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CAPILLARY GAS CHROMATOGRAPHIC PROFILING OF TOTAL LONG-CHAIN FATTY ACIDS AND CHOLESTEROL IN BIOLOGICAL MATERIALS

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SUMMARY

The profiling of total long-chain fatty acids and cholesterol in a variety of biological materials, using capillary gas chromatography with flame ionization detection, is described. The within-run precision and day-to-day precision for fifteen fatty acids and cholesterol in erythrocyte samples were investigated. Quantitative data on the analysis of amniotic fluid samples collected from women in the 30th to 38th week are given together with a correlation study on their lecithin/sphingomyelin and their palmitic acid/stearic acid ratios. In addition, the method was applied to lumbar cerebrospinal fluid, plasma, isolated leukemic blood cells and neuroblastoma tissue.

INTRODUCTION

Lipids serve both as metabolic fuel and membrane constituents of cells, and their determination as either the individual phospholipids, triglycerides, sterols,

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sterol esters and fatty acids is of importance to the understanding of many (patho)physiological processes.

Traditionally, determinations of free and total fatty acids have been performed by gas chromatography on polar stationary phases adsorbed to supporting materials (packed columns). Packed-column gas chromatography, however, is limited with respect to its separating power and in most cases requires the injection of a relatively large amount of the analyte, which is due to undesired adsorption properties. On the other hand, the development of capillary gas-chromatographic techniques using polar stationary phases has long been hampered by the lack of temperature resistance of such phases coated on the glass wall.

In this paper we describe a generally applicable method for the profiling of total long-chain fatty acids in biological materials using a CP-Sil-88-coated fused-silica capillary column. Using the same prepurified sample total cholesterol could be determined by its injection on an apolar stationary phase coated capillary gas chromatographic system.

MATERIALS AND METHODS

Standards and reagents

Certified cholesterol from the National Bureau of Standards, Washington, DC, U.S.A., was a generous gift from Dr. H.J.G.M. Derks of the R.I.V., Bilthoven, The Netherlands; 5β-cholestan-3α-ol (chola*) was from Steraloids, Wilton, NH, U.S.A., and tri-Sil-TBT from Pierce, Rockford, IL, U.S.A. 20:3c,ω9 was a generous gift from Dr. J. Kloeze, Unilever, Vlaardingen, The Netherlands. Other fatty acid standards were obtained from Applied Science, Oud Beyerland, The Netherlands, and Packard, Downers Grove, IL, U.S.A. All other reagents were from Merck, Darmstadt, F.R.G.

Samples

Blood samples (10 ml) were collected in EDTA-containing vacutainer tubes by venepuncture. Samples were immediately cooled in ice and centrifuged at 800 *g* for 10 min in a cooled centrifuge. Plasma was centrifuged again at 1400 *g* for 10 min. The buffy coat of the packed cells was removed as completely as possible and the red cells were washed three times with 5-ml portions of 0.15 mol/l sodium chloride solution adjusted to pH 7.4 by the addition of a saturated sodium hydrogen carbonate solution in water. After each washing the buffy coat was removed as completely as possible. The red cells were finally

*Abbreviations used in the text, figures and tables: 14:0 = myristic acid; 15:0 = pentadecanoic acid; 16:0 = palmitic acid; 16:1c,ω7 = palmitoleic acid; 16:1tr,ω7 = palmitelaidic acid; 17:0 = margaric acid; 18:0 = stearic acid; 18:1c,ω7 = vaccenic (*cis*) acid; 18:1c,ω9 = oleic acid; 18:2c,ω6 = linoleic acid; 18:3c,ω3 = linolenic acid; 18:3c,ω6 = γ-linolenic acid; 20:0 = arachidic acid; 20:1c,ω9 = *cis*-11-eicosenoic acid; 20:2c,ω6 = *cis,cis*-11,14-eicosadienoic acid; 20:3c,ω6 = dihomo-γ-linolenic acid; 20:3c,ω9 = all *cis*-5,8,11-eicosatrienoic acid; 20:4c,ω6 = arachidonic acid; 22:0 = behenic acid; 22:5c,ω3 = all *cis*-7,10,13,16,19-docosapentaenoic acid; 22:6c,ω3 = all *cis*-4,7,10,13,16,19-docosahexaenoic acid; 23:0 = tricosanoic acid; 24:0 = lignoceric acid; 24:1c,ω9 = nervonic acid; chole = cholesterol; chola = 5β-cholestan-3α-ol; BHT = butylated hydroxytoluene (2,6-di-*tert*.-butyl-*p*-cresol).

resuspended to a haematocrit of about 50% and counted by means of a Coulter counter.

