

University of Wollongong - Research Online

Thesis Collection

Title: Clinical analysis of plasma proteins by electrospray ionization mass spectrometry

Author: Margey Tadesse

Year: 2005

Repository DOI:

Copyright Warning

You may print or download ONE copy of this document for the purpose of your own research or study. The University does not authorise you to copy, communicate or otherwise make available electronically to any other person any copyright material contained on this site.

You are reminded of the following: This work is copyright. Apart from any use permitted under the Copyright Act 1968, no part of this work may be reproduced by any process, nor may any other exclusive right be exercised, without the permission of the author. Copyright owners are entitled to take legal action against persons who infringe their copyright. A reproduction of material that is protected by copyright may be a copyright infringement. A court may impose penalties and award damages in relation to offences and infringements relating to copyright material.

Higher penalties may apply, and higher damages may be awarded, for offences and infringements involving the conversion of material into digital or electronic form.

Unless otherwise indicated, the views expressed in this thesis are those of the author and do not necessarily represent the views of the University of Wollongong.

Research Online is the open access repository for the University of Wollongong. For further information contact the UOW Library: research-pubs@uow.edu.au

University of Wollongong Theses Collection

University of Wollongong Theses Collection

University of Wollongong

Year 2005

Clinical analysis of plasma proteins by
electrospray ionization mass
spectrometry

Margey Tadesse
University of Wollongong

Tadesse, Margey, Clinical analysis of plasma proteins by electrospray ionization mass spectrometry, MSc thesis, Department of Chemistry, University of Wollongong, 2005.
<http://ro.uow.edu.au/theses/135>

This paper is posted at Research Online.
<http://ro.uow.edu.au/theses/135>

NOTE

This online version of the thesis may have different page formatting and pagination from the paper copy held in the University of Wollongong Library.

UNIVERSITY OF WOLLONGONG

COPYRIGHT WARNING

You may print or download ONE copy of this document for the purpose of your own research or study. The University does not authorise you to copy, communicate or otherwise make available electronically to any other person any copyright material contained on this site. You are reminded of the following:

Copyright owners are entitled to take legal action against persons who infringe their copyright. A reproduction of material that is protected by copyright may be a copyright infringement. A court may impose penalties and award damages in relation to offences and infringements relating to copyright material. Higher penalties may apply, and higher damages may be awarded, for offences and infringements involving the conversion of material into digital or electronic form.

Clinical analysis of plasma proteins by electrospray ionization mass spectrometry

A thesis submitted in (partial) fulfilment of the
requirements for the award of the degree of

MASTER OF SCIENCE - RESEARCH

from



University of Wollongong

by

Margey Tadesse
Master of Science-Coursework

Department of Chemistry
2005

CERTIFICATION

I, Margey Tadesse, declare that this thesis, submitted in partial fulfilment of the requirements for the award of Master of Science-Research, in the Department of Chemistry, University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. The document has not been submitted for qualification at any other academic institution.

Margey Tadesse

16th August 2005

ABSTRACT

Electrospray ionization mass spectrometry (ESI-MS) was assessed as a tool for the analysis of different glycosylated and iron forms of transferrin (tf). Carbohydrate deficient transferrin (CDT) is clinically relevant in the diagnosis of congenital disorders of glycosylation (CDG) and in monitoring compliance in rehabilitation programs for alcoholism. The iron load of transferrin is an indicator of iron deficiency (or sufficiency). The concentration ranges (5 – 15 μ M) over which the ESI-MS response for tf was λ ive α p were determined in 0.1 M NH_4HCO_3 , pH 8.2, and in 1% formic acid. In order to develop a method for analysis by ESI-MS, optimal conditions were determined for obtaining CDT from commercial apo-tf by treatment with neuraminidase or PNGase. The resulting CDT forms were separated by HPLC and analysed by ESI-MS. The abundant human serum albumin was removed from plasma by loading 25-75 μ l of plasma onto a Micro Bio-Spin column containing Cibacron Blue-agarose. A high quality ESI mass spectrum of tf was obtained from the eluent. Using ESI-MS it was possible to distinguish between commercial holo-tf (Fe_2 -tf) and apo-tf (Fe_0 -tf). The rapid analysis of transferrin from plasma by ESI-MS lays the foundation for development of a clinical method for analysing CDT and possibly for determining the iron load of circulating tf.

ACKNOWLEDGEMENTS

I would like to thank the following people for helping to make this work possible:

- *To my supervisor Dr. Jenny Beck, without whose guidance, motivation and inspiration this work would not have been accomplished. Your enthusiasm and support are truly appreciated.*
- *To the mass spectrometry group Jihan, Michael, Karina, Linda, Jane and Aravind, thanks for your friendship and encouragement. You have made my time in the lab truly enjoyable. To Steve, Thitima, Dave and Larry, thank you for your tireless help and support.*
- *To Associate Professors Will Price and Paul Keller, for graciously helping out with practical aspects surrounding my degree over the last two years.*
- *To Professor John de Jersey, for writing comments on the thesis.*
- *To my roommates, past and present, for their friendship and for making my time in Australia special.*
- *To my family, who are my lifeline. This is for you.*

TABLE OF CONTENTS

CERTIFICATION	i
ABSTRACT	ii
ACKNOWLEDGMENTS	iii
TABLE OF CONTENTS	iv
LIST OF FIGURES	vii
LIST OF TABLES	x
ABBREVIATIONS	xi
CHAPTER 1: INTRODUCTION	1
1.1 Plasma proteins	1
1.1.1 Transferrin	2
1.1.2 The iron forms of tf	4
1.1.3 Carbohydrate deficient transferrin (CDT) and alcoholism	7
1.1.4 Congenital disorders of glycosylation (CDG)	13
1.1.5 Other plasma proteins as markers of disease	14
1.1.6 Clinical tests for CDT and tf	18
1.2 Electrospray ionization mass spectrometry	23
1.3 Aims	28
CHAPTER 2: EXPERIMENTAL SECTION	29
2.1 Materials	29
2.2 Plasma collections	29
2.3 Cerebrospinal fluid	30
2.4 Determination of protein concentrations	30

2.5 ESI-MS response of commercial apo-transferrin in different solvents	30
2.6 Treatment of transferrin with neuraminidase or PNGase	32
2.6.1 Neuraminidase	32
2.6.2 PNGase F	33
2.7 HPLC of carbohydrate- deficient transferrin (CDT)	33
2.8 Relative ESI-MS response of separated CDT	34
2.9 Fractionation of plasma using Affi-Gel[®] Blue affinity gel	35
2.9.1 Using a column	35
2.9.2 Using a Micro Bio-Spin [®] column	36
2.10 Determination of the concentration of transferrin in plasma	36
2.11 Separation of transferrin from cerebrospinal fluid	37
2.11.1 Chromatography on Sephadex-G75	37
2.11.2 Using an Affi-Gel [®] blue affinity gel Micro Bio-Spin [®] column	37
2.12 Removal of abundant plasma proteins	37
2.13 Addition of Fe³⁺ and Dy³⁺ to apo-transferrin	38
2.13.1 Iron	38
2.13.2 Dysprosium	39
2.14 Effect of pH on the iron content of holo-tf	39
2.15 ESI-MS conditions	40
CHAPTER 3: CHARACTERIZATION OF THE DEGLYCOSYLATED FORMS	
OF TRANSFERRIN	42
3.1 Introduction to ESI-MS	42
3.2 ESI-MS response of commercial apo-transferrin	43
3.2.1 The ESI-MS response of transferrin in plasma	51
3.3 Deglycosylated forms of transferrin	54

3.3.1 Neuraminidase	54
3.3.2 HPLC of deglycosylated species of tf	59
3.3.3 ESI-MS of transferrin treated with neuraminidase	62
3.3.4 Analysis of PNGase-treated apo-transferrin	64
3.4 Separation of transferrin from plasma using an Affi-Gel Blue affinity gel Column	65
3.5 Separation of transferrin from cerebrospinal fluid (CSF)	74
CHAPTER 4: TRANSFERIN WITH VARYING IRON LOAD	77
4.1 Preparing holo-tf from commercial apo-tf	77
4.2 Investigating the role of the bicarbonate ion in iron binding by tf	83
4.3 Transferrin and dysprosium	85
CHAPTER 5: DETECTION OF LOW ABUNDANCE PLASMA PROTEINS	88
CHAPTER 6: CONCLUSIONS	91
REFERENCES	95

LIST OF FIGURES

1.1	Transferrin molecule with its two globular N- and C-terminal domains	3
1.2	3D structure of human serum transferrin generated in RasMol	4
1.3	Crystal structure of the iron binding site in conformation A of htf/2N	6
1.4	Structure of a tf isoform with 5-carbohydrate antennas	8
1.5	Schematic representation of a Q-TOF <i>Ultima</i> mass spectrometer	24
3.1	Representation of Dole's charged residue model (CRM)	42
3.2	ESI mass spectra of apo-tf (10 μ M) in different solutions	44
3.3	Response curves of apo-tf in different solutions	46
3.4	The linear concentration region of apo-tf in ESI mass spectra sprayed from different solutions	48
3.5	ESI-MS response curves of apo-tf in different solutions	49
3.6	The linear response region of apo-tf in ESI mass spectra sprayed from different solutions	50
3.7	Spectra of HSA obtained from plasma and commercial HAS	52
3.8	Effect of HSA on the ion count from apo-tf in ESI mass spectra	53
3.9	ESI mass spectra of apo-tf after treatment with neuraminidase for various reaction times	55
3.10	ESI-MS spectra (converted to a mass scale) of commercial holo-tf (sprayed from 0.1 M NH_4HCO_3 , pH 8.2) and apo-tf treated with neuraminidase (fig. 3.9 (b)) (sprayed from 1% formic acid)	57
3.11	ESI mass spectra, converted to a mass scale, of commercial holo- and apo-tf and tf from plasma, treated with neuraminidase	58
3.12	The HPLC elution profile of apo-tf deglycosylated with neuraminidase	61

3.13 ESI mass spectra showing the relative intensities of a mixture of deglycosylated apo-tf species	63
3.14 Response curves obtained for a mixture of deglycosylated apo-tf species	63
3.15 ESI mass spectrum (converted to a mass scale) of apo-tf that had lost one and two sugar chains	65
3.16 Structure of Cibacron Blue	66
3.17 ESI mass spectrum of tf obtained from plasma	67
3.18 ESI mass spectrum of plasma converted to a mass scale	68
3.19 ESI mass spectra of plasma HSA eluted from the Affi-Gel Blue affinity gel column	69
3.20 ESI mass spectrum of eluent after loading plasma (75 μ l) onto a Bio-Spin Affi-Gel Blue affinity gel column	70
3.21 ESI mass spectra of different volumes of plasma loaded on an Affi-Gel Blue affinity gel Bio-Spin column	71
3.22 Standard curve for ESI-MS response of apo-tf	73
3.23 ESI mass spectra acquired of CSF that had been loaded on a Sephadex G-75 column	75
3.24 ESI mass spectrum concentrated CSF loaded on an Affi-gel Blue affinity gel Micro Bio-Spin column	76
4.1 ESI mass spectrum of commercial holo-tf in 0.1 M NH_4HCO_3 , pH 8.2 (a), and in 1% formic acid (b)	79
4.2 ESI mass spectra of commercial apo-tf and holo-tf prepared in the laboratory	80
4.3 ESI mass spectra of commercial apo- (a) and holo-tf (b) and plasma tf run in 1% formic acid	81

4.4 ESI-MS of holo-tf prepared from apo-tf in the laboratory, transformed to a mass scale	84
4.5 ESI mass spectrum of apo-tf that has bound dysprosium ions	87
5.1 ESI mass spectra of plasma obtained from the Agilent column	89

LIST OF TABLES

1.1 Factors that influence CDT values	12
1.2 Test-specific cutoffs for plasma CDT concentrations indicating chronic alcohol abuse	22
1.3 Mass spectrometric analysis of commercial iron forms of tf	26
1.4 Mass spectrometric analysis of CDT from the plasma of alcoholics	27
2.1 Preparation of tf-solutions in the presence of HSA	31
2.2 Concentration of CDT forms	34
2.3 Experimental conditions for titration of of apo-tf – 3 SA and apo-tf – 2 SA	35
2.4 Instrumental parameters on the Q-TOF-2 TM	40
2.5 Instrumental parameters on the Q-TOF <i>Ultima</i>	41
3.1 Masses observed for the species in each spectrum shown in figure 3.11	59
3.2 Masses of species in each HPLC peak observed in figure 3.12	61
3.3 Protein concentration in plasma and CSF	75
4.1 Calculated increases in mass of apo-tf with the binding of Fe ³⁺ and HCO ₃ ⁻	81
4.2 Calculated increases in mass of apo-tf with the binding of Dy ³⁺ and HCO ₃ ⁻	86

ABBREVIATIONS

A ₂₈₀	Absorption at 280 nm
A ₄₇₀	Absorption at 470 nm
Amu	Atomic mass unit
Apo-tf	Iron-free transferrin
CDG	Congenital disorders of glycosylation
CDT	Carbohydrate deficient transferrin
CID	Collision induced dissociation
CRM	Charged residue model
CSF	Cerebrospinal fluid
CZE	Capillary zone electrophoresis
Da	Daltons
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme-linked Immunosorbent Assay
ESI-MS	Electrospray ionization mass spectrometry
ExPasy	Expert protein analysis system
GDP	Guanosine diphosphate
HFBA	Heptafluorobutyric acid
Holo-tf	Iron-laden transferrin
HSA	Human serum albumin
Htf	Human serum transferrin
IEF	Isoelectric focusing
IgA	Immunoglobulin A

IgG	Immunoglobulin G
K_A/K_E	Partitioning coefficient ratio for analyte and electrolyte ions
MALDI	Matrix assisted laser desorption ionization
Mcp	multi-channel plate
PAI-1	Plasminogen activator inhibitor-1
PNGase F	N-Glycosidase F
PMI	Phosphomannose isomerase
PMM	Phosphomannomutase
PSA	Prostate specific antigen
Q-TOF	Quadrupole time-of-flight
RIA	Radioimmunoassay
rtPA	Plasminogen activator
SA	Sialic acid
SIMS	Secondary ion mass spectrometry
sTfr	Serum transferrin receptor
TFAA	Trifluoro acetic acid
TIA	Trisialo-tf
tf	Transferrin
TSAT	Transferrin saturation

CHAPTER 1: INTRODUCTION

1.1 Plasma proteins

The term plasma refers to the soluble phase of blood in which cells are suspended. In contrast to serum, which does not contain clotting proteins such as fibrinogen and prothrombin, plasma comprises the whole, soluble proteome of the blood. In addition to classical plasma proteins such as albumin, transferrin and immunoglobulins, which have an extended residence time in plasma, plasma also contains hormones such as insulin, cytokines such as interleukin 6 and non-hormone proteins such as lysosomal proteins. Proteins that normally function within cells are also leaked into plasma as a result of cell damage or death. Examples of these are cardiac troponins and myoglobin, which are markers of myocardial infarction.¹ Tumours and other diseased tissue also release proteins into plasma, including cancer markers such as cancer antigen 125.² Additionally, foreign proteins of infectious organisms or parasites are also present in plasma.

Plasma is thus in principle, the largest collection of the human proteome or “protein index” and is also the most sampled proteome, used clinically for preparation of protein therapeutic products and medical diagnosis. The diagnostic possibilities of plasma are enormous and appear to be limited only by analytical difficulties. A major goal of proteomics is the quantitative description of protein expression in disease or drug treatment. Among the clinical tests for plasma proteins employed today such as immunoassays and electrophoresis, mass spectrometry is a promising method for solving the quantitative problems associated with protein analysis.¹

Some of the plasma proteins are also present in other body fluids. For example, cerebrospinal fluid (CSF), which is the fluid that fills the ventricles of the brain and forms a thin layer around the outside of the brain and spinal cord, contains low levels of plasma proteins. The total protein concentration in CSF is much lower than in plasma. Human serum albumin (HSA), which corresponds to (approximately) 50 % of the total protein content in both fluids, has a concentration of 40 000 mg/l in plasma and a concentration of 155 mg/l in CSF.³ Relative enrichment in CSF for certain plasma proteins, such as transferrin (tf), has been observed (the ratio of HSA to transferrin is approximately 11:1 in CSF and 15:1 in plasma). Changes in the protein expression of CSF may be an indication of various diseases, most notably of neurodegenerative disorders due to the close proximity of CSF to the extracellular fluid of the brain.⁴ However, the low abundance of proteins in CSF poses a serious challenge in the isolation and analysis of proteins from this medium.

1.1.1 Transferrin

The transferrins are a class of iron binding proteins with a high affinity for binding ferric ion and other transition metals.⁵ Human serum transferrin (htf), a glycoprotein of 79 570 Da found in blood⁶, transports Fe (III) to cells where the iron is taken up via a ubiquitous transferrin receptor.⁵ The concentration of human serum transferrin is 2.5 mg/ml (35 μ M) and its diferric form has a pI of 5.6. The protein has 19 disulfide bridges. Lactoferrin, a protein of 82 400 Da, is another major member of the transferrin family of proteins, found mainly in milk, tears and saliva. Ovotransferrin, another member of the family, is found in egg white and has a molecular mass of 77 770 Da.

Human serum transferrin has 679 amino acid residues⁶ and is synthesised mainly in hepatocytes. It contains three substructural domains: a single polypeptide chain, two homologous (approximately 40%) halves (Figure 1.1) consisting of the N-terminal (residues 1-336) and C-terminal (residues 337-679) domains, each with an iron-binding site, and two complex N-linked glycan chains attached to amino acids 413 (Asn) and 611 (Asn).⁷ These glycans are suggested to play a role in maintaining the protein in a biologically active conformation and their external position in human serum transferrin might provide recognition signals.⁶

Figure 1.1. Transferrin molecule with its two globular N- and C-terminal domains. Taken from Mihas and Tavassoli.⁸

The N- and C-lobes, each consist of about 40 kDa and are joined together by a short peptide chain in a random coil (Figure 1.2). Each lobe is further divided into two equal domains consisting of a mixed β -sheet overlaid with α -helices. The cleft separating the

domains houses the metal binding site. The folding pattern orients the N-termini of the helices towards the binding cleft and their partial positive charges are thought to attract anions into the binding cleft. The domains are linked by two extended β -strands that run behind the iron site and function as backbone strands.⁶

Figure 1.2. 3D structure of human serum transferrin generated in RasMol. Based on the X-ray diffraction work of Yang *et al.*, 2000.⁹

1.1.2 The iron forms of tf

Each lobe of tf binds Fe(III) in an octahedral coordination to two Tyr, one His, one Asp and one bidentate synergistic carbonate anion. The metal-ligand bond lengths in the two binding sites are in the range 1.9-2.2 Å.⁶ In the N-lobe of transferrin, Asp63 from domain one, Tyr 188 from domain 2, and Tyr95 and His249 from the two strands that link the two domains, are the four ligands that coordinate to the metal and contribute to the reddish colour of the iron form of the protein (Figure 1.3).¹⁰ The iron is coordinated

to two oxygen atoms from the two Tyr residues, one nitrogen from the imidazole ring of His, one oxygen from Asp and two oxygens from the carbonate ion.⁶

The Asp ligand appears to be important for the metal binding site since it coordinates to the metal through one carboxylate oxygen while its other oxygen forms hydrogen bonds with the two domains of the N-lobe. Iron is released from the site when the two domains that are hinged by the backbone strands move apart to form more open conformations. Diferric transferrin or holotransferrin ($\text{Fe}_2\text{-tf}$), where both iron sites are occupied, binds strongly to the receptor on the surface of the cell. The receptor-transferrin complex is internalized and is held in membrane bound vesicles where the pH is lowered from the extracellular value of 7.4 to 5.5. The lower pH leads to release of iron. The iron free apo-transferrin remains bound to the receptor because of its high affinity to the receptor at acidic pH and is recycled back to the surface of the cell. At extracellular pH, apo-transferrin dissociates from its receptor and is released into the circulation and recycled. The half-life of transferrin in the circulation is 7.6 days and the half-life of transferrin bound Fe is 1.7 hours.⁶

The carbonate anion is found in a pocket in domain 2 and Fe^{3+} cannot bind strongly to transferrin in the absence of this synergistic anion.⁶ The pocket is formed by positively charged groups on the side chain of Arg124 and the N-terminus of helix 5, which involves residues 124-136. The *trans* nitrogen and NH_2 atoms of Arg124 are hydrogen bonded to the carbonate O_2 atom. The carbonate oxygen atom also forms hydrogen bonds with the Thr120 and the main chain amide nitrogen atoms of Ala126 and Gly127 at the N-terminus of helix 5 (Figure 1.3).¹⁰ Without the carbonate anion, the positively charged Arg side chain and the N-terminus of helix 5 might inhibit metal

binding at the site. The carbonate anion might also play a part in iron release since protonation of the carbonate could disrupt the hydrogen bonding pattern, breaking up the binding site and ensuring the reversibility of metal binding. Oxalate is another synergistic anion that can bind to transferrins, but carbonate is the synergistic anion *in vivo* and has higher affinity for promoting metal binding than most other anions. The common features of synergistic anions are a carboxylate donor and a second electron donor group that can act as a ligand for metal binding.⁶

Figure 1.3. Crystal structure of the iron binding site in conformation A of htf/2N. The red sphere indicates the iron atom and the white triangular structure indicates the carbonate anion. Ligands to the iron from Asp63, Tyr95, His249, Tyr188 and the carbonate are indicated by thin white cylinders. The dashed white lines indicate hydrogen bonds. The side chain for Arg124 is indicated and Thr120 is also shown. The main chain amide nitrogen atoms of Ala126 and Gly127 are indicated by N. Taken from MacGillivray *et al.*, 1998.¹⁰

In serum, transferrin is only about 30% saturated with iron in a healthy adult.⁶ It also binds to other metals that enter the body. About 30 metal ions have been reported to bind to transferrin with either carbonate, oxalate, or other carboxylates as synergistic anions. However, transferrin has a higher affinity for Fe^{3+} than any other ion for which the binding constant has been determined.⁶ Other ions that bind to transferrin include Bi^{3+} ,¹¹ Ga^{3+} ,¹² In^{3+} ,¹³ Al^{3+} ,¹⁴ Tl^{3+} ,¹⁵ transition-metal ions such as Mn^{2+} ,¹⁶ Cu^{2+} ,¹⁷ Ni^{2+} ,¹⁸ Zn^{2+} ,¹⁹ Ru^{3+} ,²⁰ and lanthanide ions such as La^{3+} , Ce^{3+} , Nd^{3+} , Sm^{3+} and Gd^{3+} .²¹⁻²³ The binding of these metals plays an important role in the transport and delivery of medical diagnostic radioisotopes such as Ga^{3+} and In^{3+} .²⁴

1.1.3 Carbohydrate deficient transferrin (CDT) and alcoholism

Transferrins can contain anywhere from zero to eight sialic acid moieties since the sugar chains can have a maximum of four antennae. Each antenna contains a sialic acid residue on the end. The occurrence of different isoforms of a bioactive molecule is termed microheterogeneity. Sialic acid (SA) contributes to transferrin's negative charge. A transferrin isoform with a 5-antennary carbohydrate chain and other isoforms are shown in figure 1.4. Transferrin isoforms can be separated by electrophoresis according to their different isoelectric points ($\text{pI} = 5.2\text{-}5.7$) that are a result of different numbers of sialic acid moieties. The most frequent isoform, tetrasialotransferrin, is also referred to as "normal transferrin" and comprises more than 90% of the isoforms observed in healthy subjects.⁷ The asialo- and monosialo-transferrin and octasialo-transferrin are not detectable ($<0.5\%$ asialo-tf and $<0.9\%$ monosialo-tf of total tf) under nonpathological conditions.²⁵

Figure 1.4. Structure of a tf isoform with 5-carbohydrate antennas. A, B, C, D and E represent pentasialo-, tetrasialo-, trisialo-, disialo- and asialo-tf, respectively. The dots at the end of the antennas represent sialic acid residues. Taken from Golka and Wiese.⁷

In 1976, Stibler and Kjellen first detected transferrin isoforms with pIs >5.7 in the cerebrospinal fluid and serum of alcoholics.²⁶ Increased amounts of these isoforms appeared in serum from alcoholics and disappeared after abstinence, with a half-life of about 14 days.^{7, 27} Thus, chronic alcohol consumption was correlated with a loss of carbohydrate groups of transferrin and it was shown that often entire N-glycan chains consisting of mannose, N-acetylglycosamine, galactose and sialic acid moieties were

missing.²⁸ However, transferrin isoforms can lack not only complete N-glycan chains but also parts of the N-glycan chains.²⁹ Asialo-, monosialo- and disialo-diferric transferrin came later to be referred to as carbohydrate deficient transferrin (CDT).²⁷ Trisialotransferrin which consists of two biantennary N-glycans, one with two sialic acid moieties and the other with one sialic acid moiety, is not correlated with chronic alcohol abuse.²⁸ Disialotransferrin, which comprises 2.5 % of total serum transferrin, is the most sensitive isoform with regards to chronic alcohol consumption and has the same sensitivity as total asialo-, monosialo- and disialotransferrin.³⁰

About 38 genetic variants of transferrin have been identified. The variants result from substitutions of amino acids in the polypeptide chain but only four of these are present at a prevalence of more than 1%. Tf-C, which is the most common type in Caucasians, has 16 subtypes but tf-C1 is prevalent in 95% of this population. The most frequent variants are the tf-B and tf-D variants.²⁵ Only the rare transferrin variant tf-D3 interferes with electrophoretic determination of CDT. However, the tf-D subtype may produce false positive results in other analytical methods.³¹ Coelution of the CDT isoforms of the tf-B variant with non-CDT isoforms of the tf-C variant can occur and cause false-negatives for persons who are heterozygous tf-CB and alcoholics.

