THE LIQUID CHROMATOGRAPHY OF CHOLESTEROL ESTERS

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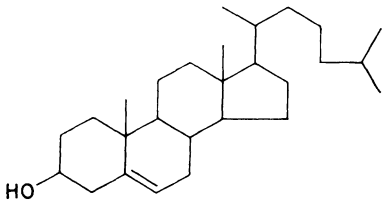
The retention characteristics were found for cholesterol and 19 of its esters with saturated and unsaturated carboxylic acids containing 1 to 22 carbon atoms using high performance liquid chromatography in the normal and reversed-phase systems. HPLC on silica gel in a mobile phase consisting of heptane and 2-propanol or ethyl acetate is especially suitable for analysis of the esters of the lower acids, while chromatography on an octadecyl silica gel sorbent in a water–tetrahydrofuran or acetonitrile–dichloromethane mobile phase can be successfully used for the separation of esters with acids containing 10—22 carbon atoms, that can differ in the presence of double bonds in the acyl or through *cis–trans* isomerism.

Cholesterol (cholest-5-en-3β-ol, *I*) and its esters with carboxylic acids constitute a group of compounds that is important in biology and technology. Primarily, it is well known that cholesterol and its esters form an important component of animal fats; technical applications include thermography and the optical properties of cholesterol-based liquid crystals, employed as temperature indicators.



I

High performance liquid chromatography is an analytical and separation method that is suitable for the determination of these high-boiling compounds. Compared with thin-layer chromatography, it is faster, has higher separation efficiency and more sensitive detection, and, compared to gas chromatography, more volatile derivatives of these substances need not be prepared. It can also be used to separate stereoisomers[1] and compounds differing in the number and positions of the double bonds in the molecule[2,3].

The literature contains a number of works in which HPLC was employed to determine cholesterol and its esters in animal tissues, blood, milk, etc.; however, mostly only selected esters or a narrow group of these compounds was considered. Systematic works dealing with the HPLC of cholesterol derivatives include the study of the behaviour of these substances on normal phases[4-6], the HPLC of cholesterol esters on reversed phases in nonaqueous eluents[3,7] and evaluation of the strategy of the determination of cholesterol and its esters in lipoproteins using HPLC on normal and reversed phases[8].

In a system with normal phases, the column packing usually consists of unmodified silica gel, or exceptionally an amine[9] or cyanogen[10] phase. Useful information on the behaviour of various cholesterol derivatives on silica gel in 30 various mobile phases can be found in a series of articles by Japanese authors[4-6]. Work with silica gel sorbents has the disadvantage that the retention of the analyzed substances depends on the water content in the mobile and stationary phases; this has also been found for the HPLC of cholesterol esters[11].

This factor no longer presents a problem in reversed-phase chromatography. Octadecyl silica gel is one of the most important sorbents for this method and the choice of mobile phase greatly depends on the number of carbons (n_c) in the esterified acid and thus on the solubility of the esters. The determination of esters with lower acids can be carried out using aqueous eluents, while it is preferable to separate the esters of higher acids in nonaqueous mobile phases. Tetrahydrofuran[12] or a mixture of methanol with propanol[13] have been found to be suitable components of the aqueous phase; however, most workers have employed nonaqueous mobile phases. These have often contained acetonitrile and 2-propanol[14-16], tetrahydrofuran and chloroform[7] or acetonitrile[17] or some other solvents[18].

This work is devoted to a study of the chromatographic behaviour of a homologous series of cholesterol esters with carboxylic acids with one to 22 carbon atoms in systems with normal and reversed phases, in aqueous and nonaqueous eluents, with refractometric detection.

EXPERIMENTAL

Instruments and Chemicals

The liquid chromatograph consisted of the high-pressure minipump MC 706 (Mikrotechna, Prague), refractometric detector RIDK 101, injection equipment LCI 20 and double pen recorder TZ 4200 (all from Laboratorní přístroje, Prague). Three chromatographic columns were employed. The first (200 × 5·5 mm) was packed with Silasorb C18, 10 μm, $V_M = 3{\cdot}1$ ml; the second (250 × 4 mm) was packed with Silasorb 300 SPH C18, 8·5 μm, $V_M = 2{\cdot}7$ ml (both sorbents from Lachema, Brno). In the former case, the specific surface area of the initial silica gel was 248 $m^2\ g^{-1}$ and the carbon content after modification with octadecyl silane prior to and after subsequent silanization with trimethyl chlorosilane was equal to 13·53% and 14·20%, respectively.

