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# SEPARATION OF ERYTHROMYCIN AND RELATED SUBSTANCES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON POLY(STYRENE–DIVINYLBENZENE) PACKING MATERIALS

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## SUMMARY

A comparative evaluation of three brands of poly(styrene–divinylbenzene) copolymers, Hamilton PRP-1 (10 μm), Rogel (8 μm) and TSK-Gel (10 μm), as column packing materials for high-performance liquid chromatographic separation of erythromycins is presented. Erythromycins A, B and C, anhydroerythromycin A, erythromycin A enol ether, N-demethylerythromycin A, anhydro N-demethylerythromycin A and N-demethylerythromycin A enol ether were chromatographed. The effects of column temperature, concentration of organic modifier in the mobile phase, concentration of phosphate buffer, the addition of quaternary ammonium salts and pH are described. The best separations were obtained on TSK-Gel with the mobile phase acetonitrile–methanol–0.2 *M* tetramethylammonium hydroxide pH 8.0–0.2 *M* phosphate buffer pH 8.0–water (30:15:25:5:25). PRP-1 and Rogel gave equally good separations but with higher retention volumes.

## INTRODUCTION

The main component of erythromycin is erythromycin A (EA), but some related substances such as erythromycins B (EB), C (EC) and D (ED) and N-demethylerythromycin A (dMeEA) are present in small amounts. Degradation products like erythromycin A-6, 9;9;12-spiroketal or anhydroerythromycin A (AEA) and 8,9-anhydroerythromycin A-6,9-hemiketal or erythromycin A enol ether (EAEN), which are formed under mildly acidic conditions, can also be present. The separation of all these compounds by thin-layer chromatography (TLC) was described recently[1]. For quantitative work, TLC is less suitable than high-performance liquid chromatography (HPLC). Several HPLC methods have been described for the separation and analysis of erythromycin components and impurities in bulk samples and solid dosage forms[2–4], in fermentation extracts[2,5] and biological fluids[6–8]. All these methods appear to be modifications of the HPLC method of Tsuji and Goetz[2], which has also found application in studies on the dissolution of erythromycin stearate[9].

The HPLC methods mentioned above used derivatized silica reversed-phase

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packing materials which are unstable above pH 7. However, mobile phases at higher pH enable better separations of erythromycins. Mobile phases at lower pH cannot be used since erythromycins are acid labile. Therefore analyses are generally performed at a compromise pH value of 7–8.

More recently, non-ionic poly(styrene–divinylbenzene) co-polymers (PSDVBs) have been introduced as packing materials in HPLC. PSDVBs are applicable over a wide pH range (0–14) and could be used for the analysis of erythromycins at higher

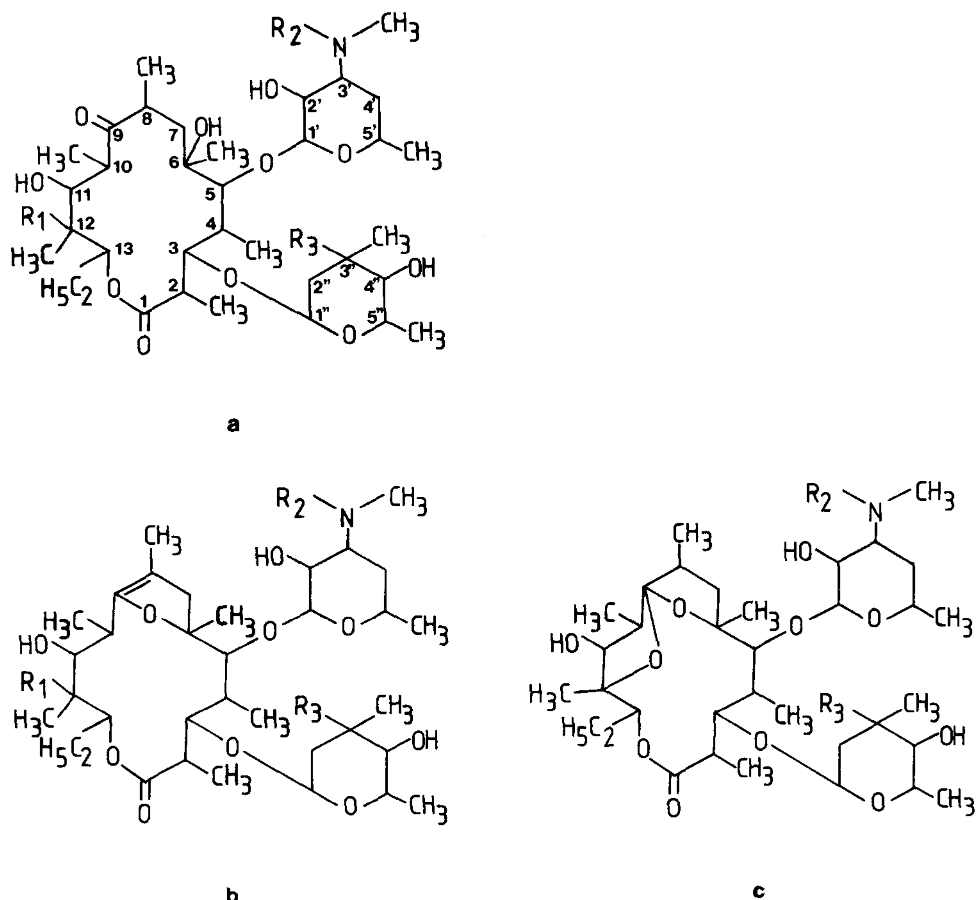


Fig. 1.

| | | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|---|
| (a) Structures of erythromycins | | | | |
| Erythromycin A | (EA) | OH | $CH_3$ | $OCH_3$ |
| Erythromycin B | (EB) | H | $CH_3$ | $OCH_3$ |
| Erythromycin C | (EC) | OH | $CH_3$ | OH |
| N-Demethylerythromycin A | (dMeEA) | OH | H | $OCH_3$ |
| (b) Structures of erythromycin enol ethers. | | | | |
| Erythromycin A enol ether | (EAEN) | OH | $CH_3$ | $OCH_3$ |
| N-Demethylerythromycin A enol ether | (dMeEAEN) | OH | H | $OCH_3$ |
| (c) Structures of anhydroerythromycins. | | | | |
| Anhydroerythromycin A | (AEA) | – | $CH_3$ | $OCH_3$ |
| Anhydro N-demethylerythromycin A | (AdMeEA) | – | H | $OCH_3$ |

pH values. A commercial brand of PSDVB, Hamilton PRP-1, has successfully been used in our laboratory for the analysis of doxycycline[10]; the columns were used for over 6 months at pH 8 without any deterioration. Recently, PSDVB packing materials have also been used for the analysis of amino acids, peptides and proteins[11,12] and for thiochrome derivatives of thiamine and some of its esters[13,14].

Here we report on the separation of erythromycin and related substances on three brands of PSDVB packing materials. Two concurrent investigations were carried out; first on the separation of erythromycins and acid degradation products present in bulk samples; secondly on the separation of the metabolites of erythromycin A found in rats. Following the administration of EA to rats, six main compounds, EA, AEA, EAEN, dMeEA, anhydro N-demethylerythromycin A (AdMeEA) and N-demethylerythromycin A enol ether (dMeEAEN) were recovered in faeces and urine[15].

## EXPERIMENTAL

### *Sample*

Pure EA was obtained by four consecutive crystallizations of a commercial sample from a 10% (w/v) solution in acetone–water (1:1). The crystals were allowed to dry in contact with the atmosphere at room temperature. EB and EC were obtained by preparative HPLC of mother-liquor concentrates from the industrial production of erythromycin[16]. AEA[17], EAEN[17] and dMeEA[18] were prepared from EA according to the described methods. AdMeEA and dMeEAEN were prepared from dMeEA according to the methods used to prepare AEA and EAEN respectively. Structures are shown in Fig. 1.

### *Instrumentation*

The chromatographic system consisted of a Waters Model M-45 solvent-delivery system (Waters Assoc., Milford, MA, U.S.A.). a Valco Model CV-6-UHPa-N60 sample-injection valve (Valco, Houston, TX, U.S.A.) equipped with a 20-$\mu$l loop, a Pye Unicam LC 3 UV variable-wavelength detector (Pye Unicam, Cambridge, U.K.) set at 215 nm and a Kipp en Zonen recorder Model BD 40 (Kipp en Zonen, Delft, The Netherlands). Columns were provided with a glass jacket and maintained at the required temperature by a circulation of water.

### *Columns and packing procedures*

Columns (25 cm $\times$ 4.6 mm I.D.) were packed with three different PSDVB materials; Hamilton PRP-1, 10 $\mu$m (Hamilton, Reno, NV, U.S.A.); TSK-Gel, 10 $\mu$m (Toyo Soda, Tokyo, Japan) and Rogel, 8 $\mu$m, an experimental sample (Laboratorium Organische Scheikunde, Rijksuniversiteit Gent, Gent, Belgium).

A 5-g amount of packing material (PRP-1 and TSK-Gel) was wetted with 8 ml acetone and slurried in 15 ml of an aqueous solution containing 10% (w/v) glycerol and 2.5% (w/v) sodium chloride. The slurry was sonicated for 4 min and quickly introduced into a slurry reservoir, 25 $\times$ 0.9 cm I.D. stainless-steel tubing fixed to the column through a 10 cm $\times$ 4.6 mm I.D. precolumn, the latter two being already filled with suspending liquid. The slurry was immediately packed into the column at a pressure of about 500 kg/cm$^2$ with 200 ml of the suspending solution as the pres-

surizing solvent, using a Haskel Pump Model DSTV-122 (Haskel, Burbank, CA, U.S.A.). For Rogel, this packing procedure resulted in columns with too large a back-pressure. A modified procedure was therefore used in which the acetone-wetted material was slurried in water which was also used as the pressurizing solvent.

