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The identification of abnormal glycoforms of serum transferrin in carbohydrate deficient glycoprotein syndrome type I by capillary zone electrophoresis

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One of the biochemical characteristics of carbohydrate deficient glycoprotein syndromes is the presence of abnormal glycoforms in serum transferrin. Both glycoform heterogeneity and variable site occupancy may, in principle, lead to the generation of a range of glycoforms which contain different numbers of sialic acid residues, and therefore variable amounts of negative charge. Capillary zone electrophoresis was used to resolve the glycoforms of normal human serum transferrin and also of a set of glycoforms which were prepared by digesting the sugars on the intact glycoprotein with sialidase. The sugars on the intact glycoprotein were also modified by a series of exoglycosidase enzymes to produce a series of neutral glycoforms which were also analysed by capillary zone electrophoresis. The oligosaccharide population of human serum transferrin was analysed by a series of mixed exoglycosidase digests on the released glycan pool and quantified using a novel HPLC strategy. Transferrin was isolated from carbohydrate deficient glycoprotein syndromes type I serum and both the intact glycoforms and released sugars were resolved and quantified. The data presented here confirm the presence of a hexa-, penta- and tetra-sialoforms of human serum transferrin in both normal and carbohydrate deficient glycoprotein syndrome type I serum samples. Consistent with previous reports carbohydrate deficient glycoprotein syndrome type I transferrin also contained a di-sialoform, representing a glycoform in which one of the two N-glycosylation sites is unoccupied, and a non-glycosylated form where both remain unoccupied. This study demonstrates that capillary zone electrophoresis can be used to resolve quantitatively both sialylated and neutral complex type glycoforms, suggesting a rapid diagnostic test for the carbohydrate deficient glycoprotein syndromes group of diseases.

Keywords: Transferrin, Capillary zone electrophoresis, Carbohydrate Deficiency Glycoprotein Syndrome, Glycoforms.

Abbreviations: CDGS: Carbohydrate Deficient Glycoprotein Syndrome; CZE: Capillary Zone Electrophoresis; hTf: human transferrin; gu: HPLC glucose units; EOF: electroosmotic flow. Nomenclature: for describing oligosaccharide structures: A(1, 2, 3, 4) indicates the number of antennae linked to the trimannosyl core; G(0–4) indicates the number of terminal galactose residues in the structure; F: core fucose; B; bisecting N-acetyl glucosamine (GlcNAc); S: sialic acid; Gal: galactose; M, Man: mannose.

Introduction

Human serum transferrin (hTf), an iron transporting glycoprotein, has two N-linked complex glycans attached to asparagine residues in the Asn413LysSer and Asn611-ValThr glycosylation sequons. Both sites are normally occupied; 85% have been shown to contain the fully sialylated, digalactosylated biantennary complex oligosaccharide, A2G2S2, while the remaining 15% are occupied by triantennary complex glycans [1–3]. Increased levels of highly branched glycans, the percentage of which is independent of hTf concentration, have been noted in pregnancy, iron deficiency anaemia and rheumatoid arthritis [4]. The role of the sugars on hTf has been

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probed by site directed mutagenesis which indicated that deletion of the N-glycosylation sites did not impair iron transport, receptor binding or circulating half-life [5–7].

Alterations in the structure of the hTf glycoforms may result from variable site occupancy and/or incomplete processing of sugars at one or both sites. Carbohydrate deficient hTf, in which one or both glycosylation sites are unoccupied, was first reported by Stibler *et al.* [8] in serum samples from alcohol abuse cases. In addition, the sugars released from individual isoforms of this hTf were shown to differ in their sialylation levels [9] indicating that both the addition of the $Glc_3Man_9GlcNAc_2$ oligosaccharide precursor and the normal processing of the complex sugars of hTf were deficient. Jaeken *et al.* [10, 11] reported the presence of carbohydrate deficient forms of hTf in some children with severe neurological disorders. Several multisystemic diseases with autosomal recessive inheritance mode were subsequently identified and designated carbohydrate deficient glycoprotein syndrome (CDGS) types I–IV [10, 11]. CDGS type I is the most common, while types II, III and IV are rare variants. All are associated with alterations to the glycosylation of serum hTf. CDGS type II, associated with a defect in N-acetyl-glucosaminyltransferase II (EC 2.4.1.143), is characterised by an increase in di-, mono- and asialo-glycoforms derived from mono-antennary complex glycans [12]. Type III, associated with reduced levels of sialic acid, galactose and N-acetylglucosamine and increased levels of mannose [13], contains an increased level of the tri-, di- and asialo-glycoforms and also a high proportion of tetra-sialylated normal hTf. Type IV CDGS gives rise to abnormal glycoforms associated with the loss of one or two sialic acid residues [14].

CDGS type I hTf has been studied in the most detail. In addition to the normal tetra-sialylated form it contains both di- and asialo- glycoforms [3, 4]. The oligosaccharides released from serum hTf from patients with type I CDGS have been analysed [15, 16]. No structurally abnormal sugar chains were identified, and the released sugars were all fully sialylated, indicating that, in contrast to other types of CDGS, the abnormal glycoforms in CDGS type I hTf relate only to site occupancy not to deficient processing of the oligosaccharides. Reasons for the under-glycosylation of glycoproteins in CDGS I include the possibility of a defect in the protein folding processes and/or in the supply of dolichol-linked oligosaccharides in the endoplasmic reticulum of the cell [11, 17, 18]. Consistent with the latter is the finding that the activity of phosphomannomutase is deficient in fibroblasts, liver and leucocytes of patients with CDGS I [19].

A diagnostic genetic probe is not yet available although the genetic locus for CDGS I has been assigned to the p13.3–p13.12 region of chromosome 16 [20]. The biochemical diagnosis of CDGS is generally made following the identification of abnormal glycoforms in serum hTf. This is usually performed by agarose electrophoresis or isoelectric focusing followed by Western blotting and immunodetection of protein bands. These methods are time consuming and, although they resolve the isoforms which arise from incompletely glycosylated hTf, the resolution depends on the state of occupation of the iron binding pockets [21, 22] which therefore needs to be controlled. The methods do not allow accurate quantitation without densitometry, neither do they lend themselves to routine analysis [8].

Capillary zone electrophoresis (CZE) [23], which offers the possibility of resolving, identifying and quantifying the relative proportions of intact glycoforms at the protein level, is potentially a powerful addition to the existing technologies. In CZE, the narrow capillary allows heat to be dissipated rapidly and field strengths up to 30 kV can be applied giving the potential for enhanced resolution over electrophoretic techniques involving gels.

The CZE separation of glycoform populations depends on amplifying the structural differences between glycans by allowing them to complex with an ionic species. The chemistry which has been used to achieve such separations has, for the most part, exploited subtle differences in the conformation of the hydroxyl groups associated with the monosaccharide residues which make up the oligosaccharides. Previously we demonstrated that CZE using borate buffer can resolve the oligomannose glycoforms of bovine pancreatic ribonuclease (RNase) B (Man-5, -6, -7, -8 and -9) in their correct molar proportions at the protein level [24]. In this case, borate ions complex with available cis diols adding charge to neutral oligosaccharides. In contrast to RNase B, normal serum hTf contains complex glycans, and in this study we have used a monosodium glutamate/tris buffer system to extend the use of CZE to analyse the glycoforms of a glycoprotein containing sialylated and neutral complex sugars.

