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Note

The use of reversed-phase high-performance liquid chromatography to analyse the cholesterol ester profiles present in lipoproteins of patients exhibiting hyperalphalipoproteinaemia or hypoalphalipoproteinaemia (Tangier disease)

D. POLL, D.R.K. HARDING and W.S. HANCOCK*

Department of Chemistry and Biochemistry, Massey University, Palmerston North (New Zealand)

E.R. NYE

Department of Medicine, University of Otago, Medical School, Dunedin (New Zealand)

E.D. JANUS

Department of Medicine, Princess Margaret Hospital, Christchurch (New Zealand)

and

S.F. HANNAN and P.J. SCOTT

Department of Medicine, Auckland University, Auckland (New Zealand)

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Recently we developed procedures for the analysis of apolipoproteins by reversed-phase high-performance liquid chromatography (HPLC) [1, 2]. These measurements were of clinical significance in view of the use of levels of C and A apolipoproteins as discriminators in patients with coronary artery disease [3].

Therefore it was thought to be of interest to extend these studies and use reversed-phase HPLC to examine the levels of cholesterol esters in the different lipoprotein fractions. The methods used in the analyses were essentially the same as published by Duncan et al. [4].

This paper will show that reversed-phase HPLC allows the highly resolutive separation of cholesterol and its esters and that the profile can readily be used to distinguish patients with lipoprotein abnormalities, such as hyperalphalipoproteinaemia and hypoalphalipoproteinaemia (Tangier disease).

EXPERIMENTAL

Apparatus

The analyses were performed on a Waters high-performance liquid chromatograph equipped with a Model M-660 solvent programmer (Waters Assoc., Milford, MA, U.S.A.). A Waters 450 UV spectrophotometer was used to monitor the effluent at 200 nm and was linked to an Omniscribe two-channel chart recorder (Houston Instruments, Austin, TX, U.S.A.). Sample injections were made with a microliter 802 syringe (Hamilton, Reno, NV, U.S.A.). The separation was achieved on either a μBondapak C_{18} column (Waters Assoc., 10 μm particle size, 30 cm × 4 mm) or a Zorbax-ODS column (DuPont, Wilmington, DE, U.S.A., 25 cm × 4.6 mm). Water was purified by distillation in an all-glass still and acetonitrile and isopropanol were purified using published procedures [5].

Methods

The lipoprotein fractions were separated from plasma obtained from fasting subjects using previously described ultracentrifugal techniques [6]. To determine the total cholesterol present in a sample, a 50-μl sample was saponified with 0.5 ml of alcoholic potassium hydroxide at 45°C for 60 min. After the reaction was complete 1 ml of water and 4 ml of pentane were added and the mixture was vortexed (30 sec). The layers were allowed to separate and 2 ml of pentane were removed and then evaporated to dryness. The residue was then taken up in 1 ml of acetonitrile—isopropanol (55:45, v/v) and injected onto the reversed-phase column. The separation was achieved on the Zorbax-ODS column with isopropanol—acetonitrile (3:2) as the mobile phase. To measure the cholesterol esters a 50-μl sample was vortexed with 250 μl of isopropanol and heated for 1 h at 60°C. To the mixture 2 ml of pentane were added and vortexed for 1 min. A 1-ml sample of the upper layer was removed, dried down under a stream of nitrogen and the residue was dissolved in 150 μl of isopropanol—acetonitrile (45:55). The ester mixture was resolved on the μBondapak C_{18} column with isopropanol—acetonitrile (45:55, v/v) as the mobile phase.

RESULTS AND DISCUSSION

In the past the separation of lipids was achieved by normal- or reversed-phase chromatography with solvents such as chloroform that absorbed below 250 nm in the UV range and therefore the solutes were detected by refractive index changes [5]. However the introduction of UV transparent mobile phases, such as acetonitrile—isopropanol, has allowed the use of UV detection at 200 nm [5]. This detection method allows the convenient monitoring of the resolved materials, with good sensitivities (from 1 to 10 μg in this study). Smith et al.

