Journal of Chromatography, 589 (1992) 165–170
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 707

Separation of soy lecithin using gel permeation chromatography

Richard E. Shansky* and Robert E. Kane

Analytical Sciences Department, Research and Development Center, Boehringer Ingelheim Pharmaceuticals, Inc., 175 Briar Ridge Road, Ridgefield, CT 06877 (USA)

(Received June 25th, 1991)

ABSTRACT

Gel permeation chromatography was applied to develop a broad relative molecular mass profile of soy lecithin. A non-aqueous mobile phase and an organic polymer-based stationary phase were found to be necessary in order to achieve interpretable chromatographic elution. Calibration of the column was performed using polystyrene standards. A broad peak was observed at a relative molecular mass of *ca.* 8000 in the two soy lecithin lots studied. This peak disappeared at sufficiently low concentrations of soy lecithin injected. The results suggested that this peak was due to the formation of reverse micelles or non-covalent aggregates of the phospholipid components and not to polymeric or protein-like high relative molecular mass component(s). No other high molecular mass (>2000) components were detected under the conditions used.

INTRODUCTION

Lecithin derived from soy beans is a complex mixture of phosphatides (chiefly phosphatidylcholine, -ethanolamine and -inositol) and other substances such as triglycerides, fatty acids and carbohydrates [1]. It has many industrial applications including use as an emulsifier and stabilizer in foods. Soy lecithin is also widely used in pharmaceutical manufacturing as a suspending agent for aerosol-delivered drugs. The establishment of procedures that adequately characterize soy lecithin for industrial uses is therefore of obvious importance. Various chromatographic methodologies have been reported and can provide information such as phospholipid ratios [2]. In this work, it was of interest to develop a test which would detect other possible lower level components such as those of higher relative molecular mass (greater than 1000). Described here is a procedure based on gel permeation chromatography (GPC) which provides information regarding the relative molecular mass distribution of soy lecithin components. In the course of the development of this GPC methodology, an anomalous chromatographic behavior was observed at high sample concentrations. As will be shown, the data suggest the source of this anomaly is the formation of reverse micelles or aggregates of the phospholipid components of soy lecithin. Injection of low concentrations (1 mg/ml or less) of soy lecithin was found to be critical in order to produce a true relative molecular mass profile.

EXPERIMENTAL

Materials

Two lots of aerosol-grade soy lecithin (designated A and B) were obtained from a commercial source. A polydivinylbenzene GPC column and polystyrene relative molecular mass markers were purchased from Alltech (Deerfield, IL, USA). L-α-Phosphatidylcholine and L-α-phosphatidylinositol were purchased from Sigma (St. Louis, MO, USA). Tetrahydrofuran was obtained from EM Science (Gibbstown, NJ, USA).

0021-9673/92/$05.00

Chromatographic conditions

A Hewlett-Packard HP 1090L automated high-performance liquid chromatographic (HPLC) system was used. Data were collected on a Hewlett-Packard HP1000 Lab Automation System utilizing a Model 18652A A/D converter. Chromatograms were reprocessed and plotted using the SigmaPlot program (version 3.1) from Jandel Scientific (Corte Madera, CA, USA). The mobile phase was tetrahydrofuran (THF) pumped at a flow-rate of 0.5 ml/min. The injection volume was 10 μl and the UV detection wavelength was 254 nm. All samples and standards were dissolved in THF for injection. Chromatography was performed at room temperature.

The column was a 250 mm × 10 mm I.D. Alltech GPC column packed with a proprietary stationary phase, described by the manufacturer as polydivinylbenzene. The particles were nominally of 5 μm diameter with pores of nominally 1000 Å.

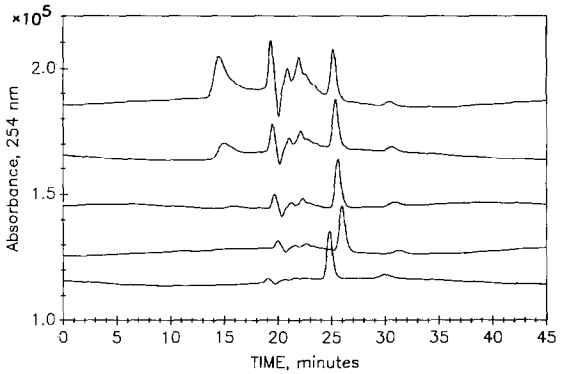

Fig. 1. GPC elution profiles of lot A. From bottom to top, sample concentration was 0.5, 1.0, 2.0, 4.0 and 8.0 mg/ml. Mobile phase, THF; flow-rate, 0.5 ml/min; injection volume, 10 μl; detection, UV at 254 nm. Chromatography was performed at room temperature. The column was a 250 mm × 10 mm I.D. Alltech GPC as described under Experimental. In this and other figures containing overlays, chromatograms are artificially offset from each other for clarity; therefore, the signal axis represents only relative (not absolute) values for each chromatogram.

