Fig. 2.

Fig. 6. Effect of the proportion of acetonitrile in the mobile phase (0.01 *M* H_3PO_4, pH 1.6) on retention of three penicillins on bare silica (Si-5). Standards in 80% acetonitrile. Symbols as in Fig. 2.



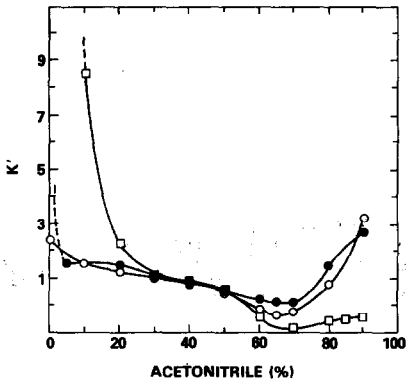


Fig. 7. Effect of acetonitrile and methanol (20%) in the mobile phase (0.01 *M* H_3PO_4, pH 1.6) on retention factors of three penicillins (2 μg of each penicillin injected) on a bonded reversed-phase column (MCH-5). Symbols as in Fig. 2.

Fig. 8. Effect of the proportion of acetonitrile in the mobile phase (0.002 *M* KH_2PO_4) on retention factors of three penicillins on a bonded ODS column (MCH-5). Symbols as in Fig. 2.

bonded silica were similar at > 50% acetonitrile, except that the same retention occurred at somewhat higher acetonitrile concentration on the bonded packings. Thus, binding of amines was weakened but not prevented by the presence of an organic coating on the silica surface. Because of steric factors, it is impossible to make all silanol groups in the silica support react during the manufacture of bonded packings[11,12].

Rather similar results were obtained when methanol was substituted for part of the acetonitrile on the bonded ODS (MCH-5) (Fig. 7). Methanol has somewhat less eluting power than acetonitrile with reversed-phase packings. Its effects were not fully investigated in the present study but it is possible that it could improve separations. However, penicillins can undergo methanolysis with prolonged exposure to methanol[9].

Retention on the MCH-5 column when 0.002 *M* KH_2PO_4 (pH 4.6) rather than 0.01 *M* H_3PO_4 (pH 1.6) was used, is shown in Fig. 8. This introduced several new effects: (a) a competing ion (K^+) and (b) an increase in pH, which increased the negative charge on the silica and which converted the carboxyl groups of the penicillins (pK_a 2.7) to the ionized form. As would be expected, binding to the organic layer at high water concentration was weakened for the ionized penicillins, which are also less soluble in organic solvents. Binding of the aminopenicillins to the silica at low water concentrations was also slightly weaker with 0.002 *M* KH_2PO_4. Binding of simple amines is increased at higher pH because of the increased negative charge on the silica surface[13,14] and, for this reason, many investigators have favored an alkaline pH for chromatography of basic compounds on silica[13–17]. On the other hand, a competing ion reduces retention[13] and the presence of an anion in the molecule interferes with binding to silica[18]. To sort out these effects, retention of a simple amine, procaine (from procaine penicillin G) and ampicillin were compared from various solvents on bare silica (Table I). Acetonitrile (80%) was used as the organic modifier. Retention of procaine and ampicillin from 0.01 *M* H_3PO_4 was quite similar.

TABLE I

EFFECT OF pH AND COMPETING IONS ON CAPACITY FACTORS (k') OF AMPICILLIN AND PROCAINE ON BARE SILICA (Micropak Si-5)

Mobile phase: acetonitrile-aqueous phase (80:20); isocratic elution.

Aqueous part of mobile phase	k'	
	Procaine	*Ampicillin*
0.01 *M* H_3PO_4 (pH 1.6)	4.52	4.08
0.002 *M* $NH_4H_2PO_4$ (pH 4.6)	15.26	5.39
0.01 *M* H_3PO_4 + 0.002 *M* KCl (pH 1.6)	3.39	3.02
0.002 *M* KH_2PO_4 (pH 4.6)	8.05	6.02

Retention was sharply decreased by addition of potassium ion (0.002 *M* KCl). Retention of procaine was much greater than that of ampicillin using either $NH_4H_2PO_4$ or KH_2PO_4, indicating that the anion in ampicillin offset increased binding of the amine cation to the silica when the molecule occurred as the zwitterion.

