Journal of Chromatography, 366 (1986) 69–78
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 753

EFFECT OF THE SILICA SUPPORT OF BONDED REVERSED-PHASE COLUMNS ON CHROMATOGRAPHY OF SOME ANTIBIOTIC COMPOUNDS

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(First received December 30th, 1985; revised manuscript received April 22nd, 1986)

SUMMARY

Chromatographic behavior of three types of antibiotics was investigated on bonded C_{18} and polymeric reversed-phase columns. With penicillins with carboxylic acid functions only, retention and separations on the two types of columns were similar. β-Lactam antibiotics with basic functions did not give as sharp peaks on the C_{18} column unless a silanol blocking agent, tetramethyl ammonium chloride (TMA) was added. In 0.01 *M* orthophosphoric acid–acetonitrile, tetracyclines were separated on the polymeric reversed-phase columns, but not on the C_{18} columns. With addition of TMA, results on C_{18} and polymeric reversed-phase columns were nearly identical. Addition of an ion pair also improved separations on the C_{18} columns, but not as much as TMA. Interaction with the silica support of C_{18} columns was used to separate tylosin from interferences in extracts of biological materials. The results demonstrate the importance of interactions with the silica support in chromatography of basic antibiotics on C_{18} packings. These interactions can be either beneficial or detrimental to separations, depending on the conditions used.

INTRODUCTION

When reversed-phase packings for liquid chromatography (LC) are prepared by bonding an organic layer onto a silica support, 50% or less of the available silanols on the silica surface react with the organic function bonded to the surface[1]. Further reaction of the silanols is inhibited by steric factors. In theory, the bonded organic layer should completely cover the surface masking any residual silanols[1]. However, it has been observed that the unreacted silanols may still interact with some solutes, especially basic compounds, giving poor chromatographic results[2–6]. When the proportion of water in the mobile phase is small, interaction with silica may be an important or dominant chromatographic mode[2]. Capping residual silanols by further treatment of the column packing has been found to reduce interference from silica[4]. Many manufacturers now employ various end-capping procedures with reversed-phase packings. The effect of the silulation method and type of silica support on interaction with basic compounds has been investigated by Köhler *et al.*[6].

0021-9673/86/$03.50

Recent studies in our laboratory of the chromatography of some antibiotic compounds have demonstrated that the silica support of even end-capped commercial reversed-phase packings may interact significantly with some antibiotics with amine functions. The importance of interaction with the silica support was established both by adding a silanol blocking agent and by using an all organic polymeric column.

MATERIALS AND METHODS

Chemicals and equipment

Chemicals. Acetonitrile, methanol, HPLC grades from several sources; tetraethyl and tetramethyl ammonium chloride were obtained from Eastman-Kodak; 1-heptane sulfonic acid, sodium salt monohydrate was obtained from Aldrich, other chemicals were reagent grade.

Antibiotic standards. The penicillins, cephapirin and tetracyclines, were obtained from Sigma, tylosin analytical standard free base was obtained from Eli Lilly. Penicillin and cephapirin standard solutions were prepared in water, tetracyclines in 0.01 *M* hydrochloric acid and tylosin in acetonitrile–water (90:10), all at a concentration of 0.01 μg/μl.

Procedure

Apparatus. A Varian Model 5000 liquid chromatograph with a Varian UV-50 variable-wavelength detector, and a Valco automatic loop injector with a 200 μl loop were used. Wavelengths used were: penicillins and cephapirin, 220 nm; tetracycline 355 nm; tylosin, 280 nm.

