*Journal of Chromatography*, 553 (1991) 285–297
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2289

# Evaluation of different packings for high-performance liquid chromatographic analysis of alkyl lysophospholipids

J. COENE*
*Laboratory of Pharmaceutical Chemistry and Drug Quality Control, State University of Ghent, Harelbekestraat 72, B-9000 Ghent (Belgium)*
M. GHIJS
*Laboratory for Organic Chemistry, State University of Ghent, Krijgslaan 281 (S4), B-9000 Ghent (Belgium)*
E. VAN DEN EECKHOUT and W. VAN DEN BOSSCHE
*Laboratory of Pharmaceutical Chemistry and Drug Quality Control, State University of Ghent, Harelbekestraat 72, B-9000 Ghent (Belgium)*
and
P. SANDRA
*Laboratory for Organic Chemistry, State University of Ghent, Krijgslaan 281 (S4), B-9000 Ghent (Belgium)*

## ABSTRACT

The analysis of the alkyl lysophospholipid 1-octadecyl-2-O-methyl-D,L-glycero-3-phosphorylcholine is currently under investigation because of its anticancer activity. The chromatographic behaviour of this compound and its 1-hexadecyl-2-O-methyl-D,L-glycero-3-phosphorylcholine homologue, which is used as an internal standard for pharmacokinetic studies, on various liquid chromatography packings gave rise to many problems. The retention and elution characteristics of both ether phospholipids were studied on silica, straight polyethyleneglycol-coated silica, reversed-phase materials, base-deactivated reversed-phase silica and polymeric resins.

## INTRODUCTION

1-Octadecyl-2-O-methyl-D,L-glycero-3-phosphorylcholine (Et-18-OMe) belongs to a family of new antitumour drugs. The group of alkyl lysophospholipids (ALPs) contains synthetic analogues of the naturally occuring 2-lysophosphatidylcholine which are selectively cytotoxic for tumour cells [1,2]. To study the influence of Et-18-OMe concentration and the effect of its metabolism in cells on antitumour activity, precise analytical methods are required. The 1-hexadecyl-2-O-methyl-D,L-glycero-3-phosphorylcholine (Et-16-OMe) homologue is an inactive derivative, which can be used as an internal standard.

Capillary gas chromatography has been investigated for the analysis of ether phospholipids. The technique appears to be very attractive because of its intrinsically high resolving power and high sensitivity. However, dephosphorylation is required and this reaction is far from being quantitative [3]. High-performance thin-layer chro-

0021-9673/91/$03.50 

matography with fluorescence detection is a fast and simple analytical technique for this class of compounds, but lacks sensitivity [4].

High-performance liquid chromatography (HPLC) is a well known technique for the differentiation of lipid and phospholipid classes. The HPLC of phospholipids is usually performed by straight-phase liquid chromatography [5–8]. The separation is based on polarity differences between the phospholipid classes and not on chain length. However, the use of normal-phase silica is associated with problems of column stability and the reproducibility of retention data. The work reported here evaluated different reversed-phase materials, and the problems encountered and the selectivity characteristics of the columns for the separation of Et-16-OMe and Et-18-OMe are described in this paper.

## EXPERIMENTAL

A Perkin-Elmer Series 4 liquid chromatograph, equipped with a Perkin-Elmer LC terminal and a Series 4 control module, was used in combination with a D.D.L. 11 light scattering detector (Sedere, France). Nebulization was performed using air at 2 bar and 40°C. Peak registration was performed with a Varian A-25 recorder or a Shimadzu C-R6A Chromatopac integrator. For refractive index determination an R401 differential refractometer (Waters Assoc., U.S.A.) coupled to a Waters M-45 pump was used. The samples were injected using a Valco valve with a 25-$\mu$l loop.

### *Columns*

Normal-phase LC was performed on a LiChroma column (A), 150 × 4.6 mm, 5 $\mu$m (Alltech, Belgium) and on polyethyleneglycol (PEG)-coated silica (B), 150 × 4.6 mm, 5 $\mu$m. The method using the PEG coating was performed according to the procedure of Ghijs *et al.*[9].

