Journal of Chromatography, 162 (1979) 185—196
Biomedical Applications

CHROMBIO. 251

SPECTRUM OF TOTAL FATTY ACIDS IN CEREBROSPINAL FLUID DETERMINED BY GAS CHROMATOGRAPHY

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SUMMARY

A simple gas—liquid chromatographic method for the determination of the spectrum of fatty acids in a small volume of cerebrospinal fluid (CSF) is presented. In a group of 49 neurological patients it has been found that in the CSF of the controls (n = 12) there are the following main fatty acids: oleic (27.28%), palmitic (23.23%), stearic (12.21%), linoleic (7.66%), myristic (5.02%), and palmitoleic (4.51%). Altogether 28 fatty acid methyl esters (FAMEs) from 12:0 to 22:2 have been tentatively identified. The majority of them appeared irregularly, sometimes in less than in 10% of cases. The composition of FAMEs in the CSF of patients with lumbar discopathy and with acute ischaemic cerebrovascular accidents does not differ from the control group. A significant difference ($P<0.01$) has been found in the group of hypophyseal adenomas in which the amounts of practically all saturated FAMEs with an odd carbon number are elevated. The same applies to the 18:0 and 20:0 compounds. In the group of atrophic and degenerative CNS processes the palmitic and stearic acids predominated to the detriment of oleic and linoleic acids.

INTRODUCTION

Lipids of the cerebrospinal fluid (CSF) and their analysis have been a relatively less well-known area in liquorology. The lipid concentration in the CSF is several thousand times lower than in the central nervous tissue and several

hundred times lower than in the serum [1]. Apart from the striking quantitative differences the CSF lipids differ from nerve tissue and from the serum in the percentage representation of lipid classes and their subfractions [2]. Free fatty acids (FAs) and phosphatidylethanolamine occur in relatively higher concentrations in the CSF than in the serum [3]; lysophosphatidylcholine is nearly absent from the CSF, and the molar ratio of esterified to free cholesterol is 1:1 [4]. A significant difference has been found between the serum and CSF cholesterol ester pattern [3]. Whereas in the serum cholesterol ester pattern cholesterol linoleate predominates, in cholesterol esters of the CSF saturated and monounsaturated FAs prevail [2,3].

The aim of this study was to ascertain the CSF total FA profile as a preliminary stage in the determination of the FA spectrum in single samples of CSF of classes of lipids other than cholesterol esters.

MATERIALS

CSF obtained by lumbar puncture has been examined in 49 neurological patients (Table I). The control group was formed by 12 patients (4 women, average age 34.5 years, and 8 men of average age 40.8 years) without any sign of organic damage to the CNS, and in whom the CSF analysis corresponds to the criteria for normal composition. In the majority there were vertebrogenic syndromes and different functional disorders of the CNS. The group of vascular cerebral ischaemic accidents was formed by 16 patients with the anamnesis of an acute cerebral accident (11 men with a mean age of 67.5 years, and 5 women of mean age 62.5 years), whose CSF was not sanguinolent and spectrophotometrically correspond to encephalomalacia [5]. Into the group of discopathies, formed by 7 patients (average age 52 years) we classified such patients whose perimyelographic examination showed a prolapse or protrusion of a disc and whose CSF gave a picture of a protein—cytological dissociation with the protein level above 41 mg/100 ml. In the group of degenerative diseases were one patient with an amyotrophic lateral sclerosis, one patient with morbus Aran-Duchene, a case of perinatal encephalopathy with an evolutional anomaly, one case of myelodysplasia, one case of cerebral atrophy and six cases of olivopontocerebellar atrophy. The adenomas of the hypophysis

TABLE I.

BASIC CLINICAL DISORDERS ON GROUPS STUDIED

Group	*n*	Age (years)	Protein (mg/100 ml)
Control	12	36	34.83
Discopathies	7	52	51.29
Vascular disorders	16	70	61.94
Degenerative disorders	11	44	33.66
Hypophyseal adenoma	3	48	47.83

(n=3) were diagnosed by X-ray, and verified through operation. There were two cases of endocrinologically silent adenomas and one case of a prolactin-producing adenoma.

