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SEPARATION OF TRANSFERRIN TYPES IN HUMAN PLASMA BY ANION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An anion-exchange high-performance liquid chromatographic procedure was developed for the separation of transferrin in human plasma. The procedure allowed the four molecular forms of transferrin, which differ with respect to bound iron, to be separated from each other and from other plasma proteins. Transferrin variants, including B and D types, could also be identified using anion-exchange high-performance liquid chromatography. The approach followed for optimizing the separation of transferrin included identification of the peaks in the chromatogram by two-dimentional polyacrylamide gel electrophoresis. This approach could be extended to other proteins in plasma or biological fluids in order to optimize their separation.

INTRODUCTION

Transferrin (Tf) is an abundant protein in human plasma. The average concentration is *ca.* 250 mg/dl, or *ca.* 3–4% of the total protein[1]. Human Tf, a monomer of 79,550 daltons, contains two homologous iron-binding domains[2]. Each iron-binding domain has an approximately equal affinity for iron. Depending on the individual plasma iron levels, four forms of Tf can be distinguished by one-dimensional polyacrylamide gel electrophoresis (1D PAGE) in the presence of 6 *M* urea[3]: apo-Tf, two monoferric transferrins (iron bound at the N- or C-terminal domain) and diferric Tf. A number of Tf variants have been detected by 1D PAGE[1] and isoelectric focusing (1EF)[4]. B-Type variants are electrophoretically more anodic while D-type variants are more cathodic than the common C-type transferrins. Recently, two-dimensional polyacrylamide gel electrophoresis (2D PAGE) has been used to detect transferrin variants in addition to variants of a number of other plasma proteins[5–7].

Anion-exchange high-performance liquid chromatography (HPLC) of serum proteins has been proposed as a rapid method for the detection of gross serum protein

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abnormalities[8–11]. Peaks corresponding to only a few plasma proteins (IgG, albumin, Tf and pre-albumin)[11] or broad globulin fractions (γ- and β-globulins) have been identified[10].

In this report we describe the separation of four Tf components, which differ in the amount of bound iron, from each other and from other plasma proteins, by anion-exchange HPLC. We also demonstrate the application of the separation procedure for the detection of Tf variants in plasma. The usefulness of utilizing 2D PAGE in the analysis of plasma protein HPLC profiles is also demonstrated.

EXPERIMENTAL

Materials

Fresh blood was drawn into tubes containing ACD* as anticoagulant. Samples were obtained from 25 families, consisting of a father, mother and newborn child. Plasma, prepared by centrifugation at 900 *g* for 5 min, was stored at −20°C. Additional plasma samples containing Tf variants were generously supplied by Drs. H. Mohrenweiser, University of Michigan Medical School, Ann Arbor, MI, U.S.A. (Tf B_1, Tf B_2, Tf C_2, Tf D_1 and Tf D_{CHI}), M. Fujita, Radiation Effects Research Foundation, Hiroshima, Japan (Tf B_1, Tf C_2, Tf D_1 and Tf D_3), and F. Kueppers, Temple University, Philadelphia, PA, U.S.A. (Tf C_2); in addition to the variant Tf, all plasma samples contained the common Tf C_1. Purified apo-Tf was obtained from Calbiochem (Palo Alto, CA, U.S.A.) and contained only the common Tf C. Thawed plasma samples were centrifuged at 2000 *g* for 10 min and used without further treatment. Alternatively, plasma containing iron-saturated Tf was utilized. To that end, ferrous sulphate (1 m*M*) was added to plasma or to purified Tf to saturate both iron binding sites. Ferrous ion was used to minimize formation of hydroxides of unbound iron at the initial conditions of pH 7.8.

Buffers were prepared with Milli-Q water (Millipore, Bedford, MA, U.S.A.) and were filtered through a 0.2 μm filter. Reagent-grade chemicals were used.

Anion-exchange HPLC

A Waters HPLC system was used. The gradient system consisted of an M6000A pump modified with Easy Prime outlet check valves, an M45 pump, a 720 System Controller, a 710B WISP automatic injector or U6K manual injector, a 440 fixed-wavelength detector with 280 nm filter and a 730 Data Module (Waters Assoc., Milford, MA, U.S.A.).