High-performance liquid chromatographic (HPLC) procedures

Penicillins and tetracyclines. Gradient elution with 0.01 *M* orthophosphoric acid–acetonitrile 85:15 to 40:60 in 20 min, flow-rate 1 ml/min. In some experiments,

TABLE I

CHROMATOGRAPHIC COLUMNS STUDIED

Column	*Guard column*	*Type*	*Size*
Varian Micropak SPC-18	Special	4-μm C_{18} bonded, end-capped	150 × 4.6 I.D. mm
Varian Micropak MCH-10	MCH-10 cartridge	10-μm C_{18} bonded	300 × 4.0 I.D. mm
Varian Micropak MCH-10-N-Cap	None	10-μm C_{18} bonded, end-capped	300 × 4.0 I.D. mm
Hamilton PRP-1	None	10-μm styrene–divinyl benzene copolymer	250 × 4.1 I.D. mm
Polymer Laboratories PLRP-S	Special	5-μm styrene–divinyl benzene copolymer	150 × 4.6 I.D. mm

0.01 *M* tetramethyl ammonium chloride (TMA) and/or 0.01 *M* sodium heptane sulfonate were added to the orthophosphoric acid as indicated in text.

Tylosin. Isocratic operation with 0.002–0.005 *M* ammonium dihydrogen phosphate–acetonitrile–methanol, proportions as indicated in text, flow-rate 1.0–1.5 ml/min.

RESULTS AND DISCUSSION

The injection volume (200 μl) used in this study is somewhat larger than is commonly used. However, in practical sample analysis, use of the larger volume improves sensitivity and precision and is essential in many cases for detection of low levels of residues. With this injection volume, the analyte must be in a solvent in which it is less mobile than it is in the carrier solvent on the column. Under these conditions the analyte is concentrated at the column inlet. Thus penicillins and tetracyclines were injected in water or 0.01 *M* hydrochloric acid, respectively, while tylosin was injected in acetonitrile with $\leqslant$ 10% water. If the analyte is injected under conditions where it is not mobile at all, it may be concentrated on the column from successive injections. If, on the other hand, the analyte is injected in a large volume of solvent in which it is more mobile than in the column carrier solvent, peak distortion, spreading, or even doubling will occur[7]. This occurred when penicillins and tetracyclines were injected in a solvent containing a high proportion of acetonitrile and when tylosin was injected in water or methanol.

On bonded reversed-phase packings, interactions of solutes with both the bonded-phase and the silica support are possible. With an all organic polymeric column, the effects of silica are unambiguously eliminated. Addition of a silanol blocking agent to the mobile phase can also suppress interaction with the silica support of bonded phase columns and confirms the magnitude of such effects.

In normal-phase LC on silica, binding is based on hydrogen bonding, except in the case of cations which are bound by ion-exchange to the weakly ionized silanols[8,9]. In reversed-phase LC, retention is based on the relative solubility of solutes in organic solvents and water[10]. The order of elution of compounds is more or less transposed in normal- and reversed-phase LC. In normal-phase LC, retention is decreased as solvent polarity is increased while the opposite is true with reversed-phase LC. In reversed-phase chromatography, retention can also be increased by adding an ion pair to the mobile phase. Retention is increased if the ion pair formed is more soluble than the original compound in the organic phase[5,11]. Kissinger[12] has suggested that the actual mechanism involved in ion-pair chromatography may be more complicated. Adjusting the pH to suppress ionization can also increase retention in a reversed-phase system[11].

These effects can be illustrated by results with the first group of compounds studied, the penicillins. A number of methods have been described for determination of penicillins by reversed-phase HPLC[13–21]. These mainly use isocratic elution. Since penicillins have a wide range of polarity from the very polar amoxicillin to the relatively non-polar cloxacillin, only a few can be recovered in a reasonable length of time at any given solvent strength. However, all can be eluted and separated nicely with an acetonitrile gradient. The ammonium salts of penicillins are more soluble in organic solvents than the sodium salts[22] and, as would be expected, addition of

ammonium ion to the mobile phase increased retention time[13]. Alkyl substituted ammonium compounds further increased retention time[13]. Ionization of carboxyl functions of penicillins can be suppressed by lowering the pH below the pK_a which is about 2.7. Amino penicillins exist as the amine cation under these conditions. Penicillins with only carboxyl functions are unionized, and the acid forms are readily soluble in organic solvents[22]. As would be expected, retention times of the acid forms are markedly increased, well beyond what could be achieved by ion pairing[13]. Retention times of the aminopenicillins could be increased by adding an anionic compound, sodium heptane sulfonate, as an ion pair.

