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Effect of detergents on the electrophoretic behaviour of plasma apolipoproteins in capillary electrophoresis

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ABSTRACT

The influence of various detergents on the capillary electrophoretic behaviour of plasma apolipoproteins was studied. Electrophoretic mobility increased in the presence of anionic detergents sodium deoxycholate (DOC) and sodium dodecyl sulfate (SDS), and decreased in the presence of non-ionic Triton X-100. Apolipoproteins from plasma low-density lipoproteins (LDLs) and high-density lipoproteins (HDLs) exhibited different affinities for DOC and SDS. Optimal separation and reproducibility of HDL and LDL apolipoproteins was obtained using high-pH buffers containing SDS. Good resolution of very-low-density lipoprotein (VLDL) apolipoproteins was obtained on addition of either SDS or cetyl trimethylammonium bromide to the running buffer. For VLDL apolipoproteins the use of polyacrylamide coated capillaries yielded better results.

INTRODUCTION

The use of capillary electrophoresis (CE) for clinical diagnostistic applications is growing in prominence. Large biomolecules can be separated rapidly in an instrumental format that delivers the resolving power of traditional electrophoresis. Furthermore, CE does not suffer from slow mass transfer rates which lead to band broadening in HPLC separations of proteins and peptides.

To date, there has been a number of papers describing the use of CE for analyzing biological samples [1–6]. Chen *et al.* [1] used CE to screen for abnormalities in serum, urine and cerebrospinal fluid. By employing high voltage gradients and high ionic strength buffers, serum proteins were separated in less than 100 s. The addition of ethylene glycol to the separation buffer was also shown to improve resolution of serum proteins [3]. Josic *et al.* [4] demonstrated the separation of intrinsic and extrinsic membrane proteins from the liver using urea-containing buffers.

We are interested in the use of CE for screening of plasma apolipoproteins. There is growing evidence that the apolipoprotein distribution in the plasma is a better marker of coronary heart disease (CHD) than the cholesterol level [7,8]. Some apolipoproteins, such as apoA-I, participate in the removal of cholesterol from the bloodstream and are therefore classified as antiatherogenic. Conversely, elevated levels of other apolipoproteins, such as apoB, indicate an increased risk of CHD, even in the individuals with normal cholesterol levels. As research into these effects of apolipoproteins grows there is increased demand for a fast and simple method for their analysis.

Recently, we reported preliminary results on the separation of plasma apolipoproteins by CE [9]. By adding the detergent sodium dodecyl sulfate (SDS) to the separation buffer, the main apolipoproteins of plasma high-density lipoproteins (HDLs) and low-density lipoproteins (LDLs) were resolved in less than 12 min. A distinct advantage to this method was that both LDL and HDL apolipoproteins could be sepa-

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rated under the same conditions in a single run. This is generally not the case for either slab gel [10] or chromatographic [11] separations of apolipoproteins.

There have been no previous reports describing free-zone CE separations of protein-detergent complexes. Capillary SDS–polyacrylamide gel electrophoresis (PAGE) separations of protein–SDS complexes have been achieved [12]. However, in these cases separation was due to differences in size and detergent binding occurred only with the addition of heat and reducing agents. In cases where low concentrations of detergents were used as buffer modifiers, the role of detergent was only to modify the surface of the capillary [13,14]. This is because in the absence of heat or prolonged equilibration times, most proteins do not complex with detergents. However, since apolipoproteins have much higher affinity for detergents, their electrophoretic behavior can be significantly influenced by using detergents as buffer modifiers.

In this paper, the influence of various detergents on apolipoprotein CE is presented. The purpose of this work is to find the optimal detergent additive for apolipoprotein resolution. The detergents chosen as buffer modifiers have been shown to undergo complexation with apolipoproteins. The effects of detergent type, detergent concentration, and pH on electrophoretic behaviour of apolipoproteins are presented. CE separations of apolipoproteins from very-low-density lipoproteins (VLDLs) as well as HDL and LDL plasma fractions are shown.

