THE BINDING ABILITY OF ALPHA-1-ACID GLYCOPROTEIN AS A MECHANISM OF RESISTANCE TO METHADONE.

Submitted by
Jennifer Lucy Behan

A thesis submitted in partial fulfilment of the

requirements of Edinburgh Napier University for the award

of Doctor of Philosophy.

October 2010

School of Life, Sport and Social Sciences

Edinburgh Napier University

Edinburgh

Declaration

It is hereby declared that this thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has lead to the award of a degree.

Signed:

Acknowledgements

Finally, as a special note of thanks, I would like to dedicate this thesis to my Gran. Our 'research' chats and her travel advice always put a smile back on my face and I hope hers too. Sadly she will not get to see the finished article but I can only hope she would be proud. I will always be thankful for her never-ending encouragement, support and great sense of humour.

Abstract

Dependence on heroin and other opioids represents a considerable problem worldwide. There is a continual need to improve therapy and/ or find more efficacious alternatives if these issues are to be addressed. The most commonly implemented pharmacological therapy in treating said dependencies is methadone; however its success is the subject of ongoing debate. Certain plasma proteins including alpha1-acid glycoprotein (AGP) bind to drugs which causes inactivation and, if low enough, may prevent a therapeutic effect being attained. The hepatic synthesis of AGP increases two- to five-fold during numerous physiological and pathophysiological conditions, becoming the most prevalent acute phase protein in the blood. Additionally, the structure of the sugar chains (glycans) attached to the surface of underlying polypeptide backbones can differ, potentially altering the functions performed.

AGP was isolated from blood samples obtained from patients undergoing various stages and types of opioid-replacement therapy and from heparinised blood samples provided by the Blood Transfusion Service. Structural analysis of the glycans was undertaken primarily through the use of high pH anion-exchange chromatography (HPAEC) and intrinsic fluorescence used as a measure of drug binding. The composition of glycans attached to the polypeptide backbone of AGP isolated from patient samples was found to markedly differ from that of a 'normal' healthy population. Levels of galactose and N-acetyl-glucosamine were amplified in all methadone treatment groups which suggested increased branching of glycans; this was supported by HPAEC analysis of complete glycan chains. Binding of methadone to all isolated AGP samples was elevated at the highest drug concentrations tested; however the degree of quenching appeared to be greater in patients.

Therefore, the glycoforms expressed by AGP appear to be associated with the subsequent binding of the glycoprotein to methadone. It is possible that altered glycosylation could increase affinity for the drug, reducing its bioactive concentration to below that required to produce the pharmacological effect. Currently, the doses of methadone used in opioid replacement therapy are primarily influenced by the expression of physical symptoms, however this preliminary study has indicated that determination of the level and glycoform expression of AGP may offer potential use when determining the most effective therapy and dosage regimen.

List of figures

List of Tables

Chapter 8

Abbreviations

AA	amino acid
AAT	α1-antitrypsin
AGP	α1-acid glycoprotein
AIDS	acquired immune deficiency syndrome
Ala	alanine
ANOVA	analysis of variance
APP	acute phase proteins
APR	acute phase response
Arg	arginine
Asn	asparagine
Asp	aspartate
B	buprenorphine
BBB	blood brain barrier
BCIP	5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt
BDZ	benzodiazepine
CAIE	crossed affinity immunoelectrophoresis
cAMP	cyclic adenosine monophosphate
CMP	caseinomakropeptide
CDG	congenital disorder of glycosylation
CDPS	Community Drug Problem Service
CE	capillary electrophoresis
CF	cystic fibrosis
CHO	aldehyde
CM	carboxymethyl
CNS	central nervous system
Con A	Concanavalin A
CRP	C-reactive protein
CTB	continuous transfer buffer
CYP	cytochrome P
Cys	cysteine
D (small)	dextrorotatory
D	Dihydrocodeine
DA	dopamine

DEAE	diethylaminoethyl
DHC	dihydrocodeine
DMSO	dimethyl sulphoxide
d-PBS	Dulbecco's phosphate buffered saline
Dol-P	dolichol pyrophosphate
DORIS	Drug Outcome Research in Scotland
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders
EDDP	2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine
ELISA	enzyme-linked immunosorbent assay
ENL	Erythema nodosum leprosum
EPO	erythropoietin
ER	endoplasmic reticulum
ESL	E-selectin ligand
ETC	endothelial cell
F	Bioavailability
FA	frontal analysis
Fe	iron
FRET	fluorescence resonance energy transfer
Fuc	fucose
GABA	γ-aminobutyric acid
Gal	galactose
GalN	galactosamine
GalNAc	N-acetylgalactosamine
GCSF	granulocyte colony stimulating factor
GDP	guanine diphosphate
Glc	glucose
GlcN	glucosamine
GlcNAc	N-acetylglucosamine
Gln	glutamine
Glu	glutamate
Gly	glycine
GP	General Practioner
H	heroin
HCl	hydrochloric acid

His	histidine
HIV	human immunodeficiency virus
H_2O	water
HPAEC	high pH anion-exchange chromatography
HPLC	high performance liquid chromatography
HR	harm reduction
HSA	human serum albumin
IEF	isoelectric focusing
IFN-γ	interferon-gamma
Ig	immunoglobin
IL-1	interleukin-1
IL-1Ra	IL-1 receptor antagonist
IL-6	interleukin-6
IL-8	interleukin-8
Ile	isoleucine
IS	internal standard
i.v	intravenous
KCl	potassium chloride
Ki	affinity constant
KSCN	potassium thiocyanate
L (small)	Levorotatory
LAAM	Levo-alpha acetyl methadol
LC	locus ceruleus
Leu	leucine
LeX	Lewis-X
LT	long-term
Lys	lysine
MAL	Macckia amurensis Leukoagglutinin
MALDI	matrix-assisted laser desorption/ionisation
Man	mannose
MDR	multi-drug resistance
MEC	minimal effective concentration
Met	methionine
$MgCL_2$	magnessium chloride
MMT	methadone maintenance therapy

MOR	mu opioid receptor
MS	mass spectrometry
MS	multiple sclerosis
NA	nucleus accumbens
NaCl	sodium chloride
NAdr	nor-adrenaline
NaOAc	sodium acetate
NaOH	sodium hydroxide
NBT	Nitro-Blue Tetrazolium Chloride
NeuAc	N-acetylneuraminic acid
NH_2	amino group
NHS	National Health Service
NMDA	N-methyl-D-aspartic acid
NMR	nuclear magnetic resonance
NTORS	National Treatment Outcome Research Study
OH	hydroxyl
OIH	opioid induced hyperalgesia
OR	opioid receptor
ORM	orosomucoid (AGP)
OST	oligosaccharyltransferase
P	phosphate
PAD	pulsed amperometric detection
PACE	pressure-assisted capillary electrophoresis
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cells
PD	pharmacodynamics
PEG	polyethylene glycol
Phe	phenylalanine
pI	isoelectric point
PK	pharmacokinetics
pKa	acid dissociation constant
PKC	protein kinase C
PMN	polymorphonuclear leukocytes
PNGase F	Peptide-N-Glycosidase F
Pro	proline

R	*Rectus*
RBC	red blood cell
R^2	Pearson correlation coefficient
RA	rheumatoid arthritis
RER	rough endoplasmic reticulum
S	*Sinister*
SA	sialic acid
SACDM	Scottish Advisory Committee on Drugs Misuse
S.D	standard deviation
SDS	sodium-dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate – polyacrylamide gel electrophoresis
Ser	serine
SF	synovial fluid
SH	steroid hormone
SLe^x	Sialyl Lewis X
SML	serum methadone level
SNA	Sambucus nigra aglutinin
SNRI	serotonin norepinephrine reuptake inhibitors
SSRI	selective serotonin reuptake inhibitors
T	titration
$t_{1/2}\beta$	elimination half-life
TBS	tris buffered saline
TCA	tricyclic antidepressant
TEMED	tetramethylethylenediamine
TFA	trifluoracetic acid
Thr	Threonine
TNF-α	tumour necrosis factor α
tPA	tissue plasminogen activator
Trp	Tryptophan
Tyr	Tyrosine
UDP	uridine diphosphate
v	volume
Val	valine
Vd	volume of distribution

w	weight
WHO	World Health Organisation
Zn	zinc

Publications

Refereed Journals

Invited Review

Behan, J. and Smith, K.D. (in press) The analysis of glycosylation: A continued need for high pH anion exchange chromatography. *Biomedical Chromatography. (published online July 2010)*

Conference Abstracts (published)

Behan, J., Bruce, M., Cruickshank, Y., Smith, K. (2008) The altered Glycosylation Pattern of Alpha-1-acid Glycoprotein may Affect Drug Binding ability to methadone. *Glycobiology* **18** (11): 966.

Conference Posters

Behan, J., Bruce, M., Cruickshank, Y., Smith, K. (2008) The altered Glycosylation Pattern of Alpha-1-acid Glycoprotein may Affect Drug Binding ability to methadone. *Glycobiology* 18 (11) 966 (abstract number 62), Fort Worth, TX, November 2008.

Behan, J., Bruce, M., Cruickshank, Y., Smith, K. (2009) The presence of an acute phase protein (α-1-acid glycoprotein) in the blood may contribute to the failure of methadone therapy in vivo altered. Royal Society of Medicine 9th Jenner Glycobiology and Medicine Symposium (abstract number 1), Brussels, September 2009.

Behan, J., Bruce, M., Cruickshank, Y., Smith, K. (2010) The existence of a plasma Acute Phase Protein (Alpha-1-Acid Glycoprotein) may contribute to the limited success of methadone therapy *in vivo*. EUROPAD 2010 (abstract number 4), Zagreb, May 2010.

Contents

Chapter 1

Introduction

1.1 Glycosylation

1.1.1 The emergence of Glycobiology

Fundamentally, Glycobiology comprises the study of biological macromolecules, namely proteins and lipids, whose surfaces are covalently modified by the enzymatic addition of oligosaccharide chains (sugars with the empirical formula $(CH_2O)_n$). Commonly known as glycosylation, it occurs co- and/or post-translation in the endoplasmic reticulum (ER) and Golgi apparatus to produce specific glycoconjugate structures belonging to the glycoprotein and glycolipid families respectively. It is a complex, ordered and energy consuming process that has become widely recognised as both functionally significant and responsible for providing huge structural diversity (Varki *et al.,* 2009; Taylor and Drickamer, 2003). The presence of oligosaccharide chains (known as glycans) had historically been given little credence in the study of these biomolecules, considered only as stores of residual energy or an embellishment of the macromolecule to which they were attached. Removal of the glycans was thus commonplace during the study of glycoproteins, instead interest focussed on the analysis of the protein backbone alone.

Technological developments have caused views regarding the importance of glycosylation to change and allowed the field of Glycobiology to grow – so named by Rademacher *et al*. in 1988. It has now become widely accepted that glycans have crucial roles in determining the properties expressed by a glycoconjugate. Through such research, numerous physiological and pathophysiological conditions in which glycan structure has huge functional significance have been identified (Varki, 1993).

1.1.2 The importance of Glycosylation

Glycosylation takes the form of variable sugar chain structures, the quantity of which is dependent on the availability of attachment sites. Through interactions forged with the surfaces of proteins or lipids, glycans affect their stability, conformation and consequently the overall function. These factors may explain why up to 70% of proteins are reportedly modified in this way (Wormald and Dwek, 1999).

2

The glycans expressed by a glycoprotein are determined by the specific enzyme clusters - particularly the glycosyltransferases – residing in a cell's ER and Golgi, thus glycosylation is not directly regulated at the genetic level. Glycosyltransferases catalyse the fusion of monomeric building blocks of the carbohydrate complexes (monosaccharides), arranging them in specific sequences which then associate with the remaining polypeptide fragment (Kobata, 1992; Sasisekharan and Myette, 2003).

If transcription of genes encoding the specific glycosyltransferases involved in glycan synthesis is altered during certain physiological and pathophysiological conditions, variations in the structure and function of the glycoconjugate is likely to occur. When specific to the causative condition, these structural alterations can offer use as biomarkers, implemented in their diagnosis and management. Studies have shown that aberrant glycosylation is expressed in various diseases but whether it is due to, or a cause of, the disease is unknown. A recent example suggested that the expression of specific glycoforms (glycoproteins with an identical amino acid sequence but variations in the surface glycosylation) of α_1-acid glycoprotein (AGP) is correlated with the inflammatory phases of leprosy (Gupta *et al.,* 2010). The glycoforms expressed by patients with ENL differed to those enduring other stages of the disease and a healthy population.

There are a specific group of inherited disorders, known as the congenital disorders of glycosylation (CDGs), which arise when the biosynthetic and processing pathways involved in glycosylation are not performed correctly (Durand and Seta, 2000; Jaeken and Matthijs 2007). In the absence of correct glycan expression various irregularities arise, particularly in neuronal development – reaffirming the importance of the structural modification. Efficiency of infectious agents and cancer progression were shown by various groups to be dependent on the presence of glycosylated structures (Bertozzi and Kiessling, 2001; Dell and Morris, 2001). Many pathogens, including *E.coli* and *N. Meningitides*, display 'normal' sugar chains on their surface allowing them to evade the immune response of infected hosts. For example, the occurrence of polysialic acids resembling those in normal developing neuronal tissues prevent the destruction of these pathogens, allowing subsequent infection of eukaryotic cells (Sears and Wong, 1998).

The occurrence of a glycocalyx (a 60-100 angstrom extracellular structure) surrounding all cells signifies that glycans are likely to function as ligands for receptors; determining the intramolecular functions of a glycoconjugate - also promoting interactions with signalling molecules and cell-to-cell and cell-to-extracellular matrix communication. Intermolecular properties such as circulating half-life and immunogenicity are further examples of the influence of glycan expression (Varki, 1993).

1.1.3 The components of Glycans

Monosaccharides represent the monomeric units of glycans, comprising two groups - the aldoses and ketoses corresponding to the expression of an aldehyde or keto functional group which are composed of a carbon covalently linked to an oxygen atom by a double bond; situated at the end (-CH=O) or within (>C=O) a chain, respectively. Upon polymerisation of eight or fewer monosaccharides, oligosaccharides are formed but if more than eight are involved in the structure they are termed polysaccharides (Bertozzi and Rabuka, 2009). Sub-classifications of monosaccharides focus upon the number of carbon atoms in the structure; the most common being six (hexose) as found in glucose (Glc), galactose (Gal) and mannose (Man). Fucose (Fuc) is termed a deoxyhexose due to the absence of the hydroxyl group (-OH) on C6. N-acetyl neuraminic acid (NeuAc), a nine carbon sugar present at the termini of glycan chains, belongs to the sialic acid family (Varki, 1992). Additionally, the hexosamines form a group of monosaccharides expressing an amino group on the second carbon and often exist in an N-acetylated form in glycan chains, for example N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc).

Simple monosaccharide structural representations use the Fischer projection implementing linear structures. However, these do not depict their existence in nature; instead they close into a cyclic hemiacetal ring to acquire increased stability. The -OH of C5 and the aldehyde group (-CHO) of C1 react as demonstrated in figure 1.1a using Man. A summary of the monosaccharides commonly found in glycans is shown in figure 1.1b; including Gal, Man, Fuc, GlcNAc and GalNAc which all adopt the chair conformation as illustrated for Man in figure 1.1a. In this more stable conformation, C1 becomes the anomeric carbon as it is a chiral centre – the –OH group joined to the anomeric C may sit below the plane of the ring (termed alpha, α) or above (beta, β).

The orientation of the -CHOH group located furthest from the functional -CHO can also exist in one of two configurations allowing them to be classed as either D-, which exists most commonly in nature, or the L- form. The monosaccharides composing glycan chains are attached through the formation of glycosidic bonds between the -OH reducing group of C1 and any other -OH of an adjacent residue (either an α or β anomeric configuration, depending on the conformation of the -OH group on C1 of the donating monosaccharide). Bond formation requires a condensation reaction; ergo a molecule of H_2O is expelled as depicted in figure 1.2, and the resultant oligosaccharide chains may be homogenous or heterogeneous in structure, as determined by the constituent monosaccharides.

The glycan structures may differ only slightly in sequence, length of chains, type of bond or number of chains that are attached, providing a wide diversity of structures (heterogeneity) from relatively few monosaccharide units. Consideration of the ability of three amino acids to produce only six tripeptides while three hexoses are capable of generating thousands of trisaccharides (27,648), illustrates how the synthesis of long glycan chains significantly increases glycoconjugate structural diversity (Maeder, 2002). There is enhanced potential for the generation of variable structures as each sugar can display different anomeric configurations – α and β. Additionally linkage position may differ thus leading to more complex structures.

These glycan chains have functions both individually important but are also involved in determining the physical properties and physiological functions of the various structures of which they become a physical component. Of particular interest to this study is the ability of oligosaccharides to covalently attach to proteins, producing glycoproteins, discussed in more detail in section 1.2.

Fischer projection of   **cyclic hemiacetal**   **Stable chair conformation**

aldehydo form (of mannose)

CHO

HO—C—H

HO—C—H

H—C—OH

H—C—OH

CH₂OH

CH₂OH

anomeric centre

O

H

OH OH

OH

OH

H H

D-Mannose

CH₂OH

HO

HO

O

OH

OH

b)

CH₂OH

O

OH H

H

OH H

H OH

H OH

α-D-Galactose

CH₂OH

O

H H

H

OH H

OH OH

H OH

α-D-Glucose

H

O

OH

H

CH₃

H OH

OH H

OH H

α-L-Fucose

CH₂OH

O

H H

H

OH

OH OH

H NH

CO

CH₃

α-D-N-acetylglucosamine
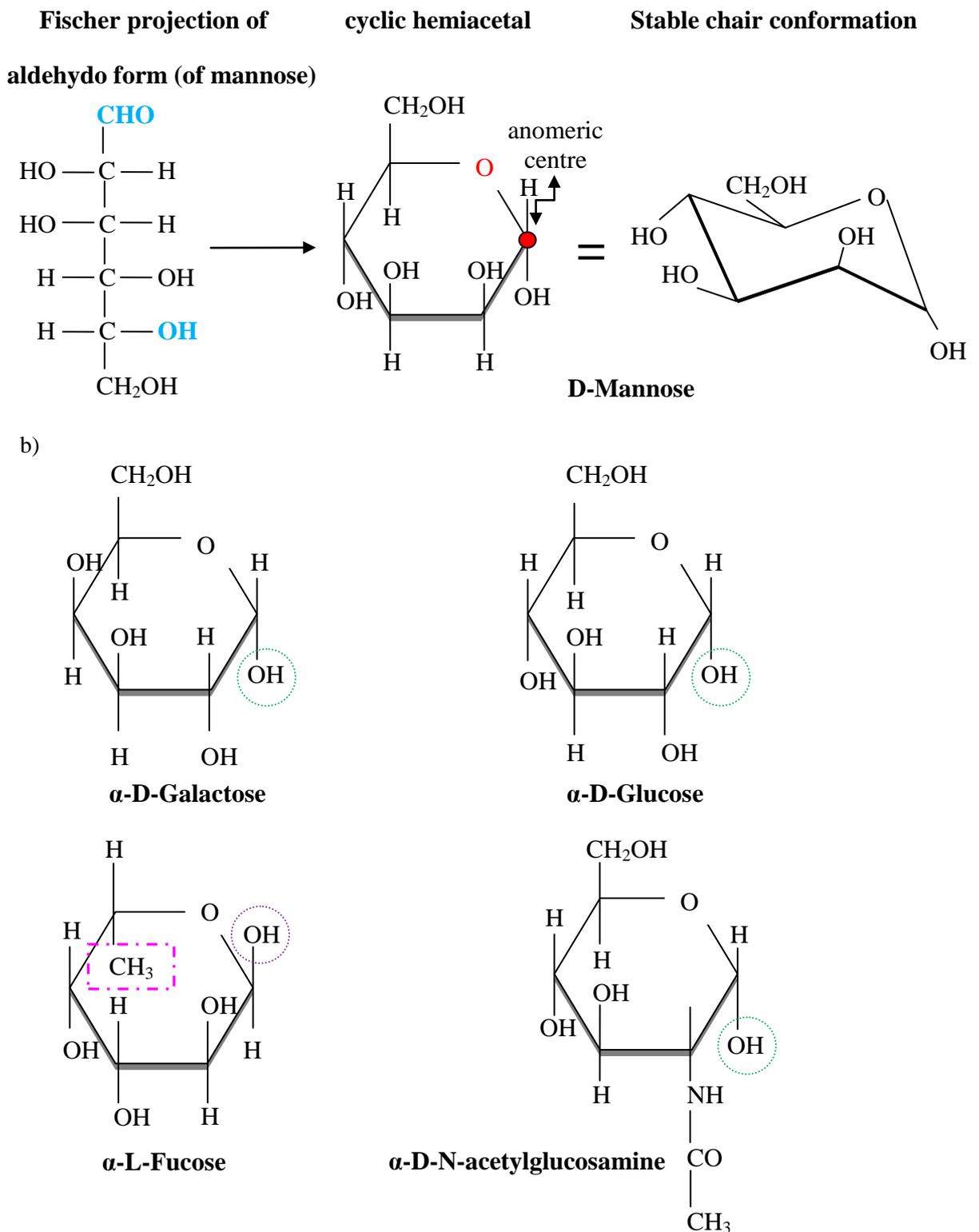
Figure 1.1.   **Summary of common glycan monosaccharides**. a) A demonstration, using Mannose, of the reaction which occurs between the -OH of carbon 5 and -CHO of carbon 1 (blue) to produce the stable ring conformation. b) Monosaccharides common to glycoprotein oligosaccharide chains represented in their stable cyclic hemiacetal configuration. The lack of an -OH group on C6 of fucose is highlighted – fucose is a deoxyhexose. α and β configurations are represented by ⬭ and ⬭ respectively.
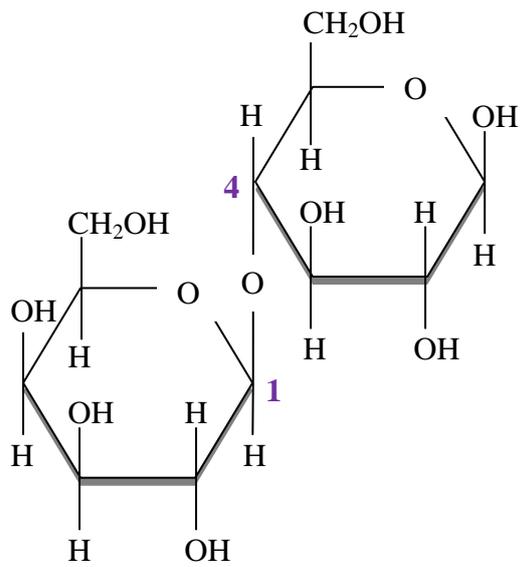
Figure 1.2. **The glycosidic linkage.** [Adapted from Ferrier and Collins, 1972]. Two monomeric units have been used to demonstrate the formation of a β1,4 glycosidic linkage which leads to the formation of the disaccharide lactose (β-D-galactopyranosyl-(1→4)-β-D-glucopyranose).

1.1.4 Glycoproteins

Glycoproteins are unique biomolecules composed of one or more glycans covalently bound to a protein 'backbone' and often terminated by sialic acid (SA) residues. The complicated synthesis and modification of glycans takes place co- and/ or post-translation and is fundamental to the determination of the function and stabilisation of the underlying polypeptide. Although it was thought to be specific to eukaryotes, glycosylation of proteins has been reported in eubacteria (Dobos *et al*., 1996) and archaea (Lechner and Wieland, 1989). In fact those discovered in bacteria display much greater variation than those in eukaryotes, some directly affecting the function of the underlying protein and others crucial to interactions with the host immune system (Benz and Schmid, 2002).

The reducing end of the oligosaccharide chain component becomes attached to the polypeptide either through N-linkages (via the amide nitrogen of specific asparagine residues) or O-linkages (via the -OH of serine/threonine). N-glycans are prevalent in plasma proteins and do not contain glucose (Glc), while the latter predominate in glycoproteins of epithelial origin and may incorporate Glc as a chain constituent (Dell and Morris, 2001).

Glycoprotein expression is dependent on the cell type to which the structures are integrated. Certain genes encode information that, once transcribed and translated, produce the protein backbone, which is subsequently responsible for directing the sites of glycosylation (Freeze, 2001). Glycoform expression promotes a high degree of heterogeneity, essentially a result of different cell types possessing a diverse array of enzymes thus catalysing a range of reactions and producing distinct glycans. Variable structures therefore not only reflect the source cell or tissue but also the physiological and biochemical conditions present during synthesis (van Dijk *et al.,* 1994). Heterogeneity can alter physical properties like stability and solubility thus affecting the conformation and ability to perform the typical function of the biomolecule including protein-protein or protein-saccharide recognition, intracellular trafficking of enzymes, cell-cell recognition, and inhibition of leukocyte rolling during the inflammatory response (Durand and Seta, 2000; Sears and Wong, 1998).

Glycans are widely distributed on the cell surface potentially linked through various combinations of N- and O-linkages, for example erythropoietin (EPO) and coagulation factor VIII. Some glycoproteins however display only one type of linkage; α1-acid glycoprotein (AGP), α1-antitrypsin, interferon β, interferon γ, and tissue plasminogen activator (TPA) are solely N-linked, whilst Interleukin 2 and GCSF express O-linked only (Lis and Sharon, 1993; Durand and Seta, 2000). The presence of a consensus sequence, Asn-X-Ser/Thr, in N-linked glycoproteins often allows predictions to be made as to which Asn residues of the protein will become glycosylated - only those situated in the sequence have this potential (Gavel and von Heijne, 1990). Kornfeld and Kornfeld (1985) reported that approximately one third of Asn residues become N-glycosylated, limited by specific requirements including a 'sufficient pool of completely assembled and glycosylated lipid-linked oligosaccharide donor, an adequate activity of oligosaccharyltransferase, and a properly oriented and accessible Asn-X-Ser/Thr sequence in the acceptor'. O-linked glycan synthesis is far simpler - numerous core structures can be generated, increasing glycan diversity thus making them more difficult to characterise than N-linked which share a core structure, $GlcNAc_2Man_3$.

Most serum proteins express glycans on their surface, particularly secretory proteins and those incorporated into the plasma membrane. Glycosylation ensures maturation of the underlying protein is correct; if absent, the function of the biomolecule may be changed or possibly lost. Some proteins like albumin do not require glycosylation to bestow functionality (Sabatini *et al.,* 1982). An example where incomplete glycosylation prevents the glycoprotein achieving optimal bioactivity is when erythropoietin (EPO), released from the kidney to regulate red blood cell (RBC) production, has reduced sialic acid (SA) content - under these circumstances it is rapidly cleared by the liver. Ordinarily sialylation prevents elimination, promoting its effectiveness in treating anaemia. For optimal solubility, biosynthesis and circulating half-life, EPO must express one O-linked glycosylation site at position 126 and three N-linked at 24, 38 and 83 (Macdougall and Eckardt, 2006; Paulson and Colley, 1989). Van Dijk and colleagues (1994) reported reduced sialylation of certain glycoproteins can also arise in rheumatoid arthritis (RA).

Despite their association with disease, the potential for glycoproteins in therapeutics has intensified with research. It has lead to the production of recombinant forms of EPO used to treat anaemia and tPA (tissue plasminogen activator - a serine protease

inhibitor), which induces fibrinolysis through the conversion of plasminogen to plasmin (Williams *et al.,* 1986; Adamson and Eschbach, 1989). More recently, the potential of recombinant glycoproteins in the treatment of Herpes Simplex Virus 2 and inflammatory bowel disease (IBD) has been indicated (Hoshino *et al.,* 2005 and Li *et al.,* 2009 respectively).

Understanding the pathways involved in the synthesis and modification of N-linked glycans, as discussed below, is of paramount importance when studying the physiological and pathological states during which these are known to be altered. Analysing the levels of glycoproteins has shown use clinically, often correlating with numerous physiological and pathophysiological conditions alongside fluctuations in the level of inflammatory cells (Fournier *et al.,* 2000). However, studying the glycan structures could offer more accurate and condition-specific markers, important when early treatment and monitoring is crucial in tackling diseases.

1.1.4.1 N-linked Glycan biosynthesis

N-glycosylation relies on a complex enzyme catalysed synthetic- (within the cytosol and ER), and processing- (in the ER and Golgi) pathway. Initially component monosaccharides are converted into activated high-energy sugar nucleotides e.g. UDP-GlcNAc and GDP-Man, and are subsequently transferred to a membrane-bound lipid dolichylphosphate (Dol-P), on the cytosolic side of the ER to form $Man_5GlcNAc_2$-Dol-P. GlcNAc-1-phosphotransferase, GlcNAc-transferase and mannosyltransferases are required to catalyse the transfer from their donor sugar nucleotide, to the Dol-P. The structure then translocates through the activity of a flippase to the lumenal side of ER. A further seven monosaccharides are donated, binding together via glycosidic bonds to produce the fourteen residue $Glc_3Man_9(GlcNAc)_2$ oligosaccharide precursor.

Dennis *et al.* (1999) effectively summarised N-glycan biosynthesis in four distinct phases occurring after the formation of this precursor and associated with different compartments of the secretory pathway. First, transfer of the oligosaccharide precursor from a dol-P donor to nascent glycoproteins occurs in the RER lumen; requiring a multimeric oligosaccharyltransferase complex (OST; Imperiali and O'Connor, 1999). Glycosidases then trim the structure while being transported in the RER and Golgi.

Substitution occurs in the medial-Golgi by GlcNAc-transferases and finally elongation takes place in the trans-Golgi network. These stages are encapsulated in figure 1.3.

As illustrated in figure 1.4, N-glycosylation of the Asn amide group requires formation of β-turns or loops in the secondary structure of the polypeptide; hydrogen-bonds form between the amide ($-NH_2$) group of Asn and the hydroxyl (-OH) group of Ser/Thr (Smith *et al,* 1997). The amino acid proline (Pro) lacks an α-amide proton; preventing the formation of loops/turns thus it is never present in the consensus sequence (Bause, 1983). A bond is formed between the anomeric -OH group of the terminal monosaccharide (GlcNAc) and the -NH group of Asn. Yet and Wold (1990) reported that interactions can occur between N-linked oligosaccharides and aromatic, un-charged, amino acid (AA) side chains within the three AAs on either side of the glycosylated Asn. The addition of N-glycans bestows protection, stability and solubility to the underlying protein. The glycan structures which are newly attached to Asn can then be processed through the competitive action of glycosidases such as glucosidases and mannosidases, and glycosyltransferases like galactosyltransferases during passage through the ER and Golgi compartments. It is the competition between enzymes for substrates that facilitates the expression of increased microheterogeneity; catalysing numerous different sets of reactions (Guile *et al.,* 1996; Couldrey and Green, 2000).

Several regulatory steps are in place to prevent the synthesis of incorrect glycoproteins; upon trimming of two Glc by α1,2-glucosidase I and α1,3-glucosidase II in the ER, the nascent glycoproteins enter the calnexin/calreticulin cycle which ensures only correctly folded proteins continue in the pathway. If folding is incorrect, a luminal glycosyltransferase adds a Glc residue onto the unfolded/misfolded molecule forcing it re-enter the calnexin/calreticulin cycle where it remains until correctly folded and the remaining Glc is removed by glucosidase II (Sousa *et al.,* 1992; Deprez *et al.*, 2005).
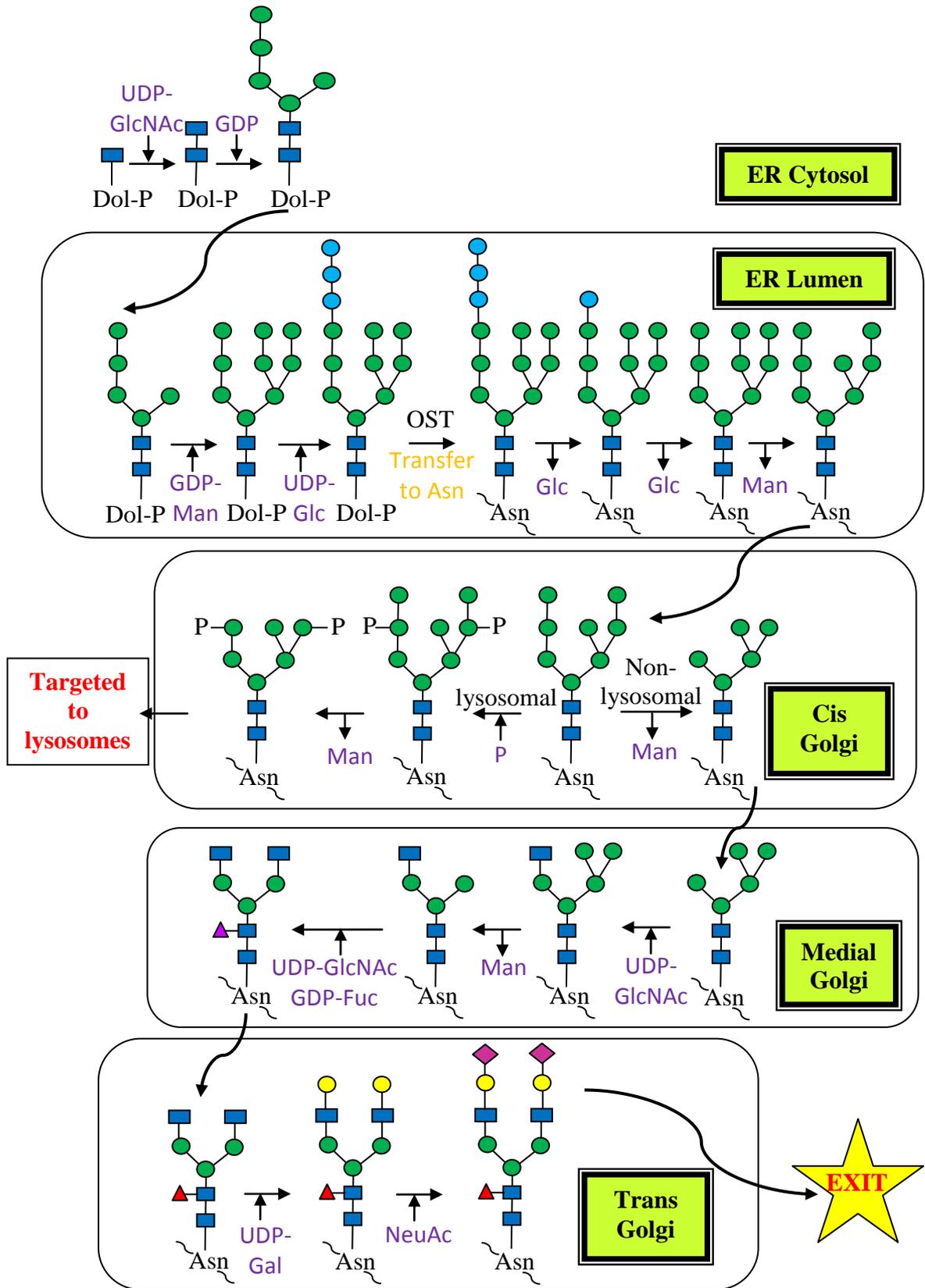
Figure 1.3. **The glycosylation pathway of N-linked glycoproteins**. [Adapted from Rich and Withers (2008) and Varki *et al.* (2009)]. A summary of the synthetic and processing pathways catalysed by enzymes residing in the ER and Golgi; these differ depending on the type of N-linked glycans expressed.

■ GlcNAc ● Man ○ Glc ○ Gal ◆ NeuAc ▲ Fuc
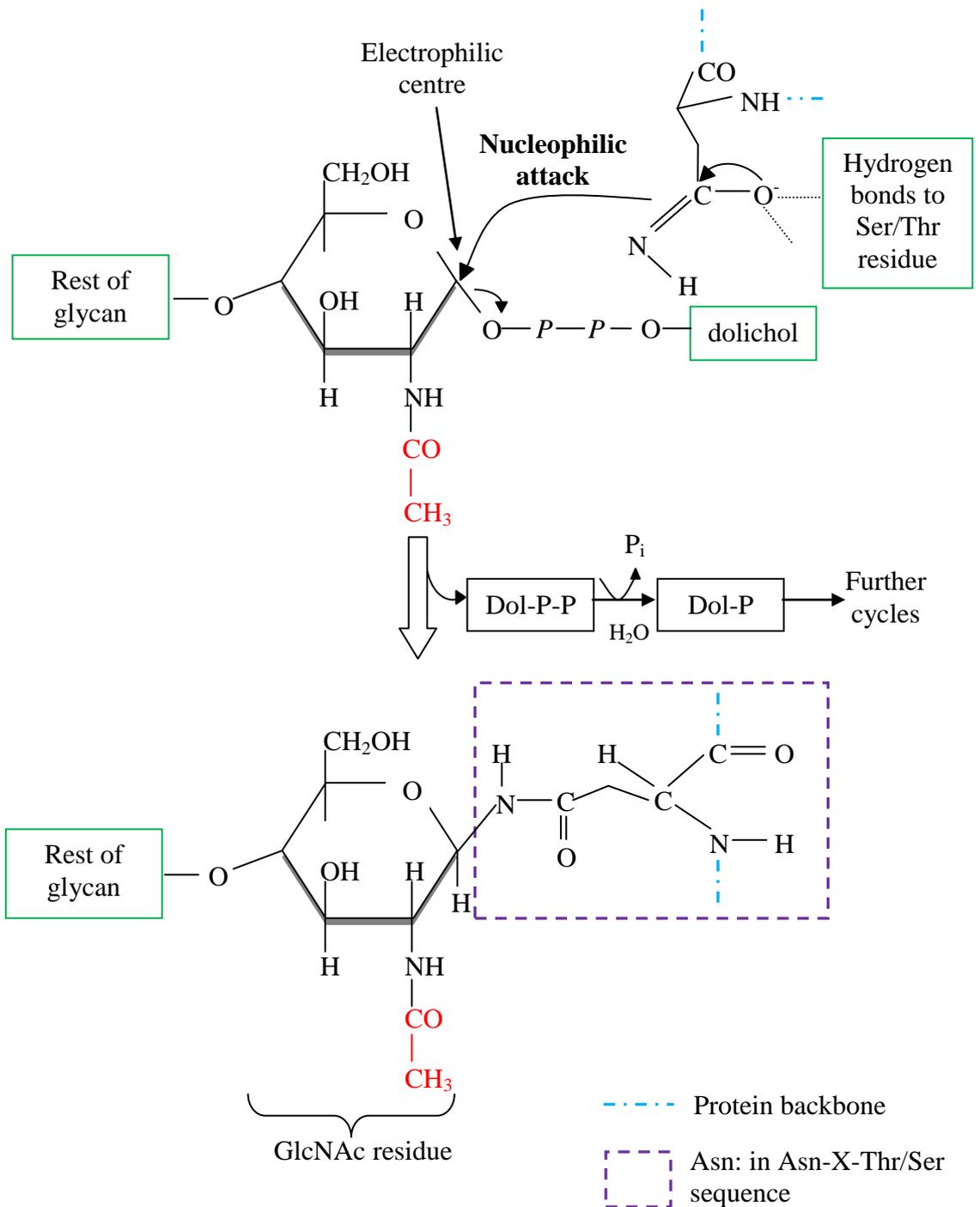
Figure 1.4. **Linkage formation between Asn and the first GlcNAc of the glycan pentasaccharide core.** [Adapted from Metzler, 2001]. OST catalyses the formation of a bond between the side-chain $-NH_2$ group of Asn and the C-1 of the first core GlcNAc residue. The hydrogen bonds formed with the Ser/Thr residue when transfer takes place from dolichol to protein are important in forming a loop. It helps polarise the $-NH_2$ group of the Asn side chain, making an electrophilic centre as shown in step 1 that can participate in a displacement reaction.

1.1.4.2 High Mannose, Complex and Hybrid N-linked glycoproteins.

Despite N-linked glycans sharing a common pentasaccharide core ($Man_3GlcNAc_2$), the overall structures can vary widely - primarily differing by the sequence and quantity of monosaccharides of which they are composed (Albani and Plancke, 1999). Enzymes residing in the ER and Golgi trim and process the glycan precursor determining whether it will be of high Man, complex or hybrid form. These structures are illustrated in figure 1.5.

Between two and six Man residues may connect to the pentasaccharide core, producing the common heptasaccharide core of high Man structures:

Man α1→6(Manα1→3)Man α1→6(Man α1→3)Manβ1→4GlcNAcβ1→4GlcNAc
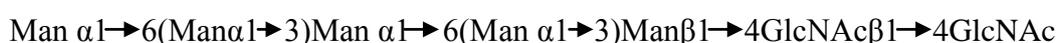
Only Man is present in the chains of these structures, up to four can bind the Man residues at the non-reducing terminal (Kobata, 1992; Imperiali and O'Connor, 1999). A common substitution of these glycans is the addition of one or more phosphate groups to the outer Man residue, producing mannose-6-phosphate in lysosomal hydrolases. It can then bind to the Man-6-phosphate receptor (Smith *et al.,* 1997).

For the biosynthesis of complex N-linked glycans more processing is required because, although the only Man residues they contain belong to the trimannosyl core, they ordinarily express a greater variety of monosaccharides and linkages. The outer GlcNAc, Gal, Fuc and SA residues typical of such structures become attached to a GlcNAc residue at the reducing end, which in turn binds to the core α-Man residues. It is also possible that a Fuc residue may become linked to the innermost GlcNAc residue, further increasing the potential structural variation (Kobata, 1992). Complex glycan structures typically express 4-6 GlcNAc residues and 5-7 neutral sugars while high Man may only have 2 GlcNAc but approximately 5-9 Man (Fan *et al.,* 1994). The complex group can be subdivided based on the number of branches present i.e. bi-, tri-, tetra-, and penta-antennary chains (figure 1.6). Neu5Ac - a SA residue - commonly terminates these glycan branches allowing them to circulate in the blood (Morell *et al.,* 1971); in their absence, the molecules may be targeted by the liver.

When oligosaccharides exhibit features of both high Man and complex type glycans, they are categorised as hybrids. Man residues can join to GlcNAc and core outer Man residues; comparable to those observed in the high Man structures. In similarity to

complex glycans, a Fuc residue may bind to the innermost GlcNAc residue (Kobata, 2000; Durand and Seta, 2000). Therefore, although they exhibit characteristics of high Man glycans with a Man $\alpha1,6$ branch, they are also similar to complex-type glycans with a Man $\alpha1,3$ branch composed of Gal and further GlcNAc residues.

An important example of a naturally occurring N-linked plasma glycoprotein is $\alpha1$ – acid glycoprotein (AGP, or orosomucoid), secreted by liver parenchymal cells. It solely expresses complex N-linked glycans and although its specific biological role/function has yet to be clearly defined it is known to bind steroids, catecholamines and certain drugs: transporting them through the plasma. AGP can also be considered a natural anti-inflammatory and immunomodulatory agent, the concentration and/or 'normal' glycosylation of which may change under various physiological and pathophysiological conditions. During the acute phase response (APR), stimulated during many of these conditions, the production of AGP is increased, possibly limiting the activity of certain drugs to which it binds (Kremer *et al*., 1988; Israili and Dayton, 2001).
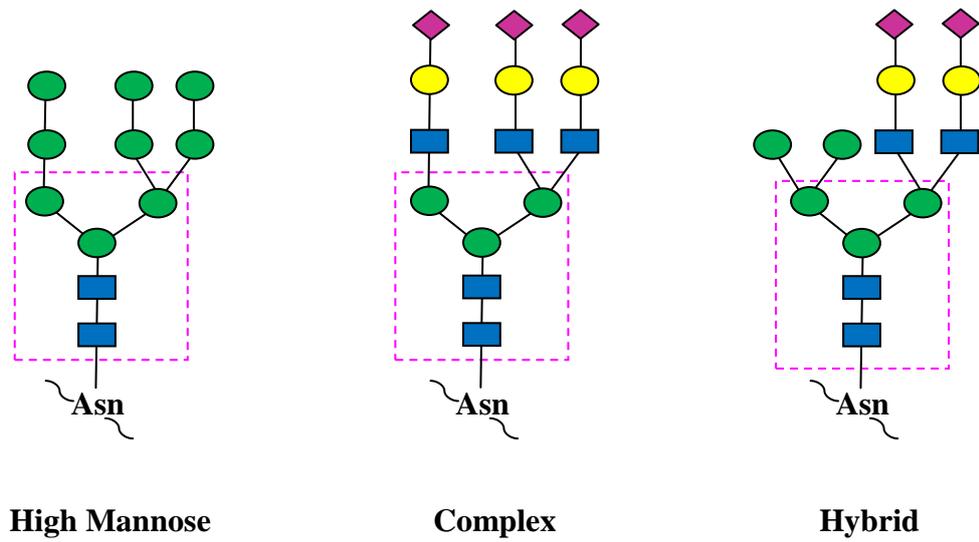
Figure 1.5. **The structure of the three types of N-linked glycan.** [Adapted from Varki *et al.,* 2009]. All share the same pentasaccharide core structure with different appendages.

■ GlcNAc ● Man ○ Gal ◆ NeuAc

a) bi-antennary

$$(NeuAc\alpha2,6)Gal\beta1,4GlcNAc\beta1,2Man$$
$$\alpha1,2$$
$$Man\beta1,4GlcNAc\beta1,4GlcNAc\beta1Asn$$
$$\alpha1,3$$
$$(NeuAc\alpha2,6)Gal\beta1,4GlcNAc\beta1,2Man$$

b) tri-antennary

$$(NeuAc\alpha2,6)Gal\beta1,4GlcNAc\beta1,2Man$$
$$\alpha1,2$$
$$Man\beta1,4GlcNAc\beta1,4GlcNAc\beta1Asn$$
$$\alpha1,3$$
$$(NeuAc\alpha2,6)Gal\beta1,4GlcNAc\beta1,2Man$$
$$\beta1,4$$
$$(NeuAc\alpha2,3)Gal\beta1,4GlcNAc$$
$$\alpha1,3$$
Fuc

c) tetra-antennary

Fuc
$$\alpha1,3$$
$$(NeuAc\alpha2,3)Gal\beta1,4GlcNAc$$
$$\beta1,6$$
$$(NeuAc\alpha2,6)Gal\beta1,4GlcNAc\beta1,2Man$$
$$\alpha1,2$$
$$Man\beta1,4GlcNAc\beta1,4GlcNAc\beta1Asn$$
$$\alpha1,3$$
$$(NeuAc\alpha2,6)Gal\beta1,4GlcNAc\beta1,2Man$$
$$\beta1,4$$
$$(NeuAc\alpha2,3)Gal\beta1,4GlcNAc$$
$$\alpha1,3$$
Fuc
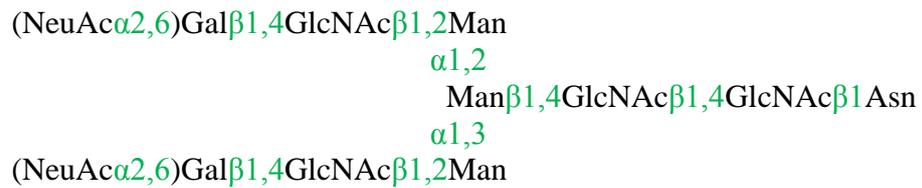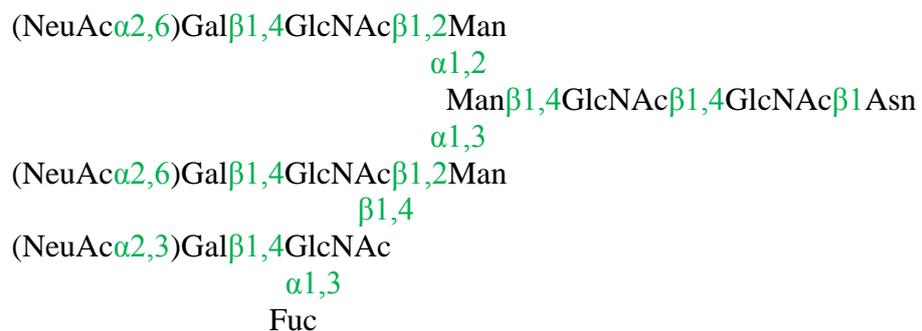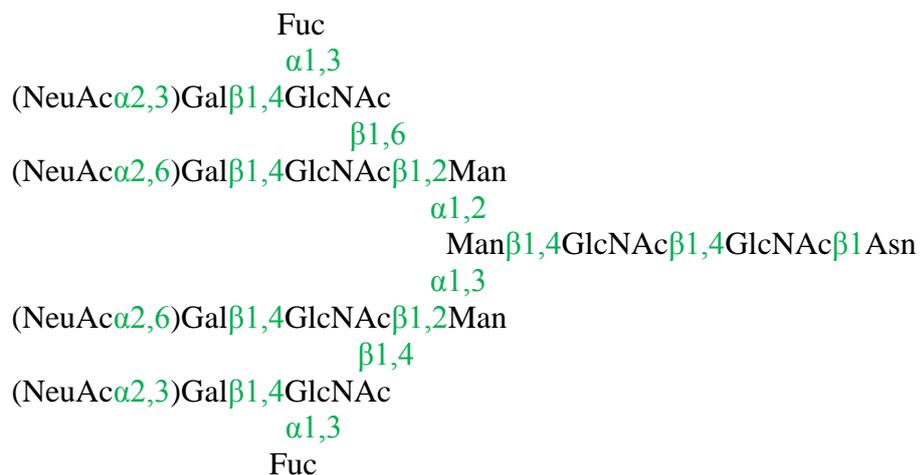
Figure 1.6.   Examples of the branching in complex and hybrid glycans. [Adapted from Hashimoto, 2004]. Different degrees of branching are exemplified with a constant core structure and linkages represented in green.

1.2 APR: Acute phase response

Initiation of the APR (summarised in figure 1.7) occurs upon systemic tissue injury, acute and chronic inflammation, and viral or bacterial infection as part of the body's defence system, attempting to regain homeostasis (Kushner and Rzewnicki, 1994). Inflammation at the site of injury results from a local immune response. Cytokine and glucocorticoid hormone release - primarily by tissue macrophages (and to a lesser degree T-lymphocytes, platelets, fibroblasts and polymorphonuclear leukocytes) - can cause a systemic immune response to follow, thereby affecting many organs. They induce the APR, disturbing the vascular system and altering the breakdown of various muscle proteins and lipids. Changes occur to the concentrations of specific plasma proteins including α_1-acid glycoprotein and albumin (Brinkmann-van der Linden *et al.,* 1998; Menkes, 1993), collectively known as acute phase proteins (APPs); the majority of which are glycoproteins (Ebersole and Cappelli, 2000). The APR is an extremely important but short-lived process, initiated minutes after the stimulus it lasts up to two days and is responsible for minimising damage to surrounding tissue and destroying foreign molecules. Anti-inflammatory cytokines are released with subsidence of the APR; IL-10 inhibits synthesis of the proinflammatory cytokines interleukin-1α (IL-1α), tumour necrosis factor α (TNF-α) and interleukin-6α (IL-6α), representing a natural feedback loop alongside the glucocorticoids. Resolution of the APR involves the restoration of membrane integrity, diuresis of excess fluid and a decrease in the catabolism of endogenous protein stores (Koj, 1998; Crestani *et al.,* 1998).

The APR reprioritises the hepatic synthesis of export proteins like albumin for an up-regulation of acute phase reactants like α_1-acid glycoprotein; a process thought to be primarily under the control of IL-6 activity on hepatocytes which induces acute phase protein transcription (Morrone *et al*., 1988). The concentration can increase from 2 fold - as reported by Voulgari *et al.* (1982) after surgery, during infections and breast, lung and ovarian cancers - to several hundred fold (Johnson and Smith, 2006; Ebersole and Cappelli, 2000). An increase has also been observed in arteriosclerosis and type 1 diabetes (Gomes *et al.,* 2003). Fibroblasts and endothelial cells are recruited, further increasing the level of cytokines produced - fevers, leukocytosis and increased hormone production can also arise (Hochepied *et al.,* 2000).
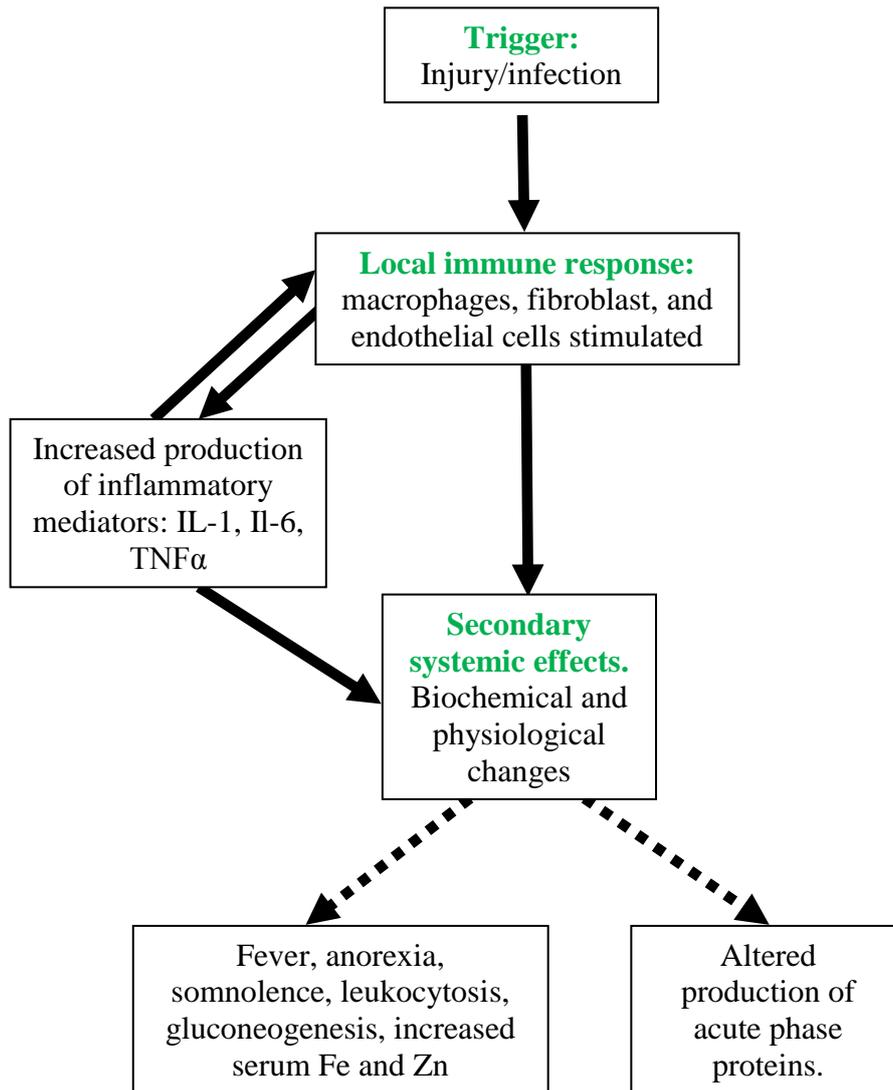
Figure 1.7. **Summary of the APR.** [Adapted from Koj, 1996].

AGP is a constitutively expressed positive APP i.e. hepatic synthesis is increased during the APR, potentially amplifying its concentration by over 25% (Ceciliani and Pocacqua, 2007). It has been suggested that a marked increase in AGP concentration may limit adverse reactions such as inflammation by providing a form of negative feedback (Crestani *et al.,* 1998). Negative APPs include albumin, α-fetoprotein and transferrin whose concentrations are correlated with a diminished hepatic synthesis during the APR. APPs can be grouped by the extent of up-regulation as illustrated in table 1.1. Alternatively it has been shown APPs can be separated into two major classes based on their response to cytokines. Type I - including AGP and serum amyloid A - are regulated by IL-1, IL-6 and glucocorticoids like dexamethasone. Type II are regulated by IL-6-type cytokines, for example the three chains of fibrinogen (Ebersole and Cappelli, 2000).

There have been studies indicating that extra-hepatic expression of APPs, although generally infrequent and less well understood than hepatic expression, can occur from human breast epithelial cells, endothelial cells and lymphocytes including the monocytes and granulocytes (Gendler *et al.,* 1982; Adam *et al.,* 2003). When increased production of AGP and other plasma proteins occur, drugs possessing a high affinity for them become increasingly bound and inactivated. Consequently, the plasma level of bioactive (unbound) drug available to the target site of action or receptor is reduced, alongside its efficacy (Kremer *et al.,* 1988). The fact that changes in APP concentration affect many aspects of health and subsequent treatment is therefore hardly surprising.

Expression of these proteins can be greatly increased in response to numerous factors therefore limiting their use as markers of specific diseases. However, additional specific alterations in glycan structure during the APR to tissue injury and inflammation can occur. In fact some studies have correlated microheterogeneity with particular diseases, therefore offering potential use as biochemical markers. Minor micro-heterogeneity for example was shown by De Graaf and colleagues (1993) to be altered during acute and chronic inflammation when there is an increase in α1-3 fucosylation of AGP; sialylation was also reported to decrease (Moule *et al.,* 1987). Additionally, Adam *et al.* (2003) reported glycosylation patterns might be useful when determining the stage of a patient's multiple sclerosis (MS); however - despite its sensitivity - they were sceptical of its specificity. The degree of branching (major microheterogeneity)

has often been reported to alter during pathological conditions; in RA for example a decrease has been detected (Pawlowski *et al*., 1989).

AGP is one of the APPs which can undergo extensive post-translational modification. It is involved in the regulation of inflammation; perhaps through the acquisition of SLe^{x} containing glycans which help AGP bind to leukocyte and endothelial cell (ETC) selectins, suppressing inflammation by preventing the movement of leukocytes, keeping the inflammatory response localised (De Graaf *et al.,* 1993).

Protein name	Class of APP	Sub-class	Function
Ceruloplasmin	Positive	I (Concentration increases by approx. 50%)	Copper transport protein
Complement C3 and C4			Opsonin
α_1-acid glycoprotein		II (2-5 fold increase)	Immunomodulation, transport
α_1-antitrypsin			Antiprotease
Fibrinogen			Coagulation
C-reactive protein		III (up to 1000-fold increase in concentration)	Opsonin, immunomodulation
Serum amyloid A			Leukocyte activation, chemotaxis, phagocytosis
Albumin	Negative	-	Transport
Transferrin		-	Transport

Table 1.1. **Classification of acute phase proteins.** Based on information from Ceciliani *et al.* (2002) and Ceciliani and Pocacqua (2007).

1.3 α_1 -Acid-Glycoprotein (AGP)

In 1882, AGP was first identified as a 'reddish-brown residue that remained in solution' (Chiu *et al.,* 1977) but the protein was not isolated or characterised until 1950 by the Weimer and Schmid groups. It is a constitutively produced glycoprotein, hepatically synthesised in parenchymal cells at a rate of 10mg/kg/day with a plasma concentration of approximately 0.3-1.46gL^{-1} in healthy young adult humans (Blain *et al.,* 1985; Ceciliani and Pocacqua, 2007). Like other APPs, its mRNA expression is influenced by cytokines and steroid hormones. Levels of AGP, as discussed in the previous section, can increase 2- to 5-fold as a result of the APR to pathological and physiological stimuli. Concentrations have been reported to increase during myocardial infarctions and stress (Duché, 2000). It is known that various factors act to influence AGP glycosylation including the inflammatory state (Van Dijk *et al.,* 1998), pregnancy (Havenaar *et al*., 1998), drug or glucocorticoid use (Pos *et al.,* 1988), and oral contraceptive use (Brinkman-van der Linden *et al.,* 1996).

Being a secondary APP, AGP should reduce the harmful systemic effects induced during the initial stages of acute inflammation (Van den Heuvel, 2000). Its ability to inhibit the complement cascade is reliant on the microheterogeneity of its glycans which in turn is dependent on the expression of (patho)physiological conditions. Microheterogeneity is described as either major, reflecting changes in the number of branches on N–linked glycans, or minor which occurs through variation in the SA or Fuc content (Kalmovarin *et al.,* 1991).

Transcription of AGP encoding genes can be both endogenous and exogenous. Although extra-hepatic expression has been reported, they are most commonly hepatically expressed (Adam *et al.,* 2003). *In vivo*, the human liver synthesises AGP from the information encoded by 3 adjacent genes, namely AGP-A, B and B', which form a cluster of 70kb on chromosome 9 (Lögdberg and Wester, 2000). All three genes exhibit six exons and five introns but code for different variants.   The hepatic expression of the genes is regulated both transcriptionally and posttranscriptionally through the activities of inflammatory cytokines including glucocorticoids, Il-1β, TNF-α and Il-6 (Chang *et al.,* 1998). Native (commercial) AGP is reported to consist of more AGP-A encoded material than AGP-B/B' but unlike the former gene, AGP-B/B' only

encodes a single variant (Eap *et al.,* 1988a). A study by Van Dijk (1991) found both gene products increased during inflammation in burn patients but the contribution made by the AGP-A gene was greater.

AGP-A codes for the predominantly monomorphic ORM-1 variant (ORM1 F1 and ORM1 S) and the identical AGP-B and AGP-B' genes encode a single ORM-2 variant (ORM2 A). They differ from AGP-A by a 22 base substitution and are expressed around 100 times less. Dente and colleagues (1985) identified the two genes using southern blotting showing they were present in normal serum in a ratio of 3:1 respectively. AGP-B and B' produce low levels of bi-antennary glycans while AGP-A primarily gives rise to tri-/tetra-antennary glycans (Dente *et al.,* 1987; Tomei *et al.,* 1989; Duché *et al.,* 2000). Normal pooled serum can contain ORM1 F1, ORM1 S and ORM2 A variants; according to Eap and colleagues (1988a) the three main human AGP phenotypes depend on whether two or three of the variants are expressed in plasma, namely F1S/A (50%), F1/A (35%) and S/A (15%).

1.3.1 Structure of AGP

AGP has a chemical nature identical to many serum components which also interact with hormones like progesterone, producing dissociable complexes (Albani, 1997). It has a molecular weight between 38.8kDa-48kDa (Israeli and Dayton, 2001); the value reported is likely to depend primarily on the method of isolation used. Unusually, it is soluble in both water and polar organic solvents (Fournier *et al.,* 2000). AGP belongs to the subfamily of immunocalins - binding proteins with immunomodulatory functions but can also be classed as a mucoprotein, seroglobulin and α-1-globulin (Kremer *et al.,* 1988). In 1973, Schmid and colleagues were the first to identify the structure of AGP, however they had believed there to be only 181 amino acids constituting the peptide backbone. This was refuted in 1985 when it became widely accepted that an extra Lys and Arg resided at positions 173 and 174 respectively, producing a 183 amino acid backbone (Dente *et al.,* 1985). The 22 sites for possible amino acid substitution which present the differences between AGP-A and AGP B/B', may lie within the glycosylation sites (positions 15, 75 and 85) but whether it causes altered glycosylation of the gene products is unknown (Van Dijk, 1991). The sequence of amino acids is illustrated in figure 1.8. Using homology modelling, Hazai *et al.,* (2006) found that serine (Ser) is

24

located at position 77 and glutamate (Glu) at 92 in ORM2 but not in ORM1. Also, at positions 98 and 115, ORM1 has phenylalanine (Phe) and aspartate (Asp) respectively but ORM2 expresses valine (Val) and tyrosine (Tyr).

The human AGP precursor consists of 201 residues; the first 18 residues become cleaved during protein processing (Ceciliani and Pocacqua, 2007). Its protein portion constitutes 23kDa of the mass and approximately 59% of the structure (Albani *et al.,* 2000; Liao *et al.,* 1985). Polymorphisms can arise; at positions 32 and 47, various amino acids can be expressed and substitutions can occur at residues 21 and 181. Despite the potential for polymorphisms, the structure of AGP still shows an 80% homology with immunoglobulin G (Kremer *et al.,* 1988).

Proportionally, the different secondary structures including α-helix, β-sheet, reverse β-turns, and unordered structures account for 15%, 41%, 12%, 8% and 24% of the AGP molecule respectively – as reported by Kopecký and colleagues (2003) upon analysis by infrared and Raman spectroscopy. The large proportion of β-sheet in the structure is common to proteins exhibiting the transport-trafficking function - lipocalins. These molecules consist of a single eight-stranded antiparallel β-sheet closed back on itself to form a continuously hydrogen-bonded β-barrel (Flower, 1996).

Four of the five AGP glycan chains are linked to Asn residues within reverse β-turns or in regions where charged and polar residues are numerous (Kremer *et al.,* 1988; Ceciliani and Pocacqua, 2007); the glycosylation process may force the nascent peptide to twist from an Asn turn to a β-turn. This has been supported by nuclear magnetic resonance (NMR) analysis (O'Connor and Imperiali, 1997). As previously described (section 1.1.3.1), proline cannot form part of the consensus sequence Asn-X-Ser(Thr) as it prevents the formation of a β-turn which is required for the glycan attachment.

Studies have shown that certain amino acids show particular preferences regarding their locations in the folded structure. There are three tryptophan (Trp) residues; Trp 25 is buried in the hydrophobic binding site while the others are closer to the surface; Trp 160 is exposed and Trp 122 lies between the hydrophobic binding site and the surface (figure 1.8). The closer the Trp residues are to the surface, the more they can rotate (Friedman *et al.,* 1985). Additionally, nearly all the Phe and 5-7 Tyr are completely or partially buried in the native state.

Glu – Ile – Pro – Leu – Cys – Ala – Asn – Leu – Val – Pro – Val – Pro – Ile – Thr –
Asn – Ala – Thr – Leu – Asp – Gln/Arg – Ile – Thr – Gly – Lys – **Trp** – Phe – Tyr – Ile
– Ala – Aer – Ala – Phe/Ala – Arg – Asn – Glu – Glu – Try – **Asn** – Lys – Ser – Val –
Gln – Glu – Ile – Gln – Ala – Thr/Ala – Phe – Phe – Try – Phe – Thr – Pro – **Asn** – Lys
– Thr – Glu – Asp – Thr – Ile – Phe – Leu – Arg – Glu – Tyr – Gln – Thr – Arg – Gln-
Asp/Asn – Gln – Cys – Ile/Phe – Try – **Asn** – Thr/Ser – Tyr – Leu – Asn- Val – Gln –
Arg – Glu – **Asn** – Gly – Thr – Ile/Val – Ser – Arg – Tyr – Val/Glu – Gly – Gly –
Gln/Arg – Glu – His – Phe/Val – Ala – His – Leu – Leu – Ile – Leu – Arg – Asp – Thr –
Lys – Thr – Tyr/Leu – Met – Leu/Phe – Ala/Gly – Phe/Ser – Asp/Tyr – Val/Leu –
Asn/Asp – Asp – Glu – Lys – Asn – **Trp** – Gly – Leu – Ser – Val/Phe – Tyr – Ala –
Asp – Lys – Pro – Glu – Thr – Lys – Glu – Gln – Leu – Gly – Phe – Tyr – Glu – Ala –
Leu – Asp – Cys – Leu – Arg/Cys – Ile – Pro – Lys/Arg – Ser – Asp – Val – Val/Met –
Tyr – Thr – Asp – **Trp** – Lys – Lys – Asp – Lys – Cys – Glu – Pro – Leu – Glu – Lys –
Gln – His – Glu – Lys – Glu – Arg – Lys – Gln – Glu – Glu – Gly – Ser – COOH

Figure 1.8. **The amino acid sequence of AGP.** [reproduced from Dente *et al.,* 1985]
The five Asn sites to which glycans attach are labelled in green and the amino acid
residues where substitution occurs are purple. Trp 25 (**red**) indicates the residue buried
in the hydrophobic pocket, Trp 160 (**orange**) is exposed and Trp 122 (**blue**) resides
between the surface and pocket.

Glycan chains account for the remaining 41-45% of the molecule. Despite such an abnormally high content, galactoglycoprotein (identified in 1980 by Schmid and colleagues) supersedes it with 76%. There are five variable, highly sialylated heteropolysaccharide complex-type glycans Asn-linked at residues 15, 38, 54, 75, and 85 - normally becoming bound in the first half of the polypeptide (Israeli and Dayton, 2001; Eap and Baumann, 1993). The presence of acidic amino acids and the -COOH group at C1 of SA (a common terminating sugar α2-3 or α2-6 linked to Gal; also called neuraminic acid-NeuAc) gives rise to the negative charge and the very low pI of 2.8-3.8 (Hochepied *et al.,* 2003; Elg *et al.,* 1997). There can be up to 16 SAs, which account for approximately 11-12% of the structure and each is reported to have a molecular weight of 314Da. If the glycoprotein is desialylated, the molecule is rendered inactive. The structures are also constructed from 14% neutral hexoses [Gal, Man and GlcN (a hexosamine)] and 1% Fuc (Albani, 2003; Kremer *et al.,* 1988; Dente *et al.,* 1987). As discussed in section 1.3, there is a region of commonality between N-linked glycans like those of AGP, namely the pentasaccharide core.

Fuc is a terminating sugar which can link to GlcNAc (α1-3) on the external branch. It can also be linked through an α1-6 bond to a core GlcNAc and α1-2 to Gal (Fournier *et al.,* 2000). It is therefore not surprising that the degree of fucosylation can vary between individuals, generally greater in structures with elevated tri- and tetra antennary chains. AGP is one of the few glycoproteins that contain tetra- as well as bi- and tri-antennary N-linked glycans. Fournier *et al.* (2000) reported that 30% of human serum proteins actually have no Fuc. Within the Byers *et al.,* (1999) study, it was shown that AGP appeared to contain 11.3% NeuAc, 0.8% Fuc, 14.9% GlcNAc, 6.3% Gal and 5.2% Man.

There is potential for the existence of 10^5 different glycoforms of AGP due to the huge structural variability provided mainly by the presence of glycans (microheterogeneity). However in normal, non-pathological conditions there are only 12-20 expressed, each exhibiting various degrees of branching, fucosylation and sialylation (Albani, 1997). The number of feasible glycoforms is reduced because the Asn residues selective for the type of glycans they express in terms of the degree of branching (figure 1.9). The first and second Asn sites (Asn 15 and 38) prefer to harbour bi-antennary glycans whilst Asn 15 will not bind a tetra-antennary chain and Asn 38 never binds fucosylated glycans. Conversely, Asn 75 and Asn 85 prefer more branched glycans; in fact site Asn 75 never carries bi-antennary chains and Asn 85 usually expresses the greatest degree of α1,3-

fucosylation (Higai *et al.,* 2005). This increased branching could explain why only these two sites potentially carry a tetra-antennary chain with more than one Fuc residue (Fournier *et al.,* 2000). It has been found that the majority of AGP glycans have a tri/tetra-antennary structure (85-90%) and the remaining 10-15% is bi-antennary glycans (Perkins *et al.,* 1985).

Anti-inflammatory properties have been shown to be dependent upon glycan composition. For example, Pos and colleagues (1990) showed that biantennary glycans must be expressed by AGP if it is to inhibit CD3-induced proliferation of lymphocytes while another group (Williams *et al.,* 1997) reported that the presence of SLe[x] groups enable it to cause amelioration of neutrophil- and complement-mediated injuries.

There are two types of microheterogeneity expressed by AGP; each can be further subclassified and are associated with the glycan chains not the peptide backbone; the peptide remains constant. For the major form, type I causes the degree of tri- and tetra-antennary branching to be reduced in preference for bi-antennary chain expression; the opposite occurs in type II, as shown in ConA studies (van Dijk *et al.,* 1994) where the most strongly retained fraction possess the greatest biantennary content while the unretained fraction represents those without any biantennary chains. According to Bayard and Kerckaert (1980), in normal plasma the three fractions account for 46%, 39.1% and 14.9% of the AGP conveyed (un-retained, weakly retained and strongly retained respectively), therefore supporting the theory that structure of AGP is highly branched.

The minor form is not directly associated with branching but instead the extent of fucosylation and sialylation of the five oligosaccharide chains (Albani *et al.,* 1997). In 2002, Smith and co-workers reported that the termini of tri- and tetra-antennary chains are more tightly bound to the polypeptide core than those of bi-antennary; this makes the glycoconjugate more metabolically stable. It is the tissue-specific regulation of glycosyltransferase genes, the availability of sugar nucleotides, and competition between enzymes during glycan processing that determines the variation in glycan microheterogeneity.
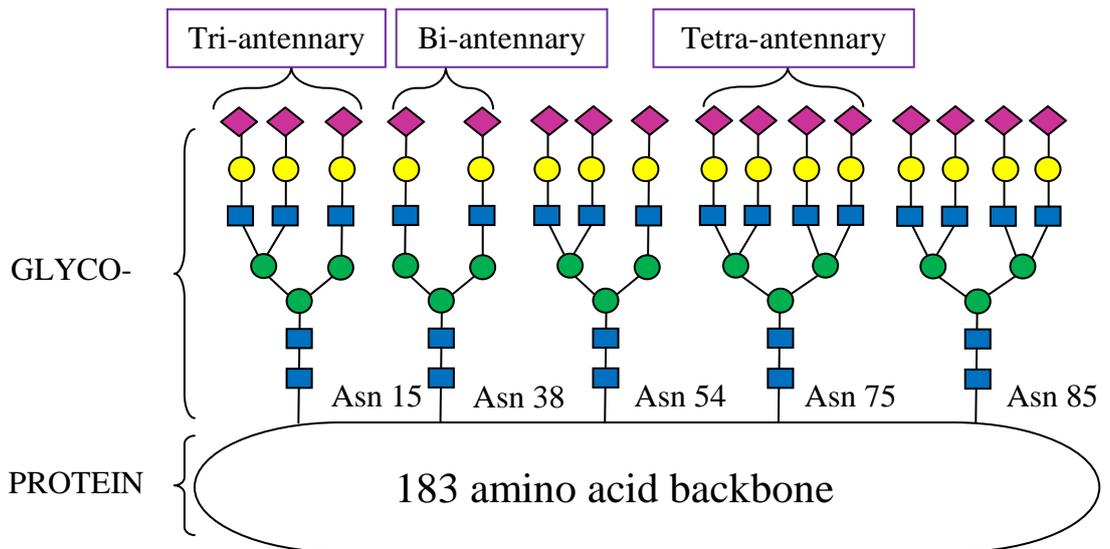
Figure 1.9. **A diagrammatic representation of possible N-linked glycosylation of AGP.** The common degrees of branching are indicated, as described in the text; the lengths of the chains can vary. Also, the specific Asn residues to which glycans become attached are labelled. Branching can be bi-, tri-, or tetra-antennary with some of the Asn sites specific in the type they will express.

■ GlcNAc   ● Man   ○ Gal   ◆ NeuAc

1.3.2 *Analysis of glycan microheterogeneity*

The functions attributed to AGP, like all glycoproteins, are largely determined by the glycans with which they are associated. During various physiological and pathophysiological conditions, the heterogeneity of the glycoforms expressed can become altered, potentially affecting the functions subsequently performed. It is therefore of interest to analyse these structures. The microheterogeneity of AGP can be ascertained using CAIE (crossed affinity-immunoelectrophoresis), which uses a Concanavalin (con A) lectin isolated from the jackbean to interact with the glycans - fractionating AGP according to the bi-antennary content, without altering the structure (Van Dijk *et al.,* 1994; Hansen *et al.,* 1989). Another highly desirable technique in the field of glycomics due to its sensitivity is high pH anion-exchange chromatography (HPAEC) which primarily separates structures based on the negative charge expressed.

Coupling HPAEC with pulsed amperometric detection (PAD) transformed the analysis of glycans by offering highly sensitive (10–100 pmol) detection and effective separation without the need for prior derivatisation. Both the monosaccharide components and whole oligosaccharides of N-linked glycans can be analysed using the technique (Townsend *et al*., 1989; Smith *et al*., 1997). More than 20 years after its introduction, the use of HPAEC remains widespread and competitive with other techniques. Recently, Adamo and colleagues (2009) concluded that its sensitivity compared favourably with capillary electrophoresis–laser induced fluorescent detection and reverse-phase HPLC coupled to electrospray mass spectrometry for the analysis of the monosaccharides of IgG glycans.

Tandem Mass Spectrometry (MS/MS) is seeing increasing use in glycoprotein analysis. It was first utilised in the analysis of N-linked glycans by Mock *et al.* (1991) but the methodology is not without problems, hindered by the potentially high proportion of peptides in a sample which can exceed that of glycopeptides. Thus, because the signals generated by peptides are often much higher, those of the glycopeptides can be masked; especially if they are terminated in negatively charged SA residues (Annesley, 2003). Difficulties also arise in its ability to distinguish oligomers of similar masses and linear isomers unlike HPAEC-PAD.

1.3.3 AGP Structure and Disease

It is unlikely that the exact cause of elevated AGP can be determined however, alterations in the microheterogeneity of the glycoprotein may offer more potential as they often correlate with specific conditions (Hansen *et al.,* 1984). Although the glycosylation of AGP is generally reproducible in healthy individuals, it is known to be altered – alongside the levels of its expression - in response to certain pathophysiological conditions like inflammation, cancer and RA (Van Dijk *et al.,* 1994).

Hashimoto (2004) for example, reported that it may be possible to analyse the glycosylation pattern of AGP to determine the progression of cancer; there were significant differences between pre-operative patients and a healthy population, indicating their clinical status. Merely analysing the levels of AGP, although significantly different between groups, did not allow the clinical status to be determined. The poorest prognosis was found in patients with advanced malignancies and highly fucosylated triantennary and tetraantennary AGP glycans for long periods after surgery. Significant changes to the level and glycosylation of AGP have also been reported by Haston (2003) during inflammation. The degree of fucosylation and sialyl Lewis X (SLe^x : Neu5Ac α2-3Galβ1-4(Fucα1-3)GlcNAc-R*)* expression is increased during acute inflammation; thought to be part of a humoral feed-back response to the interaction of leukocytes with E-selectin which is required for extravasation into inflamed tissues (van der Linden, 1994). A decrease in tri- and tetra-antennary structures but an increase in bi-antennary glycans attached to the five sites of glycosylation was reported in acute inflammation along with increased α1,3 fucosylation at most sites (Higai *et al.,* 2003; 2005). In chronic inflammatory conditions like grade III and IV RA, Mackiewicz *et al.* (1987) reported that the expression of bi-antennary glycans and sialylation decrease in preference for tri- and tetra-antennary chains occurring alongside hyperfucosylation – as demonstrated a decade later by Elliott and colleagues (1997). Asn 75 and Asn 85 (figure 1.9) show increased tri- and tetra-antennary α1,3 fucosylation. When comparing acute and chronic sera to that of healthy individuals, they did not find any significant alterations in the N-glycan composition. In 2001, Rydén *et al.* (2002) indicated that AGP fucosylation was increased with disease severity in male patients. An increase in α2-3 linked SA expression is also associated with RA (Elliott *et al.,* 1998). Smith *et al.* (2002) reported

on the possibility that AGP production is localised in rheumatoid synovial fluid (SF) because the AGP microheterogeneity differed to that in serum. The latter expressed fewer branches and a lower degree of fucosylation.