For chromatography of penicillins, a gradient of 0.01 *M* orthophosphoric acid–acetonitrile of 85:15 to 40:60 in 20 min was used. The first group studied were those with carboxyl functions only. All but carbenicillin have neutral side chains. Fig. 1 shows results on a bonded phase column (SPC-18). Peaks were sharp and all compounds were well-resolved. Fig. 2 shows results on an all organic column (PRP-1). The retention times were quite similar. Nafcillin and cloxacillin were not resolved and peaks were less sharp on the all organic column.

The better resolution on the bonded phase column was undoubtedly a result of the smaller particle size since resolution was similar on columns of comparable particle size (not shown). The similarity of results on the two types of columns indicate that the silica support had little if any effect and that retention on both columns was by a reversed-phase mechanism.

The amphoteric penicillins, ampicillin and amoxicillin occur as the amine cations at the pH of dilute phosphoric acid (1.6). These were chromatographed with a related compound, cephapirin using the same orthophosphoric acid–acetonitrile gradient as with the other penicillins. As would be expected, overall retention times were

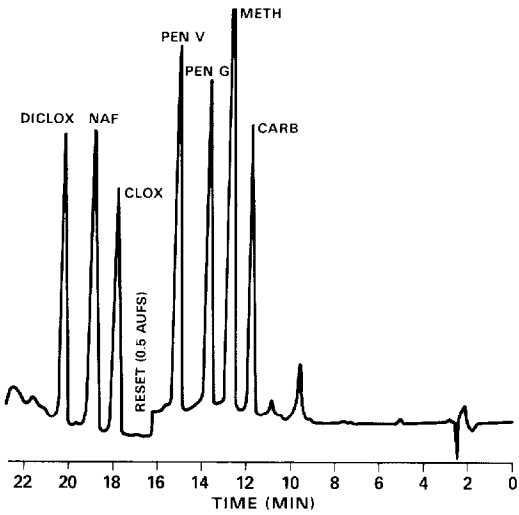

Fig. 1. Chromatographic separation of methicillin (METH), penicillin G (PEN G), penicillin V (PEN V), cloxacillin (CLOX), dicloxacillin (DICLOX), nafcillin (NAF) and carbenicillin (CARB) on a bonded reversed-phase column (Varian SPC-18). Operating conditions: flow-rate, 1 ml/min; gradient elution, 0.01 *M* orthophosphoric acid–acetonitrile (85:15 to 40:60 in 20 min); 2 μg of each injected; UV detection (220 nm); 0.2 AUFS.

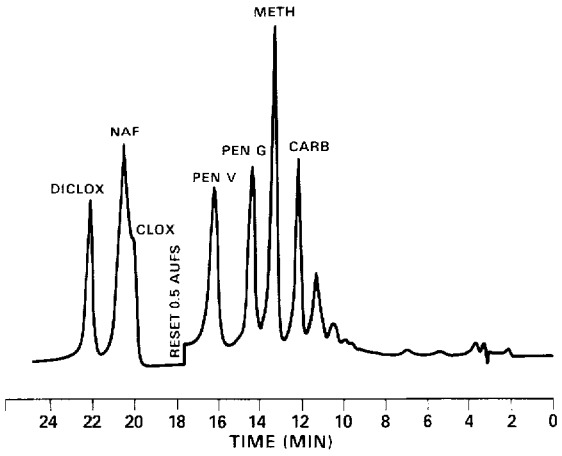

Fig. 2. Chromatographic separation of seven penicillins on an all organic polymeric column (Hamilton PRP-1). Operating conditions and abbreviations as in Fig. 1.

lower with these more polar compounds (Fig. 3). Retention times were greater on the bonded phase column and the ampicillin peak, in particular, was broadened and tailed. To establish that the differences were in fact a result of interaction with silanols, a silanol blocking agent, TMA, was added to the orthophosphoric acid in the mobile phase. Retention times on the SPC-18 column were decreased and peaks were markedly sharpened thus confirming that residual silanols were significantly affecting the results. As mentioned previously, TMA can be used as an ion pair with penicillins under less acid conditions. At the pH used, the penicillins were not in the anionic form and the tetramethyl ammonium ion functioned purely as a silanol blocking agent, decreasing retention time rather than increasing it as it would as an ion pair.

