HPLC Method Development for the Analysis of Linagliptin

By

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A thesis submitted to the Department of Pharmacy in partial fulfillment of the requirements for the degree of
Bachelor of Pharmacy (Hons)

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Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

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Approval

The thesis titled "HPLC Method Development for the Analysis of Linagliptin" submitted by Ruhani Amrin Mimiya (ID-14346010) has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy (Hons) on 29th May, 2019.

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Ethics Statement

The study does not involve any kind of animal trial and human trial.

Abstract

The purpose of this study was to develop a reliable, rapid and sensitive method based on high performance liquid chromatography (HPLC) for the determination of an anti-diabetic drug linagliptin in its pure form as well as in its pharmaceutical dosage forms. Different chromatographic conditions were investigated to optimize the parameters for the established method. In this study, the chromatographic assay involved the use of C_{18} column (150×4.6mm, packed with 5 µm particles) as the stationary phase and a combination of methanol: phosphate buffer (of pH 4.5) as the mobile phase at a ratio of 70:30 v/v pumped at a flow rate of 1mL/min in isocratic mode. The total run time was 10 min with a retention time of 3.3 min. The detection wavelength was 241 nm. Five marketed products of linagliptin were assayed by this developed method and their potency were calculated successfully, the results of which demonstrated both the feasibility and reliability of this method for the quantitation and qualification of linagliptin in pharmaceutical dosage forms as well as in its pure form.

Keywords: Diabetes mellitus; DPP-4 inhibitors; Linagliptin; High performance liquid chromatography.

Dedication

Dedicated to my parents and my supervisors

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With utmost respect and appreciation, I would like to extend my heartfelt gratitude to all the people who have directly or indirectly helped me to accomplish the completion of this paper.

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Table of contents

List of Tables

List of Figures

List of Acronyms

IDF	International Diabetes Federation
ADA	American Diabetes Association
CDA	Canadian Diabetes Association
HbA1c	Hemoglobin A1c
DCCT	Diabetes Control and Complications Trial
IFG	Impaired Fasting Glucose
IGT	Impaired Glucose Tolerance
FPG	Fasting Plasma Glucose
OGTT	Oral Glucose Tolerance Test
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
GLP-1	Glucagon Like Peptide-1
GIP	Glucose dependent Insulinotropic Polypeptide
SGLT	Sodium-Glucose Linked Transporter
GFR	Glomerular Filtration
KATP	Adenosine Tri-Phosphate sensitive Potassium
DPP-4	Di-Peptidyl Peptidase-4
PPAR	Peroxisome Proliferator Activated Receptor

CKD	Chronic Kidney Disease
CYP450	Cytochrome P450
AUC	Area Under Curve
HPLC	High Performance Liquid Chromatography
RP-HPLC	Reverse Phase- High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
RT	Retention Time
ICH	International Council for Harmonisation
USP	United States Pharmacopeia
BP	British Pharmacopeia
PBS	Phosphate Buffered Saline
SOP	Standard Operating Procedure
PDA	Photo-diode Array
DF	Dilution Factor
OPA	Ortho-Phosphoric Acid
HPLC-DAD	High Performance Liquid Chromatography- Diode Array Detector

Chapter 1

Introduction

1.1 Diabetes Mellitus

Diabetes mellitus is one of the chronic metabolic disorders that has reached to epidemic proportions worldwide (Yang et al., 2015). It may be defined as a group of physiological impairments or dysfunctions distinguished by chronic hyperglycemia as a consequence of the inability of cells to use glucose for essential biological processes. The underlying causes of diabetes mellitus include insulin insensitivity, impaired insulin secretion or excessive glucagon secretion (Mehanna, 2013).

In addition to diabetes mellitus being responsible for an increased rate of mortality and morbidity incidences in patients (Yang et al., 2015), it is usually also associated with a number of different damaging effects on various tissue, cellular and even organ functions. Some of the complications of diabetes mellitus include hypertension (Mehanna, 2013), cardiovascular diseases (Potenza et al., 2011), diabetic retinopathy (Global data on visual impairment 2010), renal nephropathy (WHO, 2010) etc. The clinical management as well as prevention of diabetes requires superlative control of factors like blood glucose, blood pressure, lipid concentrations, body weight etc. that have the potential to cause complications. This can be accomplished by maintaining a strict diet regime and regular exercise or by the use of anti-diabetic medications or a combination therapy including both (Mehanna, 2013).

1.2 Global Prevalence of Diabetes Mellitus and Current Scenario in Bangladesh

WHO declared diabetes mellitus as a major health problem around the world which is the only non-infectious disease placed in the category (WHO, 2010). According to the estimation of the International Diabetes Federation (IDF) in the years of 2013 and 2015 respectively, the prevalence of diabetes was 382 million and 415 million people globally (Cho et al., 2018). The projection of IDF also revealed that, within a period of less than 25 years, the number may go beyond 592 million (Biswas et al., 2016). In 2004 alone, the mortality rate associated with diabetes was 3.4 million as indicated by available data along with a projection that the number will expand to two-thirds by 2030 (Mehanna, 2013). Approximately, one-fifth of all the cases of diabetes worldwide are recorded in Southeast Asia, the prevalence of which is considered to inflate up to 71% by the year 2035 in this region. In addition to this, according to the projection of the 5th edition of the IDF Atlas, the prevalence of diabetes in Bangladesh will expand by more than 50%, making it the 8th most populous country with diabetic patients in the world within a period of the next 15 years (Biswas et al., 2016).

1.3 Diagnosis of Diabetes

World Health Organization published a consensus statement in 2006 where they released the current criteria with different values indicating plasma glucose level for diagnostic purposes of diabetes (WHO, 2006) that are similar to those stated in the consensus of the American Diabetes Association (ADA) in 2010 (ADA, 2010a) and the Canadian Diabetes Association (CDA) (CDA, 2013). The criteria are:

- a glucose level of ≥126 mg/dL or ≥7.0 mmol/L in plasma in fasting state on two or more occasions

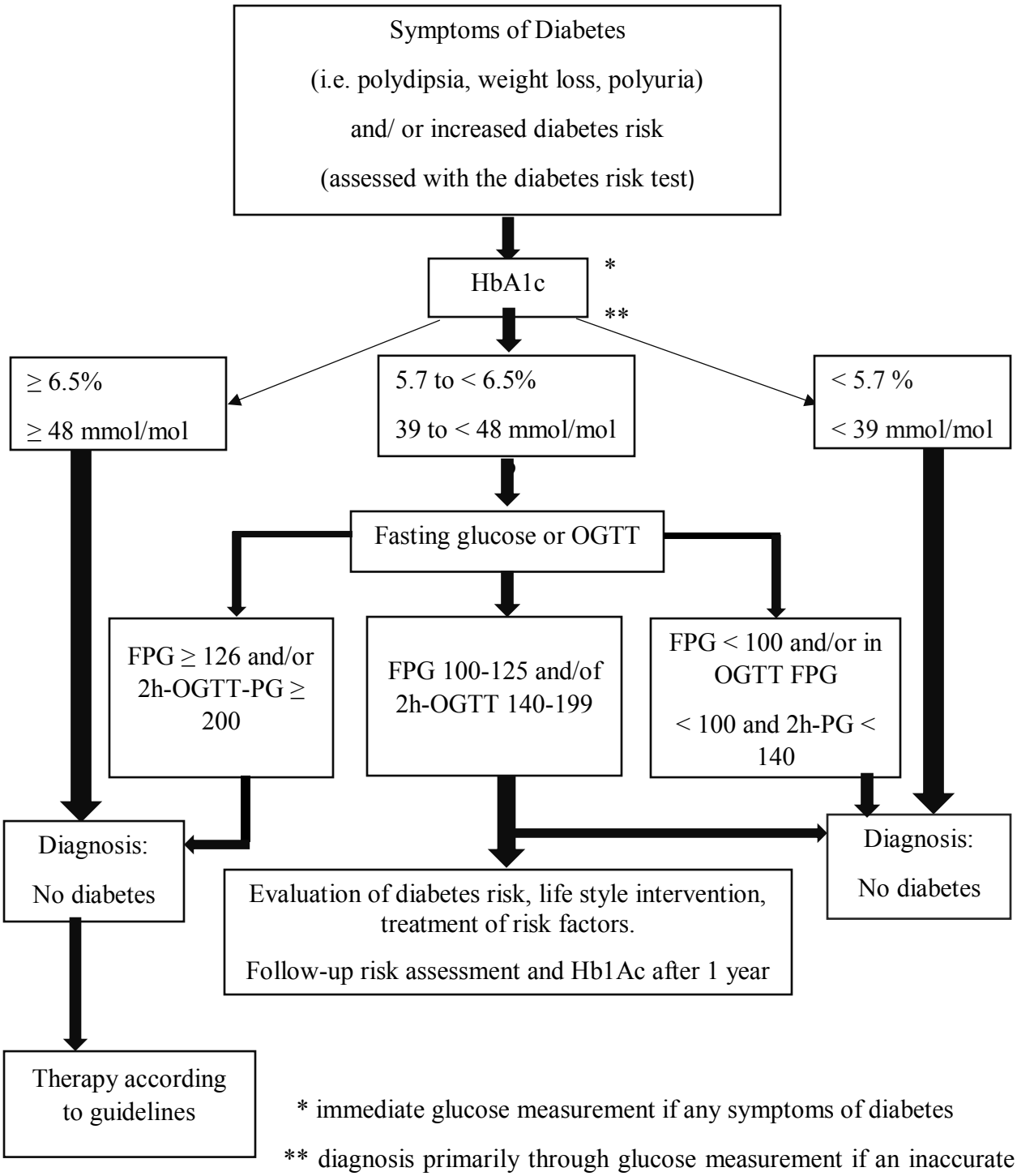
- after a glucose load of 75 g (oral glucose tolerance test, OGTT) a 2 hour glucose level of >200 mg/dL or >11.1 mmol/L in plasma
- a glucose level of ≥200 mg/dL or ≥11.1 mmol/L in plasma at random

Apart from being designated as a measure of efficacy of diabetic interventions and hyperglycemia control, hemoglobin A1c (HbA1c) has recently been incorporated in the ADA as a measure diagnostic test for diabetes mellitus. According to the certification and standardization criteria of the Diabetes Control and Complications Trial (DCCT) assay, an Hb1Ac level of ≥48 mmol/mol (≥6.5%) indicates the diagnosis of diabetes mellitus (ADA, 2010a). Some epidemiologic studies also provided data that showed that within the HbA1c concentration range of 49-53 mmol/mol (which is approximately 6.6-7.0%), microvascular complications increased markedly, specifically retinopathy. These HbA1c level also matches with those mentioned above for random, fasting and 2 hour plasma glucose levels (Mannarino et al., 2013).

The term used to collectively refer to impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT) is called "Prediabetes" (Alam et al., 2014). However, the ADA defines prediabetes as follows-

- According to ADA, following a fast kept overnight, if the glucose level in plasma is within the range of 100-125 mg/dL or 5.6 -6.9 mmol/L then it can be defined as IFG (ADA, 2010a). However, WHO recommends different values for IGF ranging from ≥110 and <126mg/dL or ≥6.1 and <7.0 mmol/L (WHO, 2006).
- According to ADA, following a fast kept overnight and an oral load of 75 g glucose or HbA1c 39-46 mmol/mol, if the 2 hour glucose level in plasma is between 140-199 mg/dL or ≥7.8 and <11.0 mmol/L then it can be defined as IGT (ADA, 2010a).

