Separation and quantitation of apolipoprotein B-48 and other apolipoproteins by dynamic sieving capillary electrophoresis

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Abstract Apolipoprotein-B-48 is a structural protein exclusively associated with post-prandial lipoproteins (chylomicrons). Apolipoprotein B-48 would be a useful marker to monitor the kinetics of chylomicrons in vivo, however, its quantitation is limited because of a low concentration in plasma and lack of specific antibodies. Dynamic sieving capillary electrophoresis (DSCE) has recently become widely available for the separation of nanomolar quantities of proteins by size and electrophoretic mobility. Here we describe the potential of DSCE to accurately quantitate apolipoprotein mass in one ml of plasma. Separation of human serum apolipoproteins was achieved through an uncoated fused silica glass capillary column with quantitation based on area response at 220 nm. The retention times for human apolipoprotein B-48, apolipoprotein B-100 and albumin were 8.96 min ± 0.57%, 10.21 min ± 0.72%, and 6.56 min ± 0.4%, respectively (phase-standardized to internal reference). A significant correlation (r = 0.99) was observed between apolipoprotein concentrations and peak area response for mass ranges of 30–400 μg/ml. DSCE provides an alternative method for quantifying apolipoprotein B-48 and therefore, may be useful for studying postprandial lipoprotein metabolism.—**Proctor, S. D., and J. C. L. Mamo.** Separation and quantitation of apolipoprotein B-48 and other apolipoproteins by dynamic sieving capillary electrophoresis. *J. Lipid Res.* 1997. **38:** 410–414.

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Atherogenesis is characterized by the deposition of lipid in arterial tissue derived from circulating lipoproteins (1, 2). Hepatically derived low density lipoproteins (LDL) (3) and intestinally derived chylomicron remnants (4, 5) are considered to be the two primary atherogenic lipoproteins that contribute to this process. Consequently, the metabolism of LDL and chylomicrons continues to be widely investigated. Compared with LDL, the metabolism of chylomicrons is difficult to study because of a rapid turnover and low plasma concentration (4). Presently, the most widely used procedure to assess chylomicron metabolism in vivo is to monitor the incorporation and clearance from plasma of dietary vitamin A (6, 7). There is, however, the possibility that retinyl esters may transfer from chylomicrons to other plasma lipoproteins making interpretation unnecessarily complex (8, 9).

An alternative approach to chylomicron–vitamin A kinetics is to monitor the clearance of apolipoprotein B-48 which is a structural protein associated exclusively with chylomicrons. However, apoB-48 quantitation is difficult because it comprises less than 1% of lipoprotein mass. Detection by standard techniques such as polyacrylamide gel electrophoresis is restricted by the amount of apoB-48 that can be recovered from a limited amount of plasma (10). Alternatively, immunodetection procedures are not widely available because of the lack of specific antibodies (11).

The commercial development of dynamic sieving capillary electrophoresis (DSCE) has enabled rapid separation of nanomolar quantities of proteins on different column supports (12, 13). Quantitation of apoB-48 by DSCE may provide a sensitive and accurate alternative to study chylomicron kinetics. However, apolipoproteins are unique in that most are amphiphylic and their separation by DSCE has not been previously described. Furthermore, the molecular weight of apoB-48 (245 kDaltons) and apoB-100 (500 kDaltons) is significantly greater than the range of proteins thus far separated by DSCE (≤200 kDaltons).

In this study we assessed the sensitivity of DSCE to quantitatively differentiate the two isoforms of apolipoprotein B. The data suggest that DSCE is sufficiently sensitive to accurately quantitate apolipoproteins including B-48.

MATERIALS AND METHODS

Apolipoprotein isolation

Human plasma was provided by the Red Cross Blood Transfusion service, Perth, Western Australia. Lipoproteins of density fraction <1.019 g/ml were isolated by ultracentrifugation, 141000 *g* for 16 h at 4°C. Lipoprotein isolates were progressively delipidated with chloroform, methanol, and anhydrous ether as previously described (14). The delipidated proteins were re-solubilized in sample buffer (0.1 M sodium phosphate, pH 7.0, 2% SDS). Apolipoprotein separation was achieved via passage through a 3.0 × 100 cm Sepharose CL-6B (Pharmacia cat# 17-0160-01) size exclusion column at a flow rate of 0.5 ml/min with running buffer (0.025 M sodium phosphate, pH 7.4, 0.1% SDS and 0.5% EDTA) (10). Note that apolipoproteins could be separated with-

Abbreviations: DSCE, dynamic sieving capillary electrophoresis; LDL, low density lipoproteins; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