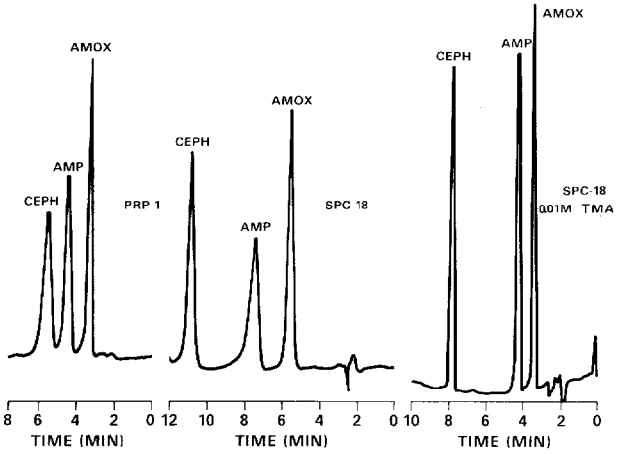

Fig. 3. Separation of amoxicillin (AMOX), ampicillin (AMP) and cephapirin (CEPH) on a polymeric (Hamilton PRP-1) and a bonded (Varian SPC-18) reversed-phase column, with and without 0.01 *M* TMA added to the 0.01 *M* orthophosphoric acid in the mobile phase. Operating conditions as in Fig. 1.

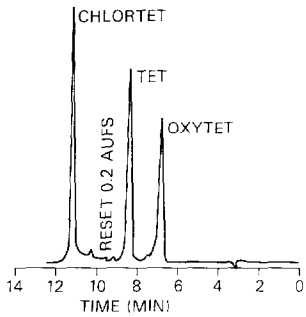

Fig. 4. Separation of oxytetracycline (OXYTET), tetracycline (TET) and chlortetracycline (CHLORTET) on an all organic polymeric column, (Polymer Labs., PLRP-S) 2-μg standards; UV detection, 355 nm; 0.5 AUFS; further operating conditions as in Fig. 1.

A second group of compounds studied was the tetracyclines. There are a number of published procedures for HPLC analysis of tetracyclines[4,5,23–29] but, in practice, it has proven difficult to reproduce results[23,24]. Mack and Ashworth[24] found that most commercial bonded columns were unsuitable for chromatography of tetracyclines. Sharma *et al.*[29] used a lengthy column conditioning procedure. Howell *et al.*[23] reported that published procedures did not give satisfactory results with tetracyclines. Knox and Jurand[5] found that compounds which functioned as ion pairs such as EDTA and nitrates improved results with tetracyclines and that capping residual silanols also improved results[4]. Knox and Jurand[5] concluded that best results with tetracyclines were obtained at pH 1–2.5 and after some investigation, we found that the 0.01 *M* orthophosphoric acid–acetonitrile gradient used for penicillins also gave optimum results with tetracyclines. At this pH, ionization of phenolic functions of tetracyclines is suppressed and the compounds exist as the dimethyl amine cations[30]. Fig. 4 shows results on a PLRP-S column. The tetracycline peaks are sharp, the compounds are well separated, and minor impurities are resolved. Fig. 5 shows results under identical conditions on a bonded column (SPC-18). The retention time is much longer, peaks are not sharp and only two peaks are evident. Addition of TMA (Fig. 6) strikingly improved results on the bonded phase column so that both separations and retention times were similar to those on the polymeric column. TMA cannot act as an ion-pairing agent with tetracycline at this pH.