Different iron load and sialic acid content as well as modifications in the polypeptide chain can affect the pI of the tf molecule. Alterations in the pI that can occur when one or two iron ions are bound or lost, can be compensated for by different genetic variants or sialic acid content. Transferrin molecules with different amounts of iron and sialic acid content can thus have equal pI values such as disialo-Fe₂(diferric)-tf, which is the main CDT isoform, and tetrasialo-Fe₁(monoferric)-tf, which is the main non-CDT

isoform. In human serum, isoelectric focusing (IEF) can detect 36 isoforms for the homozygous tf type and 72 isoforms for the heterozygous tf type. Three isoforms of the homozygous tf type and 6 isoforms of the heterozygous type are collectively referred to as CDT. Another 9 non-CDT isoforms for the homozygous type and 18 non-CDT isoforms for the heterozygous type coelute with the CDT isoforms and can lead to over estimation of CDT content.²⁵

The exact mechanism by which chronic alcohol abuse increases CDT production in the liver is not completely understood but it has been postulated that five different mechanisms might be involved:⁷

- Reduced activities of glycosyltransferases, comprising at least five enzymes (including sialyltransferase), which affects N-glycan chain synthesis in the Golgi apparatus.^{32, 33}
- Increased activity of sialidase responsible for cleaving sialic acid moieties.^{32, 33}
- Downregulated expression of the sialyltransferase genes.³⁴
- Reduced receptor-based uptake of CDT by hepatocytes.³³
- Alterations in asialoglycosylin receptors.³⁵

In a controlled drinking experiment conducted by Stibler in 1991, it was discovered that CDT values became elevated at chronic alcohol consumption of 50-80 g ethanol or more per day over a period of at least 14 days. This was the case in approximately 80% of the people examined.²⁷ In contrast, no increase in CDT values was found for the majority of men drinking alcohol only occasionally and showing normal values of γ -glutamyltransferase, another marker of alcohol abuse.³⁶ Carbohydrate deficient transferrin is correlated with regular drinking patterns and is a valuable parameter in

substantiating chronic ethanol consumption. The highest diagnostic significance of CDT is in subgroups of males with long-term alcohol intake. Determination of CDT is not suitable for screening for subjects with elevated alcohol intake in the general population but is more suitable to confirm chronic alcohol consumption.⁷

Table 1 summarizes factors that influence CDT determinations that need to be taken into account when interpreting CDT values. Total body water was found to have an effect on the alcohol consumption and CDT dose-response relationship.³⁷ Serum concentrations of CDT in healthy women are typically higher than those in healthy males.³⁸ However, increases in CDT are less significant in female alcoholics compared to males.³⁹ One reason for this is that differing drinking patterns might have a different impact on the desialylation of transferrin.⁴⁰ Premenopausal women often have elevated CDT values while postmenopausal women have lower values except for postmenopausal women on estrogen replacement therapy who show increased values.⁴¹ Pregnancy elevates CDT values⁴² while the use of contraceptives has been associated with both increases and decreases in CDT values.^{43, 42} Elevated liver iron levels cause lowering of CDT values⁴⁴ while iron deficiency in both anemic and non-anemic patients elevates CDT values.⁴⁵ Low serum transferrin levels decrease CDT values.⁴⁶ As previously mentioned, the transferrin variant tf-D3 interferes with CDT determinations by increasing CDT values. Storage or shipment of uncentrifuged whole-blood samples has also been shown to elevate CDT values.⁴⁷ Bacterial or viral contamination has also been suggested to cause false positive CDT values due to cleavage of the transferrin molecule by neuraminidase.⁵ A number of diseases have also been suggested to elevate CDT values including hepatitis⁴⁸, other liver diseases⁴⁹, congenital disorders of glycosylation⁵⁰, galactosemia⁵¹, cystic fibrosis⁵² and psychiatric disorders.⁵⁴

Table 1.1. Factors that influence CDT values.

^aAbstainers.

^bChronic alcohol abusers.

1.1.4 Congenital disorders of glycosylation (CDG)

Congenital disorders of glycosylation (CDG) are the other main class of disease, apart from alcoholism, in which levels of CDT are significantly increased. Although several plasma glycoproteins, including peptide hormones, are also aglycosylated or underglycosylated in CDG, tf is the preferred biomarker for clinical diagnosis due to its abundance in plasma. Generally, protein glycosylation affects a wide range of protein functions such as protein folding, secretion, targeting, stability in the circulation and other intercellular communication processes.⁵⁵ The function of the oligosaccharides of transferrin is not known, although deglycosylation of transferrin has been postulated to influence the receptor binding and iron donating capabilities.⁵⁶

The consequence of the non-glycosylation of tf in CDG is obscure. However, CDG has been linked to autosomal recessive disorders in children with neurodevelopmental delay. Unusual distribution of subcutaneous fat and cerebellar hypoplasia also occur in these children.⁵⁷ Psychological alterations have also been observed in patients with CDG.⁵⁸

Carbohydrate deficient transferrin forms in CDG are believed to result from the defective N-glycosylation of tf. CDG are classified as type I or II on the basis of the position of the defect in the glycosylation pathway. Type I consists of defects in the assembly and transfer to the polypeptide chain of the dolicholpyrophosphate-linked oligosaccharide. There is a decreased supply of the common oligosaccharide precursor in CDG-I. Type II consists of defects in the processing of the protein-bound N-linked glycans.

Five different enzymic defects, CDG-Ia-e, with differences in the range and severity of clinical symptoms, have been identified in CDG-I patients. In addition to being underglycosylated, the sugar chains of these proteins are processed differently from normal tf and also show different patterns of glycosylation including increased fucosylation and branching.²⁵ Congenital disorders of glycosylation II exhibits only one prominent isoform.⁵⁹

An inherited deficiency in the enzymes phosphomannomutase (PMM) and phosphomannose isomerase (PMI), is the main cause of CDG. Phosphomannomutase is required to synthesize mannose-1-phosphate, which is a precursor to GDP-mannose, the form of mannose used in glycosylation of the dolichol oligosaccharide required for N-glycosylation of proteins. The lack of mature oligosaccharide depresses the transfer of oligosaccharides to asparagine, leading to underglycosylation of tf.⁵⁷

The isoforms of CDT observed in CDG are the same as those observed in alcoholism, namely isoforms that show the absence of entire glycan chains. Besides plasma samples, CSF samples have also been analysed from patients with CDG and the same species of CDT are observed in both fluids.²⁵

1.1.5 Other plasma proteins as markers of disease

This section discusses some of the plasma proteins that have been associated with several disease states. Enzymatic assays and electrophoretic methods are common methods for the analysis of these plasma proteins.¹

Plasminogen and related proteins

Plasminogen is a precursor of the proteolytic enzyme plasmin that dissolves the fibrin in blood clots and is also called fibrinolysin. It is a single chain glycoprotein of 80-90 kDa and is mostly found in association with fibrinogen in plasma. It is changed into fibrinolysin by plasminogen activators.⁶⁰ The concentration of plasminogen in plasma is 700 mg/l and 0.25 mg/l in CSF.³ It is however, elevated plasma levels of plasminogen activator inhibitor-1 (PAI-1) which have been associated with various diseases including atherothrombotic complications, inflammation, obesity, insulin resistance and vascular risk.⁶¹

Capillary zone electrophoresis (CZE) has been generally employed for the routine analysis of intact glycoproteins and their glycoforms. The method has been applied to analyze recombinant human tissue plasminogen activator, rtPA. Although not as common as CE-HPLC methods, on-line coupling of CE with electrospray ionization mass spectrometry (ESI-MS), as well as off-line combinations of CE and MALDI-TOF-MS methods have been reported for the analysis of intact glycoproteins. However, this was reported for ovalbumin, ribonuclease B (RNase B) and a lectin from *Erythrina corallodendron*.⁶²

Fibrinogen

Fibrinogen is a plasma glycoprotein which is converted by thrombin to fibrin to form a blood clot. Fibrinogen is composed of a dimer of three polypeptide chains held together by disulfide bonds.⁶³ It has a molecular weight of 340 000 and a carbohydrate content of 3.7 %. The concentration of fibrinogen in plasma is 2600 mg/l and 0.6 mg/l in CSF.³ Elevated plasma levels of fibrinogen are associated with increased frequency of

coronary heart disease and stroke. It has even been reported that the association between plasma fibrinogen levels and cardiovascular disease is as predictive as elevated cholesterol levels.⁶⁴ Abnormal variants of fibrinogen have also been associated with diabetes mellitus, certain types of cancer and autoimmune diseases.⁶⁵

In addition to activity assays, electrophoretic and chromatographic methods, time-of-flight secondary ion mass spectrometry (TOF-SIMS), has been used to determine the composition of plasma protein film layers by comparison of their spectra with the spectra of single protein films. The limit of detection for fibrinogen was determined by comparison with independent radiolabelled fibrinogen adsorption measurements.⁶⁶

Prothrombin

Prothrombin is a plasma protein which is the inactive precursor of thrombin. It is converted to thrombin by a prothrombin activator complex. Deficiency of prothrombin is termed hypoprothrombinemia and leads to excessive bleeding.⁶⁷ Elevated levels of prothrombin increase venous thrombotic risk.⁶⁸

Prostate-specific antigen

Prostate-specific antigen (PSA) is a serine protease which is manufactured in the prostate gland. It is found in semen where its function is to liquify semen. Elevated plasma levels of PSA are associated with benign prostate hyperplasia and prostate cancer.⁶⁹ Prostate-specific antigen was found to normally be 10^{-8} times less abundant in plasma than albumin, when measured by ELISA.¹

Thioredoxin

Thioredoxin is a ubiquitous thiol reductase of 12 kDa with a redox-active dithiol/disulfide in the conserved active site. It is involved in redox-regulation of intracellular signal transduction and is also a scavenger of reactive oxygen species that lead to oxidative disease.⁷⁰ Plasma levels of thioredoxin are good biomarkers of oxidative stress. Reduced thioredoxin activity in hyperglycemia was found to increase reactive oxygen species that contributes to vascular disease and pro-atherosclerotic effects of diabetes mellitus.⁷⁰

As well as functional assay methods, measurement of intact recombinant thioredoxin from *E. coli* by matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry (MALDI-TOF-MS) has been reported.⁷¹

Ceruloplasmin and clusterin

Ceruloplasmin is a single chain molecule with a molecular weight of 135 000 and a carbohydrate content of 7% in humans. Ceruloplasmin functions as a transport protein for copper³ and binds 90% of the copper present in plasma.⁷² In addition, it can act as a ferroxidase, oxidizing ferrous to ferric ions which then bind to apo-tf, and is thus indirectly involved in the transport of iron. The concentration of ceruloplasmin in plasma is 370 mg/l and 0.9 mg/l in CSF.³ Reduced plasma levels of ceruloplasmin are indicative of Wilson's disease which increases copper levels in the liver and brain. Increased plasma abundance of ceruloplasmin is indicative of acute infection and some types of chronic liver disease.⁷²

Clusterin is a ubiquitous 80 kDa heterodimeric sulfated glycoprotein. Elevated expression of clusterin has been linked to breast cancer, endometrial cancer, myocardial infarction, renal disease and atherosclerosis.⁷³ Elevated serum levels of clusterin have been related to preeclampsia, a toxemia that occurs late in pregnancies.⁷³

Along with assays and electrophoretic methods, quantitative determination of clusterin levels in serum were carried out by MALDI-TOF-MS followed by peptide mass fingerprinting.⁷³

1.1.6 Clinical tests for CDT and tf

Some clinical tests for the various glycosylated forms of transferrin and transferrin with varying amounts of iron exploit the differences in charge of these molecules (e.g. isoelectric focusing and anion exchange chromatography).

The first hurdle to be overcome in distinguishing between transferrin with different levels of iron is in the collection of plasma. EDTA or heparin are commonly added to vacutainer tubes to prevent clotting. Further, when analyzing CDT, Fe^{3+} is often added to saturate the transferrin and obtain a uniform transferrin Fe^{3+} load (elimination of Fe_0 - and Fe_1 -tf and formation of Fe_2 -tfs). This avoids coelution of non-CDT isoforms with similar pIs to disialo- Fe_2 -tf (the main CDT form) in isoelectric focusing of plasma. Both EDTA and heparin were found to decrease *in vitro* Fe^{3+} -transferrin saturation. These factors also affect anion-exchange microcolumn non-CDT and CDT isoform fractionation, which usually follows the *in vitro* transferrin iron saturation step.²⁵ CDT isoforms are then determined by immunological procedures.

Some of the forms of transferrin that have similar pIs and therefore are difficult to separate by isoelectric focusing were mentioned above in Section 1.1.3. Additionally, there are experimental factors that must be considered in order to obtain reproducible results. For example, sample storage for 3 days at room temperature was found to cause a 30% increase in CDT. Serum storage for 30 hours at room temperature, 7 days at 4 °C, several months at -22 °C, repeated freezing and thawing, diet and common drugs taken by patients were not found to have an effect on serum CDT concentrations. Several conditions that need to be studied with regards to effects on serum CDT concentrations include positioning of the patient during blood collection, duration of congestion, *in vivo* and *in vitro* drug effects, use of EDTA- and heparin-plasma and stability of whole-blood samples.

The normal ranges (reference values or cutoffs) for absolute and relative serum CDT are method-dependant and values must always be interpreted with regard to test-specific criteria (Table 1.2). Thus the CDT value, the cutoff value and the method of analysis should always be reported. CDT is often reported as U/L (Unit/L), where one unit refers to 1 mg of transferrin.²⁵

Electrophoretic methods

Due to its high selectivity, the reference method for CDT analysis is isoelectric focusing (IEF). The transferrin isoforms are separated in a gel containing a pH gradient according to their isoelectric points. After electrophoresis, the transferrin bands are visualized by immunofixation, followed by silver staining of the CDT-antitransferrin complexes and densitometric quantification.^{7, 25} Usually 1 µL of serum or plasma

diluted 400-fold is applied to the gels. Absolute CDT concentrations can be determined with a calibration curve generated by different amounts of asialo-Fe₂-transferrin.²⁵

Capillary zone electrophoresis (CZE) has also been used to determine CDT. Analysis is usually based on narrow-bore capillaries that separate based on electric charge and molecular mass using an electropherograph with a single wavelength UV detector or a UV diode array detector and uncoated fused-silica capillaries.⁷⁴ The main advantage of this method is the separation of different isoforms of CDT. Table 1.2 summarizes the test-specific cutoff values of CDT for electrophoretic methods.

Chromatographic methods

Chromatographic methods require sample volumes of 100-500 µL and are thus less sensitive than IEF. Anion-exchange chromatography followed by immunoassay, a procedure usually used in commercial CDT tests, cannot detect genetic variants of transferrin. However, HPLC can detect genetic variants in plasma and can be used to control odd values obtained by anion-exchange chromatography/immunoassay. However, IEF is still superior to HPLC for detecting and phenotyping transferrin genetic variants. The applicability of HPLC is further reduced for a large series of CDT analyses by time-consuming HPLC column regeneration.²⁵

Lectin affinity chromatography has also been used to determine CDT. Sepharose columns coated with *Allomyrina dichotoma* (Allo A) or *Trichosanthes japaonica* (TJA) were used and CDT-Allo A and CDT-TJA, corresponding to disialo- and asialo-transferrin, respectively, were determined.⁷⁵ Table 1.2 summarises chromatographic determinations of CDT that have been reported.

Commercial CDT tests

The first commercial CDT test (CDTect-RIA, Pharmacia & Upjohn) was introduced in 1993. Except for one HPLC method supplied by Recipe, current commercial CDT tests are based on anion-exchange microcolumns. The columns adsorb transferrins with three or more sialic acid moieties and transferrins with fewer sialic acid moieties are eluted. CDT is quantified by double-antibody radioimmunoassay (CDTect-RIA). Assays based on radiolabelling of transferrin and using antibody fragments followed by separation on an ion-exchange microcolumn, are also available. The eluted antibody-transferrin complex is then detected by turbidimetric measurement (%CDT relative to total transferrin, Axis). Total transferrin is determined separately and the method does not differentiate between men and women. Enzyme immunoassay methods are also available (CDT-EIA, Axis). These methods determine asialo-, monosialo-, and disialo-transferrin. A test incorporating 50% of the trisialo-transferrin fraction has also been developed (%CDT-TIA, Bio-Rad).⁷ Table 1.2 lists the common commercial CDT tests.

Mass spectrometry

Mass spectrometry is a rapid and sensitive method for the analysis of biological samples. In proteomics analysis, proteins are usually fractionated by gel electrophoresis and digested into smaller, tractable pieces (peptides), then separated by HPLC before mass spectrometric analysis. In this project, electrospray ionization mass spectrometry (ESI-MS) will be used to analyze intact plasma and CSF proteins after simple, fractionation procedures. The aim is development of simple, relatively low-cost assays based on rapid plasma fractionation interfaced with mass spectrometric analysis.

Table 1.2. Test-specific cutoffs for plasma CDT concentrations indicating chronic alcohol abuse.²⁵

^a One DU is defined as 1% of the ratio of CDT: tf exhibited by the strongly positive control run in each gel.

1.2 Electrospray ionization mass spectrometry

The application of electrospray ionization to the mass analysis of gas phase molecules was first reported by Dole and co-workers in the late 1960's. However, it was in 1984 that electrospray ionization was first successfully interfaced to a quadrupole mass analyser and used to ionise large, non-volatile biomolecules without fragmentation. The soft ionization technique has been greatly enhanced since 1984 for the determination of the molecular weight of proteins. A series of multiply charged ions, with different mass to charge ratios are formed for the same compound, leading to the analysis of large molecules with molecular masses in excess of 100 000 Da. The molecular mass of the analyte is calculated from the average mass-to-charge ratio giving accurate molecular mass values with an accuracy of 0.01%. The amount of sample required is only in the picomole range and the time of analysis is generally less than half an hour.

In electrospray ionization, the sample flows through a stainless steel capillary with a positive or negative ion potential, resulting in a spray of charged droplets. Two major mechanisms for electrospray have been proposed. In Dole's charged residue model (CRM), which is widely thought to apply to the mechanism of ESI for proteins, a charged droplet steadily decreases in size due to solvent evaporation. Coulombic repulsion causes the parent droplet to explode into many offspring droplets and the process continues until the offspring droplet only contains one molecule.⁷⁶

The ions are then transported to the mass analyser where they are separated according to their mass-to-charge ratios giving a sequence of peaks which differ by a single charge.

Figure 1.5 is a schematic representation of the Q-TOF *Ultima* mass spectrometer used for the experiments in this project.

Figure 1.5. Schematic representation of a Q-TOF *Ultima* mass spectrometer.⁷⁷

This is a time-of-flight (TOF) instrument, which separates ions on the basis of differences in transit time through the mass analyser. The time-of-flight of the ions is dependant on the mass and charge of the ion. The quadrupole is a transmission element that in, for instance MS/MS mode, would only transmit the parent ion of interest from the ionization source and through to the hexapole collision cell, where collision induced dissociation (CID) occurs when the ions collide with argon gas. In the TOF mass analyser, a pulsed electric field is applied that pushes the ions in an orthogonal direction to their original trajectory and TOF mass separation takes place. A single-stage ion mirror or reflectron compensates for the initial energy and spatial spread of the ions and focuses them on to the detector. The Q-TOF *Ultima* achieves simultaneous detection of ions across the full mass range, giving high mass accuracy.^{77, 78} Studies in which ESI-

MS has been used to distinguish between iron-bound and iron-free transferrin (Table 1.3) and between normal transferrin and CDT (Table 1.4) are summarized below.

Table 1.3. Mass spectrometric analysis of commercial iron forms of tf.

Table 1.4. Mass spectrometric analysis of CDT from the plasma of alcoholics.

1.3 Aims

Plasma proteins present at high concentrations mask proteins present at lower concentrations. Fractionation of plasma proteins by chromatographic methods overcomes this problem. For example, magnetic beads that carry a chemical group that binds to a particular protein, could be used to remove high abundant proteins such as albumin from plasma.⁸³ Transferrin, which is approximately 20-fold less abundant in plasma, could then be analyzed by mass spectrometry. The principles established when developing these simple fractionation methods using low-medium pressure chromatography, might potentially be used to develop on-line HPLC methods where several columns will be connected in series to the mass spectrometer.

The aims of this project are:

- 1) To fractionate transferrin from biological samples such as plasma and CSF to provide samples suitable for ESI-MS of the intact protein.
- 2) To prepare CDT forms by enzymatic digestion of commercial apo-tf and characterize these by ESI-MS.
- 3) Develop a method for quantification of tf and CDT in plasma by comparing ESI mass spectra of tf in protein mixtures with internal and external standards prepared using purified tf.
- 4) Characterize the iron forms of tf by ESI-MS.
- 5) Develop a method for the analysis of low abundance plasma proteins by ESI-MS.

CHAPTER 2: EXPERIMENTAL SECTION

2.1 Materials

MilliQTM water from Millipore was used in all experiments. Human apotransferrin, human holotransferrin, human serum albumin (HSA), neuraminidase agarose, PNGase F (N-Glycosidase F) and iron (III) nitrate nonahydrate were purchased from Sigma-Aldrich (St. Louis, Mo). Ammonium acetate, ammonium bicarbonate, acetic acid, formic acid and acetonitrile were from Ajax Finechem (Seven Hills, NSW). Ammonia was obtained from Asia Pacific Specialty Chemicals Ltd (Seven Hills, NSW). Affi-Gel[®] Blue affinity gel and Micro Bio-Spin[®] chromatography columns were from Bio-Rad Laboratories (Hercules, Ca). Sephadex G-75 was purchased from Pharmacia (Uppsala, Sweden). The Agilent High Capacity Multiple Affinity Removal Spin Cartridge was obtained from Agilent Technologies (Roseville, Ca).

2.2 Plasma collection

Approximately 5 ml of whole blood was collected into an EDTA vacutainer tube from a healthy volunteer by venepuncture. Plasma was separated from cells by centrifugation at 4000 rpm and 0 °C in a benchtop Universal centrifuge. The resulting plasma was frozen in liquid nitrogen in approximately 300 µl aliquots and stored at -80 °C.

2.3 Cerebrospinal fluid

CSF from nine patients at the Mater Children's Hospital (Brisbane) was supplied by Dr Frank Bowling.

2.4 Determination of protein concentrations

The concentrations of apo-tf and holo-tf solutions were determined by measuring the A_{280} of a solution in a 1 cm pathlength cuvette using either a Shimadzu 2401-PC or Shimadzu 1700 spectrophotometer. The molar absorption coefficients (ϵ)₂₈₀ for apo- and holo-tf were 83 200⁸⁴ and 111 900⁸⁵, respectively. The concentration of HSA in plasma was taken as 0.6 mM.⁸⁵

2.5 ESI-MS response of commercial apo-transferrin in different solvents

Apo-tf solutions with concentrations ranging from 0.1-60 μ M were made up by sequential dilution of the 60 μ M solution (determined by measurement of A_{280}) in the following four solutions: (i) 50 mM ammonium acetate, pH 7.4; (ii) 0.1 M NH_4HCO_3 , pH 8.2; (iii) 1% formic acid in 50% acetonitrile and (iv) 1% formic acid. ESI mass spectra were acquired (see below for instrumental conditions) once using a Micromass Q-TOF 2TM mass spectrometer and repeated three times on a Waters (formerly Micromass) Q-TOF *Ultima* mass spectrometer. The ionization sources were cleaned before the start of each experiment. The mass spectrometric response for apo-tf in 1%

formic acid in the presence of various concentrations of HSA (in plasma), was also investigated. Tf solutions with a final concentration of 10 μM , were prepared from a stock solution of 65.8 μM (determined by measurement of A_{280}) made up to a final volume of 400 μl with the solvent and plasma. Different aliquots of plasma were added to the solutions, giving the following concentrations of HSA in the presence of tf:

Table 2.1. Preparation of tf-solutions in the presence of HSA.

* Concentration of apo-tf in each mixture was 10 μM .

† Based on the concentration of HSA in plasma of 0.6 mM.⁸⁵

2.6 Treatment of transferrin with neuraminidase or PNGase

2.6.1 Neuraminidase

A 1 mg/ml mixture of neuraminidase-agarose was prepared from a 6.54 mg/ml neuraminidase-agarose suspension supplied in 2 M $(\text{NH}_4)_2\text{SO}_4$, pH 7, by dilution in Milli-Q water. The solution was dialysed (4 x 2L) against 50 mM NH_4OAc , pH 7.4, at 4 °C and centrifuged before use. Aliquots of the dialysed enzyme mixture (50 μl and 100 μl) were added to 1 ml of 10 μM apo-tf solutions made up in 50 mM NH_4OAc , pH 7.4, and to some made up in 50 mM NH_4OAc , pH 5.5. Control mixtures (no neuraminidase) were also prepared. Half of the solutions were incubated at room temperature and half at 37 °C for different timepoints ranging from 30 minutes to 120 hours. The incubation was terminated by a 1/5 dilution into 1% formic acid before ESI mass spectra were acquired. Heat denatured apo-tf was also incubated with neuraminidase at 37 °C and ESI mass spectra acquired after 24, 48 and 120 hours. Commercial holo-tf made up in 0.1 M NH_4HCO_3 , pH 8.2, was also treated with neuraminidase in the same way as apo-tf and the mass spectrum acquired at different times.

In one experiment, transferrin separated from plasma was treated with neuraminidase. Transferrin (250 μl) that had been eluted from using the Affi-Gel Blue affinity gel column as described below, was treated with neuraminidase on agarose beads (25 μl), as above. The ESI mass spectrum of the solution was acquired after 24, 48 and 168 hours.

2.6.2 PNGase F

An apo-tf solution (0.5 ml; 10.6 μ M) made up in 0.1 M NH_4HCO_3 , pH 8.2, was heat denatured in boiling water for 5 minutes and cooled on ice after pulse centrifugation in an Eppendorf microcentrifuge. PNGase (12 μ l of 500 Unit/ml) was added to 500 μ l of the tf solution and treated at 37 °C in a water bath along with a control solution (no PNGase). Aliquots were taken after 1, 2 and 24 hours and the ESI mass spectra acquired.

