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High-performance liquid chromatographic determination of cholesteryl esters in the blood of obese children

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ABSTRACT

The serum of obese children and adolescents was analyzed for cholesteryl esters. The test substances were first separated from the sample matrix by solvent extraction and thin-layer chromatography and then resolved in a reversed-phase high-performance liquid chromatographic system involving a Separon SGX C_{18} column and a mobile phase of 2-propanol–acetonitrile (40:60, v/v), with ultraviolet detection at 206 nm. Cholesterol and 10-cholesteryl esters could be separated and determined within *ca.* 25 min at a flow-rate of 1 ml/min. The method was applied to a study of the effect of external conditions (physical stress, diet) on the content of cholesteryl esters in a test group of obese boys and girls aged from 13 to 16 years. The analyses have demonstrated that the above conditions do not affect the concentrations of the individual cholesteryl esters, although the total cholesterol concentration decreased significantly after spa treatment.

INTRODUCTION

Obesity is a common disorder in affluent countries, and a risk factor contributing to various diseases. Therefore, the study of lipids and their metabolism is of prime importance. Determination of the overall lipid content or the contents of the main components was, until recently, satisfactory for clinical purposes. However, it is now clear that this rather rough analysis is insufficient and that it is necessary to follow individual classes of lipids, and their composition and metabolism, and quantitatively determine their components. This task can no longer be solved by classical, routine biochemical methods, including the enzymic procedures, and more powerful analytical methods must be used. Chromatographic methods often meet the requirements: thin-layer chromatography (TLC) [1] and

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gas chromatography [2] have traditionally been used, and the application of high-performance liquid chromatography (HPLC) is more recent [3–22]. HPLC methods have been developed for the determination of, for example, triacylglycerols, free fatty acids and phospholipids or free and esterified cholesterol in blood [3–6], milk [8], lymph [9], amniotic fluid [10], peritoneal macrophages [7] and tissues [11–13]. To our knowledge, there have been no studies of individual cholesteryl esters in the serum of obese children.

Reversed-phase HPLC systems are mostly used for this type of analysis [6,14,15]. Mass spectrometry is useful for identification purposes [6,16,17]. UV detection [3,6,18] is used or the fluorescence intensity is measured after derivatization [6,19]. Various combinations of solvents are used as the mobile phases [20,21]. The relationship of the solute retention time to the number of double bonds in its molecule and to the *cis/trans* isomerism of the esters was also studied [20].

We dealt with obesity of children in our previous paper [16], following the composition, content and movement of phospholipids in blood under various external conditions. This paper describes the content and movement of cholesteryl esters under the same conditions.

EXPERIMENTAL

Apparatus

The HPLC measurements were performed on the Gilson gradient analytical system (Villiers-le-Bel, France). Samples were injected using a Rheodyne 7125 valve (Cotati, CA, USA) with a 20-μl loop. A Separon SGX C_{18} glass analytical column (150 mm × 3 mm I.D., 7 μm particle size, Tessek, Prague, Czechoslovakia) was preceded by a guard column (70 mm × 2.1 mm I.D., 7 μm) packed with Whatman ODS phase. A Gilson Holochrome UV detector was used at 206 nm.

The elution was carried out isocratically with 2-propanol–acetonitrile (40:60, v/v) at a flow-rate of 1 ml/min and a temperature of 20 ± 2°C.

Chemicals

The standard cholesteryl esters were obtained from Serva (Heidelberg, Germany), the HPLC-pure acetonitrile and 2-propanol from Aldrich (Milwaukee, WI, USA). Methanol and chloroform, reagent-grade, were supplied by Lachema (Brno, Czechoslovakia). All the other chemicals were of reagent-grade purity, from Lachema. The chloroform was dried and then purified by rectification. Methanol was also rectified.

Stock solutions of cholesteryl acetate, linolenate, laurate and linoleate were prepared by dissolving 1 mg of the substance in 1 ml of 2-propanol. Cholesteryl myristate, oleate, palmitate, heptadecanoate, stearate and arachidate were poorly soluble in 2-propanol alone, and thus their stock solutions were prepared by dissolving 1 mg of the substance in 2-propanol–dietyl ether (5:2, v/v). All the

