

Optimisation of the Investigation of Antibody-Mediated Dysglycaemia

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Declaration

I hereby declare that my thesis entitled:

Optimisation of the Investigation of Antibody-Mediated Dysglycaemia

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Collaborations and Contributions and specified in the text. It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution. It does not exceed the prescribed word limit for the Degree Committee of the Faculty of Clinical Medicine.

David S Church

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Summary

Optimisation of the Investigation of Antibody-Mediated Dysglycaemia

David S Church

Two rare and severe disorders of insulin action, namely insulin autoimmune syndrome (IAS) and type B insulin resistance (TB-IR), are caused by pathogenic antibodies against insulin or the insulin receptor, respectively. These may arise in isolation or may complicate management of pre-existing diabetes mellitus, and milder forms of the conditions are often suspected in patients with insulin-treated diabetes and labile glycaemic control. Antibody depletion can effectively treat either condition in many cases. This research aimed to target major limitations of existing diagnostics, specifically, that anti-insulin antibody (IA) testing alone does not establish whether antibodies alter insulin action to a clinically-significant degree, and that no clinically-accredited diagnostic test for TB-IR currently exists.

An initial collaborative study examined the ability of a panel of commercial insulin assays to quantify ten different insulin preparations. Significant variability in performance of assays against animal-derived and insulin analogues was seen, with certain insulin analogues not detected at all, with important implications for the use of insulin immunoassays in insulin-treated patients. A suite of techniques for investigation of the clinical significance of IAs were then developed and assessed. In a study of five widely-used insulin immunoassays, dilution of IAS plasma led to increased insulin recovery, and polyethylene glycol (PEG) precipitation of IAS plasma decreased insulin recovery in the majority of assays. Gel filtration chromatography (GFC) discriminated high molecular weight and monomeric insulin, while *ex vivo* addition of exogenous insulin to plasma increased sensitivity of insulin immunocomplex detection.

An observational study was performed of 7 patients, all ultimately diagnosed with IAS. IAs were measured using radioligand-binding assay and enzyme-linked immunosorbent assay (ELISA). Method comparison showed results to differ in rank order and relative magnitude. For one patient whose screening IA result was not grossly elevated using either IA assay method, immunosubtraction studies were consistent with the presence of an IgA, a class of antibody under-/not detected in the IA assays studied. Competitive radioligand-binding studies demonstrated IAs to have a range of affinities. 4 patients treated with individualised regimens of immunosuppressive therapy varied in clinical response, and 3 were managed conservatively. Plasma insulin and C-peptide measurements made using mass spectrometry demonstrated under-estimation of insulin and over-estimation of C-peptide concentration using immunoassay in IAS.

An observational laboratory and clinical study was also undertaken of 30 insulin-treated patients with diabetes and unexplained labile glycaemia. IA, and plasma insulin before and after PEG precipitation, were determined. Three groups were identified: the first were 'negative' for actionable IA; the second had demonstrable IAs of potential significance that warrant further study; and the third included 3 patients for whom immunomodulation therapy was indicated, with 1 other patient showing marked improvement of glycaemic control with close supervision and manipulation of insulin.

Finally, anti-insulin receptor antibodies were detected using a newly developed ELISA utilising Chinese hamster ovary-expressed myc-tagged wild-type human insulin receptor. 'Proof of principle' was demonstrated for the new assay, with clear scope established for future diagnostic development.

The ability to robustly prove, or conversely to rule out, the presence, of insulin-antibody complexes and/or anti-insulin receptor antibodies is invaluable in the investigation of patients with insulin resistance and/or unexplained labile glycaemia, and may decisively alter care pathways. Knowledge gained by this research has advanced understanding of the limitations of current laboratory diagnostics, and has thereby aided clinical-decision making for affected patients.

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Detection of insulin analogues in plasma using immunoassay

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Abbreviations

IA	Anti-insulin antibody
BMI	Body mass index
BSA	Bovine serum albumin
CBG	Capillary blood glucose
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1
CHO	Chinese hamster ovary
CGMS	Continuous glucose monitoring system
CSII	Continuous subcutaneous insulin infusion
CT	Computerised tomography
Da	Daltons
DELFIA®	Dissociation-enhanced lanthanide fluorescence immunoassay
DKA	Diabetic ketoacidosis
DM	Diabetes mellitus
ELISA	Enzyme-linked immunosorbent assay
FBS	Foetal bovine serum
G6P	Glucose-6-phosphate
GIP	Gastric inhibitory polypeptide
GFC	Gel filtration chromatography
GLUT2	Glucose transporter 2
GLUT4	Glucose transporter 4
GLP-1	Glucagon-like peptide-1
HMW	High molecular weight
HSA	Human serum albumin
IAS	Insulin autoimmune syndrome
IRMA	Immunoradiometric assay
kDa	Kilodaltons
LC-MS	Liquid chromatography-mass spectrometry
MRI	Magnetic resonance imaging
MS	Mass spectrometry
MSIA	Mass spectrometry immunoassay
NPH	Neutral protamine Hagedorn
OGTT	Oral glucose tolerance test
PBS	Phosphate-buffered saline
PI3K	Phosphatidylinositol 3-kinase
PAS	Protein A-Sepharose®

PGS	Protein G–Sepharose®
PEG	Polyethylene glycol
PET	Positron emission tomography
QC	Quality control
RIA	Radioimmunoassay
RL	Reference limit
SNARE	Soluble <i>N</i> -ethylmaleimide-sensitive factor attachment protein receptor
TBST	Tris-buffered saline, 0.1% Tween® 20
TB-IR	Type B insulin resistance
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
VDCC	Voltage-dependent calcium channels

CHAPTER 1: General introduction

1.1 Insulin synthesis

The primary product of the insulin gene is preproinsulin, a 12 kilodalton (kDa) biologically inactive molecule that has a 24-amino acid N-terminal signal peptide. Preproinsulin undergoes co-translational translocation from the cytoplasm into the rough endoplasmic reticulum of beta-cells of the islets of Langerhans in the pancreas [1]. The N-terminal signal peptide is immediately cleaved during this process, producing 9 kDa proinsulin. Proinsulin is trafficked in vesicles from the endoplasmic reticulum to the Golgi and is stored in secretory granules. Mature insulin has a molecular weight (MW) of 5808 daltons (Da), and is produced by post-translational modification of proinsulin that occurs in clathrin-coated vesicles [2]. Subsequently, there is maturation of coated granules into non-coated storage secretory granules [3], and these can be divided into two distinct pools: a small readily releasable pool (~1%), and a much larger reserve pool [4]. Following exocytosis of the readily releasable pool, granules are recruited from the reserve pool [5].

Insulin, along with connecting peptide (C-peptide, MW 3600 Da), is formed by endoproteolytic cleavage of proinsulin (Figure 1.1). Prohormone convertase 1 [6, 7] cleaves at the end of Arg³¹,Arg³² to produce split 32,33 proinsulin, and prohormone convertase 2 [8] cleaves at the C-terminal end Lys⁶⁴,Arg⁶⁵ to produce split 65,66 proinsulin [9, 10]. Each enzymatic hydrolysis is followed by the specific removal of the two newly exposed COOH-terminal basic amino acids by carboxypeptidase H [11, 12], and two intermediates are thus formed: des 64,65 proinsulin, and des 31,32 proinsulin. Whilst type 1 endopeptidase processes des 64,65 proinsulin and proinsulin at equal rate, type 2 endopeptidase processes des 31,32 proinsulin at a much faster rate [13], and des 31,32 proinsulin is the principal intermediate found in normal pancreatic tissue [14, 15]. Along with insulin and C-peptide release by beta-cells, a small amount of intact proinsulin, and trace amounts of intermediates (31,32 proinsulin with almost no detectable des 64,65 proinsulin (the exact difference in concentrations are difficult to elucidate [16]), are released into the circulation. Proinsulin conversion is predominantly completed prior to secretion [17] and less than 1% of proinsulin has been demonstrated to be processed in the vascular compartment [14]. Although no complete conversion of proinsulin to insulin has been noted in plasma

in vivo [18], some conversion of exogenous intact proinsulin to 64,65 proinsulin, and exogenous 64,65 proinsulin to insulin, may be extra-pancreatic (subcutaneous tissue and/or in circulation) [19, 20].

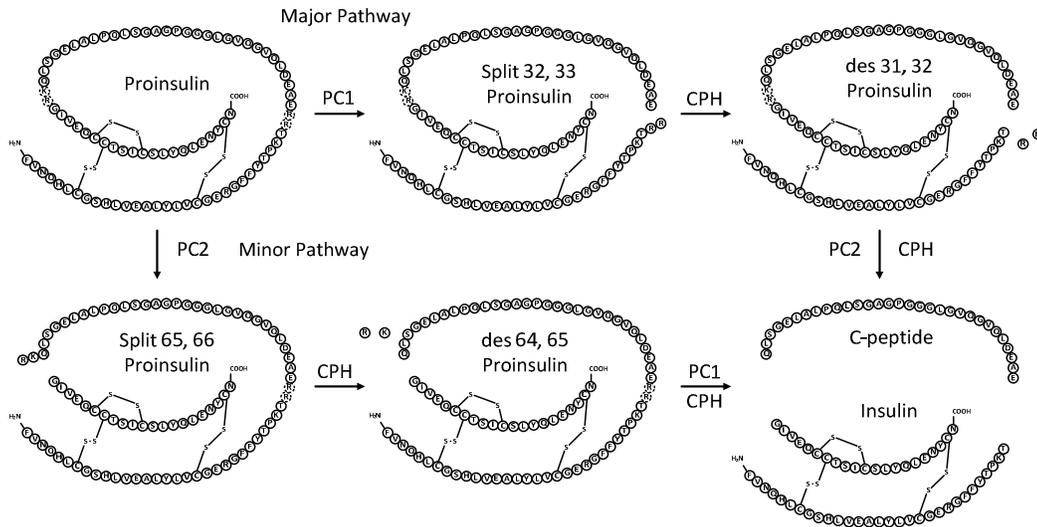


Figure 1.1 Processing of Proinsulin (reproduced from Burtis, Ashwood and Bruns (Eds.) 2006 [21]). Prohormone convertase enzymes 1 and 2 hydrolyse proinsulin to produce split proinsulins. Carboxypeptidase-H (CPH) removes two exposed C-terminal amino acids. Depending on the initial site of endopeptidic cleavage, one of two possible processing intermediates can be generated: des 31,32 or des 64,65 proinsulin. Following a second endopeptidase cleavage, insulin and C-peptide are generated.

1.2 Higher order insulin structure

Human insulin is synthesised and stored as a hexamer, however circulating bioactive insulin is monomeric. The hexameric structure of insulin consists of three dimers aggregating around two zinc ions to form a globular hexameric structure [22]. In the beta cell there is zinc-induced crystalline precipitation of insulin [23] and, following exocytosis, there is immediate insulin hexamer dissociation into monomers.

A low resolution structure of monomeric insulin was first demonstrated using X-ray crystallography in 1926 [24], however it was decades before the dimeric and zinc-containing hexameric forms of insulin were described [24–26]. The chemical structure of bovine insulin was determined by Sanger in 1951 [27, 28], and the amino acid sequence of human insulin was later published in 1960 [29].

In 1969, the three-dimensional rhombohedral structure of insulin was determined by X-ray crystallography [22].

1.3 Insulin as a regulator of blood glucose concentration

1.3.1 Glucose sensing by the pancreatic beta cell

Circulating insulin acts to lower blood glucose concentration. Effective glucose lowering requires adequate insulin production by the pancreas, interaction of insulin with its target receptor, and activation of the receptor followed by downstream signalling.

Pancreatic beta cells behave like glucose sensors, modulating insulin secretion in response to prevailing circulating blood glucose concentration. Glucose homeostasis features maintenance of plasma glucose within a narrow range despite wide variations in supply and demand [30], and in humans the normal glucose concentration threshold for glucose-stimulated insulin release is maintained close to 5 mmol/L [31]. Glucose enters the pancreatic beta cells passively by facilitated diffusion via the high capacity low affinity glucose transporter-2 (GLUT2) (Figure 1.2). Inside the beta cell, glucose is phosphorylated by glucokinase (hexokinase IV), an enzyme considered to be the beta-cell ‘glucose sensor’ [32], to form glucose-6-phosphate. Pyruvate generated from glucose by glycolysis enters mitochondria via carriers [33], and is then further metabolised by the pyruvate dehydrogenase complex and the tricarboxylic acid cycle, yielding adenosine triphosphate (ATP). Beta-cell ATP-sensitive potassium channels close leading to the depolarisation of the plasma membrane and activation of L-type voltage-dependent calcium channels, prompting influx of calcium [34–36]. The increase in free calcium in the cytosol triggers fusion of insulin granules with the plasma membrane in a soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-dependent process [5] and insulin exocytosis ensues. Other putative signalling molecules involved in glucose-sensing/insulin secretion are described, including glutamate, long chain CoA, and NADPH and their potential roles have been explored [37–39].

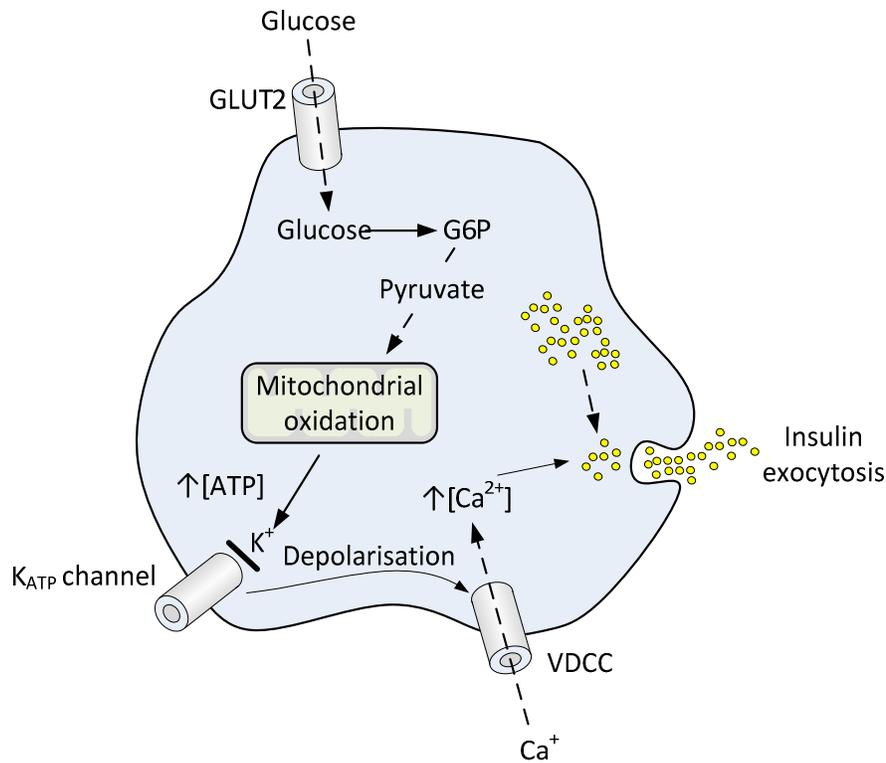


Figure 1.2 Simplified schematic of glucose sensing by the pancreatic β -cell. Glucose enters the cell via glucose transporter 2 (GLUT2). Glucose is phosphorylated to form glucose-6-phosphate (G6P) and then metabolised by glycolysis to form pyruvate which in turn is oxidised in the mitochondria. Increase in adenosine triphosphate (ATP) leads to closure of ATP-sensitive potassium channels (K_{ATP}). Membrane depolarisation activates voltage-dependent calcium (Ca^{2+}) channels (VDCC) resulting in calcium influx and insulin exocytosis.

Glucose not only regulates calcium ion signals that actuate insulin secretion, it also increases efficacy of calcium ions on the insulin secretory mechanism [40]. In addition to glucose metabolism there are other signals with influence on insulin secretion including gut-derived hormones (e.g. gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1)) [39, 41–46], and the autonomic nervous system [47, 48].

1.3.2 Profile of pancreatic insulin secretion

Exhibiting both rapid and ultradian oscillations [49, 50], insulin secretion is pulsatile [51] with an interpulse interval of 4–20 minutes [50, 52–54], and this pulsatility is demonstrably important for hepatic insulin action and signalling [55]. In response to glucose, there are two phases to insulin release [56] (Figure 1.3). The first phase is the rapid release of stored insulin and occurs 5–6 minutes after stimulation; the second phase is a gradual increase in insulin over 60 minutes [57, 58] relies on replenishment of the readily releasable pool by *de novo* insulin synthesis [59]. Amplification of insulin pulses occurs in response to hyperglycaemia [52, 60].

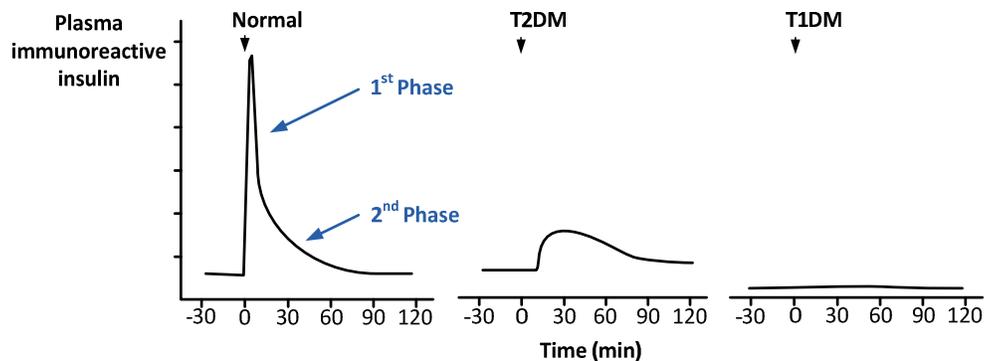


Figure 1.3 Graphical representation of insulin secretion following glucose stimulation in normal health (Normal), type 2 diabetes mellitus (T2DM), and type 1 diabetes mellitus (T1DM) (reproduced from Pfeifer, Halter, and Porte 1981 [61]). Insulin release is biphasic: an acute increase of insulin lasting approximately 10 minutes is referred to as the ‘first phase’ response, while a second phase is sustained for as long as plasma glucose concentrations are elevated. Arrows indicate time of glucose intake.

1.3.3 The insulin receptor

The action of insulin to increase permeability of cells to glucose was first described in 1950 [62], and bioactivating receptor binding of insulin was reported in 1971 [63]. The human insulin receptor was eventually sequenced in 1985 [64, 65] and was shown to be a heterodimeric transmembrane glycoprotein comprising two alpha and two beta subunits joined by disulphide bonds (Figure 1.4). The alpha subunit (MW 135 kDa) is extracellular, while the beta subunit (MW 95 kDa) includes a small extracellular portion and a transmembrane domain as well as an intracellular tyrosine kinase.

1.3.4 Cellular actions of insulin

Insulin molecules bind co-operatively to the alpha subunit of the insulin receptor leading to a conformational change, receptor activation and trans-autophosphorylation of the intracellular tyrosine kinase domains [66, 67], and tyrosine phosphorylation of a family of insulin receptor substrate proteins amongst which IRS1 and IRS2 are most convincingly implicated in insulin's metabolic action [68]. Tyrosine phosphorylated receptor and IRS proteins leads to recruitment of effector proteins, sometimes via adaptors, triggering an intracellular phosphorylation cascade (Figure 1.4). The phosphatidylinositol 3-kinase (PI3K) and AKT/PKB pathway is the key mediator of the metabolic actions of insulin, stimulating glucose uptake through translocation of glucose transporter 4 (GLUT4) to the plasma membrane in adipose tissue and muscle [69, 70]. Multiple other insulin signalling pathways have been described [71], however glucose lowering is the most pertinent action of insulin relating to this thesis.

Proinsulin, though essentially a prohormone, does have a low but measurable ability to activate the insulin receptor with around 10% of the biological potency of insulin [72]. It is suggested that blocking the A chain, rather than the B chain, may be responsible for the comparatively lower biological activity of proinsulin [73]. In decreasing order of glucose lowering effect: des 64,65 has the highest potency, then des 31,32, with intact human proinsulin having the lowest potency [74, 75]. C-peptide has not been demonstrated to exhibit significant insulin-like activity [73, 76].

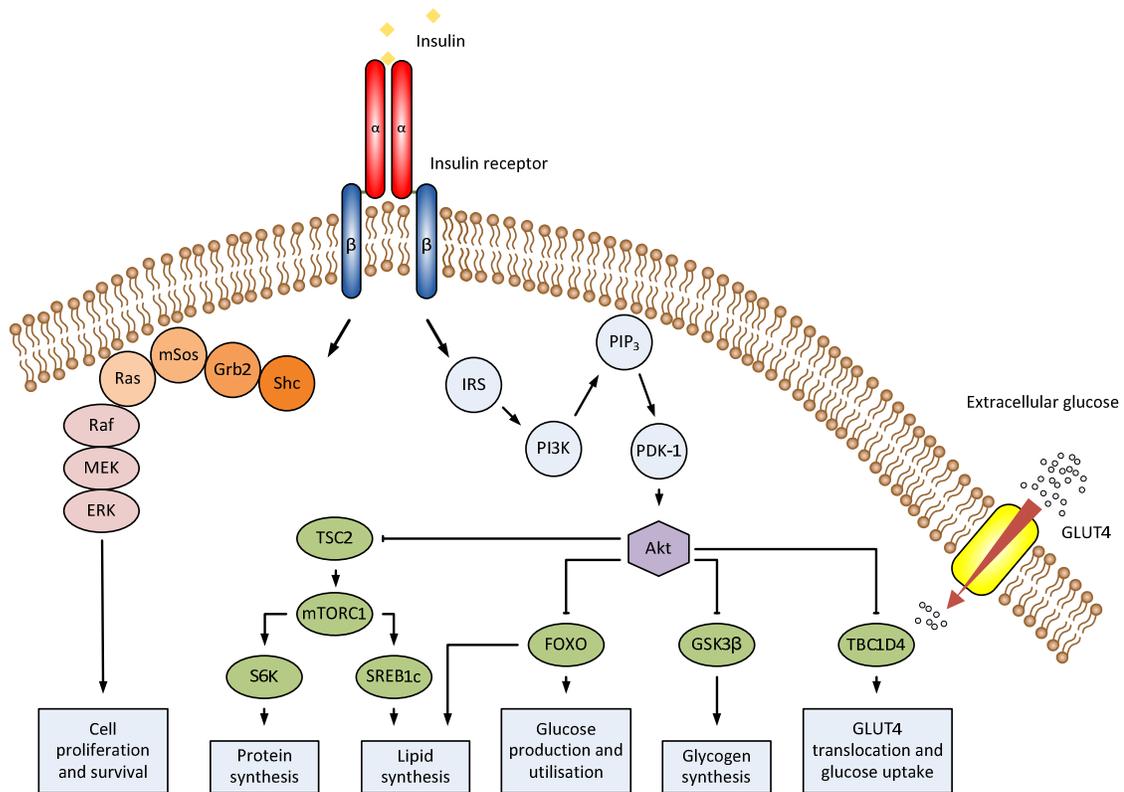


Figure 1.4 Simplified representation of the principal signalling pathways activated by the insulin receptor (adapted from [77–79]). Insulin receptor substrate (IRS) proteins mainly activate the lipid kinase PI3 (PIK3)-Akt (a serine/threonine kinase) pathway, converting the tyrosine phosphorylation signal into a lipid kinase signal by recruiting the catalytic subunit of PI3K. Phosphotyrosine sites on IRS allow binding of PI3K and activation of PI3K leads to the generation of the lipid second messenger (3,4,5)-triphosphate (PIP₃). PIP₃ recruits 3-phosphoinositide-dependent protein kinase 1 (PDK-1) activity to the plasma membrane, which in turn phosphorylates and activates Akt inducing downstream signalling. Akt phosphorylates a number of substrates at serine/threonine residues, including tuberous sclerosis complex subunit 2 (TSC2) which permits activation of mammalian target of rapamycin complex 1 (mTORC1) that leads to downstream activation of ribosomal protein S6 kinase (S6K) and sterol regulatory element-binding protein-1c (SREB1c); forkhead box transcription factor class O (FOXO) transcription factors; Glycogen synthase kinase-3 beta (GSK3 β) and Tre-2, BUB2, CDC16, 1 domain family member 4 (TBC1D4, AS160). Akt phosphorylation of TBC1D4 promotes GLUT4, the insulin-responsive hexose transporter, vesicle translocation to and fusion with the plasma membrane. Akt plays a principal role in the metabolic effects of insulin, resulting glucose production, uptake and utilisation, glycogen synthesis, lipid synthesis, and protein synthesis, and Akt also has some influence on cell cycle and survival. The adaptor protein Shc activates the Grb2/mSos/Ras/Raf/MEK/ERK pathway that is activated independently of PI3K-Akt and is involved in cell proliferation and survival.

Abbreviations: growth factor receptor bound protein 2, GRB2; mammalian Son of sevenless, MSos; Ras, G-protein with intrinsic guanosine-5'-triphosphate-ase activity; rapidly accelerated Fibrosarcoma, Raf; mitogen-activated protein kinase, MEK; extracellular signal-regulated kinases, ERK.

1.4 Insulin clearance

Insulin and C-peptide are secreted in equimolar amounts into the circulation, however 50–80% of insulin is metabolised on first pass by the liver [60, 80, 81]. Greater than 80% of total body insulin is bound to insulin receptor [82], and hepatocyte surface receptor binding of insulin triggers endocytosis of the ligand–receptor complex [83–85]. The receptor is rapidly recycled to the plasma membrane [86], however in contrast, most insulin bound to receptors is internalised and degraded [87]. Hepatocytes express carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), a transmembrane glycoprotein that is phosphorylated by the insulin receptor kinase in response to insulin, promotes receptor-mediated insulin uptake, and is internalised in combination with the insulin–receptor complex [88].

Insulin not broken down on first pass circulates via the hepatic vein to target tissues where it is internalised and ultimately undergoes lysosomal degradation, and insulin that returns to the liver via the hepatic artery is exposed to second pass degradation. During glucose infusion, the circulating insulin concentration in the portal vein can be more than thirty-fold higher than that measured in peripheral circulation [89]. The liver displays adaptive clearance rates, which increase in insulin sensitivity [90] and decrease in insulin resistance [91].

Renal extraction is the principal route of insulin clearance from the systemic circulation, with approximately 40–80% insulin delivered to the kidney being removed [92, 93]. Insulin clearance by the kidneys principally occurs by glomerular filtration followed by proximal tubular resorption and intracellular degradation, with post-glomerular peritubular clearance accounting for the majority of the remainder [94, 95]. A small amount of internalised insulin may be reabsorbed into the circulation via retroendocytosis [96]. Less than 2% of insulin filtered at the glomerulus is excreted in urine from healthy kidneys [97]. Insulin not cleared by the liver and kidneys is removed by insulin-sensitive cells via mechanisms involving internalisation and degradation [98]. However, unlike in the liver where

capillaries are fenestrated [99], the exact mechanism by which insulin transitions across the continuous microvascular endothelium of skeletal muscle and adipose tissue is yet to be established [100].

In contrast to insulin, C-peptide undergoes negligible clearance by the liver [101, 102], and around 50% of C-peptide secreted is extracted renally [81] with 5–10% excreted in urine [81, 103]. Consequently, the half-life of insulin is approximately 5 minutes [104, 105], whereas the half-life of C-peptide is approximately 35 minutes [106]. The difference in clearance is reflected in the fasting plasma insulin:C-peptide molar ratio, which is 0.03–0.25 [81, 107]. The extent of both first-pass metabolism and peripheral clearance of insulin may be variable, therefore peripheral insulin levels may not accurately reflect portal insulin secretion [101].

The rate of hepatic clearance of proinsulin, insulin, and conversion intermediates can be ranked in order from highest to lowest as: insulin, des 64,65 proinsulin, des 31,32 proinsulin, and proinsulin, respectively [108]. Concentrations of proinsulin take longer to decrease than for insulin, at least in part due to the slower hepatic extraction [109], and proinsulin clearance rate estimations range from 17–146 minutes [104, 110]. The fasting intact-proinsulin:insulin molar ratio in normoglycaemic subjects is 0.07–0.12 [111, 112], with a normal/increased proinsulin:insulin ratio demonstrated in impaired glucose tolerance [112, 113], and an increased proinsulin:insulin ratio demonstrated in type 2 diabetes mellitus (T2DM) [113–115] observed both basally and following stimulated insulin release [116–118].

1.5 Hyperglycaemia and diabetes mellitus

Increased blood glucose concentration, either acute or chronic, may result in clinical sequelae. Acute hyperglycaemia can result in polyuria and polydipsia due to osmotic diuresis that can be life-threatening in the most severe cases. Hyperglycaemia is also associated with an increased risk of infection [119]. Chronic hyperglycaemia is associated with increased risk of microvascular disease [120, 121] encompassing retinopathy, nephropathy, and neuropathy [119].

Diabetes mellitus (DM) is a group of metabolic diseases defined by hyperglycaemia. This results from defects in insulin secretion, insulin action, or both [119]. It was not until the 1930s that insulin-resistant DM was first determined as a separate clinical entity from insulin-deficient DM by Himsworth [122]. In the modern era, the diagnosis of DM is made by laboratory determination of hyperglycaemia

[123] and/or hyperglycation of haemoglobin [124], and action limits have been modified over time according to contemporary knowledge obtained from population studies of blood glucose, glycated haemoglobin and associated risk of microvascular complications [125]. Laboratory confirmation of insulin deficiency/insufficiency is not required to diagnose DM, and measurements of circulating insulin, in combination with those of C-peptide and autoantibodies targeting various beta-cell components, are used in conjunction with details of the clinical presentation to categorise the DM type [119, 126]. Individuals with T2DM have insulin resistance and/or relative insulin insufficiency, usually with loss of the first phase of insulin response due to beta-cell failure (Figure 1.3). In broad terms, therapy for T2DM either aims to improve insulin sensitivity, increase endogenous insulin secretion, or decrease renal reabsorption of glucose, but insulin replacement may be required. Individuals with type 1 diabetes mellitus (T1DM) have no, or minimal, insulin response to glucose (Figure 1.3) and require insulin therapy to prevent life-threatening ketoacidosis and hyperglycaemia. There are an estimated 4.5 million individuals with DM in the UK, with approximately 10% diagnosed with T1DM and approximately 90% diagnosed with T2DM [127]. Multiple rarer clinical subtypes of DM are described, mostly defined based on aetiology such as monogenic diabetes syndromes (<2%), e.g. maturity-onset diabetes of the young (MODY), acquired disorders of exocrine pancreas (e.g. cystic fibrosis that may exhibit features of T1DM and T2DM), and antibody-mediated diabetes (e.g. type B insulin resistance (TB-IR)) [119, 126].

1.5.1 Insulin as a medical therapy for diabetes mellitus

Prior to the discovery of insulin, the prognosis for patients with DM was extremely poor. The use of dietary restriction had only limited success, and children presenting with the disease had a very high one-year mortality rate [128–130]. Banting and Best demonstrated the glucose-lowering effect of insulin in 1921 utilising pancreatic extract from a dog [131], and as soon as the following year, the first human patient was successfully treated with purified pancreatic extract [132]. Following the original work of Abel in 1926 [24] and subsequently Scott in 1934 [133], insulin in crystalline form became available for patient use that led to largescale manufacture of animal-derived insulin of porcine and/or bovine origin. This continued until 1979, when insulin could be prepared using recombinant DNA technology [134], and in 1982, the resulting biosynthetic recombinant human insulin was approved for

clinical use [135]. Thereafter followed the production of insulin analogues with different pharmacokinetic and pharmacodynamic properties (Figure 1.5) [136–138]. In the UK today, patients newly requiring exogenous insulin are preferentially prescribed synthetic human insulin and/or insulin analogue rather than animal-derived insulin, however patients may remain on animal-derived insulin if it is their preference, such as those individuals who have taken it over many years, or when there are concerns regarding tolerance [139].

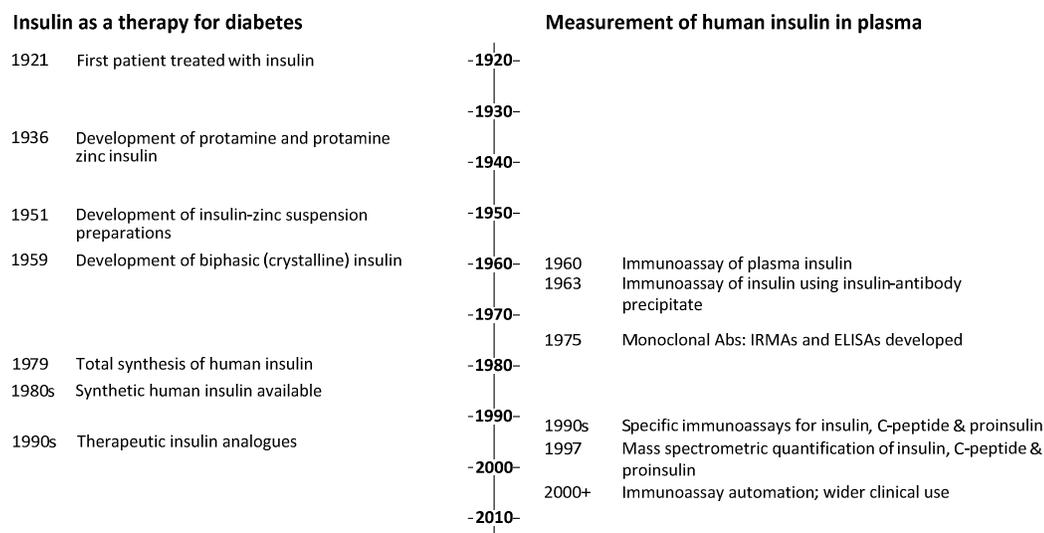


Figure 1.5 A timeline marking key events in the development of insulin therapy and insulin measurement. Abbreviations: immunoradiometric assay, IRMA; enzyme-linked immunosorbent assay, ELISA.

1.5.2 The insulin ‘unit’

A standard of bioactivity was necessary to compare insulin preparations, which was of particular importance in the early stages of insulin production when there was wide batch-to-batch variation. Initially a bioassay measurement was required as the structure of insulin was unknown, and the World Health Organisation standards and units were defined before the structure of insulin was known.

In 1922, Banting, Best, Collip, Macleod, and Noble [140] considered one insulin unit as the number of cubic centimetres of purified extracts of pancreas (‘insulin’) administered subcutaneously which caused the blood sugar of rabbits to decrease to 0.0045 % within 4 hours. This value was derived from the level at which most normal rabbits studied exhibited convulsions. A later definition specified

a reduction in glucose to a ‘convulsive level’ in rabbits of 2 kg that had been fasted for 24 hours [141]. This rabbit convulsion test was limited by the variability in correlation between blood glucose and symptoms, and the test was followed by the introduction of a rabbit blood glucose method. This gave rise to the ‘physiological unit’ from which a ‘clinical unit’ was derived. Thereafter, the first international insulin standard was prepared with one unit containing 0.125 mg of insulin. Following advances in purification, insulin standards have been prepared, and today, one unit equates to 0.035 mg human insulin. Not all insulin therapies have equal mass-per-unit ratios of insulin, as these vary depending on factors such as affinity for the insulin receptor and therapeutic potency.

1.5.3 Advances in insulin therapy: introduction of long-acting insulin

Normal endogenous insulin secretion changes according to blood glucose concentration, and varies between fasting and post-glucose load [58]. It follows that the rationale for exogenous insulin treatment is to replace/supplement the deficient hormone to optimise blood glucose concentration. Ideally, treatment would result in avoidance of hyper- and hypoglycaemia, and insulin therapies have been created to alter glucose-lowering action onset and duration to target fasting hyperglycaemia more specifically, and acute, post-prandial increases in blood glucose.

The first clinically-available insulin therapies were soluble and thus short-acting, and patients required multiple daily injections to maintain glucose control. Subsequently, attempts were made to formulate insulin suspensions that would form a subcutaneous depot that dissolved more slowly, thereby resulting in prolongation of action and mimicking basal insulin secretion more closely. Early mechanisms by which insulin action was retarded involved formulations incorporating suspension with the basic protein protamine or zinc protamine [142–144]. Compared with native insulin, sustained hypoglycaemic action with insulin protamine suspension due to retarded insulin absorption was demonstrated. Prolonged action was also demonstrated with addition of excess zinc (Lente, Ultralente insulin) and zinc with protamine (isophane; neutral protamine Hagedorn (NPH)) [142, 143]. In the 1940s, attempts were made to combine a rapid-acting and prolonged-acting insulin, and following early preparations that were unstable, a biphasic insulin preparation was developed in 1959 [145].

Insulin, an acidic molecule with an iso-electric point of approximately 5.4 [146], is ionised at the neutral pH of tissue fluids and consequently rapidly absorbed from the site of injection. Named after its inventor Hans Christian Hagedorn, NPH insulin is a compound of insulin mixed with protamine to increase the insulin pH to around 7 [142, 147]. Following multiple failed attempts, the pH was successfully increased and a precipitate was formed with the addition of zinc providing additional stability [147]. Co-precipitation of insulin and protamine occurs in a 5:1 molar ratio at pH 7.3 [148] and rather than binding to NPH, it has been suggested that protamine acts to balance molecular pH [149]. Following injection into the subcutaneous tissues, solvents in NPH crystals dissolve and diffuse into the subcutaneous tissue (Figure 1.6), and insulin hexamers and protamine are released [149]. Insulin crystals remain near the injection site, and probably macrophages, and possibly protamine-splitting enzymes, also degrade insulin crystals [150]. From hexamers, insulin dimers and monomers are released, and only insulin that has been released from dissolved NPH crystals diffuses and is then subsequently absorbed [149]. First used clinically around 1936 [151], NPH insulin allowed patients to reduce the frequency of injections necessitated by sole use of short-acting insulin. NPH has been by far the most commonly-used long-acting insulin therapy for more than 50 years, but such dominant use has somewhat diminished recently with the increased use of insulin analogue therapy.

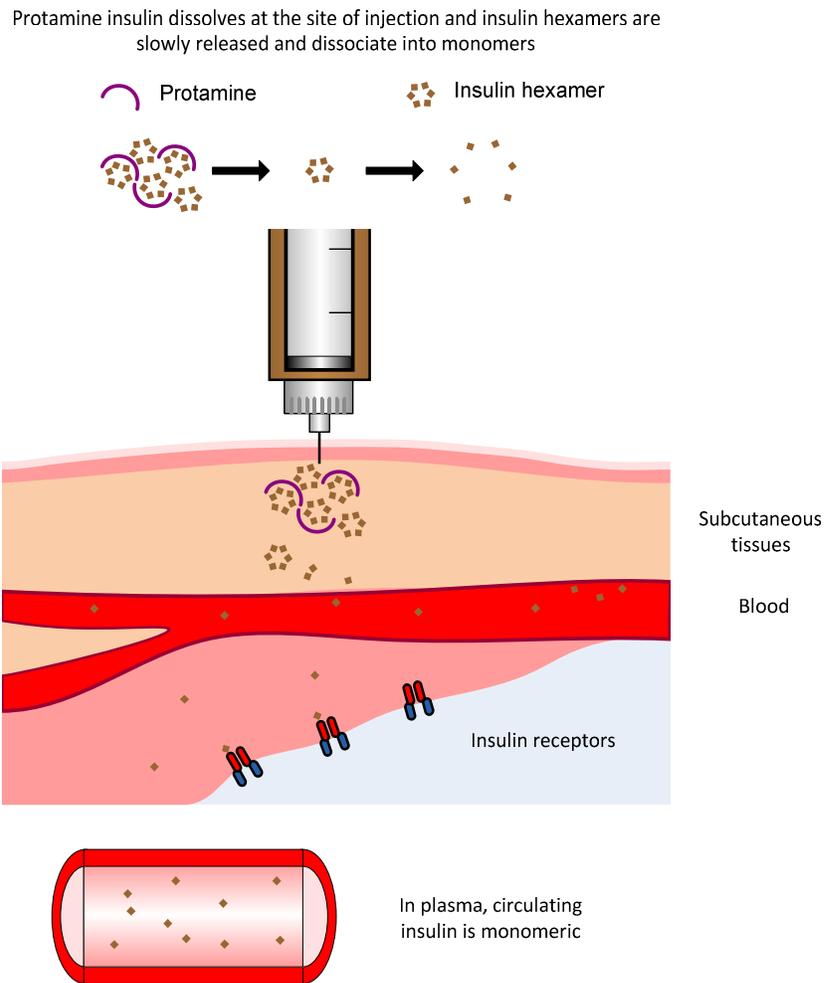


Figure 1.6 Insulin neutral protamine Hagedorn (NPH): subcutaneous dissolution, and circulation in blood. Following injection into the subcutaneous tissues, conglomerates dissolve and hexamers are released and dissociate. Monomeric insulin circulates in plasma.

1.5.4 Analogue insulin: structural modification resulting in altered insulin pharmacokinetics and pharmacodynamics

In the neutral solution used as therapy, human insulin exists as zinc-containing hexamers [22]. The rate of insulin absorption is affected by self-association of monomers, and absorption rates of insulin, ordered slowest to quickest, are hexameric, dimeric, then monomeric [152, 153]. In the 1980s, following the advent of DNA technology that enabled structural modification of insulin, synthetic preparations, that remained monomeric or dimeric at high concentrations thereby altering absorption rates [153], were produced.

Amino acid sequence changes, with/without addition of fatty acid side chains, have been used successfully to alter insulin pharmacokinetics (Table 1.1; Figure 1.7), and although insulin analogues are often referred to in clinical practice simply as ‘insulin’, compound structure can differ markedly from native insulin, and pharmacokinetics and pharmacodynamics also vary widely. To illustrate this, described below are three examples of different insulin analogues: aspart, a short-acting insulin analogue; glargine, a long-acting insulin analogue, and detemir, a long-acting insulin analogue with a fatty acid side chain and albumin-binding properties.

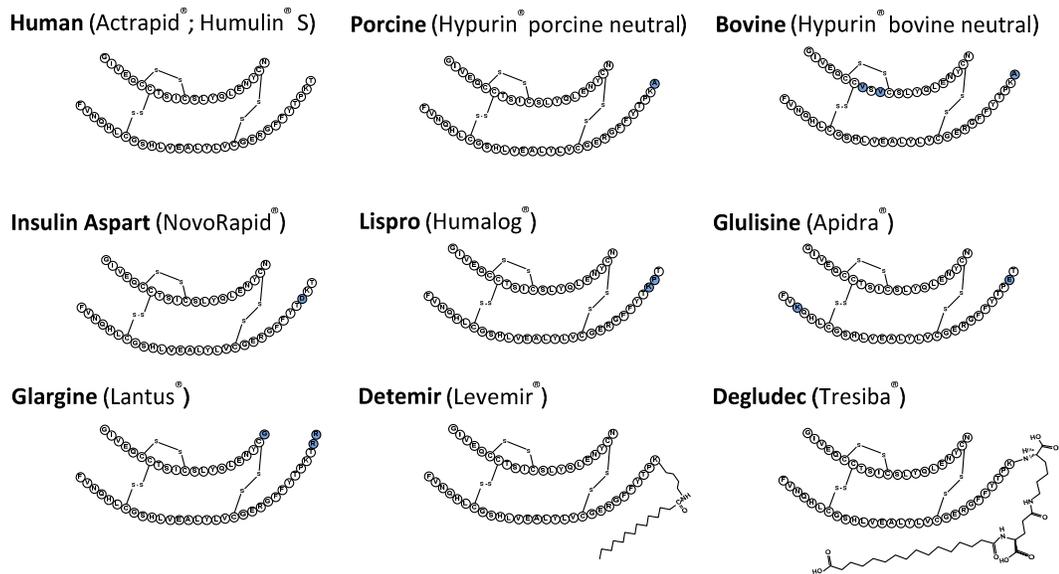


Figure 1.7 Examples of insulin therapies. Therapies may be synthetic human, animal-derived, or insulin analogue. Structural modifications to human insulin have resulted in altered pharmacokinetics and pharmacodynamics.

Table 1.1 Insulin preparations available for use in clinical practice

Insulin generic name (Trade name)	Manufacturer (Authorisation Date*)	Description	Source	Structure
Human (Actrapid®)	Novo Nordisk (07/10/2002)	Short-acting insulin	Human insulin	Homologous to human insulin
Human (Humulin® S)	Lilly	Short-acting insulin	Human insulin	Solution of human insulin
Human (Humulin® I)	Lilly	Long-acting insulin	Human insulin	Suspension of isophane human insulin
Human (Insulatard®)	Novo Nordisk (07/10/2002)	Long-acting insulin	Human insulin	Suspension of isophane human insulin
Porcine (Hypurin® porcine neutral)	Wockhardt UK	Short-acting insulin	Porcine insulin	Solution of porcine insulin. Porcine insulin is homologous to human insulin with the exception that the amino acid alanine is in place of threonine at position B30.
Porcine (Hypurin® porcine isophane)	Wockhardt UK	Long-acting insulin	Porcine	Isophane porcine insulin
Bovine (Hypurin® bovine neutral)	Wockhardt UK	Short-acting insulin	Bovine	Solution of bovine insulin. Porcine insulin is homologous to human insulin with the exception that the amino acid alanine is in place of threonine at position A8, valine is in place of isoleucine at position A10, alanine is in place of threonine at position B30.
Bovine (Hypurin® bovine isophane)	Wockhardt UK	Long-acting insulin	Bovine	Isophane bovine insulin
Aspart (NovoRapid®)	Novo Nordisk (07/09/1999)	Rapid-acting	Analogue	Homologous to human insulin with the exception of a substitution of the amino acid proline for aspartic acid in position B28
Glulisine (Apidra®)	Sanofi-Aventis (27/09/2004)	Rapid-acting	Analogue	Homologous to human insulin with the exception that the amino acid asparagine at position B3 is replaced by lysine and the lysine in position B29 is replaced by glutamic acid.
Lispro (Humalog®)	Lilly (30/04/1996)	Short-acting insulin	Analogue	Homologous to human insulin with the exception that the amino acid proline at position B28 is replaced by lysine and the lysine in position B29 is replaced by proline.
Glargine (Lantus®)	Sanofi-Aventis (09/06/2000)	Long-acting insulin	Analogue	Homologous to human insulin with the exception that the amino acid asparagine at position A21 is replaced by glycine and two arginines are added to the C-terminus of the B chain.
Detemir (Levemir®)	Novo Nordisk (01/06/2004)	Long-acting insulin	Analogue	Homologous to human insulin with the exception that the amino acid molecule in position B30 has been omitted and a 14-carbon fatty acid chain has been attached to position B29.
Degludec (Tresiba®)	Novo Nordisk (21/01/2013)	Very long-acting	Analogue	Homologous to human insulin with the exception threonine in position B30 has been omitted and where the ε-amino group of lysine B29 has been coupled with hexadecanedioic acid via a γ-glutamic acid spacer

*Date stated is that which the European Commission granted marketing authorisation, as published by the European Medicines Agency (<http://www.ema.europa.eu/ema/> accessed 13/11/2017).

1.5.5 Insulin aspart: a short-acting insulin analogue

The amino acid sequence of insulin aspart differs from native human insulin by the replacement of proline with aspartic acid at position twenty-eight of the B chain. This alteration leads to a reduction in intermolecular van der Waals forces and weakens monomer–monomer interactions [155]. This, in turn, results in the preparation existing much more readily as monomers, and the rate of absorption from subcutaneous injection is increased [153] (Figure 1.8).

