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Note

Rapid separation of plasma lipoproteins by gel permeation chromatography on agarose gel Superose 6B

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Agarose gel has been used successfully in the separation of plasma lipoproteins [1—5]. However, the conventional method of agarose gel chromatography in separating plasma lipoproteins requires a long elution time (> 16 h) and a large elution volume [1—3, 5]. The eluted fractions are usually pooled, concentrated or extracted to enable analysis of the chemical components of lipoproteins [1, 2, 5].

In this paper, a rapid separation of plasma lipoproteins using an extensively cross-linked agarose gel matrix is described. The cholesterol and triacylglycerol concentrations distributed in the different lipoproteins are also measured. A comparison is made between the separation of lipoproteins in the plasma of man, rabbit and rat.

EXPERIMENTAL

Equipment

A fast protein (peptide, polynucleotide) liquid chromatography (FPLC) system (Pharmacia Fine Chemicals, Uppsala, Sweden) was used for carrying out the separation of plasma lipoproteins on a column of Superose 6B. The system consisted of two P-500 high-precision, high-pressure pumps (only one was necessary in these experiments), a GP-250 gradient programmer, V-7 injection valve and a 5-ml sample loop. For these experiments, the system was connected to a single-path UV monitor (UV-1), a fraction collector (FRAC-100) and a chart recorder (Pharmacia Fine Chemicals).

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Superose 6B (Pharmacia Fine Chemicals) was packed onto a K 16/70 column (56 cm × 1.6 cm) equipped with a protective outer jacket.

Separation of standard and total lipoprotein fractions from human, rabbit and rat plasma was carried out on a Beckman L5-50 ultracentrifuge in a Ti 50.3 rotor.

Samples

Plasma samples were obtained from normal 12—16 h fasted men, female NZW rabbits and male Porton rats. Standard lipoprotein fractions were prepared by sequential ultracentrifugation using solid potassium bromide to obtain the required densities [6, 7]. Very-low-density lipoproteins (VLDL) were isolated as the fraction of density < 1.006 g/ml, low-density lipoproteins (LDL) in the density interval 1.006—1.063 g/ml, high-density lipoproteins (HDL) in the density interval 1.063—1.21 g/ml and the total lipoprotein as the fraction of density < 1.21 g/ml.

Gel permeation chromatography on Superose 6B

A new breed of agarose gel, Superose 6B, was used for separating plasma lipoproteins. The gel was packed onto a K 16/70 column and water was pumped into the outer protective jacket to prevent sudden temperature changes during the elution.

Total plasma lipoproteins were isolated from 2.5—3.5 ml plasma by ultracentrifugation at density 1.21 g/ml, and made up to a volume of 1.5—2.0 ml with a pre-filtered and degassed solution containing 0.15 *M* sodium chloride, 0.01% (w/v) Na_2EDTA and 0.02% (w/v) sodium azide, pH 7.2. The sample was injected onto the column through a V-7 valve attached to a 5-ml sample loop. The column was pre-equilibrated and the sample eluted with the same saline solution.

The P-500 pumps, fraction collector and recorder were controlled by the gradient programmer. A constant-elution flow-rate of 0.75 ml/min was achieved and fractions of 2.0 ml were collected after discarding the first 32 ml. The absorbance of the eluent was monitored continuously at 280 nm using the UV-1 monitor. The tracing was printed on the recorder, set at a sensitivity of 100 mV and a chart speed of 1 mm/min. Absorbance of the selected fractions was calibrated on a Gilford spectrophotometer 250 (Instrument Labs., Oberlin, OH, U.S.A.).

Chemical analyses

The cholesterol concentration in each of the fractions collected was analysed by an enzymatic method (CHOD-PAP method, Boehringer, Mannheim, F.R.G.) adapted for multiple determination in a CentrifiChem System 400 (Union Carbide, U.S.A.). Triacylglycerol concentration was analysed in the same way but with a different enzymatic method (Triglycerides GPO-PAP, Boehringer).

RESULTS

The elution profiles of lipoproteins in the plasma fraction of density < 1.21 g/ml show three major peaks which eluted with decreasing particle size

corresponding to VLDL, LDL and HDL, respectively (Fig. 1). The human HDL peak eluted later than that of rabbit and rat indicating a smaller particle size of human HDL. The peaks labelled P may represent albumin, prealbumin or potassium bromide salt used for the ultracentrifugation.

There is a species difference in the proportion of each lipoprotein class; in all cases the strongest absorbance occurred in HDL. The proportion of LDL is highest in man, lower in rabbit and negligible in rat (Fig. 1).

Cholesterol concentration was measured in each of the fractions collected. Again three distinct peaks corresponding to VLDL, LDL and HDL were observed (Fig. 2). The greatest proportion of human plasma cholesterol is found in the LDL fraction. In rabbit, LDL and HDL are the major cholesterol carriers, whereas in rat, HDL is the major carrier of plasma cholesterol.