2.7 HPLC of carbohydrate deficient transferrin (CDT)

Anion-exchange chromatography was performed in order to separate the different CDT forms. The HPLC experiments were carried out using a Beckman System Gold HPLC. CDT was prepared from intact commercial apo-tf by reacting it with neuraminidase agarose as described above. A Shodex QA-825 8 μ M SAX anion exchange column (75 x 8 mm) from Phenomenex was equilibrated with 20 mM NH_4OAc , pH 6.8, (A) at a flow rate of 1 ml min⁻¹. The sample was loaded via a Rheodyne injector. Solution B was 20 mM NH_4OAc , pH 6.8, in 1 M NaCl. A linear gradient peaking at 20% B was applied over a duration of 30 min. Prior to each HPLC run, the column was washed with 20 mM NH_4OAc , pH 3, followed by equilibration with solution A before the sample was loaded. Concentrations of tf of 1 mg/ml and 0.5 mg/ml in 1 ml of 20 mM NH_4OAc , pH 6.8, were loaded. Peaks (monitored by A_{280}) were collected from three different runs and pooled before dialysis against 20 mM NH_4OAc , pH 6.8. Formic acid was added to aliquots of the solutions to give a concentration of formic acid of 1%,

prior to ESI-MS analysis. Concentrations of each dialysed apo-tf peak were determined by measurement of A_{280} .

2.8 Relative ESI-MS response of separated CDT

Fractions containing various forms of CDT were eluted from the anion exchange column. ESI-MS analysis of the peaks showed that they corresponded to transferrin with 0, 1, 2, 3 and 4 sialic acid residues removed. Table 2.2 shows the concentration of the peaks, based on the measurement of the A_{280} .⁸⁴

Peaks two and four, corresponding to apo-tf with three and two SA residues removed, respectively, were mixed at different concentrations and ESI mass spectra acquired. Table 2.3 shows the volumes added and the final concentrations of the transferrin in the mixtures.

Table 2.2. Concentrations of CDT forms.

^a Apo-tf – 4 SA refers to apo-tf that has lost four sialic acid residues.

^b – Based on measurement of A_{280} , using ϵ_{280} for both forms of 83 200.⁸⁴

Table 2.3. Experimental conditions for titration of apo-tf – 3 SA and apo-tf – 2 SA.

Volume of apo-tf - 3 SA, (μl)	Volume of apo- tf – 2 SA, (μl)	Concentration of apo-tf – 3 SA, (μM)	Concentration of apo-tf – 2 SA, (μM)
6	94	0.1	1.2
20	80	0.3	1.0
42	58	0.7	0.75
60	40	1.0	0.5
80	20	1.4	0.3
92	8	1.6	0.1

2.9 Fractionation of plasma using Affi-Gel® Blue affinity gel

2.9.1 Using a column

A 7 x 0.9 cm column was packed with Affi-Gel Blue affinity gel and equilibrated at 4 °C with 50 mM NH₄OAc, pH 7.4. Plasma thawed from -80 °C on ice (500 μl) was loaded. Ten 400 μl fractions were collected at 0.2 ml/min over 2 minutes using a Bio-Rad Econo pump and Model 2110 fraction collector. Fractions were eluted with 50 mM NH₄OAc, pH 7.4. The ESI mass spectra of the solutions were acquired after a 1/5 dilution in 1% formic acid. Proteins that remained bound to the column after this treatment were eluted from the column with 2 M NH₄OAc, pH 7.4. The column was then re-equilibrated with the 50 mM NH₄OAc solution.

2.9.2 Using a Micro Bio-Spin[®] column

For experiments aimed at rapid fractionation of small volumes, a Micro Bio-Spin column (Millipore) was filled with 1 ml of an Affi-Gel Blue affinity gel, placed in a 1.5 ml Eppendorf tube and centrifuged for two minutes at 1000 x g. The packing solution was then discarded and the column washed four times with 500 μ l of 50 mM NH₄OAc, pH 7.4. A maximum of 75 μ l of plasma was loaded on the column and washed through with 500 μ l of 50 mM NH₄OAc, pH 7.4. The eluent was diluted 1/5 in 1 % formic acid and the ESI mass spectrum acquired.

2.10 Determination of the concentration of transferrin in plasma

Standard solutions of apo-tf with concentrations ranging from 0.01-1 μ M, were prepared in 1% formic acid by sequential dilution of the 1 μ M solution (the 1 μ M solution was diluted from a stock solution of 5 μ M). The concentrations of the solutions were determined by measurement of A₂₈₀.⁸⁴ The ESI mass spectra were acquired and the mass spectrometric response (ion count) of each apo-tf solution was obtained. A spectrum of tf that had been separated from plasma (75 μ l) on a Micro Bio-Spin column, was also acquired under the same conditions as for the standard solutions. The eluent from the column (200 μ l) was diluted 1/10 in 1% formic acid prior to ESI-MS. The overall dilution factor was approximately 1 in 27.

2.11 Separation of transferrin from cerebrospinal fluid

2.11.1 Chromatography on Sephadex G-75

A Sephadex G-75 column (4.4 x 0.9 cm) was equilibrated with 50 mM NH₄OAc, pH 7.4, at 4 °C and a flow rate of 0.2 ml/min. CSF (-80 °C) was thawed on ice, concentrated using a Microcon centrifugal filter device (from 500 µl down to 200 µl) and loaded (56 µl) onto the column. Ten 400 µl fractions were collected and diluted 1/5 with 1% formic acid prior to ESI-MS analysis. Some of the fractions (fractions 5-7) were pooled, concentrated to 100 µl (12-fold), and an ESI mass spectrum of the concentrated fractions acquired after a 1/5 dilution in 1% formic acid.

2.11.2 Using an Affi-Gel[®] Blue affinity gel Micro Bio-Spin[®] column

CSF (~2 ml) was thawed on ice, and concentrated using a Microcon centrifugal filter device (down to 100 µl). A Micro Bio-Spin column filled with 1 ml of Affi-Gel Blue affinity gel was prepared as for plasma (above) and loaded with the concentrated CSF (75 µl). The sample was diluted 1/5 in 1% formic acid prior to ESI-MS analysis.

2.12 Removal of abundant plasma proteins

Plasma (15 µl) was made up to 200 µl with “buffer A” that was supplied with an Agilent High Capacity Multiple Affinity Removal Spin Cartridge (Hu-6HC) and filtered using a spin filter device supplied by Agilent. The plasma solution was then loaded on

the spin cartridge, washed through with “buffer A” and rewashed with “buffer B”. The eluted sample was dialysed against 50 mM NH_4OAc , pH 7.4, and the mass spectrum acquired after a 1/5 dilution in 1% formic acid.

2.13 Addition of Fe^{3+} and Dy^{3+} to apo-transferrin

2.13.1 Iron

Apo-tf (10 mg) was made up to 1 ml in an Eppendorf tube with 0.1 M NH_4HCO_3 , pH 8.2. The concentration of the solution was 98.4 μM . A 1.6 mM $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ solution was prepared by dissolving 16.2 mg of the iron in Milli-Q water and then sonicating the solution for 40 minutes to ensure complete dissolution. The iron solution was then made up to 25 ml with water in a volumetric flask and used immediately. Iron solution (120 μl) was added to 900 μl of the apo-tf solution.³ The final concentrations of the iron and tf solution in the mixture were 188 μM and 87 μM , respectively, giving a tf : iron ratio of 1 : 2. The absorption at 470 nm (following incorporation of iron into tf) was monitored over 2 hours at room temperature. The resulting holo-tf solution was dialysed against the NH_4HCO_3 solution and the ESI mass spectrum acquired.

Apo-tf was made up in 0.1 M NH_4OAc , pH 8.2, and reacted with iron as described above, in order to determine if tf bound iron in the absence of the bicarbonate ion. The final concentration of tf and iron in the solution was 94.98 μM and 200 μM , respectively. Apo-tf was also made up in 1 mM, 10 mM and 20 mM NH_4HCO_3 in the presence of 0.1 M NH_4OAc , pH 8.2, in order to determine the minimum concentration of NH_4HCO_3 necessary under these experimental conditions for tf to bind

iron. Apo-tf was also prepared in 20 mM NH_4HCO_3 in 0.1 M NH_4OAc , pH 6. Iron was then added to apo-tf in each of these solutions as described above for 0.1 M NH_4HCO_3 , pH 8.2.

2.13.2 Dysprosium

A 1 mM dysprosium (III) acetate hydrate, $\text{Dy}(\text{OAc})_3\text{H}_2\text{O}$, solution was prepared in Milli-Q water. An aliquot of dysprosium acetate solution (40 μl) was added to a 100 μM apo-tf solution (160 μl) made up in 0.1 M NH_4HCO_3 , pH 8.2, giving a final concentration of Dy^{3+} of 200 μM and a final concentration of tf of 80 μM . In another experiment, 70 μl of the Dy^{3+} solution was added to 160 μl of the tf solution, giving final concentrations of Dy^{3+} and tf of 300 and 70 μM , respectively. The reactions were left to react for one hour at room temperature and the mass spectra acquired.

2.14 Effect of pH on the iron content of holo-tf

Formic acid (50 μl of 20%) was added to 1 ml of a 90 μM commercial holo-tf solution prepared in 0.1 M NH_4OAc , pH 8.2, in order to study the effect of lowering the pH on the holo-tf complex. The absorbance of the solution at 470 nm was monitored at room temperature for 40 minutes after the acid was added.

2.15 ESI-MS conditions

All ESI mass spectra were acquired in positive ion mode. Spectra were acquired over the m/z range 500-4500 on a Micromass Q-TOF 2TM and over the m/z range 500-7000 on the Waters (formerly Micromass) Q-TOF *Ultima*. Typically 15-20 acquisitions were summed to obtain raw spectra, which were then background subtracted, smoothed using a Savitsky-Golay algorithm and centred. Spectra showing electrospray series were converted to a mass scale using the transform algorithm of the MasslynxTM software. The instrument was calibrated with 1 mg/ml cesium iodide in 50% isopropanol. Samples were injected using a Harvard Model 22 syringe pump at a flow rate of 20 $\mu\text{l}/\text{min}$. Instrumental parameters used for the Q-TOF 2TM and the Q-TOF *Ultima* are listed in tables 2.4 and 2.5.

Table 2.4. Instrumental parameters on the Q-TOF-2TM

Instrumental Parameter	Value
Capillary	3000 V
Cone	70 V
Source block temp.	80 °C
Desolvation temp.	200 °C
Transport	2 V
Aperture	13 V
Collision energy	4 V
TOF	9000 V
Reflectron	36

Table 2.5. Instrumental parameters on the Q-TOF *Ultima*

Instrumental Parameter	Value
Capillary	3300 V
Cone	350 V
Source block temp.	75 °C
Desolvation temp.	250 °C
Aperture	0 V
Transport	5 V
RF Lens 1 Energy	121.2 V
Tube Lens	120 kV
TOF	9100 V
Reflectron	35.60 V
Collision energy	19.7 V
Bleed gas (Nitrogen)	1.4 x 10 ⁻¹ mBar

CHAPTER 3: CHARACTERIZATION OF THE DEGLYCOSYLATED FORMS OF TRANSFERRIN

3.1 Introduction to ESI-MS

In ESI-MS, an analyte, such as a protein in an ammonium acetate solution, is passed through a stainless steel capillary at high voltage. As the sample nebulizes (forms a spray of droplets) in the ionization chamber at atmospheric pressure, charges (NH_4^+ in this example) are present at the surface of the droplet. A stream of warm, dry nitrogen evaporates the droplets. At some point, the charge density on the surface of the droplets results in Coulombic repulsions, so that the droplets explode, forming smaller droplets. This process is repeated until the remaining droplet evaporates, and the protons from NH_4^+ are transferred to basic sites on the protein (see Dole's charged residue model in section 1.1.6).

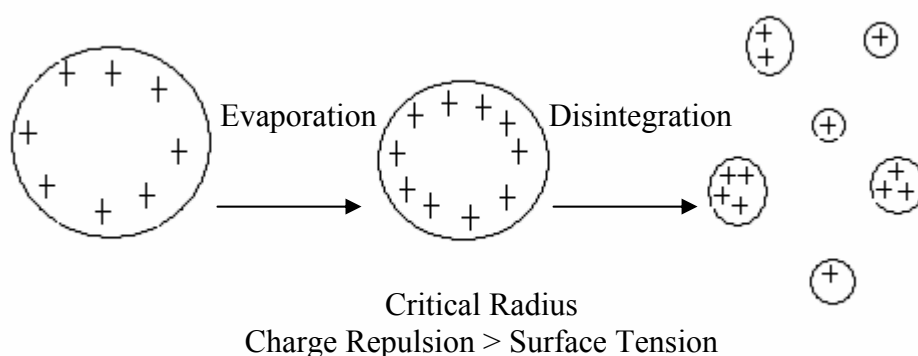


Figure 3.1. Representation of Dole's charged residue model (CRM). The parent droplet disintegrates into several offspring droplets.

The ion signal response (sensitivity) of ESI-MS is affected by many factors. These include solvent volatility, surface tension of the droplets, solvent viscosity,

conductivity, ionic strength, dielectric constant, electrolyte concentration arising from impurities in the solvent and pH, as well as analyte pKa, hydrophobicity, surface activity, ion solvation energy and gas-phase basicity.⁸⁷ Equations have been proposed that relate the observed mass spectrometric response to analyte concentration using parameters such as electrolyte concentration, partitioning coefficient ratio (K_A/K_E) for analyte and electrolyte ions between the droplet surface and bulk solution, and concentration of excess charge, which is the difference in the concentrations of the cations and anions in the electrosprayed solution. The excess charge is directly proportional to the spray current and inversely proportional to the flow rate. The concentration and set of experimental conditions at which the mass spectrometer response plateaus, is presumed to represent the condition where the concentration of available charge sites on a protein is roughly equivalent to the concentration of excess charge.^{88, 89} Experimental conditions that influence the analyte surface partitioning coefficients in electrosprayed droplets include spray position relative to the sampling orifice, spray potential, nebulizer gas flow rates, gas temperature and electrolyte concentration.⁸⁷ Therefore, in order to obtain quantitative information from mass spectrometry experiments, care must be taken to maintain constant experimental conditions and to determine the dynamic range (linear region of the response curve) under a set of experimental conditions.

3.2 ESI-MS response of commercial apo-transferrin

In order to develop clinical tests to detect and quantify transferrin by ESI-MS it will be necessary to determine the protein concentration range which produces a linear ESI-MS response under a given set of experimental conditions.

The electrospray ionization mass spectrometric response of commercial apo-tf was investigated in different solvents. Apo-tf solutions with concentrations ranging from 0.1 μM to 60 μM were made up in: (i) 0.1 M NH_4HCO_3 , pH 8.2; (ii) 50 mM NH_4OAc , pH 7.4; (iii) 1% formic acid in 50% acetonitrile, (iv) and 1% formic acid. Figure 3.2 shows the ESI mass spectra of apo-tf in 0.1 M NH_4HCO_3 , pH 8.2 (a), and in 1% formic acid (b). Charge states (ions) ranging from $[\text{M} + 18\text{H}]^{18+}$ (m/z 4423.3) to $[\text{M} + 38\text{H}]^{38+}$ (m/z 2095.5) were observed for ESI mass spectra of transferrin sprayed from 1% formic acid. The peak width at 50% ion intensity for the most abundant ion was 2.2 amu, while the value was 11.5 amu for the narrowest peak in spectra of transferrin sprayed from NH_4HCO_3 . Only charge states from $[\text{M} + 18\text{H}]^{18+}$ (m/z 4424.1) to $[\text{M} + 21\text{H}]^{21+}$ (m/z 3792.3) were observed when the apo-tf was sprayed from NH_4HCO_3 .

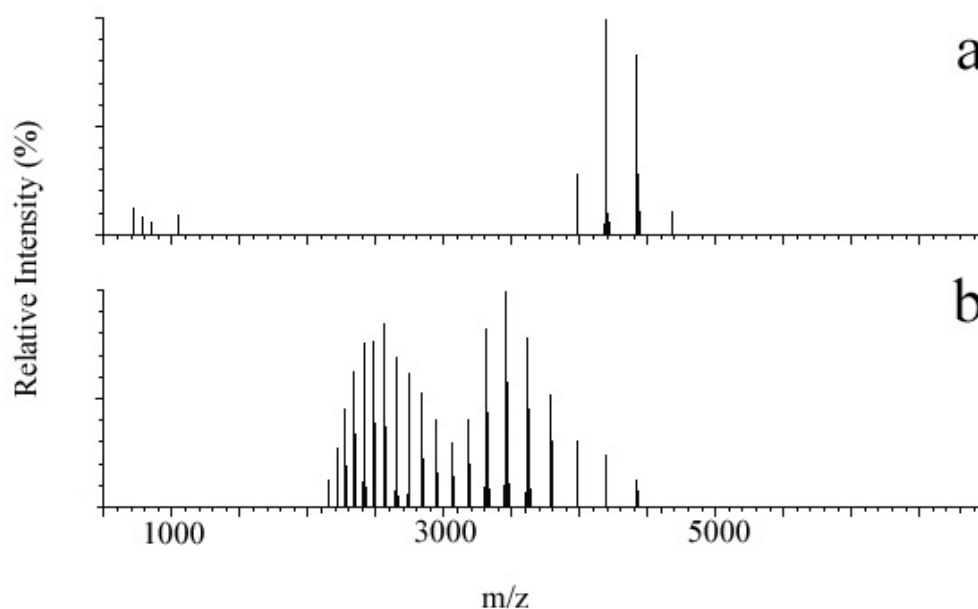


Figure 3.2. ESI mass spectra of apo-tf (10 μM) in different solutions. (a) Sprayed from 0.1 M NH_4HCO_3 , pH 8.2. (b) Sprayed from 1% formic acid.

Transformation of the spectra in figure 3.2 (a) and (b) to a mass scale gave a molecular mass for apo-tf of $79\,572 \pm 3$ Da and $79\,569 \pm 2$ Da, respectively. The masses observed are in good agreement with the calculated mass for fully glycosylated apo-tf (79 570 Da).

Since peaks in ESI mass spectra of apo-tf sprayed from solutions containing formic acid were narrower, the mass can be determined with more confidence. However, there are advantages in using a solvent at higher pH. For example, the iron in tf will be released from the protein at low pH, therefore it will not be possible to determine the mass of Fe₂-tf or Fe-tf when sprayed from formic acid (see section 4.1).

The linearity of the mass spectrometric response was tested by calculating the total ion count in each spectrum for apo-tf (combining 14 acquisitions) at different concentrations when sprayed from different solvents. Figure 3.3, shows the ion count as a function of tf concentration in NH₄HCO₃ and NH₄OAc (a), and in 1% formic acid in 50% acetonitrile and in 1% formic acid (b).

The spectra obtained when the NH₄OAc solution was used for spraying, were slightly better resolved than those obtained from NH₄HCO₃. The resolution improved noticeably in 1% formic acid in 50% acetonitrile and in 1% formic acid. Acetonitrile is a volatile solvent, which aids in the desolvation of proteins. The acid also partially denatures the proteins and breaks hydrogen bonds to the water molecules of tf, helping to desolvate the protein. More protonation sites are also made available with the more open conformation of the denatured protein. This accounts for the greater number of ions observed in figure 3.3 (b) than in 3.3 (a).

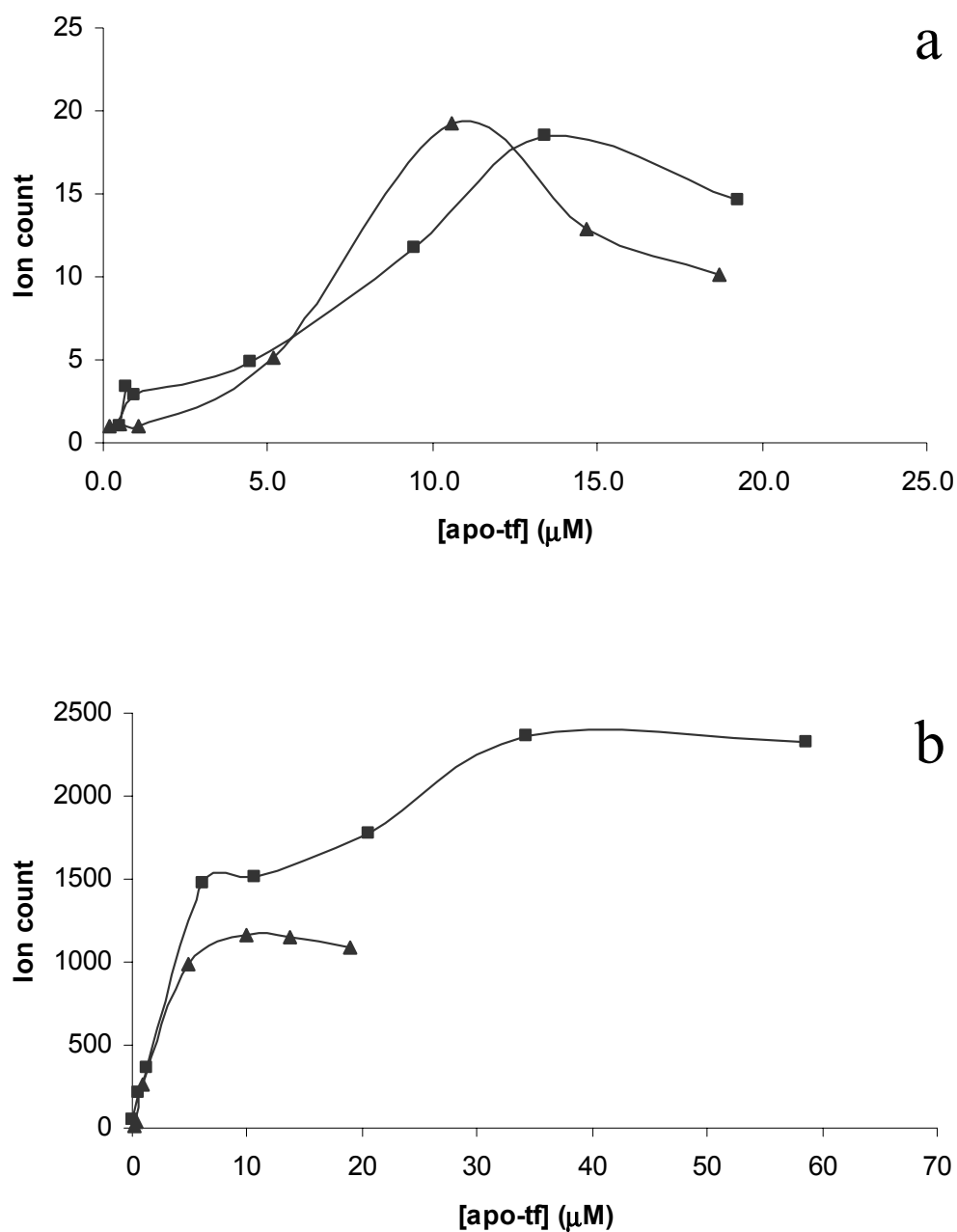


Figure 3.3. Response curves of apo-tf in different solutions. (a) Response curves of apo-tf sprayed from 0.1 M NH_4HCO_3 , pH 8.2, (■) and 50 mM NH_4OAc , pH 7.4 (▲). (b) Response curves of apo-tf sprayed from 1% formic acid in 50% acetonitrile (■) and 1% formic acid (▲). The experiments were carried out using a Micromass Q-TOF 2TM ESI mass spectrometer. Each point on the graphs represents the result from one experiment.

The response curves for apo-tf in the low pH solvents plateaued at a lower concentration ($\sim 5 \mu\text{M}$) than for the NH_4OAc and NH_4HCO_3 ($15 \mu\text{M}$). One of the contributions to the plateauing effect at high concentrations of analyte is that the multichannel plates (mcp) that detect the ions cannot distinguish between two ions that arrive at the mcp at the same time.⁹⁰ Figure 3.4 (a), (b), (c), and (d) show the concentration ranges over which the response curves were linear in the different solvents using the Q-TOF 2TM mass spectrometer. Linearity is generally observed for concentrations up to $\sim 10 \mu\text{M}$, which is consistent with the literature, where nonlinear calibration curves for proteins/peptides studied (cytochrome *c*, myoglobin, Met-Arg-Phe-Ala (MRFA)) were first observed at concentrations above $10 \mu\text{M}$, even though individual variations existed in the range of $10\text{-}150 \mu\text{M}$, with larger proteins showing non-linearity at lower concentrations.³ A fixed upper analyte limit of $10 \mu\text{M}$ for the dynamic range in ESI-MS has also been reported by several sources.⁹¹ Although acetonitrile and formic acid were the solvents that gave the best ESI-MS response, NH_4HCO_3 and NH_4OAc gave an extended linear region for apo-tf.

During the course of this project, the Q-TOF 2 was replaced by a custom built (factory modified) Waters (formerly Micromass) Q-TOF *Ultima* ESI mass spectrometer. Therefore, the experiments described above were repeated using this instrument. In figure 3.5 (a) and (b), response curves for apo-tf sprayed from NH_4HCO_3 , NH_4OAc , 1% formic acid in 50% acetonitrile and in 1% formic acid using the Q-TOF *Ultima* mass spectrometer are shown. The sensitivity for apo-tf in NH_4HCO_3 and NH_4OAc had improved more than a 100-fold on this instrument (as seen in the y-axis of figure 3.5 (a) compared to the values in figure 3.3 (a) and approximately 10-fold in 1% formic acid in 50% acetonitrile and in 1% formic acid (see y-axes of figures 3.5 (b) and 3.3 (b))

compared to ESI mass spectra obtained using the Q-TOFTM 2. This is most likely from improvements to the instrumentation and since the ion detector (multi channel plates) in the Q-TOF *Ultima* were new. The points on the graphs in figure 3.5 are from one experiment. The experiment was repeated two more times giving linear responses over the same concentration range. The ion counts at each concentration, however, varied significantly from experiment to experiment, possibly as a result of the build-up of a non-conducting coating on surfaces in the ionization source that diminished the response. This observation suggests that to enable quantitative analyses, a response curve for transferrin should be prepared prior to analysis of samples, rather than relying on a curve prepared previously.