LiChrosorb RP-2 (C) (150 × 4.6 mm, 5 $\mu$m) was supplied by Alltech; LiChrosorb RP-8 (D) and RP-18 (E) (150 × 4.6 mm, 5 $\mu$m) and Polygosil-60-D-10-CN (F) (250 × 4.6 mm, 10 $\mu$m) were purchased from Chrompack (Belgium). LiChrosorb 60 RP Select B (G) (Merck, Germany) contains base-deactivated silica with a pore size of 60 Å instead of 100 Å (150 × 4.6 mm, 5 $\mu$m). The phase was not end-capped, but specially treated to obtain the maximum bonding of methyloctyl groups [10]. An Apex Symm columm (H) (150 × 4.6 mm, 5 $\mu$m) was used which contained silica-based octadecyl reversed-phase material, which is claimed to be base-deactivated (Jones Chromatography, U.S.A.). Ultrabase $C_{18}$ (I) (250 × 4.6 mm, 5 $\mu$m) (Chromatech, France) has been developed for the same reason. Vydac 201 TP 54 (J) (250 × 4.6 mm, 10 $\mu$m) (Separations Group, Hesperia, CA, U.S.A.) is a polymeric octadecyl phase.

PRP-1 (K) is a macroporous styrene divinylbenzene copolymer (Hamilton, Switzerland). The column dimensions are 150 × 4.1 mm, 5 $\mu$m. PRLP-S (L) 100 Å (250 × 4.6 mm, 5 $\mu$,) is a rigid polystyrenedivinylbenzene without a hydrophobic ligand, and was supplied by Polymer Labs. (Separation Science Division, U.K.).

### *Reagents*

Et-18-OMe (clinical grade) was obtained from Medmark Pharma (Germany). Et-16-OMe and Et-18-OMe (Fig. 1) (analytical grade) were supplied by Sigma

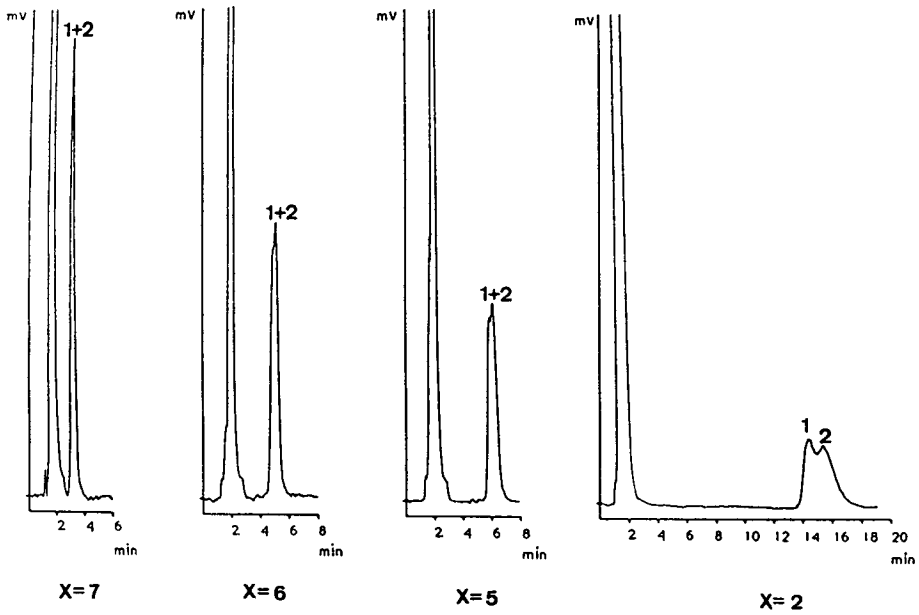


Fig. 3. Separation of (1) Et-18-OMe and (2) Et-16-OMe on column B (PEG). Conditions: mobile phase, chloroform–methanol–water (60:34:*x*, v/v); flow-rate, 1.0 ml/min; sample loop, 20 μl; and concentration mixture, *ca.* 200 μg/ml.

TABLE I

COMPARISON OF RETENTION CHARACTERISTICS OF ET-16-OME (16) AND ET-18-OME (18).

