

Sequential Purification of Human Apolipoprotein B-100, Albumin, and Fibrinogen by Immunoaffinity Chromatography for Measurement of Protein Synthesis

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A determinant of the accuracy of protein synthesis measurement using stable isotope is the purity of the protein under study. An immunoaffinity chromatographic technique to sequentially purify human plasma albumin, fibrinogen, and apolipoprotein B-100 (ApoB-100) was developed to measure isotopic enrichment in these proteins. The technique, utilizing immobilized mouse monoclonal antibodies specific to human plasma ApoB-100, albumin, and fibrinogen onto an affinity matrix, allowed purification of very low density lipoprotein (VLDL) ApoB-100, albumin, and fibrinogen from 1- to 2-ml plasma samples. Analytical sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by silver staining demonstrated consistent purity of the three purified proteins. The identity and the purity of the proteins separated by this technique were also confirmed by amino acid sequence analysis. This technique was applied to sequentially purify and measure the isotopic enrichment in those proteins by mass spectrometry from human plasma samples collected after orally ingesting L-[1-¹³C]-leucine. Reproducibility of the enrichment measurements is within 5% of the coefficient of variation. Measurements [¹³C]leucine in these proteins purified from plasma samples collected during a 10-h primed continuous intravenous infusion of L-[1-¹³C]leucine confirmed that this technique provides an efficient way to purify plasma VLDL ApoB-100, albumin, and fibrinogen for measuring their synthetic rates in human metabolism studies. © 1997 Academic Press

Measurements of synthetic rates of circulating proteins of liver origin, such as ApoB-100,² albumin, and

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² Abbreviations used: ApoB-100, apolipoprotein B-100; VLDL, very low density lipoprotein; IAC, immunoaffinity chromatography; PBS, phosphate-buffered saline; AUFS, automatic unit full scale; APE, atom percent excess.

fibrinogen, allow us to study liver protein metabolism in humans (3, 7, 9, 17, 25). Being a protein that turns over fast, isotope enrichment of VLDL ApoB-100 reaches a plateau earlier than other liver proteins such as albumin and fibrinogen (7, 19, 25). This isotopic plateau indicates that its isotopic enrichment is the same as liver amino acyl-tRNA, the obligatory precursor of protein synthesis. Therefore, enrichment of VLDL ApoB-100 has been proposed as a surrogate measure of the isotopic enrichment of hepatic amino acyl-tRNA (4, 23). Since the VLDL fraction of ApoB is composed of ApoB-100 and ApoB-48 (especially after a meal) and small amounts of many other proteins, it is important to obtain pure ApoB-100 to use its enrichment as a surrogate measure of that of liver amino acyl-tRNA.

Since different proteins are synthesized at different rates, the accuracy of the measurement of the isotope enrichment of a specific protein depends heavily on the purity of the protein separated. To obtain the pure and specific proteins for measurement of their synthesis, it is essential to have an efficient technique to purify these proteins. Immunoaffinity chromatography (IAC), employing highly specific monoclonal antibodies, has been widely used for separating proteins and biomolecules (6, 14). The technique, utilizing the specific binding between antibody and antigen, allows separation of pure and specific proteins in a single step. It requires simple equipment and fewer chemicals, and more than one protein can be obtained sequentially from the same sample. Moreover, it provides a versatile approach to purify proteins of small molecules and in low plasma concentration, which would be more difficult to purify by any other technique. Our purpose was to establish an IAC technique to purify VLDL ApoB-100, albumin, and fibrinogen for measuring synthetic rates of these proteins based on isotope incorporation into these proteins.

We describe here an IAC technique for separating these three proteins from a single plasma sample for

measuring their synthetic rates using stable isotopes. The purity of the proteins separated, the reproducibility of the technique, and the application of the technique to provide sufficient pure proteins for isotope enrichment measurement have been assessed.

MATERIALS AND METHODS

Chemicals and Equipment

Buffer reagents, such as sodium chloride, sodium phosphate, urea, and sodium bicarbonate, except where indicated, were purchased from Sigma Chemical Co. (St. Louis, MO). All electrophoresis reagents, including the silver stain kit and protein assay kit, were from Bio-Rad Laboratories (Hercules, CA). L-[1-¹³C]-Leucine (99 at.% excess) was purchased from Cambridge Isotope Laboratories (Woburn, MA).

Two low-pressure liquid chromatographic systems from Bio-Rad Laboratories were used. An automatic Econo-System (Model ES-1) was used for albumin and fibrinogen separation. A BioLogic system was used for ApoB-100 separation. All compatible columns and flow adapters were purchased from Bio-Rad. Affi-Prep 10 (Bio-Rad) was used throughout the study as affinity support due to its high flow rate and stability. These supports were chosen because of their high stability and flow rate, low leakage, and ability to couple protein ligands with a neutral or basic isoelectric point (pI).

