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Evaluation of silanol-deactivated silica-based reversed phases for liquid chromatography of erythromycin

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ABSTRACT

The suitability of eleven silanol-deactivated reversed phases for the liquid chromatography of erythromycin was investigated. The selectivity and efficiency of each stationary phase were examined. The performance was compared to that of a non-deactivated C_{18} silica-based reversed-phase material, Hypersil C_{18} (5 μm). Two types of mobile phases were used, one containing no tetrabutylammonium (TBA) and the other containing TBA. Addition of TBA as a silanol-blocking agent improved the theoretical plate number and symmetry factor of the peaks corresponding to erythromycin A (EA) and erythromycin A enol ether for all the deactivated reversed phases. These results are an indication of the presence of some residual silanol activity in these phases. Separation of erythromycin E and EA was achieved on only two of the eleven phases. The selectivity was always poorer than that obtained in a previously described method using a poly(styrene–divinylbenzene) stationary phase.

INTRODUCTION

A disadvantage of liquid chromatography (LC) of basic substances on silica-based reversed-phases is peak tailing due to interaction with residual silanols on the silica backbone. In the LC of the macrolide antibiotic erythromycin, which is a mixture of several related substances all of which are bases, similar disadvantages were observed. The separation was improved by adding tetraalkylammonium compounds to the mobile phase, such as tetramethylammonium phosphate (TMA) or tetrabutylammonium hydrogensulphate (TBA) [1,2], TMA and TBA can act as silanol-blocking agents, so that interaction between erythromycin and the silanols is weakened. It was also observed that older columns performed better [1]. Ageing of C_8- and C_{18}-derivatised silica-based reversed phases improved the chromatography. It is believed that heating a packing material, conditioned with a mobile phase containing phosphoric acid, removes metal impurities from the silica backbone and hence influences the silanol activity. Nevertheless, large differences in selectivity were seen between different brands of reversed-phase materials [2,3].

In recent years, several silica-based reversed phases have been developed that are especially suitable for the chromatography of basic substances. These "silanol-deactivated" materials are claimed to have strongly reduced residual silanol activity and to produce better peak symmetry and efficiency. As a consequence, addition of triethylamine or tetraalkylammonium compounds would become superfluous. The suitability of several silanol-deactivated stationary phases for the LC of erythromycin was investigated. A non-deactivated silica-based reversed-phase material, Hypersil C_{18} (5 μm), was used as a reference. The selectivity and efficiency of each stationary phase were determined, first with a mobile phase containing no TBA and then with a mobile phase containing 5% (v/v) of 0.2 *M* TBA. In

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a previous study, the composition of the latter mobile phase was shown to be suitable for the chromatography of erythromycin on silica-based reversed phases [4]. It was the intention in this study to examine the performance of silanol-deactivated stationary phases compared with classical silica gel derivatized reversed phases.

EXPERIMENTAL

Samples

Pure erythromycin A (EA) was obtained by crystallization of a commercial sample as described previously [5]. Anhydroerythromycin A (AEA) [6], erythromycin A enol ether (EAEN) [7] and N-demethylerythromycin A (dMeEA) [8] were prepared from EA according to the described methods. A commercial sample was available containing EA, erythromycin B, C, F, E, (EB, EC, EF, EE), dMeEA, AEA and pseudoerythromycin A enol ether (psEAEN). Structures of these substances have been shown elsewhere [9].

Instrumentation

The chromatographic system consisted of a Model 6200 pump (Merck-Hitachi, Darmstadt, Germany), a Model CV-6-UHPa N60 injection valve (Valco, Houston, TX, USA) with a 20-μl loop, a Model 441 UV detector set at 215 nm (Waters, Milford, MA, USA) and an HP 3396 integrator (Hewlett-Packard, Avondale, PA, USA). The columns were maintained at 35°C using an immersion water-bath.

Stationary phases

The columns examined are listed in Table I. Columns I-VIII were packed by the manufacturer. Columns IX to XII were packed in the laboratory following a classical slurry-packing procedure [10]. Column characteristics are reported in Table I. Stationary phase manufacturers were as follows: A, Supelco (Bellefonte, PA, USA); B, Rockland Technologies (Newport, DE, USA); C, Shandon (Runcorn, UK); D, Merck (Darmstadt, Germany); E, Phase Separations (Queensferry, UK); F, Chrompack (Middelburg, Netherlands); G, J. T. Baker (Phillipsburg, NY, USA); and H, Dr. Buszewski (Lublin, Poland) [11].

