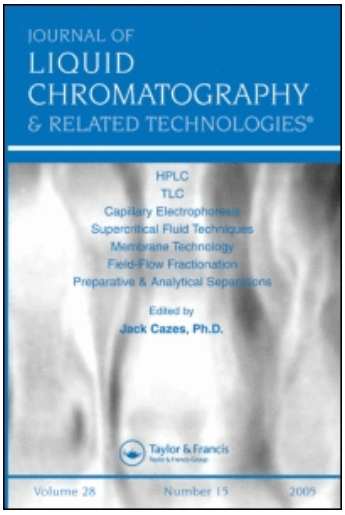

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Improved Separation of Erythromycin on aged Reversed-Phase Columns. II

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IMPROVED SEPARATION OF ERYTHROMYCIN ON AGED REVERSED-PHASE COLUMNS. II

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ABSTRACT

In a first paper it was reported that chromatography of erythromycin derivatives on short chain reversed-phases could be improved by conditioning the column by heating it at 120 °C after it had been flushed with a mobile phase methanol-water-1M phosphoric acid (50:45:5). Besides an improvement of the resolution and symmetry of the peaks, a reduction of the retention times was observed. In this paper the effect of conditioning was examined using C18 reversed-phases. Here also an improvement of the chromatography of erythromycin derivatives was achieved but reduction of retention times was only observed on a low loaded phase. It is believed that the reduction of the retention times is merely a secundary effect of the conditioning, which removes part of the bonded phase from the less stable stationary phases. The improvement of the chromatography must be related to modifications at the silica gel surface.

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INTRODUCTION

In the first part we concluded that the chromatography of erythromycin on short chain reversed-phase materials(C2,TMS and C8) can be improved in terms of resolution and peak symmetry by conditioning the column with an acidic mobile phase (1). Columns, flushed with metanol-water-1M phosphoric acid (50:45:5), were tightly closed and heated at 120 °C for up to 15 h. On most stationary phases we also observed a reduction of the retention times, especially for erythromycin A enol ether, the last eluting erythromycin component. This reduced the total analysis time.

The stationary phases were analysed prior and subsequent to conditioning. Depending on the chemical nature of these phases, an amount of bonded phase was lost during the conditioning. The shorter chain reversed-phases and materials prepared with monofunctional silylating reagents were more labile. The silanol activity, as measured by the adsorption of methyl red, increased consistently during conditioning. It was concluded that a certain level of silanol acitivity was beneficial to the chromatography of erythromycins but it was also mentioned that removal of residual metals during the conditioning may play a role since this can influence the acidity of the silanols.

Since, the conditioning procedure has been applied on C18 materials. The results are described in this paper.

EXPERIMENTAL

Apparatus

A Waters M 45 solvent delivery system (Milford, MA, USA) was used with a Valco Model CV-6-UHPa N 60 injection

valve (Houston, TX, USA), fitted with a 20 µl loop, a Waters Model 441 UV detector, set at 215 nm and a HP 3390 integrator (Hewlett - Packard, Avondale, PA,USA). The columns were kept at 35 °C by a circulation of water.

Columns

All the columns (25 cm x 4.6 mm ID) were freshly packed in the laboratory following a described slurry packing procedure (2). The reversed-phases were : Hypersil C18 (Shandon, Runcorn, UK), RSil C18 LL and RSil C18 HL (RSL - Biorad, Eke, Belgium), Spherisorb ODS 1 and ODS 2 (Phase Separations, Queensferry,UK).Characteristics of these materials are given in Table 1.

