ALTERNATIVE DELIVERY APPROACHES FOR LEVOTHYROXINE

by

Utkarshini Anand

Submitted in partial fulfilment of the requirements
for the degree of Master of Science

at

Dalhousie University
Halifax, Nova Scotia
August 2013

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This dissertation is dedicated to my mom, dad, grandfather, my extended family members and friends. Thank you for all of your love, support, and belief in me.

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ABSTRACT

Hypothyroidism affects almost 2-5% of the world population and requires lifelong thyroxine replacement therapy. Oral Administration of levothyroxine (T4) encounters enormous pathophysiological and pharmacological challenges that sometimes lead to therapeutic failure. The aim of this study was to conduct proof-of-principle studies which enables possible development of non-invasive (respiratory, transdermal) T4 delivery systems. Preformulation (solubility, stability) and biopharmaceutical (*in-vitro* absorption, gene expression) studies were conducted using Calu-3 (respiratory cell line) and Strat-M® (artificial skin membrane). Transport of T4 across Calu-3 cells suggested involvement of active transport systems which correlated with expression of thyroxine transporters (MCT8, MCT10, OATP1A2, OATP4A). However, T4 permeation across Strat-M® was non-existent even after using chemical absorption enhancers. In conclusion, data from these studies indicated that T4 may be successfully delivered via the respiratory route. However, successful transdermal delivery may require a reversible, but mildly invasive approach.

LIST OF ABBREVIATIONS USED

µg/dl	Micrograms per deciliter
µg/ml	Micrograms per milliliter
ACN	Acetonitrile
ANOVA	Analysis of variance
ASBT	Apical sodium-dependent bile acid transporter
AP-to-BL	Apical to basolateral
AA	L-Ascorbic acid
BL-to-AP	Basolateral to apical
BCS	Biopharmaceutics classification system
Calu-3	Human lung adenocarcinoma cell line
DIT	Diiodotyrosine
DMEM	Dulbecco's modified Eagles medium
DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FDA	US food and drug administration HBSS
HBSS	Hanks balanced salts
HEK-293	Human embryonic kidney cells-293
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High performance liquid chromatography
ICH	International conference on harmonization

KI	Potassium iodide
LAT	Large amino acid transporters
mIU/L	Milli-international units per liter
MCF-7	Michigan Cancer Foundation-7/ Breast cancer cell line
MCT	Monocarboxylate transporters
MDH	Mitochondrial dehydrogenase
MIT	Monoiodotyrosine
MTT	3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
NaOH	Sodium hydroxide
$NH3^+$	Ammonium cation
NTCP	Na^+/taurocholate cotransporting polypeptide
OATP	Organic anion transporting polypeptides
OECD	Organization for economic co-operation and development
P_{app}	Apparent permeability
PBS	Phosphate buffered saline
PET	Polyethylene terephathalate
PEG 400	Polyethylene glycol 400
PEG 600	Polyethylene glycol 600
QC	Quality control
RIT	Radioiodine therapy
RT-PCR	Real time polymerase chain reaction
RT	Room temperature

TPOAb	Thyroid peroxidase antibody
TRH	Thyroid regulating hormone
TSH	Thyroid stimulating hormone
T4	Thyroxine
TPGS	Tocopheryl polyethylene glycol succinate
TEER	Trans epithelial electrical resistance
TFA	Trifluoroacetic acid
T3	Trioiodothyronine
USP	United States pharmacopoeia

ACKNOWLEDGEMENTS

Although only my name appears on the cover of this dissertation, many people contributed to its realization. I owe my gratitude to all those people who have made this dissertation possible and because of whom my graduate experience has been one that I will cherish forever.

My deepest gratitude goes to my advisor, Dr. Remigius Agu. I have been amazingly fortunate to have an advisor who gave me the freedom to explore on my own and at the same time the guidance to recover when my steps faltered. Dr. Agu taught me how to question thoughts and express ideas. His patience and support helped me overcome many crisis situations that enabled me to finish this dissertation. It was a great opportunity to do my Masters under his guidance and to learn from his research expertise. I also appreciate the enormous support received from my Co-supervisor, Dr. Ali Imran. His patience, time, ideas and constructive criticism were greatly appreciated. I would like to thank Dr. Pollen Yeung for his comments and suggestions. This project would not have been completed without the support of my thesis advisory and examination committees. In this regard I acknowledge the support received from Drs. Emad and Jakeman. I would like to express my gratitude to the Director, Rita Caldwell, for making my stay in Canada financially possible. Financial support from the Dalhousie Pharmacy Endowment Fund and Capital Health District Authority was of immense help. The Faculty and Administrative staff (Tracy Jollymore, Wanda Dundas and Kate O'brien) were very kind to me and I thank them very much. I am indebted to my colleagues for their valuable suggestions and continuous co-operation throughout my research project.

I wish to express my everlasting thanks to my parents and grandfather, without whose inspiration and encouragement this work would never have been completed.

CHAPTER 1 INTRODUCTION

Thyroid gland plays a key role in controlling human metabolic functions through the action of thyroxine (T4) and trioiodothyronine (T3). The most common thyroid abnormality is hypothyroidism or underactive thyroid disease. In this condition, life-long thyroid hormone replacement is required. This therapy is available either as oral preparations or as intramuscular or intravenous injections. In most people, oral treatment provides a satisfactory form of replacement. However, in around 20 % of patients with hypothyroidism, oral replacement is either completely or partially ineffective because of poor or no hormone absorption following oral administration. It is very difficult to treat such people and they end up taking painful intramuscular or even intravenous injections on almost daily basis for the rest of their lives. This is an extremely uncomfortable and challenging scenario. In the work reported in this thesis, we conducted a series of studies that will allow us to subsequently develop alternate forms of thyroid hormone replacement that is convenient, effective and painless. In order to realize our research objectives, it is important to review the literature for important aspects of hypothyroidism such as its pathophysiology, treatment strategies and formulation development approaches and challenges.

1.1 PHYSIOLOGY OF THYROID GLAND AND HYPOTHYROIDISM

Thyroid gland is a highly vascularized butterfly-shaped organ located in the lower part of the neck inferior to the larynx[1]. It consists of two lobes present on either side of the trachea connected by an isthmus. On the posterior surface of the lobe are small, round masses of embedded tissue known as parathyroid glands (**Figure 1**). T4 and T3 are two major hormones produced and secreted by the thyroid gland[1]. The production and release of thyroid hormones are regulated by the hypothalamus and pituitary glands. The hypothalamus secretes thyrotropin releasing hormone (TRH) that stimulates the pituitary gland, which in turn secretes thyroid stimulating hormone (TSH).

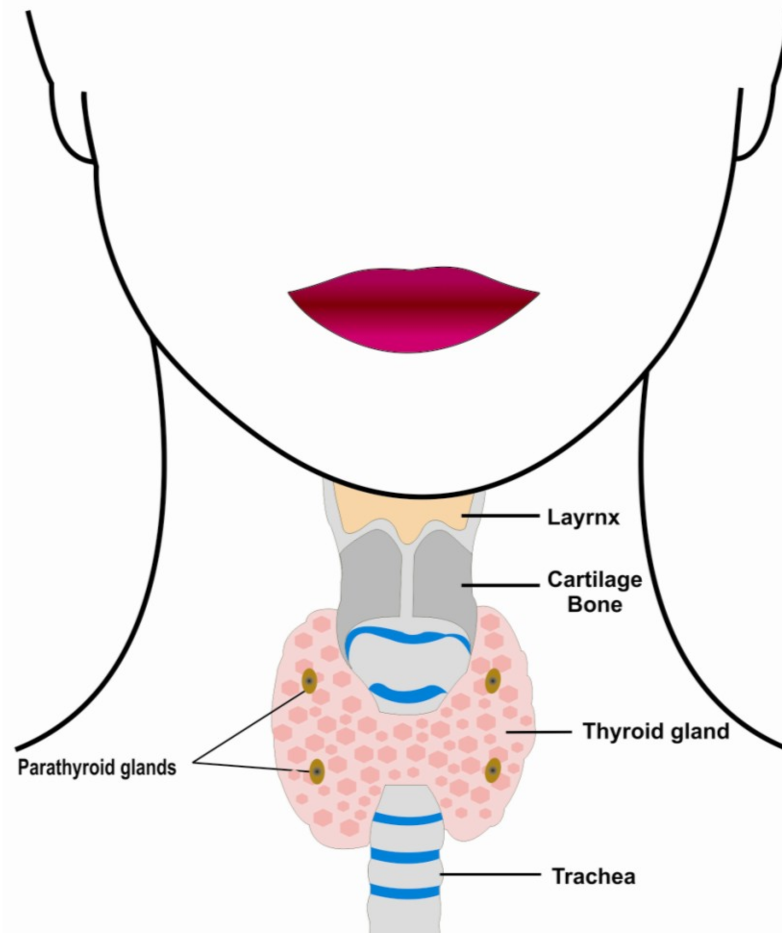


Figure 1 Anatomical location of human thyroid and parathyroid glands.

The TSH then signals thyroid follicular cells present in the thyroid gland to produce and secrete thyroid hormones. In order to produce T4 and T3, the thyroid follicular cells require iodine that is readily available in everyday diet. Thyroglobulin, a precursor of thyroid hormones already present in the thyroid gland, undergoes iodination on stimulation by the TSH to yield monoiodotyrosine (MIT) and diiodotyrosine (DIT). MIT and DIT subsequently join to yield T4 and T3. These hormones are lipid-soluble and hence diffuse out of the thyroid follicular cells into the blood stream. **Figure 2** summarizes the various steps involved in the production and secretion of T4 and T3. Although T3 is the biologically active form, T4 and T3 account for 99 % and 1 %, respectively of the thyroid hormones present in the blood. Intra-cellularly T4 is converted to T3 by deiodination. T4 exerts its effects by binding to thyroid hormone receptors found on most cells. It plays a major role in cell homeostasis (e.g. metabolism, temperature and energy regulation) and regulation of the brain, nervous, reproductive, cardiovascular and skeletal systems[1,2]. T4 is very important in controlling major physiological processes in the body and deficiency results in various adverse effects (e.g. brain damage, stunted physical growth and mental retardation).

The clinical disorder characterized by deficiency of T4 is hypothyroidism. Normally about 4.5 to 12.6 μg/dL of total T4 (bound and unbound) is present in the blood. Less than 4.5 μg/dL of total T4 can be indicative of hypothyroidism. As the pituitary gland is responsible for regulating and maintaining the production of thyroid hormones, a TSH functional analysis can be a key diagnostic test for detecting hypothyroidism. The normal range for blood TSH levels is 0.3 to 4 mIU/L. An abnormally high TSH level (i.e. higher than 4 mIU/L) is indicative of hypothyroidism. In

order to compensate for low T4 levels, the pituitary keeps secreting TSH to signal the thyroid gland to produce more T4[3]. Hypothyroidism occurs in 2-5 % of North American population[4,5] and is more prevalent amongst women, elderly and certain racial groups such as Hispanics and Whites[6]. Incidence of hypothyroidism is high amongst pregnant women and neonates (1 in every 4000 newborn babies)[7].

Hypothyroidism is predominantly classified as primary, secondary and tertiary hypothyroidism. In primary hypothyroidism, the thyroid gland is directly affected. In 50 % of the cases, the disease is due to destruction of the thyroid gland by autoimmune thyroid diseases. The remaining cases are caused by other factors such as iodine deficiency, irradiation injury and drugs (e.g. sunitinib; amiodarone) [2]. There has been an increase in the number of primary hypothyroidism cases reported in the past couple of years. One specific epidemiological study showed a nine-fold increase in new cases in UK compared to Denmark[8]. Unlike primary hypothyroidism, secondary hypothyroidism results from damage to the pituitary gland. This leads to the pituitary gland not being able to produce sufficient quantities of TSH to stimulate the thyroid follicular cells to produce T4 and T3. Damage to the pituitary gland can be due to a number of factors such as tumors, surgery, radiotherapy and head trauma[4]. Approximately 1 in every 80,000 to 120,000 individuals suffers from secondary hypothyroidism[9]. Tertiary hypothyroidism originates from the hypothalamus. When the hypothalamus is functionally impeded in its ability to produce TRH by tumors, surgery and infiltrative disorders (e.g. amyloidosis), this results in tertiary hypothyroidism[4,10]. It is prevalent in 0.0002 to 0.005 % of the general population[11].

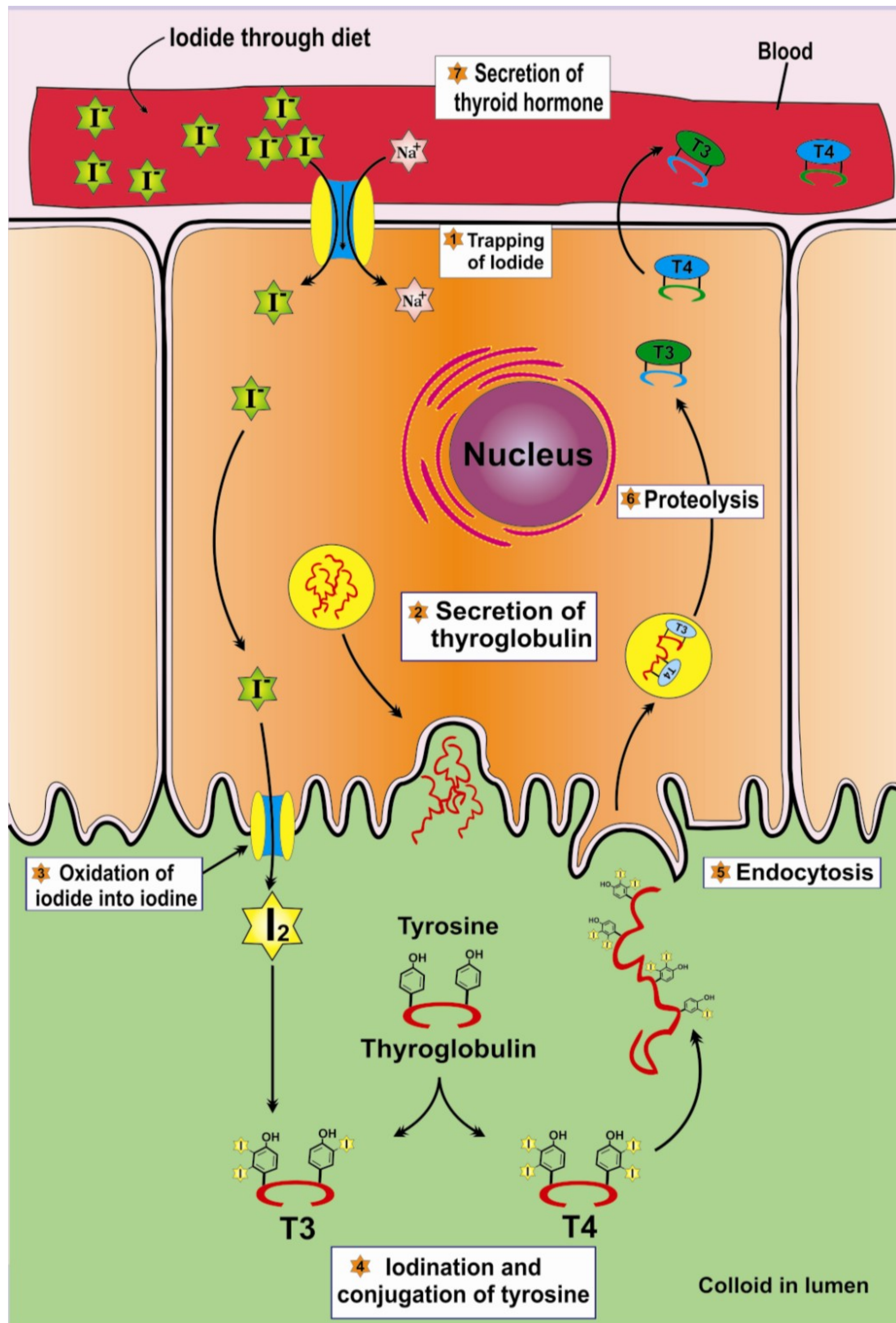


Figure 2 Steps involved in the production and secretion of thyroid hormones.

Almost 5 to 15 % of the world populations living in remote areas where staple diet is limited in iodine are likely to develop hypothyroidism[12]. In contrast, iodine

deficiency is rarely seen in developed nations such as United States, Canada and United Kingdom[13]. Although iodine deficiency remains the leading cause of hypothyroidism worldwide, in places of iodine sufficiency (e.g. North America, Europe, Japan), Hashimoto's thyroiditis is the primary cause of hypothyroidism. Almost 3 % of reported cases are caused by Hashimoto's thyroiditis[14]. Hashimoto's disease is an autoimmune disease in which thyroid hormone production is compromised by the body's immune system. The body makes antibodies that attack the thyroid follicular cells altering their ability to produce thyroid hormones. Apoptosis of the thyrocytes is observed because of lymphocytic infiltration by large numbers of B and T lymphocytes that react with thyroid specific antibodies such as thyroid peroxidase antibody (TPOAb) causing hypothyroidism[15,16,17]. Susceptibility to Hashimoto's thyroiditis increases with age and in patients already suffering from other autoimmune diseases (e.g. diabetes and pernicious anemia). Women are more likely to suffer from autoimmune thyroiditis than men[18]. Apart from Hashimoto's thyroiditis, sometimes thyroid hormone synthesis is inhibited by excess quantities of iodine available to the thyroid gland (Wolff-Chiakoff effect)[19]. The Wolff- Chiakoff effect is a short term effect where the sodium-iodide symporter in the thyroid gland shuts down in the presence of excess iodine levels in the body to prevent thyroglobulin iodinations and thus inhibiting the thyroid gland from producing excessive quantities of T4 and T3. As the intracellular iodine levels return to normal levels the "escape" phenomenon kicks in and iodification of thyroglobulin and synthesis of T4 and T3 resumes. In some individuals the body does not induce the "escape" phenomenon, thus continuing the down regulation of the sodium iodide transporter leading to a more profound and long lasting effect that results in hypothyroidism[19,20].

