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Journal of Chromatography B, 831 (2006) 63–71
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Analysis of cardiolipin in human muscle biopsy

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Received 12 August 2005; accepted 18 November 2005
Available online 6 December 2005

Abstract

Cardiolipin is a phospholipid that is specific to the inner mitochondrial membrane and essential for numerous mitochondrial functions. Accordingly, a quantitative assay for cardiolipin can be a valuable aspect of assessing mitochondrial content and functional capacity. The current study was undertaken to develop a simple and reliable method for direct analysis of the major molecular species of cardiolipin and with particular application for analysis of human skeletal muscle. The method that is presented is based on derivatization of cardiolipin in a total lipid extract with 1-pyrenyldiazomethane (PDAM), to form stable, fluorescent 1-pyrenylmethyl esters. The derivatization reaction takes 30 min on ice in a two-phase system (chloroform:methanol:H_2O:H_2SO_4) containing 0.5–1.0 mM PDAM and detergent. The contents of the major cardiolipin species in the derivatization mixture can be estimated by HPLC separation with fluorescent detection during a 20 min run on a reverse phase column and with HPLC grade ethanol/0.5 mM H_3PO_4 as the mobile phase. The recovery is about 80%. The method is specific and sensitive with quantitation limits of 0.5–1 pmol cardiolipin. The response of the fluorescence detector (peak area) is linear across a range 5–40 pmol. The assay is linear over the range between 0.3 and 3.0 mg of tissue ($R^2 = 0.998$). The assay provides good reproducibility and accuracy (within 5–10%).

Keywords: Cardiolipin; Mitochondria; Skeletal muscle; Human; Fluorescent derivatization; HPLC; 1-Pyrenyldiazomethane

1. Introduction

Cardiolipin is a phospholipid that is contained within mitochondria [1–6]. Structurally, cardiolipin contains two phosphorus and four fatty acyls and in mammalian heart or liver mitochondria, linoleic acid accounts for up to 85% of the fatty acid residues of cardiolipin [1,7]. It is synthesized in mitochondria [2,7,8] and has been found to be an essential structural component of the inner mitochondrial membrane [1,6], modulating and stabilizing activity of the complexes of the mitochondrial electron transport chain [9–13]. Phospholipid reconstitution experiments reveal that cardiolipin is integral to all basic mitochondrial functions, including electron transport, ATP synthesis, and substrate transport [1,14,15]. Cardiolipin also provides the binding site for cytochrome *c* to the inner mitochondrial membrane [16,17]. Other possible roles of cardiolipin in mitochondria include modulation of proton leak [18] and participation in the early events of apoptosis [3,19–22]. In mitochondria from rabbit myocardium, cardiolipin accounts for 16% of total lipid phosphorus [23]. Because cardiolipin is unique to the inner mitochondrial membrane, and moreover, is present within this membrane in a relatively fixed ratio relative to protein and other lipids, a determination of the amount of cardiolipin provides a quantitative assessment of the amount of inner mitochondrial membrane. In support of this concept, cardiolipin content in tissue has been found to correlate with activity of the electron transport chain [24]. In Barth's syndrome, a rare genetic disorder causing deficiency of cardiolipin, the clinical symptoms reflect impaired mitochondrial function [25]. Because of this potential to serve as a bio-marker of the inner mitochondrial membrane, a sensitive, reliable and ideally, a simple assay for quantity and composition of cardiolipin could be a valuable tool in clinical investigation of mitochondrial diseases [26], as well as in more basic research.

The objective of the current studies was to develop a cardiolipin assay for use in skeletal muscle, with sufficient sensitivity to be useful for small biopsy samples obtained in clinical research. Recently developed procedures for analysis of cardiolipin combine HPLC with mass spectroscopic detection (LC–MS) [27,28]. However, currently this instrumental com-

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doi:10.1016/j.jchromb.2005.11.031

bination is not widely available. More commonly available is HPLC chromatograph equipped with either UV- or fluorescence detector. Fluorescent derivatization provides a high sensitivity, and the possibility to monitor various molecular species of cardiolipin. Schlame and Otten [29] developed a general approach for derivatization of cardiolipin that was later evolved into a highly sensitive HPLC-based assay for cardiolipin in biological samples. That procedure uses a two-step derivatization of cardiolipin to produce a fluorescent derivative, 2-(naphthyl-1′-acetyl)-cardiolipin dimethyl ester [30]. A drawback of the first step of this procedure is diazomethane treatment of the lipid extract. Diazomethane is an unstable and potentially explosive gas that needs to be prepared before each experiment. Thus, it would be advantageous to find alternative approaches. Another limitation is that the procedure is time consuming, including two derivatization steps and two intermediary purification steps by SPE, plus the need to heat the reaction mixture for 2 h at 40 °C [30].

Therefore, the goal of the current study was to explore the possibility of achieving fluorescent derivatization of cardiolipin with a simplified procedure and without need for diazomethane, yet retaining the high sensitivity of the previously established HPLC-based method. The approach presented in this report permits direct analysis of the major molecular species of cardiolipin in a lipid extract prepared from just 1–2 mg of human muscle tissue. A key point is the use of 1-pyrenyldiazomethane for a one-step fluorescent derivatization of cardiolipin. Using this derivatization approach, cardiolipin can be analyzed by fluorescent HPLC without preliminary separation and purification of fluorescent cardiolipin derivatives.

2. Materials and methods

2.1. Reagents

Cardiolipin standards, bovine heart cardiolipin (BH-CL), tetralinoleoyl cardiolipin (TL-CL), tetramyristoyl cardiolipin (TM-CL) and tetraoleoyl cardiolipin (TO-CL) were purchased from Avanti Polar lipids Inc. (Alabaster, AL) and used without further purification. The concentration of cardiolipin in these standards was estimated on the basis of phosphorus analysis. HPLC grade chloroform, stabilized by 0.7% ethanol was obtained from Fisher Scientific (Pittsburgh, PA). Other HPLC grade solvents and reagents were purchased from Sigma Chemicals, (St. Louis, MO). 1-Pyrenyldiazomethane (PDAM) (Molecular Probes, Eugene, Oregon) was used without further purification.