RESULTS

Initial studies (data not shown) utilized a silica-based, zirconium-treated DuPont GF250 column designed for aqueous gel filtration HPLC and a mobile phase of hexane–isopropanol–acetate buffer (pH 4.2) (8:8:1). This system was found to be unsuitable, possibly owing to polar and/or ionic interactions with the negative charge of the stationary phase packing [3]. The major components of soy lecithin (phospholipids, fatty acids, etc.) were retained beyond the permeation volume of the column. Hence there was no assurance that macromolecular components were not also being retained. Under these conditions, assignment of relative molecular masses based on calibration standards could not be done with confidence.

A non-adsorptive sizing separation mechanism was desired. The major components of soy lecithin are of relatively low relative molecular mass (<1000) and would be expected to elute (poorly resolved) near the permeation volume. Any larger components (*e.g.*, proteins) could then be assigned relative molecular masses in conjunction with appropriate calibration standards. To achieve this, a column packed with a rigid organic-based gel was used.

Figs. 1 and 2 show the GPC elution profile of soy lecithin lots A and B, respectively, at several different concentrations (for clarity, the overlaid chromatograms were artificially offset; this did not affect the data or their interpretation). Qualitatively similar profiles were observed for these two soy lecithin lots because their compositions should be similar. The peak at *ca.* 25 min was not related to the sample as it remained constant in size as a function of soy lecithin concentration and also appeared in blank injections of THF alone (not shown).

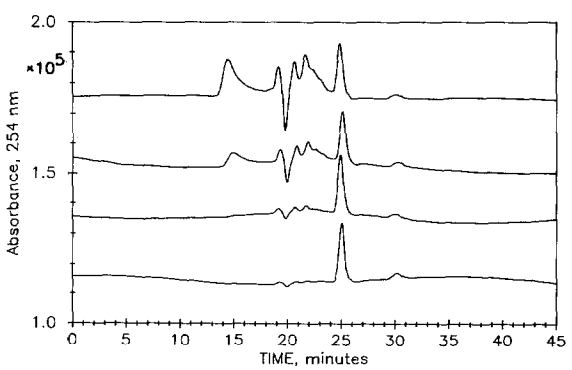

Fig. 2. GPC elution profiles of lot B. From bottom to top, sample concentration was 0.5, 1.0, 4.0 and 8.0 mg/ml. Other conditions as in Fig. 1.

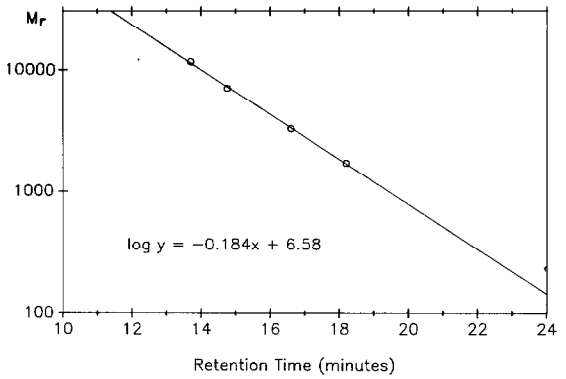

Fig 3. Relative molecular mass calibration plot based on elution of polystyrene relative molecular mass markers. Chromatographic conditions as in Fig. 1.

Four polystyrene standards of known average relative molecular mass (M_r) were injected under identical conditions. Using the resulting retention times, an M_r calibration plot for this column was constructed and is shown in Fig. 3. Linear regression yielded an equation that was used to calculate a polystyrene-equivalent M_r for each peak in the soy lecithin profiles generated using 8 mg/ml injections. This is shown in Table I.