Increased α1,3 fucosylation in hepatitis B and some cancers can cause an increase in the expression of SLe[x] on tri- and tetra-antennary glycans (Kobata., 1992; Dennis *et al.,* 1999). Studies by Kremmer *et al.,* (2004) indicated that the AGP of cancer patients expressed greater Fuc and SA content when compared to a healthy population. The increase in SA expression in cancer was shown previously by Moule (1987) who studied the microheterogeneity of serum AGP in patients with RA, myocardial infarction (MI), cancer with elevated AGP, cancer without elevated AGP and compared them to that obtained a group of healthy volunteers. Unlike the cancer groups, the level of AGP in RA patients decreased. Cystic fibrosis (CF) sufferers have decreased sialylation and increased number of Fuc residues bound via an α1,3-linkage to antennary GlcNAc. The length of the polymers affects the migration of cells; increased Fucα1,3GlcNAc and decreased SA on CF airway cells, may present a target for bacteria and leukocytes (Scanlin and Glick, 2000). It has been shown that the severity of liver diseases like cirrhosis can be reflected in the structural changes in AGP (Mooney, 2006). Increased expression of Fuc residues occurred in cirrhosis as was indicated in 1987 and 1989 by Biou and colleagues. A decrease in sialylation occurred in fibrosis. Changes - including an increase in branching and sialylation - also occur under normal physiological processes like pregnancy (Orczyk-Pawiowicz *et al.,* 2006). Any changes which occur, whether during physiological or pathophysiological conditions, can cause alterations in the functions performed by the glycoprotein because they influence the overall structure and conformation of the biomolecule.

1.3.4 Functions of AGP

It has been established that the constitutive production of AGP contributes to the maintenance of homeostasis and that the glycan composition of AGP determines the functional roles of the glycoprotein like those in immunomodulation and drug binding (Ceciliani and Pocacqua, 2007). However, its primary biological function remains elusive despite being investigated intensively since the 1950s. AGP is a member of the lipocalin family (proteins with hugely diverse sequences but highly conserved

structures), enabling it to carry out the transport of small hydrophobic molecules (Treuheit *et al.,* 1992; Flower *et al.,* 2000). As noted by Chiu *et al.* as early as 1977, many AGP functions depend on the structure of the attached glycans.

1.3.4.1 Immunomodulation

AGP has been reported to have potential physiological significance as a natural anti-inflammatory and immunomodulatory agent, both *in vitro* and *in vivo* notably with respect to anti-neutrophil and anti-complement activity (Williams *et al.,* 1997). The microheterogeneity of AGP is responsible for its fairly non-specific immunosuppressive properties; it is involved in the inhibition of neutrophil activation, platelet activation, phagocytosis and − due to interactions formed with collagen - wound healing (Costello *et al.,* 1979; Bennett and Schmid, 1980). When asialylated and agalactosylated, immunosuppression of lymphocyte function was maximised (Hochepied *et al.,* 2003; Bennett and Schmid, 1980). The number of branches and degree of sialylation is thought to be responsible for its ability to inhibit lymphocyte proliferation.

AGP can inhibit the complement cascade if the glycan composition is correct thereby reducing the harmful systemic effects induced during the initial stages of acute inflammation (Kalmovarin *et al.,* 1991). Anti-inflammatory cytokines like IL-1β, IL-6 and TNF-α, are produced in response to AGP production. Daemen *et al.*, (2000) indicated that exogenous AGP and another APP, α_1-antitrypsin (AAT), might also inhibit inflammation and the apoptosis of hepatocytes. It is possible that AGP evolved to become part of the body's defence system, limiting the damage to healthy tissues caused by inflammation. *In vivo*, normal serum AGP levels may protect against the activation of polymorphonuclear leukocytes (PMN) however, during inflammation chemotoxins and other activators could override this protection (Costello *et al.,* 1984).

The glycan moieties modulate the interaction of AGP with receptors and ligands, the extent of which depends on variations in glycosylation (Ceciliani and Pocacqua, 2007). One alteration, which occurs during acute inflammation, is the expression of the E-, L- and P- selectin ligand, SLe^x. Selectins are important for interactions between leukocytes and the endothelium thus any modification to the structure subsequently affects the interactions. For example, E- selectin binds SLe^x on AGP and therefore competes with leukocytes expressing the E-selectin ligand ESL-1. A consequence of

increased SLe^x-substituted glycan expression is the inhibition of leukocyte rolling and diapedesis into inflamed tissues, acting similarly to a negative feedback mechanism (De Graaf, *et al.*, 1993). It was also shown that the glycans of AGP are critical in suppressing lymphocyte proliferation induced by mitogens (Pos *et al.,* 1990).

When investigating the anti-inflammatory effects of AGP, Tilg *et al.,* (1993) were interested in determining if AGP and other APPs; α_1-anti-trypsin and C-reactive protein could induce IL-1β and its specific antagonist, IL-1 receptor antagonist (IL-1Ra) in peripheral blood mononuclear cells (PBMC). When incubated with AGP, the level of IL-1Ra induced was significantly less than that with CRP or AAT but the level was always preferentially induced over that of IL-1β. From this information they concluded that IL-1Ra induction may contribute to the anti-inflammatory effects of APPs. IL-1Ra prevents the binding of IL-1 (an important inflammatory mediator in fever, hypotension and the APR) to its receptors in a competitive manner both *in vivo* and *in vitro* (Arend, 1991).

In addition to the various immunological roles that AGP has, it is one of the most important non-specific binders of drugs.

1.3.4.2 Drug binding

Drug activity is dependent on a number of factors including its ability to bind to other molecules, affecting their tissue distribution. For over 50 years there has been extensive research into the binding of molecules to human serum albumin (HSA), subsequently affecting their efficacy, distribution and disposition. More recently there has been increasing interest in the study of the degree of binding to other plasma binding proteins including AGP (especially for basic drugs), to determine if binding affects their pharmacokinetics (PK) and pharmacodynamics (PD). Under non-pathological circumstances, AGP is present at lower concentrations than HSA (50-100mg/100mL compared to 4g/100mL); however its concentration may increase significantly to become the major drug binding macromolecule during certain pathophysiological conditions. Conversely, the level of HSA decreases. Such fluctuations in APP concentrations influences the ability of certain drugs to produce their effect (Ceciliani and Pocacqua, 2007; Israili and Dayton, 2001). When the drug is administered, the binding equilibrium is immediately altered between drug and proteins (Kuroda, 2003).

Binding to AGP is not universal between drugs; it depends on certain properties and therefore they are not all affected similarly. For example, AGP shows preference to basic and neutral drugs - propranolol, methadone and warfarin are among those that the glycoprotein has been shown to bind with a high-affinity and low capacity. It can also bind steroid hormones like progesterone and some acidic molecules like the retinoic acids (Israili and Dayton, 2001). This is in contrast to HSA which mainly binds acidic drugs as proven by Pike and colleagues (1983) who reported that HSA-deficient plasma showed a decrease in the binding of acidic and neutral drugs.

The products of the genes encoding AGP (namely AGP-A, AGP-B and B') show preference for the binding of different drugs. Basic drugs generally favour the ORM2 A variant, as reported using imipramine by Hervé et al. (1993), while acidic drugs like warfarin bind the ORM1 F1/S variant. Hervé and colleagues concluded that the ORM2 A variant binding site was specific for basic drugs while ORM1 F1 and/or S for acidic.

There are a number of pharmacokinetic factors associated with the binding of drugs. When bound it cannot penetrate the blood vessel walls, a process required to reach its site of action thus it is consequently rendered inactive. The ability to induce the pharmacological effect is determined by the remaining unbound drug - the free drug hypothesis (Tillement et al., 1988). In order to produce the effects associated with the drug administered, a certain free level must be present at its site of action (minimal effective concentration, MEC), if not its efficacy is reduced. Binding to AGP may prevent attainment of the MEC therefore rendering the drug ineffective if the dosage is not adjusted accordingly. The level of bioactive drug therefore varies with altered AGP concentrations despite the total drug concentration being only marginally affected (Kremer et al., 1988).

It is possible that binding to plasma proteins could be, at least in part, responsible for multi-drug resistance (MDR). The concentration of free drug that reaches the target site may be too low to induce the therapeutic effect, providing time to evolve drug resistance (Fournier et al., 2000). In some patients a commonly toxic drug dosage may not cause the associated adverse effects if the AGP concentration is elevated; although the total drug concentration is high, a large proportion will be inactivated through binding to the glycoprotein (Israili and Dayton, 2001; Silamut et al., 1991). This concept was summarised in figure 1.10.
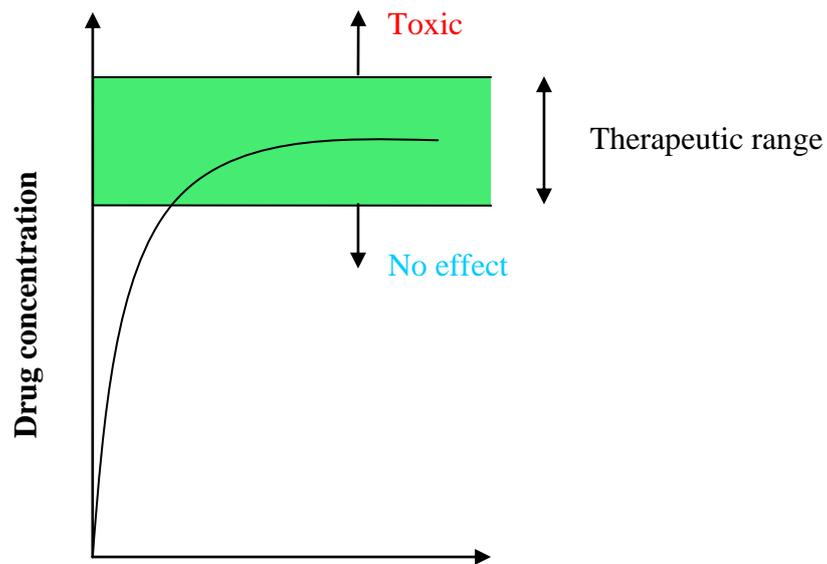
Figure 1.10.   Representation of the therapeutic range of drugs. The therapeutic range (represented in **green**) is the drug concentrations that, if present at the site of action, will produce the associated effects. Concentrations greater than this can cause toxicity (red) and those below would be ineffective (blue) in the body, possibly causing withdrawal symptoms if the drug is used regularly.

The binding sites are thought to be buried in a hydrophobic pocket, thus drug binding is generally regarded as hydrophobic. It was initially suggested that AGP expressed one wide and flexible binding site for drugs (Maruyama *et al*., 1990). However more recent studies have refuted this, reporting it to consist of at least three partially overlapping subsites; the basic ligand binding site, an acidic ligand binding site and a steroid hormone (SH) binding site (Matsumoto *et al.,* 2002). It has also been suggested that the number of binding sites expressed on AGP for a particular drug will depend on the genetic variants of the glycoprotein present (Hervé *et al.,* 1993); imipramine for example was shown to bind one site on the A variant with high affinity and to two other sites with a low affinity while warfarin exhibited binding to fewer sites. Therefore, differences in binding cannot be solely explained by variability in the affinity at a single binding site. Methadone is thought to bind AGP at a single binding site with preference to that present on the ORM 2A variant.

When AGP binds to ligands, the dynamics of the binding pocket are affected - the buried Trp residue becomes more exposed whilst the Trp at the surface becomes increasingly buried; (Fournier *et al.,* 2000; Johnson and Smith, 2006). It is the ability of Trp (and to a lesser extent, Tyr) residues to emit fluoresce between 300-400nm (max approximately 340nm) when excited at 280nm, that allows fluorimetry to be used in the analysis of drug binding – more binding causes a decreased fluorescence because it masks (quenches) the underlying fluorescing residues. However, it must be noted that fluorescence resonance energy transfer (FRET) may occur in such studies. The phenomenon is particularly problematic when the drug under investigation has absorption spectra spanning wavelengths used to determine the fluorescence emitted by AGP and other proteins. Chlorpromazine for example, is a yellow compound which is well known to absorb at wavelengths including those around 340nm. Therefore a reduction in the fluorescence is likely to be at least partly due to absorption by the drugs' (Shine & Mach, 1965).

It is also possible that in some proteins, when there is a potential change in its conformation to accommodate the ligand, the position of residues - normally far apart in the absence of drug - may be altered. Fluorescence of the residues may subsequently be reduced, not only because they become increasingly buried but due to potential transfer of fluorescence to other residues. A greater reduction would therefore be recorded than that solely due to the masking effect in the binding site brought on by the drug.

A number of factors affect the ability of AGP to successfully bind drugs *in vivo*, including physical-chemical properties and inter-individual differences. Physical-chemical properties include pH (binding increases with pH), the presence of alosteric co-operativity with proteins like albumin (Fournier *et al.,* 2000; Israili and Dayton, 2001), and altered glycoprotein concentration in various diseases like breast and liver cancers where AGP concentration increases (Jackson *et al.,* 1982). Major and minor heterogeneity, altered conformations induced by disease (for example altered sialylation, fucosylation and glycosylation pattern) and displacement by other ligands are further examples (Rydén *et al.,* 1997; Israili and Dayton, 2001).

Gender, age, ethnicity of donor and genetic polymorphisms are examples of inter-individual differences. It has been noted in a number of studies that healthy females appear to have more sialylated glycoforms than males but males generally have higher levels of AGP (Israili and Dayton, 2001). Females can show further interindividual variation, which suggests their hormones have a role in altering AGP structure. Younger females, for example those in the later stages of pregnancy and those taking the contraceptive pill, express even lower levels of AGP (Routledge, 1989). Reports regarding the levels of AGP in the elderly are in wide disagreement. Veering *et al.* (1990) for example, reported no correlation between the increased production of AGP and age, whilst Davis *et al.* (1985) found AGP increased slightly with age. However it is possible, as suggested by Israili and Dayton (2001) that factors such as weight, inflammation and nutritional status could account for the variation. Research has shown that there is a difference between Caucasians and African-Americans in the degree of binding salicylic acid and disopyramide. Caucasians appeared to have more binding initially however no significant differences were noted when the correlation factor had been accounted for (Johnson and Livingston, 1997). If any of the numerous factors cited cause alterations in the expression of AGP, the efficacy of drugs may be affected.

Various studies have been undertaken indicating differences in the ability of the AGP variants to bind drugs. Most drugs bind both AGP variants however there are some which show a preference. Disopyramide and imipramine for example, bind only the ORM2 A variant while warfarin selectively binds to ORM1 F1*S (Matsumoto *et al.,* 2002). It was Eap *et al.,* (1988c and d) who first reported that the variants could express different binding properties - these differences could explain why some studies find only a small number of high-affinity binding sites for basic drugs like methadone

(Abramson 1982). A study by Eap *et al.,* (1990) indicated the presence of the AGP genetic variants could be responsible for interindividual differences in methadone binding, favouring ORM2 A.

Increases in the concentration of AGP (and other proteins-IgA and C3) were found to occur in those with high stress perception compared to those with lower (Ebersole and Cappelli, 2000); other studies found this to occur in stress related to major depression. Also, Rostami-Hodjegan (1999) noted that heroin dependent individuals have elevated levels of AGP. These findings could be relevant to this study, titration patients are newcomers to the therapy and thus still heroin-dependent with high stress levels common especially while they are titrated onto a steady maintenance dose; as discussed further in the opiate section of this introduction.

1.4   Opiates

1.4.1 Opiate addiction: a global problem

The opiates are a family of drugs commonly abused in society, but also used medically. Members include the illicit street drug heroin and naturally occurring morphine, from which heroin is synthetically derived. A UNODC report suggested that in 2008 figures for opioid abuse ranged between 12.8 and 21.8 million individuals (World Drug Report, 2010) – commonly in conjunction with another family of CNS depressants known as the benzodiazepines (BDZs), thus it is a huge problem.

Between 0.4-0.8% of adults in Europe, USA and Australia reportedly develop opioid dependence, escalating their illegal drug habit (Hall and Mattick, 2007; Ward *et al.,* 1999). A study in 2005 estimated that over 51,500 individuals misused these substances in Scotland and that were 2,400 registered heroin users in the Lothian region alone (Hay *et al.,* 2005). Epidemiological studies performed in 2003 in the U.S.A have indicated that, after marijuana, opioid analgesics are among the most frequently abused illicit drugs in secondary school students (Johnston *et al.,* 2004). Studies of a similar age group in the UK reported that, in 2005, 6% had been offered heroin in England and Wales, and 1% of respondents had used it within the preceding year (NHS Drug Misuse and young people report, 2005). Long-term use of opioids is associated with abuse or addiction development; the term 'addict' often used to describe individuals whose repetitive drug taking behaviour stimulates the central nervous system (CNS) causing specific neurobiological changes which induce repetitive drug consumption (Scimeca *et al.,* 2000).

The death rate among heroin users is thought to be 13-fold higher than a healthy population of similar age and gender (Gossop *et al.,* 2002). Contributing factors include the spread of blood borne viruses associated with intravenous (*i.v.*) injection. Similar to the UK, Italy has a high incidence of injecting drug abuse which allowed AIDS to surpass overdose as the main cause of death among heroin users in late 1980s and early 1990s (peaking in 1991-1992). Its incidence in Italy has reduced, like much of Western and central Europe, since the early 1990s (Davoli *et al.,* 1997; Bargagli *et al.,* 2001). Perhaps this is partially due to the widespread introduction of opioid substitute

programs which reduce the need for i.v administration (instead a substitute which can be orally administered is used). However the incidence of HIV/AIDS and hepatitis C in injecting drug users has been on the rise in Eastern Europe. The occurrence of suicides in these drug users also differs among countries; in the UK it is thought to be approximately 10-15 per 10^5 of the population, similar to the USA and Australia. In Finland however it may be almost double - 30 per 10^5 (Diekstra and Gulbinat, 1993; Lynskey *et al.,* 2000).

Methadone maintenance therapy (MMT) is the most common opioid substitute treatment. The drug was first identified in the 1960s by Dole and Nyswander (1966) as a viable substitute in opioid dependence treatment despite decades of use as an alternative analgesic to morphine. The rate of achieving successful annual abstinence from heroin in the absence of therapeutic support is reportedly as low as 1-2% (Hall and Mattick, 2007). Those successful, commonly suffer relapses within 12 months if they do not receive any pharmacological or psychosocial assistance. Of those receiving methadone, abstinence from both the abused and therapeutic substitute drug may only be achieved in approximately 3% of patients annually although figures vary between studies (McKeganey, 2006). In order to improve treatment and identify viable alternatives, research must continue to enhance our understanding of why success rates may be so low.

1.4.2 The mechanism of opioid action

The general pharmacological effect of the opiates is achieved through a depression of the CNS; promoting the onset of analgesia. However, despite being a cornerstone in the treatment of moderate to severe pain, opioids can leave a patient more sensitive to pre-existing pain: this is the concept of opioid-induced hyperalgesia (OIH), for which there is accumulating evidence (Angst and Clark, 2006). Research has indicated that when compared to former heroin addicts who are not (or no longer) undergoing MMT or 'healthy' subjects, those maintained on methadone are more sensitive to cold pressor pain with some hyperalgesia. The increased pain sensitivity possibly predisposes them to recurring addiction as they attempt to prevent the onset of symptoms, thus a vicious cycle ensues (Doverty *et al.,* 2001; Compton *et al.,* 2000). It is thought that opioid tolerance and OIH coexist because they share the same neurotransmitter and receptor

systems, which may explain why it can be very difficult to distinguish between an individual displaying tolerance and another suffering from OIH (Angst and Clark 2006).

Under normal physiological conditions, the neurotransmitter γ-aminobutyric acid (GABA) is released from associated neurones and inhibits the release of dopamine (DA) from dopaminergic neurons when bound to its receptors on nerve terminals (figure 1.11a). Internally derived endogenous opioids (endorphins) may bind to μ, δ and κ opioid receptors (ORs) however, when heroin metabolites bind the μ-opioid receptors (μOR) of the nucleus accumbens (NA) and ventral tegmental area (VTA), the 'rewarding' neurobiological circuit (dependent on DA release from opioid-sensitive neurones) is enhanced, as shown in figure 1.11b. The active heroin metabolites bind to the ORs which in turn inhibit the synthesis and release of GABA, therefore DA is increasingly released into synapses and the post-synaptic membrane can be continually activated, inducing the euphoria and pleasurable feelings associated with heroin use. These 'positive' effects may explain the desire of users to repeatedly administer the drug despite the risks associated with them. Conditioned responses develop whereby individuals associate the use of drug and its 'positive' effects with particular stimuli, therefore finding it difficult to resist re-use when faced with said stimuli. In turn the users become dependent on the drug to perform activities related to daily life (Compton and Volkow, 2006).

As the dose of drug administered by an individual increases, the body creates stronger compensatory responses to its effects therefore the user requires increased doses to produce the responses previously achieved at lower levels; they become increasingly tolerant. If the concentration of bioactive drug present at receptors is insufficient to stimulate them effectively, withdrawal symptoms occur; an upregulation of the cAMP pathway and subsequent increased activity of its Noradrenaline (NAdr) secreting cells occurs in the locus ceruleus (LC) (Housová *et al.,* 2005; Kreek, 1997).
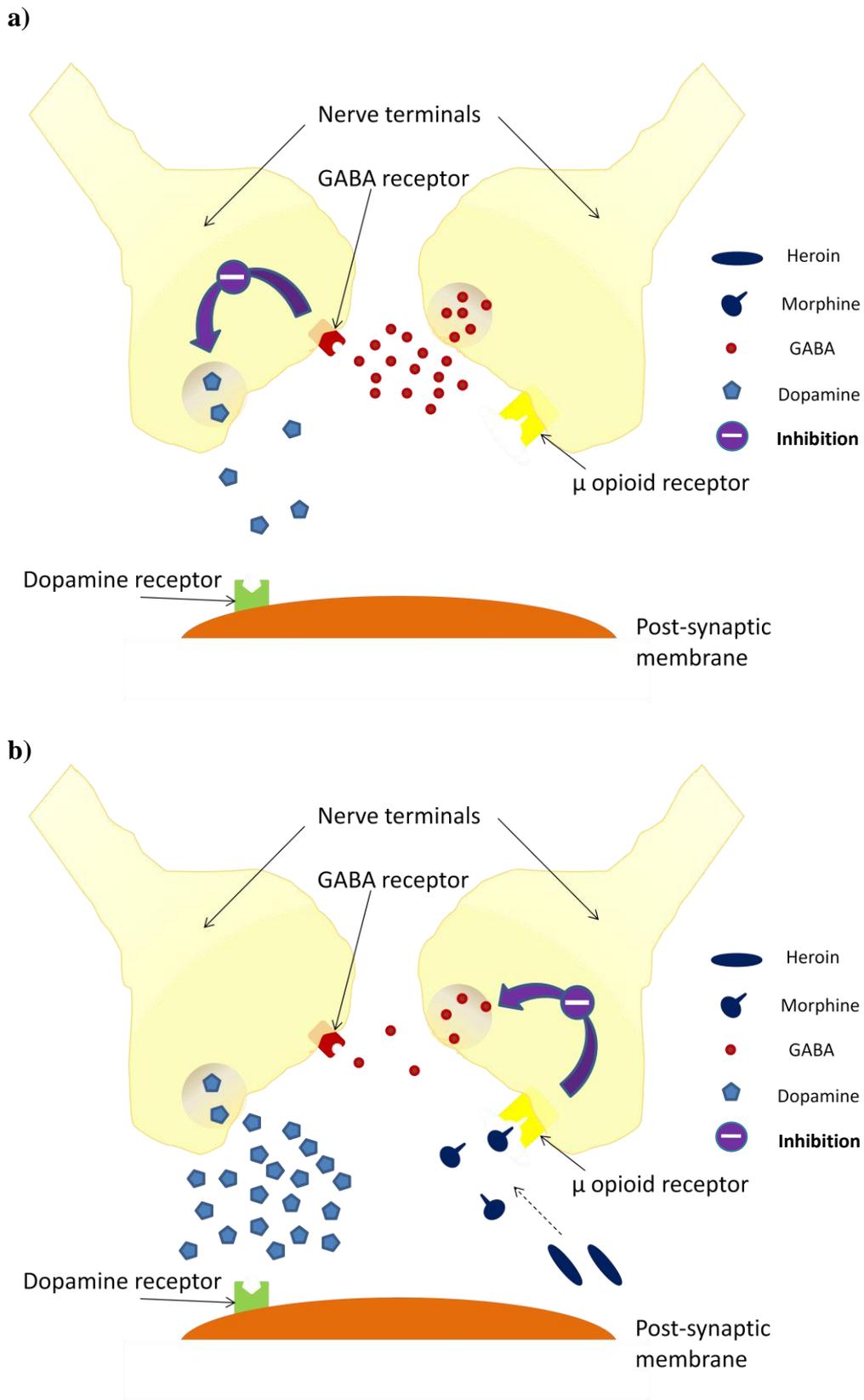
Figure 1.11. **Diagrammatic representation of synaptic activity in the absence (a) and presence (b) of heroin.** [Adapted from Schumacher *et al.,* 2009]. Introduction of μ opioid agonists inhibits the normal release of GABA, therefore allowing greater levels of dopamine to be released.

1.5   Heroin

1.5.1   Heroin addiction

Heroin is a lipophilic morphine derivative with two acetyl groups which allow it to pass through the BBB more easily than morphine (figure 1.12a and b respectively). The complete drug remains in this form for a matter of minutes before it is rapidly hydrolysed to its more stable metabolites morphine and 6-monoacetylmorphine; approximately 95% of a heroin dose is eliminated within 30minutes. The two main metabolites allow for the prolonged pharmacodynamic (PD) action of approximately 4-6hours (Salmon *et al.,* 1999; Rook *et al.,* 2005).

Essentially, increasing the rate of drug delivery to the brain is the primary 'goal' of the drug user. Therefore, because heroin has a poor oral bioavailability (the hypothetical proportion of administered dose which reaches the circulation is low), users often progress from smoking to snorting. A study undertaken by van den Brink (1999) noted many heroin users inhaled heroin vapours, produced when heating on tinfoil – commonly referred to as 'chasing the dragon'. Subsequent transfer to injecting the drug i.v. is widespread in heroin users as this provides the most direct rate of delivery to the brain ORs. Hay *et al.* (2004) reported that in 2003, over 18,500 of the ~51,500 opiate and/or BDZ misusers were injecting the drugs in Scotland. However, this route thereby increases the risk of transmitting blood-borne viruses like HIV (60% opioid-dependent persons are HIV positive according to Nadelmann and McNeely, 1996), and hepatitis C (50-70% injecting drug users infected; Krambeer *et al.,* 2001) through the sharing of needles. Holmberg (1996) reported that up to 50% of newly diagnosed cases of HIV infection are linked to injecting drug use.

It has been reported that heroin users tend to use a combination of drugs alongside heroin including BDZs (a common family of psychotropic drugs), cannabis, alcohol and amphetamines although they are not necessarily dependent on all of them (Klee, 1990). The use of alcohol and BDZs, alongside heroin is correlated with fatal and non-fatal heroin overdoses because they too are respiratory depressants, (Zador *et al.,* 1996; Darke *et al.,* 1996).

a)
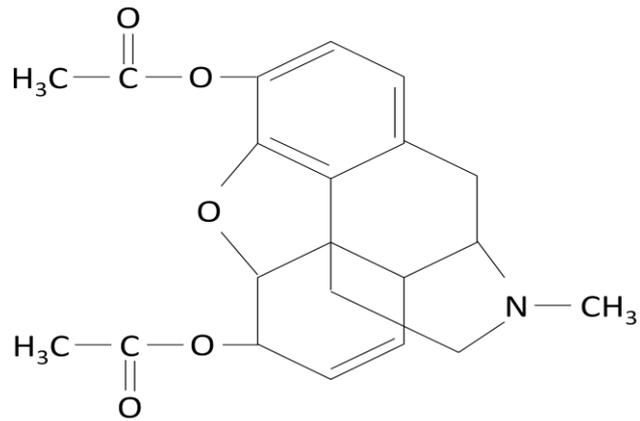
b)
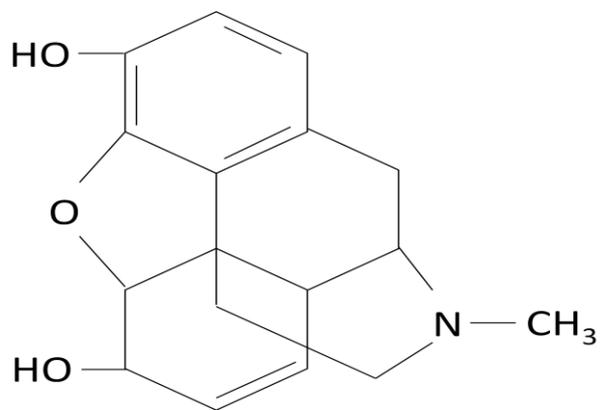
c)
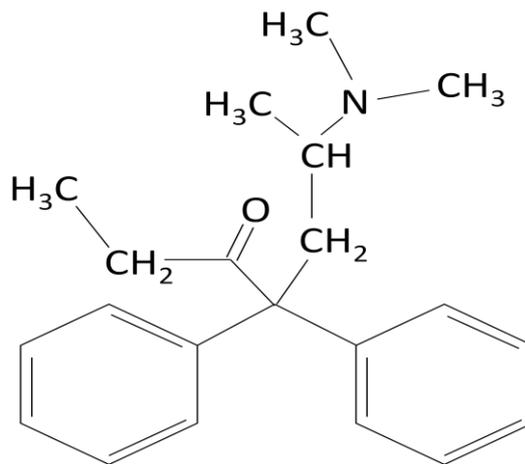
Figure 1.12.   The chemical structure of a) heroin (diacetylmorphine), b) morphine and c) methadone.   [Adapted from Rodriguez-Rosas *et al.* (2003) and Galloway and Bellet (1999)].

1.5.2   Dependence and Tolerance

All opiates have the potential to cause dependence; a complex condition involving social, psychological and biological components. Within-system adaptations that can occur in the acute reinforcing activity of drugs may involve neurochemical changes associated with certain neurotransmitters. The desire to re-experience positive 'reward' outcomes and prevent withdrawal symptoms which arise when dopaminergic and serotinergic transmission in the nucleus accumbens (NA) is reduced, affects the development of dependence. To function normally, these individuals require opioid administration resulting in compulsive drug taking with which there are significant costs to society in terms of crime, social damage, loss of earnings and productivity (Weiss *et al.,* 1992; Parsons *et al.,* 1995). Ward (1999) implied that dependence can be diagnosed if an individual persists in their use of a drug in spite of damages to health, law, social achievements and personal relationships. Various schemes are available to aid in the identification of individuals displaying physical dependence, most commonly based on recommendation of the WHO in the UK or the DSM-IV (Diagnostic and Statistical Manual of Mental Disorders) as used in the USA.

Upon initial heroin injection the drug rapidly occupies ORs to produce its effects, yet repeated administration causes a reduction in the drug's potency or efficacy due to depleted expression of these receptors (physiological tolerance). Alternatively, as suggested by Borgland and colleagues (2003), the ORs may become internalised - consequently, receptor expression is reduced. The effect is often not apparent during analysis because radiotracers used in these studies are not specific to surface ORs thus they can label internalised structures, causing the overall expression of ORs to appear relatively consistent.

Increased doses are required to reproduce the effects previously acquired with lower concentrations; a threshold is associated with tolerance to drugs, which deviates between individuals. When doses are below this level withdrawal symptoms may arise, including nausea, anxiety, tremors and dissipation of the euphoric 'high' (Drummer and Odell, 2001; Scimeca *et al.,* 2000). Conversely, if concentrations exceed the threshold too rapidly or by too much, the effects of an overdose are displayed. When at the threshold, cognitive and motor functions are normal, making it difficult to determine if those persons are abusing opioids (Dole *et al.,* 1966). Therapy for opioid dependence is

therefore based on achieving a stable and continuous brain opioid receptor occupation to prevent heroin binding, consequently avoiding the unwanted effects like craving; allowing patients to gradually return to a normal lifestyle (Scimeca *et al.,* 2000).

1.6 Methadone

1.6.1 Methadone: a brief history

Methadone - (±)-6-dimethylamino-4,4-diphenylheptan-3-one – figure 1.12c) was first produced in Germany in the late 1930s as an alternative analgesic to morphine (figure 1.12b); despite possessing a very different chemical structure, it binds the same receptors (Bernard *et al.,* 2007). Three decades later, in the 1960s, after the diligent research of Dole and Nyswander (1966), it was identified as the only viable synthetic opioid which could be substituted for abused heroin due to its low side effect expression and long half-life – criteria for treatment of opioid dependencies. At the time the primary use of methadone was in treating chronic cancer pain, but this research pioneered it as a maintenance treatment. Unlike heroin, its rapid absorption by the oral route and slow breakdown is favourable for use in therapy. The drug is now employed worldwide. In Scotland, its use as a controlled and allegedly safe heroin substitute was not realised until the 1980s (SACDM, 2007) and its use has become increasingly controversial. Some countries, including Germany, have limited methadone use opting instead for shorter acting (half-life of 4h) dihydrocodeine (Krausz, 1998).

It is a basic and lipophilic (fat soluble) drug that is mainly ionised at physiological pH (it has an ionisation constant, or pKa, of 9.0) and is primarily stored in the liver but quickly and widely distributed throughout tissues (Garrido, 1999). Like heroin metabolites, methadone binds μ, δ and κ ORs which induce the release of intracellular messengers necessary to create the associated pharmacological effects; many of these are shared by the opioid agonists e.g. analgesia. The binding of methadone to ORs prevents the attachment of heroin – the absence of its associated euphoric effects upon administration should therefore lead to a cessation of heroin use. Due to its longer half-life, the presence of methadone in the body outlives that of the abused drug. However some studies have disputed this mechanism of action, arguing that there is no significant

47

alteration in the availability of receptors when methadone is administered (Melichar *et al.,* 2005). They suggested that as methadone is a high-efficacy agonist, the number of receptors required to exert its effect may be so low that it was undetectable using the techniques available.

1.6.2 Methadone therapy: its usefulness and dosing schedule

The primary goal of the administration of the synthetic opioid methadone is to prevent abstinence syndromes. Blake and colleagues (1997) reported that it has a higher affinity constant (Ki 3.56nM±0.2) for µORs – the primary site of action - than that of other opioids like morphine (Ki 1.41nM±0.11). Methadone normalises the hormonal disruptions often existing in heroin addicts, preventing the onset of euphoric effects and reducing narcotic cravings associated with heroin use (Vetulani, 2001). It does not allow rapid abstinence from all drugs and therefore does not represent a cure (Housová, 2005). Nonetheless, the ability to orally administer the drug in a single daily dose (syrup and tablet forms) is advantageous as is its long duration of action (24-36 hours – compared to 2-3 hours for heroin) which prevents the onset of withdrawal symptoms for long periods of time (Fainsinger *et al.,* 1993). Methadone is useful because it has cross-tolerance with other opioids, enabling it to be substituted for abused drugs such as heroin and morphine. By binding the same receptors, the inhibition of DA release by GABA is prevented, similar to the effects of morphine described in section 1.5.2. DA is increasingly released from the ventral tegmental area and binding of the drug at ORs in the nucleus accumbens and prefrontal cortex is associated with the acute reinforcing effects common to heroin use. Their structural differences (refer to figure 1.12, section 1.6.1) may explain the inability of methadone to induce euphoric effects like heroin, despite being an agonist for the same receptor – they may become bound differently. An additional advantageous property of the drug is that it acts as an antagonist at NMDA receptors therefore reducing characteristics associated with the major excitatory neurotransmitter glutamate, for example anxiety.

Dole *et al.,* (1966) reported that a 100mg dose of methadone can block the effects of 80mg *i.v.* administered heroin. It is not surprising more methadone is required than heroin as the latter is injected directly into the blood and the V_d of methadone is much higher than that of morphine with only 1% reported to be present in the blood (Morley

and Makin, 1998). Therefore, in order to account for its loss, methadone must be administered in much greater doses to achieve the therapeutic plasma levels required. According to Eap *et al.* (2000), a 150-600ng/mL serum methadone level (SML) is necessitated to suppress opioid craving and a trough level ≥400ng/mL required for maintenance (to ensure sufficient blockade). Reinforcing effects of shorter acting heroin like euphoria are avoided although there is an increased possibility of methadone accumulation upon repeated dosing due to its long half-life (Dole et al., 1966; Kreek, 1992). Four or five half-lives usually pass before the elimination and amount of drug in the body are balanced (attain a steady-state SML). Considerable inter-individual variability in the PKs and PDs of methadone exist, as is the case for many drugs. Hanna and colleagues (2005) indicated that interindividual variability in methadone PK parameters was up to 8-fold greater than that exhibited intraindividually. Due to this variability in factors including metabolism, distribution, enantiomer selectivity, passage through BBB and binding to plasma proteins; the level of drug may differ and therefore adjustments must be made to the dose of orally administered methadone if the therapeutic concentration is to be attained. Defining a threshold plasma trough level capable of achieving the desired response is therefore difficult (Loimer and Schmid, 1992).

The response to a drug is directly correlated with the length of time the active form is in the body. Methadone is 'foreign' to the body (a xenobiotic) and is therefore metabolised in the liver by chemical reactions into more easily eliminated molecules. The primary metabolic pathway is sequential N-demethylation and cyclisation to inactive EDDP (2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine) chiefly involving liver cytochrome P450 (CYP450) enzymes, especially CYP3A4. The activity of these inducible enzymes affects the rate of methadone clearance from the body and varies between individuals due to genetic, environmental and/or disease-related factors. It follows that if the specific enzymes are absent or incorrectly expressed, methadone cannot be metabolised effectively. Drugs, or other agents, that induce or inhibit the activity of CYP450 enzymes – especially that of CYP3A4 – can thereby alter the PKs of methadone. Inducers including the anticonvulsant phenobarbital and the antibiotic rifampicin accelerate methadone metabolism, increase its rate of clearance, lower the SML, and potentially precipitate a withdrawal syndrome because insufficient drug reaches and blocks the ORs. Conversely, inhibitors e.g. SSRIs (sertraline etc), SNRIs (venlafaxine etc) and HIV drugs (ritinovir, zidovudine) slow metabolism, raising the

SML, leading to accumulation which may cause methadone-related toxicity with oversedation and/or respiratory depression (Leavitt *et al.,* 2000; Wolff *et al.,* 2000).

The interaction between alcohol consumption and methadone efficacy is one of great concern. It has been reported that the drugs act in a reciprocal manner thereby enhancing their metabolism. Patients in a study undertaken by Wolff *et al.* (1991) indicated that those with the lowest methadone concentrations also had long histories of alcohol abuse.

The long half-life, although variable between individuals, requires patients to follow a dosing schedule where the methadone concentration is titrated over a period of 10-14 days upon administration of the initial dose. During this stage, patients must be monitored closely as it is associated with the greatest risk of overdose and subsequent death. Doses ought to be kept low because methadone slowly accumulates in the tissues potentially causing toxicity during concurrent use of additional illicit opioids. Many patients continue unsanctioned use of heroin and other CNS depressants to combat withdrawal symptoms like abdominal cramps and diarrhoea, arising due to the low methadone doses. Wolff and colleagues (1991) reported that plasma methadone concentration is directly related to the dose of methadone administered. Once the levels between tissue storage sites and the blood have equilibrated, a maintenance dose is administered over the remainder of therapy to prevent relapses; methadone is slowly released from tissues therefore the SML remains fairly constant (Krambeer *et al.,* 2001; Borg and Kreek, 2003). Small reductions are made when the individual decides to attempt a future of abstinence. Again, monitoring patients is vital, any dramatic drop in methadone concentration may lead to a life-threatening relapse - their tolerance to the illicit drug is commonly reduced therefore toxicity could arise at lower doses than those administered prior to therapy. Studies have indicated that the risk of death when using opioids is 3-4 fold lower when undergoing MMT however it is considerably higher than in the drug free population (Caplehorn *et al.,* 1996).

Guidelines issued in 1991 by the UK Department of Health indicated methadone prescribing was to be increasingly offered as a long-term treatment than previously reported when short-term treatment with subsequent detoxification was encouraged. Support was provided by the recently updated 'Orange' Guidelines issued in 2007 (Department of Health, 1999 update 2007b).

It is unusual for the initial loading dose administered to patients to be greater than 60mg per day; between 35-100% (80% average) of a single, orally administered, methadone dose will pass into the bloodstream and can be detected there after 15-45 minutes. Therefore, if a patient vomits shortly after ingesting the dose there is uncertainty as to the extent of absorption; it takes 20-30 minutes for the drug to be fully absorbed. Similarly, if within the first few days there is strong evidence of withdrawal, doses may be increased if there is a low risk of abusing other substances. Little is known about the relationship between dose, brain levels and receptor occupation despite its widespread use for three decades.

McCarthy and colleagues (2000) demonstrated that when a group of mothers undergoing MMT consumed between 25 and 180mg of methadone per day, the level consumed by the infant was approximately 0.05mg/day if ingesting 475mL of breast milk a day. When a patients' dose of methadone is greater than 20mg/day, the American Academy of Paediatrics recommend that nursing should not be undertaken. Continuing on MMT is however associated with a number of positive qualities − it is thought to better prepare a mother for parenting and has been shown to improve obstetrical care compliance (Finnegan, 1991).

Recently, a report compiled by McKeganey (2006), concluded that giving methadone to heroin addicts has a 3% success rate (97% failed to remain free of all drugs, including that used in treatment), which was barely better than those attempting abstinence without any treatment. Subsequent to findings like these, there is a greater demand for alternatives, such as the opioid dihydrocodeine (DHC − refer to chapter 4), and increased availability of residential rehabilitation; only 2% of Scotland's drug users have adequate access to residential rehabilitation systems (Hay *et al.,* 2005). Their research did however highlight the social benefits of remaining in therapy; individuals were 7 times less likely to commit crime and more likely (59%) to be in employment or education compared to addicts. Despite the study relying on the honesty of the 695 drug users interviewed, it supports previous findings that, regardless of high relapse rates, methadone is effective in improving the patients' quality of life (Marsch, 1998).

1.6.3 Methadone has two forms administered during therapy.

In the UK methadone is commonly prescribed as a 50:50 racemic mixture of two enantiomers (molecules which share a common chemical composition but differ in their special arrangements); (R)-(-)-methadone and (S)-(+)-methadone, or levo- (l) and dextro- (d) respectively, which differ in their activity. The (R)-enantiomer has reportedly ten times higher affinity for µORs than morphine (Kristensen *et al.,* 1995) and is 50 times more potent as an analgesic than the (S)-enantiomer thus it accounts for most, if not all, the opioid pharmacological activity (Boulton and Devane, 2000; Eap *et al.,* 2002). There are a number of effects known to arise from the use of the racemic mixture including miosis (pinpoint pupils), antidiuresis, respiratory depression and analgesia.

Hallinan *et al.* (2006) performed research with MMT patients classified as 'responders' and 'non-responders', based on initial urine toxicological analysis. They reported that the optimal therapeutic daily dose in the patients was 100mg/day requiring (R)-methadone concentrations of 200ng/mL and (R,S)-methadone of 400ng/mL, thus using the R-enantiomer alone is more effective at suppressing withdrawal than the racemate. The use of S-(+)-methadone alone was shown to be ineffective at preventing withdrawal symptoms. Although certain patients require only a low dose of methadone in therapy, controlled studies have indicated that a dose-response relationship exists for methadone up to 80-100mg/day (Capelhorn *et al.,* 1993; Strain *et al.,* 1999). Thus, the higher the dose, the better the response i.e. less likely the patient is to enter withdrawal and relapse.

1.6.4 Minimal effective concentration and the role of AGP

The ability of a drug to confer a desired therapeutic effect is primarily dependent upon attainment of a certain concentration at its site of action - the minimal effective concentration, MEC. For the majority of drugs, this is accessed via the bloodstream and according to the free drug hypothesis, only free drug is capable of executing the associated effect. There are a number of mechanisms postulated to cause methadone resistance, however often overlooked is the interaction formed with plasma proteins,

such as α-1-acid glycoprotein (AGP), potentially rendering it inactive and reducing the concentration of drug to below the MEC at the target site.

Like many drugs, methadone binds to proteins in the plasma, lung, liver, kidney and spleen but as it is a basic drug, it is most strongly associated with AGP (Romach *et al.* 1981). Methadone binds the APP with an association constant of $\sim 4\times 10^5 M^{-1}$. Therefore, alterations in the concentration of the APP, like the increase reported by Rostami-Hodjegan *et al.* (1999) in heroin-dependent individuals with signs of opiate withdrawal, are likely to affect the level of unbound active drug and subsequently its ability to enforce the desired pharmacological response. In fact research by Olsen (1973) suggested that the affinity of methadone for AGP is stereoselective; greater affinity was recorded for the S-enantiomer (90% bound) than the R-form (86%), this was subsequently supported by Romach *et al.* (1981). Thus, such high binding and enantiomeric preference may explain, to some extent, why large doses (mg level) are required to provide a low (ng) therapeutic plasma level. Eap and colleagues (1988) suggested that in order to determine what concentrations of methadone are therapeutically useful; analysis of the enantiomers could be more valuable than total methadone.

It is not only enantiomeric selectivity that must be considered; methadone possesses a degree of selectivity for specific genetic variants of AGP. Therefore if the expression of the AGP-B/ B′ gene products (ORM2 A) - the genetic variant for which methadone has the greatest affinity - is increased, the active concentration of methadone decreases. Additionally, the free level of other drugs which do not bind this glycoprotein variant will increase, generating an elevated risk of toxicity from their presence alongside the onset of withdrawal from the reduction of free methadone (Marsch, 1998). Once the AGP level stabilises, the drug concentration will follow suit.

If binding is enhanced and the plasma levels are insufficient to reach the MEC, an increase in GABA release and activation of NMDA receptors causes enhanced craving and withdrawal symptoms including anxiety. An increase in the dose administered may compensate for binding but increases the risk of toxicity.

A number of drugs have been shown to displace methadone from its binding sites on AGP including propranolol and chlorpromazine. An elevation in the free plasma

concentration of methadone would arise if the drugs were taken simultaneously thus increasing the possibility of achieving toxic concentrations (Abramson, 1982). Although studies have acknowledged the ability of AGP to bind methadone, there is a paucity of research analysing the effect of glycosylation on this function.

1.6.5   Methadone therapy is not without its problems

As alluded to previously when discussing the extent of methadone use in the UK, it is not a cure for opioid dependence and a number of contraindications have been identified. The main concern regarding the use of methadone in the treatment of pain is the risk of delayed toxicity due to its long and unpredictable half-life (Manfredi *et al.,* 2001). Essentially, the adverse effects of methadone compare to those for other opioid analgesics for example nausea, vomiting, miosis, constipation, respiratory depression and possibly comas. Upon long-term use – after accumulation and stabilisation - individuals usually develop tolerance to most of these effects (Scimeca *et al.,* 2000). However they are likely to develop dependence on the opioid, becoming hesitant to reduce their dose and risk the onset of withdrawal symptoms which are reportedly worse than those for heroin. This was clear in a study by Manfredi and colleagues (2001) describing patients who had been on the therapy for as long as 10 and 20 years. Greatest risk of overdose to users therefore occurs in the first 10 days during titration as the doses are kept low - additional unsanctioned use of opioids and other CNS depressants often continues, potentially leading to toxicity. Deaths have been found to occur mainly during sleep after peak blood concentration is attained, usually three to four days after starting therapy where the risk of death is reportedly 6.7 fold greater than when not in the program (Caplehorn and Drummer, 1999). During this stage, dosing must allow for the accumulation of methadone and the additional use of CNS depressants by patients in their attempt to prevent the onset of withdrawal. In almost all deaths related to methadone, other CNS depressants are also present; the exacerbation of respiratory depression is extremely hazardous. Risk decreases after stabilisation on the therapy; it is nearly 98 times lower than in newly recruited patients (Karch and Stephens, 2000).

In the decade 1985 - 1995, it was reported that almost 50% of all UK opioid deaths were related to methadone use however whether the patients were stabilised on the therapy at

the time is unknown (Hall and Mattick, 2007). Newcombe (1996) suggested that heroin actually contributes to fewer deaths than methadone and therefore the substitute may not be as protective as first assumed.

Therefore, it can be seen that in the ongoing search for improved treatments for opioid-dependencies a firm understanding of the mechanisms influencing the efficacy of drugs is being sought. A particular aspect – specifically the structure of AGP and its potential affect on binding to, and therefore inactivation of, methadone - is the primary focus of the research detailed herein.

1.7   Research Strategy

As a result of the APR, stimulated when certain pathological and physiological states are expressed, the plasma concentration of a positive acute phase protein called AGP can be elevated 2-5 fold. Increased synthesis of AGP has been reported in highly stressed individuals and heroin users showing signs of withdrawal, ergo levels found in patients undergoing replacement therapy for heroin dependencies ought to differ from that of a 'normal' population.

Alongside overall plasma concentration, the glycosylation pattern of AGP has been shown to differ in various conditions but has not been closely studied in patients undergoing methadone therapy. Any alterations to the structure of a glycoprotein can greatly affect its function. In the case of methadone, glycosylation changes expressed by AGP could increase the binding affinity thereby reducing the level reaching the target site and subsequently increasing the risk of withdrawal symptoms developing. If binding significantly differed in treatment groups, the analyses of a patient's AGP and its binding to methadone may have potential in a clinical setting.   Identifying individuals who are less likely to respond to methadone replacement therapy and therefore will require higher doses or perhaps an alternative substitute could, and should, improve treatment retention and success.

Consequently, it was hypothesised that the level of AGP isolated from patients undergoing therapy would be greater than that from a healthy population. Also, patients were expected to express modified glycosylation for which methadone displays increased affinity. Although patients receiving higher doses of drug would be expected to express the greatest levels of AGP and glycans correlated with increased binding if any were found, these generalisations do not account for the likelihood that the methadone dose administered to patients newly recruited onto the program will be lower than that received by patients at the maintenance stage. Dosing during titration must reflect the ability of methadone to accumulate *in vivo* and the probability that the patients themselves will administer additional opiates. Therefore, in terms of relating methadone dose to AGP concentration it was expected that patients in long term therapy receiving the greatest doses, would have an increased level of AGP and/or increased affinity for the drug – more being inactivated by their AGP. Those in the titration phase

were expected to express high levels of AGP however the doses were unlikely to be correlated.

1.7.1 Aims and Objectives

1.7.1.1 Aim:

The primary aim of this research was to determine whether alterations in the level and glycosylation of AGP occurred in patients undergoing opioid replacement therapy when compared to a 'normal' healthy population. Whether such alterations could contribute to potential differences in the degree of binding was established.

Preliminary studies such as that presented within could offer use in determining the likely success of the treatments for individuals; alternative treatment programmes could be initiated immediately in those unlikely to respond well to methadone. It would prevent starting a program which is unlikely to offer any benefit to the individuals.

1.7.1.2 Objectives:

Various stages were undertaken to test the hypotheses that the level and structure of AGP in patients influenced its binding to methadone. Initial experiments were performed with commercial AGP to ensure techniques were sound and offered controls. Ethical permission was sought from both the NHS and Edinburgh Napier University.

Isolation and Purification of AGP from 'normal' (healthy individuals) blood - pooled by the blood transfusion service - adopted a method which prevented desialylation and denaturation i.e. structural integrity was not compromised and remained in the form found *in vivo*, which was important to the study. The techniques were subsequently used in the isolation and purification of AGP from the blood of consenting patients, and from the serum of an additional 'normal' population.

To determine if the glycosylation of AGP differed between subject groups, the monosaccharides and oligosaccharides were analysed. Quantitative data was generated through monosaccharide compositional analysis by high pH-anion exchange

chromatography (HPAEC). Subsequent oligosaccharide indicated if there were any changes in the branching of the glycan chains. The type of terminal sialic acid linkage was determined by ELISA.

Methadone drug binding studies with commercial AGP, normal pooled blood and patient blood were performed to determine if AGP isolated from certain patients inactivated more drug. This was then compared to data obtained during structural analysis to determine if there existed any correlations between AGP glycosylation and drug binding. Another opioid – codeine - was used in similar studies with commercial AGP to investigate whether binding occurred. Such studies provide an indication that other drugs may offer a superior choice in terms of reduced or no plasma protein binding, despite their use currently being more reserved than methadone. The study only addressed binding to AGP and there were likely to be many other factors which could render these alternatives less effective than methadone.

Overall, the project aspired to identify potential relationships between AGP glycosylation and methadone binding in patient groups so that further studies may be carried out with increased study populations.
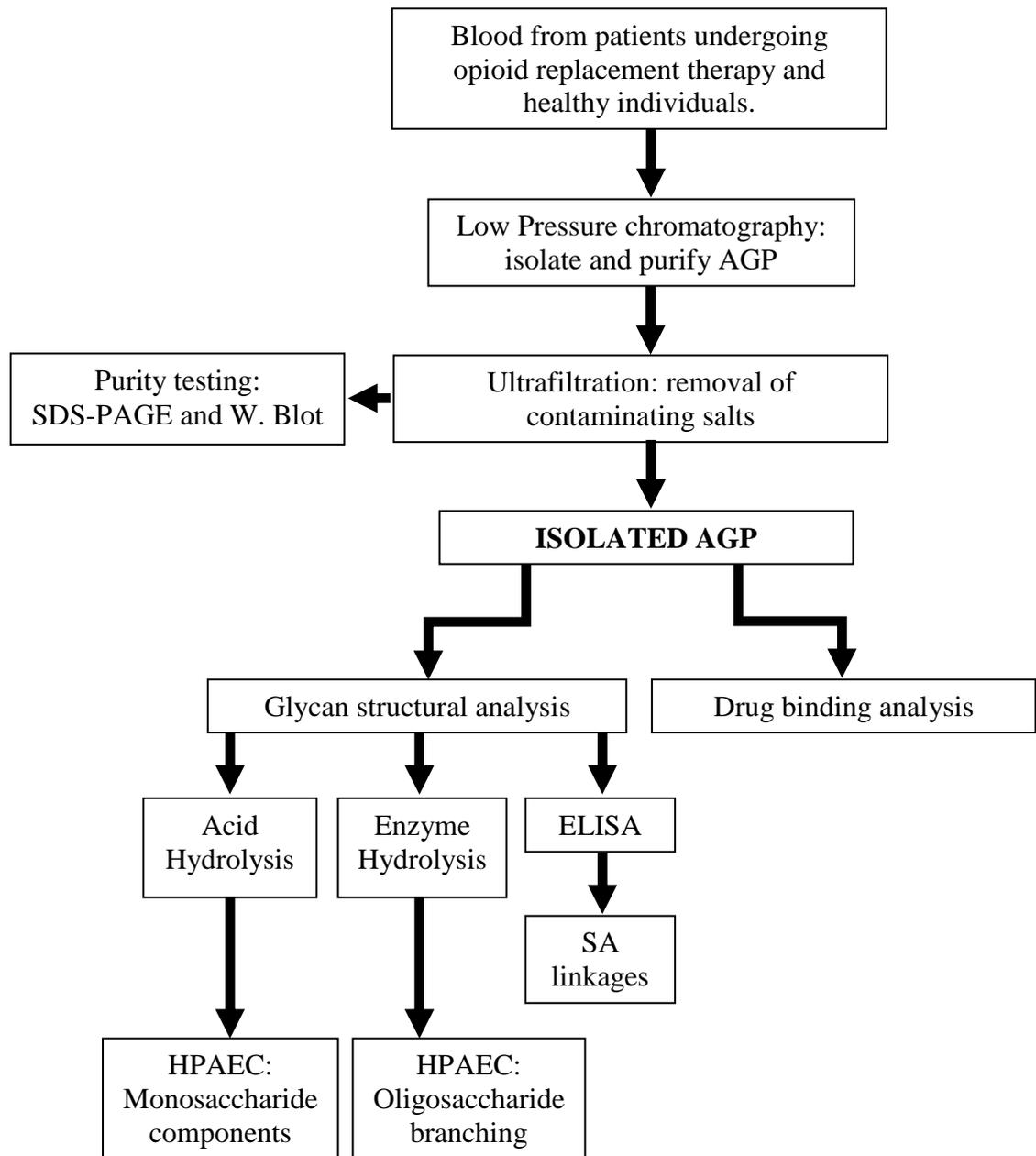
Figure 1.13.   Research Summary.

Chapter 2
Materials and Methods

2.1 Materials

2.1.1 *Patient clinical samples*

The Lothian NHS and Edinburgh Napier University Ethics committees approved the initial study where ~5mL blood samples were obtained from patients (n=12) undergoing various stages of treatment for opioid addictions at the Community Drug Problem Service (CDPS) in Edinburgh. Treatment groups included those initially recruited in the Titration phase (T), those attending the Harm Reduction scheme (HR), Long-term methadone (LT), buprenorphine (B), dihydrocodeine (D) and heroin (H) patients. An application to increase the study size and specificity by recruiting additional patients from the T and LT phases was approved. A total of twenty-six patients were recruited (table 2.1), mainly from LT maintenance therapy who were no longer required to attend the clinic daily (n=11). Samples were collected in heparinised vacutainers (Becton Dickinson, France) by a trained phlebotomist and PEG precipitated as described in the Methods section 2.2.2.1, storing the supernatants at -20ºC until required. The primary investigator discussed the study with the patients and only in the LT group was it feasible to involve their mentor in the recruitment process (Dr. Malcolm Bruce – collaborator). In future studies of a similar nature it would be useful to pursue this additional interaction, potentially improving participant recruitment due to increased trust between the care user and mentor. It would also standardise the recruitment process however careful consideration is required; it was not feasible to request the supervision of each individual's mentor during this preliminary study due to constraints on their time and perception that the investigators were most qualified to discuss the study.

Professor David George of the Glasgow Western Infirmary donated 'normal' serum samples; however upon analysis they were not deemed to offer a sound comparator as little information was known about the source population other than they were obtained from elderly cancer-free individuals. Therefore it could not be known whether other factors could have initiated an acute phase response to alter AGP levels and glycosylation. Instead, heparinised blood samples pooled from 'normal' healthy individuals supplied by the Scottish National Blood Transfusion Service were implemented throughout.

Patient number	M/F	Age	Therapy group
JB 0608 01	F	32	T
JB 0608 02	F	23	T
JB 0608 03	F	37	HR
JB 0608 04	M	28	HR
JB 0608 05	F	23	HR
JB 0608 06	F	23	HR
JB 0608 07	M	35	LT
JB 0608 08	M	33	LT
JB 0608 09	M	38	B
JB 0608 10	F	36	LT
JB 0608 11	M	29	B
JB 0608 12	F	44	LT
JB 0509 01	F	37	T
JB 0509 02	M	30	B
JB 0509 03	M	30	LT
JB 0509 04	M	51	LT
JB 0509 05	M	33	T
JB 0509 06	M	20	T
JB 0509 07	M	39	D
JB 0509 08	M	30	B
JB 0509 09	M	34	LT
JB 0509 10	M	42	LT
JB 0509 11	M	34	LT
JB 0509 12	M	31	LT
JB 0509 13	M	*	H
JB 0509 14	M	30	LT

Table 2.1   Patient Demographics.

T - Titration phase with Methadone; HR - Harm reduction program Methadone

LT – Long-term Methadone; B – Buprenorphine; D – Dihydrocodeine; H – Pure

Heroin

* Data regarding specific age was unavailable

2.1.2   AGP isolation

Glacial acetic acid, polyethylene glycol (PEG) 3350, potassium chloride, potassium thiocyanate, Reactive Blue 2 Sepharose (Cibacron Blue 3GA), Red Sepharose CL-6B, sodium acetate, sodium azide, sodium chloride and Trizma base were supplied by Sigma-Aldrich® (Poole, UK). Ethanol was purchased from Bamford Laboratories Ltd., (Norden Rochdale, UK); diluted to give a 10% (v/v) solution. HPLC-grade water was obtained from Rathburn Chemicals Ltd (Walkerburn, UK). The poly prep disposable 10mL columns were purchased from Bio-Rad, (Hemel Hempstead, UK). To load buffers and sample supernatants, a Pharmacia LKB Pump P-1 was used and the absorbance of the mobile phase was measured by a single path optical UV-1 monitor and control unit (all from Pharmacia, UK). The changing absorbance at 280nm was recorded by a Servogor 120 chart recorder, also from Pharmacia. Blood samples were initially spun in an Eppendorf Centrifuge 5415C and all eluted samples concentrated using a Christ RVC 2-18 Concentrator (SciQuip Ltd, Shrewsbury, UK).

2.1.3   De-salting

HPLC grade water was purchased from Rathburn Chemicals Ltd (Walkerburn, Scotland, UK) and Centricon YM-10 Centrifugal filter devices with a dialysis membrane MW cut off 12,000-14,000Da were obtained from Millipore (UK) Ltd. (Hertfordshire, UK). The company then replaced these with Amicon Ultra-4 centrifugal filter devices with a MW cut off of 10,000. An MSE Centaur 2 (large scale) was used to spin the samples. The Christ RVC 2-18 Concentrator was purchased from SciQuip Ltd (Shrewsbury, UK).

2.1.4   Determination of AGP level in blood

Calibration curves were produced using Commercial AGP obtained from Sigma-Aldrich® (Poole, UK) and HPLC grade water from Rathburn Chemicals Ltd. (Walkerburn, UK). Absorbance was read on a BioMate 3 spectrophotometer purchased from Thermo Fisher Scientific® (Loughborough, UK).

2.1.5 SDS-gel and Western Blot

The production and staining of the gel required glacial acetic acid, glycine, methanol, TEMED, Tris-base, and SDS purchased from Sigma-Aldrich® (Poole, UK), along with commercial AGP controls. A gel casting system VS10CBS was obtained from Geneflow (Staffordshire, UK). Bromophenol blue, Dithiothreitol, sodium chloride and Na$_2$ EDTA were purchased from New England Biolabs® Inc (Hertfordshire., UK). For the Western Blot the nitorocellulose membrane and filter paper were obtained from Whatman International Ltd., (UK). Bio-Rad Laboratories (Hemel Hempstead, UK) supplied the non-fat dry milk and Promega (Hampshire, UK) the NBT and BCIP (with dimethylformamide). The primary antibody (anti-AGP antiserum, rabbit) and secondary antibody (goat anti-rabbit IgG, AP-linked) was purchased from New England Biolabs® Inc. (Hertfordshire., UK). Tween®20 was purchased from Sigma-Aldrich® (Poole, UK). The omniPAGE gel casting system (VS10CBS) was purchased from Geneflow (UK).

2.1.6 High pH anion Exchange Chromatography

2.1.6.1 Monosaccharide Analysis

Monosaccharide standards: 2-deoxy-D-galactose, fucose, galactose, glucosamine, mannose; along with the commercial AGP, 2mL hydrolysis reaction vials, aluminium lined screw caps, Dowex®-50WX8 cation exchange resin (hydrogen form, 12% cross linked, dry mesh 50-100) and constant boiling HCl were supplied by Sigma-Aldrich® (Poole, UK). HPLC grade trifluoroacetic acid (TFA) was supplied by Thermo Fisher Scientific® (Loughborough, UK). Rathburn Chemicals Ltd. (Walkerburn, UK) supplied the HPLC-grade water and the 50% w/v sodium hydroxide was supplied by VWR International® Ltd, (Lutterworth, UK). The solutions were sparged and pressurised with helium using a Dionex Degas module. To perform HPAEC (High performance anion exchange chromatography) a CarboPac™ PA-100 analytical (4x250mm) column and guard column (4x50mm), were used on a Dionex 600™ system with pulsed electrochemical detection (PAD) (Dionex, Camberley, UK). PeakNet™ software run on a Dell OptiPlex GX110 computer controlled the GP50 gradient pump and ED40 electrochemical detector (Dionex, Camberley, UK).

2.1.6.2 Oligosaccharide Analysis

New England Biolabs[®] Inc., (Hertfordshire, UK) provided the Peptide-N-Glycosidase F (PNGase F) purified from *Flavobacterium meningosepticum*, 10% NP-40 and NE Buffer G7. HPLC-grade water was purchased from Rathburn Chemicals Ltd. (Walkerburn, UK). Ethanol was obtained from Bamford Laboratories Ltd. (Rochdale, UK) and the AGP N-linked glycan library from Prozyme[®] (Europa Bioproducts Ltd. Cambridgeshire, UK). Sigma-Aldrich[®] (Poole, UK) supplied the Sodium Acetate and the 50% w/v NaOH 50 by VWR International[®] Ltd (Lutterworth, UK). A CarboPac[TM] PA-100 analytical column (4x250mm) and guard column (4x50mm) were used on a Dionex 500[TM] system with GP50 gradient pump, ED40 electrochemical and pulsed electrochemical detection to carry out HPAEC (Dionex, Camberley, UK). Samples were analysed using PeakNet[TM] software on a Vtech 486X25 computer (Viglen, UK).

2.1.7 Determination of SA content (ELISA)

GALAB (Geesthacht, Germany) supplied the sialic acid linkage determination kit consisting of 96-well microplates, α2,3-sialic acid and α2,6-sialic acid lectin conjugates (<0.1% sodium azide), an enzyme conjugate, substrate, inhibition control, assay buffer (20x concentrated Tris buffered saline, pH8.0 – 20% Tris aminomethane, 8% Tris aminomethane, 0.1% sodium azide), detergent (Assay Buffer Additive), detection buffer (>10% diethanolamine) and equilibration buffer (2x concentrate tris buffered saline, pH 8.0 - >2% Tris aminomethane (hydrochloride), 1% Tris aminomethane, 0.1% sodium azide). Commercial AGP was purchased from Sigma-Aldrich [®] (Poole, UK). Analysis was performed using a microtitre plate reader from Dynex Technologies (Sussex, UK).

2.1.8 Drug binding

(±)-methadone, theophylline, Dulbecco's phosphate buffered saline and dimethyl sulphoxide were supplied by Sigma-Aldrich[®] (Poole, UK). Rathburn Chemicals Ltd supplied the HPLC-grade water (Walkerburn, UK). 96-well microtitre plates were purchased from Nunc (Germany). A BMG Labtech Optima fluorimeter (Germany) was used to analyse the intrinsic fluorescence of samples.

2.2 Methods

2.2.1 Clinical Samples

A trained phlebotomist obtained 2-5mL blood samples from patients undergoing methadone therapy, after informed consent from volunteers over the age of 18 was acquired. All samples were completely anonymised. Initial methodology development was carried out using commercial AGP from Sigma-Aldrich® and pooled heparinised 'normal' blood (blood transfusion service).

2.2.2 Isolation and determination of the level of AGP

Protocols similar to that reported by Elliott *et al.* (1997) were implemented in the study. The method was first published in 1994 (Smith and colleagues) however certain aspects have been altered over time to allow for reduced sample volumes.

2.2.2.1 Polyethylene Glycol Precipitation

Prior to determining the level of AGP in each patient and normal sample, the blood was separated into 1mL aliquots and protein precipitation performed by mixing with 40% (w/v) PEG 3350 (0.4g/1mL sample). They were incubated at 4°C overnight followed by a 30 minute centrifugation at 14,000 rpm. Centrifugation was repeated if remnants of the red blood cells were detected. The supernatant was collected in new eppendorfs and stored at -20°C until required for purification, the pellet was discarded.

2.2.2.2 Low Pressure Chromatography

The AGP present in the supernatant of each sample was isolated and purified through the use of two low-pressure chromatography columns, implementing dye ligand chromatography. Two 10mL Bio-Rad poly-prep disposable columns were used; one filled with ~5mL of Cibacron Blue 3GA Sepharose resin (binds and removes human serum albumin, HSA) and the second with Red Sepharose CL-6B (to remove remaining

α1-anti-trypsin, AAT). The resin slurries were kept wet at all times to ensure no air bubbles became trapped within the column, causing resin splitting which would affect the separation of plasma proteins. Buffers required in the low pressure affinity chromatography protocol were prepared according to table 2.2 and utilised when necessary.

Initially, the appropriate elution buffer was pumped through the Cibacron Blue 3GA Sepharose column at a rate of 0.5mLmin^{-1}, using a Pharmacia LKB Pump P-1, until equilibration was attained. This was illustrated on the Servogar 120 chart recorder, running at 12cm/hour, as a 'flat' base-line; recording the change in the eluent absorbance at 280nm, as measured by a UV^{-1} single path monitor optical unit. The supernatant formed upon PEG 3350 precipitation of a blood sample was applied to the column. Elution buffer was applied simultaneously and the eluted sample represented by the first peak generated by the chart recorder was collected in 15mL centrifuge tubes; this fraction contained AGP. HSA was removed from the mobile phase – its bilirubin binding sites becoming bound to the Cibacron Blue. The eluted fraction was then concentrated to approximately 2mL in a rotary evaporator.

Once entirely collected, the column was regenerated by adding the desorption buffer to dislodge the HSA bound to the column. It was re-equilibrated using elution buffer to prepare it for the addition of subsequent samples. All samples were dried under a vacuum to approximately 2mL ready for loading onto the Red Sepharose CL-6B column.

After equilibration of the Red Sepharose column with its elution buffer (table 2.2), a concentrated sample from the Cibacron Blue column was applied and further elution buffer used to collect the AGP fraction. The eluted AGP peak, the first distinguished by the chart recorder, was collected in 15mL centrifuge tubes. Any bound AAT was finally removed upon addition of the cleaning buffer (table 2.2). The pure AGP-containing fractions were again dried to 2mL in the rotary evaporator prior to desalting.

In order to prevent bacterial growth and the columns drying out after use, 10% Ethanol was added to each column and stored at 4°C.

Column	Buffer	Reagents
Cibacron Blue 3GA Sepharose	Elution	0.1mM Potassium Chloride 0.02% Sodium Azide 50mM Trizma pH 7 (albumin binds to column)
	Desorption	0.1mM Potassium chloride 500mM Potassium thiocyanate 0.02% Sodium Azide 50mM Trizma pH7 (removes bound albumin)
Red Sepharose CL-6B Column	Elution	1.15% Glacial Acetic Acid 30mM Sodium Acetate, pH 5.7 (α1-antitrypsin binds column)
	Cleaning Buffer	30mM Sodium Acetate 1M Sodium Chloride

Table 2.2.   Isolation buffers for low pressure chromatography.

2.2.3 De-salting

Salt introduced during isolation was removed from samples T1, T2, HR1 and LT1 using individual centricon® centrifugal cartridges to prevent contamination. Three drops of HPLC grade water were used to moisten the membrane. Concentrated sample (~2mL) collected after isolation was added and, by centrifugation at 4,000 rpm, any salts introduced during low-pressure chromatography, were removed by filtering through the membrane (MW cut-off 10,000). Once the salt solution passed through the membrane, the sample was washed with HPLC grade water until the filtered solution was clear. To collect the dry desalted AGP sample on the membrane, the cartridges were inverted and three drops of HPLC-grade water added. The samples were collected in fresh eppendorfs, dried to completion under a vacuum, and reconstituted in 1mL HPLC grade water to allow the level of AGP to be determined.

The remaining samples were desalted using Amicon filter devices due to a design modification made by the company (Millipore, UK). The 2mL sample from the isolating stage was added to the cartridge and made up to approximately 3.5mL using HPLC-grade water. The dissolved salt passed through the membrane (MW 10,000 cut-off) and the sample was washed several times with HPLC-grade water. Desalted samples collected in the tube were reconstituted in approximately 500μL HPLC-grade water and added to new eppendorfs; they did not require inversion like the previous design. These were then dried and reconstituted as above.

2.2.4 AGP level determination

Using the samples re-constituted in 1mL HPLC-grade water, the level of isolated AGP was determined using a spectrophotometer. Reference was made to a Standard curve produced using the absorbance values at 280nm of a series of dilutions of commercial AGP standards in HPLC-grade water (0mg/mL to 3mg/mL) − refer to section 5.2.3. This wavelength is often chosen in the determination of protein levels because proteins absorb strongly while other substances commonly in protein solutions do not. A 1mL quartz cuvette with pathlength of 1cm and an extinction coefficient of 29.7 mM^{-1} cm^{-1} was used in spectrophotometric determination.

2.2.5 SDS-gel and Western Blot

2.2.5.1 Discontinuous SDS-PAGE

To determine whether the isolation and purification procedures were effective, sodium dodecyl sulphate − polyacrylamide gel electrophoresis (SDS-PAGE) was performed. Separation of proteins present in the supernatant of a PEG precipitated blood sample and those present after the isolation process was performed alongside commercial controls and a ladder; based on their size and molecular weight. This was not used to isolate AGP because SDS is an anionic detergent which can damage sugar structures and would therefore be detrimental in the analysis of glycoproteins. When SDS is added to a sample of protein and heated to 100^oC the protein becomes denatured into separate polypeptides and a negative charge is generated when they become bound to SDS, the size of this is dependent on the length of peptide.

The large pore polyacrylamide 'stacking' gel - approximately 2pH units lower than the electrophoresis buffer − was cast over a small pour acrylamide 'resolving' gel to produce a discontinuous buffer system; their preparation is described below. SDS-bound proteins become concentrated in the stacking gel ready to migrate into the resolving gel to allow separation based upon size.

2.2.5.2 Resolving gel preparation

Spacers of 2mm and a 2mm, 12-well comb were used to set up the omniPAGE gel casting system (VS10CBS) and allowed 10cm vertical separations to be performed. Acrylamide resolving gels (10% w/v) were used because the molecular weight of AGP is ~43kDa and this gel has a resolution of ~20-75kDa, therefore it is capable of resolving proteins including albumin and AAT (MW 67kDa and 52kDa respectively).

The gel consisted of the components as stated in table 2.3; all components were added in the order stated. A final addition of 7.5µl of *N,N,N',N'*-tetramethylethyenediamine (TEMED, >99% pure) allowed polymerisation of the gel components, this was catalysed by the addition of the oxidising agent APS. To ensure no bubbles formed, the mixture was carefully mixed before loading between the two glass plates. A 3cm space was left above the gel and 1mL isopropranol layered on to reduce the gels contact with

air and ensure an even surface was formed. This layer was then decanted after a 30min polymerisation and the top of the gel was rinsed with distilled water and filter paper used to blot it dry.

2.2.5.3 Stacking gel preparation

On the addition of 6.7µl of TEMED (>99% pure) to the stacking gel components in table 2.4, the mixture was poured on top of the resolving gel. A comb was inserted and the gel was left at room temperature for 30min while the gel polymerised. Upon setting, the comb was removed and the gel rinsed with Tris-glycine electrophoresis buffer (table 2.5) to remove un-polymerised material.

The gel was transferred from the casting chamber into an electrophoresis tank and submerged in Tris-glycine electrophoresis buffer. The samples and ladder were heat denatured for 5 min at 100°C and up to 25µl loaded into appropriate wells. The molecules were concentrated in the stacking gel over 15 min at 50V then separated in the resolving gel at 100V for a further 50 min.

2.2.5.4 Protein gel staining

When the dye had run the length of the gel, it was carefully separated from the plates and any electrophoresis buffer was removed through two 10 min washes with distilled water on a slow rotating platform (50rpm). The gel was immersed in 5x volume of Coomassie blue protein stain (table 2.6) and put back on the rotating platform (50rpm) for 1h. The stain was decanted and the gel was de-stained over 6-8h with the solution recorded in table 2.7, changing the de-staining solution 3-4 times. The gel was then visualised.

10% Resolving gel	1 x 10cm gel
Distilled water	3.15mL
30% (w/v) Stock acrylamide	2.5mL
4x Resolving tris solution pH 8.8*	1.875mL
10% (w/v) Ammonium persulphate	75μL

Table 2.3.   Composition of the 10% (w/v) resolving gel for SDS-PAGE.
The APS was always added last. * (1.5M Tris-HCl; 0.4% (w/v) SDS).

Stacking gel	1 x 10cm gel
Distilled water	2.1mL
30% (w/v) Stock acrylamide	0.325mL
4x Stacking tris solution pH 6.8*	0.8mL
10% (w/v) Ammonium persulphate	3.35μL

Table 2.4.   Composition of the stacking gel.
The APS was always added last. * (0.5M Tris-HCl; 0.4% (w/v) SDS).

Reagents	1 x 10cm gel
Tris-base	5mM
Glycine pH 8.3	50mM
SDS	0.02% (w/v)

Table 2.5.   Composition of Tris-glycine electrophoresis buffer.

Reagents	Percentage present in stain
Coomassie brilliant blue R250	0.25% (w/v)
Methanol	45% (v/v)
Distilled water	45% (v/v)
Glacial acetic acid	10% (v/v)

Table 2.6.   Composition of the coomassie blue protein stain.

Reagents	Percentage present
Methanol	45% (v/v)
Distilled water	45% (v/v)
Glacial acetic acid	10% (v/v)

Table 2.7.   Composition of de-staining solution.

2.2.6   Western Blotting

2.2.6.1 Blot sandwich assembly

Filter paper – 2mm thick – and nitrocellulose membrane (0.2 μm, Optiran BA-S 83) were cut to fit the gel and pre-soaked in 1x continuous transfer buffer (CTB, table 2.8) for 15 min, alongside two fibre pads.