*Reagents, solvents and mobile phases*

Ammonium dihydrogen and diammonium hydrogen phosphate pro analysi (E. Merck, Darmstadt, F.R.G.) were used to prepare 0.2 *M* phosphate buffers. Tetramethylammonium hydroxide (TMA), 20% (w/w) solution in methanol (Janssen Chimica, Beerse, Belgium), was used to prepare a 0.2 *M* TMA solution by dilution in water. Tetrabutylammonium hydrogensulphate (TBA) from the same supplier was used to prepare 0.2 *M* TBA solutions. The pH of the quaternary ammonium solutions was adjusted to the pH of the phosphate buffer used for a particular mobile phase, with 85% phosphoric acid (E. Merck). HPLC grade methanol and acetonitrile were obtained from Rathburn (Rathburn Chemicals, Walkerburn, U.K.). Reagent grade 1-propanol (Janssen Chimica) and ethanol of pharmacopoeial quality as organic modifier were distilled from glass before use. Water was twice distilled from glass.

The mobile phases were prepared by mixing one or two organic modifiers, phosphate buffer and water. Quaternary ammonium salt solutions were added in most cases. No correction was made for volume contraction. Mobile phases were degassed by sonication.

*Chromatographic conditions*

The columns were conditioned for 1–2 h at every change of mobile phase. When using a mobile phase containing a different quaternary ammmonium salt, the column was first washed for 1 h with acetonitrile–water (1:1). Unless stated otherwise, HPLC was performed at 60°C.

Sample solutions (10 mg/ml) in acetonitrile or in acetonitrile–water (1:1) were prepared, except for EAEN and dMeEAEN (2 mg/ml). The flow-rate was set at 1 ml/min and detector sensitivity at 0.08–0.32 depending on the capacity factor, $k'$, which affected the peak heights. The chart speed was set at 5 mm/min for normal $k'$ measurements and at 0.2 mm/sec for efficiency, selectivity and resolution evaluations.

The dead volumes of PRP-1 (2.1 ml), TSK-Gel (2.2 ml) and Rogel (1.4 ml) were determined by injecting acetone.

## RESULTS AND DISCUSSION

Comparative evaluation of the chromatographic performance of the PSDVB columns was done using two groups of erythromycins. The first group is a mixture of erythromycins and derivatives detected in commercial samples such as EA, EB, EC, dMeEA, AEA and EAEN. This group is referred to collectively as erythromycin "impurities". ED was not investigated because it was not available. By TLC, ED was found to be absent from most samples recently produced[1]. The second group is a mixture of EA and other compounds identified in biological excretory fluids of rats, namely dMeEA, dMeEAEN, AdMeEA, AEA and EAEN[15]. This group is referred to as erythromycin "metabolites".

In HPLC of erythromycins, with UV detection (215 nm), many water-miscible

organic solvents cannot be used as organic modifiers due to their high UV cut-off. Also, the high viscosity of some water-miscible solvents excludes their use due to high column back-pressure. In this investigation, acetonitrile, ethanol, methanol and propanol were used. Acetonitrile was taken as the primary organic modifier because of its stronger eluting power.

Some pertinent physical and chromatographic characteristics of the PSDVB polymers used as column packing materials are given in Table I. The chromatographic efficiency of the columns was determined by chromatography of EA and dMeEA. The organic content of the mobile phase was adjusted to obtain $k'$ values of about 2–3 for dMeEA on all columns. PRP-1 and Rogel show similar efficiencies while TSK-Gel has a greater efficiency.

The comparative column performance for the separation of erythromycins has been further evaluated in terms of the influence of the column temperature, methanol content in the mobile phase, buffer concentration, the effect of adding TMA and TBA to the mobile phase and of the pH of the mobile phase.

*Column temperature*

The relationship between $k'$ values and column temperature using acetonitrile–0.2 *M* phosphate buffer pH 7.8–water (50:10:40) as the mobile phase is shown in Fig. 2. In Fig. 2c, $10 \times k'$ is used instead of $k'$. First a mobile phase at pH 7.8 was chosen because this pH is mentioned in the literature as suitable for the separation of complex mixtures[2].

In general the retention of erythromycins on the columns increases in the order TSK-Gel < PRP-1 < Rogel. EC was not investigated on Rogel and TSK-Gel because of the small amount available. For the erythromycin "impurities", an increase in temperature results in increased retention on all columns. This is in contrast to bonded silica reversed-phase columns where the retention of erythromycins varies inversely with the temperature[3]. The column temperature also affects the selectivity. On PRP-1, for example, there is loss of separation between AEA and EB upon increasing the temperature from 30 to 60°C. On Rogel, the same products are sep-

TABLE I

PHYSICAL PROPERTIES OF PACKING MATERIALS AND COLUMN CHARACTERISTICS

25 × 0.46 cm columns at 60°C; mobile phase flow-rate 1 ml/min.

