School of Pharmacy

Evaluation of the Stability of Exenatide in Aqueous Solutions

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Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

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Date: 15/1/15.....

Abstract

Introduction: Diabetes mellitus (DM) is a metabolic disorder characterised by elevated blood glucose levels, altered metabolism of carbohydrates, lipids and proteins. It also increases the risk of complications from cardiovascular diseases. In 2013 it is estimated that 6% of the world's population suffer from diabetes and this is increasing at an alarming rate. The current antidiabetic medications are effective in controlling blood glucose levels, however, they are associated with weight gain, hypoglycaemic episodes, inflammation and build-up of free radicals and toxins. Some of the current medications include metformin, sulfonylureas, glitazones, sodium-glucose co-transporter 2 inhibitors, insulin, dipeptidyl peptidase 4 inhibitors and incretin mimetics. Exenatide is a new antidiabetic drug, and one of the most commonly used Incretin Mimetics, which stimulates insulin secretion, suppresses glucagon secretion and inhibits gastric emptying, which results in reduced appetite and food intake. It also has beneficial antioxidant and antiradical effects. However, exenatide has poor oral absorption and stability profile which limits its clinical benefits in diabetes treatment. Thus, this study aimed to evaluate the stability and degradation profiles of exenatide at various pH values and temperatures.

Methodology:

An HPLC method was developed to determine the concentrations of exenatide in various aqueous formulations. Exenatide concentrations were measured using an HPLC system equipped with UV-detection set at 225nm and the mobile phase consisted of 36% acetonitrile, 64% Milli-Q water, 0.1% trifluoroacetic acid and 10mM triethylamine with a final pH of 3.00. The stability of exenatide was investigated in pH 2.00 (chloroacetate), 3.50 (citrate), 4.50 (acetate), 6.00 (citrate), 7.00 (citrate), 8.00 (Tris), and 10.00 (ethanolamine) at 40 °C and buffer concentration of 10mM. In order to obtain the shelf-life, exenatide stability was also investigated at pH 2.00, 4.50 and 10.00 at 35 °C and 45 °C. The effect of ionic strength on the stability of exenatide was also investigated.

Results and discussion: The marketed formulation of exenatide has a pH value of 4.50. However, exenatide is most stable at pH 6.00 and the reaction

rate was highly temperature dependent. Exenatide degradation followed first order kinetics under all conditions employed. At 40 °C, exenatide was most stable at pH 6.00 with a reaction rate constant of $1.11x10^{-3}$ h^{-1} while it was least stable at pH 10.00 with a reaction rate constant of $1.89x10^{-2}$ h^{-1}. The reaction rate constant at pH 4.50 is $1.15x10^{-2}$ h^{-1}, which is considerably higher than at pH 6.00. Exenatide had the highest activation energy value at pH 10.00 (138±7 kJ/mol) followed by pH 2.00 (124±3 kJ/mol) and the lowest at pH 4.50 (121±5 kJ/mol). There was no significant effect of changing the ionic strength on exenatide stability or degradation suggesting reasonable resistance to addition of electrolyte.

Conclusion: Exenatide was more stable at pH 6.00, compared with its current marketed formulation (Byetta) which is formulated at pH 4.50. The reason why exenatide was formulated at pH 4.50 has not been disclosed by the manufacturer. Future studies should aim at investigating a more stable exenatide formulation and a more convenient route of administration.

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Abbreviations

DM	Diabetes mellitus
T1D	Type 1 diabetes
T2D	Type 2 diabetes
GD	Gestational diabetes
IDDM	Insulin-dependent diabetes mellitus
NIDDM	Noninsulin-dependent diabetes mellitus
GLUT	Glucose transporter
CHD	Coronary heart disease
ESRD	End stage renal disease
DPP-4	Dipeptidyl peptidase-4
ATP	Adenosine triphosphate
TZD	Thiazolidinedione
HbA1c	Glycated haemoglobin
SGLT2	Sodium-glucose cotransporter-2
UTI	Urinary tract infection
ACE	Angiotensin converting enzyme
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide-1
GLP-1R	Glucagon-like peptide-1 receptor
CHF	Congestive heart failure
CRP	C-reactive protein
T_{max}	Time to reach peak concentration
HPLC	High performance liquid chromatography
UV	Ultraviolet
MS	Mass spectrometry
HCl	Hydrochloric acid
NaOH	Sodium hydroxide
AUC	Area under the curve
R^2	Coefficient of determination
%RSD	Percent relative standard deviation
k_{obs}	Observed rate constant

Ea	Activation energy
T_{90}	Shelf-life
mL	Millilitre
M	Molar (Mole/Litre)
v/v	Volume per volume
Min	minute
J	Joule
h	hour
T	Temperature in Kelvin
mM	Millimolar
PLGA	Poly(D,L-lactic-co-glycolic acid)

Chapter One

1 General Introduction

Diabetes mellitus

1.1 Introduction

Diabetes mellitus (DM), often referred to as diabetes, is a metabolic disorder characterised by hyperglycaemia, altered metabolism of carbohydrates, lipids and proteins, and an elevated risk of complications from cardiovascular disease (1). In DM, blood glucose levels are elevated, either because the pancreas does not produce enough insulin or because cells do not respond to existing insulin. The main symptoms include polydipsia (excessive thirst), polyuria (frequent urination), polyphagia (increased hunger), feeling lethargic, and slow healing of cuts and wounds (1). DM can lead to microvascular and macrovascular complications such as retinopathy, nephropathy and atherosclerosis (2-4).

DM is classified as Type 1 diabetes (T1D), Type 2 diabetes (T2D) or gestational diabetes (GD). T1D is an autoimmune disease manifested by the destruction of the β-cells of the pancreas resulting in a partial or complete lack of insulin production and the inability of the body to control glucose homeostasis (5). T1D is also known as juvenile-onset diabetes because it manifests at a young age. As it requires the patient to inject insulin to supplement the partial or complete lack of insulin production by the pancreas, it is also called insulin-dependent diabetes mellitus (IDDM) (6). T2D, formerly known as noninsulin-dependent diabetes mellitus (NIDDM) or adult-onset diabetes, is a metabolic disorder with onset most common in middle age and later life (7). T2D may be controlled by diet and exercise and, unlike T1D, does not always require the use of insulin (8). However, the term “noninsulin-dependent” is a misnomer since many patients require insulin therapy at some time in the course of their disease (9). T2D is often associated with obesity, hypertension and insulin resistance (10) and can result in the complete destruction of β-cells of the pancreas leading to the need to inject insulin similar to that in T1D (11). Moreover, GD occurs in pregnant women especially during third trimester (12, 13). Women who develop GD are at higher risk of developing T2D after giving birth (14, 15). An Australian study has found that

women who had GD are about 10 times more likely to develop T2D compared to those who did not develop GD (16).

In 2010, the prevalence of DM worldwide was estimated to be 285 million, which accounted for 6 % of the world's population, and by 2030, this value is expected to increase to 8% (16). The predominant form of diabetes is T2D and it accounts for 90% of all DM cases (16). Developing countries have a greater share of the increase in the prevalence of diabetes. This is possibly due to the increase in prevalence of overweight and obesity caused by the changes towards western food. Until 1990, T2D was rare in young people and in pregnant women, but this has changed and it is becoming more common.

In Australia, diabetes is the 6th highest cause of death and the fastest growing chronic illness. Diabetic patients are twice as likely to have high blood pressure, cholesterol disturbances and heart disease. In 2013, it was estimated that 1 in 4 Australian adults has either diabetes or pre-diabetes (17), with one person being diagnosed every 5 minutes. Over 3.6 million Australians are diabetic or pre-diabetic and the prevalence is increasing by 7% every year. Diabetes costs Australia more than $15 billion a year and this is not including the emotional costs arising from higher mortality rate, impaired performance of workers with diabetes and lower life expectancy.

Globally, diabetes affects more than 250 million people with more than half in the 40-65 age group (18, 19). It is predicted that the total number of diabetics will surpass all current predictions within the coming 20 years if no new or substantially better antidiabetic drugs are invented. New drugs need to be able to better control insulin secretion and glucose haemostasis, as well as being able to ameliorate diabetes-associated complications.

1.1.1 Insulin secretion and glucose regulation

Glucose is a major source of energy with the normal range in blood being 3.5-6.0 mmol/l (20). When the body is at rest (also known as the basal state), the consumption of glucose is equal to its production and there is no net gain (21). When glucose is absorbed into the systemic circulation and the body is not utilising it for energy production, it is stored in the liver and muscles in the form

of glycogen (22). In healthy individuals, glycogen synthesis (glyconeogenesis) in tissues is stimulated by insulin (23). When the amount of glucose in the blood decreases, glycogen breaks down in the liver to glucose (glycogenolysis). In healthy individuals, feedback mechanisms ensure that glucose levels are under homeostatic control by balancing glyconeogenesis and glycogenolysis (24). The liver can also convert lactate to glucose via a process known as gluconeogenesis to further supply the required glucose to the blood when levels are low (24-26). Glyconeogenesis, glycogenolysis and gluconeogenesis are controlled by anabolic hormones released from the pancreas such as glucagon (from α-cells) and insulin (from β-cells). Both hormones, glucagon and insulin, bind to specific receptors to trigger a chain of reactions that control glucose homeostasis (27-31). GLUT-2 (mainly in β-cells) and GLUT-4 (mainly in skeletal muscles) are the dominant glucose transporters. In general, insulin activates GLUT transporters to become fully functional pores that are able to transport glucose molecules into tissues (32).

The pancreas produces significant quantities of insulin which it stores in intracellular secretary granules. Upon granular stimulation from rising levels of glucose, these granules release their insulin content into the mesenteric veins (33, 34). The process of insulin secretion is different in healthy and diabetic patients. Normally, in healthy individuals, there are two phases of insulin secretion; first phase insulin secretion which starts immediately after the initial stimulus of raised glucose levels and second phase insulin secretion which starts shortly after the first phase, and has a shorter duration but greater magnitude. First phase insulin secretion occurs from β-cells of the pancreas as a direct response to high influx of extracellular glucose (35, 36). When diabetic patients lack β-cells (in T1D patients or T2D patients who lost their β-cells), both phases of insulin do not exist since there is a complete lack of functional β-cells and insulin production. In T2D patients that have remaining functional β-cells, the first phase of insulin secretion is impaired and further exposure to glucose results in a reduction in insulin secretion in the second phase due to the desensitization of β-cells to glucose (11).

1.1.2 Pathogenesis of diabetes

T2D develops in adult life most likely due to genetic and environmental factors (37) that lead to tissue desensitization to insulin. Environmental factors include high intake of saturated fat and glucose, low fibre content, lack of exercise and obesity (collectively known as the metabolic syndrome). Tissues lacking insulin sensitivity have poor expression of insulin receptors and hence have poor uptake of glucose on insulin stimulation. As a result, glucose remains free in the blood (38). Continuous stimulation of β-cells through hyperglycaemia or certain types of antidiabetic drugs can lead to tissue exhaustion and eventual cessation of insulin production due to tissue damage (39).

1.1.3 Complications of diabetes

DM increases the risk of developing complications which include gastroparesis, cardiovascular disorders such as platelet adhesion and atherosclerosis, oxidative stress, metabolic symptoms such as obesity, atherogenic dyslipidaemia, hypertension and insulin resistance, and cholecystitis (40-43). The vascular complications are caused by poor glycaemic control and may affect structure and functionality of the small blood vessels (microvascular) or the large blood vessels (macrovascular). The macrovascular complications include a two to four fold increase in the incidence of stroke, coronary heart disease (CHD) and peripheral vascular disease (44, 45). Peripheral vascular disease can lead to ulcerations, gangrene, and lower limb amputation (44). On the other hand, microvascular complication of diabetes mellitus include nephropathy, neuropathy, and retinopathy (44, 45). Diabetic nephropathy or kidney disease, is the primary cause of end stage renal disease (ESRD) (46). The biomarkers for nephropathy are elevated glomerular blood pressure and the presence and progressive increase of albumin levels in the urine (44). Approximately 15-20% of type 1 diabetes mellitus and 30-40% of T2D mellitus patients develop end stage renal disease (44). On the other hand, about 60% of diabetic patients develop diabetic neuropathy which makes it the most common long term complication of diabetes mellitus. It involves loss of sensation, weakness and pain and can lead to limb amputations (44, 47). The third microvascular diabetic complication is diabetic retinopathy which is caused by damage to

blood vessels in the retina and can lead visual impairment and blindness in the working population (48). Another complication of diabetes mellitus is periodontal and foot diseases which occur due to the impairment of the immune system caused by the diabetes. The immune system impairment lead to the body's inability to fight pathogens including bacterial, fungal, protozoal and viral infections which if not diagnosed in time can lead to gangrene and limb amputations which accounts for more than 60% of nontraumatic lower limb amputations (44, 49).

1.1.4 Drug therapy for Type 2 diabetes

Antidiabetic drugs are commonly used and are effective in minimizing variations between peaks and troughs of blood glucose levels in diabetic patients. However, weight gain, obesity and associated inflammation as well as the build-up of free radicals and toxins, remain detrimental factors in the treatment of the disease and its complications (50-52). Current antidiabetic drugs aim at inducing insulin secretion and improving tissue-response to insulin which results in better glycemic control. Biguanides, thiazolidinedione and sulphonlyureas have been available in the market for many decades while newer drugs such as dipeptidyl peptidase-4 (DPP-4) inhibitors and incretin mimetics are now available and have shown great potential in the treatment of diabetes and its complications.

1.2 Current drugs

1.2.1 Biguanides

Metformin (Figure 1.1), a drug from the biguanide class, was marketed in 1957 as an orally administered drug to lower glucose levels in T2D patients (53, 54). Metformin works by improving the utilisation of glucose in the peripheral tissue and reducing the production of glucose in the liver (54, 55). Metformin is mainly absorbed from the small intestine with a bioavailability of 50-60%. It has a half-life of 1.5-4.9 hours and a small affinity for plasma protein binding (53). The main route of elimination of metformin is via glomerular filtration rate and tubular secretion in the kidneys (53). Metformin does not undergo any hepatic metabolism and is eliminated unchanged in the urine (56). Metformin is the first line treatment for T2D. It reduces diabetes related events and mortality in

overweight diabetic patients and it does not cause hypoglycaemia and may result in weight loss which can be beneficial in diabetic patients (55).

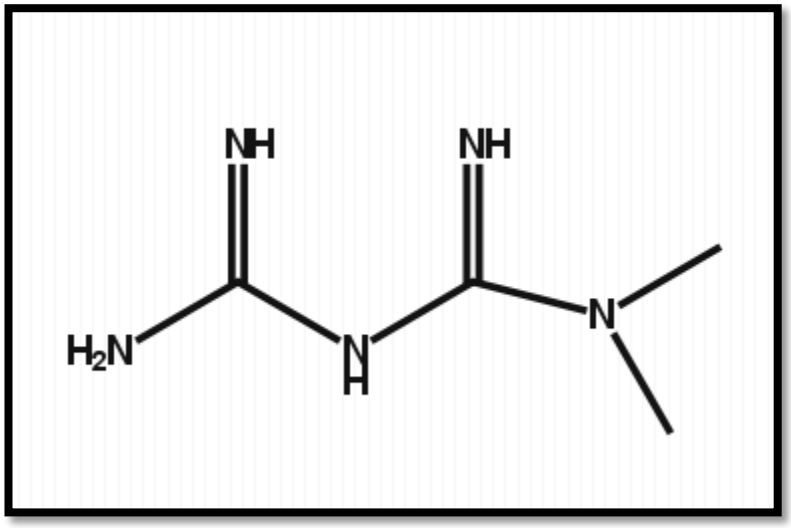

Figure 1.1: Chemical structure of metformin

1.2.2 Sulfonylureas

Sulfonylureas, chemical structure presented in Figure 1.2, were one of the first drugs to be marketed as an oral hypoglycaemic agents (57). They stimulate the β-cells in the pancreas to produce insulin (57, 58). They do that through antagonising adenosine triphosphate (ATP) sensitive potassium channels at the membrane of the pancreatic β-cells (59). They also have other effects including decreasing glucagon production, and reducing hepatic insulin clearance (51, 57). The first generation of sulfonylureas were tolbutamide, acetohexamide, chlorpropamide, and tolazamide (59). These drugs were then superseded by the second generation sulfonylureas which included gliclazide, glipizide, glibenclamide and the newer third generation agent glimepiride (58, 59). Sulfonylureas may result in weight gain and the incidence of hypoglycaemic episodes. The risk of hypoglycaemia is increased in patients with hepatic or renal failure and the elderly (55). Gliclazide and glipizide are the least likely drugs from the sulfonylurea group to cause hypoglycaemia. They are often added to metformin if metformin alone does not adequately control blood glucose levels. They can cause weight gain and expected to reduce HbA1c by 10-20 mmol/mol (55).

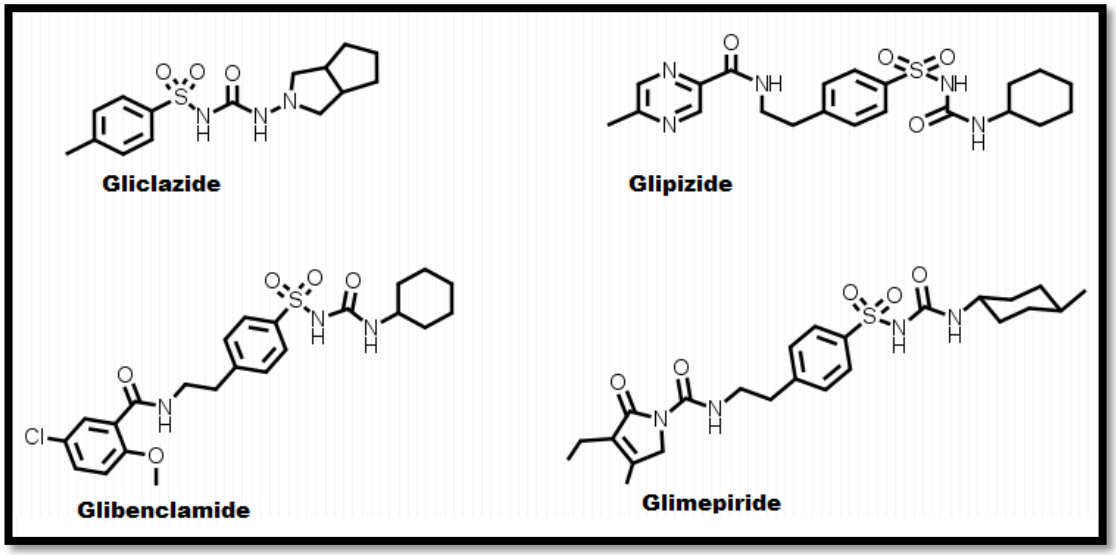

Figure 1.2: Chemical structure of selected sulfonylureas.

1.2.3 Thiazolidinediones

Thiazolidinediones (TZD) or glitazones are a class of antidiabetic drugs that improve the metabolism in patients with T2D. The chemical structures of glitazones is presented in Figure 1.3. Their novel mode of action is through the activation of peroxisome proliferator-activated receptor gamma which is responsible for regulation of genes involved in the metabolism of glucose and lipid (55). They are referred to as insulin sensitisers because they improve insulin sensitivity. They decrease insulin resistance in muscle, adipose tissue and the liver (55, 60). They may also have potential benefit on the manifestation of insulin resistance syndrome (60). The drugs included in this class are pioglitazone and rosiglitazone (55). Glitazones are normally reserved for diabetic patients unable to take other antidiabetic medications. They are ineffective in up to 30% of patients and have serious side effects such as oedema, increased fracture risk and heart failure. They do not cause hypoglycaemia if used in monotherapy and they may lead to weight gain. The expected decrease in HbA1c by glitazones is 5-15 mmol/mol. Rosiglitazone may also increase the risk of ischaemic cardiovascular events.

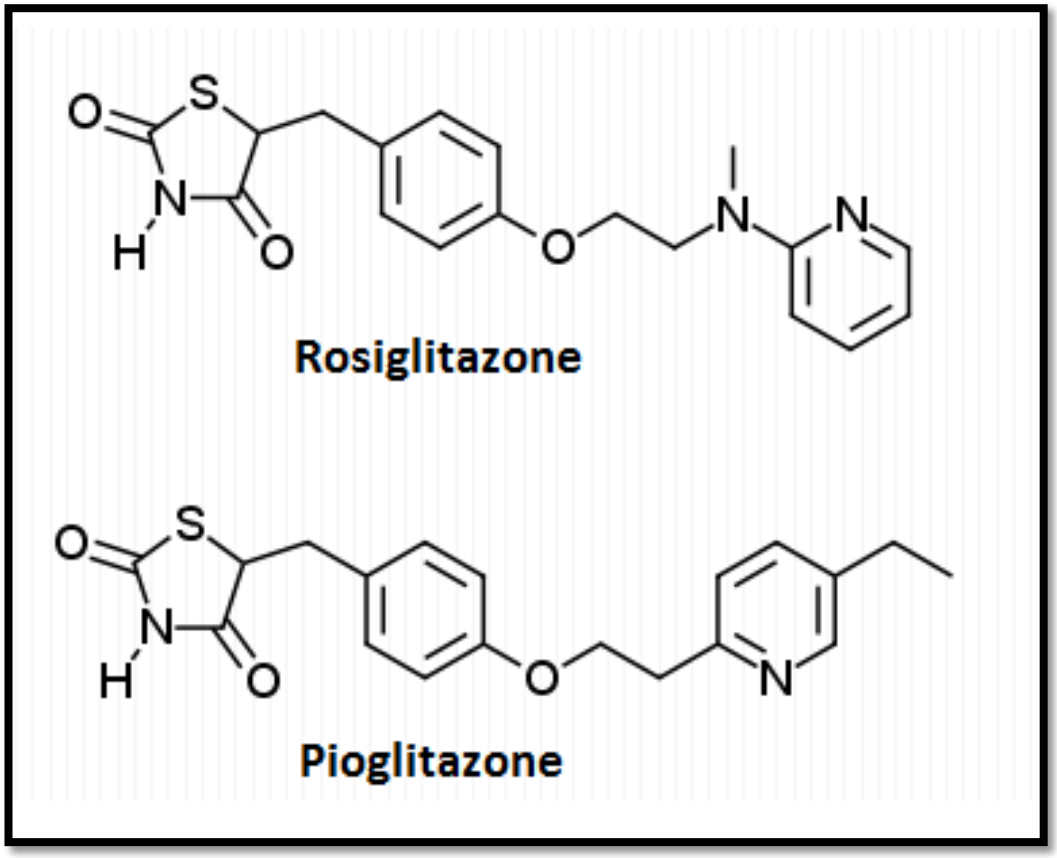

Figure 1.3: Chemical structures of pioglitazone and rosiglitazone

1.2.4 Acarbose

Acarbose is an oral alpha-glucosidase inhibitor which is used in the management of T2D. Acarbose is an oligosaccharide (Figure 1.) which is acquired from fermentation processes of a microorganism, *Actinoplanes utahensis.* It exerts its effect by delaying the digestion of ingested carbohydrates which results in a smaller rise in blood glucose levels after meals. The reduction of plasma glucose levels leads to a decrease in the levels of glycosylated haemoglobin in patients with T2D. Acarbose can produce a reduction in HbA1c by 5-10mmol/mol and does not cause hypoglycaemia if used in monotherapy. Its poorly tolerated because of its gastrointestinal side effects therefore has a limited role in diabetes therapy. Acarbose has no effect on the weight of diabetic patients (55).