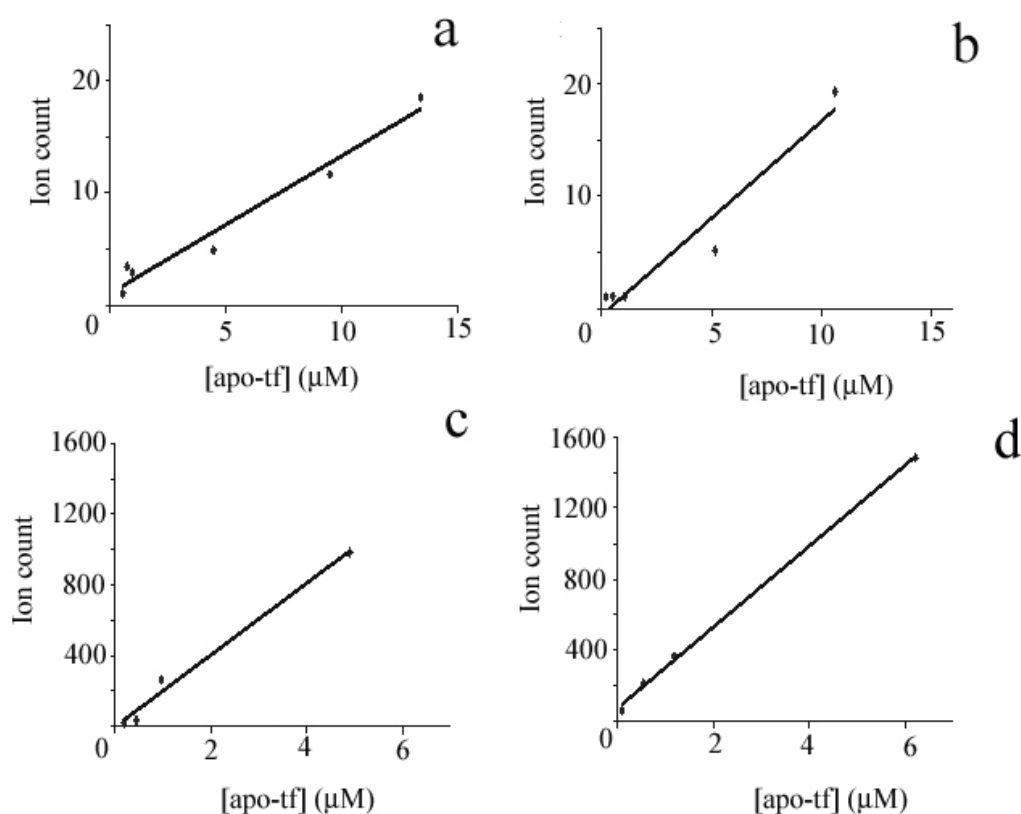


Figure 3.4. The linear concentration region of apo-tf in ESI mass spectra sprayed from different solutions. (a), (b), (c) and (d), show the linear concentration region of tf in 0.1 M NH_4HCO_3 , pH 8.2 (a), 50 mM NH_4OAc , pH 7.4 (b), 50% acetonitrile in 1% formic acid (c) and 1% formic acid (d), using the Q-TOFTM 2. Each point on the graphs represents the result from one experiment.

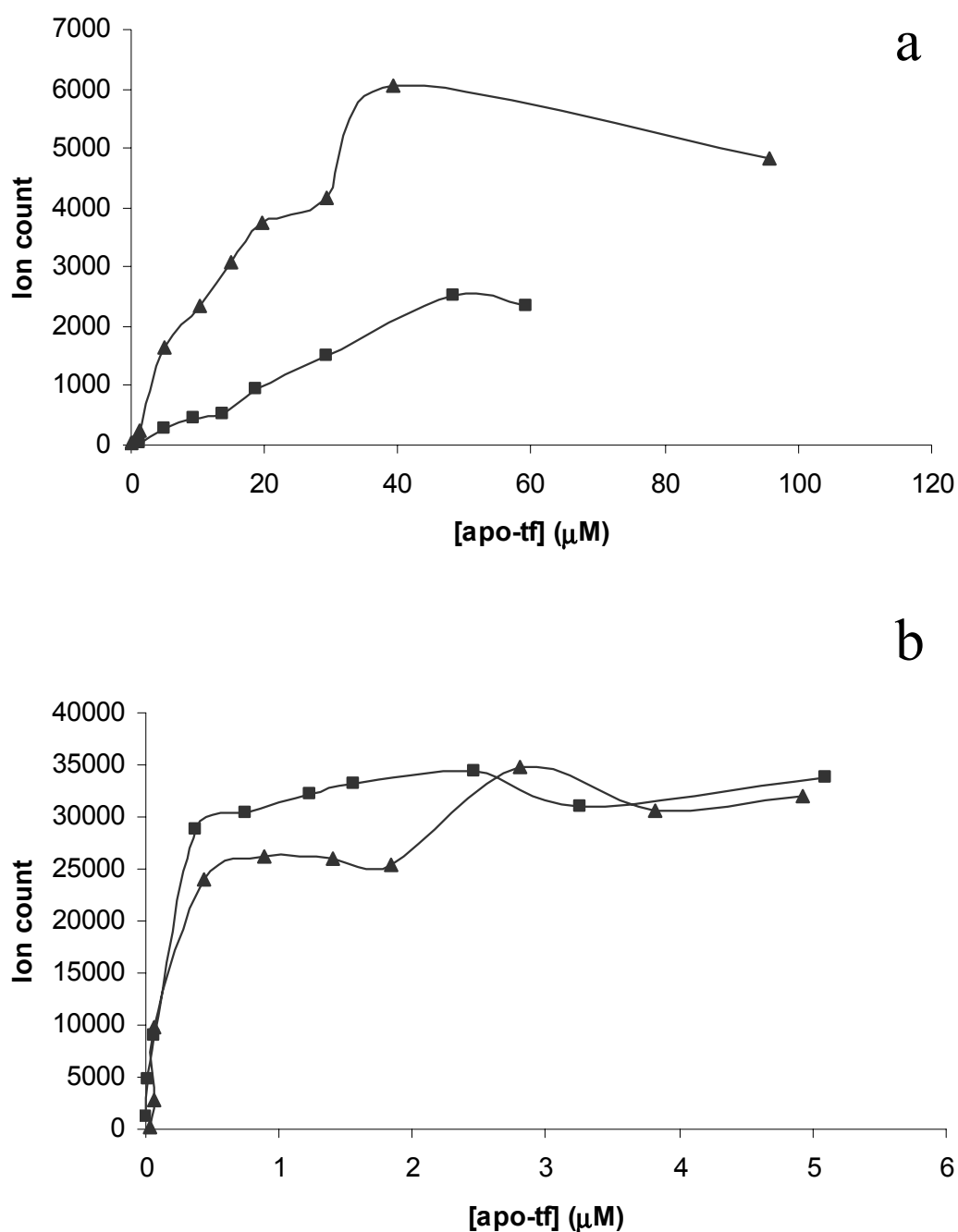


Figure 3.5. ESI-MS response curves of apo-tf in different solutions. (a) Response curves for apo-tf sprayed from ammonium bicarbonate (pH 8.2) (■) and ammonium acetate (pH 7.4). (b) Response curves of apo-tf sprayed from 1% formic acid in 50% acetonitrile (▲) and 1% formic acid (■). The experiments were conducted using a Q-TOF *Ultima* mass spectrometer from Waters. Each point on the graphs represents the result from one experiment.

The concentration ranges of apo-tf sprayed from different solvents that gave a linear ESI mass spectrometric response using the Q-TOF *Ultima*, are shown in figure 3.6.

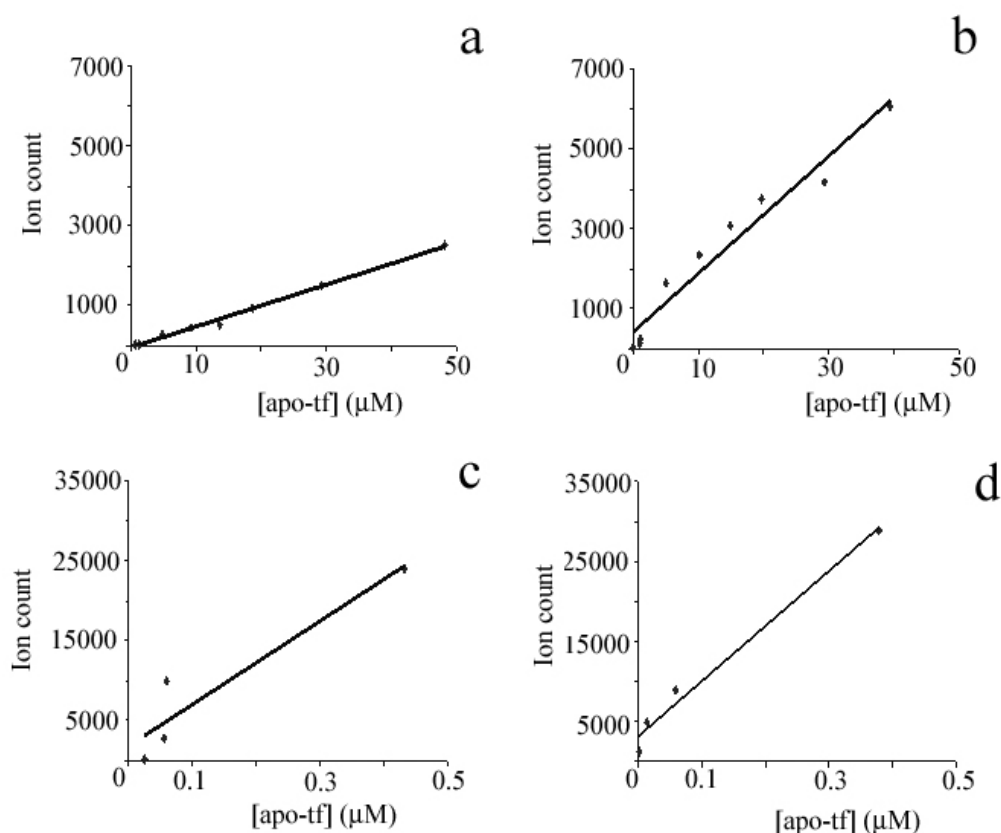


Figure 3.6. The linear response region of apo-tf in ESI mass spectra sprayed from different solutions. (a), (b), (c), and (d) show the linear concentration regions of apo-tf in 0.1 M NH_4HCO_3 , pH 8.2 (a), 50 mM NH_4OAc , pH 7.4 (b), 1% formic acid in 50% acetonitrile (c) and 1% formic acid (d), using a Q-TOF *Ultima* mass spectrometer from Waters.

The linear concentration region of apo-tf in NH_4HCO_3 and NH_4OAc , shown in (a) and (b), is extended to 40-50 μM on this instrument (cf. $\sim 10 \mu\text{M}$ on the Q-TOF 2). However, the linear region observed in 1% formic acid + 50% acetonitrile and in 1% formic acid, occurs only up to 1 μM (cf. $\sim 10 \mu\text{M}$ using the Q-TOF 2). Although spraying from formic acid gave higher resolution and sensitivity for apo-tf under these experimental conditions, it had a limited dynamic range on both instruments. Therefore, it appears that even though formic acid gives a high degree of ionization of apo-tf, the upper limit of ionization is reached at lower concentrations of tf. The

concentration of excess charge is related to solvent properties such as surface tension.⁸⁹ On the other hand, the dynamic range for apo-tf in solutions of NH_4HCO_3 or NH_4OAc , was greater. Fewer ionizable sites are available on the protein for protonation due to non-denaturing conditions. The properties of these solvents likely also influence the concentration of excess charge.

3.2.1 The ESI-MS response of transferrin in plasma

The most abundant protein in plasma is serum albumin (HSA; human serum albumin). Previously, Beck et al. showed that an ESI mass spectrum of HSA could be obtained by diluting plasma several hundred-fold into the appropriate solvent.⁹² The ESI-MS response of commercial apo-tf in a mixture with plasma, was investigated in order to establish matrix effects from other proteins on the mass spectrometric response of tf. Apo-tf was dissolved in 1% formic acid since this solution gave the best resolution and sensitivity. Different volumes of plasma were added to solutions of apo-tf with a final concentration of commercial apo-tf of 10 μM . Since HSA is so abundant in plasma compared to all other proteins, adding plasma was equivalent to adding HSA. Human serum albumin from plasma was added instead of commercial HSA since the spectrum obtained from commercial HSA was of poor quality compared to spectra from plasma HSA, as seen in figures 3.7 (a) and (b), and in agreement with earlier work.⁹² Impurities from the manufacturing process appear to affect the resolution achieved with commercial HSA more than that of commercial apo-tf.

Figure 3.8 shows the effect of concentration of HSA (calculated assuming the concentration of HSA in plasma is 0.6 mM) that was added to 10 μM apo-tf, on the ion

count of apo-tf. The mass spectrometric response of apo-tf decreased with increasing concentrations of HSA under this set of experimental conditions. This is in accordance with the expected inverse relationship between the signal of the major component and the concentration of the minor component under a similar set of conditions, i.e. low pH, high protein solubility and no other strongly basic or surface active components at high concentration.⁸⁹ In future experiments, it will be necessary to measure more data points over the concentration range 7-12 μ M HSA to determine whether this response curve can best be represented by a straight line (as in fig. 3.8) or as a curve. The response curve can be used to determine the concentration of tf in a mixture with HSA, or effectively in plasma.

Figure 3.7. Spectra of HSA obtained from plasma and of commercial HSA. The resolution for plasma HSA (a) is significantly better than for commercial HSA (b). The spectra show the $[M + 23H]^{23+}$ ion of HSA. The minor peaks in (a), are from HSA + cysteine (HSA + 120) and HSA + glucose (HSA + 162).⁹²

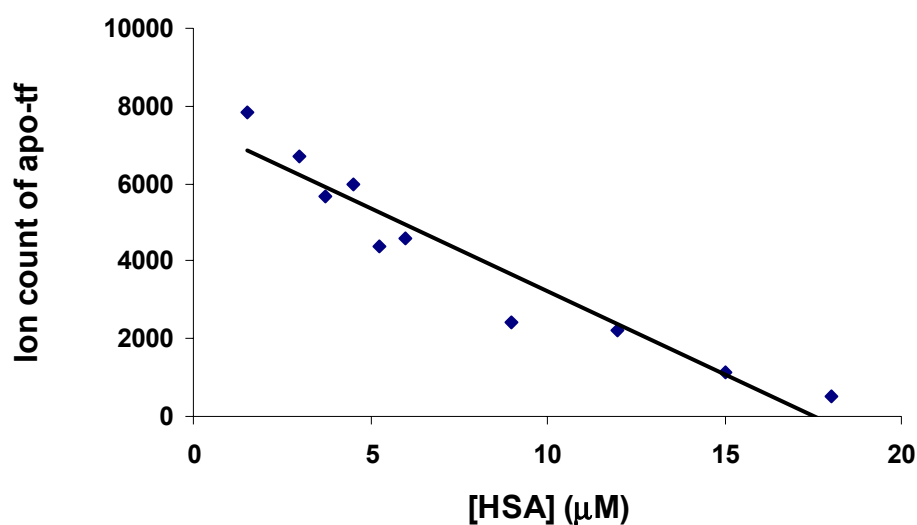


Figure 3.8. Effect of HSA on the ion count from apo-tf in ESI mass spectra. The concentration of apo-tf in each mixture was 10 μM . The solvent used was 1% formic acid.

When data are given in terms of the concentration of analyte charge sites instead of concentration of analyte molecules or charge normalized under a given set of experimental conditions, similar ESI-MS responses are observed for different proteins.⁸⁹ This suggests that it is possible to predict the concentration-dependent response of another protein based on that of apo-tf and to predict the effect of high concentrations of one protein on the response of all other proteins in a mixture. However, other data suggest that the linear dynamic range for different compounds can be affected differently by the sample complexity due to differences in ionization efficiency depending on analyte surface activity.⁹¹ Nonetheless, when sufficiently dilute sample was used or lower electrospray flow rates applied, comparable ionization efficiencies were still obtained for all the analytes in a mixture. A wider linear range is therefore expected to be routinely achievable with nano-electrospray (which uses lower flow rates), with similar ionization efficiency for any compound and ionization efficiencies approaching 100% since analytes effectively compete with solvent related

species for charge. However, a full quantitative evaluation of nano-electrospray is still not available due to technical difficulties.

3.3 Deglycosylated forms of transferrin

3.3.1 Neuraminidase

Different glycosylated forms of tf were prepared from commercial apo-tf in order to determine whether different forms of CDT could be resolved using the Q-TOF *Ultima* ESI mass spectrometer. Neuraminidase cleaves sialic acid (SA) residues from glycoproteins and was therefore used to cleave off terminal sialic acid residues from tetrasialo-tf (normal tf; see figure 1.4). Apo-tf was treated with neuraminidase in 50 mM NH₄OAc under different conditions in order to establish the effects of temperature and pH on the deglycosylation of tf. Apo-tf was incubated with neuraminidase at 37 °C and at room temperature. The reaction was also performed at pH 5.5 and at 7.4. The progress of the reactions with neuraminidase was assayed by taking samples of the reaction mixtures at different times for mass spectrometry.

Figure 3.9 shows the ESI mass spectra (converted to a mass scale) of apo-tf obtained after incubation with neuraminidase for 24, 48 and 144 hours. The masses of apo-tf obtained ($79\,278 \pm 6$, $78\,986 \pm 10$, $78\,694 \pm 11$ and $78\,402 \pm 7$ Da) were in agreement with the calculated values (79 278, 78 986, 78 694 and 78 402 Da) for sequential loss of sialic acid residues (-292) from apo-tf showing that there were protein molecules in the mixture that had lost 1, 2, 3, and 4 sialic acid residues.

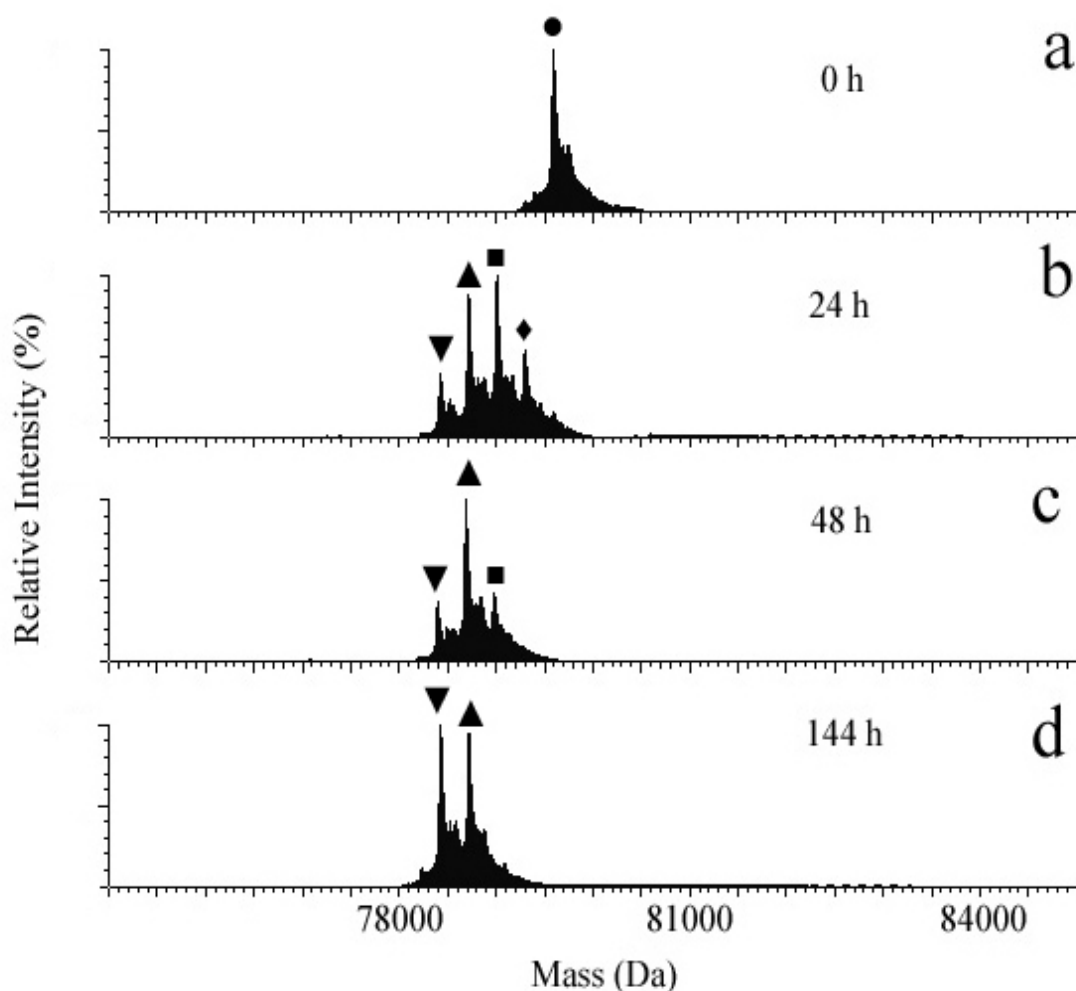


Figure 3.9. ESI mass spectra of apo-tf after treatment with neuraminidase for various reaction times. The spectra have been transformed to a mass scale. Control solution of apo-tf (a) and (b), (c) and (d) show apo-tf treated with neuraminidase after 24, 48 and 144 hours, respectively. The peaks are labelled as follows: (●) corresponds to intact apo-tf with 4 SA residues ($79\,570 \pm 13$ Da); (◆) corresponds to apo-tf with 3 SA residues obtained ($79\,278 \pm 6$ Da); (■) corresponds to apo-tf with 2 SA residues ($78\,986 \pm 10$ Da); (▲) corresponds to apo-tf with 1 SA residue ($78\,694 \pm 11$ Da) and (▼) corresponds to apo-tf with 0 SA residues ($78\,402 \pm 7$ Da). The electrospray solvent was 1% formic acid.

In initial experiments, commercial holo-tf ($\text{Fe}_2\text{-tf}$) was used to determine optimal experimental conditions for acquiring high quality ESI mass spectra of transferrin. These experiments were complicated by the presence of several peaks (corresponding to different masses) in the spectra. In contrast, spectra of commercial apo-tf comprised only one major species (figure 3.9 (a)). The spectrum acquired of neuraminidase-treated apo-tf (figure 3.9 (b)), was compared with that of commercial holo-tf. It was suspected that commercial holo-tf might contain deglycosylated forms of tf, possibly as a by-product of the manufacturing process. Figure 3.10 compares a spectrum of holo-tf sprayed from 0.1 M NH_4HCO_3 , pH 8.2, and the spectrum of apo-tf sprayed from 1% formic acid (figure 3.9(b)). The species present in the spectrum of holo-tf (a) have similar masses to the deglycosylated forms of apo-tf (b). The observed mass of intact holo-tf was $79\,678 \pm 1$ Da. This mass is 108 Da greater than the mass of apo-tf (79 570 Da) and is consistent with the binding of two iron atoms to transferrin (expected mass difference 111.7 Da; see Table 4.1). Additional peaks with masses of $79\,388 \pm 1$ Da, $79\,092 \pm 4$ Da and $78\,807 \pm 1$ Da (corresponding to the loss of one, two and three SA residues, respectively), were also observed. The holo-tf solution was prepared in 0.1 M NH_4HCO_3 , pH 8.2, to ensure the presence of the synergistic bicarbonate ion to maintain the iron-binding ability of tf (see chapter 4), therefore each of the masses is higher than the corresponding deglycosylated form of apo-tf by the mass of two iron atoms.

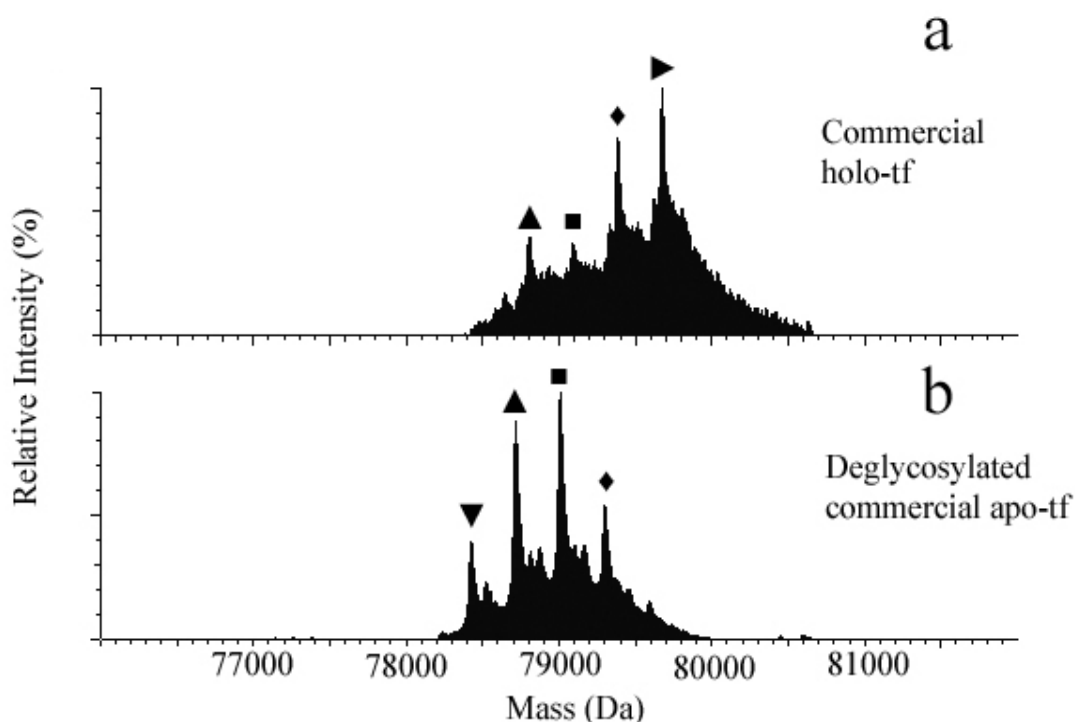


Figure 3.10. ESI-MS spectra (converted to a mass scale) of commercial holo-tf (sprayed from 0.1 M NH_4HCO_3 , pH 8.2) and apo-tf treated with neuraminidase (fig. 3.9 (b)) (sprayed from 1% formic acid). The peaks are labelled in the following way: (►) corresponds to intact holo-tf, (◆) corresponds to apo- or holo-tf with 3 SA residues, (■) corresponds to apo- or holo-tf with 2 SA residues, (▲) corresponds to apo- or holo-tf with 1 SA residue and (▼) corresponds to apo- or holo-tf with 0 SA residues.