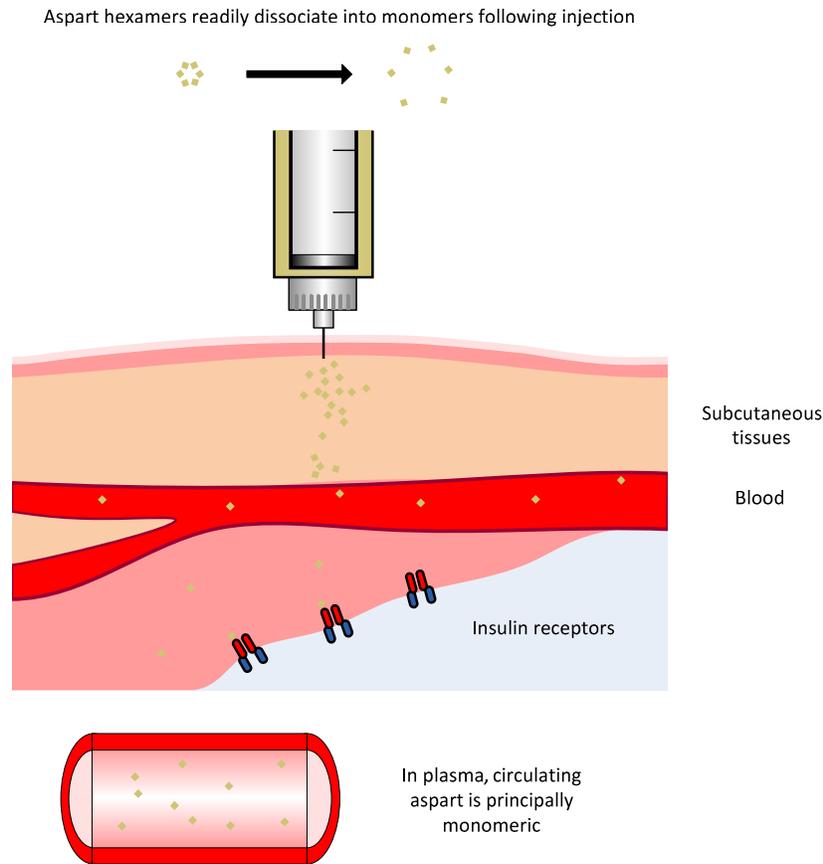


Figure 1.8 Insulin aspart: subcutaneous absorption, and circulation in blood. Following injection into the subcutaneous tissues, aspart readily dissociates into monomers. Monomeric aspart circulates in plasma.

1.5.6 Glargine: a long-acting insulin analogue with characteristics of a prohormone

Insulin glargine, Gly^{A21} Arg^{B31} Arg^{B32}, differs from human insulin by amino acid asparagine being replaced by glycine at position A21, and two arginines added to the C-terminus of the B chain. These structural changes result in an isoelectric point shift from pH 5.4 to 6.7, and decreased solubility at neutral pH [156]. Precipitation occurs in the subcutaneous tissue where, thereafter, glargine acts as a prohormone [157], as it is metabolised into two main active metabolites, M1 (GlyA21) and M2 (GlyA21, des-ThrB30). There is little or no intact glargine in circulation [158] (Figure 1.9).

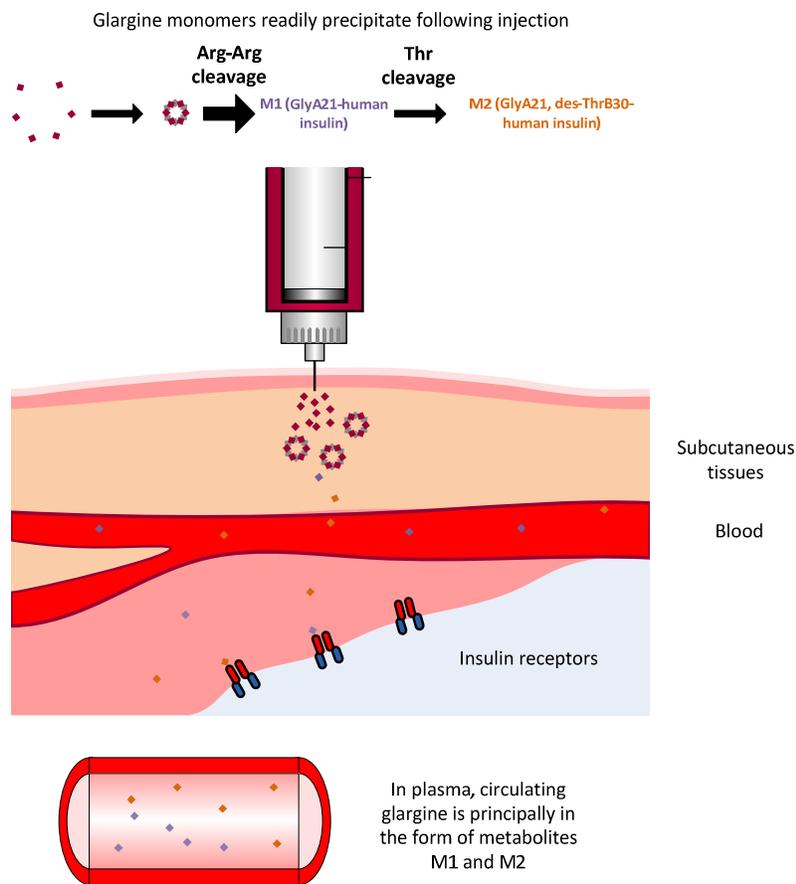


Figure 1.9 Insulin glargine: subcutaneous absorption and metabolism, and circulation of metabolites in blood. Glargine precipitates following injection and is metabolised in the subcutaneous tissues. Glargine metabolites, M1 and M2, circulate in plasma.

1.5.7 Detemir: a long-acting insulin analogue with albumin binding

Insulin detemir [Lys^{B29} (N-tetradecanoyl) des-(B30) human insulin], differs from human insulin by removal of the threonine at position B30, and the attachment of a 14-carbon myristoyl fatty acid chain to the lysine at position B29 [159]. Detemir is a long-acting insulin analogue (Figure 1.10), and its disappearance from porcine subcutaneous tissues has been studied [160]: the disappearance ($T_{50\%}$) from the injected depot was 10.2 ± 1.2 hours for insulin detemir and 2.0 ± 0.1 hours for a monomeric acylated insulin analogue preparation, suggesting that insulin action prolongation is principally a consequence of slow absorption rates of detemir into the bloodstream. Data comparing the subcutaneous disappearance of detemir with insulins of differing affinities for albumin binding and/or for hexamer-hexamer association suggested slow detemir absorption was due to hexamer association and albumin-binding in the subcutaneous tissues [160]. Removal of the C-terminal amino acid residue (B30) results in an increase in the albumin-binding affinity of Lys^{B29}(N-tetradecanoyl) insulin by 1.7 times [161]. Size-exclusion chromatography studies have confirmed detemir binding to albumin, and the albumin-bound fraction of detemir is calculated at 98–99% [162, 163].

Concurrent binding of insulin to albumin and to insulin receptor has been excluded *in vitro* [161], and albumin binding in plasma is thought to have a buffering effect on circulating detemir which may serve to smooth its pharmacokinetic profile. Experimental evidence suggests that increased binding affinity for albumin slows the distribution and elimination of insulin detemir [160], and the resultant retarded transendothelial transport may contribute to the prolonged action of detemir [163]. Reduced affinity (46%) for the insulin receptor may also contribute to prolongation of action through a decreased rate of clearance [161].

Insulin detemir has reduced molar potency [164] and, when compared with NPH human insulin, detemir formulation (1 unit contains 24 nmol) was four times the molar concentration of human insulin (1 unit contains 6 nmol), and this is an important consideration when comparing plasma measurements of different insulin analogues. During a study of detemir pharmacokinetics, total (bound plus free) serum insulin detemir was measured using a specific enzyme-linked immunosorbent assay (ELISA) which did not cross-react with human insulin or aspart [165]. The maximum picomolar concentration measured was ten-fold higher in patients receiving detemir compared with NPH insulin when given a unit-

equivalent dose (0.5 units/kg (12 nmol/kg) insulin detemir or 0.5 IU/kg (3 nmol/kg) NPH insulin, respectively), an observation previously noted at 0.30 U/kg [166]. Despite significant difference in total plasma detemir concentrations being observed, there was no clear demonstrable dose-response relationship, an observation that, in the absence of free detemir measurement, may be confounded by albumin binding itself [166].

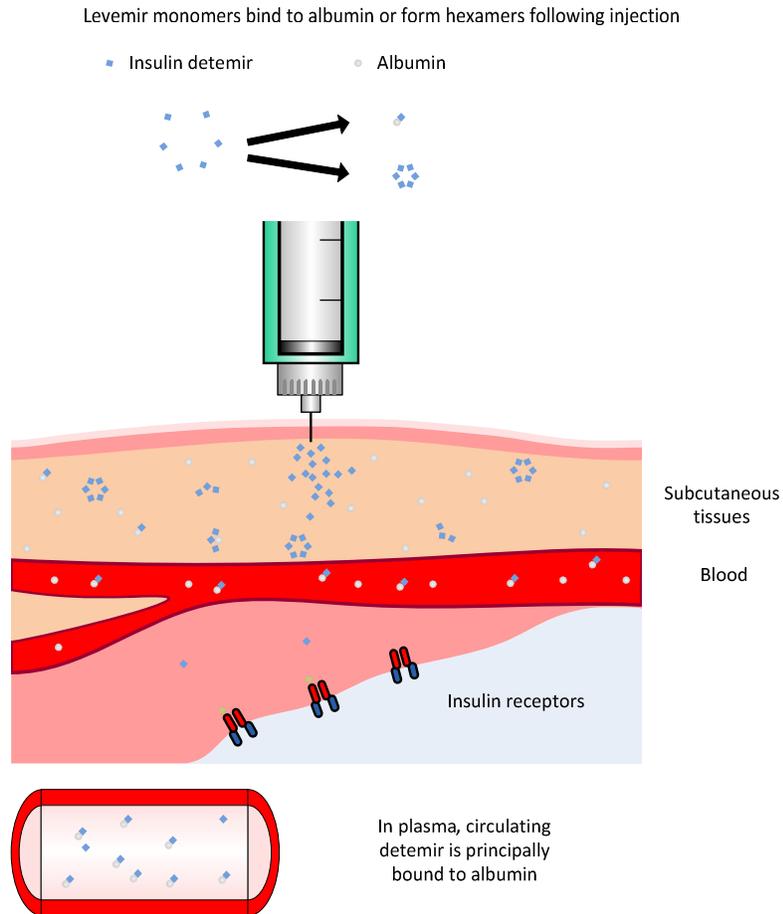


Figure 1.10 Insulin detemir: subcutaneous absorption, and circulation in blood.

Following injection, detemir absorption is protracted through hexamer formation and albumin binding. Circulating free and albumin-bound detemir circulates in plasma.

1.6 Hypoglycaemia

Clinical hypoglycaemia may be defined by Whipple’s triad of hypoglycaemic symptoms, a low blood glucose, and symptomatic improvement following glucose administration [167, 168]. Symptoms can be broadly divided into neurogenic (sympathetic response to low blood glucose), and

neuroglycopenic (clinical consequence of glucose deprivation in the central nervous system) [169]. There is no generally accepted specific biochemistry definition of hypoglycaemia [167] and there are different clinical context-specific glucose thresholds considered important. For unexplained spontaneous hyperinsulinaemic hypoglycaemia, either during a fast or postprandial, a plasma concentration of <3.0 mmol/L is considered significant [167].

For individuals with insulin-treated DM, hypoglycaemia most commonly results from an imbalance of exogenous insulin and blood glucose concentration. This situation may occur due to excessive and/or poorly-timed insulin therapy, however may also arise in states when endogenous glucose production is limited, or insulin clearance is decreased [167]. Management strategies to combat recurrent hypoglycaemia and/or labile blood glucose levels in insulin-treated DM include self-monitoring blood glucose using capillary blood glucose (CBG) measurements or continuous glucose monitoring system (CGMS), and patient training programmes in DM self-management (e.g. Dose Adjustment for Normal Eating, DAFNE). Glucose concentrations of 3.1–3.9 mmol/L stimulate epinephrine and glucagon release [170–173], and a glucose of <3.9 mmol/L has been recommended as a clinical-threshold for hypoglycaemia in treated DM. Hypoglycaemia has been categorised further: *severe*, presenting with neuroglycopenic symptoms, and requiring treatment by another individual; *symptomatic*, symptomatic hypoglycaemia and a measured plasma glucose concentration of <3.9 mmol/L (further classified as ‘probable’ when glucose is not measured), and *relative*, when there are symptoms but the glucose concentrations measured are >3.9 mmol/L [174]. The latter group may be a more common presentation in those individuals who have chronic poorly-controlled DM [170, 175].

1.7 Measurement of human plasma insulin concentration

1.7.1 Insulin bioassay

Before the advent of assays to measure concentration, attempts were made to measure insulin activity through the study of the metabolic effects of insulin. *In vivo* bioassays, such as those assessing the glucose-lowering effect in a rat preparation [176], lacked specificity and precision. However *in vitro/ex vivo* bioassays assessing glucose metabolism by rat adipose tissue [177] and diaphragm [178–181] demonstrated adequate sensitivity for measurement, but were subject to assay bias caused by the

presence of one or more atypical constituent(s) of a sample (an effect referred to as ‘assay interference’) [182, 183], and generated estimations of insulin-like activity, rather than specific quantitation.

1.7.2 Immunoassay

Using the principle of radioligand binding demonstrated by Ekins [184], Berson and Yalow demonstrated displacement of antibody binding of iodine-131-labelled insulin by unlabelled insulin led to the development of the first competitive immunoassay for human insulin [185–188]. Yalow observed that “Radioimmunoassay came into being not by directed design but more as a fall-out from our investigations into what might be considered an unrelated study” (Yalow, Nobel lecture 1977) [189]. The assay reaction involved human insulin competing with iodine-131-labelled bovine insulin for insulin-binding antibodies in guinea pig serum. The technique was then modified to utilise anti-gamma globulin antibody to pre-precipitate the anti-insulin antibody (IA), allowing rapid separation of insulin-bound antibody by filtration [190], and offering improved analytical sensitivity. Wide et al [191] developed an immunoassay method incorporating radiolabelled antibody, and soon after, Miles and Hales [192] reported the first ‘immunoradiometric’ assay for insulin. The utilisation of monoclonal antibodies as a means of ensuring predefined antibody specificity [193] allowed the development of labelled antibodies for use in assays.

Today, commercially available insulin immunoassays are used widely by hospital laboratories, and clinical insulin immunoassays are typically two-site (non-competitive) [194, 195], using either murine monoclonal capture and detection antibodies or a murine monoclonal in combination with murine polyclonal antibodies (Table 3.1). Classically, immunodetectable insulin concentrations are determined by comparison with standards of known human insulin concentration. Immunoassay technology has known limitations however, and may be susceptible to interference caused by endogenous antibodies in patient plasma, either by interacting with assay reagents or with the analyte itself (termed ‘autoantibodies’). Endogenous human anti-animal and heterophilic antibodies present in plasma, may bind assay reagents causing assay interference [196, 197], thereby altering the correct value of the result [198, 199]. Whilst the production of anti-animal antibodies follows exposure to immunogens, heterophilic antibodies are believed to occur without such exposure [197]. One-step immunometric assays (binding analyte with both capture and detection antibodies concurrently) appear

the most susceptible to positive interference by heterophilic antibodies [200]. Binding to the Fc or the F(ab')₂ fragments [201], some such antibodies may bind both immobilised and labelled immunoassay antibodies in two-site immunoassay to form a stable complex and, ultimately, yield a false positive analyte result [197, 200], decreasing detection of analyte by steric hindrance [201]. Reports of human anti-animal and heterophilic antibodies are rare and may under-represent prevalence as only results overtly discordant with the clinical picture [202] may be investigated.

A more commonly encountered limitation of clinical insulin assays is their variable detection of non-human insulin or genetically modified insulin analogue. A substance other than the analyte of interest that binds capture and detection antibodies to generate an immunoassay signal is termed a cross-reacting substance, and specificity is a term for the ability of an assay to generate a signal from the analyte of interest but no other substances. Substances such as molecules with a similar structure to the analyte of interest may cross-react in an immunoassay, and such putative interferents are studied to determine the specificity of an assay. Cross-reactivity studies are typically performed by spiking the potential cross-reactant into samples. The assay signal is compared with that of the original sample and the result expressed as the apparent percentage change in analyte concentration. In insulin immunoassay, animal/fish-derived insulin and/or insulin analogue may cross-react more or less than human insulin, or even not at all [203–208].

The advent of new insulin analogues with amino acid differences from normal human insulin (Table 1.1; Figure 1.5) coinciding with the development of more specific immunoassays for human insulin quantification, has made interpretation of insulin results more challenging, especially in cases of suspected surreptitious insulin administration. Whilst an individual clinical immunoassay cannot distinguish endogenous from exogenous human insulin, insulin:C-peptide molar ratios can be employed [209], but in the context of administered insulin analogue, a normal insulin:C-peptide ratio may not be wholly reassuring [210].

Although endogenous insulin reference ranges derived from individuals without DM are well established, there are limitations to applying these to measured exogenous insulin concentrations. For insulin-treated individuals with diabetes, one must also consider the effect of insulin analogues with protein binding properties, which may lead to higher measured concentrations of total insulin that may

not reflect free insulin concentrations. This has particular impact when attempting to establish whether a plasma insulin concentration is abnormally high in the context of hypoglycaemia, such as when it is suspected that an inappropriately high insulin dose has been administered deliberately or accidentally, by a patient or a third party. In such scenarios, assay uncertainty can be a critical hindrance in forensic investigation of maleficent insulin use, with potential implications for criminal and/or child custody proceedings.

1.7.3 Mass spectrometry

Mass spectrometry (MS) methods for the detection of human insulin were first described in 1997 [16, 211]. Although additional methods have been developed subsequently [212–214], the use of MS for the quantification of insulin has been limited by the requirement for relatively large sample volumes. Detection of insulin using mass spectrometry immunoassay (MSIA) holds much promise in being able to detect individual analogues in smaller volumes of plasma [215], however access to such assays in the routine hospital laboratory is currently limited, and the effect of heterophilic or insulin-binding antibodies on MSIA is not known.

1.8 Detection of insulin antibodies

Early laboratory techniques for the detection and quantification of IA in plasma incorporated separation of free and bound insulin by radiochromatography [185, 216], immunoelectrophoresis [217], charcoal immunoassay [218, 219], immunoprecipitation [220, 221], and polyethylene glycol (PEG) precipitation [222]. Currently, the most common techniques employed for IA detection in the clinical laboratory are RIA and ELISA. In circulation, there are five classes of immunoglobulin in humans: IgG, IgA, IgM, IgE, and IgD. These are classified by the heavy chain and they have differing physical properties (Table 1.2).

Table 1.2 Physical properties of human immunoglobulin

	Antibody Class				
	IgG	IgA	IgM	IgE	IgD
Heavy chain	γ	α	μ	ε	δ
Molecular Weight (kDa)	150	300	900	190	150
Adult serum reference range (units)	6.5–16.0 (g/L)*	0.4–3.5 (g/L)*	0.5–3.0 (g/L)*	<100 (kIU/L)*	<80 (mg/L)*
Subunits	1	2	5	1	1
Subclasses	4 (IgG1–4)	2 (IgA1 and IgA2)	-	-	-
Biological function	Secondary immune response	Immunoglobulin in mucous secretions	Primary immune response	Anti-parasite immunity	Not known

* Reference ranges [223]

1.9 Detection of insulin–antibody complexes

The existence of circulating hormones bound in hormone–immunoglobulin complexes (also known as ‘macro-hormones’) is not isolated to insulin [224]. Macroprolactin is the best-described [225], and hormone–immunoglobulin complexes have also been reported for other hormones, including luteinising hormone [226], follicular stimulating hormone [227], thyroid stimulating hormone [228], and human chorionic gonadotrophin [229]. Hormone–immunoglobulin complexes can be biologically inactive, such as macroprolactin [225], or can dissociate to cause clinical disease, such as macroinsulin [185, 230–232].

Macro-hormones can pose analytical challenges to immunoassay hormone measurement. Hormones bound in a complex may still be detected, and assay results may be clinically misleading if distinction is not made between biologically-inactive bound hormone and bioactive unbound (‘free’) hormone. Analytical adjuncts are routinely employed by specialist clinical laboratories to identify hormone–immunoglobulin complexes, the most common of which is PEG precipitation. PEG is a non-denaturing, water-soluble synthetic polymer with a protein-precipitating action which may be qualitatively explained by excluded volume effects: protein exclusion from solvent occupied by the inert

polymer results in increased protein concentration, such that solubility is exceeded and protein precipitation occurs [375]. Following centrifugation, proteins detectable in the supernatant are those which have not been precipitated by this process. PEG precipitates immunoglobulin [233] and antibody-bound insulin [222, 234], with non-precipitated (unbound) insulin remaining in solution. Consequentially, when using a PEG precipitation RIA, insulin recovery in IA-positive individuals is lower than IA-negative controls [234], and the ratio of bound/free insulin is a function of the concentration and the insulin-binding characteristics of the antibody [235]. PEG precipitation is not specific however, and analyte recovery can be variable [236] and dependent on other serum constituents. Moreover, PEG precipitation may affect the performance of some immunoassays, and an up-to-date assessment of this technique using modern clinical insulin immunoassays has not been published to date.

Investigation of possible interference caused by macro-hormones interference may include dilution studies, also referred to as parallelism studies [228, 237–243]. These are performed by diluting samples in analyte-free matrix (such as immunoassay diluent or BSA solution) and measuring the analyte concentration in the diluted sample. In an immunoassay that is linear to dilution, results may be expected to lie parallel to the calibration curve. By multiplying the result of the diluted result by the dilution factor, a calculation of the initial sample concentration can be made. Comparison of results generated from dilutions of a patient sample with those of control samples (typically selected as free from suspected interferent), a judgement is made as to whether the patient analyte dilutes in the same way as control. If the patient sample behaves differently, such as demonstrating a non-linear change in analyte concentration following dilution, this may be consistent with the presence of a constituent of the patient sample that affects immunoassay detection of the analyte. Macro-hormones may not cause non-linearity [226, 229], the presence of non-linearity is not specific to autoantibodies, and other sample interferents, including heterophilic antibodies, may also behave differently from analyte with sample dilution [237].

GFC has been used to identify HMW insulin immunoreactivity consistent with insulin–antibody complexes in patients with dysglycaemia [244]. However, the sensitivity of GFC-based approaches is limited by the dilution of sample that occurs during the filtration process, meaning the analyte must be present in plasma at a sufficiently high ambient concentration to permit assay detection post-filtration.

An additional concern is that sample dilution may alter the equilibrium between bound and unbound hormone that exists *in vivo*. In contrast, increasing insulin concentration can increase the amount of HMW insulin [185] as the quantity of insulin bound to antibody is increased. The importance of discriminating antibodies directed against assay components (heterophilic antibodies, see Section 1.7.2) [199, 202] and insulin–antibody complexes is critical, as the former entity is principally an analytical challenge, whereas presence of the latter may alter insulin kinetics/dynamics, causing dysglycaemia. Heterophilic antibody interference is assay-dependent, as is the degree to which the hormone bound to immunoglobulin is detected, and two-step assays may be more robust to such interference [239].

1.10 Insulin Autoimmune Syndrome

Insulin autoimmune syndrome (IAS), also referred to as Hirata disease, is defined as spontaneous hyperinsulinaemic hypoglycaemia due to insulin autoantibodies in individuals who have not received exogenous insulin [245, 246]. The first patient was described in 1970, and in 1982, the condition was noted to be the third-leading cause of spontaneous hypoglycaemia in Japan [245]. Insulin bound to antibody does not bind to insulin receptors, and insulin–receptor binding is dependent on the proportion of insulin that is antibody bound [247]. The clearance rate of antibody-bound insulin is impaired as hepatic and renal uptake is inhibited, and insulin is subsequently released from insulin–antibody complexes to interact with the insulin receptor, thus lower glucose concentrations at physiologically inappropriate times [248]. Classically, IAS presents with recurrent fasting and/or post-prandial hypoglycaemia), alternating with post-absorptive hyperglycaemia [248–257], a result of antibody sequestration of acutely secreted insulin, inhibiting insulin action [250], and subsequent insulin dissociation from antibody and hyperstimulation of the insulin receptor, respectively. The condition has been most widely reported in Japan, with most cases self-limiting [258], however, severe and life-threatening presentations have been described [259–265].

Detection of IAs is a cornerstone of the diagnosis of IAS [266]. Most reported cases of IAS are due to pathogenic IgG antibodies, however anti-insulin IgA has been described in the context of a patient with myeloma [244]. Attempts have been made to identify whether a structural alteration in the insulin of patients with IAS is responsible for insulin autoimmunity [267, 268]. Insulin was separated from

insulin–antibody complexes using gel and reverse-phase high performance liquid chromatography, and a hydrophobic variant of insulin, in addition to normal human insulin, was identified. However, variant insulin species were also demonstrable in the serum of insulin-treated patients with DM, and in a patient with insulin resistance but no DM or circulating anti-insulin IgG, which did not strongly suggest the presence of a variant of endogenous insulin as a precipitant for insulin autoimmunity. For one case of IAS when insulin autoantibodies were determined to be of IgG1 subclass [269], antibody cross-reactivity with animal-derived insulins varied demonstrably (Figure 1.11). Examination of the species-specific amino acid sequences of insulin led the authors to postulate the antibody’s binding site using epitope mapping.

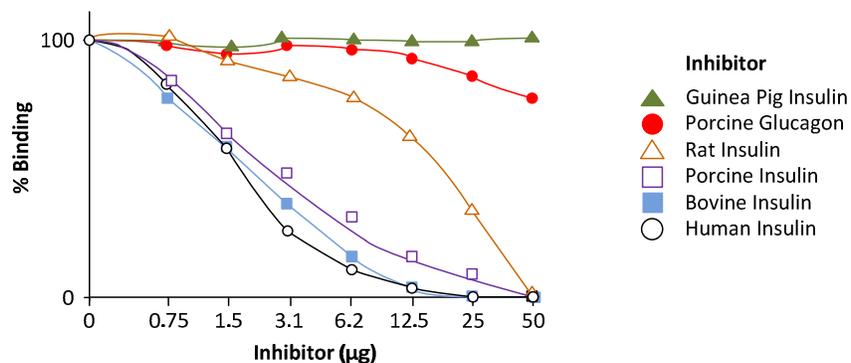


Figure 1.11 Inhibition of insulin autoantibody binding to human insulin by different animal-derived insulins and glucagon (control) (reproduced from Uchigata, Yasuko, Yao, Kenshi, Takayama-Hasumi, Sumiko, and Hirata 1989) [269].

1.10.1 Treatment of insulin autoimmune syndrome with immunomodulation therapy

Although no consensus exists about optimal approaches to therapy, individual cases of the effective use of different antibody-depletion therapies in the context of IAS have been reported, including the use of therapeutic plasma exchange (TPE) [248, 253], prednisolone [259], hydrocortisone [260], mycophenolate mofetil (MMF) [263, 264], azathioprine [261], cyclophosphamide [262], and rituximab [265].

TPE is an extracorporeal blood removal technique (Figure 1.12) used for the removal of high molecular weight (HMW) substances, such as pathogenic antibodies, from plasma [270]. The rationale of TPE is to reduce the concentration of pathogenic antibodies to ameliorate the disease process.

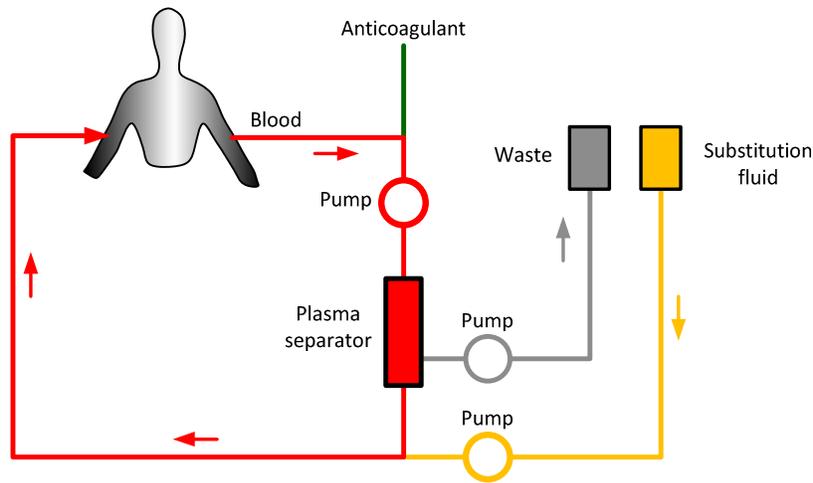


Figure 1.12 Therapeutic plasma exchange. Intravenous blood is extracted, and anticoagulant added, before separation of blood and plasma (containing antibodies), then antibody-depleted blood with plasma-substitute is returned to the body.

Generally, the effects of plasma exchange are transitory and, once a plasma exchange session is complete, there follows an increase in plasma antibody concentration over time from the post-procedure trough level. This is, in part, due to re-equilibration of immunoglobulins between the vascular space and the interstitium (approximately 80% total IgM and 45% total IgG is present in the intravascular compartment [270]) and, in part, due to the continued presence of IA-producing cells. This is an important consideration when determining the most appropriate way to monitor patients using laboratory tests. One plasma volume exchange results in a reduction in serum immunoglobulin of approximately 60%, yet the net reduction in total immunoglobulin is only about 20% [270, 271]. A course of three plasma exchange sessions on alternate days leads to an 80% reduction in IgG [271] (Figure 1.13).

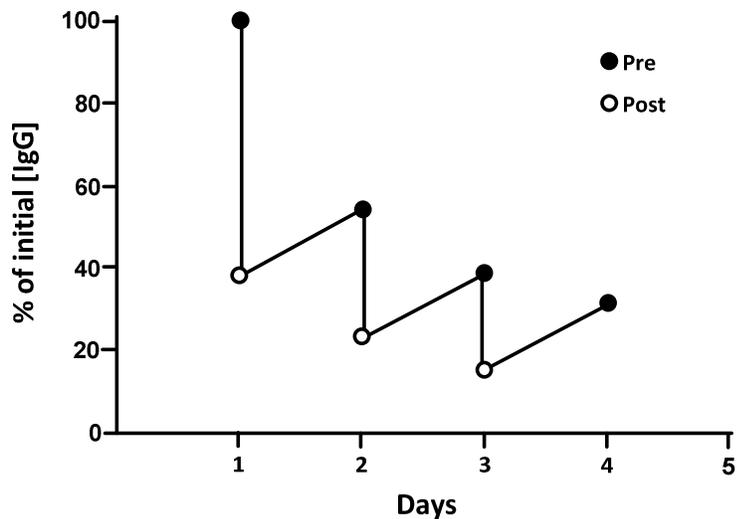


Figure 1.13 IgG extraction with plasma exchange. Progressive decrease in IgG level following three consecutive TPE treatments, each of one plasma volume. (Recreated from Kaplan (2013) [270]).

Glucocorticoid (e.g. hydrocortisone or prednisolone) therapy results in a rapid reduction of circulating T-cells and, to a lesser extent, B-cells [272]. A decrease in circulating IgG is observed [273], however the antibody synthesis by B-cells is not grossly affected by short-term administration of glucocorticoids, and the decrease in antibody titres observed appears to be a result of either decreased helper-inducer T-cells or of an increased antibody catabolism [274]. MMF, a prodrug for mycophenolic acid, inhibits inosine monophosphate dehydrogenase and in turn depletes guanine nucleotides [275]. It has immunosuppressive effects that include reduction of antibody response [276]. Azathioprine (6-(1-methyl-4-nitromidazol-5-ylthio)purine) is metabolised to 6-mercaptopurine, a purine analogue known to inhibit purine biosynthesis, and acts to suppress cellular immunity and inhibit antibody production [277]. Cyclophosphamide is a drug with immunosuppressive effects, possibly acting via a cytotoxic effect on lymphocytes [278]. Rituximab is an IgG1 chimeric monoclonal antibody, composed of murine variable and human constant regions which binds B-cell CD20 [279, 280], causing rapid depletion of certain B-cells. All the immunomodulatory agents described here have the potential to cause acute and long-term side-effects, of which long-term immunocompromise is one of the more concerning. It follows that any decision to treat using such medications should be most carefully considered.

1.11 Insulin antibodies in insulin-treated diabetes

1.11.1 Insulin antibodies and autoimmune diabetes

IAs are measured as part of a repertoire of islet cell autoantibody tests to aid classification of DM [126], to identify those individuals at increased risk of developing autoimmune DM [281, 282], and to study the natural history of the disease [283]. Insulin autoantibodies have been detected in 32–38% individuals at the onset of newly-diagnosed T1DM before insulin treatment has been initiated [284–288], and such antibodies are principally of the IgG rather than IgM class [289].

IAs that derange insulin kinetics are usually IgG, however, other classes of IA are detectable in serum of patients with diabetes, including as IgM [290] and IgE [291]. Anti-insulin IgE antibodies are most associated with cutaneous insulin allergy [292].

1.11.2 Insulin antibodies and exogenous insulin

The first clinically-effective insulin preparation was a crude acid-alcohol extract of ground bovine pancreas that was contaminated with other pancreatic substances [293]. Following more widespread clinical use of insulin, reports emerged of serious cutaneous reactions, including hypersensitivity and abscess formation, systemic reactions, including anaphylaxis [294], and severe resistance to insulin therapy [295–297]. Indeed, immunological insulin resistance became so prevalent that it emerged as a distinct clinical entity, and treatment options were limited to changing insulin preparation, high concentration insulin, and use of glucocorticoids, with variable success [295].

The immunogenicity of exogenous insulin was first reported in 1938 by Banting [298] who observed an insulin-neutralising factor in the plasma of an individual without DM following a course of insulin shock treatment, a once-common treatment for schizophrenia. In Banting's report, the patient's insulin dose increased from 20 units to 1000 units after fifty-nine treatments. Insulin-binding immunoglobulin was first demonstrated in the circulation of patients treated with exogenous insulin in 1955 [185] and there followed many studies of such antibodies in an era when clinical use of impure animal-derived insulins was widespread. Moreover, binding of iodine-131-labelled insulin to gamma-

globulin was demonstrated to be absent in exogenous insulin-naïve patients, but always present in subjects treated long-term with insulin [185].

Following repeated clinical observations of cutaneous and systemic allergy [295, 299], there followed extensive experimental work studying the antigenicity of insulin [300]. Commercial insulin preparations were found to be a heterogeneous mix of biologically active and inactive peptides [301–303] containing impurities such as pancreatic glucagon, pancreatic polypeptide, vasoactive intestinal polypeptide, and somatostatin, which were absent from highly-purified or monocomponent insulins available subsequently [304]. Although monocomponent insulin was associated with less immunogenicity, insulin allergy was still observed [305].

1.11.3 Insulin antibodies and antibody-mediated dysglycaemia in diabetes mellitus

In 1955, Berson et al postulated that fluctuations in antibody production may cause fluctuating insulin requirements in patients with so-called ‘brittle diabetes’ [185]. It was shown that patients requiring higher-dose insulin had higher insulin-binding capacities [235], however other studies did not suggest such an association [306], and indeed it was suggested that antibodies may benefit glucose control by acting as an insulin-buffer in plasma [216, 307]. For insulin-treated individuals with DM, IAs do not usually derange insulin kinetics to a clinically-significant degree [308–315]. However, some such antibodies may form reversible insulin–antibody complexes [185, 230–232], causing insulin resistance and exacerbating hyperglycaemia [316–319], with ketosis [320, 321] at one extreme, or, conversely, prolonged hypoglycaemia [316, 320–324] at the other. In some individuals, insulin sequestration by antibody may exist to such a degree that bioactive insulin is released from insulin–antibody complexes even days following cessation of insulin therapy [324, 325]. The prevalence of those insulin-binding antibodies that affect insulin kinetics and dynamics is not known.

Following the adoption of highly purified human insulin in clinical practice, the prevalence of antibodies associated with exogenous insulin use decreased [326–331], however detectable IAs in the plasma of individuals receiving exogenous insulin treatment remain common [308–311, 313, 315, 332]. Despite years of treatment with human insulin, patients previously treated with animal-derived insulin

have been shown to demonstrate higher anti-human insulin antibody levels compared with patients naïve to animal-derived insulins [332], and a decline in IAs following conversion from animal to human insulin may not be significant in the longer term [333]. It has been considered whether increasing insulin dosage could act as a stimulus to increase IA production [334], and consistent with this suggestion are reports of decreased antibody production and lower circulating insulin concentrations in T2DM following cessation of exogenous insulin [335, 336].

For rare patients with insulin-treated DM and disabling IA-mediated labile glycaemia, antibody-depletion therapy has been employed resulting in clinical benefit [316, 319, 322, 323]. Identifying such cases can be difficult, due to the varied presentation and the consideration of the more common situation of insulin manipulation. Even once insulin-binding antibodies are suggested from the clinical presentation, obtaining a definitive laboratory diagnosis can be also a challenge, and the optimal approach to investigate individuals suspected of suffering from IA-mediated dysglycaemia has not been established.

1.12 Severe insulin resistance

Insulin resistance may be defined as a subnormal glucose-lowering response to insulin and, in the context of normal pancreatic endocrine function, may result in increased insulin secretion and severe hyperinsulinaemia. Exogenous insulin resistance has been somewhat arbitrarily defined as a requirement of greater than 200 units of insulin per day for longer than two days [295, 337–339]. In 1976, Kahn et al described cases of severe insulin resistance and acanthosis nigricans as two distinct clinical subtypes: type A, who were younger, lean, female patients with hirsutism and accelerated early growth, for which an insulin receptor defect may be principally responsible; and type B, classically seen in older individuals with immunological disease who produce anti-insulin receptor antibodies [340]. ‘HAIR-AN’ is a generic collective term used for hyperandrogenism, insulin resistance, and acanthosis nigricans, and the description can encompass patients with obesity with a milder phenotype for which a single gene insulin receptor defect may not be identified [341].

1.13 Adiponectin: a plasma marker of insulin resistance

Adiponectin is expressed in white adipose tissue, and is secreted into plasma by adipocytes. The adipokine was first described in the 1990s [342–344] and to date, its physiological role is yet to be completely understood. Infusions of adiponectin [345], and transgenic overexpression of a mutant adiponectin species [346], improve insulin sensitivity in insulin resistant mice, which may indicate a role for adiponectin in some cases of insulin resistance. Studies have shown some consistent findings relating to the anthropometric and metabolic determinants of human adiponectin concentrations, which include the observed negative correlation with body mass index (BMI): circulating adiponectin concentrations are high in constitutionally-thin individuals and low in obese subjects [347, 348], however, adiponectin concentrations are lower in individuals with anorexia nervosa and bulimia nervosa [347]. Low adiponectin concentrations are associated with insulin resistance, hyperinsulinaemia, and T2DM [348–351], and very low concentrations are observed in patients with generalised lipodystrophy [352]. Contrasting this, in individuals with severe insulin resistance resulting from abnormal insulin receptor function [353], including TB-IR [354], very high adiponectin concentrations are observed.

The plasma concentrations of adiponectin in plasma from individuals with IAS has not been studied. Decreases in blood glucose (but also insulin) with fasting generally did not result in an increase in adiponectin concentration in healthy individuals [355]. Although adiponectin concentrations are higher in T1DM, which may suggest insulin deficiency plays a role [356–360], high adiponectin levels are not observed during fasting or in patients with anorexia nervosa [347, 355].

1.14 Type B insulin resistance syndrome

TB-IR is the clinical manifestation of pathogenic autoantibodies that bind the insulin cell surface receptor [361]. Initially described in middle-aged and younger female patients [340], and subsequently in males [362], it is a rare condition, however the exact prevalence of the disease is not known. It is a heterogeneous disorder and its core clinical features are manifestations of over-/under-stimulation of the insulin receptor, hirsutism, and/or virilisation in females, acanthosis nigricans (as a consequence of hyperinsulinaemia), and associated autoimmune disease, such as systemic lupus erythematosus or mixed connective tissue disease [363]. Antibodies are stimulatory at low titres, but downregulate and

inhibit the insulin receptor at high titres, therefore individuals present with hyperglycaemia and/or recurrent fasting or reactive hypoglycaemia, or severe insulin resistance, respectively [364]. Severe insulin resistance may be accompanied by features suggestive of deficient insulin action, such as hyperosmolar symptoms and rapid weight loss with ketosis [365, 366].

The best approach to treatment depends upon the principal clinical complaint and its severity at presentation. Anti-insulin receptor antibody-mediated dysglycaemia can be improved with antibody-depletion therapy [363], and successful immunodepletion therapies have been described using the combination of rituximab, dexamethasone, and cyclophosphamide [363], leading to an improvement in metabolic parameters and normalisation of testosterone [367]. To combat hyperglycaemia, exogenous insulin therapy may be used with varied effectiveness, and hypoglycaemia is combated with high-dose steroid therapy [368].

1.15 Subcutaneous insulin resistance syndrome

Labile diabetes is a term applied to glycaemic control in individuals with unpredictable insulin pharmacokinetics and/or pharmacodynamics that presents as recurrent hypoglycaemia and/or hyperglycaemia. In the face of high insulin doses, abnormal insulin absorption is sometimes considered as a cause of labile glycaemia in DM. The syndrome, often referred to as subcutaneous insulin resistance, is characterised by labile glycaemia observed at times when insulin is administered subcutaneously, with improved glycaemia when insulin is administered intravenously. When plasma insulin is measured in this context, concentrations appear inappropriately low for the subcutaneous insulin dose administered [369]. In 1979, Paulsen et al first reported subcutaneous insulin resistance in a patient in whom adipose tissue demonstrated a six-fold increase in insulin-degrading activity that underwent spontaneous remission [370]. In that report, the presenting condition was defined by three characteristics: firstly, high-dose subcutaneous insulin requirements (supported by the lack of demonstrable normal increase in plasma insulin), secondly, a good response to intravenous insulin (excessive anti-insulin and insulin receptor antibodies were excluded), and thirdly, increased insulin-degrading activity of adipose tissue. In clinical practice, subcutaneous insulin resistance is typically a diagnosis of exclusion rather than determined following demonstration of insulin sequestration or excessive insulin degradation in the

subcutaneous tissues [369]. In 1986, Duckworth and colleagues [371] set to investigate definitively the syndrome in 16 patients with a presumptive diagnosis of subcutaneous insulin resistance. From plasma free-insulin concentration profiles following insulin administration and subcutaneous insulin-degrading activity, none of the patients studied were observed to have an abnormal glucose response to insulin. The authors concluded that insulin resistance due to insulin degradation in the subcutaneous tissues was extremely rare and often misdiagnosed. Nevertheless, insulin sequestration in subcutaneous tissues has been reported [372], and topical protease inhibitor demonstrably improved subcutaneous insulin resistance by increasing insulin concentrations in plasma and increasing the glucose-lowering response [373].

Subcutaneous insulin resistance syndrome is a diagnosis sometimes considered for patients presenting with high subcutaneous insulin dose requirements. However, patients reporting high exogenous insulin requirements may not be taking the large doses which they are prescribed, and 'factitious resistance' may be unmasked following hospital admission, where patients are observed and insulin is administered by medical personnel [295].

1.16 Aims of the Project

Aims	Central hypotheses
1) To study insulin assay cross-reactivity with porcine insulin, bovine insulin, and various insulin analogues used in clinical practice	Commercial insulin assays cross-react in an equimolar fashion with all insulin therapies in plasma
2) To investigate the effect of insulin-binding antibodies on insulin immunoassays commonly used in clinical practice	Insulin immunoassay accurately detects insulin in patients with circulating IA
2a) To assess immunoassay linearity	Immunoassay plasma insulin concentration represents total insulin (antibody-bound plus free), therefore results will be linear to sample dilution with or without the presence of IA
2b) To compare immunoassay and MS insulin measurements of insulin	Immunoassay and MS detects total insulin (antibody-bound plus free), therefore immunoassay insulin concentration would be broadly similar to MS insulin concentration
2c) To investigate the effect of insulin-binding antibodies on C-peptide detection by immunoassay	Immunoassay plasma C-peptide immunoreactivity represents total C-peptide concentration and therefore would be broadly similar to MS C-peptide concentration
3) To develop novel, clinically-meaningful approaches to the assessment of insulin-binding antibodies	A single test, or streamlined panel of assays, could be used to diagnose and monitor IAS
3a) To assess of semi-quantitative IA ELISA	ELISA detection of IA could be used as a standalone test for IAS
3b) To compare quantitative ELISA and quantitative RIA IA results in a cohort of patients with IAS	ELISA or RIA quantification of IA could be used as a standalone test for IAS, and a sensitive and specific action limit could be defined for clinical purposes.
3c) To assess use of PEG precipitation	In the presence of IA in plasma, insulin recovery would be lower in PEG supernatant compared with that in supernatant of antibody-negative plasma.
3d) To develop a gel filtration chromatography (GFC) method to identify insulin-antibody complexes	GFC is the gold standard for detection of insulin-antibody complexes in plasma
3e) To use newly-developed novel diagnostic assays to assess IAs in individuals with labile insulin-treated DM	In an enriched population of insulin-treated patients with clinically unexplained erratic glycaemia, circulating IA would be commonly detectable, but that the panel of assays would exclude pathogenic insulin-binding antibodies as the principal cause of labile diabetes in most patients
3f) To study a cohort of patients with insulin-binding autoantibodies, examining burden of disease and response to immunomodulation	Assay results will provide information to explain clinical presentation and allow prediction of success of treatment intervention
4) To develop a novel ELISA method for determination of anti-insulin receptor antibodies	The novel ELISA will provide results which are as/more useful than the current gold standard Western blot assay, and the ELISA will be less laborious, allowing shorter turn-around times.

CHAPTER 2: Materials and methods

2.1 Detection of insulin analogues in plasma using immunoassay

The cross-reactivity of ten insulin preparations were tested: human (Actrapid[®], Humulin[®] S), aspart (NovoRapid[®]), porcine (Hypurin[®] porcine neutral), bovine (Hypurin[®] bovine neutral), lispro (Humalog[®]), glulisine (Apidra[®]), glargine (Lantus[®]), detemir (Levemir[®]), and degludec (Tresiba[®]). Samples were prepared by Dr Timothy McDonald's group (Blood Sciences, Royal Devon and Exeter NHS Foundation Trust, UK; NIHR Exeter Clinical Research Facility, University of Exeter, UK). Serum was collected from healthy fasted volunteers, pooled, and stored at 4°C for up to forty-eight hours, before exogenous insulin was added. Insulin preparations were extracted from their original vials and diluted to 10 nmol/L using 40 g/L BSA, then diluted with the above serum to a final calculated concentration of 1000 pmol/L and 300 pmol/L. Pooled serum (without addition of exogenous insulin) was used as a blank. Samples were divided into aliquots and stored frozen at -80°C. Samples were sent on dry ice to clinical laboratories for insulin immunoassay analysis using the assays listed in Section 2.7.

2.2 Anti-insulin antibody measurement using semi-quantitative enzyme-linked immunosorbent assay

IA measurement was performed by the SAS Peptide Hormone Section, Clinical Laboratory, Royal Surrey County Hospital, UK. Serum anti-insulin IgG was measured using the Isletest[™]-IAA (Biomercia, USA), a semi-quantitative ELISA. This assay was verified for clinical use.

2.3 Anti-insulin antibody measurement using semi-quantitative enzyme-linked immunosorbent assay

Quantitative measurement of anti-insulin IgG was performed using an in-house human insulin-specific ImmunoCAP[®] ELISA (Figure 5.1), an assay developed verified for clinical use by the Department of Immunology, Cambridge University Hospitals NHS Foundation Trust, Cambridge, UK.

2.4 Determination of anti-insulin IgG concentration by radioimmunoassay

Serum was assayed using the RiaRSRTM IAA assay, at Viapath Analytics, Clinical Immunology and Allergy Department, King's College Hospital. In this assay, serum is incubated with iodine-125-(A14)-monoiodinated insulin, before labelled insulin-antibody complexes are precipitated using anti-human IgG. The amount of radioactivity in the precipitate, which is proportional to the concentration of insulin IgG in the sample, is compared with calibrators to determine a concentration value.

2.5 Competitive anti-insulin antibody radioimmunoassays

The radioimmunoassay methods were designed in close collaboration with Claire Williams and supervisor Alistair Williams, Diabetes & Metabolism, Translational Health Sciences, University of Bristol, Southmead Hospital, Bristol, UK, and all experiments directly involving radioimmunoassay were carried out by Claire Williams and Alistair Williams. Using an RIA currently used clinically to measure IAs [374, 375], quantification of IA was undertaken. Patient serum, 5 μ L neat, or diluted with IA-negative serum, was incubated for 72 hours with 3.75 fmol A14-iodine-125-labelled human insulin (PerkinElmer[®]; diluted using 50 mmol/L Tris, 1% (v/v) Tween-20, pH 8.0 (TBT) buffer containing 1% (w/v) BSA (Sigma-Aldrich[®])) with or without unlabelled synthetic human insulin at 40 μ mol/L in a 96-well plate. A14-iodine-125-labelled human insulin-IA complexes were precipitated using 10 μ L glycine-blocked Protein A-Sepharose[®] (PAS), and then experiments performed using ethanolamine-blocked Protein G-Sepharose[®] (PGS) (GE Healthcare, Buckinghamshire, UK) [376] and/or anti-IgA agarose-antibody (Sigma-Aldrich[®]) in 50 μ L TBT. Following washing to remove unbound label, bound A14-iodine-125-labelled insulin was measured with a gamma counter. A standard curve was constructed using a serial dilution of pooled IA-positive human sera, and patient results were calculated as arbitrary units using a logarithmic curve fit. A positive result in the PAS assay was defined as >0.2 AU, a threshold determined the 97.5th percentile of 2860 healthy children [377]. This assay achieved 52% sensitivity at 97.8% specificity in the 2015 Islet Autoantibody Standardization Program (IASP) workshop, and was verified for clinical use.