The IAC technique used 20 mM phosphate with 0.15 M NaCl, pH 7.3, as application buffer; 0.1 N acetic acid with 2 N urea, pH 4.0, as elution buffer; and 0.5 M NaCl as washing buffer. For coupling antibodies onto the affinity supports, 0.1 M sodium bicarbonate, pH 7.1, was used as coupling buffer. All buffers were sterilized by filtration through a vacuum filter with 0.2 μm pore size.

Monoclonal Antibodies

Mouse monoclonal antibody against human plasma ApoB-100 was purchased from the University of Ottawa Heart Institute (Lipoproteins & Atherosclerosis Group, Ontario, Canada). The antibody is specific to human plasma ApoB-100 without cross-reaction to ApoB-48 (21, 22). Monoclonal antibody to human plasma fibrinogen was produced from hybridoma cells purchased from ATCC (ATCC HB 9740, F45J, American Type Culture Collection, Rockville, MD) at the Immunochemical Core Facility of the Mayo Clinic (Rochester, MN). Antibody was produced according to the formulation of the accompanying protocol. The antibody binds to the α-chain of human plasma fibrin and fibrinogen, but not plasmin degradation products.

Mouse monoclonal antibody to human plasma albumin was produced against pure human plasma albumin (Sigma Chemical Co.) at the Immunochemical Core Facility of the Mayo Clinic. The generation of B

cell hybridomas followed the protocol described by de St. Groth and Scheidegger (10). Ascites fluid was produced in Balb/c mice, which were primed with an intraperitoneal injection of 0.5 ml pristane oil. Three to 5 days later, $1-2 \times 10^6$ hybridoma cells in 0.5 ml PBS were injected intraperitoneally. Daily collections of ascites fluid began after 7–10 days. Ascites fluid was diluted with equal amount of PBS, stored at -20°C .

Experimental Protocol

For assessing the reproducibility of isotopic enrichment measurements of each protein, venous blood samples were drawn from a normal subject who took orally 50 ml (10 mg/ml) L-[1-¹³C]leucine 1 h before blood drawing, both at fasting state and after a large fat meal (35% fat, 15% protein, and 50% carbohydrate), into EDTA vacutainer tubes to which a cocktail of disodium EDTA and soybean protease inhibitor (per 100 ml blood, 100 mg EDTA, 20 mg sodium azide, and 20 mg soybean protease inhibitor) was added. Plasma was separated by low-speed (1500g for 25 min) centrifugation at 4°C and was stored at -20°C . All samples were used either fresh or within 2 months for separation of the proteins. To determine the isotope enrichment in plasma proteins during a primed continuous intravenous infusion, two other human subjects were given a primed continuous infusion of L-[1-¹³C]leucine at a rate of 7.5 μmol/kg/h for 10 h (20). These subjects were studied as part of a protocol, which was approved by the Institutional Review Board for Human Research to investigate the effect of aging on protein turnover.

IAC

Six milliliters of ascites fluid containing the mouse anti-human ApoB-100 antibody (3 ml 5E11 and 4G3 each) and about 10 ml ascites fluids of anti-human plasma albumin and fibrinogen antibodies were used for the IAC. Each antibody was thawed to room temperature, passed through a 0.45-μm-pore-size syringe filter, diluted to 30 ml with 0.1 M sodium bicarbonate buffer, pH 7.1, and concentrated at 1500g with the Centriprep concentrator (cut point 30,000, Amicon Inc., MA) until reaching about 5 ml. This process was repeated twice. The concentrated antibodies were coupled with 5 ml Affi-Prep 10, which was prepared according to the directions from the manufacturer (Bio-Rad Laboratories). In brief, the affinity support was washed using a sintered glass filter with 100 ml cold water, followed by 100 ml coupling buffer. The washed affinity support was then added into the concentrated antibody solution. The coupling reaction took place at pH 7.1 overnight at 4°C with constant shaking. After completion of the coupling reaction, the unoccupied sites on the affinity support were blocked by reaction with 1 ml 1.0 N ethanolamine, pH 8.0, for 1 h. The antibody coupling efficiency was assessed by measur-

ing the protein content of coupling solution before and after coupling. The capacity of the affinity columns was assessed by measuring the maximal protein content of eluted proteins when enough protein was loaded onto the column.

The coupled Aff-Prep 10 with the specific antibody was packed into a 20-ml Bio-Rad Econo-Pac column. The column was washed five more times, each with 20 ml application buffer, and stored at 4°C in the same buffer with 200 μ l 2% sodium azide added. Before each operation, the column was washed successively with application buffer, elution buffer, and washing buffer and then equilibrated with application buffer.

The plasma VLDL fraction was separated by ultracentrifugation (Beckman L50, Beckman Instruments, Inc., Fullerton, CA) at a density of 1.006 g/ml at 40,000 rpm for 18–20 h at 10°C using a 70.1 Ti fixed-angle rotor. The top layer containing VLDL along with one-third of the clear solution of the second was aspirated and stored at –20°C for later separation of ApoB-100. The remaining plasma-devoid VLDL was stored at –20°C for sequential separation of plasma albumin and fibrinogen.