Columns (100 × 4.1 mm) were packed[12] with Synchropak AX300 anion exchanger consisting of 10 μm silica particles (Synchrom, Linden, IN, U.S.A.). All columns were prepared from the same batch of ion exchanger.

Two-dimensional PAGE

In selected experiments, column effluent from chromatographic zones of interest was collected manually, either as individual peak fractions (valley to valley) or as regions between peaks. Individual fractions were dialyzed against water, lyophi-

* Trisodium citrate (1.32 g), citric acid (0.48 g) and dextrose (1.47 g), made to 100 ml in water; 0.25 ml ACD per ml of blood.

lized and analyzed by 2D PAGE[5,6]. Fractions derived from an equivalent of 3–6 μl of plasma were used for electrophoresis. The gels were initially stained with Coomassie Blue. The same gels were subsequently silver-stained by the more sensitive method of Sammons *et al.*[13].

RESULTS

Anion-exchange separation of plasma proteins and identification of Tf by 2D PAGE

Anion-exchange HPLC of plasma containing the common Tf C_1 is shown in Fig. 1. A sodium phosphate gradient at pH 7.8 was used. Peak fractions of the chromatogram were collected and analyzed for plasma proteins by 2D PAGE. A Coomassie-stained 2D gel of plasma polypeptides is shown in Fig. 2. Major Coom-

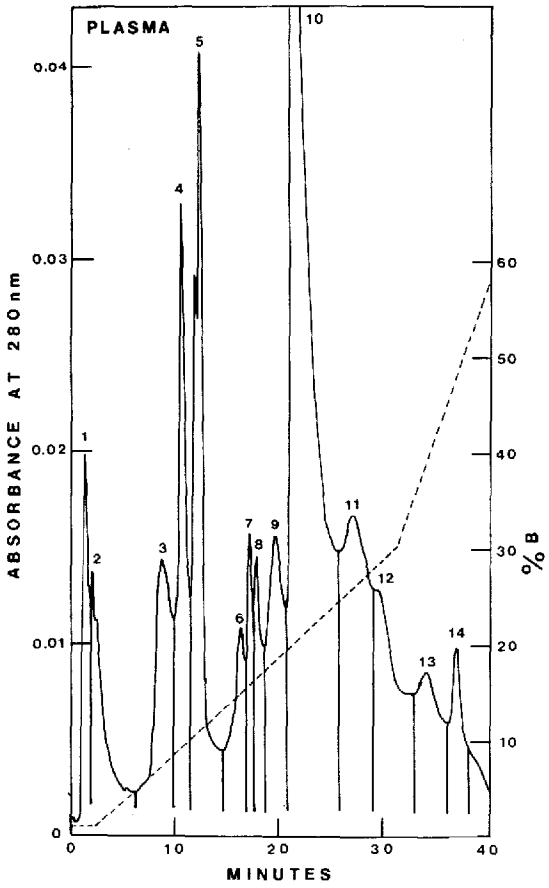

Fig. 1. Anion-exchange HPLC of total plasma. A 20-μl sample of plasma, containing Tf C_1, was diluted ten-fold with Buffer A and injected. Buffer A: 0.004 *M* sodium phosphate (pH 7.8). Buffer B: 0.4 *M* sodium phosphate (pH 7.8). Flow-rate: 0.7 ml/min. Gradient: 0–30% B in 30 min, then 30–60% B in 10 min. For reequilibration, Buffer A was passed through the column at a flow-rate of 1.5 ml/min for 10 min. The effective gradient at the detector is shown (dashed lines).