Simultaneous co-existence between IGT and IFG is possible. Additionally, both are related to the possible risk of developing cardiovascular diseases. A risk rate of 1.36 (95% cardiovascular incidences, 1.23-1.52) was reported for cardiovascular diseases by a meta-analysis conducted in 2004. Moreover, it was mentioned that women were at greater risk of cardiovascular diseases compared to women (Levitan et al., 2004). The findings of some other meta-analyses conducted in recent times have confirmed the possibility modest elevation in cardiovascular diseases which is connected to diabetes (Ford et al., 2010). Moreover, during the Oral Glucose Tolerance Test (OGTT) at 1 hour, if the concentration of glucose in plasma is at a level of ≥155 mg/dL (or ≥9.2 mmol/L) then it indicates the possibility of developing type 2 diabetes in future (DeFronzo et al., 2008).



* immediate glucose measurement if any symptoms of diabetes

** diagnosis primarily through glucose measurement if an inaccurate HbA1c level is to be expected

Abbreviation: FPG: Fasting plasma glucose, 2h-OGTT-PG: 2h plasma glucose in oral glucose tolerance test (75 g): mg/dl

Figure 1: Diagnostic Flowchart of Diabetes (glucose mg/dL) (reproduced from Kerner, 2014)

Figure 1 (reproduced from Kerner, 2014) shows the proper diagnostic criteria along with the symptoms of diabetes mellitus with different blood glucose levels or plasma glucose concentration and what the values actually indicate.

1.4 Classification of Diabetes

Diabetes mellitus can mainly be categorized in two types- type 1 diabetes mellitus or T1DM and type 2 diabetes mellitus or T2DM. Apart from these two, two more types of diabetes exist namely gestational diabetes mellitus and special type of diabetes mellitus (Mehanna, 2013).

Table 1: Types and Stages of Diabetes Mellitus (reproduced from ADA, 2010a)

Stages / Types	Normoglycemia	Hyperglycemia	
	Normal glucose regulation	Impaired Glucose Tolerance Or Impaired Fasting Glucose (Pre-diabetes)	Diabetes Mellitus Not insulin requiring / Insulin requiring for control / Insulin requiring for survival
Type 1	←	←	→
Type 2	←	←	→
Other Specific Types	←	←	→
Gestational Diabetes	←	←	→

Table 1 shows different types and stages of diabetes mellitus. Furthermore, the blockade used in the Table 1 for type 1 diabetes indicates that even though they are present in ketoacidosis in some

rare cases the patients may return to normoglycemia and the blockade used for gestational and special type indicates in special cases these patients may require insulin for achieving better glycemic control and even for survival (ADA, 2010a).

About 5-10% patients diagnosed with diabetes have T1DM. It is an autoimmune disorder which is related to the deficiency of circulating insulin resulting from gradual destruction of beta cells located in the pancreas that secrete insulin. So, insulin is a must for the treatment of T1DM. It is also designated as juvenile onset diabetes. On the contrary, T2DM may be associated with obesity and is much more common compared to type T1DM. Approximately, 90-95% diabetic patients have T2DM. A combination of defective or flawed pancreatic beta cells along with insulin resistance prompting the progressive impairment of glucose regulation in the body is the basis of T2DM. The gestational diabetes mellitus occurs mainly during pregnancy whereas, the special type of diabetes mellitus occurs as a result of uncontrolled and excessive exposure to different diseases, harmful chemicals and various immunosuppressive drugs. A more complex state of diabetes termed as insulin resistant diabetes is linked to the insensitivity or loss of sensitivity of insulin receptors towards freely circulating insulin (ADA, 2010a; Mehanna, 2013).

1.5 Medications for Diabetes

1.5.1 Medications for Type 1 Diabetes Mellitus

Insulin is a polypeptide hormone regulating the supply of glucose to every body cell except for brain cells to produce energy and to conduct different vital cellular functions (Alam et al., 2014). External insulin administration is the most popular therapy for fulfilling the purpose of clinical treatment of type 1 diabetes or T1DM (Mathieu et al., 2017) since it is caused by the deficiency of circulating insulin as a result of insulin producing pancreatic beta cell destruction (Mehanna,

2013). In this case the external insulin acts as a replacement for the beta cells associated with producing insulin and maintains the proper plasma glucose concentration. A number of different insulin analogues such as fast acting or rapid acting insulin, intermediate acting insulin, short or long acting insulin and even combination products of fast and long acting insulin are available for the treatment purpose of T1DM (Brinkman, 2017; Mathieu et al., 2017). Even though, use of insulin leads to better outcome on microcirculation, if not administered properly the rate of incidences of hypoglycemic episodes may be high upon regular intensive insulin therapy (Mehanna, 2013). In addition, the administration of insulin with hypodermic injection with hypodermic injections is associated with patient incompliance because of its invasive nature, multiple dosing frequency, pain and difficulty of administration (Schoellhammer et al., 2014).

Products like insulin pumps, insulin pens etc. are also made available to control T1DM now-a-days (Mathieu et al., 2017). Other than insulin, Pramlinitide, approved by the FDA is a comparatively new injectable agent to help manage T1DM (Frandsen et al., 2018).

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Table 2: Common Forms of Insulin Therapy for T1DM Treatment (reproduced from Alam et al., 2014)

Type of insulin	Brand names	Onset of action	Usual time of administration	Notes
Premixed	Humulin M3, Novomix 30, Mixtard 10-50*, Humalog 25, 50	Depends on the constituent insulin	Pre-meal Twice daily	Combination of intermediate-acting and short acting insulin in specific proportions
Rapid acting	Novolog, Apidra, Humalog	10-30 min	Pre-meal	_____
Long acting	Tresiba, Levemir, Ultralente, Lantus	30 min-3h	Once daily (usually bedtime)	They are often combined with rapid acting insulin
Intermediate acting	Lente, Insulatard, NPH, Humulin I	1-2 h	Pre-meal Twice daily commonly	Usually they are used after combing with rapid acting insulin
Short acting	Humalog	30 min-1h	Pre-meal	_____

*Novo Nordisk has withdrawn the insulin product Mixtard 30

Table 2 (reproduced from Alam et al., 2014) shows a list of insulin analogues used for the management of type 1 diabetes mellitus or T1DM along with their brand names, onset of action, dosing frequency and whether they should be administered pre-meal or post-meal.

1.5.2 Medications for Type 2 Diabetes Mellitus

Type 2 diabetes mellitus or T2DM is much more common compared to type 1 diabetes in recent times. It is associated with progressively defective glucose regulation because of impaired function of pancreatic beta cells on the background of insulin resistance (CDA, 2013). For the management and clinical treatment of T2DM a wide variety of drug classes are available unlike T1DM. Among these anti-diabetic agents, name of some of the classes are- sulfonylurea, biguanides, thiazolidines, α-glucosidase inhibitors, GLP-1 analogues, DPP-4 inhibitors, SGLT inhibitors, amylin analogues and even basal insulin etc. (Alam et al., 2014; Mehanna, 2013).

The attempts to discover biguanides as well as the biguanide derivatives for clinical management of diabetes mellitus began back in the middle ages. Considered to be the first-line and one of the most popular oral anti-diabetic agents, metformin- belongs to the biguanides class. As it is well tolerated among all age groups, it tends to be the main choice of drug for managing type 2 diabetes mellitus. It works by activating adenosine monophosphate-operated protein kinase which in turn inhibits mitochondrial enzyme mediated gluconeogenesis (glucose synthesis) and increases hepatic glucose uptake. In addition, it reduces the risks associated with complications as well as mortality rates while delaying the progression of diabetic symptoms in patients owing to its mechanism of action. Moreover, it enhances the activity of tyrosine kinase and also activates the expression of insulin receptors in the pancreas to achieve better insulin sensitivity (Garcia et al., 2011). Metformin provides better control over hypoglycemic episodes, is cheap and weight neutral. However, it might cause gastrointestinal side effects and also is contraindicated in renal impaired patients having an eGFR of <30 ml/h (Alam et al., 2014).

Currently, for clinical T2DM management, sulfonylureas are considered to be the second-line oral anti-diabetic agent or as an option for add-on therapy. Sulfonylureas act by blocking Adenosine Tri-Phosphate sensitive potassium (K+) channels (KATP channels) in the pancreas to increase the release of insulin and thus lowering the plasma glucose concentration. Additionally, they decrease hepatic gluconeogenesis (Chaudhury & Mirza, 2017). The breakdown of lipids to fatty acids is reduced by sulfonylureas and at the same time insulin clearance is also decreased by them. They are of two types- first generation agents and second generation agents. In comparison to second generation agents such as glyburide, glipizide, glimepiride etc., first generation agents such as tolbutamide, tolazamide, chlorpropamide etc. have some drawbacks including slower onset of action, higher risk of hypoglycemic episodes with longer elimination half-lives. On the other hand, the second generation agents show better potency with much less dosing frequency than the first generation drugs. Therefore, they are preferred and prescribed over the other ones for better management and patient compliance in type 2 diabetes (Alam et al., 2014; Proks et al., 2002). However, all the sulfonylureas have a serious side effect of hypoglycemia along with other side effects including nausea, headache, weight gain, dizziness and hypersensitivity reactions. Furthermore, their side effect of hypoglycemia may also affect the unborn child so these agents are contraindicated in pregnancy and in patients having renal or hepatic impairment issues as well (Alam et al., 2014). Diabetic patients should be cautious to avoid the risk of substantial hypoglycemia while taking drugs such as beta blockers, fibrates, aspirin, sulfonamides, allopurinol etc. that helps to prolong the effects provided by sulfonylureas and even other anti-diabetic agents and insulin (Chaudhury & Mirza, 2017).

Thiazolidines or TZDs also provide improved insulin action like biguanides. Increased glucose uptake by numerous tissues including muscle, liver and adipose tissue is promoted by them as they

are Peroxisome Proliferator Activated Receptor (PPAR) agonists. Some agents that are representatives of this class are pioglitazone and rosiglitazone. They use multiple mechanisms of action to cause β cell exhaustion which ultimately results in improved insulin resistance such as- escalation of adiponectin levels, limiting the accumulation of free fatty acid, β cell function and integrity preservation and decreasing the inflammatory cytokines (Chaudhury & Mirza, 2017). Inspite of the above mentioned benefits, this class of drugs are associated with some side effects including weight gain, anaemia, fluid retention, possible risk of developing bladder neoplasm, bone fractures etc. Some available data also connects combination therapy of insulin and TZDs with possible risk of heart failure (Alam et al., 2014). Therefore, they are rarely even considered as a step-up therapy for managing type 2 diabetes (Chaudhury & Mirza, 2017).