Mobile Phase

The mobile phase consisted of acetonitrile (x % v/v), 0.2 M ammonium phosphate buffer pH 6.5 (5 % v/v), 0.2 M

TABLE 1

Characteristics of the Investigated C18 Phases

Stationary phase	Type of modification	Carbon content (%)	Endcapping	dp (µm)	Surface area (m^2/g)	Ref.
Hypersil C18	T	9	Yes	5	170	3,4
RSil C18 LL	T	9	Yes	10	350	4
Rsil C18 HL	T	18	Yes	10	350	4
Spherisorb ODS 1	T	7	Yes	5	220	4
Spherisorb ODS 2	T	12	Yes	5	220	4

dp = particle diameter
T = trifunctional silane

tetramethylammonium phosphate (TMA) pH 6.5 (20 % v/v) and water (75-x % v/v). Acetonitrile HPLC grade was obtained from Rathburn (Walkerburn, UK). Ammonium dihydrogen phosphate and diammonium hydrogen phosphate were pro analysi from Merck (Darmstadt, GFR), 0.2 M solution of these salts were mixed to prepare the buffer. The 0.2 M TMA solution was prepared from a 20 % m/v solution of tetramethylammonium hydroxide in methanol (Janssen Chimica, Beerse, Belgium). The pH of this solution was adjusted to 6.5 with 85 % phosphoric acid pro analysi (Merck) before the solution was brought to the final volume. Water was distilled twice from glass. Mobile phases were degassed by ultrasonication and the flow rate was 1.5 ml/min.

Samples for Chromatography

A commercial erythromycin, containing erythromycin C(EC), *N*-demethylerythromycin A (dMeEA), erythromycin A (EA), erythromycin B (EB), anhydroerythromycin A (AEA) and erythromycin A enol ether (EAEN) was used throughout this study. The chemical structures of the components were shown previously (5).

Conditioning of Packed Stationary Phases

Columns were equilibrated with methanol-water-1 M phosphoric acid (50:45:5) for at least one hour. The tightly closed columns were then heated at 120 °C.

Conditioning of Stationary Phases in Bulk

To 0.5 g of reversed-phase in a 10 ml glass tube, 1 ml of conditioning solvent, methanol-water-1 M

phosphoric acid (50:45:5), was added. The glass tube was then flame-sealed and heated in an oven at 120 °C. After conditioning the content of the tube was filtered and the residue was washed with 50 ml of a mixture of methanol-water (1:1) and 20 ml of pure methanol.

Determination of the Methyl Red Adsorption Value (MRAV)

The MRAV (mg/g) was determined on 50 mg samples as described previously (6).

Loss on Ignition (LOI)

Samples (0.3 g) were first dried at 120 °C for at least 3 h and the LOI was obtained by heating at 700 °C for 4 h. Results are expressed as % m/m of the residual mass.

RESULTS AND DISCUSSION

Conditioning of C18 columns

In a first set of experiments freshly packed Hypersil C18, RSil C18 LL, Spherisorb ODS 1 and ODS 2 columns were conditioned as described in experimental. As we were seeking for both improvements of the chromatography and reduction of the analysis time as determined by the retention time of EAEN, these columns were aged for a total time of 50 to 70 h. Results are shown in Figs. 1 to 4. The total conditioning time was reached through several steps : in each step the columns