The specific surface area of the silica gel in the second column prior to modification was 300 $m^2 . g^{-1}$ and the carbon content in the final product is given by the manufacturer as minimally 12%. The third column was packed with silica gel Separon SGX, 10 μm (Laboratorní přístroje, Prague), $V_M = 2{\cdot}6$ ml, specific surface area 500 $m^2 g^{-1}$.

All the solvents were of p.a. purity; prior to use the tetrahydrofuran (Laborchemie, Apolda, G.D.R.) was freed of stabilizer by redistilling and, similarly to the other solvents, was dried using molecular sieve 5A. Acetonitrile (also from Laborchemie, Apolda, G.D.R.) was only dried and used without purification.

Chromatographed Substances

The esters of cholesterol and the substance itself are listed in Table I. The formate, valerate, laurate, palmitate, stearate, behenate, brassidate and erucate were synthesized in the authors' coworkers laboratory; the remaining esters and cholesterol were the products of Merck, F.R.G. The purity of the standards was verified by measuring the melting point and transition temperature to the isotropic liquid.

Procedure

The components of the mobile phase employed for chromatography on silica gel (heptane, 2-propanol, ethyl acetate) and on the C18 phase (tetrahydrofurane, acetonitrile,dichloromethane)

TABLE I

A list of the cholesterol esters

Name of the ester	Number of acyl carbons	Positional isomer
Formate	1	—
Acetate	2	—
Propionate	3	—
Butyrate	4	—
Valerate	5	—
Capronate	6	—
Enanthate	7	—
Caprylate	8	—
Pelargonate	9	—
Caprinate	10	—
Laurate	12	—
Myristate	14	—
Palmitate	16	—
Stearate	18	—
Oleate	18	*cis*
Elaidate	18	*trans*
Behenate	22	—
Erucate	22	*cis*
Brassidate	22	*trans*

were freed of traces of water using molecular sieve 5A activated for 3 h at a temperature of 200°C. The retention characteristics of cholesterol and its esters were measured on silica gel with heptane–2-propanol and heptane–ethyl acetate mobile phases; chromatography in the reversed-phase system on octadecyl silica gel columns was carried out using water–tetrahydrofuran and acetonitrile–dichloromethane mobile phases. Depending on the elution solutions employed, the samples of cholesterol and its esters were dissolved in tetrahydrofuran, dichloromethane or heptane, in concentrations of the order of 10^1 mg ml^{-1} and were employed using the LCI 20 stop-flow injection arrangement in volumes of 5–50 µl. The flow rate of the mobile phase through the columns was 0·5 ml min^{-1} and the eluate was studied using a refractometric detector. The retention volumes V_R of the chromatographed substances were measured and the values of their capacity ratios $k = (V_R - V_M)/V_M$ were calculated. The dead volume V_M was measured as the elution volume of heavy water D_2O in the given mobile phase. The capacity ratio values given in the tables are the arithmetic means of the results of two measurements.

RESULTS AND DISCUSSION

Reversed-Phase HPLC (RP HPLC)

Water–Tetrahydrofuran Mobile Phase

The solubilities of cholesterol esters in methanol and acetonitrile, solvents commonly used in aqueous eluents in HPLC, is very low; experiments employing mobile phases consisting only of these solvents permits the elution in an acceptable length of time and sensitive determination by the refractometric method of only cholesterol and its lowest esters (formate and acetate). Tetrahydrofuran is a suitable mobile phase component, in which all the analyzed substances are dissolved and can be successfully separated.

Table II gives the values of the capacity ratio of cholesterol and its esters determined on a Silasorb C18 column in dependence on the tetrahydrofuran content in the mobile phase, expressed by the volume fraction φ. It can be seen that the retention of these substances depends very strongly on the ratio of the two components in the mobile phase and thus reliable and reproducible results can be obtained only after careful preparation. Consequently, tetrahydrofuran was freed of traces of dissolved water by drying using a molecular sieve, as water contents in the solvent can be as great as several tenths of a percent; the results have shown that a difference of 1% (v/v) in the water content in the mobile phase produces a change in the capacity ratio of the more strongly retained esters of up to several units.

