Journal of Chromatography, 430 (1988) 135–142
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4238

Note

Improved high-performance liquid chromatographic method for the determination of apolipoproteins in serum high-density lipoproteins

MITSUYO OKAZAKI*

Laboratory of Chemistry, Department of General Education, Tokyo Medical and Dental University, Kohnodai, Ichikawa, Chiba Prefecture 272 (Japan)

MAKOTO KINOSHITA

First Department of Internal Medicine, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113 (Japan)

and

ICHIRO HARA

Scientific Instrument Division, Tosoh Co., Ltd., Hayakawa, Ayase, Kanagawa Prefecture 252 (Japan)

(First received March 11th, 1987; revised manuscript received March 16th, 1988)

In previous papers [1–3] we reported a simple and rapid method for the determination of apolipoproteins in high-density lipoproteins (HDL) by high-performance liquid chromatography (HPLC); the sample was an incubation mixture (60°C, 5 min) of HDL, the eluent consisted of 0.1 *M* sodium phosphate buffer (pH 7.0) containing 0.1% sodium dodecyl sulphate (SDS) and a gel permeation column (TSKgel G3000SW) was used. In comparison with other HPLC methods for apolipoprotein determination using urea or guanidinium chloride solution as the eluent [4, 5], this method has the advantage of eliminating the need for delipidation of HDL with organic solvents. However, the separation of the apolipoprotein (apo) A-II and apo C groups is not satisfactory, because the molecular mass of the apo C group is below the lower limit of the separation range of gel permeation chromatography in SDS solution [3].

Sodium deoxycholate (NaDOC) is used for the delipidation of serum lipoproteins, including low-density lipoproteins (LDL) in which binding of lipids and apolipoproteins is stronger than with the other lipoprotein classes, and LDL delipidated with NaDOC retain the immunological properties of the native material

0378-4347/88/$03.50

[6, 7]. Also, the application of NaDOC and Sephacryl-200 for the delipidation and separation of HDL has been reported by Robern [8].

In this paper, we examine the HPLC determination of HDL apolipoproteins using NaDOC solution as a solvent for delipidation and separation and compare the results with those given by our previous SDS system. The determination of the major apolipoprotein components of HDL, apo A-I and apo A-II, by our NaDOC system was compared with that of a single radial immunodiffusion (SRID) assay. Elution profiles of the lipid components of HDL given by HPLC with NaDOC solution were also examined.

EXPERIMENTAL

Samples

HDL was prepared from serum by the sequential flotation method of Havel et al. [9] as the fraction of density 1.063–1.21 g/ml as described previously [1, 2].

The apolipoprotein fraction of HDL (apo HDL) was prepared from HDL by delipidation with ethanol–diethyl ether [10]. Standard samples of the apo A-I, apo A-II, apo E and apo C groups were prepared using established procedures as described previously [1,2]. The proteins used for column calibration were those in a low-molecular-mass electrophoresis calibration kit (Pharmacia, Uppsala, Sweden).

Reagents

Cholesterol and choline-containing phospholipids in the effluent from the column were detected enzymatically by commercial reagent kits: Determiner TC"555" (Kyowa Medex, Tokyo, Japan) for cholesterol and PL kit K"f" (Nippon Shoji, Osaka, Japan) for choline-containing phospholipids. Quantification of apo A-I and apo A-II by an SRID assay [11] was carried out using commercially available kits: apo A-I plate and apo A-II plate (Daiichi Kagaku, Tokyo, Japan). All reagents were of the highest grade commercially available.

Sample preparation for HPLC analysis

HDL (10 μl containing 20–300 μg of protein) was added to 200 μl of 0.05 *M* ammonium hydrogencarbonate solution (pH 7.95) containing 10 m*M* NaDOC and 0.05 *M* sodium chloride, and the mixture was used as a sample for HPLC analysis. Standard proteins, purified apolipoproteins and apo HDL were dissolved in the same NaDOC solution before HPLC analysis. The protein content in the sample solution was determined by the Folin phenol method [12] using bovine serum albumin as a standard.

