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Short communication

Simple one-step procedure for the separation of apolipoproteins A1 and A2 by high-performance liquid chromatography

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Abstract

A simple assay method for apolipoproteins apo A1 and apo A2 by HPLC is introduced. The simple one-step method is based on fractionation of apo A1 and apo A2 from other serum proteins which are precipitated at 100°C and removed by centrifugation. The apo A1 and apo A2 which remain in solution can be recovered and resolved by size-exclusion chromatography without ultracentrifugation and delipidation by an organic solvent. This makes sample preparation easier. The recoveries of apo A1 and apo A2 were 104.26% and 101.04%; the precision (C.V.%) of apo A1 and apo A2 was 0.88 and 1.63 respectively © 1997 Elsevier Science B.V.

Keywords: Apolipoproteins

1. Introduction

Coronary heart disease (CHD) is a primary human health problem in most industrialized countries [1]. It is known that hyperlipidemia and some lipoproteins i.e, VLDL, LDL are meaningful risk factors below age 50. In contrast, HDL protects against development of premature atherosclerosis. More recently, much attention has focused on apolipoproteins which are protein components of serum lipoproteins. These apolipoproteins are structural constituents of lipoprotein particles and have been shown to participate in lipoprotein synthesis, secretion, processing and catabolism.

Numerous clinical and epidemiological studies suggest that the measurement of apolipoprotein would be useful for prevention, treatment and diagnosis of CHD, and would provide an insight into the molecular basis of the link between lipoproteins and atherosclerosis and contribute to the understanding of the disease [1–4].

Some investigations have shown that HDL subfractions HDL1, HDL2, apolipoprotein Al, B and their ratio have high specificity and sensitivity compared with other biochemical parameters used in the assessment of CHD.

Apolipoproteins A1 and A2 are major proteins of HDL. Al is known as an activator of the enzyme lecithin cholesterol acyltransferase (LCAT), whereas apo A2 is thought to inhibit LCAT in the presence of apo Al. Apo A1 may be a negative predictor for CHD [5].

Recently, two methods have been used to determine apolipoproteins: immunotechnology and high-performance liquid chromatography (HPLC). The use of the latter technique for the measurement of apolipoprotein has resulted in both rapid and precise analyses [5–8]. The procedure conducted by

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other workers for separation of apolipoprotein is as follows: the lipoprotein fractions of serum i.e, VLDL, LDL and HDL are separated by ultracentrifugation. Following delipidation with an organic solvent, the lipoprotein is further separated into their apolipoproteins by HPLC. The ultracentrifugation followed by organic delipidation requires expensive equipment and is a complex, time consuming procedure which can result in partial loss of apolipoproteins [9–13].

Our experiments have led to the development of a simple one-step procedure for the separation of apolipoproteins A1 and A2. This method involves analysis of serum by size-exclusion HPLC without ultracentrifugation and delipidation by an organic solvent.

After incubation of serum for 5 min at 100°C, HPLC analysis is carried out and apolipoprotein A1, A2 values are obtained with good recovery and precision within 30 min.

2. Experimental

2.1. Equipment

We used Waters HPLC equipment including: Model 1510 HPLC pump, Model 715 WISP, Model 740 data module and Model 481 LC spectrophotometer (Waters, Milford, MA, USA). The following columns were used connected in series: Bio-Sil TSK guard column (75×7.5 mm I.D., Bio-Rad, Hercules, CA, USA), Bio-Sil TSK 250 column (300×7.5 mm I.D., Bio-Rad) and Bio-Sil TSK 400 column (300×7.5 mm I.D., Bio-Rad). The ultracentrifuge used was LB-55, roto type 70.ITI (Beckman, San Ramon, CA, USA), heating block BT3 (Grant Instruments, London, UK), Mutiphor electrophoresis tank Model 2117 (LKB, Bromma, Sweden), power supply Model 2002 (LKB) and Mutitemp cooling apparatus Model 2209 (LKB).

2.2. Reagents and standards

Gel permeation molecular mass standards and Biogram Apo A1/B kit were purchased from Bio-Rad. Apolipoprotein A1 standard, apolipoprotein A2 standard, apolipoprotein A standard, guanidine hydrochloride and sodium dodecyl sulfate (SDS) were obtained from Sigma (St. Louis, MO, USA). Sodium sulphate was purchased from Laboratory (Rochester, NY, USA). Sodium dihydrogen orthophosphate and disodium hydrogen orthophosphate were purchased from Univar (Brisbane, Australia). M-partrgen apo A and lipo-partrgen apo Al were purchased from Behring (Marburg, Germany). Human pooled serum was used in all experiments.