To determine the effect of an ion-pairing agent, an anion, sodium heptane sulfonate, was added to the orthophosphoric acid in the mobile phase. On the SPC-18

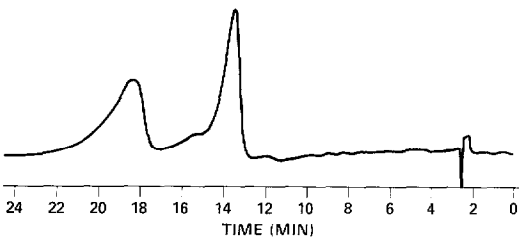

Fig. 5. Chromatography of three tetracyclines (oxytetracycline, tetracycline and chlortetracycline) on a bonded reversed-phase column (Varian SPC-18); 0.1 AUFS; further conditions in Fig. 4.

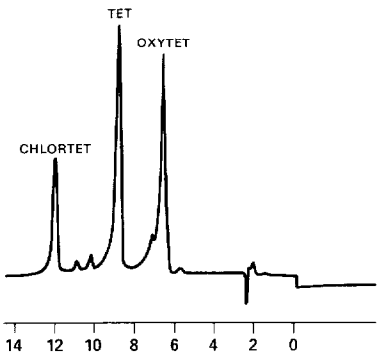

Fig. 6. Same as Fig. 5 but with 0.01 *M* TMA added to the 0.01 *M* orthophosphoric acid in the mobile phase (0.2 AUFS).

column, this improved separation of tetracyclines but peaks still tailed substantially (Fig. 7).

On the polymeric columns, sodium heptane sulfonate increased retention time, decreased separation of the tetracyclines, and had no effect on peak shape. The same results were observed on bonded phase columns if both TMA and sodium heptane sulfonate were present in the mobile phase. Bij *et al.*[3] suggested that ion-pairing reagents reduce interference from residual silanols by making the analyte less readily available for interaction. Some previous studies have been done under conditions where both ion pairing and silanol blocking can occur, confusing the two effects[31,32]. The present data clearly separate the effects of ion-pairing and silanol blocking agents. Silanol blocking agents were far more effective in improving column performances and an ion pair did not affect peak shape when a silanol blocking agent was present. The two work in opposite directions; silanol blocking decreases retention time and ion pairing increases retention time. Where significant silanol interaction is present, an ion pair may also somewhat decrease retention time.

Another antibiotic compound studied was tylosin. A method was developed for determination of tylosin in biological substrates using a reversed-phase column[33]. However the behavior of tylosin under the experimental conditions used was puzzling

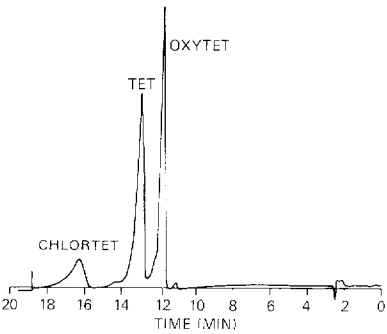

Fig. 7. Same as Fig. 5 but with 0.01 *M* sodium heptane sulfonate added to the 0.01 *M* orthophosphoric acid in the mobile phase (0.2 AUFS).

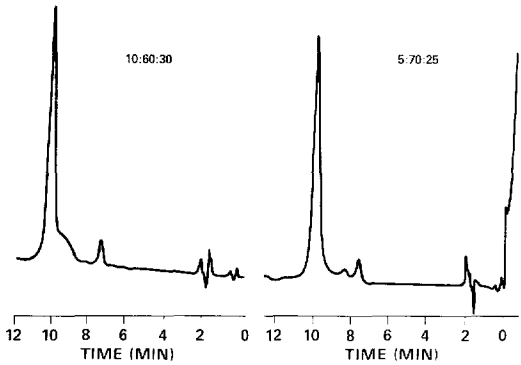

Fig. 8. Chromatography of tylosin (2 μg) on a Varian SPC-18 column with UV detection at 280 nm. Operating conditions: isocratic; solvent composition, 0.004 *M* ammonium dihydrogen phosphate–acetonitrile–methanol (10:60:30 and 5:70:25); flow-rate, 1 ml/min; 0.1 AUFS.

when an attempt was made to relate it to some type of reversed-phase mechanism. However, once it was recognized that tylosin was interacting with the silica support, the explanation of the results became clear.