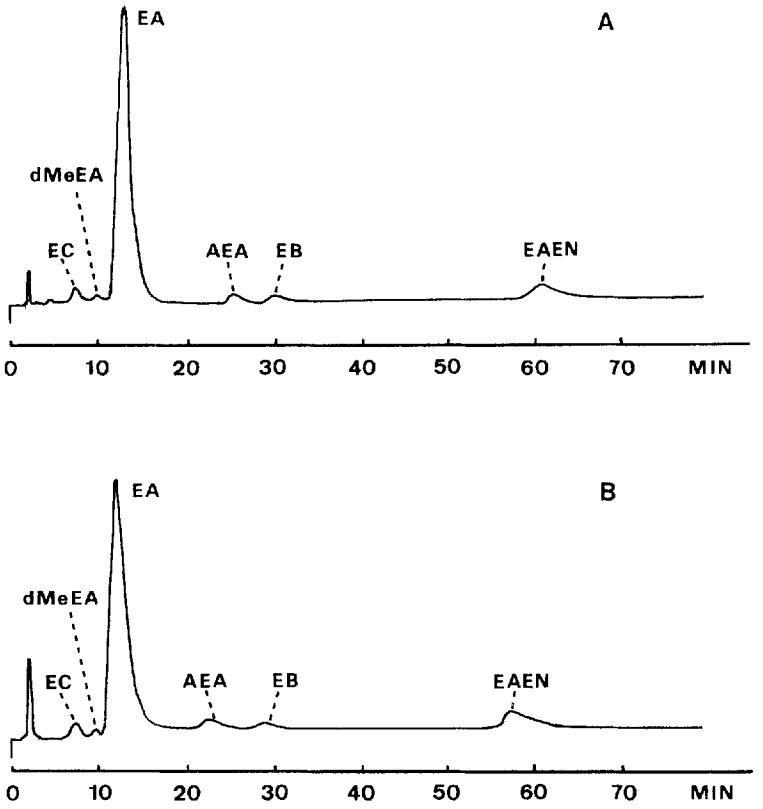

Figure 1.
Chromatograms of erythromycin on Hypersil C18. Mobile phase : acetonitrile - 0.2 M tetramethylammonium phosphate pH 6.5 - 0.2 M ammonium phosphate buffer pH 6.5 - water (30:20:5:45). Flow rate : 1.5 ml/min. Temperature : 35 °C. Detection : UV at 215 nm. Injected amount : about 250 µg.
EC = erythromycin C, dMeEA = N-demethylerythromycin A, EA = erythromycin A, AEA = anhydroerythromycin A, EB = erythromycin B and EAEN = erythromycin A enol ether.
A : new column, B : after conditioning for 50 h.

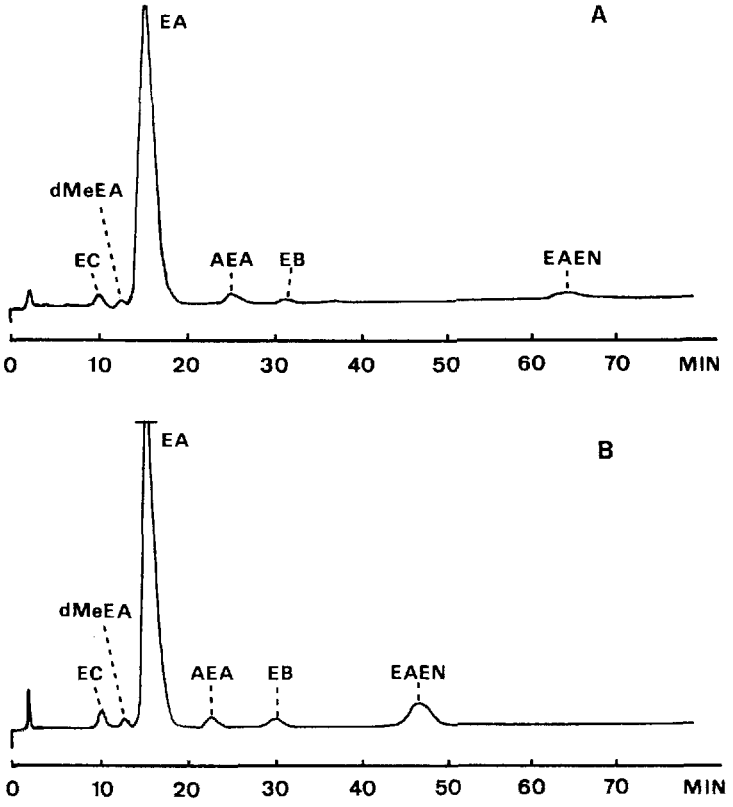

Figure 2.
Chromatograms of erythromycin on RSil C18 LL. Mobile phase : acetonitrile - 0.2 M tetramethylammonium phosphate pH 6.5 - 0.2 M ammonium phosphate buffer pH 6.5 - water (35:20:5:40). See fig. 1 for other information. A : new column, B : after conditioning for 60 h.

