notes on methodology

Silicic acid HPLC of cardiolipin, mono- and dilysocardiolipin, and several of their chemical derivatives

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Summary A silicic acid HPLC system in hexane–2-propanol–1 mM H_3PO_4 50:50:3.5 (v/v/v) is described for the analysis and/or purification of cardiolipin (CL), monolysocardiolipin (MLCL), dilysocardiolipin (DLCL), and several of their chemical derivatives. Derivatives that have been successfully analyzed include CL that is acetylated, succinylated, or tetrahydropyranylated at the 2-hydroxyl; MLCL acetylated at the 2 and 2'-hydroxyls; DLCL acetylated at the 2-hydroxyl and both 2'-hydroxyls; and MLCL tetrahydropyranylated at only the 2-hydroxyl. Water can replace 1 mM H_3PO_4 in the eluting solvent, but prior conditioning of the silicic acid column with the phosphoric acid solvent is necessary for acceptable chromatography. The most significant factor affecting the elution times of these compounds is the percentage of aqueous component, i.e., water or 1 mM H_3PO_4. —**Robinson, N. C.** Silicic acid HPLC of cardiolipin, mono- and dilysocardiolipin, and several of their chemical derivatives. *J. Lipid Res.* 1990. **31:** 1513–1516.

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Cardiolipin (CL) is a phospholipid that has recently been shown to be important for the biological activity of most electron transport complexes (1–3), as well as several translocases of the inner mitochondrial membrane (4, 5). We have been particularly interested in studying the CL requirements of bovine cytochrome *c* oxidase, the terminal electron transport complex of the inner mitochondrial membrane. In these studies it has been important to analyze and purify CL, monolysocardiolipin (MLCL), dilysocardiolipin (DLCL), and their acylated, succinylated, and/or tetrahydropyranylated derivates, preferably by a high performance liquid chromatography (HPLC) method. A number of HPLC systems are available for the analysis of phospholipid mixtures on HPLC silicic acid columns (6–12); unfortunately none of these are applicable for the analysis and/or purification of these cardiolipin derivatives. With the methods that use acetonitrile–methanol–water as the eluant, CL is not retained by the column and is eluted at the solvent front (7–9). The most successful HPLC methods for CL chromatography on HPLC silicic acid columns are modifications of the method of Geurts et al. (6) using hexane–2-propanol–water as the eluant (10–12). This type of column and solvent system has been used for the isolation of gram quantities of CL by preparative HPLC (13, 14). However, these methods are designed to separate CL from mixtures of phospholipids and have not been used for the analysis and purification of derivatives or lyso products of CL. We recently used a modification of the hexane–2-propanol–water silica gel system to purify the synthetic tetrahydropyranyl derivatives of cardiolipin (THP-CL), monolysocardiolipin (THP-MLCL), and dilysocardiolipin (THP-DLCL) (15, 16). This communication describes the details of this chromatographic system and its application to the analysis and purification of a number of chemically and enzymatically produced derivatives of CL.

MATERIALS AND METHODS

CL, monolyso-CL (MLCL) and dilyso-CL (DLCL), in chloroform and under nitrogen, were purchased from Avanti Polar Lipids. Acetylated-CL (Ac-CL), diacetylated-MLCL (Ac_2-MLCL), and triacetylated-DLCL (Ac_3-DLCL) were each prepared by acetylating either CL, MLCL, or DLCL (6 μmol) with acetic anhydride (300 μmol), in 0.6 ml dry CH_2Cl_2 with 4-(N,N-dimethylamino)pyridine (15 μmol) as a catalyst. Succinyl-CL (Suc-CL) was similarly prepared from CL with succinic anhydride. Xenon fast atom bombardment–mass spectra with negative ion detection of the unmodified, acetylated and succinylated CL, MLCL, and DLCL were as expected for specific chemical modification of the 2 and/or 2'-hydroxyl(s). The observed $[M_r\text{–H}]^-$ values (rounded to the nearest whole integer) and the expected $[M_r\text{–H}]^-$ values (in parentheses) for each compound are: CL, 1448 (1447.9); MLCL, 1186 (1185.7); DLCL, 924 (923.5); AcCL, 1490 (1489.9); Ac_2-MLCL, 1270 (1269.7); Ac_3-DLCL, 1050 (1049.5); and Suc-CL, 1548 (1547.9). Tetrahydropyranyl-CL (THP-CL) was prepared and purified as previously described (15).

