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Quantitative analysis of desmosterol, cholesterol and cholesterol sulfate in semen by high-performance liquid chromatography

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Abstract

A simple, rapid and accurate method to separate and quantify cholesterol, desmosterol and cholesterol sulfate in human spermatozoa and seminal plasma (SP) is described. This high-performance liquid chromatographic procedure is based on reversed-phase chromatography on a Inertsil ODS2 5 μm silica column with a binary gradient of mixtures of chloroform–methanol and chloroform–methanol–water as the mobile phase at a flow-rate of 0.25 ml/min. Sterols are separated with good resolution and high reproducibility. The eluted sterols are quantified using a light-scattering (mass) detector. As little as 64, 64 and 68 pmol of cholesterol, desmosterol and cholesterol sulfate, respectively, can be quantified under these conditions. Cholesterol is the predominant sterol both in spermatozoa (107 ± 7 nmol/10^8 spermatozoa) and SP (0.83 ± 0.10 μmol/ml) whereas the concentrations of desmosterol were 38 ± 6 nmol/10^8 in spermatozoa and 0.18 ± 0.02 μmol/ml in SP. Cholesterol sulfate represents about 6% of total cholesterol in the spermatozoa and SP. In conclusion, this method offers interesting perspectives for the quantitative analysis of these sterols not only in semen, but also in other biological samples.

Keywords: Desmosterol; Cholesterol; Cholesterol sulfate

1. Introduction

Cholesterol is an essential constituent of mammalian semen. It is abundant in the sperm plasma membrane and plays an important role in the fertilizing capacity of these cells.

Besides cholesterol, sperm contains desmosterol, (5,24-cholestadien-3β-ol), the direct precursor of cholesterol in the biochemical pathway, a molecule that contains one additional double bond. Desmosterol has been identified in hamster, monkey and human spermatozoa [1–3] and it may be more abundant than cholesterol [2,4]. Desmosterol was not found in important quantities elsewhere in the body except in the testis and it is proposed as a biochemical marker of puberty in monkey testis [5]. In monkey sperm, most of the desmosterol (99%) is located in the tail. This difference in membrane desmosterol composition between sperm heads and tails may be responsible for the different functions of these two parts. Desmosterol may contribute to the membrane fluidity necessary for the motility of the sperm tails [6]. A sperm suspension with a higher desmosterol to cholesterol molar ratio has a greater percentage of motile cells and a higher forward progression value [7]. In the sperm, cholesterol and desmosterol are also known to inhibit capacitation and desmosterol may be the most potent inhibitor. Among the events detected in spermatozoa as they capacitate, the loss of these two sterols in the

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environmental medium represents the initial obligatory step [8,9].

Sterol sulfates have been also found as minor compounds in the semen. Cholesterol sulfate is a normal constituent of human ejaculated spermatozoa and seminal plasma (SP), it represents more than 85% of the sterol sulfate fraction [3]. In contrast, in hamster epididymal spermatozoa, it was surprising to detect almost exclusively desmosteryl sulfate in the sulfate fraction [1]. Finally, in the monkey spermatozoa, both cholesterol and desmosterol sulfates were present [6]. Structural and functional roles of these unusual sterols have been demonstrated. Cholesterol sulfate accumulates in spermatozoa during the epididymal transit. It is located on the plasma membrane, mostly in the acrosome head region. It acts as a membrane stabiliser, it inhibits capacitation and it is also a potent inhibitor of acrosin [10,11]. Fertilization is inhibited by sterol sulfate in hamster and rabbit [12,13]. These data and the presence of sulfatase activity in the female tract, suggest a key role for this sterol sulfate during the fertilizing process [11–13].

A simple and sensitive method, allowing a quantitative analysis of samples containing markedly different amount of those three sterols is needed. In this regard, the first step in sample preparation is usually a total lipid extraction using the classic method of Folch et al. [14]. Cholesterol may be assayed by an enzymatic method or using Lieberman–Burchard reagent [15,16], however such methods did not allow to quantify specifically each sterol. In addition, most methods for cholesterol sulfate estimation do not measure the analyte intact but rather free cholesterol obtained by solvonolysis [3,6].

