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Dept. of Clinical studies,
Fac. of Veterinary Science, Univ. of Nyala, Sudan.

SEASONAL VARIATION OF SERUM PHOSPHOLIPIDS IN DONKEYS USING HPLC METHOD

(With 3 Table &7 Figures.)

H. I. Seri; M. A. H. Ghurashi; T. Hassan*; H. Y. Naim**

**Department of Med. Pharma. & Toxicology, faculty of Veterinary Medicine, University of Khartoum, Sudan.*

**Institut für Physiologische Chemie, Tierärztliche Hochschule Hannover, Bunteweg*

الاختلافات الفصلية للفوسفوليبيدات في مصل الدم في الحمير باستخدام تقنية الكروماتوغرافيا السائلة عالية الأداء

هشام إسماعيل سري، حسن نعيم، نجاني حسن، محمد أحمد حسن قرشي

في هذه الدراسة، تم استخدام تقنية الكروماتوغرافيا السائلة عالية الأداء لقياس الاختلافات الفصلية، إن وجدت، في أجزاء الفوسفوليبيدات في مصل الدم في الحمير. تم جمع عينات الدم مرتين كل شهر لعام كامل من عشرين من الحمير (عشره من الذكور وعشره من الإناث) من الفصيلة السودانية كانت موجودة في حظائر مركز المعامل والبحوث البيطرية المركزي سوبا بالسودان. تم إجراء التحليل باستخدام طريقة (ELSD) والذي أمكن بصورة ممتازة عن طريقتها من فصل كل من (PG, PE, PI, PC and SM) الطريقة المستخدمة لم تنجح في فصل (PS) عن (PI). الفوسفوليبيدات الكلية أظهرت زيادة معنوية في الفصل الممطر عند مقارنتها بالفصل البارد. في إناث الحمير أظهر تركيز الفوسفاتيديل جليسرول في مصل أناث الحمير اظهر انخفاضا معنويا في الفصل البارد عند مقارنته بالفصول الاخري. في حين ان تركيز الفوسفاتيديل ايثانولامين ذكور الحمير قد اظهر انخفاضا معنويا في الفصل البارد عند مقارنته بالفصول الاخري. وكذلك الفوسفاتيديل اينوسيتول انخفاضا معنويا في الفصل البارد عند مقارنتها بالفصل الممطر. في حين ان تركيز الفوسفاتيديل كولين لم يظهر أي اختلافات معنوية في الفصول المختلفة.

SUMMARY

In this study, an HPLC method was used to examine the seasonal variation, if any, in 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 Sudan. The analysis was carried using an evaporative laser light –scattering detector(ELSD), which enabled excellent separation of phosphatidylglycerine (PG), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylcholine (PC), and the two

phosphatiddylserine (PS), which appeared in one peak with phosphatidylinositol.Total phospholipids concentration show significant (P<0.05) increase in the rainy season when compared with the cold season. Phosphatidylglycerol (PG) concentration in female donkeys is significantly (P<0.05) low in the cold season when compared with the rainy and hot seasons. Phosphatidylethanolamine (PE) concentration is significantly (P<0.05) low in the cold season than the hot and rainy season in the male animals. Phosphatidylinositol (PI) concentration is significantly (P<0.05) low in the cold season when compared with the rainy season in the male animals. Phosphatidylcholine concentration did not show any significant difference between the three seasons. Total sphingolipids concentration is significantly (P<0.05) low in the cold season when compared with the hot and rainy seasons in the male animals.

Keywords: Phospholipids, donkeys, HPLC, seasonal variation.

INTRODUCTION

Nowadays, High Performance Liquid Chromatography (HPLC) technique is considered to be the most popular technique used for separating lipid classes. Due to their several positive activities they exert in the human body, phospholipids have received renewed interest in recent years, for example, their role in reducing blood cholesterol levels (Eckhardt *et al.*, 2002) and lastly 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).

Over the course of the past few decades, HPLC has become 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 cold – extraction procedure like that of Bligh and Dyer (1959) using chloroform – methanol (Rombaut *et al.*, 2005).

The current study was conducted to investigate the seasonal variation in phospholipids in donkeys' serum, if any.

