ADVANCING INSULIN ANALYSIS IN VARIED MATRICES FOR THE INVESTIGATION OF SUSPECTED HYPOGLYCAEMIC DEATH.

PAUL BROOKES

A thesis submitted in partial fulfilment of the requirements of the University of East London for the degree of Doctor of Philosophy

School of Health Sport and Bioscience

May 2024

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Abstract

Diabetes mellitus is a collection of glucose control disorders resulting from deficiency in insulin production/action. In 2021, 6.7 million diabetes-related deaths were reported worldwide. Since many of these deaths were due to chronic diabetes-related conditions, this figure is likely an underestimate. This study sought to improve the investigation of dysglycaemia-related deaths, particularly post-mortem analysis of recombinant human insulin and insulin analogues. It was hypothesised that a move away from traditional matrices and methods must be undertaken, along with standardisation of data interpretation provided to Coroners.

To achieve this, an end-to-end evaluation of the analytical and reporting process for post-mortem insulin was undertaken.

Roche Cobas® and Siemens Immulite® immunoassays were compared for insulin analysis. These demonstrated acceptable recoveries of endogenous insulin (106.5% and 88.3%, respectively), but poor performance for exogenous insulin compounds. Comparison of assays, e.g., Levemir® 2.0% and 12.5% ($P = < 0.0001$), respectively, and comparison of insulin compounds on a single assay, e.g., NovoRapid® and Levemir® (Cobas®), 11.8% and 2.0% ($P = 0.0007$), respectively, was poor.

A novel high-resolution accurate-mass liquid chromatography–mass spectrometry (HRAM LC–MS) vitreous humour insulin method was developed. Qualitative performance was demonstrated for five insulin compounds, with variability from expected mass/charge of ≤ 4.89 parts per million for all mass/charge groups detected

Quantitative performance for NovoRapid® demonstrated linearity up to 2000 pmol/L ($R^2 = 0.9987$), and a lower limit of quantification of 47.2 pmol/L. In one post-mortem case, two insulin compounds, Humalog® and NovoRapid®, were detected. It is unusual for these to be prescribed in combination, suggesting deliberate administration of excess insulin.

To advance the investigation of dysglycaemia-related deaths, migration away from the use of immunoassays is clearly indicated, given the poor recovery of exogenous insulin compounds. The importance of HRAM LC–MS vitreous

humour insulin analysis was highlighted by the detection of a previously-unreported potential insulin overdose. The outcomes from this study would collectively improve the investigation of dysglycaemia-related deaths, enhancing the service provided to families of the deceased.

Declaration

I, Paul Brookes, declare that I am the sole author of this thesis. No part of this thesis has been submitted, in whole or in part, in any previous application for a degree.

Except where stated otherwise, by reference or acknowledgment, the work presented is entirely my own.

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Abbreviations

Abbreviation	Full Phrase
AKA	Alcoholic ketoacidosis
ATP	Adenosine triphosphate
AUC	Area under (the) curve
β-OHB	β-hydroxybutyrate
B-R B	Blood-retinal barrier
CCG	Clinical Commission Group
CLIA	Chemiluminescence immunoassay
CO	Carbon monoxide
COVID-19	Coronavirus disease 2019
CSF	Cerebro-spinal fluid
CV	Coefficient of variation
DKA	Diabetic ketoacidosis
ELSIA	Enzyme-linked immunosorbent assay
FDA	U.S. Food and Drug Administration
ESI	Electrospray ionisation
GC–MS	Gas chromatography-mass spectrometry
HbA_{1c}	Haemoglobin A_{1c} / glycated haemoglobin
HbF	Foetal haemoglobin
HbS	Sickle cell haemoglobin
HHS	Hyperosmolar hyperglycaemic state
HRAM	High-resolution accurate-mass
HRAM LC–MS	High-resolution accurate-mass liquid chromatography-mass spectrometry
HTA	Human Tissue Authority
IAS	Insulin autoimmune syndrome
ICT	Integrated chip technology
IDE	Insulin-degrading enzyme
ISO	International Standards Organisation
LADA	Latent autoimmune diabetes in adults
LC	Liquid chromatography
LC–MS/MS	liquid chromatography – triple quadrupole mass spectrometry
LDH	Lactate dehydrogenase
LLOQ	Lower limit of quantification
m/z	Mass/charge
MALDI–ToF	Matrix-assisted laser desorption ionisation-time of flight
MCCD	Medical certificate of cause of death
MODY	Maturity-onset of diabetes of the young
NADH	Reduced form of nicotinamide adenine dinucleotide
NHS	National Health Service
NOS	Not otherwise specified
PBS	Phosphate-buffered saline
PDR	Proliferative diabetic retinopathy

Abbreviation	Full Phrase
PEG	Polyethylene glycol
PMI	Post-mortem interval
PoCT	Point of care testing
ppm	Parts per million
RCPath	Royal College of Pathologists
RH	Reactive hypoglycaemia
RIA	Radioimmunoassay
SD	Standard deviation
SGLT-2i	Sodium-glucose cotransporter-2 inhibitors
SKA	Starvation ketoacidosis
SUDIC	Sudden unexpected death in childhood
S/N	Signal to noise ratio
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TCA	Tricarboxylic acid
TIC	Total ion chromatogram
UK	United Kingdom
UKAS	United Kingdom Accreditation Service
US	United States
WHO	World Health Organisation

Glossary

Antemortem period

Defined as the time a person is alive up to death (Efe et al., 2021).

Perimortem period

Defined as the period ‘around death’, forensic anthropologist class this as ‘few hours or days before or after death’ (Kemp, 2016). As such the perimortem period can overlap both the antemortem and post-mortem periods.

Post-mortem period, and definition of death

The point that someone has deceased, i.e., death has occurred, as defined by Simpson et al. (2008) is either: ‘death following the irreversible cessation of brainstem function’ or ‘death following cessation of cardiorespiratory function’. Once at least one of these conditions is met, from this time point onwards is defined as ‘post-mortem’. Approximately four minutes after death decomposition begins (Vass, 2001), with cell metabolism continuing up to ten minutes after death, depending on environmental conditions (Vass, 2007).

Dysglycaemia

Dysglycaemia is a general definition for abnormal blood glucose concentrations. This includes hypoglycaemia (low blood glucose concentrations), and hyperglycaemia (raised blood glucose concentrations) (Aramendi et al., 2017).

Euglycaemia

Euglycaemia is defined as the condition or state in which blood glucose concentrations are within the ‘normal’ clinical range (Rawla et al., 2017).

Acknowledgements

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I would like to extend my acknowledgments to my parents, Joy and Tony, two brothers Andrew and Fred (Steven), and two children Harper and Georgia, for pushing me when needed, and helping me relax, and play golf when it was needed too.

Chapter 1: Introduction, aims, and objectives

1.0 Introduction

1.1 Diabetes mellitus

Diabetes mellitus, hereon referred to as ‘diabetes’, is a disease of inadequate control of glucose levels. Rather than being a single disorder, diabetes is a heterogeneous group of disorders that are resulting from complete or partial deficiency in insulin production or action, which if untreated results in hyperglycaemia (Alam et al., 2014). The earliest discovered description of diabetes found is in a medical text from Egypt, dating to 552 BCE (Oubré et al., 1997). With the term *diabetes* being first adopted by Greek physicians, Apollonius of Memphis around 250 BCE (Mulholland and Mckenna, 2023) and Aretaeus of Cappadocia in the 100s CE, from the Greek word for ‘siphon’. This was due to the observation that the condition caused increased urine production (Leeder, 2013). The term *mellitus* was added by Thomas Willis in 1675, from the Latin word for ‘honey sweet’ after discovering the urine of people with diabetes tasted sweet (Lakhtakia, 2013), due to the increased glucose being passed in the urine (Leeder, 2013).

As of 2021 it is estimated that 10.5% (537 million) of all 20 to 79-year-olds worldwide are living with diabetes, with an approximate 6.7 million deaths in the same year directly attributed to diabetes and diabetes-related chronic conditions. It is predicted that the number of people living with diabetes will increase to 11.3% (643 million) in 2030 and to 12.2% (783 million) by 2045 (International Diabetes Federation, 2021).

The increasing prevalence of diabetes is demonstrated in Figure 1.1.1, presenting its prevalence in England and Wales, by Clinical Commission Group (CCG) regions, as existed in 2015, and comparing this with the estimated prevalence in the respective regions in 2030. Figure 1.1.1 demonstrates that the increased prevalence will affect almost all areas, but that the prevalence of diabetes is not evenly distributed across England and Wales. In 2015, the prevalence per CCG region of diabetes ranges from 6.6 to 11.5%, (Public Health England, 2016). Some factors that are found in the areas of the highest prevalence of diabetes are those with; high levels of deprivation, and/or high proportions of elderly people. Whereas the regions with the lowest diabetes

prevalence are areas with a higher proportion of younger age groups and/or regions with lower levels of deprivation.

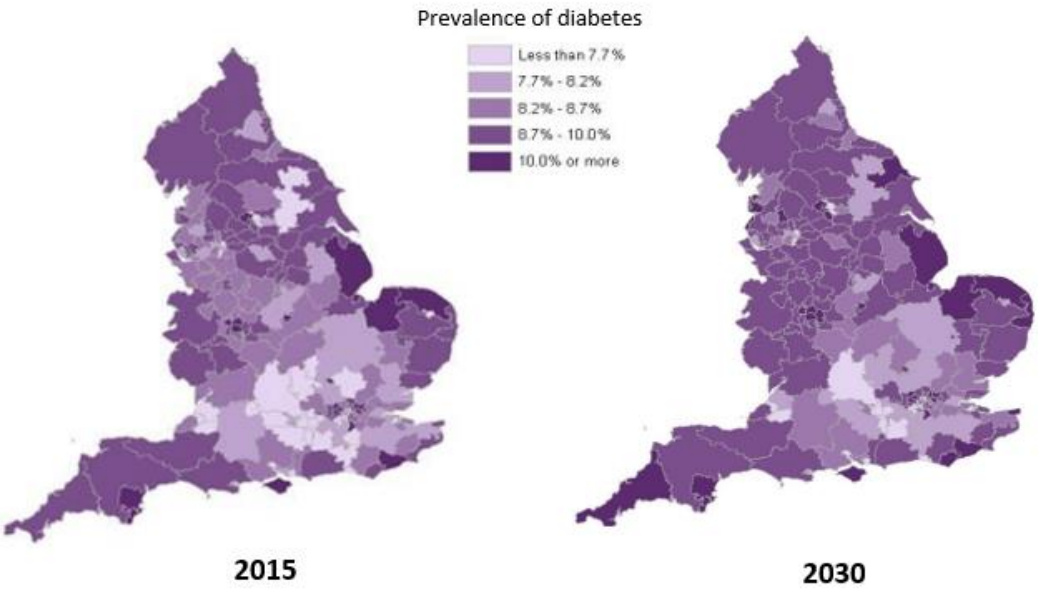


Figure 1.1.1: Prevalence of diabetes, by Clinical Commission Group in the England and Wales, comparing estimated prevalence in 2015 with estimated prevalence in 2030 (Public Health England, 2016).

1.1.1 Types of diabetes

1.1.1.1 Type 1 diabetes

Type 1 diabetes (T1DM) accounts for 1.6% of the global prevalence of diabetes worldwide, which relates to over 8 million people with T1DM in 2021 (Gregory et al., 2022). Pancreatic β-cells play a critical role in glucose homeostasis as they are the primary source of insulin synthesis, storage and release (Bartolomé, 2023). In T1DM the endocrine disease state is due to either autoimmune or idiopathic destruction of β-cells (Alberti and Zimmet, 1998). In the majority of T1DM cases leading to absolute insulin deficiency (Alberti and Zimmet, 1998), resulting in the inability to produce sufficient, or indeed any insulin, and leading to the loss of glucose regulation. Treatment of T1DM is generally with exogenous insulin compounds, with most patients monitoring their blood glucose concentrations and self-dosing in response to increasing concentrations (Thevis et al., 2012).

The most common acute complications of T1DM (and type 2 diabetes) is ketoacidosis, this condition, diabetic ketoacidosis, is explained further in Section 1.1.2.1. As well as the health implications directly caused by the hyperglycaemic episodes, people with T1DM, due to the autoimmune nature of the disorder, also have higher incidence of other autoimmune conditions. For example, Hashimoto's thyroiditis, Addison's disease, and Graves' disease (Fallahi et al., 2016), with 40% of people with T1DM presenting with further autoimmune conditions (Bao et al., 2019).

1.1.1.2 Type 2 diabetes

Type 2 diabetes (T2DM) accounts for 90–95% of the population with diabetes. As in T1DM, T2DM is related to defects in secretion and/or action of insulin, but in T2DM this is not due to autoimmune destruction of β-cells (Alberti and Zimmet, 1998). The condition is more common in people with an increased body mass index (Corbin et al., 2016), and of those people with T2DM but without increased BMIs many present with increased body fat, particularly in the abdominal region (Alberti and Zimmet, 1998). There is some β-cell compensation in early stage of the disease but as the over-nutrition and/or inactivity continues this compensation leads to β-cell dysfunction (Prentki and Nolan, 2006).

1.1.1.3 Other types of diabetes

The majority of people with diabetes will have either T1DM or T2DM. However, there are other types of diabetes, and conditions causing diabetes-like states that make up smaller percentages of the diabetes-affected population, such as:

Latent Autoimmune Diabetes Mellitus in Adults (LADA): The term LADA was introduced over 30 years ago to identify immune mediated diabetes that developed in adults (Tuomi et al., 1993). LADA shares genetic, immunological, and metabolic features with T1DM and T2DM diabetes (Zimmet et al., 1994), However, there is debate to whether LADA is a separate form of diabetes or a sub-group of T1DM (O'Neal et al., 2016), with the American Diabetes Association standard of care notes defining that all of forms of diabetes mediated by β-cell destruction as T1DM (ElSayed et al., 2023). Due to the late onset of the diabetic state, LADA is often misdiagnosed as T2DM, particularly if the patient also presents with a raised BMI (Laugesen et al., 2015). However, in cases of T2DM,

when the risk factors such as obesity are reduced, there is a good chance of control with minimal medication and non-pharmacological measures; this is not the case in LADA, due the underlying autoimmune cause of the diabetes (Rajkumar and Levine, 2020).

Gestational diabetes: Gestational diabetes refers to glucose intolerance that is first recognised, or onset is, in pregnancy and is one of the most common complications of pregnancy (McIntyre et al., 2019).

Type 3c diabetes (or pancreatogenic diabetes): Secondary diabetes due to damage to the islets of Langerhans (Vonderau and Desai, 2022), caused by exocrine pancreatic disorders, such as chronic pancreatitis, cystic fibrosis pancreatic ductal adenocarcinoma, and haemochromatosis (Hart et al., 2016).

Maturity-onset of diabetes of the young (MODY): This form of diabetes is an autosomal dominant inherited monogenic condition, resulting from mutations in a single gene (Tosur and Philipson, 2022).

Neonatal diabetes: Neonatal diabetes is defined by the occurrence of persistent hyperglycaemia within the first six months of life but may present up to 12 months (Dahl and Kumar, 2020). Most cases (80–85%) of neonatal diabetes are due to a monogenic cause (Hammoud and Greeley, 2022).

Wolfram Syndrome: A rare autosomal recessive neurodegenerative disease which presents with a number of conditions including, but not exclusive to; diabetes, diabetes insipidus (a condition relating to antidiuretic hormone) (Mutter et al., 2021), optic atrophy, and deafness (Pallotta et al., 2019).

Alström Syndrome: A monogenic condition arising from mutations of a single gene affecting the cilia. This is a rare condition, with an incident of one in a million per population (Dassie et al., 2021).

Steroid-induced diabetes: Corticosteroids are used in the treatment of disorders such as asthma (Alangari, 2014), and autoimmune disorders (Ronchetti et al., 2021). A known side effect of the treatment is the impairment of glycaemic control resulting in hyperglycaemia (Angelopoulos et al., 2014).

1.1.2 Ketoacidosis

Ketoacidosis is a metabolic acidotic state associated with high serum and urine concentrations of three main ketone bodies. During catabolic states, fatty acids are metabolised via acetyl-CoA to ketone bodies (Ghimire and Dhamoon, 2024). The quantity of acetyl-CoA produced during a ketoacidotic states exceeds the capacity of the Krebs cycle therefore converts acetyl-CoA to, acetone (a ketone), acetoacetate (a keto-/oxo- acid), and β-hydroxybutyrate (a hydroxy acid). This cycle is future described in Section 1.3.3.

1.1.2.1 Diabetic ketoacidosis

People with T1DM and T2DM diabetes can suffer from episodes of acute hyperglycaemia which result in the condition; diabetic ketoacidosis (DKA), which along with the hyperglycaemia is characterised by low blood pH, hyperkalaemia, and increased blood ketones (Dhatariya et al., 2020). DKA is the most common acute hyperglycaemic emergency in cases of diabetes, 60–70% of adult cases occurring in people with T1DM, with 20 – 30% of DKA cases occurring in people with T2DM (Nyenwe and Kitabchi, 2016). Although DKA is the most common acute complication of diabetes, deaths in the United Kingdom (UK) and United States (US) attributed to DKA are < 1% of cases (Gibb et al., 2016; Kitabchi et al., 2009). This low mortality rate however is not representative in other countries, with areas of India demonstrating a 30% mortality rate in hospitalised DKA episodes (Gibb et al., 2016).

While DKA in T1DM is predominantly due to insufficient insulin concentration/action, other reasons for hyperglycaemia in people with diabetes can trigger an DKA episode, for example, patients with well self-maintained glycaemic control presented with DKA episodes while being symptomatic with coronavirus disease 2019 (COVID-19) (de Sá-Ferreira et al., 2022).

DKA can also occur where the blood glucose levels are not significantly raised, referred to as euglycaemic DKA, this condition can be caused by a number of factors (Nasa et al., 2021), such as

- Pregnancy
- Fasting/starvation

- Bariatric surgery
- Gastroparesis
- Cocaine intoxication
- Chronic liver disease
- Glycogen storage disease
- Treatment with sodium-glucose cotransporter-2 inhibitors

Sodium-glucose cotransporter-2 inhibitors (SGLT-2i), such as empagliflozin and dapagliflozin, are used in combination with other drugs, e.g., metformin, in T2DM to control glucose levels (Hropot et al., 2023). However, the use of these compounds increases the risk of DKA, with Hamblin et al. (2019) demonstrating an incidence of DKA in people with T2DM not being treated with SGLT-2i of 0.69 per 1000, against an incidence of 1.02 per 1000 in the T2DM population being treated with SGLT-2i. To reduce the risk of DKA associated with the use of SGLT-2i the compounds should not be used when other conditions may also increase the risk of DKA, such as the patient following a very-low-calorie diet, or before major surgery (Dhatariya, 2016), and increased clinical vigilance is required in the first 180 days from starting the SGLT-2i treatment (Limenta et al., 2019).

Sodium-glucose cotransporter-2 inhibitors' glucose-lowering effect is independent of insulin secretion, and as such, these drugs are being considered as complementary to insulin treatment in cases of T1DM diabetes. Trials have demonstrated improved glycaemic outcomes (Hropot et al., 2023) in this population when treated with SGLT-2i, however due to the increased DKA risk in the T1DM when compared with T2DM population this improvement in glucose control should be considered against the increased risk of DKA.

1.1.2.2 Alcoholic ketoacidosis

While DKA is a diabetes-related condition, alcoholic ketoacidosis (AKA) is a similar ketoacidotic state found in people with a history of alcohol abuse or due to alcohol intake (Long et al., 2021). The pathophysiology of AKA is related to the underlying effects of alcohol on the body, namely, reduced glycogen, reduced nutritional stores, elevation of the reduced form of nicotinamide adenine dinucleotide (NADH) and volume depletion. The majority of AKA cases present with low or normal blood glucose concentrations, as such when ketoacidosis occurs in known alcoholics, without presenting with hyperglycaemia, then AKA can reasonably be presumed (Dhatariya et al., 2020). However, there are reported case of AKA with associated hyperglycaemia (Chandrasekara et al., 2014) and the risk of AKA with a hyperglycaemic state is increased if the patient is a person with diabetes (Garg and Garg, 2021). In cases where the patient is also a person with diabetes it can then be difficult to distinguish DKA from AKA. Although relatively simple to treat, if undiagnosed AKA can lead to death, with one study indicating mortality as high as 7% in unrecognised cases (Sidlak et al., 2022).

1.1.2.3 Starvation ketoacidosis

Another form of ketoacidosis is starvation ketoacidosis (SKA), this state forms when the prolonged calorie intake is significantly less than the metabolic requirement. SKA normally presents either with euglycaemia or hypoglycaemia (Ramesh et al., 2023) as the ketoacidosis is a result of insufficient calorie intake over a prolonged period, causing a decrease in insulin secretion and a metabolic shift to lipolysis and oxidation of fatty acids (Ghimire and Dhamoon, 2018).

1.1.2.4 Other forms of ketoacidosis

Although DKA (with hyperglycaemia or euglycaemia), AKA, and SKA account for the majority of ketoacidotic episodes, there are other conditions that may result in ketoacidosis without underlying diabetes, alcoholism, and/or starvation. Examples of these include:

- Salicylate intoxication (Espírito Santo et al., 2017)
- Calorie-sufficient but carbohydrate-restricted diets (Blanco et al., 2019)

- Sepsis (Nakamura et al., 2014)
- Use of synthetic cannabinoid compounds (Hess et al., 2015)

1.1.3 Hyperosmolar Hyperglycaemic State

Along with DKA, Hyperosmolar Hyperglycaemic State (HHS) is a serious acute complication of diabetes. While DKA accounts for up to 10% of hospital admissions of people with diabetes, the incidence of HHS is lower, accounting for < 1% (Kitabchi et al., 2009). However, whereas the mortality rate of DKA is < 1%, the mortality rate of HHS is far greater, being reported as high as 20% (Mustafa et al., 2023). While DKA is more commonly seen in people with T1DM, cases of HHS are largely from the T2DM population (Zubkiewicz-Kucharska et al., 2019). Hyperosmolar Hyperglycaemic State presents with clinical features including (Mustafa et al., 2023):

- Significant hyperglycaemia
- Significant hypovolaemia
- Hyperosmolarity
- No significant ketoacidosis

The most common underlying cause of HSS, accounting for approximately 60% of cases, is infection (Chu et al., 2001), but HSS can also be triggered due to non-compliance (omission) of antidiabetic medication, cardiovascular events, pancreatitis (Mustafa et al., 2023), undiagnosed diabetes (Stoner, 2017), and some drug compounds, such as antipsychotics (Cerimele, 2008). Hyperosmolar Hyperglycaemic State is initiated by increased levels of counter-regulatory hormones, such as catecholamines, cortisol, and/or glucagon, and therefore stimulating glucose production. High catecholamine and low insulin levels result in reduced glucose uptake, leading to hyperglycaemia with associated depletion of intracellular water concentration and subsequent osmotic diuresis (Stoner, 2017). The loss of glucose through the kidney increases water loss, while sodium is still conserved, leading to hyperosmolarity and dehydration. Unlike DKA, the insulin level can suppress lipolysis and ketogenesis (French et al., 2019).

1.1.4 Complications of hyperglycaemia

In all forms of diabetes, episodes of hyperglycaemia can occur, and these episodes of increased blood glucose, and insulin resistance, play important roles in the development of macrovascular complications (Brownlee, 2001), such as retinopathy, nephropathy, and neuropathy:

Retinopathy: diabetes–related retinopathy is a progressive disease state caused by damage to the retinal blood vessels. This condition may cause little or no symptoms in the early stages, with microaneurysm usually being the earliest visible manifestation of diabetes–related retinopathy (Mayya et al., 2021). If left untreated the condition can lead to more serious outcomes such as vitreous haemorrhaging and total loss of sight, these however can largely be prevented by laser, drug or surgical treatment (Wu et al., 2013). Diabetes was, until 2010, the leading cause of certifiable blindness in the UK, but with improvements in diabetes-related retinopathy screening programmes, and improved glycaemic control in the population with diabetes the number of cases of certifiable blindness in the UK being attributed to diabetes have been reduced (Liew et al., 2014).

Nephropathy: Nephropathy affects approximately 40% of the population with T1DM and T2DM. Diabetes causes unique damage to the kidney characterised by; increased glomerular basement membrane width, mesangial sclerosis, vascular lesions (hyalinosis and hyaline arteriosclerosis), and microaneurysm (Gross et al., 2005).

Neuropathy: Diabetes-related neuropathy is neurodegenerative disorder of the peripheral nervous system (Feldman et al., 2019). This condition can be subdivided in to focal/multifocal, such as diabetic amyotrophy, and symmetric polyneuropathies such as sensorimotor polyneuropathy (Tracy and Dyck, 2008). Diabetic sensorimotor polyneuropathy affects approximately a third of people with diabetes, as such are one of the most likely population groups to develop foot ulceration (Abbott et al., 2002). The condition can also lead to patients' feet becoming traumatised without the individual being aware (Gooday et al., 2014). Five percent of the individuals who develop diabetes related foot ulcers will die within 12 months of first ulcer, and a 42.2% die within 5 years (Walsh et al., 2016).

1.1.5 Causes of hypoglycaemia

There are number of causes of hypoglycaemia, some examples are:

Some critical illnesses: Severe liver illnesses such as severe hepatitis or cirrhosis, severe infection, kidney disease, and advanced heart disease can cause hypoglycaemia. In the case of kidney disease, the reduced renal function can affect the clearance of mediations to control diabetes (Alsahli and Gerich, 2015).

Long-term starvation: Hypoglycaemia can occur with malnutrition and starvation, when the glycogen stores that the body needs to create glucose are used up. This condition can occur in eating disorders, such as anorexia nervosa (Yanai et al., 2008).

Medications: Some medications can cause hypoglycaemia, especially in children or in people with kidney failure. One example is quinine used to treat malaria, and commonly used drugs such as paracetamol, salicylates, and β-blockers (Gama et al., 2003). However, in most cases of these examples hypoglycaemia is induced after the drugs have caused liver failure.

Reactive/postprandial hypoglycaemia (RH): This form of hypoglycaemia occurs two to five hours after a meal (Brun et al., 2000). Those affected include people with T2DM and those in pre-diabetes states (diabetic RH), people with gastrointestinal dysfunction (alimentary RH), those with some hormone deficiencies (hormonal RH), and a large percent of RH is idiopathic (Altuntaş, 2000). In many cases of idiopathic RH an inappropriate delay in the secretion of insulin which coincides with falling glucose levels.

Alcohol-induced hypoglycaemia: Alcohol-induced hypoglycaemia represents reactive hypoglycaemia caused by hepatic insulin resistance, an increase in intestinal glucose absorption, or altered lipid metabolism. Alcohol can also exacerbate hypoglycaemia induced by sulfonylurea and insulin (Marks and Teale, 1999).

Insulin overproduction: e.g., an insulinoma, a neuroendocrine tumour of the pancreas which cause inappropriate endogenous insulin excretion. Although

these are the most common form of pancreatic islet cell tumour, the incidence is still only 1 per 250,000 people per year (Grant, 2005).

1.2 Insulin

Insulin was first isolated in 1922 with an early experiment by Frederick Banting, an orthopaedic surgeon, and Charles Best, a student research assistant, working at the University of Toronto along with John Macleod and James Collip (Lewis and Brubaker, 2021). Their early work with canines showed that dogs who had had their pancreas removed developed diabetes-like states, and when injected with extract from the pancreas (defined at the time as 'isletin') would present with a lower glucose level. The team of Banting, Macleod and Collip began human trials in January 1922, with the administration of extract from bovine pancreata, to a 14-year-old boy (Quianzon and Cheikh, 2012). Although this first administration of insulin to a human didn't occur until 1922, the suspected presence of a compound that controlled glucose levels, was put forward by Oskar Minkowski and Joesph von Mering in 1889 (Bliss, 1993). The nomenclature 'insulin' was later coined by Jean de Meyer, and Edward Albert Sharpey-Schafer independently of each other in the early 1900s for, at the time, a still hypothetical molecule produced by pancreatic islets. The word 'insulin' (or 'insuline') is derived from *insula* the Latin word meaning 'island' (Lakhtakia, 2013).

Endogenous insulin is a globular polypeptide hormone chain (Figure 1.2.1) produced in the pancreas by the β-cells of the Islets of Langerhans (Da Silva Xavier, 2018), consisting of two sub-units, α chain (21 residues) and β chain (30 residues) (Weiss, 2009).

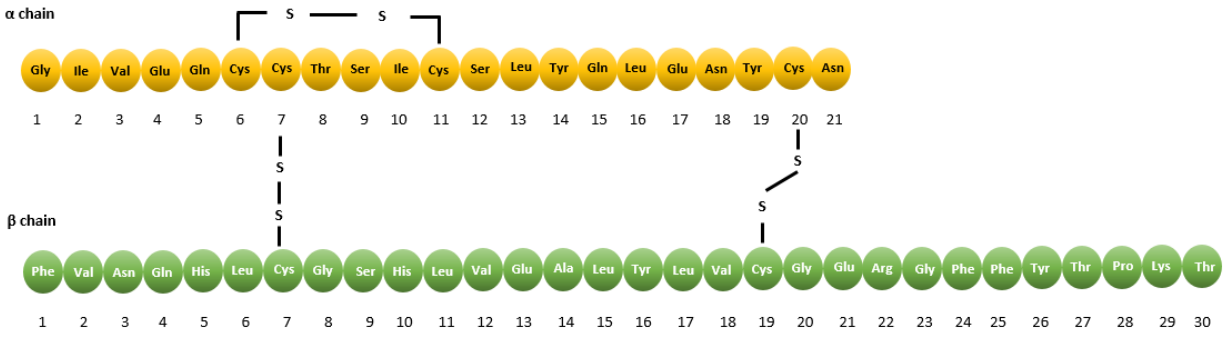


Figure 1.2.1: Amino acid sequence of α and β chains of endogenous human insulin (adapted from Hirsch et al., 2021).

Biosynthesis of insulin is via a pre-proinsulin complex (Figure 1.2.2), which is converted by signal peptidase to proinsulin. The proinsulin compound (Figure 1.2.3) consists of an insulin molecule and a C-peptide molecule (Connecting–peptide) (Yang et al., 2010). C-peptide is involved in intracellular signalling (Hills and Brunskill, 2008), as well as a role in renal function (Nordquist et al., 2009).

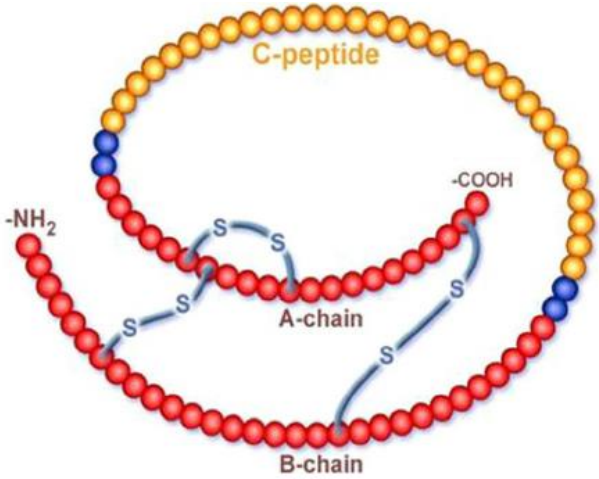


Figure 1.2.2: Pro-insulin molecule as biosynthesised in the pancreas β-cells (Akinlade et al., 2014).

Post-synthesis, insulin is sequestered as zinc-insulin complex and is released upon a trigger from β-cells in response to increased phosphorylation of monosaccharides by hexokinase IV. This occurs due to increases in circulating blood glucose levels (Wills, 1997).

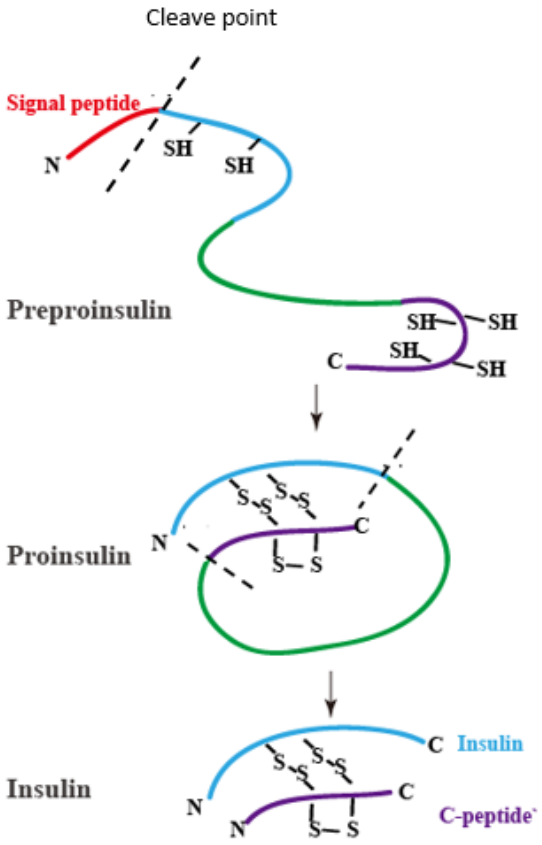


Figure 1.2.3: Biosynthesis of insulin in β-cell, synthesised from pre-proinsulin to proinsulin (Zhong, 2013).

1.2.1 Antemortem role of insulin

The roles of endogenous insulin encompass carbohydrate, lipid, and amino acid metabolism, and mRNA transcription and translation (Wilcox, 2005). Insulin has a central role in carbohydrate metabolism through the regulation of glucose uptake by adipose and muscle tissues. Here, insulin inhibits production of glucose, resulting in increased storage of glycogen. Insulin also plays a key role in lipid metabolism, whereby it exhibits an inhibitory action upon the release of fatty acids and glycerol from adipose tissues (Boden, 1996). Furthermore, insulin facilitates increased uptake of amino acids by muscle tissue and the liver and can increase polysome formation whilst also inhibiting proteolysis (Boden et al., 1994).

1.2.2 Recombinant human insulin and insulin analogue compounds

In the decades before the discovery of insulin, the treatment of diabetes was recommended by some physicians with a fasting and calorie-restricted diet (called the 'starvation diet' by detractors) (Mazur, 2011). This treatment demonstrated improvements in occurrences of glycosuria and acidosis, along with decreased incidence of coma, and delayed death among the paediatric population with diabetes. The discovery of insulin, and first administration of exogenous insulin to treat a person with diabetes (as referred to in Section 1.2), was a huge step forward in the treatment of diabetes (Quianzon and Cheikh, 2012). In the 1930s protamine, isolated from the sperm of trout (*Salmo iridius*) (Sindoni, 1937), was added to insulin administrations to prolong the action of the treatment (Bliss, 1993). This led to longer action insulins entering the market, for example, protamine-zinc insulin demonstrated glycaemic stability for 24–36 hours. This early commercially available insulin was purified from porcine or bovine pancreata (Baeshen et al., 2014). In 1978 an American Biotechnology company (Genentech) produced the first recombinant human insulin, via expression of chemically synthesised cDNA encoding for the insulin α and β chains separately in *Escherichia coli* (*E. coli*). From this step-forward in insulin synthesis, the recombinant human insulin Humulin® was marketed in 1982. Since 1986, a more efficient approach has been used commercially, which involves the single chemically synthesised cDNA encoding for proinsulin (Baeshen et al., 2014). By modifying the amino acid groups of the insulin molecule, it was found possible to change the pharmacokinetics of the molecule, increasing the absorption, and shorter half-life. The first rapid-acting insulin, lispro (Admelog®, Humalog® and Lyumjev®), was approved by the U.S. Food and Drug Administration (FDA) in 1996 (Zaykov et al., 2016). This *E. coli* production route has since been used for the production of insulin compounds, such as insulin Glulisine (Aprida®) (Walsh, 2005).

With the developments in the field of genetic engineering the following approaches have been used for the production of recombinant human insulin and analogue insulin compounds:

- Mammalian cell production
- *E. coli* expression

- Produced in yeast (*Saccharomyces cerevisiae*)
- Transgenic animals and plants as host

The distinction between a recombinant human insulin and an insulin analogue is important, recombinant human insulin being an exogenous version of endogenous human insulin, and an insulin analogue being based on the human insulin molecule, but with modifications to the amino acid groups. For example, insulin aspart, has an aspartic acid substituted for proline at position β28 of the insulin molecule (Mudaliar et al., 1999) (Figure 1.2.2.1). Further examples of recombinant human insulin, and insulin analogues used in the treatment of diabetes (Barmanray et al., 2019), are represented in Table 1.2.2.1. These exogenous insulin compounds can be classified as rapid, short-, intermediate-, and long-acting, (Labay et al., 2016), with onset of biological effects ranging from 10 mins to 4 hours (Diabetes UK, 2020). Insulin analogues allow for more accurate replication of the basal and prandial components of insulin replacement, this gives the patient more control over meal and snack timings and reduce the risks of hypoglycaemia when compared with recombinant human insulin (Hirsch, 2005).

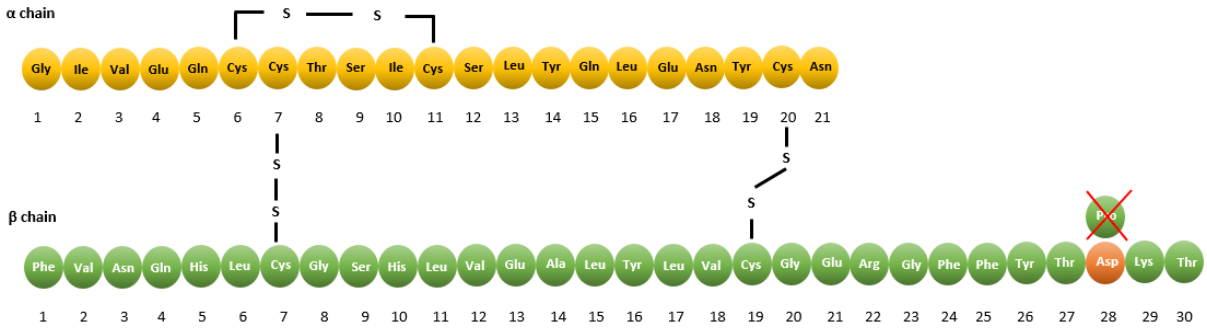


Figure 1.2.2.1: Amino acid sequence of α and β chains of insulin aspart, proline substituted for aspartic acid at position β28.

Compound Name (Insulin -)	Trade Name	Manufacturer	Molecular Weight of Insulin (Da)	Peak Action	Approximate effective Duration
Lispro	Humalog®	Lilly	5808	30 min - 1.25 hr	4 hr
Aspart	Fiasp®	Novo Nordisk	5826	30 min - 3 hr	5 hr
Aspart	NovoRapid®	Novo Nordisk	5826	30 min - 3 hr	5 hr
Glulisine	Apidra®	Sanofi	5823	30 min - 1.25 hr	3 hr
Lispro	Insulin lispro®	Sanofi	5808	30 min - 1.25 hr	5 hr
Human	Insuman Rapid®	Sanofi	5808	30 min - 3 hr	8 hr
Human	Actrapid®	Novo Nordisk	5808	30 min - 3 hr	8 hr
Human	Humulin® S	Lily	5808	30 min - 1.5 hr	8 hr
Porcine	Hypurin Porcine Neutra®	Wockhardt UK	5777	1 - 5 hr	8 hr
Human	Humulin® I	Lily	5808	1 - 8 hr	22 hr
Porcine	Hypurin Porcine Isophane®	Wockhardt UK	5777	6 - 12 hr	24 hr
Human	Insulatard®	Novo Nordisk	5808	4 - 12 hr	24 hr
Human	Insuman Basal®	Sanofi	5808	1 - 3.5 hr	20 hr
Glargine	Abasaglar®	Lily	6063	30 min - 24 hr	24 hr
Detemir	Levemir®	Novo Nordisk	5917	30 min - 24 hr	24 hr
Degludec	Tresiba® U100 & U200	Novo Nordisk	6104	30 min - 38 hr	38 hr
Glargine-yfgn	Semglee®	Mylan	6063	30 min - 24 hr	24 hr
Glargine	Lantus®	Sanofi	6063	30 min - 24 hr	24 hr
Glargine	Toujeo® U300	Sanofi	6063	30 min - 38 hr	38 hr
Degludec (and Iragutide)	Xultophy®	Novo Nordisk	6104	30 min - 38 hr	38 hr
Glargine (and Lixisenatide)	Suliqua®	Sanofi	6063	30 min - 38 hr	38 hr

Table 1.2.2.1: Common recombinant human insulin and insulin analogues used in the treatment of diabetes (Diabetes UK, 2020).

1.3 Post-mortem analysis in dysglycaemia-related death

There are a range of assays that Coroners can request to aid in the determination of cause of death in cases related to suspected antemortem/perimortem dysglycaemia, and/or unexplained death. With the most commonly required investigation being glucose and lactate levels in blood and vitreous humour. Vitreous humour is the transparent gel-like substance found in the eye (as discussed in Section 1.3.5). Other biochemical markers can be measured to provide information as to the ante- or perimortem glucose status, such as those discussed later in this Chapter, β-hydroxybutyrate (β-OHB), glycated haemoglobin (HbA_{1c}), and blood insulin concentration.

With the demonstrated increase in cases of diabetes (International Diabetes Federation, 2021), and therefore increases in diabetes-related deaths expected to follow suit, post-mortem toxicology laboratories may soon face increasing pressure to perform diabetes-related assays. Indeed, over the course of this project, there has been a notable uptake in Coroners investigations into hypo- and hyperglycaemic deaths. This trend can be observed through the increasing number of vitreous humour glucose analysis requests made by the Coroner in Norfolk, with 12 requested in 2017, 36 in 2020, and 52 in 2022 (Table 4.2.1.1).

1.3.1 Glucose

Blood concentrations are measured post-mortem to determine whether the deceased was hypoglycaemic, euglycaemic, or hyperglycaemic at death. Occurrence of hypoglycaemia in people with diabetes can be related to excess exogenous insulin administration (deliberate or accidental), or in the population without diabetes if insulin is administered. However, there are a number of other conditions that may result in a hypoglycaemic state, as discussed in Section 1.1.5.

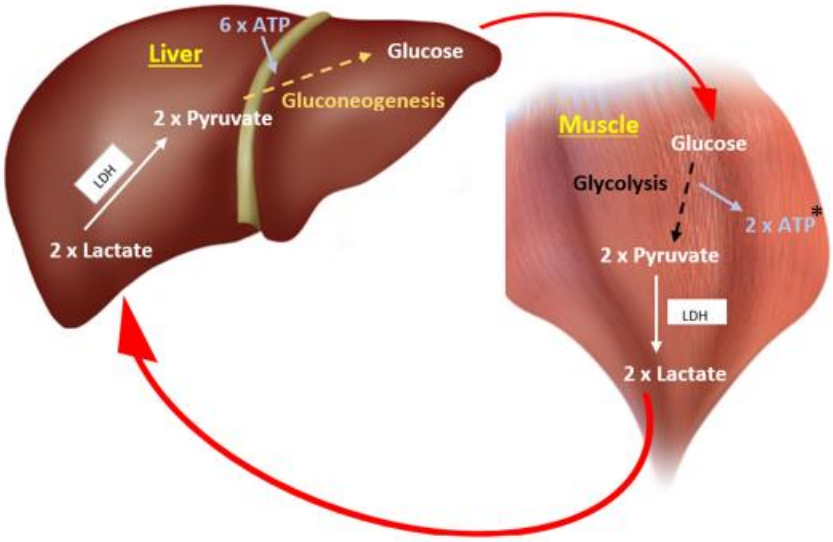
1.3.1.1 Limitations of post-mortem glucose analysis

There are limitations to the use of post-mortem blood glucose concentrations in the assessment of antemortem status as even after cardiac and respiratory function has stopped, anaerobic metabolism of glucose continues. Resulting in a post-mortem increase of lactate and decrease of glucose concentrations (Hess et al., 2011). While this means that any antemortem hyperglycaemic state can still be inferred by an elevated post-mortem blood glucose concentration, a low or 'normal' post-mortem blood glucose concentration, assessed in isolation, cannot be used as evidence to infer an antemortem hypoglycaemic state (Musshoff et al., 2011). However, when used in conjunction with other biochemical markers, a low post-mortem blood glucose may be of interest. For example, a case with a low post-mortem blood concentration compared with a normal or raised vitreous humour glucose concentration may indicate a rapid perimortem drop in blood glucose. Some evidence suggests a correction factor adjusting for the increased post-mortem lactate concentration against the decreased post-mortem blood glucose concentration can be used, known as the 'Formula of Traub' (Traub, 1969) (discussed further in Section 1.3.5.1). Any interpretation of post-mortem glucose concentrations should also consider the autopsy sampling site, as higher blood glucose concentrations are often found in hepatic vein blood, followed by the inferior vena cava, superior vena cava, and cardiac right ventricle blood (Chen et al., 2015; Palmiere, 2015).

1.3.2 Lactate

An increased lactate concentration occurs post-mortem due the increased post-mortem production from anaerobic metabolism of glucose. As stated above the post-mortem lactate concentration can be used to infer the antemortem glucose concentration using the assumption, as discussed in Section 1.3.5.1, that the calculation of post-mortem (glucose + lactate/2) closely represents the antemortem glucose concentrations (Zilg et al., 2009). In life lactate is produced by the Cori cycle by a conversion of glucose. Glycolysis in mitochondria-containing cells produces pyruvate that can then enter the citric acid cycle and undergo oxidative phosphorylation (Oexle et al., 1999). However, in non-mitochondria containing cells, such as erythrocytes, or in oxygen-depleted conditions, the pyruvate is converted to lactate by the enzyme lactate

dehydrogenase (LDH), as represented in Figure 1.3.2.1, this is defined as anaerobic glycolysis. This process occurs post-mortem in cells, and as such the antemortem and perimortem glucose concentrations will start to deplete as lactate concentrations increase.



* **Adenosine triphosphate (ATP)**

Figure 1.3.2.1: Representation of the Cori cycle, demonstrating conversion of glucose to lactate in health.

1.3.3 β-hydroxybutyrate

In DKA, there is an absence of, or insufficient concentration/action of insulin, this, along with increased stress hormones, such as cortisol, epinephrine, and norepinephrine, promotes the production of acetyl-CoA seen in Figure 1.3.3.1.

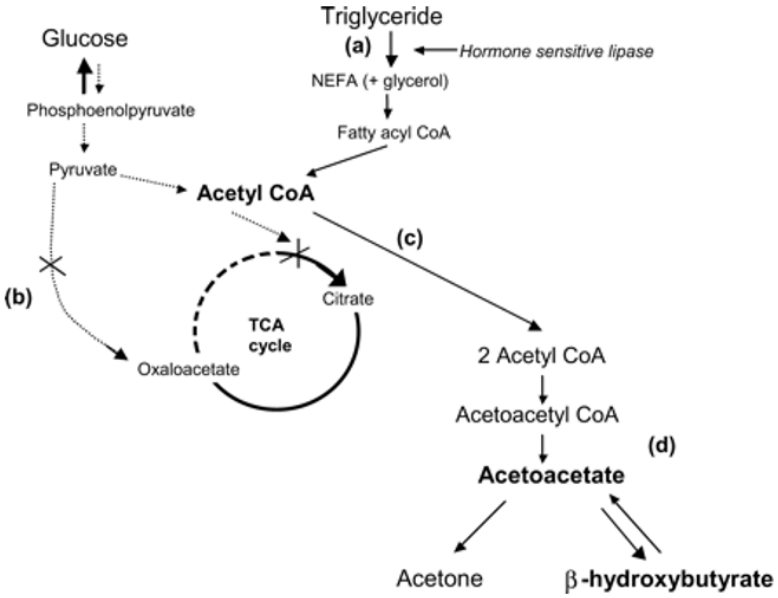


Figure 1.3.3.1: β-OHB pathway, demonstrating the increased lipolysis and decreased glucose utilisation that leads to production of β-OHB (Wallace and Matthews, 2004).

The insufficient insulin levels result in reduced utilisation of glucose and increased glycogenesis (a). This in turn decreases the production of oxaloacetate (b) and therefore reducing the production of citrate from oxaloacetate and acetyl-CoA. The reduction in citrate production diverts the acetyl-CoA from the tricarboxylic acid (TCA) pathway (c) pushing the acetyl-CoA to conversion to acetoacetate, which by reduction converts to β-OHB (as well as acetoacetate and acetone) (d) (Wallace and Matthews, 2004).

1.3.4 Glycated haemoglobin (HbA_{1c})

HbA_{1c} reflects glucose control up to 8 to 12 weeks prior to a blood sample being collected, this period relates to the life span of erythrocytes (Coelho, 2016). As demonstrated in Figure 1.3.4.1, haemoglobin in the presence of glucose will form a Schiff base with glucose binding at the N-terminal region of the Hb α or β chains. A Schiff base is a compound characterised by the presence of a N=C bond (Raczuk et al., 2022).