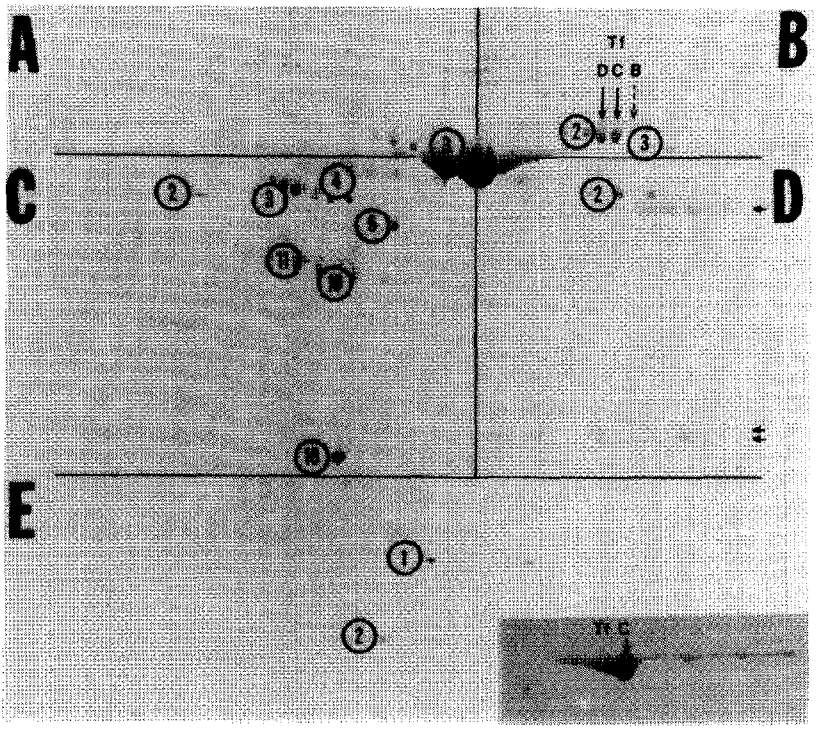

Fig. 2. Two-dimensional polyacrylamide gel electrophoresis of plasma proteins. A sample of plasma (3 μl) containing Tf C_1 and Tf D_1 was electrophoresed. The gel is subdivided into five regions (A–E) and proteins within regions are numbered to the left of the spot(s) by a previously described convention[5–7]. A horizontal string of spots corresponding to immunoglobulin γ chains (region D, upper arrow) and κ and λ chains (region D, lower arrows) are also indicated. Numbered proteins, the identity of which has been determined in 2D gels or for which genetic variation has been described[6,7,14], include the following: A-003, albumin; B-002, transferrin; B-003 unknown polymorphic protein[6,7]; C-002, α_2 HS glycoprotein; C-003, α_1 antitrypsin; C-004, Gc-globulin; C-005, fibrinogen-α chain; C-010, haptoglobin-β chain; C-011, unknown, genetic variation demonstrated[6,7,14]; C-016, apolipoprotein A-1; D-002, fibrinogen-β chain; E-001, haptoglobin-α chain; E-002, prealbumin. Tf C and D are indicated (solid arrows) within region B. The position of Tf B is indicated by a dashed arrow. Insert: Tf region (B-002) of 2D gel of Tf C_1 isolated from plasma by anion exchange HPLC: the major Tf peak of Fig. 6 was collected and electrophoresed; only the Tf region of the 2D gel contained Coomassie Blue-stained polypeptides.

assie-stained plasma proteins, which have been identified in 2D gels or for which genetic variation has been described[5–7], are indicated in Fig. 2. Individually isolated peak fractions showed only a few polypeptide spots by Coomassie staining of the gels. Silver staining of the same gels revealed numerous additional spots (*e.g.* Fig. 3) representing minor plasma proteins. The sensitivity of silver-staining provided numerous reference points within the gels for the unambiguous identification of plasma proteins in the HPLC profile.

The distinctive 2D gel pattern of Tf (Fig. 2, insert), consisting of a major spot and several minor spots on the cathodic side of the major spot, was detected in fractions 4 and 5 (Fig. 1) which were eluted at 11.1 and 12.8 min, respectively.