The precise quantification may be done by chromatographic methods. They can be successfully carried out by thin-layer chromatography (TLC). In recent attempts, to analyse cholesterol, desmosterol and cholesterol sulfate in sperm, argentation TLC was used to separate these sterols; they were then quantified simultaneously after staining, by a scanning method with a spectrodensitometer [8,17]. The quantification of these sterols was also achieved by gas–liquid chromatography (GLC) using packed or capillary columns and generally flame ionisation detection or GLC–mass spectrometry [6].

Some high-performance liquid chromatography (HPLC) methods with both normal- or reversed-phase columns were also described. However, lipid quantification by HPLC is limited due to the absence of universal detector to measure nanomolar quantities. To overcome this problem, a preliminary oxidative conversion of cholesterol and desmosterol by cholesterol oxidase may be performed, the obtained sterols then are quantified by UV absorption [18]. Another possibility is the use of light-scattering (mass) detection that presents some advantages compared to UV detection or refractive index detection. Any volatile solvents, including those of low UV transparency such as chloroform can be used since they are evaporated before the eluate reaches the light source and no baseline drift was observed with gradients. In addition, peak masses are measured directly from peak area [19–21]. The cholesterol sulfate could also be quantified by HPLC–mass spectrometric analysis [22].

We have developed a sensitive qualitative and quantitative analysis of cholesterol, desmosterol and cholesterol sulfate in human seminal plasma and spermatozoa using HPLC combined with light-scattering detection.

2. Experimental

2.1. Sample preparation

Semen samples were obtained from eight healthy men as previously described [20]. They were conformed to the limits of normality set down by the World Health Organisation [23].

After liquefaction of semen, seminal plasma and spermatozoa were obtained by successive centrifugations [20] and were stored at −80°C. Under these conditions, we verified that storage did not induced any change in stability of the analyzed sterols. Lipids were extracted by the method of Folch et al. [14] adapted for SP 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 of 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 methanol–chloroform (1:1, v/v) and just before injection solvent sample were adjusted to methanol–chloroform (9/1, v/v). Butylated hydroxytoluene (BHT) (0.01%, w/v) was added as an antioxidant in all solutions used for extraction and storage of samples.

2.2. Reagents and standards

Cholesterol, desmosterol, cholesterol sulfate and BHT were obtained from Sigma (St. Quentin Fallavier, France). Chloroform was purchased from Prolabo (Fontenay sous Bois, France), methanol was obtained from Merck (Darmstadt, Germany). Stock solutions of standards (1 mg/ml) were made either in methanol for cholesterol and desmosterol or in chloroform–methanol (50:50, v/v) for cholesterol sulfate.

2.3. Chromatographic equipment and method

The chromatographic system used in this study consists of 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).

All the quantitative assays were performed with a 250×3 mm I.D. column packed with Inertsil ODS2 5 μm silica (Interchim, Montluçon, France) and a ternary mobile phase composed of solvent A (methanol–water, 98:2, v/v), solvent B (chloroform–methanol–water, 30:60:10, v/v) and solvent C (methanol–chloroform, 50:50, v/v); all the solvents were degassed under helium before use. The solvent program employed is shown in Table 1. The last 26 min regenerated the column prior to injection of the next sample. Elution was performed at a flow-rate of 0.25 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 bars.

Table 1
Gradient elution system required for the elution of cholesterol, desmosterol and cholesterol sulfate and reactivation of the column

Time (min)	A (%)	B (%)	C (%)
0	100	0	
14	10	90	
31	10	90	
31.01	55		45
49	20		80
54	20		80
54.01	100		
70	100		

A: Methanol–water (98:2, v/v); B: chloroform–methanol–water (30:60:10, v/v); C: methanol–chloroform (50:50, v/v). A linear gradient was produced between the composition specified at each time interval.

Sterols were quantified by comparison of peak areas with those of standard curves.

3. Results and discussion

3.1. Separation

Fig. 1A shows a typical HPLC chromatogram obtained with a standard mixture of pure sterols. They were baseline separated and they eluted as single narrow peaks.The peak appearance and the retention time were highly reproducible. The gradient of 2 to 9.2% water in the solvent improves the peak shape and resolution of cholesterol sulfate. Furthermore, 9.2% water also increased the retention times of the major phospholipids of semen (phosphatidylcholine, -serine, -inositol and sphingomyelin). They are higher than 33 min (data not shown); consequently the separation of desmosterol and cholesterol from these phospholipids is improved.

