DEVELOPMENT, CHARACTERISATION AND ASSESSMENT OF CHEMICAL STABILITY OF FAST DISSOLVING ORAL LEVOTHYROXINE FILMS

DR. KAIS SHABAN

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University of Brighton

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ABSTRACT

Hypothyroidism is a condition where insufficient thyroid hormone, thyroxine, is produced in the body to meet the body's daily requirements, potentially leading to serious complications which directly or indirectly involve the thyroid gland. Levothyroxine is currently available as low dose tablets containing 25 µg, 50 µg, and 100 µg per tablet. Levothyroxine products upon storage have been reported to be susceptible to chemical instability, which alters drug stability and affects therapeutic and physicochemical properties such as bioavailability and content uniformity, respectively. Environmental factors, mainly temperature, play an important role in the production of levothyroxine breakdown products and the drug has been on the Medicines and Healthcare Product Regulatory Agency (MHRA) yellow card since 2009 due to its apparent poor formulation stability. Since 2011, the scheme has recorded a high loss in efficacy for levothyroxine tablets, with 87 negative reports on levothyroxine chemical stability. However, the precise mechanism by which the compound is subject to chemical instability remains unclear.

Levothyroxine is known to degrade into biologically active compounds. But there is insufficient data on the main reason for the declining chemical stability of levothyroxine and its content uniformity. In addition, there is not enough information on levothyroxine break down products and their toxicity. However, previous studies on APIs that are susceptible to similar chemical limitations as levothyroxine showed that oral fast dissolving film delivery systems have an ability to improve chemical stability and content uniformity profile. Therefore, the hypothesis of this research is to investigate levothyroxine stability and toxicity in accelerated conditions and stabilise it using a new formulation such as fast dissolving oral film (FDOF). The aims of this project are to investigate the influence of high temperature on levothyroxine chemical stability and structure, to develop a novel FDOF formulation of levothyroxine, thus addressing its chemical stability issues, improving its content uniformity within the formulation, and enhancing its biopharmaceutical properties and to ensure that the formulations are safe by evaluating their toxicity using an in vitro cell culture model. To investigate the effects of high temperature on levothyroxine and the mechanism of action leading to chemical instability, levothyroxine as a solid powder form was placed at different temperatures: 25°C, 40°C and 70°C, for predetermined time points (one to six months). The resulting samples were collected and analysed along with a control sample at t=0 using a novel gradient elution high performance liquid chromatography (HPLC) method developed in this research. Additionally, liquid chromatography-mass spectrometry (LC-MS) and Fourier transform infrared spectroscopy (FT-IR) were used to study the chemical stability of levothyroxine.

The idea of formulating fast dissolving films became a viable option due to the rich blood supply within the oral cavity which produces a higher dissolving environment than the tablet formulations are exposed to. FDOFs are an oral route of administration that can be used in different locations of the oral cavity: namely, the sublingual and buccal delivery systems. This administration route is characterised by a rapid disintegrating and dissolving action depending on the hydrophilic polymer and plasticiser used within the formulation and therefore FDOF provides high drug stability and rapid onset of action. Novel formulations of levothyroxine FDOFs developed in this research from different types or percentages of hydroxypropylmethylcellulose (HPMC), plasticisers and antioxidants. All formulations were validated by characterising their physiochemical properties, content uniformity using HPLC, and compatibility using FT-IR. HPLC analysis of levothyroxine sample stability indicated that the rate of drug concentration decrease was related to the duration of exposure to high temperature. By using LC-MS, three new degradation products of levothyroxine, [4-(4-ethyl-2,6-diiodophenoxy)-2,6 diiodophenol], [4-(4-ethyl-2,6-diiodophenoxy)-2 iodophenol], and [4-(2,6 diiodo-4-methylphenoxy)-2 iodophenol] were identified in samples incubated at 70°C for 2 months. These results were supported using FT-IR, where an alteration in the peaks for levothyroxine were observed. The possible cause of decreased levothyroxine concentration and subsequent change to content uniformity and chemical stability could be heat induced oxygen mediated oxidation.

Novel levothyroxine FDOF using HPMC E15 as a polymer and propylene glycol as plasticiser showed high compatibility with thyroxine spectrum using FT-IR. Acceptable drug content uniformity was confirmed using HPLC. Although, levothyroxine FDOFs and especially with BHT showed improved chemical stability when compared to the solid-state levothyroxine at three different temperatures: 25°C, 40°C and 70°C. Levothyroxine FDOF disintegrated within seconds and drug release of >95% was detected within 10 minutes into the dissolution medium. The FDOF formulations showed thin and uniform thickness, low percentage of moisture loss, low weight variation, and good flexibility (by folding endurance test). A pH test of the FDOF formulation revealed it to be neutral, making it ideal for placement in the oral cavity. As with any pharmaceutical formulation development process, toxicological properties must be observed to evaluate the safety profile of the prepared dosage form. Therefore, toxicity of levothyroxine FDOFs and levothyroxine in its solid state using oral mucosal epithelial cell line models H376 were performed. Toxicology studies were carried out by using MTT assays on the levothyroxine FDOF formulated with and without an antioxidant, and levothyroxine in its solid state. Levothyroxine fast dissolving films with BHT as an antioxidant showed better cell viability and permeability. It is essential to have *in vivo* pharmacokinetic studies as a fture work for this project to test the developed levothyroxine formulations.

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Finally, I would like to dedicate this work to my beloved city, Aleppo and I wish to Syria to come back safe and beautiful as it was.

Wish everyone who read my words the success in the life.

Author Declaration

I declare that this project is original and it was done by the author. I declare that, this research was not previously submitted to any other degree.

Kais Shaban

8/November/2018

Abbreviations

HPLC	High-Performance-Liquid-Chromatography
LC-MS	Liquid Chromatography-Mass Spectroscopy
FT-IR	Fourier transform infrared spectroscopy
DSC	Differential-Scannin-Calorimetry
BCS	Biopharmaceutical Classification System
TSH	Thyroid-Stimulating-Hormone
FDA	Food and Drug Administration
USP	United State Pharmaceopeia
MHRA	Medicine and Healthcare product Regulstory Agency
BPS	British Pharmacopeia Commission
OSD	Oral Solid Dose
API	Active Pharmaceutical Ingredient
HPMC	Hydroxy-Propyl-methyl-Cellulose
HEC	Hydroxy-Ethyl-Cellulose
PVA	Polyvinyl alcohol
CMC	Carboxymethyl cellulose
PEG	Polyethylene glycol
PG	Propylene Glycol
FDOFs	Fast Dissolving Oral Formulations
BHA	Butylated HydroxyAnisole

BHT	butylated hydroxyltoluene
ACN	Acetonitrile
QC	Quality Control
LOD	Limit Of Detection
LOQ	Limit Of Qantification
ICH	International Council for Harmonisation
STD	Standard Sample
TFA	Tri-Fluro Acetic Acid
LDH	Lactate dehydrogenase assay
MTT	colorimetric assay for assessing cell metabolic activity.
RSD	Relative Standard Deviation
BDE	Bond Dissociation Energy
NaOH	Sodium Hydroxide
UV	Ultra-Violet Spectroscopy
GLY	Glycerine
F	Code for formulations
T	Time point

1. General Introduction

1.1 Thyroid gland and its hormone

Thyroid hormones (T_3 (triiodothyronine) and T_4 (thyroxine) are secreted by the thyroid gland and modulated by thyroid stimulating hormone (**TSH**). The TSH hormone is generated from the pituitary gland in the human brain (Figure 1.1).

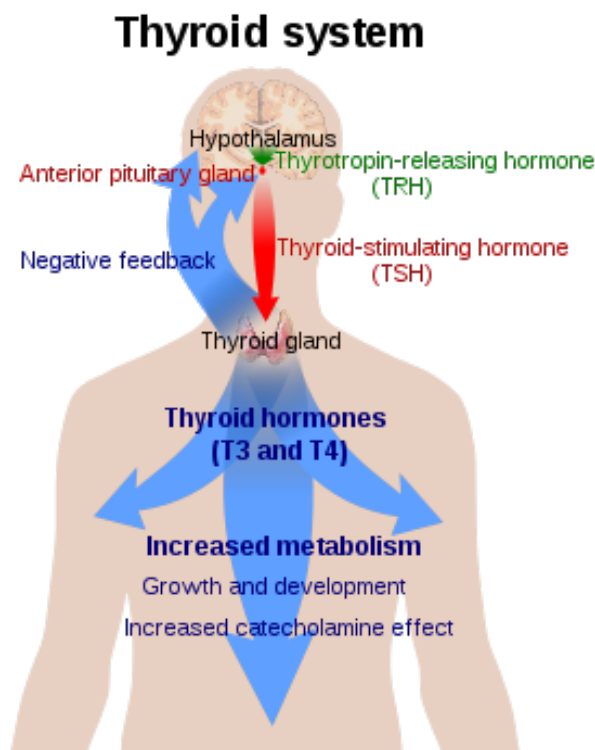


Figure 1-1: The pathway formation of Thyroid glands and its hormone. Adapted from [1]

The thyroid gland is composed of follicles containing a viscous colloid which serves as a reservoir for thyroid hormones [2]. 3,3,5-triiodo-thyronine (T_3) and 3,3,5,5-tetraiodo-thyronine (T_4) are the main thyroid hormones secreted from the thyroid gland. The difference between the two are the number of iodine molecules located in T_3 (3 iodine molecules) and T_4 (4 iodine molecules) (Figure 1.2).

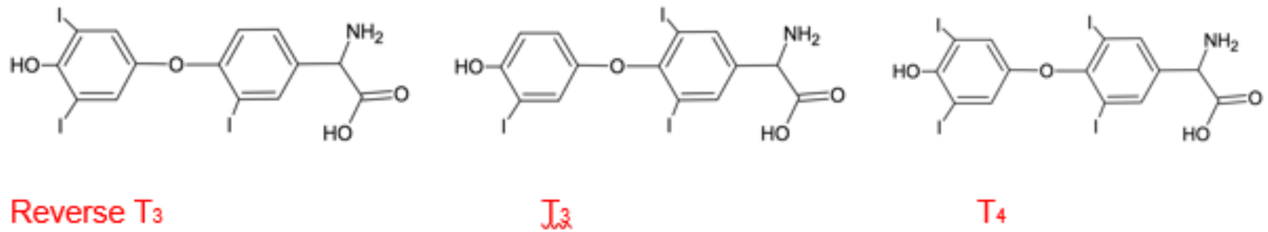


Figure 1-2: Chemical structure of T_3, T_4 and reverse T_3. Adapted from [3].

The production of T_3 and T_4 is dependent on an enzyme called iodothyronine deiodinase. However, epithelial cells are the main cells in the thyroid glands, and they are necessary to produce T_3 and T_4. These epithelial cells have the ability to absorb the iodine molecules from food and begin to form the main hormone in the thyroid glands ($T_3$and T_4). This process of $T_3$and T_4formation relies on the enzyme called iodothyronine deiodinase. Tyrosine is an amino acid and is a constituent of a protein called thyroglobulin, which is located in thyroid gland. Tyrosine then conjugates with the absorbed iodine within thyroglobulin, and produces monoiodotyrosine (MIT) or diiodotyrosine (DIT). The interaction between MIT and DIT molecules leads to the production of T_3 while the interaction between two DIT molecules leads to the production of T_4 (Figure 1.3).

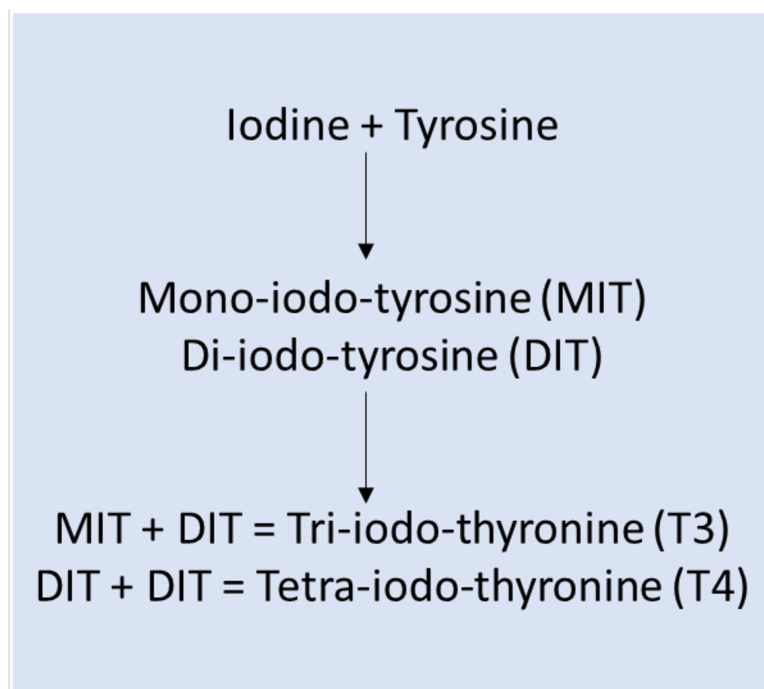


Figure 1-3: synthesis of T4 and T3 from dietary iodine and tyrosine. Adapted from [3]

Thyroid hormones have the ability to emigrate from the follicular cells into the blood circulation to reach the human target tissues [3]. Plasma proteins are necessary for transporting the thyroid hormones into the blood circulation as thyroid hormones are not soluble in water. Therefore, various thyroid hormone transporter proteins such as monocarboxylate transporter (MCT8) and organic anion-transporting polypeptide (1C1) are needed to transport the thyroid hormones into the cell membranes [4]. The concentration of T_3 and T_4 within the target tissues can be regulated by different types of deiodinase enzymes. Type 2 deiodinase enzyme yields T_3 from T_4 while type 3 deiodinase enzyme breaks down T_4 to reverse rT_3 which is (the inactive form of T_4) and converts $T_{3'}$ which is biologically more active than $T_{4'}$ to 3,3-diiodo-thyronine (T_2) which has same biological activity to T_3 and more biologically active than $T_{4.}$ [5].

1.2 Regulation of thyroid hormone secretion

The synthesis and secretion of thyroid hormones is controlled by TSH (thyroid stimulating hormone also called thyrotrophin), which is released by the anterior pituitary gland. TSH secretion is in itself stimulated by TRH (thyrotrophin releasing hormone) which is synthesized and released by the hypothalamus. TH (full form) also inhibits the synthesis of TRH and TSH subunit genes at the transcriptional level as well as the post-translational modification and release of TSH. Hence opposing TRH and TH inputs regulate the hypothalamic-pituitary-thyroid axis and the serum TSH levels (Figure 1.4) [6].

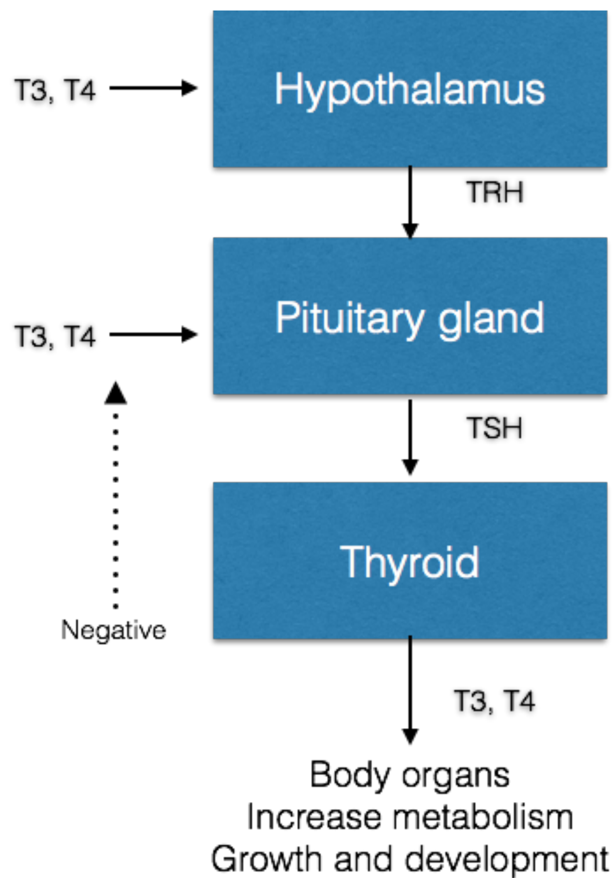


Figure 1-4: The negative feedback mechanism. Adapted from [7]

For a typical synthesis of thyroid hormone 150 µg of iodine is required in adults, 200 µg in pregnancy and between 90-120g in children every day [6]. Food is the main source of iodine with specific nutrition available as rich iodine food. The rich iodine food consists mainly of sea food, vegetables, antioxidant rich fruit such as cranberries, organic beans, organic cheese and organic yogurt. Kitchen iodinated salt is also one of the primary additional source for iodine [6] If a person does not consume enough iodine from food and the body iodine content's requirement is not met, it can lead to iodine deficiency. The inadequate amount of consumed iodine from food may lead to a lack of thyroid hormone synthesis which leads to increased TSH secretion and increased gland growth giving rise to a goitre (enlargement in the neck due to iodine deficiency). The presence of a goitre alone does not necessarily indicate a thyroid abnormality because it can be present in hypothyroidism, hyperthyroidism or even euthyroidism [8].

The thyroid hormone receptors are intracellular, and hence the cellular actions of these hormones are initiated in the plasma membrane, in the cytoplasm, and at the

mitochondrion. The biological activities of T_3 are mediated by transcriptional regulation following its binding to the thyroid hormone nuclear receptors. Transcriptional activity which is the initial step of gene expression is also regulated by a host of nuclear co-regulatory proteins. In the presence of T_3, the co-activators accelerate transcription whereas in the absence of T_3 they repress the transcriptional activity. Mutations of these receptors could lead to several abnormalities such as resistance to thyroid hormones, thyroid cancer, pituitary tumors and dwarfism [9].

Thyroid hormones affect many systems such as the sympathetic nervous system, growth, muscular function, cardiovascular system and carbohydrate metabolism. Thyroid hormones are essential not only for normal growth in children but also for neonatal brain development. Moreover, thyroid hormones have the ability to stimulate most metabolic pathways and are either catabolic or anabolic. They also exert lipolytic and lipogenic effects depending on their concentration. They act on muscles and can lead to myopathies or loss of muscle. And finally, they have effects on cardiovascular function leading to increased heart rate, contractility and cardiac output. It is necessary to note that if there is a deficiency of these hormones during the phase of brain development in children, brain damage or severe neurological impairment can result, which cannot be reversed once completed. Hence, nowadays there is a universal screening for congenital hypothyroidism in neonates. [6].

1.3 Hypothyroidism

Hypothyroidism is a condition where insufficient thyroid hormone is produced to meet requirements, potentially leading to serious complications which directly or indirectly involve the thyroid gland. Hypothyroidism can affect body growth and influence various cellular processes that can result in dry skin, hoarseness, delayed ankle reflexes, fatigue, and a ‘husky’ voice [8]. About 2% of the general women population in the United Kingdom have been diagnosed with thyroid abnormality while only 0.2% of men in the United Kingdom have been detected with thyroid issues [10]. One of the statistical studies on thyroid dysfunction in the UK showed that 4.1/1000 women suffer from hypothyroidism, while 0.8/1000 women suffer from hyperthyroidism in the UK. On the other hand, 0.6/1000 per men are suffered from hypothyroidism and the same percentage for hyperthyroidism. [11, 12].

However, there are other reasons apart from iodine deficiency that causes thyroid gland failure. These reasons are related to autoimmune disease (Hashimoto's disease), cancer of the thyroid gland and gland destruction when trying to treat hyperthyroidism (thyroid surgery, radioactive iodine therapy). The typical feature of primary hypothyroidism is a reduction in thyroxine (T_4) with an increased serum TSH. If TSH level is elevated and the T_4 level is normal, then the condition is called subclinical hypothyroidism. Secondary hypothyroidism is suspected when the T_4 level is reduced, and TSH levelly is within or below the normal range. In this case, the condition should be confirmed by investigations on the hypothalamic-pituitary function. Central causes of hypothyroidism are defined by low levels of TSH and insufficient T_3/T_4. Alternatively, the target tissues may fail to respond to thyroid hormones due to a congenital defect in the expression of their receptors. In some cases, hypothyroidism may appear due to various kinds of medicines that affect the thyroid gland. Examples of drugs that can lead to hypothyroidism are: lithium, amiodarone, interferon α, interleukin 2 and tyrosine kinase inhibitors. If left untreated, hypothyroidism can lead to hypertension, dyslipidemia, infertility, cognitive impairment and neuromuscular dysfunction [13]. It has been reported that biochemical assessment to diagnose hypothyroidism is recommended, as hypothyroidism has many signs and symptoms which need to be specified [14].

1.3.1 Consequence of hypothyroidism treatment

Depending on the hypothyroidism in the patient's situation, a treatment plan needs to be designed. In simple cases, natural treatment such as food replacement can improve the hypothyroidism situation. Diet can play important role on enhancing the situation of the thyroid patients as some types of food can interfere with iodine or thyroxine absorptions such as soya. Some calcium rich foods and supplements interfere with levothyroxine absorption.

A gap of 4 hours between the two would be adequate to ensure there is no significant impact on blood thyroxine levels. Some researchers showed that, it is better for the thyroid patient to do a diet against cabbage, cauliflower and kale as they may cause some enlargement of the thyroid gland [15].

These replacements can include iron, vitamin D., and omega three fatty acids. However, medicine treatment is one of the solutions to improve the symptoms of hypothyroidism. The most common drug used to treat hypothyroidism is thyroxine. On

the other hand, in complicated situations such as patients suffering from thyroid cancer or goiter, they need to undergo surgery to remove a part or all of the thyroid gland also known as thyroidectomy [15]. In case of removing most of the thyroid gland and the rest is not enough to produce the required thyroid functions, replacement therapy by using some drugs such as levothyroxine should be initiated adapted to TSH assay results, which is a thyroid stimulating hormone test that is necessary to indicate the required dose from the replacement therapies [16].

Regarding hyperthyroidism, food replacement as the first line of treatment is not an option because there is no deficiency in hyperthyroidism. The first line of hyperthyroidism treatment is the medical option such as carbimazole. In addition to the medical choice, radioactive iodine may be present. It is necessary to mention that these treatments for hyperthyroidism may induce hypothyroidism. Therefore; replacement therapy for hypothyroidism is required accordingly [17]. Conversely, in severe situations, thyroidectomy might be required to treat hyperthyroidism [17].

1.3.2 Consequence of untreated hypothyroidism

Thyroid hormones T_3 and the T_4 level are relatively small. Therefore, when the thyroid gland is excited to deliver sufficient levels of the hormones, it leads to an increase in the size of the thyroid gland with time (thyroid gland enlargement on the throat) and can lead to grave consequences such as goiter, dysphagia, and dyspnea [14]. Subclinical thyroid dysfunction has been linked to systolic and diastolic cardiac dysfunction and heart failure. In these patients, thyroxine replacement is reported to have improved the cardiac function [15]. As a slightly altered thyroid function is sufficient to change heart rhythm, heart rate, and ventricular function resulting in an increase in the risk of coronary diseases and cardiovascular mortality. More precisely, hypothyroidism is characterised by a combination of sinus bradycardia, low QRS complexes, prolongation of Q-T interval and inversion of the T wave. Finally, the risk is increased for myocardial infarction more than for arrhythmias [16]. Moreover, hypothyroid patients may experience hyponatremia due to a reduction in free water clearance. Sometimes when patients suffer from a reduced cardiac ejection fraction, this can lead to oedema [17]. Although hypothyroidism in the late stage may affect the prolactin level, which is responsible for human fertility. Therefore, untreated hypothyroidism may negatively affect fertility. Also, untreated hypothyroidism can

impact the regulation of the human energy by altering the levels of blood glucose, diabetes and lipid metabolisms [18].

However, TSH assay test to determine the therapeutic dose of the replacement therapies for hypothyroidism is highly required. TSH assay can provide the details needed to adjust the dose of hypothyroidism drugs such as levothyroxine. It is necessary to identify the dose of hypothyroidism medicines because one of the side effects of hypothyroidism replacement therapy overdose is hyperthyroidism.

1.4 Levothyroxine

1.4.1 Levothyroxine overview

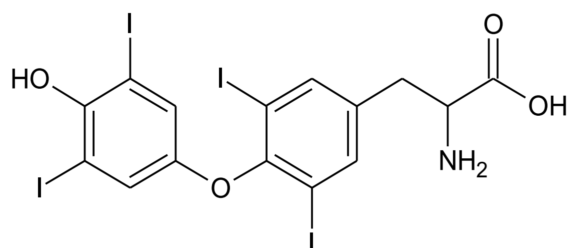


Figure 1-5: Chemical structure of levothyroxine

The chemical structure of L-thyroxine is showing in Figure 1.5. L-thyroxine is a white crystalline powder with poor solubility in both 96% ethanol and water. However, levothyroxine exhibits excellent solubility in a diluted solution of alkali hydroxide [19]. The melting point range of L-thyroxine at 5 mg/mL is between 207°C and 210°C, and it is stated as being slightly soluble in water (0.105 mg/mL at 25°C) [20].

With a chemical formula of ($C_{15}H_{11}I_4NO_4$) and anhydrous molecular weight of 776.87 g/mol, levothyroxine is mainly used as a replacement therapy for hypothyroidism disease by modulating the secretion of thyroid-stimulating hormone (TSH) and thyrotrophic releasing hormone (TRH) [21, 22]. Levothyroxine has shelf life of three years from the date of manufacture.

Following oral administration, levothyroxine is absorbed from the ileum and jejunum, which can be varied and can range from 40% to 80%. This is mainly affected by food

intake as L-thyroxine absorption can increase in the fasting state and may be reduced in malabsorption syndromes. Drug interactions with other medications such as orlistat, cholestyramine, and proton pump inhibitors can affect Na-L-thyroxine absorption [23]. As levothyroxine occurs as a naturally occurring molecule within the body, it is unclear if synthetic levothyroxine derivatives produce an equivalent therapeutic effect. It has been investigated that the process of formulating the levothyroxine in specific pharmaceutical dosage forms (tablet dosage form) has an impact on levothyroxine physicochemical properties including its dissolution rate. This is because the wet granulation techniques used to produce L-thyroxine has various steps. On of this steps are drying method and L-thyroxine is sensitive to heat. Therefore, the process of formulating L-thyroxine can strongly affect its chemical stability and hence it's content of uniformity [24].

In vivo safety investigations such as general toxicology studies are needed to develop novel pharmaceutical formulations. The assessments of novel formulations toxicity help to progress in the discovery phase of drug development. However, most of the drugs that had unsuccessful progression in early drug development were related to the toxicological issues and that can be costly exercise for many pharmaceutical companies. In addition, drug toxicity studies are necessary to increase the safety of drugs consumed by the patients and enlarge the possibility of accomplishment in non-clinical and clinical drug development [25].

The toxicity studies can be divided into two study phases: chronic toxicity studies and acute toxicity studies. The chronic toxicity test is achieved in several clinical trials to observe which of the human organs are vulnerable to drug toxicity. This study relies on the clinical and physiological chemistry signs to observe the human organs alterations. The second type of toxicity testing is acute studies in which lethal dose 50% (LD_{50}), the dose required to kill 50% of an experimental population expressed as milligrams of drug per kilogram of body weight is determined [26]

The LD_{50} of levothyroxine is 20 mg/kg of rat body weight. Levothyroxine is excreted from the kidney, so renal function testing in hypothyroidism patients is required. Levothyroxine is more efficacious than liothyronine due to its quicker onset of action (6 to 12 hours, with a peak metabolic effect occurring after 10 to 12 days) and the longer half-life (7.5 days) [23, 27]. It is recommended to take the daily therapeutic dose

of levothyroxine on an empty stomach, as food contains fibres which can affect product absorption; certain patients who should take fibres are therefore unsuitable for treatment with levothyroxine sodium tablets [28, 29].

The therapeutic index dose or the difference between the minimum therapeutic dose and the minimum toxic dose, of pure levothyroxine, remains unclear. Previous studies have reported levothyroxine as a drug with a narrow therapeutic index [30, 31]. Although the US Food and Drug Administration (FDA) has indicated levothyroxine as having a wide therapeutic index dose. Hence, the dose that can result into the drug being toxic is greatly different from the therapeutic dose, and that can classify levothyroxine as a safe drug [32]. There is insufficient data related to the influence of pH on the solubility profile of levothyroxine, increasing the complexity of applying the biopharmaceutics classification system (BCS) to levothyroxine [33, 34]. The BCS is a standard used to define drug substance permeability and solubility by classifying compounds into one of four classes. Class I includes drug substances with high permeability and high solubility, class II is drug substances with high permeability and low solubility, class III is substances with low permeability and high solubility, and class IV includes those with no efficacy in humans as they exhibit both low permeability and solubility [35].

Evidence suggests that levothyroxine sodium falls under class III (high solubility and low permeability) given its low log P, affecting its bioavailability (by increasing its variability), leading the product to exhibit poor permeability [36]. Log P is a term used to describe the lipophilicity and therefore the permeability of a compound across biological membranes. It indicates the partition coefficient between two different solvents, such as an aqueous solvent, (usually a water-based buffer) and an organic solvent (usually octanol). Other researchers have indicated that levothyroxine comes under Class I (high solubility and high permeability) based on the aqueous solubility data of levothyroxine sodium where product solubility was shown to be 150 μg/mL at 3.51 log P. Compounds belonging to BCS class I should not experience issues in formulation affecting product bioavailability but, unexpectedly, various studies have demonstrated that changes in the levothyroxine formulating process can influence its bioavailability [37].

Regarding the dissolution profile of levothyroxine, a dissolution limit for bioavailability has been shown, meaning that variable dissolution and incomplete bioavailability are characteristics of the compound which lead to bioequivalence disorders (a term used to determine the therapeutic and clinical effects of medicinal products). Also, levothyroxine exhibits low intrinsic dissolution due to the ability of its particles to affect the solution pH. Therefore, levothyroxine absorption is typically limited as its bioavailability is low and variable, negatively influencing the distribution profile. Levothyroxine thus exhibits poor pharmacokinetic properties due to the reduced location and absorption of levothyroxine sodium at the site of action [24].

Alterations in the levothyroxine manufacturing process can have an impact on stability and other physicochemical properties such as dissolution. Regarding API chemical stability, levothyroxine as a sodium salt is chemically stable at 25°C and 60% relative humidity (RH) for up to 4 years [38]. The United States Pharmacopeia (USP) assay of a levothyroxine sodium tablet formulation, indicate a sharp loss in stability and potency when stored at a temperature of 40°C and 75% RH over a 3-month period. This formulation included microcrystalline cellulose, starch, and anhydrous lactose, and was not licensed for market use due to the stability and potency loss exhibited.

USP assay data has led to the approval of a levothyroxine sodium tablet formulation following storage at a temperature of 40°C and 75% RH over a 3-month period. This formulation included ingredients such as mannitol and dibasic calcium phosphate. The 6-month stability of levothyroxine sodium in tablet form was shown to be improved following the addition of sodium bicarbonate, sodium carbonate, or magnesium oxide as pH modifiers. Levothyroxine sodium tablets typically display low stability [39], with its degradation issues well-documented and connected to chemical stability and thus therapeutic efficacy.

The drug has been on the Medicines and Healthcare Product Regulatory Agency (MHRA) yellow card since 2009 due to its apparent poor formulation stability. The MHRA yellow card scheme is a procedure for recording and evaluating adverse drug reactions and physicochemical properties of a given drug. Over the last years, the scheme has recorded a high loss in efficacy for levothyroxine sodium tablets, with 87

negative reports on levothyroxine chemical stability and its effect on decreasing effectiveness on record. The precise mechanism by which the chemical stability of the compound is reduced remains unclear [24]. Previous studies have attributed the issue to the interchangeability between the liquid and tablet form [40], which interchangeability defined as a pharmacokinetic standard used to explain the suspected biological and clinical equivalence of two pharmaceutical products. In 2012, the MHRA suspended levothyroxine 100 µg tablets from a UK based manufacturer due to the variation of interchangeability between the product and the other levothyroxine 100 µg tablets [41]. Moreover, multiple reports are available that relate the chemical stability issues to specific manufacturers' method of producing levothyroxine sodium products [24]. This compound is vital for various therapies and thus of significant strategic pharmaceutical value; the verification of product integrity and quality is thus of critical importance.

Previous researches on the chemical stability of levothyroxine were reported that, levothyroxine usually with long storage under temperature condition leads to obtain degradation products which some of these degradation compounds are reported to be having some biological activity. It will be discussed later that, levothyroxine under accelerated stability studies showed previously eight degradation compounds and in another article indicated some other breakdown compounds for the levothyroxine degradation products.

The rate of degradation in some studies on levothyroxine were showed that, within six month accelerated stability studies at 40C, levothyroxine was degraded into various levothyroxine degradation products after altering its chemical structure.

In addition to the MHRA, the British Pharmacopeia Commission (BPC) reduced the range of the potency for levothyroxine sodium from 90-110% to 95-105% for reasons of minimizing the variation of stability between different levothyroxine products. Potency is defined as the amount of compound required to produce an effect of a given intensity. High potency products thus need a low drug concentration to give the desired therapeutic effect with fewer side effects. The potency of levothyroxine has been shown to typically diminish from the acceptable range to 74.7% [24], a reduction indicating that levothyroxine has been converted from a stable form into a degraded

and unstable form, affects what the patient receives a correct therapeutic dose. Previous studies have supported the observation that levothyroxine stability is highly sensitive to factors such as light, temperature, moisture, pH, and oxygen [42].

However, exposing levothyroxine to a temperature of 40°C for six months leads to decomposition and yields eight levothyroxine degradation products that will discuss later in chapter 2 [42], despite the MHRA indicating that levothyroxine is chemically stable at 40°C (but not 50°C) for six months [24]. As mentioned previously, levothyroxine is based on a crystalline structure so exposure to temperatures above 40°C may cause the crystalline structure to convert to an amorphous structure which is more susceptible to attack by active oxygen. Regarding pH, some studies have indicated that increased pH leads to increased degradation of levothyroxine but according to the FDA (among others), the chemical stability of levothyroxine decreases with increasing pH [42, 43]. A stability issue related to levothyroxine formulation undoubtedly exists, but the main reason for this diminished stability is not clear. A lack of both potency and stability has raised concerns among physicians regarding therapeutic substitutions and the risk of failure to deliver the correct dose to patients. Manufacturers have also expressed concerns over the lack of stability which influences levothyroxine content of uniformity and solubility (and thus sub-potency) before the product expiration date has been reached [44, 45].

1.4.2 Pharmaceutical dosage form formulations

There are seven different routes for drug administration: oral, such as tablets, FDOF, capsules, powders, emulsions, gels, and suspensions; parenteral, such as injections and implants; topical, including lotions, ointments and creams; rectal and vaginal, such as suppositories, creams, and ointments; the eye route, which includes solutions, ointments and creams, the nasal route of administration, such as inhalation (the most common), sprays, aerosols, and gases; and, finally, the ear route of administration which encompasses ointments, creams, solutions and suspensions. The oral dosage form accounts for most administration routes, at about 77%, followed by parenteral at 13%, then topical, rectal and vaginal at around 3%. After that, eye preparations account for 2% and, finally, 1% for both nasal and ear preparations. The design of the

dosage form thus depends on three important factors, which are physicochemical aspects, therapeutic criteria (clinical evidence and patient compliance), and biopharmaceutical considerations including drug absorption and route of administration.

Pharmaceutical dosage forms mention to the physical display of a drug as a gas, liquid or solid that can be used to deliver the drug into the target organ in the body. The pharmaceutical dosage forms can be divided according to their route of drug delivery while the oral dosage form accounts for the most delivery system, at about 77% such as tablets, capsules, powders, granules and oral fast dissolving films. The design of the pharmaceutical dosage form depends on three important factors, which are physiochemical aspects, therapeutic criteria (clinical evidence and patient compliance), and biopharmaceutical considerations including drug absorption and the route of administration.

Drug delivery system has a different kind of aspects as it can be a syringe, infusion pump or nebulizer which holds the drug to deliver it into the target site of the action. Besides, drug delivery can refer to a design characteristic of the pharmaceutical dosage form that influences the delivery of the drug such as capsule coating. Also, drug delivery system indicates relates to the used dosage form that included to deliver the drug into the human body. There are several aspects that need to be taken into account when selecting the drug delivery system including the nature of the active pharmaceutical ingredient (API) that need to be delivered. Also, the amount of the API is necessary to be studied before choosing the type of the delivery system. The rate of drug administration, the site of drug action is also important to select the appropriate drug delivery system.

However, the production of solid oral dosage from (OSD) formulations is strongly impacted by the physical and chemical properties of the active ingredients and excipients that form the dosage form. The factors that influence the phenomenon of the (OSD) includes the powder flow properties, the content of uniformity, the pH of the final product, rigidity and mechanical properties. Also, for each dosage form, it has physical and chemical properties that varied form the other pharmaceutical dosage forms. [46,47].

However, the solid oral dosage form is the leading drug delivery that highlighted in this research to study the low dose of the sodium salt of levothyroxine. Pharmaceutical oral dosage forms are the most used dosage form in the drug market due to their safety and compliancy for the patients. The most common oral dosage forms include tablets and capsules but the issues occurred in the bioavailability, the onset of action and stability leads more pharmaceutical oral dosage forms to be developed such as fast-dissolving oral films (FDOF). Levothyroxine is an example for the issues occurring with tablet dosage forms. Levothyroxine has been produced in a small dose tablet form at 25 µg, 50 µg and 100 µg and one levothyroxine product (100 µg) has been removed from the market due to an inability to meet the standard requirements of chemical stability and content of uniformity.

1.5 Fast dissolving oral films formulations (FDOFs)

FDOFs can be administered to different regions of the oral cavity, with the common routes being the buccal delivery system when the film is placed between the cheek and the gum [48], and the sublingual delivery system, when the film is placed under the tongue [49]. The criteria for selecting one of these delivery systems over another oral dosage form depends on the rapid dissolving and disintegrating time which is required for some drugs in order to produce a rapid onset of action.

There are various barriers are located in the oral cavity and these physical and chemical barratries help to prevent toxicity and control the blood flow, hence affect the adsorption. Due to that, the blood flow from the oral cavity inhibit the hepatic first-pass effect, masticatory regions such as gingiva are covered with keratinizing epithelium.

In contrast, more elasticity regions such as buccal mucosa are no keratinizing. Therefore, owing to mechanical but also chemical factors (such as salivary turnover and production of mucus). It can be mentioned that, buccal mucosa is more permeable to drug molecules and more suitable to drug delivery.

Additionally, the high permeability that FDOF formulations offer is as a result of the high blood supply and a number of capillaries in the oral cavity which can elevate the content of uniformity of the released APIs into the system. Furthermore, drug bioavailability is considered an advantage of FDOF delivery systems over other oral dosage forms as long as FDOF formulations can avoid first pass hepatic metabolism

and directly enter the circulation because the FDOF can rapidly dissolve after insertion into the oral cavity [50, 51]. Thin-film drug delivery is known as fast dissolving oral films (FDOF) due to its rapid dissolve in the oral cavity. Moreover, the active ingredient from the FDOF is absorbed when placed in the buccal part or sublingual part of the mouth. The main reason for the rapid dissolving of the film strip in the oral cavity is the hydrophilic polymers that are used as an excipient in formulating the FDOF. Once the film is in contact with the saliva, it dissolves rapidly releasing the drug. Various FDOF have been developed and currently marketed such as Zuplenz (Ondansteron) at a concentration of 8 mg which was approved by FDA as chemotherapy [52]. In addition to this, various buccal drug delivery has been launched for the treatment of many conditions such as diabetes, cold, flu, anti-snoring and addiction like nicotine patches.

The FDOF drug delivery requires specific ingredients to formulate it which are hydrophilic polymers, plasticizer, saliva stimulating agents, thickness agents, sweetening agents, flavouring agents, and colouring agents. Buccal drug delivery of pharmaceutical formulations has a high content of uniformity, and thus, it is safer than than other oral drug pharmaceutical formulations [53]. Patient compliance is thus high with this type of drug administration [54].

Following modification of the tablet dosage form to produce fast dissolving tablets, the idea of formulating fast dissolving films became a viable option due to the rich blood supply within the oral cavity which produces a higher dissolving environment than that the tablet formulations are exposed to. FDOFs are used via the oral route of administration that can be used in different locations of the oral cavity; namely, the sublingual and buccal delivery systems (Figure 1.6).

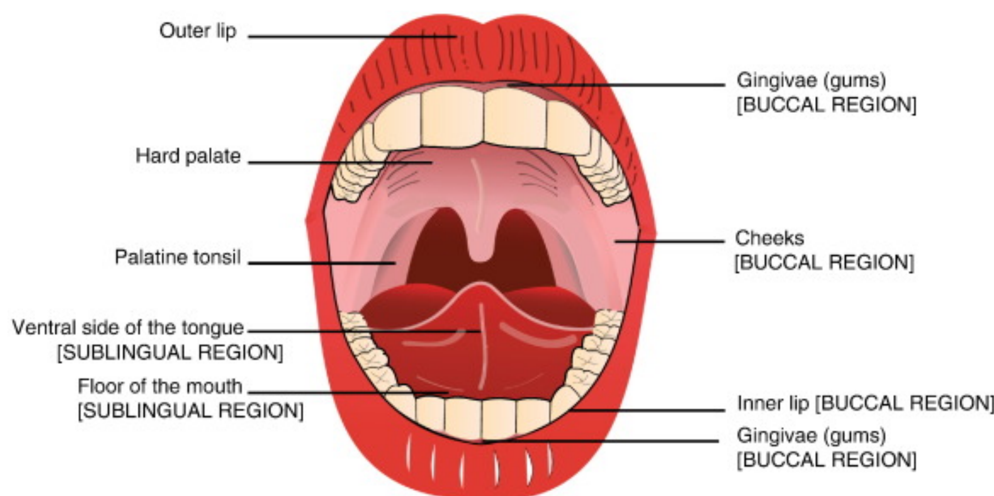


Figure 1-6: Oral cavity: the location of sublingual and buccal delivery system adapted from [50]

This administration route is characterised by a rapid disintegration and dissolution in depending on the ingredients used in the formulation [55, 56].

1.5.1 Advantages of FDOFs

- FDOFs can be formulated as thin films to allow greater flexibility and ease of handling, and thus increased convenience for the patient.
- Patient compliance is higher in this approach than with other oral dosage forms because it is easy to use as the patient need only to place it in the require part of the oral cavity. Also, FDOFs are painless.
- FDOFs are a stable dosage form which can be formulated with the high content of uniformity as they can be manufactured with a low water content and large surface area which that is missing in tablet formulations, leading to a reduction in intramolecular attraction and interaction. The dissolution profile is also enhanced, further improving the content of uniformity, increase the stability of the formulation more than other solid dosage form formulations such as tablet formulation, and reducing the dissolution and disintegrating time to provide a rapid onset of action.
- The FDOF systems ensure more accurate dosing, and effective drug delivery to the patient as each strip of the fast dissolving film will dissolve

directly along with the active pharmaceutical ingredients to the blood circulation. Therefore, the entire concentration of the drug is going to be absorbed.

- The fast onset of action is also facilitated by the generous blood supply within the oral cavity, beneficial to patients suffering from acute disease [57, 58].

However, there are some limitations of fast dissolving films such as; fast dissolving films cannot produce into dose higher than 30mg which is the maximum dose that the drug can be formulated as FDOFs. Another limitation includes difficult packaging and some variability in thickness which that can be an issue of drug content if there is a technical issue In formulation the films [4a].

1.5.2 Standard composition of FDOFs

The percentage of API and other excipients used to produce FDOFs are given in Table 1.1.

Table 1-1 Percentages of pharmaceutical ingredients in FDOF formulations [59-60]

	Excipients	Percentage
1	API	Up to 30%
2	Hydrophilic polymers	40-50%
3	Plasticiser	Up to 20%
4	Flavouring agents	Up to 10%
5	Surfactant	sufficient quantity (q.s)
6	Colours	sufficient quantity (q.s)
7	Saliva stimulating agents	Up to 6%

1.5.2.1 Active pharmaceutical ingredients (APIs)

The API is the primary active drug that is incorporated into the FDOF formulation. It can be obtained in a wide range of particle sizes and powder forms, but to achieve the optimum dissolution profile that accompanies a high content of uniformity. Conventional drugs used in FDOF technologies to release active compounds into the target organs are omeprazole, loratadine, nicotine, and salbutamol [61, 62].

1.5.2.2 Hydrophilic polymer

Oral dissolving films forming polymers produce a large chain of hydrophilic compounds. The formulation should be water soluble to allow it to dissolve in the saliva and release the drug. A wide range of polymers are available for use in formulating oral films, and it is important to select the appropriate polymer to meet formulation

requirements, as the rigidity of the film depends on the amount and type of the polymer used. Formulations should be inert in terms of toxicity and irritability, and polymers should have a high capacity for forming films in order to yield a film strip which can be classified as excellent, better, good, average, poor, and very poor which these hydrophilic polymers have achieved according to the ability of the films to form the gel that produces the films [63, 64]. More than 40% of the film formulation consists of polymers. They must, therefore, be economical regarding availability and cost. A further important consideration when choosing polymers is that they must be tasteless and colourless, as all film formulations are targeted to act in the oral cavity [65].

Hydrophilic polymers commonly used in films are either synthetic polymers such as

A. Hydroxypropylmethyl cellulose (HPMC)
B. Polyvinyl alcohol (PVA)
C. Hydroxyethyl cellulose (HEC)
D. Carboxymethyl cellulose (CMC).

On the other hand, hydrophilic polymers can be natural polymers such as:

A. Xanthan gum
B. Pullulan
C. Pectin
D. Starch gelatine [66].

1.5.2.3 Plasticiser

The elongation and flexibility properties of a good FDOF formulation are attributed to the presence of plasticisers. The main function of adding a plasticiser is to enhance polymer flow and the tensile strength of the total formulation by decreasing the glass transition temperature so that more crystal structure will be present in the formulation. Thus, the choice and percentage of plasticiser are important. As each film type uses different polymers and solvents, selection of the best plasticiser for the formulation depends on the affinity to the solvent and the existing polymer. The most common placticiser used in FDOF formulations are:

A. Glycerol

B. Polyethylene glycol 200,400,600 (PEG)
C. Propylene glycol (PG) [51].

1.5.2.4 Flavouring agent

Patient compliance is essential as it is a major consideration for the success of a given pharmaceutical product. As FDOFs are administrated in the oral cavity, it is necessary to ensure the test of the film strip is highly acceptable for the patient which that increase the formulation quality. Flavouring agents in FDOFs depend on the type of the active pharmaceutical ingredients as it is important to ensure the flavouring agent is not interfering with the drug so the flavouring agents should be pharmacologically inactive. Flavours can be extracted from different kinds of fruits, flowers and leaves. The common flavouring agents used in FDOF formulations are:

A. Menthol
B. Peppermint oil
C. Lemon oil [51].

1.5.2.5 Surfactants

Surfactants are necessary for FDOFs to ensure the drug can be dissolved within seconds. Surfactants are mainly used to enhance the solubility, dispersing and wetting the FDOFs and hence, decrease the required time to release the APIs from the films [62]. The most common surfactants used in FDOFs formulations are:

A. Tweens
B. Sodium Lauryl Sulfate (SLS)
C. Spans [67].

1.5.2.6 Saliva stimulating agents

Saliva stimulating agents may be added to facilitate a fast dissolution rate to provide rapid film disintegration. This property can be achieved by raising the rate of saliva production. Acids highly stimulate saliva and increase its rate of release; thus, acids can be used for this purpose [67]. The most common saliva stimulating agents are:

A. Ascorbic acid
B. Lactic acid
C. Citric acid [59].

1.5.2.7 Colouring agents

Colouring agents are used in FDOFs to improve patient compliance and the appearance of the product. Pigments used should be inert (i.e., should not exert any pharmacological action) [68]. The most common colouring agents used in FDOFs formulations are:

A. Sunset yellow
B. Titanium dioxide

1.5.3 Techniques used in the preparation of FDOF formulations

Four general methods are available for preparation of FDOF formulations, the solvent casting method, hot melt extrusion, solid dispersion extrusion, and the semisolid casting method. The most commonly-used of these is the solvent casting method, an easy and accurate formulation technique which yields uniform and clear gels for preparing the required films. The procedure generally involves mixing all selected ingredients together and dissolving the hydrophilic polymer in the appropriate solvent followed by homogenisation of the total mass by stirring them to produce a gel which is cast into uniform size Petri-dishes and dried at a suitable temperature to form the film formulations. The solvent casting method is suitable for heat sensitive ingredients as no temperature is applied [64]. In contrast, the hot melt extrusion method relies on producing the film using a specific temperature. This method does not require the use

of a solvent, and all ingredients (along with the API) are homogenized before the granule mass obtained is passed into an extruder at various temperatures to form the films [65]. The third method, solid dispersion extrusion, is suitable for use when the active ingredient is required to disperse in a proper solution. In this method, both solvent and temperature are needed. The temperature should not be greater than 70°C to prevent aggregation of the active ingredients and loss of uniformity. The final method, semisolid casting, is similar to the solvent casting method except that it is used when the active ingredient comprises an acid. In this method, it is necessary to prepare three separate mixtures: all excipients in mix 1, the polymer in an appropriate solvent to allow full dissolution in mix 2, and the acidic active ingredient dissolved in a solvent as mix 3 [69].

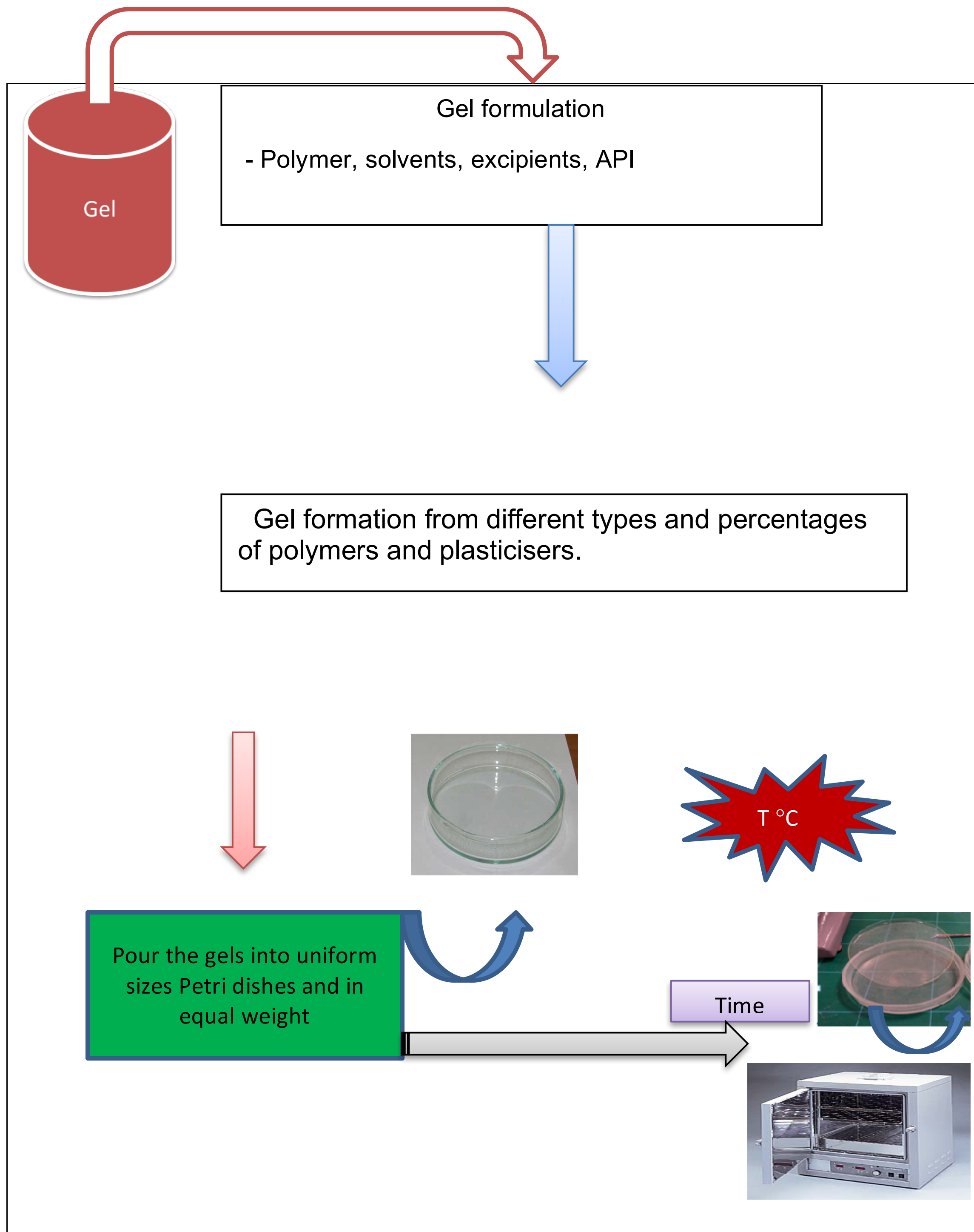


Figure 1-7: diagram showing the FDOF preparation technique (solvent casting method)

1.5.4 Evaluation of FDOFs

1.5.4.1 Weight variation

It is necessary to ensure that the FDOF formulation is uniform in weight and can deliver an accurate drug dose. This can be achieved by cutting the film into similar sized

pieces and weighing it in analytical balances to calculate the average weight. The pharmacopeia limit for the weight variation is +/-7.5% [68].

1.5.4.2 Film thickness

The delivery of an accurate active pharmaceutical dose is related to the uniformity of the film thickness. The best way to determine this is using a digital calibrator at five different regions of the formulation (four corners and the centre). The acceptable range of film thickness is 5-200 μm [70].

1.5.4.3 Folding endurance

Folding endurance is a manual test that gives an indication of film brittleness and can indicate the present of humidity as it causes folding endurance to increase. Folding endurance can be assessed by folding the strip in the same place multiple times and counting the number of folding steps before breakage appears [71].

1.5.4.4 Surface pH

FDOFs are administrated by placement in the oral cavity. To avoid oral irritation, pH of the film should not be too acidic or alkaline, as the oral cavity pH is between 6.2 and 6.8; the film should, therefore, have a pH in this range [72]. The pH testing can be performed by immersing the film quickly into distilled water. After drying the film, pH electrodes can be attached to the film's surface to record the pH reading [73].

1.5.4.5 Disintegration test

The time taken for films to disintegrate when they contact solvents such as saliva or water is called the disintegration test. Results should be rapid, in seconds, as films should be formulated to exhibit a fast onset of action. Disintegration times should range between 5-30 seconds, and the test can be performed using disintegration apparatus using an appropriate solvent which records the starting point and the point at which the film is entirely disintegrated [74].

1.5.4.6 Percentage (%) of moisture loss

This measurement determines the loss of moisture from the film following storage under conditions of relative humidity and at room temperature, using anhydrous calcium chloride. The loss of moisture can be determined by the following equation 1:

% of moisture loss = (initial weight – final weight) / initial weight X 100 [75].

1.5.4.7 In vitro dissolution test

Films formulation should dissolve very rapidly in order to be classified as fast dissolving films. This process should produce an accurate and efficient active pharmaceutical drug dose. To estimate this, a standard basket or puddle dissolution apparatus can be used to indicate the amount of active drug that releases into the medium, typically water or saliva. Different sampling time points are required in order to study drug release over the time to ensure that it is sufficiently rapid [76].

1.5.4.8 FDOFs content of uniformity

As with any pharmaceutical formulations, the content of uniformity is the principle test performed to determine whether the formulation yields a uniform dosage form and an accurate dose. The content of uniformity is assessed to ensure that the API is present in equal proportions across the film, at a percentage range between 85-115% of the target dose [77].

1.5.6 Analytical methods used to test levothyroxine

1.5.6.1 High-Performance Liquid Chromatography (HPLC)

Previous research on levothyroxine content of uniformity and the drug degradation products used HPLC as the method for providing quantitative and qualitative data. HPLC is a sensitive and accurate method, suitable for use in all dosage forms. From HPLC, the dissolution profile, content of uniformity, assay, stability study, and pharmacokinetic profiles can be estimated [78]. Six types of HPLC are available, differentiated from each other by the molecules that they are capable of separating concerning particle size, ions, molecular polarity, and mobile and stationary phase

polarities. These six HPLC types are chiral, ion exchange, ion affinity, reversed phase, normal phase, and size exclusion HPLC [79].

HPLC method comparing to another chromatographic methods have some advantages and limitations. For instance, HPLC appeared to be an accurate method that recently used for separation compounds and degradation products alon with the fact that, HPLC analytical technique is widely used as quantitative and qualitative method for protiens, amino acids and other compounds. Regarding levothyroxine, HPLC was used to determine several degradation products of levothyroxine and it was used as well to assess the maximum assay of the drug within the tablet dosage form that are now in the market.

Furthermore, in comparing HPLC to another technique such as TLC, it can be said that, HPLC use pumb instead of gravity which that provides quicker method with high sensitivity.

One of the main disadvantages of HPLC analytical method is that, HPLC is a cost effective technique. HPLC requires large quantities of expensive organics. Techniques such as solid phase extraction and capillary electrophoresis can be cheaper and even quicker, especially for analysis under good manufacturing practice. Although it is relatively easy to use existing HPLC methods, it can be complex to troubleshoot problems or to develop new methods. This is largely because of the array of different modules, columns and mobile phases.