The blot sandwich was assembled using forceps with the gel facing the negative pole. Some pre-chilled CTB was poured into the tank and a magnetic stirrer added. The module containing the blot sandwich was inserted and the tank placed in a polystyrene container of ice. The remainder of the cooled CTB was placed in the electrophoresis tank and the blot run at 75V for 100 min with a magnetic stirrer keeping the cool CTB circulating.

2.2.6.2 Blocking of the membrane (reduction of non-specific binding)

To saturate the unbound sites in the nitrocellulose membrane the blot was incubated in 50ml 3% (w/v) non-fat dry milk in 1x Tris-buffered saline (table 2.9) for 1.5 h at room temperature. The blot and solution were placed on a rotating platform (50 rpm).

2.2.6.3 Immunoprobing with primary antibody

Primary anti-AGP antibody (5μl) from the rabbit was added directly into the blocking solution (1:2,500 dilution). It was then incubated for 30 min at room temperature on a rotating platform (50 rpm) to allow the antibody to mix, and then incubated overnight at 4°C to bind. Any unbound primary antibody was removed by washing the membrane in 1x TBS/0.1% (v/v) Tween®20 for 6x 10 min washes.

2.2.6.4 Immunoprobing with secondary antibody

A 3μl volume of the secondary antibody (goat anti-rabbit IgG, AP-linked) was added into 50ml 3% (w/v) non-fat dry milk in 1x TBS (a 1:16,667 dilution) and incubated at room temperature for 1h on a rotary platform (50 rpm). Any unbound secondary antibody was washed off as described in section 2.2.6.3.

Reagents	Percentage present in stain
Tris	25mM
Glycine	192mM
Methanol	20% (v/v)

Table 2.8.   **Composition of 1x continuous transfer buffer (pH 8.3).**

Reagents	Percentage present
Tris-HCl pH 7.4	50mM
NaCl	0.9% (w/v)

Table 2.9.   **Composition of 1x Tris buffered saline.**

Reagents	Percentage present
Tris HCl (pH 9)	100mM
$MgCl_2$	1mM
NaCl	150mM

Table 2.10.   **Composition of substrate buffer.**

2.2.6.5. Immunodetection of proteins

A BCIP (5-bromo-4-cholo-3-indoyl-phosphate)/NBT (nitro blue tetrazolium) chromogenic assay was used to identify proteins on the membrane. It consists of three components; a combination of BCIP, NBT and an alkaline buffer resulting in a substrate solution for alkaline phosphatase. An insoluble NBT diformazan product is formed upon incubation with the enzyme and was identified as a purple precipitate therefore allowing colorimetric detection of alkaline phosphate-labelled molecules.

The membrane was rinsed in 50ml alkaline phosphatase buffer (table 2.10) to equilibrate the pH of the blot. NBT (50mg/ml; 70% (v/v) dimethylformamide) and BCIP (50mg/ml; 100% (v/v) dimethylformamide) were added to a further 50ml of the alkaline phosphatase buffer; 330 µl and 165µl respectively. The solution was mixed and added to the membrane, this was incubated at room temperature in the dark. Colour development occurred in approximately 30 minutes. The membrane was rinsed with distilled water to stop the colour reaction and was blotted dry with filter paper. The membrane was then visualised.

2.2.7 High pH Anion Exchange Chromatography

2.2.7.1 Acid hydrolysis

Volumes of commercial and patient AGP samples corresponding to 50µg were added to individual reacti-vials and 100µL of 2M TFA added. After replacing the aluminium lined caps, the samples were incubated at 100°C for 4 hours. The basic sugars fucose (Fuc), galactose (Gal), and mannose (Man) were hydrolysed using this treatment. Simultaneously 50µg of AGP taken from the same samples were hydrolysed with 50µL of 4M HCl, at 100°C for 6 hours to hydrolyse the amine sugar GlcNAc. Subsequently, 1cm glass wool was packed in Pasteur pipettes to act as a plug for 1mL (pH4) Dowex®- 50 WX8 H$^+$ cation-exchange resin, using separate columns for each sample to prevent contamination. To wash the column, 6mL HPLC-grade water was added and eluted, followed by the sample hydrosylate from TFA hydrolysis treatments only. HPLC-grade water (2mL) was added to collect the sample. The eluted solutions were dried to completion in the rotary evaporator. The HPLC-grade water and acid from HCl

hydrolysed samples were evaporated in a rotary evaporator (similar to the method adopted by Fan *et al.,* 1994).

All samples were then reconstituted in 20μL HPLC-grade water and 5μL internal standard (2-deoxy-D-galactose) to prepare the samples for monosaccharide analysis with high pH anion exchange chromatography (HPAEC).

2.2.7.2 *Monosaccharide analysis*

After drying to completion and reconstituting samples from the different hydrolysis conditions, the components of the glycans were analysed. The separation of the monosaccharides was performed using a Dionex 600[TM] High pH Anion Exchange Chromatography (HPAEC) system with pulsed ampertomic detection (PAD). The pulsed potentials during PAD were 0 seconds (sec), 0.05V; 0.29sec, 0.05V; 0.49sec, 0.05V; 0.5sec, 0.05V; 0.51sec, 0.6V; 0.6sec, 0.6V; 0.61sec, -0.6V; 0.65sec, -0.6V; 0.66sec, 0.05V (refer to figure 2.1). The Dx-600 was fitted with CarboPac PA-100 (containing quaternary-ammonium-bonded pellicular resins) analytical and guard columns, a GP50 gradient pump and ED40 electrochemical detector. It was operated at ambient temperature.

Before injecting any samples, the CarboPac PA-100 column was regenerated using 50% 1M NaOH/50% HPLC-grade water for 15 minutes - this was repeated. Mixtures of five monosaccharides – 5μg of Fuc, GlcNAc, Gal, Man and internal standard (2-deoxy-D-galactose) - were injected and 3% 1M NaOH / 97% HPLC-grade water was passed through the column at a flow rate of 0.5ml/min in an isocratic gradient to ensure effective separation over 35minutes. This was followed by 50% 1M NaOH/50% HPLC-grade water for 15minutes to allow regeneration of the column. The monosaccharide represented by each peak was identified by calculating the ratio of their retention times with the internal standard and comparing the values to those generated when the individual monosaccharides were run with internal standard. Before any samples were analysed, standard curves for each of the five monosaccharides were produced, plotting the peak area values against the mass of monosaccharide present (μg). The respective equations were used to quantify the monosaccharides present in the samples of AGP.
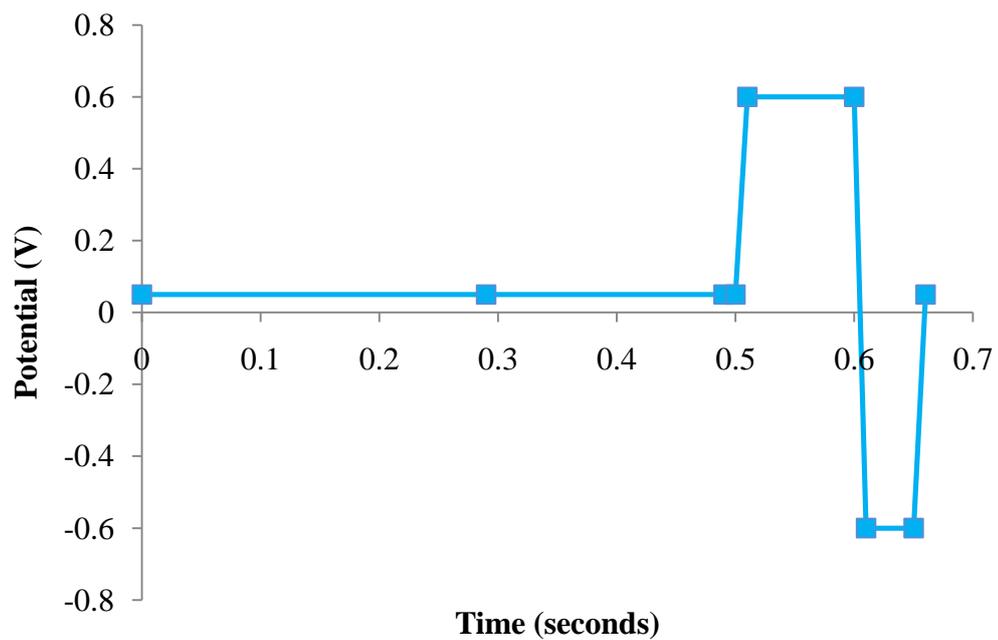
Figure 2.1. **Pulsed potentials of the PAD system during HPAEC.**

Subsequent to sample hydrolysis and reconstitution in 20µL HPLC water and 5µL of internal standard (1mg/ml), each was independently injected and separated by the column (using 3% 1M NaOH / 97% HPLC water – pH13) over 50 minutes at a flow rate of 0.5mL/min - this included a 15-minute regeneration period using 50% NaOH/ 50% HPLC. The system was then ready for the injection of the next sample. Again, the peaks generated for each sample were identified based upon the ratios of retention time with the internal standard and the monosaccharide.

2.2.7.3 Oligosaccharide analysis

2.2.7.3a Enzyme Digestion

AGP sample volumes corresponding to 100µg of the glycoprotein were dried to completion under a vacuum and reconstituted in 100µl of HPLC-grade water. The samples were transferred into separate glass vials and aluminium lined caps fitted. They were then denatured at 100°C for 3 hours and dried to completion under a vacuum.

A reaction mixture was prepared by adding the reactants stated in table 2.11 to the dry AGP samples to make a total reaction volume of 100µl. The mixtures were incubated for 24 hours at 37°C followed by a second addition of 5U of PNGase F. It was then incubated for a final 24 hours at 37°C.

To separate the protein from the glycan chains ethanol precipitation was performed. After the 48 hour incubation period, 300µl of ice-cold 80% ethanol was added to the reaction mixture to provide a ratio of 3 parts ethanol to 1 part reaction mixture. Once mixed, the samples were incubated overnight at -20°C. They were then centrifuged at 14,000 rpm for 30 minutes and the supernatant (containing the glycans) was collected in clean eppendorfs, the pellet discarded. The supernatant was dried to completion using a rotary evaporator ready to be reconstituted in 25µL HPLC-grade water for oligosaccharide analysis.

reagents	Volume present
HPLC-grade water	79µl
NE Buffer G7	10µl
NP-40	10µl
PNGaseF	5U

Table 2.11.   Composition of reaction mixture for enzyme digestion.

2.2.7.3b Oligosaccharide HPAEC Analysis

A Dionex 500[TM] system was used with the same pulsed potentials for the PAD as described in figure 2.1. A CarboPac PA-100 pellicular anion exchange column was used to separate the differently branched glycans. It was first regenerated over two 20minute cycles using 50% 1M NaOH and 50% HPLC grade water. The dry sample supernatants from the enzyme digestion stage were reconstituted in 25µL of HPLC-grade water. Each was independently applied to the PA-100 column and the glycans separated based on their degree of branching using a series of different eluent conditions, all added at a flow rate of 1mL min^{-1}. The column was equilibrated for 10 minutes using 10% NaOH/ 5% 1M NaOAc/ 85% HPLC grade water, the sample was then separated during a linear gradient over 45 minutes when the eluent proportions were 10% NaOH/ 20% NaOAc/ 70% HPLC grade water. The column was regenerated over 10minutes with an eluent composition of 50% NaOH/ 50% HPLC grade water. The final equilibration step, lasting 5 minutes, required 10% NaOH/ 5% NaOAc/ 85% HPLC grade water.

To allow comparison of sample and commercial AGP oligosaccharide composition, a human N-linked oligosaccharide library was separated, providing a reference profile. Approximately 5µg was injected.

2.2.8 Sialic acid determination ELISA

A 1:20 dilution of assay buffer (Tris buffered saline with detergent; concentration unavailable) was added to the first row of two 96-well microtitre plates (50% v/v), representing the 100% signal control i.e. where the binding of lectin enzyme conjugates would not be inhibited. In the second row the inhibition control (positive control) supplied was plated (50% v/v) to represent 0% signal – no additional preparation was required of the reagent.

Serial dilutions of AGP (5mg/mL) from a 'normal' healthy heparinised blood sample and isolated from patients undergoing titration, harm reduction and long-term methadone therapies were prepared with assay buffer and plated in triplicate (50% v/v) in the order of decreasing concentration.

To one plate, *Sambucus nigra agglutinin* (SNA) – the lectin conjugate for α2,6-linked SA - was added (50% v/v) to each well. *Macckia amurensis Leukoagglutinin* (MAL) was added in the same volumes to the wells of the second plate to detect α2,3-linked SA residues. The lectin conjugates were supplied in 100x concentrated form therefore they were diluted prior to their use.

The microtitre plates were incubated at room temperature in the dark for 2 hours. Upon removal, they were cooled at 4°C for 5minutes then washed with cold Assay buffer (~200μL in each well). The plates were then emptied and washed a further four times with cold assay buffer dropped onto paper towels between each wash to remove unbound lectin conjugate and AGP.

The enzyme conjugate, which was supplied in a 10,000-fold concentrated form, was diluted to a working concentration and added to all wells of the two plates in 100μL aliquots and both were incubated at room temperature in darkness. After 2h incubation the plates were cooled for 5minutes at 4°C and subsequently washed with cold assay buffer (200μL pre well) a total of three times.

All wells were then washed twice in diluted equilibration buffer (1:2 dilution of the 2x concentrated buffer supplied with deionised water).

Immediately prior to measurement of the absorbance of the well reagents at 405nm, 100μL of enzyme substrate was added to each well (2.8mg/mL solution in detection buffer supplied in the kit; 0.36% w/v) to catalyse the reaction which was followed colourimetrically.

Absorbance was analysed after 5, 20, 35 and 50 minutes to determine the most appropriate time interval for the reaction to take place and highlight the greatest variation between controls and samples.

2.2.9 Drug Binding Assay

2.2.9.1 Commercial AGP and Methadone standards

To conserve as much sample as possible and allow drug binding analysis to be replicated, the study was performed at the microtitre level, using Nunc 96-well microtitre plates. This technique was tested using theohylline; a drug known to bind AGP as described in the method validation chapter (section 3.4).

Initially commercial AGP (5mg/mL) was added to a microtitre plates in 10µL volumes alongside a range of methadone concentrations (25-2500µM), diluted from a 5mM stock; each at 10µL. The drug was dissolved in DMSO. D-PBS was used to make wells up to a final volume of 100µL, 80µL applied to those wells containing both AGP and drug and 90µL in those only containing AGP (0mg/mL drug). Therefore, the AGP and drug standards were diluted 10 fold on the plate. All conditions were plated in triplicate. The plates were then excited at 280nm and the fluorescence emitted at 340nm (emission maximum of AGP) was recorded using an Optima fluorimeter. The method was modified using commercial AGP at a concentration of 0.5mg/mL in order to provide data relative to that of samples; limited sample availability meant concentrations of 5mg/mL were unachievable. The concentration of drug standards remained the same because when made 10 fold lower, the fluorescence was undetected by the fluorimeter, thus the technique was ineffective.

2.2.9.2 Determination of methadone binding to sample AGP

AGP isolated from the blood of healthy volunteers and patients undergoing methadone therapy was used at a concentration of 0.5mg/mL (reconstituted in d-PBS). Methadone standards were produced using DMSO to include values representing the therapeutic level of methadone (~400ng/mL). The lowest concentration was 370ng/mL (1.07µM); 10 fold lower than the 3700ng/mL (10.7µM) plated. Concentrations much lower than this did not alter the fluorescence emitted. Higher concentrations were added; 30µg/mL, 75µg/mL and 865µg/mL (86.7, 216 and 250µM respectively) to indicate the change in binding; all were diluted ten-fold upon plating. Thus, because AGP levels were not 10 fold greater when plated, results are not indicative of the situation *in vivo*

but still represent useful data when determining differences in binding between individuals.

2.2.10 Statistical Analysis

The Pearson correlation coefficient (R^2) was calculated for the monosaccharide calibration curves, using Microsoft® Excel 2007; a linear fit represented by a value of 1. To calculate mean and standard deviations of data Microsoft® Excel 2007 was also used. Two sample t-tests and one-way ANOVAs (with Tukey's post hoc) were performed using Minitab, version 15, to determine the statistical significance of all quantitative data generated during the various analyses.

SUMMARY STATEMENT

Methods required for the structural analysis of glycans were only utilised and analysed in subsequent chapters for AGP isolated from heparinised blood samples provided by the Blood Transfusion Service and patients recruited from various stages of opioid replacement therapy. That isolated from serum samples taken from elderly individuals (cancer-free) and commercially sourced AGP were not deemed adequate representations of a 'normal' healthy population – little information regarding the potential presence of an APR in the former population was available while isolation of commercial AGP differed to that utilised in this study. The use of acidic conditions in isolation could not be avoided in the large-scale commercial methodology.

Chapter 3
Validation of Methods

3.1 Introduction

The following chapter describes the studies that were performed prior to the main investigation in order to determine the optimal conditions for monosaccharide compositional analysis and drug binding studies.

Analysis of the monosaccharide composition of AGP glycans required the use of acid hydrolysis to cleave the glycosidic bonds between the monomeric units prior to separation by HPAEC. It had been common practice in the laboratory to incubate 50µg AGP for 4 hours at 100ºC with trifluoroacetic acid (TFA) and hydrochloric acid (HCl) simultaneously. However, similar studies indicated that separate use of HCl and TFA provided optimum hydrolysis (Fan *et al.,* 1994). Therefore it was deemed necessary to investigate the most efficient methodology because the research documented in this thesis was based on structural analysis thus insufficient cleavage or monosaccharide instability under acidic conditions risks the generation of erroneous data and subsequent conclusions drawn would be inaccurate.

Additionally, adaptations to the methodology utilised previously by the group (Johnson and Smith, 2006) to analyse drug binding to AGP were required. The concept of exploiting the intrinsic fluorescence of specific amino acids (namely Trp and Tyr) in the hydrophobic binding pocket for such studies was introduced by Parikh and co-workers (2000). The fluorescence emitted is highest at 340nm for AGP due primarily to the emission by Trp residues, and is reduced (quenched) on the binding of drugs or ligands hence fluorescence studies offer potential in the detection of drug binding.

3.2   Methodology

3.2.1   Optimisation of monosaccharide release

3.2.1.1 Monosaccharide stability

Before hydrolysis was undertaken, it was deemed important to ensure the monosaccharides under investigation were not degraded when incubated at 100°C under acidic conditions for up to six hours.

Monosaccharide standards (Sigma-Aldrich), each approximately 7μg, were incubated with TFA or HCl alone, or a combination of the two. HPAEC-PAD (described in section 2.2.7.2) was performed on the samples of monosaccharide every hour (n=3) with final analysis occurring at 6h, to determine if they remained stable under the different conditions.

3.2.1.2   Development of optimal hydrolysis conditions

In order to determine which of the techniques employed by the various groups provided the optimum hydrolysis of AGP glycans, experiments were performed with 50μg commercial AGP hydrolysed for 4h with both 100 μL 2M TFA and 50μL HCl, the same conditions but incubated for 6h, 50μg hydrolysed for 4h with 100 μL 2M TFA alone and 50μg hydrolysed for 6h with 50μL 4M HCl alone.   The levels of each monosaccharide were determined after these incubation periods.   Additionally, commercial AGP samples were incubated under the same conditions but analysed each hour to determine whether the hydrolytic conditions deemed most effective in the previous experiment were not bettered if incubated for alternative time periods.

3.2.1.3 Dowex® Cation-Exchange Column study

A study was carried out to test whether Dowex®-50 H+ cation-exchange resins interfere with the elution of monosaccharide components when used to remove proteins after hydrolysis.   Three 25μg aliquots of the standard control HPAEC mixture (5μg of Fuc,

GlcNAc, Gal, Man and an internal standard of 2-deoxy-D-galactose) were passed through the Dowex® column. The mass of each monosaccharide obtained was compared to values for an identical mixture that was directly injected into the HPAEC system in order to determine any losses due solely to the resin.

Additionally, 50μg commercial AGP samples - hydrolysed using the four acidic conditions above (4M HCl alone for 4 hours, TFA alone for 6 hours, and both acids for 4 and 6 hours), but not passed through Dowex®-50 H⁺ cation exchange columns - were analysed to establish whether there were any significant effects on the component monosaccharide levels when compared to those passed through the columns. In place of the Dowex® column, the hydrolysed samples were dried in a rotary evaporator after hydrolysis and subsequently washed twice with 200μL HPLC-grade water (as reported by Fan *et al.,* 1994). Post-hydrolysis all samples, regardless of the treatment, were immediately dried and reconstituted in 20μL water and 5μL IS to allow separation by HPAEC upon injection, subsequently assisting the identification of the method least likely to compromise monosaccharide yield.

The hydrolysis conditions chosen after comparing all the results from the studies above was described in section 2.2.7.1.

3.2.2   *Microtitre plate drug binding assay*

Initial fluorescence studies were undertaken at the University of Strathclyde where fluorescence emission spectra were generated in the absence and presence of drugs known to bind AGP (refer to section 3.3.2). The studies affirmed that maximum emission occurred at approximately 340nm as has been well documented for Trp residues (Chen and Barkley, 1998). Therefore in subsequent studies at Edinburgh Napier University, where the scanning function was unavailable, a specific filter was utilised.

3.2.2.1 Testing method with Theophylline

Initially the micro-titre plate method was tested by performing studies using a range of theophylline concentrations (25-2500µM), diluted from a 5mM stock, and 5mg/mL commercial (Sigma) AGP. Both were dissolved in d-PBS. Commercial AGP (5mg/mL) was added to individual wells on a Nunc 96-well microtitre plate at a volume of 10µL (10% v/v). Each drug standard was then added to the AGP wells, also at a volume of 10µL (10% v/v) and d-PBS was used to make up a final volume of 100µL. All conditions were plated in triplicate. The plates were then excited at 280nm and the fluorescence emitted at 340nm (emission maximum of AGP) was recorded using an Optima fluorimeter. Results were graphed in figure 3.4.

The method was modified to allow for small volumes of sample AGP; 5mg/mL was unobtainable due to restrictions enforced on the project. Therefore 0.5mg/mL was used (figure 3.4). The concentration of drug standards remained consistent with the previous experiment because when made 10-fold lower, fluorescence was undetected.

3.2.2.2 Microtitre plate and Methadone

The same range of concentrations (25-2500µM) were produced with methadone, in d-PBS, and added to wells containing 10µL of either 5mg/mL or 0.5mg/mL commercial AGP. As described above (section 3.2.2.1), finite sample volumes meant 0.5mg/mL AGP was used in all sample analyses. The effects on the fluorescence of AGP in the presence of methadone in this case were graphed in figure 3.5.

3.2.2.3 Methadone dissolved in d-PBS versus DMSO

The two concentrations of commercial AGP - 0.5mg/mL and 5mg/mL were made using d-PBS, as above (section 3.2.2.1). A concentration range similar to that of theophylline was produced for methadone (25-2500µM) but using DMSO. Each drug standard was added to wells containing 10% (v/v) of either 5mg/mL or 0.5mg/mL commercial AGP and made up to 100µL with d-PBS (methadone was therefore present in the concentration range 2.5-250µM). The results (figure 3.6) were compared to those generated when d-PBS was used a solvent (figure 3.5), the reduction in fluorescence was far more evident with DMSO.

3.3 Results

3.3.1 *Optimisation of monosaccharide release methodology*

3.3.1.1 Monosaccharide stability

Overall, the neutral monosaccharides displayed decreased stability in the presence of HCl (figure 3.1a-c). When combined with TFA, the stability was generally improved on that in the presence of HCl alone. Fuc levels were most stable over longer periods in the presence of TFA alone; the other acidic conditions caused a decrease, over time. Gal and Man also appeared to become less stable as incubation periods lengthened when HCl was present, either alone or alongside TFA. The basic monosaccharide GlcN (GlcNAc is deacetylated during hydrolysis) remained largely unaffected by the acidic conditions however it was more stable in the presence of HCl than any other monosaccharide (figure 3.1d).

3.3.1.2 Acid Hydrolysis optimisation

Table 3.1 summarises the mean values of each monosaccharide obtained – according to HPAEC separation and detection – upon hydrolysis of commercially sourced AGP under four acidic conditions (n=4 for each condition). The amount of monosaccharides obtained during the combined hydrolysis technique was compared to the other conditions tested (reported in table 3.2). Tables 3.1 and 3.2 indicate that the most effective yield of neutral monosaccharides was obtained by using TFA alone (*p<0.05*). Greatest significance was found for Gal which is a monosaccharide only present in the branches of N-linked complex chains, its presence is therefore correlated with chain branching. Ensuring its optimal release was important for the subsequent structural studies.

Although the most effective method of cleaving GlcNAc residues initially appeared to be the combined use of acids (table 3.1 and 3.2), a further study was deemed necessary upon reading the literature and analysing the values generated during hydrolysis of commercial AGP, which seemed low. This involved the removal of the dowex column as described in section 3.1.3.

a)

Fuc

b)

Gal
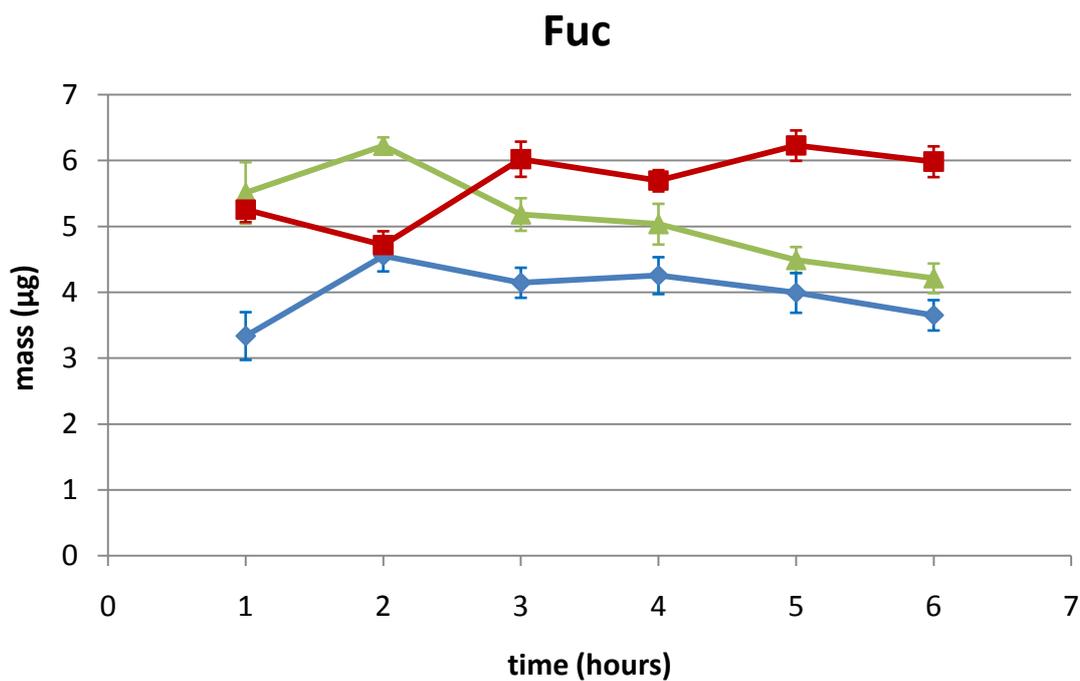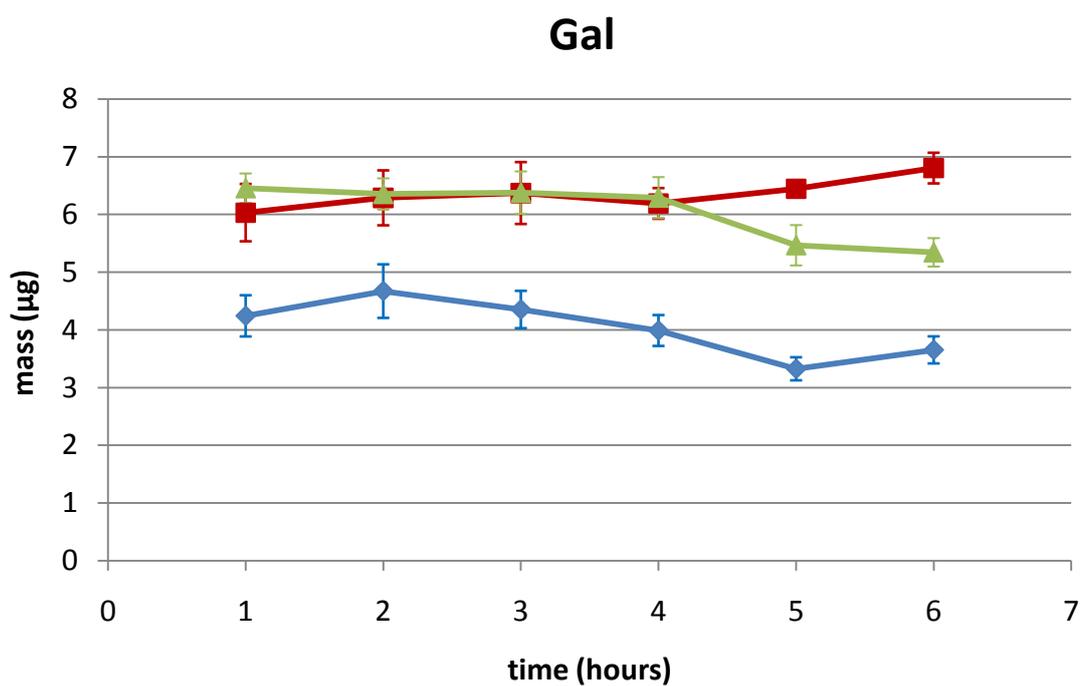
Figure 3.1.   Stability of monosaccharides.

a) Fuc and b) Gal when incubated under three acidic conditions;

TFA   HCl   TFA and HCl

90

c)

Man

d)
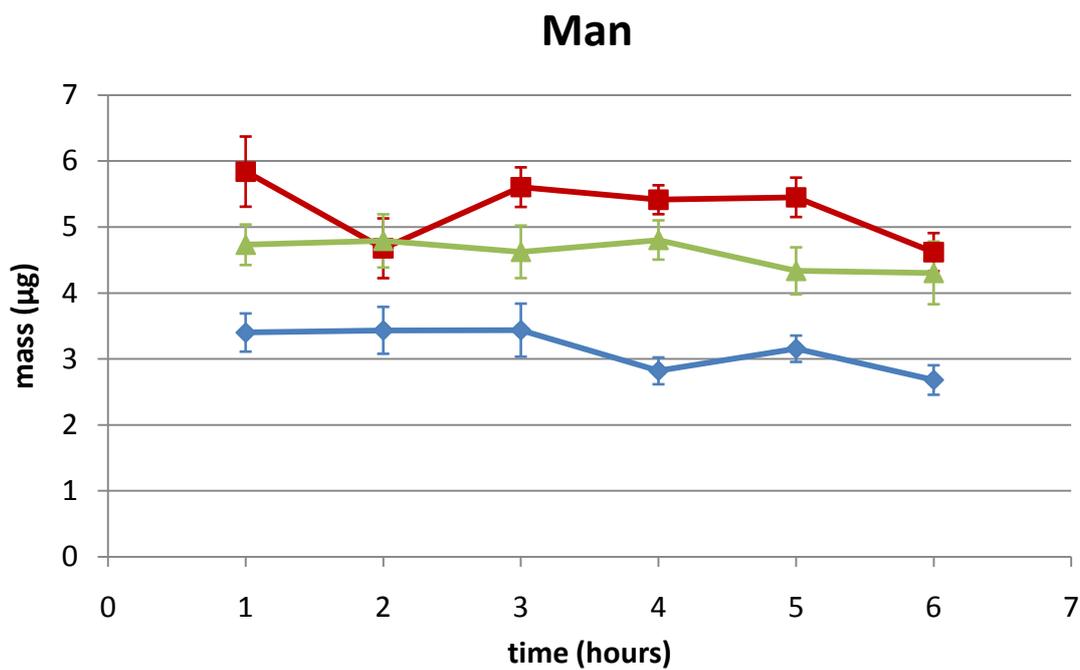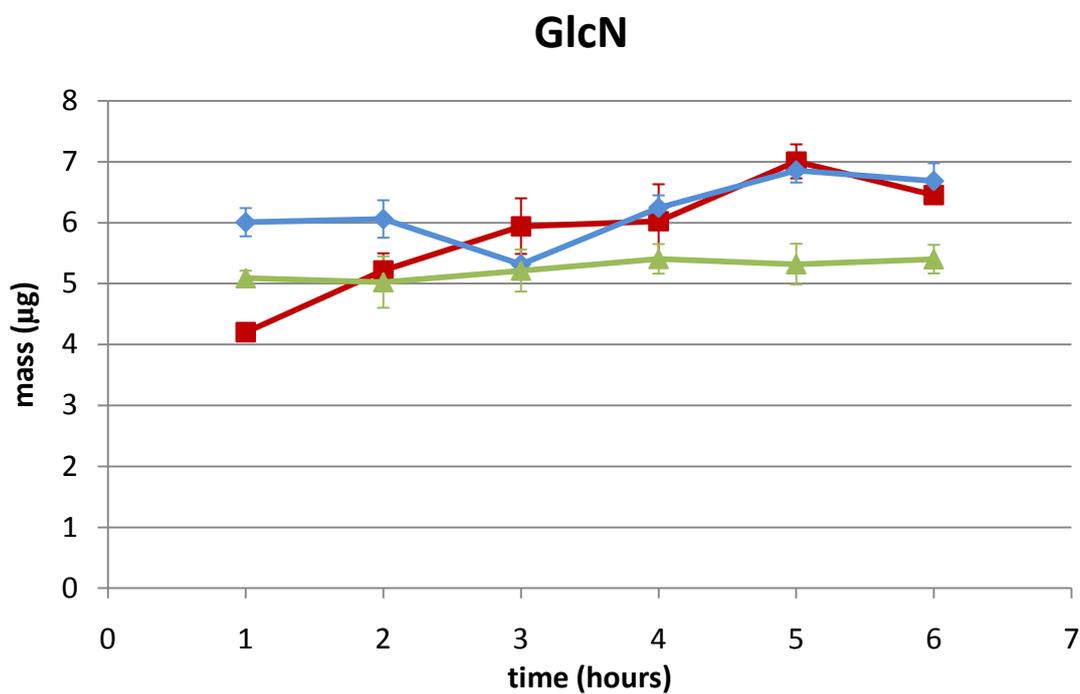
GlcN

Figure 3.1 continued.   **Stability of monosaccharides.**

c) Man and d) GlcN when incubated under three acidic conditions;

TFA   HCl   TFA and HCl

Firstly, when a mixture of the five monosaccharides of known masses (each 5µg) was passed through a dowex® column, the level of GlcN (deacetylated GlcNAc) recorded by HPAEC was significantly lower than 5µg (approximately 3.1µg, $p<0.05$). The other monosaccharides were unaffected. Subsequently 50µg (n=6) commercial AGP samples were hydrolysed using a combination of 2M TFA and 4M HCl incubated for 4h and 50µg hydrolysed using the acids alone (n=6). Three samples treated under each condition were passed through dowex® columns and the remaining six samples were washed with HPLC-grade water (2 x 200µL) and directly injected. As shown in table 3.3, it was found that when the AGP samples were hydrolysed with HCl and washed, not passed through dowex cation-exchange resins, the levels of GlcN detected were significantly greater than those in table 3.1 where the column was used (+1.434 µg, $p<0.05$). Nevertheless, the neutral monosaccharides benefited from passage through a dowex column. Therefore, the separate use of acids was found to improve the hydrolysis of the glycan chain monosaccharide components.

The above investigation only analysed samples after four or six hour incubation periods as described by the protocols tested (described by Fan *et al*. (1994) and past researchers in the Smith group). To ensure these time periods were optimal for monosaccharide cleavage, samples were incubated under the same conditions but, each hour, aliquots amounting to 100µg of AGP were analysed (in triplicate) to identify the level of the monosaccharides present at each time point; shown in figure 3.2, where peak area values are correlated with the mass of monosaccharide.

Again, it can be seen that incubating the samples separately was most effective, particularly for Gal and GlcNAc hydrolysis. Importantly, it was shown that, for Man, the use of TFA for 4h provided maximal release of the monosaccharide. Incubation with TFA for 4h also provided elevated cleavage of Fuc and Gal; although the values were not significantly different to that at 3 or 5h ($p>0.05$). When HCl was combined with TFA, the peak area values at each time point were generally lower than those when TFA was used alone. In the presence of HCl alone, hydrolysis of the neutral monosaccharides was not effective. For GlcN, the study showed that isolated use of HCl provided most effective hydrolysis at 6h, supporting the results in table 3.3.

	Mean mass of monosaccharide ± stDev (μg)			
Hydrolysis condition	Fucose	GlcNAc	Galactose	Mannose
2M TFA/ 4M HCl 4h	0.13 ± 0.02	3.04 ± 0.35	3.24 ± 0.41	1.62 ± 0.04
2M TFA/ 4M HCl 6h	0.09 ± 0.08	2.37 ± 0.13	3.13 ± 0.33	1.48 ± 0.17
2M TFA 4h	0.26 ± 0.05*	1.54 ± 0.08	4.52 ± 0.80*	2.27 ± 0.25*
4M HCl 6h	0.15 ± 0.15	2.74 ± 0.05	3.62 ± 1.27	1.57 ± 0.46

Table 3.1. **A comparison of the mean mass of monosaccharides.** Generated during hydrolysis of commercial AGP under the four different acid hydrolysis conditions ± standard deviation

	Mean difference in mass of monosaccharide compared to that generated using 2M TFA/ 4M HCl 4h (μg)			
Hydrolysis condition	Fucose	GlcNAc	Galactose	Mannose
2M TFA/ 4M HCl 4h	0.13	3.04	3.24	1.62
2M TFA/ 4M HCl 6h	- 0.06	- 0.67	- 0.10	- 0.13
2M TFA 4h	+ 0.14	- 1.50	+ 1.29	+ 0.65
4M HCl 6h	+ 0.02	- 0.30	+ 0.38	- 0.05

Table 3.2. **Summary of the difference in mass of monosaccharides obtained during hydrolysis.** Commercial AGP glycan composition under the four different acid hydrolysis conditions compared to the combined use of TFA and HCl for 4h.

	Mean mass of monosaccharide ± stDev (μg)			
Hydrolysis condition	Fucose	GlcNAc	Galactose	Mannose
2M TFA/ 4M HCl 4h	0	4.217	1.344	0.563
2M TFA 4h	0.050	4.000	2.047	0.881
4M HCl 6h	0	4.477*	1.133	0.341

Table 3.3. **Mean mass of monosaccharides obtained upon removal of the dowex cation exchange resin.** * Statistically significant improvement compared to 2M TFA/4M HCl 4h ($p < 0.05$).

a)

b)
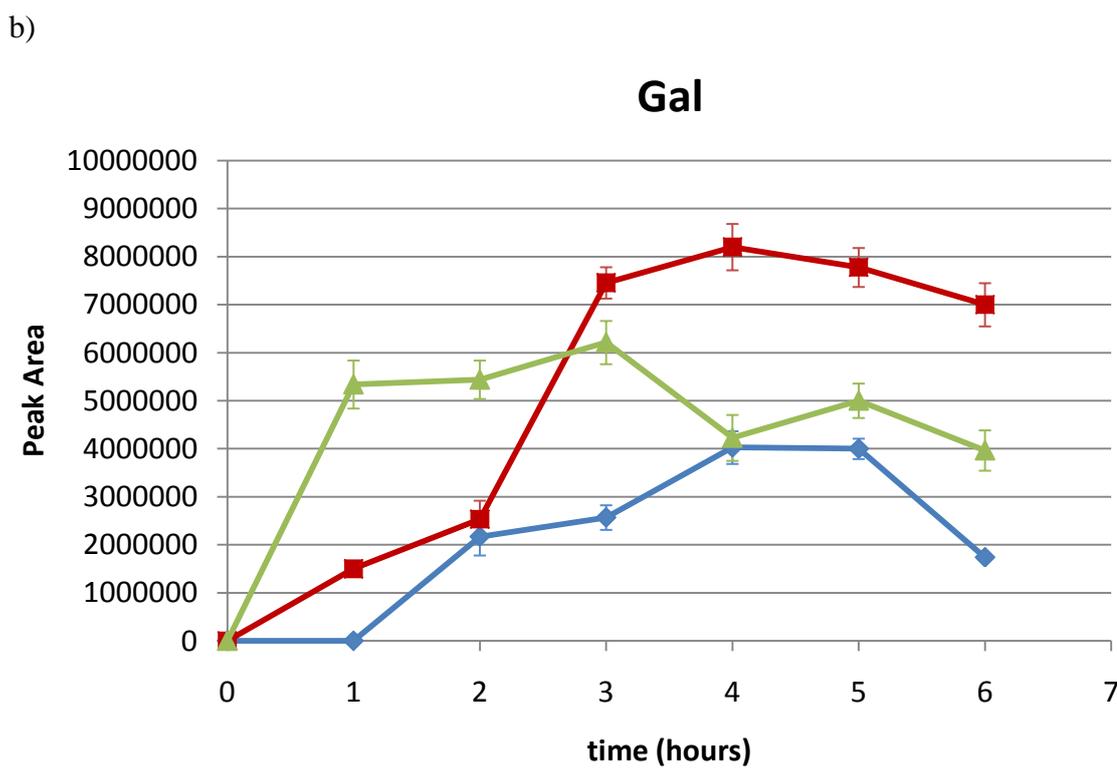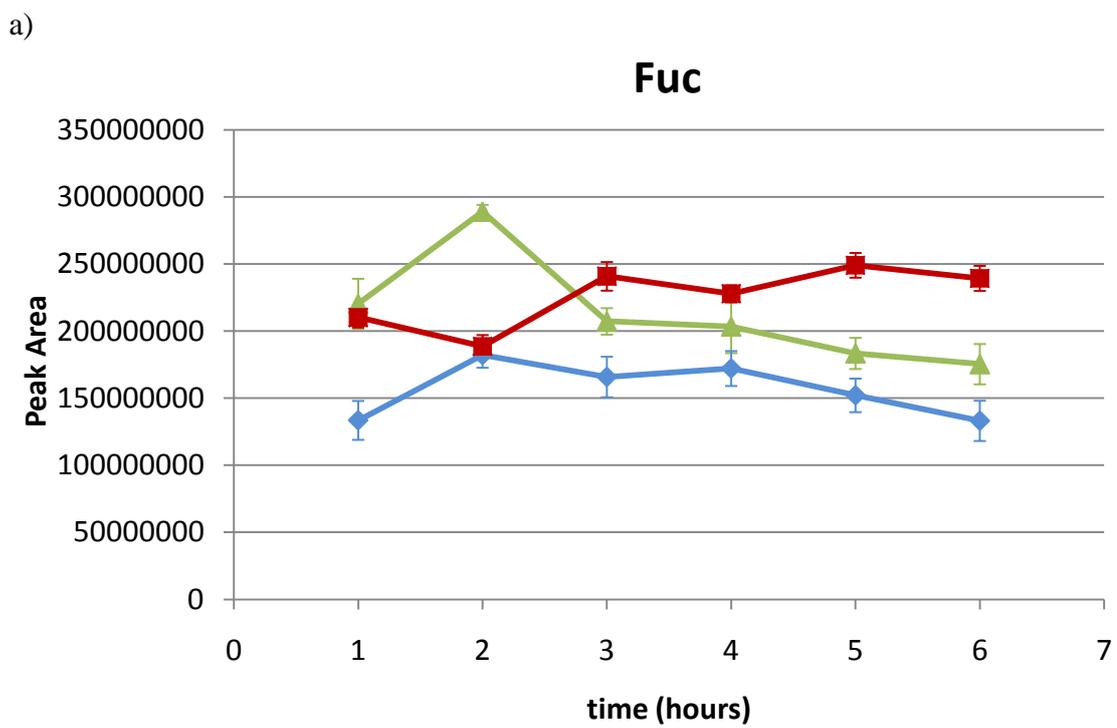
Figure 3.2. **Release of monosaccharides from commercial AGP.**

a) Fuc and b) Gal over 6h when incubated with:

TFA ━■━ HCl ━◆━ TFA and HCl ━▲━

c)

Man

d)

GlcNAc
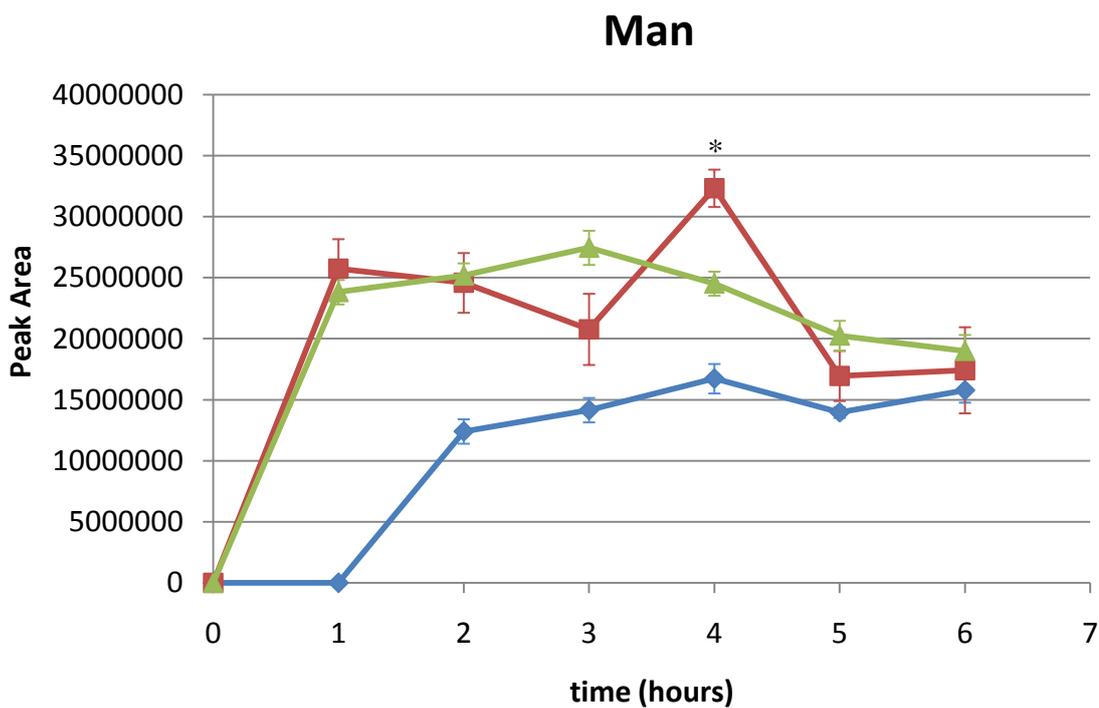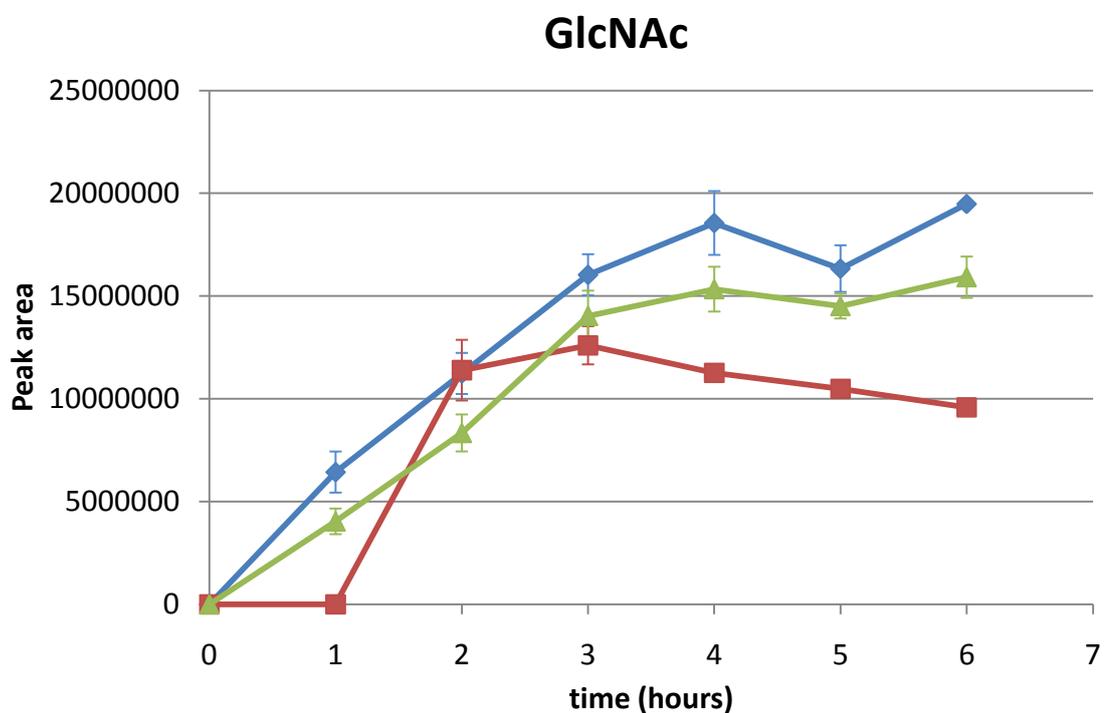
Figure 3.2 continued.   Release of monosaccharides from commercial AGP.

d) Man and d) GlcN over 6h when incubated with;

■─TFA   ◆─HCl   ▲─TFA and HCl

3.3.2 Microtitre plate drug binding assay

The excitation of AGP at 280nm generates a fluorescence emission spectra between 300-400nm, as illustrated in figure 3.3a, with the maximum fluorescence occurring at approximately 340nm. When STI571 - a drug known to bind AGP (Jørgensen *et al.*, 2002) was introduced to commercial AGP at increasing concentrations, the bell-shaped curve was found to reduce in height (figure 3.3b); the fluorescence was quenched. Conversely, when a drug was introduced which did not bind AGP, the spectrum was not affected (figure 3.3c) hence fluorescence studies are useful in the detection of drug binding. As formerly stated, emission spectra could not be generated using the equipment available and therefore a specific 340nm filter was used to detect the maximal values; important in fluorescence-based drug binding analysis.

As shown in figure 3.4, microtitre plate assays are useful in detecting fluorescence emitted by Trp and Tyr residues of AGP, a decrease occurring when a drug becomes bound. Theophylline is a drug known to bind AGP and therefore the various concentrations allow the effect of increased drug binding on intrinsic fluorescence to be determined (refer to figure 3.4). The degree of fluorescence was markedly decreased as the drug concentration was increased thus the drug was used as a positive control throughout. All drug standards were made 10-fold more concentrated but graphed as the concentration present on the plate i.e. 2.5-250μM. The values were plotted against theophylline concentration (figure 3.4) to show the reduction in fluorescence which occurred with drug binding. When the microtitre plate method was repeated using methadone in place of theophylline, no significant effect on the fluorescence emitted by AGP was detected (figure 3.5). However upon adaptation of the method, specifically by replacing d-PBS with DMSO as the solvent used for dissolving the drug, the level of binding - as indicated by a reduction in fluorescence - was more apparent, especially at high concentrations (figure 3.6). The level of binding was not as high as that seen with theophylline, but the technique remained feasible for the analysis of methadone binding.

The organic solvent DMSO was subsequently deemed the solvent of choice in which to dissolve methadone; as described in the previous chapter (section 2.2.6.1). It is capable of dissolving both polar and nonpolar compounds and although concentrated solutions can cause oxidation of -OH groups to produce ketones, it should not affect the structure of methadone which does not have free –OH groups.

a)
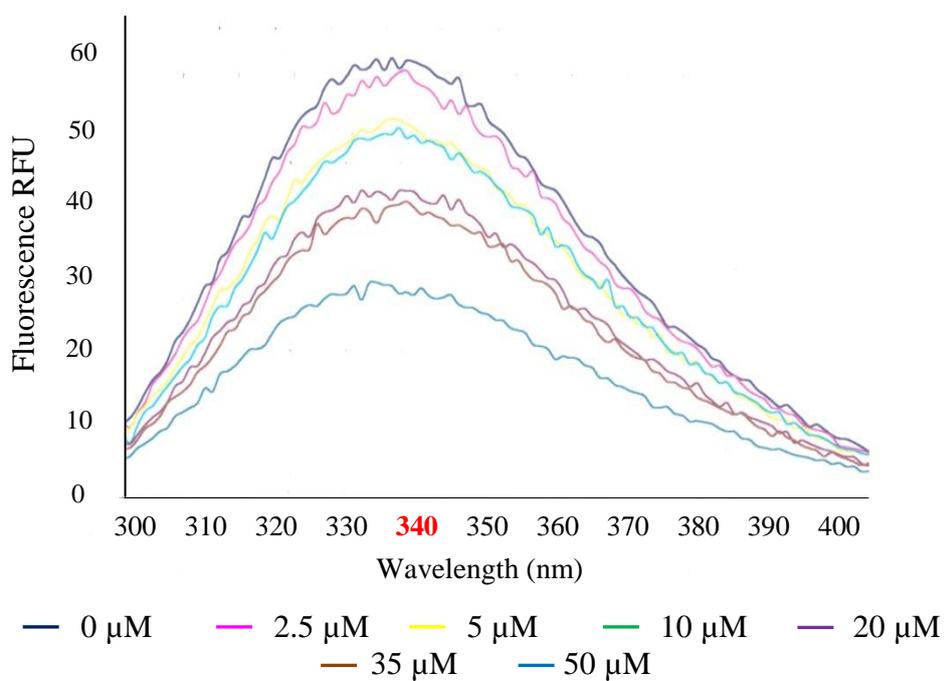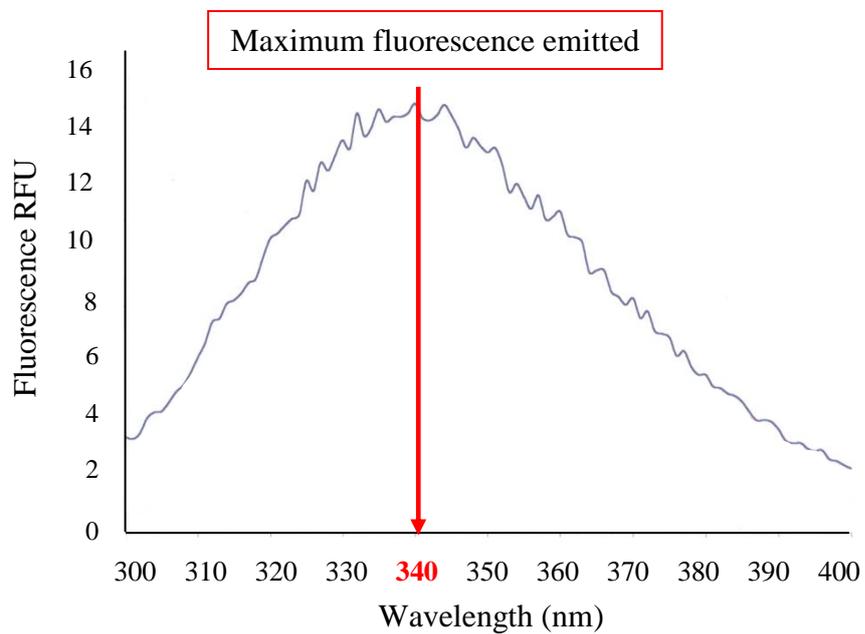
Figure 3.3. **Fluorescence spectra of commercial AGP over 300-400nm when excited at 280nm in a) the absence and b) the presence of STI571.**

c)
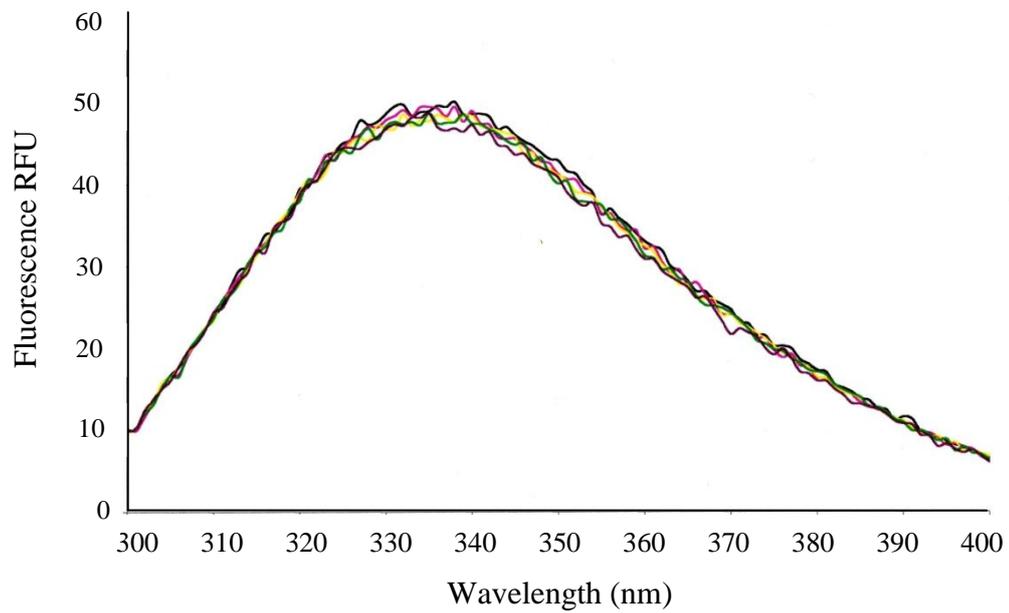
Figure 3.3 continued. **Fluorescence spectra of commercial AGP over 300-400nm when excited at 280nm in c) the presence of hydroxyurea.** [Reprinted with permission of Dr. P Mooney].

— 0 μM   — 2.5 μM   — 20 μM   — 125 μM   — 250 μM
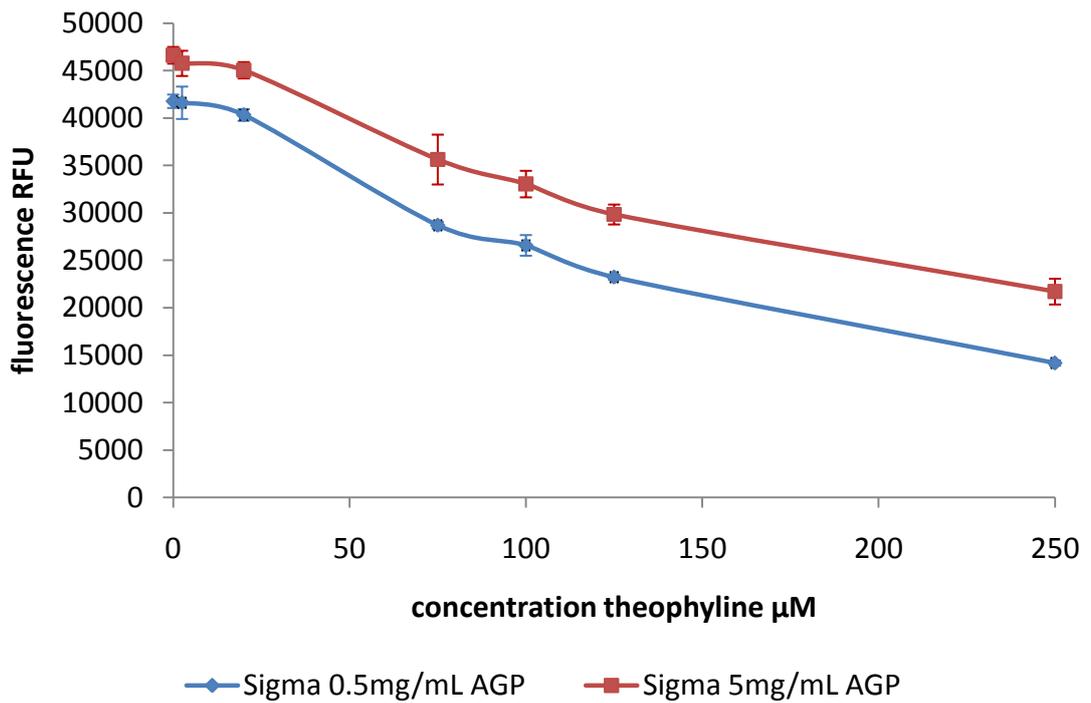
Figure 3.4. Intrinsic fluorescence at 340 nm of 5mg/mL and 0.5mg/mL commercial AGP in the presence of theophylline.
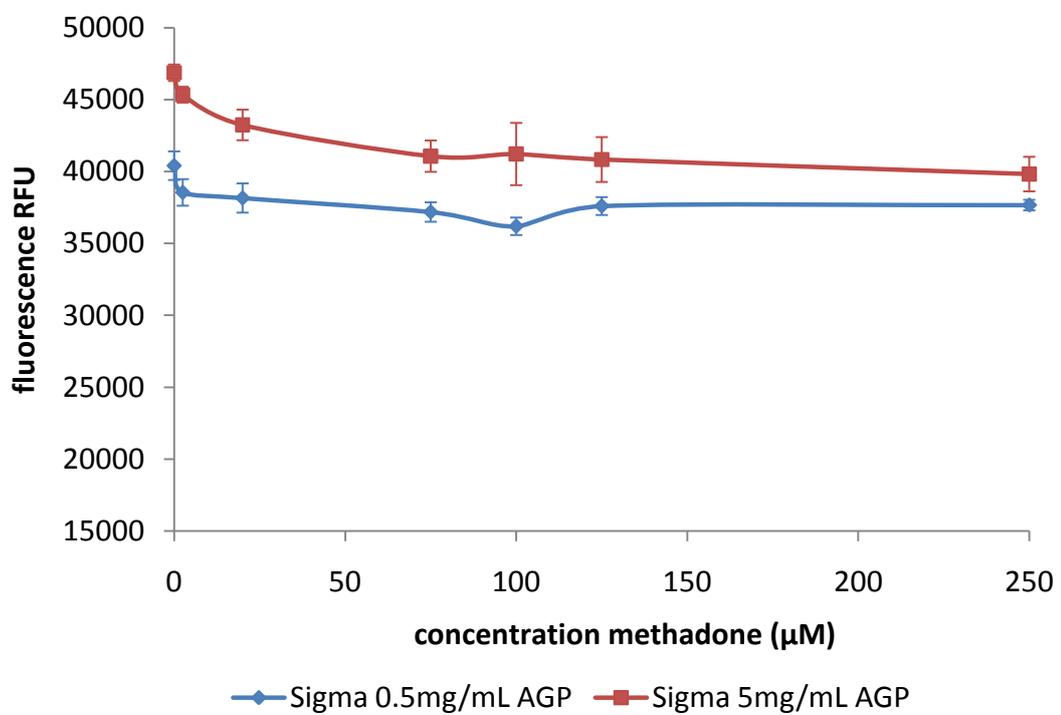
Figure 3.5. Intrinsic fluorescence at 340nm of 5mg/mL and 0.5mg/mL commercial AGP with methadone.
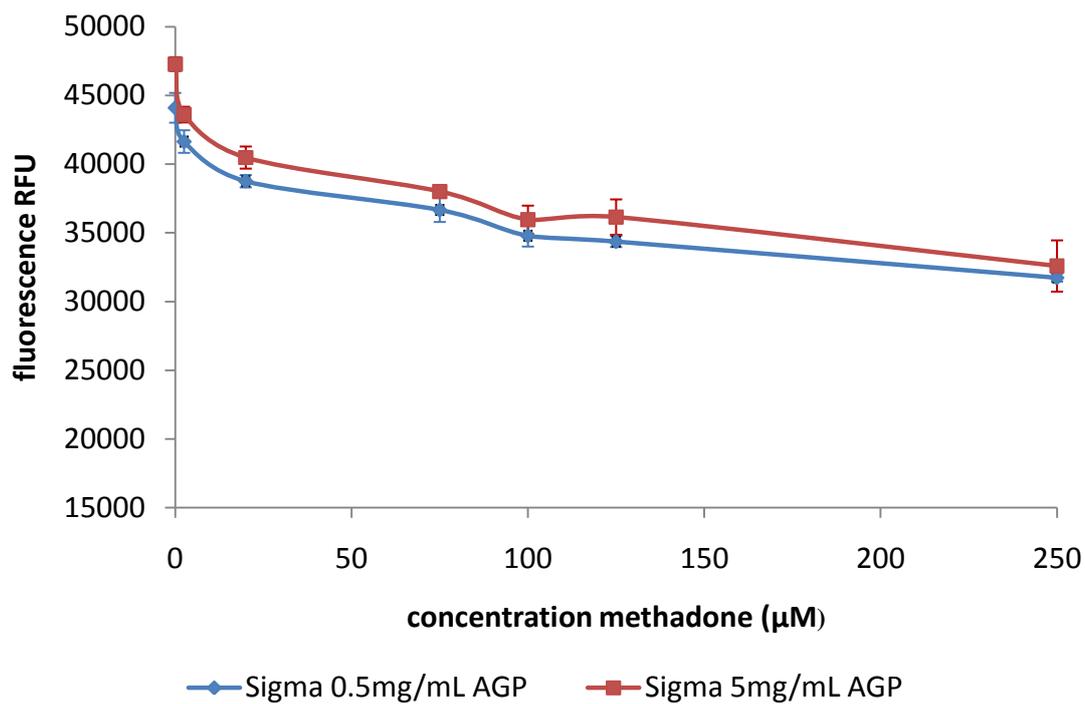
Figure 3.6. **Intrinsic fluorescence of 5mg/mL and 0.5mg/mL commercial AGP with methadone dissolved in DMSO.**

3.4   Discussion

3.4.1   *Monosaccharide release optimisation*

It is important to determine the composition of glycan chains when studying the function of AGP; their specific monosaccharide arrangements influence the overall glycoprotein structure, dynamics, and subsequently their functions. Analysis requires the identification and validation of an appropriate analytical technique i.e. acid hydrolysis. As specific monosaccharides, for example Gal, are unique to the branches of complex N-linked glycans, alterations in the monosaccharide composition can suggest changes to the branching. Confirmation requires additional analysis of complete glycans (chapter 7).

HCl cleaves the basic sugar GlcNAc (causing it to be de-acetylated to GlcN; Kim, 2000) whilst TFA is used to hydrolyse the neutral sugars Fuc, Gal and Man. In the past, TFA was used alone (e.g. Davies *et al.,* 1993; Elliott *et al.*, 1997) however it is unable to effectively cleave the basic monosaccharide GlcNAc because the monosaccharide has a higher degree of resistance to acids, the innermost GlcNAc attached to Asn residues of a polypeptide backbone particularly.

The use of HCl was introduced to provide more effective hydrolysis of this monosaccharide (French *et al.,* 2002). An earlier study performed by Fan and colleagues (1994) suggested that in combination with TFA the yield of the monosaccharides can be compromised. Therefore an experiment was undertaken within this study with commercial AGP to determine the most efficient method, it was found to compare to that described by Fan *et al.* (1994). Hardy and Townsend (1994) reported that Man in particular was destroyed with 4M HCl – as supported by this study - however in the presence of only TFA, it was effectively hydrolysed and stable, thus supporting the decision to implement separate acid hydrolysis for release of the neutral and basic monosaccharides.

Dowex® cation-exchange resins are generally used after acid hydrolysis to remove the polypeptide component of the glycoprotein; the negatively charged amino acids interact with the column and are thus removed from the monosaccharide components which

theoretically elute unhindered. Polypeptide removal is often necessary because it can interfere with the separation of monosaccharides by HPAEC-PAD; the large structures may shift the elution of monosaccharides or even prevent their resolution by eluting at the same time – particularly possible with Fuc which elutes early - rendering it difficult to determine the monosaccharide composition. During the present study, the level of GlcN collected after this stage – using commercial AGP samples - was lower than expected. To determine whether the 2mL of HPLC water used to wash the sample through the column was insufficient to elute all GlcN, a further 2mL was added and collected. However, this was not found to generate any further GlcN detected by HPAEC-PAD. It was postulated that unlike the neutral monosaccharides, GlcN may interact with the column due to its negative charge. As described above, upon removal of the column, the level of GlcN was significantly improved which was in agreement with Fan *et al* (1994).

Although it had often been reported that the level of GlcN detected by HPAEC-PAD after hydrolysis with TFA and HCl alone, or in combination, was significantly lower than that expected and a correction factor was sometimes applied, the possibility that the Dowex[®] column could reduce the levels of GlcN made available to the HPAEC-PAD detector was not considered and therefore its use continued (Smith *et al.,* 2002; Mooney *et al.,* 2006).

Interestingly, the removal of the Dowex[®] column did not significantly affect the resolution of the neutral monosaccharides; further supporting the idea that only GlcN interacted with the column. Furthermore, the pH of the Dowex[®] was taken upon each use to ensure a pH of 4 was maintained therefore it was never overly acidic so could not cause an unusual increase in sugar degradation. Finally, the detectors of the HPAEC equipment were cleaned to ensure the detection system was not at fault i.e. causing the low GlcN data. Fluctuations were minimal with no clear differences to that obtained before cleaning.

Therefore Dowex[®] cation-exchange columns were only implemented for TFA hydrolysed samples to ensure optimal generation of neutral monosaccharides, removing the protein which potentially complicates and confuses chromatograms. It was not used for the HCl hydrolysed samples to improve GlcN resolution – its retention is late on in chromatographic separation therefore any peptide present should not elute alongside.

Even if the retention time was prolonged, that of the internal standard would be similarly affected therefore the peak would still be identifiable. Despite requiring twice the mass of AGP to perform analysis of the monosaccharide composition, the enhanced effectiveness could not be ignored. This was especially true when considering the significantly improved levels of Gal and GlcN obtained (Man or Fuc were increased but statistically not significant). These two monosaccharides are present in glycan branches and thus provide useful evidence of structural changes. Gal is only present in branches and therefore it was important to ensure the separation and hydrolysis of this monosaccharide was as effective as possible. Although GlcN can provide similar information, it is also present in the pentasaccharide core and therefore it must be analysed with caution alongside Gal levels and oligosaccharide analysis. The innermost GlcNAc attached to the peptide backbone is difficult to hydrolyse as the bond is very strong (Fan *et al.,* 1994) thus higher levels in some patients are not necessarily correlated with branching but instead perhaps more efficient cleavage of the core GlcNAc residues. If an elevation coincides with that of Gal levels then it is probable that branching has increased.

In summary, it has been found that the use of a cation-exchange column, although important when analysing neutral monosaccharides, affects the level of GlcN subsequently detected by the HPAEC equipment. Therefore, for the monosaccharide analysis of AGP samples, separate hydrolytic conditions were implemented. Using these methods in the study may give an indication of, but does not definitively allow, the degree of branching of glycans attached to the AGP protein backbone to be determined however in chapter 7, the analysis of complete oligosaccharide chains is described and allows qualitative estimation of glycan branching.

3.4.2   *Drug binding analysis*

Although a more in-depth discussion will follow in chapter 8, when reporting the binding of methadone to patient AGP samples, a short discussion is necessary in order to explain why the particular assay was chosen.

All drugs must attain a specific level at their site of action in order to exert their pharmacological effect (minimal effective concentration, MEC). It is well documented

that, at physiological pH, AGP is capable of binding basic and neutral drugs with a high affinity and low capacity (Fournier, 2000; Israili and Dayton, 2001). When a drug becomes bound and therefore rendered inactive, the level present may fall below the MEC. If the effect is widespread the drug subsequently loses its ability to provide the desired pharmacological effect.

As methadone is a basic drug, it binds AGP (Kremer *et al.,* 1988; Rodriguez-Rosas *et al.,* 2003). However, whether the glycosylation of this glycoprotein affects the degree of binding and subsequent level of free active drug available, has remained largely unstudied in patients undergoing opioid replacement therapy. Analyses such as those detailed in this study are limited by the availability of sample thus performing large-scale techniques to analyse drug binding was unfeasible (even the cuvette-scale requires 10 fold more reactants), especially as samples were required for structural analysis. Therefore, the use of a microtitre plate method was useful. It was first described by Parikh and colleagues (2000) as a valuable mechanism for drug binding analysis; detecting alterations in intrinsic fluorescence. Essentially, as a drug becomes bound, the underlying Trp and Tyr residues which fluoresce when excited at 280nm are masked and therefore the fluorescence is decreased (quenched).

The studies with commercial AGP indicated that 5mg/mL AGP allowed the effects of drug binding on fluorescence to be distinguished more clearly than in the presence of 0.5mg/mL. Initially, the methodology was tested using theophylline (results graphed in figure 3.4) as its binding to AGP has been well documented (Parikh *et al.,* 2000) and so allowed the validity of the experiment to be tested. Despite this, restrictions meant the lower concentration was used in all subsequent analyses; higher concentrations were impractical. Nevertheless, 0.5mg/mL commercial AGP responded to the presence of increased concentrations of drug (theophylline and methadone) with a decreased fluorescence (figures 3.4 and 3.6 respectively). It was unsurprising as an increase in concentration renders more drug available to the binding sites therefore their occupation was enhanced thus masking increased Trp and Tyr residues. As methadone became increasingly concentrated the degree of quenching slowed – the availability of binding sites must have become limited.

Overall it can be seen that the microtitre plate method was useful in intrinsic fluorescence analysis, as reported in previous studies (e.g. Johnson and Smith, 2006)

providing an indirect indication of drug binding when samples are low in supply. Chapter 8 discusses in detail why methadone may not be able to quench the intrinsic fluorescence of AGP to the same extent as theophylline.

SUMMARY STATEMENT

It was shown that separate hydrolysis of isolated AGP with 2M TFA and 4M HCl was necessary to ensure effective glycan structural analysis, which is important in studies such as this investigating whether altered glycosylation may affect the function of a glycoprotein. Therefore these conditions were adopted throughout. Additionally, drug binding studies implementing fluorescence at the microtitre plate level were shown to be useful.

Chapter 4

Patient demographics

4.1   Patient Demographics

A trained phlebotomist obtained blood samples from consenting individuals who were enrolled in different stages or types of opioid replacement therapy at the Community Drug Problem Service (CDPS) in Edinburgh, Scotland. The subjects were grouped by type and stage of therapy (table 4.1) including those having recently become enrolled in the initial two week Titration phase of the program receiving methadone (n=5), those attending the Harm Reduction program having received maintenance doses for less than 1 year (n=4) and finally those who have been stable on methadone therapy for prolonged periods of time thus attending the clinic only occasionally (n=11). Patients receiving alternative treatments were also included; buprenorphine (n=4), dihydrocodeine (n=1) and heroin (n=1). Methadone was prescribed at the clinic as a solution with a 1mg/mL or 2mg/mL concentration.

The dose of opioid substitute administered to each patient, additionally prescribed medications and recent recreational drug use (within last week) was noted and summarised in table 4.1. New identities were assigned to the blood samples to correspond with the drug therapy they were receiving, reducing confusion in the subsequent chapters. Eighteen male and eight female volunteers were recruited into the study, ranging from 20 to 51 years of age with a mean ± standard deviation of 33.0 ± 7.0 (33.5 ± 6.7 and 31.9 ± 7.0 for males and females respectively) − refer to section 2.1.1.

Blood samples were also obtained from the Blood Transfusion Service; these consisted of a mixture of blood from a healthy 'normal' population, hence no individual patient data was available. 'Normal' serum samples were provided by Professor David George of the Glasgow Western Infirmary. These samples were taken from a cancer-free elderly female population resident in the West of Scotland. No other medical information specific to each individual was provided.

Patient Number	Stage/type of therapy	Dose (mg)	Additional prescribed drugs	Recent Recreational drug use	New Identity
JB 0608 01	Titration (T)	95	*	Heroin – smoke	**T1**
JB 0608 02		65	-	Heroin	**T2**
JB 0509 01		55	-		**T3**
JB 0509 05		40	Diazepam		**T4**
JB 0509 06		60	-		**T5**
JB 0608 03	Harm Reduction (HR)	90	*	Heroin	**HR1**
JB 0608 04		85	*	Heroin	**HR2**
JB 0608 05		150	*	Heroin	**HR3**
JB 0608 06		150	-	-	**HR4**
JB 0608 07	Long Term (LT)	95	Diazepam	Cocaine MDMA, Speed	**LT1**
JB 0608 08		150	-	-	**LT2**
JB 0608 10		40	Mirtazapine, Statin	Chlorpromazine	**LT3**
JB 0608 12		90	Diazepam	Amitryptline	**LT4**
JB 0509 03		105	Diazepam	Heroin, Crack cocaine	**LT5**
JB 0509 04		85	-	-	**LT6**
JB 0509 09		150	Diazepam	Prozac	**LT7**
JB 0509 10		50	Diazepam	Cannabis	**LT8**
JB 0509 11		150	Diazepam	Cannabis	**LT9**
JB 0509 12		200	Diazepam	-	**LT10**
JB 0509 14		90	Diazepam	-	**LT11**
JB 0608 09	Buprenorphine (B)	1.6	Diazepam, Chlorpromazine	-	**B1**
JB 0608 11		10	Mirtazapine, Amitryptiline	-	**B2**
JB 0509 02		6	Diazepam, Mirtazapine	Cannabis	**B3**
JB 0509 08		6	Diazepam, Mirtazapine	-	**B4**
JB 0509 07	Dihydrocodeine (D)	60	Amitryptiline, Olanzepine, Depixole	-	**D1**
JB 0509 13	Heroin (H)	*	*	*	**H1**

Table 4.1.   Patient data

T: Titration phase; HR: Harm Reduction; LT: Long-term methadone;

B: Buprenorphine; D: Dihydrocodeine; H: Heroin.

M/F: Male/Female; *   Data was unavailable; -   Nothing to declare

It could be seen from table 4.1 that a large number of patients undergoing opioid-replacement therapy were co-prescribed the benzodiazepine diazepam (n=12). The actual number may have been greater but data was not available for all patients. In terms of the study group for which prescribed drug data was available, this equated to 57.1% of patients. Interestingly, almost three quarters of LT patients were receiving diazepam alongside methadone (~72.7%). Mirtazapine – a tricyclic antidepressant (TCA) - was prescribed in four patients, three of which were receiving buprenorphine as the substitute opioid. Another TCA, amitryptiline, was prescribed to two individuals.