EXPERIMENTAL

Apparatus

The CE system used a Bertan Model 230R (Bertan Associates, Hicksville, NY, USA) power supply and an Isco CV^4 (Isco, Lincoln, NE, USA) detector. The output of the power supply was connected to the buffer reservoir via platinum electrodes (Bioanalytical Systems, West Lafayette, IN, USA). Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 50 μm I.D. and 375 μm O.D. were used. Electropherograms were collected on a 486DX-compatible computer using the Waters Maxima 820 (Millipore, Milford, Mass, USA) chromatography software.

Chemicals

Doubly distilled, deionized water was used for all experiments. Ultra-pure SDS, tris(hydroxymethyl)methylamine (Tris), and acrylamide were obtained from ICN Biochemicals (Montreal, Canada). Sodium tetraborate (borax) and 3-(trimethoxysilyl)propyl methacryalte were from Aldrich (Milwaukee, WI, USA). Electrophoresis grade N,N,N′,N′-tetramethylethylenediamine (TEMED) and ammonium persulfate (Biorad, Mississauga, Canada) were used. Glacial acetic acid and hydrochloric acid were purchased from J.T. Baker (Phillipsburg, NJ, USA). Sodium deoxycholate (DOC), tetredecyltrimethylammonium bromide (TDAB) and cetyltrimethylammonium bromide (CTAB) were from Sigma (St. Louis, MO, USA). Apolipoprotein A-I, A-II and B standards were purchased from Sigma and apolipoprotein C-III was purchased from Biodesign International (Kennebunkport, ME, USA).

Preparation of apolipoprotein samples

Blood from a fasting male donor was collected into tubes containing 0.01% EDTA. A hypertriglyceridemic donor was used for isolation of VLDL lipoproteins in order to increase yields. Plasma was isolated by centrifugation at 1000 *g* and 4°C for 20 min. Plasma lipoprotein fractions were obtained using standard procedures [15]. Ultracentrifugation was performed at 142 200 *g* and 5°C using a Beckman L8-80 centrifuge equipped with a Ti-50.3 rotor. The lipoproteins were isolated at the following density ranges: VLDL ($d < 1.006$ g/ml), LDL ($d = 1.019$–1.063 g/ml) and HDL ($d = 1.063$–1.21 g/ml). To remove contamination due to serum albumin, the HDL fraction was diluted 1:3 with a NaCl/NaBr solution of $d = 1.21$ g/ml and recentrifuged for 20 h. All lipoprotein fractions were dialysed against 0.9% NaCl.

The HDL fraction was delipidated using diethyl ether and ethanol [16] and dissolved in a pH 6, 0.1 *M* Tris buffer containing 6 *M* urea. The LDL and VLDL fractions were not delipi-

dated since solubility in aqueous solution decreased dramatically upon delipidation.

Preparation of polyacrylamide-coated capillaries

The method of Hjertén [17] was used with some modifications. The capillary was first conditioned using a 15-min rinse with 1 *M* NaOH followed by a 5-min rinse with water. A solution containing 30 μl of 3-(trimethoxysilyl)propyl methacrylate in 1 ml of acetic acid–water (1:1, v/v) was drawn through the capillary using house vacuum. After 1 h, this solution was removed. The polymerizing solution was prepared by adding 10 μl of TEMED and 100 μl of 10% (w/v) ammonium persulfate to 10 ml of degassed 4% (w/v) acrylamide. The capillary was filled with this solution and left in a horizontal position for about 45 min. After polymerization was completed the excess polyacrylamide was removed and the capillary was rinsed with water.

CE procedures

Uncoated fused-silica capillaries had a total length of 75 cm and a separation length of 50 cm. Polyacrylamide coated capillaries were 80 cm long and had a separation length of 55 cm. A wavelength of 220 nm was used for detection. The applied voltage was 25 kV for all separations. The pH of borax buffers was adjusted by titrating with appropriate amounts of HCl and NaOH. Bare silica capillaries were conditioned between runs by two-minute rinses with 0.1 *M* NaOH and water. Samples were injected hydrodynamically at a height of 15 cm and an injection time of 8–10 s.

