ELSEVIER

Journal of Chromatography B, 740 (2000) 101–107

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Separation and quantification of cholesterol and major phospholipid classes in human semen by high-performance liquid chromatography and light-scattering detection

G. Grizard[a,*], B. Sion[a], D. Bauchart[b], D. Boucher[a]

[a]*Biologie de la Reproduction, CECOS, CHU Hôtel-Dieu, Boulevard Léon Malfreyt, 63003 Clermont-Ferrand, France*

[b]*Nutriments et Métabolismes, INRA, 63122 Saint-Genès Champanelle, France*

Received 5 August 1999; received in revised form 2 December 1999; accepted 19 January 2000

Abstract

A high-performance liquid chromatographic method coupled with light-scattering detection for the separate and accurate quantification of cholesterol and main phospholipid classes was applied to human spermatozoa and seminal plasma (SP). This method is based on normal-phase chromatography with silica gel as stationary phase and a ternary gradient with hexane, mixtures of chloroform–methanol and water as mobile phase. Lipids are separated with a good resolution and a high reproducibility. About $5\cdot10^6$ spermatozoa or 25 μl of seminal plasma are sufficient to accurate quantitative analysis of phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidycholine (PC), sphingomyelin (SM) and cholesterol. PC is the predominant phospholipid class in spermatozoa (102 ± 8 nmol/10^8 spermatozoa) whereas SM is the major in the SP (163 ± 6 nmol/ml). Both in spermatozoa and SP, PI is the minor class of the phospholipids (12 ± 1 nmol/10^8 spermatozoa and 24 ± 2 nmol/ml). In conclusion, this method offers interesting perspectives for analysis of sperm lipid composition in semen samples with low quantities of spermatozoa. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Phospholipids; Cholesterol

1. Introduction

In spermatozoa, cholesterol and phospholipids are essential constituents. The quantity and the composition of these lipids in the plasma membrane are known to have a significant effect upon the physico–chemical properties and in turn, upon functional characteristics of the spermatozoa. For example, they are a key factor in determining phase-transition temperature, and membrane fluidity and therefore play important roles in events such as resistance to the freeze–thaw process, motility, acrosomal exocytosis or fusiogenic properties of the sperm. Some of them have been also recognized as important precursors in signal transduction pathways [1–3].

In semen, the composition of sperm membrane lipids is dependent on the exchanges of lipids between spermatozoa and seminal plasma [4].

Major phospholipid classes of human semen have been separated by thin-layer chromatography (TLC) in most studies. Then the different classes of phospholipids were quantified either by the determination of inorganic phosphorus [5,6] or after staining, by a

*Corresponding author.

E-mail address: genevieve.grizard@u-clermont1.fr (G. Grizard)

0378-4347/00/$ – see front matter © 2000 Elsevier Science B.V. All rights reserved.

PII: S0378-4347(00)00039-6

scanning method with spectrodensitometer [7,8]. The main phospholipid classes found both in spermatozoa and seminal plasma (SP) by these techniques, were sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI).

High-performance liquid chromatography (HPLC) coupled with light-scattering detection has been described to separate and accurately quantify major lipid classes in biological samples [9–12]. The light-scattering evaporative detector measures the intensity of the light scattered by the fine droplets formed by the solute upon evaporation of the column effluent. However, until now, such a procedure has not been used for quantification of lipids in human semen.

In this paper, a sensitive qualitative and quantitative analysis of cholesterol and main phospholipids in human spermatozoa and seminal plasma, using HPLC combined with light-scattering detection is presented.

2. Experimental

2.1. Semen samples

Semen samples were obtained from 14 healthy men attending our laboratory for fertility investigation. Semen samples were obtained by masturbation following a period of sexual abstinence of at least 3 days. Immediately after liquefaction (about 30 min at 37°C), ejaculate volume, sperm count, round cell count, motility and morphology were determined by routine procedures. All the semen specimens conformed to the limits of normality set down by the World Health Organization [13], i.e., sperm count $\geq 20 \cdot 10^6$/ml, motility ≥50%, spermatozoa with normal morphology ≥30% and round cells count $\leq 5 \cdot 10^6$/ml.