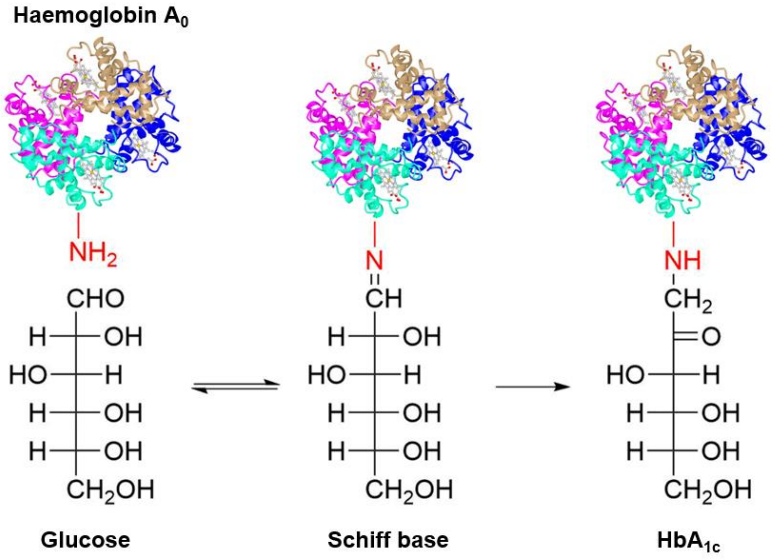


Figure 1.3.4.1: Formation of glycated haemoglobin, from the binding of glucose to haemoglobin, via Schiff base intermediary (Hörber et al., 2020).

The concentration of HbA_{1c} directly reflects the average glucose concentration, and as such, in diabetes the increased blood glucose levels relate to an increased HbA_{1c} concentration. However, caution should be taken interpreting HbA_{1c} results as the concentrations can be affected by conditions which affect the life span of the erythrocytes. For example, sickle cell anaemia decreases the life span of erythrocytes, and as such decreases the time for glycation to occur in relation to the rapid production of new erythrocytes, producing falsely lower HbA_{1c} concentrations (Alzahrani et al., 2023). Conversely, conditions that increase the erythrocyte life span, such as following a splenectomy, increase the concentrations of glycated haemoglobin, due to the disproportionate concentration of older erythrocytes (Barakat et al., 2008). Also, accurate analysis of HbA_{1c} concentrations can be affected by increased concentrations of variant

haemoglobin forms (Sacks, 2003), e.g., foetal haemoglobin (HbF) and sickle cell haemoglobin (HbS).

1.3.5 Post-mortem vitreous humour analysis

Vitreous humour is a transparent gel-like substance that fills the space between the lens and retina of the eye (Bishop, 2000), in the area described as the central vitreous region of the eye (Le Goff and Bishop, 2008), as seen in Figure 1.3.5.1. Vitreous humour comprises of approximately 99% water and contains macromolecular and low molecular weight compounds (Zilg et al., 2009).

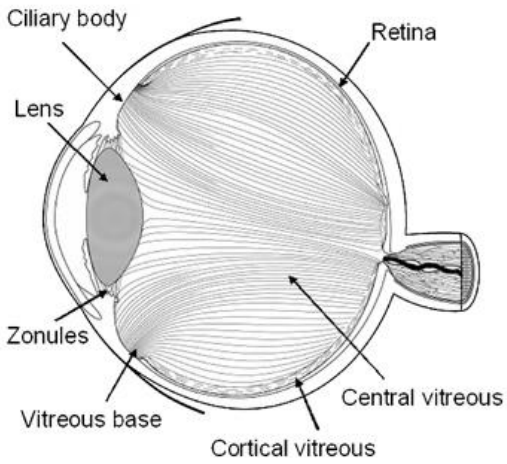


Figure 1.3.5.1: Structure of the human eye (Le Goff and Bishop, 2008).

Post-mortem vitreous humour analysis has been performed since the 1960s when Jaffe (1962) described the use of vitreous humour potassium levels as an indicator of post-mortem interval (PMI). Other biochemical analytes, such as sodium, chloride, creatinine, and lactate are considered to be stable in the vitreous humour after death and can be used to assess ante- or perimortem concentrations (Mitchell et al., 2013).

Indeed, vitreous humour is seen as the preferred post-mortem biochemical matrix, ahead of post-mortem serum, as post-mortem serum often demonstrates substantial haemolysis which precludes some analyses (such as glucose and potassium) being accurately performed. Whereas vitreous humour contains a low amount of red cells post-mortem (Zilg et al., 2022), as the structure of vitreous humour lacks vascularisation. Also, the anatomical distance from viscera, and the supporting structure of the eyeball (Bévalot et al., 2016) result in this matrix being

less affected by haemolysis. Data suggest that vitreous humour has the same physical appearance *in vivo* as *in vitro* in the period immediately after death. However, there can be some physical changes of the vitreous humour in samples where the body is maintained at room temperature, or higher, for extended periods after death (McCleskey et al., 2016), with grey-brown discoloration occurring from increased cell debris. Despite such visual physical changes, no biochemical differences are detectable, implying that the biochemical markers equilibrate before the cells have detached (Zilg et al., 2022).

Post-mortem vitreous humour glucose concentrations are generally assessed in combination with other biochemical pathways to indicate ante- or perimortem conditions in hyperglycaemia. Siddamsetty et al. (2014) demonstrated that there is a linear decrease in vitreous humour glucose with increasing PMI. From this finding Siddamsetty et al. (2014) asserted that glucose could be used as an indicator for time of death in the absence of the preferred potassium measurement. However, the linear decrease in glucose is only predictable for up to 72 hours (Siddamsetty et al., 2014) as opposed to potassium which is a suitable predictor of time of death for approximately 400 hours (Zilg, 2015). The assertion that vitreous humour glucose can be used confidently to 'back calculate' representative antemortem concentrations was published by Bray (1984). When reporting on vitreous humour results from rapidly-chilled bodies post-mortem and/or involved in fresh water immersion, but these conditions are not common to the post-mortem or perimortem circumstances of bodies.

As demonstrated in Table 1.3.5.1, there is a marked variability in the published assertions of post-mortem changes of sodium and chloride concentrations in vitreous humour, although it is accepted these analytes would not increase post-mortem. Other biochemical analytes, such as creatinine, lactate, and urea and creatinine are also relatively stable in post-mortem specimens (Flanagan and Manu, 2016). Chandrakanth et al. (2013) and Mulla et al. (2005) demonstrated that any differences in concentrations of vitreous sodium, chloride, and potassium, between right and left eyes were not statistically significant. However, any changes are negated as routine practise is to combine the vitreous from both eyes for analysis (Mitchell et al., 2013).

Author	Publication year	Vitreous humour Sodium	Vitreous humour Chloride	Time interval post death of any quoted change(s)	Comments
Madea et al.	2001	No change	No change	Up to 120 Hrs	
Tao et al.	2006	↓	↓	Less than 72 hours	
Jashnani et al.	2010	No change	No change	Up to 50 hours	
Tumram et al.	2011	No change	↓	6 hrs onwards	Tumrum et al. note that the decrease in chloride may have been subject to rapid deterioration due to 'tropical' weather conditions.
Chandrakanth et al.	2013	↓	↓	1 hours onwards	Both analytes decreased from death, Chandrakanth et al. noted this as 'marginal fall with increase time since death'
Mitchell et al.	2013	↓	↓	PMI ranged from 0 to 18 days	
Siddamsetty et al.	2014	↓	No change		
Belsey and Flanagan	2016	↓	↓		Both analytes decrease post-death at rates of up to 1 mmol/L per Hr
Zilg et al.	2016	↓	↓		Negative correlation with PMI, and Zilg et al. recommend sodium and chloride concentrations should be evaluated with PMI and/or K concentration

Table 1.3.5.1: Post-mortem changes of vitreous humour sodium and chloride, as quoted by differing authors.

While Belsey and Flanagan (2016) highlighted the current held view that vitreous humour should be viewed as the primary matrix for post-mortem analysis, the authors also highlighted two important notes of caution.

Firstly, that the time to re-equilibrate between blood and vitreous humour after changes in biochemical status in life remains unknown. However, it is understood transport, absorption, and section of proteins and solutes into the vitreous humour is regulated by the blood-retinal barrier (B-R B) (Boroumand et al., 2023), and that exchanges across the B-R B are by active and passive diffusion (Sachdeva et al., 2011). In the case of insulin, transfer across the epithelium is via receptor-mediated transcytosis, mediated by insulin receptors (Fu et al., 2021). With Sahajpal et al. (2018) demonstrating that insulin could be detected by

immunoassay, in antemortem vitreous humour samples from patients with proliferative diabetic retinopathy (PDR), but not in the patients without PDR. This indicates that 'in health' references ranges would need to be defined for vitreous humour insulin concentrations and determine how these relate to blood concentrations. This is also the case for other vitreous humour biochemical markers too (Kokavec et al., 2016).

Secondly, plasma and vitreous humour glucose concentrations rapidly decrease post-mortem, contradicting the findings of (Musshoff et al., 2011). If correct, this means that only significantly raised concentrations of vitreous humour glucose are of any use, and only then for the determination of cases of hyperglycaemia. Boulagnon et al. (2011) agreed that in cases of antemortem hyperglycaemia, degradation of matrix must be taken into consideration when assessing results. This means in cases of hypoglycaemia, a vitreous humour glucose concentration is of little use, increasing the need for robust post-mortem insulin analysis. This controversy was further questioned by Marks and Richmond (2008) who questioned the validity of vitreous humour glucose results in court. These authors justified their concern using an example of a case of very low vitreous glucose where corresponding blood insulin levels were found to be within clinical reference ranges / 'normal' concentration levels.

1.3.5.1 Formula of Traub

The Formula of Traub is a method to correct the post-mortem vitreous humour glucose result for loses of glucose from conversion to lactate, as discussed in Section 1.3.1. The formula was originally proposed by Traub (1969) for the analysis of cerebro-spinal fluid (CSF) glucose but has since been used for the correction of vitreous humour glucose concentrations (Palmiere, 2015). The formula adds 50% of the post-mortem lactate concentration to the post-mortem glucose concentration to produce a 'corrected' result accounting for post-mortem anaerobic metabolism of glucose to lactate. The Traub calculated glucose result should be considered as an estimate of the antemortem status rather than a definite corrected concentration. This is due to the formula being based on the assumptions that antemortem vitreous humour lactate concentration is relatively low compared with the corresponding glucose concentration, and that the post-mortem conversion from glucose to lactose is then linear, and only due to

glycolysis (Boulagnon et al., 2011). The formula therefore does not account for the post-mortem lactate concentration increasing due to bacterial catabolism, nor as a result of red cell haemolysis**.**

1.3.6 Post-mortem analysis of insulin

The analysis of insulin in post-mortem investigations is particularly important when excessive insulin use is suspected. Insulin, recombinant human and analogues forms, is a common means of overdose by people with diabetes (Sarkar and Balhara, 2014), which is most likely due to the ease of access to the drug (Russell et al., 2009). Administration of excessive concentrations of insulin can induce a severe hypoglycaemia state, which if not diagnosed and treated, can result in brain damage, and in some cases death (Stephenson et al., 2022). While accidental self-administered non-fatal insulin overdoses are relatively common among the population living with diabetes, fatal self-administered overdoses are much less frequent (Stephenson et al., 2022). There are also documented cases of suspicious circumstances of insulin-related hypoglycaemia due to deliberate insulin administration by a third party. Deaths in these cases are rare due to the victim often being discovered and resuscitated before irreversible brain damage occurs (Marks, 2015). In a study by Yorker et al. (2006) insulin was found to account for 11% of murders associated with deliberate poisoning by healthcare professionals. There are cases in the UK of National Health Service (NHS) staff deliberating poisoning patients with insulin including Grantham and District Hospital in 1991 (Marks, 2019), Stepping Hill Hospital in 2011 (Wise, 2011), and at the Countess of Chester Hospital between 2015 and 2016 (O'Grady, 2022).

While a number of hospitals in the UK have access to antemortem endogenous insulin analysis, required in some cases for the differential diagnosis of hypoglycaemia (Parfitt et al., 2015), currently there is only one specialist post-mortem insulin service based in the UK. With this provider using a multiple-immunoassay format. Due to the lack of specialist post-mortem insulin services within the UK, and the use of immunoassay for the determination of concentrations, The Royal College of Pathologists (RCPath) guidelines for autopsy examination states that 'sending samples abroad' for post-mortem insulin analysis should be considered. Although advice as to where to send the

samples too is not offered (Osborn et al., 2018). The scenario is similar within Europe and the US with post-mortem insulin concentrations being measured by immunoassay, this approach relies on known cross-reactions of immunoassay methods with recombinant human insulin, and insulin analogue compounds, such as those published by Parfitt et al. (2015). The measured insulin concentrations are then combined with any known clinical history (such as medical notes from primary care providers), and the circumstances of death, to propose a final interpretation of the effect of insulin on the deceased before death. This approach of multi-immunoassay platforms is open to difference in expert opinions as to the utility of the results produced. Particularly in complex/contentious cases, and/or when the insulin being measured is one of numerous recombinant human or analogue compounds. A knock-on effect of relying on one central provider is the delay in reporting turnaround to the Coronial services, in some cases in the Norfolk region this is in excess of 100 days (Table 4.2.1.4), in-turn this then delays the Coroner recording on the cause of death.

As discussed, post-mortem blood, and post-mortem vitreous humour glucose concentrations can be poor indicators of perimortem glycaemic state, particularly in cases of hypoglycaemia. As such, analysis of post-mortem insulin would provide further information to aid the investigation into the cause of death. However, blood insulin concentrations are not routinely analysed in post-mortem cases as an assessment of perimortem status from post-mortem samples for two major reasons:

Firstly, post-mortem blood sample quality is often poor, from the effects of degradation/putrefaction, and with marked haemolysis (Clark et al., 1996). Haemolysis is common in post-mortem samples due to the autolysis of cells after death from the release of intracellular enzymes, such as lytic enzymes from within the lysosomes (Cocariu et al., 2016). Haemolysis releases insulin-degrading enzyme (IDE) from the erythrocytes (Sapin et al., 1998), the action of this enzyme is to cleave the β-insulin chain. This reduces the measurable insulin molecule concentration in the sample due to the commercially available immunoassays detecting the insulin molecule rather than individual α- and β-chains of the molecule as demonstrated by Chevenne et al. (1998) and Zornitzki et al. (2015). Both groups concluded that in antemortem samples, even low haemolysis levels will result in decreased detectable insulin levels. While it was determined this

would be of negligible effect in antemortem samples, it might impact on the reliability in post-mortem sample results. This is further demonstrated by Wunder et al. (2014), by LC–MS analysis of haemolysed samples, the decreased detection of insulin molecules over a 50-hour period, with considerable loss in the first 20 hours being demonstrated against increased detection of α- and β-chains in this period. It is noted that use of this LC–MS method could be developed to calculate a 'total' insulin calculation from free α- and β-chains, but would require confirmation that no further degradation of the chains.

Secondly, as previously stated, when reviewing results for blood post-mortem insulin investigations it must also be considered that the majority of blood insulin analysis is still performed using immunoassays, rather than more specific mass spectrometry methods (Simmons et al., 2019). This is a particular issue when the insulin compound is an exogenous insulin compound (recombinant human insulin or insulin analogue) used in the treatment of diabetes (Barmanray et al., 2019), as referred to Section 1.2.2. The major immunoassays in the clinical biochemistry market display considerable variability in the detection of these exogenous insulin compounds, ranging from < 5 to 140% recovery, depending on the assay, and the exogenous insulin compound (Parfitt et al., 2015). These findings have also been demonstrated by experimental runs in this project. There are also demonstrated variability when comparing current immunoassays for the detection of endogenous insulin, with manufacturer published data showing a variation of 94.2 to 108.1% recovery of insulin (Table 2.1.1).

Rare issues/conditions that can lead to unreliable insulin immunoassay results also need to be considered, such as cases where a patient/deceased has insulin antibodies that produce falsely elevated insulin results (Halsall, 2018). These conditions, such as insulin autoimmune syndrome (IAS) or Hirata's disease (Alves et al., 2013) are rare but can be found in combination with pathologies such as diabetes (Halsall, 2018).

1.4 Mass spectrometry

To improve the service provided to the Coroners, and in turn the families of the deceased, rather than using immunoassay methods for the post-mortem analysis of insulin concentrations, the more accurate, and precise mass spectrometry-based methods should be considered. Examples of mass spectrometry analysis are matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF), liquid chromatography – triple quadrupole mass spectrometry (LC–MS/MS) (Nedelkov et al., 2018) or high-resolution accurate-mass liquid chromatography-mass spectrometry (HRAM LC–MS) (Bottinelli et al., 2019).
Mass spectrometry is now commonplace in clinical biochemistry laboratories, for the analysis of compounds such as metanephrines (van der Gugten, 2020), toxicology and/or drugs of abuse (Maurer and Meyer, 2016) and therapeutic drugs, such as tacrolimus (Shi et al., 2015).

There are three main components of mass spectrometry analyser, as represented in Figure 1.4.1, the ion source, mass analyser and ion detector.

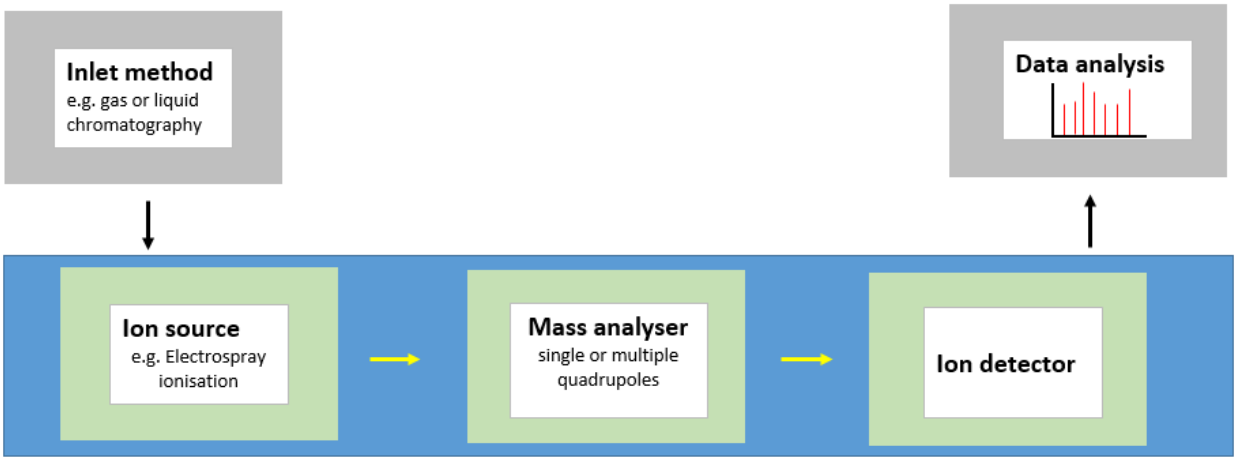


Figure 1.4.1: Components of a mass spectrometry analyser (blue zone), with required inlet for sample introduction, and post-mass spectrometry data analysis.

1.4.1 Ion source

The ion source has the function of analyte ionisation, this produces charged molecules, described by their mass-charge ratios (m/z), that can then pass into the mass analyser. There are a number of methods of ionisation such as chemical ionisation, atmospheric pressure chemical ionisation, and electrospray ionisation (ESI), ESI is represented in Figure 1.4.1.1 (Korra et al., 2023). The function of the ion source is to remove the solvent carrier phase and to charge the target

analytes. Any neutral or opposite polar charged ions are then removed in the mass analyser.

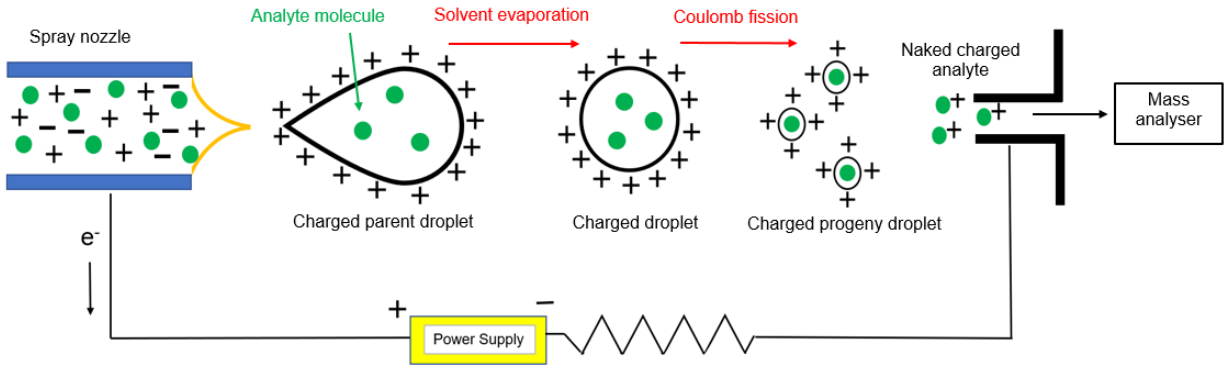


Figure 1.4.1.1: Schematic representation of electrospray ionisation, sample solution is passed through an electrical field inducing charged particles, adapted from Banerjee and Mazumdar (2012).

In the case of ESI, the ionisation mode is largely positive ionisation, i.e. adding a proton (H^+) to each molecule (M), resulting in $(M+H)^+$ ions. In the majority of positive ionisation mass spectrometry, one proton is added to each molecule, and therefore the m/z is equal to the molecular weight of M plus (one for the proton) (Siuzdak, 1994). However, in the case of large molecules such as proteins, multiple charges can occur across a molecule (Nedelkov et al.,2018), for example M plus five protons which would result in $(M+5H)^{5+}$ ions being produced. In this case the m/z is the molecular weight of M plus 5, divided by 5.

1.4.2 Mass analyser

After the charged analyte ions have been produced (the 'naked charged analyte' ions in Figure 1.4.1.1) these are transmitted into the mass analyser (Banerjee and Mazumdar, 2012). The mass analyser can consist of a single quadrupole or multiple quadrupoles in series. Each quadrupole consists of four electromagnets (Figure 1.4.2.1), a combination of radio frequency and direct current voltages are applied to the electromagnets. This 'tuning' results in target ions forming a bounded trajectory through the quadrupole while other m/z are removed, as displayed in Figure 1.4.2.1. In some mass spectrometry analysers, a group of six electromagnets rather than four are used, these are defined as a hexapole (Hannis and Muddiman, 2000).

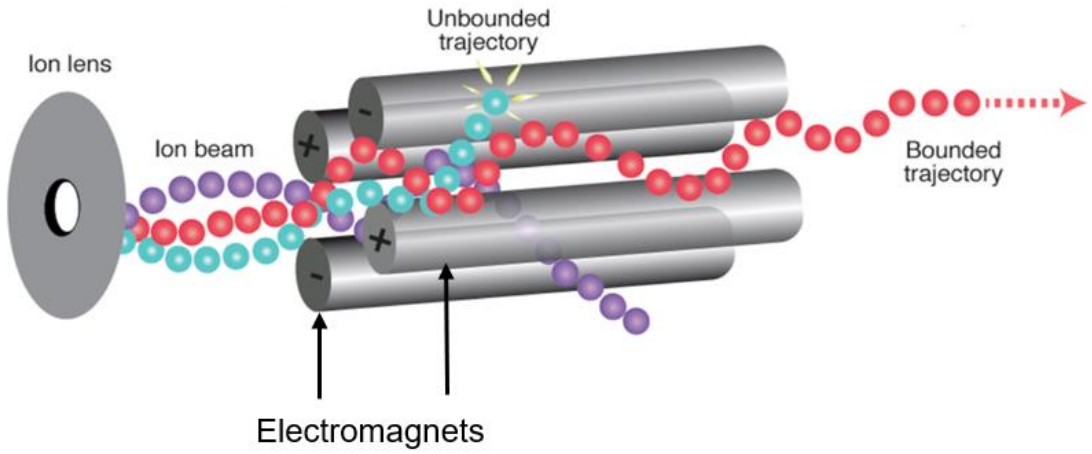


Figure 1.4.2.1: Quadrupole mass analyser, the correct magnitude of the radio frequency, and direct current voltages applied to the rods allows ions of a single m/z to maintain stable trajectories (red ions) from the ion source to the detector, whereas ions with different m/z values (blue and purple) are unable to maintain stable trajectories (Alsaleh et al., 2019).

1.4.3 Ion detection

The charged analyte ions exiting the mass analyser are then detected and transformed into a usable signal by a detector. Detectors generate an electric current proportional to the abundance of individual m/z ions. However, the actual number of ions exiting the mass analyser at any instant is relatively small, therefore amplification detectors, such as electron multipliers are required to increase the signal to the detectors (Bergin et al., 2021). In the case of an electron multiplier the single m/z ion being detected induces a cascade of serially increasing electrons, by the process of secondary electron emission. In this process the m/z strikes a surface plate that then releases electrons, these electrons then strike further plates, resulting in a usable signal (Medhe, 2018).

Mass spectrometry methods show good specificity and sensitivity for a large number of compounds and negate the disadvantages of immunoassays. Antemortem mass spectrometry analysis of insulin is available worldwide (Chen et al., 2013; Thevis et al., 2012), showing superior accuracy and sensitivity over the previous ‘gold standard’ radioimmunoassay (RIA) methods (Dong et al., 2018). In a number NHS hospitals antemortem insulin concentration is analysed by LC–MS/MS or ‘triple quad’ analysers (Figure 1.4.3.1), LC–MS/MS analysers are a combination of a liquid chromatography analyser (LC) to separate out the target compounds from biological matrix, coupled to the MS detection module.

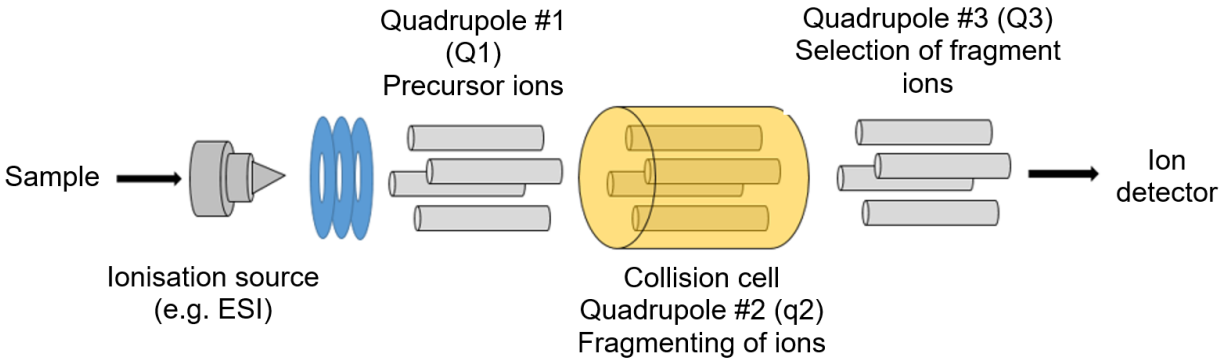


Figure 1.4.3.1: Representation of tandem mass spectrometry (MS/MS), demonstrating; ionisation source, Q1, collision cell (q2), and Q3, and their functions in the analyser.

Advances in mass spectrometry have resulted in high-resolution accurate mass-spectrometry (HRAM), such as quadrupole time of flight mass-spectrometry (QToF–MS). Resolution is the ability of the analyser to separate ions of a specific m/z, while mass accuracy is defined as the difference between the detected m/z and the true/theoretical m/z, HRAM analysers have significantly increased resolution, and mass accuracy when compared with LC–MS/MS analysis (Alsaleh et al., 2019). QToF has the same basic structure as the LC–MS/MS system, with a pre-mass spectrometry separation module, again this can be an LC unit. However, as represented in Figure 1.4.3.2, the addition of the time-of-flight tube extends the flight path of the m/z leading to further separation and increasing resolution and specificity.

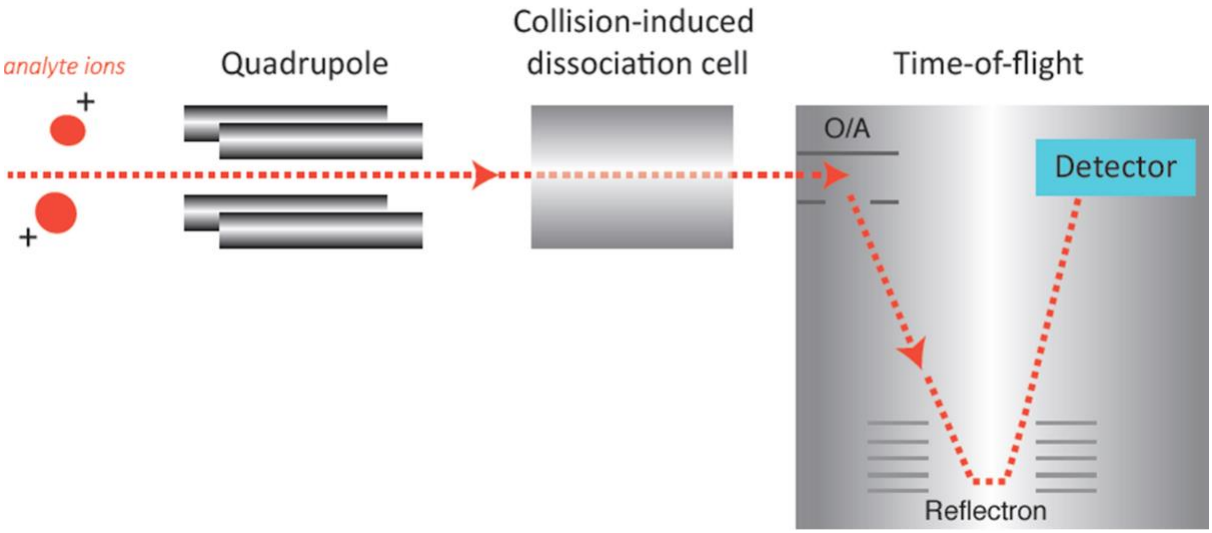


Figure 1.4.3.2: Schematic overview of QToF mass spectrometer, demonstrating the analyte ion pathway through the quadrupole, collision cell and time of flight components of the analyser (Alsaleh et al., 2019).

1.5 Dysglycaemia-related death and COVID-19

There have been significant changes to the autopsy examination process and collection of post-mortem samples due to the effect that the COVID-19 pandemic has had on the Coronial services in the UK. There has been a move away from full autopsy examinations where clinically possible, partly to reduce the risk of COVID-19 infection, but also due to the increased death rate during the pandemic in the UK causing ‘overloading’ of the mortuary and Coronial services. This change in procedure was led by the RCPath during the COVID-19 pandemic, who issued guidelines on performing autopsies on suspected COVID-19-affected patients. These guidelines advised that the invasive procedures of the autopsy process should not be performed and to remove bodily fluids solely for biochemical marker analysis identification of the cause of death (Osborn et al., 2020).

The COVID-19 pandemic also increased the number of deaths in patients with forms of diabetes, with a third of in-hospital COVID-19 deaths being people with diabetes (Barron et al., 2020). In the UK for the period February to May 2020, 1604 deaths of people with T1DM diabetes, and 36,291 deaths of people with T2DM diabetes were recorded. When compared with the same period in the years 2017 to 2019, the mean number of deaths recorded was 672 in the T1DM population and 16,071 in the T2DM populations. Presenting an increase in deaths, largely due to COVID-19, of 238.7% and 225.8% in the populations of people with T1DM and T2DM (Holman et al., 2020).

1.6 Key introductory points

As discussed in this Chapter, diabetes is significant worldwide health issue, and there is considerable data to show that good control of the disorder makes a difference in reducing the risk of developing acute and chronic complications (McGill and Felton, 2007), such as those covered in Sections 1.1.2 to 1.1.5. People with T1DM and an increasing number of people with T2DM are being treated with exogenous insulin compounds (Cahn et al., 2015), recombinant human and increasingly, insulin analogues, to improve good glucose control and reduce episodes of hypoglycaemia (Lefever et al., 2020). However, insulin is a drug commonly used in deliberate overdose (Russell et al., 2009), as well as accidental overdose from over administration.

While current assays can detect human endogenous insulin and recombinant human insulin, analysis of insulin analogues remain difficult to detect using conventional methodologies. This work looks at using newer methodologies to help detect and quantify these insulin analogues. From this, it can be hypothesised that to advance the investigation of dysglycaemia-related deaths a move away from the traditional analysis matrix (blood), and methods (immunoassays) must be undertaken. It should be considered that post-mortem biochemistry and toxicology be seen as specialist disciplines and as such methods, techniques, and interpretation should not be based solely on the antemortem practices. It is hypothesised that for this to progress, a change from post-mortem blood insulin analysis by immunoassay to the use of vitreous humour insulin concentrations, with mass spectrometry-based methods, as the primary indicator of perimortem/post-mortem status will improve the investigations into dysglycaemia-related deaths.

It can also be hypothesised that the reduction in IDE concentrations in the vitreous humour as compared with blood should increase the stability of the insulin concentrations within the matrix.

Further to this, that standardisation of the biochemical data generated from laboratory analysis, and the interpretation of these data, would improve the Coroner’s understanding of the investigations.

The knowledge gaps this project is to address are the use of immunoassay for the determination of post-mortem requests, and the lack of current determination of recombinant human insulin and/or insulin analogues in post-mortem cases. All contributing to the possibility of inappropriate cause of death being attributed to cases by a Coroner.

There is an onus on the histopathologist, biomedical scientists, and/or clinical biochemist presenting the data to the Coroner to understand fully the implications of incorrect post-mortem insulin results, the limitations of some post-mortem sample matrix and interferents. As well as the limitations in sensitivity, and specificity of immunoassay methods for the analysis of post-mortem insulin.

1.7 Aims

1.7.1 Evaluation of insulin quantification using HRAM LC–MS analysis

Evaluation of a novel diagnostic approach for insulin quantification using HRAM LC–MS analysis, for the measurement of blood and vitreous humour insulin concentrations. To be compared against currently used analytical techniques.

Currently there is no publicly available mass spectrometry based post-mortem blood insulin service in the UK, and very limited services in the rest of Europe and US. Data derived from this evaluation will address this gap in knowledge and skills. The produced novel analysis for vitreous humour insulin with quantification of exogenous insulin compounds, and differentiation from endogenous insulin, will improve the analytical service provided to the Coroners' regions. It is also essential for the interpretation of the analytical data to be in the context of the circumstances of death, and clinical history, and in some rare cases, potentially suspicious circumstances.

Consideration must be taken into possible differences in the transport of exogenous insulin into the vitreous humour compared with endogenous. As well as possible concentration differences between corresponding blood and vitreous humour insulin.

This approach provides improved techniques for investigation into dysglycaemia-related deaths, by increased accuracy and enhanced interpretations for Coroners, in turn improving the quality of service provided to families of the deceased.

To establish long-term quality of service to users the project will provide the necessary data to pave the way to gain United Kingdom Accreditation Service (UKAS) recognition against the International Standards Organization (ISO) standards ISO/IEC 15189 (Medical Laboratory) and ISO/IEC 17025 (Forensic Services).

1.7.2 Audit reporting of dysglycaemia-related deaths by HM Coroners

To audit changes in reporting of dysglycaemia-related deaths by HM Coroners in England, Wales and Northern Ireland, and Procurator Fiscal in Scotland, (from herein these are collectively referred to as the Coroners).

This audit would highlight any discrepancies in use of sample matrices, analytes and analytical methods that could be standardised and consequently improve the post-mortem analytical service.

1.8 Objectives

1.8.1 To undertake quantitative comparative evaluation of insulin measurements in vitreous humour compared with blood

In cases of suspected hyperglycaemia, and hypoglycaemia related-deaths, glucose has been routinely measured in vitreous humour since the 1980s (Péclet et al., 1994). Despite this, there has been limited research conducted into the validity of insulin concentration levels in the vitreous humour. With the first report of vitreous humour insulin being considered as a possible indicator for insulin poisoning not being published until 2013 (Hess et al., 2013). This report only considered cases of insulin poisonings, the insulin concentrations in these cases would be seen as clinically significant increases in the detectable insulin. These insulin concentrations may or may not include exogenous compounds as the method could not distinguish endogenous from exogenous insulin compounds. This method was deemed not suitable for cases of dysglycaemia-related death not involving exogenous insulin administration because of concerns over measuring very low insulin levels using immunoassay methods. With the advent of novel mass spectrometry methods, it is timely to evaluate if these concerns have been negated. Development of HRAM LC–MS analytical methods would increase specificity and expand the detectable concentration range of insulin.

1.8.2 Assessment of long-term sample storage and impacts upon quality affecting insulin concentration levels.

The interval between time of death and performing the autopsy examination can vary from a few days, with the body stored at 4°C for much of this time, to in excess of ten weeks, without controlled temperatures, this needs to be assessed for any variations in results this may induce. Evaluation of pre-analytical storage also is required to enable effective storage post autopsy, as well as post-analytical storage. Therefore, sample stability for storage post-analysis of a minimum of 12 months needs to be assessed.

1.8.3 Comparative evaluation of insulin immunoassays against the developed HRAM LC–MS method

Comparative evaluation of insulin immunoassay methods against the corresponding mass spectrometry method for analysis of post-mortem blood samples. And to consider the advantages and disadvantages of using HRAM LC–MS or LC–MS/MS as the mass spectrometry method of choice.
The use of mass spectrometry analysis will be critically assessed in parallel with immunoassays of differing concentrations of endogenous and exogenous insulin for accurate and precise analysis of blood samples.

1.8.4 Bridge the knowledge gaps in the reporting of T1DM deaths

Bridge the knowledge gap regarding the accurate and precise concentration measurements of the numerous recombinant human insulin, and insulin analogues compounds presently used in treatment of T1DM.
The immunoassays are targeted for antemortem endogenous insulin detection, and as such when used for the analysis of post-mortem insulin concentrations, any exogenous insulin present cannot be accurately measured.
There appears to be a ‘disconnect’ in the reporting of post-mortem insulin results to the Coroners, and this should be rectified by a better understanding of their needs, and their current understanding of the laboratory investigations in to dysglycaemia-related deaths.

Chapter 2: Immunoassay analysis of insulin

2.1 Introduction

The first immunoassay for the determination of human blood insulin concentrations was described in 1960 by Yalow and Berson using a RIA (Yalow and Berson, 1960) and was part of the works that led to Yalow being awarded the Nobel Prize in Physiology or Medicine in 1977 (Nobelprize.org, 2023). With the development of enzyme linked immunosorbent assay (ELISA), and chemiluminescence immunoassay (CLIA) insulin assays, which can be converted for use on high throughput platforms (Shen et al., 2019), antemortem clincal insulin analysis is now commonly performed in clinical biochemistry laboratories.

Table 2.1.1 demonstrates the analytical performance of some of these auto-analyser insulin assays. When comparing manufacturer data in Table 2.1.1, the assays appear precise for endogenous insulin, with assay precision being defined by the coefficient of variation (CV). CV is normally presented as a percentage, and is calculated from the determined concentration mean and standard deviation (SD) values., i.e.: CV (%) = (SD/Mean)*100 (Reed et al., 2002)

In the case of endogenous insulin, the assays demonstrate CV values ranging from 0.6 to 5.3%. With CV values less than 20% representing acceptable assay precision, and less than 10% representing good precision (Reed et al., 2002). However, there is variation in the detection of insulin, with recovery experiments demonstrating mean recoveries of 92.2% (Siemens Immulite®) to 108.1% (Siemens Atellica®). Therefore, clinical assay results generated between these systems may not be directly comparable.

		Spiked insulin recovery			Assay precision		
Manufacturer	**Analyser**	**Total of endogenous insulin plus spike (pmol/L)**	**Observed (pmol/L)**	**% recovery**	**Mean (pmol/L)**	**SD (pmol/L)**	**CV (%)**
Abbott (Abbott, 2010)	Architect®	154.7	147.2	95.2	44.8	1.6	3.6
		400.1	377.6	94.4	230.1	4.7	2.0
		1232.0	1144.4	92.9	716.5	12.8	1.8
		Mean		94.2			
Abbott (Abbott, 2022)	Alinity®	**No Data**			59.0	1.1	1.8
					276.1	4.1	1.5
					864.3	14.1	1.6
					1078.3	17.6	1.6
Roche (Roche, 2017)	Cobas®	**No Data**			47.6	2.3	4.9
					116.0	4.3	3.7
					383.0	12.9	3.4
					2949.0	69.6	2.4
Siemens (Siemens, 2022b)	Atellica®	171.6	181.8	105.9	89.4	0.4	0.5
		183.0	199.2	108.9	312.0	1.6	0.5
		202.8	210.6	103.8	970.2	5.8	0.6
		351.6	400.2	113.8			
		Mean		108.1			
Siemens (Siemens, 2022a)	ADVIA Centaur®	171.6	181.8	105.9	82.9	2.5	3.0
		183.0	199.2	108.9	459.7	14.7	3.2
		323.4	350.4	108.3	853.9	28.2	3.3
		Mean		107.7			
Siemens (Siemens, 2006)	Immulite®	223.8	220.8	98.7	75.0	3.7	5.0
		427.8	385.8	90.2	158.4	7.9	5.0
		738.0	648.0	87.8	1746.0	93.0	5.3
		Mean		92.2			

Table 2.1.1: Comparison of manufacturer supplied data for endogenous insulin detection, and precision of insulin assays.

The manufacturers listed in Table 2.1.1 only comment on few possible/known interferences to the individual assays. Cross-reactivity with insulin compounds that are used in the treatment of diabetes is only being commented on by one manufacturer, Roche, for the Cobas® assay. In this case, insulin aspart, insulin glargine and insulin lispro all demonstrated undetectable concentrations (< 1.39 pmol/L) when samples were spiked with 6000 pmol/L of each analogue

(Roche, 2017). Parfitt et al. (2015) demonstrated cross-reactivity with some exogenous insulin compounds, particularly recombinant human insulin compounds, such as Humulin® and Actrapid®. However, this is not always the case when the insulin compound structurally differs from human insulin. For example, in the case of insulin detemir none of the compound could be detected by several of the immunoassays Parfitt et al. assessed.

2.2 Assessment of sample storage stability at –20°C

2.2.1 Methods and materials: assessment of sample storage stability at –20°C

Storage conditions of blood samples prior to analysis can impact on analytical quality and therefore impact on clinical relevance of any results (Kift et al., 2015), as such should be taken into consideration when undertaking an analytical service. Large NHS laboratories may have access to –80°C freezers for the storage of non-routine clinical samples, such as clinical trial/research samples, sudden unexpected death in childhood (SUDIC) and CSF samples. With some specialist laboratories having facilities for nitrogen cooling. However, smaller NHS sites will predominately only have –20°C units. With the aim that this service will be used by sites across the NHS and out-side the UK it would be preferable for samples to be stored in –20°C units and transported frozen (either using 'dry ice' as a coolant, or frozen at –20°C and then transported over a short distance/time period).
Long term post analytical storage of samples will also be at –20°C, stability of insulin under these conditions is considered suitable for up to 5 years (Retnakaran et al., 2019),

2.2.1.1 Antemortem blood samples

Antemortem samples for the experiments in this Chapter were collected from patients with clinical analysis requests for endogenous insulin, and from a mixed population containing people with; T1DM and T2DM, as well as people with no clinical history of diabetes. Samples were stored as part of the NNUH Biorepository.

Sample types collected were:

- Serum or plasma (supernatant from whole blood samples post centrifuging at 3605 *g* for 10 mins), these samples were then frozen at –20°C. Collected into in serum tubes with silica separating gel, BD-SST® II vacutainer specimen tubes (Becton, Dickinson and Company, Franklin Lakes, US) (BD®, product reference: 367954) or BD® lithium heparin preservative plasma tubes with separating gel (BD®, product reference: 367375).

- Where feasible, a whole blood sample was collected at the same time as the serum/plasma from the patient group quoted above, preserved with the anti-clotting agent Ethylenediaminetetraacetic acid (EDTA). Samples collected into BD® vacutainer tubes, EDTA preservative (BD®, product reference: 367838). These samples were not centrifuged, stored between 4 and 8°C.

2.2.1.2 Siemens Immulite® and Roche Cobas® insulin assays

The insulin immunoassay quantifications for the experiments document in this Chapter were performed using two differing immunoassay auto-analysers, commonly found in clinical biochemistry laboratories, the Immulite® (Siemens Healthcare Diagnostics, Erlangen, Germany), and Cobas® e601 (Roche Diagnostics, Meylan, France) analysers. The samples were assayed using the manufacturer analyser specific reagents, Siemens insulin 100 reagent packs (Siemens, product reference: 6606384) with the assay calibrated using Siemens insulin adjustor (Siemens, product reference: L2KIN2). The Cobas® insulin assay (Roche, product reference: 12017547-122) was calibrated using the Cobas® insulin calibrator set (Roche, product reference: 105210). Both analysers were calibrated; at least monthly, when a new insulin assay reagent lot was used, and/or when sample batch quality assessment indicated recalibration was required. The decision to accept or reject/rerun insulin assay batches, and following from this to recalibrate assays if required, was performed by assessing assay precision via the results from internal quality control samples run at the start and end of each sample batch. External quality control from a national scheme was processed monthly to ensure assay accuracy.

2.2.1.3 Assessment of sample storage stability at –20°C

For the assessment of post-analytical storage conditions, samples were analysed on day 0 using a Siemens Immulite®, and then stored as four separate 250 µl aliquots of each sample, at –20°C for periods of 44 and 97, 730 (+/- 10) and 1490 days (+/- 10). Four aliquots were required to ensure that a fresh aliquot was used at each time period to remove any variability due to multiple freeze-thaw processes.

2.2.1.4 Sample storage, antemortem and post-mortem samples

The refrigeration and freezer units used for storage of all the project samples were fitted with the RF512® continuous temperature monitoring system (Comark Instruments, Norwich, UK). Temperatures at multi-points in the units are monitored in 'real time', with alert criteria set in the temperature monitoring software so that any variance +/- 2°C from the target temperatures for single time periods of greater than 30 mins triggered visual and audible alarms. In the event of a unit failure samples were moved to secondary units, which are also fitted with the RF512® system.

2.2.1.5 Data handling

Graphs have been produced using Microsoft Excel® (Microsoft Corporation, Washington, US) with Analyse-it® software (Analyse-it Software Ltd, Leeds, UK). Paired with *t*-test analysis has been performed using an on-line calculator, available from: www.graphpad.com/quickcalcs/ttest1/.

2.2.2 Results: assessment of sample storage stability at –20°C

Figures 2.2.2.1 to 2.2.2.8 and Table 2.2.2.1 present data from the assessment of sample storage stability. Insulin concentrations were determined at day 0, 44, 97, 730 and 1490 days after the initial analysis. Each time period is presented as Passing & Bablok graph and Bland-Altman difference plot to assess any variance in insulin concentration results at each time period compared to the original day 0 concentrations.

The Passing & Bablok graph demonstrates both the Passing & Bablok regression (green line) and the line of identity (grey line). The Passing & Bablok method calculates the regression line from the median slope of straight lines between any two points on the graph (Passing and Bablok, 1983). Whereas the line of identity is the ideal line of best fit where to two data sets would be equal, therefore y = x.

Bland-Altman difference plots demonstrate the difference between corresponding results from two methods/assays (y axis) against the mean of the two corresponding results (x axis) (Bland and Altman, 1986). The plot demonstrates bias between two methods/assays, this can either be fixed (or constant) bias, or proportional bias. Fixed bias, where the bias is a constant concentration value

difference across differing value data points, is presented on the plot as a constant departure of the mean differences (y axis) from zero. A proportional bias is where the difference in the corresponding results is similar in percentage difference but not actual concentration value across differing data points. Any proportional bias presents as departure from zero of the regression line of the differences of the means (x axis) (Ludbrook, 2010). The combination of these two forms of bias is considered the ‘total bias. For clinical assays a bias within the 95% confidence range is deemed acceptable (Giavarina, 2015)

It is important to confirm the stability of insulin, both for pre-analytical and post analytical storage. Coroners verdicts can take up to nine months, and with COVID-19 this was extended further. Therefore, insulin analysis can be requested or added at any time during the process to aid the Coroner in attributing the cause of death.

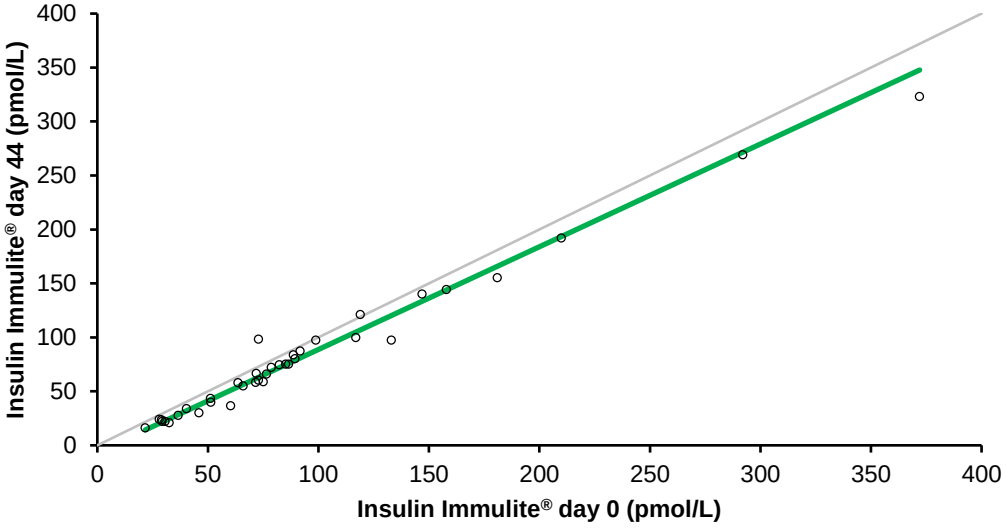


Figure 2.2.2.1: Antemortem insulin single freeze thaw process (pmol/L) day 0 to 44 – Passing & Bablok fit (green) and line of identity, y = x, (grey).