As expected, an increase in the tetrahydrofuran content in the mobile phase leads to a decrease in the ester retention; the relationship between $\log k$ for these substances and φ for tetrahydrofuran in the eluent is linear in the measured range, reflected in the values of the correlation coefficients r in Table II, which are mostly smaller than $-0{\cdot}995$. The ester retention increased with increasing number of carbon atoms in the esterified acid; the only exception was a slightly greater retention of the formate than the acetate, probably as a result of the more intense interaction of the

acetate with the eluent. The highest k value found in 90% tetrahydrofuran for cholesteryl laurate and the low correlation coefficient value must be attributed to imprecisions in the measurement of the capacity ratios in this mobile phase. It can be seen from Fig. 1 that the dependences of log k for the esters on the number of carbon atoms in the corresponding acid are practically linear and that the k–n_c dependence is also almost linear. The correlation coefficient values for these two dependences and for the water–tetrahydrofuran mobile phase are also given in Table II.

Differences were found in the retention of esters differing in the presence of a double bond in the ester acyl (n_c = 18 and 22) and in the *cis* and *trans* isomers (Table II). As expected, the unsaturated esters were eluted sooner, first the *cis* and then the

TABLE II

Dependence of the capacity ratio (k) of cholesterol esters on the volume fraction of tetrahydrofuran (φ) in the water–tetrahydrofuran mobile phase on the Silasorb C18 column

Ester (or cholesterol)	Capacity ratio k for φ (THF), %					r_1[a]
	65	70	75	80	90	
Cholesterol	2·53	1·72	—	0·55	0·27	−0·995
Formate	7·03	3·70	2·04	1·04	0·39	−0·997
Acetate	6·86	3·74	1·86	0·99	0·39	−0·999
Propionate	8·91	4·26	2·44	1·12	0·23	−0·998
Butyrate	9·92	5·25	2·45	1·21	0·26	−0·999
Valerate	10·84	5·81	2·79	1·34	0·22	−0·997
Capronate	11·41	6·72	3·14	1·46	0·30	−0·998
Enanthate	12·90	7·29	3·51	1·56	0·30	−0·998
Caprylate	15·45	8·56	3·67	1·70	0·30	−0·998
Pelargonate	—	8·99	3·85	1·88	0·35	−0·999
Caprinate	—	10·26	4·25	1·98	0·32	−0·999
Laurate	—	12·11	4·50	2·23	0·53	−0·997
Myristate	—	14·06	5·63	2·44	0·45	−0·999
Palmitate	—	14·75	5·89	3·04	0·41	−0·997
Stearate	—	17·21	6·83	3·02	0·42	−0·999
Oleate	—	—	6·54	2·29	0·38	−0·999
Elaidate	—	—	6·72	2·66	0·40	−0·999
Behenate	—	—	8·35	3·69	0·47	−0·998
Erucate	—	—	7·70	3·63	0·46	−0·998
Brassidate	—	—	7·88	3·68	0·45	−0·998
r_2	0·981	0·984	0·986	0·989	0·634	
r_3	0·977	0·990	0·993	0·979	0·939	

[a] r_1, r_2, r_3 correlation coefficients in the dependences $\log k = a_1 + b_1\varphi$, $\log k = a_2 + b_2 n_c$, $k = a_3 + b_3 n_c$.

trans isomer, while the esters of saturated acids were retained more strongly. Figure 2 gives the chromatogram for the separation of a mixture of cholesterol and some of its esters.

The Acetonitrile–Dichloromethane Mobile Phase

The main advantages of nonaqueous mobile phases are the high solubility of cholesterol, its esters and related compounds in these solvents and also the fact that the retention of the chromatographed substances does not depend on the content of the effective elution component in the mobile phase to the same extent as in aqueous solvents. This was also confirmed by experiments in acetonitrile–dichloromethane mobile phase, the results of which are given in Table III. The behaviour of the analyzed substances on Silasorb C18 in this mobile phase were similar as in aqueous eluent, i.e. their retention increases with increasing number of carbon atoms in the ester acyl and with decreasing content of dichloromethane in the mobile phase. The dependence of log k on the volume fraction of dichloromethane is prac-