Two chromatographic conditions can be implemented using HPLC. The first of which is isocratic and is used in the analysis of compounds with a single peak; this method is not suitable for degradation compounds as the width of the peak increases with retention time and is therefore not constant. The isocratic method is ideal for determining the content of uniformity of the product or dissolution profile but not in samples under stress conditions or in accelerated or long-term stability studies. In contrast, the second set of conditions is gradient and is suitable for products susceptible to degradation or those likely to produce complex peaks. Peak width remains constant, even with increased retention time, so this method is critical for studies with longer retention times. Samples are partitioned using gradient reversed phase HPLC according to the polarity of the eluent (mobile phase) along with the

sample's affinity for the stationary phase which is non-polar (C_{18}); hence, depending on the sample's chemical properties such as pK_a, pH, and polarity, the retention time will differ and separation time and signal shape (peak) will subsequently be different for each molecule [80].

To date, however, no studies have incorporated quantitative-qualitative assessments of levothyroxine with investigating the factors contributing to instability and production of degradation products; neither have attempts been made to resolve these issues in parallel by modifying the formulation (either in a solid or liquid state) and retesting it to determine if the chemical stability profile improved.

1.5.6.2 Liquid Chromatography-Mass Spectrometry (LC-MS)

LC-MS is an analytical procedure that incorporates two sensitive analytical methods, liquid chromatography, and mass spectrometry; this technique can identify the mass of a compound and its physical separation and is thus highly accurate and robust. It demands on separation processes and impurities identifications. Particles mass can be detected using mass spectrometry as it quantifies the ratio of mass to charge (*m/z*) of particles by using an ion source in the instrument to produce charged molecules through their ionization. LC-MS is widely used to determine the chemical impurities of compounds and degradation products, depending on the peaks generated by the separated ions. The resulting separation is the result of different ratios of mass to charge of ions, represented as signals that a specific detector in the instrument recognizes and converts into mass spectra. The key elements common to all mass spectrometry are the sample separation method, ion source, mass analyser and detector. The sample separation method (e.g., HPLC) partitions the samples depending on the proportion and retention time of the sample between the eluent (mobile phase) and the stationary phase (column) which is then connected to the MS. The second element is the ion source which yields ions in the gas phase by exposing the separated LC samples to atmospheric pressure. LC-MS flow rates and mobile phases can vary. Thus various LC-MS types can be distinguished per different separation in the LC step. The third element is the mass analyser, responsible for distinguishing between the ions depending on mass, following exposure to

electromagnetic fields. Finally, the detector registers the abundance data of the existing ions. In summary, this method can be considered a quantitative and qualitative approach [81].

1.6 Hypothesis of the research

Previous studies on APIs that are susceptible to the same chemical limitations as levothyroxine showed that oral dissolving film delivery systems have a proven ability to improve chemical stability and content of uniformity profiles of several pharmaceutical formulations. However, a full knowledge is required of the chemical stability profile of levothyroxine, its chemical structure changes, and the challenges related to the dissolution properties of the active ingredients' properties. Novel formulations also require validation of their pharmacology, toxicology, and physicochemical characteristics.

1.7 Aims of the research

The hypothesis is tested in the current research following specific aims and objectives:

a) To investigate the influence of high temperature on levothyroxine chemical stability and chemical structure;
b) To develop a novel FDOF formulation of levothyroxine, thus addressing its chemical stability issues, improving its content uniformity within the formulation, and enhancing its biopharmaceutical properties by improving its solubility and permeability profiles; and
c) To ensure that the novel FDOF formulations are safe by evaluating their toxicity using oral mucosal cell line cultures.

1.8 Originality of the research

Levothyroxine differs from other drugs in that the origin of its chemical stability issues, which are known to affect its efficacy, are largely unknown. The originality of this research lies in the design of novel analytical methods for investigating the chemical

factors influencing levothyroxine chemical stability and the development of a novel technique for producing levothyroxine products. The novel technologies will be evaluated under stress conditions (at 25°C, 40°C and 70°C) to investigate the influence of chemical factors on levothyroxine chemical stability. The new FDOF formulations should ensure a final product with high quality regarding its solubility, chemical stability and its content of uniformity which overcomes the foregoing limitations of the levothyroxine product. If successful, hypothyroidism patients may ultimately receive the appropriate therapeutic dose of levothyroxine which does not have any formulation stability issues. The desired outcome and goal thus lies in improving the quality of life of hypothyroidism patients globally.

1.9 Contribution to science

The outcomes of this project will benefit pharmaceutical companies to produce stable levothyroxine products. In addition, the novel technology used in the development of new levothyroxine FDOF formulations may be utilised as a starting point for the development of formulations of other compounds with similar benefits. Therefore, this research has the possibility to have marked impact and a valuable contribution to research in pharmaceutical sciences.

2. Chemical stability of solid state levothyroxine and its analytical validation

2.1 Introduction

Temperature is one of the main factors that affect the chemical stability of a compound, especially in compounds that are highly temperature-sensitive (such as levothyroxine) [82]. As mentioned in Chapter 1, previous studies on levothyroxine chemical stability using HPLC method has reported eight degradation products of levothyroxine when stored at 40°C for six months. Therefore, a required dose of levothyroxine might not have been correctly delivered to the patients due to the lack of levothyroxine stability [83].

Many studies have related the poor chemical stability of levothyroxine to exposure or storage at high temperature [84,85]. The main reason for levothyroxine decreased stability remains unknown, but chemical and environmental factors such as temperature undoubtedly affect levothyroxine chemical stability [86]. However, previous research on the chemical stability of levothyroxine has investigated the process of levothyroxine degradation and it was found that, chemically, de-iodination has been reported as the main mechanism of action for levothyroxine degradation [87].

In order to produce an active compound of high quality it is essential to determine its solid state stability and hence a detailed stability study for this compound should be considered. It is evident from previous literature that stability studies should be widely applied to products such as levothyroxine as the regulatory authorities have reported many consequences related to levothyroxine bioequivalence and levothyroxine stability [87]. Therefore, such studies on the drug stability may provide information on the appropriate expiration date of the product, the content uniformity, precise dose determination, and any impurities and degradation in the product that might afford a toxic effect and produce biologically active compounds. Three types of stability studies

are commonly used to determine the stability of drug substance and a drug product: long term, intermediate term, and accelerated, as described in Table 2.1. In accelerated stability study, it is expected to observe significant alterations on the tested samples ant therefore, it is recommended to have three time points of study, for example, at t=0, t= 3 months and t= 6 months. In terms of the intermediate stability condition, it is following the results have obtained from the accelerated stability study and It is recommended to test the samples under four different time points. However, the long terms stability study should have frequent time points to test the samples and it is recommended to test the samples very three months [88].

Table 2-1: General stability studies used to evaluate pharmaceutical products [88]

Stability Study	Storage condition	duration of the storage
Accelerated	40°C and above at 75% RH	6 months
Intermediate term	30°C at 65% RH	6 months
Long term	25°C at 60% RH 30°C at 65% RH	12 months

The aim of the studies performed in this chapter is to provide credible data on levothyroxine chemical stability by inducing stress conditions on levothyroxine storage such as expose the drug into different temperatures for six months (accelerated stability study). Hence, this can provide a rigid platform to understand the mechanism of action that altered the chemical structure of levothyroxine. In addition, it is essential to design and develop a novel levothyroxine formulation that can overcome the chemical stability issues related to this drug.

2.2 methodology

2.2.1 Levothyroxine stability studies

An accelerated stability study was designed to examine chemical degradation and changes in levothyroxine structure. This study was conducted by analysing levothyroxine samples in solid state at t=0 and in stress conditions. The process included an accelerated stability study of levothyroxine which was performed at different storage conditions: 25°C, 40°C and 70°C for six months and analysed after t= 30 days, t= 60 days, t= 90 days, and t=180 days' incubation using HPLC, LC-MS, and FTIR. To have enough samples for any repeating of the experiments, levothyroxine powder has been weighed (100mg) placed in a ml beaker and stored at 25°C, 40°C, and 70°C. At each time point, a small aliquot was removed from the beaker for the required analytical test. For HPLC and LC-MS analysis, samples were dissolved in a suitable solvent and analysed and for FTIR measurements samples were analysed in a solid state. The stability studies were studied at same intraday time points to make sure the samples are studied at low intraday variability.

2.2.1.1 HPLC analytical method development (gradient method) for levothyroxine analysis

This method was developed to identify the levothyroxine peak and quantify levothyroxine under normal and stressed conditions. This approach was adapted from a previous study that used an HPLC gradient method to evaluate levothyroxine degradation products [89]. But the HPLC method in this research was modified suitably for the analysis. The solvent, mobile phase, sample preparation, detector (wavelength), column conditions, and standard concentration were similar to the published method (table 2.2). The mobile phase consisted of A: 1000 ml of distilled water with 1 ml trifluoroacetic acid and B: 1000 ml acetonitrile (ACN) with 1 ml trifluoroacetic acid. The mobile phase was manually mixed for 2 min and then degassed by sonication for 40 minutes. The gradient consisted as follows; (0-25 minutes) 20-80% B, (25-30 minutes) mobile phase B remained at 80% B and then decreased to 20% at (30-35 minutes). Then it remained 20% at (35-40 minutes).

- Preparation of the calibration standard

A stock solution of levothyroxine (20 µg/ml) was prepared by weighing 5 mg of levothyroxine into a 250-ml of the solvent, which according to the US monograph, is 0.01 M of methanolic sodium hydroxide. The stock solution was diluted suitably to prepare levothyroxine calibration standards from 1 µg/ml to 20 µg /ml.

- Preparation of the quality control standards

It is imperative to validate the analytical method by using suitable quality control (QC) samples. It is ideal if the QC standards were within the calibration range. Therefore, 8, 10, and 12 µg/ml concentrations were used for the analysis. All samples were prepared from a separate levothyroxine stock solution using the same method of preparation.

- Validation

According to the International Conference on Harmonisation (ICH), the required validation parameters successfully achieved were linearity, accuracy, precision, robustness, limit of detection (LOD) and limit of quantification (LOQ). Linearity in response was evaluated on standards with concentrations between 1-20 µg. The relative standard deviation of the slope relative standard deviation (%RSD) was also calculated.

Accuracy was measured by testing different control samples with the drug standards to verify a lack of interference. Repeatability of injecting the standards of levothyroxine samples at a different concentration was achieved to show the method was precise. Robustness was also tested to demonstrate the system suitability and was achieved by testing two different levothyroxine standards (STD A and STD B) from different stock solutions of levothyroxine. LOD and LOQ were mathematically calculated to determine the lowest concentration of drug that this method can detect and quantify. These parameters were calculated as per ICH guidelines using the following equation 1.

$$LOD = \frac{3\ X\ SD\ of\ the\ lowest\ concentration}{slope}$$

$$LOQ = \frac{10\ X\ SD\ of\ the\ lowest\ concentration}{slope}$$

Table 2-2: Developed HPLC gradient method parameters

HPLC method	Gradient method
Column	5 µm Fortis C_{18} (150 x 4.6 mm)
Detector	Parkin Elmer Series 200 UV/VIS detector (223 nm)
HPLC injector	Water 717 plus autosampler
Mobile phase	0.1% TFA/ACN/(gradient)%
Solvent	0.01 M Methanolic sodium hydroxide
Standard	1-20 µg of levothyroxine from stability and standard studies
Flow rate	0.8 ml/min
Run-time	40 min

2.2.1.2 Liquid chromatography-mass spectrometry LC-MS

Samples were dissolved in 5% methanol in water at a concentration of 20 mg/ml and stored at 4°C until analysis by LC-MS. Samples (20 µL) were injected onto a C_{18} reverse phase column (C_{18}, 100 x 2.1 mm, 5 µ particle size) equipped with a guard column using a gradient at a flow rate of 0.35 mL/min (Binary Pump, Agilent 1200). The gradient consisted of distilled water, 0.1% formic acid (eluent A) and acetonitrile, 0.1% formic acid (eluent B), as follows; (0-4 minutes) 2% B, (4-20 minutes) 2-70% B, (20-22 minutes) 70-100% B, (22-26 minutes) 100% B, 26-27 minutes) 100-2% B, and equilibrated at 2% B for 8 minutes. Data was recorded on an ion trap mass

spectrometer (Esquire HCT Plus, Bruker Daltonics) in the positive mode using ESI. Full scale (150 – 800 m/z) data recorded on an ion trap mass spectrometer. Threshold: 50%) with exclusion after 1 spectrum (release after 2 min.). Some analysis parameters were as follows; nebulizer gas at 40 psi, dry gas at 8 L/min, dry temp. 300°C, Cap. voltage -4000 V, Skimmer at 40 V, Cap. exit at 150 V. Maximum acquisition time was set to 200 ms with an ICC target of 200, 000. Data was viewed and analysed using DataAnalysis (V 3.3, Build 149) Software (Bruker Daltonics).

2.2.1.3 Fourier transform infrared spectroscopy (FTIR)

The study was carried out using a Spectrum 65 FT-IR spectrometer to determine the differences in chemical structure and to characterise functional groups in all fresh and stability samples of levothyroxine. The abscissa unit used in this experiment was wavelength while the ordinate unit was %T. The wavelength scan started from 4000 cm^{-1} to 650 cm^{-1} with a resolution of 4 $cm^{-1.}$

A small amount of levothyroxine samples was analysed by placing it on the cleaned diamond sample holder. The swing arm was then screwed down on to the levothyroxine sample to view the spectra. The %transmission was between 30-40%. The drug spectra were obtained by selecting the ‘scan the sample’ and the ‘data tune up' was performed to yield the optimum baseline. The method was successfully repeated on all levothyroxine solid samples (fresh and stability samples).

2.3 Results

2.3.1 Physical appearance of levothyroxine solid powder

An accelerated stability study of levothyroxine at 25°C, 40°C and 70°C for six months was conducted. It was observed that there was a change in the colour of the levothyroxine solid powder stored at 40°C and 70°C. On the other hand, no colour change occurred on levothyroxine powder when it was stored at room temperature for six months.

Levothyroxine powder initially was white and crystalline without applying a thermal stress condition. Figure 2.1 shows the change in levothyroxine powder physical appearance. The white crystalline powder of levothyroxine has been altered to become a brown coloured powder when the levothyroxine sample was exposed to a high temperature of 70°C for 30 days. However; a dark brown colour was formed when the levothyroxine sample was incubated at high temperature of 70°C for 180 days.

Furthermore, comparing the physical appearance of levothyroxine solid when exposed to 40°C against levothyroxine sample which was exposed to 70°C for six months (Figure 2.2), it was observed that the colour of levothyroxine powder appeared yellowish white instead of white crystalline powder at 40°C and dark brown at 70°C when stored for six months. The stability studies were studied at same intraday time points to make sure the samples are studied at low intraday variability.

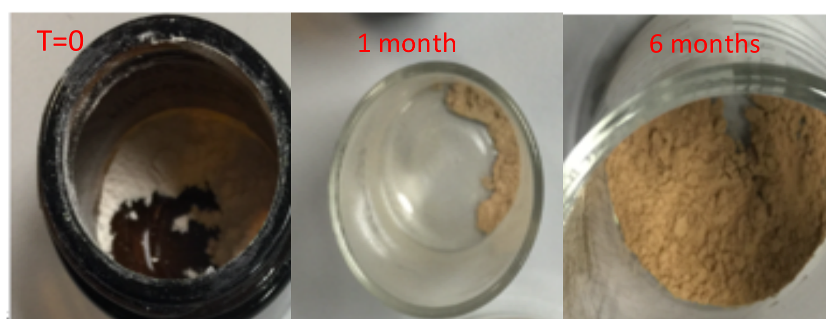


Figure 2-1: Photographs illustrating the colour change of three samples of levothyroxine. L to R: Levothyroxine t=0, levothyroxine following stress condition of t=30 days at 70°C, levothyroxine following stress state of t=180 days at 70°C

Figure 2-2: The difference between levothyroxine stored at 40°C (Left), and levothyroxine stored at 70°C after t=180 days (Right)

2.3.2 Development of gradient (HPLC) method

HPLC gradient method was developed to evaluate the levothyroxine chemical stability during the accelerated stability study. Figure 2.3. Shows the chromatogram obtained for the gradient method as it described in the methods section of this chapter. The peak for levothyroxine at 20 µg/mL eluted at 15.65 min (± 20 sec) and the run time was 40 min. Also, the solvent front for the sample representing 0.01 M methanolic sodium hydroxide was observed at 1.6 min. It can be noticed from figure 2.3 that, there is a slope on the baseline and that appeared on all the HPLC baseline in this study. This is possible due to some software issues on the HPLC machine

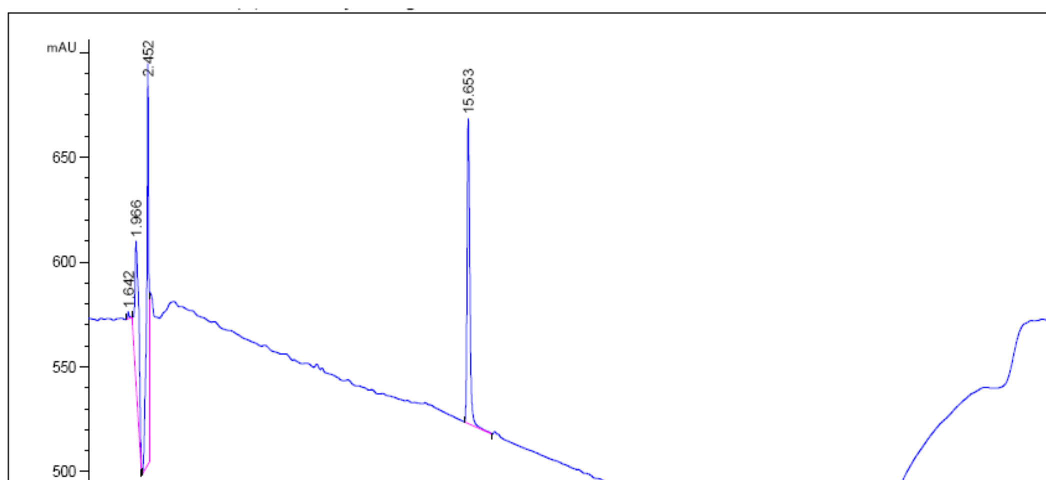


Figure 2-3: Levothyroxine, 20 µg/ml peak, obtained using a gradient HPLC method.

Table 2.3 shows the calculations of three different standards for levothyroxine (10 µg/ml – 20 µg/mL) analysed using the gradient HPLC method. Levothyroxine concentrations were calculated along with the percentage recovery for all standards.

The percentage recovery of levothyroxine samples from its standards was obtained by the following equation 2.

% Recovery = {(obtained levothyroxine concentration / levothyroxine standards concentration) x 100}.

Table 2-3: **the average and concentration showing for the developed HPLC method.**

Conc(µg/mL)	% Recovery	SD
20	100.6	0.8
18	99.6	1.5
16	99.8	1.8
14	100	0.9
12	99.1	3.8
10	97.4	4.4

In this research, a novel HPLC method was developed to investigate the chemical stability of levothyroxine. Repeatability is necessary on developing an analytical method such as HPLC, to indicate the selectivity of the method. Therefore, Levothyroxine has been injected for more than ten times at the same concentration, solvent, and run conditions to shown that the peak represented is levothyroxine; all runs verified that levothyroxine was eluted in this method after 15.56 min (+/- 20 seconds). After demonstrating that levothyroxine could be correctly eluted using this method, a calibration curve and standards samples were prepared and calculated to test for analytical method validation. The standards sequence had a maximum concentration of 10 µg/ml and minimum concentration of 1 µg/ml.

Linearity was established for the analytical calibration range and least 10 calibration standards with blanks were created, and the average of three samples of each

concentration was calculated. Three samples injections were performed at each concentration point, and the peak areas were plotted against concentration to obtain a linear correlation (R2) = 0.9999. This value suggested a positive correlation between concentration and the peak area following absorption. Intercept and slope of the linear regression were included to allow the unknown levothyroxine sample concentrations and their content of uniformity to be calculated.

The relative standard deviation of the slope (%RSD) was also calculated by taking the average slope of n=3 from three different stock solutions and dividing it by the mean standard deviation. %RSD of the calibration curve was 1.20%, less than the 5% threshold required to show that the standard deviation between levothyroxine samples was acceptable.

Accuracy was determined by running three quality control samples of different levothyroxine concentrations in triplicate, 8 μg/ml, 10 μg/ml, and 12 μg/ml as recommended by ICH guidelines. The quality control samples were compared to the standard concentrations of 8 μg/ml, 10 μg/ml, and 12 μg/ml to determine the percentage recovery of levothyroxine in the quality control samples. Accuracy was 97.6% for the 8 μg/ml concentration, 97.3% for 10 μg/ml, and 97.9% for 12 μg/ml. These data indicate that the method was accurate and that the quality control samples of levothyroxine, when compared to the standard samples, were in the acceptable range of accuracy.

Robustness was tested to demonstrate the system's suitability by changing the pH of the mobile phase by +0.2 over the actual pH of the mobile phase. The system robustness test was performed by adding one drop of TFA and adjusting the solution with water and a pH meter to obtain a +0.2 difference from the original pH of 3 to test the system's suitability. Therefore, the mobile phase pH was 3.2. This adjustment did not affect the elution of levothyroxine.

Limit of detection (LOD) and limit of quantification (LOQ) were calculated to measure the lowest concentration that this advanced method can detect and quantify [93]. LOD for this method was 0.1 μg/ml while the LOQ was 0.35 μg/ml.

2.3.3 Chemical stability study of levothyroxine determined using (HPLC)

Samples of levothyroxine from the accelerated stability study stored at three different storage conditions (at room temperature, 40°C, 70 °C) were tested by HPLC as described under the methods section of this research (levothyroxine stability studies). The samples were also observed for any physical changes in appearance. The study of levothyroxine chemical stability showed the differences in peak areas of levothyroxine 10 µg/ml following 30 days, 90 days and 180 days under stress conditions and the concentration of the levothyroxine samples was calculated using the standard curve of levothyroxine.

2.3.3.1 Separation and identification of levothyroxine and its degradation products using the gradient (HPLC) method

Figures 2.4 to 2.11 show the chromatograms of the eight known degradation products of levothyroxine [8]. The eight degradation products of levothyroxine are: tyrosine which has a retention time of 1.734 min (figure 2.4), di-iodo tyrosine with 6.689 min of elution time (figure 2.5), triiodo tyrosine with 8.549 min of elution time (figure 2.6). Figure 2.7 shows the retention time of thyronine, which is one of the levothyroxine degradation products, as 8.670 min. The remaining levothyroxine degradation products that were previously identified are: diiodo thyronine with a retention time of 11.852 min (figure 2.8), tri-iodothyronine which eluted at 13.996 min (figure 2.9). Tri-iodo-thyroacetic acid another degradation product of levothyroxine had a retention time of 17.285 min (figure 2.10). In addition to the previous levothyroxine degradation products, tetra-iodo-thyroacetic acid was analysed, which had a retention time of 19.221 min (figure 2.211). All the eight degradation products of levothyroxine were separated and identified in a single HPLC run using the method developed (figure 2.12).

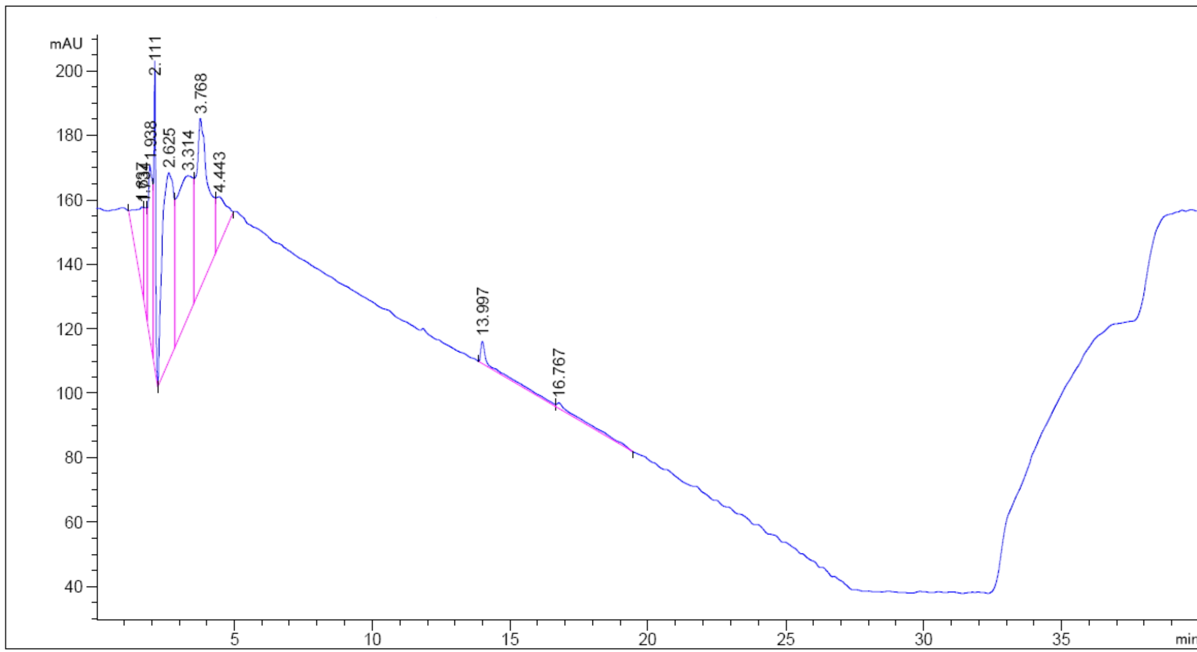


Figure 2-4: Tyrosine 20 µg/ml sample. The retention time was 1.734 min. Some impurity peaks appeared at 13.997 min and 16.767 min

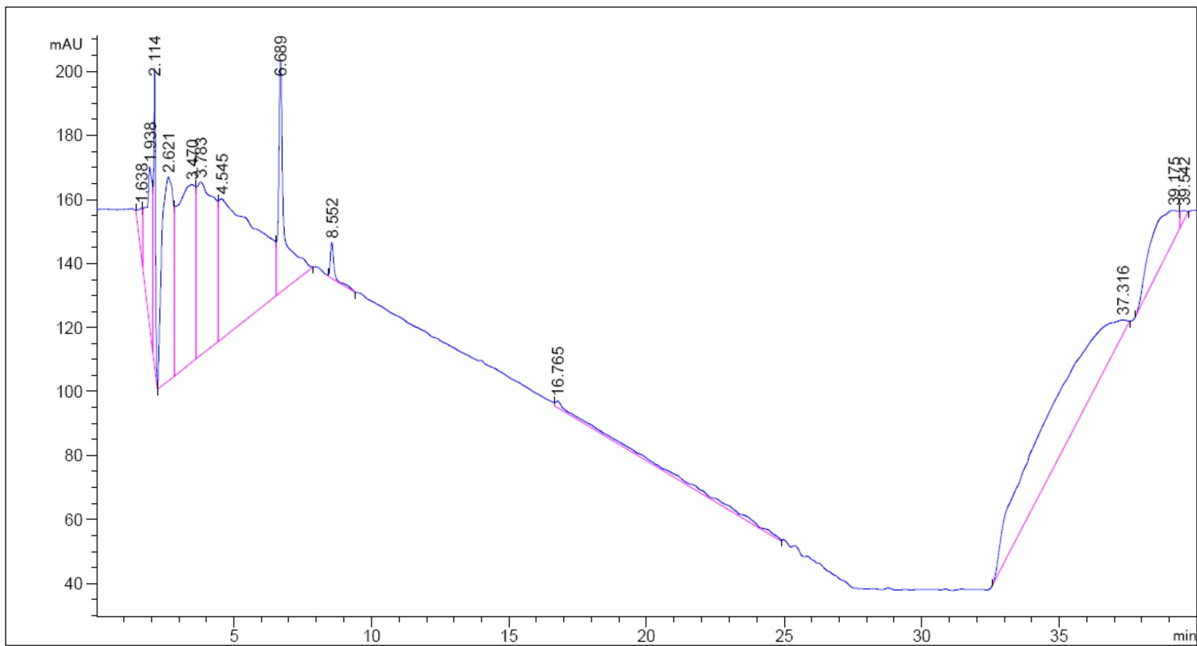


Figure 2-5: Diiodo tyrosine 20 µg/ml sample. The retention time was 6.689 min and impurity peak appeared at 16.765 min

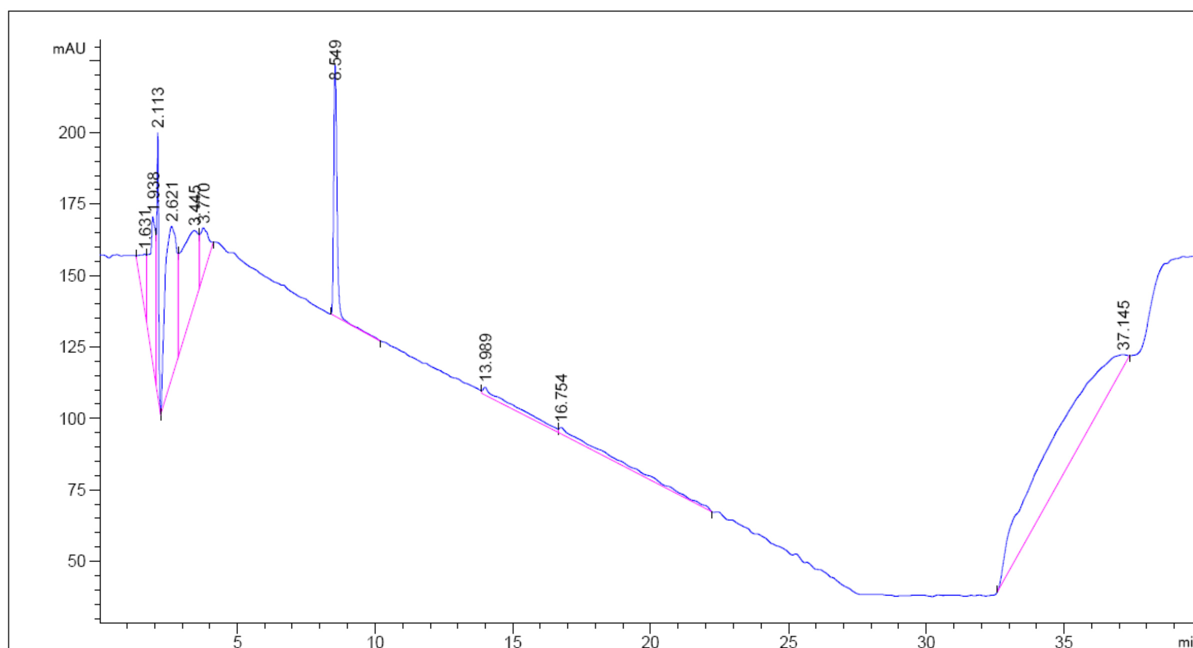


Figure 2-6: tri-Iodo-tyrosine 20 µg/ml sample. The retention time was 8.549 min and impurity peaks apperad at 13.989 min and 16.754 min

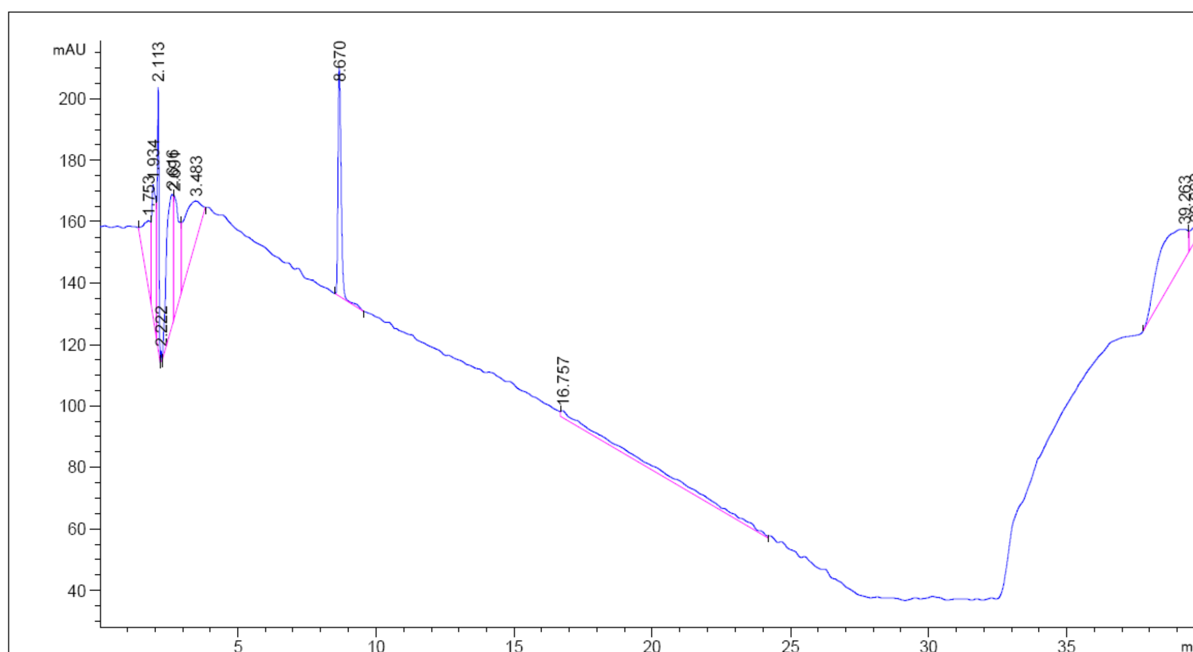


Figure 2-7: Thyronine 20 µg/ml sample. The retention time was 8.670 min and impurity peak appeared at 16.757 min.

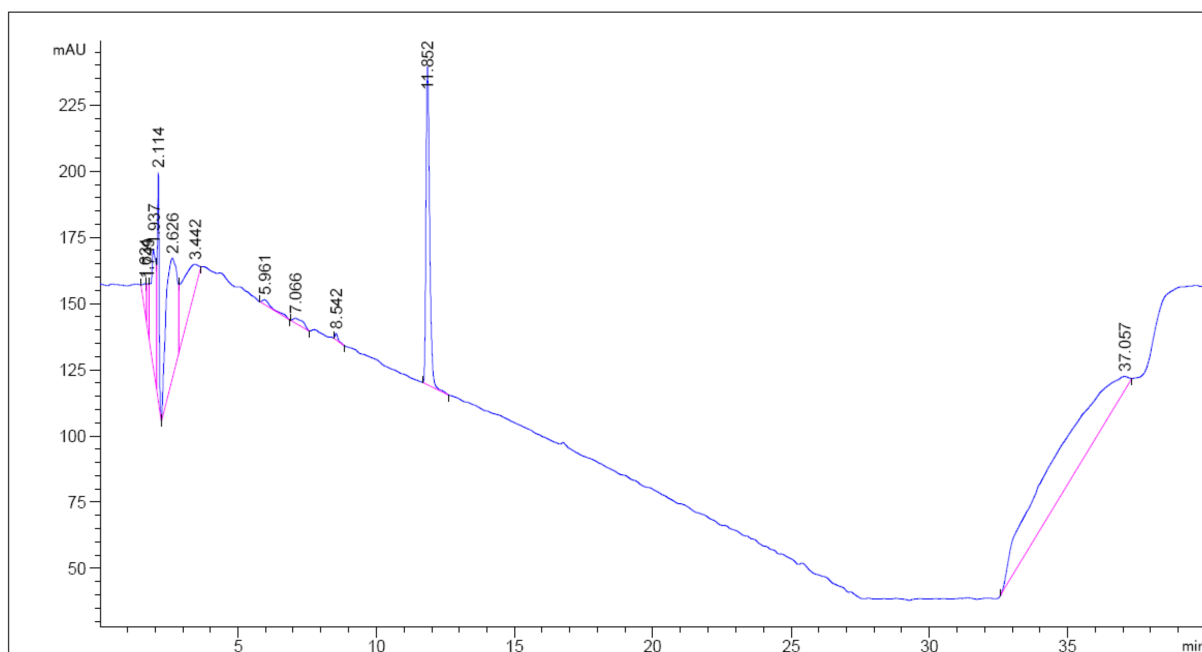


Figure 2-8: Di-Iodo-thyronine 10 µg/ml sample. The retention time was 11.852 min

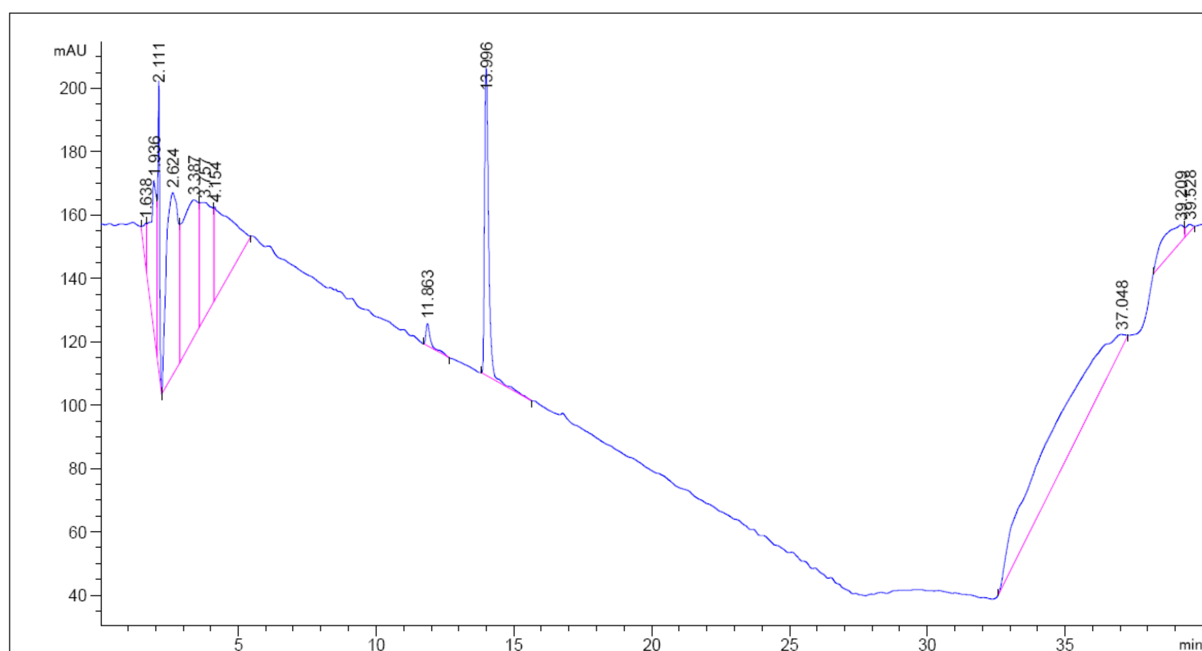


Figure 2-9: Tri-Iodo-thyronine 20 µg/ml sample. The retention time was 13.966 min and impurity peak appeared at 11.883 min

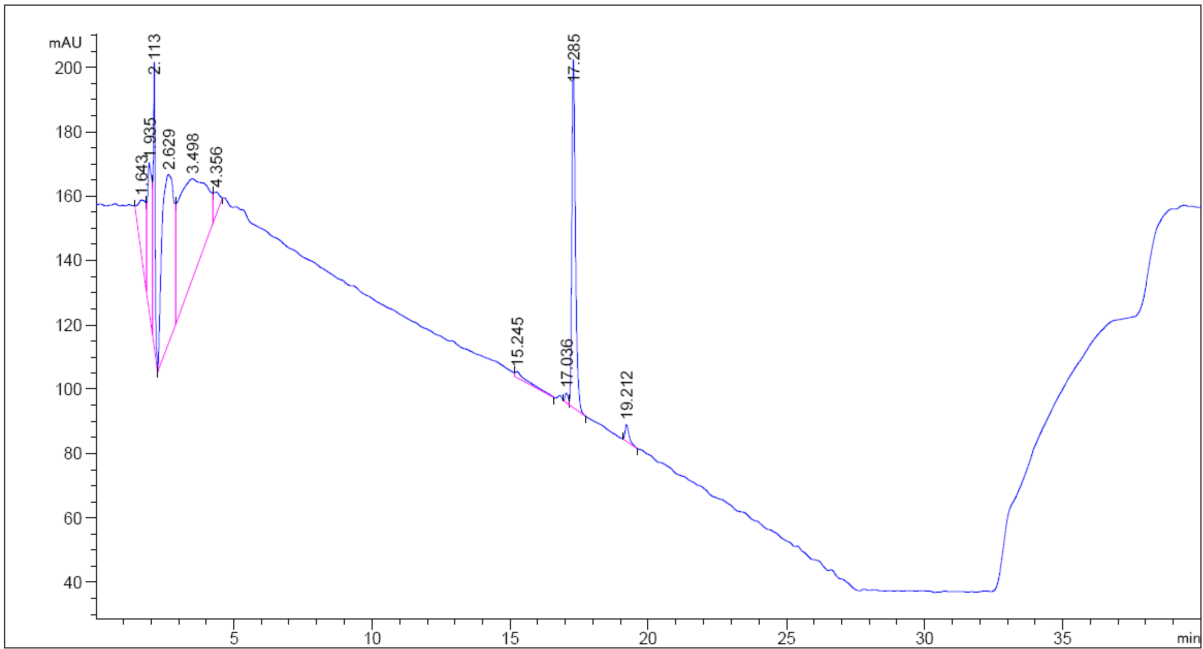


Figure 2-10: Triiodo thyroacetic acid 20 µg/ml sample. The retention time was 17.285 min and impurity peaks appeared at 15.245 min, 17.036 min and 19.212 min.

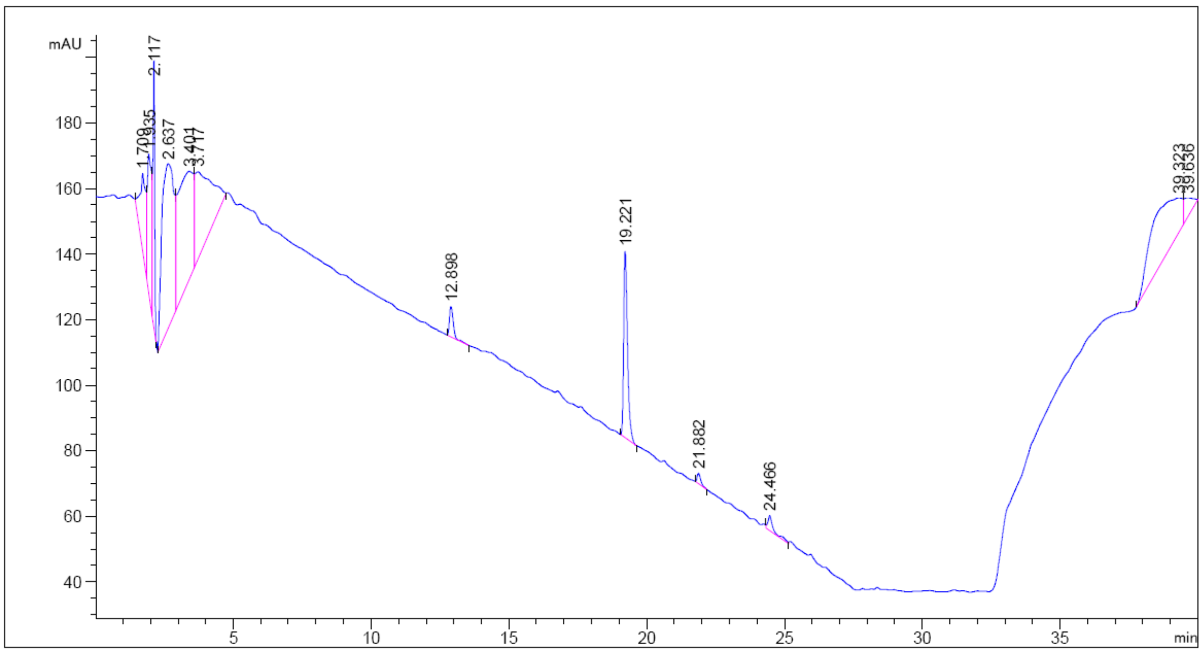


Figure 2-11: Tetra-iodo thyroacetic acid 20 µg sample. The retention time was 19.221 min and impurity peaks appeared at 12.898 min, 21.882 min and 24.468 min

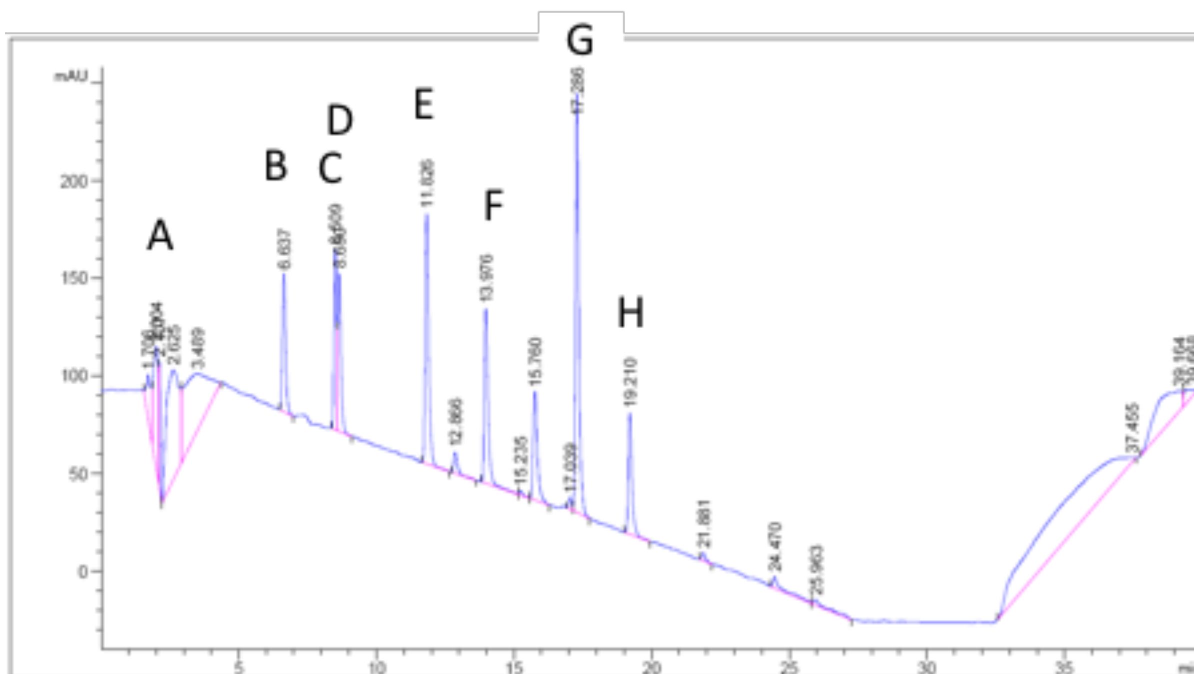


Figure 2-12: All eight known degradation products of levothyroxine separated and identified in a single HPLC sample. A) Thyrosine 1.706 mins, B) Di-iodotyrosine 6.637 mins, C) Tri-iodotyrosine 8.509 mins, D) Thyronine 8.622 mins, E) Di-iodothyronine 11.826 mins, F) Tri-iodothyronine 13.970 mins, G) Tri-iodothyroacetic acid 17.286 mins, H) Tetra-iodothyroacetic acid 19.210 mins.* The separation parameters such as theoretical plates and resolution have not compared to previous HPLC method as all the tested peaks were analysed individually and the main aim was to prove the peaks obtained individually from the retention time

- Stability of levothyroxine stored at 25°C

Following 30 days of exposure of levothyroxine sample to 25°C, there were no impurity peaks found in the sample and the peak purity for the levothyroxine was 100%.

Following 90 days of exposure of levothyroxine samples to 25°C, it can be seen clearly that, there was one impurity peak detected from the average of three individual runs at 22.994 min (± 4 seconds). The average purity percentage of the observed impurity peak of this injection was 1.950%± 0.341%) (n=3) while 98.050% was the average of the peak purity for the drug peak.

Levothyroxine sample stored at 25°C for 180 days showed the presence of two impurity peaks found in this run which consists together (3.312% ± 0.200%) (n=3) of

the total peak purity. On the other hand, levothyroxine has formed about 96.687%± 0.890%) (n=3) of peak purity in this sample. (figure 2.13).

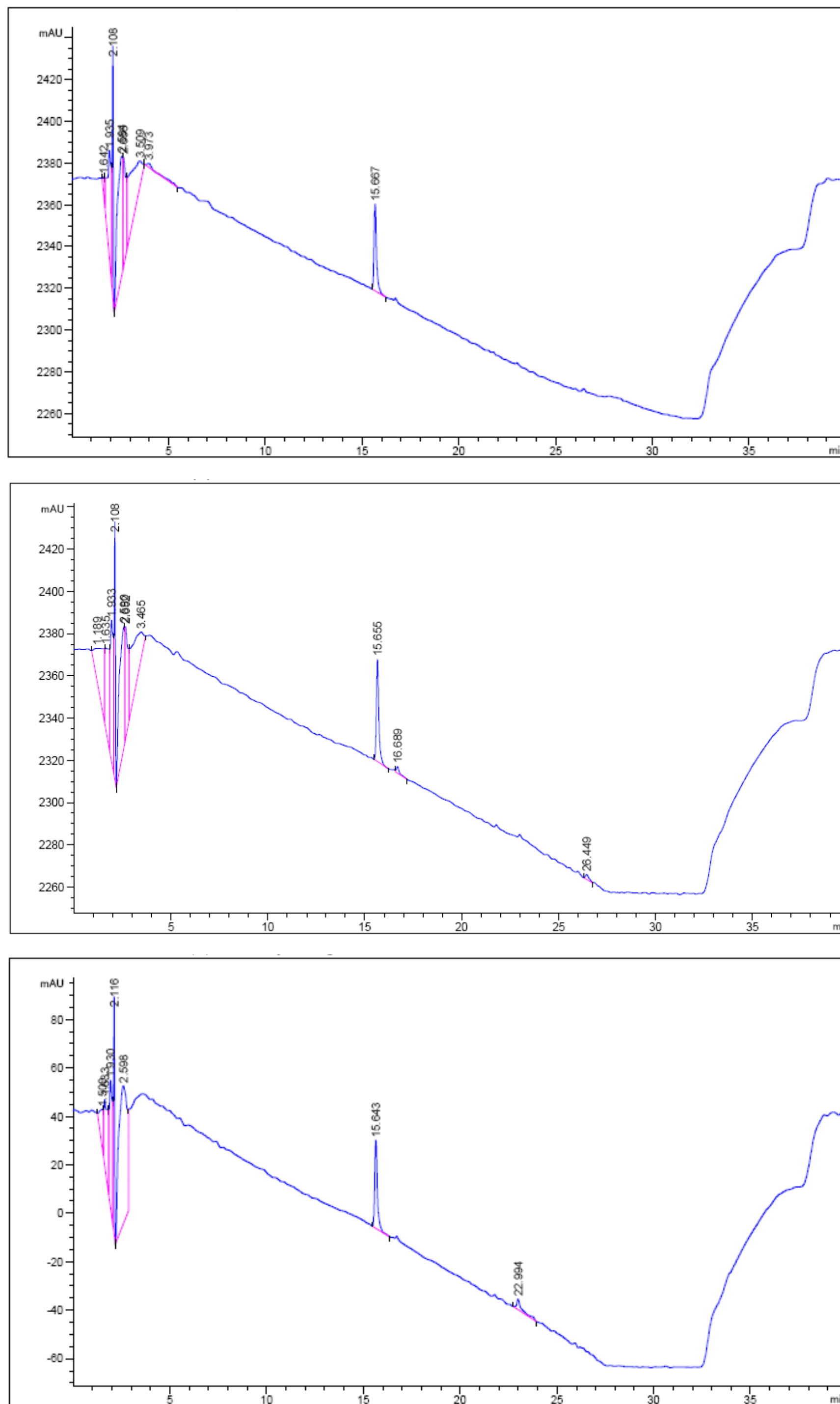


Figure 2-13: Levothyroxine 10 µg/ml sample following exposure to RT for t = 30 days (Above) the retention time was 15.667 min. RT for t = 90 days (middle)* The retention time was 15.643 *min. RT for t = 180 days (Bottom) The retention time was 15.655 min.

Table 2.4 is comparing the percentage recovery achieved from the levothyroxine solid state samples that were stored at room temperature for six months. However, levothyroxine samples that were stored at 25°C for 30 days showed about 95.81% recovery.

In terms of the levothyroxine sample that was stored at 25°C for 90 days, the mean percentage recovery of levothyroxine content in solution was 92.88 %. About 7.12% was the percentage loss of the levothyroxine sample when exposed to 25°C for 90 days.

Regarding the levothyroxine samples stored at 25°C for 180 days, the average levothyroxine concentration in solution decreased when compared to the samples stored under the same conditions for a shorter time (30 days, 90 days). The percentage recovery of levothyroxine content in solution was 90.753%.

Table 2-4: the % recovery of levothyroxine content in solution of stress condition sample (t = 30 days, 90 days, 180 days at room temperature

Sample	% recovery (Mean of n=3)	SD
Levothyroxine t=30 days, RT	95.81 %	0.032
Levothyroxine t=90 days, RT	92.88 %	0.041
Levothyroxine t=180 days, RT	90.75 %	0.060

- Stability of levothyroxine stored at 40°C

Levothyroxine samples stored at 40°C showed an increase in the impurity peaks with a decrease in the concentration of levothyroxine compared to the levothyroxine

samples stored at room temperature. Following 30 days of exposure of levothyroxine samples to 40°C, six impurity peaks were detected (figure 2.14).

The total of the average percentage purity of the observed impurity peaks in this sample was 26.744%± 2.112%) (n=3) while around 65.487% was the average peak purity for the drug peak. It can be noticed from figure 2.14 that, four impurity peaks were detected in levothyroxine sample stored at 40°C for 90 days, less than the number of impurity peaks found in levothyroxine sample that stored for 30 days at the same temperature. Nevertheless, in comparing the samples at 40°C for 90 days to the sample stored at 25°C for 90 days, the total average percentage recovery and the total number of impurity peaks in this sample was more than the number observed in levothyroxine sample stored at 25°C for 90 days.

The total mean percentage peak purity of the impurity peaks detected in this sample was 34.891%± 1.600%) (n=3) while around 65.09% was the mean percentage peak purity for the drug peak. Levothyroxine sample exposed to 40°C for 180 days showed that, about 38.186%± 5.930%) (n=3) was the mean percentage peak purity of the observed impurity peaks in this sample. However, the mean percentage peak purity of the drug was 61.812% when levothyroxine sample has exposed to 40°C for 180 days. (figure 2.14).

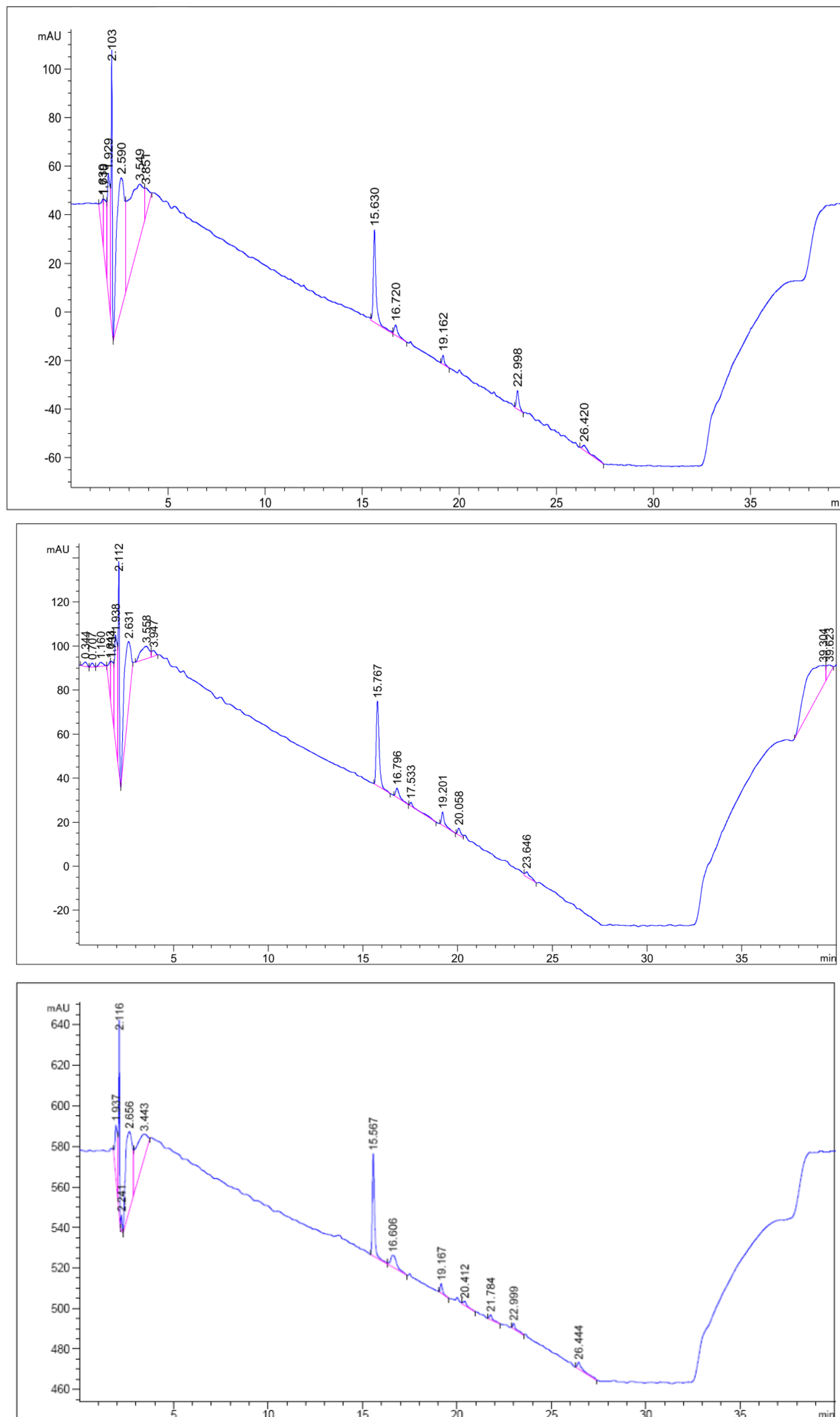


Figure 2-14: Levothyroxine 10 µg/ml sample following exposure to 40°C for t= 30 days *(Above.* The retention time was 15.567 min. 40°C *for t = 90 days (middle)* The retention time was 15.630 *min.* 40°C *for t = 180 days (Bottom) The retention time was 15.767 min.*

Table 2.5 shows that, the mean percentage recovery of levothyroxine content in solution was 76.06%. In terms of the levothyroxine sample that was stored at 40°C for 90 days, the mean percentage recovery of levothyroxine content in solution was

60.33%. In the levothyroxine sample stored at 40°C for 180 days, the percentage recovery of the drug content in solution was only 58.24%. These results indicate that there is an increase in the loss of drug upon prolonged storage at elevated temperature.