MATERIALS AND METHODS

Experimental animals: Serum samples were collected bimonthly for a whole year from 20 (10 male and 10 female) non-working donkeys from breeds available in the Sudan aging 4 – 10 years, kept at separate pens in the premises of 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.

Time schedule: Blood samples were collected bimonthly for a whole year (February 2004 to January 2005). Months from March to June were considered as hot season, July to October as rainy season, and November to February as cold season.

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 Lichtstreudetector, 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 125X4.0 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 was 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 in (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 suked 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 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 in the chromatographic system.

Statistics: SPSS 11.5 for Windows Computer Package was utilized in assessing significant differences, if any. Analysis of variance (ANOVA) was used to compare between means.

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 phosphoatidylserine from phosphatidylinositol. In the present study, phosphatidylcholine forms the major part of the phospholipids in donkeys serum followed by phosphatidylglycerol.

As shown in table (1), total phospholipids concentration show significant ($P<0.05$) increase in the rainy season when compared with the cold season. Phosphatidylglycerol (PG) concentration in female donkeys is significantly ($P<0.05$) low in the cold season when compared with the rainy and hot seasons as shown in table (3). In table (2), we could observe that phosphatidylethanolamine concentration is significantly ($P<0.05$) low in the cold season than the hot and rainy seasons in the male animals. As shown in table (2) phosphatidylinositol concentration is significantly ($P<0.05$) low in the cold season when compared with the rainy season in the male animals. From table (1), phosphatidylcholine concentration did not show any significant difference between the three seasons. As shown in table (2) total sphingolipids concentration is significantly ($P<0.05$) low in the cold season when compared with the hot and rainy seasons in the male animals.

DISCUSSION

Several methods are described for the separation of lecithin and derivatives (Mounts *et al.*, 1992; Abidi *et al.*, 1996). However, these methods are less applicable in our case. Phosphatidylserine, 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.

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; and 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, 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 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, as described by other researchers (Christie *et al.*, 1987; Becart *et al.*, 1990; Vaghela and Kilara, 1995). And this presumably may be due to the heterogeneity of SM fatty acid residues (saturated and unsaturated fatty acids) (Breton *et al.*, 1989). The

method was unable to separate phosphoatidylserine from phosphatidylinositol.

Phosphatidylserine, which is only present in trace amounts in lecithin fractions, is often poorly separated from other phospholipids. 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 the present study, phosphatidylcholine forms the major part of the phospholipids in donkeys serum followed by phosphatidylglycerol.

This may be attributed to hormonal effects (T_3, T_4 and glucocorticoides). The difference between male and female animals may attributed to the effect of testerone and the increase in metabolism by about 10% and build of muscle mass and increased body protein.

It is to conclude that, an HPLC method is able to demonstrate a seasonal significant difference in phospholipids fractions in donkey's serum, although it is within the reference values reported by other researcher, but the method remains of value in quantification and separation of phospholipids in serum.

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Table (1): Means and standard deviation of the total phospholipids concentration (mmol/l) in donkeys' serum.

Season	PG Mean ± SD	PE Mean ± SD	PI Mean ± SD	PC Mean ± SD	SM Mean ± SD	Total Mean ± SD
Hot	0.101 ± 0.041	0.027 ± 0.012[a]	0.034 ± 0.015[a]	1.222 ± 0.379	0.11 ± 0.05	1.49 ± 0.42
Rainy	0.110 ± 0.048	0.027 ± 0.007[a]	0.040 ± 0.014[b]	1.225 ± 0.270	0.11 ± 0.03	1.51 ± 0.32[a]
Cold	0.087 ± 0.112	0.022 ± 0.008[b]	0.036 ± 0.015	1.151 ± 0.357	0.10 ± 0.12	1.38 ± 0.38[b]
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 a different asterisk are significantly different.

Table (2): Means and standard deviation of the total phospholipids concentration (mmol/l) in male donkeys' serum.