2.2. HPLC equipment

A Shimadzu high performance liquid chromatograph (model LC-10AT *vp*) equipped with an autosampler (model SIL-10AD) and a tray cooler, and a Shimadzu fluorescence detector (model RF-10Axl) and a Waters UV–vis detector (model 486) was used for these studies. The analog signal of the detectors was processed and stored in digital form with Shimadzu Class-VP software (Shimadzu Scientific Instruments Inc., Columbia, MD).

2.3. Research volunteers

Volunteers were recruited and medically screened. Participants were of stable weight, in good general health and with normal values for hematological, renal, thyroid and hepatic function. The University of Pittsburgh Institutional Review Board approved the investigation, and all volunteers gave informed written consent. Tissue from the *vastus lateralis* portion of quadriceps femoris muscle was obtained after an overnight fast using the percutaneous biopsy technique of Bergstrom [31]. Samples were frozen in liquid nitrogen and kept at −80 °C until analysis.

2.4. Creatine kinase assay

Activity of creatine kinase was measured using an HPLC-based assay as previously described [32].

2.5. Estimation of lipid phosphorus

An aliquot of a lipid solution was dried in a Pyrex tube and after mineralization of the residue by $HClO_4$ at 190 °C for 40 min, the phosphomolybdate/ascorbate assay was used to estimate inorganic phosphate [33].

2.6. Lipid extraction

For lipid extraction we used particulate fraction that was prepared from tissue homogenate as described earlier [32,34]. An aliquot of the particulate fraction, corresponding to 1–3 mg of tissue (wet weight) and containing more then 95% of tissue mitochondria [35], was re-suspended in 500 μl of washing medium containing 10 mM EDTA and 0.1 mg/ml BSA, (pH 8.3 at 21 °C) and then was centrifuged for 20 min at 4 °C at 22 000 rpm (45 000 × *g*) in an Heraeus Stratos Biofuge. BSA was added to washing medium to decrease the content of free fatty acid in final lipid extract because free fatty acids can react with derivatization reagent (PDAM). After centrifuging the supernatant was discarded, and 40 μl of methanol, 10 μg BHT (1 μl, 10 mg/ml in methanol) with 100–300 pmol of TO-CL as an internal standard (1–3 μl, 100 μM in ethanol) was added to the pellet for chloroform/methanol extraction or 20 μl of 5% *n*-dodecyl β-D-maltoside in H_2O with the same amount of TO-CL and BHT was added for 2-propanol/hexane extraction. The pellet was sonicated under nitrogen for 2–3 min at 4 °C using a horn probe connected to a Torbeo 36810 (Cole-Parmer, www.coleparmer.com) generator that was set to maximum power (20 dB). Then the pellet was extracted by chloroform/methanol mixture (2:1; total volume 300 μl) according to modified Folch et al. [36] procedure that did not include a final wash, or the pellet was extracted by 2-propanol/hexane mixture (2:3; total volume 500 μl) according to the procedure of Kolarovic and Fournier [37]. The lipid film obtained after extraction was dissolved in 330 μl of a chloroform: methanol mixture (2:1) and kept at −80 °C under nitrogen.

Second 2-propanol/hexane extraction after treatment of solid residue by proteinase K. To address whether cardiolipin was being fully extracted, additional experiments were performed

using the solid residue remaining after the first lipid extraction (2-propanol/hexane extraction in the presence of dodecyl maltoside). This solid residue was dried in a Speed Vac. This dry pellet was incubated with 20 μl of the solution of proteinase K (250 U/ml) for 120 min at 30 °C under nitrogen and then extracted by 2-propanol/hexane mixture in the presence of internal standard. The lipid film was re-extracted using Folch et al. procedure to remove residual products of proteolysis.

2.7. Preparation of red cell membranes and red cell lipid extract

Fresh blood from healthy human donor was collected and used immediately. All manipulations were on ice. Plasma and buffy coat were separated from the cells after a 15 min centrifugation at 1500 × *g*. The cells were washed three times in 5 volumes of buffer, which contained: 150 mM NaCl, 5 mM NaPi, (pH 8.0). Cell membranes (ghost) were prepared by lysis of washed cells in hypotonic phosphate buffer (5 mM NaPi, pH 8.0) according procedure [38]. Lipids from red cell membranes were extracted by using chloroform/methanol procedure.

2.8. Derivatization of cardiolipin in total lipid extract

The lipid film obtained after a lipid extraction was dissolved in 330 μl of a chloroform:methanol mixture (2:1). Two 150 μl aliquots of the lipid solution were transferred to glass tubes. To each aliquot, 6 μl of 50 mM H_2SO_4 was added and the mixture was vortexed to form a single phase. The tubes were allowed to stand for 5 min on ice to complete ion-exchange, following which, the solution was mixed with 100 μl H_2O or with 100 μl of water solution of *n*-dodecyl β-D-maltoside (10 mg/ml). The addition of this detergent is essential for derivatization of lipid extracts prepared in the absence of *n*-dodecyl β-D-maltoside. If lipids were extracted in the presence of *n*-dodecyl β-D-maltoside, the final lipid extract already contained a sufficient amount of detergent. The presence of a water phase extracts sulfuric acid and sulfates from the chloroform phase and provides conditions for derivatization. The detergent facilitates mixing of the two phases: aqueous and organic (see Section 3). The mixture was vortexed and then centrifuged for 2 min in a Speed Vac (at ∼3000 rpm, no vacuum) to achieve separation of the two phases and to collect the bulk of chloroform at the bottom of the tube. Both phases were left in the tube for the next step. A chloroform solution of PDAM (2.5–5.0 μl; 1.0 mg/ml) was added to the chloroform phase on the bottom of the tube. The tubes were agitated manually for 2–3 s, vortexed for 20 s, and then placed for 30 min on ice, under nitrogen, to complete the derivatization. Two microliters of concentrated acetic acid were added at the end of this incubation. The resulting mixture was vortexed and kept on ice for 5 min to eliminate the excess of PDAM in the reaction with acetic acid. Then, 100 μl of chloroform plus 500 μl of 50% methanol in water was added, tubes were vortexed, and then centrifuged for 2 min in a Speed Vac (at ∼3000 rpm, no vacuum). After phase separation, the chloroform phase was transferred to glass tubes and the solvent was evaporated using a Speed Vac vacuum concentrator. The solid residue was re-dissolved in 80 μl of tetrahydrofuran. At this point, samples can be stored at −80 °C under nitrogen without deterioration for several weeks. Prior to HPLC, the samples were diluted by 20 μl of H_2O, and transferred to autosampler glass vials using a Pasteur pipette.