2.6 Anti-insulin antibody affinity determination using radioimmunoassay

Serum was analysed for IA affinity using a PAS radioligand binding assay [377, 378]. Samples were diluted in IA-negative sera (ratios of sample:IA-negative serum were 1:9, 1:49, and 1:99), based on the binding demonstrated in the PAS RIA described in Section 2.5, to improve discrimination between samples. In preparation for the IA affinity assays, samples were incubated with A14-iodine-125-labelled human insulin alone, or with a range of concentrations of soluble human insulin (Actrapid®) at 5.5×10^{-11} , 2.2×10^{-10} , 1.7×10^{-9} , 7×10^{-9} , 2.8×10^{-8} , 2.8×10^{-7} , and 4.0×10^{-5} mol/L for 72 hours. Insulin dilutions were made using TBT buffer containing 1% (w/v) BSA. To detect all possible IA-reactive IgG autoantibodies based on the standard PAS assay results, insulin-antibody complexes were precipitated and measured with a 50:50 mixture of PAS and PGS to include all possible IA-reactive IgG antibodies. Due to limited sample volume, standard curves were not made, but three IA-positive serum samples were used as intermediate, and high affinity, antibody controls. Antibody binding was determined by radioactivity detection with a gamma counter. Using non-linear regression analysis (GraphPad Prism6, GraphPad Software Inc., San Diego, CA, USA), inhibition concentration at 50% (IC₅₀), Kd calculations (mol/L) and reciprocal Kd (L/mol) were calculated. Antibodies were characterised according to Kd (mol/L), calculated using a one-site model [377].

2.7 Insulin immunoassays

Quantitative insulin measurements were undertaken using commercially-available insulin immunoassays, namely DiaSorin LIAISON® XL (Saluggia, Italy), PerkinElmer® (PE) AutoDELFIA® (Coventry, UK), Siemens ADVIA Centaur® (Surrey, UK), and Siemens IMMULITE® 2000, Abbott ARCHITECT (Illinois, USA), Beckman Access® Ultrasensitive (High Wycombe, UK), Mercodia Insulin (Uppsala, Sweden), Mercodia Iso-Insulin, and Roche Elecsys® (Cobas®) (Rotkreuz, Switzerland). All assays were verified for clinical use.

2.8 C-peptide immunoassays

Quantitative insulin measurements were undertaken using commercially-available insulin immunoassays, namely DiaSorin LIAISON[®] XL, Siemens IMMULITE[®] 2000, Abbott ARCHITECT, and Mercodia C-peptide ELISA. All assays were verified for clinical use.

2.9 Adiponectin measurement

Plasma adiponectin concentration was made using an in-house solid-phase, two-site dissociation-enhanced lanthanide fluorescence immunoassay (DELFI[®]A). This assay was verified for clinical use.

2.10 Assays of immunoglobulin and albumin

Measurements of IgA, IgM, IgG, and albumin concentration were performed using the Siemens ADVIA 2400[®] (Siemens). All assays were verified for clinical use.

2.11 Quantitative mass spectrometric measurement of plasma insulin and C-peptide

Methods were designed in close collaboration with Dr Richard Kay, The University of Cambridge Metabolic Research Laboratories, Wellcome Trust-MRC Institute of Metabolic Science, Cambridge, UK, and experiments directly involving mass spectrometry were carried out by Richard Kay. Human plasma was thawed from frozen storage and enriched with insulin lispro (Humalog[®]) and C-peptide (Bachem, Bubendorf, Switzerland) to generate final peptide concentrations of 8610 pmol/L and 16,548 pmol/L, respectively. To generate a standard curve, plasma was diluted in the same pooled plasma to generate insulin concentrations of 6890, 1720, 861, 172, 86, 34, and 17, pmol/L and paired C-peptide concentrations of 13245, 3307, 1655, 331, 165, 65, and 33 pmol/L, respectively. Each sample of known peptide concentration, patient plasma, and blank (non-fortified) pooled plasma were transferred, at a volume of 250 μ L to a 96-well plate. Patient, and control samples were extracted with quality control (QC) material and calibration samples. Protein precipitation solvent (80% (v/v) acetonitrile in water, with 1 ng/mL bovine insulin (Sigma-Aldrich[®])), at a volume of 1 mL was added,

followed by thorough mixing to precipitate plasma proteins, with subsequent centrifugation at 2900 *g* for 10 minutes at 4°C. Supernatant was transferred to a 96-well plate (Eppendorf™ Protein LoBind Deepwell™ Fisher Scientific, Loughborough, UK), and evaporated under a stream of nitrogen gas at 45°C. To reconstitute the residue, 200 µL of 0.1% (v/v) formic acid was added, then samples loaded directly onto a plate (Oasis® PRiME HLB µElution, Waters, Elstree, UK) that was then transferred to a positive-pressure solid-phase extraction manifold (Waters), and the solutions passed through the sorbent slowly under pressure. Cartridges were washed with 200 µL of 0.1% (v/v) formic acid, followed by 200 µL of 5% (v/v) methanol with 1% (v/v) acetic acid. The peptides were then eluted with 2 x 30 µL of 60% (v/v) methanol with 10% (v/v) acetic acid in water. Prior to injecting 50 µL sample onto the liquid chromatography-mass spectrometry (LC-MS) system, 75 µL of 0.1% (v/v) formic acid was added. At a flow rate of 300 µL per minute (UltiMate™ 3000 system, Thermo Fisher Scientific, Hemel Hempstead, UK), peptides were loaded onto a 50 x 2.1 mm reverse-phase column (AQUITY UPLC® HSS T3 C₁₈, Waters). Starting conditions were 22% B (0.1% ACN with 0.1% formic acid v/v) and 78% A (0.1% formic acid in water v/v), then B was increased to 32% after 6.4 minutes. Before returning to starting conditions for the duration of 2 minutes, the column was washed at 90% B for 1.6 minutes.

MS was performed using a Q Exactive Plus Orbitrap system (Thermo Fisher Scientific) using a heated electrospray ionisation source (positive electrospray mode), with a needle voltage of 3 kV, gas flow rates of 55 and 10 for sheath gas and aux gas. The aux gas was set to a temperature of 350°C, the transfer capillary to 350°C, and an s-lens value set to 70V. MS data were acquired from *m/z* 700-1600, with a resolution of 70,000, and an automatic gain control target of 3e6 ions. Using the standards described above, insulin and C-peptide calibration curves were generated using *m/z* values for the [M+5H]⁵⁺ charge states relating to the monoisotopic and multiple ¹³C isotopes of human insulin (1161.7362), and for the [M+3H]³⁺ charge state of C-peptide (1007.1783). After correcting for endogenous analyte, calibration curves for insulin and C-peptide gave a linear fit with R² values of 0.995 and 0.994, respectively, and calibration standards and QC samples were all within ±25% of expected values. Regression between immunoassay and MS control plasma values were linear for insulin (0.8727x-27.025; R²=0.974), and C-peptide (1.317x-56.86; R² = 0.997).

2.12 Plasma dilution (insulin immunoassay linearity) studies

Insulin was measured using immunoassay in neat plasma, and then following a plasma:diluent dilution of 1:4, and in parallel 1:49 where stated, with assay-specific diluent, and initial insulin concentration back-calculated using the dilution factor.

2.13 Polyethylene glycol precipitation of plasma

A 25% (w/v) solution of PEG 6000 (BDH Prolabo, UK) was prepared using deionised water. 0.9% (w/v) saline was prepared using 18% (w/v) sodium chloride solution (BDH Prolabo) and deionised water. Samples were diluted in a ratio 1:1 with 25% (w/v) PEG, mixed for 10 seconds using a vortex, then centrifuged at 13,200 *g* for 15 minutes.

Analyte was measured in PEG supernatant, except when a sampling error was reported by the assay platform (likely triggered by the viscosity of PEG supernatant), in which case, PEG supernatant was diluted in saline prior to analysis. Matched dilutions of plasma using saline were used as control.

2.14 Immunosubtraction using class-specific anti-human immunoglobulin–agarose

Anti-human IgA–agarose antibody, anti-human IgM–agarose antibody, and anti-human IgG–agarose antibody, and Protein G (immunoglobulin (IgG)-binding bacterial cell wall protein isolated from group G streptococcal strain)–Sepharose[®] (Fast Flow, P3296, Sigma-Aldrich[®]) were each washed three times with 0.9% (w/v) saline and stored at 4°C until use. Plasma:agarose ratios were based on in-house data [379], and the same dilutions of plasma were made in saline to act as control for insulin recovery calculation. Volume ratios of plasma:agarose antibody were as follows: anti-human IgA–agarose antibody 5:1 with plasma; anti-human IgM–agarose antibody 29:20 with plasma; anti-human IgG–agarose antibody 32:3 with plasma; Protein G–Sepharose[®] 29:20 with plasma. Using the same samples, equal mixtures were made with saline for use as control matrix. Samples were then mixed on a tube roller mixer for 60 minutes prior to centrifugation at 13,200 *g* for 15 minutes. To overcome sampling error on the DiaSorin LIAISON[®] XL (likely caused by increased sample viscosity), agarose supernatant was diluted at a ratio of sample 4:1 with saline prior to analysis.

2.15 Gel filtration chromatography of plasma

The GFC protocol for the separation of insulin species was modified and optimised, from an existing GFC standard operating procedure for macroprolactin [380]. An ÄKTAprime plus liquid chromatography system (GE Healthcare) was used in conjunction with a HiLoad 16/60 Superdex 75 (120 mL) size exclusion column (GE Healthcare) and a laptop (Latitude D620, Dell, Texas, USA; PrimeView 5.0, Amersham Bioscience, Buckinghamshire, UK, 2004). The equipment was set-up and calibrated in accordance with the manufacturer's instructions. 500 μ L of sample was loaded onto the column in combination with a 25 mmol/L Tris/0.52 mol/L NaCl buffer mobile phase at pH 7.4, with a flow rate of 1 mL/min.

Six millilitre elution volume fractions with 1 mL BSA (final volume 7 mL, calculated BSA concentration 40 g/L) were collected in polypropylene tubes (Cellstar[®], Greiner Bio-One). 36–114 mL eluted volume was collected. Insulin analysis was performed using the DiaSorin LIAISON[®] XL.

2.16 Gel filtration chromatography with *ex vivo* insulin addition

990 μ L of neat plasma was mixed with 10 μ L of human insulin/synthetic insulin analogue of the desired concentration. The samples were incubated on a roller mixer at 24°C for 24 hours before being eluted through the GFC protocol described in Section 2.15, in parallel with samples prior to exogenous insulin addition.

2.17 Column recovery of blue dextran

Blue dextran 2000 (Amersham Bioscience) solutions of 5 mg/mL and 0.25 mg/mL were prepared in GFC buffer. 38 mL was run to waste, and volume 38–58 mL was collected in a polypropylene tube (Centrifuge Tubes, CELLSTAR[®], Greiner Bio-One). The collected eluted volume was scanned from 800 nm to 200 nm (Cary 100 Bio UV-Visible Spectrophotometer; Cary WinUV scan application Software, version 3.00). Based on peak absorbance, absorbance was then measured at 610 nm.

2.18 Effect of bovine serum albumin on insulin recovery in gel filtration chromatography buffer

Human insulin (rDNA, Actrapid[®]) was diluted four times into 2 mL plastic tubes (Micro tube, Sarstedt, Nümbrech, Germany) in series: the first two dilutions were in saline followed by a further dilution into GFC buffer, and a final dilution in GFC buffer containing bovine serum albumin (BSA, Sigma-Aldrich[®], Dorset, UK) at a calculated concentration of 0 g/L, 5 g/L, 10 g/L, 20 g/L, or 40 g/L. Samples were stored at -80°C overnight prior to analysis. Samples were analysed for insulin using immunoassay.

2.19 Effect of vessel material, freeze-thaw cycle, and bovine serum albumin on insulin recovery in gel filtration chromatography buffer

Human insulin (rDNA, Actrapid[®], Novo Nordisk) was diluted into three plastic 2 mL (Micro tube, Sarstedt) tubes before further dilution into a fourth tube, either plastic (Sarstedt tube, as above) or glass (test tube medium wall rimless borosilicate glass, grade 3.3 wall thickness 1.0 mm, ISO 4142 75 mm x 12 mm, Fisherbrand[®], Thermo Fisher Scientific; covered in Parafilm PM996 Wrap, Cole-Parmer[®], London, UK). The dilutions were performed in series: the first two dilutions were in 0.9% (w/v) saline, followed by a further dilution into GFC buffer, either neat or with 40 g/L BSA. The calculated final insulin concentration added was 362 pmol/L. The samples were stored for 12 hours at either +4°C or -80°C. The samples were then warmed to room temperature prior to analysis. Measurement of insulin was then performed using immunoassay. Two separate preparations of insulin were measured in singleton in GFC buffer with BSA 40 g/L (without addition of human insulin).

2.20 Serum protein electrophoresis

Protein electrophoresis of patient B30 serum was performed at the Department of Immunology, Cambridge University Hospitals NHS Foundation Trust, using the Helena V8 Nexus capillary zone

electrophoresis (Helena Biosciences, Tyne and Wear, UK) method. This assay was verified for clinical use.

2.21 Culture of CHO Flp-IN cells, stably expressing C-terminal myc-tagged human insulin receptor

The cell culture protocol was designed with close collaboration with Gemma Brierley, Research Associate, Department of Clinical Biochemistry, WT-MRC Institute of Metabolic Science; and cell culture experiments were performed under the supervision of Rachel Knox, Research Assistant, The University of Cambridge Metabolic Research Laboratories. CHO Flp-IN cells, stably expressing C-terminal myc-tagged human insulin receptor (CHO Flp-IN hINSR WT; generated and kindly donated by Gemma Brierley) were maintained at 37°C in a humidified incubator in F-12 Ham nutrient mixture supplemented with 10% (w/v) foetal bovine serum (FBS), 1,000 U/L penicillin, 0.1 g/L streptomycin, and 4 mmol/L L-glutamine (Sigma-Aldrich®). Hygromycin B (Thermo Fisher Scientific) at a concentration of 200 µg/mL was used to continue selection for insulin receptor-expressing cells, as the expression cassette contains the hygromycin B phosphotransferase (hph) resistance gene.

After long-term cryostorage of cells in 90% (w/v) FBS and 10% dimethyl sulfoxide (v/v), cells were thawed rapidly in a water bath at 37°C, added to 20 mL of the above media in a T75 flask (omitting hygromycin B for the initial recovery passage), and grown until confluence two days later. Cells were then passaged by removing media and washing with warmed phosphate-buffered saline (PBS, Sigma-Aldrich®), prior to adding 3 mL 1x trypsin-EDTA (Sigma-Aldrich®) and incubating at 37°C/5%CO₂ for five minutes. Cells were then visualised under the microscope to ensure all cells had detached from the flask base, and cells were transferred into five new flasks at a split ratio of 1:7 with the media described above. Upon confluence two days later, cells were harvested by removing the media, washing twice with PBS, and snap-freezing in liquid nitrogen. The flasks were stored at –80 °C until use.

2.22 Preparation of cell lysate

The flasks containing snap-frozen confluent CHO Flp-IN hINSR WT cell monolayers, were defrosted on wet ice. To each, 12 mL chilled lysis buffer (Table 2.1) was added, ensuring full and even

coverage of the monolayer, and the flasks were incubated at 4°C for one hour to allow chemical lysis and solubilisation of the cell membrane. Cells were then scraped, collected into 15 mL falcon tubes, and centrifuged at 4,000 g for 15 minutes at 4°C. The cell debris pellet was discarded, and the cleared supernatants of lysate were combined and stored in 2 mL aliquots at –80°C until required to prepare assay plates.

2.23 Insulin receptor antibody enzyme-linked immunosorbent assay

ELISA experiments were carried out with benchwork assistance from Cornelia Gewert, Research Associate, The University of Cambridge Metabolic Research Laboratories. A schematic representation of the insulin receptor antibody ELISA design is given in Figure 7.1. The assay was performed as follows:

On day one, anti-myc antibody (Millipore, Clone 9E10) was diluted to a concentration of 2.5 µg/mL in 100 mmol/L bicarbonate/carbonate buffer pH 9.6. A LUMITRAC™ 96-well white microplate (Greiner Bio-One International) was coated with 100 µL/well anti-myc antibody solution (250 ng/well) and incubated overnight at 4 °C.

On day two, the microplate wells were washed three times with 200 µL/well of Tris-buffered saline with 1% (v/v) Tween®20 (TBST), and emptied, before the addition of 200 µL of blocking solution (2% w/v BSA/TBST). The plate was incubated for 2 hours at 23°C. Microplate wells were then washed three times with 200 µL/well of TBST, emptied, and 100 µL/well of either neat CHO-WT-hINSR-myc cell lysate, or blocking solution, was added to each well. The plate was incubated overnight at 4°C.

On day 3, microplate wells were washed three times with 200 µL/well of TBST. Sample was then added, 100 µL/well, and the plate incubated for 2 hours at 23°C. Each sample was analysed in duplicate. Microplate wells were then washed three times with 200 µL/well of TBST, emptied, and incubated in 100 µL/well of specific IgG conjugate (β-galactosidase–anti-IgG (approximately 1 µg/mL) mouse monoclonal antibody, Thermo Fisher Scientific) for 30 minutes at 23°C. Microplate wells were then washed three times with 200 µL/well of TBST, emptied, and incubated in 100 µL/well of

Development Solution (4-methylumbelliferyl- β -galactoside 0.01%, Thermo Fisher Scientific) for 9 minutes at 23°C. Then, 100 μ L/well of Stop Solution (Sodium carbonate 4% (alkaline), Thermo Fisher Scientific) was added, and the plate incubated for 9 minutes at 23°C. Fluorescence was then measured, at an excitation wavelength of 365 nm and an emission wavelength of 455 nm, using a microplate reader (Infinite[®] M1000 PRO, Tecan, Männedorf, Switzerland).

Table 2.1 CHO cell lysis buffer components

Component	Concentration	Weight/volume for 500 mL	Company	Comments
HEPES	20 mmol/L	2.383 g	Sigma-Aldrich [®]	EGTA was added first and pH adjusted to 8 to allow it to fully dissolve. Other components were then added, dissolved and the pH was adjusted to 7.5.
NaCl	150 mmol/L	4.383 g	Sigma-Aldrich [®]	
MgCl ₂	1.5 mmol/L	0.071 g	Sigma-Aldrich [®]	
Glycerol	10% v/v	50 mL	Sigma-Aldrich [®]	
Triton X	1% v/v	5 mL	Sigma-Aldrich [®]	
EGTA	1 mmol/L	0.1902 g	Sigma-Aldrich [®]	
Phenylmethylsulfonyl fluoride (PMSF)	1 mmol/L	-	Sigma-Aldrich [®]	A stock solution at 100mmol/L was prepared in isopropanol and stored at -20°C. 500 μ L was added fresh on day of lysis to a 50 mL aliquot of stock lysis buffer.
Complete EDTA-free protease inhibitor tablets	1 tablet per 10-15 mL	-	Sigma-Aldrich [®]	4 tablets added fresh on day of lysis to a 50 mL aliquot of stock lysis buffer.
Sodium orthovanadate (Na ₃ VO ₄)	2 mmol/L	-	Sigma-Aldrich [®]	A stock solution at 200mmol/L was prepared and stored at -20°C. 500 μ L was added fresh on day of lysis to a 50 mL aliquot of stock lysis buffer.

CHAPTER 3: Detection of insulin analogues in plasma using immunoassay

3.1 Background

In the investigation of recurrent unexplained hypoglycaemia, insulin measurements are made to confirm whether low blood glucose can be attributed to inappropriately raised (non-suppressed) insulin production. Incidents of deliberate insulin self-overdose and poisoning have been reported since the 1950s [381], and an understanding of immunoassay specificity is required to interpret insulin results appropriately from such cases [210]. When insulin measurements are used in the investigation of unexplained hypoglycaemia, accurate detection of animal-derived insulin and insulin analogue is required before exogenous insulin can be excluded as a cause.

It is almost a century since exogenous insulin therapy was first used to treat an individual with DM. Thereafter followed large-scale production of animal-derived insulin that led to widespread availability of insulin therapy. After the later introduction of synthetic human insulin, new insulin analogue preparations have been, and continue to be, developed. Insulin analogues possess structural differences from native human insulin that are preparation-specific, and these modifications may include amino acid substitutions with/without the addition of fatty acid side chains designed to alter insulin pharmacokinetics and/or pharmacodynamics (Section 1.5.4). Concurrent to the increased use of insulin analogue preparations in clinical practice was the development of insulin immunoassays exhibiting improved specificity for native human insulin detection (Figure 1.5). Cross-reactivity data are not always provided by assay manufacturers, however data have been published showing insulin analogue detection by modern day clinical insulin immunoassay is variable [204–207].

Insulin determination is also of value in clinical contexts other than hypoglycaemia, for example when confirming insulin resistance, when detection of an unequivocally-high plasma insulin concentration is often sought. In addition to such cases, marked hyperinsulinaemia (whether endogenous or exogenous) may be demonstrable when insulin action is disturbed by the presence of antibodies that bind either insulin itself or the insulin cell surface receptor (see sections 1.10 and 1.14, respectively).

Confirming the presence of hyperinsulinaemia (by measurement of endogenous and exogenous insulin) is instructive in these contexts, following which, further laboratory investigations may be undertaken.

Previous published studies of assay cross-reactivity have limitations, including failure to provide cross-comparison of current commonly-used clinical insulin assays (as variation in methodologies restrict direct comparison of different study results), and failure to study animal-derived and/or insulin analogues recently introduced into clinical practice (e.g. insulin degludec). In this chapter, to test the hypothesis that commercial insulin assays cross-react in an equimolar fashion with all insulin therapies in plasma, the ability of six clinical insulin immunoassays to detect animal-derived insulin, synthetic human insulin, and insulin analogue in plasma was examined.

3.2 Materials and methods

The cross-reactivity of ten insulin preparations were tested: human (Actrapid[®], Humulin[®] S), aspart (NovoRapid[®]), porcine (Hypurin[®] porcine neutral), bovine (Hypurin[®] bovine neutral), lispro (Humalog[®]), glulisine (Apidra[®]), glargine (Lantus[®]), detemir (Levemir[®]), and degludec (Tresiba[®]). Samples were prepared by Dr Timothy McDonald's group (Blood Sciences, Royal Devon and Exeter Foundation Trust, UK; NIHR Exeter Clinical Research Facility, University of Exeter, UK). Serum was collected from healthy fasted volunteers, pooled, and stored at 4°C for up to forty-eight hours before exogenous insulin was added. Insulin preparations were extracted from their original vials and diluted to 10 nmol/L using 40 g/L BSA, then diluted with the above serum to a final calculated concentration of 1000 pmol/L and 300 pmol/L. Pooled serum (without addition of exogenous insulin) was used as a blank. Samples were divided into aliquots and stored frozen at -80°C. Samples were sent on dry ice to clinical laboratories for analysis. Laboratories were blinded to sample contents.

Samples were analysed in singleton for insulin in Cambridge using the DiaSorin LIAISON[®] XL, PerkinElmer[®] (PE) AutoDELFIA[®], Siemens ADVIA Centaur[®], and Siemens IMMULITE[®] 2000. Samples were also analysed at clinical laboratories elsewhere, using the Abbott ARCHITECT, Beckman Access[®] Ultrasensitive, Mercodia Insulin, Mercodia Iso-Insulin, and Roche Elecsys[®]. Each method, as stated in the assay kit/calibrator documentation provided by the manufacturer, was referenced to the 1st WHO International Reference Preparation 66/304, a lyophilised impure preparation of human insulin [382]. Each assay was subject to local verification and sera were analysed using the same protocol as clinical samples. Results were reported to Dr Timothy McDonald's group for analysis. Each result was blank-corrected against the insulin concentration value obtained from analysis of blank pooled serum. Insulin recovery was calculated as the mean percentage recovery at 1000 pmol/L and 300 pmol/L. Cross-reactivity was categorised arbitrarily as poor/no cross-reactivity if <20%, moderate if 20–79%, and good if >80%.

3.3 Results

Insulin immunoassays were two-site, non-competitive, and differed in both design and measurable range (Table 3.1).

3.3.1 Cross-reactivity of human insulins

Performance of different assays across a panel of insulins is summarised in Table 3.1. Synthetic human insulin (Actrapid[®], Humulin[®] S) demonstrated $\geq 80\%$ cross-reactivity in all assays tested, with the exception of the Beckman Access[®] that demonstrated cross-reactivity of 79% with Actrapid[®] (however 103% with Humulin[®] S), and the Siemens IMMULITE[®] 2000 that demonstrated cross-reactivity of 66% and 74% with Actrapid[®] and Humulin[®] S, respectively. Insulin recovery varied by more than 60% across the assays.

3.3.2 Cross-reactivity of porcine and bovine insulins

Insulin detection differed across assays. Porcine insulin, which has one B chain amino acid difference from human insulin (Table 1.1), demonstrated good cross-reactivity using the Abbott ARCHITECT, DiaSorin LIAISON[®] XL, Mercodia Insulin, Mercodia Iso-Insulin, PE AutoDELFI[®]A, and Siemens ADVIA Centaur[®]; moderate cross-reactivity using the Beckman Access[®] Ultrasensitive; and poor cross-reactivity using the Roche Elecsys[®], and Siemens IMMULITE[®] 2000. Bovine insulin, which has three amino acid differences, that include two on the A chain and one on the B chain, from human insulin (Table 1.1), demonstrated good cross-reactivity using the DiaSorin LIAISON[®] XL, and Mercodia Iso-Insulin; moderate cross-reactivity using the Abbott ARCHITECT, Beckman Access[®] Ultrasensitive, Mercodia Insulin, and PE AutoDELFI[®]A; and poor using the Roche Elecsys[®], Siemens ADVIA Centaur[®], and Siemens IMMULITE[®] 2000.

Table 3.1 Insulin assay characteristics

Assay	Principle	Capture Antibody	Detection Antibody	Sensitivity (pmol/L)	Assay range (pmol/L)
Abbott ARCHITECT	One-step chemiluminescent microparticle immunoassay	Mouse monoclonal anti-human insulin (coated paramagnetic microparticles)	Mouse monoclonal acridinium-labelled anti-human insulin antibody	7.2	2152
Beckman Access® Ultrasensitive	Simultaneous one-step immune-enzymatic assay	Mouse monoclonal IA bound to para-magnetic particles	Mouse monoclonal anti-insulin alkaline phosphatase conjugate	0.21	2100
DiaSorin LIAISON® XL	Two-site chemi-luminescent immunoassay	Mouse monoclonal antibody coated onto magnetic particles	Monoclonal antibody linked to an isoluminol derivative	1.18– 3.47*	3473*
Mercodia Insulin	Solid-phase two-site colorimetric enzyme immunoassay	Mouse monoclonal coated plate	Peroxidase conjugated mouse monoclonal anti-insulin	6	1200
Mercodia Iso-Insulin ELISA	Solid-phase two-site colorimetric enzyme immunoassay	Mouse monoclonal coated plate	Monoclonal IA conjugated to peroxidase	12	600
PE AutoDELTA®	Solid-phase, two-site fluorometric assay	Mouse monoclonal anti-insulin microtitration strips	Mouse monoclonal anti-insulin- Eu tracer	3	1080
Roche Elecsys®	Two-site electrochemiluminescence immunoassay	Biotinylated mouse monoclonal IA (streptavidin-coated microparticles)	Mouse monoclonal IA-labelled with ruthenium complex	1.39	6945
Siemens ADVIA Centaur®	Two-site chemi-luminescent immunoassay	Monoclonal mouse IA covalently couples to paramagnetic particles	Monoclonal mouse IA labelled with acridinium ester	3.5*	2084*
Siemens IMMULITE® 2000	Solid-phase enzyme-labelled chemi-luminescent immunometric assay	Monoclonal murine IA-coated bead	Polyclonal sheep IA conjugated to alkaline phosphatase (bovine intestinal calf) and monoclonal murine IA conjugated to alkaline phosphatase (bovine calf intestine)	14.4	2165

*Conversion factor 1 µIU/ml:6.945 pmol/L [383] where needed. Otherwise values taken directly from manufacturers' instructions. Manufacturers' reported sensitivity and assay range for human insulin given.

Table 3.2 Insulin preparation cross-reactivity in insulin immunoassays

Assay	Mean insulin recovery (1000 pmol/L and 300 pmol/L; %)											
	Human insulin		1 amino acid difference			2 amino acid differences		3 amino acid differences			Fatty acid side chain	
	Actrapid®	Humulin® S	Aspart (NovoRapid®)	Porcine (Hypurin® porcine neutral)	Lispro (Humalog®)	Glulisine (Apidra®)	Bovine (Hypurin® bovine neutral)	Glargine (Lantus®)	Detemir (Levemir®)	Degludec (Tresiba®)		
Abbott ARCHITECT	108	124	110	90	129	10	27	140	30	24		
Beckman Access® Ultrasensitive	79	103	108	67	98	2	26	124	1	3		
DiaSorin LIAISON® XL	104	124	2	128	1	0	88	16	1	1		
Mercodia Insulin	95	113	0	116	0	0	62	7	0	0		
Mercodia Iso-Insulin ELISA	140	140	140	108	139	140	95	93	46	44		
AutoDELFIA®	93	115	1	140	1	1	55	10	1	1		
Roche Elecsys®	107	134	0	19	0	0	19	20	0	4		
Siemens ADVIA Centaur®	120	140	140	137	140	0	0	140	24	13		
Siemens IMMULITE® 2000	66	74	12	16	10	0	19	54	8	2		

Cross-reactivity calculated as the mean percentage recovery of spiked insulin at 1000 pmol/L and 300 pmol/L. Cross-reactivity values were truncated at >140%. Cross-reactivity categorised as poor/no cross-reactivity if <20% (■), moderate if 20–79% (■), and good if >80% (■).

3.3.3 Cross-reactivity of synthetic insulin analogues with substituted amino acids only

Insulin analogue detection differed across assays. DiaSorin LIAISON[®] XL, Mercodia Insulin, PE AutoDELFI[®], Roche Elecsys[®], and Siemens IMMULITE[®] 2000, demonstrated higher specificity for human insulin than insulins aspart, lispro, glulisine, and glargine. The Mercodia Iso-Insulin assay demonstrated broad specificity, with the highest cross-reactivity across all insulin analogues tested. With the exception of the Mercodia Iso-Insulin assay, glulisine, which is homologous to human insulin but for two amino acid substitutions on the B chain (Table 1.1), demonstrated poor cross-reactivity across all assays tested.

3.3.4 Cross-reactivity of synthetic insulin analogues including a fatty acid moiety

Insulins detemir and degludec have an amino acid omission at B30, and addition of a fatty acid side chain (Table 1.1). With the exception of the Abbott ARCHITECT, Mercodia Iso-Insulin assay, and Siemens ADVIA Centaur[®], which showed moderate cross-reactivity, detemir demonstrated poor cross-reactivity across all assays tested. For degludec poor cross-reactivity was seen across all assays tested except the Abbott ARCHITECT and Mercodia Iso-Insulin assay which demonstrated moderate cross-reactivity.

3.4 Discussion

Immunoassays tested demonstrated good recovery of human insulin (although somewhat lower for the Siemens IMMULITE[®] 2000), however varied in cross-reactivity with animal-derived insulin and insulin analogue. Commercial insulin assays did not cross-react in an equimolar fashion with all insulin therapies in plasma, and cross-reactivity characteristics were assay-dependent, whilst for some insulin analogues, such as detemir and degludec, cross-reactivity was moderate or poor across all assays. Certain analogues, such as aspart, had good cross-reactivity in some assays (Abbott ARCHITECT, Beckman Access[®] Ultrasensitive, Mercodia Iso-Insulin, and Siemens ADVIA Centaur[®]), and poor cross-reactivity in the others (DiaSorin LIAISON[®] XL, Mercodia Insulin, PE AutoDELFI[®], Roche Elecsys[®], and Siemens IMMULITE[®] 2000). This suggests that the single amino acid alteration of aspart (at position B28) to native human insulin (Table 1.1) can result in loss of detection, and this is likely a consequence of distortion/loss of the normal binding site of the assay antibodies to native insulin. Although information detailing assay antibodies and/or antibody epitopes are not generally available from manufacturers, the identity and approximate binding sites of the monoclonal antibodies used in the PE AutoDELFI[®] assay (HUI018 and OXI005)[194, 384] are known (Figure 3.1). The site of amino acid substitution of insulin aspart (Figure 3.2), could account for the loss of detection of the assay resulting from the failure of OXI005 to bind. Other insulin analogues that have modifications affecting B28, including insulin lispro which also has little or no cross-reactivity in the PE AutoDELFI[®] assay. However, although insulin glulisine, which incorporates a modification at B29, has little or no cross-reactivity in the PE AutoDELFI[®] assay, porcine insulin, which incorporates an amino acid difference at B30, demonstrated good cross-reactivity. Alterations to the C-terminal end of the B chain, including addition of a fatty acid chain, may inhibit or preclude OXI005 binding.

To examine cross-reactivity more robustly, one would ideally determine cross-reactivity at more concentrations of insulin therapy across each assay range, as cross-reactivity may be concentration-dependent. This may be of particular concern for the Siemens IMMULITE 2000 insulin assay where polyclonal antibodies are used. In addition, to reduce the risk of generating a false cross-reactivity result caused by pipetting error, it would be desirable to prepare more than one sample at each concentration to be tested. However, the study design enabled conclusions to be drawn broadly concerning detection

of insulin therapy in plasma and which assay(s) may be more suitable for use in insulin-treated individuals.

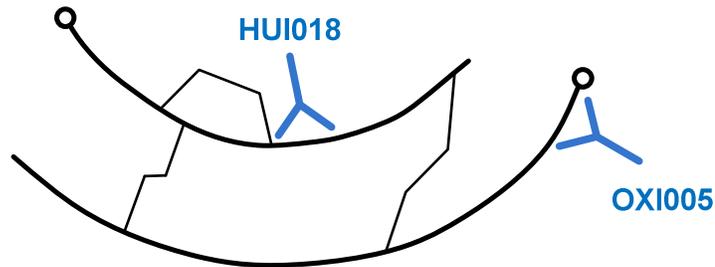


Figure 3.1 PE AutoDELFIACapture/detection antibodies. The epitope of monoclonal antibody HUI018 and OXI005 are found on the A chain and B chain of native human insulin, respectively.

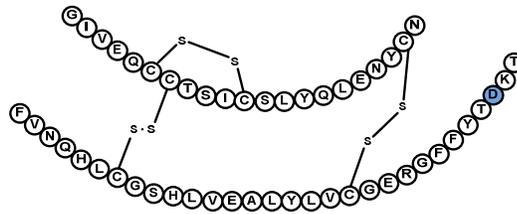


Figure 3.2 Insulin aspart. Homologous to human insulin with the exception of a substitution of the amino acid proline for aspartic acid in position B28.

Although immunoassay cross-reactivity studies using *ex vivo* insulin addition of plasma are limited by design, particularly relating to long-acting insulin analogue that have complex *in vivo* pharmacokinetics, these data are currently the best available to use when interpreting insulin measurements by immunoassay. Generally, immunoassay calibrators are chosen for optimal quantification of the analyte of interest. However, although cross-reacting substances may generate an assay signal, they may not possess the same characteristics to bind both capture and/or detection antibodies to the same extent as the analyte of interest, and therefore generate an assay signal to a different extent from analyte. What is not examined in this study is the potential for a cross-reacting substance to bind either capture or detection antibody only, such as to prevent binding and detection of

human insulin/other insulin species. Although all preparations tested are typically referred to as ‘insulin’ in clinical practice, clinical insulin assays are designed and optimised to measure human rather than non-native insulin, and assay detection is not equivalent for human and non-native insulin. The principal implication of this is that some clinical assays fail to detect insulin analogues in plasma. It follows, therefore, that the use of insulin:C-peptide ratios may be unreliable in identifying surreptitious insulin administration, as both endogenous insulin and C-peptide will be suppressed in response to hypoglycaemia in normal physiology. To determine the presence of insulin analogues in circulation, the difference in reported insulin concentrations between a specific human insulin assay (e.g. DiaSorin LIAISON[®]) and a broad-specificity assay (e.g. Mercodia Iso-Insulin) can be used, although such an approach will not specifically identify the particular analogue(s) in circulation.

Concerning individuals with insulin-treated diabetes, interpretation of insulin results may be particularly complex, as there are analogue-specific differences in molar concentrations per unit of insulin preparation (Section 1.5.7). In addition, assay antibodies may exhibit different affinities for different insulin species, and therefore detection of an insulin therapy may be affected by other insulins present in plasma. These considerations are particularly pertinent when investigating those with suspected poor insulin adherence, where plasma measurements are sometimes used. It likely follows that there exists a broad range of concentrations that could be considered ‘normal’ for insulin-treated individuals, that are dependent on the individual, analogue, and assay.

For immunoassay measurement of ‘total’ insulin (human/animal-derived/analogue) in insulin-treated individuals, the Mercodia Iso-Insulin was the most suitable assay tested, due to its broad specificity (Table 3.2). It was this assay that was used in this research to investigate insulin-treated individuals suspected clinically of having antibody-mediated dysglycaemia (Chapter 6). The Mercodia Iso-Insulin was more laborious than a platform-based, random access, assay; a plate-based ELISA, the assay required controls and calibrators to be analysed with each assay run, and had a narrow analytical range (6–600 pmol/L) [385] thus typically required each plasma analysis to be analysed concurrently with some plasma dilutions, to avoid the requirement of repeating the assay, should the ambient plasma insulin concentration exceed the assay limits.

Plasma analysis using MS may confirm the presence of non-native human insulin in plasma, which is of particular benefit in cases of suspected insulin poisoning. However, although MS affords new opportunity for more specific measurements of insulin [212–214], the challenges involved in correctly deciding what constitutes a ‘normal’ insulin analogue concentration, including consideration of molar concentration versus activity of insulin, total (including antibody- and albumin-bound) versus bioactive insulin, and bioactive insulin metabolites [158], will require further study to overcome.

CHAPTER 4: Laboratory diagnosis of insulin autoimmune syndrome

4.1 Background

The presence of circulating IAs is a defining factor of IAS (Section 1.10) although not specific for the disease (Section 1.11). It was hypothesised that ELISA detection of IA could be used as a standalonetest for IAS, and this was tested alongside adjunctive assays used to identify the presence of insulin–antibody complexes in plasma. In this chapter, a study of different insulin assays in the context of plasma dilution and PEG precipitation studies is presented. It was hypothesised that immunoassay plasma insulin concentration represents total insulin (antibody-bound plus free), therefore results will be linear to sample dilution, with or without the presence of IA, and as such, insulin immunoassay accurately detects insulin in patients with circulating IA. Based on published data from studies of immunoassay, it was hypothesised that in the presence of IA in plasma, insulin recovery would be lower in PEG supernatant compared with that in supernatant of antibody-negative plasma. Also, in this chapter a protocol for the detection of insulin–antibody complexes in plasma using GFC, incorporating addition of insulin to demonstrate concentration increases of antibody-bound insulin and/or exchangeability of insulin, is described. It was hypothesised that GFC of plasma incubated with exogenous insulin would increase detection sensitivity of insulin–antibody complexes in plasma. The overarching rationale of performing this study was to understand the behaviour of these techniques when using modern, clinically-utilised immunoassays, and thereafter, use of the newly-developed methods to investigate plasma from individuals with suspected IAS to discriminate those patients with IAs that were unlikely to cause clinical sequelae.

4.2 Materials and methods

4.2.1 Patients studied

Three patients who presented with hyperinsulinaemic hypoglycaemia, without pre-existing DM, were evaluated. Samples were referred for investigation of insulin antibody dysglycaemia to the UK Severe Insulin Resistance Supraregional Assay Service, Cambridge University Hospitals NHS

Foundation Trust, Cambridge, as part of clinical investigation for which the patients gave their consent. Laboratory studies and sample management were undertaken in accordance with the World Medical Association Declaration of Helsinki (2000).

4.2.2 Sample collection

Venous blood samples were collected on wet ice, and plasma/serum were promptly separated and frozen at -80°C until analysis. Surplus plasma from patient A1 was used for the comparison study of insulin assays.

4.2.3 Investigative approach

Samples were investigated, as outlined in Figure 4.1. The following studies were undertaken: measurement of IAs to detect the presence of IgG in serum, insulin immunoassay dilution studies to investigate the effect of IAs on assay linearity, insulin assay PEG precipitation studies to examine the effect of PEG on immunoassay insulin recovery, and GFC studies to identify the presence of HMW insulin immunoreactivity consistent with insulin-binding antibodies.

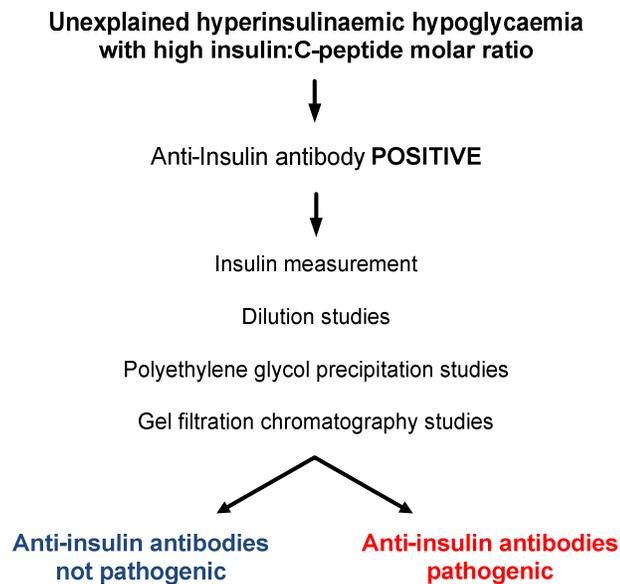


Figure 4.1 Laboratory investigations. Studies were performed on serum/plasma to identify the presence of insulin-binding antibodies and thus discriminate those patients with IAS from individuals with circulating IAs not of clinical concern.

4.2.4 Anti-insulin antibody measurement

IA measurement was performed by the SAS Peptide Hormone Section, Clinical Laboratory, Royal Surrey County Hospital, UK. Serum anti-insulin IgG was measured using the Isletest™-IAA (Biomercia), a semi-quantitative ELISA. This clinical assay was chosen as it was previously used for routine diagnostic purposes at Cambridge University Hospitals NHS Foundation Trust for many years as a screening test for T1DM (accessed via the Supraregional Assay Service [386]). As an established NHS clinical laboratory test, this United Kingdom Accreditation Service (UKAS) accredited assay had been subject to verification locally, hence the analytical performance was not critically examined in this study.

4.2.5 Insulin immunoassays

As part of the dilution studies and PEG precipitation studies outlined below, insulin was measured in pooled plasma from patient A1 using a panel of insulin immunoassays, namely Siemens ADVIA® Centaur, Siemens IMMULITE® 2000, DiaSorin LIAISON® XL, PE AutoDELFLIA®, and the Beckman Coulter Access® 2. Insulin analysis was performed in singleton based on known assay performance characteristics and consistent with routine diagnostic laboratory practice (Appendix A: Assay performance characteristics). Measurement of venous plasma insulin (DiaSorin LIAISON® XL), C-peptide (DiaSorin LIAISON® XL), and glucose (Siemens ADVIA® 2400 Chemistry System) was also performed for all three patients. Measurement of insulin in GFC fractions was performed using the DiaSorin LIAISON XL assay, however for patient A3, the Beckman Coulter® 2 assay was also used, due to its ability to detect insulin aspart (Table 3.2). It was previously established that the Beckman Coulter® 2 assay could detect insulin in GFC fractions (data not shown).

4.2.6 Plasma dilution studies

A singleton measurement of insulin in neat plasma and then following a 1:4 ratio dilution with assay-specific diluent was performed, and initial insulin concentration back-calculated using the dilution factor. Surplus pooled IA-negative plasma from exogenous-insulin naïve individuals was used as a control.

4.2.7 Polyethylene glycol precipitation of plasma

PEG precipitation protocol was based on that used to investigate serum for macroprolactinaemia [387], and that used for many years to investigate macro-hormones, such as macroprolactin and macro-TSH, at Cambridge University Hospitals NHS Foundation Trust. A 25% (w/v) solution of PEG 6000 (BDH Prolabo) was prepared using deionised water. 0.9% (w/v) saline was prepared using 18% (w/v) sodium chloride solution (BDH Prolabo) and deionised water. Ten non-IAS plasma samples were diluted in a ratio 1:1 with 25% (w/v) PEG, mixed for 10 seconds using a vortex, then centrifuged at 13,200 g for 15 minutes. To overcome the sampling error reported by the assay platform (likely triggered by the viscosity of PEG supernatant), PEG supernatant was diluted in a ratio of 1:1 with saline prior to analysis. Matched saline dilutions of the same plasma were prepared and used as control. Singleton measurements of IgA, IgM, IgG, and albumin concentration were performed using the Siemens ADVIA 2400® (Siemens), in the diluted supernatant and the saline-diluted control samples, were undertaken and individual percentage analyte recoveries calculated. Subsequently, the mean and standard deviation of analyte concentration recovery were calculated.

The same pooled plasma sample from patient A1 analysed in the dilution studies was diluted in a ratio 1:1 with 25% (w/v) PEG and mixed for 10 seconds using a vortex, then centrifuged at 13,200g for 15 minutes. A singleton measurement of insulin concentration in the neat supernatant was undertaken using the panel of insulin immunoassays. To overcome sampling error with the DiaSorin LIAISON® XL (likely triggered by the increased sample viscosity due to PEG) a 1:1 ratio dilution of PEG supernatant was also analysed in singleton for insulin.

Insulin recovery was then determined in ten IA-negative control samples, and in non-fasting plasma for all three patients. Insulin analysis was performed using the DiaSorin LIAISON® XL because it demonstrated specificity for human insulin (Table 3.2).

4.2.8 Gel filtration chromatography method development

The GFC protocol for the separation of insulin species was modified and optimised, from an existing GFC laboratory protocol for macroprolactin [380]. An ÄKTAprime plus liquid chromatography system (GE Healthcare) was used in conjunction with a HiLoad 16/60 Superdex 75 (120 mL) size

exclusion column (GE Healthcare) and a laptop (Latitude D620, Dell, Texas, USA; PrimeView 5.0, Amersham Bioscience, 2004). The equipment was set-up and calibrated in accordance with the manufacturer's instructions. 500 μ L of sample was loaded onto the column in combination with a 25 mmol/L Tris/0.52 mol/L NaCl buffer mobile phase at pH 7.4, with a flow rate of 1 mL/min.

4.2.9 Column recovery of blue dextran

Blue dextran 2000 (Amersham Bioscience) solutions of 5 mg/mL and 0.25 mg/mL were prepared in GFC buffer. 38 mL was run to waste, and volume 38–58 mL was collected in a polypropylene tube (Centrifuge Tubes, CELLSTAR[®], Greiner Bio-One). The collected eluted volume was scanned from 800 nm to 200 nm (Cary 100 Bio UV-Visible Spectrophotometer; Cary WinUV scan application Software, version 3.00). Based on peak absorbance, absorbance was then measured at 610 nm. Experiments were performed in singleton.

4.2.10 Effect of bovine serum albumin on insulin recovery in gel filtration chromatography buffer

Human insulin (rDNA, Actrapid[®]) was diluted four times into 2 mL plastic tubes (Micro tube, Sarstedt) in series: the first two dilutions were in saline followed by a further dilution into GFC buffer, and a final dilution in GFC buffer containing bovine serum albumin (BSA, Sigma-Aldrich[®], Dorset, UK) at a calculated concentration of 0 g/L, 5 g/L, 10 g/L, 20 g/L, or 40 g/L. Samples were stored at -80°C overnight prior to analysis. Samples were analysed for insulin using the Beckman Coulter Access[®] 2.

