Journal of Chromatography, 432 (1988) 103–111
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4338

SEPARATION OF TRANSCOBALAMIN II ISOPROTEINS BY MEANS OF CHROMATOFOCUSING

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(First received February 16th, 1988; revised manuscript received June 7th, 1988)

SUMMARY

Transcobalamin II, the principal cobalamin- binding protein in human plasma, expresses a genetic polymorphism. Four more or less common alleles, denoted by X, S, M and F, have been defined earlier by means of gel electrophoretic techniques followed by autoradiography. This technique is less suitable for the analysis of individual samples and requires long exposure times. This paper describes the analysis of transcobalamin II phenotypes by means of fast protein liquid chromatofocusing. This technique has the advantage that the results of the analysis of several samples can be obtained within a day, and it also seems applicable to the preparative separation of transcobalamin II isoproteins. The sequence of elution of the isoproteins was in complete accordance with the banding pattern obtained by electrophoretic separation. The characteristic doublet bands found with polyacrylamide gel eletrophoresis were less obvious in the chromatofocusing elution pattern.

INTRODUCTION

Transcobalamin II (TC II) is the principal transport protein for vitamin B_{12} or cobalamin (Cbl) in plasma and distributes Cbl over the tissues through specific cellular surface binding sites [1–4]. It is functionally and immunologically different from the other plasma Cbl carriers, the R-binders, which seem to function as Cbl scavengers rather than as specific transport proteins [5].

A genetic polymorphism of TC II has been described by Daiger et al. [6] and Fràter-Schröder and co-workers [7,8]. Four more or less common alleles, denoted

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by X, S, M and F, have been observed with polyacrylamide gel electrophoresis (PAGE) of serum saturated with [^{57}Co]Cbl and subsequent autoradiography of the gel [9]. These electrophoresis patterns display two remarkable phenomena. Individuals homozygous for a TC II allele present two bands of equal density and heterozygotes display four or sometimes three bands when overlap between two of the bands occurs. The distance between the two bands in a doublet is equal between different isotypes, but the density of the two isotypes in a heterozygote differs in such a way that the faster variants tend to show more intense bands, which correlates with a higher Cbl-binding capacity. Porck et al. [10] have demonstrated that these differences represent different concentrations of protein rather than different affinities for Cbl.

The electrophoretic method for the identification of the phenotypes in individual serum samples appears reliable in experienced hands and is very suitable for large numbers of serum samples, as is the case in population studies. However, it is time-consuming because of ten days of exposure time required for the autoradiographs. This makes this method less useful for more solitary analysis in clinical situations and, moreover, it is not possible to apply it for preparative purposes. Therefore a technique that is especially suited for those situations was developed. Assuming that the mobilities of the different isoproteins in PAGE are the result of different isoelectric points, which has been confirmed in a previous study on the isolation of pure human TC II [11], we applied the technique of fast protein liquid chromatofocusing (FPLC) to the separation and identification of the TC II isoproteins.

EXPERIMENTAL

Cyano [^{57}Co] cobalamin (CN[^{57}Co]Cbl), with a specific activity of 284.5 μCi/nmol (10.5 μCi/ml), was obtained from the Radiochemical Centre (Amersham, U.K.). A Mono P HR 5/20 chromatofocusing column, Polybuffer 74, activated CH-Sepharose-4B and CM-Sephadex C-50 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Ultrafilter holder sets of the Amicon micropartition system (MPS-1) supplied with 14-mm YMT membranes were purchased from Amicon (Danvers, MA, U.S.A.). Millex 0.22-μm filter units type GV (25 mm) and Durapore 0.22-μm microporous membranes type GVWP (47 mm) were purchased from Millipore (Bedford, MA, U.S.A.). Acro LC 13, HPLC-certified, disposable 0.45-μm filter units were obtained from Gelman Sciences (Ann Arbor, MI, U.S.A.) Pure human holo-transcobalamin II (holo-TC II) and partially purified apo-transcobalamin II (apo-TC II) concentrate with specific Cbl-binding capacities of 0.98 and $6.2 \cdot 10^{-5}$ mol Cbl per mol TC II, respectively, were prepared from Cohn fraction III as previously described [11]. Antibodies raised against pure human apo-TC II in rabbits were purified and covalently bound to CH-Sepharose as previously described [12]. All reagents used were of analytical-grade purity.