After liquefaction the semen was centrifuged for 10 min at 500 *g*. The supernatant was collected, recentrifuged at 7000 *g* for 30 min at 4°C to eliminate all possible contaminating cells and final supernatant, referred to as SP and stored at −80°C. The sedimented sperm pellet was washed twice with 0.9% saline solution and centrifuged at 800 *g* for 10 min between washings. The final sperm pellet was suspended in 0.9% saline solution and a sperm count was made before storage at −80°C. Under these conditions, storage did not induced any change in stability of cholesterol and major phospholipid classes of both spermatozoa and SP (data not shown).

2.2. Reagents and standards

Cholesterol and sphingomyelin were obtained from Sigma (St. Quentin Fallavier, France), the other phospholipids (PE, PC, PI, PS) were obtained from Interchim (Montluçon, France). Chloroform and formic acid were purchased from Prolabo (Fontenay sous Bois, France), methanol was obtained from Merck (Darmstadt, Germany) and hexane was obtained from BDH (UK).

2.3. Instrumentation

HPLC was carried out on a Kontron system (Milan, Italy) interfaced with chromatography data system software and equipped with a programmable ternary gradient pump (system 325), an automatic injector with a 100-μl loop (autosampler 360) and a light-scattering detector (DDL 21 Eurosep Instruments, Cergy Pontoise, France).

2.4. Preparation of samples for lipid analysis

Cholesterol and phospholipids were extracted by the method of Folch et al. [14] adapted for seminal plasma and spermatozoa. Briefly, an aliquot (0.2 ml) of sperm suspension or SP was mixed with 8 ml chloroform–methanol (2:1, v/v), the mixture was vortex-mixed, 1.5 ml distilled water was added and the mixture was again vortex-mixed and allowed to stand at room temperature for 1 h before centrifugation at 500 *g* for 10 min. The upper layer was resuspended in 8 ml chloroform–methanol–water (86:16:1, v/v) and centrifuged at 500 *g* for 10 min, after which the chloroform extracts were pooled. Finally, the organic layers were evaporated under nitrogen. The dried material was dissolved in a convenient volume of chloroform–methanol (4:1,

v/v) depending on the techniques used to assay lipids.

2.5. Cholesterol and phospholipid separation by HPLC

All the quantitative assays were performed with a 250×3 mm I.D. column packed with Inertsil 5 μm silica (Interchim) and a ternary mobile phase composed of solvent A (hexane), solvent B (chloroform–methanol, 100:8, v/v) and solvent C (methanol–0.05 *M* formic acid in water, 100:4, v/v); all the solvents were degassed under helium. The solvent program employed is shown in Table 1. The non polar lipids eluted during the first 28 min and the polar lipids eluted during the following 68 min. The last 28 min regenerated the column prior to injection of the next sample. Elution was performed at a flow-rate of 0.35 ml/min. The lipids were separated at room temperature and quantified by a light-scattering detector, the temperature in the evaporator was adjusted to 70°C and internal nitrogen pressure was set at 2 bar. Lipid components were quantified by comparison of peak areas with those of standard curves.

Table 1
Ternary gradient elution system required for the elution of cholesterol and phospholipid classes and reactivation of the column[a]

Time (min)	% Solvent		
	A	B	C
0	100		
6	100		
6.01	95	5	
25		100	
28		100	
51		58	42
73		32	68
94		10	90
96		10	90
96.01		100	
107		100	
107.01	100		
124	100		

[a] A: Hexane; B: chloroform–methanol (100:8, v/v); C: methanol–0.05 *M* formic acid in water (100:4, v/v). A linear gradient was produced between the composition specified at each time interval.

2.6. Identification of phospholipids species by high-performance thin-layer chromatography (HPTLC)

HPTLC plates were used after prewashing with a mixture of chloroform–methanol (1:1, v/v) followed by heating at 125°C for 5 min. The plates were developed to 1 cm from the bottom either with methyl acetate–chloroform–*n*-propanol–methanol–0.25% KCl in water (25:25:25:10:9, v/v) or with chloroform–methanol–water (65:25:4, v/v). Then, the plates were sprayed with 10% (w/v) cooper sulfate in 8% (v/v) phosphoric acid solution and heated at 180°C for 15 min to stain all the phospholipids, and finally these ones were identified by reference to standards [15].