Additional major plasma proteins were identified in the chromatogram. Table

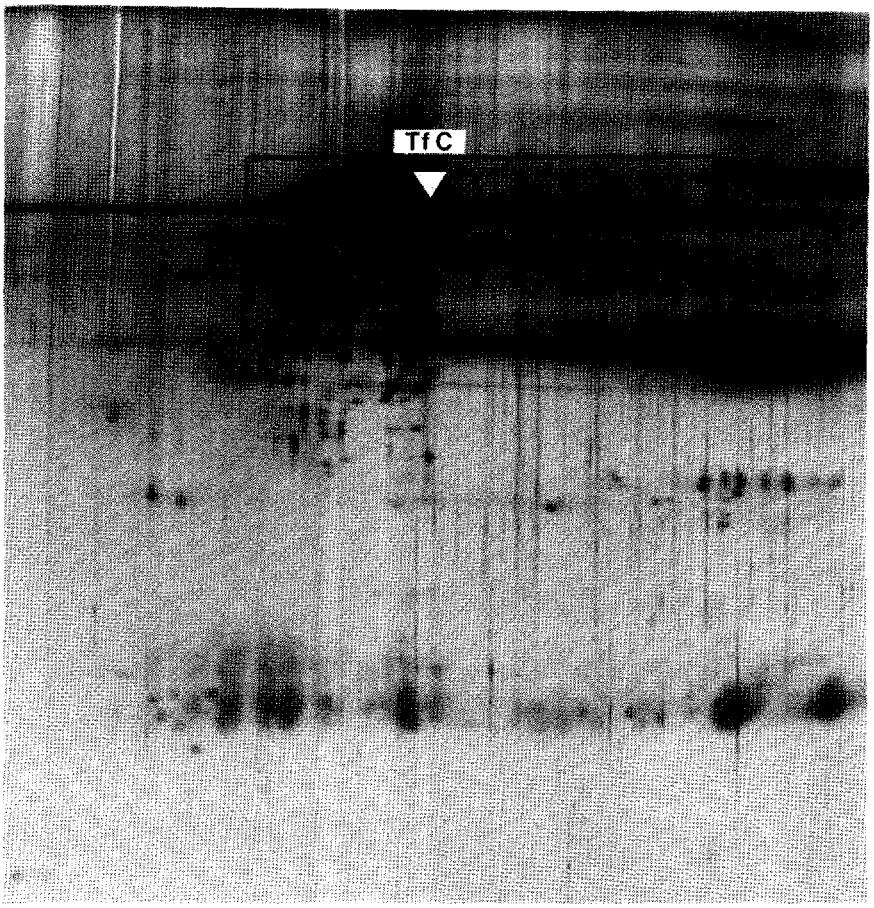

Fig. 3. Silver stained 2D gel of Tf from plasma. The major Tf peak which was eluted at 25.3 min in Fig. 6 was collected and electrophoresed. The Tf region of this gel (enclosed area) which had been stained with Coomassie is shown in the Insert to Fig. 2.

I lists the chromatographic elution position of the major plasma proteins which are labeled in Fig. 2 and which were identified by 2D PAGE.

Separation of multiple iron binding forms of Tf

By using a combination of a weaker buffer, Tris-HCl instead of phosphate, and a decreasing pH gradient, Tf was eluted well after the immunoglobulins and before the other plasma proteins. Four distinct peaks were eluted at 23.9, 25.4, 27.5 and 29.5 min (Fig. 4, peaks a–d, respectively). Use of 2D PAGE indicated that each of these peaks contained predominantly Tf with a small amount of other contaminating proteins. Each Tf peak (Fig. 4, peak a–d) had similar patterns on Coomassie-stained 2D gels. No significant differences were observed in the relative amount of the major Tf spot and its associated minor spots in the Tf 2D gel pattern of peaks a–d. HPLC analysis of 30 individual plasma samples revealed a considerable degree of quantitative variability within this region (*cf.* Fig. 5, top tracing). The heteroge-

TABLE I

HPLC ELUTION POSITION OF SOME PLASMA PROTEINS, IDENTIFIED BY 2D PAGE

Protein	*Protein or subunit spot designation**	*Anion-exchange HPLC fraction*
Albumin	A-003	10, 11
α_1 Antitrypsin	C-003	9, 10
Apolipoprotein-A1	C-016	9–11
Fibrinogen** (α chain)	C-005	11–14
(β chain)	D-002	11–14
Gc-Globulin	C-004	6, 7
Haptoglobin (α chain)	E-001	8, 9
(β chain)	C-010	8, 9
α_2 HS Glycoprotein	C-002	11, 12
Immunoglobulin (γ chain)	–***	1–5
(κ, λ chains)	–§	1–5
Prealbumin	E-002	13
Transferrin	B-002	4, 5
Unknown§§, polymorphic	B-003	12
Unknown§§§, genetic variant	C-011	9–11

* Proteins or their subunits are numbered by a previously described convention[5–7].

