Journal of Chromatography, 425 (1988) 169–174
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3991

Note

Rapid quantification of apolipoprotein E enriched very-low-density lipoproteins by heparin–Sepharose chromatography

PETER WEISWEILER

Institute for Clinical Pharmacology Munich, P.O. Box 1165, D-8011 Höhenkirchen-Siegertsbrunn (F.R.G.)

(First received July 27th, 1987; revised manuscript received September 28th, 1987)

Ultracentrifuged plasma very-low-density lipoproteins (VLDLs; $d < 1.006$ kg/l) include groups of particles that are polydisperse in size and composition and heterogeneous in origin [1]. The commonly used methods applied to isolate VLDL subfractions are based on differences in size and hydrated density [2]. Shelburne and Quarfordt [3] introduced the separation of VLDLs into two subfractions by means of affinity chromatography on heparin–Sepharose [3]. The unbound subfraction contained very little apolipoprotein E, whereas the subfraction bound to heparin–Sepharose was enriched with this apolipoprotein. Investigation of the function of both VLDL subfractions showed that only the bound subfraction was able to interact with the low-density lipoprotein (LDL) receptor [4].

However, previous methods of lipoprotein subfractionation are limited because of need for large sample volumes, a long separation time, delipidation procedures or difficulties in the correct quantification of the separated fractions [3–7]. Here we describe a new approach, involving use of fast protein heparin–Sepharose affinity chromatography, for subfractionating and quantifying VLDLs.

EXPERIMENTAL

Materials

A Model L 5-75 ultracentrifuge (Beckman Instruments, Munich, F.R.G.) with a 50.3 Ti rotor was used, with a fast protein liquid chromatographic system from Pharmacia (Uppsala, Sweden). For electrophoresis, the LKB Multiphor appa-

0378-4347/88/$03.50

ratus (LKB, Gräfelfing) was used. Heparin–Sepharose CL-6B was obtained from Pharmacia. All other chemicals were analytical grade.

Methods

Preparation of VLDLs. Tubes containing 1 g/l EDTA were used to collect blood from fasting subjects either with a Type III (apolipoprotein E-2/2 phenotype) or Type IV (apolipoprotein E-3/3 phenotype) lipoprotein pattern [8,9]. After low-speed centrifugation, 3 ml of plasma were once diluted with saline ($d = 1.006$ kg/l) in a polyallomer tube and ultracentrifuged at 160 000 *g* for 24 h at 4 °C [10]. VLDLs were obtained by slicing the tube and removing 2 ml of the upper layers.

Fast protein affinity chromatography on heparin–Sepharose. About 7 ml of swollen gel were packed into a small column (10 × 1.0 cm I.D.) and equilibrated with 2 mmol/l sodium phosphate buffer (pH 7.4) containing 0.1 mol/l sodium chloride. The column was run at a flow-rate of 1 ml/min at room temperature, and 1 mg of VLDL protein was applied. The unbound subfraction was eluted with 17 ml of equilibrating buffer and the bound subfraction was eluted with 17 ml of the phosphate buffer, containing 0.64 mmol/l sodium chloride. Column fractions were monitored at 280 nm and calculated by setting a threshold level of 2% relative to the full scale deflection. VLDL and lipoprotein subfractions were further quantified in milligrams of total cholesterol.

Analytical procedures. We determined total cholesterol enzymatically, using the sensitive oxidation reaction of iodine for detection of hydrogen peroxide (Merck kit, Darmstadt, F.R.G.). Further chemical analysis included free cholesterol (Merck kit), phospholipid and triglyceride (Boehringer kits, Mannheim, F.R.G.) by enzymatic methods, and protein by the Lowry procedure [11]. The albumin content was determined by radial immunodiffusion, using commercially available plates from Behringwerke (Marburg, F.R.G.). The apolipoproteins were identified by horizontal 10% polyacrylamide gel electrophoresis in 1% sodium dodecyl sulphate (SDS) [12] and by double immunodiffusion in 2% agar [13].

The apolipoprotein E phenotype was assessed by horizontal isoelectric focusing on 6% polyacrylamide gels with 8 mol/l urea and 2% ampholytes, pH 3.5–9.5 [14]. We prepared pure apolipoprotein E and a monospecific antiserum against this apolipoprotein, as described previously [15].

Differences between means were evaluated using Student's unpaired *t*-test.