Reagents, solvents and mobile phases

Ammonium dihydrogenphosphate and diammonium hydrogen phosphate of analytical-reagent grade (Merck) were used to prepare a 0.2 *M* phosphate buffer (pH 6.0). Tetrabutylammonium (TBA) hydrogensulphate (Janssen Chimica, Beerse, Belgium) was used to prepare a 0.2 *M* TBA solution, adjusted to pH 6.0 with 40% (w/v) sodium hydroxide, before the solution was made up to the final volume. LC-grade acetonitrile (grade S) and metha-

TABLE I

COLUMNS EXAMINED

Column No.	Stationary phase	Manufacturer	Particle size (μm)	Column[a] length (cm)	Laboratory packed (L) or prepacked (P)
I	Supelcosil LC-ABZ	A	5	25	P
II	Zorbax R_x-C_{18}	B	5	25	P
III	Zorbax SB-C,	B	5	15	P
IV	Hypersil BDS C_{18}	C	5	25	P
V	LiChrospher 60 RP-Select B	D	5	25	P
VI	Spherisorb ODS-B	E	5	25	P
VII	Polyspher RP- 18	D	10	15	P
VIII	Chromspher B	F	5	25	P
IX	Bakerbond BDC	G	5	25	L
X	Mono PB	H	5	10	L
XI	Mono-C,,	H	7	10	L
XII	Hypersil C_{18}	C	5	25	L

[a] All 4.6 mm I.D.

TABLE II

COLUMN PARAMETERS

N_{EA}, S_{EA}, N_{EAEN}, S_{EAEN} = theoretical plate number (N) and symmetry factor (S) for EA and EAEN. − TBA = results obtained with mobile phase acetonitrile-0.2 M phosphate buffer (pH 6.0)–water [x:5:(95 − x)]; +TBA = results obtained with mobile phase acetonitrile-0.2 M phosphate buffer (pH 6.0)–0.2 M TBA (pH 6.0)–water [x:5:5:(90 − x)].

Column No.	Acetonitrile content, x (%)		N_{EA}/m		S_{EA}		N_{EAEN}/m		S_{EAEN}	
	-TBA	+ TBA	-TBA	+ TBA	− TBA	+ TBA	− TBA	+ TBA	-TBA	+ TBA
I	23	20	890	3600	2.2	0.9	25000	45000	1.4	1.1
II	28	25	440	2900	1.4	0.85	8600	23000	2.2	2.2
III	28	21	920	4830	10	1.5	7900	12400	2.0	1.6
IV	28	23	1080	3200	2.5	1.0	8200	41200	2.6	2.2
V	38	28	900	3320	6.3	1.2	460	7000	6.1	4.0
VI	28	22	1420	10400	5.3	1.1	8800	20100	6.2	4.9
VII	40	21	ND	1180	ND	1.1	ND	470	ND	1.7
VIII	40	20	820	3700	3.3	1.8	2130	13600	3.3	1.9
IX	30	25	240	2640	3.2	1.1	ND	2140	> 10	2.9
X	27	24	3740	6180	0.9	0.7	17650	19000	2.9	1.5
XI	40	30	770	4650	2.8	1.5	1760	3260	3.0	2.3
XII	38	23	740	2140	2.8	1.4	2540	2920	2.4	2.0

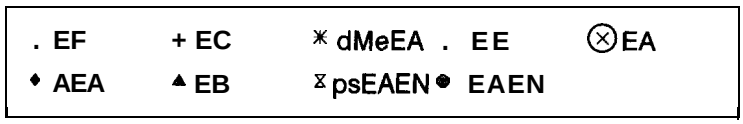
[a] ND = not determined.

nol were from Rathburn Chemicals (Walkerburn, UK). Water was distilled twice from glass apparatus. Samples were dissolved in methanol-water (1:1). Mobile phase were prepared by adding the organic modifier (x ml) to 0.2 M phosphate buffer (pH 6.0)–0.2 M TBA (pH 6.0)–water [x:5:5:(90 − x)] or [x:5:0:(95 − x)]. No correction was made for volume contraction. Mobile phases were degassed by ultrasonication. The concentration of acetonitrile in the mobile phase was adjusted for each stationary phase in order to obtain comparable capacity factors for EA (Table II). The flow-rate was 1.5 ml/min for all columns except VII, X and XI (0.6 ml/min). In mobile phases containing TBA the amount of acetonitrile had to be decreased. The components of the mobile phases were the same as described previously for the analysis of erythromycin on C_{18} silica-based reversed phases [4].