Sample preparation

Blood (10 ml) from healthy laboratory workers was obtained by venepuncture with evacuated blood collection tubes containing 0.1 ml of 15% K_3EDTA as an-

ticoagulant. Within 30 min after venepuncture, plasma was prepared by centrifugation at 1500 *g* for 10 min at 4 °C and then immediately centrifuged at 48 000 *g* for 30 min at 4 °C. Plasma was decanted, immediately frozen and stored at −20 °C. Plasma samples were phenotyped for TC II isoproteins by PAGE as previously described [13].

Preparation of TC II concentrate

About 3 ml of plasma were filtered through a Millex filter unit, and 2 ml of the filtrate were transferred to a 5-ml conical glass tube. The plasma Cbl-binding proteins were saturated by adding 3.7 pmol of CN[^{57}Co]Cbl (0.1 ml). After incubation for 30 min at room temperature, 1 ml of 0.4 mol/l glycine–HCl buffer (pH 3.0) containing 4 mmol/l K_2EDTA and 0.15 mmol/l potassium cyanide was carefully added, resulting in a final pH of 5.6. Subsequently, 3 mg of dry CM-Sephadex C-50 were added, followed by continuous rotation overnight at room temperature. The swollen beads were collected by centrifugation at 2000 *g* for 5 min at 37 °C, washed four times with 4 ml of cold 25 mmol/l sodium phosphate buffer (pH 5.6) containing 0.175 mol/l sodium chloride, 4 mmol/l K_2EDTA, 0.15 mmol/l potassium cyanide and 0.85 mol/l betaine, and the ^{57}Co radioactivity of the pellet was counted in a Packard Multiprias gamma counter. The efficiency of TC II adsorption by this procedure was evaluated by comparison with the results of the measurement of unsaturated TC II by immuno adsorption assay [12]. The amount of TC II–CN[^{57}Co]Cbl adsorbed on the CM−Sephadex pellet was expressed as a percentage of the amount of TC II–CN[^{57}Co]Cbl bound to the immunoreactive beads. CN[^{57}Co]Cbl-saturated TC II was eluted from the CM-Sephadex pellet by adding 0.5 ml of 0.1 mol/l triethanolamine–iminodiacetic acid buffer (pH 8.0) containing 0.75 mol/l sodium chloride, 4 mmol/l K_2EDTA, 0.15 mmol/l potassium cyanide and 0.85 mol/l betaine (buffer A), followed by an incubation for 30 min at room temperature on a rotary mixer. After centrifugation for 10 min at 2000 *g*, the pellet was washed with another 0.5 ml of buffer A and both supernatants were transferred into the sample reservoir of an MPS-1 ultrafiltration holder. The remaining ^{57}Co radioactivity in the wet CM-Sephadex C-50 pellet was counted. Concentration of the combined supernatants to ca. 0.1 ml was achieved by centrifugation at 2000 *g* for 30 min at 4 °C in a Sorvall RC2-B centrifuge, using a fixed-angle rotor type SS-34 with delrin adaptors. The medium of the concentrated TC II–CN[^{57}Co]Cbl complex was changed to buffer B (buffer A devoid of sodium chloride and K_2EDTA) by adding two 0.9-ml portions of this buffer and repeated centrifugation. The ^{57}Co radioactivity lost in the ultrafiltrates was counted and expressed as a percentage of the total CN[^{57}Co] Cbl radioactivity eluted from the ion exchanger.

Pure human holo-TC II and partially purified CN[^{57}Co]Cbl-saturated apo-TC II preparations from Cohn fraction III were concentrated and changed to buffer B following the above concentration procedure. The obtained micro-concentrate of ca. 0.1 ml, containing the TC II–CN[^{57}Co]Cbl complex, was collected into a 1.0-ml syringe supplied with a polypropylene tube, tightly fitted onto a needle (25 mm × 0.6 mm I.D.). The MPS-1 sample reservoir was washed three times with 0.1 ml of buffer B. The ^{57}Co-radioactivity in the collected portions of concentrate was counted by direct measurement of the total radioactivity in the syringe.