2.3. Procedure

A 2-ml volume of pooled serum was incubated in boiling water for 5 min. Alternatively, the serum was heated in a heating block for 10 min. After centrifugation at 780 *g* for 5 min the supernatant was filtered through a 0.22-mm filter (Millipore, Newstead, Brisbane, Australia). A 20-μl sample of filtered supernatant was injected onto the column. The HPLC conditions were: flow-rate 1.0 ml/min at ambient temperature, eluent solution consisted of 0.05 *M* Na_2SO_4, 0.02 *M* NaH_2PO_3, containing 0.1% SDS and 0.05% NaN_3 (pH 6.8). Protein eluted from the column was detected at 280 nm. Quantity of apolipoprotein was measured by external standard calibration. For these determinations we used pure apo A1 and pure apo A2 as external standard calibrators and the pretreated apo A as a control.

Radial immunodiffusion (RID) and immunoelectrophoresis (IEP) were carried out using standard methods as described by the manufacturer.

3. Results and discussion

We examined the relationship between elution time and the logarithm of molecular masses for the gel permeation column using commercially available molecular mass standards. Fig. 1 illustrates this relationship.

The standard molecular masses of apo Al and apo A2 were 28 000 Da and 17 000 Da respectively. The elution times for apo Al and apo A2 were approximately 18 min and 20 min respectively. Figs. 2–4 show the eluent pattern of pure standard apo A1, apo A2, HDL and pretreated serum.

We collected eluates of pooled serum from HPLC at 18 and 20 min elution time. After concentration, these collected samples were placed in radial im-