Table I indicates that the series of overlapping peaks between 19 and 23 min in Figs. 1 and 2 have M_r in the range 300–1100. A calculation of the formula weight of the most common C_{16}–C_{18} phospholipids yields a range from 660 to 860. The corresponding fatty acids have a formula weight of 250–290. Injection of authentic phosphatidylinositol and phosphatidylcholine standards produced peaks of

TABLE I

POLYSTYRENE-EQUIVALENT RELATIVE MOLECULAR MASSES OF SOY LECITHIN GPC PEAKS

Lot A		Lot B	
Retention time (min)	M_r	Retention time (min)	M_r
14.43	8400	14.52	8060
19.15	1100	19.37	1030
20.68	590	20.89	540
21.70	390	21.94	350
22.23	310		
24.89	100	25.19	90

TABLE II

RELATIVE MOLECULAR MASS OF BROAD PEAK AS A FUNCTION OF SOY LECITHIN CONCENTRATION

Lot A		Lot B	
Concentration (mg/ml)	M_r	Concentration (mg/ml)	M_r
2	7100	2	5000
4	6550	4	6720
8	8400	8	8060
15	10 480	15	7530
167	10 100	197	10 300

M_r 490 and 230, respectively. Therefore, it is very likely that the series of overlapping peaks between 19 and 23 min correspond to the major phospholipid and/or fatty acid components of soy lecithin.

As mentioned earlier, the peak at *ca.* 25 min was found in a blank injection of THF alone. Table I indicates that this peak has M_r 90–100. As THF itself has a formula weight of 72, this peak most likely represents the elution of THF (or contaminant of THF) at the permeation volume of the column.

For the purpose of this work, there was a need to characterize components over a broad M_r range to ensure the consistency of the soy lecithin as a raw material for pharmaceutical purposes. In this regard, it was of interest to focus on the broad peak eluting at 14–15 min, which has an apparent M_r of 8000–9000. The magnitude of this peak is directly proportional to the injected soy lecithin concentration, strongly suggesting that it is related to the component(s) of the sample. The retention time and therefore the apparent relative molecular mass were also found to vary as a function of injected soy lecithin concentration, as shown in Table II.

Because of the broadness of the peak, assignments of the retention times vary. This results in some variability in relative molecular mass assignment. For both lots, however, there appears to be a distinct shift to higher M_r at high concentrations as a result of shorter retention times. It is unlikely that this shift in M_r was due to any chemical changes (*e.g.*, polymerization) in the soy lecithin under the chromatographic conditions employed. Indeed, harsh treatments are necessary to modify soy lecithin chemically [4]. Further, such a high M_r compo-

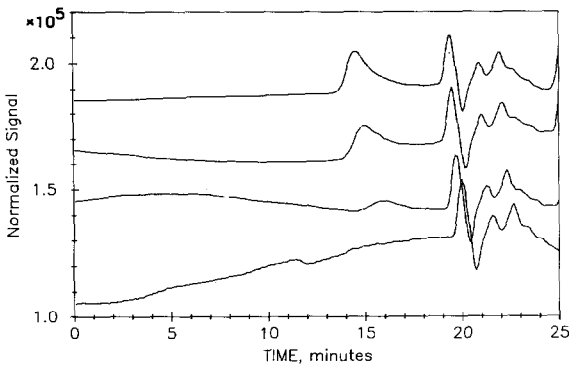

Fig. 4. Normalized elution profile of lot A. The signal (ordinate) values in each chromatogram from Fig. 1 were multiplied by a factor which compensates for the difference in injected sample concentration. From bottom to top, sample concentration was 1.0, 2.0, 4.0 and 8.0 mg/ml. Other conditions as in Fig. 1.

nent is not known to be present in the proportions indicated by the profile (five runs ranging from 2 to 15 mg/ml showed an average relative abundance of 28% according to peak integration). Therefore, the presence of this band would appear to be some type of artifact arising from the separation of the components of soy lecithin in this system.

A more detailed examination of the profile as a function of sample concentration was done. Figs. 4 and 5 are derived from the data shown in Figs. 1 and 2 with two changes: (1) the high-M_r region has been expanded by truncation of the elution time axis at 25 min; and (2) the absorbance values have been normalized to sample concentration. Under ideal conditions, concentration-normalized chromatograms should be essentially superimposable. As shown in Figs. 4 and 5, the broad peak at 14–15 min diminishes in size as the sample concentration is reduced whereas the phospholipid peaks at 19–23 min remain essentially unchanged. At the lowest concentration employed, the broad peak has completely disappeared and no other high-M_r (>2000) components are detectable.