According to a randomised control trial (STOP-NIDDM) published in 2002, Acarbose can be used to prevent or delay the conversion of impaired glucose

tolerance into T2D (61). Acarbose has similar efficacy and tolerability to metformin in Chinese patients with a new diagnosis of type two diabetic patients (62).

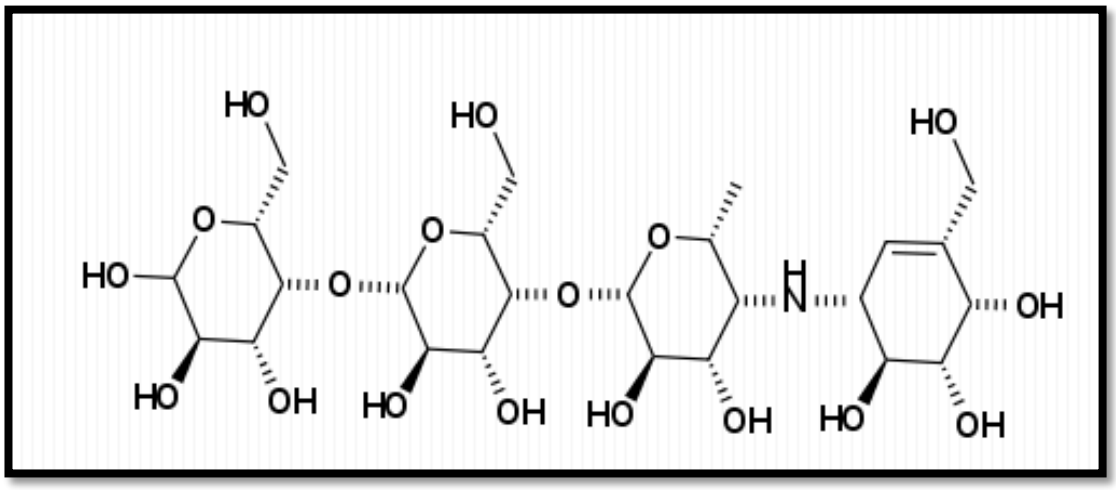

Figure 1.4: Chemical structure of Acarbose.

1.2.5 Sodium-Glucose cotransporter-2 (SGLT2) inhibitors

1.2.5.1 Dapagliflozin

Dapagliflozin (Forxiga®), Figure 1.4, selectively inhibits the sodium-glucose cotransporter 2 (SGLT2) which results in inhibition of renal glucose reabsorption and prompting the emission of glucose in the urine (63, 64) as a result of urinary glucose excretion, hyperglycaemia may improve while also providing the benefits of weight control and weight loss (64). The drug was developed to be used as therapy for T2D that is independent from insulin (63, 64). SGLT2 inhibitors may increase the risk of hypoglycaemia if used with insulin or sulfonylureas, however, they have a low risk if the drug is used alone. SGLT2 inhibitors should be avoided in patients with renal impairment. They can also increase the risk of urinary adverse effects such as urinary tract infection (UTI) and vaginal candidiasis due to the increased of glucose concentration in the urine. They can reduce HbA1c by 5-10 mmol/mol and can also lead to weight loss in diabetic patients (55). Dapagliflozin was studied as monotherapy in previously untreated type 2 diabetic patients whose hyperglycaemia was inadequately controlled by diet and exercise. The study

showed that there was a significant reduction in glycated haemoglobin (HbA1c) from base line in the treatment group compared to placebo (65). The Australian approval for dapagliflozin permits it to be used as monotherapy only in diabetic patients who cannot tolerate metformin, however, it can be employed as initial treatment in combination with metformin (66). The effect of dapagliflozin was investigated in type 2 diabetic patients who had insufficient glycaemic control with metformin alone. The finding of the study revealed a significant difference in the effect of dapagliflozin in the treatment group on HbA1c and the placebo group (67).

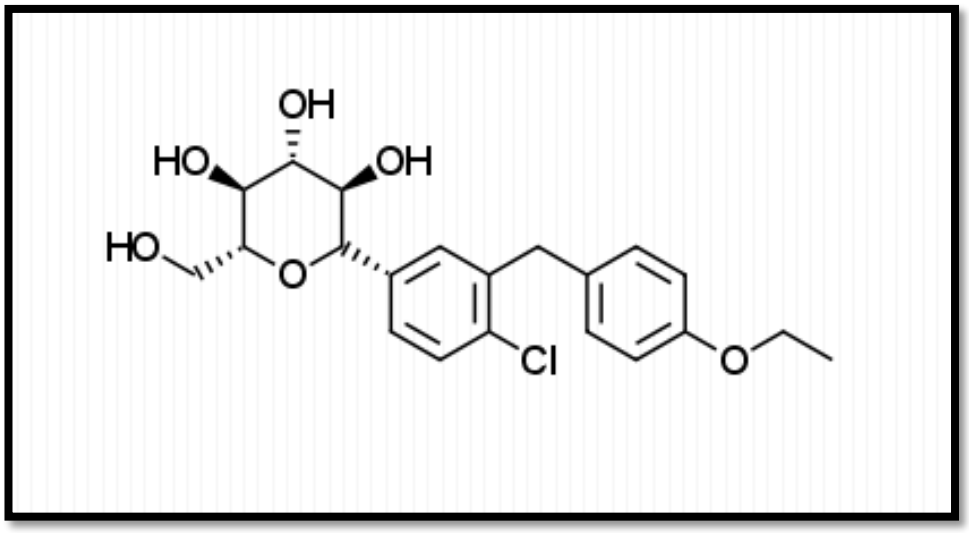

Figure 1.4: Chemical structure of dapagliflozin

1.2.5.2 Canagliflozin

The second sodium-glucose co-transporter inhibitor approved in Australia is Canagliflozin (Invokana®). The chemical structure of canagliflozin the presented in Figure 1.5. Like dapagliflozin, it increases the excretion of glucose in the urine through the reduction of glucose reabsorption which leads to a fall in the blood glucose levels. The bioavailability of canagliflozin is around 65% and even though food does not affect bioavailability, it is recommended to take canagliflozin before breakfast to potentially decrease postprandial glucose levels (68). Canagliflozin increases the risk of hyperkalaemia in patients with moderate renal impairment, especially if they are taking ACE inhibitors or potassium-sparing diuretics (68). The safety and efficacy of canagliflozin as

monotherapy has been investigated in 584 T2D patients that has not been controlled by diet and exercise. The study concluded that treatment with canagliflozin enhanced glycaemic control, produced weight loss and was generally well tolerated in this group of patients (69). Another study explored the effect on canagliflozin as add on therapy to metformin. It was concluded that canagliflozin was associated with significant improvement in glycaemic control, weight loss and a low incidence of hypoglycaemia in type 2 diabetic patients (70).

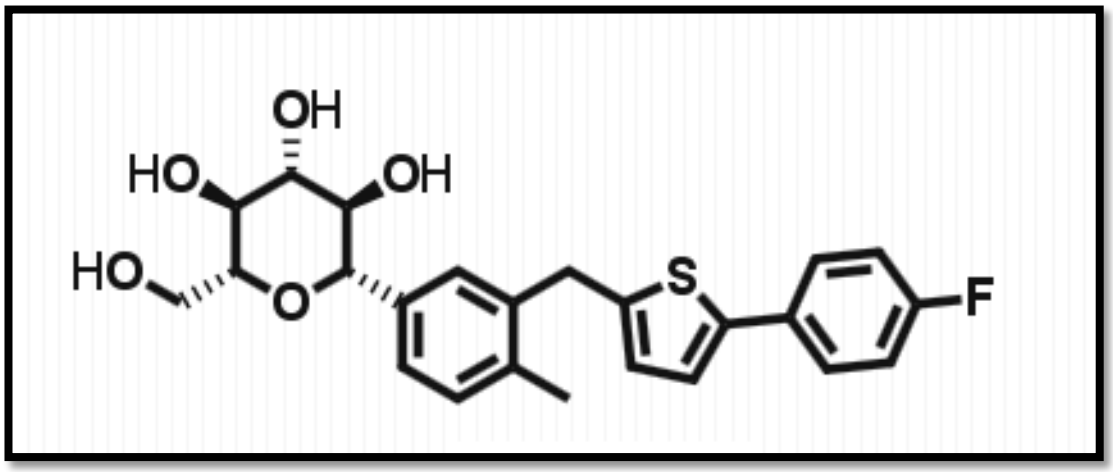

Figure 1.5: Chemical structure of canagliflozin

1.2.6 Insulin

Insulin is a protein that consists of 51 amino acids which are divided in to two peptide chains that are linked by disulphide bridges. It was discovered in 1921 by Banting and Best, and since then it has become one of the most extensively studied molecules in biochemistry (71, 72). The chemical structure of insulin is shown in Figure 1.6.

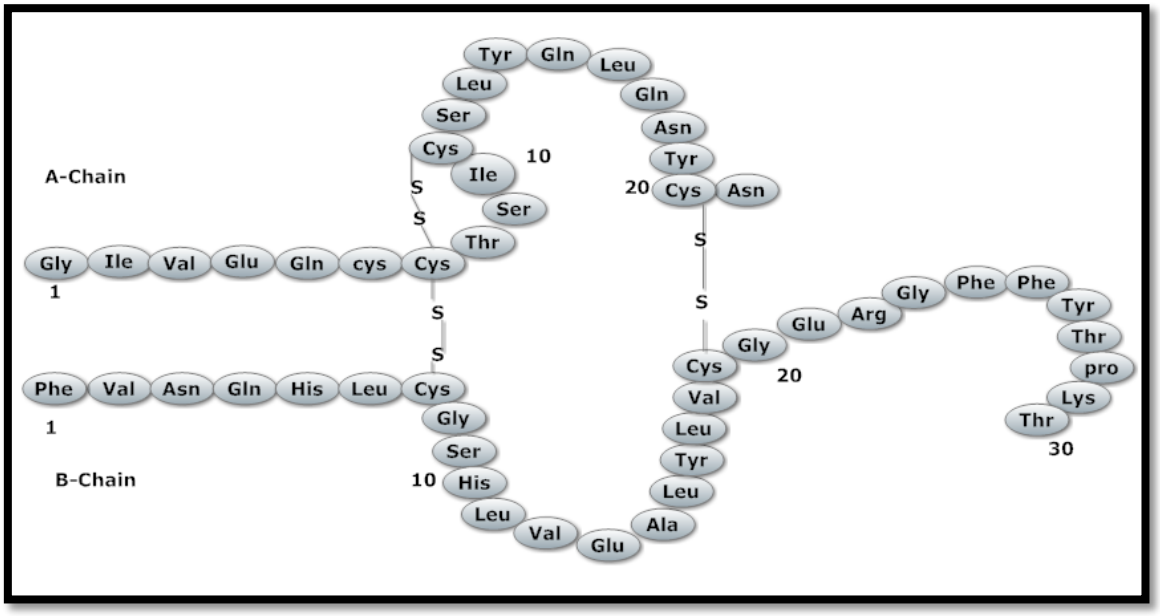

Figure 1.6 Primary structure of human insulin

Insulin is synthesised in the β cells of the pancreas as proinsulin, which is then enzymatically converted in the storage vesicle to insulin (72).

In T2D patients, insulin is either given as a monotherapy or as a combination with other antidiabetic medications. Monotherapy regimens combine short and long acting insulin and given subcutaneously in a single dose or twice daily before morning and evening meals. If combined with other antidiabetic medications, long acting insulin is normally given once a day (55).

1.2.7 Incretin system

Incretin hormones which include glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1(GLP-1) play an important role in the disposal of absorbed glucose by stimulating the secretion of insulin from the pancreas (73, 74). They also increase B cell mass by promoting B cell proliferation and inhibiting apoptosis (75). GIP consists of 4- amino acids and is synthesised by the enteroendocrine K cells in the duodenum and jejunum while GLP-1 is made in the ileal and colonal enteroendocrine L cells (74). The amount of insulin secreted after an oral glucose load was three to four folds

that after an intravenous glucose load (76). This effect was attributed to the incretin hormones which are GIP and GLP-1 that are released from the gut (77). GLP-1 also controls glycaemia through other pathways such as the effect on glucose sensors, inhibition of glucagon secretion, inhibition of gastric emptying and reducing food intake (75). In the fasting state, GLP-1 levels are low but increase rapidly after eating. Enzymatic deactivation mainly by dipeptidyl peptidase-4 (DPP-4) and renal clearance causes a rapid decline in the concentration of GIP and GLP-1 (74).

The incretin effect is lost or greatly reduced in patients with T2D (73, 76, 77). The secretion of GIP in T2D is normal but it lacks the glucose lowering effect due to the loss of normal GIP induced potentiation of second phase insulin secretion. The insulinotropic response to GLP-1 is intact and the circulating levels of postprandial GLP-1 are reduced (76, 77).

To overcome the rapid degradation of GLP-1, two approaches have been developed. One is to increase the concentration of endogenous GLP-1 by inhibiting DPP-4 which is responsible for degradation of GLP-1, and the other is using agents that mimic the action of the incretin hormone GLP-1 (78).

1.2.8 Dipeptidyl Peptidase-4 (DPP-4) Inhibitors

DPP-4 extensively and rapidly degrades the intestinal incretin hormone GLP-1 (74, 79). It is therefore postulated that inhibiting DPP-4 enzyme can be used as a treatment for T2D by protecting the circulating levels of GLP-1 from degradation (79). Experiments have shown that administration of DPP-4 inhibitors to pigs have protected exogenous and endogenous GLP-1 from degradation which improved insulin response to hyperglycaemia (79). DPP-4, also known as CD26 is serine peptidase which is widely present on many tissues such as kidney, liver, lung, lymphocytes, intestinal brush-border membranes, and endothelial cells (74, 77, 79). The 766 amino acids enzyme is a membrane –associate peptidase but also available in a soluble circulating form in the plasma (74, 80). It preferentially cleaves peptide with an alanine or proline moiety in the second N-terminal position (74, 79). Substrates for DPP-1 include neuropeptides, gastrointestinal hormones, cytokines, and chemokines (74). Inhibitors of DPP-4 imitate many of the actions attributed

GLP-1 receptor agonists, including inhibition of glucagon and stimulation of insulin secretion, stimulation of βcells proliferation and inhibition of apoptosis (81). However, DPP-4 inhibitors do not reduce gastric emptying or cause weight loss, which could be due to the modest protection of postprandial levels of intact plasma GLP-1 seen after inhibition of DPP-4. Pharmaceutical companies have developed small-molecule DPP-4 inhibitors that exclusively and potently hinder DPP-4 activity after an oral dose. More than 80% of DPP-4 activity is inhibited and some of the inhibition is maintained for 24 hours on once daily treatment (74, 82). Sitagliptin (Januvia®) was the first marketed DPP-4 inhibitor followed by vildagliptin (Galvus®) which reached the European Union market in 2008. The bioavailability of both drugs is good and they have a reasonably long duration of action (76). Sitagliptin and vildagliptin can be used as a monotherapy where they have shown a considerable antidiabetic action, or in combination where they also improved the glycaemic control when added to other antidiabetic drugs such as sulfonylureas, metformin and thiazolidinediones. Studies have shown that metformin may improve GLP-1 biosynthesis and secretion, and the combination with DPP-4 inhibitor may produce a more significant increase in GLP-1 concentration than that if either of the drugs is used alone (74).

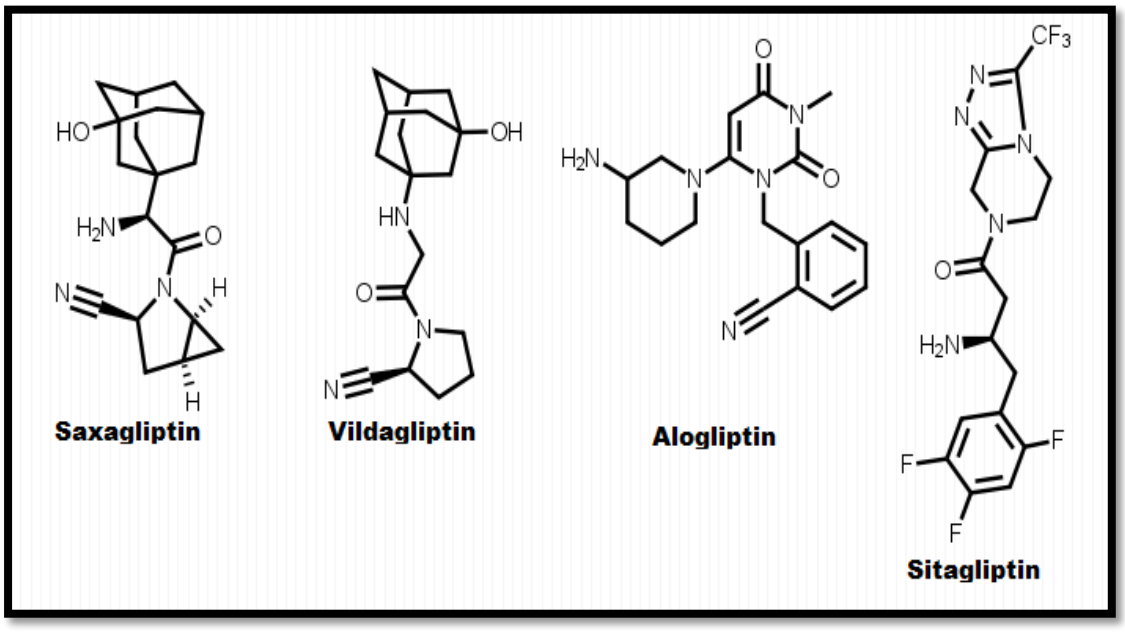

Figure 1.7: Chemical structure of the four DPP-4 inhibitors: sitagliptin, vildagliptin, saxagliptin, and alogliptin.

1.2.9 Incretin Mimetics

Incretin mimetics are a new class of antihyperglycaemic agents that mimic the actions of endogenous incretin hormones such as glucagon-like peptide-1 (GLP-1) (74, 77). GLP-1 is a gut derived hormone that stimulates insulin secretion and suppresses the secretion of glucagon. GLP-1 also inhibits gastric emptying and reduces appetite and food intake (83). Several GLP-1 receptor (GLP-1R) agonists or GLP-1 analogues that are resistant to degradation by the enzyme DPP-4 have been developed. Liraglutide was developed by Novo Nordisc, and contains Arg34Lys substitution and glutamic acid and 16-C fatty acid addition to Lys26 (84, 85). Another GLP-1 receptor agonist, Lixisenatide, is manufactured by Sanofi, under licence from Zealand Pharma A/S, and marketed under the brand name Lyxumia® (86, 87). It is administered subcutaneously once daily and has a half-life of 2.8 hrs. It is a synthetic 44 amino acid peptide derived from exendin-4 and its resistance to degradation by DPP-4 is achieved by removing proline from the 38 position and adding 6 lysine residues. Lixisenatide has approximately four times the affinity for the human GLP-1R compared to the native GLP-1 peptide (88). There are other incretin mimetic drugs either available on the market or still under development by drug companies, but the first drug to come on the market in the incretin mimetic class is exenatide.

1.3 Exenatide

Exenatide was discovered by Amylin Pharmaceutical Inc., and is under license to Elli Lilly. It is marketed under the brand name Byetta®. Exenatide is the synthesized form of a peptide venom found in the saliva of the Gila Monster lizard and is degradation resistant (77, 89). Figure 1.8 shows the differences between the chemical structures of human GLP-1, liraglutide, exenatide and lixisenatide (90).