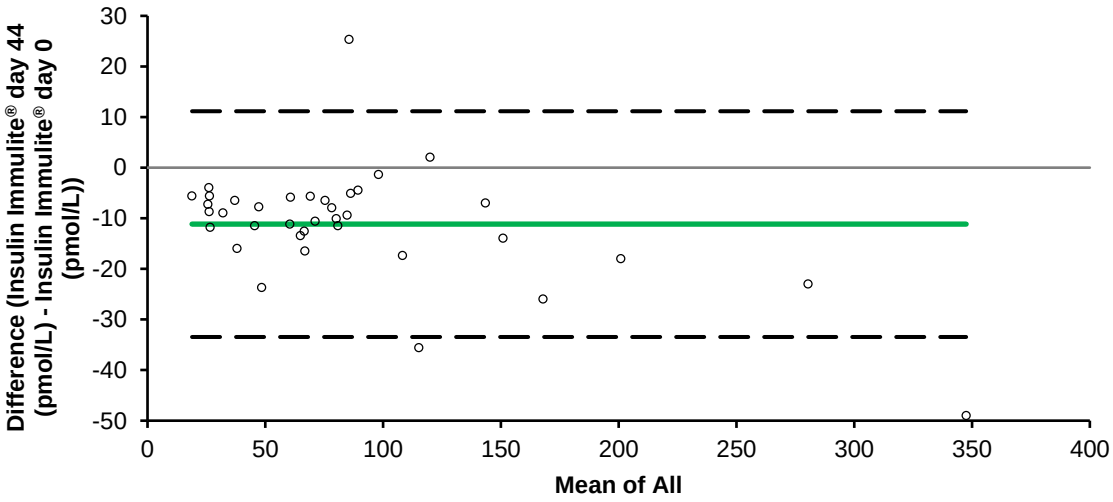


Figure 2.2.2.2: Antemortem insulin single freeze thaw process (pmol/L) day 0 to 44 – Bland-Altman difference plot.

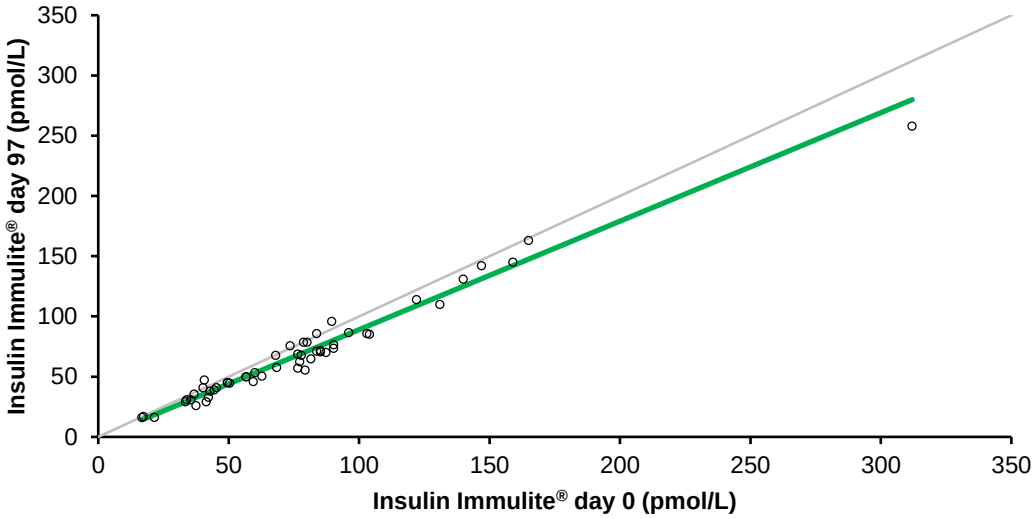


Figure 2.2.2.3: Antemortem insulin single freeze thaw process (pmol/L) day 0 to 97 – Passing & Bablok fit (green) and line of identity, y = x, (grey).

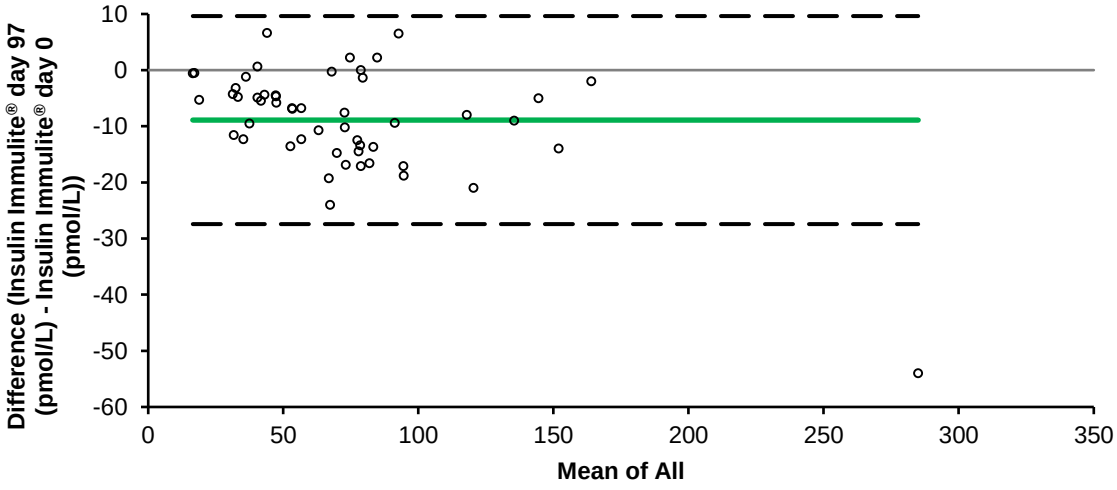


Figure 2.2.2.4: Antemortem insulin single freeze thaw process (pmol/L) day 0 to 97 – Bland-Altman difference plot.

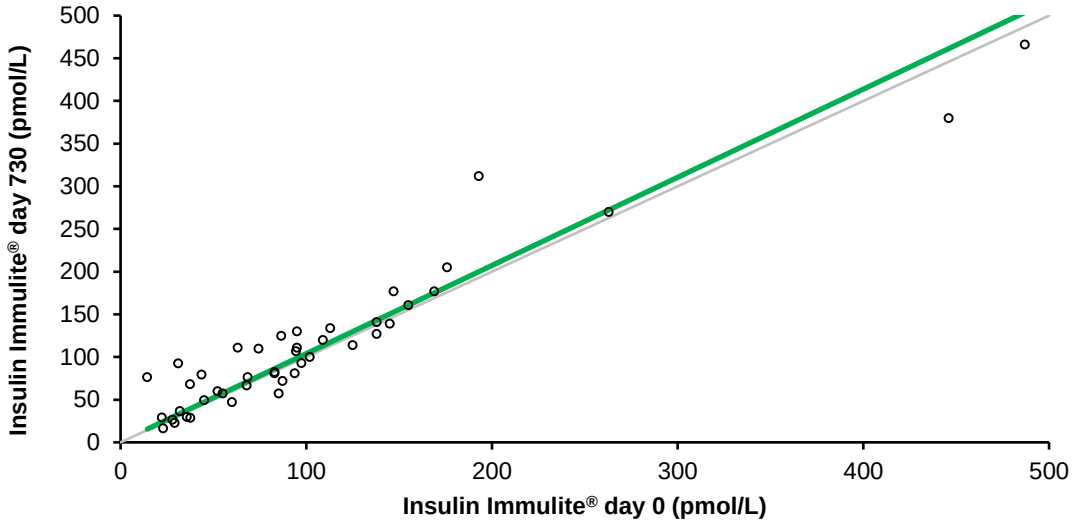


Figure 2.2.2.5: Antemortem insulin single freeze thaw process (pmol/L) day 0 to 730 – Passing & Bablok fit (green) and line of identity, y = x, (grey).

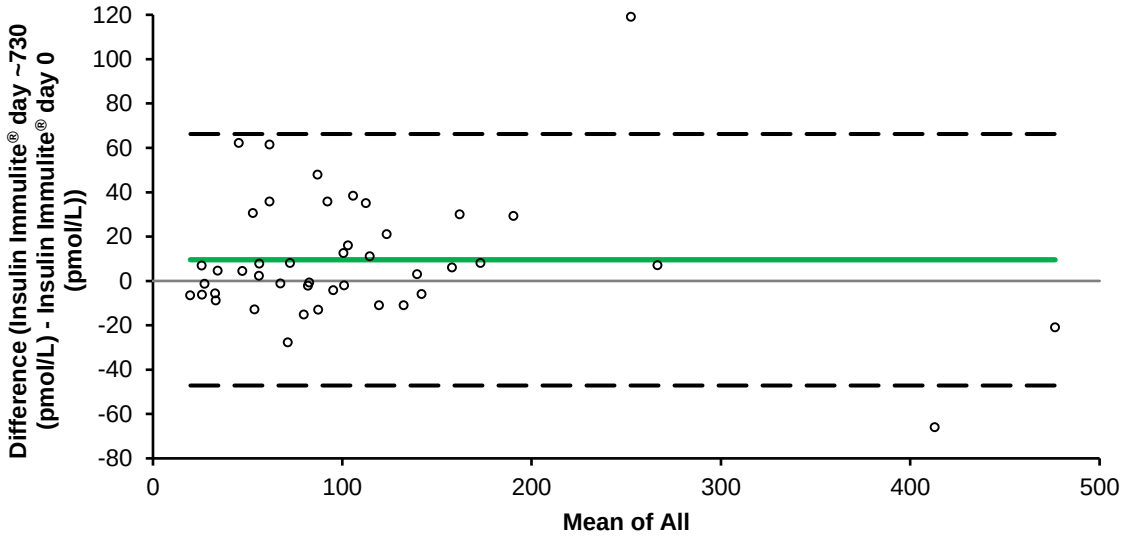


Figure 2.2.2.6: Antemortem insulin single freeze thaw process (pmol/L) day 0 to 730 – Bland-Altman difference plot.

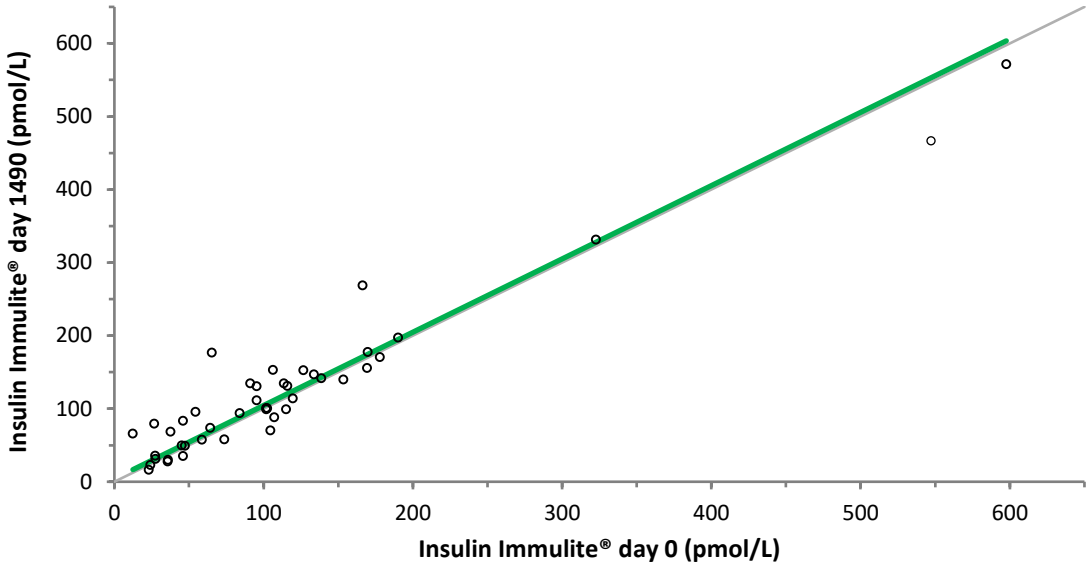


Figure 2.2.2.7: Antemortem insulin single freeze thaw process (pmol/L) day 0 to 1490 – Passing & Bablok fit (green) and line of identity, y = x, (grey).

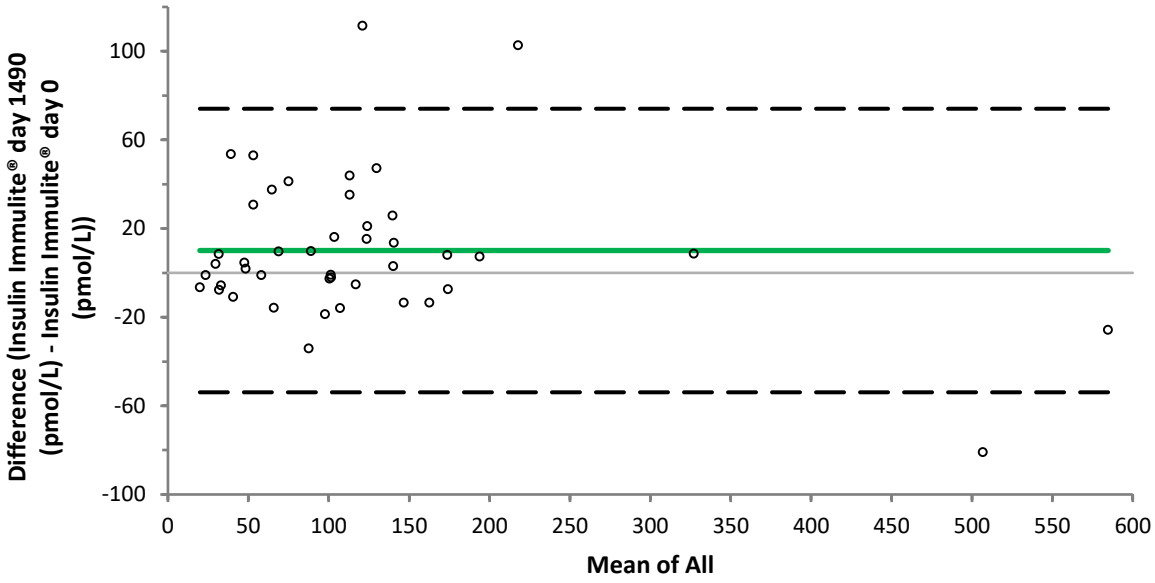


Figure 2.2.2.8: Antemortem insulin single freeze thaw process (pmol/L) day 0 to 1490 – Bland-Altman difference plot.

	Insulin concentration (pmol/L)				
	day 0	**day 44**	**day 97**	**day 730**	**day 1490**
Mean	93.47	82.32	67.79	114.72	126.01
SD	72.83	66.53	42.48	92.05	108.24
% CV	77.9	80.8	62.7	80.2	85.9

Table 2.2.2.1: Mean insulin concentration when samples stored at –20°C, days 0 to 1490.

2.2.3 Discussion: assessment of sample storage stability at –20°C

The minimum required storage for forensic/post-mortem cases at the NNUH is currently 12 months, as agreed with the Norfolk Coroner. The RCPath does not recommend any minimum or maximum retention of post-mortem samples but suggest that these should be set in agreement with Coroner (Wilkins, 2015). As the post-mortem insulin service is aimed at being at least UK wide, individual Coroners will order/dictate retention of samples as per their requirements and protocols. Reporting scientists should consider; the stability of the sample and/or analyte, and 'likelihood' of report requesting or additional testing need, when advising the Coroners.

There are, however, also cases where longer storage is required by UK law, e.g., a minimum of three years, two such cases are:

- Retention of samples requested by the Police under the remit of the Police and Criminal Evidence Act 1984 (Police and Criminal Evidence Act 1984).

- Possible negligence claims by the family of the deceased, which can be made up to 3 years post date of death.

Post-mortem samples for insulin analysis may need to be retained in either of the above two cases, as such a minimum post-analysis storage period of three years would be preferable.

When looking at relatively short-term storage, within one-year post analysis, Figures 2.2.2.1 to Figures 2.2.2.4, demonstrate results of duplicate samples run at time intervals of 44, and 97 days. There is a relatively small systematic bias of –6.4 pmol/L, and –1.0 pmol/L and good agreement with no statistically significant difference between the day 44 or 97 storage from the results on day 0, ($p = < 0.0001$ for both groups). However, it is clear from Figures 2.2.2.1 and 2.2.2.3 that there is increased scatter from the fit line at the lower concentrations, below 100 pmol/L. This would not be deemed to have clinical significance for the interpretation of insulin induced hypoglycaemia, where the insulin concentrations can be significantly above 1000 pmol/L (Hess et al., 2013). The Bland-Altman plots of this data (Figures 2.2.2.2 and 2.2.2.4) present bias of –11.15 pmol/L, and –8.9 pmol/L, but as can be seen in Figure 2.2.2.3 concentration results of

approximately 100 pmol/L are demonstrating differences of –28 to 62 pmol/L. For the assessment of insulin in cases of peri-mortem hyperglycaemia these changes could make significant difference in the interpretation of results, particularly at the extreme of these differences, 72 against 162 pmol/L, this must be taken into account when presenting data to the Coroners.

When considering longer-term storage, 730- and 1490-days' post original analysis, the results from the stability assessment demonstrated that three years is a feasible storage length at –20°C. The Passing & Bablok fit represents a good correlation and a relatively small systematic bias of -0.63 pmol/L (Figure 2.2.2.5) and 4.14 pmol/L (Figure 2.2.2.7), with the mean difference represented by Bland-Altman plot of 9.56 pmol/L (Figure 2.2.2.6) and 10.09 pmol/L (Figure 2.2.2.8), representing good agreement. Paired *t*-test of these samples indicates there is a statistical difference in the samples from the day 0 results (P = 0.0399 and 0.0464). It should be noted that higher insulin concentrations demonstrate an underestimation in the 730 and 1490-day storage groups, as demonstrated in Figures 2.2.2.5 and 2.2.2.7. This is also represented on the Bland-Altman plot with both data sets showing negative bias from the y axis (Figures 2.2.2.6 and 2.2.2.8).

These variations require clarification when presenting results to the Coroner. It is important that insulin concentrations are not seen in 'isolation' and the storage conditions are considered. From this data, insulin concentrations may show variation, particularly around 100 pmol/L range. It is important that unlike antemortem which are interpreted against 'fixed' reference ranges, post-mortem insulin concentrations are interpreted in conjunction with factors such as storage conditions and storage duration.

2.3 Pre-analytical separation of whole blood and insulin for determination of effect of IDE

2.3.1 Methods and materials: pre-analytical separation of whole blood and insulin for determination of effect of IDE

Blood samples were collected into BD® lithium heparin preservative plasma tubes with separating gel (BD®, product reference: 367375), to prevent clotting, and were collected from patients with antemortem insulin requests. Each sample was well mixed (for 5 minutes via roller mixer), 1 ml of mixed whole blood was then removed and centrifuged (3605 *g*, 5 minutes at room temperature).

The remaining sample (the 'primary' sample) was then centrifuged as above, and the plasma and red cells separated and stored at –20°C.

Plasma from the 1 ml aliquots were analysed for insulin concentrations on the Siemens Immulite® analyser. The insulin concentrations of the aliquots were then used to sort the primary samples in to three groups:

- < 75 pmol/L ('low' pool),
- 250 to 350 pmol/L ('medium' pool)
- > 500 pmol/L ('high' pool)

Upon testing, red cells and plasma, were defrosted at room temperature and then remixed together. The reconstituted samples in each group were then mixed to give three pool samples, when analysed at t = 0 min of 50.6, 286.4 and 728.7 pmol/L.

Each of the three pool samples were separated into 17 aliquots of 500 µl each (51 aliquots in total). This covered the 17 time points from t = 0 to t = 360 min. At each time point an aliquot of each pool level was centrifuged (as per conditions above) and analysed immediately in triplicate for insulin concentration using the Siemens Immulite® method.

2.3.2 Results: pre-analytical separation of whole blood and insulin for determination of effect of IDE

As stated in Section 1.3.6, IDE is known to cause an *in vitro* decrease of the insulin concentration. As such the effect of the IDE was determined by assessing insulin concentration reduction related to the time from sample collection to the plasma being separated from the red cells.

Table 2.3.2.1 presents the mean insulin concentrations from the samples run in triplicate, after the plasma was removed, by centrifugation from the red cell component for each time point.

Figures 2.3.2.1, 2.3.2.2 and 2.3.2.3 present the data showing the loss of insulin with increasing separation time of the plasma from the red cells.

Time (min)	Mean insulin concentration (pmol/L)	% variation from t = 0	Mean insulin concentration (pmol/L)	% variation from t = 0	Mean insulin concentration (pmol/L)	% variation from t = 0
0	50.6		286.4		738.7	
10	51.3	1.38	279.4	-2.4	734.8	-0.5
20	51.4	1.58	279.3	-2.5	732.7	-0.8
30	49.7	-1.78	280.1	-2.2	733.9	-0.6
40	50.1	-0.99	276.5	-3.5	732.5	-0.8
50	50.2	-0.79	277.8	-3.0	730.5	-1.1
60	48.7	-3.75	274.9	-4.0	726.9	-1.6
90	47.2	-6.72	273.2	-4.6	712.6	-3.5
120	46.7	-7.71	271.2	-5.3	709.3	-4.0
150	46.4	-8.30	263.4	-8.0	703.8	-4.7
180	46.1	-8.89	260.8	-8.9	684.3	-7.4
210	46	-9.09	243.8	-14.9	677.3	-8.3
240	45.7	-9.68	236.4	-17.5	674.2	-8.7
270	45.6	-9.88	227.8	-20.5	662.3	-10.3
300	45.3	-10.47	221.4	-22.7	657.2	-11.0
330	44.4	-12.25	219.6	-23.3	650.9	-11.9
360	39.2	-22.53	210.3	-26.6	619.2	-16.2

Table 2.3.2.1: Mean plasma insulin concentrations of three levels, measured after plasma was separated from red cells, and % variation over time from t = 0 concentration.

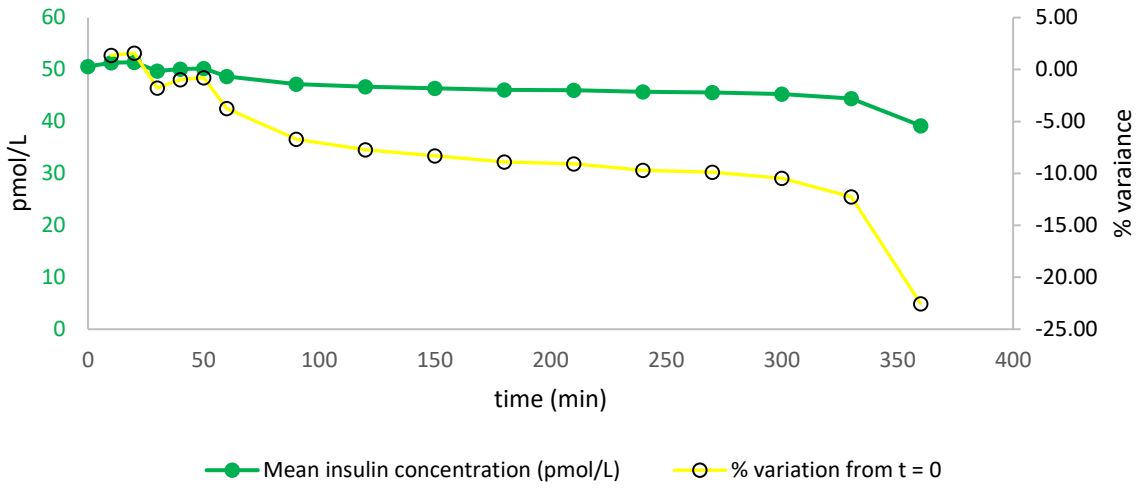


Figure 2.3.2.1: Mean plasma insulin concentrations, and % concentration variance from t = 0 value, of sample with mean concentration of 50.6 pmol/L (at time 0) after separation from red cells at set times from 10 to 360 minutes.

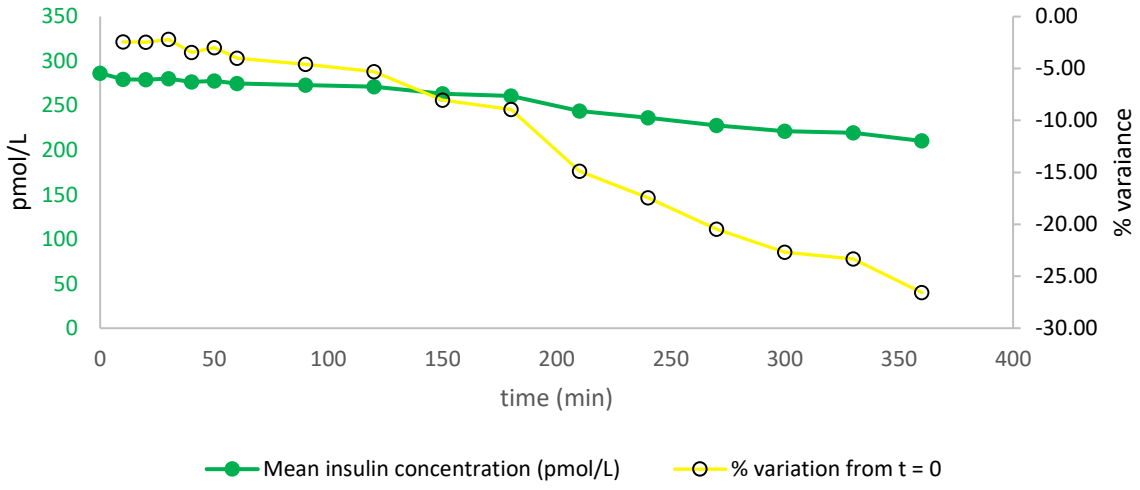


Figure 2.3.2.2: Mean plasma insulin concentrations, and % concentration variance from t = 0 value, of sample with mean concentration of 286.4 pmol/L (at time 0) after separation from red cells at set times from 10 to 360 minutes.

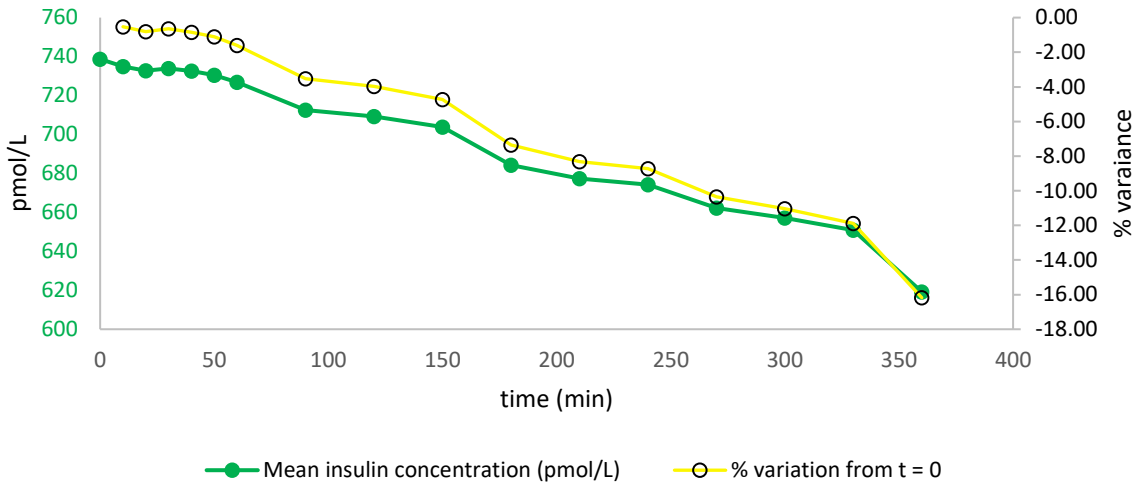


Figure 2.3.2.3: Mean plasma insulin concentrations, and % concentration variance from t = 0 value, of sample with mean concentration of 728.7 pmol/L (at time 0) after separation from red cells at set times from 10 to 360 minutes.

2.3.3 Discussion: pre-analytical separation of whole blood and insulin for determination of effect of IDE

These findings demonstrate that in addition to the pre- and post- analytical storage discussed in Section 2.2.3, the time that is taken for the sample to be removed from the body and then centrifuged must also be considered as a factor of possible fluctuation in concentration. As discussed in Section 1.3.6, the depletion of insulin due to the action of IDE should be considered when the sample is haemolysed. This is demonstrated in Table 2.3.2.1 and Figures 2.3.2.1, 2.3.2.2, and 2.3.2.3, where there are marked loses of insulin concentration over time, with a reduction by up to 26.6% of the insulin concentration demonstrated within six hours. This percentage loss at 6 hours appears to be consistent across differing concentration values. These findings agree with works by Sadagopan et al. (2003).

The samples used for the experiments in this Section were un-haemolysed, this was confirmed by analysis using the Abbott auto-analyser plasma/serum haemolysis indicator assay, which assesses the presence of red cells. The practise of assessing the haemolysis-index in the sample (Yin and Herskovits, 2022) is not common practise when processing post-mortem samples. PMI, as per Section 1.3.5, can be assessed to confirm age of sample, with longer term

PMI values being presumed to indicate increased insulin degradation, and with haemolysis considered (Stephenson et al., 2022), when there is considerable red coloration of the plasma.

It is suspected that with the increased release of IDE from red cells in haemolysed samples this reduction of insulin concentration *in vitro* would be accelerated. While in antemortem sampling it is sometimes possible for a non-haemolysed sample to be collected from the patient at a later date, this is clearly not feasible in post-mortem cases.

2.4 Comparison of immunoassay insulin assays - antemortem blood samples

2.4.1 Methods and materials: comparison of immunoassay insulin analysis - antemortem blood samples

Samples were from the collection described in Section 2.2.1.1. The analysis of insulin required either serum or plasma to be used i.e., the whole blood was centrifuged and the extracellular fluid (plasma or serum, dependent on collection tube type), was removed. No other sample preparation is required for analysis on the Immulite® or Cobas® auto-analysers.

2.4.2 Results: comparison of immunoassay insulin analysis - antemortem blood samples

To assess if there is variability in the measurement of endogenous insulin by the two immunoassay auto-analyser available in the NNUH laboratory, the Siemens Immulite® and Roche Cobas® platforms. Plasma insulin samples were analysed on both immunoassay platforms on the same day, to remove variability produced by factors, such as storage conditions, freeze-thaw processes, and/or laboratory changes in temperature. Figures 2.4.2.1 and 2.4.2.2, and Table 2.4.2.1 represent the data from the comparison of the two auto-analysers.

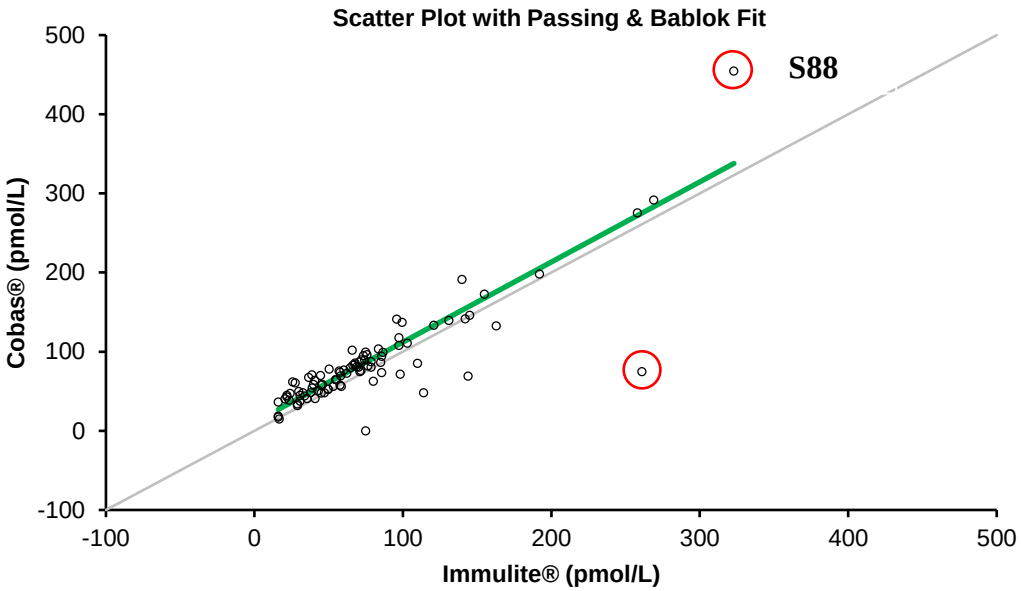


Figure 2.4.2.1: Antemortem plasma insulin concentrations (pmol/L) of samples measured on both Siemens Immulite® and Roche Cobas® immunoassay auto-analysers – Passing & Bablok fit (green) and line of identity, y = x, (grey).

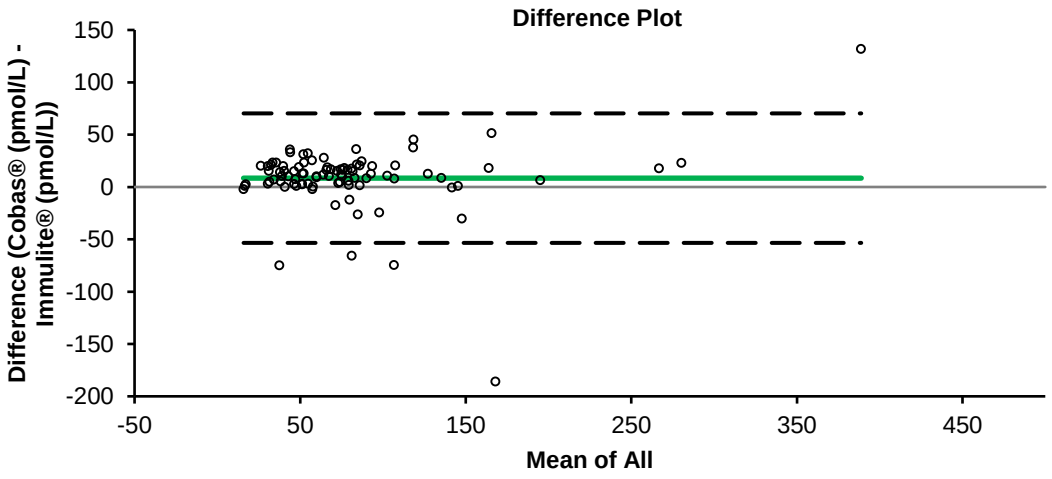


Figure 2.4.2.2: Antemortem plasma insulin concentrations (pmol/L) of samples measured on both Siemens Immulite® and Roche Cobas® immunoassay auto-analysers – Bland-Altman difference plot.

Sample	Analyser	1st Analysis	2nd Analysis	Mean	SD
S39	Immulite®	261	223	242	26.87
	Cobas®	74.9	57.3	66.1	12.47
S88	Immulite®	323	341.2	332.1	12.87
	Cobas®	454.7	438.2	446.4	11.66

Table 2.4.2.1: Re-analysis of samples S39 and S88 on Siemens Immulite® and Roche Cobas® analysers.

2.4.3 Discussion: comparison of immunoassay insulin analysis - antemortem blood samples

The two immunoassay platforms show good comparison with a Passing & Bablok fit of 10.73+1.01x (Figure 2.4.2.1), representing a good correlation. The Bland-Altman plot (Figure 2.4.2.2) further supports this good correlation between the two immunoassay methods with a demonstrated total bias of 10.73 pmol/l, this is within the 95% confidence range deemed acceptable for clinical assays (Giavarina, 2015). When considered against a clinical reference range of 67.1 – 210.0 pmol/l this bias of 10.73 pmol/L represents 16.0% (of 67.1 pmol/l) and 5.1% (of 210.0 pmol/l) at either extremity of the range. Although the *t*-test indicates a statistically significant difference between the two analytical groups (P = 0.0123), in terms of clinical interpretation there is no difference in assay groups.

However, this data also includes two samples, of the 103 analysed, from different individuals with results which significantly differ between the analysers, S39 (P = 0.0139) and S88 (P = 0.0113). The samples were re-assayed on both platforms, and the repeat results are presented in Table 2.4.2.1. The re-analysis of the samples confirmed the original results were consistent for each analyser but did not correlate well between the two immunoassay methods. The clinical history related to sample S39 (M, 38 yr) indicates no use of any prescription or non-prescription drugs. In the case of S88 (M, 67 yr) the only prescribed drug stated in the clinical history is atorvastatin, this drug is used to control low-density lipoprotein concentrations (van Leuven and Kastelein, 2005). However, there are no published reports of statins as an interfering substance for insulin immunoassays. When considering that statins are one of the most prescribed drugs in the UK, with approximately 25% of people over 40 years of age in the prescribed statins (García Rodríguez et al., 2022), these compounds would have almost certainly been reported as factor for erroneous insulin results.

As such, from the clinical history of these two samples there are no clear drug cross-reaction related reasons that may have caused this difference in results. It is possible that other causes for spurious immunoassay results, such as auto insulin-antibodies (Kim et al., 2011), or the presence of biotin (Samarasinghe et al., 2017), discussed in Section 2.5.3, may be the cause for these differing results.

2.5 Comparison of immunoassay insulin assays – post-mortem blood samples

While the works described in this Chapter have been utilising antemortem blood samples to assess suitability of the immunoassay analyser and sample stability, it is also important to assess post-mortem plasma insulin using the immunoassay auto analyser.

2.5.1 Methods and materials: comparison of immunoassay insulin analysis – post-mortem blood samples

2.5.1.1 Post-mortem blood samples

Samples for toxicology and/or biochemical analysis from autopsy examinations performed at the NNUH, James Paget University Hospital (Gorleston) and Queen Elizabeth Hospital (King's Lynn) are routinely sent to the Toxicology-Endocrinology section of the NNUH for analysis. As such, these samples were stored, post-reporting of results to the Coroners service. Samples were then assigned to one of three sample collection groups dependant on the case/clinical history:

- Control group: samples collected from post-mortem cases where neither hypo- nor hyperglycaemia conditions were suspected perimortem.

- Samples collected from post-mortem cases where hypoglycaemia was suspected in the circumstances of death.

- Samples collected from post-mortem cases where hyperglycaemia was suspected in the circumstances of death.

These samples are used in the experiments performed in this Chapter and Chapter 3.

2.5.1.2 Post-mortem blood sample types collected

- Whole blood, no preservative collected into Sterilin® 30 mL polystyrene universal containers (Sterilin Ltd, Newport, UK) (Sterilin, product reference: 128B/FS). Samples were centrifuged at 3605 *g* for 10 mins and supernatant removed from red cells and then frozen at –20°C.

- Whole blood collected into BD-SST® II vacutainer specimen tubes (BD®, product reference: 367954). Samples were centrifuged at 3605 *g* for 10 mins; supernatant removed and added to a separating cup in the original sample tube, samples were then frozen at –20°C.

- Whole blood collected into BD® vacutainer tubes plasma, 2 ml tube containing, fluoride oxalate preservative (BD®, product reference: 368920). Samples were centrifuged at 3605 *g* for 10 mins, supernatant removed and added to a separating cup in the original sample tube. Samples stored at –20°C.

Post-mortem samples collected for the project were only from cases where consent from the family has been received for tissues and samples to be 'retained as part of the medical record & use of the tissues in research' this means that the samples can be used for research without any further requirement for consent under HTA guidelines (Human Tissue Act 2004).

2.5.1.3 Post-mortem vitreous humour samples

Vitreous humour samples received from post-mortem cases were collected into BD® vacutainer plasma tubes, 5 mg fluoride oxalate preservative (BD® reference: 3368920) samples were frozen for a minimum of 24 Hrs at –20°C, thawed at room temperature for a minimum of one hour, and then centrifuged at 7378 *g* for 5 mins.

Post-primary analysis the supernatant was then split into three separate aliquots (i, ii and iii) of approximate equal measures, minimum of 400 µl, or two aliquots if less than 1.2 ml supernatant in total.
Two aliquots (i and ii) were stored in the same –20°C freezer unit.

Aliquot i from each case was then later thawed and assayed for insulin concentrations by the Siemens Immulite® and Roche Cobas® assays.
Aliquot ii was stored in preparation for analysis by the developed HRAM LC–MS method.
Aliquot iii for each case (where possible) was then stored in a second –20°C freezer. This was to ensure that were there any breakdowns or prolonged temperature increases of the primary sample freezer which would possibly affect sample integrity; the third aliquot could be used for mass spectrometry analysis.

2.5.1.4 Post-mortem blood immunoassay

A comparison of the two aforementioned Siemens and Roche systems was performed using 107 post-mortem plasma samples from diabetes and non-diabetes-related deaths, the data for this analyser comparison is presented in Figures 2.5.2.1 and 2.5.2.2. From these 107 post-mortem cases 87 also had three other biochemical markers analysed: blood glucose (where sample quality was suitable), vitreous humour glucose, and vitreous humour sodium, as described in Section 2.5.1.2. Of these 87 cases, 35 were chosen as they had considerable clinical history from around the time of death provided. The data from the analysis of these 35 samples are presented in Table 2.5.2.2.

2.5.1.5 Vitreous humour glucose, vitreous humour sodium, and blood glucose analysis

Following the freeze-thaw sample preparation as described in 2.5.1.3 the primary analysis of glucose and sodium was performed on the vitreous humour, these along with the blood glucose were performed using the immunoassay (glucose) and integrated chip technology (ICT) (sodium), on the Abbott Architect® c16000 analyser (Abbott Laboratories, Chicago, US). This auto analyser requires no additional sample preparation.

- Glucose using the Abbott Architect® c16000 glucose hexokinase method (Abbott, product reference: 3L82).

- Sodium using the Abbott Architect® c16000 ion selective electrode method (Abbot, product reference: 9D28)

Vitreous humour sodium was used as a reference marker for the sample quality (Mitchell et al., 2013) with markedly raised or depressed sodium levels indicating possible degradation of the sample.

2.5.1.6 Polyethylene glycol extraction

A polyethylene glycol (PEG) solution was produced by adding 12.5 g of PEG 6000 (VWR®, Radnor, Pennsylvania, US) (VWR®, product reference: 26610.290), to 50 ml phosphate buffered solution (pH 7.4) (Thermo Fisher Scientific®, Massachusetts, US) (Thermo Fisher Scientific®, product reference: 10010023) giving a 25% (w/v) solution.

The PEG extraction is performed to remove any insulin:IgG molecule complexes that may form in the samples (discussed further in Section 2.5.3). For this extraction 300 µl of PEG solution was added to 300 µl of patient sample into a micro centrifuge tube. The solution was vortex mixed for 30 secs, and then centrifuged in a microfuge at 15,000 *g* for 5 min at room temperature. The supernatant was removed and pipetted into a sample Immulite® sample cup for analysis.

2.5.2 Results: comparison of immunoassay insulin analysis – post-mortem blood samples

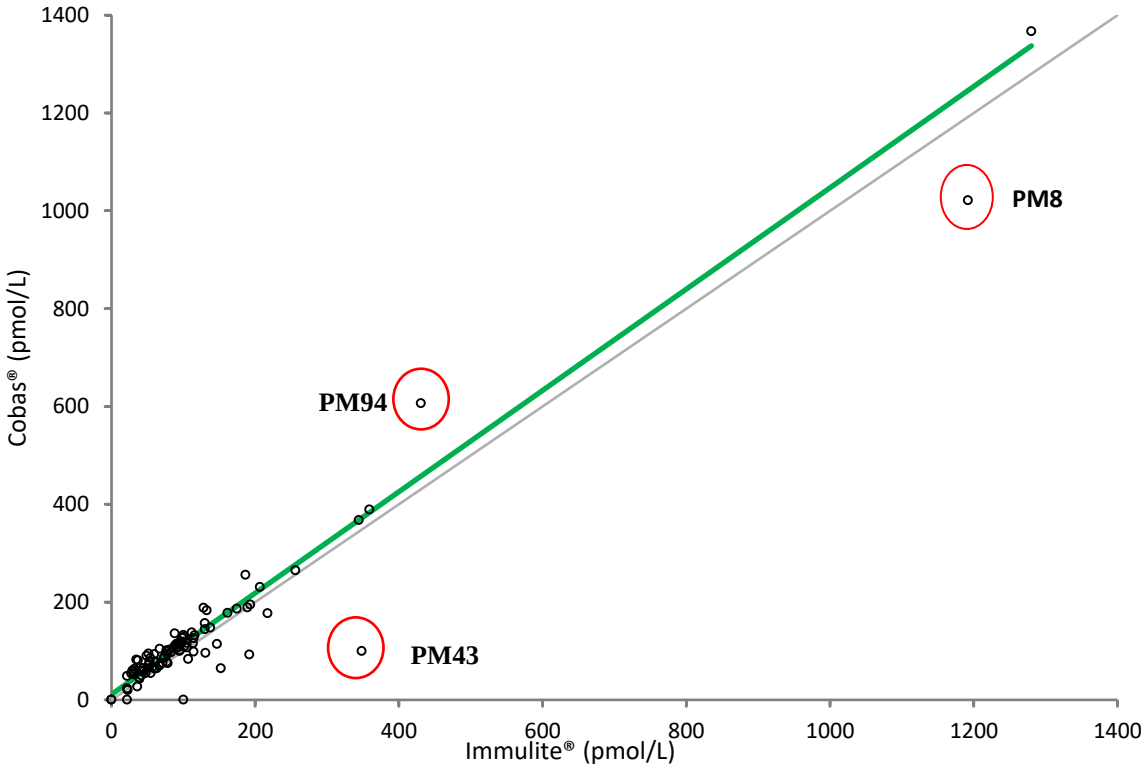


Figure 2.5.2.1: Post-mortem blood insulin Cobas® v Immulite® immunoassays – Passing & Bablok fit (green) and line of identity, y = x, (grey).

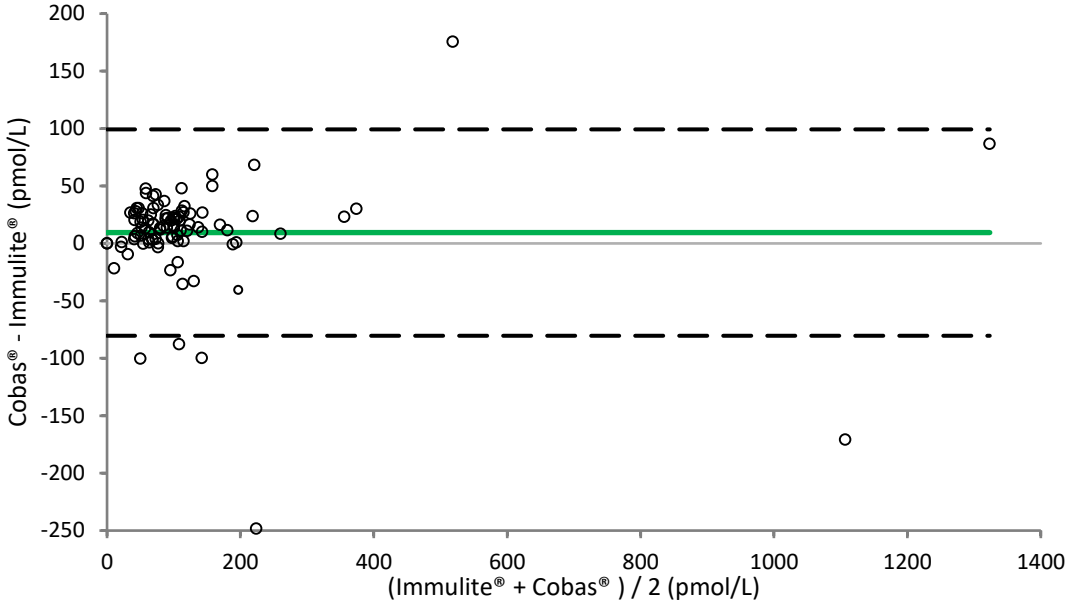


Figure 2.5.2.2: Post-mortem blood insulin Cobas® v Immulite® immunoassays – Bland-Altman difference plot.

Sample	Analyser	Insulin concentration without PEG extraction (pmol/L)	Insulin concentration post-PEG extraction (pmol/L)	Mean (pmol/L)	SD	CV (%)
PM8	Immulite®	1192	1162	1177	21.2	1.80
	Cobas®	1021	1007	1014	9.9	0.98
PM43	Immulite®	348	346	347	1.4	0.41
	Cobas®	100	87	93.5	9.2	9.83
PM94	Immulite®	431	426	428.5	3.5	0.83
	Cobas®	606	603	604.5	2.1	0.35

Table 2.5.2.1: Insulin concentration results for samples PM8, PM43 and PM94, without PEG extraction and post-PEG extraction.