METHODS

A 3-ml volume of centrifuged CSF was extracted with chloroform—methanol (1:1) [6]. The total lipid extract was evaporated to dryness in a stream of nitrogen, dissolved in 0.5 ml of benzene—methylacetate (1:4, v/v) and boiled for 20 min in a water-bath in sealed test-tubes with 4.5 ml of 10—12% boron trifluoride in methanol (w/v). The methylesters (FAMEs) obtained after shaking four times with 5 ml of light petroleum (b.p.<40°) were isolated on a micro-column of silicic acid (1 g of Kieselgel (Merck) 80—120 mesh) and eluted with 5 ml of 2% ethyl ether in light petroleum. After evaporation of the solvent in a stream of nitrogen the residue was dissolved in 20 μl of chloroform and 1—2 μl were injected into the glass column (1.8 m × 4 mm I.D.) of a Perkin Elmer F-30 gas chromatograph. The stationary phase was 13% DEGS on Chromaton N AW DMCS, 0.125—0.160 mm (Lachema, Brno, Czechoslovakia). The carrier gas was nitrogen at a flow-rate of 75 ml/min. The analysis was at 175°; the temperature of the injector block and of the detector was 250°. The recorder was set at 5 mV, the chart speed was 0.5 cm/min. The chromatograms were evaluated by means of an integrator SIP 1. Tentative identification of the individual FAMEs was made from the graphs on the basis of the logarithms of the various retention times compared with those of commercial standards (Serva, Heidelberg, G.F.R.). For the structural identification of FAMEs that were not always present or present only in small quantities, suitable techniques such as mass spectrometry were not available. Calculation of the FAME mass percentage was done without any internal standards or corrections.

RESULTS

Table II gives the reproducibility of the quantitative analysis and the standard deviation (S.D.) of fourteen determinations of a mixture of five standard saturated FAMEs. The chromatogram of the qualitative mixture of the commercial standards is shown in Fig. 1, whereas Fig. 2 shows the chromatogram

TABLE II

THE REPRODUCIBILITY OF THE STANDARD FAME MIXTURE ANALYSIS

FAME	Given	Found	S.D. (n=14)	S.D. (%)
15:0	15.5	15.57	0.601	3.86
16:0	17.2	17.20	0.563	3.27
17:0	19.7	19.56	0.465	3.38
18:0	22.8	22.83	0.568	2.49
19:0	24.8	24.83	0.488	1.97