Columns used were C to L with mobile phases of methanol and methanol containing 0.5% (v/v) strong ammonia solution. Number of experiments = 3.

| Column | Methanol | | | Ammonia solution–methanol (0.5:99.5, v/v) | | |
|---|---|---|---|---|---|---|
| | $k'_{16}$ [a] | $k'_{18}$ [b] | $\alpha$ [c] | $k'_{16}$ [a] | $k'_{18}$ [b] | $\alpha$ [c] |
| C | ∞ | ∞ | – | 4.356 | 4.356 | 1.000 |
| D | ∞ | ∞ | – | 1.258 | 1.348 | 1.072 |
| E | ∞ | ∞ | – | 1.528 | 2.067 | 1.353 |
| F | 0.961 | 0.961 | 1.000 | 1.046 | 1.046 | 1.000 |
| G | 1.103 | 1.224 | 1.110 | 1.103 | 1.224 | 1.110 |
| H | ∞ | ∞ | – | ∞ | ∞ | – |
| I | 2.607 | 3.607 | 1.384 | 2.643 | 3.786 | 1.432 |
| J | ∞ | ∞ | – | ∞ | ∞ | – |
| K | 1.371 | 1.924 | 1.404 | 1.289 | 1.871 | 1.452 |
| L | 1.377 | 1.926 | 1.399 | 1.372 | 1.936 | 1.411 |

[a] Capacity factor for Et-16-OMe.
[b] Capacity factor for Et-18-OMe.
[c] Selectivity, as defined in text.

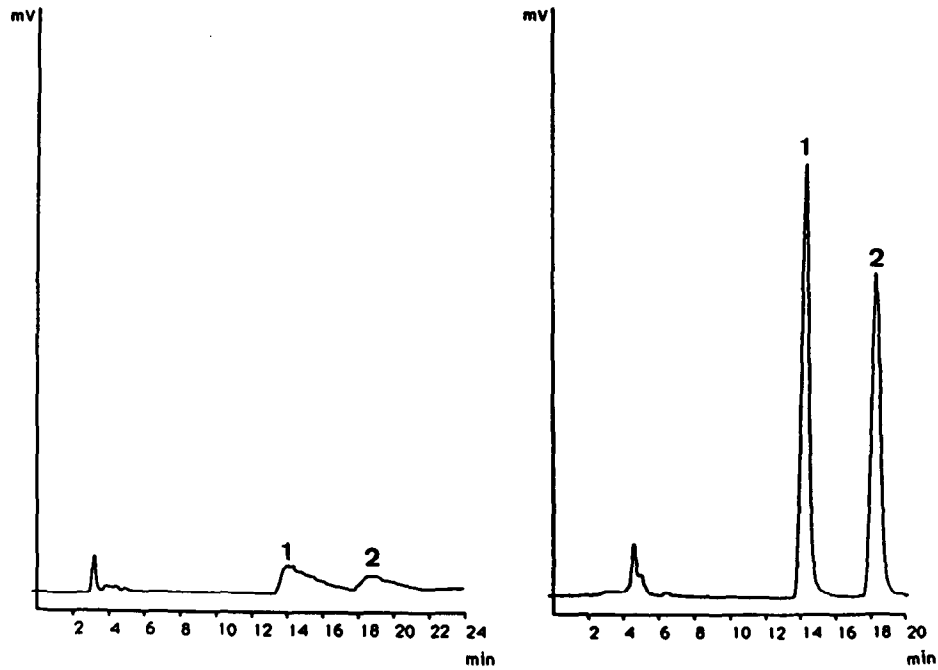


Fig. 4. Separation of (1) Et-16-OMe and (2) Et-18-OMe on column H (left) (Apex Symm RP18) and I (right) (Ultrabase C18). Conditions: mobile phase, 100% methanol; flow-rate 1.5 ml/min; sample loop, 20 $\mu$l; and concentration mixture, *ca.* 250 $\mu$g/ml.

of silica. The separation mechanism is attributed to hydrophobic interactions between the solutes and the alkyl chains of the chemically modified silica gel.

Isocratic elution with methanol on RP-2 (C), RP-8 (D) and RP-18 (E) phases was insufficient for the separation of the two studied compounds. This can be attributed to interactions with free silanol groups. These silanols are highly acidic and are very reactive for the quaternary ammonium group. The adsorption or desorption of the phosphocholine moiety by the silanols has slow reaction kinetics. The effect of such silanophilic interactions on retention has been tested for other recently introduced HPLC materials. Manufacturers claim that their materials posses the benefits of silica-based reversed phases but with a minimized silanol activity.