Amniotic fluid samples from patients in the 30th to 38th week were collected by amniocentesis for diagnostic purposes. After centrifuging for 10 min at 800 *g* the lecithin/sphingomyelin ratio was determined by thin-layer chromatography, and creatinine by means of an Automatic Clinical Analyzer (Dupont).

Lumbar cerebrospinal fluid samples were obtained from patients with a variety of neurological diseases, and centrifuged for 10 min, at 800 *g*.

Peripheral leukemic blood cells were isolated from patients in a leukemic phase by a Lymphoprep (Nijegaard, Oslo, Norway) density gradient. The cells were resuspended in isotonic phosphate-buffered saline pH 7.4 to a concentration of about 10^7 per 200 μl, and counted by means of a Coulter counter.

Neuroblastoma tissue was obtained from paediatric patients after (partial) surgical removal. In each case the diagnosis was confirmed by pathological anatomical examination. Tissue samples were either freeze-dried or frozen at −20°C before use.

TABLE I

RELATIONSHIP BETWEEN THE AMOUNT OF SAMPLE, ANTIOXIDANT (BHT), INTERNAL STANDARDS (17:0, 5β-CHOLESTAN-3α-OL) AND THE FINAL VOLUME FOR THE GAS CHROMATOGRAPHIC DETERMINATION OF TOTAL FATTY ACIDS AND CHOLESTEROL IN DIFFERENT KINDS OF BIOLOGICAL MATERIAL

Material	Amount (μl)	Additions			Final volume (μl)
		BHT (mg)	17:0 (μg)	Chola (μg)	
Cerebrospinal fluid	1000	0.2	2.5	5.0	20
Amniotic fluid*	1000	0.4	10.0	10.0	80
Plasma/serum	100	1.0	50.0	200.0	400
Erythrocytes**	200	1.0	50.0	100.0	400
Leucocytes***	200	0.4	10.0	10.0	80
Neuroblastoma§	1.5 mg	0.2	20.0	20.0	160

*Samples obtained between the 30th and 40th week of gestation.
**Suspension with a haematocrit of about 50% (ca. 10^9 cells per 200 μl).
***Suspension containing ± 10^7 cells per 200 μl.
§Dry tissue (ca. 10 mg wet tissue).

Transesterification of fatty acids and hydrolysis of cholesterol esters

Table I shows the amounts of biological material together with those of the internal standards and antioxidant used for the determination of the total fatty acid profile and cholesterol. Indicated amounts were pipetted or put into 15 ml Sovirel tubes using stock solutions of butylated hydroxytoluene (BHT; 10 g/l) margaric acid (17:0; 0.5 g/l) and 5β-cholestan-3α-ol (chola; 0.5 g/l) in methanol. The volumes of cerebrospinal fluid and amniotic fluid were reduced to at least 200 μl by evaporation at 40°C under a stream of nitrogen. Two milliliters of a methanol—hydrochloric acid solution, prepared by adding 50 ml

of methanol to 10 ml of a 6 mol/l hydrochloric acid solution in water, were added. The tubes were flushed with a stream of nitrogen, tightly capped and heated at 90°C for 4 h. After cooling the samples were extracted with 2 × 2 ml of hexane and the combined hexane layers evaporated to dryness at 40°C under a stream of nitrogen.

Trimethylsilylation

To the dry residues were added 150-μl aliquots of tri-Sil-TBT and the tubes heated at 80°C for 30 min in a heating block. After the addition of 4 ml of water, fatty acid methyl esters and trimethylsilylated sterols were extracted into 4 ml of hexane. The hexane layer was evaporated to dryness at 40°C under a stream of nitrogen and the residues redissolved in the indicated amounts of hexane (Table I).

Gas chromatography

Profiling of total fatty acids. Aliquots of 2 μl were automatically injected into a Hewlett-Packard Model 5880 gas chromatograph equipped with a Model 7672 A automatic injection system and a 25 m × 0.25 mm I.D. CP-Sil-88-coated (film thickness 0.25 μm) fused-silica column (Chrompack, Middelburg, The Netherlands). Helium gas flow-rate was 0.65 ml/min, split ratio 1:15, flame ionization detector temperature 300°C, and injector temperature 220°C. The oven temperature program was: 150°C, 1°C/min to 200°C, 10 min at 200°C.

Determination of total cholesterol. Amounts of 0.5—2.0 μl were automatically injected into the same gas chromatograph equipped with a 15 m × 0.20 mm I.D. cross-linked methylsilicone-coated (film thickness 0.11 μm) fused-silica column (Hewlett-Packard). Helium gas flow-rate was 0.39 ml/min, split ratio 1:15, flame ionization detector temperature 300°C, injector temperature 280°C, and oven temperature 265°C.