4.2.11 Effect of vessel material, freeze-thaw cycle, and bovine serum albumin on insulin recovery in gel filtration chromatography buffer

Human insulin (rDNA, Actrapid[®], Novo Nordisk) was diluted into three plastic 2 mL (Micro tube, Sarstedt) tubes before further dilution into a fourth tube, either plastic (Sarstedt tube, as above) or glass (test tube medium wall rimless borosilicate glass, grade 3.3 wall thickness 1.0 mm, ISO 4142 75 mm x 12 mm, Fisherbrand[®], Thermo Fisher Scientific; covered in Parafilm PM996 Wrap, Cole-Parmer[®], London, UK). The dilutions were performed in series: the first two dilutions were in 0.9% (w/v) saline,

followed by a further dilution into GFC buffer, either neat or with 40 g/L BSA. The calculated final insulin concentration added was 362 pmol/L. The samples were stored for 12 hours at either +4°C or –80°C. The samples were then warmed to room temperature prior to analysis. Measurement of insulin was then performed using the Beckman Coulter Access[®] 2. Two separate preparations of insulin were measured in singleton in GFC buffer with BSA 40 g/L (without addition of human insulin).

4.2.12 Insulin recovery in gel filtration chromatography eluted volume using optimised chromatography protocol

To reduce bacterial contamination, 0.78 g sodium azide (Sigma-Aldrich[®]) was added to the NaCl when preparing the buffer. A stock solution of BSA was made to a concentration of 0.28 g/mL in GFC buffer using a 50 mL centrifuge tube (Corning[®], New York, USA), and 1 mL of this solution was added to each of the fractions prior to each GFC run. A plasma sample with an endogenous insulin concentration of 764 pmol/L was processed, the insulin concentrations measured in eluted fractions, and percentage recovery calculated. The chromatography method was repeated 30 times and absorbance of eluate measured at 280 nm. Based on the earliest demonstrable absorbance peak, the elution volume of immunoglobulin was recorded.

4.2.13 Gel filtration chromatography of patient plasma

Six millilitre elution volume fractions with 1 mL BSA (final volume 7 mL, calculated BSA concentration 40 g/L) were collected in polypropylene tubes (Cellstar[®], Greiner Bio-One). 36–114 mL eluted volume was collected. Insulin analysis was performed using the DiaSorin LIAISON[®] XL immunoassay, as in-house data demonstrated sufficiently high analytical sensitivity (1.2 pmol/L) and acceptable coefficient of variation at lower insulin concentrations (8.6% at 34 pmol/L; n=244).

4.2.14 Gel filtration chromatography with *ex vivo* insulin addition

990 µL of neat plasma was mixed with 10 µL of human insulin/synthetic insulin analogue of the desired concentration. The samples were incubated on a roller mixer at 24°C for 24 hours before being eluted through the GFC protocol described in Section 4.2.13, in parallel with samples prior to exogenous insulin addition.

4.3 Results

The three patients studied were female and presented with recurrent spontaneous hyperinsulinaemic hypoglycaemia (venous plasma glucose level below 2.5 mmol/L confirmed by laboratory analysis), ‘positive’ IAs by the IsletestTM-IAA ELISA, and were not treated for DM at the time of blood sampling. Non-fasting samples were analysed for insulin, C-peptide, and glucose (Table 4.1). All three patients had detectable plasma C-peptide and a high insulin:C-peptide molar ratio [209] at times when hypoglycaemia was not present. The samples were analysed to determine whether insulin-binding antibodies could cause the presenting metabolic disorder.

Table 4.1 Demographic characteristics and initial biochemical profile of patients studied

Patient	Non-fasting plasma glucose (mmol/L) (<7.8)	Insulin (pmol/L) (<60)	Insulin recovery following PEG precipitation (>102%)	C-peptide (pmol/L) (174-960)
A1	4.3	7020	8	3297
A2	7.7	1650	63	3240
A3	8.0	69,000	4	4960

Insulin recovery following PEG precipitation calculated as percentage insulin concentration in PEG supernatant/saline dilution-matched control was calculated.

4.3.1 Dilution studies

To assess the effect of IA on insulin immunoassay linearity, measurement of insulin following dilution of pooled plasma from patient A1 was performed. To avoid an initial dilution that could affect the equilibrium of insulin–antibody binding, thus reducing the observable effect on linearity of subsequent dilutions, a neat plasma sample with an insulin concentration within assay reportable limits was chosen for this assessment. Pooled IA-negative plasma was used as a control. Five different immunoassays were studied, namely Siemens ADVIA[®] Centaur, Siemens IMMULITE[®] 2000, DiaSorin LIAISON[®] XL, PE AutoDELFIA[®], and the Beckman Coulter Access[®] 2. Concordance among insulin assays was consistent with known method bias [195, 388]. Plasma insulin concentration was then

measured following a dilution of ratio 1:4 with assay diluent. All insulin assays displayed linear insulin recovery in control plasma (Figure 4.2a), however there was increased recovery of insulin (Mann-Whitney test $p < 0.05$) in the IAS plasma following dilution using all five assays (Figure 4.2b).

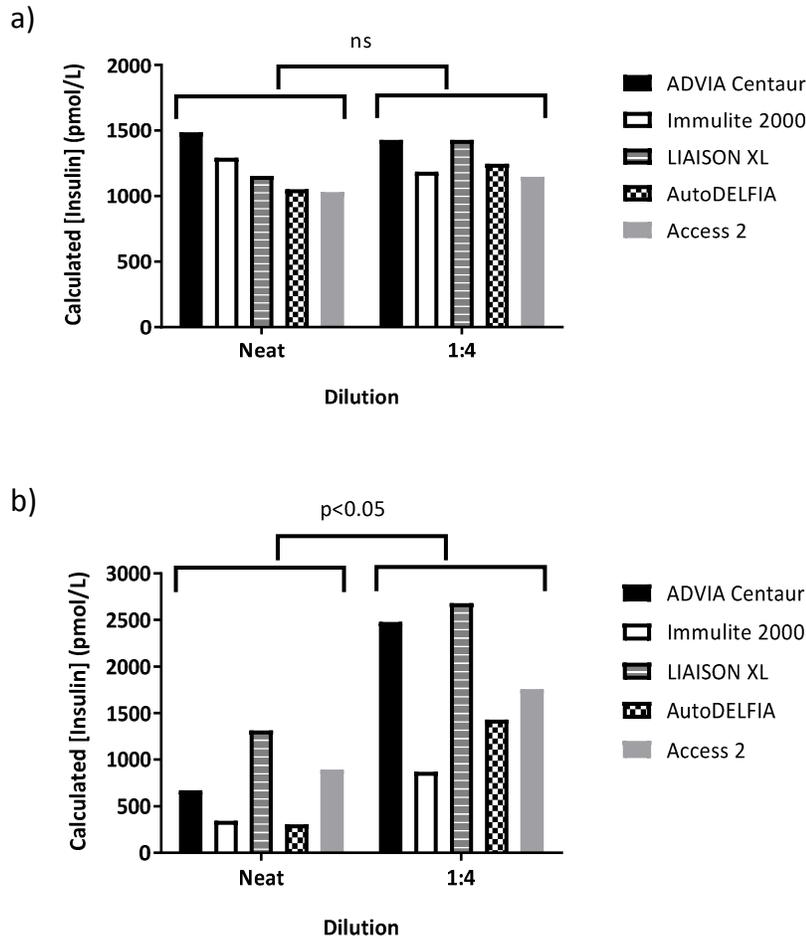


Figure 4.2 Effect of plasma dilution and IA on insulin determination by a panel of insulin immunoassays. Calculated insulin concentration plotted against plasma dilution (ratio plasma 1:4 assay diluent) for antibody-negative control plasma (a) and IAS plasma from patient A1 (b). Insulin measurements were made using a panel of assays (Siemens ADVIA® Centaur, Siemens IMMULITE® 2000, DiaSorin LIAISON® XL, PE AutoDELFLIA® and the Beckman Coulter Access® 2, as indicated). Neat control plasma concentrations and corresponding calculated starting concentrations derived from assay of diluted samples were compared using the Mann-Whitney U test.

4.3.2 Polyethylene glycol precipitation

To determine the normal recovery of immunoglobulin and albumin following PEG precipitation, a study was performed of control plasma. Ten samples were diluted in a ratio 1:1 with 25% (w/v) PEG and, following centrifugation to avoid sampling error, a dilution of ratio 1:1 with 0.9% (w/v) saline was performed in order to reduce the viscosity of the supernatant. Recovery was calculated as percentage analyte concentration in PEG supernatant/saline dilution-matched control. The mean (and standard deviation) IgA, IgM, IgG, and albumin recoveries were 33% (10%), 5% (4%), 0% (0%), 104% (4%), respectively (Figure 4.3).

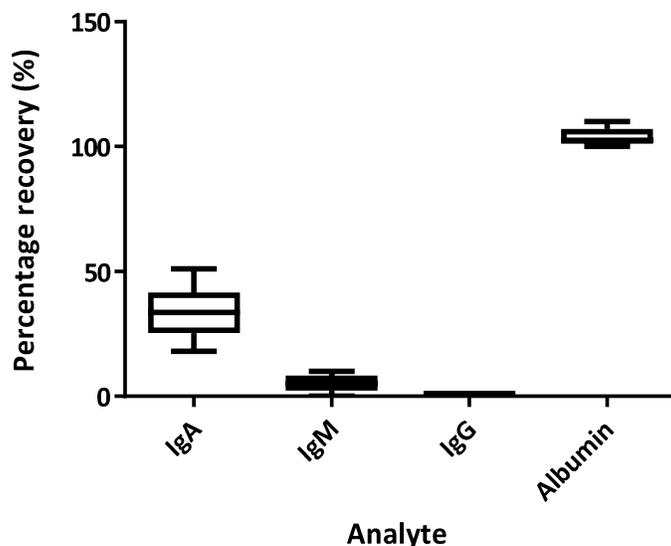


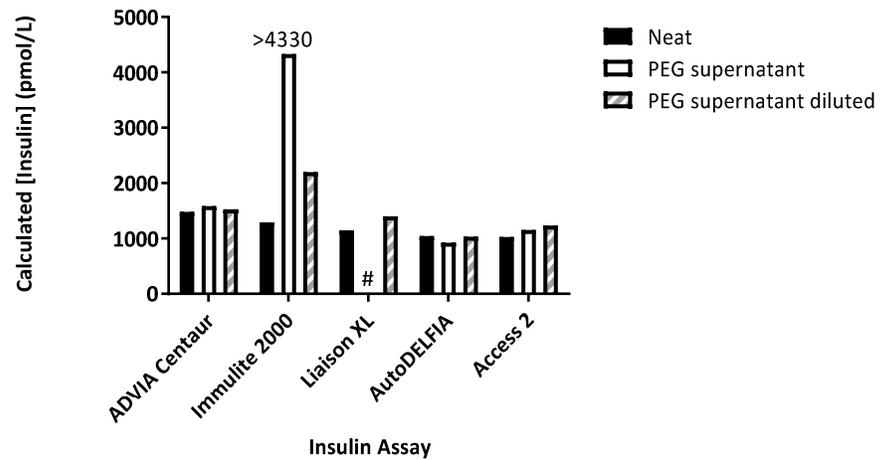
Figure 4.3 Immunoglobulin and albumin recovery measured in plasma supernatant following PEG precipitation.

The PEG supernatant insulin concentration (assayed following a 1:1 ratio dilution with 0.9% (w/v) saline) expressed as percentage insulin concentration in PEG supernatant/saline dilution-matched sample was calculated for ten control plasma samples, a lower reference limit (RL) of 102% was defined for the DiaSorin LIAISON[®] XL assay (median 107%; 95% confidence interval 102–112%). To establish whether PEG precipitation of plasma can be utilised to screen plasma for the presence of insulin-antibody complexes using the different insulin assays, insulin recovery in supernatant following PEG precipitation of plasma was then studied. The same neat plasma samples analysed in the dilution studies

outlined above were diluted in a 1:1 ratio with PEG, and following centrifugation, insulin was measured in the supernatant. Insulin concentrations in the PEG supernatant of control plasma were consistent with a 50% dilution. There were two exceptions: firstly, the Siemens IMMULITE® 2000 paradoxically demonstrated increased insulin immunoreactivity following dilution with PEG (Figure 4.4a). Secondly, the DiaSorin LIAISON® XL assay which repeatedly reported a sample error. This error was avoided by performing a 1:1 ratio dilution of supernatant with assay-specific diluent, thereby reducing the viscosity. Except for the Siemens IMMULITE® 2000, the assays demonstrated linearity in PEG supernatant.

Using plasma from patient A1 with IAS, insulin recovery in PEG supernatant from three of the five assays was low, however the Siemens IMMULITE® 2000 assay, as previously, exhibited an increased insulin immunoreactivity following dilution with PEG (Figure 4.4b), and the DiaSorin LIAISON® XL, as previously, reported a sample error that was avoided by performing a 1:1 ratio dilution with assay-specific diluent. Measurement of insulin in diluted PEG supernatant using the Siemens ADVIA® Centaur, PE AutoDELFA® , and the Beckman Coulter Access® 2 (which had measured insulin in the neat supernatant), demonstrated linearity in relation to the PEG supernatant itself, but not to the neat plasma. Measurement of insulin following a further 1:1 dilution demonstrated a much lower insulin recovery than expected, given the insulin concentration in the undiluted PEG supernatant using the Siemens IMMULITE® 2000.

a)



b)

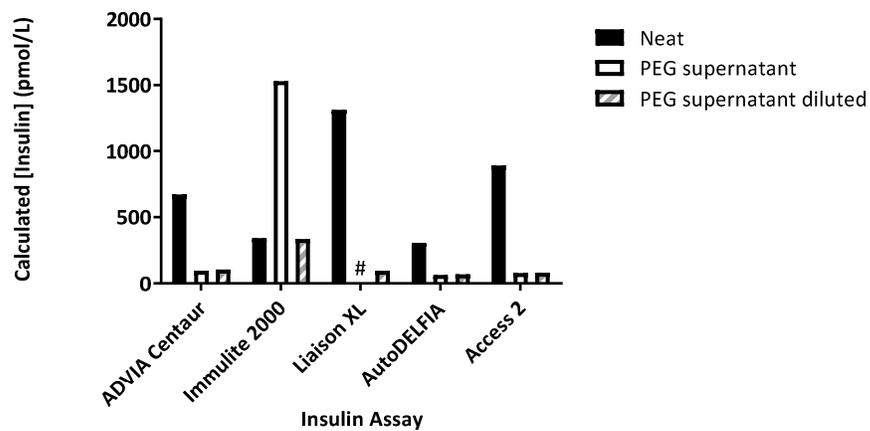


Figure 4.4 Effect of PEG precipitation and IA on insulin determination by a panel of insulin immunoassays. Calculated insulin concentration made using measured concentrations in neat plasma, PEG supernatant, and PEG supernatant following 1:1 ratio dilution with assay buffer for IA-negative control plasma (a) and IAS plasma from patient A1 (b), are shown. Insulin measurements were made using a panel of assays (Siemens ADVIA[®] Centaur, Siemens IMMULITE[®] 2000, DiaSorin LIAISON[®] XL, PE AutoDELFLIA[®], and the Beckman Coulter Access[®] 2), as indicated. The DiaSorin LIAISON[®] XL was unable to analyse neat PEG supernatant and reported a sample error (#).

4.3.3 Gel filtration chromatography method development

4.3.3.1 Column recovery of blue dextran

Blue dextran was used to estimate GFC column recovery because, due to its HMW (average 2,000 kDa), it would be excluded from the column. Recovery was 73% at 5 mg/mL and 74% at 0.25 mg/mL in GFC buffer.

4.3.3.2 Effect of bovine serum albumin on insulin recovery in gel filtration chromatography buffer

It was considered whether the presence of low protein concentrations in the chromatography fractions may adversely affect immunoassay insulin recovery, irrespective of the true analyte concentration (i.e. a 'matrix effect'), and therefore account for repeatedly low insulin recovery from the GFC column. The effect of albumin concentration of eluted fluid on insulin recovery was examined. Insulin was added, to a calculated final insulin concentration of 90.4 pmol/L, and the samples stored at -80° C overnight, prior to analysis. Approximate insulin recoveries in GFC buffer with albumin concentrations 0, 5, 10, 20 or 40 g/L, were 0%, 15%, 52%, 39%, and 57%, respectively. BSA 40 g/L in GFC buffer did not demonstrate cross-reactivity in the insulin assay (insulin <0.21 pmol/L). Following these findings, the addition of BSA to fractions at a calculated concentration of 40 g/L, (a concentration in keeping with that of albumin in human plasma), was used for subsequent GFC experiments.

4.3.3.3 Effect of vessel, freeze-thaw cycle, and bovine serum albumin on insulin recovery in gel filtration chromatography buffer

To identify additional causes of low insulin recovery from the GFC column, the effect on insulin concentrations of a freeze-thaw cycle compared with refrigeration, plastic compared with glass tubes, and the presence or absence of 40 g/L BSA, was studied (Figure 4.5). A comparison of insulin concentrations measured in singleton demonstrated that the presence of BSA had the greatest effect on insulin recovery (Wilcoxon matched-pairs signed rank test, $p<0.05$).

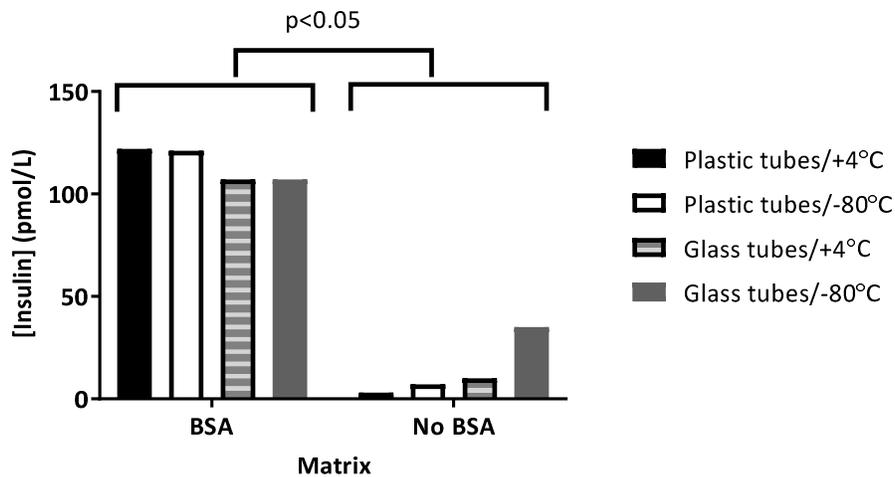


Figure 4.5 Effect of storage temperature, sample vessel material, and addition of BSA on immunoassay insulin recovery. The measured insulin in GFC buffer with BSA was for plastic tubes stored at +4°C: 123 pmol/L; plastic tubes stored at -80°C: 122 pmol/L; glass tubes stored at +4°C: 108 pmol/L; and glass tubes stored at -80°C: 108 pmol/L. The measured insulin in GFC buffer without BSA was: for plastic tubes stored at +4°C: 3.3 pmol/L; plastic tubes stored at -80°C: 7.0 pmol/L; glass tubes stored at +4°C: 11.0 pmol/L; glass tubes stored at -80°C: 35.4 pmol/L.

4.3.3.4 Insulin recovery following gel filtration chromatography method optimisation

A high endogenous insulin IA-negative sample was processed using the optimised GFC method, that included addition of sodium azide to the GFC buffer, and addition of BSA (to give a final concentration of 40 g/L in each fraction) to the GFC fractions before the chromatography process, use of polypropylene tubes, and inclusion of one freeze-thaw cycle prior to analysis. Insulin recovery was 75%, calculated as amount of insulin recovered in eluted fractions as a percentage of amount of insulin loaded onto the column. Insulin recovery in excess of this value was achieved for IAS plasma. For ten IAS plasma samples that underwent GFC, median insulin recovery in eluted fractions was 153%, with a range of 79–1503%. The chromatography method demonstrated good precision with an elution volume coefficient of variation of 6% for immunoglobulin (n=30; mean elution volume 49 mL).

4.3.4 Gel filtration chromatography with insulin addition/exchange studies of patient plasma

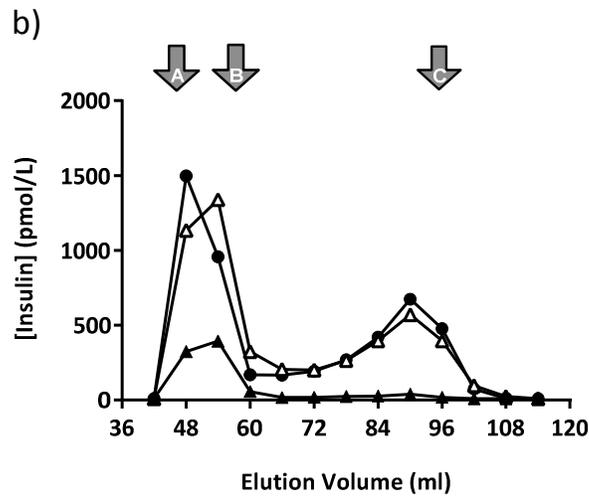
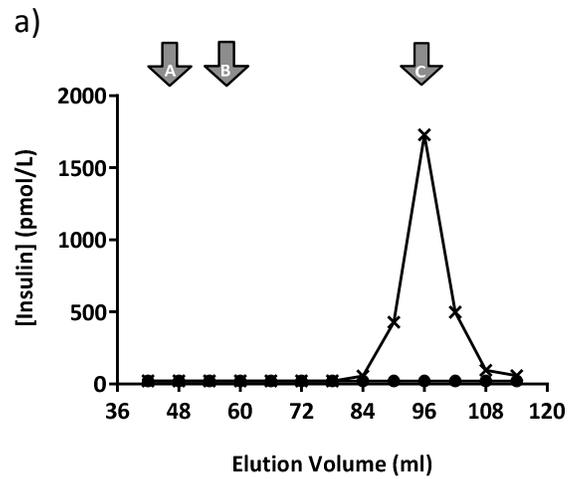
Plasma analysis was then performed using GFC to separate insulin species according to size and to identify the presence of insulin–antibody complexes. In a control sample with a measured neat plasma insulin concentration of 14 pmol/L, no insulin peaks were discernible in the column eluate (Figure 4.6a), an expected finding resulting from insulin concentrations in the eluted fractions being below the assay limit of quantification. After addition of exogenous synthetic human insulin to increase the measured insulin concentration to 7655 pmol/L, the peak of eluted insulin was consistent with monomeric (free) insulin only. This method, of GFC pre- and post-addition of exogenous insulin, was then applied to plasma from the three patients.

A non-fasting sample with a measured insulin concentration of 7480 pmol/L from patient A1 demonstrated peaks of insulin immunoreactivity consistent with principally two insulin species: a monomeric, and a HMW insulin immunoreactivity peak consistent with insulin–antibody complexes. A fasting sample with a measured insulin concentration of only 774 pmol/L in neat plasma from patient A1 was next used to challenge the discriminatory power of the GFC method at this lower insulin level. Even at this lower measured insulin concentration, insulin–antibody complexes were demonstrable, and the insulin bound to immunoglobulin was the principal insulin species identified (Figure 4.6b). Exogenous insulin was then added to the same fasting sample from patient A1, increasing the measured insulin concentration to 7840 pmol/L, and GFC performed on this insulin-enriched sample. The insulin concentrations in the HMW fractions increased markedly compared with those demonstrable in the fasted sample, findings consistent with excess antibody insulin-binding capacity. These findings demonstrated that with increasing plasma insulin concentration, the sensitivity of the GFC method to clearly demonstrate insulin binding by antibody increased, and may additionally provide information on the binding capacity of the antibody.

GFC of neat plasma from patient A2, with an insulin concentration of 198 pmol/L, did not demonstrate HMW insulin immunoreactivity (Figure 4.7a). Furthermore, the addition of exogenous insulin to increase the measured insulin concentration to 8720 pmol/L markedly increased the

monomeric insulin peak, however no HMW insulin peak was identified. This finding was consistent with the plasma antibody having insufficient affinity and/or concentration to form a HMW peak using this GFC method. It follows that the antibody detected using the ELISA is unlikely to sequester sufficient insulin to derange insulin kinetics/dynamics and cause clinical sequelae.

The plasma insulin concentration of patient A3 was 69,000 pmol/L and required multiple dilutions before an insulin concentration within the assay detection limits could be recorded. GFC of plasma demonstrated HMW insulin immunoreactivity as the principal insulin species (Figure 4.7b). This result may be consistent with the presence of IAs with high affinity and/or concentration, and high insulin binding capacity. However an alternative explanation was HMW insulin immunoreactivity caused by heterophilic antibody interference. To discriminate between the two entities, a study was performed to assess the ability of the IAs to exchange endogenous insulin for exogenous insulin analogue. Insulin aspart (NovoRapid®) was the analogue chosen as it had been demonstrated to exhibit very low cross-reactivity with the DiaSorin LIAISON® XL assay (Table 3.2). Following addition of aspart to plasma, insulin concentrations measured by the DiaSorin LIAISON® XL in GFC fractions resulted in a decrease in HMW insulin immunoreactivity and a marked increase in monomeric insulin (Figure 4.7b). This result may be consistent with insulin aspart displacing endogenous (human) insulin from the antibody, thereby decreasing HMW insulin immunoreactivity using the DiaSorin LIAISON® XL. The increase in monomeric insulin represents the displaced native insulin and the (very low) cross-reactivity of large amounts of unbound insulin aspart. A qualitative comparison of data from GFC of non-fasting plasma and non-fasting plasma spiked with aspart using the Beckman Coulter® 2 assay was undertaken, both sets of samples being inherently subject to similar matrix effects during GFC. Analysis of GFC fractions pre- and post-addition of insulin aspart using the Beckman Coulter Access® 2 that has higher cross-reactivity with insulin aspart (Table 3.2) confirmed native insulin could be displaced from the antibody using insulin aspart (Figure 4.8).



- ▲ Fasting neat plasma
- Non-fasting neat plasma
- ▲ Fasting plasma human insulin spike
- ✕ Non-fasting plasma human insulin spike

Figure 4.6 Demonstration of reversible insulin binding to immunocomplexes using gel filtration chromatography of plasma. Results of insulin assay after GFC of IA-negative control plasma or patient plasma are shown: plasma pre- and post-human insulin spike of IA-negative control (a); non-fasting plasma, or fasting plasma pre- and post-human insulin spike of patient A1 (b). Elution volumes of immunoglobulin (A), albumin (B) and monomeric insulin (C) are shown. Insulin concentrations were measured using the DiaSorin LIAISON® XL.

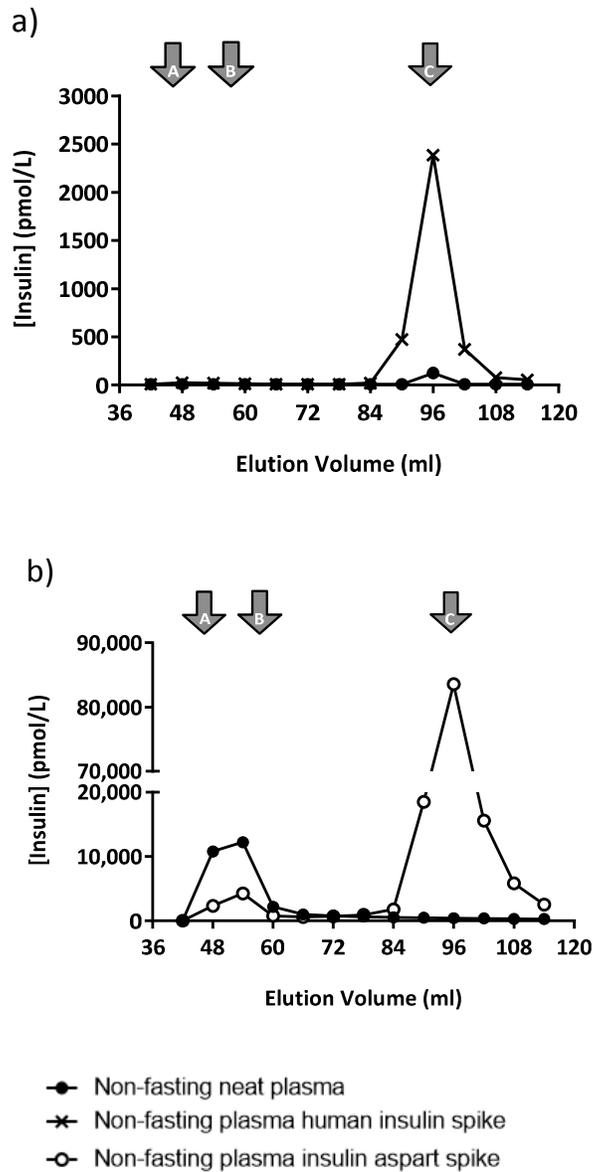


Figure 4.7 Demonstration of reversible insulin binding to immunocomplexes using gel filtration chromatography of plasma. Results of insulin assay after GFC of patient plasma are shown: non-fasting plasma pre- and post-human insulin spike of patient A2 (a); non-fasting plasma pre- and post-insulin aspart spike of patient A3 (b). Elution volumes of immunoglobulin (A), albumin (B) and monomeric insulin (C) are shown. Insulin concentrations were measured using the DiaSorin LIAISON® XL.

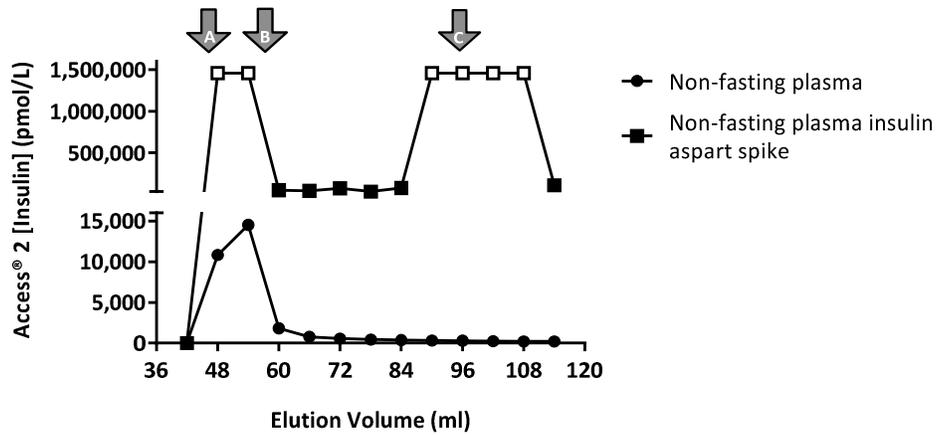


Figure 4.8 Demonstration of insulin aspart binding to immunocomplexes using gel filtration chromatography of plasma. Non-fasting plasma pre- and post *ex vivo* insulin aspart incubation of patient A3. Elution volumes of immunoglobulin (A), albumin (B) and monomeric insulin (C) are shown. Insulin concentrations were measured using the Beckman Coulter Access[®] 2 assay, which exhibits cross-reactivity with aspart (Table 3.2). Results were truncated at 1,500,000 pmol/L (truncated data points indicated by □).

4.4 Discussion

Macro-hormones are a cause of increased total plasma hormone concentrations, however for the most part, the existence of these hormone–immunoglobulin complexes is not of physiological significance, as the hormones remain biologically inactive. However, even when macro-hormones are not bioactive, they may still have clinical impact, leading to high measured hormone concentrations that may be incorrectly interpreted by clinicians who are misled to investigate or to treat patients for an endocrinopathy. Detection of such complexes can explain abnormal endocrine results, and has been shown to have great diagnostic utility [225, 242, 389]. In addition to the analytical challenges that insulin-binding antibodies pose [390, 391], some such IAs also can cause aberrant insulin action. This is because the ability of some such IAs to acutely sequester secreted (or injected) insulin leading to relative bioactive insulin insufficiency and hyperglycaemia, and subsequently release bound insulin at inappropriate times causing bioactive insulin excess and hypoglycaemia, adversely affect insulin kinetics and dynamics. Although the detection of IAs in serum has clinical utility in the investigation of unexplained hyperinsulinaemic hypoglycaemia, ELISA detection of IA using the Isletest-IAA™ could not be used as a robust standalonetest for IAS as results are less informative on the diagnostic utility of a ‘positive’ antibody result. This consideration was the stimulus to study laboratory techniques for identifying clinically-significant IAs in the context of insulin immunoassays used by clinical laboratories.

Concordance between the insulin assays employed was considerably reduced in the presence of insulin autoantibodies, highlighting a method-dependent sensitivity to IA interference. Data from plasma dilution studies suggested measured concentrations of insulin in neat plasma will not necessarily reflect either total (antibody-bound plus unbound), or unbound (free) insulin, as the binding equilibrium between insulin and antibody is likely to be disturbed due to factors including dilution in assay reagents and assay incubation times, factors which are assay-dependent. The degree to which immunoassays detect insulin complexed with antibody may be affected by the binding characteristics of endogenous antibody and the propensity of the insulin–antibody complex to dissociate in the assay, and possibly by the insulin-binding epitope(s) of the assay antibodies (those that may compete with binding of plasma insulin by endogenous antibody) (Figure 4.9).

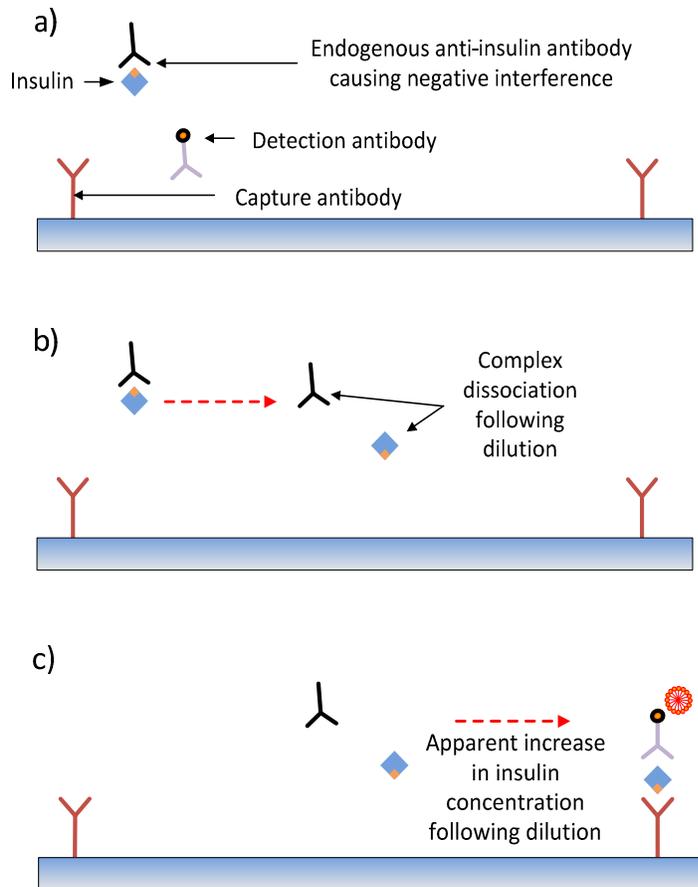


Figure 4.9 Possible mechanism of insulin antibody immunoassay interference on insulin measurement. Endogenous IA binding may affect the interaction of insulin in the immunoassay (a). Following dilution of the sample (b), measuring insulin with back-calculation of estimated original insulin concentration (c) reveals increased insulin recovery following dilution compared with the result in neat plasma.

Demonstration of assay non-linearity following sample dilution is used as a non-specific indicator of immunoassay interference. Following dilution of a sample containing insulin–antibody complexes, all insulin assays studied were non-linear, including the PE AutoDELFI[®], a two-step assay (see Table 3.1) considered to be more robust to such interference. To provide additional evidence for assay interference caused by IAS plasma, further samples were examined using the DiaSorin LIAISON XL assay, and non-linearity with increased insulin recovery with dilution demonstrated (Chapter 5; Table 5.2). The sensitivity of dilution studies to detect the presence of certain antibodies, namely very

low affinity IAs that release insulin readily, or very high-affinity IAs that bind insulin tightly, may be questionable but, where fixed concentrations of these antibodies exist, the clinical relevance of such antibodies may also be uncertain. Some unbound insulin is present even when there are high concentrations of insulin-binding antibody, suggesting the occurrence of some dissociation of the insulin–antibody complex [334]. However, the relative amounts of antibody-bound and free insulin detected by immunoassay, and how much insulin dissociates from antibody during the assay procedure, is not known. It follows that it is not clear how immunoassay insulin concentrations truly reflect acutely bioavailable insulin, and it follows that this may be dependent on the binding characteristics of the circulating IA.

PEG precipitation of plasma is used commonly to screen for the presence of macro-hormones. Assay performance using this technique is assay-dependent, and data from this chapter highlight the importance of examining performance and determining reference limits for each assay. PEG precipitation of plasma containing insulin-binding antibodies resulted in decreased insulin recovery in PEG supernatant for all but the Siemens IMMULITE® 2000, which had increased insulin recovery, likely to be a matrix effect (i.e. an effect independent of true insulin concentration and an analytical artefact due to other sample components). Using the Siemens IMMULITE® 2000, the calculated insulin concentration in the 1:1 diluted PEG supernatant corresponded exactly to the value measured in the neat plasma, however the above study data from the control plasma suggest that this may only be coincidental, and potentially misleading in a clinical diagnostic context, given the over-recovery of insulin in this assay in the presence of PEG. To provide additional evidence for low insulin recovery following PEG precipitation of plasma from different individuals, further samples were studied using the DiaSorin LIAISON XL assay (Chapter 5).

PEG precipitation can exhibit sample-specific matrix effects [236], and the sensitivity of the technique is dependent on the ability to quantify insulin accurately and precisely post-precipitation if the ambient plasma insulin concentration is low.

PEG precipitation can exhibit sample-specific matrix effects [236], and the sensitivity of the technique is dependent on the ability to quantify insulin accurately and precisely post-precipitation if the ambient plasma insulin concentration is low. Although IgG was essentially completely removed

using the PEG precipitation method, other immunoglobulin classes demonstrated differential precipitation. Notably, IgA was not completely removed, consistent with previous reports [240]. Antibodies of the IgG class are most commonly expected in IAS, however, non-IgG antibodies have been reported in the context of myeloma [244], and the presence of antibodies that are not IgG should be considered in situations of hyperinsulinaemic hypoglycaemia where insulin recovery post-PEG precipitation is equivocal. In general, providing the limitations of this technique are appreciated, PEG precipitation can be used as a method to screen for the presence of insulin-binding antibodies in plasma.

A GFC method was developed and optimised to discriminate insulin–antibody complexes from monomeric insulin. Investigations were performed to examine BSA concentration, vessel material, and storage temperature as possible contributors of poor insulin recovery in eluted fractions. Study findings were consistent with previous observations, noting insulin binding to laboratory plasticware [392] and glassware [393]. Insulin recovery was improved with addition of BSA irrespective of sample tube material, or whether the sample was stored refrigerated or frozen. A BSA concentration of 40 g/L in plastic tubes failed to recover spiked insulin fully, however, compared with the calculated insulin spike concentration, insulin recovery is likely to be diminished by the initial serial dilution of human insulin that was performed in plastic tubes with saline. Adding BSA directly to solutions of saline-diluted insulin led to increased insulin recovery (data not shown). Over-recovery of insulin in eluted fractions may be expected in IAS plasma as the phenomenon was observed with sample dilution of plasma (Section 4.3.1). Although over-recovery of insulin in eluted fractions can be considered additional evidence for the presence of insulin–antibody complexes in plasma, determination of the recovery had limited value in some cases as the ambient plasma insulin concentration was in excess of the assay reportable range, requiring plasma dilution before analysis, thus potentially disturbing the insulin–antibody interaction and magnitude of effect of interference.

HMW and monomeric insulin immunoreactivity peaks were unequivocally distinguishable using the GFC method, and when insulin exceeds the binding capacity of antibody, excess insulin will be demonstrable at the position of monomeric insulin [250]. However, due to sample dilution, immunocomplex dissociation may have occurred during the filtration process: there was a ‘tail’ notable between the end of the HMW and start of the monomeric peak in some GFC results (e.g. Figure 4.6b,

Figure 5.6b, Figure 5.7a, Figure 5.7b, Figure 5.8) that may represent complex dissociation during chromatography. Therefore, GFC results may underrepresent total HMW insulin amount and overestimate total monomeric insulin amount in ambient plasma. Unlike macroprolactin [394], there was no clear negative correlation between insulin recovery following PEG precipitation and HMW insulin immunoreactivity determined by GFC. This finding may be a consequence of variable detection of insulin in immunocomplexes by immunoassay in neat plasma, the incomplete resolution of HMW and monomeric insulin immunoreactivity peaks in some cases, and the possibility of dissociation of immunocomplexes during the gel filtration chromatography process.

The GFC method was combined with *ex vivo* insulin binding/exchange that served both to increase the sensitivity of detection of insulin-binding antibodies, and to provide evidence to refute the presence of heterophilic antibody interference as a cause of HMW insulin immunoreactivity. This offers invaluable information for clinical decision-making; whilst the presence of heterophilic antibodies is essentially an analytical artefact, the presence of insulin-binding antibodies may derange insulin kinetics and cause clinical harm. The method has the advantage of greater convenience over methods requiring radiolabelled insulin [248].

For patients with large amounts of circulating insulin, insulin–antibody complexes could be readily identified using GFC and, used in conjunction with the clinical presentation findings and other biochemistry results, a determination could be made that the IAs were clinically-significant. For samples with lower insulin concentrations, such as from a blood collection during a fast, or following insulin degradation prior to analysis, particularly when the insulin–antibody complexes may dissociate during filtration, the sensitivity of the GFC method is limited. However, the addition of exogenous insulin increases sensitivity to detect insulin-binding antibodies by increasing HMW (and monomeric) insulin. This was of particular use in patient A2, for whom increasing plasma insulin concentration did not reveal HMW insulin, and in whom advanced liver disease offered an alternative explanation for fasting hypoglycaemia, rather than labile glycaemia due to an insulin-binding antibody.

Adding insulin analogue to plasma prior to GFC can be used to demonstrate insulin-exchange by antibody, when results are compared between immunoassays with different cross-reactivity characteristics. The addition of insulin analogue to plasma, in combination with an analogue-specific

insulin immunoassay to demonstrate insulin exchange by antibody, was demonstrated to have diagnostic utility for patient A3, where predominantly endogenous HMW insulin immunoreactivity was demonstrable using GFC. Insulin exchange from endogenous human insulin to aspart in the HMW fractions was consistent with insulin-binding antibodies, and effectively ruled out heterophilic antibodies as the principal cause of HMW insulin immunoreactivity. In theory, this technique may be limited if the endogenous antibody of concern does not bind the analogue (due to analogue structural differences from native insulin), and although monoclonal antibodies with specificity to human insulin appear to be incorporated in immunoassays [204–207], such an antibody with such specificity has not been clearly identified in human plasma thus far.

This study extends previous reports of the use of dilution studies, PEG precipitation, and GFC in the context of widely used commercial immunoassays, identifying strengths and limitations of each approach. Comprehensive understanding of the performance of these techniques, and development of efficient diagnostic algorithms, will require their application to a larger population of patients with unexplained dysglycaemia.

CHAPTER 5: Assessment and management of anti-insulin autoantibodies in varying presentations of insulin autoimmune syndrome

5.1 Background

IAS forms part of the differential diagnosis of spontaneous adult hypoglycaemia [167], and for adult patients presenting with unexplained hypoglycaemia, IA measurements are recommended [167]. However, different assays are used by different clinical laboratories, and IA assays are not standardised between methods [395]. Reported results may be only qualitative, or semi-quantitative at best, and for quantitation, a variety of different units are used. These factors complicate direct comparison of results between assays, which is of particular relevance when seeking to use thresholds to diagnose disease or guide treatment. Furthermore, the presence of plasma IAs is not specific for IA-mediated dysglycaemia (Section 1.11), and detection of IAs does not confirm the presence of demonstrable insulin–antibody complexes in circulation (Chapter 4). Successful removal of IAs using multimodal immunosuppression has been reported in cases of disabling dysglycaemia (Section 1.10.1).

To bind insulin in such a way as to derange insulin kinetics to a clinically-significant degree, IAs must be of sufficient affinity and/or concentration. Radioligand binding assays, which involve antibody incubation at a range of concentrations of radiolabelled ligand, are typically used to assess antibody affinity and demonstrate IA-binding characteristics [230, 289, 377, 378, 396–401], and in IAS antibodies with higher [265, 398] and lower [255, 402] affinities have been reported.

In IAS, a high plasma insulin:C-peptide molar ratio is usually demonstrated, as insulin clearance is impaired by antibody [248], while C-peptide clearance rate is typically unaffected. Insulin:C-peptide ratios are also widely used to identify those individuals with hyperinsulinaemia caused by surreptitious administration of exogenous insulin (either by the individual under medical investigation, or by a third party) [209]. In situations of exogenous insulin poisoning, exogenous insulin lowers blood glucose, thereby reducing endogenous insulin (and therefore C-peptide) secretion. In insulin poisoning, as in IAS, a high insulin:C-peptide molar ratio is classically seen. Therefore, an optimal investigative

approach is important to obtain the correct diagnosis and avoid delay, as very different clinical interventions should be made in each clinical scenario.

Immunoassay detection of insulin is susceptible to interference by heterophilic antibodies (Section 1.7.2) and IA (Chapter 4). By avoiding the use of antibodies for capture or detection of insulin, MS obviates interference by endogenous antibodies and offers more robust quantification in the face of such interference [403]. This considered, a comparison of insulin concentrations determined by immunoassay and MS was undertaken, as it was hypothesised that if immunoassay plasma insulin immunoreactivity represents total insulin (antibody-bound plus free) concentration results by both methods would be broadly similar. A comparison of C-peptide concentrations determined by immunoassay and MS was also undertaken, and it was hypothesised that immunoassay plasma C-peptide immunoreactivity represents total C-peptide concentration, and again, therefore would be broadly similar to MS C-peptide concentration.

In this chapter, seven patients with hyperinsulinaemic hypoglycaemia are presented, who were diagnosed with IAS following application of methods described in Chapter 4. Measurements of glycaemia, serum IA quantification (by ELISA and RIA) and IA affinity, immunoassay plasma insulin and C-peptide concentration before/after PEG precipitation, GFC, MS insulin quantification, and MS C-peptide quantification were undertaken. It was hypothesised that ELISA or RIA quantification of IA could be used as a standalone test for IAS, and a sensitive and specific action limit could be defined for clinical purposes. Studies were repeated, when required, to guide management during and after varied treatment regimens encompassing diazoxide, glucocorticoids, MMF, and rituximab. It was hypothesised that determination of IA affinity and insulin binding capacity using a radioligand binding assay would provide information on antibody characteristics to predict type of clinical presentation (fasting versus post-absorptive hypoglycaemia), and symptom burden (thus provide evidence for immunomodulation). In addition, it was hypothesised that one of these assays (IA, insulin, PEG precipitation of plasma, or GFC of plasma), or a streamlined panel of these assays, could be used to diagnose and robustly monitor response to antibody-depletion therapy in IAS.

5.2 Materials and methods

5.2.1 Patients studied

Seven individuals presenting with hyperinsulinaemic hypoglycaemia and detectable IA were studied. Samples were referred for investigation of insulin antibody dysglycaemia to the UK Severe Insulin Resistance Supraregional Assay Service, Cambridge University Hospitals NHS Foundation Trust, Cambridge, as part investigation for which the patients gave their consent. Laboratory studies and sample management were undertaken in accordance with the World Medical Association Declaration of Helsinki (2000).

5.2.2 Samples

Blood samples were collected on wet ice and centrifuged without delay. Plasma and serum were stored frozen at -80°C until analysis. Samples were taken at presentation, and the collection repeated to monitor treatment response when appropriate. IA radioligand-binding studies were performed on surplus serum from patient presentation at Diabetes and Metabolism, Southmead Hospital.

5.2.3 Immunoassays

Quantitative measurement of anti-insulin IgG was performed using an in-house human insulin-specific ImmunoCAP[®] ELISA (Figure 5.1), an assay developed by the Department of Immunology, Cambridge University Hospitals NHS Foundation Trust, Cambridge, UK. As an established NHS clinical laboratory test, this assay had been subject to verification locally, hence the analytical performance was not critically examined in this study.

