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Anion-exchange high-performance liquid chromatography assays of plasma lipoproteins and modified low-density lipoproteins using a ProtEx-DEAE column

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Abstract

Previously [Anal. Biochem., 232 (1995) 163–171], we reported a high-performance liquid chromatography (HPLC) assay method for human plasma lipoproteins using a diethylaminoethyl (DEAE)-glucomannan column, which is not commercially available. In this study, HPLC assay methods for lipoproteins in plasma samples of human and experimental animals, and modified low-density lipoproteins (LDLs) of rabbits have been developed using a commercially available anion-exchange ProtEx-DEAE column. For the assays of plasma lipoproteins, the method includes complete separation of high-density lipoproteins, LDLs and very low-density lipoproteins within 20 min using stepwise elution, and determination by post-column reaction with an enzymatic cholesterol reagent as the total cholesterol (TC) level. Similarly, mild oxidative and artificially oxidised LDLs were separated into their subfractions using stepwise elution, and determined based on the TC level. The methods using the DEAE-glucomannan and ProtEx-DEAE columns were cross-validated. There was an excellent correlation between the two methods. The obtained results reveal that the anion-exchange HPLC method using the ProtEx-DEAE column could be useful for the assays of plasma lipoproteins and modified LDLs.

Keywords: Lipoproteins; Modified low-density lipoproteins

1. Introduction

The major plasma lipoproteins are known as high-, low- and very low-density lipoproteins (HDLs, LDLs and VLDLs, respectively). High plasma LDL and low HDL levels are well-known to accelerate the progression of atherosclerosis and related diseases [1]. Additionally, several studies have revealed that modified LDL plays an important role in the initiation and development of atherosclerosis [2–4]. Notably, oxidatively modified LDL was demonstrated to be present in atherosclerotic plaques in vivo [5]. We have reported a high-performance liquid chromatography (HPLC) method for the assays of lipoproteins in plasma samples of human [6] and experimental animals [7]. The method included complete separation of HDLs, LDLs and VLDLs within 20 min using a diethylaminoethyl (DEAE)-gluco-

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mannan gel with stepwise elution, enzymatic oxidation of cholesterol (following ester hydrolysis) followed by a two-step reaction to form a fluorogenic product, and fluorometric detection as the total cholesterol (TC) level of each lipoprotein. Further, the method was applied to the assay of artificially modified LDLs by $CuCl_2$ [8], the search for evidence for the presence of oxidatively modified LDLs in the plasma of Watanabe heritable hyperlipidemic (WHHL) rabbits [8], and the detection of cigarette smoke-modified LDLs [9]. However, the method has a disadvantage that the DEAE-glucomannan gel is not commercially available. We tried to find a commercially available anion-exchange HPLC column, which is applicable to the separation of plasma lipoproteins and modified lipoproteins, and to develop an accurate and precise HPLC method for the assays of them. Among the commercially available anion-exchange HPLC columns, the MCI GEL ProtEx-DEAE column could separate three lipoproteins reproducibly.

In this study, we deal with an anion-exchange HPLC assay method for lipoproteins in plasma samples of human and experimental animals, and mildly modified LDLs in the plasma of WHHL rabbits and artificially oxidised LDLs of WHHL rabbits using a ProtEx-DEAE column.

2. Experimental

2.1. Reagents and chemicals

Cholesterol oxidase (CO; EC 1.1.3.6; 18 U/mg) from *Streptomyces* sp., peroxidase (PO; EC 1.11.1.7; 134 U/mg) from horseradish and cholesteryl ester hydrolase (CEH; EC 3.1.1.13; 144 U/mg) from *Pseudomonas* sp. were purchased from Toyobo (Osaka, Japan), homovanillic acid (HVA) from Tokyo Kasei (Tokyo, Japan), Triton X-100 and disodium ethylenediaminetetraacetic acid (EDTA) from Nacalai Tesque (Kyoto, Japan). The other chemicals of analytical-reagent grade were purchased from Nacalai Tesque. Water purified with a Milli-Q Jr. (Millipore, Tokyo, Japan) was used for the preparation of the eluents.