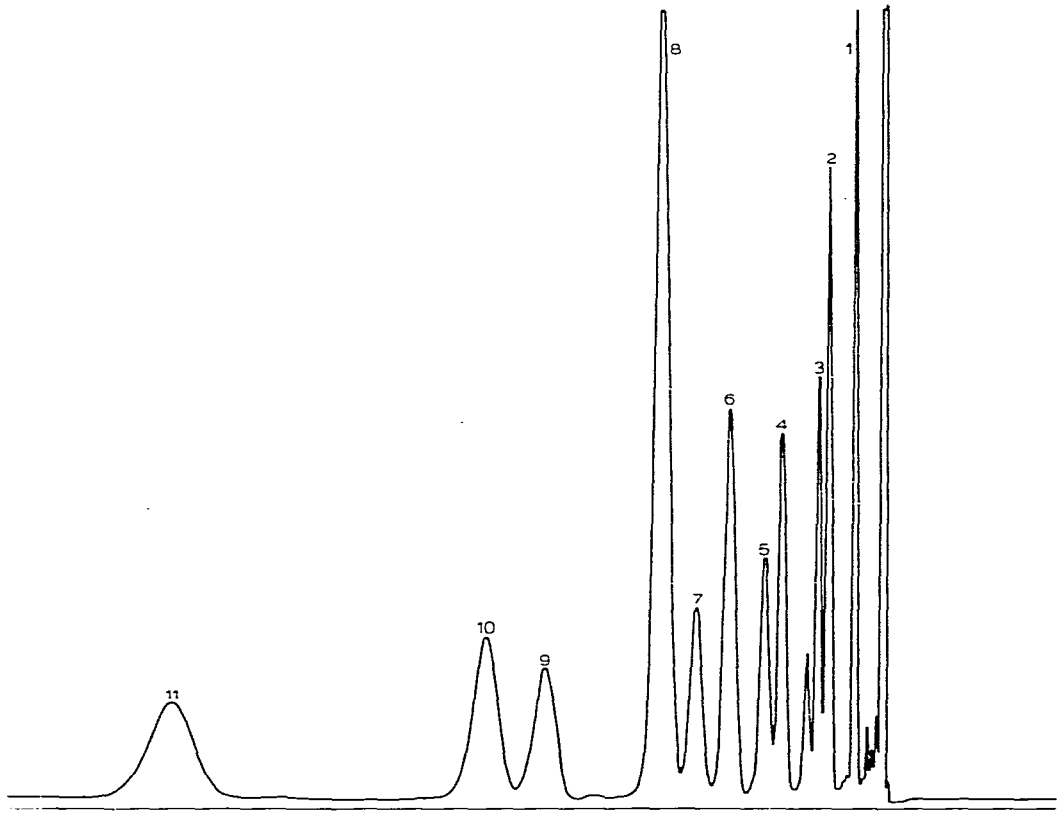

Fig. 1. Chromatogram of the qualitative mixture of commercial standards (Serva, Heidelberg, G.F.R.). 1=14:0, 2=16:0, 3=16:1, 4=18:0, 5=18:1, 6=18:2, 7=20:0, 8=18:3, 9=22:0, 10=22:1, 11=24:1.

of the quantitative commercial mixture of 15:0, 16:0, 17:0, 18:0 and 19:0 standards. Fig. 3 shows the trace of GC analysis of FAMEs in normal CSF. Fig. 4 shows the trace of the GC analysis of FAMEs in CSF from a patient with hypophysical adenoma. A graphic representation of the FAMEs in all examined groups (excluding the discopathies which are identical with the controls) is shown in Fig. 5. The spectrum of FAMEs in the groups under study is given in Table III.

Owing to the large variability and small number of cases it was not possible to use the Student's *t*-test for statistical evaluation. We therefore used the non-parametric sequential test as described by Wilcoxon [7]. The significant values on the 0.05 level are marked ↑ or ↓, those on the 0.01 level by ⇑ or ⇓. We have identified a total of 28 FAMEs. Although the group of ischaemic vascular cerebral accidents does not differ in the distribution of the constantly occurring FAMEs, we have found certain differences in the distribution of short FAMEs; this will be presented elsewhere [8].

The discopathy group does not differ essentially from the controls. The degenerative diseases show a statistically highly significant reduction of 18:1 and 18:2, and an increase in 16:0 and 18:0 and in some odd-numbered FAMEs

