Note

Isolation of hydroxy fatty acids from livers of carbon tetrachloride-treated rats by thin-layer chromatography

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Non-enzymatic auto-peroxidation of polyunsaturated fatty acid moieties of glycerophosphatides has been a proposed mechanism of membrane damage induced by several toxins and reactive oxygen intermediates[1–5]. Most studies on the relationship of lipid peroxidation to toxicity have depended either on indirect and non-specific methods for quantification of peroxidation[1–4] or on the identification of some parts of the oxidation products. Hughes *et al.*[5] have identified conjugated double-bond containing peroxidation products of one class of glycerophosphatides, phosphatidylcholines, from the liver of carbon tetrachloride-treated mouse. Consequently, the structure of the *in vivo* lipid peroxidation products and the underlying mechanism are still mostly obscure.

The objective of our work was to isolate all long-chain products of *in vivo* non-enzymatic auto-peroxidation of glycerophosphatide fatty acids which are formed following carbon tetrachloride poisoning. Since the work of Hughes *et al.*[5] indicated that peroxidized lipids were rapidly converted *in vivo* to the corresponding hydroxy acids, we developed a method for isolation of all 12–22 carbons long hydroxy fatty acids, conjugated and not conjugated, from all classes of glycerophosphatides without *in vitro* peroxidation during chemical and chromatographic manipulations of the tissue lipids.

EXPERIMENTAL

All thin-layer chromatography (TLC) plates were pre-coated and were obtained from Analtech, Newark, DE, U.S.A. TLC was carried out at ambient laboratory temperatures (about 22°C) in standard TLC glass chambers (inside dimensions: 7.3 × 27.5 × 26 cm) lined with filter paper. Before chromatography, the solvents were allowed to saturate the chambers for about 10–20 min. Unless otherwise stated, the plates were developed until the solvent front reached the top, about 18 cm from the origin.

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Extraction of liver lipids[6]

Male Sprague-Dawley rats, weighing 200–250 g, were obtained from Harlen, Houston, TX, U.S.A. Two rats were fasted 12 h overnight prior to receiving carbon tetrachloride (10 mmol/kg) dissolved in mineral oil (1:1, v/v) by gavage. The animals were killed 60 min after dosing by decapitation, exsanguinated, and their livers were excised and kept on ice prior to extraction of lipids. The livers were weighed and total lipids were extracted with 20 volumes of chloroform–methanol (2:1, v/v) containing 0.05% (w/v) 2,6-di-*tert*-butyl-*p*-cresol (BHT). After filtration, the filtrate was washed with 0.2 volume of 0.8% aqueous sodium chloride and centrifuged at 9500 g at 4°C to separate the chloroform and water phases. The chloroform layer was removed and concentrated to 2 ml on a rotary evaporator under reduced pressure in oxygen-free nitrogen at 20°C. Water was removed from the lipid extract by repeated addition and evaporation of chloroform. The last 2 ml of chloroform was removed from the lipid extract by addition and evaporation of 50 ml of toluene. The extraction and evaporation procedures were performed in the dark.

Hydrogenation of total-liver lipids

To hydrogenate 700 mg of total-liver lipid extract, 500 mg catalyst (5 or 10 % platinum on charcoal) was placed in a micro hydrogenerator (Supelco, Bellefonte, PA, U.S.A.) and 5 ml of acetic acid–toluene (1:1, v/v) was added. The mixture was stirred by a magnetic stirrer for 15 min under 344.7 kPa (50 lb/in.^{2}) of hydrogen to activate the catalyst. Then, the lipids dissolved in 15 ml of acetic acid–toluene (1:1, v/v) were transferred into the micro hydrogenerator and were hydrogenated at room temperature for 24–48 h under 344.7 kPa (50 lb/in.^{2}) of hydrogen. After hydrogenation, the catalyst was removed by centrifugation and the solvents were removed under reduced pressure by addition and evaporation of toluene.

Isolation of liver phospholipids

Phospholipids were isolated as described earlier[7]. Briefly, to a 2 mm thick, 20 × 20 cm silica gel G TLC plate, about 150–180 mg of hydrogenated total-lipid mixture (0.16 mmol lipid-phosphorus) was applied as a 1 or 2 cm wide band. The plates were developed 3 times by using acetone–chloroform–methanol–acetic acid (50:39:10:1, v/v) as developing solvent. Between developments, the plates were dried for 30 min.

To recover the phospholipids, silica gel was scraped from the place of application and from a 2-cm band above the origin and the phospholipids were eluted with chloroform–methanol–water–acetic acid (50:39:10:1, v/v). After elution, to 100 ml elution solvent, 28 ml of chloroform and 17 ml of 0.85% sodium chloride were added. The mixture was shaken and centrifuged for 40 min at 9500 g. The upper phase was removed and the lower phase was evaporated under reduced pressure.