| | *PRP-1* | *Rogel* | *TSK-Gel* |
|---|---|---|---|
| Particle size (μm) | 10 | 8 | 10 |
| Particle shape | Spherical | Spherical | Spherical |
| Specific surface area ($m^2/g$) | 415 | 1200 | 205 |
| Average pore diameter (Å) | 75 | 30 | 250 |
| Capacity factor, $k'$, for dMeEA | 2.96 | 3.11 | 2.19 |
| Plates per metre for dMeEA | 4500 | 4240 | 9900 |
| Capacity factor, $k'$, for EA | 6.38 | 6.62 | 5.66 |
| Plates per metre for EA | 4240 | 4000 | 6280 |
| Mobile phase: acetonitrile–methanol–0.2 *M* TMA pH 8.0–0.2 *M* phosphate buffer pH 8.0–water | 30:35:15:5:15 | 35:35:15:5:10 | 30:20:15:5:30 |

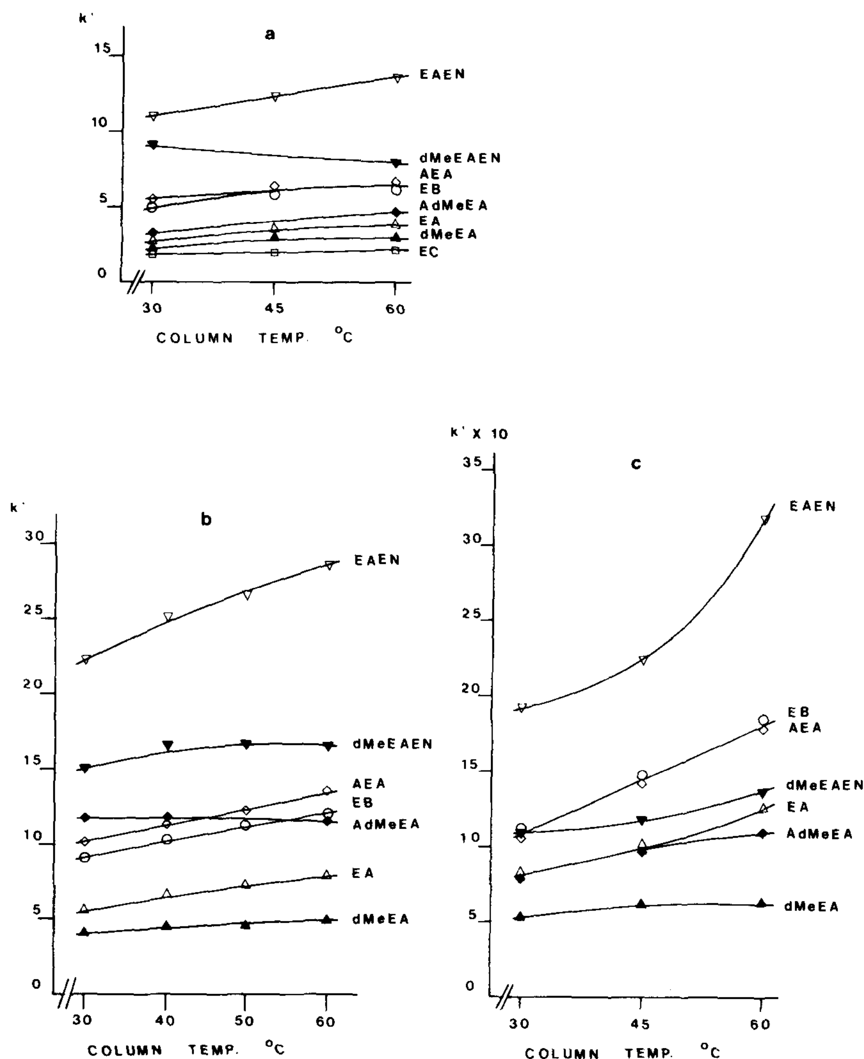


Fig. 2. Effect of column temperature on the capacity factor, $k'$, of erythromycins. Columns: a, Hamilton PRP-1; b, Rogel; c, TSK-Gel. Mobile phase: acetonitrile–0.2 $M$ phosphate buffer pH 7.8–water (50:10:40) at a flow-rate of 1 ml/min. For TSK-Gel, $k' \times 10$ is used instead of $k'$.

arated throughout this range, but they are co-eluted on TSK-Gel. The other erythromycin impurities show clear separations.

The compounds in the "metabolites" group however show contrasting selectivities on the different columns. On PRP-1, $k'$ for dMeEAEN decreases with increasing temperature. On TSK-Gel, the separation between AdMeEA and EA improves with increasing temperature. AdMeEA and EA are well separated on Rogel but closely eluted on PRP-1. There is also a variation in the elution order of dMeEAEN on the columns. In general, despite increased retentions, the peak shapes

are better at high temperatures. A temperature of 60°C was chosen for further investigations. This also allowed operation at back-pressures of about 1000 p.s.i. (PRP-1) to 2500 p.s.i. (TSK-Gel). Rogel gave intermediate pressures.