Triacylglycerol concentration profiles also show the presence of three

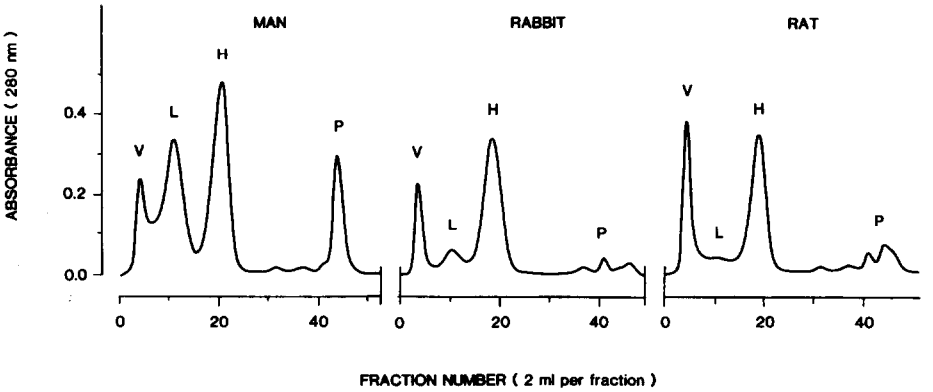

Fig. 1. Elution profile of lipoproteins on Superose 6B. Total lipoproteins were isolated by ultracentrifugation at density 1.21 g/ml from 3.5 ml plasma. A final volume of 1.5—2.5 ml was injected onto a column of Superose 6B (56 cm × 1.6 cm) and eluted at 0.75 ml/min with 0.15 *M* sodium chloride, 0.01% Na_2EDTA and 0.02% sodium azide, pH 7.2. Fractions (2 ml) were collected after discarding the first 32 ml. The monitor sensitivity was set at 0.1, the recorder sensitivity at 100 mV and the chart speed 1 mm/min. The operation was carried out at room temperature (ca. 23°C). Peaks: V = VLDL, very-low-density lipoproteins; L = LDL, low-density lipoproteins; H = HDL, high-density lipoproteins; P = unidentified peaks.

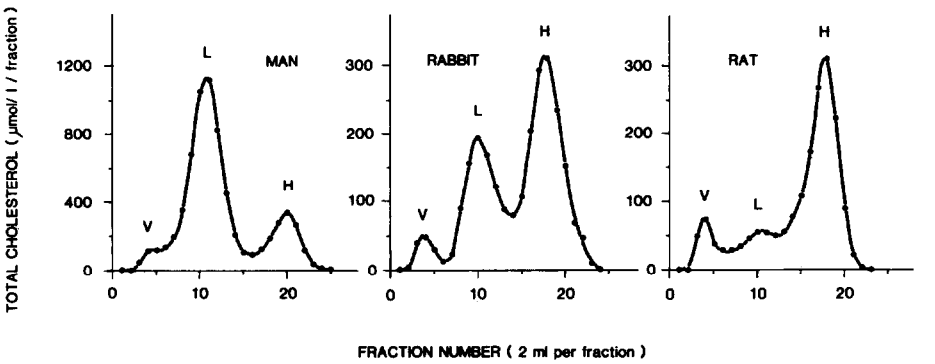

Fig. 2. Cholesterol profiles of lipoproteins. Cholesterol concentration in each of the lipoprotein fractions shown in Fig. 1 was measured enzymatically as described in Experimental. The concentration scale for rabbit and rat is different from that of man.

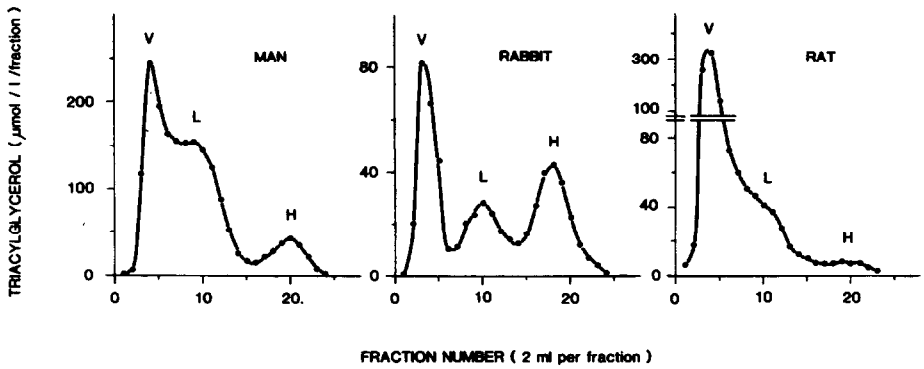

Fig. 3. Triacylglycerol profiles of lipoproteins. Triacylglycerol concentration in each of the lipoprotein fractions shown in Fig. 1 was measured enzymatically as described in Experimental. The concentration scale is different for each of the three animal species.