RESULTS AND DISCUSSION

The stationary phases I-XI (Table I), except VII, are silanol-deactivated silica-based reversed-phase materials, which are claimed to be suitable for the chromatography of basic compounds. Column VII contains a polymer-based C_{18} reversed phase. Most columns had a length of 25 cm. The commercial columns III and VII were available only in a 15-cm length. Laboratory-packed columns X and XI were packed in a 10-cm length owing to the limited amount of stationary phase available. The residual silanol activity of these packings should be strongly reduced or absent. If so, the presence of a quaternary ammonium compound such as TBA in the mobile phase should have no effect on the peak shape of the basic substances, as TBA is used to block interactions with residual silanols. The chromatography of the antibiotic erythromycin (pK_a = 8.8) using silica-based reversed phases is influenced by the residual silanol activity. It was seen previously that the chromatography could be improved by adding TMA [1] or TBA [4] to the mobile phase and by using aged stationary phases [1–3]. Ageing was said to eliminate metal impurities from the silica backbone, hence influencing the silanol activity. Loss of bonded phase and the consequent increase in silanol activity observed with some of the reversed-phase materials was merely seen as a secondary effect of the ageing procedure [3].

In the present study the performance of the silanol-deactivated materials in the LC of erythromycin was examined. As a reference a non-deactivated C_{18} reversed-phase material, Hypersil C_{18} (5 μm) was used (column XII). This column was not condi-