TABLE I

TEST GROUP OF OBESE CHILDREN

Patient	Age	Weight (kg)				Total cholesterol content in serum (mg/ml)		
		Before therapy	After therapy	Difference		Before therapy		After therapy
				kg	%	At rest	After exercise	At rest
Boys (n = 4)								
1	13	61.0	56.2	4.8	7.9	5.17	5.13	4.26
2	13	59.9	51.8	8.1	13.7	5.07	5.07	3.67
3	14	78.0	69.0	9.0	11.5	3.65	3.50	3.46
4	14	74.7	70.4	4.3	6.1	4.33	4.31	3.93
Mean		68.4	61.9	6.6	9.8	4.56	4.50	3.83
Girls (n=6)								
1	13	52.2	47.2	5.0	9.1	4.06	4.89	–
2	14	85.6	79.4	6.2	7.7	3.40	2.91	3.62
3	16	108.0	104.0	4.0	3.7	4.66	4.36	3.25
4	14	72.3	67.0	5.3	6.9	2.86	2.88	–
5	13	66.5	60.2	6.3	10.1	4.54	4.74	2.99
6	15	87.8	82.9	4.9	5.8	3.40	3.60	2.90
Mean		78.7	73.5	5.3	7.2	3.82	3.90	3.19
Total test group								
Mean		74.6	68.8	5.8	8.3	4.11	4.14	3.51
S.D.		16.3	16.8	1.6	2.9	0.77	0.86	0.46

stock solutions were stored at −24°C and were appropriately diluted with the solvent(s) used for their preparation immediately before use.

Sample pretreatment

A group of four boys and six girls with various degrees of obesity was randomly selected (Table I). The cholesterol content values refer to the state prior to treatment for obesity, after a physical stress and after the treatment. The children were hospitalized for the purpose at the IVth Children Clinic, Charles University (Prague, Czechoslovakia). After fourteen days at the hospital, with a diet of 5 MJ per day and increased physical exercise, the patients were transferred to a spa with the same diet and physical exercise. Blood samples were taken before the treatment and after it, when the patients were at rest after a three-day special diet.

The effect of physical stress was studied with the same group of children. The patients underwent a test on a bicycle ergometer with variable load, in three 6-min periods separated by a 1-min rest. The load was selected individually, and 90% of the maximum was attained in the last minute. Blood samples were taken at rest prior to the ergometric test and 5 min after completion of the rest.

The serum obtained was always immediately frozen and stored at −24°C until analysis.

Lipid extraction

A 1-ml sample of plasma was shaken for 20 min with 20 ml of freshly prepared chloroform–methanol (2:1, v/v). The mixture was then filtered into a separating funnel, the vessel was rinsed with three 4-ml portions of the extraction mixture, and 7 ml of a 5% sodium chloride solution were added to the combined filtrates. The mixture was shaken in the funnel and allowed to stand for 3 h at laboratory temperature. The bottom layer was drained into a flask, and 10 ml of the extraction mixture were added to the layer remaining in the funnel. The mixture was allowed to stand for 1 h, and the bottom layer was again drained into the flask and the combined extracts were evaporated to dryness *in vacuo*. The raw lipids prepared in this way are stable for up to three months at −24°C.

TLC fractionation

Cholesteryl esters were isolated on a silica gel thin-layer plate (Merck silica gel H + $HF_{254+366}$ plate, 1:1, w/w, 250 μm), using the procedure of Mareš *et al.* [23]. The plates were activated by heating for 30 min at 110°C. The raw lipid residue obtained from the extraction was dissolved in 500 ml of methanol. A 250-μl aliquot was applied to the activated plate, and the chromatogram was developed with *n*-heptane–diethyl ether–acetic acid (84:15:1, v/v). The plate was dried, and the cholesteryl esters detected under a UV lamp, mechanically removed and transferred to a column containing Florosil (60–100 mesh) and silica gel (Merck, 70–325 mesh) at a ratio of 1:1 (w/w). The cholesteryl esters were eluted from the column with three 2-ml portions of chloroform–methanol (1:1, v/v). The solvents were then evaporated *in vacuo*, the dry residue was dissolved in 250 μl of 2-propanol–diethyl ether, (5:2, v/v) and a 20-μl aliquot was injected into the HPLC system.

RESULTS AND DISCUSSION

This study of cholesteryl esters in the blood of obese children is based on the data provided by HPLC analyses preceded by a TLC fractionation. Our procedure is derived from published works (for the references see the Introduction) and optimized for our particular conditions.

Of a number of mobile phases tested, 2-propanol–acetonitrile (40:60, v/v) permits the best separation of cholesteryl esters. The resolution of the individual esters improves with increasing acetonitrile content, but the retention increases. An increase in the flow-rate decreases the retention time, but the peak area becomes smaller (the peak height, however, remains constant). The above composition of the mobile phase and a flow-rate of 1 ml/min, represent the best compromise.

TABLE II

CAPACITY RATIOS OF THE SUBSTANCES STUDIED

For conditions, see Experimental.