The various retention characteristics are summarized in Table I, in which capacity factors and selectivities are given. Capacity factors ($k'$) are measured by the relationship $(t_R - t_0)/t_0$, where $t_R$ is the retention time of the compound of interest and $t_0$ is the column void volume measured using the solvent disturbance peak when methanol is injected onto the column. Selectivities ($\alpha$) for the studied ALPs are calculated by the formula $k'_2/k'_1$, where $k'_2$ is the capacity factor of the compound with the highest retention and $k'_1$ is the capacity factor of the compound with the lowest retention.

It should be noted that the Apex Symm column (H), in contrast to the Ultrabase C 18 analogue (I), shows a low efficiency (Fig. 4). To obtain a good selectivity, an octadecyl coating is necessary. The hydrophobic affinity between the octyl chains and

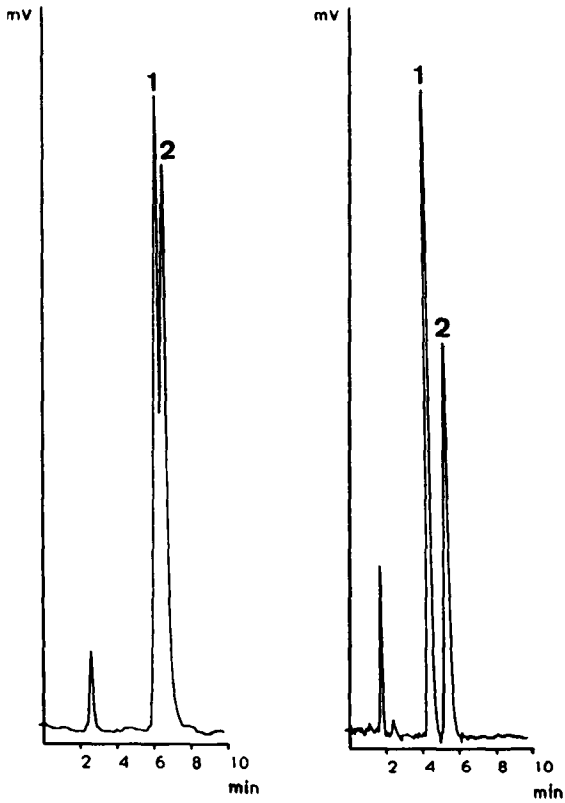


Fig. 5. Separation of (1) Et-16-OMe and (2) Et-18-OMe on column G (left) (LiChrosorb 60 RP Select B) and E (right) (LiChrosorb RP-18). Conditions: mobile phase 100% methanol (column G), strong ammonia solution–methanol (0.5:99.5, v/v) (column E); flow-rate 1.0 ml/min; sample loop, 20 μl; and concentration mixture *ca.* 200 μg/ml.

the long alkyl chain of the ALPs is too low to provide sufficient separation (Fig. 5).

Current methods to reduce the base–silanol interaction not only consist in modifying the stationary phase, but also in mobile phase modification. This includes pH changes or increasing the ionic strength of the mobile phase to suppress ionization and ionic interactions and the addition of low-molecular-weight amines to compete with the analyte base for adsorption on to the acidic silanols. In fact, good chromatographic practice often requires masking the surface silanols by doping the eluent with low concentrations of an aliphatic amine, such as triethylamine (TEA) to reduce peak tailing. The use of such 'modifiers' is restricted because there are limitations on the range of materials which can be added to the mobile phase when light scattering is used as a detection method. For the same reason, ion-pair reagents such as butanesulphonic acid could not be used in the ion-pair chromatographic separation of these quaternary ammonium compounds. Only ammonia and acetic acid could be added to the eluent without the mass detector moving off-scale. Doping methanol with 0.5% acetic acid (v/v) to suppress the ionization of the silanols was not succesful.