RESULTS AND DISCUSSION

CE behaviour of apolipoprotein–detergent complexes

To understand the nature of interaction between apolipoproteins and detergents a closer look at apolipoprotein assembly within lipoprotein particles is necessary. A diagram of a typical lipoprotein particle is shown in Fig. 1 [18]. The apolipoproteins normally exist on the periphery of the lipoprotein particle. One side of the apolipoprotein is in contact with the aqueous plasma environment and is therefore hydrophilic. The other side is hydrophobic since it is associated with the lipid core of the lipoprotein particle. This dual nature allows apolipoproteins to readily associate with other amphiphiles such as detergents.

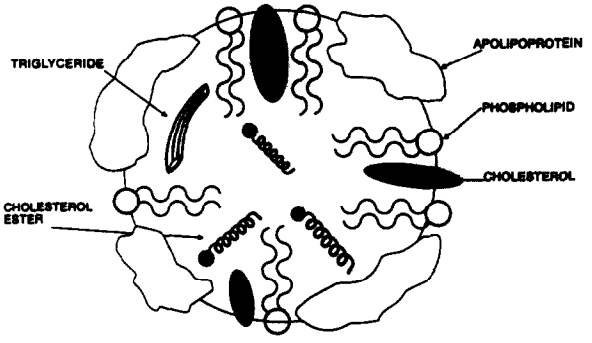

Fig. 1. Schematic of lipoprotein particle.

Discrete, non-interacting detergent binding sites on apolipoproteins have been observed at low detergent concentrations ($<10^{-4}$ *M*) [19]. These sites are hydrophobic in nature and involve only the monomeric detergent. Formation of the apolipoprotein–detergent complex can therefore be represented by:

$$A + nD \rightarrow AD_n \quad (1)$$

where A is the apolipoprotein, D is the detergent and n is the number of bound detergent monomers.

The number of discrete binding sites and maximum amount of bound detergent for HDL and LDL apolipoprotein detergent complexes are shown in Table I [19,20]. The values for Triton X-100 and DOC are expecially interesting since hydrophilic proteins do not bind these detergents even after prolonged equilibration. The binding of detergent to apolipoprotein molecules alters the Stokes radius and in the case of ionic detergents, the charge of the protein. This causes a change in the electrophoretic mobility of the protein since [21]:

$$\mu_e = \frac{Q}{R_S}\left(\frac{1}{6\pi\eta}\right) \quad (2)$$

where μ_e is the electrophoretic mobility, Q is the total charge, R_S is the Stokes radius and η is the viscosity. Changes in electrophoretic mobility

TABLE I

NUMBER OF PROTEIN BINDING SITES AND AMOUNT OF DETERGENT BOUND IN APOLIPOPROTEIN–DETERGENT COMPLEXES

Apolipoprotein	Detergent	n	Detergent/protein (w/w)
Apo-AI[a]	SDS	4	1.4
	DOC	1	0.4
	TDAB	4	1.1
Apo-AII[a]	SDS	4	1.4
	DOC	1	0.4
	TDAB	10	1.1
Apo-B[b]	DOC		0.64
	Triton X		0.52

[a] From ref. 19, detergent binding data obtained by equilibrium dialysis.
[b] From ref. 20, detergent binding data obtained by gel filtration of the proteins in the presence of detergent.

upon detergent binding to apolipoproteins can be used therefore to estimate the effect of detergent on protein size and charge. The value of μ_e can be experimentally determined using:

$$\mu_e = \frac{l_d L}{V}\left(\frac{1}{t_{eo}} - \frac{1}{t}\right) \quad (3)$$

where l_d is the detection length of the capillary, L is the total length, V is the applied voltage, t_{eo} is the retention time of the neutral marker and t is the analyte retention time.

The effect of three surfactants on resolution of HDL and LDL apolipoproteins is illustrated in Fig. 2. The concentrations of added detergent ensured maximum binding to apolipoproteins. The presence of anionic detergents SDS and DOC resulted in complete resolution of all components. Addition of the neutral detergent Triton X-100 resulted in separation of A and B apolipoproteins. Resolution of apoA-I, apoA-II and apoB-100, apoB-48 pairs however, was not possible. The extra peaks in Fig. 2C were found to be due to sample buffer components.

