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Separation of dipalmitoyl phosphatidyl choline, cholesterol and their degradation products by high-performance liquid chromatography on a perfluorinated stationary bonded phase

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Abstract

The behaviour of a perfluorinated stationary phase, Fluofix, has been investigated for HPLC of lipid compounds. The lipid compounds investigated were those usually composed of liposomes, including dipalmitoyl phosphatidyl choline (DPPC), cholesterol, their impurities and degradation products. Some other phospholipids and lipids of interest like aliphatic amines and detergents were included. Because of the specific behaviour of the perfluorinated bonded phase towards hydrogenated compounds, a great variety of lipids can be separated using simple ethanol–water mobile phases. This method separates DPPC and cholesterol from their degradation products and also separates phospholipids differing by their polar head group or their fatty acid residues. It could a very useful tool for chromatographic analysis, stability studies and formulation characterisation of liposome suspensions.

Keywords: Stationary phases, LC; Fluorinated stationary phase; Phospholipids; Cholesterol

1. Introduction

The use of perfluorinated bonded silica phases in HPLC has been a subject of research for the last twenty years.

In 1979 De Galan and co-workers reported the synthesis of a perfluoroalkyl bonded silica support showing a specific interaction for fluorine-containing molecules and a decreased retention of hydrogenated compounds when compared to a classic reverse phase silica support [1].

Billiet and co-workers [2] reported that the decreased retention of solutes was due to the extremely low polarity of the perfluorinated stationary phase and these trends were rationalised in terms of a model based on the Hildebrand solubility parameter.

In other works [3], this chromatographic behaviour was compared to the segregation phenomenon between perfluorinated and perhydrogenated surfactants as described by Mukerjee et al. [4]. Studies performed by Kruger et al. [5] indicate that the internal rotation energetic barrier of perfluorinated compounds is much higher than for their hydrogenated homologues. This means that the mobility of perfluorinated chains is much lower than that of perhydrogenated chains and this seems to be a determining factor in the reciprocal non-solubility. In chromatography, the stationary perfluoroalkyl bonded phase then behaves as a ‘rigid brush’,

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limiting the penetration of hydrogenated solutes whilst showing increased retention for fluorinated compounds [6].

This specific retention of fluorinated compounds and lower retention of hydrogenated solutes is not the only specific characteristic of perfluorinated stationary phases. An increased retention of solutes containing polar groups has been described [7,8] and specific separations of aromatic polycyclic hydrocarbons could be due to interactions between the π-electrons and the C–F dipole on the stationary phase and also to the more-or-less-planar structure of these molecules [9,10].

The reduced retention of solutes on the perfluorinated bonded phase has sometimes been described as a drawback for its use in chromatography. However, this property has been successfully exploited for the chromatography of proteins and macromolecules, such as nucleic acids, where the reduced interaction improves separation and recovery using less denaturant mobile phases [11,12].

Surprisingly, the use of this property for the chromatography of highly hydrophobic lipids has not yet been investigated. The chromatography of lipids and phospholipids is of interest in pharmaceutics and cosmetics (liposome analysis) and also in food analysis. Usually, phospholipids are separated by normal-phase chromatography on silica or aminated silica supports using mobile phases including chloroform, methanol, water, tetrahydrofuran (THF) and ammonia or phosphoric buffers [13,14]. Validation of these methods in pharmaceutical analysis is difficult as silica supports are rapidly impaired by impurities.

Reverse phase chromatography of phospholipids and highly hydrophobic lipids is difficult to perform as highly non-polar mobile phases are needed. In addition, when compounds differing in hydrophoby or polar heads are analysed, different mobile phase systems are needed for each [15,16].

In this work we show that the use of perfluorinated stationary phases for the analysis of lipids allows the separation of a great variety of lipids including phospholipids, cholesterol and their degradation products, detergents and some other lipids of interest. The mobile phases used are easy to handle ethanol–water compositions.

This chromatographic method, together with the use of a universal Evaporative Light Scattering Detector (ELSD), enables the analysis of complex liposomal compositions by direct injection of the aqueous liposome suspension into the chromatographic system.

2. Experimental

2.1. Reagents

Ethanol was obtained from Carlo Erba (France). Pure water with a conductivity of 18.2 MΩ cm^{-1} and a Total Organic Carbon (TOC) of 7 ppb was obtained from a Milli-Q Synthesis A-10 system (Millipore, Molsheim, France).

