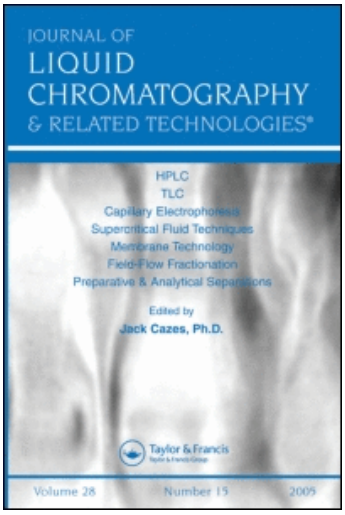

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF CHOLESTERYL ESTERS

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Abstract

The separation of cholesteryl esters by high performance liquid chromatography was facilitated by cooling of the column, followed by spontaneous return to ambient conditions. The resolution of the esters was increased. The method was applied to detection of the cholesteryl esters in plasma.

Introduction

The role of cholesteryl esters and cholesterol metabolism in various physicological disorders has been the subject of much investigation. Hyperlipidemic and elevated triglyceride levels have been implicated in diabetes (1). The possible role in cardiac and vascular disease has been investigated and many studies have evolved to quantitate the cholesteryl esters. Cholesterol is found either as free or esterified

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with long chain fatty acids. It is found 75% as esterified steroid in the serum. Many methods use hydrolysis and subsequent analysis of the free steroid. Kuo and Yeung (2) and Smith etal (3) have reported studies on these separations.

The work reported here was stimulated by the fact that there does not appear to viable methods for separation of cholesteryl esters. Previous work from this laboratory showed that cholesteryl esters may be indicative of fetal lung status (4). Cholesteryl esters are readily found in extracts of plasma and easily separated from cholesterol by high performance liquid chromatograpy using detection at 213 nm. Described are results of HPLC of plasma samples and the results of studies in manipulation of the mobile phases in order to achieve the desired results.

Experimental

Materials and Instruments

All solvents were EM Omnisolv, purchased from E.M. Science. They were filtered and degassed before use. Cholesterol (C), Cholesteryl Linolenate (C-18:3), Cholesteryl Arachidonate (C-20:4), Cholesteryl Linoleate (C-14:0), Cholesteryl Oleate (C-18:1) and Cholesteryl Palmitate (C-16:0) were purchased from SIGMA Chemical Co. (St. Louis, MO). Cholesteryl Eicosenoate (C-20:1), Cholesteryl 11, 14-Eicosadienoate (C-20:2), Cholesteryl 11, 14, 17-Eicosatrienoate (C-20:3) and Cholesteryl Myristoleate (C-14:1) were purchased from NU CHEK PREP INC. (Elysian, MN).

A modular liquid chromatograph equipped with an LDC pump and a variable wavelength UV spectroflow 773 detector, was interfaced with a Hewlett Packard HP 3385A integrator. A Rheodyne loop injector was used. The column was a Whatman Partisil 5 ODS-3 (250 x 4.6 mm) column. A 4 cm x 4.6 mm column was packed with C18 for use as a guard column.

Methods

1. Extraction: 0.5 ml of plasma as vortexed with 2 ml of chloroform and 1 ml of methanol, the chloroform layer (lower) was aspirated and centrifuged. The water layer was again extracted. The extracts were combined and dried with nitrogen stream in a water bath (65 °C). This was reconstituted in tetrahydrofuran (THF) and aliquots taken for injection into the HPLC.

2. HPLC analysis: The Whatman Partisil 5ODS-3 column was cooled by bleeding liquid nitrogen into a glass jacket to give the desired temperature. The mobile phase was acetonitrile-THF-hexane (90:10:10). Flow was 2 ml/min, wavelength: 213 nm, and absorption range: 0.1 au. Aliquots of extract or standards were injected. The temperature (15 °C) of the column was maintained for 40 min. The cooling was then stopped and the temperature allowed to spontaneously rise to ambient (25 °C).

Results and Discussion

Since it is difficult even using an integrator to determine base line leads for individual peaks, the efficiency of separation or resolution in a system is suggested. The formula for resolution requires that the width of the base line be determined. As seen in Table 1 a concept using the height ratios gives some indication of resolution. Thus the ratio peak height of analyte versus valley height appears to be of some value in showing how a separation is progressing. The ratios indicated show the progress of separation as methodology improves resolution.

Fig. 1 shows that there is a correlation between the number of double bonds in the molecule and the retention times. A linear regression is followed. The molecules with the fewer double bonds were retained the longest. Due to the lack of availability of some of the necessary compounds some of the curves in the figure are not complete. These data are shown to present the possiblility of using the result in identification of unsaturated cholesteryl esters. Possibly

Table 1
Peak Height - Depth of Valley Ratio (%)

Temperature °C	C-18:3 - C-20:4	C-18:2 - C-16:1	C-16:1 - C-14:0	C-18:1 - C-16:0
25	45.6	67.3	19.1	9.4
20	27.3	40.5	8.6	6.1
15	16.7	37.9	8.7	2.2
10	25.6	36.0	4.9	0.7
15*	16.7	22.5	5.9	0.7

* 40 min at 15°C, then return to ambient.