Identification and quantification

Fatty acid methyl esters were identified on the basis of their retention times using two different standard solutions containing even- and odd-carbon-numbered saturated fatty acids (14:0—24:0) and (mono- and poly)unsaturated fatty acids. 18:1c,ω7 and 22:5c,ω3 were identified on the basis of the erythrocyte membrane total phospholipid fatty acid profiles reported by Heckers et al. [1], who used a similar capillary gas-chromatographic system. Peak area ratios were calculated using a Tracor 812 analytical processing data system. Concentrations were calculated by the following equation:

$$\text{Concentration of fatty acid} = \frac{\text{peak area of fatty acid}}{\text{peak area of 17:0}} \times \frac{\text{amount of 17:0}}{\text{molecular weight of fatty acid}} \times \frac{1}{\text{sample size}}$$

in which the amount of 17:0 is given in g, and sample size may either have the dimension of l, g, mol of creatinine, or number of cells.

Cholesterol concentrations were calculated by comparing the peak area ratios of cholesterol and 5β-cholestan-3α-ol of samples with those of a standard containing equal amounts of both compounds.

RESULTS AND DISCUSSION

In Fig. 1 the time-dependent changes in the yields of methylated long-chain fatty acids (expressed as peak area ratio to the internal standard 17:0) and free cholesterol (expressed as peak area ratio to 5β-cholestan-3α-ol) are shown during heating in the methanol—hydrochloric acid solution at 90°C. Both erythrocytes and plasma were chosen as models to investigate the completeness of transesterification and cholesterol ester hydrolysis, as erythrocytes (composed of glycerophospholipids, sphingolipids and free cholesterol) and plasma (mainly composed of triglycerides and cholesterol esters) provide a natural source of variability in lipid class composition. From Fig. 1 it can be concluded that for both types of samples a steady state was reached after at least 3 h. For routine purposes a transesterification—hydrolysis time of 4 h was selected. On account of the presence of 16.7% of water in the methanol—hydrochloric acid mixture no dehydration or methylation of cholesterol, nor the formation of dimethylacetals from long-chain aldehydes, liberated from potentially present plasmalogens, was experienced.

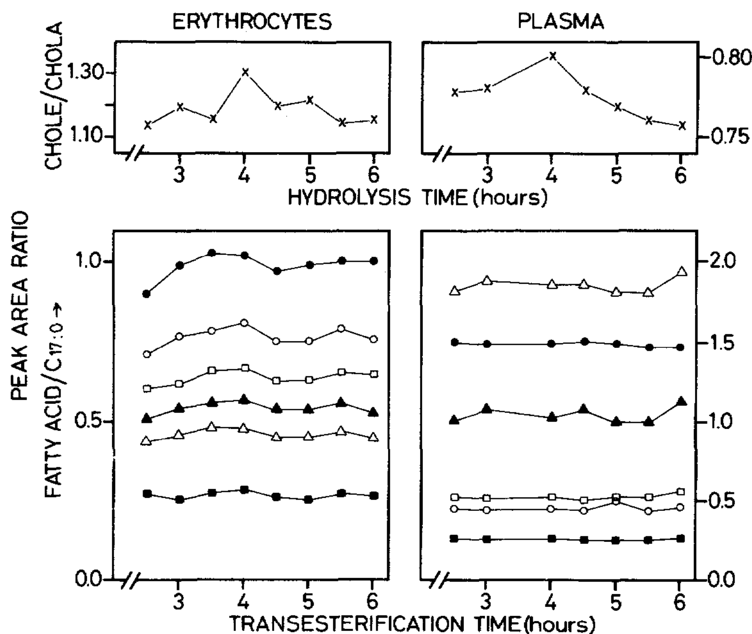

Fig. 1. Time-dependent changes in the yields of methylated fatty acids and free cholesterol for erythrocytes and plasma during heating in the methanol—hydrochloric acid solution at 90°C. Data are expressed as peak area ratios of the quantitatively most important analytes relative to their internal standards. (×), cholesterol; (•), 16:0; (○), 18:0; (□), 20:4c,ω6; (▲), 18:1c,ω9; (△), 18:2c,ω6; (■), 22:6c,ω3.