All HPLC elutions were performed at room temperature with a Waters RCM 100 Radial Module and a Radial Pak Resolve Silica 5μ cartridge (0.8 × 10 cm) at a flow

Abbreviations: Ac-Cl, 2-acetylcardiolipin or 1,3-bis-(3-*sn*-phosphatidyl)-2-acetyl-*sn*-glycerol; Ac_2-MLCL, diacetylated-monolysocardiolipin or 1-(3-*sn*-phosphatidyl)-3-(3-*sn*-phosphatidyl-2'-acetyl)-2-acetyl-*sn*-glycerol; Ac_3-DLCL, triacetylated-dilysocardiolipin or 1,3-bis-(3-*sn*-phosphatidyl-2'-acetyl)-2-acetyl-*sn*-glycerol; CL, cardiolipin; DLCL, dilysocardiolipin; HPLC, high performance liquid chromatography; MLCL, monolysocardiolipin; Suc-CL, 2-succinylcardiolipin or 1,3-bis(3-*sn*-phosphatidyl)-2-succinyl-*sn*-glycerol; THP-CL, 2-tetrahydropyranylcardiolipin or 1,3-bis(3-*sn*-phosphatidyl)-2-tetrahydropyranyl-*sn*-glycerol.

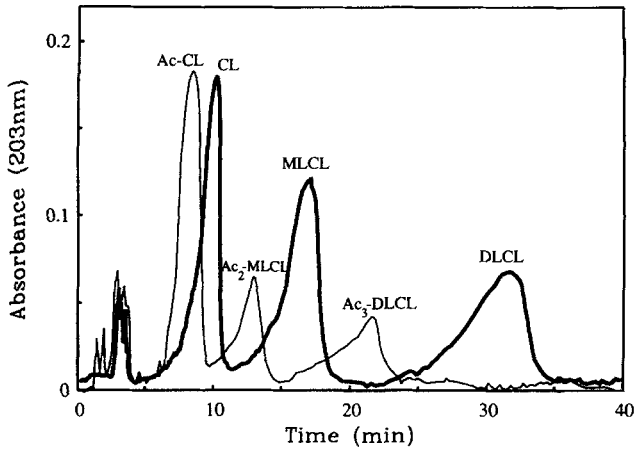

Fig. 1. Separation of CL and derivatives of CL on a Waters Silica Resolve Radial-Pak HPLC column in cyclohexane–2-propanol–water 45:50:3.5 (v/v/v). The heavy line is the elution obtained with a mixture of CL, MLCL, and DLCL; the thin line is the elution obtained with a mixture of Ac-CL, Ac_2-MLCL, and Ac_3-DLCL. In each case, a mixture containing 40–60 nmol of each compound was injected in 100 μl of solvent. The flow rate of the column was 1 ml per min.

rate of 1 ml per min with a Laboratory Data Control HPLC pump and controller. Detection of CL elution and the elution of its derivatives was monitored at 203 nm with a Gilson variable wavelength detector. Radioactivity of [^{14}C]AcCL was quantified in a Beckman model LS 230 scintillation counter after adding 5 ml of Triton X-100–toluene scintillation liquid to each dried sample.

2-Propanol, cyclohexane, and phosphoric acid (each HPLC grade) were purchased from Fisher Scientific Co. Deionized water was further purified by distillation. All solvent mixtures were filtered through a 0.45-μm Alpha 450 Metricel Membrane filter before they were used for HPLC elution.

