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A high performance liquid chromatography (HPLC) method with evaporative light scattering detector for quantification of major phospholipids classes of donkey serum

Hisham I. Seri[1]*, Hassan Y. Naim[2], and Tigani Hassan[3]

[1]*Department of Clinical Studies, Faculty of Veterinary Medicine, University of Sudan of Science and Technology (SUST), Sudan*

[2]*Institut für Physiologische Chemie, Tierärztliche Hochschule Hannover, Hannover, Germany*

[3]*Department of Medicine Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Khartoum, Sudan*

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ABSTRACT

An HPLC method with an evaporative laser scattering detector was used to quantify major phospholipids fractions in donkeys' serum. Blood samples were collected bimonthly for a whole year from 20 donkeys (10 male and 10 female) from the Sudanese breed kept at the premises of the Central Veterinary Research Laboratory (CVRL), Soba. The method used made the excellent separation possible of phosphatidylglycerine (PG), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylcholine (PC), and sphingomyelin (SM). in 27 minutes, including the regeneration of the column. The SM resulted in two peaks (saturated and unsaturated fatty acids), as described by other researchers. The method was unable to separate phosphatidylserine (PS), which appeared in one peak with phosphatidylinositol. There is a significant difference in the level of PG, PE, PI and SM between females and males.The method fully discussed in this study and the obtained values of major phospholipids can be used for health control and diagnosis of diseases.

Key words: donkeys, ELSD, HPLC, phospholipid, sphingolipid, Sudan

Introduction

Nowadays, the high performance liquid chromatography (HPLC) technique was considered to be the most popular technique used for separating lipid classes. Due to the various positive activities they exert in the human body, phospholipids have received renewed interest in recent years. For example, their role in reducing blood cholesterol

*Corresponding author:

Hisham Ismail Seri, Ph.D., Department of Clinical Studies, Faculty of Veterinary Medicine, University of Sudan of Science and Technology, P.O. Box 204 Khartoum North, Sudan, Phone: +249 129 356 040; E-mail: hishamseri@Yahoo.com

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levels (ECKHARDT et al., 2002) and to enhance brain functioning (PEPEU et al., 1996), their antioxidative properties (SAITO and ISHIHARA, 1997) their bacteriostatic properties (SPRONG et al., 2002), and the inhibitory effect of sphingolipids on colon cancer have been studied intensively (VESPER et al., 1999).

Lipids are precursors for numerous second messengers. For example, sphingolipids are enzymatically broken down into ceramides, which are important signaling molecules involved in the modulation of cell proliferation, cell cycle arrest and senescence (CAMERA et al., 2004). Phospholipids are precursors to arachidonic acid, which can be enzymatically broken down to leukotrienes, prostaglandins and thromboxanes, all important modulators of inflammation. Phospholipids are also precursors to platelet activating factors and inositol triphosphate (ZEMSKI BERRY and MURPHY, 2004).

In addition, the pharmaceutical industry has implemented the use of synthetic phospholipids as drug carrier systems (URAN et al., 2001). This further necessitates analytical methods that have the ability to discriminate between endogenous and exogenous phospholipids in the biological system so pharmacokinetic and toxicokinetic data can be obtained.

Phospholipids constitute 60% of the lipid mass of a eukaryotic cell membrane (HAN and GROSS, 2004). Therefore, minute changes in phospholipids can lead to changes in the membrane that have major consequences on cell function and viability. The cell membrane has an asymmetrical distribution of phospholipids. The outer leaflet is mainly composed of phosphatidylcholine (PC) while phosphatidylethanolamine (PE) and phosphatidylserine (PS) are the primary phospholipids found in the inner cytosolic membrane (BEVERS et al., 1998).

In this study, an HPLC method used for the analysis of phospholipids in the serum of donkeys is discussed. Aspects that are addressed are sample preparation, time of analysis, detector, and ability to quantitate. Major phospholipids classes in donkey serum were quantified and compared between male and female donkeys.

Materials and methods

Experimental animals. Serum samples were collected bimonthly for a whole year from 20 (10 male and 10 female) donkeys from breeds available in the Sudan aged 4-10 years, kept in separate pens (according to sex) in the premises of the Central Veterinary Research Laboratories (CVRL), Soba, Sudan. The animals were housed and kept on water from taps and straw *ad libitum*. They were provided two times every week with a calculated amount of Dura (Sorghum bicolar). Whenever blood samples were collected they were allowed to clot and then serum was harvested and stored at -20 °C until analysis.