2.7. Miscellaneous

In standard solutions and in different lipid class fractions obtained by HPLC, phospholipids were determined by measuring the amount of inorganic phosphorus [16] but using HNO_3 as oxidant instead of H_2O_2 and cholesterol was assayed using Liberman Burchard reagent [17].

3. Results

3.1. Separation

Fig. 1 shows a typical HPLC chromatogram obtained with a standard mixture of pure lipid classes. The cholesterol and phospholipids classes were separated with a good resolution. The peak appearance and the polarity-dependent retention time were highly reproducible. Peaks of cholesterol and PE are high and very narrow. PI, PC and PS eluted as broad peaks probably because the time retention for molecular species belonging to the same phospholipid class slightly varies with the acyl group of the molecule. SM was eluted as a double peak presumably because of partial separation of molecular species; such a result has been previously described by several authors [9–11,18].

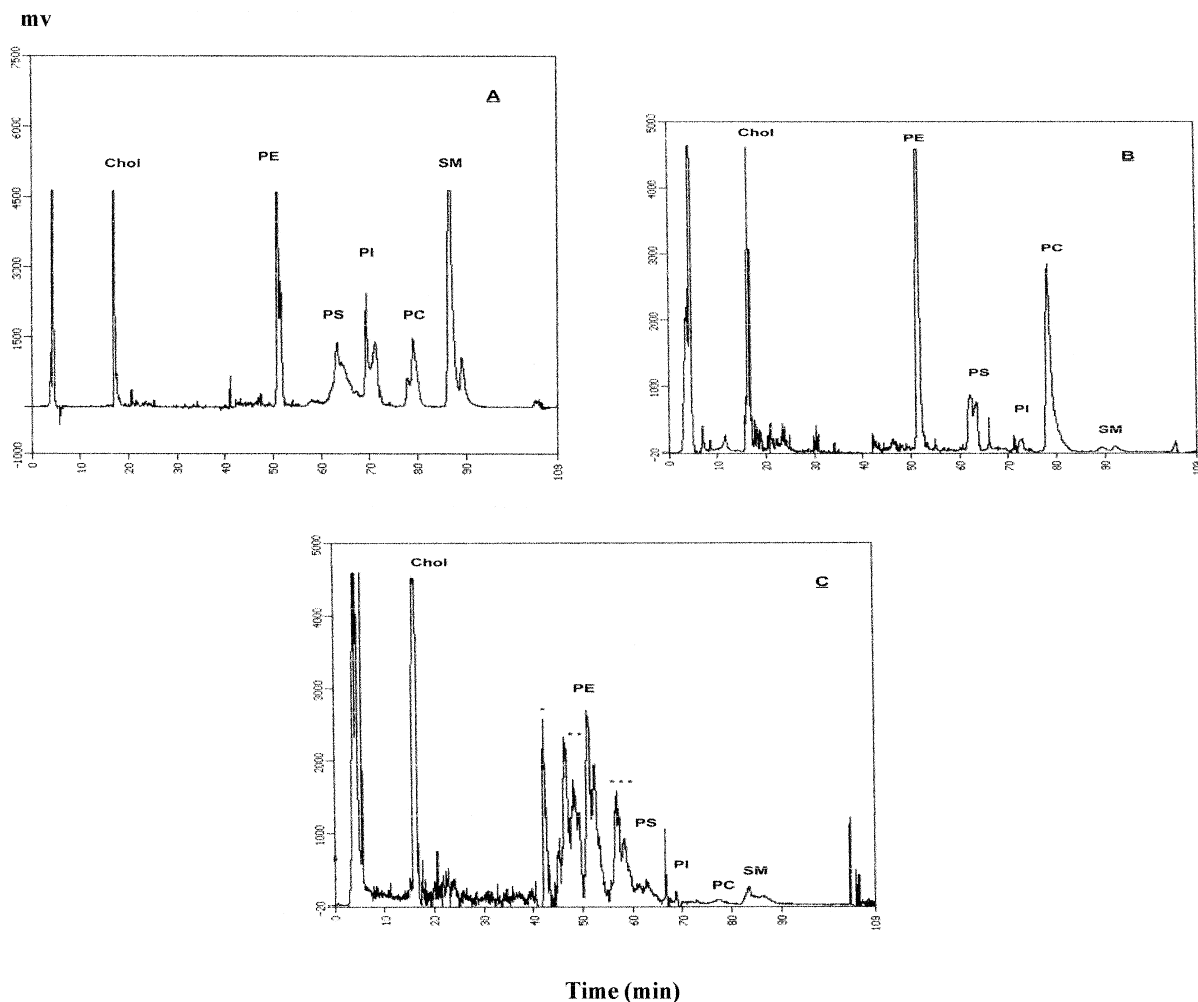

Fig. 1. HPLC separation of cholesterol and phospholipid classes using an Inerstil column and light-scattering detection. Peaks refer to the following components: Chol: cholesterol, PE: phosphatidylethanolamine, PS: phosphatidylserine, PI: phosphatidylinositol, PC: phosphatidylcholine, SM: sphingomyelin. Experimental conditions are given in Experimental. (A) Chromatogram of a mixture of lipid standards: (50 nmol of Chol, 3.6 nmol of PE, 6.7 nmol of PS, 7.5 nmol of PI, 7.0 nmol of PC and 17 nmol of SM). (B) Chromatogram of a lipid extract of $6 \cdot 10^6$ spermatozoa. (C) Chromatogram of a lipid extract of 25 μl seminal plasma. The marked peaks have the same retention times that * cerebroside, ** cardiolipin, *** cholesterol sulfate.

3.2. Quantitation

Due to the nature of the applied detection technique, the response of the light-scattering detector is non-linear with the amount of lipids eluting from the column. Therefore calibration curves are required in order to quantify the separated components (Fig. 2). The amount of each lipid was calculated from calibration curves fitted with the equation: $y=ax^2+bx+c$ with x: amount of lipids expressed in nmol of cholesterol or phosphorus, y: detector response (mV×min) and a, b, c: constants which are different for each standard.

The limit of detection (twice the noise level) was 0.15 nmol for cholesterol, 0.2 nmol for PE, 1 nmol for PC and SM, 1.5 nmol for PS and PI.

Experiments were performed to determine the reproducibility of the method. Five measurements of

