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Development of a Pharmaceutical Apotransferrin Product for Iron Binding Therapy

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Abstract. High-dose chemotherapy of patients with haematological malignancies results in extracellular iron accumulation and appearance of non-transferrin-bound iron, which is thought to predispose the patients to septic infections and contribute to organ toxicity. We describe the development of a human plasma-derived apotransferrin product for iron binding therapy. The product is purified from Cohn fraction IV of human plasma by two ion exchange chromatography steps and ultrafiltration. The process comprises solvent detergent treatment as the main virus inactivation step and 15 nm virus filtration and polyethylene glycol precipitation as removal steps for physico-chemically resistant infectious agents. Product characterization by electrospray and MALDI-TOF mass spectrometry indicated no other chemical modifications than N-linked glycan chains and disulphide bonds, except minor oxidation. The purity of the product was more than 98%, main impurities being IgG, IgA and hemopexin. The product had intact iron binding capacity and native conformation. A stable liquid formulation for the finished product was developed. The product has proved safe and well tolerated in early clinical trials in iron binding therapy.

Introduction

Transferrin is the major iron-carrier protein in human plasma and extracellular space in tissues. It binds two ferri-ions with high affinity and delivers iron to dividing cells, particularly to the erythroid progenitor cells in the bone marrow. The transferrin receptor mediates the internalization of iron-saturated transferrin into an endosome inside the cell, where iron is taken up by the cell and iron-free apotransferrin is recycled back to the extracellular space.[1] Under normal conditions, the iron saturation of transferrin is only 20–35% and the level of low-molecular weight, non-transferrin-bound iron is very low. Transferrin-bound iron is in a redox-inactive state and, unlike unbound iron, does not catalyse hydroxyl radical formation.[2] Transferrin also keeps the iron inaccessible from most bacteria and fungi.[3] Therefore, transferrin is an important antioxidant and anti-microbial protein in human plasma.

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Patients with leukaemia and other malignancies typically have a low serum transferrin concentration and diminished capacity to sequester iron. During high-dose chemotherapy, iron is accumulated in the circulation and the iron binding capacity of transferrin becomes exceeded, which results in the appearance of non-transferrin-bound iron in the patient serum.[4,5] The occurrence of non-transferrin-bound iron is particularly common in patients undergoing myeloablative therapy and bone marrow stem cell transplantation.[6,7] Non-transferrin-bound iron in plasma and extracellular space catalyses hydroxyl radical formation, which may contribute to organ toxicity, such as mucosal and liver injuries.[4,8] Additionally, non-transferrin-bound iron promotes the growth of bacteria and fungi and may thus predispose the patients, who have a low count of neutrophilic granulocytes, to septic infections by opportunistic bacteria and fungi.[2,9,10] Therefore, iron chelation therapy has been suggested for the prevention of toxic effects of non-transferrin-bound iron[5] and protection against septic infections.[10]

The low-molecular weight iron chelator deferoxamine, which is currently in clinical use, has limited

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efficacy in the binding of non-transferrin-bound iron and displays dose-related toxicity.[11] Theoretically, administration of iron-free apotransferrin to bone marrow transplantation patients could be beneficial by binding the accumulating iron into a non-toxic form and by providing the iron to the recovering erythropoiesis, once engraftment of the transplant has taken place. An apotransferrin product that could be safely given to patients must be free of potentially harmful impurities and viruses and other infectious agents. As the doses of apotransferrin required for iron chelation therapy are in the order of several grams, the production costs must remain feasible. We have developed a high-purity virus-safe apotransferrin product, which is identical to plasma iron-free transferrin and has full iron-binding capacity. In this paper we describe the manufacturing of the product from human plasma and its detailed characterization.

Materials and methods

Manufacturing of apotransferrin from Cohn fraction IV

The apotransferrin was produced from fraction IV paste of the Cohn cold ethanol plasma fractionation process.[12] The starting plasma pool was made up from ca. 7000–10 000 plasma units, each tested negative for HbsAg, anti-HIV-1 and -2 and anti-HCV. The plasma pools were also tested negative for HCV RNA and parvovirus B19 by PCR tests.

The fraction IV paste was dissolved with water for injection. The protein solution was collected by filtration in the presence of filter aids in a filter press. 5% (w/v) polyethylene glycol (PEG) 4000 (Merck) was added and the precipitate was removed with a depth filter. EDTA was added to the solution to a concentration of 5 mM. The clarified solution was applied to a chromatography column packed with SP Sepharose (Amersham Pharmacia Biotech) gel and pre-equilibrated with 40 mM sodium acetate buffer, pH 5·9. Other proteins and impurities were flushed away with the equilibration buffer. Transferrin was eluted with 40 mM Tris–HCl buffer, pH 7·5, and the pH of the collected transferrin-containing fraction was adjusted to pH 7·3 with the elution buffer. 1% (w/w) of Tween 80 and 0·3% (w/w) of tri-(n-butyl)phosphate were added for the solvent detergent (SD) treatment, and the solution was incubated at 26° C for 10 h for inactivation of enveloped viruses.[13] The partially purified apotransferrin solution was applied to a chromatography column packed with Q Sepharose gel (Amersham Pharmacia Biotech) pre-equilibrated with 40 mM Tris–HCl buffer, pH 7·5. The SD chemicals and protein impurities were flushed away with the equilibration buffer. Apotransferrin was eluted using 40 mM Tris–HCl buffer, pH 7·5, with 85 mM sodium chloride. The buffer of the protein solution was changed by diafiltration in an ultrafiltration unit, and the solution was concentrated to a protein concentration of 55 g/l. The pH of the pure apotransferrin solution was adjusted to pH 6–7. The solution was prefiltered using a 0·1 μM filter and filtered using a Planova 15N filter (Asahi Industries) for removal of viruses. The pure and virus-safe product was sterile filtered and aseptically filled into infusion bottles. The product was stored as a liquid formulation at refrigerator temperature or, in early development batches, optionally lyophilized.