Dipalmitoyl phosphatidyl choline (DPPC) and dipalmitoyl phosphatidyl glycerol (DPPG), were purchased from Lipoid KG (Mannheim, Germany).

Dipalmitoyl phosphatidyl ethanolamine (DPPE), dioleoyl phosphatidyl choline (DOPC), octyl-β-D-glucopyranoside (OGP), stearyl amine (STA), palmitic acid, cholesterol, desmosterol, lathosterol, 7-β-hydroxycholesterol, 7-β-ketocholesterol and lysophosphatidyl choline were purchased from Sigma (St. Louis, USA).

2.2. Material and methods

The chromatographic system was composed of a Spectra System P400 ternary pump (Thermoseparation Products, France), a HPLC 360 Autosampler (Kontron Instruments, France), a Eurosas HPLC column oven (Eurosas, France), an Evaporative Light Scattering Detector DDL21 (Eurosep, France) and a PE-Nelson Model 1020 Integrator (Perkin Elmer, France).

The column was a Fluofix 25 cm×4.6 mm from Neos Company Ltd. (Japan).

Chromatographic conditions were: flow-rate 1ml min^{-1}, gradient from ethanol–water (50:50) to 100% ethanol with different gradient profiles. The column was thermostated at 40°C. Injection volume was 30 μl.

For the Light Scattering Detector, the carrier gas was air at a pressure of 1.8 bars and the spray chamber temperature was 55°C.

All pure samples were prepared at 1 g/l in ethanol

or in water–ethanol (10:90) depending mainly on the phospholipid solubility.

Liposomes (SUV) were prepared at 1 g/l by the ethanol injection method with a composition in DPPC–cholesterol of 70:30 w/w or a composition DPPC:DOPC:DPPE:DPPG:Cholesterol of 20:20:20:20:20 w/w. The lipid mixture was dissolved in ethanol at 50°C at a concentration of 25 mg/ml. One millilitre of the mixture was injected into 10 ml of water maintained at 60°C with vigorous stirring. The liposome suspension was allowed to cool down at room temperature and was injected without further purification.

3. Results and discussion

The HPLC analysis of lipids is a growing research field as many problems are encountered when mixtures of lipids of very different natures have to be analysed. In the field of liposomes, the analysis of phospholipids has been the subject of many publications, concerning both the chromatographic analysis and the detection procedures.

Liposomes are usually composed of phospholipids that can be of different natures, ranging from pure synthetic phospholipids such as DPPC, DMPC, DOPC, DPPG, DPPE to mixtures of natural origin like egg yolk phospholipids or soya phospholipids. They can also contain a variety of non-polar lipids such as cholesterol, vitamins, triglycerides etc. mainly for stability reasons.

The main chromatography problem concerns the separation of phospholipids. Their high hydrophobicity and the presence of head polar groups make their chromatography very difficult either on reverse bonded phases or by normal-phase chromatography. The use of short chain bonded phases has been reported for the separation of phospholipids of different acyl chain lengths [15]. This work showed that concentrated phosphoric acid had to be added to the mobile phase in order to control non-specific interactions between the polar head of phospholipids and the uncovered silica support. Other work showed that separation of lipids and phospholipids on short alkyl bonded phases could be optimised by systematic approaches using complex ternary solvent mixtures; however, different solvent systems are needed to separate and identify properly neutral or polar lipids [16].

More usually, phospholipids are analysed by normal-phase HPLC using silica supports but mobile phases such as hexane–propanol–water [17] or chloroform–methanol–ammonium hydroxide [18] need to be used. In that case, the performance of the columns is rapidly degraded as silica is affected by impurities and is very difficult to regenerate.

Fluorinated phases have been described as showing reduced retention of hydrophobic compounds in comparison with their hydrogenated homologues [1–3]. As has been described above, attempts to rationalise this trend in terms of solubility parameters have been made. However, for the following reasons we think that the structure of the fluorinated phase should play an important role in retention. Firstly, the segregation observed between fluorinated and non-fluorinated surfactants has been attributed to the differences in rigidity between fluorinated and non-fluorinated carbon chains [4,5]. Secondly, the chromatographic theory developed by Horvath and Melander [19], indicates that the monolayer stationary phase bonded on the silica support cannot be considered as a free liquid and that the structure of the bonded phases is important to understand their chromatographic behaviour. Further work performed by Berendsen et al. [20] has shown that the penetration of solutes in the alkyl bonded phases was an important factor in their retention in reverse phase chromatography.