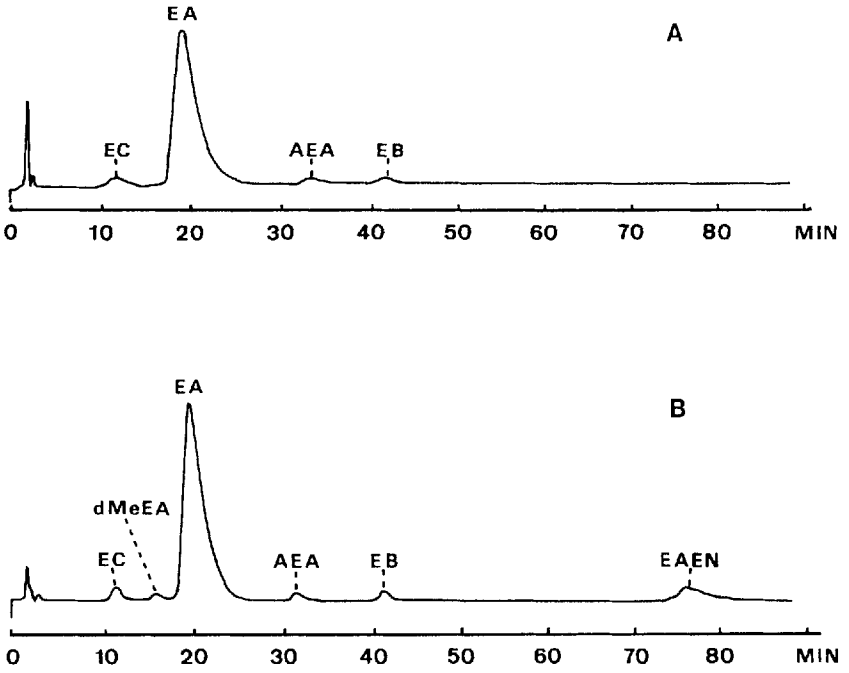

Figure 3.
Chromatograms of erythromycin on Spherisorb ODS 1. See fig. 2 for other information. A : new column, B : after conditioning for 60 h.

were flushed and heated for maximum periods of about 15 h. The evolution of the conditioning was assessed by HPLC of erythromycin. Within the first period of conditioning an improvement of the chromatography was observed on Spherisorb ODS 1, ODS 2 and RSil C18 LL, but not on Hypersil C18. On further conditioning we observed only with RSil C18 LL a slight decrease in retention times, while on Spherisorb ODS 1 and ODS 2 the retention times remained almost unchanged.

In a second set of experiments we focussed on the improvement of the separation, obtained by conditioning

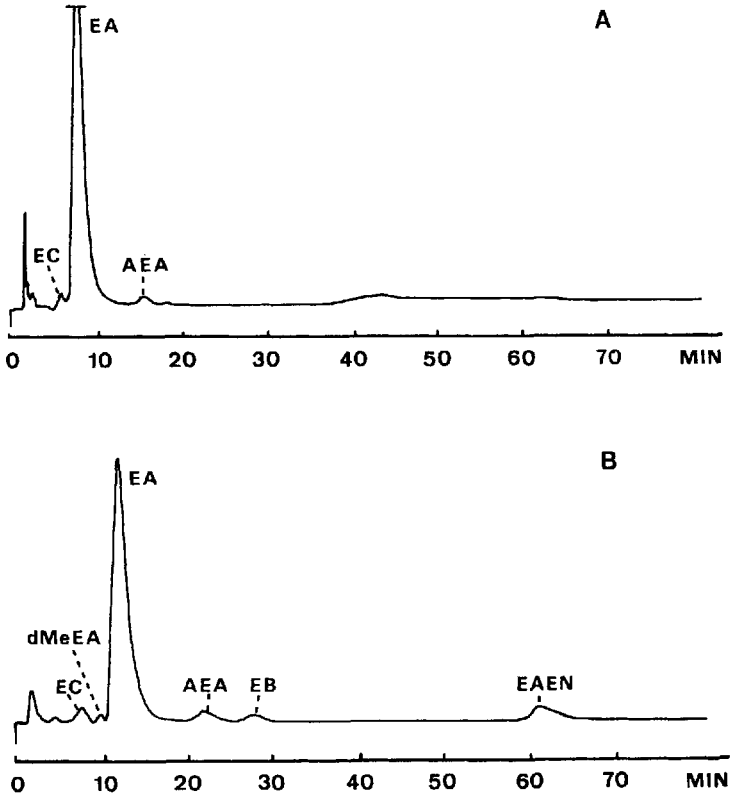

Figure 4.
Chromatograms of erythromycin on Spherisorb ODS 2. See fig. 2 for other information. A : new column, B : after conditioning for 70 h.