Cases Nº	Vitreous humour glucose (mmol/L)	Vitreous humour sodium (mmol/L)	Blood glucose (mmol/L)	Blood insulin (pmol/L)	Clinical Information (including any known drugs prescribed)
PM1701	< 0.3	142	< 0.3	2100 / 1808	T1DM
PM1702	7.2	138	8.4	83.4	T2DM, hanging, Rx citalopram
PM1703	12.2	141	10.8	26.9	Polypharmacy overdose
PM1704	48.1	147	56.7	70.6	Insulin & citalopram
PM1705	< 0.3	144		410.0	Humlog® (lispro), zopiclone
PM1706	> 48.0	145	56.6	< 14.4	DKA on admission
PM1707	7.8	126		58.4	Found deceased in alley
PM1708	12.2	143	16.1	74.3	Polytrauma (No Rx history)
PM1709	4.6	148		125.0	Bipolar, Rx zopiclone, amitriptyline, and diazepam
PM1710	< 0.3	139		265.0	Oromorph, alendronic acid, co-codamol
PM1711	9.3	142	16.8	76.5	Use of heroin confirmed at scene
PM1712	< 0.3	144		228.0	Head trauma prior to death
PM1713	5.8	146		45.3	T2DM, Rx metformin
PM1714	16.4	142		< 14.4	CO poisoning
PM1715	< 0.3	139	<0.3	321.0	Prostate cancer, naproxen, tamsulosin, and zopiclone
PM1717	68.5	144	87.4	< 14.4	T1DM, DKA on admission
PM1718	< 0.3	145		634.0	T1DM
PM1719	26	138	18.5	26.5	T2DM
PM1720	< 0.3	140		310.2	? history of opiate abuse
PM1721	< 0.3	146	1.8	658.2	T1DM, history of mental health conditions
PM1722	7.8	126		58.4	Found deceased in street
PM1723	12.2	143	16.1	74.3	Poly-trauma (no Rx history)
PM1724	4.6	148		125.0	Bipolar, Rx: sertraline and diazepam
PM1725	< 0.3	139		265.0	Oromorph
PM1726	15.6	142		23.4	History of heroin use
PM1727	< 0.3	144		228.0	Head trauma prior to death
PM1728	< 0.3	145		365.3	Cardiac arrest
PM1729	< 0.3	146		32.8	Shot gun injury
PM1730	No result	143	5.8	136.2	History of ethanol and opiate abuse
PM1731	69.8	158	69.4	< 14.4	T1DM, Rx NovoRapid®, known heroin and methadone user
PM1732	50.1	117		164.1	Overdose (unknown drug(s))
PM1733	< 0.3	144	5.7	184.3	Methadone
PM1734	3.1	143	4.8	67.4	Cardiac arrest
PM1735	24.5	124	36.4	18.5	Polypharmacy overdose

Table 2.5.2.2: Post-mortem sample results with clinical information. Results from vitreous humour glucose, vitreous humour sodium, blood glucose, and blood insulin (from Siemens Immulite® assay).

2.5.3 Discussion: comparison of immunoassay insulin assays – post-mortem blood samples

The two immunoassay platforms show good comparison with a Passing & Bablok fit of 11.24 + 1.036x (Figure 2.5.2.1), representing a good correlation and a relatively small systematic bias of 11.24 pmol/L. The Bland-Altman plot (Figure 2.5.2.2) further support this with a demonstrated bias of 9.45 pmol/L between the two immunoassay methods.

However, this data also includes three samples with considerably differing results for each sample between the immunoassays, PM8, PM43 and PM94. All three samples were re-assayed, and the original findings were confirmed. Re-analysis of the samples confirmed consistency for each analyser, but do not correlate well between the two immunoassay methods. PM94 is discussed in Chapter 5. Samples PM8 and PM43 were both taken from cases where there was no clinical indication that insulin was prescribed. Consequently, this may represent cross-reactivity, such as auto insulin-antibodies (Kim et al., 2011), or the presence of biotin (Samarasinghe et al., 2017). Auto insulin-antibodies can produce hormone-monomer:IgG molecule complexes. These complexes are not biologically active but interfere with immunoassays resulting in falsely raised results (Vaishya et al., 2010). Although immunoassays to assess auto insulin-antibodies are available they are variable in yield, not standardised, and not commonly in use. However, it is possible to account for the auto insulin-antibodies by performing a PEG extraction of samples, similar to the procedure described in Section 2.5.1.6. the PEG extraction of the macro-hormone complex to gain a ‘corrected’ concentration which better reflects the concentration of the bioactive hormone. In the cases of PM8, PM43 and PM94 there was no marked variation between the insulin concentrations without and post-PEG extraction, indicated by CV values < 10% (Table 2.5.2.1).

It is advised by Church et al. (2018) that a PEG extraction is performed on all antemortem samples where the patient presents with hyperinsulinemic hypoglycaemia and with a high insulin/C-peptide ratio. The presence of auto insulin-antibodies will cause falsely increased insulin concentrations (Church et al., 2018). Biotin is available ‘over the counter’ as a health supplement, but some

immunoassays use a streptavidin/biotin-based target molecule binding complex, as such biotin in the sample interferes with the immunoassay (Samarasinghe et al., 2017). These biotin inference has been particularly marked in Roche immunoassays (Trambas et al., 2018).

Although the *t*-test indicates significant statistical difference between the two analytical groups (P = 0.0461) in terms of clinical interpretation there is only relatively small consistent bias of 11.24 pmol/l. When considered against a clinical reference range of 67.1 – 210.0 pmol/l, a bias of 11.24 pmol/l represents 16.7% (of 67.1 pmol/L) and 5.4% (of 210.0 pmol/L) at either extremity of the range. These agree with the antemortem assessment comparison study which, as shown in Section 2.4.3 demonstrated a bias of 10.73 pmol/L, representing 16.0% (of 67.1 pmol/L) and 5.1% (of 210.0 pmol/L) at either end of reference range.

When assessing post-mortem serum samples assayed via immunoassay for insulin concentrations, (as in Table 2.5.2.2) there are a number of interesting cases:

PM1701, this sample was collected from a deceased patient who was prescribed an exogenous insulin, the circumstances of death were an overdose using prescription drugs. Due to the demonstrable increased insulin concentration (2100 pmol/L), the sample was re-analysed post-PEG precipitation, and markedly raised bioavailable insulin was still recorded (1808 pmol/L). The blood insulin result correlates with the low vitreous humour and blood glucose results of < 0.3 mmol/L and indicates inappropriate antemortem exogenous insulin administration. The vitreous humour was analysed using the Siemens Immulite® method and gave an insulin result of 40.1 pmol/L.

PM1704, hyperglycaemia (indicated by both blood and vitreous humour glucose) with depressed insulin (Palmiere, 2015). As the deceased was prescribed insulin this would indicate suboptimal control of diabetes with insufficient administration of prescription.

PM1711, PM1726, and PM1731; hyperglycaemia has been associated with intravenous heroin use (Vallecillo et al., 2018), with the hyperglycaemic state

being induced by hypoxia of brain cells (Solis et al., 2017); this appears to be demonstrated in samples PM1711 and PM1726. In the case of sample PM1731 hyperglycaemia after intravenous heroin is again demonstrated but with added complication that the deceased was a person with T1DM. With blood insulin levels of < 14.4 pmol/L and significantly raised blood and vitreous humour glucose both of > 60 mmol/L this indicates suboptimal diabetes control by the deceased.

PM1714, unfortunately there was insufficient blood sample to determine blood glucose concentrations, but the raised vitreous humour glucose and corresponding supressed blood insulin could be attributed to the carbon monoxide (CO) poisoning, Huang et al. (2022), from a combination of human epidemiologic studies, and reviews of animal studies, suggest that CO poisoning increases the risk of hyperglycaemic crisis in people with diabetes. Although the mechanisms of how this occurs are still not confirmed.

PM1721, clinical history indicates suboptimal diabetes control in T1DM. In this case hypoglycaemic state was confirmed by low vitreous humour and blood glucose concentrations, with raised blood insulin indicating excess administration of exogenous insulin.

2.6 Cross-reactivity of recombinant human insulin, and insulin analogues using differing immunoassay platforms

2.6.1 Methods and materials: cross-reactivity of recombinant human insulin, and insulin analogues using differing immunoassay platforms

Antemortem samples from the sample storage study, collected as per Section 2.2.1, were retained. Twenty of those samples, from patients fasting prior to blood sample collection, with insulin concentrations of < 100 pmol/L, were pooled to create a 'standard' pool of human sera. Separate aliquots of this pool were then spiked with one of five insulin compounds used in the treatment of diabetes, Actrapid® (recombinant human insulin) (Novo Nordisk, Bagsværd, Denmark), Humulin® S (recombinant human insulin) (Eli Lilly & Company, Indianapolis, US), NovoRapid® (insulin aspart) (Novo Nordisk, Bagsværd, Denmark), Humalog® (insulin lispro) (Eli Lilly & Company, Indianapolis, US) and Levemir® (insulin detemir) (Novo Nordisk, Bagsværd, Denmark). All insulin compounds where medical grade, sourced and supplied by the Pharmacy department at NNUH.

The five insulin compounds were spiked in at four concentrations: 250, 500, 750 and 1000 pmol/L. These concentrations were achieved by dilution of stock solutions to 20 nmol/L (Tables 2.6.1.2 to 2.6.1.6), and the 20 nmol/L solutions then diluted as per Table 2.6.1.8.

All dilutions were with Optima® LC–MS grade water, (Thermo Fisher Scientific®, product reference: 10095164).

The five insulin compounds were chosen, from the over twenty different insulin compounds, available for the treatment of diabetes at the start of this experiment (Joint Formulary Committee, 2018). These five insulin compounds include recombinant human insulin, which have molecular weights the same as endogenous insulin, and insulin analogues differing from the human insulin structure by varying in amino acid numbers (Table 2.6.1.1).

Along with these five insulin compounds, bovine insulin (Sigma-Aldrich®, Massachusetts, US), (Sigma-Aldrich®, product reference: I0516) was also as analysed at the same concentrations. Dilution from the stock concentration of 1744 µmol/L to 20 nmol/L was a per Table 2.6.1.7.

Each insulin compound and spike concentration aliquot were analysed 20 times and the mean percentage recovery was calculated after analysis on both Immulite® and Cobas® platforms.

Trade name / Compound name	Recombinant human insulin / insulin analogue	Molecular weight	Difference from endogenous insulin (Kramer et al., 2021)
Actrapid®/ human insulin	Human	5807.6	No primary structure difference
Humulin® S / human insulin	Human	5807.6	No primary structure difference
NovoRapid® / insulin aspart	Analogue	5829.7	1 amino acid difference proline substituted for aspartic acid at β28 (Figure 1.2.2.1)
Humalog® / insulin lispro	Analogue	5811.7	Lysine and proline swapped at β28 and 29 (Figure 2.6.1.1)
Levemir® / insulin detemir	Analogue	5914.8	Threonine removed at β30 and molecule acylated with a 14-carbon fatty acid (Figure 2.6.1.2)

Table 2.6.1.1: Five insulin compounds used for the treatment of diabetes, used for the cross-reactivity of immunoassay experiments.

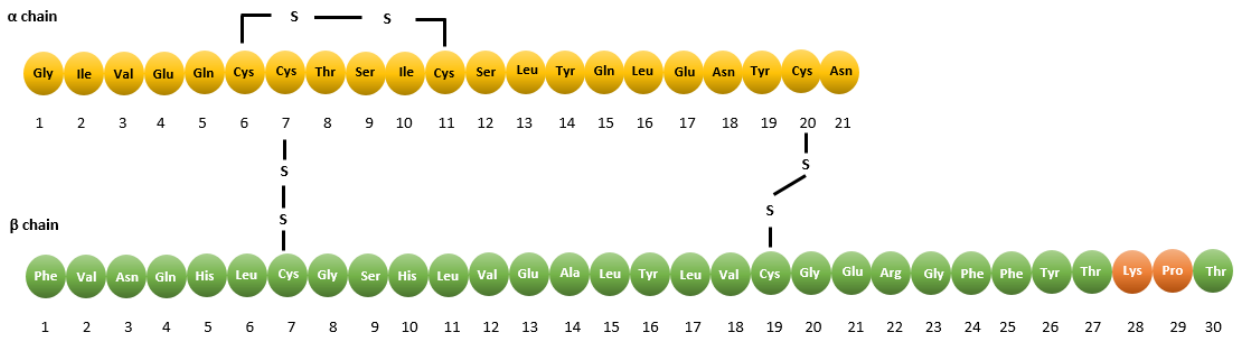


Figure 2.6.1.1: Amino acid sequence of α and β chains of insulin lispro, lysine and proline swapped at positions β28 and 29.

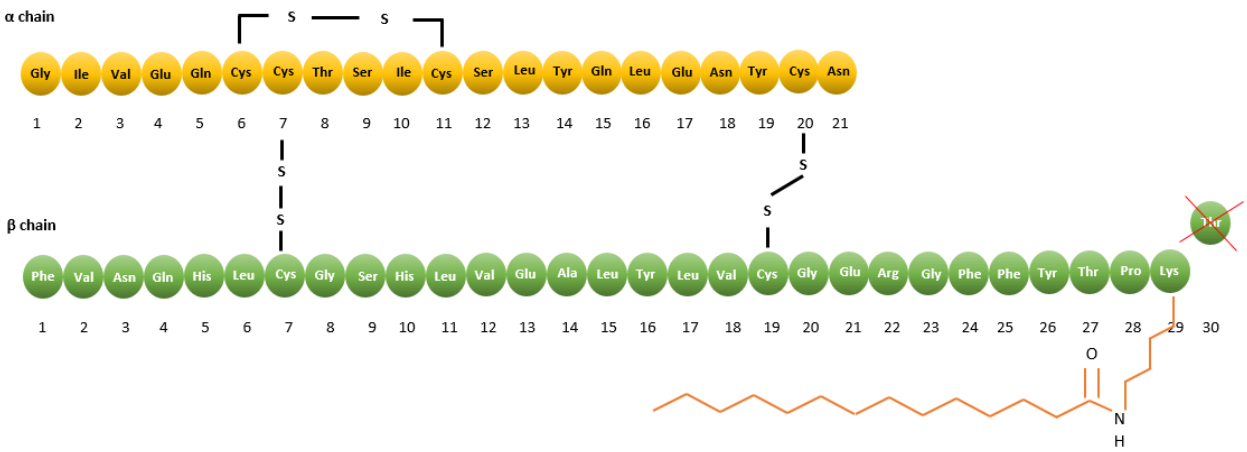


Figure 2.6.1.2: Amino acid sequence of α and β chains of insulin detemir, threonine removed at β30 and molecule acylated with a 14-carbon fatty acid.

Actrapid® stock solution	602.7	µmol/L

Starting concentration (µmol/L)	Dilution	Concentration (µmol/L)	Concentration (nmol/L)
602.7	1 in 300	2.009	2008.9
2.009	1 in 100.5	0.020	20.0

Table 2.6.1.2: Dilution of Actrapid® stock solution (602.7 µmol/L) to 20 nmol/L solution.

Humulin® S stock solution	602.9	µmol/L

Starting concentration (µmol/L)	Dilution	Concentration (µmol/L)	Concentration (nmol/L)
602.9	1 in 300	2.010	2009.6
2.010	1 in 100.5	0.020	20.0

Table 2.6.1.3: Dilution of Humulin® S stock solution (602.9 µmol/L) to 20 nmol/L solution.

NovoRapid® stock solution	600.4	µmol/L

Starting concentration (µmol/L)	Dilution	Concentration (µmol/L)	Concentration (nmol/L)
600.4	1 in 300	2.00	2001.3
2.0	1 in 100.0	0.020	20.0

Table 2.6.1.4: Dilution of NovoRapid® stock solution (600.4 µmol/L) to 20 nmol/L solution.

Humalog® stock solution	602.2	µmol/L

Starting concentration (µmol/L)	Dilution	Concentration (µmol/L)	Concentration (nmol/L)
602.2	1 in 305	1.975	1974.5
1.975	1 in 98.5	0.020	20.0

Table 2.6.1.5: Dilution of Humalog® stock solution (602.2 µmol/L) to 20 nmol/L solution.

Levemir® stock solution	591.7	µmol/L

Starting concentration (µmol/L)	Dilution	Concentration (µmol/L)	Concentration (nmol/L)
591.7	1 in 305	1.940	1940.1
1.940	1 in 97	0.020	20.0

Table 2.6.1.6: Dilution of Levemir® stock solution (591.7 µmol/L) to 20 nmol/L solution.

Bovine insulin stock solution	1744.0	µmol/L

Starting concentration (µmol/L)	Dilution	Concentration (µmol/L)	Concentration (nmol/L)
1744	1 in 100	17.44	17440
17.44	1 in 80	0.218	218
0.218	1 in 10.9	0.02	20

Table 2.6.1.7: Dilution of bovine insulin stock solution (1744.0 µmol/L) to 20 nmol/L solution.

Insulin compound concentration (nmol/L)	Dilution	Insulin compound concentration (pmol/L)
20	1 in 80	250
20	1 in 40	500
20	1 in 26.7	750
20	1 in 20	1000

Table 2.6.1.8: Dilution of 20 nmol/L insulin compound solution to produce solutions with concentrations of: 250, 500, 750, and 1000 pmol/L.

2.6.2 Results: cross-reactivity of recombinant human insulin, and insulin analogues using differing immunoassay platforms

Human insulin	250 pmol/L	500 pmol/L	750 pmol/L	1000 pmol/L	Mean % Recovery
Immulite®	83	94	85	91	88.3
Cobas®	101	105	112	108	106.5
Actrapid®	**250 pmol/L**	**500 pmol/L**	**750 pmol/L**	**1000 pmol/L**	
Immulite®	79	78	86	74	79.3
Cobas®	118	125	104	126	118.3
Humulin® S	**250 pmol/L**	**500 pmol/L**	**750 pmol/L**	**1000 pmol/L**	
Immulite®	68	75	79	74	74.0
Cobas®	135	132	141	126	133.5
NovoRapid®	**250 pmol/L**	**500 pmol/L**	**750 pmol/L**	**1000 pmol/L**	
Immulite®	10	9	13	15	11.8
Cobas®	12	8	11	17	12.0
Humalog®	**250 pmol/L**	**500 pmol/L**	**750 pmol/L**	**1000 pmol/L**	
Immulite®	6	4	8	12	7.5
Cobas®	4	8	4	4	5
Levemir®	**250 pmol/L**	**500 pmol/L**	**750 pmol/L**	**1000 pmol/L**	
Immulite®	1	2	4	1	2.0
Cobas®	15	12	11	12	12.5
Bovine insulin	**250 pmol/L**	**500 pmol/L**	**750 pmol/L**	**1000 pmol/L**	
Immulite®	0	8	6	1	3.8
Cobas®	11	22	16	15	16.0

Table 2.6.2.1: Mean % recovery from 20 replicates of recombinant human insulin, and insulin analogues from spiked antemortem. Bovine insulin to indicate cross-reactivity with other insulin forms but is not commonly used in treatment of diabetes.

2.6.3 Discussion: cross-reactivity of recombinant human insulin, and insulin analogues using differing immunoassay platforms

Table 2.6.2.1 demonstrates there is a significant difference in the results generated by the two immunoassay platforms for corresponding concentrations of recombinant human insulin ($P = 0.0117$). In antemortem assessment of endogenous insulin this may affect patient treatment pathways, particularly at the higher concentrations. For post-mortem blood analysis this adds another variable to be considered when interpreting results for human insulin by immunoassay, particularly in cases where only endogenous insulin was implicated in the circumstances of death and/or clinical history.

From the data it can also be seen there is poor detection of bovine insulin by both immunoassay platforms, with mean recoveries of 3.75% and 16.0% (Immulite® and Cobas®, respectively) the results were also significantly different when using the recombinant human insulin as the baseline ($P = 0.0001$ in both cases). This is to be expected, as although both human and bovine insulins are structurally similar, having the same tertiary structure, their primary sequences differ by 3 amino acids (Mauri et al., 2015). Bovine insulin, which is rarely found in human samples as it is not used in the treatment of diabetes, was used in the project as internal standard for the mass spectrometry method (Section 3.3.1.4). However, in this experiment bovine insulin was assessed to further demonstrate that there would be representable differences between differing insulin compounds. This pattern is also seen with the insulin analogues which have one amino acid or greater difference from human insulin: NovoRapid® (insulin aspart), Humalog® (insulin lispro) and Levemir® (insulin detemir). With these compounds demonstrating experimental percentage recoveries of $< 15\%$ of the actual concentration present in the samples, and $P = 0.0001$ when compared to the recombinant human insulin.

When considering the recombinant human insulin compounds; Actrapid® and Humulin® S, these have identical amino acid primary structures to human insulin and show greater recovery in the spike experiments with mean recoveries of 79.25 and 118.25% (Actrapid®) and 74.0 and 133.5% (Humulin® S), compared to human insulin mean recoveries of 88.3 and 106.5%. These results do demonstrate the need to assess analyser performance, and the clinical relevance

of any bias when assessing post-mortem overdose cases. When considering the mean recoveries at 500 pmol/L the Immulite® demonstrates a negative bias of 16% for Actrapid® and 20% for Humulin® S, whereas the Cobas® demonstrates positive bias for Actrapid® and Humulin® S, 20% and 26% respectively.

As well as the demonstrated differing analytical bias, when comparing the mean recoveries of exogenous insulin compounds, there is significant difference between the analysers. In the case of Levemir® 2.0% of the compound was recovered by the Siemens Immulite® and 12.5% by the Roche Cobas® method ($P = < 0.0001$). There are also significant differences in the recoveries between compounds by the same analyser, e.g., NovoRapid® and Levemir®, 11.8% and 2.0% ($P = 0.0007$) by the Siemens Immulite®.

The differences in the recovery from endogenous insulin, and recombinant human insulin compounds, with the same amino acid primary structures could be related to differences in the three-dimensional (tertiary) structures affecting antibody binding. For example, exogenous insulin formulations contain antimicrobial preservatives, and these have been demonstrated to influence the insulin tertiary structure (Wollmer et al., 1987).

Chapter 3: Mass spectrometry analysis of insulin compounds

3.1 Introduction

Mass spectrometry methods are commonplace in clinical laboratories, with techniques such as MALDI–ToF, LC–MS/MS, and gas chromatography–mass spectrometry (GC–MS), being used for a range of applications, such as detection of SARS-CoV2 virus (Rocca et al., 2020), steroid screens (Boggs et al., 2016), and opiate identification (Meadway et al., 2002). However, HRAM LC–MS analysers are far less common in NHS laboratories, largely due to cost. In clinical biochemistry laboratories LC–MS/MS systems are generally used for the analysis of compounds such as 25OH-vitamin D, and are both specific and sensitive, whereas HRAM LC–MS analysers are utilised for the accurate mass determination of larger molecules (Pitt, 2009). LC–MS/MS analysis of antemortem insulin compounds has been shown to be an advantage in the investigation of dysglycaemia-related disorders (Ackermans et al., 2022).

At the start of the study, the NNUH had a single available mass spectrometry system, the Varian 320–MS® LC–MS/MS, this instrument model has been used in published studies for the analysis of insulin (Sundararajan et al., 2010). The purchase and installation of the Waters® Xevo® G2-XS QToF (Waters Corporation, Milford, US) in early 2020, lead the to the development of the novel HRAM LC–MS method, but unfortunately, this period coincided with the COVID-19 pandemic in the UK, which had the negative effect of slowing down the development period, as NHS pressures took precedence.

3.2 Insulin LC–MS/MS mass spectrometry

3.2.1 Methods and materials: insulin LC–MS/MS mass spectrometry

A solution of recombinant human insulin (1377.5 pmol/L) was produced from a stock of 17.2 μmol/L (Sigma-Aldrich®, product reference: I-034), diluted in Optima® LC–MS water (Thermo Fisher Scientific®, product reference: 10095164) as per Table 3.2.2.1. The 1377.5 pmol solution was infused directly into the Varian 320–MS® (Agilent Technologies, Santa Clara, US) mass spectrometer ion source. Therefore, not via the LC unit of the LC–MS/MS analyser, this removes variations in analysis that may occur due to the LC section of the analyser, such as column changes, buffer polarity and concentrations (Honour, 2011).

Starting concentration (μmol/L)	Dilution	Concentration (μmol/L)	Concentration (nmol/L)	Concentration (pmol/L)
17.2	1 in 100	0.1722	172.2	
0.1722	1 in 100	0.001722	1.722	1721.9
0.001722	4 in 5	0.001378	1.3775	1377.5

Table 3.2.2.1: Dilution of stock recombinant human insulin to 1377.5 pmol/L

Scanning was performed using quadrupole 3, and set to scan for m/z ratios between 100 and 1250 m/z. The MS/MS parameters are presented in Table 3.2.2.2.

Varian 320–MS® LC–MS/MS parameters	
Needle voltage positive	5.0 kV
Spray shield voltage positive	600 V
Spray chamber temperature	50 °C
Drying gas temperature	350 °C
Collision-induced dissociation gas pressure	2 mTorr
Nebulising gas pressure	45 psi
Drying gas pressure	22 psi
Dwell time	0.333 s
Cone Voltage	50 V
Collision energy	40 eV

Table 3.2.2.2: Varian 320–MS® settings used for the direct infusion of recombinant human insulin solution.

3.2.2 Results: insulin LC–MS/MS mass spectrometry

The total ion chromatogram (TIC) spectra from continuous infusion of the insulin solution is shown in Figure 3.2.2.1, it is possible to view the mass spectra that generate these TIC peaks, and these are presented in Figures 3.2.2.2, 3.2.2.3, 3.2.2.4 and 3.2.2.5.

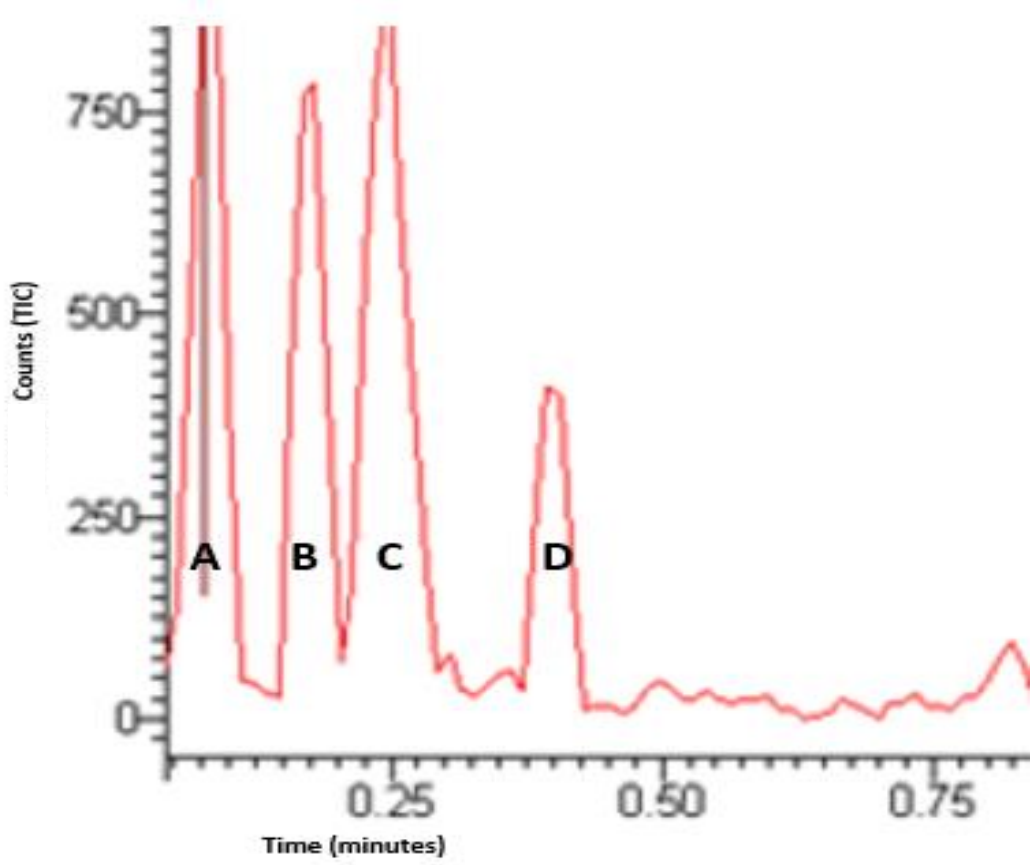


Figure 3.2.2.1: The total ion chromatogram generated from recombinant human insulin standard using direct infusion into quadrupole 3, Varian 320–MS®.

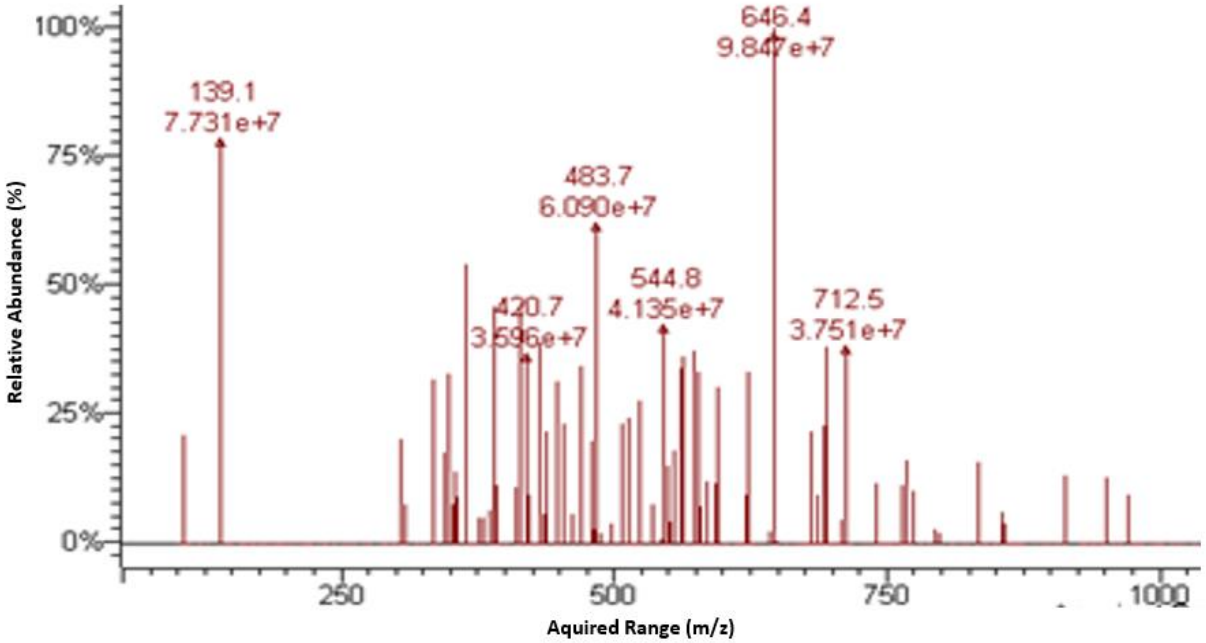


Figure 3.2.2.2: MS spectra generated from infusion of recombinant human insulin directly into MS/MS - peak A in Figure 3.2.2.1.

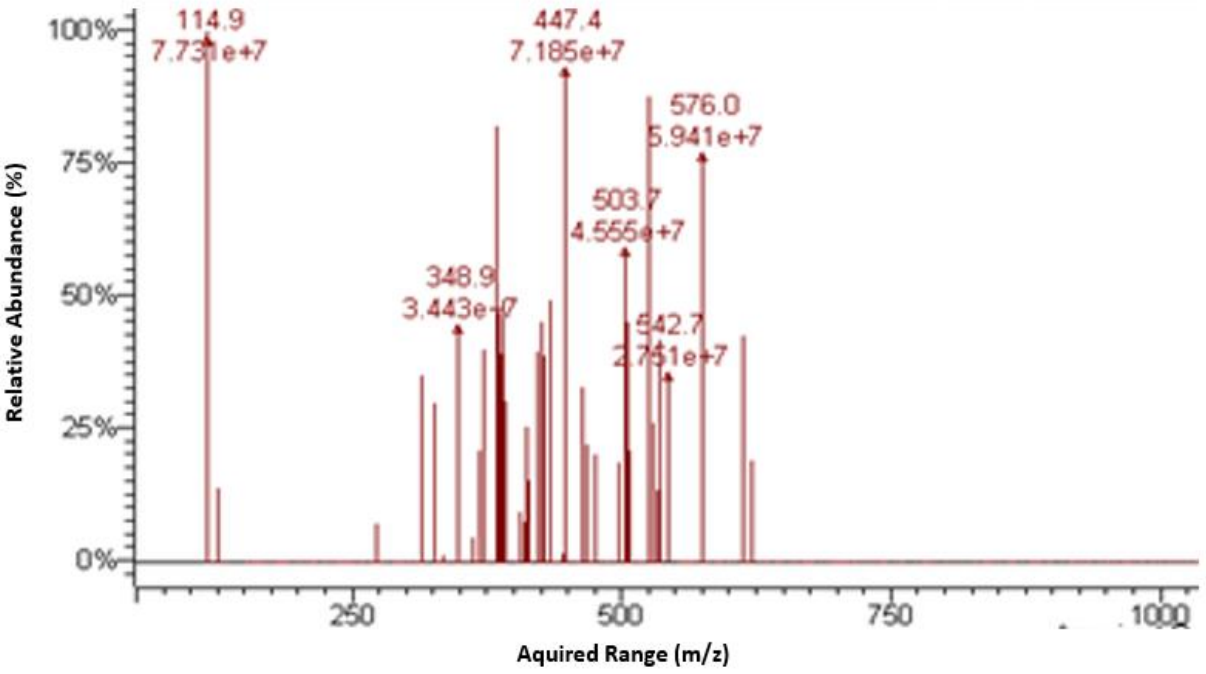


Figure 3.2.2.3: MS spectra generated from infusion of recombinant human insulin directly into MS/MS - peak B in Figure 3.2.2.1.

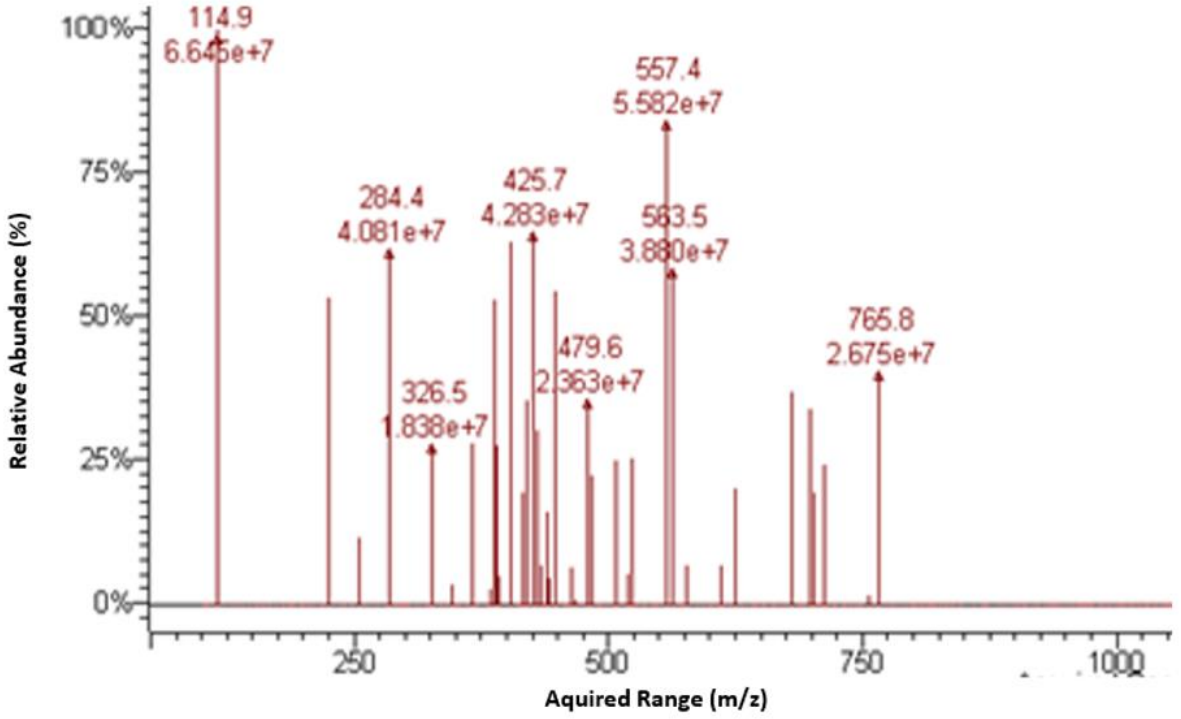


Figure 3.2.2.4: MS spectra generated from infusion of recombinant human insulin directly into MS/MS - peak C in Figure 3.2.2.1.

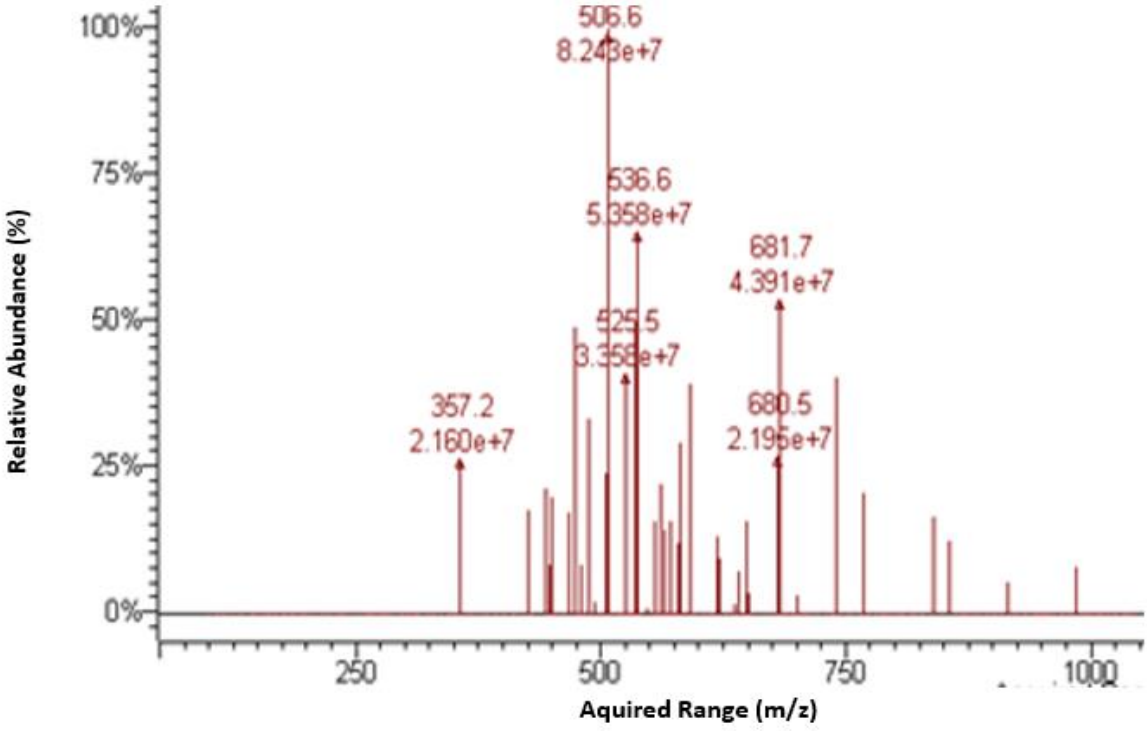


Figure 3.2.2.5: Spectra generated from infusion of recombinant human insulin directly into MS/MS - peak D in Figure 3.2.2.1.

3.2.3 Discussion: insulin LC–MS/MS mass spectrometry

The m/z spectra from Section 3.2.2 (Figures 3.2.2.2 to 3.2.2.5) do not demonstrate detection of the infused insulin from multiple assay runs of insulin solution infusion, consequently the sensitivity of the analyser was found to be insufficient for this analysis. The infused concentration 1377.5 pmol/L is more than five times higher than the upper limit of the clinical reference range for endogenous insulin. The Varian 320–MS®, was a relatively old instrument at the start of the experiments (> 15 years old) and these findings indicate the analyser had limited sensitivity and cast doubt that this system was going provide a robust method to generate data for a post-mortem insulin service.

A further consideration from this data was whether to continue with the development of a LC–MS/MS method or move to a higher resolution HRAM LC–MS analyser method. There are documented disadvantages of using LC–MS/MS in protein analysis (Karpievitch et al., 2010), partly due to the high molecular masses of proteins which result in poor ionisation efficiency in MS/MS analysis. Multiple charges can also occur across the protein molecule under ionisation, leading to incomplete m/z identification (Bronsema et al., 2012). Along with these the limits of LC–MS/MS analysis, the significantly increased resolution (Birhanu, 2023), and increased m/z detection range of HRAM LC–MS analysis results in superior detection of proteins and large molecules (Franke et al., 2020). As such the LC–MS/MS method development was not continued, with the development of an HRAM LC–MS insulin method undertaken from this point.

3.3 HRAM LC–MS analysis of insulin compounds

3.3.1 Methods and materials: HRAM LC–MS analysis of insulin compounds

3.3.1.1 Analytical set up of HRAM LC–MS analysis

The HRAM LC–MS analysis of the post-mortem vitreous humour and blood samples were analysed using the Waters® G2 Xevo® XS QTof analyser, with the settings as follows:

The Acquity® I Class UPLC chromatographic system (Waters Corporation) was equipped with a Waters® Acquity® UPLC HSC C18 column (150 mm × 2.1 mm, 1.8 µm), (Waters Corporation, product reference: 186003534) maintained at 50 °C. The mobile phases consisted of: Optima® LC–MS water (Thermo Fisher Scientific®, product reference: 10095164) with 5 mM ammonium formate (Thermo Fisher Scientific®, product reference: 014517.18) (mobile phase A), and LC–MS grade acetonitrile (Thermo Fisher Scientific®, product reference: 047138.M1) with 0.1% formic acid (Thermo Fisher Scientific®, product reference: 270480250) (mobile phase B). Runtime was set at 5 minutes and sample injection volume was 5 µL.

The Waters® Xevo® G2 XS Q-Tof mass spectrometer system equipped with an ESI source, was coupled to the Waters® Acquity® I class UPLC chromatographic system (Waters Corporation).

The settings for the ion source were as per Table 3.3.1.1.1.

Waters® G2 Xevo® XS QTof ion source parameters	
Ionisation mode	Positive
Capillary voltage	0.8 kV
Sampling cone voltage	25 V
Extraction cone voltage	± 4.0 V
ESI source temperature	149 °C
Desolvation temperature	581 °C
Cone gas flow	19 L/h
Desolvation gas flow	793 L/h
Collision gas flow	0.5 mL/min
Scan time	0.100 s
Collision energy: Low-energy scan	6.0 eV
Collision energy: High-energy scan	10 – 40 eV

Table 3.3.1.1.1: Waters® G2 Xevo® XS QTof ion source settings used for HRAM LC–MS analysis of insulin compounds.

A leucine-enkephalin solution (3 ml of 2 ng/µL, 27 ml acetonitrile/water 50:50, v/v (Thermo Fisher Scientific®, product reference: 047138.M1 and Thermo Fisher Scientific®, product reference: 10095164) was used to ensure reproducibility and mass accuracy detection during acquisition period, as a 'lock-mass' control (m/z 556.2766 in ESI+). The MS calibration was performed by sodium formate solution (Thermo Fisher Scientific®, product reference: A17813.30), 0.5% w/v in acetonitrile/water (Thermo Fisher Scientific®, product reference: 047138.M1 and Thermo Fisher Scientific®, product reference: 10095164).

3.3.1.2 HRAM LC–MS data analysis

UNIFI® 1.9.4 software and UNFI® large molecule licence (Waters Corporation) was used in the processing of the raw analytical data generated by the HRAM LC–MS instrument as per parameters in Table 3.3.1.2.1.

Waters® G2 Xevo® XS QTof parameters for peak detection	
m/z range	50 – 5500
Extract ion chromatogram window	0.05 Da
Intensity threshold	1000 counts
Mass window	0.05
Retention time window	0.10 min

Table 3.3.1.2.1: Waters® G2 Xevo® XS QTof peak detection settings used for HRAM LC–MS analysis of insulin compounds.

3.3.1.3 Preparation of calibrators for insulin analysis

Insulin aspart (NovoRapid®) calibrators to generate a standard curve for the quantification of the compound in samples, were produced by dilution of a stock solution (600.4 µmol/L) as described in Table 2.6.1.4, to a concentration of 20 nmol/L.

From this 20 nmol/L solution, further dilutions were performed to produce calibrator concentrations of: 200, 400, 600, 800, 1000, 1200, 1400, 1600, 1800 and 2000 pmol/L (Table 3.3.1.3.1).

As per Section 2.6.1, all dilutions were performed using Optima® LC–MS grade water (Thermo Fisher Scientific®, product reference: 10095164).

Starting insulin aspart concentration (nmol/L)	Dilution	Calibrator concentration (pmol/L)
20.0	1 in 100	200
20.0	1 in 50	400
20.0	3 in 100	600
20.0	1 in 25	800
20.0	1 in 20	1000
20.0	3 in 50	1200
20.0	7 in 100	1400
20.0	2 in 25	1600
20.0	9 in 100	1800
20.0	1 in 10	2000

Table 3.3.1.3.1: Dilution of 20 nmol/L solution of insulin aspart to produce calibrator concentrations between 200 and 2000 pmol/L.

3.3.1.4 HRAM LC–MS analysis bovine insulin

To achieve accurate and precise quantification of insulin compounds, an internal standard is required (Tan and Awaiye, 2013), the ratio of the target analyte and internal standard responses is used to create a calibration curve and quantify unknown samples (Fouque, et al., 2018). By using the ratio of these responses, rather than solely the target analyte response to quantify, any sample-to-sample variations in pre-analytical and analytical conditions is corrected for (Fouque, et al., 2018). Any internal standard for HRAM LC–MS analysis is required to be structurally similar to the target compound, but also a compound that would not be present in the sample prior to addition as an internal standard. This is so there is a known, consistent, amount of internal standard in each sample.

For HRAM LC–MS analysis of recombinant human insulin and insulin analogues, bovine insulin is considered a good internal standard (Egan et al., 2022). With a molecular weight 5733.5 Da it is similar to human insulin (5808 Da) (Olsen et al., 2022), but is structurally distinct from human insulin, and as previously commented on, is not commonly used in the treatment of diabetes.

A stock solution of 499.7 pmol/L of bovine insulin, (Sigma-Aldrich®, product reference: I0516), was produced to use as an internal standard, to be added to each calibrator and sample. This stock solution was produced by multiple dilutions using Optima® LC–MS grade water (Thermo Fisher Scientific®, product reference: 10095164) as per Table 3.3.1.4.1.

Starting concentration (µmol/L)	Dilution	Concentration (µmol/L)	Concentration (nmol/L)	Concentration (pmol/L)
1744	1 in 100	17.44	17440	
17.44	1 in 100	0.1744	174.4	
0.1744	1 in 100	0.001744	1.744	1744.0
0.001744	1 in 3.45	0.000500	0.500	499.7

Table 3.3.1.4.1: Dilution of bovine insulin stock solution of to produce internal standard solution of 499.7 pmol/L.

3.3.1.5 Lower limit of quantification of the quantitative insulin aspart HRAM LC–MS method

To assess the lower limit of quantification (LLOQ) there are two relevant accepted methods; for the assessment of bioanalytical methods the LLOQ can be defined as the lowest mean concentration of replicates with a CV below 20% (Moein et al., 2017). And particularly when assessing LLOQ of mass spectrometry-based methods, the lowest analyte concentration where the analyte signal to noise (S/N) ratio is equal to/greater than 10:1 (UKAS, 2023).
To assess the LLOQ of the method, sample PM1701 quantified by the insulin aspart method discussed in Section 3.5.3 to have a concentration of 1326.4 pmol/L, was diluted via serial 1:2 dilutions from 1326.4 to 10.4 pmol/L.
All dilutions were performed using Optima® LC–MS grade water (Thermo Fisher Scientific®, product reference: 10095164).

Each concentration in the dilution series was analysed using the insulin aspart method in replicates of 10, and the CV for each concentration level calculated, as explained in Section 2.1. Once the CV for these dilutions were calculated, the lowest insulin aspart concentration with a CV value less than 20% was then further diluted in serial dilutions of 19:20. Each sample concentration was analysed 10 times, and the CV and S/N ratio were calculated.

The S/N ratio was calculated from, the analyte signal, the change of detected response due to the presence of the target analyte. And noise, the fluctuation in the background signal of the mass spectrometry analyser (Muddiman et al., 1997). An example of analyte signal and noise is presented in Figure 3.3.1.5.1.

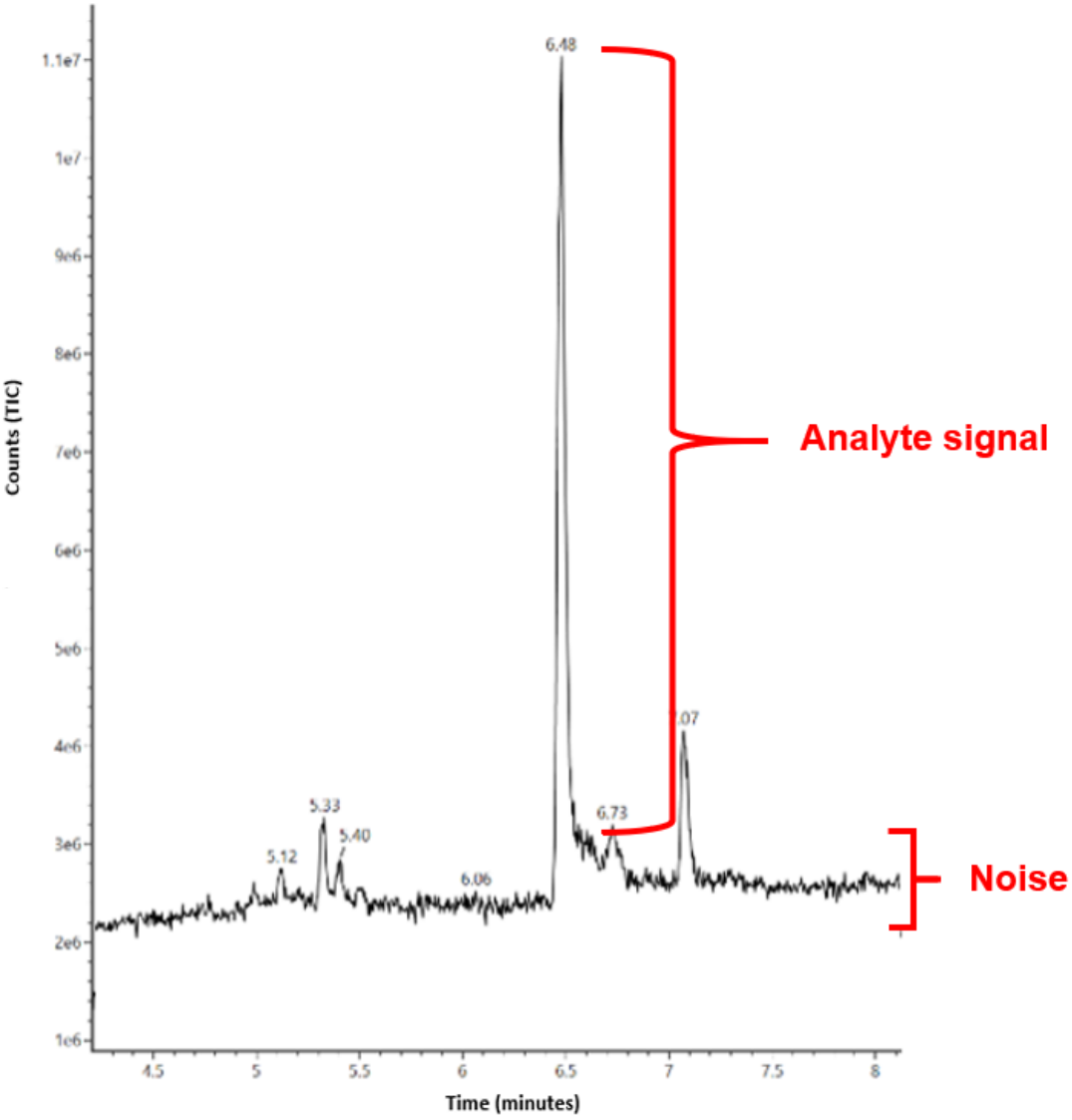


Figure 3.3.1.5.1: HRAM LC–MS chromatogram of insulin aspart indicating analyte signal and noise in mass spectrometry analysis.