Two series of hTf glycoforms were prepared by modifying the natural population of normal human hTf. One series consisted of hTf glycoforms containing two complex biantennary structures with from 0–6 sialic acid residues. The second set consisted of hTf glycoforms containing two neutral complex glycans successively modified by exoglycosidase digestions. Capillary zone electrophoresis was able to resolve glycoforms containing both sialylated and neutral complex oligosaccharides, suggesting that this may be a useful technique for diagnosing all types of CDGS. Here CZE was used to resolve and analyse quantitatively the hTf glycoforms isolated from CDGS type I serum, showing that the asialo and disialylated glycoforms which result from partial site occupancy can be distinguished from those

asialo and disialylated glycoforms which contain two sugars but are deficient in sialic acid.

Materials and methods

Enzymes

Arthrobacter ureafaciens neuraminidase and *Streptococcus pneumoniae* β-hexosaminidase were obtained from Oxford GlycoSystems, *S. pneumoniae* β-galactosidase was prepared by Dr T. Butters (Oxford Glycobiology Institute), *S. (diplococcus) pneumoniae* endoglycosidase D was obtained from Boehringer Co.

Standard human serum hTf

This was obtained from Sigma Chemical Co. Ltd and shown by them to be >99% pure by agarose electrophoresis and to contain <0.005% of iron. Product number: T0519. Lot number: 105F9476. Cas no: 11095-37-0

Purification of human serum hTf

hTf was purified from serum by the method adapted from [25]. Serum (250 μl) was diluted with 175 μl of distilled water and 175 μl of 3% Rivanol (6,9-di-amino-2-ethoxyacridine lactate, Sigma Chemical Co.) in distilled water was added dropwise while vortexing. The sample was centrifuged at 10 000 rpm in a microfuge for 10 min. A 500 μl aliquot of the supernatant was withdrawn and mixed with 25 mg of NaCl and re-centrifuged. The resulting supernatant was mixed with an equal volume of saturated aqueous ammonium sulphate solution to precipitate immunoglobulins and allowed to stand for 10 min at room temperature. The sample was then centrifuged and the supernatant, which contained hTf, was separated and dialysed against 0.85% NaCl. Iron enriched hTf was prepared by dialysing hTf against 0.85% sodium chloride saturated with $FeCl_3$. Iron depleted transferrin was prepared by extensively dialysing hTf against 0.1% trifluoroacetic acid (pH 2.15).

Immunoelectrophoresis of serum and serum transferrin

The sample (3 μl serum diluted 1:10 or transferrin 120 $\mu g\,ml^{-1}$) was loaded to an agarose gel in pH 8.6 buffer. After electrophoretic separation, anti-whole human serum antibody was added to the central trough and allowed to diffuse overnight at room temperature. The gel was then washed with PBS × 3 and stained with Coomassie Blue. Arcs were assigned by comparison with known standards.

Estimation of hTf levels in serum by Radial Immuno Diffusion

Anti-hTf serum (0.5 ml, Atlantic Antibodies Catalogue no. 80265) was added to 10 ml of agarose (1% in PBS) containing 3% w/v PEG 6000 and 25 μl red ink. The gel solution was poured into an immunodiffusion plate and allowed to gel. Samples of serum were analysed without prior dilution. The plate was calibrated using calibrator 1 (Atlantic Antibodies Catalogue no. 844000) diluted 1/5, 2/5, 3/5, 4/5 and undiluted.

Release and analysis of hTf glycans

Glycans were released from hTf using the GlycoPrep 1000 (Oxford GlycoSystems). The sugars were fluorescently labelled with 2-amino-benzamide using an (Oxford GlycoSystems) kit and analysed by HPLC using the (Oxford GlycoSystems) GlycoSepN column [26]).

Multiple oligosaccharide sequencing on the released glycan pool

Enzyme digests were performed at 37 °C for 16–24 h in 100 mM citrate/phosphate buffer pH 4.5, 0.2 mM zinc acetate, 0.15 M sodium chloride. Conditions for the individual enzymes were as follows: *A. ureafaciens* neuraminidase: 1–2 $U\,ml^{-1}$; substrate concentration 5–30 μM. Bovine testes β-galactosidase (Oxford GlycoSystems): 1–2 $U\,ml^{-1}$; substrate concentration 20 μM. Jack bean N-acetyl β-hexosaminidase (Oxford Glycobiology Institute): 10 $U\,ml^{-1}$; substrate concentration: 20 μM. *Charonia lampas* α-fucosidase (Oxford Glycobiology Institute): 10 $U\,ml^{-1}$ per 1 $mg\,ml^{-1}$ BSA; substrate concentration: 20 μM; Jack bean α-mannosidase (Oxford Glycobiology Institute) 10 $U\,ml^{-1}$ substrate concentration: 20 μM; *Helix pomatia* β-mannosidase (Oxford Glycobiology Institute): 3 $U\,ml^{-1}$; substrate concentration: 20 μM.

Normal phase separations of neutral and acidic oligosaccharides

Normal phase separations were performed on a Glycosep-N chromatography column (Oxford GlycoSystems Ltd) (4.6 × 250 mm). Samples were applied in 80% acetonitrile and 20% water. Gradient conditions were as follows: Solvent A was 50 mM ammonium formate (pH 4.4) and solvent B was acetonitrile. Initial conditions were 20% A at a flow rate of 0.4 $ml\,min^{-1}$. This was followed by a linear gradient of 20–58% A at a flow rate of 0.4 ml min^{-1} for 152 min, followed by 100% A at 0.4 $ml\,min^{-1}$ for 3 min, followed by 100% A at 1 $ml\,min^{-1}$ for 17 min. This gradient was used to resolve both neutral and acidic sugars.

High Performance CZE

Separations were carried out on a P/ACE System 2100 (Beckman Instruments) using a fused silica capillary (107 cm × 75 μm ID). The detection was by UV at 200 nm. Injections of 1–2 $mg\,ml^{-1}$ for 1.5–2 s were performed (5 ng) under high pressure. The buffer was 50 mM Tris, 50 mM mono-sodium glutamate ($COOHCH_2CH_2CHNH_2COONa$) (pH 8.54–8.64). The voltage was 10 kV. Several types of capillaries were tested; uncoated capillaries were selected since coated capillaries

are either expensive, if obtained from commercially available sources, or difficult to reproduce in other laboratories, if coated in-house. CZE elution times are not sufficiently reproducible as a basis for assigning peaks. Factors which contribute to the variability include minor variations in the pH and absolute composition of the sample loading buffer, and the inherent variation of the surface of uncoated fused silica capillaries with time. To address this problem a standard sample (hTf obtained from Sigma) was used in over 50 individual separations. Each time a new running buffer batch was prepared, the separation of the standard was assessed, and each peak identified by the elution position relative to the negative injection spike associated with each electropherogram. The injection spike is caused by the difference in absorbance between the running buffer and the sample loading buffer and makes the use of a separate electrophoretic marker unnecessary.