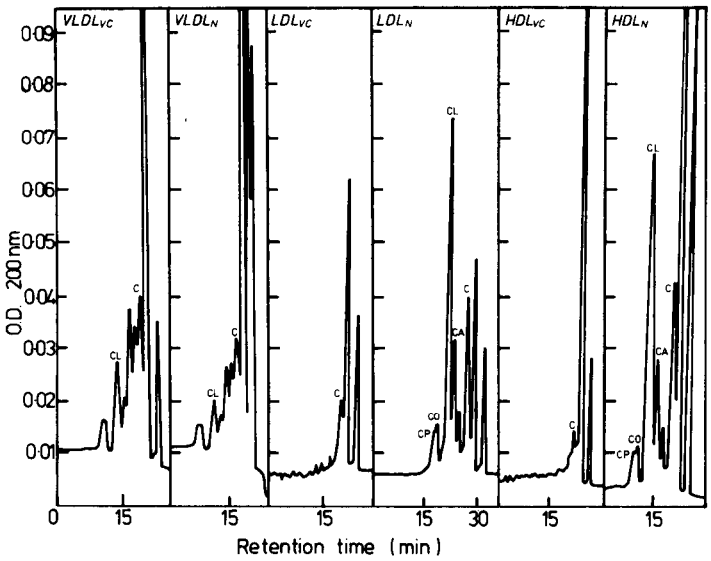

Fig. 1. The elution profile for the cholesterol and cholesterol ester fraction of the different lipoprotein classes isolated from a patient (VC) exhibiting Tangier disease (hypoalphalipoproteinaemia) and a normolipaemic control (N). The separation was achieved on a μBondapak C_{18} column with a mobile phase consisting of acetonitrile—isopropanol (55:45) at a flow-rate of 1 ml/min. In these separations 15 μl of the LDL and 25 μl of the VDL and HDL fractions were injected. The following abbreviations are used: VLDL = very low density lipoprotein; LDL = low density lipoprotein; HDL = high density lipoprotein; C = cholesterol; CA = cholesterol acetate; CL = cholesterol linoleate; CO = cholesterol oleate; and CP = cholesterol palmitate.

[7] and Carroll and Rudel [8] used a Zorbax-ODS column for the quantitation of triglycerides and cholesterol esters in plasma samples obtained from rats and monkeys, respectively. However, in this study on lipoproteins fractionated from human plasma samples, it was found that the Zorbax-ODS column gave a more complex elution profile with individual triglycerides eluting close to some of the cholesterol esters. For this reason the μBondapak C_{18} column used by Duncan et al. [4] was preferred. The Zorbax-ODS column was used for determination of total cholesterol levels (see Methods section) after the sample had been hydrolysed under alkaline conditions.

Fig. 1 shows the separations achieved for the mixture of cholesterol and cholesterol esters extracted from the lipoprotein classes isolated from a normolipaemic plasma sample (N). These were confirmed by the chromatography of authentic standards and by calibration of the separation with injections of cholesterol (1.0 and 2.5 μg). The studies indicate that cholesterol is readily separated from the corresponding acetate, linoleate, oleate and palmitate esters. In addition, these analyses are consistent with the observation that low density lipoprotein (LDL) constitutes the predominant fraction for cholesterol transport (15 μl analysed), although high density lipoprotein (HDL) also contains significant levels (25 μl analysed) [9]. The other main lipoprotein fraction is very low density lipoprotein (VLDL), in which triglycerides form the major constituent [9].

Tangier disease is a rare autosomal recessive disorder that is characterised by

the accumulation of cholesterol esters in the reticuloendothelial system and other tissues and by the absence or deficiency of normal HDL in plasma, with the presence of very low levels of apolipoproteins A-I and A-II [10, 11]. The low levels of HDL in the subjects of this study were demonstrated by Frith et al. [10]. These authors used polyacrylamide gel electrophoresis to demonstrate the absence of apolipoprotein A-I in the plasma sample.

