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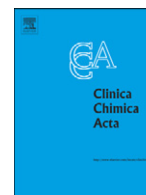

Quantitative profile of lipid classes in blood by normal phase chromatography with evaporative light scattering detector: Application in the detection of lipid class abnormalities in liver cirrhosis

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ABSTRACT

Background: The lack of analytical methods specific for each lipid class, particularly for phospholipids and sphyngolipids, makes necessary their separation by preparative techniques before quantification. LC–MS would be the election method but for daily work in the clinical laboratory this is not feasible for different reasons, both economic and time consuming. In the present work, we have optimized an HPLC method to quantify lipid classes in plasma and erythrocytes and applied it to samples from patients with cirrhosis.
Methods: Lipid classes were analyzed by normal phase liquid chromatography with evaporative light scattering detection. We employed a quaternary solvent system to separate twelve lipid classes in 15 min.
Results: Interday, intraday and recovery for quantification of lipid classes in plasma were excellent with our methodology. The total plasma lipid content of cirrhotic patients *vs* control subjects was decreased with diminished CE (81 ± 33 *vs* 160 ± 17 mg/dL) and PC (37 ± 16 *vs* 60 ± 19 mg/dL). The composition of erythrocytes showed a decrease in acidic phospholipids: PE, PI and PS.
Conclusion: Present methodology provides a reliable quantification of lipid classes in blood. The lipid profile of cirrhotics showed alterations in the PC/PE plasma ratio and in the phospholipid content of erythrocytes, which might reflect alterations in hepatocyte and erythrocyte membrane integrity.
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1. Introduction

Although central for the understanding of human pathophysiological processes, current knowledge of lipid class changes related to disease conditions in the clinical setting is scarce. This is, in part, because of analyses of other lipid classes beyond typical measurements of plasma cholesterol and triglyceride levels are seldom performed in clinical panels. However, quantification of lipid classes in plasma and other human tissues might, not only expand our diagnostic capabilities but also, improve our ability to stratify and follow-up many disease conditions [1]. A few examples could be the association of subclinical atherosclerosis with sphingomyelin (SPM) concentrations in plasma [2], the relationship of the phosphatidylethanolamine (PE) to phosphatidylcholine (PC) ratio with the transition of NAFLD (nonalcoholic fatty liver disease) to NASH (nonalcoholic steatohepatitis) [3], the measurement of free fatty acid (FFA) levels as a surrogate marker of insulin resistance in the context of metabolic syndrome [4], and the use of the free cholesterol (FC) to cholesteryl ester (CE) ratio in the diagnosis of lecithin–cholesterol acyltransferase (LCAT) deficiency [5].

A reliable analysis of different lipid classes is difficult to carry out, because the traditional approach to quantify lipid classes (thin layer chromatography—TLC) lacks the accuracy and throughput necessary to work with clinical samples [6]. There also exist enzymatic methodologies that allow quantitation of some lipid classes in plasma: FC, CE, triglycerides (TG), free fatty acids (FFA) and choline containing

Abbreviations: MS, mass spectrometry; LC-MS, liquid chromatography mass spectrometry; ESI-MS, electrospray ionization mass spectrometry; NPLC, normal phase chromatography; ELSD, evaporative light scattering detector; HPLC, high performance liquid chromatography; HILIC, Hydrophobic interaction liquid chromatography; THF, tetrahydrofuran; CE, cholesteryl ester; TG, triglyceride; FC, free cholesterol; FFA, free fatty acids; ISTD, bathyl alcohol internal standard; MG, monoglycerides; PE, phatidylethanolamine; PG, phosphatidylglicerol; CL, cardiolipin; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; SPM, sphyngomyelin; Rt, retention time; CV, coefficient of variation; LOQ, limit of quantification; LOD, limit of detection; CT, total cholesterol; PL, total phospholipids; MELD, model of end stage liver disease.

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phospholipids (PC, SPM) and new enzymatic methods have been recently developed to measure plasma levels of phosphatidylserine (PS), PE and SPM [7–9]. These methodologies offer the advantage of simplicity and the chance of automation (when commercialized in kits), but they imply multiple steps of enzymatic reactions, which make them prone to interferences. Neither do they allow analysis on a single run nor provide a full profile of all lipid classes.

On the other hand, the development of mass spectrometry (MS) in the field of lipids (lipidomics), either by direct infusion (shotgun lipidomics) [10] or coupled with liquid chromatography (LC–MS) [11], provides quantification of individual molecular lipid species and having a full picture of the lipidome in a biological sample [12]. But despite their huge capabilities, MS methods still face some disadvantages when the objective is to quantify the lipid content of a sample on the basis of a class alone not as individual species. Ion suppression, differences in ionization efficiency of lipid classes regarding acyl chain composition [13] and the wide dynamic range concentration in which the different molecular species can be found in a sample are major concerns in electrospray MS (ESI-MS) quantification of lipids [14,15]. This can be overcome with careful selection of a full set of lipid standards for each class, previous knowledge of the total lipid concentration of the extract and also, selection of experimental conditions for each individual molecular species to work at a low range concentration [16]. Still, there is not a single MS procedure enabling, with a single injection, to get a quantitative measure of the most abundant lipid classes present in human plasma and erythrocytes [17]. Moreover, costs, technical complexity, the need of specially trained technical staff and the need of laborious maintenance procedures, restrict the widespread of MS in clinical laboratories.