Exenatide, liraglutide and lixisenatide are representatives of the currently market available agents belonging to the Glucagon-Like Peptide-1 (GLP-1) analogues or GLP-1 receptor agonists (Chaudhury & Mirza, 2017). Increased resistance to enzymatic degradation mediated by Dipeptidyl peptidase-4 or DPP-4 is exhibited by some agents of this class (Harris & McCarty, 2015). Their mechanism of action include inhibition of glucagon synthesis, slowing down of gastric emptying and decreasing postprandial plasma glucose concentration (Alam et al., 2014). For the treatment of patients with abnormal metabolic profile, central obesity or for young patients whose diagnosis confirmed T2DM recently, choosing GLP-1 analogues can be considered beneficial for improving metabolic dysfunctions, reducing body weight (Chaudhury & Mirza, 2017) and for maintaining normal blood pressure. However, if concurrently used with sulfonylurea or insulin, there is a possibility of developing hypoglycemia with GLP-1 analogues. Additionally, they are contraindicated to be used by renal failure patients. Moreover, the probability of facing gastrointestinal side effects frequently and the lack of established long term drug safety profile for

these agents raise concerns for them to be used in type 2 diabetes mellitus treatment (Alam et al., 2014).

Another class of anti-diabetic agents are amylinomimetics which show efficacy against both T1DM as well as T2DM. They have multiple mechanisms of action including inhibition of glucagon synthesis, slowing down of gastric emptying and decreasing postprandial plasma glucose concentration. They provide the benefit of weight loss as well. Nevertheless, the disadvantage of being associated with frequent gastrointestinal side effects and lack of established long term drug safety profile, the use of this class of drug in the treatment of diabetes is limited. Pramlintide is a representative of this class of drug (Alam et al., 2014).

Sodium-glucose linked or co-transporter 2 inhibitors or SGLT2 inhibitors block the reabsorption of glucose taking place in the proximal tubules of the kidney and provide insulin-independent glucose lowering effect and are referred to as the new class of glucosuric agents (Taylor & Harris, 2013). Some examples of this class of drugs include- empagliflozin, canagliflozin, dapagliflozin etc. These drugs are considered to provide similar efficacy when there is a possibility of permanently losing the insulin producing pancreatic beta cell reserves in advanced stages of type 2 diabetes since they have the unique insulin-independent glucose lowering mechanism of action (Chaudhury & Mirza, 2017). The advantages of this class include- improved lipid parameters, modest weight loss and reduction of blood pressure. However, urinary tract infections that results in either pyelonephritis or urosepsis and genital mycosis may occur as side effects while using SGLT2 inhibitors (Alam et al., 2014). Concurrently, they may cause ketoacidosis even though the incidences are rare. Nevertheless, if any patient being treated with this class of drugs experience any type of ketoacidosis symptoms like non-specific features such as abdominal discomfort or

fatigue, or vomiting or nausea etc. they are suggested to stop taking those and seek immediate medical attention for their safety (Chaudhury & Mirza, 2017).

Meglitinides are a class of drug that were approved back in 1997 for the treatment or management of T2DM. Despite being known as non-sulfonylurea secretagogues (Chaudhury & Mirza, 2017), they facilitate the release of pro(insulin) by binding to the same receptor located in pancreatic beta cells as sulfonylureas, thus sharing their mechanism of action with sulfonylureas (Alam et al., 2014). Since the binding manner of these drugs are weaker, they are less effective compared to sulfonylureas and can also be referred to as short-acting insulin secretagogues. Additionally, they are not weight neutral and might cause increase in weight as a side effect. Both of these drawbacks combined with the fact that for this class of drug to instigate insulin secretion from pancreatic beta cells a higher blood sugar level is required, contribute to their limited use in type 2 diabetes mellitus management. However, in patients developing late postprandial hypoglycemia as a side effect of using sulfonylurea and those who do no follow regular meal schedules, rapid-acting secretagogues or meglitinides such as nateglinide or repaglinide may be prescribed as an alternative treatment (Chaudhury & Mirza, 2017; Alam et al., 2014).

Another class of anti-diabetic agents, that can be used both in combination therapy (with other T2DM agents) or as monotherapy called Dipeptidyl Peptidase-4 Inhibitors or DPP-4 inhibitors (Richard et al., 2011) were introduced back in 2006. Some representatives of this class are vildagliptin, sitagliptin, linagliptin, saxagliptin, alogliptin etc. (Alam et al., 2014). These agents are an area of interest for the management of T2DM as unlike many other oral anti-diabetic agents they are not associated with higher risk of hypoglycemic episodes rather are known for their safe and meaningful demonstration of anti-hyperglycemic effect. They are also weight neutral with

good tolerability profiles and do not cause gastrointestinal side effects in patients (Barnett, 2011; Richard et al., 2011).

DPP-4 is a serine protease enzyme of the cell surface that is found in the liver, pancreas, placenta, thymus, spleen, epithelial cells, vascular endothelium, lymphoid and myeloid cells, but they are present in the bone marrow, intestines as well as in kidney in highest concentration. The DPP-4 inhibitors cause blood glucose optimization in diabetic patients following a mechanism of action which suppresses the enzymatic (DPP-4 mediated) degradation and in turn enhances the circulation of active incretin hormones- glucose dependent insulinotropic polypeptide or GIP as well as glucagon like peptide-1 or GLP-1 derived from the gut and encompasses the glucoregulatory functions of these hormones (Barnett, 2011; Grunberger, 2013).

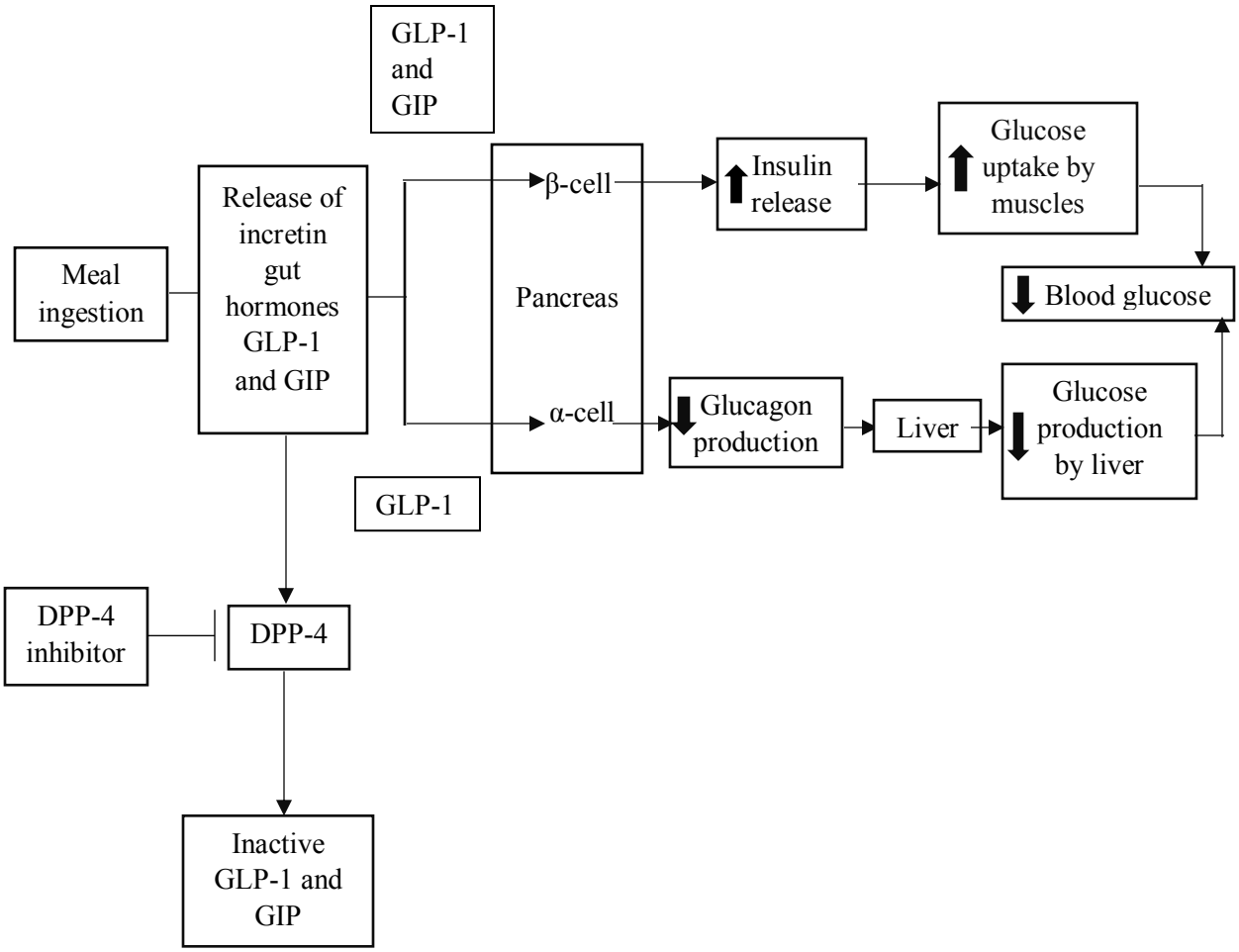

Figure 2: Mechanism of Action of DPP-4 Inhibitors (reproduced from Grunberger, 2013)

Figure 2 (reproduced from Grunbuger, 2013) shows the mechanism of action followed by DPP-4 inhibitors for providing better blood glucose control for the management of diabetes which is to increase the level of incretin hormones in systemic circulation by limiting the degradation of these hormones. Inhibition of DPP-4 enzyme mediated degradation or breakdown of these hormones restricts glucagon secretion and causes enhanced insulin release, ultimately reducing liver glucose output. Since both insulin secretion as well as restriction on glucagon release are dependent on glucose level and DPP-4 inhibitors decrease the glucose production by liver- risk of hypoglycemic

episodes are less when treated with them. Additionally, pancreatic beta cell function may also be improved owing to the enhanced level of active GLP-1 hormone (Grunberger, 2013).

Despite sharing a common mechanism of action, the DPP-4 inhibitors' differ from each other in structural heterogeneity and even in terms of various pharmacokinetic aspects such as bioavailability, metabolism, protein binding, half-life, excretion routes, systemic exposure etc. (Baetta & Corsini, 2011) which could eventually translate into dissimilarities in their clinical efficacy profiles and pharmacological properties (Grunberger, 2013).

1.6 Potential Benefits of Linagliptin over Other DPP-4 Inhibitors

Both the United States and Europe approved linagliptin as a T2DM medication. Linagliptin is a long-acting, xanthine based and potent DPP-4 inhibitor having a target-mediated non-linear pharmacokinetic profile that is administered orally in patients. It is highly selective with minimal renal clearance and exhibits concentration dependent protein binding (Deacon & Holst, 2009; Graefe-Mody et al., 2012; Heise et al., 2009; Roth et al., 2008).

Linagliptin exhibits clinically meaningful glucose lowering effect both when used in combination therapy or as monotherapy, which is distinguished by reduction in HbA1c level ranging from -0.13% to -1.6% similar to that of the other medications of the DPP-4 inhibitor class (Haak et al., 2012; Dugi et al., 2011). In addition, in therapeutic dose range it exhibits a unique non-linear pharmacokinetic profile unlike other currently available members of DPP-4 inhibitors family who have linear pharmacokinetic profiles. Linagliptin represents the non-linear pharmacokinetics as a characteristic property in both preclinical as well as clinical pharmacokinetics studies. This distinct property results from the drug's higher binding affinity to DPP-4. As a result, it dissociates slowly

from DPP-4 when compared to other DPP-4 inhibitors providing a drug disposition that is target mediated (Graefe-Mody et al., 2012).