2.9. Preparation of 1-pyrenylmethyl ester derivative of TO-CL

1-Pyrenylmethyl tetraoleoyl cardiolipin was prepared by derivatization of 250 μg (0.167 μmol) of tetraoleoyl cardiolipin by PDAM in a molar ratio of 1:10. The standard procedure was scaled upward by a factor of 10, except that the quantity of detergent in the assay was kept the same. The derivative was purified using a SPE procedure on a C-18 reverse phase cartridge (Sep-Pak Cartridge C18, Waters, www@waters.com). The cartridge was first washed with 100% tetrahydrofuran and then preconditioned with 60% tetrahydrofuran. The chloroform phase from the derivatization mixture was evaporated and solid residue was re-dissolved in 0.6 ml of tetrahydrofuran. This solution was then mixed with 0.4 ml of water and applied onto the pre-conditioned cartridge. After application of the sample, the cartridge was washed by 5 ml of 60% tetrahydrofuran. The cardiolipin derivative was eluted from the cartridge by 2 ml of 100% tetrahydrofuran. The product showed essentially a single peak under HPLC reverse phase separation, with absorbance detection at 205 or 342 nm (purity 95% at 342 nm) (Fig. 1B). The calculated yield of 1-pyrenylmethyl tetraoleoyl cardiolipin was approximately 70% of the theoretical.

2.10. HPLC separation

Aliquots of prepared samples (2–10 μl) were injected onto a C-18 reverse phase column (Zorbax XDB-C18, 4.6 mm × 150 mm; Agilent Technologies, www.agilent.com/chem). The column was protected by a guard cartridge (Eclipse XDB-C18; Agilent Technologies) and was connected to fluorescence and UV detectors. The column was eluted (1 ml/min) by a mobile phase composed of HPLC grade ethanol and 0.5 mM H_3PO_4. In the eluate, fluorescence of 1-pyrenylmethyl ester derivatives of cardiolipin was monitored at an emission of 395 nm, after excitation at 340 nm. The UV detector was set at 205 or 342 nm. All separations were performed at a stable ambient temperature (21 °C).

3. Results and discussion

3.1. Fluorescent derivatization of cardiolipin

The first objective was to assess whether a fluorescent derivatization of cardiolipin could be obtained using 1-pyrenyldiazomethane. PDAM readily reacts with carboxylic acids, including prostaglandins, at room temperature and without need for a catalyst [39–41]. The resulting esters are highly fluorescent and stable [39]. The diazomethyl analog of PDAM, 9-anthryldiazomethane, has been used to obtain a fluorescent derivative of polyphosphoinositides and phosphatidic acid [42,43]. Initial attempts at the incubation of

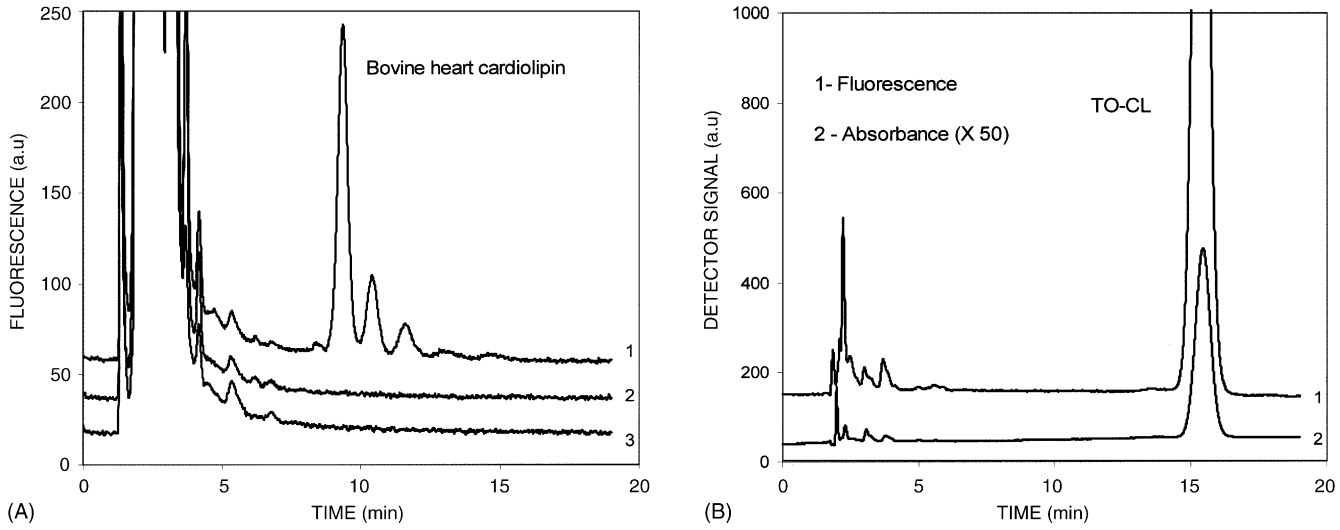

Fig. 1. (A) Fluorometric detection of 1-pyrenylmethyl ester derivatives of bovine heart cardiolipin (trace 1) in the derivatization mixture using HPLC separation on reverse phase column. Twenty nanograms of derivatized bovine heart cardiolipin standard was injected. Derivatization was performed without addition of cardiolipin (trace 2), or PDAM was preliminarily exhausted in reaction with acetic acid (trace 3). (B) Reverse phase separation of purified 1-pyrenylmethyl ester derivative of tetraoleoyl cardiolipin. Injected amount: 100 pmol. Absorbance at 342 nm.