** Fibrogen γ-chain is not readily visualized on Coomassie-stained gels.

*** Region D upper arrow of Fig. 2.

§ Region D lower arrows of Fig. 2.

§§ Polymorphic plasma protein described by Rosenblum and co-workers[6,7].

§§§ Plasma protein with genetic variation described by Tracy *et al.*[14] and Rosenblum and co-workers[6,7].

neity and quantitative variability seen in plasma Tf may be explained if each Tf peak represented different amounts of bound iron.

To test this hypothesis, the four Tf forms were individually isolated and saturated with iron prior to rechromatography (Fig. 5). Saturation with iron resulted in a shift in retention time of each form of Tf except the form eluted at position b. Samples of Tf from peaks a and b were contaminated with small amounts of material eluted between 8 and 11 min. Peak c contained a component the retention time of which was not altered by added iron. Purified apo-Tf containing undetectable iron (< 0.004 mole of Fe per mole of Tf) was eluted at position c. Saturation of purified Tf with iron resulted in a shift in retention time to position b. The addition of subsaturating amounts of iron to apo-Tf resulted in the appearance of all four molecular forms with the progressive decrease of peak c and increase of peaks a, b and d as the level of added iron was increased to give increasing percentage saturation of Tf. We conclude that diferric-Tf was eluted at position b while apo-Tf was eluted at position c. The monoferric forms of Tf were eluted at positions a and d.

Chromatography of iron-saturated Tf in total plasma is shown in Fig. 6. A major peak which was eluted at 25.3 min contained predominantly Tf. The Coomassie-stained 2D gel of this peak is shown in Fig. 2 (insert). The silver-stained gel is shown in Fig. 3. Tf was also detected in the regions which were eluted at *ca.* 21.5 and 28.8 min. With the exception of the Tf region, the 280-nm absorbance profile of plasma was unaffected by the addition of iron.