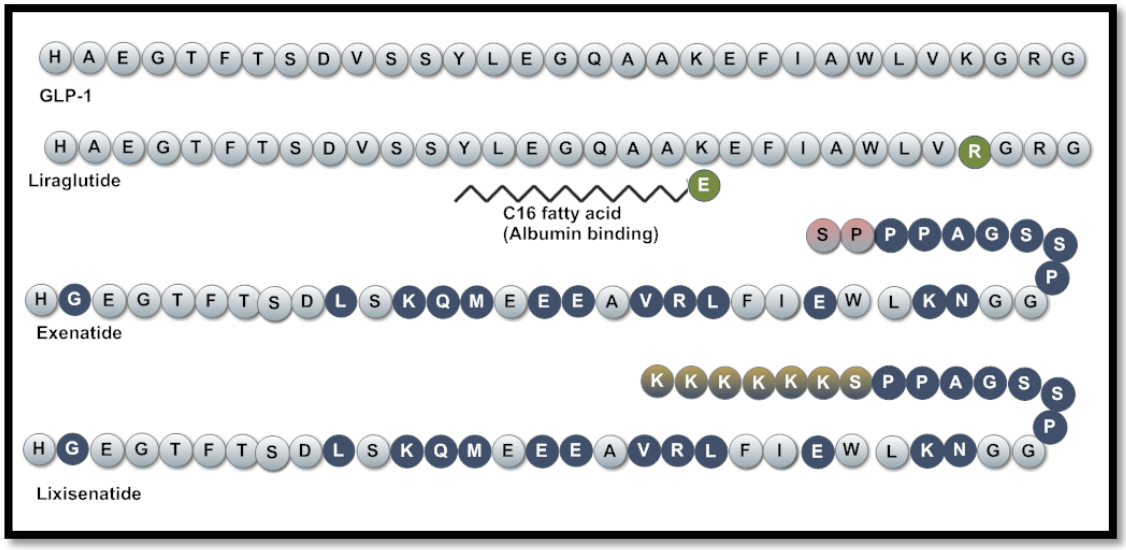

Figure 1.8: The difference in amino acid sequence between the human glucagon-like peptide-1 (GLP-1), liraglutide, exenatide and lixisenatide and other formulation changes to affect its bioavailability and elimination.

Exenatide is a degradation resistant GLP-1 receptor agonist that is based on exendin-4 which is a 39 amino acid peptide found in the saliva of the *Heloderma suspectum* lizard (75). Roughly, 50% of the amino acid sequences are the same in both exendine- 4 and GLP-1 hormones, found in humans (74). The presence of the amino acid glycine at position 2 in the sequence of exenatide protects the peptide from being degraded by DPP-4 (75).

Due to its improved pharmacokinetic profile, the potency of exenatide for glucose lowering is 3000 times more than native GLP-1 after intravenous administration (75, 91). Exenatide can activate the GLP-1 receptor and exert its glucoregulatory effect at a much lower concentration compared to endogenous GLP-1 (92). The effect of exenatide is dependent on glucose concentration, which means an extended release formulation is a suitable option in the treatment of T2D (92). Exenatide can be administered subcutaneously twice a day within 60 minutes of a meal, or once weekly using the extended release formulation (2). The extended release formulation of exenatide (exenatide QW or Bydureon (93)) incorporates the peptide into a matrix of a biodegradable polymer (92). The biodegradable polymer (poly(D,L-lactide-co-glycolide; PLG)) is known to be safe for use in humans, and will

degrade over time producing a prolonged release of exenatide. The median peak plasma concentration of exenatide is reached 2.1 hours after subcutaneous administration. Exenatide has a volume of distribution of 28.3 L and a half-life of 2.4 hours. It is predominantly eliminated by glomerular filtration with subsequent proteolytic degradation and the apparent clearance of the drug is 9.1 L/hr (94). The iso-electric point of exenatide is reported to be 4.2 (95).

As an incretin mimetic or a GLP-1 receptor agonist, exenatide exerts glucoregulatory effects similar to that of GLP-1. These effects include glucose-dependent improvement of insulin secretion, suppression of glucagon secretion, increased insulin sensitivity, reduction of food intake and promoting weight loss through the slowing of gastric emptying (96).

There are many studies that have investigated the effectiveness of exenatide in conjunction with other antidiabetic drugs. A study by DeFronzo *et al.*, investigated the effect of exenatide in patients taking the maximum therapeutic dose of metformin (96)(96). The study showed that exenatide was generally well tolerated and reduced HbA_{1c} without causing any weight gain and incidents of hypoglycaemia. Another study, conducted by Buse *et al*., found that when exenatide was given to T2D patients failing to get optimal glycaemic control with the maximum effective dose of sulfonylureas, it significantly reduced HbA1c, was generally well tolerated and resulted in weight loss (97). In a placebo-controlled trial which included 217 T2D patients for more than three years, were given placebo, 5µg exenatide, or 10µg exenatide twice a day. This study has shown that adjunctive exenatide treatment resulted in sustained improvements in glycaemic control, cardiovascular risk factors, and hepatic biomarkers associated with progressive weight reduction (98). A study by Wu *et al*., investigated the effects of exenatide on inflammatory and oxidative stress markers in T2D patients. It concluded that treatment with exenatide twice daily led to a reduction in body weight and BMI, improvement in HbA1c, decrease in daily mean glucose, limiting of glycaemic excursion, and reduction in inflammatory and oxidative stress markers in patients with insufficient glycaemic control (99). Furthermore, Varanasi *et al*, concluded that treatment of T2D patients with exenatide has positively durable and persistent

effect on weight, HbA1c, systolic blood pressure, CRP, and the concentration of triglycerides. These beneficial effects were reversed within 6 months of ceasing the treatment (100).

The effect of exenatide on T2D patients with congestive cardiac failure was investigated by D. Nathanson *et al*. and concluded that infusion of exenatide in male T2D patients with CHF increased cardiac index as a consequence of chronotropy. It also had a positive effect on pulmonary capillary wedge pressure and the drug was well tolerated (101).

In addition, Linnebjerg *et al.* concluded that, exenatide slows gastric emptying in T2D patients (102). They also highlighted the importance of this effect on the absorption of medications in patients who are taking exenatide. They recommend those patients take their medications at a time when the effect of exenatide is minimum (i.e. midday or bedtime). However, in a study evaluating the effect of exenatide on digoxin concentration, exenatide decreased the mean plasma concentration of digoxin and delayed T_{max} by 2.5 hours but did not change the steady state pharmacokinetics of digoxin or a clinical significance (103). Another study examined the effect of exenatide twice daily on warfarin pharmacokinetics, and found no significant effect (104).

Exenatide has demonstrated *in vitro* to have a neuroprotective and neurotropic properties, however, the detail mechanisms of action remained unclear. Due to exenatide's good safety profile in T2D patients and the encouraging results from Parkinson disease models in animals, recruitment of patients to evaluate the effect of exenatide on Parkinson's disease has been completed (105). This suggests wide implications of the drug beyond glycemic control, and a huge potential in treating not only T2D but many associated complications.

Despite all the potential benefits of exenatide in the treatment of T2D and other medical conditions, its route of administration and dosing frequency represent a significant drawback on its clinical use. The twice daily dosing and the pain and irritation cause by the injection may have a significant effect on patient compliance. Microencapsulation and other route of administration methods such as oral and sublingual formulation could significantly improve the clinical outcomes for the patients.

As a protein-based drug, exenatide is susceptible to degradation mainly by deamidation, oxidation and aggregation.

1.4 Proteins

Proteins are molecules constructed from amino-acids through peptide bonds (106). A protein chain can consist of 50 to 2000 amino acid and each chain of amino acids forms a secondary structure through folding (107, 108). The secondary structure can be either an α-helix or β-pleated sheets. Hydrogen bonds are the force that maintains the secondary structure. When the protein chain further folds on itself to form a three dimensional shape, it is referred to as a tertiary structure. The forces that hold the dimensional structure together are disulphide bridges, hydrogen bonds, ionic interactions and van der Waals forces (109).

1.4.1 Degradation of proteins

The pathways of degradation of proteins can be categorised into two different classes, physical and chemical degradation. Physical degradation involves changes to the higher structure (i.e. secondary structure and higher) of the proteins. These changes include denaturation, aggregation, adsorption to surfaces and precipitation (110). On the other hand, chemical degradation involves covalent bond breaking or formation resulting in new chemical entities. These chemical changes and instabilities include hydrolysis, deamidation and oxidation.

1.4.1.1 Deamidation

One of the most common routes of degradation of proteins is deamidation which occurs in proteins containing asparagine (Asn) moieties especially in the pH range 4-9 (111, 112). For deamidation in acidic conditions, direct hydrolysis of Asn takes place to produce only Asp and the reaction is subject to acid catalysis. On the other hand, for neutral to basic conditions (higher than pH 6.00) deamidation occurs via an intramolecular cyclisation reaction (succinimide) with the emission of ammonia (113). The cyclic intermediate compound then hydrolyses to produce either normal aspartic acid (Asp) instead of Asn or iso-aspratic acid as shown in Figure 1.10 (112, 114). The

deamidation reaction is a two-step reaction and each step has a different reaction rate. Deamidation can also occur in glutamine (Gln) residue but at a lot slower rate than asparagine (115).

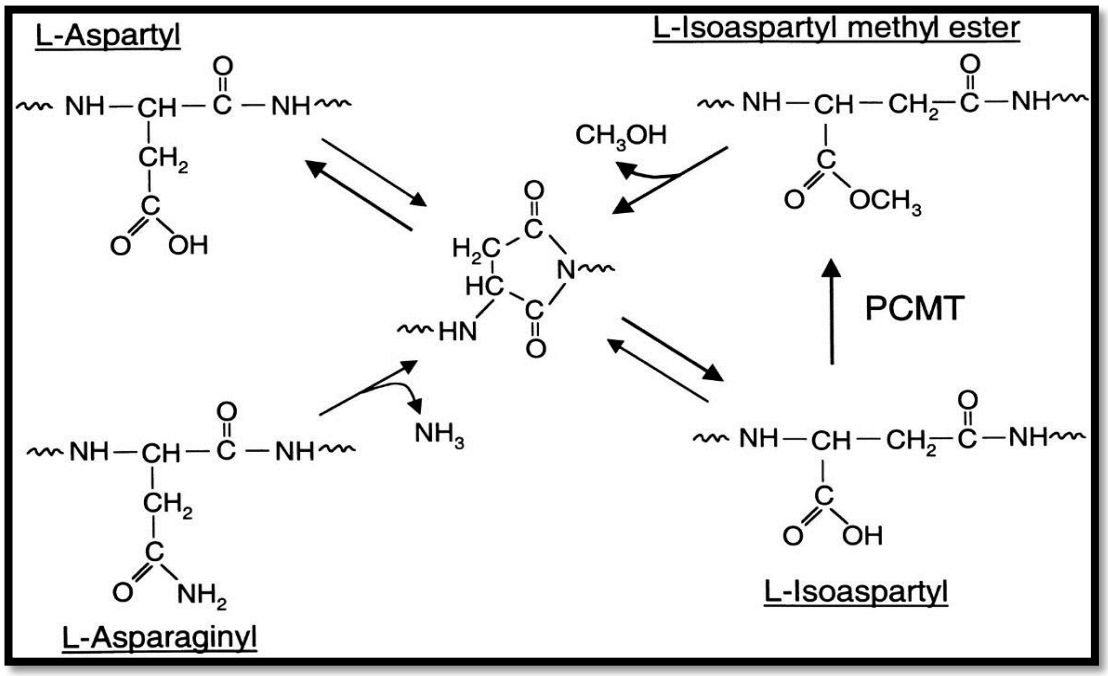

Figure 1.9: Pathways for spontaneous deamidation, isomerization, and racemization for aspartyl and asparaginyl peptides (111).

1.4.1.2 Oxidation

Oxidation is another major chemical degradation pathway for proteins. The most susceptible amino acids to oxidation are methionine, cysteine, histidine tryptophan and tyrosine (113). The susceptibility of these amino acids to oxidation is due to their elevated reactivity to oxygen species. The presence of oxidising contaminants catalysed by light and transition metal ions is a major inducer of oxidation in proteins during manufacturing and storage (116). Methionine oxidation can result in the formation of sulfoxides which form under mild oxidative conditions or sulfones under harsher oxidation conditions (117).

1.4.1.3 Aggregation

One of the major problems that faces the development of pharmaceutical proteins is aggregation. Aggregation can lead to the production of antibodies and the development of serious side effects (118, 119). Protein aggregates

can range from dimers to sub-visible and visible particles (120). Aggregation and conformation of proteins can be influenced by pH conditions, different ions, and heat (121).

1.5 Possible degradation sites for exenatide

Figure 1.10 shows that exenatide has three sites, which are susceptible to degradation. Asp (red) and Met (blue) are susceptible to hydrolysis and oxidative degradation respectively. Asn (green) is known to be vulnerable to deamidation reactions.

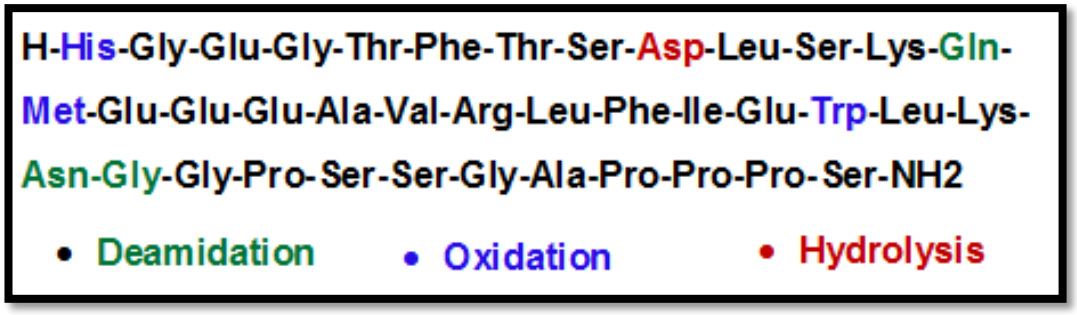

Figure 1.10: Chemical structure of exenatide with possible degradation sites.

Thus, in order to quantitate degradation, stability testing can be carried out examining the effects of ionic strength, temperature and pH, *in vitro*.

1.6 Formulation and stability of exenatide

An investigation of the stability profile of exenatide in aqueous solutions is yet to be published. However, there are a few studies that have recently been published which investigate the stability of exenatide in microspheres and microcapsules. The most common method of microencapsulation of exenatide used poly(D,L-Lactic-co-glycolic acid) commonly referred to as PLGA. These studies investigated the effect of PLGA on the bio-stability and the pharmacokinetics of the drug. Qi *et al.* compared the properties of exenatide loaded PLGA microspheres, that are formed by either ultrasonication or homogenisation methods. They concluded that PLGA microspheres formed by

the homogenisation method were more appropriate for two-week sustained release. On the other hand, he found the ultrasonication method to be more suitable for longer time release, and may be used once monthly (122). Liang *et al.* studied the effect of PLGA and solution media on the degradation of exenatide. The study concluded that solutions containing solvents such as dimethyl sulfoxide has a strong impact on the conformation state and the stability of exenatide. Furthermore, higher pH accelerates the degradation of exenatide, due to promoting the interaction between PLGA and the peptide leading to the formation of acylated products (123). However, none examined the underlying stability of exenatide.

1.7 Ionic strength

Ionic strength of a solution is a measure of the concentration of all ions in that solution. The ionic strength is calculated using the following equation (Equation 1.1)

$$\mu = 1/2 \sum c_i z^2_i \qquad \textbf{Equation (1.1)}$$

Where μ is ionic strength, C_i is the molar concentration of ion i (M), Z_i is the charge number of that ion, and the sum is for all the ions in the solution. The ionic strength of a solution can influence the rate of degradation of a drug according to the following equation (Equation 1.2)

$$\log k = \log k_0 + 1.02\, Z_A\, Z_B \sqrt{\mu} \qquad \textbf{Equation (1.2)}$$

Where Z_A and Z_B are the charges carried by the reacting species in solution, μ is the ionic strength, k is the rate constant of degradation, k_0 is the rate constant at infinite dilution (124).

By plotting the logarithm of the reaction rate constant against the square root of the ionic strength, it can be determine whether an increase in ionic strength can lead to an increase, decrease, or has no effect on the rate of degradation. This effect is shown in Figure 1.11.

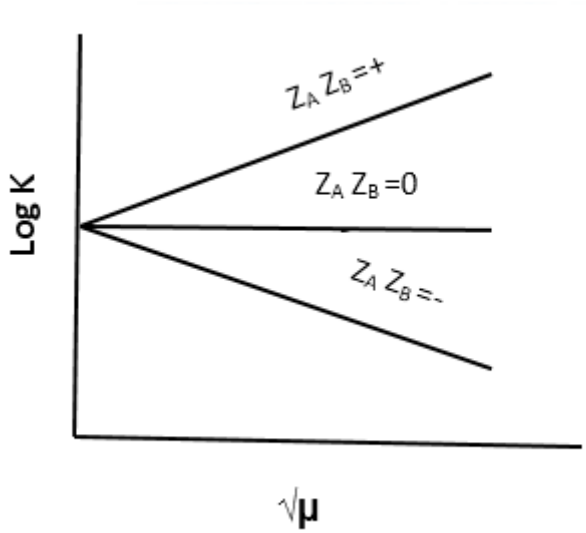

Figure 1.1: Effect of ionic strength on degradation rate

The ionised species of a buffer may also have a catalytic effect on the rate of reaction above any pH effect exerted by the buffer. Such an effect is temperature-dependant.

1.8 Effect of temperature on kinetics of degradation

Data obtained under accelerated temperatures for temperature-dependent reactions allows the prediction of the stability of a drug at determined shelf-life temperatures. The relationship between temperature and reaction rate is expressed in the Arrhenius Equation:

$$k=Ae^{-(Ea/RT)} \qquad \textbf{Equation (1.3)}$$

Where, k is the specific rate constant, A is the frequency factor (total number of collisions) and is an index of reactive species in the activated state, R is the universal gas constant (8.314 joules degree^{-1} mol), T is the temperature in Kelvin, E_a is the activation energy. $e^{-Ea/RT}$ indicates the probability that a given collision will produce a reaction.

Taking logarithms of the Arrhenius equation, the following equation can be used (Equation 1.4).

log k=log A-(Ea/2.303)(1/RT) **Equation (1.4)**

The shelf-life has a reciprocal relationship to the rate constant. In addition, the activation energy can be calculated by determining k at a number of temperatures and plotting log k against 1/T. The activation energy exemplifies the minimum energy required for reactants to undergo a reaction. The higher the activation energy, the more temperature-dependent is the reaction (125).

In summary, a good approach to improving the treatment of diabetes is through optimising formulations of newly marketed drugs that have shown potential in treating diabetes but have formulation-based limitations which compromise their efficacy and safety. Such limitations include poor stability and absorption profiles. Accordingly, improving diabetes treatment can be achieved by studying a new drug that has shown excellent antidiabetic properties in controlling insulin release from β-cells, as well as having desirable extrapancreatic effects that include slowing down gastric emptying, delaying glucose absorption, reducing appetite, weight loss, supressing glucagon release in response to eating, as well as anti-toxic effects that help alleviating diabetes-associated complications. A good example of such a drug that has great extrapancreatic effects in diabetes, but poor stability and an undesirable route of administration is exenatide (126-129).

1.9 Aim of thesis

The overall aim of this thesis is to investigate the stability of exenatide in aqueous solution. This will be achieved by:

1. Developing and validating an HPLC stability indicating method.
2. Investigating the effect of temperature, pH and ionic strength on the rate of degradation of exenatide.
3. Determining if refrigeration of the aqueous solution of exenatide can provide an improved shelf-life compared with current formulation.

Chapter Two

2 Method Development and Validation

2.1 Introduction

Stability and formulation issues can be addressed through measuring the shelf-life, degradation pathways and reaction rate constants at various pH and temperature values. This depends on reliable and sensitive analytical methods to measure the concentrations of the drug in different solutions. Various analytical methods available, including high performance liquid chromatography (HPLC) with detection by ultraviolet (UV) spectrophotometry or mass spectrometry (MS). The analytical method must be validated for specificity, sensitivity, linearity, accuracy, precision (inter- and intra-day variation), recovery and stability under the experimental conditions used (130). This chapter describes in-depth the development and validation method of an HPLC method for the determination of exenatide in solution.

2.2 Exenatide analysis by HPLC

Materials

Pure exenatide acetate powder was purchased from Aemon Chemical Technology Co. Limited (China) with lot number 141732-76-5 and batch number 12AC0816. Exenatide injection 250µg/mL (Byetta®), was purchased from Amylin Pharmaceuticals, Inc. (Australia).

Acetonitrile HPLC grade was purchased from Fisher Scientific (Australia). Triethylamine hydrochloride was purchased from Fluka Chemika (Switzerland). Trifluoroacetic acid was purchased from SIGMA-ALDRICH (USA). The HPLC grade water was purified using a Milli-Q Ultrapure system manufactured in Australia by Ibis Technology in Mount Hawthorn Western Australia. The thermometers used in the experiments were the Zeal Strengthened 76 mm immersion (England). The water baths were manufactured by Grant Instruments (Cambridge) Ltd, Barrington; Cambridge. To reduce water evaporation from the water baths, polypropylene spheres were used. The pH meter used in the laboratory was Hanna instruments HI 8519N (Portugal).

The HPLC system consisted of a Waters 501 pump by Millipore, Waters 484 Millipore tuneable absorbance detector set at 225nm, and Hewlett Packard HP 3396 Series III Integrator used as a chart recorder. A Vydac 214TP C4, 5um, 150mm, and 4.6mm column made by Grace Davidson Discovery Sciences (USA). The assay was undertaken under isocratic conditions at a flow rate of 1 mL per minute. Rheodyne, Model 7125 loop injector 20µL (USA).

2.3 HPLC analysis

The mobile phase consisted of 64% v/v HPLC-grade water, 36% v/v acetonitrile, and 0.1% v/v trifluoroacetic acid. Triethylamine hydrochloride was added to the mobile phase at a concentration of 10 mM to act as an anti-tailing agent. The mobile phase has a pH value of 3.00. The mobile phase was thoroughly mixed and filtered using 0.45µm Millipore filter and degassed.

A stock solution was made using 5mg of exenatide powder in a 100mL volumetric flask, made up to volume using Milli-Q water. From this stock solution, a series of dilutions were made to produce six concentrations: 2.5, 5, 10, 12.5, 20 and 25 µg/mL. All the solutions were stored in the refrigerator (2-8°C) and analysed by HPLC within 24 hour of preparation.

2.4 Method development and validation

A stability indicating method for measuring exenatide concentrations has been developed and validated. Initially, a method developed by Bachhav and Kalia was adapted (131). However, it showed a high signal-to-noise ratio and had to be refined further. A new method was adapted based on the work of Tsai *et al.* (132). This method was further refined and optimised to enable sensitive, accurate, robust and reproducible analysis of the drug in the buffers as well as in the marketed formulation.

