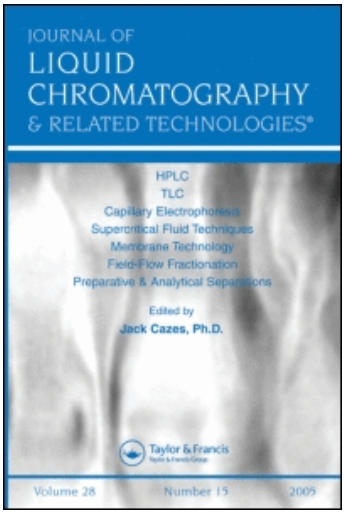

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ISOLATION OF CHOLESTERYL PALMITATE FROM HUMAN TERM AMNIOTIC FLUID

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ABSTRACT

The characterization of cholesteryl palmitate from human term amniotic fluid using thin layer chromatographic methods, nuclear magnetic resonance and mass spectrometry is described.

INTRODUCTION

Current methods for the assessment of fetal lung maturity involve the detection by thin layer chromatography (TLC) of the phosphorous moiety of phospholipids present in amniotic fluid after acetone extraction (1-2). This methodology excludes from the analysis other lipids that lack phosphorous but that may otherwise be somewhat related to the synthesis of the fetal lung surfactant. Ongoing work in this laboratory has been focused on the analysis of lipids in amniotic fluid by TLC using copper sulfate/copper acetate as the detection reagent and densitometry for quantitation. This methodology allows precise detection of polar and non-polar lipids in the nanogram range. Negligible manipulation of the sample is required since extraction is

performed directly on solid phase with the sample applied on the TLC plate (4). During our investigation of the lipids present in term amniotic fluid it was observed that the non-polar lipids represented a substantial fraction of the total lipid content. In this study evidence is presented for the identification of the major component of the neutral lipid fraction from human term amniotic fluid as cholesteryl palmitate.

MATERIALS AND METHODS

The synthetic standards 1,2-dipalmitoyl-Sn-glycerol-3-phosphocholine (DPPC), 1,2-dipalmitoyl-Sn-glycerol-3-phosphoethanolamine (DPPE) 1,2-dipalmitoyl-Sn-glycerol-3-phosphoglycerol (DPPG), sphingomyelin (SP), phosphatidylinositol (PI) and phosphatidylserine (PS) were obtained from Avanti Biochemicals (Birmingham, Alabama). Cholesterol, cholesteryl oleate, cholesteryl palmitate, cholesteryl linoleate, cholesteryl linolenate, cholesteryl arachidonate, glyceryl tristearate and glyceryl tripalmitate were purchased from Sigma Chemical Co (St. Louis, Missouri). Phospholipid standards solutions were prepared at concentrations between 0.5 and 2 mg/ml in chloroform/methanol (1:1, v/v). Cholesterol, cholesterol esters and triglycerides were prepared at concentrations between 0.1 and 0.5 mg/ml in chloroform. The purity of the standards was verified by TLC with different mobile phases. Solvents were EM Science Omnisolve. Precoated silica LK5 plates (250 μm thick) with preadsorbent zone of 500 μm thickness were obtained from Whatman Inc. (Clifton, New Jersey). Inorganic salts were from J. T. Baker (Phillisburg, New Jersey) and of the highest purity available.

Thin Layer Chromatography of Amniotic Fluid lipids

Whatman LK5 silica gel plates (20 x 20 cm; 250 um thick with preadsorbent zone) were washed by continuous development overnight in chloroform-methanol (1:1, v/v). Those plates used for scanning purposes were scored on a Schoeffel scoring device to give 1 cm lanes prior to the washing procedure. Development was carried out in standard size tanks.