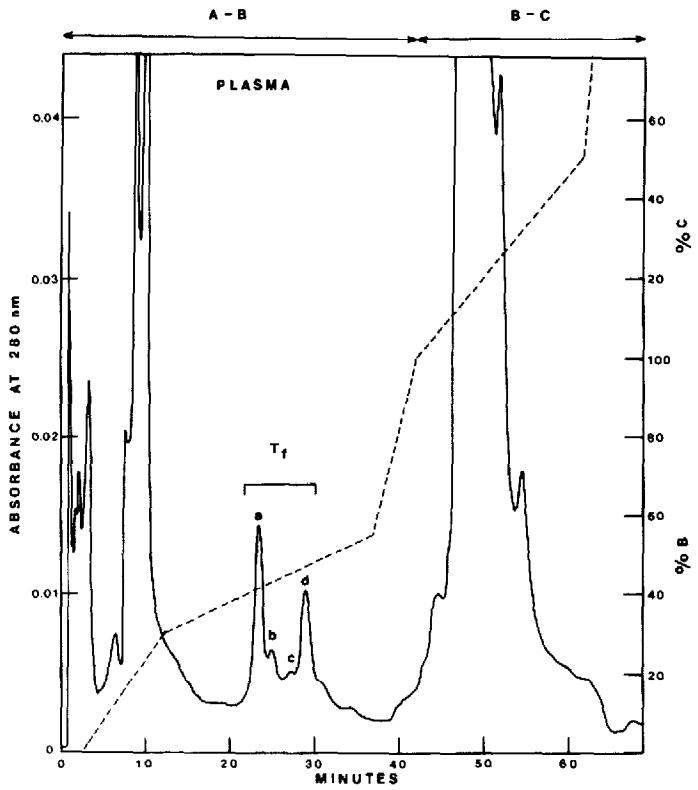

Fig. 4. Anion-exchange HPLC of plasma with a modified gradient elution. A 20-μl sample of plasma, containing Tf C_1, was diluted 10-fold with buffer A and injected. Buffer A: 0.02 *M* Tris-HCl (pH 8.0). Buffer B: 0.02 *M* Tris-HCl and 0.2 *M* sodium chloride (pH 7.0). Buffer C: 0.4 *M* sodium phosphate (pH 7.8). Flow-rate: 1 ml/min. Gradient A–B: 0–30% B in 10 min, 30–55% B in 25 min, 55–100% B in 5 min. Gradient B–C: 0–50% C in 20 min, 50–100% C in 2 min. For reequilibration, Buffer A was passed through the column at a flow-rate of 1.5 ml/min for 10 min. Individual forms of Tf are labeled (a–d).

A gradual decrease in retention time for Tf was observed with prolonged use of the column. After *ca.* 50 injections of plasma, diferric Tf was eluted *ca.* 1 min earlier (Fig. 5) than on a relatively new column (Figs. 6 and 7).

Detection of Tf variants in plasma

Plasma samples containing known variants of Tf were chromatographed after saturation of Tf with iron (Fig. 7). All plasma samples from individuals heterozygous for Tf C_1 and for either a B- or D-type Tf variant showed a new peak in an amount approximately equal to that of Tf C_1. D-Type electrophoretic variants were eluted earlier than Tf C_1. Tf D_1 and Tf D_{CHI} were incompletely separated from Tf C_1. We could unambiguously identify them as D-type Tf variants, but we could not distinguish between D_1 and D_{CHI}. Tf D_3 was eluted immediately after the IgG region and was separated from Tf C_1 by 6.4 min. B-Type electrophoretic variants were eluted later than Tf C_1. Tf B_1 was eluted 4.4 min later than Tf C_1 and was well separated. Tf B_2 was incompletely separated from Tf C_1 and was eluted 1.5 min later. Tf C_2 and Tf C_1 could not be resolved.

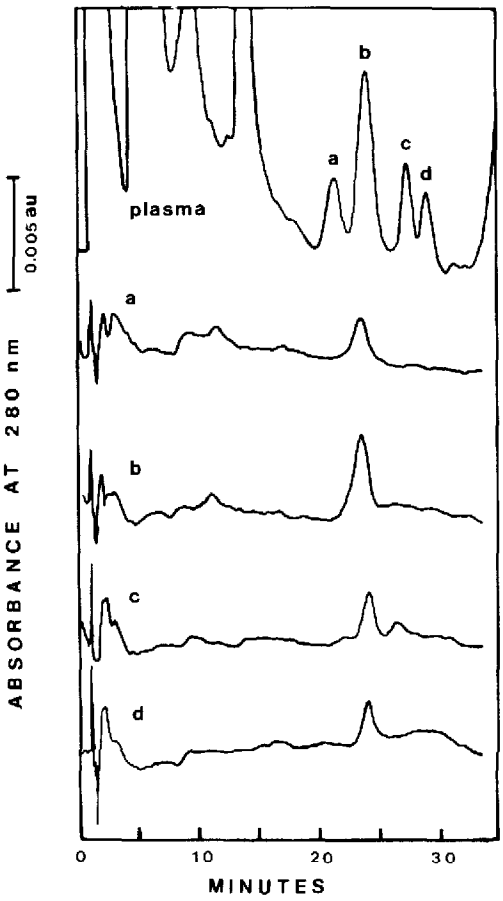

Fig. 5. Effect of iron saturation on retention times of individual forms of transferrin. Untreated plasma was chromatographed (top tracing), individual forms of transferrin (peaks a–d) were collected, and iron(II) sulphate (1 m*M* final concentration) was added to saturate Tf. Each sample was then diluted with an equal volume of water and rechromatographed. Tracings a–d correspond to rechromatography of material isolated from peak regions a–d, respectively.

Additional plasma samples were obtained from 25 families (father, mother and newborn child) to test the ability of anion-exchange HPLC to detect Tf variants in previously untyped plasma samples. Plasma Tf was saturated with iron prior to analysis. Tf variants were detected in five genetically unrelated individuals. Three Tf B_1, one Tf B_2 and one Tf D (Tf D_1 or Tf D_{CHI}) variants were found. Inheritance was demonstrated for the three Tf B_1 variants. Tf B_1 was found in the plasma of the child in three families. In two of these families, the variant was inherited from the mother and in one from the father.