Protein and peptide sequencing

Sequence analysis was performed on an Applied Biosystems 494A Procise™ sequencer (Perkin Elmer). For N-terminal sequencing of apotransferrin fragments, the fragments were separated in 12% SDS–PAGE followed by electroblotting on to ProBlott™-membrane (Applied Biosystems) in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid, pH 11, 10% methanol using a constant voltage of 50 volts for 2 h 45 min.[14] The apotransferrin fragments were cut out after visualization with Coomassie Brilliant Blue and loaded to the sequencer.

Mass spectrometry

MALDI-TOF mass spectrometry was performed on a Biflex™ time-of-flight instrument (Bruker-Franzen Analytik) equipped with a nitrogen laser operating at 337 nm. Peptides and glycans were measured in the reflector mode or in the delayed extraction (200 nsec delay) reflector mode. All spectra were obtained in the positive ion mode. Peptides from proteolytic digestions were separated by RP-HPLC before MALDI analysis. RP-HPLC was carried out using a 1·0 × 150 mm C4-column (5 μM, 300Å, LC-Packings) and linear gradients of 0–40% B in 120 min followed by 40–80% B in 50 min. Solvent A was 0·1% trifluoroacetic acid and solvent B 0·075% trifluoroacetic acid in acetonitrile. Flow rate was 50 μl/min and UV-detection was at 214 nm. The RP-HPLC isolated peptides were analysed by using a saturated solution of α-cyano-4-hydroxy cinnamic acid in acetone as the matrix.[15] For peptide analysis the MALDI mass spectra were calibrated using standard peptides. For glycan analysis calibration was done with Dextran 1000 and the matrix

used was 10 mg/ml 2,5-dihydroxybenzoic acid in 10% acetonitrile.

Electrospray mass spectra were collected using an API 300 triple quadrupole mass spectrometer (Perkin–Elmer Sciex Instruments). Samples were dissolved in 50% methanol in H_2O containing 0·5% acetic acid to a final concentration of 0·2–5 pmol/μl and directly injected into the electrospray with a syringe pump (Harvard Instruments) at a flow rate of 3–5 μl/min. The instrument was calibrated with polypropyleneglycol mixture as supplied by the instrument manufacturer.

Proteolytic digestions

The native and deglycosylated apotransferrin product was reduced and alkylated before proteolytic digestion by dissolving protein samples (160–1000 μg) in 80 μl of 6 M guanidine hydrochloride in 0·5 M Tris–HCl, pH 7·5, containing 2 mM EDTA and 5 μl 0·66 M dithiothreitol. After incubation for 20 min at room temperature, 1 μl of 4-vinylpyridine was added and the solution was incubated for 20 min. The reaction was stopped by adding 5 μl of 0·66 M dithiothreitol. The reduced and alkylated protein was then desalted with RP-HPLC using a 4·6 × 30 mm Poros R1-column with the same solvents as in the other RP-HPLC used for mass spectrometry samples. Elution was done with a flow rate of 5 ml/min and a linear gradient of 0–100% B in 15 min. The protein containing fractions were dried in a vacuum centrifuge. For the endoproteinase LysC digestions, the dried protein sample was dissolved in 100 μl of 0·1 M Tris–HCl, pH 9·2, containing 10% acetonitrile, 4 μg endoproteinase LysC (Wako Chemicals GmbH) was added and the solution was incubated overnight at 37° C.

Glycan analysis

N-glycans from the apotransferrin product and from the IEX-HPLC fractions of the product were removed by enzymatic hydrolysis with N-glycosidase F. The protein sample (50–160 μg) was dissolved in 10 μl 20 mM sodium phosphate, pH 7·2 and 1 μl 10% SDS, and the mixture was boiled for three minutes. The mixture was cooled to room temperature and 75 μl 20 mM sodium phosphate, pH 7·2, 10 μl 10% octyl glucopyranoside and 5 μl 0·2 U/μl N-glycosidase F (recombinant N-glycosidase F, Boehringer Mannheim) were added and the mixture was incubated at 37° C overnight. The released N-glycans were purified from proteins and detergents with a BondElut® C18-column (1 ml, Analytichem International). The sample was diluted to a final volume of 300 μl with water before loading it to the column. The glycans were eluted from the column with 1·5 ml water, and dried down in a vacuum centrifuge. The glycans were desalted by gel filtration on a Superdex® Peptide PC 3·2/30-column (Amersham Pharmacia Biotech) eluted with 50 mM NH_4HCO_3 at a flow rate of 40 μl/min and monitored at 205 nm. The glycan fractions were evaporated to dryness in a vacuum centrifuge. The purified glycans were subjected to MALDI-TOF analysis before and after permethylation.[16]