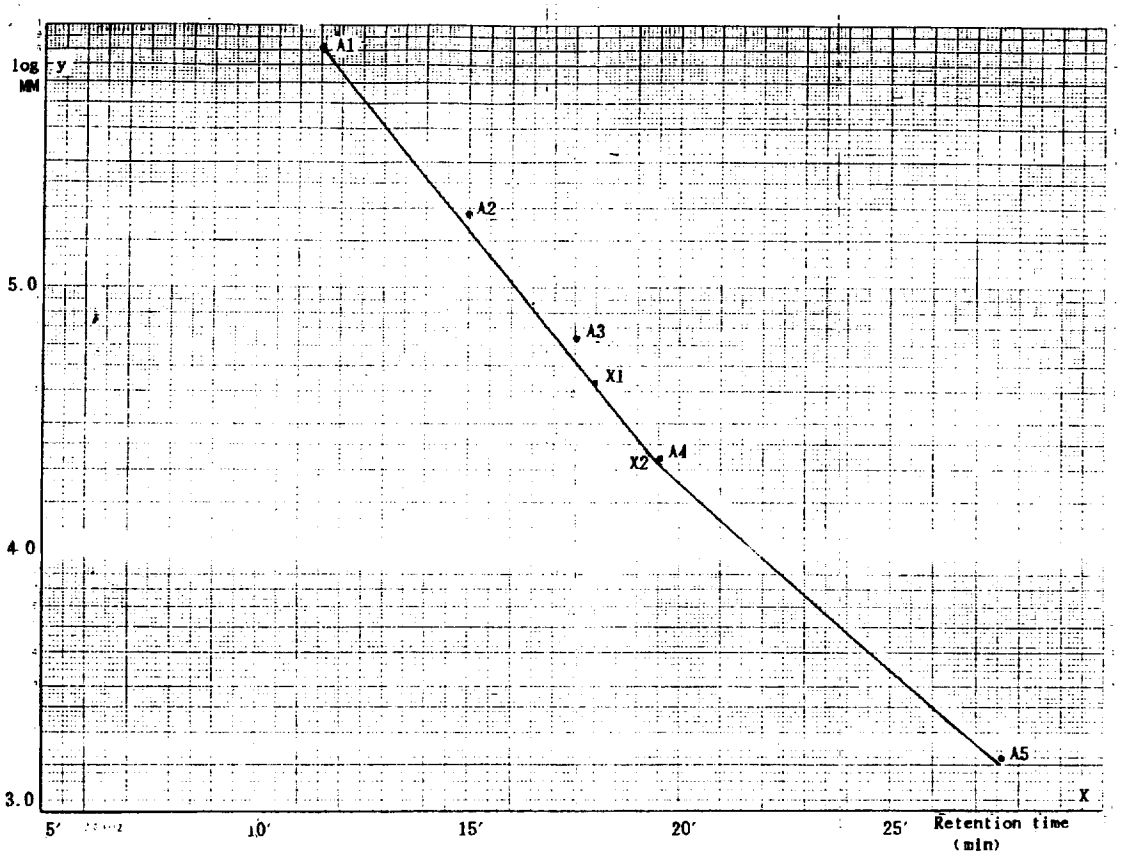

Fig. 1. Curve of log M_r versus retention time.

Annotation	Molecular mass (Da)
A1 Thyroglobulin	670 000
A2 γ-globulin	158 000
A3 Bovine albumin	44 000
A4 Myoglobulin	17 000
A5 Vit B12	1350
X1 Apo A1	28 000
X2 Apo A2	17 000

munodiffusion wells in lipo-partrgen apo A1 and M-partrgen apo A plates. A ring of precipitation appeared in both RID plates where the sample collected from the HPLC at 18 min was applied. However, no precipitation ring appeared in the lipo-partrgen apo A1 and a precipitation ring appeared in the M-partrgen apo A plates when sample collected at 20 min was applied.

There are two ways to estimate recovery: (1) Assessing analytical recovery by adding pure apo A1 and pure apo A2 to pooled serum. To each 1.0 ml of serum we added 0.15 ml pure apo A1 (200 mg/dl), 0.15 ml apo A2 (100 mg/dl) and 0.15 ml elute. Using the same preparation, the sample was applied to the HPLC column. Table 1 illustrates the results obtained. The recovery for apo A1 was 104.26%, and for apo A2 101.04%. (2) Using a pooled human serum for which apo A1 and apo A2 values were determined. We conducted an experiment by using commercial standard. To each 1 ml of unknown

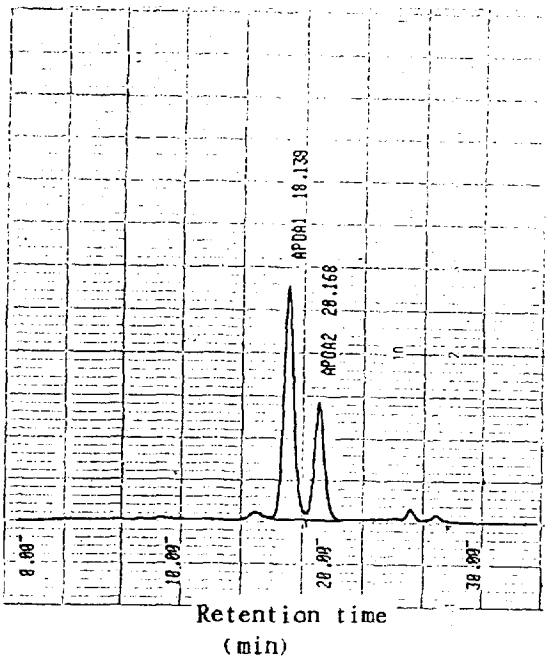

Fig. 2. Eluent pattern of pure apo A1 and apo A2 — for chromatography conditions see Section 2.3.

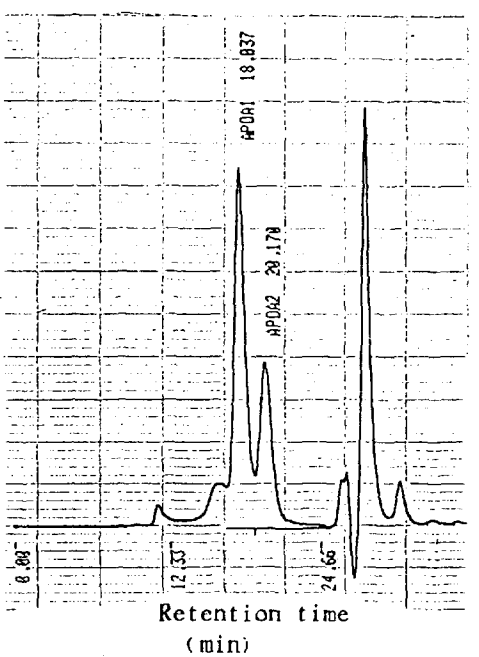

Fig. 3. Eluent pattern of pure HDL — for chromatography conditions see Section 2.3.