Linagliptin's efficacy is comparable to other members of the DPP-4 inhibitors family. Additionally, considering its effect on glycemic parameters, linagliptin was found to be non-inferior to the biguanide metformin as well as to the sulfonylureas (such as glimepiride) in clinical studies. According to studies, it has a safety profile much better than many sulfonylureas, such as glimepiride. In fact, available clinical data indicates that patients treated with linagliptin achieved significant optimization of blood-glucose level or a composite endpoint (without being associated with the risk of hypoglycemia or gaining weight with an HbA1c level of <7%) more frequently compared to the patients receiving glimepiride for treatment. Moreover, unlike linagliptin, patients treated with glimepiride are at potential risk of drug accumulation (if renally impaired) and their risk of experiencing hypoglycemic incidences are higher too (Gallwitz, 2015; Lewin et al., 2012; Taskinen et al., 2011).

Currently, linagliptin is the only representative agent of DPP-4 inhibitor family having a small but effective oral dosage regimen of 5 mg- once daily that is well tolerated in patients of all age groups (Barnett, 2011; Grunberger, 2013). In addition, unlike other oral anti-diabetic agents dosage adjustment is not required for linagliptin while prescribing it to geriatric patients or even in patients whose livers or kidney functions are compromised (Graefe-Mody et al., 2012; Grunberger, 2013). In fact, according to clinical studies, linagliptin can be prescribed without performing any type of dosage adjustment in a whole spectrum of renal diseases which even covers Chronic Kidney Diseases (CKD) up to stage 5 where the Glomerular Filtration Rate (GFR) is as low as <15 mL/min/1.73 m^2 which makes it unique compared to others (Gallwitz, 2015).

1.7 Chemical Properties and Pharmacology of Linagliptin

Linagliptin is described as 1H-Purine-2,6-dione, 8-[(3R)-3-amino-1-piper-idinyl]-7-(2-butyn-1-yl)-3,7-dihydro-3-methyl-1-[(4-methyl-2-quinazolinyl)methyl]- chemically (Figure 3). It is a small molecule having a molecular weight of 472.6 g/mol and was developed by Boehringer Ingelheim Pharmaceuticals (Eckhardt et al., 2008; Gallwitz, 2011).

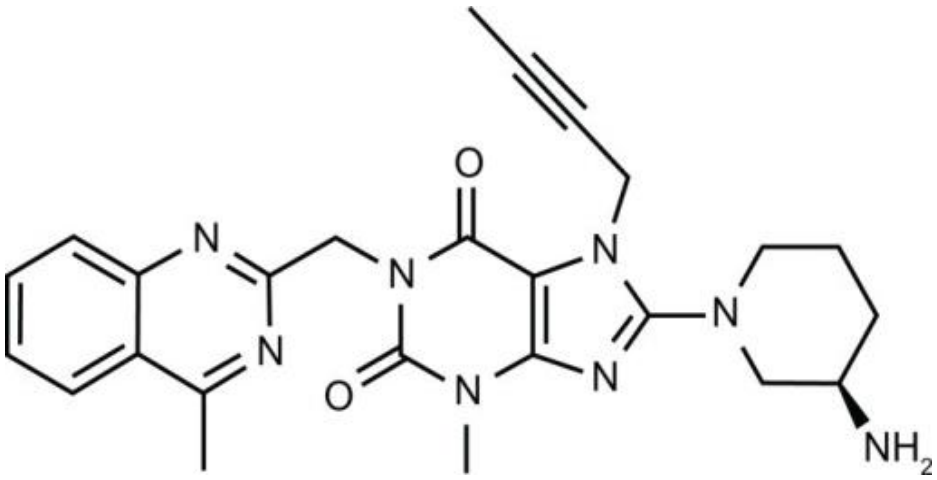

Figure 3: Chemical Structure of Linagliptin (Mourad et al., 2016)

Figure 3 (collected from Mourad et al., 2016) shows the chemical structure of linagliptin, the xanthine based DPP-4 inhibitor in which the purine and quinazoline rings are visible.

Amongst all the members of DPP peptidase family, linagliptin is approximately 40,000 fold more selective towards the enzyme DPP-4. After binding, its dissociation rate from the active site of DPP-4 enzyme is also slow owing to its sustained release. Linagliptin mediates competitive DPP-4 inhibition which is reversible at the same time (Eckhardt et al., 2008; Gallwitz, 2011). Even though with once daily administration of a 5 mg oral dose, it takes linagliptin about 2 to 5 days to

reach a steady state concentration to provide therapeutic efficacy, it provides up to 90% DPP-4 inhibition which is long lasting. The fact that, the inhibition rate is maintained at 85 % even after a period of 24 hours proves its potential of providing sustained effect (Heise et al., 2009). However, linagliptin does not prevent any cytochrome P450 (CYP450) enzyme (Eckhardt et al., 2008; Gallwitz, 2011) and at therapeutic concentration, the plasma protein binding of linagliptin is almost complete (Gallwitz, 2015).

Linagliptin is safe as it doesn't usually have side effects like hypoglycemia, weight gain etc. In addition, reportedly, it doesn't show any drug interaction with commonly used medications. Linagliptin is eliminated from the body by hepatobiliary excretion since only 1-6% leaves the body following renal elimination whereas approximately 90% linagliptin is eliminated from the body unmetabolized via the feces (Heise et al., 2009; Hüttner et al., 2008; Roth et al., 2008).

1.7.1 Solubility

Linagliptin-the purine and quinazoline derivative has been identified as a class 3 drug of Biopharmaceutics Classification System based on its low in vitro/in vivo absorption/permeability as well as its high solubility profile over a pH range of 1-7.5. It is a lipophilic drug as it has an estimated logP of 2.85. It is sparingly soluble in ethanol, very slightly soluble in isopropanol, alcohol and soluble in methanol. It's solubility in water is 0.9 mg/mL (Graefe-Mody et al., 2012; The Merck Index, 2014).

1.7.2 Absorption

Typically, after administering a single 5 mg once daily oral dose of linagliptin, the geometric mean C_{max} of approximately 8-10 mmol/L and steady state concentration of approximately 11-12 mmol/L is achieved (Hüttner et al., 2008). Whereas, AUC_{24} (the area under the curve at 24 hours)

and AUC_{ss} (area under the curve at steady state) recorded after the administration of that single 5 mg oral dose were approximately 120 nmol/L and 150 nmol/L respectively (Graefe-Mody et al., 2012).

In addition, while conducting a randomized study with 36 healthy males it was found that linagliptin has an absolute bioavailability of approximately 30% (Jungnik et al., 2010). On the other hand, the results of a 5 mg single dose crossover study that was open-label and randomized conducted with 32 healthy female and male subjects suggested that there is no clinically significant effect of food on linagliptin absorption. Furthermore, without any alteration in AUC, reduction of C_{max} by approximately 15% and increase in t_{max} by approximately 2 hours was observed from the results of this same study. However, since the drug maintained an adequate drug exposure over a period of 24 hours for clinically meaningful DPP-4 inhibition after the single dose administration, these changes were considered clinically insignificant (Graefe-Mody et al., 2012).

1.7.3 Distribution

In a study conducted with 36 healthy males, the results indicated linagliptin is extensively distributed in tissues as the steady state apparent volume of distribution (Vss) of the drug after a 5 mg intravenous infusion was found to be 110 L. The dose dependent behavior of linagliptin may be linked to its characteristic target-mediated drug disposition (Graefe-Mody et al., 2012).

1.7.4 Metabolism

Unlike other medications, the effect of metabolism in eliminating linagliptin from the body as well as in overall drug disposition is minor. The results of a study, where all the healthy volunteers received a <1mSv whole body radioactive dose of linagliptin indicated that, the drug does not undergo extensive metabolism in vivo as approximately 90% of the radioactivity that was

recovered was in the form of the unchanged parent compound. Among the identified metabolites CD1790 the S-3-hydroxypiperidinyl derivative of linagliptin was marked as the principal metabolite. Following, a 5 mg once daily oral linagliptin dosing, over a period of 24 hours approximately 13.3% of the molar drug plasma exposure was comprised by this metabolite alone even though it is pharmacologically inactive. Several in-vitro experiments concluded that CD1790 metabolite formation is a complex two-step process, involving the formation of a ketone derivative initially which then undergoes stereoselective reduction to form CD1790. The ketone formation and reduction processes are catalyzed by CYP3A4 and aldo-keto reductases respectively. Following intravenous or oral dosing, the presence of low level of several minor metabolites were reported to be found in urine, plasma or feces sample (Blech et al., 2010; Graefe-Mody et al., 2012).

1.7.5 Elimination

After the administration of a 5 mg oral dose, a rapid decline in the plasma linagliptin concentration is observed initially which is usually followed by a long terminal elimination phase indicating, the drug's elimination takes place in a biphasic manner. After multiple 5 mg dosing, linagliptin is found to have a steady state plasma terminal half-life of >100 hours. The accumulation half-life achieved after a 5 mg dosing of the drug is found to be approximately 10 hours in patients since the drug exhibits rapid accomplishment of steady state (Graefe-Mody et al., 2012; Heise et al., 2009).

Available data also suggest that, in healthy subjects administered with linagliptin radioactive doses only a small portion of approximately 5% of that linagliptin was recovered in urine that too after 4 days whereas approximately 85% of the drug was recovered unchanged from feces indicating

the clearance of the drug occurs mainly via biliary pathway. The clearance of linagliptin is also dose-dependent which can be explained by the fact that the relationship between changes in plasma drug concentration and changes in unbound fraction of the drug is proportional. Following a 5 mg oral dosing, linagliptin shows a low (70mL/min) and dose dependent renal clearance whereas, the apparent total body clearance is approximately 1200mL/min at steady state. Therefore, it can be prescribed to hepatic or renal impaired patients without requiring any dose adjustment making it unique among DPP-4 inhibitors (Graefe-Mody et al., 2012).

1.8 Analysis of Linagliptin

Despite having a significantly unique pharmacokinetic and pharmacodynamics profile, the number of investigation reports available for the bio-analysis of linagliptin is low. Among the reliable analytical reports, only a few were related to the quantitation of linagliptin in biological fluids such as plasma, in pharmaceutical dosage forms or in bulk in. These include RP-HPLC methods spectrophotometric assays and HPTLC methods for the determination of linagliptin present in combination with metformin in binary mixtures in pharmaceutical dosage forms and in bulk as well as in biological fluids such as plasma (El-Bagary et al., 2013; El-Kimary et al, 2016; Kavitha et al., 2013; Mourad et al., 2016).

1.8.1 Spectrophotometric Assay

Many drugs have been analyzed using spectrophotometric method since it is both cheap and simple. However, after extensive literature review, it was found that there are no available spectrophotometric analytical reports for the determination of the drug linagliptin alone in its pure form as well as when it is present with metformin in a binary mixture. Thus, the purpose of the study conducted by El-Bagary et al. was to develop different spectrophotometric methods for

simultaneously determining both linagliptin and metformin combinedly present in the binary mixture.

In the study, linagliptin was successfully determined from the binary mixture using two different spectrophotometric methods designated as first derivative and zero order spectrophotometric method without any interferences from metformin. The absorbance were recorded at 311 nm and 299 nm respectively for the two methods where linagliptin was determined in 5-30 μg/mL range and the obtained spectra from each method indicated that the results were acceptable in terms of accuracy as well as linearity (El-Bagary et al., 2013).