2.4.1 Stability indicating assays

The stability indicating method was carried out by exposing standard exenatide solutions to a range of stress conditions. These conditions included acidic, basic, and oxidative environments. The acid stress test was carried out by adding 2mL of 0.1M hydrochloric acid (HCl) solution to 2mL of 25µg/mL standard exenatide solution. A 40^{o}C temperature was used for all stability studies as the standard-reaction temperature because it has been used by other studies on exenatide and also it high enough speed up the rate of reaction without degrading the drug (133). Degradation studies were not carried out at higher temperature to avoid unfolding and consequent thermal denaturation of the peptide drug. The mixture was kept in a water bath for 3 hours and samples were taken at 30 minute intervals. The samples collected were treated with the required amount of sodium hydroxide (NaOH) to neutralise the acid in the sample to prevent any damage to the column. On the other hand, the alkaline stress test was performed by adding 2 mL of 0.1M of sodium hydroxide (NaOH) solution to 2mL of 25µg/mL standard exenatide solution. The mixture was then kept in a water bath for three hours and samples were collected at 30 minute intervals. The collected samples then treated with the required amount of hydrochloric acid to neutralise NaOH in the sample. For an oxidative stress test, 0.2mL of 30% hydrogen peroxide solution (H_2O_2) was added to 2mL of 25µg/mL standard exenatide solution. The mixture was maintained in a water bath for three hours and samples were collected at 30 minute intervals. All samples were injected immediately onto the HPLC system and analysed for exenatide concentrations. All measurements were carried out in triplicate, and data are expressed as mean ± SD.

2.4.2 Linearity measurements

A calibration curve was constructed to investigate the linearity of the method used. Standards were prepared and analysed using six exenatide concentrations in buffer, 2.5, 5, 10, 12.5, 20 and 25 µg/ml. Concentrations were plotted against Area Under the Curve (AUC).

A least square fitted line was constructed and all measurements were carried out in triplicate and data are expressed as mean ± SD.

2.4.3 Precision measurements

The precision of this method was determined by performing triplicate assays on three samples of different concentrations on the same day to confirm intraday precision. To confirm inter-day precision, the procedure used in intraday validation was used on three different days. From the data collected, the mean, standard deviation and the percent relative standard deviation (%RSD) were determined. The formula used to calculate %RSD was as follows:

%RSD= Standard deviation / mean x 100 Equation (2.1)

For the method to be adequately precise for this study, the percent relative standard deviation (%RSD) value for the intra and inter day variation should be less than 3 %(134).

2.5 Results

The method was specific and chromatographic peaks in blank and calibration samples were free of interference (Figure 2.1). The retention time for exenatide was 3.2 min and the run time was 8 min.

2.5.1 Assay Validation

To identify the exenatide peak in the chromatogram, a sample of 60µg/mL of exenatide was injected into HPLC and was compared to a blank injection as shown in Figure 2.1. The chromatogram in Figure 2.1(A) shows the retention time of exenatide is 3.246 minutes and it is clear of any interference, while Figure 2.1 (B) represents a blank injection and only shows the solvent front.

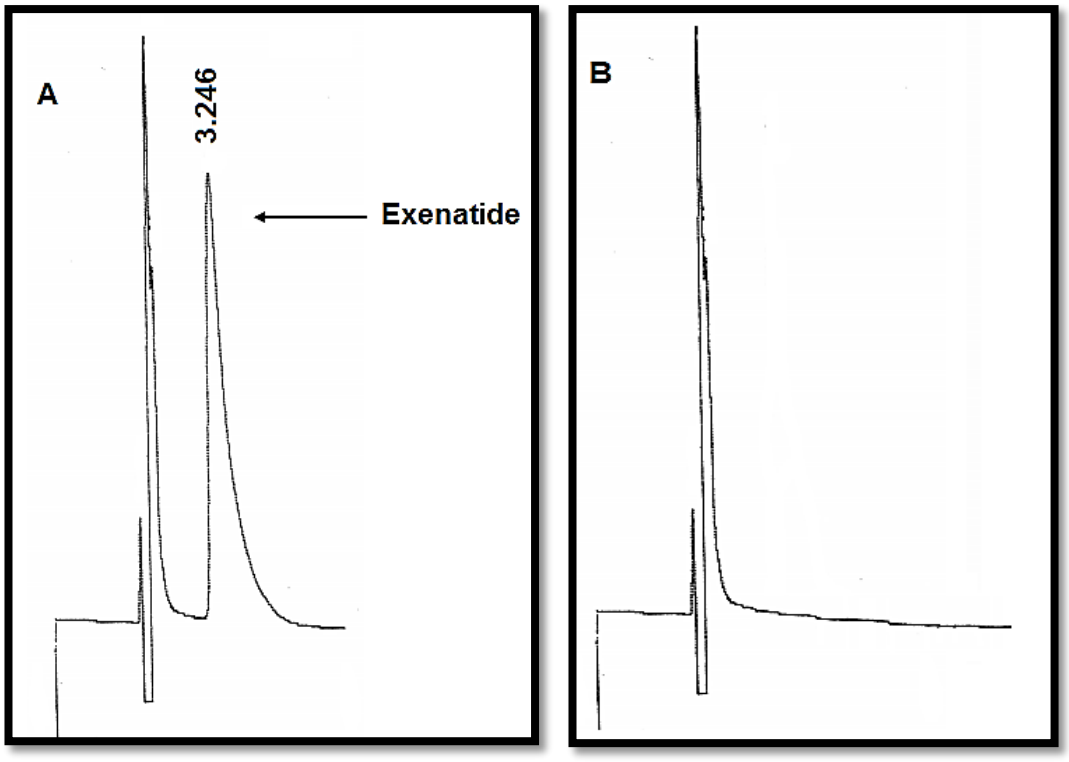

Figure 2.1: Chromatograms representative of exenatide in aqueous solution (A) and a Blank run (B).

2.5.2 Linearity measurements

The linearity of the method was validated according to the standards specified in the literature (134). All AUC values of the six standard concentrations were plotted against the concentration values to produce a standard curve. The coefficient of determination (R^2) of the line of best fit was 0.987 and the intercept was not significantly different from zero. The equation of the slope is Y = 7474x+3917. This confirms the linearity of the selected method. The standard curve of exenatide is shown in Figure 2.1.

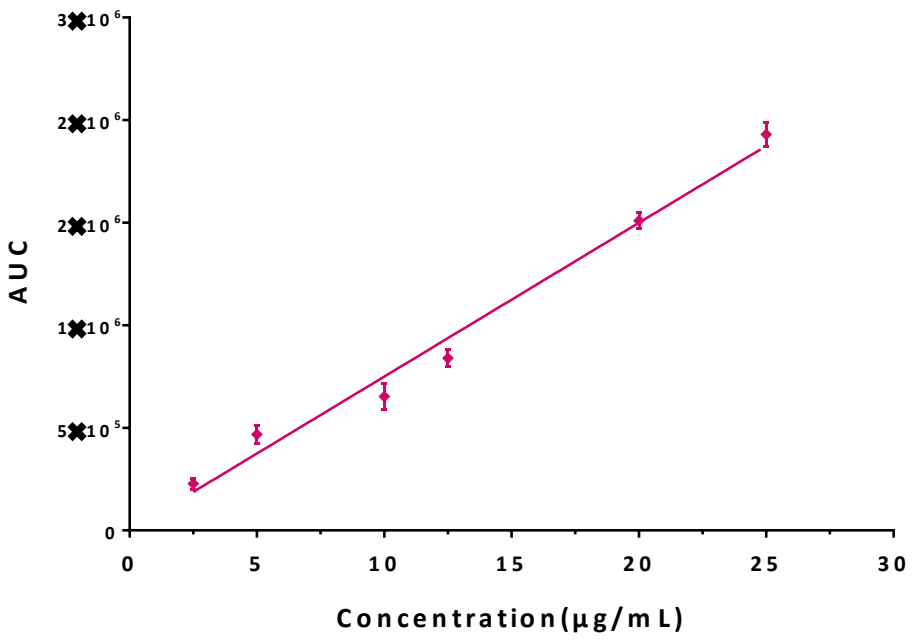

Figure 2.1: Standard curve of exenatide (mean ± SD, n=3).

2.5.3 Precision measurements

The other important parameter that was validated in this assay was precision. For the three tested concentrations (58µg/mL, 29µg/mL and 14.5µg/mL), the intra and inter day readings gave a percentage relative standard deviation (RSD) values within the acceptable limit of 3% (134). The values for the intra and inter day precision values are shown in Table 2.1. For days 1, 2 and 3, three reading of each concentration was taken and the percentage of the mean for each concentration is listed for the corresponding day. The inter-day precision for each concentration is then calculated from the mean and the standard deviation and are listed in column 5 of Table 2.1.

Table 2.1: Intra and inter day assay precision.

Conc. µg/mL	Day 1 % of mean	Day 2 % of mean	Day 3 % of mean	Inter-day precision %RSD	Intra-day precision %RSD
58.0	98.6-100.6	99.6-101.2	99.7-100.3	0.3-1.1	1.05
29.0	99.5-100.6	98.2-102.2	99.4-100.5	0.5-2.1	2.74
14.5	98.8-102	99.6-101	99.5-100.7	0.6-1.8	0.75

The method was specific and chromatographic peaks in blank and calibration samples were free of interference. Standard curve was linear (R^2 >0.987) in the range 2.5-25 µg/ml (Figure 2.1). The precision of the method was established since all the intra and inter day variations were < 3%.

This chapter presents a selective and reproducible HPLC method for the analysis of exenatide. There were no detectable degradation peaks which suggests method sensitivity, and the drug was stable throughout the analysis. This method, was deployed in the analysis of exenatide concentrations in

aqueous solutions at different pH, ionic strength and temperatures (Chapter 3).

2.5.4 Experimental variation

The observed variation in findings including the reaction rate constants was possibility due to instrumental error and/or fluctuation of temperature in the laboratory. Temperature control during experiments offer an accuracy in the range of ± 0.02°C, resulting in maximum error of ±1%. The Hanna pH-meter specified an accuracy of ± 0.01 pH unit, therefore the measurement of pH involved an error of ± 0.005 of a pH unit. Standardisation techniques could introduce an error of ± 0.05. Therefore any measurement could have involved a maximum error of ±0.055 of a pH unit. The HPLC analysis of samples compared to standards involved a maximum error of ± 3%. Therefore, the reaction rate constant could have a maximum error of ±3%. When experiments were repeated, the reaction rate constants were reproducible within this range.

Chapter Three

3 The effect of temperature, ionic strength and pH on the stability and degradation of exenatide

3.1 Introduction

Exenatide is currently marketed in injectable formulations, and needs to be stored under refrigeration. The stability of these formulations is expected to be mainly affected by temperature, ionic catalysis and pH change. In order to investigate these effects, exenatide concentrations at various temperatures, pH and ionic strength values were investigated. Initially, a wide range of temperatures and pH values were examined in order to determine most reliable experimental conditions to use for exenatide stability testing. Eventually, exenatide concentrations were determined using temperatures of 35, 40 and 45 ^{o}C, and pH values of 2.00, 3.50, 4.50, 6.00, 7.00, 8.00 and 10.00, as well as ionic strength effect with different concentrations of sodium chloride (50, 100 and 250 mM) at different pH values (2.00, 4.50 and 10.00). These values represent a pH value below, near and above the reported isoelectric point (pI) of 4.2 (123). Under these experimental conditions, exenatide shelf life, rate of degradation and reaction rate constants were measured (Figure 3.1). The stability of aqueous exenatide solution at actual laboratory temperature (20±1 °C) was also examined over a period of 6 weeks.

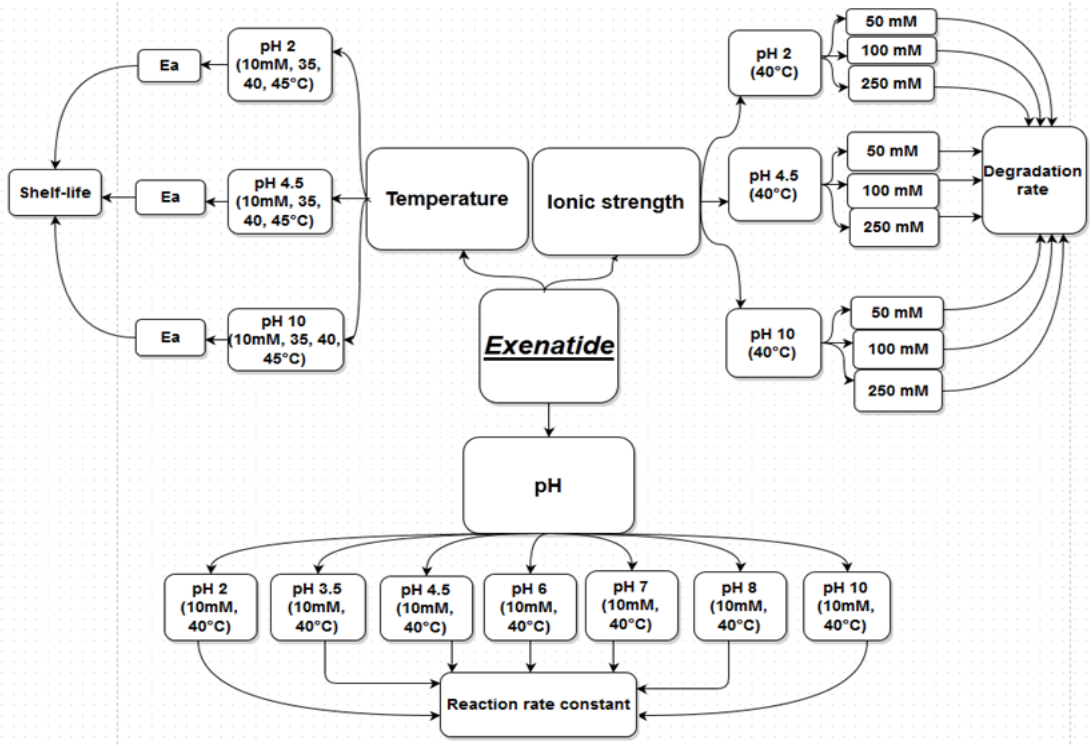

Figure 3.1: A diagram outlining the study design investigating the effects of temperature, ionic strength and pH on the stability of exenatide.

3.2 Reaction rate order measurements

For each experiment, the weighed amount of exenatide was dissolved in the specified buffer solution to produce the required concentration. Samples were collected for analysis as soon as temperature equilibration occurred (time = 0) and then at a regular time intervals. The samples are then analysed by HPLC and the logarithm (log_{10}) on the remaining concentration percentage is plotted against time to obtain the reaction rate constant for each condition.

The first order reaction can be expressed in the following equation.

$$-dc/dt=kc \qquad \textbf{Equation (3.1)}$$

$$\ln C-\ln C_0=-kt \qquad \textbf{Equation (3.2)}$$

$$\log C=\log C_0-(kt/2.303) \qquad \textbf{Equation (3.3)}$$

The linear relationship of Equation 3.3 indicates that a plot of log of the remaining concentration percentage against time is linear with a slope ($-k_{obs}/2.303$) from which k_{obs} is determined.

The shelf-life can be calculated using Equation 3.4 for a first order reaction.

$$T_{90}=0.105/k_{obs} \qquad \textbf{Equation (3.4)}$$

3.2.1 Stability indicating HPLC method.

To determine whether or not the assay method was stability indicating, exenatide was subjected to a range of stress conditions to accelerate the degradation which included acidic (HCl), alkaline (NaOH) and oxidative stress (H_2O_2) conditions at 40°C. The method was also investigated to ensure the separation of the peak from any interference.

3.2.2 Acid degradation

The acid stress test on exenatide was performed as described in section 2.4.1. The chromatogram (Figure 3.1) showed the detectable exenatide peaks at zero and 180 minutes. There was no interference from any degradants with the exenatide peak at the retention time. At the end of the experiment (180 min) only 50% of the peak area remained intact. No new peaks or interference with the analytical peak was detectable.

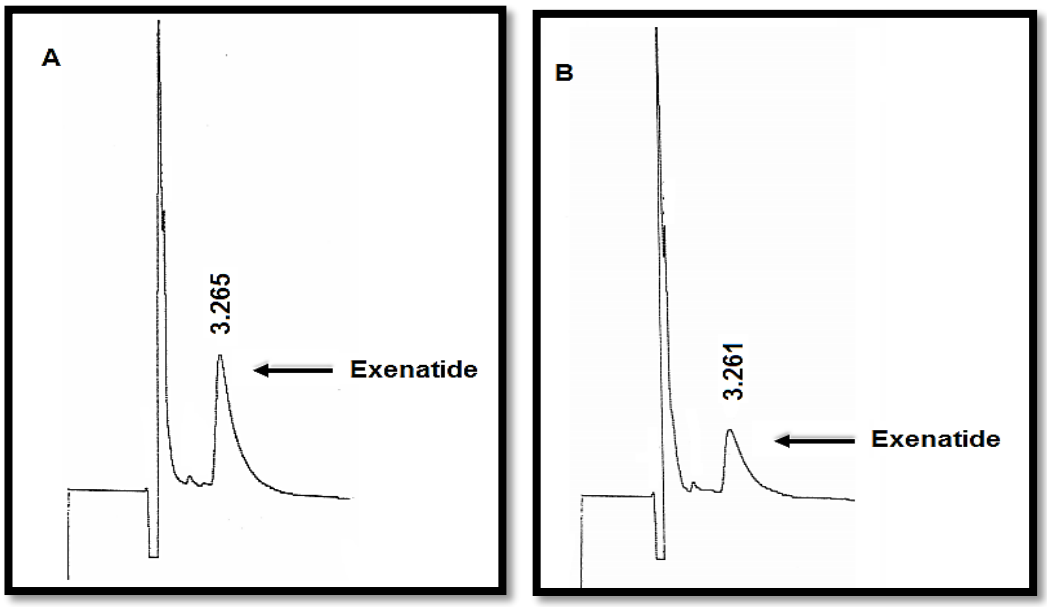

Figure 3.1: Chromatograms representative of degradation of exenatide in 0.1M HCl (pH 1.00) at 40°C. (A) At zero time and (B) after 180 minutes.

3.2.3 Alkaline degradation

An alkaline stress test on exenatide was performed as described in section 2.4.1. As evident from Figure 3.2, exenatide essentially completely degraded in the alkaline conditions and new peaks emerged which were not present initially. These peaks are degradants of exenatide in the alkaline solution. These peaks did not interfere with the exenatide peak.

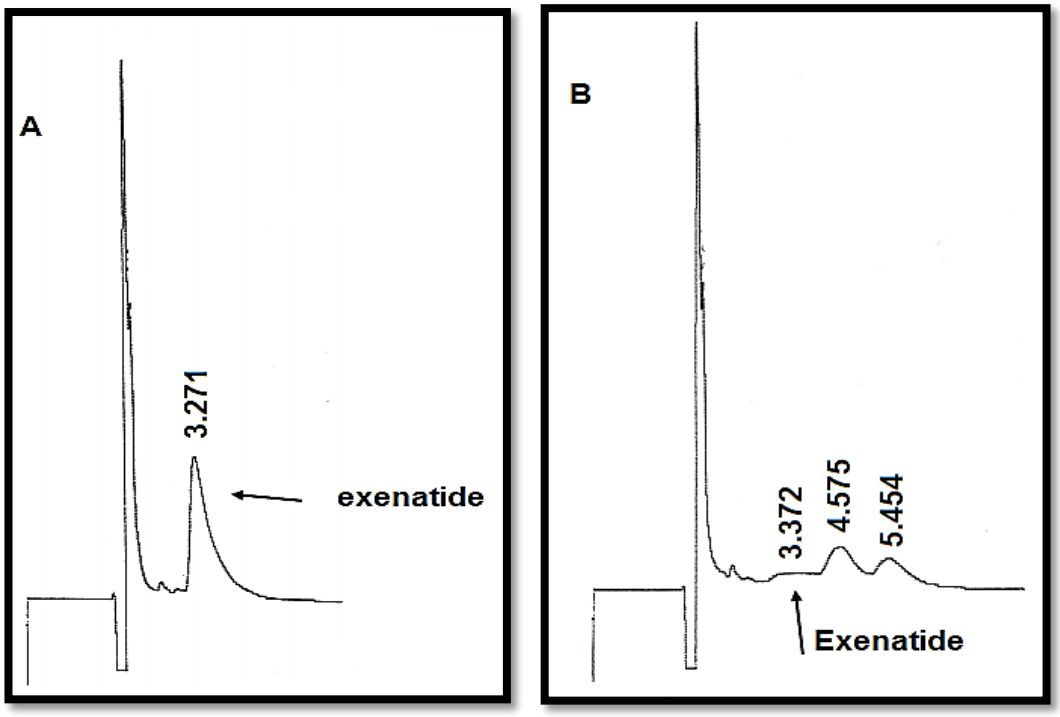

Figure 3.2: Chromatograms representative of degradation of exenatide in 0.1M NaOH (pH 13.00) at 40°C. (A) At zero time and (B) After 180 minutes.

3.2.4 Oxidative stress degradation

The method used to perform the oxidative stress test on exenatide was described in section 2.4.1. After 180 minutes, exenatide had completely degraded and there was no peak visible, which indicated exenatide was very susceptible to oxidation (Figure 3.3).