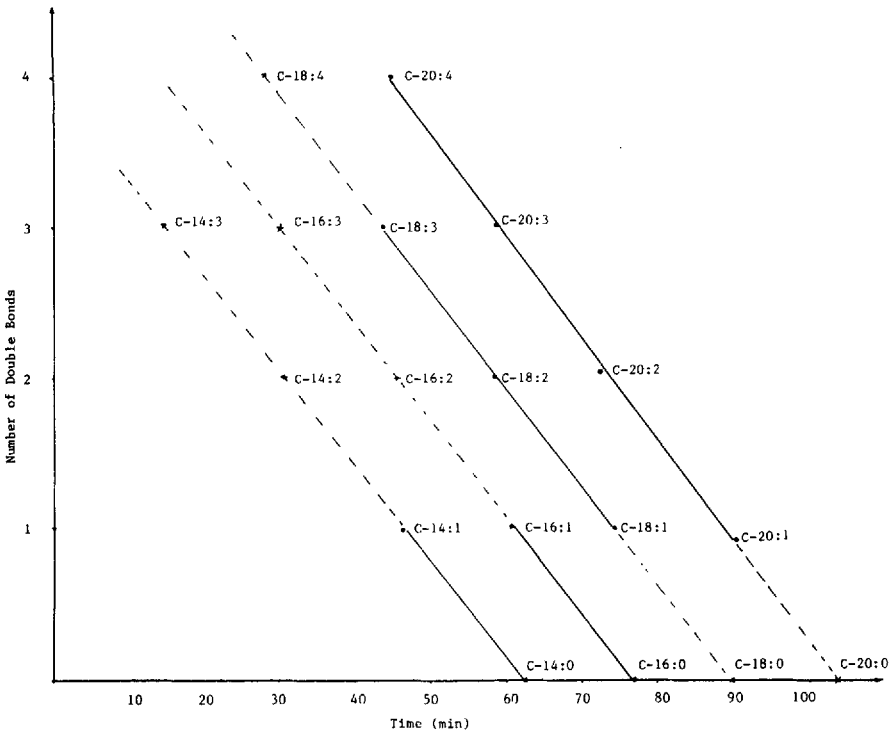

Figure 1. Correlation between the number of double bonds in the fatty acid moiety of the cholesteryl ester and the retention times. Conditions: as in text. (X-unavailable)

the scheme may prove useful in other situations where double bonds are present.

Based on the earlier work of Smith et. al (3) an investigation was made of the possibility of using acetonitrile-isopropanol mixtures as the mobile phase. Different proportions of these two solvents did not give complete resoultion of the cholesteryl esters of interest. Therefore, changes in the mobile phases based on these earlier works followed. This appeared necessary since the short wavelength used

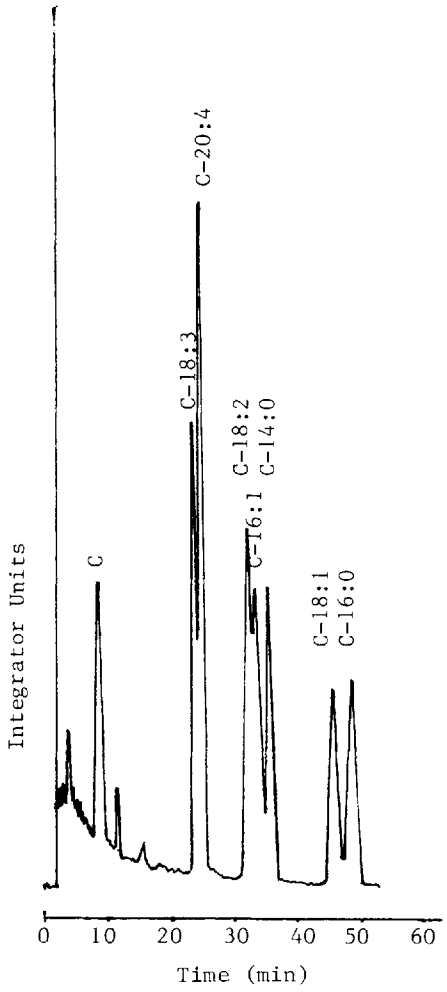

Figure 2. Separation of cholesteryl esters as noted. This was carried out with the mobile phase acetonitrile-THF-hexane (90:10:10) at ambient temperature.