Thyroid gland destruction by radioactive iodine or surgery may cause hypothyroidism. Patients suffering from hyperthyroid conditions such as Grave's disease, thyroid cancer, thyroid nodules and head or neck lymphomas[21] are treated with either radioiodine therapy or surgery, which can result in hypothyroidism[18, 22]. In one study, it was found that almost 57 % of patients investigated in the study developed hypothyroidism after radioiodine therapy (RIT)[23]. Patients also develop hypothyroidism upon partial or total removal of the thyroid gland[24]. Sometimes injury, development of imflammatory diseases and formation of tumors in the pituitary or hypothalamus hamper TRH and TSH production resulting in either secondary or tertiary hypothyroidism[9]. Occasionally, hypothyroidism can be caused by medications used to treat other diseases. Most of the time drugs used for treating hyperthyroidism indirectly induce hypothyroidism. Medicines such as antithyroid agents (methimazole, propylthiouracil)[25]; psychiatric medications (lithium); tyrosine kinase inhibitors (sunitinib)[18]; somatostatin analogs (lanreotide); and glucocorticoids (cortisol)[26] cause hypothyroidism. Drugs that contain a large amount of iodine (e.g. amiodarone, potassium iodide, Lugol's solution) can also cause decreased production of thyroid hormones.

1.2 CURRENTLY AVAILABLE THERAPIES FOR HYPOTHYROIDISM

Although T4 monotherapy is widely accepted as the core treatment for hypothyroidism, other therapies such as desiccated thyroid, T3 monotherapy and combinations of T4 and T3 are also available. Various studies have found that hypothyroid patients treated with T4 achieve euthyroid conditions with similar circulating T3 levels. The strategy for disease treatment is to achieve normal blood levels of T4 and TSH and to improve patients' quality of life. Many synthetic T4 formulations are available on the market. Some of the US Food and Drug Administration (FDA) approved T4 oral preparations are Unithroid®, Synthroid®, L-Thyroxine® and Levoxyl®. Various other brand names and generic versions are also available worldwide. Tirosint®, a novel soft gelatin T4 capsule that uses only gelatin, glycerin and water as excipients has been approved by the FDA[27]. Unlike T4, Cytomel® is the only FDA-approved T3 replacement therapy available in United States and Canada. Some healthcare professionals prefer prescribing T3 to patients suffering from hypothyroidism. However, half-life of T3 in the blood is very short thus necessitating multiple doses in a day. It has been found that after taking T3 tablets, the blood levels of the hormone rises rapidly and can lead to unpleasant side effects. Generally, T3 tablets are used as adjuvants to T4 monotherapy[27]. Thyrolar® is a T4-T3 combination hormone preparation available in the US. Various studies regarding the benefits of T4-T3 combination preparations have been conducted, but no proven advantages have yet been documented compared to T4 monotherapy[28,29]. It has been found that combination T4-T3 preparations contain high amount of T3 than produced in the body, which can lead to similar side effects to T3 monotherapy. Therefore, doctors tend to prescribe Cytomel® tablets in addition to T4 to determine if

combination of T4 and T3 helps some patients who are not comfortable taking T4 alone[27].

Armour® is a dried and powdered animal thyroid mainly obtained from pigs. It was used as a treatment option for people suffering from hypothyroidism before the discovery and availability of synthetic thyroid hormones. The extract is available as pills. As the pills are formulated using animal thyroid gland extracts, they may contain hormones and proteins that are not present in human species. Furthermore, the ratio of T4 and T3 in animals may vary from that of humans and batch-to-batch pill variation are common. So far no study has shown any significant advantage of desiccated thyroid over synthetic T4[27].

Regarding injectable formulations, Levothroid® and Triostat® are two of the FDA- approved preparations available in the United States. Levothyroxine sodium for injection by Pharmaceutical Partners of Canada, Inc is approved by Health Canada for sale within Canada. Levothyroid® is a synthetic T4 preparation and Triostat® is a synthetic T3 preparation. These injectable preparations are prescribed as an alternative to the oral hormones when a rapid effect is needed or when the use of oral formulations is not possible (e.g. allergy to excipients, malabsorption)[30].

1.3 HURDLES AGAINST EFFECTIVE T4 AND T3 THERAPIES

Currently, hormone replacement therapy for hypothyroidism comprises T4 and T3 formulations administered as oral or parenteral formulations. However, some factors hamper effective delivery of the drugs to the patients. These factors can be classified into three categories namely: drug-related, physiological, and miscellaneous factors.

1.3.1 Drug-related Factors

Solubility of a compound affects its dissolution profile. In one study the intrinsic dissolution rate of levothyroxine sodium was extremely slow ($\cong$ 0.0002mg/min/cm^2)[31]. In order for a compound to be considered as rapidly dissolving, it should have an intrinsic dissolution rate greater than 0.1mg/min/cm^2 [32]. Based on the discrepancies in levothyroxine sodium solubility data, log *P* and dissolution profiles; it cannot be categorized as Biopharmaceutics Classification System (BCS) Class 1 or Class 3 drug[33,34]. The presence of three ionisable hydrophilic functional groups in T4 structure greatly affects its solubility and dissolution profiles at different pH. Between the pH ranges expected physiologically (1-6); the solubility of the compound seems to decrease. Sodium salt of T4 is amphiphilic as there are both hydrophobic and hydrophilic groups in the structure. These structural characteristics greatly affect the aqueous solubility of the molecule. The presence of three pKa's and the tendency of the compound to exist as a cation, anion, zwitterion and dianion greatly affect its stability and susceptibility to interactions and chemical reactions with multiple agents[35].

1.3.2 Physiological Factors

T4 undergoes drug-drug, drug-food and drug-disease interactions. Drugs containing calcium or magnesium form insoluble complexes with T4 and render it unavailable for absorption. Drugs such as antacids or proton pump inhibitors alter gastric pH and secretion, which affects T4 solubility and absorption[36,37]. Some medications that affect T4 absorption include sevelamer hydrochloride, chromium picolinate, raloxifene, lanthanum carbonate and ciprofloxacin[38,39]. Certain food and supplements such as soy protein[40], ferrous sulphate[41], dietary fiber[42], coffee[43], walnut[44], papaya fruit[45], and grapefruit juice[46] interact with T4 negatively. Certain pathophysiological conditions such as celiac disease[47], helicobacter pylori infection[48], atrophic gastritis, inflammatory bowel disease, lactose intolerance, coelic sprue and gastric surgery also affect T4 absorption[49,50]. Normal physiological condition such as gastric acidity is one of the major culprits responsible for T4 malabsorption. The complexity of the acid-producing machinery in the stomach contributes to individual variability observed in daily thyroxine requirement[51]. For instance in *H. pylori* infection, bacterial production of urease neutralizes gastric pH, impairing the absorption of many drugs[52].

1.3.3 Miscellaneous Factors

FDA regulates the protocols for conducting pharmacokinetic, stability, bioavailability and dissolution testing of T4 products[53]. This is not surprising because there are many reports of T4 formulations potency loss. FDA wanted to reduce the variability observed in the stability profiles of levothyroxine sodium formulations and

hence, decided to tighten the potency and shelf-life specifications of these products. The potency specification of 90-110 % was reduced to 95-105 % until the expiration date[54]. Poor patient compliance to therapeutic regimes is a major cause of hypothyroidism treatment failure. Pseudomalabsorption is a term used for describing poor T4 absorption due to issues with a patient's unwillingness and disability to adhere to dosing instructions[55].

1.4 Drug development: role of biopharmaceutics

Drug development is a term used to describe all the processes that a drug candidate undergo once a molecule is discovered. These steps are important to successfully advance a product to the market. Biopharmaceutics plays an important role in fast-tracking drug development. Considering the need for alternative delivery forms of thyroid hormones, we shall be adopting some biopharmaceutics screening methods to characterize T4 stability and absorption potential across the respiratory and transdermal surfaces. Prior to presenting the data generated using these methods, it is important to review the key elements of biopharmaceutics principles and their applications in drug development. In this section, the importance of biopharmaceutics in drug development and the roles of *in vitro* cell culture models as drug absorption screening tools will be discussed.

The aim of preclinical drug development is to acquire important data on the physicochemical properties of a drug candidate; it's pharmacological (pharmacokinetic and pharmacodynamic) and toxicological profiles. During various stages of drug development, biopharmaceutics plays a fundamental role in speeding up drug screening

and formulation development. Biopharmaceutics is the study of the interdependency between physicochemical properties of a drug and biological aspects of a living organism[56]. *In vitro* biopharmaceutics techniques are used to identify metabolic liabilities, drug interactions, and possible transport mechanisms across biological membranes. The methods are also important for estimating the rate and extent of drug absorption. Various physicochemical properties of a drug candidate such as solubility, stability, lipophilicity, particle size and salt form, influence *in-vivo* drug performance and must be considered while establishing its efficacy. Therefore, biopharmaceutical parameters are of high importance in establishing drug safety, clinical effectiveness and formulation development[56]. Knowledge of the biopharmaceutical properties of a drug or a drug candidate helps the pharmaceutical scientist to overcome major hurdles in the development or reformulation of a drug. The biopharmaceutical properties of a drug also allow us to design and establish the material properties and manufacturing variables that assist us to produce high quality products[56].

1.5 NEED FOR IN VITRO CELL MODELS

In vitro cell culture models have gained importance in the past couple of years as drug development tools and have become an integral part of drug development process. *In vitro* cell culture models offer various advantages over traditional *in vivo* models. They are simple and easy to use, have obvious ethical advantages compared to animal testing, and are cost effective. Furthermore, they are less complex than *in vivo* models and data variability is less common. Using *in vitro* models carcinogenic and mutagenic properties

of drugs can be screened without the need to use animals. With these models, pharmacokinetic parameters such as rate and extent of absorption can be estimated, especially during early stages of drug candidate selection and development. These models make it easier to measure the impact of a drug in a well-controlled system. Numerous studies have found good correlation between *in vitro* and *in vivo* drug screening data. Epithelial cells grown on permeable inserts are used for drug permeation studies. These studies in various epithelial cell culture systems has provided us with our current knowledge regarding active and passive drug transport mechanisms[57,58,59]. Caco-2, Calu-3, HEK 293, MCF-7 and other validated cell culture models are commonly used during drug development. For the purpose of our study, we will focus on Calu-3 cell line as a model for human airway epithelium. Calu-3 cells were originally derived from lung adenocarcinomas and are well differentiated and characterized. The respiratory tract is one of the preferred routes of delivery for many drugs due to its large surface area, relatively low enzymatic activity, highly vascularized blood supply, ease of accessibility for both local and systemic action, rapid onset of action and circumvention of hepatic first- pass metabolism[60]. Studies have indicated that Calu-3 cells are useful for studying drug effect on bronchial epithelial cells and mechanisms of drug delivery to the respiratory epithelium[61].

1.6 PHYSICOCHEMICAL PROPERTIES OF LEVOTHYROXINE

In order to develop new formulations of T4 for respiratory and transdermal routes, it is important to review the physicochemical properties of the compound. T4 is the synthetic levo isomer of thyroxine (**Figure 3**). It is commonly available in its sodium salt

form[62]. The molecular weight, degree of ionization at physiological pH, and partition coefficient (log *P*) has to be optimal for clinically relevant plasma concentrations to be reached. Sodium salt of T4 is a weakly acidic drug with 50 % ionization when pH equals pKa. Hence the drug is only approximately 1 % ionized at blood pH (7.4)[63]. Other physicochemical properties of the drug relevant for respiratory and transdermal delivery are as follows: pKa of ionized form = 8.72, log *P* (neutral form) = 3.21, log *P* (ionized form) = 0.35, intrinsic solubility = 42.8 µg/ml, solubility in experimental buffer (HBSS) = 10µM at pH 7.4 and molecular weight = 798.86 (anhydrous)[64].

T4 is a narrow therapeutic index drug and is commonly available in powder form. There is great variation in the published scientific literature about the physicochemical properties of the compound. In one study the solubility of levothyroxine sodium was estimated to be 150 µg/ml (log *P* of 3.51)[65].

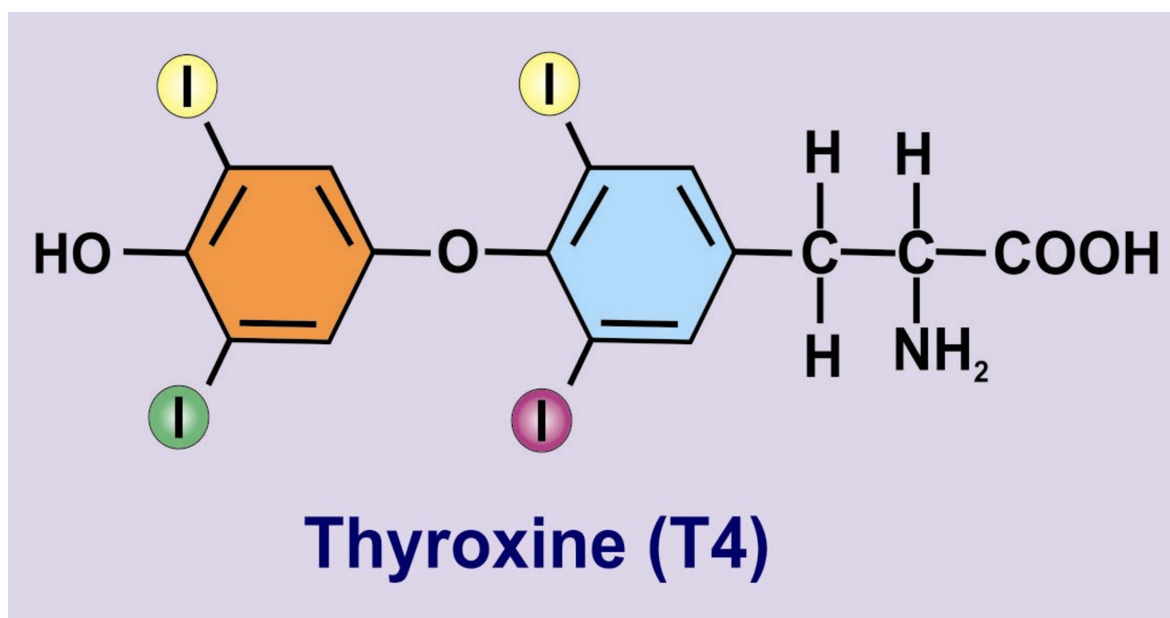


Figure 3 Chemical Structure of Thyroxine.

Based on the high aqueous solubility and permeability estimates the compound was classified as Biopharmaceutics Classification System (BCS) Class 1 drug (highly soluble, highly permeable). However, other authors classified the compound otherwise. For instance, T4 was also classified as a BCS Class 3 drug (high solubility, low permeability)[33,34]. Bioavailabilities of drugs belonging to BCS Class 1 are generally independent of formulation and process variables. However, this does not seem to be the case with levothyroxine sodium[66]. Structurally, T4 contains a hydrophobic benzoyl moiety attached to three hydrophilic moieties – carboxylate, phenol and amine groups. The three hydrophilic groups are ionisable and hence contain three pKa values (carboxyl, pKa = 2.2, phenolic group, pKa = 6.7 and amino group, pKa = 10.1)[67]. As a result of the presence of three ionisable moieties, T4-sodium can exist as a cation, anion, dianion and zwitterion, depending on the pH of the solution[35]. The presence of both hydrophobic and hydrophilic moieties makes it amphiphilic. Despite the log P and solubility discrepancies reported for T4, a number of variable stability issues have been reported for the compound. The plethora of inconsistencies in these reports may be explained by the fact that T4 is labile to heat, pH, moisture, light, oxidative conditions and chemical reactions[66,68]. These variables pose enormous challenges to successful formulation of the compounds and difficult to control in many laboratories.

CHAPTER 2 OBJECTIVES AND SCOPE

The aim of this study was to conduct a proof-of-principle studies that will enable possible development of non-invasive respiratory and transdermal delivery systems for T4. The delivery systems will hopefully be useful for patients with gastro-intestinal auto-immune diseases such as inflammatory bowel disease (e.g. Crohn's disease, ulcerative colitis) or patients with intestinal resection in whom absorption of orally administered thyroid hormones is significantly impaired. Injectable alternative preparations are extremely inconvenient because of life-long need for deep intramuscular or intravenous injections. It has been documented that major failures in thyroid hormone replacement is due to patient compliance. Many patients inadvertently take the hormones with meals or supplements containing compounds that bind and inactivate the drug. Alternative delivery routes for the hormone will be useful clinically in solving the above-mentioned problems. The formulations will neither be invasive nor prone to food-drug interactions. Furthermore, inter-changeability of thyroid hormones has been challenging due to stability and bioequivalence issues.

The following hypotheses were tested:

a. T4 when applied to the respiratory mucosa permeates the mucosa at a reasonable rate and quantity to potentially elicit systemic effects.

b. T4 permeation across artificial human skin substitute (Strat-M®) can be used to predict its permeation across the human skin.