VLDL for separating ApoB-100 was injected into the BioLogic system via a dynaloo injection. The system allows automatic control of buffer change, loading speed, and elution collection. The sample loading was set at 0.5 ml/min and the washing and elution at 1 ml/min. Eluted ApoB-100 fractions were collected at 2-ml intervals, and the tubes containing the ApoB-100 were pooled.

Albumin and fibrinogen were separated from 1 ml plasma (plasma after removal of VLDL) by passing through the corresponding column containing either anti-human plasma albumin antibody or anti-human plasma fibrinogen antibody with the aid of the Econo-System. The two columns were connected in series so that the plasma samples passed sequentially through the two columns. When no uv absorbance appeared on the monitor, the two columns were disconnected such that elution of each protein could be separately accomplished. The sensitivity of uv absorbance of the Econo-System was set at the most sensitive point, 0.01 automatic unit full scale (AUFS), and the speed of sample loading was set at 2.0 ml/min. The eluted protein in about 4–5 ml elution buffer was collected and precipitated as described later. Thus, three proteins were separated sequentially from the same samples. The entire process was performed at room temperature.

Eluted ApoB-100 was collected and subsequently precipitated with 50% isopropanol at room temperature overnight. The precipitate was again washed twice with 50% isopropanol, once with 100% isopropanol, and air dried. The separated albumin and fibrinogen were precipitated with 10% trichloroacetic acid. The precipitates were hydrolyzed in 1 ml 6 N HCl at 110°C for 18–20 h. The hydrolysates were

further cleared up by passing through a cation-exchange column containing 3.0 ml Bio-Rad AG 50W-X8, 100–200 mesh, H⁺ form. The dried amino acid was ready to be derivatized for mass spectrometric measurement of [¹³C]leucine enrichment, otherwise stored at –80°C in 0.1 ml 0.01 N HCl.

The purity of separated ApoB-100, albumin, and fibrinogen was checked by analytical SDS-PAGE and silver staining (Bio-Rad) and compared with some other techniques. SDS-PAGE was performed on 4–15% Tris-glycine graded gels (Bio-Rad Laboratories) under both denaturing and nondenaturing conditions. The purity of ApoB-100 separated by IAC was compared with that obtained by precipitation with 50% isopropanol as described by (11).

The identity of the purified ApoB-100 and fibrinogen was also checked by amino acid sequence analysis through the Protein Core Lab of the Mayo Clinic. Protein sequencing was performed with a Perkin-Elmer/Applied Biosystems Procise Model 492A protein sequencer system (Perkin-Elmer/Applied Biosystem Division, Foster City, CA). The proteins, which had been electroblotted to the Applied Biosystem Problott membrane, were run on the sequencer in gas-phase mode. Sequence assignments were made using the Applied Biosystem Model 610A (version 2.1) data analysis software running on a Macintosh Quadra 650.

The maximal binding capacity and percentage recovery were tested by passing different amounts of pure protein through the affinity columns. The binding or saturating profile of each column was calculated by the amount of protein eluted from the affinity column. The percentage recovery was calculated as the ratio of the amount of protein eluted and total protein applied. Measurement of protein concentration was carried out by a protein assay kit from Bio-Rad.

Isotope enrichments of L-[1-¹³C]leucine in the hydrolysates of VLDL ApoB-100 were measured by gas chromatography/mass spectrometry (GC/MS) (26), and albumin and fibrinogen were measured by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) described by (2). To test the reproducibility of the technique, a blood sample was drawn from a normal subject (no pathological lipid metabolism) 1 h after a mix meal (30% lipid, 20% protein, and 50% carbohydrate). The reproducibility of the procedure for measurement of [¹³C]leucine enrichment of the separated three proteins was tested individually on five aliquots of the same sample. The isotope enrichment of [¹³C]leucine in the three proteins over time during 10 h primed continuous intravenous infusion of L-[1-C¹³]leucine was also measured in order to determine the potential application of this technique for measurement of protein synthesis.

RESULTS

Assuming half of the proteins in the ascites fluid is antibody and an antibody-affinity support coupling

efficiency of 50%, which was estimated by measurement of protein concentration in the ascites fluids before and after coupling, each affinity column for albumin and fibrinogen contained about 5–10 mg of antibody, and the affinity column for ApoB-100 contained 3 mg of antibody. The capacity of the affinity column, in terms of the largest amount of proteins obtained by this affinity technique, is more than 1 mg for VLDL ApoB-100, up to 6 mg for albumin, and 2.5 mg for fibrinogen.