Electrophoretic and chromatographic methods

SDS–PAGE was carried out in 10% gels (10 × 10 cm) or in 4–15% Phast gels (Amersham Pharmacia Biotech) under reducing and non-reducing conditions. The gels were stained either with silver (PlusOne™ Silver Staining kit Protein, Amersham Pharmacia Biotech) or Coomassie Brilliant Blue. For Western blotting, the proteins were electroblotted on to nitrocellulose membranes (Trans-Blot Transfer medium, Bio-Rad). The membrane was blocked with 0·5% Tween in PBS overnight and incubated with rabbit anti-human transferrin (Dako A/S) in PBS containing 1% BSA and 0·05% Tween 20 for 2 h at room temperature. The blots were washed and incubated with anti-rabbit IgG conjugated with alkaline phosphatase (Jackson Immuno Research Laboratories Inc.) for 1 h at room temperature. After washes the blots were stained with the BCIP/NBT colour development solution (Immuno-Blot, Alkaline phosphatase assay kit, Bio-Rad).

Immunoelectrophoresis was done using polyclonal antibodies raised against normal human serum, IgG and IgA (DADE Behring) and against human transferrin and hemopexin (Dako A/S). Cellulose acetate electrophoresis was done for quantitation of β-globulins.

Urea-polyacrylamide gel electrophoresis (urea–PAGE) was carried out as described before[17] using 6% gels (10 × 10 cm) with 6 M urea; 15–20 μg of transferrin was applied and the electrophoresis was run at a constant voltage of 170 V for 2 h. The gels were stained with Coomassie Brilliant Blue. High resolution ion exchange chromatography (IEX-HPLC) was carried out using a Mono Q HR 5/5 column (Amersham Pharmacia Biotech). The sample was injected on to the column equilibrated with 0·02 M bis–Tris, pH 6·0 and eluted with a linear gradient of 0–10% in 10 min and 10–100% in two minutes of 0·02 M bis–Tris, pH 6·0, containing 1·0 M NaCl. The flow rate was 1 ml/min and UV-detection

was at 280 nm. Molecular size distribution was determined by size exclusion liquid chromatography (SE-HPLC) according to the Ph. Eur. method for human albumin[18] using a TSK-GEL G3000 SW column (10 μM, 7·5 × 600 mm, Toso Haas).

Determination of iron binding capacity

The iron binding capacity of transferrin was determined by titration with ferric nitrilotriacetic acid.[19] 100 μl aliquots of ferric nitrilotriacetate solution (1–1000 μM) were added to 900 μl samples of apotransferrin (3·3 g/l) in 0·3 M Tris–HCl, pH 8, to give final concentrations of ferric nitrilotriacetate ranging from 0 to 100 μM. After incubation for one hour at 20–25° C, samples of 350 μl were transferred to a microtitre plate and the absorbance was measured at 450 nm using a microplate spectrophotometric reader (Titertek Multiscan RC, Labsystems). The amount of iron required for full saturation was determined from the inflection point of the absorbance plot, and the iron binding capacity (%) was calculated by dividing this amount with the theoretical amount of iron required to fully saturate the added transferrin. The molar concentration of transferrin was calculated using a molecular mass of 79 570 g/mol for transferrin.

Determination of total protein, protease activities and process derived impurities

Total protein was determined by the biuret method using human serum albumin as a standard. Apotransferrin concentration was determined by absorbance at 280 nm using the extinction coefficient of 11·1.[20] Quantitation of the impurity proteins was carried out by an immunoturbidimetric assay for IgG, an enzyme immunoassay for IgA[21] and immunodiffusion for hemopexin (NOR-Partigen Hemopexin, DADE Behring). Prekallikrein activator (PKA) activity was measured according to the Ph. Eur. method[22] measuring the hydrolysis of the chromogenic substrate S-2302 (Chromogenix) in the presence of prekallikrein substrate. The same method without prekallikrein was used for the determination of the amidolytic activity of other proteases able to directly hydrolyse the chromogenic substrate, such as kallikrein, thrombin, plasmin and factor Xa. The result of this amidolytic activity (IU/ml) was obtained from the reference curve of the PKA assay. The plasmin/plasminogen activity was measured as hydrolysis of the chromogenic substrate S-2251 (Chromogenix) without and with streptokinase (Kabikinase, Pharmacia Upjohn), respectively.[23]

Iron was determined by the colorimetric ferrozine method (Boehringer Mannheim Diagnostics). Aluminium and zinc were measured by atomic absorption spectrometry. Tri-*n*-butyl phosphate (TNBP) was assayed with a gas chromatographic method.[24] Tween 80 was measured by a colorimetric method.[25]

Parvovirus B19 PCR assay

DNA was purified and amplified as previously described.[26] PCR products were transferred on to streptavidin-coated wells and hybridized to a digoxigenin-labelled 364 bp probe.[26] The labelled hybrids were visualized with alkaline phosphatase-conjugated anti-digoxigenin Fab fragments (Boehringer–Mannheim) and Lumi Phos 538-substrate (Lumigen, Inc.) in a Luminoskan meter (Labsystems). Samples were studied by end-point titrations with positive and negative controls in each series. The detection limit was 10^2 gen. eq./ml.