Commercial holo-tf was also treated with neuraminidase in order to obtain a sample that contained no protein with its full complement of sialic acid residues (4). Figure 3.11 (a) shows a spectrum converted to a mass scale of deglycosylated holo-tf (sprayed from 1% formic acid), which contained peaks with masses the same as those obtained for deglycosylated apo-tf (the same spectrum shown in figures 3.9 and 3.10 (b) is reproduced in fig. 3.11 (b) for comparison). The iron dissociated from tf at this pH, and therefore tf molecules with iron bound were not observed in the spectrum seen in figure 3.11 (a), however, the deglycosylated species were readily resolved.

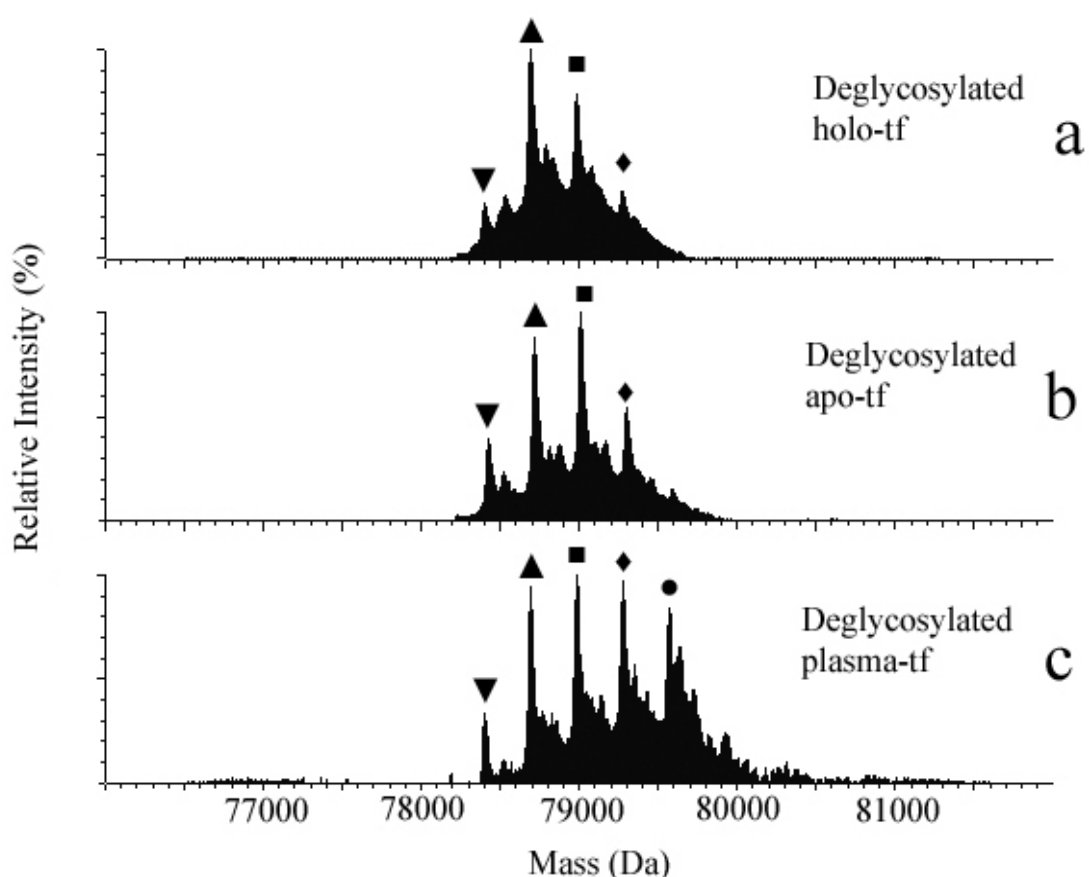


Figure 3.11. ESI mass spectra converted to a mass scale of commercial holo- and apo-tf, and tf from plasma treated with neuraminidase. The spray solvent in each case was 1% formic acid. This solvent is expected to remove iron from holo-tf (see Chapter 4). The peaks are labelled as follows: (●) corresponds to intact tf with 4 SA residues; (◆) corresponds to tf with 3 SA residues; (■) corresponds to tf with 2 SA residues; (▲) corresponds to tf with 1 SA residue and (▼) corresponds to tf with 0 SA residues.

Plasma that had been fractionated using an Affi-Gel Blue affinity gel column to remove HSA (see section 3.4), was also treated with neuraminidase in an attempt to deglycosylate plasma tf. Deglycosylated species of tf from plasma (sprayed from 1% formic acid), were observed in the spectrum after treatment with neuraminidase for 24 hours. The same masses were observed as for commercial apo-tf, as seen in figure 3.11 (c). The masses observed for the species in each spectrum and the calculated masses of transferrin after removal of one, two, three and four sialic acid residues are shown in table 3.1.

Table 3.1. Masses observed for the species in each spectrum shown in figure 3.11. The calculated masses of intact apo-tf and various deglycosylated species of apo-tf, are also listed.

Fig. 3.11 (a) Observed masses (Da)	Fig. 3.11 (b)¹ Observed masses (Da)	Fig. 3.11 (c) Observed masses (Da)	Calculated masses (Da)
		79 573 ± 4	79 570 Intact apo-tf
79 276 ± 3	79 278 ± 6	79 279 ± 2	79 278 Apo-tf – 1SA ²
78 987 ± 4	78 986 ± 10	78 987 ± 2	78 986 Apo-tf – 2SA
78 695 ± 2	78 694 ± 11	78 696 ± 2	78 694 Apo-tf – 3SA
78 407 ± 6	78 402 ± 7	78 402 ± 5	78 402 Apo-tf – 4SA

¹This is the same spectrum shown as shown in figure 3.9 (b) and figure 3.10 (b).

²SA = Sialic acid residue

3.3.2 HPLC of deglycosylated species of tf

The results of the experiments above show that the forms of transferrin with varying numbers of sialic acid residues can be resolved using the Q-TOF *Ultima* mass spectrometer. In order to compare the mass spectrometric response of the various forms, it was necessary to first purify them by HPLC. Anion exchange columns are most frequently used to separate the various forms of carbohydrate-deficient transferrin (CDT),⁹³⁻⁹⁵ however, affinity columns such as graphite Glyco H columns,⁹⁶ which are specific for the N-glycans of proteins, and Poros HQ10 columns,⁹⁷ have also been used. In the following experiments a Shodex IEC QA-825 8u SAX anion exchange column from Phenomenex was used.

Intact, commercial apo-tf was partially deglycosylated with neuraminidase as described earlier (treatment of 10 µM apo-tf for 24 hours) and the different deglycosylated forms

separated by anion exchange HPLC. The aim was to separate the different deglycosylated forms of tf, collect each peak and then mix them at different concentrations in order to obtain an ESI-MS response curve for each species. Normal intact tf which contains 4 (negatively charged) sialic acid residues would be expected to be most tightly bound to the anion exchange column and elute last, while asialo-tf, which has had all the sialic acid residues cleaved off, would elute first. An elution profile of apo-tf, deglycosylated by treatment with neuraminidase, is shown in figure 3.12. The concentrations of the different forms of transferrin eluted from the column and dialysed prior to ESI-MS were low. Nevertheless it was possible to differentiate between the various glycosylated forms. Intact apo-tf eluted at 17.6 minutes. The first peak gave a poor quality ESI mass spectrum (low signal-to-noise) and contained a deglycosylated species of apo-tf with a mass of $78\,426 \pm 18$ Da, corresponding to the loss of 4 SA residues (calculated mass 78 401). This species of apo-tf was present at a low concentration as judged from the absorption at 280 nm, which explains the poor resolution and high error in mass achieved for this species. The species that eluted in the HPLC peaks 2 and 3 had the same mass within experimental error correlating to apo-tf missing three SA residues. This may be the result of removal of a different combination of sialic acid residues giving modified transferrin molecules with slightly different conformations that altered their interactions with the chromatographic resin. Confirmation of this will require complete sequence analysis of the different forms of transferrin eluted in peaks 2 and 3. Alternatively, it is possible that there was an irregularity in column packing or flow rate that contributed to this split peak. The next peak corresponded to apo-tf with the loss of 2 SA residues, while the last two peaks to elute corresponded to transferrin with the loss of one SA residue and intact apo-tf.

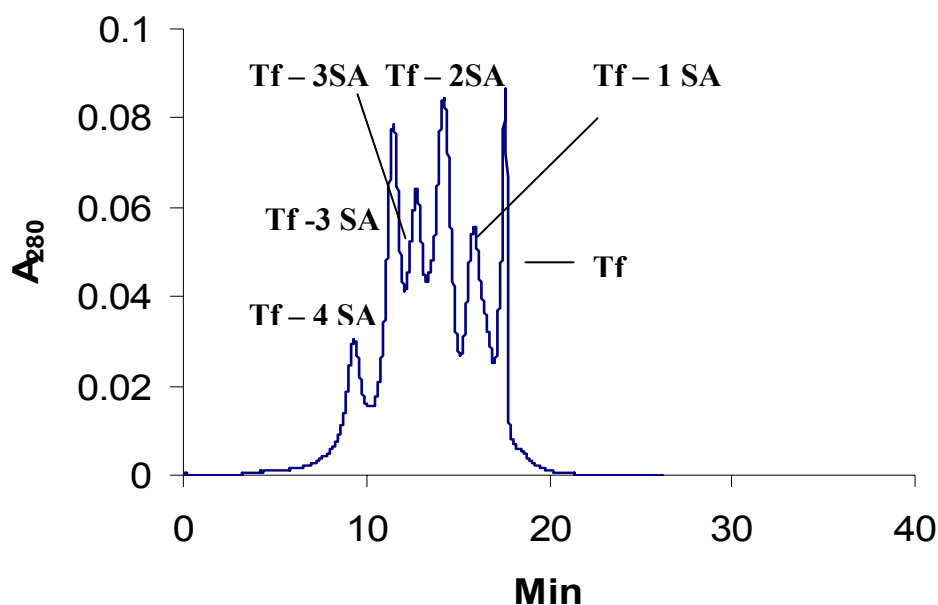


Figure 3.12. The HPLC elution profile of apo-tf deglycosylated with neuraminidase. The column was a Shodex IEC QA-825 8u SAX anion exchange column from Phenomenex. The column was equilibrated with 20 mM NH_4OAc , pH 6.8, and the protein eluted using 20 mM NH_4OAc , pH 6.8, in 1 M NaCl (2% over 30 min). The flow rate was 1 ml min^{-1} .

Table 3.2. Masses of species in each HPLC peak observed in figure 3.12. The calculated mass of each species is also listed.

Peak in figure 3.12	Observed mass (Da)	Calculated mass (Da)
tf	$79\,571 \pm 8$	79 570
tf – 1 SA	$79\,278 \pm 11$	79 278
tf – 2 SA	$78\,986 \pm 14$	78 986
tf – 3 SA	$78\,707 \pm 13$	78 694
tf – 3 SA	$78\,694 \pm 17$	78 694
tf – 4 SA	$78\,394 \pm 12$	78 402

3.3.3 ESI-MS of transferrin treated with neuraminidase

The concentration of each HPLC peak was measured by determination of the absorbance at 280 nm. Apo-tf fractions containing molecules where three and two SA residues were cleaved off, were mixed together in different amounts to determine whether the relative amounts of the different forms in a single mixture could be measured by ESI-MS.

Figure 3.13 shows the spectra transformed to a mass scale that were acquired at different concentrations. The deglycosylated forms of apo-tf were mixed in the ratios (apo-tf – 2 SA/apo-tf – 3 SA) shown on the right hand side of the figure. There is reasonable correlation between the relative amounts of the two forms based on protein concentration (A_{280}), and the relative intensities of peaks in the mass spectra, except in figures 3.13 (c) and (d). This is most likely due to inaccuracies in determining the A_{280} of the dialysed fractions (~ 0.1). Nevertheless, the data show that relative amounts of these two forms of deglycosylated transferrin can be distinguished by ESI-MS.

In figure 3.14, a response curve obtained for each species in the mixture, is shown. As mentioned in section 3.2.2, matrix effects from other species present in a mixture can lead to a decrease in the intensity of ions of other species that are also present. Under these experimental conditions, however, the concentration of both species can be determined since the relationship between concentration determined by measurement of A_{280} and the ESI-MS response was linear.

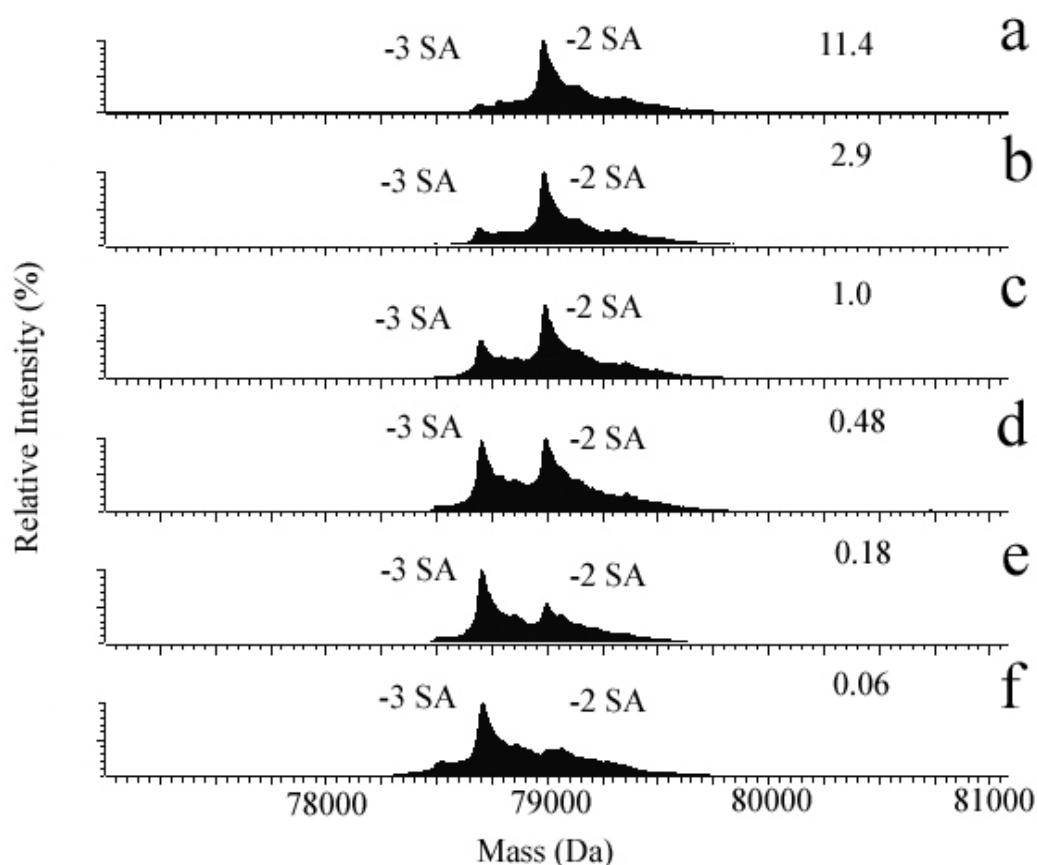


Figure 3.13. ESI mass spectra showing the relative intensities of a mixture of deglycosylated apo-tf species. Apo-tf – 2 SA and apo-tf – 3 SA were mixed in ratios so that apo-tf – 2 SA/apo-tf – 3 SA (based on A_{280} measurements) were: (a) 11.4; (b) 2.9; (c) 1.0; (d) 0.48; (e) 0.18; (f) 0.06.

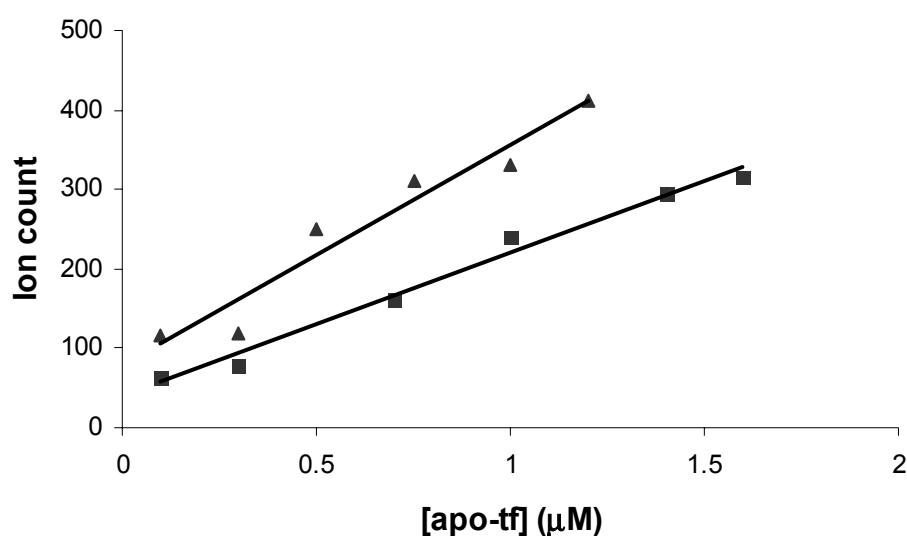


Figure 3.14. Response curves obtained for a mixture of deglycosylated apo-tf species. Apo-tf – 3 SA is denoted with (■) and apo-tf – 2 SA is denoted with (▲).

3.3.4 Analysis of PNGase-treated apo-transferrin

In the experiments described above, it was shown that apo-tf that had lost one sialic acid residue (292 Da) could easily be resolved in ESI mass spectra from intact apo-transferrin. The deglycosylated forms of tf that are most frequently observed in alcoholism and CDG, correspond to transferrins where entire sugar chains are absent rather than just the terminal sialic acid residues. Therefore, it was expected that these forms will be readily distinguished by ESI-MS. PNGase, which is an enzyme specific for removing asparagine-linked sugar chains, was used to prepare *in vitro* these clinically relevant species of CDT. A method was developed where heat denatured commercial apo-tf was treated with PNGase at 37 °C for 24 hours. Tf was made up in 0.1 M NH_4HCO_3 , pH 8.2. The expected masses of the resulting apo-tf species were 77 365 Da and 75 157 Da⁵⁹, which correspond to tf missing one and two sugar chains, respectively.

Figure 3.15 shows an ESI mass spectrum (converted to a mass scale) of apo-tf deglycosylated with PNGase as described above. The two species in the mass spectrum had masses that were $77\,365 \pm 6$ and $75\,156 \pm 5$ Da; 2205 and 4414 Da, respectively, less than the mass of apotransferrin. This is in agreement with the calculated mass difference of 2205 Da⁵⁹ for the loss of one sugar chain which is within an acceptable experimental error. Since it is possible to prepare these clinically relevant CDT forms in the laboratory, standard solutions can be prepared to quantify absolute concentrations of CDT in clinical samples in the same way as demonstrated for neuraminidase-treated apo-tf.

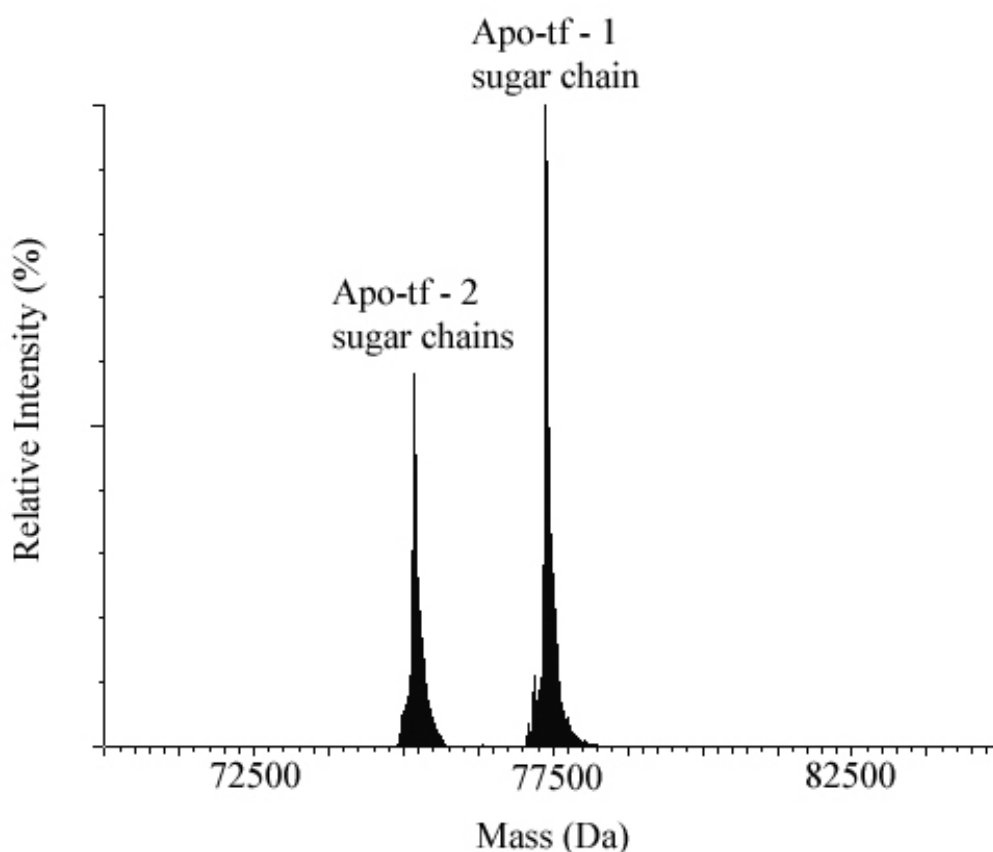


Figure 3.15. ESI mass spectrum (converted to a mass scale) of apo-tf that had lost one and two sugar chains. Denatured apo-tf was treated with PNGase at 37 °C for 24 h. Formic acid (1%) was added to the reaction mixture for ESI-MS analysis. The two species in the mass spectrum had masses of $77\,365 \pm 6$ and $75\,156 \pm 5$ Da.

3.4 Separation of transferrin from plasma using an Affi-Gel Blue affinity gel column

The methods developed above for obtaining high quality spectra of various glycosylated forms of transferrin and the determination of the concentration ranges over which linear responses could be obtained for analysis of transferrin by ESI-MS sets the stage for analysis of transferrin from plasma. The experiments described below were aimed at developing a rapid, simple and inexpensive method for separation of transferrin from plasma.

In order to be able to analyse transferrin, HSA had to be removed, since this protein is by far the most abundant in plasma.³ In the current work, a method was developed to obtain transferrin free of the more abundant plasma proteins. This is an important first step towards development of a simple, rapid method for analysing plasma transferrin by ESI-MS.

Cibacron Blue, which is the functional group of the Affi-Gel Blue affinity gel, is a dye with an aromatic triazine structure that has a high affinity for HSA. The dye can bind proteins by electrostatic, hydrophobic, aromatic and stereoselective interactions.⁹⁸ In Affi-Gel Blue affinity gel, Cibacron Blue is covalently attached to a crosslinked agarose gel. The structure of Cibacron Blue is shown in figure 3.16.

Figure 3.16. Structure of Cibacron Blue.⁹⁸

Plasma was loaded onto an Affi-Gel Blue affinity gel column equilibrated with 50 mM NH_4OAc , pH 7.4, and eluted fractions were analysed by ESI-MS. The sixth fraction contained tf at approximately a 5-fold dilution of plasma. The ESI mass spectrum of tf that was acquired is shown in figure 3.17. Acetonitrile and acetic acid were added to the fraction to aid in the desolvation and ionization of the protein, giving much better resolution and sensitivity. The mass of tf obtained was $79\,570 \pm 3$ Da. This is in agreement with the calculated mass of 79 570 Da for apo-tf consistent with dilution into this solvent resulting in dissociation of iron from the protein.

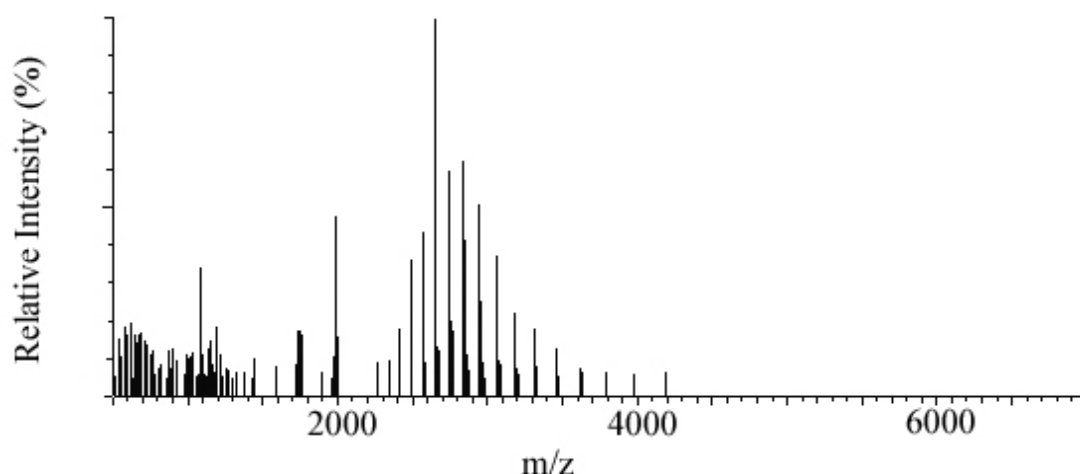


Figure 3.17. ESI mass spectrum of tf obtained from plasma. The spectrum was acquired after plasma was loaded on the Affi-Gel Blue affinity gel column and acetonitrile and acetic acid were added to improve the resolution.