Except where stated otherwise, insulin and C-peptide measurements were performed using the DiaSorin LIAISON[®] XL assay. Plasma adiponectin concentration was made using an in-house solid-phase, two-site dissociation-enhanced lanthanide fluorescence immunoassay (DELFI[®]).

5.2.4 Immunoprecipitation of plasma with polyethylene glycol

PEG precipitation studies were performed as outlined in Chapter 4, and insulin and C-peptide determinations in supernatant were performed. Insulin recovery was determined as the PEG supernatant

insulin concentration (assayed following a dilution 1:1 with 0.9% (w/v) saline-diluted sample) expressed as a percentage insulin concentration in PEG supernatant/saline dilution-matched sample. C-peptide recovery was calculated in the same way. Insulin dilutions were made with assay diluent, and dilutions for C-peptide analysis were made using 0.9% (w/v) saline as in-house studies demonstrated assay bias using DiaSorin LIAISON[®] Endocrinology Diluent in the C-peptide immunoassay. C-peptide recovery was determined in all seven patients in non-fasting plasma and eight control plasma samples.

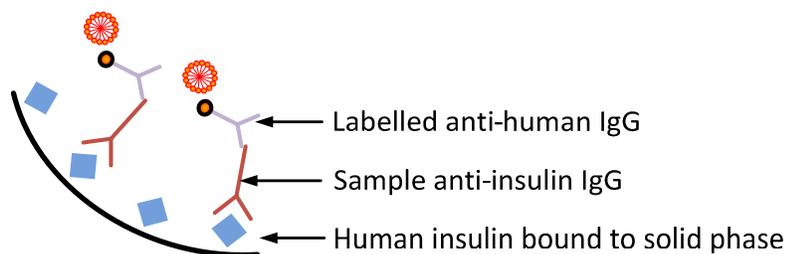


Figure 5.1 Test Principle of the ImmunoCAP[®] Specific method for IA (Modified from Thermo Fisher Scientific 2012) [404]. In this assay, endogenous IAs bind to immobilised human insulin, and following a wash step, a β -galactosidase–anti-IgG mouse monoclonal IgG subclass 1 conjugate (that binds to an epitope common to all human IgG subclass Fc regions) is added. Following incubation, unbound conjugate is washed away, then through addition of substrate 4-methylumbelliferyl- β -galactoside, fluorescence is measured using an excitation wavelength of 365 nm and an emission wavelength of 455 nm.

5.2.5 Gel filtration chromatography of plasma

GFC was performed as described in Chapter 4.

5.2.6 Competitive anti-insulin antibody radioimmunoassays

The radioimmunoassay methods were designed in close collaboration with Claire Williams and supervisor Alistair Williams, Diabetes & Metabolism, Translational Health Sciences, University of Bristol, Southmead Hospital, Bristol, UK, and all experiments directly involving radioimmunoassay were carried out by Claire Williams and Alistair Williams. Using an RIA currently used clinically to measure IAs [374, 375], quantification of IA was undertaken. Patient serum, 5 μ L neat, or diluted with IA-negative serum, was incubated for 72 hours with 3.75 fmol A14-iodine-125-labelled human insulin

(PerkinElmer®; diluted using 50 mmol/L Tris, 1% (v/v) Tween-20, pH 8.0 (TBT) buffer containing 1% (w/v) BSA (Sigma-Aldrich®)) with or without unlabelled synthetic human insulin at 40 µmol/L in a 96-well plate. A14-iodine-125-labelled human insulin–IA complexes were precipitated using 10 µL glycine-blocked Protein A–Sepharose® (PAS), and then experiments performed using ethanolamine-blocked Protein G–Sepharose® (PGS) (GE Healthcare) [376] and/or anti-IgA agarose–antibody (Sigma-Aldrich®) in 50 µL TBT. Following washing to remove unbound label, bound A14-iodine-125-labelled insulin was measured with a gamma counter. A standard curve was constructed using a serial dilution of pooled IA-positive human sera, and patient results were calculated as arbitrary units using a logarithmic curve fit. A positive result in the PAS assay was defined as >0.2 AU, a threshold determined the 97.5th percentile of 2860 healthy children [377]. This assay achieved 52% sensitivity at 97.8% specificity in the 2015 Islet Autoantibody Standardization Program (IASP) workshop.

5.2.7 Anti-insulin antibody affinity determination using radioimmunoassay

Serum was analysed for IA affinity using a PAS radioligand binding assay [377, 378]. This clinical laboratory assay was chosen as radioligand binding assays may have advantageous performance characteristics when used in the diagnosis of T1DM [395], and it was considered whether quantitative IA results could provide additional information about IA insulin-binding capacity and add value in the diagnostic process. Samples were diluted in IA-negative sera (ratios of sample:IA-negative serum were 1:9, 1:49, and 1:99), based on the binding demonstrated in the PAS RIA described in Section 2.5, to improve discrimination between samples.

In preparation for the IA affinity assays, samples were incubated with A14-iodine-125-labelled human insulin alone, or with a range of concentrations of soluble human insulin (Actrapid®) at 5.5×10^{-11} , 2.2×10^{-10} , 1.7×10^{-9} , 7×10^{-9} , 2.8×10^{-8} , 2.8×10^{-7} , and 4.0×10^{-5} mol/L) for 72 hours. Insulin dilutions were made using TBT buffer containing 1% (w/v) BSA. To detect all possible IA-reactive IgG autoantibodies based on the standard PAS assay results, insulin–antibody complexes were precipitated and measured with a 50:50 mixture of PAS and PGS to include all possible IA-reactive IgG antibodies. Due to limited sample volume, standard curves were not made, but three IA-positive serum samples were used as intermediate, and high affinity, antibody controls. Antibody binding was determined by radioactivity detection with a gamma counter. Using non-linear regression analysis (GraphPad Prism6,

GraphPad Software Inc.), inhibition concentration at 50% (IC_{50}), K_d calculations (mol/L) and reciprocal K_d (L/mol) were calculated. Antibodies were characterised according to K_d (mol/L), calculated using a one-site model [377].

5.2.8 Immunosubtraction using class-specific anti-human immunoglobulin–agarose

Insulin concentrations were estimated in plasma from patient A8, control plasma containing insulin-binding IgG, and three antibody-negative plasma control samples with similar ambient insulin concentrations, using the DiaSorin LIAISON[®] XL. Synthetic human insulin (Actrapid[®], diluted in 5% (w/v) BSA), 5 pmol, was added to 990 μ L (final volume 1000 μ L) of each sample before incubating on a roller mixer for 24 hours at 24°C. PEG precipitation of the insulin-spiked plasma from patient A8 was performed using the method outlined above. Insulin concentrations were measured for each insulin-spiked plasma sample using the DiaSorin LIAISON[®] XL assay, following a sample dilution of 1:4 with assay diluent.

Anti-human IgA–agarose antibody, anti-human IgM–agarose antibody, and anti-human IgG–agarose antibody (Table 5.1), were each washed three times with 0.9% (w/v) saline and stored at 4°C until use. Plasma:agarose ratios were based on in-house data [379], and the same dilutions of plasma were made in saline to act as control for insulin recovery calculation. Volume ratios of plasma:agarose antibody were as follows: anti-human IgA–agarose antibody 5:1 with plasma; anti-human IgM–agarose antibody 29:20 with plasma; anti-human IgG–agarose antibody 32:3 with plasma. Anti-human IgA–agarose antibody experiments for patient A8 were performed in triplicate. Using the same plasma samples, equal mixtures were made with saline for use as control matrix. Samples were then mixed on a tube roller mixer for 60 minutes prior to centrifugation at 13,200 g for 15 minutes. To overcome sampling error on the DiaSorin LIAISON[®] XL (likely caused by increased sample viscosity), agarose supernatant was diluted at a ratio of sample 4:1 with saline prior to analysis. Insulin recovery in supernatant was calculated as percentage insulin recovery (supernatant/saline, %). For the experiments using class-specific agarose, mean and standard deviation values for antibody-negative control plasma were calculated.

Table 5.1 Anti-human agarose–antibody preparations used for the class-specific immunosubtraction of anti-insulin antibodies

Name	Reference	Biological source	Antibody form	Clone	Conjugate
Anti-human IgA (α -chain specific)–agarose antibody	A2691 Sigma	Goat	Affinity-isolated antibody	Polyclonal	Agarose conjugate
Anti-human IgM (μ -chain specific)–agarose antibody	A9935 Sigma	Goat	Affinity-isolated antibody	Polyclonal	Agarose conjugate
Anti-human IgG (Fc Specific)–agarose antibody	A3316 Sigma	Goat	Affinity-isolated antibody	Polyclonal	Agarose conjugate

5.2.9 Quantitative mass spectrometric measurement of plasma insulin and C-peptide

Methods were designed in close collaboration with Dr Richard Kay, The University of Cambridge Metabolic Research Laboratories, Wellcome Trust-MRC Institute of Metabolic Science, Cambridge, UK, and experiments directly involving mass spectrometry were carried out by Richard Kay. Human plasma was thawed from frozen storage and enriched with insulin lispro (Humalog[®]) and C-peptide (Bachem) to generate final peptide concentrations of 8610 pmol/L and 16,548 pmol/L, respectively. To generate a standard curve, plasma was diluted in the same pooled plasma to generate insulin concentrations of 6890, 1720, 861, 172, 86, 34, and 17 pmol/L, and paired C-peptide concentrations of 13245, 3307, 1655, 331, 165, 65, and 33 pmol/L, respectively. Each sample of known peptide concentration, patient plasma (except that from patient A4), and blank (non-fortified) pooled plasma were transferred, at a volume of 250 μ L to a 96-well plate. Six patient, and 34 control samples were extracted with QC material and calibration samples. Protein precipitation solvent (80% (v/v) acetonitrile in water, with 1 ng/mL bovine insulin (Sigma-Aldrich[®])), at a volume of 1 mL was added, followed by thorough mixing to precipitate plasma proteins, with subsequent centrifugation at 2900 g for 10 minutes at 4°C. Supernatant was transferred to a 96-well plate (Eppendorf[™] Protein LoBind Deepwell[™] Fisher Scientific), and evaporated under a stream of nitrogen gas at 45°C. To reconstitute the residue, 200 μ L of 0.1% (v/v) formic acid was added, then the samples loaded directly onto a plate

(Oasis® PRiME HLB μ Elution, Waters) that was then transferred to a positive-pressure solid-phase extraction manifold (Waters), and the solutions passed through the sorbent slowly under pressure to facilitate extraction. Cartridges were washed with 200 μ L of 0.1% (v/v) formic acid, followed by 200 μ L of 5% (v/v) methanol with 1% (v/v) acetic acid, and peptides eluted with 2 x 30 μ L of 60% (v/v) methanol with 10% (v/v) acetic acid in water. Prior to injecting 50 μ L sample onto the liquid chromatography–mass spectrometry (LC–MS) system, 75 μ L of 0.1% (v/v) formic acid was added. At a flow rate of 300 μ L per minute (UltiMate™ 3000 system, Thermo Fisher Scientific), peptides were loaded onto a 50 x 2.1 mm reverse-phase column (AQUITY UPLC® HSS T3 C₁₈, Waters). Starting conditions were 22% B (0.1% (v/v) ACN with 0.1% (v/v) formic acid) and 78% A (0.1% (v/v) formic acid in water), then B was increased to 32% after 6.4 minutes. Before returning to starting conditions for the duration of 2 minutes, the column was washed at 90% B for 1.6 minutes.

MS was performed using a Q Exactive Plus Orbitrap system (Thermo Fisher Scientific) using a heated electrospray ionisation source (positive electrospray mode), with a needle voltage of 3 kV, gas flow rates of 55 and 10 for sheath gas and aux gas. The aux gas was set to a temperature of 350°C, the transfer capillary to 350°C, and an s-lens value set to 70V. MS data were acquired from m/z 700-1600, with a resolution of 70,000, and an automatic gain control target of 3e6 ions. Using the standards described above, insulin and C-peptide calibration curves were generated using m/z values for the [M+5H]⁵⁺ charge states relating to the monoisotopic and multiple ¹³C isotopes of human insulin (1161.7362), and for the [M+3H]³⁺ charge state of C-peptide (1007.1783). After correcting for endogenous analyte, calibration curves for insulin and C-peptide gave a linear fit with R² values of 0.995 and 0.994, respectively, and calibration standards and QC samples were all within $\pm 25\%$ of expected values. Regression between immunoassay and MS control plasma values were linear for insulin (0.8727x-27.025; R²=0.974), and C-peptide (1.317x-56.86; R² = 0.997).

5.3 Results

Clinical characteristics of the seven patients studied are listed in Appendix B: Clinical characteristics of patents without pre-existing DM. Individual case histories are described in more detail below. (The clinical features and results for patient A2 are given in Section 4 and are not further described in this section.)

5.3.1 Patient A1:

A 56-year old Caucasian woman with a BMI of 26.2 kg/m² presented with a 20-month history of tremor, sweating, pallor, and confusion, typically occurring 1-2 hours following meals, symptoms that ceased following carbohydrate consumption. Since the onset of symptoms, she had noted a 7 kg bodyweight increase. She had a past medical history of autoimmune thyroiditis, asthma, and factor XI deficiency, and was not taking any regular medications. Except for livedo reticularis on both legs, clinical examination findings were normal.

During emergency department attendance the patient was confirmed to have hyperinsulinaemic hypoglycaemia, with a plasma glucose concentration of 1.7 mmol/L (RL 4.0–6.1), insulin 267 pmol/L (RL <60) (Siemens ADVIA Centaur[®]) and C-peptide 899 pmol/L (RL 174–960) (Siemens IMMULITE[®] 2000). Calculated insulin:C-peptide molar ratio was increased at 0.30 (RL 0.03–0.25) [81, 107]. The patient underwent further endocrine investigation, including a 72-hour fast and mixed meal test that did not cause hypoglycaemic symptoms, and the glucose nadir was not significant at 3.3 mmol/L. A 75-g oral glucose tolerance test (OGTT) did provoke symptomatic hypoglycaemia, with a glucose nadir of 2.2 mmol/L at 240 minutes (Figure 5.2a), prior to which, rescue with intravenous glucose was required to prevent the patient losing consciousness. During a typical day, CGMS of interstitial fluid confirmed glycaemic lability, notably late postprandial hypoglycaemia (Figure 5.3a). Imaging investigations, namely computerised tomography (CT), magnetic resonance imaging (MRI), and endoscopic ultrasound, did not reveal a pancreatic mass. During one of the episodes of hypoglycaemia, beta-hydroxybutyrate concentration was 0.1 mmol/L (RL 0.03–0.3), and serum sulphonylurea screen was negative. Insulin-like growth factor-binding protein 3, insulin-like growth factor 1, insulin-like growth factor 2, gastrin, glucagon, and pituitary function tests, were normal. IA level was 722.40 U/mL (RL

<0.4) (RiaRSR® IAA, Cardiff, UK) and anti-insulin receptor, anti-islet cell, anti-GAD65, and anti-IA2 antibodies were not detected.

Samples were referred to the Institute of Metabolic Science for specialist biochemical investigation. Gross hyperinsulinaemia was confirmed in non-fasting plasma using MS, and immunoassay insulin recovery was very low following PEG precipitation (Table 5.2), consistent with HMW insulin immunoreactivity and suggestive of insulin–antibody complexes. Plasma adiponectin concentration was within reference limits. IAs were detectable using the human insulin-specific ImmunoCAP® ELISA (Table 5.2), and GFC with preincubation of exogenous human insulin demonstrated an increase in HMW insulin immunoreactivity from baseline consistent with insulin-binding by antibody, confirming the diagnosis of IAS (Figure 4.6b).

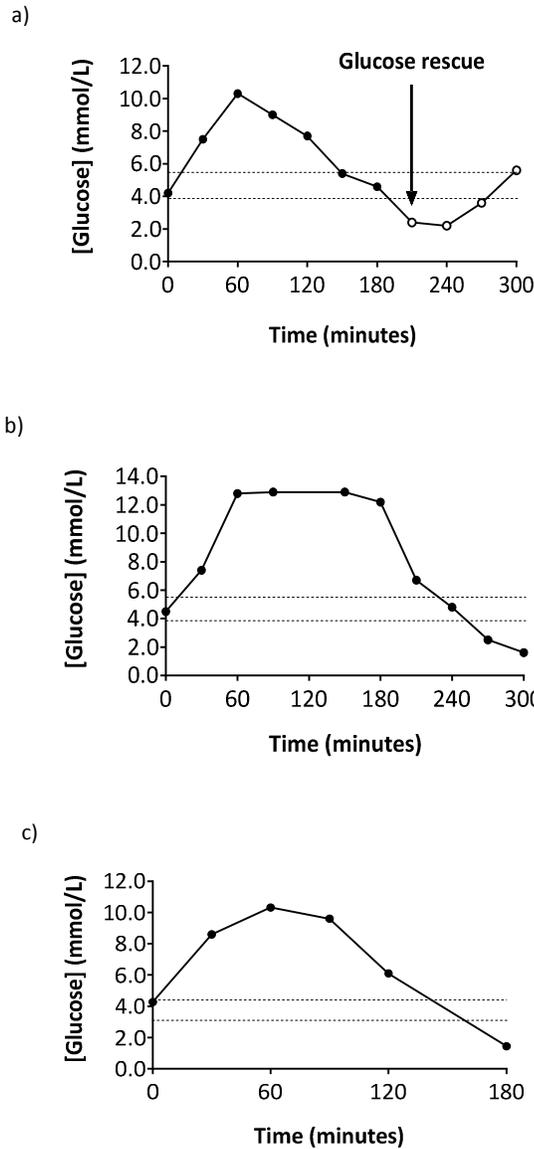
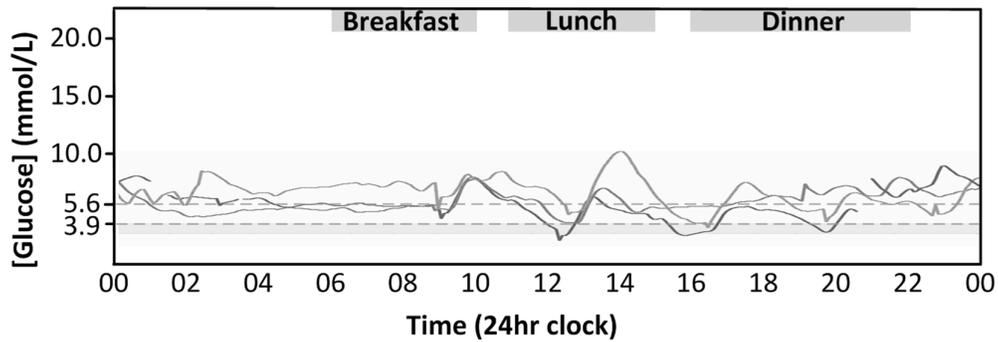


Figure 5.2 Reactive hypoglycaemia in patient A1, patient A6, and patient A8. (a) Venous plasma glucose concentrations against time during 75-g OGTT at presentation of patient A1; ○ represents venous plasma glucose measurement following glucose rescue. Glucose nadir was 2.2 mmol/L. (b) Demonstration of reactive hypoglycaemia in patient A6 at presentation. Venous plasma glucose concentrations against time during a mixed meal test. The peak glucose concentration was 12.9 mmol/L with hypoglycaemia at 300 minutes with a glucose nadir of 1.6 mmol/L. (c) Demonstration of reactive hypoglycaemia in patient A8 at presentation. Venous plasma glucose concentrations against time during a 75-g OGTT at presentation (●). Glucose nadir was 1.4 mmol/L.

a)



b)



Figure 5.3 Glycaemia of patient A1 pre- and post-immunomodulatory therapy. (a) Demonstration of labile glycaemia in patient A1 at presentation by Continuous Glucose Monitoring System. (b) Demonstration of normoglycaemia in patient A1 following immunomodulation therapy.

RIA studies confirmed very high insulin-binding capacity by antibody (Table 5.2). Results from competitive insulin-binding studies using a 1 in 10 serum dilution were consistent with high-affinity antibodies with sub-nanomolar dissociation constant (Table 5.2).

The patient was initially treated with two 1 g intravenous doses of methylprednisolone, one day apart monthly, for four months, before reducing the dose, and ultimately stopping the drug. There was a modest decrease in IA concentration from 16 mg/L at presentation to 7 mg/L, and insulin recovery

increased from 8% to 19%, however without clinical improvement. Symptoms continued for a further two years, and hypoglycaemia was demonstrable with follow-up OGTT and CGMS. In view of this, rituximab was administered intravenously 50 mg/m² body surface area in two consecutive doses two weeks apart, with good clinical response. The patient recorded only two CBG readings <3.1 mmol/L and CGMS demonstrated decreased glycaemic lability (Figure 5.3b). IA measurement was repeated at the patient's local hospital and demonstrated a decreased level of 152.9 U/ml (RL<0.4) (RiaRSR[®] IAA), and corresponding fasting plasma insulin of 173 pmol/L (RL <60) (Siemens ADVIA Centaur[®]), and C-peptide concentration 500 pmol/L (RL 174–960) (Siemens IMMULITE[®] 2000), respectively.

5.3.2 Patient A3:

A 37-year old Caucasian woman with a BMI of 28.5 kg/m² presented to the emergency department with loss of consciousness. Emergency attendants measured CBG, and glucose concentration was below the glucose meter detection limit. Following administration of intravenous and oral glucose, venous blood was taken and a laboratory plasma glucose measurement of 2.8 mmol/L was recorded. Previously, the patient had had six pregnancies: during the last two she had been treated for gestational diabetes with aspart (NovoRapid[®]), glargine (Lantus[®]), and metformin. For ten months, and since the birth of her last child, the patient had experienced recurrent episodes of dizziness, during some of which she had taken CBG readings, recording glucose concentrations of 2–3 mmol/L. Symptoms resolved with oral carbohydrate intake. The patient had intentionally lost weight, achieving a 15 kg reduction with diet and exercise, and had ascribed the low CBG readings to reduced dietary intake. She had a past medical history of persistent lupus anticoagulant following a pulmonary embolism during a previous pregnancy. She did not have any relevant family history, was not prescribed any medication, and denied taking insulin. Except for hypertension, with a blood pressure of 151/83mmHg, clinical examination findings were normal.

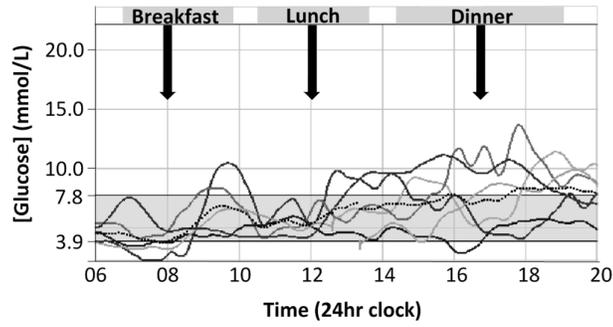
During hospital admission, CGMS demonstrated matutinal hypoglycaemia and marked postprandial hyperglycaemia (Figure 5.4a), and the patient had decreased awareness of hypoglycaemia. During an episode of hypoglycaemia, venous blood samples were analysed by the laboratory, and reported a plasma insulin of 39,181 pmol/L (Abbott ARCHITECT) and a C-peptide of 1046 pmol/L (Abbott ARCHITECT). Calculated insulin:C-peptide molar ratio was markedly increased at 37.46 (RL

0.03–0.25). Urine sulphonylurea screen was negative. Full blood count, renal function tests, thyroid function tests, liver enzymes, and C-reactive protein were all within reference limits, and cortisol response was normal during a short tetracosactide (synACTHen®) test. HbA_{1c} concentration was normal at 41 (20–42) mmol/mol. The patient was provided with CGMS that included a hypoglycaemia alert, glucagon for injection in case of severe hypoglycaemia, and advised a low glycaemic-index carbohydrate diet. Despite these interventions, hypoglycaemia persisted, and CBG readings were between 2.0 mmol/L to 3.5 mmol/L.

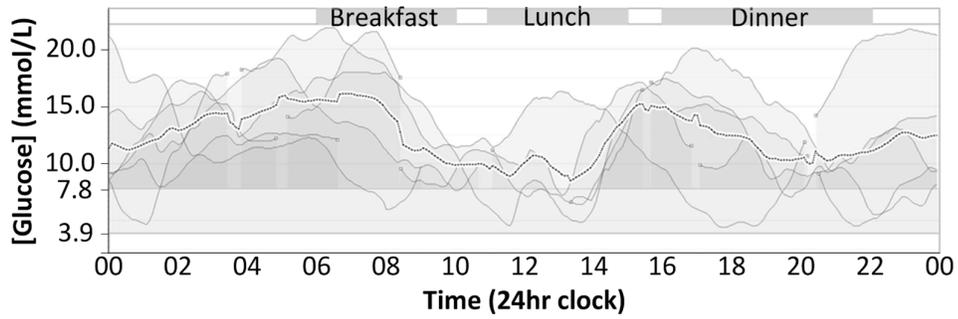
Samples were referred to the Institute of Metabolic Science for specialist biochemical investigation. Gross hyperinsulinaemia was confirmed in non-fasting plasma using MS, and immunoassay insulin recovery was very low following PEG precipitation (Table 5.2), consistent with HMW insulin immunoreactivity and suggestive of insulin–antibody complexes. Plasma adiponectin concentration was within reference limits. IAs were detectable using the human insulin-specific ImmunoCAP® ELISA (Table 5.2), and GFC with preincubation of insulin aspart demonstrated insulin exchange/insulin binding by antibody, confirming the diagnosis of IAS (Figure 4.7b; Figure 4.8).

RIA studies undertaken at Translational Health Sciences, University of Bristol, Southmead Hospital, confirmed extremely high insulin-binding capacity by antibody (Table 5.2), such that it exceeded the standard curve at serum dilutions of 1 in 50. Results from competitive insulin-binding studies were consistent with high-affinity antibodies with sub-nanomolar dissociation constant (Table 5.2).

a)



b)



~ Sensor trace ~ Interrupted ~ Average

c)

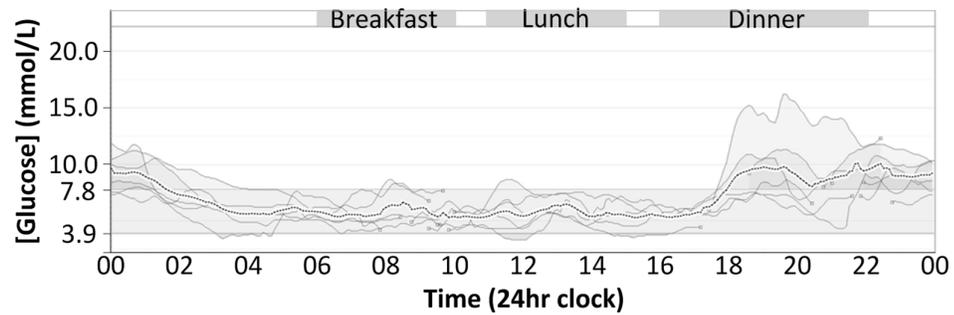


Figure 5.4 Variable patterns of dysglycaemia of patient A3. Continuous glucose monitoring at presentation demonstrating labile glycaemia (a); day 59 post-rituximab, concurrent with prednisolone therapy demonstrating continued labile glycaemia but reduced hypoglycaemia (b); and day 271 concurrent with prednisolone therapy demonstrating a marked improvement in daytime glucose fluctuation but with nocturnal hyperglycaemia (c).

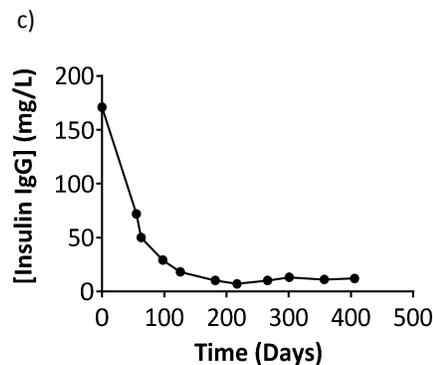
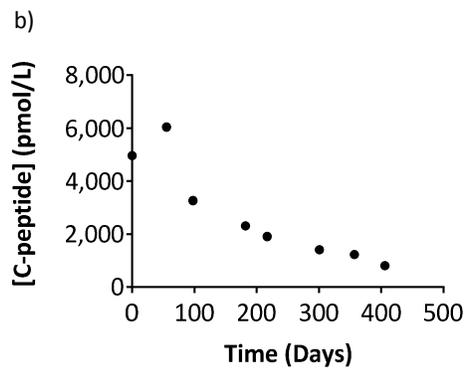
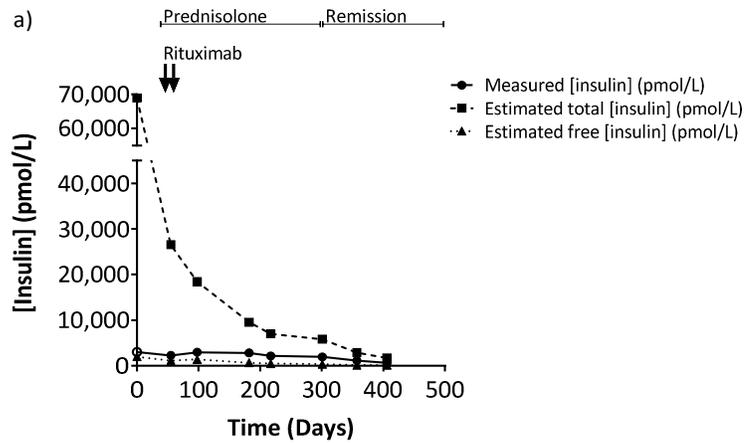


Figure 5.5 Treatment and progression timeline with plasma insulin (a), C-peptide (b) and anti-insulin antibody concentration (c). Day 0 is the day of presentation; high-dose prednisolone was commenced on day 44; rituximab was administered on days 44 and 58. Insulin measurement in neat plasma (measured insulin concentration); insulin concentration following measurement after sample dilution (ratio sample:diluent of 1:49) to combat negative interference by antibodies (see Section 4.3.1), and back-calculation; insulin measurement in PEG supernatant and back-calculation (estimated free insulin). C-peptide measurement in neat plasma. Anti-insulin IgG concentration measured in neat serum.

Following the diagnosis of IAS, the patient was treated with 80 mg prednisolone daily, and two 1 g Intravenous rituximab infusions were administered two weeks apart. The glucocorticoid was then dose-reduced as hypoglycaemic symptoms improved. To re-evaluate glucose control, CGMS was undertaken on day 60 concurrent with glucocorticoid therapy, which showed labile glycaemia (Figure 5.4b), although the frequency of severe hypoglycaemic episodes possibly reduced. Over the following 14 months, the IA, insulin, and C-peptide concentration decreased (Figure 5.5a–c), and there was a reduction in the frequency of hypoglycaemic episodes. Six months following rituximab, CGMS was repeated and showed markedly decreased glycaemic lability (Figure 5.4c), hypoglycaemia was a rare event, but nocturnal hyperglycaemia was notable and may be attributable to evening glucocorticoid therapy. At ten months, prednisolone was stopped. Although her bodyweight had increased by around 16 kg since starting glucocorticoid therapy, the patient was able to lose this extra weight subsequently. To date, the patient has had no further hypoglycaemia.

5.3.3 Patient A4:

A 52-year old Thai woman with a body mass of 35.0 kg/m² presented with hypoglycaemic symptoms, that included syncope, occurring during fasting. She had no past medical history, and a family history of T2DM. She was not taking any medications. Except for acanthosis nigricans of the neck skinfolds, clinical examination findings were normal. During a prolonged fast, the patient became symptomatically hypoglycaemic after 10 hours, and laboratory testing of plasma determined a venous plasma glucose of 1.9 mmol/L, insulin concentration of 68,123 pmol/L (Siemens IMMULITE® 2000), and C-peptide of 3690 pmol/L (Siemens IMMULITE® 2000). Calculated insulin:C-peptide molar ratio was markedly increased at 18 (RL 0.03–0.25). Sulphonylurea screen was negative. Imaging studies, that included a ⁶⁸Ga-DOTATATE positron emission tomography (PET) scan did not identify a neuroendocrine tumour. Anti-nuclear (ANA), anti-extractable nuclear antigen (ENA), anti-neutrophil cytoplasmic (ANCA) and anti-double stranded DNA (dsDNA) antibodies were negative.

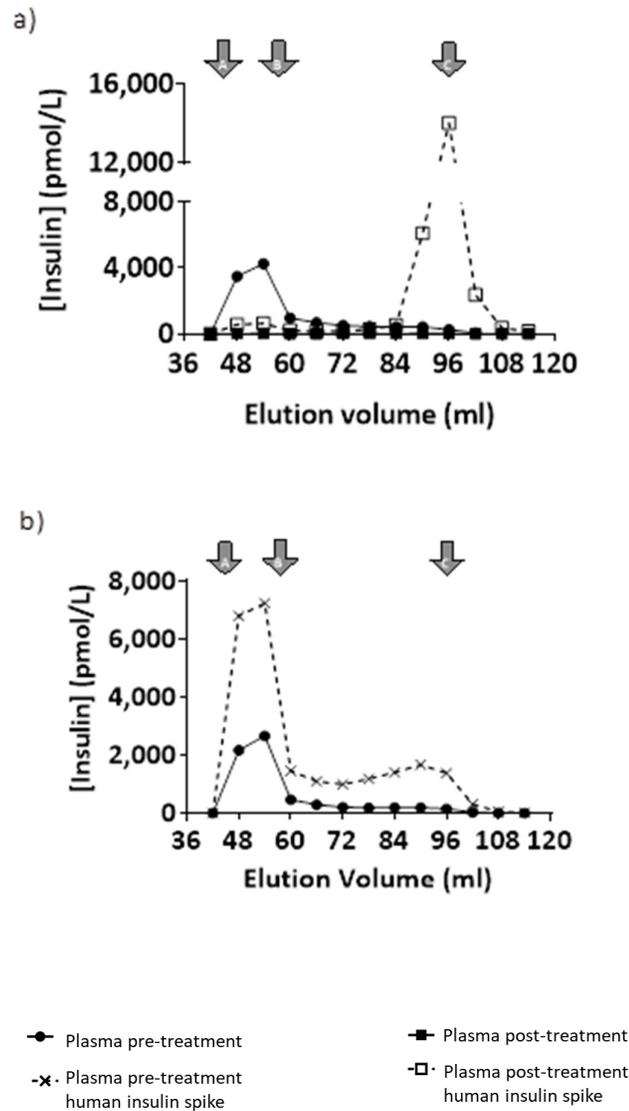


Figure 5.6 Demonstration of insulin–antibody complexes using gel filtration chromatography. Elution volumes of immunoglobulin (A), albumin (B) and monomeric insulin (C) are shown. Insulin concentrations were measured using the DiaSorin LIAISON® XL. Results of insulin assay after GFC of non-fasting plasma. Results from plasma at presentation, and from plasma pre- and post-human insulin addition following immunomodulation, from patient A4 are shown (a). Results from plasma at presentation pre- and post-insulin addition from patient A5 (b) are also shown.

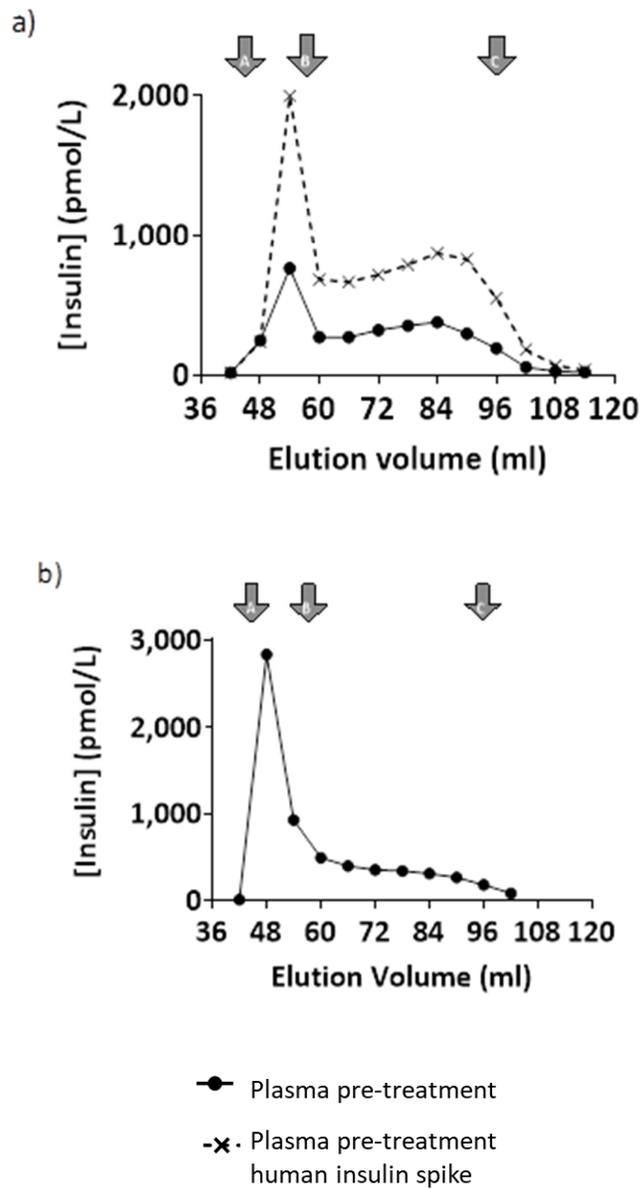


Figure 5.7 Demonstration of insulin-antibody complexes using gel filtration chromatography. Elution volumes of immunoglobulin (A), albumin (B) and monomeric insulin (C) are shown. Insulin concentrations were measured using the DiaSorin LIAISON® XL. Results of insulin assay after GFC of non-fasting plasma. Results from plasma at presentation pre- and post-insulin addition from patient A6 (c), and from plasma at presentation from patient A7 (d) are shown.

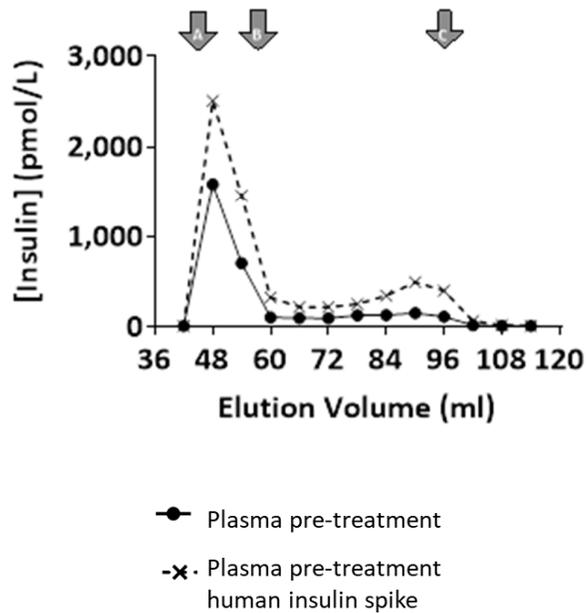


Figure 5.8 Demonstration of insulin–antibody complexes using gel filtration chromatography. Elution volumes of immunoglobulin (A), albumin (B) and monomeric insulin (C) are shown. Insulin concentrations were measured using the DiaSorin LIAISON[®] XL. Results of insulin assay after GFC of non-fasting plasma. Results from plasma at presentation pre- and post-insulin addition from patient A8 are shown.

Samples were referred to the Institute of Metabolic Science for specialist biochemical investigation. Based on clinical findings and degree of hyperinsulinaemia, investigations for TB-IR were undertaken in the first instance, however neither anti-insulin receptor IgG nor IgM were detected using the Western blot-based method described in Chapter 7 (25). Gross hyperinsulinaemia was confirmed in non-fasting plasma using MS, and immunoassay insulin recovery was very low following PEG precipitation (Table 5.2) consistent with HMW insulin immunoreactivity and suggestive of insulin–antibody complexes. Plasma adiponectin concentration was increased. IAs were detectable using the human insulin-specific ImmunoCAP[®] ELISA (Table 5.2), and GFC demonstrated HMW insulin immunoreactivity consistent with insulin-binding by antibody, confirming the diagnosis of IAS (Figure 5.6a).

RIA studies undertaken at Translational Health Sciences, University of Bristol, Southmead Hospital, confirmed very high insulin-binding capacity by antibody (Table 5.2). Results from

competitive insulin-binding studies using a 1 in 10, or 1 in 50, dilution of serum, were consistent with high-affinity antibodies with nanomolar dissociation constant (Table 5.2).

Pending the definitive diagnosis, the patient was initially treated with diazoxide, but this did not result in clinical improvement and led to neutropenia, prompting its discontinuation. Following diagnosis, prednisolone 30mg daily was commenced with MMF added as a steroid-sparing agent. After 4 weeks of therapy, hypoglycaemic symptoms resolved and the blood tests were repeated. IgG IAs had reduced to 5 mg/L, plasma insulin to 322 pmol/L, and C-peptide to 1210 pmol/L, however insulin recovery following PEG precipitation, although at 17% had somewhat increased from presentation, remained below normal. Following resolution of symptoms, GFC with and without preincubation of plasma with exogenous human insulin demonstrated a decrease in HMW insulin immunoreactivity from presentation consistent with a reduction of insulin binding by antibodies (Figure 5.6a). The patient has not had further hypoglycaemia for 12 months, remaining off all immunosuppressive therapy for 6 months thus far.

5.3.4 Patient A5:

A 28-year old Caucasian woman with a BMI of 25.1 kg/m² presented with recurrent episodes of anxiety, confusion, and perioral paraesthesia with generalised diaphoresis, that principally occurred in the fasting state. She would awaken from sleep with feelings of terror. Symptoms would terminate rapidly following carbohydrate ingestion. Concurrent with symptoms, emergency medical attendants recorded CBG readings of 2.0 and 2.4 mmol/L. She had no past medical history, and was not taking any medication. Clinical examination findings were normal. During a prolonged fast, the patient became symptomatically hypoglycaemic after 4 hours, with a venous plasma glucose of 2.2 mmol/L, plasma insulin of 17,800 pmol/L (Mercodia Iso-Insulin ELISA) and C-peptide concentration of 409 pmol/L (Mercodia C-peptide ELISA). Calculated insulin:C-peptide molar ratio was markedly increased at 44 (RL 0.03–0.25). HbA_{1c} concentration was normal at 35 mmol/mol (RL 20–42). Findings from a ⁶⁸Ga-DOTATATE PET/CT scan did not reveal a neuroendocrine tumour.

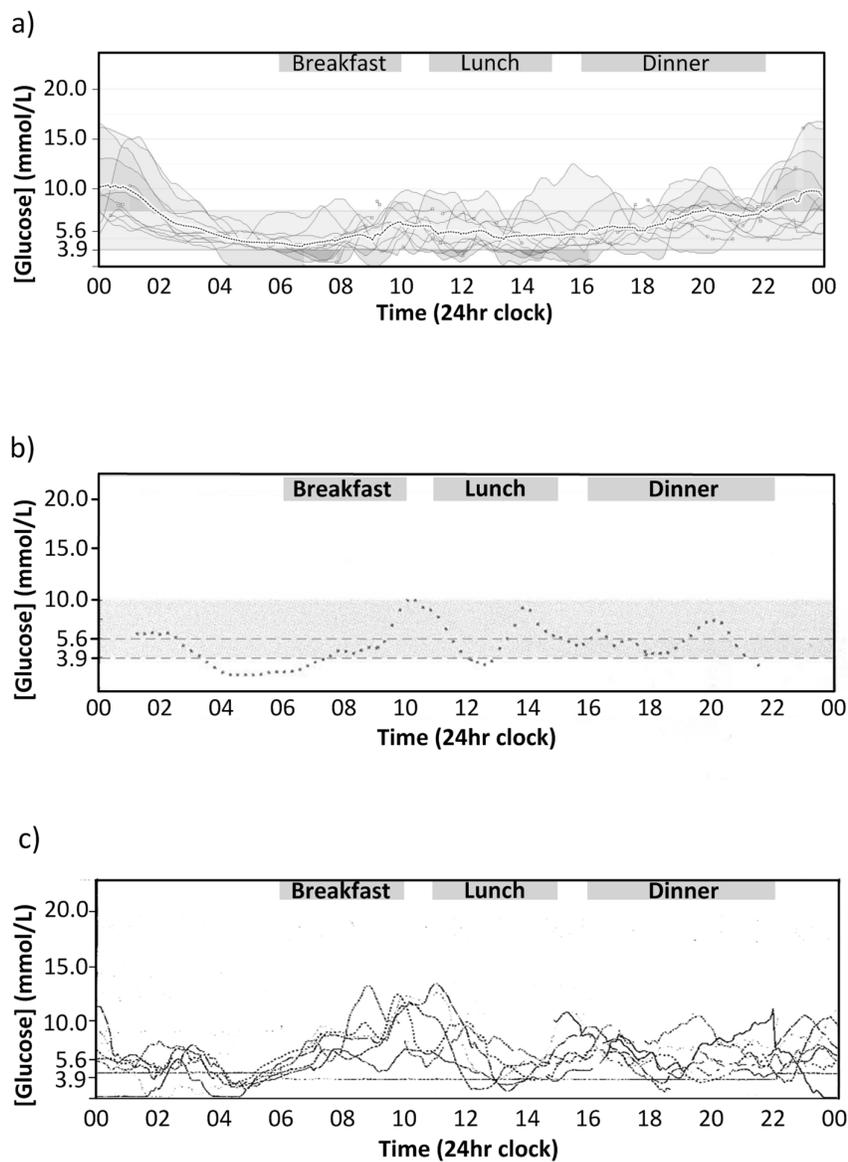


Figure 5.9 Variable patterns of dysglycaemia at presentation of patient A5, patient A6, and patient A8. Demonstration of labile glycaemia in patient A5 at presentation (a). Demonstration of reactive and nocturnal hypoglycaemia in patient A6 at presentation (b). Demonstration of labile glycaemia in patient A8 at 4 months following presentation concurrent with glucocorticoid therapy (c).

Samples were referred to the Institute of Metabolic Science for specialist biochemical investigation. Gross hyperinsulinaemia was confirmed in non-fasting plasma using MS, and immunoassay insulin recovery was non-linear with dilution and very low following PEG precipitation (Table 5.2) consistent with HMW insulin immunoreactivity and suggestive of insulin-antibody complexes. Plasma adiponectin concentration was above reference limits. IAs were detectable using the

human insulin-specific ImmunoCAP® ELISA (Table 5.2), and GFC with preincubation of exogenous human insulin demonstrated an increase in HMW insulin immunoreactivity from baseline consistent with insulin binding by antibody, confirming the diagnosis of IAS (Figure 5.6b).

RIA studies undertaken at Translational Health Sciences, University of Bristol, Southmead Hospital, confirmed extremely high insulin-binding capacity by antibody (Table 5.2) such that it exceeded the standard curve at serum dilutions of 1 in 100. Results from competitive insulin-binding studies were consistent with high-affinity antibodies with sub-nanomolar dissociation constant (Table 5.2).

The patient was initially treated with diazoxide, but this did not result in clinical improvement and the drug led to a rise in serum liver enzyme levels resulting in its discontinuation. Following diagnosis of IAS, prednisolone 60 mg daily was commenced and later changed to dexamethasone 8 mg twice daily, and MMF was later added as a steroid-sparing agent. Labile glycaemia, with both hyper- and hypoglycaemia, was demonstrable on CGMS (Figure 5.9a). MMF led to nausea and raised serum liver enzyme levels, and the drug was stopped and replaced with azathioprine 50 mg twice daily. Similar to patient A3 but in contrast to the response by patient A4, this patient required high-dose glucocorticoid treatment to reduce the frequency of hypoglycaemic episodes. She developed complications of the steroid treatment, including Cushingoid appearance, depression, and avascular necrosis of the hip. In view of the significant side effects of glucocorticoid treatment, 1 g rituximab was given for two doses, and dexamethasone weaned to 1 mg daily. Follow-up blood test results did not support the depletion of IAs (Figure 5.10), and dysglycaemia was still present with the patient continuing to have repeated episodes of symptomatic hypoglycaemia, and as a result, dexamethasone was up-titrated. In view of this, and to confirm the clinical effect of antibody-depletion in this individual to help guide further therapy, the patient underwent TPE (3 times weekly, on three occasions), that led to complete resolution of fasting hypoglycaemia, and removal of ELISA-detectable anti-insulin IgG, increased insulin recovery following PEG-precipitation, and improved insulin immunoassay linearity (Figure 5.11). This demonstrable clinical and biochemical improvement was transient but served as evidence to support the rationale for further immunodepletion therapy. Rituximab, at a dose of 750 mg/m² for 4 doses, was then administered and some improvement in hypoglycaemic symptoms did follow, however this patient did

not experience complete resolution of symptoms, and after a period of 6 months, a further 8 TPE sessions were performed, and rituximab 750 mg/m² for 4 doses was administered. Six months following rituximab, the patient no longer requires glucocorticoid therapy, but continued taking azathioprine, and no longer experienced fasting hypoglycaemia, however may experience reactive hypoglycaemia that is managed with dietary alterations.