4.2   Discussion

As discussed with some depth in section 1.5.1, the identification of effective treatment strategies is central to addressing the huge problem opioid dependence poses not only to the UK but worldwide. A recent Scottish Government study (SACDM, 2007) used information provided by the country's NHS boards to illustrate that there has been an increase in GP-based patient treatment; approximately 22,224 (including 1,093 in prison) were receiving substitute therapy – an increase of 1,904 on the figures in 2004 (19,227 - not including prisoners).

As reported in table 4.1, not all patients recruited into this study were receiving methadone as the substitute treatment, instead four received buprenorphine, one dihydrocodeine and another individual was prescribed pure heroin. Generally, alternatives are only considered when methadone has proved unsuccessful in aiding an individual to abstain from illicit heroin use. Initially it was hoped to focus sample collection to the long-term and titration methadone groups however this was virtually impossible, certainly for the latter group where recruitment was hindered by their apprehension. Therefore it was decided an interesting alternative for this preliminary study was to recruit patients undergoing therapy at the clinic with the alternative drugs. Limitations put in place by the NHS ethics board regarding the number and volume of blood samples rendered it difficult to recruit a larger number of individuals in each category.

A report by Strang *et al.* (2005) indicated that DHC is the most commonly prescribed drug after methadone in the UK although the number of studies and trials undertaken with the drug are few. As for patient D1, it is generally implemented if methadone is unsuccessful. Within the four year period of 1995-1999, prescriptions for DHC increased by 11% in the West of Scotland alone. It has been shown that the sedative effects and dangers of toxicity associated with methadone mean patients would often prefer the shorter acting DHC (Robertson *et al.,* 2006). Within the same period of the late 1990s, an increase in the number of DHC-related deaths was also reported; 55% of deaths in a study population of 89 individuals were a direct result of DHC use (Seymour *et al.,* 2001; Power *et al.,* 2002).

Buprenorphine is a partial opioid agonist which is demonstrating increased usage and competes with full agonists like heroin for the opioid receptor. It carries a decreased risk of overdose due to its longer $t_{1/2}$ and helps achieve stabilisation of neurobiological pathways (Auriacombe *et al.,* 2001). Efficacy can therefore be maintained with less frequent dosing than methadone, as reported by patients B1-B4 in this study. The bioavailability of buprenorphine is lower than methadone with much of it lost during first pass metabolism therefore it is administered sublingually rather than orally, allowing diffusion into the tissues under the tongue. Therefore, lower doses of buprenorphine are required in comparison to methadone, as demonstrated in table 4.1.

It can potentially increase the number of patients seen by a single clinic as the number of visits required is decreased (making it more convenient for the patient) and the need for take-home doses is lessened. Accumulation of the drug should also be less likely. At high doses the euphoric effects are low thus it has potential in the long-term treatment of opioid dependence as a substitute but also in the short-term management of withdrawal (Walsh and Eissenberg, 2003). In France it has been used as a substitute therapy since 1996 with less monitoring required than that associated with methadone therapy because it is only available from specialised centres. It does however carry a greater risk of adverse effects and diversion than methadone. In 2001, Auriacombe and co-workers estimated that there were 1.4 times more buprenorphine-related deaths in France during 1994-1998 than those due to methadone however, to put this in perspective, 14 times more patients received buprenorphine than methadone. It was found in the current study that 75% of the patients receiving buprenorphine were also prescribed diazepam which was similar to those having received methadone long-term however only four patients were recruited from this treatment group.

Despite the widespread recreational use of impure heroin, research has shown the potential of pure heroin as a means of treating illicit heroin use. Studies performed in Switzerland (Rehm *et al.,* 2001) suggested 70% of individuals treated with pure injectable heroin can be retained on the treatment over a year which may make it a useful alternative treatment in those where MMT is unsuccessful, as was the case for patient H1. It was found that this heroin-assisted treatment was more effective than methadone and a feasible treatment for reducing physical, mental and social problems. However, the research did not account for any psychological treatment received by patients. A more recent German study (Haasen *et al.,* 2007) subsequently examined the

effectiveness of medically prescribed heroin and supervised heroin injection in an open-label randomised controlled trial while addressing the problems identified in the Swiss study. Some of the participants had not responded sufficiently to MMT and others were not in a drug misuse program. It was found that heroin-assisted treatment of people with severe opioid dependence and treatment resistance, had significantly improved health and decreased illicit drug use than MMT individuals, supporting the results seen by previous studies like Rehm *et al.* (2001) and Perneger *et al.* (1998). Pilot studies undertaken in England and the Netherlands indicated the combined prescription of pure heroin and methadone could provide a 25% improvement in physical and mental health compared to methadone alone (SACDM, 2007). Recently news headlines indicated the Scottish government have been contemplating the introduction of pure heroin treatment and clinics where users can inject heroin safely.

As can be seen from the tables in section 4.1, despite receiving substitute treatment, patients are often discovered to continue a degree of illicit heroin drug use, certainly in the earlier stages (T and HR). Such behaviour is partly expected due to the low doses of methadone administered during the initial titration phase while treatment teams determine the degree of tolerance possessed by a patient - the doses are rarely sufficient to prevent the onset of withdrawal symptoms. However it is important that treatment is administered in this manner and patients are closely monitored because - although the effects of methadone take a number of days to be expressed - the drug slowly accumulates in tissues therefore additional opioids can exacerbate its effect potentially causing overdose.

Heroin addicts are also commonly reported to co-abuse BDZs, particularly flunitrazepam (Woods and Winger, 1997; Peles *et al.,* 2007) probably due to their anti-anxiolytic properties; individuals often become extremely anxious as part of withdrawal. In one study 35-41% of patients undergoing MMT reported use of BDZs three or more times a week (DuPont, 1988). Therefore, when undergoing substitute therapy patients are often prescribed a BDZ e.g. diazepam in an attempt to treat both addictions; unless the patient is pregnant in which case diazepam should not be administered as it is known to cause birth defects (Laegreid *et al.,* 1992). Similar treatment regimens with both a substitute opioid and a BDZ were recorded for the patients recruited in this study. More than half the patients, for which data was available, were prescribed diazepam, the majority receiving approximately 5mg along

with their daily methadone dose. As a BDZ, it enhances the activity of γ-aminobutyric acid (GABA), causing sedative effects which help stabilise the patient's sleep patterns and – alongside other drugs including the anti-psychotics chlorpromazine and olanzapine - it has anxiolytic properties. Darke and Ross (1997) administered the BDZ diazepam (10 or 20mg per 70kg) to patients on MMT and found that their subsequent methadone consumption was reduced, supporting earlier research by Preston *et al.* (1984) where a combination of diazepam and methadone enhanced the opioid effects. Thus less opioid was required to achieve similar effects as that previously attained with higher doses of methadone alone. When comparing the patients in the current study there was no clear reduced dosing in those co-prescribed diazepam.

The TCAs, co-prescribed in some patients, are normally given to treat mood-disorders such as depression. Euphoria is not the only effect produced by heroin, like other opioids and the BDZs, its administration is correlated with the occurrence of depression in frequent users (Havard *et al.,* 2006) perhaps explaining why certain individuals require a TCA alongside their opioid substitute.

While the current study was interested in the possibility the efficacy of methadone was affected by the presence and structure of a specific protein in the blood, it was only possible to compare patients receiving opioid-replacement therapy to a 'normal' healthy population. A useful comparative group would be heroin dependent individuals whom are not receiving therapy to determine whether any effects detected in the patients were a direct result of therapy or perhaps due to underlying stresses associated with the prior illicit drug use. This was impossible in the current study. Instead the structure and level of AGP isolated from patient blood was analysed, its ability to bind methadone determined and compared to all other patients and a 'normal' healthy population. Results pertaining to these studies are described in the following chapters.

SUMMARY STATEMENT

Numerous opioids can be utilised as substitutes in the treatment of opioid dependencies. Methadone is the most commonly prescribed in the UK with huge variability in doses required to prevent withdrawal symptoms. Increasing our understanding as to why methadone may be less effective in certain individuals is important and investigated further in the following chapters.

Chapter 5

AGP isolation and level determination

Results and Discussion

5.1   Introduction

5.1.1   Isolation of AGP

Prior to the 1980s it was commonplace to disregard sugar components during investigations into the functions performed by glycoproteins - instead interest was focussed on the underlying polypeptides which were more easily analysed. It is now well established that glycans are vital in the determination of overall structure and subsequent function of these biomolecules, often uniquely altered during some physiological and pathophysiological states. Thus it is not only the hepatic synthesis of AGP which is augmented during the APR. Consequently, a prerequisite of studies investigating the structure and subsequent roles of glycoproteins like AGP is that their *in vivo* glycan structures must remain uncompromised during isolation from other plasma proteins.

Despite the availability of a number of well documented isolation techniques developed for proteins and glycoproteins, the majority require harsh acidic conditions thereby causing a loss of glycan structural integrity - desialylation and other forms of degradation common under acidic conditions. In the first reported methods for AGP isolation, described by Schmid (1950) and Weimer *et al.* (1950), ammonium sulphate was used to precipitate AGP at low pH. The first large-scale fractionation of AGP-containing Cohn Fraction V supernatant, described by Hao and Wickerhauser (1973) also implemented acidic conditions to promote the removal of HSA. AGP became bound to diethylaminoethyl (DEAE)-Sephadex and the DEAE elate was then introduced to a carboxymethyl (CM)-cellulose column to allow separation of the Cohn Fraction V by ion-exchange chromatography. Before these techniques, AGP had often been contaminated with HSA, its removal is therefore important especially as HSA generally represents the main proportion of plasma proteins - over 50% (by weight) - thus complicating isolation of other proteins, especially if they share similar MW or charge.

In 1978 Plancke *et al.* implemented ion-displacement chromatography, gel filtration chromatography and ion-exchange chromatography to purify AGP, a method later repeated by Albani *et al.* (2000). In the preparation of the glycopeptides from AGP, the

samples were in brief contact with HCl. Therefore to ensure the structure remained unchanged, the use of methods similar to those cited was discouraged.

Methods like those utilised in this study where AGP isolation and purification was undertaken without the use of acids, were previously employed in the mid 1980s by Laurent and colleagues (1984), using a three-stage purification procedure; Cibacron Blue F3GA and Procion Red HE3B pseudo-ligand chromatography columns followed by isoelectric focusing (IEF). HSA was removed through strong interactions formed by its bilirubin binding sites to the Cibacron Blue resin. It had previously been suggested by Wilson and colleagues (1976) that weak interactions were formed with hydrophobic fatty acid-anion-binding sites or dinucleotide-binding domains. IgA, IgG, ceruloplasmin, most transferrin and prealbumin were then removed during passage through the Procion Red column with NaOAc buffer. Any remaining contaminants were eliminated by isoelectric focussing (IEF) to ensure the final 88% AGP yield obtained was of enhanced purity as indicated by gel electrophoresis and immunoelectrophoresis. ConA crossed affinity immunoelectrophoresis (CAIE) demonstrated that the glycans remained intact with no loss of structural integrity - probably because acids were not required.

Both Cibacron blue and Procion Red sepharose columns were fundamental to the methodology reported by Smith and colleagues (1994). However, eluent from the Blue column was passed through a Q-sepharose column before its introduction to Red sepharose in an attempt to improve the purity of isolated AGP. The intermediary Q-sepharose column removed α_1-antitrypsin (AAT) by anion-exchange chromatography thus reducing potential contamination. They considered this a useful alternative to the Laurent method, the results of which they were unable to reproduce. Purity was demonstrated through the use of SDS-PAGE and immunodiffusion; indicating the presence of AGP at each stage and the efficiency of the purification process.

Therefore, in terms of glycan analysis, isolation through low pressure chromatography has proven efficiency; the absence of acidic conditions prevents any structural degradation. The levels of isolated AGP can then be determined; values thus representing only the glycoprotein, not other contaminants. Although other methodologies may provide a greater yield, they also risk altering the glycosylation of

AGP, rendering them useless when glycan structure is the primary focus of studies such as that detailed herein.

5.1.2 Level determination

It is well understood that the hepatic production of AGP, a positive acute phase protein (APP), increases during the APR. The levels present under 'normal' disease-free states can rise 2-5 fold during conditions such as rheumatoid arthritis and cancer (Fournier *et al.,* 2000).

In order to perform comparative studies of the structure of AGP between different groups of individuals, it was important to determine the level present in an isolated sample. The volume required to ensure mass consistency in each analysis could then be determined. If not, it would be impossible to ascertain whether variations were structural or due to disparity in the level of AGP between samples.

There are numerous techniques utilised in the determination of AGP concentration. Commonly an immunoturbimetric assay technique was used by the group (French *et al.,* 2002) which relies on the spectrophotometric measurement of the formation of an immunoprecipitate with AGP antiserum at 340nm. However, these studies were indulged by the availability of large volumes of sample which could be distributed to other groups for the relevant analyses. Alternative means were sought in the current study to accommodate the sample availability. It was shown that absorbance of AGP is dependent on the Beer Lambert Law (Albani, 2004) thus absorbance values of AGP standards at 280nm (primarily due to Trp residues) can be used to produce standard curves and determine the relative level of AGP isolated from blood samples.

5.2 Results

5.2.1 Isolation and purification of AGP from samples

For the purpose of this study, an adapted form of the large-scale low pressure chromatography procedure established by Smith *et al.* (1994) to isolate and purify AGP from patient blood samples was used, thereby conserving structural integrity through the absence of acidic buffers. Cibacron Blue 3GA and Procion Red CL6B sepharose columns were used but the Q-sepharose intermediary step was removed to minimise loss of AGP. SDS-PAGE and Western Blots were performed to indicate that the two column technique preserved AGP purity, as reported in the following section (5.2.2).

Upon precipitation with PEG3350, AGP isolation and purification was initiated by introducing the remaining soluble proteins to a Cibacron Blue 3GA Sepharose low-pressure chromatography column. The first fraction, containing AGP, eluted after approximately 5 minutes and was illustrated by the chart recorder as a peak extending from the baseline (figure 5.1a). The fraction was collected until the baseline was retained, usually within 45- 55 minutes. Removal of HSA bound to the Cibacron Blue Sepharose beads by desorption buffer was represented by the second peak (figure 5.1a). Complete column regeneration occurred within approximately 30 minutes. The concentrated fraction collected during the previous stage was applied to the Red Sepharose CL-6B column. The first AGP-containing fraction eluted within approximately 10 minutes of application and, again, was represented by a peak (figure 5.1b) which required roughly 20 minutes to elute. Impurities such as α_1-antitrypsin (AAT) were removed from the sample, binding to the column. They were eluted upon application of a cleaning buffer and represented by the smaller peak (figure 5.1b) allowing regeneration of the column after approximately 40 minutes.

All samples were desalted and concentrated using Amicon or Centricon filter devices to prevent sample contamination and any subsequent interference with the absorbance of AGP at 280nm during spectrophotometry which was used to calculate the relative AGP level isolated from samples.
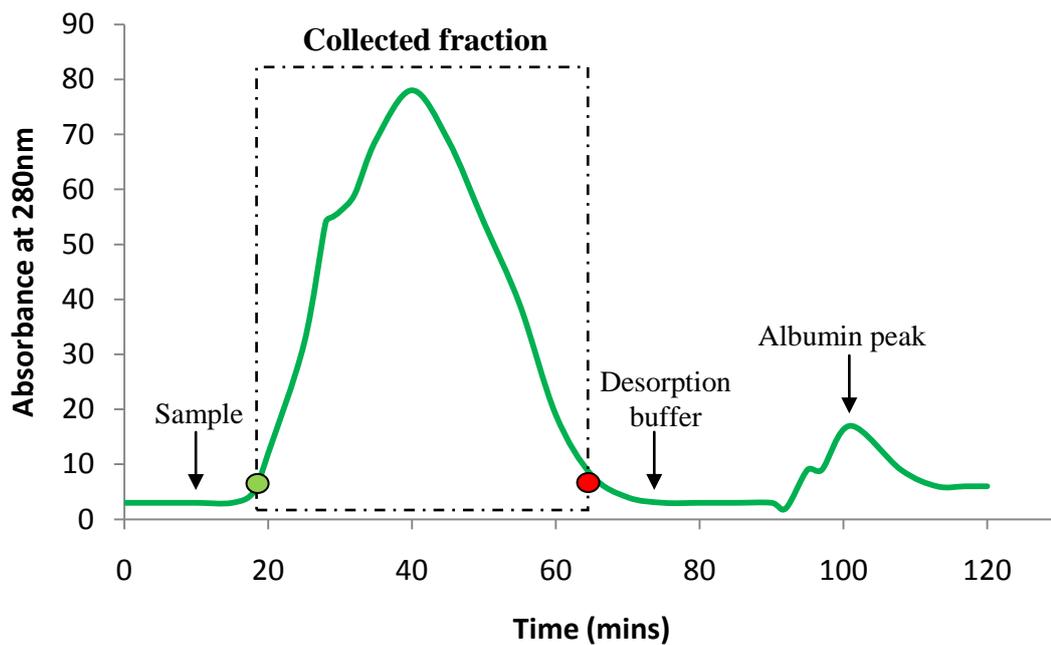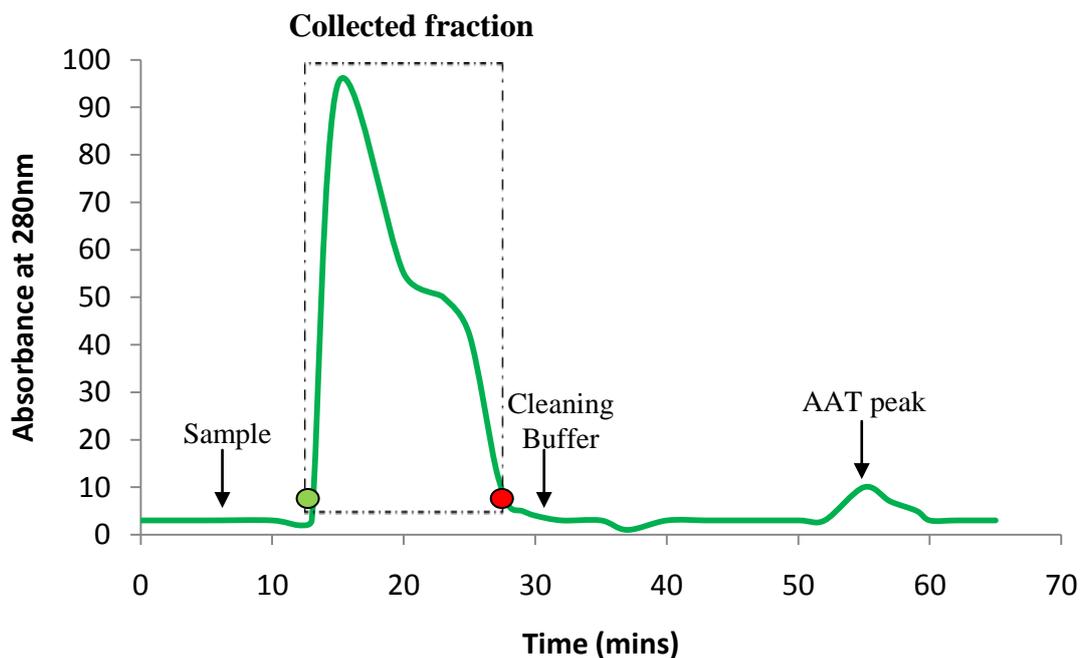
Figure 5.1. **Traces generated during the isolation of AGP by low pressure chromatography** using a Cibacron Blue Sepharose (a) and Red Sepharose (b) column.

○ collection started ● collection stopped

5.2.2 *Evaluation of isolated AGP purity*

Isolated and purified AGP was analysed using SDS-PAGE and Western Blots to determine the effectiveness of the two-column low-pressure chromatography technique. It was necessary to ensure that the adaptations made to the large-scale methodologies of Smith and colleagues (1994) did not compromise the final purity of the glycoprotein.

The Coomassie brilliant blue-stained SDS-PAGE gel and Western Blot (figure 5.2a and b respectively) indicated that PEG3350-precipitated blood samples contained numerous highly soluble proteins, demonstrated by a number of bands in lane 2. HSA, ATT and AGP are the three most common (MW 67, 52 and 41-43kDa, respectively). The inclusion of commercial AGP (lane 1) in the analysis further supported the presence of the glycoprotein in the PEG3350-precipitated and isolated samples; each lane contained a band which migrated the same distance, corresponding to the ladder marker at ~43kDa. The two isolated samples tested (which had also been desalted), appeared to be relatively pure; only one band was present in lanes 3 and 4 with no others evident. On performance of the Western Blot using anti-AGP antibody, the bands present in lanes representative of isolated and desalted samples were confirmed to be AGP.

Importantly, the SDS-PAGE performed in this study was used only to determine whether AGP was successfully isolated using the techniques of section 2.2.2.2; indicated through a reduction in the number of bands visualised when compared to the PEG-precipitated sample (lane 3). The band densities could not be used as a means of determining whether the mass was compromised during isolation. Although the same level of AGP was used for the commercial and isolated samples, it was not possible to determine the specific level of AGP present in the supernatant of PEG-precipitated protein.

Overall, the use of an SDS-PAGE and Western Blot indicated that the isolation process generated pure AGP and therefore the two-stage low pressure chromatography was a valid technique at the micro-scale. Analysis of the eluted components after each stage of isolation was deemed unnecessary as the final isolated samples showed no indication of contamination by other proteins. Also, the group recently implemented immunodiffusion to indicate that albumin was removed by the Blue sepharose column and most of the remaining contaminants like AAT, by the Procion Red column

(Gallacher, 2010). Subsequent spectophotometric analysis at 280nm, the results of which are provided in the following section, therefore allowed the level of AGP present in samples to be determined. The absence of contaminating trace proteins assured that values were not skewed.

a)

b)
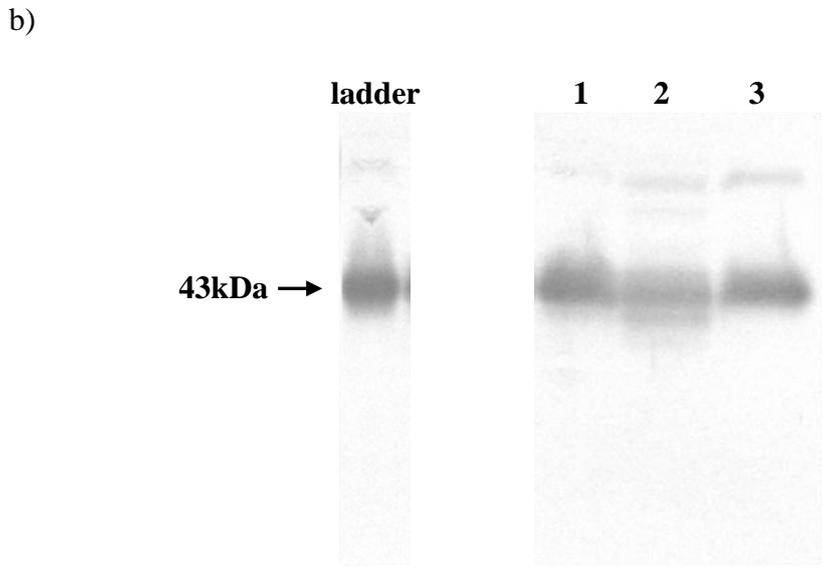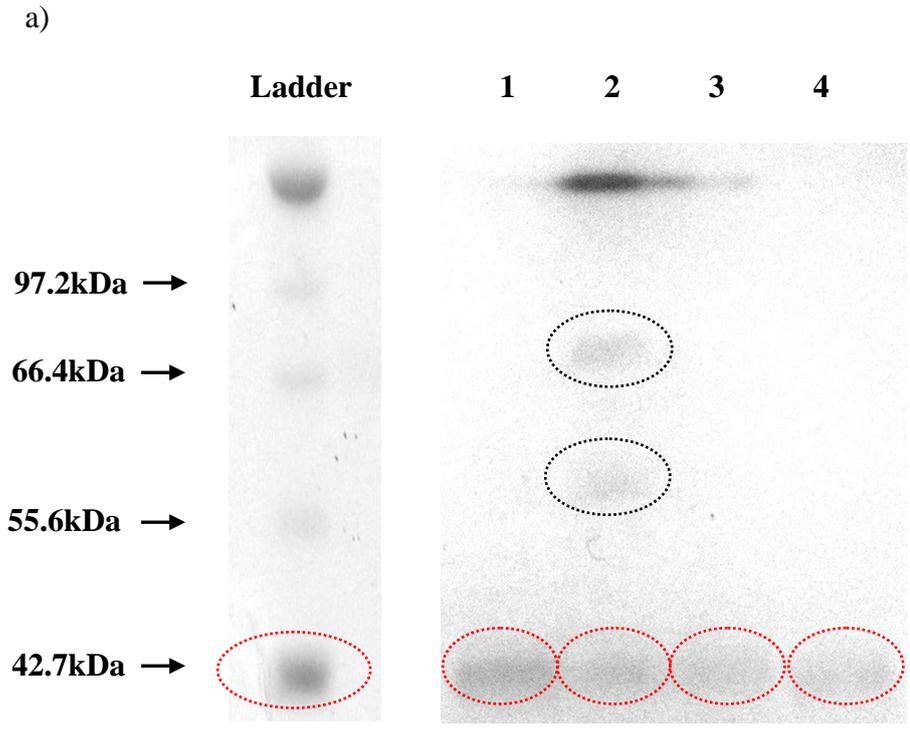
Figure 5.2. **The effectiveness of low pressure chromatography in isolating and purifying AGP** as shown by a) an SDS-PAGE gel and b) a Western Blot with anti-AGP. Ladder – ladder of MW markers, lane 1 – Commercial (Sigma) AGP positive control, Lane 2 - PEG-precipitated normal blood sample; Lane 3 - isolated AGP sample no.1; Lane 4 (SDS-PAGE only) - isolated AGP sample no.2.

5.2.3 AGP level determination

Absorbance values of the commercial AGP standards (0-3mg/mL, n=5) at 280nm, produced a line of best fit (figure 5.3) with equation $y = 0.8316x$ ($R^2 = 0.99$). Table 5.1 summarises the level of AGP isolated from normal blood samples pooled by the blood transfusion service (n=4), those from 'normal' serum samples obtained from an unknown group of elderly patients (n=4), and from each patient undergoing various stages of opioid replacement therapy (n=26). The values were reported as the sum of four 1mL blood aliquots, the mean in each aliquot and finally the difference in the level of AGP per mL of blood compared to that isolated from 'normal' blood. Samples were separated into more manageable 1mL volumes to ensure that if errors in the isolation procedure occurred, the whole sample would not be compromised. Statistical analysis of the difference in level between each patient group and a 'normal' population was undertaken with two sample t-tests.
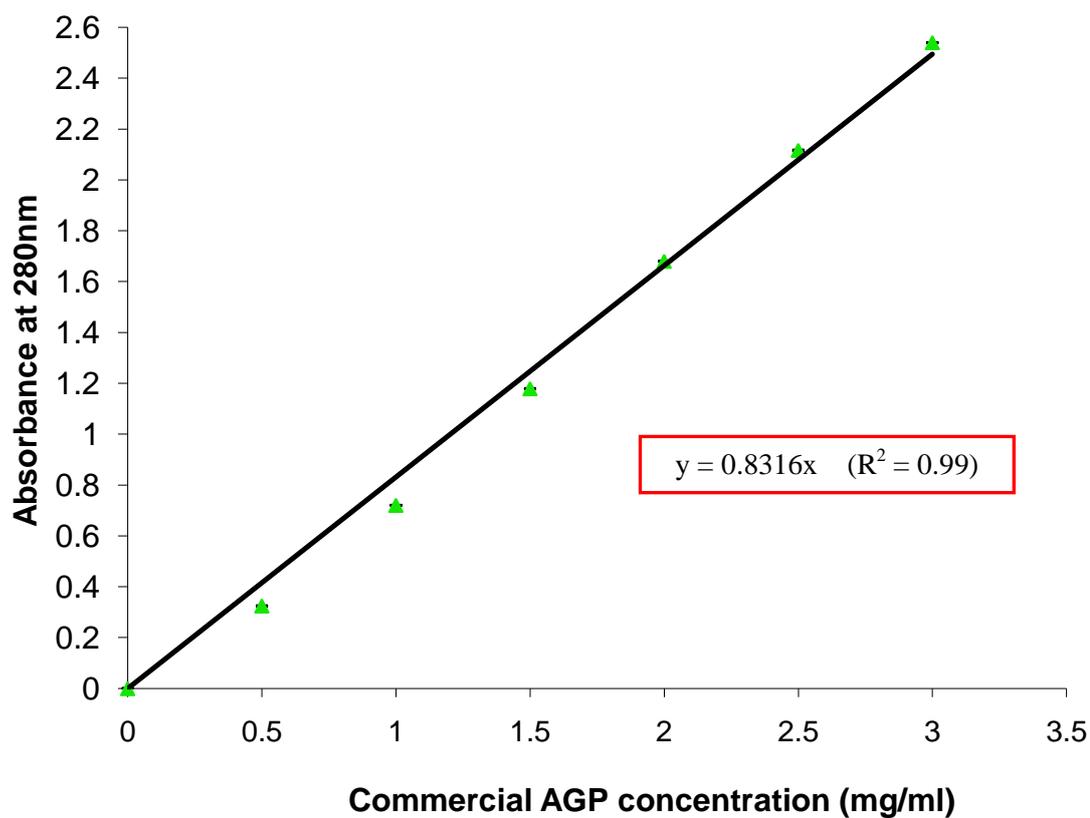
Figure 5.3. **Standard curve produced using commercial AGP** (Sigma-Aldrich) standards (n=3). $R^2>0.9$. Relative amount of AGP in a sample (x):

x = Abs at 280nm/0.8316.

Sample	Mean level of isolated AGP (mg/mL)	Level compared to 1mL normal blood
Normal pooled blood 1 ⇧	0.076	-
Normal pooled blood 2 ⇧	0.080	-
Normal pooled blood 3 ⇧	0.066	-
Normal pooled blood 4 ⇧	0.081	-
Normal pooled blood 5 ¥	0.072	-
Normal pooled blood 6 ¥	0.084	-
Normal pooled blood 7 ¥	0.086	-
MEAN normal blood	**0.078**	-
T1	0.337	+0.261
T2	0.141	+0.065
T3	0.248	+0.172
T4	0.131	+0.055
T5	0.175	+0.099
MEAN T group	**0.210***	
HR1	0.227	+0.151
HR2	0.169	+0.093
HR3	0.258	+0.182
HR4	0.143	+0.067
MEAN HR group	**0.190***	
LT1	0.152	+0.076
LT2	0.125	+0.049
LT3	0.333	+0.257
LT4	0.196	+0.120
LT5	0.208	+0.132
LT6	0.173	+0.097
LT7	0.083	+0.007
LT8	0.078	+0.002
LT9	0.172	+0.096
LT10	0.113	+0.037
LT11	0.132	+0.056
MEAN LT group	**0.163***	
B1	0.111	+0.035
B2	0.168	+0.092
B3	0.272	+0.196
B4	0.231	+0.155
MEAN B group	**0.138***	
D1	0.178	+0.102
MEAN D group	**0.178**	
H1	0.078	+0.002
MEAN H group	**0.078**	

Table 5.1.   Summary of the levels of AGP in blood samples.

*p<0.05, ⇧ isolated from same source and using same techniques as patient

samples. ¥ Data obtained from previous studies (unpublished).

The mean level of AGP in each group was also highlighted in table 5.1 and graphed in figure 5.4 to summarise the differences in expression between groups of individuals. Approximately 0.08mg/mL AGP was isolated from the healthy 'normal' blood group; this was significantly lower than that isolated from 'normal' serum. However, values derived from the blood samples were deemed the most appropriate representation of a 'normal' population when compared to that from serum and commercially sourced AGP.

The T, HR, LT and B treatment groups expressed significantly higher levels of isolated AGP than that in a 'normal' population ($p<0.05$). Recruitment of a single patient from the D and H treatment populations rendered statistical analysis somewhat uncertain as it was unknown if the values were representative of the groups as a whole. The parametric two-sample t-test could not be applied using single values. When a one-way ANOVA with Tukey's post hoc test was performed to allow all groups to be compared to one another, not just to the 'normal', the H1 and D1 individuals were included. No significant difference in the levels isolated were identified between the treatment groups ($p>0.1$), excepting H1. The level of AGP isolated from the single patient receiving heroin (0.078mg/mL) was indistinguishable from the 'normal' group (0.076mg/mL). Finally, the levels of AGP within each group were similar; no statistically significant difference was found between the patients within a single group.

In summary, the levels of AGP isolated from patients undergoing T, HR, LT and B opioid-replacement therapies were significantly higher than that from a 'normal' population but showed no significant variation from each other. Despite the indication that H1 expressed significantly lower levels of AGP than other treatment groups, a single sample was not deemed reliable enough on which to base conclusions.
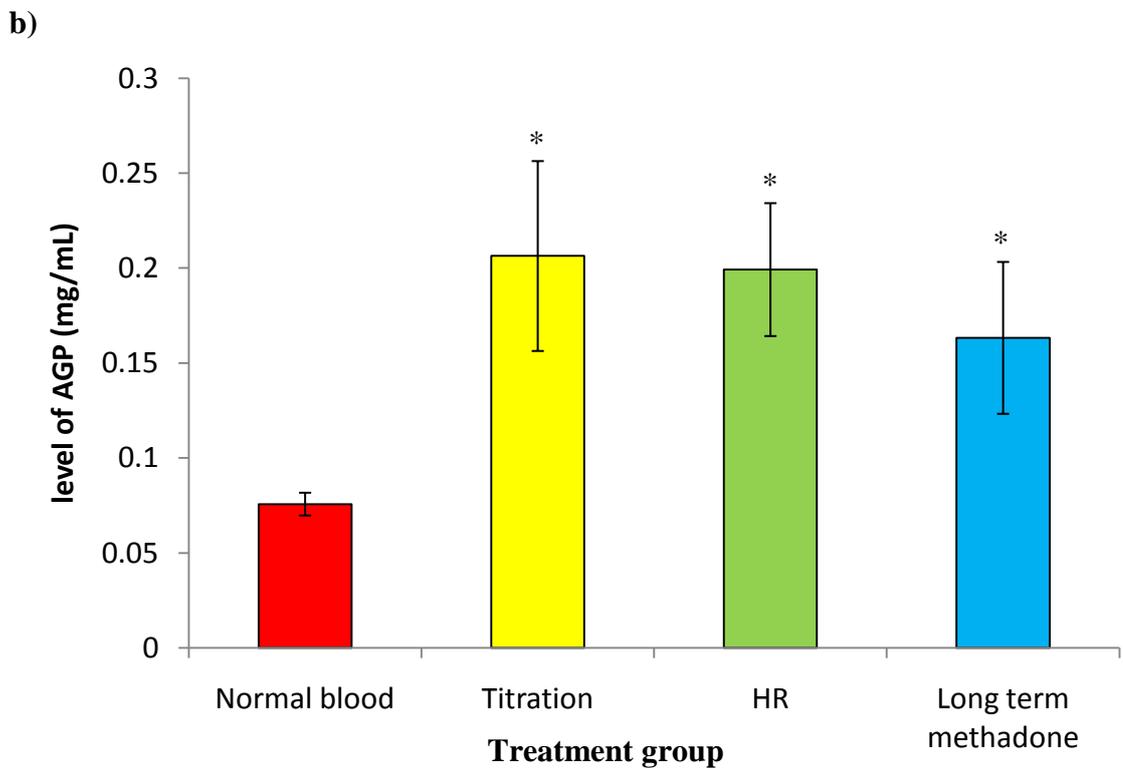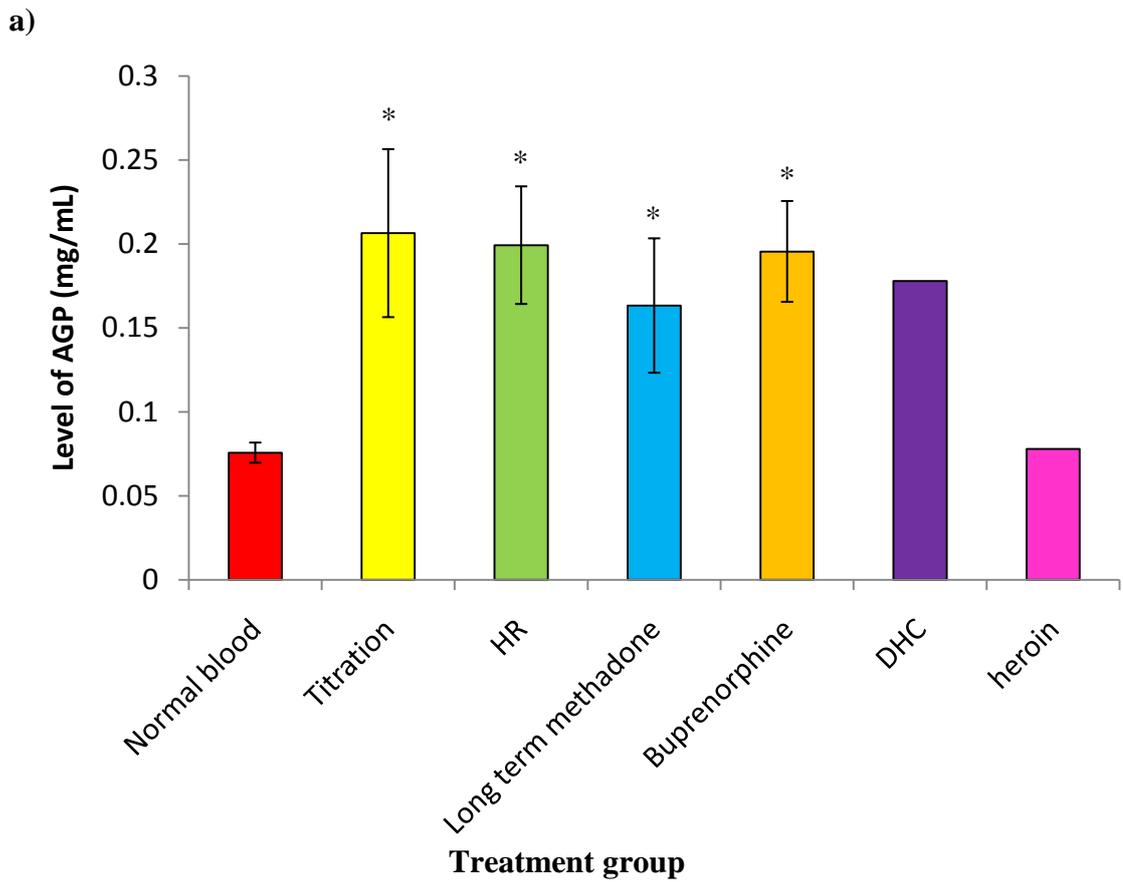
Figure 5.4.   Summary of the mean level of AGP isolated.

from a) all treatment groups and b) patients at different stages of methadone therapy.

* statistically different to 'normal' blood (*p<0.05*).

5.3 Discussion

As described by Gelpi (2003), both purity and integrity of biologically active analytes are the most important prerequisites in structural and functional studies. It was therefore essential that the glycoprotein of interest to this study (AGP) was isolated from patient samples using methods which retained its structural integrity. Although methods available for the isolation of AGP from blood or plasma are plentiful, many of these utilise harsh acidic conditions which can compromise the structure of the glycans. These are therefore unacceptable as any results pertaining to subsequent structural analysis would be erroneous - in order to determine the degree of branching of AGP glycans for example, SA residues must remain intact which is unlikely at low pH.

Therefore, low pressure chromatography (based upon the Smith *et al.,* 1994 method) using two consecutive columns was implemented without the need for harsh acidic conditions; it was also sufficiently sensitive to isolate AGP from other plasma proteins. Despite the common use of SDS-PAGE in isolating specific proteins from samples with variable content (Zdebska and Koscielak, 1999), the acidic conditions rendered it of use only when testing the purity of fractions eluted from the low-pressure chromatography columns in this study. The small masses required were sacrificed from the 'normal' blood sample to ensure the techniques were effective in generating pure AGP before patient samples were subjected to the treatment. The Western Blot (figure 5.2b) confirmed that the single band in lane 3 (figure 5.2a) was AGP as the same sample was run in both SDS-PAGE and Western Blot. AAT, which had previously been shown to be present in trace levels by Gallacher (2010), was not found during this study; a band in lanes 3 and 4 of the SDS-PAGE gel (figure 5.1) corresponding to its molecular weight (approximately 67kDa) would have been expected had it been a contaminant. Its absence was important in this study as it too is a glycoprotein therefore it could have affected structural analysis. Other contaminants could include albumin with a molecular weight of approximately 67kDa; the presence of either of the proteins would have suggested incomplete removal by the blue and red sepharose columns respectively. However the absence of bands corresponding to the MW of the protein in lanes 3 and 4 of the SDS-PAGE gel (figure 5.1) suggested there was no such contamination.

The Western Blot appeared to indicate other proteins or contaminants were present on the membrane in addition to AGP that were not picked up by the coomassie stain (the amount of protein was probably below detectable levels). Due to the nature of the antibody used, non-specific binding was possible; polyclonal antibodies are not directed towards AGP solely. Also, it is possible that not all proteins were transferred onto the membrane, affecting the clarity of the blot and as experiments were preliminary, the protocol requires optimisation. Restaining the gel to confirm the transfer of AGP would potentially allow this to be taken into consideration.

As anticipated, the PEG-precipitated sample generated numerous bands because the polymer does not exhibit specificity to a single plasma protein. PEG 3350 was used at 40% w/v to separate soluble proteins like AGP from less soluble proteins and blood cells. Proteins precipitate in its presence because the water soluble polymer boosts the number of protein-protein interactions by drawing water molecules away from the protein; when these interactions become sufficiently extensive, the protein can no longer dissolve and therefore they concentrate – or 'precipitate' (Ingham, 1990). An additional quality of PEG was that it does not denature the proteins it comes into contact with, which is extremely important if the *in vivo* structure is to be maintained. A sample which had been precipitated with PEG3350 was included in the blot to demonstrate the success of two-stage low pressure chromatography in removing other soluble proteins, leaving a single anti-AGP reactive band in the lane 3 representing samples from which AGP had been isolated. Commercial AGP was included as a control, migrating the same distance as corresponding bands in the other lanes. It was not deemed necessary to analyse the eluted samples after each stage of low-pressure chromatography because the final isolated sample was shown to be AGP thus the technique was effective.

Isolated samples were run in the desalted form to reduce any possibility that the presence of salt (primarily introduced by sodium acetate buffer) could interfere with the migration of the proteins. It was removed by Amicon or Centricon filter devices (molecular weight cut off 10,000Da); molecules greater than 10kDa (AGP) are collected on the filter while those with a lower MW (including the salt) dissolve in HPLC-grade water and pass through the filter, thus removing them from the sample.

The potential of Cibacron Blue sepharose in HSA removal from serum has been acknowledged for decades; Travis and colleagues (1976) reported that 98% of HSA

could be removed from a sample through its use – sodium thiocyanate (NaSCN) is required to cause desorption and collection of the protein. Leatherbarrow and Dean (1980) showed this was possible because the dye binds to the bilirubin binding sites of HSA – like bilirubin, cibacron blue contains planar aromatic ring systems with negative charges in analogous positions. A similar interaction was not found for other serum albumins sourced from bovine, horse or rabbit; instead a low level of binding occurred at long-chain fatty acid binding sites in these species. It should be noted that Cibacron blue is capable of binding other proteins, not just HSA; in 1978, Angal and Dean reported the elution of 11 proteins from the column using a pH gradient. For the purpose of this study, the value of the Cibacron blue column was in the ability to separate AGP from its common contaminant HSA, the latter formed interactions while the former eluted unhindered.

Smith *et al.* (1994) indicated the removal of the protein by subjecting the first eluted fraction to immunodiffusion, highlighting the presence of AGP and AAT but no HSA. Subsequently, the second fraction representing the proteins which were bound to the column surface was eluted and reacted with anti-albumin in immunodiffusion experiments. Further research by the group using SDS-PAGE and immunodiffusion indicated that PEG precipitation, low-pressure and dye-ligand chromatography with Cibacron Blue Sepharose, Q-sepharose and Red Sepharose columns yielded pure AGP (Smith *et al.,* 1994; Elliott *et al.,* 1997; Haston *et al.,* 2003). However this was for isolation on a relatively large scale and recently, it had been proposed that the slight improvement in purity bestowed upon isolated AGP by the Q-sepharose column did not justify the reduction in the level of protein isolated. Gallacher (2010) indicated that when the Q-sepharose column was removed the AGP isolated was relatively pure with virtually undetectable levels of contaminants in immunodiffusion; the blue and red sepharose columns were still useful at the micro-scale. Although it was found that HSA was incompletely removed after passage through the blue column, comparable to the study by Travis and colleagues (1976), the subsequent use of Red Sepharose columns removed additional HSA along with most other contaminants. The purity of AGP after two-stage low-pressure chromatography was supported by the current study using SDS-PAGE and Western Blots.

There have been other combinations of techniques described since the early 1990s offering variable success in yielding pure AGP. Non-miscible solvent extraction

demonstrated potential in the isolation of AGP from serum (Kremmer *et al.,* 1995) with a recovery over 95%; however the actual yield was approximately 89% due to loss during phase separation. Hervé and colleagues (1996) also utilised IEF techniques, alongside Cibacron blue F3GA cross-linked to agarose and chromatography on hydroxyapatite (buffers pH 5.8-5), achieving an 80% yield. Acidic conditions were not required but the techniques used are more appropriate on a larger scale which was not feasible in the current study.

More recently, low-pressure chromatography has demonstrated consistent effectiveness in the isolation and purification of AGP e.g. Elliott *et al.* (1997), Jørgensen *et al.* (2002) and Haston *et al.* (2003); although the exact methodology often deviates depending upon sample availability. A similar yield to that of Hervé *et al.* (1996) was obtained by Azzimonti *et al.* (2003) using a single-step precipitation with Cibacron Blue F3GA, but use of acidic buffers (pH 4.0 and 4.95 respectively) render such a technique invalid for investigations of this nature. It was however useful in the separation of the genetic variants of AGP. To isolate AGP from patients with cancer and acute inflammation, Szollosi and colleagues (2004) also implemented Cibacron blue F3GA dye-ligand chromatography using Toyopearly AF-Blue HC-650M columns that had been equilibrated with 10mM Phosphate buffer at a pH of 5.8, collecting UV detectable fractions which were then desalted and lyophilised. They also compared methods for isolating AGP; deducing that 100% recovery was feasible using solvent extraction and precipitation while affinity chromatography, using a new generation of dye-ligand phase, yielded over 85%. Ethanol was used by the group to eliminate the bulk of serum proteins (mainly albumin), and/or solvents and low molecular contaminants.

Contamination of isolated AGP by salts primarily introduced during low pressure chromatography would interfere with and skew the calculation of AGP levels because they too absorb at 280nm, therefore affecting all subsequent analyses which were reliant on the use of consistent masses of AGP to ensure valid interindividual comparisons. The presence of salt could explain why the level of AGP in patient T1 (0.337mg/mL) was considered higher than in other patients in this group; perhaps it was not completely removed thus absorption figures were inaccurately high. However, later analysis of glycan composition proved this not to be the case; the levels of components were not significantly low in comparison to other glycans of AGP samples which would be expected if AGP was contaminated with salt (chapter 7). The high values may instead

be due to contamination by other proteins which also contain the Trp and Tyr residues responsible for absorbance at 280nm. As most plasma proteins are glycosylated any contamination would be problematic in structural analysis if wrongly assumed to be AGP. The tests used to determine purity of AGP samples (section 5.1.2) indicated the technique, which was common to all samples, provided pure AGP, thus contamination by other glycoproteins was unlikely to be noteworthy. The patient must therefore express a higher level of AGP.

Although the construction of a calibration curve with commercial standards was not the most accurate way to determine the level of AGP in a sample, it was the most appropriate considering the restrictions in place, such as sample availability. Importantly, the terms 'concentration' and 'level of AGP isolated' were not used interchangeably within this study because some of the glycoprotein may have been lost during isolation and purification, therefore it was inaccurate to suggest that the levels obtained were identical to the *in vivo* concentrations. As the same procedures were implemented in the isolation and purification of AGP from all samples, the results themselves were comparable. The partial desialylation which can occur during large-scale isolation, like that used by Sigma-Aldrich (based on that originally described by Hao and Wickerhauser, 1973), would not affect the reliability of the standard curve in the determination of patient sample AGP levels because it is the Tyr and Trp residues of the protein backbone responsible for absorbance at 280nm − which are unaffected (Stoscheck, 1990). The correlation between absorbance and AGP level was demonstrated by an R^2 value >0.9.

Alternative methods used in the determination of AGP concentration include a sandwich-type ELISA using antihuman AGP and horseradish peroxidise-conjugated antihuman AGP (Hashimoto, 2004). Despite the requirement of minimal levels of sample in techniques such as this and immunoturbimetric assays, it still reduces that available for use in structural and drug-binding analysis. Spectrophotometry was useful because it did not require the removal of any AGP or cause its degradation.

The levels of AGP determined in table 5.2 were found to be at least two-fold higher which is comparable with the APR (Ceciliani and Pocacqua, 2007). In a clinical setting, levels of C-reactive protein (CRP) are commonly used to gauge the expression of inflammatory conditions. As described in section 1.2, the concentration of the class III

positive acute phase protein is increased during inflammatory conditions when the APR is initiated. It is normally present at concentrations lower than 10mg/L but levels may be amplified up to 1000-fold during the APR to bacterial infections, as described by Clyne and Olshaker (1999). Although levels were not so grossly elevated in patients, levels over 10mg/L supported the probability that an APR was expressed in the patients. In some cases, the level of AGP appeared to be correlated to dose (summarised in chapter 4). However it was not possible to include the titration phase patients within this generalisation; the individuals commonly received low doses of methadone or substitute opioid to allow for the accumulation and the continued administration of illicit opioid sources. The risk of overdose is high during this stage so precautions must be enforced. Patients with high levels of AGP in this group would perhaps be expected to require higher doses of methadone to become stable due to its potential removal by AGP; however such follow-up information was unavailable due to the nature of the study.

The five samples taken from the titration phase participants indicated a significant increase in the level of AGP isolated, perhaps a result of the APR. Commonly, in the clinical setting, levels of C-reactive protein (CRP) are used to gauge the presence of an APR – as described in section 1.2, the concentration of class III positive acute phase protein is increased during the APR

Rostami-Hodjegan and colleagues (1999) reported that the levels of AGP in heroin dependent individuals with signs of opiate withdrawal (a common problem in the initial stages of therapy when methadone doses are kept low) were greater than in a drug free population and also showed a higher proportion binding to the glycoprotein when analysed *ex vivo*. Therefore over time, as treatment-related stresses are expected to decrease, the stimulation of the APR may lessen in order to regain baseline homeostasis. For example, anti-inflammatory cytokines may be released in order to stop the APR (negative feedback) and therefore the levels of AGP stabilise or even decline during this period; possibly explaining why patients LT7 and LT8 for example, had lower levels of AGP – they have been maintained on the therapy for over 4 years and with generally less chaotic lifestyles than those faced with the sudden change from short acting heroin to long acting methadone. However, this was not true for the majority of other patients in the LT group, with levels similar to those in the titration phases, thus there must be other explanations. Perhaps a sustained response by the body to the xenobiotic

occurred, at least partly explaining why all patients had elevated levels. This would also correlate with the fact that patients in LT therapy were often receiving high doses of the drug to remain withdrawal-free even if a decrease was expected - AGP levels may remain high as a mechanism to remove the drug from the system. A cycle could ensue - like tolerance an increase in AGP in response to the drug could lead to the requirement of higher doses if the drug binds with a high affinity, removing it from the active concentration. Thus the levels required to induce the desired effect were not attained. It may be possible that the stimulation of dopamine (DA) release during heroin use causes an increase in the synthesis of AGP. DA is a neurotransmitter commonly released during stress; perhaps the increase in production deceives the body, causing further stimulation of an APR (Pruessner *et al.,* 2004). The concept of drug binding is described more thoroughly in chapter 8.

Alternatively, the elevated levels of AGP in patients may be completely unrelated to stress or the presence of opioid substitute; smoking for example has been shown to increase level of AGP (Benedek *et al.,* 1984b). The possibility that factors such as smoking may be responsible cannot be ignored and should be investigated in more detail. Recruitment of non-smoking patients, in an already hesitant study population, was near impossible.

It must therefore be acknowledged in studies such as this that various factors may be responsible for the disparity seen in levels of AGP. Thus it would not be valid to presume all data collected from analyses is due to the therapy alone.

SUMMARY STATEMENT

The level of AGP isolated from heparinised blood samples taken from a 'normal' population (the Blood Transfusion Service) differed significantly to that isolated from patients undergoing various stages of opioid replacement therapy. The level between treatment groups did not differ. If methadone exhibits considerable binding to AGP, the increase in its expression detected may affect the drug's efficacy. However, important to the current study is that alterations in the glycosylation can affect the function of a glycoprotein – this concept was investigated further in the following chapters.

Chapter 6

AGP Monosaccharide Analysis

Results and Discussion

6.1 Introduction

Certain physiological or pathological conditions can alter the structure of glycans attached co- or post-translation to a polypeptide backbone thereby causing the conformation of the glycoprotein to be incorrect, rendering it unable to perform the functions normally attributed to the particular glycan expression. Structural analysis of the glycan chains and their components (microheterogeneity) is therefore important. Some of the monomeric units used in the construction of complex N-linked glycans, like those in AGP, are exclusive to branches - for example galactose (Gal). These offer potential indicators of altered branching if distinctly different between two populations.

A common technique for the analysis of glycan monosaccharide composition is HPAEC-PAD, the use of which has been well documented since its conception (for a recent review refer to Behan and Smith, 2010). Although a number of alternative analytical methods are available to investigate glycan structural composition, including lectin ELISAs (Rydén *et al.*, 1999), nuclear magnetic resonance (van Halbeek *et al.,* 1981) and Mass Spectrometry (Imre *et al.,* 2008), HPAEC remains one of the most effective (Sim *et al.,* 2009). The implementation of HPAEC in oligosaccharide analysis of AGP is discussed in the following chapter, supporting the versatility of the technique. Its use in this study was valuable as a means of providing sensitive analysis (it is capable of detecting 10-100 picomolar quantities) without the requirement for large samples or prior derivatisation as is the case in gas chromatography and other liquid chromatography-based methods (Hardy *et al.,* 1988; Townsend *et al.,* 1989; Smith *et al.,* 1997). Techniques like MS are commonly undertaken offline due to the high salt concentration of the mobile phase. However the coupling of the technique to HPAEC has been reported (Okinaga *et al.,* 1992).

Prior to separation by HPAEC-PAD, component monosaccharides must be released. Commonly implemented are TFA and HCl for the cleavage of the different monosaccharides respectively. Zdebska and Koscielak (1999) hydrolysed samples from SDS-PAGE with 0.2M TFA for 1h at 80°C to release sialic acids, then 2M TFA for a further 4h at 100°C – releasing the neutral sugars. Finally 6M HCl was utilised to remove the amino acids maintaining the temperature at 100°C. TFA and HCl were also

used in the current study, in order to prepare the neutral and basic monosaccharides for HPAEC-PAD analysis respectively - optimal conditions were discussed in chapter 3.

Analysis of the released monosaccharides by HPAEC required the use of a stationary phase with high mechanical and chemical stability - usually a column with polymer based pellicular resin, the active component of which is a quaternary ammonium ion (for a recent review of columns available that allow selective analysis see Behan and Smith, 2010). An alkaline mobile phase (0.03M NaOH) and detection by pulsed amperometry were also important (McGuire *et al.,* 1999). NaOH was used in an isocratic gradient, to cause electrocatalytic oxidation of weakly acidic sugars, ionising the hydroxyl groups (-OH) and forming oxyanions when the pH is 12 or higher (figure 6.1). There are therefore several potentially ionisable –OH groups and due to variation in the position of these groups in monosaccharides, each exhibit different dissociation constants (pKa 12-14). Anomeric –OH groups are the most acidic, the others follow a hierarchy of acidity: 1-OH>2-OH>>6-OH>3-OH>4-OH. The strength with which oxyanions bind to pellicular anion-exchange resins of CarboPac[TM] PA-100 columns commonly used in HPAEC analysis to separate monosaccharides, subsequently depends on their pKa values; those with higher pKa form weaker interactions with the resin and are therefore eluted more easily (Lee, 1990; McGuire *et al.,* 1999). Fuc oxyanions for example have a high pKa and are therefore less well retained compared to Man thus they elute early in a chromatogram. Sugars expressing anomeric -OH groups are less well retained than essentially the same sugar with normal -OH groups at this position; this effect is exemplified by the long retention times of inositols which do not contain anomeric -OH groups. Generally, the greater the collative negative charge of a monosaccharide, the stronger the interaction formed with the column.

Fouling of the gold electrode occurs as the oxides build up and form a coating. However the addition of PAD in the 1980s, as first documented by Rocklin and Pohl (1983) and highlighted during research by the Johnson group (Neuburger and Johnson, 1987; Johnson *et al.,* 1992), was shown to vastly improve the technique. Pulsed potentials (figure 2.1, chapter 2) allow oxides to form as the potential is increased but follow this by lowering the potential to remove the layer of oxide from the electrode surface. Therefore, reproducibility is ensured as the electrode is constantly 'cleaned'.
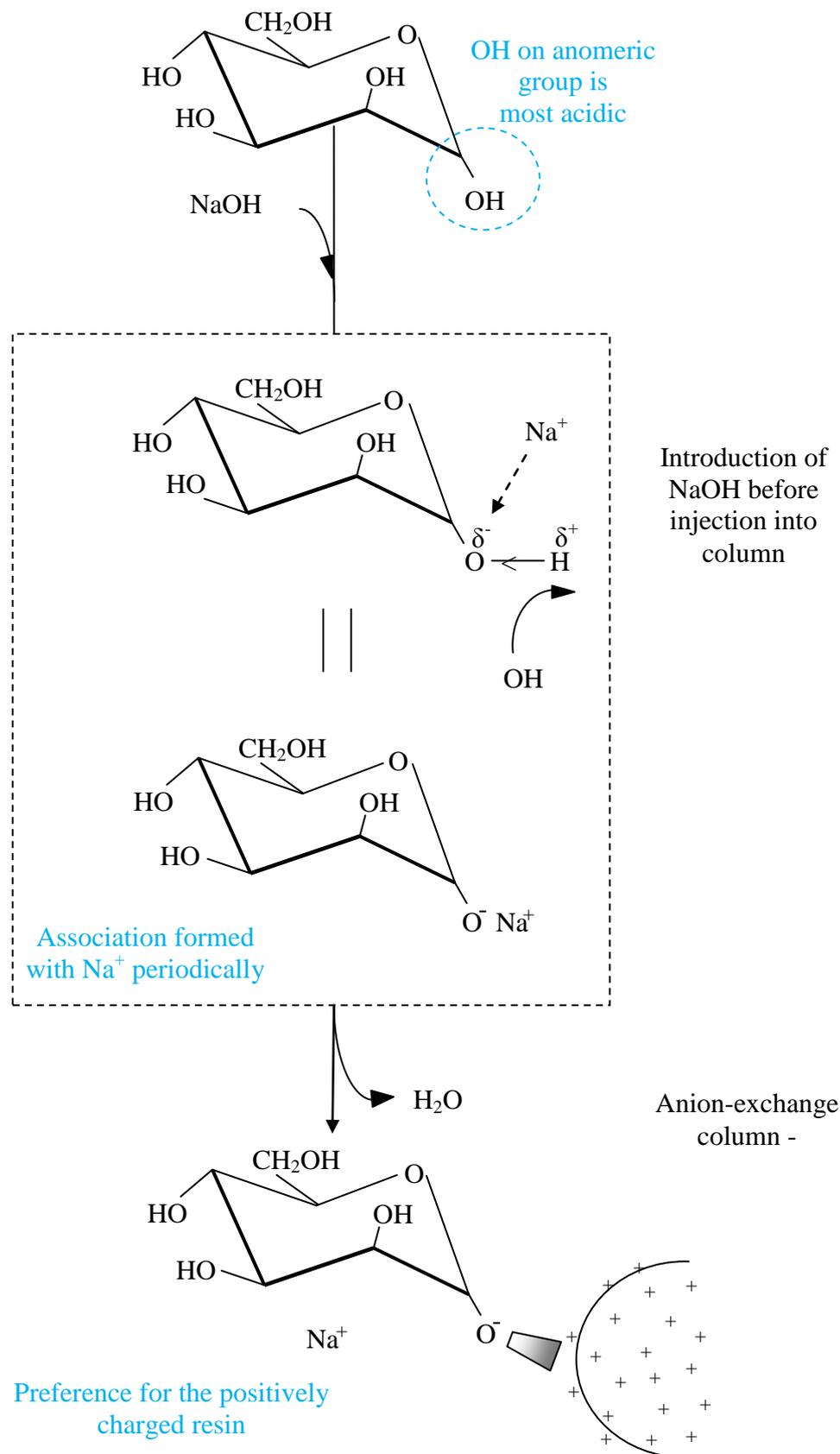
Figure 6.1. **Oxyanion formation at C1 (mannose).** The presence of NaOH causes basic separating conditions (pH 13) where the monosaccharide oxyanion interacts with positively charged Na^+ ions until it is introduced to the positively charged resin. The strength of interaction – based on the negative charge of the monosaccharide – determines when it will elute.

6.2 Results

In any study of the structure of glycans it is important to ensure efficient cleavage and subsequent separation of components - degraded or un-cleaved monosaccharides are not detected, thus compromising the accuracy of the analysis.

HPAEC separation of two mixtures of standard monosaccharides is shown in figure 6.2a and 6.2b; the former a mixture of monosaccharides common to glycans in general and the latter those representing the components of complex N-linked glycans. The traces demonstrated the ability of the technique to separate stereoisomers; isomers that differ in the orientation of their atoms in space e.g. Glc and Man (figure 6.2a), the structures of which were illustrated in figure 1.1 (refer to section 1.1.2, chapter 1). HPAEC resolves monosaccharides, which can be identified by the ratio of their retention times in relation to that of an internal standard (2-deoxy-D-galactose). Standards for GlcNAc and GalNAc were glucosamine (GlcN) and galactosamine (GalN) respectively, representing the deacetylation of the N-acetylated hexosamines which occurs during acid hydrolysis with HCl and subsequent HPAEC under alkaline conditions (NaOH).

However, as mentioned, Glc and GalNAc are not routinely present in N-linked complex glycans - AGP was no exception. The retention ratios of the unknown peaks generated on separation of commercial AGP glycan monosaccharide components (figure 6.3) with the IS were compared to those generated during the separation of six standard monosaccharides (figure 6.2a) and no values were ascertained to represent Glc or GalN. Therefore, a mixture of five monosaccharides Fuc, IS, GlcN, Gal and Man were used as a standard solution injected prior to samples; not only ensuring the effective operation of equipment, but also to assist in the identification of peaks generated upon HPAEC analysis of samples (figure 6.2b).

Figures 6.3 and 6.4 demonstrate the need for separate use of TFA and HCl, the former effectively hydrolysed neutral monosaccharides (figure 6.3a and 6.4a) but that of GlcNAc (represented as a small GlcN peak) was incomplete. HCl (figures 6.3b and 6.4b) was required as investigated in greater detail in chapter 3.
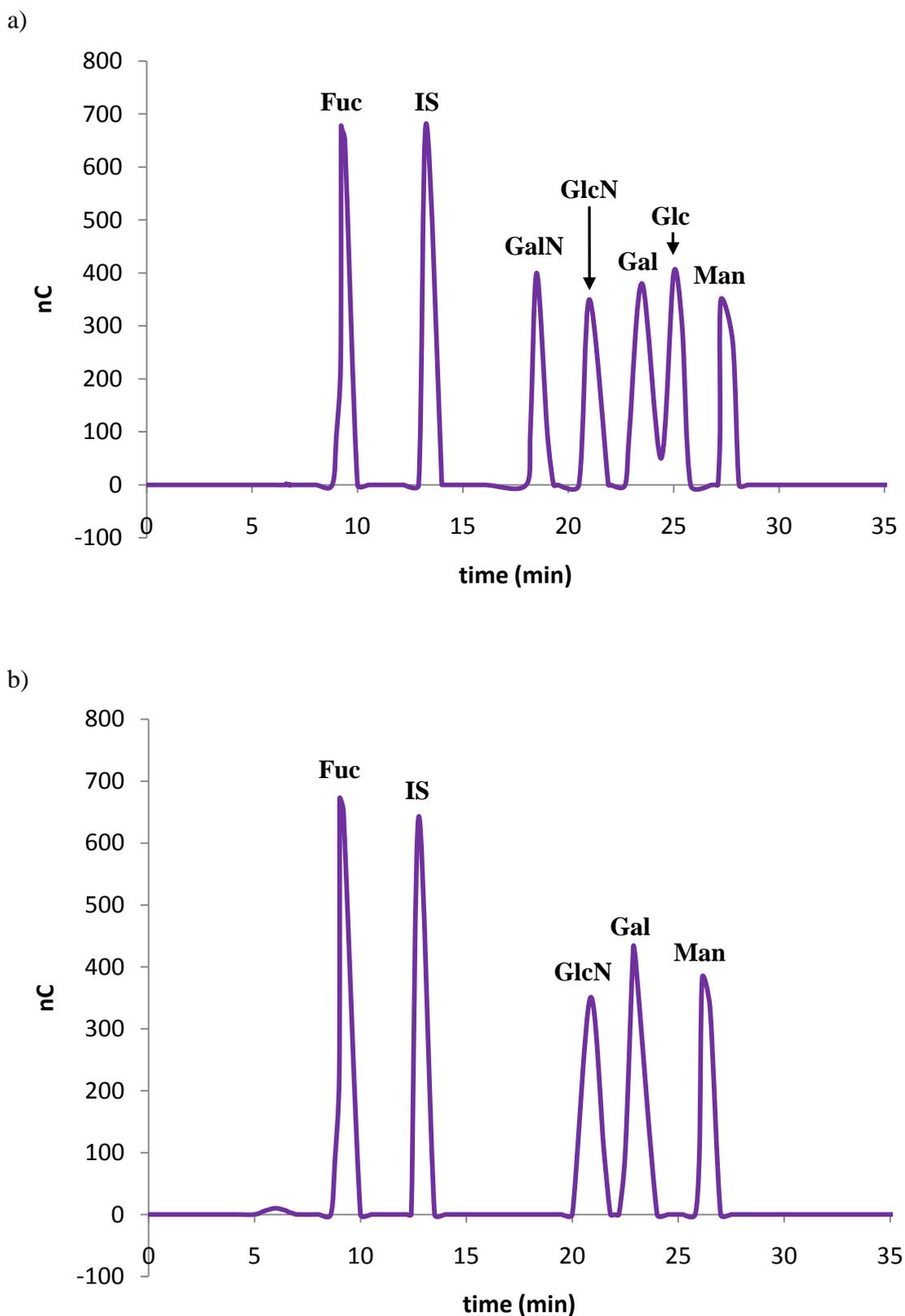
Figure 6.2. **Traces generated during HPAEC separation** of a) a mixture of monosaccharides common to glycoproteins, separating isomeric monosaccharides and b) the 4 monosaccharides commonly found in AGP alongside an I.S.

Fucose (Fuc), 2-deoxy-D-galactose (I.S), Galactosamine (GalN), Glucosamine (GlcN), Galactose (Gal), Glucose (Glc), Mannose (Man). Nano Culon (nC)
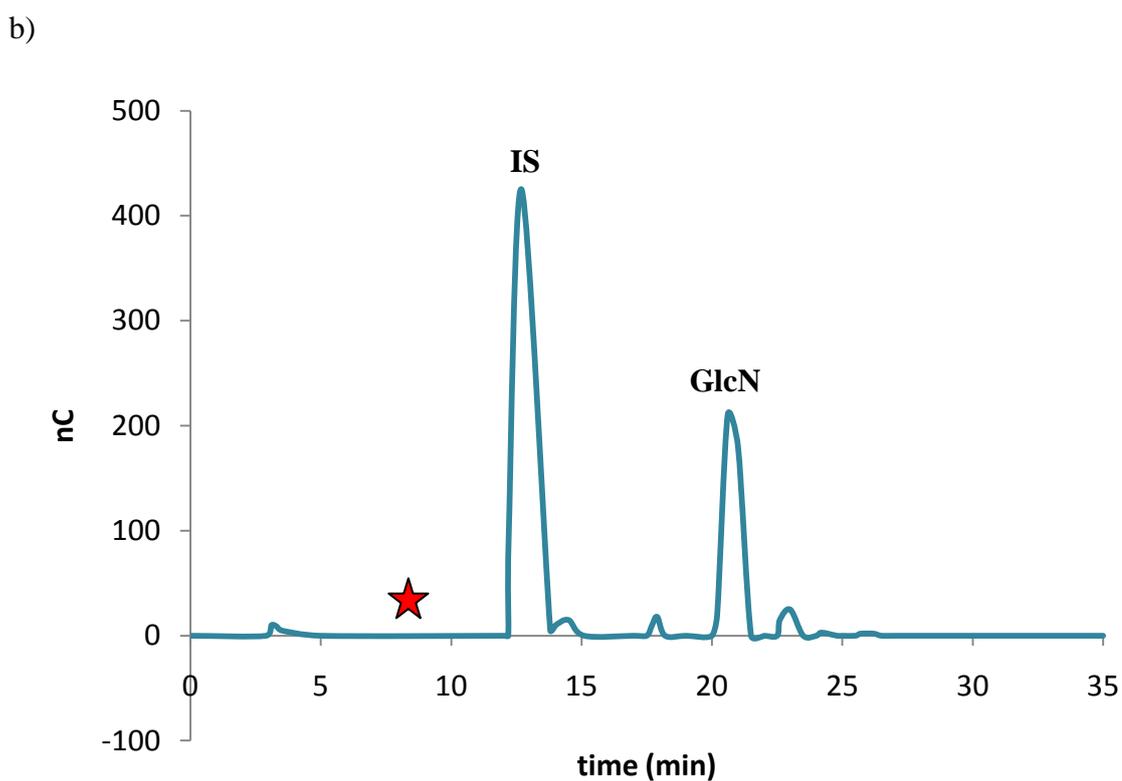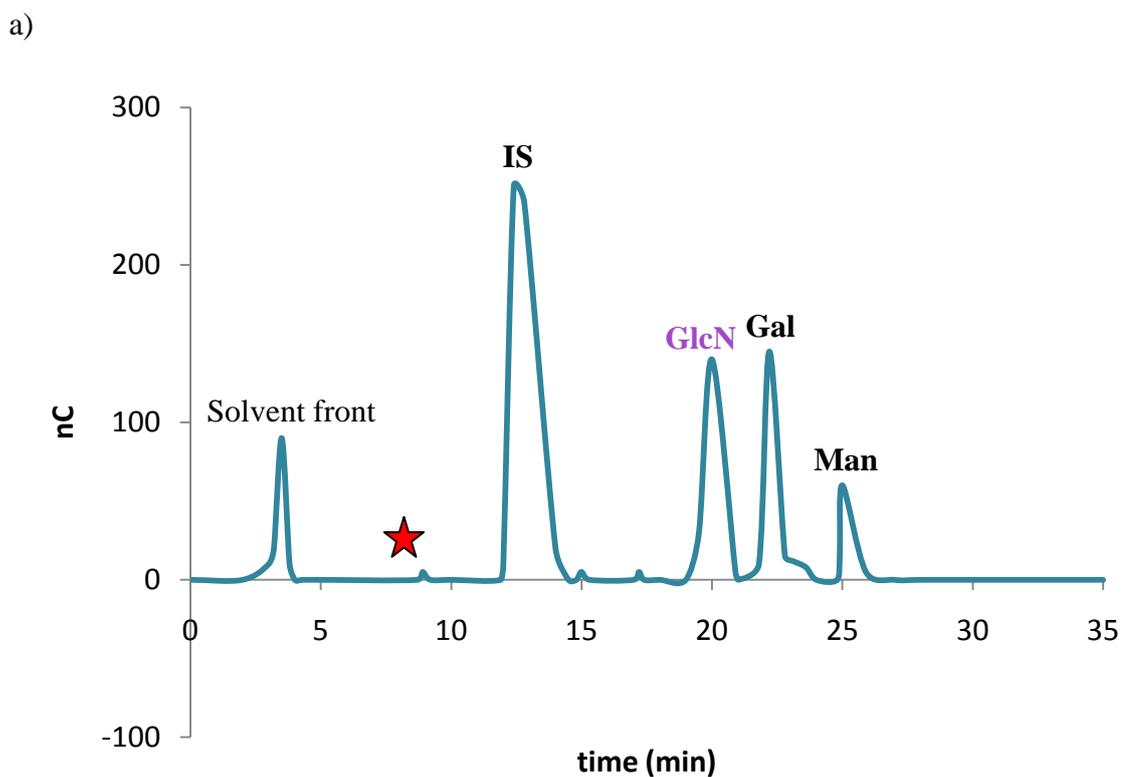
Figure 6.3. **Traces generated during HPAEC separation of a) neutral and b) basic monosaccharides from commercial AGP alongside an I.S**.

⭐ No Fuc detected at the expected retention time.

a)

b)
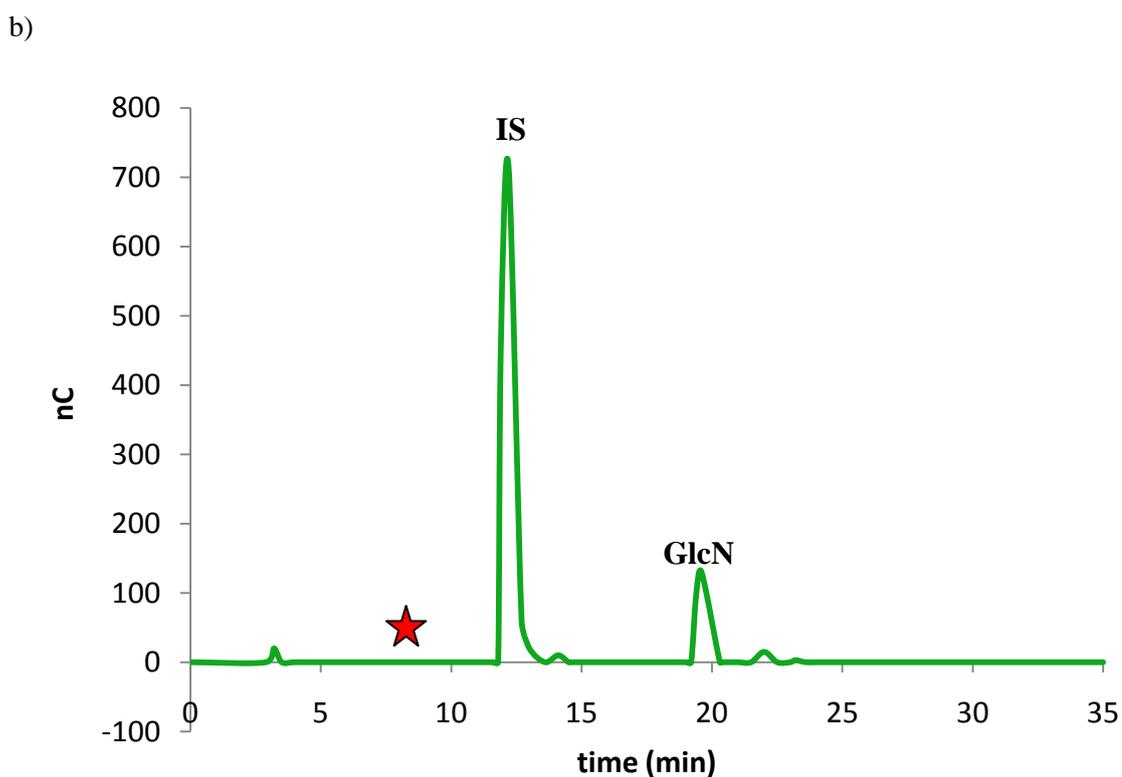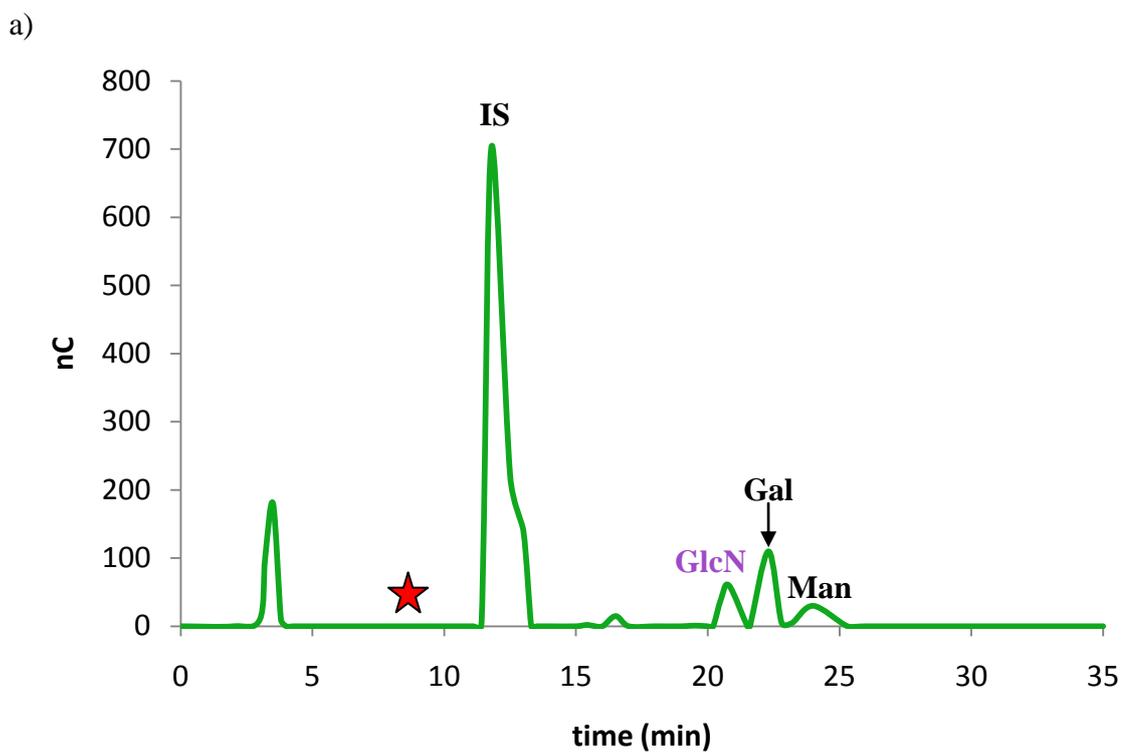
Figure 6.4.   Traces generated during HPAEC separation of a) neutral and b) basic monosaccharides from patient sample AGP alongside an I.S.