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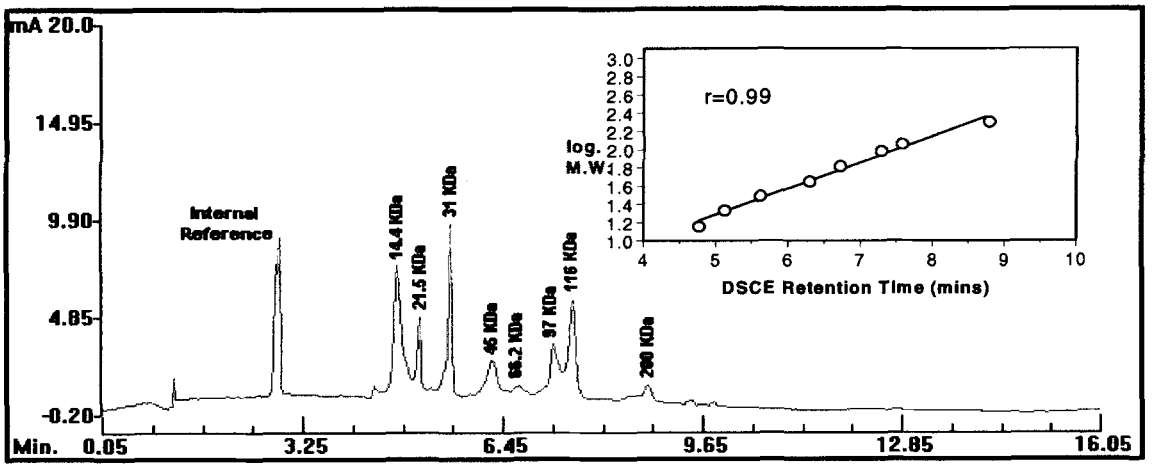

Fig. 1. Separation of the CE-SDS protein standards by dynamic sieving capillary electrophoresis. From left to right, benzoic acid (internal reference elution at approximately 3 min), lysozyme (14.4 kDa), trypsin inhibitor (21.5 kDa), carbonic anhydrase (31.0 kDa), ovalbumin (45.0 kDa), serum albumin (66.2 kDa), phosphorylase B (97.0 kDa), β-galactosidase (116.0 kDa), and myosin (200.0 kDa). Separation conditions: uncoated capillary 24 cm × 50 μm; polarity: negative to positive; injection: 200 Psi/sec for 5 sec; run buffer 1.0% SDS; voltage: 15 kv; detection: 220 nm; capillary temperature: 20°C. Inset depicts correlation of logarithm molecular weight (log MW) of separated proteins with corresponding retention time (r = 0.99).

out delipidation of lipoproteins (data not shown); however, resolution often became erratic because of excess lipid debris. Fractions containing apoB-48, B-100, and albumin were identified according to molecular weight after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by the Lammeli method (15).

Capillary electrophoresis

Dynamic sieving capillary electrophoresis was done using a BioFocus® 3000 Capillary Electrophoresis System (Bio-Rad). DSCE protein standards provided by the manufacturer (cat#148-2015) were prepared as described in the instructions except 1% SDS was replaced with 5% SDS. Benzoic acid was used as an internal standard to monitor inter-run variation. The molecular weight standards were heated at 100°C for 10 min in the presence of 10% mercaptoethanol.

To selected apolipoprotein fractions, 2-mercaptoethanol, (v/v) benzoic acid solution (1 mg/ml), and Bio-Rad sample buffer were added to achieve final volumes of 13:1:1:4 (v/v), respectively. Similar amounts of reducing agent, internal standard, and sample buffer were added to bovine serum albumin dissolved in 0.025 M sodium phosphate (pH 7.4, 0.1% SDS and 0.5% EDTA). In preliminary experiments we found it was not necessary to heat the apolipoprotein solution at 100°C. Apolipoproteins were eluted through a 25-cm × 50-μm uncoated silica capillary (Bio-Rad cat#148-3060) with run buffer (cat#148-5032) increased to 1.0% SDS. Injection time was 200 psi/sec for 5 sec with polarity direction negative to positive and voltage 15 kV for 20 min at 20°C. Proteins were detected at 220 nm.

Chromatogram analysis

The separation of proteins by DSCE can vary depending on the molecular resistance between each run. To adjust for inter-run variations, separate runs are phase-standardized utilizing the DSCE integration software (BioFocus® 3000). Phase standardization adjusts each chromatogram based on the retention time of an internal standard (benzoic acid). Thereafter, the molecular weights of semipurified apolipoproteins calculated by DSCE are determined based on the retention times of proteins with increasing molecular weight (14.4–200 kDa, **Fig. 1**) and expressed as area/migration time.

