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ISOLATION OF APOLIPOPROTEIN-B-48 FROM CHYLOMICRONS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Apolipoprotein-B-48 (B-48) is a component of chylomicrons and chylomicron remnants and originates in the human gut wall. Its molecular weight represents 48% of the hepatic apolipoprotein-B-100 (B-100). So far, immunochemical assays are only available for B-100 and do not permit the specific determination of B-48. As a convenient immunochemical method is necessary for clinical and biochemical investigations of chylomicron remnants, we isolated B-48 in amounts sufficient to permit the production of monoclonal antibodies. After delipidation of the chylomicron fraction from 3 l of human chylous ascites, size-exclusion chromatography was performed by using two serially connected TSK columns. B-48 was eluted as a second peak at about 35% of the total volume, well separated from B-100 (eluted as a first peak). After detection by UV absorption at 280 nm, the fractions collected from 25 automated runs were combined, ultrafiltered and examined for their protein composition by sodium dodecyl sulphate polyacrylamide gel electrophoresis. This indicated that we were able to isolate rapidly 3.4 mg of B-48 in a purity sufficient to perform immunization.

INTRODUCTION

Previously, apolipoprotein-B has been thought to be a homogeneous protein. However, it has been shown by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) that in fact it consists of several species with different molecular weights. The physiologically most important forms are apolipoprotein-B-100 (B-100) and apolipoprotein B-48 (B-48), produced by the liver and the gut wall, respectively. B-100 is the major lipoprotein of very-low- and low-density lipoproteins (VLDL and LDL), whereas B-48 is found in chylomicrons and their catabolic products, the chylomicron remnants[1,2].

A knowledge of the distribution of these apolipoprotein-B subfractions within lipoproteins would permit more detailed insights into the lipoprotein metabolism in normal and hyperlipidaemic states[3]. Such clinical and biochemical investigations could be performed most effectively by specific immunoassays. So far, immunochemical assays are available only for B-100 and do not permit the specific determination

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of B-48[4]. Therefore, we planned to produce monoclonal antibodies for an immunoassay.

The large difference in the molecular weights of the B-100 (about 500 000[1]) and B-48 (about 240 000[1]) should allow their separation by gel permeation chromatography (GPC). We therefore designed a rapid and highly reproducible method for the preparation of B-48 on a micropreparative scale using GPC over a TSK column system.

EXPERIMENTAL

Ultracentrifugation

Chylomicrons were prepared from 3 l of chylous ascites of a tumour patient by ultracentrifugation as follows.

(1) After centrifugation for 30 min at 25 000 rpm (75 500 *g*) at 10°C at their own density, the upper layer was collected.

(2) This fraction from step 1 was transferred to another tube, overlayered by 0.9% sodium chloride (= 2:1) and centrifuged again at 25 000 rpm at 10°C for 60 min.

(3) The upper fraction collected from step 2 was centrifuged for a further 20 h at 25 000 rpm (75 000 *g*) at 10°C and the resulting upper layer was collected again.

The total cholesterol content of the upper layer (200 ml) collected from step

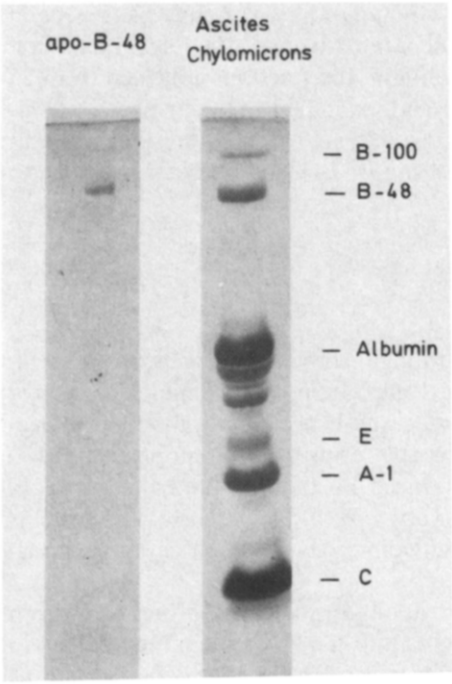

Fig. 1. SDS-PAGE (4–30% gradient gel) of purified B-48 and other apoproteins from ascites chylomicrons.

3 was 174 mg/dl and its total protein content was 74.7 mg/dl. An aliquot was examined by SDS-PAGE for protein composition (Fig. 1).