1.8.2 HPTLC Assay

A comprehensive literature review revealed the lack of high performance thin layer chromatography or HPTLC methods utilizing which the quantitation and qualification of linagliptin and other gliptins (saxagliptin, vildagliptin) alone or in combination with the drug metformin in bulk or in pharmaceutical preparations can be accomplished. Therefore, the aim of the study conducted by El-Kimary et al. was the development and validation of a single method that would be simple yet selective and could be utilized to determine either vildagliptin, linagliptin or saxagliptin from their binary mixture without any interference from metformin in pharmaceutical dosage forms. In this study, the wavelength of 225 nm was selected for densitometric measurement of the spots. Whereas an environmentally preferable mobile phase having a combination of aqueous ammonium sulfate (0.5% w/v) and methanol in a 2:8 v/v ratio and Merck HPTLC aluminium sheets made out of silica gel 60 F254 were used for the analytical separation. For the linagliptin-metformin binary mixture, the regression line was obtained using the analytical data of linear regression in a range of 0.05-0.5 μg/band. Additionally, the results of

inter and intraday precision study performed for the method suggested that both percentage relative standard deviation values and percentage relative error values were in acceptable range. Moreover, a good performance was exhibited by the developed method during validation in terms of accuracy, specificity, linearity, precision, selectivity and limits of detection as well as quantitation. Therefore, the researchers concluded that, the developed method was accurate, specific and could be satisfactorily applied in the bio-analysis as well as in quality control studies for the successful simultaneous determination of the drugs in their pharmaceutical dosage forms (El-Kimary et al., 2016).

1.8.3 HPLC Assays

An extensive literature review suggested that, there were many articles available regarding the establishment of methods with high performance liquid chromatographic or HPLC analysis for simultaneously estimating linagliptin in combination with metformin in pharmaceutical preparations (Kavitha et al., 2013; Mourad et al., 2016; Swamy & Baba, 2013).

The purpose of the study conducted by Kavitha et al. was to establish a simple and precise RP-HPLC method that would contribute to the estimation of linagliptin and metformin hydrochloride simultaneously in their pharmaceutical formulations as well as in their pure forms. C_8 column and a combination of acetonitrile, water and methanol in the ratio of 25:50:25 (v/v) with their pH adjusted to 4.1 (using 0.1% orthophosphoric acid) were selected as the stationary phase and mobile phase respectively to carry out the separation of linagliptin, metformin along with their degradation products in this study. A diode array detector performed the analytical detection at 243 nm. A good analytical separation was achieved in the study and the recorded retention time for metformin and linagliptin were 2.9 minutes and 7.0 minutes respectively. Linearity was found in 5-30 µg/mL

range for linagliptin and in 10-100 µg/mL range for metformin while performing validation studies following ICH guidelines. Therefore, it was concluded that the established method is capable of successful determination of both drugs in pharmaceutical dosage forms and in their pure form (Kavitha et al., 2013).

In another study, an accurate, rapid, cost effective but at the same time highly sensitive RP-HPLC method was for simultaneously estimating both linagliptin and metformin was established by researchers Swamy and Baba. A Hypersil C_{18} column with the specifications 250×4.6 mm and having a 5µ particle size and a combination of acetonitrile, pH 5.6 phosphate buffer (diluted with orthophosphoric acid) and methanol in the ratio of 55:40:5 (v/v) were selected as the stationary phase and mobile phase in this study respectively to carry out the analytical separation. The wavelength of 233 nm was selected for monitoring the eluents. With a 1.0 mL/min flow rate the recorded retention time for metformin and linagliptin were 6.6 minutes and 5.4 minutes respectively. The method showed good performance in terms of accuracy, limit of detection and quantification, precision, specificity, linearity as well as robustness while carrying out ICH guidelines recommended validation procedures. During validation studies, the linearity for linagliptin was found in 0.625-3.75 µg/mL range and the linearity for metformin in 125-750 µg/mL range. Additionally, the linearity of both drugs had notable regression coefficients or R^2 values of 0.999. Therefore, it was concluded that the proposed method could successfully determine both linagliptin and metformin in bulk or in pharmaceutical dosage forms (Swamy & Baba, 2013).

Another study conducted by Mourad et al. proposed a high performance liquid chromatography-diode array detector mediated or HPLC-DAD method to investigate the degradation kinetics of linagliptin when subjected to oxidative forced degradation conditions, or in alkaline or acidic conditions and to perform quantitation of linagliptin in its tablet dosage form. In this particular

study, linagliptin was analyzed with its various degradation products and to do so the drug samples were diluted after being extracted from the tablet dosage form. Following the dilution, it is subjected to several ICH recommended degradation or stress conditions for further investigation related to degradation kinetics. A C_{18} column and a combination of water and methanol containing 0.3 % TEA at a pH of 4.5 and in 60:40 ratio were selected as the stationary phase and isocratic mobile phase respectively to carry out the analytical separation in this study. With a 1.0 mL/minute flow rate of the drug, detection occurred at a wavelength of 225 nm whereas the recorded retention time was 11 minutes. The study exhibited good performance linearity while carrying out ICH guidelines recommended validation procedures. Therefore, it was concluded that the proposed method could successfully separate and determine linagliptin in the presence of various degradation products and at the same time expanded the scope to further study the degradation kinetics of the drug (Mourad et al., 2016).

1.9 Purpose of the Study

A comprehensive literature review suggested that, only a few reports are available regarding the quantitation of linagliptin. Even though, the studies were conducted with a view to determine linagliptin concentration in bulk or in pharmaceutical dosage forms, the reported assays showed either longer retention times or lacked detailed information about the estimation of drug absorption. Moreover, most of developed or proposed methods were for simultaneous determination of linagliptin along with metformin in their pharmaceutical formulations containing linagliptin-metformin combination or their binary mixture rather than focusing solely on the determination of the content of linagliptin alone in its pure or in pharmaceutical dosage forms.

A unique aspect of the study is that, currently no written or analytical monographs are available for the powder or dosage forms of the drug linagliptin in United States Pharmacopeia (USP) or in the British Pharmacopeia (BP) (Mourad et al., 2016) despite having such unique and beneficial pharmacological properties. Therefore, after reviewing previous trails, the aim of the current study was to establish a simple, rapid, sensitive and accurate HPLC method to analyze and quantify the linagliptin in its pure form as well as in pharmaceutical preparations or dosage forms, in addition to estimating the absorption profile of the drug in biological fluid.

Chapter 2

Materials and Methods

2.1 Chemicals and Reagents

Linagliptin standard was obtained from Beximco Pharmaceuticals Ltd. Bangladesh, in the form of standard API powder which was used as standard in the study. HPLC grade Methanol and 37% Hydrocloric acid were purchased from Active Fine Chemicals Ltd, Bangladesh. Potassium dihydrogen phosphate and Disodium hydrogen phosphate were purchased from Scharlab S.L., Spain. Sodium chloride and Potassium chloride were purchased from Merck group, India. Samples of linagliptin were obtained from local pharmacies of Dhaka city, Bangladesh.

2.2 Instruments

1. Electronic balance (ATY 224) – It was used for weighing different chemicals and other substances accurately.

2. UV-Vis Spectrophotometer (UV 1800) – it was used for scanning and initial identification of the standard drug and to check the solubility of the standard in two different solvent systems (Figure 4).

Figure 4: UV-Vis Spectrophotometer, Model-UV-1800, Shimadzu, Japan

3. Digital pH meter bench top (S220) – It was used for adjusting pH of different buffer solutions.

4. Shimadzu HPLC LC-20 AT series (Figure 5) binary gradient pump (Tokyo, Japan) with

- SPD-M20A PDA detector
- Solvent delivery system (SIL-20 AHT)
- Auto injector
- Online Line 5 Channel degasser
- HPLC filtration unit 1000 mL

It was used for qualification and quantification of the standard and samples of linagliptin.

Figure 5: High Performance Liquid Chromatography (HPLC) Machine, Model-LC-20 AT, Shimadzu, Japan.

5. Ultra-sonic sound bath (LUC 405) – It was used for degassing solutions or solvents (removal of bubbles from them) and for achieving homogenous solutions.

2.3 Preparation of Phosphate Buffer Solution for Mobile Phase

To prepare 300 mL 0.05 M potassium dihydrogen phosphate buffer at first, 2.04135 g potassium dihydrogen phosphate (KH_2PO_4) was accurately weighed and transferred into 150 mL of filtered distilled water in a beaker and more of the water was added to increase the volume to 280 mL. Then the pH was checked and found to be 4.5. The volume was then adjusted upto 300 mL with more of filtered distilled water. The prepared buffer was filtered by using 0.45µ membrane filter again and sonicated for 5 minutes using ultra-sonic bath at a temperature of 28ºC.

2.4 Preparation of PBS (or Phosphate Buffered Saline) as Diluent

To prepare 500 mL of 0.1M phosphate buffered saline or PBS (to be used as the diluent to dissolve the standard and sample linagliptin), at first the required amount of NaCl (400 mg), KCl (10 mg), Na_2HPO_4 (72 mg), KH_2PO_4 (12 mg) were accurately weighed and transferred to 200 mL of filtered distilled water and stirred well with a glass rod to dissolve all the salts. The pH was checked and found to be 7.95. But the required pH of the buffer is 7.4. Therefore, the pH was adjusted to 7.42 with diluted HCl (37%) solution (3-4 drops of HCl was taken in 40 mL of filtered distilled water to prepare the diluted HCl solution (Appendix 2, 2000) which was then added drop-wise with the help of a pipette, while performing simultaneous pH check as the pH fluctuates a lot. Around 8 mL of diluted HCl was required to reach to the pH of 7.42). After fixing the pH, the volume of the buffer was adjusted to 500 mL with filtered distilled water. Then, buffer was filtered using 0.45µ membrane filter again and sonicated for 5 min using ultra-sonic bath at a temperature of 28ºC.

2.5 Preparation of Standard and Sample Solutions

2.5.1 Preparation of Standard Stock Solution

To prepare the standard linagliptin stock solution, at first 10 mg of standard linagliptin was accurately weighed and transferred in a 100 mL volumetric flask. Small amount of PBS was taken into the flask. The mixture was sonicated for 2 min to obtain a uniform solution. The volume was then adjusted up to 90 mL with PBS and sonicated again for 3 min. After obtaining a uniform, clear solution the volume was adjusted up to 100 mL with PBS to prepare the standard stock solution of linagliptin having the concentration of 0.1 mg/mL or 100 µg/mL. From this stock solution, working solutions having five different concentrations were prepared by serial dilution for being assayed in HPLC and for preparing calibration curve later on.

2.5.2 Preparation of Standard Working Solutions

Working solutions having the concentrations- 0.625 µg/mL, 1.25 µg/mL, 2.5 µg/mL, 5 µg/mL and 10µg/mL were chosen for the convenience of the study. For preparing the standard working solutions of the above mentioned concentrations, at first about 5 mL of the standard stock solution was filtered and 1 mL of it was transferred into a 10 mL volumetric flask. The volume was then adjusted up to 10 mL with PBS to obtain the concentration 10 µg/mL. From this solution (conc. 10 µg/mL), 5 mL was withdrawn into another 10 mL volumetric flask and the volume was adjusted up to 10 mL with PBS to obtain the the concentration 5 µg/mL. Similarly, to obtain the concentration 2.5 µg/mL, 5 mL solution (having the conc. of 5 µg/mL) was withdrawn in a 10 mL volumetric flask and the volume was adjusted up to 10 mL with PBS. Later on, 5 mL solution (having the conc. of 2.5 µg/mL) was withdrawn into another 10 mL volumetric flask and the volume was adjusted up to 10 mL with PBS to obtain the the concentration 1.25 µg/mL. Lastly, 5

mL solution (having the conc. of 1.25 µg/mL) was withdrawn into another 10 mL volumetric flask and the volume was adjusted up to 10 mL with PBS to obtain the the concentration 0.625 µg/mL.