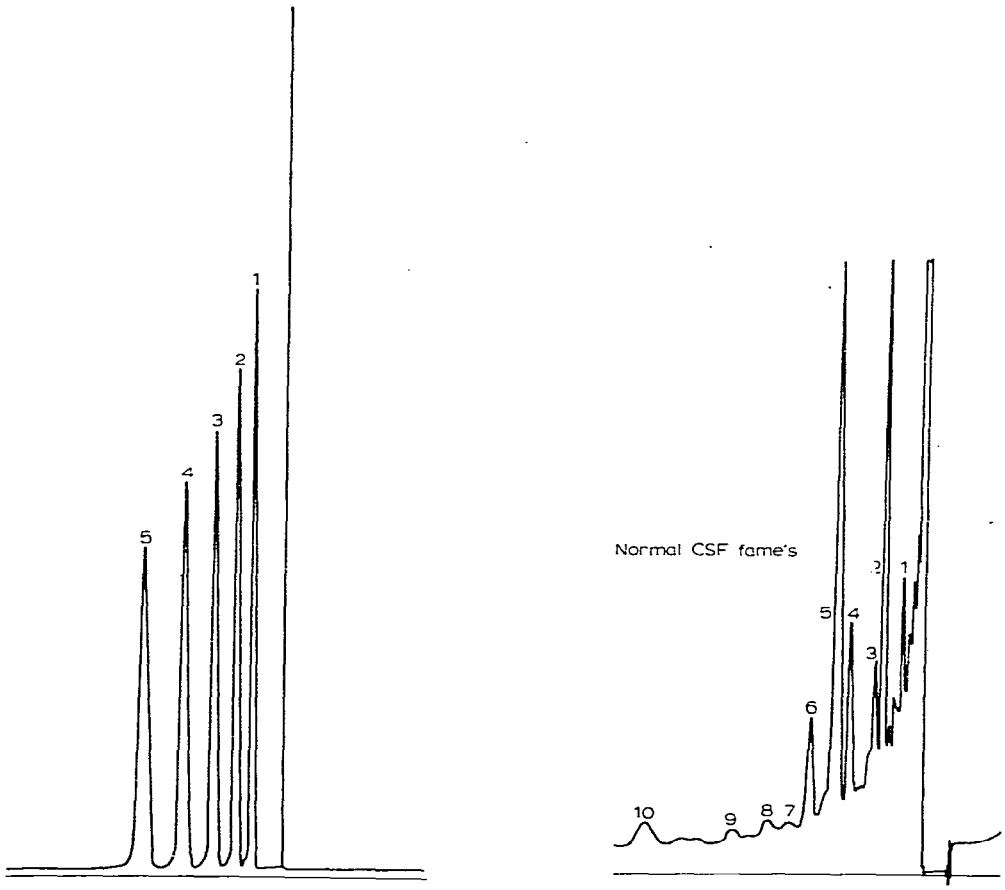

Fig. 2. Chromatogram of 2.5 μg of a quantitative mixture of commercial standards (Applied Science Labs.). 1=15:0, 2=16:0, 3=17:0, 4=18:0 and 5=19:0.

Fig. 3. Chromatogram of a normal CSF. 1=14:0, 2=16:0, 3=16:1, 4=18:0, 6=18:2, 7=20:0, 8=18:3, 9=20:1, 10=22:1.

15:0, 17:0). Still more significant were the changes in FAMEs in the small group of hypophyseal adenomas. There were increases in 15:0, 17:0, 19:0, 20:0 and 21:0 and a marked decrease in 18:1 and 18:2.

The average values presented (Table III) do not reflect, however, the true profile of FAME occurrence, because many FAs do not appear constantly. Fig. 6 shows the frequency of occurrence of the individual FAMEs in the set studied. We can see that the regular occurrence of FAME is proved for the following acids: myristic, palmitic, plamitoleic, stearic, oleic and linoleic acids, which appear in disease groups such as those of the controls, vascular accidents and degenerative diseases. Some FAMEs appear in less than 10% of examined cases.

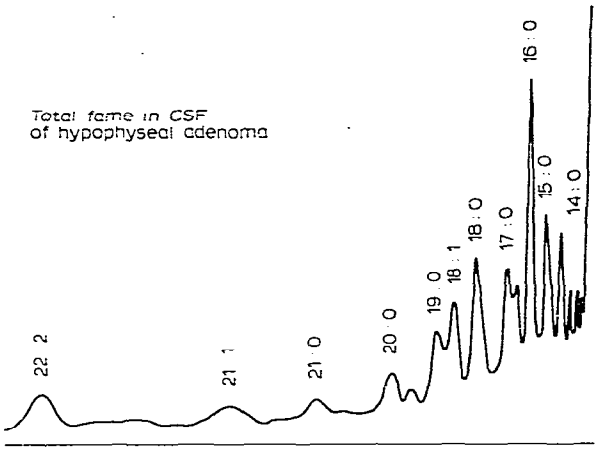

Fig. 4. Chromatogram of CSF total fatty acids from a patient with hypophyseal adenoma.