*Effect of methanol content of the mobile phase*

The effect of methanol was investigated using acetonitrile–methanol–0.2 *M* TMA, pH 7.8–0.2 *M* phosphate buffer pH 7.8–water (40:$x$:25:5:30 − $x$) as a mobile phase. The results are shown in Fig. 3. There is a general decline in $k'$ values with increasing methanol content. On PRP-1 and TSK-Gel, EB is eluted after AEA but on Rogel it is eluted before AEA. On PRP-1 and Rogel AEA and dMeEAEN are co-eluted, while on TSK-Gel, EA and dMeEAEN are co-eluted. An increase in methanol content seems to have less effect on the separation of the erythromycin "impurities" or "metabolites" on the three columns.

Hence the methanol content in the mobile phase can be used to alter $k'$ without compromising the selectivity. The peak shapes were slightly better in the presence of methanol.

*Effects of the phosphate buffer and of tetraalkylammonium salts on k', efficiency, selectivity, resolution and peak symmetry*

In a number of experiments, erythromycin impurities were chromatographed on the three columns in order to investigate the effect of the buffer concentration and of quaternary ammonium salts on the retention behaviour. A mobile phase consisting of 30% acetonitrile, 30% methanol and 5% 0.2 *M* phosphate buffer pH 8.0 was used. An increase in the concentration of buffer in the mobile phase resulted in lower $k'$ values on the three columns.

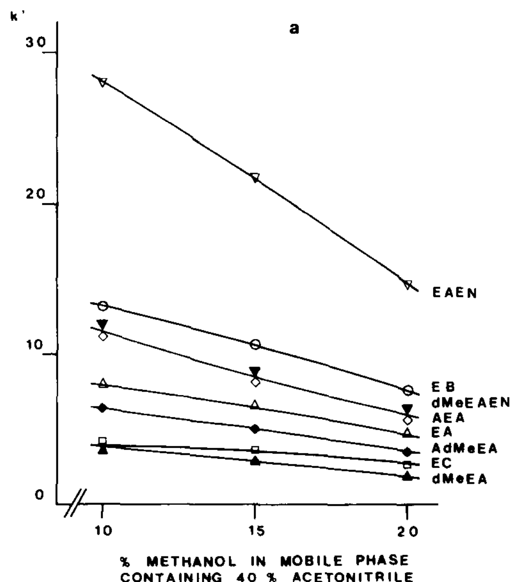


Fig. 3.

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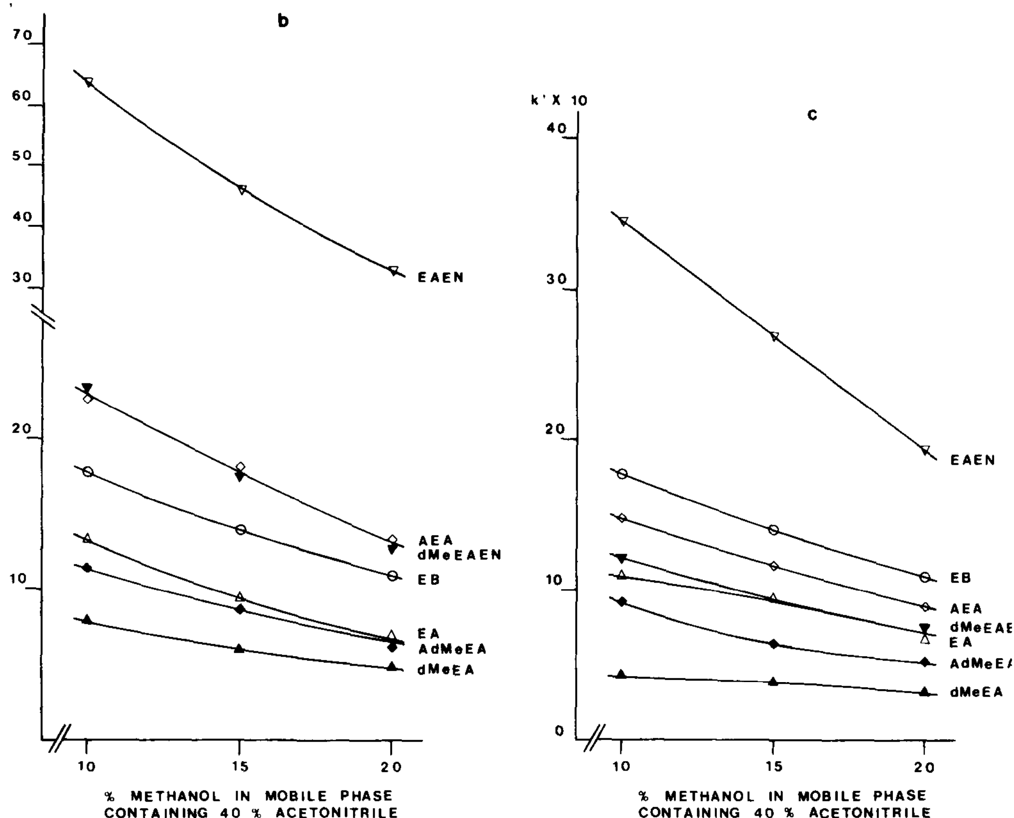