Table 2-5: The % recovery of levothyroxine content in solution of stress condition sample (t = 30 days, 90 days and 180 days at 40°C

Sample	% recovery (Mean of n=3)	SD
Levothyroxine t=30 days, 40°C	76.06 %	0.085
Levothyroxine t=90 days, 40°C	60.33 %	0.091
Levothyroxine t=180 days, 40°C	58.24 %	0.070

- Stability of levothyroxine stored at 70°C

Levothyroxine samples stored at high temperature (at 70°C), lead to the formation of a significant number of impurity peaks. Figure 2.15 shows the HPLC chromatogram of levothyroxine sample stored for 30 days at 70°C. About 17 impurity peaks were detected when the sample exposed to 70°C for 30 days. The impurity peaks found in this sample amounted to 68.68%± 7.111%) (n=3) from the mean percentage peak purity while only 31.320% was the mean percentage peak purity for the drug peak.

Figure 2.15 shows the chromatogram of levothyroxine sample stored for 90 days at 70 °C. It was observed that, the mean percentage peak purity of the observed impurity peaks was 66.393%± 5.654%) (n=3) while 33.607% was the average peak purity for the drug peak.

When levothyroxine sample was stored at 70°C for 180 days, seven degradation peaks were detected by HPLC. The mean percentage peak purity of the degradation

peaks was 68.784%± 4.880%) (n=3), while 31.216% was the average peak purity for the levothyroxine peak (figure 2.15).

Observations from the stability studies indicate that levothyroxine samples exposure to elevated temperatures leads to a decrease in the percentage recovery of the drug. Figure 2.15 shows that after 30 days' exposure at 70°C, percentage recovery of the drug dropped sharply. This phenomenon of reduction in the drug content when stored at higher temperature was observed after 90 days and 180 days too.

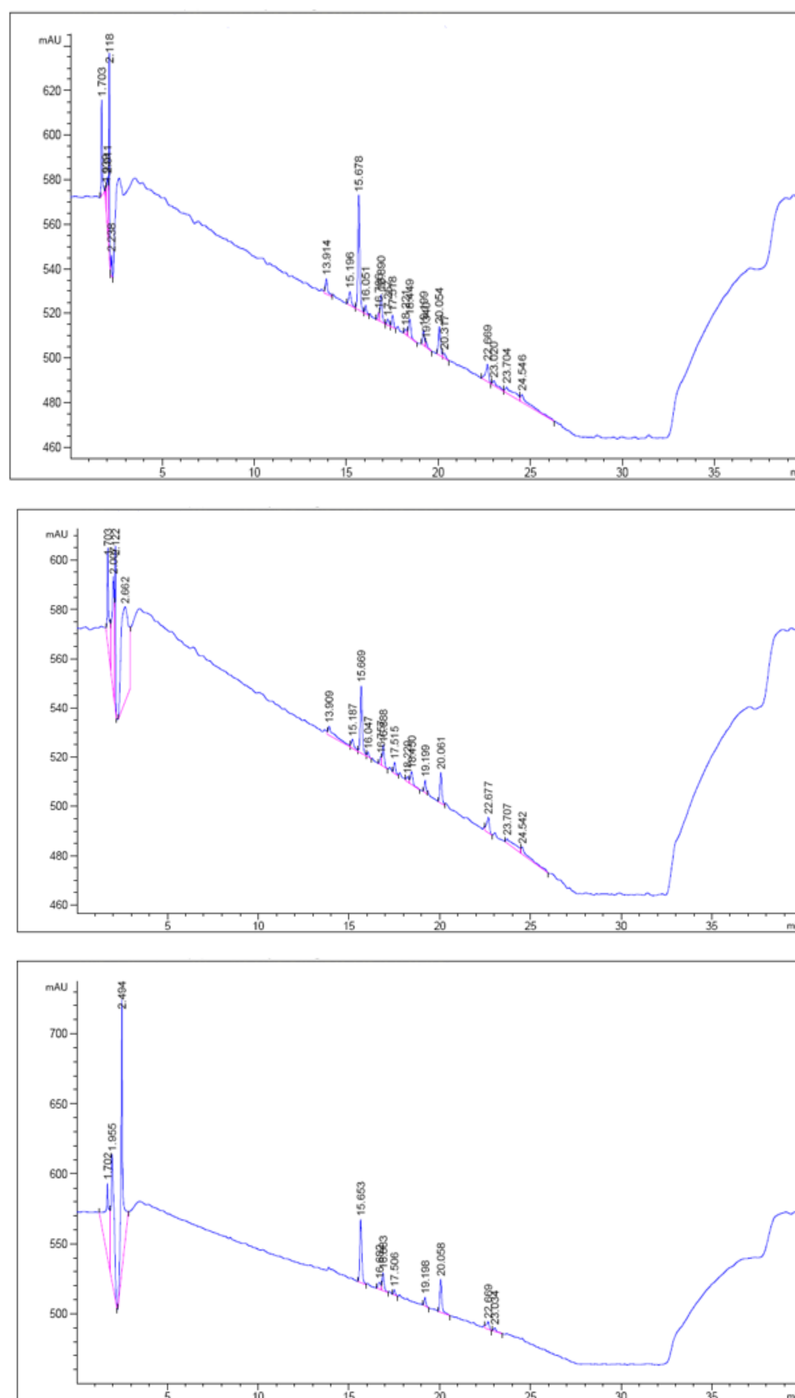


Figure 2-15: Levothyroxine 10 µg sample following exposure to 70°C for t= 30 days (Above), The retention time was 15.678 min, ***70°C for t= 90 days (Middle), the retention time was 15.669, min, 70°C for t= 180 days (Bottom), the retention time was 15.653 min.***

Table 2.6 shows that, the mean percentage recovery of levothyroxine content in solution was 61.73 %. In terms of the levothyroxine sample that have been stored at 70°C for 90 days, the mean percentage recovery of levothyroxine content in solution was 54.53 % However, in terms of the levothyroxine sample that have been stored at

70°C for 180 days, the percentage recovery of the drug content in solution was only 35.7%.

Table 2-6: the % recovery of levothyroxine content in solution of stress condition sample (t = 30 days, 90 days and 180 days at 70°C

Sample	% recovery (Mean of n=3)	SD
Levothyroxine t=30 days, 70°C	61.73 %	0.132
Levothyroxine t=90 days, 70°C	54.53 %	0.121
Levothyroxine t=180 days, 70°C	35.7 %	0.087

2.3.4 Chemical stability study of levothyroxine using LC-MS

- Analysis of the eight known degradation compounds of levothyroxine

Eight degradation compounds have previously been reported for levothyroxine. Therefore, the samples analysed using LC-MS in this research included these eight impurities for comparison and identification of unknown impurities to the previously reported degradation compounds of levothyroxine. Figures 2.22 shows the LC-MS spectrum for thyronine which has a molecular mass of 273.28 g/mol. Figure 2.23 represents the LC-MS injection for 3,5 di-iodo-thyronine, with a molecular mass of 525.077 g/mol. 3,3', five tri-iodo thyronine with a molecular mass of 650.977 g/mol is shown in figure 2.24. Tyrosine is one of the degradation products of levothyroxine, and it has a molecular mass of 181.19 g/mol (figure 2.25) while the spectrum for mono-iodo-tyrosine is shown in figure 2.26 which has a molecular mass of 307.085 g/mol. The molecular mass of di-iodo tyrosine is 432.289 g/mol shown in figure 2.27. Figure 2.28 shows the spectrum for 3,3', five tri-iodo thyroacetic acid which has a molecular mass of 620 g/mol while the mass spectrum for 3,3',5,5' tetra-iodo-thyroacetic acid is shown in figure 2.29 which has a molecular mass of 747 g/mol.

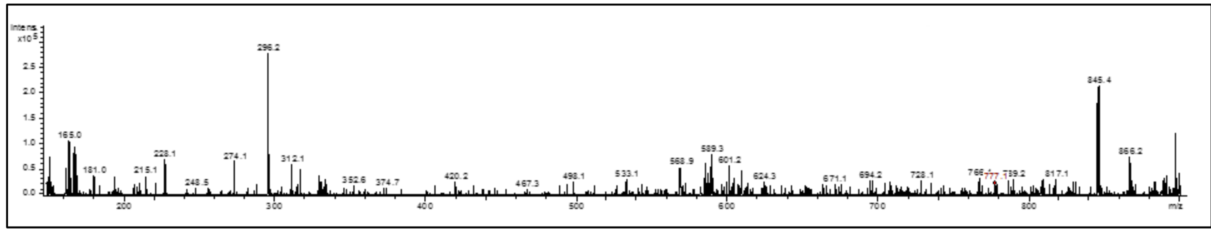


Figure 2-16: LC-MS run showing the detection of thyronine solid-state sample.

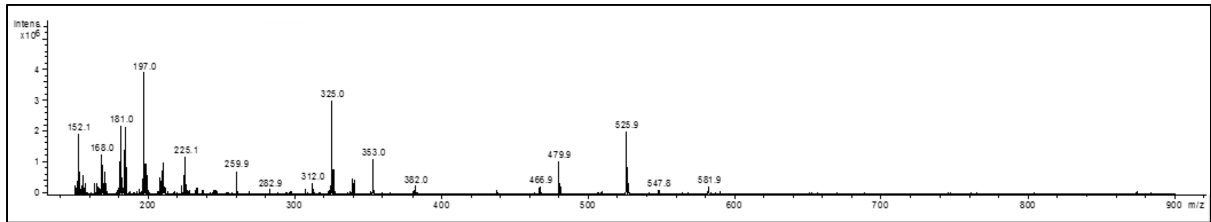


Figure 2-17: LC-MS run showing the detection of di-iodo-thyronine solid-state sample.

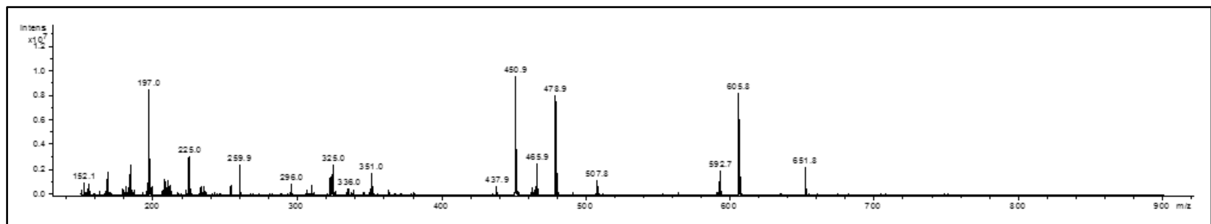


Figure 2-18: LC-MS run showing the detection of tri-iodo-thyronine solid-state sample.

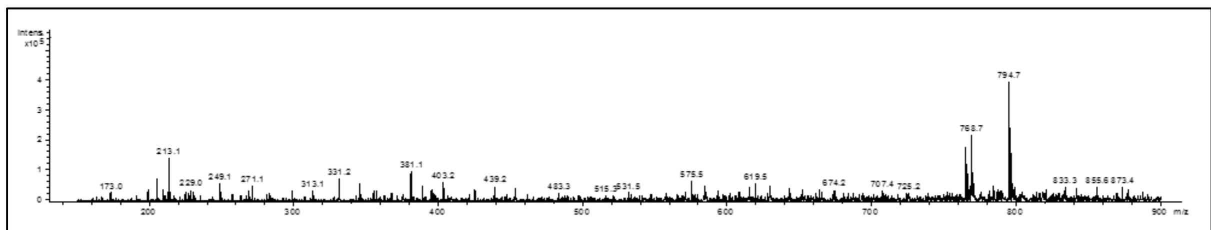


Figure 2-19: LC-MS run showing the detection of tyrosine solid-state sample.

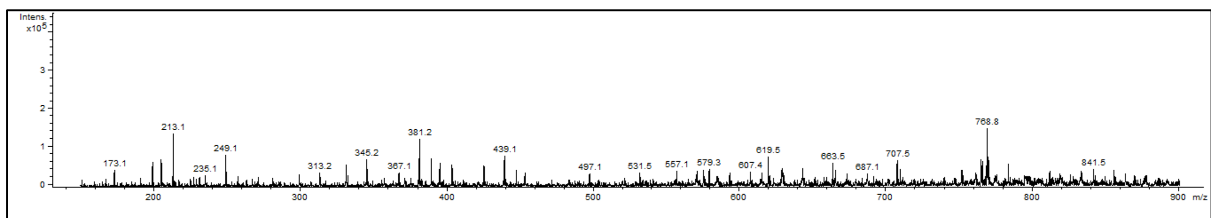


Figure 2-20: LC-MS run showing the detection of mono-iodo-tyrosine solid-state sample.

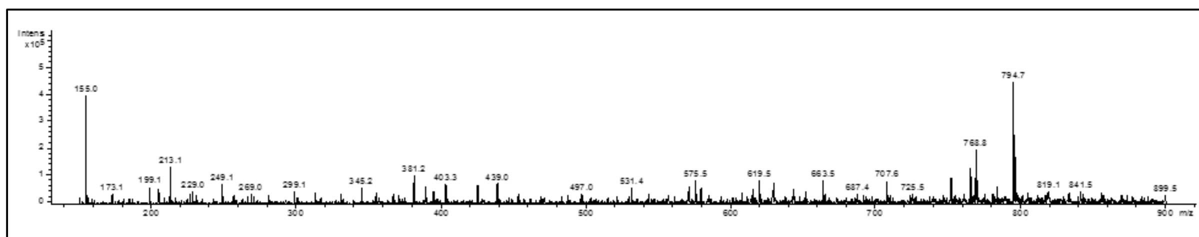


Figure 2-21: LC-MS run showing the detection of di-iodo-tyrosine solid state sample

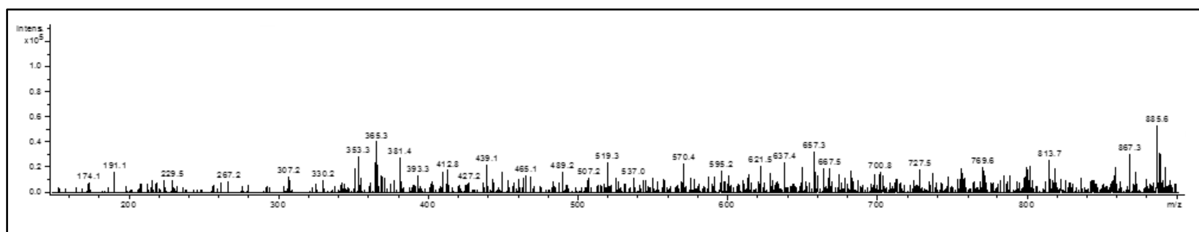


Figure 2-22: LC-MS run showing the detection of the tri-iodo-thyroacetic acid solid-state sample.

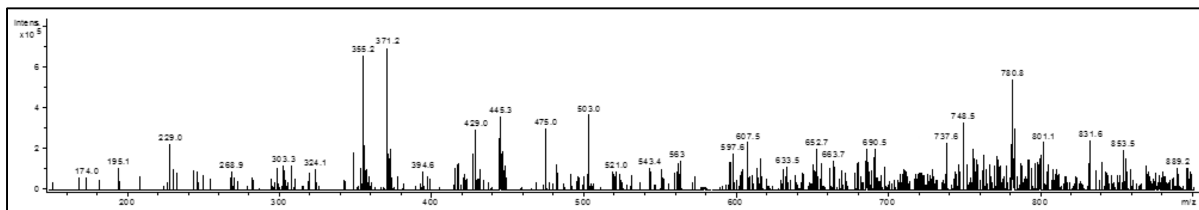


Figure 2-23: LC-MS run showing the detection of the tetra-iodo-thyroacetic acid solid-state sample.

- Stability of levothyroxine stored at 25°C

Studying the chemical stability of levothyroxine solid state using LC-MS, the main ion observed and analysed was thyroxine with a molecular ion at m/z 777.7 with a small number of fractures being created.

Figure 2.24 shows the mass spectral analysis of levothyroxine sample in its solid-state at t=0, and it can be clearly seen that the molecular mass to charge (m/z) ratio of thyroxine was observed to be 777.7.

The m/z value at 731.7 fragment is potentially an indicator of decarboxylation of levothyroxine molecule. Hence it appears in all the samples even at t=0. Comparing the drug samples stored at room temperature to t=0, it can be clearly seen that the chemical stability of levothyroxine had not altered. No impurity peaks were observed even when the drug samples have been kept for six months (t=180 days) (figure 2.24).

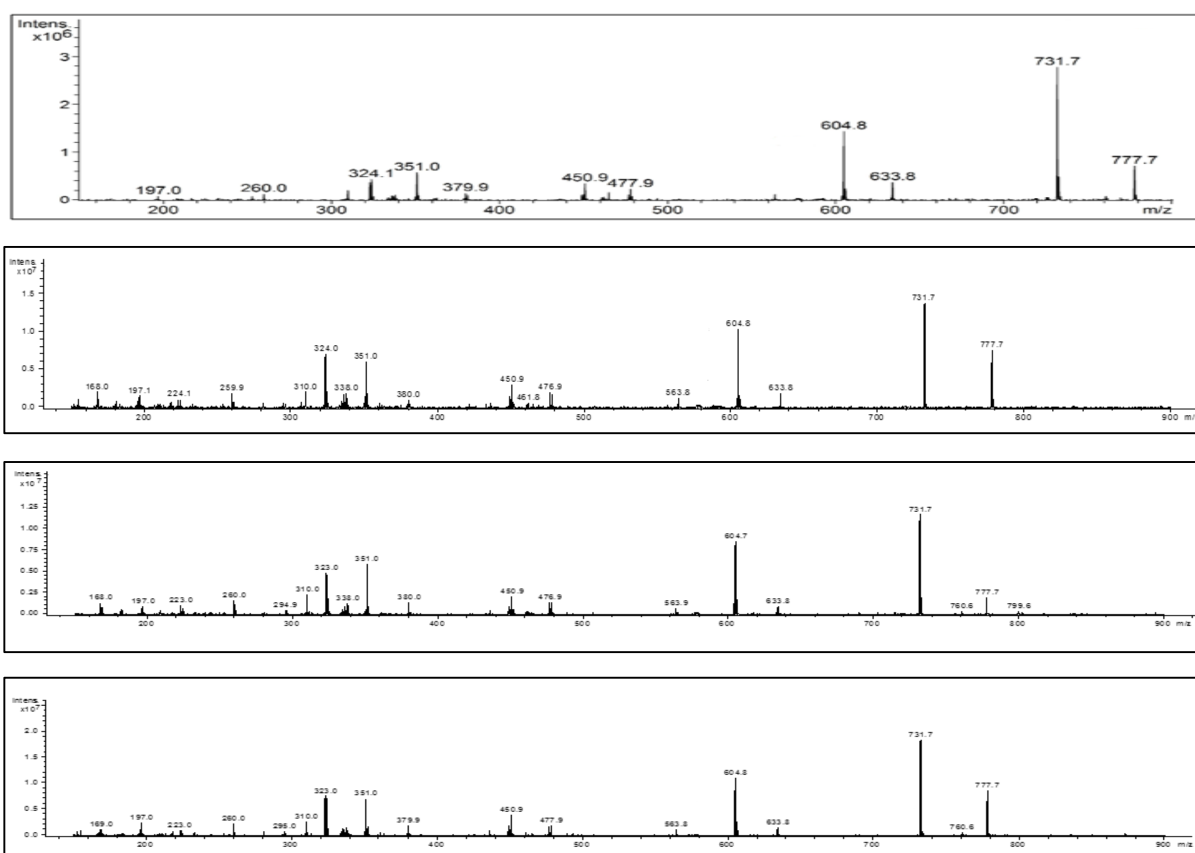


Figure 2-24: LC-MS run showing the detection of levothyroxine solid state sample at different time points (From top to bottom: at t=0/30/90/180 days at 25C

- Stability of levothyroxine stored at 40°C

Figure 2.25 shows the chromatogram of a fresh sample of levothyroxine (in black) compared to levothyroxine sample stored at 40°C for 180 days (in red). It can be

observed that three new peaks have been detected when the levothyroxine sample was stored at 40°C. These observations are further confirmed by LC-MS analysis.

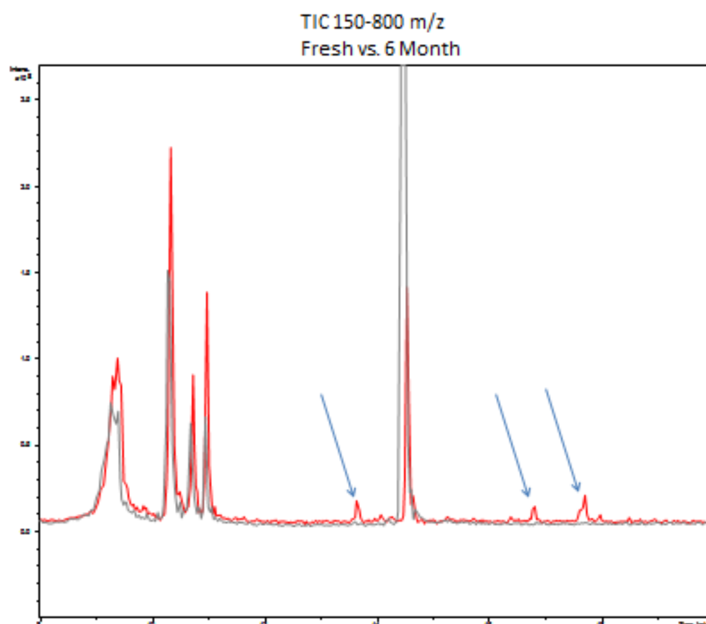


Figure 2-25: Chromatogram showing the difference between new levothyroxine (black) and levothyroxine after six months at 40°C under stress conditions (red). Three new peaks were observed

After 30 days of storing the levothyroxine sample at 40°C, three new degradation products of levothyroxine have been noticed which had different (m/z) ratios and various retention time. The first impurity observed was [4-(2,6 diiodo-4-methylphenoxy)-2 iodophenol] at 577 (m/z) while the second impurity peak has been identified as [4-(4-ethyl-2,6-diiodophenoxy)-2 iodophenol] at 592 (m/z). In addition to the previous two identified impurity peaks, [4-(4-ethyl-2,6-diiodophenoxy)-2,6 diiodophenol] was found at 717 (m/z) (figure 2.26).

Figure 2.27 and figure 2.28 show levothyroxine samples that have been stored at 40°C for t=90 days and t=180 days. It can be observed that the same three degradation products at the same mass to charge ratio were obtained. (The chemical structures of the degradation products are explained and shown in the discussion section of this chapter).

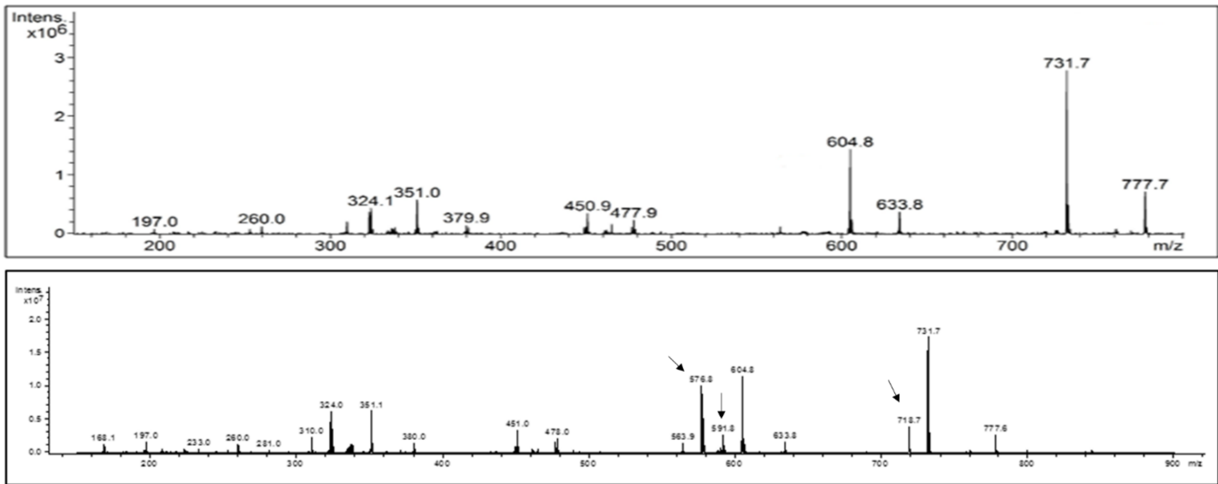


Figure 2-26: LC-MS run showing the detection of impurity peaks according to their masses (black arrows). The top baseline is levothyroxine solid state sample when t=0 and the lower baseline is levothyroxine sample when t= 30 days at 40°C

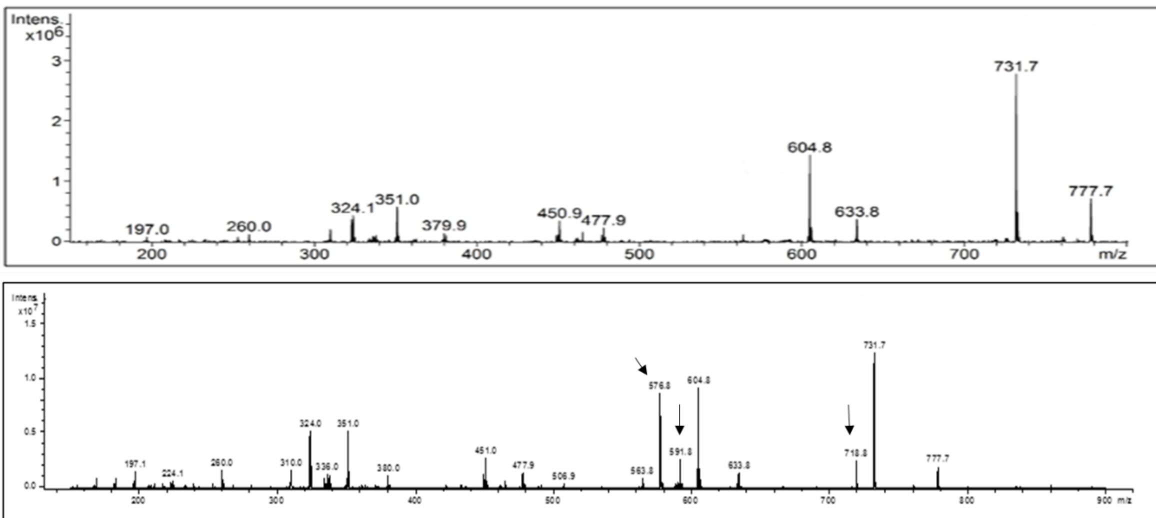


Figure 2-27: LC-MS run showing the detection of impurity peaks according to their masses (black arrows). The top baseline is levothyroxine solid state sample when t=0 and the lower baseline is levothyroxine sample when t= 90 days at 40°C

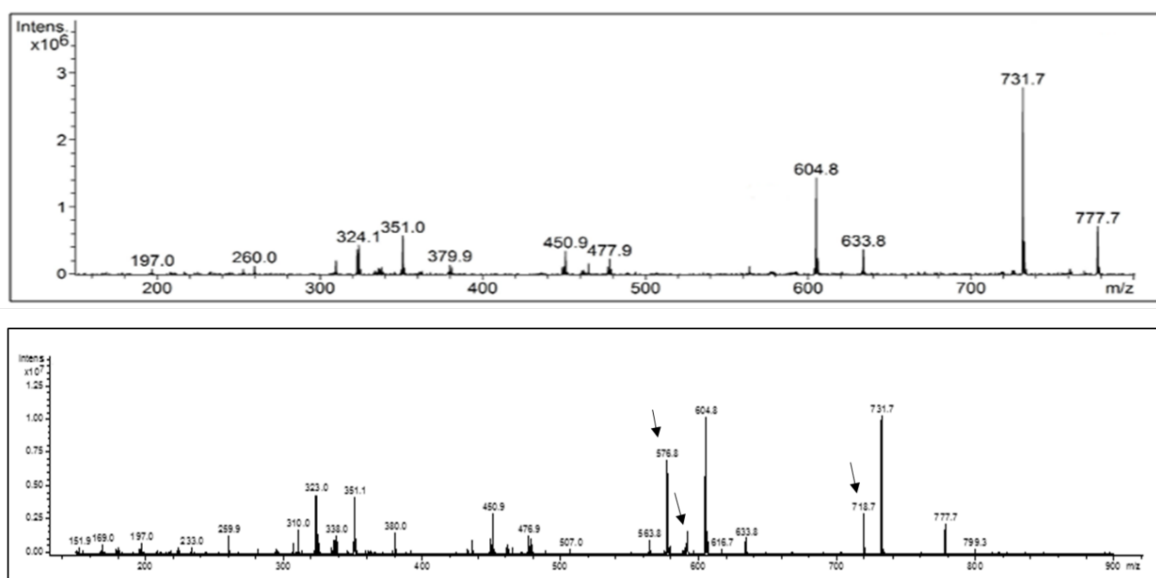
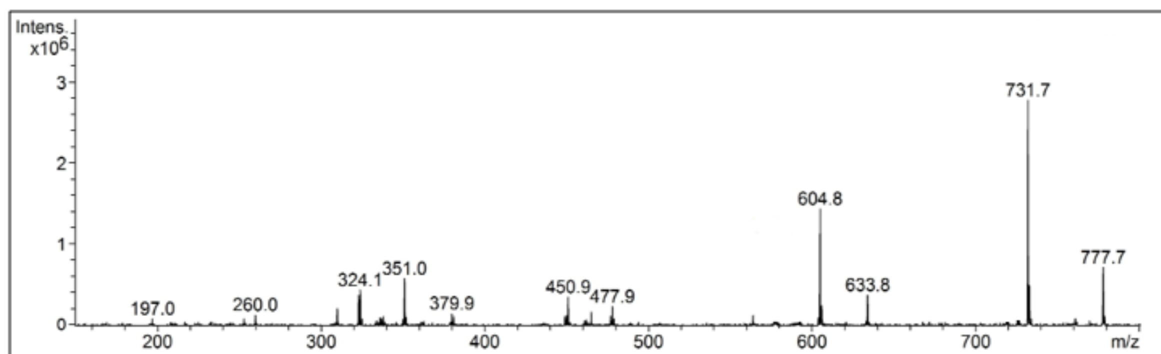


Figure 2-28: LC-MS run showing the detection of impurity peaks according to their masses (black arrows). The top baseline is levothyroxine solid state sample when t=0 and the lower baseline is levothyroxine sample when t= 180 days at 40°C

- Stability of levothyroxine stored at 70°C

When the storage condition of the levothyroxine samples has been changed to a higher temperature (70°C), identical degradation products to ones observed at 40°C were observed with the drug sample that was stored at 70°C for t=30 days (figure 2.29). In contrast, after 90 days, the three new impurity peaks have degraded further and therefore, the chromatogram baseline showed many impurities along with small peaks. This phenomenon is possibly as a result of further break down of the impurity peaks of the levothyroxine sample. The outcome of levothyroxine exposure to higher heat at 70°C resulted in the formation of 3,5 diiodo thyronine compound at 525.3 (m/z). 3,5 Diiodo thyronine has previously been reported as one of the degradation products for levothyroxine (The chemical structure is shown in the discussion section of this chapter) (figure 2.30). After 180 days of exposing the levothyroxine sample to 70°C, nearly all the peaks related to levothyroxine have been degraded further. Also, the number of peaks obtained for all the samples has sharply reduced and converted to different residues of thyroacetic acid. It has been noticed from this spectrum that, 3,3', 5 tri-iodo-thyroacetic acids at 620.8 (m/z) was detected. In addition, 3,3',5,5' tetra-iodo-thyroacetic acid at 747 (m/z) has also been detected for this sample (The chemical structures of the thyroacetic acid compounds are shown in the discussion section of

this chapter). These acids have previously been identified as the degradation products for levothyroxine (figure 2.31).



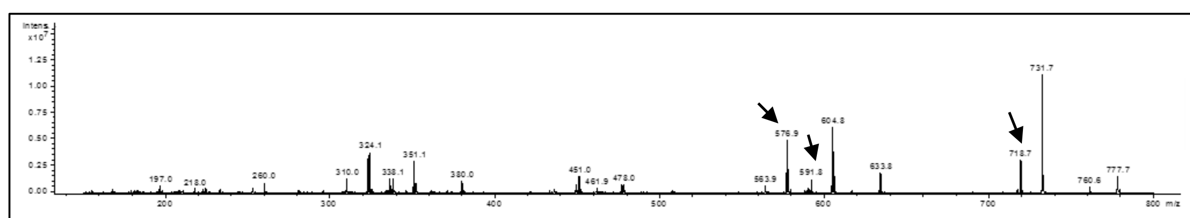
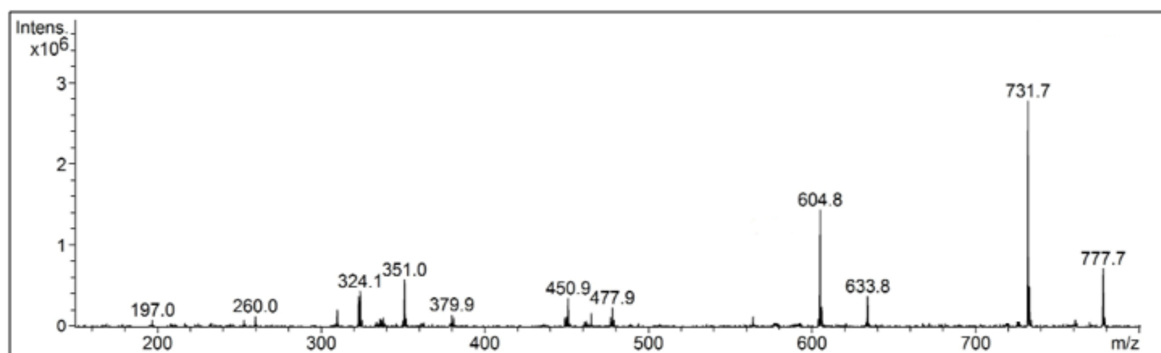


Figure 2-29: LC-MS run showing the detection of impurity peaks according to their masses (black arrows). The top baseline is levothyroxine solid state sample when t=0 and the lower baseline is levothyroxine sample when t= 30 days at 70°C



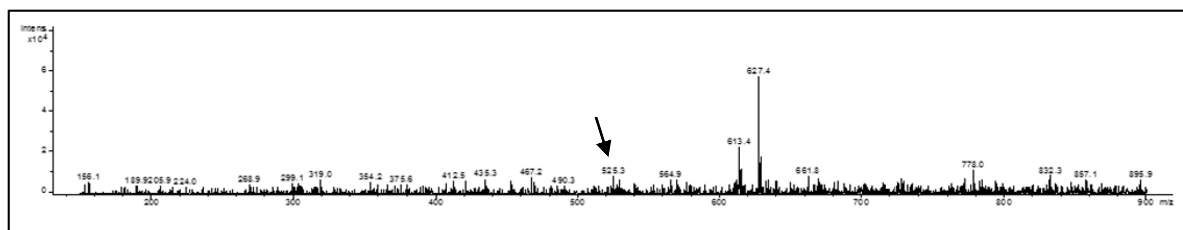


Figure 2-30: LC-MS run showing the detection of impurity peaks according to their masses (black arrows). The top baseline is levothyroxine solid state sample when t=0 and the lower baseline is levothyroxine sample when t= 90 days at 70°C

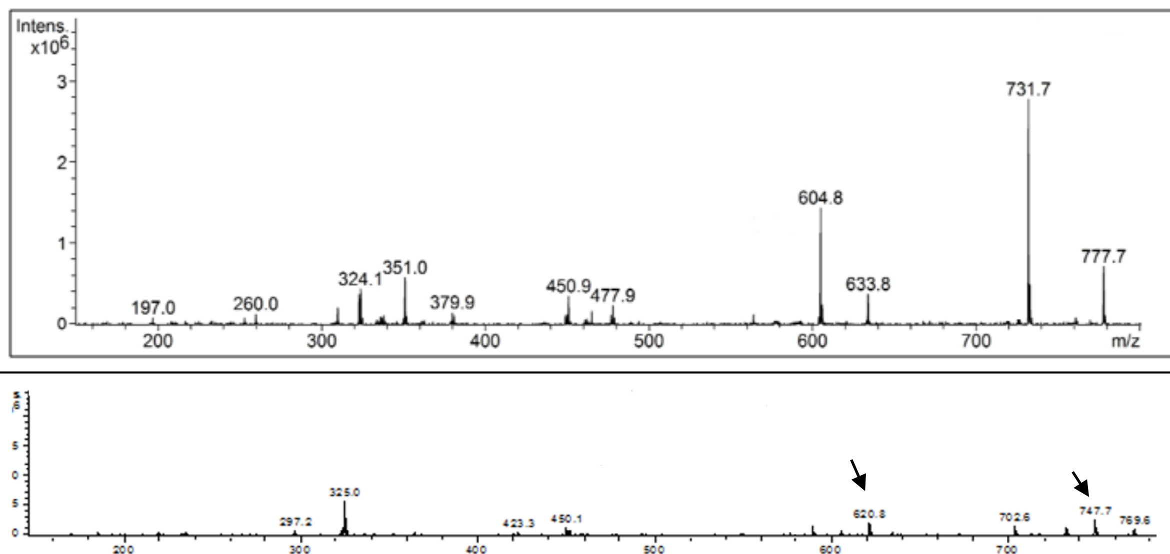


Figure 2-31: LC-MS run showing the detection of impurity peaks according to their masses (black arrows). The top baseline is levothyroxine solid state sample when t=0 and the lower baseline is levothyroxine sample when t= 180 days at 70°C

2.3.5 Fourier Transform Infrared Spectroscopy (FT-IR)

A solid-state levothyroxine sample at t=0 and its chemical stability standards were tested using FT-IR to find the changes in the chemical structure after exposing the standards samples to three different temperatures (25°C,40°C,70°C).

All the drug standards samples were compared to the t=0 solid state levothyroxine sample, and correlation values were obtained to show the similarities in the chemical structures of the tested samples. However, the 100% similarity in the composition of the functional groups of the stress condition samples with the solid-state levothyroxine sample is equal to a correlation value of 1.

FT-IR of the t=0 levothyroxine sample has performed and compared to the FT-IR spectrum of the levothyroxine stability samples in figures (2.32). The peaks obtained have correlated to the molecule's spectrum. In addition, the FT-IR chromatogram showed that the FT-IR of levothyroxine sample at t=0 was distinguished by N-H stretching at 3266.70 cm^{-1}, O-H stretching at 3076 cm^{-1}, and C=O stretching at 1603 cm^{-1}, thus confirming COOH and amine in the structure. Ether C-O stretching was at 1183cm^{-1} while aromatic rings stretching was at 880 cm^{-1} and 847 cm^{-1}.

In comparing the t=0 levothyroxine sample to the sample following 90 days of storage at 25°C, no significant difference was observed. In addition, the correlation between the spectra was 0.99 rather than 1 (obtained when comparing spectra of similar structure). It has been noticed (figure 2.30) that storage at room temperature had no influence on the chemical structure of levothyroxine even when the duration time of exposure was 180 days. The correlation between the chemical structure of t=0 and t=180 days at 25°C was somewhat similar, at 0.99

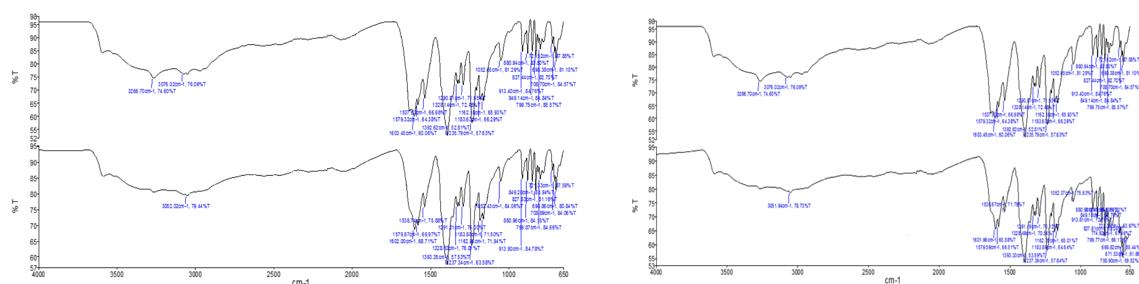


Figure 2-32: (Left): FTIR spectra of the levothyroxine t=0 sample and levothyroxine standard following three months at room temperature. The top spectrum is the levothyroxine t=0 sample while the bottom spectrum is the levothyroxine at RT after three months. (Right): FTIR spectra of the levothyroxine t=0 sample and levothyroxine standard following six months at RT. The top spectrum is the levothyroxine fresh sample while the bottom spectrum is the levothyroxine at RT after six months.

In contrast, figure 2.33 indicate that at a temperature of 40°C a slight alteration has occurred after 90 days, but significant influence was observed after 180 days. The most frequent change obtained was on the aromatic ring area where the 880 cm^{-1} and 847 cm^{-1} stretching changed. At 40°C, t= 90 days, the correlation of the compared spectra dropped to 0.70, so the large structural changes had started to appear after three months at 40°C.

The levothyroxine standard following 180 days at stress condition of 40°C produced sharply altered spectrum on the aromatic ring. Besides, it has shown a high change on the levothyroxine functional groups. The correlation between levothyroxine t=0 sample and levothyroxine six-months sample at 40°C was only 0.67.

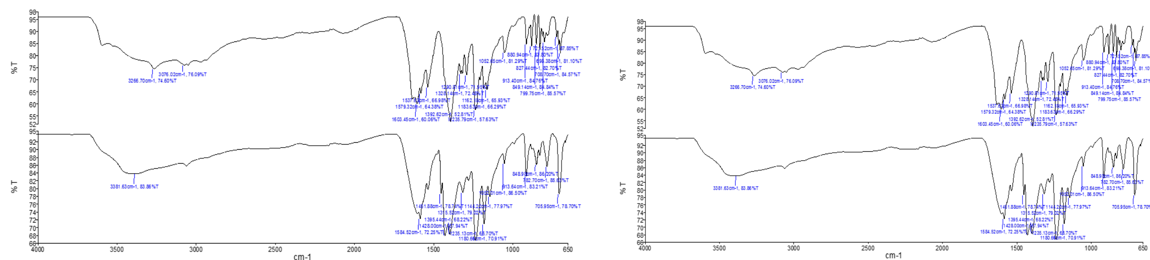


Figure 2-33: (Left): FTIR spectra of the levothyroxine t=0 sample and levothyroxine standard following three months at 40°C. The top spectrum is the levothyroxine fresh sample while the bottom spectrum is the levothyroxine at 40°C after three months. (Right): FTIR spectra of the levothyroxine t=0 sample and levothyroxine standard following six months at 40°C. The top spectrum is the levothyroxine fresh sample while the bottom spectrum is the levothyroxine at 40°C after six months.

However, the spectra of levothyroxine standards had the same alterations after 90 days at 70°C, by which time all peaks started to decrease. This observation also was noted in the samples after 180 days of the stress conditions at 70 °C

The correlations calculated between levothyroxine t=0 sample and levothyroxine standards were 0.64 after three months and 0.62 after six months (Figure 2.34).

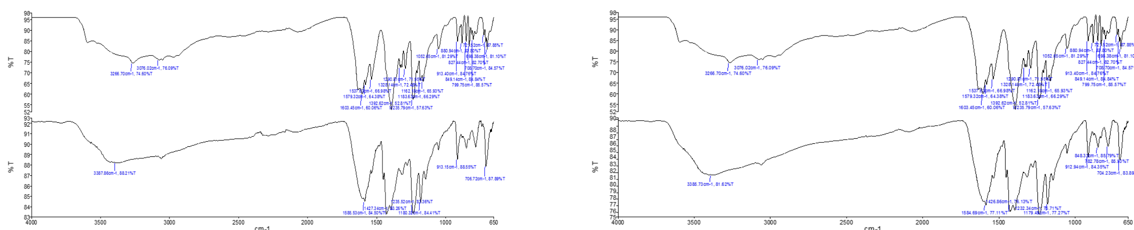


Figure 2-34: (Left): FTIR spectra of the levothyroxine t=0 sample and levothyroxine standard following three months at 70°C. The top spectrum is the levothyroxine fresh sample while the bottom spectrum is the levothyroxine at 70°C after three months. (Right) FTIR spectra of the levothyroxine t=0 sample and levothyroxine standard following six months at 70°C. The top spectrum is the levothyroxine fresh sample while the bottom spectrum is the levothyroxine at 70°C after six months.

2.4 Discussion

2.4.1 Physical appearance of levothyroxine solid state stored at different temperatures

Samples of levothyroxine stored at three different temperatures 25°C, 40°C, 70°C(figure 2.5 and figure 2.6), displayed changes to the physical appearance of levothyroxine samples when the samples were exposed to high temperature. Besides, the white crystalline powder had changed to yellowish white colour at 40°C after 180 days and to brown colour at 70°C after 30 days, which had converted to darker brown after 180 days.

Previous research has indicated that a brown colour is obtained following chemical interactions under thermodynamic conditions. Appearance of the sample's brown colour could be due to free oxygen interaction with the compounds [90]. Levothyroxine under high temperature and in the presence of atmospheric oxygen lead to oxidation of the sample. This oxidation can lead to the formation of different compounds [87].

Another study on the investigation of paprika degradation products found that a non-enzymatic degradation of the sample lead to the formation of brown colour (the brown colour obtained was due to water activity and not due to the presence of any enzymatic reaction). The brown colour formed has related to the water activity (amount of water available for hydration) and that produced degradation products of paprika powder. It has been found that water activity plays an important role on product shelf life and chemical stability as water behaves as a solvent and its content affects the chemical reaction rate, as the water can be a reactant itself. Therefore, when the sample with the absorbed water content was exposed to high temperatures for a long time, there was an acceleration of the chemical reaction rate leading to the deterioration of the sample. Hence, the formation of brown colour has been attributed to non-enzymatic reaction at this point [91, 92].

It was thus important to test levothyroxine stability samples using various analytical instruments such as HPLC, LC-MS, and FTIR to detect and identify any changes in functional groups present in the structure. These techniques were also used to observe and analyse molecules or compounds included in the levothyroxine chemical structure and degradation products that has formed from it. Furthermore, it was

important to take into account these changes in powder colour as they can be indicative of levothyroxine degradation products.

2.4.2 Chemical stability study of levothyroxine using (HPLC, LC-MS, FTIR)

Previous studies on the chemical stability of levothyroxine found that, levothyroxine contains a radical initiator within its structure which is capable of significantly modifying the chemical structure of levothyroxine following exposure to stress conditions such as high temperatures [94]. These radicals lead to the initiation of three mechanisms (which occur when two electrons from the original bond are distributed equally between the resulting fragments) in the levothyroxine compound. The outcomes of these mechanisms are;

1) I-C bond cleavage of the benzyl ring and ether cleavage, 2) hydroquinone formation, 3) oxidative decarboxylation and acid formation [94].

- **Scenario 1: Loss of iodine group(s) and ether bridge breakdown in the chemical structure of levothyroxine**

Under stress conditions of temperature, the C-I bond on the phenyl ring typically experiences a breakage resulting in an extremely active phenyl radical that abstracts a hydrogen atom from levothyroxine. Loss of iodine is mainly dependent on the activity of the oxygen radical. During exposure of drug samples to a particular degree of heating, water in the crystal will withdraw entirely due to the high affinity of the crystal structure to the water [94].

The excessive abstraction of the hydrogen radical produces an alteration in the chemical structure of levothyroxine. This modification appears as an ether bridge breakage linking the aromatic rings on the chemical structure of levothyroxine. The outcome of this chemical reaction is formation of tyrosine [94].

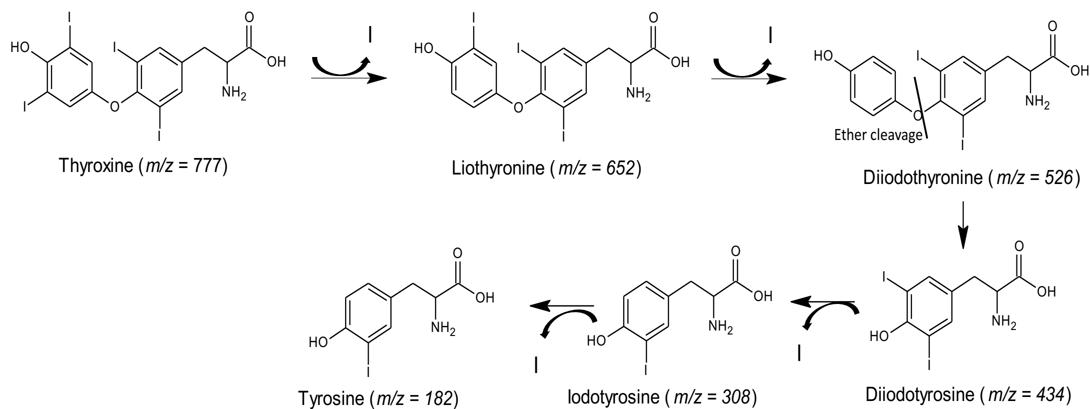


Figure 2-14: Levothyroxine degradation pathway with the loss of iodine groups and breakage of the ether bond of the chemical structure (m = mass, z = charge) [13].

- **Scenario 2: Formation of hydroquinone compounds from the breakdown of levothyroxine**

In some cases, more than one iodine group leaves the chemical structure of levothyroxine before ether cleavage can take place. All four groups have the potential to be removed from levothyroxine to form thyronine. Ether cleavage subsequently takes place to form the hydroquinone and phenylalanine compounds [95].

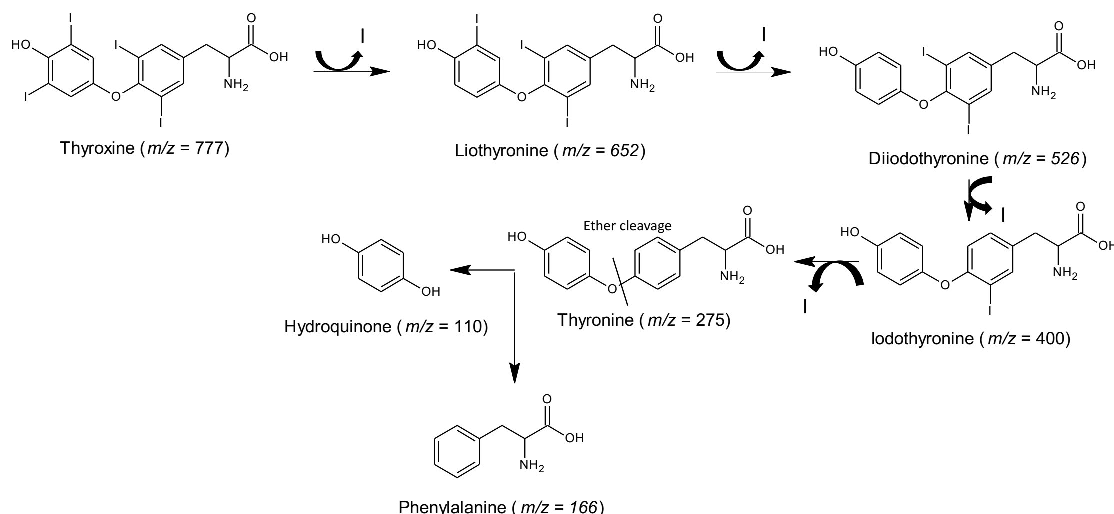


Figure 2-15: Levothyroxine degradation pathway was producing hydroquinone and phenylalanine.

- **Scenario 3: Oxidative deamination process can occur from the chemical structure of the drug, leading to acid formation**

Intramolecular loss of water can occur following an attack by excess oxygen atoms and hydrogen radical abstraction from the chemical structure of levothyroxine. This process leads to oxidative deamination of the levothyroxine chemical structure which, alongside abstraction of the hydrogen radical, leads to the cleavage of the hydrogen bridge between the benzyl and the amino groups. The amino group is thus removed from the chemical structure of levothyroxine. If the water of crystallization is available at this point, an acid (particularly tetra-iodo thyroacetic acid) can form. Iodine loss due to free oxygen attack can subsequently lead to the production of tri-iodo thyroacetic acid [95].

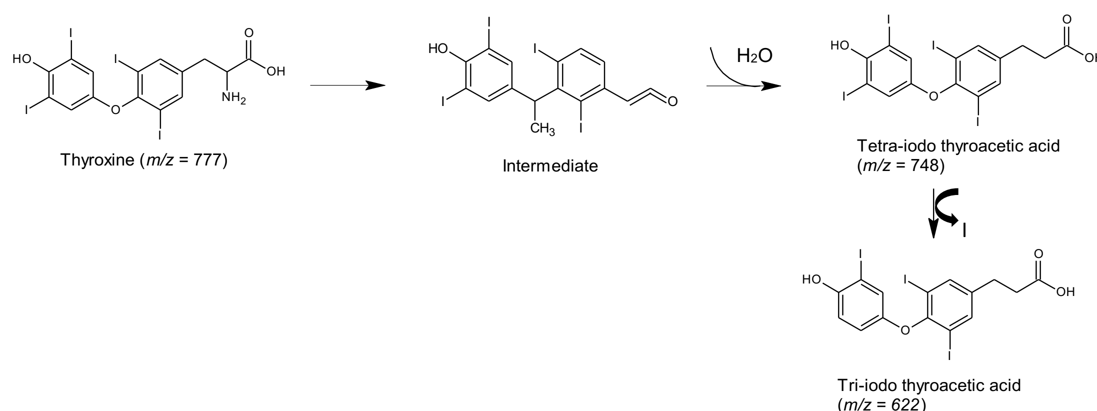
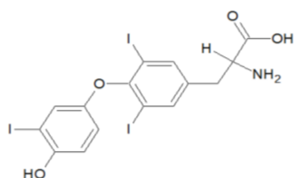


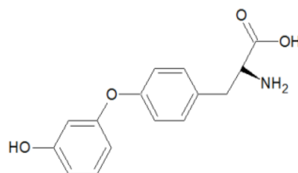
Figure 2-16: Oxidative deamination process of levothyroxine chemical structure degradation.

It is necessary to highlight that, the free radical that attach the chemical structure of levothyroxine and the degradation products that can be obtained from levothyroxine compound mainly relies on the bond dissociation energy (BDE), which is the energy required to degrade the bond of the chemical molecule from the structure. In terms of levothyroxine chemical structure, the bond dissociation energy (BDE) for thyroxine C-I is only 285 [kJ/mol] [94] which is lower than most of the (BDE) of the levothyroxine molecules. This clearly describe that, the degradation products of levothyroxine observed in losing one iodine or more.

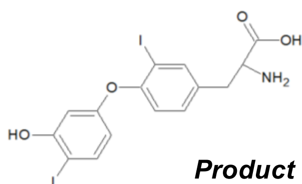
In addition, another study on the degradation products of levothyroxine has reported eight degradation products of levothyroxine which are shown in figure 2.4. These eight degradation products have been analysed on the HPLC to compare them to any impurity or degradation product that observed in the levothyroxine stress condition samples [89].



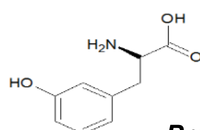
Product A: 3,3´,5-tri-iodo-L-thyronine



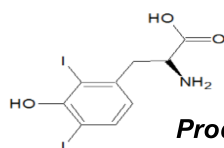
Product B: Thyronine



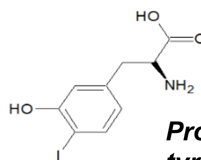
Product C: Di-iodo-thyronine



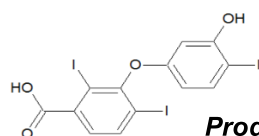
Product D: Tyrosine



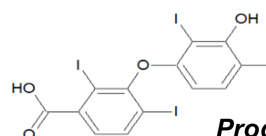
Product E: Di-iodo tyrosine



Product F: Mono-iodo tyrosine



Product G: 3,3´,5-tri-iodo-L-thyroacetic acid



Product H: 3,3´,5,5´-tetra-iodo-L-thyroacetic acid

Figure 2-17: The eight degradation products of-levothyroxine. Absorption and metabolism of thyronine and its metabolic products (A, B and C) have serious implications on patient safety while the other degradation products display no pharmacological activity but do affect product quality (e.g., organoleptic properties such as odor and colour) [8].