Season	PG Mean ± SD	PE Mean ± SD	PI Mean ± SD	PC Mean ± SD	SM Mean ± SD	Total Mean ± SD
Hot	0.082 ± 0.029	0.025 ± 0.008[a]	0.032 ± 0.011[a]	1.193 ± 0.353	0.10 ± 0.07[a]	1.43 ± 0.39
Rainy	0.090 ± 0.043	0.025 ± 0.007[a]	0.036 ± 0.010[a]	1.237 ± 0.271	0.10 ± 0.02[a]	1.49 ± 0.32[a]
Cold	0.087 ± 0.112	0.020 ± 0.006[b]	0.032 ± 0.008[b]	1.112 ± 0.345	0.07 ± 0.02 [b]	1.32 ± 0.34[b]
Total	0.086 ± 0.071	0.024 ± 0.007	0.033 ± 0.010	1.181 ± 0.327	0.09 ± 0.04	1.42 ± 0.36

Means in the same column with a different asterisk are significantly different.

Table (3): Means and standard deviation of the total phospholipids concentration (mmol/l) in female donkeys' serum

Season	PG Mean ± SD	PE Mean ± SD	PI Mean ± SD	PC Mean ± SD	SM Mean ± SD	Total Mean ± SD
Hot	0.119 ± 0.043[a]	0.029 ± 0.016	0.036 ± 0.019	1.251 ± 0.406	0.12 ± 0.03	1.55 ± 0.44
Rainy	0.130 ± 0.044[b]	0.029 ± 0.008	0.043 ± 0.017	1.213 ± 0.272	0.12 ± 0.03	1.53 ± 0.32
Cold	0.088 ± 0.029[c]	0.024 ± 0.009	0.040 ± 0.019	1.189 ± 0.369	0.12 ± 0.16	1.44 ± 0.41
Total	0.112 ± 0.043	0.027 ± 0.011	0.040 ± 0.018	1.218 ± 0.352	0.12 ± 0.09	1.51 ± 0.39

- Means in the same column with a different asterisk are significantly different.

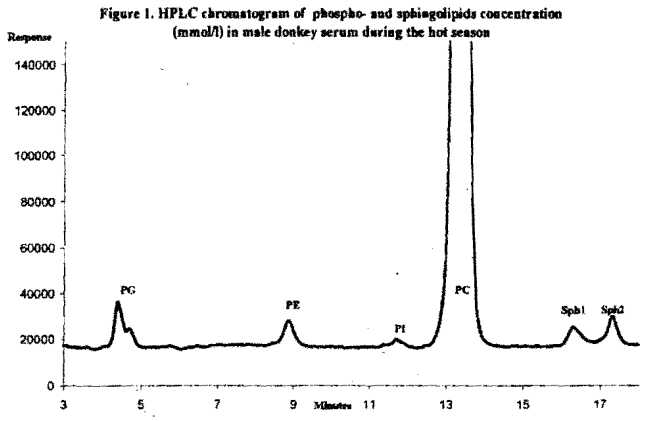

Figure 2. HPLC chromatogram of phospho- and sphingolipids concentration (mmol/l) in male donkey serum during the rainy season

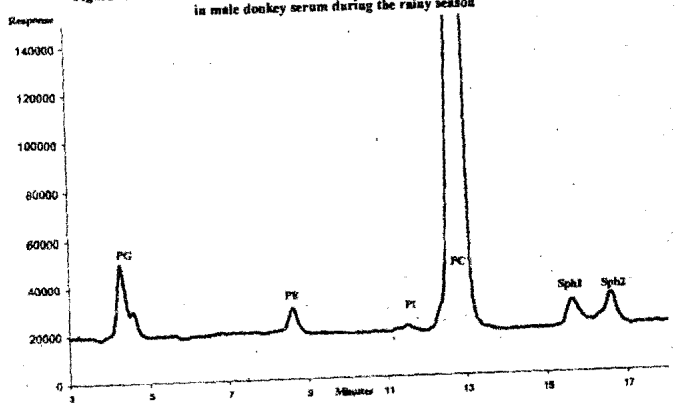

Figure 3. HPLC chromatogram of phospho- and sphingolipids concentration (mmol/l) in male donkey serum during the cold season