1-pyrenyldiazomethane with cardiolipin standards, including bovine heart cardiolipin obtained from Avanti Polar Lipids as a sodium salt, did not result in the formation of 1-pyrenylmethyl ester derivatives of cardiolipin. This was regardless of the type of solvent that was used for the derivatization mixture (chloroform, ethyl acetate or chloroform/methanol mixture). However, if cardiolipin derivatization takes place within in a two-phase solvent system (chloroform and H_2O/methanol), and in the presence of H_2SO_4, it was found that the 1-pyrenyldiazomethane easily reacts with cardiolipin to form fluorescent derivatives, and that this occurs even carrying out the reaction on ice. The derivatives of the various molecular species of cardiolipin can be monitored by an isocratic, reverse-phase HPLC separation with fluorescence or UV detection, using HPLC grade ethanol containing 0.5 mM H_3PO_4 as a mobile phase.

In Fig. 1A (trace 1), a typical chromatogram is presented, obtained after reverse-phase separation of a 10 μl aliquot of the derivatization mixture, containing 20 ng of a highly purified preparation of commercially obtained bovine heart cardiolipin. Several peaks with retention times from 8 to 15 min, corresponding to the different molecular species of cardiolipin can be identified. To verify that these peaks corresponded to cardiolipin derivatives, it was shown that there were not peaks in this region when derivatization was performed without either the addition of cardiolipin (trace 2), or if PDAM was preliminarily exhausted in reaction with acetic acid (trace 3). Tetraoleoyl cardiolipin was used in these experiments as an internal standard, and it was observed that following its derivatization, there was a single fluorescence peak (and a single absorbance peak) with a retention time of approximately 15 min. This is illustrated in Fig. 1B, in which a chromatogram for 1-pyrenylmethyl-TO-CL is shown that was prepared by derivatization and purification on an SPE cartridge. The response of the fluorescence or UV detector (peak area) was linear across a range from 5 to 40 pmol of injected 1-pyrenylmethyl-TO-CL ($R^2 = 0.999$).

Derivatized lipid extracts from human skeletal muscle revealed the same set of cardiolipin peaks as did bovine heart cardiolipin standards, though in slightly different proportions for the molecular species. In Fig. 2A (trace 1), a typical chromatogram is presented, obtained with fluorescent detection during reverse phase separation of a 10 μl aliquot of the derivatization mixture. This mixture contained the total lipid extract from ~0.15 mg (wet weight) of human skeletal muscle (0.75 nmol lipid phosphorous) plus 13 pmol of tetraoleoyl cardiolipin as an internal standard. The retention times for the major peaks (peaks I–III) corresponding to the 1-pyrenylmethyl ester derivative of cardiolipin species in tissue lipid extract, are in a range from 8 to 14 min and are located between TM-CL and TO-CL standards (Fig. 2B, trace 1). These same peaks can be seen on the chromatogram obtained with UV detection at 342 nm (Fig. 2A, trace 2). The identity of the cardiolipin peaks was established on the basis of retention times. The major cardiolipin peak (peak I) in the tissue extract had the same retention time (9.3 min) as tetralinoleoyl cardiolipin (Fig. 2B, trace 2). The finding that tetralinoleoyl cardiolipin is the major peak in human skeletal muscle is consistent with a previous analysis of cardiolipin species in this tissue [30]. The other prominent peaks (II and III), also identified from relative retention times and height, are trilinoleoyl-oleoyl cardiolipin and dilinoleoyl-dioleoyl cardiolipin, respectively [30].

The cardiolipin assay that was developed combines two procedures, conversion of cardiolipin to an acidic form followed by derivatization. The first step, that of the conversion of cardiolipin to an acidic form, was achieved in a one phase mixture by the addition of a small aliquot of 50 mM H_2SO_4 to the lipid solution in chloroform/methanol. The presence of methanol was found to be essential for a high yield of cardiolipin derivatives. This is because methanol helps to form a one-phase mixture needed for effective ion exchange between cardiolipin and H_2SO_4. The effect of H_2SO_4 concentration on the yield of 1-pyrenylmethyl