This makes us to think that the best explanation for the non-retention of hydrogenated compounds on fluorinated phases could be that their extreme rigidity prevents the penetration of compounds in the stationary phase, as was described elsewhere [6].

We could expect three benefits from this. The lower retention should permit the chromatography of lipid compounds with moderately hydrophobic mobile phases and the fluorinated barrier should prevent non-specific interactions of the polar head groups with the non-covered silica support. This could result in a pure mechanism of mild hydrophobic chromatography. A third benefit from the use of unbuffered volatile alcohol–water mobile phases would be an improvement in detection capabilities either by UV absorption at low wavelengths or by the use of other type of detectors.

Concerning the detection, the absence of chromophoric groups in most lipids implies the use of UV detection at very low wavelengths, the use of electrochemical detection or post-column treatments for fluorescence detection [16,21–23].

As an interesting alternative, the use of an ELS detector has been reported [18]. The emergence of new generations of ELS detectors eliminates their drawback of low sensitivity and makes them very interesting as universal detectors for non-volatile compounds. The only condition is the use of volatile mobile phases and the avoidance of buffers or salts even at low concentrations.

We have then evaluated the use of fluorinated stationary phases together with the use of an ELS detector as an alternative for the chromatography of lipid compounds.

The first interesting results were obtained for the separation of DPPC and related degradation products, mainly lyso-phosphatidyl choline and palmitic acid. Fig. 1 shows that, using a mild ethanol–water gradient, DPPC is eluted at about 95% of ethanol and its degradation products are well separated. We can observe two peaks of lyso-phosphatidyl choline corresponding to the elimination of the acyl chain on the first or second position.

A slightly different gradient profile allows the separation of cholesterol and its more usual impurities (desmosterol, lathosterol) and degradation products (ketocholesterol and hydroxycholesterol). The chromatographic profile is shown in Fig. 2.

Fig. 3 shows the chromatographic profile of liposomes of DPPC–Cholesterol containing impurities and degradation products. This chromatogram was obtained by direct injection of the aqueous liposomes suspension.

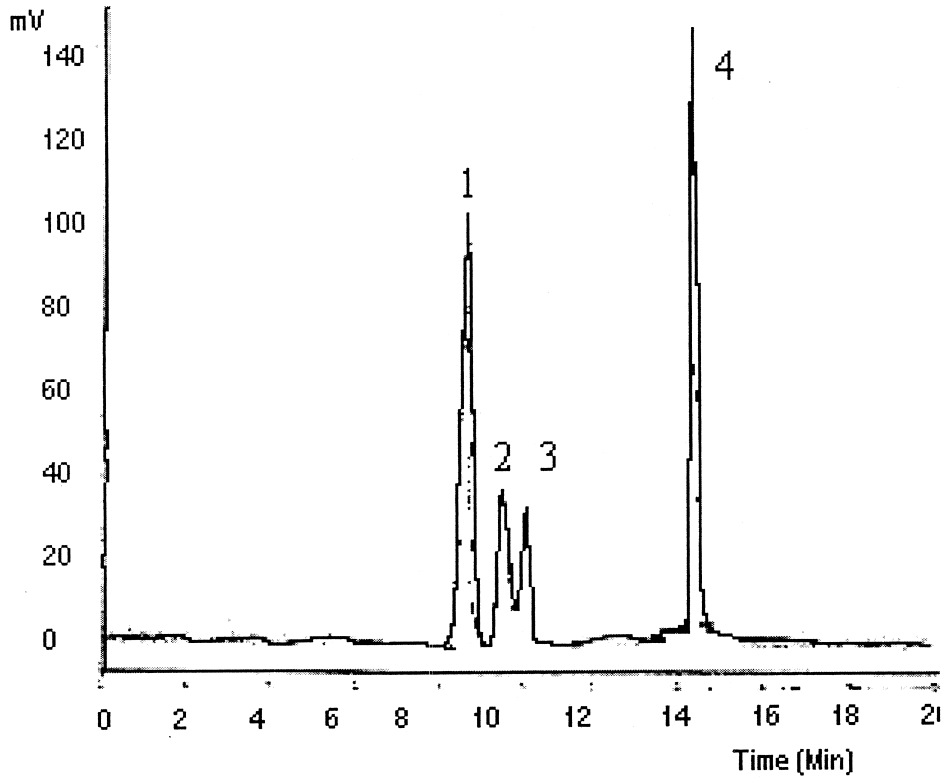

Fig. 1. Chromatographic profile of the separation of DPPC and corresponding degradation products. Column Fluofix 25 cm×4.6 mm. Flow-rate 1 ml min^{-1}. Mobile phase ethanol–water. Gradient profile 50–100% ethanol in 15 min, plateau at 100% ethanol for 5 min. Light Scattering Evaporating Detector (1) lysophosphatidyl choline 1; (2): Lysophosphatidyl choline 2; (3) Palmitic acid; (4) DPPC).