As split injection of samples containing mixtures of compounds characterized by a large range in boiling points may lead to day-to-day variation in the yields of the individual analytes on the column (split-dependent fractionation), this possible source of imprecision was studied by calculating the day-to-day coefficient of variation in peak areas obtained from the standard containing a mixture of equal amounts of even- and odd-carbon-numbered saturated fatty acid methyl esters (14:0–24:0). In Fig. 2 the carbon number

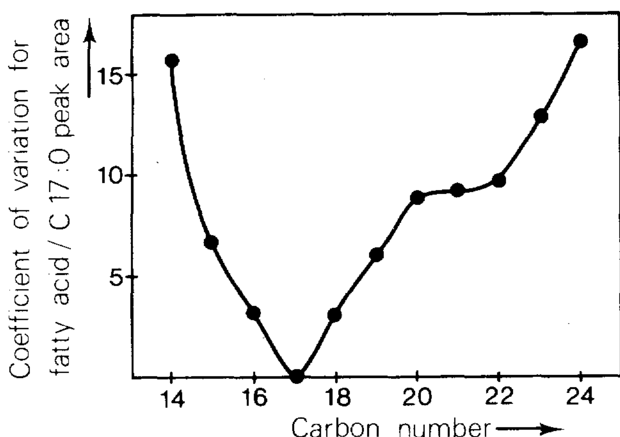

Fig. 2. Relationship between the carbon numbers of even and odd saturated fatty acid methyl esters and the day-to-day coefficient of variation of their respective gas chromatographic peak area ratios relative to 17:0. The coefficient of variation was calculated for fourteen determinations, equally divided over a period of nine months.

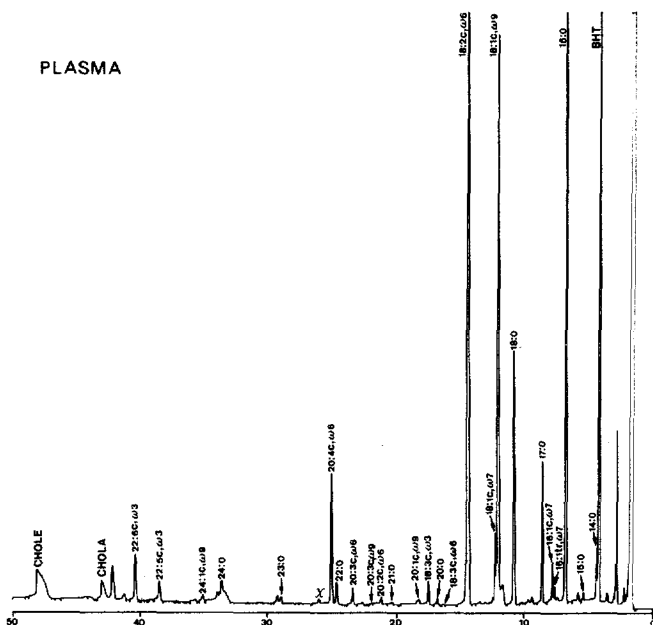

Fig. 3.