Exo- and endo-glycosidase digestions of glycans on intact transferrin

A. ureafaciens neuraminidase: the time course was performed over 260 min using an optimised dilution of the *A. ureafaciens* neuraminidase at 4 mU:2 mg hTf 200 μl^{-1} 100 mM sodium acetate (pH 5.0) at 37 °C. Aliquots were removed from the reaction mixture during the digestion and the reaction stopped by freezing at −20 °C. *A. ureafaciens* neuraminidase cleaves NeuNAc-α2−6 > 3,8 R.

S. pneumoniae β-galactosidase: digestion of asialo glycans − 2 mg ml^{-1} of hTf were incubated with 300 mU ml^{-1} of β-galactosidase in 50 mM citrate/phosphate (buffer pH 6) for 18 h at 37 °C. The enzyme cleaves Galβ1−3,4 > 6 GlcNAc R.

S. pneumoniae β-hexosaminidase: 2 mg ml^{-1} of desialylated and de-galactosylated hTf were incubated with 500 mU ml^{-1} of β-hexosaminidase in 50 mM citrate/phosphate buffer (pH 6). Incubation was for 18 h at 37 °C. *S. pneumoniae* β-hexosaminidase cleaves GlcNAc β1−2,4,6 Man or Glcβ1− 3,6Gal.

S. pneumoniae β-galactosidase + *S. pneumoniae* β-hexosaminidase + *S. (diplococcus) pneumoniae* Endoglycosidase D: hTf (2 mg ml^{-1}) was incubated with 300 mU ml^{-1} of β-galactosidase + 500 mU ml^{-1} of β-hexosaminidase + 150 mU of endoglycosidase D at 37 °C in 50 mM citrate/phosphate (buffer pH 6). Endoglycosidase D cleaves between the GlcNAc residues in the chitobiose core.

Results and discussion

Purification and analysis of normal human serum hTf

hTf was isolated from normal human serum (present at 3.1 g l^{-1}) and from two patients with CDGS type I (Patient 1: 2.65 g l^{-1} and patient 2: 2.6 g l^{-1}) using rivanol and ammonium sulphate precipitation. The protein was analysed by immunoelectrophoresis (Fig. 1). The samples contained <5% of serum albumin (which is not glycosylated) and IgG was not detected. This was consistent with the analysis of the released sugars (Figs 2 and 3 below). None of the biantennary, fucosylated, agalactosyl (A2G0F) sugar, which is associated with IgG and not hTf, was detected. The batch of commercial human hTf obtained from Sigma Chemical Company was confirmed by them to be >99% pure by agarose electrophoresis (data not shown).

Analysis of the pool of glycans released from normal human serum Tf

Glycans were released from normal transferrrin by hydrazinolysis, fluorescently labelled with 2-aminobenzamide and analysed by normal phase HPLC (Figs 2a and 3b). The elution positions of the peaks were calculated in HPLC glucose unit (gu) values, which were obtained by comparison with the elution positions of the components of a standard dextran ladder shown at the top of Fig. 3a). Structures (Table 1) were assigned from the gu values using the elution positions of standard sugars together with experimentally determined incremental values for the addition of monosaccharide residues to standard glycan cores (for full details of this method see [26]). The assignments were confirmed by simultaneously sequencing the pool of sugars with a set of exoglycosidase enzyme arrays (Table 2). The data, (Fig. 3c−g, and Table 1) which indicated that the major glycan was the complex biantennary glycan containing two α2,6-linked sialic acid residues, A2G2S2(2,6;2,6) were generally consistent with earlier reports [2, 27]. A population of the monosialylated glycan A2G2S1 [2, 6] (12.2%) was found; this is not consistent with previous data and may indicate loss of sialic acid during storage of the glycans. Minor populations included 7% of the fucosylated glycan A2G2FS2(2,6;2,6), the triantennary glycan A3G3 (8%) and, in addition, 2% of a tetra-antennary sugar was detected.

Capillary zone electrophoresis resolution of glycoforms of normal human serum hTf

Normal human hTf, from a commercial preparation and from pooled serum, was resolved into four glycoforms (Fig. 4a,b respectively) of which the major species in both cases was the tetra-sialylated glycoform, (peak 4). Further samples of the hTf isolated from pooled serum were analysed following depletion of iron and after saturation with iron (Fig. 4c,d). The occupancy of the iron binding sites did not affect the CZE elution profile.

Preparation and analysis of a series of variably sialylated hTf glycoforms

Capillary zone electrophoresis separations depend on the fact that, in most buffer systems, the inner walls of the

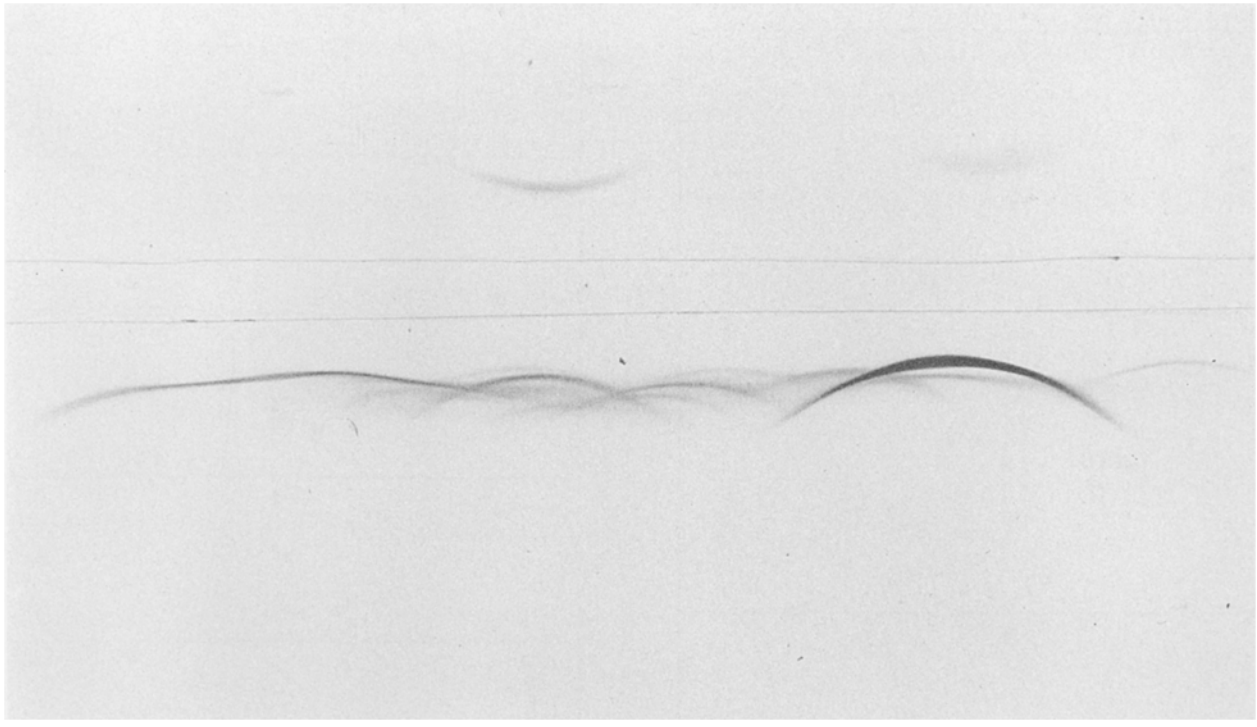


Figure 1. Immunoelectrophoresis of normal human serum transferrin (upper trace) and normal human serum (lower trace). Anti-whole human serum antibody diffused into the gel from a central trough. Arcs were assigned by comparison with known standards. The two arcs in the upper trace are transferrin (on the left) and albumin (right). IgG was not detected.