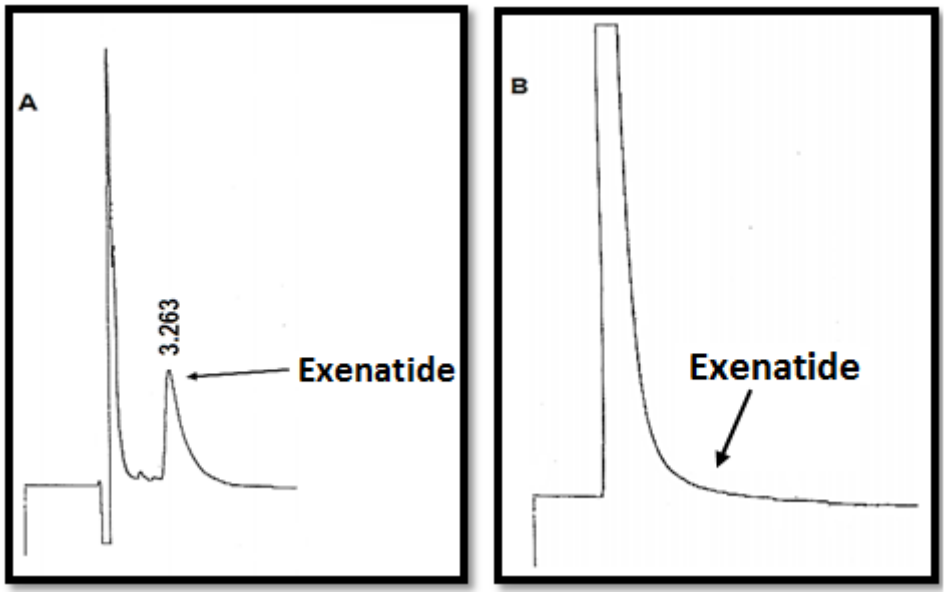

Figure 3.3: Chromatograms representative of degradation of exenatide in 30% H_2O_2 at 40°C. (A) At zero time and (B) After 180 minutes.

3.3 Determination of pH rate profile

Most drugs are salts of either weak acids or bases depending on their pK_a values. As a result, drug molecules will dissociate partially or completely in aqueous solution which will have an effect on the stability of the drug. Often, there is general acid-base catalysis and/or specific hydrogen ion [H^+] or hydroxide ion [OH^-] catalysis in aqueous solutions.

At the lower pH's, hydrogen ion catalysis is often the predominant reaction, while hydroxide ion catalysis is predominant at higher pHs. At the intermediate range, the reaction can be catalysed by either hydrogen or hydroxide ion, or it can be independent of pH. The pH stability profile can be obtained from plotting the logarithm of the rate constant versus pH. The pH-rate profile has different forms which are typical of reactions susceptible to specific acid-base catalysis.

The degradation of exenatide was investigated at different pH values ranging from 2.00 to 10.00. The buffers used to achieve these pH values are listed in (Table 3.1). The final pH of the buffer was adjusted to the required value after

adding exenatide to the solution, to account for any effect on the pH of the buffer. The pH of the mixtures were recorded at the start and the end of the experiment. There was no significant change in the pH of the mixture over the run of the experiments. The concentration of all buffers in this study were 10mM. The experiments were run until at least 20% of the drug was degraded.

The rate of degradation of exenatide at each pH was determined from plotting the logarithm of the percentage of drug remaining against time. A least squares line of best fit was used to fit the slope of the line from which the reaction rate constant (k_{obs}) was determined. All the assays were done on triplicates.

Table 3.1: Buffers used in the project

pH	Buffer	pKa
2.00	Chloroacetate buffer	2.88
3.50	Citrate buffer	3.14
4.50	Acetate buffer	4.76
6.00	Citrate buffer	6.39
7.00	Citrate buffer	6.39
8.00	Tris buffer	8.06
10.00	Ethanolamine buffer	9.50

3.3.1 Degradation of exenatide in aqueous solution at pH 2.00.

The degradation of exenatide at pH 2.00 was studied by adding the drug to chloroacetate buffer. The mixture was maintained at 40°C and samples were taken and injected onto the HPLC, until the concentration of the drug in the solution reached less than 80% of the original concentration and the data plotted as shown in Figure 3.4. The reaction rate constant was calculated from the slope according to Equation 3.3 and it was $1.44x10^{-03}$ h^{-1} as listed in Table 3.2.

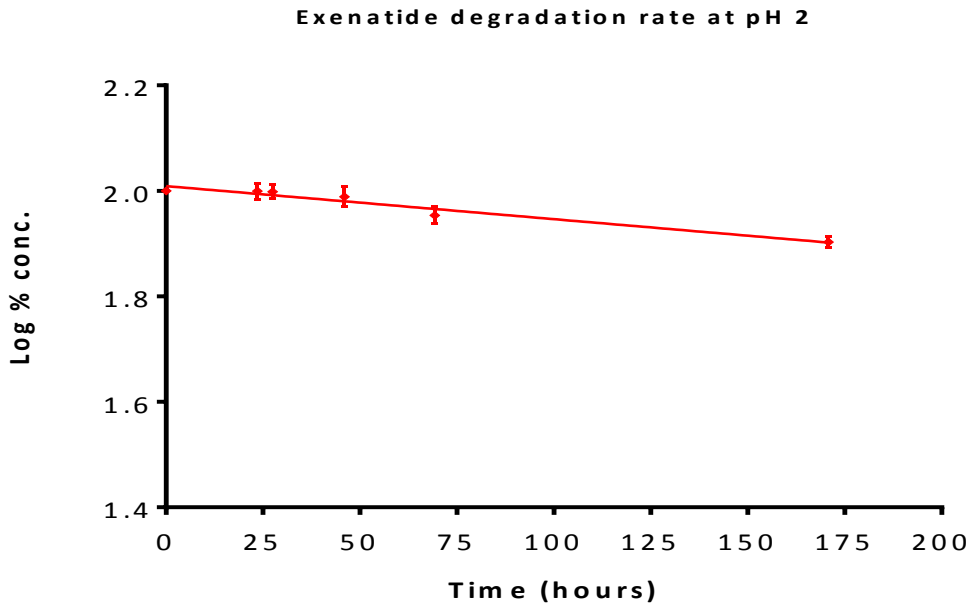

Figure 3.4: Degradation of exenatide at pH 2.00 (n=3, data average ± SD)

3.3.2 Degradation of exenatide in aqueous solution at pH 3.50.

To investigate the rate of degradation of exenatide at pH 3.50, citrate buffer (pKa 3.14) was employed. The mixture was maintained at 40°C until at least 20% of exenatide was degraded. The plotted data showed a linear relationship which indicate a first order reaction as shown in Figure 3.5 and the reaction rate constant calculated to be $9.73x10^{-03}$ h^{-1}.

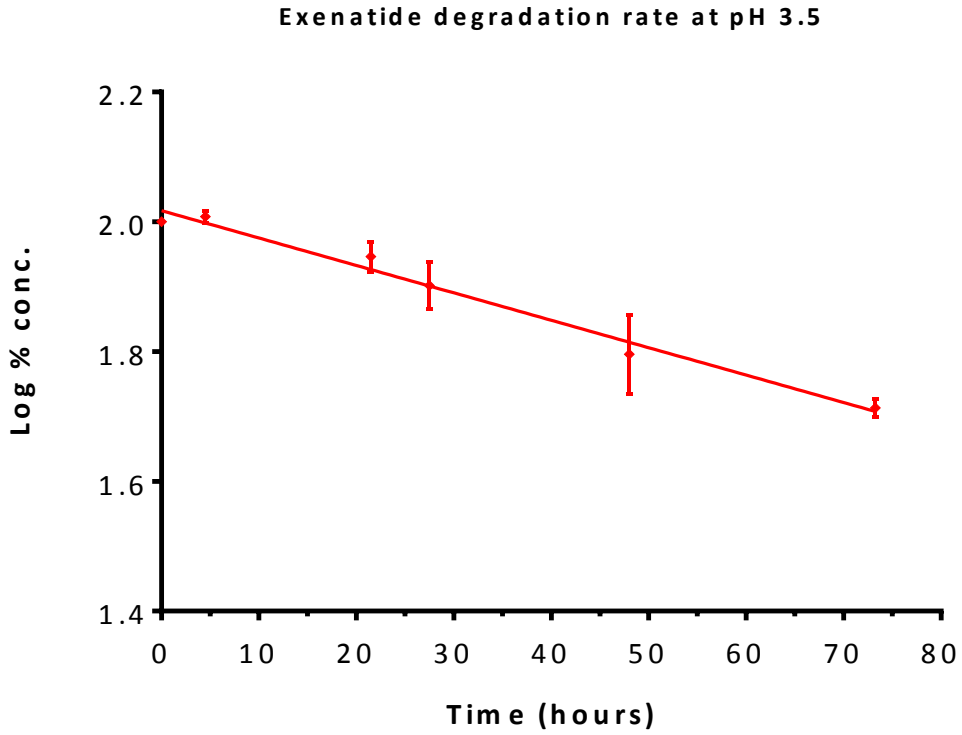

Figure 3.5: Degradation of exenatide at pH 3.50 (n=3, data average ± SD)

3.3.3 Degradation of exenatide in aqueous solution at pH 4.50.

Acetate buffer (pKa 4.76) was used to investigate the degradation of exenatide at pH 4.5 using the method described in section 3.3.1. The plot of the data represented a linear relationship indicating a first order reaction with a reaction rate constant of 1.15×10^{-02} h^{-1}. The plot of the data is represented in Figure

3.6.

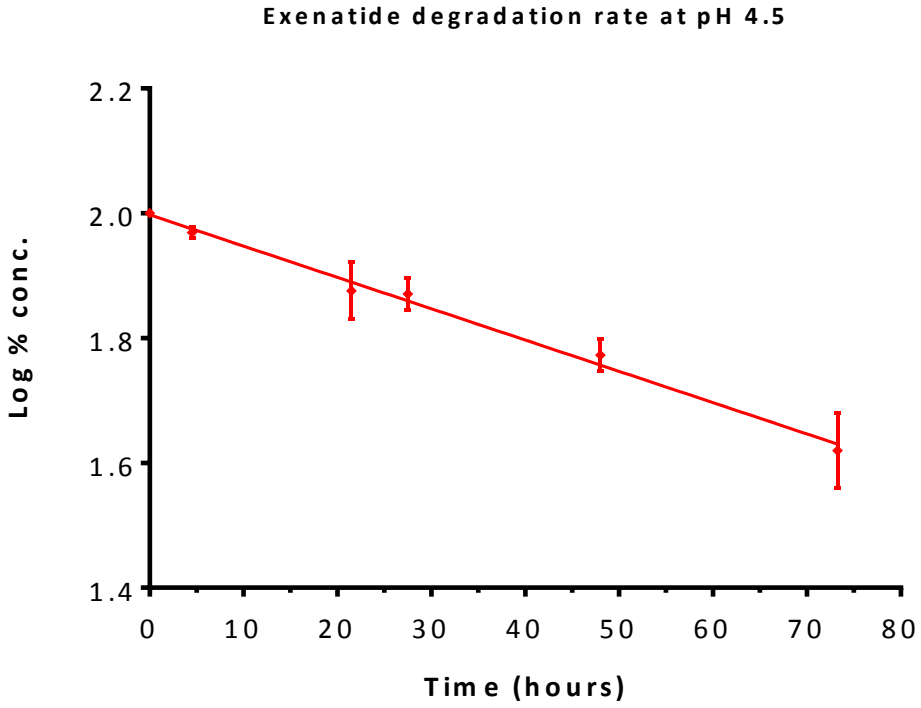

Figure 3.6: Degradation of exenatide at pH 4.50 (n=3, data average ± SD)

3.3.4 Degradation of exenatide in aqueous solution at pH 6.00 and 7.00.

To investigate the degradation of exenatide in pH 6.00 and pH 7.00, citrate buffer (pK_a 6.39) was used and the pH was adjusted using HCl and NaOH to arrive at the required pH values. The two exenatide and buffer mixtures were placed in water bath at a temperature of 40°C and samples were taken at regular intervals. The collected samples were injected onto HPLC and the process continued until the remaining concentration exenatide was less than 80% of the starting concentration. The data for both experiments produced a linear relationship which point to a first order reaction rate for both experiments. The data for citrate buffer at pH 6.00 is represented in Figure 3.7 and the reaction rate constant is $1.05x10^{-03}$ h^{-1}. On the other hand, the reaction rate constant for pH 7.00 is $2.10x10^{-03}$ h^{-1} and the data represented in Figure 3.8.

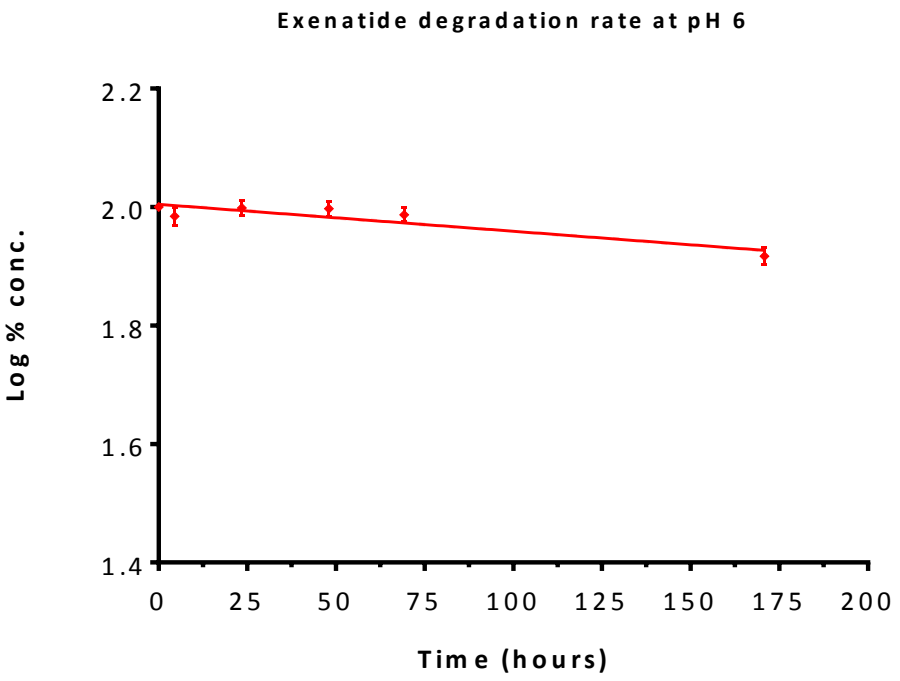

Figure 3.7: Degradation of exenatide at pH 6.00 (n=3, data average ± SD)

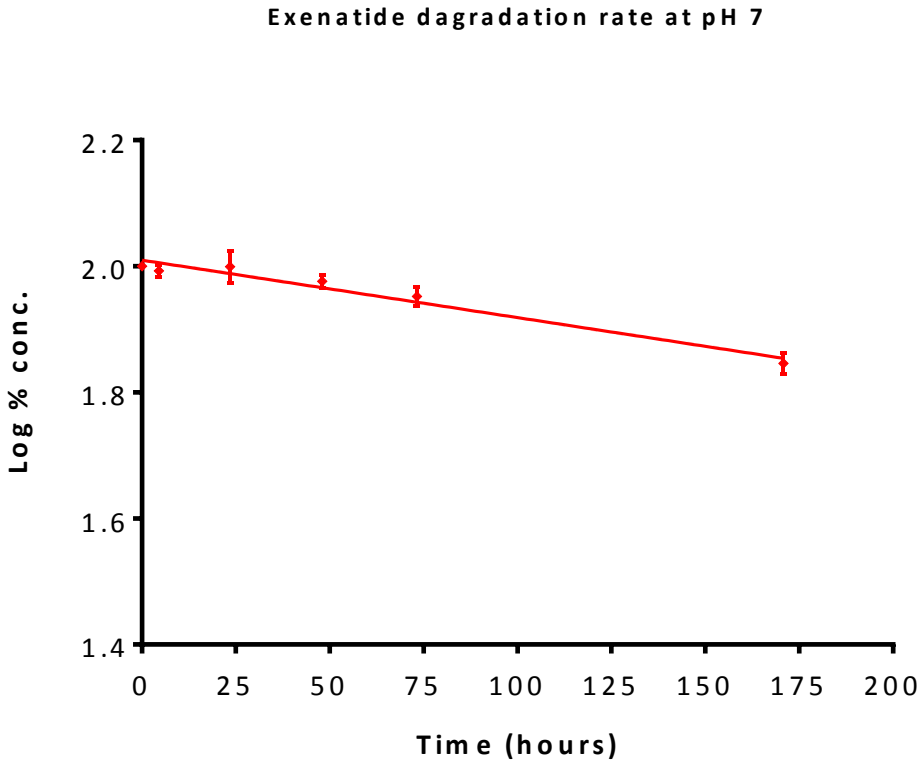

Figure 3.8: Degradation of exenatide at pH 7.00 (n=3, data average ± SD)

3.3.5 Degradation of exenatide in aqueous solution at pH 8.00.

The stability of exenatide in pH 8.00 was investigated an evaluated using Tris buffer (pKa 8.06) using the method described in section 3.3.1. The data were plotted and a linear relationship resulted as shown in Figure 3.9 pointing to a first order reaction. The reaction rate constant of the degradation of exenatide in a pH 8.00 buffer was 5.50×10^{-03} h^{-1} which was calculated from the slope of the line of best fit.

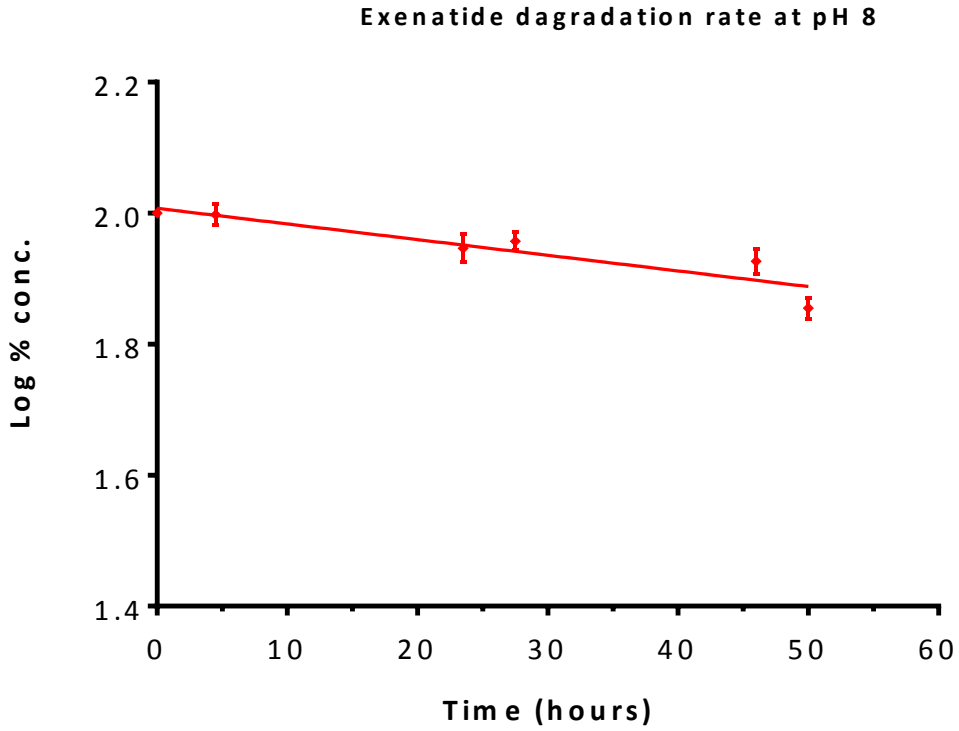

Figure 3.9: Degradation of exenatide at pH 8.00 (n=3, data average ± SD)

3.3.6 Degradation of exenatide in aqueous solution at pH 10.00.

The degradation of exenatide was also investigated in pH 10.00 buffer solution using ethanolamine (pKa 9.5). The mixture was placed in a hot water bath at 40°C and samples were collected at a regular intervals. The collected samples were analysed using HPLC and the process was repeated until at least 20% of the initial exenatide concentration at the start of the experiment degraded.

The logarithm of the percentage of the remaining concentration was plotted against time as shown in Figure 3.10. Since the plot shows a linear relationship, it can be concluded that the degradation of exenatide in pH 10.00 buffer is first order with a reaction rate constant of 2.05×10^{-02} h^{-1}.

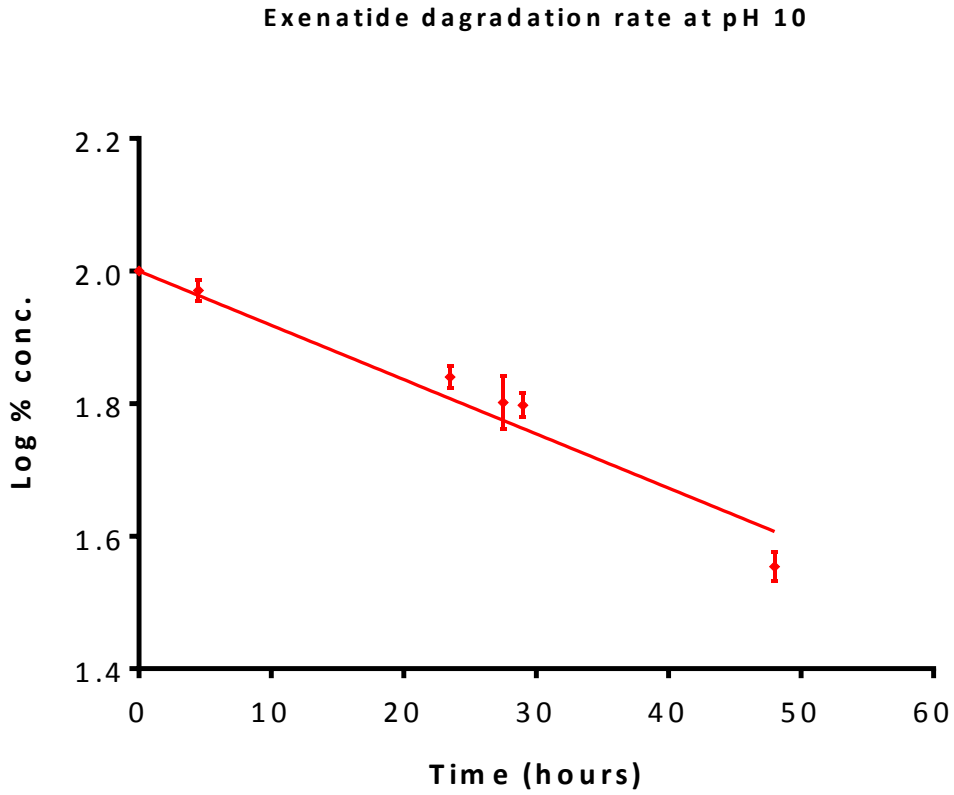

Figure 3.10: Degradation of exenatide at pH 10.00 (n=3, data average ± SD)

The reaction rate constants of the degradation of exenatide in aqueous solutions at the investigated pH values are listed in Table 3.2. This clearly shows that exenatide is most stable at pH 6.00 with reaction rate constant of 1.11×10^{-3} compared to all the investigated pH values while pH 10.00 is the least stable with reaction rate constant of 1.89×10^{-2}. The rate constants are also presented graphically in Figure 3.11.