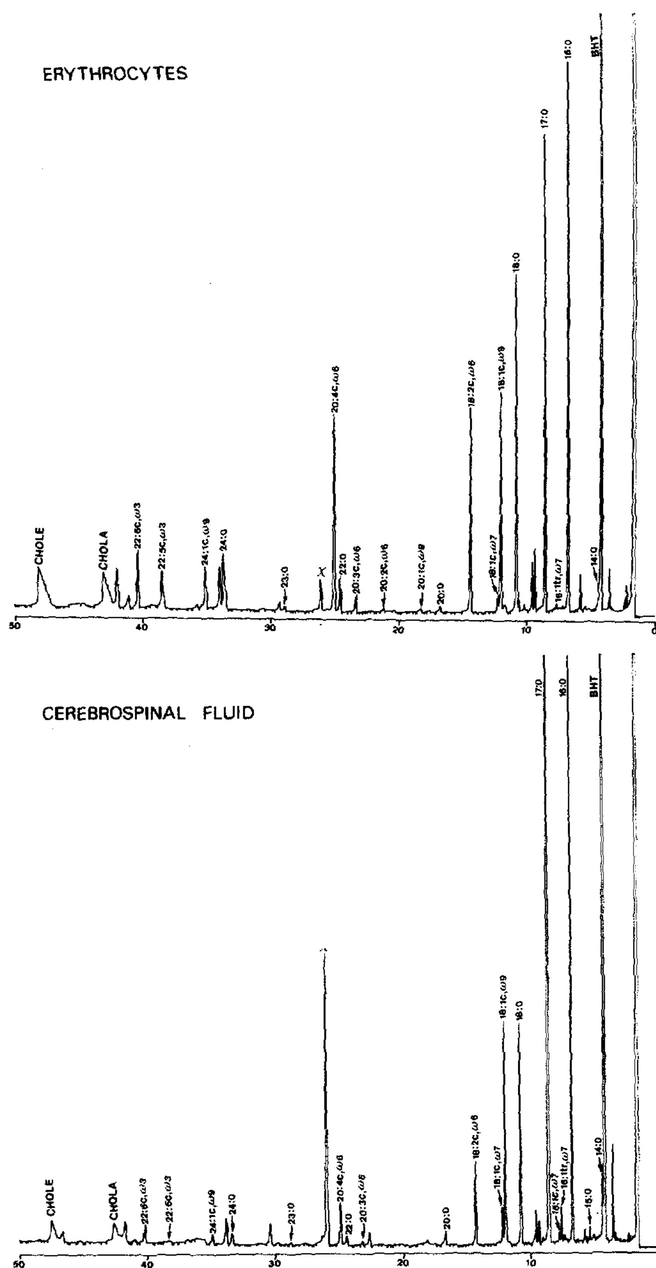

Fig. 3. Typical gas chromatograms of methylated total fatty acids from plasma, erythrocytes and cerebrospinal fluid prepared on a CP-Sil-88-coated fused-silica capillary column. In the case of plasma, 25 μg of 17:0 were added to 100 μl of sample, For erythrocytes and cerebrospinal fluid 50 and 2.5 μg of 17:0 were added to 200 μl (haematocrit ± 50%) and 1 ml, respectively. X = compound of unknown identity (possibly a phthalate) introduced during the clean-up procedure. Time axis in minutes.

dependent coefficients of variation of the respective peak area ratios relative to 17:0 are depicted. It can be concluded that, using 17:0 as an internal standard, notably fatty acid chain lengths smaller than 15:0 and longer than 22:0 suffer from the mentioned phenomenon. Uninvestigated improvements that may (partly) solve this source of imprecision include the use of on-column injectors or splitless injectors, and the additional employment of "low"- and "high"-boiling internal standards.

Fig. 3 shows typical gas chromatograms of methylated total fatty acids from plasma, erythrocytes and cerebrospinal fluid. The original set-up of our method, to measure simultaneously the total fatty acid profile and cholesterol in a single gas chromatographic run, could not be realized as the trimethylsilylated sterols showed poor gas chromatographic properties on the polar CP-Sil-88 stationary phase, giving rise to unacceptable quality control data. As notably polyunsaturated fatty acids are difficult to obtain in a highly purified form we decided to quantify all acids on the basis of the peak area of the internal standard 17:0, by assuming an equal ratio between the amount (expressed in g) and its peak area for each of the individual fatty acids. With respect to possible fractionation introduced by split injection, this approach

TABLE II

QUALITY CONTROL DATA ON THE DETERMINATION OF THE TOTAL FATTY ACID AND CHOLESTEROL COMPOSITION OF A FROZEN ERYTHROCYTE SUSPENSION

Compound	Within-run precision		Day-to-day precision	
	Mean* (nmol per 10^8 cells)	C.V. (%)	Mean** (nmol per 10^8 cells)	C.V. (%)
16:0	17.50	3.5	18.14	4.0
18:0	13.36	4.8	12.88	4.0
18:1c,ω9	8.36	3.0	8.40	1.1
18:1c,ω7	0.67	10.0	0.78	9.0
18:2c,ω6	9.01	1.9	8.93	3.7
20:0	0.32	7.8	0.32	11.0
20:2c,ω6	0.22	10.2	0.26	15.5
20:3c,ω6	1.16	6.5	1.03	10.3
20:4c,ω6	10.96	3.1	10.45	5.3
22:0	1.61	4.5	1.50	13.4
22:5c,ω3	1.78	4.6	1.70	8.0
22:6c,ω3	3.90	7.0	3.06	14.9
23:0	0.23	12.0	0.25	12.6
24:0	3.58	4.0	3.20	16.9
24:1c,ω9	2.35	2.9	2.23	17.6
Total fatty acids	74.94	2.3	73.13	2.1
Cholesterol	29.57	4.5	n.d.***	n.d.
Total fatty acids/ cholesterol	2.54	4.4	n.d.	n.d.

*Mean of twelve determinations.
**Mean of six determinations equally divided over a period of four months.
***n.d. = not determined.

leads to a slight underestimation of analytes with lower boiling points than 17:0 and a slight overestimation of analytes with higher boiling points.

In Table II some quality control data are presented for a 50% erythrocyte suspension prepared from a blood sample of a normal healthy adult. For the determination of the day-to-day precision, 200-μl amounts were stored at −20°C under nitrogen and subsequently analyzed over a period of four months. Due to the large quantitative differences between the individual fatty acids, relatively large coefficients of variation were calculated for the minor ones. Striking discrepancies were found between the within-run and the day-to-day coefficients of variation for 22:0, 24:0, and 24:1c,ω9. This was explained to be caused by the previously mentioned split-dependent fractionation and further by small, but significant, day-to-day changes in the relative retention times of these compounds, leading to incomplete separation from neighbouring peaks. During intensive use of the column over a period of six months a gradual decrease in its separating power and a subsequent worsening of the within-run quality control data for each of the individual fatty acids was experienced. The data shown in Table II should therefore only be considered as indicators of the quality control that can be achieved by the present method.