⭐ No Fuc detected at the expected retention time.

The denaturation of the peptide backbone and desialylation which occurs at low pH does not hinder the analysis of glycan structure at this stage because the individual monosaccharides of interest are uncompromised and removed from the protein. Exclusion of terminal SA residues at this stage was unimportant; their presence was not essential until the analysis of complete glycan chains (chapter 7).

Unknown peaks generated by HPAEC-PAD separation for each 'normal' heparinised blood, commercial and patient sample were identified through comparison to those obtained during separation of the mixture of standard monosaccharides (figure 6.2b). Generally, every sample was analysed in triplicate.

To quantify the newly identified monosaccharides in patient (titration, HR, long-term methadone, buprenorphine, DHC or heroin) and 'normal' heparinised blood samples; standard curves were produced for Fuc, GlcN, Gal and Man (figure 6.5). Analysis of each mass (0-5 µg) was repeated five times to determine the mean values and standard deviations (error bars). The peak area data generated by HPAEC analysis indicated that the area increased with mass of each monosaccharide. Each R^2 value was >0.9 therefore there was a strong linear association between the two variables.
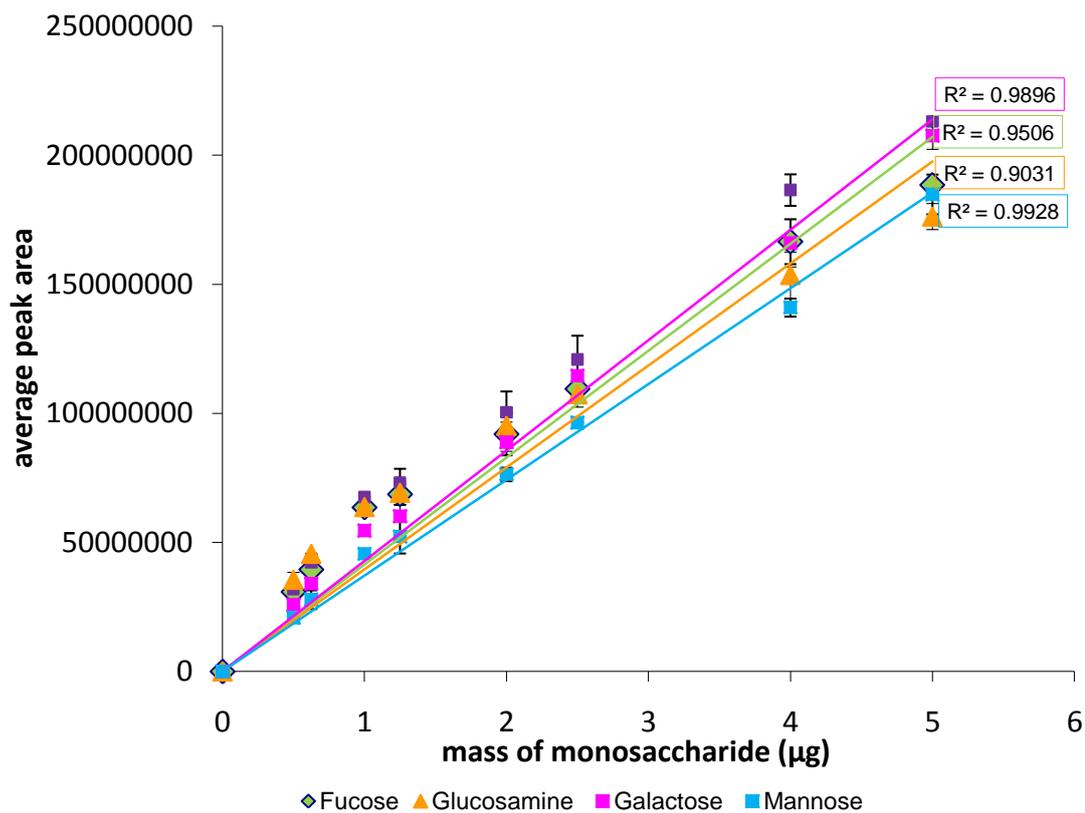
Figure 6.5. **The five monosaccharide standard curves**; mass of monosaccharide versus the mean peak area values (n=5) - error bars signify the standard deviation. All correlation coefficients (R^2) >0.9.

Following the identification of unknown peaks in the HPAEC chromatograms generated for all patient AGP samples, the relative mass of each monosaccharide present was finally converted to moles of each sugar / mol AGP. Values were recorded as the mean ± standard deviation in table 6.1, 6.2 and 6.3 ('normal' heparinised blood; titration, harm reduction and long-term phase patients; non-methadone therapies respectively). This allowed comparisons to be made between the glycosylation of AGP expressed in patient blood samples with that from a 'normal' healthy population. It must be remembered that data for the glycan composition of commercial AGP and that isolated from 'normal' serum was not included – as summarised previously in chapter 2, these were not deemed adequate representatives of 'normal' AGP.

Four 'normal' heparinised blood samples were hydrolysed using the two acids individually. All patient samples were similarly treated with the exception of LT3, LT9 (with HCl), B4 (TFA) and HR4 (HCl and TFA) where only single analysis for each acid was possible.

Fuc was detected in some 'normal' heparinised blood samples. Other monosaccharides common to complex N-linked glycans were detected in the patient, 'normal' and commercial samples. Statistical analysis of the data was performed using one-way ANOVA with a Tukey's post hoc to allow comparison not only with a 'normal' population but also between treatment groups. Whether variation in the data generated achieved statistical significance was indicated in table 6.2 and 6.3. The levels of the monosaccharides in commercial AGP were greater ($p < 0.05$) than those in AGP isolated from 'normal' blood. Direct comparisons were not deemed adequately valid, despite the analysis of consistent masses of AGP because the large-scale techniques used in the isolation of commercial AGP differed to those used in this study. Therefore, in making comparisons between patient samples and 'normal' populations it was considered more appropriate to use the blood values as comparisons as they were isolated using a common technique. Also, serum samples were donated by individuals who, although known to be cancer free, were an elderly group and therefore may have an APR expressed; the samples may not be an accurate representation of a truly healthy population.

One-way ANOVA analysis indicated that the level of Gal and GlcN (GlcNAc in the *in vivo* structure) were significantly greater in patients from the various stages of

methadone therapy (T, HR and LT treatments) compared to 'normal' blood (*p<0.05)* which may suggest an increase in chain branching. The *p*-value was slightly improved upon removal of the outlying value in GlcN analysis (representative of patient LT6) however, significance was gained without its removal. Although some of the AGP isolated from individuals receiving buprenorphine therapy displayed apparently higher levels of the two monosaccharides than that in the 'normal' group, the values were not found to reach significance upon statistical analysis.

The level of the two monosaccharides remained relatively constant within and between treatment groups. Patient T1 and LT6 expressed higher levels of GlcN (also Gal in patient T1) compared to other members of their respective groups, making the deviation from the mean relatively high in comparison to other groups. Levels of Gal varied between 3.87-5.97 mols/mol AGP, 2.55-4.47 mols/mol AGP, 3.27-4.63 mols/mol AGP, 2.24-4.06 mols/mol AGP for T, LT, HR and B treatment groups respectively. GlcN, which is not exclusive to branches like Gal, was higher at 6.52-10.2 mols/mol AGP, 5.47-9.03 mols/mol AGP (both groups indicating a large variation about the mean due to patients T1 and LT6), 6.70-8.84 mols/mol AGP and 4.99-8.99 mols/mol AGP in the groups above.

Although patient D1 and H1 had a significantly lower level of Gal (*p<0.05*) than the titration phase group but no significant differences in the level of either monosaccharide to that of 'normal', it could not be known if these levels were representative of others on the therapy thus it would have been inaccurate to assume so.

The level of Man was relatively consistent – no statistically significant differences were found in the AGP of patient and 'normal' samples - suggesting the presence of pentasaccharide core structures remained consistent and therefore, as required, the approximate level of the glycoprotein analysed was equivalent; Man is only present in core structures of complex N-linked glycans.

Therefore, as the only significant alterations occurred in the Gal and GlcN levels, there was a suggestion of increased branching of glycans in the AGP of patients, although it did not appear to be dependent upon stage or types of opioid replacement therapy as the groups do not significantly differ to each other.

	Mols monosaccharide/mol AGP			
Sample	Fuc	GlcN	Gal	Man
Normal 1	0.0	2.55	2.36	0.905
Normal 2	0.57	3.7	1.02	0.98
Normal 3	0.7	4.27	2.41	1.22
Normal 4	0.0	3.86	1.47	0.75
Normal 5 ¥	0.7	6.27	2.41	1.22
Normal 6 ¥	0.55	4.62	2.23	1.08
Normal 7 ¥	0.57	5.70	3.00	0.75
Normal 8 ¥	1.29	5.60	1.29	0.60
Mean 'normal' heparinised blood	**0.32±0.37**	**3.60±0.74**	**1.82±0.68**	**0.96±0.20**

Table 6.1. Monosaccharide composition of 'normal' AGP samples.

	Mols monosaccharide/mol AGP		
Sample	GlcN	Gal	Man
Mean 'normal' heparinised blood	**3.60±0.74**	**1.82±0.68**	**0.96±0.20**
T1	9.99±0.51▪	8.24±0.46▪	2.77±0.24
T2	6.79±0.87▪	3.51±0.61▪	1.53±0.33
T3	10.69±0.24▪	3.65±0.26▪	1.03±0.43
T4	7.45±0.08▪	4.21±0.49▪	1.65±0.05
T5	6.88±0.54▪	4.99±0.10▪	2.11±0.48
Mean T	**8.36±1.84 ♦**	**4.92±1.05 ♦**	**1.82±0.66**
HR1	9.24±0.31▪	3.60±0.27▪	1.14±0.02
HR2	7.63±2.04▪	3.81±0.06▪	1.44±0.09
HR3	7.22±1.02▪	4.87 [a]	2.07 [a]
HR4	6.98*	3.51*	1.52*
Mean HR	**7.77±1.07 ♦**	**3.95±0.68 ♦**	**1.54±0.47**
LT1	6.21±3.82▪	4.64±0.42▪	2.17 ±0.13
LT2	4.03±1.02 ‡	1.67 [a] ‡	1.00 [a]
LT3	7.38 *	3.45±0.70▪	2.60±0.19
LT4	7.08±1.95▪	4.46±0.37▪	2.21±0.07
LT5	5.56±0.43▪	3.30±1.00‡	1.12±0.20
LT6	10.57±0.36 ▪	4.65±1.89▪	1.93±0.22
LT7	7.95±0.76 ▪	2.27±0.04‡	0.49±0.02
LT8	7.38±0.51▪	3.03± 0.41▪	1.32± 0.09
LT9	9.62 *	3.52 [a]	1.73 [a]
LT10	7.08±1.95▪	4.21±0.37▪	2.01±0.16
LT11	6.92±0.49▪	3.40±0.26▪	1.98±0.05
Mean LT	**7.25±1.78 ♦**	**3.51±0.96 ♦**	**1.45±0.96**

Table 6.2a. Monosaccharide composition of AGP from 'normal' blood and patients undergoing titration (T), harm reduction (HR) and long-term (LT) methadone therapies.

Sample	Mols monosaccharide/mol AGP		
	GlcN	Gal	Man
Mean normal heparinised blood	3.60±0.74	1.82±0.68	0.96±0.20
B1	9.62±0.51▪	4.18±0.21▪	1.11±0.09
B2	6.92±0.49▪	1.97±0.35	0.56±0.18
B3	7.38±1.90▪	3.31±0.07▪	0.81±0.17
B4	4.03±1.02	3.13*	1.20*
Mean B	6.99±2.00	3.15±0.91	0.83±0.29
D1	3.81±0.9	3.26±1.00	1.38±0.58
H1	5.56±0.75	1.01±0.32	0.95±0.42

Table 6.2b.   Monosaccharide composition of AGP from 'normal' and patients undergoing Buprenorphine (B), DHC (D) and heroin (H) opioid replacement treatments.

Data in tables 6.2a and 6.2b is reported as the mean number of moles of monosaccharide / mole AGP ± the standard deviation (n=3). Exceptions are those where the monosaccharide was only detected in a single run due to equipment malfunctions (ª) or where there was insufficient sample to duplicate analysis (*). Mean values for each group are highlighted in green; ♦ Mean value significantly different (p<0.05) to 'normal' heparinised blood. ‡ Individual level expressed in patient glycan not significantly different to normal. ▪ Level significantly different to 'normal' mean value (*p*<0.05). ¥ Data obtained from analysis of monosaccharides during a previous study using the same techniques.

The mean monosaccharide levels were then graphed in figure 6.6a however because there was only a single patient representing the treatment groups for DHC and heroin, these values were removed (figure 6.6b); it could not be known if they represented the majority of patients on the respective therapies. To allow closer inspection of the difference in the level of sugar in each group, a series of boxplots were created (figure 6.7), indicating the distribution of the sugar in each group and highlighting the outlier in the LT group mentioned previously. Again, patients D1 and H1 were not included as there was only one sample and therefore only variation in the levels detected in it, not within a group. Both the graphs and boxplots illustrated the findings of statistical analysis, with an apparent increase in the Gal and GlcN (therefore GlcNAc in the *in vivo* structure) components in the methadone patients, suggesting an increase in the branching of glycans in patients undergoing treatment for opioid dependencies.
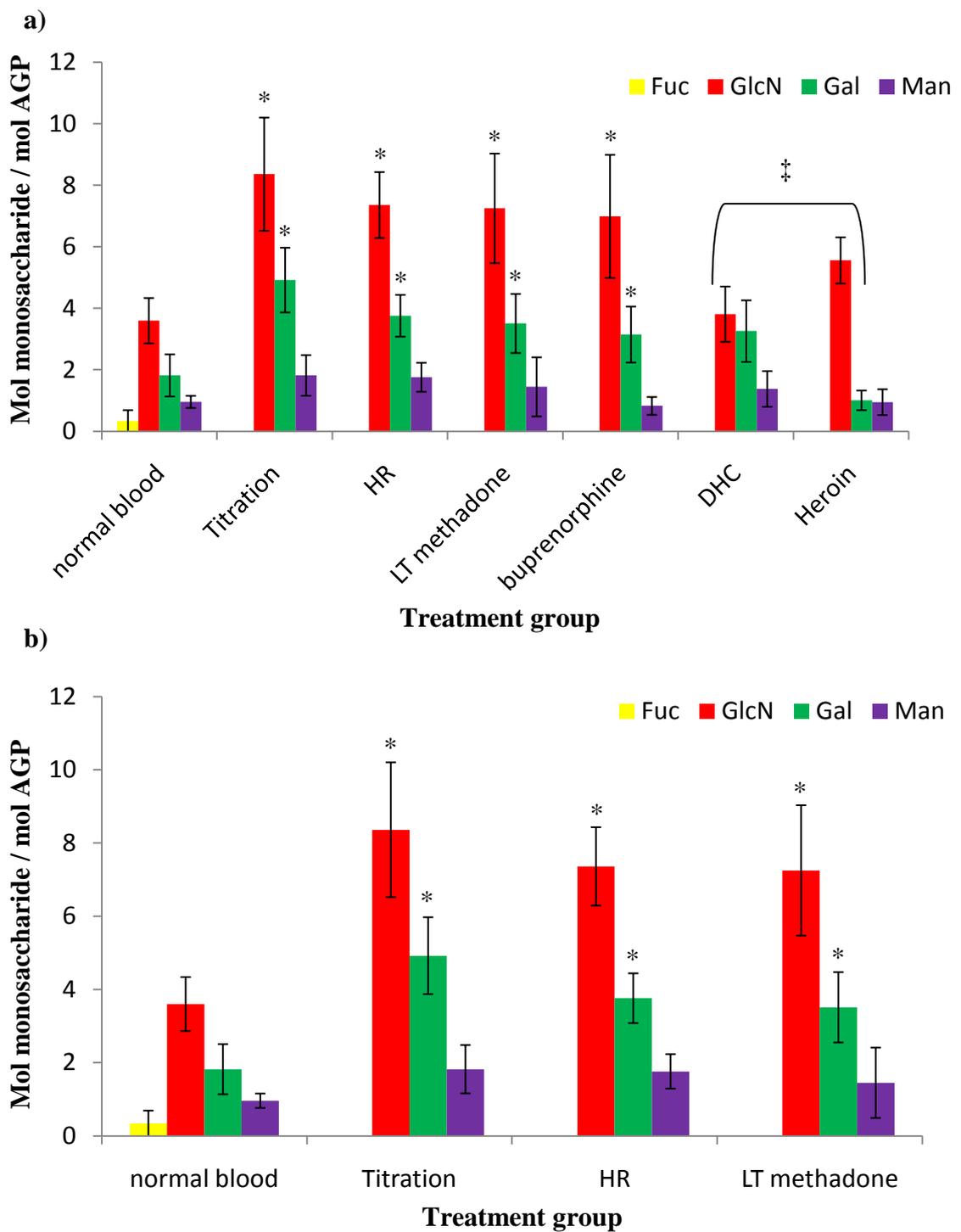
Figure 6.6.   Bar chart summarising the mean level of monosaccharide / mol AGP in a) the separate treatment groups and b) in the different stages of methadone therapy, where 'normal blood' represents heparinised blood from the Blood Transfusion Service

* Statistically significant difference compared to normal heparinised blood sample (p<0.05) HR – harm reduction; LT – long term treatment. ‡standard deviation calculated from triplicate analysis of a single sample while others were calculated from data from more than one patient (n = 4/+).

a)
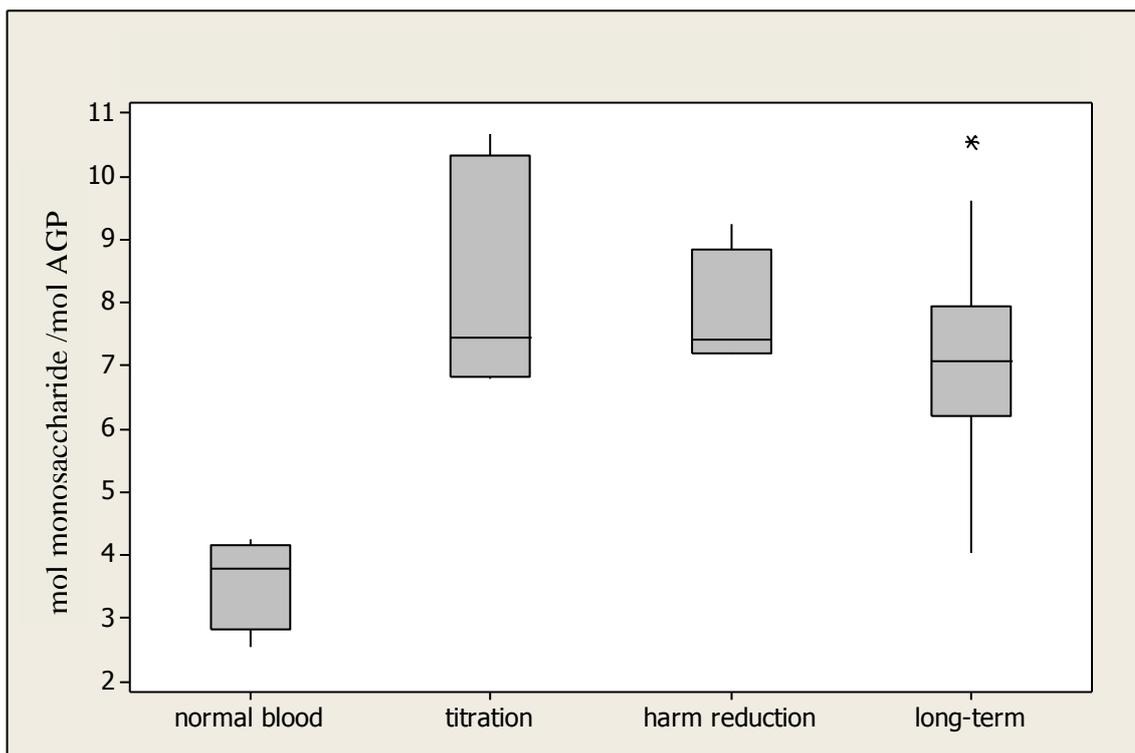
b)
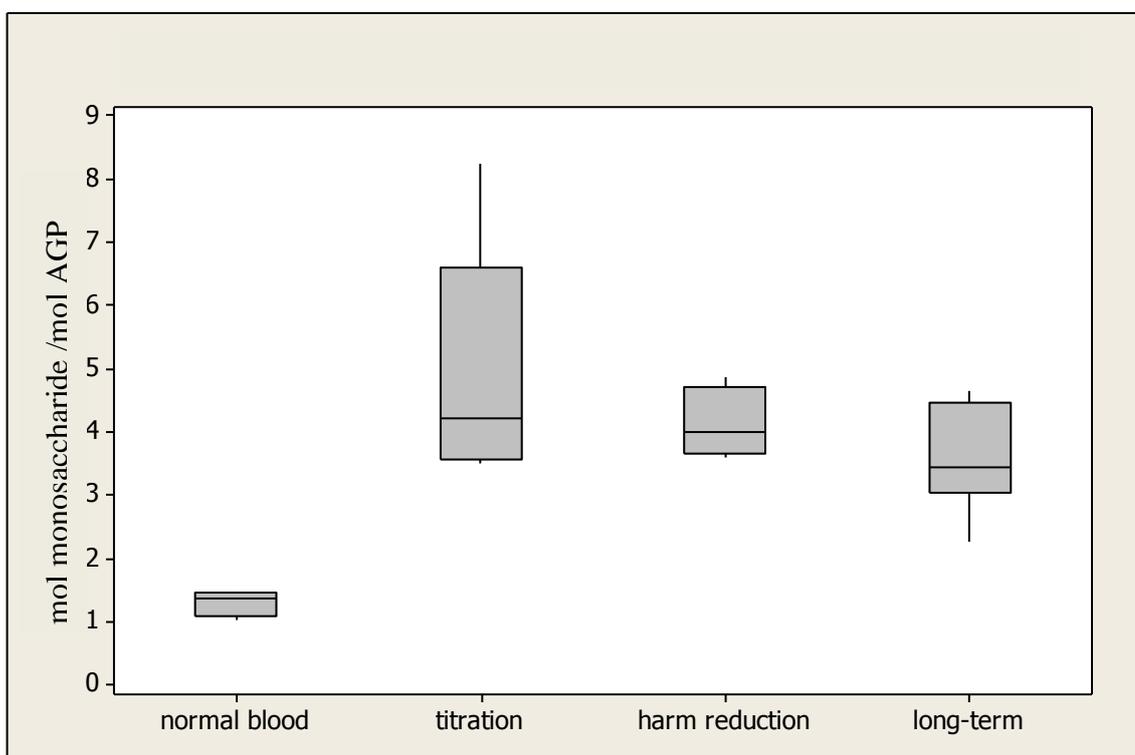
Figure 6.7.   **Box plot of monosaccharide distribution** - a) GlcN (and by extension GlcNAc) and b) Gal in AGP isolated from 'normal' heparinised blood, patients undergoing titration, long-term methadone, HR and buprenorphine treatment for opioid dependence.

c)
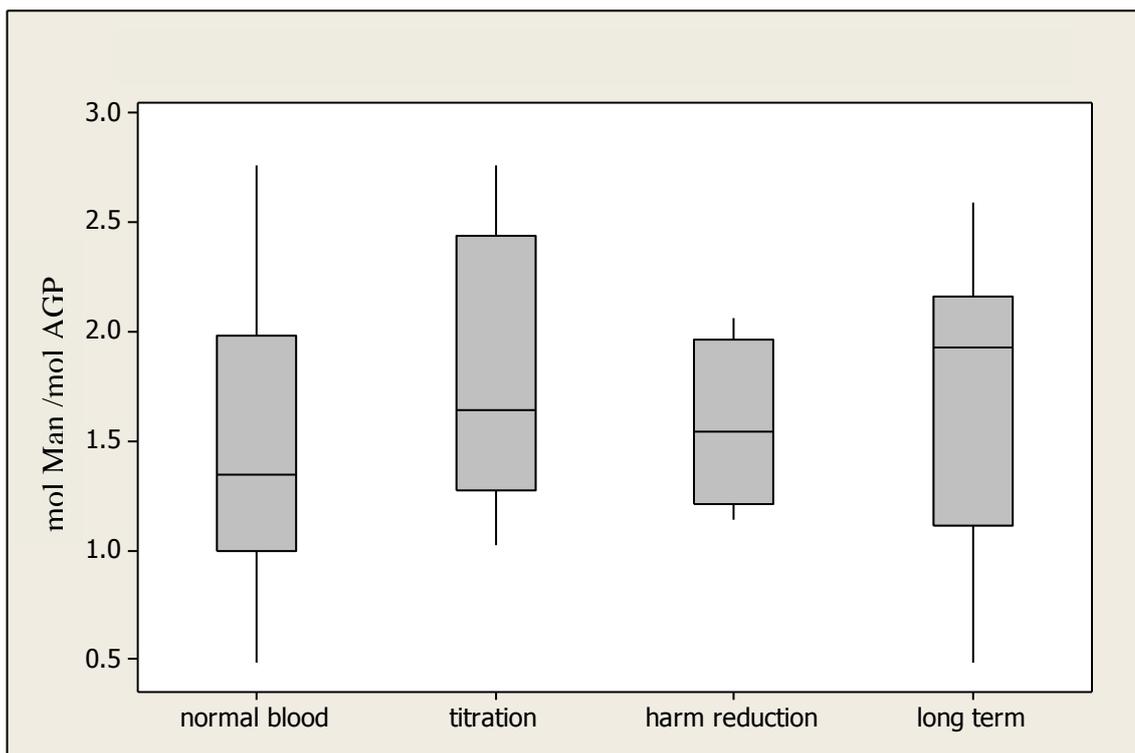
Figure 6.7 continued.   Box plot of monosaccharide distribution c) Man in AGP isolated from 'normal' heparinised blood, patients undergoing titration, long-term methadone, HR and buprenorphine treatment for opioid dependence.

6.3   Discussion

It is not only the *in vivo* concentration of AGP which can be altered as a result of the APR, structural alterations may also arise in the glycosylation, potentially affecting the ability of the glycoprotein to perform functions for which it was intended. Müller (1989) reported that AGP glycans are not critical in the interaction with drugs at the binding site and instead it is solely dependent on the formation of the correct tertiary structure; describing the folding of the protein into its three dimensional structure which is essentially determined by the sequence of amino acids (primary structure). However, it has become increasingly understood that glycans play an important role in determining the ability of glycoproteins to bind drugs and other ligands by ensuring the correct folding of the polypeptide backbone and therefore without it, the binding site would not form correctly. Therefore it is important to analyse glycan structures during studies interested in the functions of glycoproteins, such as that undertaken and documented.

Although cleavage of glycan chains into component monosaccharides does not allow the exact sequence to be determined, they can provide an indication of structural changes; especially when coupled to data gathered during analysis of the complete glycans (chapter 7). Gal is a key example because its presence is limited to the branches of complex N-linked glycans (Hashimoto *et al.,* 2004). GlcNAc is another however its levels have the potential to be misleading – it is not unique to the branches of these structures as two residues also form part of each pentasaccharide core alongside three Man ($GlcNAc_2Man_3$). As it is difficult to ensure the strong covalent interaction between the innermost GlcNAc and the Asn residues of the polypeptide chain is cleaved, an increase in its detection does not automatically denote changes in branching but perhaps improved cleavage of this bond. Nevertheless, when levels are increased in conjunction with Gal it is likely they signify structural changes in glycans.

Commercially sourced AGP was rendered an inappropriate representation of 'normal' AGP in this study because it was isolated using alternative methodologies to those implemented in this study – adapted from the large-scale methodology developed by Hao and Wickerhauser in 1973. All factors considered, it was deemed most suitable to use AGP isolated from 'normal' blood as the source material and isolation techniques

were common to those for patient samples, reducing the number of factors which may induce structural changes.

Results generated after acid hydrolysis and subsequent separation by HPAEC-PAD indicated a significant change in the glycosylation of AGP in individuals receiving methadone as an opioid replacement therapy compared to that from a 'normal' healthy population. All had significantly greater levels of Gal and GlcNAc. In the buprenorphine group, the levels appeared to be similar to the HR and LT groups but it did not reach significance. Patients D1 and H1 expressed significantly lower levels of Gal when compared to the titration group but there was no difference when compared to those of the 'normal' population. Although the increased expression of Gal and GlcNAc could infer increased branching, additional analysis of intact glycans was required (chapter 7). It could not be established without said analysis whether expression was a result of branching or perhaps elongation of the branches already present. Unlike AGP isolated from 'normal' blood, the monosaccharide Fuc was not detected in any patient sample. A negligible level or apparent absence of Fuc is typical of the glycans expressed by the AGP in healthy individuals; its expression is generally increased in pathophysiological conditions such as inflammation and cancer (Turner *et al.,* 1985; Listinsky *et al.,* 1998).

Also, as was mentioned in section 6.2, no patient AGP glycans contained GalNAc or Glc; this was expected as these monosaccharides are not associated with complex N-linked glycans. Instead, GalNAc is a common constituent of O-linked glycans while Glc expression generally targets the structure to the calnexin/calreticulin cycle because it should be cleaved prior to transfer from the ER lumen to the *cis* Golgi (refer to figure 1.3, section 1.1.3.1).

The separate use of TFA and HCl in achieving optimal hydrolysis of neutral and basic monosaccharides respectively had been discussed in chapter 3 with cleavage of Gal and GlcNAc being especially improved. If the monosaccharides were not effectively cleaved or became denatured, they would not have been detected during HPAEC separation because formation of correct oxyanion structures would be hindered thus an accurate picture of the glycan composition would not be obtained.

Zdebska and Koscielak (1999) reported that a low ratio of Gal or SA to Man in glycoproteins may reflect either underglycosylation of the outer portions of glycan or an excess of core Man. The current study suggested the opposite occurred in the AGP of patients studied as there was always a high level of Gal compared to Man.

It must be remembered that although suggestions of altered branching after analysis of this type were apparent, even if levels of Gal and GlcN were extremely high, it could not be unequivocally ascertained whether this was due to an increase in the number of branches or perhaps an increase in the length of the branches already present. Sialic acid (SA) residues may provide more detailed information as the residues terminate branches and therefore an increase in their presence suggests an increase in the number of branches. However, it is well known that acid hydrolysis causes the SA of glycans to become unstable and destroyed therefore undetected by HPAEC-PAD (Smith *et al.,* 1997). During monosaccharide analysis, it was sufficient to analyse the other components because not only is it accepted that the majority of branches are terminated by SA (if not, the exposure of Gal targets the structures for degradation or repair), but in the following chapter, when complete glycans were analysed, the SA residues remained intact to allow determination of the relative level of branching using HPAEC and an ELISA.

The moles mono/mol AGP data was compared to determine whether any disparity existed in the glycosylation of patient samples and 'normal' blood samples. Patients T1, T3 and LT6 had the greatest mol GlcN/mol AGP however only T1 also had a higher level of Gal compared to other individuals in the treatment group. Although the level of AGP isolated from patients T1 and T3 (table 5.2) were relatively high (0.34 and 0.25mg/mL respectively) for the group, that of LT6 was comparatively similar to others of the LT group (0.173mg/mL). An increased synthesis during the APR could not explain the elevations in the monosaccharide because the analysis was performed on a common mass of AGP (50µg) as calculated from the levels isolated thus any difference could only be due to glycan expression. Support of this concept was provided by the relatively consistent level of Man detected in all samples; the number of pentasaccharide cores should not change.

Interestingly, the two oldest patients (LT4 and LT6 - 44 and 51 years old respectively) presented high levels of Gal (4.46±0.37 and 4.64±1.89 mol/mol AGP respectively)

compared to other members of the group (mean 3.51 ± 0.91mol/mol AGP) however, the high standard deviation for LT6 demonstrated that the value was not necessarily as high as first suggested. Only LT6 presented much higher than average GlcN levels (10.57 ± 0.36 mol/mol AGP), in fact this was the highest value out of all the patients, that of LT4 was similar to the mean.

Various teams have indicated the presence of altered glycosylation of AGP with age when individuals were considered healthy while others disagreed (Davis *et al.,* 1985; Veering *et al.,* 1990). In those who suffer from inflammatory conditions, the incidence of which often increases with age, the appearance of alterations in the glycosylation are well documented (Smith *et al.,* 1994; Rydén *et al.,* 2001). It was therefore unlikely that the alterations in AGP glycosylation suggested by HPAEC-PAD data were due to age, especially as most participants had levels of GlcN and Gal consistent with others from their respective treatment groups. Moreover, a study with only two patients >40 years old does not provide enough data to ensure they are representative of this population.

Similarities were found between the single patient receiving pure heroin as a replacement treatment and the 'normal' population, not only with respect to the level of AGP isolated but also in the level of all the monosaccharides constituting the glycans, and indicated that a change in type of drug administered may stimulate, or at least augment changes in AGP glycosylation. H1 remained on a similar type of drug to that which was abused - the stresses associated with factors such as a new drug dosage regimen may have been more manageable and prevented the onset of an APR. Also, the continuation with a similar drug, albeit in a more pure form, would not necessarily stimulate a reaction as the body had become accustomed to it over the duration of illicit drug use. However it could only be speculated upon as H1 was the only patient undergoing such treatment. Rostami-Hodjegan *et al.* (1999) reported that individuals abusing heroin had greater levels of AGP however these individuals were reported to be exhibiting withdrawal unlike patient H1. Even if monosaccharide levels were similar, it did not necessarily signify a common structure, merely that the overall composition was analogous, the order and linkages binding the monomeric units could vary widely. Such information could not be gathered using the technique described in this chapter.

There are no known studies which have investigated the potential that AGP glycosylation may be altered in individuals undergoing opioid replacement therapy,

potentially affecting the efficacy of treatments. Therefore it was not known if the data generated in this small preliminary study was representative of patients on the whole. The results from monosaccharide analysis in this study have indicated that although altered glycan composition of AGP did not appear to be associated with any particular treatment stage, all methadone patients had a demonstrable change in glycosylation hence there was potential that its subsequent ability to bind basic drugs like methadone may have been affected, altering its efficacy. Prior determination of the glycosylation of patient AGP may be useful when considering the type of therapy to be administered. For example, if patient AGP expressed a high level of branching – as indicated by Gal and GlcNAc residues – and this was found to be associated with increased binding to methadone, it would be beneficial to consider an alternative therapy as high doses of methadone would be required to allow for inactivation by AGP, subsequently increasing the risk of toxicity.

It was important to perform subsequent analysis of complete glycans as it is the whole structure which determines the ability of a glycoprotein to execute its functions.

SUMMARY STATEMENT

The monosaccharide composition of glycans cleaved from the polypeptide backbone of AGP differs significantly (in terms of Gal and GlcNAc content) between that isolated from 'normal' heparinised blood and from individuals undergoing various stages of opioid replacement therapy. There was no significant difference between individuals receiving methadone as a substitute. Results suggested an increase in branching therefore additional oligosaccharide profiling was required – as discussed in the following chapter.

Chapter 7

AGP Oligosaccharide Analysis

Results and Discussion

7.1   Introduction

Understanding the close relationship between the specific structure of AGP glycans and the influence they have on the functions performed by the glycoprotein is paramount to the hypothesis of this study.

HPAEC is a highly reliable method used in the detection and separation of glycans thus it was implemented to separate those generated upon cleavage of the isolated AGP samples by the enzyme PNGaseF. Similar to monosaccharide compositional analysis, the separation of whole glycans relies upon the presence of weakly acidic sugars in a basic media (pH13) which undergo electrocatalytic oxidation when in contact with the gold electrode to form oxyanions. The conditions required for its optimal performance differ slightly to account for the new analytes of interest. Along with negatively charged terminal 9-carbon carboxylated SA residues (specifically N-acetylneuraminic acid, NeuAc), the negative charge contributed by component monosaccharides under alkaline conditions determine the strength with which the glycans interact with the stationary phase - a pellicular anion exchange resin (ammonium-bonded beads) ionically attached to large cation-exchange spheres. In the late 1980s Townsend and colleagues demonstrated that glycans could be separated solely by the presence of SAs, using relatively acidic pHs as low as 4.6 (Townsend *et al.,* 1988; 1989). The formation of oxyanions under strongly alkaline conditions enhances separation, allowing isomers to be recognised as structurally distinct based on the differing pKa values of constituent monosaccharides (Lee, 1990).

The sensitivity of HPAEC-PAD technology (shown to be 10-100pmol by Hardy and Townsend, 1988) owes itself to the detection and separation of glycan chains in addition to that of monosaccharide analysis. Again, the introduction of pulsed amperometric detection (PAD) to HPAEC vastly improved the sensitivity of detection by ensuring periodic decontamination of the sugar oxide coating which formed on the electrode under the highly alkaline conditions. Throughout the late 1980's and early 1990's, HPAEC was by far the most commonly used and sensitive technique available for glycan structural analysis after enzymatic or chemical release of the oligosaccharides from the underlying protein. One of the primary advantages of HPAEC-PAD was that

sensitivity does not require prior derivatisation to enhance hydrophobicity as is necessary in many other techniques such as MS (Lee, 1990).

Removal of glycans bound to the anion-exchange resin requires an increasing linear gradient of a pushing agent, commonly sodium acetate (50-250mM NaOAc over 30min) which competes with the glycans for the column surface thereby displacing the oligosaccharide chains. The gradient of NaOAc ensures that all structures are released, the most difficult being highly branched sialylated structures. NaOH is used at 100mM to provide an alkaline pH. There are two major forms of SA, N-glycolyl- and N-acetylneuraminic acid, which differ by the presence or absence of a single -OH group. Despite this small difference, HPAEC-PAD can successfully separate them by more than ten minutes when the NaOAc gradient is used (Behan and Smith, 2010). Without sodium acetate it would not be possible to dislodge the glycans from the column.

If two similar oligosaccharide structures differing only in degree of sialylation were analysed, elution would occur in separate charge bands because the collative negative charge differs substantially (figure 7.1). The size of the glycans subequently determine at what stage they elute within a given charge band; glycans with a greater monosaccharide composition elute later than glycans with the same sialylation but fewer monosaccharides. Sugars with more carbon atoms have an increased number of -OH groups and negative charge, thus they are retained for longer (Paskach *et al.,* 1991). For example, a mixture of bi-sialylated bi-antennary, tri-antennary and tetra-antennary structures would elute in this order within the bi-sialylated charge band because the structures increase in size and therefore in strength of interactions formed with the column. By extension, it is possible for a highly branched, but incompletely sialylated, glycan to elute before a fully sialylated structure with fewer branches e.g. a bi-sialylated tetra-antennary glycan would elute earlier than a tri-sialylated tri-antennary structure. Such circumstances are relatively uncommon because the absence of SA at branch termini causes exposure of the Gal to which it is attached. This would normally target the structure for degradation, unless another structure is expressed in its place.

Townsend and colleagues (1988 and 1989) also indicated the possibility of separating oligosaccharide chains by manipulating the ability of Gal to bind the pentasaccharide core via a β(1, 4) or β(1, 3) linkage to GlcNAc. The former elutes faster because the

-OH groups are less able to associate with the column surface when converted to oxyanions at pH 13 (Townsend *et al*., 1988; Townsend *et al*., 1989).

It is also possible to distinguish glycans with a common degree of sialylation by the types of linkage existing within the chains. NeuAc for example is capable of binding to the outer Gal residue through an α(2, 6) or α(2, 3) linkage; the greater the expression of the latter, the stronger is the interaction with the column (Townsend *et al.,* 1988). This is because α(2, 6) causes elimination of the oxyanion effect at the C6 –O⁻ group of Gal (formed at the –OH group under alkaline conditions); it then becomes involved in the linkage to NeuAc. Therefore, the overall charge is decreased causing it to be more easily eluted by NaOAc than a similar structure expressing an α(2, 3) linkage which does not impede the interaction of the oxyanion group of C6 (Townsend *et al.,* 1989). The two types of linkage can be detected using specific lectin conjugates and performing an ELISA; the relative level can thereby be investigated.

7.2   Results

7.2.1   *HPAEC oligosaccharide analysis*

The generation of distinct peaks in the chromatograms is thought to be due to the absence of tautomerisation, epimerisation and other Lobry de Buyn-van Ekenstein transformations; reactions which carbohydrate structures may undergo at high pH subsequently interfering with chromatography. However at room temperature and during the time-course of the chromatography, it is reported that these do not arise (Lee, 1990; Dionex Corp., 1994).

HPAEC-PAD firstly separated glycans into specific charge bands. Those glycans expressing a single SA residue elute first (10-20 minutes), followed by bi-sialylated (20-30minutes), tri-sialylated (30-40minutes) and finally tetra-sialylated, eluting between 40-50 minutes. Figure 6.1, demonstrates this with an oligosaccharide library containing bi-, tri- and tetra-sialylated chains; each group of peaks representing the different degrees of sialylation as labelled. The library was used as a comparison trace for patient

samples, allowing identification of the sialylation of branches. Although monosaccharides can be easily quantified through the use of calibration curves, this was not possible for oligosaccharides because there was no similar availability of pure forms to use as reference material.

A commercial AGP sample was run and compared to the library, generating peaks within the same charge bands however there was a reduction in the variability of tetra-sialylated structures, indicated by fewer peaks (figure 7.2a). Similar results were found for AGP isolated from 'normal' heparinised blood samples (figure 7.2b). Although some tetra-sialylated structures appeared to be present, favouritism was demonstrated for bi-sialylated forms. There were fewer peaks than in commercial sample suggesting less variability.
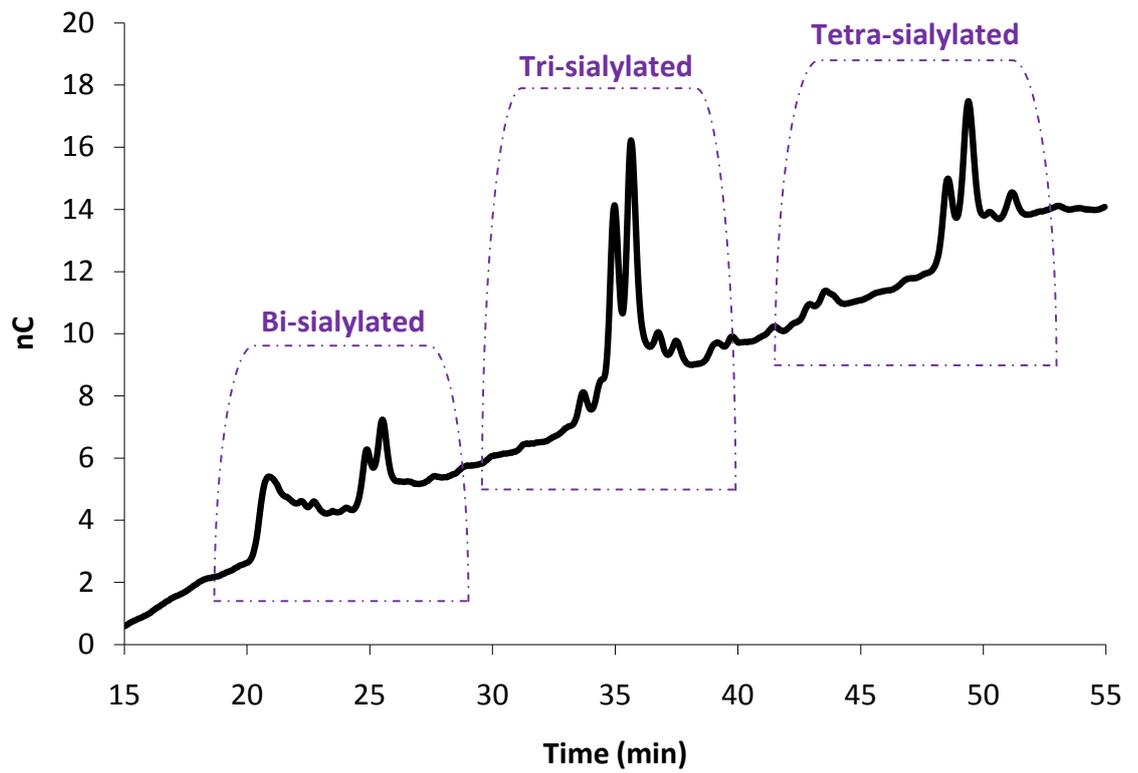
Figure 7.1. Oligosaccharide library trace generated during separation by HPAEC. Separation based on the charge of oligosacchiarde chains as measured using nano culons (nC). Degree of sialylation highlighted in **purple.**
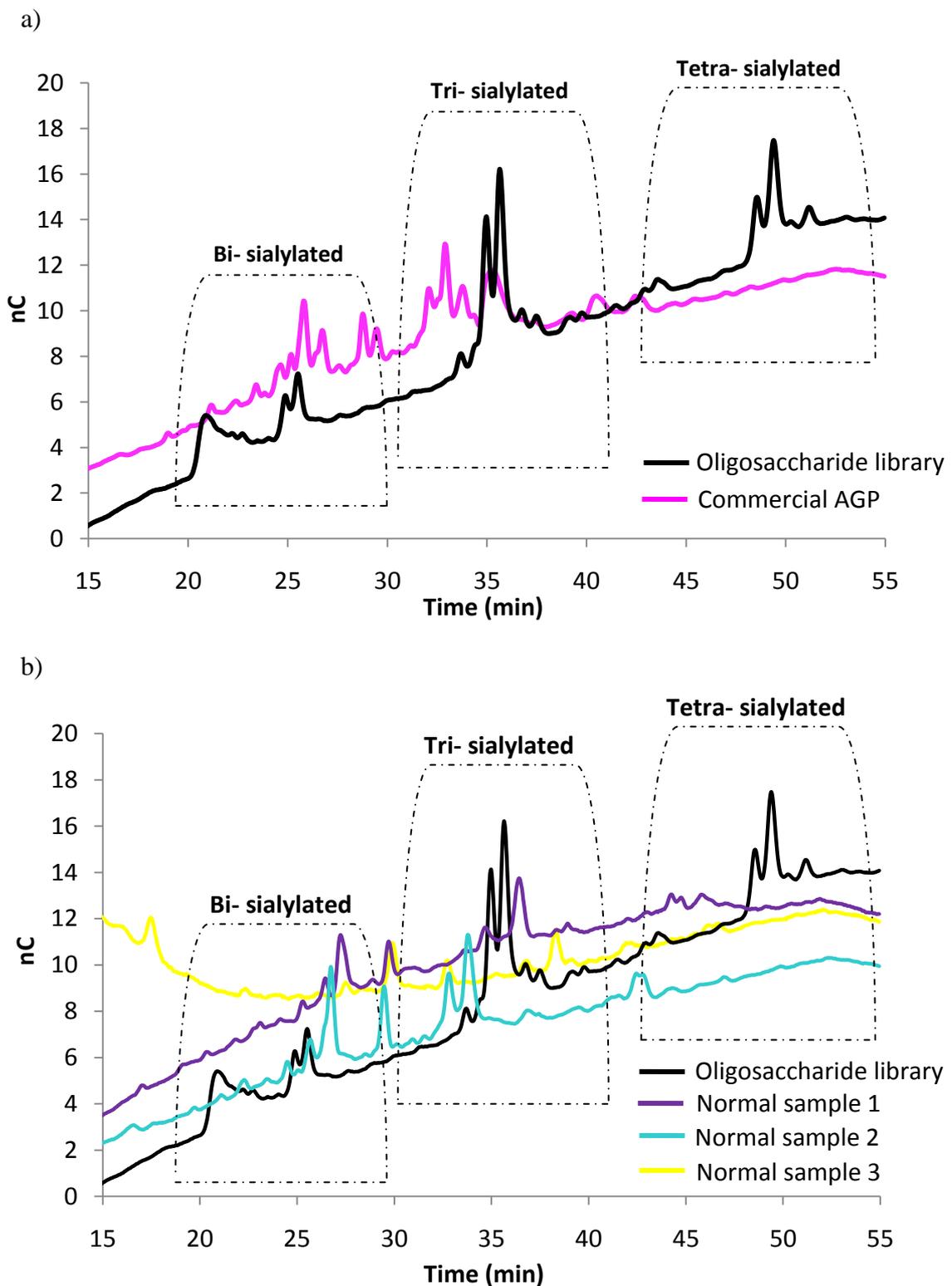
Figure 7.2. **HPAEC chromatograms of oligosaccharide library versus a) commercially sourced AGP and b) AGP form 'normal' heparinised blood samples.** Separation based on the charge of oligosaccharide chains as measured using nano culons (nC). Degree of sialylation highlighted in dashed boxes. 'Normal' sample data represented the oligosacchairde profiles of AGP isolated from heparinised blood samples obtained from the Blood Transfusion Service.

Patient samples from the titration phase of therapy (n = 5) were analysed and each chromatogram generated during HPAEC was graphed alongside the library (figure 7.3). In general it could be seen that, when compared to the library, these glycans displayed a preference for the charge bands representing bi- and tri-sialylated structures with very few peaks in the tetra- sialylated region. This appeared to be common with the 'normal' and commercial samples of figure 7.2 suggesting the AGP of these individuals had low level branching. However, all titration samples displayed some, albeit relatively few, peaks in the tetra-sialylated charge band. The most noteworthy being T3 and the least T2. Additionally, the chromatograms of T1 and T3 displayed higher levels of branhes terminated in three SA residues, with much larger distinct peaks compared to the other samples within the group; peaks were more comparable with those in the library.

The two main peaks within the tri-sialylated charge band of T1 (34 and 35 minutes) were almost identical to the library in size and shape and probably represented similar structures, however they eluted slightly earlier. Perhaps this could explain why the peak at approximately 42 minutes lay at the interface of the tri- and tetra-sialylated bands; it may be a tetra-antennary structure that eluted earlier than expected in line with the previous peaks. A similar pattern was identified at this time point in the chromatogram of patient T2 but the remainder of the profile was composed of smaller peaks. There was a novel peak at approximately 53 minutes in T3 AGP, unlike any of the other titration group samples where glycans tended to elute earlier in the tetra-siaylated region representing shorter chains. HPAEC-PAD separation of T3 glycans also generated numerous peaks in the tetra-sialylated region, more so than any other titration pateint.

There were also two small peaks (17-18minutes) in the chromatogram of patient T1 and a relatively large peak at approximately 16 minutes in that of T2 which may indicate the presence of monosialylated oligosaccharides. This was unusual and unexpected because the absence of terminal SA residues causes the underlying Gal to be exposed which should cause its subsequent degradation.

Overall the glycans expressed by AGP isolated from those in the titration phase of therapy appeared to show preference for branching of the bi- and tri-sialylated type, much like that in the 'normal' and commercial samples (figure 7.2).
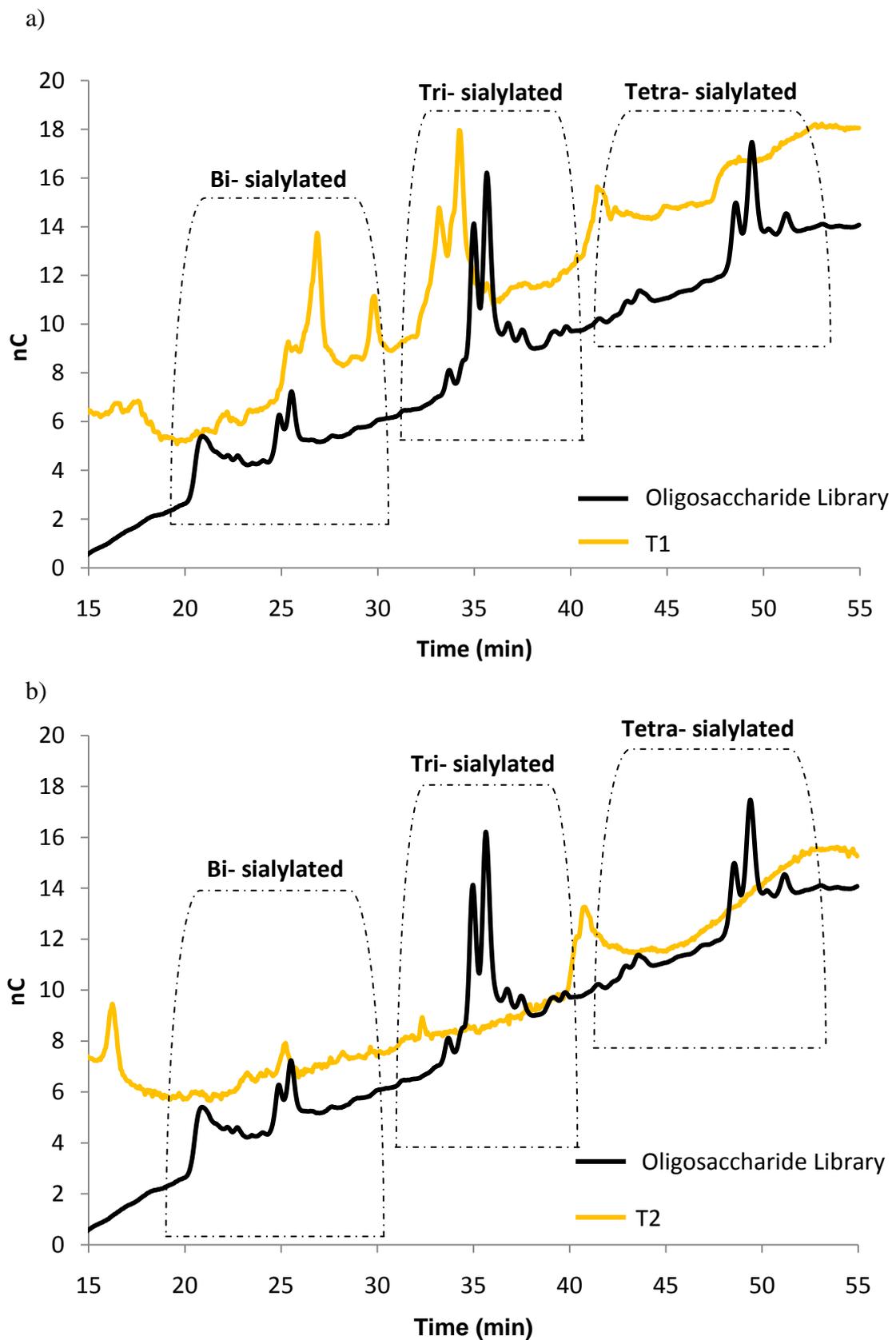
Figure 7.3. **Chromatograms of oligosaccharide library versus titration patients** a) T1 and b) T2 (orange). Separation based on the charge of oligosaccharide chains as measured using nano culons (nC). Degree of sialylation highlighted in dashed boxes.

c)

d)
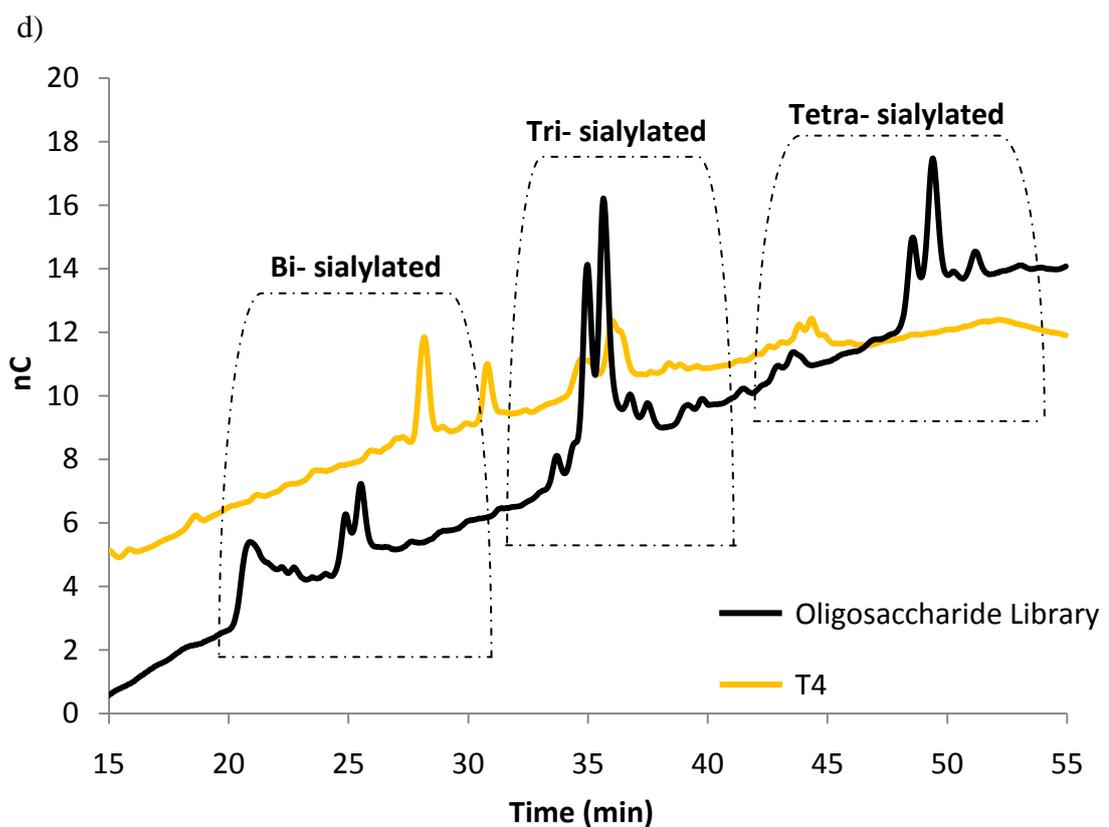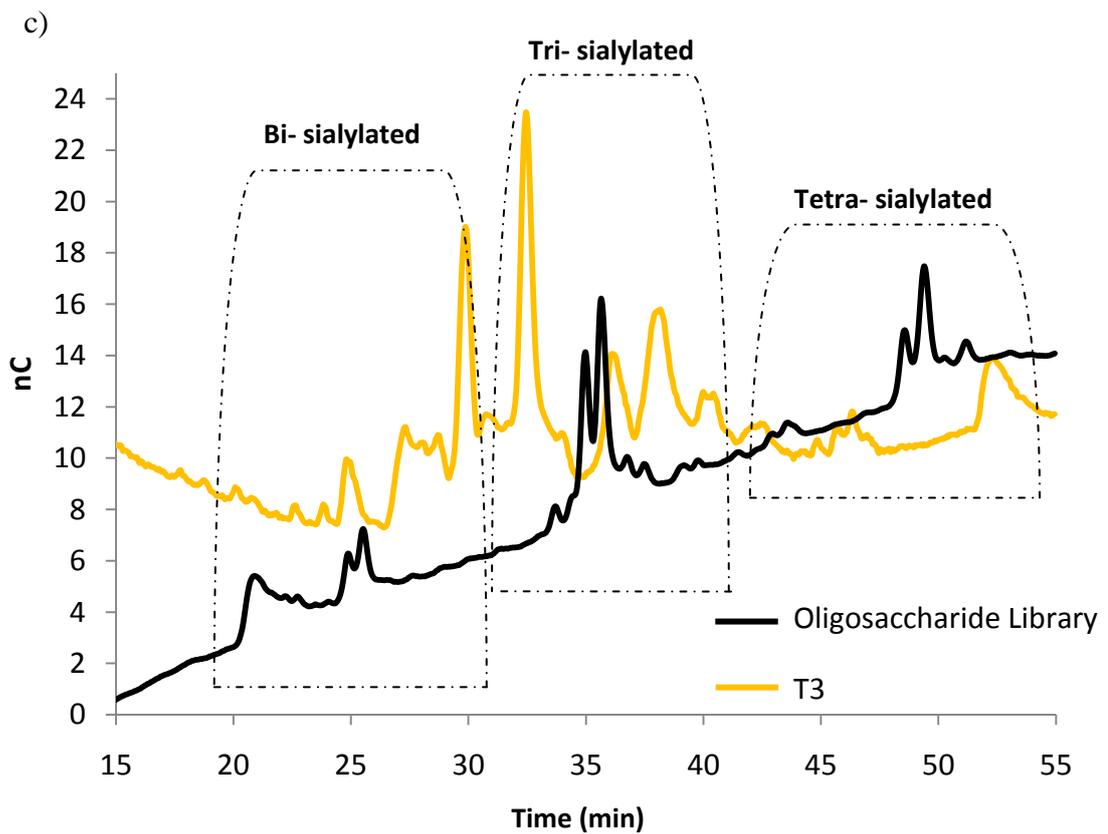
Figure 7.3 continued. Chromatograms of oligosaccharide library versus titration patients c) T3 and d) T4 (orange). Separation based on the charge of oligosaccharide chains as measured using nano culons (nC). Degree of sialylation highlighted in dashed boxes.

e)
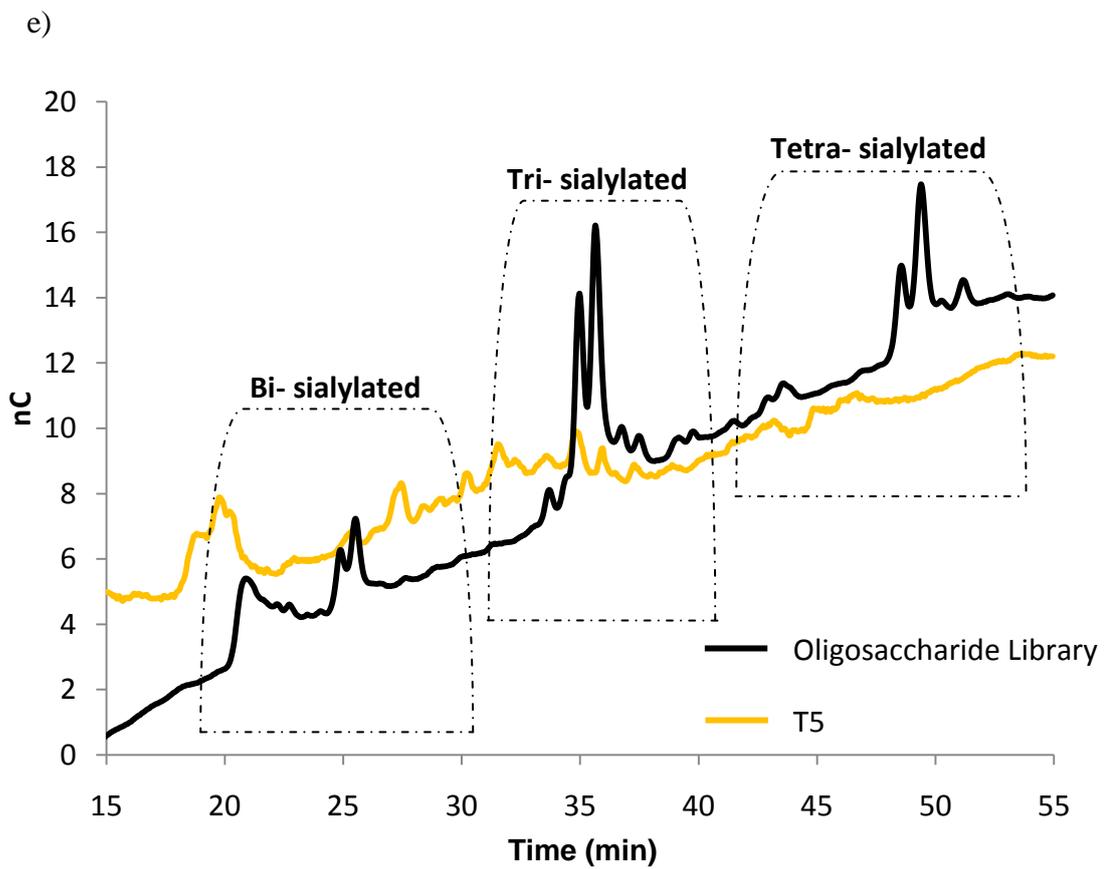
Figure 7.3 continued. **Chromatogram of oligosaccharide library versus titration patient** e) T5 (in orange). Separation based on the charge of oligosaccharide chains as measured using nano culons (nC). Degree of sialylation highlighted in dashed boxes.

Figure 7.5 illustrates the HPAEC-PAD separation of AGP glycans from patients in the harm reduction (HR) scheme alongside that of an oligosaccharide library. The only commonality between the chromatograms obtained for this group was that all displayed bi-sialylated structures. Apart from this, the three samples representing the HR phase of opioid replacement therapy shared very few similarities with respect to their oligosaccharide profiles.

HR1 displayed few peaks in any other charge band, with no discernable tetra-sialylated structures and limited evidence of tri-sialylated, although there was a small but broad peak at approximately 39 minutes which suggested there were some glycans terminated in three SA residues. The large peak at 15 minutes followed by another smaller peak may represent a large number of mono-sialylated AGP glycans.

Although there were few peaks spread throughout the chromatogram in pateints HR1 and HR3, there were some in HR2, albeit much more broad than that of the library or patients from the other therapy groups, suggesting more of this particular glycan structure was expressed. It should be noted that the chromatogram of sample HR3 may show more peaks if the scale was truncated; the peak in the tri-sialylated region was amost twice the height of the library therefore dwarfs the smaller peaks because the y-axis scale accounted for the highest value.

a)

b)
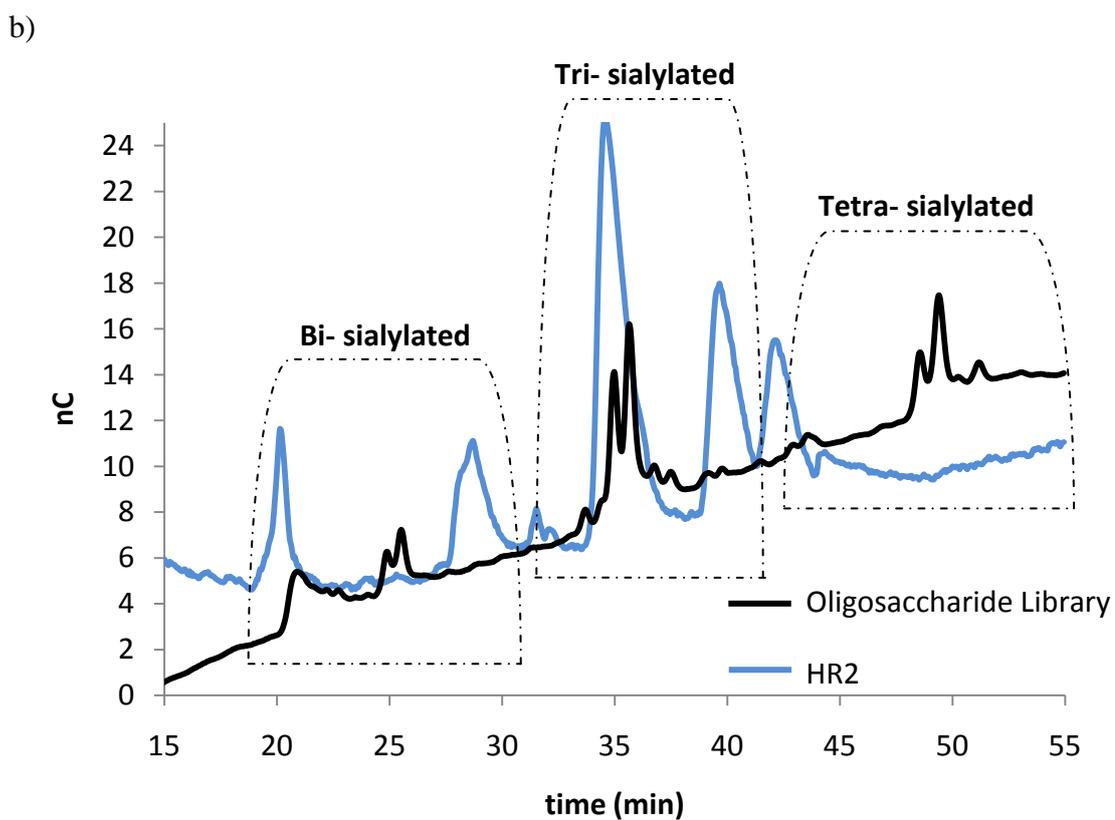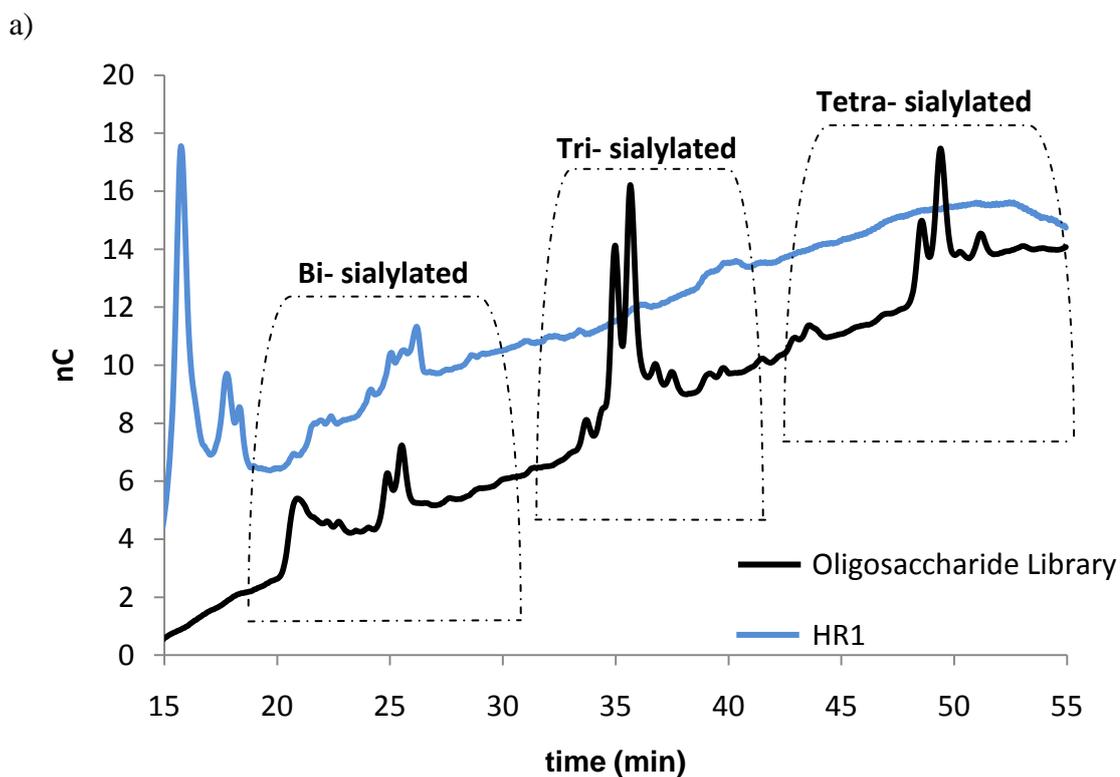
Figure 7.4. Chromatograms of oligosaccharide library versus harm reduction methadone patients a) HR1 and b) HR2 (in **blue**). Separation based on the charge of oligosaccharide chains as measured using nano culons (nC). Degree of sialylation highlighted in dashed boxes.

c)
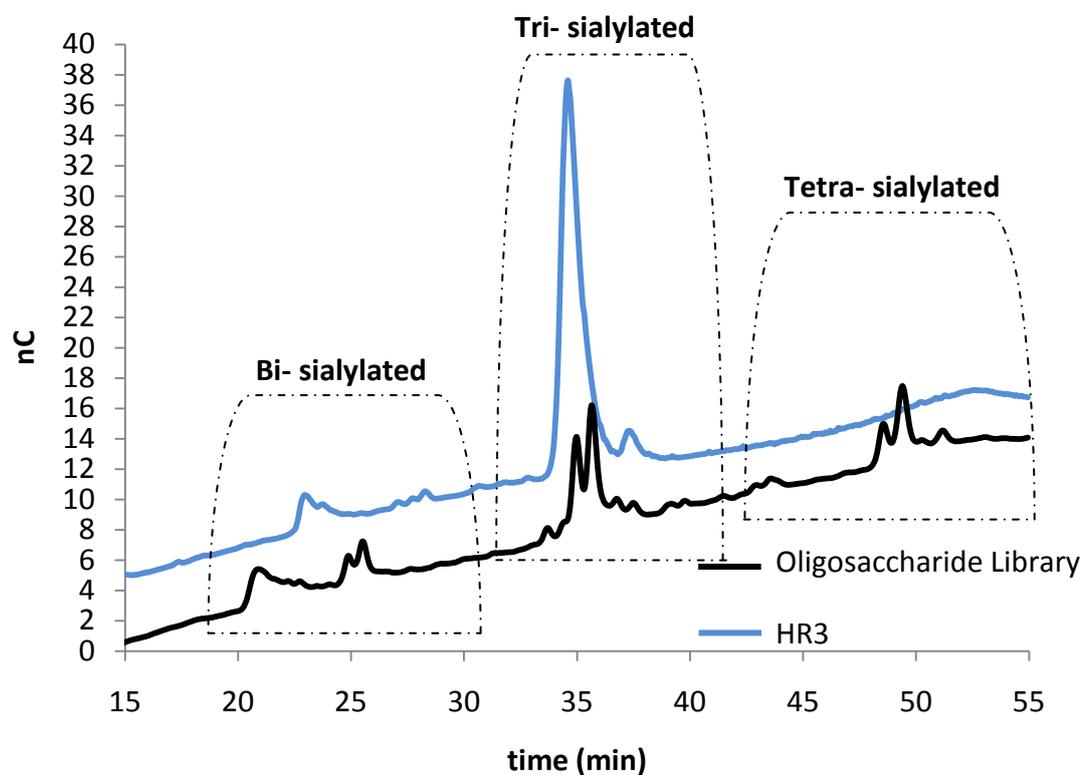
Figure 7.4 continued. Chromatograms of oligosaccharide library versus harm reduction methadone patients c) HR3 (in **blue**). Separation based on the charge of oligosaccharide chains as measured using nano culons (nC). Degree of sialylation highlighted in dashed boxes.

Subsequently, chromatograms generated during the HPAEC-PAD analysis of glycans cleaved from the AGP of long-term methadone therapy (LT) patients were graphed alongside the oligosaccharide library (figure 7.5).

Like the AGP isolated from patients within the titration phase of opioid-replacement therapy, the glycans of LT patients appeared to be mainly bi- and tri-sialylated. The peaks of LT1, LT3, LT5, LT9 and LT10 chromatograms were large and more distinct than in other members of this treatment group such as LT4, LT6 and LT7. Dissimilar to the titration phase group however, the peaks representing tetra-sialylated structures in the chromatograms of some LT patients (for example LT4, LT9 and LT10) were more defined. There was one main peak present in the chromatogram of LT1 within the tetra-sialylated region eluting at approximately 43 minutes suggesting most tetra-antennary glycans existing in their AGP were of a common structure. Comparison of LT9 and LT10 chromatograms suggested similar glycans were expressed, the main difference being that peaks in LT10 were generally much larger.

LT1, LT3 and LT5 all expressed peaks in the latter region of the bi-sialylated charge band along with the early part of that corresponding to tri-sialylated structures, suggesting numerous long bi- and short tri-antennary structures were present. The intial peaks detected in figure 7.4g (LT7) could represent mono-sialylated glycans (~17minutes).

The chromatogram of patient LT8 had a high frequency of peaks, albeit small, in the tri-sialylated region. There appeared to be a preference for increased branching of the glycoforms of this AGP sample; very few peaks were recorded in the bi-sialylated region but some in the tetra-sialylated region. The glycans of AGP isolated from patients LT9 and LT10 were also predominantly tri-sialylated.