The solvent system used was a ternary one using 0.002–0.005 *M* ammonium dihydrogen phosphate–acetonitrile–methanol containing $\leqslant 10\%$ aqueous phase. The proportions were varied as necessary to separate tylosin from intererfences in sample extracts[33]. Figs. 8 and 9 illustrate the effect of increasing the proportion of acetonitrile on retention times of tylosin and on interferences in an extract of pork muscle, respectively. The retention time of tylosin was unchanged while that of the interferences was greatly decreased. Thus it can be concluded that tylosin and the interferences were chromatographing by different mechanisms. The interferences showed the behavior expected on a reversed-phase column. The retention time of tylosin on the other hand was unchanged and actually increased as the concentration of acetonitrile

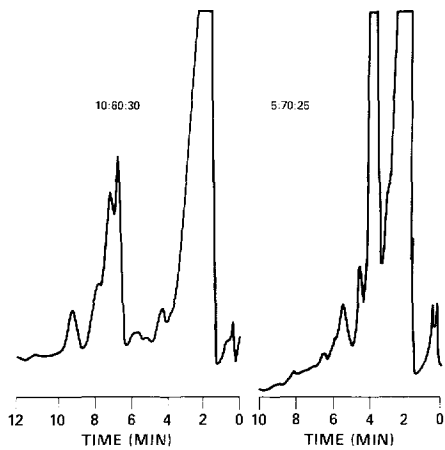

Fig. 9. Extract from 0.4-g pork muscle prepared as previously described[20]. Operating conditions as for Figure 8 (0.02 AUFS).

was increased further[33]. This is the exact opposite expected of a reversed-phase mechanism. Further support of the observation that tylosin is chromatographing by interactions with the silica support is given by (1) tylosin was not retained at all on an all organic polymeric column under the same conditions and (2) addition of TMA decreased retention of tylosin. With use, retention times of tylosin on reversed-phase columns tended to decrease and this can be explained on the basis that residual silanols gradually become blocked by substances in sample extracts.

A dual retention mechanism has been noted with other compounds also and appears to occur in solvents with a low proportion of water[2]. Under these conditions, the silica support becomes accessible to interaction with some types of compounds. In other solvents with a higher proportion of water, tylosin shows the expected reversed-phase behavior on either type of column. The dual retention mechanism is particularly useful in separating tylosin from interferences in bilogical materials.

Unger *et al.*[1] found that a maximum of about 50% of available silanols on a silica surface were blocked when an organic material (trimethyl silane chloride) was bonded to the surface and that even fewer reacted with bulkier bonding agents. They noted that further reaction of silanols with bonding agents was sterically blocked. They also concluded that the bonded layer should completely cover the surface and mask residual silanols from interaction with solutes. This is obviously not the case although it is not clear how the silica support becomes accessible to solutes. Manufacturers of bonded reversed-phase columns have attempted to block or "end-cap" residual silanols and with the columns used in the present study, this approach was not totally satisfactory. However, addition of an alkylamine to the mobile phase was clearly effective in blocking access to remaining silanols. Bij *et al.*[3] concluded that interaction with the carbonaceous layer stabilized the blocking effects of alkylamines and that the blocking was strengthened as chain length of the alkylamine was increased.

Although it is widely recognized that interaction with the silica support of bonded-phase columns occurs, the magnitude of such effects is not always fully appreciated by chromatographers. The present study presents evidence of such interactions in a group of antibiotic compounds. Such interactions may be detrimental or beneficial in separations. Published reports suggest considerable variation in silanol effects with different commercial bonded packings. It is not clear whether such effects can be totally avoided by manufacturing procedures. Silanol effects may be readily avoided by using an all organic polymeric column or by adding an alkylamine to the mobile phase. It therefore seems questionable if extensive efforts to modify bonded packings to eliminate silanol effects are warranted.

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