CHAPTER 3 MATERIALS AND METHODS

3.1 CHEMICALS

Levothyroxine sodium salt hydrate (T4) and High Performance Liquid Chromatography (HPLC) grade trifluoroacetic acid (TFA) were purchased from Acros Organics BVBA (Geel, Belgium). 3, 3′, 5-Triiodo-L-thyronine sodium salt (T3), caffeine, benzoic acid, polyethylene glycol 400 (PEG 400), polyethylene glycol 600 (PEG 600), sodium fluorescein, isopropyl alcohol, D-(+)- glucose and 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolinium bromide (MTT) were all purchased from Sigma-Aldrich (Oakville, ON, Canada). HPLC grade acetonitrile (ACN), HEPES, L-ascorbic acid (AA), dimethylsulfoxide (DMSO), agarose and ethidium bromide was purchased from Fischer Scientific (Ottawa, ON, Canada). HPLC grade methanol and sodium bicarbonate were purchased from EMD Millipore (Billerica, MA, USA). Sodium hydroxide (NaOH), glycerol, ethylenediaminetetraacetic acid (EDTA) and sodium chloride (NaCl) were purchased from VWR (Mississauga, ON, Canada).

3.2 CELL CULTURE MEDIA AND OTHER COMPONENTS

Fetal bovine serum (FBS), GlutaMAX®, penicillin-streptomycin, DMEM F-12, TRIzol® and primers for thyroid hormone transporters (MCT8, MCT10, OATP1A2, OATP4A, LAT1, LAT2 and CD98) were purchased from Life technologies Inc (Burlington, ON, Canada). Phosphate buffered saline (PBS, 10X, Bioreagent suitable for

cell culture) and Hanks Balanced Salts (HBSS), with and without $Ca^{2+}$and Mg^{2+} were purchased from Sigma-Aldrich (Oakville, ON, Canada). Ultracruz® 24 well tissue culture plates were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). Corning PET 24 well Transwell® inserts were purchased from Fisher Scientific (Ottawa, ON, Canada). Strat-M® membrane was purchased from EMD Millipore (Billerica, MA, USA).

3.3 METHOD OPTIMIZATION AND VALIDATION

Stock solutions and calibration curves were prepared using the method described in the United States Pharmacopoeia (USP 34)[62]. A stock solution of 1.0 mg/ml was prepared for both T4 and T3. Subsequently, calibration sample concentrations of 0.1, 0.5, 1, 10 and 100 µg/ml for each of the hormones was prepared from the stocks. The vehicle used to dissolve the hormones was a mixture of methanol and 20 mM sodium hydroxide (50:50). The HPLC method used for quantifying the compounds was validated for linearity, range, precision and accuracy according to the International Conference on Harmonization (ICH) Guidelines for HPLC method validation[69]. Peak resolution was calculated using the formula:

$RS = (RT_{T4} - RT_{T3}) \div [0.5*(W_{T4} + W_{T3})]$…..***Equation 1***

Where: RT_{T4}= Retention time of T4; $RT_{T3=}$ Retention time of T3; W_{T4}= Width of peak for T4; $W_{T3=}$ Width of peak for T3

3.3.1 HPLC Method Validation: Linearity and Range

Standard calibration curves were prepared with five calibrators over a concentration range of 0.1–100 μg/mL (0.1, 0.5, 1, 10 and 100 μg/mL) for T4 and T3. The concentration range was chosen based on expected drug concentration in the sample solutions. The data was plotted as peak area versus drug concentration. The analytical range was established by the highest and lowest concentrations of analyte where acceptable linearity, accuracy and precision were obtained.

3.3.2 HPLC Method Validation: Precision

Precision of the method was determined for T4 and T3 by analyzing the Quality Control (QC) standard samples at three concentrations (low QC = 0.1 μg/mL, intermediate QC = 1 μg/mL, and high QC = 100 μg/mL). The method was checked for intermediate precision and repeatability. For intermediate precision, the three QC standard samples were injected in triplicates on three different days to measure interday variability. For repeatability studies, the above-mentioned QC standard samples were injected in triplicates on the same day. Precision was expressed as percentage coefficient of variation (CV %) of the analyte peaks.

3.3.3 HPLC Method Validation: Accuracy

Accuracy was determined for the three QC standards (5, 30 and 75 μg/mL) and each of the concentrations was injected into the HPLC in triplicates and percentage recovery was measured.

3.4 SOLUBILITY STUDIES

Solubility studies were conducted to determine the solubility of the thyroid hormones in various solvents. Pre-solubility studies were conducted using water, HBSS (with and without calcium), PBS and colourless DMEM/F12 media. Based on the results obtained from these studies, further solubility studies were conducted in the above-mentioned media to optimize the best vehicle to use for further studies. T4 and T3 solubility enhancement studies were conducted using PEG-400, PEG-600, polypropylene glycol, glycerol, DMSO, Tween 20 and ethanol at various concentrations.

3.5 STABILITY STUDIES

Stability studies were conducted to test the effect of different conditions on T4 stability. To test the effect of temperature, 10 μM T4 solutions were kept at 4°C, room temperature (RT, 23.5°C) and 37°C, respectively for 2 hours. Samples were aliquoted at different time points and analyzed with the HPLC. For the effect of pH on stability, 10 μM aliquots of T4 were kept at five different pH's (i.e. 5.0, 6.0, 7.0, 8.0 and 9.0) for a period of two hours at 37°C. Effect of glass and plastic materials on T4 stability was also tested. For these studies, 10 μM T4 solutions were kept in borosilicate glass test tubes and in a 24-multi-well polystyrene plate for 2h. Samples were aliquoted and analyzed by HPLC. Effect of stability enhancers such as potassium iodide (KI), ethylenediaminetetraacetic acid (EDTA) and ascorbic acid (AA) was also tested.

3.6 RESPIRATORY STUDIES

3.6.1 Cell Culture

The Calu-3 cells were grown according to a standard protocol. The cells were cultured in 1:1 D-MEM/F-12 supplemented with 10 % FBS, 1 % GlutaMax™, 100U/ml penicillin, and 100 mg/ml streptomycin. Initially, they were grown submerged in the culture medium for 4 days. After this period, they were maintained at an air–liquid interface by not including culture medium on the donor compartment of the inserts (cells for transport studies). Except for cells that were used for transport studies all cell batches were grown on Santa Cruz Biotech clear polyester membrane flasks. For transport studies cells were grown on polyester (PET) Transwell inserts. Only cells with Transepithelial electrical resistance (TEER) above 400 $\Omega.cm^2$ were used for experiments. The cells were fed every other day with a DMEM-F12 containing 10 % FBS, 1 % Glutamax®, 1 % 10,000 units/mL penicillin and 1 %, 100 mg/ml streptomycin. The cells were maintained at 95 % O_2 and 5 % CO_2 environment. Cells within passages 15-20 were used for the studies. **Figure 4** shows the cell culture scheme used for the studies.

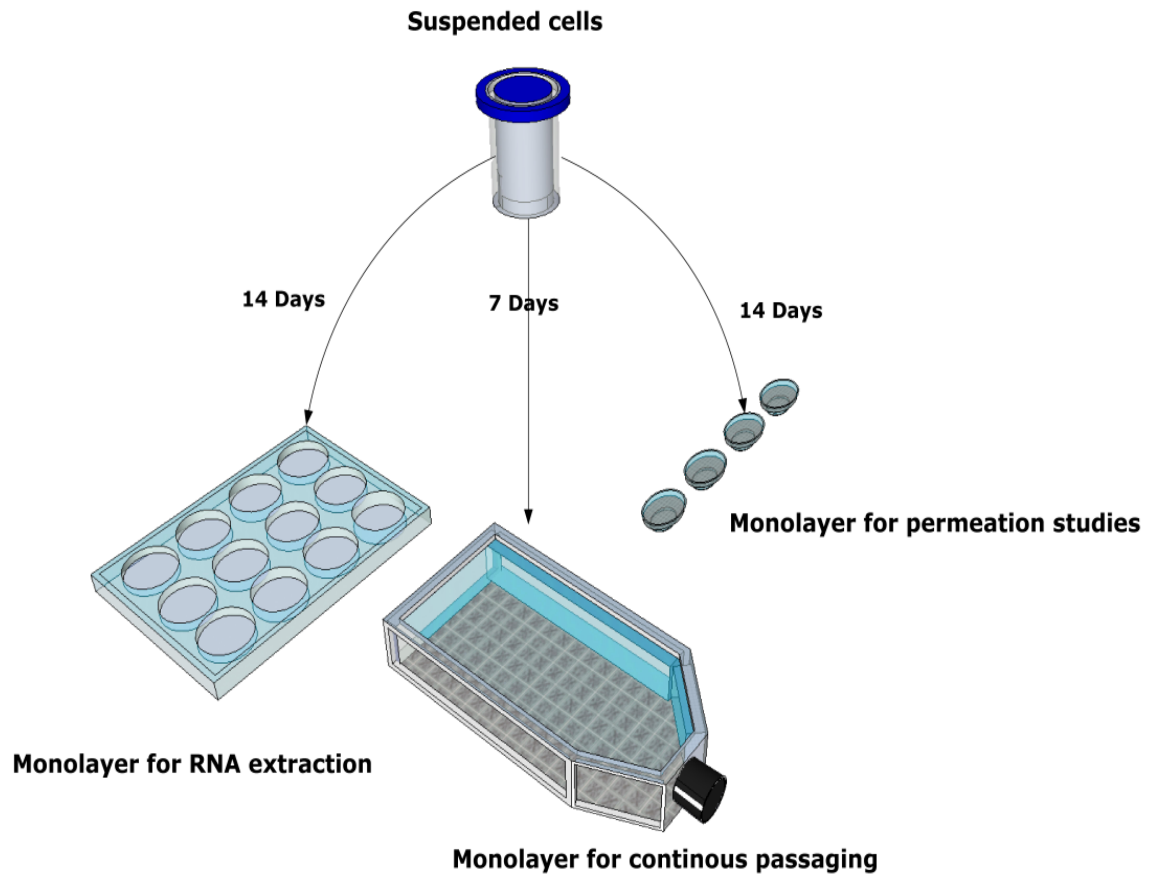


Figure 4 Summary of cell culture approaches used for growing Calu-3 cells

3.6.2 RNA Extraction

Cells were grown on 12-well plates for 14 days. The cells were washed twice with RNAse-free PBS. 1ml of TRIzol® was added to each well and incubated for 5 minutes. The media was then transferred to sterile centrifuge tubes and 200 µl of chloroform was added to each tube. The cells were then vortexed for 15 seconds and then incubated at

room temperature for 3 minutes. The tubes were then centrifuged at 4°C (13000 rpm) for 15 minutes. 400 µl of the top colourless phase was removed and added to 500 µl of isopropyl alcohol. The solution was then vortexed and the tubes were allowed to stand for 10 minutes. The cells were centrifuged again at the above- mentioned conditions and the supernatant was removed. The pellet was then washed thrice with 75 % chilled ethanol. After that the ethanol in the tubes was allowed to evaporate and 30 µl of RNAase-free water was added to the pellet and stored at -80°C. RNA concentration and purity were quantified using Cary 50 UV-Vis spectrophotometer (Varian Inc., CA, USA) and ratio of A260/280 was analyzed. RNA samples with A260/280 ratio less than 2 were discarded.

3.6.3 Real Time Polymerase Chain Reaction (RT-PCR) Studies

Real time PCR studies were conducted on PTC-100 programmable thermal controller (Bio-Rad Laboratories, ON, Canada.) using the protocol prescribed by iScript™ One-Step RT-PCR Kit with SYBR® Green (Bio-Rad Laboratories, ON, Canada). Primers used were for MCT8, MCT10, OATP1A2, OATP4A, LAT1, LAT2 and CD98 thyroid hormone transporters. 18S gene was used as housekeeping gene. Mastermix devoid of any primers was used as negative control. A 50 µl aliquot of master mix was prepared containing 25 µl of 2X SYBR® Green RT-PCR Reaction Mix, 10 µM of forward primer and 10 µM of reverse primer, 1 pg of RNA template and 1 µl of iScript reverse transcriptase for One-Step RT-PCR. The remaining volume was made up with nuclease-free water. Cycling conditions were as follows: 10 min at 50 °C, 5 min at 95 °C, and 40 cycles of 10 s at 95°C, plus 30s at 60 °C.

3.6.4 Transport Studies

The effect of time, concentration (25, 50, 75, 100 µM), temperature (4°C and 37°C), pH (5.5 and 7.4), sodium and polarity (apical to basolateral, AP-to-BL and basolateral to apical, BL-to-AP) on T4 transport were investigated. Prior to the experiments, the cells were washed twice and pre-incubated with transport media, which consisted of 0.9 % saline supplemented with 25 mM glucose, 10 mM HEPES, 30 µM KI, 17 µM EDTA and 15 µM AA (pH 8.0) for 15 min at 37°C. For the effect of sodium studies, the transport medium consisted of 9.25 % sucrose supplemented with 10 mM HEPES buffer with pH of the medium maintained at 8.0. Transport studies were initiated by adding 250 µl of test solutions to the donor compartment and 400 µl of transport medium to the receiver compartment. At stipulated time points (0–180 min), 100 µl aliquots were sampled from the receiver compartment and were replaced immediately with an equal volume of transport media. Cells on each insert were checked for epithelial integrity before and after permeation experiments by TEER measurement and sodium fluorescein permeation assay. Inserts with TEER values below 400 $\Omega.cm^2$ prior to experiments were not used for transport studies. Sodium fluorescein was analyzed using Cary 50 UV-Vis spectrophotometer (Varian Inc., CA, USA) at 490 nm wavelength. Similarly, data from inserts with TEER readings below 400 $\Omega.cm^2$ and sodium fluorescein permeation above 1.0 % after 1 h incubation following transport experiments were discarded.

3.6.5 Toxicity Studies

TEER was used for evaluating the toxicity of cosolvents. Solutions tested were 0.9 % saline with 25 mM glucose and 10 mM HEPES (pH8.0); T4 solutions (100 µM, 50 µM) in 0.9 % saline with 25 mM glucose and 10 mM HEPES, glycerol, PEG 600, polypropylene glycol at three concentrations (1 %, 5 %, 10 %) in 0.9 % saline containing 25 mM glucose and 10mM HEPES. A 200 µl sample solutions were added to the apical side and 400 µl of 0.9 % saline containing 25 mM glucose and 10 mM HEPES was added to the basolateral side. At specific time points (0, 5, 15, 30, 60, 120 min) TEER of the cells was measured and percentage decrease calculated.

MTT assay was used to monitor cell viability after incubating the cells with different concentrations of the cosolvents. At the end of the incubation, the Calu-3 cells were washed twice with HBSS. Control group for the experiment consisted of cells incubated with HBSS alone. Subsequently, 600 µL of MTT (2 mg/mL) was added to each well to completely submerge the cells. The cells were then incubated for 3 h at 37 °C. At the end of the incubation period, the MTT solution was removed. The purple formazan crystals were dissolved in 200 µl of DMSO. Formazan absorbance was measured spectrophotometrically at 560 nm using a Cary 50 Bio UV-Visible Spectrophotometer.

3.7 TRANSDERMAL STUDIES

3.7.1 Strat-M® Validation

Validation of Strat-M, a synthetic membrane similar in structure to the human *stratum corneum* was carried out as prescribed by the OECD Guideline 428 for testing skin absorption using *in vitro* methods[70]. The diffusion studies were carried out using NaviCyte® horizontal diffusion chamber (Warner Instruments, Hamden, CT, USA, **Figure 5)**. Caffeine was used as reference test substance. A stock solution of 10 mg/ml was prepared in ethanol/water (1:1) and diluted in 0.9 % saline to achieve a final concentration of 4 mg/cm^2. Receptor solution consisted of 0.9 % saline. A 100 µl aliquot of the receptor fluid was sampled at different time points (1, 2, 4, 6, 8, 24 h) for analysis. The receptor solution withdrawn at each time point was replaced with exact quantity of saline in the receptor compartment. At the end of the experiment, the donor solution was kept for analysis. The experiments were conducted at 33°C as prescribed by the OECD Guideline 428[70]. The receptor samples were analyzed by HPLC.

3.7.2 Levothyroxine Permeation Across Strat-M®

T4 permeation studies were also conducted with NaviCyte® horizontal diffusion chambers **(Figure 5)**. The donor solution consisted of 0.9 % saline containing 25 mM glucose, 10 mM HEPES, 30 µM KI, 17 µM EDTA, 15 µM AA (pH 8.0) with and without 30 % PEG 600 or 30 % polypropylene glycol or 15 % Vitamin E-tocopheryl polyethylene glycol succinate(TPGS). Strat-M® was used as a synthetic transdermal membrane that mimics barrier properties of *stratum corneum*. 1ml of 100 µM T4 solution containing 30

% PEG 600 or 30 % polypropylene glycol or 15 % Vitamin E-TPGS was added to the donor side of the chamber. In solutions containing only transport medium, T4 concentration was 25 μM and 2.5 ml of transport medium was added to the receiver side. At specified time points (1, 2, 4, 6, 8, 24 h) 75 μl of sample solutions were aliquoted and diluted with 25 μl of mobile phase. At the end of the experiment, donor solutions were collected for mass balance analysis. Samples were analyzed using HPLC.