Analysis by HPLC

The separation of apolipoproteins in HDL was carried out by HPLC (HLC 803D, Tosoh, Tokyo, Japan) with an aqueous gel permeation column (TSKgel, Tosoh). The HPLC conditions were as follows: column, G3000SW (600 mm × 7.5 mm I.D.); eluent, 0.05 *M* ammonium hydrogencarbonate solution (pH 7.95) containing 10 m*M* NaDOC and 0.05 *M* sodium chloride; flow-rate, 0.53 or 0.33 ml/

min; sample volume, 100 μl. Proteins in the effluent from the column were detected at 280 nm. Cholesterol or choline-containing phospholipids in the effluent from the column were detected at 550 or 500 nm, respectively, after on-line enzymatic reaction of our method for lipoprotein analysis [13].

RESULTS AND DISCUSSION

Of the various grades of gel permeation columns (TSKgel SW type), a G3000SW column was used for the separation of HDL apolipoproteins in the light of the results obtained with our previous method [3]. The composition of the NaDOC solution was determined from the study of Robern [8] to be 10 m*M* NaDOC–0.05 *M* ammonium hydrogencarbonate–0.05 *M* sodium chloride; we used 0.05 *M* ammonium hydrogencarbonate (pH 7.95) instead of 0.05 *M* sodium carbonate (pH 10) in order to protect the column, as the lifetime of the column (TSKgel SW type) is well known to decrease with increase in the pH of the eluent.

Elution patterns of the purified apolipoproteins of the apo E, apo A-I, apo A-II and apo C groups obtained with the above column and eluent system are shown in Fig. 1. These standard apolipoproteins were eluted in order of their molecular masses, and the separation of the apo A-II and apo C groups was improved in comparison with that in our previous system using SDS solution [1–3]. Moreover, the elution pattern of the apo C group showed heterogeneity of molecular mass due to the subfractions: apo C-I, 6500; apo C-II, 8800; apo C-$III_{0,1,2}$, 8900.

The relationship between the molecular mass and the elution volume for the G3000SW column in NaDOC solution was examined using standard proteins and apolipoproteins of known molecular mass. The two peaks of the apo C group were identified as follows: the first peak was apo C-II plus apo C-III, the molecular mass adopted being the mean of the two (8850), and the second peak was apo C-I. As shown in Fig. 2, the plot of the elution volume against the logarithm of molecular mass is linear for all compounds, and the slope for the molecular mass range below 20 000 is larger than that for the range above 20 000. In our previous SDS system, the linear relationship broke down at molecular masses below 15 000 [3]. These results indicate that the separation of the apo A-II and apo C groups, which was impossible with our previous system, can be achieved with the use of NaDOC solution as the eluent.

The HPLC pattern of the apolipoprotein fraction in HDL (apo HDL) prepared by delipidation with organic solvents (ethanol–diethyl ether) from HDL was compared with that obtained for HDL using the same preparation procedure as with our previus SDS system, i.e., application of a mixture of HDL and NaDOC solution incubated at 60°C for 5 min. Elution patterns monitored at 280 nm for the above two samples from the same subjects are presented in Fig. 3. The elution positions of the apo E, apo A-I, apo A-II and apo C groups determined from the calibration graph in Fig. 2 are shown as arrows with numbers. For the delipidated sample (apo HDL), two peaks of the apo C group similar to those of the purified standard sample were observed. However, the elution pattern of the apo C group for an HDL sample without delipidation with organic solvents was different from that for a standard sample or apo HDL. Moreover, another peak behind the apo

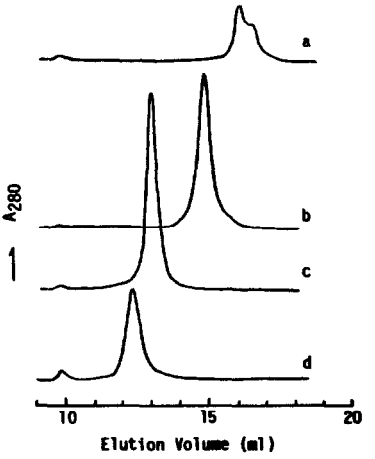

Fig. 1. Elution pattern of standard apolipoproteins. Column: G3000SW (600×7.5 mm I.D.). Eluent: 0.05 *M* ammonium hydrogencarbonate (pH 7.95) containing 10 m*M* sodium deoxycholate and 0.05 *M* sodium chloride. Flow-rate: 0.53 ml/min. Sample: purified apolipoproteins dissolved in the eluent. a, Apo C group; b, apo A-II; c, apo A-I; d, apo E. Sample volume: 100 μl. Detection: 280 nm, 0.04 absorbance units, 10 mV.