Independent analysis of the same 25 families by 2D PAGE confirmed these results, but 2D PAGE could not readily distinguish among the Tf B, Tf C and Tf D subtypes. No other Tf variants were detected by 2D PAGE in the remaining 67 individuals which were typed by anion-exchange HPLC as having only the Tf C

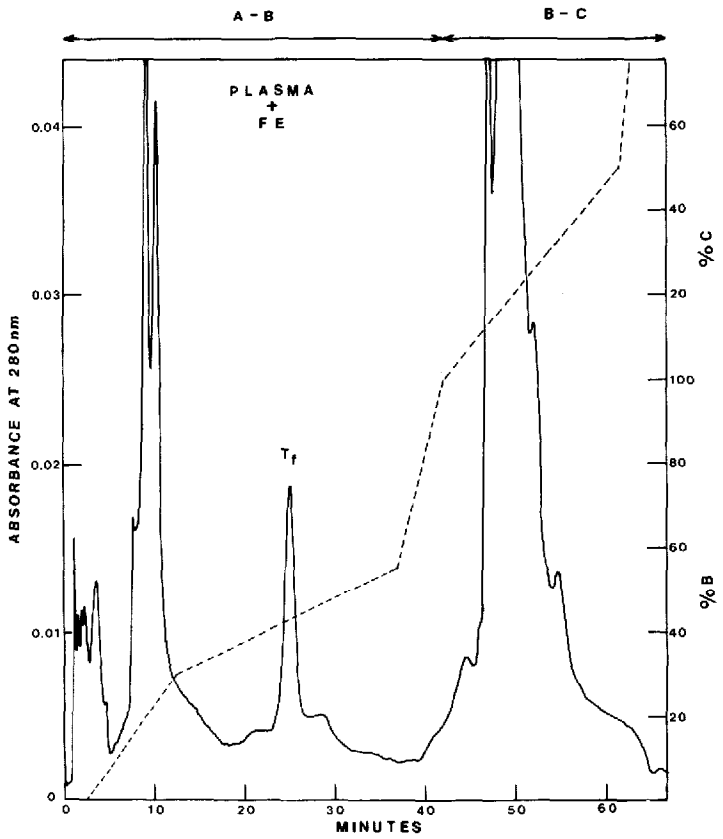

Fig. 6. Anion-exchange HPLC of iron-saturated transferrin in plasma. Chromatographic conditions as in Fig. 4. Iron(II) sulphate was added to plasma and diluted ten-fold with Buffer A to give a final concentration of 1 m*M* iron(II) sulphate.

allele. The Tf B subtyping was confirmed by conventional 1D PAGE. Our Tf D variant was identified as Tf D_1 by 1D PAGE.

DISCUSSION

Quantitation of the different molecular iron-bound forms of Tf is presently accomplished by PAGE in the presence of urea followed by protein staining and densitometry[15]. Anion-exchange HPLC can provide an alternative means of measuring the level of iron-saturation of plasma Tf. In addition, the different iron-bound forms can be recovered for use in studies of the iron-donating efficiency of each iron-binding domain in iron transfer to cells. This subject has recently been reviewed[16].

The complex absorbance profile seen in the anion-exchange HPLC of Tf should alert others to the limitations of using purified proteins alone to identify elution position. Purified apo-Tf was previously used to identify Tf in human serum protein absorbance profiles by anion exchange[11]. Our results show that a previously