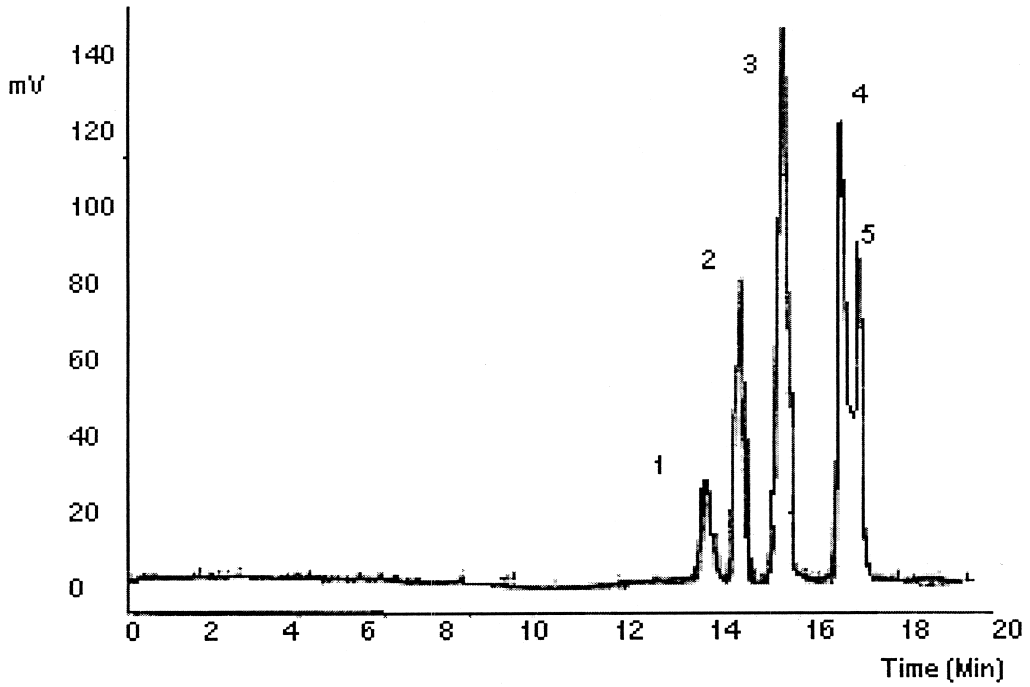

Fig. 2. Chromatographic profile of the separation of cholesterol, impurities and degradation products. Column Fluofix 25 cm×4.6 mm. Flow-rate 1 ml min^{-1}. Mobile phase ethanol–water. Gradient profile 50%–80% ethanol in 17 min, 80–100% ethanol in 3 min, plateau at 100% ethanol for 5 min. Light Scattering Evaporating Detector. (1) Hydroxycholesterol; (2) Desmosterol; (3) ketocholesterol; (4) Cholesterol; (5) Lathosterol.

The results described above are interesting for several reasons. Due to the decreased retention of DPPC on the mobile phase system used, one would expect a strongly decreased retention and lack of resolution for cholesterol or lysophospholipids as it has been described on short chain reverse phase bonded phases [15,16]. Instead, retention is high enough to separate compounds with a big variety of polarities and resolution between isomers and close structures is quite good.

Cholesterol (Cholest-5-en-3β-ol) and lathosterol (Cholest-7-en-3β-ol) are quite close isomers and their separation by reverse phase HPLC is compromised (non published results). Results in Fig. 2 show that this separation is possible on the fluorinated phase. The resolution is not very high but the process can be further optimised.

All of this probably indicates that the mechanism of retention is not merely hydrophobic but that the structure of the fluorinated phase participates in the separation, resulting in an improvement of the chromatographic properties thus indicating that useful separations could be obtained with this type of stationary phase.