Samples of amniotic fluid 20 and 40 μl aliquots were applied directly to the preadsorbent zone of scored plates. Synthetic or purified lipid standards were applied in adjacent lanes for lipid localization. Isolation of lipids from the TLC plate was carried out by applying 125 μl aliquots of amniotic fluid samples directly on the preadsorbent zone of unscored plates. Synthetic or purified standards were applied to the two edges of the plate for location of the unknown lipids. The plates were dried under a stream of warm air for 10 min. After drying the layers were predeveloped three times in chloroform-methanol (1:1,v/v) to the interface of the preadsorbent zone. Between each predevelopment, the layers were air dried for a time sufficient to assure complete evaporation of the solvent. This procedure extracts the phospholipid from the sample and deposits it as a line on the starting point of the chromatogram. The mobile phase for phospholipids was chloroform/ethanol/triethylamine/water (30:34:30:8, v/v/v/v) (6). For neutral lipids the mobile phase was hexane/ether (96:2,v/v). Development proceeded until the mobile phase reached 2 cm from the top of the plate; this usually required 1.5 hr for the former, and 15 min. for the latter. After development the plates were dried and, when lipid extraction was indicated, 1-inch strips from both edges of the plates, where sample and standards had been applied, were cut and dried in an oven at 170°C for two min. to remove residual solvent. Then half of the strips were sprayed with a 10% solution of copper sulfate in 8% H_3PO_4; the other half were sprayed with a 5% solution of copper acetate in 8% H_3PO_4. This allows the detection of both saturated and unsaturated moieties respectively (4). The plates were then dried for 5 min at room temperature, heated in an oven at 110°C for 5 min and finally placed in an oven at 170°C for the chromatograms sprayed with $CuSO_4$ and at 180°C for the copper acetate sprayed chromatograms. These procedures gave optimal development for detection.

The chromatograms were scanned in a Kontes Fiber Optic Scanner (Model 800) using a 440 nm filter. A Hewlett-Packard 3390A integrator provided

integration of the absorbance bands. The scanning was carried out in the transmission mode using double beam operation. For this reason, when chromatograms were to be obtained, samples were applied on alternate lanes of scored plates. Amounts of phospholipids between 0.5-5 μg gave linear standard curves. Those of neutral lipids were between 0.1 and 1 μg.

Spectroscopic Analysis of Amniotic Fluid Neutral Lipid(s).

The major component of the "neutral lipid fraction" of term amniotic fluid was isolated directly from the plates by scraping off the zone (see Fig 1) located by copper sulfate and copper acetate staining. Lipid release from the silica gel was facilitated by adding a few drops of water, followed by extraction with 3 ml of chloroform. The suspension was homogenized with a Vortex Mixer, then centrifuged at 600 xg for 5 min. to remove silica. The liquid phase was separated and the silica resuspended in 3 ml of chloroform and extraction repeated. The chloroform extract then was filtered through a Millipore 0.45 μm membrane to remove residual silica and dried under nitrogen until the solvent was completely removed. Recovery was 90%. Samples were then subjected to analysis by spectrometry.

Nuclear magnetic resonance spectrometry (^{1}H-NMR) analysis was carried out in a Brucker WH-300 spectrometer. All samples were dissolved in $CDCl_3$. Chemical shifts are quoted in ppm downfield from the internal tetramethylsilane (TMS) standard and are accurate to within 0.005 ppm. The spectrometer was operated at 360 MHz. Gas chromatography-mass spectrometry was performed with a Hewlett Packard 5890 gas chromatograph interfaced with a Hewlett Packard 5970 mass spectrometer.