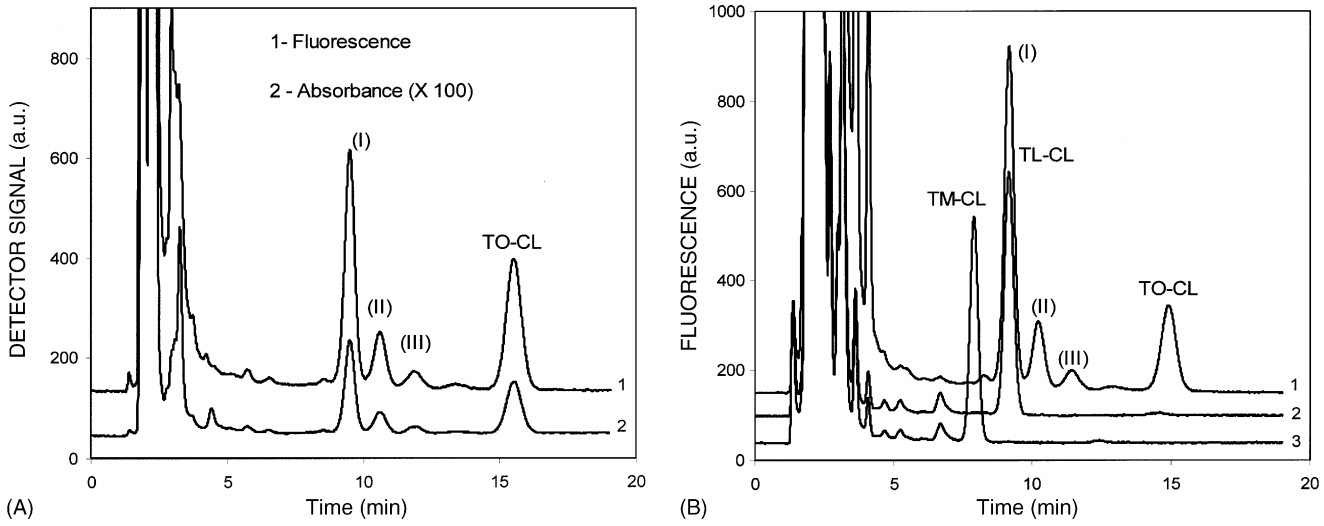

Fig. 2. (A) Detection of cardiolipin in lipid extract from human skeletal muscle. Aliquot of derivatized lipid extract (corresponding ~0.17 mg tissue wet weight) and containing 13 pmol of tetraoleoyl cardiolipin (TO-CL) as internal standard was applied on the column. Absorbance at 342 nm. Peaks I–III are cardiolipin molecular species corresponding to the 1-pyrenylmethyl ester derivative of tetralinoleoyl-, trilinoleoyl-oleoyl- and dilinoleoyl-dioleoyl cardiolipin, respectively. (B) 1-Pyrenylmethyl ester derivatives of cardiolipin standards and cardiolipin molecular species in lipid extract from human muscle. (1) Lipid extract corresponding to 0.1 mg of tissue (wet weight) plus 10 pmol of tetraoleoyl cardiolipin (TO-CL) as internal standard; (2) 15 pmol of tetralinoleoyl-cardiolipin and (3) 15 pmol of tetramyristoyl-cardiolipin. Peaks I–III are cardiolipin molecular species in the lipid extract corresponding to the 1-pyrenylmethyl ester derivative of tetralinoleoyl-, trilinoleoyl-oleoyl- and dilinoleoyl-dioleoyl cardiolipin, respectively.

ester derivatives of cardiolipin in a two-phase system can be seen in Fig. 3. In the absence of H_2SO_4, the product yield is low. Product yield is sharply increased after the addition of 3 μl of 50 mM H_2SO_4. An increase in H_2SO_4 concentration by factor of two or three had no additional effect on yield (Fig. 3). Hydrochloric or phosphoric acids, when tested, showed significantly less effect to increase yield of product than did H_2SO_4.

The second step is cardiolipin derivatization. A necessary condition for a smooth derivatization of cardiolipin by PDAM is the formation of two phases (chloroform and H_2O/methanol). Even after conversion of cardiolipin to an acidic form by H_2SO_4-treatment, a derivatization reaction carried out in a one-phase system (chloroform or ethyl acetate) during 2 h on ice, or at 30 °C, was found to yield a negligible amount of cardiolipin derivatives. Formation of a two-phase system was achieved by the addition of an excess of water (see Section 2) to a solution of lipid in a chloroform/methanol/H_2SO_4 mixture.

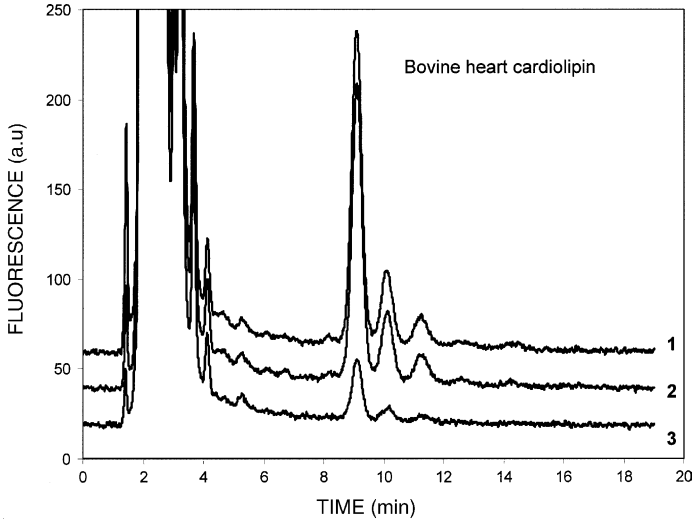

Fig. 3. Effect of H_2SO_4 on derivatization of bovine heart cardiolipin by 1-pyrenyldiazomethane. Two hundred nanograms of bovine heart cardiolipin standard was derivatized by 1-pyrenyldiazomethane in the absence of H_2SO_4 in the derivatization mixture (3) or in the presence of 3 or 6 μl aliquot of 50 mM H_2SO_4 (1 and 2, respectively) and then the aliquot equivalent to 10% of derivatization mixture was applied on the column.

Tetraoleoyl cardiolipin was used as an internal standard in our method. Human muscle normally contains only trace amounts of this molecular form of cardiolipin, making it a good choice to use as an internal standard. Another choice is tetramyristoyl cardiolipin, and this is also commercially available, and not normally present in tissue (Fig. 2B). A major problem of analytical methods that are based on fluorescent derivatization can be interfering peaks caused by derivatization reagents [41]. Fortunately, because of the high molecular mass of cardiolipin, interferences due to the derivatization mixture were found to not be an issue because these components have substantially shorter retention times than do the cardiolipin derivatives. The column load and accordingly, the sensitivity of the assay can be increased with a reduction in PDAM concentration in the derivatization mixture from 5.0 to 2.5 μg per assay.