2.2. Chromatography

The experimental setup used in this study is as described in a previous report [6]. Four pumps used were as follows: two PU-980 intelligent pumps for delivering eluents (eluents A and B), and two 880-PU intelligent pumps (all from Japan Spectroscopic, Tokyo, Japan) for delivering an enzymatic cholesterol reagent and a sodium hydroxide solution. The injector was a Rheodyne 7125 loop injector with a 50-μl loop (Cotati, CA, USA). A DEAE-glucomannan gel (particle diameter 30–90 μm) [10,11], which was kindly donated by Kurita Industries (Tokyo, Japan), is packed into a 50×4.6 mm I.D. stainless steel column. MCI GEL CQA31S (particle diameter 10 μm; column size, 75×7.5 mm I.D.) and ProtEx-DEAE (particle diameter 5 μm; column size, 50×4.6 mm I.D.) columns, both of which are anion-exchange columns having DEAE groups, were kindly donated by Mitsubishi (Tokyo, Japan). After post-column reaction with an enzymatic cholesterol reagent followed by alkalization by a sodium hydroxide solution, lipoproteins were detected with excitation wavelength at 325 nm and emission wavelength at 420 nm using a RF-535 spectrofluorimeter (Shimadzu, Kyoto, Japan). Chromatographic data were collected with a CBM-10A interface, transmitted to COMPAQ computer, and integrated using CLASS-LC10 software version 1.41 (Shimadzu). The eluents used were as follows: eluent A, 20 mmol/l sodium phosphate buffer (pH 7.0) containing 1 mmol/l EDTA; eluent B, 500 mmol/l sodium chloride containing 1 mmol/l EDTA. Lipoproteins in plasma samples of human and experimental animals, mildly modified LDLs in the plasma of WHHL rabbits and artificially oxidised LDLs of WHHL rabbits were separated by stepwise elution using eluents A and B. The flow-rate was maintained at 1.0 ml/min. The separation was carried out at 25°C using a water bath (Thermo Minder Lt-100; Taitec, Saitama, Japan). The stepwise elution conditions used for CQA31S and ProtEx-DEAE columns are specified in the legends of the figures and tables. The separation conditions used for DEAE-glucomannan column were as described in previous reports [6,7].

For post-column reaction with an enzymatic

cholesterol reagent, which includes CEH, 5 μg/ml; CO, 20 μg/ml; PO, 50 μg/ml; and HVA, 500 mg/ml, were dissolved in 20 mmol/l sodium phosphate buffer (pH 7.0) containing 0.2% Triton X-100, and delivered at a flow-rate of 0.5 ml/min. The reaction coil (15 m×0.5 mm I.D.) used for the enzymatic reactions was immersed in the water bath (Thermo Minder, Lt-100) at 45°C in order to keep the enzymatic reaction temperature maintained at 37°C, because the enzymatic cholesterol reagent was kept at 4°C. After the enzymatic reaction, 0.1 mol/l sodium hydroxide solution was delivered into the reaction coil (0.5 m×0.5 mm I.D.) at a flow-rate of 0.5 ml/min to alkalize the effluent. The cholesterol level was estimated using a known concentration of a free cholesterol solution as a standard.

2.3. Preparation of blood samples

Blood samples were obtained from human and experimental animals (rabbit, rat and mouse). Blood was drawn into tubes containing EDTA at a final concentration of 1 mg/ml. Plasma was separated by centrifugation (1500 *g* for 10 min) from blood, stored at 4°C and used within 7 days.

2.4. Method validation: repeatability, intermediate precision and calibration graph

The repeatability and intermediate precision data were obtained with the assay of rat plasma samples. The samples were stored at 4°C until assay. The repeatability and intermediate precision investigations were performed over five and three replicates, respectively.

For calibration standards, the plasma samples were prepared at varied concentration from 5 to 1000 μg/ml as the TC level, and assessed by five replicate determinations at each concentration. The calibration graphs were constructed by plotting the peak area to the TC level, using a least-squares regression program (Stat View software package, Abaxus Concepts, Calabasas, CA, USA).

2.5. Cross-validation data between two methods based on DEAE-glucomannan and ProtEx-DEAE columns

Three lipoprotein levels in plasma samples of human and experimental animals were analysed by two methods based on DEAE-glucomannan and ProtEx-DEAE columns. Their values were compared using a least-squares regression program (Stat View software package).

2.6. Preparation of artificially oxidised LDL

An aliquot of LDL, which is obtained from WHHL rabbit plasma, was oxidised by incubation with 5 μmol/l $CuCl_2$ at 37°C for 3 h. The reaction was terminated by addition of 10 μmol/l EDTA. After the termination of the reaction, artificially oxidised LDL was dialyzed with phosphate buffer saline at 4°C, and used as experimental samples.