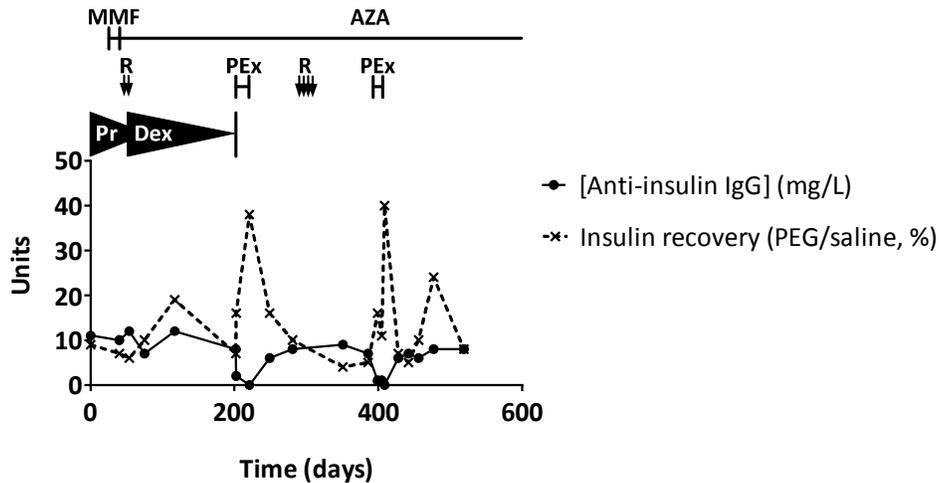


Figure 5.10 Cumulative results for patient A5 following treatment. Anti-insulin IgG concentrations (in-house human insulin-specific ImmunoCAP[®]), and insulin recovery following PEG precipitation, over time. PEG precipitation studies were performed to illustrate insulin binding by antibody. Insulin recovery is presented as percentage concentration of PEG supernatant/saline dilution. Insulin concentrations were measured using the DiaSorin LIAISON[®] XL. Abbreviations: azathioprine, AZA; dexamethasone, Dex; mycophenolate mofetil, MMF; plasma exchange, PEx; prednisolone, Pr; rituximab, R.

5.3.5 Patient A6:

A 76-year old Caucasian man with a BMI of 29.5 kg/m² presented following an 8-month history of episodes of severe sweating, headache, hunger, and decreased mental clarity. He had a past medical history of ischaemic heart disease, chronic obstructive pulmonary disease, and glaucoma. His regular medications were fluoxetine, omeprazole, naproxen, and timolol eye drops. Initially, he had consulted a neurologist who identified hypoglycaemia, and the patient was referred to an endocrinologist for investigation. A few days following initial consultation with endocrinology, the patient had a myocardial infarction and underwent coronary artery bypass surgery, and was then prescribed frusemide, spironolactone, bisoprolol and losartan for chronic heart failure.

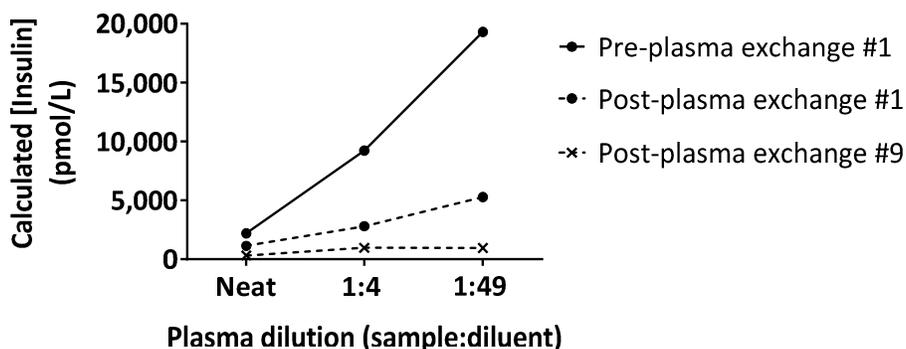


Figure 5.11 Effect of plasma exchange on insulin immunoassay linearity to dilution. Calculated insulin concentration plotted against plasma dilution of patient A5 plasma before plasma exchange and following cycle 1 and cycle 9. Insulin measurements were made using the DiaSorin LIAISON® XL.

Concomitant with an episode of spontaneous hypoglycaemia, an insulin of 1732 pmol/L (Roche Cobas® 6000), and a C-peptide of 794 pmol/L (Siemens IMMULITE® 2000) was measured. The calculated insulin:C-peptide molar ratio was markedly increased at 2.18 (RL 0.03–0.25). Over the course of two separate 72-hour fasts, a venous plasma glucose nadir of 2.5 mmol/L was recorded. During a mixed meal test, post-challenge hyperglycaemia with a peak plasma glucose of 12.9 mmol/L, and hyperinsulinaemic hypoglycaemia at 300 minutes (Figure 5.2b) with an insulin concentration of >6945 pmol/L (Roche Cobas® 6000) and a glucose concentration of 1.6 mmol/L (C-peptide was not assayed), was measured. To alert the patient of hypoglycaemia, he was given a portable glucose sensor. CGMS confirmed labile glycaemia, demonstrated by recurrent post-prandial hyperglycaemia and nocturnal hypoglycaemia (Figure 5.9b). An insulinoma was not identified with MRI, endoscopic pancreatic ultrasound, or octreotide scanning with single photon emission CT. A left parotid gland pleomorphic adenoma was identified using PET with fluorodeoxyglucose, but there was no pathological signal in the pancreas or elsewhere.

Samples were referred to the Institute of Metabolic Science for specialist biochemical investigation. Gross hyperinsulinaemia was confirmed in non-fasting plasma using MS, and immunoassay insulin recovery was non-linear with dilution and very low following PEG precipitation

(Table 5.2) consistent with HMW insulin immunoreactivity and suggestive of insulin–antibody complexes. Plasma adiponectin concentration was within reference limits. IAs were detectable using the human insulin-specific ImmunoCAP® ELISA (Table 5.2), and GFC with preincubation of exogenous human insulin demonstrated an increase in HMW insulin immunoreactivity from baseline consistent with insulin binding by antibody, confirming the diagnosis of IAS (Figure 5.7a).

RIA studies undertaken at Translational Health Sciences, University of Bristol, Southmead Hospital, demonstrated low insulin-binding capacity by antibody using the PAS assay (Table 5.2), but with high levels using PGS (data not shown), and these findings are consistent with IAS due to insulin-binding IgG3. Results from competitive insulin-binding studies were consistent with low affinity antibodies with micromolar dissociation constant (Table 5.2).

The patient was initially treated with 50 mg diazoxide three times daily, and the frequency and severity of hypoglycaemic episodes were reduced. After 6 months, hypoglycaemia continued to adversely affect the man’s life, and once monthly subcutaneously lanreotide 60 mg was added. The medication was stopped after it caused significant gastrointestinal side effects, and the patient was also unable to tolerate acarbose. Over the course of three years, the patient received 100 mg diazoxide three times daily, and a HbA_{1c} concentration of 55 mmol/mol (RL 20–42) was measured. The patient developed chronic heart failure that responded to diuretic therapy. Although prednisolone and rituximab treatment were initially declined, immunomodulation is currently being considered by the patient.

5.3.6 Patient A7:

An 89-year old Caucasian woman with a BMI of 19.4 kg/m² presented to hospital with recurrent falls, cognitive decline, and chronic venous leg ulcers. She had a past medical history of small B cell lymphoma, IgM paraprotein, and anaemia, and was taking frusemide, fexofenadine, and ferrous fumarate. During the initial in-patient stay, borderline low CBG concentrations down to 2.8 mmol/L, as well as concentrations as high as 13.8 mmol/L consistent with DM, were recorded during admission, however no hypoglycaemic symptoms were reported. Concurrent with a blood glucose of 2.1 mmol/L there was an insulin concentration of 1024 pmol/L (Abbott ARCHITECT) with concomitant C-peptide of 679 pmol/L (Abbott ARCHITECT). Calculated insulin:C-peptide molar ratio was markedly increased

at 1.51 (RL 0.03–0.25). Serum cortisol concentration increase in response to synthetic ACTH-stimulation was normal.

Samples were referred to the Institute of Metabolic Science for specialist biochemical investigation. Gross hyperinsulinaemia was confirmed in non-fasting plasma using MS, and immunoassay insulin recovery was non-linear with dilution and very low following PEG precipitation (Table 5.2) consistent with HMW insulin immunoreactivity and suggestive of insulin–antibody complexes. Plasma adiponectin concentration was increased. IAs were detectable using the human insulin-specific ImmunoCAP® ELISA (Table 5.2), and GFC of plasma demonstrated HMW insulin immunoreactivity consistent with insulin-binding by antibody, confirming the diagnosis of IAS (Figure 5.7b).

RIA studies undertaken at Translational Health Sciences, University of Bristol, Southmead Hospital, demonstrated high insulin-binding capacity by antibody using the PAS assay (Table 5.2), and increased levels with PGS (data not shown). Results from competitive insulin-binding studies were consistent with low affinity antibodies with micromolar dissociation constant (Table 5.2).

The patient was closely monitored as an in-patient, and with avoidance of long periods of fasting, CBG concentrations were satisfactory and the patient remained asymptomatic. She declined further investigation and was discharged to residential care with a CBG meter and advice to avoid prolonged fasting. Four months later, the patient was admitted to hospital with decreased consciousness, confusion, slurred speech, and a CBG of 1.3 mmol/L was recorded. Blood glucose was normalised with administration of intravenous 10% dextrose. A sulphonylurea screen was negative and no further investigations were undertaken. The patient commenced prednisolone 10 mg daily, was provided with glucose gel in case of hypoglycaemia, and she was discharged from in-patient care with advice for routine CBG monitoring.

5.3.7 Patient A8:

A 50-year old Caucasian man with a BMI of 22.3 kg/m² presented with two episodes of loss of consciousness due to hypoglycaemia, both times at which emergency medical attendants recorded low CBG. He had no past medical history and was not taking any medications. Liver enzymes, and tests of

renal and pituitary function, were within normal limits. Full blood count was normal but for a low haemoglobin concentration of 136 g/L (137–172). Hypoglycaemia was not provoked during either of two 72-hour fasts, and the lowest blood glucose concentration recorded was 4.0 mmol/L. Glucagon was administered after the second fast that did not markedly increase plasma glucose concentrations (rise from 4.1 mmol/L to 4.6 mmol/L). At 180 minutes during OGTT, a plasma glucose nadir of 1.4 mmol/L (Figure 5.2c) was recorded, concomitant with an insulin of 1285 pmol/L (Siemens IMMULITE® 2000 XPi) and a C-peptide of 1006 pmol/L (Siemens IMMULITE® 2000 XPi). Calculated insulin:C-peptide molar ratio was markedly increased at 1.28 (RL 0.03–0.25). The patient required intravenous dextrose administration to normalise blood glucose. Sulphonylurea screen was negative, and CT scan did not reveal any pancreatic masses. Based on clinical features and raised insulin:C-peptide ratio, IAS was suspected, daily prednisolone 60 mg with diazoxide 300 mg was commenced, and the patient was advised to avoid carbohydrate intake completely. To monitor for episodes of hypoglycaemia, the patient was provided a CGMS device. IA results were negative by two RIA methods (the first a PEG precipitation method; the second a protein A/G precipitation method).

Samples were referred to the Institute of Metabolic Science for specialist biochemical investigation. Gross hyperinsulinaemia was confirmed in non-fasting plasma using MS, and immunoassay insulin recovery was non-linear with dilution and low following PEG precipitation (Table 5.2) consistent with HMW insulin immunoreactivity and suggestive of insulin–antibody complexes. Plasma adiponectin concentration was increased. IAs were detectable using the human insulin-specific ImmunoCAP® ELISA (Table 5.2) although were within reference limits, and IAs were within RIA reference limits. GFC with preincubation of exogenous human insulin demonstrated an increase in HMW insulin immunoreactivity from baseline consistent with insulin binding by antibody, confirming the diagnosis of IAS (Figure 5.8). RIA studies undertaken at Translational Health Sciences, University of Bristol, Southmead Hospital, demonstrated raised insulin-binding capacity by antibody using the PAS assay (Table 5.2), but the increase was only small to that expected for an antibody with high binding capacity.

In view of the high insulin concentration, in the context of a low (but not *very* low) insulin recovery in PEG supernatant with a within-reference range IAs using 4 clinical assays, the presence of

IgA IAs was considered as this antibody class may be under-represented using PEG precipitation studies (Section 4.2.7; Figure 4.3) and the human insulin-specific ImmunoCAP® ELISA is designed to detect IgG IAs (Figure 5.1). Using antibody class-specific agarose–antibodies, immunosubtraction studies were performed. Patient A8 plasma was studied and compared with control plasma with insulin-binding IgG, and three insulin concentration-matched control plasma samples. Insulin concentrations in neat plasma were as follows: patient A8: 257 pmol/L; insulin-binding IgG control: 248 pmol/L; and insulin-matched antibody-negative control plasma: 276 pmol/L, 241 pmol/L, and 247 pmol/L. To increase the sensitivity of the method to detect insulin–antibody complexes, each plasma sample was incubated with synthetic human insulin (Actrapid®) to drive the binding equilibrium in favour of bound insulin. Following incubation with human insulin and sample dilution (ratio 1:4 with assay diluent), were: patient A8: 4076 pmol/L; anti-insulin IgG control: 5290 pmol/L; insulin-matched antibody-negative controls: 4770 pmol/L, 4388 pmol/L, and 5270 pmol/L. To determine the effect of increasing insulin concentration on the proportion of antibody-bound insulin, PEG precipitation of insulin-spiked plasma from patient A8 was performed. Insulin recovery was 73%, and consistent with the insulin recovery following PEG precipitation of the sample prior to addition of exogenous insulin (Table 5.2). Estimations of plasma insulin recovery in each of the samples following immunosubtraction, using antibody class-specific agarose–antibodies, are shown in (Figure 5.12). Following immunosubtraction using anti-human IgA agarose–antibody, the mean insulin recovery from patient A8 plasma was lower compared with that of control samples and the IgG IA positive control consistent with the presence of IgA IAs in patient A8. Following immunosubtraction using anti-human IgG agarose–antibody, the insulin recovery of the IgG IA positive control plasma was lower compared with that of control plasma and patient A8 plasma.

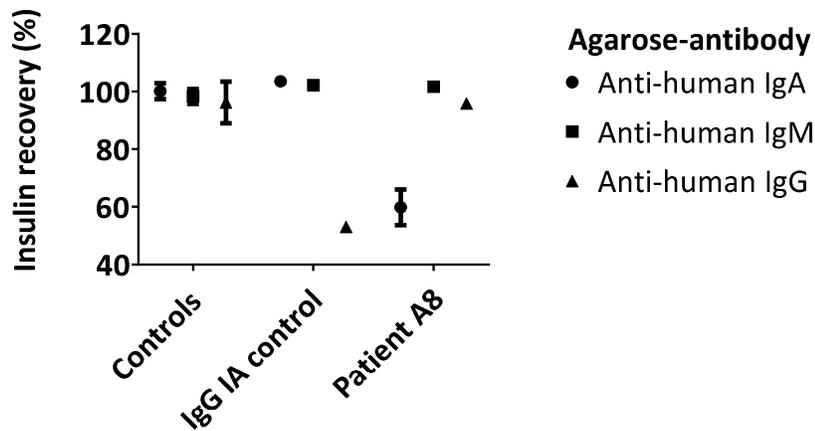


Figure 5.12 Insulin recovery following immunosubtraction using antibody class-specific agarose-antibodies compared with matched saline-diluted plasma from patient A8, control plasma with insulin-binding IgG, and three insulin concentration-matched controls. Control insulin recovery: anti-human IgA, mean 100% (95% confidence interval (CI): 93–107%); anti-human IgM, mean 98% (95% CI: 92–105%); anti-human IgG, mean 96% (95% CI: 78–114%). Insulin-binding IgG control insulin recovery: anti-human IgA, 104%; anti-human IgM, 102%; anti-human IgG, 53%. Patient A8 plasma insulin recovery: anti-human IgA, mean 60% (95% CI: 44–75%); anti-human IgM: 102%; anti-human IgG: 96%. Abbreviation: anti-insulin antibody, IA.

To provide further evidence to support the presence of IgA IA, anti-human IgA-agarose antibody was again used to precipitate IgA-specific IA, using the standardised PAS RIA, and increased IgA binding was demonstrated supporting the presence of IgA IAs in this patient (data not shown). Results from competitive insulin-binding studies could not be interpreted due to the very low binding in the PAS assay.

No further symptomatic hypoglycaemia was recorded, and the prednisolone dose was reduced to 40mg daily. However, four months after glucocorticoids were commenced, blood tests confirmed the continued presence of insulin-binding antibodies. CGMS was performed and matutinal hyperglycaemia with postprandial hyperglycaemia observed (Figure 5.9c). In view of the on-going glycaemic lability despite glucocorticoids, the patient is considering immunomodulatory therapy.

5.3.8 Polyethylene glycol precipitation studies of immunoassay C-peptide

From the six patients who had it measured, C-peptide was detected by immunoassay in samples taken concurrent with hypoglycaemia. To investigate this, PEG precipitation of IAS plasma, and 8 control plasma samples with ambient C-peptide concentrations across the DiaSorin LIAISON® XL assay range, was undertaken.

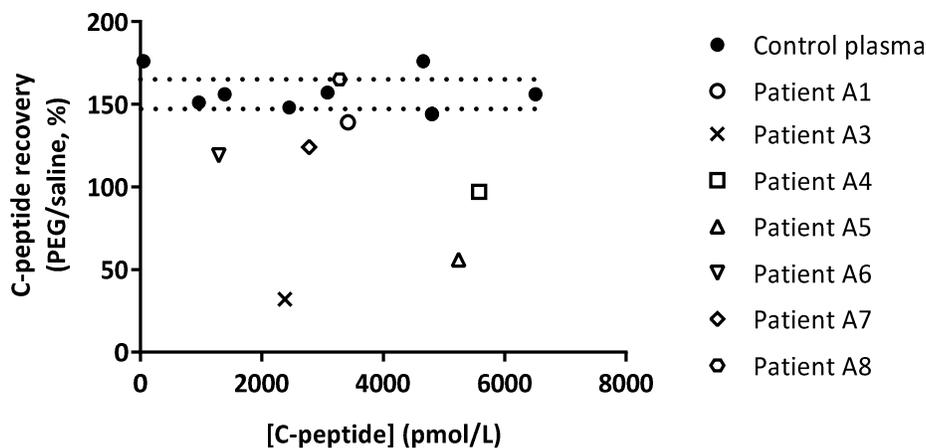


Figure 5.13 Immunoassay C-peptide recovery following PEG precipitation. C-peptide recovery (PEG supernatant/saline dilution, %) against neat plasma C-peptide concentration. The dotted lines indicate 95% confidence interval of mean C-peptide recovery of control plasma (147–165%).

The mean control C-peptide recovery (PEG supernatant/saline, %) was 156%, and 95% confidence interval 147–165 %. Except for patient A8, all patients had a C-peptide recovery below the 95% confidence interval for control plasma, and C-peptide recovery in patients 2, 3, and 7 appeared markedly low (Figure 5.13).

5.3.9 Liquid chromatography–mass spectrometry quantification of insulin and C-peptide

Insulin and C-peptide concentrations, as quantified by MS, are given in Table 5.2. There was insufficient plasma volume from patient A4 for LC–MS analysis. The ratio of the MS-derived insulin:C-

peptide ratios showed that for the 34 control samples the ratio varied from 0.16 to 1.48 with one outlier of 0.02, and for the patient samples, the ratio ranged from 3.70 to 273.21.

Table 5.2 Biochemical evaluation of patients

Patient	Anti-insulin IgG mg/L (0–5)*	Anti-insulin autoantibody IA units (<0.2)	Kd mol/L	Insulin pmol/L (<60)		Insulin recovery after PEG precipitation % (>102)	GFC of insulin	C-peptide pmol/L (174–960)	Insulin: C-peptide molar ratio	Adiponectin mg/L (**)	MS insulin (pmol/L)	MS C-peptide (pmol/L)	Insulin: C-peptide molar ratio
				>3000	Dilution ratio (Plasma:diluent)								
A1	16	2408	3.42×10^{-10}	>3000	7020	8	HMW insulin present	3750	1.87	4.5 (3.5–15.5)	5278	1428	3.70
A3	171	>10,000	7.88×10^{-10}	>3000	68,950	3	HMW insulin present	4960	13.90	10.1 (3.5–15.5)	139,882	512	273.21
A4	38	8738	1.16×10^{-9}	>3000	11,585	6	HMW insulin present	5580	2.07	20.3 (2.6–14.9)	Ins	Ins	-
A5	11	>10,000	4.68×10^{-10}	782	4601	9	HMW insulin present	2380	0.33	19.2 (3.5–15.5)	1583	215	7.36
A6	>200	4.0	6.55×10^{-6}	1340	3912	11	HMW insulin present	1190	1.13	6.5 (2.4–10.6)	2912	348	8.37
A7	89	300	8.55×10^{-7}	2781	7805	3	HMW insulin present	3110	0.89	19.5 (4.4–17.7)	6589	880	7.49
A8	5	0.1	-	2906	5630	65	HMW insulin present	3280	0.86	13.0 (2.6–12.6)	4012	750	5.35

Abbreviations: gel filtration chromatography, GFC; high molecular weight, HMW; anti-insulin antibody, IA; mass spectrometry, MS; polyethylene glycol, PEG. *The reference limit of 0–5 mg/L used for the anti-insulin IgG assay was provided by a reference laboratory using the same method (Sheffield Protein Reference Unit, Sheffield, UK). Testing 28 of the 34 control samples used in the quantitative mass spectrometric analysis of insulin and C-peptide in Section 5.2.9 yielded a 75% percentile IA concentration of 4.8 mg/L. **Reference ranges for adiponectin are 5th–95th percentiles from sex- and BMI-matched non-diabetic controls, and are indicated for each patient in brackets.

5.4 Discussion

Classically, IAS presents as hyperinsulinaemic hypoglycaemia that may occur during fasting or postprandially. The case histories in this chapter report symptoms ranging from daytime postprandial loss of consciousness, to nocturnal modest hypoglycaemia with fasting. In patients A1, A6, and A8 hypoglycaemia was reactive, in patients A4 and A5 hypoglycaemia occurred during fasting, and in patient A3, a mixture of both reactive and fasting hypoglycaemia was observed. There was absence of hypoglycaemia <3 mmol/L [167] during prolonged fasting in patients A1, A6, and A8, as reported in some other cases of IAS [265, 398, 405].

IAS is most commonly reported in Japan [246] with cases infrequently reported in the West, however there continues to be a lack of awareness of the disease amongst endocrinologists. IAS is sometimes considered in legal proceedings relating to suspected insulin poisoning, where the interpretation of hyperinsulinaemic hypoglycaemia with a high insulin:C-peptide ratio in the absence of testing for IAS, is questioned. During the investigation of each of the patients in this report, the primary finding that led to consideration of the diagnosis of IAS was the markedly raised plasma insulin concentration and high insulin:C-peptide molar ratio in samples collected concurrently with a hypoglycaemic episode. Notably, samples taken in the non-fasting, non-hypoglycaemic state were of value for laboratory analysis as insulin-antibody complexes persisted. The heterogeneity in presentation, disease rarity, and limited access to specialist biochemistry investigations, may all contribute to delayed diagnosis. Out of the seven case histories described here, four patients underwent specialist imaging in a comprehensive attempt to identify a secretory tumour.

IAs were originally reported in patients receiving exogenous insulin [185, 406] and this association was viewed in early literature as being strong enough for the presence of detectable IAs in ostensibly insulin-naïve individuals presenting with hypoglycaemia to be regarded as nearly diagnostic of surreptitious insulin administration [407]. Although identification of IAs is central to the diagnosis of IAS (Section 1.10), the presence of circulating IAs is not specific to the condition (Section 1.11) and it is therefore important to discriminate those antibodies whose affinity and/or concentration is such that insulin kinetics are altered such to cause dysglycaemia. There is no validated IA assay for the diagnosis

of IAS. The diagnostic utility of IA assays in isolation for IAS is limited by lack of standardisation between assays and lack of diagnostic result thresholds. Methods used for detection of IAs include ELISA and RIA [408–411], and agreement between assay results remains poor [395, 401], despite attempts to harmonise them over many years [395, 412]. Detection of IAs varies depending on methodology, and consistent with the results of this study, ELISA and RIA may generate different results from the same sample [408, 413]. Except in the case of monoclonal gammopathies, IAs are most commonly polyclonal and heterogeneous [256, 260, 263, 265, 269, 364, 400, 414–416], and are likely to recognise different epitopes on insulin. Detection may be limited to a particular class of antibody, such as is the case for the ELISA used in this study. In all but patient A8, and patient A6 using RIA, IAs were markedly above the reference limit in both assays. Although both ELISA and RIA determined ‘positive’ results for patients A1, and A3-7, they differed in their relative result magnitude. Most notably for patient A6, there was a clear discrepancy between the high concentration IgG IA measured by ELISA, a result in keeping with the GFC findings (Figure 5.7a), and the RIA result which was only marginally positive. RIAs rely on an effective means of immunosubtraction, and the latter finding was possibly attributable to under-representation of IgG3 by PAS assays suggested by the high result using PGS. High ambient insulin concentration is a possible interferent [219] for both assay designs, and the degree to which endogenous insulin competes with assay reagents is not certain (Figure 5.14).

The high insulin:C-peptide molar ratios seen in all patients in this study are characteristic of IAS and are attributed to impaired plasma clearance of immunoreactive insulin due to the presence of complexes. In the four patients for which dilution studies were undertaken, immunoassay insulin recovery was non-linear, consistent with insulin assay non-linearity in the presence of IAs demonstrated in Section 4.3.1 (Figure 4.2), and such analytical interference may therefore artefactually lower insulin:C-peptide ratio. Total insulin concentration measured in neat plasma by MS confirmed under-recovery of insulin by immunoassay. Of additional interest, C-peptide was measured in all seven patients at the time of hypoglycaemia, consistent with some previous IAS reports [256, 257, 421, 422, 265, 398, 402, 405, 417–420], and there was lower C-peptide recovery after PEG precipitation, in particular for patients A3, A4, and A5. There are several possible contributors to this observation, including differential kinetics of the decay in plasma insulin (plasma half-life of 5 minutes) and C-peptide (plasma

half-life 35 minutes) concentrations after acute suppression of insulin secretion; or true presence of some HMW C-peptide immunoreactivity, that could be HMW C-peptide, cross-reactivity of HMW proinsulin and/or HMW insulin precursors, or other HMW interferents in the C-peptide assay. Proinsulin is known to cross-react at approximately 100% in the DiaSorin LIAISON® C-peptide assay (in-house data, Core Biochemical Assay Laboratory (CBAL), Cambridge University Hospitals NHS Foundation Trust), however, standards for des 31,32 and des 64,65 proinsulin were unavailable for testing. Consistent with (non C-peptide) interferents in C-peptide immunoassay, even after considering assay bias, immunoassay C-peptide measurements in IAS appear to be over-estimations relative to MS total C-peptide concentrations. Collectively, the effect of under-estimating insulin and over-estimating C-peptide by immunoassay in IAS may therefore artefactually decrease the insulin:C-peptide ratio.

In Section 4.3.2, it was shown that when using PEG, insulin immunoassay performance varies between immunoassay methods, and there are intrinsic limitations to the use of PEG in this way, including incomplete removal of antibodies that are not IgG class. Patient A8 presented a particular analytical challenge, with within-reference range antibody levels using four assays (three RIAs and one IgG IA ELISA), and a low insulin recovery in PEG supernatant albeit not as low as patients A1, A3–7. Patient A8 had an IgA IA, demonstrated by the results of immunosubtraction studies. This finding could account for the discordant PEG and GFC results, the latter consistent with an immunoglobulin with a high capacity to bind insulin (Figure 5.8). Results from PEG precipitation studies (Figure 4.3) showed that only around 70% of IgA is removed using the PEG precipitation method, and highlights that PEG precipitation has perennial potential to generate a ‘false negative’ result in the case of IgA antibodies. IgA insulin-binding antibodies that cause dysglycaemia have been reported in the context of IgA- κ myeloma [244], but in that case PEG recovery was grossly abnormal (5%, versus >70% for control samples). By virtue of detectable circulating IAs not being a finding specific for IAS (Section 1.11), and in some cases circulating IAs not detected at all, a more sensitive and definitive means to detect insulin-antibody complexes is required for a confident diagnosis. To that end, in Chapter 4, a GFC method with incorporation of incubation of exogenous insulin was used to increase sensitivity to detect insulin binding by antibody, and ultimately confirm the diagnosis of IAS.

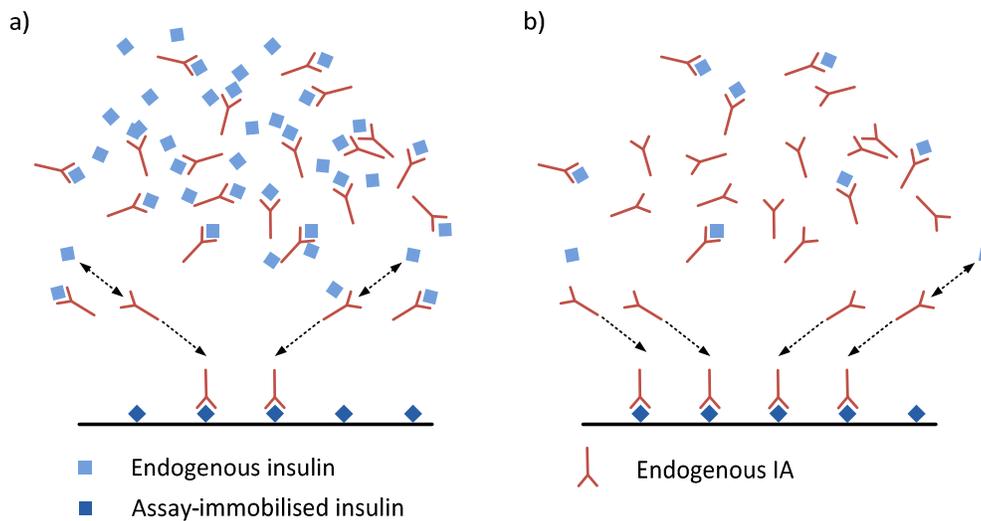


Figure 5.14 Possible limitation to measurement of IA in the presence of a high ambient plasma insulin concentration. In the same way that IAs can adversely affect immunoassay insulin measurement, endogenous insulin that is not removed from the sample prior to analysis could affect detection of endogenous IAs. It follows that in the presence of insufficient assay-bound insulin/absence of sufficient incubation time, endogenous antibodies may remain bound to endogenous insulin in plasma and may thus be under-represented in IA measurement. Abbreviation: anti-insulin antibody, IA.

Results of radioligand binding studies demonstrated a range of antibody affinities and concentrations, consistent with other reports [248, 255, 425, 426, 262, 263, 265, 322, 336, 398, 423, 424]. Neither antibody level nor affinity appeared to have a clinical correlation, however there are theoretical and practical limitations when using radioligand binding assays to determine affinities of polyclonal antibodies [427], as antibodies may exhibit contrasting affinities and bind different epitopes on the insulin molecule [428].

Four patients in this study had a raised plasma adiponectin concentration. This finding is in contrast to what is classically observed in most forms of hyperinsulinaemia secondary to insulin resistance [429]. In severe insulin resistance secondary to a genetic or acquired insulin receptor dysfunction, hypoglycaemia, and gross hyperinsulinaemia with high adiponectin concentrations are also seen [353, 354]. Although considered to be highly predictive of insulin receptor dysfunction [353], gross hyperinsulinism with hyperadiponectinaemia may instead be due to insulin-binding antibodies

consistent with the findings in this current study. However, the adiponectin concentrations were not raised in all IAS patients, and when raised were not grossly elevated as seen in some cases of TB-IR.

Based for the most part on Japanese reports that IAS typically resolves spontaneously [258] within three months [246] is the notion that the majority of IAS patients require little or no treatment. However, the case histories in this report describe severe and disabling hypoglycaemia, requiring prompt medical intervention and symptoms lasting months or years. As the dysglycaemia observed in IAS is a consequence of altered insulin kinetics due to antibody binding, it follows that the most effective therapies are those that suppress IA production. Consistent with this, diazoxide, a potassium channel activator drug that inhibits insulin secretion and adjunct medication commonly used in the management of insulinoma, failed to establish long-term clinical improvement in any of the four patients on whom it was used. To date, four of the patients have been treated with immunomodulatory therapy, demonstrating varied responses. Patient A1 was treated with pulsed intravenous methylprednisolone, and symptoms persisted, despite treatment over two years, and consequently rituximab therapy was undertaken. Patient A3, who presented with recurrent severe hypoglycaemia, was successfully treated with a combination of glucocorticoids and rituximab, although it was many months before the patient experienced symptomatic benefit. In this case, monitoring antibody and insulin concentration showed utility in demonstrating to the patient and clinicians that the immunomodulatory therapy was taking effect. With overt clinical improvement, although insulin concentration had markedly reduced from baseline, insulin recovery in PEG supernatant remained below normal, suggesting some persistence in insulin binding by antibody. Patients A4 and A5 were both initially treated with oral glucocorticoid therapy and MMF, but while response to treatment was successful in patient A4, patient A5 failed to demonstrate clinical improvement, culminating in the use of rituximab and plasma exchange. Although plasma exchange was the only intervention that unequivocally alleviated the symptoms of patient A5, the effects were transitory, however provided evidence for the efficacy of immunodepletion, prompting the further use of rituximab with plasma exchange. Therapeutic responses to immunomodulation in IAS are variable, and dependent upon the effectiveness of antibody reduction, which depends on both the time required for clearance of antibody, and the longevity of antibody-producing cells.

In summary, IAS is a rare and likely underdiagnosed condition. It presents as spontaneous recurrent hypoglycaemia with hyperinsulinaemia and a high insulin:C-peptide molar ratio. Detection of IAs is critical to diagnosis; however, sensitivity and specificity of IA assays is limited, and assay-dependent. PEG precipitation of plasma may be used to screen individuals suspected of having IAS, although limitations of PEG precipitation techniques should be noted. When monitoring response to immunomodulatory therapy, measurement of IA, insulin, and PEG precipitation studies, may have some utility, especially when there is a lag time before clinical response.

CHAPTER 6: Investigation of insulin antibody-mediated labile glycaemia in insulin-treated diabetes mellitus

6.1 Background

Insulin-binding antibodies can cause, or contribute to, labile glycaemia in DM [316–324], and cases of clinical improvement following successful depletion of pathogenic IAs have been reported [316, 319, 322, 323]. Although necessary for the diagnosis, detection of IAs in plasma *per se* does not confirm the condition (Chapters 4 & 5). Furthermore IAs are not specific to the disorder either, as IAs may be present in individuals with a diagnosis of, or who are at risk of developing, T1DM (Section 1.11.1), in individuals treated with insulin (Section 1.11.2), in healthy blood donors [430], and in those with autoimmune disease unrelated to DM [431, 432]. Unlike exogenous insulin-naïve individuals, the use of insulin:C-peptide ratios (Chapters 4 & 5) as an indicator of insulin sequestration has significant limitations in the context of insulin-treated DM when there is endogenous insulin (and therefore C-peptide) deficiency/insufficiency, and exogenous insulin present in plasma. Although PEG precipitation methods are currently used to identify circulating hormone–antibody complexes (Section 4.3.2), there are analytical challenges to investigating insulin-treated individuals with diabetes, as many insulin assays fail to cross-react with insulin analogues (Chapter 2). In addition, IAs can interfere with insulin measurement by immunoassay (Chapter 4). These factors, present either in isolation or in combination, can lead to reported insulin concentrations that do not reflect amounts of *in vivo* bioactive insulin. The optimal approach to investigate individuals for suspected IA-mediated dysglycaemia has not been established, and, outside a research laboratory setting, there is limited access to analytical techniques that are able to confirm the presence of insulin–antibody complexes. Furthermore, the performance of PEG precipitation studies and GFC studies has not been comprehensively tested for insulin analogues, and existing data are often proprietary information.

Following on from the study of a cohort of individuals with IAS (Chapter 5) using a panel of laboratory investigations (Chapter 4), a similar approach was adopted using an insulin immunoassay that demonstrated wide cross-reactivity with insulin analogues (Chapter 3) for the assessment of

individuals with labile glycaemia in insulin-treated DM, in whom IA-mediated dysglycaemia was suspected. It was hypothesised that in an enriched population of insulin-treated patients with clinically unexplained erratic glycaemia, circulating IA would be commonly detectable, but that the panel of assays would exclude pathogenic insulin-binding antibodies as the principal cause of labile diabetes in most patients.

6.2 Materials and methods

6.2.1 Patients

Thirty insulin-treated patients, presenting with varying patterns of dysglycaemia, including unexplained exogenous insulin resistance (high subcutaneous and/or intravenous insulin requirements), and/or unexplained daytime hyperglycaemia with nocturnal/matutinal hypoglycaemia, and/or unexplained recurrent diabetic ketoacidosis (DKA), were studied. Samples were referred for investigation of insulin antibody dysglycaemia to the UK Severe Insulin Resistance Supraregional Assay Service, Cambridge University Hospitals NHS Foundation Trust, Cambridge, as part of investigation for which the patients gave their consent. Laboratory studies and sample management were undertaken in accordance with the World Medical Association Declaration of Helsinki (2000).

6.2.2 Samples

At presentation, non-fasting blood samples were collected on wet ice and plasma/serum was promptly separated and frozen at -80°C until analysis.

6.2.3 Immunoassays

Quantitative measurement of serum anti-insulin IgG was performed using the insulin-specific ImmunoCAP[®] assay (Figure 5.1). Plasma insulin was measured in duplicate, using the Mercodia Iso-Insulin ELISA (performance characteristics given in Appendix A: Assay performance characteristics) that was shown to cross-react with insulin analogues *ex vivo* (Chapter 2), and insulin concentration determined by the mean value of two optical density readings. Dilutions were made, where necessary, using the Mercodia Iso-Insulin ELISA Calibrator 0. For patient B7, B14, B19, B22, B23, B26–B29, neat plasma C-peptide was measured in singleton using the DiaSorin LIAISON[®] XL assay. Measurement of insulin in GFC fractions was performed using the DiaSorin LIAISON XL assay, however for patient B30 the Mercodia Iso-Insulin assay, due to its ability to detect insulin analogues (Table 3.2), was also used. It was previously established that the Mercodia Iso-Insulin assay could detect insulin in GFC fractions with a limit of detection of 15 pmol/L (data not shown).

6.2.4 Polyethylene glycol precipitation of plasma

Insulin PEG precipitation studies based on the method described in Section 4.2.7 were performed on plasma from the 30 patients, and 10 IA-negative samples as control with ambient plasma insulin concentrations covering the assay analytical range. Using the Mercodia Iso-Insulin assay, insulin concentration was measured in the neat PEG supernatant. Insulin recovery was calculated as the percentage insulin concentration in PEG supernatant/insulin concentration measured in a matched saline-dilution of plasma.

PEG precipitation of detemir-spiked (approximately 6,000 pmo/L) plasma, and detemir-spiked (approximately 6,000 pmo/L) human serum albumin (HSA, 50g/L in GFC buffer), was also undertaken and immunoreactive insulin recovery calculated as above.

6.2.5 Gel filtration chromatography of patient plasma

GFC was performed pre- and post-addition *ex vivo* of synthetic human insulin, as described earlier (Section 4.2.8).

6.2.6 Immunosubtraction using class-specific anti-immunoglobulin–agarose

Insulin concentrations were estimated in plasma from patient B30, and two control samples from patients receiving detemir: one with, and one without, ImmunoCAP[®] detectable anti-insulin IgG, using the Mercodia Iso-Insulin assay. Dilutions were made, where necessary, using Mercodia Iso-Insulin ELISA Calibrator 0.

Anti-human IgA agarose–antibody, anti-human IgM agarose–antibody, anti-human IgG agarose–antibody (Table 5.1) were prepared as outlined in Section 5.2.8, and Protein G (immunoglobulin (IgG)-binding bacterial cell wall protein isolated from group G streptococcal strain)–Sepharose[®] (Fast Flow, P3296, Sigma-Aldrich[®]) was prepared in the same manner. Plasma:agarose volume ratios were based on in-house data [379], and the same dilutions of plasma were made in saline to act as control matrix for recovery calculation. Volume ratios were Protein G–Sepharose[®] 29:20 plasma, and the rest as outlined in as outlined in Section 5.2.8. Using the same plasma samples, equal mixtures were made with saline for use as control matrix. Samples were then mixed on a tube roller

mixer for 60 minutes prior to centrifugation at 13,200 *g* for 15 minutes. Insulin recovery in supernatant was calculated as percentage insulin recovery (agarose supernatant/saline, %). Due to limited sample volume, analyses were performed in singleton.

6.2.7 Serum protein electrophoresis

Protein electrophoresis of patient B30 serum was performed at the Department of Immunology, Cambridge University Hospitals NHS Foundation Trust, using the Helena V8 Nexus capillary zone electrophoresis (Helena Biosciences) method.

6.2.8 Determination of anti-insulin IgG concentration by radioimmunoassay

Patient B30 serum was assayed using the RiaRSR[™] IAA assay, at Viapath Analytics, Clinical Immunology and Allergy Department, King's College Hospital. In this assay, serum is incubated with iodine-125-(A14)-monoiodinated insulin and then labelled insulin-antibody complexes are precipitated using anti-human IgG. The amount of radioactivity in the precipitate, which is proportional to the concentration of insulin IgG in the sample, is compared with calibrators to determine a concentration value.

6.3 Results

Thirty patients, 7 male and 23 female, with labile insulin-treated DM (26 T1DM; 4 T2DM), were studied. Clinical characteristics of the 30 patients are listed in Appendix C: Clinical characteristics of patients with insulin-treated DM. Laboratory investigation aimed to establish whether these IAs principally explained the presenting lability in blood glucose.

6.3.1 Anti-insulin IgG concentration

IA results determined for all patients using the human insulin-specific ELISA ranged from <0.02 to 132 mg/L (Table 6.1): twenty-eight patients had detectable IA, and 17 results were above the 0–5 mg/L reference limit. In a group of 28 control plasma samples (Table 5.2), results ranged from 1.6 to 7.7 mg/L, and 6 results were above the reference limit.

6.3.2 Plasma insulin concentration

Insulin was measured in non-fasting plasma using the Mercodia Iso-Insulin (Table 6.1), an immunoassay with broad specificity to detect animal-derived insulin and insulin analogues (Table 3.2). There were 4 clear outliers with extremely high plasma insulin concentrations – patient B27: 15,700 pmol/L; patient B28: 12,050 pmol/L; patient B29: 87,800 pmol/L; and patient B30: 38,300 pmol/L. Excluding these 4, the remaining 26 patients had an insulin concentration within the range 39–2488 pmol/L.

6.3.3 Immunoprecipitation with polyethylene glycol precipitation

Insulin recovery, calculated as the percentage of the insulin concentration measured in PEG supernatant/saline, was then determined in all 30 patients and ten control samples (Table 6.1). For IA-negative controls, the median insulin recovery was 140% and the 95% confidence interval range was 126–167%.

Table 6.1 Non-fasting blood test results at presentation

Case	Anti-insulin IgG, mg/L (0–5)*	Insulin concentration, pmol/L (12–150)**	PEG precipitation recovery, % (>126%)	C-peptide, pmol/L (174–960)
B1	9	335	56	-
B2	8	189	75	-
B3	2	77	71	-
B4	15	2158	74	-
B5	2	118	116	-
B6	<0.02	540	108	-
B7	3	39	109	388
B8	3	906	107	-
B9	3	449	109	-
B10	3	93	152	-
B11	10	2488	95	-
B12	7	100	104	-
B13	7	318	85	-
B14	2	1578	78	<9
B15	2	403	119	-
B16	6	1192	72	-
B17	6	977	93	-
B18	4	131	159	-
B19	2	361	102	1140
B20	13	84	176	-
B21	4	1495	115	-
B22	6	198	29	<9
B23	18	1545	7	246
B24	10	1758	20	-
B25	10	2257	8	-
B26	20	2246	12	894
B27	18	15700	1.2	18
B28	25	12050	5	<9
B29	132	87800	0.4	3230
B30	<0.02	38300	1.5	-

*The reference limit of 0–5 mg/L used for the anti-insulin IgG assay was provided by a reference laboratory using the same method (Sheffield Protein Reference Unit, Sheffield, UK). **Mercoxia Iso-Insulin reference range provided by assay manufacturer [385].

6.4 Examination of group data

After correlating patient plasma insulin recovery in PEG supernatant against measured insulin concentration, three arbitrary populations could be identified (Figure 6.1) – Group 1: 21 individuals with insulin concentrations <2500 pmol/L and insulin recovery >50%; Group 2: 5 individuals with insulin concentrations <2500 pmol/L and insulin recovery <50%; Group 3: 4 individuals with insulin concentrations >2500 pmol/L and insulin recovery <50% (Figure 6.2).

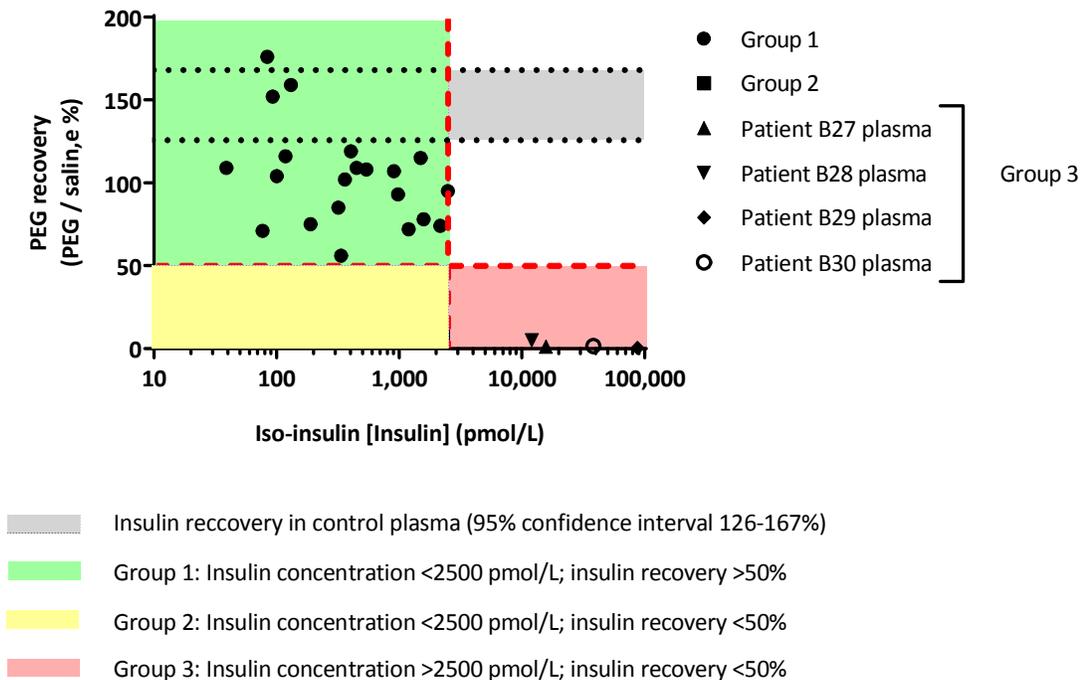


Figure 6.1 Insulin recovery in PEG supernatant (PEG/saline, %) against \log_{10} insulin concentration. Groups 1, 2 and 3 were populations identified arbitrarily and are delineated by the red dashed lines. The 95% confidence interval of control median insulin recovery is indicated by the dotted lines at 126 and 167%.