RESULTS

Heparin–Sepharose affinity chromatography

Using the method described here, we were able to resolve an intact VLDL sample into two major subclasses in less than 40 min. After a small peak, eluted in the void volume of the column, the unbound subfraction was eluted with the equilibrating buffer (0.1 mol/l sodium chloride) whereas the bound subfraction was eluted by increasing the sodium chloride concentration to 0.64 mol/l (Fig. 1). In a series of ten experiments with the same VLDL sample from a Type IV subject, both subfractions accounted for 68.7 ± 3.8% [mean ± S.D.; coefficient of

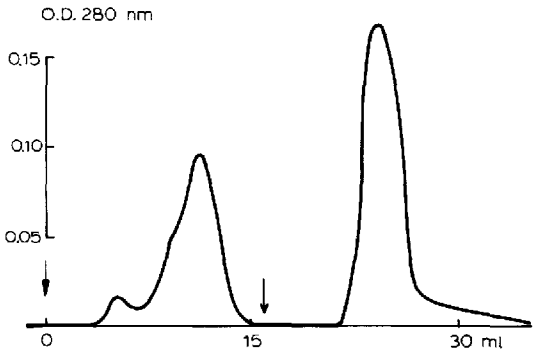

Fig. 1. Fast protein affinity chromatography on heparin-Sepharose of VLDLs from a Type III subject. The arrows indicate the buffers containing 0.1 mol/l sodium chloride (unbound subfraction) and 0.64 mol/l sodium chloride (bound subfraction), respectively.

variation (C.V.) = 5.5%] and 29.3 ± 1.9% (C.V. = 6.4%), respectively, according to calculations from monitoring at 280 nm. The peak areas increased linearly with concentration for samples containing 0.5, 1.0 and 1.5 mg of protein, respectively. Total recovery from the column was 89.1 ± 5.6% (expressed in terms of total protein after application of 1.0 mg). When the material of the two major peaks was dialysed against saline ($d = 1.006$ kg/l) and rechromatographed, the unbound subfraction was again eluted entirely with the equilibrating buffer, and the bound subfraction again bound and was eluted entirely, with 0.64 mol/l sodium chloride, as a single peak. Addition of human albumin (up to 5% in the bound fraction) did not affect the binding properties at rechromatography.

Composition of VLDL subfractions

The two VLDL subfractions differed in their lipid and protein contents (Table I): the bound subfraction contained more cholesteryl ester and protein (Lowry

TABLE I

PERCENTAGE COMPOSITION OF HEPARIN-UNBOUND AND -BOUND VLDL SUBFRACTIONS

Values are expressed as mean ± S.D.; Type III subjects $n = 4$, Type IV subjects $n = 10$.

	Free cholesterol	Cholesteryl ester	Phospholipid	Triglyceride	Protein*
Type III subjects					
Unbound VLDLs	11.0 ± 6.1	5.8 ± 1.7	11.5 ± 3.0	63.1 ± 12.9	8.6 ± 2.2
Bound VLDLs	8.5 ± 1.9	17.4 ± 6.9**	18.8 ± 4.3	41.2 ± 15.9**	14.1 ± 0.7**
Type IV subjects					
Unbound VLDLs	8.5 ± 3.2	5.3 ± 2.4	11.2 ± 2.8	67.2 ± 12.5	7.8 ± 2.5
Bound VLDLs	7.5 ± 1.0	10.1 ± 2.2**	13.8 ± 6.9	54.1 ± 8.4**	14.4 ± 4.8**

*Protein = Lowry protein minus albumin.
**Significantly different from unbound VLDLs at $p < 0.05$.

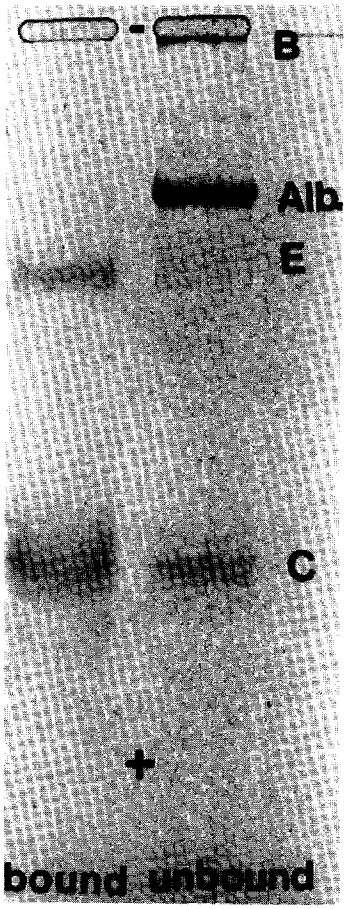

Fig. 2. SDS polyacrylamide gel electrophoresis of VLDL subfractions (ca. 15 μg protein).

TABLE II

ABSOLUTE AND PERCENTAGE DISTRIBUTIONS OF CHOLESTEROL IN HEPARIN-UNBOUND AND -BOUND VLDL SUBFRACTIONS

Values are expressed as mean ± S.D.; Type III subjects $n = 4$, Type IV subjects $n = 10$.