3.2. The specificity of cardiolipin assay

Cardiolipin is not only lipid that that can be derivatized by PDAM. Other lipids containing free phosphate or carboxyl group also can react with PDAM and therefore, in the development of this assay it a question that needed to be addressed was whether such derivatives might interfere in the HPLC identification of cardiolipin. To probe the possibility of interference in

the cardiolipin assay, red blood cells, which do not contain mitochondria but do contain all basic membrane lipid classes except cardiolipin [44], were used. A lipid extract from red cell membranes (ghosts) was derivatized in the presence or in the absence of internal cardiolipin standard (TM-CL). Derivatized lipids from red cells (20 μg) do not show interfering peaks with retention times close to those for cardiolipin. In the lipid extract from red cells to which an internal cardiolipin standard, was added, the cardiolipin peak can be identified, which demonstrates the specificity of this assay for cardiolipin (data not shown).

3.3. Calibration of fluorescence and absorbance detectors

To quantitate cardiolipin molecular forms with fluorescence or UV detector, a purified (HPLC purity ∼95%) 1-pyrenylmethyl ester derivative of TO-CL was prepared as described in Section 2. A solution of 1-pyrenylmethyl ester derivative of TO-CL dissolved in ethanol/tetrahydrofuran mixture (10:1, v/v) was used to calibrate fluorescence or UV detector. Analysis of the phosphorus content was used to estimate the concentration of the 1-pyrenylmethyl ester derivative of TO-CL in the stock solution. In Fig. 4, the absorbance spectrum of a 1-pyrenylmethyl ester derivative of TO-CL is presented. The spectrum is typical for pyrenyl derivatives, with characteristic peaks at 276 and 344 nm. The specific fluorescence peak area for 1-pyrenylmethyl-TO-CL that was estimated from the calibration curve is 87 a.u./pmol and the specific absorbance peak area at 342 nm is 0.34 a.u./pmol. Comparison of the areas of fluorescent and A_{342} peaks obtained for different molecular species of cardiolipin showed that the specific fluorescent peak area does not depend on the fatty acid composition of cardiolipin. The sensitivity of the Shimadzu fluorescence detector model RF-10Axl and Waters UV–vis detector model 486 is sufficient to detect 1-pyrenylmethyl ester derivatives equivalent to 0.5–1 pmol and 5–10 pmol (S/N > 3) of cardiolipin, respectively.

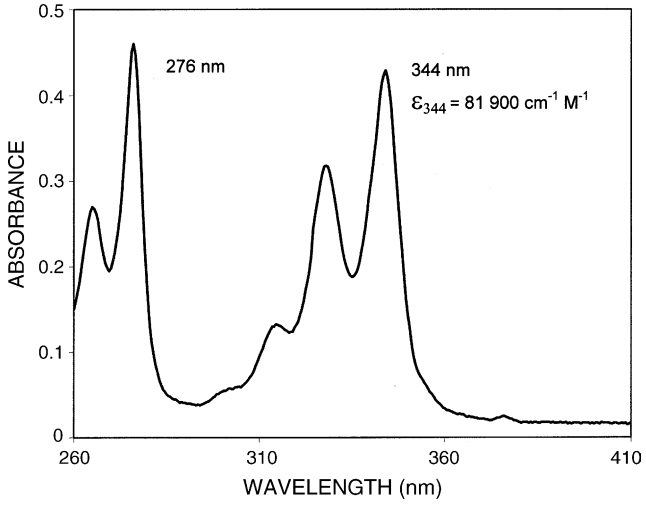

Fig. 4. Absorbance spectra of 1-pyrenylmethyl tetraoleoyl-cardiolipin. Solvent: ethanol/tetrahydrofuran (10:1, v/v). Concentration of 1-pyrenylmethyl-tetraoleoyl cardiolipin ester: 5.03 μM. The concentration of 1-pyrenylmethyl-tetraoleoyl cardiolipin ester was calculated from analysis of phosphorus.

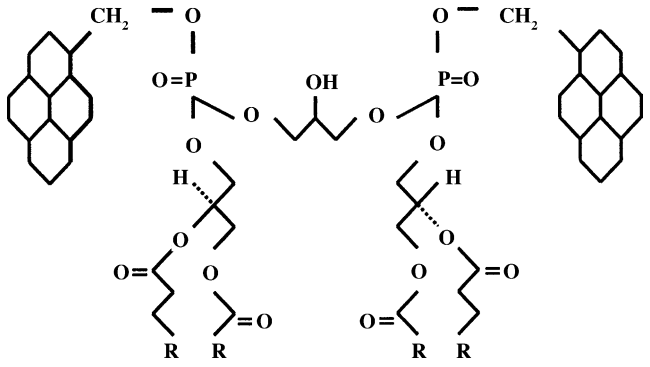

Fig. 5. Suggested structure of di-(1-pyrenylmethyl) ester derivative of cardiolipin.

3.4. Stoichiometry of reaction between PDAM and cardiolipin

The stock solution of 1-pyrenylmethyl ester derivative of TO-CL was also used to estimate the molar extinction coefficient at 344 nm (ε_{344}) (Fig. 4). The analysis of phosphorus and pyrenyl absorbance showed that the molar extinction coefficient of 1-pyrenylmethyl ester derivative of TO-CL at 344 nm is 81 900 cm^{-1} M^{-1}. This value is approximately two-fold higher than the corresponding value for various types of molecular probes that contain only one pyrenyl residue per molecule (35–41 000 cm^{-1} M^{-1}) [45]. A two-fold higher molar extinction coefficient for cardiolipin derivatives indicates that the derivatization product of cardiolipin obtained with PDAM is actually a di-(1-pyrenylmethyl) ester derivative, one that contains a 1-pyrenylmethyl on both phosphates of cardiolipin (Fig. 5). Furthermore, this derivatization converts cardiolipin from an acidic to a neutral form and consistent with this, the addition of phosphoric acid to the mobile phase did not affect the retention time for 1-pyrenylmethyl-cardiolipin on a reverse phase column (data not shown).