Table 3.2: Exenatide reaction rate constants at selected pH values at 40°C.

pH	k h^{-1}	±SE	Shelf-Life (Hour)
2.00	$1.44x10^{-3}$	$6.91x10^{-5}$	72.92
3.50	$9.73x10^{-3}$	$6.36x10^{-4}$	10.79
4.50	$1.15x10^{-2}$	$4.84x10^{-4}$	9.13
6.00	$1.11x10^{-3}$	$6.91x10^{-5}$	94.59
7.00	$2.10x10^{-3}$	$1.61x10^{-4}$	50.00
8.00	$5.40x10^{-3}$	$4.61x10^{-4}$	19.44
10.00	$1.89x10^{-2}$	$1.18x10^{-3}$	5.56

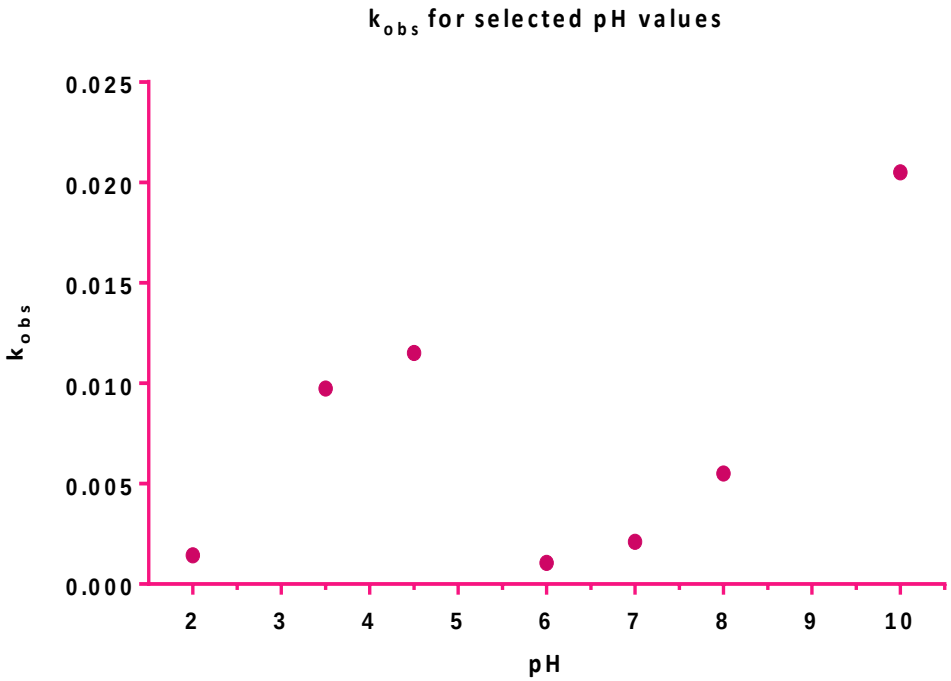

Figure 3.11: Graphical presentation of reaction rate constants for selected pH values at 40 °C.

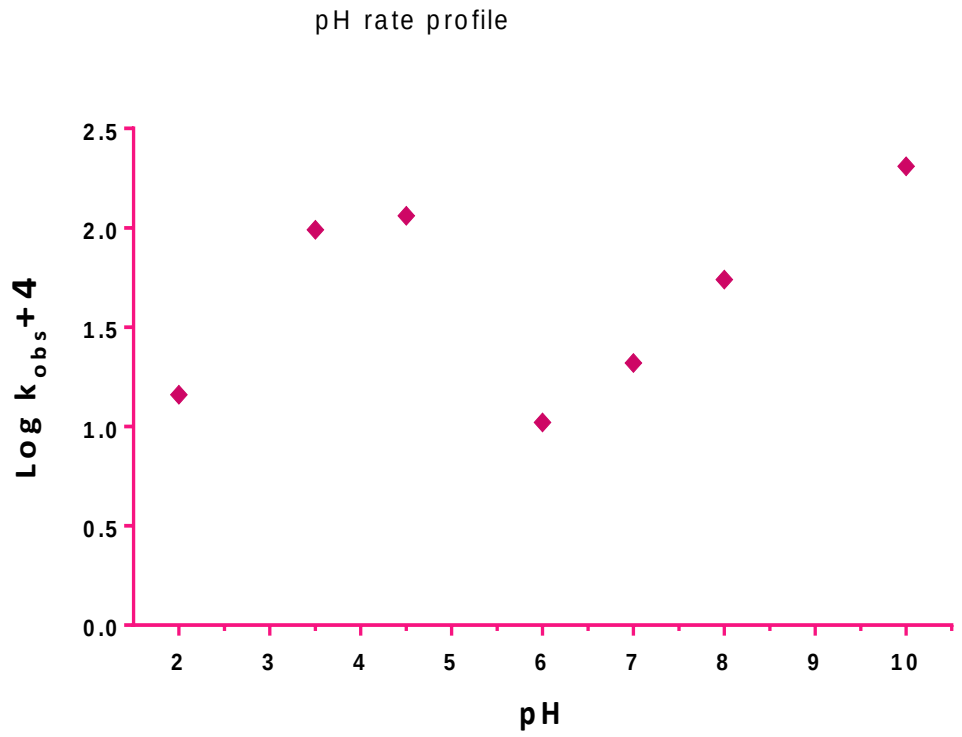

Figure 3.12: Graphical presentation of log k versus pH.

The slope of the points from pH 6.00 to pH 10.00 inclusive is 0.33 which is significantly different from +1 indicating that the degradation in the alkaline region is not a simple hydroxide ion catalysis. Furthermore, the slope indicates that more complex reactions are taking place in the degradation of exenatide and this may involve multiple degradation pathways taking place at the same time. This could relate to the formation of the cyclic intermediate in the pathway to deamidation at higher pH values.

On the other hand, there is no clear pattern of degradation under acidic condition. The reaction rate appears to increase until the pH reaches 3.50 and then remains constant until pH 4.50 before decreasing to the lowest point at pH 6.00. This could be due to the changes in the ionisation of the drug as the pH changes influencing it rate of degradation. The isoelectric point of exenatide is approximately 4.20 (123). The manufacturers of commercially available exenatide injection (Byetta®) have formulated its product at pH 4.50, which from data in Table 3.2 is less stable than pH 6.00. Exenatide will exist

mainly as the protonated species to pH 3.20 and above, where the zwitterionic form will predominate from pH 3.20-5.20, above which the anionic form will be dominant. It would appear that the zwitterionic form is more reactive than the individually charged species.

3.4 The effect of ionic strength on the rate of degradation of exenatide

Another facet of the stability of exenatide is the effect of ionic strength on the rate of degradation. The effect of ionic strength was investigated by altering the ionic concentration in the buffer solutions via the addition of sodium chloride (NaCl). The ionic strength of the buffer was calculated using Equation 1.1. The buffers investigated in this study were chloroacetate (pH 2.00), acetate (pH 4.50) and ethanolamine (pH 10.00), and the ionic strengths were 50, 100 and 250mM. The three ionic strengths of each buffer were placed in a water bath at 40°C and samples were collected at regular intervals. The collected samples were then injected onto HPLC for analysis and the process repeated until less than 80% of the starting exenatide concentration remained in solution. The logarithm of the reaction rate constants for each ionic strength of each buffer is plotted against the square root of the ionic strength as shown in Figure 3.13. The graph shows that there is marginal increase in the degradation rate of exenatide as the ionic strength increases. This would imply that no species of exenatide is subjected to significant ionic strength effects. A value of 3 was added to the values in Figure 3.13 to move them to the positive region.

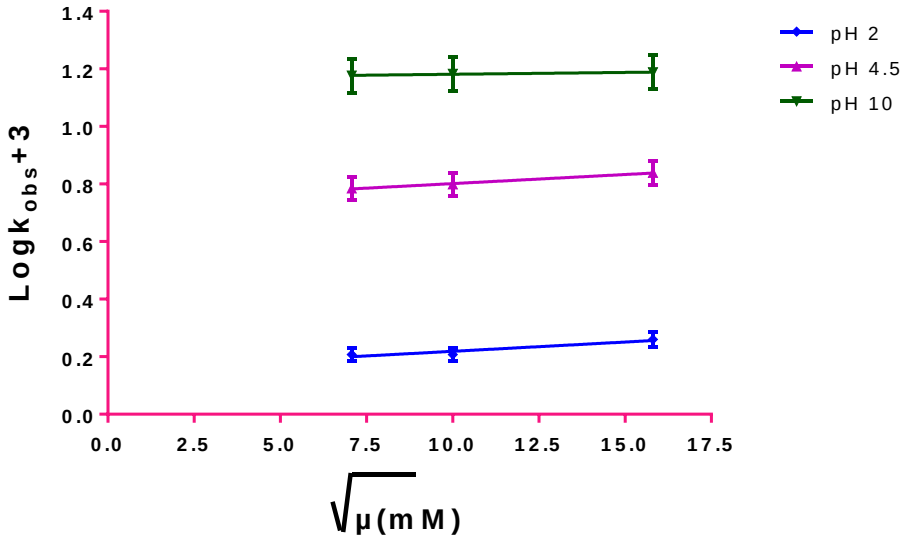

Figure 3.13: A plot of the logarithm of the reaction rate constant for the three buffers against the square root of the ionic strength of the buffer.

3.5 Temperature dependence studies

The effect of temperature on exenatide was investigated at three temperatures (35, 40, and 45 °C). The pH values selected were pH 2.00, 4.50, and pH 10.00. The rate constants (k) were obtained from plotting the log of the remaining concentration percentage versus time. The degradation rate at each pH value increased with temperature as expected.

The exenatide solution at pH 2.00, 4.50 and 10.00 were incubated at 35, 40 and 45°C. Samples were collected at the start of the experiment (time = 0) and then at selected time intervals.

The log of the remaining concentration percentage versus time was plotted for each test and the reaction rate constant was obtained. The log of the reaction rate constant at each temperature was then plotted against the reciprocal of temperature in Kelvin (1/T) to determine the activation energy. The activation energy is calculated using the following equation

E_a=-slope (2.303.R) **Equation (3.5)**

Table 3.3 shows the rate constant values for pH 2.00, 4.50 and 10.00 at temperatures 35, 40 and 45 °C. When the log of the rate constant (log k) is plotted against the reciprocal of temperature (1/T) in Kelvin the activation energy (E_a) of exenatide is obtained using the Arrhenius plot.

The plots of the graphs for all temperatures produced linear relationships; hence, the degradation of exenatide follows the Arrhenius relationship.

Table 3.3: The observed rate constants values of exenatide at pH 2.00, 4.5 and 10 and temperatures 35, 40 and 45 °C.

Temp. °C	k h^{-1} (pH 2.00)	k h^{-1} (pH 4.50)	k h^{-1} (pH 10.00)
35	1.20E-03	5.53E-03	8.52E-03
40	1.38E-03	1.17E-02	2.12E-02
45	5.52E-03	1.84E-02	2.95E-02

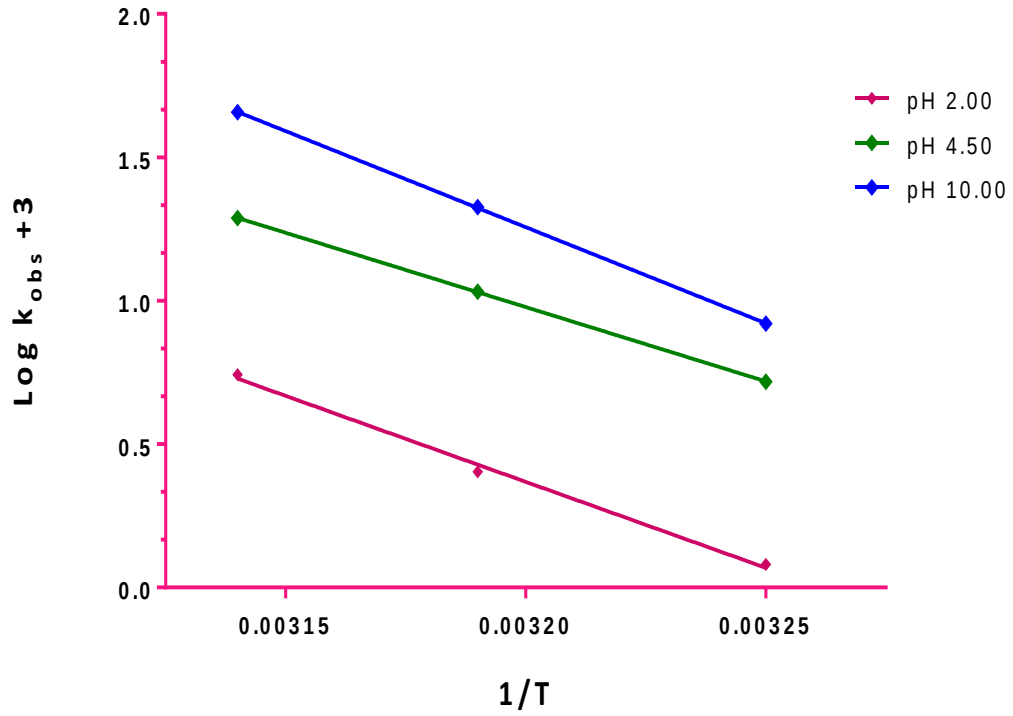

Figure 3.14: Graphical presentation of log k versus the reciprocal of temperature in kelvin.

The activation energy for exenatide at pH 2.00 was calculated using Equation 3.5 to be 124.0 $kJmole^{-1}$ as shown in Table 3.4. The activation energy for pH 4.50 and 10.00 were 121.0 and 138.0 $kJmol^{-1}$ respectively.

Table 3.4: Activation Energy at selected pH values. Data expressed as mean ± SD, n=3.

pH	E_a (kJ/mol)
2.00	124±3
4.50	121±5
10.00	138±7

The preservative (metacresol) in the marketed formulation of exenatide interfered with the analysis so an exenatide formulation was prepared identically to that of the marketed formulation, but without metacresol. This formulation was left at room temperature (20±1 °C) to investigate exenatide degradation. The exenatide solution was studied for 6 weeks with samples taken at regular intervals for analysis by HPLC. The data of the study are reported in Figure 3.15. The logarithm of the remaining concentration was plotted against time. The relationship is linear with R^2 of 0.99 and the reaction rate constant was calculated and found to be $3.80x10^{-04}$ h^{-1}. The pH at which the exenatide was formulated was 4.50 to replicate that of the Byetta® marketed injection. The shelf-life of exenatide was 276 hours.

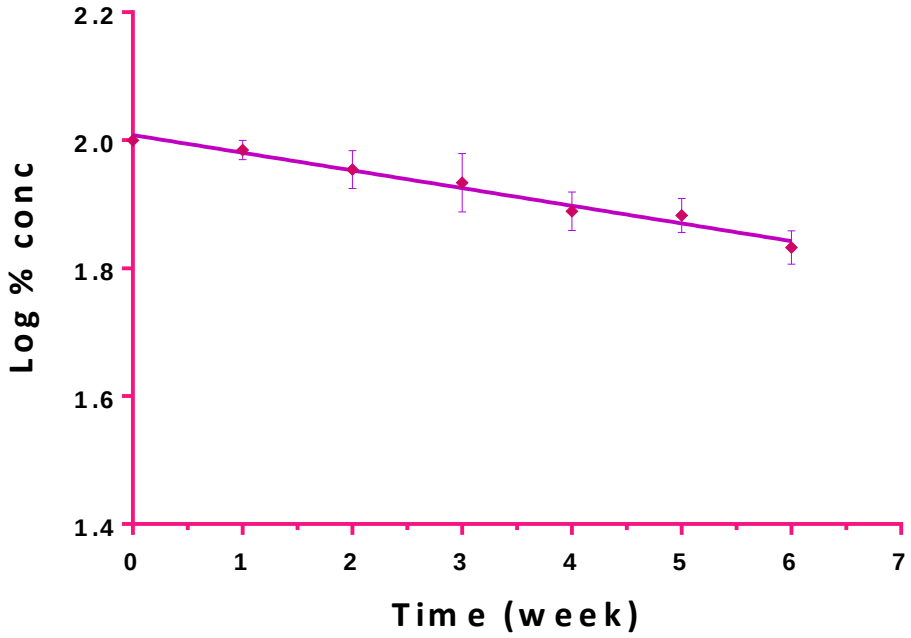

Figure 3.15: Six weeks degradation profile of exenatide at room temperature (20°C) and pH 4.50. R^2=0.99.

Using the activation energy for pH 4.50 found in (Table 3.4) and the reaction rate constant for pH 4.50 from Table 3.3, the theoretical reaction rate constant for pH 4.50 at 20 °C was also calculated. The theoretical (extrapolated) reaction rate constant calculated to be $4.83x10^{-4}$ h^{-1} which is higher than the observed reaction rate constant calculated from the 6 weeks experiment ($3.80x10^{-04}$ h^{-1}), however, there was no significant difference if we calculated the theoretical reaction rate constant at the higher end of the activation energy (i.e. 126 kJ/mol) which gives a reaction rate constant of $4.24x10^{-04}$ h^{-1}. The slight improvement in the degradation rate after the six weeks experiment compared to the calculated reaction rate constant could be due to the presence of mannitol in the formulation of the six weeks experiment which may have a stabilising effect of proteins (135). The reaction rate constants for pH 4.50 at a selected number of temperatures is listed in Table 3.5. However, compared with the calculated shelf life for pH 6.00 (Table 3.6), it is obvious that the shelf-life of the formulation at pH 4.50 is markedly lower than if it was formulated at pH 6.00. Therefore, it worth doing more research on formulating the drug at pH 6.00, rather than pH 4.50 to improve its stability and prolong its shelf life.

Table 3.5: The reaction rate constants (k) and shelf life (hours) for pH 4.50 and Ea (121 kJ/mol) at selected temperatures.

Temp. (°C)	Temp. (Kelvin)	k (hr^{-1})	t_{90}(hours)
2.00	275.15	$1.878x10^{-5}$	5591.00
4.00	277.15	$2.750x10^{-5}$	3818.20
8.00	281.15	$5.805x10^{-5}$	1808.10
20.0	293.15	$4.830x10^{-4}$	217.40
25.0	298.15	$1.110x10^{-3}$	94.59
30.0	303.15	$2.480x10^{-3}$	42.00
35.0	308.15	$5.411x10^{-3}$	19.41
40.0	313.15	$1.150x10^{-2}$	9.13
45.0	318.15	$2.387x10^{-2}$	4.40

Table 3.6: The reaction rate constant (k) and shelf life (hours) for pH 6.00 and Ea (138 kJ/mol) at selected temperatures.

Temp. (°C)	Temp. (Kelvin)	k (hr^{-1})	t_{90}(hours)
2.00	275.15	$7.357x10^{-7}$	142721.20
4.00	277.15	$1.137x10^{-6}$	92348.28
8.00	281.15	$2.667x10^{-6}$	39377.46
20.0	293.15	$2.986x10^{-5}$	3516.41
25.0	298.15	$7.716x10^{-5}$	1360.81
30.0	303.15	$1.932x10^{-4}$	543.50
35.0	308.15	$4.698x10^{-4}$	223.50
40.0	313.15	$1.110x10^{-3}$	94.60
45.0	318.15	$2.553x10^{-3}$	41.13

This chapter also investigated the effects of pH, ionic strength and temperature on the stability of exenatide.

The pH stability profile shows that degradation rate increases linearly from pH 6.00 to pH 10.00. However, there is no clear pattern in the acidic region (pH 2.00 to 6.00) as the rate of degradation is low at pH 2.00 then it rises at pH 3.50 and peaks at pH 4.50 before dropping to the lowest rate at pH 6.00.

At the currently marketed formulation (pH 4.50), exenatide shelf-life was highest (5591 hr) at refrigeration temperature (2°C) compared with 45^{o}C (4.40 hr) as expected. At room temperature (25°C), shelf-life was low (94.6 hr) suggesting raised degradation outside the refrigerator. Interestingly, the stability of exenatide at 40^{o}C (pH 6.00) was significantly higher than at its marketed formulation pH 4.50. Compared with pH 4.50, the pH 6.00 formulation could have a shelf-life of 142721.20 hours (5946.7 days) at 2°C while at 25°C it would expire in 1360.81 hours (56.7 days). The pH 6.00 formulation has a 25.5 fold higher shelf-life compared to the pH 4.50 formulation at 2°C and 14.4 fold higher at 25 °C. The number fold deference between the shelf-lives of exenatide at pH 6.00 and 4.5 widens as the temperature decreases. The activation energy of exenatide at pH 6.00 could be similar to pH 10.00 since they fall on the same trend line which makes the reaction more temperature dependant. Taking into account, the greater variation between refrigerated and non-refrigerated temperatures at pH 6.00, it still offered a much improved shelf-life compared to pH 4.50. Improving the shelf-life of exenatide formulation will makes it more convenient for both pharmacies and patients. The shorter shelf-life could lead to the product reaching its expiry date before the pharmacy can supply it to patients which leads to financial loss. On the other hand, patients who are living or visiting in a temperate climate with unreliable access to power will benefit from the longer expiry date of the improved formulation.