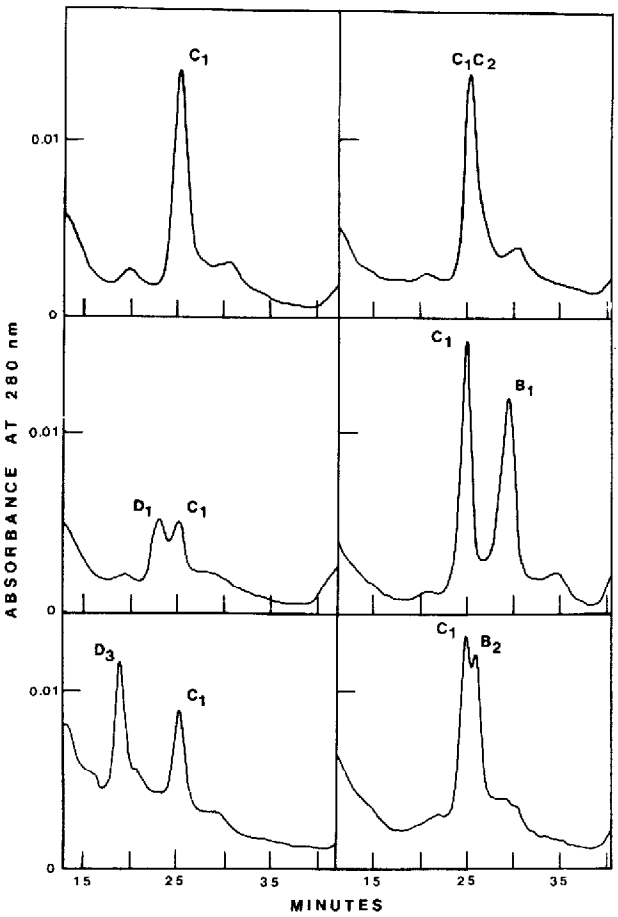

Fig. 7. HPLC of transferrin varients saturated with iron. Chromatographic conditions as in Fig. 4. Plasma contained known Tf variants as follows: Tf C_1 only (top left); Tf C_1 and D_1 (middle left); Tf C_1 and D_3 (bottom left); Tf C_1 and C_2 (top right); Tf C_1 and B_1 (middle right); Tf C_1 and B_2 (bottom right). Plasma samples were from adults, except for the sampling containing Tf D_1 which was from a neonate.

unidentified peak and shoulder represent diferric and one of the monoferric forms of Tf. A combination of 2D PAGE and chromatography of Tf genetic variants assisted in the analysis. Monospecific antibodies would also be useful in the analysis of complex protein mixtures.

The relative elution times of the different bound iron forms of Tf did not agree with an elution order based on electrophoretic mobility. The monoferric forms of Tf are intermediate in mobility between apo-Tf and diferric Tf by electrophoresis, whereas apo-Tf and diferric Tf are intermediate in elution time between the monoferric forms by anion-exchange HPLC. The relative elution times of the Tf variants which we studied agreed with the predicted order of elution based on electrophoretic mobility with one exception. Tf B_2 is electrophoretically more anodic than Tf B_1 but Tf B_2 was eluted before Tf B_1 by anion-exchange HPLC. Electrophoretic mobility

in 1D PAGE systems reflects not only the charge of the protein but also the shape[17], and is an unreliable criterion for predicting relative elution times in ion-exchange chromatography.

In our initial studies we attempted to separate known Tf variants in untreated plasma. Five to seven peaks and major shoulders were observed in the Tf region. It was clear from the absorbance profile that the Tf region of plasma was more complex than normal plasma but no reliable peak identifications could be made. Iron saturation of plasma Tf was essential to phenotype the Tf variants accurately.

We consistently found lower amounts of Tf in the plasma of newborn children in the families we examined (*cf* Fig. 7, middle, left). This is in agreement with the reported lower levels of plasma Tf in neonates[18].

We found that plasma protein carry-over from previous chromatograms was not a problem with relatively new columns (*ca.* 20–30 samples). This corresponds to the application of *ca.* 400–500 μl of total plasma to the column. When a cumulative volume of 1 ml of plasma had been applied to the column, significant protein carry-over was observed for minor plasma polypeptides as revealed by silver-staining of 2D gels[19]. The protein carry-over could not be detected in Coomassie-stained gels. The 2D gels of plasma are so complex that a selective loss, *e.g.* irreversible binding or low recoveries of some minor proteins, may have been overlooked in the 2D PAGE analysis of individual fractions from anion-exchange chromatography. Improved column clean-up procedures other than high salt (0.4 *M* phosphate) must be devised, if minor plasma proteins are to be studied.

ACKNOWLEDGEMENT

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