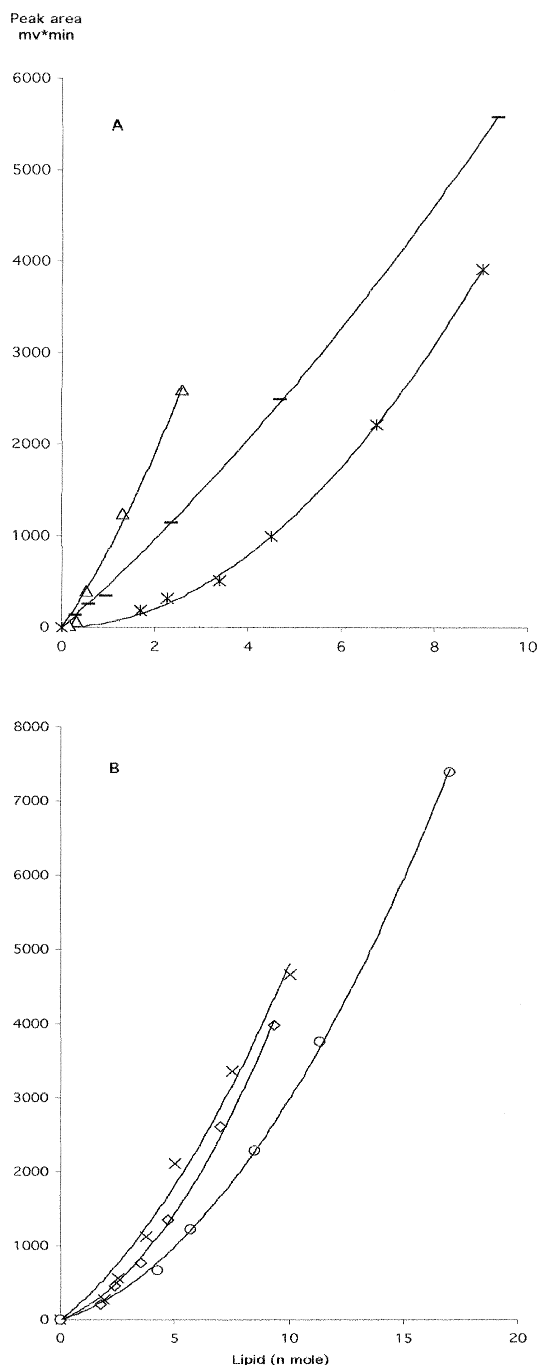

Fig. 2. Representative calibration curves for lipid classes analyzed by HPLC and light-scattering detection. The peak area expressed in mV×min for each lipid class is plotted against absolute injected amount. Legends: (A) Chol –△–, PE – - -–, PS –★–; (B) PI –×–, PC –◊–, SM –○–. For abbreviations see legend of Fig. 1.

the same biological sample were studied in the same assay and in five different assays to calculate intra- and inter-assay relative standard deviations (RSDs), respectively. For phospholipids, intra-assay RSDs ranged from 3% (PC, SM) to 8% (PS) and inter-assay RSDs ranged from 6% (PE) to 14% (PS), for cholesterol, they were of 11% and 12%, respectively (Table 2).

Recoveries of sample preparation were determined by adding lipid standards (PE: 1.6 nmol, PS, PI, PC: 3.5 nmol, SM: 8.5 nmol and 2.5 nmol of cholesterol) to five different biological samples and exceeded 90% except for PI (85%) (Table 2).

3.3. Lipid composition of spermatozoa and seminal plasma

Fig. 1B and C show chromatograms of lipid extracts from spermatozoa and SP. Each phospholipid class with a retention time equal to that of the standard lipids were collected directly from the HPLC. They were submitting to HPTLC using the two solvent systems previously described. All lipid peaks were pure and they have identical R_F than those of corresponding standards. In addition, quantification of cholesterol and phospholipid from calibration curves obtained by both HPLC or Liberman Burchard reagent and phosphorus estimation, respectively, gave similar results (between 95% and 105% of the values).

This technique was highly sensitive and enabled accurate quantitative analysis of phospholipids (PE, PC, PI, PS, SM) and cholesterol from as few as $5 \cdot 10^6$ spermatozoa or 25 μl of SP. However, lipid

Table 2
Intra- and inter-assay relative standard deviations (RSDs) of measurements and recoveries of cholesterol and phospholipid classes[a]