silica capillary column carry a negative charge. Positive ions in the electrolyte are attracted to the wall and, under high voltage, migrate towards the cathode creating a bulk flow of liquid called the electroosmotic flow (EOF). If a sample is introduced into the capillary at the anode all the components are carried by the EOF towards the cathode. Simultaneously the current flow will retard negatively charged species so that the positive species elute first, followed by the neutral and finally the negative. In the case of transferrin the least sialylated species elute first.

Limited digestion of normal hTf with neuraminidase resulted in a series of glycoforms containing from 0–6 sialic acid residues which could be resolved by CZE (Fig. 5). After further incubation (260 min) these were digested to one major species. By analogy with the enzyme digestions shown in Fig. 3, and consistent with previous findings, this single glycoform contained two asialo biantennary glycans (Fig. 5: 260 min, peak 0), An envelope of smaller peaks eluting between the asialo (peak 0) and the monosialylated species (peak 1) was also present. These minor peaks may contain asialo hTf glycosylated with tri- and tetra-antennary glycans. The time course analysis of the digest indicated that as sialic acid residues are successively removed from the sugars the glycoforms elute earlier, consistent with a decrease in negative charge.

These data indicated that all sialic residues in N-linked glycans attached to native human hTf were accessible to the action of neuraminidase. However, the observed differences in the resistance of different glycoforms to the enzyme (for example the mono-sialylated form takes longer to digest than the tetra sialylated-form) suggests that the kinetics of interaction of the enzyme with the terminal sialic acid residues on the glycans attached to the protein alters as the molecule becomes less charged.

Analysis of a series of neutral hTf glycoforms

Four neutral hTf glycoforms, which could be resolved by CZE on the basis of size by complexing the oligosaccharide moieties with tris/glutamate buffer, were prepared by modifying the carbohydrate residues with four different arrays of glycosidase enzymes (as depicted in Fig. 6). These enzymes cleave terminal galactose, N-acetylglucosamine and the chitobiose core sugar ($Man_3GlcNAc$) from the desialylated hTf in a stepwise manner, leaving a single N-acetylglucosamine residue attached to the protein [28]. The protein linked- N-acetylglucosamine was not removed because the cleavage of Asn-GlcNAc bond with the enzyme PNGase F converts the asparagine residue into aspartic acid and introduces a charge to the de-glycosylated protein, which would alter the elution behaviour of the molecule on CZE. Although the pH optimum is

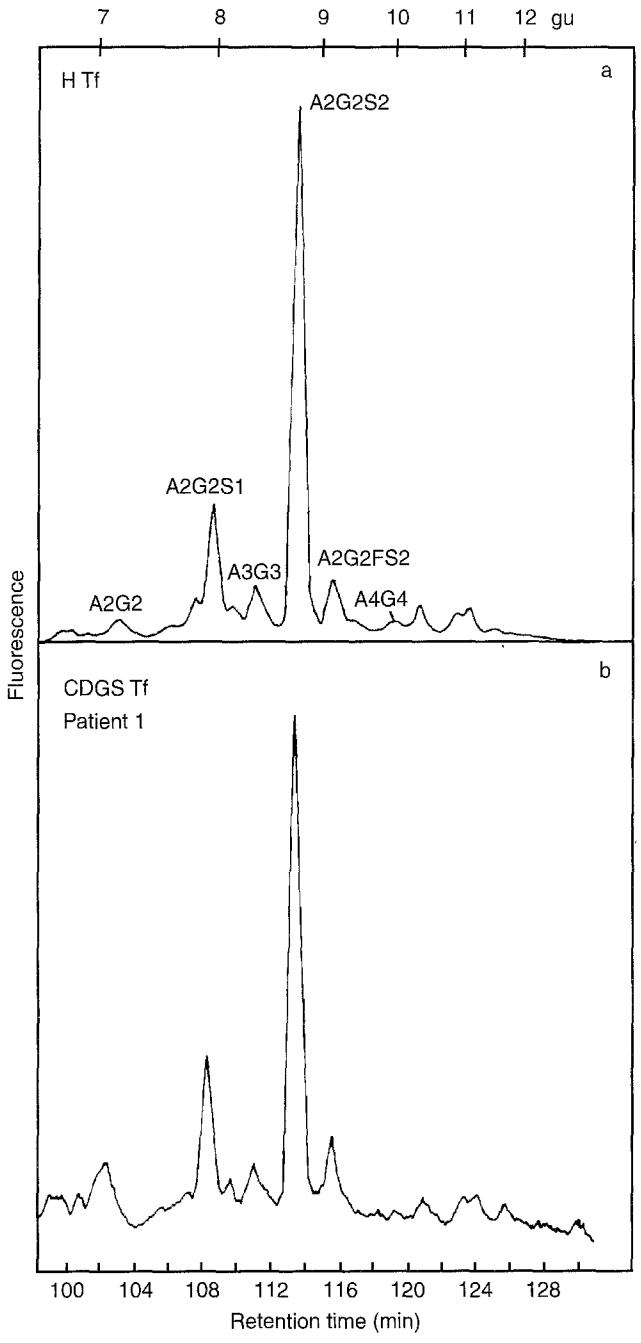


Figure 2. The analysis of the sugars released from a control individual and CDGS serum hTf. The fluorescently labelled glycan pools were analysed by normal phase HPLC. The gu values of the peaks were calculated from the standard dextran ladder shown at the top of the profile. Structures were assigned (Table 1) using the incremental values for monosaccharide additions to standard glycan cores as described in Guile *et al.* [26]. The relative proportions of the glycan populations are shown in Table 1.