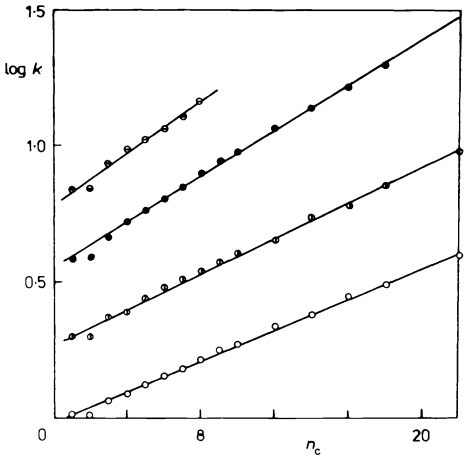


FIG. 1

The dependence of the logarithm of the capacity ratio (k) of cholesterol esters on the number of carbon atoms (n_c) in the esterified acids on a Silasorb C18 column in the water–tetrahydrofuran mobile phase. φ(THF) in % (v/v): ⊖ 65%, ● 70%, ⊙ 75%, ○ 80%

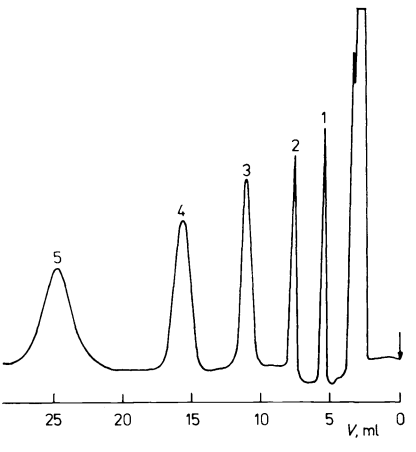


FIG. 2

The separation of a mixture of cholesterol esters on a Silasorb C18 column. Mobile phase: water–tetrahydrofuran, φ(THF) = = 80%, F_m = 0·5 ml min^{-1}. RI detection. Compounds: 1 cholesterol, 2 acetate, 3 enanthate, 4 laurate, 5 stearate. V eluate volume

TABLE III

Dependence of the capacity ratio for cholesterol esters (k) on the volume fraction of dichloromethane (φ) in the acetonitrile–dichloromethane mobile phase on the Silasorb C18 column

Ester (or cholesterol)	Capacity ratio k for φ (CH_2Cl_2), %												r_1 [a]
	5	10	20	30	40	45	50	60	70	80	90	100	
Cholesterol	—	—	—	—	9·57	6·43	4·63	2·51	1·31	0·82	0·78	0·52	−0·965
Formate	20·23	13·55	7·19	4·00	2·33	1·65	1·22	0·65	0·31	0·18	—	—	−0·932
Acetate	26·11	19·59	9·15	4·67	2·40	1·92	0·87	0·63	—	—	—	—	−0·995
Propionate	27·49	19·74	9·31	5·00	2·34	1·80	0·97	0·82	—	—	—	—	−0 995
Butyrate	30·94	20·22	9·31	4·17	2·46	1 93	1·12	0·67	—	—	—	—	−0·999
Valerate	—	21·61	9·80	5·33	2·64	1·88	1·17	0·79	—	—	—	—	−0·997
Capronate	—	23·20	10·20	5·83	2·53	1·85	1·29	0·80	—	—	—	—	−0·997
Enanthate	—	—	11·67	6·50	2·69	2·00	1·38	0·87	—	—	—	—	−0 995
Caprylate	—	—	14·63	7·67	2·93	2·51	1·55	0·88	—	—	—	—	−0·696
Pelargonate	—	—	19·18	8·67	3·30	2·58	1·72	0·94	—	—	—	—	−0·996
Caprinate	—	—	23·17	10·67	3·67	2·80	1·76	1·05	—	—	—	—	−0·993
Laurate	—	—	—	14·46	4·66	3·59	2·18	1·20	—	—	—	—	−0·989
Myristate	—	—	—	16·23	5·56	4·29	2·44	1·27	—	—	—	—	−0·994
Palmitate	—	—	—	20 07	7·54	4·82	2·79	1·41	—	—	—	—	−0·997
Stearate	—	—	—	25·80	9·49	6·17	2·75	1·73	—	—	—	—	−0·986
Oleate	—	—	—	16·88	7·12	4·06	2·82	1·32	—	—	—	—	−0·998
Elaidate	—	—	—	18·99	7·91	4·51	3·16	1·36	—	—	—	—	−0·998
Behenate	—	—	—	39 04	14·54	7·64	5·02	2·08	0·70	0·42	—	—	−0·996
Erucate	—	—	—	—	11·46	7·70	4·70	1·69	—	—	—	—	−0·999
Brassidate	—	—	—	36·18	12·85	7·70	5·01	1·76	—	—	—	—	−0·998
r_2	0·974	0·861	0·980	0·972	0·994	0·934	0·860	0·958					