	Type III subjects		Type IV subjects	
	mg	%	mg	%
Unbound VLDLs	0.53 ± 0.08	41.0 ± 6.3*	0.51 ± 0.08	64.7 ± 10.1
Bound VLDLs	0.76 ± 0.03	59.0 ± 2.5*	0.28 ± 0.06	35.3 ± 6.9

*Significantly different from Type IV subjects at $p < 0.05$.

protein minus albumin) and less triglyceride than the unbound. VLDL subfractions from the Type III subjects did not differ from that from the Type IV subjects. The small peak, eluted in the void volume of the column, contained unresolved VLDLs. The protein profiles of the two subclasses of VLDLs were

displayed by SDS polyacrylamide gel electrophoresis, as shown in Fig. 2. Apolipoproteins B, E and C and albumin were identified by co-electrophoresis with authentic standards. Unlike the unbound subfraction, the bound subfraction contained most apolipoprotein E. In addition, only this fraction contained immunodetectable apolipoprotein E, as determined with a monospecific apolipoprotein E antiserum.

Distribution of VLDL subfractions from Type III and Type IV subjects

Because of the albumin contamination within VLDLs we routinely determined the concentrations of total cholesterol in VLDL and its subfractions. Table II shows absolute and percentage distributions between both VLDL subfractions from Type III and Type IV subjects. The portion of the bound subfraction from Type III subjects was 1.7 times greater than that from Type IV subjects.

DISCUSSION

Recent advantages in development of fast protein liquid chromatographic techniques provide an alternative method for isolation of lipoprotein fractions within a very short time and with high protein yields [16]. In this study we show that a small sample of human VLDLs can be easily separated into two distinct subfractions by affinity chromatography on heparin–Sepharose. The high resolving power is combined with a very high reproducibility and linearity of response over an adequate concentration range.

The chemical composition and the apolipoprotein distribution of the VLDL subfractions confirm the results of previous investigators [3,4,6] that cholesteryl ester and apolipoprotein E are in particularly high concentrations in the bound subfraction. The determination of cholesterol with a sensitive method allows the direct quantification of this VLDL subfraction.

The different percentage amounts of apolipoprotein E enriched VLDLs from Type III and Type IV subjects is consistent with the idea of Nestel et al. [17] that these VLDLs may represent partially catabolised triglyceride-rich lipoproteins (remnants), which one would expect to accumulate in Type III dyslipoproteinemia [18]. This method does not allow the differentiation of remnants derived from liver or intestine. However, the binding of these lipoproteins to cell receptors is only dependent on apolipoprotein E [19]. The rapid and precise assessment of apolipoprotein E enriched VLDLs allows, therefore, further insight in the atherogeneity of hypertriglyceridemia.

ACKNOWLEDGEMENTS

This study was supported by the Deutsche Forschungsgemeinschaft We 955/1-4. The author wishes to thank Christine Friedl and Margrit Ungar for their excellent laboratory work.

REFERENCES

1 R. Jackson, J.D. Morrisett and A.M. Gotto, Physiol. Rev., 56 (1976) 259.

2 T. Sata, R.J. Havel and A.L. Jones, J. Lipid Res., 13 (1972) 757.
3 F.A. Shelburne and S.H. Quarfordt, J. Clin. Invest., 60 (1977) 944.
4 E. Trezzi, C. Calvi, P. Roma and A.L. Catapano, J. Lipid Res., 24 (1983) 790.
5 K.H. Weisgraber and R.W. Mahley, J.Lipid Res., 21 (1980) 316.
6 M.W. Huff and D.E. Telford, Biochim. Biophys. Acta, 796 (1984) 251.
7 J. Borensztajn, T.J. Kotlar and S.C. Meredith, J. Biol. Chem., 260 (1985) 13047.
8 J.L. Beaumont, L.A. Carlson, G.R. Cooper, Z. Fejfar, D.S. Fredrickson and T. Strasser, Bull. WHO, 43 (1970) 891.
9 W.J. Schneider, P.T. Kovanen, M.S. Brown, J.L. Goldstein, J.G. Utermann, W. Weber, R.J. Havel, L. Kotite, J.P. Kane, T.L. Innerarity and R.W. Mahley, J. Clin. Invest., 68 (1981) 1075.
10 R.J. Havel, H.A. Eder and J.H. Bragdon, J. Clin. Invest., 34 (1955) 1345.
11 O.H. Lowry, N.J. Rosenbrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265.
12 K. Weber and M. Osborn, J. Biol. Chem., 244 (1969) 4406.
13 Ö. Ouchterlony, Ark. Kemi B, 26 (1948) 1.
14 A. Görg, W. Postel and R. Westermeier, Anal. Biochem., 89 (1978) 60.
15 P. Weisweiler and P. Schwandt, Clin. Chim. Acta, 124 (1982) 45.
16 P. Weisweiler, C. Fried and P. Schwandt, Biochem. Biophys. Acta, 875 (1986) 48.
17 P. Nestel, N. Tada, T. Billington, M. Huff and N. Fidge, Metabolism, 31 (1982) 398.
18 R.J. Havel and J.P. Kane, Proc. Natl. Acad. Sci. U.S.A., 70 (1973) 2015.
19 D.Y. Hui, T.L. Innerarity, R.W. Milne, Y.L. Marcel and R.W. Mahley, J. Biol. Chem., 259 (1984) 15060.