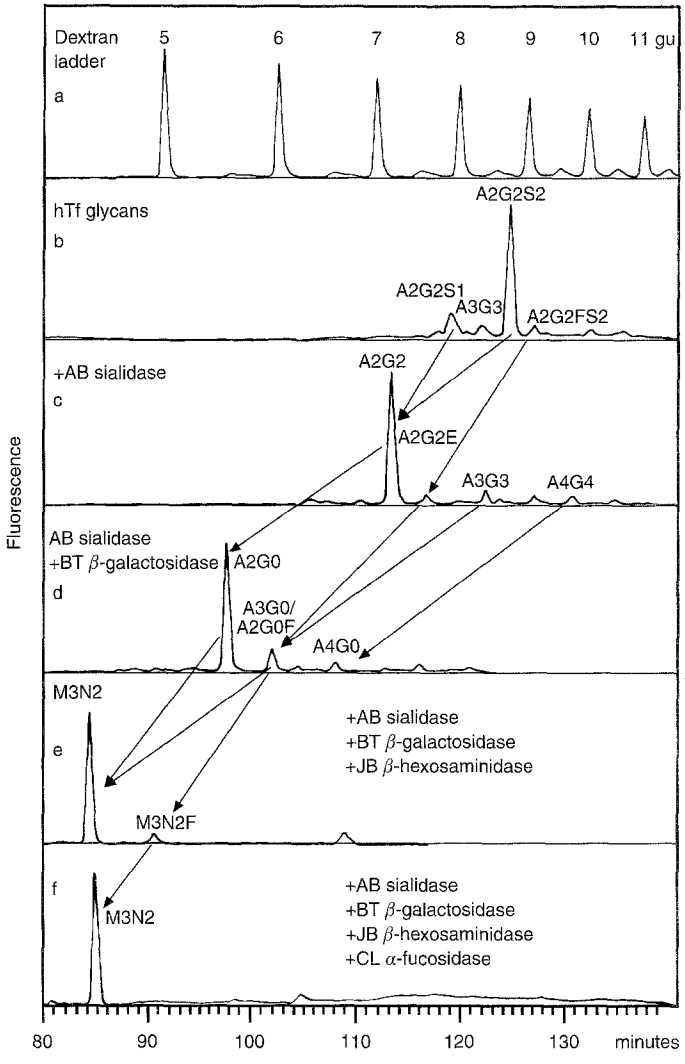


Figure 3. HPLC analysis of the products of the multiple exoglycosidase enzyme digests of the pool of fluorescently labelled sugars released from hTf from a control individual. The products of the enzyme arrays were compared. The data are summarised in Table 1; the assignments were consistent with the products of the digests and the known specificities of the enzymes. Panel: (a) Dextran ladder (b) total glycan pool (c) glycan pool + *A. ureafaciens* sialidase (d) glycan pool + *A. ureafaciens* sialidase + Bovine testes β-galactosidase (e) *A. ureafaciens* sialidase + Bovine testes β-galactosidase + jack bean β-hexosaminidase (f) *A. ureafaciens* sialidase + Bovine testes β-galactosidase + jack bean β-hexosaminidase + *Charonia lampas* α-fucosidase.

different for each enzyme, all of the enzymes used are active over a range of pH values, including pH 6 which was used for the mixed digests. The principle of using enzyme mixtures for sequencing oligosaccharides (Reagent Array Analysis Method) has been established previously in this laboratory [29]. This current study demonstrates that the method can be extended to modify complex oligosaccharides attached to glycoproteins such as hTf, where the glycans are exposed. Enzymatic digestions of the oligomannose sugars on an intact glycoprotein have been used previously in this laboratory to prepare single, pure glycoforms of RNase B [30].

Each digest was followed through a time course. For example, Fig. 7 shows the time course of the digestion of desialylated and degalactosylated transferrin with jack

Table 1. Oligosaccharide analysis of glycans released from normal pooled serum hTf

hTf		*Panel b total*	*Panel c ab*	*Panel d abs/btg*	*Panel e abs/btg/ jbh*	*Panel f abs/btg/ jbh/clf*
gu value	*Per cent*	*Peaks*	*Peaks*	*Peaks*	*Peaks*	*Peaks*
4.4					M3N2	M3N2
4.9					M3N2F	
5.5				A2G0		
5.8				A3G0/A3G0F		
6.5				A4G0		
7.1			A2G2			
7.74	2.4	A2G2F	A2G2F			
7.91	12.2	A2G2S1(2,6)				
8.33	7.8	A3G3	A3G3			
8.72	58.1	A2G2S2(2,6;2,6)				
9.1	6.6	A2G2FS2(2,6;2,6)				
9.75	2.1	A4G4	A4G4			
10.05	4.2	NA	NA			
10.62	6.6	NA	NA			

The columns refer to the HPLC profiles in Figs 2 and 3, panels c–e.
Column 1 shows the HPLC gu values of the major peaks in the hTf glycan pool. Column 2 shows the percentage of each population of glycans and column 3 shows the assignments.
Columns 4–7 show the major peaks in the products of the digestions with the enzyme arrays c–f respectively (Table 2).
All gu values were obtained by comparison with the elution positions of a standard dextran ladder. Peak assignments were made by comparison with the elution positions of known standards. They are consistent with the incremental values for the addition of monosaccharide units to basic oligosaccharide cores, and with the known specificities of the enzymes. Full details of this technology are in Guile *et al.* [26].
abs: *A. ureafaciens* sialidase; btg: Bovine testes galactosidase; jbh: jack bean β-hexosaminidase; clf: *C. lampas* fucosidase. NA – not assigned.

Table 2. The enzyme arrays used to sequence the pool of hTf sugars simultaneously. Four aliquots of the hTf glycan pool were each digested with one of the arrays. The HPLC analyses of the digestion products are shown in Fig. 2, panels c–f

Panel c: ABα2,3-/α2,6-sialidase
Panel d: ABα2,3-/α2,6-sialidase + BTβ-galactosidase
Panel e: ABα2,3-/α2,6-sialidase + BTβ-galactosidase + JBβ-hexosaminidase
Panel f: ABα2,3-/α2,6-sialidase + BTβ-galactosidase + JBβ-hexosaminidase + CLα-fucosidase

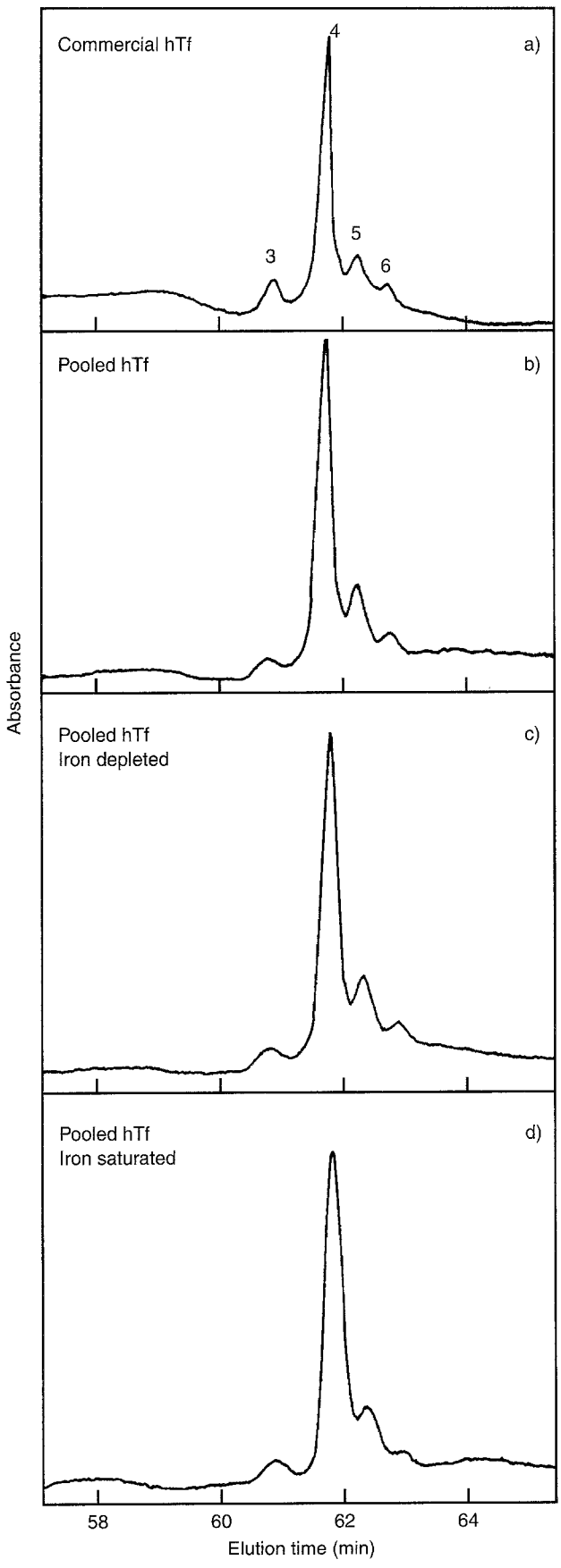
NDV: Newcastle Disease Virus; AB: *A. ureafaciens*; BT: bovine testes; JB: jack bean; CL: *C. lampas*