As shown in Figure 2.50 some degradation products of levothyroxine are pharmacologically harmful with serious medical implications. Products A and B interfere with normal metabolic functioning while product C can stimulate mitochondrial respiration and affection exchange. Product C can also modulate the transcription of certain genes, while other levothyroxine degradation products display no pharmacological activities [89].

It can be said that, the above three scenarios and the reported degradation products of levothyroxine confirmed what was observed in the stability studies of levothyroxine samples by using HPLC, LC-MS and FT-IR. As on HPLC, levothyroxine samples at room temperature demonstrated that, levothyroxine peak height decreased with increased exposure time of the solid samples to increased storage temperature conditions. Also, when comparing all samples to the t=0 injection sample, the baseline was shown to contain several impurity peaks which are also related to the exposing time of levothyroxine samples to the high temperature which indicated that levothyroxine at room temperature is not chemically stable when stored for more than three months.

However, as levothyroxine stored at 40°C and 70°C for various duration times, the drug concentration and its percentage recovery decreased. Besides, the proportion of the peak impurity increased. In addition, when the levothyroxine sample at this storage condition compared to the eight degradation products of levothyroxine, it has been found that two degradation compounds (tri-iodo-thyroacetic acid at a retention time of about 17 min and tetra-iodo-thyroacetic acid at a retention time of about 19 minutes) have been detected but more investigations on the levothyroxine samples have been done by using LC-MS.

However, at 70°C, t=180 days, the number of impurity peaks has decreased to 7 impurities as they were 17 impurity peaks after 30 days and 13 impurity peaks after 90 days. This is possibly because some of the degradation peaks have fully degraded after long time of storing the samples at 70°C. It has been shown that the number of impurity peaks is unrelated to the storage time of drug samples to high temperature but a reduction in drug sample concentration can be seen in proportion to duration at high temperature, demonstrating how strongly levothyroxine solid samples are affected by high temperatures.

To gain an entirely understandable point of view of the alterations obtained on the levothyroxine samples, LC-MS analysis has become a part of this research. Various impurity compounds or metabolites in drugs samples have been successfully analysed by using mass spectrometry. The most common convenient analytical technique is LC-MS.

LC-MS method is widely used to determine any structural changes, impurities in compounds and degradation products in pharmaceuticals [96,97]. The purpose of using different mobile phase in LC-MS method from the mobile phase to the one employed in HPLC method is because LC-MS mobile phase should be volatile. The LC-MS mobile phase requires to be evaporated prior the ions of the tested samples are passed into the vacuum part of the mass spectrometer. Therefore, acetic acid was used in LC-MS technique [96].

The aim of using LC-MS technique was to characterise the chemical structure of the impurities in the samples that were identified in the levothyroxine samples analysed using the HPLC method. In addition, LC-MS method was useful to detect the effect of high temperature on levothyroxine chemical stability. A solid sample of levothyroxine and its stability standard samples were injected into the LC-MS. The storage conditions of this study were exposure the drug to room temperature, 40°C, and 70°C for t=30 days, t=90 days and t=180 days.

Depending on the three above scenarios that were explained about the degradation products of levothyroxine and the BDE of the levothyroxine bonds, the hypothesis suggests that exposure of levothyroxine to a high temperature of 40°C causes oxidative deamination to occur with no residual crystal water available to penetrate the structure and produce a carboxylic group required to form an acid degradation product (tetra-iodo-thyroacetic acid).

Figure 2.51 shows the identification of three new degradation products from levothyroxine sample that was stored at 40 °C and 70 °C.

A deamination/decarboxylation reaction occurs due to exposure to high temperature leading to the formation of an iodophenol. The BDE for the benzyl C-C bond has been reported to be 285 kJ/mol [94] and the bond is likely to cleave at elevated temperatures leading to the formation of a functional methyl group. A further oxidative reaction due

to free molecular oxygen on the iodophenol leads to the removal of the iodine group and formation of the new three degradation compounds, pathway shown in figure 2.51. The products identified from the degradation of the levothyroxine chemical structure have not yet been investigated or evaluated regarding biological activity in any previous research.

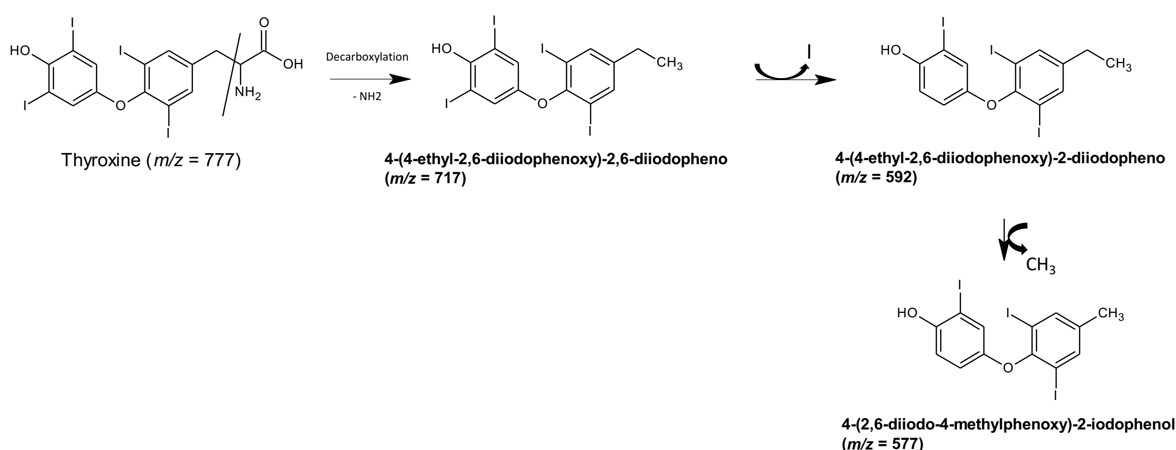


Figure 2-18: Levothyroxine breakdown products elucidated using LC-MS which have not been detected previously

Moreover, when the temperature was increased to 70°C, at short time (t=30 days), the mechanism of action remained the same as the reactions happened when the drug was stored at 40°C. On the other hand, after exposing the drug sample to 70°C for a longer time (t=90 days), levothyroxine by free radical influences had formed diiodthyronine when two iodine groups were removed from the chemical structure of levothyroxine due to iodine low BDE. In addition, following calculation of the molecular masses and at 70°C after 180 days, oxidative decarboxylation still appeared to be present.

The outcome of this chemical stability study showed that levothyroxine underwent oxidative deamination/decarboxylation from the chemical structure. A second potential

reaction is due to extra crystal water present in the molecule that lead to the formation of an acid (tetra-iodo-thyroacetic acid) which was further modified by the removal of iodine group leading to the formation of triiodo thyroacetic acid.

It has been highlighted by the FT-IR data that the degradation of levothyroxine samples occurred slowly, as the changes in the levothyroxine chemical structure at room temperature were limited but at 40°C and 70°C, the aromatic rings and their functional groups were affected by losing some peaks. The data from FT-IR followed similar trend to what was seen on HPLC and LC-MS, which is that levothyroxine is chemically unstable under a stress condition of high temperature. It is necessary to note that, the storage condition of the accelerated stability that performed in this research was achieved under different temperature while humidity was not included. Therefore, future work can be performed under same storage condition that used in this research but with including high humidity condition to observe if the detected degradation peaks can be obtained.

2.1 Conclusion

In this chapter, the main aim was to study the chemical stability of levothyroxine in its solid state and detect any degradation products of levothyroxine. Levothyroxine samples were incubated at three different stress conditions which are 25°C, 40°C, and 70°C for30 days, 90 days and 180 days and analysed using HPLC, LC-MS, and FT-IR.

Oxidation reaction reported clearly on the levothyroxine samples stored at (70°C) which the sample colour changed to brown. This phenomenon was not observed when the levothyroxine sample stored at 25°C but in long period storage at 25°C, levothyroxine was chemically unstable when the sample analysed using HPLC.

The stability study of levothyroxine using HPLC and LC-MS method represented two previously reported degradation products of levothyroxine which are ne (tri-iodo-thyroacetic acid and tetra-iodo-thyroacetic acid) and three new degradation products of levothyroxine that were not reported before. These degradation products of levothyroxine observed when the samples stored at 40°C and 70°C. The hypothesis for these thermal degradation for levothyroxine samples is due to the BDE of the levothyroxine molecules especially the iodine bond as it has low BDE, which can be easily attacked by a free radical and remove the bond from the chemical structure of levothyroxine.

The data obtained from FT-IR confirmed an alteration on the chemical structure of levothyroxine especially in the aromatic rings that contains Iodine groups. The next chapter in this research will focus on the design of levothyroxine fast dissolving oral films formulations that can overcome the issues related to the chemical stability of levothyroxine.

3. Levothyroxine Fast Dissolving Oral Film Formulations (FDOF)

3.1 Introduction

The oral route of the drug administration is classified as one of the most patient compliant due to its convenience and ease of use by patients and inexpensive cost of production. In some cases, oral dosage forms can be difficult for swallowing by a certain group of patients such as geriatric and paediatric patients. Generally, many patients complain about difficulty in swallowing due to unpleasant taste for some tablets and this has been recorded in one study where 26% of 1576 patients suffered from administrating the tablets [98,99].

Specifically, as mentioned earlier in this research (Chapter 1), levothyroxine tablet dosage forms are reported to have chemical instability and low content of uniformity. Therefore, there is a demand to formulate levothyroxine in a different oral dosage form to overcome the issues related to its chemical stability and improve patient compliance.

However, there are many types of oral pharmaceutical dosage forms. One such dosage form that has recently increased in demand is the fast dissolving oral films. The main reasons for such interest in developing these fast dissolving oral film formulations (FDOFs) is due to its fast dissolution and disintegration and rapid administration without using water, which is more convenient to use for the patients and the potential for the drug to be absorbed through the buccal cavity [100,101].

FDOFs were initially developed in 1970’s mainly as mouth fresheners. The idea of designing FDOFs depended on the technology that was used to develop transdermal formulations. While transdermal formulations need to diffuse through the skin layers, FDOFs are administrated orally and dissolve in the oral cavity [102]. The mechanism of action for delivering FDOFs is depends mainly hydrating the formulation by the oral saliva when in contact with the thin layer of the film formulations leading to its disintegration. The FDOFs is then rapidly dissolved and absorbed through the rich capillary network in oral cavity to reach the target site of action [103-105].
Fast dissolving films are mainly designed by using hydrophilic polymers that enhance the solubility of the formulations in the oral cavity and release the active

pharmaceutical ingredient from the formulation. One such hydrophilic polymer is the hydroxypropylmethylcellulose (HPMC), which is available in many different grades [106, 107]. In addition to the hydrophilic polymers, incorporating a plasticiser is necessary for the formulation for the film to possess some degree of flexibility and minimize brittleness. Incorporating a plasticiser can increase film strength of the hydrophilic polymer used in the formulation by reducing the glass transition temperature for the hydrophilic polymers. There are a wide range of pharmaceutical ingredients that can be used as plasticisers such as propylene glycol and glycerine [108].

However, while designing FDOFs, it is necessary to take into account that the formulation requires heat for evaporating the solvent used for preparing the formulation. This can be a main issue in formulating the films as it has been reported that, rapid or uncontrolled evaporation of the solvent from the FDOFs may lead to some cracking on the surface of the films, which would affect the APIs content uniformity. Therefore, it is necessary to monitor the heat and moisture content in order to maintain the formulation stability and provide uniform film formulations [109].

The aim of the study reported in this chapter was initially to develop different types of placebo FDOFs and perform the different characterisation tests to select the ideal combinations of the hydrophilic polymer, plasticizer and other excipients. Depending on these observations, FDOFs containing the active drug (levothyroxine), were then developed and characterised for its physical and chemical properties as well as the drug concentration within the FDOFs. Development of FDOFs would hence overcome the issues related to levothyroxine formulations such as chemical instability and improve drug content of uniformity.

3.2 Methodology

3.2.1 Preparation of levothyroxine FDOFs (Screening of components for placebo FDOF formulation)

Various film-forming polymers and plasticisers were tested by preparing the placebo and active formulations, using the solvent casting technique, for comparing and selecting the appropriate excipients. In solvent casting method, the polymer should be added to the required solvent and all the other excipients dissolved in proper solutions to obtain a homogenous gel. The gel was then casted in petri-dishes and dried at a consistent temperature to form the films [110, 111].

The placebo formulations of fast dissolving films were prepared using three different film weights (10 g 15 g and 20 g) and different compositions of the hydrophilic polymers and plasticizers. Using a solvent mixture of 66.7% water and 33.3% of 0.01M ethanolic sodium hydroxide (400 g NaOH dissolved in 500 ml of water 250 ml of ethanol was added to this solution and mixed thoroughly) with different drying temperatures (25°C, 30°C and 40°C). All the formulations were prepared by solvent casting method. Gels were poured (10 g, 15 g and 20 g) into uniform sized Petri dishes (area 50 cm^2), and weight variation was assessed after drying to select the most appropriate weight. All preliminary evaluations to select the ideal composition and drying temperatures were performed on placebo films. Table 3.1 shows all the placebo formulations developed and tested with respect to drying temperatures, weights and solvent percentages used in the development of active formulations.

Table 3-1: Scanning of component for place fast dissolving formulations

Films code	Polymer(s)	Plasticiser (10% w/w)	Flavouring agent 0.5% (w/w)	Concentration (%) of polymers (w/w)
F1	HPMC(E15)	PG	Menthol	3.0
F2	HPMC(E15)	PG	Menthol	5.0
F3	HPMC(E15)	PG	Menthol	8.0
F4	HPMC(E15)	PEG 400	Menthol	5.0
F5	HPMC(E15)	Glycerol	Menthol	5.0
F6	Xanthan gum	PG	Menthol	3.0
F7	Xanthan gum	PG	Menthol	5.0
F8	Xanthan gum	PEG 400	Menthol	5.0
F9	Xanthan gum	Glycerol	Menthol	5.0
F10	HEC	PG	Menthol	5.0
F11	Carbopol Ultrez 10	PG	Menthol	0.25
F12	HPMC(E50)	PG	Menthol	5.0
F13	HPMC (E50)	PEG 400	Menthol	5.0
F14	HPMC(E15)+ Xanthan gum	PG	Menthol	4.0(HPMC)+1.0(XTN)

The placebo formulations were prepared by adding the hydrophilic polymer into a 400 mL beaker and mixing it with the selected solvent. Plasticiser and flavouring agent were then added with continuous stirring using the overhead stirrer) to obtain a

homogenous viscous solution. The viscous solution was left overnight at room temperature for the polymer to hydrate and form a gel. Any air bubbles that may have formed during the mixing process will also be eliminated in this equilibration process. The gel was then slowly poured into plastic 50 (cm^2) Petri dishes using an analytical balance (AE-Adam-PW124) to achieve the required weight of 10 g. The formulations were then dried at three different temperatures, RT, 30°C and 40°C for 24 h.

The placebos films were characterised regarding mainly their physical appearance such as thickness and gel flow-ability. The gels were poured to uniform petri-dishes and observe if the gels have suitable viscosity to form the films. In addition to that, the films were visually observed after drying at different temperatures. This was achieved by observing the surface of the films to record any crack that can affect the films uniformity.

3.2.1.1 Use of HPMC (E15/E50) and HEC as the polymers:

Plasticiser (10 g), menthol (0.5 g dissolved in 10 g of ethanolic sodium hydroxide, 0.01M) and varying amounts of the polymer HPMC E15, E50 and HEC were used (3% w/w 5% w/w, 8% w/w of HPMC E15), (5% w/w of HPMC E50 and 5% w/w of HEC) to prepare the gels. The formulation mixture was made up to 100 g using ethanolic sodium hydroxide (0.01 M) and mixed using an overhead stirrer. The hydrophilic polymer was added slowly to prevent clumping and stirred until dissolved. A pre-weighed quantity of Plasticiser, glycerol, PEG 400, or PG, was added dropwise, followed by menthol and the mixture was stirred for 30-40 min at room temperature. The solution was removed, labelled, covered and stored at room temperature for 24 h. The next day, the resulting gel was poured into uniform sized of plastic Petri dishes (50 cm^2), to the required weights of 10 g, 15 g or 20 g. The final step was drying of the plates at three different temperatures: which were room temperature, or in an oven at 30°C or 40°C for 24 hr.

3.2.1.2 Use of Xanthan gum as the polymer:

The only difference from the previous preparation (in section 3.2.1.1) is that xanthan gum is freely soluble in water but slightly soluble in the organic solvent; therefore, the

main solvent in this preparation was water instead of the ethanolic sodium hydroxide 0.01M. Therefore, xanthan gum (3% and 5%) was dissolved in water as the main solvent and followed the same preparation of the films as mentioned earlier in the preparation of films using HPMC and HEC.

3.2.1.3 Use of Carbopol Ultrez 10 as the polymer:

Carbopol (0.25 g) was dissolved in 89 g water and stirred until all contents were dissolved followed by the addition of 10 g of PG to the solution. A few drops of 0.1 M sodium hydroxide (NaOH) was added to the solution, and the viscosity was continuously monitored. When a clear gel was formed addition of NaOH was stopped and the beaker was covered and left to rest at room temperature for 24 h. The gel was poured into uniform sized (50 cm^2) of plastic Petri dishes to the required weight of 10 g, 15 g, or 20 g. The final step was drying the plates at three different temperatures as mentioned in Section 3.2.1.1.

3.2.1.4 Use of a combination of HPMC and Xanthan gum (4:1) as the polymer:

Ethanolic sodium hydroxide solution (0.01M: 50 g) was measured accurately into a 400 mL beaker to make up the weight to 100 g followed by the addition of 4 g HPMC and 1 g of xanthan gum, while constantly stirring using an overhead stirrer. In addition, PG (10 g) and menthol (1 g) were accurately weighed and added to the solution, while constantly mixing. The solution was stirred for 30 min in order to dissolve all the solid components and produce a clear viscous solution. The beaker was covered with Parafilm®, and stored at room temperature for 24 h for gel formation. The following day the gel was accurately weighed into uniform sized (50 cm^2) of plastic Petri dishes to the required weights of 10 g, 15 g or 20 g. The Petri dishes were placed at three different temperatures (room temperature, 30°C and 40°C) to dry the gels.

3.2.2 Preparation of levothyroxine FDOFs (Screening of components for formulation of levothyroxine FDOFs)

Three formulation prototypes were selected from the initial placebo FDOFs formulations developed, based on physical appearance, weight variation, texture, and viscosity. The drying temperature selected was 30°C, the drying period was 24 h, and the solvent was ethanolic (33.3%) sodium hydroxide 0.01M. The preparation conditions of FDOFs containing levothyroxine are shown in Table 3.2.

Table 3-2: Composition of levothyroxine FDOFs at a drug loading of 100 µg/cm^2 prepared using menthol (0.5% w/w) as a flavouring agent. The gels were dried for 24 h at 30°C

Polymer	Plasticiser (10%) w/w	Concentration of polymers % w/w
HPMC(E15)	PG	5.0
HPMC(E15)	PEG 400	5.0
HPMC(E50)	PG	5.0

The total weight of the levothyroxine FDOFs was prepared in 100 g gel by the solvent casting technique. Menthol (0.5 g) was dissolved in 10 g of ethanolic sodium hydroxide 0.01M.

Ethanolic sodium hydroxide solution (0.01M: 74.495 g) was measured accurately into a 400 mL beaker to make up the weight to 100 g followed by the addition of 5 g of the polymers and, while constantly stirring using an overhead stirrer. In addition, propylene glycol or poly ethylene glycol (10 g) and menthol (0.5 g) were accurately weighed and added to the solution, while constantly mixing. The solution was stirred for 30 min in order to dissolve all the solid components and produce a clear solution. Levothyroxine (5 mg) was added to the solution and it was stirred for another h to dissolve the API. The beaker was covered with Parafilm®, and stored at room temperature for 24 h for gel formation. The following day the gel was accurately

weighed into uniform sized (50 cm^2) plastic Petri dishes to the required weights of 10 g and were placed at 30°C to dry the gels for 12 h, 18 h and 24 h. The drying period of 24 h was selected as the films after 24 h drying showed better physical appearance.

3.2.3 Levothyroxine FDOFs evaluation

3.2.3.1 Compatibility study using FTIR

Levothyroxine solid state at t=0 samples and levothyroxine FDOFs were studied using FT-IR to investigate excipient compatibility. The study was performed using a Spectrum 65 FT-IR spectrometer with wavelength between from 650 cm^{-1} and 650 $cm^{-1.}$ The FT-IR resolution used in this experiment was cm^{-1} and background scan was run at an average of 12 scans. Besides the background scan, a scan of monitoring the samples was run before each levothyroxine sample.

Before placing any drug or the formulation, the stainless-steel disc of the FT-IR was cleaned using 100% of methanol and the disk was completely dried before analysis. In terms of the solid state levothyroxine samples, a small amount of the pure API was placed into the diamond of the disk and the swing arm was positioned near the sample to be tested and screwed down to touch the sample to view the spectra. The %T was between 30-40%. In the final step, the drug spectra were obtained by selecting the scan icon. When the spectrum had been displayed, and the instrument had completed the scan process, 'data tune up' was performed to yield the optimum baseline. The same performance of the experiment work was done on the levothyroxine FDOFs formulations by taking approximately 1 cm x 3 cm and placed it in the centre of the disc part of the FT-IR. Three levothyroxine FDOFs formulations were tested by FTIR which are; F1: levothyroxine FDOFs formulation using 5% of HPMC E15, F2: levothyroxine FDOFs formulation using 8% of HPMC E15 and F3: levothyroxine FDOFs formulation using 5% of HPMC E50.

3.2.3.2 Levothyroxine FDOF uniformity of weight:

An analytical weighing balance (AE-Adam-PW124) was used to test the weight variation of the films formulations. 1 x 1 cm^2 was cut from three different batches of each levothyroxine formulation and the average weight of these levothyroxine FDOFs along with standard deviation was calculated [112].

3.2.3.3 Film thickness measurement:

The thickness of levothyroxine FDOFs was measured using a Vernier caliper on three separate film batches of the same formulation; the mean film thickness and standard deviation were then calculated. The test was performed by measuring the thickness along the four different edges of each film A homogenous film thickness indicates that the drying process of the film has been uniform [113].

3.2.3.4 Folding endurance of FDOFs:

A folding endurance test provides an indication of film brittleness, which is calculated by the number of times a film is folded without breaking is expressed as the “folding endurance value”. Films were repeatedly folded by hand in opposite directions from the same central point until a crack or fracture was observed. The number of folds that the film can withstand before the film breaks should be more than 300 times, indicating the flexibility of the film [112, 114]. Three batches from each formulation of strip size 1 cm x 3 cm were tested (folded from the centre of each film by using the right hand), and the average number of times before the film cracked or fractured was calculated along with the standard deviation.

3.2.3.5 Surface pH of FDOFs:

The purpose of this experiment was to avoid oral mucosal irritation, given that the films were intended for use in the oral cavity. FDOFs were therefore required to have a pH close to neutral levels. The experiment was done by immersing the film formulations of levothyroxine (n= 3 for each levothyroxine FDOFs) from each formulation in 0.5 ml of water for 30 sec and measuring the pH [115, 116]. Each film was placed in the (50 cm^2) plastic Petri dishes followed by the addition of water and placing the pH electrode on the surface of the films and the pH reading was recorded.

3.2.3.6 Determination of FDOF *in vitro* disintegration time:

This test was used to determine the time needed for the films to disintegrate and release the API into the oral cavity. However, this experiment was visually determined using a 1 cm x 3 cm film strip (n=3 of each levothyroxine film), which was placed in (50 cm^2) of plastic Petri dish containing 10 ml of deionised water. The disintegration time was recorded at the point that the film piece started to disintegrate [112].

3.2.3.7 Percentage moisture loss from FDOF upon storage:

This assessment was performed by weighing three film batches of 1 cm x 3 cm in a beaker containing anhydrous calcium chloride for three days and then reweighing the films. This experiment was conducted under normal temperature conditions (room temperature) and relative humidity [117]. The percentage moisture loss was then calculated using the following formula:

$$\% \; moisture \; loss = \frac{initial \; weight \; of \; film - final \;\; weight \; of \; film}{initial \; weight \; of \; film} \; X \; 100$$

3.2.3.8 levothyroxine FDOFs drug content:

Levothyroxine FDOFs containing 0.5 g/film (theoretical amount) were dispersed in 500 ml ethanolic sodium hydroxide (0.01M) in a volumetric flask with constant stirring to obtain a solution with 1 mg/ml and diluted to obtain a solution with 10 µg/ml. Once the film had completely dissolved, drug concentration in the solution was measured using UV spectrophotometry at 225 nm. Standard solutions of levothyroxine were prepared in the same solvent at a range of concentrations between 1 µg/ml to 10 µg/ml. Levothyroxine FDOFs concentrations were calculated using the calibration curve prepared. The acceptance criteria for content of uniformity levothyroxine in FDOFs is 85% to 115% [118, 119].

3.2.3.9 Determination of *in vitro* dissolution profile of levothyroxine from FDOFs

A study of the dissolution profile of the prepared FDOFs is essential to investigate the percentage of drug released over a period of time from the formulations. An *in vitro* dissolution test was performed using USP II paddle dissolution apparatus [20]. Each levothyroxine FDOFs formulations that has 0.5 mg of levothyroxine (n =3) was placed in a solution containing pH 6.8 phosphate buffer (300 ml) at 37°C. An aliquot of 5 ml of the dissolution medium was taken at different time points for analysis and replaced with an equal aliquot of buffer. The time points used in this analysis were 1, 2, 3, 4, 6, 8, and 10 min. Samples were filtered using 0.22μm filters and diluted with 4 ml of (33%) ethanoloc sodium hydroxide 0.01M. All the diluted samples then were vortexed for 1 min and were analysed using UV spectrometry at 225 nm.

3.2.3.10 Mechanical properties of levothyroxine fast dissolving films

- **Tensile strength of FDOFs:**

Tensile strength is the maximum stress applied to the point at which the film breaks. Tensile strength was determined for both the placebo and active formulations. The experiment was performed by cutting each of the levothyroxine FDOF formulations (n=3) into 1 cm x 3 cm pieces and were located between the clamps of the texture analyser, where the distance in the position of the clamps was 3 cm. During the test, the film piece was stretched until it broke and the stress applied in this point was recorded. The average reading of three fast dissolving films was taken as the tensile strength. Tensile strength was calculated by the following equation [112]:

$$Tensile\ strength = \frac{(break\ force)}{elongated\ length\ of\ the\ film}$$

- **% Elongation of FDOFs:**

Percentage elongation is the increase in length of a film when stretched. It refers to the strain that can be imposed on the film, and is a valuable tool for determining the concentration of the plasticiser that can be used for developing the formulations. The test is mainly based on the tensile strength determination of the film as discussed in the previous section. The test was performed by placing a levothyroxine FDOF formulations (1 cm x 3 cm pieces, n = 3) between the clamps of the texture analyser and stretched. The length that the film can be stretched before it broke was recorded. Percentage elongation is calculated using the following equation [112]:

$$\% \, Elongation = \frac{increase \ in \ film \ length}{original \ length \ of \ the \ film} \ X \ 100$$

3.3 **Results**

3.3.1 Preparation of placebo formulations of FDFOs

Different hydrophilic polymers and various plasticisers were investigated in the development of fast dissolving films formulations (FDOFs) that can be used to produce levothyroxine FDOFs. The main hydrophilic polymers investigated were; HPMC E15, HPMC E50, xanthan gum, HEC and carbopol Ultrez 10 at different concentrations (0.25% w/w, 3% w/w, 5% w/w and 8% w/w) in the formulation to optimize visually from the physical appearance of the gel to good flow-ability of gel that can form clear transparent films.

The main plasticisers used in this research were Polyethylene glycol 400, propylene glycol and Glycerol which were prepared firstly in small scale in a mixture with different hydrophilic polymer percentages (5% w/w, 10% w/w and 20% w/w with respect to the amount of plasticiser used in the formulation). Visual observations indicated that 10% w/w of plasticiser was essential to form a flow-able gel for most formulations especially when using HPMC E15 and HPMC E50 as the hydrophilic polymers.

Drying temperatures investigated in the developmental stage for preparing the films were 25°C, 30°C and 40°C and it was found that, at 25°C the film took a long time to dry, approximately 2 days while at 40°C, the film showed cracks on the surface after 24 h, which was not observed when the films were dried at 30°C for 24 h.

Table 3.3 shows the number of placebo formulations that were investigated for their physical properties from the visual observations such as gel flow-ability and dry film appearance. The observations indicated that the films formed using HPMC resulted in the formation of viscous solutions with mostly a medium and high flow which was visually observed and that is important to form the gel that yields the films. The term of low gel flow ability referred to the gel which has viscosity level that significantly difficult to pour the gel into the uniform petri dish. While the medium gel flow ability is the gel that has viscous properties but as well it can be poured into the uniform petri dish. The high gel flow ability is the gel which is low viscous and it can be easily poured into the uniform petri dishes to form the films However, 3% w/w of HPMC in the formulations resulted in a visually less viscous solution (low flow) while 5% w/w

of HPMC resulted in the formation of a slightly more viscous solution (medium flow). In contrast, xanthan gum and carbopol Ultrez 10 form gel with very high and very low flow, respectively. The physical appearance for most of the HPMC formulations showed the formation of clear gels compared to the opacity observed with gels prepared using xanthan gum and carbopol Ultrez 10. However, using hydroxyl ethyl cellulose has resulted in the formation of opaque gel, but with low viscosity that was visually determined. Hence, two different types of HPMC were used to prepare the gels containing levothyroxine for FDOFs: HPMC E15 and HPMC E50.

Table 3-3: Physical appearance of placebo FDOF formulations by using the visual observation

Polymer + plasticiser used	Concentration (% w/w)	Appearance	Visual determination of gel viscocity properties
HPMC(E15) + PG	3.0	Clear	Low
HPMC(E15) + PG	5.0	Clear	Medium
HPMC(E15) + PG	8.0	Clear	Medium
HPMC(E15) + GLY	5.0	Clear	High
HPMC(E15) + PEG400	5.0	Clear	High
XANTHAN GUM + PG	5.0	Opaque	Zero flow
XANTHAN GUM + PG	3.0	Opaque	Zero flow
XANTHAN GUM + PEG400	5.0	Opaque	Very low
XANTHAN + GLY	5.0	Opaque	Very low
CARBOPOL + PG	0.25	Opaque	Very high
HEC + PG	5.0	translucent	Very High
HPMC(E50) + PEG400	5.0	Translucent	High
HPMC(E50) + PG	5.0	Translucent	Medium
HPMC(E15)/Xanthan gum + PG	4.0(HPMC)+1.0(XTN)	Translucent	High

Menthol was used in the development fast dissolving film formulations as a flavouring agent. In the early stage of development of the films, menthol was used at 1 %w/w. However, the concentration of menthol was found to be unsuitable as it was observed that upon drying of the gel at 1 %w/w menthol concentration, presence of crystals was observed on the film's surface, as shown in the optical microscope picture Figure 3.1. Therefore, the percentage of the menthol in the FDOFs formulations was decreased to 0.5 %w/w.

Figure 3-1: : 5 %w/w HPMC (E15) placebo film showing the presence of large crystal structures on the surface indicating that menthol at 1 %w/w concentration might have precipitated.

Figure 3.2 compares the formulations of placebos FDOFs containing different hydrophilic polymers and plasticisers. It was visually observed that using HPMC in the formulation of FDOFs showed better film physical appearance than when the films were prepared using xanthan gum or HEC.

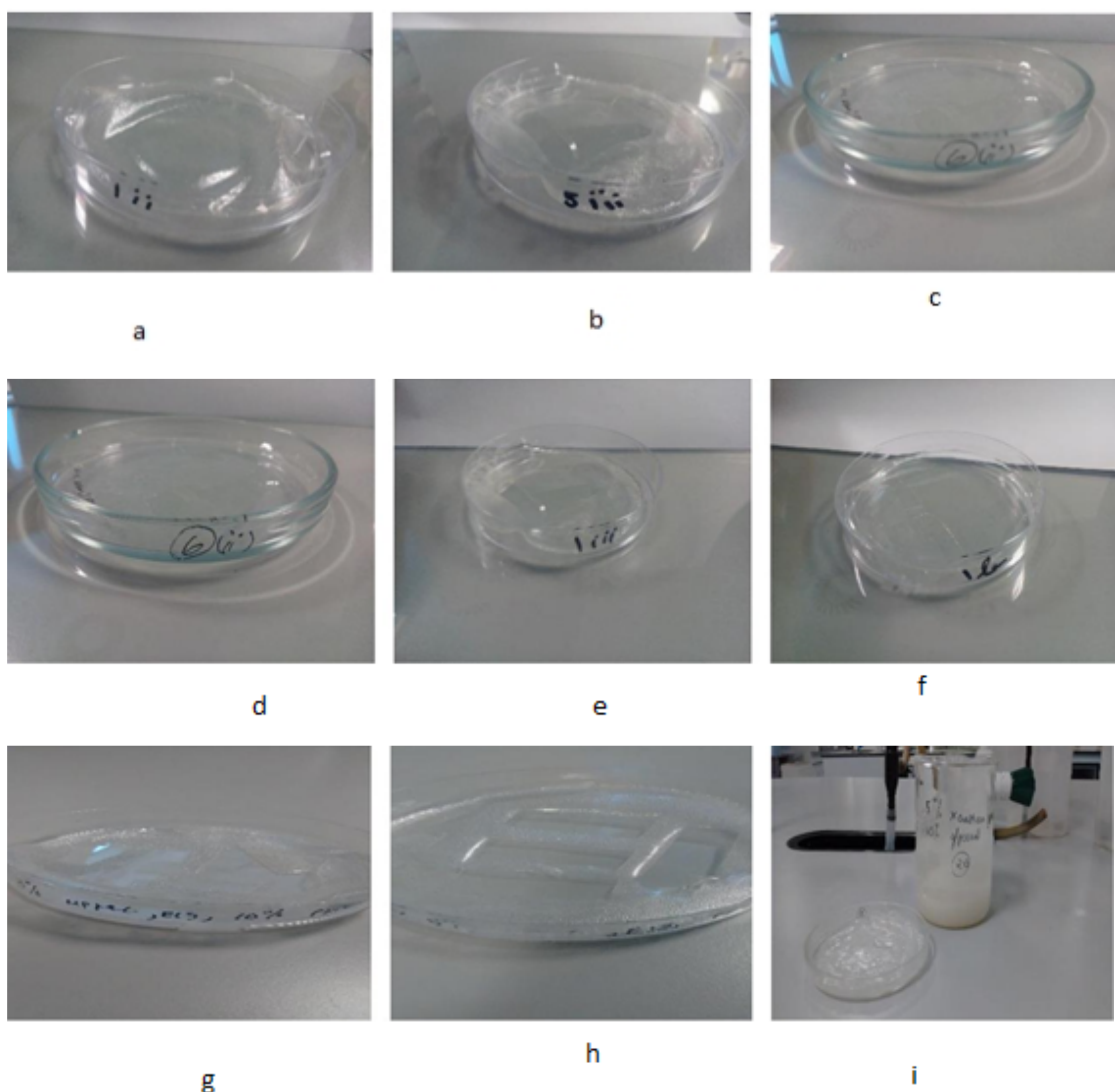


Figure 3-2: Development of placebo FDOF formulations. Individual photographs show the visual appearance of the films formed when using different hydrophilic polymers along with different plasticisers. a: 5% HPMC (E50) + PG; which looks uniform b: 5% HPMC (E15) + PG which looks thicker than (a) but transparent and uniform ; c: 3% HPMC (E15) + PG which looks very thin; d: 5% HPMC (E15) + glycerol which is viscous film and not completly uniform; e: 5% HPMC (E15) + PEG400 which looks transparent but very thick due to high viscocity; f: 8% HPMC (E15) + PG which looks transparent ; g: 5% HPMC (E50) + PEG400 which is approximately transparent and not thick film; h: 5% HEC + PG which is not uniform and not transparent; i: 4% HPMC (E15)/ 1% xanthan + PG which is not transparent and not uniform. Visual observations indicate that films formed using HPMC as the polymer had a clear physical appearance and good texture.

3.3.2 Preparation of levothyroxine FDOFs formulations

Based on the physical appearance of the placebo formulations and the visual observations, levothyroxine fast dissolving films were developed using three placebo formulations for guidance. The formulations developed were: (F1) 8%w/w HPMC E15, (F2) 5%w/w HPMC E15 and (F3) 5%w/w HPMC E50. All the developed formulations

had the same amount of propylene glycol (PG) as the plasticiser (10%w/w) and menthol as the flavouring agent (0.5%w/w).

Figure 3.3 shows the three active formulations of levothyroxine fast dissolving oral films (FDOFs) formed by drying the gels at 30°C for 24 h, resulting in the formation of a clear flexible film with no visual appearance of any cracks on the film surface. However, all the developed levothyroxine FDOFs resulted in the formation of a clear film with fully dissolved drug particles, observed visually at this stage before performing further FDOFs characterisation tests. In addition, the films were observed to be very thin and showed a good flexibility when folded manually. Therefore, these formulations of levothyroxine FDOFs were ready to undergo further investigations by performing the required film characterisation tests.

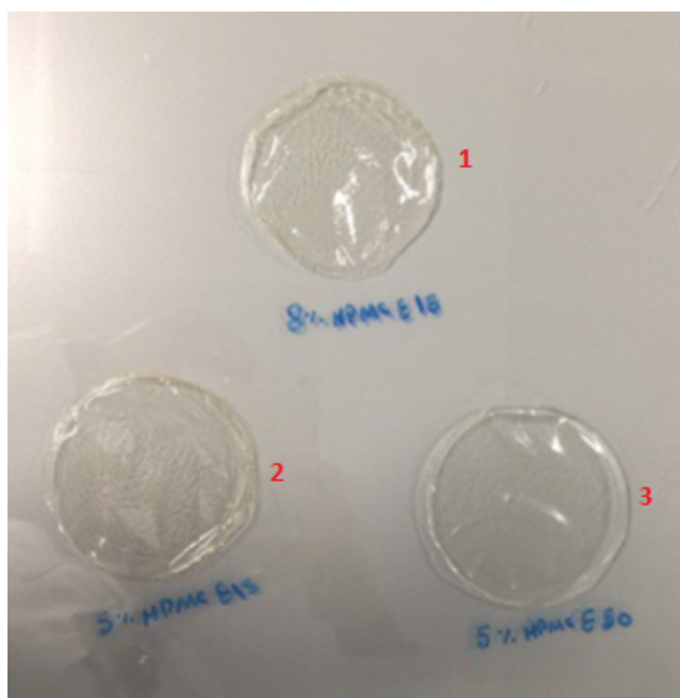


Figure 3-3: Levothyroxine FDOFs. (1): 8%w/w HPMC E15, (2): 5%w/w HPMC E15, (3): 5%w/w HPMC E50. Visual observations indicated that films formed were clear in physical appearance and texture

3.3.3 Evaluation of levothyroxine FDOFs formulations

3.3.2.1 Investigation into the Compatibility of levothyroxine with polymers using FTIR

Drug-polymer interaction between levothyroxine and the polymer was studied using FTIR. The three levothyroxine fast dissolving film formulations were analysed by FTIR and the results are shown in Figures 3.4. The terms F1 refers to the formulation of 5% HPMC E15- levothyroxine FDOFs, F2 refers to the formulation of 8% HPMC E15- levothyroxine FDOFs and F3 refer to the formulation of 5% HPMC E50- levothyroxine FDOFs.

The spectrum of levothyroxine at t=0 was compared against the spectrum of 5% HPMC E15 containing levothyroxine FDOF against the spectrum of 8% HPMC E15 containing levothyroxine FDOF and against the spectrum of 5% HPMC E50 containing levothyroxine FDOF.

The FT-IR of the levothyroxine at t=0 was characterised by the N-H stretching at 3266 cm^{-1}, O-H stretch at 3076 cm^{-1}, and C=O stretching at 1603 cm^{-1} to confirm the carboxylic acid and amine groups in the chemical structure of levothyroxine. Ether stretching was observed at 1183 cm^{-1}, and the aromatic rings in the pure drug chemical structure were observed at 827 cm^{-1} and 849 cm^{-1}. The FT-IR spectra of levothyroxine FDOFs formulation containing 5%w/w of HPMC E15 polymer showed N-H stretching at 3415 cm^{-1}, O-H stretching at 2930 cm^{-1}, C=O stretching at 1603 cm^{-1}, and two aromatic rings stretching at 828 cm^{-1} and 849 cm^{-1}.

The FT-IR spectra of the levothyroxine FDOFs formulation containing 8%w/w HPMC E15 showed N-H stretching at 3413 cm^{-1}, O-H stretching at 2930 cm^{-1}, C=O stretching at 1604 cm^{-1} and two aromatic rings stretching at 828 cm^{-1} and 849 cm^{-1}.

However, The FT-IR spectra of the levothyroxine FDOFs that was formulated using 5%w/w HPMC E50 indicated N-H stretching at 3432 cm^{-1}, O-H stretching at 2907 cm^{-1}, C=O stretching at 1603 cm^{-1} and two aromatic rings stretching at 829 cm^{-1} and 850 cm^{-1}.

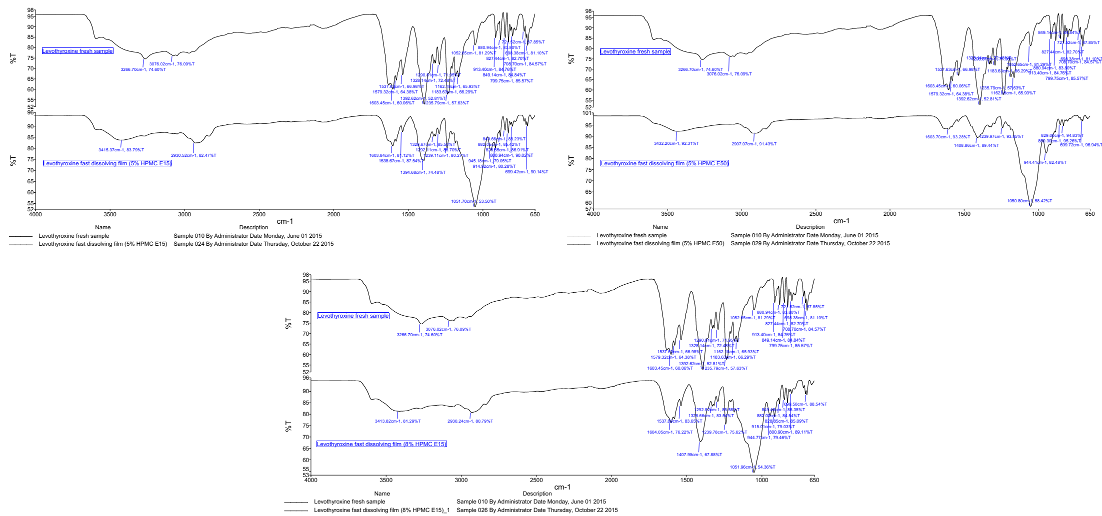


Figure 3-4: (Left): FT-IR spectra of pure drug (levothyroxine) and levothyroxine FDOF formulation containing 5%w/w HPMC E15. Identical spectral profile for levothyroxine was observed for the drug and the FDOF. (Right): FT-IR spectra of pure drug (levothyroxine) and levothyroxine FDOF formulation containing 8%w/w HPMC E15. Identical spectral profile for levothyroxine was observed for the drug and the FDOF. (Bottom): FT-IR spectra of pure drug (levothyroxine) and levothyroxine FDOF formulation containing 5%w/w HPMC E50. Identical spectral profile for levothyroxine was observed for the drug and the FDOF.

The three FDOF formulations were further characterised by testing for the drug content, weight variation, thickness measurement, folding endurance, surface pH, *in vitro* disintegration and percent moisture content.

3.3.2.2 Determination of uniformity of weight of levothyroxine FDOFs:

Each piece of 1 cm x 1 cm should have a theoretical weight of 41 mg/ cm^2 (after film dried, about 2.050 g should be the weight of the API and the excipients in 50 cm^2 of the petri dish). with low variation in weight between the batches of the same formulation. it can be observed that the actual film weight for the formulation F1 was 39 ± 1 mg/ cm^2 while for the formulation F2 it was 39.12 ± 1.8 mg/ cm^2 and it was 38.98 ± 2.2 mg/ cm^2 was the uniformity of film weight for the formulation F3. The data shows that F1 has the least variation in film weight while F3 shows the higher variation. All formulations were tested as n=3. However, it was observed that there was no statistically significant difference in the amount of drug content between the three formulations ($p > 0.05$, T-test, two tailed).

3.3.2.3 Determination of film thickness:

Thickness of levothyroxine FDOFs was determined using a Vernier callipers. Thickness of each levothyroxine FDOF formulation was measured at the four corners of the film (n=3). Figure 3.8 shows that the thickness of the developed levothyroxine FDOFs was slightly different between each formulation. As shown in Figure 3.7, the average thickness for the F1 formulation was 0.10 ± 0.005 mm while the average thickness for the F2 formulation was 0.15 ± 0.02 mm and the average thickness for the F3 formulation was 0.11 ± 0.007 mm. However, all the formulations showed small variation in thickness measurements with formulation F2 being thicker than the other two formulations.

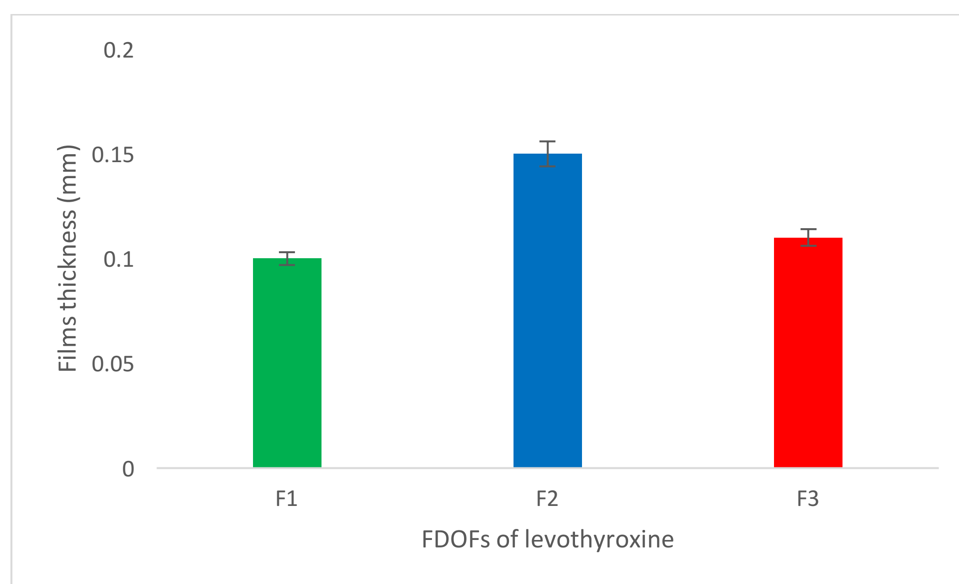


Figure 3-5: Thickness of levothyroxine FDOFs for three developed formulations. F1 is 5% HPMC E15- levothyroxine. F2 is 8% HPMC E15- levothyroxine. F3 is 5% HPMC E50- levothyroxine (mean ± SD, n = 3). Statistically, one-way ANOVA showed p value is 0.0072, considered very significant difference film thickness between the developed formulations

3.3.2.4 Folding endurance of FDOF formulations:

Folding endurance for the three FDOF formulations of levothyroxine is shown in Figure 3.9. The test was done manually (n=3) for each formulation and all the formulations showed good flexibility as the crack appeared on the centre of the film after the film was folded more than 300 times. Figure 3.8 shows the folding endurance for F1 was

344 ± 1 while for F2 it was 341.3 ± 1.5 times to fold the film until it cracked and for F3 it had a folding endurance of 339.7 ± 2.08 times.

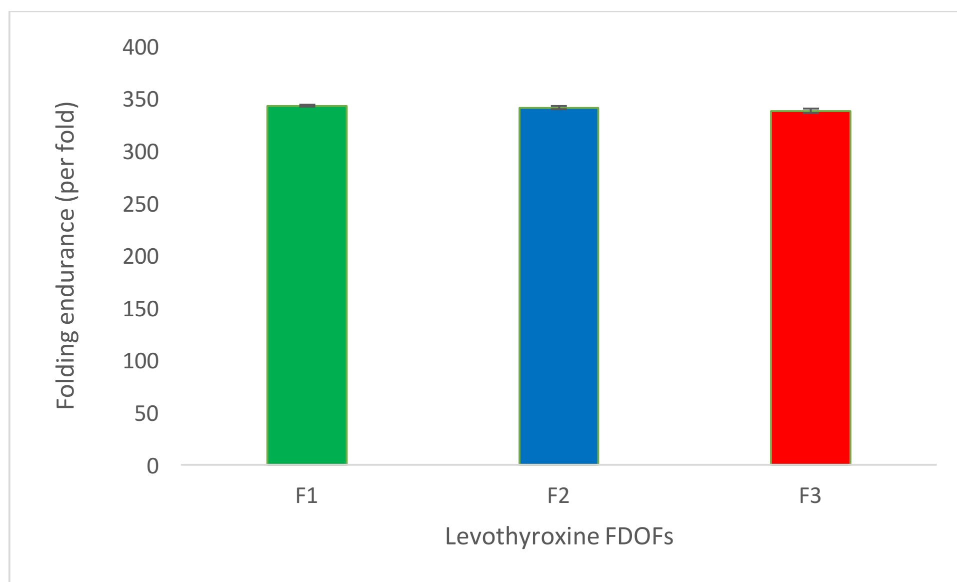


Figure 3-6: folding endurance of levothyroxine FDOFs for three developed formulations. F1 is 5% HPMC E15- levothyroxine. F2 is 8% HPMC E15- levothyroxine. F3 is 5% HPMC E50-levothyroxine (mean ± SD, n = 3). Statistically, one-way ANOVA showed p value is > 0.9999 considered no significant different films regarding the folding endurance test.

3.3.2.5 Surface pH of FDOF formulations:

All the developed formulations of levothyroxine FDOFs indicated neutral pH value which is ideal to place the film inside the oral cavity. Figure 3.9 shows that the surface pH of F1 was 6.45 ± 0.02 while for formulation F2 it was 6.40 ± 0.08 and for F3 the surface pH was 6.60 ± 0.09. All formulations were tested as n=3

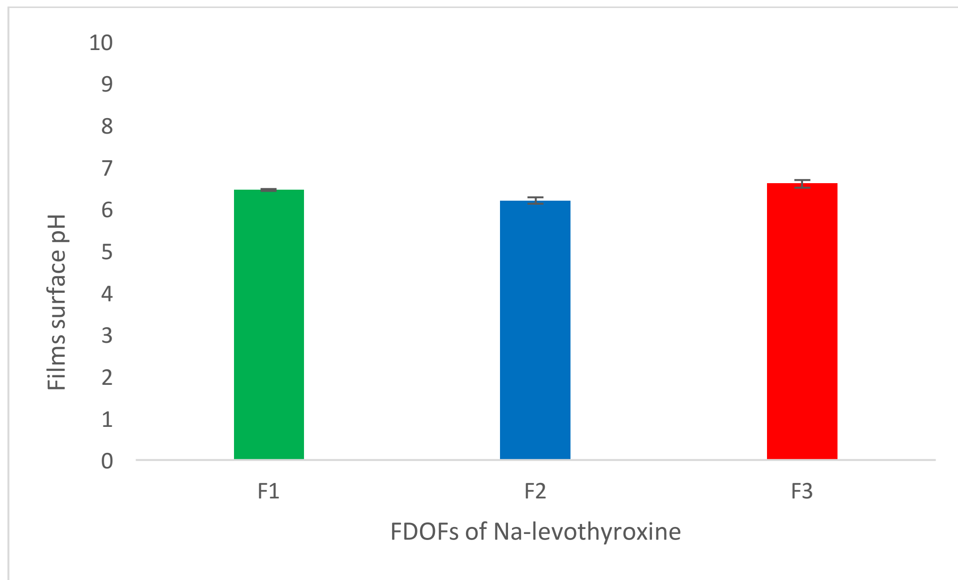


Figure 3-7: Surface pH study of levothyroxine FDOFs for three developed formulations. F1 is 5% HPMC E15- levothyroxine. F2 is 8% HPMC E15- levothyroxine. F3 is 5% HPMC E50- levothyroxine (mean ± SD, n = 3). Statistically, one-way ANOVA showed p value is > 0.9999 considered no significant different films regarding the surface pH.

3.3.2.6 *In vitro* disintegration time of FDOF formulations:

In vitro disintegration time of the developed formulations was determined by immersing a 1 cm x 3 cm piece of each film (n=3) in 10 ml of water. All the films started to disintegrate in less than one min, which indicates a fast disintegration period. Figure 3.10 shows that formulation F1 disintegrated within 18 ± 1 sec, while formulation F2 disintegrated within 19.67 ± 1.55 sec and for formulation F3 it required 19.13 ± 0.58 sec to disintegrate.

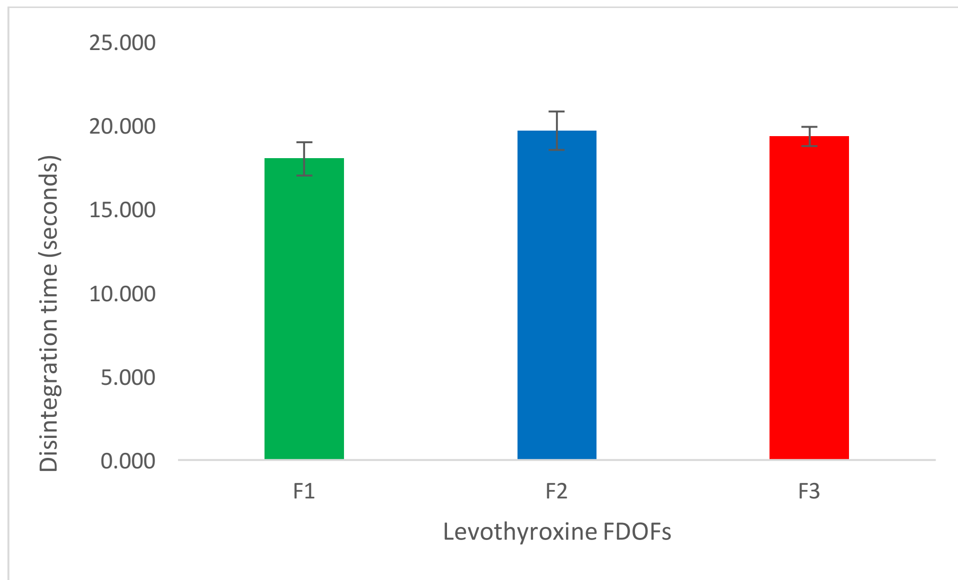


Figure 3-8: In vitro disintegration study of levothyroxine FDOFs for three developed formulations. F1 is 5% HPMC E15- levothyroxine. F2 is 8% HPMC E15- levothyroxine. F3 is 5% HPMC E50- levothyroxine. (mean ± SD, n = 3). Statistically, one-way ANOVA showed p value is > 0.9999 considered no significant different films regarding the *In Vitro* disintegration time

3.3.2.7 Percentage moisture loss from FDOF formulations upon storage:

The percentage moisture loss in the developed formulations was determined as explained in section 3.2.3.7. and the results are shown in figure 3.11. Formulation F1 showed a moisture loss of 1.417 ± 0.015 % while formulation F2 revealed a moisture loss of 1.917 ± 0.055 % and formulation F3 revealed a moisture loss of 1.517 ± 0.015 % when the formulations were incubated in a beaker containing anhydrous calcium chloride for three days and then reweighing the films. All formulations were tested as n=3

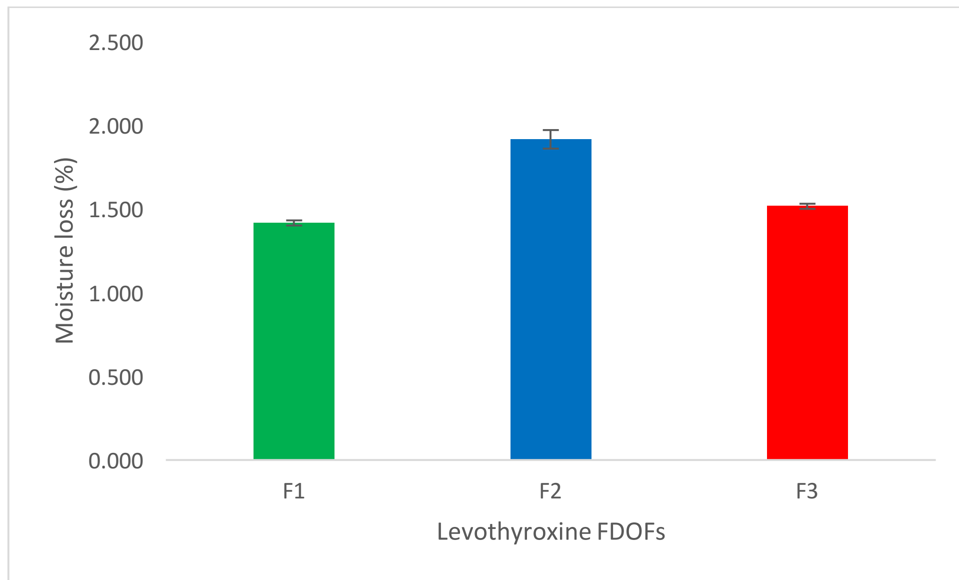


Figure 3-9: Moisture percentage loss study of levothyroxine FDOFs for three developed formulations. F1 is 5% HPMC E15- levothyroxine. F2 is 8% HPMC E15- levothyroxine. F3 is 5% HPMC E50- levothyroxine (mean ± SD, n = 3). Statistically, one-way ANOVA showed p value is > 0.9999 considered no significant different films regarding moisture loss92.65.