3.3.2 Results: HRAM LC–MS analysis of insulin aspart

3.3.2.1 HRAM LC–MS peak and detection confirmation of insulin aspart, and bovine insulin

The HRAM LC–MS insulin method was tested by running a 600.4 pmol/L calibrator of insulin aspart, diluted 1:5 with Optima® LC–MS water (Thermo Fisher Scientific®, product reference: 10095164). The MS results were generated using the Waters® Xevo® G2 XS QTof analyser, running the method quoted in Sections 3.3.1.1 and 3.3.1.2.

Detection of insulin aspart and bovine insulin by the HRAM LC–MS method is demonstrated in Figures 3.3.2.1.1 and 3.3.2.1.4, with confirmed TIC peaks at 6.48 min (6 min 29 sec) for insulin aspart, and 6.34 min (6 min 21 sec) for bovine insulin. The m/z, and intensities of these m/z, that contribute to the TIC peaks can be identified as seen in Figures 3.3.2.1.2 and 3.3.2.1.5. With further investigation of the $(M+4H)^{4+}$ peak shown in Figures 3.3.2.1.3 and 3.3.2.1.6

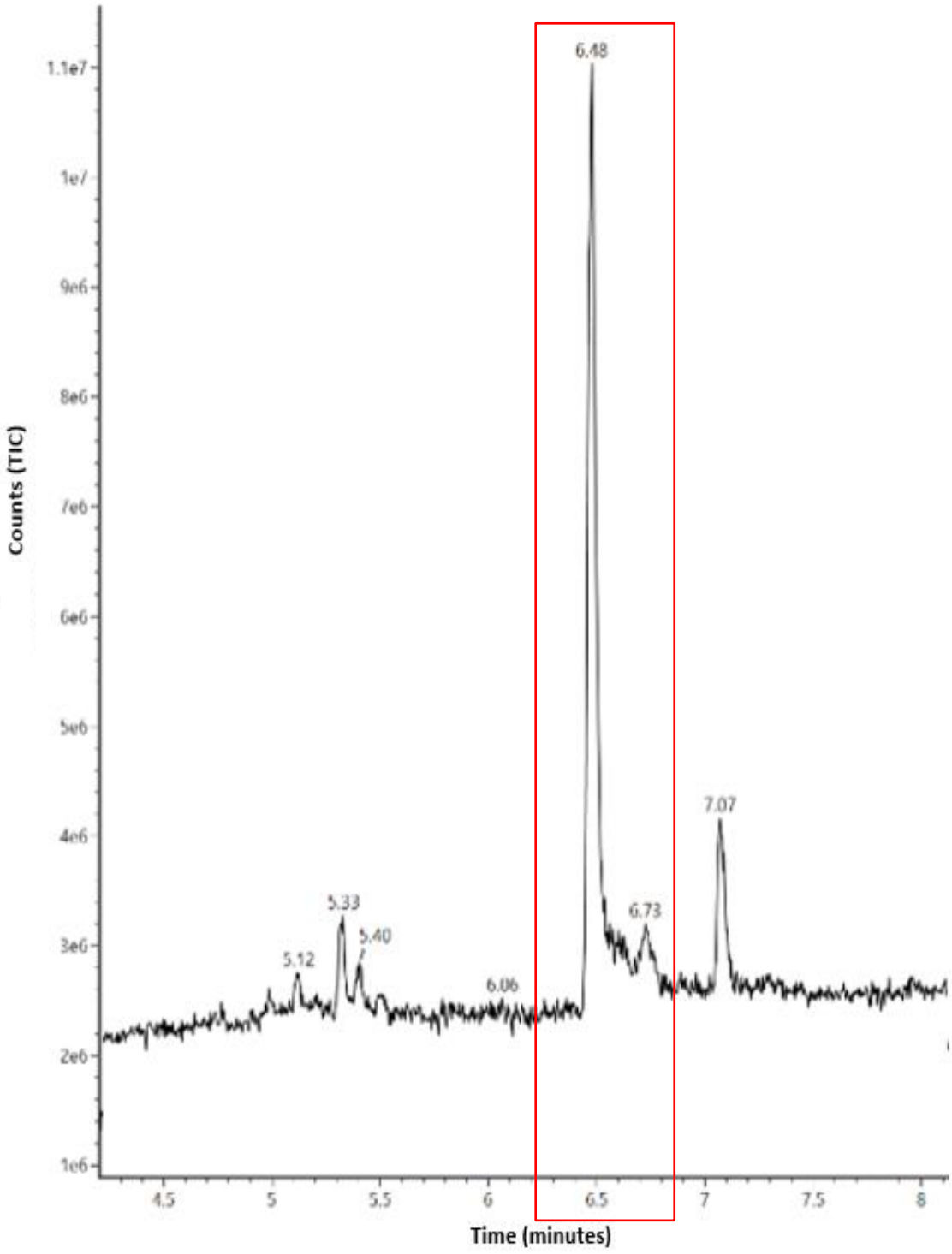


Figure 3.3.2.1.1: Total ion chromatogram generated from insulin aspart, by HRAM LC–MS analysis, indicating a LC retention time of 6.48 min (6 min 29 sec).

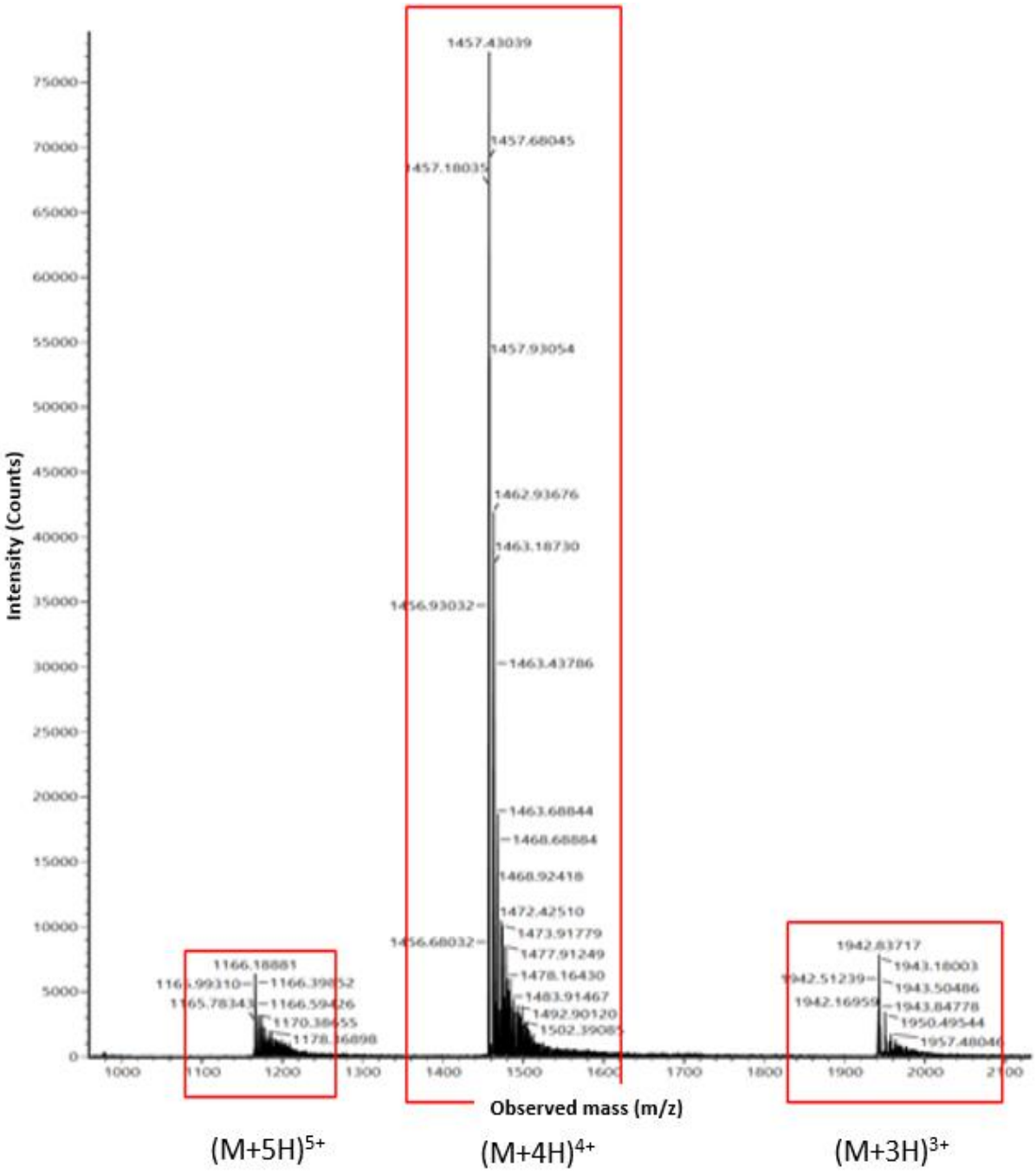


Figure 3.3.2.1.2: HRAM LC–MS full scan of m/z 1000 to 2100 of insulin aspart. Mass spectrum acquired under the chromatographic elution profile at time period 6.48 min (6 min 29 sec). The insets show the narrow mass range around the M+XH^{X+} precursor charge state for the three charge variants, 5+, 4+ and 3+.

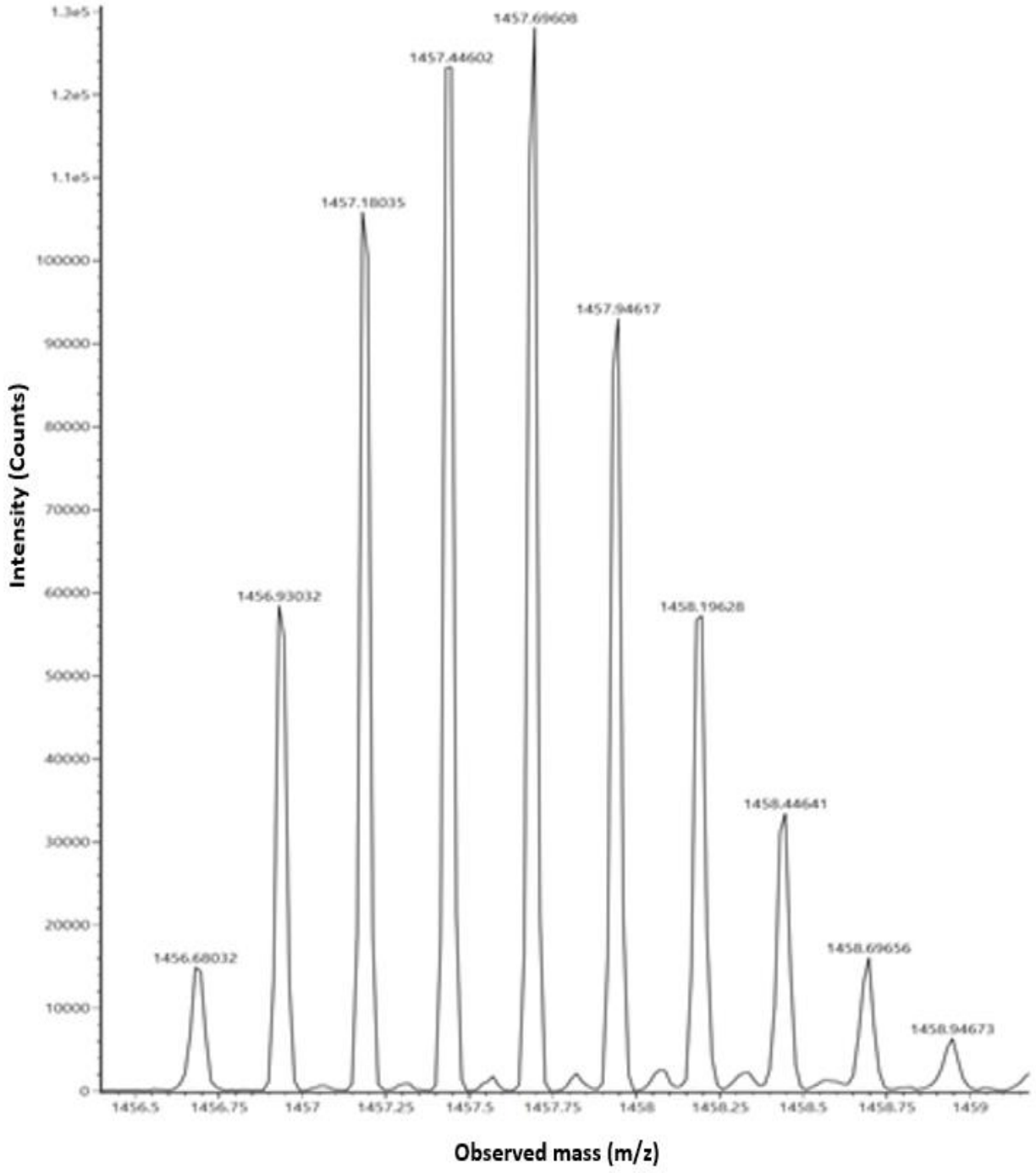


Figure 3.3.2.1.3: HRAM LC–MS mass spectrum of m/z 1456.5 to 1459 of insulin aspart, indicating the $(M+4H)^{4+}$ spectra.

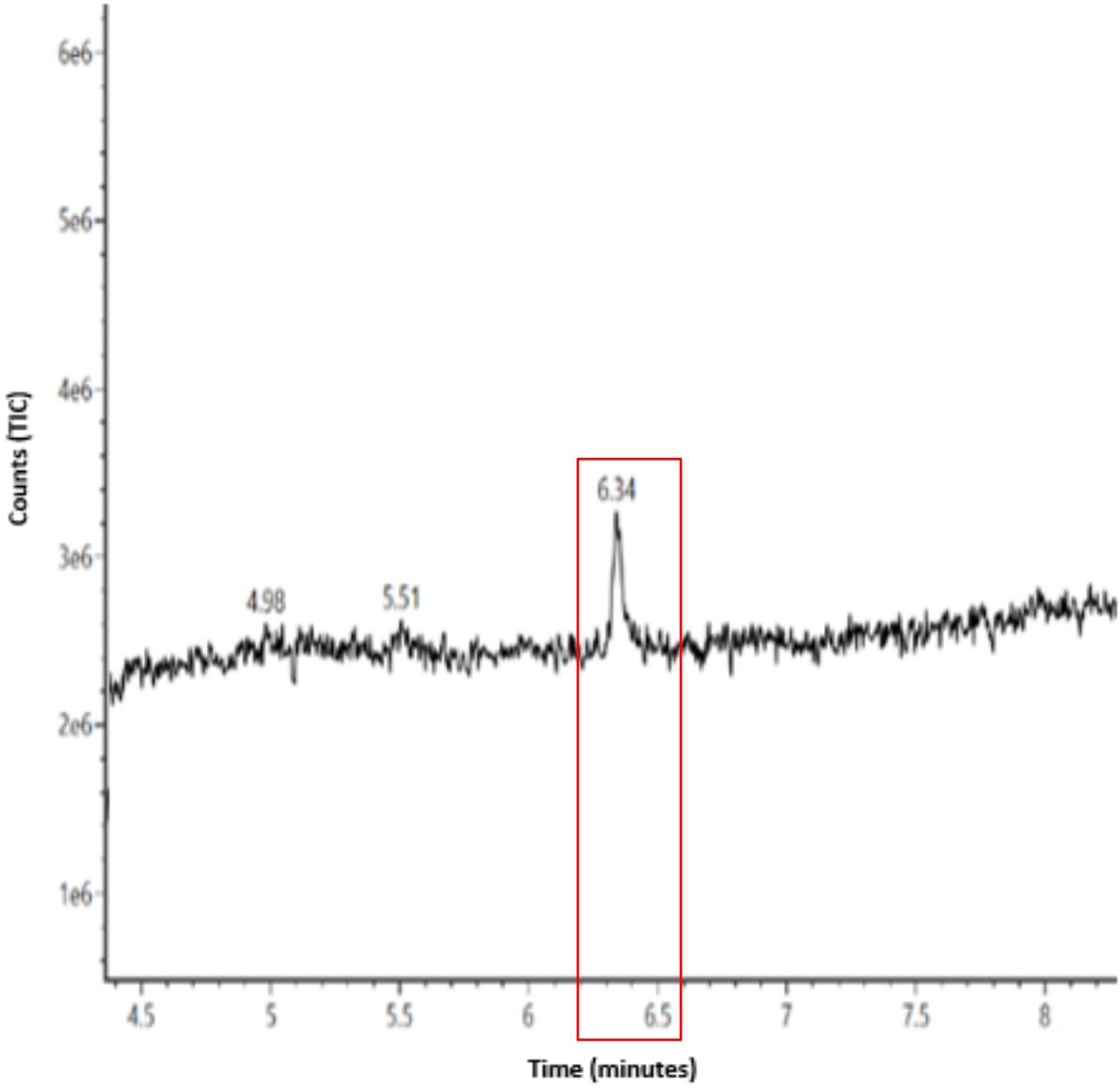


Figure 3.3.2.1.4: Total ion chromatogram generated from bovine insulin, by HRAM LC–MS analysis, indicating a LC retention time of 6.34 min (6 min 21 sec).

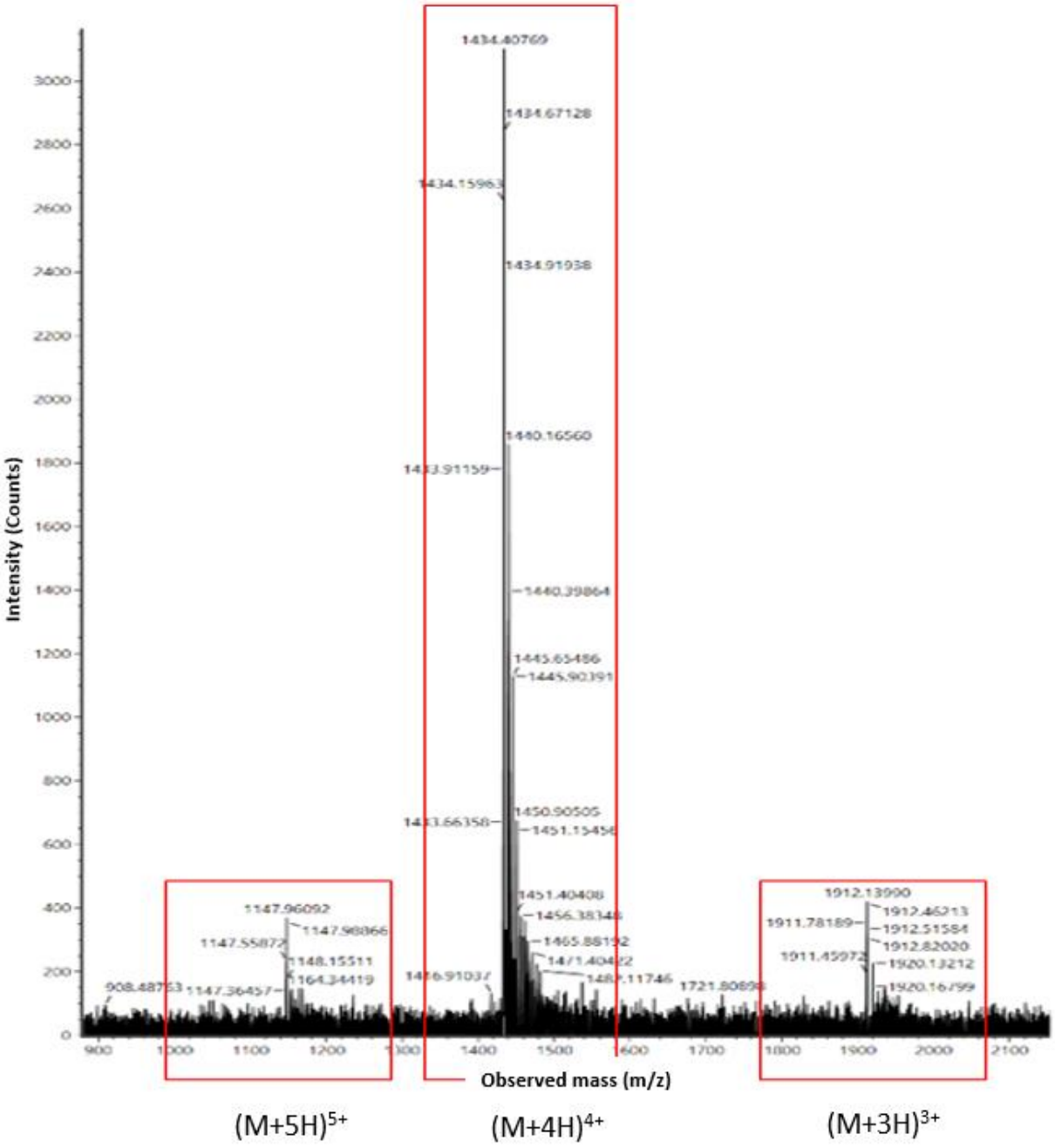


Figure 3.3.2.1.5: HRAM LC–MS full scan of m/z 900 to 2100 of bovine insulin. Mass spectrum acquired under the chromatographic elution profile at time period 6.34 min (6 min 21 sec). The insets show the narrow mass range around the M+XH^{X+} precursor charge state for the three variants, 5+, 4+ and 3+.

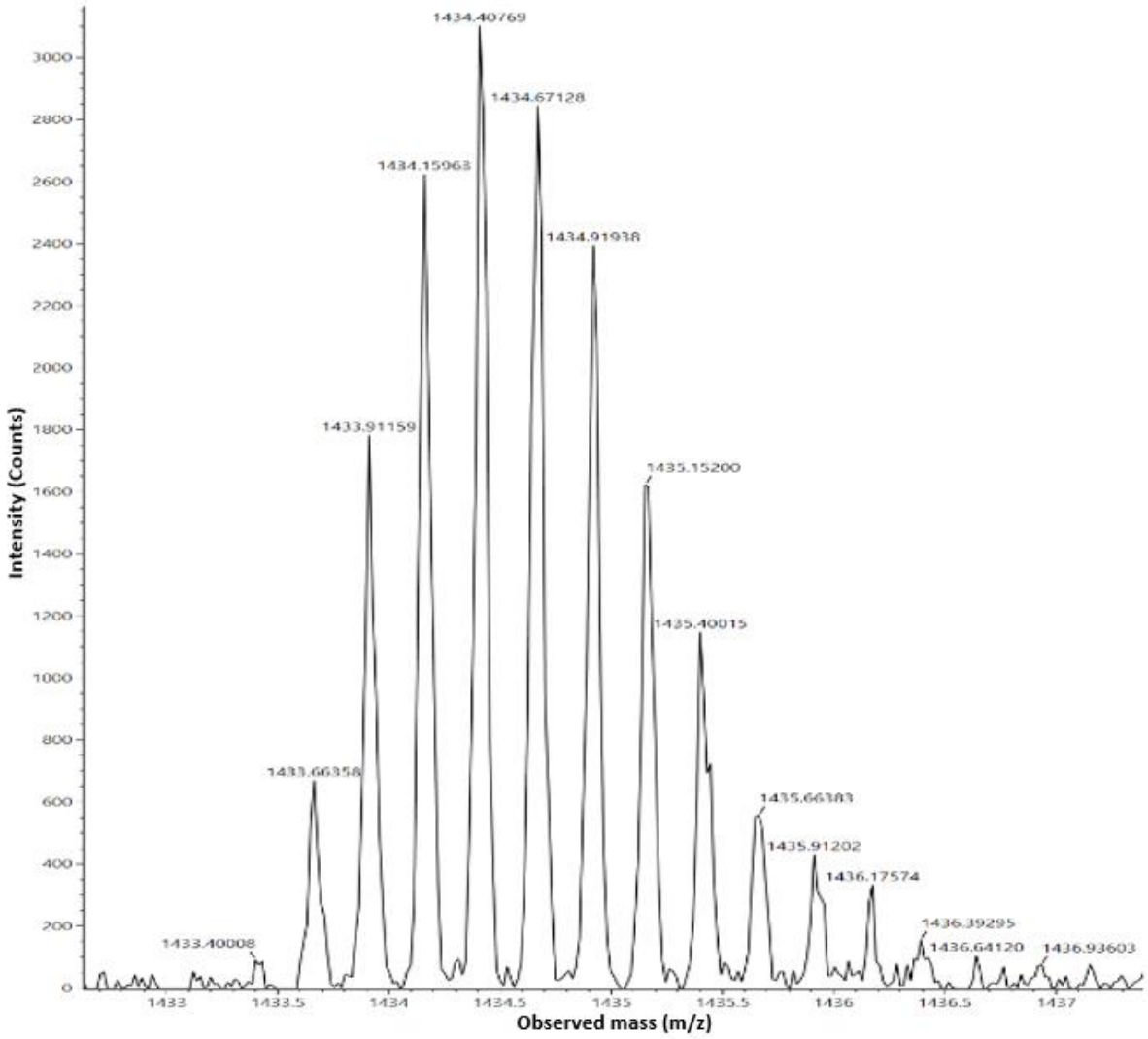


Figure 3.3.2.1.6: HRAM LC–MS mass spectrum of m/z 1433 to 1437 of bovine insulin, indicating the $(M+4H)^{4+}$ spectra.

3.3.2.2 HRAM LC–MS quantitative analysis of insulin aspart

Figure 3.3.2.2.1 presents the calibration data for insulin aspart, comparing the calibration curves generated for insulin aspart from the combined area under the curve (AUC) of all m/z groups, against the AUC for only $(M+4H)^{4+}$ peaks, as seen in Figure 3.3.2.1.3. In both calibration set-ups bovine insulin, AUC of all m/z groups, and AUC for only $(M+4H)^{4+}$ peaks respectively, was used as an internal standard to negate any sample preparation and/or analytical errors or differences. The data in Figure 3.3.2.2.1 is presented as a ratio of insulin aspart against bovine insulin peak areas.

● **Combined AUC values for all $(M+XH)^{X+}$ as ratio of insulin aspart against bovine insulin internal standard.**

● **Calibration calculated from $(M+4H)^{4+}$ data peak integration against bovine insulin internal standard.**

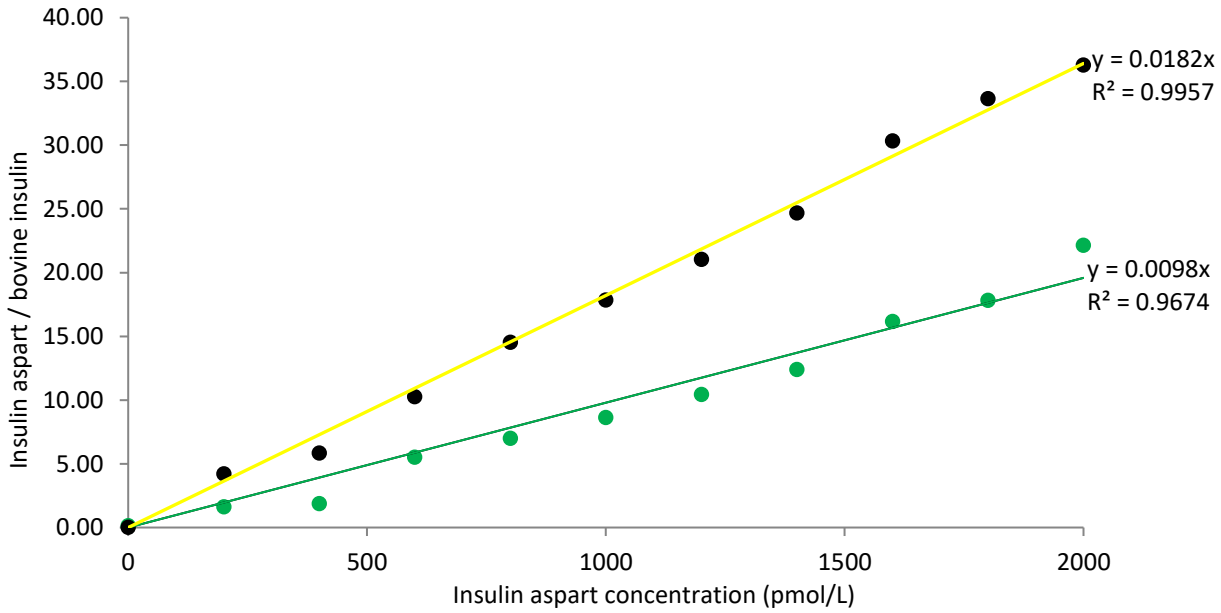


Figure 3.3.2.2.1: Insulin aspart calibration curves generated from insulin aspart peak area and against bovine insulin peak area. Showing $(M+XH)^{X+}$ where x= 3, 4 and 5 (yellow) and $(M+XH)^{4+}$ only (green).

Tables 3.3.2.2.1 and 3.3.2.2.2 present the data to calculate the LLOQ for the insulin aspart HRAM LC–MS method. As discussed in Section 3.3.1.5 there are two methods relevant to this project to determine the LLOQ, as such the LLOQ for this method was defined as the lowest concertation of insulin aspart that

complies with both criteria (a CV of less than 20% and S/N ratio of equal too/greater than 10:1).

Table 3.3.2.2.1 demonstrates that the LLOQ was between the two highlighted concentration values of 82.9 and 41.5 pmol/L. From this, further serial dilutions from the 41.5 pmol/L sample, as described in Section 3.3.1.5, were produced and the CV and S/N ratio were calculated from each concentration. The data from this is presented in Table 3.3.2.2.2.

Concentration (pmol/L)	CV (%)
1326.4	2.88
663.2	3.42
331.6	3.20
165.8	8.11
82.9	14.79
41.5	23.19
20.7	32.49
10.4	38.62

Table 3.3.2.2.1: Mean CV (%) for insulin aspart concentrations of 1326.4 to 10.4 pmol/L, values calculated from 10 replicates of each concentration.

Concentration (pmol/L)	CV (%)	S/N ratio
82.9	14.79	33 : 1
78.8	15.10	26 : 1
74.8	16.36	21 : 1
71.1	16.62	18 : 1
67.5	17.83	17 : 1
64.1	18.98	16 : 1
60.9	18.20	16 : 1
57.9	18.56	15 : 1
55.0	19.64	14 : 1
52.2	19.89	14 : 1
49.6	19.91	13 : 1
47.2	19.96	12 : 1
44.8	21.68	11 : 1
42.6	22.86	9 : 1
40.4	23.62	8 : 1

Table 3.3.2.2.2: Mean CV (%) and S/N ration for insulin aspart concentrations of 82.9 to 40.4 pmol/L, values calculated from 10 replicates of each concentration.

3.3.3 Discussion: HRAM LC–MS analysis of insulin aspart

The analysis of both insulin aspart and bovine insulin produced considerable TIC peaks (Figures 3.3.2.1.1 and 3.3.2.1.4) indicating good sensitivity to the detection of the molecules. The generated multiple m/z peak patterns $(M+XH)^{X+}$ where X = 3, 4 and 5, as seen in Figures 3.3.2.1.2 and 3.3.1.5, are due to the insulin molecules maintaining multiple charges across the molecular structure (Nedelkov et al., 2018). As well as m/z fragmentation, HRAM LC–MS compound analysis is confirmed by isotope detection (Evans et al., 2014). In the case of insulin compounds, this would be the relative abundance of carbon isotopes ^{12}C and ^{13}C. When considering the $(M+4H)^{4+}$ m/z group, as seen in Figures 3.3.2.1.3 and 3.3.2.1.6, the m/z presents with replicating patterns of greater than eight peaks. The m/z peaks should be considered as multiple groups of four peaks, due to both carbon ^{12}C and ^{13}C being detected by the analyser.

When assessing the data generated using the Waters® Xevo® G2 XS QTof analyser, it can be derived that there is a decrease in variance from the expected concentration data points when using the combined area under the curve (AUC) analysis for all m/z charge peaks compared with single m/z area analysis. This is demonstrated by the R^2 values for the analysis of solely the $(M+4H)^{4+}$ data ($R^2 = 0.9674$), chosen as this group has the largest peak intensity of the three m/z groups, compared to the combined AUC of all m/z peaks ($R^2 = 0.9987$). When quantifying using the combined AUC for all m/z peaks the developed insulin aspart HRAM LC–MS quantitative method was determined to have a LLOQ of 47.2 pmol/L, as shown in Table 3.3.2.2.2. This is the lowest concentration where both criteria for LLOQ as set out in 3.3.1.5 was satisfied.

3.4 HRAM LC–MS analysis of exogenous insulin compounds (recombinant human insulin, and insulin analogues)

3.4.1 Methods and materials: HRAM LC–MS analysis of exogenous insulin compounds

Vitreous humour from cases with biochemical markers, such as raised vitreous humour glucose, indicating a hyperglycaemic status at death, were pooled together. The premise being that this group of vitreous humour would largely have suppressed endogenous insulin results.

The pool was then split in to five aliquots, and five exogenous insulin compounds, Actrapid® (recombinant human insulin), Humulin® S (recombinant human insulin), NovoRapid® (insulin aspart), Humalog® (insulin lispro) and Levemir® (insulin detemir), were spiked in at a concentration of 1000 pmol/L into the separate vitreous humour aliquots.

The 1000 pmol/L concentrations were achieved by repeating the dilutions in Tables 2.6.1.2 to 2.6.1.6, but with vitreous humour as the diluent. Then diluting the 20 nmol/L samples to 1000 pmol/L further in vitreous humour as per Table 2.6.1.8.

The HRAM LC–MS analysis of the exogenous insulin compounds involved 20 batch runs each of five exogenous insulin compounds.

Acceptable method performance was set at the threshold of ≤ 5 parts per million (ppm) difference between the expected/theoretical and detected m/z values (Mihaleva et al., 2008).

3.4.2 Results: HRAM LC–MS analysis of exogenous insulin compounds

		$(M+3H)^{3+}$		
Insulin compound	**Expected m/z**	**Mean detected m/z**	**Da difference**	**ppm difference**
Actrapid®	1935.8667	1935.8621	-0.0046	-2.38
Humulin® S	1935.8667	1935.8641	-0.0026	-1.34
NovoRapid®	1943.2000	1943.1943	-0.0057	-2.93
Humalog®	1937.2333	1937.2260	-0.0073	-3.77
Levemir®	1971.6000	1971.6085	0.0085	4.31
		$(M+4H)^{4+}$		
Insulin compound	**Expected m/z**	**Mean detected m/z**	**Da difference**	**ppm difference**
Actrapid®	1451.9000	1451.8929	-0.0071	-4.89
Humulin® S	1451.9000	1451.8974	-0.0026	-1.79
NovoRapid®	1457.4000	1457.3987	-0.0013	-0.89
Humalog®	1452.9250	1452.9182	-0.0068	-4.68
Levemir®	1478.7000	1478.7064	0.0064	4.33
		$(M+5H)^{5+}$		
Insulin compound	**Expected m/z**	**Mean detected m/z**	**Da difference**	**ppm difference**
Actrapid®	1161.5200	1161.5162	-0.0038	-3.27
Humulin® S	1161.5200	1161.5182	-0.0018	-1.55
NovoRapid®	1165.9200	1165.9194	-0.0006	-0.51
Humalog®	1162.3400	1162.3382	-0.0018	-1.55
Levemir®	1182.9600	1182.9636	0.0036	3.04

Table 3.4.2.1: HRAM LC–MS generated mean m/z of 20 replicates of exogenous insulin compounds ions, compared with theoretical m/z.

3.4.3 Discussion: HRAM LC–MS analysis of exogenous insulin compounds

It was demonstrated that all five exogenous insulin compounds were detectable to a high degree of accuracy (Table 3.4.2.1). When assessing the three major generated m/z ions, $(M+XH)^{X+}$ with x= 3, 4 or 5, the mean detected m/z compared to the expected/theoretical value for all five insulin compounds, all were within a tolerance of ≤ 5 ppm.

Further development is required to produce a quantitative method for all five exogenous insulin compounds in vitreous humour. This development would involve repeating the processes in Section 3.3 for each individual compound to determine suitable quantification and LLOQ values. However, the results from this Chapter give confidence that developing this method is feasible.

3.5 HRAM LC–MS analysis of post-mortem blood and vitreous humour samples

3.5.1 Methods and materials: HRAM LC–MS analysis of post-mortem blood and vitreous humour samples

3.5.1.1 Post-mortem blood and vitreous humour sample preparation for HRAM LC–MS analysis

Blood samples from a total of 87 post-mortem examinations with paired vitreous humour samples were available for analysis. For vitreous humour samples, 100 µL of the bovine insulin (internal standard) (500 pmol/l in water) and 500 µL of LC–MS grade water were added to each 500 µL aliquots of vitreous humour or blood. After gently mixing, 800 µL of acetonitrile was added, mixed and centrifuged for 10 min at 13000 *g* (at room temperature). The supernatant was removed, and the liquid was concentrated using a nitrogen sample evaporator (at approximately 45^{o}C). When the solvent was near dryness 500 µL of anti-mouse IgG secondary antibody-coated magnetic bead solution (Thermo Fisher Scientific®, product reference: 11201D) and 500 µL of sterile phosphate-buffered saline (PBS) (Thermo Fisher Scientific®, product reference: 10010002) were added. These samples were incubated, with gentle stirring, for 2 hours at room temperature to extract the insulin. The supernatant was discarded while the pellet was retained using a handheld magnet and residual beads were washed twice by adding and discarding 500 µL of fresh PBS. The antigen–antibody complexes were dissolved by adding 100 µL of acetic acid (4%) (Thermo Fisher Scientific®, product reference: 035572.AP). After removing the beads with the magnetic separator, the supernatant was transferred to a polypropylene tube prior to injection on the HRAM LC–MS system.

3.5.1.2 HRAM LC–MS qualitative identification of insulin compounds

Post-mortem cases were processed for vitreous humour sodium and glucose (as per Section 2.5.1.5), blood insulin on both the immunoassay systems (as per Section 2.5.1.4), and the vitreous humour was processed for HRAM LC–MS determination of exogenous insulin (as per Section 3.3.1). The results were then assessed along with the clinical information available.

3.5.2 Results: HRAM LC–MS analysis of post-mortem blood and vitreous humour samples

Case Nº	Vitreous humour glucose (mmol/L)	Vitreous humour sodium (mmol/L)	Blood glucose (mmol/L)	Blood insulin (pmol/L) Cobas®	Blood insulin (pmol/L) Immulite®	HRAM LC–MS vitreous humour insulin	Clinical Information (including any known drugs prescribed)
PM1701	< 0.3	142	< 0.3	2196 (post-PEG 1862)	2100 (post-PEG 1808)	Insulin aspart (1326 pmol/L) ? insulin lispro	T1DM
PM1704	48.1	147	56.7	71.2	70.6	Human insulin	Insulin & citalopram
PM1705	< 0.3	144		452.1	410.0	Insulin lispro	Humlog® (lispro), zopiclone
PM1706 (right eye)	> 48.0	145	56.6	< 14.4	< 14.4	None detected	DKA on admission
PM1706 (left eye)	56.2	145	56.6	< 14.4	< 14.4		
PM1707	7.8	126		59.6	58.4	None detected	Deceased in alley
PM1710	< 0.3	139		258.7	265	Human insulin	Oromorph, alendronic acid, co-codamol
PM1712	< 0.3	144		227	228.0	Human insulin	Head trauma prior to death
PM1713	5.8	146		45.2	45.3	None detected	T2DM, Rx metformin
PM1714	16.4	142		< 14.4	< 14.4	None detected	CO poisoning
PM1715	< 0.3	139	< 0.3	321.8	321.0	Human insulin	Prostate cancer, naproxen, tamsulosin, and zopiclone
PM1717	68.5	144	87.4	< 14.4	< 14.4	None detected	T1DM
PM1718	< 0.3	145		688.1	634.0	Insulin aspart	T1DM
PM1719	26	138	18.5	26.8	26.5	None detected	T2DM
PM1720	< 0.3	140		318.2	310.2	Human insulin	? history of opiate abuse
PM1721	< 0.3	146	1.8	681.9	658.2	Insulin aspart	T1DM, history of mental health conditions
PM1724	4.6	148		126.2	125.0	Human insulin	Bipolar, Rx: sertraline and diazepam
PM1731	69.8	158	69.4	21.1	< 14.4	None detected	T1DM, Rx NovoRapid®, Heroin and methadone

Table 3.5.2.1: HRAM LC–MS qualitative confirmation/identification of insulin compounds detected in post-mortem samples, along with confirmatory biochemical markers.

3.5.3 Discussion: HRAM LC–MS analysis of post-mortem blood and vitreous humour samples

Of these 87 cases with paired post-mortem blood and vitreous humour, 17 cases also had detailed antemortem clinical history and/or drug history information. Table 3.5.2.1 presents the data from these 17 cases. There are number of interesting cases in Table 3.5.2.1, particularly; PM1701, PM1706, PM1714, PM1717, PM1718 and PM1721.

Sample PM1701 was a case of a person with diabetes presenting with undetectable blood glucose concentration. Both immunoassays reported significantly raised blood insulin levels (greater than 2000 pmol/L). A PEG extraction was performed on the sample (as described in Section 2.5.1.6) In the case of this sample the PEG extractant demonstrated similar results to the non-extracted sample, indicating that the result was due to endogenous and/or exogenous insulin compound(s), and not macro-complexes. HRAM LC–MS qualitative analysis of the vitreous humour indicated the presence of both insulin aspart (NovoRapid®) and insulin lispro (Humalog®). As shown in Table 2.6.2.1 the recoveries for both these insulin compounds via the immunoassay methods are poor. With mean recoveries of 5% and 12%, insulin aspart and insulin lispro respectively, this indicates the actual concentration of the exogenous insulin compounds in the sample was significantly above the 1862 – 2196 pmol/L detected by immunoassay. When analysed using the quantitative method described in Section 3.3.1 the insulin aspart concentration was found to be 1326.4 pmol/L.

Samples from case PM1706 (two separate samples taken from the left and right eyes), PM1714, and PM1717 all presented with no detectable blood insulin levels by immunoassay, as well as no detectable insulin compounds in the vitreous humour by HRAM LC–MS analysis. In all three cases there were clinically significant raised vitreous humour glucose concentrations. In the case of PM1706 the clinical indication on admission was of DKA, further indicating that the ‘negative’ HRAM LC–MS insulin compound screen result is an accurate representation of the perimortem conditions. Sample PM1717 was related to a clinical history of sub-optimal glucose control, and several incidences of hospitalisation due to hyperglycaemia.

Insulin aspart was confirmed in two other cases (PM1718 and PM1721); in both cases vitreous humour glucose was undetectable and raised blood insulin concentrations was detected by immunoassay.

Recombinant human (exogenous)/endogenous insulin was detected in several cases, these also correlate well with the clinical history

3.6 Conclusion

The HRAM LC–MS detection of insulin compounds showed greater detection potential than the LC–MS/MS analysis. The Varian 320–MS® LC–MS/MS method did not demonstrate sufficient resolution or sensitivity for the identification of human insulin at considerably above the endogenous clinical reference range. As previously mentioned, the benefit of HRAM LC–MS compared with LC–MS/MS is the increase in resolution and sensitivity. The developed novel HRAM LC–MS method has been shown to quantify the insulin aspart compound with a suitable LLOQ for post-mortem analysis. As well as the method being demonstrated as suitable for the qualitative analysis of five exogenous insulin compounds.

Chapter 4: Investigation into the UK Coroners service reporting of dysglycaemia-related deaths

4.1 Introduction

Under UK law a death must be reported in one of two ways. In the first way, a medical doctor signs a medical certificate of cause of death (MCCD), stating a natural cause of death, enabling the deceased's family/next of kin to register the death. In the second, the death is referred to the Coroner (England, Wales, and Northern Ireland) or Procurator Fiscal (Scotland) (Parks and Maskel, 2022).

However, the definition of 'natural death' or 'unnatural death' is not clearly defined in the Coroners and Justice Act 2009 which establishes the duties and roles of the Coroner. 'Natural death' in medicine may be defined as

- Death from internal morbidity, where the deceased suffered from a disease from which death was expected; the death occurred entirely independently of any external factors of legal significance (Madea and Rothschild, 2010)

Deaths that are classed as 'natural' are coded using the International Classification of Diseases (ICD-10) system, for example, deaths where the underlying cause is diabetes are covered by codes E10 – E14, as per Table 4.1.1.

ICD-10 Code	Condition	Including	Excluding
E10	Diabetes mellitus -T1DM	Brittle Juvenile on-set Ketosis-prone	Malnutrition-related Neonatal Pregnancy, childbirth Glycosuria
E11	Diabetes mellitus – T2DM	Adult-onset Maturity-onset Non-ketotic Stable	Malnutrition-related Neonatal Pregnancy, childbirth Glycosuria
E12	Malnutrition-related diabetes mellitus	T1DM T2DM	Pregnancy, childbirth Glycosuria Impaired glucose tolerance Neonatal diabetes mellitus Postsurgical hypoinsulinaemia
E13	Other specified diabetes mellitus		Malnutrition-related Neonatal Pregnancy, childbirth T1DM T2DM Glycosuria Impaired glucose tolerance Postsurgical hypoinsulinaemia
E14	Unspecified diabetes mellitus	Diabetes - Not Otherwise Stated (NOS)	Malnutrition-related Neonatal Pregnancy, childbirth T1DM T2DM Glycosuria Impaired glucose tolerance Postsurgical hypoinsulinaemia

Table 4.1.1: List of diabetes related International Classification of Diseases (ICD-10) codes.

If the criteria for MCCD are not fulfilled, then the death is referred to the relevant regional Coroner (Ministry of Justice, 2022). Circumstances that are defined as being ‘unnatural’ are set out in the guidance for doctors completing medical certificates of cause of death (Lishman, 2020), for example:

- Industrial disease
- Death during prison/police custody or shortly after (including natural deaths when under a mental health section or ‘Deprivation of Liberty’ order)
- Suicide
- During or after an abortion
- During a surgical operation or procedure involving anaesthetic
- Neglect, either self or by third party
- Adverse effects of treatment or of standards of care, about which a complaint has been raised. This is advisable in these circumstances in England and Wales, but is compulsory in Scotland
- Accident, e.g., road traffic incident
- Cause unknown or no doctor attending the death
- The certifying doctor has not seen/treated the deceased in the 14 days prior to death

A Coroner’s investigation into cause of death often requires an autopsy to be performed (Harris, 2017), and for various samples to be collected, e.g. for biochemical analysis, histology, and/or toxicology. A Coroner’s investigation — often, but not always — results in an inquest being held. At an inquest, the Coroner will assign cause of death and adjudge learning outcomes. These may be case-related, or general advice to improve outcomes for patients and/or the general public. Coroners differ as to whom they address their final outcome reports, and this inconsistency may result in the reports having differing influences (Ferner et al., 2018). Coroners’ reports are often sent to local authorities and/or hospital trusts, and this results in a very ‘local’ outcomes, with fewer national improvements. While Coroners can express concerns, they are not permitted to rule how these concerns are negated. Ferner et al. (2023) highlighted several cases where a national view would result in greater

improvements in patient care, rather than the outcomes being addressed only at a regional level.

The reporting of dysglycaemia-related deaths appears highly variable, with a wide range of reported information and biochemical analytes used to infer the perimortem conditions. This is potentially further complicated by the inherent variability while investigating insulin by immunoassay (as described in Chapter 2). Clearly there is also variability in both the information provided to the Coroner being presented in different formats, and also resulting from the lack of standardisation of assays being used for the determination of dysglycaemia-related deaths.

The aim of the Coroners audit was to investigate the level, if any, of standardisation across the UK in the investigation of dysglycaemia-related deaths. With reference to the tests performed, the understanding of the importance of the test results and the variability of interpreting these results.