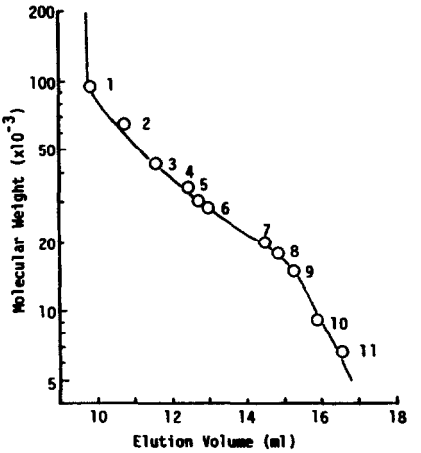

Fig. 2. Relationship between elution volume and molecular mass for the gel permeation column (G3000SW). Sample: standard proteins (low-molecular-mass calibration kit) and purified apolipoproteins dissolved in the eluent. 1, Phosphorylase b; 2, bovine serum albumin; 3, ovalbumin; 4, apo E; 5, carbonic anhydrase; 6, apo A-I; 7, soybean trypsin inhibitor; 8, apo A-II; 9, α-lactalbumin; 10, apo C-II and apo C-III; 11, apo C-I. Other HPLC conditions as in Fig. 1.

C group was observed in the case of HDL. The elution profile, except for the apo C group, was the same for the two samples. Therefore, without delipidation with organic solvents, the determination of HDL apolipoproteins was found to be performed equally well with the NaDOC system and our previous SDS system. The separation of the apo A-II and apo C groups was found to be better with the NaDOC system than with the previous SDS system, which made it possible to quantify apo A-II from the peak area in the HPLC pattern, as described later.

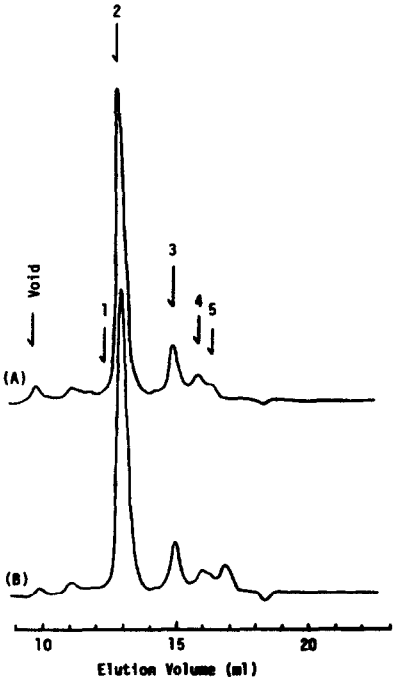

Fig. 3. Elution pattern of HDL apolipoproteins. Sample: (A) apo HDL dissolved in the eluent and (B) HDL incubated with the eluent at 60°C for 5 min. Elution positions: 1, apo E; 2, apo A-I; 3, apo A-II; 4, apo C-II and apo C-III; 5, apo C-I. Other HPLC conditions as in Fig. 1.

The procedure of sample preparation for HPLC analysis using the NaDOC system was examined for the following three conditions: (1) incubation of a mixture of HDL and NaDOC solution at 60°C for 5 min; (2) mixing of HDL and the NaDOC solution; (3) no pretreatment. The HPLC patterns monitored at 280 nm were the same for all three conditions (results not shown). The effect of the concentration of HDL applied to the column on the HPLC pattern monitored at 280 nm was examined for the three conditions, using HDL samples containing 16–400 μg of protein. The apparent peak area of apo A-II increased with increase in the HDL concentration applied to the column for more than 200 μg of protein, but not with apo A-I, for all three conditions. This seems to be due to the decrease in the peak resolution of the apo A-II and apo C groups by the elution of lipid micelles with NaDOC in a similar molecular mass range to the apo A-II and apo C groups, as described later. Then, the critical determination of HDL apolipoproteins must be carried out on HDL samples containing less than 200 μg of protein if the delipidation procedure with organic solvents is eliminated.