3.3.2.8 Content of uniformity of levothyroxine FDOFs

The percentage range of levothyroxine content in the developed formulations was 91.42 ± 0.72% to 97.32 ± 0.81%. F1 contains the highest drug content while F3 has the lowest drug content (Figure 3.12). All formulations were tested as n=3

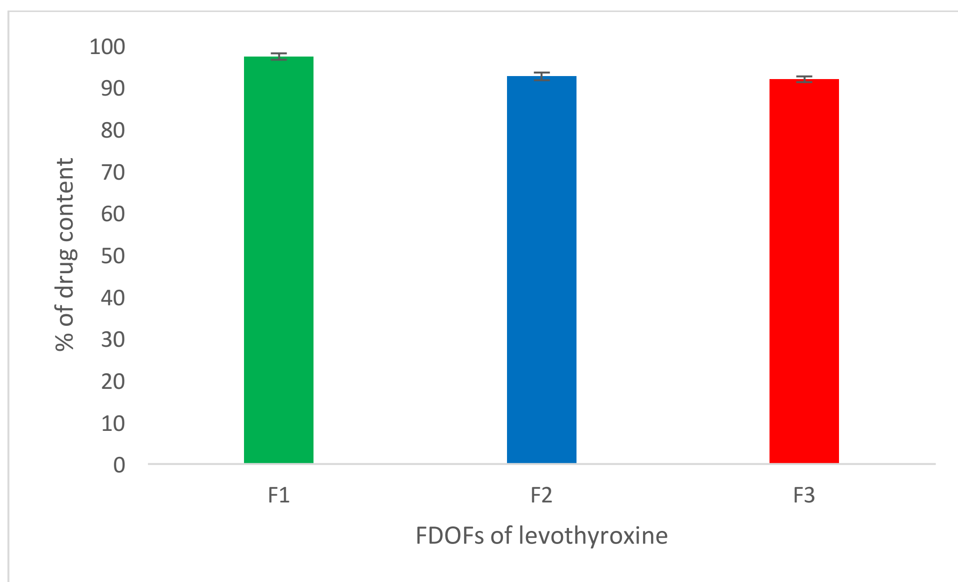


Figure 3-10: drug content of levothyroxine FDOFs for three developed formulations. F1 is 5% HPMC E15- levothyroxine. F2 is 8% HPMC E15- levothyroxine. F3 is 5% HPMC E50- levothyroxine (mean ± SD, n = 3). Statistically, one-way ANOVA showed p value is > 0.0003 considered extremely significant different films regarding the drug content.

3.3.2.9 In vitro dissolution profile of levothyroxine FDOFs formulations

In vitro dissolution profile of levothyroxine FDOFs is shown in figure 3.14. All three formulations disintegrated in the dissolution medium within the first min. However, it took 10 min for the entire drug to go into solution. All three formulations showed identical release profiles. The mean percentage release of the drug from the formulations at 10 min was between 95% and 100% for all three formulations. From figure 3.14 it can be observed that Formulation F1 shows the amount of drug released after 10 min was 99.31% while for formulation F2 it was only 95.2% drug released after 10 min and for formulation F3 it was 98.01% drug release after 10 mins. In terms of the first min, the amount of drug released from F1 was 33.03% while in 28.47% and around 20% in F3. All formulations were tested as n=3

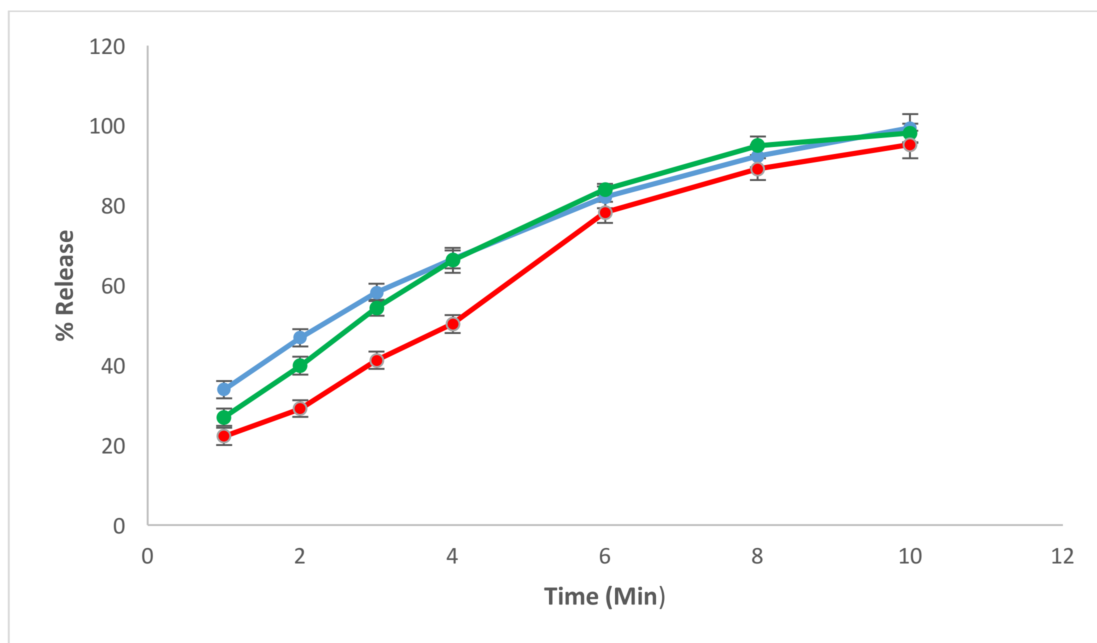


Figure 3-11: Dissolution profile of levothyroxine FDOF formulations. Percentage drug release from the formulations. (*F1, *F2, *F3) (mean ± SD, n = 3). Statistically, one-way ANOVA showed p value is > 0.9999 considered no significant different films regarding the dissolution study.

3.3.2.10 Mechanical properties of levothyroxine FDOFs formulations

The mechanical properties (percentage elongation and tensile strength) of levothyroxine FDOFs were also determined. This particular test gives information on the physical properties about durability, strength, and stress and strain when the film might be handled by an end user. The percentage elongation and tensile strength were determined by placing the FDOFs in a texture analyser and stretching the film until it breaks. Formulation F1 had a tensile strength value of 15.80 N/mm ± 2.7 and a percentage elongation of 14.37% ± 2.2. Formulation F2 had a tensile strength of 18.11 N/mm ± 3.1 and percentage elongation of 16.28% ± 1.9 while for formulation F3 had a tensile strength of 16.08 N/mm ± 1.1 and percentage elongation of 13.40% ± 1 (Table 3.4). All formulations were tested as n=3

Table 3-4: The mechanical properties of the levothyroxine fast dissolving oral films formulations (mean ± SD, n = 3).

Formulations	Tensile strength(N/mm)	% elongation
F1	15.80 ± 2.7	14.37 ± 2.2
F2	18.11 ± 3.1	16.28 ± 1.9
F3	16.08 ± 1.1	13.40 ± 1

3.4 Discussion

The hydrophilic polymers tested included the commonly used excipients HPMC E50 and E15, HEC, xanthan gum, carbopol, and a mixture of HPMC and xanthan gum. All these polymers have suitable gel forming abilities and are used for oral, topical, parenteral and other routes of drug administration [120]. HPMC, xanthan gum, polyvinyl alcohol and other polymers have previously been reported in the development of FDOFs prepared by solvent casting method containing different drugs such as the antihypertensive drug, Telmisartan [121], anticholinergic drug Dicyclomine [122] and a sedative Diazepam [123] to name a few examples. In addition to FDOF formulations hydrophilic polymers are also widely used as a rate controlling film in matrix for controlled release [124] and sustained release [125] tablets and as a mucoadesive polymer for intranasal drug delivery [126].

Plasticisers investigated in the development of FDOFs include PG, PEG400 and glycerol. Plasticisers afford the film flexibility and durability during production, storage and usage by reducing the glass transition temperatures of the polymers used in the production of the films [127]. A wide range of plasticisers such as propylene glycol, glycerol, pectic, polyvinyl alcohol etc. have been investigated for their ability to afford film flexibility and durability during the production of FDOFs [121, 126].

Organoleptic properties such as taste and smell are key parameters while evaluating the acceptability of a formulation by the end user, which is patient compliance. Artificial and synthetic sweeteners, fruit flavours both natural and artificial play a predominant role in the development of oral dosage forms. This is particularly important when developing a dispersible tablet or fast dissolving oral film where the patient will taste the medication. Therefore, it is critical to select a flavouring agent that is widely used and accepted in the food industry. The flavouring agent investigated in this study was menthol, which has been reported to be used in the development of FDOF formulations containing an antihypertensive drug [121]. It was decided to select these specific polymers, plasticisers and flavouring agent due to their ability to form gels that could then be formulated as FDOFs.

Water is a commonly used primary solvent in the development of FDOFs formulations mainly due to ease of hydration of the hydrophilic polymers leading to the formation of

viscous solutions and subsequently gels. [128]. While hydrophilic polymers are freely soluble in water, levothyroxine solubility in water is only 0.105 mg/ml, but freely soluble in alkaline solutions [128, 129]. Therefore, to prepare an aqueous solution of levothyroxine, the main solvent to solubilise the drug recommended under FDA guidelines was 0.01 M of methanolic sodium hydroxide.

However, oral consumption of methanol is associated with severe hepatotoxicity and blindness. As the levothyroxine FDOFs formulations are expected to be taken orally, using methanol as the solvent for developing the formulations is therefore not an option. Methanol is metabolised in the body to formic acid and formaldehyde which sharply attack the body cells and the vital organs [130]. In contrast ingestion of ethanol is safer as it can be easily metabolised in the body to acetic acid and acetaldehyde, sources of energy in the Kreb's cycle [131].

Therefore, a solvent of 0.01M ethanolic sodium hydroxide was chosen as the solvent for developing the placebo and active formulations. Owing to the solubility of levothyroxine in ethanol, the volume of ethanol that can be used to prepare the mixture of 0.01M of ethanolic sodium hydroxide is half of the volume recommended in the FDA guidelines to prepare 0.01M of methanolic sodium hydroxide. A mixture of water with an organic solvent such as ethanol is also ideal for the quick formation of transparent gels due to the ease with which the solvent can evaporate leading to the formation of films [131].

However, during the developmental stage of formulating placebo FDOF formulations, various temperatures for drying the films were tested. It was noticed that, drying the films under a higher temperature of 40°C, films required less time to dry than at 30°C while the physical appearance of the films showed surface waves at 40°C due to faster drying, leading to cracks on the film's surface. This observation influenced film thickness and thus the content uniformity. Therefore, the placebo films were tested at 30°C and observed after 6, 12, 18 and 24 h of drying. From the physical appearance of the films, they were fully dry after 24 h showing uniform shape, good flexibility by folding manually the films and visually they were dry. In addition to the visual observations of the films, the weight of the dried films after 24 h was approximately the same weight of added excipients apart from the weight of the solvent used in the formulation.

On constituent ingredients, polymers such as HPMC, xanthan gum, carbopol ultrez 10, HEC, and a mixture of two or more polymers are widely used to formulate FDOFs. The essential plasticisers used in pharmaceutical formulations are polyethylene glycol 400 and propylene glycol, while menthol typically used as a flavouring agent for films [132].

From previous literature of preparing FDOFs formulations using HPMC E15 with plasticisers such propylene glycol showed that, films were uniform, stable and flexible [132]. It was observed from the placebo formulations that HPMC (E15) yielded clear gels at different percentages (3%, 5%, or 8%w/w). Also, propylene glycol showed better flow for forming films than glycerol or polyethylene glycol 400, as gels require moderate flowability to allow them to be poured into uniformly sized Petri-dishes for the last drying stage of FDOF production. Gels developed for the preparation of FDOFs were not assessed for their flow properties and viscosity as these two parameters were considered to be independent of the final film that was formed. Therefore, the different compositions of the excipients were used to prepare the gels and were dried to form the films.

Gels with low flowability are difficult to pour and obtain uniform FDOFs, while high gel flowability is associated with gels that cannot produce films. 5%w/w polymer was essential to produce satisfactory films when using HPMC polymers (E15 and E50). The films produced using HPMC as the polymer resulted in the formation of clear, flexible, transparent films with uniform film thickness. Therefore, from these physical appearance and visual observations, three formulations out of the placebo FDOFs prepared were considered for further development to produce levothyroxine FDOFs. These were: 5% HPMC (E15) + PG, 8% HPMC (E15) + PG, and 5% HPMC (E50) + PG. The required levothyroxine FDOFs will be administrated to the oral cavity; therefore, a flavouring agent is required for oral pharmaceutical formulations to cover the unpleasant taste and increase the patent compliance [133]. Due to the pleasant taste of menthol, it was chosen as the flavouring agent in all formulations.

Excipient compatibility of levothyroxine with HPMC E15 and HPMC E50 polymers was performed using FT-IR to study the compatibility of the drug with the polymers used in the FDOF formulations. These tests show any changes to the chemical structure of the API (levothyroxine) when formulated in FDOFs using different percentages and

types of HPMC polymer. In this test, any chemical interaction that might take place can be observed on the spectrum of FTIR as additional peaks of some functional groups due to the interaction between the excipients presents in the formulation and the active pharmaceutical ingredients. However, these addition peaks observed in the spectrum can be an indication of any chemical degradation in the APIs which confirm that the API is chemically unstable. On the other hand, if there were no chemical interactions between the formulation and the API or there was no any chemical degradation for the API, the spectrum of the FTIR shows the peaks of the excipients present plus the peaks of the functional groups of the API.

The study of the FT-IR spectra for the levothyroxine pure drug and the FDOF (HPMC E15) did not show the presence of any additional functional groups. The absence of an interaction between the drug and the formulation ingredients was evident due to the distinguished peaks for levothyroxine chemical structure being remarkably similar to the peaks obtained with the FDOF formulation. Furthermore, the levothyroxine FDOF produced using HPMC E50 behaved in a similar manner to the formulations that were designed to include HPMC E15 polymer in the film. All the samples were analysed using the FT-IR, and the peaks observed indicate that the pure drug was compatible with the excipients of the three advanced levothyroxine FDOF formulations. Similar excipient compatibility studies reported in the literature state that there was no significant interaction between the polymers used for the preparation of FDOF and an antihypertensive drug metaprolol, as determined using FTIR. [134].

Distribution of the drug within the formulation and its weight uniformity are necessary to obtain an accurate drug dose. This can be assessed using a drug content test and consistency of weight test, which was performed on the three formulations. It has been reported in previous research on FDOF formulations that the films weight can be uniform when the average weight of the films is not significantly different from each tested film [135] and that is what was observed from the developed formulations. There was no significant different as it mentioned under the results section of the uniformity of weight test.

However, in order to show that, the developed levothyroxine FDOFs were prepared uniformly, film thickness test was performed. This test indicates, weather the casting method was accurately achieved or not. As the gel casting method was a manual

process, a certain amount of variation is expected. Therefore, this test which depends on the thickness variation between the randomly tested films will give an indication of the success of the process. The test measures uniformity of distribution of the prepared gel and the drying step of the gel to produce a uniform film. It was reported that the variation in the thickness of the FDOF formulations should be less than 5% for it to be acceptable [136]. As observed in the designed levothyroxine FDOF formulations, the standard deviation of four separate measurements of the four corners of the films was less than 5%, indicating that the thickness of the developed levothyroxine FDOFs formulations is acceptable.

A folding endurance test provides an indication of film flexibility and rigidity. The number of times a film is folded without breaking is calculated as the folding endurance value. For all the formulations developed, FDOF folding endurance was more than 300 folding times which showed that the formulations are flexible and the concentration of the plasticiser in the formulation is adequate for the purpose [112]. The FDOFs should have a pH close to that of the oral cavity to avoid oral mucosal irritation [137]. The developed formulations of levothyroxine showed an acceptable pH ranging from 6.2 to 6.6, a range close to the natural pH. Hence, the possibility of irritation and side effects in the oral cavity were minimal for these formulations.

The time required for the films to disintegrate in the oral cavity and release the API should be rapid (in the order of sec). The time required to disintegrate the film is higher in formulations carrying more polymer content [138]. Hence, a relationship exists between polymer concentration and disintegration time. Formulation F2 film showed a higher disintegration time than the other films, once again indicating that the amount of polymer used in the development of the FDOF formulations affect the disintegration time. However, all three formulations showed acceptable disintegration times as it is recommended to have the films disintegration time between 5-30 sec [139, 140].

In contrast to the disintegration time, higher polymer concentrations were associated with low moisture loss [141]. Percentage of moisture loss from pharmaceutical formulations provides information on the stability of formulations under normal storage condition. As a small percentage moisture loss theoretically indicates formulations to be more stable than those with a higher percentage moisture loss [142]. In this research, the data obtained from the percentage moisture loss was less than 2%

similar to the values reported in the literature. As increasing the percentage of the hydrophilic polymer in the film increased, the moisture loss is less indicating that polymer quantities can effect film stability.

Uniformity of drug content test for the amount of levothyroxine per a unit area of the developed films. This test determines whether the drug concentration in the film had uniformly distributed during the gel preparation, gel casting and gel drying stages to form the film. Hence, the concentration of the drug should be uniform in all the parts of the same film [143]. From the results observed in this research, it was seen that all the developed films had uniformity of drug content between the limits that were recommended for drug content uniformity. These observations indicate that the uniformity for the drug concentration within the all batches of the developed levothyroxine formulations were within the acceptable limits. A second conclusion that can be drawn from these observations is that, although the casting process was a manual process, the procedures followed resulted in the formation of a uniform film – both in its physical properties as well as in drug content.

Determining the films' mechanical properties helps in understanding the durability, flexibility, handling ability, physical stability and ability of the film to withstand the rigours during packaging, transport and usage. Therefore, it is essential to determine the films' mechanical properties such as tensile strength and elongation ability, also known as stretch ability. Tensile strength was determined to observe the elasticity of the formulations and the percentage elongation test was performed to predict the maximum elongation of the film until it breaks. These mechanical tests of levothyroxine films provide details about the toughness and stretch ability before breakage, so these parameters of the automated tests give the instructions for handling the formulations [136, 144]. It was reported from previous research on evaluation of FDOFs of Domperidone that, there is a relation between the concentration of the hydrophilic polymers in the formulation with increasing the tensile strength and the percentage elongation of the film [141]. The FDOF formulations developed were tested for their tensile strength and percentage elongation. Formulation F2 with 8%w/w of HPMC E15 showed higher tensile strength and percentage of elongation for the film than the formulations F1 and F3 which both have 5%w/w of HPMC E15 for formulation F1 and HPMC E50 for formulation F3. Therefore, it can be said that, increasing the

concentration of the hydrophilic polymer in the formulation can improve the mechanical properties of the film formulations.

However, the higher percentage of hydrophilic polymers such as HPMC can affect the drug release from the film formulations. Using HPMC in the film formulations reduced the rate of drug release due to the formation of a rigid layer of matrix structure within the formulation that can affect the mobility of the drug particles [145]. In formulation F2 with 8%w/w of HPMC showed lower release of drug particles from the formulations than the films formulations that were designed with 5%w/w of HPMC concentration in the formulations. However, HPMC is a hydrophilic polymer that is widely used in the preparation of gels and films, mainly due to it is stability in air, light and temperature. In addition, HPMC plays an important role in preventing the drug degradation in the formulation by forming solid matrix inside the formulation. HPMC in different grades has been recommended for use between 2-20%w/w in film formulations [146]. As at 8%w/w, it affected the drug release and at 5%w/w it showed films with good physical and chemical properties, 5%w/w of HPMC concentration in the levothyroxine FDOFs is preferable than the films formulated with 8%w/w of HPMC.

3.5 Conclusion

In this chapter, levothyroxine was formulated as Fast Dissolving Oral Film (FDOF) to improve the active pharmaceutical ingredient (API) chemical stability and drug uniformity in the formulation.

To produce FDOF formulation, hydrophilic polymers were used such as HPMC in different grades and it is necessary that, the used polymer has no interference with the chemical prosperity of the API in the formulation. Therefore, the developed of levothyroxine formulations were tested using FT-IR.

Moreover, three levothyroxine FDOFs formulations using HPMC E15 and HPMC E50 showed acceptable properties regarding the drug content (between 90%-100% and the drug release (less than 10 minute). In addition to this, the three developed formulations of levothyroxine in this chapter represented good flexibility, good physical appearance, rigidity and not varied in thickness. Furthermore, acceptable pH values which is necessary to obtain the film in neutral pH due to the fact that, the films will be placed in the oral cavity.

In general, the formulations developed for levothyroxine as FDOF using HPMC E15 and HPMC E50 succeed to provide a formulation with acceptable physical and chemical properties for the API.

4. Stability study of levothyroxine FDOFs using an antioxidant as a formulation stabiliser

4.1 Introduction

The hypothesis behind the investigation into the chemical stability of levothyroxine in this research was that oxidation of the drug leads to the production of levothyroxine degradation products. Therefore, levothyroxine FDOF formulations should be developed such that the chemical stability of the drug can be improved. However, as discussed in chapters 2 and 3, levothyroxine FDOFs require protective additives in order to retain the chemical stability of levothyroxine in the developed formulations, especially when the formulations were stored at various storage conditions. Addition of antioxidant excipients to the formulations may solve the issues of levothyroxine chemical stability. Efficacy of antioxidants as protective additives in improving the chemical stability of different formulations has previously been reported in the literature [147-149].

Antioxidant excipients are generally used in pharmaceutical products that suffer from chemical instability due to exposure of the formulation to environmental factors such as heat, extreme pH and light. One of the main reactions of chemical instability of the pharmaceutical products in such conditions is oxidation. These oxidative reactions occur due to the production of free radicals leading to the decomposition of the active pharmaceutical ingredient (drug) in the formulation [150]. In addition to the oxidation reactions, there are other chemical reactions can cause the chemical instability of the active pharmaceutical ingredient such as: decarboxylation, hydrolysis and photolytic reactions [151].

The main mechanism of action for the antioxidant excipients is by preventing or reducing the oxidation reaction that can occur in the formulation due to the oxidative stress produced by the free radicals. Oxidative stress can be successfully mitigated by using an antioxidant excipient that has a higher oxidation potential than the substrate. Therefore, the antioxidant excipient competes with the oxidisable substrate for the free radical and is readily oxidised thereby sparing the formulation [6]. This mechanism of action for the antioxidant compounds has different phenomenon that relies on the excipients used for antioxidant purposes [152].

Antioxidants are widely used to prevent or decrease the oxidation reaction in a formulation. Therefore, producing formulations with antioxidant excipients is ideal to increase the chemical stability of drugs. Various researchers have investigated the efficacy of commonly used antioxidants ingredients such as butylated hydroxyltoluene (BHT), butylated hydroxyanisole (BHA), ascorbic acid and sodium metabisulfite (SMB).

Antioxidants such as ascorbic acid, butylated hydroxyltoluene (BHT) and butylated hydroxyanisole (BHA) are phenols that can produce antioxidant effects by donating electrons to the free radicals that are capable of attacking the chemical structures (Figure 4.1, Figure 4.2, Figure 4.3). Previous research has been shown that BHT is the most efficient ingredient that can be used as an antioxidant in the pharmaceutical formulations (used at 0.5% and 0.1% w/w) for increasing chemical stability [150].

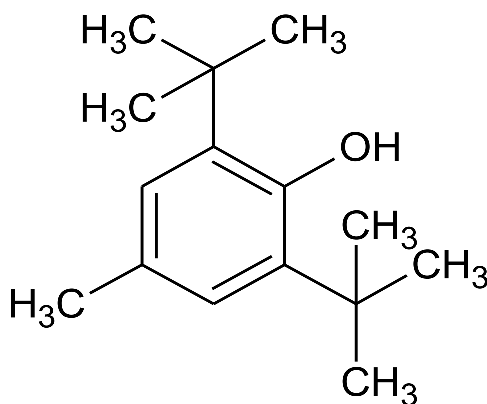


Figure 4-1: Chemical structure of butylated hydroxytoluene (BHT)

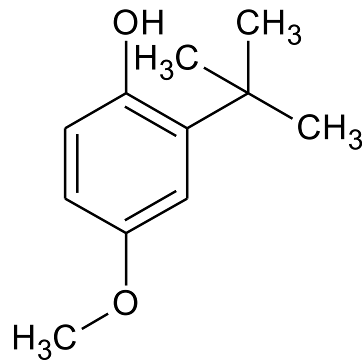


Figure 4-2: The chemical structure of butylated hydroxyanisole (BHA)

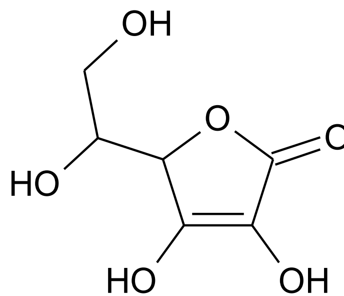


Figure 4-3: The chemical structure of ascorbic acid

In addition, the antioxidant active compounds such as sodium metabisulfite (SMB) has redox potential properties due to its chemical structures. The redox potential formulations allow compounds to gain an electron and therefore redox the oxidation interactions from the free radicals [150] (Figure 4.4).

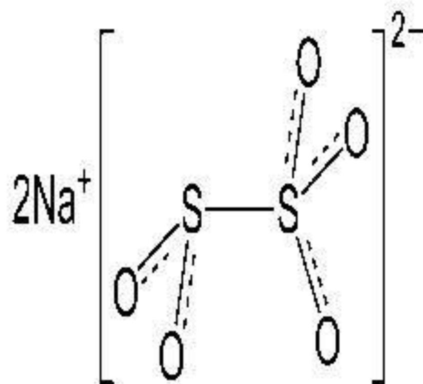


Figure 4-4: The chemical structure of sodium metabisulfite

4.2 Methodology

4.2.1 Preparation of antioxidant levothyroxine FDOFs

To improve the chemical stability of levothyroxine, the drug FDOFs containing antioxidants were formulated. The method of preparation is similar to the procedure used in chapter 3 for producing the levothyroxine FDOFs. Three antioxidant formulations: ascorbic acid at 1% w/w, butylated hydroxytoluene (BHT) at 0.1% w/w and sodium metabisulfite at 0.1% w/w were incorporated into FDOF prepared using HPMC E15 at 5% w/w. the percentage of antioxidants used is referred to previous researched used these antioxidants in their formulations to improve the formulation stability [150]. Ascorbic acid was dissolved in water and added to the formulation while BHT and SMB have been added to the formulations by dissolving them in an organic solvent (ethanol 95%). Table 4.1 shows the formulations of levothyroxine FDOFs with different antioxidants.

Table 4-1: The levothyroxine FDOFs formulations with different antioxidants (BHT, SMB and ascorbic acid)

Polymer(s)	Plasticiser (10%) w/w	Flavouring agent (0.5%) w/w	Concentration (%) of polymers w/w	Drug loaded	Antioxidant	Drying temperature (°C)	Drying period (hr)
HPMC(E15)	PG	Menthol	5.0	Levothyroxine (100 µg/cm2 of the film	Ascorbic acid 1%	30	24
HPMC(E15)	PG	Menthol	5.0	Levothyroxine (100 µg/cm2 of the film	BHT 0.1%	30	24
HPMC(E15)	PG	Menthol	5.0	Levothyroxine (100 µg/cm2 of the film	SMB 0.1%	30	24

4.2.2 Evaluation of levothyroxine FDOFs using antioxidants

Levothyroxine FDOFs incorporating different antioxidants were analysed using the same methods that were used for evaluation the levothyroxine FDOFs in chapter 3,

which are: weight variation, thickness measurement, folding endurance, surface pH, in vitro disintegration time, Percentage moisture loss, content uniformity, in vitro dissolution study, tensile strength and percentage elongation.

4.2.3 Chemical stability of levothyroxine FDOFs formulations

The main aim for this chapter is to study the chemical stability of levothyroxine FDOFs containing an antioxidant and compare it to the formulations that were designed without an antioxidant and with levothyroxine in its solid state used as a comparator. Levothyroxine FDOFs with antioxidants were stored at three different temperatures (25°C, 40°C, 70°C) for six months to test their chemical stability and the time points for analysis were t=0, t=1 months, t=3 months and t=6 months.

4.3 Results

4.3.1 Levothyroxine FDOFs with antioxidants.

Levothyroxine fast dissolving films were formulated using three different antioxidants to understand the chemical and physical stability study of the films. Figure 4.5 shows three different formulations of levothyroxine FDOFs. A: Levothyroxine fast dissolving film containing 5% HPMC E15 with 0.1% SMB, B: Levothyroxine fast dissolving film containing 5% HPMC E15 without antioxidant, C: Levothyroxine fast dissolving film containing 5% HPMC E15 with 0.1% BHT. All the developed formulations in the figure 4.5 showed a uniform film surface with no wavy shape after drying.

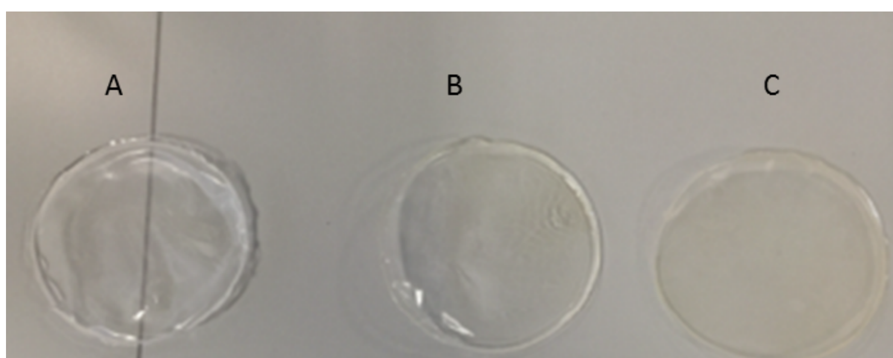


Figure 4-5: The developed levothyroxine fast dissolving films formulations in this research.

The developed formulations using antioxidants were stored at three different temperatures (25°C, 40°C and 70°C, at t= 30 days, 90 days and 180 days) and compared with formulations of levothyroxine FDOFs developed without any antioxidant. It can be seen from figure 4.6 that, there is a change in the physical appearance of the developed film formulations without an antioxidant when the films were exposed to high temperature indicated by change in film colour from clear white colour films to light yellow films. Levothyroxine FDOFs with BHT (F1) and SMB (F2) as antioxidants, it was noticed that there is a small change in the film colour after 90 and 180 days of the films' storage at 70°C for 180 days, but less than what was observed with the levothyroxine FDOFs without antioxidant (F3).

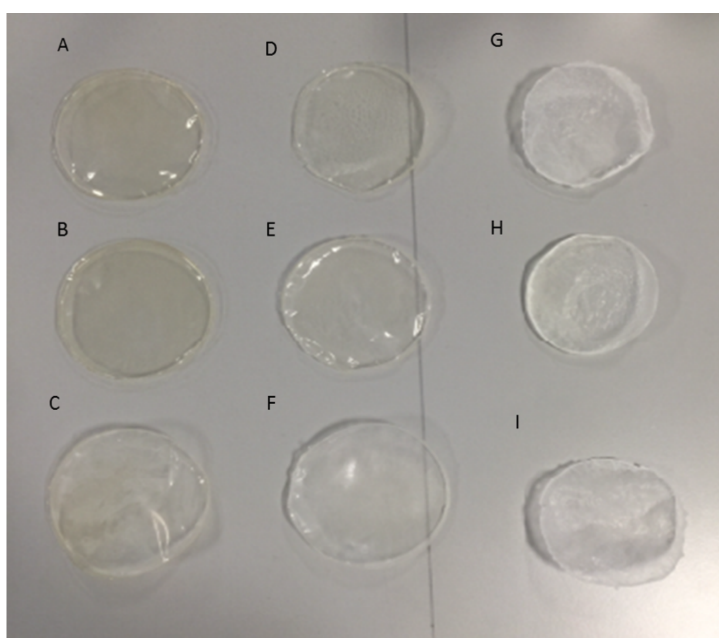


Figure 4-6: Stability study of the developed levothyroxine fast dissolving films with and without antioxidants. A: levothyroxine fast dissolving film without antioxidant at t=180 days, 25°C, B: levothyroxine fast dissolving film without antioxidant at t=180 days, 40°C, C: levothyroxine fast dissolving film without antioxidant at t=180 days, 70°C, F: levothyroxine fast dissolving film with BHT antioxidant at t=180 days, 25°C, E: levothyroxine fast dissolving film with BHT antioxidant at t=180 days, 40°C, D: levothyroxine fast dissolving film with BHT antioxidant at t=180 days, 70°C, I: levothyroxine fast dissolving film with SMB antioxidant at t=180 days, 25°C, H: levothyroxine fast dissolving film with SMB antioxidant at t=180 days, 40°C, G: levothyroxine fast dissolving film with SMB antioxidant at t=180 days, 70°C.

A third formulation of the levothyroxine film containing ascorbic acid as the antioxidant was also formulated. Figure 4.7 shows the stability of levothyroxine FDOF containing ascorbic acid when stored at different temperatures. It can be observed from Figure 4.7 that, levothyroxine film with ascorbic acid as an antioxidant when stored at under the same conditions as the other developed levothyroxine films, indicated a drastic change in the colour of the films from white to yellow and orange colours.

The figure 7.4 shows a levothyroxine film (A) with ascorbic acid as an antioxidant at t=0 and it was white in colour. The film colour changed to a yellow colour after storing the film for 30 days at 25°C (film D). However, storing the levothyroxine films with ascorbic acid for 30 days at 40°C (film C), the film colour changed from white colour to a brown colour which is darker than the formulation stored at 25°C. When the film was stored at 70°C for 30 days, the film colour changed to a much darker brownish yellow colour (film B). The results clearly indicate some instability in the levothyroxine

FDOF containing ascorbic acid as the antioxidant. This formulation was not tested further.

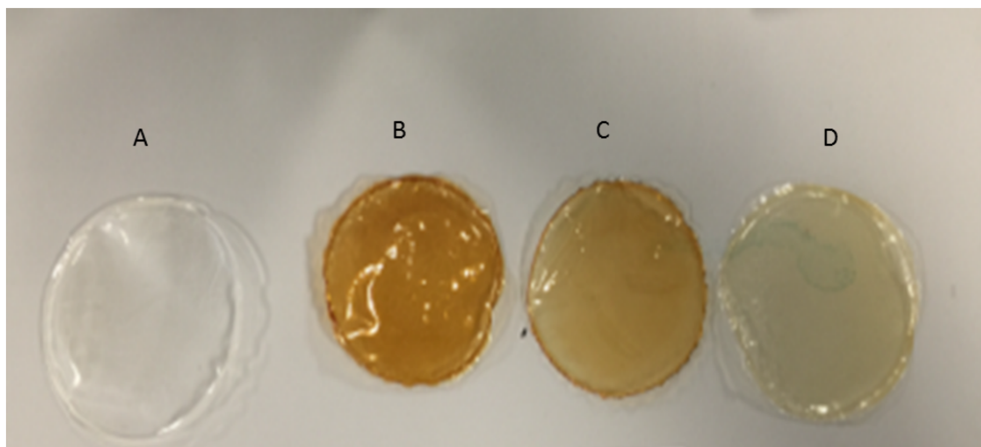


Figure 4-7: stability study of levothyroxine fast dissolving film with ascorbic acid as an antioxidant. (A) Levothyroxine film with ascorbic acid as an antioxidant at t=0. (B): Levothyroxine film with ascorbic acid at 70°C, 30 days. (C): Levothyroxine films with ascorbic acid at 30 days, 40°C. (D): Levothyroxine film with ascorbic acid at 30 days, 25°C.

Table 4.2 indicates the analysis of levothyroxine fast dissolving films with BHT (F2) and SMB (F3) as antioxidants compared with levothyroxine fast dissolving film without an antioxidant (F1) at t=0.

On comparing the data in the table below it can be observed that the drug content of the three formulations ranged between 97% and 98%. In terms of the weight variation for the three developed formulations, table 4.1 shows that, there was a slight different between F1, F2 and F3. However, thickness was similar between the formulations and folding endurance was higher on F2 than F1 and F3.

In addition, the surface pH of the formulation has SMB as an antioxidant was higher at 7.92 compared to the formulation that had BHT as an antioxidant and higher than the formulation without an antioxidant.

The *in vitro* disintegration time for the levothyroxine fast dissolving films formulations was between 32 seconds and 34 seconds, which confirms that there was no different on the disintegration time between the developed levothyroxine FDOFs formulations.

The % moisture loss for the levothyroxine FDOFs formulations was between the three formulations as well as the mechanical properties (tensile of strength and % elongation) for levothyroxine formulations.

Table 4-2: The physico-chemical properties of levothyroxine fast dissolving films with and without antioxidant F1: Levothyroxine FDOF without anti-oxidant; F2: Levothyroxine FDOF with 0.1 %BHT; F3: Levothyroxine FDOF with 0.1% SMB. All results are shown as mean ± SD (n=3)

FDOFs	F1	F2	F3
Drug content %	98.53 ± 0.25	98.62 ± 0.41	97.44 ± 0.68
Weight variation (mg)	24. ± 1.5	25 ± 2.1	23 ± 1.4
Thickness (mm)	0.10 ± 0.02	0.11 ± 0.03	0.10± 0.04
Folding endurance	280.3	287 ± 2	277 ± 3
Surface pH	6.45 ± 0.02	7.11 ± 0.05	7.92 ± 0.09
In vitro disintegration time (seconds)	33.33 ± 2.1	32.25 ± 2.7	34.11 ± 3.4
% moisture loss	2.42 ± 0.17	2.39 ± 0.33	2.44 ± 0.54
Tensile strength(N/mm)	16.890	16.950	16.055
% elongation	15.284	15.447	15.631

4.3.2 Dissolution profile of levothyroxine FDOFs

The test for drug release of the developed levothyroxine FDOFs formulations has been determined using the dissolution apparatus. At t=0, levothyroxine release from the formulation containing BHT as an antioxidant was about 98.55% +/- 0.012 after 10 min which is similar to the percentage of levothyroxine released from the formulation that has no antioxidant after 10 min as 98.08% +/- 0.023 of levothyroxine has released from this formulation (5% of HPMC). From the levothyroxine FDOFs that included SMB as antioxidant, the percentage drug release was slightly less than the other levothyroxine formulations as the levothyroxine release after 10 min at 97.25% +/-

0.032. However, it can be observed from the drug release profile given in Table 4.3, more than 40% of levothyroxine had released from all the formulations after 1 minute (table 4.3).

Table 4-3: the percentage of drug release from the developed formulations of levot hyroxine FDOFs at t=0

Time point (mins), t=0	FDOFs with BHT (%)	FDOFs with SMB (%)	FDOFs without AOX (%)
1	45.35	49.66	45.43
2	60.61	62.67	59.90
3	72.54	74.33	69.61
4	81.33	79.58	79.91
6	89.65	87.21	89.18
8	94.36	92.33	95.42
10	98.55	97.25	98.08

Figures 4.8, 4.9 and 4.10 show the percentages of drug release after 10 min when the formulations were stored at 25°C, 40 °C and 70°C for 1 month. When the samples of levothyroxine FDOFs were exposed to different storage conditions, drug release after 10 min from the samples stored at 25°C for 1 month was 97.88% +/- 0.11 for FDOF formulation containing BHT as an antioxidant, 97.3% +/- 0.18 for FDOF formulation that has no antioxidant and 96.02% +/- 0.23 for the formulation that contains SMB as an antioxidant. It was observed that the percentage of drug release dropped when the formulations were stored at 40°C for 1 month. Drug release was 92.14% +/- 0.42 for the FDOFs with BHT, 90.65% +/- 0.4 for the FDOFs without antioxidant and 88.67% +/- 0.49 for the FDOFs with SMB. Levothyroxine FDOF formulations stored at 70°C for one month showed a drug release of 87.94% +/- 0.56 for FDOFs with BHT, 81.74% +/- 0.38 for FDOFs without antioxidant and 80.15% +/- 0.39 for the FDOFs with SMB as antioxidant after 10 min.

Figure 4.8 shows percentage levothyroxine released from the fast dissolving oral films when the samples exposed to 25°C for 1 month. From the graph it can be observed that all three formulations had a similar release profile. However, drug release from

the formulation containing SMB as the antioxodant was marginally slower at 1 minute than the other two formulations.

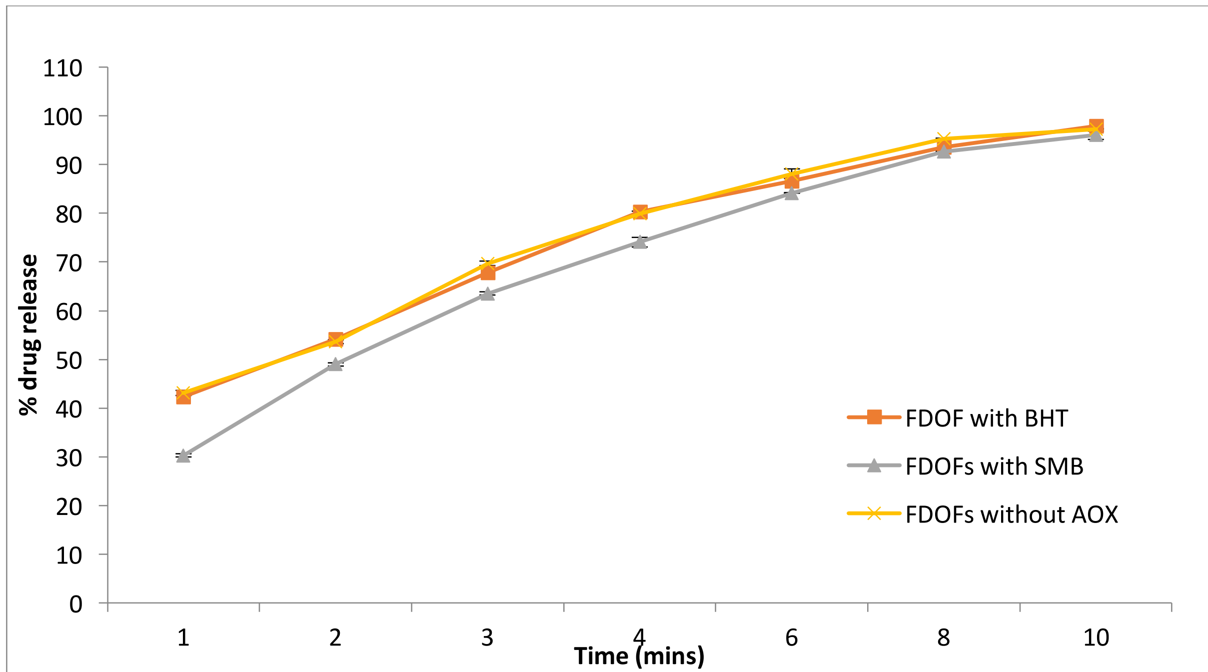


Figure 4-8: the percentage of drug release from FDOFs of levothyroxine at t=30 days, 25°C n=3. Statistically, one-way ANOVA showed p value is 0.8966 considered no significant different films regarding the percentage of drug release under this condition.

Figure 4.9 shows the percentage release of levothyroxine from the developed formulations when the formulations were stored at 40°C for 1 month. The figure shows that there was no significant difference in the drug release profile from the three formulations.

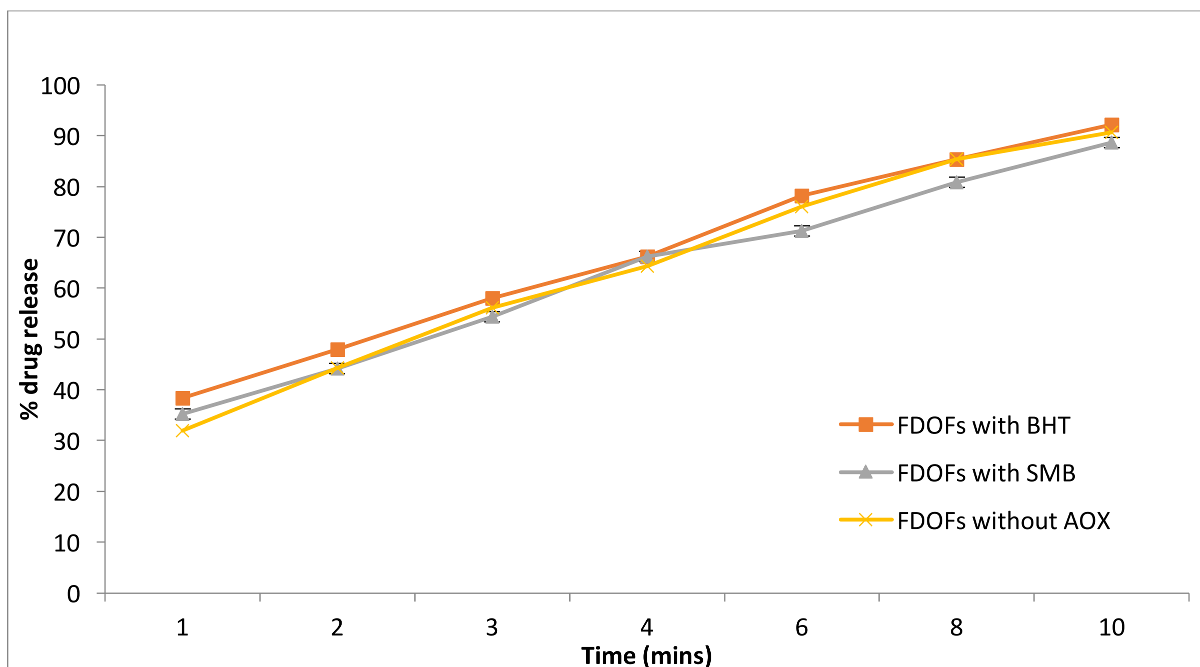


Figure 4-9: the percentage of drug release from FDOFs of levothyroxine at t=30 days, 40°C n=3. Statistically, one-way ANOVA showed p value is 0.9521 considered no significant different films regarding the percentage of drug release under this condition.

Levothyroxine release profile from the FDOF formulations with and without the antixoidants stored at 70°C for 30 days is shown in Figure 4.10. While there was no difference in the drug release profile from FDOFs with the antioxidants, the release was initially slower up to six min from the formulation without an antioxidant.The drug release from the FDOFs formulation at 10 min from all three formulations was however, silightly similar.

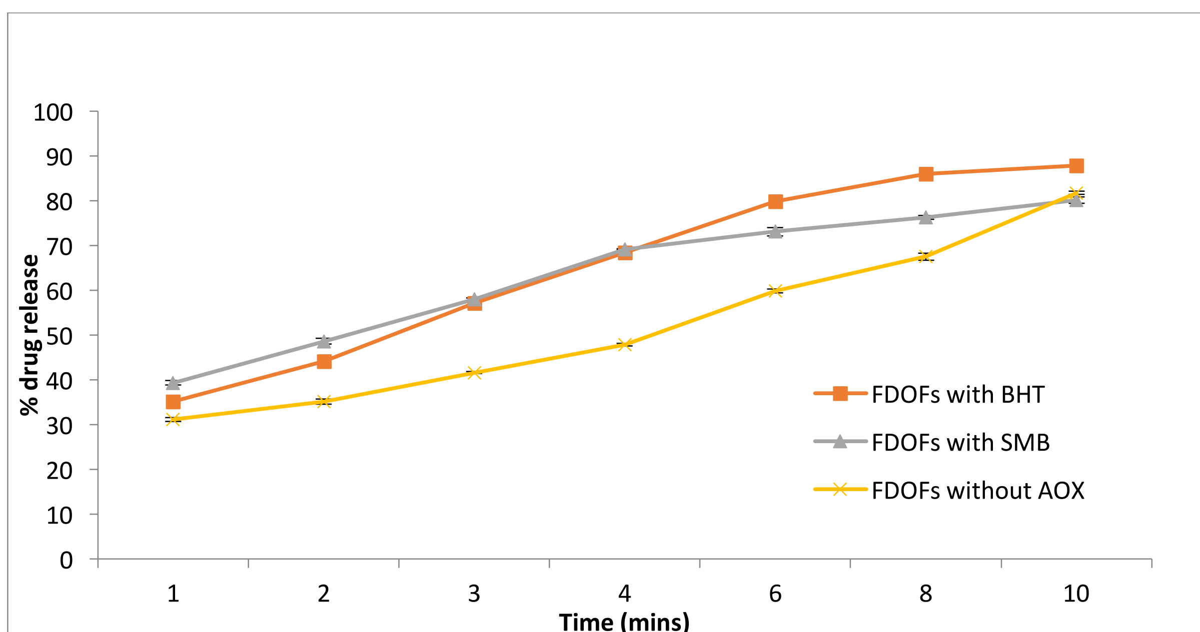


Figure 4-10: the percentage of drug release from FDOFs of levothyroxine at t=30 days, 70°C n=3. Statistically, one-way ANOVA showed p value is 0.3472 considered no significant different films regarding the percentage of drug release under this condition.

Levothyroxine FDOF formulations when stored for longer duration (t=90 days) at three different temperatures (25°C,40°C and 70°C) the drug release from all the developed formulations was comparatively less than when stored for 30 days. FDOF formulation containing BHT, the drug release after 10 min at 25°C was 96.77% +/- 0.67 while the drug release was 90.47% +/- 0.42 when the formulation was stored at 40°C and levothyroxine release from the formulation stored at 70°C was 85.94% +/- 0.6, indicating that the drug had probably degraded.

FDOF formulations containing SMB drug release after 10 min from the sample stored at 25°C was 95.14% +/- 0.33, about 87.58% +/- 0.58 was released from the FDOF formulations stored at 40°C and was 79.44% +/- 0.54 from the FDOF formulations stored at 70°C. Data shows that, even with an antioxidant incorporated into the formulation, there was a downward trend in the amount of drug released from the formulations when they were stored at a higher temperature.

From the FDOF formulations that did not include an antioxidant, drug release was 95.73% +/- 0.69 when stored at 25°C for 90 days, 83.31% +/- 0.41 was the drug release from the formulation stored at 40°C and 77.14% +/- 0.40 was the drug release from the formulation stored at 70°C. The amount of drug release was similar to the

amounts released from the formulations containing an antioxidant. Figures 4.11, 4.12 and 4.13 shows levothyroxine release pattern from the FDOF formulations stored for 90 days at 25°C, 40°C & 70°C. The data shows that there is no significant difference in the release profile of the drug from the formulations with or without an antioxidant.

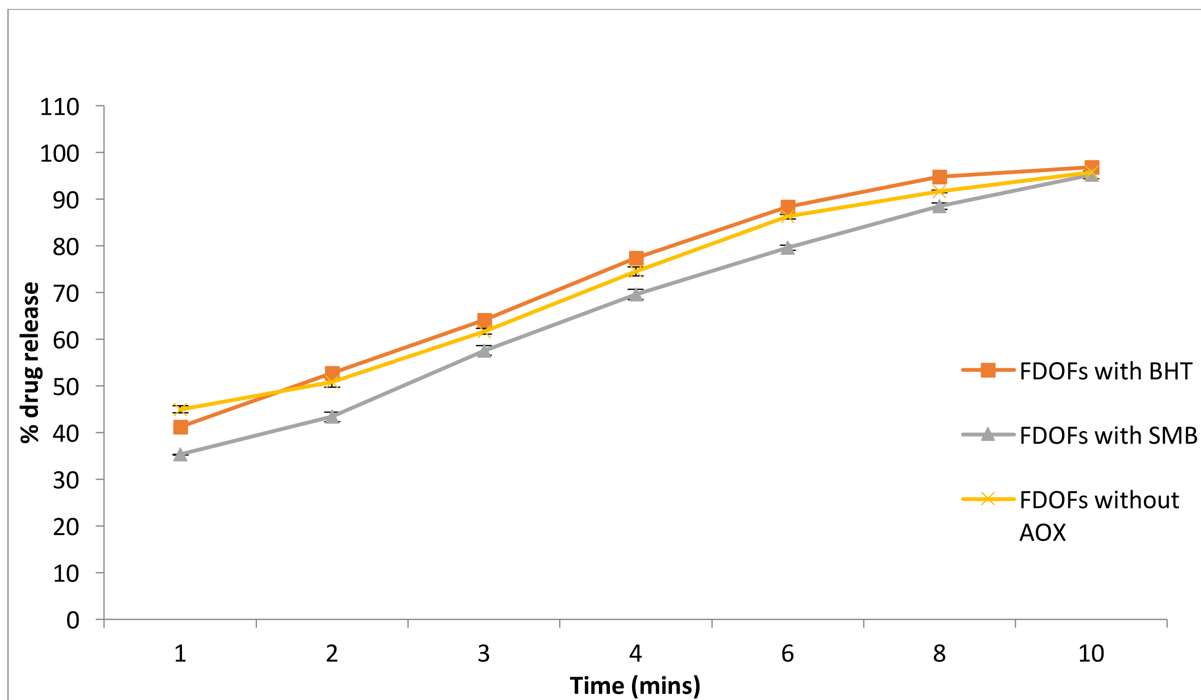


Figure 4-11: the percentage of drug release from FDOFs of levothyroxine at t=90 days, 25°C n=3. Statistically, one-way ANOVA showed p value is 0.9243 considered no significant different films regarding the percentage of drug release under this condition.

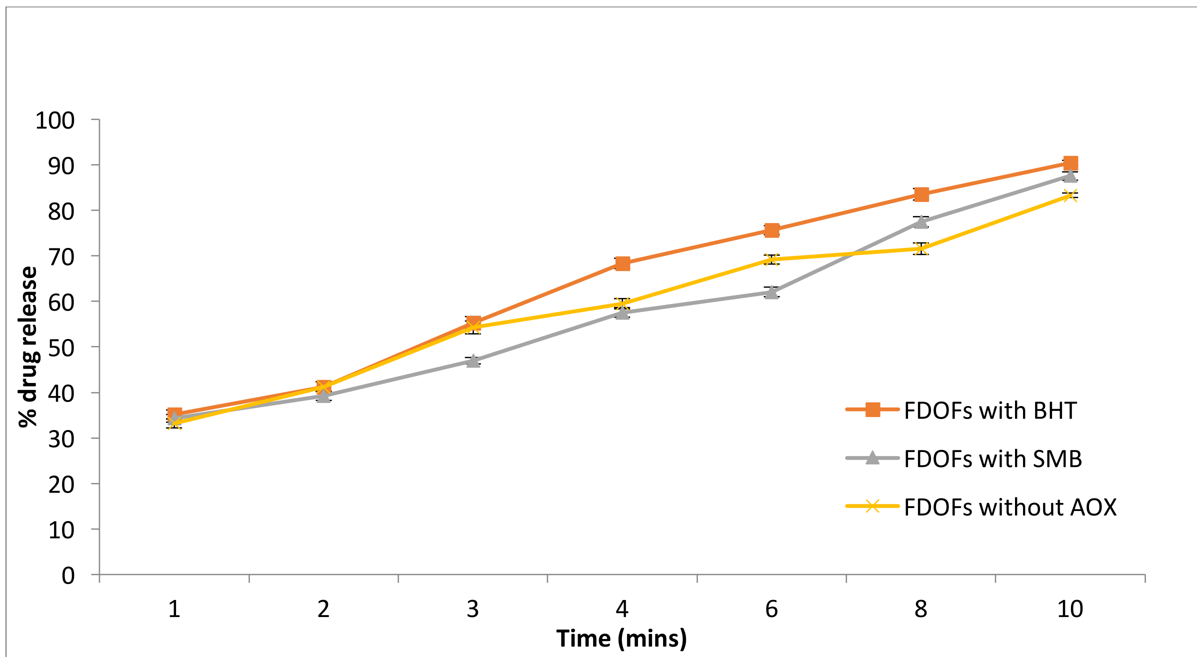


Figure 4-12: the percentage of drug release from FDOFs of levothyroxine at t=90 days, 40°C n=3. Statistically, one-way ANOVA showed p value is 0.8933 considered no significant different films regarding the percentage of drug release under this condition.