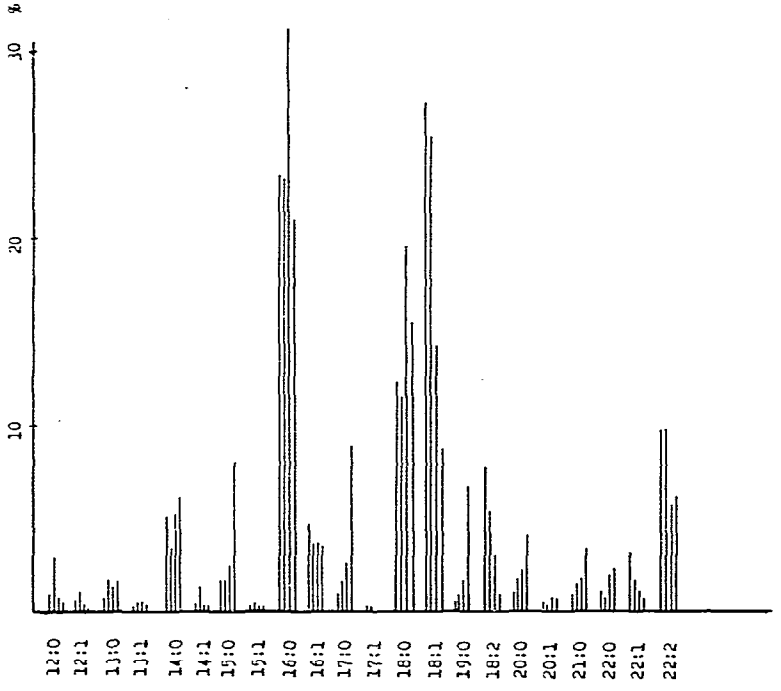

Fig. 5. Relative distribution of FAMEs in four of the groups studied: (from left to right) controls, vascular diseases, degenerative diseases and hypophyseal adenoma.

DISCUSSION

FAMEs for determination by gas—liquid chromatography (GLC) can be prepared by acid- or base-catalysed esterification or transesterification. The methanolic solutions of boron trifluoride and hydrochloric and sulphuric acid form

TABLE III

RELATIVE DISTRIBUTION OF FAMEs IN THE CSF IN CONTROLS AND PATIENTS WITH NEUROLOGICAL DISORDERS

FAME	Controls (n=12)	Discopathies (n=7)	Vascular disorders (n=16)	Degenerative diseases (n=11)	Hypophyseal adenoma (n=3)
12:0	0.87.	1.61	2.89	0.65	0.42
12:1	0.37	0.83	1.66	0.28	0.00
13:0	0.63	0.68	1.61	1.16	1.51
13:1	0.08	0.18	0.31	0.39	0.00
14:0	5.02	4.83	3.38	5.10	6.07
14:1	0.26	0.87	1.19	0.11	0.00
15:0	1.58	1.21	1.53	2.35 ↑	7.87 ⇑
15:1	0.07	0.40	0.28	0.03	0.00
16:0	23.24	19.64	23.51	31.16 ⇑	21.12
16:1	4.51	4.55	3.43	3.59	3.40
17:0	0.83	0.63	1.50	2.59 ⇑	8.67 ⇑
17:1	0.05	0.00	0.08	0.00	0.00
18:0	12.21	9.48	11.44	19.45 ⇑	15.33
18:1	27.28	29.48	25.53	14.33 ⇓	8.56 ⇓
19:0	0.39	0.55	0.72	1.60	6.69 ⇑
19:1	0.00	0.71	0.00	0.00	0.00
18:2	7.66	6.63	5.22	2.89 ⇓	0.65 ⇑
20:0	0.85	0.93	1.55	2.19	4.03 ⇓
18:3	0.03	0.00	0.08	0.00	0.00
20:1	0.29	0.43	0.19	0.50	0.47
20:2	0.00	0.05	0.00	0.00	0.00
21:0	0.58	1.24	1.22	1.56	3.28 ⇑
21:1	0.00	0.09	0.00	0.00	1.39
22:0	0.08	1.08	0.56	1.79	2.17
22:1	2.87	2.33	1.48	0.98	0.70
21:2	0.06	0.67	0.00	0.00	0.00
23:0	0.04	0.00	0.00	0.00	1.56
22:2	9.44	11.02	9.70	5.59	6.06

↑ = $P<0.05$
⇑ = $P<0.01$

on heating FAMEs from free FAs and their esters. Under these conditions the different fractions of lipids react differently. MEs are formed most quickly from free FAs and from trigiycerides, and most slowly from cholesterol esters and sphingomyelins. The mixtures of lipid fractions react according to the proportional representation of the individual components. A higher efficiency can be achieved only by prolonging the heating period, for instance in a sealed ampoule on a boiling water-bath. The longer the heating the higher the probability of forming artefactual compounds: methoxymethylesters of unsaturated FAs and methoxymethylcholesterol. Their quantity is also proportional to the concentration of mineral acid in the methanol [9—12].