Results

Manufacturing of apotransferrin from Cohn fraction IV

The manufacturing method was developed to fulfil the requirements for a large scale production of a pharmaceutical apotransferrin product suitable for intravenous administration. The process is outlined in Figure 1. Each purification step was optimized for high throughput and yield, and the number of process steps were kept to a minimum. The first cation exchange chromatography removed the bulk of the impurities, and the final purification took place in the second anion exchange step. By use of pH dependent elution in the first ion exchange step, buffer exchange before the second chromatography step could be avoided. In a pilot scale production with a batch size of 500 g of the purified product, over 90% of transferrin could be recovered from the dissolved fraction IV paste.

Several virus inactivation and removal steps were used in the manufacturing process. The dissolved fraction IV paste was treated with 5% polyethylene glycol, which precipitated both lipoprotein impurities and viruses while transferrin remained in the solution. The major virus inactivation step was the SD treatment. The SD chemicals, Tween 80 and TNBP, were removed from the product in the subsequent ion exchange chromatography step. A 15 nm hollow fibre filter with a high retention of small non-enveloped viruses was chosen for virus

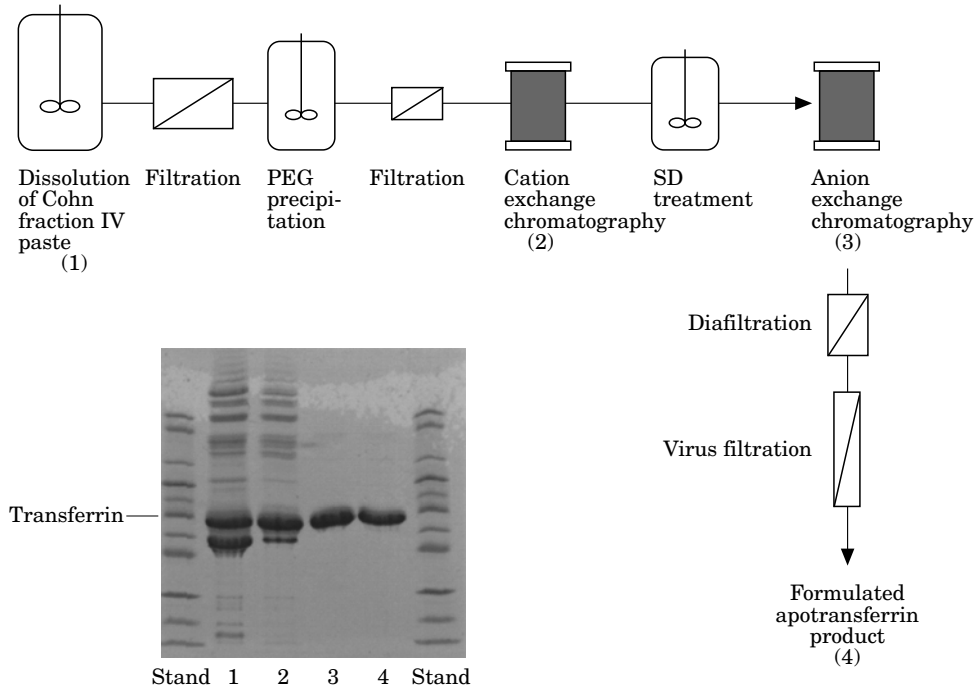

Figure 1. Flow scheme of the apotransferrin manufacturing process and SDS–PAGE (non-reduced, Coomassie stained) of samples taken from the purification. Lane 1, dissolved fraction IV; lane 2: partially purified apotransferrin after cation exchange; lane 3: purified apotransferrin after anion exchange; and lane 4: formulated finished product.

filtration of the formulated bulk solution. The filtration conditions were optimized for high protein throughput, and apotransferrin solution corresponding up to 4 kg protein/m^2 has been filtered successfully. The high filtration capacity makes the use of the expensive virus filters cost-effective in large scale manufacturing.

Virus validation studies indicated rapid inactivation of at least 4·7 $\log_{10}$ of HI virus and at least 5·1 $\log_{10}$ of BVD virus in the SD treatment. Removal of small non-enveloped viruses was studied in the down-scaled process steps by spiking with a high titre parvovirus B19 positive serum, resulting in a parvovirus level of at least 10^6 gen. eq./ml in the starting material, and by using PCR for detection of virus DNA in the product before and after the step. These studies indicated that 3·7 $\log_{10}$ of parvovirus was removed in the PEG precipitation step and more than 4·5 $\log_{10}$ in the virus filtration step with a protein load of 4 kg/m^2. The total clearance of parvovirus in these two steps was thus more than 8·2 $\log_{10}$.

Plasma transferrin is normally partially iron saturated and the manufacture of iron-free apotransferrin requires displacement of iron from transferrin. In the described process, iron was mostly dissociated from transferrin in the dissolved fraction IV paste, which was evidently due to the low pH and citrate buffer used for fraction IV precipitation. By adding EDTA to the solution and applying the solution to the first ion exchange column at the low pH, iron was effectively removed with the wash fractions, and apotransferrin with low iron content could be recovered (Table 1).