Therefore, there were similarities with the titration, 'normal' and commercial groups in that all appeared to have a high proportion of tri-sialylated structures however, within the LT, variations existed. Some patients showed apparent preference for more highly branched structures (e.g. LT4 and LT10) while others for less branched (e.g. LT3). Those with peaks in the most highly charged band displayed more distinctive peaks than any in the titration phase where peaks were noted.
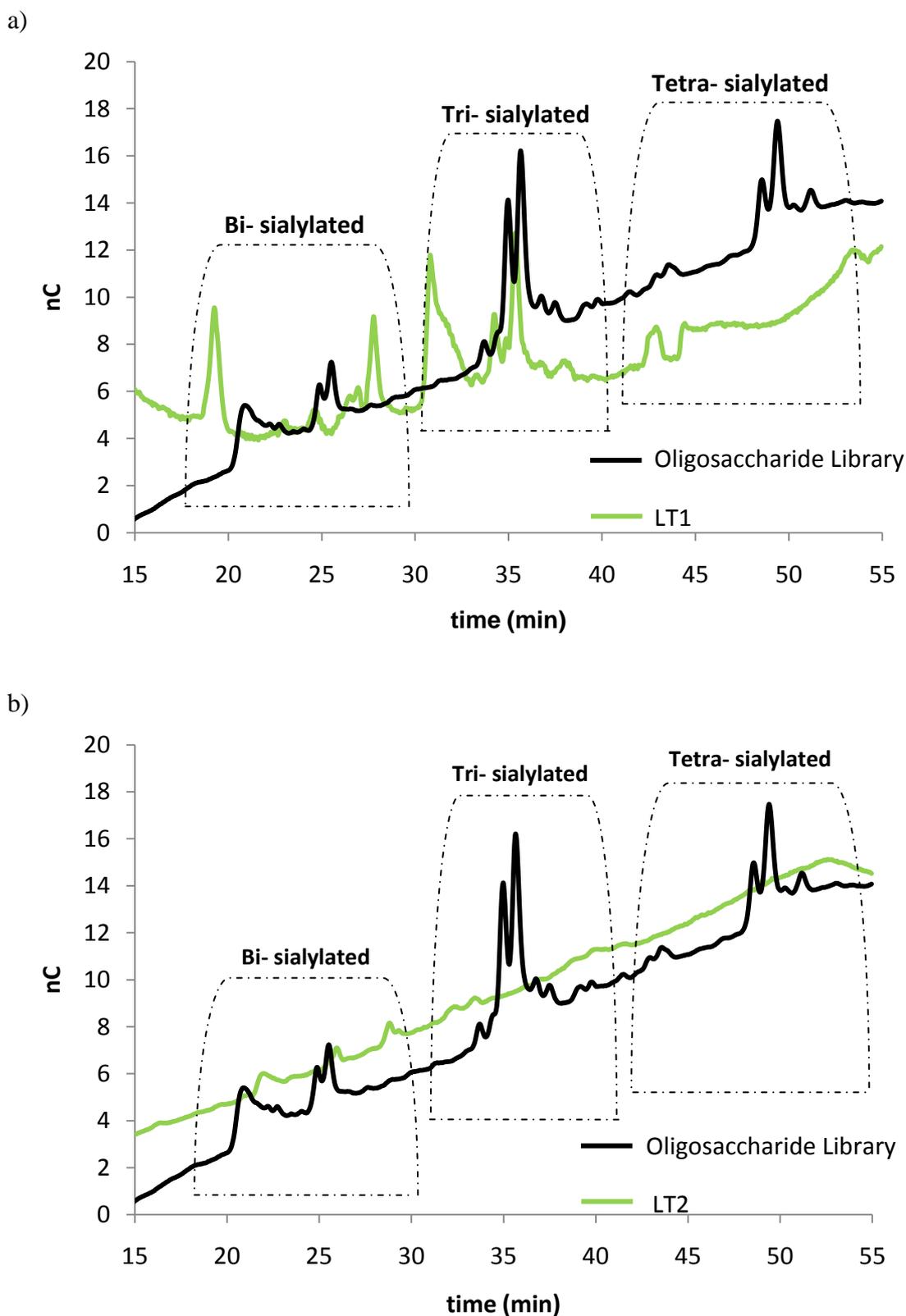
Figure 7.5. Chromatograms of oligosaccharide library versus long term methadone patients a) LT1 and b) LT2 (in green). Separation based on the charge of oligosaccharide chains as measured using nano culons (nC). Degree of sialylation highlighted in dashed boxes.

c)

d)
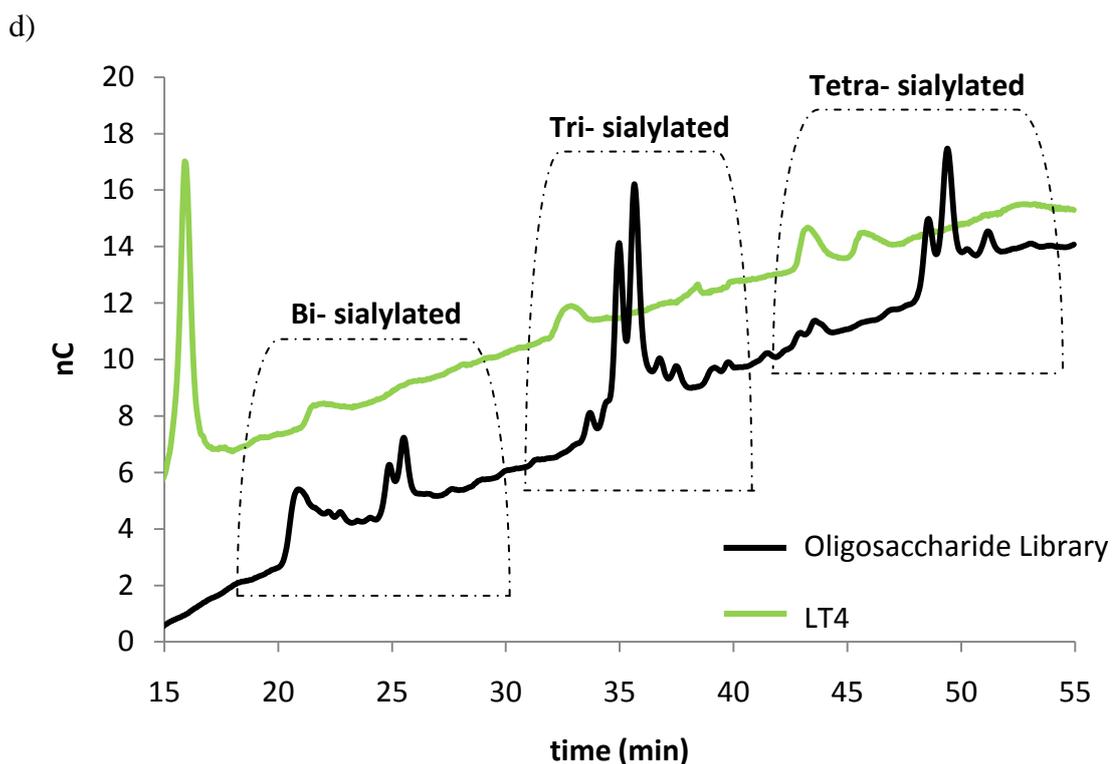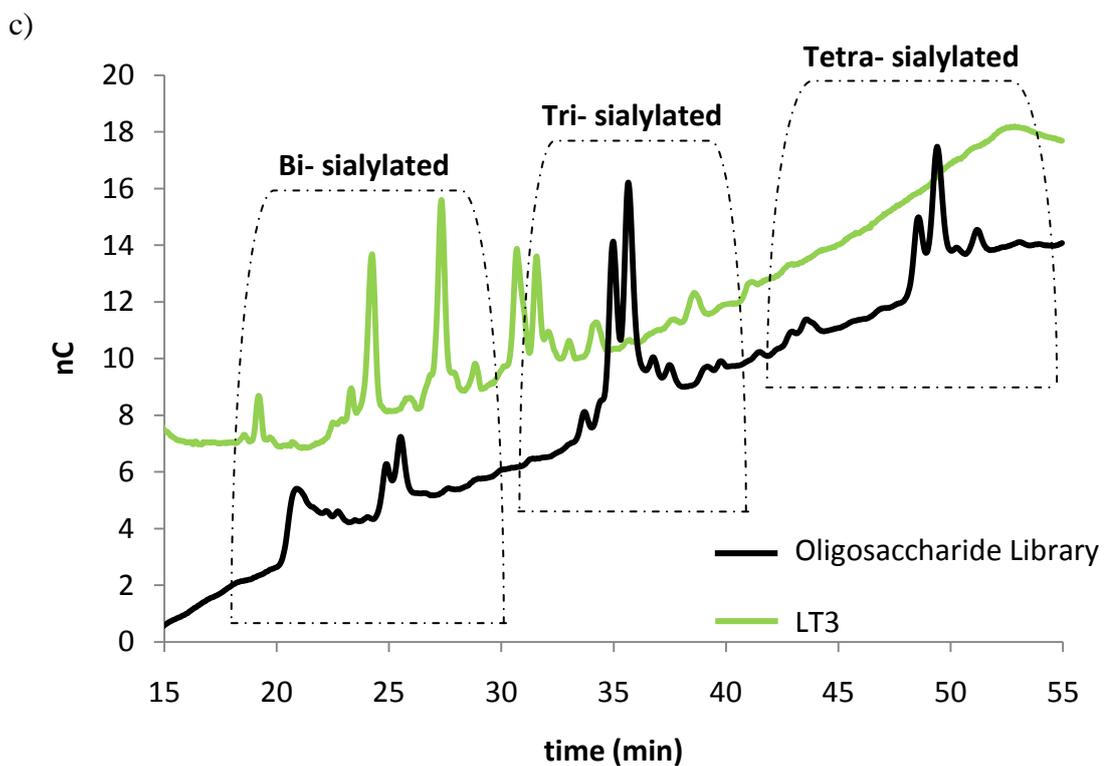
Figure 7.5 continued. Chromatograms of oligosaccharide library versus long term methadone patients c) LT3 and d) LT4 (in **green**). Separation based on the charge of oligosaccharide chains as measured using nano culons (nC). Degree of sialylation highlighted in dashed boxes.

e)

f)
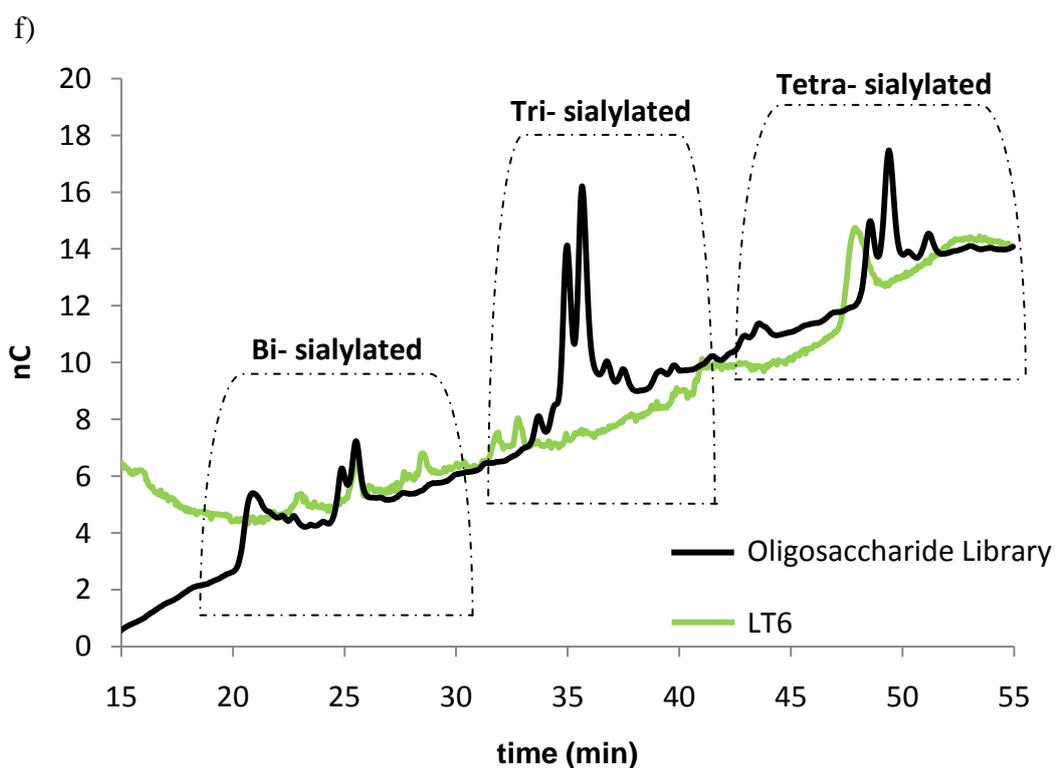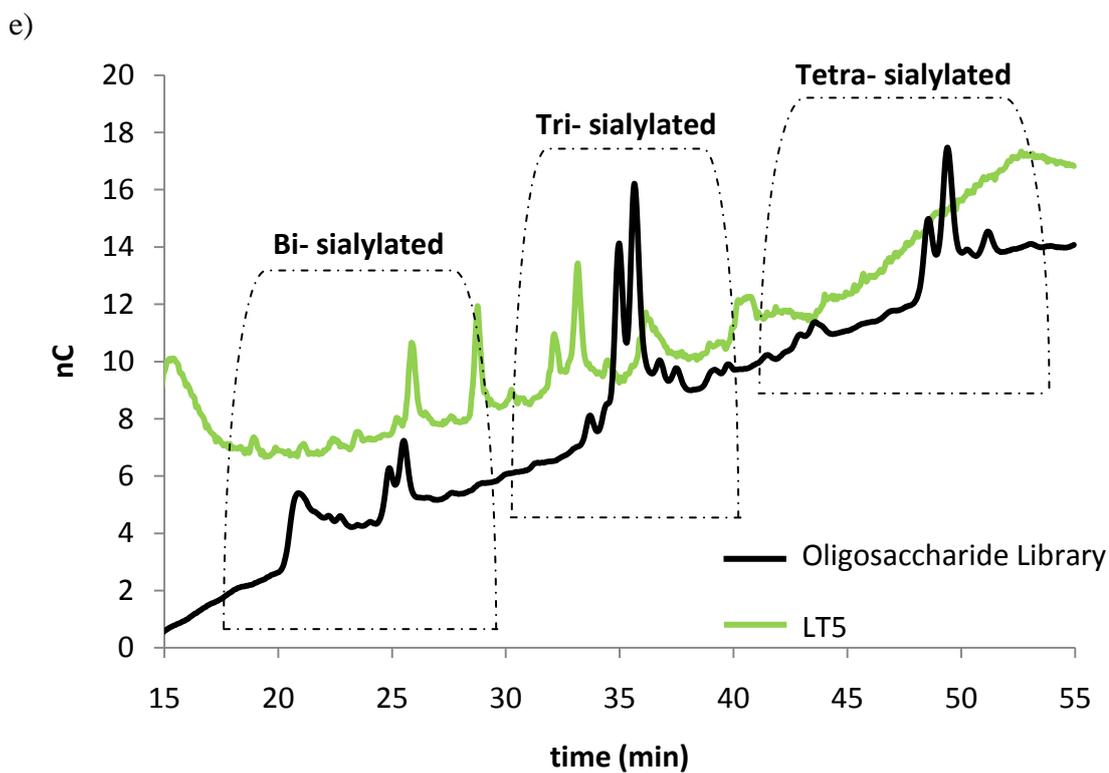
Figure 7.5 continued. Chromatograms of oligosaccharide library versus long term methadone patients e) LT5 and f) LT6 (in green). Separation based on the charge of oligosaccharide chains as measured using nano culons (nC). Degree of sialylation highlighted in dashed boxes.

g)

h)
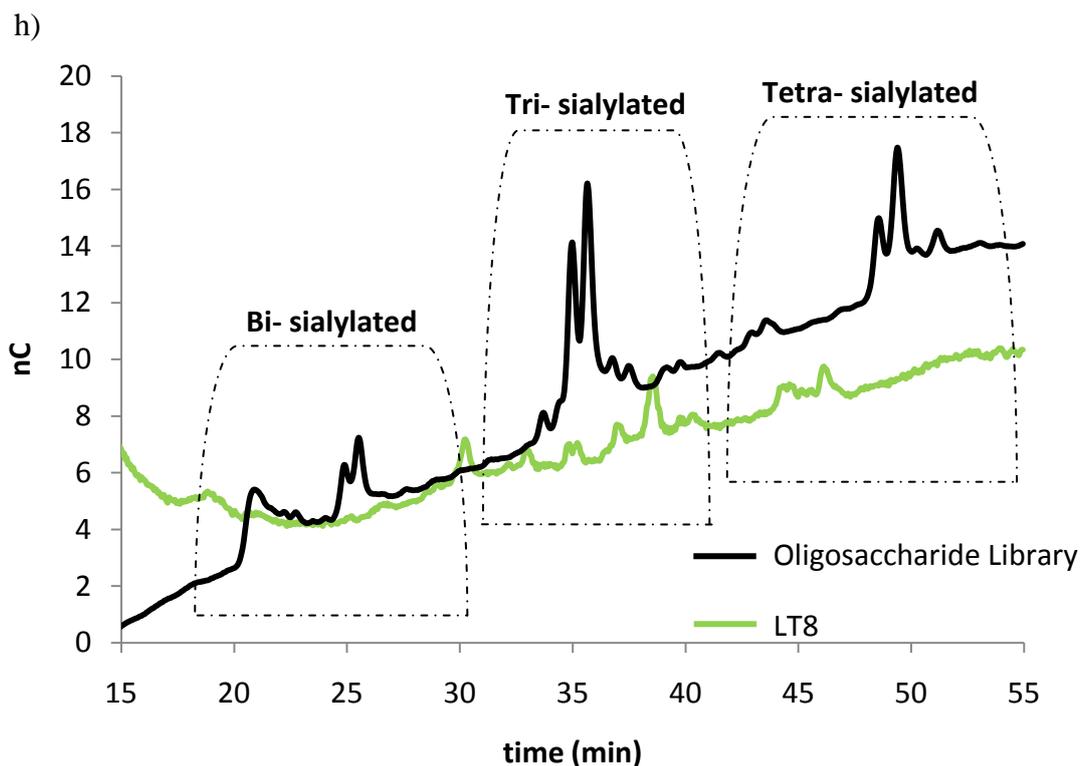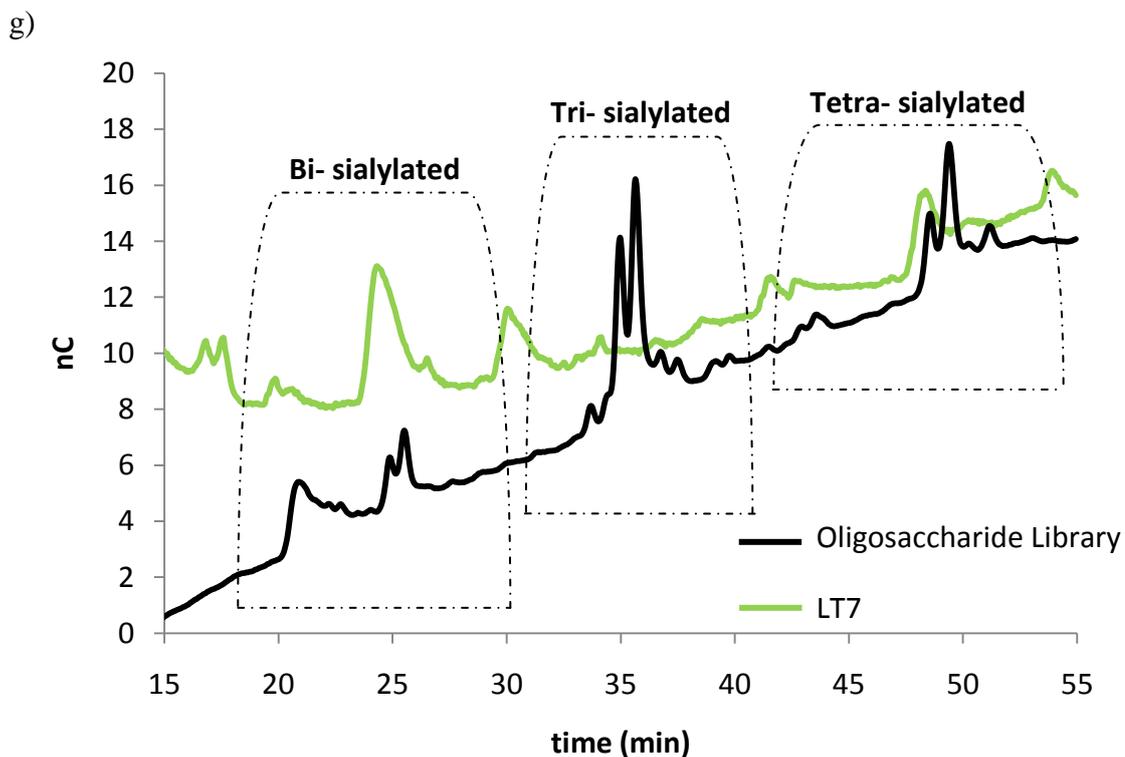
Figure 7.5 continued. Chromatograms of oligosaccharide library versus long term methadone patients g) LT7 and h) LT8 (in **green**). Separation based on the charge of oligosaccharide chains as measured using nano culons (nC). Degree of sialylation highlighted in dashed boxes.

i)
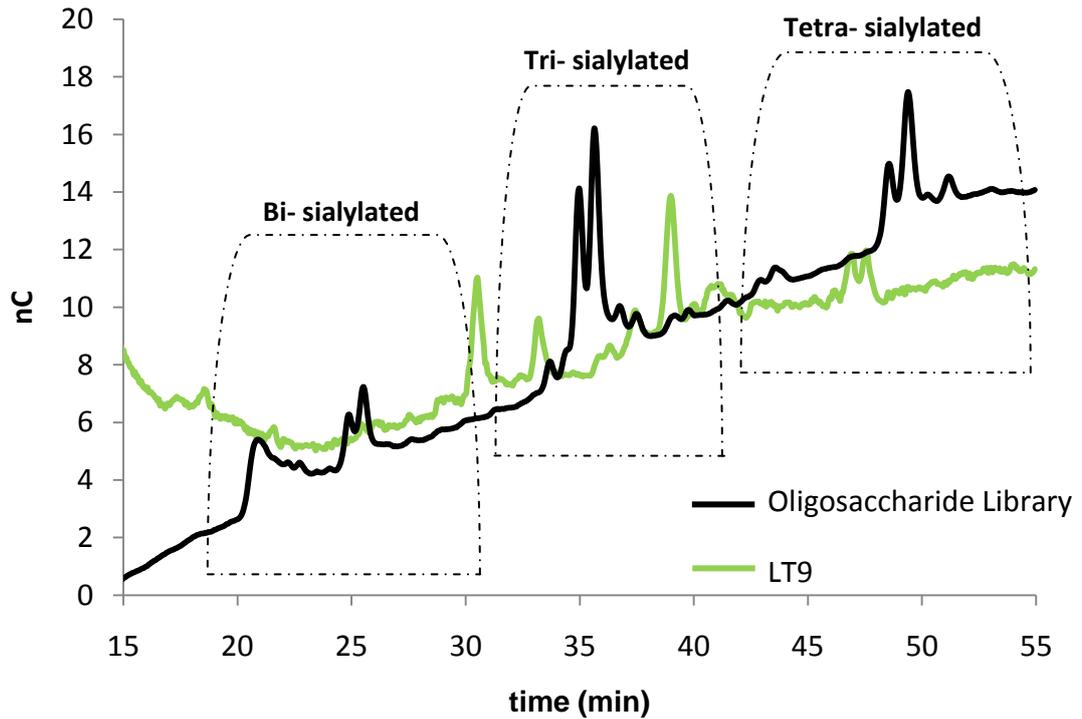
j)
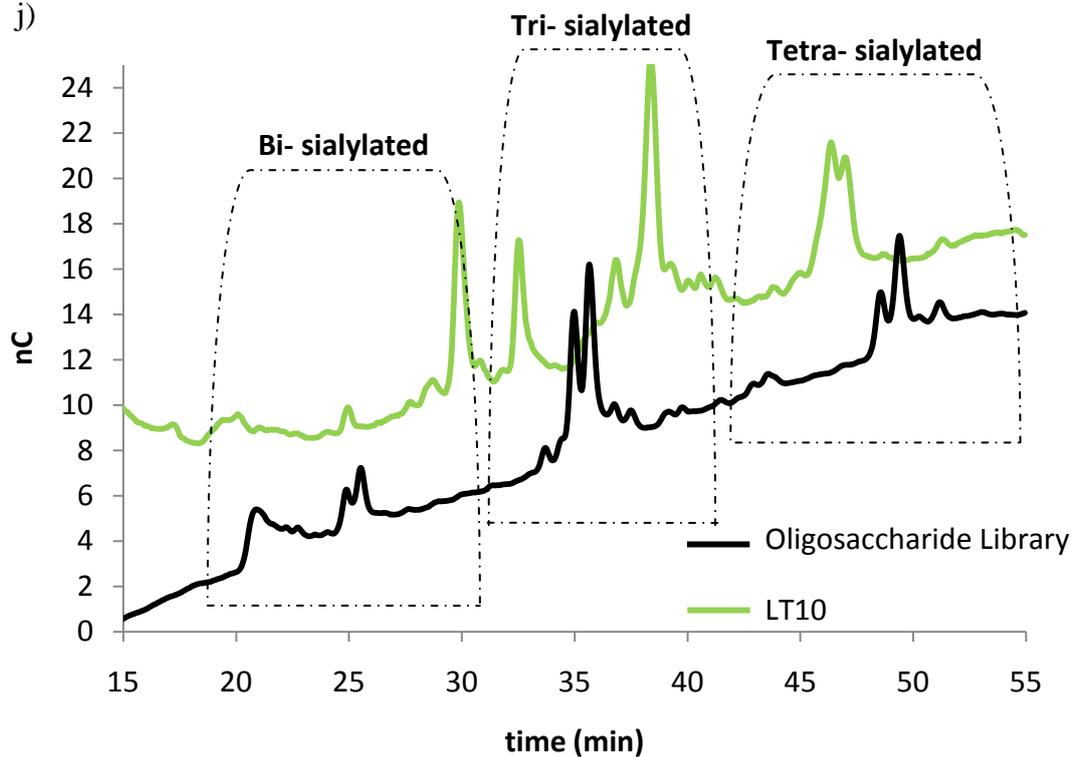
Figure 7.5 continued. Chromatograms of oligosaccharide library versus long term methadone patients i) LT9 and j) LT10 (in green). Separation based on the charge of oligosaccharide chains as measured using nano culons (nC). Degree of sialylation highlighted in dashed boxes.

The remaining samples analysed from patients undergoing alternative therapies were graphed in figure 7.6.

Patient B1 (receiving buprenorphine) displayed evidence of all types of sialylation as did B2. However the chromatograms differed widely with small peaks (B1) representative of strucutres eluting during the later stages of the bi-sialylated charge band and early on in the tri-sialylated band. Sample B2 had large peaks at approximately 20, 25 and 30 minutes – suggesting two main bi-sialylated and one main tri-sialylated structure were expressed by this AGP. The proflie of B1 was more comparable to B3, where peaks were of similar size in all charge bands suggesting similar levels of structures. An exception was that AGP isolated from B1 appeared to display tetra-sialylated structures.

Although the AGP isolated from pateint B1 and B4 expressed some tetra-sialylated glycans, the structures must vary because the peaks which represented them eluted at different stages within the charge band. The chromatogram of B4 indicated structures were mainly tri-sialylated but all peaks were very small. It was quite similar to that obtained for LT6 in figure 7.5f.

The chromatogram generated upon HPAEC-PAD analysis of AGP isolated from the patient undergoing heroin therapy (H1), showed a large quantity of peaks throughout all charge bands. Peaks in the bi-sialylated band were initially broad but as retention time increased, the peaks narrowed. It would suggest that the AGP from this pateint displayed numerous different glycan structures terminated in various degrees of sialylation. Most structures were bi- and tri-sialylated which was common with many samples analysed during this study. Finally, patient D1 (receiving dihydrocodeine as a treatment) displayed peaks in the region preceeding the bi-sialylated charge band suggesting there may be some glycans terminated in SA at a single branch. Although the trace displayed fewer peaks than H1, there were still examples of each type of sialylation.
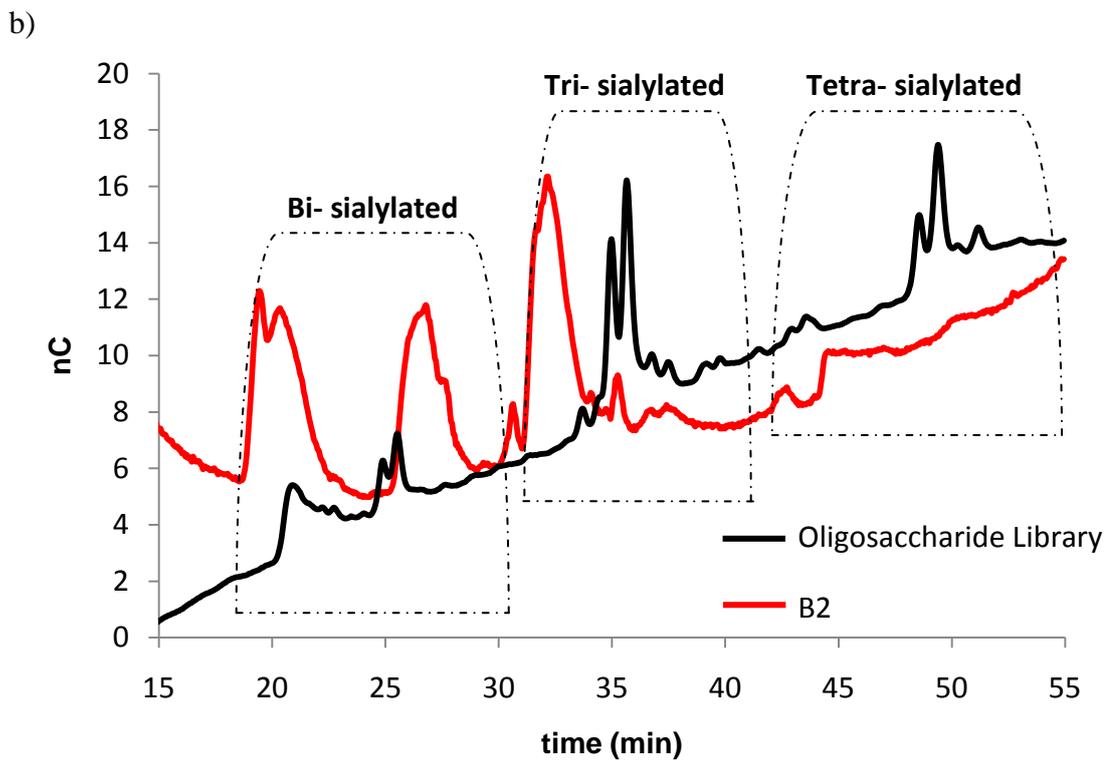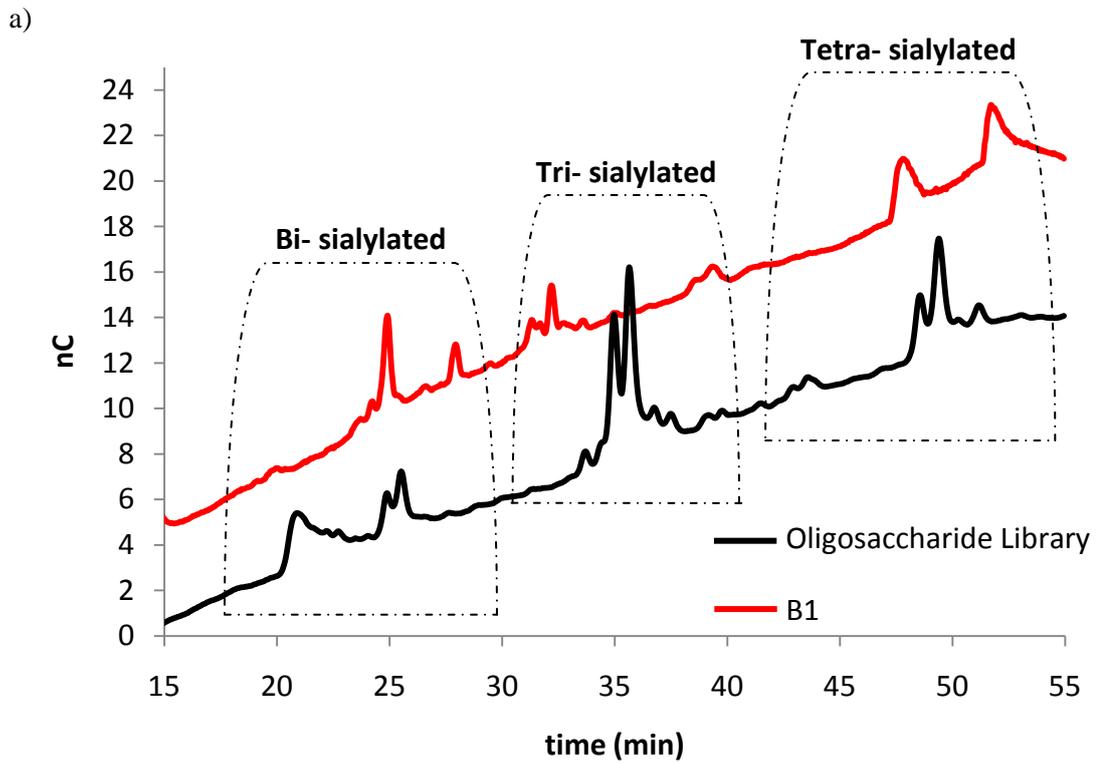
Figure 7.6. Chromatograms of oligosaccharide library versus patients on alternative therapies a) B1 and b) B2 (in red). Separation based on the charge of oligosaccharide chains as measured using nano culons (nC). Degree of sialylation highlighted in dashed boxes.

c)
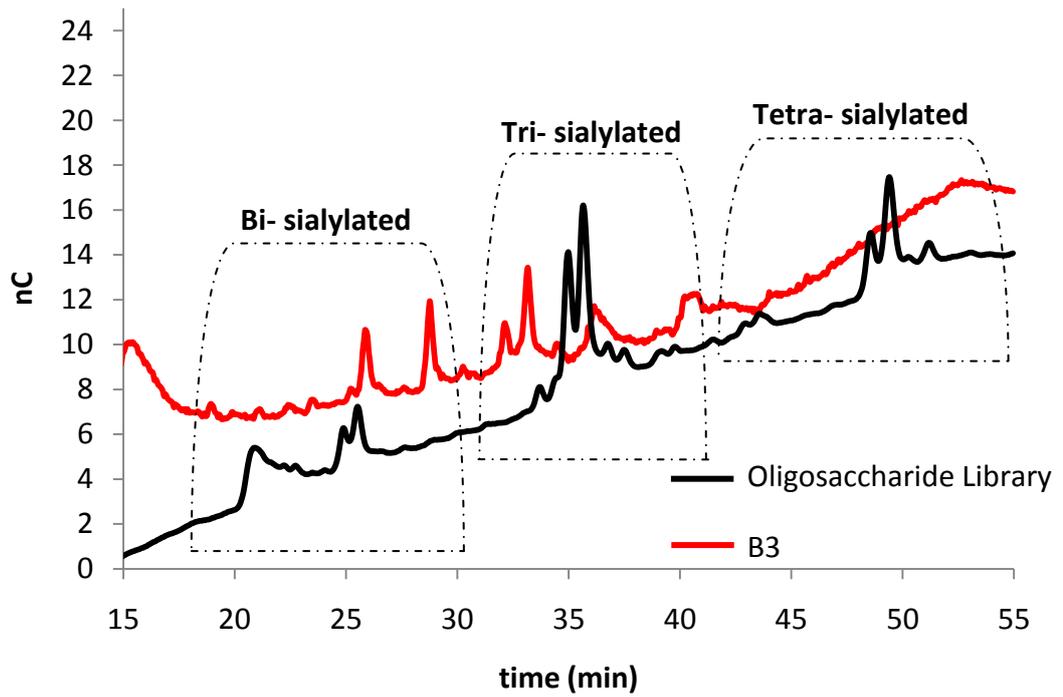
d)
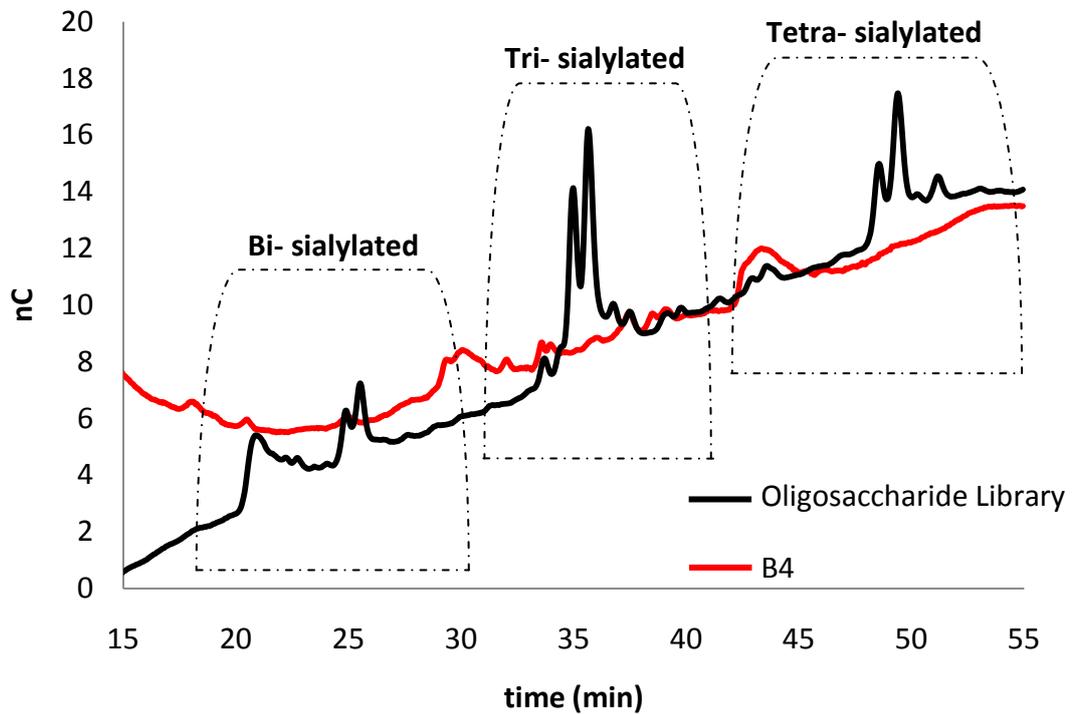
Figure 7.6. continued. Chromatograms of oligosaccharide library versus patients on alternative therapies a) B3 and b) B4 (in red). Separation based on the charge of oligosaccharide chains as measured using nano culons (nC). Degree of sialylation highlighted in dashed boxes.

e)
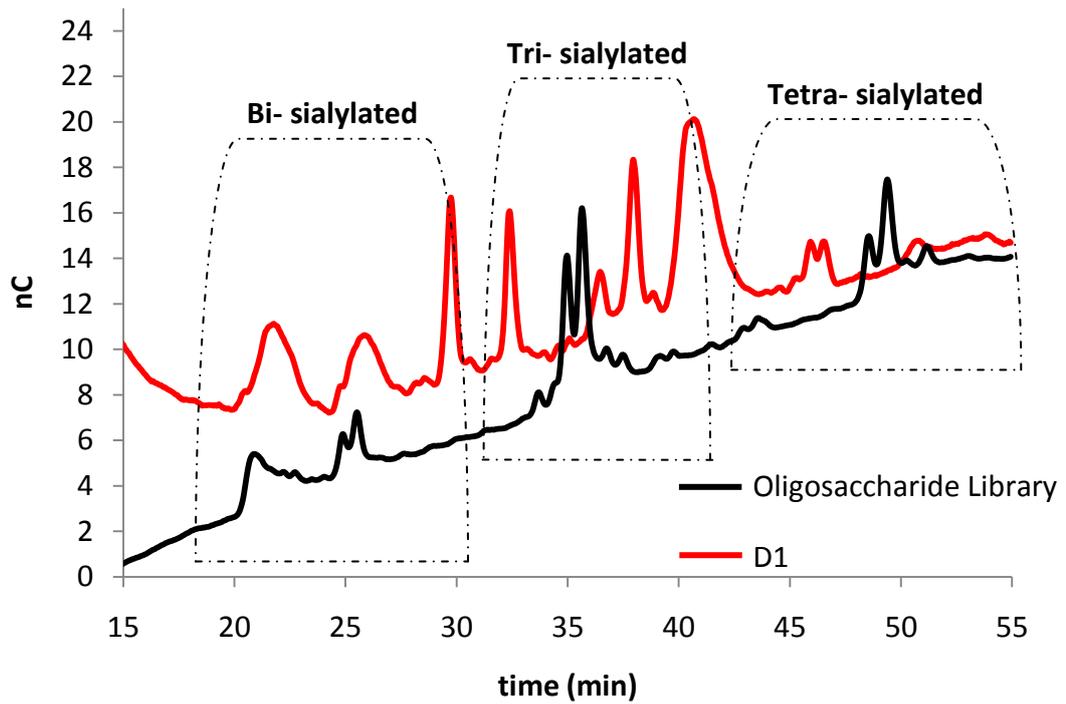
f)
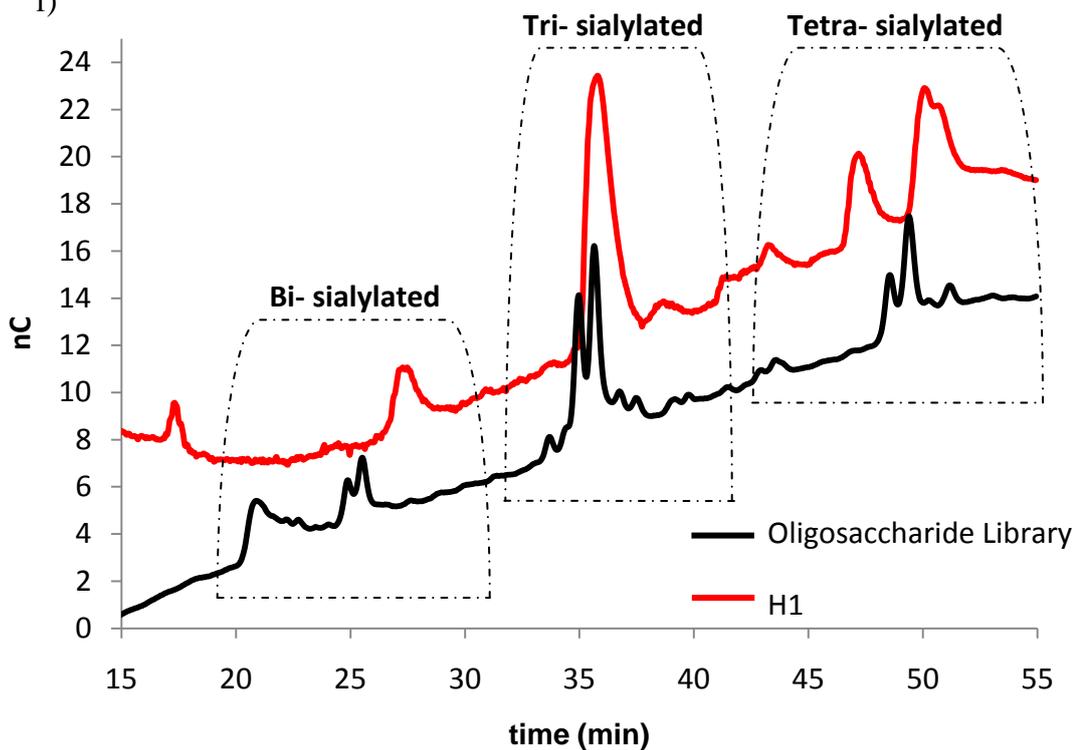
Figure 7.6 continued. Chromatograms of oligosaccharide library versus patients on alternative therapies e) D1 and f) H1 (in red). Separation based on the charge of oligosaccharide chains as measured using nano culons (nC). Degree of sialylation highlighted in dashed boxes.

7.2.2   Siailic Acid ELISA

SA residues present at the termini of glycan chains were investigated in more detail by performing an ELISA with specific lectin conjugates allowing the detection of α2,6- and α2,3- linked NeuAc. The kit was developed by Galab primarily to analyse the sialic acid activity of milk and milk-derived samples but as the glycans of AGP are also terminated in these strucutres it was decided to perform the analysis and determine if the particular assay offered potential. Not including the 'normal' heparised blood sample control, it was only possible to anaylse three AGP samples therefore data pertaining to the analysis was to be incomplete from the start. However, as part of a preliminary study it was considered useful.

The ELISA only generated useable absorbance data for α2,3 linkages therefore it was impossible to establish total SA content and compare to HPAEC analysis to determine if results were associated. The α2,6-linked SA experiment did not provide absorbance values that differed to that of the inhibition control or 100% signal i.e the inhibition control was erroneous. It was deemed useful to record and discuss the results of the α2,3 data which is increasingly expressed in highly branched glycans (Bierhuizen *et al.,* 1988).

The use of an inhibition control allowed the application of the assay to be gauged – sufficient glycoprotein should be used to out-compete the glycosylated plate surface thus providing absorbance values similar to the inhibition control (i.e. when inhibition is 100%). AGP was used at a concentration of 0.5mg/mL which was 10-fold lower than that suggested for the milk protein caseinomakropeptide (CMP) for which the assay was developed. However, it was found that this concentration was capable of producing inhibition relatively similar to that of the control.   As the concentration of the glycoprotein decreased, there was less inhibition hence absorbance values became increasingly similar to the 0% inhibition control. Values were recorded on addition of the enzyme conjugate and substrate after increasing incubation intervals to ensure data used in determining the relative α2,3-linked SA activity of each sample of glycans was the most efficient.

Theoretically, the technique was based on the principle that the addition of AGP caused competition with the glycosylated plate suface for the lectin conjugate (specific to the

linkage type under investigation). The removal of the lectin conjugate with AGP upon washing caused the reaction catalysed by the enzyme conjugate to be inhibited hence the inhibition was greatest when the glycoprotein of highest concentration was added. As the concentration decreased, more lectin was able to bind therefore the enzyme activity was increased.

Figure 7.7 illustrates the relative signal intensity of SA recorded at 405nm over the range of AGP concentrations tested (to 0.5mg/mL) in the α2,3 assay. It appeared that most α2,3 linkages were present in the T and LT sample. Even at the highest concentrations, the intensity of the HR AGP sample did not pass through 0.5 units which represents 50% inhibition and was necessary for the calculation of the SA activty. As shown in the bar chart of figure 7.8, there was a distinct lack of activity of the sample SA residues to the α2,3 lectin conjugate MAL which suggested this type of sialylation was too low to be detected thus α2,6 was perhaps more predominant however the inability to obtain data from the ELISA prevents this being supported experimetally. Higher concentrations of the HR AGP may be required for detection. Conversely, there was clear activity in the T and LT samples, however only the LT sample demonstrated levels greater than the 'normal' sample. Despite these apparent differences, the technique was only performed on few randomly selected samples therefore whether observations made were relatvie to all others in the study population could not be confirmed.

a)
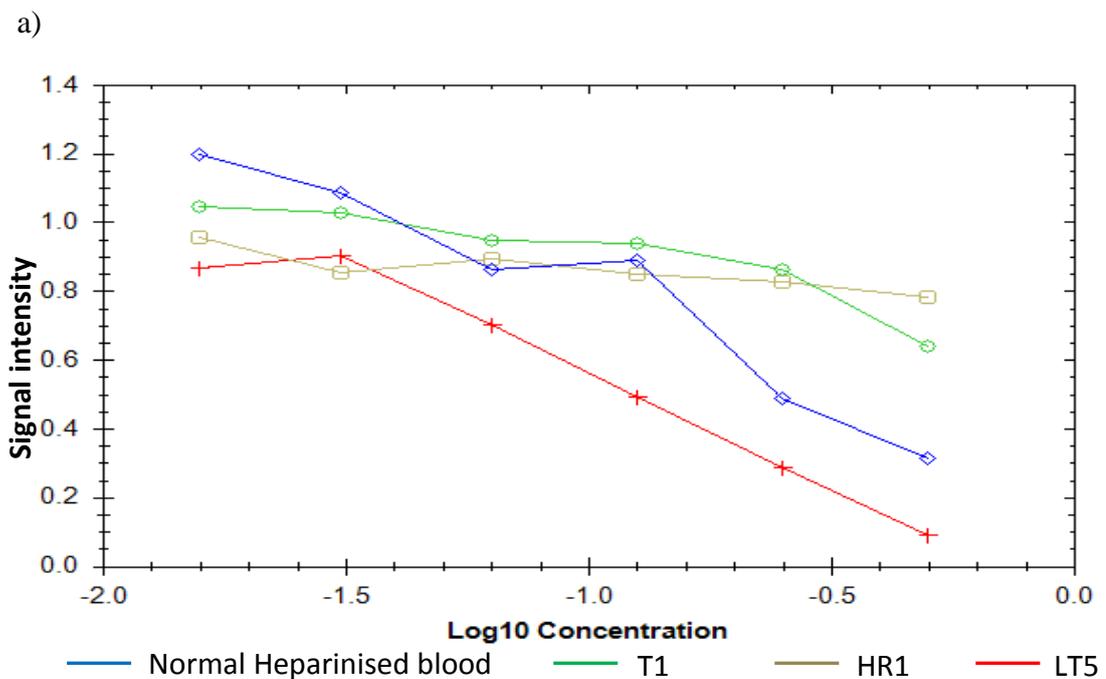
Figure 7.7.   Signal intensity at 405nm over a range of AGP concentrations. The concentration of each sample at a signal intensity of 0.5 which represents 50% inhibition, was used to calculate sialic acid content – only when a declining linear trend was apparent. Intensities which remained above 0.5 represented sample concentrations which did not cause sufficeint inhibition.

b)
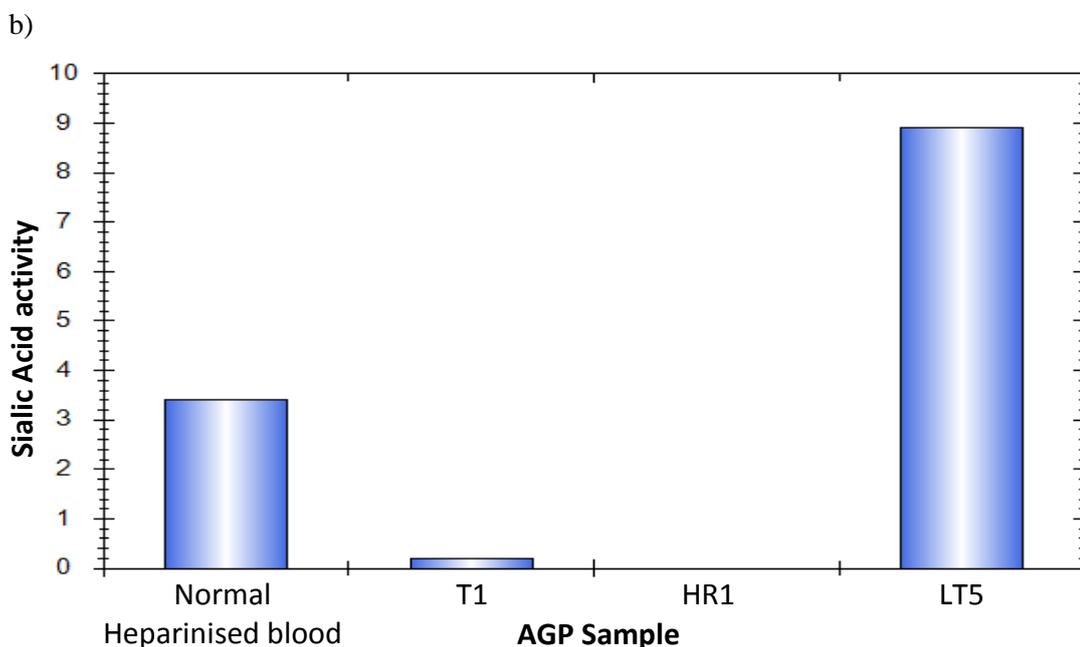
Figure 7.8.   α2,3 – linked sialic acid activity. A 2-D column graph representing the sialic acid activity of randomly selected samples from each methadone treatment group, calculated using the IC50 values from figure 7.7 i.e. the concentration at 50% inhibition of lectin binding.

7.3   Discussion

The analysis of intact glycan structures expressed by glycoproteins first requires their release from an underlying polypeptide backbone; the structure must first be denatured (chapter 2). Denaturing agents such as SDS could not be used as they destroy glycans (Hermentin *et al.,* 1992).   Heat denaturation improves the subsequent hydrolytic cleavage by the endoglycosidase PNGaseF therefore it was utilised to denature AGP. The enzyme was used because it only cleaves the bond between the Asn residue of the denatured polypeptide backbone and the initial GlcNAc of the pentasaccharide core; generating complete glycan chains. It ensured the release of all AGP glycans because the Asn residues to which they were attached were in turn linked to other amino acids (the consensus sequence) at each termini; a pre-requisite for the enzyme (Plummer *et al.,* 1984).   Deamination of the Asn residue forms aspartic acid, leaving the glycans unstable, subsequently becoming converted to a reduced structure. Unlike the acidic conditions used to cleave component monosaccharides, the enzyme prevented desialylation of the glycan branches which was crucial to the HPAEC-PAD analysis utilised in this phase of the study; it relied on the presence of the negative charge of SA residues as a means of separating glycans.

Figures 7.2 to 7.6 provided evidence that PNGase F was capable of cleaving glycan chains and that HPAEC-PAD separated them into charge bands based on the negative charge produced primarily by the SA content as labelled in the library trace (figure 7.1). As described above, the oligosaccharide chains were separated by HPAEC based upon their charge and size but also the constituting monosaccharides and linkages holding them together (Hardy and Townsend, 1988). Strong alkaline conditions (pH13) were required to induce oxyanion formation allowing improved resolution than that possible using SA expression alone – isomeric structures can be distinguished when oxyanions are generated.

It has been shown that reducing glycan chains before analysis by HPAEC could improve the resolution of peaks however it required additional steps and may itself lead to the epimerisation and degradation of the structures if they are sensitive to the alkaline conditions utilised e.g. 0.5M Sodium borohydride ($NaBH_4$) in 0.1M NaOH (Lee, 1996).

Chromatograms generated on analysis of isolated AGP samples were plotted with the library to aid identification of the degree of sialylation; charge bands could be easily distinguished. Although it was possible to identify general features common to the oligosaccharide profiles generated for each AGP sample belonging to the individual study groups, selecting one to represent their respective groups was deemed inappropriate as variations were also clear. Instead, comparisons were made between the general observations made in section 7.2. As discussed previously, commercial AGP was not used to represent a 'normal' trace because the isolation techniques implemented may have caused partial desialylation, rendering the HPAEC analysis inaccurate. The chromatogram of commercial AGP (figure 7.2a) may support this as there were no detectable tetra-antennary peaks while the normal sample appeared to have some small peaks. However, both were in agreement with previous studies (Kimura *et al.,* 2006), where the branching of AGP appeared to favour bi- and tri-antennary structures as demonstrated by the peaks in the corresponding charge bands. The general peak distribution was relatively consistent within the titration treatment group, generally displaying a large proportion of bi- and tri-sialylated structures. Preference for similar charge bands was displayed by the LT group. Chromatograms generated for the HR group differed considerably. Additionally, the 'normal' AGP samples often displayed a peak prior to the bi-sialylated charge band which may represent glycans with only one branch terminated in a SA residue. There are very few reports suggesting the AGP of healthy populations would express such structures, mainly because the absence of a SA residue causes the underlying Gal to become exposed. This would normally act as a signal for the destruction or repair of the structure; targeted towards lysosomes or calnexin/calreticulin cycles respectively.

If representative profiles are to be generated, further analysis must be undertaken. A limitation of the current study was the restrictions on patient recruitment enforced by the NHS ethics board. Preliminary investigations of a wide group of individuals have indicated the glycosylation of AGP may be altered in individuals undergoing opioid replacement therapy. The study would benefit from focussing interest on particular groups to investigate this further.

In general, the glycosylation of AGP isolated from patients undergoing opioid replacement therapy showed preference for bi- and tri-sialylated branching. Some patients demonstrated the presence of peaks in the tetra- and mono-sialylated regions

but, on the whole, it was less prevalent than other types. Although AGP glycans isolated from 'normal' heparinised blood also tended to express mostly bi- and tri-sialylated glycans, data generated during monosaccharide (chapter 6) and oligosaccharide analysis has highlighted that the glycosylation of AGP in patients undergoing methadone therapy appears to differ. Therefore, as all patients it was important to determine if functional changes occurred.

The monosaccharide data (table 6.2) for the 'normal' heparinised blood samples suggested there was a low degree of branching because the level of Gal and GlcN was low while Man was relatively constant – the number of core structures remained common as expected because comparable levels of AGP was analysed. However, as the Gal and GlcN levels were far lower than the commercial AGP data, it would be expected that the chromatogram would show more peaks in the bi-sialylated charge band. This was not the case and instead commercial AGP had more peaks in this area. Perhaps desialylation caused during isolation of the commercial sample resulted in the premature elution of glycans due to the reduction in negative charge - the degree of branching suggested in the profile may be misleading, instead the commercial sample may express higher levels of branching but without terminal SA residues. The peaks corresponding to the glycans of commercial AGP were therefore expected to elute later in the charge bands due to the larger structures suggested by monosaccharide analysis however this was not the case, certainly within the tri-sialylated charge band.

Interestingly the data obtained from monosaccharide analysis of many patient samples in chapter 6 was in agreement with the findings during glycan analysis i.e. a high content of Gal and GlcN, the monosaccharides present in glycan branches, often correlated with oligosaccharide profiles with peaks eluting in the tetra-sialylated charge band. For example, patient T3 displayed significantly higher levels of GlcN and Gal compared to the 'normal' heparinised sample suggesting highly branched structures. T3 recorded the highest level of GlcN (table 6.2) and a relatively similar level of Gal compared to other patients in the treatment group. When the glycans were subsequently resolved using HPAEC-PAD, a higher degree of branching was evident; this patient was the only one to show a relatively large number of peaks in the tetra-sialylated region. Conversely, samples like LT2 which generated relatively 'smooth' chromatograms more akin to the 'normal' profiles of figure 7.2b than to others of their respective group also appeared to have low levels of Gal and GlcN; the levels did not show a significant

difference when compared to those for the 'normal' population. It would have been useful to repeat oligosaccharide analysis of AGP glycans for said samples to determine whether the profiles were reproduced however limited sample availability rendered this impossible.

It should be noted that Fuc was not detected in any patient samples and therefore it could not have been responsible for reducing the retention time of glycans as described by Pfeiffer *et al.* (1990). Such modifications have been reported in diseases such as cancer or RA where fucosylation increased (Mackiewicz and Mackiewicz, 1995; Smith *et al.,* 2002). It is also known to be increased as a result of liver damage, like that which occurs during hepatitis or due to drug taking (Anderson *et al.,* 2002). There was no evidence suggesting this.

Overall, HPAEC-PAD has been shown by this study and numerous others before to represent a useful sensitive technique in the analysis of glycan structures. PAD detects only compounds with functional groups which are oxidised at the detection voltage. Similar to monosaccharide analysis, alterations in the structure of glycans were apparent in comparison to a normal population.

The SA ELISA indicated that although the kit was produced to analyse the SA activity of milk and milk-derived proteins, it could be adapted for human AGP. The determination of SA was based upon a competitive lectin assay with the plate presenting sialylated structures which bind specific lectin conjugates – SNA and MAL for $\alpha(2,6)$ and $\alpha(2,3)$ respectively. The sialylated glycoprotein AGP should inhibit this binding.

Patients randomly selected from the T and LT phases of therapy were found to demonstrate $\alpha 2,3$ activity however only the LT AGP glycans had higher activity compared to the 'normal' AGP. Although the level of $\alpha 2,6$-linked NeuAc was not determined, the ELISA was useful as part of a preliminary study. The data may correlate with that of HPAEC-PAD analysis of the oligosacchairdes, as the T and LT patients expressed a high level of branching however, with the lack of information regarding $\alpha 2,6$ SA activity and therefore total SA content, whether the ELISA truly mirrored the results of HPAEC-PAD analysis could not be determined.

Relatively high levels of α2,3 sialylation would be expected because the linkage is common to AGP glycans (Bierhuizen *et al.,* 1988). Its presence generally causes later elution of a glycan when compared to a similar structure but with α2,6 linkages because the latter has a lower collative negative charge due to the absence of the C6 oxyanion - this effect could not be clearly detected in the chromatograms generated. However, it has been reported that an increased expression of α2,3-linked neuraminic acid residues is correlated with more highly branched structures, while α2,6 is more common to bi-antennary glycans (Bierhuizen *et al.,* 1988). This is in agreement with the oligosaccharide analysis by HPAEC-PAD, where the LT samples (figure 7.5) commonly demonstrated evidence of tetra-sialylated structures.

Overall, HPAEC-PAD indicated that the glycosylation of AGP isolated from patients undergoing various stages of opioid-replacement therapy was different to that expressed by the AGP isolated from a 'normal' heparinised blood sample. For many patients the analysis supported, and had been supported by, data obtained during monosaccharide compositional analysis (chapter 6).

SUMMARY STATEMENT

The glycans of AGP isolated from heparinised 'normal' blood and individuals undergoing opioid substitute therapies were shown to differ. All groups appeared to show a preference for bi- and tri-sialylated structures however tetra-sialylated structures were only found in AGP isolated from patients, most commonly in the LT group. The results often provided support for the data generated during analysis of monosaccharide composition in chapter 6. Whether these structural differences could alter the ability of the glycoprotein to bind drugs was therefore of interest.

Chapter 8

Drug Binding

Results and Discussion

8.1   Introduction

Preceding chapters have focussed upon the structural analysis of successfully isolated AGP from patients undergoing opioid replacement therapy. In the present chapter the focus shifts towards a particular function performed by the glycoprotein, namely binding to basic drugs - primarily, to methadone.

The magnitude of the role performed by AGP and other APPs in determining the level of active drug present *in vivo* is often incompletely appreciated during studies into a drug's effectiveness. There are no known studies which have investigated the presence of the glycoprotein and more specifically the structure of its glycans in individuals undergoing opioid-replacement therapy. Instead, those actually acknowledging AGP mainly concentrated on individuals administering heroin (e.g. Rostami-Hodjegan *et al.,* 1999) or those prescribed methadone for pain management (e.g. Abramson, 1982; Duché *et al.,* 2000), however the studies did not considered altered glycosylation.

Numerous techniques have shown use in the investigation of drug binding to the APPs like AGP. Equilibrium dialysis and ultrafiltration had been the most commonly implemented methods during the 1980s and 1990s, enabling the straightforward analysis of various *in vitro* and *ex vivo* systems (Oravcova *et al.,* 1996). The former relies upon the use of a permeable membrane (for low molecular weight structures) which separates two compartments - one containing the protein of interest and the second accommodating buffer. The short analysis time and availability of commercial kits rendered ultrafiltration a useful technique in the clinical analysis of active drug levels. Ultracentrifugation (Matsushita and Moriguchi, 2003), calorimetry (Aki and Yamamoto, 1994), liquid chromatography (Zhang *et al.,* 2000), capillary electrophoresis (Jia *et al.,* 2002) and fluorescence quenching analysis (Epps *et al.,* 1999) are other valuable techniques.

Increasingly implemented are forms of capillary electrophoresis (CE) - for example Jia and colleagues (2002) used pressure-assisted capillary electrophoresis (PACE) in conjunction with frontal analysis (FA). The percentage of protein-bound drug can be ascertained based on the level of free drug which in turn is indicated by the frontal peak height. Interest has also developed in chromatographic-based analysis e.g. HPLC (Hage,

2001) where immobilisation of the protein in columns is necessary to determine the retention of solute on the stationary phase and ascertain the percentage of drug which becomes bound. CE has often been reported to allow greater throughput of samples however adsorption can occur at the capillary wall and it is generally considered less sensitive than HPLC - especially with the progress which has been made in reducing analysis time and the requirement of radio-labelled ligands (Oravcova *et al.,* 1996; Rundlett and Armstrong, 2001). Even more recent techniques were described in a paper by Xuan and Hage (2005) where AGP was immobilised onto columns to mimic the native form. Such techniques have increased potential in the current climate because they are reusable unlike many of the column and membrane-based techniques; they have improved precision but still provide information regarding equilibrium and kinetics (Frostell-Karlsson *et al.,* 2000). In the current study however, it was decided to utilise intrinsic fluorescence techniques as reported by Parikh *et al.* (2000), although minor adaptations were required as described in more detail in chapter 3.

The benefit of fluorescence-based studies over those above include its sensitivity, accuracy and ability to be used without prior separation of bound and unbound fractions (Epps *et al.,* 1999). Although not the most direct method to analyse binding interactions, only 30μg aliquots were required to perform triplicate analysis of AGP in the absence or presence of each drug concentration, therefore in this study less than 200μg was needed. Also, the methodology was preferred over dialysis techniques which risk the binding of hydrophobic drugs to the membrane thus reducing the accuracy of the method (Parikh *et al.,* 2000). The quick and simple fluorescence-based technique could also offer a basis on which to develop an assay with potential in a clinical setting. As described in section 3.4, intrinsic fluorescence studies detect the extent to which drugs bind proteins like AGP. Trp (and to a lesser extent Tyr) residues of the polypeptide backbone fluoresce when excited at 280nm. The residues become masked upon the binding of a drug therefore causing a reduction in the florescence emitted i.e. it becomes 'quenched'. While HSA binds acidic drugs, AGP binds those basic or neutral in character, including methadone (Eap *et al.,* 1990). However, as indicated in section 1.3.4.2, caution must be applied as fluorescence resonance energy transfer (FRET) may occur in such studies, especially if the drug under investigation is known to absorb at wavelengths used in the analysis.

8.2   Results

Previously in chapter 3 it was demonstrated using theophylline that the analysis of intrinsic fluorescence using a microtitre plate method adapted from the Parikh *et al.* (2000) study was useful in the investigation of drug binding to AGP. A reduction in fluorescence of the Trp and Tyr residues was detected as the concentration of drug – and therefore binding – increased. The method was subsequently repeated with methadone. Advantageously, it required only 30μg of analyte to triplicate each AGP-drug concentration analysis. Therefore, all analyses were undertaken by excitation of the AGP and drug reaction components at 280nm, recording the emission at 340nm – the wavelength with which maximum fluorescence is correlated (refer to figure 3.1, section 3.1).

Figure 8.1a indicates the reduction in fluorescence alongside increasing methadone concentration, thus illustrating that the level of quenching was increased but that it was not capable of completely quenching all fluorescence. Figure 8.2 illustrated the effect the aforementioned methadone concentrations had on AGP isolated from 'normal' and patient samples from the titration (T), harm reduction (HR), long-term methadone (LT), buprenorphine (B) and dihydrocodeine (D) treatment groups (figure 8.2a and 8.2b-f respectively). All graphs were represented with the concentrations 50-200μM removed to allow a clearer representation of the effect low concentrations had on fluorescence. It was discovered that in all individuals, the level of quenching was correlated with the concentration of methadone – quenching increased (i.e. fluorescence intensity decreased) as the glycoprotein samples were exposed to elevated concentrations of drug. Nonetheless, this effect on AGP isolated from the 'normal' and patient blood samples attained a plateau at very high concentrations (> ~100μM) which was unlike the continual reduction in fluorescence detected when theophylline was used (refer to figure 3.4, section 3.3.2).

The fluorescence values recorded for AGP isolated from all available samples in the absence of methadone, with 1.07μM and with 250μM of the drug were summarised in Table 8.1 to highlight the similarity of samples (except D1) with 'normal' AGP when analysed alone without the introduction of drug which was difficult to detect in the respective figures. Reporting the values after the introduction of methadone at both a

193

low and high concentration showed there were differences in binding between the patient samples and 'normal' AGP.

The rate at which intrinsic fluorescence emitted by AGP isolated from patient samples declined was most pronounced at low concentrations, steadying after the introduction of 22μM methadone. There was therefore an indication that the binding sites available on AGP became saturated relatively quickly. In spite of this, methadone did not bind all AGP binding sites – the continued detection of fluorescence in the presence of drug excess (e.g. 250μM) suggested binding sites remained available.

The extent of reduction did not differ widely between patients within each group or between treatment groups (as illustrated in figure 8.3). Nonetheless, the emission was generally lower upon the introduction of methadone than that of the 'normal' (graphed alongside all samples) and 0.5mg/mL commercial sample (figure 8.1) suggesting that more binding occurred in individuals undergoing opioid-replacement therapy – fluorescence was quenched more effectively.

Finally, analysis of AGP binding to another opioid - codeine (2.5-250μM) - was undertaken with commercial and normal serum AGP. The drug was found to dissolve most effectively in the solvent DMSO. Although insufficient patient AGP samples were available to undertake further drug binding studies, those performed using commercial AGP and a range of drug concentrations identical to that used previously for methadone and theophylline (25-2500μM) signified that the glycoprotein was unable to bind codeine (figure 8.4).
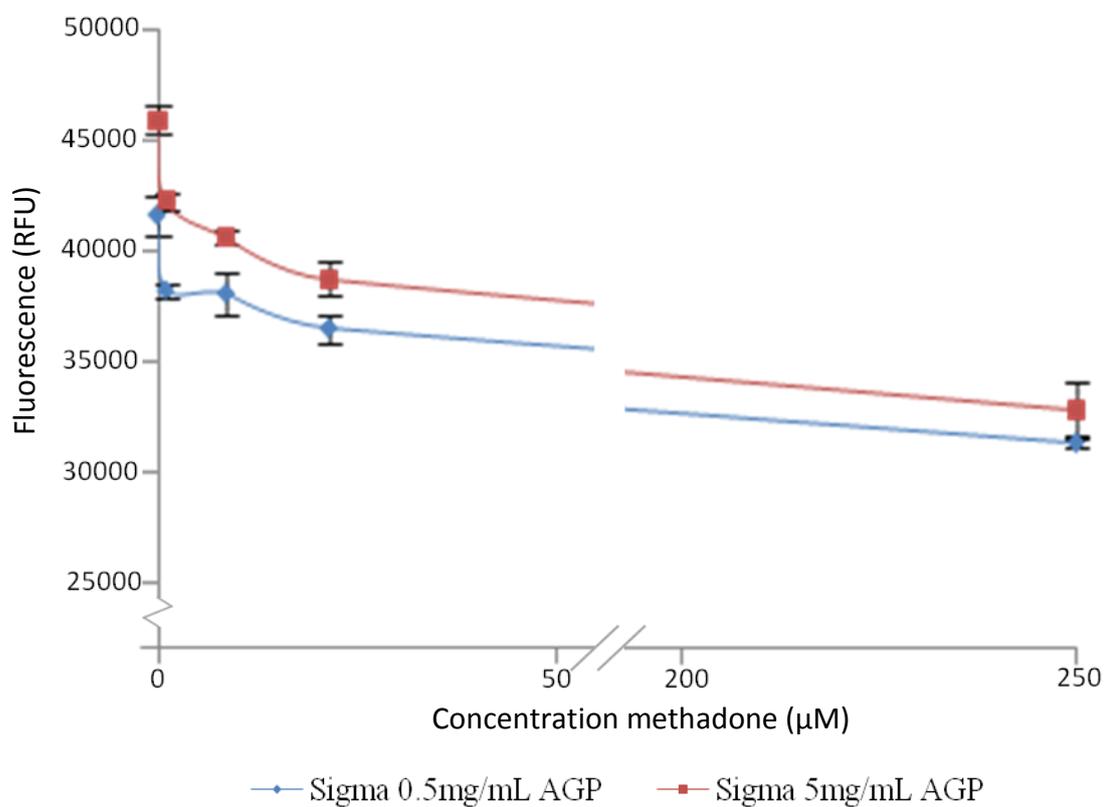
Figure 8.1. **Intrinsic fluorescence at 340nm of 0.5mg/mL and 5mg/mL commercial AGP.** AGP samples, alongside increasing concentrations of methadone (1.07-250μM), were excited at 280nm and the fluorescence emitted was recorded, the extent of reduction indicating the degree of binding.
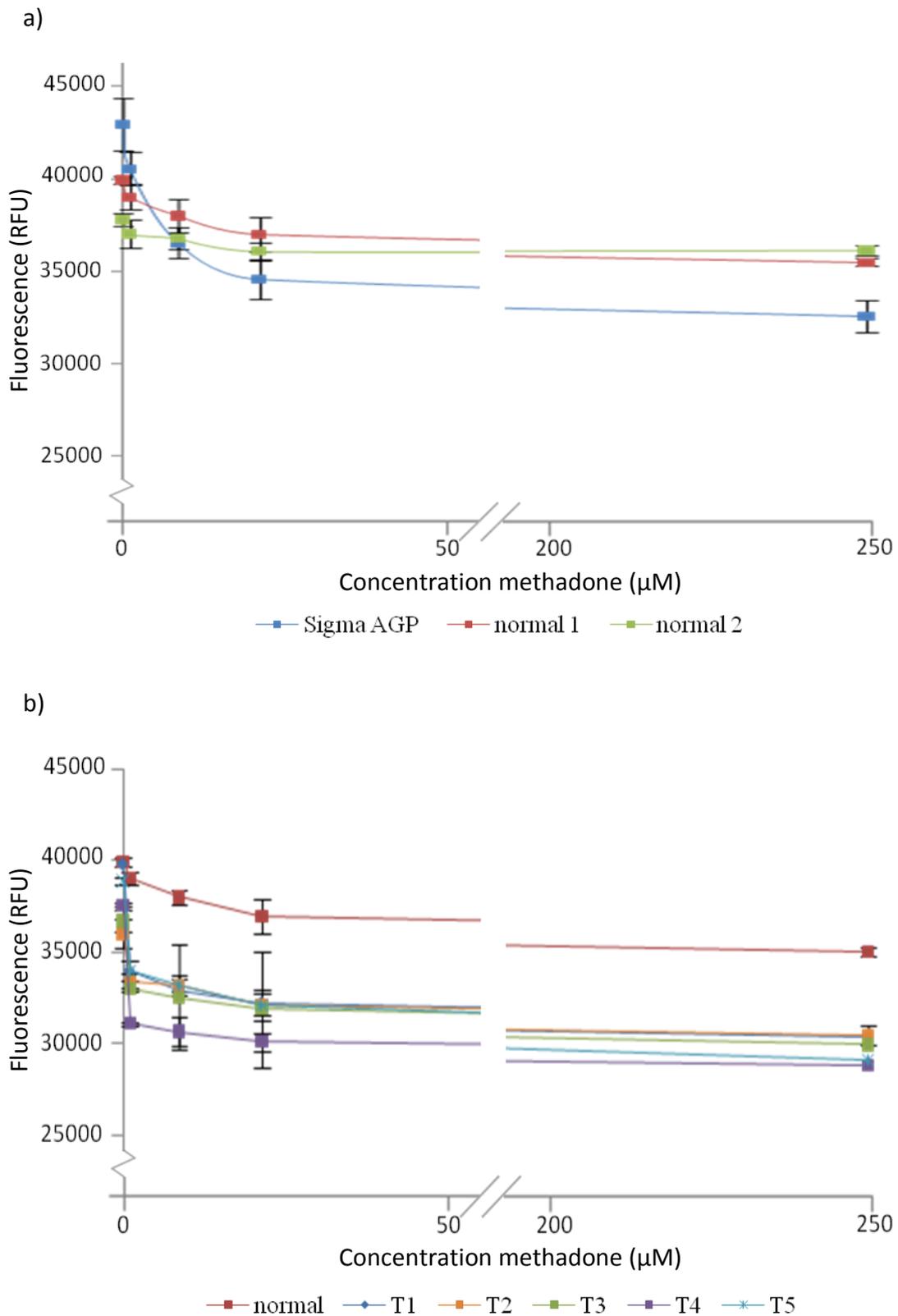
Figure 8.2. Intrinsic fluorescence at 340nm of AGP isolated from 'normal' individuals (a) and patients undergoing the titration phase of therapy (b) in the presence of methadone. AGP samples, alongside increasing concentrations of methadone (1.07-250μM), were excited at 280nm and the fluorescence emitted was recorded, the extent of reduction indicating the degree of binding.

c)

d)
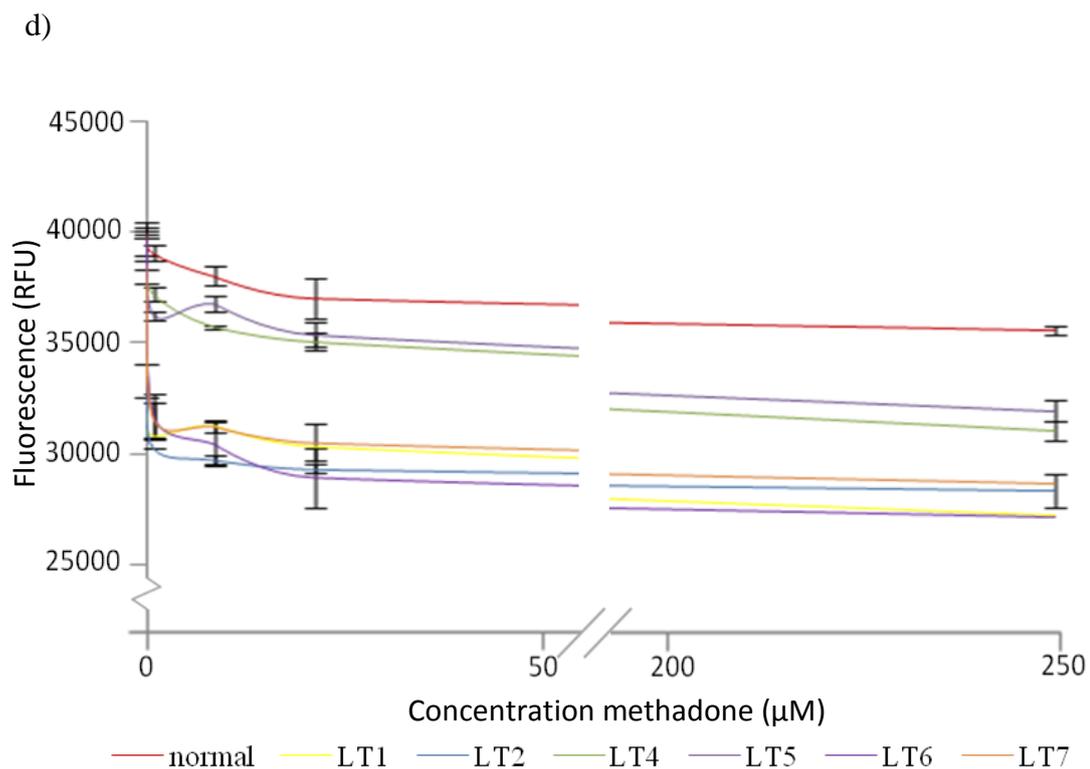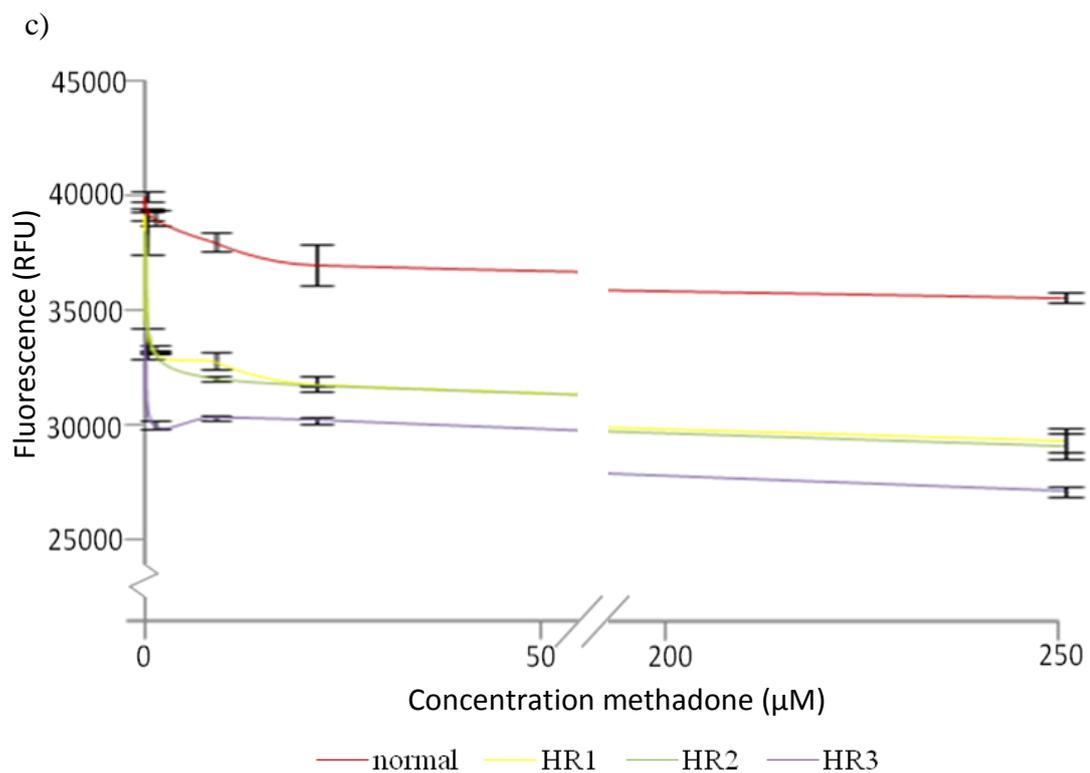
Figure 8.2 continued. Intrinsic fluorescence at 340nm of AGP isolated from patients undergoing harm reduction (c) and long term methadone therapy (d) in the presence of methadone. AGP samples, alongside increasing concentrations of methadone (1.07-250µM), were excited at 280nm and the fluorescence emitted was recorded, the extent of reduction indicating the degree of binding.

e)

f)
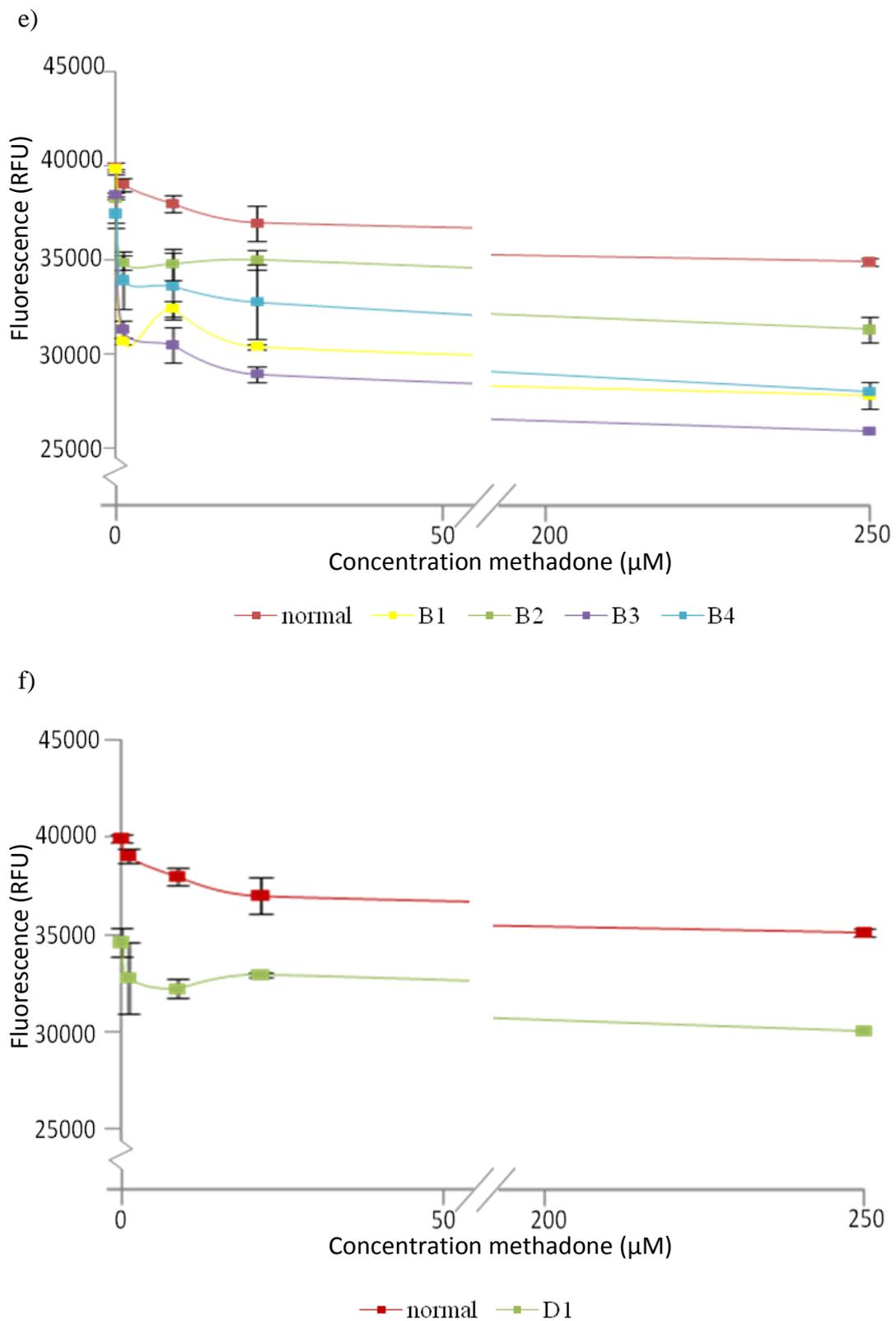
Figure 8.2 continued. Intrinsic fluorescence at 340nm of AGP isolated from patients undergoing buprenorphine (e) and dihydrocodeine therapy (f) in the presence of methadone. AGP samples, alongside increasing concentrations of methadone (1.07-250μM), were excited at 280nm and the fluorescence emitted was recorded, the extent of reduction indicating the degree of binding.
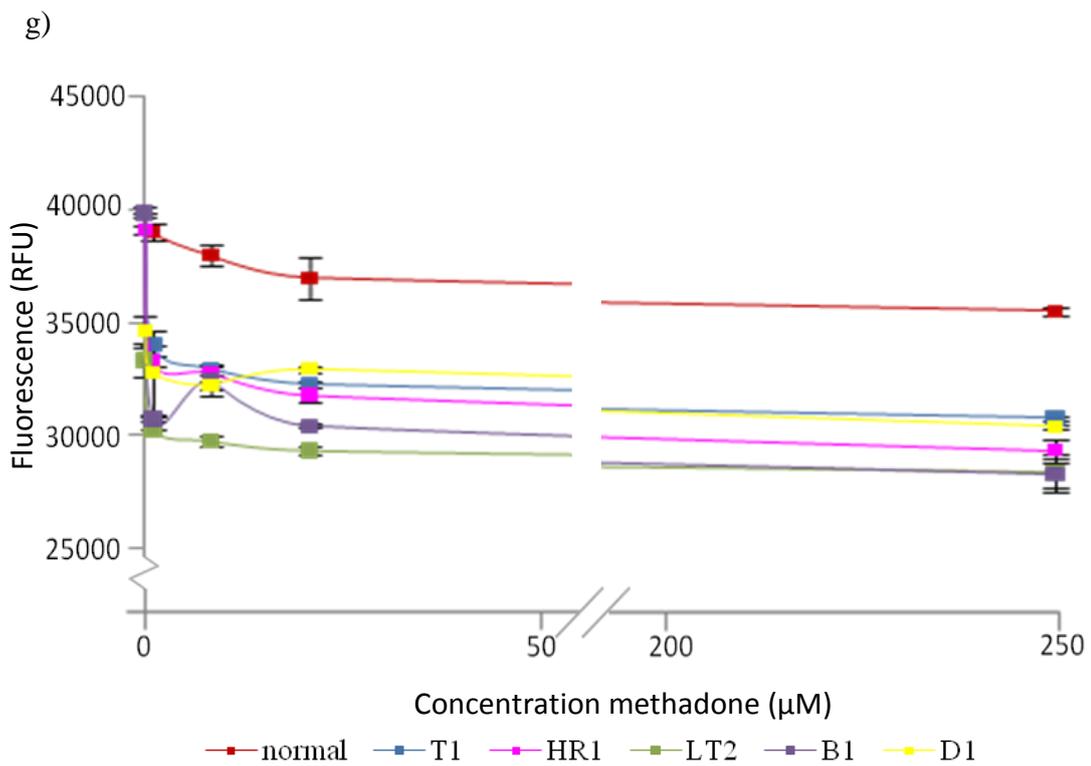
Figure 8.3.   Intrinsic fluorescence at 340nm of AGP isolated from 'normal' heparinised blood and opioid-replacement patients in the presence of methadone. AGP samples, alongside increasing concentrations of methadone (1.07-250μM), were excited at 280nm and the fluorescence emitted was recorded, the extent of reduction indicating the degree of binding.