The base-catalysed transesterification of FA esters by sodium methoxide proceeds at normal temperatures about 1000 times more quickly than the

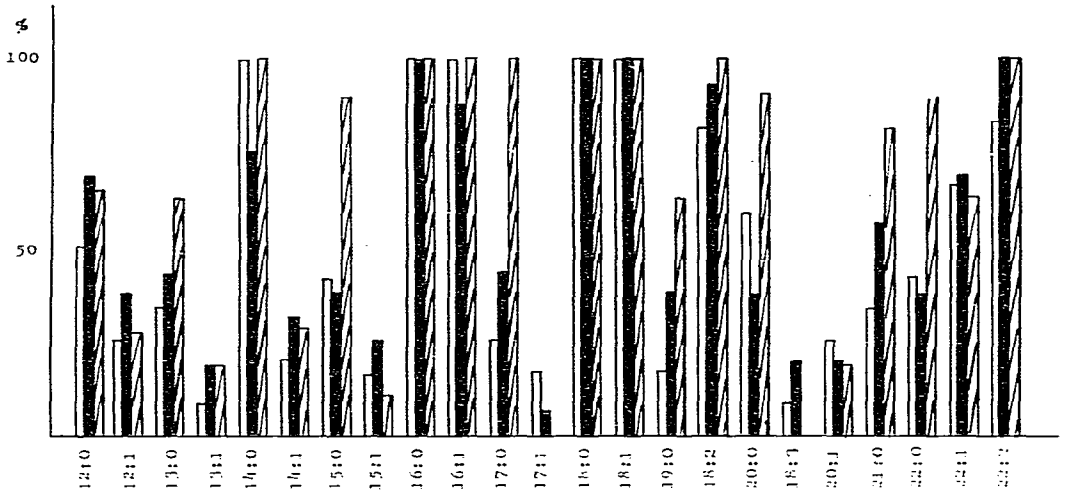

Fig. 6. Frequency of the individual FAMEs of CSF in the groups of controls (▫) and cerebrovascular (▪) and degenerative (▨) disorders.

saponification of these esters. Saponification of the FA esters can be accelerated by raising the temperature in the case of cholesterol esters and sphingomyelins. At temperatures higher than 90° and in alkaline conditions cumulation of isolated double bonds of unsaturated FAs takes place [13].

The data on transmethylation or on the preparation of FAMEs for GLC analysis are very numerous and often contradictory. We can state that if the more gentle transmethylation process is chosen, the more incomplete is the transmethylation, especially of sphingomyelins and cholesterol esters, as well as of FFAs and triglycerides (Fig. 5). If more drastic processes are chosen, especially with a high temperature, artefacts are easily formed (Fig. 7). Boron trifluoride was chosen for the preparation of MEs for its universal character and recommended efficiency especially with FFAs. The transmethylation process has been modified so that all lipids in an environment rich in methyl groups are brought into solution. This has enabled us to use methylacetate with a 20% admixture of benzene as the solvent for lipid extraction prior to transmethylation. The completeness of transmethylation with boron trifluoride depends on the age of the reagent and on the quality of the methanol. The reaction does not run satisfactorily if the environment is not completely water-free.

Comparison of the results of different laboratories that have used different methods of transmethylation is always problematic, particularly for the different preparatory procedure of FAMEs. Blomstrand et al. [14] were the first to study the FA spectrum in liquor lipids by menas of GLC. They ascertained that, contrary to serum, the CSF contains predominantly palmitic and oleic acids. We have ascertained, first by paper chromatography [15] and later by thin-layer chromatography [2,3] that in the CSF cholesterol esters predominate over saturated and monounsaturated fatty acids, contrary to the serum, in which cholesterol linoleate is the main ester. Tuna et al. [16] compared the composition of FAs in serum and liquor. Fourteen FAMEs were determined by