Fig. 3. Effect of methanol concentration in a mobile phase containing 40% acetonitrile on the capacity factor, $k'$, of erythromycin. Columns: a, Hamilton PRP-1; b, Rogel; c, TSK-Gel, at 60°C. Mobile phase: acetonitrile–methanol–0.2 $M$ tetramethylammonium phosphate pH 7.8–0.2 $M$ phosphate buffer pH 7.8–water ($40:x:25:5:30 - x$) at a flow-rate of 1 ml/min. For TSK-Gel, $k' \times 10$ is used instead of $k'$.

When 15% 0.2 $M$ TMA was introduced into the mobile phase, lower $k'$ values were recorded on PRP-1 and Rogel, but increased $k'$ on TSK-Gel. Increasing the 0.2 $M$ TMA content to 25% resulted in a further reduction in retention on PRP-1, but with a levelling-off effect. On TSK-Gel there was no further increase in $k'$, and no change in $k'$ was observed on Rogel. When TBA was substituted for TMA, a reduction in retentions was recorded on all columns.

The effect of the buffer concentration and TMA addition on the chromatographic efficiency, selectivity, resolution and peak symmetry was calculated for several erythromycin "impurities". The plate number increases with the concentration of buffer and also on addition of TMA. Addition of TMA increases the resolution by an increase in the plate number and selectivity. It was decided to use 25% 0.2 $M$ TMA and 5% 0.2 $M$ phosphate buffer in the mobile phases for further experiments.

*Mobile phase pH*

The influence of the pH of the mobile phase on the separation of erythromycins

on the three columns is shown in Fig. 4. For the TSK-Gel column, the methanol content of the mobile phase was lowered in order to obtain sufficient retention. For all erythromycins, an increase in retention is recorded with increasing pH. This behaviour is similar to that observed on bonded silica reversed-phase materials[2]. The separations generally improve with increasing pH.

In the group of erythromycin "impurities" on the three columns, the pairs EC–dMeEA and EB–AEA show reversal of elution order with increasing pH. In the group of "metabolites" the curves for pair AdMeEA–EA on PRP-1 and TSK-Gel show reversal of elution with increasing pH.

The pH of the mobile phase affects the stability of erythromycins especially below 6.5. As demonstrated, the separations improve with increasing pH but this effect seems to level off at about pH 8. With the ammonium phosphate buffer system, about pH 8 is the limit since the 0.2 *M* diammonium hydrogenphosphate solution has a pH of about 8.2. A pH value of 8 was therefore chosen for further chromatography.

The chromatographic behaviour of erythromycins on PSDVB packing materials shows that optimization to obtain a HPLC system capable of separating erythromycins is possible. It is evident that the three types of PSDVB, used here require different amounts of organic modifiers for reasonable analysis times. In this respect, TSK-Gel has obvious advantages over PRP-1 and Rogel, since it results in less retention, and also affords a better separation between EA and AEA. AEA is one of the important components found in both erythromycin "impurities" and "metabolites". The HPLC conditions established on TSK-Gel for impurities and metabolites were acetonitrile–methanol–0.2 *M* TMA pH 8.0–0.2 *M* phosphate buffer pH 8.0–water (30:15:25:5:25) at 60°C.

Fig. 5 shows chromatograms for erythromycin A spiked with dMeEA, EB, EC, AEA and EAEN, and for a reference mixture of "metabolites". Under the chro-

TABLE II

RELATIVE RETENTIONS OF ERYTHROMYCIN AND DERIVATIVES ON VARIOUS CHROMATOGRAPHIC SYSTEMS

Values in columns 1–4 are obtained from literature. Key to HPLC methods: 1, ref. 2; 2, ref. 4; 3, ref. 7; 4, ref. 8; 5, TSK-Gel, 10 μm (25 cm × 4.6 mm) at 60°C; mobile phase, acetonitrile–methanol–0.2 *M* tetramethylammonium phosphate pH 8.0–0.2 *M* phosphate buffer pH 8.0–water (30:15:25:5:25); flow-rate 1 ml/min; 6, as 5, except mobile phase ratio 35:10:5:5:45. NA = not available; ND = not determined.