bean β-hexosaminidase (the end point is shown in Fig. 8c). Figure 8 shows the end points of each enzyme digest, and these are aligned with respect to the negative elution peaks (Fig. 7). The data indicate that the series of neutral glycoforms eluted earlier than the series of sialylated glycoforms (Fig. 5). There was an increase in retention time following sequential removal of monosaccharide residues from the desialylated hTf.

The data indicate that, within the neutral charge band, CZE resolves glycoforms on the basis of differences in their oligosaccharide composition and may therefore be a useful technique for probing the glycosylation of hTf from types of CDGS which involve changes in the neutral oligosaccharide structure. The data also demonstrate that the N-linked glycans attached to human hTf are accessible to all the exoglycosidases used in this study, and are therefore not shielded by the protein structure, nor do they interact strongly with the protein surface.

Capillary zone electrophoresis resolution of glycoforms of serum hTf from CDGS type 1

The CZE elution profile of normal hTf was compared with those of serum hTf from two patients with CDGS type 1 (Fig. 9a–c). These data are quantified in Table 3 and indicate that CDGS type I serum hTf contains tetra-, penta- and hexasialoforms (peaks (S)4, 5 and 6) in approximately the same proportion as normal hTf. In addition, the CDGS type I samples also contain hTf eluting as peaks (S)0 and (S)2. The elution profile of CDGS type I (patient 1) mixed with normal hTf (Fig. 9d) demonstrates that (S)2 and (S)0 peaks are unique to the



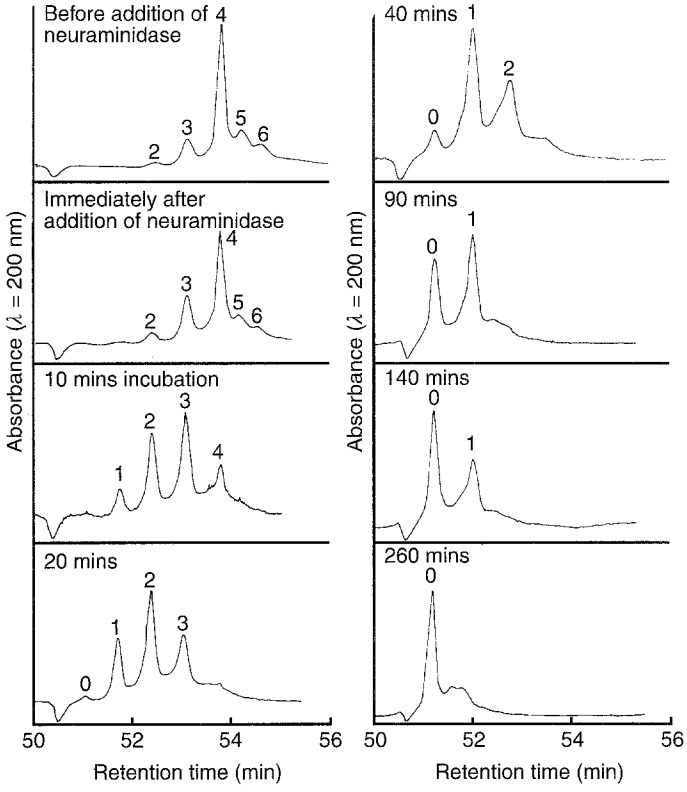


Figure 5. The time course following the neuraminidase digestion of the glycoform populations of normal hTf monitored by CZE. The original five populations containing 2, 3, 4, 5 and 6 sialic acid residues were reduced to one major and two minor populations containing only asialo hTf. Peak 4 contains the major glycoform (S_4) which is glycosylated with two di-sialylated biantennary complex glycans. The other peaks contain hTf with 2, 3, 5 or 6 sialic acid residues attached to bi- or tri-antennary complex glycans (S_2, S_3, S_5, S_6). The profiles have been aligned using the negative injection spike. The transferrin was from a commercial preparation (>99% pure).

CDGS type 1 and that (S)2 peak elutes earlier than the (S)3 peak of the normal hTf. This indicates that the (S)2 peak in CDGS type 1 contains the disialo glycoform and that it constitutes a major glycoform component. The material referred to as (S)0, is not found in either control nor is it present in either of the enzymatically digested normal hTf samples.

CZE resolution of desialylated glycoforms of CDGS type I hTf

Figure 10 shows the overlay of the separations of the native (solid line) and desialylated (broken line) CDGS type I hTf. Peaks S_4 and S_2 contain the tetra- and di-sialoforms, containing two and one sialylated complex

Figure 4. The iron content of hTf does not affect the CZE separation of the glycoform populations (a) commercial hTf (b) normal pooled hTf (c) normal pooled serum hTf depleted of iron (d) normal pooled hTf saturated with iron.