3. Results and discussion

3.1. Selection of a commercially available anion-exchange HPLC column for lipoprotein separation

We have reported an anion-exchange HPLC method for the assays of lipoproteins and oxidatively modified LDLs using a DEAE-glucomannan column [6–8]. Glucomannan is a heteropolysaccharide, composed of mannose and including glucose [12]. Thus, it seemed that DEAE-glucomannan gels have more hydrophilic surfaces than organic polymer-based anion-exchange columns. Fig. 1A and B show the separation of three lipoproteins, HDLs, LDLs and VLDLs, in human plasma on the commercially available anion-exchange CQA31S and ProtEx-DEAE columns, respectively, with stepwise elution by increasing in the concentration of eluent B to 25, 40 and 100%. Eluents A and B, whose composition was 20 mmol/l sodium phosphate buffer (pH 7.0) and 500 mmol/l sodium chloride (both including 1 mmol/l EDTA), respectively, were used for the stepwise elution. On the CQA31S column, three lipoproteins were coeluted and gave smaller peak area than on the ProtEx-DEAE column. Furthermore,

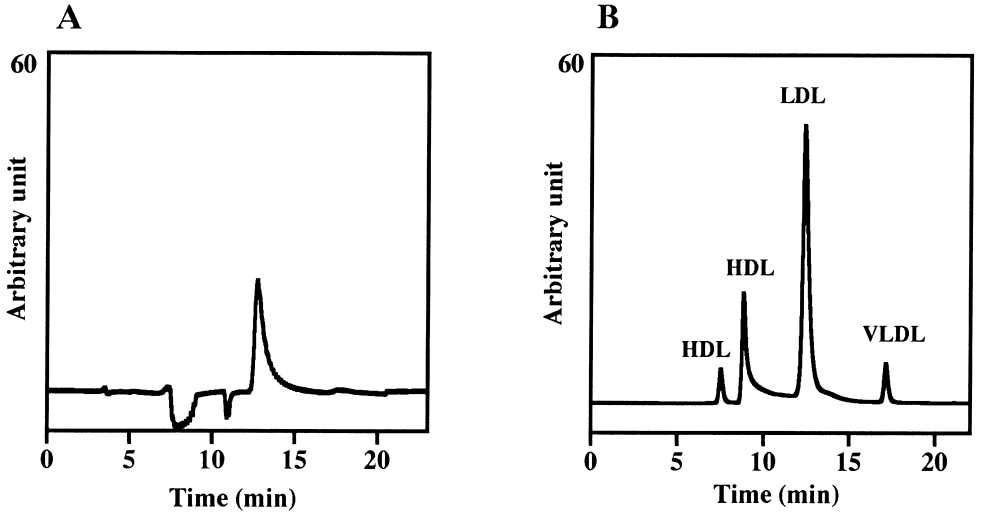

Fig. 1. Chromatograms of human plasma sample on CQA31S (A) and ProtEx-DEAE (B) columns. A 10-μl aliquot of a plasma sample was injected. Column size: 75×7.5 mm I.D. CQA31S column and 50×4.6 mm I.D. ProtEx-DEAE column. Stepwise elution conditions as in Table 1.

reproducible results were not obtained. Since the CQA31S gels were organic polymer-based hydrophobic anion-exchange resins, lipoproteins could be denatured on the surface of the gels. On the other hand, three lipoproteins were separated in the order of HDL, LDL and VLDL within 20 min on the ProtEx-DEAE column. The elution order of HDL, LDL and VLDL was the same with those on the DEAE-glucomannan gel and the migration order on a polyacrylamide gel electrophoresis [6]. Since the ProtEx-DEAE gels [13] have consisted of hydrophilic base materials and hydrophilic crosslinked surface layers as well as the DEAE-glucomannan, it could have almost no serious hydrophobic interactions with lipoproteins. These results suggest that the ProtEx-DEAE column could be useful for the assays of lipoproteins. Though on the ProtEx-DEAE column HDL split into two peaks, the reason has not yet been clarified.