Figure 1a represents a typical protein purification profile by using the Econo-System. As shown in the chart, separating one protein took about 20–30 min when the sensitivity of the uv monitor was set at the highest scale, 0.01 AUFS, and the speed of sample loading at 2.0 ml/min. The first peak in the chart indicates the unbound proteins that went through the affinity column along with the application buffer. Two proteins were sequentially purified, as indicated in this instance; the second peak was albumin and the third was fibrinogen. The affinity columns for albumin and fibrinogen were repeatedly used for 200 cycles and a small, about 20%, decrease in their capacity was noted.

Figure 1b provides the purification profile for ApoB-100 by using the BioLogic system. The eluted ApoB-100 fraction is shown in the upper right corner of the figure.

The maximal binding capacity of the affinity column for albumin was about 7.6 mg when 10 mg albumin was applied to the column. When the amounts of albumin applied to the column were less than 2 mg, nearly 100% albumin was bound to the column. When more than 3 mg albumin was applied to the affinity column, an increased amount of albumin was washed off, but the total amount of albumin bound to the affinity column increased. The percentage recovery ranged between 100 and 76% when the amount of protein applied to the column increased from 0.5 to 10 mg. The recovery of albumin from 1 ml plasma was 6 mg. The binding capacity for fibrinogen was 0.7 mg when 1 ml plasma was used, the percentage recovery being less than 50%. The binding capacity for ApoB-100 was 2.0 mg when 2 ml plasma was applied to the column, while 1.4 mg was recovered from 1 ml plasma and 0.71 mg was recovered from 0.5 ml plasma. Since the total amount of ApoB-100 in the testing plasma could not be determined, the percentage recovery was not calculated.

Figure 2 shows the purity of VLDL ApoB-100 separated by IAC subsequently precipitated with 50% isopropanol, compared with the isopropanol-precipitated VLDL as reported by Egusa *et al.* (11). When the separated proteins were run on the 4–15% graded gel, the bands at the top were ApoB-100, which was also confirmed by amino acid sequence analysis that matches the N-terminal 10 amino acids. A small band corresponding to the M_r 200 kDa appears in lane 3, the VLDL ApoB-100 which was separated by the anti-

ApoB-100 antibody available in our laboratory. No other bands were noted in lane 4; VLDL ApoB-100 was separated by IAC with the specific antibody to ApoB-100. In another lane, however, the isopropanol-precipitated VLDL shown in lane 2 contains three major bands, which include ApoB-100, ApoB-48, and a protein with a M_r of 66 kDa, possibly albumin. The specificity of the ApoB-100 also confirmed by amino acid sequence analysis, which matches the N-terminal of ApoB (sequence: E-E-E-M-L-E-N-N-V-S-L-V-X-P-X-D).

The purity of albumin is shown in Fig. 3. Only one band appears on the lane of albumin at the 66-kDa position, indicating that the separated albumin is pure (lane 1). In comparison, albumin separated by absolute alcohol extraction is not pure. Lane 2 shows human albumin separated by this method. Lane 3 is pig albumin separated by alcohol extraction.