Ammonia obviously masked the silanols in the same way as it did in the

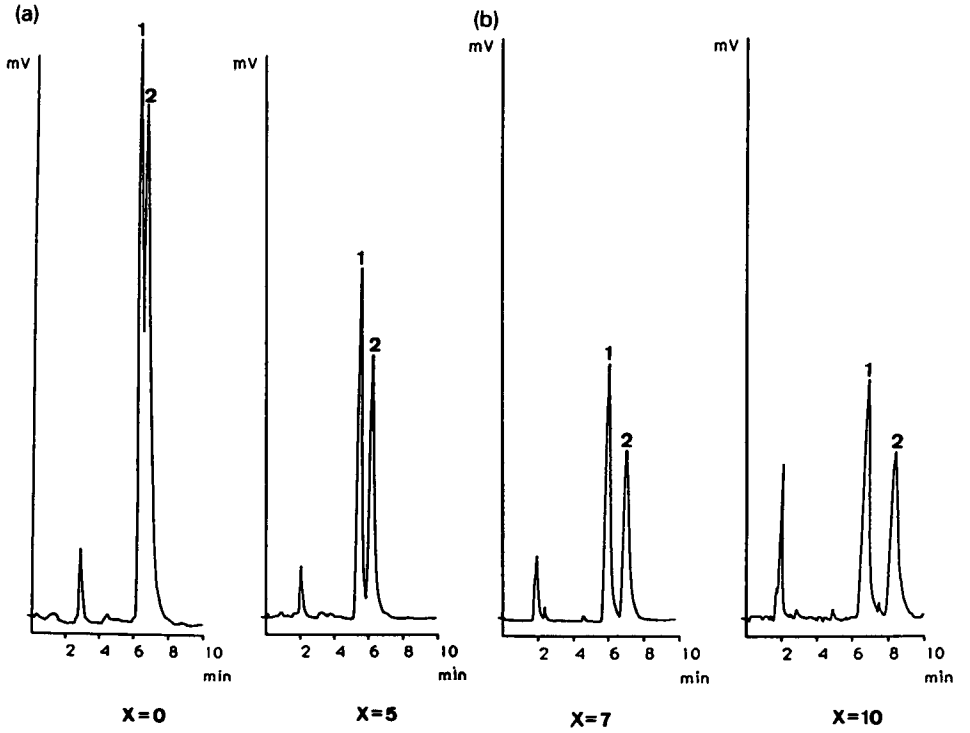


Fig. 6. Separation of (1) Et-16-OMe and (2) Et-18-OMe on Column D (Lichrosorb RP-8). Conditions: mobile phase, strong ammonia solution–water–methanol (0.5:variable, v/v); flow-rate, 1.5 ml/min; sample loop, 20 $\mu$l; and concentration mixture, *ca.* 200 $\mu$g/ml. $x$ = % water (v/v).

straight-phase mode. The results are shown in Table I. Most of the reversed phase columns, which showed adsorption without ammonia, now eluted with ammonia. The adsorption problem on the Vydac (I) and base-deactivated Apex Symm column (H) was not solved. This proves again that the optimization and standardization of silica packing materials in LC is difficult. The possible correlation between ammonia concentration and the reversibility of adsorption of the ALPs was investigated. Improvements in elution and selectivity were observed when the RP-8 column (D) was gradually "loaded" by increasing the amount of ammonia. However, it was difficult to see such a dependence because the elution is obtained only after loading the column with an excess of ammonia.

The ethyl (C), octyl (D, G) and cyanopropyl (F) phases are not sufficiently hydrophobic to create an affinity for the long ether chain; the selectivities are summarized in Table I. The resolution on the octyl reversed-phase column could be improved by adding water to the eluent. Fig. 6 shows four chromatograms obtained after elution on a classical RP-8 column (D) with increasing amounts of water. The effect of increasing the polarity on the retention and separation on RP-8 (D) and base-deactivated RP-8 (G) is summarized in Table II.

When methanol was replaced by acetonitrile on the RP-8 and RP-18 phases, the good elution profiles were no longer observed. This is not due to a detection problem

TABLE II

INFLUENCE OF WATER CONTENT IN THE MOBILE PHASE ON THE RETENTION MECHANISMS OF ET-16-OME (16) AND ET-18-OME (18) ON RP-8 (D) AND LICHROSORB 60RP SELECT B (G) COLUMNS

Number of experiments = 3. Symbols as in Table I.

| Water (%, v/v) | Column D | | | Column G | | |
|---|---|---|---|---|---|---|
| | $k'_{16}$ | $k'_{18}$ | $\alpha$ | $k'_{16}$ | $k'_{18}$ | $\alpha$ |
| 0 | 1.040 | 1.161 | 1.116 | 1.103 | 1.224 | 1.110 |
| 5 | 1.698 | 2.073 | 1.221 | 1.810 | 2.293 | 1.267 |
| 7 | 1.929 | 2.449 | 1.269 | 2.478 | 3.400 | 1.372 |
| 10 | 2.544 | 3.422 | 1.345 | 3.690 | 5.466 | 1.481 |

because the same phenomenon was observed when refractive index detection was used instead of light scattering. This observation can be explained by the fact that ALPs are not very soluble in acetonitrile–water.