3.8 PREPARATION OF SOLUTIONS

Solutions used during the experiments were prepared by dissolving required amounts of T4 or T3 in the appropriate solutions as demanded by the study and sonicated for 20 minutes. Final concentrations were expressed in micromolar (μM) or weight per volume (μg/ml) and pH of the solutions was maintained at 8.0. Where applicable, pH of the solutions was adjusted with NaOH (1.0 %) or HCl (1.0 %). T4 stability was enhanced by the addition of 30 μM KI, 17 μM EDTA and 15 μM AA. Samples were weighed using Toledo AE 240 balance (Mettler, Zurich, Switzerland) and pH of the solutions was measured with a Hanna pH meter (Hanna Instruments, RI, USA).

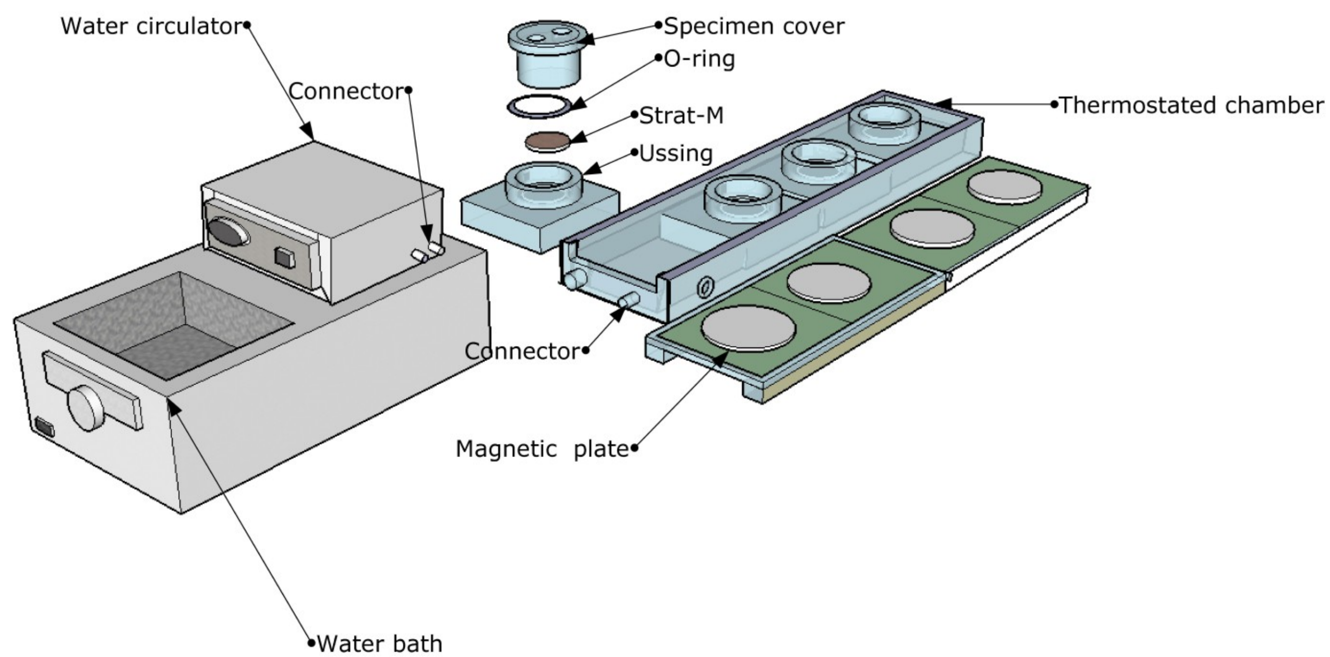


Figure 5 Experimental setup for permeation studies using NaviCyte® horizontal diffusion chambers and Strat-M®

3.9 SAMPLE ANALYSIS

Thyroid hormones (T3, T4) were analyzed with a Varian 920-LC chromatography unit (AgilentTechnologies, Mississauga ON, Canada) equipped with a low pressure quaternary gradient pump with built-in 4-channel degasser, UV-fluorescence detector combo and refrigerated auto sampler. Acetonitrile and deionised water containing 0.1 % trifluoroacetic acid was used as mobile phase in a ratio of 70:30. The mobile phase was filtered through a 0.22 µm nylon filter (Chromatographic specialties Inc., ON, Canada) and degassed in an ultrasonic bath sonicator (Branson, CT, USA) for 20 minutes. A Spherisorb ODS2 column (4.6 mm x 250 mm, 5 µm size, Waters, Mississauga, ON, Canada) was used for resolving the hormone peaks. The autosampler temperature was maintained at 4°C during analysis. The injection volume was 50 µl and the HPLC was run at a flow rate of 1.0 ml/min. The samples were detected at a wavelength of 225 nm. For caffeine, the injection volume was 20 µl and the mobile phase for the analysis consisted of methanol and water in a ratio of 70:30. The flow rate was maintained at 1.1 ml/min and the compound was detected at a wavelength of 254 nm using a UV detector. A standard curve was prepared in ethanol/water (1:1) using six concentrations (0.5, 1, 5, 10, 50, 100 µg/ml). The chromatographic data were analyzed with Galaxie software® (Agilent Technologies Inc., Mississauga, ON, Canada).

3.10 STATISTICAL AND DATA ANALYSES

Unless stated otherwise, all experiments were performed three times and data presented as mean±SD. Where appropriate, statistical significance of the results were determined using analysis of variance (ANOVA) followed by post-test analysis. InStat® 3.0 (GraphPad, San Diego, CA, USA) was used for the analysis. A $p < 0.05$ was considered significant.

The cumulative concentrations (C_n) of T4 and caffeine that appeared in the receiver compartment were estimated by equation 2:

$C_n = C'_n + V_s/V_t.\ (C'_{n-1} + \text{-----} + C'_1)$…..***Equation 2***

Where, C_n, C'_{n-1}, C'_1 = concentration at n, previous sample, and first sample, respectively.

V_s, V_t = Volumes of sample in the donor and receiver compartments, respectively.

Apparent permeability coefficients, P_{app} (cm/s) for T4 transport was calculated using equation 3:

$$P_{app} = \frac{dQ}{dt} x \frac{V}{AC_0}$$ …..***Equation 3***

Where, $\frac{dQ}{dt}$ (µg/sec or µM/sec) = steady rate of appearance of T4 to the receiver side,

C_0 (µg/ml) = initial concentration in the donor chamber, A (cm^2) = effective growth surface area of the inserts (1.0 cm^2), V (ml) = volume of the receiver compartment.

CHAPTER 4 RESULTS

4.1 METHOD OPTIMIZATION AND VALIDATION

An HPLC method was developed and optimized to obtain a complete separation of T4 and T3. It was important that our analytical conditions resolved both peaks because T3 is the major pharmacologically active metabolite of T4. **Figure 6** is a representative chromatogram of the separation achieved after injecting 50µg/ml of T4 and T3, respectively. T3 eluted at 4.7minutes followed by T4 at 6.3 minutes. The resolution achieved was 2.35. The HPLC method was optimized for the organic solvent in the mobile phase, additives and composition of mobile phase and run time. Initially an isocratic solvent consisting of 40:60 (acetonitrile: water) was used. The solvent contained 0.05 % phosphoric acid. However, T3 and T4 were not detected with the solvent. Upon interchanging the mobile phase composition to 60:40 (acetonitrile: water) containing 0.05 % phosphoric acid, the peaks were separated. However, the chromatograms showed peak broadening and poor resolution. This problem was resolved by using trifluoroacetic acid instead of phosphoric acid[71]. With this substitution, good resolution was observed between T4 and T3 with no peak tailing. The retention times were 12.16 and 10.34 min for T4 and T3, respectively. The method was further optimized to reduce the retention times of the compounds by changing the mobile phase compositions. The ratios of organic phase to water tested were 65:35, 70:30 and 80:20. Out of the three, the mobile phase composition of 70:30 (acetonitrile: 0.1 % TFA in deionized water) was optimum

for generating chromatograms with sharp peaks and reasonable retention times for T4 and T3 (6.3 and 4.7 min, respectively).

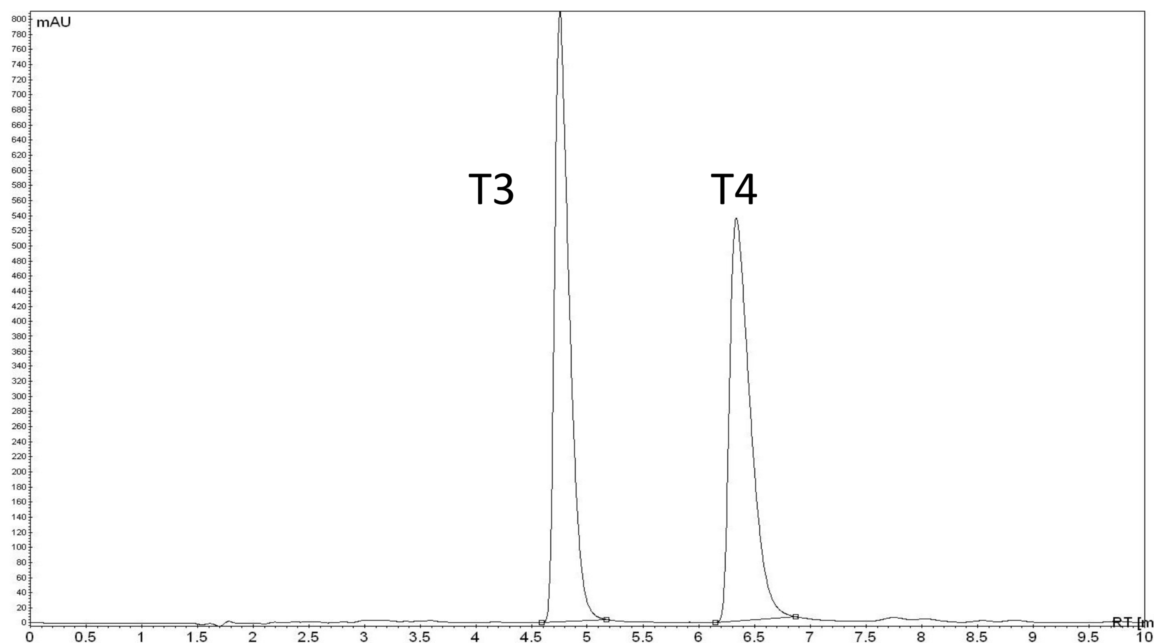
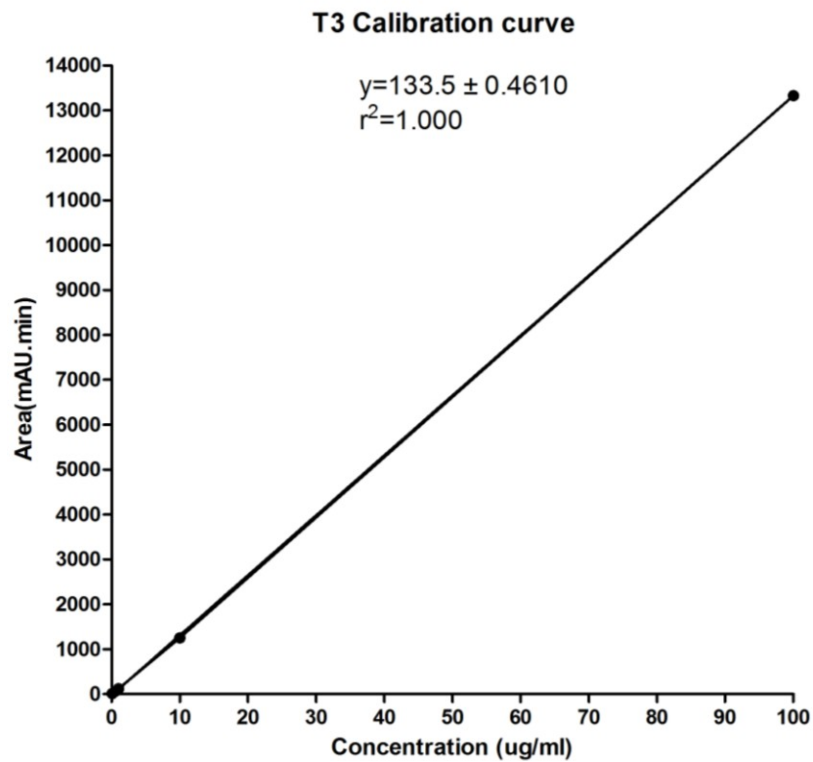


Figure 6 HPLC chromatogram for 50μg/ml T4 and T3. Retention times observed for T4 and T3 were 6.3 and 4.7 minutes, respectively. Run time was 10 min.

4.1.1 Linearity

Linearity of the HPLC method was confirmed for T4 and T3 using the analytical range of 0.1–100μg/mL. A standard curve was then prepared for each compound by plotting known drug concentrations (x-axis) against peak area (y-axis) of standard solutions. A linear correlation between analyte peak area and concentration of T3 and T4 was observed. The R^2 (y = 133.5x ± 0.4610 for T3 and y = 131.3x ± 0.4278 for T4) was 1.00 for both compounds. The linearity data are summarized in **Figure 7**. Range was optimized based on the concentrations of T4 and T3 expected in samples during experiments.



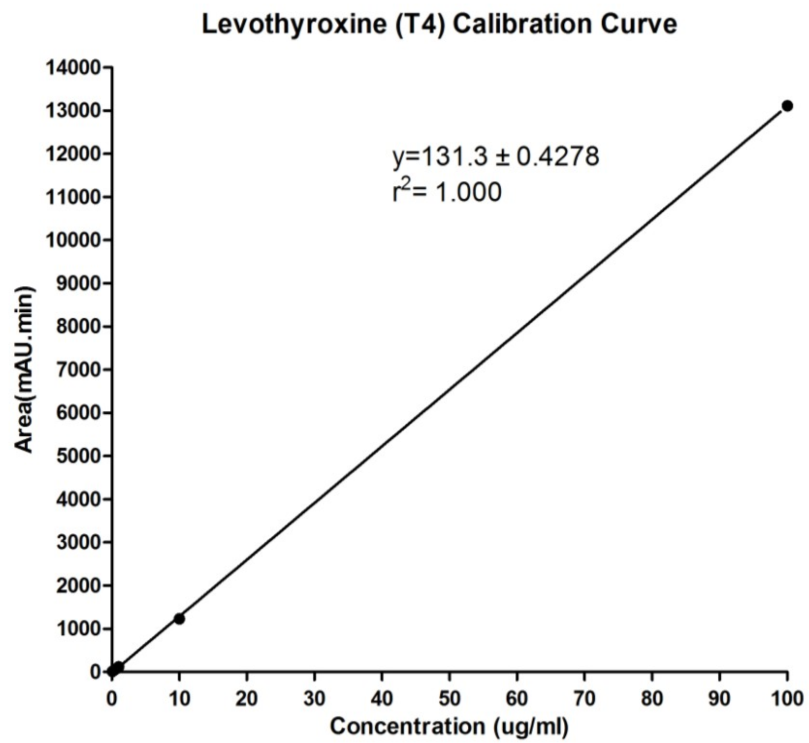


Figure 7 Linearity of T3 and T4 within a concentration range of 0.1–100 μg/mL.

4.1.2 Accuracy

Accuracy was established across the linear analytical range for T3 and T4. Results for the intra-day accuracy of T3 and T4 are summarized in **Table 1** and **Table 2.** The tables show the percentage recovery of both hormones, which were within the 95 % - 105 % acceptable range for early stages of drug development activities.

Table 1 Accuracy of T3 quantification with Varian 920 LC system (n=3).

Nominal Concentration	5 µg/ml	30 µg/ml	75 µg/ml
Amount Recovered µg/ml	4.43	28.82	77.59
	4.42	28.85	77.77
	4.41	28.78	77.81
Mean	4.4	28.8	77.72
Standard Deviation	0.01	0.03	0.12
% RSD	0.23	0.12	0.15
% Recovery	99.79	99.98	100.17

Table 2 Accuracy of T4 quantification with Varian 920 LC system (n=3).

Nominal Concentration	5 µg/ml	30 µg/ml	75 µg/ml
Amount Recovered (µg/ml)	4.87	29.03	77.75
	4.95	29.01	77.91
	5.06	28.96	77.75
Mean	4.99	29.00	77.80
Standard Deviation	0.05	0.033	0.094
% RSD	1.09	0.11	0.12
% Recovery	99.9	96.66	103.73

4.1.3 Precision

In repeatability studies, the mean percent coefficient of variation for low (0.1 µg/ml), intermediate (1 µg/ml) and high standard (100 µg/ml) QC T3 solutions was 4.05, 1.58 and 1.56 %, respectively. Similarly for T4, the mean percent coefficient of variation for low (0.1 µg/ml), intermediate (1 µg/ml) and high (100 µg/ml) QC standards was 4.1, 3.1 and 1.9 %, respectively. The mean percent coefficient of variation for interday studies for T3 and T4 was 2.4 and 2.9 %, respectively. All the results obtained were within the acceptable limit of 5 %[74]. Precision data are summarized in **Tables 3 and 4.**

Table 3 Repeatability and interday precision for T3 (n=3).