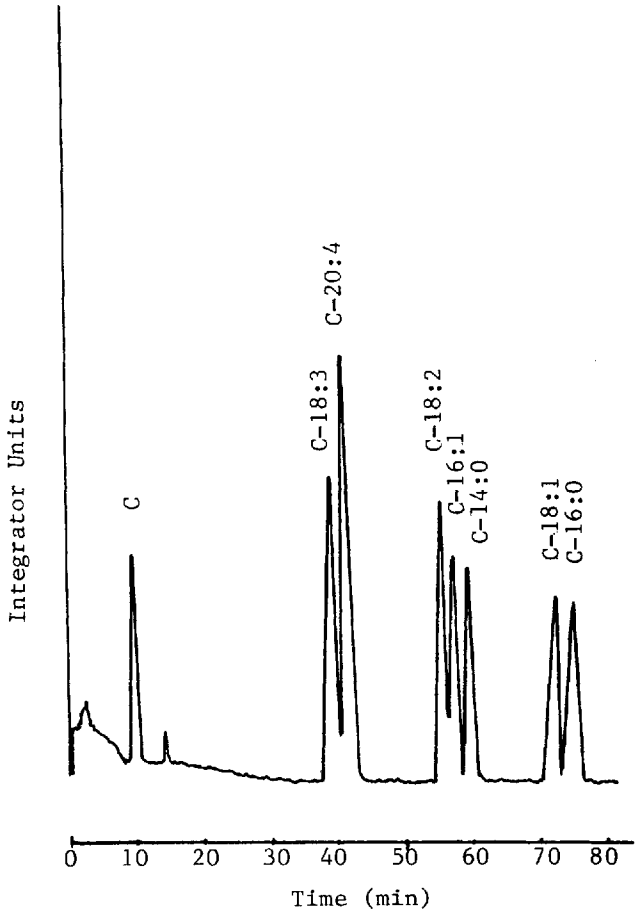

Figure 3. The same separation as in Figure 2 but using cooling: 40 min at 15 °C, then spontaneously increasing the temperature to ambient by shut off of cooling. The rise in temperature from 15 °C to 23 °C occurred over 35 min period. Refer to methods for peak designations.

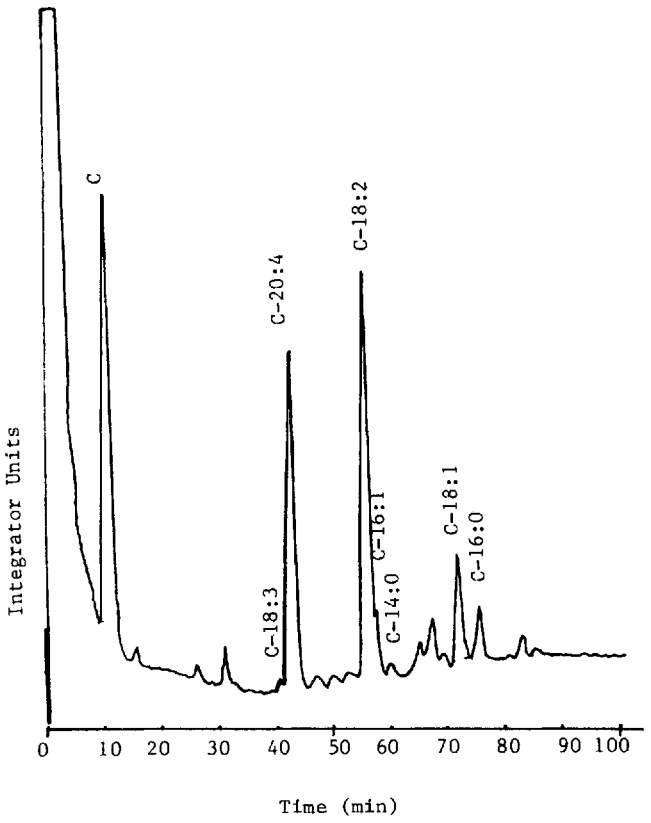

Figure 4. Separation of cholesteryl esters from plasma from a pregnant woman. The cholesteryl palmitate (C-16:0) is readily seen as well as same of the common cholesteryl esters. The identity of the peaks not labeled is unknown at this point.

would detect interfering compounds. The use of the short wavelength would eliminate those esters of the saturated fatty acids. The modification of the mobile phase by addition of hexane to the mixtures of acetonitrile and THF increased the resolution as indicated in the figures that follow. Also, as seen in Table 1, the use of cooling below ambient enhances the separation. Figure 2 shows the

separation of eight different lipids used as reference compounds during the investigation of mobile phase changes. Using the mobile acetonitrile-THF-hexane (90:10:10) without cooling the chromatogram seen in Figure 2 resulted, the resolution obtained was present but was low. Figure 3 shows the separation obtained when the column was cooled at 15 °C for 40 min, followed by warming spontaneously to ambient over 35 min peroid by shut down of the cooling. As can be seen the effect of cooling is to increase the resolution. The effect of cooling on the separation of steroids using a C18 column has been reported previoulsy from this laboratory (6, 7).

Following the procedure described in methods, plasma extracts were prepared for separation using the described method including the cooling. Figure 4 shows that the eight cholestryl esters used as reference compounds as seen in Figure 3 are readily seen in the plasma extracts. Cholesterol. which is abundant in plasma is readily seen in the chromatogram. In further experiments attempts were made to use the method for asssay of cholesteryl palmitate in amniotic fluid. Because of the inherent low sensitivitiy of the detection at 213 nm, it was not possible to determine cholesteryl palmitate in spite of the fact that it is readily seen by thin layer chromatographic methods.

The method is presently being used for a survey of cholesteryl esters in plasma of subjects with cardiac pathology. Also it is being used to determine whether the level of detection maybe suitable for the assay of cholesteryl palmitate in plasma in problem pregnancies.

Acknowledgements

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