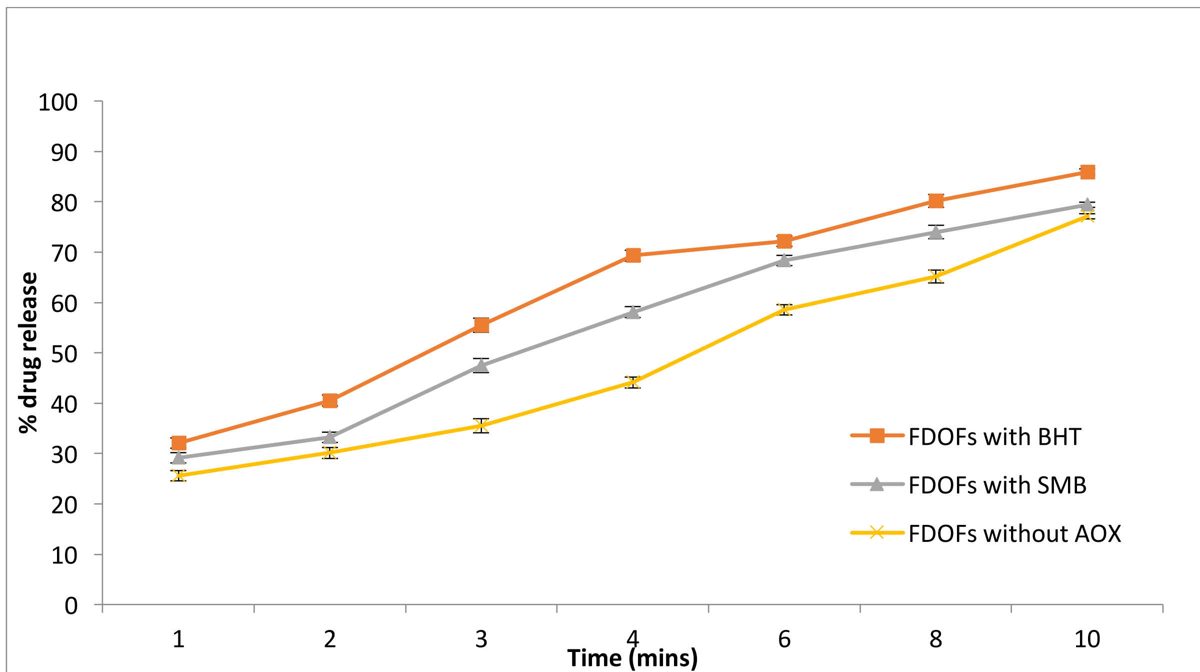


Figure 4-13: the percentage of drug release from FDOFs of levothyroxine at t=90 days, 70°C n=3. Statistically, one-way ANOVA showed p value is 0.3632 considered no significant different films regarding the percentage of drug release under this condition.

Levothyroxine FDOF formulations when stored for 180 days at 25°C,40°C and 70°C the total drug release from all the formulations was comparatively less than when stored for 30, but similar to the total drug release observed with formulations stored for 90 days. FDOF formulation containing BHT, the drug release after 10 min at 25°C was 96.04% +/-0.67 while the drug release was 90.33% +/- 0.34 when the formulation was stored at 40°C and levothyroxine release from the formulation stored at 70°C was 82.58% +/- 0.49, indicating that the drug had probably degraded.

From the FDOF formulations containing SMB as the antioxidant, drug release after 10 min from the formulation stored at 25°C was 94.28% +/- 0.32, about 85.46% +/- 0.65 was released from the FDOF formulations stored at 40°C and was 75.55% +/- 0.29 from the FDOF formulations stored at 70°C. Data shows that, even with an antioxidant incorporated into the formulation, there was a downward trend in the amount of drug released from the formulations when they were stored at a higher temperature. The total release profile and amount is, however, similar to the observations made from samples stored for 90 days.

From the FDOF formulations that did not include an antioxidant, drug release was 95.44% +/- 0.37 when stored at 25°C for 180 days, 82.97% +/- 0.58 was the drug release from the formulation stored at 40°C and 71.22% +/- 0.61 was the drug release from the formulation stored at 70°C. The amount of drug release was similar to the amounts released from the formulations containing an antioxidant.

Figures 4.14, 4.15 and 4.16 shows levothyroxine release pattern from the FDOF formulations stored for 180 days at 25°C, 40°C & 70°C. The data shows that there is no significant difference in the release profile of the drug from the formulations with or without an antioxidant. However, the release profile is similar to what was observed with formulations stored for 90 days at the same temperature conditions.

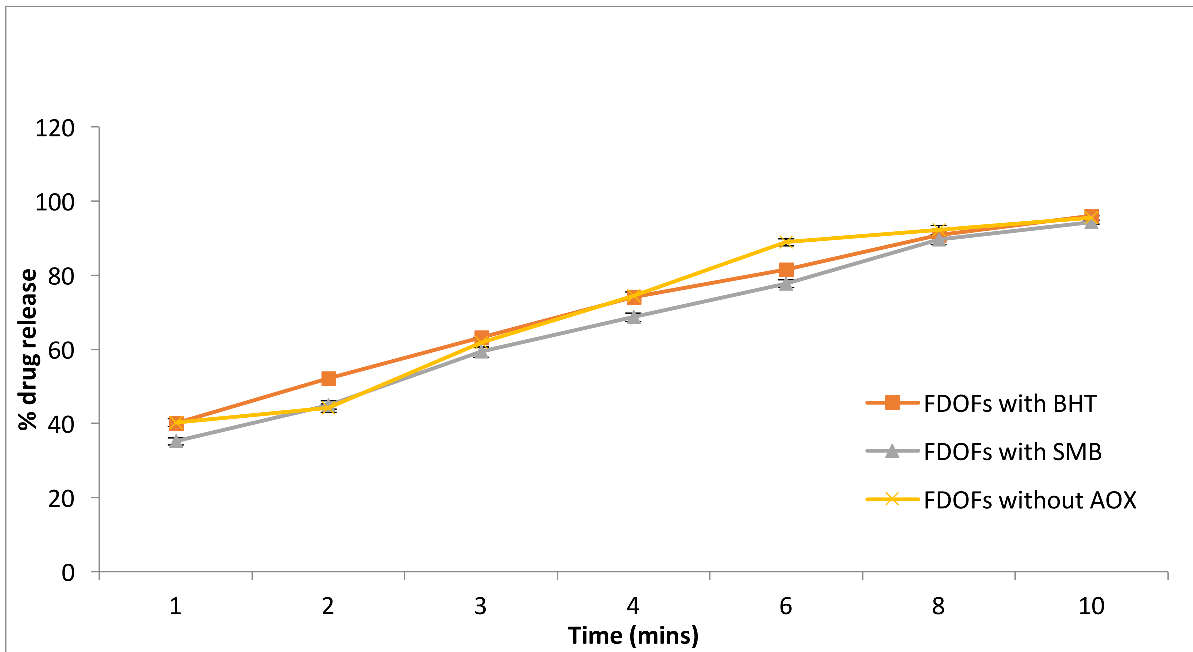


Figure 4-14: the percentage of drug release from FDOFs of levothyroxine at t=180 days, 25°C (n=3). Statistically, one-way ANOVA showed p value is 0.9988 considered no significant different films regarding the percentage of drug release under this condition.

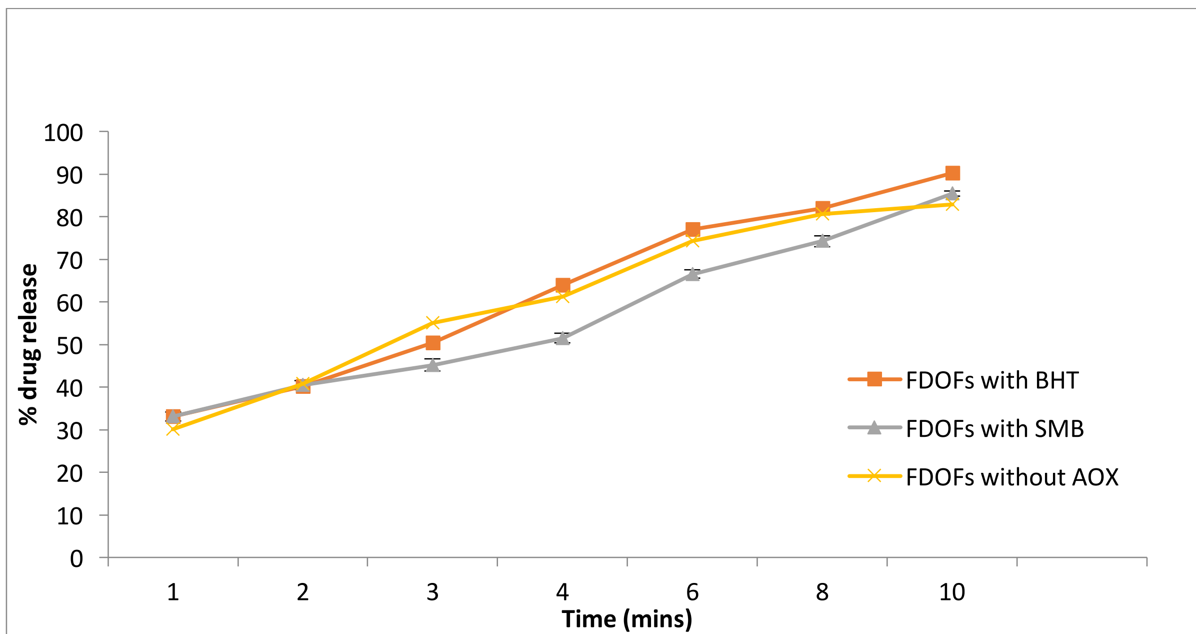


Figure 4-15: the percentage of drug release from FDOFs of levothyroxine at t=180 days, 40°C (n=3). Statistically, one-way ANOVA showed p value is 0.4982 considered no significant different films regarding the percentage of drug release under this condition.

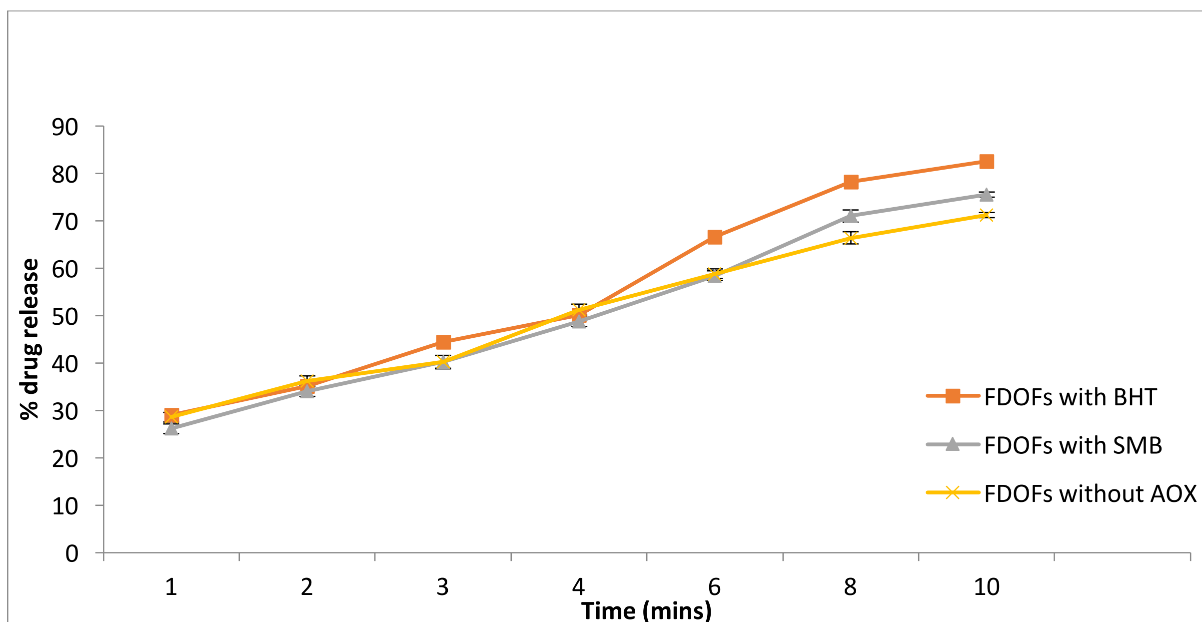


Figure 4-16: the percentage of drug release from FDOFs of levothyroxine at t=180 days, 70°C (n=3). Statistically, one-way ANOVA showed p value is 0.3998 considered no significant different films regarding the percentage of drug release under this condition.

4.3.3 Levothyroxine FDOFs stability study using HPLC

In addition to the dissolution test for the developed levothyroxine FDOFs, stability study using HPLC was investigated for the levothyroxine films formulations with antioxidants (BHT and SMB). Therfore, the following chromatographs are showin the formulations when BHT and SMP included and compared to levothyroxine with no antioxidant at same storage conditions. Figure 4.17 shows the HPLC chromatogram of levothyroxind FDOFs with BHT. It can be seen that the drug had eluted after 15.619 min with a minor peak observed around 16.1 min, which was too small for integration. The peak was present in the levothyroxine standard sample too, and hence it was considered as an artefact. Chromatogram for levothyroxine FDOF with SMB is shown in figure 4.17. It can be seen that the drug peak eluted at 15.551 min along with the small artefact at 16.619 min. The third developed levothyroxine FDOFs formulation tested under the same condition of the other developed FDOFs formulations in this research, it can be seen from figure 4.17 as well that, the levothyroxine peak eluted after 15.615 min and a small peak at 16.655 min.

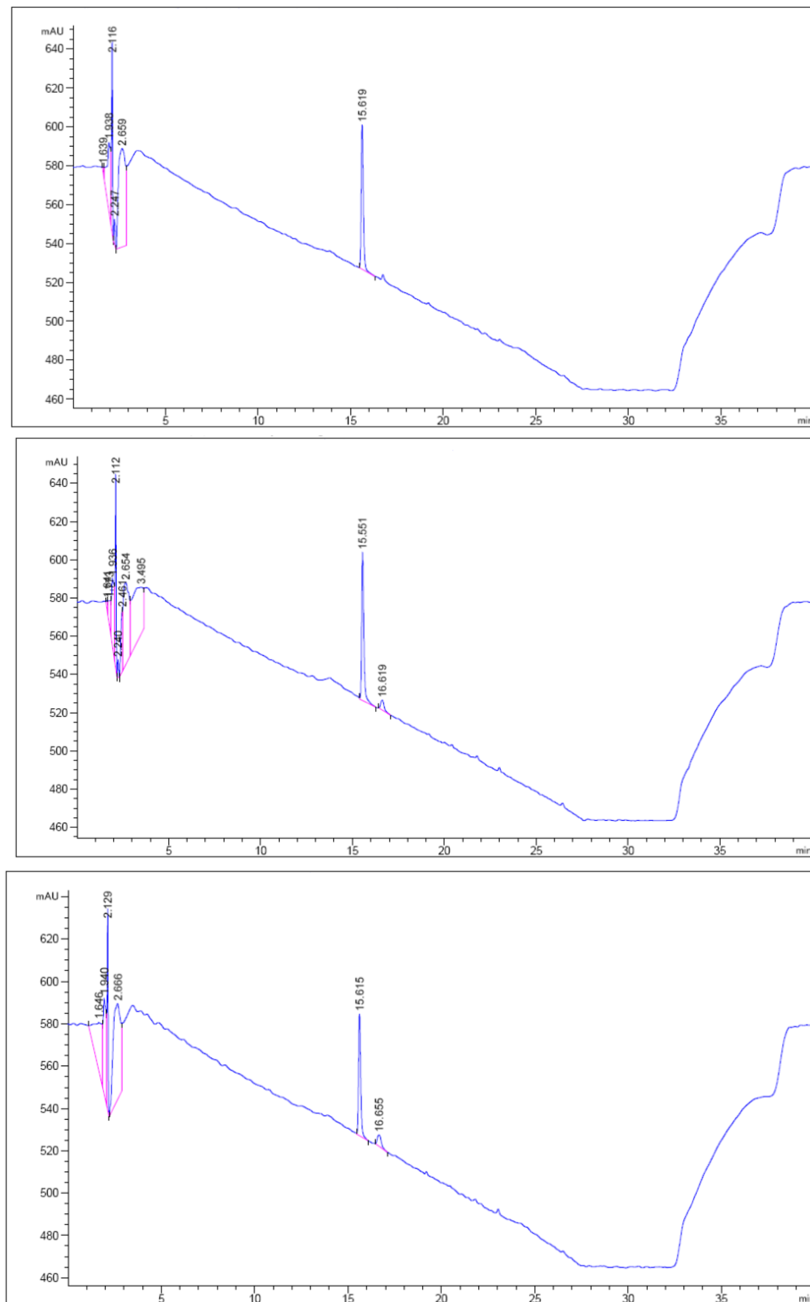


Figure 4-17: (Left) HPLC of levothyroxine FDOFs with BHT as antioxidant at a concentration of 10µg/mL, T=0. (Right): HPLC of levothyroxine FDOFs with SMB as antioxidant at a

To compare the three-development levothyroxine FDOFs formulations at t=0, figure 4.18 shows the percentage recovery of levothyroxine from FDOF formulation with BHT is higher than the percentage recovery of the other developed levothyroxine FDOFs formulations.

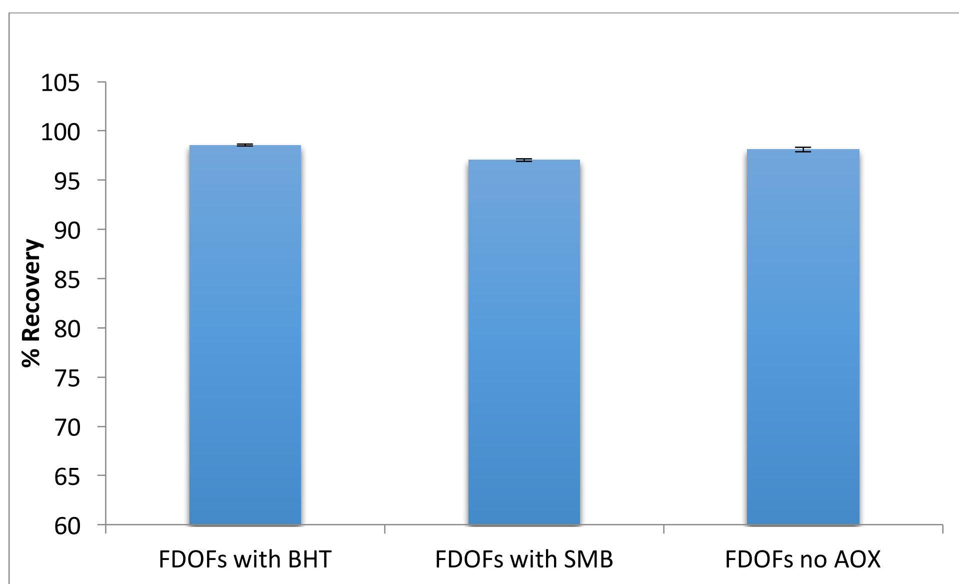


Figure 4-18: The percentage recovery of levothyroxine from FDOFs formulations at t=0. The mean of FDOFs with BHT is 98.55% +/- 0.017 (n=3). Statistically, one-way ANOVA showed p value is > 0.9999 considered no significant different films regarding the percentage of drug release under this condition.

It can be seen from the HPLC injections for the sample of levothyroxine FDOFs, the peak purity is not 100% as there were other peaks observed on the chromatograms. The peak purity of levothyroxine FDOFs with BHT is 100% while the peak purity for the drug on the FDOFs formulation with SMB was 97.6% and 98.22% for the run obtained for levothyroxine FDOFs without antioxidant.

After 1 month of storing the levothyroxine FDOFs formulations at room temperature, it was noticed that, the sample of levothyroxine FDOFs with BHT had eluted from the HPLC after 15.605 min. The peak of the drug is the only peak observed on the run obtained for levothyroxine FDOFs with BHT (figure 4.19, Top graph). The small peak next to the drug peak was too small for integration. While it can be observed that the levothyroxine peak with SMB (Middle graph) eluted at 15.622 min. In addition to the drug peak in this run, four small peaks have been recorded after 16.709min, 21.799min, 23.017 min and 26.457 min. Figure 4.19 represents the HPLC chromatogram of levothyroxine FDOFs formulation without antioxidant (Bottom graph) in which the sample had eluted after 15.625 min with no any other peaks observed in this run.

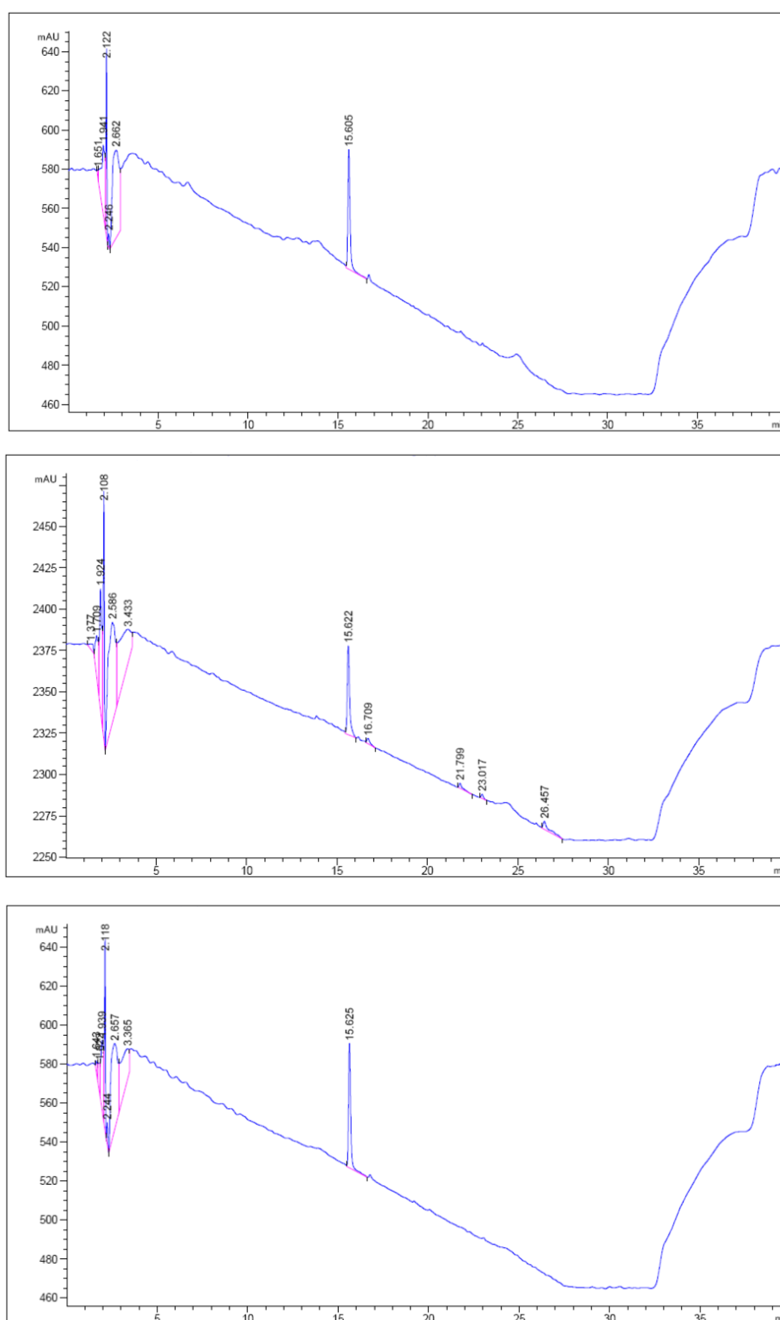


Figure 4-19: (Top): HPLC of levothyroxine FDOFs with BHT as antioxidant at a concentration of 10µg/mL, T=30 days, room temperature. (Middle): HPLC of levothyroxine FDOFs with SMB as antioxidant at a concentration of 10µg/mL, T=30 days, room temperature. (Bottom): HPLC of levothyroxine FDOFs without antioxidant at a concentration of 10µg/mL, T=30 days, room temperature.

The peak purity of levothyroxine FDOFs with BHT and without antioxiant was 100% while the peak purity was 88.3% for the sample of levothyroxine FDOFs with SMB due to the appearance of four impurity peaks.

Increasing the storage temperature to 40° for 30 days, three small peaks have been observed from the levothyroxine FDOFs with BHT formulation sample after 11.978min, 16.710 min and 23.002 min. However, the drug has been eluted after 15.636 min (figure 4.20 , Top graph). levothyroxine FDOFs sample stored for 30 days at 40℃ was observed after 15.593 min. The impurity peaks observed in this run have eluted after 16.678min, 21.059min, 21.791min, 25.402min, 26.007 min and 26.443 min. Figure 4.20 (Bottom graph) represents the HPLC chromatogram of levothyroxine FDOFs without antioxidant which shows two impurity peaks along with the drug peak. The drug in this run had eluted after 15.611 min while the impurities peaks have been observed at 16.724 min and 19.193 min. Figure 4.20 (Middle graph) represents the HPLC chromatogram of levothyroxine FDOFs with SMB which shows six impurity peaks along with the drug peak. FDOFs formulation with SMB was only 80.62% and 90.24% for the levothyroxine FDOFs formulation without antioxidant.

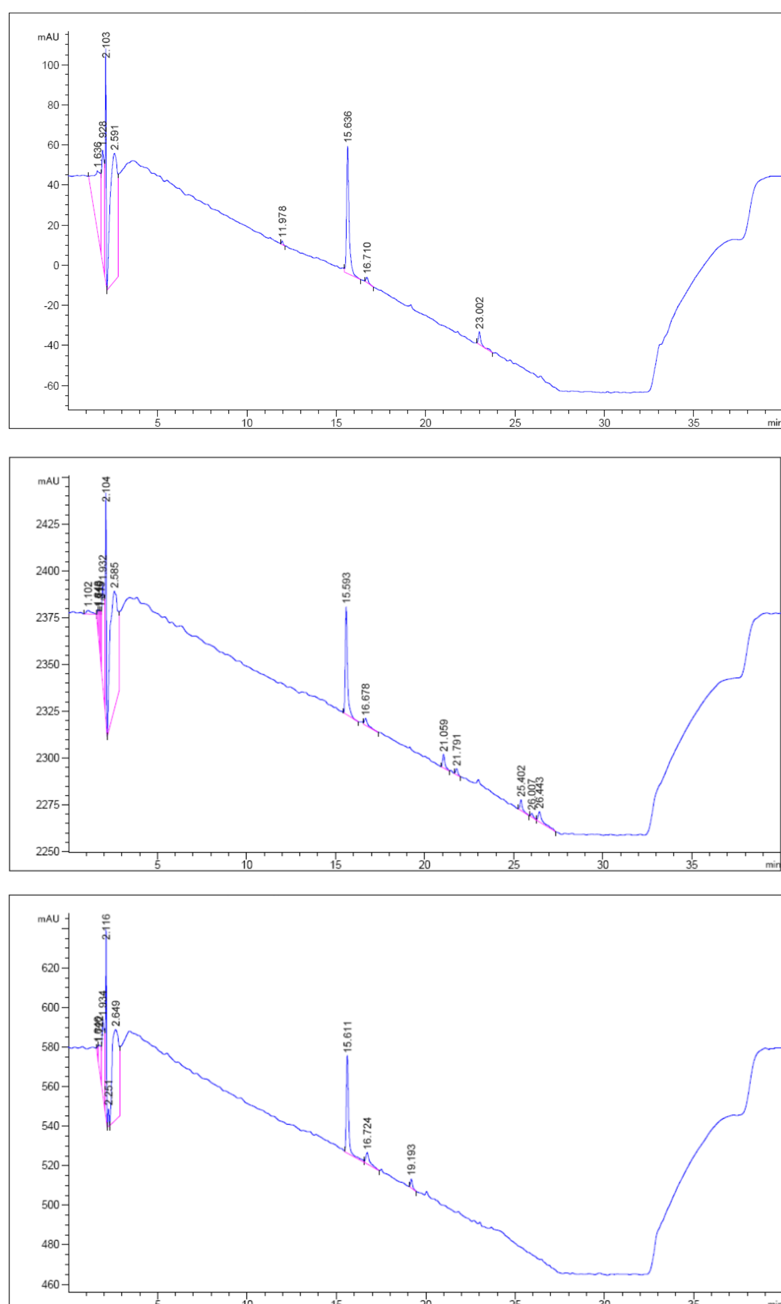


Figure 4-18: (Top): HPLC of levothyroxine FDOFs with BHT as antioxidant at a concentration of 10µg/mL, T=30 days, 40°C. (Middle): HPLC of levothyroxine FDOFs with SMB as antioxidant at a concentration of 10µg/mL, T=30 days, 40°. (Bottom): HPLC of levothyroxine FDOFs without antioxidant at a concentration of 10µg/mL, T=30 days, 40°.

It can be observed that, the number of impurity peaks are higher in the levothyroxine FDOF containing SMB compared to the other formulations. The percentage of the drug purity on the levothyroxine FDOFs with BHT at 40°C for 30 days was 92.7% while the percentage of peak purity under the same storage condition for the levothyroxine

Increasing the storage temperature to 70° for 30 days, three small degradation peaks were observed from the levothyroxine FDOFs with BHT formulation sample at 21.061, 25.411 and 26.455 min. The drug peak had eluted at 15.596 min (Figure 4.25, top graph). (Figure 4.25 middle graph) shows the chromatogram obtained from the FDOF formulation containing SMB stored at the same temperature for the same duration of time. Levothyroxine peak eluted at 15.767 min along with four degradation peaks being observed at 16.789, 19.169, 21.119 and 25.425 min. Levothyroxine FDOFs formulation without an antioxidant was also stored for 30 days at 70°C. Figure 4.29 shows the chromatogram obtained with this samples, where it can be seen that levothyroxine eluted at 15.58 min along with two degradation peaks at 16.733 min and 19.169 min.

The percentage of peak purity on the sample of levothyroxine FDOFs with SMB (Figure 2.25, bottom graph) was lower compared to the other levothyroxine FDOF formulations. The percentage of peak purity for levothyroxine FDOFs sample with SMB was about 84.8% while the peak purity was 88.7% for the levothyroxine FDOF formulation without an antioxidant and it was 93.54% levothyroxine FDOF formulation containing BHT as the antioxidant.

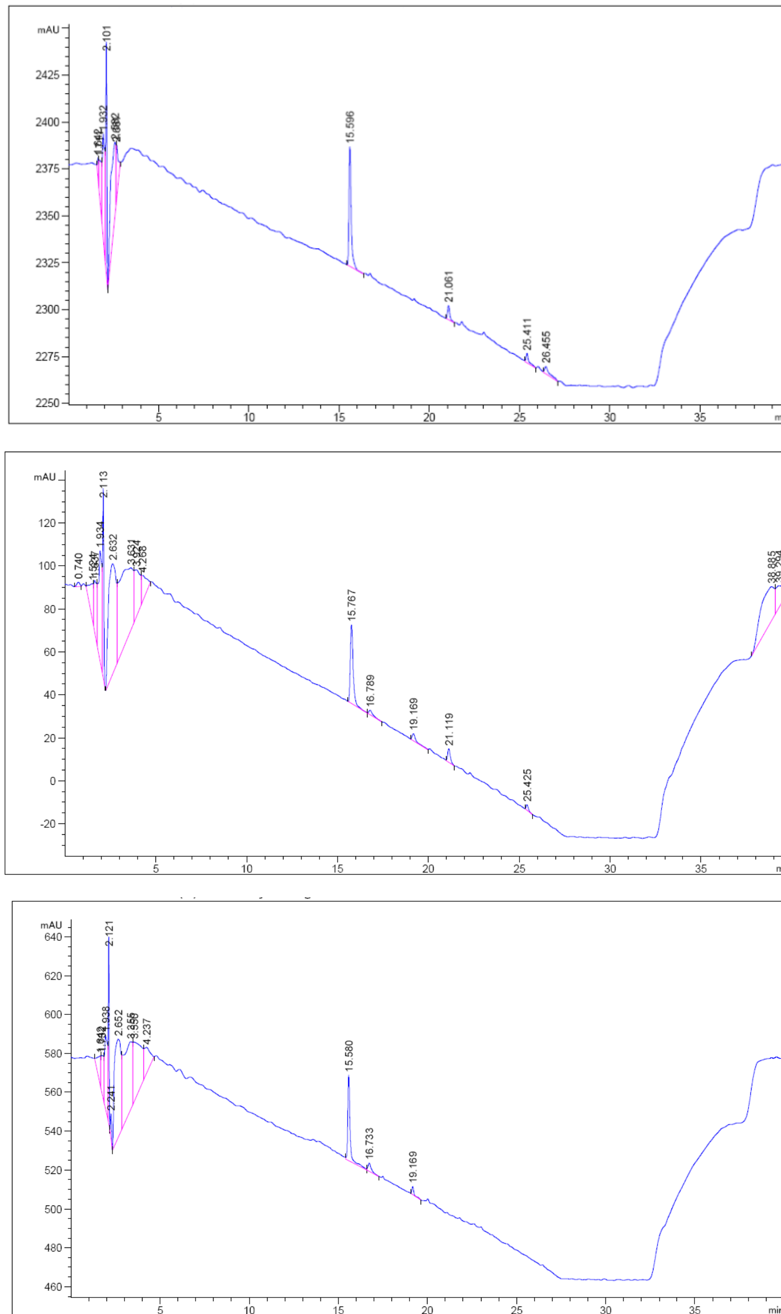


Figure 4-25: (Top): HPLC of levothyroxine FDOFs with BHT as antioxidant at a concentration of 10µg/mL, T=30 days, 70°C. (Middle): HPLC of levothyroxine FDOFs with SMB as antioxidant at a concentration of 10µg/mL, T=30 days, 70°C. (Bottom): HPLC of levothyroxine FDOFs without antioxidant at a concentration of 10µg/mL, T=30 days, 70°C.

Data comparing the three levothyroxine FDOF formulations at t=30 days in terms of their drug percentage recovery is shown in Figure 4.26. It can be seen from the figure that, at all the tested temperatures, levothyroxine FDOFs with BHT has the higher drug percentage recovery than the other formulations. In addition, the drug percentage

recovery has decreased when the storage temperature has increased in all the formulations.

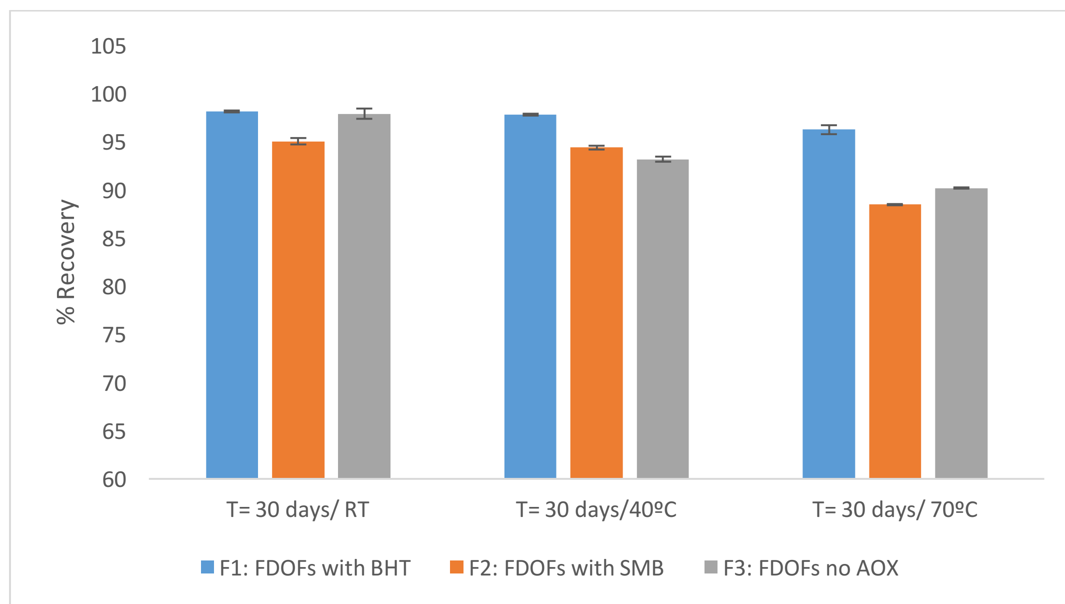


Figure 4-26: A graph comparing the percentage recovery of the developed formulations of levothyroxine FDOFs at t=30 days under three different temperatures: room temperature, 40°C and 70°C. The means of F1 at RT is (98.12% +/- 0.09), at 40°C (97.77% +/- 012) and at 70°C (96.22% +/- 0.45). The means of F2 at RT is (95% +/- 0.32), at 40°C (94.36% +/- 022) and at 70°C (88.44% +/- 0.086). The means of F3 at RT is (97.86% +/- 0.53), at 40°C (93.14% +/- 026) and at 70°C (90.16% +/- 0.097). Statistically, at different storage temperatures for, 30 days, one-way ANOVA showed p value is < 0.0001 considered extremely significant different between the formulations.

However, storing the formulations for 90 days at 25°C shows a drug peak after 15.606 min from the levothyroxine FDOFs formulation that has BHT as antioxidant (Figure 4.27, top graph). In addition, there were no impurity peaks observed in this chromatogram. Figure 4.27, (middle graph) represents the peak of the levothyroxine FDOF formulation containing SMB stored at 25°C for 90 days. The chromatogram below shows the drug peak elution at 15.667 min and there were no impurity peaks in this run either. Figure 4.27 (bottom graph) shows drug peak at 15.599 min from the FDOF formulation without antioxidant when stored at 25°C for 90 days. In addition, there were four impurity peaks in this run which had different retention times compared to the ones observed previously. The four degradation peaks in this run eluted at 19.161min, 21.063min, 25.412 min and 26.459 min.

The percentage of peak purity for the levothyroxine FDOFs formulation with BHT and with SMB was 100%. On the other hand, the percentage peak purity for the levothyroxine FDOFs formulation without antioxidant was 82%.

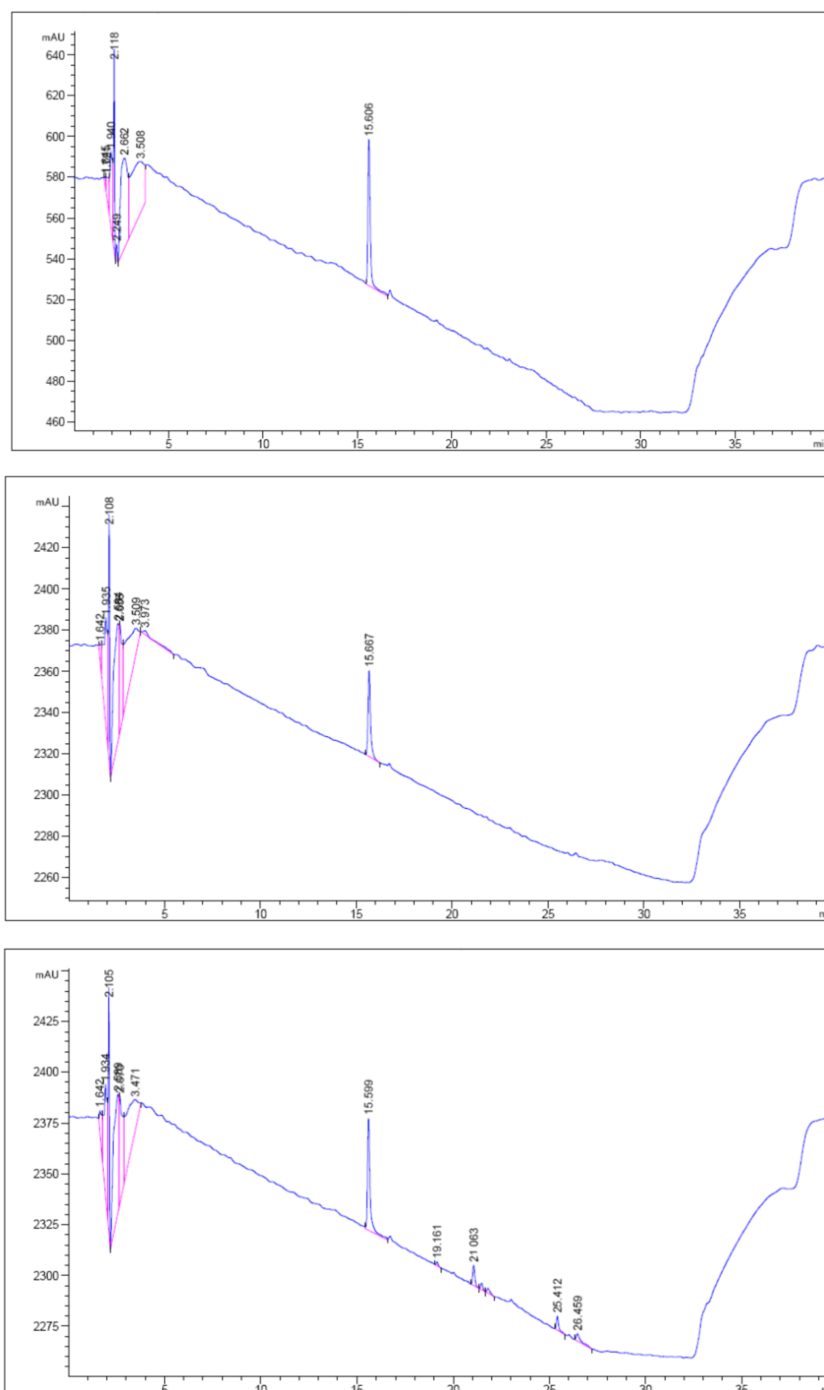


Figure 4-27: Top: HPLC of levothyroxine FDOFs with BHT as antioxidant at a concentration of 10µg/mL, T=90 days, 25°C. Middle: HPLC of levothyroxine FDOFs with SMB as antioxidant at a concentration of 10µg/mL, T=90 days, 25°C. Bottom: HPLC of levothyroxine FDOFs without antioxidant at a concentration of 10µg/mL, T=90 days, 25°C.

Figure 4.28 (Top graph) shows the chromatogram of levothyroxine FDOF formulation with BHT when it was stored at 40°C for 90 days. The drug peak in this run eluted after 15.765 min but with two small impurity peaks. The impurity peaks that were observed in the current run eluted after 6.785 min and 16.806 min. However, figure 4.28 (Middle graph), four impurity peaks were seen from the run of levothyroxine FDOF formulation

with SMB when stored at 40°C for 90 days. The drug peak in this run eluted after 15.644 min while the impurity peaks were observed after 16.743 min, 19.164 min, 22.998 min and 26.409 min. When the levothyroxine FDOF formulation without an antioxidant was stored at 40°C for 90 days, five impurity peaks were observed when analysed by HPLC (Figure 4.28, bottom graph). The drug peak in this run eluted after 15.610 min while the impurity peaks eluted at 13.848 min, 16.671 min, 21.780 min, 22.974 min and 26.374 min.

The percentage peak purity for the levothyroxine FDOF formulation with BHT was 96.22% while it was about 84.5% for the levothyroxine FDOFs formulation with SMB. The percentage peak purity for levothyroxine FDOF formulation without antioxidant was 84.17%.

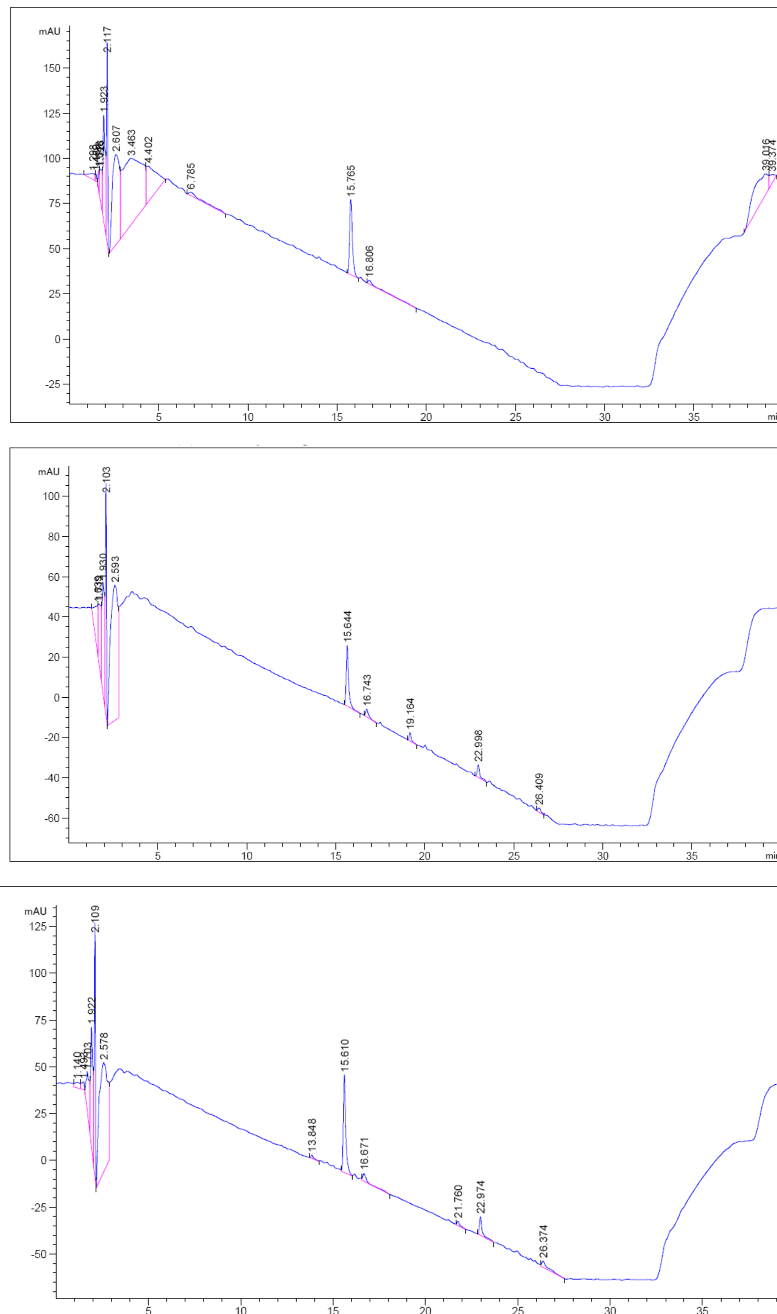
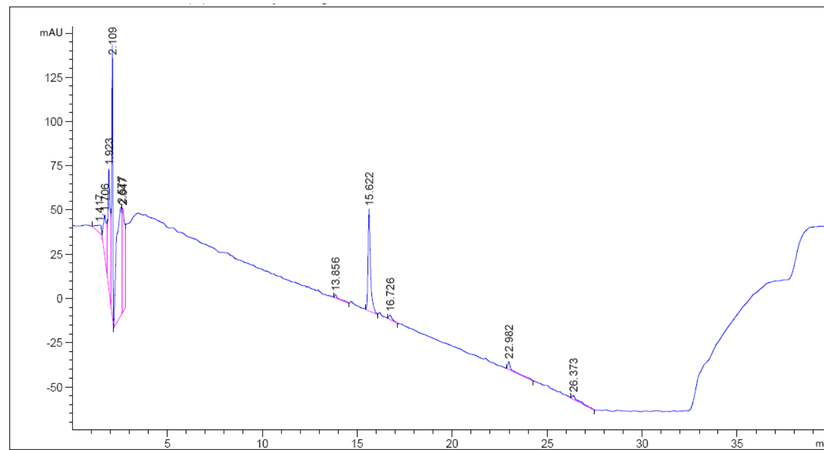


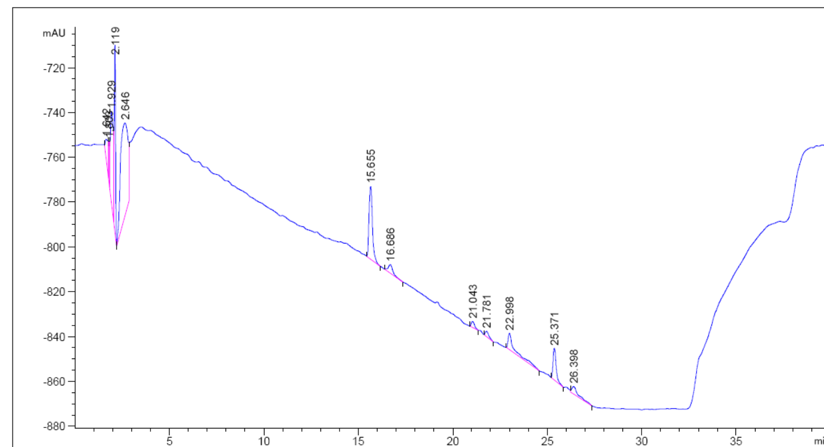
Figure 4-28: Top: HPLC of levothyroxine FDOFs with BHT as antioxidant at a concentration of 10µg/mL, T=90 days, 40°C. Middle: HPLC of levothyroxine FDOFs with SMB as antioxidant at a concentration of 10µg/mL, T=90 days, 40°C. Bottom: HPLC of levothyroxine FDOFs without antioxidant at a concentration of 10µg/mL, T=90 days, 40°C.

Levothyroxine FDOF with BHT when stored at 70°C for 90 days and analysed by HPLC is shown in figure 4.29 (Top graph). The drug peak eluted after 15.622 min along with four small impurity peaks which eluted at 13.856 min, 16.726 min, 22.987 min and 26.373 min. Figure 4.29 (Middle graph) illustrates the chromatogram of levothyroxine FDOF with SMB when the formulation was stored at 70°C for 90 days.

The drug peak was seen at 15.655 min. In addition, there were six impurity peaks observed in this run, which eluted at 16.686 min, 21.043 min, 21.781 min, 22.998 min, 25.371 min and 26.398 min. Figure 4.29 (Bottom graph) illustrates the chromatogram of levothyroxine FDOF formulation without an antioxidant when stored at 70°C for 90 days. The drug peak eluted at 15.622 min along with four impurity peaks that eluted at 16.610 min, 21.749 min, 23.011 min and 26.450 min.

The percentage peak purity for the levothyroxine FDOFs formulation with BHT was 90.76% while it was 67.4% for the levothyroxine FDOF formulation with SMB. The percentage peak purity for the levothyroxine FDOFs formulation without an antioxidant was 74.45%.





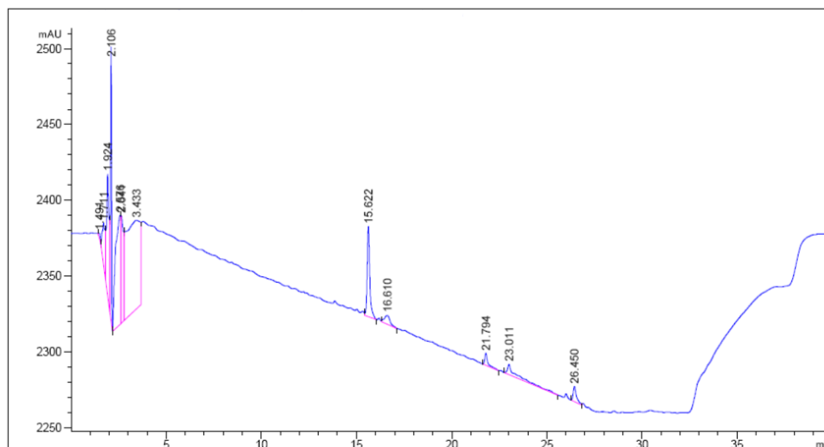


Figure 4-29: Top: HPLC of levothyroxine FDOFs with BHT antioxidant at a concentration of 10µg/mL, T=90 days, 70°C. Middle: HPLC of levothyroxine FDOFs with SMB antioxidant at a concentration of 10µg/mL, T=90 days, 70°C. Bottom: HPLC of levothyroxine FDOFs without antioxidant at a concentration of 10µg/mL, T=90 days, 70°C.

Figure 4.30 shows a comparison of the stability of the three levothyroxine FDOF formulations stored at t=90 days expressed in terms of their drug percentage recovery. It can be seen from the data below that, at all the tested temperatures, levothyroxine FDOFs with BHT had a higher drug percentage recovery compared to the other

formulations. In addition, the drug percentage recovery had decreased when the storage temperature was increased for all the formulations.

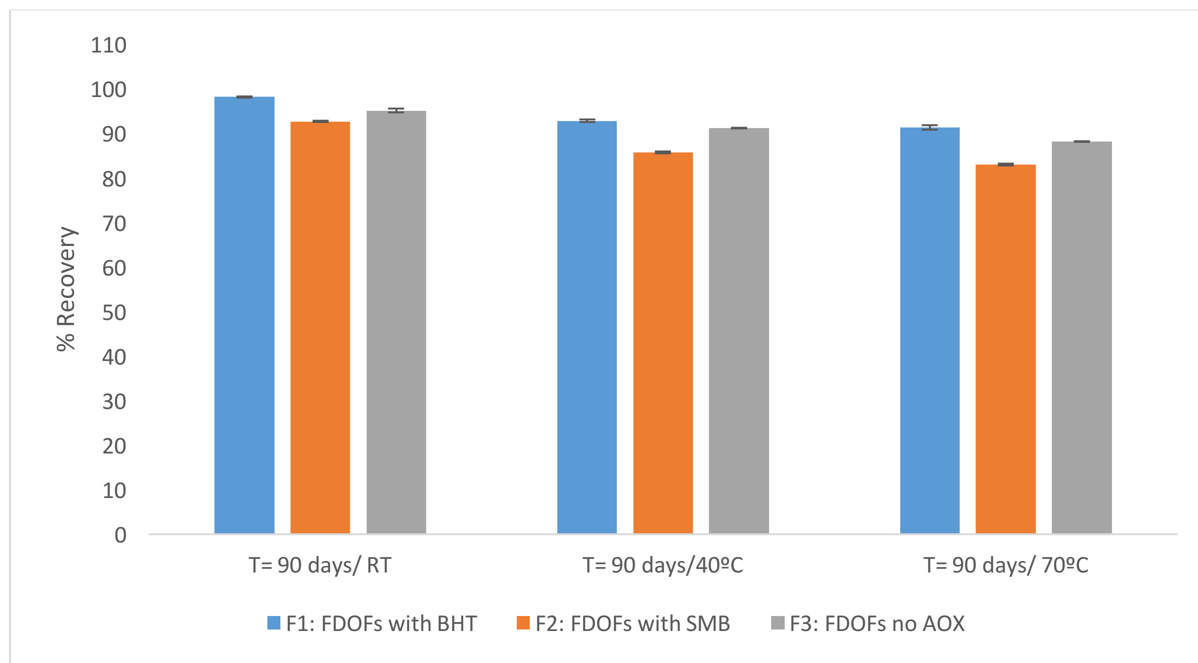


Figure 4-19: graph comparing the percentage recovery of the developed formulations of levothyroxine FDOFs at t=90 days under three different temperatures: room temperature, 40°C and 70°C. The means of F1 at RT is (98.15% +/- 0.31), at 40°C (92.8% +/- 0.11) and at 70°C (91.33% +/- 0.12). The means of F2 at RT is (92.66% +/- 0.08), at 40°C (85.76% +/- 0.41) and at 70°C (82.99% +/- 0.26. The means of F3 at RT is (95.12% +/- 0.61), at 40°C (91.16% +/- 0.12) and at 70°C (88.12% +/- 0.44). Statistically, at different storage temperatures for, 90 days, one-way ANOVA showed p value is < 0.0001 considered extremely significant different between the formulations.

Figure 4.31 (Top graph) shows the chromatogram of levothyroxine FDOF formulation with BHT when stored at room temperature for 180 days. It can be noticed that the levothyroxine peak eluted at 15.584 min along with three impurity peaks eluting at 21.056 min, 25.403 min and 26.442 min. Figure 4.31 (Middle graph) illustrates the chromatogram of levothyroxine FDOF formulation with SMB when stored at 25°C for 180 days. The levothyroxine peak eluted at 15.648 min along with five impurity peaks eluting at 11.307 min, 11.985 min, 16.748 min, 19.179 min and 23.014 min. Figure 4.31 (Bottom graph) shows the chromatogram of levothyroxine FDOF formulation without an antioxidant when stored at room temperature for 180 days. The levothyroxine peak eluted at 15.622 min along with two impurity peaks at16.756 min and 19.199 min.

The percentage peak purity for the levothyroxine FDOF formulation with BHT was 90.75%, while it was about 86.4% for the levothyroxine FDOFs formulation with SMB. The percentage peak purity for the levothyroxine FDOFs formulation without antioxidant was around 94.08%.