Substance	Capacity ratio, k'
Cholesterol	4.9
Cholesteryl acetate	4.9
Cholesteryl linolenate	11.9
Cholesteryl laurate	13.1
Cholesteryl linoleate	14.6
Cholesteryl myristate	17.0
Cholesteryl oleate	20.0
Cholesteryl palmitate	22.3
Cholesteryl heptadecanoate	25.7
Cholesteryl stearate	29.6
Cholesteryl arachidate	39.5

As indicated in Table II, the capacity ratios of the esters depend on the chain length of the corresponding bound fatty acids and the number of multiple bonds in their molecules. The smaller the carbon number of the bound fatty acid, and the greater the number of double bonds, the lower is the capacity ratio of the ester. This is in agreement with the theory of reversed-phase HPLC [20].

The test substances absorb radiation at very low wavelengths and thus high demands are placed on the purity of the mobile phase (2-propanol–acetonitrile). We observed that UV detection became impossible at wavelengths below 205 nm. Therefore, we used a wavelength of 206 nm in all the measurements.

The dependence of the peak area on the amount of solute injected was measured for cholesterol. It was linear from *ca.* 0.3 μg to the highest tested amount, 51 μg in a volume of 20 μl. The regression straight line has a correlation coefficient of 0.9995 and the limit of detection, calculated as four times the standard deviation of the regression plot divided by the sensitivity (the slope of the plot), equals 0.32 μg per injected volume.

The sensitivity of detection of cholesteryl esters increases with increasing number of double bonds in the molecule (linolenate $\geqslant$ linoleate $>$ oleate), as can be seen from Fig. 1: *e.g.*, the detection limit for cholesteryl linolenate is 50 ng. For this reason it is impossible to use an internal standard and a calibration plot must be measured for each ester. The reproducibility of determination is then satisfactory. Typical relative standard deviations are 8.0% for 1.3 μg, 3.6% for 10.2 μg and 1.6% for 38.2 μg. Fig. 1 depicts an analysis of a mixture of ten standard cholesteryl esters: their capacity ratios are listed in Table II. It is evident that cholesterol cannot be separated from cholesteryl acetate using this procedure.

The peaks were identified on the basis of matching their retention times to those of the standards. It was shown by gas chromatographic and mass spectro-

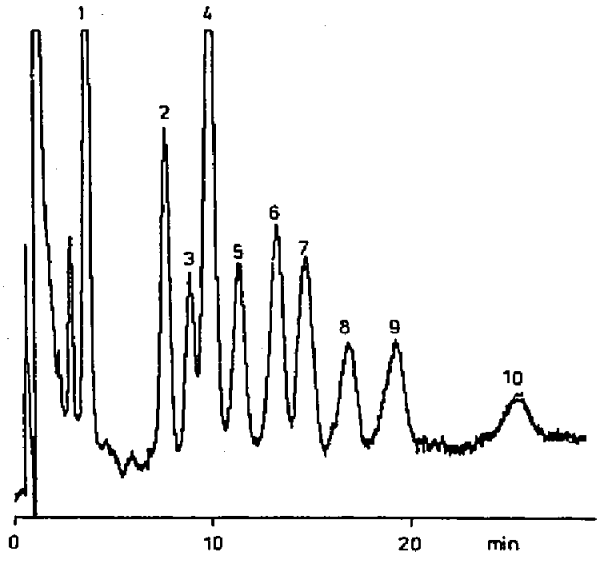

Fig. 1. Chromatogram of cholesteryl ester standard substances (20-μg amounts). For conditions see Experimental. Peaks: 1 = cholesterol + cholesteryl acetate; 2 = cholesteryl linolenate; 3 = cholesteryl laurate; 4 = cholesteryl linoleate; 5 = cholesteryl myristate; 6 = cholesteryl oleate; 7 = cholesteryl palmitate; 8 = cholesteryl heptadecanoate; 9 = cholesteryl stearate; 10 = cholesteryl arachidate.

metric measurements that the sample pretreatment described above excludes all possible interferences, and thus the peak assignment can be considered unambiguous.