2.5.3 Preparation of Sample Stock Solutions

Linagliptin 5 mg marketed by 5 different pharmaceutical companies were collected as samples and designated as M, N, O, P and Q to be assayed by HPLC following the developed method and chromatographic conditions. To do so, 10 tablets of each sample were taken and crushed to make fine powder. Then, from those powders a calculated amount equivalent to 10 mg of linagliptin was transferred in 5 different 100 mL volumetric flasks. Small amount of PBS was taken into each of the flasks and the powder was dissolved to make a solution. The resulting solutions were sonicated for 2 min. The volumes were adjusted up to 90 mL with PBS. Then they were subjected to sonication for another 3 min and volume of each solution was adjusted up to 100 mL with PBS to obtain the concentration of 100 µg/mL for each sample (which were used as the sample stock solutions).

2.5.4 Preparation of Sample Working Solutions

Small amount was filtered from each stock solution. 1 mL from each solution was taken into 10 mL volumetric flask. Volume was adjusted up to 10 mL with PBS to make the concentration 10 µg/mL. All the HPLC vials and the syringe used to fill them were washed with the PBS 3-4 times and rinsed once with very minute amount of the working solution of the specific concentration prior insertion. All of the solutions were filtered using 0.22 µm Restek syringe filter while filling the vials. The vials were filled gradually, labeled and inserted into the HPLC machine for analysis.

2.6 Chromatographic Conditions

HPLC analysis of the standard and sample working solutions of linagliptin having different concentrations were performed with the following chromatographic conditions (Table 3).

Table 3: Chromatographic Conditions

Parameters	Chromatographic conditions
Stationary Phase	HPLC column: Phenomenex Luna C_{18} (150× 4.6mm) (USA) packed with 5 µm particles.
Mobile Phase	Methanol: Phosphate buffer (pH 4.5) = 70:30 (v/v)
Mode	Isocratic elution
Column temperature	Ambient (25±2 ºC)
Detector	SPD-M20A PDA detector
Detection wavelength	241 nm
Diluent	0.1 M PBS (pH 7.4)
Flow rate	1 mL/min
Injection volume	10 µL
Run time	10 min
Retention time	Approximately 3.3 min

Table 3 represents the summary of chromatographic conditions which includes stationary phase, mobile phase, flow rate, injection volume, run time, retention time, detector information etc. used for this particular method development for the quantitation and qualification of linagliptin.

2.7 Validation Procedure

2.7.1 Robustness

The robustness of this method was studied by measuring the effects of deliberate changes in the method like alterations in the pH of the mobile phase (pH= 3, 4.6 and 6) and variations in the ratio of mobile phase components (methanol: phosphate buffer of pH 4.5 in 60:40 v/v ratio and 50:50 v/v ratio).

2.8 Potency Calculation of the Marketed Products of Linagliptin

Equation from the curve, $y = 101640x + 516232$

So, $m = 101640$ and $c = 516232$

y = peak area of sample,

x= conc. of the sample

1. For sample M,

   y = 1521391

   $x = ((y - c) \div m)$

Therefore, $x = ((1521391 - 516232) \div 101640)$

$= 9.9$

Amount of drug per tablet = ((x × Dilution Factor × average weight per tablet) ÷ (amount of powder taken × 1000))

= ((9.9 × 1000 × 83.2) ÷ (160.52 × 1000))

= 5.13 mg

Potency = ((found amount ÷ declared amount) × 100) %

= (5.13 ÷ 5) × 100 %

= 102.6 %

2. For sample N,

y = 1489325

x = ((y - c) ÷ m)

Therefore, x = ((1489325 - 516232) ÷ 101640)

= 9.57

Amount of drug per tablet = ((x × DF × average weight per tablet) ÷ (amount of powder taken × 1000))

= ((9.57 × 1000 × 104.38) ÷ (208.76 × 1000))

= 4.81 mg

Potency = ((found amount ÷ declared amount) × 100) %

= (4.81 ÷ 5) × 100 %

= 96.2 %

3. For sample O,

y = 1550008

x = ((y - c) ÷ m)

Therefore, x = ((1550008 - 516232) ÷ 101640)

= 10.2

Amount of drug per tablet = ((x × DF × average weight per tablet) ÷ (amount of powder taken × 1000))

= ((10.2 × 1000 × 105.6) ÷ (205.2 × 1000))

= 5.24 mg

Potency = ((found amount ÷ declared amount) × 100) %

= (5.24 ÷ 5) × 100 %

= 105 %

4. For sample P,

y= 1493286

x = ((y - c) ÷ m)

Therefore, x = ((1493286 - 516232) ÷ 101640)

= 9.6

Amount of drug per tablet = ((x × DF × average weight per tablet) ÷ (amount of powder taken × 1000))

= ((9.6 × 1000 × 195.14) ÷ (386.28 × 1000))

= 4.85 mg

Potency = ((found amount ÷ declared amount) × 100) %

= (4.85 ÷ 5) × 100 %

= 97 %

5. For sample Q,

y = 1503137

x = ((y - c) ÷ m)

Therefore, x = ((1503137 - 516232) ÷ 101640)

= 9.7

Amount of drug per tablet = ((x × DF × average weight per tablet) ÷ (amount of powder taken × 1000))

= ((9.7 × 1000 × 119.74) ÷ (231.48 × 1000))

= 5.02 mg

Potency = ((found amount ÷ declared amount) × 100) %

= (5.02 ÷ 5) × 100 %

= 100.4 %

Chapter 3

Results

3.1 Solubility Study of Linagliptin

Before selecting phosphate buffered saline or PBS as the final diluent to prepare standard and sample solutions of linagliptin for HPLC analysis, it's solubility in different solvent systems were checked following articles regarding similar studies. To establish the solubility profile of linagliptin, the drug's solubility in methanol and ethanol was tested. Findings of the solubility study matches with those mentioned in PubChem 2019, which are- the drug is soluble in methanol and sparingly soluble in ethanol (The Merck Index, 2014). After that, linagliptin's solubility was studied in PBS.

Phosphate buffered saline or PBS is a type of buffer utilized for accomplishing biological research purposes as it is considered innocuous or non-toxic to majority of cells. It contains a variety of salts and therefore is a water-based salt solution by nature (Martin et al., 2006). Even though, the solubility of linagliptin was studied in different solvent systems, PBS 0.1M of pH 7.4 was selected as the optimum diluent for preparation of standard and sample solutions of linagliptin. The reason behind this is, in terms of osmolality and ion concentrations PBS solution is equivalent to the human body fluids (isotonic) (Sigma-Aldrich, 2019), specifically blood as the pH of blood is also 7.4. Therefore, PBS was selected as the optimum diluent in the study to estimate the absorption of this drug in human blood after oral administration. In addition, the optimum mobile phase for the experiment was decided to be the combination of methanol and potassium dihydrogen or KH_2PO_4 phosphate buffer (pH 4.5) in a ratio of 70: 30.

3.2 Choice of Mobile Phase

At the beginning of the study, various compositions of mobile phases were considered for carrying out the HPLC analysis of linagliptin. Among those, selected few are mentioned below:

- Methanol and water containing 0.3% tri-ethyl amine in a ratio of 40:60 and pH 4.5 (Mourad et al., 2016)
- Phosphate buffer (pH 5.6, diluted with OPA or orthophosphoric acid) and methanol and acetonitrile in the ratio of 40:5:55 (Swamy and Baba, 2013)
- Acetonitrile and water and methanol in the ratio of 25:50:25 to pH 4.1 with 0.1% OPA or orthophosphoric acid (Kavitha et al., 2013)

The first mobile phase could not be examined due to the unavailability of tri-ethyl amine in the laboratory whereas, the other methods required longer run times of 15 and 20 minutes respectively, which was both time consuming and expensive. After vigorous studying, initially the mobile phase containing acetonitrile and KH_2PO_4 buffer of pH 4.6 obtained with OPA (orthophosphoric acid) in the ratio of 70:30 v/v and 60:40 v/v were used for linagliptin assay. The resulting peaks however split. Therefore, the trial and error phase continued until the combination of methanol and KH_2PO_4 buffer of pH 4.5 in the ratio of 70:30 v/v was examined to assay the drug which produced sharp peaks in a relatively shorter run time of 10 minutes. In addition, the retention time was also shorter (approximately 3.3 minutes) than any achieved by the above mentioned studies.

While examining robustness of the study, alterations in the pH of the mobile phase and also the ratio were brought and the observation indicated with increase in the pH and changes in the ratio both the area of the peak and retention time got affected. Therefore, the composition of methanol

and KH_2PO_4 buffer of pH 4.5 in the ratio of 70:30 v/v was selected as the optimum mobile phase for carrying out the assay of linagliptin.

3.3 Construction of Calibration Curve

Retention Time (RT) and Area Under the Curve (AUC) were selected as the primary form of data collection in this study. Here, RT was used for qualitative or identification purpose and AUC was used as a quantitative measure. Readings were taken in triplicates for solutions of each concentration the average RT and AUC of which is shown in Table 4. The data were further used to create calibration curve shown in Figure 6 by plotting average area or AUC (in X- axis) against concentration (in Y-axis) for the standard linagliptin, in order to determine the unknown concentration of linagliptin present in each of the samples and to estimate their absorption profile. For the first three runs the run time was set at 20 minutes. Since the retention time of the standard was unknown, a longer runtime creating a bigger window was considered to be safe for the optimum mobile phase. In the optimum chromatographic conditions, the RT for linagliptin was found to be approximately 3.3 minutes. Although, the retention time for the standard was found to be under 10 minutes, the run time was set to 15 minutes this time to evaluate the consistency of the study. After ensuring the consistency in RT from the third time and onwards, the run time was fixed at 10 minutes since no peaks were observed after 4 minutes. The following chromatogram was obtained for the analysis of the standard linagliptin which is shown in Figure 6.

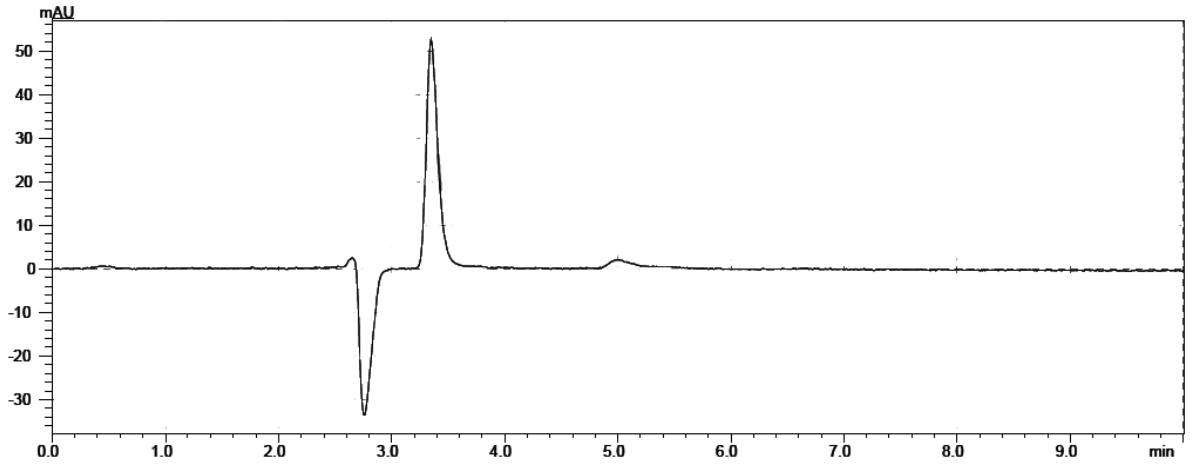

Figure 6: Chromatogram of Standard Linagliptin

Figure 6 shows a chromatogram obtained by HPLC analysis of the standard linagliptin following the developed method.