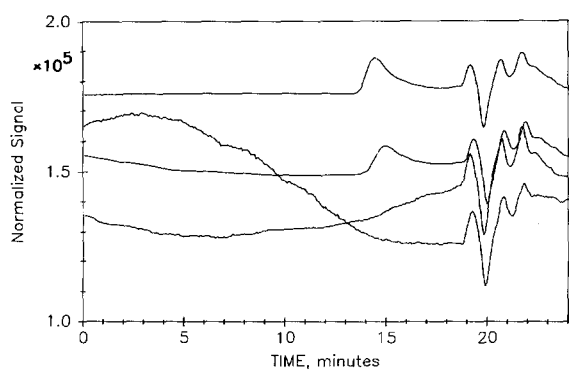

Fig. 5. Normalized elution profile of lot B. The signal (ordinate) values in each chromatogram in Fig. 2 were multiplied by a factor which compensates for the difference in injected sample concentration. From bottom to top, sample concentration was 0.5, 1.0, 4.0 and 8.0 mg/ml. Other conditions as in Fig. 1.

DISCUSSION

An increase in sample concentration generally results in an increase, rather than a decrease, in retention volume in GPC [5]. This effect has been studied for polystyrene [6,7] and becomes more pronounced as the relative molecular mass of the polystyrene increases, apparently owing to a decrease in the effective dimensions of the polymer [5]. The high viscosity of concentrated solutions can also cause tailing of solute bands due to viscous streaming effects [5]. For both large and small solutes, high sample loads can induce changes in the distribution coefficient as the solute proceeds through the column [8]. However, this change is also in the direction of increased retention volume as a function of sample concentration. From this discussion, it would appear that the most commonly reported sample overload artifacts in GPC do not yield reductions in retention volume. This suggests that a different effect may be operating in this work.

A possible explanation for the presence of a component of high relative molecular mass in soy lecithin is that the broad peak represents phospholipid aggregates. A high soy lecithin concentration would tend to favor their formation. These aggregates are not completely broken up by dilution through the chromatographic system until a sufficiently low concentration of soy lecithin is used. Although the exact nature of these aggregates was not determined, the formation of high-M_r aggregate species [9] and/or micelles [10] of phospholipids in organic solvents has been documented. In a different chromatographic system, a concentration-dependent aggregation of one particular protein has been demonstrated [11].

The concept that micelles can elute intact through a gel permeation column has long been rec-

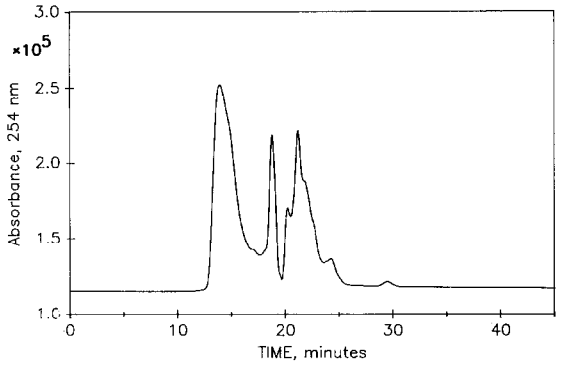

Fig. 6. GPC elution profile of lot B. Sample concentration, 167 mg/ml. Other conditions as in Fig. 1.

ognized and led to a theoretical elaboration by Coll [12]. Inclusion of the surfactant under study in the mobile phase at a concentration just above its critical micelle concentration (CMC) can be employed to suppress equilibrium-induced dissociation of micelles [13]. However, this has been shown more recently not to be necessary if the surfactant is injected at a concentration far above its CMC [14,15]. In this instance, the hydrodynamic volume of micelles may be adequately determined [15]. Of relevance to the work presented here, reversed micelles and other molecular aggregates have been observed in, and can be studied by, size-exclusion chromatography with organic solvents [16–18].

Given the probable identification of the broad peak at 14–15 min as phospholipid aggregate(s), it is of interest to examine the remainder of the high-

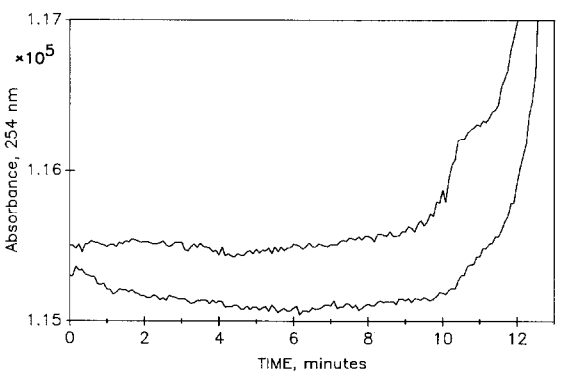

Fig. 7. High-sensitivity comparison of two soy lecithin lots in the region corresponding to $M_r > 15\,000$. Upper trace, lot A; lower trace, lot B. Other conditions as in Fig. 1.