Fig. 4 shows the chromatographic profile obtained with liposomes composed of DPPG, DPPE, DPPC, DOPC and cholesterol. All the lipids are separated with good resolution.

Two unexpected results should be noticed on this chromatogram which are the lack of retention obtained for DPPG and the inversion of retention between DOPC and DPPC. Usually DPPG is more retained than DPPC on normal-phase chromatography because its adsorption on the stationary phase is stronger [21]. The same phenomenon is observed in reversed-phase chromatography [15], indicating a non-specific interaction of DPPG with the silica support. We have no explanation for the little retention of DPPG in terms of hydrophobic interaction, but this result suggests that the fluorinated bonded

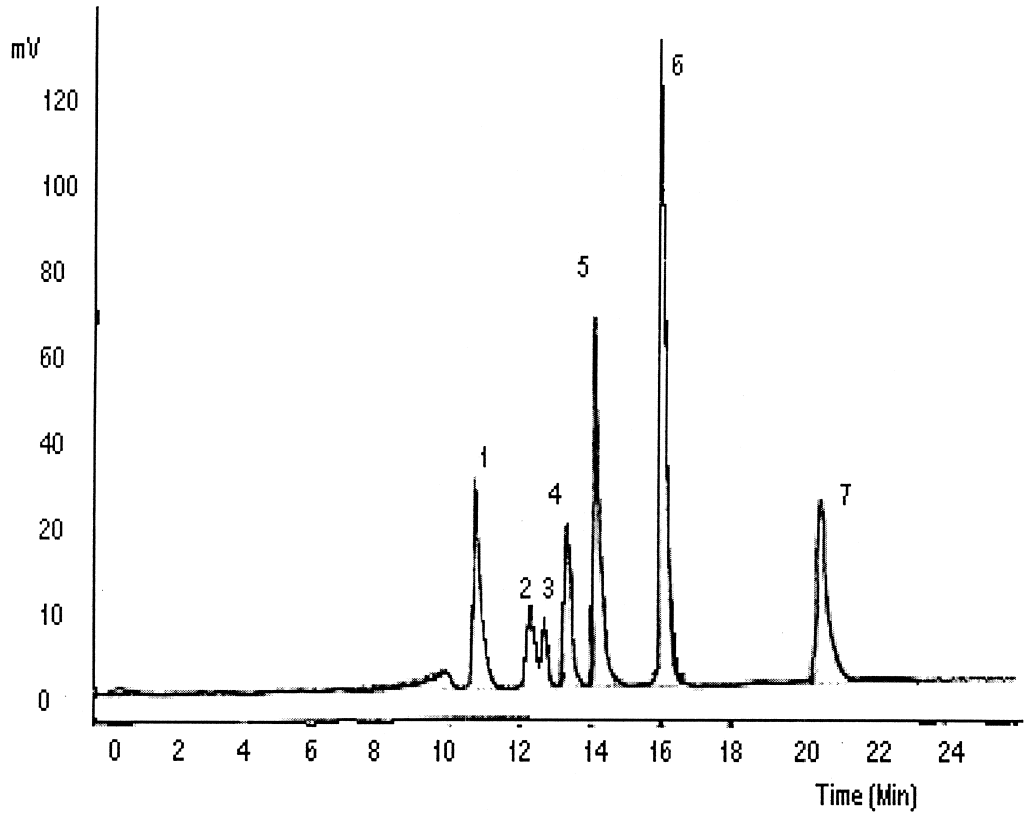

Fig. 3. Chromatographic profile of partially degraded liposomes of DPPC–Cholesterol (70:30, w/w). Chromatographic conditions as in Fig. 2 (1) Lysophosphatidyl choline 1; (2) Lysophosphatidyl choline 2; (3) hydroxycholesterol; (4) Desmosterol; (5) Ketocholesterol; (6) Cholesterol; (7) DPPC.

phase effectively prevents the interaction of the compound with the silica surface.

The higher retention of DPPC compared to DOPC is also interesting as the aliphatic chain length of DOPC is higher. This inversion of retention may be related to the DOPC unsaturation (C_{18}:1) which results in a higher mobility of the aliphatic chains. However, further studies would be necessary in order to correctly understand the retention mechanism.

In a further experiment, a variety of phospholipids and other lipids were injected, including usual detergents like Octyl-β-glucopyranoside (OGP). Table 1 shows the retention time observed for a variety of compounds.