Table 4: Average AUC for Different Concentrations of Standard Linagliptin

Serial no.	**Concentration (µg/mL)**	**Average retention time (in minutes)**	**Average AUC ± Standard deviation**
1.	0.625	3.353 min	564073.7 ± 45688.1
2.	1.25	3.355 min	656056 ± 33148.7
3.	2.50	3.351 min	774245.3 ± 50891.1
4.	5	3.362 min	1021115 ± 29405.6
5.	10	3.357 min	1531947.3 ± 19758.8

Table 4 provides information about average RT and AUC for different concentration of linagliptin solution prepared by serial dilution. The average were calculated by analyzing each solution in triplicates.

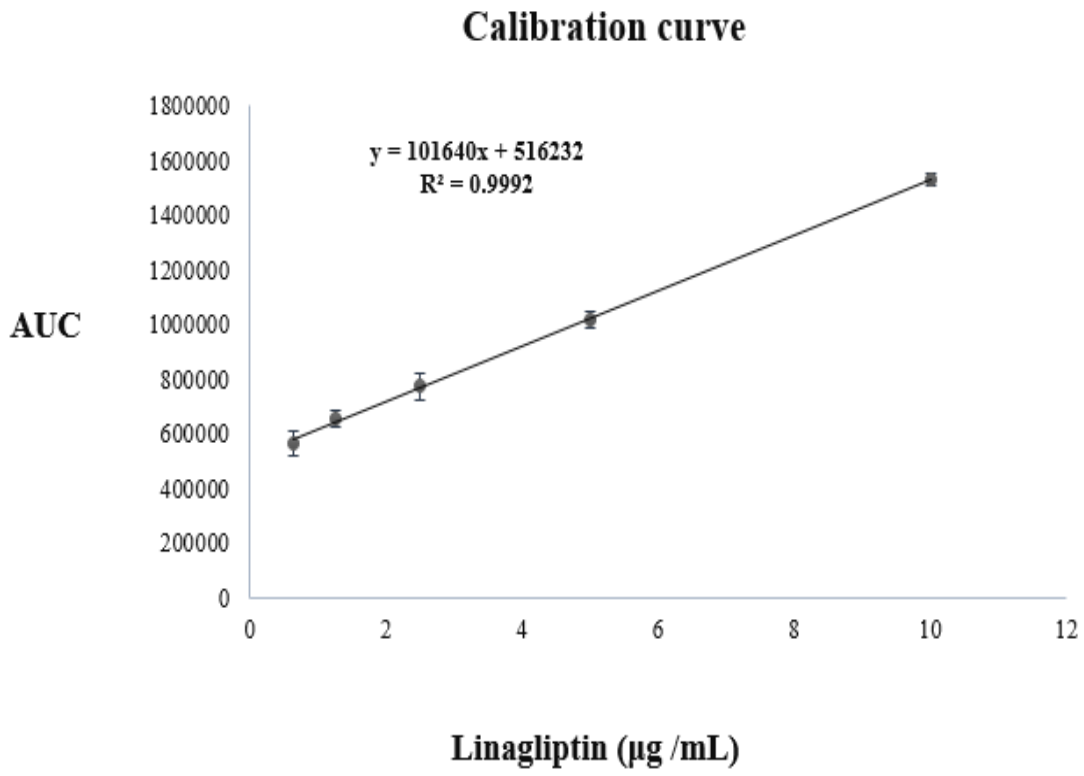

Figure 7: Calibration Curve of Standard Linagliptin, where n=3

Figure 7 represents the calibration curve (along with regression co-efficient or R^2 value and equation representing y = mx + c) prepared using the analytical results of the standard solutions of linagliptin having different concentrations, where each solution was analyzed thrice for minimizing errors. The vertical bars are error bars obtained during the analysis of each standard solution having different concentration (performed thrice; n=3) which shows the variability of data from the mean.

3.4 Study of Physical Properties of Samples

The linagliptin 5 mg tablets marketed by different pharmaceutical companies were collected from the local markets of Dhaka city, Bangladesh. 10 tablets of each sample were used for the analysis for ensuring the sensitivity of the analytical results.

Table 5: Physical Properties of Marketed Samples of Linagliptin

Serial no.	Sample ID	Dosage form	Average weight (mg)	Color
1.	Sample M	Tablet	83.2	Pink
2.	Sample N	Tablet	104.38	Light green
3.	Sample O	Tablet	105.6	Light green
4.	Sample P	Tablet	195.14	Brick red
5.	Sample Q	Tablet	119.74	Pink

The properties of the analyzed marketed samples of linagliptin such as average weight, color etc. were compiled in Table 5.

3.5 Analysis of Sample and Potency Determination

For the convenience of the study, the concentration 10 µg/ml was selected for preparing working solutions of the five different samples to be analyzed following the optimum chromatographic conditions. Readings were taken in triplicates for each solution of the same concentration which is shown in Table 6. Retention Time (RT) and AUC were selected as the primary form of data collection.

Table 6: HPLC Analysis of Marketed Samples of Linagliptin

Serial no.	Sample ID	Concentration (µg/mL)	Average retention time (in minutes)	Average AUC ± Standard deviation	Potency
1.	Sample M	10	3.34	1521391 ± 12062.1	102.6%
2.	Sample N	10	3.34	1489325 ± 18515.5	96.2%
3.	Sample O	10	3.36	1550008 ± 10308	105%
4.	Sample P	10	3.36	1493286 ± 9044.11	97%
5.	Sample Q	10	3.36	1503137 ± 21135.5	100.4%

Table 6 shows the average RT, AUC and standard deviation along with potency related data of the 5 analyzed samples of linagliptin collected from local markets of Dhaka. The average peak area (among three areas) of each sample was taken to calculate the concentration, amount of linagliptin present in each sample and potency using the equation obtained from the standard calibration curve shown in Figure 7.