The cationic detergent TDAB was also studied as a buffer modifier since the values in Table I indicate that it undergoes significant binding with apolipoproteins. The resulting electropherogram (not shown) exhibited a noisy baseline and protein peaks were not observed after 30 min. It is possible that under these conditions the electrophoretic and electroosmotic velocities are similar in magnitude but in opposite directions. Addition of another cationic detergent, CTAB, also gave poor results.

Changes in apolipoprotein electrophoretic mobility on addition of surfactants are shown in Table II. Triton X-100 caused a decrease in the

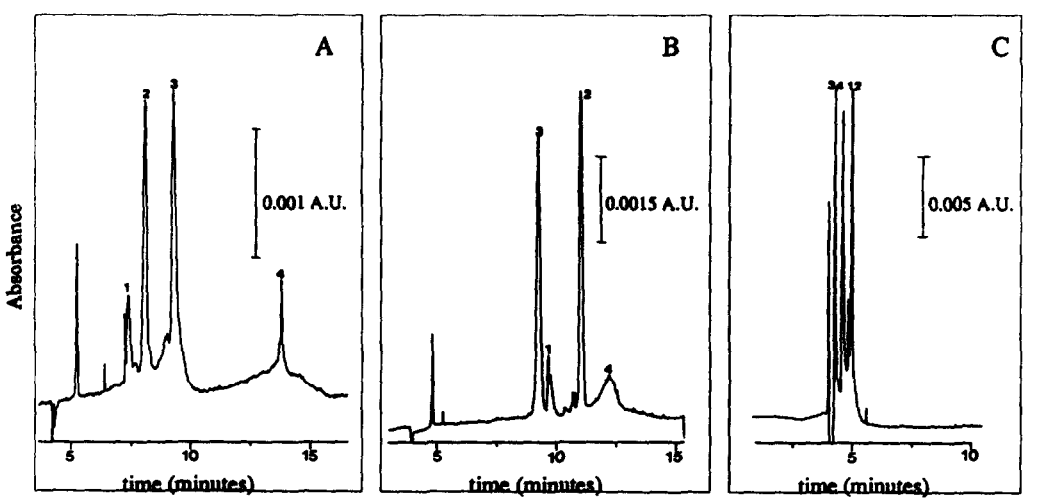

Fig. 2. Effect of various detergents of resolution of apolipoproteins. Conditions: 30 mM borate buffer pH 9. (A) 10 mM DOC; (B) 3.5 mM SDS; (C) 3.5 mM Triton X-100. Peaks: 1 = apoA-II; 2 = apoA-I; 3 = apoB-100; 4 = apoB-48.

TABLE II

EFFECT OF DETERGENTS ON ELECTROPHORETIC MOBILITIES OF APOLIPOPROTEINS

CE conditions as in Fig. 2.

Protein	Mobility, μ_e (10^{-4} cm^2/V s)				$(\mu_{e2} - \mu_{e1})$ (10^{-4} cm^2/V s)		
	Borate buffer (μ_{e1})	SDS (μ_{e2})	DOC (μ_{e2})	Triton X (μ_{e2})	SDS	DOC	Triton X
ApoA-I	1.40	4.06	2.82	0.92	2.66	1.42	−0.48
ApoA-II	1.40	3.74	2.57	0.92	2.34	1.17	−0.48
ApoB-100	1.40	3.64	3.21	0.84	2.24	1.81	−0.06

electrophoretic mobility for all three proteins. This is because binding of this detergent causes an increase in the protein's Stokes radius and a concomitant decrease in total charge. The net result, as shown by eqn. 2, is decreased mobility.

Both anionic detergents caused a significant increase in apolipoprotein electrophoretic mobility. The effect of SDS was more pronounced, however, for all proteins. Mikano *et al.* [19] showed that Stokes radii of apoA–DOC and apoA–SDS complexes were similar. However, since four times as much SDS as DOC is bound to the protein, the SDS complexes exhibit a higher negative charge and consequently increased electrophoretic mobility.