Fast protein liquid chromatofocusing of human TC II isoproteins

A Pharmacia P-500 high-precision pump, a V-7 injection valve supplied with a 0.5-ml sample loop, a Frac-100 fraction collector and an Isco UA5 UV absorbance monitor (280-nm filter) were used throughout the study. Prior to each FPLC separation of pure or partially purified human TC II isoproteins, the Mono P column was washed with freshly prepared bidistilled water, at a flow-rate of 60 ml/h and regenerated by injecting successively 1.5 ml of 2 mol/l sodium iminodiacetate (pH 7.0), 1.5 ml of 70% acetic acid, 1.5 ml of 5 mol/l sodium hydroxide and 1.5 ml of 2 mol/l sodium iminodiacetate (pH 7.0). Then the column was equilibrated with 25 mmol/l triethanolamine–iminodiacetic acid buffer (pH 6.60), containing 0.85 mol/l betaine, until the pH of the effluent was $\leqslant 6.9$. Prior to each FPLC procedure all solvents were freshly prepared, filtered by vacuum filtration through a Durapore GVWP filter and thoroughly degassed by vacuum. During FPLC, solvent degassing was achieved by continuous helium purging at a flow-rate of 20 ml/min in each reservoir.

The TC II-containing concentrate was injected through an acrodisc filter, directly connected to the injection valve by means of a polypropylene adapter, and subsequently applied to the column at a flow-rate of 30 ml/h. While 2.5-ml fractions were being collected, the column was further eluted with equilibration buffer until the UV absorption baseline at 0.05 a.u.f.s. setting was stable. The ^{57}Co radioactivity in these fractions was counted and expressed as a percentage of the actual ^{57}Co radioactivity applied to the column, which was given by the difference between the total ^{57}Co radioactivity of the MPS-1 concentrate and the remaining ^{57}Co radioactivity in the syringe, acrodisc filter and loop connections after the injection of the concentrate. In order to start chromatofocusing of TC II isoprotein fractions, the elution buffer was immediately changed to 1.75% (v/v) Polybuffer 74, adjusted to pH 5.15 with iminodiacetic acid and containing 0.85 mol/l betaine. At this stage the pH of the effluent rapidly fell to ca. 6.10, after which a shallow descending gradient with a ΔpH of 0.02/ml was formed automatically. About 100 fractions of 0.5 ml were collected and the ^{57}Co radioactivity was counted. Finally the pH in the fractions was measured by means of a Radiometer PHM 84 research pH meter, supplied with a combined electrode type GK 2321C.

RESULTS

Evaluation of the preparation of TC II-[^{57}Co] Cbl concentrate

In four different serum samples the amount of TC II adsorbed on the CM–Sephadex ranged from 99.3 to 99.8% of the amount of TC II measured by the immunoadsorption assay. TC II-[^{57}Co]Cbl in the CM-Sephadex eluate was purified 80 ± 6 fold ($n = 10$). The radioactivity retained by the CM-Sephadex after elution was $4.5 \pm 1.4\%$, and another $1.4 \pm 0.5\%$ was lost in the combined ultrafiltrates. The mean recovery of radioactivity after concentration by MPS-1 ultrafiltration was $87.5 \pm 2.8\%$. On average, the ^{57} Co radioactivity injected amounted to 850 000 cpm; the total amount of injected protein was ca. 2 mg.