Fig. 1B and C show chromatograms of lipid extracts from $5 \cdot 10^6$ spermatozoa and 10 μl of SP. HPLC peaks assignments were made in comparison with the reference standards. Each sterol was quantified by integrated peak area analysis.

3.2. Quantification

Due to the nature of the applied detection tech-

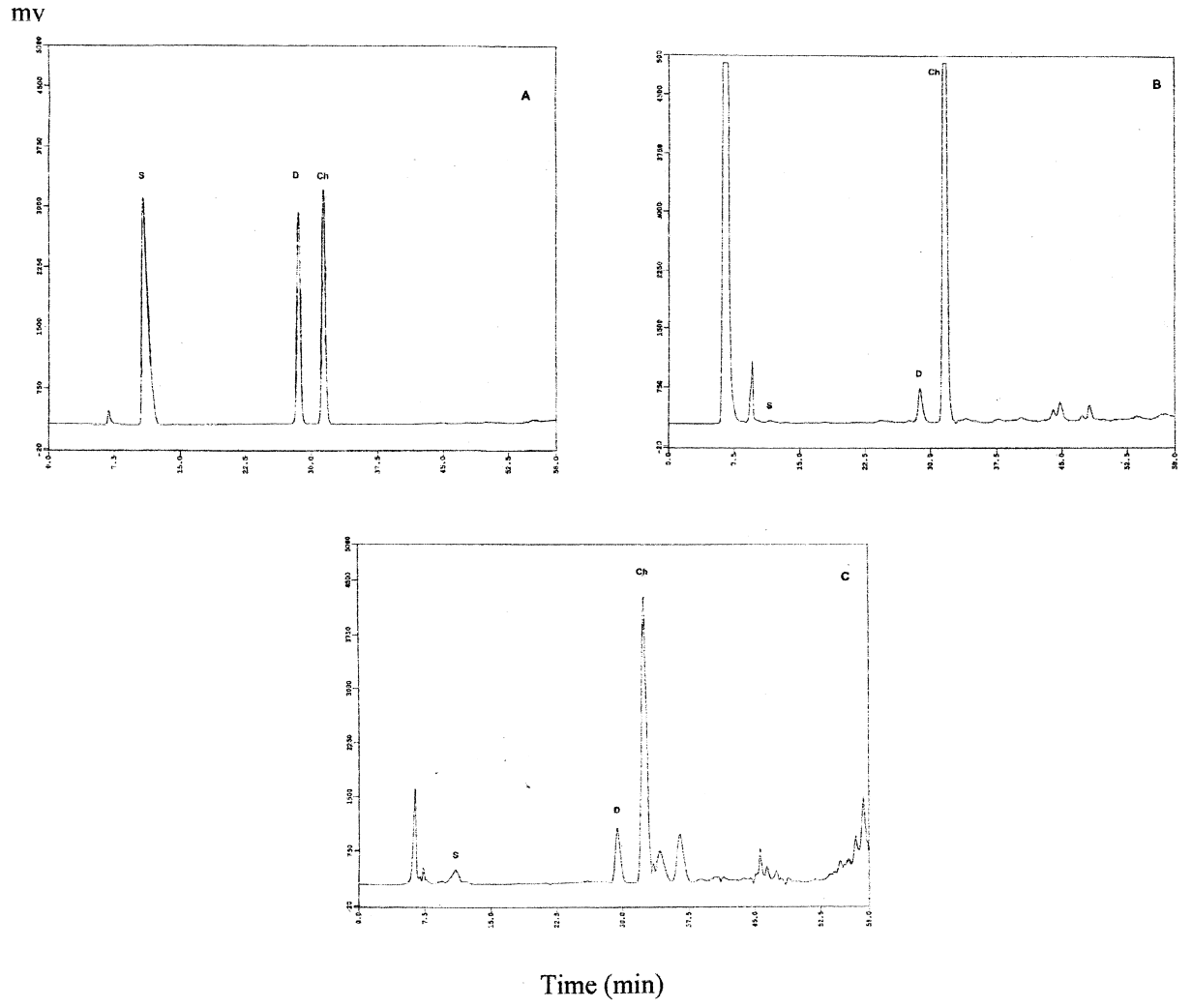

Fig. 1. HPLC separation of cholesterol (Ch), desmosterol (D) and cholesterol sulfate (S), using an Inertsil ODS 2 5 μm silica column and light-scattering detection. Experimental conditions are given in the text. (A) Chromatogram of a mixture of sterol standards: 3.2, 3.2, 5.1 nmol of cholesterol, desmosterol and cholesterol sulfate, respectively. (B) Chromatogram of a lipid extract of 10 μl of seminal plasma. (C) Chromatogram of a lipid extract of $5 \cdot 10^6$ spermatozoa.

nique, the response of the light-scattering detector is non-linear with the amount of lipids eluted from the column. The best fit was found with log–log straight lines regression: log $y = a \log x + b$ with a and b constants, x the amount of sterols expressed in nmol and y the detector response (mV/min) in accord with Stolyhwo et al. [19]. The R values for curve fitting are higher than 0.99. In order to investigate the precision of the calibration curves, a dilution series containing the three sterols was prepared and the calibration curves for each compounds was generated. The average and standard deviation of the intercepts and slopes of these curves are given in Table 2. The curve was linear over the range 0.10 nmol to 3.25 nmol of cholesterol and desmosterol and 0.15 nmol to 5.10 nmol of cholesterol sulfate. The response coefficients were closely the same for these three compounds contrary to UV detection in which the number of double bonds is crucial. The background level is low. The limit of detection

Table 2
Coefficient of regression of standard curves

	Intercept	Slope	*R*
Cholesterol	−1.446±0.028	0.636±0.011	0.998
Desmosterol	−1.445±0.097	0.645±0.015	0.996
Cholesterol sulfate	−1.280±0.033	0.597±0.012	0.997