The purified apotransferrin was adjusted to 5% protein concentration in a sodium chloride solution at pH 6–7. This formulation could be stored at refrigerator temperature after sterile filtration and aseptic filling. The general characteristics of the finished apotransferrin product as a liquid formulation are shown in Table 1. To fulfil the requirements for intravenous use, the product was sterile, had a low endotoxin content (<2 IU/ml), was pyrogen-free and passed the abnormal toxicity test in mouse and guinea-pig according to the European Pharmacopoeia.

Primary structure and chemical modifications of the product

Sequencing of the 25 N-terminal amino acid residing showed identity of the product with human

Table 1. General characteristics for a liquid formulation of the apotransferrin product, mean of four batches

Characteristic	Result
Total protein	49·7 g/l
Iron content	0·24 mg/l (0·3% saturation)
Iron binding capacity	94%
Purity and integrity	
CA electrophoresis	99·1% β-globulins
SDS-PAGE	*
IEX-HPLC	*
Urea-PAGE	*
SE-HPLC: Monomer	98·5%
Dimer/Other proteins	1·5%
Polymers	0%
Process derived impurities	
Tween 80	32 mg/l
Tri-(*n*-butyl)-phosphate	<1 mg/l
Aluminium	<10 μg/l
Zinc	<30 μg/l

*Conforms to reference material (Figs 2A, 3B and 4A–D).

transferrin (identified sequence VPDKTVRWX-AVSEHEATKCQSFRDH, X being an unidentified amino acid). Peptide mass mapping with MALDI-TOF mass spectrometry of the product after cleavage with endoproteinase-LysC revealed more than 90% of the expected apotransferrin peptides (data not shown). Some of the peptides had masses about 16 or 32 Da higher than expected, suggesting oxidation of one or two methionine residues.

A high resolution IEX-HPLC method was developed, which separated apotransferrin into four peaks (Fig. 2A). The distribution of the peaks was reproducible in different production batches of apotransferrin, the proportions of the three main peaks (peaks 2–4) being 7–9%, 79–83% and 9–12%, respectively. The molecular weight of the whole protein was determined by electrospray mass spectrometry either directly or after separation of the product into the four peaks by the IEX-HPLC (Fig. 2A). The predicted mass of transferrin was calculated by taking into consideration the polypeptide chain (75 181·4 Da) with 19 disulphide bonds (-38 Da) and glycan chains (-18 Da for N-linkage). The mass obtained for peak 1 was close to the calculated mass of human apotransferrin carrying one biantennary disialylated glycan with two fucose residues (Fig. 2B). The mass of peak 2 was close to the calculated mass of human transferrin carrying one biantennary monosialylated glycan and one biantennary disialylated glycan. The third major peak gave a mass which was closely similar to the calculated mass of human transferrin carrying two biantennary disialylated glycan chains. The fourth peak gave two masses which corresponded to the calculated masses of human transferrins carrying one biantennary disialylated glycan and one triantennary trisialylated glycan chain without or with a fucose residue. Some of the peaks in the mass spectral analysis showed a mass shift of about 9–14 Da, which may have been due to sodium adducts of glycan moieties or oxidation of a single amino acid residue in the protein.

For more detailed glycan analysis, glycan chains were removed from apotransferrin enzymatically with N-glycosidase F and characterized by MALDI-TOF mass spectrometry after permethylation. Glycan analysis confirmed the presence of the glycan structures in the IEX-HPLC peaks as predicted by electrospray mass spectrometry, i.e. biantennary disialylated glycans in the major third IEX-HPLC peak and additionally, biantennary monosialylated glycans in the second peak and triantennary trisialylated glycans in the fourth peak. Glycan analysis also revealed traces of glycan structures carrying fucose in all IEX-HPLC peaks. The identified glycan structures are summarized in Figure 2B. Taken together, the mass spectrometric studies revealed no chemical modifications in apotransferrin other than glycosylation and the presence of