6.4.1 Group analysis

Group 1 results were defined as ‘negative’ for actionable antibodies, and no further action was taken. Plasma from Group 2 and Group 3 patients underwent GFC studies utilising addition *ex vivo* of synthetic human insulin.

Group 2 included the 4 patients B22–B25. Patient B22 had a low insulin recovery following PEG precipitation at 29%, however the IA concentration was only slightly above the reference limit at 6 mg/L (RL 0–5 mg/L) and the insulin concentration was only 198 pmol/L. Therefore, despite the low insulin recovery, the insulin concentration appeared too low compared with unequivocal cases of IA-mediated labile glycaemia successfully treated with antibody depletion therapy and, although some interference with insulin kinetics could not be excluded, there was insufficient evidence to instigate immunomodulatory treatment. The patient received closely supervised insulin management as an out-patient and the plan is to investigate the patient's adherence with insulin therapy in the next instance.

GFC of patient B23 (Figure 6.3a), B24 (Figure 6.3b), and B25 (Figure 6.5a) plasma following insulin addition demonstrated principally monomeric insulin immunoreactivity in eluted fractions, consistent with IAs of insufficient affinity and/or concentration for detection of immunocomplexes by the GFC method (Chapter 2). IA concentration was 18 mg/L (RL 0–5 mg/L), 10 mg/L and 10 mg/L, respectively, and insulin recovery in PEG supernatant was low at 7%, 20% and 8%, respectively, and although some derangement of insulin kinetics could not be excluded, in the absence of large concentrations of insulin–antibody complexes demonstrably by GFC following *ex vivo* addition of insulin, there was insufficient evidence that immunodepletion would result in a clinical improvement in diabetic control for these patients.

GFC of B26 plasma following insulin addition demonstrated principally HMW insulin immunoreactivity in eluted fractions, pre- and post-insulin addition (Figure 6.5b), consistent with IAs of sufficient affinity and/or concentration for detection of immunocomplexes by the GFC method. However, the clinical history, that included prolonged periods of insulin resistance followed by prolonged periods of increased insulin sensitivity/recurrent hypoglycaemia was equivocal for IA-mediated dysglycaemia, and further investigation of this patient was undertaken. On examination, there was acanthosis nigricans, and during in-patient admission, intravenous insulin administration, and supervision of subcutaneous insulin administration, no hypoglycaemia was demonstrated. On follow-up IAs decreased to 13 mg/L and insulin recovery in PEG supernatant increased to >22% (further dilutions not performed), and there was insufficient evidence that immunodepletion would

result in any clinical improvement in diabetic control for this patient and the at follow-up, the patient was responding positively to closely supervised insulin management as an out-patient.

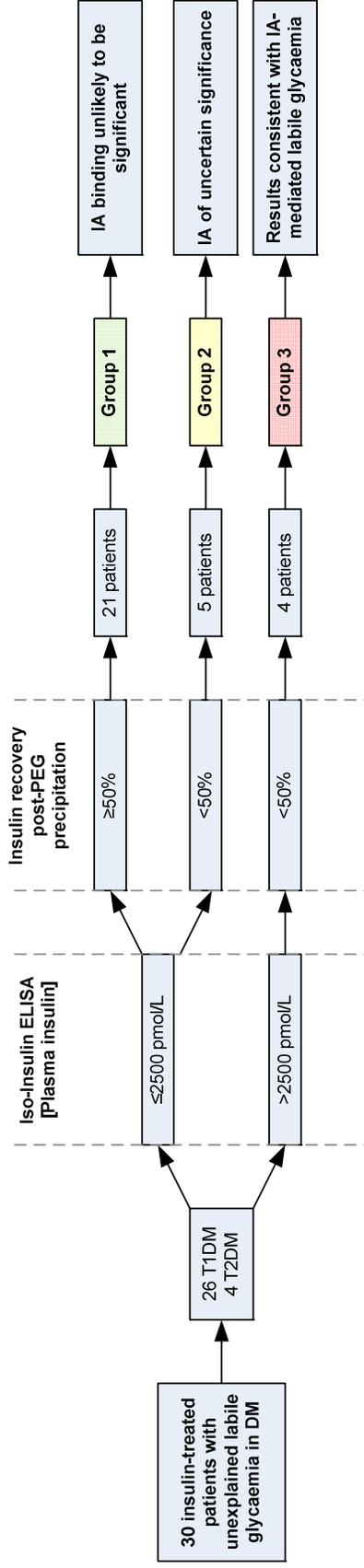


Figure 6.2 Division of the patient cohort into three groups according to insulin concentration and insulin recovery in PEG supernatant

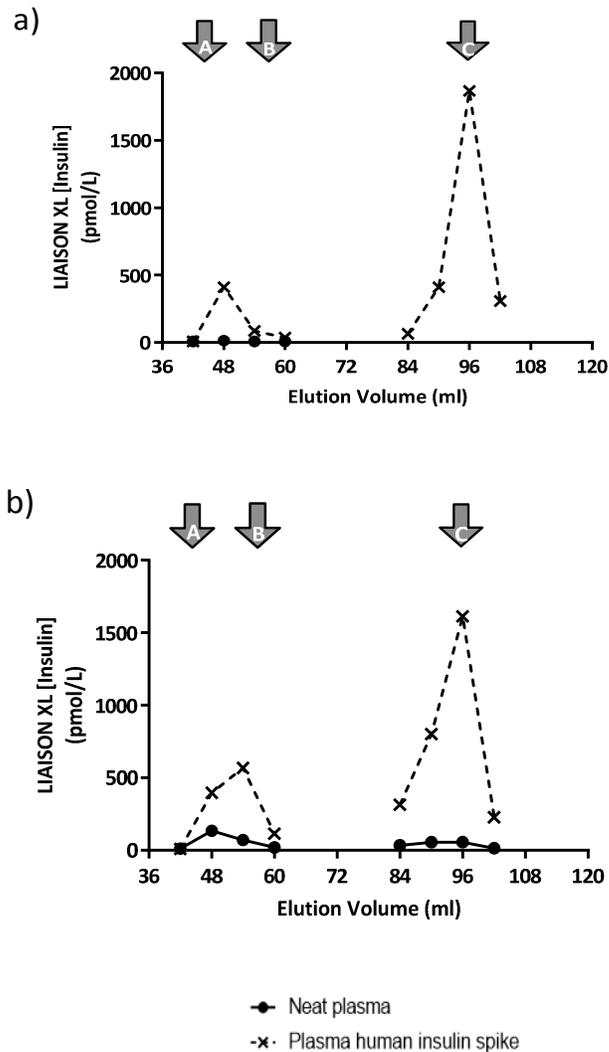


Figure 6.3 Demonstration of insulin–antibody complexes using gel filtration chromatography. Results of insulin assay after GFC of non-fasting plasma. Elution volumes of immunoglobulin (A), albumin (B) and monomeric insulin (C) are shown. The principal HMW and monomeric fractions were analysed. Insulin concentrations were measured using the DiaSorin LIAISON® XL, an assay with specificity for human insulin. Results from plasma at presentation, pre- and post-insulin addition (insulin concentrations 10 pmol/L and 5745 pmol/L, respectively) from patient B23 (a); pre- and post-insulin addition (insulin concentrations 408 pmol/L and 7825 pmol/L, respectively) from patient B24 (b) are shown.

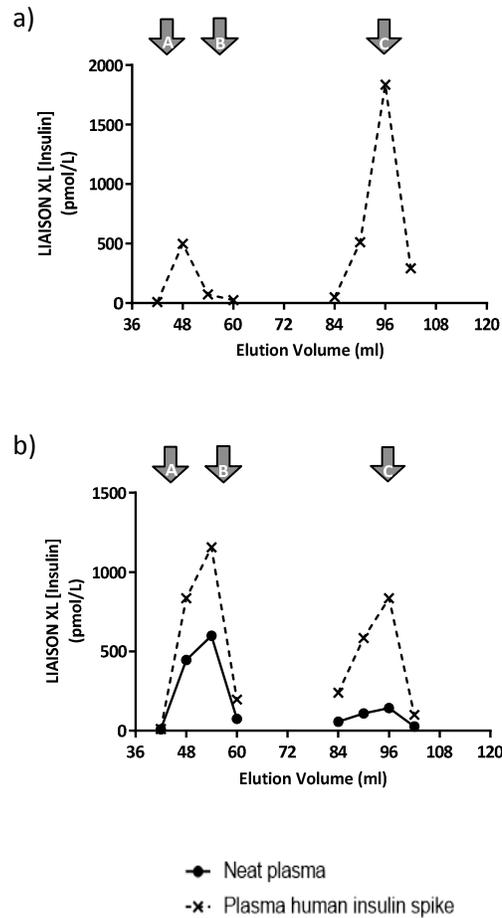


Figure 6.4 Demonstration of insulin-antibody complexes using gel filtration chromatography. Results of insulin assay after GFC of non-fasting plasma. Elution volumes of immunoglobulin (A), albumin (B) and monomeric insulin (C) are shown. The principal HMW and monomeric fractions were analysed. Insulin concentrations were measured using the DiaSorin LIAISON® XL, an assay with specificity for human insulin. Results from plasma at presentation post-insulin addition (insulin concentrations <3 pmol/L and 7085 pmol/L pre- and post-insulin addition, respectively) from patient B25 (a); and pre- and post-insulin addition (insulin concentrations 1161 pmol/L and 7390 pmol/L, respectively) from patient B26 (b) are shown.

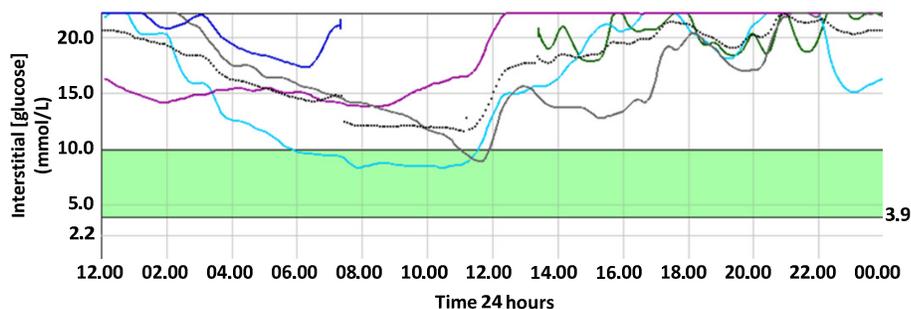
Group 3 included the 4 patients B27–B30. The four patients in Group 3 were defined by very high insulin immunoreactivity and very low insulin recovery in PEG supernatant (Figure 6.1). Patients B27, B28, and B29 had unequivocally raised anti-insulin IgG concentrations using the human insulin-

specific ELISA, whereas patient B30 antibody concentration was below assay reportable range. The four patient histories are given in more detail below.

6.4.2 Patient B27

A 58-year old man with a diagnosis of T1DM since the age of 2 presented with chronic labile glycaemia (Figure 6.5a) characterised by delayed-onset of insulin action and extremely variable exogenous insulin requirements. Any increase in the infusion of insulin took 7–9 hours to have any glucose-lowering effect, and insulin dose differed by as much as 20 units per day. He would take carbohydrate snacks regularly throughout the day to avoid hypoglycaemic symptoms and eat at night to avoid nocturnal hypoglycaemia. Since the age of 18 years, glucose control had been a challenge, but for the past 12 years, his blood sugars had been particularly labile despite meticulous glucose monitoring, structured insulin dose-adjustment training, and continuous subcutaneous insulin infusion (CSII). In the past, the patient had tried using insulin analogues, such as glulisine, aspart and glargine, all of which failed to improve glycaemic control in the long-term. At presentation, he was treated with porcine CSII as this had achieved the best glucose control. He had a past medical history of diabetic background retinopathy, peripheral vascular disease, diabetic peripheral neuropathy, ischaemic heart disease, and mild psoriasis. Associated with labile diabetes were symptoms of emotional lability and loss of mental clarity.

a)



b)

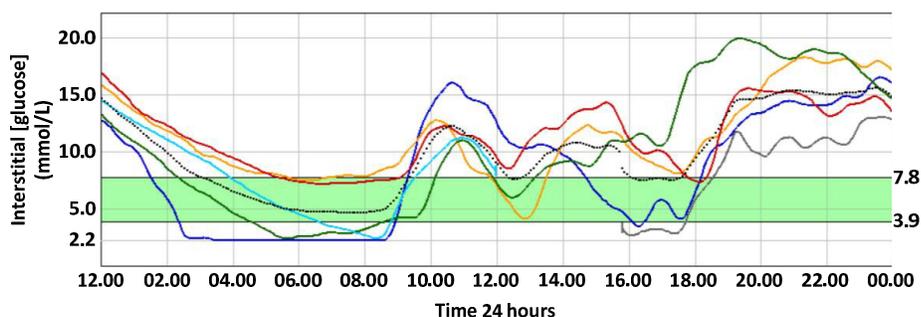


Figure 6.5 Variable patterns of dysglycaemia at presentation of patient B27 and patient B28. Demonstration of hyperglycaemia and matutinal decrease in blood glucose in patient B27 (a). Demonstration of labile glycaemia in patient B28 (b).

Samples were referred to the Institute of Metabolic Science for specialist biochemical investigation. Using the Mercodia Iso-Insulin assay, gross hyperinsulinaemia was confirmed in non-fasting plasma, and insulin recovery was very low following PEG precipitation (Table 6.1) consistent with HMW insulin immunoreactivity and suggestive of insulin-antibody complexes. IAs were detectable using the human insulin-specific ELISA (Table 6.1), and measuring insulin in fractions from GFC with preincubation of exogenous human insulin demonstrated an increase in HMW insulin immunoreactivity from baseline consistent with insulin-binding by antibody, confirming the presence of antibody with a high capacity to exchange and bind insulin (Figure 6.6a). The patient has been referred for, and is awaiting, immunomodulation therapy.

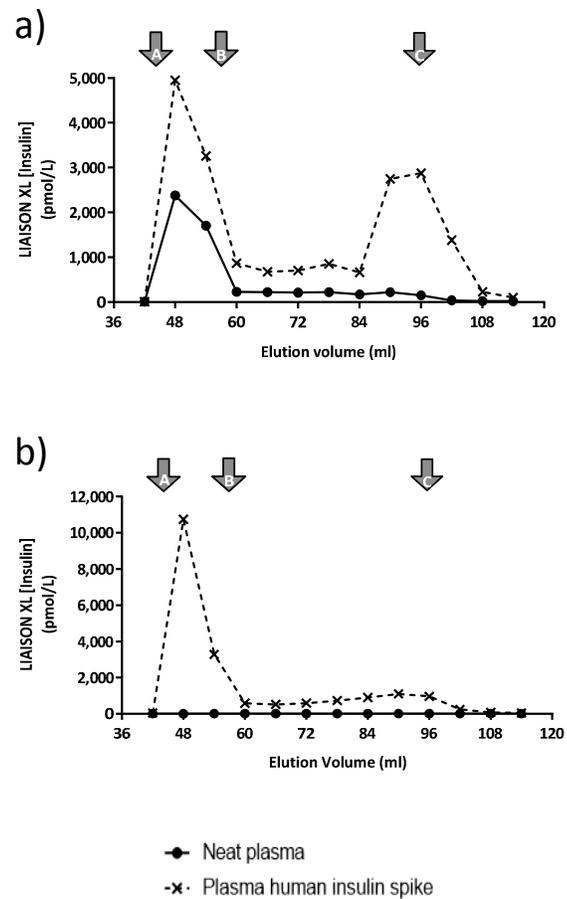


Figure 6.6 Gel filtration chromatography of patient plasma pre- and post-insulin addition. Results of insulin assay after GFC of non-fasting plasma. Elution volumes of immunoglobulin (A), albumin (B) and monomeric insulin (C) are shown. Insulin concentrations were measured using the DiaSorin LIAISON® XL, an assay with specificity for human insulin. Results are shown from plasma at presentation, pre- and post-insulin addition (insulin concentrations 13,080 pmol/L and 27,300 pmol/L, respectively) from patient B27 (a); pre- and post-insulin addition (insulin concentrations <3 pmol/L and 27,340 pmol/L, respectively) from patient B28 (b).

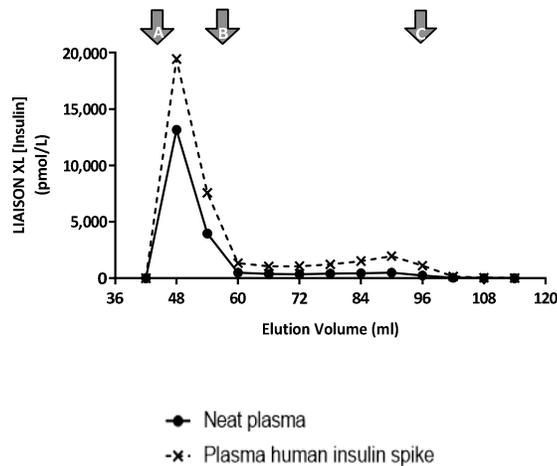


Figure 6.7 Gel filtration chromatography of patient plasma pre- and post-insulin addition. Results of insulin assay after GFC of non-fasting plasma. Elution volumes of immunoglobulin (A), albumin (B) and monomeric insulin (C) are shown. Insulin concentrations were measured using the DiaSorin LIAISON® XL, an assay with specificity for human insulin. Results are shown from plasma at presentation pre- and post-insulin addition (insulin concentrations 35,760 pmol/L and 76,300 pmol/L, respectively) from patient B29.

6.4.3 Patient B28

A 52-year old woman with a diagnosis of T1DM since the age of 9 years presented with chronic labile glycaemia characterised by unpredictable insulin action, daytime/postprandial hyperglycaemia and nocturnal hypoglycaemia (Figure 6.5b). At presentation, DM was treated with once daily biphasic insulin lispro (25% insulin lispro, 75% insulin lispro protamine). Different insulin regimes, including dosing and timing of insulin administration, had been tried in an attempt to improve glycaemic control without success. She had a past medical history of hypertension and had very poor hypoglycaemic awareness, but no microvascular complications of diabetes.

Samples were referred to the Institute of Metabolic Science for specialist biochemical investigation. Using the Mercodia Iso-Insulin assay, gross hyperinsulinaemia was confirmed in non-fasting plasma, and insulin recovery was very low following PEG precipitation (Table 6.1) consistent with HMW insulin immunoreactivity and suggestive of insulin–antibody complexes. IAs were detectable using the human insulin-specific ELISA (Table 6.1), and measuring insulin in fractions from

GFC with preincubation of exogenous human insulin demonstrated an increase in HMW insulin immunoreactivity from baseline consistent with excess insulin binding by antibody, confirming the presence of an antibody with a high capacity to bind insulin (Figure 6.6b). The patient, while grateful for the explanation for her chronically labile glycaemia, declined immunomodulatory therapy.

6.4.4 Patient B29

A 56-year old woman with a diagnosis of T2DM for 14 years, exhibited good glycaemic control for 12 years with oral anti-diabetic medications (metformin and gliclazide). Then followed significant deterioration in blood glucose control, and HbA_{1c} increased from 57 mmol/mol to 115 mmol/mol prompting the commencement of subcutaneous insulin therapy. Despite increased insulin doses to 4 units per kg, blood glucose concentrations remained elevated. During in-patient admission, she demonstrated insulin resistance and hyperglycaemia that persisted with intravenously administered insulin at 6 units per hour. At presentation, the patient was taking U300 glargine and U200 lispro with a total daily insulin dose of >200 units. Previously, she had tried a biphasic soluble insulin/isophane insulin, and also a biphasic soluble insulin lispro/insulin lispro protamine suspension preparation without significant clinical improvement. She had a past medical history of acute lymphoblastic leukaemia with allogenic bone marrow transplant 21 years earlier, and chronic kidney disease (estimated glomerular filtration rate 26 mL/min). The patient was taking aspirin, candesartan, carvedilol, ferrous fumarate, simvastatin, and furosemide. IAs were >200 mg/L measured using the human insulin-specific ImmunoCAP[®] ELISA at Sheffield Protein Reference Unit.

Samples were referred to the Institute of Metabolic Science for specialist biochemical investigation. Using the Mercodia Iso-Insulin assay, gross hyperinsulinaemia was confirmed in non-fasting plasma, and insulin recovery was very low following PEG precipitation (Table 6.1), consistent with HMW insulin immunoreactivity and suggestive of insulin-antibody complexes. IAs were detectable using the human insulin-specific ELISA (Table 6.1) and measuring insulin in fractions from GFC with preincubation of exogenous human insulin demonstrated an increase in HMW insulin immunoreactivity from baseline consistent with insulin binding by antibody, confirming the presence of antibody with a high capacity to bind insulin (Figure 6.8). This patient is being managed currently with high dose insulin, and immunomodulatory therapy is being considered.

6.4.5 Patient B30

A 12-year old girl with a history of T1DM, presented acutely with recurrent hypoglycaemia on the background of chronic labile diabetes. She had recently changed from aspart CSII to an aspart/detemir basal-bolus regime. Excess exogenous insulin was initially suspected, and the patient was admitted to hospital, blood samples taken, and insulin therapy stopped for 72 hours. Plasma insulin was 31,878 pmol/L using an assay with detemir and aspart cross-reactivity (Abbott ARCHITECT; Table 3.2). During the admission, she was persistently hyperglycaemic, and the basal-bolus insulin regime was re-started. Whilst an out-patient, the patient had close supervision of insulin administration. During this time further blood samples were taken, and plasma insulin concentrations were extremely high at 15,327–32,243 pmol/L in the absence of hypoglycaemia.

Samples were referred to the Institute of Metabolic Science for specialist biochemical investigation. Using the Mercodia Iso-Insulin assay which cross-reacts with detemir, gross hyperinsulinaemia was confirmed in non-fasting plasma. Insulin recovery was very low following PEG precipitation (Table 6.1), which was not seen in detemir-spiked plasma or HSA (data not shown), consistent with HMW insulin immunoreactivity in patient plasma, however IAs were not detectable using the human insulin-specific ELISA (Table 6.1).

In the absence of ELISA-detectable anti-insulin IgG, and as circulating detemir is principally albumin-bound (Section 1.5.7), it was considered whether HMW insulin immunoreactivity could be a result of abnormal albumin binding. Albumin was shown not to be removed with PEG precipitation (Section 4.3.2), and serum protein electrophoresis did not identify an alloalbumin.

Table 6.2 Findings of gel filtration chromatography of Patient B30 plasma

Sample	Insulin therapy	Insulin spike	GFC (Mercodia Iso-Insulin)*	GFC (DiaSorin LIAISON)**
Plasma 1	detemir, aspart	-	HMW insulin	-
Plasma 2	detemir, aspart	-	HMW insulin	No insulin immunoreactivity
Plasma 2	detemir, aspart	synthetic human	-	HMW insulin
Follow-up plasma	aspart	synthetic human	-	HMW insulin

The Mercodia Iso-Insulin assay cross-reacts with insulins detemir and aspart; ** The DiaSorin LIAISON assay does not cross-react with insulins detemir and aspart (Table 3.2).

A qualitative comparison of data from GFC of Plasma 1 and Plasma 2 using the Mercodia Iso-Insulin assay was undertaken, both sets of samples being inherently subject to similar matrix effects during GFC. Measuring insulin in fractions from GFC using the Mercodia Iso-Insulin assay (Table 6.2; Figure 6.8), demonstrated HMW insulin immunoreactivity in plasma from admission when the patient was receiving insulins detemir and aspart (Plasma 1), and after stopping these insulins/re-starting under supervision (Plasma 2). Results demonstrated a decrease in insulin immunoreactivity in HMW fractions consistent with a decrease in ambient plasma immunoreactivity. To further corroborate that the HMW insulin immunoreactivity detected using the Mercodia Iso-Insulin assay was not albumin-bound detemir, and to refute the presence of heterophilic antibody interference, GFC of Plasma 2 pre- and post-addition of synthetic human insulin was undertaken using the DiaSorin LIAISON assay which does not cross-react with insulins aspart or detemir (Table 3.2). This demonstrated an increase in HMW insulin immunoreactivity consistent with human insulin bound to antibody. Albumin binding could not account for the increase in HMW insulin immunoreactivity following addition of human insulin, as human insulin does not bind to albumin and GFC studies of human insulin in IA-negative plasma generated a peak of eluted insulin consistent with monomeric (free) insulin only (Figure 4.6a). To establish whether IAs were present in the absence of detemir therapy, GFC of plasma taken concurrently with only insulin aspart therapy (Follow-up plasma) post- incubation with exogenous human insulin was undertaken and

demonstrated HMW insulin immunoreactivity consistent with the continued presence of insulin binding/exchange by antibody.

6.4.6 Immunosubtraction of patient B30 plasma using class-specific anti-immunoglobulin–agarose

As the in-house assay is designed to detect only IgG IAs (Figure 5.1), to confirm the presence of non-IgG IA, immunosubtraction of plasma from patient B30 and 2 detemir-treated control patients was undertaken. In addition to antibody class-specific agarose used in Section 5.2.8, Protein G was used for subtraction of IgG, and allowed use of a lower agarose:plasma volume ratio than that used for anti-human IgG agarose-antibody, thereby reducing the risk of adverse dilutional or matrix effects on analysis. Insulin was measured using the Mercodia Iso-insulin as this assay has demonstrable cross-reactivity with detemir *ex vivo* (Table 3.2). Initial insulin concentrations were 5860 pmol/L for the patient, 490 pmol/L and 452 pmol/L for controls. Insulin recovery was lower in anti-insulin IgG agarose–antibody supernatant and protein G supernatant from patient B30 plasma compared with that from control plasma (Figure 6.9), findings consistent with IgG IA in patient B30 plasma.

Table 6.3 Cumulative results of patient B30, comparing aspart monotherapy and detemir/aspart therapy

	Treatment	Insulin concentration, pmol/L (12–150)*	PEG precipitation recovery, % (>126%)	ImmunoCAP® anti-insulin IgG, mg/L (0–5)	RiaRSRTM IAA anti-insulin IgG, U/ml (<0.4)
Pre-presentation plasma	Aspart only	914	70		44.2
Plasma 1	Detemir/aspart	29,500	3	<0.02	62.9
Plasma 2	Detemir/aspart	9100	<1	-	-
Follow-up plasma	Aspart only	563	25	6	57.6

*Mercodia Iso-Insulin reference range provided by assay manufacturer [385]

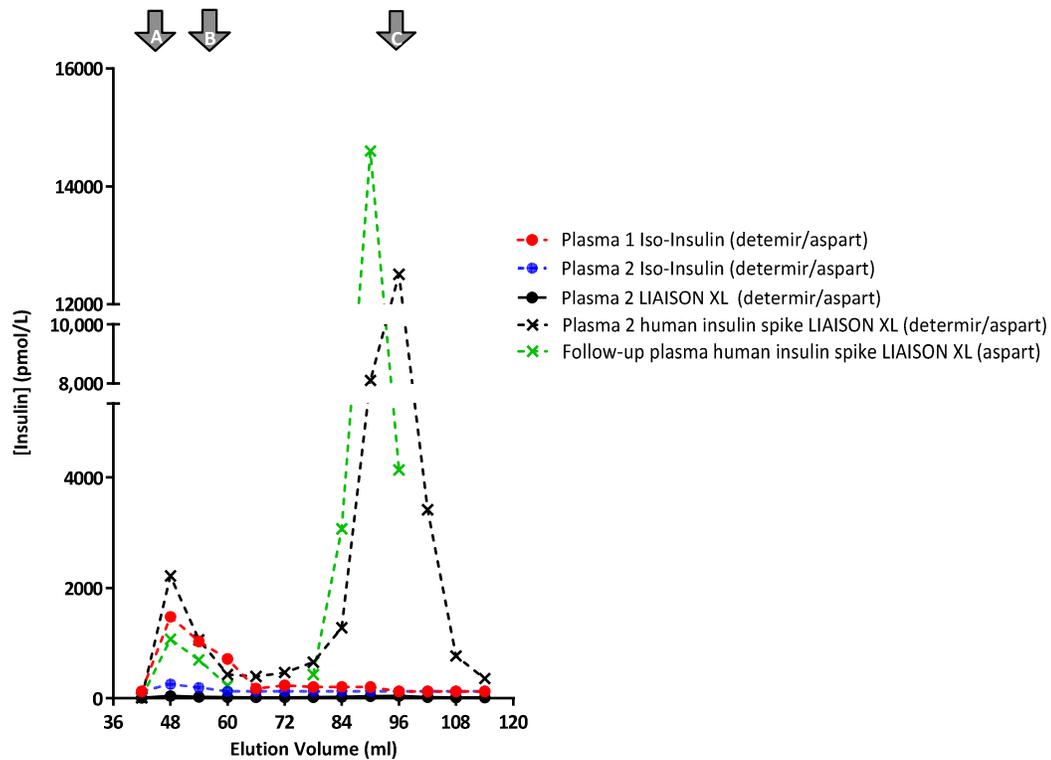


Figure 6.8 Gel filtration chromatography of Patient B30 plasma pre- and post-insulin addition. Results of insulin assay after GFC of non-fasting plasma. Elution volumes of immunoglobulin (A), albumin (B) and monomeric insulin (C) are shown. Insulin concentrations were measured using insulin assays, as indicated above. Results from plasma at presentation whilst the patient was taking insulin detemir and aspart (Plasma 1); at presentation after stopping exogenous insulin pre- and post-insulin addition as indicated (Plasma 2); and follow-up whilst taking aspart only post-insulin addition (Follow-up plasma) from Patient B30 are shown. Mercodia Iso-Insulin assay demonstrates cross-reactivity with aspart and detemir (Table 3.2). Diasorin LIAISON® XL assay does not cross-react with aspart nor detemir, but cross-reacts with human insulin. Plasma 1, Mercodia Iso-Insulin: principally HMW insulin. Plasma 2, Mercodia Iso-Insulin: principally HMW insulin immunoreactivity, although lower initial ambient insulin concentration than Plasma 1. Plasma 2, DiaSorin LIAISON® XL: no HMW insulin cross-reactivity, however, HMW insulin is demonstrable following human insulin addition. Follow-up plasma human insulin spike, DiaSorin LIAISON® XL: Demonstrable HMW insulin immunoreactivity (initial ambient plasma concentration <3pmol/L).

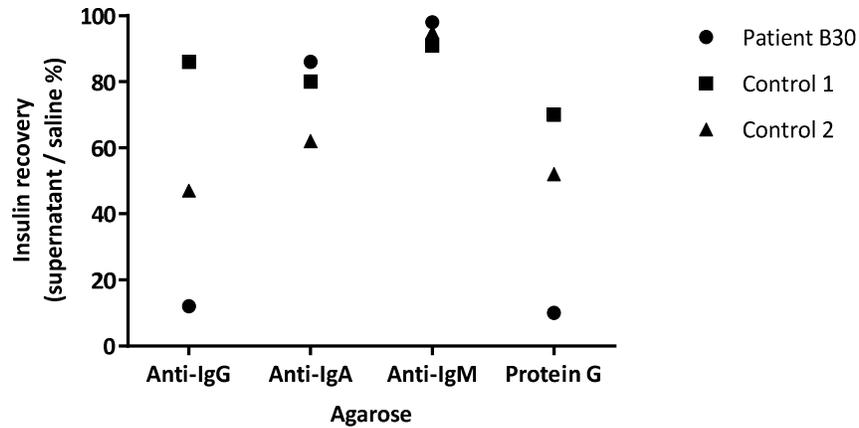


Figure 6.9 Insulin recovery following immunosubtraction of plasma. Merckodia Iso-Insulin immunoreactive insulin recovery (agarose supernatant/saline, %) of patient B30, and two control plasma samples from patients receiving detemir: control 1 (anti-insulin IgG concentration below the ImmunoCAP® detection limit), and control 2 (anti-insulin IgG concentration 10 mg/L). Insulin recovery in anti-human IgA agarose–antibody supernatant of patient B30 was 86%, compared with 80% and 62% for control 1 and 2, respectively. Insulin recovery in anti-human IgM agarose–antibody supernatant of patient B30 was 98%, compared with 91% and 95% for control 1 and 2, respectively. Insulin recovery in anti-human IgG agarose–antibody supernatant of patient B30 was 12%, compared with 86% and 47% for control 1 and 2, respectively. Protein G supernatant of patient B30 was 10%, compared with 70% and 52% for control 1 and 2, respectively.

Insulin was measured in a sample taken prior to presentation and starting detemir, during aspart monotherapy, and the concentration was much lower and recovery in PEG supernatant much higher (Table 6.3). The patient was changed back to insulin aspart and insulin was re-measured at a similar order of magnitude to pre-detemir.

6.4.7 Determination of anti-insulin IgG concentration in patient B30

plasma by radioimmunoassay

Although large plasma HMW immunoreactive insulin concentrations were detected concurrently with detemir therapy, and results from immunosubtraction studies were consent with IgG IA, IAs were not detectable using the human insulin-specific ELISA. One hypothesis to explain this

observation was the co-existence of antibodies with a high affinity for detemir, the effect of which caused high measured insulin concentrations and inhibited the detection of IAs using the ELISA but which had no demonstrable consequence for the dysglycaemia. To explore this further, anti-human insulin IgG concentration was then determined using a radioligand binding assay, that incorporates a longer incubation time (16–20 hours) than the ELISA, allowing additional time for dissociation of the insulin–IA complexes and for endogenous IAs to interact in the assay. Anti-insulin IgG concentration was strongly positive in samples irrespective of concurrent detemir treatment (Table 6.3), with data consistent with the continued presence of anti-human insulin antibodies irrespective of insulin therapy preparation administered.

GFC experiments were undertaken pre- and post-addition of exogenous detemir, however recovery of detemir in eluted fractions was unsuccessful and, in the absence of a suitable assay, detection of detemir-specific antibody was not undertaken.

The patient was switched back to aspart CSII and the insulin concentration was 563 pmol/L. She has remained on aspart therapy and there have been no further concerns regarding hypoglycaemia thus far.

6.5 Discussion

‘Anti-insulin activity’ demonstrable in the serum of patients with insulin resistance acquired following repeated insulin administration was reported as early as 1938 [298]. Insulin-binding globulin was later demonstrated [185], and it was considered that fluctuations in antibody production may be a cause of the variable insulin dose requirements noted in patients with so-called ‘brittle diabetes’ [185]. Since the advent of widespread use of purified synthetic insulin therapy, clinically overt IA-mediated dysglycaemia is a far less commonly reported clinical phenomenon today. Although anecdotal case reports describe improvements in chronic glycaemic control following a change in insulin therapy in the face of high IA levels [433], the specificity of the detected IA, and the effect of switching therapy on acute glycaemic lability and insulin kinetics has not been extensively investigated. Whilst there are data showing IAs produced by an individual (human anti-beef and pork insulin sera) has variable affinity for different insulin species (human, beef, pork, horse, sheep) [434], the effect of changing insulin therapy on an individual’s antibody production (i.e. affinity, concentration, and specificity) in the longer term is not known, and further study in this regard is needed.

As expected, higher antibody binding of insulin is associated with higher serum insulin concentrations [231, 315, 391, 435–437], and, as in IAS, endogenous insulin clearance is delayed [230, 231, 248, 437–439]. Clearance of insulin injected subcutaneously [230] or intravenously [250, 438, 439] is also delayed in the presence of insulin-binding antibodies.

This study presents an investigation of 30 individuals with varying forms of labile glycaemia potentially attributable to IA. Consistent with observations in autoimmune and insulin-treated DM, IAs were more prevalent in the patient samples than control plasma [126, 185, 281, 406], supporting the untested notion that the number of patients experiencing the burden of IA-mediated dysglycaemia in insulin-treated diabetes may be higher than currently recognised. Due to the nature of sample referral, prevalence of antibody-mediated dysglycaemia in insulin-treated DM cannot be made from this study, but this research provides sufficient evidence to suggest population studies in this area are warranted. As the index of clinical suspicion was high and patients were often thoroughly investigated prior to referral, this is likely to result in a high pre-test probability, and grossly overestimate the prevalence of the condition in the general patient population. Previously described laboratory methods (Chapter 4)

identified three patients with unequivocal IA-mediated labile glycaemia with sufficient confidence to recommend immunosuppressive therapy. They also ruled out actionable antibodies in 21 patients, and identified a further five patients that had evidence of antibody-bound insulin to a lesser degree, currently of uncertain clinical significance. This latter 'intermediate' group is the most challenging, as whilst evidence is presented to demonstrate the presence of IAs which may affect insulin pharmacokinetics, current laboratory methods do not provide persuasive enough evidence to instigate immunomodulatory therapy in this group. Further study of insulin kinetics using more sophisticated analytical methods [440–442] or modelling [82, 443] is warranted in this group to provide a more detailed analysis of antibody affinity and kinetics; this would allow the generation of kinetic models for insulin clearance, which could be supported by *in vivo* glucose and insulin clearance studies [248].

Patient B30 presented a unique analytical challenge, with labile glycaemia, gross hyperinsulinaemia with low insulin recovery by PEG precipitation, and HMW insulin immunoreactivity demonstrable with GFC, consistent with insulin-antibody complexes. However, IA results were conflicting depending on the method used (negative or equivocal using the ELISA, but positive with RIA). This could be explained by the presence of a blocking effect of detemir on the ELISA, or by the presence of a high-affinity IA circulating as immunocomplexes, and thus unable to interact with the insulin bound to the solid-phase of the ELISA. The results of immunosubtraction experiments supported the presence of anti-insulin immunoreactive IgG antibodies in plasma from patient B30, although interpretation may be limited due to the low number of both positive and negative controls from detemir-treated patients. Although assays cannot discriminate between cases of IAS and cases of exogenous insulin-induced IA production, high ambient levels of immunoreactive insulin were present only when the patient was receiving detemir and aspart, and immunoreactive insulin concentrations decreased markedly when aspart alone was administered. The patient was successfully managed with close supervision of insulin administration. It follows that this patient had a pre-existing antibody which was revealed by changing insulin therapy. In the context of labile glycaemia, the pre-presentation plasma insulin concentration of 914 pmol/L, with insulin recovery of 70% following PEG precipitation, concurrent with aspart monotherapy, the patient would have been placed in group 1 until they were treated with detemir. Although this case is anecdotal evidence that changing insulin analogue can have

unpredictable consequences in the face of IA, further studies are needed to confirm whether detemir-specific binding by antibody that is not clinically significant could account for the biochemistry results observed.

A major conclusion from this chapter is that whilst IA-mediated dysglycaemia should be considered as a cause of unexplained labile diabetes, identifying actionable antibodies is complex and requires more than measurement of antibody alone. Data presented in this thesis demonstrate that direct measurement of anti-human insulin IgG is neither sensitive nor specific enough to identify all patients with those antibodies causing deranged insulin kinetics, and, taking into account assay cross-reactivity, that insulin concentration is a more specific indicator. Possible causes for the lack of ELISA utility may be explained by IA ELISA design. Non-IgG antibodies are not likely to be detected, as the antibody ELISA format uses an anti-IgG conjugate (Section 4.2.4). Human insulin is the assay antigen, therefore antibodies with specificity for non-native insulin, such as those antibodies with epitopes present in a particular insulin analogue, may be poorly detected, if at all. The ELISA attempts to measure endogenous antibody that may be bound as insulin–antibody complexes, thus antibody may be inhibited from interacting in the antibody assay, and therefore be under-represented (Figure 4.9). Proponents of RIA to measure IAs have described the superiority of RIA in this context [444, 445], however, the performance of this method is dependent upon the agent used to precipitate radiolabelled insulin binding complexes (Chapter 5). Neither the ELISA nor RIA assays can provide kinetic data that may be of value in predicting antibody pathogenicity (i.e. insulin-IA association rate and dissociation-rate). Modern methods, such as surface plasmon resonance can directly interrogate analyte and ligand kinetics, providing a real-time readout of immunocomplex formation and dissociation. This has been used to study IA–insulin interaction in diabetes and a presumptive case of IAS [440–442]. Such analyses have derived values of kinetic constants in addition to data of affinity and concentration, however, the technique has yet to be proven robust against the effect of high endogenous insulin on endogenous IA–assay ligand interaction, and the method is challenging in complex matrices such as human plasma.

Due to differences in insulin sensitivity and beta-cell reserve, considerable inter-individual variability in exogenous insulin absorption exists [149]. This is affected by change in depth and site of injection [446], pharmacokinetics, and pharmacodynamics [447], and there is substantial complexity in

the clinical determination of aberrant plasma glucose excursions in an insulin-treated individual with diabetes. For the three patients with actionable antibodies presented in this thesis, the presentations encompassed hyperglycaemia with delayed onset of insulin action, unpredictable insulin action with daytime hyperglycaemia and nocturnal hypoglycaemia, and resistance to exogenous insulin. Such presentations are not specific for IA-mediated dysglycaemia, and for the population of individuals with labile diabetes, glycaemic lability may occur more frequently due to insulin-carbohydrate mismatch that may be more appropriately treated with insulin management and dietary manipulation. Insulin antibody studies, as detailed in this thesis, may provide useful data to exclude IAs as a contributory factor in this context.

For individuals with insulin-treated diabetes, laboratory investigation is also complex, more so than for IAS, as IAs are more common, and insulin:C-peptide ratios cannot be used. In both cases, immunoassay of insulin has limitations in the presence of IA, as antibodies can interfere with insulin measurement by immunoassay (Section 4.3.1). The limitations of insulin analogue assays need to be considered, which compromises the integrity of the results presented in this chapter: Firstly, the Mercodia Iso-insulin has a narrow analytical range, and only 15 patients had an ambient insulin concentration within that range and all others required further dilution. Secondly, immunoassay linearity (optimised for measurement of human insulin) may also be affected by the insulin analogue(s) present in plasma due to insulin analogue cross-reactivity (Chapter 2). Invariably, there is more than one insulin species in circulation (e.g. long-acting analogue, short-acting analogue, and endogenous insulin present concurrently) that may compete to differing degrees for assay antibodies. Thirdly, insulin analogue absorption and metabolism can differ markedly from human insulin, and it may be the analogue metabolites that are present in plasma rather than the native molecule (e.g. glargine) (Section 1.5.6) and/or the analogue may be subject to albumin-binding (e.g. detemir; Section 1.5.7). Finally, the molar concentration of insulin therapies differs per insulin unit (Section 1.5.7). It follows that plasma insulin measurement as an estimation of bioavailable insulin analogue appears simplistic compared with the situation *in vivo*. Although MS provides a more specific means of detecting insulin analogues (Section 1.7.3), quantification of total insulin will not provide information on the relative proportion of free:IA-

bound insulin in plasma using such methods, unless a size-exclusion technique is employed prior to MS analysis.

Whilst it is generally accepted that detectable circulating IAs in insulin-treated DM usually have little or no clinical consequence for insulin kinetics [448, 449], data in this chapter demonstrate that for rare individuals, the presence of insulin-binding antibodies can restrict the opportunity to achieve optimal glucose control with exogenous insulin, and identification of such antibodies may allow the understanding of the mechanism by which insulin action is deranged and thus open new care pathways. For clinicians involved in the management of patients with diabetes, it is noteworthy that antibody-mediated dysglycaemia in insulin-treated diabetes may occur in the current era, including those insulin-treated individuals prescribed modern synthetic insulin analogues. Clinical clues may be identified from a clinical history of unexplained erratic glycaemia, particularly on the background of previously well-controlled diabetes. The patient may report aberrant/unpredictable insulin action that may encompass delayed onset of insulin action, prolonged insulin action, prolonged hypoglycaemia in the face of exogenous insulin cessation, and/or highly variable insulin requirements not explained by carbohydrate intake/activity. There may be unexplained severe exogenous insulin resistance (hyperglycaemia/ketosis/DKA) in the face of high insulin doses and/or unexplained recurrent hypoglycaemia in the face of low insulin doses. On examination of blood glucose measurements taken throughout the day (e.g. CBG or CGM), a recurrent pattern of nocturnal/matutinal hypoglycaemia and daytime hyperglycaemia may be seen. Laboratory investigation should include blood analysis by a panel of assays: measurement of anti-insulin antibodies – although not a specific test, a very high insulin concentration is more suggestive of the condition; measurement of insulin using a broad-specificity assay; and, when sample ambient insulin immunoreactivity is high (>2500 pmol/L using the Mercodia Iso-Insulin), GFC following sample incubation with recombinant human insulin. Samples can be taken in clinic, and while no special preparation is required, it is desirable for patients to take insulin therapy in their usual way. To aid result interpretation, an appropriate assessment of glucose variability throughout the day should be sought and a record of insulin treatment, dosage, and last dose taken.

While an approach to investigating suspected antibody mediated labile diabetes has been tested in this thesis, full understanding of the limitations of the analytical techniques will require a larger

population study. Whilst the analytical approach remains laborious, employing multiple techniques enhances diagnostic sensitivity, results from adjunctive assays were shown to help alter clinical management for patients that may have suffered poor glycaemic control for many years. However, the benefit of such investigations extends beyond solely the identification of rare patients, but more commonly to be able to rule out IA-mediated dysglycaemia confidently, thus allowing the clinician and patient to seek alternative explanation for the dysglycaemia.

CHAPTER 7: Development of a novel clinical assay for the detection of anti-insulin receptor antibodies

7.1 Background

Type B insulin resistance (TB-IR), a rare condition caused by pathogenic insulin receptor antibodies (Section 1.14), is a cause of significant morbidity for affected patients. When individuals with pathogenic antibodies are identified, antibodies can be successfully treated with immunodepletion therapy [363]. In addition to identifying such cases, ruling-out the presence of these antibodies can have great clinical utility. Currently, the ‘gold standard’ insulin receptor antibody assay is a Western blot method, that utilises non-purified cell lysates of receptor-expressing Chinese hamster ovary (CHO) cells [367]. This assay employs immunoprecipitation of solubilised native human insulin receptors bound to endogenous antibody, and through capture with goat anti-human IgG–agarose beads, endogenous insulin receptor antibody is detected by immunoblotting using insulin receptor beta subunit-specific antibody. Although this research-based assay has proved to be robust in detecting pathogenic anti-insulin receptor IgG over many years of diagnostic use, the assay has two key limitations as a clinical assay: firstly, the assay is totally manual and highly laborious, taking a trained technician three days to complete, and secondly, the assay is only qualitative (or semi-quantitative, at best).

The aim of this part of the project was to begin development of a new assay for the detection of anti-insulin receptor antibodies; one that is less laborious yet maintains the diagnostic capability of the current Western blot method, ideally with improved sensitivity, so that the new assay may be used to detect and quantify insulin receptor antibodies for clinical purposes. A solid-phase ELISA design was chosen for its practical simplicity, its suitability to use in a clinical laboratory (by not requiring the use of specialised research-based equipment or radioactive materials), and the utilisation of hardware or materials that, where appropriate, could be shared by other more commonly-used commercial antibody assays. A CHO Flp-IN cell line, over-expressing myc-tagged human insulin receptors (CHO Flp-IN hINSR WT) was used to produce antigen and offered potential advantages of targeted anchoring and increased antigen purity over CHO-cell lysates from the Western blot method (that contain receptors

without a myc-tag). It was hypothesised that novel ELISA will provide results which are as/more useful than the current gold standard Western blot assay, and the ELISA will be less laborious, allowing shorter turn-around times.

7.2 Materials and methods

7.2.1 Patient samples

Samples were taken at clinical presentation from patients with labile glycaemia suspected clinically to produce pathogenic insulin receptor antibodies. Two pools of anonymised samples that were assayed using the Western blot method as part of clinical investigation via the UK Severe Insulin Resistance Supraregional Assay Service, one pool with strongly detectable and one pool without detectable anti-insulin receptor antibodies, were used as positive and negative control material respectively. Blood samples were collected on wet ice and serum was promptly separated then frozen at -80°C until analysis. All studies were performed in accordance with the World Medical Association Declaration of Helsinki (2000).