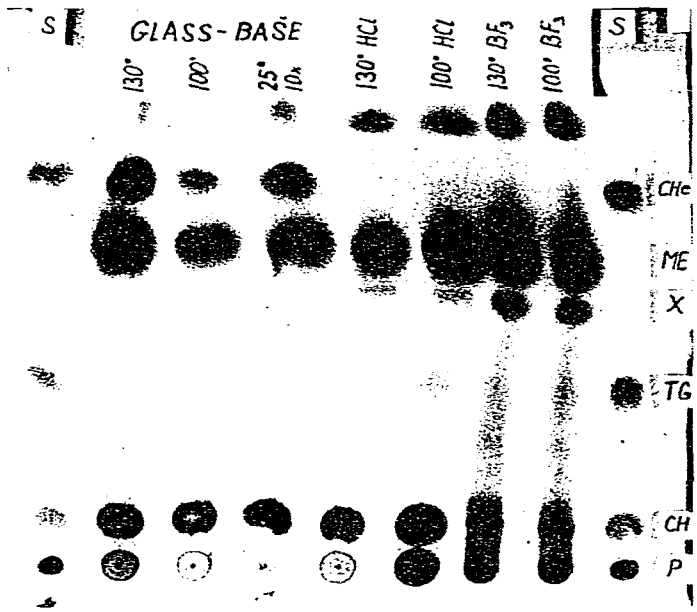

Fig. 7. Thin-layer chromatography of different procedures for preparing methyl esters from serum total lipids. Silica gel G; solvent, *n*-heptane—ethyl ether—acetic acid (85:15:1, v/v); detection by charring after $(NH_4)_2SO_4$ spray. CHe= esterified cholesterol, ME= methylesters, TG= triglycerides, CH= free cholesterol, P= phospholipids, X= artefacts (methoxymethylesters?).

means of GLC. The acids of highest concentration in the liquor were verified as palmitic, oleic, palmitoleic and stearic acids. Polyenoic FAs showed very low concentrations: linoleic acid approximately 4%, and arachidonic acid approximately 1.5%. The same authors found no substantial differences when comparing the FA spectrum in demyelinating diseases with that in controls. In the degenerative diseases they found a similar spectrum to that of the controls (Tuna et al. [17]). The composition of FAs in liquor has been treated in various papers by Farstad and Skaug [18]. They found that the highest concentration is of 18:1 (22.8—43.8%), and a constant further occurrence of 14:0 (2.6—7.7%), 16:0 (9.7—18.7%) and 18:0 (4.2—9.0%). Of the remaining FAs the following showed a measurable concentration: 14:1, 15:0, 15:1, 17:0, 18:2 and 18:3. The identification of single FAs, especially of the critical pairs 16:0—16:1 and 18:0—18:1, is not convincing enough, however. Moreover, at the end of the chromatogram a high fraction occurs, characterized as 22:6, which the author [19] identified later as an artefactual one. In our chromatograms as well, obviously owing to the drastic transmethylation ratio by 10—12% boron trifluoride, artefactual MEs with cumulated double bonds appeared.

Farstad's paper [19] established, by identification of 15 different FAs, that the physiological oscillation of individual FAs in the CSF is enormous. The importance of their determination in different neurological and psychiatric diseases has also been evaluated by Farstad in a subsequent paper [20]. He evaluated the aliphatic FAMEs up to 22:0 in 84 patients but he did not identify the branched ones. Each diagnostic group has shown a significant variation

of values for individual FAMEs. It was not possible to ascertain a significant change of individual fatty acids in any of the examined groups of psychoneuroses, headaches, muscle diseases, psychoses, intra cerebral tumours, epilepsy and encephalopathy. Oleic and linoleic acids were lower in the liquor than in the serum. However, myristic acid was five times more frequent in the CSF, comprising 6—9%. Seidel and Lindlar [21] found a striking and surprisingly high percentage of myristic acid in all examined groups of liquor lipids: in cholesterol esters over 21%, in triglycerides over 17%, and in phosphatides over 25%. However, oleic acid was surprisingly depressed; in all examined lipids it formed approximately only 10%. We have examined the spectrum of total FAs in the CSF of 100 neurological patients. In a number of cases the FAME spectrum differed significantly; a detailed report is being presented elsewhere (Skorkovská et al. [22]).