It is interesting to note that elution order of apoA-I and apoB-100 is different in DOC and SDS. This can be explained by the relative hydrophobicities of the proteins. LDL lipoproteins contain a higher fraction of lipid compared to HDL particles and the main protein of LDL, apoB, is much more hydrophobic than apoA-I which is found in HDL. Since DOC is derived from cholesterol it more closely resembles the natural lipoprotein environment. Consequently, apoB binds more strongly to DOC and experiences a greater change in its electrophoretic mobility.

On examination of the electropherograms in Fig. 2, addition of either DOC or SDS to the running buffer appears to give good resolution of apolipoproteins. However, reproducibility of the separation must also be considered. This is especially true for routine clinical diagnostic applications. We found that the behaviour of apoB was not reproducible in the DOC system. The apoB peak tended to degrade over time. This may have been due to adsorption of the protein complex to the capillary wall. As illustrated in Fig. 3, electropherograms obtained in SDS-containing buffers were found to be highly reproducible in retention time and peak area. Further studies were therefore concentrated on SDS buffers.

Fig. 4 shows the retention profile of apolipoproteins at different SDS concentrations. When the buffer contained less than 2.7 m*M* SDS, the individual apolipoproteins could not be distinguished. Instead the mixture appeared as a broad series of peaks between 6 and 12 min. Injection of individual apolipoprotein standards produced similar electrophoretic patterns. This behavior is most likely due to the binding characteristics of SDS–apolipoprotein complexes. Reynolds and Simon [22] showed binding to

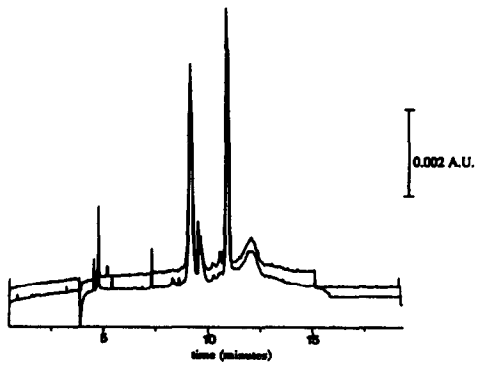

Fig. 3. Overlay showing reproducibility of first and eleventh run in SDS containing buffers. Conditions and peaks as in Fig. 2B.

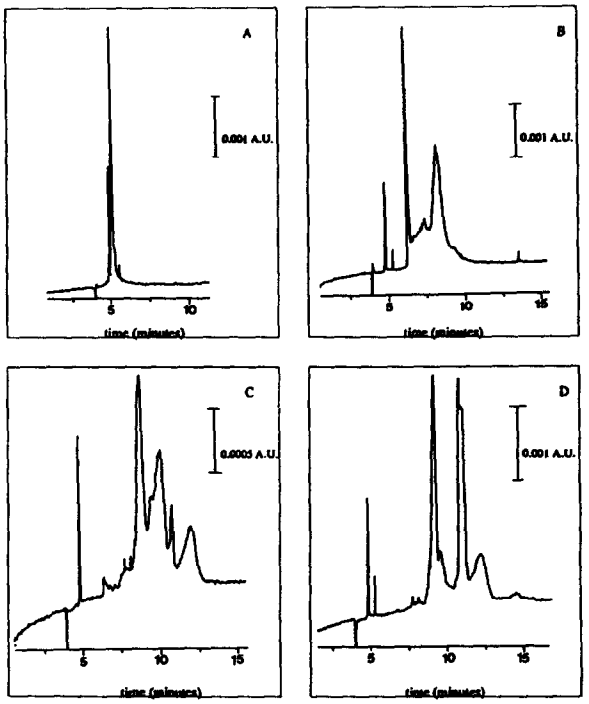

Fig. 4. Effect of SDS concentration on apolipoprotein CE. (A) No SDS; (B) $9 \cdot 10^{-4}$ *M* SDS; (C) $1.7 \cdot 10^{-3}$ *M* SDS; (D) $2.6 \cdot 10^{-3}$ *M* SDS. Other conditions as in Fig. 2.

apoA-I begins at about 0.1 m*M* SDS and plateaus at about 3 m*M* SDS. Therefore, the elution patterns obtained in Fig. 4 are likely due to the existence of apolipoproteins with varying degrees of binding to SDS.