| *Compound* | *HPLC system* | | | | | |
|---|---|---|---|---|---|---|
| | *1* | *2* | *3* | *4* | *5* | *6* |
| N-Demethylerythromycin A | NA | NA | 0.81 | 0.88 | 0.46 | 0.55 |
| Erythromycin C | 0.72 | 0.78 | 0.72 | NA | 0.55 | 0.64 |
| Anhydro N-demethylerythromycin A | NA | NA | NA | NA | 0.70 | 0.84 |
| Erythromycin A | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| N-Demethylerythromycin A enol ether | NA | NA | NA | NA | 1.08 | 1.18 |
| Erythralosamine | 1.16 | 1.32 | 1.16 | NA | ND | ND |
| Anhydroerythromycin A | 1.22 | 1.38 | 1.29 | 1.38 | 1.37 | 1.45 |
| Erythromycin B | 1.40 | 1.46 | 1.48 | 1.19 | 1.64 | 1.51 |
| Erythromycin A enol ether | 1.90 | 2.20 | 2.30 | 2.70 | 3.67 | 3.07 |

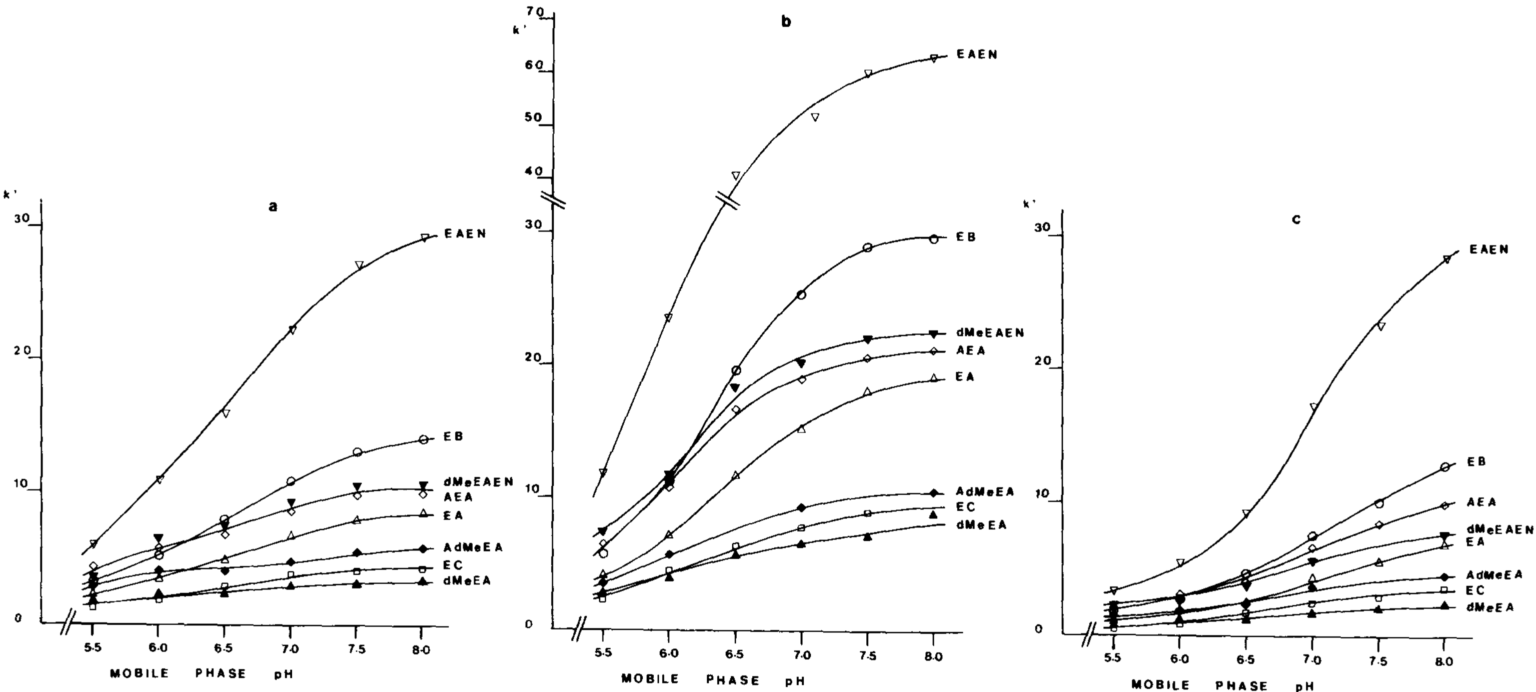


Fig. 4. Effect of mobile phase pH on the capacity factor, $k'$, of erythromycins. Columns: a, Hamilton PRP-1; b, Rogel; c, TSK-Gel, at 60°C. Mobile phases: acetonitrile–methanol–0.2 *M* tetramethylammonium phosphate pH *x*–0.2 *M* phosphate buffer pH *x*–water (30:30:25:5:10) for a and b, (30:15:25:5:25) for c. Flow-rate: 1 ml/min.