3.2. Separation of HDL, LDL and VLDL in plasma samples of experimental animals

Though we have reported the separation of lipoproteins of experimental animals on the DEAE-glucomannan column [7], the concentration of sodium chloride required for elution of HDL and LDL varied with the animal species. In this study, we tried to separate each lipoprotein in plasma samples of the experimental animals on the ProtEx-DEAE column with a slight modification of the method for human plasma lipoproteins described above. As shown in Table 1, the HDL, LDL and VLDL of rabbits were eluted with 35, 60 and 100% eluent B, respectively; 55, 65 and 100% eluent B for rats and 45, 55 and 100% eluent B for mice. The HDL, LDL and VLDL were separated completely within 20 min, as shown in Fig. 2.

Table 1
Stepwise elution conditions for human and experimental animals[a]

Time (min)	Eluent B (%)			
	Human	Rabbit	Rat	Mouse
0–2	0	0	0	0
2–7	25	35	55	45
7–12	40	60	65	55
12–22	100	100	100	100

[a] Eluent A, 20 mmol/l sodium phosphate buffer (pH 7.0) containing 1 mmol/l EDTA; eluent B, 500 mmol/l sodium chloride containing 1 mmol/l EDTA. The initial condition is 100% eluent A.

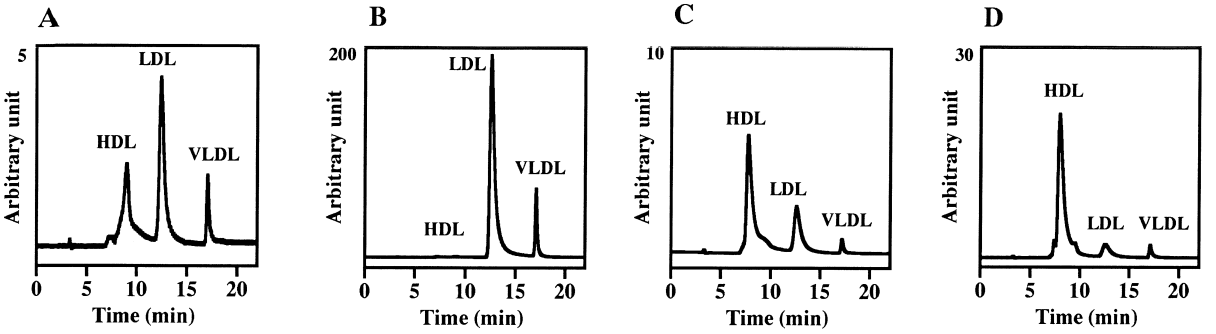

Fig. 2. Chromatograms of plasma samples from JW rabbit (A), WHHL rabbit (B), rat (C) and mouse (D) on a ProtEx-DEAE column. Stepwise elution conditions as in Table 1.

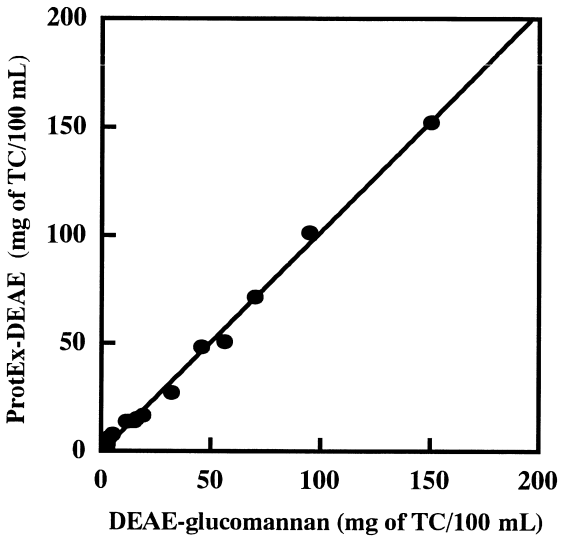

Fig. 3. Cross-validation data between two methods based on DEAE-glucomannan and ProtEx-DEAE columns. A 10-μl aliquot of a plasma sample was injected.

3.3. Cross-validation between two methods based on DEAE-glucomannan and ProtEx-DEAE columns

Fig. 3 shows cross-validation data for the assays of three lipoproteins between the two methods using the DEAE-glucomannan and ProtEx-DEAE columns. The regression equation obtained was $y=1.019x-0.477$ with a correlation coefficient of 0.997, where y is the lipoprotein level estimated using a ProtEx-DEAE column and x is that using a DEAE-glucomannan column. There was an excellent correlation between the two methods.