RESULTS

Conditioning of the Waters Radial Pak Silica Resolve HPLC column with phosphoric acid was necessary for acceptable chromatography of CL or its chemical derivatives. Without prior conditioning of the column, CL is eluted as a broad asymmetric peak in the cyclohexane–2-propanol water 45:50:5.0 (v/v/v) solvent system described by Small et al. (13). Conditioning of the Silica Resolve column with cyclohexane–2-propanol–50 mM H_3PO_4 in water 45:50:5 (v/v/v) for 5–12 h at 0.5–1.0 ml/min caused CL to be eluted much sooner (7–10 ml) as a sharp symmetric peak, about 1 ml wide (**Fig. 1** and **Fig. 2A**). The H_3PO_4 column conditioning was monitored by determining the elution behavior of CL; once CL began to elute satisfactorily, phosphoric acid was removed from the aqueous component of the eluant without affecting subsequent elution behavior. Inclusion of 50 mM H_3PO_4 in the eluant for long periods of time decreased the elution time for CL and its derivatives and eventually made the silicic acid column unusable.

Phosphoric acid (or phosphate) bound to the silicic acid column during the conditioning procedure since phosphate was detected in the eluant several months after it was eliminated from the eluting solvent. After long periods of use, depletion of bound phosphate eventually caused CL to elute later as a more skewed and asymmetric peak. This deterioration of column performance was usually reversed by subsequent column conditioning with the phosphoric acid-containing solvent. Alternatively, 1–10 mM phosphoric acid can be included in the aqueous component during all chromatographic elutions. This improves the useful lifetime of the column to about 6–9 months of frequent use and results in less skewed elution peaks, but care must be taken that column poisoning with the resultant early elution of CL does not occur.

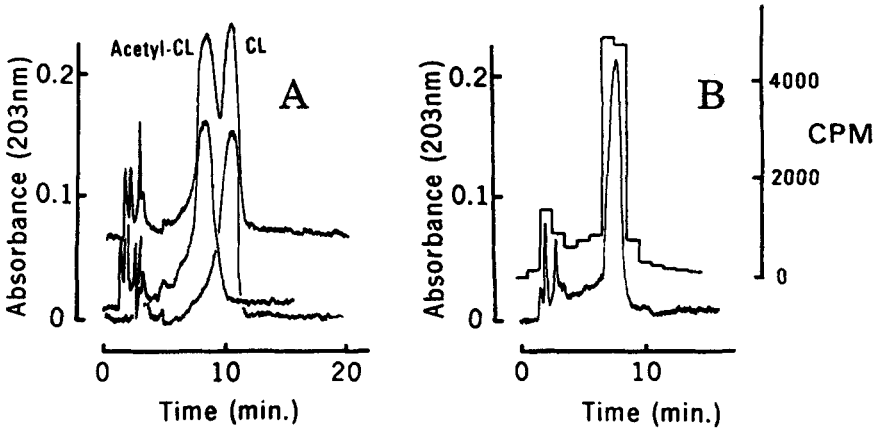

Fig. 2. Elution of acetylcardiolipin from an HPLC silicic acid column with cyclohexane–2-propanol–water 45:50:3.5 (v/v/v) as the eluant. Panel A: comparison of the elution of CL and Ac-CL: the two bottom traces are separate chromatographic elutions of 50 nmol Ac-CL and 50 nmol CL (Ac-CL at 8 min, CL at 10 min). The offset top trace is the chromatographic elution of a mixture of 50 nmol Ac-CL and 50 nmol CL. Panel B: purification of 38 nmol [^{14}C]Ac-CL (0.25 μCi/μmol). The lower tracing is the absorbance at 203 nm, the histogram represents the CPMs in each 1 ml fraction.