Small amounts of phospholipids transferred into the sample during the blood treatment do not interfere, as they elute with the solvent front. As pointed out by

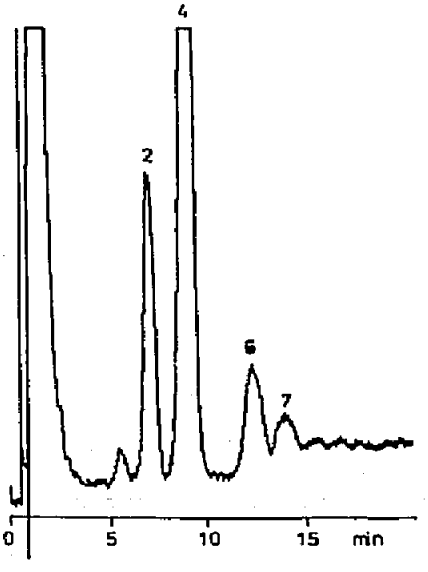

Fig. 2. Analysis of a blood sample. For the procedure and conditions see Experimental. Peaks as in Fig. 1; amounts: 2, 1.20 μg; 4, 6.94 μg; 6, 3.51 μg; 7, 1.83 μg.

Duncan *et al.* [3], some triacylglycerols, *e.g.* trilaurin, trimyristin, etc., exhibit retention times very similar to those of cholesteryl esters. However, this interference is prevented by the TLC separation described above.

Fig. 2 depicts a typical chromatogram of a serum sample pretreated as described. The peak with the retention time of 5.47 min is probably cholesteryl arachidonate; however, the identification is uncertain because the standard substance was not available, and therefore its area was not evaluated in further experiments.

Application of the method

The results summarized in Tables III and IV were obtained by the method described. Table III lists the changes in the cholesteryl linolenate, linoleate, oleate and palmitate content in the blood of the test group after physical stress.

The cholesteryl linolenate concentration in the blood of boys amounted, on average, to 96.2 μg/g in the absence of physical stress, which is 8.03% of the total cholesterol concentration. Physical stress did not cause a great change in its absolute amount, but its percentage decreased. The blood of girls contained, on average, 81.5 μg/g of this substance, which is 6.15% of the total cholesterol concentration. In contrast to the boys, physical stress caused in the girls a decrease in both the percentage and the absolute amount of cholesteryl linolenate. The average values in the whole group (*i.e.* boys and girls) slightly decreased after physical stress, both the absolute amount and the relative content.

The results for cholesteryl linoleate were similar. With the boys, there was a decrease in both the amount and the percentage after physical stress: in the girls, the absolute amount decreased and the percentage was virtually unchanged. The trend for cholesteryl oleate and palmitate was opposite with the boys: physical stress led to an increase in both the absolute amounts and percentages of the substances. In the girls, the amount of cholesteryl oleate was unchanged and its percentage increased, while both the values decreased for cholesteryl palmitate. The content of cholesteryl oleate and palmitate generally slightly increased after physical stress.

The weight of all the patients decreased after the clinical and spa therapy and the total cholesterol content in their blood decreased (Table I). The values for the cholesteryl esters studied after the spa treatment are given in Table IV. The content of cholesteryl linolenate remained virtually unchanged in the boys, whereas both its amount and percentage slightly increased in the girls and, on average, in the whole test group. The amount of cholesteryl linoleate increased in the boys, but its percentage remained constant: there was virtually no change in the girls or in the test group as a whole. The amount of cholesteryl oleate in the boys was unchanged after the spa treatment and its percentage slightly decreased: both these values mildly increased in the girls, although the whole test group exhibited very little change.