At pH 10.00, the reaction rate constant was the highest. The linear relationship from pH 6.00 suggests that the degradation is partially catalysed by hydroxide ions. Plotting the log of the reaction rate constants versus the pH value from pH 6.00 to 10.00 produces a straight line with a slope of 0.33. This is significantly lower than 1.00 which is the value expected for the slope of a simple hydroxide ion catalysis. This indicated the involvement of multiple reaction pathways with different reaction rates in the degradation process. This

indicates that the rate of degradation in the alkaline region does not follow simple hydroxide ion catalysis but rather more complex reactions involving multiple steps and reaction pathways. These degradation pathways most likely involve deamidation as well as possibly oxidation and reduction reactions. The deamidation reaction is a two-step process and each process has its own rate parameters, which are related to the formation of the cyclic intermediate in the pathway to deamidation. It is possible that the rate limiting step could vary with pH and temperature and that this is one of the reasons that the alkaline degradation of exenatide does not follow simple hydroxide ion catalysis.

As shown in Figure 1.11, exenatide has asparagine (Asn) in its structure which is prone to deamidation reactions through the formation of the cyclic intermediate (succinimide) and the release of ammonia (113). The presence of glycine (Gly) immediately after asparagine in the structure of exenatide increases the rate of deamidation of asparagine as Asn-Gly is a sequence with the highest reactivity in polypeptides (113, 136). Even though deamidation of glutamine is not as common as for asparagine (113), it may still contribute to the degradation of exenatide. As previously stated, the plot of the log of the reaction rate constant versus pH value from pH 6.00 to 10.00 produced a slope of 0.33. This is consistent with published studies showing that an increase in the hydroxide ion concentration does not always lead to a proportional increase in the rate of deamidation (136).

Oxidation is the other degradation pathway that can potentially affect any proteins containing the amino acids His, Met, Cys, Tyr, and Trp (113). Exenatide has three of the above listed amino acids in its structure (His, Met and Trp) which makes it potentially susceptible to oxidation. Histidine (His) is sensitive to metal-catalysed oxidation and can result in various species with 2-oxo-His as the main product. This product has been detected in human growth hormone, relaxin and prolastin (113). Methionine which is important for conformational stability and the function of the protein can be oxidised by molecular oxygen to produce a sulfoxide. The rate of oxidation of Met is almost independent of pH, however, its rate is increased with increased accessibility of the solvent to the residue. The other amino acid in the structure of exenatide susceptible to oxidation is Trp, which can be oxidized to produce kynurenine

derivatives even in the absence of light, especially when iron-based oxidants are present (113). This confirms our findings at section 3.2.4 that exenatide is sensitive to oxidation supported by the disappearance of exenatide peak when subjected to hydrogen peroxide oxidation.

The isoelectric point (pI) of exenatide is approximately 4.2 (123). Exenatide will exist mainly as the protonated form (cationic) at pH 3.20. From pH 3.20 to 5.20, the zwitterionic form will predominate, while above pH 5.20 the deprotonated form (anionic) will dominate. It would appear the zwitterionic form is more reactive, than the individually charged species. Figure 4.1 shows the different structures of the functional groups of exenatide in different pH solutions.

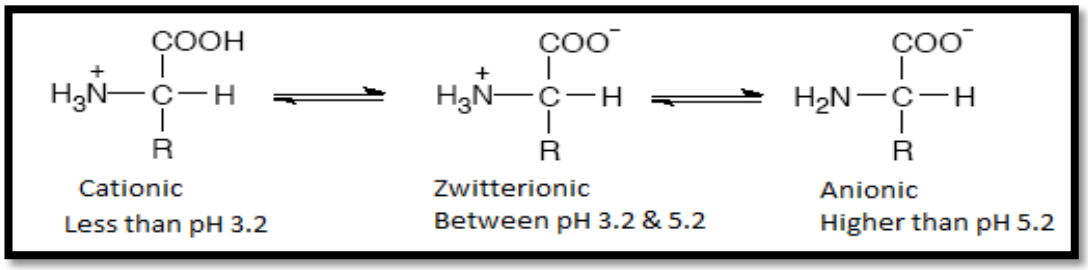

Figure 3.16: The structure of functional groups of exenatide in solutions at different pHs.

As expected, the rate of degradation of exenatide with increased temperature followed the Arrhenius model. Exenatide had the highest activation energy at pH 10.00 (138±7 kJ/mol) and the lowest at pH 4.50 (121±5 kJ/mol) which suggests that the formulation was most influenced by change in temperature at pH 10.00, and was least influenced by change in temperature at pH 4.50. Even though our findings showed the activation energy of exenatide to be relatively high, it is still comparable with the activation energies of other polypeptides such as oxytocin (137, 138). The poly-peptide drug, pramlintide, has an activation energy of 21.9 kcal/mol at pH 4.00 which equals to 91.63 kJ/mol (139) whereas, the activation energy of alteplase is 34.2 kcal/mol which equals to 143.2 kJ/mol (140). Furthermore, the activation energy may vary for the same peptide drug according to the type of reaction taking place. For insulin, the activation energy of neutral deamidation is 80 kJ/mol while the aminolysis reactions have an activation energy of 119 kJ/mol (141). Thus, the activation energy of a peptide drug can change depending on the pH and the

structural conformation. In the case of the anti-stroke peptide, NR2B9c, the lowest activation energy is 81.87 kJ/mol at pH 7.50 (Tris buffer) and the highest activation energy is 128.37 kJ/mol at pH 8.00 (Tris buffer), due to altered reaction mechanism (142). Oxytocin, also has an activation energy of 116.3 kJ/mol at pH 4.50 (143) which is similar to that of exenatide at the same pH value suggesting that our findings are in line with other drug peptides, such as oxytocin.

Ionic strength stability profiles showed that the rate of degradation of exenatide did not significantly change when the ionic strength was set at 50, 100 and 250mM using NaCl at pH 2.00, 4.50 and 10.00. The results suggest that changing the ionic strength of the buffer solution does not have a significant effect on the rate of degradation within each pH value. This is in line with previous studies which have demonstrated little change in degradation pathways of protein-drugs at different ionic strength concentrations when pH is maintained constant (144-146). This could also be due to the fact that ionic strength does not affect the rate of deamidation of the asparagine residue (136).

The degradation rate of exenatide in the Byetta® replica formulation, over 6 weeks, when stored at 20±1 °C (actual laboratory temperature) was slower than that extrapolated from the activation energy and the reaction rate constant in pH 4.50 buffer at 40°C. However, the difference between the rates of degradation were very close to being within the experimental margin of error of the activation energy data. The difference between Byetta formulation and the pH 4.50 buffer used, was the presence of mannitol in Byetta, which is an osmolarity-regulator. This could be due to the stabilising effect of mannitol (135) on the degradation of exenatide or could be due to the slower denaturation of the protein. It is possible to exclude the effect of mannitol on the stability of exenatide in the future by inclusion of mannitol in all the stability studies of exenatide to remove the difference from the marketed formulation.

The degradants and by products of the degradation reactions were not determined, as it is outside the scope of the study.

Chapter Four

4 Conclusions and recommendations for further research.

4.1 Conclusions

Type 2 diabetes mellitus (T2D) is a complex disease that is characterised by uncontrolled glycaemia and is associated with multiple complications including toxin build up and compromised gut movement, obesity and high cholesterolemia. An excellent antidiabetic drug will be able to exert positive antidiabetic effects and will have a convenient route of administration and good patient compliance. The oral route is the most convenient for administration since dosing is non-invasive and favoured by most patients (147). Clearly, good bioavailability and low intra- and inter-patient variability are desirable for oral drug delivery.

Exenatide is a newly marketed antidiabetic drug, for T2D. In addition to its insulin-stimulating effects on β-cells of the pancreas, it has many positive antidiabetic effects. These positive effects include antioxidant effects, slowing gastric emptying rate, appetite reduction, weight loss, suppression of glucagon release resulting in optimised glycaemic control as well as protective effects on β-cells, which slows down their damage and eventual insulin requirements (126-129, 148).

Exenatide is currently available as injectable formulation which requires refrigeration. The requirement for refrigeration is a major limitation to its use, especially in countries where refrigeration or power is limited (133). Thus, in order to optimise its current formulation, there is a need to investigate the effect of temperature on its stability profile. Ideally, this should be investigated and optimised *in vitro*, followed by *in vivo* preclinical and clinical studies.

The chemical analysis of peptide-based drugs is often complex and method development and validation requires skills to accurately and sensitively isolate the analyte from excipients, degradation products, impurities, and matrix components. This is in addition to the drug's potential chirality, secondary structures, conformations and the states of any aggregates (149). In the case of exenatide, this was further complicated by the potential reactivity, hydrolysis or bond-cleavage and racemisation and modifications of the amino acid chains. In addition, there is also potential interactions with the containers and equipment used, when carrying out sample analysis (133). It is likely that with

polypeptides a range of degradation products of various chemical characteristics will occur. Hydrophobic chromatographic techniques such as RP-HPLC are potentially useful in these separations (150). Accordingly, in this project, multiple RP-HPLC methods for exenatide analysis were initially examined. The initial HPLC analysis of exenatide in its marketed injectable formulation was unsuccessful due to significant interference from excipients, particularly the preservative, meta-cresol. During method development, multi-gradient mobile phase, double-column separation, gradient-pressure, and multiple flow rates were examined. When the pure drug was sourced the analytical HPLC-method was further developed, refined and validated before a final method was established and the study commenced.

In chapter two, the validity of the established method for studying the degradation of exenatide in aqueous solution was investigated. Reverse phase HPLC was used for the analysis of the stability of exenatide. The method was examined for its accuracy and precision and for its stability indicating capability and the method was suitable for different temperature and pH conditions. The method showed good selectivity, sensitivity and results were consistent throughout the analysis. However, degradation products were not examined as this was outside the scope of this project.

In chapter three, exenatide was examined at different conditions which include different temperatures (35, 40 and 45^{o}C), pH values (2.00, 3.50, 4.50, 6.00, 7.00, 8.00 and 10.00) and ionic strengths (50, 100 and 250 mM). From that study, the reaction rate constants, effect of ionic strength, and the activation energies at selected pH values and the shelf-life was determined.

4.2 Conclusions and recommendation for future directions of research

In conclusion, it is clear from this study that the lowest degradation rate occurs when exenatide is formulated at pH 6.00. Whereas, the highest reaction rate constant occurs when exenatide is formulated at pH 10.00. The reaction rate constant at pH 4.50 was considerably higher compared to that of pH 6.00 and 7.00. The shelf-life calculations of exenatide shows a significantly improved shelf life when formulated at pH 6.00 compared to pH 4.50. The manufacturing

company has kept all stability data for its product, Byetta, confidential. Therefore, the rationale behind formulating Byetta at pH 4.50 instead of pH 6.00 is speculated to be based on excipient compatibility. Accordingly, future research should examine new formulations of exenatide that will provide better stability, such as formulating at pH 6.00 instead of 4.50, and a better route of administration including sublingual or the oral route which may lead to a better patient compliance and a better clinical outcomes. This may include innovative technologies such as novel microencapsulation using bile acid-based platforms which can offer protection against enzymatic degradation and accurately controlling drug release from the microcapsules. This can be examined in preclinical as well as clinical studies in T2D.

5 References

1. Laurence L. Brunton, John S. Lazo, Keith L. Parker. Goodman & Gilman's The Pharmacological Basis Of Therapeutics. 11th ed: The McGraw-Hill Companies; 2006.
2. Nauck MA. Unraveling the science of incretin biology. Am J Med. 2009;122(6 Suppl):S3-S10.
3. Reagan LP. Diabetes as a chronic metabolic stressor: causes, consequences and clinical complications. Exp Neurol. 2012;233(1):68-78.
4. Clark CM, Jr., Lee DA. Prevention and treatment of the complications of diabetes mellitus. N Engl J Med. 1995;332(18):1210-7.
5. Barbeau WE, Bassaganya-Riera J, Hontecillas R. Putting the pieces of the puzzle together - a series of hypotheses on the etiology and pathogenesis of type 1 diabetes. Med Hypotheses. 2007;68(3):607-19.
6. Ludvigsson J. Why diabetes incidence increases--a unifying theory. Ann N Y Acad Sci. 2006;1079:374-82.
7. Ferrannini E. Insulin resistance versus insulin deficiency in non-insulin-dependent diabetes mellitus: problems and prospects. Endocr Rev. 1998;19(4):477-90.
8. Belcher G, Lambert C, Goh KL, Edwards G, Valbuena M. Cardiovascular effects of treatment of type 2 diabetes with pioglitazone, metformin and gliclazide. Int J Clin Pract. 2004;58(9):833-7.
9. DeFronzo RA, Ferrannini E, Simonson DC. Fasting hyperglycemia in non-insulin-dependent diabetes mellitus: contributions of excessive hepatic glucose production and impaired tissue glucose uptake. Metabolism. 1989;38(4):387-95.
10. Weiss R, Caprio S. Development of type 2 diabetes in children and adolescents. Minerva Med. 2006;97(3):263-9.
11. Phillips P. Type 2 Diabetes - failure, blame and guilt in the adoption of insulin therapy. Rev Diabet Stud. 2005;2(1):35-9.
12. Mahmoud F, Abul H, Dashti A, Al-Jassar W, Omu A. Trace elements and cell-mediated immunity in gestational and pre-gestational diabetes

mellitus at third trimester of pregnancy. Acta medica academica. 2012;41(2):175-85.

13. Huidobro A, Prentice A, Fulford T, Parodi C, Rozowski J. Gestational diabetes, comparison of women diagnosed in second and third trimester of pregnancy with non GDM women: Analysis of a cohort study. Revista medica de Chile. 2010;138(3):316-21.

14. Gunderson EP, Jacobs DR, Jr., Chiang V, Lewis CE, Feng J, Quesenberry CP, Jr., et al. Duration of lactation and incidence of the metabolic syndrome in women of reproductive age according to gestational diabetes mellitus status: a 20-Year prospective study in CARDIA (Coronary Artery Risk Development in Young Adults). Diabetes. 2010;59(2):495-504.

15. Luengmettakul J, Boriboonhirunsam D, Sutantawibul A, Sunsaneevithayakul P. Incidence of large-for-gestational age newborn: a comparison between pregnant women with abnormal and normal screening test for gestational diabetes. J Med Assoc Thai. 2007;90(3):432-6.

16. Nolan CJ, Damm P, Prentki M. Type 2 diabetes across generations: from pathophysiology to prevention and management. Lancet. 2011;378(9786):169-81.

17. Dunstan DW, Zimmet PZ, Welborn TA, De Courten MP, Cameron AJ, Sicree RA, et al. The rising prevalence of diabetes and impaired glucose tolerance: the Australian Diabetes, Obesity and Lifestyle Study. Diabetes Care. 2002;25(5):829-34.

18. Moaven LD. Is Australia ready to use glycated haemoglobin for the diagnosis of diabetes? Med J Aust. 2011;195(9):514.

19. Taylor AR, Brownsill RD, Grandon H, Lefoulon F, Petit A, Luijten W, et al. Pharmacokinetics and pharmacodynamics of gliclazide in Caucasians and Australian Aborigines with type 2 diabetes. Br J Clin Pharmacol. 2000;49(3):223-30.

20. Clausen JO, Borch-Johnsen K, Ibsen H, Bergman RN, Hougaard P, Winther K, et al. Insulin sensitivity index, acute insulin response, and glucose effectiveness in a population-based sample of 380 young healthy Caucasians. Analysis of the impact of gender, body fat, physical fitness, and life-style factors. J Clin Invest. 1996;98(5):1195-209.

21. Cardin S, Walmsley K, Neal DW, Williams PE, Cherrington AD. Involvement of the vagus nerves in the regulation of basal hepatic glucose production in conscious dogs. Am J Physiol Endocrinol Metab. 2002;283(5):E958-64.
22. Suh SH, Paik IY, Jacobs K. Regulation of blood glucose homeostasis during prolonged exercise. Mol Cells. 2007;23(3):272-9.
23. Frosig C, Rose AJ, Treebak JT, Kiens B, Richter EA, Wojtaszewski JF. Effects of endurance exercise training on insulin signaling in human skeletal muscle: interactions at the level of phosphatidylinositol 3-kinase, Akt, and AS160. Diabetes. 2007;56(8):2093-102.
24. Boden G. Gluconeogenesis and glycogenolysis in health and diabetes. J Investig Med. 2004;52(6):375-8.
25. Staehr P, Hother-Nielsen O, Beck-Nielsen H, Roden M, Stingl H, Holst JJ, et al. Hepatic autoregulation: response of glucose production and gluconeogenesis to increased glycogenolysis. Am J Physiol Endocrinol Metab. 2007;292(5):E1265-9.
26. Krebs M, Brehm A, Krssak M, Anderwald C, Bernroider E, Nowotny P, et al. Direct and indirect effects of amino acids on hepatic glucose metabolism in humans. Diabetologia. 2003;46(7):917-25.
27. Galassetti P, Mann S, Tate D, Neill RA, Wasserman DH, Davis SN. Effect of morning exercise on counterregulatory responses to subsequent, afternoon exercise. J Appl Physiol (1985). 2001;91(1):91-9.
28. Laurent D, Hundal RS, Dresner A, Price TB, Vogel SM, Petersen KF, et al. Mechanism of muscle glycogen autoregulation in humans. Am J Physiol Endocrinol Metab. 2000;278(4):E663-8.
29. Borghouts LB, Keizer HA. Exercise and insulin sensitivity: a review. Int J Sports Med. 2000;21(1):1-12.
30. Hansen BF, Asp S, Kiens B, Richter EA. Glycogen concentration in human skeletal muscle: effect of prolonged insulin and glucose infusion. Scand J Med Sci Sports. 1999;9(4):209-13.
31. Magnusson I, Rothman DL, Jucker B, Cline GW, Shulman RG, Shulman GI. Liver glycogen turnover in fed and fasted humans. Am J Physiol. 1994;266(5 Pt 1):E796-803.

32. Kokk K, Verajankorva E, Laato M, Wu XK, Tapfer H, Pollanen P. Expression of insulin receptor substrates 1-3, glucose transporters GLUT-1-4, signal regulatory protein 1alpha, phosphatidylinositol 3-kinase and protein kinase B at the protein level in the human testis. Anat Sci Int. 2005;80(2):91-6.

33. Liu GJ, Simpson AM, Swan MA, Tao C, Tuch BE, Crawford RM, et al. ATP-sensitive potassium channels induced in liver cells after transfection with insulin cDNA and the GLUT 2 transporter regulate glucose-stimulated insulin secretion. FASEB J. 2003;17(12):1682-4.

34. McClenaghan NH, Flatt PR, Ball AJ. Actions of glucagon-like peptide-1 on KATP channel-dependent and -independent effects of glucose, sulphonylureas and nateglinide. J Endocrinol. 2006;190(3):889-96.

35. Straub SG, Sharp GW. Hypothesis: one rate-limiting step controls the magnitude of both phases of glucose-stimulated insulin secretion. Am J Physiol Cell Physiol. 2004;287(3):C565-71.

36. Christin L, Nacht CA, Vernet O, Ravussin E, Jequier E, Acheson KJ. Insulin. Its role in the thermic effect of glucose. J Clin Invest. 1986;77(6):1747-55.

37. Moore PA, Zgibor JC, Dasanayake AP. Diabetes: a growing epidemic of all ages. J Am Dent Assoc. 2003;134 Spec No:11S-5S.

38. Stone NJ, Schmeltz LR. Metabolic syndrome management. Expert Opin Pharmacother. 2007;8(13):2059-75.

39. Fajans S. [Classification, pathogenesis and course of various types of diabetes mellitus]. Verh Dtsch Ges Inn Med. 1987;93:139-44.

40. Madsen D, Beaver M, Chang L, Bruckner-Kardoss E, Wostmann B. Analysis of bile acids in conventional and germfree rats. J Lipid Res. 1976;17(2):107-11.

41. Zhang L, Krzentowski G, Albert A, Lefebvre PJ. Risk of developing retinopathy in Diabetes Control and Complications Trial type 1 diabetic patients with good or poor metabolic control. Diabetes Care. 2001;24(7):1275-9.