Calibration curves were obtained by log–log transformation of the values. The following dilutions 1/2, 1/4, 1/8, 1/16, 1/32 and 1/64 of standard solutions containing 6.5, 6.5, 10.2 nmol/100 μl of cholesterol, desmosterol and cholesterol sulfate, respectively, were analysed in triplicate. The calibration curves were linear on these ranges of concentration. Means±standard error were reported.

(twice the noise level) was of 68 pmol for cholesterol sulfate, 64 pmol for desmosterol and 64 pmol for cholesterol. These results show that mass detection has the same sensitivity as UV detection.

Experiments were performed to determine the reproducibility of the method. Three measurements of the same biological sample were performed in the same assay to calculate intra-relative standard deviation (RSD). In addition, three aliquots of the same biological sample were analyzed in three separate assays at different days to calculate inter-RSD (Table 3). The small amount of cholesterol sulfate in biological sample analyzed may explain that the RSDs of this compound were slightly higher than RSDs of the two other sterols.

To estimate the accuracy of the method, we performed a recovery experiment. Thus sterol standards were added to three different biological samples either before or after extraction. From sterol concentrations found, we subtracted the endogenous sterol concentration and calculated the recovery of added sterols after both total procedure and analytical assays by HPLC (Table 3). They were slightly lower for cholesterol sulfate (88% for the total procedure recovery) than for desmosterol and cholesterol, but the efficiencies of extraction procedure and HPLC assay are good for the three sterols. Using an another procedure for extraction (Sep-Pak cartridge and methanol extraction), a recovery of 93% was reported for plasma cholesterol sulfate [22]. No data of recovery were found in the other studies.

3.3. Sterol composition of spermatozoa and seminal plasma

Sterol composition of spermatozoa and SP is shown in Table 4. Cholesterol is the dominant sterol in human spermatozoa and SP. However, we also found a large amount of desmosterol. The desmosterol/cholesterol molar ratio was 0.34 and 0.23 in spermatozoa and SP, respectively. Cholesterol sulfate represented about 6% of the total cholesterol both in spermatozoa and SP.