**Abbreviations*: HPLC - High performance liquid chromatography; ELSD - evaporative light scattering detector; PG - phosphatidylglycerine; PE - phosphatidylethanolamine; PI - phosphatidylinositol; PS - phosphatidylserine; PC - phosphatidylcholine, and SM - sphingomyelin.

Reagents and chemicals. Chloroform and methanol used for extraction were 99% HPLC grade and obtained from (Sigma-Aldrich, Taufkirchen, Germany). Chloroform, methanol, ammonia (NH_3) and water for the mobile phase were of HPLC grade and obtained from (Sigma-Aldrich, Taufkirchen, Germany). The phospholipids standards of PG, PE, PI, PS, PC, and SM were obtained from (Fluka, Sigma-Aldrich, Taufkirchen, Germany)

Chromatographic analysis. Phospholipids separation was performed on a Kaneur Maxi Star HPLC system with 4 solvent lines, a degasser SEDEX 55 evaporating light detector (SEDEX 55 Lichtstreu detector, S.E.D.E.R.E., France) which was coupled with Apex M 625 software (Autochrom, USA). As the nebulizing gas, N_2 was used at a flow rate of 4 L/min, and a nebulizing temperature of 40 °C. The gain was set at 8 and 2.0 bar N_2.

A 125×4.0 mm Si - 60 column with 5 µm particle diameter (Lichrospher) was used. The elution program was a linear gradient with 80: 19.5: 0.5 (v/v) chloroform: methanol: ammonia (NH_3) at t = 0 min to 60: 34: 5.5: 0.5 (v/v) chloroform: methanol: water: ammonia (NH_3) at t = 22 min and the column was allowed to equilibrate until the next injection at t = 27 min. The injection volume was 50 µL.

Phospholipids extraction. A liquid phase extraction procedure adapted from the method described by BLIGH and DYER (1959) was used to extract the serum samples. Briefly, 50 µL of sample were diluted with 750 µL of deionised water and mixed well, and then 2.0 mL of methanol and 1.0 mL of chloroform were added to the sample and mixed well. Then the mixture was homogenised (Rotary mixture 34526, Snijders) for 15 minutes. The mixture was centrifuged for 5 minutes by 4000 rpm (Varifuge 3.0 R, Heraeus-Sepatech, Omnilab). After centrifugation the supernatant was transferred to another tube then 1.0 mL of chloroform and 1.0 mL of water were added to the tube, mixing and centrifugation for 10 minutes at 4000 rpm (Varifuge 3.0 R, Heraeus-Sepatech, Omnilab). The methanol: water phase was sucked up with a water stream pump. Under a stream of nitrogen the $CHCl_3$ - phase was evaporated to dryness. The crude lipids were re-dissolved in 0.4 mL $CHCl_3$: MeOH (2:1 v/v) transferred into a capped test tube and before injection the samples were filtered using 0.2 µm filter unit (Restek, Germany). Finally, from this filtrate 50 µL were injected intothe chromatographic system.

Statistical analysis. The raw data of the different phospholipids fraction were analyzed using the independent sample T test. An analysis of data was performed, using a statistical software programme (SPSS 16, for Windows).

Results

The method used in this study is able to separate PG, PE, PI, PC, and SM and this was in 27 minutes, including the regeneration of the column. The SM resulted in two peaks. The method was unable to separate phosphatidylserine from phosphatidylinositol.

As shown in Table 1, although they were non significant ($P>0.05$) the levels of PC and total phospholipids were higher in female donkeys than in males, while levels of PG, PE, PI and SM were significantly ($P<0.05$) higher in females than males.

Table 1. Means and standard deviation of the total phospho- and sphingolipids concentration (mmol/L) in donkey serum

Animal gender	PG Mean ± SD	PE Mean ± SD	PI Mean ± SD	PC Mean ± SD	SM Mean ± SD	Total Mean ± SD
Female	0.112 ± 0.043*	0.027 ± 0.011*	0.040 ± 0.018*	1.218 ± 0.352	0.12 ± 0.09*	1.51 ± 0.39
Male	0.086 ± 0.071*	0.024 ± 0.007*	0.033 ± 0.010*	1.181 ± 0.327	0.09 ± 0.04*	1.42 ± 0.36
Total	0.102 ± 0.064	0.025 ± 0.010	0.037 ± 0.015	1.199 ± 0.339	0.11 ± 0.08	1.46 ± 0.38