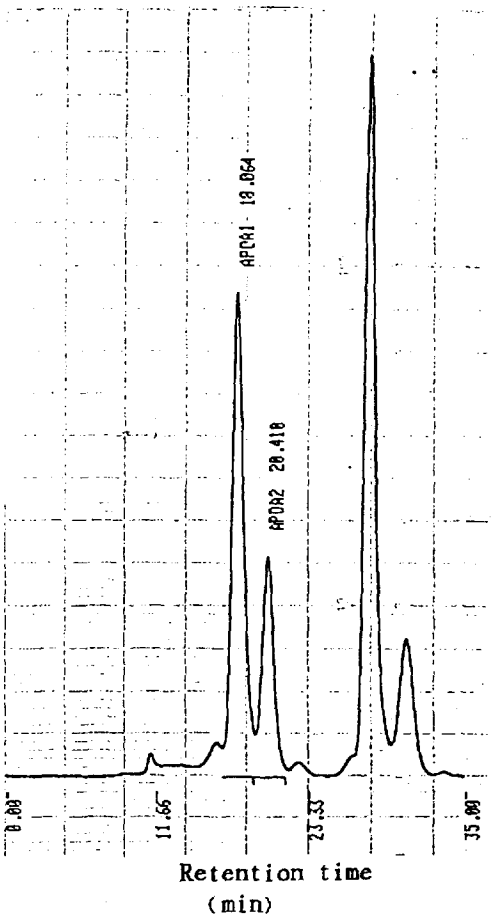

Fig. 4. Eluent pattern of the serum supernatant after incubation for 5 min at 100°C — for chromatography condition see Section 2.3.

serum, we added varying volumes of determined serum. The linear regression lines for the relationships between apo A1 and added apo A2 and concentrations determined were: $y=70.03+0.766x$ ($r=0.999$) and $y=30.88+0.691x$ ($r=0991$), respectively.

The samples of pooled serum were pretreated under similar conditions. The mean concentration of apo A1 was 192.1±1.68 mg/dl, the coefficient of variation (C.V.) was 0.88%, and the mean concentration of apo A2 was 93.4±1.53 mg/dl, the coefficient of variation (C.V.) was 1.63%.

We compared the results of our HPLC experiments with those obtained by IEP and RID. The correlation coefficient between IEP and our method

Table 1
Recoveries of pure apo A1 and apo A2

	Apo A1 (μg/dl)			Apo A2 (μg/dl)		
	Detected	Expected	Recovery	Detected	Expected	Recovery
1 ml Serum A+150 μl buffer	173.29			91.31		
1 ml Serum A+150 μl apo A1 (200 μg/dl)	207.8	199.37	104.26%			
1 ml Serum A+150 μl apo A2 (100 μg/dl)				105.45	104.36	101.04%

was 0.71% ($n=6$, $P<0.025$). The correlation coefficient between our method and RID was 0.15% ($n=6$, $P>0.1$).

Without pretreatment, the interference from other proteins in serum would affect the determination of apo A1 and apo A2. The principle of our method is based on the coagulation temperature of apo A1 and apo A2 which is above 100°C. The coagulation temperature of most proteins is below 100°C.

The other function of the incubation of serum at 100°C is delipidation. Traditional methods of delipdation use an organic solvent [6–8]. Other workers used heating at 60°C for 5 min to delipidate the serum [9,10]. After experimentation by using fat red staining electrophoresis results, we found that incubation of serum at 100°C for 5 min is an optimal and complete delipidation procedure compared with delipidation using an organic solvent.

We used several eluents previously used by other workers [6–11], i.e, 6 *M* urea buffer, 4 *M* guanidine hydrochloride buffer, phosphate buffer (pH 7.0) and sodium phosphate buffer. Various pH values were tested. Urea buffer and guanidine hydrochloride buffer of high concentration did not provide satisfactory results. As other workers observed, 0.1 *M* phosphate buffer provided adequate resolution, but the buffer also caused precipitation; 0.05 *M* Na_2SO_4, 0.02 *M* NaH_2PO_4 (pH 6.8) elution solvent provided adequate resolution of apolipoprotein. With the addition of 0.1% SDS to this eluent, the peak resolution was improved and quantification is achieved. We chose a flow-rate of 1.0 ml/min in this case, and obtained satisfactory results within 35 min.

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