RESULTS AND DISCUSSION

Both isoforms of apolipoprotein B have molecular weights greater than the published resolving capacity of DSCE. To explore whether these large proteins could be separated by DSCE, apolipoproteins B-48 and B-100 were semipurified from plasma and separated by DSCE. Purity of each apolipoprotein fraction was initially assessed by SDS-PAGE. We found that when separated by DSCE, both apoB-48 and apoB-100 remained on the linear portion of the molecular weight standard graph (Fig. 1 inset). The retention time of apoB-48 separated by DSCE **(Fig. 2)** standardized against the internal reference (benzoic acid 3.07 min) was 8.96 min ± 0.57%. ApoB-100 has a retention time of 10.21 min ± 0.72%

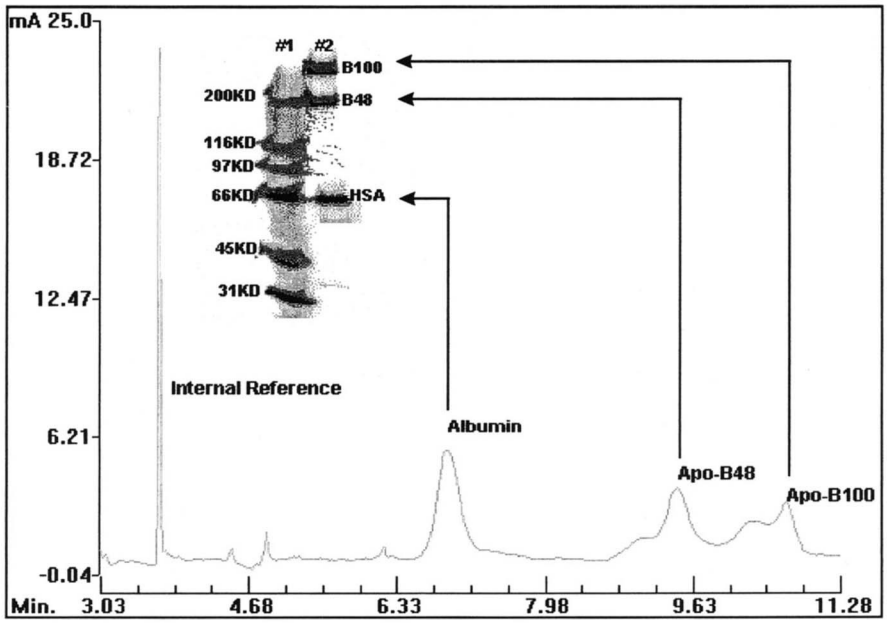

Fig. 2. Separation of apolipoproteins by dynamic sieving capillary electrophoresis. Typical chromatogram of sample containing human serum albumin (HSA), apoB-48 and B-100 in ≤5.0% SDS. Samples were separated by DSCE over period of 12 min. Separation conditions: uncoated capillary 24 cm × 50 μm; polarity: negative to positive; injection: 200 Psi/sec for 5 sec; run buffer: 1.0% SDS; voltage: 15 kv; detection: 220 nm; capillary temperature: 20°C. Insert corresponds to same sample separated by 5–20% SDS-PAGE.