		Conc. (µg/ml)	Repeatability			Inter day		
			Mean	SD	CV (%)	Mean	SD	CV (%)
Control 1 0.1 µg /ml	**Day 1**	0.11 0.10 0.11	0.11	0.003	2.81	0.11	0.001	1.17
	Day 2	0.10 0.11 0.11	0.11	0.007	6.87			
	Day 3	0.11 0.11 0.11	0.11	0.003	2.49			
Control 2 1 µg/ml	**Day 1**	1.01 1.01 1.02	1.02	0.005	0.31	0.99	0.021	2.10
	Day 2	0.97 0.99 0.97	0.98	0.013	1.28			
	Day 3	1.02 0.96 1.00	1.00	0.032	3.16			
Control 3 100 µg/ml	**Day 1**	106.60 105.24 108.59	106.81	1.687	2.79	102.13	4.141	4.05
	Day 2	98.41 103.01 100.50	100.65	2.306	2.29			
	Day 3	99.72 98.11 98.99	98.94	0.804	0.81			

Table 4 Repeatability and interday precision for T4 (n=3).

		Conc. µg/ml	Repeatability			Interday		
			Mean	**SD**	**CV (%)**	**Mean**	**SD**	**CV (%)**
Control 1 0.1µg/ml	**Day 1**	0.10 0.10 0.11	0.11	0.003	3.29	0.10	0.002	2.14
	Day 2	0.11 0.10 0.11	0.11	0.004	4.03			
	Day 3	0.10 0.10 0.11	0.10	0.005	4.98			
Control 2 1 µg/ml	**Day 1**	1.05 1.06 1.06	1.06	0.009	0.86	1.01	0.044	4.32
	Day 2	1.01 0.95 1.028	1.00	0.040	4.09			
	Day 3	0.99 0.92 1.00	0.97	0.042	4.34			
Control 3 100 µg/ml	**Day 1**	101.10 103.53 104.75	103.12	1.859	1.80	1.02	2.181	2.14
	Day 2	98.46 100.43 98.65	99.18	1.088	1.09			
	Day 3	104.59 102.84 100.86	102.77	1.868	1.82			

4.2 SOLUBILITY STUDIES

4.2.1 Pre-solubility Studies

Pre-solubility studies of T3 and T4 were conducted using deionized water, PBS (1X) and HBSS with and without calcium (Ca^{2+}) and magnesium (Mg^{2+}), and colorless DMEM-F12 at three different concentrations (50, 100 and 150 µg/ml).These studies were conducted to find the media that may be used for *in vitro* biopharmaceutic studies. Results from the studies are shown in **Table 5**. T4 and T3 precipitates were observed in HBSS with calcium and magnesium and colorless DMEM-F12. No precipitates were observed in deionised water, PBS (1X) and HBSS without calcium and magnesium.

Table 5 Pre-solubility data for T4 and T3 (n = 2).

Conc. (µg/ml)	Deionised Water	PBS (1X)	HBSS with Ca^{2+}/Mg^{2+}	HBSS without Ca^{2+}/Mg^{2+}	Colorless DMEM-F12 media
150	Clear solution with undissolved compound	Clear solution with undissolved compound	Precipitates observed	Clear solution with undissolved compound	Precipitates observed
100	Clear solution with undissolved compound	Clear solution with undissolved compound	Precipitates observed	Clear solution with undissolved compound	Precipitates observed
50	Clear solution with undissolved compound	Clear solution with undissolved compound	Precipitates observed	Clear solution with undissolved compound	Precipitates observed

4.2.1 Further Solubility Studies

This batch of experiments was conducted to find the percentage of T4 and T3 soluble in water, PBS and HBSS without Ca^{2+}/Mg^{2+}. Solubility of T4 and T3 in methanol: 20mM sodium hydroxide (50:50) was used as a control. The results are shown in **Figure 8**. The Solubility of both compounds (T4 and T3) was higher in deionised water compared to PBS and HBSS without Ca^{2+}/Mg^{2+}. Solubility of T4 was independent of the concentrations used; but this was not the case for T3. Highest T3 solubility was seen at 100µg/ml.

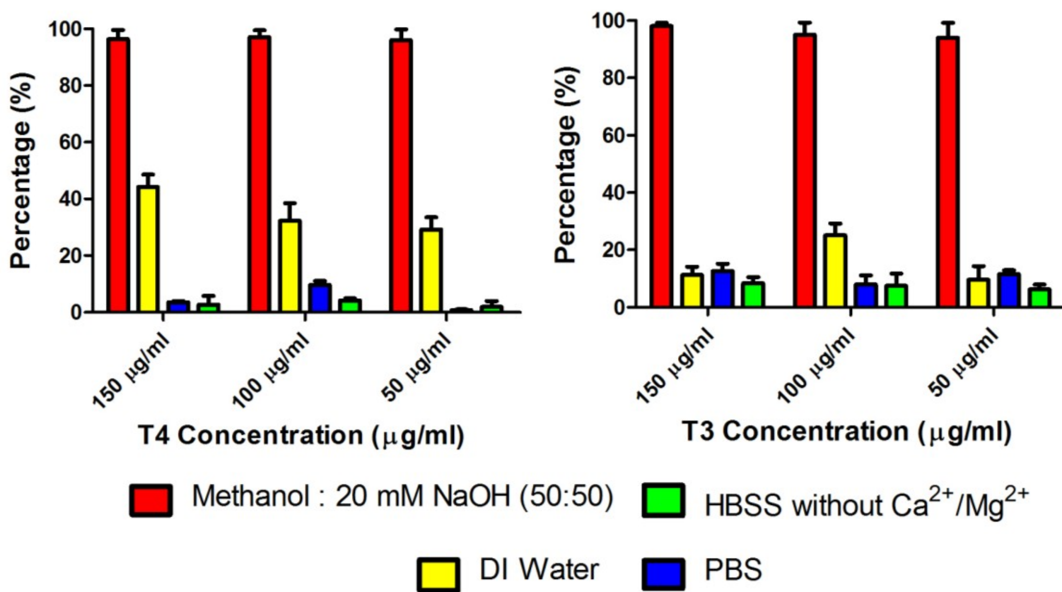


Figure 8 Solubility of T4 and T3 in deionized water, PBS and HBSS without Ca^{2+}/Mg^{2+}. Data represent mean ± SD, n = 3.

Furthermore, the effect of cosolvents (PEG 400, PEG 600, glycerol and polypropylene glycol) on T4 solubility was also investigated. The concentration range that was investigated covered the range recommended by the FDA for respiratory and

transdermal formulations[92]. PEG 400, PEG 600 and glycerol enhanced T4 solubility by 2-fold, 6-fold and 2-fold, respectively (**Figure 9**). However, polypropylene glycol, DMSO, ethanol and Tween 20 did not show any significant effect ($p > 0.05$).

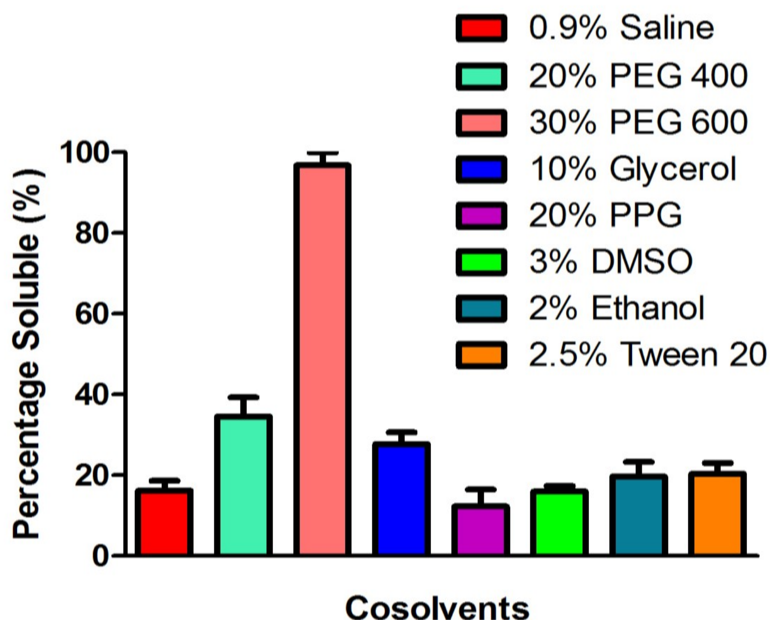


Figure 9 Effect of cosolvents on T4 stability. Vehicle optimization studies were conducted using cosolvents (PEG 400, PEG 600, glycerol, polypropylene glycol, DMSO, ethanol and Tween 20). Data represent mean ± SD, n = 3.

Further experiments were conducted with the cosolvents that resulted in significant solubilization effect (**Figure 10**). The studies were conducted to find the minimum concentration of a particular cosolvent needed to achieve maximum T4 solubility. The maximum percentage T4 solubility achieved in 30 % glycerol, PEG 400 and PEG 600 was 42.07 ± 4.57 %, 73.25 ± 3.62 % and 102.18 ± 4.23 %, respectively. This implies that 30 % PEG 600 had the maximum solubilizing effect. The solubility of T4 in the vehicles was proportional to PEG 600 and PEG 400 concentrations. The data suggest that PEG

600 (30 %) can be used as solubility and absorption enhancer for respiratory and transdermal formulations.

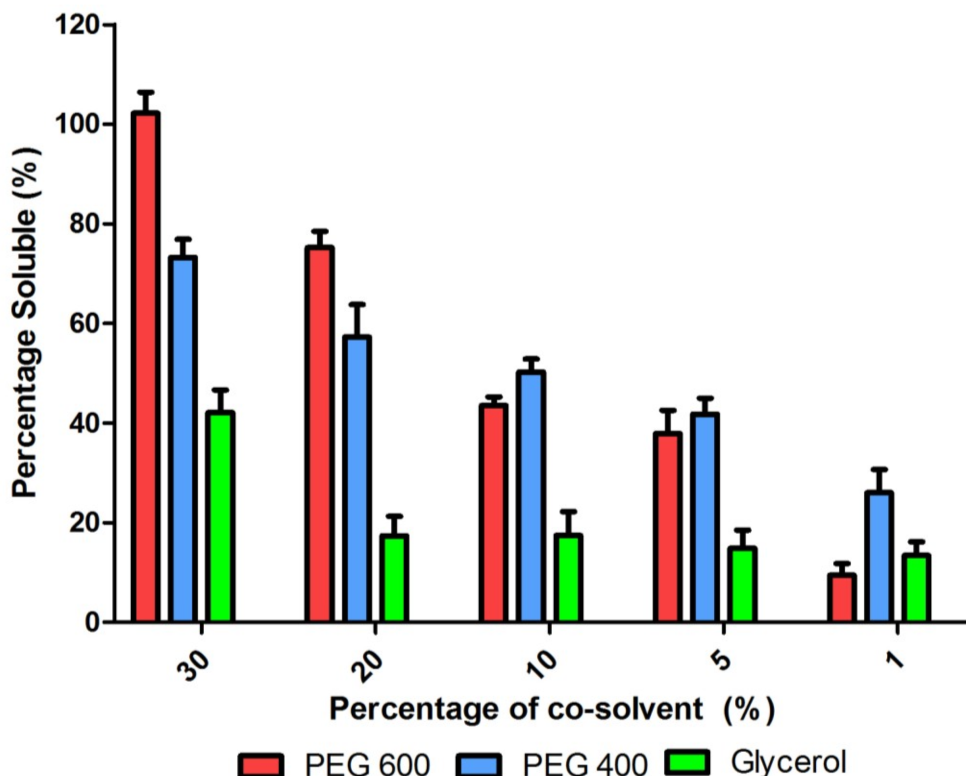


Figure 10 Effect of various cosolvent concentrations on T4 solubility. Data represent mean ± SD, n = 3.

4.3 STABILITY STUDIES

4.3.1 Effect of pH on T4 Stability

Stability of T4 is affected by changes in pH due to presence of three functional groups (carboxylic acid, phenol and amine) that undergo oxidation in the presence of protons. Therefore, it was necessary to optimize the pH of solutions to be used for further studies to prevent T4 degradation. The effect of pH on stability of T4 shown in **Figure 11**

The figure shows the percentage of drug remaining over 2 hours after exposure to five pH conditions (5.0, 6.0, 7.0, 8.0, 9.0). Percentage of drug remaining at pH 9.0, 8.0, 7.0, 6.0 and 5.0 was 91.04 ± 6.24, 82.49 ± 1.64, 66.03 ± 7.37, 27.28 ± 2.39 and 33.67 ± 1.13 %, respectively. Based on the data, it appears that T4 was relatively less stable in acidic environments compared to alkaline pH.

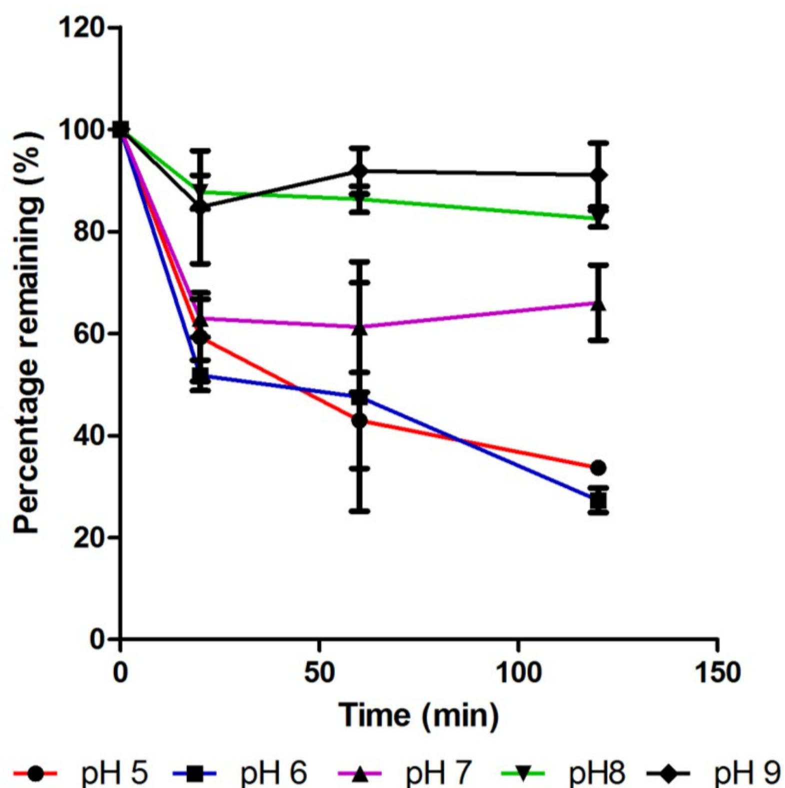


Figure 11 Effect of pH on stability of T4 over a 2-h period. Data represent mean ± SD, n = 3

4.3.2 Effect of Temperature on T4 Stability

The effect of temperature on T4 stability is shown in **Figure 12**. T4 solutions were maintained at 4°C, RT (23.5°C) and 37°C over a 2-h period. The amount of drug

remaining decreased as temperature of the solutions increased. The percentage of the hormone remaining at 4°C, RT (23.5°C) and 37°C, respectively was approximately 99.22 ± 4.30, 93.66 ± 2.14 and 82.49 ± 1.65 %, respectively. The maximum decrease in T4 was about 15 % (37°C).

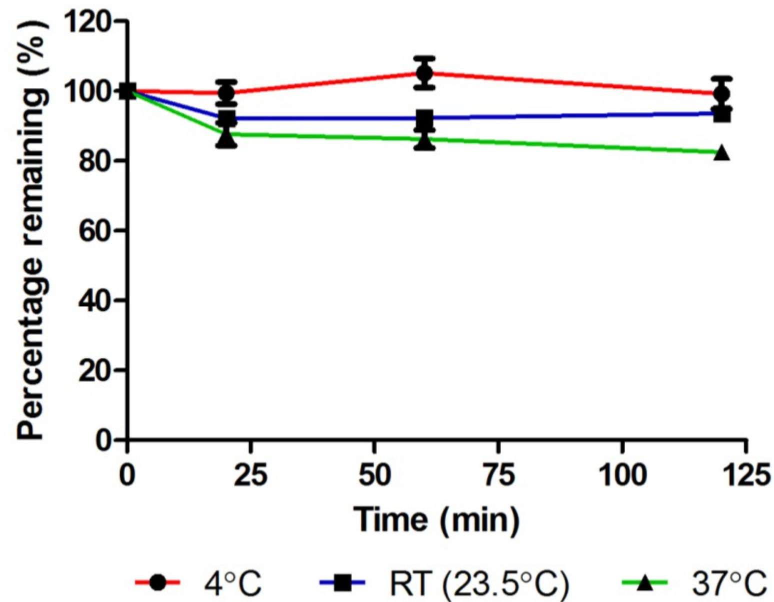


Figure 12 Effect of temperature on stability of T4 over a 2-h period. Data represents mean ± SD, n = 3

4.3.3 Effect of Stability Enhancers on T4 Stability

Potential stability enhancing effects of KI, EDTA and AA are shown in **Figure 13.** The figure shows percentage of T4 remaining in solution over a 2h period in the presence of the additives. For the purpose of the study, solutions were maintained at RT (23.5°C). The presence of KI, EDTA and AA had some stability enhancing effect on T4. The percentage of the hormone remaining in solution was highest in KI+EDTA+AA

(89.61 ± 3.41 %) followed by AA (85.23 ± 2.67 %), KI (80.85 ± 3.21 %) and EDTA (73.67 ± 2.18 %).