RESULTS AND DISCUSSION:

Total lipid and neutral lipid profiles of human term amniotic fluid, as determined by TLC with $CuSO_4$ staining, are shown in Fig. 1. and 2 respectively. The predominant component of the neutral lipid fraction of term amniotic fluid, comigrated with cholesteryl palmitate, using hexane-ether (96:2) as the mobile phase. Isolation of this component by extraction from

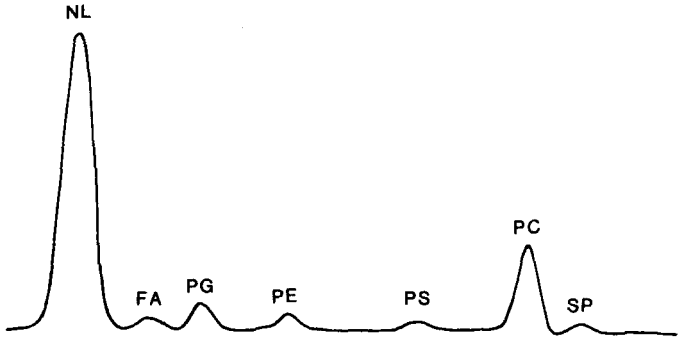

Fig 1. Chromatographic profile of total lipids in human term amniotic fluid after copper sulfate reaction.

NL - neutral lipids
FA - fatty acids
PG - phosphatidyl glycerol
PE - phosphatidyl ethanolamine
PS - phosphatidyl serine
PC - phosphatidyl choline
SB - sphingomyelin

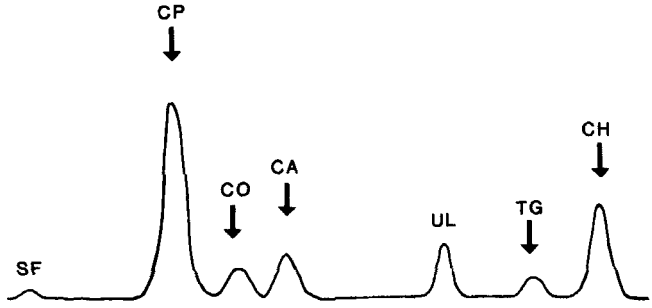

Fig 2. Chromatography of NL isolated from the chromatogram of Fig. 1

CP - cholesteryl palmitate
CO - cholesteryl oleate
CA - cholesteryl arachidonate
UL - unknown
TG - triglycerides
CH - cholesterol

Arrows indicate the position to which the reference substance migrated in the same system.