In Table III the total fatty acid composition of eight amniotic fluid samples obtained from women in the 34th and 35th week of gestation is shown. As the interindividual amniotic fluid volume is subject to large variation it was

TABLE III

MEAN TOTAL FATTY ACID COMPOSITION CALCULATED FOR EIGHT AMNIOTIC FLUID SAMPLES OBTAINED IN THE 34th (FOUR SPECIMENS) AND 35th (FOUR SPECIMENS) WEEK OF GESTATION

Fatty acid	Concentration				Relative amount*	
	μmol/l	C.V. (%)	mmol/mol creatinine	C.V. (%)	mol per 100 mol	C.V. (%)
15:0	1.53	21.6	18.9	38.8	1.37	80.8
16:0	82.80	69.0	907.9	61.1	46.69	25.3
16:1c,ω7	3.66	72.2	39.7	56.0	2.17	21.2
18:0	13.52	39.8	155.3	33.6	9.35	22.7
18:1c,ω9	23.04	57.8	246.9	30.8	14.96	22.9
18:1c,ω7	3.14	53.9	34.2	35.2	2.02	13.6
18:2c,ω6	13.30	51.8	146.0	26.5	9.30	34.6
20:0	0.71	10.2	8.8	32.9	0.66	78.0
20:3c,ω6	1.66	47.6	19.4	56.4	1.10	58.0
20:4c,ω6	10.27	51.5	116.9	48.9	6.44	20.8
22:0	1.57	16.1	19.0	30.0	1.37	72.4
22:6c,ω3	2.91	57.7	32.4	45.5	1.85	25.6
24:0	1.89	16.4	22.8	25.2	1.59	63.6
24:1c,ω9	1.17	30.2	14.5	43.4	1.14	87.2
Total	161.18	55.7	1782.7	44.6	100.01	—

*Trace amounts (⩽ 0.5 mol per 100 mol) of 23:0 were detectable in five samples, 22:5c,ω3 and 18:3c,ω6 in two samples, and 20:1c,ω9, 20:2c,ω6 and 21:0 in one sample. 14:0 and 16:1tr,ω7 were detectable in all samples, but not included in the calculations.

investigated whether expressing the fatty acid concentrations as a ratio to that of creatinine would lead to a somewhat more uniform picture. This was in some degree found to be the case, but except for the quantitatively minor acids the smallest coefficient of variation was calculated for the relative amounts.

Correlation studies using the data obtained from analyses of twelve amniotic fluid samples that were well defined with respect to gestational age, revealed high correlation coefficients between their lecithin/sphingomyelin ratios and the concentrations (in mmol/mol of creatinine) for the following fatty acids: 16:0 (correlation coefficient = 0.92), 22:6c,ω3 (0.90), 18:1c,ω9 (0.84), 20:4c,ω6 (0.81) and 18:0 (0.79). These data suggested that besides the 16:0/18:0 ratio other fatty acid/18:0 ratios might be of use as indicators of fetal lung maturity. In the left panel of Fig. 4, 16:0 and 18:0 concentrations are depicted as a function of gestational age, while the graphs shown in the right panel allow a visual comparison between the gestational age-dependent increases of the different fatty acid/18:0 ratios mentioned above. From this figure it is clear that during the last two months of gestation the 16:0/18:0 ratio undergoes the most notable increase.