The effect of adding detergent to apolipoprotein samples was also studied. In this case the running buffer contained no detergent and the sample contained 3.5 m*M* SDS. There was no significant improvement in resolution compared to the electropherogram in Fig. 3. The retention time of the peak did increase, however, by over two minutes. This indicates that SDS remains in a complex with the proteins throughout the run. However, the absence of SDS in the running buffer means that protein-protein interactions can still occur. This causes the proteins to elute as a single peak. When both the sample and running buffer contained SDS, electropherograms similar to those in Fig. 3 were obtained.

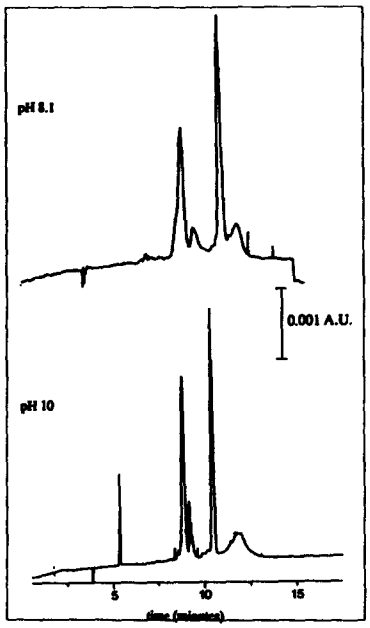

Fig. 5. Effect of buffer pH on resolution. Conditions: borate buffer, 25 kV, 40 μA for both runs.

Effect of pH

The effect of pH on apolipoprotein resolution is illustrated in Fig. 5. The ionic strength of the borate buffer was adjusted to maintain the same current in both runs. Both resolution and efficiency were improved at the higher pH. This can be partially attributed to decreased adsorption. It is now well known that proteins undergo pH-dependent association with silanol groups of fused silica. It is also possible that SDS–apolipoprotein association is weakened at lower pH values.

CE of VLDL apolipoproteins

The main apolipoproteins found in plasma VLDL particles are apoB-100 (M_r 500 000), apoB-48 (M_r 250 000), apoE (M_r = 35 000), apoC-III (M_r 8750), apoC-II (M_r = 8800) and apoC-I (M_r 6500) [18]. The apolipoproteins in this fraction are the most heterogeneous with respect to their size and hydrophobicity. This makes their separation by chromatographic techniques difficult. The effect of detergent addition on the separation of VLDL apolipoproteins is

shown in Fig. 6. As in the case of HDL and LDL fractions, the VLDL apolipoproteins migrate as a single species in the absence of detergent in the buffer.

All three detergents had a dramatic effect on the elution profile of VLDL apolipoproteins. Addition of DOC (Fig. 6B) appears to result only in the bulk separation of hydrophobic apoB from the more hydrophilic apoC proteins. Results obtained with SDS and CTAB are more promising. At least four major components can be distinguished when either of these detergents is added to the buffer. The effect of CTAB is especially interesting since we had found it to be a poor choice for resolution of HDL and LDL apolipoproteins.

Separations of apolipoproteins in polyacrylamide capillaries are shown in Fig. 7. A significant increase in resolution was observed in these capillaries. This is another difference from CE separations of HDL and LDL apolipoproteins where resolution was not affected by coating the capillary [9]. In both the SDS- and CTAB-containing buffers over 7 different components of VLDL could be distinguished.