The content and percentage of cholesteryl palmitate increased in the boys and

TABLE III

EFFECT OF PHYSICAL STRESS ON THE CHOLESTERYL ESTER CONTENT IN THE SERUM

Patient	Linolenate				Linoleate				Oleate				Palmitate			
	Rest		Exercise		Rest		Exercise		Rest		Exercise		Rest		Exercise	
	µg/ml	%	µg/ml	%	µg/ml	%	µg/ml	%	µg/ml	%	µg/ml	%	µg/ml	%	µg/ml	%
Boys (n=4)																
1	62.1	5.62	64.0	5.19	611.7	55.37	615.9	49.92	280.0	25.35	349.1	28.29	150.9	13.66	204.8	16.60
2	118.0	9.82	108.0	9.61	682.2	56.76	552.1	49.12	265.8	22.12	297.1	26.43	136.8	11.30	166.8	14.84
3	88.0	7.38	92.3	7.24	626.9	52.60	652.1	51.19	307.4	25.79	321.0	25.18	169.5	14.22	209.4	16.43
4	116.6	9.29	119.5	8.89	643.2	51.26	682.4	50.76	324.0	25.82	354.6	26.37	170.9	13.62	188.1	13.99
Mean	96.2	8.03	96.0	7.73	641.0	54.00	625.6	50.24	294.3	24.77	330.5	26.57	156.8	13.20	192.3	15.47
Girls (n=6)																
1	52.0	4.10	52.4	4.15	783.0	61.75	760.4	60.16	288.6	22.76	302.1	23.90	144.4	11.39	149.1	11.80
2	107.0	7.36	109.0	8.04	764.9	52.65	754.3	55.66	377.6	25.99	339.5	25.05	203.4	14.00	152.4	11.25
3	56.6	4.71	49.5	4.12	665.1	55.37	660.0	54.97	328.9	27.38	330.5	27.53	150.6	12.54	160.6	13.38
4	49.8	3.97	41.6	3.42	737.1	58.75	705.3	57.93	325.6	25.95	329.8	27.09	142.1	11.33	140.9	11.57
5	107.1	8.40	94.0	7.12	637.2	49.95	665.3	50.39	392.7	30.79	390.9	29.61	138.6	10.87	170.1	12.88
6	116.5	8.35	103.6	8.17	776.2	55.65	709.7	55.96	309.1	22.16	317.2	25.01	193.0	13.84	137.8	10.86
Mean	81.5	6.15	75.0	5.84	727.3	55.69	709.2	55.85	337.1	22.85	335.0	26.37	162.0	12.33	151.8	11.96
Total test group																
Mean	87.4	6.90	83.4	6.60	692.8	55.08	675.8	53.60	320.0	25.41	333.2	26.45	159.8	12.68	168.0	13.36
S.D.	29.2	2.16	28.7	2.20	66.3	3.56	65.2	3.82	40.2	2.63	27.4	1.74	23.5	1.33	25.3	2.08

TABLE IV

EFFECT OF CLINICAL AND SPA THERAPY ON THE CHOLESTEROL ESTER CONTENTS IN THE SERUM

Patient	Linolenate				Linoleate				Oleate				Palmitate			
	Before cure		After cure		Before cure		After cure		Before cure		After cure		Before cure		After cure	
	μg/ml	%	μg/ml	%	μg/ml	%	μg/ml	%	μg/ml	%	μg/ml	%	μg/ml	%	μg/ml	%
Boys (n=4)																
1	61.1	5.62	63.0	5.08	611.7	55.37	708.1	57.05	280.0	25.35	289.7	23.34	150.9	13.66	180.4	14.53
2	118.0	5.82	114.0	9.93	682.2	56.76	658.2	57.32	265.8	22.12	232.5	20.25	135.8	11.30	143.6	12.51
3	88.0	7.38	89.0	7.28	626.9	52.60	644.4	52.69	307.4	25.79	310.8	25.41	169.5	14.22	178.9	14.63
4	116.6	9.29	119.8	8.89	643.2	51.26	694.1	51.52	324.0	25.82	350.5	26.02	170.9	13.62	182.8	13.57
Mean	96.2	8.03	96.5	7.80	641.0	54.00	676.2	54.65	294.3	24.77	295.9	23.76	156.8	13.20	171.4	13.81
Girls (n=6)																
1	52.0	4.10	90.0	6.94	783.0	61.75	770.2	59.36	288.6	22.76	296.4	22.84	144.4	11.39	140.9	10.86
2	107.0	7.36	104.5	7.15	764.9	52.65	749.6	51.27	377.6	25.99	384.6	26.30	203.4	14.00	223.5	15.29
3	56.6	4.71	82.1	6.55	665.1	55.37	682.8	54.44	328.9	27.38	335.4	26.74	150.6	12.54	153.9	12.27
4	49.8	3.97	61.2	4.86	737.1	58.75	729.4	57.97	325.6	25.95	336.4	26.73	142.1	11.33	131.3	10.43
5	107.1	8.40	98.8	7.75	637.2	49.95	639.4	50.16	392.7	30.79	394.2	30.92	138.6	10.87	142.3	11.16
6	116.5	8.35	104.8	7.65	776.2	55.65	752.5	54.89	309.1	22.16	358.1	26.12	193.0	13.84	155.4	11.34
Mean	81.5	6.15	90.2	6.82	727.3	55.69	720.7	54.68	337.1	25.84	350.9	26.61	162.0	12.33	157.9	11.89
Total test group																
Mean	87.4	6.90	92.7	7.21	692.8	55.01	702.9	54.67	320.0	25.41	328.9	25.47	159.8	12.68	163.3	12.66
S.D.	29.2	2.16	19.8	1.54	66.3	3.56	46.9	3.19	40.2	2.63	48.3	2.85	23.5	1.33	28.0	1.75

decreased in the girls. The average of the whole test group remained constant.

It can be concluded that physical stress and clinical and spa treatment did not significantly alter the amounts and percentages of the studied cholesteryl esters in the test group. The total cholesterol amount was not changed after a short-time physical stress, but it significantly decreased after the spa treatment, especially in the boys.

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