[a] The significance of the symbols is given in Table II.

tically linear and the log k vs n_c dependence can also be considered linear (see r_2 in Table III, Fig. 3). The different behaviour of cholesterol in the two studied mobile phases is interesting. While cholesterol was retained less than its esters in the aqueous phase, as expected, the opposite results were obtained in some nonaqueous media. This is probably a result of the interactions of cholesterol and its esters with components of the eluents. Because of its OH group, cholesterol is replaced on the surface of the stationary phase in nonaqueous medium more intensely than its esters.

In contrast to the results obtained in aqueous mobile phase, formate was retained far less strongly than acetate in aqueous mobile phase.

It was found in a study of the effect of double bonds in acyl of esters and of the *cis* and *trans* isomers and their retentions that, similarly as in the aqueous phase, the more polar unsaturated esters are eluted sooner than the saturated and the *cis* isomers are eluted prior to *trans* isomers; these differences can be utilized to separate these substances (Fig. 4). Figure 5 gives an example of the separation of higher cholesterol esters.

HPLC on Normal Phases

The removal of traces of water from the eluent constitutes a basic condition for the obtaining of reliable, reproducible and practically useful results in chromatography on normal phase systems on silica gel sorbent. When water is not removed, the activity of the stationary phase and the selectivity of the system are so low that it is even difficult to separate esters from the opposite ends of the homologous series (i.e. differing by 20 carbons in the acyl) even in undried heptane alone; this is in agreement with the results described in an earlier publication[11].

Tables IV and V give the capacity ratio values for cholesterol esters measured on a Separon SGX silica gel column in heptane–2-propanol and heptane–ethyl acetate mobile phases. The retention characteristics for cholesterol are not given as it was retained so strongly in the mobile phases suitable for the esters that its retention could not be found under the given conditions. Even though the retention characteristics of the esters in heptane–ethyl acetate and sometimes also in heptane–2-propanol mobile phases were determined for only three eluents differing only in the content of ethyl acetate or 2-propanol, the results can be interpreted unambiguously. It follows that the retention of the esters depends strongly on the content of 2-propanol in the mobile phase, especially at contents below 0·125% (v/v), where a difference of 0·025% leads to a change in the capacity ratio by up to several units. It is thus necessary to measure the 2-propanol added to the mobile phase very carefully. This dependence is not as sharp for mobile phases containing ethyl acetate (the log k vs log φ lines have a lower slope). As expected, the retention of the esters decreases with increasing content of 2-propanol or ethyl acetate in the eluent and

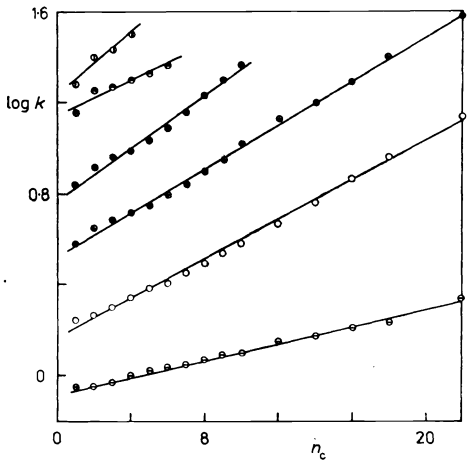


FIG. 3

The dependence of the logarithm of the capacity ratio (k) of cholesterol esters on the number of carbon atoms (n_c) in the esterified acid on a Silasorb C18 column in acetonitrile–dichloromethane mobile phase. $\varphi(CH_2Cl_2)$ in % (v/v): ⊙ 5%, ◓ 10%, ● 20%, ⊕ 30%, ○ 40%, ⊖ 50%