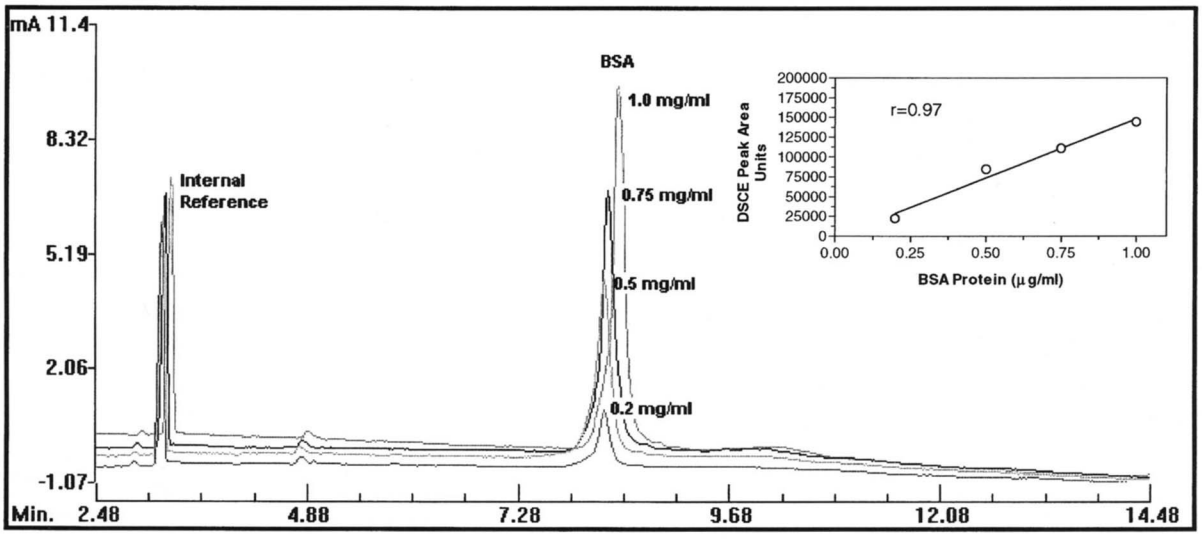

Fig. 3. Separation of bovine serum albumin by dynamic sieving capillary electrophoresis. Separation of pure BSA (1.0–0.2 mg/ml in 0.3% SDS) by DSCE. Separation conditions: uncoated capillary, 24 cm × 50 μm; polarity: negative to positive; injection: 400 Psi/sec for 5 sec; Bio-Rad CESDS run buffer 0.1% SDS; voltage: 15 kv; detection: 220 nm; capillary temperature: 20°C. Inset depicts bovine serum albumin concentration with integrated peak area ($r = 0.97$). Peak area units are given as integrated area/migration time to reduce influence caused by significantly different retention times.

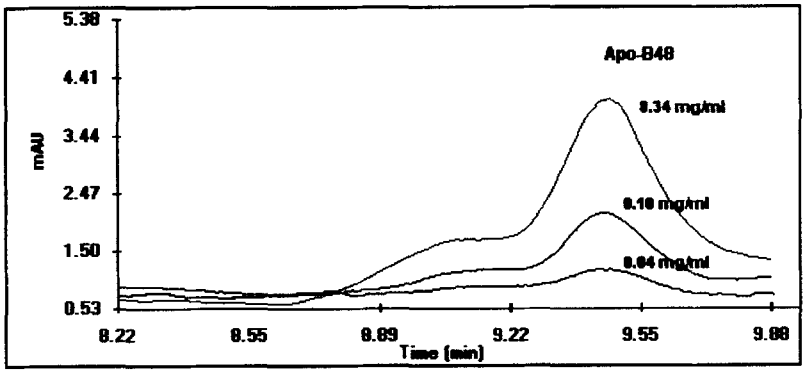

Fig. 4. Separation of apoB-48 by dynamic sieving capillary electrophoresis. Selective region of chromatograms displaying separation of apoB-48. Separation conditions: uncoated capillary 24 cm × 50 μm; polarity: negative to positive; injection: 400 Psi/sec for 5 sec; Bio-Rad CESDS run buffer 0.1% SDS; voltage: 15 kv; detection: 220 nm; capillary temperature: 20°C. The increasing concentration of apoB-48 of range 40–340 μg/ml correlated significantly to integrated peak area (r = 0.99).

and human serum albumin 6.56 min ± 0.40% (Fig. 2). The retention time of apoB-48 corresponded to a molecular weight of approximately 245 kDa and apoB-100 to a molecular weight of approximately 500 kDa, both similar to their mass determined by amino acid analysis. DSCE clearly has the potential to resolve proteins with molecular weights significantly greater than that presently reported. Furthermore, the small error associated with each mean shows that there was little variation between separate apolipoprotein isolations and/or repetitive runs.

In order to assess the relationship between protein mass loaded and area response, increasing quantities of BSA were resolved by DSCE **(Fig. 3).** A linear relationship between peak area and protein concentration was observed for the range 200–1000 μg/ml (r = 0.97) (Fig. 3 inset). The mass of protein loaded was chosen to encompass the physiological concentrations of plasma apolipoproteins (4).

Increasing quantities of apolipoprotein B-48 were separated by DSCE **(Fig. 4).** There was a significant relationship between mass loaded and area response (r = 0.99). The significance of the correlation between peak area and protein content reaffirms that apoB-48 quantification can be readily determined by DSCE. Recently, we reported the concentration of apoB-48 in fasting plasma of normal subjects and hyperlipidemic subjects to be on the order of 60 μg/ml and 200 μg/ml, respectively (16). The mass of apoB-48 previously reported in one milliliter of plasma falls within the linear portion of the apoB-48 standard curve presented in this study. Therefore, the sensitivity of the DSCE would suggest that it may be useful for studying postprandial lipoprotein metabolism.

Quantitation of apoB-100 is substantially easier than for apoB-48, because of the increased plasma mass. Nevertheless, co-quantitation of both isoforms may be useful for some metabolic studies. There is substantial interest in the potential impact that chylomicron-derived lipid might have on hepatic production of lipoproteins (17, 18). DSCE might provide a simplified procedure for monitoring postprandial lipid metabolism, while simultaneously gauging the concentration of apoB-100-containing lipoproteins.

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