for a short period (3 h). This time we used columns, freshly packed with RSil C18 LL, RSil C18 HL, Spherisorb ODS 1 and Spherisorb ODS 2. Results of these experiments are summarized in Table 2.

Plate numbers and symmetry factors were calculated for the peaks corresponding to EA, the main component of erythromycin and to EAEN, the last eluted component. The

TABLE 2

Influence of Conditioning on the Efficiency and Symmetry

	EA		EA[a]		EAEN		EAEN[a]	
Stationary phase	N/m[b]	Symmetry factor[c]	N/m[b]	Symmetry factor[c]	N/m[b]	Symmetry factor[c]	N/m[b]	Symmetry factor[c]
RSil C18 LL	4340	1.9	5880	1.5	800	> 3	12760	1.9
RSil C18 HL	3560	2.2	3430	1.5	5190	> 3	11720	1.8
Spherisorb ODS 1	3260	1.5	4500	1.5	810	> 3	6820	2.3
Spherisorb ODS 2	3540	1.5	7970	1.5	1875	> 3	6950	1.5

[a], Mobile phase : acetonitrile - 0.2 M tetramethylammonium phosphate pH 6.5 - 0.2 M ammonium phosphate buffer pH 6.5 - water (35:20:5:40) after conditioning in a single operation of 3 h.

[b], N/m = theoretical plates per meter calculated from the formula $n = 5.54\ (t_R/b_{0.5})^2 \times 4$ with t_R = retention time of the peak and $b_{0.5}$ = peak width at half height of the peak.

[c], calculated from the expression : $b_{0.05}/2A$ with $b_{0.05}$ = peak width at one twentieth of the peak height and A = distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one - twentieth of the peak height.

gain in plate number was most spectacular for EAEN with an increase from 800 to 12760 plates per meter on RSil C18 LL. On all columns the symmetry factor decreased for both peaks but here also the largest improvement was observed for EAEN.

Conditioning in bulk

As measuring the LOI and MRAV of the packing materials needs destruction of the columns, we developed a method for conditioning stationary phases in bulk (1). The C18 phases were thus aged in glass tubes for 50 h. Methyl red adsorption (MRAV) was used to determine the relative amount of the accessible free silanol groups (6,7). The loss on ignition (LOI) is related to the amount of bonded phase in the reversed-phase material. Table 3 summarizes the MRAV and LOI data obtained before and after treatment. More details on the determination and on the use of LOI to calculate the bonded phase content were described previously (1). The figures for bonded phase content correspond quite well with the figures for carbon content reported in Table 1. The conditioning period of 50 h does not affect the C18 phases much in terms of loss of bonded phase and consequent increase of silanol activity (MRAV) except for RSil C18 LL. This is consistent with the decrease of retention times of erythromycin components upon aging (Fig. 2). The important increase in MRAV upon aging can be explained by the fact that this low loaded material loses a major part of its endcapping upon aging whereafter an important part of its surface can interact with methyl red. The loss of bonded phase is smaller for the highly loaded RSil C18 HL where less of the easily removable endcapping is present and consequently less of the surface is liberated for interaction with methyl red.

RSil has a larger specific surface than Spherisorb (See Table 1). This is confirmed by the MRAV values of parent silicas in Table 3. A lower carbon content will be sufficient to ensure a denser coverage of the Spherisorb surface, which is therefore less easily set free for interaction with methyl red. This is illustrated by the fact that even a loss of 8 % of the bonded phase of Spherisorb ODS 2 does not lead to any adsorption of methyl red. The Hypersil is perfectly stable against the aging procedure.