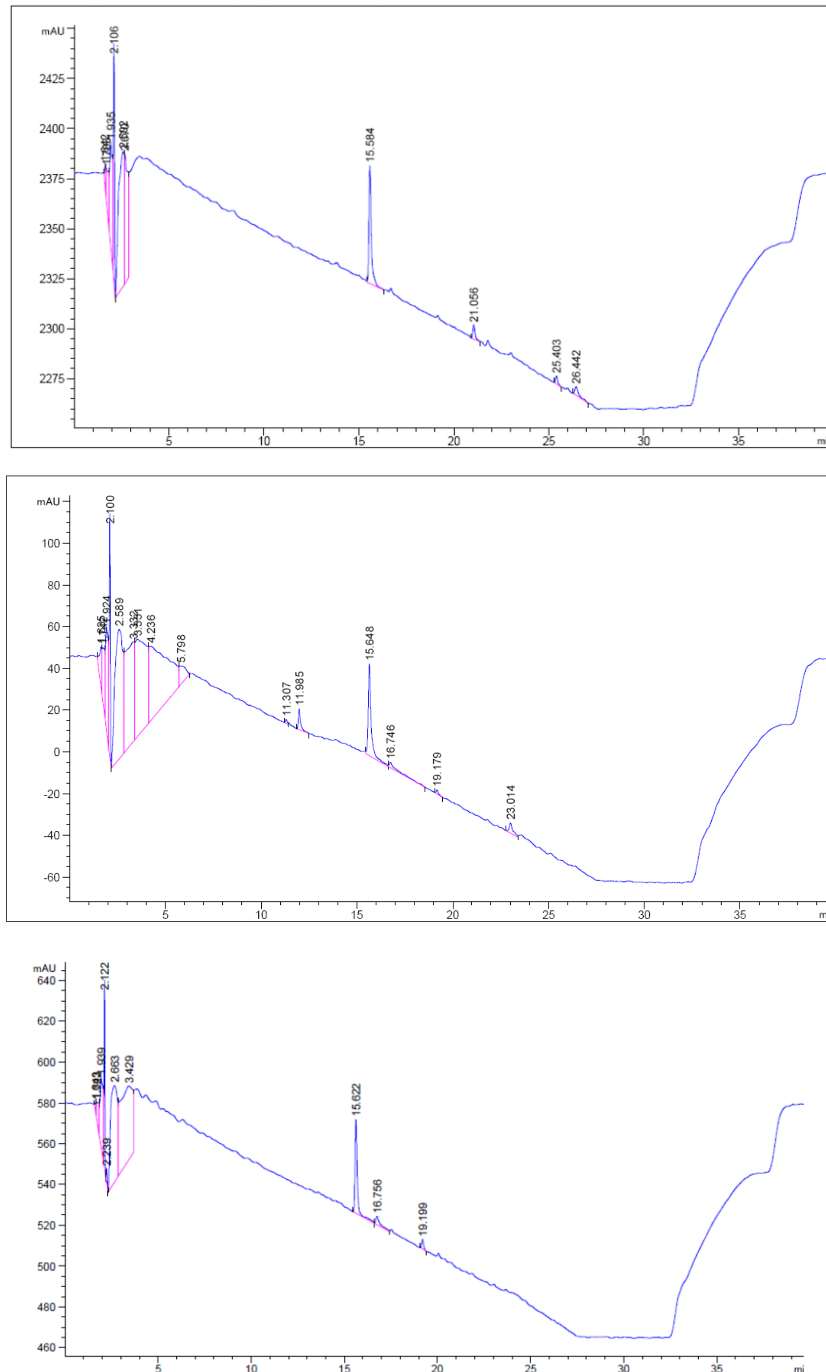


Figure 4-20: Top: HPLC of levothyroxine FDOFs with BHT antioxidant at a concentration of 10µg/mL, T=180 days, 25°C. Middle: HPLC of levothyroxine FDOFs with SMB antioxidant at a concentration of 10µg/mL, T=180 days, 25°C. Bottom: HPLC of levothyroxine FDOFs without antioxidant at a concentration of 10µg/mL, T=180 days, 25°C.

Figure 4.32 (Top graph) represents the chromatogram of levothyroxine FDOF formulation with BHT when stored at 40°C for 180 days. The levothyroxine peak eluted at 15.627 min along with three impurity peaks being observed at 16.697 min, 22.985 min and 26.376 min. Figure 4.32 (Middle graph) represents the chromatogram of levothyroxine FDOF formulation with SMB when stored at 40°C for 180 days. The levothyroxine peak eluted at 15.613 min along with seven impurity peaks eluting at 16.661 min, 19.156 min, 21.050 min, 21.770 min, 22.981 min, 25.371 min and 26.393 min. The drug peak from the formulation of levothyroxine FDOF without an antioxidant (40°C, 180 days) is shown in figure 4.32 (Bottom graph). The drug peak eluted at 15.618 min along with five impurity peaks seen at 16.710 min, 21.050 min, 22.985 min, 25.375 min and 26.385 min.

The percentage peak purity for the levothyroxine FDOF formulation with BHT was about 93.71% while it was about 75.5% for the levothyroxine FDOFs formulation with SMB. The percentage peak purity for the levothyroxine FDOFs formulation without an antioxidant was around 81.87%.

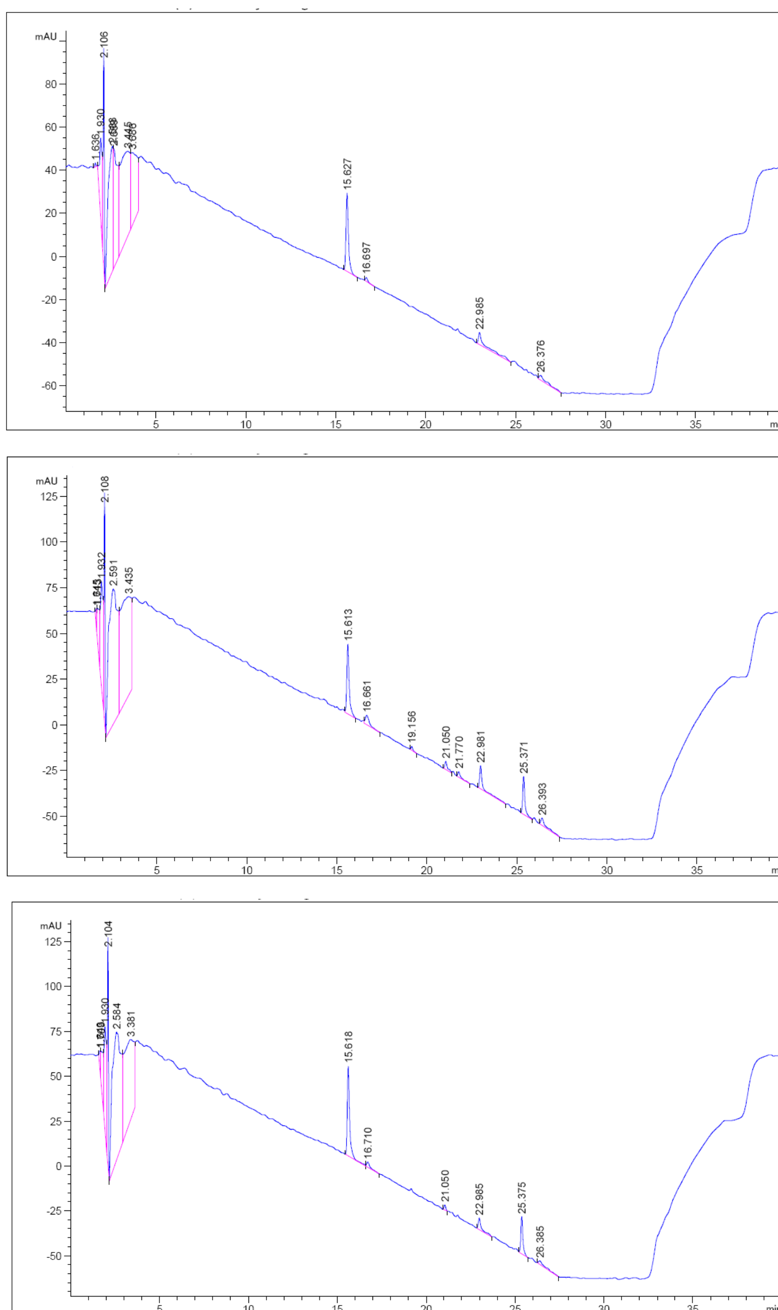


Figure 4-32: Top: HPLC of levothyroxine FDOFs with BHT antioxidant at a concentration of 10µg/mL, T=180 days, 40°C. Middle: HPLC of levothyroxine FDOFs with SMB antioxidant at a concentration of 10µg/mL, T=180 days, 40°C. Bottom: HPLC of levothyroxine FDOFs without antioxidant at a concentration of 10µg/mL, T=180 days, 40°C.

Figure 4.33 (Top graph) shows the chromatogram of levothyroxine FDOF formulation with BHT when stored at 70°C for 180 days. The drug peak had eluted after 15.780 min with two impurity peaks being observed at 16.757 min and 19.173 min. Figure 4.33 (Middle graph) shows the chromatogram obtained for the levothyroxine FDOFs with SMB when stored at 70°C for 180 days. The drug peak eluted at 15.619 min with

six impurity peaks observed at 16.722 min, 19.157 min, 21.052 min, 22.980 min, 25.369 min and 26.377 min. Figure 4.33 (Bottom graph) shows the chromatogram obtained with levothyroxine FDOFs without and antioxidant when stored at 70°C for 180 days. The drug peak was detected at 15.791 min along with six impurity peaks observed at 16.832 min, 17.573 min, 19.233 min, 20.092 min, 21.125 min and 23.686 min.

The percentage peak purity for the levothyroxine FDOFs formulation with BHT was about 97.42% while it was about 83.3% for the levothyroxine FDOFs formulation with SMB. The percentage peak purity for the levothyroxine FDOFs formulation without an antioxidant was around 84.59%.

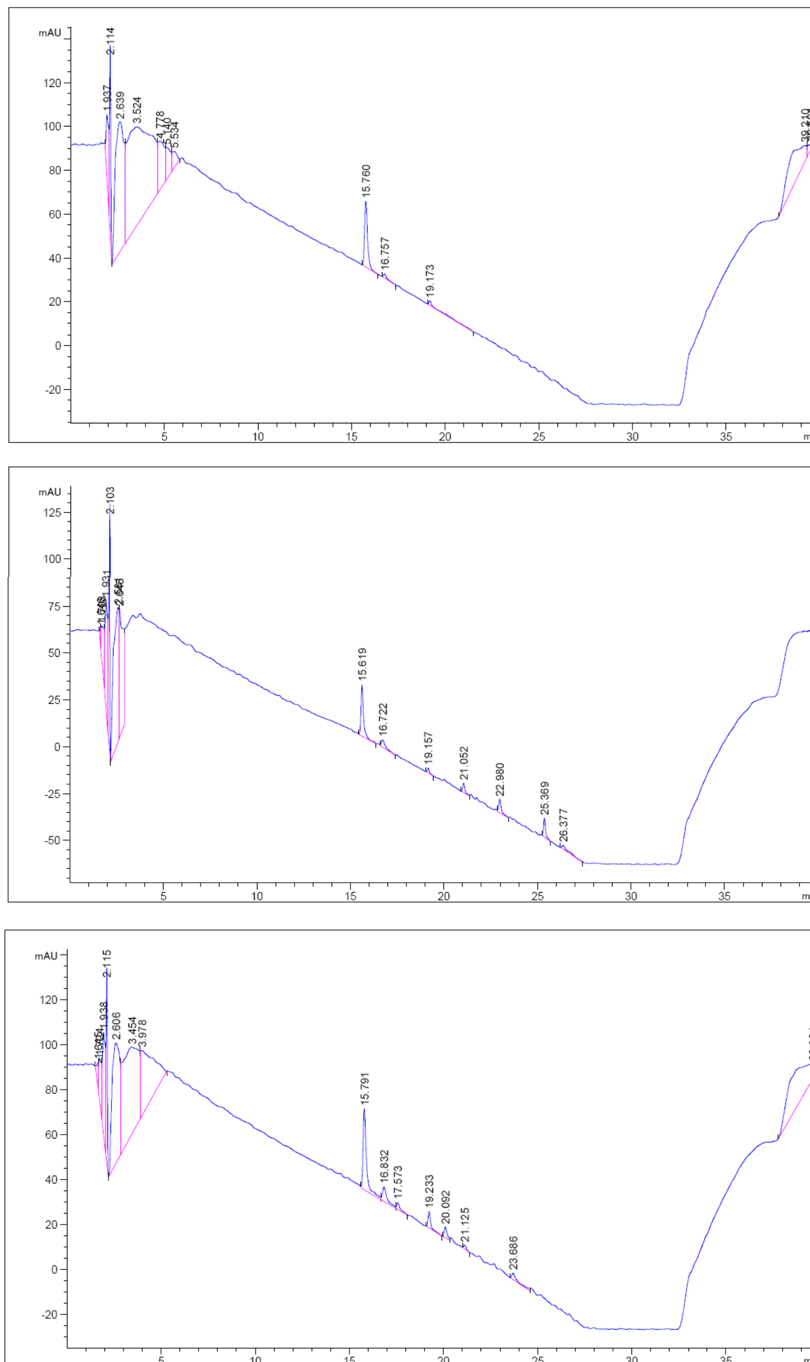


Figure 4-33: Top: HPLC of levothyroxine FDOFs with BHT antioxidant at a concentration of 10µg/mL, T=180 days, 70°C. Middle: HPLC of levothyroxine FDOFs with SMB antioxidant at a concentration of 10µg/mL, T=180 days, 70°C. Bottom: HPLC of levothyroxine FDOFs without antioxidant at a concentration of 10µg/mL, T=180 days, 70°C.

Figure 4.34 shows the comparison between the three levothyroxine FDOF formulations stored for 180 days at different temperatures in terms of their drug percentage recovery. It can be seen from the data below that, at all the tested temperatures, levothyroxine FDOFs with BHT had higher drug percentage recovery

compared to the other formulations. In addition, the drug percentage recovery had decreased when the storage temperature was increased for all the formulations.

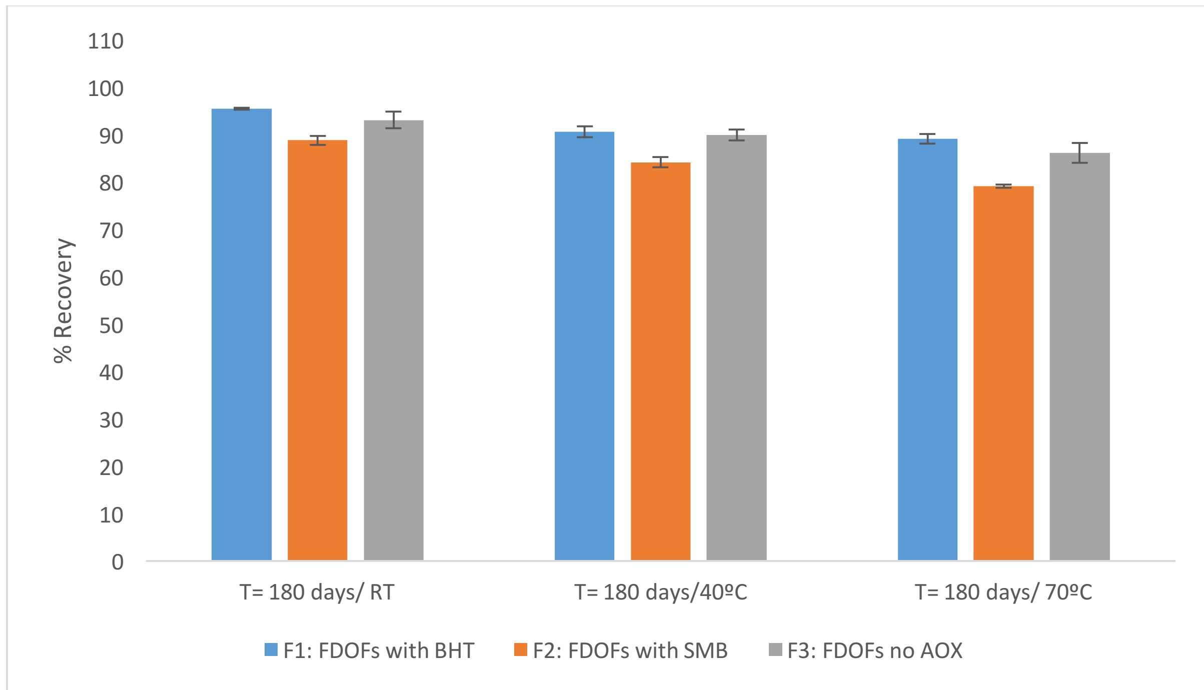


Figure 4-34: A graph comparing the percentage recovery of the developed formulations of levothyroxine FDOFs at t=180 days under three different temperatures: room temperature, 40°C and 70°C. The means of F1 at RT is (95.55% +/- 0.16), at 40°C (90.66% +/- 0.08) and at 70°C (89.2% +/- 0.21). The means of F2 at RT is (88.87% +/- 0.39), at 40°C (84.22% +/- 025) and at 70°C (79.13% +/- 0.38). The means of F3 at RT is (93.16% +/- 0.25), at 40°C (90.01% +/- 0.17) and at 70°C (86.24% +/- 0.61). Statistically, at different storage temperatures for, 180 days, one-way ANOVA showed p value is < 0.0001 considered extremely significant different between the formulations.

4.3.4 LC-MS Analysis of FDOF stability samples

In order to investigate the breakdown products of levothyroxine formulations observed in the HPLC analysis in the previous section, formulation stability samples were analysed by LC-MS.

Figure 4.35, shows the LC-MS spectrum for the levothyroxine FDOFs formulations at t=0. Figure 4.51 (Top) represents the LC-MS spectrum for the levothyroxine FDOF with BHT; Figure 4.51 (Middle) represents the LC-MS spectrum for the levothyroxine FDOF with SMB while Figure 4.51 (Bottom) represents the LC-MS spectrum for the levothyroxine FDOFs without an antioxidant. The peak of 777.7 m/z represents the levothyroxine peak while 737.7 is the decarboxylated levothyroxine.

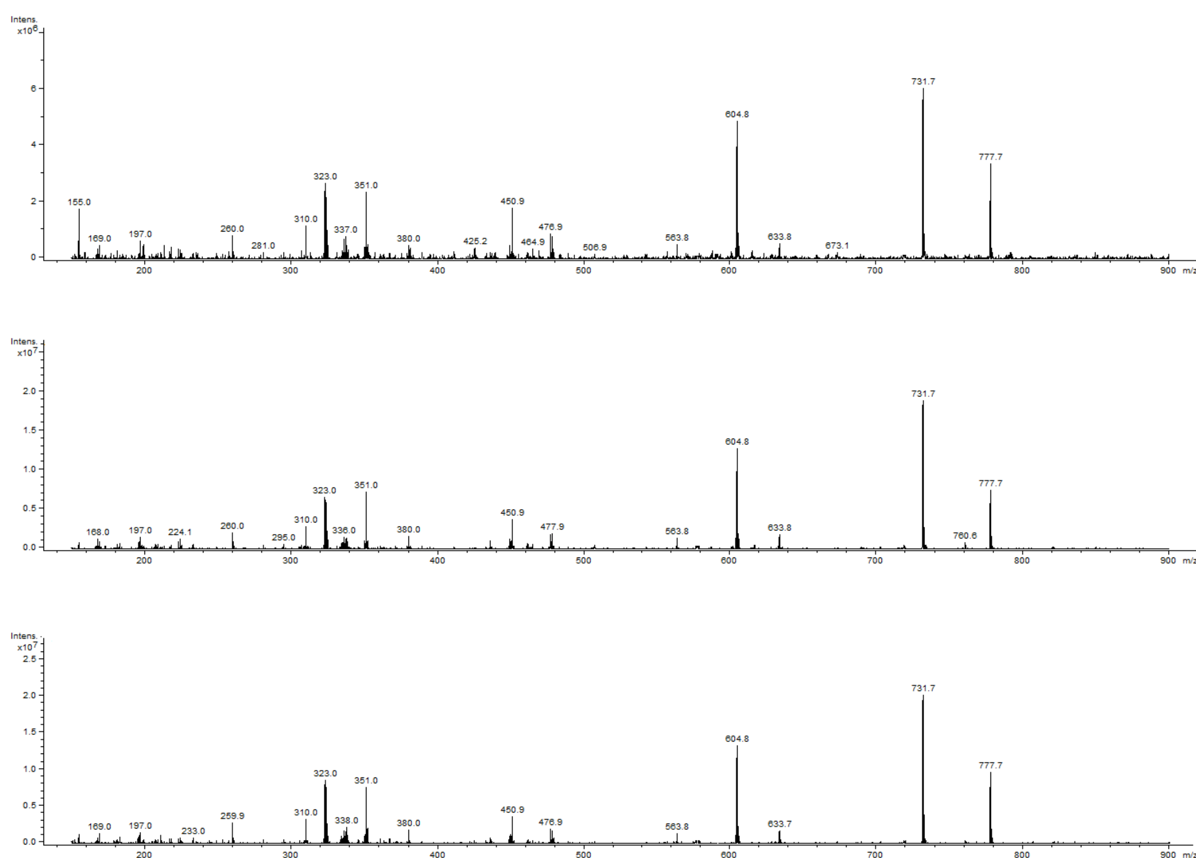


Figure 4-35: LC-MS of three samples which are A: FDOFs with BHT as antioxidant (Top graph), B FDOFs with SMB as antioxidant (Middle graph), C: FDOFs without antioxidant (Bottom graph). The storage condition of the samples is: t=0

Figure 4.36 shows the LC-MS spectrum for the developed levothyroxine FDOF formulations at t=30 days, room temperature. Figure 4.52 (Top) represents the LC-MS for the levothyroxine FDOFs with BHT, Figure 4.52 (Middle) represents the LC-MS spectrum for the levothyroxine FDOFs with SMB and Figure 4.52 (Bottom) represents the LC-MS spectrum for the levothyroxine FDOFs without an antioxidant. Levothyroxine peak was observed at 777.7 m/z and there are no degradation peaks noticed in this run.

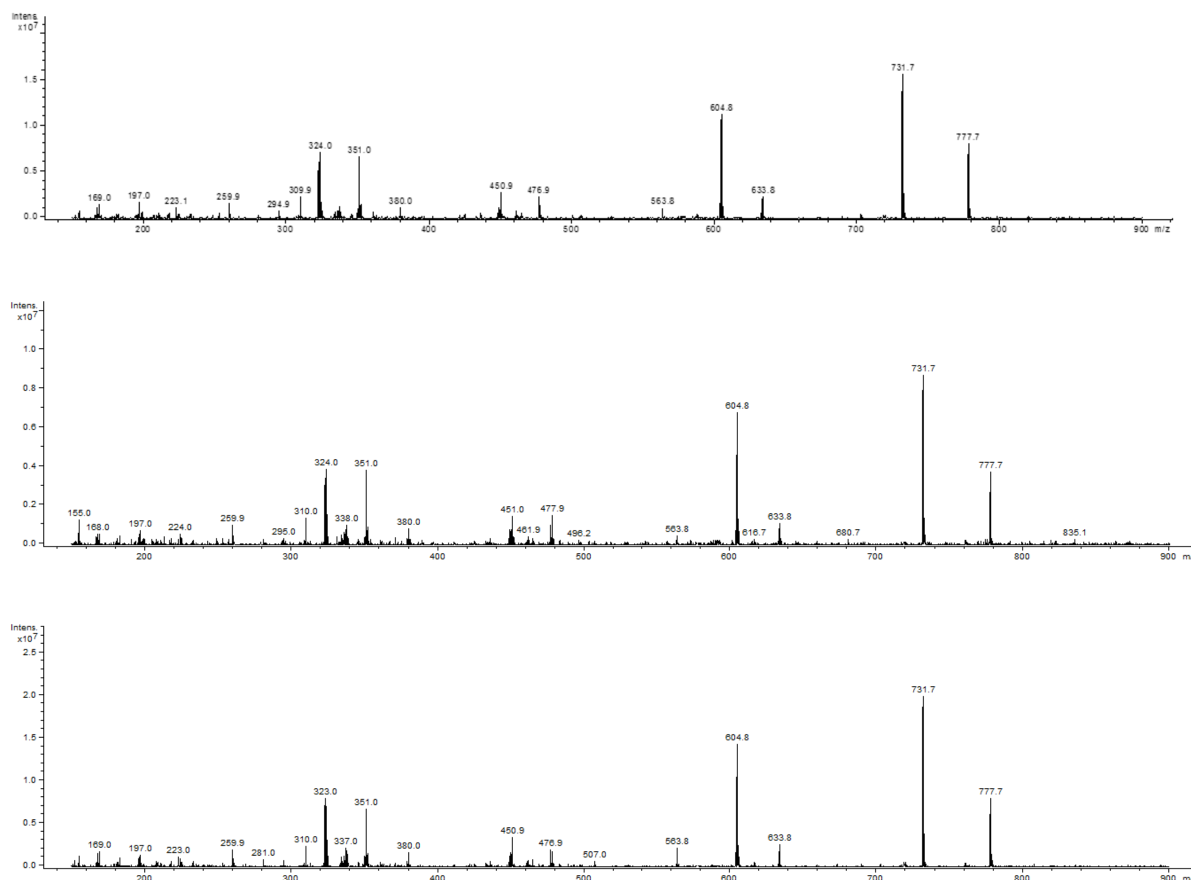


Figure 4-36: LC-MS of three samples which are 1: FDOFs with BHT as antioxidant (Top graph), 2: FDOFs with SMB as antioxidant (Middle graph), 3: FDOFs without antioxidant (Bottom graph). The storage condition of the samples is: t=30 days at 25°C

By storing the samples of the levothyroxine FDOFs at higher temperature of 40°C for 30 days, the formulations were found to remain stable and no degradation peaks were observed. Figure 4.34 (Top) represents the LC-MS for the levothyroxine FDOFs with BHT, Figure 4.34 (Middle) represents the LC-MS for the levothyroxine FDOFs with

SMB while Figure 4.34 (Bottom) represents the LC-MS for the levothyroxine FDOFs without an antioxidant. The drug peaks were observed at 777.7m/z.

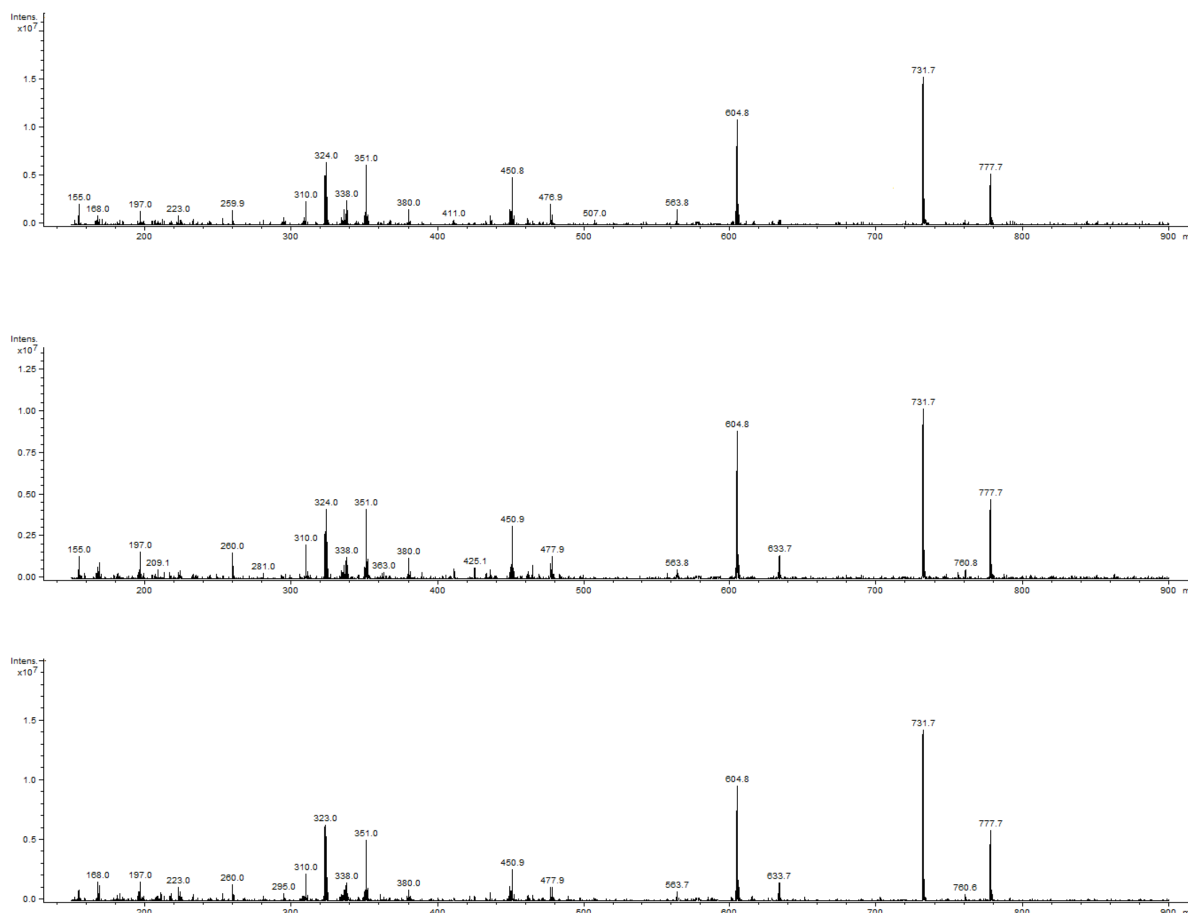


Figure 4-34: LC-MS of three samples which are 1: FDOFs with BHT as antioxidant (Top graph), 2: FDOFs with SMB as antioxidant (Middle graph), 3: FDOFs without antioxidant (Bottom graph). The storage condition of the samples is: t=30 days at 40°C

The levothyroxine FDOFs formulations were stored at a higher temperature of 70°C for 30 days and were analysed by LC-MS. From figure 4.35, it can be seen that, the high temperature had affected the formulation F2 (Levothyroxine FDOFs with SMB) and formulation F3 (Levothyroxine FDOFs without antioxidant). In contrast, formulation F1 had remained stable and no degradation peaks have been observed. The degradation peaks for F2 were observed at 577 m/z, 747 m/z while the degradation peak recorded for F3 was at 577 m/z, 592 m/z, 718 m/z and 747m/z.

These degradation peaks have been discussed earlier in chapter 2 and they will be discussed in the discussion section of this chapter.

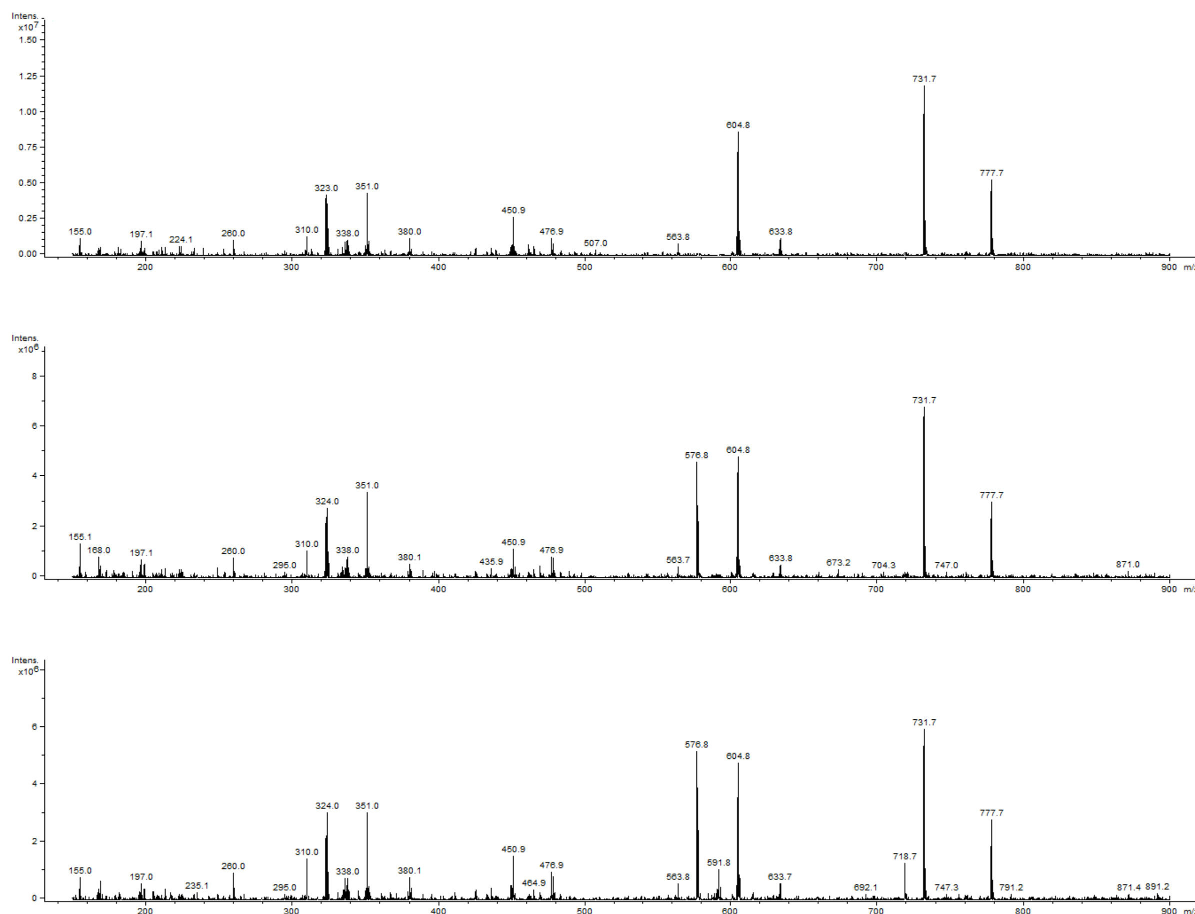


Figure 4-35: LC-MS of three samples which are 1: FDOFs with BHT as antioxidant (Top), 2: FDOFs with SMB as antioxidant (Middle), 3: FDOFs without antioxidant (Bottom). The storage condition of the samples is: t=30 days at 70°C.

All three formulations were stored for 90 days at three different storage conditions: 25C, 40C and 70C. Figure 4.56 shows the LC-MS of levothyroxine FDOFs formulations when the samples stored at 25°C for 90 days. From the spectrum shown in Figure 4.36 it can be seen that F1 (Top) had remained stable and F2 (Middle) had small peaks but not a recognized degradation product of levothyroxine while F3 (Bottom) had a degradation peak at 592 m/z.

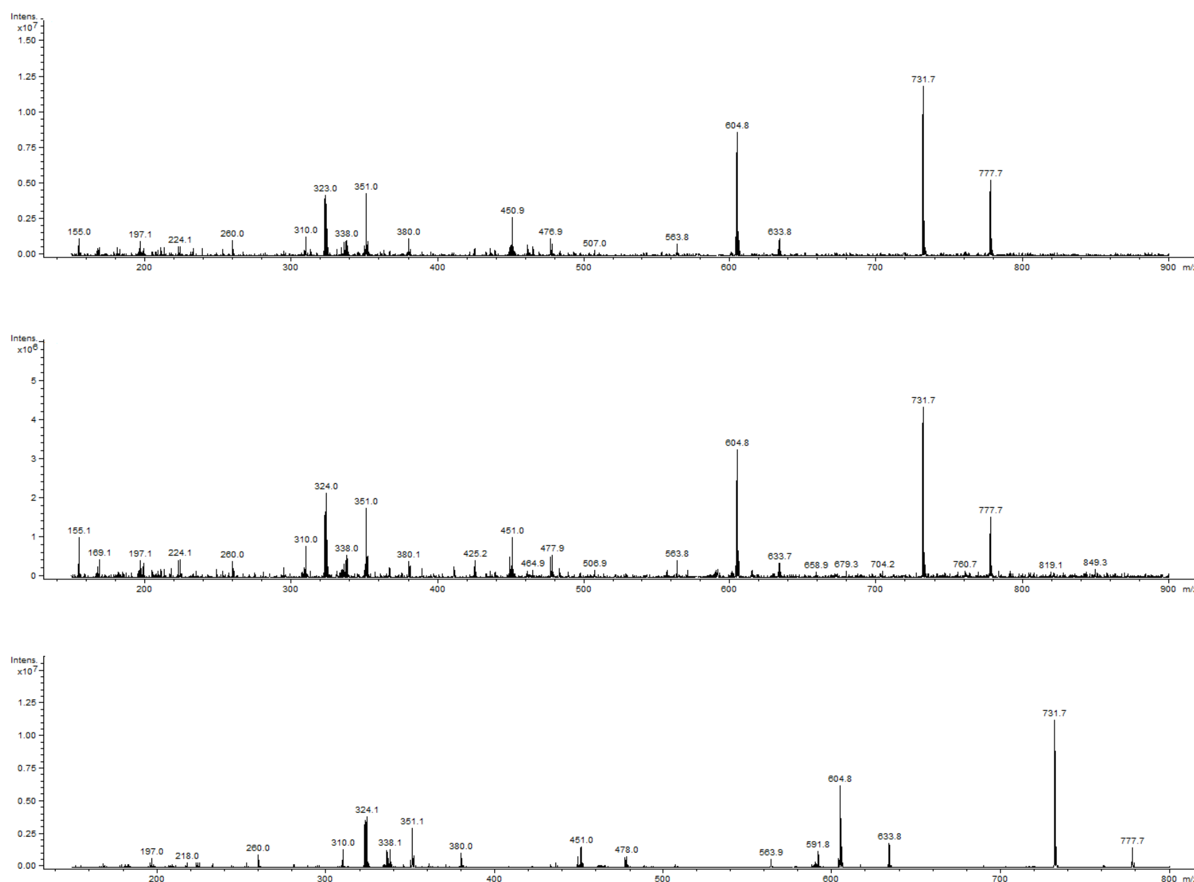


Figure 4-36: LC-MS of three samples which are 1: FDOFs with BHT as antioxidant (Top graph), 2: FDOFs with SMB as antioxidant (Middle graph), 3: FDOFs without antioxidant (Bottom graph). The storage condition of the samples is: t=90 days at 25°C

When the formulations were stored at high temperature of 40°C for 90 days, Figure 4.37, F1 (TOP) and F2 (Middle) had remained stable and no any degradation products were observed. In contrast, F3 (Bottom) yielded a degradation product at 577 m/z along with a number of small peaks noticed in this analysis.

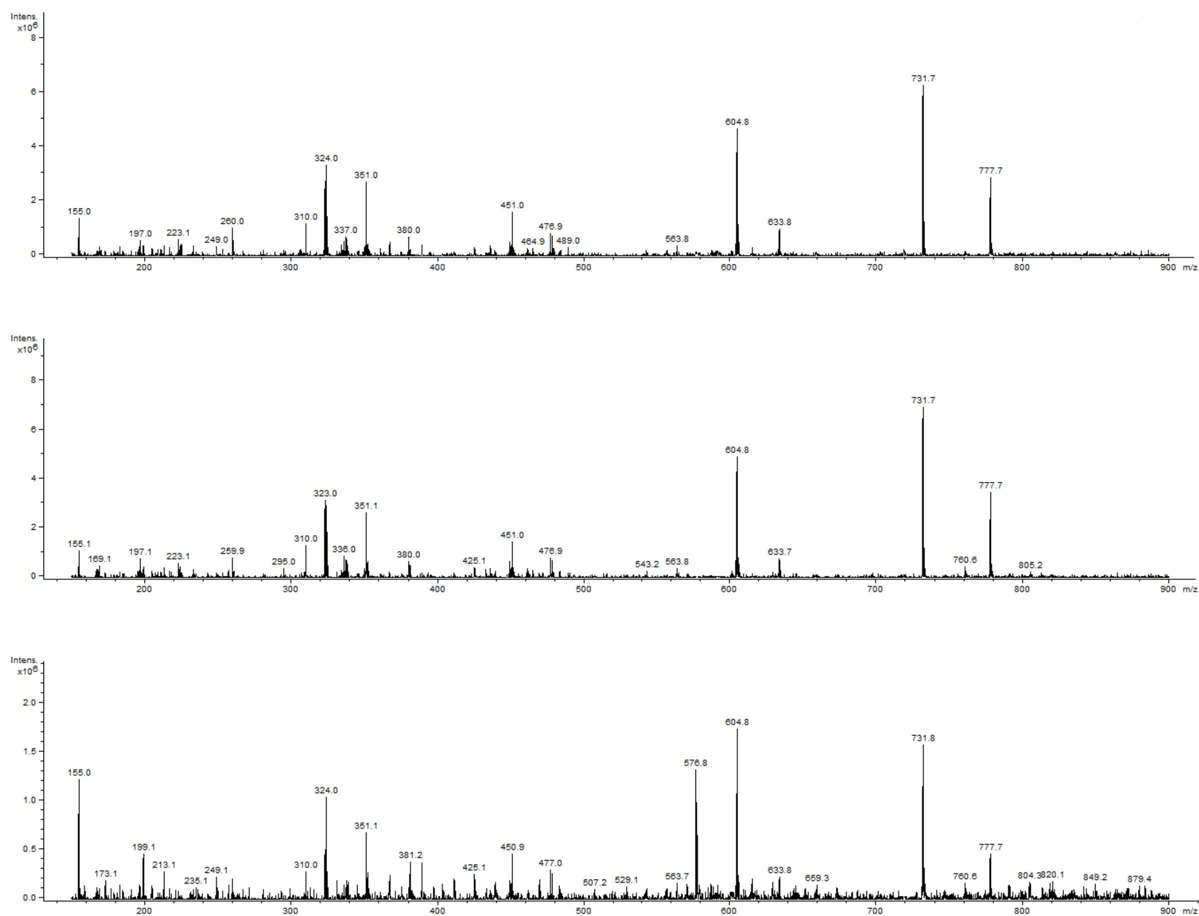


Figure 4-37: LC-MS of three samples which are 1: FDOFs with BHT as antioxidant (Tp graph), 2: FDOFs with SMB as antioxidant (Middle graph), 3: FDOFs without antioxidant (Bottom graph). The storage condition of the samples is: t=90 days at 40°C.

Figure 4.38 shows the LC-MS analysis of formulations stored at 70°C for 90 days' storage. F1 (Top) had remained stable with no degradation peaks observed while F2 (Middle) shows a degradation peak at 577 m/z along with a number of small impurities recorded in this analysis. F3 (Bottom) shows a degradation peak at 592 m/z.

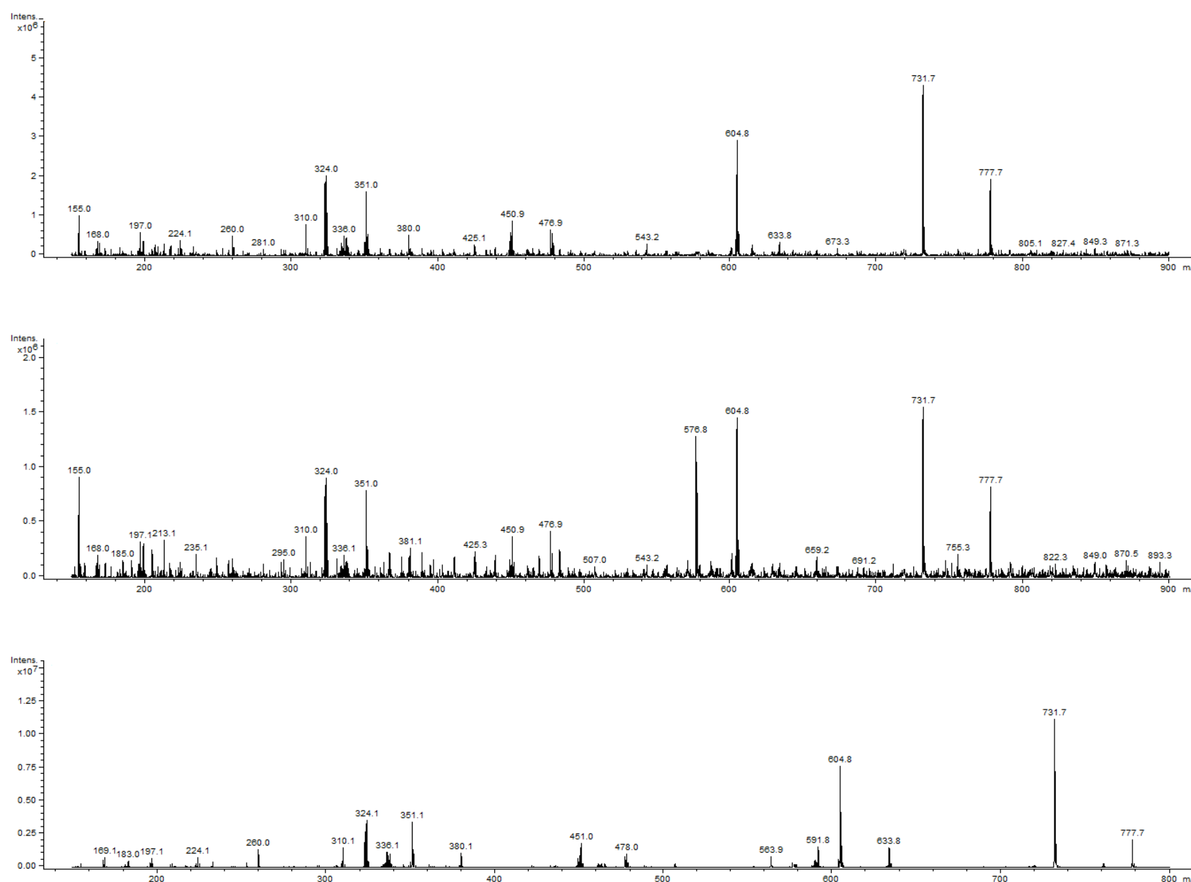


Figure 4-38: LC-MS of three samples which are 1: FDOFs with BHT as antioxidant (Top graph), 2: FDOFs with SMB as antioxidant (Middle graph), 3: FDOFs without antioxidant (Bottom graph). The storage condition of the samples is: t=90 days at 70°C

The formulations of levothyroxine FDOFs were also stored for 180 days at three different temperatures which are 25°C, 40°C and 70°C. Figure 4.39 shows the stability study of levothyroxine FDOFs when the samples were stored at 25°C for 180 days. It can be seen that, F1 (Top), F2 (Middle) and F3 (Bottom) did not show any degradation peaks in this analysis.

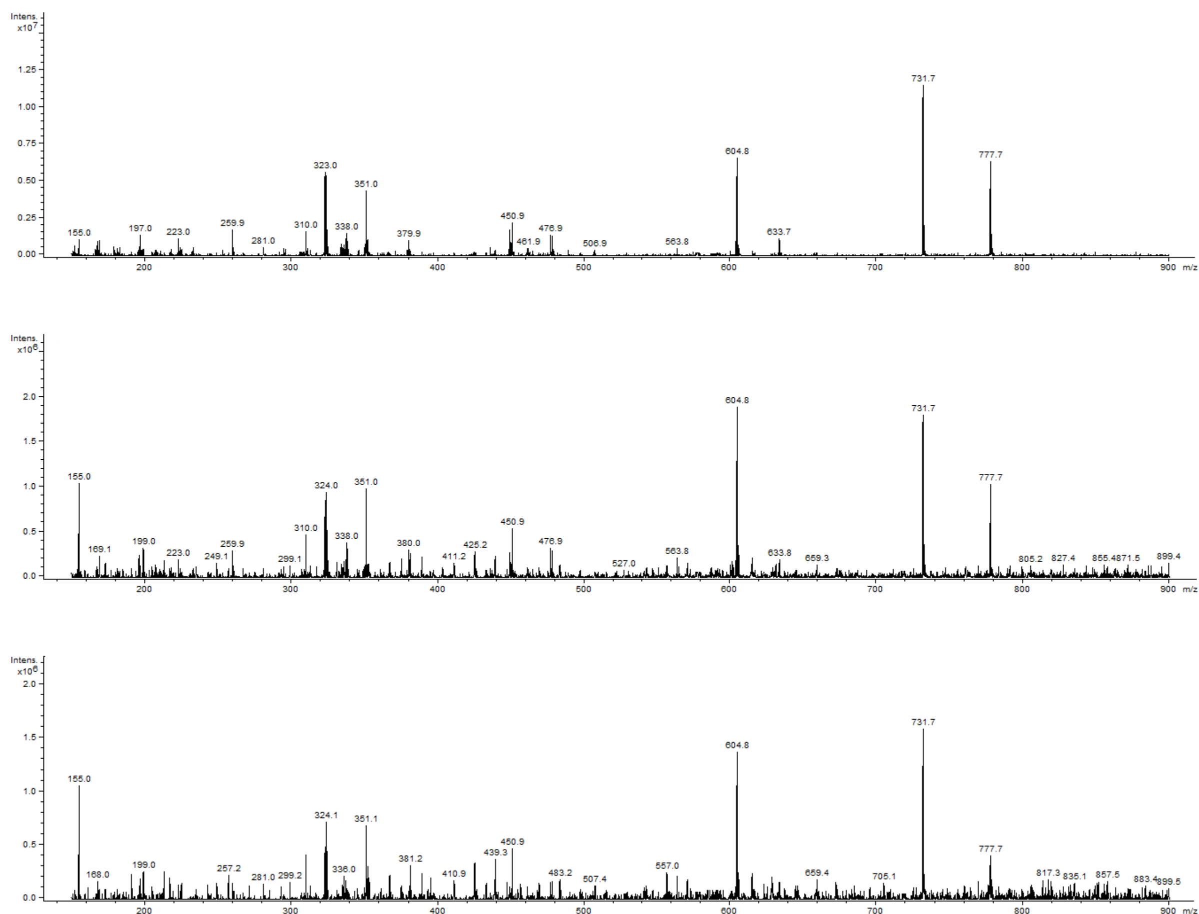


Figure 4-39: LC-MS of three samples which are 1: FDOFs with BHT as antioxidant (Top graph), 2: FDOFs with SMB as antioxidant (Middle graph), 3: FDOFs without antioxidant (Bottom graph). The storage condition of the samples is: t=180 days at 25°C.

Figure 4.40 shows the stability study of levothyroxine FDOFs when the samples were stored at 40°C for 180 days. There were no degradation peaks noticed for F1 (Top) and F2 (Middle) while F3 (Bottom) has shown degradation peaks at 577 m/z and 718 m/z.

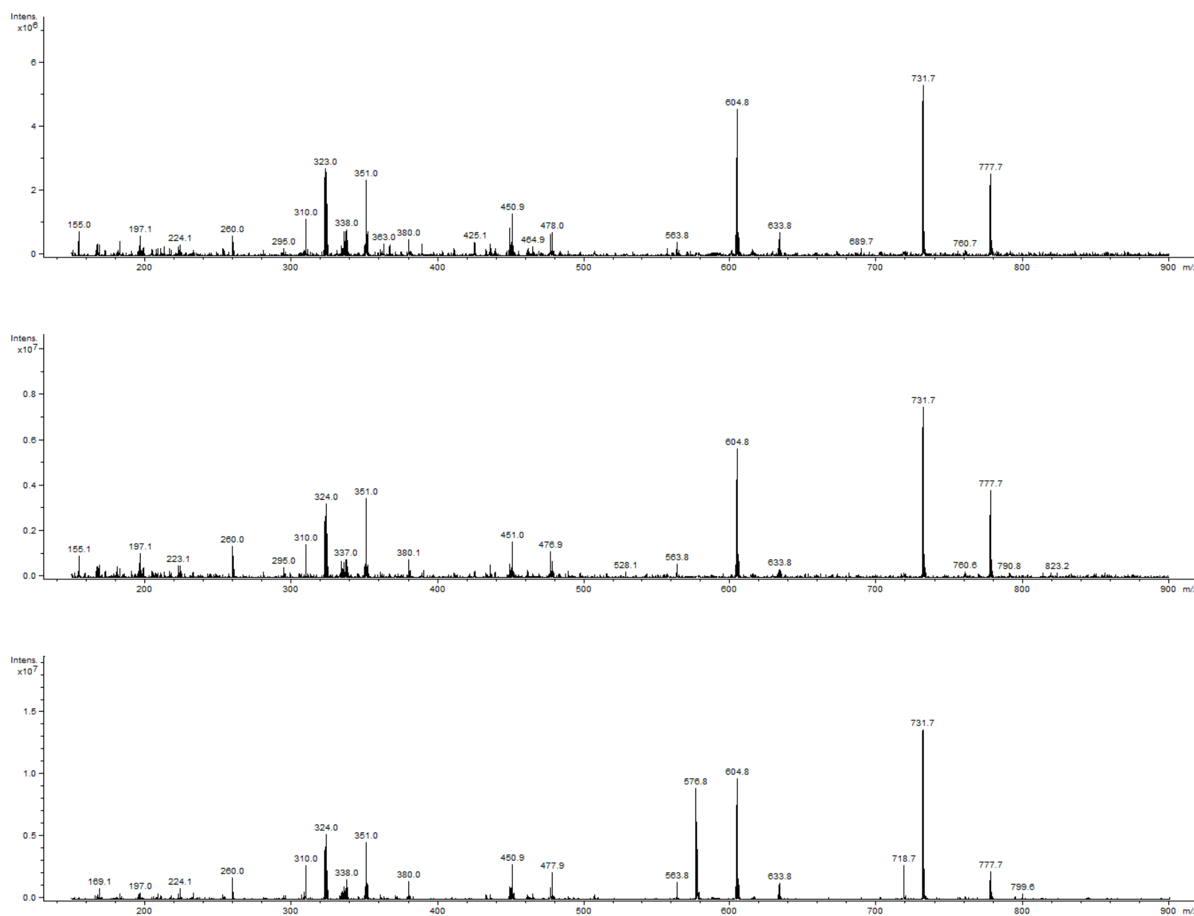
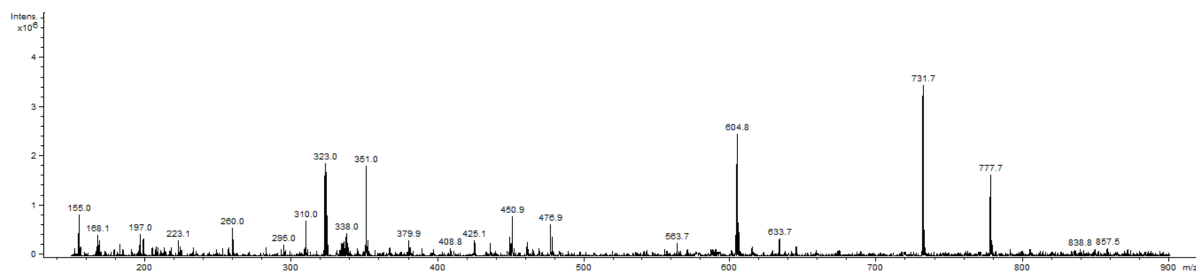
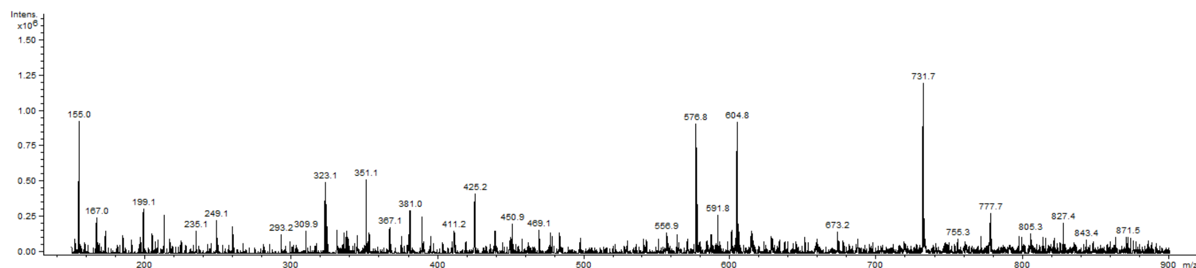


Figure 4-40: LC-MS of three samples which are 1: FDOFs with BHT as antioxidant (Top graph), 2: FDOFs with SMB as antioxidant (Middle graph), 3: FDOFs without antioxidant (Bottom graph). The storage condition of the samples is: t=180 days at 40°C

LC-MS analysis of the samples stored at 70°C for 180 days, formulation F1 (Top) did not show any degradation peaks while F2 (Middle) resulted in two degradation peaks at 577 m/z and 592 m/z. Formulation F3 (Bottom), also showed two degradation peaks but at 577 m/z and 718 m/z.





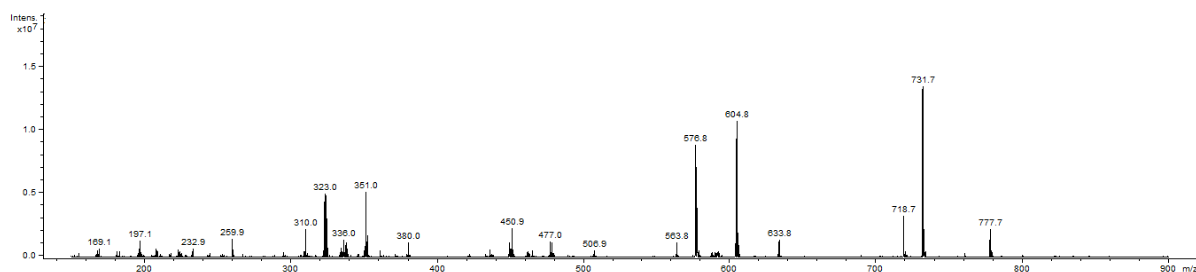


Figure 4-41: LC-MS of three samples which are 1: FDOFs with BHT as antioxidant (Top graph), 2: FDOFs with SMB as antioxidant (Middle graph), 3: FDOFs without antioxidant (Bottom graph). The storage condition of the samples is: t=180 days at 70°C.

4.4 Discussion

In this chapter, four formulations of levothyroxine FDOFs were developed to examine their chemical stability under three different temperatures 25°C,40°C, and 70°C, for a storage period of 30 days, 90 days and 180 days. The formulations that were developed are the FDOFs with and without an antioxidant and all of them were developed using 5% w/w HPMC as the polymer. The antioxidants used to potentially enhance the stability of the active ingredient were BHT, SMB and ascorbic acid. The main aim in this chapter was to improve the chemical stability of the levothyroxine formulations by adding an antioxidant to prevent the oxidation reaction, which is the main concern for levothyroxine when stored at different temperatures for prolonged period of time.

Results obtained in this chapter showed that the formulation developed without an antioxidant had changed its colour when exposed to a high temperature for a long period. However, it was discussed earlier in this research (chapter 2) that a change in the colour of levothyroxine is related to the chemical interaction of the drug with the free oxygen under thermodynamic conditions and due to presence of the water activity in the formulation.

Furthermore, the change in the FDOFs colour sharply reduced in the formulations that were developed with BHT and SMB, which clearly indicated that BHT and SMB have mostly prevented the oxidation reaction. It was reported previously in another research that, BHT has a very strong effect on inhibiting the oxidation reaction [153]

In terms of the formulation that was developed with ascorbic acid, this formulation has not succeeded due to formation of a yellow-brown colour on exposing the sample to high temperature even for a short period of storage time (30 days). A previous study on ascorbic acid has indicated that, non-enzymatic browning reaction can take place with ascorbic acid and it will degrade into dehydroascorbic acid [154]. Therefore, further development of the formulation using ascorbic acid as antioxidant was stopped due to the presence of the brown coloration.