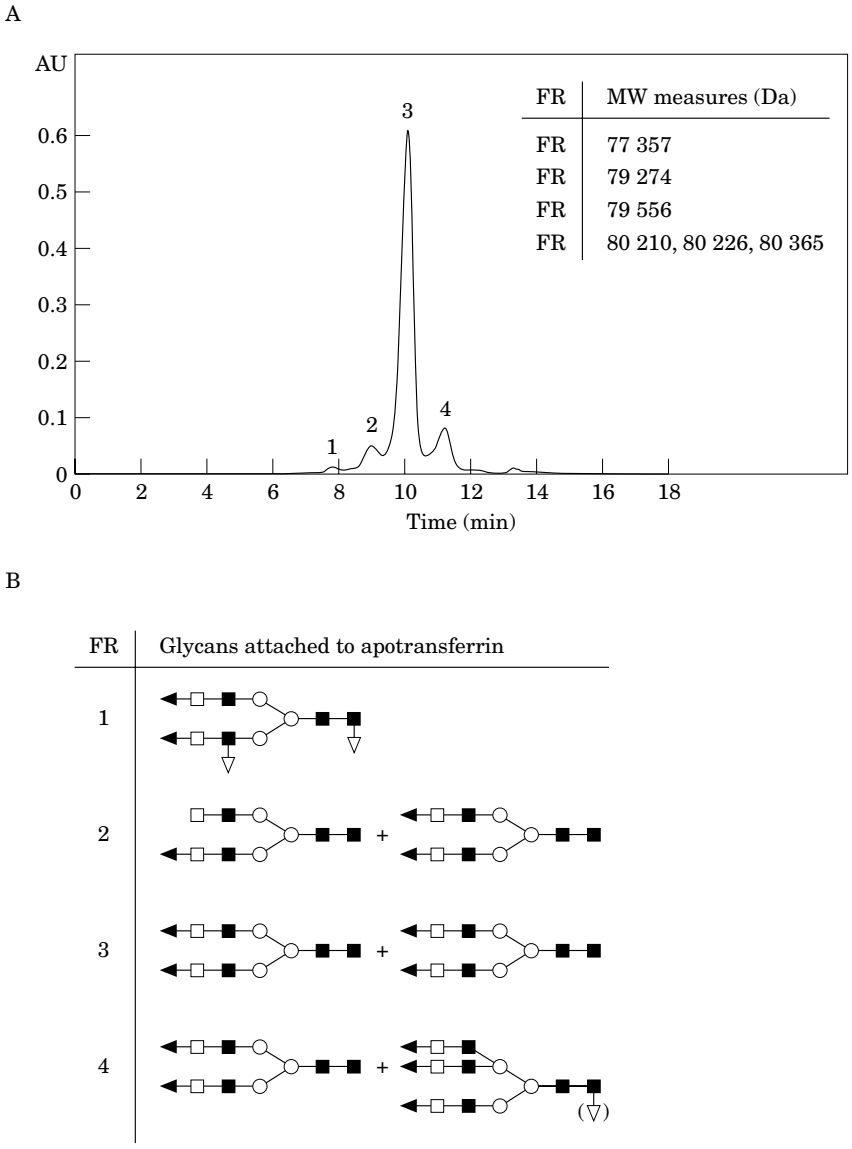

Figure 2. (A) Separation of apotransferrin into four glycoforms by high resolution ion exchange chromatography. The molecular weight of apotransferrin in the IEX-HPLC peaks was measured by electrospray mass spectrometry. (B) The proposed glycan structures of apotransferrin in the IEX-HPLC peaks. The symbols used for the monosaccharide residues are: (▼) N-acetylneuraminic acid; (□) galactose; (■) N-acetylglucosamine; (○) mannose; and (▽) fucose.

the predicted 19 disulphide bonds, except minor oxidation.

Assessment of native conformation of the product

The conformation and integrity of apotransferrin was studied by several assays, comprising size exclusion liquid chromatography (SE-HPLC), urea-polyacrylamide gel electrophoresis (urea–PAGE) and iron binding capacity. SE-HPLC separated the product into a major peak corresponding to transferrin monomer and a minor peak, which corresponded to a molecular size area of about 180–290 kDa. In the product lyophilized under optimized conditions and in the liquid formulation, the proportion of the minor peak was about 1%. On the basis of Western blotting of the isolated peak with specific antibodies and immunochemical quantitation, it could be concluded that the minor peak consisted mainly of monomeric IgA and IgG and of a small amount of transferrin dimer. The transferrin dimer migrated as monomeric transferrin in SDS–PAGE under non-reducing conditions.

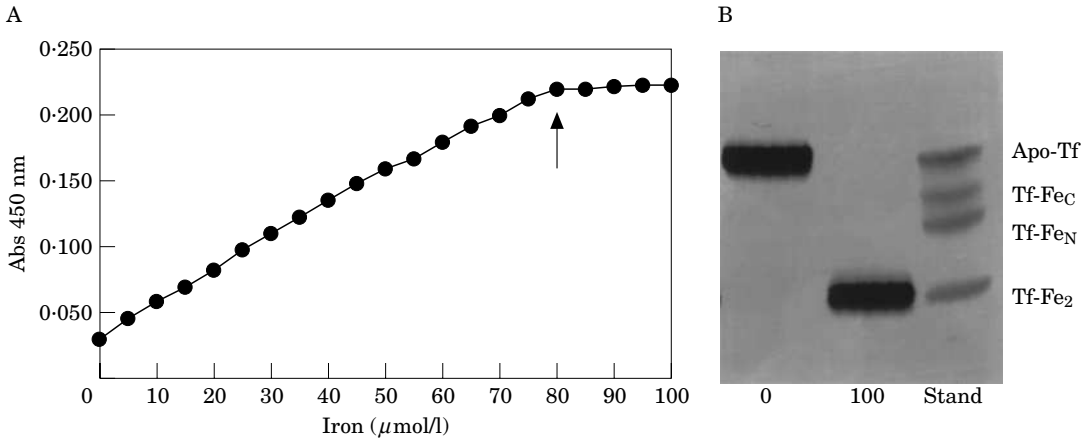

Figure 3. (A) Determination of iron binding capacity of transferrin with the full saturation point indicated. (B) Urea–PAGE showing the apotransferrin product and its conversion into the iron saturated form after addition of 100 μmol/l iron. The standard shows the four iron forms of transferrin: Apo-Tf, iron free transferrin; Fe_C-Tf and Fe_N-Tf, monoferric transferrins with iron in the C and N lobe, respectively; Fe_2-Tf, iron saturated transferrin.