The spectrum (converted to a mass scale) in figure 3.18, shows that the Affi-Gel Blue affinity gel column was highly effective at removing HSA, which was absent from the spectrum.

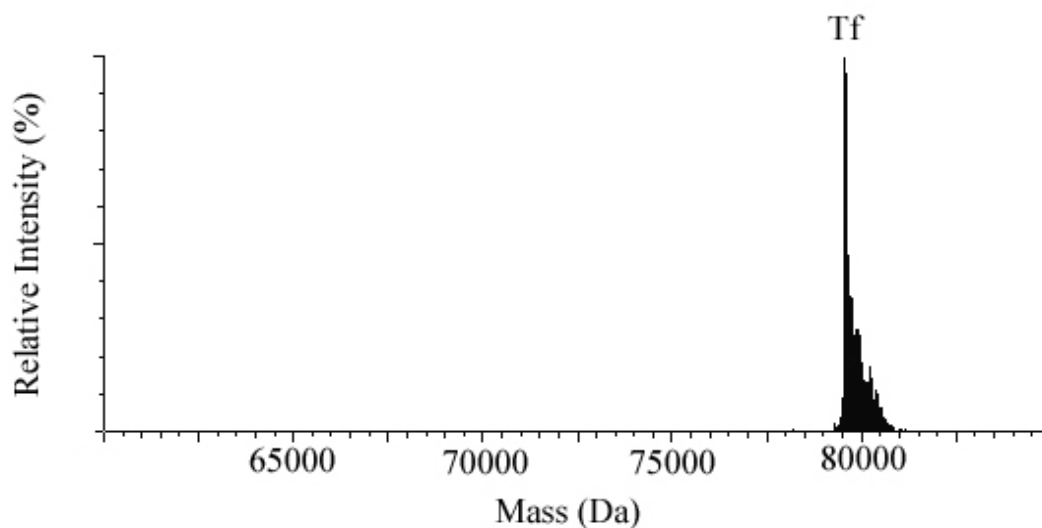


Figure 3.18. ESI mass spectrum of plasma tf converted to a mass scale. The plasma was loaded on an Affi-Gel Blue affinity gel column, which had removed all traces of HSA.

Step elution of HSA from the Affi-Gel Blue affinity gel column using increasing concentrations of ammonium acetate was attempted in order to assess how strongly the dye binds HSA. Elution buffers with concentrations ranging from 5 mM to 2 M of NH_4OAc , pH 7.4, were used. Ammonium acetate, rather than sodium chloride, was used since it is compatible with electrospray, and ESI mass spectra were directly obtained from eluted fractions. HSA did not elute prior to application of 2 M NH_4OAc to the column. Figure 3.19(a) shows the spectrum that was obtained from the eighth fraction collected with 2 M NH_4OAc . The mass was consistent with the calculated mass (66 446 Da) of HSA. Under these conditions (high salt, near neutral pH), there were two abundant ions, corresponding to $[\text{HSA} + 17\text{H}]^{17+}$ and $[\text{HSA} + 16\text{H}]^{16+}$. The width at half height of these ions was broad (40 and 42 m/z) since the ESI mass spectrum was not obtained under optimal conditions for HSA. Figure 3.19(b) shows the spectrum in (a) converted to a mass scale.

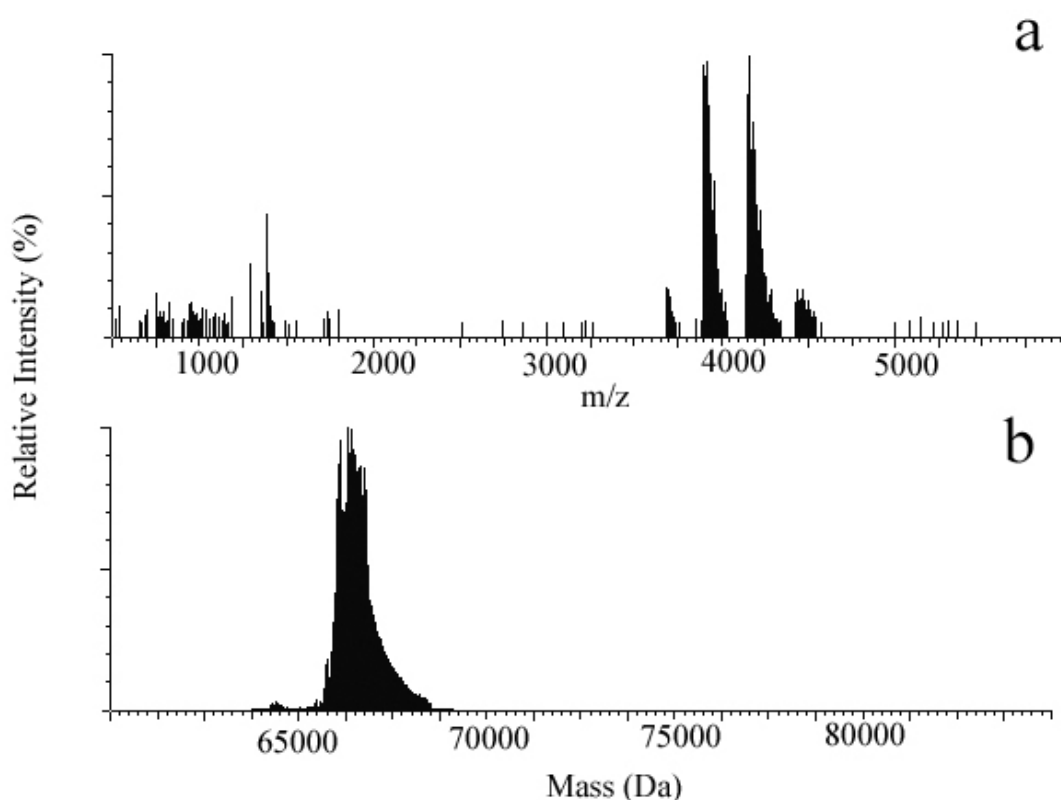


Figure 3.19. ESI mass spectra of plasma HSA eluted from the Affi-Gel Blue affinity gel column. (a) ESI mass spectrum of HSA. (b) Spectrum of HSA converted to a mass scale. HSA was eluted from the column with 2 M NH_4OAc and the column equilibrated with 50 mM NH_4OAc .

Since HSA was successfully eluted from the Affi-Gel Blue affinity gel column, the same column can in principle be reused after washing to separate tf from fresh plasma samples.

Given that Cibacron Blue-agarose was so effective at removing HSA from plasma, an attempt was made at developing a simpler and faster method for small scale purification of tf from plasma using the Affi-Gel Blue affinity gel resin. Initially, 300 μl of Affi-Gel Blue affinity gel was mixed gently with 200 μl of plasma in an Eppendorf tube and the mass spectrum of the supernatant after centrifugation was acquired. HSA was present in the spectrum and tf could not be conclusively identified in these initial experiments.

This suggests that, under these conditions, the HSA binding sites on the Affi-Gel Blue affinity gel were saturated, leaving substantial amounts HSA remaining in the supernatant.

Bio-Rad Micro Bio-Spin columns are small chromatographic columns with a built in frit, that fits inside Eppendorf tubes. Affi-Gel Blue affinity gel (1 ml) was added to the column, 75 μ l of plasma loaded and the column centrifuged for 4 minutes. An ESI mass spectrum was acquired of the flowthrough. The flow-through was diluted 1/5 in 1% formic acid prior to mass spectrometry in order to improve the quality of the mass spectra. The Bio-Spin column did not remove all traces of HSA; both HSA and tf were observed as shown in the spectrum (converted to a mass scale) in figure 3.20. Although, HSA was present in the eluent along with tf, the treatment (Affi-Gel Blue affinity gel in Bio-Spin column) greatly reduced the amount of HSA, so that tf could be detected. This method could therefore be used to analyse transferrin from the plasma of patients. The minimum time taken for the procedure (spinning of plasma, ESI-MS analysis) was 10 minutes.

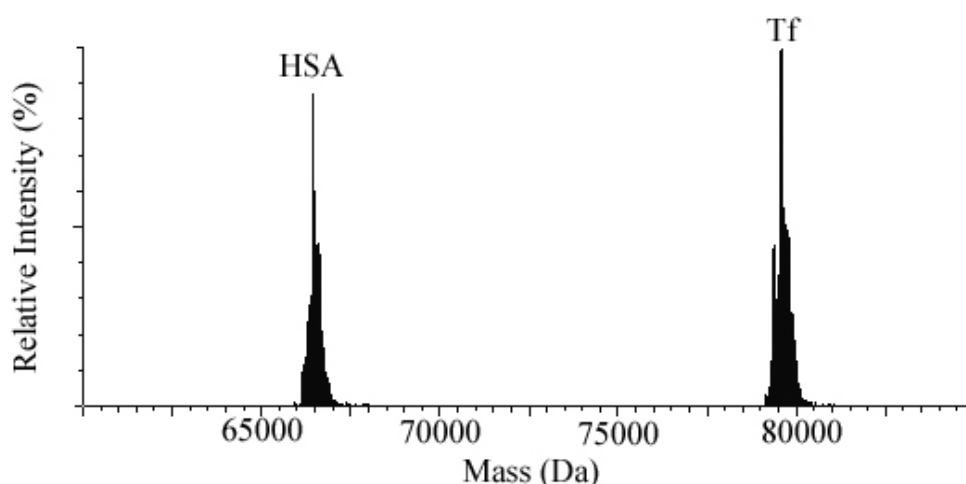
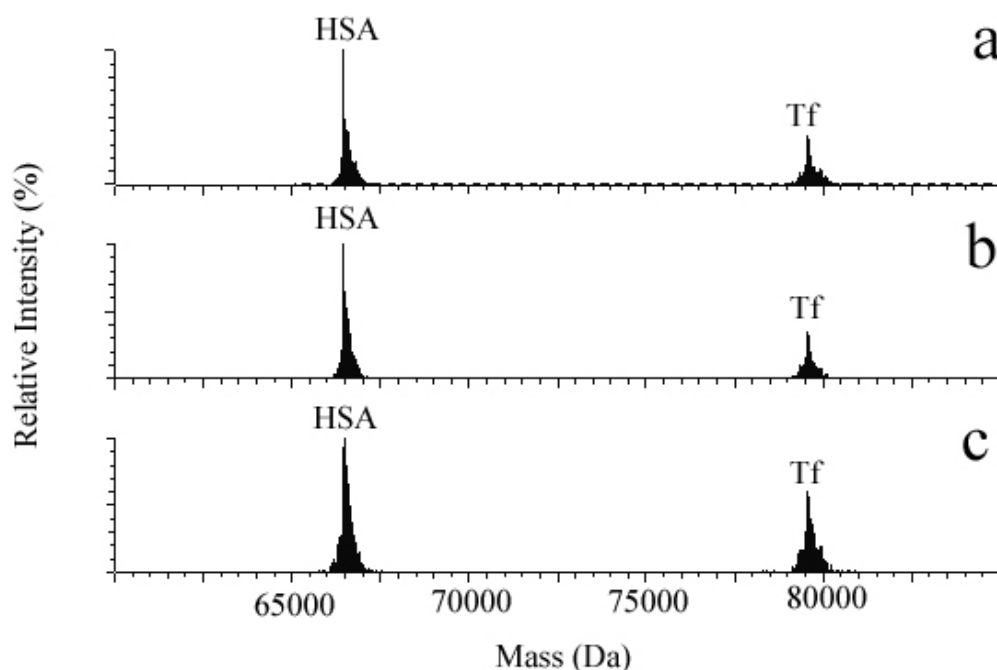


Figure 3.20. ESI mass spectrum of eluent after loading plasma (75 μ l) onto a Bio-Spin Affi-Gel Blue affinity gel column. The masses of HSA and tf were in agreement with the calculated masses for each protein.

Different amounts of plasma were loaded onto the Bio-Spin Affi-Gel Blue affinity gel column in order to establish the smallest amount of plasma that could be used to analyse CDTs. Figure 3.21 shows the ESI mass spectra (converted to a mass scale) acquired after passage of 75 (maximum sample volume), 50 and 25 μ l of plasma through the Bio-Spin column. The 25 μ l aliquot was the minimum amount of sample that gave a good working spectrum of tf. The relative amounts of HSA and transferrin present in the column eluents as judged by ESI-MS in this experiment were not the same as in the experiment shown in figure 3.20, suggesting that there was some variability from column to column. Even though HSA was observed at a higher ratio than tf in the spectra in figure 3.21, this experiment confirms that this small scale fractionation method is a fast and convenient way to separate tf from plasma for analysis by ESI-MS. The method could be used to detect carbohydrate deficient transferrin in plasma.



Figures 3.21. ESI mass spectra of different volumes of plasma loaded on an Affi-Gel Blue affinity gel Bio-Spin column. Figures (a), (b) and (c), show the spectra (transformed to a mass scale) that were acquired when 75, 50 and 25 μ l aliquots of plasma were loaded on the Bio-Spin column, respectively. The smallest amount of sample that could be loaded on the column was 25 μ l. HSA was observed at higher abundance than tf.

Three previous studies were found in the literature that have used ESI-MS to distinguish between the different glycosylated forms of transferrin present in plasma.^{57, 59, 81} All of these methods required extensive sample preparation steps. For example, in one study, three chromatographic columns were linked online to a mass spectrometer, including an immunoaffinity column that had to be prepared in the laboratory. Plasma samples had to be iron-saturated and air-dried on a blood-spot cartridge with an adsorptive membrane (which was the first chromatographic step) and finally eluted through a post-concentration cartridge prior to ESI-MS.⁸¹ In another study, plasma samples were diluted in water (1:5) and loaded onto an immunoaffinity column in a LC-MS procedure that took 10 minutes.⁵⁷ The most recent work, required no iron-saturation procedures.⁵⁹ The following three step analytical procedure was developed: anion-exchange chromatography (first step), followed by SpeedVac concentration (second step) and finally desalting and analysis by HPLC-ESI-MS (third step).⁵⁹

The advantage of the method proposed in the current work compared to the methods described above, is that it requires less extensive preparation steps such as preparation of an immunoaffinity column and iron-saturation of plasma, as well being a rapid method. The use of HPLC can be avoided and a working spectrum of CDT acquired from plasma through a simple spin chromatographic step using Affi-Gel Blue affinity gel. The method is also less expensive as it only requires low-cost equipment such as the affinity gel and the Micro Bio-Spin chromatography column, apart from the mass spectrometer.

The concentration of tf that had been fractionated from plasma on an Affi-Gel Blue affinity gel Bio-Spin column, was determined from a standard curve. Figure 3.22

shows the standard curve that was obtained from solutions of commercial apo-tf in 1% formic acid with known concentrations (judged by A_{280}). Tf from plasma eluted from the Bio-Spin column using 50 mM NH_4OAc , pH 7.4, was diluted 1/10 in 1% formic acid prior to ESI-MS. The ion count of plasma tf was obtained and the corresponding concentration of tf calculated using the standard curve. The concentration of tf in the fraction, after compensating for the 1/10 dilution, was 0.36 μM . Since 75 μl of plasma was loaded on the column, and 200 μl of flow-through obtained, this accounts for another 2.7-dilution, giving an initial concentration of tf in the sample of 9.7 μM . The literature value for tf in plasma is 35 μM .⁶ The inconsistency in the two values can very likely be attributed to a loss of tf during the fractionation step. It is necessary to estimate this loss in order to quantify tf species in plasma by this method, but this was not attempted in the current work due to time constraints. Clearly this experiment needs to be repeated alongside an experiment in which an aliquot of a known quantity of apo-tf is treated in the same way to estimate losses. Nevertheless, the relative amounts of different forms transferrin will be determined as long as one form is not preferentially retained on the column over another.

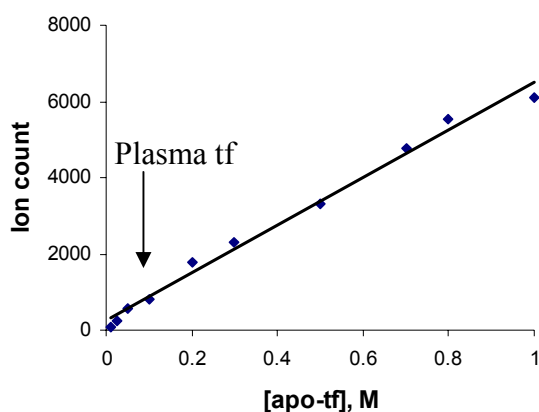


Figure 3.22. Standard curve for ESI-MS response of apo-tf. The ion count versus concentration curve obtained for commercial apo-tf in 1% formic acid, was used to quantify the concentration of tf in the eluent obtained by passing plasma through an Affi-Gel Blue affinity Micro Bio-Spin column. The value was later adjusted for the 1/10 dilution prior to ESI-MS.

3.5 Separation of transferrin from cerebrospinal fluid (CSF)

Experiments were also conducted in order to separate tf from CSF. CSF contains significantly lower concentrations of proteins compared to plasma, as shown in table 3.3. The fractionation methods applied to plasma may therefore not be appropriate for CSF or a concentration step may be required prior to analysis. In a preliminary experiment, CSF was diluted 30-fold with a 2.5 mM NH_4OAc and 3% acetic acid solution in order to determine whether the masses of abundant proteins in CSF could be determined with minimal treatment. High quality ESI mass spectra of HSA can be acquired by 600-fold dilution of plasma.⁹² It was not possible to resolve a spectrum of any protein by dilution of CSF. Further, there were abundant peaks that most likely arose from man-made polymers as evidenced by a constant mass difference between the peaks. In a subsequent experiment, CSF was chromatographed on a Sephadex G-75 column and spectra of HSA and tf were obtained from concentrated fractions, as seen in the spectrum transformed to a mass scale in figure 3.23. The spectra of the pooled, concentrated fractions were less resolved and had broader peaks than those obtained from plasma.

Although the spectrum in figure 3.23 shows only two proteins, it should be noted that the spectrum contained many minor peaks and there was a low signal-to-noise ratio. The transformation software revealed only the major species since it was set up to ignore peaks of low intensity among the noise. The low signal-to-noise accounts for the broadness of the peaks.

Table 3.3. Protein concentration in plasma and CSF. Data from reference 3.

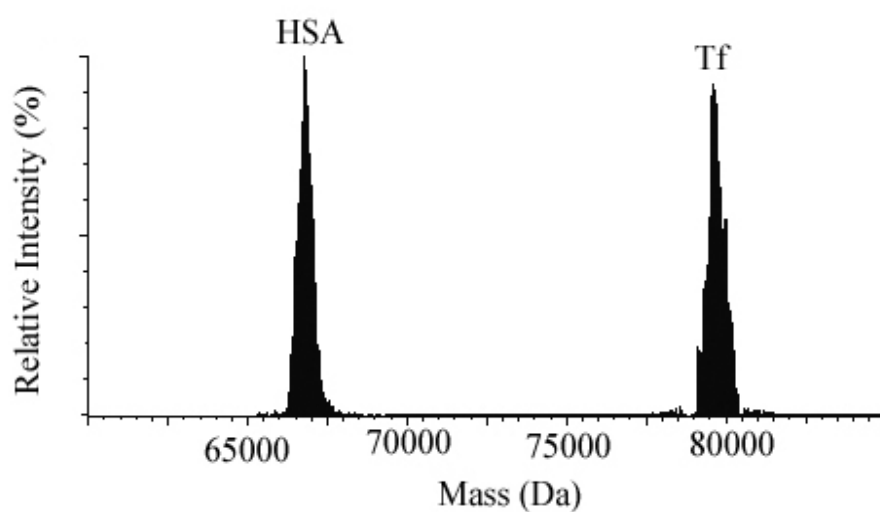


Figure 3.23. ESI mass spectra acquired of CSF that had been loaded on a Sephadex G-75 column. Three x 400 μ l fractions were pooled and concentrated to 100 μ l (12-fold concentration) and diluted 1 in 5 in 1% formic acid for ESI-MS. HSA and tf were observed in the spectrum (converted here to a mass scale).

In order to improve ESI-MS analysis of CSF, CSF was concentrated ~20-fold (75 μ l) and loaded onto a Micro Bio-Spin column containing Affi-Gel Blue affinity gel as for plasma (above). The eluent from the column was diluted 1 in 5 into 1% formic acid for ESI-MS. Figure 3.24 shows the spectrum that was acquired.

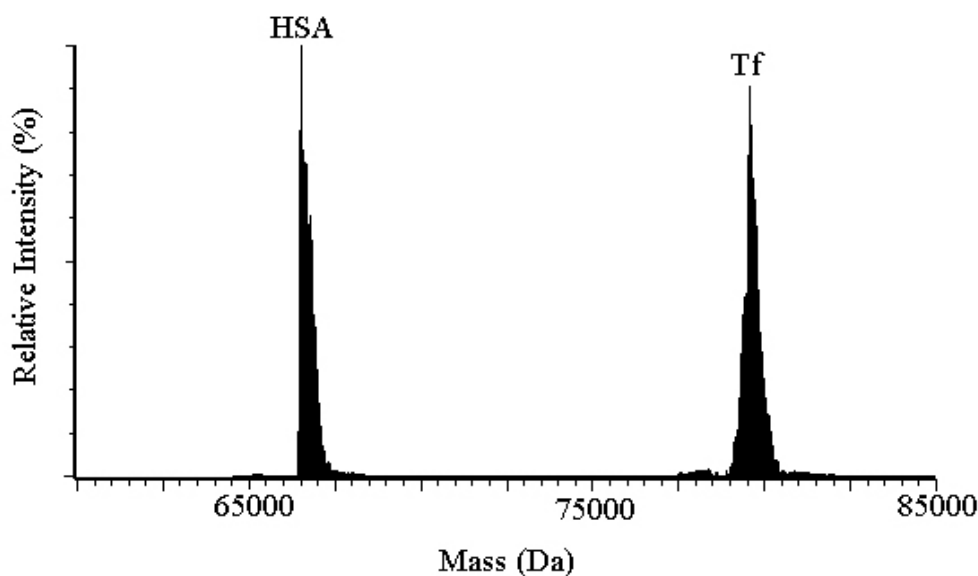


Figure 3.24. ESI mass spectrum of concentrated CSF loaded on an Affi-Gel Blue affinity gel Micro Bio-Spin column. The spectrum has been converted to a mass scale. CSF was sprayed from 1% formic acid.

Since transferrin is 186-fold higher in concentration in plasma than in CSF (table 3.3), the 20-fold concentration step leaves the concentration of transferrin in the CSF eluted from the column approximately 9-fold lower than the concentration of transferrin when plasma was loaded onto the column. Accordingly, the spectrum was of poorer quality. Nevertheless, this experiment shows that it is possible to analyse tf in CSF using ESI-MS after rapid fractionation of concentrated CSF on an Affi-Gel Blue affinity gel Micro Bio-Spin column. However, further method development with CSF is necessary in order to improve the spectra so that CDT forms that might be present can be distinguished and quantitative data obtained.

CHAPTER 4: TRANSFERRIN WITH VARYING IRON LOAD

It is clinically important to be able to assess the status of an individual's iron reserves in the diagnosis, prognosis and management of diseases such as iron deficiency anemia¹⁰⁰ and hemochromatosis,¹⁰¹ which is a disease characterized by progressive iron overload leading to clinical complications such as diabetes mellitus, liver dysfunction and a bronze pigmentation of the skin.¹⁰² Transferrin is one of the proteins commonly used for the diagnostic measurement of the body's iron stores. Inflammation down-regulates the synthesis of transferrin and is often taken into account when interpreting tf iron measurements.¹⁰³ Frequently, iron in transferrin is not measured directly from holo-tf but concentrations of serum transferrin receptor (sTRF) are measured^{100, 104} which is effective at detecting iron deficiency associated with anemia in chronic diseases such as rheumatoid arthritis. The results are often given as the percentage of iron saturation (TSAT), which is computed from levels of tf and free iron in plasma.^{100, 101, 103} Transferrin immunoassay kits are used to measure tf levels.¹⁰³

The following experiments were carried out to determine whether ESI-MS could be used to distinguish between apo-tf, Fe-tf and Fe₂-tf, potentially providing a simpler method of estimating TSAT in plasma samples.

4.1 Preparing holo-tf from commercial apo-tf

In early experiments, an ESI mass spectrum of holo-tf from Sigma (spray solvent, 0.1 M NH₄HCO₃, pH 8.2) gave a mass for fully glycosylated transferrin of $79\,678 \pm 1$ Da

(figure 3.10 (a)). When Sigma holo-tf was sprayed from 1% formic acid (where iron is expected to be lost), the mass of fully glycosylated transferrin was $79\,571 \pm 1$ Da (figure 4.1 (b)). The difference in mass is 107 Da. The mass of two iron atoms is 111.7 Da (Table 4.1). Taking into account the errors in masses, comparison of these spectra therefore suggest that Fe₂-tf (holo-tf) will be able to be resolved from apo-transferrin in ESI mass spectra. The spectra of Sigma holo-tf were complicated, however, by the presence of forms of transferrin with various numbers of sialic acid residues (figure 3.10). Therefore, holo-tf was prepared from commercial apo-tf in the laboratory in order to compare resolution of holo-tf and apo-tf in ESI mass spectra without the complication of the presence of various glycosylated forms of the protein.

Apo-tf was reacted with ferric nitrate in 0.1 M NH₄HCO₃, pH 8.2, at a protein to iron ratio of 1:2. Some methods for preparing Fe₂-tf⁹⁹ complex iron with nitrilotriacetic acid (NTA) in order to prevent precipitation of ferric hydroxides. However, the solution remained stable and did not precipitate when used immediately after preparation and there was no need to add NTA.