These results were confirmed by Ouchterlony immunodiffusion studies (with apolipoprotein A-I anti-serum) and by reversed-phase HPLC (data not shown). Fig. 1 shows that the cholesterol and the cholesterol ester content of the HDL fraction is markedly depressed in the Tangier subject (T) when compared with a normolipaemic subject (N). In addition the cholesterol and cholesterol ester content of the LDL fraction is significantly reduced. This observation is consistent with other studies which attribute the LDL deficiency to a decreased net esterification by cholesterol in these subjects [9].

Recently a new variation of Tangier disease was discovered by Frith et al. [10] in which the patients exhibited a heterogeneous syndrome arising from a combination of Type II hyperlipoproteinaemia and Tangier disease. Since the presence of the Type II form of hyperlipaemia results in an elevation of the LDL fraction [10], the combination of this together with Tangier disease can explain the presence of normal levels of cholesterol and its esters in the LDL_T fraction (see Fig. 2). However the presence of Tangier disease in the patient (K.H.) is clearly determined by the very low level of cholesterol and its esters in the HDL fraction. Fig. 3 identifies two other family members (S.H. and C.H.) as exhibiting the same syndrome as K.H., with very low levels of cholesterol and its esters being observed in the corresponding HDL fractions.

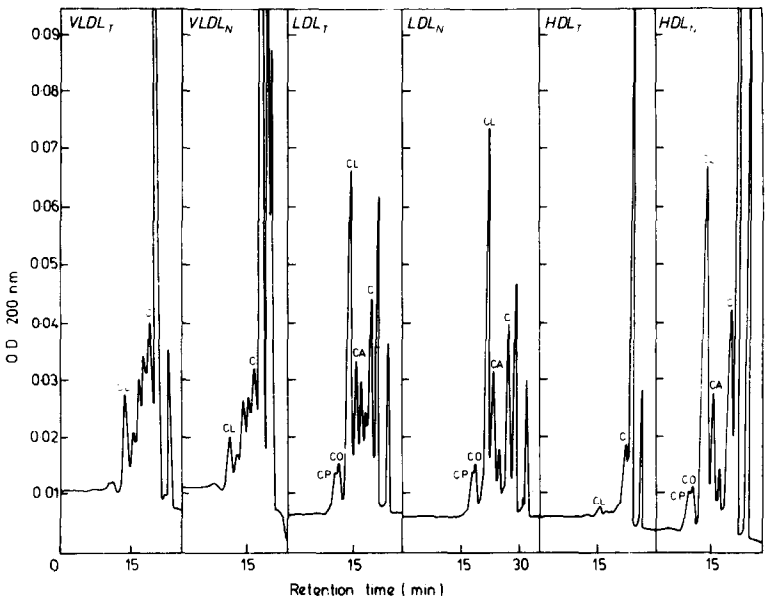

Fig. 2. The elution profile for the cholesterol and cholesterol ester fractions of the different lipoprotein classes isolated from a patient (K.H., denoted as T) exhibiting a mixed syndrome of Tangier disease and of Type II hyperlipoproteinaemia. The separation conditions and abbreviations are the same as for Fig. 1.