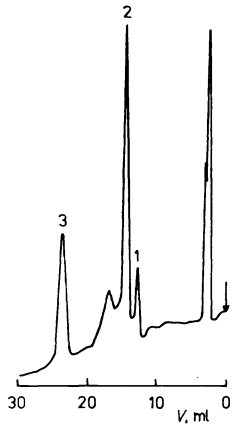


FIG. 4

Separation of a mixture of cholesterol esters of saturated and unsaturated acids on a Silasorb 300 SPH C18 column. Mobile phase: acetonitrile–dichloromethane, $\varphi(CH_2Cl_2) = 40\%$, $F_m = 0{\cdot}5$ ml min^{-1}. RI detection. Compounds: 1 oleate, 2 elaidate, 3 stearate. V eluate volume

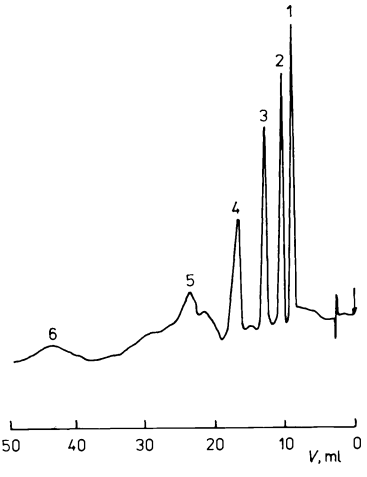


FIG. 5

Separation of a mixture of cholesterol esters of higher alkane acids on a Silasorb 300 SPH C18 column. Mobile phase: acetonitrile–dichloromethane, $\varphi(CH_2Cl_2) = 40\%$, $F_m = 0{\cdot}5$ ml min^{-1}. RI detection. Compounds: 1 caprinate, 2 laurate, 3 myristate, 4 palmitate, 5 stearate, 6 behenate. V eluate volume

TABLE IV

Dependence of the capacity ratios (k) of cholesterol esters on the volume fraction of 2-propanol (φ) in the mobile phase heptane–2-propanol on the Separon SGX column

Ester	Capactiy ratio k for φ (2-PrOH), %				r^a
	0·1	0·125	0·2	0·5	
Formate	—	2·26	1·40	0·35	−0·996
Acetate	—	4·79	3·11	0·64	−0·991
Propionate	5·77	2·90	1·54	0·27	−0·996
Butyrate	4·38	2·19	1·12	0·17	−0·995
Valerate	3·77	1·85	0·97	0·08	−0·993
Capronate	3·37	1·70	0·84	—	−0·981
Enanthate	3·11	1·41	0·77	0·07	−0·993
Caprylate	2·92	1·36	0·70	0·03	−0·989
Pelargonate	2·60	1·32	0·65	0·01	−0·977
Caprinate	2·53	1·27	0·62	—	−0·982
Laurate	2·24	1·17	0·58	—	−0·984
Myristate	2·05	1·12	0·55	—	−0·988
Palmitate	1·88	1·10	0·54	—	−0·992
Stearate	1·85	1·03	0·53	—	−0·985
Oleate	—	1·27	0·61	—	—
Elaidate	—	1·12	0·66	—	—
Behenate	1·77	0·97	0·46	—	−0·991
Erucate	—	0·95	0·54	—	—
Brassidate	—	0·98	0·57	—	—

[a] r correlation coefficient of the function $\log k = a + b \log \varphi$.

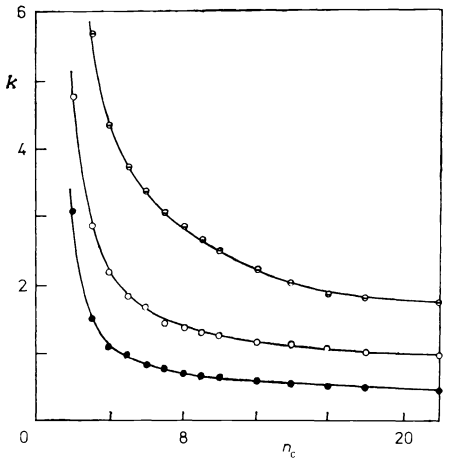


FIG. 6

Dependence of the capacity ratio (k) for cholesterol esters on the number of carbon atoms (n_c) in the esterified acid on a Separon SGX column in heptane–2-propanol mobile phase. φ(2-PrOH) in % (v/v): ⊖ 0·1%, ○ 0·125%, ● 0·2%