Concerning peak shape, the number of plates and symmetry factors have not been calculated but it seems from figures that peaks are thin and quite symmetric. It is known that peak symmetry in gradient conditions is usually good because the solvent strength increases during the elution process. Peak shape and resolution characteristics should be tested in isocratic conditions for a given separation problem to be representative.

Repeated injections of different types of lipids showed very good reproducibility. Isocratic methods for the separation of cholesterol, DPPC and their degradation products are being developed by our Analytical Development Department. These methods are presently being validated for pharmaceutical analysis purposes.

The fluorinated bonded phase has been extensively used for routine analysis and validation for the last

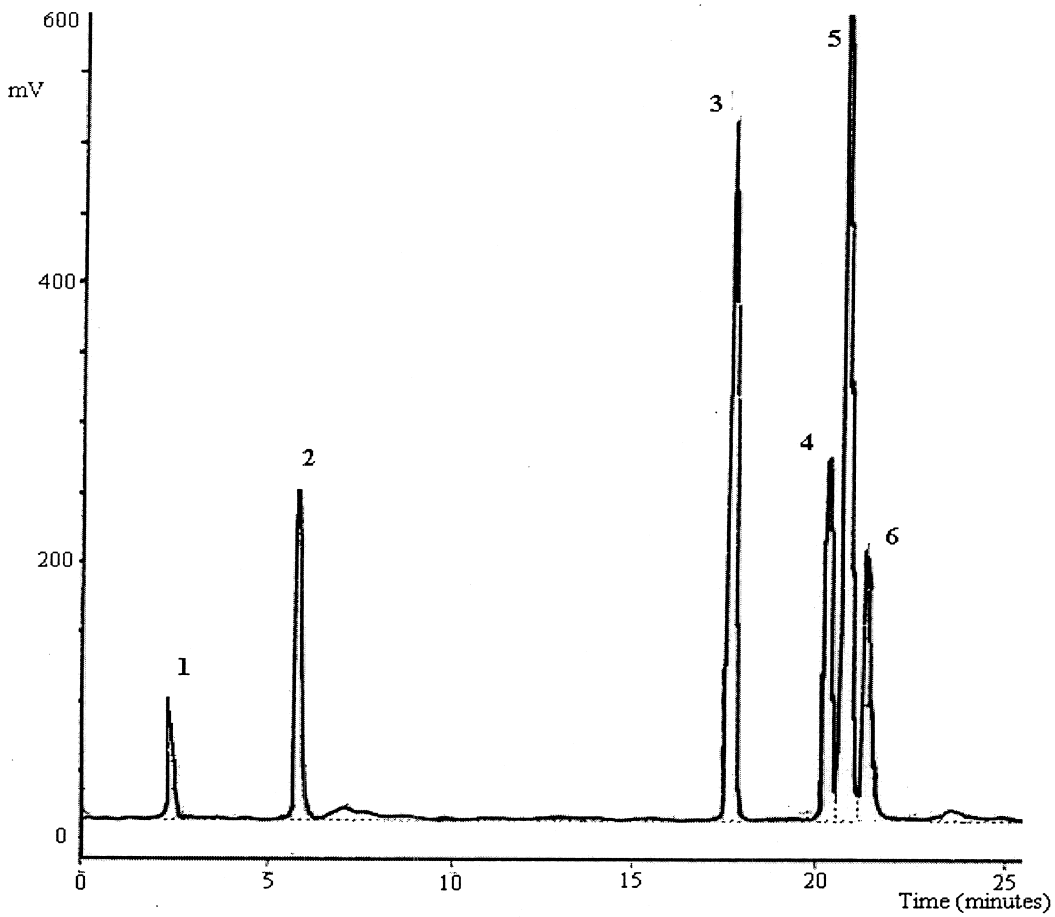

Fig. 4. Chromatographic profile of multi-component liposomes containing OGP. Chromatographic conditions as described in Fig. 2 (1) DPPG; (2) OGP; (3) Cholesterol; (4) DPPE; (5) DOPC; (6) DPPC.

six months and to date no degradation of the performance or the chromatographic properties of the column has been observed (unpublished results).

4. Conclusion

In this work it has been shown that a HPLC fluorinated bonded phase, Fluofix, can be used satisfactorily for the separation of a great variety of lipids including phospholipids, cholesterol and their main degradation products.