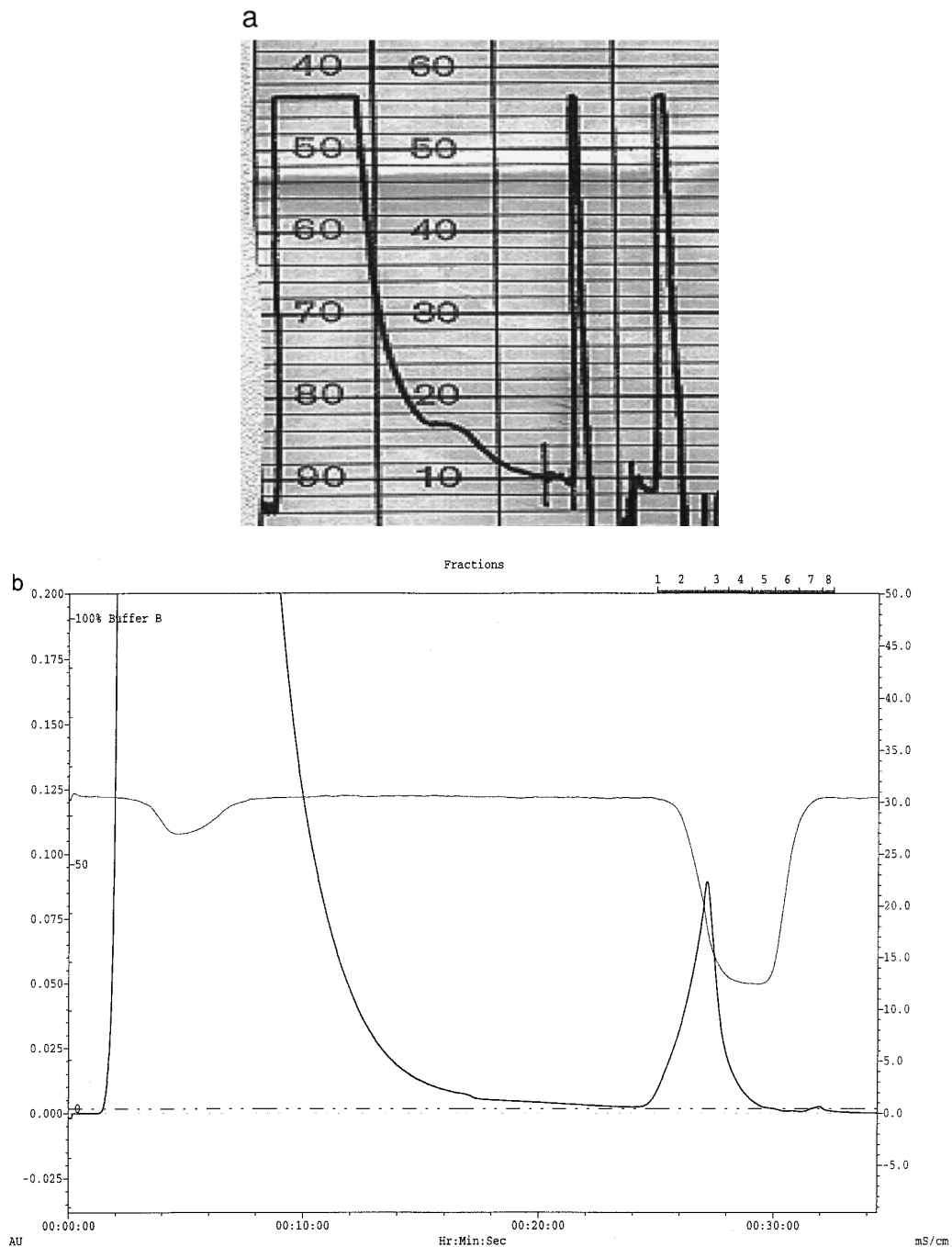
The purity of fibrinogen is indicated in Fig. 4. A heavy band and a light band appeared on the 4–20% graded gel when it was run under nonreduced conditions (lane 1) and three bands when it was run under reduced conditions (lane 2). The three bands correspond to alpha, beta, and gamma monomers of fibrinogen. The purity of the fibrinogen separated by IAC was the same as fibrinogen separated by five-peptide affinity chromatography (27) (lane 3). The specificity of fibrinogen was also confirmed by amino acid sequence analysis of the alpha band (sequence: A-D-S-G-E-G-D-F-L-A).

Table 1 shows the reproducibility of the technique when measuring the isotopic enrichment of the three proteins on five aliquots of enriched plasma samples by mass spectrometry. The coefficients of variation (CV) of atom percent excess (APE) for all the three proteins are all less than 5%.

Figure 5 depicts the [^{13}C]leucine enrichment in VLDL ApoB-100, fibrinogen, and albumin during a primed continuous infusion of L-[1- ^{13}C]leucine in a normal subject. The isotope enrichment of VLDL ApoB-100 increased much faster than that of fibrinogen and albumin, which reaches an early plateau at about 8 h of infusion. A linear increase in isotope incorporation was noted for albumin and fibrinogen. Fibrinogen has a higher isotope enrichment than that of albumin, suggesting that fibrinogen turns over faster, while the isotope enrichment of albumin is much slower. The isotope enrichments of the three proteins in the present study are compatible with that reported in other studies (8, 19, 25).

DISCUSSION

An immunoaffinity chromatographic technique to sequentially purify ApoB-100, albumin, and fibrinogen from 1–2 ml of plasma sample for measurement of rates of these protein syntheses is described. The amount of protein separated from 1 to 2 ml of plasma



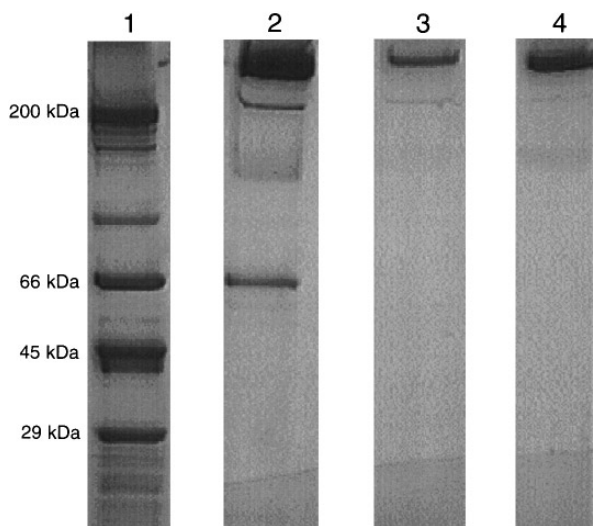


FIG. 2. SDS-PAGE of VLDL ApoB-100 was performed on 4–15% graded gel under reduced conditions. Lane 1 is the M_r standard with M_r indicated on the left of the chart. Lane 2, VLDL precipitated with 50% isopropanol. This lane shows several bands, indicating that the contamination of ApoB-100 was obtained by this technique. In lane 3 was VLDL ApoB-100 separated using monoclonal antibody developed in our lab. Minimal contamination with ApoB-48 was noted as a faint band which appeared at about 200 kDa. Lane 4 is VLDL ApoB-100 separated by monoclonal antibody which is highly specific to ApoB-100. Only one band is clearly shown, and significantly less ApoB-48 was shown.

is sufficient for measurement of isotope enrichment by mass spectrometry. The technique is highly reproducible in measuring isotope enrichment (less than 5% coefficient of variation). This technique, because of its simplicity to sequentially separate three proteins with high purity from a small amount of plasma samples, in a relatively short period of time (20 to 30 min per protein), offers potential applications in protein turnover studies.