As transferrin binds iron, it takes on a characteristic intense red-brown colour (λ_{max} 470 nm) arising from tyrosinate \rightarrow Fe (III) charge transfer bands. The absorption of the solution at A₄₇₀ was monitored over 2 hours. The final concentrations of tf and iron in the solution were 87 μ M and 188 μ M, respectively. Using a molar absorption coefficient at 470 nm of 2310 M⁻¹ cm⁻¹ for holo-tf,⁸⁶ the expected change in absorbance at 470 nm if two iron atoms bound to the protein, was 0.200. The observed change in absorbance was 0.198, suggesting that apo-tf had successfully bound two irons.

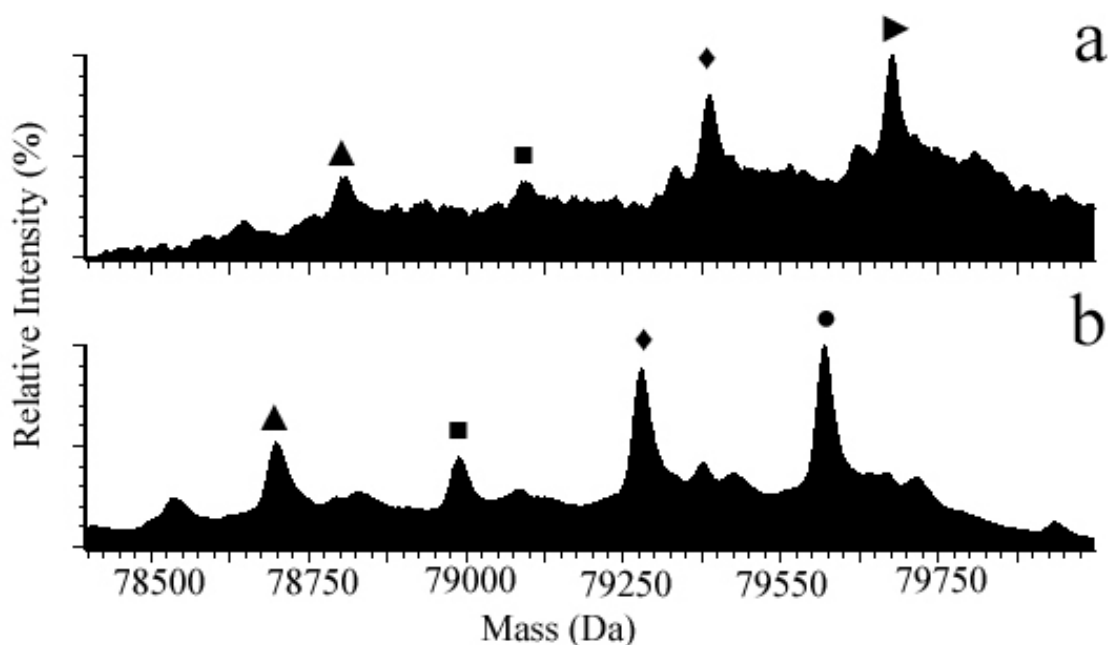


Figure 4.1. ESI mass spectrum of commercial holo-tf in 0.1 M NH_4HCO_3 , pH 8.2 (a), and in 1% formic acid (b). Holo-tf reverted to apo-tf in acid. The figures are labelled in the following way: (▶) corresponds to intact holo-tf, (●) corresponds to intact apo-tf, (◆) corresponds to apo- or holo-tf with 3 SA residues, (■) corresponds to apo- or holo-tf with 2 SA residues and (▲) corresponds to apo- or holo-tf with 1 SA.

The holo-tf solution that was prepared in the laboratory was dialysed against 0.1 M NH_4HCO_3 , pH 8.2, to determine whether the iron was stably bound to the protein. After dialysis, the mixture retained its colour and ESI mass spectra were acquired to determine the mass of the protein. The spectra were acquired using 0.1 M NH_4HCO_3 , pH 8.2 as solvent, rather than acid, in an attempt to ensure that the iron would remain bound to the protein. Figures 4.2 (a) and (b) show the spectra converted to a mass scale, of commercial apo-tf and holo-tf that was prepared in the laboratory, respectively. Both solutions were prepared and sprayed in the NH_4HCO_3 solution. The peak in the spectrum of holo-tf was broader than that of apo-tf.

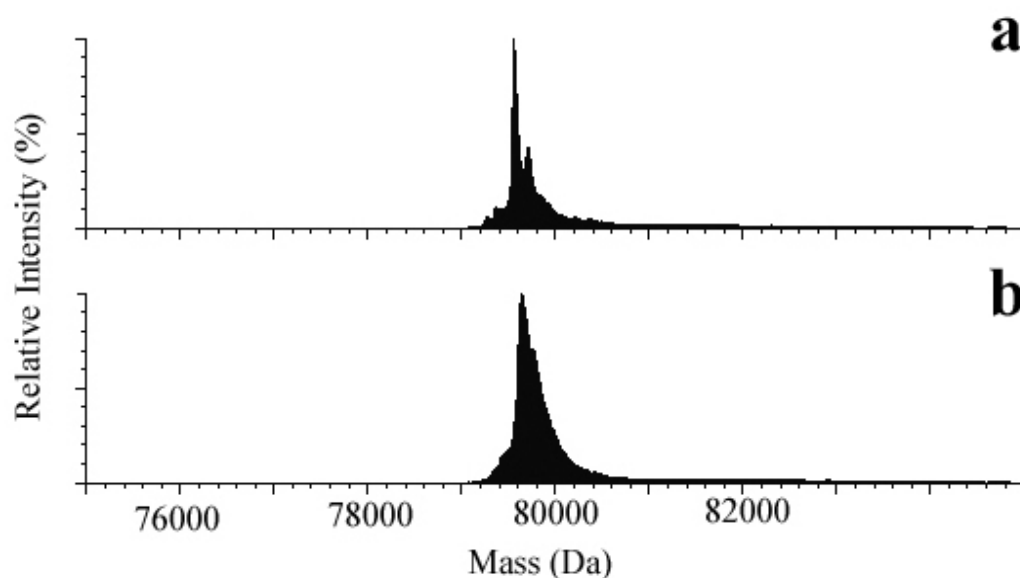


Figure 4.2. ESI mass spectra of commercial apo-tf and holo-tf prepared in the laboratory. The spectra are converted to a mass scale. (a) Apo-tf with a mass of $79\,569 \pm 1$ Da. (b) Holo-tf that was made in the lab, with a mass of $79\,680 \pm 3$ Da. Both apo- and holo-tf were sprayed from 0.1 M NH_4HCO_3 , pH 8.2.

The mass obtained for apo-tf of $79\,569 \pm 1$ Da was in agreement with the calculated value for apo-tf. An unidentified shoulder was observed on the apo-tf peak (to higher m/z) with a mass ~ 147 Da higher than apo-tf in the spectrum shown in figure 4.2 (a). This peak was also present when apo-tf was dissolved in 1% formic acid (figure 4.3 (a)) but did not appear to be present or was not resolved in tf fractionated from plasma (figure 4.3 (b)). The peak was however present in commercial holo-tf prepared in 1% formic acid as shown in figure 4.3 (c), where the iron has dissociated from the protein due to the low pH value. Since the peak appeared to be absent from plasma tf, it suggests that the presence of the peak in commercial apo- and holo-tf might be a side product of the commercial preparation.

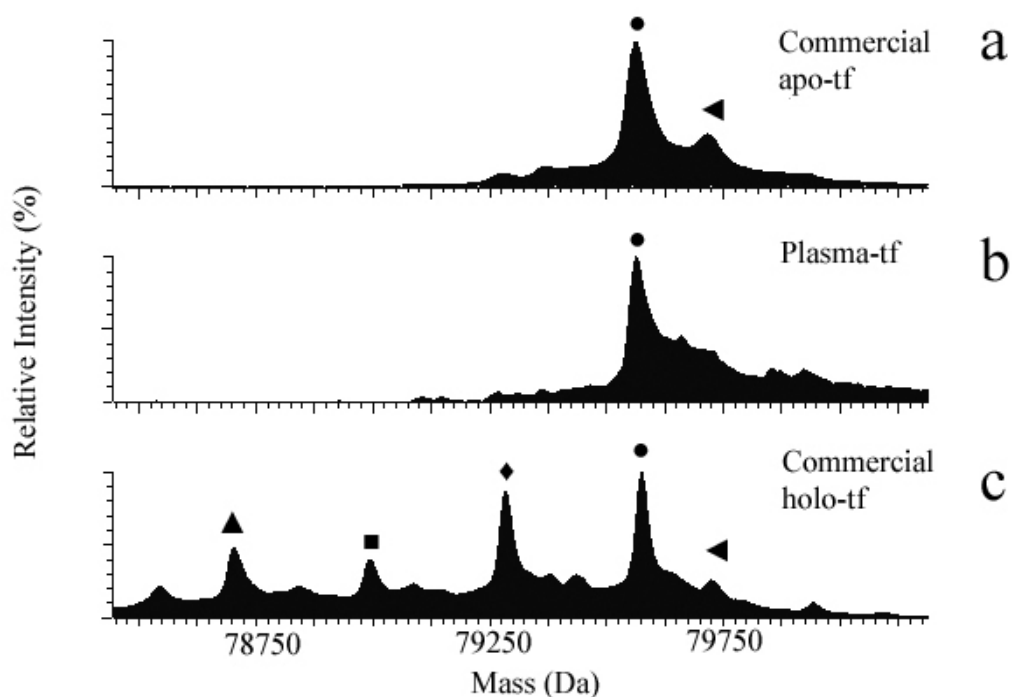


Figure 4.3. ESI mass spectra of commercial apo- (a) and holo-tf (c) and plasma tf (b) run in 1% formic acid. (a) Shows that the extra peak (◄) from a species of commercial apo-tf with a mass of 147 Da higher than the mass of apo-tf, is also observed in 1% formic acid, while (b) shows that the peak was absent from tf fractionated from plasma and (c) shows that the peak was present in commercial holo-tf prepared in 1% formic acid. The peaks are labelled in the following way: (●) corresponds to intact tf with 4 SA residues, (◆) corresponds to tf with 3 SA residues, (■) corresponds to tf with 2 SA residues and (▲) corresponds to tf with 1 SA residue.

The mass obtained for holo-tf prepared in the laboratory was $79\,680 \pm 3$ Da (figure 4.2) and was 111 Da higher than the mass obtained for apo-tf. Table 4.1 shows the increases in mass of apo-tf expected for the binding of various combinations of iron and bicarbonate.

Table 4.1. Calculated increases in mass of apo-tf with the binding of Fe^{3+} and HCO_3^- .

	+ Fe^{3+}	+ HCO_3^-	+ Fe^{3+} + HCO_3^-	+ 2 Fe^{3+}	+ 2 Fe^{3+} + HCO_3^-	+ 2 Fe^{3+} + 2 HCO_3^-
Mass Difference (Da)	55.85	61.02	116.86	111.69	172.71	233.72

The mass difference therefore corresponds to two iron atoms. In solution, transferrin has been shown to bind iron only in the presence of bicarbonate.⁶ Our data suggest that bicarbonate dissociates from the transferrin in the mass spectrometer. Conclusions based on the spectra shown in figure 4.2 should be considered with caution, however, since the peaks were broad. In a previous study by Gumerov and Kaltashov,⁷⁹ they stated that at low desolvation temperature and minimal collisional activation in the cone region (zero skimmer potential), they were able to detect the synergistic oxalate ion which proceeds to dissociate from the protein at higher skimmer potentials. In a later work,⁸⁰ they specified that due to wide peak widths, it was difficult to differentiate whether they were observing tf with two bound iron atoms or tf bound to a synergistic oxalate and one iron atom. They concluded that it was most likely that the synergistic anion (oxalate in this case) dissociated in the mass spectrometer.

In our experiment (figure 4.2), the iron appears to remain bound to tf for the time frame necessary for detection. The poor resolution achieved under these conditions makes an accurate mass determination of holo-tf difficult. While lowering the pH is expected to result in narrowing of peak widths by enhancing desolvation, it is also expected to result in dissociation of iron from the protein. Holo-tf only exists *in vivo* at the extracellular, physiological pH of 7.4. Inside the cell, where the pH is lower, iron is released from holo-tf which reverts into apo-tf (see chapter 1). This effect of pH was confirmed in the laboratory by adding acid to commercial holo-tf.

The initial absorbance of a holo-tf solution at 470 nm was 0.348. Formic acid was added to holo-tf giving a final concentration of 1% (v/v). Within seconds of adding the acid, the red colour of holo-tf disappeared. The absorbance flattened out at 0.012,

suggesting that holo-tf had instantaneously lost both its irons. Therefore, as expected, there would be no holo-tf present in solutions of tf sprayed from 1% formic acid.

Decreasing the pH of the spraying solution increased sensitivity and resolution of ESI mass spectra of tf (Section 3.2.1). These conditions are incompatible with the stable ligation of iron to transferrin. The following experiments were carried out to determine the lowest pH and the lowest bicarbonate concentration that could be used to prepare holo-tf from apo-tf as judged by measuring A_{470} values of transferrin/iron mixtures.

4.2 Investigating the role of the bicarbonate ion in iron binding by tf

The bicarbonate ion is a synergistic ion necessary for the binding of iron to tf.⁶ The minimum concentration of NH_4HCO_3 necessary for tf to bind iron was investigated. Apo-tf (0.1 mM) was prepared in a 0.1 M NH_4OAc solution at pH 8.21 containing 0.1 mM NH_4HCO_3 , and iron added as described earlier. The absorbance at 470 nm, showed that there had been no iron binding at this concentration of NH_4HCO_3 . No iron binding was observed when the NH_4HCO_3 concentration was 10 mM. Binding of iron to tf was not observed before the concentration of NH_4HCO_3 was increased to 20 mM NH_4HCO_3 . The calculated value of A_{470} for holo-tf was 0.224, which is in good agreement with the experimental value of 0.208 (1.86 iron atoms per tf molecule).

This sample was dialysed against 0.1 M NH_4OAc at pH 8.21 containing 20 mM NH_4HCO_3 and an ESI mass spectrum obtained using this spray solvent is shown in figure 4.4 (a). Even though the resolution had improved, as indicated by the slight reduction in peak width observed in this spectrum compared to that observed when

holo-tf was prepared in and dialysed against 0.1 M NH_4HCO_3 (figure 4.4 (b)), the improvement was not adequate to determine the difference between the binding of two iron atoms or one iron and one bicarbonate. The mass difference between apo-tf and tf treated in this way, was 112 ± 14 Da (the error is the sum of the error of each value), while the mass difference between apo-tf and Fe_2 -tf prepared in 0.1 M NH_4HCO_3 was 111 ± 4 Da.

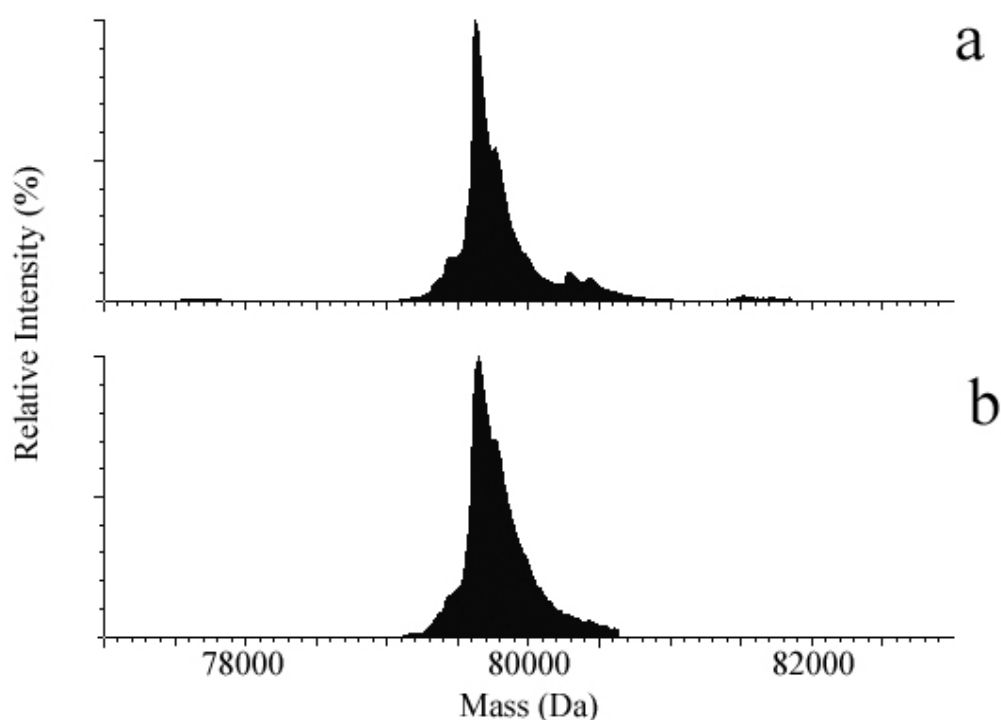


Figure 4.4. ESI-MS of holo-tf prepared from apo-tf in the laboratory, transformed to a mass scale. (a) Shows holo-tf that was prepared in 0.1 M NH_4OAc with 20 mM NH_4HCO_3 , pH 8.2 and (b) shows holo-tf prepared in 0.1 M NH_4HCO_3 , pH 8.2. The peak width at half height of each ion in the spectrum in (a) was 181 amu and 309 amu in (b). The peak width at half height in the transformed spectrum in (a) was 12 amu and 15 amu in (b).

An attempt was also made at binding iron to apo-tf in the 20 mM NH_4HCO_3 in 0.1 M NH_4OAc solution at a lower pH value, in an effort to narrow the peak width and improve sensitivity. Iron was added to apo-tf in the above solution at pH 6. The

calculated absorbance at 470 nm for this concentration of holo-tf was 0.224 and the observed value was 0.170, indicating that 1.5 atoms of iron had been bound.

The ESI mass spectrum (not shown), gave a mass for tf that was consistent with the proposal that the transferrin did not carry its full complement of iron, with an observed increase in mass of 49 ± 19 Da (corresponding to 1 Fe-tf). The mass spectrum did not show any evidence that the bicarbonate ion was bound. Previous ESI-MS work investigating the pH-induced release of iron from holo-tf,⁸⁰ indicated that the protein fully retains both irons until a pH of 5.5. Although this appears to be refuted by both the spectrophotometric and mass spectrometric findings of the current work, the two experiments are investigating different issues: the retention of iron compared with the binding of iron.

4.3 Tf and dysprosium

An experiment was designed in order to confirm that the bicarbonate ion was not being observed in the ESI mass spectrum of holo-tf due to dissociation in the gas phase. If the bicarbonate ion did not dissociate in the mass spectrometer, there was a possibility that what was observed was one iron molecule and one bicarbonate ion, since they have similar masses of 55 and 60, respectively, and not necessarily two iron molecules. Another metal with a mass that differed significantly from the bicarbonate ion was added to tf in order to investigate whether the bicarbonate ion would then be observed. Since tf binds lanthanides, dysprosium which has a mass of 162 was added to apo-tf. Tf binds lanthanides at the iron binding site in the presence of the synergistic bicarbonate ion.¹⁰⁵ A dysprosium solution (300 μ M) was added to an apo-tf solution

(70 μ M) prepared in 0.1 M NH_4HCO_3 , pH 8.2. Table 4.2 shows the calculated mass increases of apo-tf for the binding of various combinations of dysprosium and bicarbonate. The ESI mass spectrum that was acquired gave masses of tf that corresponded to tf binding one Dy^{3+} ion (a mass increase of 158 ± 13 Da) and two Dy^{3+} ions (mass increase of 330 ± 13 Da), as shown in the spectrum (transformed to a mass scale) in figure 4.5. The synergistic bicarbonate ion was not observed, confirming that the bicarbonate ion does indeed appear to dissociate from tf in the gas phase in the mass spectrometer.

Table 4.2. Calculated increases in mass of apo-tf with the binding of Dy^{3+} and HCO_3^- .

	+ Dy^{3+}	+ HCO_3^-	+ Dy^{3+} + HCO_3^-	+ 2 Dy^{3+}	+ 2 Dy^{3+} + HCO_3^-	+ 2 Dy^{3+} + 2HCO_3^-
Mass Difference (Da)	162.50	61.02	226.52	325.00	392.02	453.04

Although this experiment supports the hypothesis that metal-transferrin molecules lose the HCO_3^- in the gas phase, it is possible that the Dy^{3+} bound at non-specific sites (sites other than the iron-binding sites), and therefore HCO_3^- was not necessary for binding. This seems unlikely, since only a 3-fold excess of Dy^{3+} was present and UV-vis spectroscopy and stopped-flow spectrophotometry have previously shown that tf binds lanthanides at its iron-binding site.¹⁰⁵

The results of these experiments may provide the bases for another method for determining the extent of iron binding by tf in plasma based on the results that showed a dysprosium-tf complex could be detected by ESI-MS. Dysprosium could be added to

plasma, where it is known that species of tf are present with zero, one or two bound irons. It might be possible to observe if dysprosium reacts at the metal-free binding sites, giving new peaks corresponding to tf with one iron and one dysprosium ion or tf with two dysprosium ions. The success of this method will rely on the results of experiments aimed at determining whether dysprosium displaces iron. If dysprosium does not displace iron, then this may form the basis of a method for obtaining a measure of the percentage saturation of tf with iron.

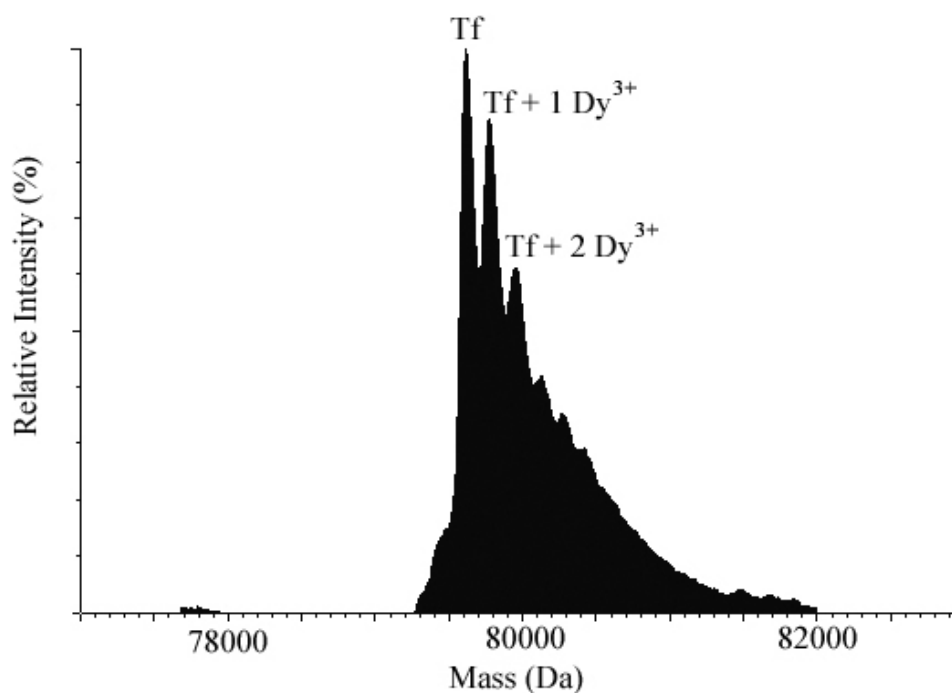


Figure 4.5. ESI mass spectrum of apo-tf that has bound dysprosium ions. The spectrum has been transformed to a mass scale. A mass increase of 158 ± 13 Da was observed for apo-tf that had bound one Dy^{3+} ion and a mass increase of 330 ± 13 Da was observed for apo-tf that had bound two Dy^{3+} ions. The bicarbonate ion was not observed.

CHAPTER 5: DETECTION OF LOW ABUNDANCE PLASMA PROTEINS

Previous research⁹² and the current work show that HSA and tf can be rapidly analysed in plasma by ESI-MS simply by dilution (HSA) or by spinning through Affi-Gel Blue affinity gel (transferrin). It will be useful to determine whether proteins in the next tier of abundance in plasma can also be rapidly analysed. Agilent Technologies have recently developed a spin cartridge for removing abundant proteins from plasma.¹⁰⁶ This product was tested in the following experiment.

Plasma was loaded on a high capacity multiple affinity removal spin cartridge from Agilent. This column specifically removes six of the most abundant plasma proteins: HSA, IgG, α_1 -antitrypsin, IgA, tf and haptoglobin from plasma, allowing analysis of the less abundant plasma proteins. The protein fraction eluted from the column was dialysed against 50 mM NH_4OAc , pH 7.4, and diluted 1/5 in 1% formic acid prior to ESI-MS.

Figure 5.1 (a) shows the ESI mass spectrum that was acquired, while (b) shows the spectrum transformed to a mass scale. The spectrum features three species with masses of $28\,131 \pm 5$ Da (A), $17\,296 \pm 9$ Da (B) and $51\,212 \pm 11$ Da (C).