Sample	Fluorescence in the absence of methadone (RFU)	Fluorescence in the presence of 1.07μM methadone (RFU)	Fluorescence in the presence of 250μM methadone (RFU)
Commercial (Sigma)	41636	38236	30620
Normal 1	**39943.5**	**39021**	**35476**
Normal 2	**37784.5**	**37032**	**36138**
T1	39859	34006	30712
T2	35992	33371	30840
T3	36715	33020	30284
T4	37571	31089	29182
T5	38882	33959	29412
HR1	39107	33280	29289
HR2	38438	33215	29025
HR3	33531	30023	27072
LT1	31770	30775	27196
LT2	33324	30252	28346
LT4	38688	37189	31008
LT5	39937	36241	31893
LT6	39691	31681	27137
LT7	38484	31538	28658
B1	39862	30718	28227
B2	38273	34872	31803
B3	38492	31337	26303
B4	37482	33940	28412
D1	34608	32789	30363

Table 8.1. Intrinsic fluorescence of AGP in absence and presence of 1.07μM and 250μM methadone.
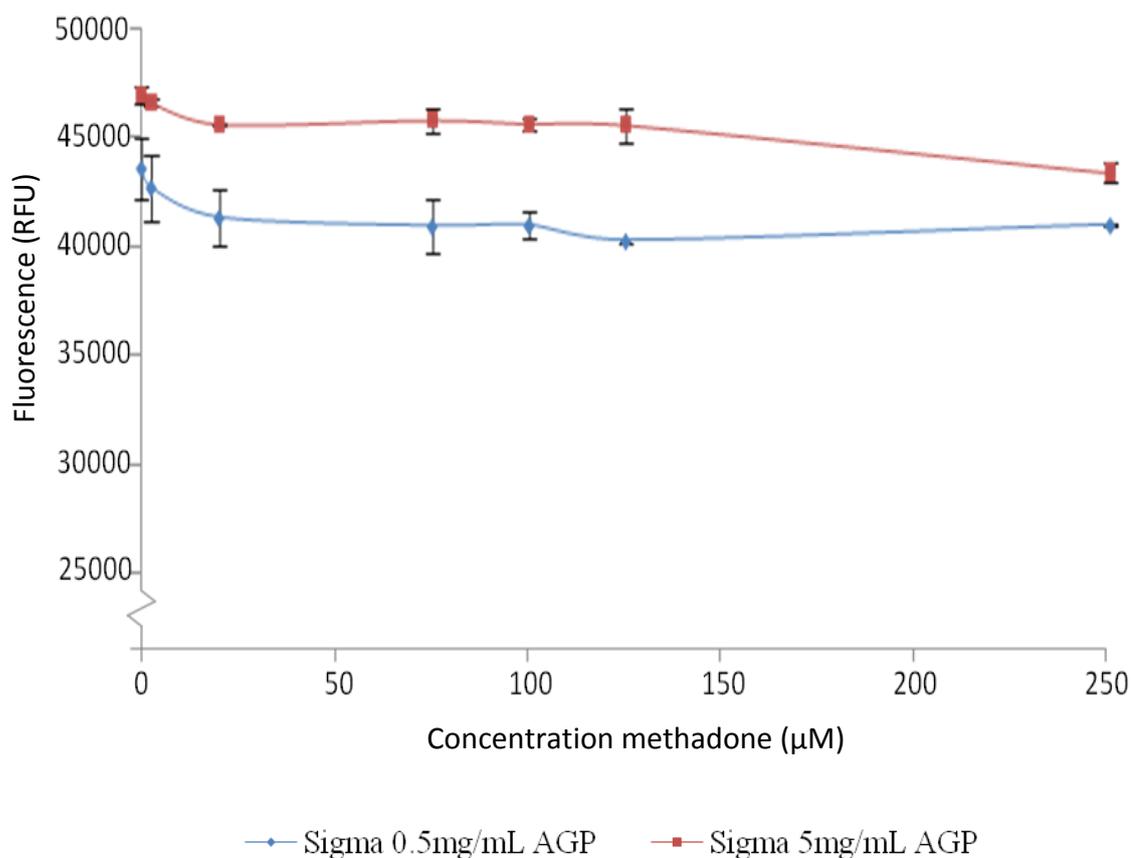
Figure 8.4.   **Intrinsic fluorescence at 340 nm of commercial AGP in the presence of codeine.** AGP samples, alongside increasing concentrations of codeine (2.5-250μM), were excited at 280nm and the fluorescence emitted was recorded, the extent of reduction indicating the degree of binding.

8.3   Discussion

Methadone is an opioid substitute therapy implemented globally in the treatment of opioid dependencies and has been well documented as a favourable means of reducing crime and the spread of infectious disease (Ward *et al.,* 1999). There is still disagreement over its success in terms of its ability to assist individuals in their quest to achieve complete drug abstinence. Four years ago it was suggested that as few as 7% of patients may actually achieve this state annually in Scotland (McKeganey, 2006). Many more remain on methadone maintenance therapy long-term – their study did not consider this to signify those in which the treatment was successful.

An explanation as to why the treatment has more success in certain individuals than others is essential if the therapy is to encourage and improve abstinence rates. It is no easy task with numerous pharmacokinetic and pharmacodynamic factors fundamental in the determination of the drug's effect. Differing rates of metabolism and elimination, which ultimately reduce the level of active drug in the blood, may be partly responsible for the variability in treatment success. For example, individuals expressing greater activity of cytochrome P450 enzymes (particularly CYP3A4) will possess lower levels of free active drug.

Of interest to this study is the presence of *in vivo* drug-binding proteins such as AGP which forms predominantly hydrophobic interactions with basic and neutral drugs at a single binding site (Jia *et al.,* 2002). AGP binds methadone with a high affinity but low capacity at physiological pH. Investigations into the binding of imipramine to AGP by Hervé and colleagues (1993) suggested that AGP may display variable numbers of binding sites, primarily determined by the variants expressed. They reported that one high affinity site and two lower affinity binding sites may exist on the ORM-2A variant (the variant for which methadone expresses a high affinity at a single hydrophobic binding site). The apparent correlation with the AGP variants expressed, suggests there is likely to exhibit interindividual variation in the binding of drugs like methadone to AGP.

As many drugs reach their target site of action via the bloodstream, the presence of such proteins may have a significant effect on the availability of active drug; some becoming

bound upon contact, rendering it inactive. The subsequent alteration of equilibrium between tissues affects other PK parameters including the volume of distribution (V_d) and elimination etc. If binding is substantial, the level of active (unbound) drug may be insufficient to stimulate the pharmacological effect. The concept has been widely studied for numerous drugs (for example Friedman *et al.,* 1985; Paterson *et al.,* 2003; Johnson and Smith, 2006 who studied chlorpromazine, tamoxifen and anti-tuberculosis drugs respectively). It must also be remembered that the effectiveness of drugs which bind to AGP and other APPs will be affected during the APR to various physiological and pathophysiological conditions where synthesis is altered (Benedek *et al.,* 1984) and atypical glycans may be expressed. Increases in the methadone dose may be necessary to allow for enhanced AGP binding during the APR which, in the case of methadone, may prevent attainment of the MEC at the ORs causing the emergence of withdrawal symptoms. Upon termination of the APR, swift dosage adjustments must be made accordingly to prevent toxicity.

It was hypothesised that alterations in the level and structure of this glycoprotein, shown to occur in chapters 5, 6 and 7, may enhance the affinity for methadone - inactivation of the drug would be exacerbated, rendering individuals susceptible to withdrawal and ensuing relapses. AGP isolated from all patient blood samples did appear to bind more methadone, as shown by greater fluorescence quenching. Rostami-Hodjegan and colleagues (1999) reported that the levels of AGP in heroin dependent individuals with signs of opiate withdrawal were greater than in a drug free population and also showed a higher proportion binding to the glycoprotein when analysed *ex vivo*. There have however been no notable studies published which analysed the level and structure of AGP in patients undergoing opioid-replacement therapy. Those in the initial weeks of titration therapy represent a study population similar to that central to the study by Rostami-Hodjegan and colleagues (1999); individuals commonly continue to co-administer heroin at least until a maintenance dose of methadone is attained.

As documented in chapter 5, the level of AGP isolated was greater in all patients compared to a 'normal' population. Fundamentally patients expressing the greatest levels of AGP would be expected to require the highest doses of methadone as binding would be expected to increase. Instead, within the LT group, patients LT4 and 6 demonstrated the greatest binding but were receiving 90 and 60 mg / day respectively, by no means the greatest doses. One explanation could be that those with apparently

high levels of binding but receiving comparably low doses of methadone expressed fewer cytochrome P450 enzymes involved in its metabolism than those where binding was relatively similar but whom required much greater doses like LT2 and LT7 (150-200mL). The greatest reduction when comparing representative group members was found for LT2 and B1. However, not all patients in these groups displayed such large reductions for example, LT4, LT6, and B2.

Studies undertaken thus far have suggested that the glycosylation of AGP was altered in all patients however it was unspecific to a particular treatment group. It was possible that these structural changes increased the affinity for methadone – in the absence of drug, fluorescence of Trp and Tyr residues of the polypeptide backbone was consistent with that of a 'normal' AGP sample isolated from heparinised blood which suggested the structural alterations alone were not affecting fluorescence or location of Trp and Tyr therefore FRET was unlikely. Also, methadone does not absorb at wavelengths implemented in the intrinsic fluorescence studies so any reduction detected should be indicative of the binding of the drug to AGP and the effect altered glycosylation may have had.

Additionally, the fluorescence reduction detected in all patients may be partly due to an increased expression of the ORM2A variant of AGP – the variant to which methadone is thought to exhibit preference (Eap *et al.,* 1990). As stated in the results, the reduction in intrinsic fluorescence was most pronounced at low concentrations suggesting relatively rapid uptake by the plasma protein with an apparent limit to the quenching effect, indicating saturation of the available binding sites. There was very little variation in the extent of quenching between patients. Unlike theophylline etc, the fluorescence was far from eradicated – this would have been expected had methadone been unspecific in its binding to the glycoprotein. Binding sites must remain available otherwise the fluorescence would continue to be quenched, as shown for chlorpromazine and theophylline (refer to figures 3.1b and 3.4 respectively) which bind highly to AGP. The relative proportions of AGP variants could not be determined for the patients due to insufficient samples but there was no reason not to conclude that the glycosylation changes which occur to this particular variant of AGP would increase its affinity for methadone. The culmination of increased synthesis (as shown in chapter 5) and altered glycoform expression *in vivo* may cause substantial reduction in the active drug concentration in treatment groups thus necessitating dosage adjustments.

Selectivity is not only seen in terms of which AGP variants methadone binds but also in the chirality of the ligand (i.e. enantiomer) for which the glycoprotein exhibits preference (Muller, 1988), potentially contributing to the limitation of quenching when compared to that of theophylline and chlorpromazine. Eap and colleagues (1990) have also documented that the enantiomers bind AGP genetic variants differently - similar to the traits of warfarin studied by Hervé *et al* (1998). The (R) enantiomer of methadone expresses affinity for AGP. However at concentrations of 250µM it is likely that there would be enough of the (R) enantiomer to cause a more significant decrease from the preceding 21µM which is approximately 10-fold lower. In most countries, the (R) form of the drug is administered alongside the (S) enantiomer as a racemate which may partially explain why doses are so much higher (mg level) that the plasma level required to induce the pharmacological effect (ng level).

Whether the single patient receiving dihydrocodeine was representative of all individuals receiving this treatment, was unknown. Nonetheless, it was the only sample to have significantly different fluorescence to normal in the absence of drug suggesting that the structure of the molecule was altered or less was analysed. Binding still occurred because the fluorescence reduced however not to the same degree as the other samples, again suggesting the binding site had lower affinity for the drug.

It was seen that codeine was unable to bind AGP. Although this opioid (a natural isomer of methylated morphine) is used in pain management and not usually in opioid-replacement therapy for drug abuse due to its short duration of action of 2.51-3.15h (Yue *et al.,* 1991), it was deemed of interest as it belongs to the same family and DHC could not be sourced (essentially codeine with two additional –H atoms and no double bond, making it more stable with greater metabolic resistance). If considering the influence of AGP alone on the efficacy of methadone, a drug like codeine but with a longer half-life – if capable of reproducing the same effects such as withdrawal prevention – may be of use as an alternative treatment in those expressing high levels of AGP glycoforms with affinity for methadone.

Finally, as reported by Parikh *et al.* (2000), the multi-well technique is well suited for high-throughput screening of drug binding at the binding site close to the integral Trp and Tyr residues. The technique could be adapted for use in a clinical setting, allowing the clinician to perform a rapid binding assay without prior derivatisation or separation

of bound and unbound drug or genetic variants thus aiding in the determination of the likely effectiveness of the drug at doses under consideration. It may also offer use as a monitoring tool because it would be easy to detect alterations in the level of AGP. However, the technique would ultimately require adaptations for urine analysis, is likely to be expensive if specific structures are to be detected but also, there are likely to be many factors influencing the bioactive concentration of methadone including the expression of CYP450 enzymes involved in its metabolism thus further analysis needs to be undertaken regarding the interaction of all these factors if therapies are to be improved.

SUMMARY STATEMENT

Binding of methadone to AGP isolated from all sources was shown - using intrinsic fluorescence measurements - to increase with its concentration; the most apparent reduction in fluorescence was found at low concentrations of drug. The degree of quenching was subsequently reduced at high concentrations suggesting binding sites on AGP became saturated. There did not appear to be any clear interindividual variation in the binding of methadone to AGP isolated from patient blood samples however the quenching was greater than that for AGP isolated from heparinised 'normal' blood.

Chapter 9

Conclusions

9.1   Conclusions

Opioid dependencies represent a worldwide problem not only in terms of the health of an abuser but to society as a whole due to associated illegal drug-seeking activities and unemployment. Substitute drugs are commonly implemented in programmes targeted towards fighting these issues, aiding the achievement of abstinence through a safe and controlled manner while attaining a more stable lifestyle for the individual. The use of orally administered substitute therapies also minimises the spread of potentially deadly blood borne viruses.

Although numerous therapies are available, methadone remains that most commonly prescribed due to its low cost and long half-life. The drug prevents the onset of withdrawal symptoms which would normally occur in the absence of the abused opioid and, as it belongs to the same family, it has many of the same effects as heroin therefore eliminating cravings and preventing the onset of withdrawal. It does not similarly induce euphoria thus the 'reward' associated with heroin is removed.

The success of drug therapies relies on the presence of specific concentrations of its bioactive form (MEC) at the corresponding site of action. Numerous factors determine whether this can be achieved, however often overlooked is the binding to plasma proteins. Of interest to this study was AGP, a positive acute phase protein known to bind basic drugs including methadone (Kremmer *et al.,* 1988). During the APR the hepatic synthesis of this glycoprotein is known to increase 2-5 fold, which is well correlated with numerous pathological conditions including rheumatoid arthritis, inflammation and cancer (Fournier *et al.,* 2000). Alterations in its concentration are therefore not specific to a particular condition, unlike the glycosylation of AGP which may be uniquely altered. Additionally, any structural change has the potential to affect the functions performed.

The current preliminary study aimed to investigate whether AGP has a significant role in the determination of methadone activity. Of primary interest was whether the level and glycoform expression of AGP isolated from patients undergoing various stages and types of substitute therapy for opioid-dependence differed to a 'normal' healthy

population. It was supposed that alterations may correlate to variations in the binding of the glycoprotein to methadone. Patients were recruited at the CDPS clinic in Edinburgh, including those undergoing titration, harm reduction, long-term methadone, buprenorphine, dihydrocodeine and heroin replacement. It was hypothesised that AGP isolated from patients would exhibit higher levels and structural changes causing increased binding to methadone when compared to a 'normal' AGP sample. Greater binding would at least partially explain why high doses of the drug are required – accounting for that which is bound and therefore inactivated.

AGP was isolated using a two-column low-pressure chromatography technique that had been adapted from the methodology devised by Smith *et al.* (1994) to allow for low sample volumes and prevent degradation of the *in vivo* structure. SDS-PAGE and a Western Blot were performed and indicated that the technique generated pure AGP.

Similar to research undertaken by Rostami-Hodjegan *et al.* (1999), the current study detected increased levels of AGP expressed in individuals who displayed signs of withdrawal from heroin (represented by the titration group). Recruitment of heroin-dependent individuals who were not receiving substitute therapy was not possible. The levels isolated from the patients in the remaining treatment groups – excluding the individual receiving heroin - were also greater than that isolated from the 'normal' population. An explanation may be that the presence of a xenobiotic, or the increase in DA release caused by the opioid agonist administered, deceives the body; causing stimulation of the APR or increased synthesis of AGP to aid in removal of methadone.

The general hypothesis regarding heroin therapy is that maintaining the same drug in therapy as that abused - albeit in a more pure form, as was the case for patient H1 - may have prevented such a strong induction of the APR hence the low AGP level isolated compared to the other treatment groups. However, the recruitment of a single patient was insufficient to determine whether it represented the whole group, therefore the accuracy of any conclusions made from the corresponding data could not be gauged.

Although it is well understood that structural changes to AGP glycosylation can significantly affect the functions of the biomolecule, there is currently a paucity of research data pertaining to this effect in patients undergoing opioid-replacement therapy. It is well understood that AGP generally exhibits a degree of selectivity for the ligands

to which it binds and in terms of drugs, they are generally of the neutral or basic variety (Kremer *et al.,* 1988). If binding to methadone is altered by structural modifications to the glycan chains present on AGP, the free active concentrations and subsequent pharmacological effect could be changed. A reduction in efficacy would become apparent when the affinity increases or vice versa if the affinity was reduced.

The variability in the binding of methadone to AGP may partly explain why some individuals require more drug than others to produce the effect. That is not to forget the roles of metabolism and elimination which display interindividual variability due to differences in gene expression.

The analysis of glycan structure and composition initially required the most effective means of cleaving glycans into component monosaccharides to be identified. Neutral monosaccharides became unstable in the presence of HCl and GlcNAc was less effectively cleaved by TFA, requiring instead a stronger acid. Combining the acids, as performed by previous studies undertaken by the group (Smith *et al.,* 2002; Mooney *et al.,* 2006), did not provide adequate hydrolysis of the monosaccharides when analysed in this study therefore the methodology was adapted. Optimal monosaccharide cleavage of the basic and neutral sugars was achieved by separating the use of HCl and TFA (and incubating for 6 and 4h respectively). In order to detect the greatest levels of GlcN, hydrolysis with HCl was followed by washes with HPLC-grade water, the treated sample was not passed through a dowex cation-exchange column as it hindered the elution of the GlcN residues. It was postulated that the additional negative charge displayed by the sugar after deacetylation, increased the interactions formed with the column. The separate use of the acids TFA and HCl ensured the levels of Gal and GlcNAc were not compromised; these monosaccharides are correlated with glycan branching and therefore effective cleavage was important.

The level of mannose remained constant which supported that differences detected in the other monosaccharide levels represented alterations in the structure of the chains. Mannose is only present in the core of complex N-linked glycans and should therefore remain constant when the same level of AGP from each patient was analysed; AGP expresses five complex N-linked glycans on the polypeptide backbone. Glycan structural analysis indicated that the composition of other monosaccharides was altered in the majority of patients. Elevations in the level of Gal and GlcNAc in the AGP of all

patient groups (except D1 and H1) inferred an increase in branching occurred in the glycans of patient AGP. Such an effect had not been documented previously therefore whether the results obtained represent other individuals undergoing similar stages and types of substitute therapy was unknown.

There was no significant difference in the monosaccharide levels of glycans in patient AGP with respect to the commercial equivalent. Nonetheless it was deemed more appropriate to use the 'normal' heparinised blood samples as a control because commercially sourced AGP was isolated using a method that caused partial desialylation. The alternative - 'normal' serum - was sourced from an elderly population of patients hence the expression of an APR was possible due to supplementary inflammatory conditions that were perhaps un-noted or undiagnosed and could therefore change the level and structure of AGP.

Increased branching was supported in qualitative oligosaccharide analysis where the number of branches appeared to be higher in some AGP isolated from patients than in the 'normal' AGP glycans. A number of the glycans of patient AGP expressed tetra-sialylated chains; this was not the case in the normal samples. The ELISA supported the finding to a degree, indicating greater SA expression for the LT patient than in the 'normal' population – the α2,3 linkage is associated with more highly branched structures. However, the AGP isolated from patient HR1 did not provide sufficient activity to be detected and that of T1 was lower. The level of α2,6 could not be ascertained therefore the complete SA content was not known. The study indicated the possibility that such analytical kits could be adapted for AGP SA analysis but many more samples must be tested and the technique perfected.

Drug binding analysis based on the methodology developed by Parikh and colleagues (2000) - exploiting intrinsic fluorescence on the microtitre plate scale -was shown to be effective. In terms of patients, the binding increased in all when the concentration of methadone was increased. The binding appeared to be enhanced when compared to a 'normal' sample however there was no clear difference between groups. Additionally, in the absence of drug, fluorescence was relatively consistent to 'normal' suggesting it was unlikely that the conformation of the binding site was altered by the presence of the different glycan structures. However, the fluorescence upon drug administration

decreased more substantially than in the 'normal' sample suggesting that binding was affected by glycosylation.

A useful comparative group would be heroin dependent individuals who are not receiving therapy; it may be possible to determine whether any effects seen in the patients were a direct result of therapy or perhaps due to underlying stresses associated with the illicit drug use. The apparent increase in glycan branching or length of branches already present may increase the affinity of AGP for the drug thereby explaining why some patients may require higher doses than others despite other pharmacokinetic parameters remaining constant.

Currently treatment is based on a titration phase where individuals are given low doses of drug to allow for its slow accumulation. Finally, a maintenance dose is determined. However, with investigations like these it may be possible to determine the relative level of glycoforms expressed by an individual's AGP to identify those where it is less likely to be effective or where higher doses will be required. Also, assays may have potential in monitoring individuals to prevent relapses. Therefore it may reduce the high risk of overdose often seen especially in the first two weeks of therapy. However, any clinical test based on such analysis is likely to be expensive and potentially time-consuming unless advances are made. The need for blood samples from which to isolate AGP would introduce difficulties; not only would it be invasive but a common consequence of *i.v* heroin use is collapsed veins, complicating venipuncture. However, this preliminary study has shown that a potential correlation exists between the structure of AGP glycans and its ability to bind the basic drug methadone; although a larger patient population is required to address other influential factors and determine whether the interaction could significantly affect the efficacy of methadone in the treatment of opioid dependencies.

9.2   Future work

The study presented within has highlighted the potential for the glycosylation of AGP to be altered in patients undergoing therapy for opioid dependence which can be correlated to an increased binding to methadone. However, the study was limited by ethical approval as to the size of the patient cohort. It has provided a useful preliminary study which could be expanded to determine if the accuracy of conclusions could be improved. Also, the study could be expanded in various ways to examine other aspects of the effect of AGP on treatment of opioid dependence.

- Increase the patient population studied to determine if results from this preliminary study are supported.
  - Analyse female and male groups only to determine if the effect of gender is significant, few females were recruited as few were undergoing therapy.
  - Isolate AGP from a population of healthy individuals age-matched to the study sample to ensure all differences are due to the conditions of therapy.
  - Increase numbers from different therapies to determine if AGP plays a significant role in determining the levels of drug available.
- Perform other forms of analysis for example Mass Spectrometry to compare the techniques perhaps gathering information about the peptide binding site.
- Further in-depth analysis of the binding site conformation in the presence and absence of methadone to study the binding process.
- Perform more drug binding studies using the scanning fluorescence mode to enable calculation of binding coefficients.
- Drug binding studies with other treatments including dihydrocodeine alongside AGP isolated from all groups. This will determine if those on methadone would actually benefit from another therapy because binding is less although should carry out studies with albumin etc to see if instead they bind to these proteins.
- Isolate and separate the genetic variants of AGP and determine if binding greater to specific variant and if related to glycosylation of that variant.
- Repeat the sialic acid ELISA with AGP samples isolated from a larger group of patients and determine the $\alpha(2,6)$ content.

Unfortunately in the current study it was not possible to recruit individuals whom had relapsed within the preceding 24 hours as they did not re-visit the clinic the following day. Those that did were unwilling to participate in the study. They had been considered a potentially interesting study group to determine whether the AGP level correlated with an increased risk of withdrawal when compared to that from individuals maintained long-term.

Perhaps an ELISA could be developed similar to that utilised in this study to allow rapid determination of AGP concentration and relative branching of glycans using specific lectin conjugates, binding to multiwell plates. However, no specific glycosylation patterns were determined for a particular treatment group thus perhaps it is most important to perform a drug binding assay like that described to attain information regarding how strongly the binds to the aberrant glycoprotein structures.

This study determined the quantitative and qualitative changes in the glycosylation of AGP between normal and patient populations and their correlation with the degree of binding to methadone. Although preliminary, results to date support the existence of a method of drug resistance linked to plasma protein binding and also may explain the often observed lack of response to methadone therapy. Further research may aid in the development of improved treatment regimens to tackle the reportedly low success of current therapies.

Chapter 10
Bibliography

Abramson, F.B. (1982) Methadone plasma protein binding: Alterations in cancer and displacement from alpha 1-acid glycoprotein. *Clinical Pharmacology and Therapeutics.* **32**: 652-658.

Adam, P., Sobek, O., Táborský, L., Hildebrand, T., Tutterová, O. and Žáček, P. (2003) CSF and serum orosomucoid (α-1-acid glycoprotein) in patients with multiple sclerosis: a comparison among particular subgroups of MS patients. *Clinica Chimica Acta.* **334**: 107-110.

Adamo, M., Qiu, D., Dick, Jr. L.W., Zeng, M., Lee, A-H. and Cheng, K-C. (2009) Evaluation of oligosaccharide methods for carbohydrate analysis in a fully human monoclonal antibody and comparison of the results to the monosaccharide composition determination by a novel calculation. *Journal of Pharmaceutical and Biomedical Analysis.* **49**: 181–192.

Adamson, J.W. and Eschbach, J.W. (1989) Management of the anaemia of chronic renal failure with recombinant erythropoietin. *Quarterly Journal of Medicine.* **73**: 1093-1101.

Aki, H. and Yamamoto, M. (1994) Thermodynamic characterization of drug binding to human serum albumin by isothermal titration microcalorimetry. *Journal of Pharmaceutical Sciences.* **83**(12): 1712-1716.

Albani, J.R. (1997) Binding effect of progesterone on the dynamics of α1-acid glycoprotein. B*iochimica et Biophysica Acta.* **1336**: 349-359.

Albani, J.R. and Plancke, Y.D. (1999) Interaction between calcofluor white and carbohydrates of α_1-acid glycoprotein. *Carbohydrate Research.* **318**: 194-200.

Albani, J.R., Sillen, A., Plancke, Y.D., Coddeville, B. and Engelborghs, Y. (2000) Interaction between carbohydrate residues of a1-acid glycoprotein (orosomucoid) and saturating concentrations of Calcofluor White. A fluorescence study. *Carbohydrate Research.* **327**: 333-340.

Albani, J.R (2004) In: *Structure and dynamics of macromolecules: absorption and fluorescence studies*, Elsevier, ISBN 0-444-51449-X

Albani, J.R. (2003) Relation between the secondary structure of carbohydrate residues of α_1-acid glycoprotein (orosomucoid) and the fluorescence of the protein. *Carbohydrate Research.* **338**: 1097-1101.

Anderson, N., Pollacchi, A., Hayes, P., Therapondos, G., Newsome, P., Boyter, A. and Smith, K. (2002) A preliminary evaluation of the differences in the glycosylation of alpha-1-acid glycoprotein between individual liver diseases. *Biomedical Chromatography.* **16**: 365-372.

Angal, S. and Dean, P.D. (1978) The use of immobilised cibacron blue in plasma fractionation. *FEBS Letters.* **96**(2): 346-348.

Angst, M.S. and Clark, J.D. (2006) Opioid-induced hyperalgesia. *Anaesthesiology.* **104**: 570-587.

Annesley, T.M. (2003) Ion suppression in mass spectrometry. *Clinical Chemistry.* **49**:1041–1044.

Arend, W.P. (1991) Interleukin 1 receptor antagonist. A new member of the interleukin 1 family. *The Journal of Clinical Investigation*. **88**: 1445-1451.

Auriacombe, M., Franques, P. and Tignol, J. (2001) Deaths Attributable to Methadone vs Buprenorphine in France. *Journal of the American Medical Association.* **285**(1): 39-45.

Azzimonti, F., Atchley, D.H., Morrision, C.A., Dodd, S., Boulton, D.W., DeVane, C.L. and Arnaud, P. (2003) One step purification of alpha1-acid glycoprotein from human plasma-fractionation of its polymorphic allele products. *Journal of Chromatography B*. **784**: 33-38.

Bargagli, A.M., Sperati, A., Davoli, M., Forastiere, F., and Perucci, C.A. (2001) Mortality among problem drug users in Rome: an 18-year follow-up study, 1980–97. *Addiction.* **96**: 1455-1463.

Bause, E. (1983) Structural requirements of N-glycosylation of proteins. *Biochemical Journal*. **209**: 331-336.

Bayard, B. and Kerckaert, J.P. (1980) Evidence for uniformity of the Carbohydrate Chains in Individual Glycoprotein Molecular Variants. *Biochemical and Biophysical Research Communications.* **95**: 777-784.

Behan, J. and Smith, K.D. (in press) The analysis of glycosylation: A continued need for high pH anion exchange chromatography. *Biomedical Chromatography. (published online July 2010)*

Benedek, I.H., Blouin, R.A. and McNamara, P.J. (1984) Serum protein binding and the role of increased α-1-acid glycoprotein in moderately obese male subjects. *British Journal of Clinical Pharmacology*. **18**: 941-946.

Benedek, I.H., Blouin, R.A. and McNamara, P.J. (1984b) Influence of Smoking on Serum Protein Composition and the Protein Binding of Drugs. *Journal of Pharmacy and Pharmacology*. **36**: 214-216.

Bennett, M. and Schmid, K. (1980) Immunosuppression by human plasma α1-acid glycoprotein: Importance of the carbohydrate moiety. *Proceedings of the National Academy of Sciences*. **77**: 6109-6113.

Benz, I. and Schmidt, M.A. (2002). Never say never again: protein glycosylation in pathogenic bacteria. *Molecular Microbiology*. **45**(2): 267-276.

Bernard, J., Opdal, M.S., Karinen, R., Morland, J. and Khiabani, H.Z. (2007). Relationship between methadone and EDDP (2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine) in urine samples from Norwegian prisons. *European Journal of Clinical Pharmacology.* **63**: 777-782.

Bertozzi, C.R. and Kiessling, L.L. (2001). Chemical glycobiology. *Science.* **291**(5512): 2357-2364.

Bertozzi, C.R. and Rabuka, D. (2009) Structural Basis of Glycan Diversity. **In** Varki, A., Cummings, R.D., Esko, J.D., Freeze, H.H., Stanley, P., Bertozzi, C.R., Hart, G.W. and Etzler, M.E (Eds.) *Essentials of Glycobiology*, *2nd Edition*. Cold Harbor Laboratory Press.

Bierhuizen, M.F., De Wit, M., Govers, C.A., Ferwerda, W., Koeleman, W., Pos. O. and Van Dijk, W. (1998) Glycosylation of three molecular forms of human alpha 1-acid glycoprotein having different interactions with concanavalin A. Variations in the occurrence of di-, tri-, and tetraantennary glycans and the degree of sialylation. *European Journal of Biochemistry*. **175**(2): 387-394.

Biou, D., Chanton, P., Konan, D., Seta, N., N'Guyen, H., Feger, J. and Durand, G (1989) microheterogeneity of the carbohydrate moiety of human alpha 1-acid glycoprotein in two benign liver diseases: Alcoholic cirrhosis and acute hepatitis. *Clinica Chimica Acta.* **186**(1): 59-66.

Biou, D., Konan, D., Féger, J., Agneray, J., Leroy, Y., Cardon, P., Fournet, B. and Durand, G. (1987) Alterations in the carbohydrate moiety of alpha-1-acid glycoprotein purified from human cirrhotic ascetic fluid. *Biochimica et Biophysica Acta*. **913**: 308-312.

Blain, P.G., Mucklow, J.C., Rawlins, M.D., Roberts, D.F., Routledge, P.A. and Shand, D.G. (1985) Determinants of plasma α1-acid glycoprotein (AAG) concentrations in health. *British Journal of Clinical Pharmacology*. **20**: 500-502.

Blake, A., Bot, G., Freean, J.C. and Reisine, T. (1997) Differential Opioid Agonist Regulation of the Mouse μ Opioid Receptor. *Journal of Biological Chemistry*. **272**(2): 782-790.

Borg L. and Kreek M. (2003) The pharmacology of opioids. **In** Graham, A.W., Schultz, T.H., Mayo-Smith, M.F., Ries, R.K., Wilford, B.B. (Eds). *Principles of Addiction Medicine, 3rd edition*, *American Society of Addiction Medicine.* Chevy Chase, MD; 141–153.

Borgland, S.L., Connor, M., Osborne, P.B., Furness, J.B. and Christie, M.J. (2003) Opioid Agonists Have Different Efficacy Profiles for G Protein Activation, Rapid Desensitization, and Endocytosis of Mu-opioid Receptors. *Journal of Biological Chemistry*. **278**(21): 18776–18784.

Boulton, D.W. and Devane, C.L. (2000) Development and Application of a Chiral High Performance Liquid Chromatography Assay for Pharmacokinetic Studies of Methadone. *Chirality.* **12**: 681.

Brinkmann-van der Linden, E.C.M., de Haan, P.F., Havenaar, E.C. and van Dijk, W. (1998) Inflammation-induced expression of sialyl Lewisx is not restricted to α1-acid glycoprotein but also occurs to a lesser extent on α1-antichymotrypsin and haptoglobin. *Glycoconjugate Journal*. **15**: 177-182.

Brinkman-Van der Linden, E.C.M., Havenaar, E.C., Van Ommen, E.C.R., Van Kamp, G.J., Gooren, L.J.G. and van Dijk, W. (1996) Oral estrogen treatment induces a decrease in expression of sialyl Lewis x on α1-acid glycoprotein in females and male-to-female transsexuals. *Glycobiology*. **6**: 407-412.

Byers, H.L., Tarelli, E., Homer, K.A. and Beighton, D. (1999) Sequential deglycosylaton and utilization of the N-linked, complex-type glycans of human α_1-acid glycoprotein mediates growth of *Streptococcus oralis*. *Glycobiology.* **9**(5): 469-479.

Capelhorn, J.R.M., Bell J., Kleinbaum D.G. and Gebski V.J. (1993) Methadone dose and heroin use during maintenance treatment. *Addiction.* **88**: 119-124.

Ceciliani, F., Giordano, A. and Spagnolo, V. (2002) The systemic reaction during inflammation: the acute phase proteins. *Protein and Peptide Letters*. **9**: 211-223.

Ceciliani, F. and Pocacqua, V. (2007) The acute phase protein α1-acid glycoprotein: a model for altered glycosylation during diseases. *Current Protein and Peptide. Science*. **8**: 91-108.

Chang, C., Chen, Y., Lee, S. (1998) Coactivator TIF1β Interacts with Transcription Factor C/EBPβ and Glucocorticoid Receptor To Induce α1-Acid Glycoprotein Gene Expression. *Molecular and Cellular Biology*. **18**(10): 5880-5887.

Chen, Y. and Barkley, M.D. (1998) Toward Understanding Tryptophan Fluorescence in Proteins. *Biochemistry.* **37**: 9976-9982.

Chiu, K.M., Mortensen, R.F., Osmand, A.P. and Gewurz, H. (1977) Interactions of alpha1-acid glycoprotein with the immune system. *Immunology.* **32**: 997-1005.

Clyne, B. and Olshaker, J.S. (1999) The C-Reactive Protein. *The Journal of Emergency Medicine.* **17**(6): 1019–1025.

Compton, P., Charuvastra, V.C., Kintaudi, K. and Ling, W. (2000) Pain responses in Methadone-Maintained Opioid Abusers. *Journal of Pain and Symptom Management*. **20**: 237-245.

Compton, W.M. and Volkow, N.D. (2006) Major increases in opioid analgesic abuse in the United States: Concerns and strategies. *Drug and Alcohol Dependence.* **81**: 103–107.

Connock, M., Juarez-Garcia, A., Jowett, S., Frew, E., Liu, Z., Taylor, R.J., Fry-Smith, A., Day, E., Lintzeris, N., Roberts, T., Burls, A. and Taylor, R.S. (2007) Methadone and buprenorphine for the management of opioid dependence: a systematic review and economic evaluation. *Health Technology Assessment.* **11**(9): 1-171.

Costello, M., Fiedel, B.A. and Gewurz, H. (1979) Inhibition of platelet aggregation by native and desialised alpha-1 acid glycoprotein. *Nature.* **281**: 677-678**.**

Costello M.J., Gewurz, H. And Siegel, J.N. (1984) Inhibition of neutrophil activation by al-acid glycoprotein. *Clinical and Experimental Immunology.* **55**: 465-472.

Couldrey, C. and Green J.E. (2000) Metastases: the glycan connection. *Breast Cancer Research*. **2**: 321-323.

Crestani, B., Rolland, C., Lardeux, B., Fournier, T., Bernuau, D., Poüs, C., Vissuzaine, C., Li, L., Aubier, M. (1998) Inducible Expression of the α_1-Acid Glycoprotein by Rat and Human Type II Alveolar Epithelial Cells. *Journal of Immunology*. **160:** 4596-4605.

Daemen, M.A.R.C., Heemskerk, V.H., Cornelis, V.V., Denecker, G., Wolfs, T.G.A.M., Vandenabeele, P. and Buurman, W.A. (2000) Functional protection by acute phase

proteins α1-acid glycoprotein and α1-antitrypsin against ischemia/reperfusion injury by preventing apoptosis and inflammation. *Circulation*. **102**: 1420-1426.

Darke, S. and Ross, J. (1997). Polydrug dependence and psychiatric comorbidity among heroin injectors. *Drug and Alcohol Dependence.* **48**: 135-141.

Darke, S., Ross, J., Hall, W (1996) Overdose among heroin users in Sydney, Australia: 1. Prevelance and correlates of non-fatal overdose. *Addiction.* **91**: 405-411.

Davies, M.J., Smith, K.D., Carruthers, R.A., Chai, W., Lawson, A.M. and Hounsell, E.F. (1993) Use of a porous graphitised carbon column for the high-performance liquid chromatography of oligosaccharides, alditols and glycopeptides with subsequent mass spectrometry analysis. *Journal of Chromatography.* **646**: 317-326.

Davis, D., Grossman, S.H., Kitchell, B.B., Shand, D.G. and Routledge, P.A. (1985) The effects of age and smoking on the plasma protein binding of lignocaine and diazepam. *British Journal of Clinical Pharmacology.* **19**: 261-265.

Davoli, M., Perucci, C.A., Rapiti, E., Bargagli, A.M., D'Ippoliti, D., Forastiere, F., Abeni, D (1997) A Persistent Rise in Mortality among Injection Drug Users in Rome, 1980 through 1992. *American Journal of Public Health.* **87**: 851-853.

De Graaf, T.W., Van der Stelt, M.E., Anbergen, M.G. and van Dijk, W. (1993) Inflammation-induced expression of Sialyl Lewis X-containing glycan structures on α1-acid glycoprotein (orosomucoid) in human sera. *Journal of Experimental Medicine*. **177**: 657-666.

Dell, A. and H. R. Morris (2001). Glycoprotein Structure Determination by Mass Spectrometry. *Science*. **291**(5512): 2351-2356.

Dennis, J.W., Granovsky, M., Warren, C.E. (1999) Glycoprotein glycosylation and cancer progression. *Biochimica et Biophsica Acta.* **1473**: 21-34.

Dente, L., Ciliberto, G. and Cortese, R. (1985) Structure of the human α1-acid glycoprotein gene: sequence homology with other human acute phase protein genes. *Nucleic Acids Research.* **13**: 3941-3952.

Dente, L., Pizza, M.G., Metspalu, A. and Cortese, R. (1987) Structure and expression of the genes coding for human α1-acid glycoprotein. *EMBO Journal*. **6**: 2289-2296.

Department of Health (England) and the devolved administrations (1999) *Drug Misuse and Dependence: Guidelines on Clinical Management*. London: Department of Health (England), the Scottish Office Department of Health Welsh Office Department of Health and Social Services, Northern Ireland.

Department of Health (England) and the devolved administrations (2007) *Drug Misuse and Dependence: UK Guidelines on Clinical Management*. London: Department of Health (England), the Scottish Government, Welsh Assembly Government and Northern Ireland Executive.

Deprez, P., Gautschi, M. and Helenius, A. (2005) More Than One Glycan Is Needed for ER Glucosidase II to Allow Entry of Glycoproteins into the Calnexin/Calreticulin Cycle. *Molecular Cell.* **19**: 183-195.

Dionex Corporation, (1994) Dionex Corporation, Analysis of carbohydrates by high performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD). *Technical Note* **20**.

Diekstra, R.F. and Gulbinat, W. (1993) The epidemiology of suicidal behaviour: a review of three continents. *World Health Statistics Quarterly.* **46**(1):52-68.

Dobos, K.M., Khoo, K.H., Swiderek, K/M., Brennan, P.J., Belisle, J.T (1996) Definition of the full extent of glycosylation of the 45-kilodalton glycoprotein of Mycobacterium tuberculosis. *Journal of Bacteriology.* **178**: 2498-2506.

Dole, V.P. and Nyswander, M.E. (1966) Rehabilitation of heroin addicts after blockade with methadone. *New York State Journal of Medicine.* **66**:2011–2017.

Dole, V.P., Nyswander, M.E. and Kreek, M.J. (1966) Opioid Blockade. *Archives of Internal Medicine.* **118**(4): 304-309.

Doverty, M., Somogyi, A.A., White, J.M., Bochner, F., Beare, C.H., Menelaou, a., Ling, W (2001) Methadone maintenance patients are cross-tolerant to the antinociceptive effects of morphine. *Pain.* **93**: 155-163.

Drummer, O. and Odell, M. (2001) **In** Drummer, O.H. (Ed.) *The Forensic Pharmacology Drugs of Abuse.* Hodder Arnold.

Duché, J., Urien, S., Simon, N., Malaurie, E., Monnet, I. and Barré, J. (2000) Expression of the genetic variants of human alpha-1-acid glycoprotein in cancer. *Clinical. Biochemistry.* **33**: 197-202.

DuPont, R.L. (1988) abuse of Benzodiazepines: The Problems and the Solutions. *American Journal of Drug and Alcohol Abuse.* **14** (1): 1-69.

Durand, G. and Seta, N (2000) Protein Glycosylation and Diseases: Blood and Urinary Oligosaccharides as Markers for Diagnosis and Therapeutic Monitoring. *Clinical Chemistry.* **46**(6): 795-805.

Eap, C.B. and Baumann, P. (1993) The α1-acid glycoprotein: structure and possible functions in the acute phase response. *Acute Phase Proteins: Molecular Biology, Biochemistry and Clinical Applications*, CRC Press, Chapter 6: 107-116.

Eap, C.B., Bourquin, M., Martin, J-L., Spagnoli, J., Livoti, S., Powell, K., Baumann, P. and Déglon, J-J. (2000) Plasma concentrations of the enantiomers of methadone and therapeutic response in methadone maintenance treatment. *Drug and Alcohol Dependence*. **61**: 47–54.

Eap, C., Buclin, T. and Baumann, P. (2002) Interindividual Variability of the Clinical Pharmacokinetics of Methadone: Implications for the Treatment of Opioid Dependence. *Clinical Pharmacokinetics.* **41**(14): 1153-1193.

Eap, C.B., Cuendet, C. and Baumann, P. (1988a) Orosomucoid (alpha-1 acid glycoprotein) phenotyping by use of immobilized pH gradients with 8*M* urea and immunoblotting. *Human Genetics*. **80**(2):183-185.

Eap, C.B., Cuendet, C. and Baumann, P. (1988b) Selectivity in the binding of psychotropic drugs to the variants of alpha-1 acid glycoprotein. *Archives of Pharmacology*. **337**: 220-224.

Eap, C.B., Cuendet, C. and Baumann, P. (1988c) Binding of amitriptyline to alpha 1-acid glycoprotein and its variants. *Journal of Pharmacy and Pharmacology*. **40**: 767-70.

Eap, C.B., Cuendet, C. and Baumann, P. (1990) Binding of d-methadone, l-methadone, and dl-methadone to proteins in plasma of healthy volunteers: Role of the variants of α_1-acid glycoprotein. *Clinical Pharmacology*. **47**: 338-346.

Ebersole, J.L. and Cappelli, D. (2000) Acute-phase reactants in infections and inflammatory diseases. *Periodontology*. **23**: 19-49.

Elg, S.A., Mayer, AR., Carson, L.F., Twiggs, L.B., Hill, R.B. and Ramakrishnan, S. (1997) α-1 acid glycoprotein is an immunosuppressive factor found in the ascites from ovarian carcinoma. *Cancer*. **80**: 1448-1456.

Elliot, M.A., Elliot, H.G., Gallagher, K., McGuire, J., Field, M. and Smith, K.D. (1997) Investigation into the Concanavalin A reactivity, fucosylation and oligosaccharide microheterogeneity of α1-acid glycoprotein expressed in the sera of patients with rheumatoid arthritis. *Journal of Chromatography B*. **688**: 229-237.

Elliot, M.A., Jørgensen, H.G., Smith, K.D. (1998) Hypersialylation of α1-acid glycoprotein in rheumatoid arthritis. *Pharmacy and Pharmacology Communications*. **4**: 545-547.

Epps, D.E., Raub, T.J., Caiolfa, V., Chiari, A., Zamai, M (1999) Determination of the Affinity of Drugs toward Serum albumin by Measurement of the Quenching of the Intrinsic Tryptophan Fluorescence of the protein. *Journal of Pharmacy and Pharmacology*. **51**(1): 41-48.

Fan, J-Q., Namiki, Y., Matsuoka, K., Lee, Y.C. (1994) Comparison of Acid Hydrolysis Conditions for Asm-Linked Oligosacchairdes. *Analytical Biochemistry*. **219**: 375-378.

Fainsinger, R., Schoeller, T. and Bruera, E. (1993) Methadone in the management of cancer pain: a review. *Pain*. **52**: 137-147.

Ferrier, R.J. and Collins, P. M. (1972) *Monosaccharide Chemistry*. Penguin Books Ltd, pg 235.

Fiellin, D. A., O'Connor, P. G., Chawarski, M., Pakes, J.P., Pantalon, M.V. and Schottenfeld, R.S. (2001). Methadone Maintenance in Primary Care. *Journal of the American Medical Association*. **286**(14): 1724-1731.

Finnegan, L.P., Haga, T., Kaltenbach, K.A (1991) Treatment issues for opioid-dependent women during the perinatal period. *Journal of Psychoactive Drugs*. **23**:191- 201.

Flower, D.R., North, A.C.T. and Sansom, C.E. (2000) The lipocalin protein family: structural and sequence overview. *Biochimica et Biohysica Acta*. **1482**: 9-24.

Flower D.R (1996) The lipocalin protein family: structure and function. *Biochemical Journal.* **318**: 1-14.

Fournier, T., Medjoubi-N, N. and Porquet, D. (2000) Alpha-1-acid glycoprotein. *Biochimica et Biophysica Acta*. **1482**: 157-171.

Freeze, H. H. (2001). Update and perspectives on congenital disorders of glycosylation. *Glycobiology.* **11**(12): 129R-143R.

French, D., Watson, J., McCahill, B., Taggart, I. and Smith, K.D. (2002) A preliminary evaluation of the functional significance of alpha-1-acid glycoprotein glycosylation on wound healing. *Biomedical Chromatography.* **16**: 412-419.

Friedman, M.L., Schlueter, K.T., Kirley, T.L., Halsall, H.B. (1985). Fluorescence quenching of human orosomucoid. *Biochemical Journal.* **232**: 863-867.

Frostell-Karlsson, A., Remaeus,A., Roos, H., Andersson, K., Borg, P., Hamalainen, M., Karlsson, R. (2000) Biosensor analysis of the interaction between immobilized human serum albumin and drug compounds for prediction of human serum albumin binding levels, *Journal of Medicinal Chemistry.* **43**: 1986–1992.

Gallacher, G. (2010) Alpha-1-acid glycoprotein as a biomarker for early breast cancer. *PhD thesis.*

Galloway, F.R. and Bellet, N.F. (1999) Methadone conversion to EDDP during GC-MS analysis of Urine Samples. *Journal of Analytical Toxicology.* **23**: 615.

Garrido, M.J. and Trocóniz, I.F. (1999) Methadone: a review of its pharmacokinetic/ pharmcodynamic properties. *Journal of Pharmacology and Toxicology.* **42**: 61-66.

Gavel, Y. and von Heijne, G. (1990) Sequence differences between glycosylated and non-glycosylated Asn-X-Thr/Ser acceptor sites: implications for protein engineering. *Protein. Engineering.* **3**: 433-442.

Gelpi, E. (2003) Contributions of liquid chromatography–mass spectrometry to ''highlights'' of biomedical research. *Journal Chromatography A.* **1000:** 567-581.

Gendler, S.J., Dermer, G.B., Silverman, L.M. and Tökés, Z.A (1982) Synthesis of α1-antichymotrypsin and α1-acid glycoprotein by human breast epithelial cells. *Cancer Research.* **42**: 4567-4573.

Gomes, M.B., Piccirillo, L.J., Nogueira, V.G., Matos, H.J. (2003). Acute-phase proteins among patients with type 1 diabetes. *Diabetes and Metabolism.* **29**: 405-411.

Gossop, M., Stewart, D., Treacy, S, Marsden, J (2002) A prospective study of mortality among drug misusers during a 4-year period after seeking treatment. *Addiction*. **97**: 39-47.

Guile. G.R., Rudd. P.M., Wing. D.R., Prime. S.B., Dwek. R.A. (1996). A Rapid High-Resolution High-Performance Liquid Chromatographic Method for Separating Glycan Mixtures and Analyzing Oligosaccharide Profiles. *Analytical Biochemistry.* **240**(2): 210-226.

Gupta N, Shankernarayan NP, Dharmalingam K (2010) alpha1-acid glycoprotein as a putative biomarker for monitoring the development of the type II reactional stage of leprosy. *Journal of Medical Microbiology.* **59**: 400–407.

Haasen, C., Verthein, U., Degkwitz, P., Berger, J., Krausz, M., Naber, D. (2007) Heroin-assisted treatment for opioid dependence. *The British Journal of Psychiatry.* **191**(1): 55-62.

Hage, D.S. (2001) Chromatographic and electrophoretic studies of protein binding to chiral solutes. *Journal of Chromatography A.* **906**: 459–481.

Hall, W. D. and Mattick R. P. (2007). Clinical Update: Codeine Maintenance in Opioid Dependence. *Lancet.* **370**(9587): 550-552.

Hallinan, R., Ray, J., Byrne, A., Agho, K., Attia, J. (2006) Therapeutic thresholds in methadone maintenance treatment: A receiver operating characteristic analysis. *Drug Alcohol and Dependence.* **81**: 129-136.

Hanna, J., Foster, D.J.R., Salter, A., Somogyi, A.A., White, J.M., Bochner, F (2005) Within- and between- subject variability in methadone pharmacokinetics and pharmacodynamics in methadone maintenance subjects *British Journal of Clinical Pharmacology.* **60**(4): 404-413.

Hansen, J.E., Bøg-Hansen, T.C., Pedersen, B., Neland, K. (1989). Microheterogeneity of orosomucoid in pathological conditions. *Electrophoresis.* **10**(8-9):574-578.

Hansen, J.S., Larsen, V.A. and Bøg-Hansen, T.C. (1984) The microheterogeneity of α1-acid glycoprotein in inflammatory lung disease, cancer of the lung and normal health. *Clinica Chimica Acta.* **138**: 41-47.

Hao, Y. and Wickerhauser, M. (1973) Development of large-scale methods IV. A simple method for the large-scale preparation of α1-acid glycoprotein. *Biochimica. et Biophysica Acta.* **322**: 99-108.

Hardy, M.R. and Townsend, R.R. (1988) Separation of positional isomers of oligosaccharides and glycopeptides by high-performance anion-exchange chromatography with pulsed amperometric detection. *Proceedings of the National Academy of Sciences of the United States of America.* **85**: 3289-3293.

Hardy, M.R., Townsend, R.R. and Lee, Y.C. (1988) Monosaccharide analysis of glycoconjugates by anion exchange chromatography with pulsed amperometric detection. *Analytical Biochemistry.* **170**: 54-62.

Hardy, M.R. and Townsend, R.R. (1994) High-pH anion-exchange chromatography of glycoprotein-derived carbohydrates. *Methods in Enzymology.* **230**: 208-225.

Harvey, D.J. (2005) Structural determination of *N*-linked glycans by matrix-assisted laser desorption/ionization and electrospray ionization mass spectrometry. *Proteomics.* **5**: 1774-1786.

Hashimoto, S., Asao, T., Takahashi, J., Yagihashi, Y., Nishimura, T., Saniabadi, A.R., Poland, D.C.W., van Dijk, W., Kuwano, H., Kochibe, N. and Yazawa, S. (2004) α1-acid glycoprotein fucosylation as a marker of carcinoma progression and prognosis. *Cancer*. **101**: 2825-2836.

Haston, J.L., FitzGerald, O., Kane, D. and Smith, K.D. (2003) The influence of α1-acid glycoprotein on collagenase-3 activity in early rheumatoid arthritis. *Biomedical Chromatography*. **17**: 361-364.

Havard, A., Teesson, M., Darke, S., Ross, J (2006) Depression among heroin users: 12-Month outcomes from the Australian Treatment Outcome Study (ATOS). *Journal of Substance Abuse Treatment*. **30**: 355– 362.

Havenaar, E.C., Hoff, R.C., van den Eijnden, D.H., van Dijk, W (1998) Sialyl Lewis(x) epitopes do not occur on acute phase proteins in mice: relationship to the absence of α3-fucosyltransferase in the liver. *Glycoconjugate Journal*. **15:** 389-395.

Hay, G., Gannon, M., McKeganey, N., Hutchison, S., Goldberg, D. (2005). Estimating the National and Local Prevalence of Problem Drug Misuse in Scotland. Glasgow, Centre for Drug Misuse Research, University of Glasgow. Scottish Centre for Infection and Environmental Health: 1-57.

Hazai, E., Visy, J., Fitos, I., Bikádi, Z., Simonyi, M. (2006) Selective binding of coumarin enantiomers to human *a*1-acid glycoprotein genetic variants. *Bioorganic and Medicinal Chemistry*. **4**: 1959-1965.

Hermentin, P., Witzel, R., Doenges, R., Bauer, R., Haupt, H., Patel, T., Parekh, R.B. and Brazel, D. (1992) The mapping by high-pH anion-exchange chromatography with pulsed amperometric detection and capillary electrophoresis of the carbohydrate moieties of human plasma α1-acid glycoprotein. *Analytical Biochemistry*. **206**: 419-429.

Hervé, F., Gomas, E., Duche, J-C., Tillement, J-P. (1993) Evidence for differences in the binding of drugs to the two main genetic variants of human α1-acid glycoprotein. *British Journal of Clinical Pharmacology*. **36**: 241-249.

Hervé, F., Millot, M., Eap, C.B., Duché, J. and Tillement, J. (1996) Two-step chromatographic purification of human plasma α1-acid glycoprotein. Its application to the purification of rare phenotype samples of the protein and their study by chromatography on immobilized metal chelate affinity adsorbent. *Journal of Chromatography B*. **678**: 1-14.

Hervé, F., Caron, G., Duché, J., Gaillard, P., Rahman, N.A., Tsantili-Kakoulidou, A., Carrupt, P-A., D'Athis, P., Tillement, J-P. and Testa, B. (1998) Ligand Specificity of the Genetic Variants of Human a1-Acid Glycoprotein: Generation of a Three-Dimensional Quantitative Structure-Activity Relationship Model for Drug Binding to the A Variant. *Molecular Pharmacology*. **54**: 129-138.

Higai, K., Azuma, Y., Aoki, Y. and Matsumoto, K. (2003) Altered glycosylation of α1-acid glycoprotein in patients with inflammation and diabetes mellitus. *Clinica Chimica Acta*. **329**: 117-125.

Higai, K., Aoki, Y., Azuma, Y. and Matsumoto, K. (2005) Glycosylation of site-specific glycans of α1-acid glycoprotein and alterations in acute and chronic inflammation. *Biochimica et Biophysica Acta*. **1725**: 128-135.

Hochepied, T., Berger, F.G., Baumann, H. and Libert, C. (2003) α1-acid glycoprotein: an acute phase protein with inflammatory and immunomodulating properties. *Cytokine and Growth Factor Reviews.* **14**: 25-34.

Hochepied, T., Van Molle, W., Bergeri, F.G., Baumann, H. and Libert, C. (2000) Involvement of the Acute Phase Protein α1-Acid Glycoprotein in Nonspecific Resistance to a Lethal Gram-negative Infection. *Journal of Biological Chemistry.* **275**(20): 14903-14909.

Holmberg, S.D. (1996) The Estimated Prevelance and Incidence of HIV in 96 Large US Metropolitan Areas. *American Journal of Public Health.* **86**: 642-654.

Home Office (2006) Drug use, smoking and drinking among young people in England in 2005: headline figures. *A survey carried out for the Health and Social Care Information Centre and the Home Office by the National Centre for Social Research and the National Foundation for Educational Research.*

Hoshino, Y., Dalai, S.K., Wang., K., Pesnicak, L., Lau, T.Y., Knipe, D.M., Cohen, J.I., Straus, S.E. (2005) Comparative Efficacy and Immunogeneicity of Replication-Defective, Recombinant Glycoprotein, and DNA Vaccines for Herpes Simplex Virus 2 infections in Mice and Guinea Pigs. *Journal of Virology.* **79**(1): 410-418.

Housová, J., Wilczek H., Haluzík MM., Křemen J., Křížová J., Haluzík M (2005) Adipocyte-Derived Hormones in Heroin Addicts: the Influence of Methadone Maintenance Treatment. *Physiological Research.* **54**: 73-78

Imperiali, B. and O'Connor, S.E. (1999) Effect of N-linked glycosylation on glycopeptides and glycoprotein structure. *Curent5 Opinion in Chemical Biology*. **3**: 643-649.

Imre, T., Kremmer, T., Héberger, K., Molnár-Szöllösi, E., Ludányi, K., Pócsfalvi, G., Malorni, A., Drahos, L. and Vékey, K. (2008) Mass spectrometric and linear discriminant analysis of N-glycans of human serum alpha-1-acid glycoprotein in cancer patients and healthy individuals. *Journal of Proteomics.* **71**: 186-197.

Ingham, K.C. (1990) Precipitation of proteins with polyethylene glycol. *Methods in Enzymology*. **182**: 301-306.

Israili, Z.H. and Dayton, P.G. (2001) Human alpha-1-glycoprotein and its interactions with drugs. *Drug Metabolism Reviews*. **33**: 161-235.

Jackson, P.R., Tucker, G.T., Woods, H.F. (1982). Altered plasma drug binding in cancer: Role of α1-acid glycoprotein and albumin. *Clinical Pharmacology and Therapeutics*. **32**(3):295-302.

Jaeken, J. and Matthijs, G. (2007) Congenital disorders of glycosylation: a rapidly expanding disease family. *Annual Review of Genomics and Human Genetics.* **8**: 261-278.

Jia, Z., Ramstad, T. and Zhong, M. (2002) Determination of protein–drug binding constants by pressure-assisted capillary electrophoresis (PACE)/frontal analysis (FA) *Journal of Pharmaceutical and Biomedical Analysis*. **30**: 405–413.

Johnson, J.A. and Livingston, T.N. (1997). Differences between blacks and whites in plasma protein binding of drugs. *European Journal of Clinical Pharmacology*. **51**: 485-488.

Johnson, D.C. and LaCourse, W.R. (1992) Pulsed electrochemical detection at noble metal electrodes in liquid chromatography. *Electroanalysis.* **4**(4): 367-380

Johnson, D. and Smith, K.D. (2006) The efficacy of certain anti-tuberculosis drugs is affected by binding to α-1-acid glycoprotein. *Biomedical Chromatography*. **20**: 551-560.

Johnston, L.D., O'Malley, P.M., Bachman, J.G., Schulenberg, J.E (2004) Monitoring the Future National Results on Adolescent Drug Use. *National Institute on Drug Abuse.* NIH Publication number 05-5726.

Jørgensen, H.G., Elliott, M.A., Allan, E.K., Carr, C.E., Holyoake, T.L., Smith, K.D. (2002) α1-Acid glycoprotein expressed in the plasma of chronic myeloid leukemia patients does not mediate significant in vitro resistance to STI571. *Blood*. **99:**713-715.

Kalmovarin, N., Friedrichs, W.E., O'Brien, H.V., Linehan, L.A., Bowman, B.H., Yang, F (1991) Extrahepatic expression of plasma protein genes during inflammation. *Inflammation.* **15**: 369-379.

Karch, S.B. and Stephens, B.G. (2000). Toxicology and pathology of deaths related to methadone: retrospective review. *The Western Journal of Medicine*. **172**:11-14.

Kim, S., Kim, S., Ha1, K-S., Leem, S-H. (2000) An improved method for quantitative sugar analysis of glycoproteins. *Experimental and Molecular Medicine.* **32**(3): 141-145.

Kimura, T., Shibukawa, A. and Matsuzaki, K. (2006) Biantennary Glycans as well as Genetic Variants of α$_1$-Acid Glycoprotein Control the Enantioselectivity and Binding Affinity of Oxybutynin. *Pharmaceutical Research*. **23**(5): 1038-1042.

Klee, H. (1990) A new target for behavioral research—amphetamine misuse. *British Journal of Addiction.* **85**: 1125-1132.

Kobata, A. (1992) Structure and functions of the sugar chains of glycoproteins. *European Journal of Biochemistry.* **209**(2): 483-501.

Kobata, A. (2000) A journey to the world of glycobiology. *Glycoconjugate Journal.* **17**: 443-464.

Koj, A. (1996) Initiation of acute phase response and synthesis of cytokines. *Biochimica et Biophysica Acta*. **1317**: 84-94.

Koj, A. (1998). Termination of Acute-Phase Response: Role of Some Cytokines and Anti-Inflammatory Drugs. *General Pharmacology*. **31**(1): 9-18.

Kornfeld, R. and Kornfeld, S. (1985) Assembly of Asparagine-Linked Oligosaccharides. *Annual Review Biochemistry.* **54**: 631-664.

Kopecký, V., Ettrich, R., Hofbauerová, K. and Baumruk, V. (2003) Structure of human α₁-acid glycoprotein and its high-affinity binding site. *Biochemical and Biophysical Research Communications.* **300**(1): 41-46.

Krambeer, L., Von-McKnelly, W., Gabrielli, W.F., Penick, E. (2001). Methadone Therapy for Opioid Dependence. *American Family Physician.* **63**(12): 2404-2410.

Krausz, M., Verthein, U., Degkwitz, P., Haasen, C., Raschke, P. (1998) Maintenance treatment of opiate addicts in Germany with medications containing codeine - results of a follow-up study. *Addiction.* **93**(8): 1161- 1167.

Kreek, M. J. (1997). Opiate and Cocaine Addictions: Challenge for Pharmacotherapies. *Pharmacology Biochemistry and Behaviour.* **57**(3): 551-569.

Kreek, M.J. (1992). Rationale for maintenance pharmacotherapy of opiate dependence *Research Publications-Association for Research in Nervous and Mental Disease.* **70**:205-230.

Kremer, J.M.H., Wilting, J., Janssen, L.H.M. (1988). Drug Binding to Human Alpha-1-acid Glycoprotein in Health and Disease. *The American Society for Pharmacology and Experimental Therapeutics.* **40**:1-47.

Kremmer, T., Szöllösi, E., Boldizsár, M., Vincze, B., Ludányi, K., Imre, T., Schlosser, G., Vékey, K (2004) Liquid chromatographic and mass spectrometric analysis of human serum acid alpha-1-glycoprotein. *Biomedical Chromatography.* **18**(5): 323-329.

Kremmer, T., Boldizsár, M., Kovács, J., Paulik, E., Bencsik, K., Szajáni, B (1995) Determination and Analysis of Human Serum Alpha-1-acid Glycoprotein by Liquid Chromatographic Methods. *Journal of Liquid. Chromatography.* **18**: 1207-1218.

Kristensen, K., Christensen, C.B., Christrup, L.L. (1995) The mu1, mu2, delta, kappa opioid receptor binding profiles of methadone stereoisomers and morphine. *Life Sciences.* **56**(2): PL 45-50.

Kuroda, Y., Matsumoto, S., Shibukawa, A., Nakagawa, T (2003) Capillary electrophoretic study on pH dependence of enantioselective disopyramide binding to genetic variants of human a1-acid glycoprotein. *Analyst.* **128:** 1023-1027.

Kushner, I. and Rzewnicki, D.L. (1994) The acute phase response: general aspects. *Baillière's Clinical Rheumatology.* **8**: 513-530.

Kuster, B., Wheeler S.F., Hunter A.P., Dwek R.A., Harvey D.J. (1997) Sequencing of N-Linked Oligosaccharides Directly from Protein Gels: In-Gel Deglycosylation Followed by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry and Normal-Phase High-Performance Liquid Chromatography. *Analytical Biochemistry.* **250**(1): 82-101.

Laegreid, L., Hagberg, G., Lundberg, A. (1992) The Effect of Benzodiazepines on the Fetus and the Newborn. *Neuropediatrics.* **23**(1): 18-23.

Laurent, P., Miribel, L., Bienvenu, J., Vallve, C. and Arnaud, P. (1984) A three-step purification of human α1-acid glycoprotein. *Federation of European Biochemical Societies Letters.* **168**: 79-83.

Leatherbarrow, R.J. and Dean, P.D.G. (1980) Studies on the mechanism of binding of serum albumins to immobilized Cibacron Blue F3GA. *Biochemical Journal.* **189**: 27-34.

Leavitt, S., M. Shinderman, Maxwell, S., Eap, C.B, Paris, P. (2000). When 'enough' is not enough. *Mount Sinai Journal of Medicine.* **67**(5): 404-411.

Lechner, J. and Wieland, F. (1989) Structure and biosynthesis of prokaryotic glycoproteins. *Annual Review of Biochemistry.* **58**:173–194.

Lee, Y.C. (1990) High-performance anion-exchange chromatography for carbohydrate analysis. *Analytical Biochemistry.* **189**: 151-162.

Lee, Y.C. (1996) Carbohydrate analyses with high-performance anion-exchange Chromatography. *Journal of Chromatography A.* **720**: 137-149.

Li, X., Wei, B., Goodglick, L., Wen, T., Xia, L., Braun, J. (2009). Investigating Therapeutic Approach of IBD Using Recombinant Glycoprotein Mucin2. *FASEB Journal.* **23**: 570.1.

Liao, Y.J., Taylor, J.M., Vannice, J.L., Clawson, G.A., Smuckler, E.A. (1985). Structure of the rat alpha 1-acid glycoprotein gene. *Molecular and Cellular Biology.* **5**(12): 3634-3639.

Lis, H. and Sharon, N. (1993) Protein glycosylation: structural and functional aspects. *European Journal of Biochemistry.* **218**: 1-27.

Listinsky, J.J., Siegal, G.P., Listinsky, C.M. (1998) α-L-fucose. A potentially critical molecule in pathologic process including neoplasia. *American Journal of Clinical Pathology.* **110**: 425-440.

Lögdberg, L. and Wester, L. (2000) Immunocalins: a lipocalin subfamily that modulates immune and inflammatory responses. *Biochimica et Biophysica Acta.* **1482**: 284-297.

Loimer, N. and Schmid, R. (1992). The use of plasma levels to optimize methadone maintenance treatment. *Drug and Alcohol Dependence.* **30**(3):241-246.

Lynskey, M., Degenhardt, L., Hall, W. (2000) Cohort trends in youth suicide in Australia 1964–1997. *Australian and New Zealand Journal of Psychiatry.* **34**: 408-412.

Macdougall, I.C., Eckardt, K. (2006) Novel strategies for stimulating erythropoiesis and potential new treatments for anaemia. *Lancet.* **368**: 947-953.