Discussion

The C18 stationary phases examined here are definitely more stable during conditioning than the short chain phases which were examined previously (1). This can be concluded from the conditioning experiments in bulk but also from the stability of the retention times of the different erythromycin components observed in the chromatographic experiments.

It is remarkable that the improvement of the chromatography upon conditioning is realised during a first, short conditioning period. Taking into account the observed stability of C18 phases it is improbable that a relatively large loss of bonded phase and consequent increase of the silanol activity occurs within this short period. This indicates that 'degradation' of the phase is not the key to the improvement of the chromatography. In the previous part of this paper it was already pointed out that this phenomenon is possibly related to the removal of metal impurities (1). These metal impurities are considered by several authors to be responsible for deleterious effects during chromatography (8 - 12). Metal impurities would act either directly on the chromatographed solutes or indirectly by influencing

TABLE 3

Modification of the Characteristics of C18 Phases upon Conditioning in Bulk

Stationary phase	Conditioning time (h)	LOI[a]		LOI of parent silica	Bonded phase content[b]		Loss of bonded phase[c] (%)	MRAV[d] (%)		Typical MRAV of parent silica
		I	II		I	II		I	II	
Hypersil C18	50	13.5	13.5	2.2	10.2	10.2	0	0	0	97
RSil C18 LL	50	14.9	13.5	4.1	9.7	8.6	11	17	56	212
RSil C18 HL	50	26.9	25.3	4.1	18.6	17.5	6	1	12	212
Spherisorb ODS 1	50	10.6	10.4	1.3	8.5	8.3	2	5	17	91
Spherisorb ODS 2	50	15.9	14.6	1.3	12.7	11.7	8	0	0	91

[a], LOI = loss on ignition, expressed as a percentage of the final mass, I = before conditioning, II = after conditioning.

[b], Calculated from the LOI of the reversed-phase and the LOI of a parent bare silica. The values are expressed now as a percentage of the original mass of the packing material. See ref. 1 for more details. See a for I and II.

[c], Difference in % between the bonded phase content I and II.

[d], MRAV = Methyl Red Adsorption Value (mg methyl red/g phase). See a for I and II.

the acidity of neighbouring silanol groups (9 - 12). Verzele has shown that metal impurities can be partly removed by acid washing with hydrochloric acid (8). It has been hypothesized that the acid treatment to remove a fraction of metal impurities from silica gel prior to silanization results in a more homogeneous surface and a better support for chromatography (9).

In the light of the results obtained with the C18 phases, it is indicated to explain the effects of a short conditioning with an acidic mobile phase and the consequent improvement of the chromatography of erythromycin in terms of removal of interfering metal impurities. The loss of bonded phase and the consequent increase of silanol activity, which was observed to occur more rapidly on short chain reversed-phase materials, then should be considered merely as a secundary consequence of the conditioning procedure. This secundary effect is most striking for the low loaded RSil LL phase. The lack of any effect on the Hypersil column can be explained by its very good stability and by the absence of metal impurities. This is sustained by the good quality of the chromatography, in terms of separation of dMeEA and EA, obtained with the unconditioned Hypersil column.

CONCLUSIONS

The chromatography of erythromycin on C18 phases may be improved by conditioning as was described previously for short chain phases. This conditioning procedure, which consists of flushing the column with methanol-water-1M phosphoric acid (50:45:5) and heating the closed columns at 120 °C for a short period, does not reduce the

retention times of the erythromycin derivatives in the same way as was observed for short chain reversed-phases. This reduction in retention time seems to be a secundary effect of the conditioning and is due to partly removal of the bonded phase which occurs more easily with the less stable short chain phases. The improvement of the chromatography must be primarily related to modifications at the silica gel surface. It is possible that the conditioning procedure removes metal impurities from the silica backbone and hence influences the silanol activity. It seems interesting to examine whether such conditioning can also improve the chromatography of other substances.

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