The identities of these peaks have not been positively determined. An initial ExPasy¹⁰⁷ protein database search found that the proteins that were closest in mass to peaks A, B and C in figure 5.1 (b) were, respectively: (i) Microfibrillar-associated protein 5 precursor (17 294 Da); (ii) tissue-type plasminogen activator precursor protein (28 122

Da), and (iii) complement C1r subcomponent precursor protein (51 191 Da). The last two proteins are plasma proteins,¹⁰⁷ while microfibrillar-associated protein 5 precursor is secreted from tissues such as skeletal muscle into the extracellular matrix.¹⁰⁷

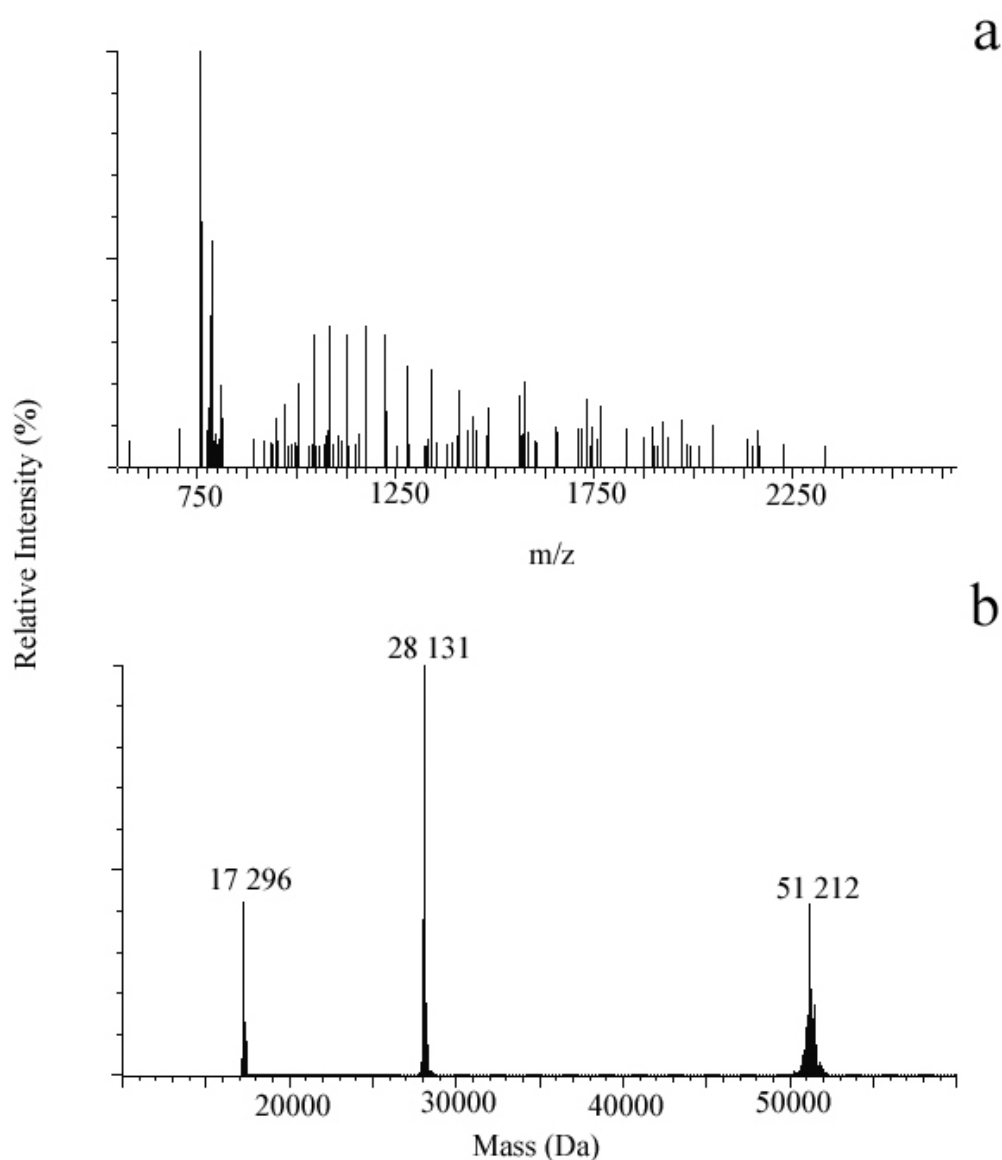


Figure 5.1. ESI mass spectra of plasma obtained from the Agilent column. Figure (a) shows the spectrum acquired, containing three low abundant plasma species with masses of $17\,296 \pm 9$ Da (A), $28\,131 \pm 5$ Da (B) and $51\,212 \pm 11$ Da (C), and (b) shows the spectrum in (a) converted to a mass scale.

While the masses of these proteins agree with the masses of the species shown in figure 5.1 (b) considering the quality of the original spectrum (figure 5.1 (a)), and that these proteins are glycosylated, the observed masses would be expected to be considerably higher. The identities of these proteins will need to be unequivocally determined by sequence analysis by tryptic digestion and tandem mass spectrometry of the resulting peptides.

CHAPTER 6: CONCLUSIONS

Investigation of the ESI mass spectrometric response of commercial apo-tf in different solutions, showed that 1% formic acid gave spectra of highest quality, providing the most reliable mass determination. However, the dynamic range of apo-tf in 1% formic acid was limited compared to that obtained when apo-tf was sprayed from ammonium bicarbonate and ammonium acetate.

ESI-MS was shown to resolve the various forms of deglycosylated forms of tf. The optimal deglycosylation conditions determined for commercial apo-tf, were successfully applied to tf separated from plasma. Furthermore, CDT forms were purified by HPLC, mixed together at known concentrations and a response curve obtained for each CDT form. This is the first step in the determination of a method for quantifying the relative amounts of carbohydrate deficient forms of transferrin in plasma.

Optimal conditions for the deglycosylation of commercial apo-tf with PNGase were also determined, showing that it is possible to prepare clinically relevant CDT forms in the laboratory for use as standards. In future experiments, these CDT forms will be purified by HPLC and response curves for mixtures of these proteins will be determined.

ESI-MS analysis showed that Affi-Gel Blue affinity gel effectively removes HSA from plasma making it possible to analyse tf. The method was robust and inexpensive as it was shown that HSA can be eluted and the column reused for fresh plasma samples. A

rapid analytical method for the small scale fractionation of tf from plasma was also developed using Micro Bio-Rad Bio-Spin columns filled with Affi-Gel Blue affinity gel. The whole process takes 10 minutes and is a convenient method to rapidly analyse tf from plasma samples. This work differs from other studies since tf and CDT in plasma can be separated from other proteins simply and inexpensively, without the use of rigorous methods such as HPLC.

The Affi-Gel Blue affinity gel Micro Bio-Rad Bio-Spin column was also used in an attempt to estimate the concentration of tf in plasma by ESI-MS. Taking into account the approximate dilution factor, the result differed from the literature value due to the loss of tf on the column. In future work, the loss of tf on the column will be quantified by loading a known amount of tf on the column and calculating the difference in concentration between the eluent and the tf sample that was loaded.

Gel filtration of CSF on a Sephadex G-75 column resulted in fractions containing transferrin as judged by ESI-MS. However, a higher quality spectrum was obtained when CSF was concentrated several-fold and applied to an Affi-Gel Blue affinity gel Micro Bio-Rad Bio-Spin column. In future work, ion-exchange or immunoaffinity chromatography will be used to purify and concentrate the tf for analysis of clinical samples.

ESI-MS was shown to be a good method for resolving commercial holo-tf that had retained its iron in a bicarbonate solution and holo-tf that had lost its iron in acid (figure 4.1), demonstrating that mass spectrometry can distinguish between Fe_0 -tf (apo-tf) and Fe_2 -tf (holo-tf). However, the resolution between apo-tf and holo-tf that was prepared

in the laboratory, was not complete (figure 4.3). The ions were broad perhaps as a result of incomplete desolvation and the binding of salts to the protein. Furthermore, it is possible that some iron was lost during dialysis prior to ESI-MS. In future studies, the iron load of tf in plasma will be tested by acquiring ESI mass spectra of plasma tf in an ammonium bicarbonate solution and comparing it with a spectrum of plasma tf acquired in acid. If incomplete iron binding is observed in a plasma sample, for instance, if a mass difference is observed corresponding to the binding of one iron atom, then this could potentially have clinical implications.

The Agilent High Capacity Multiple Affinity Removal Spin Cartridge was an effective method for the detection of the next tier of most abundant plasma proteins by ESI-MS. The original spectrum contained many peaks of low abundance, but three proteins of relatively high abundance were observed. In order to unequivocally identify these proteins, sequence analysis (tandem mass spectrometry of tryptic digests) will be carried out in future experiments. Further method development is necessary in order to detect additional proteins and positively identify them. Ion-exchange and immunoaffinity might be required for this purpose.

Overall, this work shows that ESI-MS may be an effective tool for the qualitative and quantitative analysis of tf and CDT in plasma samples. More work is, however, required in order to acquire good quality spectra of tf in CSF with the methods used in this work. The Affi-Gel Blue affinity gel Bio-Spin column proved to be an efficient tool for the rapid analysis of tf in plasma. ESI-MS was found to have limitations as a tool for the study of the iron forms of tf. In future work, rapid methods for removing

the more abundant plasma proteins from plasma will be explored so that the next tier of most abundant plasma proteins can be analysed by ESI-MS.

REFERENCES

- 1) N. Leigh Anderson and Norman G. Anderson. 2002. *Molecular & Cellular Proteomics*. **1**. 845.
- 2) Bast, R. C., Jr.; Klug, T. L.; St. John, E.; Jenison, E.; Niloff, J. M.; Lazarus, H. Berkowitz, R. S.; Leavitt, T.; Griffiths, C. T.; Parker, L.; Zurawski, V. R., Jr.; Knapp, R. C. 1983. *N. Engl. J. Med.* **309**. 883.
- 3) Schreiber, Gerhard. 1987. *The plasma proteins*. Putnam, Frank W. Ed. Academic Press, Inc. **V**. 294.
- 4) Takenaka, K. V.; Sakai, N.; Murase, S.; Kuroda, T.; Okumura, A.; Sawada, M. 2000. *Neur. Res.* **22**. 797.
- 5) Yang, A. H. W.; Macgillivray, R. T. A.; Chen, J.; Lou, Y.; Wang, Y.; Brayer, G. D.; Mason, A. B.; Woodworth, R. C.; Murphy, M. E. P. 2000. *Protein science*. **9**. 49.
- 6) Sun, H.; Li, H.; Sadler, P. J. 1999. *Chem. Rev.* **99**. 2817.
- 7) Golka, Klaus and Wiese, Andreas. 2004. *Journal. toxicol. and environm. health, Part B*. **7**. 319.
- 8) Mihas, A. A. and Tavassoli, M. 1992. *Am. J. Med. Sci.* **303**. 415.
- 9) Yang, H.-W., Macgillivray, R. T. A., Chen, J., Luo, Y., Wang, Y., Brayer, G. D., Mason, A., Woodworth, R. C., Murphy, M. E. P. 2000. *Protein Sci.* **9**. 49.
- 10) Macgillivray, R. T. A.; Moore, S. A.; Chen, J.; Andersen, B. F.; Baker, H.; Luo, Y.; Bewley, M.; Smith, C. A.; Murphy, M. E. P.; Wang, Y.; Mason, A. B.; Woodworth, R. C.; Brayer, G. D. and Baker, E. N. 1998. *Biochem.* **37**. 7919.
- 11) Li, H.; Sadler, P. J.; Sun, H. 1996. *J. Biol. Chem.* **271**. 9483.
- 12) Harris, W. R. and Pecoraro, V. L. 1983. *Biochem.* **22**. 292.
- 13) Evans, R. W. and Ogowang, W. 1988. *Biochem. Soc. Trans.* **16**. 833.
- 14) Donovan, J. W. and Ross, K. D. 1975. *J. Biol. Chem.* **250**. 6022.
- 15) Bertini, L.; Luchinat, C.; Messori, L. 1983. *J. Am. Chem. Soc.* **105**. 1347.
- 16) Harris, W. R. and Chen, Y. 1994. *J. Inorg. Biochem.* **54**. 1.
- 17) Hirose, J.; Fujiwara, H.; Magarifuchi, T.; Iguti, Y.; Iwamoto, H.; Kominami, S.; Hiromi, K. 1996. *Biochim. Biophys. Acta.* **1296**. 103.

- 18) Harris, W. R. 1986. *J. Inorg. Biochem.* **27**. 41.
- 19) Harris, W. R. 1983. *Biochem.* **22**. 3920.
- 20) Kratz, F.; Hartmann, M.; Keppler, B. K.; Messori, L. 1994. *J. Biol. Chem.* **269**. 2581.
- 21) Harris, W. R. 1986. *Inorg. Chem.* **25**. 2041.
- 22) O'Hara, P. B. and Koenig, S. H. 1986. *Biochem.* **25**. 1445.
- 23) Zak, O. and Aisen, P. 1988. *Biochem.* **27**. 1075.
- 24) Ward, S. G. and Taylor, R. C. 1988. *Metal-Based Antitumour Drugs*. Gielen, M. F., Ed. Freund Publishing House Ltd. 1.
- 25) Arndt, Torsten. 2001. *Clin. Chem.* **47**. 13.
- 26) Stibler, H and Kjellin, K. G. 1976. *J. Neurol. Sci.* **30**. 269.
- 27) Stibler, H. 1991. *Clin. Chem.* **37**. 2029.
- 28) Landberg, E.; Pahlsson, P.; Lundblad, A.; Arnetorp, A.; Jeppeson, J. O. 1995. *Biochem. Biophys. Res. Commun.* **210**. 267.
- 29) Stibler, H. and Borg, S. 1986. *Alcohol Clin. Exp. Res.* **10**. 61.
- 30) Martensson, O.; Harlin, A.; Brandt, R. 1997. *Alcohol Clin. Exp. Res.* **21**. 1710.
- 31) Bean, P. and Peter, J. B. 1994. *Clin. Chem.* **40**. 2078.
- 32) Xin, Y.; Rosman, A. S.; Lasker, J. M.; Lieber, C. S. 1995. *Hepatology.* **22**. 1462.
- 33) Whitfield, J. B.; Fletcher, L. M.; Murphy, T. L.; Powell, L. W.; Halliday, J.; Heath, A. C.; Martin, N. G. 1998. *Clin. Chem.* **44**. 2480.
- 34) Lakshman, M. R.; Rao, M. N.; Marmillot, P. 1999. *Alcohol.* **19**. 239.
- 35) Ghosh, P.; Okoh, C.; Lui, Q. H.; Lakshaman, M. R. 1993. *Alcohol Clin. Exp. Res.* **17**. 576.
- 36) Lesch, O. M.; Walter, H.; Antal, J.; Heggeli, D. E.; Kovacz, A.; Leitner, A.; Neumeister, A.; Stumpf, I.; Sundrehagen, E.; Kasper, S. 1996. *Alcohol Alcohol.* **31**. 265.
- 37) Martinez, L. D.; Baron, A. E.; Helander, A.; Conigrave, K. M.; Tabakoff, B. 2002. *Alcohol Clin. Exp. Res.* **26**. 1097.
- 38) Figlie, N. B.; Benedito-Silva, A. A.; Monteiro, M. G.; Souza-Formigoni, M. L. *Alcohol Clin. Exp. Res.* **26**. 1062.

- 39) Anton, R. F. and Moak, D. H. 1994. *Alcohol. Clin. Exp. Res.* **18**. 747.
- 40) Oslin, D. W.; Pettinati, H. M.; Luck, G.; Semwanga, A.; Cnaan, A.; O'Brien, C. P. 1998. *Alcohol. Clin. Exp.* **22**. 1981.
- 41) Stauber, R. E.; Vollman, H.; Pessler, I.; Jack, B.; Lipp, R.; Halwachs, G.; Wilders-Truschnig, M. *Alcohol. Clin. Exp.* **20**. 1114.
- 42) Sillanaukee, P.; Alho, H.; Strd. N.; Jousilahti, P.; Vartiainen, E.; Olsson, U.; Sillanaukee, P. 2000. *Alcohol. Clin. Exp.* **24**. 1505.
- 43) Lott, J. A.; Curtis, L. W.; Thompson, A.; Gechlik, G. A.; Rund, D. A. 1998. *Clin. Chim. Acta.* **276**. 129.
- 44) Jensen, P. D.; Peterslund, N. A.; Poulsen, J. H.; Jensen, F. T., Christensen, T.; Ellegard, J. 1994. *Br. J. Haematol.* **88**. 56.
- 45) De Fao, T. M.; Fargion, S.; Duca, L.; Mattioli, M.; Cappellini, M. D.; Sampietro, M.; Cesana, B. M.; Fiorelli, G. 1999. *Hepatology.* **29**. 658.
- 46) Lesch, O. M.; Walter, H.; Freitag, H.; Heggeli, D. E.; Kovasc, A.; Leitner, A.; Mader, R.; Neumeister, A.; Passweg, V.; Pusch, H.; Semler, B.; Sundrehagen, E.; Kasper, S. 1996. *Alcohol. Clin. Exp. Res.* **20**. 249.
- 47) Arndt, T. and Kropf, J. 2002. *Med. Sci. Monit.* **8**. BR61.
- 48) Dimartini, A.; Day, N.; Lane, T.; Beisler, A. T.; Dew, M. A.; Anton, R. 2001. *Alcohol. Clin. Exp. Res.* **25**. 1729.
- 49) Sharpe, P. C.; McBride, R.; Archbold, G. P. 1996. *Q. J. Med.* **89**. 137.
- 50) Stibler, H.; Holzbach, U.; Kristiansson, B. 1998. *Scand. J. Clin. Lab. Invest.* **58**. 55.
- 51) Stibler, H.; von Döbeln, U.; Kristiansson, B.; Guthenberg, C. 1997. *Acta Paediatr.* **86**. 1377.
- 52) Larsson, A.; Flodin, M.; Kollberg, H. 1998. *Ups. J. Med. Sci.* **103**. 231.
- 53) Reif, A.; Keller, H.; Schneier, M.; Kamolz, S.; Schmidtke, A.; Fallgatter, A. J. 2001. *Alcohol. Clin. Exp. Res.* **25**. 603.
- 54) Stibler, H.; Borg, S.; Jousilahti, M. 1986. *Alcohol. Clin. Exp. Res.* **10**. 535.
- 55) Wong, Chi-Huey. 2005. *J. Org. Chem.* **70**. 4219.
- 56) Hoefkens, P.; Huijskens-Heins, M. I. E.; de Jeu-Jaspers, C. M. H.; van Noort, W. L.; van Eijik, H. G. 1997. *Glycoconjugate J.* **14**. 289.

- 57) Lacey, J. M.; Bergen, R.; Magera, M. J.; Naylor, S.; O'Brien, J. F. 2001. *Clin. Chem.* **47**. 513.
- 58) Mills, P.; Mills, K.; Clayton, P.; Jonson, A.; Whitehouse, A.; Wnchester, B. 2001. *Biochem. J.* **359**. 249.
- 59) Kleinert, P.; Kuster, T.; Durka, S.; Ballhausen, D.; Bosshard, N. U.; Steinmann, E. H.; Jaeken, J.; Heizmann, C. W.; Troxler, H. 2003. *Clin. Chem. Lab. Med.* **41**. 1580.
- 60) Princeton University. 2001. Plasminogen. <http://www.websters-online-dictionary.org/definition/english/Pl/Plasminogen.html>. Retrieved October, 2004.
- 61) Juhan-Vague, I.; Alessi, M.-C.; Mavri, A.; Morange, P. E. 2003. *J. Throm. Haem.* **1**. 1575.
- 62) Pantazaki, A.; Taverna, M.; Vidal-Madjar, C. 1999. *Anal. Chim. Acta.* **383**. 137.
- 63) Princeton University. 2001. Fibrinogen. <http://www.websters-online-dictionary.org/definition/fibrinogen>. Retrieved October, 2004.
- 64) Montalescot, G.; Collet, J. P.; Thomas, D. 1998. *European Heart Journal.* **19**. H11.
- 65) Henschen-Edman, A. H. 2001. *Annals of the New York Acad. Science.* **936**. 580.
- 66) Wagner, M. S.; Horbett, T. A.; Castner, D. G. 2003. *Biomaterials.* **24**. 1897.
- 67) Princeton University. 2001. Prothrombin. <http://www.websters-online-dictionary.org/definition/english/Pl/Prothrombin.html>. Retrieved October, 2004.
- 68) Jennings, I. and Cooper, P. 2003. *Brit. J. Biomed. Science.* **60**. 39.
- 69) Princeton University. 2001. Prostate specific antigen. <http://www.websters-online-dictionary.org/definition/prostate+specific+antigen>. Retrieved October, 2004.
- 70) Schulz, P. C.; Yoshioka, Y.; Takahashi, T.; Ziheng, H.; King, G. L.; Lee, R. T. 2004. *J. Biol. Chem.* **279**. 30369.
- 71) Lin, M.; Campbell, J. M.; Mueller, D. R.; Wirth, U. 2003. *Rapid Commun. in Mass Spec.* **17**. 1809.
- 72) Oxbridge Solutions Ltd. 2005. General Practice notebook, ceruloplasmin. <http://www.gpnotebook.co.uk/simplepage.cfm?ID=241893406>. Retrieved October, 2004.
- 73) Waranabe, H.; Hamada, H.; Yamada, N.; Sohda, S.; Yamakawa-Kobayashi, K.; Yoshikawa, H.; Arinami, T. 2004. *Proteomics.* **4**. 537.

- 74) Fermo, I.; Germagnoli, L.; Solfarini, A.; Dorigatti, F.; Paroni, R. 2004. *Electrophoresis*. **25**. 469.
- 75) Yoshikawa, K.; Umetsu, K.; Shinzawa, H.; Yuasa, I.; Maruyama, K.; Ohkura, T. *FEBS Lett*. **458**. 112.
- 76) Dole, M.; Mack, L. L.; Hines, R. L.; Mobley, R. C.; Ferguson, L. D.; Alice, M. B. 1968. *J. Chem. Phys.* **49**. 2240.
- 77) Waters corporation. 2005. Waters. <http://www.waters.com/WatersDivision/Contentd.asp?watersit=CEAN-5KUSS8>. Retrieved July, 2005.
- 78) Chernushevich, I. V.; Loboda, A. V.; Thomson, B. A. 2001. *J. Mass Spectrom.* **36**. 849.
- 79) Gumerov, D. M. and Kaltashov, I. A. 2001. *Anal. Chem.* **73**. 2565.
- 80) Gumerov, D. M.; Mason, A. B.; Kaltashov, I. A. 2003. *Anal. Chem.* **42**. 5421.
- 81) Bergen, H. R.; Lacey, J. M.; O'Brian, J. F.; Naylor, S. 2001. *Anal. Biochem.* **296**. 122.
- 82) Sanz-Nebot, V.; Gonzalez, P.; Toro, I.; Ribes, A.; Barbosa, J. 2003. *J. Chrom. B.* **798**. 1.
- 83) Odabasi, F.J. and Denizli, A. 2004. *Poly. Int.* **53**. 332.
- 84) Chasteen, N. D. 1983. *Advanced Inorg. Biochem.* **5**. 201.
- 85) Zweier, J. L. and Aisen, P. 1977. *J. Biol. Chem.* **252**. 6090.
- 86) Baldwin, D. A. and Desousa, D. M. R. 1981. *Biochem. Biophys. Res. Commun.* **99**. 1101.
- 87) Sjoberg, P. J, R.; Bokmann, C. F.; Bylund, D.; Markides, K. E. 2001. *J. Am. Soc. Mass Spectrom.* **12**. 1002.
- 88) Consantopoulos, T. L.; Jackson, G. S.; Enke, C. G. 1999. *J. Am. Soc. Mass Spectrom.* **10**. 625.
- 89) Pan, Peng and McLuckey, Scott A. 2003. *Anal. Chem.* **75**. 1491.
- 90) Eland, J. H. D. 1994. *Meas. Sci. Technol.* **5**. 1501.
- 91) Tang, K.; Page, J. S.; Smith, R. D. 2004. *J. Am. Soc. Mass Spectrom.* **15**. 1416.
- 92) Beck, J. L.; Ambahera, S.; Yong, S. R.; Sheil, M. M.; Jersey, J. d.; Ralph, S. F. 2004. *Anal. Biochem.* **325**. 326.
- 93) Jeppsson, JO; Kristensson, H.; Fimiani, C. 1993. *Clin. Chem.* **39**. 2115.

- 94) Turpeinen, U.; Methuen, T.; Alftan, H.; Laitinen, K.; Salaspuro, M.; Stenman, U.-H. 2001. *Clin. Chem.* **47**. 1782.
- 95) Helander, A.; Husa, A.; Jeppson, J.-O. 2003. *Clin. Chem.* **49**. 1881.
- 96) Mills, P.; Mills, K.; Clayton, P.; Jonson, A.; Whitehouse, A.; Wnchester, B. 2001. *Biochem. J.* **359**. 249.
- 97) Bean, P.; Liegmann, K.; Lovli, T.; Westby, C.; Sundrehagen, E. 1997. *Clin. Chem.* **43**. 983.
- 98) Bio-Rad Laboratories. 2005. Affi-Gel® Blue Affinity Chromatography Gel for Enzyme and Blood protein Purifications. http://www.bio-rad.com/LifeScience/pdf/Bulletin_1107.pdf. Retrieved June, 2005.
- 99) Baldwin, D. A. and Desousa, D. M. R. 1981. *Biochem. Biophys. Res. Commun.* **99**. 1101.
- 100) Margetic, S.; Topic, E.; Ruzic, D. F.; Kvaternik, M. 2005. *Clin. Chem. Lab. Med.* **43**. 326.
- 101) de Gobbi, M.; D'Antico, S.; Castagno, F.; Testa, D.; Merlini, R.; Bondi, A.; Camaschella, C. 2004. *Haematalogica*. **89**. 1161.
- 102) MedicineNet, Inc. 2002. Hemochromatosis (Iron Overload) http://www.medicinenet.com/iron_overload/article.htm. Retrieved August, 2005.
- 103) Ritchie, R. F.; Palomaki, G. E.; Neveux, L. M.; Navolotskaia, O.; Ledue, T. B.; Craig, W. Y. 2002. *J. Clin. Lab. Anal.* **16**. 246.
- 104) Lok, C.; Loh, T. T. 1998. *Biol. Signals Recept.* **7**. 157.
- 105) Du, X.-I.; Zhang, T.-I.; Yuan, L.; Zhao, Y.; Li, R.; Wang, K.; Yan, S. C.; Zhang, L.; Sun, H. and Qian, Z. 2002. *FEBS*. **269**. 6082.
- 106) Agilent Technologies. 2005. Library. <http://www.chem.agilent.com/scripts/Library.asp>. Retrieved August, 2005.
- 107) Swiss Institute of Bioinformatics. 2003. TagIdent Tool. <http://tw.expasy.org/tools/tagident.html>. Retrieved August, 2005.