7.2.2 Cell culture

The cell culture protocol was designed with close collaboration with Gemma Brierley, Research Associate, Department of Clinical Biochemistry, WT-MRC Institute of Metabolic Science; and cell culture experiments were performed under the supervision of Rachel Knox, Research Assistant, The University of Cambridge Metabolic Research Laboratories. CHO Flp-IN cells, stably expressing C-terminal myc-tagged human insulin receptor (CHO Flp-IN hINSR WT; generated and kindly donated by Gemma Brierley) were maintained at 37°C in a humidified incubator in F-12 Ham nutrient mixture supplemented with 10% (w/v) foetal bovine serum (FBS), 1,000 U/L penicillin, 0.1 g/L streptomycin, and 4 mmol/L L-glutamine (Sigma-Aldrich[®]). Hygromycin B (Thermo Fisher Scientific) at a concentration of 200 $\mu\text{g}/\text{mL}$ was used to continue selection for insulin receptor-expressing cells, as the expression cassette contains the hygromycin B phosphotransferase (hph) resistance gene.

After long-term cryostorage of cells in 90% (w/v) FBS and 10% (v/v) dimethyl sulfoxide, cells were thawed rapidly in a water bath at 37°C , added to 20 mL of the above media in a T75 flask (omitting hygromycin B for the initial recovery passage), and grown until confluence two days later. Cells were then passaged by removing media and washing with warmed phosphate-buffered saline (PBS, Sigma-Aldrich[®]), prior to adding 3 mL 1x trypsin-EDTA (Sigma-Aldrich[®]) and incubating at $37^{\circ}\text{C}/5\%\text{CO}_2$ for

five minutes. Cells were then visualised under the microscope to ensure all cells had detached from the flask base, and cells were transferred into five new flasks at a split ratio of 1:7 with the media described above. Upon confluence two days later, cells were harvested by removing the media, washing twice with PBS, and snap-freezing in liquid nitrogen. The flasks were stored at -80°C until use.

7.2.3 Preparation of cell lysate

The flasks containing snap-frozen confluent CHO Flp-IN hINSR WT cell monolayers, were defrosted on wet ice. To each, 12 mL chilled lysis buffer (Table 2.1) was added, ensuring full and even coverage of the monolayer, and the flasks were incubated at 4°C for one hour to allow chemical lysis and solubilisation of the cell membrane. Cells were then scraped, collected into 15 mL falcon tubes, and centrifuged at 4,000 g for 15 minutes at 4°C . The cell debris pellet was discarded, and the cleared supernatants of lysate were combined and stored in 2 mL aliquots at -80°C until required to prepare assay plates.

7.2.4 Insulin receptor antibody enzyme-linked immunosorbent assay

ELISA experiments were carried out with benchwork assistance from Cornelia Gewert, Research Associate, The University of Cambridge Metabolic Research Laboratories. A schematic representation of the insulin receptor antibody ELISA design is given in Figure 7.1. The assay was performed as follows:

On day one, anti-myc antibody (Millipore, Clone 9E10) was diluted to a concentration of 2.5 $\mu\text{g}/\text{mL}$ in 100 mmol/L bicarbonate/carbonate buffer pH 9.6. A LUMITRACTM 96-well white microplate (Greiner Bio-One International) was coated with 100 $\mu\text{L}/\text{well}$ anti-myc antibody solution (250 ng/well) and incubated overnight at 4°C .

On day two, the microplate wells were washed three times with 200 $\mu\text{L}/\text{well}$ of Tris-buffered saline with 1% (v/v) Tween[®]20 (TBST), and emptied, before the addition of 200 μL of blocking solution (2% (w/v) BSA/TBST). The plate was incubated for 2 hours at 23°C . Microplate wells were then washed three times with 200 $\mu\text{L}/\text{well}$ of TBST, emptied, and 100 $\mu\text{L}/\text{well}$ of either neat CHO-WT-hINSR-myc cell lysate, or blocking solution, was added to each well. The plate was incubated overnight at 4°C .

On day 3, microplate wells were washed three times with 200 μL /well of TBST. Sample was then added, 100 μL /well, and the plate incubated for 2 hours at 23°C. Each sample was analysed in duplicate. Microplate wells were then washed three times with 200 μL /well of TBST, emptied, and incubated in 100 μL /well of specific IgG conjugate (β -galactosidase–anti-IgG (approximately 1 $\mu\text{g}/\text{mL}$) mouse monoclonal antibody, Thermo Fisher Scientific) for 30 minutes at 23°C. Microplate wells were then washed three times with 200 μL /well of TBST, emptied, and incubated in 100 μL /well of Development Solution (4-methylumbelliferyl- β -galactoside 0.01%, Thermo Fisher Scientific) for 9 minutes at 23°C. Then, 100 μL /well of Stop Solution (Sodium carbonate 4% (alkaline), Thermo Fisher Scientific) was added, and the plate incubated for 9 minutes at 23°C. Fluorescence was then measured, at an excitation wavelength of 365 nm and an emission wavelength of 455 nm, using a microplate reader (Infinite[®] M1000 PRO, Tecan).

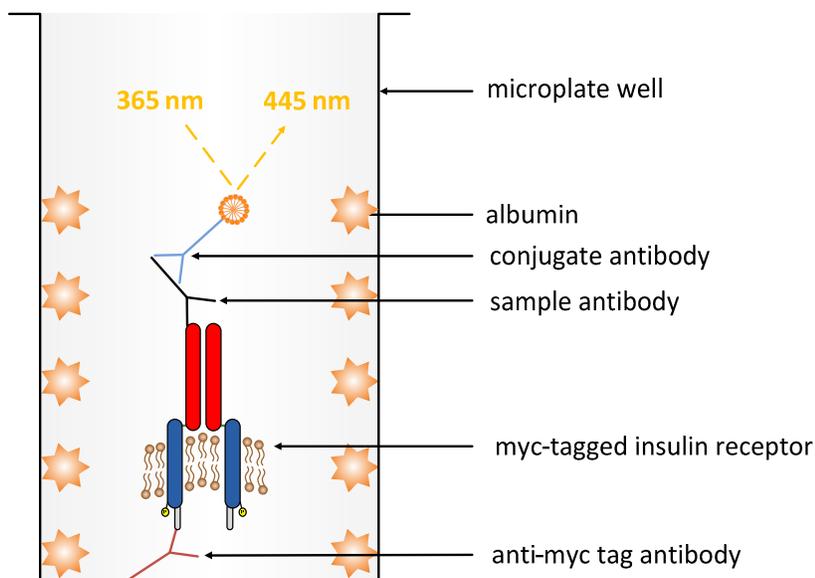


Figure 7.1 Schematic representation of the anti-insulin receptor antibody ELISA.

Microplate wells are coated with anti-myc antibody, blocked with BSA, and further coated with myc-tagged native human insulin receptor (CHO Flp-IN hINSR WT). Anti-insulin receptor IgG antibodies present in the sample bind to insulin receptor and are detected by specific IgG conjugated to β -galactosidase. 4-methylumbelliferyl- β -galactoside used as a substrate for β -galactosidase and, when excited at 365 nm, the released methylumbelliferone is monitored at 445 nm.

7.2.5 Examination of non-specific binding

The assay was performed as above analysing 2% (w/v) BSA ('blank'), neat positive control serum, neat negative control serum, neat positive control serum without CHO Flp-IN hINSR WT lysate, and neat negative control serum without CHO Flp-IN hINSR WT lysate. Analyses were repeated four times (analysis in duplicate on two separate assays).

7.2.6 Examination of assay linearity with sample dilution

Positive control serum, and negative control serum, was analysed neat, and following dilutions with EIA diluent (Thermo Fisher Scientific), serum 1:9 with diluent, with varying dilutions of CHO Flp-IN hINSR WT lysate, and dilutions of specific IgG conjugate, as outlined below. Analyses were performed in duplicate and mean fluorescence calculated.

7.2.7 Examination of assay linearity with CHO Flp-IN hINSR WT lysate dilution

The assay was performed with neat CHO Flp-IN hINSR WT lysate, and following dilution ratios: lysate 1:1, 1:3, and 1:7 with EIA diluent, and dilutions of serum, as outlined above. Analyses were performed in duplicate and mean fluorescence calculated.

7.2.8 Examination of assay linearity with specific IgG conjugate dilution

The assay was performed with neat specific IgG conjugate, and following dilution ratios: specific IgG conjugate 1:1, 1:3, and 1:7 with EIA diluent, and dilutions of serum, as outlined above. Analyses were performed in duplicate and mean fluorescence calculated.

7.3 Results

7.3.1 Examination of non-specific binding

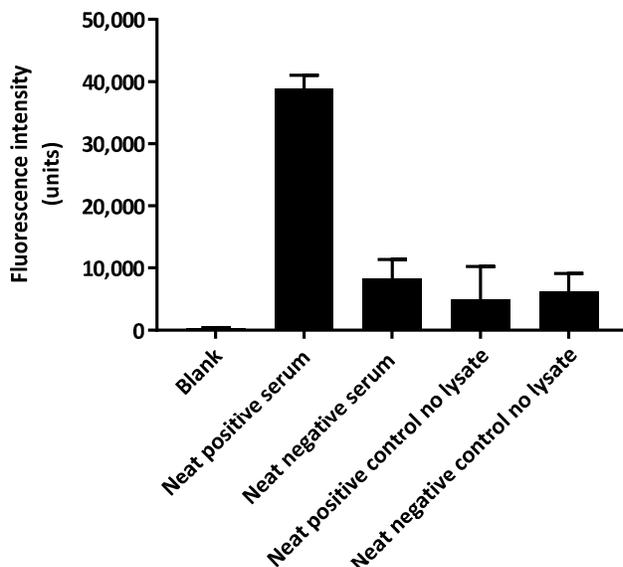


Figure 7.2 Examination of non-specific binding in the insulin receptor antibody ELISA. Mean and standard deviation of fluorescence intensity from four analyses. Blank: 2% (w/v) BSA; neat positive control serum: insulin receptor antibody positive by Western blot method; neat negative control serum: insulin receptor antibody negative by Western blot method; neat positive control serum without CHO Flp-IN hINSR WT lysate: insulin receptor-free well; and neat negative control serum without CHO Flp-IN hINSR WT lysate: insulin receptor-free well.

To examine non-specific binding, 2% (w/v) BSA ('blank'), neat positive control serum, neat negative control serum, and neat positive control serum without CHO Flp-IN hINSR WT lysate, and neat negative control serum without CHO Flp-IN hINSR WT lysate, were assayed (Figure 7.2). Analyses were repeated four times (analysis in duplicate from two separate assays). Mean (and standard deviation) of fluorescence intensity was calculated as: Blank: 2% (w /v) BSA; 292 units (134 units); neat positive control serum: 38,879 units (2121 units); neat negative control serum: 8218 units (3116 units); neat positive control serum without CHO Flp-IN hINSR WT lysate: 4886 units (5400 units); neat negative control serum without CHO Flp-IN hINSR WT lysate: 6204 units (2888 units). Fluorescence

was approximately four-times higher in neat positive compared with neat negative serum. Fluorescence from the blank was lowest, however neat positive control serum without CHO Flp-IN hINSR WT lysate, and neat negative control serum without CHO Flp-IN hINSR WT lysate was comparable with neat negative serum.

7.3.2 Examination of assay linearity with sample dilution and with CHO Flp-IN hINSR WT lysate dilution

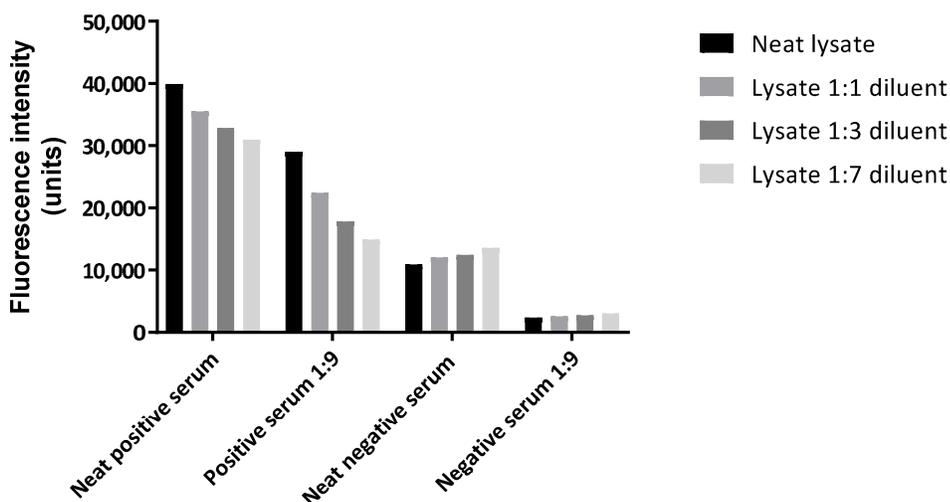


Figure 7.3 Assay linearity with sample dilution and CHO Flp-IN hINSR WT lysate dilution. Neat positive control serum: insulin receptor antibody positive by Western blot method: neat negative control serum: insulin receptor antibody negative by Western blot method. Sample dilution with EIA diluent, serum 1:9 diluent. Lysate dilutions with EIA diluent, lysate 1:1, 1:3 and 1:7 with diluent. Analyses were repeated in duplicate and mean calculated.

Experiments were undertaken to examine the effect of sample dilution, CHO Flp-IN hINSR WT lysate dilution, and specific IgG conjugate dilution. In neat serum, fluorescence was highest using neat lysate, and decreased with lysate dilution. Using neat lysate, the difference in signal observed between positive and negative serum was greater between samples diluted 1:9 with EIA diluent than between neat samples (units ratio 12.4 versus 3.7, respectively) (Figure 7.3). In negative serum, serum dilution had a more pronounced effect on lowering fluorescence than lysate dilution.

7.3.3 Examination of assay linearity with specific IgG conjugate dilution

In neat serum, fluorescence was highest using neat conjugate, and decreased with conjugate dilution. Using neat conjugate, the difference in signal observed between positive and negative serum was greater between samples diluted 1:9 with EIA diluent than between neat samples (ratio 14.6 versus 6.8, respectively) (Figure 7.4). In negative serum, fluorescence decreased with serum dilution and lysate dilution.

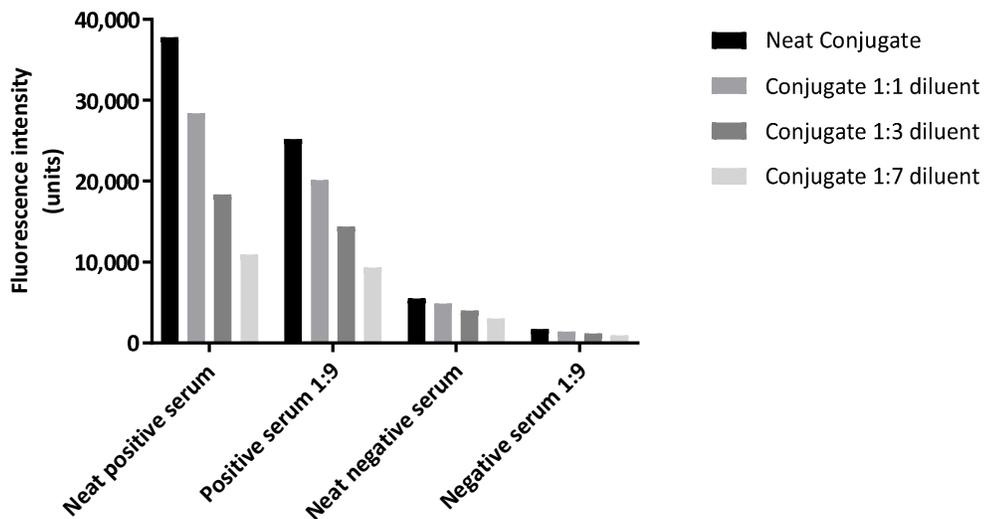


Figure 7.4 Assay linearity with sample dilution, and specific IgG conjugate dilution. Neat positive control serum: insulin receptor antibody positive by Western blot method; neat negative control serum: insulin receptor antibody negative by Western blot method. Sample dilution with EIA diluent, serum 1:9 with diluent. Neat specific IgG conjugate, and following dilutions with EIA diluent, conjugate 1:1, 1:3 and 1:7 with diluent.

7.4 Discussion

A solid-phase ELISA was developed for the detection of anti-insulin receptor IgG antibodies. The preparation of the assay plate was relatively short and simple, such that the total labour time was less compared with the gold standard Western blot method. Although requiring two overnight incubations per single assay run, in a situation when the assay may need to be performed on a routine basis, batch antibody coating of microplates could be undertaken in advance, and the plates stored in blocking buffer, thus requiring just one overnight incubation to capture receptor before sample analysis. The new ELISA discriminated the positive and negative control samples, and fluorescence was positively-associated with sample, conjugate, and insulin receptor concentration in positive control serum. From those variables tested, optimal assay conditions to discriminate positive control serum and negative control serum were neat CHO Flp-IN hINSR WT lysate, with neat specific IgG conjugate, and with serum diluted 1:9 with assay diluent.

A notable signal was seen in both neat positive control serum without CHO Flp-IN hINSR WT lysate, and neat negative control serum without CHO Flp-IN hINSR WT lysate. Moreover, these fluorescence signals were of similar magnitude to the negative serum with lysate (Figure 7.2), and were of a higher magnitude than detected in blank wells. This finding may be consistent with non-specific binding, such as that due to insufficient washing/blocking, and/or cross-reactivity of serum constituents with the assay plate/BSA/coating antibody. A marked decrease in negative control background signal was not observed with dilution of conjugate, but was observed with dilution of sample (and, therefore, any potential serum interferents). Although BSA is a commonly-used blocking agent for biochemistry assays of human serum, BSA cross-reactivity could be examined further, and if significant interference is observed, alternative blocking agents, such as a non-mammalian protein blocker (e.g. salmon serum), or a protein-free matrix, could be used.

Fluorescence decreased for positive serum with lysate dilution, conjugate dilution, and for negative serum conjugate dilution, however, signal appeared unchanged, or even increased with lysate dilution for negative serum. To determine whether the latter finding is analytically significant requires further investigation to determine analytical variability.

Now the assay design has been demonstrated as ‘proof of principle’, further work will be undertaken to adapt the ELISA to incorporate DELFIA[®] technology, increase assay sensitivity, increase dynamic range, and reduce background signal (Appendix D: Plan of further development of anti-insulin receptor antibody assay). Anti-insulin receptor antibodies represent an important mechanism for disordered glucose metabolism that may present in isolation, or may complicate pre-existing DM. A rare cause of dysglycaemia, TB-IR may lead to significant morbidity in affected individuals, compounded in situations where the diagnosis is overlooked, or if considered, is delayed due to the current unavailability of a clinically-validated diagnostic assay. Given the complex clinical presentation of dysglycaemia and the multitude of causes, exclusion of this condition can be as useful as a positive diagnosis. In the UK the Western blot assay is currently offered for the diagnosis of this condition on a semi-research basis; as discussed, this is semi-quantitative at best, and, due to the complexity of the assay, suffers from long turn-around times. A more robust, validated assay would not only reduce turn-around times and provide more provenance to the results generated, but would also allow access to the assay in a wider patient group to investigate potential roles for anti-insulin receptor antibodies in more common presentations of dysglycaemia. Improved sensitivity of insulin receptor antibody detection will have utility in detecting those antibodies of low concentration responsible for hypoglycaemia. In addition, the combination of an increased dynamic range and precise antibody quantification will offer scope to detect changes in insulin receptor antibody concentration to monitor response to immunomodulatory treatment and detect disease relapse. Of wider importance, such an assay will allow greater access for clinicians to screen patients for insulin receptor antibodies, adding to the existing repertoire of screening tests used to investigate unexplained labile DM.

CHAPTER 8: Summary and General Discussion

During a dinner at Caius College Cambridge, the word ‘hormone’ was first coined by Starling [450], and later his lecture delivered to the Royal College of Physicians introduced the term to the English language in 1905. Within two decades, Banting and Best had treated the first diabetic patient with insulin [132], and this success revolutionised the management of individuals with DM. The first immunoassay was used to measure the deficient/insufficient diabetes hormone, insulin, in 1959 by Berson and Yalow [185–188], and this paved the way for the serial measurement of hormones that has shaped modern clinical endocrinology. Immunoassays remain central to endocrine laboratories today, yet in diabetology, insulin is rarely measured.

IAS and TB-IR are two rare and severe disorders of insulin action that may complicate pre-existing diabetes and may be treated with multimodal immunosuppression. Milder forms of the conditions are often suspected in patients with insulin-treated DM and labile glycaemic control. This research aimed to target the major limitations of existing assays to diagnose these conditions: existing IA testing does not yield sufficient information about the likelihood that the IAs detected were altering insulin kinetics and/or dynamics to a clinically-significant degree, and there lacked a readily accessible clinically-accredited diagnostic test for TB-IR.

Plasma insulin is measured as part of the investigative work-up of individuals with unexplained spontaneous hypoglycaemia and/or exogenous insulin resistance, and this research began by evaluating the cross-reactivity of insulin therapy in insulin immunoassay. First generated in the 1980s, insulin analogues, which exhibit antigenic differences from native hormone, are prescribed to treat diabetes, yet those clinical immunoassays used to measure insulin demonstrate specificity for human insulin. Consequently, some clinical immunoassays fail to quantify correctly or to detect circulating insulin analogue therapy, and this requires important consideration to avoid misinterpretation of insulin results in individuals presenting (reportedly) with exogenous insulin resistance and/or suspected insulin misadministration.

Described in this thesis is an approach to the evaluation of IAs in patients with unexplained spontaneous hypoglycaemia. The question was critical, not only because pathogenic antibodies may

effectively be treated with antibody-depleting therapies, curing life-threatening metabolic derangement, but also because immune-mediated hypoglycaemia is a major differential diagnosis in cases of suspected surreptitious insulin administration or insulin poisoning. Additionally, in rare instances, the immune system itself can complicate immunoassay detection of hormones, when heterophilic antibodies block or bind capture/detection antibodies, generating misleading assay results that can lead to unnecessary clinical intervention. Included in this research is a report on the performance of commercially available insulin immunoassays in the context of dilution and PEG precipitation studies. A novel protocol for detecting insulin-antibody complexes using GFC, with incorporation of *ex vivo* insulin binding, is described. In addition to demonstrating the presence of insulin binding by antibody and providing some information regarding the insulin-binding capacity of IA, the GFC method offered evidence to refute the presence of HMW insulin immunoreactivity caused by heterophilic antibody interference. Use of total MS insulin and C-peptide assays obviated possible interference by antibody, and there is now scope to explore further the use of MS technology in cases of insulin immunoassay interference, and for quantification of insulin analogues in plasma. As standard MS methods will only detect the total insulin concentration, further development is needed to discriminate insulin that is free versus IA-bound.

The existence of insulin-binding immunoglobulin has been known for more than half a century [298], but the major unmet need arises from poor specificity of direct measurement of IAs for identifying those with insulin-binding antibodies provoking clinically-significant aberration of insulin kinetics/dynamics. The evolution of clinical IA measurement has involved some divergence from the use of laborious RIA, requiring long incubation times, to the use of fast turn-around, random-access ELISA platforms that do not use radiolabelled materials. However, RIA remains the preferred assay design for studies of autoimmune DM [395, 444, 445, 451]. This research is the first report of an evaluation of RIA and ELISA in the context of diagnosing IAS, and neither RIA nor ELISA proved universally sensitive in identifying the disorder. Merely detecting IA does not establish them to be clinically significant, which is why collateral studies including PEG precipitation and chromatography to demonstrate complexes are of value. Elucidation of immunocomplex association and dissociation rates may have diagnostic value, and surface plasmon resonance may provide this information in combination with measurements of affinity and concentration using just a single platform. However, the

effect of endogenous insulin in plasma on IA–assay ligand interaction must be determined before the utility of this technique can be fully exploited.

As it has been demonstrated that individuals treated with immunomodulation may have symptomatic benefit and objective improvements in glucose control with some antibodies still present (Chapter 5), it follows theoretically that, in the face of immunosuppression, antibodies could remain detectable, even as their ability to sequester insulin diminishes. For each patient described in this thesis, careful consideration was given as to which index of circulating insulin–antibody complexes to use for monitoring. In all cases it was critical to keep dysglycaemia firmly in mind as the disorder being treated, rather than relying on total depletion of antibodies as a treatment target. Following the advancement in understanding of the varied presentation of dysglycaemia, and the IA-binding characteristics, derived from the findings in this research, further examination of insulin clearance and glucose excursion rates can be justified.

Outside the extreme clinical phenotype, the influence of IAs on glycaemic control in insulin-treated individuals with DM in the current era is believed usually to be clinically insignificant [448, 449]. From population studies, there is some suggestion IAs cause relative postprandial hyperglycaemia in T1DM [452], but there is no clear correlation between IA levels and average glycaemic control [230, 435, 460, 436, 453–459]. There is evidence for [235, 461, 462], and against [295, 454, 459], an association of IAs with insulin dose, with limited data available on the study of IAs and onset of insulin action [463]. All such studies are limited by clinical inclusion/exclusion criteria and the robustness of the chosen laboratory investigative approach. Following this research, there is scope for a case–control study using the methods outlined in this thesis to compare a cohort of individuals with labile glycaemia with a cohort with good glycaemic control, and to look for a statistically significant difference in “positivity” for a slimmed-down panel of investigations for IAS.

Diagnosing IA-mediated labile glycaemia in insulin-treated DM is complex; poor glycaemic control in DM may have multiple contributory factors, circulating IAs in insulin-treated DM is common, and the association of antibodies with dysglycaemia may not be causal. It followed that, in addition to the detection of large circulating pools of antibody-bound insulin, careful assessment of insulin management and objective measures of glycaemia were required to confirm IAs as the principal cause

of an individual's labile diabetes. Use of insulin kinetic modelling in the context of insulin-binding antibodies to provide a more robust determination of aberrant insulin kinetics will help in this regard and is a possible future area of study.

Anti-insulin receptor antibodies can be detected using time-consuming and laborious research tests, but there is no reliable and rapid means available in hospital laboratories. Part of this research project set a foundation for a new test for anti-insulin receptor antibodies that is to undergo further development with the intention that testing for these antibodies can become more accessible. Successful detection of anti-insulin receptor antibodies incorporating CHO-cell-expressed myc-tagged human insulin receptor using ELISA was achieved. Further development is required to optimise the assay before rolling out in hospital laboratories. In addition to its use to diagnose TB-IR, further study of the new assay in a broader population of individuals with labile diabetes may help to identify patients with a lower concentration of antibodies not identified using the Western blot method, and thus possibly identify a broader disease phenotype.

8.1 Concluding comments

Yalow stated during her Nobel Lecture in 1977 concerning RIA [189], "Only if we can detect and measure can we begin really to understand", and the measurement of hormones began with the paradigm of insulin immunoassay that advanced endocrinology. With clinical descriptions reported as far back as the introduction of insulin therapy, although less commonly reported today, antibody-mediated insulin resistance and labile diabetes, like TB-IR, continue to prove to be a physiological burden to afflicted individuals. The new knowledge gained through this research has already been instrumental in the diagnosis and management of individuals with antibody-mediated dysglycaemia in situations that, until investigated in Cambridge, had been diagnostic dilemmas to clinicians. There now exists the challenge to endocrine biochemists to increase access to such robust diagnostics, and through population studies, to understand the prevalence of antibody-mediated dysglycaemia in individuals with insulin-treated DM.

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APPENDICES

Appendix A: Assay performance characteristics

Assay	Precision studies		Spiking recovery (mean, %)	Linearity (Mean dilution recovery, %)	Reference
	Within-run coefficient of variation (mean, %)	Total coefficient of variation (mean, %)			
Siemens ADVIA® Centaur	3.7	4.4	108.4	94.6	Insulin (IRI). Siemens ADVIA® Centaur and ADVIA® Centaur XP Systems, Siemens Healthcare Diagnostics, 10629880_EN Rev. L, 2011-07 © 2008
Siemens IMMULITE® 2000	4.0	5.1	91.6	104.4	Immulite® 2000 Insulin, Siemens Healthcare Diagnostics, PIL2KIN-29, 2009-10-06
DiaSorin LIAISON® XL	3.3	4.3	99.0	105.3	LIAISON® Insulin, REF 310360, DiaSorin, DiaSorin S.p.A, EN - 200/007-911, 02 - 2013-03
PE AutoDELFIA®	3.1	4.6	96	100	B080-101 AutoDELFIA® Insulin, Time-resolved fluoroimmunoassay, Wallac Oy, 13904213-15 Revised April 2011
Beckman Coulter Access® 2	2.6	4.0	97	101.3	ULTRASENSITIVE INSULIN, REF 33410, Beckman Coulter Access Immunoassay Systems, Beckman Coulter, Inc., A83694B © 2010
Mercodia Iso-Insulin	3.0*	4.5*	101*	115**	* Merckodia Iso-Insulin ELISA, Merckodia AB, 31-3112, version 9.0; **in-house data
Mercodia Insulin	3.4	4.5	104	-	Mercodia Insulin ELISA, Merckodia AB, 31-3107, revised 2007-01-16
Roche Elecsys®	1.1	3.6	-	-	Elecsys Insulin, Roche Diagnostics GmbH, 2017-06, V 15.0
Abbott ARCHITECT	2.5	2.3	95.5	-	ARCHITECT Insulin REF 8K41-27, Abbott Laboratories Diagnostics Division, Insulin 8K41, F5-Y302-2/ R03, B8K4Y0, revised 2015-09

Appendix B: Clinical characteristics of patents without pre-existing

DM

Patient	Age (years)	Sex	Ethnicity	BMI (kg/m ²)	Past medical history	Medications	Presentation
A1	56	Female	Caucasian	26.2	Autoimmune hypothyroidism Asthma Factor XI deficiency	None	Postprandial hypoglycaemia
A2	28	Female	Caucasian	25.1	Autoimmune hypothyroidism Alcoholic hepatic cirrhosis Systemic lupus erythematosus	Hydroxychloroquine Propranolol Ranitidine Lansoprazole Levothyroxine	Postprandial hypoglycaemia
A3	37	Female	Caucasian	28.5	Pulmonary embolism (lupus anticoagulant) Gestational DM	None	Recurrent severe hypoglycaemia
A4	52	Female	Thai	35.0	None	None	Fasting hypoglycaemia
A5	28	Female	Caucasian	25.1	None	None	Fasting hypoglycaemia
A6	76	Male	Caucasian	29.5	Type 2 diabetes Ischemic heart disease Parotid pleomorphic adenoma Glaucoma	Spirolactone Furosemide Losartan Aspirin Bisoprolol Atorvastatin Omeprazole Fluoxetine	Postprandial/ nocturnal hypoglycaemia
A7	89	Female	Caucasian	19.4	Small B cell lymphoma	Fruzemide Fexofenadine Ferrous fumarate	Low capillary blood glucose readings
A8	50	Male	Caucasian	22.3	None	None	Postprandial hypoglycaemia

Appendix C: Clinical characteristics of patents with insulin-treated DM

Case	Age	Sex	Diabetes type	Other medical history	Insulin 1	Insulin 2	Other medications	Presenting Features
B1	44	Female	T1DM	Myasthenia gravis, vitiligo, Budd-Chiari syndrome, thrombophilia	Aspart, CSII	-	-	Cutaneous reaction to insulin, unpredictable insulin action
B2	16	Female	T1DM	-	Glargine, BD	Apidra	Metformin BD	Insulin resistance (increasing insulin dose failed to lower blood glucose)
B3	29	Male	T1DM	Hypertension, hypoglycaemic seizures	Human insulin, OD	Aspart, PRN (glucose >8.5 mmol/L)	Acarbose, with meals	Recurrent hypoglycaemia, unpredictable hyperglycaemia
B4	44	Female	T1DM	Lumbar spine surgery, hysterectomy, deep vein thrombosis, hypothyroidism	Detemir, OM	Aspart, TDS	Sitagliptin OD	Recurrent severe hypoglycaemia
B5	28	Female	T1DM (subtotal pancreatic resection)	-	Aspart, CSII	-	Propranolol OD, naproxen PRN, fluoxetine OD, iron supplements OD	Recurrent hypoglycaemia
B6	56	Male	T2DM	-	Detemir, OD	Lispro, TDS	Metformin BD, simvastatin OD	Early morning hypoglycaemia, protracted insulin response
B7	47	Female	T2DM	Sensorineural deafness, haemangioma, benign liver cyst	Soluble insulin aspart/protamine insulin aspart 30%/70%, TDS	-	Acarbose BD, pioglitazone OD, metformin OD, lisinopril OD, simvastatin OD, omeprazole OD, amitriptyline OD	Labile glycaemia
B8	12	Female	T1DM	-	Glulisine	-	-	Very labile glycaemia, recurrent hypoglycaemia despite low insulin doses
B9	23	Male	T1DM	-	Glargine, OD	Lispro, OD	-	Recurrent hypoglycaemia currently treated with glucocorticoids, low insulin requirements
B10	32	Male	T1DM	-	Glargine, OM	Aspart, with meals	-	Recurrent prolonged hypoglycaemia
B11	43	Male	T1DM	-	Detemir, BD	Lispro, TDS	Dapagliflozin OD, atorvastatin OD	Severe insulin resistance syndrome with high HbA _{1c} , 44 kg weight loss
B12	59	Female	T1DM	Diabetic gastroparesis, Raynaud's phenomenon, hypothyroidism	Aspart, CSII	-	-	Labile glycaemia
B13	45	Female	T1DM	-	Human insulin U-500, QDS	-	-	Severe insulin resistance syndrome, hyperglycaemia

(continued)

Clinical characteristics of patents with insulin-treated DM (continued)

Case	Age	Sex	Diabetes type	Other medical history	Insulin 1	Insulin 2	Other medications	Presenting Features
B14	17	Female	T1DM	-	Degludec	Glulisine, CSII	-	Extremely challenging labile glycaemia, insulin resistance (multiple insulin preparations tried including lispro and aspart), followed by periods of prolonged hypoglycaemia requiring carbohydrate rescue, evidence of transient HbA _{1c} improvements when insulin is changed (supported by CGMS).
B15	20	Female	T1DM	Significant band-like lipodystrophy on either side but no cutaneous allergy	Detemir, OM	Aspart, with meals	-	Recurrent severe nocturnal hypoglycaemia.
B16	67	Female	T1DM	Asthma	Detemir, OM	Aspart TDS	Sertraline OD, ferrous sulphate OD, amitriptyline OD, simvastatin, OD, salmeterol xinafoate inhaler, BD	Labile glycaemia, recurrent hypo-unawareness > 10yrs, autonomic neuropathy of the gastrointestinal tract.
B17	15	Female	T1DM	-	Glargine, OD	Aspart	-	Poor glycaemic control, high SC insulin doses (four times intravenous insulin dose requirements).
B18	45	Female	T1DM	Depression	Lispro, TDS	-	Sertraline OD	Unexplained severe hypoglycaemic episode.
B19	46	Female	T2DM	Asthma, hypercholesterolaemia, hypertension, painful peripheral neuropathy	Glargine, BD	Aspart, TDS (with meals)	Aspirin OD, frusemide OD, salbutamol, ipratropium bromide BD-TDS, carvedilol BD, atorvastatin OD, pregabalin, perindopril OD, spironolactone OD, tramadol PRN	High subcutaneous insulin requirements (ten-times intravenous insulin requirements).
B20	46	Male	T1DM	Sleeve gastrectomy, intolerance of detemir and aspart (both resulting in palpitations)	Glargine, OM	Glulisine, TDS (with food)	-	Labile glycaemic control (possibly multifactorial).
B21	20	Female	T1DM	Primary hypothyroidism, pulmonary embolism	Lispro, CSII	-	Levothyroxine OD, ferrous sulphate TDS, enoxaparin OD	Autonomic neuropathy (diabetic gastroparesis, neurogenic bladder), no hypo awareness, recurrent DKA then persistent hypoglycaemia for 48 hours despite no exogenous insulin.
B22	43	Female	T1DM	Diabetic gastroparesis, recurrent urinary tract infections, cerebral vascular accident (secondary to air embolism)	Glargine, ON	Insulin aspart, PRN via CSII	Atorvastatin OD, clopidogrel OD, codeine phosphate QDS PRN, lansoprazole BD, paracetamol QDS PRN, pregabalin OD, quinidine sulphate ON	Recurrent admission with DKA, insulin requirements previously high, with recent marked reduction.

(continued)

Clinical characteristics of patents with insulin-treated DM (continued)

Case	Age	Sex	Diabetes type	Other medical history	Insulin 1	Insulin 2	Other medications	Presenting Features
B23	16	Female	T1DM	Migraines, chronic back pain	Soluble insulin aspart/protamine insulin aspart 30%/70%, BD	Aspart, PRN (hyperglycaemia/ ketosis)	-	Insulin resistance, severe cutaneous hypersensitivity to insulin with lipatrophy.
B24	47	Female	T1DM	Long QT syndrome, irritable bowel syndrome	Human isophane insulin, OM	Lispro, with meals	Glucophage SR OD, omeprazole OD, simvastatin OD	Severe hyperglycaemia, unpredictable hypoglycaemia.
B25	29	Female	T1DM	Celiac disease	Detemir	Aspart	-	Variable insulin sensitivity.
B26	45	Female	T2DM	Diabetic neuropathy, hypercholesterolaemia, hypertension,	Human isophane insulin, BD	-	Dapagliflozin OD, exenatide extended-release weekly, ramipril OD, simvastatin ON, zopiclone ON, pregabalin TDS, sertraline OD, pramipexole OD, beclomethasone dipropionate HFA inhaler BD, lithium carbonate ON, salbutamol inhaler BD.	Recurrent episodes of hyperglycaemia with high insulin requirements then recurrent hypoglycaemia requiring marked reduction in insulin requirements.
B27	58	Male	T1DM	Ischaemic heart disease, hypertension, hypercholesterolaemia, eczema	Porcine neutral, CSII	-	Simvastatin OD, aspirin OD, candesartan OD, sitagliptin OD, glyceryl trinitrate spray PRN	Labile glycaemia, hyperglycaemia not responding to increased insulin doses, tried multiple insulin preparations without benefit, delay in insulin action onset.
B28	51	Female	T1DM		Soluble insulin lispro solution/protamine insulin lispro 25%/75%, OD	-	Lisinopril OD, mirtazapine OD	Recurrent unpredictable hypoglycaemia inappropriate for insulin action profile, marked hypo-unawareness.
B29	56	Female	T2DM	Previous allogeneic bone marrow transplant for acute lymphocytic leukaemia, chronic renal impairment (eGFC 26ml/min), unexplained eosinophilia, mild elevated serum free light chains.	Glargine U-200	Lispro U-200	Candesartan OD, carvedilol BD, ferrous fumarate, simvastatin OD, frusemide	Deterioration in glucose control (57 mmol/mol to 115 mmol/mol) requiring insulin treatment, subcutaneous insulin ineffective (up to 4 units/kg). Intravenous insulin resistance (hyperglycaemia with 6 units of insulin per hour).
B30	12	Female	T1DM	-	Detemir	Aspart	-	Labile diabetes, unexplained hypoglycaemia despite stopping insulin.

Abbreviations: OD, *omne in die*, once daily; OM, *omni mane*, every morning; ON, *omne nocte*, every night; BD, *bis in die*, twice daily; TDS, *ter die sumendum*, three times daily; QDS, *quater die sumendum*, four times daily; PRN, *pro re nata*, as required; CSII, continuous subcutaneous insulin infusion.

Appendix D: Plan of further development of anti-insulin receptor

antibody assay

Aim: Convert ELISA to DELFIA® assay format

Objective: Compare fluorescence between positive and negative serum

Principle: Replace specific IgG conjugate (β -galactosidase-anti-IgG) label with DELFIA® Europium-labelled anti-human IgG (PE 1244-330)



Aim: optimise DELFIA®

Objectives:

- 1) Study of non-specific binding, e.g. comparing anti-myc antibody to anti-myc antibody plus CHO Flp-IN hINSR WT lysate
- 1) Study the effect of different anti-myc antibody concentrations, e.g. 5.0 $\mu\text{g/mL}$, 2.5 $\mu\text{g/mL}$, 1.25 $\mu\text{g/mL}$
- 2) Study the effect of different CHO Flp-IN hINSR WT lysate concentrations, e.g. neat, 1/2, 1/4
- 3) Study the effect of different sample dilutions (e.g. 1/2, 1/10, 1/20, 1/100)
- 4) Study the effect of different incubation times (conjugate)
- 5) Determine calibrators e.g. anti-insulin receptor alpha antibody (83-14 or 83-7)



Aim: assay validation

Objectives: confirmation, by obtaining evidence, that the assay requirements are met

Precision

Intra-assay imprecision: repeat sample measurements (ideally 20 times, on samples at a range of antibody concentrations) then determine standard deviation and coefficient of variation

Inter-assay (intermediate) imprecision: repeat measurements at different times/on different days then determine standard deviation and coefficient of variation

Accuracy (Bias)

May be limited assessment to assess due to limited access to robust quantitative methods

Compare DELFIA® quantities result to existing Western blot method

Comparison to existing method

Compare DELFIA® quantities result to 'gold standard' Western blot method

Measurement uncertainty

Determined by calculation, expressed as ± 1.96 of the long-term coefficient of variation

Analytical range (linearity)

Examine a range of concentrations of anti-insulin receptor antibody and assay signal to determine if relationship is linear

Note: linearity to dilution of samples is not essential in this assay, as sample antibodies are unlikely to match the affinity and/or binding capacity of calibrants, it is generally accepted that assays of antibody are unlikely to generate concentrations that are linear to dilution.

Limit of detection

Determination of the lowest concentration obtained from the measurement of a sample containing anti-insulin receptor antibodies that is distinguishable from the concentration generated from the measurement of a sample not containing anti-insulin receptor antibodies. Deduced by repeated measurements of low concentration blank samples and analysis of the distributions of values.

Limit of quantitation

Determination of the lower limit of the reportable range, it is the concentration of an analyte in a sample that can be determined with acceptable precision and accuracy

Specificity

Examine effect of interfering substances, e.g. insulin, insulin analogue, rheumatoid factor

Stability (reagents and samples)

Appendix E: Publications and presentations resulting from this work to date

E.1 Publications

2014 Semple R, Church D. Lessons from the ‘diabetes hormone’: insulin’s triumphs and challenges. *The Endocrinologist* 2014; 111: 10–11.

2015 Parfitt C, Church D, Armston A, Couchman L, et al. Commercial insulin immunoassays fail to detect commonly prescribed insulin analogues. *Clin Biochem* 2015; 48: 1354–1357.

2017 Church D, Cardoso L, Bradbury S, Clarke C, et al. Diagnosis of insulin autoimmune syndrome using polyethylene glycol precipitation and gel filtration chromatography with ex vivo insulin exchange. *Clin Endocrinol* 2017; 86: 347–353.

Church D, Halsall D. The endocrine laboratory. *Medicine (Baltimore)* 2017; 45: 453–455.

Church D, Hunter RW, Lyall M, Clarke C, et al. Resolution of Hypoglycemia and Cardiovascular Dysfunction After Rituximab Treatment of Insulin Autoimmune Syndrome. *Diabetes Care* 2017; dc170264.

2018 Church D, Semple R. Biochemical Endocrinology. In: Baynes J and Dominiczak M, eds. *Medical Biochemistry (Fifth Edition)*. 2018, pp. 369–396.

Church D, Cardoso L, Kay R, Williams C, et al. Assessment and Management of Anti-insulin Autoantibodies in Varying Presentations of Insulin Autoimmune Syndrome. *JCEM*. Epub ahead of print 2018. DOI: 10.1210/jc.2018-00972. [Epub ahead of print].

David S Church, The Insulin Antibody Study Group, Julie Harris, Keith A Burling, et al. Insulin autoantibody-induced labile glycemia in insulin-treated diabetes. *In Preparation*.

E.2 Presentations

2013 Poster presentation. **Church D**, Halsall D, Barker P, Burling K, Semple R. Identification of Insulin–Antibody Complexes in the Management of Patients with Positive Anti-Insulin Antibodies. (*Insulin Club, Leonardo Royal Hotel Berlin, Germany, UK*)

Poster Presentation. **Church D**, Isaac I, Barker P, Burling K, Halsall D, Semple R. Identification of Insulin–Antibody Complexes and Anti-Insulin Receptor Antibodies in the Management of Patients with Hyperglycaemia. (*IMS Away Day, Robinson College, Cambridge, UK*)

2014 Poster presentation. **Church D**, Bradbury S, Barker P, Oddy S, Burling K, Cardoso L, Cronin M, Halsall D, Semple R. Insulin–Antibody Complexes Cause Negative Interference in 5 Commonly Used Clinical Insulin Assays. MRL Student Symposium. (*Institute of Metabolic Science, Addenbrooke’s Hospital, Cambridge, UK*).

Oral presentation David Church. Insulin: Assays, Analogues and Antibodies. (MEDIC Meeting, *Institute of Metabolic Science, Addenbrooke’s Hospital, Cambridge*)

Poster presentation. Luís Cardoso, **David Church**, Dírcea Rodrigues, Márcia Alves, Daniela Guelho, Joana Saraiva, Carolina Moreno, Rosa Dantas, Nuno Vicente, Robert K Semple & Francisco Carrilho 2014. Hirata disease: a rare form of hyperinsulinemic hypoglycemia. (*Presented at 16th European Congress of Endocrinology ESE 2014, Wroclaw, Poland. Endocrine Abstracts 35 P327*)

2015 Poster presentation. **Church DS**, Halsall DJ and Semple RK. Development of a laboratory approach to anti-insulin antibody-mediated dysglycaemia to guide clinical decision making. (*Diabetes UK Annual Professional Conference 2015, ExCeL, London, UK*)

Poster presentation. McDonald TJ, Parfitt C, Armston A, **Church D**, Couchman L, Evans C and Wark G. Commercial insulin immunoassays fail to detect commonly prescribed insulin analogues. (*Diabetes UK Annual Professional Conference 2015, ExCeL, London*)

Poster presentation. Raj SC, Herring R, Davidson S, Tuthill B, Wark G, **Church D**, Semple RK and Karim Y. The novel use of a rituximab and methylprednisolone regimen for the

management of autoimmune insulin syndrome. (*Diabetes UK Annual Professional Conference 2015, ExCeL, London, UK*)

Poster presentation. **Church D**, Bradbury S, Houghton S, Harris J, Halsall D, Semple R. Insulin-binding antibodies and labile glycaemia in diabetes mellitus – What have we learnt so far? (*IMS Away Day, Robinson College, Cambridge, UK*)

2016 Oral communication. Myuri Moorthy, Bernard Freudenthal, **David Church**, Judith Kisalu, Emma Woolman, Amy Hale, Huw Beynon, Efthimia Karra, Mark Cohen & Bernard Khoo 2016. Two contrasting cases of spontaneous severe hypoglycaemia secondary to anti-insulin antibodies (Insulin Autoimmune Syndrome / Hirata disease). (*Presented at Society for Endocrinology BES 2016, Brighton, UK. Endocrine Abstracts 44 OC5.4*)

Poster presentation. Bayman EM, Kiff SE, **Church DS**, Semple RK, Noyes KJ, Clark C, Bath LE. Unexplained hypoglycaemia in an adolescent with type 1 diabetes – simple insulin overdose or abnormal insulin binding? (*ISPAD (International Society for Pediatric and Adolescent Diabetes) 2016, Valencia, Spain*)

2017 Poster presentation. Moorthy M, Freudenthal B, **Church D**, Semple RK, Kisalu J, Woolman E, Hale A, Beynon H, Karra E, Cohen MA and Khoo BCE. Two Contrasting Cases of Spontaneous Severe Hypoglycaemia Secondary to Anti-Insulin Antibodies (Insulin Autoimmune Syndrome / Hirata disease). (*ENDO 2017, The Endocrine Society's 99th Annual Meeting and Expo, Orlando Florida, USA*)

Poster presentation. **Church D**, Macfarlane D, Karamat M, Semple R. Anti-insulin antibodies causing labile glycaemia in insulin-treated diabetes. (*Diabetes UK Professional Conference 2017, Manchester Central Convention Complex, Manchester, UK*)

2018 Oral communication. Ruth Ronneberger, Afizah Nobeebux, Yuliya Manova, Gill Rumsby, Francis Lam, Gary Woodward, David Isenberg, Michael Ehrenstein, David Halsall, **David Church**, Robert Semple & Helen Simpson 2018. Recurrent severe hypoglycaemic episodes in the context of insulin receptor antibodies. (Presented at Society for Endocrinology BES 2018, Birmingham, UK. Endocrine Abstracts 55 OC8)

2018 Oral presentation (x2). **David Church**. Insulin: assays, analogues and antibodies; Antibodies that block/stimulate the insulin receptor. (*Barts/UCLH endocrine meeting. UCLH, London, UK*)