Another chromatographic approach could be the use of polymeric particles such as polystyrenedivinylbenzene. No data for the chromatography of phospholipids with these materials have yet been reported. The chromatographic separation of Et-16-OMe and Et-18-OMe was therefore tried on two commercial phases, PRP-1 (K) and PRLP-S (L). The columns seem to perform very well for this type of separation because no adsorption was observed (see Table I) and the chromatographic efficiency was better than with octyl reversed phases. The microporosity of this resin explains the greater hydrophobic properties of polystyrenedivinylbenzene compared with the classical octyl or octadecyl reversed phases. The influence of the addition of water which causes a dramatic increase in the retention of the ALPs because of the reduced hydrophobicity in the microporous cavities, is also seen.

However, when water was added to methanol, a strange phenomenon occurred. Although there is no obvious explanation for this behaviour, it is reported here. The results are summarized in Table III and chromatograms are shown in Fig. 7. When

TABLE III

INFLUENCE OF WATER CONTENT IN THE MOBILE PHASE ON THE RETENTION MECHANISMS OF ET-16-OME (16) AND ET-18-OME (18) ON A PRLP-S- COLUMN (L)

Number of experiments = 3. Symbols as in Table I.

| Water (%, v/v) | $k'_{16}$ | $k'_{18}$ | $\alpha$ |
|---|---|---|---|
| 0 | 1.373 | 1.926 | 1.399 |
| 1 | 1.500 | 2.182 | 1.455 |
| 2 | 1.831 | 2.712 | 1.481 |
| 3 | 2.049 | 3.223 | 1.573 |
| 4 | 2.466 | 4.095 | 1.661 |
| 5 | 2.769 | 4.758 | 1.718 |
| 6 | 2.826 | 4.871 | 1.724 |
| 8 | 4.776 | 9.265 | 1.940 |
| 10 | 6.197 | 12.826 | 2.070 |

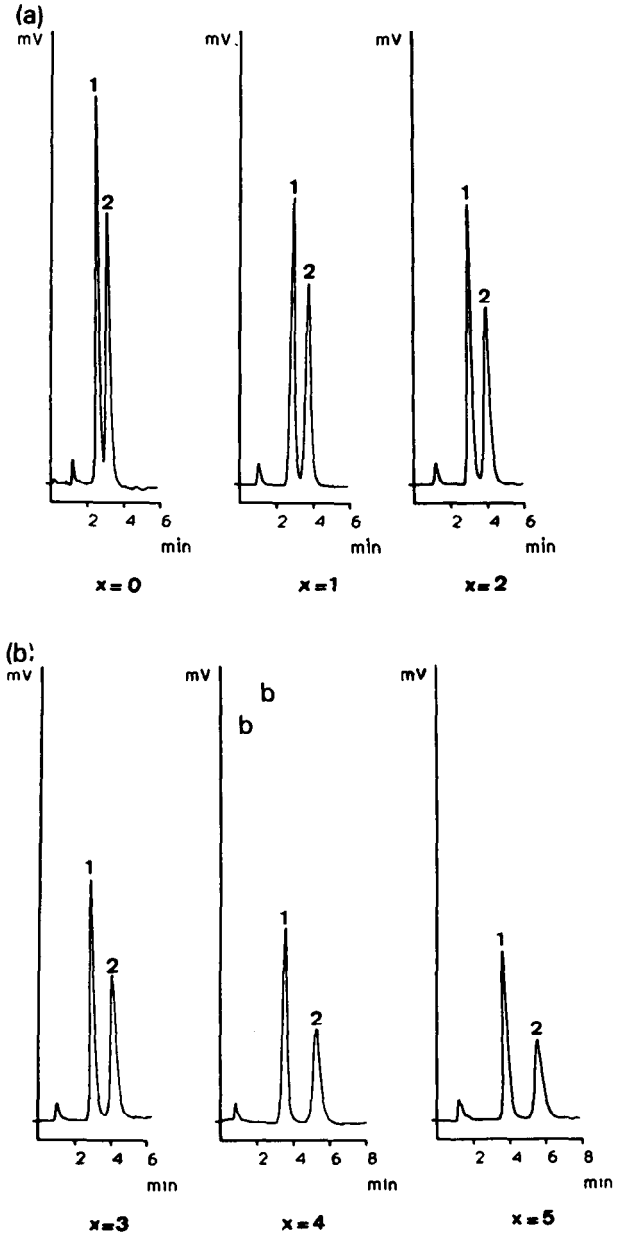
(a)
mV
1
2
2 4 6
min
x = 0
mV
1
2
2 4 6
min
x = 1
mV
1
2
2 4 6
min
x = 2
(b)
mV
1
2
2 4 6
min
x = 3
mV
b
b
1
2
2 4 6 8
min
x = 4
mV
1
2
2 4 6 8
min
x = 5

Fig. 7.