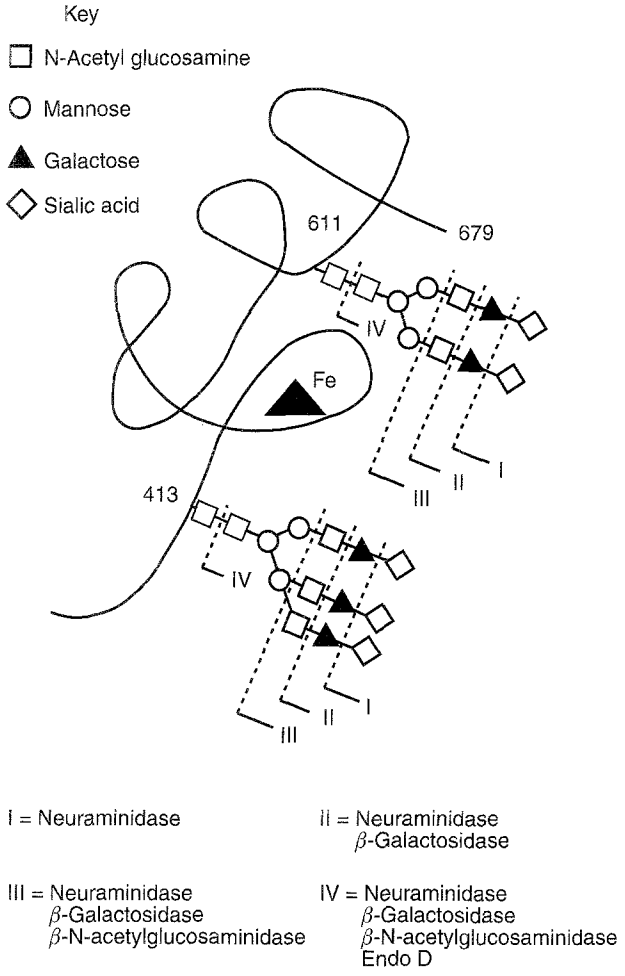


Figure 6. A schematic figure showing the C_t domain of one of the glycoforms of hTf (S_5) with the cleavage positions of the exoglycosidase enzymes used to prepare a series of neutral glycoforms of transferrin. The enzymes were (I) *A. ureafaciens* neuraminidase (II) Jack Bean β-galactosidase (III) Jack Bean β-hexosaminidase (IV) Endoglycosidase D. hTf contains two domains, the N_t domain is not glycosylated.

biantennary N-linked glycans respectively. S_4 elutes later than S_2, consistent with the data shown above (Fig. 5) and the fact that more negatively charged species are relatively retarded in the EOF because they are attracted towards the anode. S_4' and S_2' are the desialylated forms of S_4 and S_2 respectively. S_4' is larger than S_2' and elutes earlier, consistent with the trend observed in Fig. 8 which indicated that the larger neutral glycoforms of hTf have a shorter retention time.

Peaks S_0 and S_0' elute later than either of the de-

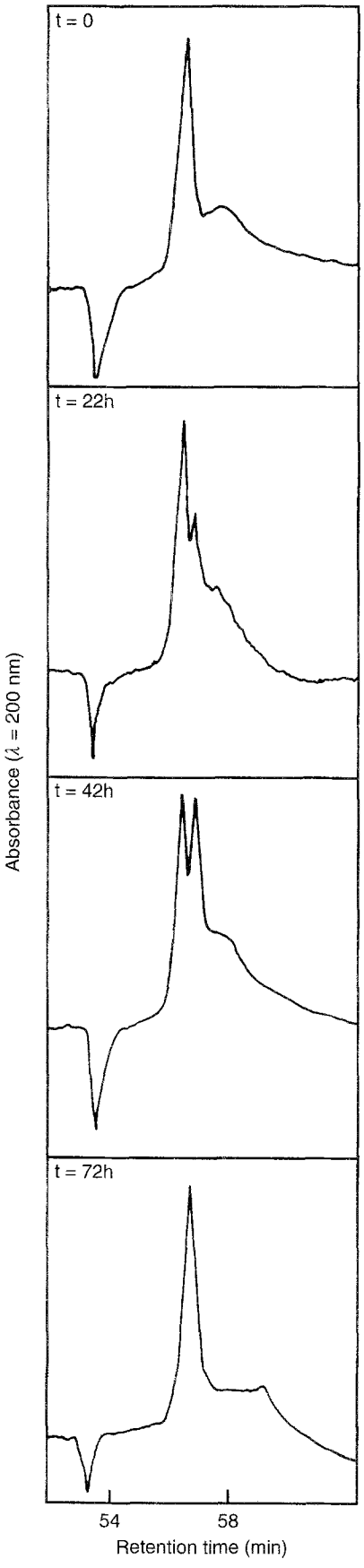


Figure 7. The time course for the digestion of hTf glycoforms with neuraminidase, β-galactosidase and β-hexosaminidase. The details of this digest (end point shown in Fig. 8c) is a typical example of the time courses of the digestions end points are shown in Fig. 8.

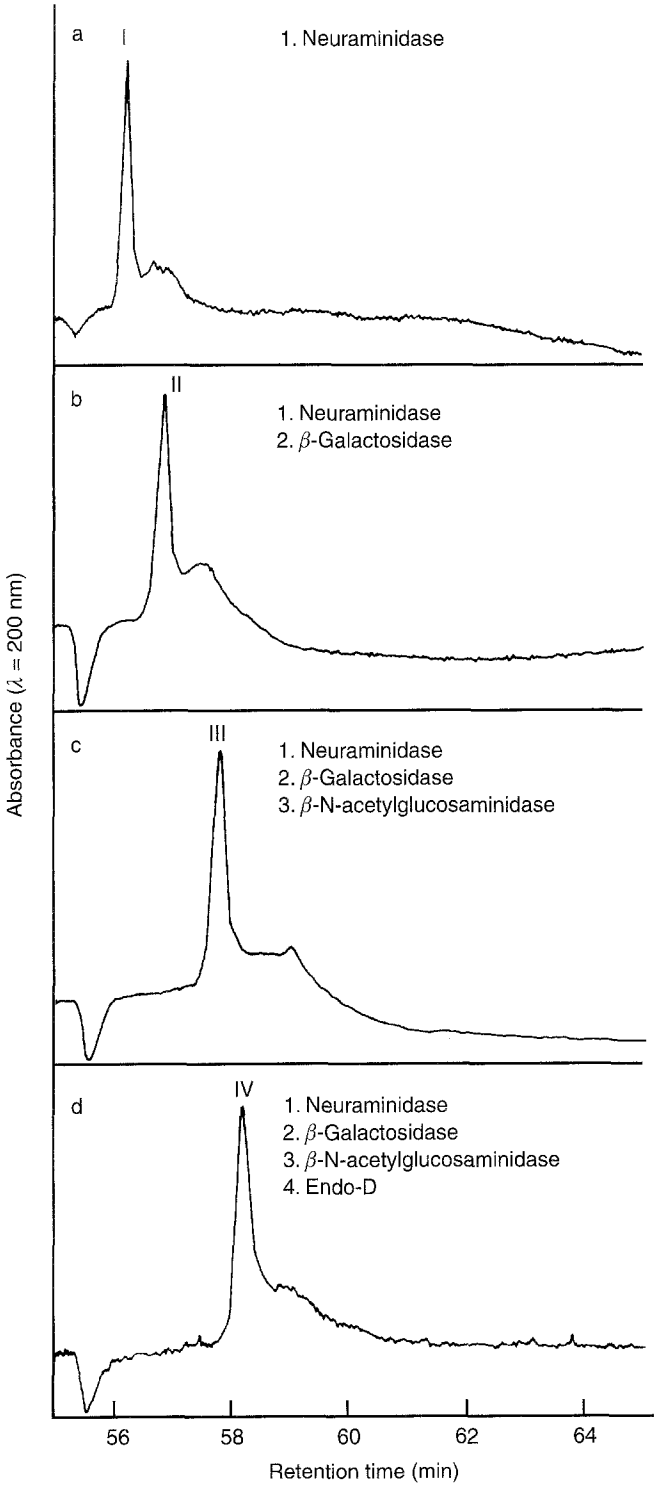


Figure 8. (a–d) The sequential digestion of the oligosaccharides attached to normal hTf showing the elution positions of the series of glycoforms generated by the digestions shown schematically in Fig. 6. The profiles have been aligned using the negative injection spike. The transferrin was from a commercial preparation (>99% pure).

sialylated glycoforms, indicating that these glycoforms contain less oligosaccharides. They elute earlier than any of the charged natural glycoforms, indicating that they contain less negative charge. In addition, the elution position is unaffected by the neuraminidase digestion. These findings are consistent with the assignment of this peak to aglycosylated hTf, the glycoform in which neither glycosylation site is occupied.