Westerlund *et al.*[19] found that k' for benzylpenicillin decreased sharply when the pH of the mobile phase was increased from 2 to 3.5 which represents the transition from the acid to the salt form. Amphoteric compounds showed a similar decrease in k' in this pH range but, unlike benzylpenicillin, retention increased above pH 4.5. This parallels the increased retention of amines on silica with increasing pH by other authors[13,14]. Above pH 7, the increase in retention leveled off for three amphoteric penicillins studied which had pK_2 values of 7.2–7.5 but continued to increase with mecillanam with a pK_2 of 8.9, indicating that increased retention was related to the presence of the cationic form of the amine as well as the charge on the silica. Further studies using polymeric and bare silica packings would be helpful to establish the effect of the silica on the retentions observed.

Binding of cations to silica occurs by ion-exchange, the electronegative surface being produced by ionization of silanols in water[3,13–18]. Binding of other compounds is by hydrogen bonding. Retention of penicillin G on the bonded columns under the conditions studied occurred purely by a reversed-phase mechanism, as it was not retained to any extent on bare silica even from 100% acetonitrile. A slight increase in retention on silica using 100% 0.01 *M* phosphoric acid indicates hydrophobic binding to the silica. With amoxicillin or ampicillin, which have amine functions, a dual retention mechanism was evident. Although binding to the organic layer was dominant at high water concentrations, the silica still affected both retention time and peak shape[1]. Similarly, at low water concentration where binding to silica was dominant, the organic layer still affected the relative retentions of amoxicillin and ampicillin. On bare silica, the retentions of amoxicillin and ampicillin were nearly identical, indicating that ion exchange was the dominant effect. A mathematical treatment for a dual retention mechanism was described by Nahum and Horváth[2].

Bare silica has been widely used for thin-layer chromatographic analysis of penicillins but its use for high-performance liquid chromatographic analysis of these compounds has not been reported[20]. However, several investigators have found that bare silica is highly satisfactory for chromatography of basic compounds[13–17], amox-

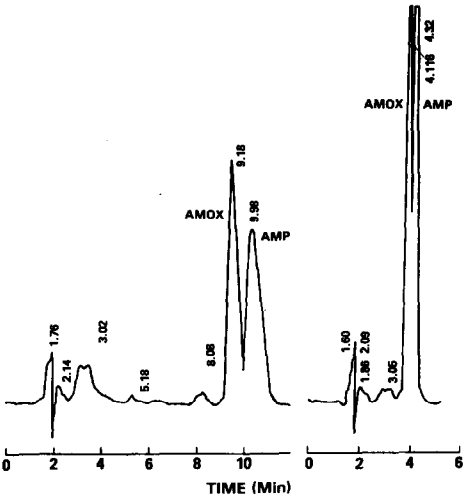


Fig. 9. Separation of ampicillin (AMP) and amoxicillin (AMOX) by normal-phase chromatography on a bonded ODS column. Mobile phase: (A) 0.01 *M* H_3PO_4–acetonitrile (20:80), and (B) as A, but containing 0.002 *M* KCl in the aqueous phase.

icillin and ampicillin also gave sharp peaks on bare silica with 0.01 *M* H_3PO_4–acetonitrile with *ca.* 20% aqueous phase but the two were not separated. They were somewhat separated on bonded ODS under the same conditions with slightly less aqueous phase (Fig. 9). However, peaks were not particularly sharp. Substituting part of the acetonitrile by methanol did not improve peak shape. However, addition of a competing ion (0.002 *M* K^+ from KCl to the aqueous phase markedly improved peak shape and also reduced retention time but did not improve resolution. The retention time could be increased by lowering the water concentration, but this did not improve resolution.

A calculation of the number of theoretical plates showed that the apparent increase in peak sharpness was not solely a result of decreased retention time. Ampicillin (1295 theoretical plates) showed more spreading than amoxicillin (2289 theoretical plates) in 0.01 *M* H_3PO_4. In the presence of 0.002 *M* KCl, the number of theoretical plates was increased to 2843 for ampicillin and 2796 for amoxicillin, values which are identical within the limits of measurement.

The presence of an organic surface layer bonded to silica will weaken but not prevent interactions between unreacted silanols and solutes with basic functions. At low water concentrations, normal-phase chromatography of basic compounds is possible on either bonded silica or bare silica. Peaks are sharper on bare silica. However, the bonded organic layer may aid in separations that are not possible on either bare silica or by means of a pure reversed-phase mechanism.

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