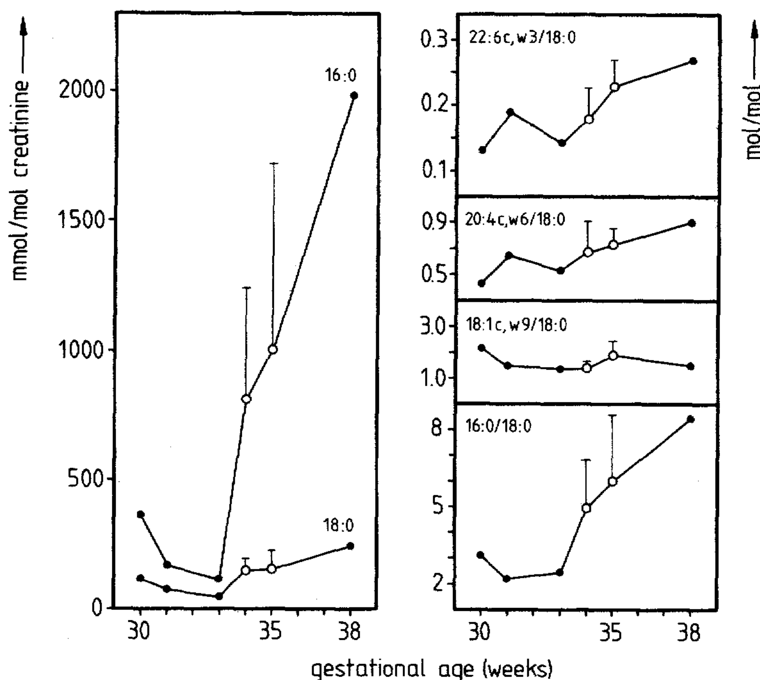

Fig. 4. Gestational age-dependent concentrations of 16:0 and 18:0 (left) and fatty acid/18:0 ratios (right) in twelve amniotic fluid samples. (○), mean ± 1 S.D. of three data.

In Fig. 5 the results of a correlation study between the lecithin/sphingomyelin ratio and the 16:0/18:0 ratio for 22 amniotic fluid samples are depicted. We found a linear relationship between the two ratios with a correlation coefficient of 0.908, which observation is somewhat different from that of Lavoinne et al. [2]. Apart from the differences in analysis time, quality control and degree of difficulty between the two methods, there is, in our opinion, no preference for one of them.

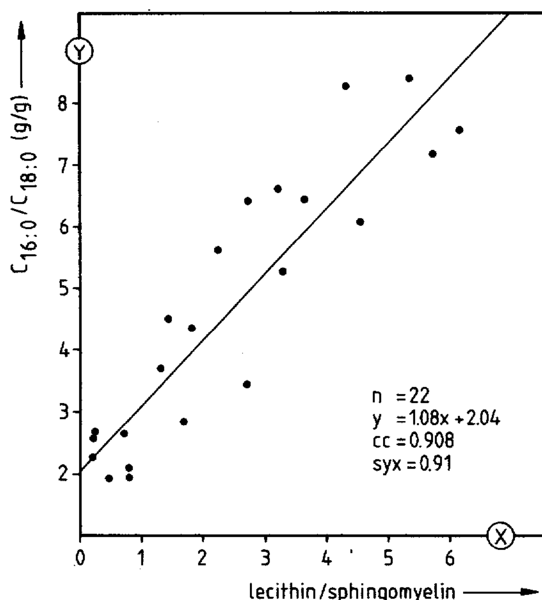

Fig. 5. Correlation between the lecithin/sphingomyelin ratio and the 16:0/18:0 (g/g) ratio for 22 amniotic fluid samples.

TABLE IV

MEAN TOTAL FATTY ACID COMPOSITION CALCULATED FOR SEVEN CEREBROSPINAL FLUID SAMPLES

Fatty acid	Concentration		Relative amount*	
	μmol/l	C.V. (%)	mol per 100 mol	C.V. (%)
15:0	0.67	9.5	2.26	24.4
16:0	8.50	15.6	27.95	15.5
18:0	4.36	18.9	14.22	11.9
18:1c,ω9	7.44	17.8	24.13	6.5
18:1c,ω7	1.01	17.2	3.30	12.8
18:2c,ω6	4.12	46.4	12.78	31.3
20:0	0.39	37.4	1.25	29.3
20:3c,ω6	0.24	8.5	0.79	19.6
20:4c,ω6	1.73	27.9	5.53	16.7
22:0	0.52	20.2	1.69	19.8
22:6c,ω3	0.93	31.2	2.01	25.6
24:0	0.44	34.8	1.39	22.1
24:1c,ω9	0.53	32.2	1.70	23.0
Total	30.86	18.4	100.00	—

*Trace amounts (⩽ 0.7 mol per 100 mol) of 21:0 and 23:0 were detectable in three samples and 22:5c,ω3 in one sample. 14:0 and 16:1tr,ω7 were detectable in all samples, but not included in the calculations.