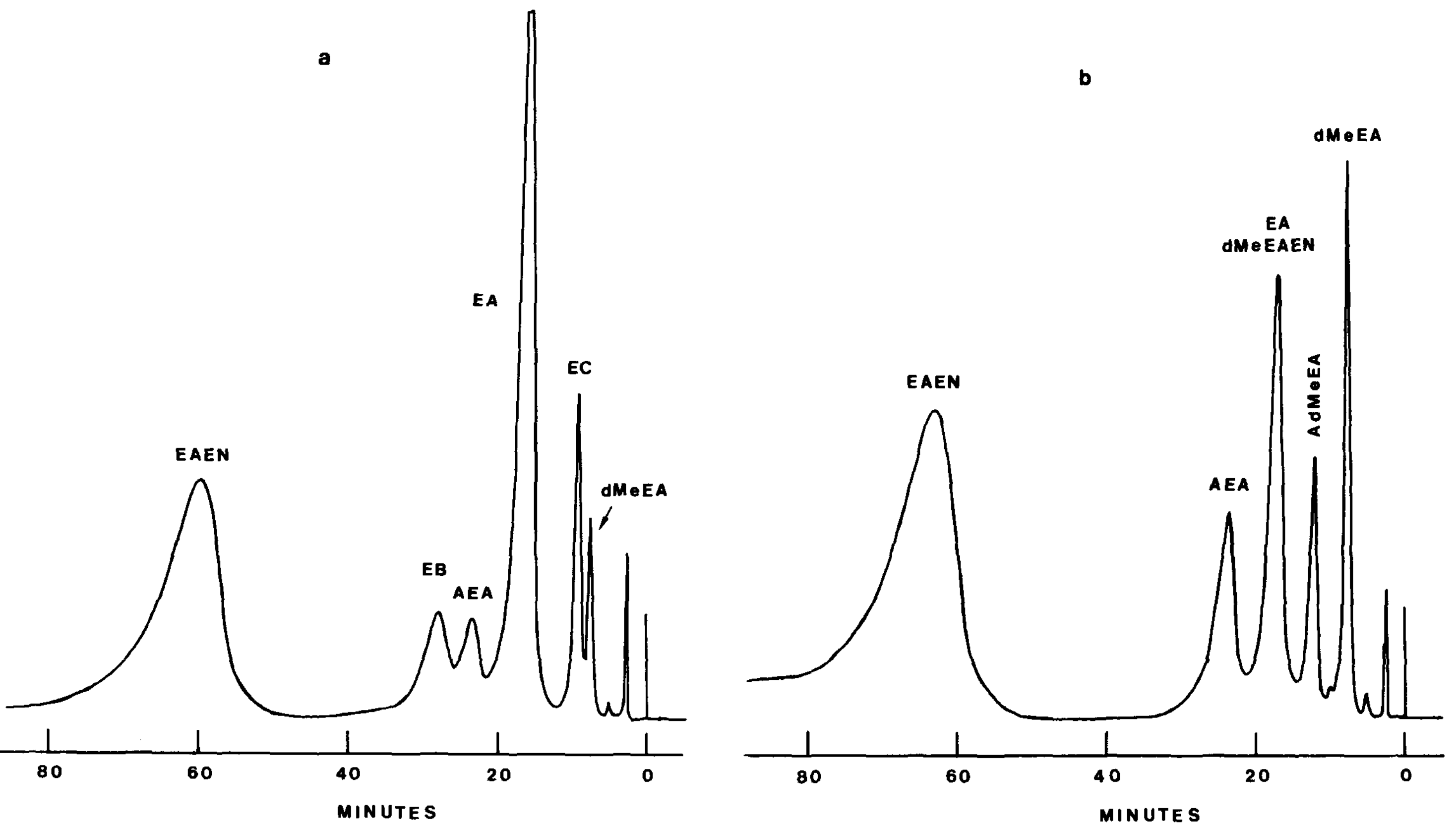


Fig. 5. HPLC chromatograms of erythromycin A spiked with impurities (a) and a mixture of erythromycin "metabolites" (b) on TSK-Gel (10 $\mu$m) at 60°C. Mobile phase: acetonitrile–methanol–0.2 *M* tetramethylammonium phosphate pH 8.0–0.2 *M* phosphate buffer pH 8.0–water (30:15:25:5:25). Flow-rate: 1 ml/min.

matographic conditions mentioned, dMeEAEN and EA are co-eluted. The optimum separation between the metabolites was obtained by reducing the methanol and 0.2 *M* TMA contents to 10 and 5% respectively, and increasing the acetonitrile content to 35%. With this solvent, EA and dMeEAEN are separated, but AdMeEA is eluted closer to EA (Table II). Since dMeEAEN was observed to be the minor metabolite[15] the system as presented in Fig. 5 seems to be the most suitable.

As shown in Table II, HPLC on TSK-Gel offers a comparable separation in terms of relative retention times to those obtained on bonded silica reversed-phase packing materials[2,4,7,8]. The latter packing materials offer better chromatograms in terms of baseline separation and peak shapes, but the former shows much higher stability under extreme conditions (pH 8, 60°C). The columns used in this work showed no deterioration in performance over the 6–8 months of investigations.

The HPLC method developed is capable of separating the impurities of erythromycin commonly present in commercial samples, as well as the most important metabolites of erythromycin. Hamilton PRP-1 or Rogel can be used instead of TSK-Gel but small changes in organic modifier content are needed.

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