Lipids	Intra-assay RSD (%)	Inter-assay RSD (%)	Recovery (%)
Chol	11	12	97±3
PE	5	6	99±3
PS	8	14	92±2
PI	7	11	85±3
PC	3	10	95±2
SM	3	9	93±1

[a] For abbreviations, see legend of Fig. 1. Recovery (mean±S.E.M.) is determined from five measurements.

Table 3
Cholesterol and phospholipids composition in human spermatozoa and seminal plasma[a]

	Spermatozoa (nmol/10^8 cells)	Seminal plasma (nmol/ml)
Cholesterol	95±11	433±28
Phospholipids		
PE	88±6	70±8
PS	23±2	79±6
PI	12±1	24±2
PC	102±8	60±3
SM	57±2	163±6

[a] Values are means±S.E.M. (n=14). For abbreviations, see legend of Fig. 1.

detection and quantification depend on the lipid class studied. Consequently, two different quantities of each extract have to be injected in order to obtain peaks within the quantification limit (about a two-fold lower quantity to detect cholesterol and PE than other phospholipids).

The relative composition of phospholipid classes are listed in Table 3. In spermatozoa, PC was the predominant phospholipid class (36% of total phospholipids measured) whereas SP contained a large amount of SM which represented 42% of total phospholipids. Both in SP and spermatozoa, PI is the minor class of phospholipid (about 5%).

4. Discussion

The results of this study have demonstrated that HPLC coupled with light-scattering detection can be applied to the separation and quantification of a wide range of lipid classes in semen.

Light-scattering detection (also denoted mass-detection) is more and more used in lipid analysis because in contrast to the ultraviolet (UV) detection it is possible to perform gradient elution even with solvents of low UV transparency such as chloroform and it is not dependent upon the degree of unsaturation of fatty acids [9,18]. In addition our data show that the use of a column with a low I.D. (3 mm), which allows a low flow-rate of eluting solvents (0.35 ml/min) increased the sensitivity of the method without significant loss of the resolution and permitted nanomolar quantification of lipids.