Since different proteins are not synthesized at the same rate in the human bodies, the purity of the separated proteins is of primary importance for measurement of isotope enrichment of a specific protein. As shown in Figs. 3 through 5, all three proteins separated by IAC in the present study attained higher purity compared with the frequently used techniques, as assessed by analytical SDS-PAGE followed by silver staining.

The purity of separated VLDL ApoB-100 was further confirmed by comparing with that of isopropanol-precipitated VLDL, a technique described by Egusa *et al.* (11), and was used by several studies that measured isotope enrichments (1, 5, 7). ApoB-48 was consistently present with ApoB-100 when VLDL was precipitated with 50% isopropanol. As shown in Fig. 2, isopropanol precipitation does not allow purification of ApoB-100 from ApoB-48 as well as from other proteins such as albumin. In comparison of the two techniques, our

method allows a substantial improvement in purification of ApoB-100, which showed very little ApoB-48 contamination in comparison with isopropanol precipitation.

The selectivity of a specific monoclonal antibody to ApoB-100 is a primary factor that determines the purity of ApoB-100 separated by IAC in this study. In this instance, the ApoB-100 separated by an affinity column that employed one monoclonal antibody against ApoB (actually both ApoB-100 and ApoB-48) still contained a small amount of ApoB-48. It is thus recommended that in studies when the ApoB-48 level is likely to be high, a very specific monoclonal antibody should be used.

To our knowledge, separating albumin by IAC with immobilized specific monoclonal antibody has not been previously reported. It has been shown that albumin purified through other techniques, such as affinity chromatography with immobilized fatty acids (24, 27), bilirubin (16), and some other ligands (13) were generally used, but contaminations may be present for some techniques. Precipitation with ethanol (15) and polyethylene glycol (12) may not be highly specific; also, albumin separated by alcohol extraction is not pure. In comparison, the albumin separated in the present study is highly pure, and the technique is also simple and efficient (Fig. 3).

The purity of fibrinogen separated by the current technique is comparable to that separated by using a chromatographic procedure with immobilized five peptides which bind to the alpha chain of fibrinogen (18). Both techniques used an affinity chromatographic procedure and reached similar purity. The current technique may provide another approach for fibrinogen purification, in addition to the frequently used other techniques, such as precipitation with thrombin, ethanol, ammonium sulfate, β -alanine, or glycine. Also, IAC has the additional advantages of sequentially purifying two proteins as shown in the present study.

The IAC technique described here shows no evidence of antibody leakage or antibody contamination of the separated proteins as demonstrated by a single band purity of the three proteins separated on SDS-PAGE gel samples followed by silver staining. This may be attributed to the highly stable Affi-Prep gel and to the thorough washing of the column with elution buffer before each use. Although the longevity of the column could not be determined, the columns for albumin and fibrinogen have been used more than 200 times, the two columns appeared to decrease their capacity progressively, but are still in useful condition.

The efficiency of the coupling of antibodies onto the affinity supports is the first step in the current technique which decides the capacity of the affinity column. Although purification of monoclonal antibody in ascites fluids for IAC purposes was generally performed (14), our experience proved this may not be necessary. Prep-

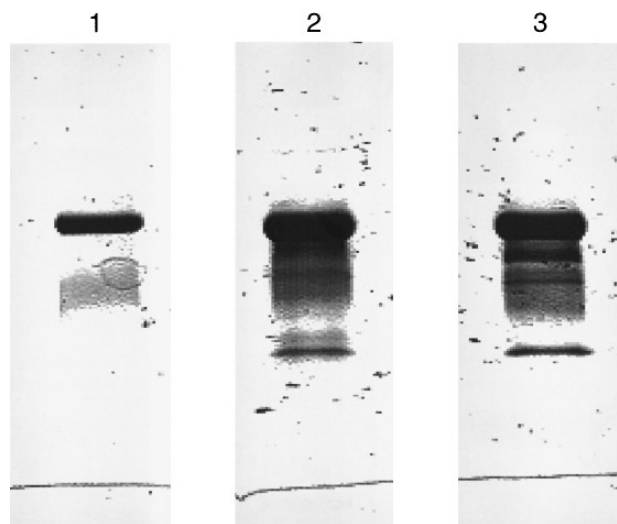


FIG. 3. SDS-PAGE of albumin separated through IAC was run on 4–15% graded gel. Lane 1, albumin separated by the current method; lane 2, albumin (human); and lane 3, pig albumin separated by ethanol extraction.

paration of antibody for coupling by simply concentrating with a Centrprep filter allowed us to achieve a 50% coupling efficiency. The coupling efficiency may be increased by further purification of the antibodies through other ways, such as precipitation with ammonium sulfate.