We examined the elution profiles of the lipid components of HDL in this analytical system. Cholesterol or choline-containing phospholipids in the effluent from the column was detected at 550 or 500 nm, respectively, by our established on-line lipid monitoring method [13]. Elution patterns monitored at 280 nm (protein), 550 nm (cholesterol) and 500 nm (choline-containing phospholipids) for HDL samples dissolved in NaDOC solution are shown in Fig. 4. Bile salts are

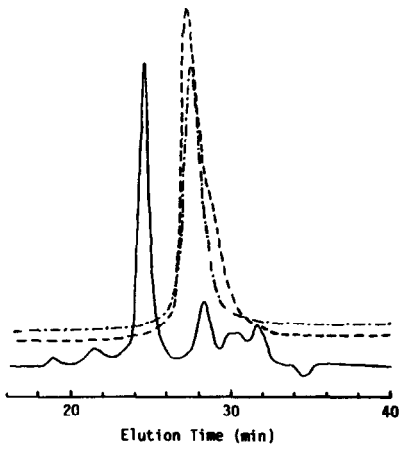

Fig. 4. Elution profile of protein (—), cholesterol (- - -), and choline-containing phospholipids (-·-·-). Sample: HDL dissolved in the eluent. Detection: 280 nm (—), 0.04 absorbance units, 10 mV; 550 nm (- - -), 0.04 absorbance units, 10 mV; 500 nm (-·-·-), 0.08 absorbance, 10 mV. Other HPLC conditions as in Fig. 1.

known to form small mixed micelles with lipids such as cholesterol, phospholipids and glycerides [14,15]. In our system, both cholesterol and choline-containing phospholipids eluted in the molecular mass range between apo A-I and apo A-II to form small mixed micelles with NaDOC, as shown in Fig.4. As these mixed micelles of lipids and NaDOC have no significant absorption at 280 nm, the determination of apolipoproteins is not disturbed by the coelution of the mixed micelles. On the other hand, the peak after the apo C group, in which no lipids or apolipoproteins can be detected, interferes with the determination of the apo C group, and this influence is emphasized by low content of the apo C group. This peak may be due to the elution of NaDOC micelles, which elute at elution volumes from 17 to 19 ml. This peak is not observed with delipidated samples such as apo HDL and purified apolipoproteins. Therefore, precise information about the apo C group cannot be obtained with the NaDOC system using HDL samples without the delipidation procedure with organic solvents.

For fifteen normal human subjects, the concentration of apo A-I and apo A-II in HDL was measured using our HPLC method and an SRID assay [11]. The concentration of apo A-I and apo A-II obtained with the HPLC method was determined from peak areas by using a calibration graph prepared using a purified sample of apo A-I and apo A-II. A good correlation between the two methods was obtained for both apo A-I and apo A-II. The concentrations of apo A-I and apo A-II in the HDL of these subjects were 2–25 and 1–10 mg/ml, respectively. The correlation coefficients between the two methods were 0.973 and 0.971 for apo A-I and apo A-II, respectively. Therefore, the quantification of apo A-I and apo A-II was comfirmed to be possible by using our HPLC method with an HDL sample without the delipidation procedure with organic solvents.

HPLC patterns monitored at 280 nm using a G3000 SW column for HDL samples from various subjects dissolved in NaDOC solution are shown in Fig. 5. A