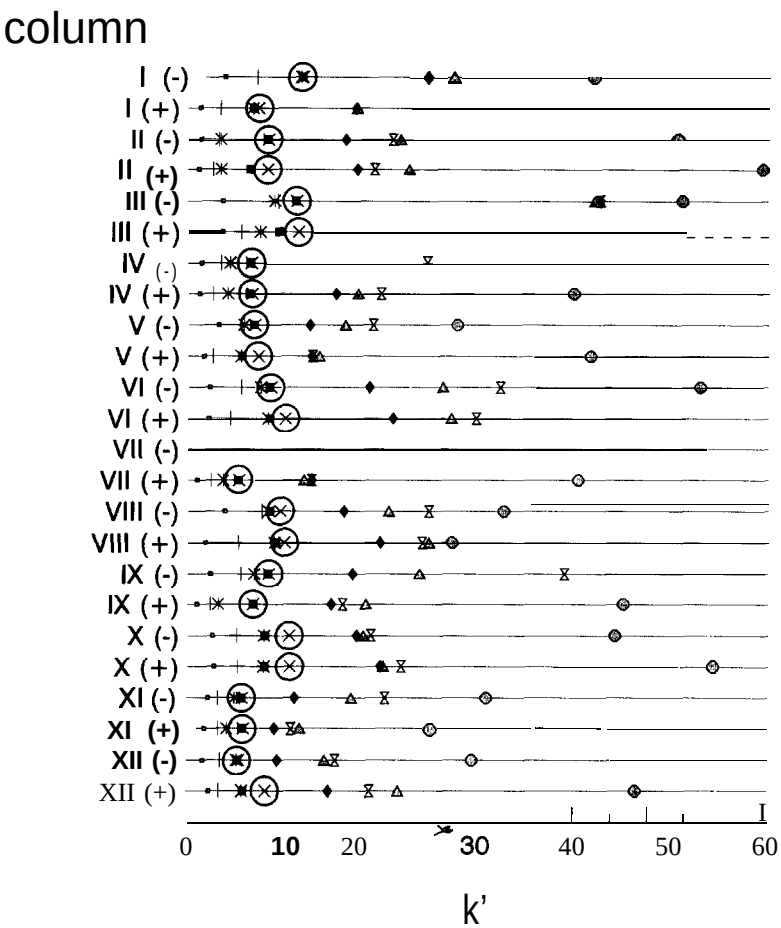


Fig. 1. Capacity factors. EA, EB, EC, EE and EF = erythromycin A, B, C, E and F, respectively; dMeEA = N-demethylerythromycin A; AEA = anhydroerythromycin A; EAEN = erythromycin A enol ether; psEAEN = pseudoerythromycin A enol ether. Mobile phase: (-) = acetonitrile-0.2 *M* phosphate buffer (pH 6.0)–water [x:5:(95 − *x*)]; (+) = acetonitrile-0.2 *M* phosphate buffer (pH 6.0)–0.2 MTBA (pH 6.0)–water [x:5:5:(90 − *x*)]. Flow rate: 1.5 ml/min except for columns X and XI (0.6 ml/min). Temperature: 35°C. Detection: UV at 215 nm.

tioned by ageing. In previous experiments it was observed that the chromatography of erythromycin was not affected much by ageing of Hypersil C_{18} [3]. The mobile phase used in this study was that previously shown to ensure separation of EE from EA on silica-based reversed phases [4]. Therefore, the type of organic modifier, the buffer and the pH of the mobile phase were not further adapted in these experiments. Indeed, it was the intention of this study to compare the performance of silanol-deactivated stationary phases with that of classical silica gel derivatized reversed phases. Capacity factors for EF, EC, dMeEA, EE, EA, AEA, psEAEN, EB and EAEN were determined on the twelve stationary

phases. First a mobile phase without TBA was used (Fig. 1). The amount (x) of acetonitrile was adjusted for each stationary phase to obtain similar capacity factors for EA and to optimize the separation of EA (see Table II).

The sequence of elution of the compounds eluted before the main peak EA was the same on all the columns as on the Hypersil C_{18} columns viz., EF, EC, dMeEA, except for column III (a C_8 material), where EC was eluted after dMeEA. Results for column VII were not included because the selectivity and peak shapes were very poor, so that capacity factors, symmetry factors and theoretical plate numbers could not be determined. It should be emphasized that this column contained a polymer-based stationary phase. The separation of dMeEA and EE from EA is most difficult to achieve. Satisfactory separation between dMeEA and EA was obtained on columns II, III, IV, V, VI, IX and X, while partial separation was obtained on columns I and VIII. On columns XI and XII dMeEA was not separated from the main peak. Separation between EE and EA was obtained only on columns I and VIII (partial separation of the pair EE + dMeEA as a shoulder on the main peak) and on column X (satisfactory separation between the pair EE + dMeEA and EA).

The order of elution for AEA, EB, psEAEN and EAEN was not the same on all the columns. On columns II and XI, EB was eluted after psEAEN. AEA was eluted close to EB and psEAEN on column III. EAEN was always retained strongly ($k' > 30$). On several columns k' for EAEN exceeded 60.

Fig. 1 also shows the results obtained with mobile phases containing TBA. Addition of TBA to the mobile phase caused the retention times to decrease, so the amount of organic modifier had to be adjusted in order to maintain similar capacity factors. Separation between EE, dMeEA and EA was improved on columns II, III, V, VI and XII. Overall, the pair EE + dMeEA was separated completely from EA on columns VI and X. Columns I, III, VIII and XII gave a partial separation. The order of elution EB–psEAEN was reversed on columns III and V compared with the results obtained with the TBA-free mobile phase.

Of course, the separation is governed not only by the selectivity but also by the theoretical plate number (N) per metre and the symmetry factor (S), which were calculated for EA and for the strongly retained EAEN according to the prescriptions of the European Pharmacopoeia [12]. EAEN was shown previously to be very sensitive to changes in silanol activity, caused by ageing of the stationary phase [3]. In Table II it can be seen that the symmetry factor for EA and EAEN always decreased on adding TBA to the mobile phase. On columns III, V, VI and IX an important improvement in the symmetry was observed. Compared with the non-deactivated column XII, S_{EA} + TBA was better on all the columns, except on columns VIII and XI. The theoretical plate number for EA (N_{EA}) always increased using a mobile phase containing TBA. The highest N_{EA} values were found for the columns that gave the best separation between EE and EA (columns VI and X). N_{EAEN} + TBA was higher on all the deactivated phases than on the non-deactivated Hypersil C_{18} column, except for column IX. Column VII, the polymer-based C_{18} stationary phase, which does not have residual silanols, showed a poor efficiency. The reason for this is not clear.