4.2 Audit of UK Coroners-investigation of dysglycaemia-related deaths: stage 1

4.2.1 Methods and materials: audit of UK Coroners-investigation of dysglycaemia-related deaths: stage 1

Three meetings were held with representatives from the Norfolk Coroner's office to discuss the format and questions used in stage 2 of the audit, as well as the likely ease of accessing the information by the Coroner's Officers. These initial meetings were also to investigate the number of cases per annum processed by the Norfolk Coroner, and the number that had diabetes related to the cause of death. Further meetings were then held to investigate the number of the diabetes-related deaths that had vitreous humour glucose, blood β-OHB, and/or blood insulin tests requested. This process involved a mixture of trawling computerised records of the deceased for demographic data, such as name and/or case number, and then paper records for the biochemical assays requested and the corresponding results. Direct contact with the Norfolk Coroner continued for the duration of the project.

4.2.2 Results: audit of UK Coroners-investigation of dysglycaemia-related deaths: stage 1

Data from the Norfolk Coroner is shown in Table 4.2.1.1, the data was then updated throughout the project timeline. Data referring to the registration of deaths by the Coroner, and total number of autopsy examinations performed in the Norfolk Coroner's region in 2016, are presented in Table 4.2.1.2.

Year	Cases referred to Coroner	Cause of death related to diabetes	% of cases related to diabetes	Vitreous humour requests	Blood β-OHB	Blood insulin assayed
2011	3807	300	7.9	8	4	1
2012	3718	317	8.5	9	1	1
2013	3866	340	8.8	9	0	2
2014	3964	366	9.2	11	1	1
2015	4421	442	10.0	24	0	4
2016	4209	504	12.0	21	1	1
2017	4258	448	10.5	12	1	1
2018	4793	587	12.2	28	4	7
2019	2923	178	6.1	18	15	1
2020	3026	163	5.4	24	18	5
2021	2976	244	8.2	36	24	2
2022	3304	413	12.5	52	36	11

Table 4.2.1.1: Total number of deaths reported to the Norfolk Coroner's region (2011 to 2018), and tests requested in relation to investigation of dysglycaemia-related deaths.

Year	All registered deaths	Deaths reported to Coroners	% of Deaths reported to Coroners	No of autopsy examinations	% of autopsy examinations
2016	9456	4047	42.4	1,560	37.1

Table 4.2.1.2: Total reported deaths in reported the Norfolk in 2016, and number referred to the regional Coronial service.

The data in Table 4.2.1.3 were obtained from the published Coroners' statistics from the UK government (Ministry of Justice, 2024), presenting the total number of registered deaths, those registered by doctors, those referred to the Coroners, and how many autopsy examinations were performed.

Year	All registered deaths in UK	Deaths reported to Coroners	% of Deaths reported to Coroners	No of autopsy examinations	% of autopsy examinations (of those deaths referred to Coroners)
2016	525,048	241,211	45.9	86,545	35.9
2017	533,253	229,786	43.1	85,552	37.2
2018	541,589	220,648	40.7	85,593	38.8
2019	530,841	210,912	39.7	82,072	38.9
2020	607,922	205,438	33.8	79,357	38.6
2021	586,213	195,180	33.3	84,599	43.3
2022	577,160	208,340	36.1	90,191	43.9
2023	581,367	194,999	33.5	86,014	44.1

Table 4.2.1.3: Total reported deaths in England and Wales from 2016 to 2023 (Ministry of Justice, 2024).

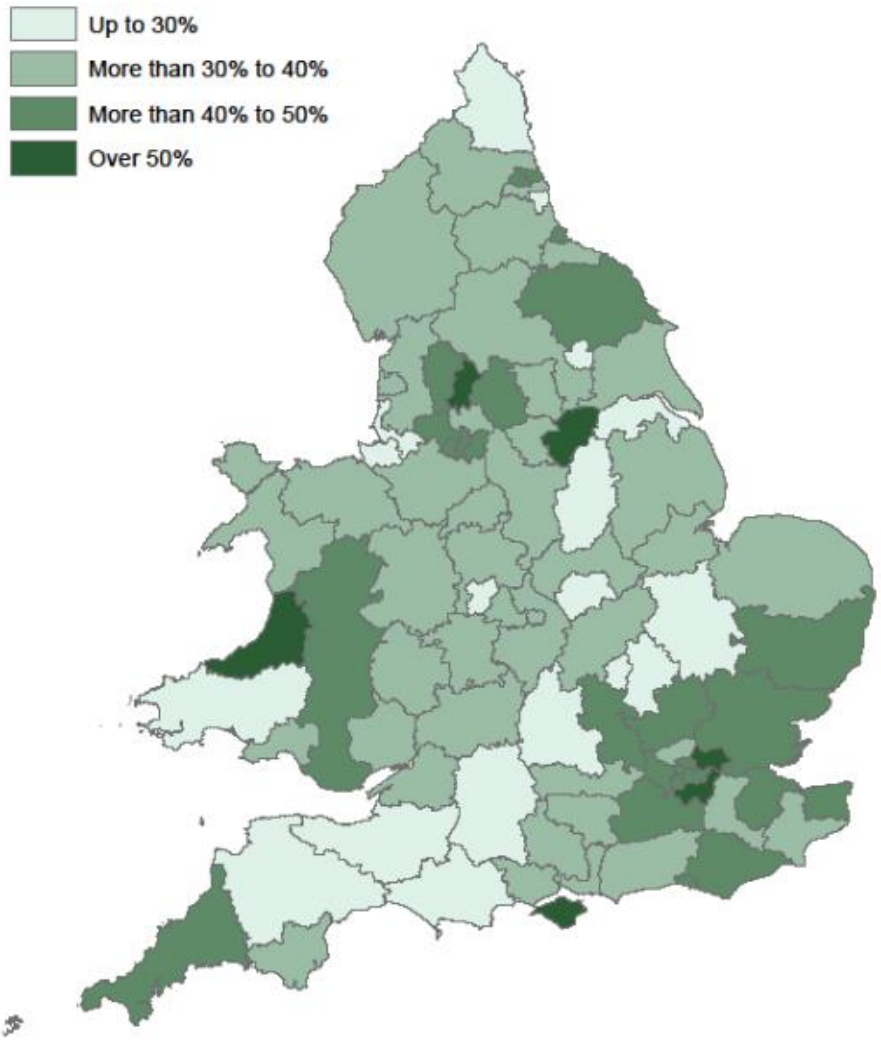


Figure 4.2.1.1: Post-mortem investigations performed as a percentage of deaths reported to Coroners in England and Wales 2016 (Ministry of Justice, 2017).

The data below, Table 4.2.1.4, were from an audit of the turnaround time for reports for post-mortem blood insulin results being received by the NNUH from the date of sending the sample to the third-party referral laboratory.

Date sent	Report received	Turnaround time in days
29/01/2014	03/07/2014	155
02/06/2015	12/10/2015	132
25/06/2015	09/10/2015	106
01/09/2015	09/10/2015	38
03/09/2015	09/10/2015	36
21/09/2015	09/11/2015	49
29/09/2015	04/01/2016	97
14/10/2015	04/01/2016	82
17/10/2016	07/12/2016	51
17/10/2016	07/12/2016	51
17/08/2017	09/01/2018	145
11/07/2019	16/11/2020	494
11/03/2020	09/04/2020	29
29/04/2020	22/05/2020	23
05/08/2020	10/03/2022	582
24/11/2020	10/03/2022	471
22/12/2020	12/10/2021	294
24/02/2022	10/08/2022	167
21/09/2022	05/11/2022	45
Average time (days)		160.4

Table 4.2.1.4: Turnaround time in days of post-mortem blood insulin result reports being received by the NNUH from date of referring sample to third-party laboratory.

4.2.3 Discussion: audit of UK Coroners-investigation of dysglycaemia-related deaths: stage 1

In the meetings with the Coroner for Norfolk it was highlighted that approximately 40% of all registered deaths in Norfolk were processed by the Coroner's Officers (see Table 4.2.1.2), with the remaining cases being directly registered by representatives of the deceased at the local council Registry Offices. This approximate 40:60 ratio is also demonstrated nationally, as highlighted in Table 4.2.1.3, for the deaths registered in England and Wales in the period 2016 to 2023. For the years 2020 and 2021 (Table 4.2.1.3) the number of deaths referred to the Coroner dropped to approximately 33%; this can be attributed to the COVID-19 pandemic, where a large number of deaths across the UK were certified with COVID-19 as the cause of death. From the start of the pandemic in the UK, in March 2020, until December 2022, the number of deaths stating COVID-19 on the MCCD, therefore indicating the deceased was deemed to be infected with the SARS-CoV-2 virus within 28 days of death, was 212,247 (UK Health Security Agency, 2023). It should also be noted that Coronial services were also affected by the UK-wide 'lockdown', restricting the ability to perform inquests and confirm causes of death. However, the percentage of cases referred to the Coroners did not return to the approximate 40% in the post-COVID-19 period of 2023. The number of cases involving autopsy in the period 2016 to 2023, with the exception of 2022, remains consistently above 80,000 per annum (Figure 4.2.1.4), but as can been seen from Figure 4.2.1.1, the percentage is not consistent across the Coroners' regions with it ranging from < 30% to > 50%.

As referred to in Chapter 1, the majority of post-mortem insulin analysis in the UK is performed by one laboratory. Table 4.2.1.4 demonstrates the average turnaround time, the time period between the laboratory receiving samples and a result report being returned to the requestor, for samples sent from the NNUH to the referral laboratory in the period 2014–2022. The average turnaround time in this period is 160 days, and when allowing for the period of COVID-19, (removing requests after 2019), the average turnaround is still 85 days. This 85-day wait for results to be reported to the NNUH, with further time then required to report to the Coroner, may delay an inquest possibly causing increased stress to the families of the deceased (Gregory, 2014).

4.3 Audit of UK Coroners-investigation of dysglycaemia-related deaths: stage 2

4.3.1 Methods and materials: audit of UK Coroners-investigation of dysglycaemia-related deaths: stage 2

Requests for information were sent in April 2018 to the 82 Coroners' districts/regions of England and Wales, the three Coroners in Northern Ireland, and the two Procurator Fiscals in Scotland, with a request for replies within 10 weeks.

As a consequence of some sharing of Coroners' administration resources between adjoining regions, this resulted in 78 requests for information being sent out. The communication formats were:

- 61 requests sent by email using contact addresses from the individual websites of the county/region.
- 6 requests sent via direct messenger links from the individual websites of the county/region.
- 11 requests sent by hard copy via Royal Mail due to no electronic contact details available.

The request for information questions as per full request letter, Appendix 1 AP 1.1, was as follows:

1. What is the total number of deaths reported in your region in each year from 2011 to 2017 (inclusive), and how many of these are related to diabetes mellitus?
2. Approximately what percentage of deaths in the region would be processed by the Coroner's Office, and what percentage would be processed by the Register Office?
3. In the years 2011 to 2017 (inclusive), how many insulin results have been requested by, or reported to, the Coroner's Office?
4. If this information is available, would it be possible to comment on the laboratory analysis that is undertaken regularly in cases of diabetes-

related deaths? Are any of the following test results provided by the laboratory?

- Glucose – Y/N
- Insulin – Y/N
- Other blood chemistry (e.g. β-hydroxybutyrate) – Y/N
- Vitreous humour analysis – Y/N

5. Would you like to receive a follow-up questionnaire/survey that will also contain feedback from this study? – Y/N

4.3.2 Results: audit of UK Coroners-investigation of dysglycaemia-related deaths: stage 2

The 78 responses from the 87 UK Coroners are displayed in Table 4.3.2.1. Forty-seven regions provided full or partial responses, representing a 60% return rate. This was a greater return rate than reported in similar information requests to the Coroners (Roberts et al., 2000). Twelve of the Coroners' regions replied that IT or administration issues precluded them from fully responding to all questions. It was a noted that six of these returns stated responses were limited due to the stored records solely being in paper form, making collation of this data prohibitively expensive in staff time.

Response type	Nº of returns
Total Coroners' regions	87
Requests for information	78
Total regions that provided responses to questions	29
Provided full responses to questions	14
Provided partial responses to questions	15
Unable to obtain data (IT or administration issues)	22
No response (bar initial acknowledgement)	36

Table 4.3.2.1: Request for information from the UK Coroners service.

Figure 4.3.2.1 indicates the Coroners' regions that completed, either fully or partially, the questions referring to sample types collected at autopsy for biochemical analysis. The results from the 14 Coroners that gave direct 'yes' or 'no' responses are represented Table 4.3.2.2.

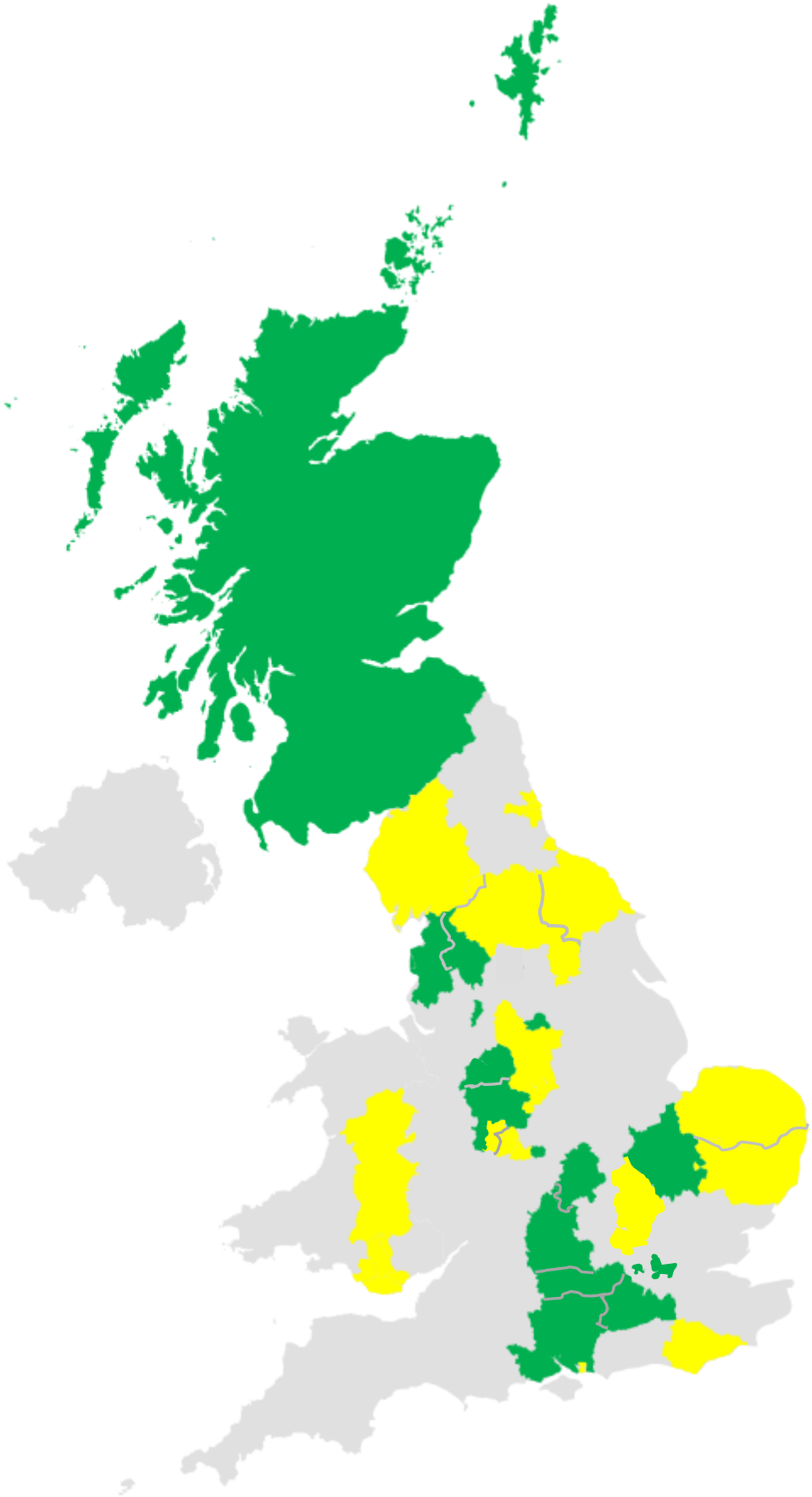


Figure 4.3.2.1: Request for information from the UK Coroners service: Coroners' regions returning full (yellow) and partial (green) responses.

Coroners' Region	Blood glucose	Blood insulin	Blood β-OHB	Vitreous Humour sampled
Bedfordshire and Luton	Y	N	Y	Y
Birmingham and Solihull	Y	Y	Y	Y
Cumbria	Y	Y	Y	Y
Derby and Derbyshire	Y	Y	Y	Y
East Sussex	Y	Y	Y	Y
Gateshead and South Tyneside	Y	Y	Y	N
Hartlepool	Y	N	Y	N
Norfolk	Y	Y	Y	Y
North Tyneside	Y	Y	Y	Y
North Yorkshire Eastern District	Y	Y	Y	Y
Portsmouth and South East Hampshire	Y	Y	Y	Y
South Wales Central	Y	N	Y	N
Staffordshire South	Y	N	Y	Y
Suffolk	Y	Y	Y	Y/N

Table 4.3.2.2: Information supplied from the UK Coroners service in reply to the data request stated in 4.3.1 – stage 2.

4.3.3 Discussion: audit of UK Coroners-investigation of dysglycaemia-related deaths: stage 2

The Coroners that returned a partial or full response were spread across England, Scotland, and Wales are presented in Figure 4.3.2.1. It is clear from the responses seen in Table 4.3.2.2 that there was a marked difference in the assays being requested by the Coronial services to provide information relating to perimortem glucose status. The data demonstrate that it is commonplace for the histopathologist performing an autopsy to request, on behalf of the Coroner, β-OHB and blood glucose concentrations. With all 14 regions reporting that both are routinely requested, and indeed, in the case of blood glucose, it is used across Europe as a marker of diabetes-related deaths (Lukočiūtė et al., 2019). However, it is not universal for blood insulin concentrations to be measured, which may be because there is a lack of awareness that post-mortem blood insulin analysis is available, or there is an awareness of the difficulty obtaining meaningful results. The underlying reasons for blood insulin not being requested by all Coroners regions would require further investigation, and to be incorporated into any learning outcomes and education provided to the Coroners.

There are also Coroners' regions that reported not collecting vitreous humour samples at autopsy, but this also could be due to a lack of awareness of the potential use of this sample type. The Coroners in the UK are largely qualified lawyers, and in the case of the 14 Coroners' regions returning replies all but one of the Senior Coroners were from legal backgrounds, with one from a primarily medical background; two further regions had assistant Coroners who had medical backgrounds. These three regions, with Coroners who trained as medical doctors, answered 'yes' for all four questions. From the data, there appears to be a misunderstanding of the requirement to assess the blood insulin in some of the Coroners' regions, with one indicating that they believed the analysis of insulin was not possible in post-mortem blood samples. Vitreous humour is requested in some Coroners regions but is not seen as a 'front line' analysis matrix. It can be seen in Table 4.3.2.2 that the response from the Suffolk Coroner to whether vitreous humour was collected was both yes and no. This is due to autopsies for the Suffolk region being sent to two different histology services, with one histology service routinely collecting vitreous humour at autopsy, but not the other.

This highlights the lack of standardisation of the biochemical analysis Coronial services request. Within antemortem medicine there is a great degree of standardisation, for example, the World Health Organisation (WHO) has issued 'rules' for the diagnosis of both T1DM and T2DM. The key to standardisation from these cases is a single, national or international 'voice' putting forward the message, and education on the need for standardisation. However, in post-mortem medicine, there is very little standardisation in the biochemical, or indeed toxicology, markers used to infer perimortem conditions. On assessing the current published works, only limited guidelines for the interpretation of post-mortem β-OHB and vitreous humour glucose are available, such as those authored by Belsey and Flanagan (2016), these guidelines are discussed further in Section 5.5.

4.4 Audit of UK Coroners-investigation of dysglycaemia-related deaths: Stage 3

4.4.1 Methods and materials: audit of UK Coroners-investigation of dysglycaemia-related deaths: stage 3

Follow-up information was requested from 18 of the Coroners that returned information in stage 2. Along with this, a request for information was emailed to three forensic toxicology referral laboratories in the UK. This stage was to be a more comprehensive follow-up, involving site-visits. However, due to the COVID-19 pandemic, the Coronial services were unable to provide the time for on-site or online meetings to provide the further data requested. The secondary request for information required more specific data than in stage 1, as detailed below, (full request letter in Appendix 1, AP 1.2):

1. Are samples routinely collected for insulin analysis – Y/N
2. If yes would these be blood, vitreous humour, or both
3. Is there any documentation relating to sample collection times and laboratory interventions? (such as, centrifugation)
4. Are there samples rejected/no results provided for plasma insulin? –Y/N
5. If yes does this affect the interpretation of other results?
6. What are the main stated reason for no result be reported?
7. Haemolysed sample
8. Insufficient sample collected
9. Inappropriate storage
10. Other – please comment
11. Is the Coroner and/or Coroner's officer aware of the protocol in the investigation of hyperglycaemic (high blood glucose) related deaths, to have a β-hydroxybutyrate and glucose result reported?
12. Does the Coronial service believe there is a need for the standardisation of dysglycaemia-related deaths?
13. Would you like a copy of the final thesis, and/or be involved in implementing learning outcomes?

4.4.2 Results: audit of UK Coroners-investigation of dysglycaemia-related deaths: stage 3

	Coroners' region			Referral laboratories		
	Yes	No	Don't know/ no comment	Yes	No	Don't know/ no comment
Are samples routinely collected for insulin analysis	14		4	3		
Blood insulin analysis only	13			3		
Blood and vitreous humour insulin analysis	1					
Is there any documentation relating to sample collection times and laboratory interventions	1	11	6	3		
Are there samples rejected/no results provided for plasma insulin	13		5	3		
If yes does this affect the interpretation of other results	1	4	9		3	
What are the main stated reasons for results not being reported:						
• Haemolysed sample	10		3	3		
• Insufficient sample collected	2		11	3		
• Inappropriate storage			18	2		1
Is the Coroner and/or Coroner's officer aware of the protocol in the investigation of hyperglycaemic (high blood glucose) related deaths, to have a β-OHB and glucose result reported?	4	10	4	3		
Does the Coronial service believe there is a need for the standardisation of dysglycaemia-related deaths?	12		2			3

Table 4.4.2.1: Information supplied 18 UK Coroners and 3 referral laboratory services in reply to the data request stated in 4.4.1 – stage 3.

4.4.3 Discussion: audit of UK Coroners-investigation of dysglycaemia-related deaths: stage 3

When looking at the data from Table 4.4.2.1, the disconnect between the specialist laboratories performing the testing and the Coroner services is highlighted. Ten of the returning Coroners' regions stated that they were not aware of the β-OHB protocol, whereas the three referral laboratories all returned that they believed that the Coroners services were aware of the β-OHB protocol. This discord between the data seems to be due to a lack of communication between the Coroners and the referral laboratories. As previously stated, the Coroners are largely from legal rather than medical backgrounds, and whilst the specialist services are aware of this, they are not involved in the education of the service users. This is particularly important when considering the referral laboratories are rarely involved in sample collection at autopsy, and for them to request extra sampling adds further delays to time-dependent sample collection. While interrogating the data, it is worth noting that the grade of staff completing the data return was rarely noted, with three returns stating that they did not see post-mortem laboratory reports, but it is feasible that a more senior or a designated member of the Coroner's team may have been aware of the laboratory reports.

Only one return indicated that they had access to documentation relating to sample collection. When considering that the majority of regions stated that they request insulin concentrations, this indicates that the Coroners' officers are largely unaware of pre-analytical factors that need to be considered when interpreting these results. Such as the need for rapid centrifugation of samples, and separation of the plasma and redundant erythrocyte components. However, the majority of Coroners' regions that returned data were aware of reports stating that haemolysis was a reason for a concentration not being reported. This is important, as discussed in Chapter 1, haemolysis commonly occurs in post-mortem blood samples.

Two of the referral laboratories, and one of the Coroners' regions, stated that when blood insulin analysis was requested, these samples were analysed at the current only specialist post-mortem insulin service in the UK. One Coroner stated a desire to have access to vitreous humour insulin analysis, however, for clarity,

this was the Norfolk Coroner, who is aware of the project from all three stages of the audit. It is worth noting that this last point may be seen as an example of the specialist scientific service influencing the knowledge of the Coroner's office, through ongoing engagement and communication.

Thus, there is an onus is on the scientific and medical experts presenting data, either in court or via report, to explain the data in the most relevant format, whilst remembering that the Coroner and family of the deceased (if present) are not likely to be experts in the field. Standardisation of the tests requested, and how these are presented, would enable clearer advice to the Coroners, particularly in complicated cases where the blood glucose and/or insulin concentrations are not indicative of the suspected conditions of death.

Chapter 5: Discussion

5.1 Discussion

The starting hypothesis for this thesis was that there was little, if any, standardisation in the investigation of dysglycaemia-related deaths, and that the analysis of blood insulin is of limited use due to inherent pre-analytical and analytical errors. It was hypothesised that these issues were exacerbated as the most common immunoassay-based insulin methods are designed for clinical analysis of endogenous insulin analysis rather than specifically for post-mortem analysis (Labay et al., 2016). As such, in general these immunoassays do not account for any exogenous insulin compounds present. These combined factors, as demonstrated in Chapter 2, could lead to the Coroners being influenced by misleading or incomplete results.

Indeed, it has been demonstrated herein that there is little standardisation in the use of protocols when investigating dysglycaemia-related deaths. The thesis has shown that while blood β-OHB and blood glucose are commonly requested in these cases, the use and limitations of these assays are not necessarily understood by the Coroner, as the 'end service user' (see Chapter 4). Similarly, the advantage of using vitreous humour as the preferred matrix for post-mortem analysis is not widely realised by the Coroners' services. A considerable education and communication 'gap' was demonstrated between the Coroners and the specialist laboratories undertaking the analysis of samples from dysglycaemia-related deaths. This should be a collaborative effort, with the bulk of the responsibility to fill this gap falling with specialist biochemists and toxicologists.

Another important outcome of this thesis was the development of a novel method for the analysis of vitreous humour insulin by HRAM LC–MS, which was demonstrated to be able to quantify one of the common exogenous insulin compounds used for treatment of diabetes, insulin aspart (NovoRapid®). Furthermore, it was possible to identify four other exogenous insulin compounds; two recombinant human insulin compounds (Actrapid® and Humulin® S), and two insulin analogues, insulin lispro (Humalog®) and insulin detemir (Levemir®). It was also demonstrated that automated insulin immunoassays, such as those supplied by Roche and Siemens, for the quantification of blood insulin concentrations, have varied sensitivity to exogenous insulin compounds (see Chapter 2). Therefore, the use of the HRAM LC–MS vitreous humour methods developed

herein for post-mortem investigation of dysglycaemia-related deaths would give a more accurate indicator of insulin status at death than blood insulin, as well as identifying the administration of exogenous insulin compounds prior to death. One post-mortem case of the 17 that were reviewed in Chapter 3 indicated a possible unreported overdose of insulin compounds. Without the vitreous humour insulin analysis this would have remained undetected.

By improving the analysis with the aforementioned vitreous humour insulin HRAM LC–MS method, and better communication with, and understanding from, the Coroners, the investigation of dysglycaemia-related deaths can be improved and standardised, ultimately resulting in an improved service for the families of the deceased.

5.2 Post-mortem sample integrity and storage

One of the aims of this research was to improve the reliability and reproducibility of this post-mortem service provided to Coroners services. For this, it must be demonstrable that pre-analytical steps, such as storage conditions of samples prior to analysis, do not affect the concentrations of insulin, and if there was to quantify the extent of any degradation observed. Most clinical laboratories have –20°C storage, but smaller laboratories may not have the ability to store at –40°C or lower. Transportation of clinical samples that need to remain frozen are often delivered by courier, using 'dry-ice' (solid carbon dioxide) packaging to maintain sample integrity. As such, the premise for this investigation was that for a national UK, European and indeed worldwide service, the requirement for frozen storage was to be at –20°C.

When looking at relatively short storage period post-analysis, e.g., fewer than 100 days, it was demonstrated that the samples results were not statistically different compared with the day 0 analysis. But, as discussed in Chapter 2, there was increased variability at approximately 100 pmol/L, this may be clinically important when considering cases of hyperglycaemia, where blood insulin would be expected at the lower end of, or below, the clinical reference range.

When assessing long-term sample storage at –20°C, insulin concentrations remained stable for the maximum period assessed in this thesis, 1490 days (4 years and 30 days). At this period the mean insulin concentration increased in the sample group from 115.92 pmol/L to 126.01 pmol/L, a *t*-test assessment of the concentrations results at 1490 days when compared with day 0 demonstrated that there was a significant statistical difference ($P = 0.0461$) between the day 1490 insulin concentrations from the day 0 results. However, this trend as demonstrated in Figure 2.2.2.8, is of little clinical relevance, the variability is greater at the lower insulin concentrations (<100 pmol/L), but still within the 95% confidence range. As previously stated, this variability at concentrations below 100 pmol/L should be a consideration in cases of hyperglycaemia. In cases of hypoglycaemia with an associated increase in insulin concentration, the variability at lower insulin concentrations will be of little consequence, as the expected insulin secretion or intake is expected to be raised.

As discussed in Chapter 2, a delay of over five hours in separating the plasma from the whole blood may result in an *in vitro* reduction of up to 20% of the true insulin concentration. This has an impact on the collection of these samples by the histopathologist or mortuary technicians. Samples must either be centrifuged and separated in the mortuary or transferred to the biochemistry/toxicology laboratory within this time frame, so that the sample is centrifuged and the serum/plasma separated promptly. The audit findings suggest that there are currently no protocols for monitoring or 'time stamping' the samples from collection to centrifugation and then to storage. From the Coroners and referral laboratories, none could supply data for sample times and date, bar the initial collection information (Table 4.4.2.1). From this, it can be inferred that none of current interpretations supplied to the Coroners for plasma insulin results consider the possibility of reduced insulin concentrations due to IDE action from delayed separation of samples. This again seems to be an issue that would benefit from shared learning from clinical analytical processes, where the requirement of rapid removal of the plasma from erythrocytes has been in place for many years (Arnqvist et al., 1987). A point of care testing (PoCT) device could be considered, and there are several ongoing studies on the development of PoCT insulin devices, using faradic electrochemical impedance spectroscopy (Khanwalker et al., 2022) and based on the principles of current immunoassay analysis (Soffe et al., 2019). The use of these systems reduces the time period from sample collection to analysis by removing the need to transport the sample to the laboratory to be separated and analysed. However, the proposed solution would be for each mortuary to have access to their own centrifuge. These are relatively inexpensive, but a standardised protocol would need to be in place to ensure that the specification of the centrifuging conditions, i.e., relative centrifugal force (*g*), length of centrifugation time, and centrifugation temperature are standardised to ensure the samples are suitable for analysis. Storage conditions of samples for the subsequent analysis of plasma insulin would also need to be clearly defined and based on the works in Chapter 2.

This approach is simple, inexpensive (particularly when compared with standard PoCT costs), and a relatively low work burden per case, but it would ensure that on requirement of plasma insulin by the Coroner, the samples would be of the best possible quality to analyse. Again, this does not account for IDE action from death to autopsy but does remove any further delay.

5.3 Insulin immunoassay analysis

Further heterogeneity in the insulin results can arise from the use of immunoassays when exogenous insulin compounds are encountered, these immunoassays were found to have considerable variability as demonstrated in Table 2.6.2.1. These factors affect the quality of the data that are provided to the Coroners, such as the interpretation of 'total' insulin, without clearly demonstrating the presence of endogenous versus exogenous components. It is important that scientists providing data to the Coroners make the information as clear and as accessible as possible. Study findings revealed the shortcomings of these immunoassay assays, leading to the conclusion that they are not suitable for the true assessment of blood insulin concentrations when exogenous compounds have been administered, as demonstrated by Parfitt et al. (2015). As discussed, in Chapter 2, the percentage of exogenous insulin compounds detected by the current insulin immunoassays is not concentration dependent, adding to the works of Parfitt et al. (2015), and Glenn and Armston. (2010). As well as the variability due to the presence of exogenous insulin compounds, there are a number of other factors to consider, such as auto insulin-antibodies (Kim et al., 2011), or the presence of biotin (Samarasinghe et al., 2017). The need to consider differing cross-reactivity/recovery of exogenous insulin compounds for various immunoassay platforms is highlighted by sample PM94, Figure 2.5.2.1. The patient information provided states that they were prescribed Insuman® (recombinant human insulin); the results from the Cobas® and Immulite® analysers (606 and 431 pmol/L) are markedly different, with the 606 pmol/L being approximately three times the upper limit of the clinical reference range and 431 pmol/L approximately being twice the upper limit. Both of these insulin concentrations, in the absence of insulin resistance (Wallace and Matthews, 2002), could be related to the patient presenting in a hypoglycaemic state, or post-insulin administration to correct a hyperglycaemic state. The severity of the hypoglycaemia, or change in glucose concentrations if post-administration, would be assessed against the insulin concentrations. For these results to be viewed in 'isolation', i.e., without being aware that the insulin result may not appropriately reflect the actual biological total insulin concentration, could affect the investigation of a hypo- or hyperglycaemic state post-mortem. This case also highlights that in the investigation of antemortem glycaemic status, the presence

of exogenous insulin compounds may also give a false clinical picture, and any reports to clinicians acting on these results should reflect this.

It is important in the analysis of clinical samples that there should be a distinct detection of endogenous from exogenous insulin levels, as otherwise the patient's removal from treatment would be required. Manufacturers are required to inform the user of known cross-reactions, and in the cases of the Roche Elecsys® (Sapin et al., 2001) and Beckmann DxI® (Glenn and Armston, 2010) insulin assays, these data have been published. For the assessment of antemortem insulin concentrations, the Roche Cobas® was the preferred platform, as demonstrated herein, and by Parfitt et al. (2015), due to the near 100% recovery of endogenous insulin and low recovery of exogenous insulin compounds used for treatment of diabetes (≤ 20%). Using this assay, endogenous insulin levels can be accurately assessed without the need for the patient to stop treatment. Conversely, this assay lacks the ability to detect exogenous insulin compounds accurately, needed for post-mortem analysis (or indeed, if this is required in antemortem conditions), as the recovery can be as low as 1% of the true concentration. The immunoassay shows variation between different exogenous insulin compounds, and without a clear clinical history indicating the likelihood of exogenous insulin use, it is not possible to comment on the 'true' insulin results.

Manufacturers of drug compounds must demonstrate quality and performance of the drug as per requirements of regulatory bodies, such as the Medicines and Healthcare products Regulatory Agency (in the UK) and Food Drug Administration (in US) (Aziza, 2022). Four of the major manufacturers of exogenous insulin compounds, Eli Lilly and Company Limited, Novo Nordisk Ltd, Sanofi Medical, and Wockhardt, were contacted regarding the assays/methods and performance material used for assaying their products (as per Appendix 2). However, all four replied that due to this being commercially sensitive information, they could not supply any data. Prior reports related to the immunoassay measurement of exogenous insulin, for example, insulin lispro (Simmons et al., 2019; Heise et al., 2020), and insulin aspart (Andersen et al., 2000) are available. This demonstrates an awareness to identify and quantify exogenous insulin compounds in antemortem care. These immunoassays will still have several of

the issues raised in Chapter 2, e.g., auto-antibody interactions. Also, if using immunoassays for specific exogenous insulin compounds, multiple of these immunoassays may be needed in each post-mortem case to determine the presence or absence of a number of exogenous insulin compounds.

To bridge this gap, a more sensitive and specific analytical method was explored, in this case, mass spectrometry for post-mortem analysis of endogenous and exogenous insulin. In the case of determining clinical insulin concentrations, mass spectrometry analysis is still not common, but is feasible (Bottinelli et al., 2020; Beckett et al., 2021).

5.4 Mass spectrometry insulin analysis

The Varian 320–MS® LC–MS/MS system initially used in this project was not of a high enough specification to assess insulin within the desired concentration ranges. Although determination of the m/z was theoretically feasible, Figures 3.2.2.1 to 3.2.2.5 demonstrated that even at a high concentration of insulin, > 1000 pmol/L, no distinct insulin m/z could be detected. To try and optimise the method above this concentration may have been possible, but to operate the method above this clinically significant concentration, and with the lack of diagnostic sensitivity, would compromise the utility of the method in the laboratory setting.

As the LC–MS/MS method was not pursued, and due to the described complexity of the pre-analytical process and immunoassay variability of blood insulin analysis, a novel method for vitreous humour endogenous and exogenous insulin was developed. This method was developed using a HRAM LC–MS analyser, the Waters® Xevo® G2 XS QTof (as described in Chapter 3). HRAM LC–MS technology is accepted as the preferred platform for qualitative mass spectrometry (Beckett et al., 2021). Particularly in the case of large molecular weight compounds, such as insulin, with molecular weights in excess of 5000 Da (Nedelkov et al., 2018), and as these macromolecules also often produce multiple m/z groups which result in complications in identifying target m/z in LC-MS/MS analysis (Hamidli et al., 2022). As demonstrated in Section 3.4, when exogenous insulin compounds were spiked into vitreous humour samples detection of three ion forms was demonstrated $(M+3H)^{3+}$, $(M+4H)^{4+}$, and $(M+5H)^{5+}$. All five exogenous insulin compounds, two recombinant human insulins (Actrapid® and Humulin® S), insulin aspart (NovoRapid®), insulin lispro (Humalog®), and insulin detemir (Levemir®), demonstrated little variation, < 5 ppm, of detected m/z from the expected/theoretical values. The vitreous humour HRAM LC–MS method was optimised for the quantification of one of these compounds, insulin aspart. Acceptable linearity across an analytical range up to 2000 pmol/L was demonstrated (R^2 = 0.9987) when calculating combined AUC for all $(M+XH)^{X+}$ peaks, and using bovine insulin as an internal standard. The LLOQ was determined as 47.2 pmol/L; this is lower than the LLOQ of similar assays for blood insulin compound quantification (Bottinelli et al., 2021). For this HRAM LC–MS method to be suitable for a post-mortem vitreous humour insulin service the

method will need to be developed to identify and quantify all insulin compounds currently used in the treatment of diabetes. With this method development continuing for when new insulin compounds are released for use, this continuing development could be best achieved by working in conjunction with the insulin manufacturers.

The importance of identifying and quantifying any exogenous insulin compound(s) present in post-mortem samples is demonstrated in the cases presented in Table 3.5.2.1. Particularly in case PM1701, the plasma sample contained a clinically significant insulin concentration, and the case also presented with supressed vitreous glucose concentrations, indicating a possible insulin-induced hypoglycaemic state. When analysing the vitreous humour for insulin compounds using the HRAM LC–MS method, two exogenous insulin compounds were detected, insulin aspart and insulin lispro. The total post-PEG blood insulin concentration, reported from the Immulite® immunoassay, was 1808 pmol/L, this insulin concentration is markedly above the clinical reference range. However, when reviewing the demonstrated cross-reactivity of these two exogenous insulin compounds using the Immulite® immunoassay (Table 2.6.2.1), the mean recovery for insulin aspart (NovoRapid®) was 11.8% and insulin lispro (Humalog®) 7.5%. This indicates that the post-PEG insulin value of 1808 pmol/L result is likely to be considerably lower than actual combined concentration of the two exogenous insulin compounds present in the sample. Indeed, the analysis of the vitreous humour sample using the developed method determined an insulin aspart concentration of 1326 pmol/L. As well as the elevated insulin concentration is it also is significant that insulin aspart and insulin lispro were detected in the sample. While the detection of a single insulin compound at increased value may be from accidental inappropriate administration of prescribed insulin, it is highly unusual for a person with diabetes to be prescribed two different rapid-acting insulin compounds in combination (Racsa et al., 2017). Therefore, this finding is suggestive of deliberate administration of excess insulin.

By being able to define the constituent insulin compounds this information will aid the Coroners services in their role to define cause of death. This agrees with Beckett et al. (2021) who have put forward exogenous insulin analysis in vitreous humour as a promising advancement in the Australian Coronial system. Along

with Beckett et al. (2021), there is a body of work, including Ojanperä et al. (2013), who assert that vitreous humour should be the preferred matrix for insulin analysis. With the development of the HRAM LC–MS endogenous and exogenous insulin method this should be included in a standardised protocol for the assessment of dysglycaemia-related deaths.

It is important however to develop vitreous humour endogenous insulin clinical ranges, to compare with the current accepted blood insulin clinical ranges. Vitreous humour samples for this study would need to be sourced from people without a disease state. For this the removal of vitreous humour in live patients (vitrectomy) could be considered. This requires removal by an ophthalmic surgeon and is normally carried out in conditions such as retinal detachment, raised intraocular pressure, and cataracts (Meleth and Carvounis, 2014). Although some of these aforementioned conditions are also symptoms of both T1DM and T2DM and as such might not be truly reflective of the reference range of the non-disease state population. It would, however, act as a sample pool for direct correlation studies between vitreous humour and blood insulin concentrations (as well as glucose and lactate) without the variables due to post-mortem sampling.

5.5 Reporting of post-mortem results to the Coroners

The assessment of a hypoglycaemic death is complicated by a number of factors, the condition of the matrix, IDE activity, and the measurement of endogenous and exogenous insulin. These all have implications on the data that are then presented to the Coroner. This required improvement in the investigation in dysglycaemia-related deaths was further highlighted with the onset of the COVID-19 pandemic in 2020. At this time in the UK 0.4% and 4.7% of patients registered with a General Practitioner were diagnosed with either T1DM or T2DM, respectively, with a further 0.1% having other types of diabetes, such as gestational diabetes (Barron et al., 2020). However, of the total hospital deaths related to COVID-19, 31.4% of the deaths were people with T2DM, and 1.5% and 0.3% of deaths related to people with T1DM and other diabetes types, respectively (Barron et al., 2020). It is worth noting that the distribution of deaths was highly age-related, 92.3% of COVID-19 deaths affecting people aged 60 or over (UK Health Security Agency, 2023), with the majority of T2DM cases also in this age group (Bellary et al., 2021). This increase in deaths, and the advice from the RCPath in the UK to reduce post-mortem examinations due to staffing pressures and risk to staff, increased the reliance on biochemical analysis.

Another effect of the COVID-19 pandemic was to limit access to the Coroners services, due to increased workload and changes in working practices, such as working from home. As seen in Table 4.2.1.3, the percentage of all deaths that were reported to the Coroners dropped to 33%, from the approximate 40% seen in the years previous to this. This is likely due to the Chief Coroner's communication (Teague, 2022) relaxing guidelines for the referral of deaths to the Coroner. In addition, when COVID-19 had been diagnosed before death, this allowed medics to complete a MCCD (James et al., 2023), rather the death being reported to the Coroner.

The findings in Chapter 4 demonstrate that there is a marked difference across the UK in the investigation of dysglycaemia-related death by different Coroners, and the assays/tests used to support the diagnosis. The role of the reporting scientist in these cases, be it a toxicologist, histopathologist, or biomedical scientist, is to present relevant data in an accessible format that can be

understood by people in a Coroner's court, and indeed by the Coroners themselves. These people attending Coroners' courts often lack a scientific professional background, and often include families of the deceased (George et al., 2016). For the most part, in medical science, standardisation is the key to this type of data, covering; presentation format, tests used to confirm cause of death, and interpretation of test results. There are multiple cases in current UK medicine where non-standardisation has been shown to have been detrimental to patient care, such as highlighted in the Mid-Staffordshire NHS Foundation Trust public inquiry, in which Francis (2013) raised 290 recommendations related to 'appalling care' received by patients at the Trust in part due to lack of formal and correct processes.

In the investigation of dysglycaemia-related deaths there is very little standardisation of approach, as shown in the findings of the audit of Coroners' practices in Chapter 4. Importantly, in the areas where there is agreement on investigation pathways and/or published guidelines, these are not all in line with recent developments in the antemortem care of diabetes. Such a case is the use of blood β-OHB in conjunction with either the blood or vitreous glucose to distinguish between AKA and DKA, as per guidelines published by Belsey and Flanagan (2016) (Figure 5.5.1). These guidelines do not account for all possible causes and presentations of DKA, and if followed could misclassify perimortem conditions.

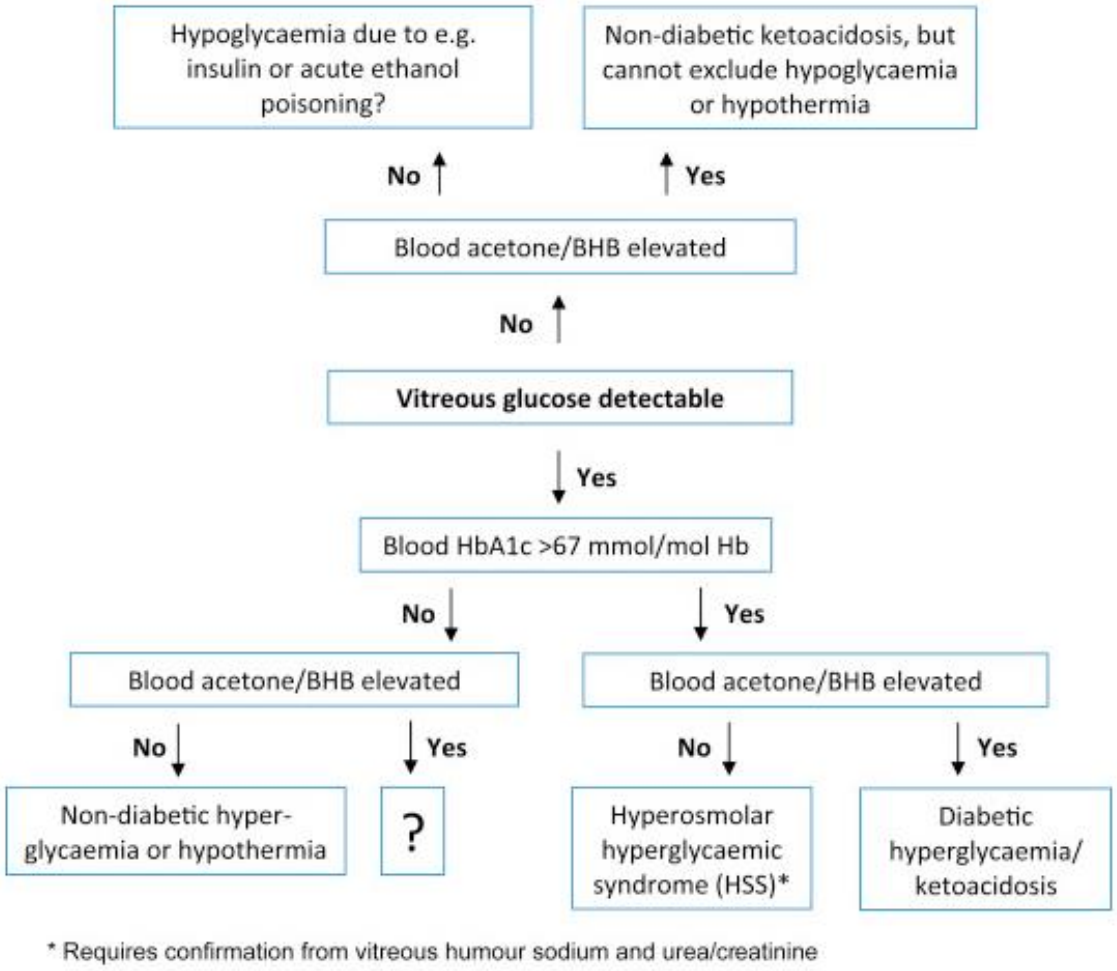


Figure 5.5.1: Pathway for the interpretation of raised vitreous humour glucose, blood β-OHB as recommended by Belsey and Flanagan (2016).

There are number of issues with this pathway, as it is based on the theory that if the vitreous glucose is not detectable, with a raised blood β-OHB, then this is related to a non-diabetic ketoacidosis. However, this does not account for cases of euglycaemic DKA, as discussed in Chapter 1. Nor are there any recommendations to account for a possible post-mortem drop in glucose concentration in the vitreous humour that could result in a spurious undetectable glucose concentration. In the case of HSS, the pathway states that this condition should only be considered if presenting with an elevated HbA_{1c}. However, this scenario does not account for people with newly diagnosed diabetes, where the HbA_{1c} may not be raised, with HbA_{1c} being a long-term (three month) marker of glucose status. Indeed, in the case of HSS, the recommendations of Belsey and Flanagan (2016) go on to state that HHS 'normally occurs in patients with T2DM who are often nursing home residents aged 55–70 years'. While the majority of HSS cases are indeed in the over 45-year age group, HSS can occur in younger

people (Zubkiewicz-Kucharska et al., 2019). From these examples alone, it can be seen that it is possible that following this pathway could lead to the misclassification of dysglycaemia-related deaths.

The data from the Coroners' audit (Table 4.3.2.2) demonstrate that the β-OHB levels are required by all 14 Coroners in the case of dysglycaemia-related death. However, when subsequently following-up with the Coroners to determine whether they were aware of the tests requirement/use in relation to hyperglycaemia, only 4 of the 14 Coroners agreed they were (Table 4.4.2.1). Indeed, the three referral laboratories believed that the Coroners' areas they cover do understand the importance of β-OHB. As such, there is a clear disconnect, as discussed in Section 4.3.3, and this can be attributed to the lack of engagement from the laboratories performing assays for the Coroners. Of the three Coroners' regions where either the senior Coroner or an assistant Coroner had medical backgrounds, two of them stated that they were aware of the protocol to request β-OHB, and possibly this greater understanding of medicine and/or biochemistry has aided this situation. As discussed, regarding insulin, one option to increase the reporting of post-mortem blood β-OHB concentrations is the use of PoCT devices for the measurement of blood β-OHB while performing the autopsy examination (Mitchell and McCleskey, 2023). However, there is considerable variability in sensitivity between PoCT devices, and analytical error at higher concentrations (Bjerg et al., 2022) that could lead to a false assertion that DKA is not a possible cause of death. From the Coroners audit, it is clear that the protocol of requesting/requiring blood β-OHB needs to be better used and understood, and this should be achieved from education on the need and importance of measurement of blood β-OHB, with the laboratory still the mainstay for analysis.