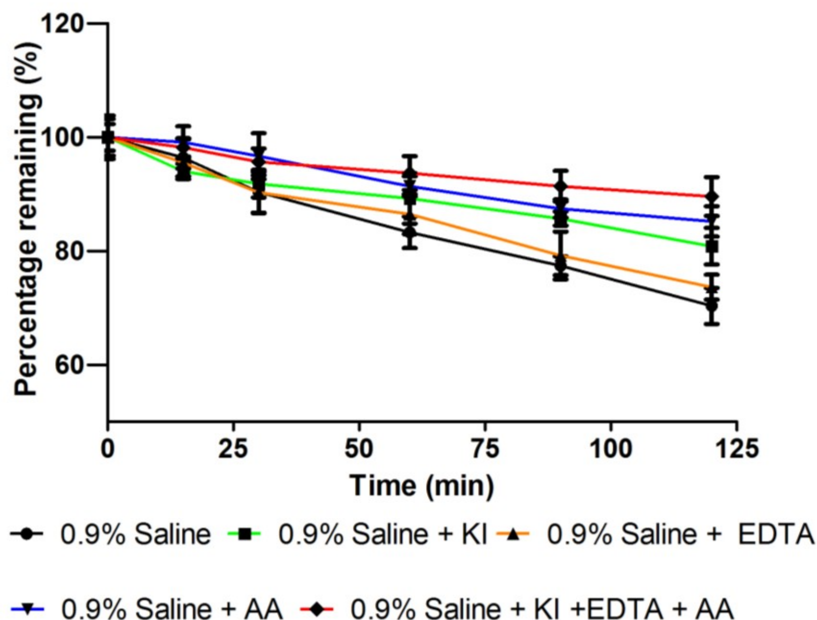


Figure 13 Effect of stability enhancers on degradation of T4 over a 2-h period. Enhancers used were KI, EDTA and AA. Data represents mean ± SD,n=3.

4.3.4 Effect of Glass and Plastic Surfaces on T4 Stability

A study conducted by Frennette et al., (2011)[80] showed that T4 stability is affected by the type of container in which the hormone is stored. Furthermore, lipophilic compounds are non-specifically adsorb to plastic surfaces. It was therefore necessary to investigate the effect of glass and plastic on T4 stability. The effect of glass (class1 Type A borosilicate glass) and plastic (polystyrene) plates on T4 stability were tested. Percentage of T4 remaining in solutions over a period of 2 hours is shown in **Figure 14**. The quantity remaining in solution at each sampling point was found to be similar.

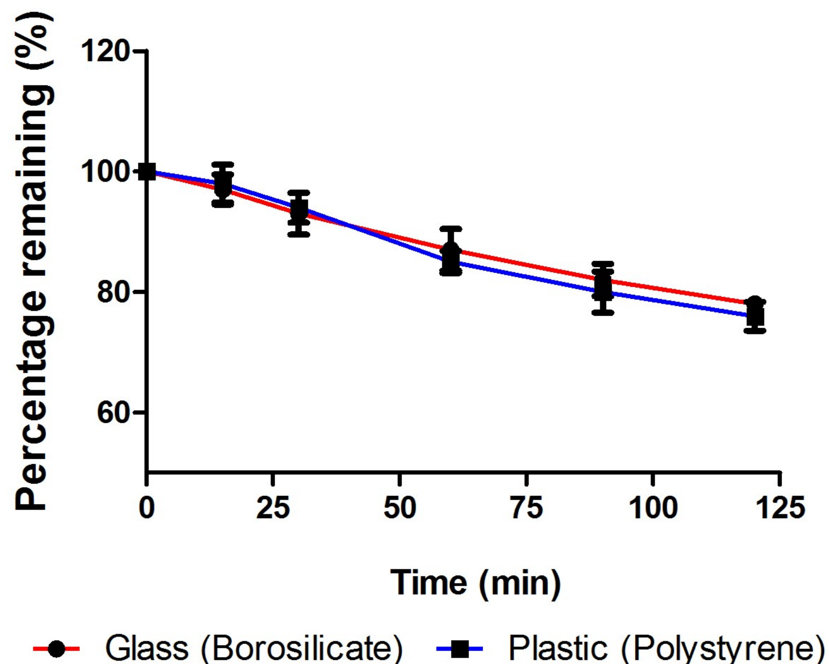


Figure 14 Effect of glass and plastic on T4stability. Data represents mean ± SD, n = 3. No statistically significant difference was seen between glass and plastic (P > 0.05).

4.4 RESPIRATORY STUDIES

4.4.1 Epithelial Permeation Studies

Calu-3 cells were used to investigate the permeation of T4 across respiratory cells. **Figure 15** represents the cumulative amount of T4 transported across Calu-3 epithelial cell membrane. The mean cumulative amount of the drug transported in 3 hours was about 3 times higher in the BL-to-AP compared to AP-to-BL (**Figure 15A**). Correspondingly, the apparent permeability (P_{app}) of the drug for the BL-to-AP (43.45 ± 5.29 X 10^{-6} cm/s) was higher than that observed for the AP-to-BL direction (19.76 ± 4.89 X 10^{-6} cm/s) (**Figure 15B**)

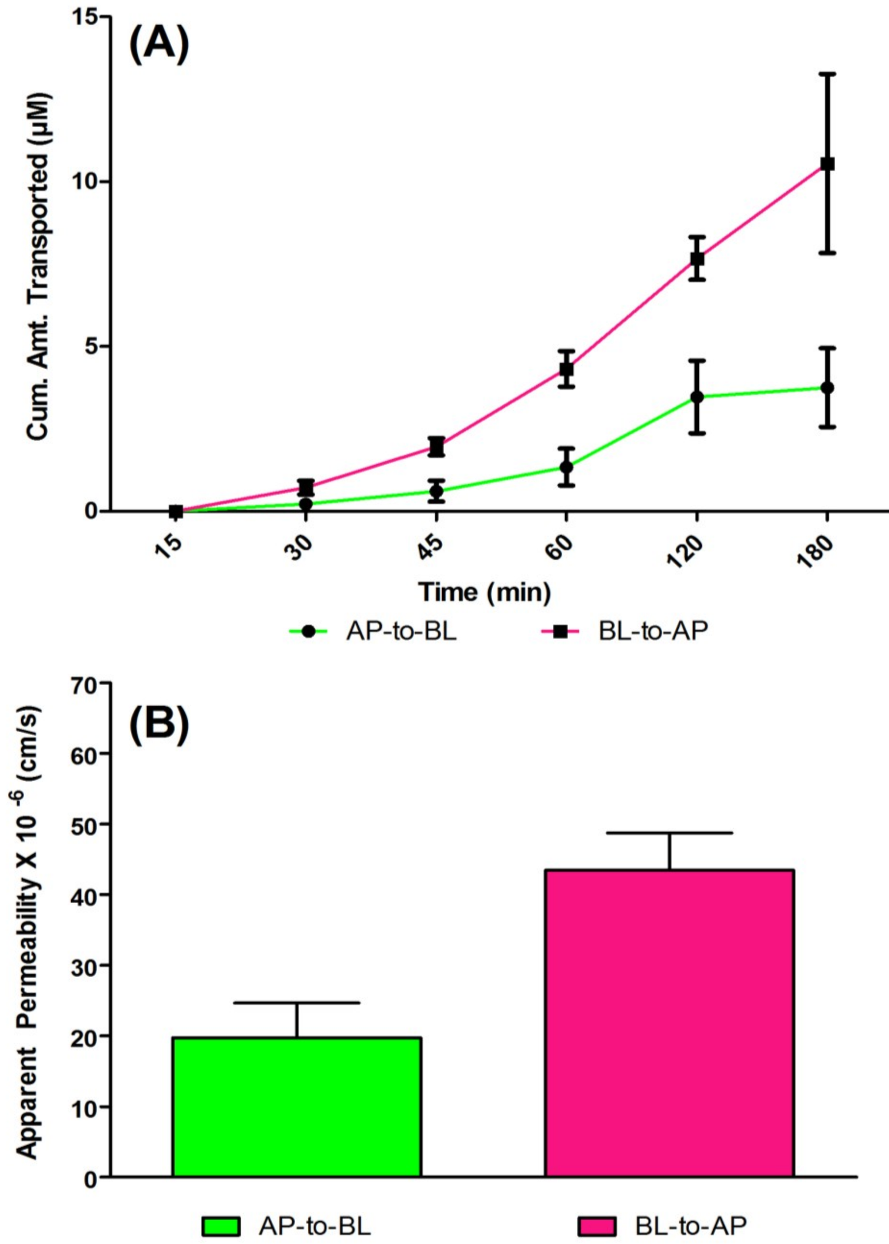


Figure 15 Cumulative amount of T4 transported across Calu-3 cells (A) and corresponding apparent permeability coefficient (B). Data represent mean ± SD, n = 3.

The apparent permeability coefficients indicate that significant amount of T4 passed through the cells. In order to understand the mechanisms involved in its transport, we investigated the effect of pH, temperature and concentration on T4 transport. **Figure 16** shows the effect of sodium ion on T4 transport. The mean cumulative amount transported in 3 hours from the BL-to-AP direction (33.86 ± 6.43 μM) was 5 times higher than that in AP-to-BL direction (6.85 ± 1.89 μM) in the absence of sodium. Correspondingly, the mean P_{app} of the compound was considerably higher in the BL-to-AP direction compared to that observed in the AP-to-BL direction in both studies with and without sodium. We observed a 2-fold decrease in T4 P_{app} values in the AP-to-BL direction compared to transport of the compound in the presence of sodium. Absence of sodium, however had no effect on the BL-to-AP transport and P_{app} obtained were found to be comparable to those obtained in the presence of sodium.

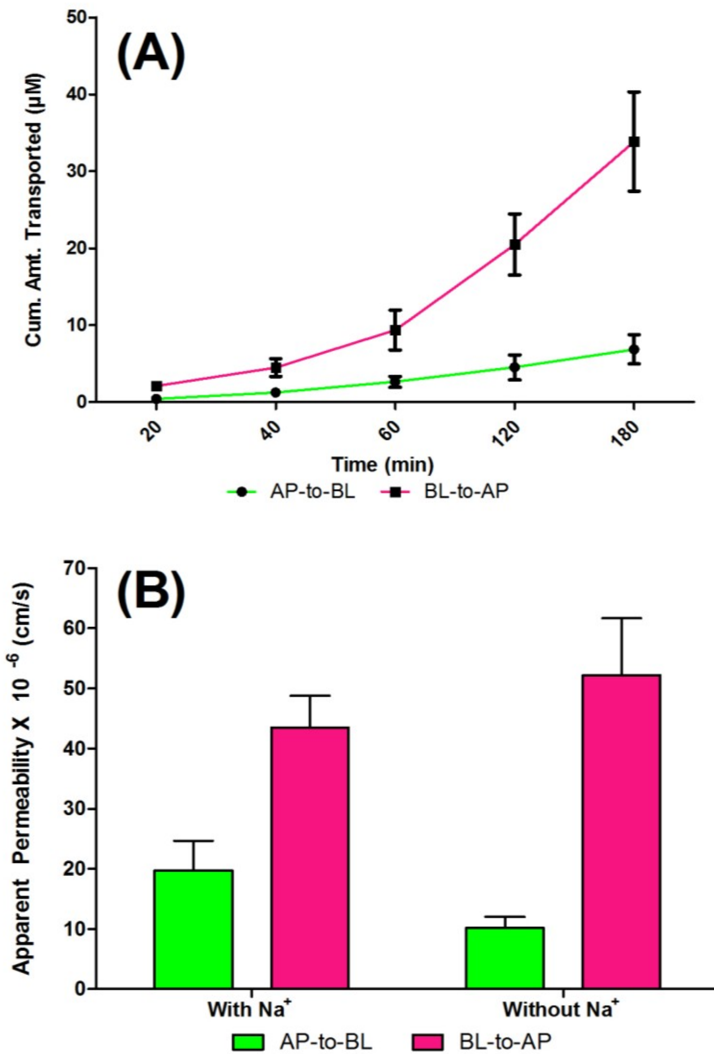


Figure 16 Cumulative transport of T4 with and without sodium (A) and corresponding permeability coefficient (B). Data represent mean ± SD, n = 3.

Figure 17 shows the effect of temperature on T4 transport through the Calu-3 cells. The mean cumulative amount transported over a three-hour period in the BL-to-AP direction (1.19 ± 0.43 µM) was higher than the amount transported in the AP-to-BL direction (0.90 ± 0.53 µM). However, when compared to the mean cumulative amount transported at 37°C there was a 9-fold decrease in the amount transported in the BL-to-AP direction and a 4-fold decrease in amount transported in the AP-to-BL direction. Also

as seen in **Figure 17** and **Table 6** there was a considerable decrease in transported amounts at 4°C in both directions (6- and 10-fold decrease in P_{app} was observed in the AP-to-BL and BL-to-AP direction, respectively).

Figure 18 shows the effect of T4 concentration (25-100 μM) on transport across the Calu-3 cells. The cumulative amount of the hormone transported increased as the concentrations increased in both AP-to-BL and BL-to-AP directions. The amount of T4 transported was higher in BL-to-AP compared to AP-to-BL direction. Within the concentration range that was investigated, the P_{app} of T4 did not vary with increasing concentrations in both AP-to-BL and BL-to-AP directions.

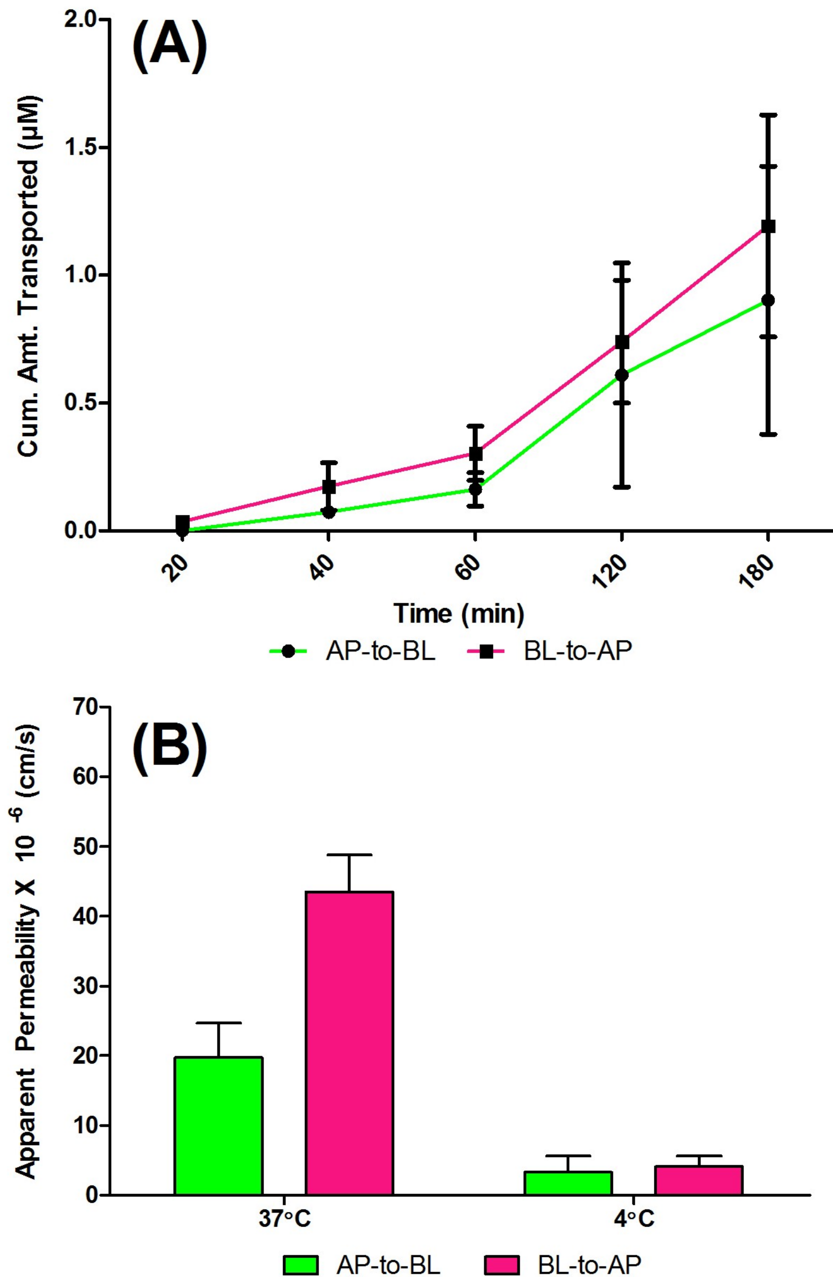


Figure 17 Effect of temperature on cumulative transport (A) and permeability coefficients (B) of T4 transport across Calu-3 cells. Data represent mean ± SD, n = 3.