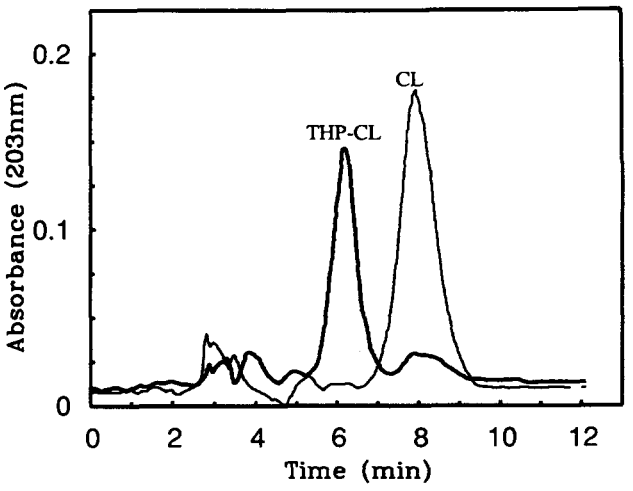

Fig. 3. Comparison of the elution of CL and THP-CL from an HPLC silicic acid column in cyclohexane–2- propanol–1 mM H_3PO_4 in water 50:50:3.0 (v/v/v). The thin line and heavy lines are the elutions obtained with CL and THP-CL, respectively. Approximately 50 nmol of each compound was injected in 100 μl of solvent. The flow rate was 1 ml per min. THP-CL was prepared as described (16).

CL, MLCL,, and DLCL are readily separated with cyclohexane–2-propanol–water 45:50:3.5 (v/v/v) as the eluant once the column is conditioned with phosphoric acid (Fig. 1). Each of the three peaks has an obvious leading edge, but the shape of each peak is much better than was observed prior to conditioning with H_3PO_4. A mixture containing Ac-CL, Ac_2-MLCL, and Ac_3-DLCL can also be separated with the same HPLC solvent (Fig. 1). In these separations, the more polar derivatives are eluted later than the more apolar ones, i.e., DLCL > MCLC > CL and Ac_3-DLCL > Ac_2-MLCL > Ac-CL. The more polar, nonacetylated compounds are also eluted later than the corresponding acetylated derivative, i.e., CL is eluted later than Ac-CL, MLCL later than Ac_2-MLCL, and DLCL later than Ac_3-DLCL. Ac-CL and CL are eluted quite closely to each other, but their separation is sufficient to resolve the two when they are loaded as a mixture (Fig. 2A). We have used this separation of Ac-CL and CL to purify synthetic [^{14}C]Ac-CL (Fig. 2B). Similar elution conditions have also been used to purify synthetic THP-CL from unmodified CL (**Fig. 3**). Normally, 1–5 μmol of the CL derivatives can be purified with an 80–90% yield from the silicic acid column.

Minor adjustments in the percent of the aqueous component are needed for each application in order to maximize separations and maintain reasonable peak shape. For example, separation of CL from Ac-CL (Fig. 2A) usually requires a lower percentage of the aqueous component than separation of monolyso-Ac-CL from Ac-CL and dilyso-Ac-CL. In each application, the most critical variable is the percentage of the aqueous component in the eluant, not the exact composition of the organic phase. Little difference in the elution behavior of CL was found with cyclohexane–2-propanol 50:50 (v/v) (Fig. 3) as the organic phase rather than cyclohexane–2-propanol 45:50 (v/v) (Fig. 1). However, even small changes in the volume of aqueous component greatly altered elution times. **Fig. 4A** summarizes the elution behavior of CL, MLCL,