Separation of glycerophosphatides and sphingomyelin[8]

To a 2 mm thick, 20 × 20 cm silica gel H TLC plate, about 100 mg of hydrogenated phospholipids (0.13 mmol lipid-P) were applied. The plates were developed by using chloroform–methanol–water (80:35:5, v/v/v). The position of phospholipids on the plate was detected by spraying the plates with water and comparing the lipid pattern with another plate which was charred after spraying with methanol–sulfuric acid (1:1, v/v) at 100°C.

To recover the glycerophosphatides, silica gel was scraped from the plate from the bottom of the phosphatidylcholine band to the top of the plate and the phospholipids were eluted from the silica gel as described above.

Mild alkaline hydrolysis of glycerophosphatides[9]

An amount of 50 mg of phospholipids was dissolved in 8 ml of chloroform–methanol (1:1, v/v) and 2 ml of 0.5 M sodium methoxide in methanol was added. Trans-esterification of glycerophosphatides was allowed to proceed at room temperature for 60 min and then, 2 ml of 0.5 M HCl, 1 ml water and 4 ml chloroform were added. The mixture was shaken and centrifuged for 5 min at 9500 g. The upper aqueous layer was discarded and the lower phase was evaporated. Water was removed from the lipid residue by repeated addition and evaporation of chloroform.

Isolation of hydroxy fatty acids

The products of alkaline hydrolysis of 100 mg liver glycerophosphatides were applied to a 1 mm thick, 20 × 20 cm silica gel G plate. Both sides of the glass plate were scored 2.5 cm from the vertical edge (Prep-scored uniplate, Analtech, Newark, DE, U.S.A.). The lipids to be fractionated were applied between the two score marks in the middle part of the plate. A mixture of C_{12} and C_{24} hydroxy fatty acid methyl esters was placed on both side of the plate about 1.2 cm from the vertical edge to serve as standards. The plates were developed by using hexane–ether (1:1, v/v) as developing solvent. During the first development, the solvent was allowed to migrate to about 10 cm from the origin. The plates were dried and then developed in the same solvent system once more. After the second development, the 2.5 cm wide strips were snapped off, charred at 100°C after spraying with methanol–sulfuric acid (1:1, v/v) and were compared with the middle part of the plate. The hydroxy fatty acid fraction of the glycerophosphatide hydrolysate, located between the hydroxy fatty acid standards, were scraped off and were eluted from the silica gel with chloroform–methanol, (9:1, v/v).

Synthesis of hydroxy fatty acid methyl ester standards

The hydroxy fatty acid methyl esters used as chromatographic standards were synthesized as described earlier.[10]

RESULTS AND DISCUSSION

Fig. 1 shows the sequence of procedures used for isolation of hydroxy fatty acid moieties of rat liver glycerophosphatides. The described methods had two principle objectives:

(1) To assure that the isolated oxygenated fatty acids of liver glycerophosphatides were peroxidized *in vivo* and not after the lipids were extracted from the livers.

(2) To ensure that the isolated hydroxy fatty acids originated from the glycerophosphatides and not from other lipids of the livers.

To minimize peroxidation of liver lipids during the extraction procedures, the lipids were protected by (i) adding antioxidant (BHT), and (ii) performing the procedures in the dark under nitrogen atmosphere at temperatures below 20°C.

The optional step of hydrogenation of the total-lipid mixture was included to
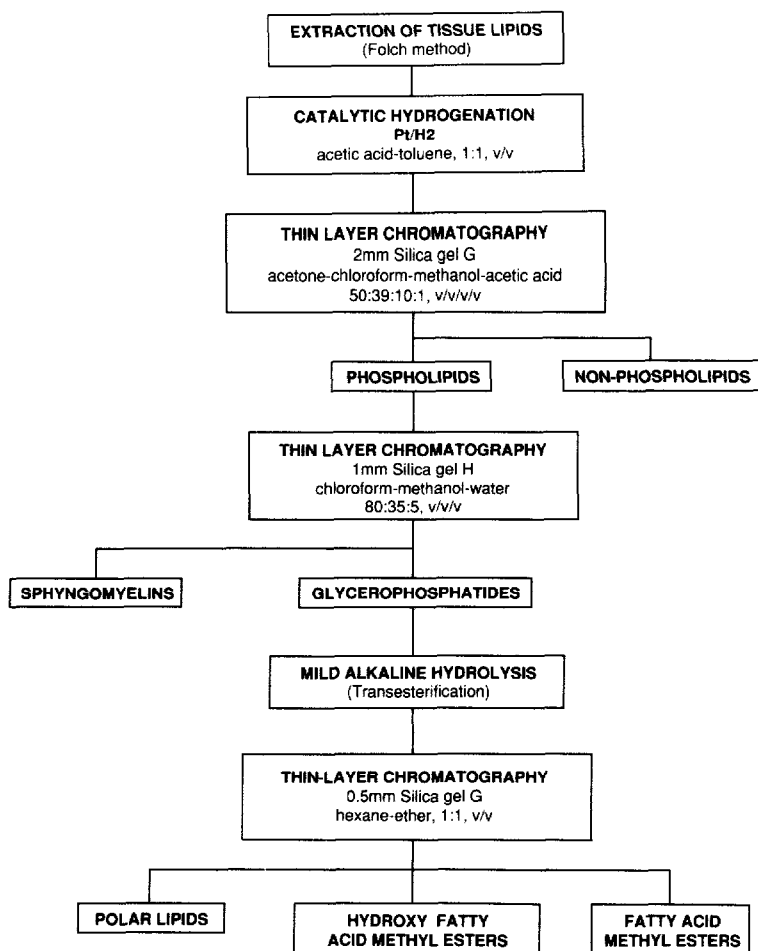
Fig. 1. Isolation of hydroxy fatty acids from rat liver glycerophosphatides.