An iron titration assay was developed for the determination of iron binding capacity of transferrin (Fig. 3A). The assay had an inter-assay precision of 3% and an intra-assay precision of less than 1%. Under the standardized conditions, the assay gave values between 92% and 96% for the finished product of the different apotransferrin batches.

Iron-free apotransferrin, the two monoferric transferrin forms and diferric transferrin can be separated by urea–PAGE on the basis of their different conformational stability.[27] Urea–PAGE can also detect genetic variants of transferrin.[28] The apotransferrin product ws analysed in urea–PAGE before and after iron saturation with ferric nitrilotriacetic acid. The detection limit of the assay was less than 5%. Urea–PAGE revealed a single band in all production batches, which had identical mobility with apotransferrin standard and was fully converted into the diferric form after iron saturation (Fig. 3B).

The integrity of the polypeptide chain was assessed by SDS–PAGE and Western blotting under non-reducing and reducing conditions. Silver staining of SDS–PAGE and Western blotting with transferrin specific antibodies consistently visualized a major band of 76 kDa and minor bands with lower molecular weights in reduced samples of the product with a high sample load (Fig. 4). The same bands were also visualized in Western blotting of fresh human plasma samples (data not shown), which indicated that the minor transferrin bands were already present in normal plasma. They probably derived from intra-chain fragmentation of transferrin and their amount both in the purified product and in plasma transferrin was very low. The different transferrin bands seen in the non-reduced samples were most probably due to the microheterogeneity in the glycosylation of transferrin, as their intensity varied in the different IEX-HPLC fractions.

Identification of impurities in the product

The purity of the finished product was at least 98% as assessed by the different electrophoretic and chromatographic methods. Immunoelectrophoresis of the finished product with polyclonal antibodies raised against normal human serum revealed four precipitation arcs in all batches. The major arc corresponded to transferrin and the other arcs were identified as hemopexin, IgA and IgG by using the corresponding specific antibodies. According to the quantitative immunochemical assays, the proportions of impurity proteins were 0·3–0·8% of total protein (Table 2). Of the possible plasma-derived protease impurities, the level of prekallikrein activator was less than 1 IU/ml in all apotransferrin batches and the levels of the other protease contaminants were also very low (Table 2).

The amount of iron in the finished product was very low and the calculated iron saturation value was typically 0·3% (Table 1). The level of zinc and aluminium was below the detection limit of atomic absorption spectrometry (Table 1).

Stability of the product

To develop a stability indicating profile of the product, the liquid formulation was stored at accelerated temperature. The most informative methods

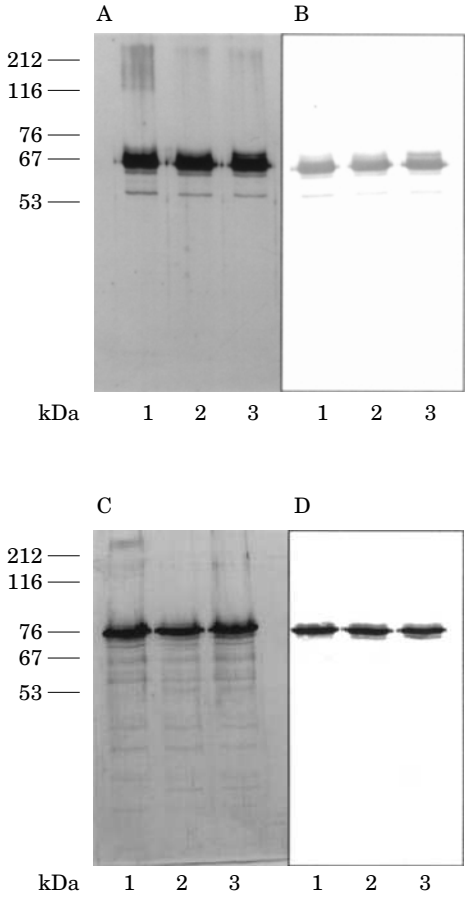

Figure 4. SDS–PAGE of the apotransferrin product. Lane 1, lyophilized apotransferrin from early development; Lane 2, lyophilized apotransferrin produced according to the described method; Lane 3, apotransferrin in a liquid formulation. (A) Non-reduced, silver stain, protein load 0·125 μg; (B) non-reduced, Western blot with anti-transferrin, load 0·25 μg; (C) reduced, silver stain, load 0·5 μg; and (D) reduced, Western blot with anti-transferrin, load 0·25 μg.

Table 2. Plasma proteins and protease activities in the finished product containing 50 g/l apotransferrin, mean of four batches

Protein	Amount
IgG	0·14 g/l
IgA	0·37 g/l
Hemopexin	0·3 g/l
Prekallikrein activator (PKA)	<1 IU/ml
Amidolytic activity without prekallikrein substrate	8 IU/ml
Plasmin/plasminogen	<0·03/<0·05 CU/ml