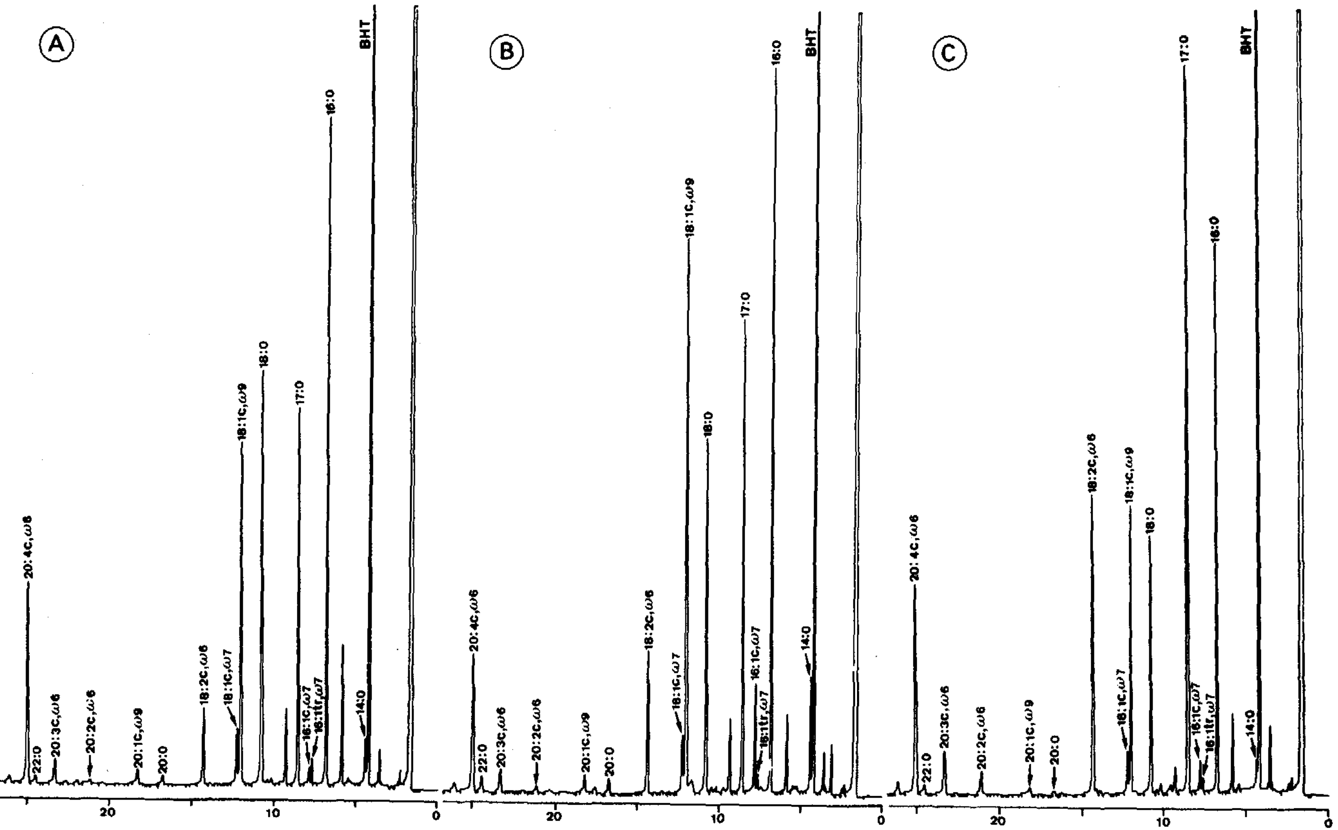

Fig. 6. Parts of total fatty acid profiles of neuroblastoma tissue obtained from three different paediatric patients. Time axis in minutes.

In Table IV the total fatty acid concentrations determined for seven cerebrospinal fluid samples of patients with various non-degenerative neurological diseases are presented. As is the case with amniotic fluid, the (patho)physiological significance of total fatty acid profiling of cerebrospinal fluid is poorly understood and has therefore not yet found its application in clinical diagnosis. The large interindividual variations in total fatty acid concentrations and their relative amounts found by others [3] and in the present study may have hampered further investigations in this field.

In Fig. 6 parts of strongly differing total fatty acid profiles prepared from neuroblastoma tissue of three different paediatric patients are shown. Recently Dawson and Golomb [4] reported fatty acid differences amongst isolated leukemia cell types, suggesting a possible application in terms of the diagnosis of analytical subtypes and the development of new therapeutic strategies. In addition, in vitro experiments revealed differences between normal and tumor cells in their preference to incorporate certain fatty acids [5], while changes in fatty acid metabolism have been observed after the induction of tumor cell differentiation [6, 7]. It is nowadays widely accepted that the phospholipid fatty acid composition influences the fluidity of the membrane bilayer, which in its turn influences, for example, membrane-associated enzymatic activity, receptor activity and transport mechanisms. Although we realize that statements on tissue samples which are poorly defined with respect to their homogeneity are at the least tentative, the observed variance in the fatty acid composition of the neuroblastoma samples may be explained by differences in fatty acid uptake and/or differences in fatty acid synthesis associated with pathologic-anatomically indistinguishable subtypes.

In conclusion, the present method has proven its usefulness for total long-chain fatty acid profiling and the determination of total cholesterol in a variety of biological materials. The results obtained from its application to a study of erythrocyte and plasma samples collected from normal healthy adults after an overnight fast will be presented in a separate paper [8].

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