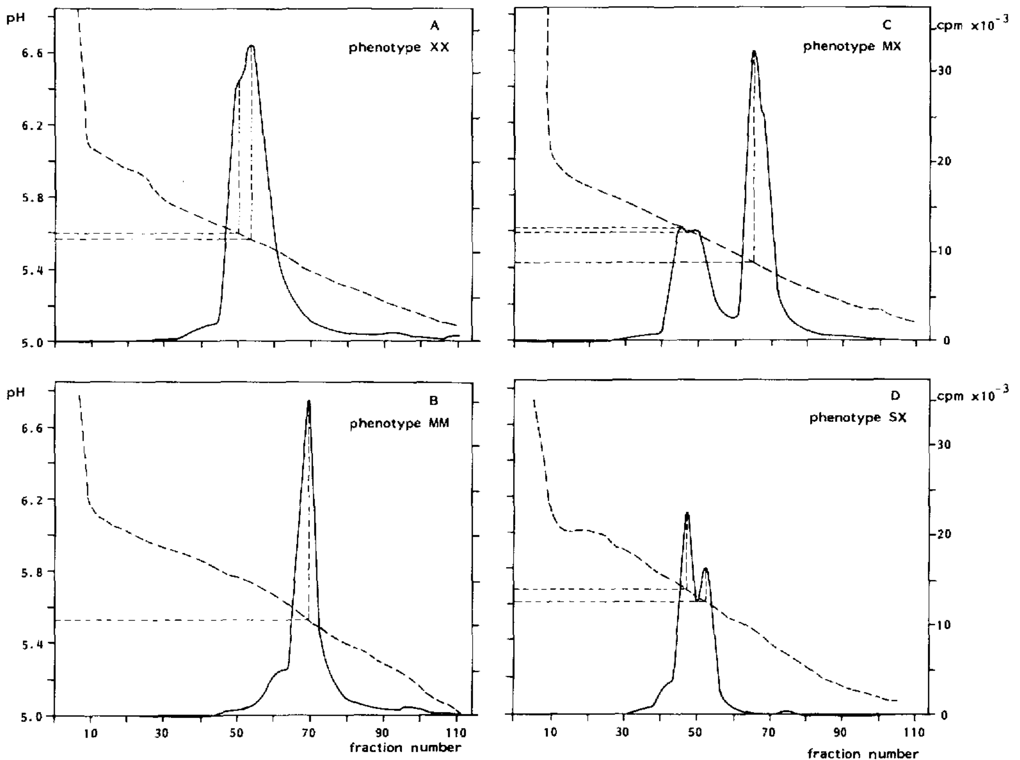

Fig. 1. Chromatofocusing elution patterns of four different TC II phenotypes (solid line). The pH of each of the peak fractions is indicated. The broken line represents the course of the pH gradient.

Fast protein liquid chromatofocusing of TC II isoproteins

In preliminary experiments using a TC II preparation from a mixture of plasma samples or pure human holo-TC II, the conditions for optimal separation of isoproteins by chromatofocusing were established. Sample preparation had to take place at pH 8.0, because at the low ionic strength used TC II tends to aggregate at a lower pH. In addition 0.85 mol/l betaine was added to the sample and all eluents to further stabilize the protein. Various start buffers were evaluated on the basis of the resulting shallowness of the gradient in combination with the eluent buffer. A starting buffer of less than 20 mmol/l resulted in an unstable pH at the start of the gradient, and therefore a 25 mmol/l buffer concentration was chosen. We preferred a triethanolamine buffer to the recommended Bis–Tris buffer because of better solubility of TC II in this buffer. The selected eluent buffer was 1.75% Polybuffer 74, brought to pH 5.15 with concentrated iminodiacetic acid and containing 0.85 mol/l betaine. With this combination a linear pH gradient was consistently obtained with a ΔpH of 0.02/ml from pH 6.1 to 5.2. The separation pattern was not improved by the addition of urea, EDTA or non-ionic detergents as advocated in other studies [11,13].

The results of four typical examples of elution patterns are presented in Fig. 1. The homozygous M phenotype is present as a sharp single peak with sometimes