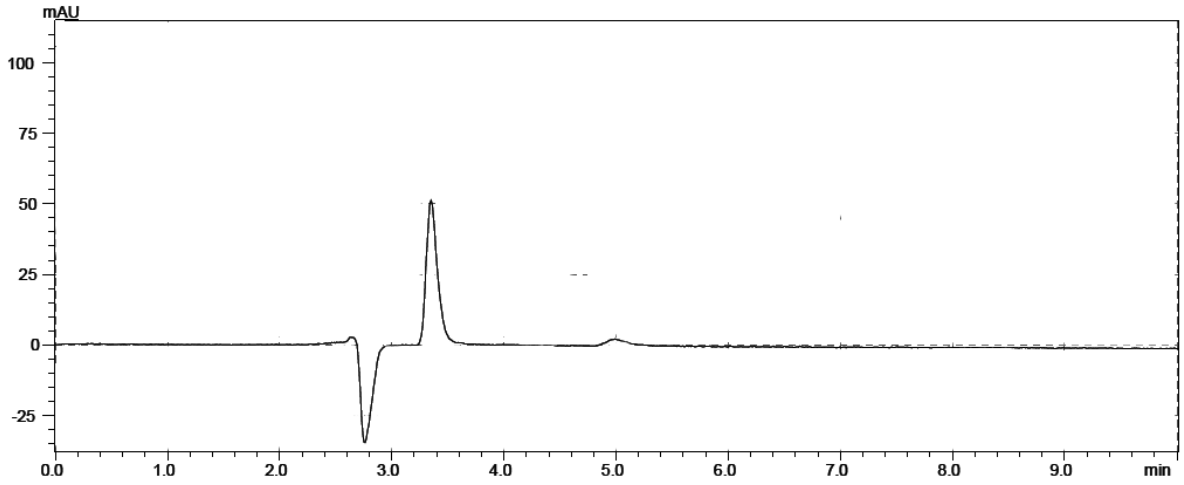

Figure 8: Chromatogram of Sample M

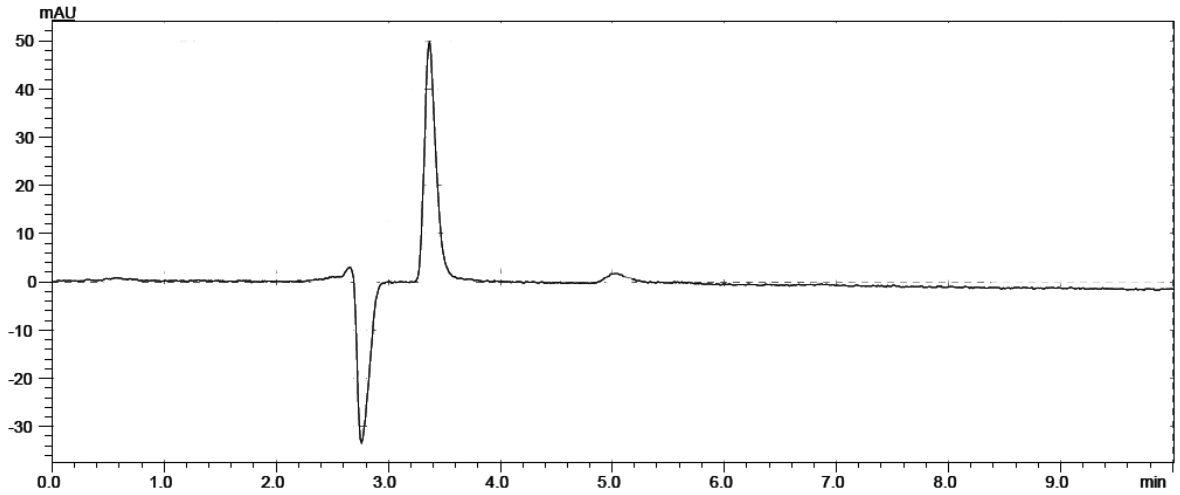

Figure 9: Chromatogram of Sample N

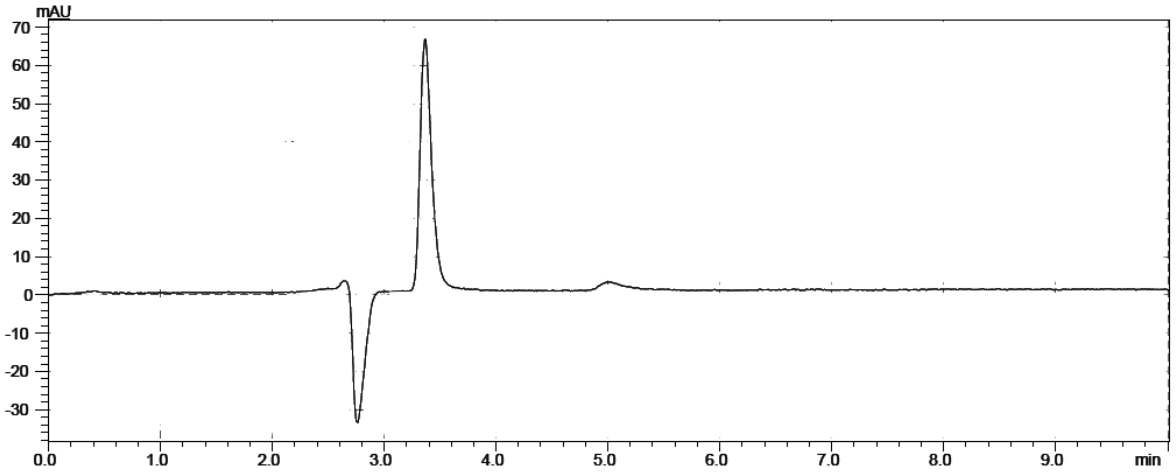

Figure 10: Chromatogram of Sample O

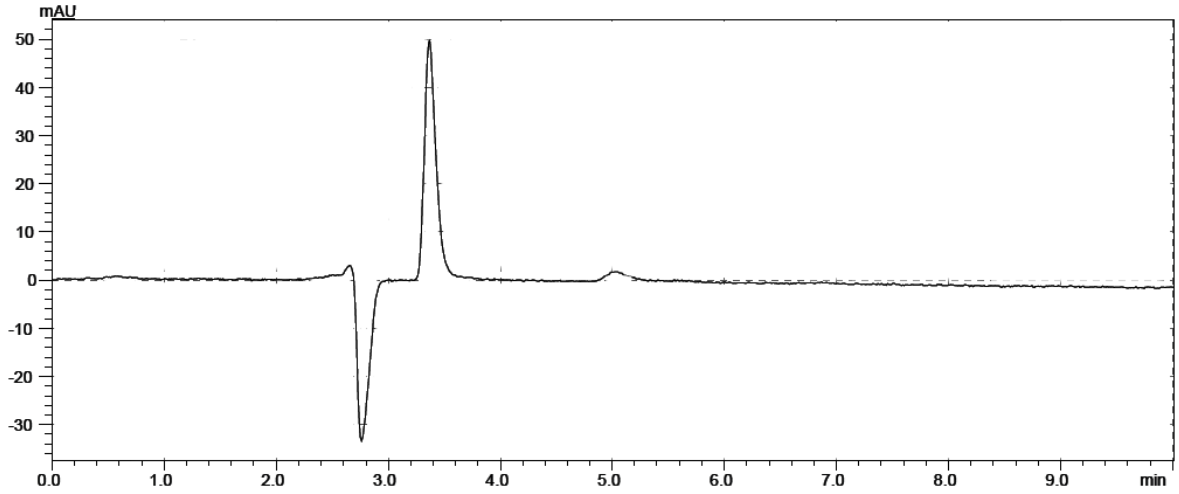

Figure 11: Chromatogram of Sample P

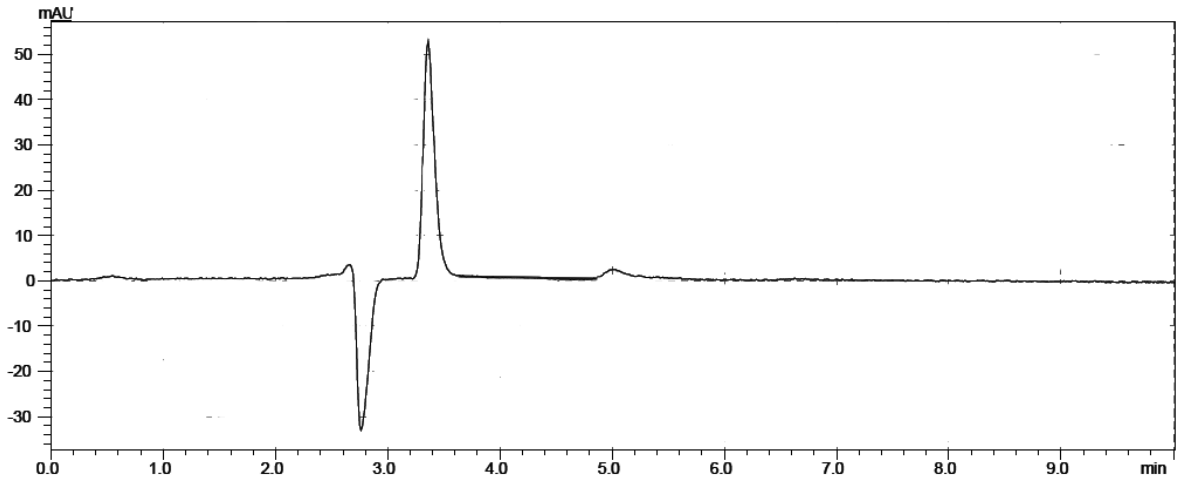

Figure 12: Chromatogram of Sample Q

Figure 8-12 represent the chromatograms obtained by the analysis of 5 marketed samples (M, N, O, P and Q respectively) of linagliptin using the developed HPLC method. In these chromatograms, x axis represents the retention time and y axis represents the peak height.

Chapter 4

Discussion

Since type 2 diabetes mellitus or T2DM has become an increasing global concern due to the morbidity and mortality rate associated with it, the need for safe and efficacious novel anti-hyperglycemic medications that will not interact with other medicines prescribed for controlling T2DM in poly-pharmacy practices nor will it be contraindicated for common co-existing diseases such as hypoglycemia or renal (kidney) diseases are valued and recommended to patients. Linagliptin, the dipeptidyl peptidase-4 inhibitor possesses a pharmacokinetic and pharmacodynamics profile that meets these medical requirements of diabetic patients. Additionally, in terms of important pharmacokinetic parameters, the drug is different compared to other DPP-4 inhibitors (Graefe-Mody et al., 2012). Clinically meaningful efficacy in terms of glycemic control has been demonstrated by linagliptin with an acceptable profile of safety and tolerability even during its phase III clinical trials. An oral dose of linagliptin once daily can successfully inhibit the plasma DPP-4 activity by >80% owing to its prolonged plasma elimination half-life as well as its high potency. This in turn results in a clinically meaningful depletion in the elevated level of plasma glucose and also in the increase of the plasma glucagon like peptide-1 levels providing better glycemic control. In addition, it can be prescribed to patients with compromised kidney functions without any sort of dosage adjustment, as it has a unique and primarily non-renal excretory pathway that possibly is the outcome of its high affinity for binding with proteins (Graefe-Mody et al., 2010). In spite of being such a significant oral anti-hyperglycemic agent, it does not have any written analytical monograph in the British Pharmacopeia or United States Pharmacopeia. Additionally, the extensive literature reviews

suggested the absence of reliable, simple methods for determining linagliptin alone in single pharmaceutical dosage forms rather than in combinations with other medications such as metformin.

Therefore, this study was designed to try and establish a precise as well as simple method for determining the drug linagliptin in pharmaceutical preparations and in its pure form. In addition, the aim of the study was also to estimate the absorption of the drug at the same time by HPLC analysis. In this study, the combination of methanol and KH_2PO_4 phosphate buffer of pH 4.5 in a ratio of 70:30 v/v was selected as the mobile phase to analyze the drug instead of other mobile phase combinations such as acetonitrile: water or acetonitrile: phosphate buffer as it produced sharp peaks in a relatively shorter amount of time (retention time was approximately 3.3 minutes) within a run time of 10 minutes unlike any of the combinations mentioned above. In fact, in case of the other combinations, the resulting peaks broke. The diluent PBS 0.1M of pH 7.4 was chosen to be the optimum diluent for linagliptin keeping its osmolality and ion concentrations in mind. Since these properties of PBS are similar to those of biological fluids such as blood, therefore by analyzing the drug in the diluent, the absorption profile or bioavailabilty of the drug can be estimated in vitro. To observe the absorption of linagliptin, standard linagliptin solutions having five different concentrations were prepared and analyzed to collect the data – retention time and peak area and a calibration curve (Figure 7) was prepared. The collected samples were analyzed using the same method and their concentrations and potencies were calculated using the calibration curve equation which are presented in the Table 6.

From the final results it was evident that the sample O and sample M produced higher average peak areas than that of the standard drug, indicating they provide higher concentrations of the drug in blood following oral administration, than that of the standard drug. On the other hand, the

sample Q produced peak area almost equivalent to that of the standard. Sample N and sample P however produced average peak areas that were in close proximity to that of the standard indicating they provide similar drug concentrations in the systemic circulation following oral administration as the standard.

The results of potency determination suggested that the sample O had the highest potency of approximately 105%, followed by the potency of 102.6% provided by sample M and 100.4% provided by sample Q. On the other hand, the samples N and P had the potency of approximately 96.2% and 97% respectively. According to the potency range established for most compounded products by USP, all of these values are acceptable as they fall into the range of ±10 %, indicating the potency should stay within 90% - 110% (USP, 2008). It can thus be said that the samples analyzed by the developed method provided acceptable potency and are patient compliant. In addition, the observations indicated that the samples O, Q and M produce higher concentration in systemic circulation and as a result provide higher potency which may be related to the source, storage condition and quality of the API that directly affect the absorption or bioavailability of these drugs and in turn, patient compliance. The remaining samples N and P also provided acceptable potency and helped to estimate that the bioavailability profile of these will also be acceptable. Therefore, it can be said that the study provides a comparative base for the marketed samples of linagliptin in terms of their potency as well as their absorption profile or bioavailability.

In total six chromatograms are represented in this study which were obtained from the HPLC analysis of standard and sample linagliptin (sample M, N, O, P and Q). All of the chromatograms produced by the standard (Figure 6) and sample linagliptin (Figure 8-12) shows two distinctive peaks one of which is in the positive range whereas the other one is in the negative range. The distinctive peak obtained in the positive range belonged to the drug linagliptin which was

confirmed by comparing with chromatograms obtained during trial and error phase. The other negative peak (or system peak) that is visible can be because of the choice of the diluent (Srbek et al., 2005). The possible explanation behind this can be the usage of a diluent (phosphate buffered saline) to dissolve the drug which is different from the mobile phase (combination of methanol and potassium dihydrogen phosphate buffer) used for the study. To confirm whether the negative peak belonged to the diluent or not the chromatograms were matched with the chromatograms obtained during the trial and error phase where methanol and acetonitrile were used as the diluent instead of PBS and it was found that in those chromatograms the negative peak was absent. Therefore, it was concluded that the negative peak in fact was obtained because of the choice of the diluent that is different from the mobile phase.

Chapter 5

Conclusion

The proposed method for the HPLC based determination of linagliptin is sensitive, reliable, precise and simple. Additionally, the method is fast and feasible. Therefore, this method can be successfully used for the estimation of the absorption profile of linagliptin after oral administration and determination of the drug in its pure form as well as in pharmaceutical preparations without interference from co-formulated drugs.

Chapter 6

Future Directions

The developed method was used only for the analysis of tablet dosage forms of linagliptin and to estimate their absorption profiles in this study. Therefore, this method can be used to analyze other dosage forms or formulations of linagliptin likewise and can be utilized to estimate their bioavailability as well in future. The developed method can also be followed for simultaneously determining the drug linagliptin present in binary mixture with other drugs.

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