Comparison of the sugars released from normal and CDGS hTf

The HPLC analysis of the total pool of sugars released from normal and CDGS hTf is shown in Fig. 2b. These data show that there are no significant differences either in the type or the relative proportions of the oligosaccharides associated with hTf. This is consistent with previous reports that CDGS I Tf contains no abnormal glycoforms and suggests that the glycosylating enzymes process hTf sugars in a regulated manner which is independent of the number of occupied N glycan sites. Interestingly, in a study of the variations in hTf structure and glycan synthesis in various physiological states including pregnancy, iron deficiency anaemia, and rheumatoid arthritis De Jong *et al.* [4] showed that the variation between individuals in controls and groups with established stable diseases was very limited.

Conclusion

In this study two sets of hTf glycoforms, one charged and one neutral, were prepared by enzymatic modifications of the natural population and resolved quantitatively by CZE coupled with UV detection. The natural glycoforms of normal and CDGS hTf were also resolved by CZE. Peaks were assigned with reference to the elution positions of the enzymatically prepared glycoforms and to the analysis of the released sugars, which was based on a novel strategy using HPLC. Consistent with previous findings [11] CDGS hTf was shown to contain three populations of molecules containing 0, 1 or 2 occupied sugar sites. Analysis of the released sugars indicated that, in the samples analysed, CDGS hTf contained no abnormal sugars. In addition the relative populations of the oligosaccharides were similar to those of normal hTf, indicating that the processing of transferrin sugars is independent of the number of occupied glycosylation sites, and suggesting that the processing of the N-linked sugars attached to hTf is a strictly controlled process.

This study suggests that CZE may be a useful addition to the existing technologies for diagnosing type I CDGS, in which the abnormal processing leads to glycoforms which lack sialic acid, because either one or both glycosylation sites are unoccupied. In addition, a comparison of the CZE elution profiles of native and desialylated samples of CDGS type I hTf showed that,

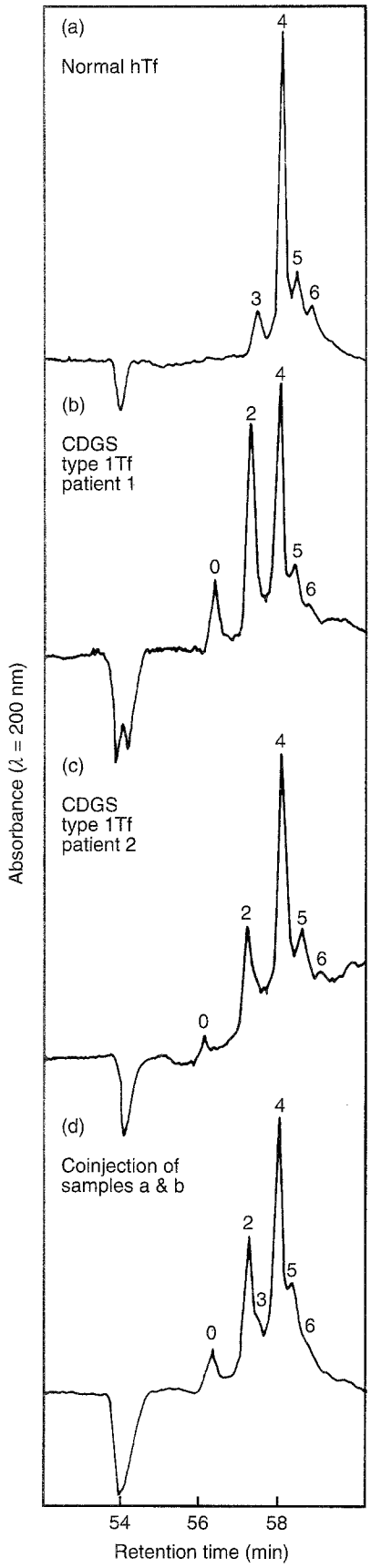


Table 3. The ratio of glycoforms in normal and CDGS type I hTf.

	Percent ratios of hTf glycoforms					
	S_0	S_2	S_3	S_4	S_5	S_6
Normal hTf	0	2	12	67	13	6
CDGS type I Tf						
Patient 1	11	34	4	41	8	2
Patient 2	4	22	0	57	14	5

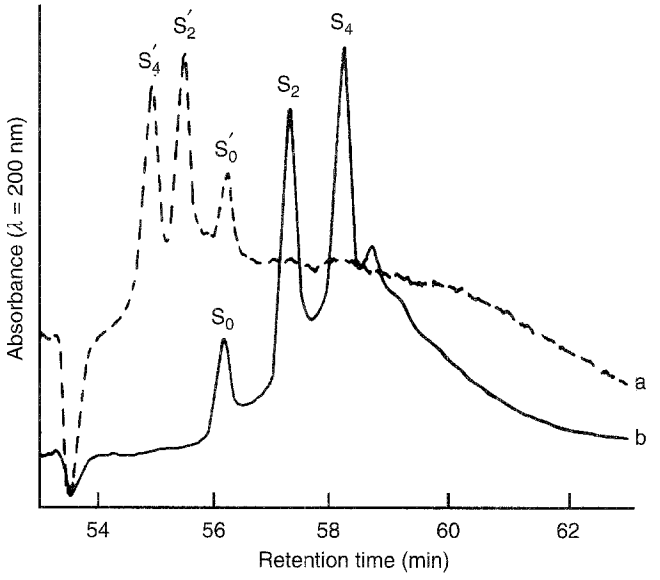


Fig. 10. An overlay of native CDGS type I hTf (b) and the same sample after de-sialylation with *A. ureafaciens* neuraminidase (a). S_4 and S_2, contain two and one sialylated complex biantennary N-linked glycans respectively. S_4' and S_2' are the desialylated forms of S_4 and S_2. The elution position of S_0 (the non-glycosylated isoform of hTf) is unchanged following desialylation (S_0'). The profiles have been aligned using the negative injection spike.

in contrast to the mono and di-glycosylated species, non-glycosylated hTf did not alter its elution position. This procedure may also provide a rapid analysis of relative proportions of different sialylated and neutral glycoforms of this glycoprotein. We have also shown that CZE can resolve neutral glycoforms; this suggests that this technology may provide a useful direct means of identifying other types of CDGS where the alterations in glycosylation are a result of incomplete processing rather than under-glycosylation.

Figure 9. (a–d) The analysis of hTf from two patients with type I CDGS (panels b,c) compared with normal hTf from a single individual (panel a). A co-injection of the CDGS sample in (panel b) with normal hTf from (panel a) is shown in Fig. 5d. The profiles have been aligned using the negative injection spike.

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