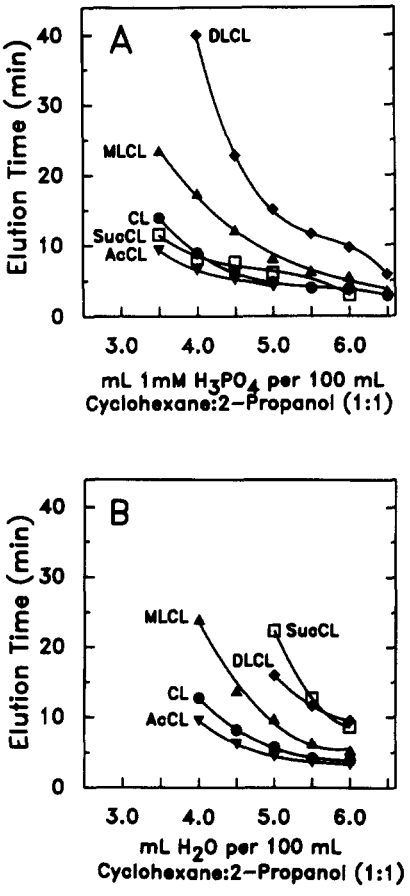

Fig. 4. Effect of changing the volume of the aqueous component upon the elution of CL and derivatives of CL from an HPLC silicic acid column. For each solvent mixture 100 ml of cyclohexane–2-propanol 50:50 (v/v) was mixed with 3.5–6.5 ml of 1 mM H_3PO_4 or water. (Note that the composition of the organic phase is identical to that used in Fig. 3, but is slightly different from that used in Figs. 1 and 2.) Elution times were measured after injecting a mixture containing two or three of the compounds, 10 nmol of each, in 100 μl. The flow rate was 1 ml per min. The absolute elution time shown in each panel is very dependent upon the column history, e.g., amount of conditioning with eluant containing 50 mM H_3PO_4, how long the column has been used with water as the aqueous component, total length of time the column has been in service, etc. For these reasons, the elution of CL in Fig. 3 at about 8 min (obtained with a column that had been used for more than 1 year) is earlier than would be expected from the data in panel A (obtained with a separately conditioned column that had been in service for less than a month). The relative elution times shown in both panels do accurately reflect the effect of varying the volume of the aqueous component since all of the data were collected within 2 days. With each solvent change, the column was equilibrated for at least 1 h at 1 ml/min prior to determining elution times for each compound.

DLCL, and their acetylated and succinylated products when the volume of 1 mM phosphoric acid is increased from 3.5 to 6.5 ml per 100 ml of cyclohexane–2-propanol 50:50 (v/v). Fig. 4B summarizes the elution of these same compounds from a H_3PO_4-equilibrated column as a function of the volume of water in the eluant, rather than the volume of 1 mM phosphoric acid. Without H_3PO_4 in the eluant, high percentages of water had to be avoided to prevent decreased column performance, presumably due to elution of bound phosphate from the column. When either the percentage of water or 1 mM H_3PO_4 was increased, the elution times of the CL compounds decreased and peaks became sharper. The differences in elution times as water was replaced by 1 mM H_3PO_4 were slight except for Suc-CL; retention times of Suc-CL in 1 mM H_3PO_4 were greatly decreased, probably due to protonation of the free carboxyl group.

DISCUSSION

Chemical derivatives of CL can be successfully separated and purified on a silicic acid HPLC column that has been conditioned with phosphoric acid and eluted with either cyclohexane–2-propanol–water 50:50:3.5–5.0 (v/v/v), or cyclohexane–2-propanol–1 mM H_3PO_4 in water 50:50:3.0–6.5. Conditioning of the column with phosphoric acid together with careful adjustment of the percentage of the aqueous phase permitted us to separate a number of cardiolipin derivatives rapidly and in relatively high yield, e.g., CL was separated from mono- and dilyso-CL (Fig. 1), Ac-CL from CL (Fig. 2A), and THP-CL from CL (Fig. 3). Separations of this type are very useful for preparing chemically modified CLs, e.g., Ac-CL, Suc-CL, and THP-CL; in monitoring phospholipase A_2 digestion of CL, Ac-CL, or THP-CL; or in purifying the monolyso products. We have recently used the latter application, purification of THP-monolyso-CL, during the synthesis of 2'-(12-[N-4-azido-2-nitrophenyl)] aminododecanoyl-CL (16). We have also used the method to advantage in monitoring the addition of a tetrahydropyranyl group, acetyl or succinyl group to the hydroxyl group of CL (15–17) (refer to Fig. 4). Others who synthesize chemical derivatives of CL and study CL chemistry should find the method extremely useful.

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