TABLE V

Dependence of the capacity ratios (k) of cholesterol esters on the volume fraction of ethyl acetate (φ) in the heptane–ethyl acetate mobile phase on the Separon SGX column

Ester	Capacity ratio k for φ (EtOAc), %			r^a
	0·5	1·0	1·5	
Formate	—	3·41	1·71	—
Acetate	9·62	5·76	3·11	−0·980
Propionate	5·29	2·90	1·53	−0·986
Butyrate	4·16	2·22	1·34	−0·976
Valerate	3·75	1·74	1·13	−0·999
Capronate	3·30	1·57	1·00	−0·999
Enanthate	2·95	1·31	0·87	−0·999
Caprylate	2·54	1·19	0·77	−0·935
Pelargonate	2·28	1·08	0·69	−0·999
Caprinate	2·03	1·00	0·64	−0·999
Laurate	1·90	0·88	0·55	−0·999
Myristate	1·84	0·90	0·46	−0·991
Palmitate	1·79	0·82	0·44	−0·996
Stearate	1·76	0·79	0·42	−0·997
Oleate	2·54	0·92	0·61	−0·996
Elaidate	2·39	0·78	0·59	−0·983
Behenate	1·58	0·64	0·36	−0·999
Erukate	1·98	0·69	—	—
Brassidate	1·72	0·71	—	—

[a] r correlation coefficient of the function $\log k = a + b \log \varphi$.

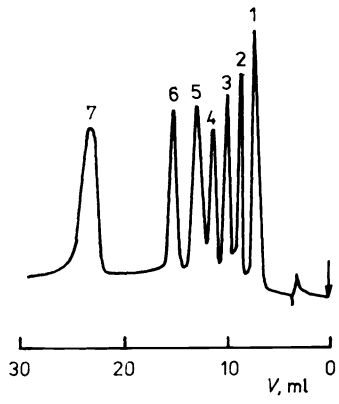


FIG. 7

Separation of a mixture of cholesterol esters on a Separon SGX column. Mobile phase: heptane–2-propanol, φ(2-PrOH) = 0·125%, F_m = 0·5 ml min^{-1}. RI detection. Compounds: 1 behenate, 2 caprinate, 3 enanthate, 4 valerate, 5 butyrate, 6 propionate, 7 acetate. V eluate volume

the log k vs log φ dependences are linear with 99% probability (a poorer quality correlation was found only for the caprylate). The results also confirmed that, in contrast to reversed-phase chromatography, there is not a linear relationship between the capacity ratio or its logarithm and the number of carbons in the ester acyl in systems with normal phases on a silica gel adsorbent (Fig. 6). The retention of the esters greatly depends on the number of carbon atoms in the acyl of the esters of the lower acids (for 0·1% (v/v) 2-propanol in heptane $n_c \leqq 12$). The differences between the capacity ratios for the higher esters increase in mobile phases with low contents of 2-propanol or ethyl acetate ($\varphi < 0{\cdot}1$ and 0·5%, respectively); however, even under these conditions, the selectivity of the system will probably still not be sufficient for the separation of esters with $n_c = 16-22$. In addition, the retention times increase to such a degree that the analysis would take a long time. Consequently, this tested system of normal phases can be recommended for the separation of low cholesterol esters; under isocratic elution conditions, esters differing in one carbon atom in the acyl can be separated, beginning with the acetate and ending approximately with the capronate. Figure 7 gives an example of the separation of such a mixture. Low esters can, of course, readily be separated from high esters.

The retentions of the saturated and unsaturated esters and their *cis* and *trans* isomers differed only slightly in the studied mobile phases and this system is thus not suitable for the separation of the isomeric esters of higher acids.

In conclusion, high-performance liquid chromatography is a suitable method for the analysis of mixtures of cholesterol esters; the advantages of systems with normal or reversed phases can be utilized for concrete separation problems. The former are preferable for the analysis of the esters of the lower alkane acids ($n_c \leqq 10$), while reversed phases can be recommended for the efficient separation of higher esters ($n_c \geqq 10$), that can also differ in the presence of double bonds in the acyl chain or *cis–trans* isomerism, using both aqueous and nonaqueous mobile phases.

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