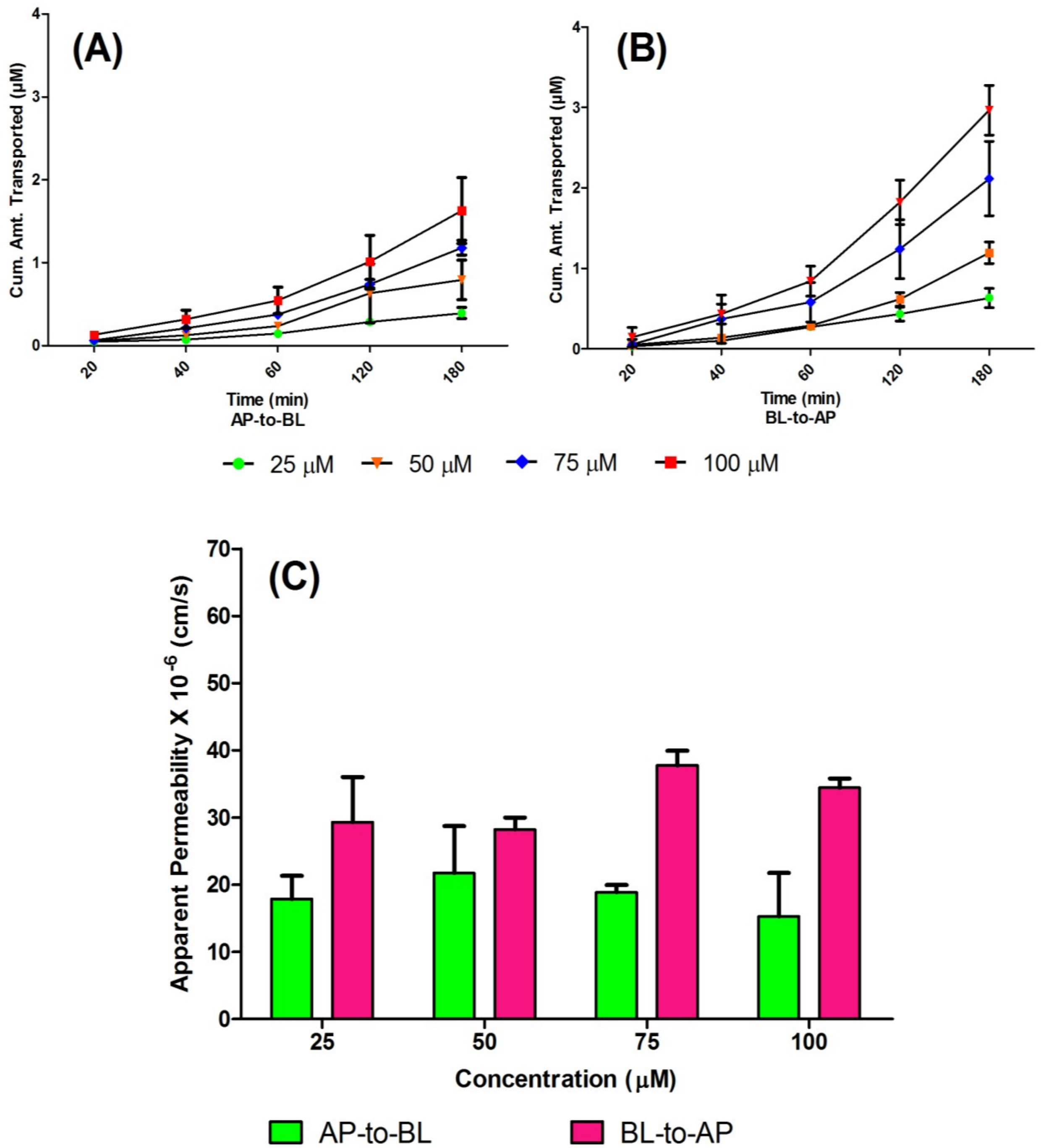


Figure 18 Effect of concentration on cumulative transport of T4 in apical to basolateral direction (A), basolateral to apical direction (B) and corresponding apparent permeability coefficients (C). Data represent mean ± SD, n = 3.

Following oral administration, the absorption of T4 occurs in the jejunum and upper ileum. The pH of these areas are relatively alkaline (6-7.4)[72] compared to the stomach (pH 1-3). It was also important to investigate if changes in pH affected T4 transport across the respiratory cells. The pH of the donor solution was kept at 8.0 to maintain T4 solubility and stability. Thus, only the pH of the receiver solution was changed. **Figure 19** shows the effect of receiver solution pH on the cumulative amount of T4 transported and P_{app} across the Calu-3 cell monolayer. Cumulative amount transported was higher in the AP-to-BL direction in the receiver solution of pH 7.4 ($143.09 \pm 10.00 \times 10^{-2}$ μM) compared to the solution with pH of 5 ($81.68 \pm 2.13 \times 10^{-2}$ μM). Cumulative amount transported was found to be similar in the BL-to-AP direction at both pH 7.4 and pH 5.5. Although P_{app} in the AP-to-BL direction was not affected by the changes in pH, there was a significant decrease in the BL-to-AP direction when pH of the receiver solution was changed.

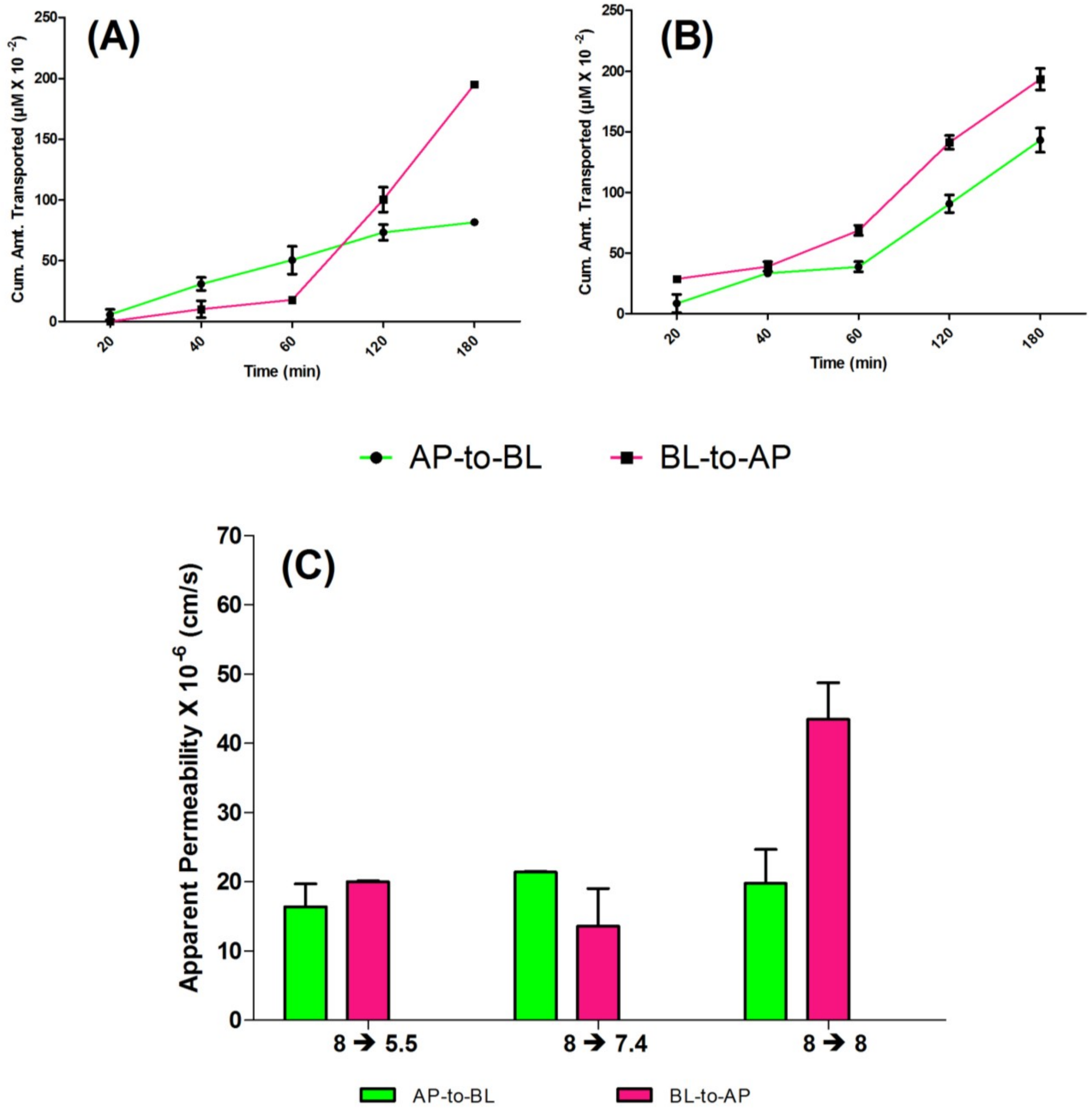


Figure 19 Effect of pH on T4 cumulative transport at receiver pH 5.5 (A) and 7.4 (B), and corresponding permeability coefficients (C) across Calu-3 cells. Data represent mean ± SD, n = 3.

4.4.2 RT-PCR Studies

Functional transport data (polarity of transport, effect of sodium and temperature) suggested the involvement of active transport processes in T4 transport.To confirm this observation, we investigated the expression of T4 transporters in the Calu-3 cells using PCR. Transcripts for MCT8 (150bp), MCT 10 (77bp), OATP1A2 (87bp), OATP4A (70bp), LAT-1 (73bp), LAT-2 (87bp) and CD98 (73bp) were observed (**Figure 20**). There was no genomic DNA contamination in the transcription process as no band was detected for the control (transcribed RNA template) in lane 1. Based on the intensity of the bands, LAT-1, LAT-2 and CD-98 appeared to have lower expression than MCT8, MCT10, OATP1A2 and OATP 4A. Amongst the thyroid hormone transporters, MCT8 seems to have the highest expression level in the Calu-3 cells. Quantitative PCR will be needed to confirm this observation.

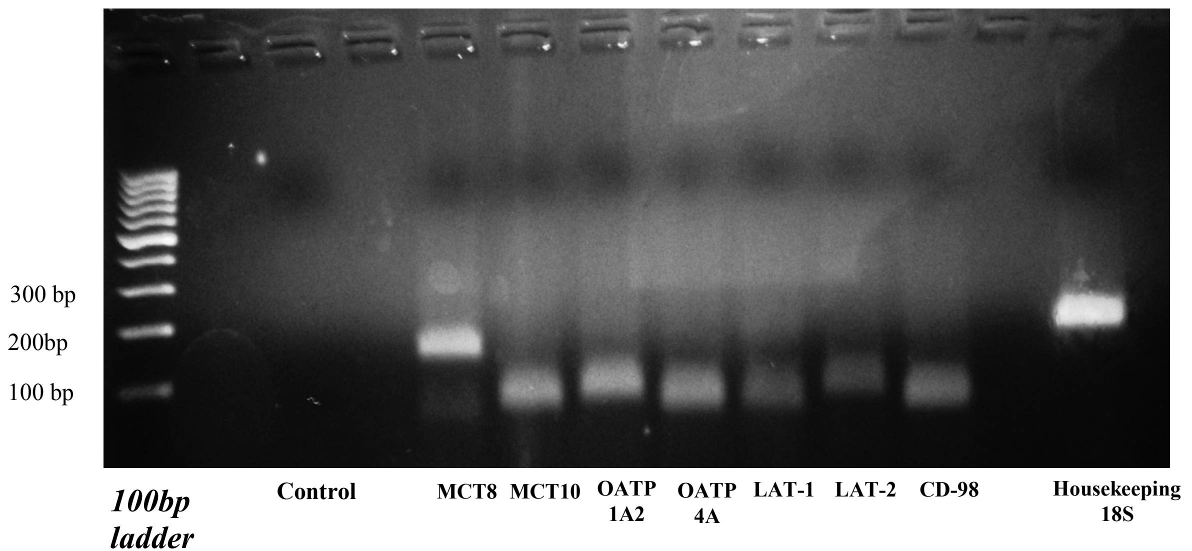


Figure 20 Qualitative mRNA expression of thyroid hormone transporters in Calu-3 cells. Bands depict expected transcripts as follows- MCT8 (150 bp), MCT 10 (77 bp), OATP1A2 (87 bp), OATP4A (70 bp), LAT-1 (73 bp), LAT-2 (87 bp), CD98 (73 bp) and housekeeping 18S (187 bp).

4.4.3 Toxicity Studies: Transepithelial Electrical Resistance and MTT studies

Toxicity studies were conducted using 0.9 % saline (with 25mM Glucose, 10mM HEPES, 30 µM KI, 17 µM EDTA and 15µM AA), T4 (100 µg/ml, 50 µg/ml), glycerol (1 %, 5 %, 10 %), PEG 600 (1 %, 5 %, 10 %) and polypropylene glycol (1 %, 5 %, 10 %). This study was conducted to find out if the cosolvents caused any significant toxicity on the cells via tight junctions opening and cytotoxicity. Effect of buffered saline (0.9 % saline) and T4 on TEER were also tested to know if the vehicle and hormone caused any toxicity to the cells. The effect of these solutions on TEER over a period of 3 hours was investigated **(Figure 21).** A decrease of 80 % was seen in TEER from initial readings in wells containing 10 % polypropylene glycol. Lower concentration of polypropylene glycol (1 %) showed a 50 % decrease in TEER. Similar results were observed at higher concentration of glycerol (10 %), which also showed an 80 % decrease in TEER. The percentage decrease in TEER for polypropylene glycol and glycerol was directly proportional to the concentration of cosolvent in the solution. Solutions containing 0.9 % saline, T4 and PEG 600 showed a decrease of about 50 % in TEER readings over the period. This implies that even though 0.9 % saline and T4 showed an inherent 50 % decrease in TEER, 10 % PEG 600 did not show any significant toxicity as TEER decrease was comparable to that obtained without PEG 600.

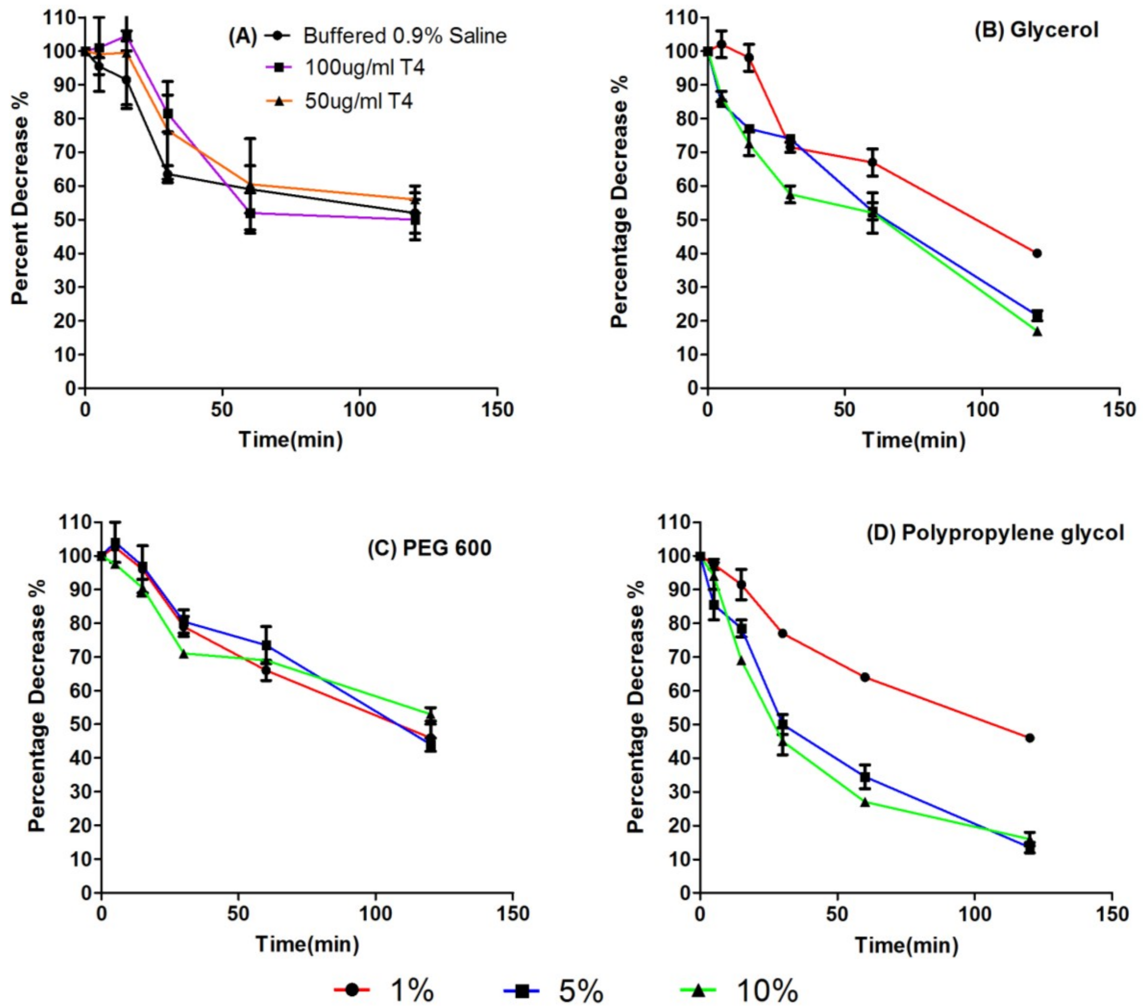


Figure 21 Concentration-dependent effect of solubility enhancers on TEER. Data represent mean ± SD, n = 3

Although TEER studies showed a decrease in TEER in the presence of each solvent tested, we wanted to use the MTT assay to know if there was any significant effect on cell viability and death. Results obtained from this study are shown in **Figure 22**. Calu-3 cells incubated with HBSS with Ca^{2+} and Mg^{2+} was used as control. The MDH activity observed in the presence of these solvents was not statistically significant ($p>0.05$) from the control (HBSS). This indicates that TEER decrease was not associated with similar loss in cell viability. However, higher percentages of polypropylene glycol

(5 %, 10 %) and glycerol (5 %, 10 %) showed a significant decrease in TEER which corresponds to the TEER decrease observed in the presence of these solvents.