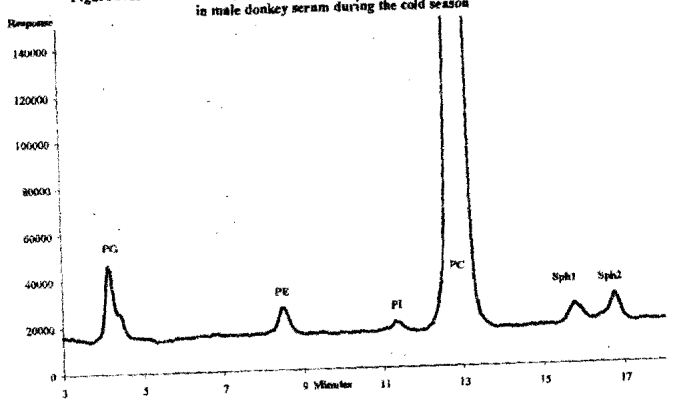

Figure 4. HPLC chromatogram of phospho- and sphingolipids concentration (mmol/l) in female donkey serum during the hot season.

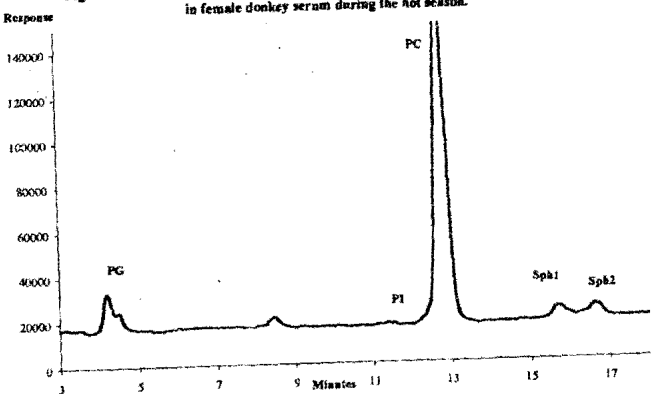

Figure 5. HPLC chromatogram of phospho- and sphingolipids concentration (mmol/l) in female donkey serum during the rainy season.

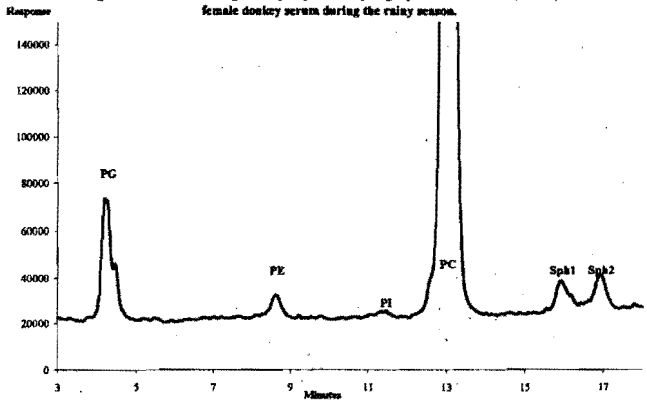

Figure 6. HPLC chromatograph of phospho- and sphingolipids concentration (mmol/l) in female donkey serum during the cold season.

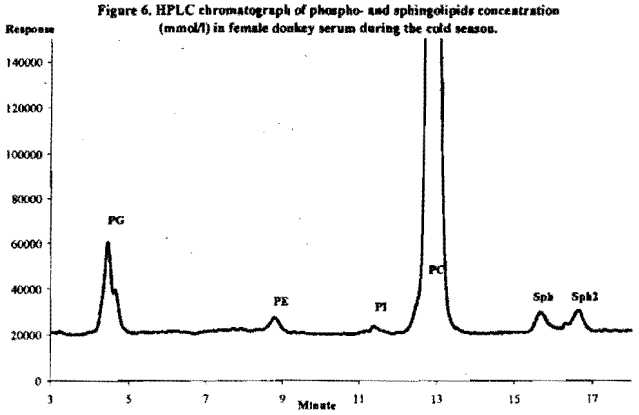

Figure 7. HPLC chromatogram of phospho- and sphingolipids concentration (mmol/l) in male donkey serum during the hot season with abnormal phosphatidylglycerine level.