The primary objective of the current method is to obtain a sufficient amount of pure protein for isotopic

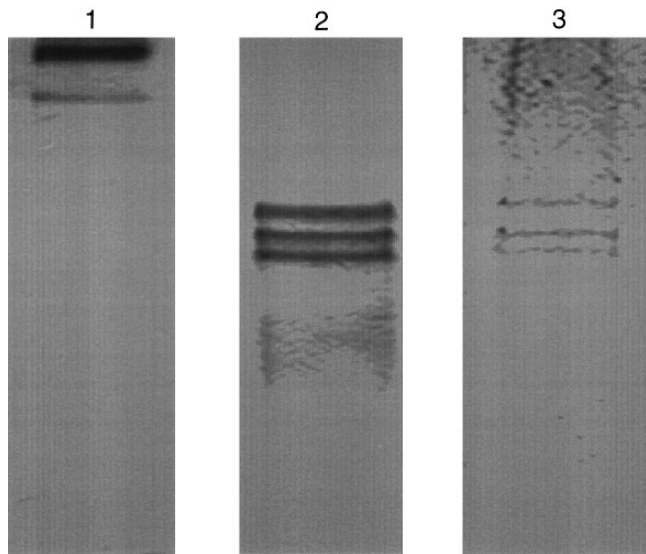


FIG. 4. Fibrinogen separated by IAC compared with that separated by five-peptide affinity chromatography. Lanes 1 and 2 show human fibrinogen separated by the current technique. Lane 1, SDS-PAGE under nonreducing conditions; lane 2, SDS-PAGE under reducing conditions; and lane 3, fibrinogen separated by five-peptide affinity chromatography and SDS-PAGE under reducing conditions.

TABLE 1

Reproducibility of Measurements of Five Aliquots of Plasma Samples

Sample No.	Albumin (APE) ^b	VLDL ApoB-100 (APE)	Fibrinogen (APE)
1	0.0974	3.4948	0.0724
2	0.0962	3.1315	0.0663
3	0.0941	3.3629	0.0703
4	0.0910	3.4575	0.0668
5	0.0990	3.2430	0.0723
Mean + SE	0.0955 ± 0.0014	3.3379 ± 0.0676	0.0696 ± 0.0013
CV (%) ^a	3.2494	4.5225	4.2091

^a CV, coefficient of variation = 100 × standard deviation/mean.

^b APE, atomic percentage excess.

enrichment measurement. We accomplished this objective by purifying 6.0 mg albumin, 2.0 mg fibrinogen, and 1 mg ApoB-100 from 1- to 2-ml plasma samples. The amounts of proteins we obtained are sufficient for appropriate mass spectrometry analysis. Although we did not accomplish a complete recovery of these proteins from plasma, we demonstrated that when a smaller amount of albumin (2 mg) was injected, 100% recovery was achieved. A larger column with higher capacity could accomplish higher recovery from plasma. For accomplishing 100% recovery, the amount of protein injected into the column should be less than the binding capacity of the column. It is also possible that the recovery of albumin is less when plasma is used, which contains higher concentration of albumin and many other proteins. For the purpose of obtaining sufficient amounts of albumin or other proteins for isotopic measurements, the yield from 1 ml of plasma using this technique is adequate.

As we demonstrated in this study, the IAC technique performed after separation of the VLDL fraction by ultracentrifugation allowed us to separate ApoB-100 from ApoB-48. Since ApoB-48 is synthesized in the gut and its synthesis is stimulated by meal (especially fat meal), it is important to use a technique that allows complete separation of ApoB-100 from ApoB-48. To use the isotope enrichment of ApoB-100 at plateau as a surrogate measure of amino acyl-tRNA in liver to measure liver protein synthesis, it is critical to purify ApoB-100 from ApoB-48. In the fasted state, ApoB-48 concentration may be small but synthesis increases during meals, which contribute significantly to the VLDL fraction of plasma proteins. The current technique allows us to use ApoB-100 enrichment at a plateau as a surrogate measure of amino acyl-tRNA enrichment in human study during fasting and following meals. This technique allows us to measure ApoB-100 synthesis in humans.