Fig. 2 shows typical chromatograms obtained on columns XII and VI. For comparison, the separation obtained on a wide-pore poly(styrene–divinylbenzene) stationary phase (PLRP-S, 8 μm, 1000 A) using a previously described method is shown [9]. A special mobile phase was developed for this column. A major advantage of this polymer phase is that an alkaline mobile phase, in this instance of pH 9, can be used with no effect on the stability. Alkaline mobile phases allow a better separation of erythromycin.

The addition of TBA to the mobile phase clearly improved the quality of the separation. It was stated previously that erythromycin and related substances are very sensitive to small changes in the sorbent surface, especially to changes in the content of residual silanols [2]. TBA can act as a shielding agent for these silanols. At the same time a positively charged TBA layer can be formed on the stationary phase surface, causing repulsion of the positively charged erythromycin molecules. In order to avoid ionization of silanols it is better to use mobile phases of low pH. However, the major concern in this study was to compare the performance of these phases with that of a non-deactivated silica-based reversed phase for the chromatography of erythro-

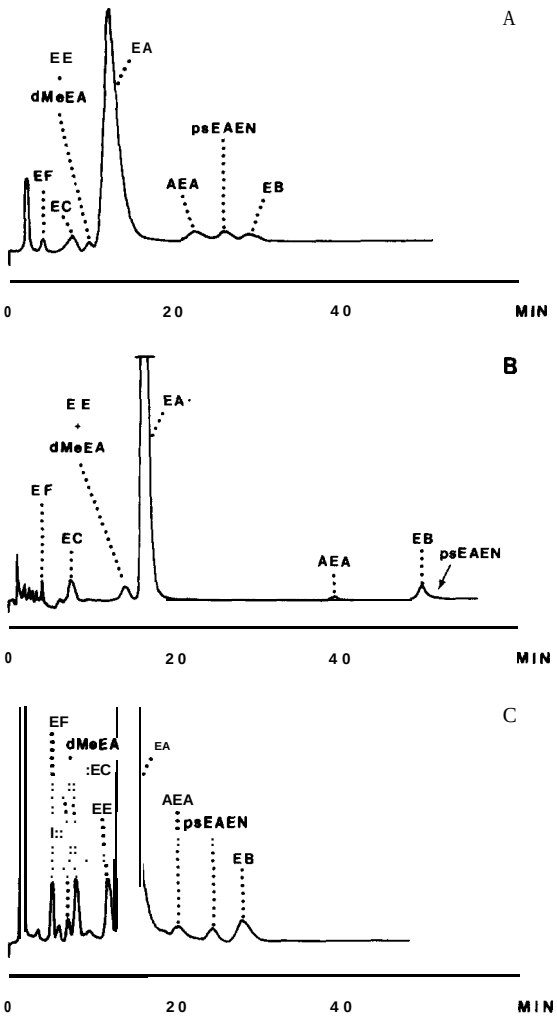


Fig. 2. Typical chromatograms. (A) Hypersil C_{18}, 5 μm (column XII); (B) Spherisorb ODS-B, 5 μm (column VI); (C) PLRP-S. 8 μm, 1000 Å [8].

mycin. Therefore, it was necessary to use a mobile phase comparable to that developed for the non-deactivated phases. On these phases, statisfactory separation of erythromycin could not be achieved using an acidic mobile phase. The results obtained in this study show that residual silanol activity still exists with these silanol-deactivated stationary phases when used with a mobile phase of neutral pH.

CONCLUSION

The different deactivated silica-based reversed phases examined showed variable performance in the chromatography of erythromycin A and related substances. Addition of TBA improved the peak symmetry and the efficiency, although this was not expected when using silanol-deactivated reversed phases. Compared with a non-deactivated reversed phase, only two columns (VI and X) out of eleven gave a better separation of EA when using a similar mobile phase. A method involving a wide-pore poly (styrene-divinylbenzene) stationary phase (PLRP-S, 8 μm, 1000 A) and a specially developed mobile phase has been shown to be a better alternative for the LC of erythromycin. This method has been discussed elsewhere [9]. The fact that some stationary phases did not perform well in these experiments does not mean that they cannot be very suitable for solving other chromatographic problems.

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