An alternative to obtain a quantitative profile of lipid classes is normal phase chromatography (NPLC) coupled with evaporative light scattering detector (ELSD). This methodology was first introduced by Christie [18] enabling the separation of lipid classes taking advantage of subtle differences in their polarity. Several NPLC methods based on the original have been published varying the column package employed, the solvent gradient used and the methodology [19,20]. They have been successfully applied to determine the lipid composition of different samples and also in the purification of lipid classes [21]. The principles and practice of the ELSD are well known [22,23] and, although it is not as sensitive as the MS detectors, the response is independent of the fatty acyl chain within a lipid class making easier the calibration with a simple mixture of lipid standards.

Nonetheless, NPLC–ELSD has been seldom applied to study the lipid class composition of human samples in clinical studies [24]. This is because the feeling exists that NPLC conditions are difficult to reproduce in order to be used in clinical studies in which a high within and between day reproducibility is required to cope with a large number of samples. In this work we have validated a normal phase HPLC method with ELSD for the quantification, on a single run, of lipid classes which are not routinely measured with standard clinical panels. The methodology has been applied to show abnormalities in the lipid class composition of plasma and erythrocytes in patients with decompensate chronic liver disease.

2. Materials and methods

2.1. Reagents

Cholestesteryl heptanoate, triheptadecanoin, tricosanoic acid and monoheptadecanoic acid were purchased from Nu-Check Prep, Inc (USA) and bathyl alcohol (1-O-octadecylglycerol) was from Sigma-Aldrich. Dimyristoyl phosphatidylserine, diheptadecanoyl phosphatidylcholine, dioleyl phosphatidylethanolamine, phosphatidylglicerol, phosphatidylinositol, cardiolipin and sphingomyelin were from Larodan. Lipoprotein-deficient human plasma was obtained by ultracentrifugation in KBr (1.21 kg/L) for 40 h. Apo A1, Apo B1 and albumin were measured on a BNII nephelometer (Siemens). Total cholesterol, triglyceride, LDL-cholesterol, HDL-cholesterol and other assays were performed in a c16000 analyzer (Abbott Diagnostics). Choline containing phospholipids (Kemia) and free fatty acids (Wako) were analyzed on a Pentra 200 (Olympus). Only HPLC grade solvents were used: isooctane and isopropanol (VWR); acetone, dichloromethane, chloroform and ethanolamine (Sigma-Aldrich); and triethylamine (Teknokroma).

2.2. Preparation of lipid extracts

Plasma was separated from erythrocytes after centrifugation (1900 *g* for 15 min) and frozen at −80 °C. Erythrocytes were washed twice with NaCl (0.88 %) and stored at −80 °C until extraction. Lipids from plasma were extracted following the method described by Folch et al. [25] with minor modifications. Briefly, 200 μL of plasma ($EDTA-K_3$) were mixed vigorously with 300 μL of 0.88% NaCl and 2 mL of Cl_3CH/MeOH (2:1 vol/vol) containing 25 μg/mL of internal standard (ISTD, bathyl alcohol) for 2 h. The ISTD was added to correct from losses in the extraction procedure. After centrifugation (1900 *g*, 5 min, 4 °C), the lower phase was taken with a Pasteur pipette through the protein disk and washed with 1 mL of MeOH/H_2O (1:1 vol/vol). Following vortexing (2 min) and centrifugation (1900 *g*, 5 min, 4 °C), the lower phase was collected into a conical glass tube and evaporated under N_2 current.

Erythrocyte lipids (100 μL) were extracted as described previously [26] with 4.9 mL of isopropanol/hexane (2:3 vol/vol) containing 10.2 μg/mL of ISTD. After vortexing (5 min), the samples were sonicated for 30 min at 50 °C and mixed with an aqueous solution of Na_2SO_4 (0.47 M). Following centrifugation (1900 *g*, 5 min, 4 °C) the upper phase was withdrawn in a new tube and the lower phase extracted again with 4 mL of isopropanol/hexane (2:7 vol/vol). Once vortexed, the upper phase was put together on the same collection tube and evaporated to dryness under N_2 current.

The dried lipid extracts were always redissolved in 100 μL of Cl_3CH before HPLC. For plasma 2 μL of reconstituted extract was injected and 10 μL for erythrocytes. The recovery of the ISTD in the extraction of plasma samples ranged between 80 and 104% and was between 72 and 108% in the protocol used for erythrocytes. The later value was obtained directly from the analysis of erythrocyte samples from controls and patients with cirrhosis.

2.3. HPLC system

The Agilent HPLC system consisted of a 1260 LC quaternary pump, a 1260 injector, a 1260 oven set at 40 °C, and an Agilent 1200 evaporative light scattering detector set to gain 4, drift tube temperature 45 °C and 3.0 bar internal nitrogen pressure. The column employed was a Kinetex-HILIC (2.6 μm, 100 × 4.6 mm, Phenomenex) with a guard cartridge (HILIC, 4.6 mm, Phenomenex).

A quaternary gradient of mixed solvents as described in Table 1 was used. Solvent A, isooctane:THF (99:1 vol/vol); Solvent B, acetone: dichlorometane (4:1 vol/vol); Solvent C, isopronanol:chloroform (4:1 vol/vol); and Solvent D, isopropanol:water (1:1 vol/vol), containing acetic acid (10 mM), etanolamine (5 mM), and triethylamine (5 mM). Once mixed, Solvent D was adjusted to pH = 5.0.

2.4. Patients

Forty decompensate hospitalized cirrhotic patients (22 Child-Pugh B and 18 Child-Pugh C) with different etiologies (HBV (5), HCV (12), ethanol (10), mixed (HCV/HBV + ethanol) (13), and 25 healthy controls were enrolled in the study. Blood samples (4 mL, $EDTA_3K$) were obtained in the fasting state from the antecubital vein, once the patients were stabilized. The ethics committee of the Hospital Ramon y Cajal approved the study protocol and informed consent was obtained from

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