M_r region of the GPC elution profile for the presence of other species. Fig. 6 shows the profile obtained for lot B at the highest concentration tested, 167 mg/ml. No additional peaks appear in this chromatogram relative to those at lower concentration (Figs. 1 and 2). In Fig. 7, a high sensitivity/high sample concentration comparison is made between lots A and B for the portion of the chromatograms corresponding to $M_r > 15\,000$. No additional peaks are observed in either sample (the rising baseline at 10–12 min corresponds to the front of the aggregate-like peak).

The results presented here could be further confirmed using static light-scattering experiments or with an on-line low-angle laser light-scattering (LALLS) detector [19]. Indeed, light scattering has been applied to the study of lecithin micelles in apolar solvents, a system designed to mimic the delivery of therapeutic aerosols from pressurized metered dose inhalers (MDIs) [10]. Using GPC and LALLS separately or in combination, it might be possible to study in more detail aggregate or micelle formation with specified solvents. Experiments along these lines are in progress.

ACKNOWLEDGEMENTS

We thank Dr. Daniel McNamara for helpful discussions and the management of Boehringer Ingleheim Pharmaceuticals, R&D, for their support during this project.

REFERENCES

1 *Food Chemicals Codex*, National Academy Press, Washington, DC, 3rd. ed., 1981, p. 166–167.
2 A. Nasner and L. Kraus, in J. N. Hawthorne and D. Lekim (Editors), *Soya Lecithin Dietetic Applications: Proceedings of the Second International Colloquium on Soya Lecithin, Brighton, April 3, 1982*, Semmelweis-Verlag, Hoya, 1983, pp. 26–34.
3 R. M. Sheeley, W. J. Hurst, D. M. Sheeley and R. A. Martin, *J. Liq. Chromatogr.*, 10 (1987) 3173.
4 M. Ghyczy, in B. F. Szuhaj (Editor), *Lecithins: Sources, Manufacture and Uses*, American Oil Chemists' Society, Champaign, IL, 1989, Ch. 8.
5 W. W. Yau, J. J. Kirkland and D. D. Bly, *Modern Size Exclusion Chromatography: Practice of Gel Permeation and Gel Filtration Chromatography*, Wiley, New York, 1979, pp. 241–246.
6 S. Mori, *J. Appl. Polym. Sci.*, 21 (1977) 1921.
7 J. Janca, *J. Chromatogr.*, 134 (1977) 263.
8 J. J. Kirkland and P. E. Antle, *J. Chromatogr.*, 15 (1977) 137.

9 M. Schneider, in B. F. Szuhaj (Editor), *Lecithins: Sources, Manufacture and Uses*, American Oil Chemists' Society, Champaign, IL, 1989, pp. 117 and 123.
10 R. M. Evans, D. Attwood, S. M. Chatham and S. J. Farr, *J. Pharm. Pharmacol.*, 42 (1990) 601.
11 N. Grinberg, R. Blanco, D. M. Yarmush and B. L. Karger *Anal. Chem.*, 61 (1989) 514.
12 H. Coll, *Sep. Sci.*, 6 (1971) 207.
13 P. Schmidt and H. Sucker, *Fresenius' Z. Anal. Chem.*, 250 (1970) 384.
14 H. H. Teo, M. G. Styring, S. G. Yeates, C. Price and C. Booth, *J. Colloid Interface Sci.*, 114 (1986) 416.
15 M. G. Styring, H. H. Teo, C. Price and C. Booth, *J. Chromatogr.*, 388 (1987) 421.
16 J. H. Lyngaae-Joergensen, *Makromol. Chem.*, 167 (1973) 311.
17 P. L. Dubin, in T. Provder (Editor), *Size Exclusion Chromatography (GPC)* (*ACS Symposium Series*, Vol. 138), American Chemical Society, Washington, DC, 1980, pp. 225–238.
18 P. Spacek and M. Kubin, *J. Appl. Polym. Sci.*, 30 (1985) 143.
19 H. H. Stuting I. S. Krull, R. Mhatre, S. C. Krzysko and H. G. Barth, LC · GC, 7 (1989) 402.