*Means in the same column with asterisk are significantly different at $P<0.05$

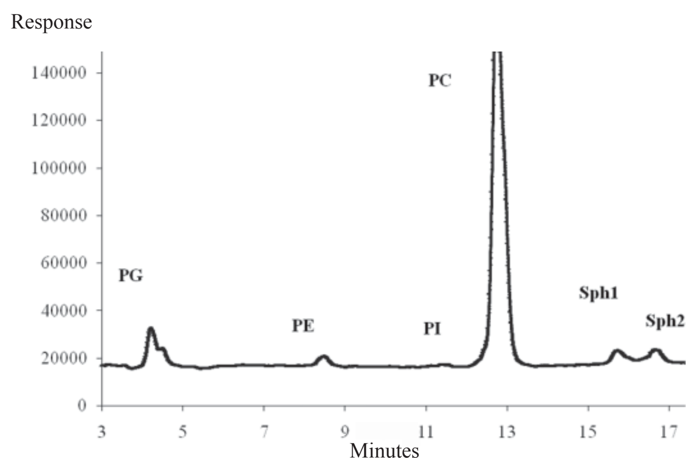

Fig. 1. HPLC chromatogram of phospo- and sphingolipids concentration (mmol/L) in female donkey serum

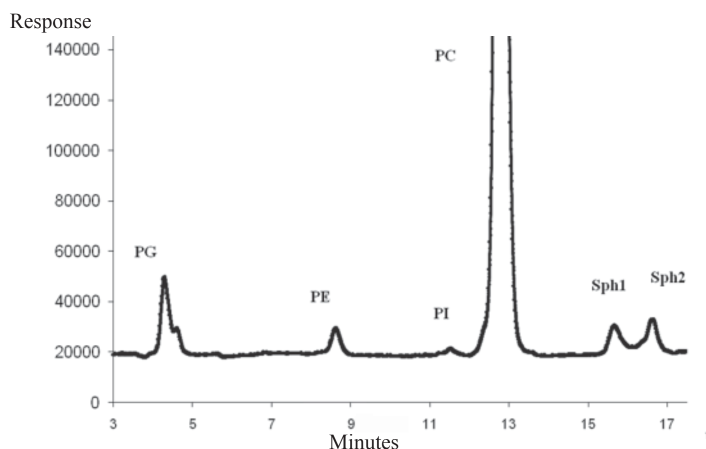

Fig. 2. HPLC chromatogram of phospo- and sphingolipids concentration (mmol/L) in male donkey serum

Discussion

Over the course of the past few decades, HPLC has become the preferred method for the determination of phospholipids, as quantitative and qualitative analysis can readily be obtained at a relative low cost compared with ^{31}P-nuclear magnetic resonance. As some methods of extraction may lead to oxidation and hydrolysis of phospholipids, it is preferable to use a cold - extraction procedure like that of BLIGH and DYER (1959), using chloroform-methanol (ROMBAUT et al., 2005). Several methods are described for the separation of lecithin and derivatives (MOUNTS et al., 1992; ABIDI et al., 1996; CARELLI et al., 1997; BONEKAMP and FIEBIG, 1999). However, these methods are less applicable in our case. Phosphatidylserine (PS), which is only present in trace amounts in lecithin fractions, is often poorly separated from other phospholipids. Moreover, these methods do not consider the presence of sphingomyelin and cerebrosides.

Despite all this, the concentration of phospholipids obtained in this study is in close agreement with the findings of JORDANA et al. (1998), though slightly lower. In comparison with the results obtained in this study, JORDANA et al. (1998), reported that phospholipid concentrations showed a significant sex effect ($P<0.01$) in the Catoalonian donkey population, where the values for the male population (mean = 1.82 ± 0.54) were significantly higher ($P<0.01$) than those for females (mean = 1.55 ± 0.53). We could attribute this to the small number of animals examined in our study.

In the present study, phosphatidylcholine (PC) forms the major part of the phospholipids in donkeys serum, followed by phosphatidylglycerol (PG). The SM resulted in two peaks, as described by other researchers (CHRISTIE et al., 1987; BECART et al., 1990; VAGHELA

and KILARA, 1995). This presumably may be due to the heterogeneity of SM fatty acid residues (BRETON et al., 1989).