The cholesterol amounts determined in this study are generally lower than that obtained by colorimetric or enzymatic methods [16,24,25]. In fact, such methods are not very specific and they do not allow

Table 3
Intra- and inter-assay relative standard deviations (RSDs) of measurements and recoveries of cholesterol, desmosterol and cholesterol sulfate added in the extract or in biological samples

Lipid	Intra-assay $m\pm\sigma$ μmol/ml (RSD, %)	Inter-assay $m\pm\sigma$ μmol/ml (RSD, %)	Recovery of the HPLC assay (%)	Recovery after total procedure (%)
Cholesterol	0.62±0.05 (6)	0.648±0.062 (10)	100±4	100±7
Desmosterol	0.118±0.006 (6)	0.124±0.008 (8)	96±5	95±5
Cholesterol sulfate	0.036±0.003 (10)	0.032±0.004 (14)	95±4	88±5

The RSDs ($\sigma\times100/m$) were determined by analysis of 5 μl of seminal plasma. The recovery of the HPLC assay was determined by addition of 0.10, 0.12, 0.16 nmol of standard in extract of 5 μl seminal plasma just before each injection. The recovery after extraction and quantification procedures were made by addition of 24, 23, 23 nmol of standard in 100 μl of seminal plasma. Values (means±SD) were determined from three measurements.

Table 4
Cholesterol, desmosterol and cholesterol sulfate in human spermatozoa and seminal plasma

	Seminal plasma, μmol/ml	Spermatozoa, nmol/10^8 cells
Cholesterol	0.83±0.10 (0.36–1.15)	107±7 (63–161)
Desmosterol	0.18±0.02 (0.11–0.24)	38±6 (16–70)
Cholesterol sulfate	0.054±0.011 (0.02–0.08)	6.2±3.1 (ND–10.3)

Values are means±S.E.M.(n=8). Ranges in parentheses. ND: Not detectable.

to measure separately cholesterol and desmosterol. However, in human spermatozoa, the amounts of cholesterol are in the range of values reported by other authors using different chromatographic procedures (Table 5). Nevertheless, we observed large variations between the different studies. They can be explained, in part, by the origin of the spermatozoa. Measurement may be performed with spermatozoa obtained from ejaculate, without selection [3,7,20,26] or after selection on gradient density [27,28] or swim up [8,29]. In addition, the quality of the semen was not always mentioned. Differences in the methodology at each step (extraction, separation, and quantification) may also be variation factors. Unfortunately, no comparative analysis using these different procedures was reported. SP is a complex biological fluid especially in its lipid composition [20,30,31] and it can modulate the properties of the spermatozoa. In SP, except for cholesterol, data about analysis of other sterols are scarce. Lalumière et al. [3] reported that ratio of desmosterol/cholesterol was 0.04 but these authors did not give the absolute value of cholesterol and desmosterol. With normal-phase HPLC, the same range of cholesterol was reported [20].

The method described in this report is a rapid and accurate procedure for the separation and quantification of cholesterol, desmosterol and cholesterol sulfate in semen. It may also be applicable to other biological samples which containing these three particular sterols.

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Table 5
Cholesterol, desmosterol and cholesterol sulfate concentrations in human spermatozoa determined by chromatographic techniques, in the literature

	Cholesterol, nmol/10^8 cells	Desmosterol, nmol/10^8 cells	Cholesterol sulfate, nmol/10^8 cells	Technique
Lalumière et al. [3] (n=5)	na	na	3.2±0.6	GLC
Connor et al. [7] (n=8)	29±10[a]	10±5[a]	na	GLC
Osheroff et al. [8] (n=3)	119±2	67.0±0.4	3.2±0.1	HPTLC
Grizard et al. [20] (n=14)	95±11	na	na	HPLC
Hoshi et al. [26] (n=8)	125±41	na	na	GLC
Parinaud et al. [27] (n=10)	39±25	16±11	na	GLC
Cross [28] (n=9)	35±2	25±2	na	GLC
Alvarez and Storey [29] (n=10)	133±4	78.5±2.5	na	HPTLC

Values are mean±S.E.M. n: Number of sample. [a]Median and interquartiles ranges. na: Non analyzed. GLC: Gas–liquid chromatography, HPTLC: high-performance thin-layer chromatography, HPLC: high-performance liquid chromatography.

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