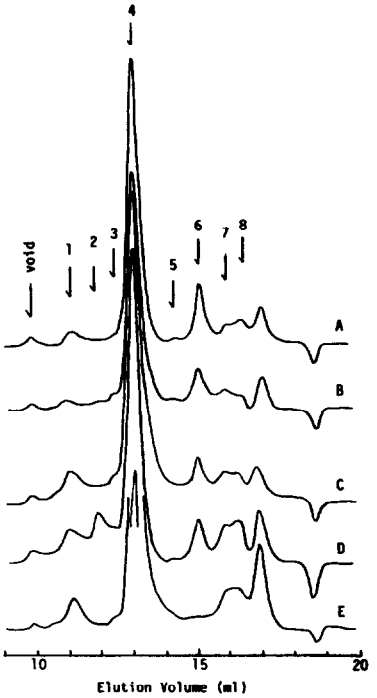

Fig. 5. Elution pattern of HDL apolipoproteins for various subjects. Sample: HDL from (A–D) human serum and (E) dog serum dissolved in the eluent. A, Normal male; B, lecithin:cholesterol acyltransferase deficiency; C, acute hepatitis; D, hyper-α-lipoproteinaemia. Elution positions: 1, serum albumin; 2, apo A-IV; 3, apo E; 4, apo A-I; 5, apo D; 6, apo A-II; 7, apo C-II and apo C-III; 8, apo C-I. Flow rate: 0.33 ml/min. Other HPLC conditions as in Fig. 1.

small void peak, a broad peak due to albumin contaminated in the ultracentrifugation procedure, a large peak of apo A-I and variously shaped peaks of the apo C group were commonly observed for all subjects, and a peak of apo A-II was observed for all subjects except for the dog. A small shoulder peak of apo E was observed in front of the apo A-I peak for some subjects. Also, the peaks at elution volumes corresponding to molecular masses of 46 000 and 20 000 from the calibration graph in Fig. 2 may be due to apo A-IV and apo D, respectively. Compared with the HPLC patterns obtained with our previous SDS system using two G3000SW columns (see Fig. 4 in ref. 3), the peak resolution was improved with the NaDOC system even though a single G3000SW column was used. As shown in Fig. 5, HPLC patterns obtained with a small amount of HDL (less than 100 μg of protein) without the delipidation procedure with organic solvents were found to give useful qualitative and quantitative information about HDL apolipoproteins in a short experimental time (less than 50 min), and the NaDOC system was found to be superior to the previous SDS system.

Our method for the determination of HDL apolipoproteins using NaDOC solution is useful for the study of lipoprotein metabolism because of the simple experimental procedure, the short experimental time and the small amount of

sample required for analysis. We are examining the application of this NaDOC system to the determination of apolipoproteins in other lipoprotein classes.

ACKNOWLEDGEMENTS

The authors gratefully appreciate the gifts of serum from and useful discussions with Dr.Y. Matsuzawa of Osaka University and Dr. T. Teramoto and Dr. H. Kato of Tokyo University.

REFERENCES

1 M. Kinoshita, M. Okazaki, H. Kato, T. Teramoto, T. Matsushima, C. Naito, H. Oka and I. Hara, J. Biochem., 94 (1983) 615.
2 M. Kinoshita, M. Okazaki, H. Kato, T. Teramoto, T. Matsushima, C. Naito, H. Oka and I. Hara, J. Biochem., 95 (1984) 1111.
3 M. Okazaki, M. Kinoshita, C. Naito and I. Hara, J. Chromatogr, 336 (1984) 151.
4 D. Polacek, C. Edelstein and A.M. Scanu, Lipids, 16 (1981) 927.
5 C.T. Wehr, R.L. Cunico, G.S. Ott and V.G. Shore, Anal. Biochem., 125 (1982) 386.
6 A.Helenius and K. Simons, J. Biochem., 10 (1971) 2542.
7 M.T. Walsh and D. Atkinson, Biochemistry, 22 (1983) 3170.
8 H. Robern, Experientia, 38 (1982) 437.
9 R.J. Havel, H.A. Eder and J.H. Bragden, J. Clin. Invest., 34 (1955) 1345.
10 A. Scanu, J. Toth and C. Edelstein, Anal. Biochem., 44 (1971) 576.
11 J.J. Albers, P.W. Wahl, V.G. Cavana, W.R. Hazzard and J.J. Hoover, Metabolism, 25 (1976) 633.
12 O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265.
13 I. Hara and M. Okazaki, Methods Enzymol., 129 (1986) 57.
14 F.P. Woodfor, J. Lipid Res., 10 (1969) 539.
15 W. Shankland, Chem. Phys. Lipids, 4 (1970) 109.