3.5. Improving the yield and reproducibility of the assay

The reaction between PDAM and cardiolipin was found to take place efficiently within a two-phase system. We postulated that improving inter-mixing of two phases might further improve the yield and reproducibility of the assay. To facilitate inter-mixing of the chloroform and methanol-water phases, we added 1 mg of *n*-dodecyl β-D-maltoside (grade "Ultra"), a high quality, neutral detergent (Sigma Chemicals), to the derivatization mixture. The yield of derivatization did increase significantly with addition of this detergent, as did reproducibility of the assay. At a temperature of 4 °C (on ice), the yield of the derivative of the internal standard (TO-CL) reached approximately 80% of the theoretical maximum yield within the first 30 min of incubation. Increasing the incubation time for up to 3.0 h had no significant additional effect. In this analytical approach, cardiolipin quantity in the muscle lipid extract was calculated as the ratio between the sum of the peak areas of major molecular cardiolipin species (peaks I–III in Fig. 2) and the peak area of the internal standard. This ratio remained constant regardless of incubation time. The

use of an internal standard provides good reproducibility and accuracy (within 5–10%) of assay. The recovery of the internal standard (TO-CL) across the whole procedure, including lipid extraction and derivatization, is approximately 80%.

The cardiolipin assay was observed to have a wide dynamic range. The amount of muscle TL-CL detected by the assay was highly linear across a range from 2.5 to 25 nmol of total tissue lipid phosphorus ($R^2 = 0.998$). With respect to human skeletal muscle, this range corresponds to 0.3–3 mg of tissue wet weight.

3.6. Probing a possible effect of adventitious cations of transition metals on cardiolipin assay

In the preparation of the tissue samples there is a potential for contamination by adventitious cations of transition metals, entering the sample from sources such as the stainless steel shearing assembly of the Polytron homogenizer. Cations of iron or nickel could interfere with the cardiolipin assay if these became tightly bound to the cardiolipin phosphate moiety since this would hinder derivatization. To examine the sensitivity of the cardiolipin assay to the presence of transition metals, $FeSO_4$ was used as a source of transition metal and 8-hydroxy-5-quinolinesulfonic acid was added as a chelating agent [46]. The addition of $FeSO_4$ to the chloroform/methanol solution in a molar ratio to cardiolipin of 1:1 had no effect on the yield of the cardiolipin derivative. Also, the addition to the water phase of 2.5 mM of 8-hydroxy-5-quinolinesulfonic acid, additional to H_2SO_4, had no significant effect to improve the yield of cardiolipin derivatives. These experiments suggest that the cardiolipin assay is highly resistant to possible contamination by trace amounts of transition metals, and is not improved by the addition of a chelating agent.

3.7. Cardiolipin extraction

Cardiolipin is an acidic phospholipid and has capacity to form a complex with basic proteins. A classic example of this interaction is that of cardiolipin with cytochrome *c* [47]. However, binding of cardiolipin with proteins could potentially reduce its extraction and thereby cause an underestimation of its tissue content. Our observations support this suggestion. Mild acidification (1 mM HCl) of the chloroform/methanol mixture, sufficient to converts proteins to a positively charged form, significantly reduced the yield of cardiolipin in lipid extracts. We compared different methods of lipid extraction for recovery of cardiolipin from tissue. To reduce electrostatic interactions between proteins and cardiolipin, the mitochondria fraction was washed by a 10 mM solution of EDTA at pH 8.3 prior to lipid extraction. The presence of a high concentration of EDTA ensures the removal of divalent cations. These can cross-link negatively charged proteins with negatively charged cardiolipin. The chloroform/methanol procedure achieves approximately a 90% extraction of cardiolipin from an EDTA-washed particulate fraction as was tested by second extraction using *n*-dodecyl β-D-maltoside/2-propanol/hexane mixture. Nearly complete extraction of cardiolipin (more than 97%) can be achieved by adding *n*-dodecyl β-D-maltoside to a 2-propanol/hexane mixture. Digestion of the protein residue, obtained after first 2-propanol/hexane extraction by proteinase K [48], did not release additional cardiolipin (Fig. 6). The 2-propanol/hexane procedure can be recommended for extraction of isolated sub-cellular fractions. The chloroform/methanol procedure can be recommended for extraction of tissue homogenates and cell cultures. The chloroform/methanol procedure provides lower fluorescent background in the chromatograms, probably as result of better separation of lipids from non-lipid contaminates.

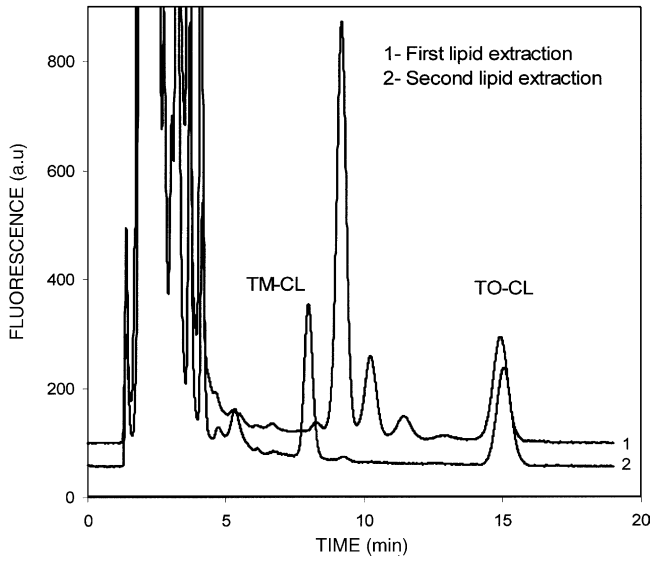

Fig. 6. Extraction of cardiolipin by 2-propanol/hexane mixture in the presence of *n*-dodecyl β-D-maltoside. Aliquot of derivatized lipid extract (corresponding 0.1 mg of tissue wet weight) and containing 10 pmol of tetraoleoyl cardiolipin (TO-CL) or containing the mixture of 10 pmol of tetraoleoyl cardiolipin (TO-CL) plus 10 pmol of tetramyristoyl cardiolipin (TM-CL) was applied on the column. (1) First lipid extraction and (2) second lipid extraction after digestion of residue from first extraction by proteinase K (second extract reveals only traces amount of cardiolipin).