The fluorinated bonded phase shows a decreased retention that allows the analysis of all lipids in ethanol–water mobile phases. The resolution capacity of the column remains very good and isomers and close structures can be separated with high efficiency. The use of a universal Light Scattering Evaporation Detector allows the detection of all types of lipids with good sensitivity.

Analyses of liposomes of different compositions by direct injection of the aqueous liposomes suspension were performed satisfactorily.

The results obtained show that the chromatographic properties of the fluorinated phase cannot be explained by a simple hydrophobic mechanism and that the specific properties of the fluorinated phase participate in the chromatographic process.

Table 1
Retention times observed for various products. Column Fluofix 25 cm×4.6 mm. Flow-rate 1 ml min^{-1}. Mobile phase ethanol–water. Gradient profile 50–80% ethanol in 17 min, 80–100% ethanol in 3 min, plateau at 100% ethanol for 5 min. Light Scattering Evaporating Detector. The number of plates is calculated by the usual formula

Product name	Retention time (min)
Dipalmitoyl phosphatidyl glycerol	3
Octyl-β-D Glucopyranoside	5.8
Lysophosphatidyl choline (1)	10.8
Stearyl amine	11.7
Lysophosphatidyl choline (2)	12.2
7-β-Hydroxycholesterol	12.8
Ketocholesterol	13.4
Desmosterol	14.2
Cholesterol	16.2
Lathosterol	16.7
Dipalmitoyl phosphatidyl ethanolamine	20.0
Dioleoyl phosphatidyl choline	20.7
Dipalmitoyl phosphatidyl choline	21.2

Fluorinated bonded phases seem to be an interesting tool for performing lipid separations in mild mobile phase conditions.

References

[1] G.E. Berendsen, K.A. Pikaart, L. De Galan, C. Olieman, Anal. Chem. 52 (1980) 1990–1993.
[2] H.A.H. Billiet, P.J. Schoenmakers, L. De Galan, J. Chromatogr. 218 (1981) 443–454.
[3] I. De Miguel, S. Exbrayat, D. Samain, Chromatographia 24 (1987) 849–853.
[4] P. Mukerjee, A.Y.S. Yang, J. Phys. Chem. 80 (1976) 1383.
[5] D.G. Kruger, S. Rajan, C.A. Kingsbury, J. Sol. Chem. 11 (1982) 719.
[6] A. Haas, J. Kohler, Chromatographia 14 (1981) 341.
[7] P.C. Sadek, P.W. Carr, J. Chromatogr. 288 (1984) 25–41.
[8] P.C. Sadek, P.W. Carr, M.J. Ruggio, Anal. Chem. 59 (1987) 1032–1039.
[9] W. Ecknig, B. Trung, R. Radeglia, U. Gross, Chromatographia 16 (1982) 178–182.
[10] K. Jinno, H. Nakamura, Chromatographia 39 (1994) 285–293.
[11] G. Xindu, P.W. Carr, J. Chromatogr. 269 (1983) 96–102.
[12] H. Itoh, T. Kinoshita, N. Nimura, J. Chromatogr. A 662 (1994) 95–99.
[13] P. Juaneda, G. Rocquelin, P.O. Astorg, Lipids 25 (1990) 756–759.
[14] M. Grit, W.J.M. Underberg, D.J.A. Crommelin, J. Pharm. Sci. 82 (1993) 362–366.
[15] B.J. Compton, W.C. Purdy, Anal. Chim. Acta 141 (1982) 405–410.
[16] M.S. McCraken, N.H. Holt, J. Chromatogr. 348 (1985) 221–227.
[17] L.L. Dugan, P. Demediuk, C.E. Pendley, L.A. Horrocks, J. Chromatogr. 378 (1986) 317–327.
[18] J. Becart, C. Chevalier, J.P. Biesse, J. High Res. Chrom. 13 (1990) 126–129.
[19] C. Horvath, W. Melander, J. Chrom. Sci. 15 (1977) 393–404.
[20] G.E. Berendsen, L. De Galan, J. Chromatogr. 196 (1980) 21–37.
[21] A. Rastegar, A. Pelletier, G. Duportail, L. Freysz, C. Leray, J. Chromatogr. 518 (1990) 157–165.
[22] W. Korytowsky, G.J. Bachowsky, A.W. Girotti, Anal. Biochem. 213 (1993) 111–119.
[23] W. Bernhard, M. Linck, H. Creutzburg, A.D. Postle, A. Arning, I. Martin-Carrera, K. Sewing, Anal. Biochem. 220 (1994) 172–180.