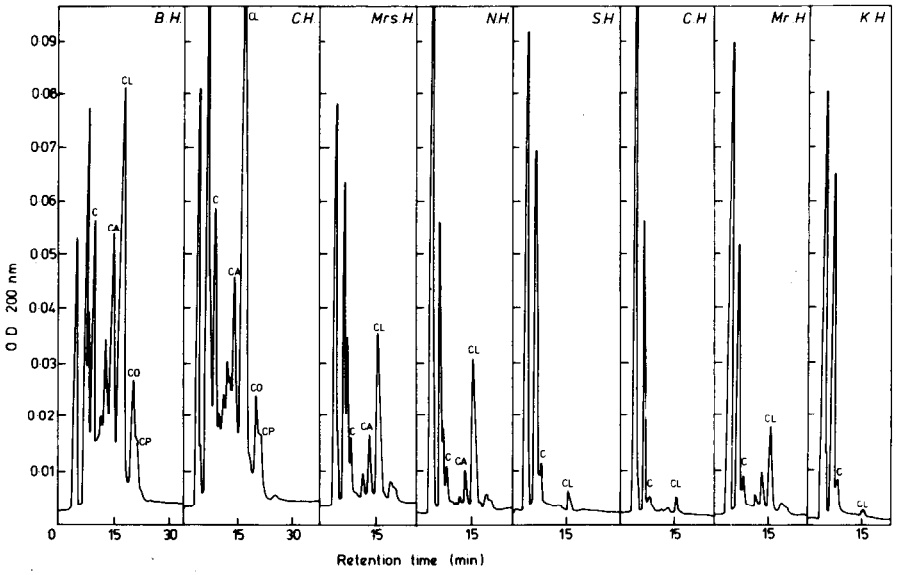

Fig. 3. The elution profile for the cholesterol and cholesterol ester fractions of the lipoprotein classes exhibited from a familial study of Tangier disease. The separation conditions and abbreviations are the same as for Fig. 1.

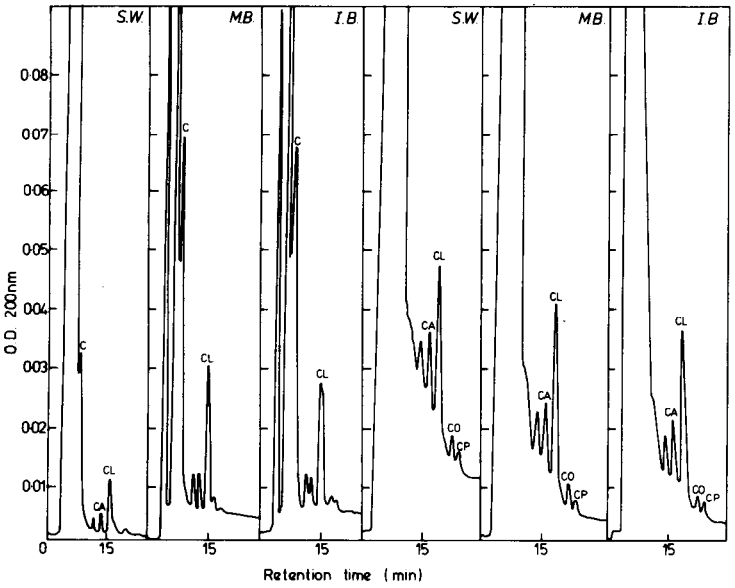

Fig. 4. The elution profile for the cholesterol and cholesterol ester fractions of the lipoprotein classes isolated from related individuals in a familial study on hyperalphalipoproteinaemia. The samples I.B. and M.B. were diluted 1:5 to allow for the presence of elevated levels of HDL in these samples. The three analyses on the left-hand side of the figure were from the HDL fraction, while the right-hand side shows the analysis of the corresponding LDL fraction. The separation conditions and abbreviations are the same as for Fig. 1.

The opposite syndrome, namely hyperalphalipoproteinaemia or an elevation of HDL is not associated with premature vascular disease [12]. Fig. 4 shows that the cholesterol profile is similar to the normolipaemic profile shown in Figs. 1 and 2. The elevation of the HDL fraction was also seen by the increased levels of the protein constituents as well as cholesterol and its esters [12].

In conclusion, these studies have demonstrated that the method developed by Duncan et al. [4] for plasma samples can be successfully applied to lipoprotein fractions isolated by ultracentrifugation. In addition, recent studies [11] have indicated that dyslipoproteinaemias can exhibit a complex aetiology and simple cholesterol and apolipoprotein measurements are not adequate to distinguish different varieties. This study shows that analysis of the cholesterol and cholesterol ester fractions can give useful additional information in the analysis of abnormal lipoproteins.

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