In terms of the drug dissolution, the samples of the developed FDOFs of levothyroxine were investigated for drug release after storage for 6 months at three different

temperatures 25°C, 40°C and 70°C. The outcomes of the dissolution study for the levothyroxine FDOFs can be explained by relying on different observations that are discussed below.

Applying a stress condition on the stored samples by exposing them to high temperature had a negative influence on the percentage of the drug release from all the formulations but to a different extent. It can be calculated that, after six months, the percentage drug release that decreased from the samples when they were stored at 25°C was only 2% from the levothyroxine FDOFs with BHT, around 5.8% from the levothyroxine FDOFs with SMB and 4.6% from the levothyroxine FDOFs without antioxidants.

At 40°C for six months, the percentage of drug release declined by 9.7% from the levothyroxine FDOFs with BHT, 14.6% from the levothyroxine FDOFs with SMB and 17% from the levothyroxine FDOFs without antioxidants. At 70°C for six months, the percentage of drug release had reduced by 17.5% from the levothyroxine FDOFs with BHT, 24.5% from the levothyroxine FDOFs with SMB and 28.8% from the levothyroxine FDOFs without antioxidants.

The date presented above highlights that a higher temperature has affected the percentage of the drug release of the levothyroxine FDOFs and this influences has been decreased when the antioxidant BHT has been included in the preparation of the films. BHT showed an improvement in the percentage levothyroxine release when the FDOFs with BHT was compared to the levothyroxine FDOFs that had no antioxidant. In contrast, SMB had not shown any positive alteration on the percentage of levothyroxine release from the formulations. Previous studies on the drug stability and drug delivery have been showing that, BHT can decrease the oxidative stress phenomenon (can produces from exposing the drug to high temperature [150].

From the data represented in the dissolution profile, storage of FDOF with or without an antioxidant has lead to a lower amount of drug being released. The results indicate that the active drug might have degraded over the storage period. These observations are further investigated with the use of HPLC as the analytical technique. Howwver, the statistical nalaysis for the formulations regarding the drug release has calcualted to compare the signifant difference between the formulations. It can be mentioned from the statistical part of the drug release data that, there were no significant

differences between the developed levothyroxine FDOFs formulations when One way Anova was used for this purpose. This means that, the developed levothyroxine FDOFs formulations showed slightly same drug release pattern under different storage and stress conditions. That was not the case when the percentage recovery was statisticaly analysed by one way anova as the formulations showed extremely significant difference regarding their percentage of recovery. That support the data obtained from HPLC as mentioned below in this section.

In addition to this, including BHT in the pharmaceutical formulations such as FDOFs improves the drug release profile which was observed in this research. However, in referred to the graphs that were presented in the results section of this chapter (dissolution study), it can be seen that, levothyroxine FDOFs with BHT showed an ideal drug release from the films formulations and it is a higher drug release than the other levothyroxine FDOFs that were developed in this research.

In addition to the dissolution studies, the drug content studies have been performed using the developed HPLC method in this research to observe the levothyroxine percentage recovery from the levothyroxine FDOFs formulations. The data that was obtained from the HPLC analysis indicated that, the fast dissolving films formulations showed significant increase on the levothyroxine content on the samples stored at all the studied temperature (25°C, 40°C, 70°C) than the samples stored at the same temperatures for six months when the sample were in solid state (chapter 2). Previous studies on the fast dissolving films formulations represents that, the FDOFs enhance the drug content and that what it can be observed in this research [155].

However, the percentage drug recovery and the peak purity of levothyroxine from the FDOF formulations was higher on the formulations that included BHT as antioxidant. Furthermore, the percentage recovery of levothyroxine and its peak purity from the FDOFs formulations was higher than the percentage of drug recovery and its peak purity that was observed in the stability study of levothyroxine solid state at 25°C, 40°C and 70°C for six months' study. A study on the storage of Tween 20, BHT has indicated an activity of preventing the formation of peroxide which that inhibit the oxidation reaction on the compound and hence, improve its chemical stability. That leads to an improvement in the drug content and prevent drug degradation [156].

Another study on BHT had shown that, using BHT on the dental sealants produced a positive impact in preventing the oxidative reaction of the sealants and BHT had removed the free radicals that increased the possibility of degradation. Hence, BHT in the published study had increased the shelf life of the sealants [157].

However, in comparing the HPLC chromatograms of the levothyroxine FDOFs formulations and the eight degradation products of levothyroxine that were analysed and reported in chapter 2, it can be observed that, the retention time of tri iodo thyroacetic acid was about 17 min and tetraiodo-thyroacetic acid had a retention time of around 19 min were seen to different extents in levothyroxine FDOFs with SMB and levothyroxine FDOFs without antioxidant. Furthermore, there were other impurity peaks on the run obtained on levothyroxine FDOFs formulations but more detailed investigation of these samples is needed possibly using LC-MS-MS.

In addition, it can be observed from the HPLC chromatograms of levothyroxine FDOFs during the stability study that, there were some impurity peaks that were detected but the peak purity of the impurity peaks had no relationship with the period of exposing the samples to the high temperature. Therefore, more investigation has been done on the levothyroxine FDOFs samples by using LC-MS.

LC-MS method was essential in this research to observe any degradation products obtained during the stress study of levothyroxine FDOFs formulations. As already mentioned in chapter 2 of this research, there were in total six degradation products of levothyroxine observed from levothyroxine samples in their solid state. These degradation products of levothyroxine have been detected using LC-MS. In terms of levothyroxine FDOF formulations, they have been stored for six months at three different temperatures. It can be see that, the formulation with BHT has shown a stable formulation during the full period of six months' storage under stress conditions.

In contrast, the formulation without an antioxidant and the formulation with SMB as the antioxidant have shown three new degradation products that have been discovered in this research (chapter 2) which are; 4(4-ethyl-2,6-diiodophenoxy)-2,6-diiodophenol (m/z 718), 4(4-ethyl-2,6-diiodophenoxy)-2-iodophenol (m/z 592), 4(2,6-diiodo-4-methylphenoxy)-2-iodophenol (m/z 577). In addition, tetraiodo-thyroacetic acid has been detected from the samples of the both formulations at m/z of 747.

The results confirm that the formulations of levothyroxine FDOFs helps in improving the percentage recovery of levothyroxine and an antioxidant such as BHT assists further in preventing any oxidation reactions. In contrast, incorporating SMB in the levothyroxine FDOFs formulations did not help to overcome the degradations that took place in levothyroxine samples under stress conditions.

However, in comparing the levothyroxine FDOFs sample with BHT and the levothyroxine FDOFs without an antioxidant, there is a significant difference in terms of the observed impurity peaks. Therefore, the formulation of levothyroxine with 0.1% BHT [4] is essential formulation in terms of giving a levothyroxine dosage form with high drug percentage recovery, better drug release and improved chemical stability even under a storage within a stress condition.

However, further investigation into the stability of levothyroxine FDOFs formulations at different relative humidity as well as in a packaging material is essential as these levothyroxine formulations are novel. It is necessary to ensure that, the levothyroxine FDOFs are safe for use and therefore, toxicity studies have been included in this research, which will be discussed in the subsequent chapter 5.

4.5 Conclusion

Levothyroxine FDOFs formulations in this chapter were developed using BHT, SMB and ascorbic acid as antioxidants to increase the chemical stability of the formulations.

The developed levothyroxine FDOFs formulations with antioxidants were investigated by dissolution studies and chemical stability studies using HPLC and LC-MS. Therefore, the samples of levothyroxine FDOFs formulations with antioxidants were stored for six months under different temperatures degrees (25°C, 40°C and 70°C).

It can be mentioned that, levothyroxine FDOFs formulations with BHT as antioxidant showed better results than the other formulations in regarding to the drug release and stability in the formulation. In addition to this, the FDOFs formulations of levothyroxine with and without antioxidant obtained more chemical stability for the API in comparing the formulations with the solid state of levothyroxine.

As the formulations are novel, safety profile of the formulations is necessary to be investigated ad therefore, the next chapter will cover the toxicity studies on the developed levothyroxine formulations that performed in this research.

5. Cytotoxicity study of levothyroxine FDOFs using epithelial cell culture

5.1 Introduction

The novel levothyroxine FDOF formulations were investigated for their toxicity profile. In addition, levothyroxine FDOFs are designed for administration through the oral cavity, *in-vitro* toxicity tests should be performed using cells that are located orally. This chapter will highlight the observations related to the human oral mucosa, cell culture techniques that were used and the assays implemented to test the toxicity profile of the drug and the formulations.

5.1.1 Human oral mucosa

The human oral mucosa is rich with blood capillaries and tissues that makes the oral cavity an ideal route to administer the drugs to reach the target site of action [158]. The orifice of the mouth is where the oral mucosa starts and it is the passage of air and nutrition into the body. The oral mucosa contains three different linings: gingiva (gum), labial mucosa (lips) and the buccal mucosa (cheek). It is necessary to mention that, the epithelial cells (the simple squamous cells that isolates the oral cavity from the environment) are the main connectors between the teeth and the oral mucosa.

The oral mucosa includes all the posterior area of the oral cavity until the oesophagus with various types of oral cavity tissues that gives the oral cavity its distinct morphology and functions [158]. In addition, the epithelial cells located in the oral cavity can be either keratinised or non-keratinised, depending on their location inside the oral cavity. For instance, the oral mucosa that covers the areas of cheek and buccal area is non-keratinised while the oral mucosa that surrounds the gingiva is keratinised. In terms of the third type of the oral mucosa, it is a mixture between the previous two types and it is mainly located in the human lips and tongue [159].

The main aim of the different oral mucosal types is preserving the oral tissues form the microorganisms, toxins and antigens. These oral mucosae differ in their morphology as they have a different way of saving the oral tissues [159].

The epithelial cell line is necessary in this research as they will be used to determine the toxicity of the developed levothyroxine FDOFs. They can be cultured in the lab and can be used to investigate the cell viability after exposure to the drug and formulations at different concentrations and using specific assays.

5.1.2 Cell culture

The demand of cell culture is increased recently research as it provides enough data *in vitro* that can explain what the drugs toxicity, drug metabolism and drug permeability might be when administered *in vivo.* Therefore, cell culture is widely used in determining the safety profile of the drug and understand their mechanism of actions [160, 161].

However, in order to perform cell culture studies, the required cells need to be selected and that relies on the origin and the types of cell that needs to be tested [162,163], as there are many types of cell in the human and animals. There are two main cell line banks that widely used from research to buy the tested cells which are a European cell line bank {The European Collection of Authenticated Cell Cultures (ECACC)} and the second cell line bank is American {The American Type Culture Collection (ATCC)}. [164,165].

There are two different cell lines according to the cell culture which are; normal cell line, as this type is the main type for all the cells, it is called diploid cells. The diploid cells are mostly not permeant as these cells normally die after 50 times of culturing. In some cases, the diploid cells after 50 times of culturing can produce neoplastic transformation that yield persist culture. Therefore, the persist culture can form the permeant cell line (polyploid) when it has over then 70 times of cell cultures [166].
In terms of isolating the cells, they need to be under the same conditions of the human blood to be viable. Therefore, for culturing the cells, they are incubated at a temperature of 37°C and 5% CO_2 [167]. In order to culture the cells and keep them alive, they need nutrition which is a combination of sugars, vitamins, amino acid, gases and some minerals which is called as a culture medium. Furthermore, the culture

medium, contains a regulated physio-chemical environment such as pH, osmotic pressure, and temperature to maintain the same situation as that of an *in vivo* condition, which ensures that the cells can keep dividing and obtain the required confluence. The cells confluence refers to the cells that are isolated and cultured which makes them o grow into a certain cell density or percentage that is ready for investigations by exposing them to the formulation or drugs to be tested [160]. It is necessary to note that, each cell type has specific culture media. The culture media are different from each other in terms of the percentage and the type of the nutrition. [168].

Cell culture is useful in determining the toxicity profile of the formulations as they might be sensitive to oxidative stress induced by some anions, redox metals, free molecular oxygen and hydrogen peroxide [169].

5.1.3 cytotoxicity assays of cell culture

There are various types to assays to test the cells toxicity and the most common ones are bromodeoxyuridine (BrdU), Lactate dehydrogenase (LDH) and enzymatic reaction of (MTT) [170]. The BrdU assay (synthetic nucleoside of thymidine) is mainly used for observign cell proliferation by colouring reaction. The reaction in this assay take place when the colourless tetramethylbenzidine substrate changes its colour to blue and the resultant colour is related to bromodeoxyuridine, as the intensity of the colour increases with the presence of BrdU in the cells.

However, the second assay (LDH) is enzymatic reaction that is used to determine cellular toxicity. The LDH assay is mainly a colouring reaction that shows formation of a red colour of a formazan compound after the reaction takes place. This red coloured formazan compound is an indication of presence of LDH in the culture media after the cells are damaged which signifies cellular toxicity. The MTT assay is also an enzymatic reaction that provides knowledge about the metabolic activity of the cells in the presence of the drug/formulation. The mechanism of action in the MTT assay depends on converting the yellow colour of a tetrazolium salt into the red colour of a formazan in the living cells indicating cell viability. The resultant formazan can be measured using spectrophotometry to determine cellular viability [171,172]. In this study only the MTT assay was performed.

In this project, novel levothyroxine FDOFs formulations were developed by using different excipients such as HPMC E15 as a hydrophilic polymer and propylene glycol as a plasticiser. The formulations of levothyroxine FDOFs were designed with and without antioxidants to improve the formulation chemical stability. However, studying the toxicity profile for any developed pharmaceutical formulation is a necessary requirement to ensure the safety of using the drug in the formulation [173, 174]. There are two different types of studying the toxic profile of the developed formulations which are; human toxicity studies and non-clinical toxicity studies. Human toxicity studies can start with small trial and then increase the group of tested people and the consumed dose of the active pharmaceutical ingredient by the participants, while the non-clinical trials can be performed on *in-vitro* samples that were prepared for this purpose [175, 176].

H376 monolayer cell line was fully investigated in previous researches and it was mentioned that, H376 cell is essential cells to be used in studies required non-keratinised oral epithelium cells [177, 178]. In addition to this, it is necessary to mention that, non-keratinised tissues exhibited in buccal mucosa which it considered as the major tissues in the human oral cavity. This is because the largest number of tissues in the human oral cavity are non-keratinised tissues [179-181]. Furthermore, H376 was reported to be one of the most sensitive cells for detecting the toxicity and permeability profile of the tested materials [182-184]. In order to provide an accurate data regarding the cell viability against the developed formulations, it is essential to select to suitable assay for this purpose. MTT assay was reported to be the most accurate assay for toxicity studies and cell viability detection [185-188].

The cell viability of H376 epithelial cells was investigated by using MTT reduction assay as the cytotoxicity assay to study the toxic profile of the developed levothyroxine FDOFs. The MTT assay is a reduction assay which one of the first cytotoxicity reduction assays that produced to test the cell viability on the 69-well plate [189]. The MTT assay is a simple assay that depends on detect the cell viability by enzymatic reaction that reduces the soluble tetrazolium to insoluble formazan [190, 191]. The main advantage of using the MTT assay is that, most of the cells including the H376 cells have ability to metabolise the MTT and produce accurate results of cell viability. In addition to this, the MTT assay is a robust assay [192].

Cytotoxicity assays such as MTT are in demand for early investigation of the safety profile for the developed pharmaceutical compounds as it can give essential information on whether the tested product is chemically unstable by having biological activity compounds that might harm the patients [194]. The reduction reaction of the MTT assay can be seen on figure 5.1.

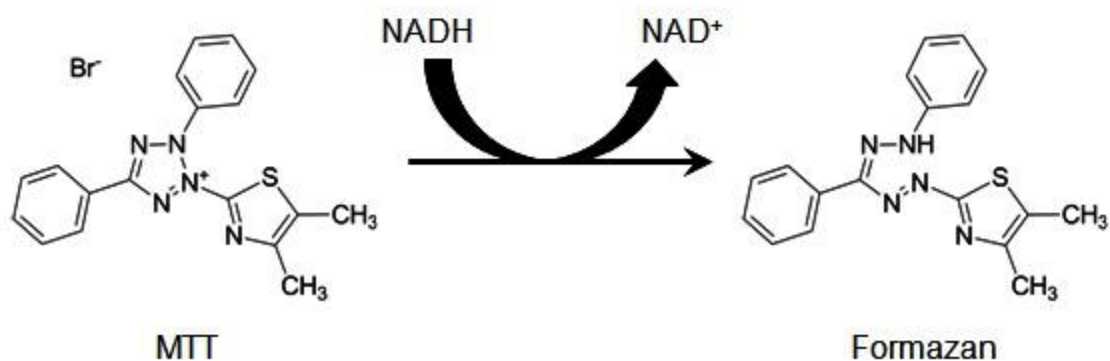


Figure 5-1: The reduction reaction of the MTT assay. Taken from [193].

5.2 Methodology

5.2.1 H376 oral mucosa cell line

The cells used in this research were H376 non-keratinised cells which were isolated from a human sublingual tissue. The H376 cells are a derivate from human squamous cell carcinoma and to ensure the avoidance any alteration on the phenotype of the H376 cells *in vitro*, the cells were kept in the culture medium for 10 passages [173]. The H376 cells were purchased from Sigma-Aldrich and it was mentioned that, the H376 cells reaches a confluence of about 70-80% after around 5 days by using its culture medium.

5.2.2 H376 culture medium

The culture medium used for growing the H376 cells in this research is Dulbecco's modified eagle medium -F12 (DMEM-F12). This culture medium contains growth factor, fatal bovine serum (FBS, 2500 IU/mL) in a percentage of 10% v/v, 0.5 ug/mL of hydrocortisone, pencilline and 2 mM glutamine. The cultured medium was purchased from PAA laboratories in the UK.

5.2.3 Cell passage

For culturing the cells, the cell passage was performed on the H376 cells to reach the cells confluence of 70-80% before treating the cells with the developed levothyroxine FDOFs formulations. The cell passage for H376 cells was achieved by using the cell culture flasks (polystyrene flask with t-75 of surface). The cells first were cultured with the culture medium at a seeding density of 3×10^3 cells/cm^2. The cells were then incubated at 37°C/5% CO_2.

The cell passage was done on the H376 cells every 5 days and the medium was changed after two days from each cell passage. The cell passage was performed by removing the medium from the t-75 flask and the flask was washed using 10 mL of phosphate buffer saline (PBS)., PBS was removed 1 minute after adding it to remove the floating medium from the flask. After that, in order to de-attach the cells from the flask wall, about 5 mL of trypsin was used for this purpose. The flask was then placed for 5 minute in the cell culture oven at a temperature of 37°C and atmospheric humidity of 5% CO_2. In order to confirm that the cells were de-attached in the flak, the flask was

always visually checked at this point to see the cells floating in the flask and it was confirmed under the microscope.

The next step in cell passage was performed by adding 10mL of the media into the flask as the trypsin without medium will keep the cells de-attached and kill them. Therefore, 10 mL of the culture medium was added to the t-75 flask and all the volume was transferred directly after adding the culture medium to the centrifuge tube and centrifuged for 5 minutes.

The supernatant solution was withdrawn from the centrifuge tube and the cells remained in the tube. Then finally, the cells that remained in the tube were re-suspended with 1 mL of the culture medium and mixed 25 times with the culture medium to ensure homogeneity. After that, in a new t-75 flask, 12 mL of the culture medium was added and the re-suspended cells were added to a new t-75 flask that had fresh culture media. The new flask was then incubated at 37°C/5% CO_2 and after two days, the medium was changed to provide the nutrition for the cells, and in each 4-5 days when the confluence about 70-80%, another passage was performed under the same conditions.

5.2.4 Cell treatment

Cell treatment refers to the drug or the pharmaceutical formulation added to the cells at the required confluence between 70-80%. In this research, the cells were treated with the developed levothyroxine FDOFs formulations (F1, F2, F3 and F4; refer to Chapter 4). After the cell passage, the cell viability study on levothyroxine solid state and levothyroxine fast dissolving oral films formulations was performed at n=6. Levothyroxine stored at three different temperatures: 25°C, 40°C and 70°C for six months was exposed to H376 cells, also treated with levothyroxine fast dissolving oral film formulations and tested after 6 hr (early cell viability study) and after 24 hours (late cell viability study) to obtain the full knowledge of the cellular toxicity. Furthermore, levothyroxine samples were prepared in an increasing concentration range to treat the H376 cells (20 ng/mL, 40 ng/mL, 60 ng/mL, 80 ng/mL, 100 ng/mL, 20 µg/mL).

5.2.5 Cytotoxicity assay using MTT

MTT was prepared at a concentration of 0.2mg/ml which has been incubated for four hours before the toxicity experiment. 0.5 mL of MTT (0.2mg/mL) was needed in each well of the 24 well plate (MTT cell viability assay, Life Technologies, UK). However, the treatment of the drug in the 24 well plate was prepared by re-suspending the cells in 1mL media then 47mL of media was added to reach to the volume of 48mL which is enough for two plates. After 24 hours, the media was removed and washed with PBS then the drug was loaded (1ml) and incubated for 6 hours and 24 hours. The drug solution was subsequently removed and 0.5mL of MTT added. After the addition of MTT, the plates were incubated for 1 hr and 125µl of dimethyl-sulfoxide (DMSO) was added, mixed gently and then transferred to the 96 well plate (MTT cell viability assay, life technologies, UK) to read the absorbance using the plate reader (Thermo Multiskan Ascent 354) at a wavelength of 540 nm. The calculation for the cell viability was performed by calculating the percentage of the absorbance according to the percentage of the blank (without treating the cells).

5.3 Results

5.3.1 cell passage and treatment

To study cell viability of the H376 cells, the cells were passaged to reach the required confluence for loading the levothyroxine samples to the cells. It was noticed that, the cells reached a confluence of 70-80% after each 5 days of passage. Figure 5.1 shows the morphology of the H376 cells when they reached to 70%-80% of the confluence, where the cells showed normal growth. This means that the cell growth showed no dead cells as no floating cells were noticed on day 5 from the passage. In addition, the cells distributed in the flask in uniform shape.

After the H376 cells reached a confluence of 70%-80%, levothyroxine samples were loaded. The tested levothyroxine samples were F1: Levothyroxine in its solid state, F2: levothyroxine FDOFs with 5% of HPMC E15 and without antioxidant, F3: levothyroxine FDOFs with 5% of HPMC E15 with Butylated hydroxytoluene as antioxidant and F4: levothyroxine FDOFs with 5% of HPMC E15 and Sodium metabisulfate as antioxidant.

It can be seen from figure 5.2 that, the morphology of the H376 cells remained the same after loading one of the levothyroxine samples into the cells (F3 at T=0).

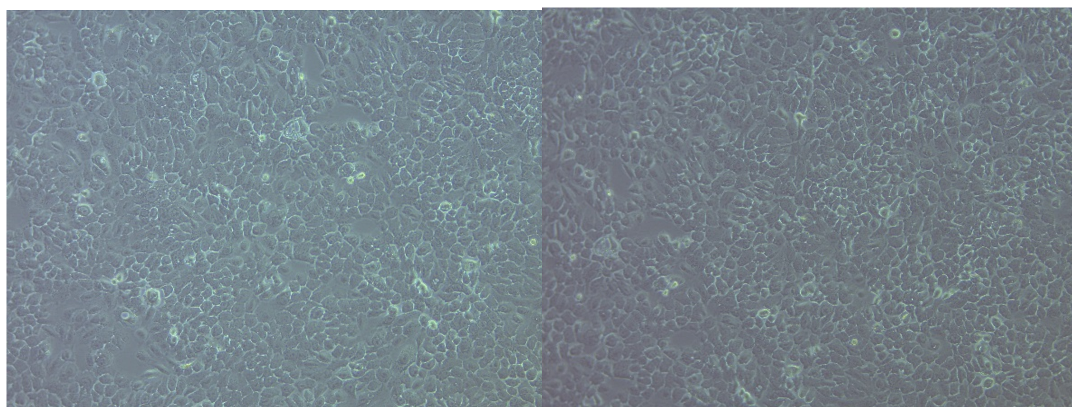


Figure 5-2: The morphology of H376 at 500x. The H376 cells confluence of 80-90% left: untreated, right: treated

5.3.2 Cell viability study using MTT assay

The cytotoxicity study to H376 cells when exposed to levothyroxine and its formulations was determined using MTT assay. Stability study has been included in this work as levothyroxine samples (F1, F2, F3 and F4) were tested at early stage (after 6 hours of loading the levothyroxine samples to the H376 cells) and late study (after 24 hours of loading the levothyroxine samples to the H376 cells). In addition, the cytotoxicity study was performed on the levothyroxine samples that were stored at three different temperatures (25°C, 40°C and 70°C) for t=30 days, t=90 days and t=180 days.

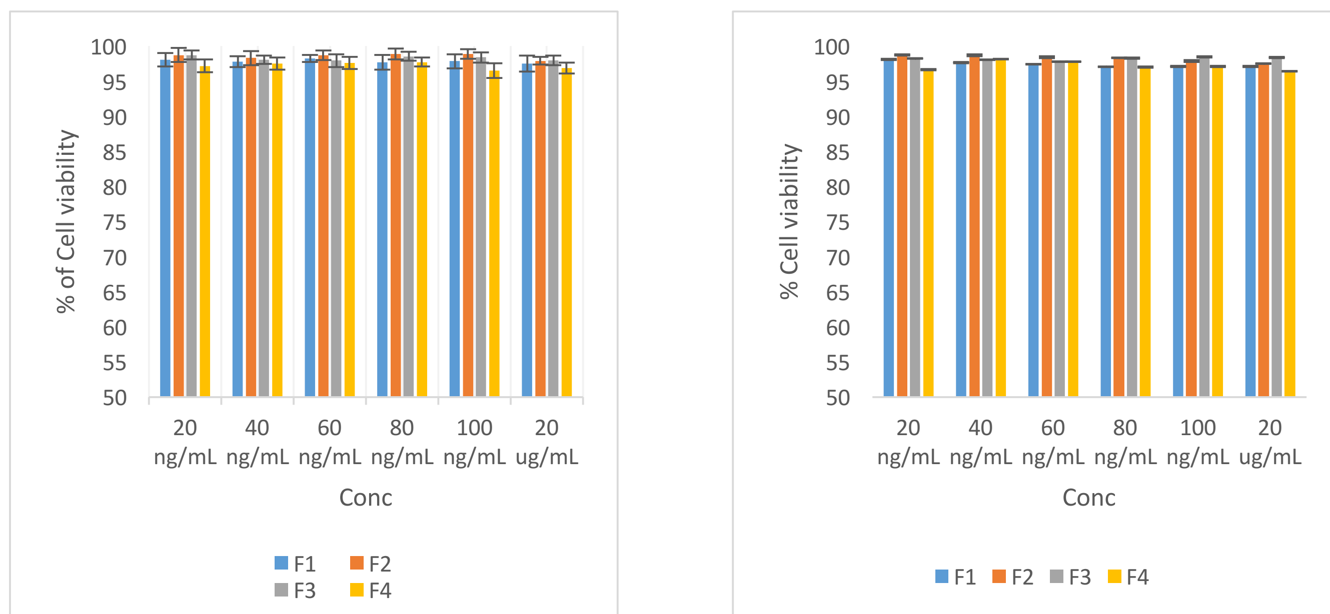


Figure 5-3: A graph showing the cytotoxic response of the levothyroxine solid state and levothyroxine fast dissolving films formulations after treating the H376 monolayers at a range of concentrations between 20 ng/mL and 20 µg /mL w/v of levothyroxine using DMEM-F12 media and incubated for 5 minutes at a temperature of 37°C/ 5% CO2. The percentage cell viability as determined using MTT assay is the outcome of six repeats (n=6). Data shown mean ± SD. The study was performed after (Left: 6 hours, Right: 24 hours) of treating the cells with the drug. The samples were studied at t=0. one-way ANOVA showed p value is > 0.05 considered no significant difference between the controlled sample and F1, F2 and F3 while one-way ANOVA showed p value is < 0.001 considered extremely significant different between the F4 especially at higher concentration when compared to the control sample.

It can be seen from Figure 5.3 that the cell viability remained un affected after treating the H376 cells with levothyroxine samples at t=0 for 6 hr and 24 hr at all the tested drug concentrations. The cell viability in these studies was approximately between 97% and 99%. In addition, the four FDOD formulations F1, F2, F3 and F4 were also tested for cytotoxic response. In terms of storing the levothyroxine at 25°C for 30 days, the cell viability remains unaffected with more than 95% for all the four drug samples at all the drug concentrations.

However, the stability study of the drug samples using the MTT assay showed that, there was a dose dependence in regards to the cell viability when the drug samples were stored at 40°C for t=30 days, and the cell viability decreased with increasing concentrations of the drug loaded on to the H376 cells. Formulation F3 showed the higher cell viability in all the tested drug concentrations (cell viability between 95%-97% after 6 hours and cell viability between 94%-96% after 24 hours) while F1 showed the lowest cell viability in all the tested drug concentrations (cell viability between 88%-89% after 6 hrs and cell viability between 87%-88% after 24 hrs). Furthermore, increasing the exposure time of the drug to the cells had reduced the cell viability as it can be noticed with formulation F1. The cell viability after 24 hrs was less than the cell viability after 6 hr of testing the drug samples in all the drug concentrations. However, in terms of the IC_{50}, the cell viability was more than 50%.

In addition, the storage temperature of the levothyroxine samples had affected the percentage cell viability. It can be observed that, storing the levothyroxine samples at 70°C for 30 days had decreased the cell viability when compared to the cell viability after treating the H376 cells with the levothyroxine samples stored at 25°C and 40°C. Formulation F1 had showed the lowest cell viability (cell viability between 74%-76% after 6 hours and cell viability between 63%-71% after 24 hours) while F3 showed the higher cell viability (cell viability between 78%-82% after 6 hours and cell viability between 72%-78% after 24 hours). It can be seen that, there is a sharp difference between the cell viability of the levothyroxine FDOFs formulations and the cell viability of the levothyroxine in its solid state. (Figure 5.4)

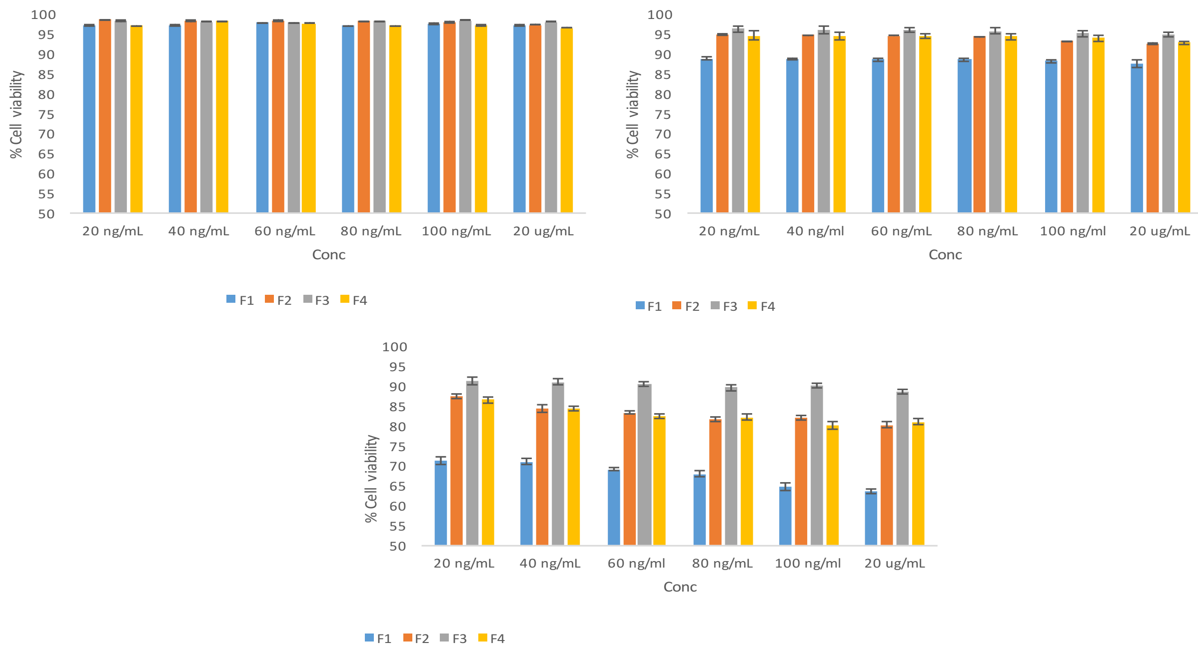


Figure 5-4: A graph showing the cytotoxic response of the levothyroxine solid state and levothyroxine fast dissolving films formulations after treated the H376 monolayers at a range of concentrations between 20 ng/mL and 20 µg /mL w/v by using DMEM-F12 media and incubated for 5 minutes at a temperature of 37°C/ 5% CO_2. The used assay is MTT assay and the percentage of the cell viability is the outcome of six repeats (n=6). Data shown mean ± SD. The study was performed after 24 hours from treating the cells with the drug. The samples have studied at t=30 days/25°C (Left) 40°C (Right) 70°C (Bottom). For t=30 days/25°C one-way ANOVA showed p value is > 0.05 considered no significant difference between the controlled sample and F2 and F3 while one-way ANOVA showed p value is < 0.001 considered extremely significant different between the F1 and F4 especially at higher concentration when compared to the control sample. For t=30 days/40°C and t=30 days/70°C, one-way ANOVA showed p value is < 0.001 considered extremely significant different between all the formulations when compared to the control sample.

Levothyroxine samples were tested for cytotoxic response when stored for 90 days at three different temperatures. At 25°C, mainly F3 had higher cell viability than the other formulations (cell viability between 96%-98% after 6 hr and 24 hr) while the effect of increasing the storage period of the levothyroxine samples on the cell viability was significant on F1. The stress condition of levothyroxine samples storage played important role on the percentage cell viability. It can be seen that (F1, F2, F3 and F4) stored at 40°C for 90 days, cell viability had decreased in comparison to the cell viability of the samples stored at 25°C for 90 days. However, the levothyroxine samples had not shown any cytotoxic response in terms of cell viability (IC_{50}) and F3 had shown a higher cell viability in this assay (cell viability between 93%-96% after 6 hours and cell viability between 92%-95% after 24 hours) while formulation F1 had shown the lowest cell viability (cell viability between 80%-85% after 6 hours and 24 hours.

Furthermore, increasing the storage condition of the levothyroxine samples to 70°C for 90 days had supported the data before showing that the decrease in the cell viability is related to the high storage temperature of levothyroxine samples. It can be seen that, the cell viability on F1 was strongly affected by the high temperature (cell viability between 63%-71% after 6 hours and cell viability between 60%-71% after 24 hours) and the cell viability on F3 had remained the highest cell viability from all the tested levothyroxine samples (cell viability between 87%-90% after 6 hours and cell viability between 86%-90% after 24 hours). In addition, the late stage of the cytotoxicity study (after 24 hours) showed a decrease on cell viability when compared to the early stage of the cytotoxicity study (after 6 hours). (Figure 5.5).

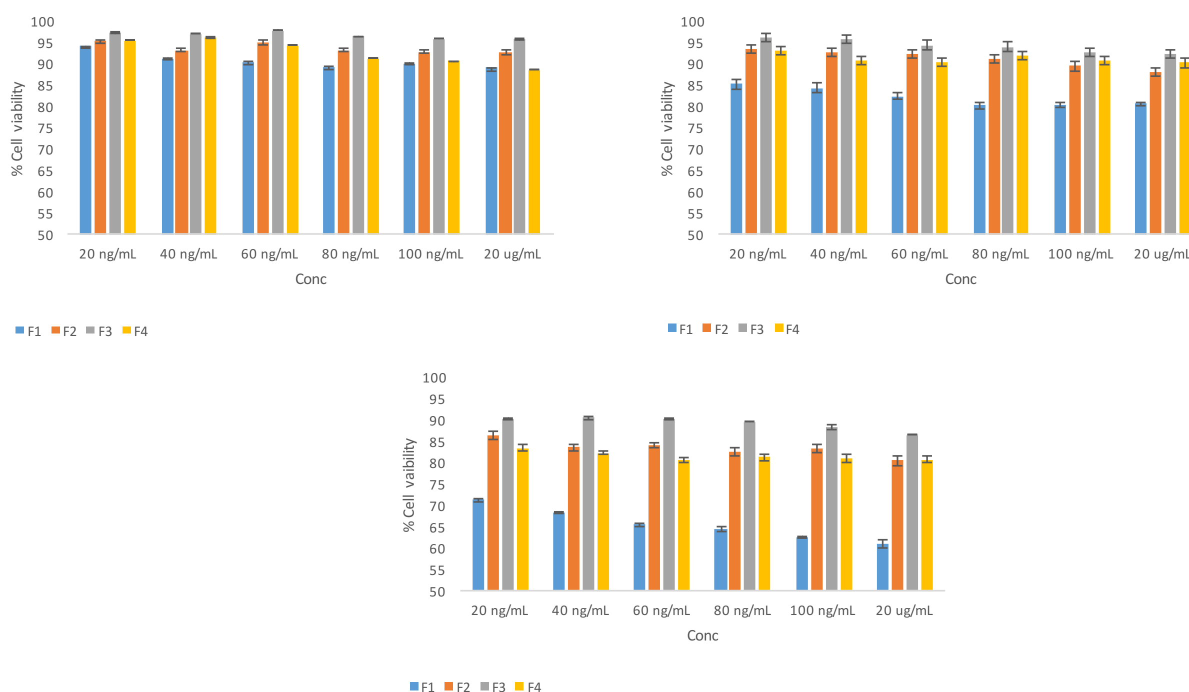


Figure 5-5: A graph showing the cytotoxic response of the levothyroxine solid state and levothyroxine fast dissolving films formulations after treated the H376 monolayers at a range of concentrations between 20 ng/mL and 20 µg /mL w/v by using DMEM-F12 media and incubated for 5 minutes at a temperature of 37°C/ 5% CO2. The used assay is MTT assay and the percentage of the cell viability is the outcome of six repeats (n=6). Data shown mean ± SD. The study was performed after 24 hours from treating the cells with the drug. The samples have studied at t=90 days/25°C (Left), t=90 days/40°C (right), t=90 days/70°C (bottom) one-way ANOVA showed p value is < 0.001 considered extremely significant different between all the formulations when compared to the control sample under all the storage conditions.

Levothyroxine samples kept under stress condition for 180 days at three different temperatures (25°C, 40°C and 70°C) were also tested for their effects on cell viability. Figures 5.5 show the levothyroxine samples stored at 25°C had influenced the cell viability but all the formulations at this storage temperature have high cell viability (in early and late stage study) even after 180 days of levothyroxine samples storage. The cell viability of F1 was recorded as the lowest cell viability (cell viability between 90%-93% after 6 hours and cell viability between 88%-92% after 24 hours) compared to the other levothyroxine FDOFs formulations while F3 showed the highest cell viability (cell viability between 94%-97% after 6 hours and 24 hours).

In addition, the data of the formulations stored at 40°C for 180 days showed the same phenomenon as the previous observations, as exposing the levothyroxine samples to 40°C for longer time affected negatively on the cell viability but for all the formulations cell viability was more than 50% (IC_{50}). In addition, F1 had the less effect on the cell

viability (cell viability between 92%-96% after 6 hours and 24 hours) while the lowest cell viability was still observed with cells exposed to F1 (cell viability between 75%-83% after 6 hours and cell viability between 73%-82% after 24 hours).

Finally, the last assay on this experiment was performed on the levothyroxine samples that were stored at 70°C for 180 days. This assay represented the same aspects that the previous assays of this experiment have obtained. It can be seen from that, the average cell viability for F1 was between 58%-65% after 6 hours and cell viability between 52%-63% after 24 hours) which is close to the IC_{50} limit and it is the lowest cell viability recorded in all the assays performed for the levothyroxine formulations. On the other hand, the formulation F3 showed in all the assays the highest cell viability which in this run was between 85%-88% after 6 hours and cell viability between 83%-88% after 24 hours), which is different to the cell viability at t=0 that was over than 95% but it remained far from the toxic limit. (Figure 5.6).

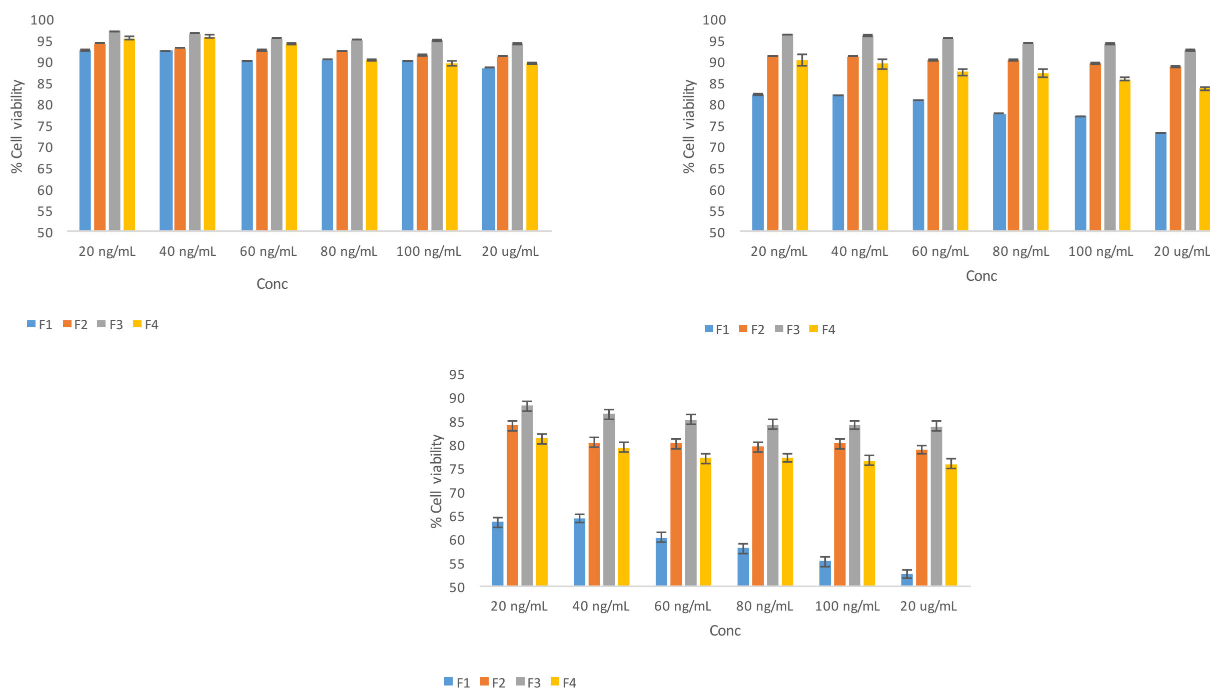


Figure 5-6: A graph showing the cytotoxic response of the levothyroxine solid state and levothyroxine fast dissolving films formulations after treated the H376 monolayers at a range of concentrations between 20 ng/mL and 20 µg /mL w/v by using DMEM-F12 media and incubated for 5 minutes at a temperature of 37°C/ 5% CO2. The used assay is MTT assay and the percentage of the cell viability is the outcome of six repeats (n=6). Data shown mean ± SD. The study has performed after 24 hours from treating the cells with the drug. The samples have studied t=180 days/25°C (Left), t=180 days/40°C (Right), t=180 days/70°C (bottom) one-way ANOVA showed p value is < 0.001 considered extremely significant different between all the formulations when compared to the control sample under all the storage conditions.

5.4 Discussion

The cytotoxicity study of levothyroxine on its solid state (F1) and on fast dissolving film formulations (F2, F3, F4) was determined. The aim of this study was to determine the safety profile for the developed levothyroxine FDOFs. MTT assay was shown in previous researches for its capability to provide the cytotoxic details for many compounds when the yellow colour of the MTT compound reduces to the colour of formazan which is purple [195]. Therefore, H376 monolayer oral epithelial cell has taken place in this study as it has been used before to investigate the toxicity effect of some drugs that are located in the epithelial cells [196].

Cytotoxicity study discussed in this chapter had been performed on the levothyroxine at three different temperatures of the stress condition (25°*C*, 40°*C* and 70°*C*). Furthermore, the study on MTT assay was used at the early stage study (after 6 hr) and late stage study (after 24 hr).

FDA had reported that levothyroxine has issues related to the bioequivalence (study the pharmacokinetics of two preparations for the same drug) which FDA was reported that, some of the levothyroxine products in terms of their *in vivo* biological response are not same. It was mentioned in the bioequivalence study of levothyroxine that, levothyroxine carries narrow therapeutic index [197] which means, the toxic level of the levothyroxine is close to the maximum therapeutic dose and that was achieved by comparing the concentration of levothyroxine that leads to provide the therapeutic response with the levothyroxine concentration that give the adverse reactions [198] Therefore, it is necessary to monitor the serum plasma level when levothyroxine is the choice of the treatment. Hence, in this project, the developed levothyroxine FDOFs formulation and levothyroxine in its solid state have been studied at a wide range of concentrations between 20 ng/ml and 20 μg/ml.

From the figures presented above in this chapter, it has been shown that, the morphology of the H376 had not altered when the levothyroxine sample (solid state at t=0) has treated the cells. The morphology of H376 cells should be a polygonal epithelial cells and that was clearly observed from the figure 5.1 even after treated the cells with the levothyroxine samples at t=0. Therefore, the levothyroxine at normal

condition (without stress condition such as high temperature storage condition), is not toxic for the H376 cells.

However, as discussed in chapter 2, levothyroxine solid state under stress conditions of high temperature have been reported with degradation products [199] that are unknown if they have any biological activity. In addition, it was mentioned in this research that, some of the previously detected degradation products of levothyroxine were reported to possess some biological activity. This means that, exposing levothyroxine to high temperature may reduce the safety profile for the drug to induce some toxic affect. This phenomenon has been proven in this study as the cell viability of H376 cells had sharply decreased when treated with levothyroxine solid state samples (F1) that were previously exposed to high temperatures. The cell viability of the H376 cells has reached at the end to slightly over than 50% (IC50) when it has treated with levothyroxine solid state samples that were stored at 70°C for six months.

Moreover, the cell viability of the H376 cells started to significantly decrease since treated with the levothyroxine solid state samples (even after t= 30 days, at 40°C and 70°C) while this decrease was not noticed on the levothyroxine samples stored at 25°C. This clearly indicates that, the high temperature is strongly influencing the stability of levothyroxine and induces degradation products, which may produce toxic effect as observed in the cell viability of H376 cells.

In terms of the developed levothyroxine FDOFs, (F2, F3, F4), as discussed in chapter 4, levothyroxine fast dissolving oral films have been designed and developed with high stability and that has been presented especially when the stability study samples of levothyroxine FDOFs were compared to the levothyroxine solid state. In addition, it was reported in chapter three of this research that, many articles were shown the improvement that occurred on the stability of some medicines when they have been designed as fast dissolving films. This is related to the observations discussed in this chapter, as the cell viability on the levothyroxine fast dissolving film formulations (F2, F3 and F4) was higher than the cell viability that was observed on the cells that were treated with levothyroxine solid state samples (F1).

The data shown on LC-MS in chapter 4 shows the disappearance of the degradation products that were noticed on levothyroxine solid state samples. Also the data of LC-MS in chapter 4 presented that, the levothyroxine FDOFs with BHT as antioxidant (F3)

had much less impurity peaks even at high temperature storage condition. This is explained why in this chapter, the cell viability on (F3) was higher than the other developed formulations of levothyroxine. It can be mentioned that, the antioxidant BHT plays important role in improving the levothyroxine formulation stability and safety and that what it was observed in this research.

5.5 Conclusion

The aim of this chapter was to investigate the safety profile of the developed levothyroxine FDOFs formulations by testing the cell viability of simple epithelial cell line (H376) when the formulations and the API added to the cells. The test was performed in two phases, early stage after 6 hr of treating the cells with the developed levothyroxine FDOFs formulations and at late stage after 24 hr of treating the cells with the levothyroxine FDOFs formulations. The cell viability assay used was MTT and the samples stored at 3 different temperatures which are 25°C, 40°C and 70°C for 180 days.

It was observed that, at 25°C, the samples of levothyroxine FDFOs formulations showed no significant effect on the cell viability but as the storage temperature of the developed levothyroxine FDOFs increase, the cell viability decrease which is an indication of the chemical instability of levothyroxine at higher temperatures (40°C and 70°C) that leads to produce some toxic effect on H376 cells.

In addition to this, including BHT as antioxidant in the developed levothyroxine formulations enhanced the safety profile of the formulations even at higher temperature which that shows, BHT inhibited or reduced the degradation of levothyroxine at higher temperature.

Therefore, in order to produce a fast dissolving film formulation of levothyroxine with higher chemical stability, it is recommended to include BHT at 0.1% as antioxidant in the formulation.

6. General conclusions and future work

The main aim on this project was to improve the chemical stability of levothyroxine. This drug has been reported to experience chemical instability issues that affected both its chemical and physical properties. Temperature was reported as the main impact on the chemical instability of levothyroxine. Therefore, the focus of this project was to study levothyroxine under stress conditions, which was done by placing the drug in its solid state into three different temperatures for six months of stability study (the stress conditions of different temperatures were 25°C, 40°C, and 70°C). The duration of storage under stressed conditions for levothyroxine samples was 30 days, 90 days and 180 days. All the stress state samples were compared to the drug sample, at t=0. All the samples were studied under three analytical methods, which were; HPLC, LC-MS, and FT-IR. It is necessary to report that, humidity was not considered when the tested formulations for stability study were placed into different temperatures. In addition, all the formulations statistically were studied by one way anova as discussed above in each chapter of this research.

The physical appearance for the stress condition samples of levothyroxine, changed in color of the solid state powder of levothyroxine samples have been observed to be darker in the samples that were stored at 70°C (brown color). This phenomenon could potentially be due to oxidation of the chemical structure of levothyroxine. It has been recorded that, levothyroxine samples at room temperature decreased their stability when they were stored for longer than 30 days, when analysed by HPLC. In addition, it was noticed that, two of the previously reported compounds as degradation products for levothyroxine (tri-iodo-thyroacetic acid and tetra-iodo-thyroacetic acid) have been detected in the samples of levothyroxine that were stored at 40°C and 70°C (high temperature) which that clearly indicates that levothyroxine is chemically unstable at high temperature.

Further investigation on levothyroxine stress condition samples was performed to gain full knowledge of the degradation mechanism of action for levothyroxine under stress conditions. This was performed using LC-MS. From the LC-MS data, at high temperature, the two degradation products of levothyroxine (tri-iodo-thyroacetic acid

and tetra-iodo-thyroacetic acid) were detected besides one of the previously reported degradation products of levothyroxine, di-iodo thyronine. In addition to previously detected degradation products of levothyroxine, LC-MS analysis lead to the discovery of three new degradation products of levothyroxine that have not been detected before. The hypothesis for this thermal degradation of levothyroxine is due to the BDE of the levothyroxine molecules especially for the C-I bond. As the carbon-iodine bond has low BDE which can easily be susceptible to free radical attack and removal of the atom from the chemical structure of levothyroxine. Levothyroxine is strongly affected by heat where new degradation products that could potentially be biologically active are produced, the drug content sharply decreased with temperature when analysed by HPLC, and primary chemical structure of levothyroxine had altered and lost the main functional groups such as iodine, which was observed by using FT-IR.

The second Aim in this project was to improve the chemical stability of levothyroxine by developing a novel FDOF formulation of levothyroxine which was previously reported as an efficient pharmaceutical formulation to overcome chemical instability issues of various compounds. Hydrophilic polymers which were used to formulate the levothyroxine FDOF formulations were compatible with the API, as verified by FT-IR. The three FDOF formulations of levothyroxine displayed an acceptable content of uniformity within the range of 85% to 115% with high drug release within 10 minutes.

The physical and chemical properties for the developed levothyroxine films showed good flexibility, good physical appearance, rigidity and uniform thickness. All films were tested for surface pH in order to avoid any potential oral mucosal irritations and the results showed that all the films were approximately of neutral pH. The developed films of levothyroxine have proven the hypothesis about enhancing the physical and chemical properties of levothyroxine by formulating the drug in fast dissolving oral films. However, as these formulations are novel, it is required to test the levothyroxine FDOFs with respect to the chemical stability and toxicity, which is discussed in chapters 3.

To ensure higher stability of levothyroxine formulations, the levothyroxine FDOF formulations developed in this research include the addition of different antioxidants: BHT, SMB and ascorbic acid. These developed levothyroxine formulations are F1: levothyroxine fast dissolving oral film formulation with BHT as antioxidant, F2:

levothyroxine fast dissolving oral film formulation with SMB as antioxidant, F3: levothyroxine fast dissolving oral film formulation without antioxidant. The developed levothyroxine FDOFs formulations were stored for six months at different storage conditions (25°C, 40°C and 70°C).

Levothyroxine FDOFs with BHT as the antioxidant showed better drug release, drug percentage recovery and drug stability than the other levothyroxine FDOFs formulations. In terms of the other levothyroxine FDOFs formulation containing SMB as antioxidant or the formulation without antioxidant, they have shown better drug percentage recovery than the levothyroxine samples when they were stored in a solid state under the same storage conditions. This clearly indicates the formulating the drug in fast dissolving oral film incorporated with an antioxidant, improves the stability of levothyroxine, even in accelerated conditions.

Levothyroxine FDOFs formulations improved the drug percentage recovery with BHT in the formulation, levothyroxine samples have shown no degradation products which provide more efficient and safe dosage form for levothyroxine than the tablets dosage forms and the levothyroxine solid state.

The third aim in this research was to ensure that the developed FDOF formulations are not toxic and that was determined by performing in vitro toxicity test using a cell culture model, H376 cells. After developing the levothyroxine FDOFs formulations with an antioxidant, which showed a promising chemical stability, the safety profile for the levothyroxine formulation was determined in a cell culture model. The aim of this chapter was to test the *in vitro* cytotoxicity profile for the developed levothyroxine FDOFs formulation and ensure that, the levothyroxine formulations are safe under a stress condition of storage at high temperature for six months.

The cytotoxicity study has shown that, levothyroxine is not toxic under normal conditions as determined by MTT assay. In addition, levothyroxine at room temperature had no significant effect on the cell viability and did not show any toxic response. In contrast, storing the levothyroxine solid state samples at high temperature, induced a significant effect on the cell viability as the high temperature influenced the chemical stability of levothyroxine. This was observed when levothyroxine stored at three different temperatures for stability studies (25°C, 40°C and 70°C) and the samples stored at 40°C and 70°C showed less chemical stability

and cell viability. Levothyroxine fast dissolving films improved the cell viability and the chemical stability of levothyroxine but have shown some alteration on the cell viability of H376 especially with the samples stored for longer time at higher temperatures. This issue was improved when the levothyroxine fast dissolving film was designed to include BHT as an antioxidant. Therefore, chapter 5 showed that, the fast dissolving films and the antioxidant BHT are the key facts that improved the chemical stability and the toxicity profile of levothyroxine. Levothyroxine FDOFs with BHT is essential formulation to be developed in terms of the chemical stability and safety profiles.

6.1 Future work

The future work for this project can be answering the outstanding questions regarding the work was done in this research. The outstanding questions can be as follows:

what is the chemical stability of the developed levothyroxine FDOFs formulations in long term of storage conditions and study include into the consideration the humidity for comparisons?

To investigate the chemical stability of the developed levothyroxine formulations in this research under long terms storage conditions, levothyroxine FDOFs formulation with and without antioxidants should be stored at 25°C at 60% RH and 30°C at 65% RH for 12 months [200] and test the samples at defined time points regarding the drug release and the chemical stability of the formulations and the API using the same method created in this project for HPLC, LC-MS and FTIR.

What are the chemical and physical properties of the new degradation products of levothyroxine that observed in this project?

This work is necessary to investigate the biological activity of the detected degradation products. This can be achieved by synthesise the degradation compounds and study their physical and chemical properties. It is possible to perform a chemical stability studies on these compounds to indicate the degradation compounds can be observed from these compounds. Therefore, this work can include, chemical synthesis of the degradation compounds of levothyroxine, study the physical and chemical properties of the degradation compounds such as melting point and glass transition temperature using DSC, Chemical structure using FTIR, texture and odour of the compounds,

assay the compounds using HPLC. In addition to this, study these compounds under UV spectrum is essential to indicate the wavelength of the degradation compounds. Furthermore, LC-MS is essential in this study to study the single peaks in these compounds. After that, perform a chemical stability studies are in demand and it should be under the same accelerated stability condition that was performed in this project.

What is the influence of the developed levothyroxine FDOFs *in vivo*?

As levothyroxine FDOFs formulation with BHT showed better drug release, drug content and high cell viability, it is essential to perform *in vivo* pharmacokinetic and pharmacodynamics studies on the developed levothyroxine FDOFs that included in this project. The films can be prepared the same method of preparation in this research and study the influence of the organism on the tested formulations such as ADME (Administration, Distribution, Metabolism, Excretion) and study the drug half-life and the maximum drug concentration. [201, 202]. In addition to the *in vivo* pharmacokinetic study, *in vivo* pharmacodynamics study is necessary to detect the influence of the drug on the body which is related on the dose and drug concentration in the organism [203].

7. References

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