42. Bonadonna RC, Cucinotta D, Fedele D, Riccardi G, Tiengo A. The metabolic syndrome is a risk indicator of microvascular and macrovascular

complications in diabetes - Results from Metascreen, a multicenter diabetes clinic-based survey. Diabetes Care. 2006;29(12):2701-7.
43. Kilpatrick ES, Rigby AS, Atkin SL. Insulin resistance, the metabolic syndrome, and complication risk in type 1 diabetes - "Double diabetes" in the Diabetes Control and Complications Trial. Diabetes Care. 2007;30(3):707-12.
44. Kaul K, Tarr J, Ahmad S, Kohner E, Chibber R. Introduction to Diabetes Mellitus. In: Ahmad S, editor. Diabetes. Advances in Experimental Medicine and Biology: Springer New York; 2013. p. 1-11.
45. Huang P, Huang P. Diabetes Mellitus and the Metabolic Syndrome. In: Gaggin HK, Januzzi JJL, editors. MGH Cardiology Board Review: Springer London; 2014. p. 120-32.
46. Assogba FG, Couchoud C, Hannedouche T, Villar E, Frimat L, Fagot-Campagna A, et al. Trends in the epidemiology and care of diabetes mellitus-related end-stage renal disease in France, 2007-2011. Diabetologia. 2014;57(4):718-28.
47. Sytze Van Dam P, Cotter MA, Bravenboer B, Cameron NE. Pathogenesis of diabetic neuropathy: focus on neurovascular mechanisms. Eur J Pharmacol. 2013;719(1-3):180-6.
48. Mohan A, Moorthy K. Early detection of diabetic retinopathy edema using FCM. International Journal of Science and Research (IJSR), India. 2013.
49. Control CfD, Prevention. National diabetes fact sheet: national estimates and general information on diabetes and prediabetes in the United States, 2011. Atlanta, GA: US Department of Health and Human Services, Centers for Disease Control and Prevention. 2011;201.
50. Cani PD, Neyrinck AM, Fava F, Knauf C, Burcelin RG, Tuohy KM, et al. Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. Diabetologia. 2007;50(11):2374-83.
51. Al-Salami H, Butt G, Tucker I, Fawcett PJ, Golocorbin-Kon S, Mikov I, et al. Gliclazide reduces MKC intestinal transport in healthy but not diabetic rats. Eur J Drug Metab Pharmacokinet. 2009;34(1):43-50.
52. Al-Salami H, Butt G, Tucker I, Mikov M. Influence of the semisynthetic bile acid MKC on the ileal permeation of gliclazide in vitro in healthy and

diabetic rats treated with probiotics. Methods Find Exp Clin Pharmacol. 2008;30(2):107-13.
53. Bailey CJ, Turner RC. Metformin. N Engl J Med. 1996;334(9):574-9.
54. Bailey CJ. Biguanides and NIDDM. Diabetes Care. 1992;15(6):755-72.
55. Rossi S. Australian medicines handbook 2013: Australian Medicines Handbook; 2013.
56. Nasim A. Pharmacology of Metformin Drugs Used In Diabetes2012 [cited 2014 07/07/2014]. Available from: http://www.about-pharmacology.com/2012/09/09/pharmacology-of-metformin/.
57. Thule PM, Umpierrez G. Sulfonylureas: a new look at old therapy. Curr Diab Rep. 2014;14(4):473.
58. Al-Salami H, Butt G, Fawcett JP, Tucker IG, Golocorbin-Kon S, Mikov M. Probiotic treatment reduces blood glucose levels and increases systemic absorption of gliclazide in diabetic rats. Eur J Drug Metab Pharmacokinet. 2008;33(2):101-6.
59. Rendell M. The role of sulphonylureas in the management of type 2 diabetes mellitus. Drugs. 2004;64(12):1339-58.
60. Hauner H. The mode of action of thiazolidinediones. Diabetes Metab Res Rev. 2002;18 Suppl 2(S2):S10-5.
61. Chiasson JL, Josse RG, Gomis R, Hanefeld M, Karasik A, Laakso M, et al. Acarbose for prevention of type 2 diabetes mellitus: the STOP-NIDDM randomised trial. Lancet. 2002;359(9323):2072-7.
62. Gallo M, Candido R, De Micheli A, Esposito K, Gentile S, Ceriello A, et al. Acarbose vs metformin for new-onset type 2 diabetes. Lancet Diabetes Endocrinol. 2014;2(2):104.
63. Komoroski B, Vachharajani N, Boulton D, Kornhauser D, Geraldes M, Li L, et al. Dapagliflozin, a novel SGLT2 inhibitor, induces dose-dependent glucosuria in healthy subjects. Clin Pharmacol Ther. 2009;85(5):520-6.
64. Strojek K, Yoon KH, Hruba V, Elze M, Langkilde AM, Parikh S. Effect of dapagliflozin in patients with type 2 diabetes who have inadequate glycaemic control with glimepiride: a randomized, 24-week, double-blind, placebo-controlled trial. Diabetes Obes Metab. 2011;13(10):928-38.
65. Ferrannini E, Ramos SJ, Salsali A, Tang W, List JF. Dapagliflozin monotherapy in type 2 diabetic patients with inadequate glycemic control by

diet and exercise: a randomized, double-blind, placebo-controlled, phase 3 trial. Diabetes Care. 2010;33(10):2217-24.

66. prescriber A. New drugs (dapagliflozin) 2013 [cited 2014 18/08/2014]. Available from: http://www.australianprescriber.com/online-first/17/dapagliflozin.

67. Bailey CJ, Gross JL, Pieters A, Bastien A, List JF. Effect of dapagliflozin in patients with type 2 diabetes who have inadequate glycaemic control with metformin: a randomised, double-blind, placebo-controlled trial. Lancet. 2010;375(9733):2223-33.

68. Fala L. Invokana (Canagliflozin): First-in-Class SGLT2 Inhibitor Approved for the Treatment of Type 2 Diabetes.

69. Stenlof K, Cefalu WT, Kim KA, Alba M, Usiskin K, Tong C, et al. Efficacy and safety of canagliflozin monotherapy in subjects with type 2 diabetes mellitus inadequately controlled with diet and exercise. Diabetes Obes Metab. 2013;15(4):372-82.

70. Rosenstock J, Aggarwal N, Polidori D, Zhao Y, Arbit D, Usiskin K, et al. Dose-ranging effects of canagliflozin, a sodium-glucose cotransporter 2 inhibitor, as add-on to metformin in subjects with type 2 diabetes. Diabetes Care. 2012;35(6):1232-8.

71. Brange J, Langkjcer L. Insulin Strueture and Stability. Stability and Characterization of Protein and Peptide Drugs: Case Histories. 1993;5:315.

72. Derewenda U, Derewenda Z, Dodson GG, Hubbard RE, Korber F. Molecular structure of insulin: the insulin monomer and its assembly. Br Med Bull. 1989;45(1):4-18.

73. Vilsboll T, Krarup T, Sonne J, Madsbad S, Volund A, Juul AG, et al. Incretin secretion in relation to meal size and body weight in healthy subjects and people with type 1 and type 2 diabetes mellitus. J Clinical Endocrinol Metab. 2003;88(6):2706-13.

74. Drucker DJ, Nauck MA. The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. Lancet. 2006;368(9548):1696-705.

75. Drucker DJ. The biology of incretin hormones. Cell Metab. 2006;3(3):153-65.

76. Holst JJ, Gromada J. Role of incretin hormones in the regulation of insulin secretion in diabetic and nondiabetic humans. Am J Physiol Endocrinol Metab. 2004;287(2):E199-206.
77. Nielsen LL. Incretin mimetics and DPP-IV inhibitors for the treatment of type 2 diabetes. Drug Discov Today. 2005;10(10):703-10.
78. Triplitt C, Wright A, Chiquette E. Incretin mimetics and dipeptidyl peptidase-IV inhibitors: potential new therapies for type 2 diabetes mellitus. Pharmacotherapy. 2006;26(3):360-74.
79. Holst JJ, Vilsboll T, Deacon CF. The incretin system and its role in type 2 diabetes mellitus. Mol Cell Endocrinol. 2009;297(1-2):127-36.
80. Barnett A. DPP-4 inhibitors and their potential role in the management of type 2 diabetes. Int J Clin Pract. 2006;60(11):1454-70.
81. Wang Y, Zhao L, Huang Q, Peng Y. Effects of dipeptidyl peptidase-4 inhibitors in a type 2 diabetes patient with failure of glucagon-like peptide-1 receptor agonists. J Diabetes. 2014;6(2):164-6.
82. Wong MC, Wang HH, Kwan MW, Zhang DD, Liu KQ, Chan SW, et al. Comparative effectiveness of dipeptidyl peptidase-4 (DPP-4) inhibitors and human glucagon-like peptide-1 (GLP-1) analogue as add-on therapies to sulphonylurea among diabetes patients in the Asia-Pacific region: a systematic review. PLOS ONE. 2014;9(3):e90963.
83. Madsbad S, Schmitz O, Ranstam J, Jakobsen G, Matthews DR, Group NNIS. Improved glycemic control with no weight increase in patients with type 2 diabetes after once-daily treatment with the long-acting glucagon-like peptide 1 analog liraglutide (NN2211): a 12-week, double-blind, randomized, controlled trial. Diabetes Care. 2004;27(6):1335-42.
84. Watson E, Jonker DM, Jacobsen LV, Ingwersen SH. Population pharmacokinetics of liraglutide, a once-daily human glucagon-like peptide-1 analog, in healthy volunteers and subjects with type 2 diabetes, and comparison to twice-daily exenatide. J Clin Pharmacol. 2010;50(8):886-94.
85. Harder H, Nielsen L, Tu DT, Astrup A. The effect of liraglutide, a long-acting glucagon-like peptide 1 derivative, on glycemic control, body composition, and 24-h energy expenditure in patients with type 2 diabetes. Diabetes Care. 2004;27(8):1915-21.

86. Christensen M, Knop FK, Vilsboll T, Holst JJ. Lixisenatide for type 2 diabetes mellitus. Expert Opin Investig Drugs. 2011;20(4):549-57.
87. Horowitz M, Rayner CK, Jones KL. Mechanisms and clinical efficacy of lixisenatide for the management of type 2 diabetes. Adv Ther. 2013;30(2):81-101.
88. Seino Y, Takami A, Boka G, Niemoeller E, Raccah D, investigators PDY. Pharmacodynamics of the glucagon-like peptide-1 receptor agonist lixisenatide in Japanese and Caucasian patients with type 2 diabetes mellitus poorly controlled on sulphonylureas with/without metformin. Diabetes Obes Metab. 2014;16(8):739-47.
89. Triplitt C, Chiquette E. Exenatide: from the Gila monster to the pharmacy. J Am Pharm Assoc. 2006;46(1):44.
90. Bauer A, Bronstrup M. Industrial natural product chemistry for drug discovery and development. Nat Prod Rep. 2014;31(1):35-60.
91. Joy SV, Rodgers PT, Scates AC. Incretin mimetics as emerging treatments for type 2 diabetes. Ann Pharmacother. 2005;39(1):110-8.
92. Malone J, Trautmann M, Wilhelm K, Taylor K, Kendall DM. Exenatide once weekly for the treatment of type 2 diabetes. Expert Opin Investig Drugs. 2009;18(3):359-67.
93. Ballav C, Gough S. Bydureon: long-acting exenatide for once-weekly injection. Prescriber. 2012;23(1-2):30-3.
94. Bank D. Exenatide 2014 [cited 2014 16/10/2014]. Available from: http://www.drugbank.ca/drugs/DB01276.
95. Skovgaard M, Kodra JT, Gram DX, Knudsen SM, Madsen D, Liberles DA. Using evolutionary information and ancestral sequences to understand the sequence-function relationship in GLP-1 agonists. J Mol Biol. 2006;363(5):977-88.
96. DeFronzo RA, Ratner RE, Han J, Kim DD, Fineman MS, Baron AD. Effects of exenatide (exendin-4) on glycemic control and weight over 30 weeks in metformin-treated patients with type 2 diabetes. Diabetes Care. 2005;28(5):1092-100.
97. Buse JB, Henry RR, Han J, Kim DD, Fineman MS, Baron AD, et al. Effects of exenatide (exendin-4) on glycemic control over 30 weeks in

sulfonylurea-treated patients with type 2 diabetes. Diabetes Care. 2004;27(11):2628-35.

98. Klonoff DC, Buse JB, Nielsen LL, Guan X, Bowlus CL, Holcombe JH, et al. Exenatide effects on diabetes, obesity, cardiovascular risk factors and hepatic biomarkers in patients with type 2 diabetes treated for at least 3 years*. Curr Med Res Opin. 2008;24(1):275-86.

99. Wu JD, Xu XH, Zhu J, Ding B, Du TX, Gao G, et al. Effect of exenatide on inflammatory and oxidative stress markers in patients with type 2 diabetes mellitus. Diabetes Technol Ther. 2011;13(2):143-8.

100. Varanasi A, Chaudhuri A, Dhindsa S, Arora A, Lohano T, Vora MR, et al. Durability of effects of exenatide treatment on glycemic control, body weight, systolic blood pressure, C-reactive protein, and triglyceride concentrations. Endocr Pract. 2011;17(2):192-200.

101. Nathanson D, Ullman B, Lofstrom U, Hedman A, Frick M, Sjoholm A, et al. Effects of intravenous exenatide in type 2 diabetic patients with congestive heart failure: a double-blind, randomised controlled clinical trial of efficacy and safety. Diabetologia. 2012;55(4):926-35.

102. Linnebjerg H, Park S, Kothare PA, Trautmann ME, Mace K, Fineman M, et al. Effect of exenatide on gastric emptying and relationship to postprandial glycemia in type 2 diabetes. Regul Pept. 2008;151(1-3):123-9.

103. Kothare PA, Soon DK, Linnebjerg H, Park S, Chan C, Yeo A, et al. Effect of exenatide on the steady-state pharmacokinetics of digoxin. J Clin Pharmacol. 2005;45(9):1032-7.

104. Soon D, Kothare PA, Linnebjerg H, Park S, Yuen E, Mace KF, et al. Effect of exenatide on the pharmacokinetics and pharmacodynamics of warfarin in healthy Asian men. J Clin Pharmacol 2006;46(10):1179-87.

105. Aviles-Olmos I, Limousin P, Lees A, Foltynie T. Parkinson's disease, insulin resistance and novel agents of neuroprotection. Brain. 2013;136(Pt 2):374-84.

106. Haggerty LM. Protein Science and Engineering : Protein Structure. Hauppauge, NY, USA: Nova Science Publishers, Inc.; 2011.

107. Lin MM, Mohammed OF, Jas GS, Zewail AH. Speed limit of protein folding evidenced in secondary structure dynamics. Proc Natl Acad Sci U S A . 2011;108(40):16622-7.

108. Zhang H, Zhang T, Gao J, Ruan J, Shen S, Kurgan L. Determination of protein folding kinetic types using sequence and predicted secondary structure and solvent accessibility. Amino acids. 2012;42(1):271-83.
109. Clark J. The Structure of Proteins 2004 [updated October 2014; cited 2014 21/10/2014]. Available from: http://www.chemguide.co.uk/organicprops/aminoacids/proteinstruct.html.
110. Manning MC, Patel K, Borchardt RT. Stability of protein pharmaceuticals. Pharm Res. 1989;6(11):903-18.
111. Xie M, Schowen RL. Secondary structure and protein deamidation. J Pharm Sci. 1999;88(1):8-13.
112. Stratton LP, Kelly RM, Rowe J, Shively JE, Smith DD, Carpenter JF, et al. Controlling deamidation rates in a model peptide: effects of temperature, peptide concentration, and additives. J Pharm Sci. 2001;90(12):2141-8.
113. Manning MC, Chou DK, Murphy BM, Payne RW, Katayama DS. Stability of protein pharmaceuticals: an update. Pharm Res. 2010;27(4):544-75.
114. Chavous DA, Jackson FR, O'Connor CM. Extension of the Drosophila lifespan by overexpression of a protein repair methyltransferase. Proc Natl Acad Sci U S A. 2001;98(26):14814-8.
115. Yoshioka S, Stella VJ. Stability of Drugs and Dosage Forms: Springer; 2000.
116. Li S, Schoneich C, Borchardt RT. Chemical instability of protein pharmaceuticals: Mechanisms of oxidation and strategies for stabilization. Biotechnol Bioeng. 1995;48(5):490-500.
117. Hayes CS, Illades-Aguiar B, Casillas-Martinez L, Setlow P. In vitro and In vivo oxidation of methionine residues in small, acid-soluble spore proteins from bacillus species. J Bacteri. 1998;180(10):2694-700.
118. Pearlman R, Bewley T. Stability and Characterization of Human Growth Hormone. In: Wang YJ, Pearlman R, editors. Stability and Characterization of Protein and Peptide Drugs. Pharmaceutical Biotechnology. 5: Springer US; 1993. p. 1-58.
119. Rosenberg AS. Effects of protein aggregates: an immunologic perspective. AAPS J. 2006;8(3):E501-7.

120. Mahler HC, Jiskoot W. Analysis of Aggregates and Particles in Protein Pharmaceuticals: Wiley; 2011.
121. Jiskoot W, Crommelin D. Methods for Structural Analysis of Protein Pharmaceuticals: American Assoc. of Pharm. Scientists; 2005.
122. Qi F, Wu J, Hao D, Yang T, Ren Y, Ma G, et al. Comparative studies on the influences of primary emulsion preparation on properties of uniform-sized exenatide-loaded PLGA microspheres. Pharm Res. 2014;31(6):1566-74.
123. Liang R, Zhang R, Li X, Wang A, Chen D, Sun K, et al. Stability of exenatide in poly(D,L-lactide-co-glycolide) solutions: a simplified investigation on the peptide degradation by the polymer. Eur J Pharm Sci. 2013;50(3-4):502-10.
124. Lachman L, Lieberman HA, Kanig JL. The Theory and Practice of Industrial Pharmacy. . Third ed. Philadelphia: Lea and Febiger; 1986.
125. Peleg M, Normand MD, Corradini MG. The Arrhenius equation revisited. Crit Rev Food Sci Nutr. 2012;52(9):830-51.
126. Sze L, Purtell L, Jenkins A, Loughnan G, Smith E, Herzog H, et al. Effects of a single dose of exenatide on appetite, gut hormones, and glucose homeostasis in adults with Prader-Willi syndrome. J Clin Endocrinol Metab. 2011;96(8):E1314-9.
127. Hutton SF. Byetta (exenatide): what's your gut feeling? S D Med. 2006;59(1):16-7.
128. Ding X, Saxena NK, Lin S, Gupta NA, Anania FA. Exendin-4, a glucagon-like protein-1 (GLP-1) receptor agonist, reverses hepatic steatosis in ob/ob mice. Hepatology. 2006;43(1):173-81.
129. Tushuizen ME, Bunck MC, Pouwels PJ, van Waesberghe JH, Diamant M, Heine RJ. Incretin mimetics as a novel therapeutic option for hepatic steatosis. Liver Int. 2006;26(8):1015-7.
130. Aburuz S, Millership J, McElnay J. The development and validation of liquid chromatography method for the simultaneous determination of metformin and glipizide, gliclazide, glibenclamide or glimperide in plasma. J Chromatogr B Analyt Technol Biomed Life Sci. 2005;817(2):277-86.

131. Bachhav YG, Kalia YN. Development and validation of a rapid high-performance liquid chromatography method for the quantification of exenatide. Biomed Chromatogr. 2011;25(7):838-42.
132. Tsai CW, Kao WH, Chang LC, Ruaan RC, Chen WY. Structural stability-chromatographic retention relationship on exenatide diastereomer separation. Anal Bioanal Chem. 2012;404(8):2437-44.
133. Zhang B, He D, Fan Y, Liu N, Chen Y. Oral delivery of exenatide via microspheres prepared by cross-linking of alginate and hyaluronate. PLOSE ONE. 2014;9(1):e86064.
134. Hagan RL. High-performance liquid chromatography for small-scale studies of drug stability. Am J Hosp Pharm. 1994;51(17):2162-75.
135. Cleland JL, Jones AJS. Stable Formulations of Recombinant Human Growth Hormone and Interferon-γ for Microencapsulation in Biodegradable Mircospheres. Pharm Res. 1996;13(10):1464-75.
136. Tyler-Cross R, Schirch V. Effects of amino acid sequence, buffers, and ionic strength on the rate and mechanism of deamidation of asparagine residues in small peptides. J Biol Chem. 1991;266(33):22549-56.
137. Mozziconacci O, Schoneich C. Photodegradation of oxytocin and thermal stability of photoproducts. J Pharm Sci. 2012;101(9):3331-46.
138. Kaushal G, Sayre BE, Prettyman T. Stability-indicating HPLC method for the determination of the stability of oxytocin parenteral solutions prepared in polyolefin bags. Drug Discov Ther. 2012;6(1):49-54.
139. Kenley RA, Tracht S, Stepanenko A, Townsend M, L'Italien J. Kinetics of pramlintide degradation in aqueous solution as a function of temperature and pH. AAPS PharmSciTech. 2000;1(2):E7.
140. Nguyen T, Ward C. Stability Characterization and Formulation Development of Alteplase, a Recombinant Tissue Plasminogen Activator. In: Wang YJ, Pearlman R, editors. Stability and Characterization of Protein and Peptide Drugs. Pharmaceutical Biotechnology. 5: Springer US; 1993. p. 91-134.
141. Brange J. Chemical stability of insulin. 4. Mechanisms and kinetics of chemical transformations in pharmaceutical formulation. Acta pharm nord. 1991;4(4):209-22.

142. Li R, Wang F, Chen L, Zhu S, Wu L, Jiang S, et al. Stability of an anti-stroke peptide: driving forces and kinetics in chemical degradation. Int J Pharm. 2014;472(1-2):148-55.
143. Hawe A, Poole R, Romeijn S, Kasper P, van der Heijden R, Jiskoot W. Towards heat-stable oxytocin formulations: analysis of degradation kinetics and identification of degradation products. Pharm Res. 2009;26(7):1679-88.
144. Papaneophytou CP, Grigoroudis AI, McInnes C, Kontopidis G. Quantification of the effects of ionic strength, viscosity, and hydrophobicity on protein-ligand binding affinity. ACS med chem lett. 2014;5(8):931-6.
145. Ma Y, Dong J, Bhattacharjee S, Wijeratne S, Bruening ML, Baker GL. Increased protein sorption in poly(acrylic acid)-containing films through incorporation of comb-like polymers and film adsorption at low pH and high ionic strength. Langmuir . 2013;29(9):2946-54.
146. Moller J, Schroer MA, Erlkamp M, Grobelny S, Paulus M, Tiemeyer S, et al. The effect of ionic strength, temperature, and pressure on the interaction potential of dense protein solutions: from nonlinear pressure response to protein crystallization. Biophys J. 2012;102(11):2641-8.
147. Werle M, Hoffer M. Glutathione and thiolated chitosan inhibit multidrug resistance P-glycoprotein activity in excised small intestine. J Control Release. 2006;111(1-2):41-6.
148. Couto FM, Minn AH, Pise-Masison CA, Radonovich M, Brady JN, Hanson M, et al. Exenatide blocks JAK1-STAT1 in pancreatic beta cells. Metabolism. 2007;56(7):915-8.
149. Kelley WP, Chen S, Floyd PD, Hu P, Kapsi SG, Kord AS, et al. Analytical characterization of an orally-delivered peptide pharmaceutical product. Anal Chem. 2012;84(10):4357-72.
150. Riggin RM, Dorulla GK, Miner DJ. A reversed-phase high-performance liquid chromatographic method for characterization of biosynthetic human growth hormone. Anal Biochem. 1987;167(1):199-209.

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