However, each phospholipid class is composed of individual molecular species with different saturated and unsaturated fatty acids in their molecules which cannot be separated from each other using this normal-phase chromatographic method. But, recently a new method using reversed-phase HPLC on a RP 18 column and a light-scattering detector has allowed, with success, a quantitative analysis of PC molecular species from a variety of biological samples including boar sperm [19].

HPLC elution profiles of lipid extracts from LS and spermatozoa are different. Both extracts contained cholesterol, PE, PC, PI, PS and SM but whereas no other major lipid classes are detected in spermatozoa, the elution profile of SP is more complex. Preliminary studies suggest that LS contains significant amounts of cerebrosides cardiolipin and cholesterol sulfate as indicated by peaks marked with an asterisk in Fig. 1C, however further analysis are needed to confirm these preliminary identifications.

Differential analysis of phospholipids reveals that PC and PE are the dominant phospholipids in spermatozoa while the predominant phospholipid in seminal plasma is SM. Such data are in accordance with previous studies using other evaluation techniques [5–7].

In conclusion, HPLC and light-scattering detection can detect nanomolar quantities of cholesterol and different classes of phospholipids that are commonly present in human semen in a less-time consuming manner than other techniques. This method may be very useful to analyze lipid composition of spermatozoa in semen of poor quality, especially oligozoospermic ones.

Acknowledgements

The authors thank Professor Claire Lartigue for her advice in editing the manuscript and Claudine Nouailles for her technical assistance. This work was supported by a grant from the Department of Health and Social Security.

References

[1] V. Hinkovska-Galcheva, D. Petkova, K. Koumanov, Cryobiology 26 (1989) 70.
[2] J.E. Parks, D.V. Lynch, Cryobiology 29 (1992) 255.
[3] S. Hamamah, G. Grizard, B.M. Gadella, C. Barthélémy, D. Royère, in: S. Hamamah, R. Mieusset (Eds.), Research in Male Gametes, INSERM, Paris, 1996, p. 187.
[4] L. Huacuja, N. M Delgado, L. Calzada, A. Wens, R. Reyes, N. Pedron, A. Rosado, Arch. Androl. 7 (1981) 343.
[5] A. Poulos, I.G. White, J. Reprod. Fertil. 35 (1973) 265.
[6] S.M. Sebastian, S. Seharaj, M.M. Arnldhas, P. Govindarajulu, J. Reprod. Fertil. 79 (1987) 373.
[7] J.G. Alvarez, B.T. Storey, J. Androl. 13 (1992) 232.
[8] G. Haidl, C. Opper, Hum. Reprod. 12 (1997) 2720.
[9] W.W. Christie, J. Lipid Res. 26 (1985) 507.
[10] H. Bunger, U. Pison, J. Chromatogr. B 672 (1995) 25.
[11] C. Silversand, C. Haux, J. Chromatogr. B 703 (1997) 7.
[12] L. Landi, M.C. Galli, L. Cabrini, G. Hakim, C. Carru, D. Fiorentini, Biochem. Mol. Biol. Int. 44 (1998) 1157.
[13] World Health Organization Laboratory Manual For the Examination of Human Semen and Sperm–Cervical Mucus Interaction, Cambridge University Press, Cambridge, 1992.
[14] J. Folch, M. Lees, G.H. Sloanne-Stanley, J. Biol. Chem. 226 (1957) 497.
[15] F. Vitiello, J.P. Zanetta, J. Chromatogr. 166 (1978) 637.
[16] G.R. Barlett, J. Biol. Chem. 234 (1959) 466.
[17] R.W. Burke, B.I. Diamondstone, R.A. Velapoldi, O. Menis, Clin. Chem. 20 (1974) 794.
[18] P. Juaneda, G. Rocquelin, P.O. Astorg, Lipids 25 (1990) 756.
[19] J.F. Brouwers, B.M. Gadella, L.M. Van Golde, A.G. Tielens, J. Lipid Res. 39 (1998) 344.