Mackiewicz, A. and Mackiewicz, K. (1995) Glycoforms of serum α1-acid glycoprotein as markers of inflammation and cancer. *Glycoconjugate Journal.* **12**: 241-247.

Mackiewicz, A., Pawlowski, T., Mackiewicz-Pawlowski, A., Wiktorowicz, K. and Mackiewicz, S. (1987) Microheterogeneity forms of alpha$_1$-acid glycoprotein as indicators of rheumatoid arthritis activity. *Clinica Chimica Acta.* **163**: 185-190.

Maeder, T. (2002). Sweet Medicines. *Scientific American.* **287**(1): 40-47.

Manfredi, P.L., Gonzales, G.R., Cheville, A.L., Kornick, C., Payne, R. (2001) Methadone Analgesia in Cancer Pain Patients on Chronic Methadone Maintenance Therapy. *Journal of Pain and Symptom Management.* **21**(2): 169-174.

Maremmani, I., Pacini, M., Lubrano, S., Giuntoli, G. and Lovrecic, M. (2002) Harm reduction and specific treatments for heroin addiction. Different approaches or levels of intervention? An illness-centred perspective. *Heroin Addiction & Related Clinical Problems.* **4**(3): 5-11.

Maremmani, I., Pacini, M. Lubrano, S.. and Lovrecic, M. (2003) When "enough" is still not "enough". Effectiveness of high-dose methadone in the treatment of heroin addiction. *Heroin Addiction & Related Clinical Problems*. **5**(1): 17-32.

Marsch, L. A. (1998). The efficacy of methadone maintenance interventions in reducing illicit opiate use, HIV risk behaviour, and criminality: a meta-analysis. *Addiction.* **93**(4): 515-532.

Maruyama, T., Otagiri, M., Takadate, A. (1990). Characterization of drug binding sites on alpha 1-acid glycoprotein. *Chemical and Pharmaceutical Bulletin*. **38**(6): 1688-1691.

Matsumoto K., Sukimoto, K., Nishi, K., Maruyama, T., Suenaga, A., Otagiri, M. (2002) Characterization of Ligand Binding Sites on the α_1-Acid Glycoprotein in Humans, Bovines and Dogs. *Drug Metabolism Pharmacokinetics.* **17**(4): 300-306.

Matsushita, Y. and Moriguchi, I. (2003) Measurement of protein binding by ultracentrifugation. *Chemical and Pharmaceutical Bulletin.* **33**: 2948-2955.

McCarthy, J.J. and Posey, B.L. (2000) Methadone Levels in Human Milk. *Journal of Human Lactation.* **16**(2): 115-120.

McGuire, J.M., Stewart, Y.M. and Smith, K.D. (1999) The Effect of pH on the High pH Anion-Exchange Chromatography Elution of monosaccharides. *Chromatographia.* **49**(11/12): 699-702.

McKeganey, N., Bloor ,M., Robertson, M., Neale, J., MacDougall, J. (2006). Abstinence and drug abuse treatment: Results from the Drug Outcome Research in Scotland study. *Drugs: education, prevention and policy.* **13**(6): 537-550.

Melichar, J.K., Hume, S.P., Williams, T.M., Daglish, R.C., Taylor, L.G., Ahmad, R., Malizia, A.L., Brooks, D.J., Myles, J.S., Lingford-Hughes, A. and Nutt, D.J (2005) Using [11C]Diprenorphine to Image Opioid Receptor Occupancy by Methadone in Opioid Addiction: Clinical and Preclinical Studies. *Journal of Pharmacology and Experimental. Therapeutics.* **321**(1): 309-315.

Menkes, C. (1993). Effects of disease-mdifying anti-rheumatic drugs, steroids and non-steroidal anti-inflammatory drugs on acute-phase proteins in rheumatoid arthritis. *British Journal of Rheumatology.* **32**:14-18.

Metzler, D.E. and Metzler, C.M. (2003) Sugars, Polysaccharides and Glycoproteins, *Biochemistry: the chemical reactions of living cells.* Volume 2 (Edited by Metzler, D.E.) Academic Press.

Mock, K.K., Davey, M. and Cottrell, J.S. (1991) The analysis of underivatized oligosaccharides by matrix-assisted laser desorption mass-spectrometry. *Biochemical and Biophysical Research Communications*. **177**: 644–651.

Mooney, P., Hayes, P. and Smith, K. (2006) The putative use of α-1-acid glycoprotein as a non-invasive marker of fibrosis. *Biomedical Chromatography.* **20**: 1351-1358.

Morell, A.G., Gregoriadia, G. and Scheinberg, I.H. (1971) The Role of Sialic Acid in Determining the Survival of Glycoproteins in the Circulation. *Journal of Biological Chemistry.* **246**(5):1461-1467.

Morley, J.S. and Makin, M.K. (1998) The use of methadone in cancer pain poorly responsive to other opioids. *Pain Reviews.* **5**: 51–58.

Morrone, G., Ciliberto , G., Oliviero, S., Arcone, R., Dente, L., Content, J., Cortese, R. (1988) Recombinant interleukin 6 regulates the transcriptional activation of a set of human acute phase genes. *Journal of Biological Chemistry*. **263**: 12554-12558.

Moule, S.K., Peak, M., Thompson, S. and Turner, G.A. (1987) Studies of the sialylation and microheterogeneity of human serum α1-acid glycoprotein in health and disease. *Clinica Chimica Acta*. **166**: 177-185.

Muller, W.E. (1988). **In** I.W. Wainer and D.E. Drayer (Eds), Drug Stereochemistry. *Analytical Methods and Pharmacology*, Marcel Dekker, New York and Basel, p. 227

Neuburger, G.G. and Johnson, D.C. (1987) Pulsed amperometric detection of carbohydrates at gold electrodes with a two-step potential waveform. *Analytical Chemistry.* **59**(1): 150-154.

Newcombe, R. (1996). Live and let die: is methadone more likely to kill you than heroin? *Druglink*. January-February issue. 9-12.

O'Connor, S.E. and Imperiali, B. (1997) Conformational Switching by Asparagine-Linked Glycosylation. *Journal of the American Chemical Society.* **119**: 22295-96.

O'Connor, P.G., Oliveto, A.H., Shi, J.M., Triffleman, E.G., Carroll, K.M., Kosten, T.R., Rounsaville, B.J., Pakes, J.A., Schottenfeld, R.S. (1998) A randomized trial of buprenorphine maintenance for heroin dependence in a primary care clinic for substance users versus a methadone clinic. *American Journal of Medicine.* **105**(2): 100-105.

Okinaga, T., Ohashi, Y. and Hoshi, M. (1992) A novel saccharide structure, Xyl 1→3 Gal 1→ (SO3−)3, 4 Fuc→, is present in acrosome reaction-inducing substance of the starfish, *Asterias amurensis. Biochemical and Biophysical Research Communications*. **186**(1): 405-410.

Olsen, G. (1973). Methadone binding to human plasma proteins. *Clinical Pharmacology and Therapeutics.* 14: 338-343.

Orczyk-Pawiłowicz, M., Hirnle, L. and Kątnik-Prastowska, I. (2006) Alterations of N-glycan branching and expression of sialic acid on amniotic fluid alpha-1-acid glycoprotein derived from second and third trimesters of normal and prolonged pregnancies. *Clinica Chimica Acta*. **367**: 86 – 92.

Oravcova, J., Sojkova, D. Lindner W. (1996) Comparison of the Hummel-Dreyer method in high-performance liquid chromatography and capillary electrophoresis conditions for study of the interaction of *(RS)-, (R)-* and (S)-carvedilol with isolated plasma proteins. *Journal of Chromatography B*. **682**: 349-357.

Parikh, H.H., McElwain, K., Balasubramanian, V., Leung, W., Wong, D., Morris, M.E. and Ramanathan, M. (2000) A Rapid Spectrofluorimetric Technique for Determining Drug-Serum Protein Binding Suitable for High-Throughput Screening. *Pharmaceutical Research*. **17**(5): 632-637.

Parsons, L.H., Koob, G.F. and Weiss, F. (1995) Serotonin dysfunction in the nucleus accumbens of rats during withdrawal after unlimited access to intravenous cocaine. *Journal of Pharmacology and Experimental Therapeutics.* **274**: 1182-1191

Paskach, T.J., Lieker, H-P., Reilly, P.J., Thielecke, K. (1991) High-performance anion-exchange chromatography of sugars and sugar alcohols on quaternary ammonium resins under alkaline conditions. *Carbohydrate Research*. **215**(1): 1-14.

Paterson, S.C., Lim, C.K., Smith, K.D. (2003). Analysis of the interaction between alpha-1-acid glycoprotein and tamoxifen and its metabolites. *Biomedical Chromatography.* **17**: 143-148.

Paulson, J.C. and Colley, K.J. (1989) Glycosyltransferases: structure, localization and control of cell type-specific glycosylation. *Journal of Biological Chemistry.* **264**: 17615-17618.

Pawlowski, T., Mackiewicz, S.H. and Mackiewicz, A. (1989) Microheterogeneity of alpha1-acid glycoprotein in the detection of intercurrent infection in patients with rheumatoid arthritis. *Arthritis and Rheumatism.* **32**: 347-351.

Peles, E., Schreiber, S., Naumovsky, Y., Adelson, M. (2007) Depression in methadone maintenance treatment patients: Rate and risk factors. *Journal of Affective Disorders.* **99**: 213–220.

Perkins, S.J., Kerckaert, J-P., Loucheux-lefebvre, M (1985) The shapes of biantennary and tri/tetraantennary α_1-acid glycoprotein by small angle neutron and X-ray scattering. *European Journal of Biochemistry.* **147**: 151-171.

Perneger, T.V., Giner, F., del Rio, M. and Mino, A. (1998) Randomised trial of heroin maintenance programme for addicts who fail in conventional drug treatments. *Biomedical Journal*. **317**: 13-18.

Pfeiffer, G., Geyer, H., Geyer, R., Kalsner, I. and Wendorf, P. (1990) Separation of glycoprotein-*N*-glycans by high-pH anion-exchange chromatography. *Biomedical Chromatography*. 4(5): 193-199.

Pike, E., P. Kierulf, Skuterud, B., Bredesen, J.E., Lunde, P.K.M. (1983). Drug binding in sera deficient in lipoproteins, albumin or orosomucoid. *British Journal of Clinical Pharmacology*. **16**: 233-239.

Plancke, Y., Dautrevaux, M. And Biserte, G. (1978) Human serum hemopexin : direct evidence for change of its isoelectric point upon heme binding. A new serum protein fractionation. *Biochimie.* **60**: 171–175.

Plummer, T.H., Elder, J.H., Alexander, S., Phelan, A.W. and Tarentino, A.L. (1984) Demonstration of Peptide:N-Glycosidase F Activity in Endo-β-N-acetylglucosaminidase F Preparations. *Journal of Biological Chemistry*. **259**(17): 10700-10704.

Pos, O., van Dijk, W., Ladiges, N., Linthorst, C., Sala, M., van Tiel, D., Boers, W. (1988) Glycosylation of four acute-phase glycoproteins secreted by rat liver cells in vivo and in vitro. Effects of inflammation and dexamethasone. *European Journal of Cell Biology.* **46**: 121-128.

Pos, O., Oostendorp, R.A.J., Van der Stelt, M.E., Scheper, R.J. and van Dijk, W. (1990) Con A-nonreactive human α1-acid glycoprotein (AGP) is more effective in modulation of lymphocytes proliferation than Con A-reactive AGP serum variants. *Inflammation*. **14**: 133-141.

Power, A., Mackie, M., Thompson, A., Carroll, J. (2002). Dihydrocodeine, Detoxification and DVTs. *NHS: Glasgow Prescriber.*

Preston, K.L., Griffiths, R., Stitzer, M.L., Bigelow, G.E. and Liebson, I.A. (1984) Diazepam and methadone interactions in methadone maintenance. *Clinical Pharmacology and Therapeutics.* 36: 534–541.

Pruessner, J.C., Champagne, F., Meaney, M.J. and Dagher, A. (2004) Dopamine Release in Response to a Psychological Stress in Humans and Its Relationship to Early Life Maternal Care: A Positron Emission Tomography Study Using [^{11}C]Raclopride. *The Journal of Neuroscience*. **24**(11):2825-2831.

Rademacher, T.W., Parekh, R.B. and Dwek, R.A. (1988) Glycobiology. *Annual Review of Biochemistry.* **57**: 785-838.

Rehm, J., Gschwend, P., Steffen, T., Gutzwiller, F., Dobler-Mikola, A., Uchtenhagen, A. (2001). Feasibility, safety, and efficacy of injectable heroin prescription for refractory opioid addicts: a follow-up study. *Lancet.* **358**(9291): 1417-1420.

Robertson, J. R., Raab, G. M., Bruce, M., McKenzie, J.S., Storkey, H.R., Salter, A. (2006). Addressing the efficacy of dihydrocodeine versus methadone as an alternative maintenance treatment for opiate dependence: a randomized controlled trial. *Addiction.* **101**(12): 1752-1759.

Rocklin, R.D. and Pohl, C.A. (1983) Determination of Carbohydrates by Anion Exchange Chromatography with Pulsed Amperometric Detection. *Journal of Liquid Chromatography and Related Technologies.* **6**(9): 1577-1590.

Rodriguez Rosas, M.E., Preston, K.L., Epstein, d.H., Moolchan, E.T. Wainer, I.W. (2003) Quantitative determination of the enantiomers of methadone and its metabolite (EDDP) in

human saliva by enantioselective liquid chromatography with mass spectrometric detection. *Journal of Chromatography B*. **796**: 355-370.

Romach, M.K., Piafsky, K.M., Abel, J.G., Khouw, V., Sellers, E.M. (1981) Methadone binding to orosomucoid (α_1-acid glycoprotein): Determinant of free fraction in plasma. *Clinical Pharmacology and Therapeutics*. **29:** 211–217.

Rook, E.J., Hillebrand, M.J.X., Rosing, H., van Ree, J.M. and Beijnen, J.H. (2005) The quantitative analysis of heroin, methadone and their metabolites and the simultaneous detection of cocaine, acetylcodeine and their metabolites in human plasma by high-performance liquid chromatography coupled with tandem mass spectrometry. *Journal of Chromatography B*. 824(1-2):213-221.

Rostami-Hodjegan, A., Wolff, K., Hay, A.W., Raistrick, D., Calvert, R. and Tucker, G.T. (1999) Population pharmacokinetics of methadone in opiate users: characterization of time-dependent changes. *British Journal of Clinical Pharmacology*. **48**:43-52.

Routledge, P.A. (1989). Clinical Relevance of Alpha$_1$ Acid Glycoprotein in Health and Disease. *Progress in Clinical and Biological Research*. **300**: 185-198.

Rundlett, K. L. and Armstrong, D. W. (2001) Methods for the determination of binding constants by capillary electrophoresis**.** *Electrophoresis*. **22:** 1419–1427.

Rydén, I., Skude, G., Lundblad, A., Pâhlsson, P. (1997**).** Glycosylation of α1-acid glycoprotein in inflammatory disease: analysis by high-pH anion-exchange chromatography and concanavalin A crossed affinity immunoelectrophoresis**.** *Glycoconjugate Journal*. **14:**481-488.

Rydén, I., Lundblad, A. And Pâhlsson, P. (1999) Lectin ELISA for Analysis of a1-Acid Glycoprotein Fucosylation in the Acute Phase Response. *Clinical Chemistry*. **45**(11): 2010-2012.

Rydén, I., Pâhlsson, P., Lundblad, A. and Skogh, T. (2002) Fucosylation of a1-acid glycoprotein (orosomucoid) compared with traditional biochemical markers of inflammation in recent onset rheumatoid arthritis. *Clinica Chimica Acta*. **317**: 221–229.

Sabatini, D.D., Kreibich, G., Morimoto, T., Adesnik, M. (1982) Mechansims for the incorporation of proteins in membranes and organelles. *Journal of Cellular Biology*. **92**: 1-22.

SACDM. (2007*). Reducing harm and promoting recovery: a report on methadone treatment misuse in Scotland*. [internet]. PLACE: SACDM methadone Project Group (Published 2007). Available at: http://www.scotland.gov.uk/Publications/2007/06/22094730/0 [accessed 20 Aug 2008].

Salmon, A.Y., Goren, Z., Avissar, Y., Soreq, H. (1999) Human erythrocyte but not brain acetylcholinesterase hydrolyses heroin to morphine. *Clinical and Experimental Pharmacology and Physiology*. **26**: 596-600.

Sasisekharan, R. and Myette, J.R. (2003). The Sweet Science of Glycobiology. *The American Scientist.* **91**: 432-441.

Scanlin, T.F. and Glick, M.C. (2000) Terminal glycosylation and disease: Influence on cancer and cystic fibrosis. *Glycoconjugate Journal.* **17**: 617-626.

Schmid, K. (1950) Preparation and properties of an acid glycoprotein prepared from human plasma. *Journal of the American Chemical Society.* **72**: 2861.

Schmid, K., Kaufmann, H., Isemura, S., Bauer, F., Emura, J., Motoyama, T., Ishiguro, M. and Nanno, S. (1973) Structure of α1-acid glycoprotein. The complete amino acid sequence, multiple amino acid substitutions and homology with the immunoglobulins. *Biochemistry.* **12**: 2711-2724.

Schmid, K., Mao, S.K.Y., Kimura, A., Hayashi, S. and Binette, J.P. (1980) Isolation and characterization of a serine-threonine-rich galactoglycoprotein from normal human plasma. *Journal Biological Chemistry.* **255**: 3221-3226.

Schumacher, M.A., Basbaum, A.I. and Way, W.L. (2009) **In** Katzung, B.G., Masters, S.B. and Trevor, A.J. (Eds.) Opioid Analgesics and Antagonists. *Basic and Clinical Pharmacology, 11th Edition.* P 531-552.

Scimeca, M., Savage, S.R., Portenoy, R., Lowinson, J. (2000). Treatment of Pain in Methadone-Maintained Patients. *Mount Sinai Journal of Medicine.* **67**(5 and 6): 412-422.

Scottish Office Home and Health Department, (1994). *Drugs in Scotland: meeting the challenge. Report of Ministerial Drugs Task Force.* Scottish Office Home and Health Department. Edinburgh: Scottish Office.

Sears, P. and Wong, C.H. (1998) Enzyme action in glycoprotein synthesis. *Cell and Molecular Life Sciences.* **54**: 223-252.

Sees, K. L., Delucchi, K.L., Masson, C., Rosen, A., Clark, H.W., Robillard, H., Bany, P., Hall, S.M. (2000). Methadone Maintenance vs 180-Day Psychosocially Enriched Detoxification for Treatment of Opioid Dependence. *Journal of the American Medical Association.* **283**(10): 1303-1310.

Seymour, A., Black, M., Jay, J. And Oliver, J.S. (2001).The role of dihydrocodeine in causing death among drug users in the west of Scotland. *Scott Med J* 46(5): 143-146.

Shine, H. J., and Mach, E. E. (1965) Ion Radicals. V. Phenothiazine, phenothiazine-5-oxide, and phenothiazine-3 in acid solutions. *J. Org. Chem.* **30**: 2130-2139.

Silamut, K., Molunto, P., Ho, M., Davis, T.M.E., White, N.J. (1991). Alpha 1-acid glycoprotein (orosomucoid) and plasma protein binding of quinine in falciparum malaria. *British Journal of Clinical Pharmacology.* **32**: 311-315.

Sim, H-J., Jeong, J-S., Kwon, H-J., Kang, T.H., Park, H.M., Lee, Y-M., Kim, S.Y., Hong, S-P (2009) HPLC with pulsed amperometric detection for sorbitol as a biomarker for diabetic neuropathy. *Journal of Chromatography B.* **877**: 1607-1611.

Smith, K.D., Elliot, M.A., Elliot, H.G., McLaughlin, C.M., Wightman, P. and Wood, G.C. (1994) Heterogeneity of α1-acid glycoprotein in rheumatoid arthritis. *Journal of Chromatography B*. **661**: 7-14.

Smith, K.D., Hounsell, E.F., McGuire, J.M., Elliot, M.A. and Elliot, H.G. (1997) Structural elucidation of the N-linked oligosaccharides of glycoproteins using high pH anion-exchange chromatography. *Advances in Macromolecular Carbohydrate Research.* **1**: 65-91.

Smith, K.D., Pollacchi, A., Field, M. and Watson, J. (2002) The heterogeneity of the glycosylation of alpha-1-acid glycoprotein between the sera and synovial fluid in rheumatoid arthritis. *Biomedical Chromatography.* **16**: 261-266.

Sousa, M.C., Ferrero-Garcia, M.A. and Parodi, A.J. (1992) Recognition of the oligosaccharide and protein moieties of glycoproteins by the UDP-Glc:glycoprotein glucosyltransferase. *Biochemistry.* **31**: 97-105

Strain, E.C., Bigelow, G.E., Liebson, I.A., Stitzer, M.L. (1999) Moderate- vs High-Dose Methadone in the Treatment of Opioid Dependence. *Journal of the American Medical Association.* **28:** 1000-1005.

Strang, J., Sheridan, J., Hunt, C., Kerr, B., Gerada, C., Pringle, M. (2005). The prescribing of methadone and other opioids to addicts: national survey of GPs in England and Wales. *The British Journal of General Practice.* **55:** 444-451.

Stoscheck, CM. (1990) Quantitation of Protein. *Methods in Enzymology. 182:* 50-69.

Sullivan, L.E., Metzger, D.S., Fudala, P.J. Fiellin, D.A. (2005) Decreasing international HIV transmission: the role of expanding access to opioid agonist therapies for injection drug users. *Addiction.* 100(2) 150-158.

Szöllosi, É., Kremmer, T., Ludányi, K., Imre, T., Schlosser, G., Boldizsár., Vincze, B. and Vékey. K. (2004) A Novel Method for the Separation and Purification of Human Serum Acid Alpha-1-Glycoprotein. Liquid Chromatographic and Mass Spectrometric Investigation of Tryptic Fragments. *Chromatographia.* **60**(1): S213-S219.

Taylor, M.E. and Drickamer, K (2003) *Introduction to Glycobiology*. Oxford University Press Limited.

Tilg, H., Dinarello, C.A., Mier, J.W (1993) Antiinflammatory Properties of Hepatic Acute Phase Proteins: Preferential Induction of Interleukin 1 (IL-1) Receptor Antagonist over IL-1β Synthesis by Human Peripheral Blood Mononuclear Cells. *The Journal of Experimental Medicine.* **178**: 1629-1636.

Tillement, J-P., Urien, S., Chaumet-Riffaud, P., Riant, P., Bree, F., Morin, D., Albengres, E., Barre, J. (1988) Blood Binding and Tissue Uptake of Drugs. Recent Advances and Perspectives. *Fundamental and Clinical Pharmacology.* **2**: 223-238.

Tomei, L., Eap, C.B., Baumann, P., Dente, L. (1989) Use of transgenic mice for the characterization of human alpha 1-acid glycoprotein (orosomucoid) variants. *Human Genetics.* **84**: 89-91.

Townsend, R.R., Hardy, M.R., Hindsgaul, O. and Lee, Y.C. (1988) High-performance anion-exchange chromatography of oligosaccharides using pellicular resins and pulsed amperometric detection. *Analytical Biochemistry.* **174**: 459-470.

Townsend, R.R., Hardy, M.R., Cumming, D.A., Carver, J.P. and Bendiak, B. (1989) Separation of branched sialylated oligosaccharides using high-pH anion-exchange chromatography with pulsed amperometric detection. *Analytical Biochemistry.* **182**: 1-8.

Travis, J., Bowen, J., Tewksbury, D., Johnson, D. and Pannell, R. (1976) Isolation of albumin from whole human plasma and fractionation of albumin-depleted plasma. *Biochemical Journal*. **157**: 301-306.

Treuheit, M.J., Costello, C.E. and Halsall, H.B. (1992) Analysis of the five glycosylation sites of human α1-acid glycoprotein. *Biochemical Journal.* **283**: 105-112.

Turner, G.A., Skillen, A.W., Buamah, P., Guthrie, D., Welsh, J., Harrison, J. and Kowalski, A. (1985) Relation between raised concentrations of fucose, sialic acid and the acute phase proteins in serum from patients with cancer: choosing suitable serum glycoprotein markers. *Journal of Clinical Pathology*. **38**: 588-592.

Van den Brink, W., Hendriks, V.M. and van Ree, J.M. (1999) Medical co-prescription of heroin to chronic, treatment-resistant methadone patients in the Netherlands. *Journal of Drug Issues.* **29** (3): 587–608.

Van den Heuvel, M.M., Poland, D.C.W., De Graaf, C.S., Hoefsmit, E.C.M., Postmus, P.E., Beelen, R.H.J. and van Dijk, W. (2000) The degree of branching of the glycans of α1-acid glycoprotein in asthma. *American Journal of Respiratory and Critical Care Medicine.* **161**: 1972-1978.

van der Linden, E.C.M., De Graaf, T.W., Anbergen, M.G., Dekker, R.M., van Ommen, E.C.R., van den Eijnden, D.H. and van Dijk, W. (1994) Preparative affinity electrophoresis of different glycoforms of serum glycoproteins: application for the study of inflammation-induced expression of sialyl-LewisX groups on α1-acid glycoprotein (orosomucoid). *Glycosylation and Disease*. **1**: 45-52.

van Dijk, W., Pos, O., van der Stelt, M.E., Moshage, H.J., Yap, S.H., Dente, L., Baumann, P. and Eap, C.B. (1991) Inflammation-induced changes in expression and glycosylation of genetic variants of α1-acid glycoprotein. *Biochemical Journal.* **276**: 343-347.

van Dijk, W., Turner, G.A. and Mackiewicz, A. (1994) Changes in glycosylation of acute-phase proteins in health and disease: occurrence, regulation and function. *Glycosylation and Disease.* **1**: 5-14.

Van Dijk, W., Brinkman-Van der Linden, E.C.M. and Havenaar, E.C. (1998) Glycosylation of ~ 1-Acid Glycoprotein (Orosomucoid) in Health and Disease: Occurrence Regulation and

Possible Functional Implications. *Trends in Glycoscience and Glycotechnology.* **10**: 235-245.

van Halbeek, H., Dorland, L. and Vliegenthart, J.F.G. (1981) Characterization of the microheterogeneity in glycoproteins by 500-MHz 1H-NMR Spectroscopy of glycopeptides preparations. *Journal of Biological Chemistry.* **256**: 5588-5590.

Varki, A. (1993) Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology.* **3**: 97-130.

Varki, A. (1992) Diversity in the sialic acids. *Glycobiology.* **2**: 25-40.

Varki, A., Cummings, R.D., Esko, J.D., Freeze, H.H., Stanley, P., Bertozzi, C.R., Hart, G.W. and Etzler, M.E. (2009) *Essentials of Glycobiology*, 2nd Edition. Cold Spring Harbour Laboratory Press.

Veering, B.TH., Burm, A.G.L., Souverijn, J.H.M., Serree, J.M.P., Spierdijk, J. (1990). The effect of age on serum concentrations of albumin and alpha 1-acid glycoprotein. *British Journal of Clinical Pharmacology*. **29**: 201-206.

Vetulani, J. (2001) Drug Addiction. Part I. Psychoactive Substances in the past presence. *Polish Journal of Pharmacology.* **53**: 303-317.

Voulgari, F., Cummins, P., Gardecki, T., Beeching, N.J., Stone, P.C.W., Stuart, J. (1982). Serum levels of acute phase and cardiac proteins after myocardial infarction, surgery, and infection. *British Heart Journal.* **48**: 352-356.

Walsh, S. L. and T. Eissenberg (2003) The clinical pharmacology of buprenorphine: extrapolating from the laboratory to the clinic. *Drug and Alcohol Dependence.* **70**(2): S13-S27.

Ward, J., Hall, W., Mattick, R.P. (1999). Role of maintenance treatment in opioid dependence *Lancet.* **353**: 221-226.

Wasan K.M and Cassidy SM (1998) Role of plasma lipoproteins in modifying the biological activity of hydrophobic drugs. *Journal of Pharmaceutical Sciences.* **87** (4) 411-423.

Weimer, H.E., Mehl, J.W. and Winzler, R.J. (1950) Studies on the mucoproteins of human plasma: isolation and characterization of a homogenous mucoprotein. *Journal of Biological chemistry*. **185**: 561-568.

Weiss, F., Markou, A., Lorang, M.T., Koob, G.F. (1992) Basal extracellular dopamine levels in the nucleus accumbens are decreased during cocaine withdrawal after unlimited-access self-administration. *Brain Research.* **593**: 314-318.

WHO Expert Committee on Drug Dependence. Meeting (34th: 2006: Geneva, Switzerland) WHO Expert Committee on Drug Dependence: thirty-fourth report.

Williams, J.P., Weiser, M.R., Pechet, T.T.V., Kobzik, L., Moore, F.D. and Hechtman, H.B. (1997) α1-acid glycoprotein reduces local and remote injuries after intestinal ischemia in the rat. *American Journal of Physiology – Gastrointestinal and Liver Physiology.* **273**: 1031-1035.

Williams, D.O., Borer, J., Braunwald, E., Chesebro, J.H., Cohen, L.S., Dalen, J., Dodge, H.T., Francis, C.K., Knatterud, G. and Ludbrook, P. (1986) Intravenous recombinant tissue-type plasminogen activator in patients with acute myocardial infarction: a report from the NHLBI thrombolysis in myocardial infarction trial. *Circulation*. **73**: 338-346.

Wilson, J. E. (1976) Applications of blue dextran and cibacron blue F3GA in purification and structural studies of nucleotide-requiring enzymes. *Biochemical and Biophysical Research Communications*. **72**, 816-823.

Wolff, K., Sanderson, M., Hay, A.w.m., Raistrick, D (1991) Methadone Concentrations in Plasma and Their Relationship to Drug Dosage. *Clinical Chemistry.* **37**(2): 205-209

Wolff, K., Rostami-Hodjegan, A., Hay, A.W.M., Raistrick, D. and Tucker, G. (2000). P-based pharmacokinetic approach for methadone monitoring of opiate addicts: potential clinical utility. *Addiction.* **95**(12): 1771-1783.

Woods, J.H and Winger, G (1997) Abuse liability of flunitrazepam. *Journal of Clinical Psychopharmacology.* **17**:1S-57S

World Drug Report 2010. UNODC. UNODC, *World Drug Report 2010* (United Nations Publication, Sales No.E.10.XI.13)

Wormald, M.R. and Dwek, R.A. (1999) Glycoproteins: glycan presentation and protein-fold stability. *Structure.* **7**: R155-R160.

Xuan, H. and Hage, D.S. (2005) Immobilization of α_1-acid glycoprotein for chromatographic studies of drug–protein binding. *Analytical Biochem*istry. **346**(2): 300-310.

Yet, M-G. and Wold, F. (1990) The distribution of glycan structures in individual N-glycosylation sites in animal and plant glycoproteins. *Archives of Biochemistry and iophysics.* **278**: 356-364.

Yue, Q.Y., Hasselström, J., Svensson, J.O. and Säwe, J. (1991) Pharmacokinetics of codeine and its metabolites in Caucasian healthy volunteers: comparisons between extensive and poor hydroxylators of debrisoquine. *British Journal of Clinical Pharmacology.* **31**(6): 635-642.

Zador, D., Sunjic, S. and Darke, S**.** (1996) Heroin-related deaths in New South Wales, 1992: toxicological findings and circumstances. *The Medical of Journal of Australia.* **164:** 204-207.

Zdebska, E. and Kościelak, J. (1999) A Single-Sample Method for Determination of Carbohydrate and Protein Contents glycoprotein Bands by Sodium Dodecyl sulphate-Polyacrylamide Gel Electrophoresis. *Analytical Biochemistry*. **275**: 171-179.

Zhang, Y., Leonessa, F., Clarke, R. And Wainer, I.W. (2000) Development of an immobilized P-glycoprotein stationary phase for on-line liquid chromatographic determination of drug-binding affinities. *Journal of Chromatography B*. **739**: 33–37.

Appendix

The analysis of glycosylation: a continued need for high pH anion exchange chromatography

Jennifer L. Behan and Kevin D. Smith*

ABSTRACT: An appreciation of the structures of the oligosaccharide chains which become attached to biomolecules (the process known as glycosylation), and their relevance to the biological function of the molecule concerned, has progressed rapidly in recent years with developments in site-selective protein glycosylation, oligosaccharide synthesis and *in vivo* targeting of oligosaccharides. These developments have necessitated the parallel development of effective analytical tools for the determination of the structures of glycosylation. The conclusion of studies in the 1980s and 1990s was that high pH anion exchange chromatography (HPAEC) was the most effective HPLC mode for the analysis of glycosylation. It allowed the fractionation of complex mixtures of monosaccharides or oligosaccharides, the latter in terms of charge, size, composition, anomerity and intra-chain linkages. This review reinvestigates whether HPAEC still appears to offer the most effective means of analysing glycosylation. Copyright © 2010 John Wiley & Sons, Ltd.

Introduction

Glycosylation is a complex, ordered and energy-consuming process that has become recognized as both functionally significant and responsible for presenting huge structural diversity. Intra- and intermolecular properties such as circulatory lifespan, immune modulation, solubility and conformation stability have been shown to be dependent upon the expression of oligosaccharides by glycoconjugates (Lis and Sharon, 1993; Varki, 1993).

Oligosaccharide chains are ordered structures comprising various monosaccharides, primarily the hexoses, mannose (Man), galactose (Gal), the deoxyhexose fucose (Fuc), and the hexosamines, glucosamine and galactosamine (formed by the addition of an amino group on carbon 2 of a hexose). The hexosamines are commonly *N*-acetylated to form *N*-acetylglucosamine (GlcNAc) and *N*-acetylgalactosamine (GalNAc) respectively. Finally, *N*-acetylneuraminic acid (NeuAc), a nine-carbon sialic acid, with a formal negative charge, is commonly the terminal component of oligosaccharide chains (Varki, 1992).

An oligosaccharide chain is synthesized by individual attachment of monosaccharide components in specific sequences to the protein or lipid biomolecule initially in the endoplasmic reticulum (ER) lumen, then in the *cis* Golgi where the final structural modifications occur (Kobata, 1992; Sasisekharan and Myette, 2003). This synthesis and modification of oligosaccharide structures is enzymatic in origin and is regulated by glycosyltransferases in the ER and Golgi apparatus (Rademacher *et al*., 1988, Lis and Sharon, 1993).

Glycoproteins are classified by the mechanism of attachment to the protein backbone. Glycans attached to the amide nitrogen of asparagine (Asn) amino acids are termed N-linked structures. This type of glycosylation only occurs when the Asn residue is part of a consensus sequence Asn–X–Ser/Thr where X is never proline because it hinders the formation of the important secondary structure: β turns (Avanov, 1991). O-linked glycosylation occurs when the oligosaccharide chain is attached to the biomolecule through the hydroxyl group of either a serine or threonine amino acid. N-linked oligosaccharide chains all contain a common pentasaccharide core ($Man_3GlcNAc_2$), as indicated in Fig. 1. The monosaccharides attached to this can differ, causing the generation of three main families—high mannose, complex and hybrid N-glycans (Kobata, 1992; Hashimoto *et al*., 2004). O-linked glycans however are not restricted by the need for the presence of a specific core structure; instead synthesis is commonly initiated in the Golgi by the transfer of GalNAc (catalysed by an *N*-acetyl galactosaminyltransferase) to a Ser or Thr residue of the peptide, followed by the action of specific transferases which lead to the formation of various core structures. The process does not require a preceding assembly pathway in the ER, which is required during N-glycan synthesis (Hounsell *et al*., 1996). The mucin-type O-glycans are thought to be the largest group; GalNAc is the initiating sugar and there are six core oligosaccharide structures possible (Van den Steen *et al*., 1998; Sears and Wong, 1998).

One of the key sources of variability is the expression of glycoconjugates with the same protein or lipid structure but differing

* Correspondence to: K. D. Smith, School of Life Sciences, Edinburgh Napier University, 10 Colinton Road, Edinburgh EH10 5DT, UK. E-mail: k.smith@napier.ac.uk

School of Life Sciences, Edinburgh Napier University, 10 Colinton Road, Edinburgh EH10 5DT, UK

a)

Monosaccharides attached to the outer Man residues can differ widely. All residues may be Man (high mannose), may have a selection of GlcNAc, Gal, Fuc etc but no Man (complex), or a mixture of both (hybrid). Branches are usually terminated in NeuAc	β1,2**Man**
	     α1,2
	     Manβ1,4**GlcNAc**β1,4**GlcNAc**β1**Asn**
	     α1,3
	β1,2**Man**

b)

O-linked do not have a common core and the chains can also be highly substituted. Glc may also be a component	**NeuAc**
	     α2,3
	     Gal
	       β1,4
	       GalNac-Ser/Thr
	       β1,3
	     Gal
	   α2,3
	NeuAc
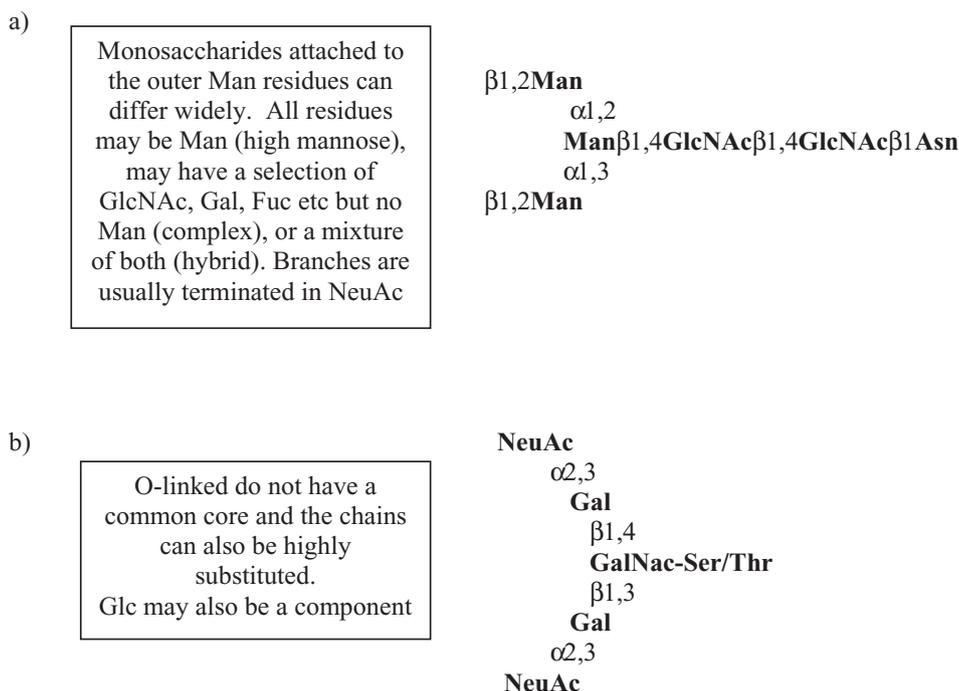
Figure 1. Simplified glycan structures. The pentasaccharide core of N-linked glycans (a) and an example O-linked (b). Adapted from Hashimoto *et al.* (2004) and Lo-Guidice *et al.* (1994).

oligosaccharide components (glycoforms). Their expression promotes a high degree of heterogeneity which can be cell-, organ- or tissue-specific and occurs prominently in disease. This 'glycopathology' (Kobata, 2000) is normally a result of alterations to the transcription of genes encoding glycosyltransferases by cytokines produced during the acute phase response to injury and infection as part of the body's defence system. Understanding the potential for the expression of disease-specific glycoforms could be important in the development of future treatments; perhaps offering use as biomarkers for certain pathological conditions.

HPLC of Glycosylated Structures

A consideration of the various HPLC stationary phases and mode of detection has been the subject of numerous reviews including two excellent papers in the tenth anniversary edition of this journal (Davies and Hounsell, 1996; Lo-Guidice and Lhermitte, 1996). A further detailed consideration is outwith the scope of this review and instead we intend to follow on from where the latter articles concluded in that high pH anion exchange chromatography (HPAEC) appeared to provide the most optimum and effective means of resolving and detecting monosaccharides and oligosaccharides. As shown by Lee (1990), HPAEC is able to detect sugars as low as the femtomole level and indeed pleas have been made for lower levels of detection (Davies and Hounsell, 1996).

Recently, a major area of glycobiological research has been in the evaluation of biomarkers or substances that can be indicative of a biological state. HPAEC has shown great potential in highlighting alterations in oligosaccharide microheterogeneity which may offer use as prognostic and diagnostic biomarkers of disease (Anderson *et al.*, 2002; Mooney *et al.*, 2006).

High pH Anion Exchange Chromatography

The accurate analysis of the glycosylation pattern of glycoproteins is essential when investigating potential disease-specific changes and their subsequent evaluation as biomarkers of disease diagnosis, prognosis and progression. HPAEC is capable of providing information on both the monosaccharide and oligosaccharide composition of N-linked oligosaccharides at a sensitivity of 10–100 pmol (Hardy *et al.*, 1988; Townsend *et al.*, 1989; Smith *et al.*, 1997). A major advantage of the technique is that it does not require prior derivitization or clean-up of carbohydrate samples in order to allow quantification (Lee, 1990).

The essence of HPAEC is a combination of a polymer-based pellicular resin (the active component of which is a quaternary ammonium ion), equilibrated by an alkaline mobile phase with detection by pulsed amperometry. A large number of columns are available containing specific modifications of the stationary phase (Table 1) in order to impart a distinctive selectivity of analysis. This stationary phase is a characterized by a high mechanical and chemical stability. One of the first columns developed, the CarboPAc PA1® was a multipurpose column which could be used for the analysis of monosaccharides, disaccharides and specific oligosaccharides. Thereafter refinements have resulted in columns that are more sensitive for the analysis of monosaccharides, disaccharides and sialic acids (CarboPAc PA10® and CarboPAc PA20®), oligosaccharides (CarboPAc PA100® and CarboPAc PA200®) and reduced monosaccharides and disaccharides (CarboPAc MA1®).

Given that the effectiveness of resolution and detection is dependent upon the ionization of monosaccharides either individually or as part of a larger moiety, the composition of the mobile phase is critical to the sensitivity, selectivity and speed of the technique. Normally the mobile phase is sodium hydroxide

Table 1.	Summary of column composition
Column	**Composition**
CarboPAc PA1®	10 μm-diameter substrate (polystyrene 2% cross-linked with divinylbenzene) agglomerated with 500 nm MicroBead quaternary ammonium functionalized latex (5% cross-linked)
CarboPAc PA10®	10 μm-diameter substrate (ethylvinylbenzene 55% cross-linked with divinylbenzene) agglomerated with 460 nm MicroBead™ difunctional quaternary ammonium ion (5% cross-linked)
CarboPAc PA20®	6.5 μm-diameter substrate (ethylvinylbenzene 55% cross-linked with divinylbenzene) agglomerated with 130 nm MicroBead difunctional quaternary ammonium ion (5% cross-linked)
CarboPAc PA100®	8.5 μm-diameter ethylvinylbenzene/divinylbenzene substrate (55% cross-linking) agglomerated with 275 nm MicroBead™ quaternary amine functionalized latex (6% cross-linked)
CarboPAc PA200®	5.5 μm-diameter ethylvinylbenzene/divinylbenzene substrate (55% cross-linked) agglomerated with 43 nm MicroBead quaternary amine functionalized latex (6% cross-linked)
CarboPAc MA1®	7.5 μm-diameter vinylbenzyl chloride/divinylbenzene macroporous substrate fully functionalized with an alkyl quaternary ammonium group (15% cross-linked)
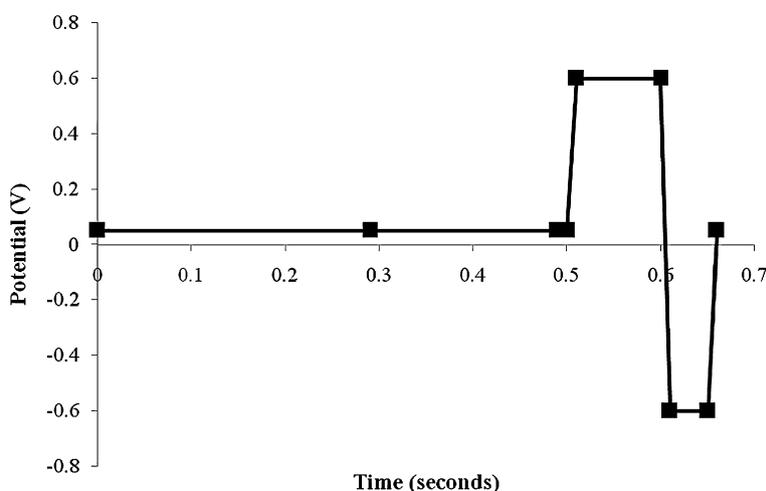
Figure 2.   Pulsed electrochemical detection.

with the exact concentration used being dependent on the nature of the molecules being analysed. The analysis of oligosaccharides and individual sialic acids, both of which interact more strongly with the stationary phase, often requires the inclusion of sodium acetate (or similar) which act as pushing agents to reduce retention to the column. The inclusion of barium acetate in the mobile phase is suggested to remove the need for column regeneration between runs and also eliminates carbonate formation (Cataldi *et al.*, 1999).

In terms of detection, the use of pulsed potentials is important because it ensures that the gold electrode does not become coated in, and therefore fouled by, oxides. Electrocatalytic oxidation of weakly acidic sugars occurs when in contact with the gold electrode. These oxidized sugars coat the electrode surface, hindering detection; therefore they must be removed to ensure reproducibility. The potential is increased and a layer of gold oxide can form at the electrode surface thereby removing the sugars. Upon lowering the potential, the coating is removed because the oxide is reduced (LaCourse and Johnson, 1993). The system consists of the application of a triple electrical pulse to the surface of a gold electrode (Fig. 2). The first pulse, of 0.5 s duration (0.05 V) allows electrode equilibration which is followed by 0.29–0.49 s of sampling time. The second pulse, 0.51–

0.6 s (0.6 V) causes full oxidation of the species on the electrode surface to their most highly oxidized and most soluble forms, allowing a 'washing away' of any contaminating species. Surface oxidation of the electrode is inevitable for this process, thus from 0.61–0.65 s a reducing potential is applied for the electrode surface regeneration (−0.6 V). This final pulse of three immediately precedes the electrode re-equilibration time of 0.0–0.29 s (0.05 V).

HPAEC of Monosaccharides and Oligosaccharides

Under alkaline conditions, the hydroxyl groups of a monosaccharide, either individually or as part of a sequence, are ionized to exist as negatively charged oxyanions. A monosaccharide possesses several potentially ionizable hydroxyl groups with the following descending hierarchy of acidity: 1-OH > 2-OH >> 6-OH > 3-OH > 4-OH. The varying location of the OH groups results in slight differences in the pK_a value (ranging from 12 to 14) of individual monosaccharides under these conditions. The differences in ionization are unique both with respect to individual monosaccharides and also cumulatively; a sequence of monosaccharides can be exploited chromatographically, using the anion
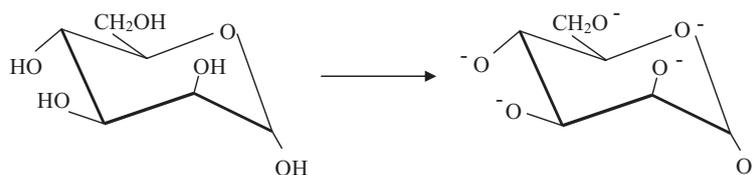
Figure 3. An example of an oxyanion (mannose).
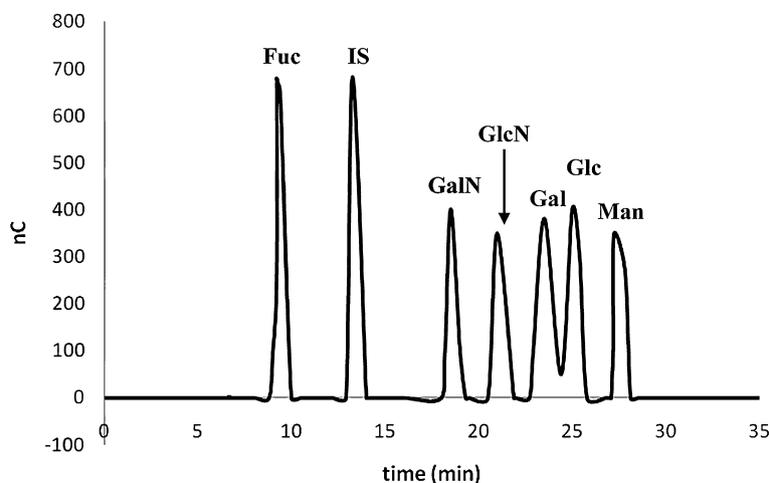
Figure 4. An example of HPAEC trace for separation of known monosaccharide standards. Fuc, fucose; IS, internal standard (2-deoxy-D-galactose); GalN, galactosamine; GlcN, glucosamine. Gal, galactose; Glc, glucose; Man, mannose.

exchange stationary phases and the electrochemical detector based on pulsed potentials. The use of eluents with a pH of 12 or higher stimulates electrocatalytic oxidation of weakly acidic sugars when in contact with the gold electrode.

Monosaccharides

For a typical monosaccharide analysis, an alkaline environment of 0.03 M sodium hydroxide causes ionization and results in the formation of oxyanions unique to individual monosaccharides (Fig. 3). The interaction of the charged oxyanions with the strong anion-exchange CarboPac™ column and the slight differences in relative pK_a values aids the chromatographic separation of individual monosaccharides. The general rule is 'the lower the pK_a; the longer it will be retained by the column' (Lee, 1990). Additionally, a monosaccharide with an anomeric OH group, which is more acidic than other OH groups, is less well retained than those that do not contain anomeric OH groups, e.g. the inositols.

Typical conditions for the separation of monosaccharides using HPAEC would be a Dionex 600™ HPAEC system (Dionex, Camberley, UK) comprising a CarboPac PA-100 analytical 4 × 250 mm and guard 4 × 50 mm columns, a GP50 gradient pump and ED40 electrochemical detector (Johnson and Smith, 2006). The pulsed potentials for the waveform are 0 s, 0.05 V; 0.29 s, 0.05 V; 0.49 s, 0.05 V; 0.5 s, 0.05 V; 0.51 s, 0.6 V; 0.6 s, 0.6 V; 0.61 s, −0.6 V; 0.65 s, −0.6 V; and 0.66 s, 0.05 V, as shown in Fig. 2. A typical separation of monosaccharide standards is shown in Fig. 4, which was achieved using an isocratic separation of 0.03 M sodium hydroxide.

In biomarker research, monosaccharide compositional analysis has become a key tool for the rapid elucidation of changes in the monosaccharide content of oligosaccharides or free oligosaccharides that are associated with a disease or pathological condition. Alterations in the oligosaccharide component of alpha-1-acid glycoprotein (AGP) occur in a number of disease conditions (Ceciliani and Pocacqua, 2007). In rheumatoid arthritis, HPAEC monosaccharide compositional analysis has been utilized to demonstrate that hyperfucosylation, hypersialylation and increased chain branching of AGP glycans occur, differing from early acute stages when the content of biantennary chains is increased (Elliott *et al.*, 1997; Jørgensen *et al.*, 1998). In other studies by the same group, the determination of an increased fucosylation of the oligosaccharide chains in rheumatoid arthritis or burn injury either inhibited collagen fibril formation (Yule *et al.*, 1999) or resulted in the atypical collagen deposition associated with hypertrophic scarring (French *et al.*, 2002). HPAEC monosaccharide compositional analysis has proved that it is possible to distinguish individual liver diseases on the basis of their AGP glycosylation, e.g. in hepatitis C the oligosaccharide chains express more fucose (Fuc) residues in comparison to the other liver diseases and also contain the rare monosaccharide N-acetylgalactosamine (GalNAc) (Anderson *et al.*, 2002; Mooney *et al.*, 2006). Further work has established not only that AGP in chronic myeloid leukaemia has unique glycosylation that can be correlated with severity and prognosis, but also that these alterations influence the extent that AGP is able to bind to administered drugs in the plasma and reduce their therapeutic effect (Jørgensen *et al.*, 2002).
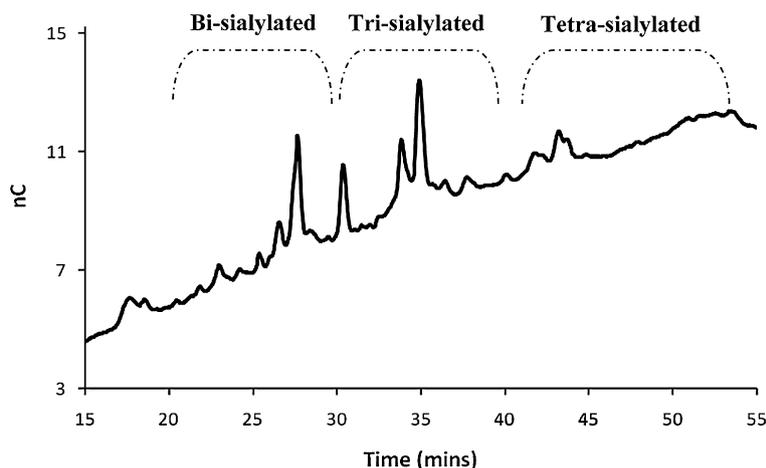
Figure 5. A standard HPAEC trace of AGP.

Reduced Monosaccharides

Historically, the application of HPAEC to glycosylation analysis faltered when the analyte of interest either existed, or was released, in a reduced form. Recently Sim *et al.* (2009) have proposed that the HPAEC determination of sorbitol could be a screening tool to identify diabetic neuropathy. The author tested four anion-exchange columns (CarboPac PA1, CarboPac PA20 and CarboPac MA1 all from Dionex, and a MetrocepCarb1 from Metrohm) and concluded that the MetrocepCarb1 column was the most efficient for the analysis of sorbitol. They also determined that the most effective PAD waveform consisted of six potentials.

Bardotti *et al.* (2000) used the CarboPac MA1 column to quantify ribitol in a conjugate vaccine for *Haemophilus influenzae type b* (Hib-CRM; PRP oligosaccharides linked to cross reacting material of non-toxic diphtheria toxin variant) after TFA hydrolysis and subsequent chromatographic separation. This analysis allowed accurate monitoring of free saccharide in the formulated conjugate both alone and when combined with other vaccines—due to the requirement of only nanomole levels.

Sialic Acids

Sialic acids are a group of sugars which terminate O-linked and N-linked complex and hybrid glycan chains, two common to such glycans are *N*-acetyl- and *N*-glycolyl-neuraminic acid, which differ only by the presence or absence of one hydroxyl (-OH) group. These residues carry a formal negative charge which results in a strong interaction with anion-exchange resins. The subsequent removal from the strong charge interaction with the stationary phase requires more robust conditions; an increasing sodium acetate linear gradient is useful (50–250 mM over 30 min in addition to sodium hydroxide (100 mM). Such conditions are able to separate *N*-acetyl- and *N*-glycolyl-neuraminic acid but not individual N- or O-acetylated variants (Smith *et al.*, 1997).

Oligosaccharides

In this instance, the alkaline environment created during HPAEC allows the efficient separation of oligosaccharide chains on the basis of their size, formal charge, monosaccharide composition

and intra-chain linkages (Hardy and Townsend, 1988). The application of oligosaccharide chains to an anion-exchange column results in the negatively charged oligosaccharides binding strongly to the positively charged beads of the column resin. Subsequent removal from the column requires the addition of an eluent that can compete with the oligosaccharides for the positively charged beads and displace them from the resin. The strong interaction of the negatively charged NeuAc with the quaternary ammonium-bonded pellicular resin used in HPAEC ensures that stronger elution conditions are required to displace the charged molecules from the column (Smith *et al.*, 1997). This is achieved through the addition of a sodium acetate gradient; the greater the negative charge (greater the number of NeuAc), the higher the sodium acetate concentration required to displace the oligosaccharides from the column. It is possible for the oligosaccharides to be separated at a lower pH (4.6) purely on the basis of their NeuAc content (Townsend *et al.*, 1988), but the additional oxyanion charge at alkaline pH enhances resolution of the oligosaccharide mixture by allowing separation of the chains based on isomeric differences (Townsend *et al.*, 1989).

Sialylated Oligosaccharides

The sialylated portion of the oligosaccharide allows separation of the chains into distinct charge bands; the greater the number of sialic acids, the greater the overall negative charge, resulting in a longer retention time. Within these charge bands, the oligosaccharides can be separated according to size with larger structures demonstrating greater retention times. On this basis, bi-antennary, bi-sialylated structures will elute earlier than tri-antennary, bi-sialylated structures (Fig. 5). It is also possible for this technique to detect and successfully separate structures within charge bands which differ in one linkage. NeuAc can be linked either α (2, 6) or α (2, 3) to an outer galactose (Gal) residue. The presence, or increased proportion, of α (2, 6) NeuAc-linked chains results in a reduction in retention time (Townsend *et al.*, 1988). It is thought that, when NeuAc is added with α (2, 6), the oxyanion effect of the 6-OH of Gal is blocked, resulting in an overall reduction in charge and a decrease in the retention time when compared with α (2, 3)-linked NeuAc (Townsend *et al.*, 1989).

Within each charge band, further separation based on the neutral portion of the oligosaccharide can also be achieved. This is demonstrated by the greater retention time of sialylated structures containing Galβ (1,3) linked to GlcNAc compared with Galβ (1,4)-linked (Townsend *et al.*, 1988, 1989). It was suggested that this difference in retention time was due to the conformation of the OH groups in the Galβ (1,3)-GlcNAc, allowing for greater interaction with the column. The presence of Fuc on an oligosaccharide chain has also been shown to affect retention time, reducing it quite considerably. It is thought that this is due to a 'masking' effect of the charges present on surrounding monosaccharides and therefore a reduction in the overall charge of the oligosaccharide (Hardy and Townsend, 1988; Lee, 1990).

In general the analysis of oligosaccharides by HPAEC is very similar to monosaccharide analysis except with respect to the mobile phase. The column is initially regenerated over two 20 min cycles using 50% 1 M NaOH and 50% HPLC-grade water and then equilibrated for 10 min using 10% NaOH–5% 1 M NaOAc–85% HPLC-grade water (v/v). The sample then separates during a linear gradient over 45 min when the eluent proportions are 10% NaOH–20% NaOAc–70% HPLC-grade water (v/v). The column is regenerated over 10 min with an eluent composition of 50% NaOH–50% HPLC-grade water (v/v). The final equilibration step, lasting 5 min, requires 10% NaOH–5% NaOAc–85% HPLC-grade water (v/v) (Elliott *et al.*, 1997; Smith *et al.*, 1997).

Neutral Oligosaccharides

In 1988, Hardy and Townsend reported on the ability of HPAEC-PAD to separate neutral oligosaccharides by their molecular size, sugar composition and linkage type. Two identical pentasaccharide chains, differing only by one residue were resolved; one had a glucose (Glc) residue and the other an *N*-acetylglucosamine (GlcNAc). The Glc-containing chain eluted more slowly than its GlcNAc-containing counterpart. They explained this by suggesting that the 3-OH group of GlcNAc was less acidic than the 2-OH of Glc, or perhaps the Glc group was able to interact with the stationary phase more readily than GlcNAc. When an oligosaccharide expressed more 1,6 linkages, it was suggested that its increased rotational freedom allowed it to interact with the pellicular resin more strongly. It is possible using the HPAEC-PAD technique to detect picomole levels of underivitized oligosaccharides. Hardy and Townsend were able to separate positional isomeric oligosaccharides and glycopeptides, and also detect reducing and non-reducing underivitized carbohydrates in the pmol range. The use of an alkyl- or amine-bonded stationary phase allows the separation of neutral oligosaccharides differing by one monosaccharide; however, if they differ only in the position of the linkage to a single monosaccharide, it is far harder to separate effectively (Dua and Bush, 1984; Tomiya *et al.*, 1987; Mellis and Baenziger, 1983; Blanken, *et al.*, 1985).

Anumula and Taylor (1991) separated acidic (asialylated) and neutral (asialo-complex and high-mannose) oligosaccharide chains chromatographically, after cleavage from polypeptide backbones using peptide *N*-glucosidase F, endo-*β*-*N*-acetylglucosaminidase F and endo-*β*-*N*-acetylglucosaminidase H. The separation required a polymeric anion-exchange column HPIC-AS6/CarboPac PA-1 (Dionex), protected by a CarboPAc guard column and 0.5 M NaOH and 3% acetic acid–NaOH pH 5.5 as eluents, producing a varied gradient when mixed with water. The oligosaccharides were quantified—the technique sensitive enough to detect <5 pmol—through the use of PAD.

When Fuc and GlcNAc are both present in a molecule, it is likely that the hydrophobic methyl and acetyl groups, respectively, prevent the interaction of the oligosaccharide anion with the column matrix.

Routier *et al.* (1998) compared the use of chromatography and lectin-based assays in the quantification of oligosaccharides released from IgG which were purified from the serum of patients suffering from rheumatoid arthritis. This was to determine the percentage of glycoforms expressing no Gal, one Gal or two Gal. In some experiments they used reverse-phase or normal-phase columns for HPLC—after protease digestion and glycosaminidase A oligosaccharide release, or hydrazinolysis treatment by exoglycosidases and a Biogel P4 chromatography of 2-aminobenzamide derivatives, or release of oligosaccharides using PNGaseF and Biogel P4 chromatography of 2-AB derivatives or HPAEC-PAD of native oligos. The change in glycosylation profile of IgG can be useful in developing a diagnostic test for the rheumatic diseases.

The ability of HPAEC to resolve neutral oligosaccharides is perfectly demonstrated (McGuire *et al.*, 1996) when applied to the resolution of the asialylated and agalactosylated biantennary present on immunoglobulin G (IgG).

McGuire *et al.* (1996) investigated the resolution of neutral oligosaccharides in IgG by analysing the effect of alterations to the sodium acetate concentration gradient; the less extreme the gradient, the better the resolution of the neutral oligosaccharides (Fig. 6). The most effective was an isocratic mixture of 25% A–0.5% B–4.5% C (where A denotes 1 M sodium hydroxide, B is 1 M sodium acetate, and C is HPLC-grade water) for 40 min followed by regeneration by elution with 50% A–50% C, which enabled resolution of peaks indistinguishable under more severe acetate conditions at a linear gradient, e.g. 10% A–2% B–88% C to 10% A–14% B–76% C over 57 min or 10% A–5% B–85% C to 10% A–20% B–70% C over 40 min.

Glycoforms

When glycoproteins possess identical amino acid sequence but different glycan components, they are known as glycoforms. A DNA Pac™ PA-100 column has been proposed that allows the separation of glycoforms of the same glycoprotein which, like the columns described for oligosaccharide and monosaccharide analysis, is a pellicular anion-exchange column. Its ability to distinguish between different levels of sialic acid expression is useful in distinguishing sialylated glycoforms of glycoprotein and glycopeptides (Rohrer and Avdalovic, 1996; Rohrer, 1994). Those with low sialylation are less well retained by the column and thus elute earlier. The study by Rohrer (1994) indicated that glycoforms of fetuin can be separated by the structure of the sialylated glycans attached.

It is also possible to resolve glycoforms with the same degree of sialylation, but expressing the residues on different branches. The use of CarboPac™ PA-100 columns for the resolution of structures within a group of glycoforms is dependent on the differing sialylation. It is possible to initially separate sialylated glycoforms with a DNA Pac™ PA-100 column and treat the fractions with PNGaseF to release the oligosaccharides, resolving the sialylated structures within the glycoform fraction (Townsend and Hardy, 1991; Rohrer *et al.*, 1993).

This technique was applied to human serum transferrin glycoforms (Dionex application note 105) where they reported that, after digested of fractions eluted from the DNA Pac™ PA-100
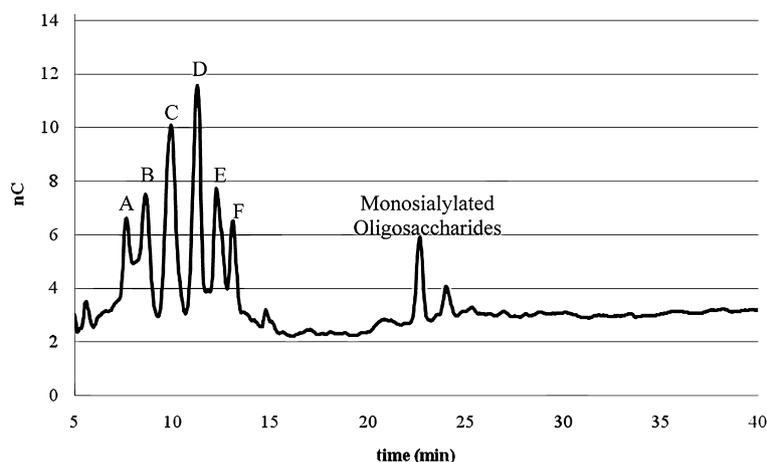
Figure 6. Separation of oligosaccharide chains from IgG. A, unknown; B, agalactosylated fucosylated biantennary; C, agalactosylated, biantennary; D, monogalactosylated biantennary (with bisecting GlcNAc); E, bigalactosylated biantennary (with fucose and bisecting GlcNAc); F, bigalactosylated biantennary (with bisecting GlcNAc).

column with PNGaseF, the sialylated oligosaccharides were resolved by degree of sialylation, as seen by the retention times of each peak. Neuraminidase was used to remove sialic acid residues from PNGaseF F digestions to show that the peaks represent sialylation of glycans during HPAEC-PAD analysis. The retention times of sialylated glycans were reduced.

The Future

The development and emergence of high pH anion exchange chromatography coupled with pulsed amperometry has revolutionized the analysis of the glycosylated components of biomolecules by offering highly sensitive detection and effective separation without the need for derivitization. Even at the present time of writing, more than 20 years after its introduction and without any major change in the mechanisms of separation or detection, the use of HPAEC is widespread and still competitive with other techniques. A recent study (Adamo *et al.*, 2009) concluded that sensitivity of HPAEC in monosaccharide and oligosaccharide compositional analysis of IgG was almost identical to, and compared favourably with, both capillary electrophoresis–laser induced fluorescent detection and reverse-phase HPLC coupled to electrospray mass spectrometry. The coupling of HPAEC with mass spectrometry has been accomplished and utilized in several studies (Okinaga *et al.*, 1992; Barr *et al.*, 1991); however, MS is still usually undertaken offline due the high salt concentration of the mobile phase.

To date, HPAEC still appears to be the most commonly used and sensitive technique available in the investigation of the oligosaccharide chains of glycoconjugates after enzymatic or chemical release. Not only has it revolutionized the analysis of monosaccharides and oligosaccharides in general, but it has also provided the technology required to investigate and identify alterations in glycosylation that are diagnostically significant as biomarkers of disease processes.

Recently, a major area of interest for researchers seeking the identification of biomarkers for breast cancer has involved the 'mapping' of a tissue (Dwek and Alaiya, 2003). Although the proteomic (mapping a tissue or cell according to the proteins it

expresses) approach to cancer biomarkers has yielded important and fruitful results, recent research has also started to focus on post-translational modifications of proteins, rather than protein expression. Given that more than half of all proteins present in serum are glycosylated and the same glycoprotein can exist as a glycoform population, there is a real requirement for an analytical tool which is able to identify subtle but significant alterations in glycosylation consistent with the pathogenesis of a disease. HPAEC satisfies this requirement both for the analysis of glycosylation in general and in the investigation of disease biomarkers in particular and the potential is still ongoing and appears positive.

References

Adamo M, Qiu D, Dick Jr LW, Zeng M, Lee A-H and Cheng K-C. Evaluation of oligosaccharide methods for carbohydrate analysis in a fully human monoclonal antibody and comparison of the results to the monosaccharide composition determination by a novel calculation. *Journal of Pharmaceutical and Biomedical Analysis* 2009; **49**: 181–192.

Anderson NE, Pollacchi A, Hayes P, Therapondos G, Newsome P, Boyter A and Smith KD. A preliminary evaluation of the differences in the glycosylation of alpha-1-acid glycoprotein between individual liver diseases. *Biomedical Chromatography* 2002; **16**: 365–372.

Anumula KR and Taylor PB. Rapid characterization of asparagine-linked oligosaccharides isolated from glycoproteins using a carbohydrate analyzer. *European Journal of Biochemistry* 1991; **195**: 269–280.

Avanov. Conformational aspects of glycosylation. *Molecular Biology* 1991; **25**: 237–249.

Bardotti A, Ravenscroft N, Ricci S, D'Ascenzi S, Guarnieri V, Averani G and Constantino P. Quantitative determination of saccharide in *Haemophilus influenzae* type b glycoconjugate vaccines, alone and in combination with DPT, by use of high-performance anion-exchange chromatography with pulsed amperometric detection. *Vaccine* 2000; **18**: 1982–1993.

Barr JR, Anumula KR, Vettese MB, Taylor PB and Carr SA. Structural classification of carbohydrates in glycoproteins by mass spectrometry and high-performance anion-exchange chromatography. *Analytical Biochemistry* 1991; **192**: 181.

Blanken WM, Bergh MLE, Koeppen PL and Van den Eijnden DH. High-pressure liquid chromatography of neutral oligosaccharides: effects of structural parameters. *Analytical Biochemistry* 1985; **145**: 322–330.

Cataldi TDR, Campa C, Angelotti M and Bufo SA. Isocratic separations of closely-related mono- and disaccharides by high-performance anion-exchange chromatography with pulsed amperometric detection

using dilute alkaline spiked with barium acetate. *Journal of Chromatography A* 1999; **855**: 539–550.

Ceciliani F and Pocacqua V. The acute phase protein α1-acid glycoprotein: a model for altered glycosylation during diseases. *Current Protein and Peptide Science* 2007; **8**: 91–108.

Davies MJ and Hounsell EF. Carbohydrate chromatography: towards yoctomole sensitivity. *Biomedical Chromatography* 1996; **10**: 285–289.

Dua VK and Bush CA. Resolution of some glycopeptides of hen ovalbumin by reverse-phase high-pressure liquid chromatography. *Analytical Biochemistry* 1984; **137**(1): 33–40.

Dwek MV and Alaiya A. Proteome analysis enables separate clustering of the normal breast, benign breast and breast cancer tissues. *British Journal of Cancer* 2003; **89**: 305–307.

Elliott MA, Elliott HG, Gallagher K, McGuire J, Field M and Smith KD. Investigation into the Concanavalin A reactivity, fucosylation and oligosaccharide microheterogeneity of α1-acid glycoprotein expressed in the sera of patients with rheumatoid arthritis. *Journal of Chromatography: Biomedical Applications* 1997; **688**: 229–237.

French D, Watson J, McCahill B, Taggart IM and Smith KD. A preliminary evaluation of the functional significance of alpha-1-acid glycoprotein glycosylation on wound healing. *Biomedical Chromatography* 2002; **16**: 412–419.

Hardy MR and Townsend RR. Separation of positional isomers of oligosaccharides and glycopeptides by high-performance anion-exchange chromatography with pulsed amperometric detection. *Proceedings of the National Academy of Science* 1988; **85**: 3289–3293.

Hardy MR, Townsend RR and Lee YC. Monosaccharide analysis of glycoconjugates by anion exchange chromatography with pulsed amperometric detection. *Analytical Biochemistry* 1988; **170**: 54–62.

Hashimoto S, Asao T, Takahashi J, Yagihashi Y, Nishimura T, Saniabadi AR, Poland DCW, van Dijk W, Kuwano H, Kochibe N and Yazawa S. α_1-Acid glycoprotein fucosylation as a marker of carcinoma progression and prognosis. *Cancer* 2004; **101**: 2825–2836.

Hounsell EF, Davies MJ and Renouf D. O-linked protein glycosylation structure and function. *Glycoconj Journal* 1996; **13**(1): 19–26.

Johnson D and Smith KD. The efficacy of certain anti-tuberculosis drugs is affected by binding to α-1-acid glycoprotein. *Biomedical Chromatography* 2006; **20**, 551–60.

Jørgensen HG, Elliott MA, Watson J and Smith KD. α1-Acid glycoprotein derived from the human hepatoma cell line HepG2, and which overexpresses fucose, can function as a ligand for E-selectin. *Pharmaceutical and Pharmacological Communications* 1998; **4**: 123–127.

Jørgensen HG, Elliott MA, Paterson S, Holyoake TL and Smith KD. Binding of imatinib by α_1-acid glycoprotein. *Blood* 2002; **100**: 367–369.

Kobata A. Structures and functions of the sugar chains of glycoproteins. *European Journal of Biochemistry* 1992; **209**(2): 483–501.

Kobata A. A journey to the world of glycobiology. *Glycoconjugate Journal* 2000; **17**: 443–464.

LaCourse WR and Johnson DC. Optimization of waveforms for pulsed amperometric detection of carbohydrates based on pulsed voltammetry. *Analytical Chemistry* 1993; **65**: 50–55.

Lee YC. High-performance anion-exchange chromatography for carbohydrate analysis. *Analytical Biochemistry* 1990; **189**: 151–162.

Lis H and Sharon N. Protein glycosylation: structural and functional aspects. *European Journal of Biochemistry* 1993; **218**: 1–27.

Lo-Guidice J-M and Lhermitte M. HPLC of oligosaccharides in glycobiology. *Biomedical Chromatography* 1996; **10**: 290–296.

Lo-Guidice J-M, Wieruszeski J-M, Lemoine J, Verbert A, Roussel P and Lamblin G. Sialylation and sulfation of the carbohydrate chains in respiratory mucins from a patient with cystic fibrosis. *Journal of Biology Chemistry* 1994; **269**(29) 18794–18813.

McGuire JM, Douglas M and Smith KD. The resolution of the neutral *N*-linked oligosaccharides of IgG by high pH anion-exchange chromatography. *Carbohydrate Research* 1996; **292**(4): 1–9.

Mellis SJ and Baenziger JU. Size fractionation of anionic oligosaccharides and glycopeptides by high-performance liquid chromatography. *Analytical Biochemistry* 1983; **134**(2): 442–449.

Mooney P, Hayes P and Smith KD. The putative use of α-1-acid glycoprotein as a non-invasive marker of fibrosis. *Biomedical Chromatography* 2006; **20**: 1351–1358.

Okinaga T, Ohashi Y and Hoshi M. A novel saccharide structure, Xyl 1$\rightarrow$3 Gal 1$\rightarrow$ (SO$_3^-$)3, 4 Fuc$\rightarrow$, is present in acrosome reaction-inducing substance of the starfish, *Asterias amurensis*. *Biochemistry and Biophysics Research Communications* 1992; **186**(1): 405–410.

Rademacher TW, Parekh RB and Dwek RA. Glycobiology. *Annual Reviews of Biochemistry* 1988; **57**: 785–838.

Rohrer J. Improved fractionation of sialylated glycopeptides by pellicular anion-exchange chromatography. *Journal of Chromatography A* 1994; **667**: 75–83.

Rohrer J and Avdalovic N. Separation of human serum transferrin isoforms by high-performance pellicular anion-exchange chromatography. *Protein Expression and Purification* 1996; **7**: 39–44.

Rohrer JS, Cooper GA and Townsend RR. Identification, quantification, and characterization of glycopeptides in reversed-phase HPLC separations of glycoprotein proteolytic digests. *Analytical Biochemistry* 1993; **212**: 7–16.

Routier FH, Housell EF, Rudd PM, Takahashi N, Bond A, Hay FC, Alavi A, Axford JS and Jefferis R. Quantitation of the oligosaccharides of human serum IgG from patients with rheumatoid arthritis: a critical evaluation of different methods. *Journal of Immunological Methods* 1998; **213**: 113–130.

Sasisekharan R and Myette JR. The sweet science of glycobiology. *The American Scientist* 2003; **91**: 432–441.

Sears P and Wong C-H. Enzyme action in glycoprotein synthesis. *Cellular and Molecular Life Science* 1998; **54**: 223–252.

Sim H-J, Jeong J-S, Kwon H-J, Kang TH, Park HM, Lee Y-M, Kim SY and Hong S-P. HPLC with pulsed amperometric detection for sorbitol as a biomarker for diabetic neuropathy. *Journal of Chromatography B* 2009; **877**: 1607–1611.

Smith KD, Hounsell EF, McGuire JM, Elliott MA and Elliott HG. Structural elucidation of the N-linked oligosaccharides of glycoproteins using high pH anion-exchange chromatography. In *Advances in Macromolecular Carbohydrate Research*, Volume 1, Sturgeon RJ (ed.). Elsevier: Amsterdam, 1997.

Tomiya N, Kurono M, Ishihara H, Tejima S, Endo S, Arata Y and Takahashi N. Structural analysis of N-linked oligosaccharides by a combination of glycopeptidase, exoglycosidases, and high-performance liquid chromatography. *Analytical Biochemistry* 1987; **163**(2): 489–499.

Townsend RR and Hardy MR. Analysis of glycoprotein oligosaccharides using high-pH anion exchange chromatography. *Glycobiology* 1991; **1**: 139–147.

Townsend RR, Hardy MR, Hindsgaul O and Lee YC. High-performance anion-exchange chromatography of oligosaccharides using pellicular resins and pulsed amperometric detection. *Analytical Biochemistry* 1988; **174**: 459–470.

Townsend RR, Hardy MR, Cumming DA, Carver JP and Bendiak B. Separation of branched sialylated oligosaccharides using high-pH anion-exchange chromatography with pulsed amperometric detection. *Analytical Biochemistry* 1989; **182**: 1–8.

Van den Steen P, Rudd PM, Dwek RA and Openakker G. Concepts and principles of O-linked glycosylation. *Critical Reviews in Biochemistry and Molecular Biology* 1998; **33**(3): 151–208.

Varki A. Diversity in the sialic acids. *Glycobiology* 1992; **2**: 25–40.

Varki A. Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology* 1993; **3**: 97–130.

Yule FSB, Watson J and Smith KD. In-vitro effects of α_1-acid glycoprotein on collagen fibril formation in rheumatoid arthritis. *Pharmaceutical and Pharmacological Communications* 1999; **5**: 507–510.