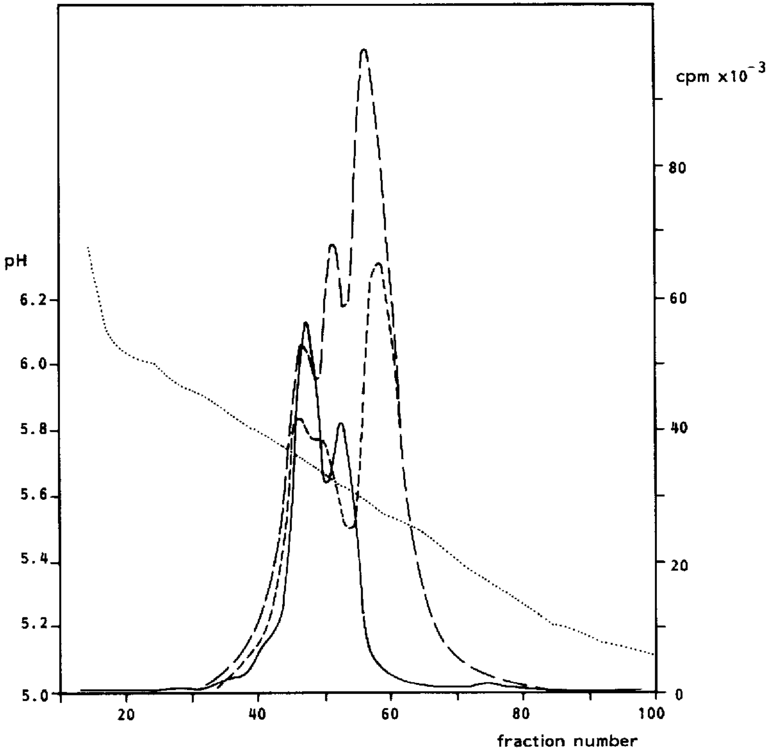

Fig. 2. Overlay of three consecutive chromatofocusing elutions of a suspected heterozygous SX phenotype (——), a heterozygous MX phenotype (--------) and a combination of both (----). The dotted line represents the course of the pH gradient.

a small shoulder, whereas the homozygous X phenotype is always represented by a double peak. The heterozygous MX phenotype is characterized by a double X and a single M peak, of which the former contains ca. 40% and the latter ca. 60% of the total radioactivity. It turned out to be very difficult to discern an SX or SM heterozygous from a MX heterozygous TC II phenotype. Only by simultaneous elution with a known MX, XX or MM phenotype it appeared possible to define these rare heterozygous variants in chromatofocusing patterns (Fig. 2).

The variability in the pH at which the various isoproteins are eluted from the column is rather small. The first peak of the X variant has its maximum in a fraction with a pH of 5.70 ± 0.03 (mean $\pm$ S.D., $n=7$); the M peak reaches its maximum at pH 5.55 ± 0.02 (mean $\pm$ S.D., $n=7$).

In a pool of numerous individual plasma samples all phenotypes are probably represented, and the areas under the individual peaks will be a rough reflection of the distribution of phenotypes in the population. Fig. 3A shows the elution pattern of a TC II concentrate from a pool of normal plasma, derived from ca. 5000 individuals. In addition to the already known peaks of the X and M isoproteins a fourth peak with an even lower isoelectric point is found, presumably representing the F variant. The low frequency of the S phenotype in the population ($<2\%$) precludes the detection of a separate peak from this isoprotein. From