3.8. Analysis of cardiolipin in biopsy from human skeletal muscle

The derivatization of cardiolipin with PDAM provides a sensitivity that is sufficient to estimate cardiolipin content in a 0.5–1 mg wet weight sample of human skeletal muscle. It can be estimated that this amount of tissue contains 150–300 ng (100–200 pmol) of cardiolipin. It is important to know the minimal weight of the tissue sample that can be used for an accurate analysis of cardiolipin content in muscle. To estimate heterogeneity in the distribution of cardiolipin in small samples of tissue, three fragments, each of ∼1–4 mg wet weight, were taken randomly from the same biopsy sample. The tissue samples were homogenized and separated by centrifugation into particulate and soluble fractions, as described earlier [32,34]. The supernatant was used for determination of creatine kinase activity and the pellet was used for lipid extraction to determine cardiolipin content. In Table 1, data on cardiolipin content are shown in these three replicate samples. Also shown in Table 1 are values for cardiolipin content, expressed either on the basis of tissue wet weight or normalized to tissue creatine kinase activity, the

Table 1
Analysis of cardiolipin content in the tissue samples taken randomly from the same biopsy

Tissue sample[a] (mg w-w)	Creatine kinase (U/sample)	Cardiolipin (pmol/sample)	Cardiolipin (pmol/mg w-w)	Cardiolipin (pmol/U CK)
1.0	4.5	190.0	190.0	42.4
1.8	11.2	516.8	287.1	46.1
3.9	16.5	851.7	218.4	51.7

[a] Wet weight.

latter reflecting the content of muscle fibers in the biopsy sample [49]. As revealed in this comparison, expressing cardiolipin content relative to CK activity has an advantage compared to using tissue wet weight in the denominator. The variance in the replicate determination of cardiolipin content in tissue is significantly smaller when normalized to tissue CK activity, which may be useful for statistical comparisons across groups and the heterogeneity of cardiolipin content, even for small fragments of tissue (1–4 mg), does not exceed ±10%.

Table 2 shows the relative content of the major molecular forms of cardiolipin (tetralinoleoyl cardiolipin, trilinoleoyl-oleoyl and dilinoleoyl-dioleoyl cardiolipin) in these tissue samples, each processed from the same biopsy. As can be seen in Table 2, there is strong consistency in the relative distribution of the different cardiolipin species. The major molecular form of cardiolipin is tetralinoleoyl cardiolipin and this represents ∼81% of total cardiolipin in human skeletal muscle. The sensitivity of the assay even at a column load of only 10% of the total volume of the derivatization mixture is sufficient to reliably estimate cardiolipin content in an aliquot of lipid extract from just 0.5–1 mg muscle tissue containing 150–300 pmol cardiolipin, as shown in Table 1. However, from a practical point of view, it seems reasonable that the amount of tissue used to prepare homogenate and the particulate fraction should be 10–20 mg of wet weight. The particulate fraction prepared from this amount of tissue can be used not only for analysis of cardiolipin, but also for assessment of functional activity of the electron transport chain [32]. Also, increasing the amount of tissue reduces the potential for sample heterogeneity in mitochondrial content.

In summary, this analysis shows that a two-phase reaction system containing sulfuric acid and a detergent achieves reliable derivatization of cardiolipin, provides excellent reproducibility, and a high yield, that attains ∼80% of theoretical. The derivatization of cardiolipin by PDAM yields essentially one product, di-(1-pyrenylmethyl)-cardiolipin ester, and this was found to be stable and highly fluorescent. Combined with a good method for lipid extraction, the approach that was achieved was found to attain accurate quantitation of cardiolipin and delineation of cardiolipin species even when used with quite small amounts of tissue. With UV detection, the procedure provides the same sensitivity as the previously developed technique [30]. Fluorescence analysis increases sensitivity by more than 10-fold. We suggest that the significant improvement in assay sensitivity with both UV and fluorescence detectors is due to the attachment of two pyrenyls to cardiolipin. The assay takes fewer steps then the previous procedure and does not expose cardiolipin to a long incubation at elevated temperature [30]. The robust quality of this derivatization procedure has the further advantage that it can be conducted under mild conditions with samples placed on ice. This decreases the possibility for oxidation of cardiolipin. Overall, because of its sensitivity and reliability, the cardiolipin assay developed in these experiments can be used as a valuable tool in clinical and basic research studies of mitochondrial function.

Table 2
The relative content of cardiolipin molecular species in the tissue samples taken randomly from the same biopsy

Tissue sample[a] (mg w-w)	Cardiolipin molecular form (% of total)		
	Tetralinoleoyl cardiolipin	Trilinoleoyl-oleoyl cardiolipin	Dilinoleoyl-dioleoyl cardiolipin
1.0	80.7	14.7	4.4
1.8	81.5	13.7	4.6
3.9	81.6	13.7	4.5

[a] Wet weight.

Acknowledgements

Funding for this study was provided by the Department of Veteran Affairs Merit Award, and by grant RO1 DK49200-06. We would like to gratefully acknowledge the valuable contributions of Carol Kelley, RN, the research coordinator, and the nursing staff of the University of Pittsburgh General Clinical Research Center for their efforts in carrying out the clinical investigation component of this project. We would also like to express our appreciation to the research volunteers who participated in these studies.

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