Most of the recent chromatographic methods used for the separation of phospholipids are based on the method of BECART (1990) using a gradient mixture of chloroform, methanol and a buffer at high pH (>7) with an alkali modifier, like triethylamine or ammonium hydroxide, on a plain silica column (BECART et al., 1990; CABONI et al., 1996). The modifier is used to enhance peak shape and resolution. Although enabling a fair separation of most of the phospholipids, the high pH quickly dissolves the silica packing, thereby seriously reducing column life (ROMBAUT et al., 2005). For the chromatographic analysis of fats and oils, as in our case, the use of evaporative light scattering detection is generally preferred, in this type of detector; the elution solvent from the column is nebulized by the aid of pressurized gas (compressed air, helium, or nitrogen) in a heating tube. The analyte is not evaporated and passes as an aerosol through a beam of conventional or laser light, which is reflected or refracted. The scattered light is detected by a photomultiplier or a photodiode, which is placed at a fixed angle and is directly related with the quantity of the analyte and the droplet size. The evaporative light - scattering detector is a universal detector that responds to any analyte that is less volatile than the mobile phase. However, the droplet size (and thus the response) is highly dependent on the flow of the nebulizing gas, the temperature of the evaporating tube and the flow rate, and on the composition and physical characteristics of the mobile phase.

The mobile phase should be of the highest quality, as non-volatile impurities would result in an increased background signal, and could alter analyte droplet formation and consequently detector sensitivity.

The role of lipids in cellular biochemistry is only partly understood, but our knowledge is rapidly expanding. Apoptosis involves an alteration of the plasma membrane such that phospholipids with the phosphatidylserine (PS) head group are translocated to the outer leaflet of the cellular plasma membrane, and the appearance of PS at the outer leaflet, in large part, is responsible for the recognition of the cell by the macrophage and subsequent phagocytosis (FADOK et al., 2001). Furthermore, alterations in phospholipid composition have been noted in certain disease states, such as decreased plasmalogens in the gray matter of patients with Alzheimer's disease (HAN et al., 2001). Therefore, a detailed analysis of lipid structure and molecular species composition has emerged as an active area of research in cellular biochemistry, an area that is important in understanding cellular metabolism, signalling, and membrane trafficking.

Changes in lipid metabolism are known to occur in cases of thyroid carcinoma and hypothyroidism. DAS and ISICHEI (1989) found higher concentrations of triglycerides, free fatty acids, free cholesterol, esterified cholesterol, phosphatidylcholine and sphingomyelin in cancerous thyroid tissue, compared with normal thyroid tissue. Furthermore, the blood

plasma of hypothyroid subjects had higher levels of HDL2, LDL and phospholipids than that of euthyroid subjects (ENGLER and RIESEN, 1993; HYLANDER and ROSENQVIST, 1982; FRANKLYN et al., 1993).

It is to be concluded that, these data generated using the HPLC method discussed above could be used easily and effectively in quantification of major phospholipid classes in the serum of equine in healthy and disease states.

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SAŽETAK

Visokotlačna tekućinska kromatografija s detektorom raspršenja svjetla u uparenom uzorku rabljena je za određivanje količine glavnih frakcija fosfolipida u magarećem serumu. Uzorci krvi bili su uzeti od 20 životinja (10 magaraca i 10 magarica sudanske pasmine) držanih u prostorima Središnjega veterinarskoga istraživačkoga laboratorija, Soba. Rabljena metoda omogućila je izvrsno odvajanje fosfatidilglicerola (FG), fosfatidiletanolamina (FE), fosfatidilinozitola (FI), fosfatidilkolina i sfingomijelina (SM) za 27 minuta uključujući i regeneraciju kolone. Sfingomijelin je pokazivao dva vrška (zasićene i nezasićene masne kiseline) kao što je opisano od drugih autora. Rabljenom metodom nije se moglo odvojiti fosfatidilserin od fosfatidilinozitola. Ustanovljena je signifikantna razlika u razini FG, FE, FI i SM u mužjaka i ženki. U radu se iscrpno raspravlja o metodi, a dobivene vrijednosti glavnih fosfolipida mogu se upotrijebiti za kontrolu zdravlja i dijagnosticiranje bolesti.

Ključne riječi: magarci, visokotlačna tekućinska kromatografija, fosfolipid, sfingolipid, Sudan

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