The potential application of this IAC technique in the measurement of isotope enrichment in protein

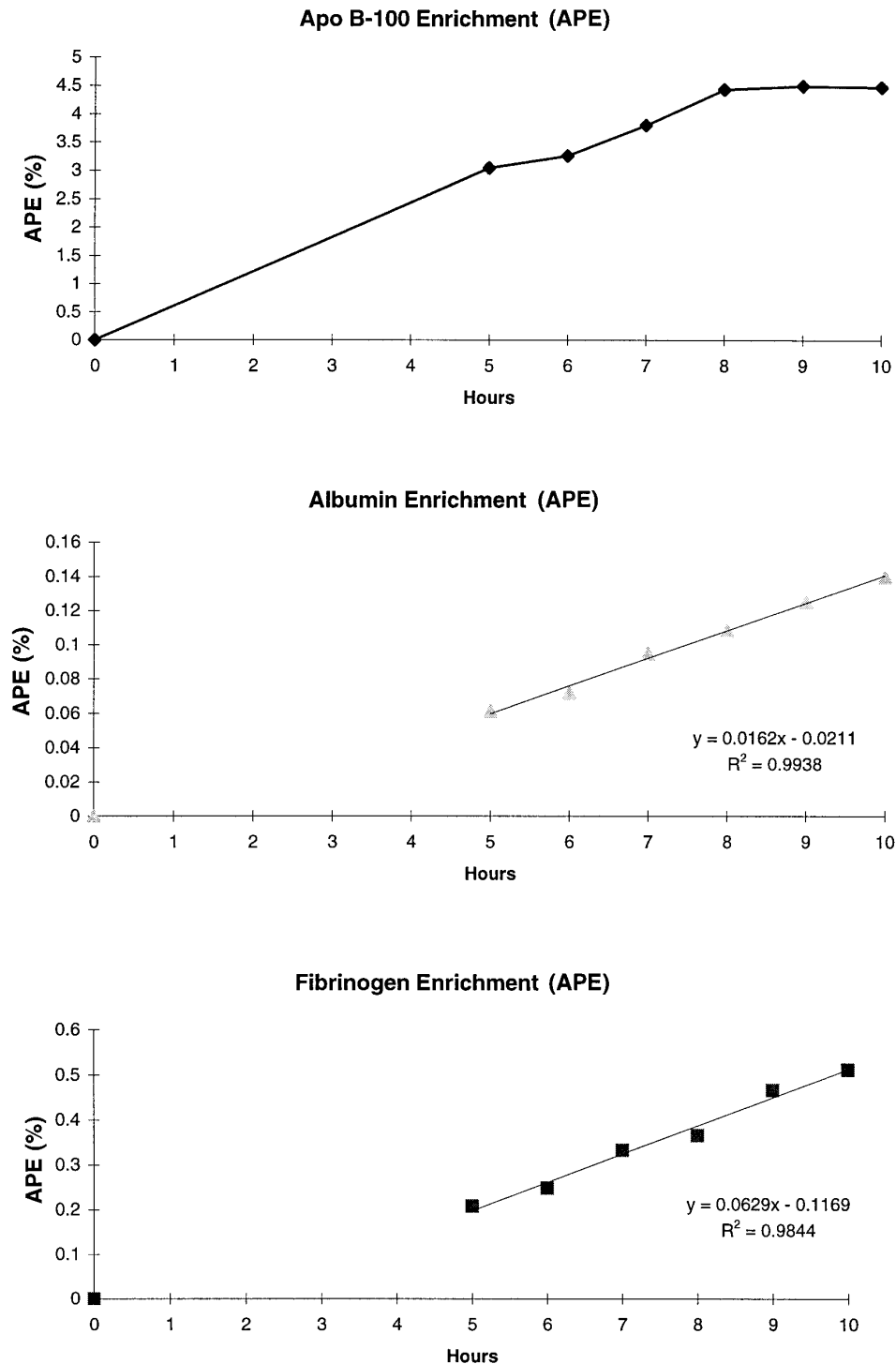


FIG. 5. Measured isotope enrichment of VLDL ApoB-100, albumin, and fibrinogen from plasma samples collected during a primed continuous infusion of L-[^{13}C]leucine in a normal human subject. Isotope enrichment of VLDL ApoB-100 reached a plateau at about 8 to 9 h after starting infusion of isotope. A linear increase in isotope enrichment during 10 h isotope infusion was noted for albumin and fibrinogen.

turnover studies has been demonstrated. As shown in Fig. 4, [^{13}C]leucine enrichments in the separated albumin and fibrinogen displayed a linear increase over a

10-h infusion period. The [^{13}C]leucine enrichment of VLDL ApoB-100 attained an early plateau within 8 h of infusion. Thus, samples collected between 8 and 10

h are sufficient to measure synthesis rates of albumin and fibrinogen.

In summary, we described here an immunoaffinity chromatographic technique to sequentially purify ApoB-100, albumin, and fibrinogen from plasma samples and measure their isotopic enrichments. The major advantage of the method is the efficiency of the technique to sequentially purify sufficient amounts of these liver proteins from 1- to 2-ml plasma samples for isotopic enrichment measurement. In addition, this technique allows us to obtain higher purity ApoB-100 and albumin than currently used techniques. The technique has the potential for application in separating other proteins of interests.

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