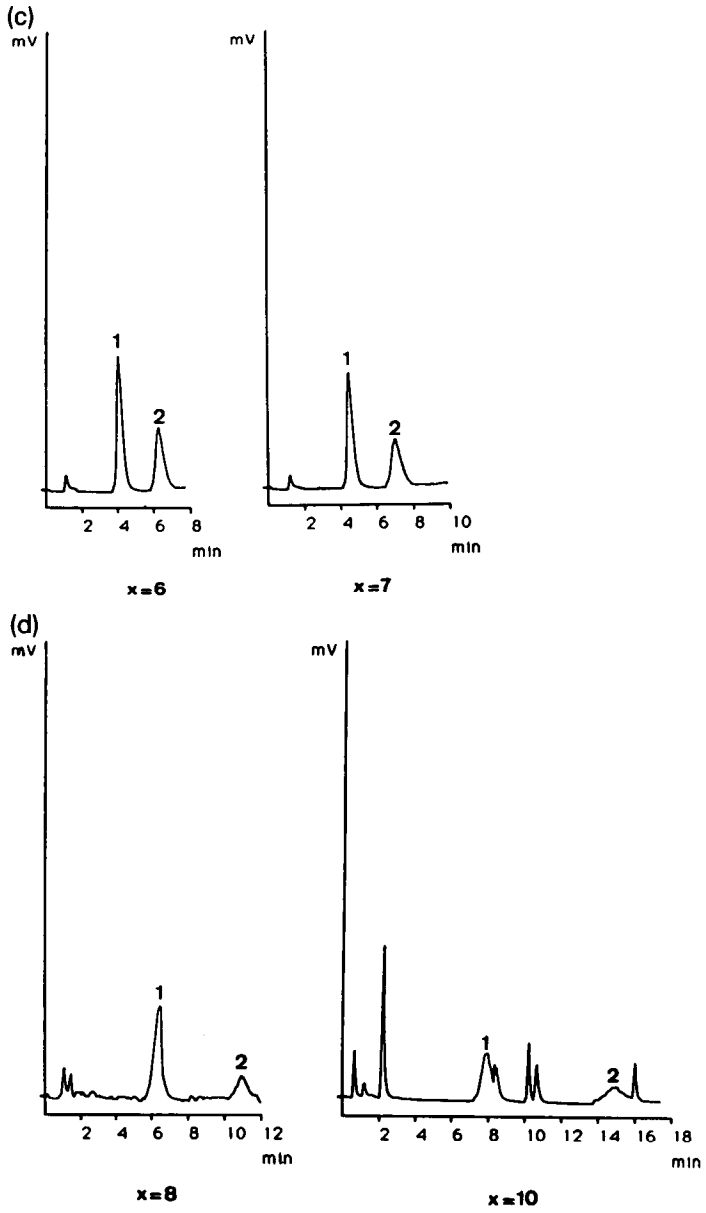


Fig. 7. Separation of (1) Et-16-OMe and (2) Et-18-OMe on Column L (PRLP-S). Conditions: mobile phase, water–methanol (variable); flow-rate, 1.0 ml/min; sample loop, 20 $\mu$l; and concentration mixture, *ca.* 200 $\mu$g/ml. $x$ = % water (v/v).

the water content is increased, the resolution is improved but the observed eluted amount decreases. The last chromatographic run with 10% (v/v) water in methanol yielded a peak distortion. Fig. 8 shows the dependence of the addition of water to the mobile phase on the detection signal. The peak height and surface area decreased