Blood glucose, as described in Chapter 1, and to a lesser degree vitreous humour (Zilg, 2015), can be affected by post-mortem conversion of glucose to lactate, presenting with a decreased concentration compared with perimortem conditions. Mitchell and McCleskey (2023) have suggested the use of HbA_{1c} as an alternative to vitreous glucose for the confirmation of DKA. However, as discussed in Section 1.3.4, HbA_{1c} is directly linked to the lifespan of erythrocytes (Little and Sacks, 2009), and as such, is not a marker of acute changes in glycaemic status

(Keltanen et al., 2013), and indeed, may not even be raised in the aforementioned case of DKA in a person with new-onset diabetes. Also, other considerations of underlying conditions, such as splenectomy (Steveling et al., 2009), alcohol intake (Wiss, 2019), and chronic renal failure (Kang et al., 2015) affect the HbA_{1c} concentration. It is possible that the analysis of post-mortem HbA_{1c} would be of use to assess glucose status over a period prior to death, possibly assisting in the determination of undiagnosed diabetes, or as part of a comprehensive investigation pathway that includes vitreous or blood glucose and β-OHB.

It is suspected that dysglycaemia/diabetes-related deaths are under-reported in the UK, particularly when other causes of death, such as cardiovascular disease, are also present (Thomason et al., 2005). This under-reporting, or reduced investigation of diabetes-related deaths, is also seen in other parts of the world. Coppell et al. (2004) put forward that there is under-reporting of diabetes-related deaths due to differing interpretations of the World Health Organisation cause of death coding system. In their study, based in New Zealand, 45% of those deceased with documented diabetes had no mention of this on their death certificate. This suggests that little or no post-mortem investigation into the disorder was conducted (Coppell et al., 2004). This under-reporting to the New Zealand Coronial service was further highlighted by Chen et al. (2004), who again surmised that this should be seen as a worldwide problem.

To achieve improvements in the investigation of dysglycaemia-related deaths there must be paradigm shifts in practices, and this would be best led by a collaboration of scientists and clinicians who between them understand the implications of the assays, sample conditions, results variability due to cross-reactivity, limitations of the data provided and disease conditions. However, change must be also led by the Coroners themselves, who ultimately have the responsibility for the correct and accurate reporting of cause of deaths.

Chapter 6: Conclusion, and future works

6.1 Conclusion

The aims and objectives of this thesis were established to lead to improvements in the investigation of dysglycaemia-related deaths. This was a multifaceted study, spanning pre-analytical collection and storage of suitable samples, through to evaluation and improvements in the analysis of post-mortem samples, and interpretation of data collected and presented to Coroners.

This study has demonstrated shortcomings in the identification of exogenous insulin compounds, and determination of their concentrations. With respect to the cases involved in this thesis, at least one case (PM1701) indicated the possibility of accidental or deliberate administration of exogenous insulin that previously had not been detected.

The importance of a move to HRAM LC–MS analysis is underscored by the poor performance of insulin immunoassays when used for post-mortem sample analysis. Currently, the majority of post-mortem insulin analysis in the UK and Europe is performed using immunoassays produced for the determination of antemortem insulin concentrations. The data herein have clearly demonstrated that these assays are not suitable for the determination of exogenous insulin compounds. If solely used for the assessment of insulin concentrations, both exogenous and endogenous, there is a serious concern that the concentrations of biologically available insulin at the time of death would be grossly underestimated, potentially resulting in erroneous conclusions being drawn.

It has been demonstrated that with a move to HRAM LC–MS identification and quantification of the exogenous insulin compounds is possible, with this analysis being performed using vitreous humour rather than blood as the preferred sample type to negate the effects of IDE activity.

It is important, when presenting data for Coroners, that the interpretation of results includes an assessment of any insulin concentration changes due to pre-analytical conditions. The work in this thesis sought to improve post-mortem investigation of dysglycaemia-related death, but the laboratory work, although very important, is only the data-gathering part of any investigation. How these data are presented, and indeed the tests that are performed, are led by the Coronial services. With the exception of the histopathology teams, there is little

input from other disciplines, and as stated above, some biochemical results should be reported as a collaborative venture between the histopathology and biochemistry/toxicology teams. The findings from this thesis indicate that collaborative working would improve the lack of standardisation currently seen across the Coronial service.

The findings presented suggest the following improvements for the investigation of dysglycaemia-related deaths:

- Vitreous humour insulin concentrations to be analysed using HRAM LC–MS technology, to determine endogenous and exogenous insulin concentrations in cases of suspected dysglycaemia-related deaths.

- A review of the use of other biochemical markers, e.g., blood β-OHB, HbA_{1c}, and vitreous humour glucose concentrations. This review needs to be led by experts in post-mortem analysis and clinical experts in diabetes, to ensure that when guidelines are produced, they do not have the shortcomings such as those shown by the pathway in Table 5.5.1. This process also needs to lead to education/information being generated for Coroners and histopathologists to explain the need and importance of the β-OHB and glucose protocol.

- A national protocol for the immediate centrifugation and separation of post-mortem blood samples to minimise the effect of insulin-depleting enzymes when blood insulin concentrations are required.

- Standardised result reporting with cut-off values and interpretative comments. This should be coupled with a training/educational programme to inform the Coroners services how these data should be interpreted.

Collectively these would improve the investigation of dysglycaemia-related deaths, enhancing the service provided to families of the deceased.

These changes need to be led by the scientific community, such as the RCPath, Association for Laboratory Medicine, and the Coronial services, in collaboration

with experts in clinical diabetes. The data presented herein clearly show that novel analytical methods, such as the developed HRAM LC–MS endogenous and exogenous insulin method, greatly improve the investigation of dysglycaemia-related death and would considerably improve our diagnostic service in the UK.

6.2 Future Works

The future works from this thesis:

- Collaborative work with experts from other areas, such as histopathologists, Coroners, experts in post-mortem biochemistry & toxicology, and experts in antemortem dysglycaemia-related disorders. To produce pathways to define post-mortem analysis and standardisation of result interpretation.

- Assessment of IDE in red cells, to determine whether the degradation of insulin is linear and, if any, the effects of environmental factors (e.g., temperature of storage, or of body at death) on the rate of insulin degradation.

- To perform the same validation of storage conditions detailed in Section 2.2.2 for vitreous humour samples, to be analysed using the HRAM LC–MS endogenous and exogenous insulin method. This would require 'paired' samples of blood and vitreous, collected at the same time, and then stored in aliquots so that there is only one freeze-thaw process for each aliquot.

- Further development of the HRAM LC–MS vitreous humour insulin method to identify and quantify all insulin compounds currently used for the treatment of diabetes. With this being a continuous process as new insulin compounds are released by manufacturers for treatment of diabetes.

- Establishment of vitreous humour antemortem analyte clinical ranges, particularly insulin, and comparison studies with paired blood analyte concentrations.

The findings from this thesis have demonstrated that there are a number of improvements to be made in how dysglycaemia-related deaths are currently investigated. Several of these, such as the continuing development of the HRAM LC–MS method for new insulin compounds, and standardisation of result interpretation to the Coroners, require collaborative working between public and private bodies. However, with this partnership-working, the service provided to the families of the deceased would be greatly improved.

References

Abbott (2010). *Architect® Insulin assay*. [Assay regent kit insert].

Abbott (2022). *Alinity® i Insulin reagent kit*. [Assay regent kit insert].

Abbott, C. A., Carrington, A. L., Ashe, H., Bath, S., Every, L. C., Griffiths, J., Hann, A. W., Hussein, A., Jackson, N., Johnson, K. E., Ryder, C. H., Torkington, R., Van Ross, E. R. E., Whalley, A. M., Widdows, P., Williamson, S., and Boulton, A. J. M. (2002). The North-West Diabetes Foot Care Study: Incidence of, and risk factors for, new diabetic foot ulceration in a community-based patient cohort. *Diabetic Medicine*, *19*(5), 377–384. https://doi.org/10.1046/j.1464-5491.2002.00698.x

Akinlade, A. T., Ogbera, A. O., Fasanmade, O. A., and Olamoyegun, M. A. (2014). Serum C-peptide assay of patients with hyperglycemic emergencies at the Lagos State University Teaching Hospital (LASUTH), Ikeja. *International Archives of Medicine*, *7*(1), 1–26. https://doi.org/10.1186/1755-7682-7-50

Alam, U., Asghar, O., Azmi, S., and Malik, R. A. (2014). 'General aspects of diabetes mellitus', in D. W. Zochodne and R. A. Malik (eds.) *Handbook of Clinical Neurology 3rd* edition (126). London: Elsieveir B.V, 211–222. https://doi.org/10.1016/B978-0-444-53480-4.00015-1

Alangari, A. A. (2014). Corticosteroids in the treatment of acute asthma. *Annals of Thoracic Medicine*, *9*(4), 187–192. https://doi.org/10.4103/1817-1737.140120

Alberti, K. G. M. M., and Zimmet, P. Z. (1998). Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: Diagnosis and classification of diabetes mellitus. Provisional report of a WHO consultation. *Diabetic Medicine*, *15*(7), 539–553. https://doi.org/10.1002/(SICI)1096-9136(199807)15:7<539::AID-DIA668>3.0.CO;2-S

Alsahli, M., and Gerich, J. E. (2015). Hypoglycemia in patients with diabetes and renal disease. *Journal of Clinical Medicine*, *4*(5), 948–964. https://doi.org/10.3390/jcm4050948

Alsaleh, M., Barbera, T. A., Andrews, R. H., Sithithaworn, P., Khuntikeo, N., Loilome, W., Yongvanit, P., Cox, I. J., Syms, R. R. A., Holmes, E., and Taylor-Robinson, S. D. (2019). Mass Spectrometry: A Guide for the Clinician. *Journal of Clinical and Experimental Hepatology*, *9*(5), 597–606. https://doi.org/10.1016/j.jceh.2019.04.053

Altuntaş, Y. (2000). Postprandial reactive hypoglycemia. *Diabetes and Metabolism*, *26*(5), 215–220. https://doi.org/10.14744/semb.2019.59455

Alves, C., Constança, J., De León, D., Snider, K., and Stanley, C. (2013). A novel atypical presentation of insulin autoimmune syndrome (Hirata's disease) in a child. *Journal of Pediatric Endocrinology and Metabolism*, *26*, 11–12.

Alzahrani, B. A., Salamatullah, H. K., Alsharm, F. S., Baljoon, J. M., Abukhodair, A. O., Ahmed, M. E., Malaikah, H., and Radi, S. (2023). The effect of different types of anemia on HbA1c levels in non-diabetics. *BMC Endocrine Disorders*, *23*(1), 1–9. https://doi.org/10.1186/s12902-023-01280-y

Andersen, L., Jørgensen, P. N., Jensen, L. B., and Walsh, D. (2000). A New Insulin Immunoassay Specific for the Rapid-Acting Insulin Analog, Insulin Aspart, Suitable for Bioavailability, Bioequivalence, and Pharmacokinetic Studies. *Clinical Biochemistry*, *33*(8), 627–633. https://doi.org/10.1016/S0009-9120(00)00183-1

Angelopoulos, T. G., Tentolouris, N. K., Bertsias, G. K., and Boumpas, D. T. (2014). Steroid-induced diabetes in rheumatologic patients. *Clinical and Experimental Rheumatology*, *32*(1), 126–130.

Aramendi, I., Burghi, G., and Manzanares, W. (2017). Dysglycemia in the critically ill patient: Current evidence and future perspectives. *Revista Brasileira de Terapia Intensiva*, *29*(3), 364–372. https://doi.org/10.5935/0103-507X.20170054

Arnqvist, H., Olsson, P. O., and von Schenck, H. (1987). Free and total insulin as determined after precipitation with polyethylene glycol: analytical characteristics and effects of sample handling and storage. *Clinical Chemistry*, *33*(1), 93–96. http://www.ncbi.nlm.nih.gov/pubmed/3542298

Aziza, F. N. (2022). Review and Comparison of Food Drug Administration (FDA) and Medicines and Healthcare products Regulatory Agency (MHRA) on Good Manufacturing Practice (GMP) Implementation. *Majalah Farmaseutik*, *18*(2), 236–240. https://doi.org/10.22146/farmaseutik.v18i2.73520

Baeshen, N. A., Baeshen, M. N., Sheikh, A., Bora, R. S., Morsi, M., Ahmed, M., Ramadan, H. A. I., Saini, K. S., and Redwan, E. M. (2014). Cell factories for insulin production. *Microbial Cell Factories, 13*(1), 1–9. http://www.microbialcellfactories.com/content/13/1/141

Banerjee, S., and Mazumdar, S. (2012). Electrospray Ionization Mass Spectrometry: A Technique to Access the Information beyond the Molecular Weight of the

Analyte. *International Journal of Analytical Chemistry*, *2012*, 1–40. https://doi.org/10.1155/2012/282574

Bao, Y. K., Weide, L. G., Ganesan, V. C., Jakhar, I., McGill, J. B., Sahil, S., Cheng, A.-L., Gaddis, M., and Dress, B. M. (2019). High prevalence of comorbid autoimmune diseases in adults with type 1 diabetes from the HealthFacts database. *Journal of Diabetes*, *11*(4), 273–379.

Barakat, O., Krishnan, S. T. M., and Dhatariya, K. (2008). Falsely low HbA1c value due to a rare variant of hemoglobin J-Baltimore. *Primary Care Diabetes*, *2*(3), 155–157. https://doi.org/10.1016/j.pcd.2008.05.003

Barmanray, R., Chiang, C. Y., Yeoh, K., and Yates, C. J. (2019). A Case of Surreptitious Glargine Overdose Confirmed by Insulin Pharmacokinetic Time Curves. *Journal of Analytical Toxicology*, *43*(6), E4–E6. https://doi.org/10.1093/jat/bkz025

Barron, E., Bakhai, C., Kar, P., Weaver, A., Bradley, D., Ismail, H., Knighton, P., Holman, N., Khunti, K., Sattar, N., Wareham, N. J., Young, B., and Valabhji, J. (2020). Associations of type 1 and type 2 diabetes with COVID-19-related mortality in England: a whole-population study. *The Lancet Diabetes and Endocrinology*, *8*(10), 813–822. https://doi.org/10.1016/S2213-8587(20)30272-2

Bartolomé, A. (2023). The Pancreatic Beta Cell: Editorial. *Biomolecules*, *13*(3), 1–3. https://doi.org/10.3390/biom13030495

Beckett, N., Tidy, R., Douglas, B., and Priddis, C. (2021). Detection of intact insulin analogues in post-mortem vitreous humour—Application to forensic toxicology casework. *Drug Testing and Analysis*, *13*(3), 604–613. https://doi.org/10.1002/dta.2974

Bellary, S., Kyrou, I., Brown, J. E., and Bailey, C. J. (2021). Type 2 diabetes mellitus in older adults: clinical considerations and management. *Nature Reviews Endocrinology*, *17*(9), 534–548. https://doi.org/10.1038/s41574-021-00512-2

Belsey, S. L., and Flanagan, R. J. (2016). Postmortem biochemistry: Current applications. *Journal of Forensic and Legal Medicine*, *41*, 49–57. https://doi.org/10.1016/j.jflm.2016.04.011

Bergin, M., Ward, D. J., Lambrick, S. M., von Jeinsen, N. A., Holst, B., Ellis, J., Jardine, A. P., and Allison, W. (2021). Low-energy electron ionization mass spectrometer for efficient detection of low mass species. *Review of Scientific Instruments*, *92*(7), 1–13. https://doi.org/10.1063/5.0050292

Bévalot, F., Cartiser, N., Bottinelli, C., Fanton, L., and Guitton, J. (2016). Vitreous humor analysis for the detection of xenobiotics in forensic toxicology: a review. *Forensic Toxicology*, *34*(1), 12–40. https://doi.org/10.1007/s11419-015-0294-5

Birhanu, A. G. (2023). Mass spectrometry-based proteomics as an emerging tool in clinical laboratories. *Clinical Proteomics*, *20*(1), 1–20. https://doi.org/10.1186/s12014-023-09424-x

Bishop, P. N. (2000). Structural macromolecules and supramolecular organisation of the vitreous gel. *Progress in Retinal and Eye Research*, *19*(3), 323–344. https://doi.org/10.1016/S1350-9462(99)00016-6

Bjerg, L. N., Thomsen, H. H., Madsen, J. B., and Sandfeld-Paulsen, B. (2022). Evaluation of the point-of-care devices KetoSureTM and StatStrip Express® blood ketone tests using β-hydroxybutyrate spiked samples. *Scandinavian Journal of Clinical and Laboratory Investigation*, *82*(3), 226–231. https://doi.org/10.1080/00365513.2022.2054027

Blanco, J. C., Khatri, A., Kifayat, A., Cho, R., and Aronow, W. S. (2019). Starvation ketoacidosis due to the ketogenic diet and prolonged fasting - A possibly dangerous diet trend. *American Journal of Case Reports*, *20*, 1728–1731. https://doi.org/10.12659/AJCR.917226

Bland, J. M., and Altman, D. G. (1986). Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet*, *327*(8476), 307–310. http://www.ncbi.nlm.nih.gov/pubmed/2868172

Bliss, M. (1993). The History of Insulin. *Diabetes Care*, *16*(3), 4–7.

Boden, G. (1996). Role of Fatty Acids in the Pathogenesis of Insulin Resistance and NIDDM. *Diabetes*, *46*, 3–10.

Boden, G., Chen, X., Ruiz, J., White, J. V., and Rossetti, L. (1994). Mechanisms of fatty acid-induced inhibition of glucose uptake. *Journal of Clinical Investigation*, *93*(6), 2438–2446. https://doi.org/10.1172/JCI117252

Boggs, A. S. P., Bowden, J. A., Galligan, T. M., Guillette, L. J., and Kucklick, J. R. (2016). Development of a multi-class steroid hormone screening method using Liquid Chromatography/Tandem Mass Spectrometry (LC–MS/MS). *Analytical and Bioanalytical Chemistry*, *408*(15), 4179–4190. https://doi.org/10.1007/s00216-016-9512-1

Boroumand, M., Grassi, V. M., Castagnola, F., De-Giorgio, F., d'Aloja, E., Vetrugno, G., Pascali, V. L., Vincenzoni, F., Iavarone, F., Faa, G., and Castagnola, M. (2023). Estimation of postmortem interval using top-down HPLC–MS analysis of

peptide fragments in vitreous humour: A pilot study. *International Journal of Mass Spectrometry*, *483*. https://doi.org/10.1016/j.ijms.2022.116952

Bottinelli, C., Cartiser, N., Bévalot, F., Fanton, L., and Guitton, J. (2020). Is insulin intoxication still the perfect crime? Analysis and interpretation of postmortem insulin: review and perspectives in forensic toxicology. *Critical Reviews in Toxicology*, *50*(4), 324–347. https://doi.org/10.1080/10408444.2020.1762540

Bottinelli, C. J.Guitton, J. Fanton, L. and Bévalot, F. (2019). Characterization of suicide involving insulin analog injection: Interest of injection site analysis by LC/HRMS. *Toxicologie Analytique et Clinique*, *31*(2), S34–S35.

Bottinelli, C., Nicoli, R., Bévalot, F., Cartiser, N., Roger, C., Chikh, K., Kuuranne, T., Fanton, L., and Guitton, J. (2021). Development and validation of a method for quantification of human insulin and its synthetic analogues in plasma and post-mortem sera by LC–MS/HRMS. *Talanta*, *225*, 1–13. https://doi.org/10.1016/j.talanta.2020.122047

Boulagnon, C., Garnotel, R., Fornes, P., and Gillery, P. (2011). Post-mortem biochemistry of vitreous humor and glucose metabolism: an update. *Clinical Chemistry and Laboratory Medicine*, *49*(8), 1265–1270. https://doi.org/10.1515/CCLM.2011.638

Bray, M. (1984). The Effect of Chilling, Freezing, and Rewarming on the Postmortem Chemistry of Vitreous Humor. *Journal of Forensic Sciences*, *29*(2), 404–411. https://doi.org/10.1520/JFS11686J

Bronsema, K. J., Bischoff, R., and Van de Merbel, N. C. (2012). Internal standards in the quantitative determination of protein biopharmaceuticals using liquid chromatography coupled to mass spectrometry. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *893–894*, 1–14. https://doi.org/10.1016/j.jchromb.2012.02.021

Brownlee, M. (2001). Biochemistry and molecular cell biology of diabetic complications. *Nature*, *414*, 813–820.

Brun, J. F., Fedou, C., and Mercier, J. (2000). Postprandial reactive hypoglycemia. *Diabetes and Metabolism*, *26*, 337–351.

Cahn, A., Miccoli, R., Dardano, A., and Del Prato, S. (2015). New forms of insulin and insulin therapies for the treatment of type 2 diabetes. *The Lancet Diabetes and Endocrinology*, *3*(8), 638–652. https://doi.org/10.1016/S2213-8587(15)00097-2

Cerimele, J. B. A. (2008). Hyperosmolar Hyperglycemic State in a Patient Taking Risperidone. *Primary Care Companion to the Journal of Clinical Psychiatry*, *10*(3), 255.

Chandrakanth, H. V., Kanchan, T., Balaraj, B. M., Virupaksha, H. S., and Chandrashekar, T. N. (2013). Postmortem vitreous chemistry-An evaluation of sodium, potassium and chloride levels in estimation of time since death (during the first 36 h after death). *Journal of Forensic and Legal Medicine*, *20*(4), 211–216. https://doi.org/10.1016/j.jflm.2012.09.001

Chandrasekara, H., Fernando, P., Danjuma, M., and Jayawarna, C. (2014). Ketoacidosis is not always due to diabetes. *BMJ Case Reports*, *2014*(1), bcr2013203263–bcr2013203263. https://doi.org/10.1136/bcr-2013-203263

Chen, F., Florkowski, C. M., Dever, M., and Beaven, D. W. (2004). Death Certification and New Zealand Health Information Service (NZHIS) statistics for diabetes mellitus: An under-recognised health problem. *Diabetes Research and Clinical Practice*, *63*(2), 113–118. https://doi.org/10.1016/j.diabres.2003.09.002

Chen, J. H., Michiue, T., Inamori-Kawamoto, O., Ikeda, S., Ishikawa, T., and Maeda, H. (2015). Comprehensive investigation of postmortem glucose levels in blood and body fluids with regard to the cause of death in forensic autopsy cases. *Legal Medicine*, *17*(6), 475. https://doi.org/10.1016/j.legalmed.2015.08.004

Chen, Z., Caulfield, M. P., McPhaul, M. J., Reitz, R. E., Taylor, S. W., and Clarke, N. J. (2013). Quantitative insulin analysis using liquid chromatography-tandem mass spectrometry in a high-throughput clinical laboratory. *Clinical Chemistry*, *59*(9), 1349–1356. https://doi.org/10.1373/clinchem.2012.199794

Chevenne, D., Letailleur, A., Trivin, F., and Porquet, D. (1998). Effect of hemolysis on the concentration of insulin in serum determined by RIA and IRMA. *Clinical Chemistry*, *44*(2), 354–356. https://doi.org/10.1093/clinchem/44.2.354

Chu, C. H., Lee, J. K., Lam, H. C., and Lu, C. C. (2001). Prognostic factors of hyperglycemic hyperosmolar nonketotic state. *Chang Gung Medical Journal*, *24*(6), 345–351. http://www.ncbi.nlm.nih.gov/pubmed/11512365

Church, D., Cardoso, L., Kay, R. G., Williams, C. L., Freudenthal, B., Clarke, C., Harris, J., Moorthy, M., Karra, E., Gribble, F. M., Reimann, F., Burling, K., Williams, A. J. K., Munir, A., Jones, T. H., Führer, D., Moeller, L. C., Cohen, M., Khoo, B., Semple, R. K. (2018). Assessment and management of anti-insulin autoantibodies in varying presentations of insulin autoimmune syndrome.

Journal of Clinical Endocrinology and Metabolism, *103*(10), 3845–3855. https://doi.org/10.1210/jc.2018-00972

Clark, M. A., Worrell, M. B., and Pless, J. E. (1997). 'Forensic Taphonomy: The Postmortem Fate of Human Remains', in W. D. Haglund and M. H. Sorg (eds.) *Forensic taphonomy: the postmortal fate of human remains*. Florida: CRC Press, 151–164.

Clark, M., Worrel, M., and Pless, J. (1996). Postmortem changes in soft tissues. *Forensic Taphonomy*, 151–164.

Cocariu, E. A., Mageriu, V., Stăniceanu, F., Bastian, A., Socoliuc, C., and Zurac, S. (2016). Correlations Between the Autolytic Changes and Postmortem Interval in Refrigerated Cadavers. *Romanian Journal of Internal Medicine = Revue Roumaine de Médecine Interne*, *54*(2), 105–112. https://doi.org/10.1515/rjim-2016-0012

Coelho, S. (2016). What is the Role of HbA1c in Diabetic Hemodialysis Patients?. *Seminars in Dialysis*, *29*(1), 19–23. https://doi.org/10.1111/sdi.12408

Coppell, K., McBride, K., and Williams, S. (2004). Under-reporting of diabetes on death certificates among a population with diabetes in Otago Province, New Zealand. *The New Zealand Medical Journal*, *117*(1207), U1217. http://www.ncbi.nlm.nih.gov/pubmed/15608810

Corbin, L. J., Richmond, R. C., Wade, K. H., Burgess, S., Bowden, J., Smith, G. D., and Timpson, N. J. (2016). BMI as a modifiable risk factor for type 2 diabetes: Refining and understanding causal estimates using mendelian randomization. *Diabetes, 65*(10), 3002–3007. https://doi.org/10.2337/db16-0418

Dahl, A., and Kumar, S. (2020). Recent advances in neonatal diabetes. *Diabetes, Metabolic Syndrome and Obesity*, *13*, 355–364. https://doi.org/10.2147/DMSO.S198932

Dassie, F., Favaretto, F., Bettini, S., Parolin, M., Valenti, M., Reschke, F., Danne, T., Vettor, R., Milan, G., and Maffei, P. (2021). Alström syndrome: an ultra-rare monogenic disorder as a model for insulin resistance, type 2 diabetes mellitus and obesity. *Endocrine*, *71*(3), 618–625. https://doi.org/10.1007/s12020-021-02643-y

de Sá-Ferreira, C. O., da Costa, C. H. M., Guimarães, J. C. W., Sampaio, N. S., de Moraes Lopes Silva, L., de Mascarenhas, L. P., Rodrigues, N. G., dos Santos, T. L., Campos, S., and Young, E. C. (2022). Diabetic ketoacidosis and COVID-19: what have we learned so far? *American Journal of Physiology -*

Endocrinology and Metabolism, *322*(1), E44–E53. https://doi.org/10.1152/AJPENDO.00244.2021

Dhatariya, K. (2016). Blood ketones: Measurement, interpretation, limitations, and utility in the management of diabetic ketoacidosis. *Review of Diabetic Studies*, *13*(4), 217–225. https://doi.org/10.1900/RDS.2016.13.217

Dhatariya, K. K., Glaser, N. S., Codner, E., and Umpierrez, G. E. (2020). Diabetic ketoacidosis. *Nature Reviews Disease Primers*, *6*(1), 40. https://doi.org/10.1038/s41572-020-0165-1

Diabetes UK. (2020). *diabetes-uk-meds-and-kit-2020*. Available at: https://www.diabetes.org.uk (Accessed: 03 October 2023).

Dong, S., Gu, Y., Wei, G., Si, D., and Liu, C. (2018). Direct comparison of LC–MS/MS and RIA methods for the pharmacokinetics assessment of human insulin in preclinical development. *Biomedical Chromatography*, *32*(11). https://doi.org/10.1002/bmc.4323

Efe, J. O. J., Igho, O. E., and Mamuyovwi, U. J. (2021). Investigating antemortem, perimortem and postmortem injuries: Forensic implication. *Bangladesh Journal of Medical Science*, *20*(1), 50–57. https://doi.org/10.3329/bjms.v20i1.50345

ElSayed, N. A., Aleppo, G., Aroda, V. R., Bannuru, R. R., Brown, F. M., Bruemmer, D., Collins, B. S., Gaglia, J. L., Hilliard, M. E., Isaacs, D., Johnson, E. L., Kahan, S., Khunti, K., Leon, J., Lyons, S. K., Perry, M. Lou, Prahalad, P., Pratley, R. E., Seley, J. J., Stanton, R. C., and Gabbay, R. A. (2023). 2. Classification and Diagnosis of Diabetes: *Standards of Care in Diabetes—2023*. *Diabetes Care*, *46*(Supplement_1), S19–S40. https://doi.org/10.2337/dc23-S002

Egan, A. M., Galior, K. D., Maus, A. D., Fatica, E., Simha, V., Shah, P., Singh, R. J., & Vella, A. (2022). Pitfalls in Diagnosing Hypoglycemia Due to Exogenous Insulin: Validation and Utility of an Insulin Analog Assay. *Mayo Clinic Proceedings*, *97*(11), 1994–2004. https://doi.org/10.1016/j.mayocp.2022.07.021

Espírito Santo, R., Vaz, S., Jalles, F., Boto, L., and Abecasis, F. (2017). Salicylate Intoxication in an Infant: A Case Report. *Drug Safety - Case Reports*, *4*(23), 1–5. https://doi.org/10.1007/s40800-017-0065-9

Evans, A. M., Bridgewater, B. R., Liu, Q., Mitchell, M. W., Robinson, R. J., Dai, H., Stewart, S. J., DeHaven, C. D., and Miller, L. A. D. (2014). High Resolution Mass Spectrometry Improves Data Quantity and Quality as Compared to Unit Mass Resolution Mass Spectrometry in High-Throughput Profiling

Metabolomics. *Journal of Postgenomics Drug and Biomarker Development*, *4*(2), 1–7. https://doi.org/10.4172/2153-0769.1000132

Fallahi, P., Ferrari, S. M., Ruffilli, I., Elia, G., Biricotti, M., Vita, R., Benvenga, S., and Antonelli, A. (2016). The association of other autoimmune diseases in patients with autoimmune thyroiditis: Review of the literature and report of a large series of patients. *Autoimmunity Reviews*, *15*(12), 1125–1128.

Feldman, E. L., Callaghan, B. C., Pop-Busui, R., Zochodne, D. W., Wright, D. E., Bennett, D. L., Bril, V., Russell, J. W., and Viswanathan, V. (2019). Diabetic neuropathy. *Nature Reviews Disease Primers*, *5*(41), 1–18. https://doi.org/10.1038/s41572-019-0092-1

Ferner, R. E., Brittain, R., Cox, A. R., Heneghan, C., Richards, G. C., and Aronson, J. K. (2023). Recognition of Coroners' Concerns to Prevent Future Deaths from Medicines: A Systematic Review. *Pharmaceutical Medicine*, *37*(5), 357–363. https://doi.org/10.1007/s40290-023-00486-8

Ferner, R. E., Easton, C., and Cox, A. R. (2018). Deaths from Medicines: A Systematic Analysis of Coroners' Reports to Prevent Future Deaths. *Drug Safety*, *41*(1), 103–110. https://doi.org/10.1007/s40264-017-0588-0

Flanagan, R. J., and Manu, P. (2016). 'Forensic Investigation of Antipsychotic-Related Deaths', in P. Manu, R. J. Flanagan, and K. J. Ronaldson (eds.) *Life-Threatening Effects of Antipsychotic Drugs*. London: Academic Press, 333–349. https://doi.org/10.1016/B978-0-12-803376-0.00016-2

Fouque, D. J. D, Maroto, A., and Memboeuf, A. (2018). Internal Standard Quantification Using Tandem Mass Spectrometry of a Tryptic Peptide in the Presence of an Isobaric Interference. *Analytical Chemistry*, *90*(24), 14126–14130. https://doi.org/10.1021/acs.analchem.8b05016

Francis, R. (2013). *Report of the Mid Staffordshire NHS Foundation Trust Public Inquiry.* Available at: https://www.gov.uk/government/publications/report-of-the-mid-staffordshire-nhs-foundation-trust-public-inquiry (Accessed: 15 November 2023).

Franke, A. A., Li, X., Dabalos, C., and Lai, J. F. (2020). Improved oxytocin analysis from human serum and urine by orbitrap ESI-LC-HRAM–MS. *Drug Testing and Analysis*, *12*(6), 846–852. https://doi.org/10.1002/dta.2783

French, E. K., Donihi, A. C., and Korytkowski, M. T. (2019). Diabetic ketoacidosis and hyperosmolar hyperglycemic syndrome: Review of acute decompensated

diabetes in adult patients. *The BMJ*, *365*, 1–15. https://doi.org/10.1136/bmj.l1114

Fu, J., Yu, M. G., Li, Q., Park, K., and King, G. L. (2021). Insulin's actions on vascular tissues: Physiological effects and pathophysiological contributions to vascular complications of diabetes. *Molecular Metabolism*, 52, 1-11. https://doi.org/10.1016/j.molmet.2021.101236

Gama, R., Teale, J. D., and Marks, V. (2003). Best Practice No 173. Clinical and laboratory investigation of adult spontaneous hypoglycaemia. *Journal of Clinical Pathology*, *56*(9), 641–646. https://doi.org/10.1136/jcp.56.9.641

García Rodríguez, L. A., Cea Soriano, L., de Abajo, F. J., Valent, F., Hallas, J., Gil, M., Cattaruzzi, C., Rodriguez-Martin, S., Vora, P., Soriano-Gabarró, M., and Gaist, D. (2022). Trends in the use of oral anticoagulants, antiplatelets and statins in four European countries: a population-based study. *European Journal of Clinical Pharmacology*, *78*(3), 497–504. https://doi.org/10.1007/s00228-021-03250-6

Garg, S. K., and Garg, P. (2021). Differential diagnosis of ketoacidosis in hyperglycemic alcoholic diabetic patient: The role of insulin. *Indian Journal of Critical Care Medicine*, *25*(10), 1201–1202. https://doi.org/10.5005/jp-journals-10071-23988

George, B., Nicholls, K., Pompeus, S., and Vasu, V. (2016). Providing effective evidence for the coroner. *Archives of Disease in Childhood: Education and Practice Edition*, *101*(2), 82–86. https://doi.org/10.1136/archdischild-2015-308584

Ghimire, P., and Dhamoon, A. S. (2018). *Ketoacidosis*. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15998156 (Accessed: 16 July 2021)

Giavarina, D. (2015). Understanding Bland Altman analysis. *Biochemia Medica*, *25*(2), 141–151. https://doi.org/10.11613/BM.2015.015

Gibb, F. W., Teoh, W. L., Graham, J., and Lockman, K. A. (2016). Risk of death following admission to a UK hospital with diabetic ketoacidosis. *Diabetologia*, *59*(10), 2082–2087. https://doi.org/10.1007/s00125-016-4034-0

Glenn, C., and Armston, A. (2010). Cross-reactivity of 12 recombinant insulin preparations in the Beckman Unicel DxI 800 insulin assay. *Annals of Clinical Biochemistry*, *47*(3), 264–266. https://doi.org/10.1258/acb.2010.010002

Gooday, C., Murchison, R., and Dhatariya, K. (2014). Complex relationships requiring long-term follow-up: Obesity, bariatric surgery-induced diabetic remission and the diabetic foot. *Diabesity in Practice*, *3*(2), 59–66.

Grant, C. S. (2005). Insulinoma. *Best Practice and Research: Clinical Gastroenterology*, *19*(5), 783–798. https://doi.org/10.1016/j.bpg.2005.05.008

Gregory, M. J. (2014). Managing the homicide-suicide inquest the practices of coroners in one region of England and Wales. *International Journal of Law, Crime and Justice*, *42*(3), 237–250. https://doi.org/10.1016/j.ijlcj.2014.01.009

Gregory, G. A., Robinson, T. I., Linklater, S. E., Wang, F., Colagiuri, S., de Beaufort, C., Donaghue, K. C., Harding, J. L., Wander, P. L., Zhang, X., and Li, X. (2022). Global incidence, prevalence, and mortality of type 1 diabetes in 2021 with projection to 2040: a modelling study. *Lancet Diabetes Endocrinology*, *10*(10), 741–760.

Gross, J. L., de Azevedo, M. J., Silveiro, S. P., Canani, L. H., Caramori, M. L., and Zelmanovitz, T. (2005). Diabetic Nephropathy: Diagnosis, Prevention, and Treatment. *Diabetes Care*, *28*(1), 164–176. https://doi.org/10.2337/diacare.28.1.164

Halsall, D. (2018). Insulin assay presented at the Society for Endocrinology BES. *Endocrine Abstracts*, *59*, MTE3.

Hamblin, P. S., Wong, R., Ekinci, E. I., Fourlanos, S., Shah, S., Jones, A. R., Hare, M. J. L., Calder, G. L., Epa, D. S., George, E. M., Giri, R., Kotowicz, M. A., Kyi, M., Lafontaine, N., Macisaac, R. J., Nolan, B. J., O'Neal, D. N., Renouf, D., Varadarajan, S., Wong, J., Xu, S., and Bach, L. A. (2019). SGLT2 Inhibitors Increase the Risk of Diabetic Ketoacidosis Developing in the Community and during Hospital Admission. *Journal of Clinical Endocrinology and Metabolism*, *104*(8), 3077–3087. https://doi.org/10.1210/jc.2019-00139

Hamidli, N., Pajaziti, B., Andrási, M., Nagy, C., and Gáspár, A. (2022). Determination of human insulin and its six therapeutic analogues by capillary electrophoresis – mass spectrometry. *Journal of Chromatography A*, *1678*. https://doi.org/10.1016/j.chroma.2022.463351

Hammoud, B., and Greeley, S. A. W. (2022). Growth and development in monogenic forms of neonatal diabetes. *Current Opinion in Endocrinology and Diabetes and Obesity*, *29*(1), 65–77.

Hannis, J. C., and Muddiman, D. C. (2000). A Dual Electrospray Ionization Source Combined With Hexapole Accumulation to Achieve High Mass Accuracy of

Biopolymers in Fourier Transform Ion Cyclotron Resonance Mass Spectrometry. *Journal of American Society of Mass Spectrometry, 11,* 876–883.

Harris, A. (2017). 'Natural' and 'Unnatural' medical deaths and coronial law: A UK and international review of the medical literature on natural and unnatural death and how it applies to medical death certification and reporting deaths to coroners. *Medicine, Science and the Law*, *57*(3), 105–114. https://doi.org/10.1177/0025802417708948

Hart, P. A., Bellin, M. D., Andersen, D. K., Bradley, D., Cruz-Monserrate, Z., Forsmark, C. E., Goodarzi, M. O., Habtezion, A., Korc, M., Kudva, Y. C., Pandol, S. J., Yadav, D., and Chari, S. T. (2016). Type 3c (pancreatogenic) diabetes mellitus secondary to chronic pancreatitis and pancreatic cancer. *The Lancet Gastroenterology and Hepatology*, *1*(3), 226–237. https://doi.org/10.1016/S2468-1253(16)30106-6

Heise, T., Linnebjerg, H., Coutant, D., LaBell, E., Zijlstra, E., Kapitza, C., Bue-Valleskey, J., Zhang, Q., Dellva, M. A., and Leohr, J. (2020). Ultra rapid lispro lowers postprandial glucose and more closely matches normal physiological glucose response compared to other rapid insulin analogues: A phase 1 randomized, crossover study. *Diabetes, Obesity and Metabolism*, *22*(10), 1789–1798. https://doi.org/10.1111/dom.14094

Hess, C., Musshoff, F., and Madea, B. (2011). Disorders of glucose metabolism-post mortem analyses in forensic cases: Part I. *International Journal of Legal Medicine*, *125*(2), 163–170. https://doi.org/10.1007/s00414-010-0509-6

Hess, C., Stockhausen, S., Kernbach-Wighton, G., and Madea, B. (2015). Death due to diabetic ketoacidosis: Induction by the consumption of synthetic cannabinoids? *Forensic Science International*, *257*, e6–e11. https://doi.org/10.1016/j.forsciint.2015.08.012

Hess, C., Thomas, A., Thevis, M., Stratmann, B., Quester, W., Tschoepe, D., Madea, B., and Musshoff, F. (2012). Simultaneous determination and validated quantification of human insulin and its synthetic analogues in human blood serum by immunoaffinity purification and liquid chromatography-mass spectrometry. *Analytical and Bioanalytical Chemistry*, *404*(6–7), 1813–1822. https://doi.org/10.1007/s00216-012-6271-5

Hills, C. E., and Brunskill, N. J. (2008). Intracellular signalling by C-peptide. *Experimental Diabetes Research*, *2008*, 1-8. https://doi.org/10.1155/2008/635158

Hirsch, I. B. (2005). Insulin Analogues. *New England Journal of Medicine, 352*(2), 174–183. www.nejm.org

Hirsch, I. B., Juneja, R., Beals, J. M., Antalis, C. J., and Wright, E. E. (2021). The evolution of insulin and how it informs therapy and treatment choices. *Endocrine Reviews*, *41*(5), 733–755. https://doi.org/10.1210/ENDREV/BNAA015

Holman, N., Knighton, P., Kar, P., O'Keefe, J., Curley, M., Weaver, A., Barron, E., Bakhai, C., Khunti, K., Wareham, N. J., Sattar, N., Young, B., and Valabhji, J. (2020). Risk factors for COVID-19-related mortality in people with type 1 and type 2 diabetes in England: a population-based cohort study. *The Lancet Diabetes and Endocrinology*, *8*(10), 823–833. https://doi.org/10.1016/S2213-8587(20)30271-0

Honour, J. W. (2011). Development and validation of a quantitative assay based on tandem mass spectrometry. *Annals of Clinical Biochemistry*, *48*(2), 97–111. https://doi.org/10.1258/acb.2010.010176

Hörber, S., Achenbach, P., Schleicher, E., and Peter, A. (2020). Harmonization of immunoassays for biomarkers in diabetes mellitus. *Biotechnology Advances*, *39*, 1–15. https://doi.org/10.1016/j.biotechadv.2019.02.015

Hropot, T., Battelino, T., and Dovc, K. (2023). Sodium-Glucose Co-Transporter-2 Inhibitors in Type 1 Diabetes: A Scoping Review. *Hormone Research in Paediatrics*, *96*(6), 620–630. https://doi.org/10.1159/000527653

Huang, C. C., Chen, T. H., Ho, C. H., Chen, Y. C., Chen, R. J., Wang, Y. J., Hsu, C. C., Lin, H. J., Wang, J. J., Chang, C. P., and Guo, H. R. (2022). Risks of Developing Diabetes and Hyperglycemic Crisis Following Carbon Monoxide Poisoning: A Study Incorporating Epidemiologic Analysis and Animal Experiment. *Clinical Epidemiology*, *14*, 1265–1279. https://doi.org/10.2147/CLEP.S380990

Human Tissue Act 2004. (2004). Available at: https://www.legislation.gov.uk/ukpga/2004/30/contents (Accessed: 05 April 2019).

International Diabetes Federation. (2021). *IDF Diabetes Atlas, 10th edition.* Available at: *https://www.diabetesatlas.org (Accessed: 28 September 2023).*

Jaffe, F, A. (1962). Chemical postmortem changes in the intraocular fluid. *Journal of Forensic Sciences*, *7*, 231–237.

James, J., Robinson, C., Mason, C., Richards, C., West, K., and Morgan, B. (2023). Impact of the COVID-19 pandemic on a post-mortem CT service for adult non-

suspicious death. *Clinical Radiology*, *78*(11), 822–831. https://doi.org/10.1016/j.crad.2023.03.013

Jashnani, K. D., Kale, S. A., and Rupani, A. B. (2010). Vitreous humor: Biochemical constituents in estimation of postmortem interval. *Journal of Forensic Sciences*, *55*(6), 1523–1527. https://doi.org/10.1111/j.1556-4029.2010.01501.x

Jia, W., Wang, C., Wang, Y., Pan, G., Jiang, M., Li, Z., and Zhu, Y. (2015). Qualitative and quantitative analysis of the major constituents in Chinese medical preparation lianhua-qingwen capsule by UPLC-DAD-QTOF–MS. *Scientific World Journal*, *2015*, 1–19. https://doi.org/10.1155/2015/731765

Joint Formulary Committee. (2018). *British National Formulary, 82*. London: BMJ and Pharmaceutical Press..

Kang, S. H., Jung, D. J., Choi, E. W., Cho, K. H., Park, J. W., and Do, J. Y. (2015). HbA1c levels are associated with chronic kidney disease in a non-diabetic adult population: A nationwide survey (KNHANES 2011-2013). *PLOS ONE*, *10*(12), 1–11. https://doi.org/10.1371/journal.pone.0145827

Karpievitch, Y. V., Polpitiya, A. D., Anderson, G. A., Smith, R. D., and Dabney, A. R. (2010). Liquid chromatography mass spectrometry-based proteomics: Biological and technological aspects. *Annals of Applied Statistics*, *4*(4), 1797–1823. https://doi.org/10.1214/10-AOAS341

Keltanen, T., Sajantila, A., Valonen, T., Partanen, T., and Lindroos, K. (2015). HbA1c method evaluation for postmortem samples. *Forensic Science, Medicine, and Pathology*, *11*(1), 35–39. https://doi.org/10.1007/s12024-014-9638-4

Keltanen, T., Sajantila, A., Valonen, T., Vanhala, T., and Lindroos, K. (2013). Measuring postmortem glycated hemoglobin - A comparison of three methods. *Legal Medicine*, *15*(2), 72–78. https://doi.org/10.1016/j.legalmed.2012.09.002

Kemp, W. L. (2016). Postmortem Change and its Effect on Evaluation of Fractures. *Academic Forensic Pathology*, *6*(1), 28–44. https://doi.org/10.23907/2016.004

Khanwalker, M., Fujita, R., Lee, J., Wilson, E., Ito, K., Asano, R., Ikebukuro, K., LaBelle, J., and Sode, K. (2022). Development of a POCT type insulin sensor employing anti-insulin single chain variable fragment based on faradaic electrochemical impedance spectroscopy under single frequency measurement. *Biosensors and Bioelectronics*, *200*, 1–17. https://doi.org/10.1016/j.bios.2021.113901

Kift, R. L., Byrne, C., Liversidge, R., Babbington, F., Knox, C., Binns, J., and Barth, J. H. (2015). The effect of storage conditions on sample stability in the routine clinical laboratory. *Annals of Clinical Biochemistry*, *52*(6), 675–679. https://doi.org/10.1177/0004563215580000

Kim, S., Yun, Y. M., Hur, M., Moon, H. W., and Kim, J. Q. (2011). The effects of anti-insulin antibodies and cross-reactivity with human recombinant insulin analogues in the E170 insulin immunometric assay. *Korean Journal of Laboratory Medicine*, *31*(1), 22–29. https://doi.org/10.3343/kjlm.2011.31.1.22

Kitabchi, A. E., Umpierrez, G. E., Miles, J. M., and Fisher, J. N. (2009). Hyperglycemic crises in adult patients with diabetes. *Diabetes Care*, *32*(7), 1335–1343. https://doi.org/10.2337/dc09-9032

Kokavec, J., Min, S. H., Tan, M. H., Gilhotra, J. S., Newland, H. S., Durkin, S. R., Grigg, J., and Casson, R. J. (2016). Biochemical analysis of the living human vitreous. *Clinical and Experimental Ophthalmology*, *44*(7), 597–609. https://doi.org/10.1111/ceo.12732

Korra, T., Perumal, T. N., and Thera, U. K. (2023). 'Multiomics analysis of rhizosphere and plant health', in J. A. Parray, N. Shameem, D. Egamberdieva, and R. Z. Sayyed (eds.) *Rhizobiome: Ecology, Management and* Application. London: Elsevier, 433–444. https://doi.org/10.1016/B978-0-443-16030-1.00022-5

Kramer, C. K., Retnakaran, R., and Zinman, B. (2021). Insulin and insulin analogs as antidiabetic therapy: A perspective from clinical trials. *Cell Metabolism*, *33*(4), 740–747. https://doi.org/10.1016/j.cmet.2021.03.014

Labay, L. M., Bitting, C. P., Legg, K. M., and Logan, B. K. (2016). The Determination of Insulin Overdose in Postmortem Investigations. *Academic Forensic Pathology*, *6*(2), 174–183. https://doi.org/10.23907/2016.019

Lakhtakia, R. (2013). The History of Diabetes. *Sultan Qaboos University Medical Journal*, *13*(3), 368–370. https://doi.org/10.1007/BF01831214

Laugesen, E., Østergaard, J. A., and Leslie, R. D. G. (2015). Latent autoimmune diabetes of the adult: Current knowledge and uncertainty. *Diabetic Medicine*, *32*(7), 843–852. https://doi.org/10.1111/dme.12700

Le Goff, M. M., and Bishop, P. N. (2008). Adult vitreous structure and postnatal changes. *Eye*, *22*(10), 1214–1222. https://doi.org/10.1038/eye.2008.21

Leeder, S. R. (2013). Medical Journal of Australia - 2013 - Leeder - The history of insulin the mystery of diabetes. *The Medical Journal of Australia*, *199*(4), 227.