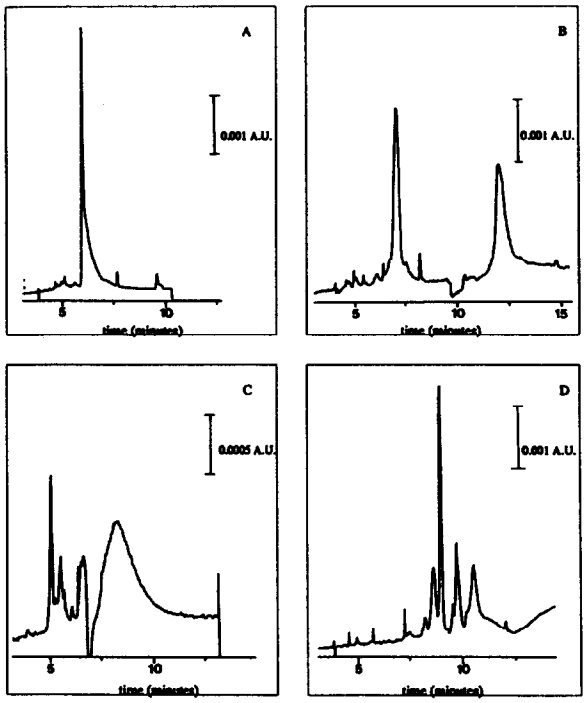

Fig. 6. Effect of detergent addition on VLDL apolipoprotein resolution in fused-silica capillaries. Conditions: 30 m*M* borate buffer pH 9; (A) no detergent added; (B) 10 m*M* DOC; (C) 3.5 m*M* CTAB; (D) 3.5 m*M* SDS.

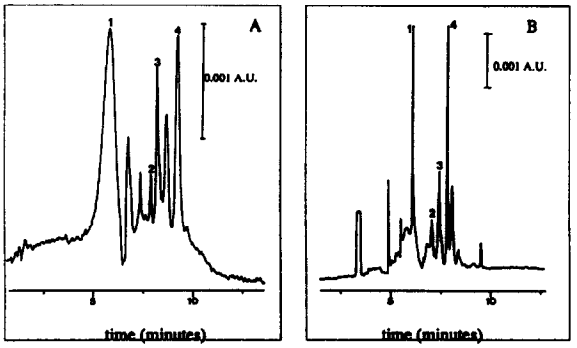

Fig. 7. Separation of VLDL apolipoproteins in acrylamide-coated capillaries. Conditions: 30 m*M* borate pH 9, (A) 3.0 m*M* CTAB; (B) 3.5 m*M* SDS. Peaks: 1 = apoB; 2, 3 = apoCIII variants; 4 = albumin.

Since standards of all VLDL apolipoproteins were not available, it was difficult to assign all of the peaks in Fig. 7. This also made it difficult to draw conclusions regarding which detergent is better for these separations. The efficiency in SDS-buffers was somewhat higher. However, as illustrated in Fig. 8, CTAB showed better results for resolving charged variants of apoC-III. The isoforms of apoC-III occur as a result of varying degrees of sialization. For now, it appears that

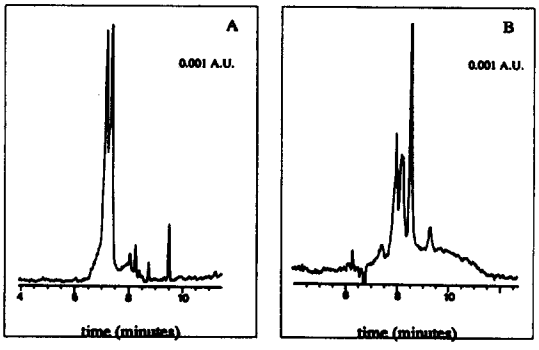

Fig. 8. Separation of apoC-III charged variants. (A) 3.5 m*M* SDS; (B) 3.0 m*M* CTAB buffer. Other conditions as in Fig. 7.

either detergent is very effective for obtaining VLDL profiles.

CONCLUSIONS

The electrophoretic behaviour of apolipoproteins can be modified by addition of surfactant to the running buffer. Addition of anionic detergents results in optimal resolution of HDL and LDL proteins. Both cationic and anionic detergents improve the resolution of VLDL apolipoproteins. The CE procedures in this paper are advantageous since one buffer system can be used for resolving all apolipoproteins. Furthermore, detergent modified CE of apolipoproteins does not require extensive sample treatment as in the case of chromatography or slab electrophoresis. CE therefore appears to be well-suited for rapid screening of all major plasma apolipoproteins. Future work is being directed at using CE to identify apolipoprotein abnormalities in plasma.

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