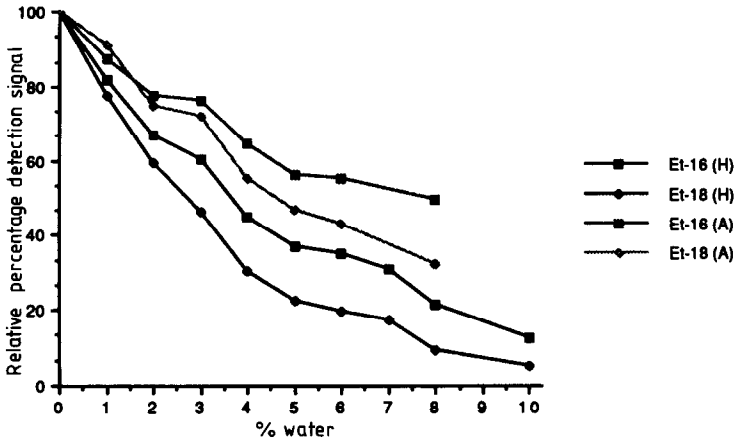


Fig. 8. Relative percentage detection signal compared to the values obtained in the chromatogram on column L (PRLP-S) with 0% water. (H) peak height; (A) area under the curve.

significantly as a function of water content. Chromatography on polystyrenedivinylbenzene is impossible with the mobile phase methanol–water (100:10, v/v) whereas it is still possible on reversed-phase materials (Fig. 6).

## CONCLUSIONS

The best results for the separation of the two ALPs studied were obtained on the Ultrabase column (I), where the selectivity was highest and where no silanol activity was observed. The proposed HPLC method with light scattering detection can be used for studies of relatively high-concentration ALP formulations. However, as it is intended to develop an analytical method for the determination of ALPs in pharmacokinetic studies, where sensitivity is of prime importance, the possibilities of capillary gas chromatography are currently being investigated.

## ACKNOWLEDGEMENT

Support by the National Fonds of Scientific Research under Grant No. 3.0026.90 is greatefully acknowledged.

## REFERENCES

1 *Lipids*, 22 (1987) 775.
2 W. E. Berdel, R. Andreesen and P. G. Munder, in J. F. Kuo (Editor), *Phospholipids and Cellular Regulation*, Vol. 2, CRC Press, Boca Raton, FL, 1985, p. 41.
3 J. Coene, F. David, E. Van den Eeckhout, W. van den Bossche and P. Sandra, *Abstracts Pittsburgh Conference 1990, New York*, p. 835.
4 J. Coene, M. E. Ramos, E. van den Eeckhout, P. Sandra and W. van den Bossche, *Chromatographia*, 30 (1990) 414.
5 H. K. Mangold, G. Zweig and J. Sherma (Editors), *CRC Handbook of Chromatography. Lipids*, Vol. I, CRC Press, Boca Raton, FL, 1984.

6 G. V. Marinett (Editor), *Lipid Chromatographic Analysis*, Vol. I, Marcel Dekker, New York, 1976.
7 A. Kuksis (Editor), *Chromatography of Lipids in Biomedical Research and Clinical Diagnosis. Journal of Chromatography Library*, Vol. 37, Elsevier, Amsterdam, 1987.
8 W. W. Christie, *Lipid Analysis*, Pergamon Press, Oxford, 2nd. ed., 1982.
9 M. Ghijs, D. Repka and P. Sandra, *Abstracts Internation Symposium on Chromatography*, Amsterdam 1990, Tu-P-056.
10 W. Jost, R. Gasteier, G. Schwinn, M. Tueylue and R. E. Majors, *Int. Lab. Spectrosc. Bioanal. Tech.*, May (1990) 46.
11 A. Nahum and C. Horvath, *J. Chromatogr.*, 203 (1981) 53.
12 K. E. Bij, C. Horvath, W. R. Melander and A. Nahum, *J. Chromatogr.*, 203 (1981) 65.
13 A. Sokolowski and K.-G. Wahlund, *J. Chromatogr.* 189 (1980) 299.
14 S. G. Weber and W. G. Tramposch, *Anal. Chem.*, 55 (1863) 1771.
15 G. B. Cox and R. W. Stout, *J. Chromatogr.*, 381 (1987) 315.
16 E. Bayer and A. Paulus, *J. Chromatogr.*, 400 (1987) 1.
17 T. L. Ascah, B. Feibush, R. C. Ludwig and L. T. Peters, *Abstracts Pittsburgh Conference, Atlanta, CA, 1989*, p. 1450.
18 B. M. van Liedekerke, H. J. Nelis, W. E. Lambert and A. P. de Leenheer, *Anal. Chem.*, 61 (1989) 728.