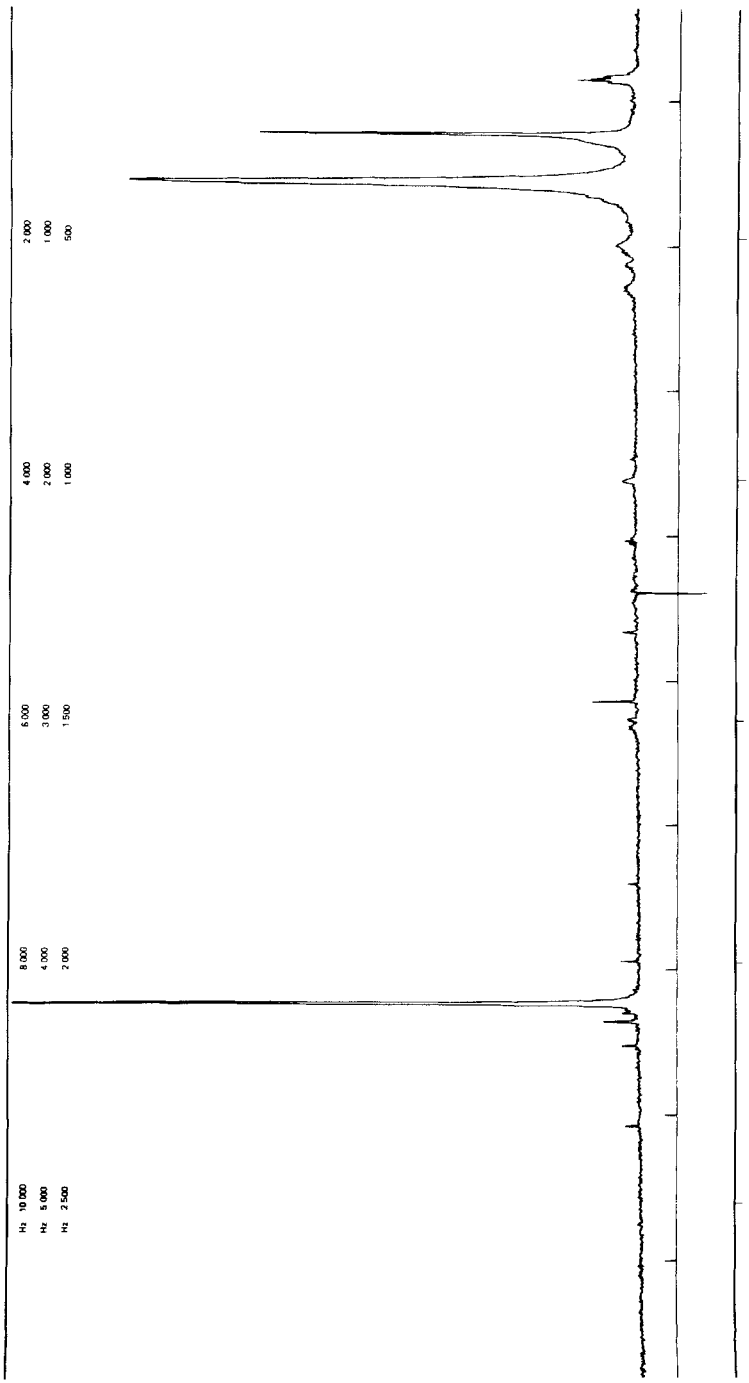

Fig 3. ^{1}H-NMR spectrum of "cholesteryl palmitate" isolated from NL of human term amniotic fluid.

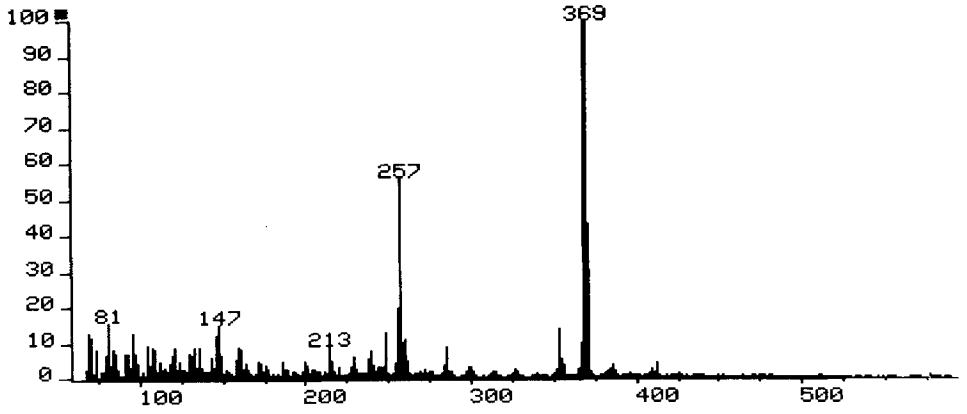

Fig 4. Mass Spectrum of the cholesteryl palmitate isolated from human term amniotic fluid.

the silica gel and subsequent in situ treatment on a thin layer plate with 2N NaOH resulted in two distinct bands after chromatography in chloroform-ether (90:10) that coincided with cholesterol and palmitic acid respectively, as determined by TLC with $CuSO_4$ staining. ^{1}H-NMR analysis provided additional evidence for a cholesterol ester (Fig. 3). Further analysis by GC-MS indicated the presence of a major peak (data not shown) which yielded the mass spectrum shown in Fig. 4. The ion at m/z 369 corresponded to cholesterol and the ion at m/z 257 to palmitic acid. These results provided positive evidence for the identification of cholesteryl palmitate as a major component of the neutral lipid fraction of term amniotic fluid. To our knowledge this is the first report of the identification of cholesteryl palmitate in human amniotic fluid.

Investigation underway indicates that the concentration of cholesteryl palmitate in amniotic fluid increases with gestational age and may provide quantitative means for the assessment of fetal lung maturity.

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