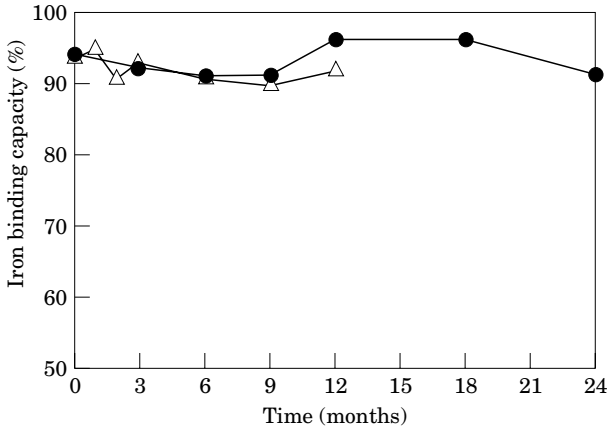

Figure 5. Iron binding capacity of apotransferrin in the liquid formulation during storage at refrigerator (●) and room temperature (△).

in the detection of changes in the product during storage proved to be the iron binding capacity, urea–PAGE directly and after iron-saturation, IEX-HPLC and SE-HPLC. Different lyophilised formulations have been stable for four years at refrigerator temperature. The liquid formulaiton has been stable at least for two years in refrigerator (2–8° C) and 12 months at room temperature (25° C) (Fig. 5).

Discussion

The manufacturing process described in the present paper produces pure and virus-safe apotransferrin with high yield and only a few process steps. The process starts from Cohn fraction IV, which is a by-product of albumin production from human plasma. Transferrin is maintained in the iron-free apoform from the beginning of the process and it is therefore not necessary to have a separate iron-removal step. Apotransferrin is purified by a combination of a cation and anion exchange chromatography and only one ultrafiltration step is needed for concentration and buffer exchange of the pure apotransferrin solution. The process yields a pharmaceutical composition of apotransferrin, which has a purity of at least 98% and contains less than 1% transferrin dimers and no detectable polymers or aggregates. The developed formulation can be lyophilized or stored as a solution and it is suitable for intravenous infusion. Several assays were applied for the assessment of the iron binding capacity and integrity of the pure apotransferrin product, which proved to be sensitive indicators of the stability of the product. The lyophilized formulations have been stable for four years and the

developed liquid formulation for two years in refrigerator and six months at room temperature.

The structural characterization indicated that the pure apotransferrin product is identical with human plasma iron-free transferrin. Electrospray mass spectrometry did not indicate chemical modifications in the apotransferrin molecule other than the N-linked glycans and the predicted 19 disulphide bonds, except minor oxidation most probably of single methionine residues. The identified glycan chains were structurally closely similar to the structures described before for human plasma transferrin.[29] The minor transferrin variant with only one biantennary glycan chain has not been identified in the earlier studies.

As in the case of other plasma products, the viral safety of the apotransferrin product is based on reduction of potential virus load in the starting material by donor selection and testing for infection markers and by effective virus inactivation and removal during the manufacturing. The major virus inactivation step in the process is SD treatment, which very reliably inactivates all enveloped viruses. No transmission of enveloped viruses, including HIV and hepatitis B and C viruses, has been reported during more than 10 years of clinical use of SD-treated plasma products.[30] Virus filtration with a 15 nm pore size hollow fibre filter effectively removes even the small non-enveloped viruses, such as HAV and parvovirus.[31] Parvovirus B19 currently remains a problem with plasma products because its level in the starting plasma may be very high (up to 10^9 gen. eq./ml) and a single virus filtration step may not be capable of removing the potential virus load from the plasma pools.[32] Although clinically significant parvovirus transmissions by plasma products are apparently very rare, parvovirus transmission might be harmful to immuno-compromised patients receiving high-dose chemotherapy, who are a target population of apotransferrin administration. Therefore, a twofold strategy has been followed in the new product. First, the potential virus load in the starting plasma has been restricted by PCR testing of parvovirus B19 DNA. Second, both PEG precipitation and 15 nm virus filtration were implemented to the process in order to achieve two effective removal steps for parvovirus. Considering the potential level of parvovirus in the plasma pools after PCR screening and the clearance of parvovirus observed in virus removal studies of the process, it can be concluded that the finished product is with high confidence safe also with respect to parvovirus transmission.

The therapeutic use of transferrin has been studied before in three patients undergoing myeloablative chemotherapy and bone marrow transplantation.[33] Two of the three patients developed acute renal failure, which may have been associated with the administration of the transferrin preparation. This study did not demonstrate efficacy of the transferrin preparation in the binding of free iron in the patients or other benefits but rather suggested that the therapeutic use of transferrin may not be safe. Details of the quality of the transferrin preparation used were not described, except that it contained a high amount of aluminium. Another study described manufacturing processes for plasma transferrin that yielded a product with high zinc content.[34] Potentially harmful impurities described in other transferrin preparations include aggregates and polymers formed during pasteurization.[35] As with other plasma, contaminating plasma proteases present a further possible cause for adverse effects in patients. The level of all these plasma and process derived impurities were very low in the present product.

An investigational apotransferrin product, which meets the same specifications as the current product, has been safely used in iron chelation therapy in new-born infants with a fatal inherited metabolic disorder affecting iron metabolism.[36] The new product has proved safe in early clinical trials in bone marrow transplantation patients[37] and it should also be suitable for transferrin substitution therapy in atransferrinaemia patients with inherited transferrin deficiency.[38] Further, the feasibility to produce high quantities of the product with reasonable production costs makes it possible to give apotransferrin to patients in gram quantities, which would be needed for the prevention of the appearance of non-transferrin-bound iron in patients receiving high-dose chemotherapy.

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