3.4. Method validation: repeatability, intermediate precision and calibration graph

Table 2 shows the repeatability and intermediate precision of HDL, LDL and VLDL assays in rat plasma samples. The relative standard deviation (RSD) of each lipoprotein assay was highly reproducible, as less than 3%. The established method

Table 2
Repeatability and intermediate precision data for the assay of rat plasma[a]

Lipoprotein	Repeatability[b]			Intermediate precision[c]		
	Mean peak area ($\cdot\mu v\cdot s\cdot 10^5$)	Estimated TC level (mg/100 ml)	RSD (%)	Mean peak area ($\cdot\mu v\cdot s\cdot 10^5$)	Estimated TC level (mg/100 ml)	RSD (%)
HDL	6.96	45.42	0.63	7.00	45.53	0.49
LDL	1.05	6.84	2.70	1.03	6.71	2.11
VLDL	0.67	4.35	1.37	0.66	4.33	0.46

[a] Experimental conditions are given in the text.
[b] Five replicates.
[c] Three replicates.

using a ProtEx-DEAE column was more precise than that using a DEAE-glucomannan column. This could be due to the fact that the former column consists of a spherical 5 μm gel, while the latter an irregular 30–90 μm gel. The calibration graph, constructed from peak area versus TC level, was linear with a correlation coefficient of 0.999 over the concentration ranges 5–1000 μg/ml as the TC level. The quantitation limit was 5 μg/ml with a 20-μl injection, as less than 10% in RSD. The detection limit was 2.5 μg/ml at a signal-to-noise ratio of 3 with a 20-μl injection.

3.5. Application to the separation of mild oxidative and artificially oxidised LDLs

Fig. 4A and B shows chromatograms of plasma samples of Japanese white (JW) and WHHL rabbits, respectively, on a ProtEx-DEAE column by stepwise elution with 35, 43, 52, 60, 70 and 100% eluent B. Those fractions were labelled as HDL, LDL1, LDL2, LDL3, VLDL1 and VLDL2, respectively. Since three lipoproteins, HDL, LDL and VLDL, of rabbit plasma samples were eluted with 35, 60 and 100% eluent B. Thus, LDL and VLDL were divided into three and two fractions, respectively. In JW rabbit plasma, LDL1 gave the largest peak area among the LDL subfractions, while LDL2 gave the largest in WHHL rabbit plasma. We have reported that more oxidatively modified LDL should elute with a higher concentration of sodium chloride, and that LDL in WHHL rabbit plasma might undergo mild oxidative modification [8]. The obtained result agrees well with that reported previously [8].

The HPLC method was applied to the assays of artificially oxidised LDL with Cu(II) ions for 3 h at 37°C. Fig. 5A and B shows native and artificially oxidised LDLs, respectively, of WHHL rabbits, eluted with 43, 52, 60, 70 and 100% eluent B. The first four peaks were labelled with LDL1, LDL2, LDL3 and VLDL1, respectively. Native and artificially oxidised LDLs gave the largest peak area with LDL2 and LDL3 fractions, respectively. The artificially oxidised LDL for 24 h at 37°C was not eluted on the ProtEx-DEAE column, but eluted on the DEAE-glucomannan column [8]. Since extent of denaturation of modified LDL for 24 h is larger than that for 3 h, the modified LDL for 24 h could be retained on the ProtEx-DEAE column, which could be more hydrophobic than the DEAE-glucomannan column. The results obtained above reveal that the anion-exchange HPLC method using a ProtEx-DEAE column could be applicable for the assays of mildly modified LDL in the plasma of WHHL