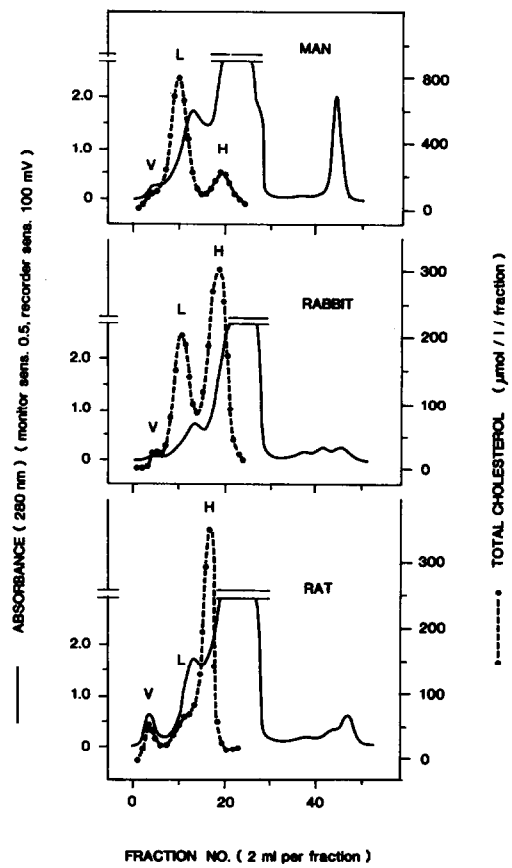

Fig. 4. Elution profiles of whole plasma. A 3-ml volume each of fasted rat and rabbit plasma and 2.5 ml of human plasma were chromatographed on a column of Superose 6B as described in Fig. 1. The absorbance was monitored continuously. The monitor sensitivity was set at 0.5 and the recorder sensitivity at 100 mV. The fractions collected were assayed for cholesterol concentration using enzymatic methods as described in Experimental.

lipoprotein regions, but the VLDL and LDL peaks overlapped in the case of man and rat owing to the high concentration of triacylglycerol in VLDL (Fig. 3). While most of the plasma triacylglycerol is found in VLDL and LDL in all three animal species, there is a marked difference in the proportion of triacylglycerol distributed in HDL. The proportion of triacylglycerol in HDL is highest in rabbit, less in man and negligible in rat (Fig. 3).

The absorbance profiles of whole plasma of the three animal species are shown in Fig. 4. While the VLDL peak is visible in all cases, there are no obvious peaks corresponding to LDL and HDL, as these are masked by the bulk of plasma proteins. However, the individual lipoprotein peaks can be identified by measuring the total cholesterol concentration in each fraction collected (Fig. 4).

DISCUSSION

The classification of plasma lipoproteins by their hydrated densities after ultracentrifugation has its drawbacks, especially when inter-species comparison is concerned. Often, the density range defined for human plasma lipoprotein classes may not correspond exactly to that of rabbit or rat, e.g. the overlapping of LDL and HDL_1 density range in rat [8]. The difference in the particle size of human HDL and that of rabbit or rat (Fig. 1) indicates that the density range of HDL may vary betweeen these animal species. The other disadvantage of prolonged ultracentrifugation is the dissociation of some apoproteins from HDL [9—11]. For these reasons, a more gentle separation of plasma lipoproteins according to their particle size by gel permeation chromatography offers a viable alternative.

The separation of lipoprotein classes by conventional agarose gel chromatography offers a considerable saving in time compared to the sequential ultracentrifugation; however, a typical chromatographic run still requires 16 h or longer [1—5]. Furthermore, the fractions collected are often too dilute for direct measurement of the chemical components. The present method uses a new agarose gel matrix, superose 6B, which greatly reduces the separation time and improves the analysis of lipoproteins. The highly cross-linked nature of the individual agarose bead ensures its overall rigidity. As a consequence of the small bead size (20—40 nm), an increase in eluent flow-rate can be achieved. A flow-rate of 0.75 ml/min is possible without encountering excessive back-pressure and a good lipoprotein separation can be achieved within 2—3 h (Fig. 1). The fractions collected from the column are concentrated enough to be assayed directly for cholesterol (Fig. 2) and triacylglycerol (Fig. 3) without any requirement for prior concentration of the sample. Unless the absorbance profile of lipoproteins is required, lipoproteins can be separated from whole plasma directly without prior separation by ultracentrifugation (Fig. 4). Because lipoproteins can be separated rapidly by this method, the procedure can be carried out at room temperature with reduced chances of protein denaturation. The control of the operation by the FPLC system enables automation and reproducibility.

This method is particularly well suited for metabolic studies of lipoproteins. The distribution of lipid or protein components of lipoprotein classes can be

compared directly [12]. Examples of such usage is shown in this study: the smaller human HDL particle size compared to those of rabbit and rat is obvious from the difference in elution volumes (Fig. 1). The cholesterol distribution among the lipoproteins is quite different in the three animal species (Fig. 2). The predominence of triacylglycerol in rabbit and human HDL compared to that of rat (Fig. 3) is consistent with the reported lipid transfer protein activity in these animal species [13, 14].

Despite the usefulness of this technique, one should be aware of its limitations. An obvious disadvantage of the method is that only one lipoprotein sample can be handled at a time. The recovery of samples from the process is about 90% owing to a small extent of non-specific absorption by the agarose gel. However, these are minor problems when compared to other available techniques. This method should provide a useful additional technique for studies of plasma lipoprotein metabolism.

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