eliminate the possible peroxidation of the unsaturated fatty acids during chemical and chromatographic procedures. This step is especially useful to determine the trace amounts of hydroxy fatty acids in livers of normal rats[5]. The hydrogenation step, however, could be eliminated if the objective was to determine the position of double bonds in the peroxidized fatty acids.

Following the hydrogenation, vigorous purification of the glycerophosphatides was achieved by two chromatographic procedures. These steps were necessary to assure that hydroxy fatty acids were isolated from glycerophosphatides only. The purification could not be achieved in a single TLC procedure because the rat liver lipids were found to contain trace amounts of some polar compounds. These compounds seemed to contain neither phosphorous nor carbohydrate moieties but they overlapped with glycerophosphatides during TLC[7]. These compounds were removed, together with the neutral lipids, from the glycerophosphatides by the first chroma-

tographic solvent system which contained acetone to prevent the phospholipids migrating away from the origin[7].

Sphingomyelins were separated from the glycerophosphatides because these compounds were known to contain hydroxy fatty acids[11] and because we could not confirm the complete stability of sphingomyelins during the mild alkaline hydrolysis as reported[9]. After repeated purification of the sphingomyelins by combined mild alkaline hydrolysis and TLC, we found trace amounts of unknown compounds that were still released from sphingomyelins during the alkaline hydrolysis.

Fig. 2 demonstrates the isolation of hydroxy fatty acids from alkaline hydrolysate of purified glycerophosphatides. The hydroxy fatty acids thus isolated can be fractionated according to chain length and according to the position of the hydroxyl group by reserved-phase and adsorption high-performance liquid chromatography
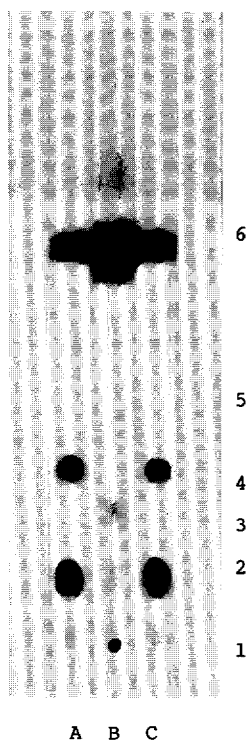

Fig. 2. Isolation of hydroxy fatty acid methyl esters from alkaline hydrolysate of rat liver glycerophosphatides. Developing solvent: hexane–diethyl ether (1:1, v/v). Plate: 0.25 mm thick silica gel G. The plate was charred at 100°C after spraying with methanol–sulfuric acid (1:1, v/v). Lanes A and C are mixtures of chromatographic standards containing C_{12} and C_{24} hydroxy fatty acid methyl esters (methyl 12-OH-dodecanoate and methyl 16-OH-tetracosanoate, respectively) and methyl oleate. Lane B is a sample from the alkaline hydrolysate of glycerophosphatides of carbon tetrachloride dosed rats. (1) is the origin of the chromatogram, containing compounds more polar then C_{12} hydroxy fatty acid methyl ester; (2) in lanes A and B is C_{12} hydroxy fatty acid methyl ester; and (3) in lane B are hydroxy fatty acid methyl esters from the hydrolyzed glycerophosphatides; (4) in lanes A and B is C_{24} hydroxy fatty acid methyl ester; (5) in lane B is trace amounts of unesterified fatty acids; (6) in lanes A and C is methyl oleate and in lane B fatty acid methyl esters from the hydrolyzed glycerophosphatides.

(HPLC), respectively as described earlier[10,12]. The combined chemical and chromatographic properties of the isolated fatty acids (before and after derivatization, TLC purification and reversed-phase HPLC analysis) confirmed the presence of both acyl and hydroxyl groups in the isolated compounds.

The method described for isolation of *in vivo* peroxidized acyl moieties of glycerophosphatides is elaborate but it is more useful than the existing methods because (i) the possible peroxidation of unsaturated fatty acids during manipulation of the lipids is kept to a minimum by hydrogenation of the total liver lipids immediately after extraction from the tissues, (ii) the detection of oxygenated fatty acids does not depend on the presence of conjugated double bonds. The method is able to isolate all long-chain (C_{12}–C_{24}) hydroxy fatty acids, (iii) the procedure can be used for both qualitative and for quantitative analysis (with added internal standards) of the isolated hydroxy fatty acids and (iv) the method can be modified to isolate short-chain hydroxy fatty acids, as esters of butanol, at both micro and preparative scales.

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