Extraction

A 20-ml volume of the material obtained following step 3 was incubated overnight at −20°C with a 10-fold volume of ethanol–diethyl ether (2:1). The resulting precipitate was centrifuged at 3000 rpm at 4°C for 20 min and the pellet was resuspended in 200 ml of diethyl ether. This washing procedure was repeated twice and the final pellet was reconstituted in 5 ml of buffer (10 m*M* Tris–0.9% sodium chloride buffer (pH 7.4) containing 1 m*M* EDTA, 2% SDS, 0.5 m*M* phenylmethylsulphonyl fluoride (PMSF) and 0.01% sodium azide.

HPLC

GPC was performed by using two serially connected TSK columns (TSK G 5000 PW and TSK G 3000 SW, each 600 × 7.5 mm I.D.; Toyo Soda, Tokyo, Japan), and a pre-column (BioSil TSK 400, 75 × 7.5 mm I.D.; Bio-Rad Labs., Munich, F.R.G.). The column calibration is indicated in Fig. 1.

The eluent contained 0.9% sodium chloride, 2% SDS, 1 mmol EDTA and 0.05% sodium azide. The flow-rate of the pump (Type 420; Kontron, Eching, F.R.G.) was 0.7 ml/min. The resulting pressure was 34 bar.

Proteins were detected by UV absorption at 280 nm and 0.02 a.u.f.s. Injection of 200 μl of the reconstituted protein mixture was automated by use of an autosampler (WISP 710 B; Waters Assoc., Königstein, F.R.G.). Fractionated peaks were collected on a fraction collector (FRAC 100; Pharmacia, Uppsala, Sweden) connected to a UV detector and a magnetic switching valve for operation in a signal-controlled "peak-cutting mode". Suitable timing of the autosampler and the fraction collector allowed automated overnight system operation. Each one had a duration of 120 min. Fractions 1 and 2 were concentrated by ultrafiltration using an Amicon PM 30 filter (Amicon, Wittenruhr, F.R.G.) at 3 bar. SDS-PAGE was performed in a 4–30% gradient gel.

RESULTS

The chromatographic reproducibility of the method is characterized by mean retention times of 29.7 ± 0.3 min [$\bar{x}$ ± 1 S.D.; coefficient of variation (C.V.) = 1%] for peak 1 and 33.9 ± 0.3 min ($\bar{x}$ ± 1 S.D.; C.V. = 0.9%) for peak 2. Peak 1 corresponds to B-100 and peak 2 to B-48 (Fig. 1, left). The elution volume of B-48 represented about 35% of the total column volume. The peaks eluted later corresponded to the other apolipoproteins present in chylomicrons, albumin and aggregates.

On the basis of the column calibration, the apparent molecular weight of B-100 was determined to be *ca.* 600 000 and that of B-48 *ca.* 275 000.

The fractions of peak 2 collected from 25 runs within two days yielded 3.4 mg of B-48 of high purity (Fig. 1, left). This amount corresponded to about 2% of the total protein content of the chylomicrons isolated from the ascitic fluid.

DISCUSSION

The separation of apoproteins from chylomicrons from human chylous ascites by GPC gives a complex peak pattern. The first peak corresponds to B-100 and the second to B-48. These apolipoproteins were well separated and were free from lower-molecular-weight components or other impurities. The molecular weights, determined by our method, are 600 000 for B-100 and 275 000 for B-48. These are in good agreement with the values of 650 000 and 278 000 found by Schwandt *et al.*[5]. The high portion of lower molecular weight peaks from chylomicrons observed agrees also with the results of Schwandt *et al.*[5]. Compared with a VLDL-derived apoprotein mixture, they found a much higher portion of low-molecular-weight proteins in chylomicrons. In addition to albumin, these lower-molecular-weight proteins represented the apolipoproteins apo-E, apo-AI and apo-C as well as their aggregates. Hence it should be possible to separate these apoliproteins by this method.

Usually, the production of monoclonal antibodies requires 2–3 mg of antigen of over 90% purity. Hence the amount and purity of B-48 obtained by our method are suitable for this purpose.

In conclusion, the method described here for the purification for B-48 from an apolipoprotein mixture of chylomicrons is rapid, effective and reproducible. The semi-automated operation and the speed of this chromatographic system permits the isolation of enough B-48 of sufficient purity for subsequent procedures in about two days in a very convenient and effective way.

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