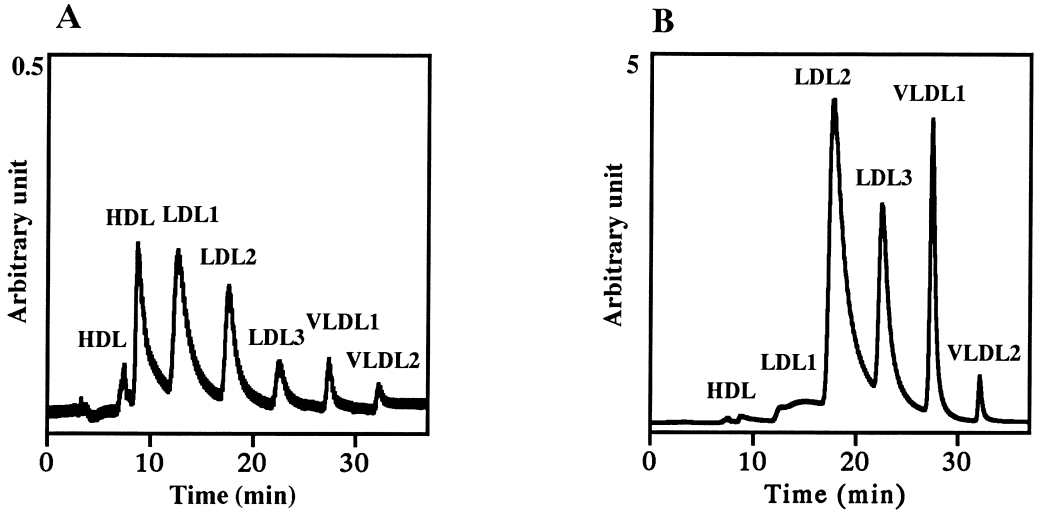

Fig. 4. Chromatograms of plasma samples of JW (A) and WHHL (B) rabbits by stepwise elution on a ProtEx-DEAE column. Stepwise elution conditions: 0% of eluent B for 0–2 min, 35% of eluent B for 2–7 min, 43% of eluent B for 7–12 min, 52% of eluent B for 12–17 min, 60% of eluent B for 17–22 min, 70% of eluent B for 22–27 min and 100% of eluent B for 27–35 min.

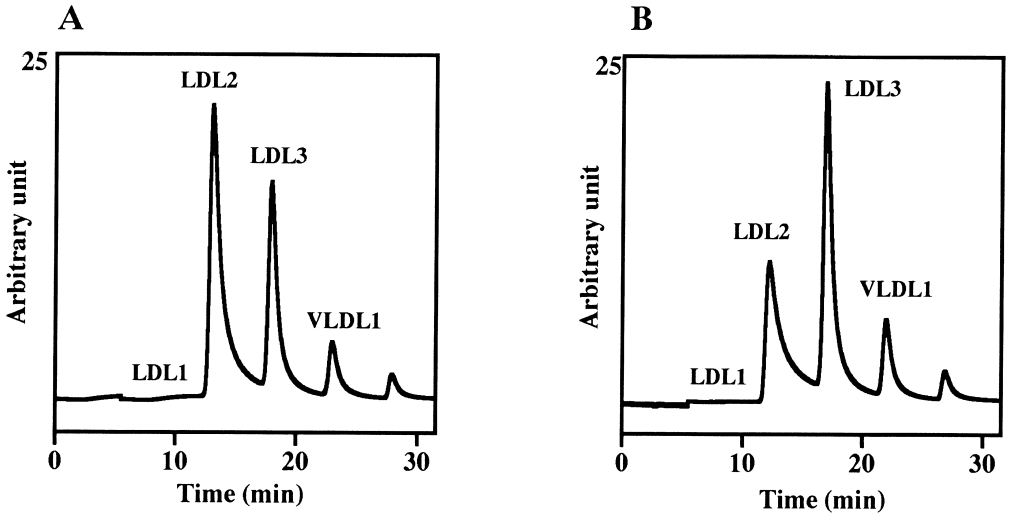

Fig. 5. Chromatograms of native (A) and artificially oxidised (B) LDLs of WHHL rabbit plasma on a ProtEx-DEAE column. Artificially oxidised LDL was obtained with incubation of native LDL, which is obtained from WHHL rabbits, with 5 μmol/l $CuCl_2$ at 37°C for 3 h. Stepwise elution conditions as in Fig. 4.

rabbits and artificially oxidised LDLs of WHHL rabbits.

In conclusion, we developed an anion-exchange HPLC method using a commercially available ProtEx-DEAE column for the assays of lipoproteins. The methods using the DEAE-glucomannan and ProtEx-DEAE columns were cross-validated. There was an excellent correlation between the two methods. Further, the method was applied to the assays of mildly modified LDLs in the plasma of WHHL rabbits and artificially oxidised LDLs of WHHL rabbits. The obtained results reveal that an accurate and precise anion-exchange HPLC method using the ProtEx-DEAE column could be useful for the assays of lipoproteins and modified lipoproteins.

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