Lefever, E., Vliebergh, J., and Mathieu, C. (2021). Improving the treatment of patients with diabetes using insulin analogues: current findings and future directions. *Expert Opinion on Drug Safety*, *20*(2), 155–169. https://doi.org/10.1080/14740338.2021.1856813

Lewis, G. F., and Brubaker, P. L. (2021). The discovery of insulin revisited: Lessons for the modern era. *Journal of Clinical Investigation*, *131*(1). https://doi.org/10.1172/JCI142239

Liew, G., Michaelides, M., and Bunce, C. (2014). A comparison of the causes of blindness. *BMJ Open*, *4*, 1–6.

Limenta, M., Ho, C. S. C., Poh, J. W. W., Goh, S. Y., and Toh, D. S. L. (2019). Adverse Drug Reaction Profile of SGLT2 Inhibitor-Associated Diabetic Ketosis/Ketoacidosis in Singapore and their Precipitating Factors. *Clinical Drug Investigation*, *39*(7), 683–690. https://doi.org/10.1007/s40261-019-00794-5

Little, R. R., and Sacks, D. B. (2009). HbA1c: How do we measure it and what does it mean? *Current Opinion in Endocrinology, Diabetes and Obesity*, *16*(2), 113–118. https://doi.org/10.1097/MED.0b013e328327728d

Lishman, S. (2020). *Cause of death list*. Available at: https://www.rcpath.org/static/c16ae453-6c63-47ff-8c45fd2c56521ab9/G199-Cause-of-death-list.pdf (Accessed: 14 August 2022).

Long, B., Lentz, S., and Gottlieb, M. (2021). Alcoholic Ketoacidosis: Etiologies, Evaluation, and Management. *Journal of Emergency Medicine*, *61*(6), 658–665. https://doi.org/10.1016/j.jemermed.2021.09.007

Ludbrook, J. (2010). Confidence in Altman-Bland plots: A critical review of the method of differences. *Clinical and Experimental Pharmacology and Physiology*, *37*(2), 143–149. https://doi.org/10.1111/j.1440-1681.2009.05288.x

Lukočiūtė, G., Ginčienė, K., Chmieliauskas, S., Laima, S., Stasiūnienė, J., Fomin, D., and Jasulaitis, A. (2019). The post-mortem evaluation of glucose concentration in blood and its diagnostical value. *CBU International Conference Proceedings*, *7*, 1–7. https://doi.org/10.12955/cbup.v7.1452

Madea, B., Kreuser, C., and Banaschak, S. (2001). Postmortem biochemical examination of synovial fluid - a preliminary study. *Forensic Science International*, *118*(2), 29–35.

Madea, B., and Rothschild, M. (2010). Medical mortuary examination: Determination of the cause and manner of death. *Deutsches Arzteblatt*, *107*(33), 575–588. https://doi.org/10.3238/arztebl.2010.0575

Marks, V. (2015). Insulin and the law. *Annals of Clinical Biochemistry*, *52*(6), 696–698. https://doi.org/10.1177/0004563215592652

Marks, V. (2019). *Murder and Attempted Murder with Insulin by Carers*. Florida: CRC Press.

Marks, V., and Richmond, C. (2008). Kenneth Barlow: the first documented case of murder by insulin. *Journal of the Royal Society of Medicine*, *101*(1), 19–21. https://doi.org/10.1258/jrsm.2007.071002

Marks, V., and Teale, J. D. (1999). Drug-induced hypoglycemia. *Endocrinology and Metabolism Clinics of North America*, *28*(3), 555–577. https://doi.org/10.1016/S0889-8529(05)70088-8

Maurer, H. H., and Meyer, M. R. (2016). High-resolution mass spectrometry in toxicology: current status and future perspectives. *Archives of Toxicology*, *90*(9), 2161–2172. https://doi.org/10.1007/s00204-016-1764-1

Mauri, S., Pandey, R., Rzeźnicka, I., Lu, H., Bonn, M., and Weidner, T. (2015). Bovine and human insulin adsorption at lipid monolayers: A comparison. *Frontiers in Physics*, *3*, 1–6. https://doi.org/10.3389/fphy.2015.00051

Mayya, V., Kamath S., S., and Kulkarni, U. (2021). Automated microaneurysms detection for early diagnosis of diabetic retinopathy: A Comprehensive review. *Computer Methods and Programs in Biomedicine Update*, *1*, 1–15. https://doi.org/10.1016/j.cmpbup.2021.100013

Mazur, A. (2011). Why were “starvation diets” promoted for diabetes in the pre-insulin period? *Nutrition Journal*, *10*(23), 1–9. https://doi.org/10.1186/1475-2891-10-23

McCleskey, B. C., Dye, D. W., and Davis, G. G. (2016). Review of Postmortem Interval Estimation Using Vitreous Humor: Past, Present, and Future. *Academic Forensic Pathology*, *6*(1), 12–18. https://doi.org/10.23907/2016.002

McGill, M., and Felton, A-M. (2007). New global recommendations: A multidisciplinary approach to improving outcomes in diabetes. *Primary Care Diabetes*, *1*(1), 49–55. https://doi.org/10.1016/j.pcd.2006.07.004

McIntyre, H. D., Catalano, P., Zhang, C., Desoye, G., Mathiesen, E. R., and Damm, P. (2019). Gestational diabetes mellitus. *Nature Reviews Disease Primers*, *5*(47), 1–19. https://doi.org/10.1038/s41572-019-0098-8

Meadway, C., George, S., and Braithwaite, R. (2002). Interpretation of GC–MS opiate results in the presence of pholcodine. *Forensic Science International*, *127*(1–2), 131–135. https://doi.org/10.1016/S0379-0738(01)00643-0

Medhe, S. (2018). Mass Spectrometry: Detectors Review. *Sharad Medhe. Mass Spectrometry: Detectors Review. Chemical and Biomolecular Engineering*, *3*(4), 51–58. https://doi.org/10.11648/j.cbe.20180304.11

Meleth, A. D., and Carvounis, P. E. (2014). Outcomes of Vitrectomy for Tractional Retinal Detachment in Diabetic Retinopathy. *International Ophthalmology Clinics*, *54*(2), 127–139. https://doi.org/10.1097/IIO.0000000000000021

Mihaleva, V. V., Vorst, O., Maliepaard, C., Verhoeven, H. A., de Vos, R. C. H., Hall, R. D., and van Ham, R. C. H. J. (2008). Accurate mass error correction in liquid chromatography time-of-flight mass spectrometry based metabolomics. *Metabolomics*, *4*(2), 171–182. https://doi.org/10.1007/s11306-008-0108-4

Ministry of Justice. (2017). *Coroners Statistics Annual 2017*. Available at: https://www.gov.uk/government/statistics/coroners-statistics-2016 (Accessed: 03 October 2020).

Ministry of Justice. (2022). *Guidance for registered medical practitioners on the Notification of Deaths Regulations.* Available at: https://www.gov.uk/government/publications/guidance-notes-for-completing-a-medical-certificate-of-cause-of-death/guidance-for-doctors-completing-medical-certificates-of-cause-of-death-in-england-and-wales-accessible-version (Accessed: 21 June 2023).

Ministry of Justice. (2024). *Coroners statistics 2023 England and Wales*. Available at: https://www.gov.uk/government/statistics/coroners-statistics-2023 (Accessed: 07 February 2024).

Mitchell, C., and McCleskey, B. (2023). Utility of a Handheld Blood Ketone Meter as a Postmortem Indicator of Diabetic Ketoacidosis. *American Journal of Forensic Medicine and Pathology*, *44*(1), 17–20. https://doi.org/10.1097/PAF.0000000000000794

Mitchell, R., Charlwood, C., Thomas, S. D., Bellis, M., and Langlois, N. E. I. (2013). An audit of the contribution to post-mortem examination diagnosis of individual analyte results obtained from biochemical analysis of the vitreous. *Forensic Science, Medicine, and Pathology*, *9*(4), 515–520. https://doi.org/10.1007/s12024-013-9469-8

Mitchell, R., Thomas, S. D., and Langlois, N. E. I. (2013). How sensitive and specific is urinalysis “dipstick” testing for detection of hyperglycaemia and ketosis? An audit of findings from coronial autopsies. *Pathology*, *45*(6), 587–590. https://doi.org/10.1097/PAT.0b013e3283650b93

Moein, M. M., El Beqqali, A., and Abdel-Rehim, M. (2017). Bioanalytical method development and validation: Critical concepts and strategies. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *1043*, 3–11. https://doi.org/10.1016/j.jchromb.2016.09.028

Moriyama, M., Hayashi, N., Ohyabu, C., Mukai, M., Kawano, S., and Kumagai, S. (2006). Performance Evaluation and Cross-Reactivity from Insulin Analogs with the ARCHITECT Insulin Assay. *Clinical Chemistry*, *52*(7), 1423–1426. https://doi.org/10.1373/clinchem.2005.065995

Mudaliar, S. R., Lindberg, F. A., Joyce, M., Beerdsen, P., Strange, P., Lin, A., and Henry, R. R. (1999). Insulin Aspart (B28 Asp-Insulin). *Diabetes Care*, *22*(9), 1501–1506.

Muddiman, D. C., Huang, B. M., Anderson, G. A., Rockwood, A., Hofstadler, S. A., Weir-Lipton, M. S., Proctor, A., Wu, Q., and Smith, R. D. (1997). Application of sequential paired covariance to liquid chromatography-mass spectrometry data. Enhancements in both the signal-to-noise ratio and the resolution of analyte peaks in the chromatogram. *Journal of Chromatography A*, *771*, 1–7.

Mulholland, O., and Mckenna, K. (2023). The bronze diabetic. *British Journal of Dermatology*, *188*(4), 113–115.

Mulla, A., Massey, K. L., and Kalra, J. (2005). Vitreous Humor Biochemical Constituents. *American Journal of Forensic Medicine and Pathology*, *26*(2), 146–149. https://doi.org/10.1097/01.paf.0000164001.50097.4c

Musshoff, F., Hess, C., and Madea, B. (2011). Disorders of glucose metabolism: Post mortem analyses in forensic cases-part II. *International Journal of Legal Medicine*, *125*(2), 171–180. https://doi.org/10.1007/s00414-010-0510-0

Mustafa, O. G., Haq, M., Dashora, U., Castro, E., and Dhatariya, K. K. (2023). Management of Hyperosmolar Hyperglycaemic State (HHS) in Adults: An updated guideline from the Joint British Diabetes Societies (JBDS) for Inpatient Care Group. *Diabetic Medicine*, *40*(3), 1–12. https://doi.org/10.1111/dme.15005

Mutter, C. M., Smith, T., Menze, O., Zakharia, M., and Nguyen, H. (2021). Diabetes Insipidus: Pathogenesis, Diagnosis, and Clinical Management. *Cureus*. https://doi.org/10.7759/cureus.13523

Nakamura, K., Inokuchi, R., Doi, K., Fukuda, T., Tokunaga, K., Nakajima, S., Noiri, E., and Yahagi, N. (2014). Septic ketoacidosis. *Internal Medicine*, *53*(10), 1071–1073. https://doi.org/10.2169/internalmedicine.53.1791

Nasa, P., Chaudhary, S., Shrivastava, P. K., and Singh, A. (2021). Euglycemic diabetic ketoacidosis: A missed diagnosis. *World Journal of Diabetes*, *12*(5), 514–523. https://doi.org/10.4239/wjd.v12.i5.514

Nedelkov, D., Niederkofler, E. E., Oran, P. E., Peterman, S., and Nelson, R. W. (2018). Top-down mass spectrometric immunoassay for human insulin and its therapeutic analogs. *Journal of Proteomics*, *175*, 27–33. https://doi.org/10.1016/j.jprot.2017.08.001

Nobelprize.org. (2023). *Rosalyn Yalow Facts*. Available at:
https://www.nobelprize.org/prizes/medicine/1977/yalow/facts/ (Accessed: 19 October 2023).

Nordquist, L., Brown, R., Fasching, A., Persson, P., and Palm, F. (2009). Proinsulin C-peptide reduces diabetes-induced glomerular hyperfiltration via efferent arteriole dilation and inhibition of tubular sodium reabsorption. *American Journal of Physiology-Renal Physiology*, *297*, 1265–1272. https://doi.org/10.1152/ajprenal.00228.2009.-C

Nyenwe, E. A., and Kitabchi, A. E. (2016). The evolution of diabetic ketoacidosis: An update of its etiology, pathogenesis and management. *Metabolism: Clinical and Experimental*, *65*(4), 507–521. https://doi.org/10.1016/j.metabol.2015.12.007

Oexle, H., Gnaiger, E., and Weiss, G. (1999). Iron-dependent changes in cellular energy metabolism: influence on citric acid cycle and oxidative phosphorylation. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, *1413*(3), 99–107. https://doi.org/10.1016/S0005-2728(99)00088-2

O'Grady, C. (2022). Unlucky numbers. *Science*, *379*(6629), 228–233.

Ojanperä, I., Sajantila, A., Vinogradova, L., Thomas, A., Schänzer, W., and Thevis, M. (2013). Post-mortem vitreous humour as potential specimen for detection of insulin analogues by LC–MS/MS. *Forensic Science International*, *233*(1–3), 328–332. https://doi.org/10.1016/j.forsciint.2013.10.009

Olsen, C., Wang, C., Abadpour, S., Lundanes, E., Hansen, A. S., Skottvoll, F. S., Scholz, H., and Wilson, S. R. (2023). Determination of insulin secretion from stem cell-derived islet organoids with liquid chromatography-tandem mass spectrometry. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *1215*. https://doi.org/10.1016/j.jchromb.2022.123577

O'Neal, K. S., Johnson, J. L., and Panak, R. L. (2016). Recognizing and appropriately treating latent autoimmune diabetes in adults. *Diabetes Spectrum*, *29*(4), 249–252. https://doi.org/10.2337/ds15-0047

Osborn, M., Howard, M., Morley, S., and McCarthy, H. (2018). *Guidelines on autopsy practice: Autopsy when drugs or poisoning may be involved*. Available at: https://www.rcpath.org/static/f855f467-c352-40a0-aba5c51fdcd4f876/G169-Guidelines-on-autopsy-practice-when-drugs-or-poisoning-may-be-involved.pdf (Accessed: 16 October 2022)

Osborn, M., Lucas, S., Stewart, R., Swift, B., and Youd, E. (2020). Briefing on COVID-19 Autopsy practice relating to possible cases of COVID-19 (2019-nCov, novel coronavirus from China 2019 / 2020). *The Royal College of Pathologists*, *19*(02), 1–14.

Oubré, A. Y., Carlson, T. J., King, S. R., and Reaven, G. M. (1997). From plant to patient: An ethnomedical approach to the identification of new drugs for the treatment of NIDDM. *Diabetologia*, *40*(5), 614–617. https://doi.org/10.1007/s001250050724

Pallotta, M. T., Tascini, G., Crispoldi, R., Orabona, C., Mondanelli, G., Grohmann, U., and Esposito, S. (2019). Wolfram syndrome, a rare neurodegenerative disease: From pathogenesis to future treatment perspectives. *Journal of Translational Medicine*, *17*(238), 1–12. https://doi.org/10.1186/s12967-019-1993-1

Palmiere, C. (2015). Postmortem diagnosis of diabetes mellitus and its complications. *Croatian Medical Journal*, *56*(3), 181–193. https://doi.org/10.3325/cmj.2015.56.181

Parfitt, C., Church, D., Armston, A., Couchman, L., Evans, C., Wark, G., and McDonald, T. J. (2015). Commercial insulin immunoassays fail to detect commonly prescribed insulin analogues. *Clinical Biochemistry*, *48*(18), 1354–1357. https://doi.org/10.1016/j.clinbiochem.2015.07.017

Parks, C., and Maskell, P. D. (2022). Medico-Legal Death Investigation Systems – Scotland. *Sri Lanka Journal of Forensic Medicine, Science and Law*, *13*(0), 47. https://doi.org/10.4038/sljfmsl.v13i0.7921

Passing, H., and Bablok, W. (1983). A New Biometrical Procedure for Testing the Equality of Measurements from Two Different Analytical Methods. Application of linear regression procedures for method comparison studies in Clinical

Chemistry, Part I. *Clinical Chemistry and Laboratory medicine*, *21*(11), 709–720. https://doi.org/10.1515/cclm.1983.21.11.709

Péclet, C., Picotte, P., and Jobin, F. (1994). The use of vitreous humor levels of glucose, lactic acid and blood levels of acetone to establish antemortem hyperglycemia in diabetics. *Forensic Science International*, *65*(1), 1–6. https://doi.org/10.1016/0379-0738(94)90293-3

Police and Criminal Evidence Act 1984. Available at: https://www.legislation.gov.uk/ukpga/1984/60/contents (Accessed: 18 June 2019).

Prentki, M., and Nolan, C. J. (2006). Islet β cell failure in type 2 diabetes. *Journal of Clinical Investigation*, *116*(7), 1802–1812. https://doi.org/10.1172/JCI29103

Public Health England. (2016). *Diabetes Prevalence Model*. Available at: https://assets.publishing.service.gov.uk/media/5a82c07340f0b6230269c82d/Diabetesprevalencemodelbriefing.pdf (Accessed: 12 July 2022).

Puchalska, P., and Crawford, P. A. (2017). Multi-dimensional Roles of Ketone Bodies in Fuel Metabolism, Signaling, and Therapeutics. *Cell Metabolism*, *25*(2), 262–284. https://doi.org/10.1016/j.cmet.2016.12.022

Quianzon, C. C., and Cheikh, I. (2012). History of insulin. *Journal of Community Hospital Internal Medicine Perspectives*, *2*(2), 18701. https://doi.org/10.3402/jchimp.v2i2.18701

Racsa, P. N., Meah, Y., Ellis, J. J., and Saverno, K. R. (2017). Comparative Effectiveness of Rapid-Acting Insulins in Adults with Diabetes. *Journal of Managed Care and Specialty Pharmacy*, *23*(3), 291. www.jmcp.org

Raczuk, E., Dmochowska, B., Samaszko-Fiertek, J., and Madaj, J. (2022). Different Schiff Bases—Structure, Importance and Classification. *Molecules*, *27*(3), 1–24. https://doi.org/10.3390/molecules27030787

Rajkumar, V., and Levine, S. N. (2020). *Latent Autoimmune Diabetes*. Available at: https://www.ncbi.nlm.nih.gov/books/NBK557897/ (Accessed: 27 December 2022)

Ramesh, R., Kanagasingam, A., Sabrina, S., and Anushanth, U. (2023). Starvation Ketoacidosis in a Young Healthy Female After Prolonged Religious Fasting. *Cureus*. https://doi.org/10.7759/cureus.39962

Rawla, P., Vellipuram, A. R., Bandaru, S. S., and Pradeep Raj, J. (2017). Euglycemic diabetic ketoacidosis: a diagnostic and therapeutic dilemma.

Endocrinology, Diabetes and Metabolism Case Reports, *17*, 1–4. https://doi.org/10.1530/edm-17-0081

Reed, G. F., Lynn, F., and Meade, B. D. (2002). Use of coefficient of variation in assessing variability of quantitative assays. *Clinical and Diagnostic Laboratory Immunology*, *9*(6), 1235–1239. https://doi.org/10.1128/CDLI.9.6.1235-1239.2002

Retnakaran, R., Ye, C., and Kramer, C. K. (2019). Stability of insulin and C-peptide measurement with long-term frozen storage of serum: Implications for diabetes research studies. *Diabetes, Obesity and Metabolism*, *21*(4), 1058–1060. https://doi.org/10.1111/dom.13617

Roberts, I. S. D., Gorodkin, L. M., and Benbow, E. W. (2000). What is a natural cause of death? A survey of how coroners in England and Wales approach borderline cases. *Journal of Clinical Pathology*, *53*(5), 367–373. https://doi.org/10.1136/jcp.53.5.367

Rocca, M. F., Zintgraff, J. C., Dattero, M. E., Santos, L. S., Ledesma, M., Vay, C., Prieto, M., Benedetti, E., Avaro, M., Russo, M., Nachtigall, F. M., and Baumeister, E. (2020). A combined approach of MALDI-TOF mass spectrometry and multivariate analysis as a potential tool for the detection of SARS-CoV-2 virus in nasopharyngeal swabs. *Journal of Virological Methods*, *286*, 1–7. https://doi.org/10.1016/j.jviromet.2020.113991

Roche (2017). *Elecsys Cobas® Insulin reagent kit*. [Assay regent kit insert].

Ronchetti, S., Ayroldi, E., Ricci, E., Gentili, M., Migliorati, G., and Riccardi, C. (2021). A Glance at the Use of Glucocorticoids in Rare Inflammatory and Autoimmune Diseases: Still an Indispensable Pharmacological Tool? *Frontiers in Immunology*, *11*, 1–11. https://doi.org/10.3389/fimmu.2020.613435

Russell, K. S., Stevens, J. R., and Stern, T. A. (2009). Insulin overdose among patients with diabetes: A readily available means of suicide. *Primary Care Companion to the Journal of Clinical Psychiatry*, *11*(5), 258–262. https://doi.org/10.4088/PCC.09r00802

Sachdeva, N., Yashoda, R., Ritu, S., and Atul, M. (2011). Estimation of Post-Mortem Interval from the Changes in Vitreous Biochemistry. *Journal of Indian Academy of Forensic Medicine*, *33*(2), 171–174.

Sacks, D. B. (2003). Hemoglobin Variants and Hemoglobin A 1c Analysis: Problem Solved? *Clinical Chemistry*, *49*(8), 1245–1247. https://academic.oup.com/clinchem/article/49/8/1245/5641785

Sadagopan, N. P., Li, W., Cook, J. A., Galvan, B., Weller, D. L., Fountain, S. T., and Cohen, L. H. (2003). Investigation of EDTA anticoagulant in plasma to improve the throughput of liquid chromatography/tandem mass spectrometric assays. *Rapid Communications in Mass Spectrometry*, *17*(10), 1065–1070. https://doi.org/10.1002/rcm.1023

Sahajpal, N. S., Jain, S., Vig, V., Singh, P., Singh, R., Singh, H., Singh, K., Goel, R., Yasuda, J., Wright, D., and Chaubey, A. (2018). Insulin in the vitreous humor of patients with proliferative diabetic retinopathy. *Investigative Ophthalmology and Visual Science*, *59*(9), 5366.

Samarasinghe, S., Meah, F., Singh, V., Basit, A., Emanuele, N., Emanuele, M. A., Mazhari, A., and Holmes, E. W. (2017). Biotin interference with routine clinical immunoassays: Understand the causes and mitigate the risks. *Endocrine Practice*, *23*(8), 989–996. https://doi.org/10.4158/EP171761.RA

Sapin, R., Le Galudec, V., Gasser, F., Pinget, M., and Grucker, D. (2001). Elecsys Insulin Assay: Free Insulin Determination and the Absence of Cross-Reactivity with Insulin Lispro. *Clinical Chemistry*, *47*(3), 602–605.

Sapin, R., Ongagna, J. C., Gasser, F., and Grucker, D. (1998). Insulin measurements in haemolysed serum: Influence of insulinase inhibitors. *Clinica Chimica Acta*, *274*(1), 111–117. https://doi.org/10.1016/S0009-8981(98)00051-5

Sarkar, S., and Balhara, Y. P. S. (2014). Diabetes mellitus and suicide. *Indian Journal of Endocrinology and Metabolism*, *18*(4), 468–474. https://doi.org/10.4103/2230-8210.137487

Shen, Y., Prinyawiwatkul, W., and Xu, Z. (2019). Insulin: a review of analytical methods. *The Analyst*, *144*(14), 4139–4148. https://doi.org/10.1039/C9AN00112C

Shi, R. Z., El Gierari, E. T. M., Manicke, N. E., and Faix, J. D. (2015). Rapid measurement of tacrolimus in whole blood by paper spray-tandem mass spectrometry (PS–MS/MS). *Clinica Chimica Acta*, *441*, 99–104. https://doi.org/10.1016/j.cca.2014.12.022

Siddamsetty, A. K., Verma, S. K., Kohli, A., Puri, D., and Singh, A. (2014). Estimation of time since death from electrolyte, glucose and calcium analysis of postmortem vitreous humour in semi-arid climate. *Medicine, Science and the Law*, *54*(3), 158–166. https://doi.org/10.1177/0025802413506424

Sidlak, A. M., Trautman, W. J., Lynch, M. J., and Marino, R. T. (2022). Alcoholic Ketoacidosis: Review of Current Practice and Association of Treatments to Improvement. *American Journal of Therapeutics*, *29*(4), 481–483. https://doi.org/10.1097/MJT.0000000000001323

Siemens (2022a). *ADVIA Centaur® CP Insulin (IRI)*. [Assay regent kit insert].

Siemens (2022b). *Atellica® IM Insulin (IRI)*. [Assay regent kit insert].

Siemens-DPC (2006). *Immulite® Insulin assay*. [Assay regent kit insert].

Simmons, E., Johnson, D., Delhaye, R., Colwell, J., Clor, E., Santa, P., and Bowsher, R. R. (2019). Development and validation of a noncompetitive electrochemiluminescence-based immunoassay (ECLIA) for specific determination of insulin lispro (Humalog®) in human serum to support pharmacokinetic assessments. *Journal of Immunoassay and Immunochemistry*, *40*(3), 314–327. https://doi.org/10.1080/15321819.2019.1596950

Simpson, P., Bates, D., Bonner, S., Costeloe, K., Doyal, L., Falvay, S., Gaffin, J., Howard, R., Kane, N., Kennedy, C., Kennedy, I., Kerr, S., Manara, A., Pickard, J., Rolles, K., and Short, A. (2008). *A code of practice for the diagnosis and confirmation of death*. Available at: https://www.aomrc.org.uk/wp-content/uploads/2016/04/Code_Practice_Confirmation_Diagnosis_Death_1008.pdf (Accessed: 18 December 2022).

Sindoni, A. (1937). Protamine insulin versus ordinary insulin. *Journal of the American Medical Association*, *108*(16), 1320. https://doi.org/10.1001/jama.1937.02780160012004

Siuzdak, G. (1994). Review The emergence of mass spectrometry in biochemical research. *Proceedings of the National Academy of Sciences*, *91*(24), 11290-11297. https://www.pnas.org

Soffe, R., Nock, V., and Chase, J. G. (2019). Towards Point-of-Care Insulin Detection. *ACS Sensors*, *4*(1), 3–19. https://doi.org/10.1021/acssensors.8b01253

Solis, E., Cameron-Burr, K. T., Shaham, Y., and Kiyatkin, E. A. (2017). Intravenous heroin induces rapid brain hypoxia and hyperglycemia that precede brain metabolic response. *ENeuro*, *4*(3). https://doi.org/10.1523/ENEURO.0151-17.2017

Stephenson, L., van den Heuvel, C., Humphries, M., and Byard, R. W. (2022). Characteristics of fatal insulin overdoses. *Forensic Science, Medicine, and Pathology*, *18*(4), 429–441. https://doi.org/10.1007/s12024-022-00511-3

Steveling, A., Völzke, H., Nauck, M., and Wallaschofski, H. (2009). HbA1c determination – A new diagnostic criterion?. *LaboratoriumsMedizin*, *33*(1). https://doi.org/10.1515/jlm.2009.003et

Stoner, G. D. (2017). Hyperosmolar Hyperglycemic State. *American Family Physician*, *96*(11), 729–736. www.aafp.org/afp

Sun, H., Saeedi, P., Karuranga, S., Pinkepank, M., Ogurtsova, K., Duncan, B. B., Stein, C., Basit, A., Chan, J. C. N., Mbanya, J. C., Pavkov, M. E., Ramachandaran, A., Wild, S. H., James, S., Herman, W. H., Zhang, P., Bommer, C., Kuo, S., Boyko, E. J., and Magliano, D. J. (2022). IDF Diabetes Atlas: Global, regional and country-level diabetes prevalence estimates for 2021 and projections for 2045. *Diabetes Research and Clinical Practice*, *183*, 1–13. https://doi.org/10.1016/j.diabres.2021.109119

Sundararajan, C., Besanger, T. R., Labiris, R., Guenther, K. J., Strack, T., Garafalo, R., Kawabata, T. T., Finco-Kent, D., Zubieta, J., Babich, J. W., and Valliant, J. F. (2010). Synthesis and Characterization of Rhenium and Technetium-99m Labeled Insulin. *Journal of Medicinal Chemistry*, *53*(6), 2612–2621. https://doi.org/10.1021/jm100096c

Tan, A., and Awaiye, K. (2013). 'Use of Internal Standards in LC-MS Bioanalysis', in Wenkui, L., Zhang, J., and Tse, F.L.S. (eds.) *Handbook of LC-MS Bioanalysis*. Hoboken: John Wiley & Sons Inc, 217–227. https://doi.org/10.1002/9781118671276.ch17

Tao, T., Xu, J., Luo, T.-X., Liao, Z.-G., and Pan, H.-F. (2006). Contents of vitreous humor of dead body with different postmortem intervals. *Journal of Sichuan University. Medical Science Edition*, *37*(6), 898–900, 927. http://www.ncbi.nlm.nih.gov/pubmed/17236589

Teague, T. (2022). *Chief Coroner's Guidance No.34 COVID-19*. Courts and Tribunals Judiciary. Available at: https://www.judiciary.uk/guidance-and-resources/chief-coroners-guidance-no-34-covid-19/ (Accessed: 26 May 2023).

Thevis, M., Thomas, A., Schänzer, W., Östman, P., and Ojanperä, I. (2012). Measuring insulin in human vitreous humour using LC–MS/MS. *Drug Testing and Analysis*, *4*(1), 53–56. https://doi.org/10.1002/dta.368

Thomason, M. J., Biddulph, J. P., Cull, C. A., and Holman, R. R. (2005). Reporting of diabetes on death certificates using data from the UK Prospective Diabetes Study. *Diabetic Medicine*, *22*(8), 1031–1036. https://doi.org/10.1111/j.1464-5491.2005.01584.x

Tosur, M., and Philipson, L. H. (2022). Precision diabetes: Lessons learned from maturity-onset diabetes of the young (MODY). *Journal of Diabetes Investigation*, *13*(9), 1465–1471. https://doi.org/10.1111/jdi.13860

Tracy, J. A., and Dyck, P. J. B. (2008). The Spectrum of Diabetic Neuropathies. *Physical Medicine and Rehabilitation Clinics of North America*, *19*(1), 1–26. https://doi.org/10.1016/j.pmr.2007.10.010

Trambas, C., Lu, Z., Yen, T., and Sikaris, K. (2018). Characterization of the scope and magnitude of biotin interference in susceptible Roche Elecsys competitive and sandwich immunoassays. *Annals of Clinical Biochemistry*, *55*(2), 205–215. https://doi.org/10.1177/0004563217701777

Traub, F. (1969). Methode zur Erkennung von tödlichen Zuckerstoffwechselstörungen an der Leiche. *Zbl Alla Path*, *112*, 390–399.

Tumram, N. K., Bardale, R. V., and Dongre, A. P. (2011). Postmortem analysis of synovial fluid and vitreous humour for determination of death interval: A comparative study. *Forensic Science International*, *204*(1–3), 186–190. https://doi.org/10.1016/j.forsciint.2010.06.007

Tuomi, T., Groop, L. C., Zimmet, P. Z., Rowley, M. J., Knowles, W., and Mackay, I. R. (1993). Antibodies to Glutamic Acid Decarboxylase Reveal Latent Autoimmune Diabetes Mellitus in Adults With a Non—Insulin-Dependent Onset of Disease. *Diabetes*, *42*(2), 359–362. https://doi.org/10.2337/diab.42.2.359

UK Health Security Agency. (2023). *COVID-19 confirmed deaths in England (to 31 December 2022): report*. Available at: https://www.gov.uk/government/publications/covid-19-reported-sars-cov-2-deaths-in- england/covid-19-confirmed-deaths-in-england-to-31-december-2022-report (Accessed: 06 January 2024).

UKAS. (2023). *UKAS accreditation of laboratories performing analysis of toxicology samples*. Available at: https://www.ukas.com/wp-content/uploads/2021/06/LAB-51-UKAS-Accreditation-of-Laboratories-Performing-Analysis-of-Toxicology-Samples.pdf (Accessed: 22 January 2024).

Vaishya, R., Gupta, R., and Arora, S. (2010). Macroprolactin; a Frequent Cause of Misdiagnosed Hyperprolactinemia in Clinical Practice. *Journal of Reproduction and Infertility*, *11*(3), 161–167. http://www.ncbl.nlm.nih.gov

Vallecillo, G., Robles, M. J., Torrens, M., Samos, P., Roquer, A., Martires, P. K., Sanvisens, A., Muga, R., and Pedro-Botet, J. (2018). Metabolic Syndrome among Individuals with Heroin use Disorders on Methadone Therapy:

Prevalence, Characteristics, and Related Factors. *Substance Abuse*, *39*(1), 46–51. https://doi.org/10.1080/08897077.2017.1363122

van der Gugten, J. G. (2020). Tandem mass spectrometry in the clinical laboratory: A tutorial overview. *Clinical Mass Spectrometry*, *15*, 36–43. https://doi.org/10.1016/j.clinms.2019.09.002

van Leuven, S. I., and Kastelein, J. J. P. (2005). Atorvastatin. *Expert Opinion on Pharmacotherapy*, *6*(7), 1191–1203. https://doi.org/10.1517/14656566.6.7.1191

Vass, A. A. (2001). Beyond the grave – understanding human decomposition. *Microbiology Today*, *28*, 190–193.

Vass, A. A. (2007). *After a person's pulse and breathing stop, how much later does all cellular metabolism stop.* Available at: https://www.scientificamerican.com/article/experts-cell-metabolism-after-death/ (Accessed: 03 January 2024).

Vonderau, J. S., and Desai, C. S. (2022). Type 3c: Understanding pancreatogenic diabetes. *Journal of the American Academy of Physician Assistants*, *35*(11), 20–24. https://doi.org/10.1097/01.JAA.0000885140.47709.6f

Wallace, T. M., and Matthews, D. R. (2002). The assessment of insulin resistance in man. *Diabetic Medicine*, *19*(7), 527–534. https://doi.org/10.1046/j.1464-5491.2002.00745.x

Wallace, T. M., and Matthews, D. R. (2004). Recent advances in the monitoring and management of diabetic ketoacidosis. *QJM: An International Journal of Medicine*, *97*(12), 773–780. https://doi.org/10.1093/qjmed/hch132

Walsh, G. (2005). Therapeutic insulins and their large-scale manufacture. *Applied Microbiology and Biotechnology*, *67*(2), 151–159. https://doi.org/10.1007/s00253-004-1809-x

Walsh, J. W., Hoffstad, O. J., Sullivan, M. O., and Margolis, D. J. (2016). Association of diabetic foot ulcer and death in a population-based cohort from the United Kingdom. *Diabetic Medicine*, *33*(11), 1493–1498. https://doi.org/10.1111/dme.13054

Weiss, M. A. (2009). Proinsulin and the genetics of diabetes mellitus. *Journal of Biological Chemistry*, *284*(29), 19159–19163. https://doi.org/10.1074/jbc.R109.009936

Wilcox, G. (2005). Insulin and Insulin Resistance. *Clinical Biochemistry Review*, *26*, 19–39.

Wilkins, B. (2015). *The retention and storage of pathological records and specimens (5th edition)*. Available at: https://www.rcpath.org/static/049ea966-df5c-4a9f-9353ba24a69bb808/The-retention-and-storage-of-pathological-records-and-specimens-5th-edition.pdf (Accessed: 23 November 2020).

Wills, E. (1997). in Gillham, B., Papachristodoulou, D., and Thomas, J. H. (eds.), *Will's biochemical basis of medicine 3rd edition*. Oxford: Taylor and Francis.

Wise, J. (2011). Police investigate hospital deaths after contamination of saline solution. *BMJ (Clinical Research Ed.)*, *343*(d4707), 1. https://doi.org/10.1136/bmj.d4596

Wiss, D. A. (2019). The Relationship between Alcohol and Glycohemoglobin: A Biopsychosocial Perspective. *BioResearch Open Access*, *8*(1), 146–154. https://doi.org/10.1089/biores.2019.0009

Wollmer, A., Rannefeld, B., Johansen, R. R., Hejnaes, K. R., Balschmidt, P., and Hansen, F. B. (1987). Phenol-Promoted Structural Transformation of Insulin in Solution. *Biological Chemistry Hoppe-Seyler*, *368*(2), 903–912. https://doi.org/10.1515/bchm3.1987.368.2.903

Wu, L., Fernandez-Loaiza, P., Sauma, J., Hernandez-Bogantes, E., and Masis, M. (2013). Classification of diabetic retinopathy and diabetic macular edema. *World Journal of Diabetes*, *4*(6), 290–294. https://doi.org/10.4239/wjd.v4.i6.290

Wunder, C., Kauert, G. F., and Toennes, S. W. (2014). Factors leading to the degradation/loss of insulin in postmortem blood samples. *Forensic Science International*, *241*, 173–177. https://doi.org/10.1016/j.forsciint.2014.06.003

Yalow, R. S., and Berson, S. A. (1960). Immunoassay of endogenous plasma insulin in man. *Journal of Clinical Investigation*, *39*(7), 1157–1175. https://doi.org/10.1172/JCI104130

Yanai, H., Yoshida, H., Tomono, Y., and Tada, N. (2008). Severe hypoglycemia in a patient with anorexia nervosa. *Eating and Weight Disorders - Studies on Anorexia, Bulimia and Obesity*, *13*(1), e1–e3. https://doi.org/10.1007/BF03327785

Yang, Y., Hua, Q. X., Liu, J., Shimizu, E. H., Choquette, M. H., Mackin, R. B., and Weiss, M. A. (2010). Solution structure of proinsulin: Connecting domain flexibility and prohormone processing. *Journal of Biological Chemistry*, *285*(11), 7847–7851. https://doi.org/10.1074/jbc.C109.084921

Yin, T., and Herskovits, A. Z. (2022). The Impact of Hemolysis-Index Thresholds on Plasma and Serum Potassium Measurements. *Journal of Applied Laboratory Medicine*, *7*(3), 788–793. https://doi.org/10.1093/jalm/jfab156

Yorker, B. C., Kizer, K. W., Lampe, P., Forrest, A. R. W., Lannan, J. M., and Russell, D. A. (2006). Serial murder by healthcare professionals. *Journal of Forensic Sciences*, *51*(6), 1362–1371. https://doi.org/10.1111/j.1556-4029.2006.00273.x

Zaykov, A. N., Mayer, J. P., and Dimarchi, R. D. (2016). Pursuit of a perfect insulin. *Nature Reviews Drug Discovery*, *15*(6), 425–439. https://doi.org/10.1038/nrd.2015.36

Zhong, J. (2013).'Endoplasmic Reticulum (ER) Stress in the Pathogenesis of Type 1 Diabetes', in Escher, A., and Long, S. A. (eds.), *Type 1 Diabetes*. London: InTech Open. https://doi.org/10.5772/52644

Zilg, B. (2015). *Postmortem analyses of vitreous fluid*. [Doctoral thesis], Solna, Sweden: Karolinska Institutet.

Zilg, B., Alkass, K., Berg, S., and Druid, H. (2009). Postmortem identification of hyperglycemia. *Forensic Science International*, *185*(1–3), 89–95. https://doi.org/10.1016/j.forsciint.2008.12.017

Zilg, B., Alkass, K., Berg, S., and Druid, H. (2016). Interpretation of postmortem vitreous concentrations of sodium and chloride. *Forensic Science International*, *263*, 107–113. https://doi.org/10.1016/j.forsciint.2016.04.006

Zilg, B., Alkass, K., Kronstrand, R., Berg, S., and Druid, H. (2022). A rapid method for postmortem vitreous chemistry—deadside analysis. *Biomolecules*, *12*(1). https://doi.org/10.3390/biom12010032

Zimmet, P. Z., Tuomi, T., Mackay, I. R., Rowley, M. J., Knowles, W., Cohen, M., and Lang, D. A. (1994). Latent autoimmune diabetes mellitus in adults (LADA): the role of antibodies to glutamic acid decarboxylase in diagnosis and prediction of insulin dependency. *Diabetic Medicine*, *11*(3), 299–303.

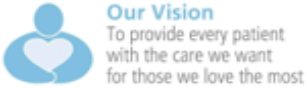
Zornitzki, T., Blaychfeld-Magnazi, M., Knobler, H., and Ulman, M. (2015). The effect of phlebotomy induced haemolysis on insulin level. *Endocrine Practice: Official Journal of the American College of Endocrinology and the American Association of Clinical Endocrinologists*, *21*(10), 1093–1097. https://doi.org/10.4158/EP15698.OR

Zubkiewicz-Kucharska, A., Chrzanowska, J., and Noczyńska, A. (2019). Hyperosmolar hyperglycaemic state (HHS) as the first manifestation of type 2

diabetes in a child. *Pediatric Endocrinology, Diabetes and Metabolism*, *25*(2), 85–89. https://doi.org/10.5114/pedm.2019.85819

Appendix 1: Request for information from Coroners

AP 1.1 Coroners audit stage 2 (as per Section 4.2.1)



Norfolk and Norwich University Hospitals NHS
NHS Foundation Trust

Toxicology & Endocrinology Section,

Department of Clinical Biochemistry,

Norfolk and Norwich University Hospital,

Colney Lane,

NR4 7UY.

(01603) 287430

Paul.brookes@nnuh.nhs.uk

Dear Sir or Madam,

RE: PhD project investigating changes in case numbers and biochemical analysis for diabetes related deaths

My name is Paul Brookes, Chief Biomedical Scientist for the Toxicology & Endocrinology section, Department of Biochemistry, at the Norfolk and Norwich University Hospital.

In the past five years we have seen a significant change in the total number of cases related to the investigation of glucose/diabetes-related deaths, and as a result, in the type of biochemical markers that have been requested for testing.

I am currently working on a part-time PhD at the University of East London related to my work in this area, and I would be most grateful if you could please spare some time to help with this project.

The project is split into two sections:

(i.) to develop a more accurate analytical method for the measurement for insulin concentrations in post-mortem samples

(ii.) to assess whether the changes in case numbers and test requests noticed locally are reflected UK-wide, or vary with the characteristics of each region in the UK (i.e. rural, city, etc.).

I understand how busy the Coroners' Offices are in the UK, but your help would be most appreciated, answering a few questions, and also if you would be willing to participate in the second stage of the audit.

The follow-up questionnaire will involve some short questions, using a platform such as Survey Monkey, to gather further information regarding the demographics of the region you cover, and the tests used to confirm diabetes mellitus-related deaths. This second questionnaire is not intended to be sent out until, or after, February 2019.

However, if you could provide answers/data for all, or some, of the following questions it would be a great help:

- What is the total number of deaths reported in your region in each year from 2011 to 2017 (inclusive), and how many of these are related to diabetes mellitus?

- Approximately what percentage of deaths in the region would be processed by the Coroner's Office, and what percentage would be processed by the Register Office?

- In the years 2011 to 2017 (inclusive), how many insulin results have been requested by, or reported to, the Coroner's Office?

- If this information is available, would it be possible to comment on the laboratory analysis that is undertaken regularly in cases of diabetes-related deaths? Are any of the following test results provided by the laboratory?
    - Glucose – Y/N
    - Insulin – Y/N
    - Other blood chemistry (e.g. β-hydroxybutyrate) – Y/N
    - Vitreous humour analysis – Y/N
- Would you like to receive a follow-up questionnaire/survey that will also contain feedback from this study? – Y/N

If you are happy to participate in the follow-up questionnaire please supply a suitable email address on which I may contact you.

Thank you very much for any help you can provide, I am happy to receive response to this letter either by post or via my email address. If you have any questions regarding the project please do not hesitate to contact me

Yours sincerely,

Paul Brookes

AP 1.2 Coroners audit stage 3 (as per Section 4.2.1)

Analytical and Specialist Chemistry (ASC) section,

Norfolk and Norwich University Hospital,

Colney Lane,

NR4 7UY.

(01603) 287430

paul.brookes@nnuh.nhs.uk

paulbrookes@nhs.net

Dear Sir or Madam,

RE: PhD project investigating changes in case numbers and biochemical analysis for diabetes related deaths.

Firstly, thank you for returning information in the first phase of my audit of Coroner's practises related to the investigation of dysglycaemia-related deaths. I appreciate the first contact was in 2018 and at the time you or your office indicated a willingness to be involved in a follow-up request for data, but the 'world has changed' since then, with Covid. I have slimmed down from the planned site-visits and appreciate that staff are also not available to access large amounts of data, so hope the following questionnaire can be completed without too much inconvenience to yourselves.

- Are samples routinely collected for insulin analysis – Y/N

    - If yes would these be blood, vitreous humour, or both

- Is there any documentation relating to sample collection times and laboratory interventions? (such as, centrifugation)
- Are there samples rejected/no results provided for plasma insulin? –Y/N
    - If yes does this affect the interpretation of other results?
- What are the main stated reason for no result be reported?
    - Haemolysed sample
    - Insufficient sample collected
    - Inappropriate storage
    - Other – please comment
- Is the Coroner and/or Coroner's officer aware of the protocol in the investigation of hyperglycaemic (high blood glucose) related deaths, to have a β-hydroxybutyrate and glucose result reported?
- Does the Coronial service believe there is a need for the standardisation of dysglycaemia-related deaths?
- Would you like a copy of the final thesis, and/or be involved in implementing learning outcomes?

Any questions, please do not hesitate to contact me either by email or phone.

Yours sincerely,

Paul Brookes

Appendix 2: Correspondence with insulin manufacturers

AP2.1: Initial contact email

Thu 18/01/2024 16:36

Brookes, Paul (NNUHFT)

Post-mortem insulin PhD project: request for information

To UKMedInfo@lilly.com; enquiries@wockhardt.co.uk; uk-medicalinformation@sanofi.com; reception@viatris.com

Hello,

Sorry to bother you, I am a Biomedical Scientist at the Norfolk and Norwich University Hospital, and am completing a PhD at the University of East London. My thesis project is to develop and evaluate the use of QToF-MS method for the analysis of post-mortem vitreous humour insulin, particularly insulin analogues. I have also engaged a number of Coroner's offices and the hope is that the learning outcomes from this thesis could help improve the investigation of glucose related-deaths.

I am contacting you to enquire what assays, methods and/or platforms are used in the evaluation and confirmation of your insulin products are? I understand some of this information may be commercially sensitive, but any information regarding the methods and assays would help and be gratefully received.

Thanks

Paul

Paul Brookes

Chief Biomedical Scientist - Analytical and Specialist Chemistry section | Honorary Lecturer - UEA
Eastern Pathology Alliance (EPA)
Norfolk and Norwich University Hospital | James Paget University Hospital | Queen Elizabeth Hospital Kings Lynn
01603 287430 | Ext 3430
Norfolk & Norwich University Hospital, Level 1, East Block, Colney Lane, Norwich, Norfolk, NR4 7UY, England

Appendix 3: Ethical approval

AP 3.1: Original approval from NNUH & UEA ethics committee

Faculty of Medicine and Health Sciences Research Ethics Committee

University of East Anglia

Research & Enterprise Services
West Office (Science Building)
University of East Anglia
Norwich Research Park
Norwich, NR4 7TJ

Telephone: +44 (0) 1603 591490

Email: fmh.ethics@uea.ac.uk

Web: www.uea.ac.uk/researchandenterprise

Paul Brookes
NNUH

12/5/16

Dear Paul,

Project title: Impact of post mortem insulin analysis in varied matrix on the confirmation of suspected hypoglycaemic related death
Reference: 2015/16 77 HT

The submission of your above proposal for the use of Human Tissue has been considered by a Sub-Committee of the Faculty Research Ethics Committee and we can confirm that your proposal has been approved.

Please could you ensure that any further amendments to either the protocol or documents submitted are notified to us in advance and also that any adverse events which occur during your project are reported to the Committee. Please could you also arrange to send us a report once your project is completed.

Can I remind you that any tissue used should be destroyed at the end of the experiment, or, with prior arrangement returned to the Tissue Bank.

We would like to remind you that following clarification of the NRES approval for the Biorepository/tissue bank to collect tissue, it is now a requirement that researchers such as yourself collecting tissue using the NNUH biorepository process are obliged to bank some of their sample with the tissue bank. This banking will allow other researchers to potentially access and use these samples. Therefore, you will be required to bank some of the tissue that you are collecting as a result of your amendment. Mark Wilkinson will be able to offer you guidance as to how much and in what form you should bank this tissue.

The Committee would like to wish you good luck with your project

Yours sincerely

[redacted]

Linda Harvey
Chair Human Tissue - FMH Research Ethics Committee

AP 3.2: Change of project title

University of East Anglia
Norwich Research Park
Norwich. NR4 7TJ

Email: ethicsapproval@uea.ac.uk
Web: www.uea.ac.uk

Study title: Impact of post mortem insulin analysis in varied matrix on the confirmation of suspected hypoglycaemic related deaths

Application ID: ETH2223-0877 (Amendment prior to EM)

Dear Paul,

Your amendment to your study was considered on 18th November 2022 by the FMH S-REC (Faculty of Medicine and Health Sciences Research Ethics Subcommittee).

The decision is: **approved**.

Researcher	Mr Paul Brookes Mr Paul Brookes
Date	21 Nov 2022
Academic year	2022 - 2023
Ethics reviewers	FMH S-REC (Faculty of Medicine and Health Sciences Research Ethics Subcommittee)
Project title	Advancing insulin analysis in varied matrices for the investigation of suspected hypoglycaemic death
Original UEA ethics review body	FMH S-REC (Faculty of Medicine and Health Sciences Research Ethics Subcommittee)