Sastry and Stancer [23] who studied the FAME pattern in isolated fractions of phospholipids (lecithins, phosphatidylethanolamines, sphingomyelins) in children, concluded that the main acid in lecithins is palmitic acid (44.3%); stearic acid amounted to 16.5% and oleic acid to 14.1%. Myristic acid only accounted for 2.7%. In the phosphatidylethanolamines there was 37.5% of palmitic acid and 25.1% of stearic acid. In sphingomyelins palmitic acid contributed 30.6% and stearic acid 35.1%. The oleic acid in phosphatidylethanolamines formed 6.2% and in sphingomyelins 2.2%.

In 1970 we compared the FA profiles of some main classes of lipids in the CSF of healthy persons and of chronic alcoholics. We studied the spectrum of cholesterol esters, triglycerides, phosphatidylethanolamines and phosphatidyl cholines by means of GLC after their prior separation on thin layers of silica gel G. Owing to a small concentration of lipids it was necessary to work with pooled liquor samples from six healthy and nine alcoholic subjects. Each class of lipids studied showed its own specific spectrum. The distribution of FAMEs in triglycerides and in cholesterol esters is shown in Table IV.

In the spectrum of phosphatidylcholines palmitic and oleic acids predomi-

TABLE IV

FATTY ACID COMPOSITION OF CHOLESTERYL ESTERS AND TRIGLYCERIDES IN CSF AND SERUM (S) OF CONTROLS

Fatty acid	Cholesteryl esters		Triglycerides	
	CSF	S	CSF	S
16:0	23.2	11.0	41.9	24.8
16:1	11.2	3.9	5.2	5.2
18:0	0.6	1.0	14.1	5.1
18:1	37.0	20.9	27.9	36.9
18:2(*n*-6)	21.4	53.4	2.8	14.3
18:3(*n*-6)	0.2	0.5	0.5	0.3
18:3(*n*-3) + 20:1	0.4	1.0	0.7	3.2
20:3(*n*-6)	0.3	0.5	0.2	0.6
20:4(*n*-6)	4.7	5.2	4.6	3.6
22:6(*n*-3)	0.4	0.7	1.7	2.0

nated, together comprising more than 70% of all the FAMEs. In the FAME spectrum of the phosphatidylethanolamines a high percentage of 20:6 and 22:6 has been found (Tichý et al. [24]). Arnetoli et al. [25] determined the FAs in CSF by GLC. In agreement with other authors they found the following highest concentrations for the acids: 16:0 (36.21%), 18:1 (23.74%), 16:1 (6.6%) and 18:2 (4.52%). Amaducci et al. [26] shared their experience in examining liquors of more than 300 neurological patients. They found a considerable variability, especially in the percentage representation of 18:2. Its increase was related to the reduction in 14:0. Owing to the fact that both FAMEs have quite different concentrations in CSF and serum, the authors came to the conclusion that the change in the amounts of these acids in liquor may well demonstrate damage to blood—brain barrier (in this case the linoleic acid content increases). A comprehensive survey of the problems of CSF lipids and FAs has been made by Pilz [1] in his monograph.

The group which we studied is a small one and not very homogeneous. In the control group particularly, minority fractions show considerable variation, resulting in a lack of statistical significance of some deviations found. The FAME spectrum in discopathies with increased proteinosis and in the acute ischaemic cerebrovascular accidents do not differ either in the main FAs or in those acids which appear in the CSF only irregularly. The most striking finding appears to be the statistically significant increase in the odd-numbered FAMEs, 15:0, 17:0, 19:0 and 21:0, in three cases of hypophyseal adenoma, where the increase is compensated by a statistically significant decrease in 18:1 and 18:2. The insufficiently homogeneous group of degenerative processes showed an increase in 16:0 and 18:0 to the debit of 18:1 and 18:2. All charges were statistically highly significant.

It would be premature to try to explain these findings. It remains a fact that in the CSF there are some changes in the pattern of total FAs in some neurological disorders.

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