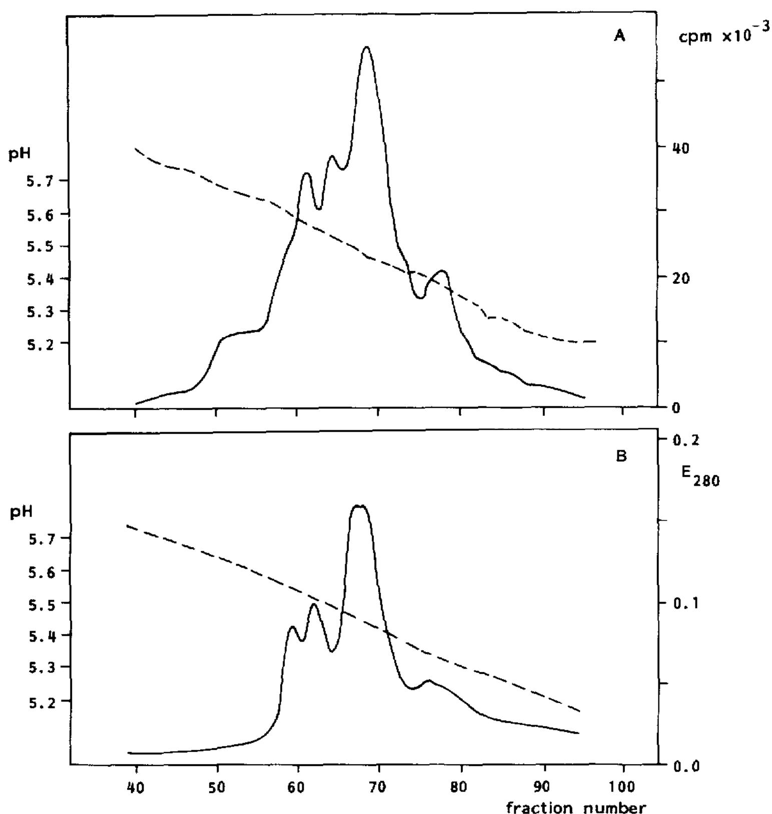

Fig. 3. Chromatofocusing elution patterns of (A) [^{57}Co]Cbl-saturated TC II from pooled human plasma and (B) pure, human holo-TC II isolated from the same plasma pool. The broken line represents the course of the pH gradient.

the same pool of plasma, TC II was isolated to homogeneity [11] and subjected to chromatofocusing (Fig. 3B). This elution pattern, on the basis of UV absorbance by the proteins, agrees well with the radioactivity distribution of partially purified transcobalamin TC II–[^{57}Co]Cbl presented in Fig. 3A.

DISCUSSION

This paper describes a new method for the analytical separation of the four TC II isoproteins most commonly found in human plasma. The sequence of elution of the isoproteins is in accordance with the banding pattern of TC II variants found with PAGE, as described by Fràter-Schröder et al. [7] and Porck [13], and which was recently confirmed by Hansen and Fràter-Schröder [14] using polyacrylamide gel isoelectric focusing (IEF). In a pH gradient with decreasing pH, the variant that moves slowest in PAGE is eluted first and the fast-moving variant F is eluted last. In a previous study we demonstrated that the isoelectric points of the TC II variants ranged from pH 6.8 to 6.2, and similar values were reported by Hansen and Fràter-Schröder [14]. The pH of the elution buffer in

which the isoproteins appeared from the chromatofocusing column is obviously not identical with the pH value of the isoelectric points of the respective variants. The shift to pH 5.8–5.3 in chromatofocusing is caused by electrostatic forces on the surface of the matrix (Donnan potential), which are responsible for a slightly higher pH in the matrix than in the surrounding eluent. This effect becomes stronger when the concentration of the eluent buffer is lower and has less buffer capacity [15].

The sample preparation procedure serves three purposes: a reduction of the total amount of protein applied to the column, the removal of R-binder Cbl-binding capacity and reduction of the sample volume to an injectable amount of 500 μl. CM- Sephadex ion-exchange chromatography has proved its value as a first purification step in the isolation of TC II and for the removal of R-binder [11], and the method proved equally suitable as a small-scale sample preparation procedure in this study.

The most remarkable difference between the three techniques for the identification of TC II phenotypes now available is that the duplicate bands, which are found in PAGE, do not occur in PAGE–IEF and are visible only in the X and possibly S phenotype but almost not in the M phenotype using chromatofocusing. In PAGE–IEF the protein bands might be too vague for doublet forms to be discerned. In chromatofocusing the distance between the two bands of the X-doublet is only ca. 0.03 pH unit and much less for the M-variant. Therefore the assumption seems justified that the doublet banding of TC II isopeptides in PAGE is caused not so much by different isoelectric points of the two partners of the doublet, as by conformational differences between them. This links up with the now generally accepted idea that TC II is not composed of two peptide subunits as suggested earlier [16], but consists of a single polypeptide chain [11]. Doublet formation might be a general phenomenon of Cbl-binding proteins, as desialysed R-binders also demonstrate duplicate banding in PAGE–IEF [17]. The observed difference in Cbl-binding capacity between the X and the M phenotype reflected by their respective peak surfaces correlates with the earlier observation of Porck [13] that the slower TC II variants showed less intense bands in PAGE–autoradiography.

The value of the method presented here is further illustrated by the demonstrated separation of TC II isoproteins from a preparation of pure human TC II, originating from a large pool of human plasma. This procedure will make it possible to isolate milligram amounts of pure TC II of a defined subtype for further analysis of the molecular differences between the various isoproteins. On an analytical scale the method will be of value for the identification of the TC II phenotype of individual plasma in paternity testing, for the detection of chimerism after allogeneic bone marrow or liver transplantation and for family studies of patients with an aberrant TC II.

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