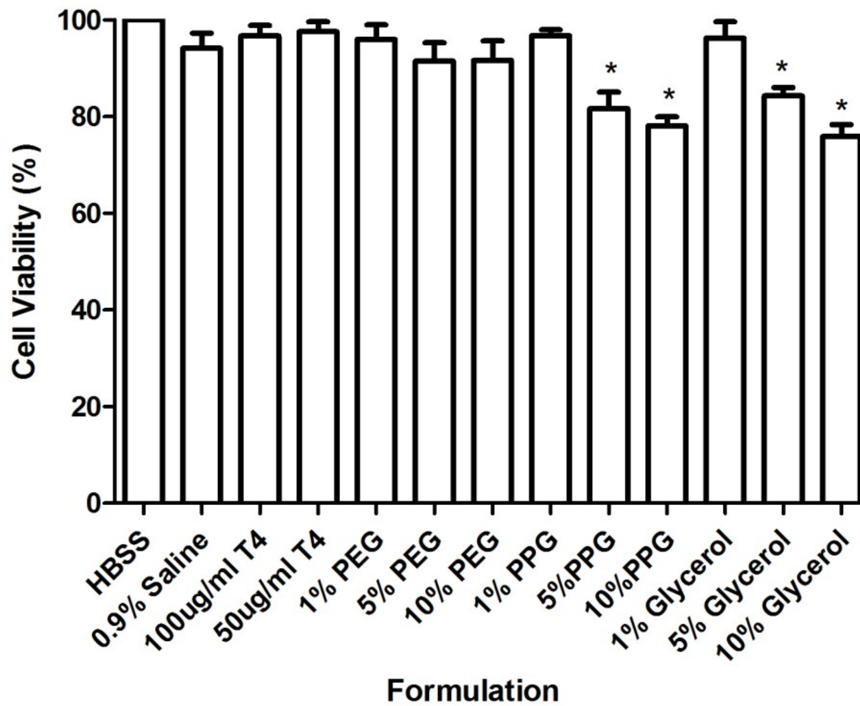


Figure 22 Effect of different cosolvents on viability of Calu-3 cells. Data represent mean ± SD, n = 3. PEG, polyethylene glycol; PPG, polypropylene glycol, *significantly different from control ($P < 0.05$)

4.5 TRANSDERMAL STUDIES

4.5.1 Strat-M® Validation

The cumulative amount of caffeine that permeated through Strat-M® (μg/cm^2) per hour is shown in **Figure 23**. The mean maximum absorption rate obtained was 6.58 ± 0.86 μg/cm^2-hr. The time to maximal rate was 2.06 ± 0.92 h. Within 1 hour caffeine started to permeate through Strat-M®. Beyond 4 h, caffeine was found to permeate Strat-M® at a fairly constant rate upto 24 h.

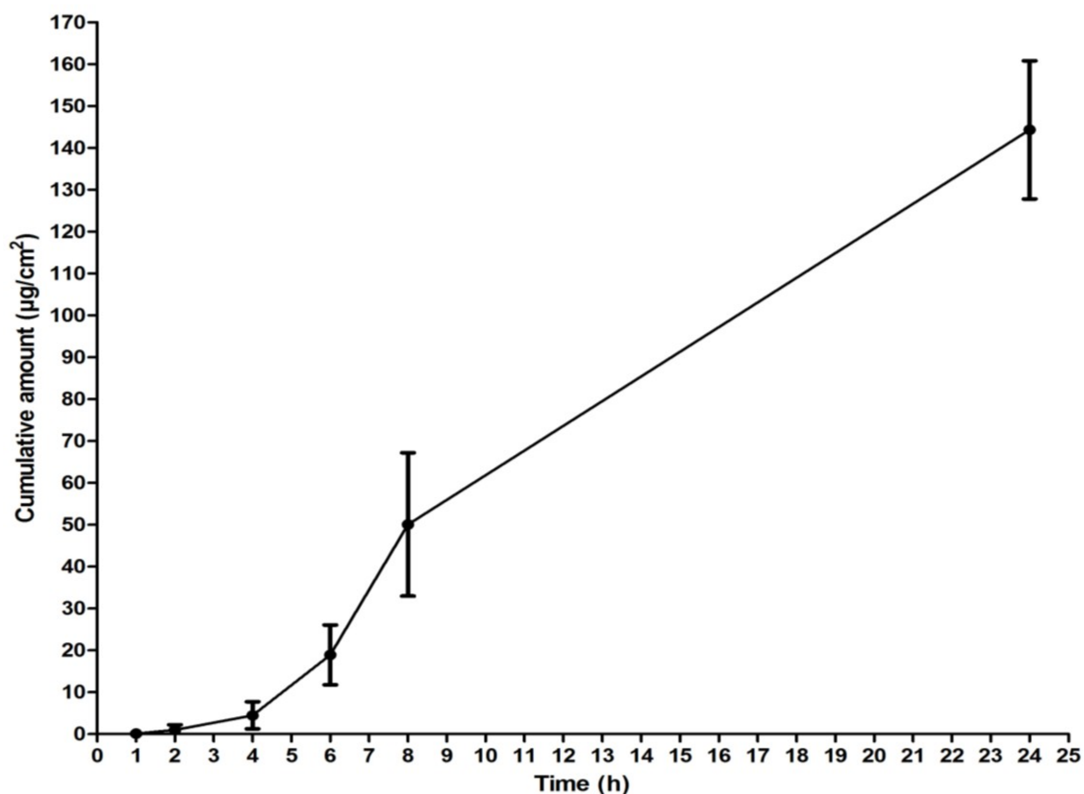


Figure 23 Cumulative amount of caffeine that passed through Strat-M® (μg/cm^2) vs. Time (h). Data represent mean ± SD, n = 3

4.5.2 Transdermal Levothyroxine Permeability Studies

Permeation of T4 across Strat-M® membrane was conducted using buffered 0.9 % saline as donor and acceptor solutions. pH of the solutions was maintained at 8.0. No transport of T4 was observed over 24 hours across Strat-M® membrane (**Figure 24**). Initial and 24 h donor concentrations were also measured to estimate if T4 remained stable over 24 h at 37°C. Donor concentrations were also estimated to compare the amount of T4 that passed through the Strat-M® membrane. It was observed that 75% of T4 was still remaining in the donor solution after 24h, which proved that the T4 was available for absorption during the permeation study. As no permeation was observed in 0.9% saline alone, transdermal absorption enhancers (PEG 600, polypropylene glycol and TPGS) were added to to the donor solution at various concentrations. Nevertheless, no T4 permeation was observed over 24 h (**Figure 25**). However, as indicated in **Figure 25** about 80% of T4 was still remaining in all three donor solutions containing absorption enhancers over 24h. This proved that T4 was available for absorption during the permeation study.

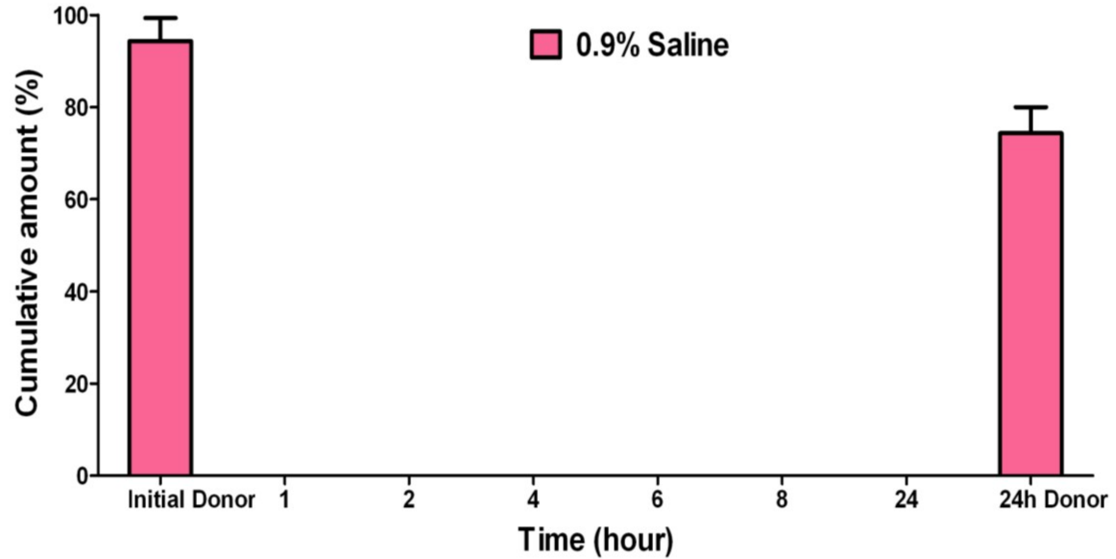


Figure 24 Cumulative amount (%) of levothyroxine that passed through Strat-M® (μg/cm^2) over a 24 hour period. Data represent mean ± SD, n = 3

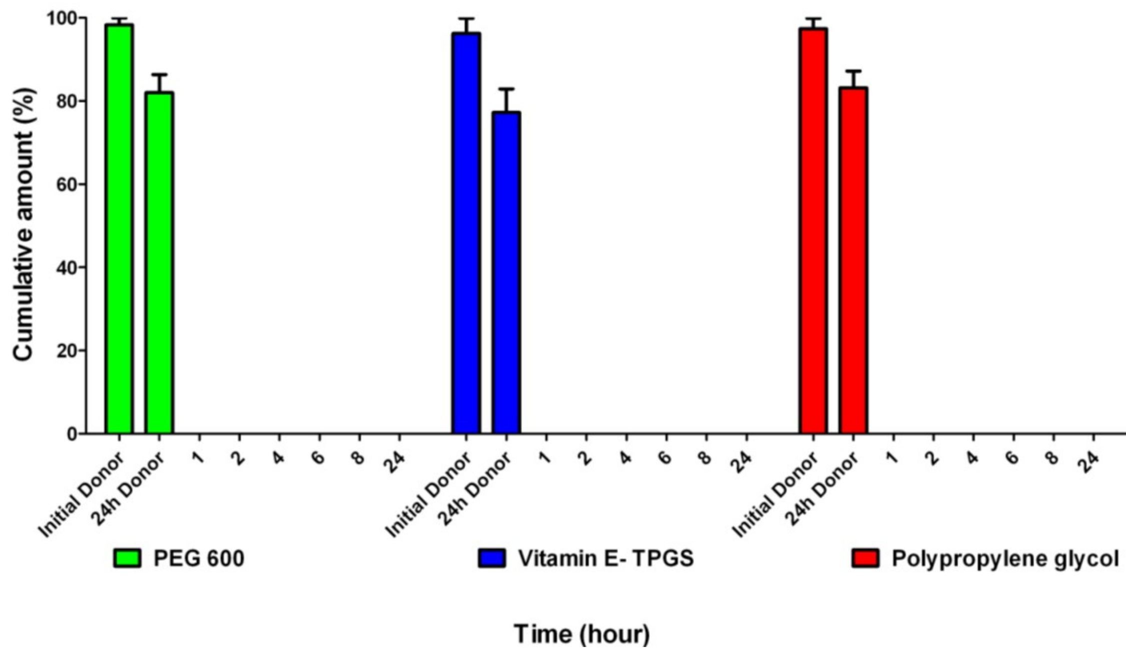


Figure 25 Effect of absorption enhancers (PEG 600, Vitamin E-TPGS, Polypropylene glycol) on cumulative amount of levothyroxine that passed through Strat-M® (μg/cm^2) over a 24 hour period. Data represent mean ± SD, n = 3.

CHAPTER 5 DISCUSSION

The aim of the studies described in this thesis was to conduct a proof-of-principles studies necessary for the possible development of respiratory and transdermal non-invasive delivery systems for T4. T4 is poorly soluble in water with a log *P* of approximately 3.51, which implies that the compound is lipophilic. Structurally, T4 is amphiphilic due to the presence of both hydrophobic and hydrophilic moieties[15].The compound has a moderate size (776.87 g mol^{-1}) and three pKAs. These characteristics make the compound challenging to formulate in a dosage form from solubility and stability perspectives, especially in aqueous environments.

5.1 ANALYTICAL METHOD DEVELOPMENT AND VALIDATION

Based on the non-polar nature of T4 and T3, reversed-phase liquid chromatography was chosen for the detection and quantitation of the compounds. The resolution factor obtained for T4 and T3 was 2.35, which was greater than the acceptable resolution factor of 1.5 for baseline separation between two peaks[73]. Repeatability and interday studies designed to detect the precision of the method confirmed the reproducibility of the assay method (CV < 5 % for intraday and interday precision studies for both T4 and T3). Furthermore, the HPLC method was accurate as the percentage recovery of T4 and T3 was within 95 % - 105 % range (acceptable range for early drug development). Accuracy studies were conducted to measure the closeness of agreement between the sample values obtained and the true accepted values. The relative standard deviation was less than 2 %, which was also within the acceptance criteria limits for

accuracy studies[74]. The fact that the analytical method was accurate and precise for the detection and quantification of both T4 and T3, subsequent studies were conducted with the assurance that sample detection and quantification would not negatively affect the experimental data.

5.2 SOLUBILITY AND FORMULATION STUDIES

Although T4 is amphipathic, it is sparingly soluble in aqueous solutions. Solubility studies were very important for not only selecting the solvents that were used in this study, but for screening solvents for future formulation development for respiratory and transdermal delivery systems. In studies conducted to optimize the vehicles for *invitro* biopharmaceutical studies, we found that T4 interacted with HBSS with calcium and magnesium, as well as with colorless DMEM F-12 and PBS to form insoluble complexes that precipitated out of the solutions. Formation of precipitates in HBSS with calcium and magnesium, and DMEM- F12 could be explained based on the presence of divalent ions such as Ca^{2+} and Mg^{2+} ions in the media[75,76]. Based on these studies, deionized water, PBS and HBSS without Ca^{2+} and Mg^{2+} were chosen as media for solubilising T4. Subsequently, additional solubility studies were conducted to determine if T4 was more soluble in these vehicles or solvents devoid of divalent cations. **Figure 8** shows that the solubility of T4 was higher in deionized water compared to PBS and HBSS without Ca^{2+} and Mg^{2+}. Low solubility in PBS and HBSS without Ca^{2+} and Mg^{2+} may be ascribed to the fact that T4 can exist as a zwitterions, which can cause the amino group to extract a proton and form a positive ammonium cation (NH_3^+). The NH_3^+

can react with phosphate, dihydrogen phosphate and bicarbonate anions present in PBS and HBSS without Ca^{2+} and Mg^{2+} resulting in lower solubility. As all these functional groups are bulky, there is a possibility that this interaction might reduce the solubility of T4 in these solutions. Therefore, for *in vitro* biopharmaceutical studies with Calu-3 cells, the only solvent we were left to work with was water. Because of water's high osmotic pressure and low buffering capacity, we supplemented it with 0.9 % NaCl and 10 mM HEPES buffer to form buffered-physiological saline. This solvent served as our control solvent for formulation, stability, permeation and toxicity studies.

Formulation studies were conducted to optimize the cosolvent systems necessary to increase the solubility of T4 to enable respiratory and transdermal formulations development. PEG 600, PEG 400, polypropylene glycol, glycerol, DMSO, ethanol and Tween 20 were tested at the maximum concentrations recommended by the FDA[92] (**Figure 9**). PEG 600 resulted in the highest solubility followed by PEG 400, polypropylene glycol and glycerol in decreasing rank order. DMSO, ethanol and Tween 20 did not seem to have any effect on T4 solubility. Increased solubility in PEG can be attributed to the fact that PEGs contain both hydrophilic and hydrophobic moieties. Because of the lipophilic nature of T4, it may be dissolved in lipophilic regions of PEGs. Furthermore, the solubility of T4 in PEG 600 was higher than PEG 400 because as the PEG molecular weight increases, its lipophilic nature increases as well[77]. Lower solubility in polypropylene glycol could be explained by the fact that polypropylene glycol is hydrophilic at room temperature, thus T4 show slow affinity towards the excipient[78]. Similarly, glycerol is a hydrophilic compound based on the presence of three OH- groups in its chemical structure, which explains T4's relatively lower solubility

compared to the PEGs in the solvent. Although the PEGs solubilized T4, it was important to optimize the concentrations of the cosolvents to achieve maximum aqueous solubility enhancement. As expected, higher concentrations of PEGs resulted in higher T4 solubility. As discussed previously, the molecular weight of PEGs is proportional to their lipophilicity. So at equal concentration, the solubility of T4 was higher in PEG 600 than in PEG 400. Unlike the PEGs, the quantity of glycerol used had no effect on T4 solubility, which was consistent with the hydrophilic nature of glycerol. Ethanol did not show any solubility enhancing effect, an observation that was in line with previous studies that indicated T4 has poor solubility in ethanol[79].

5.3 STABILITY STUDIES

Considering the fact that *in vitro* drug absorption studies using cell culture models typically last 2-3h, it was important to ascertain the stability of T4 over this period of time under various experimental conditions (pH, temperature, environment, formulation additives). Furthermore, future development of aqueous liquid formulation dictates that information on stability of the compound in aqueous environments be provided.

A study by Frenette *et al*. (2011)[80] showed that container materials used for storing levothyroxine sodium injection had an effect on its stability. The compound was more stable in glass and polyolefin bags, but when it was stored in polyvinyl chloride (PVC) containers it degraded by 10 % in the first hour. Furthermore, many studies have shown that lipophilic drugs have a tendency to adsorb to plastic by non-specific binding

interactions[81,82,83]. As T4 is lipophilic we wanted to see if the materials used for storing and handling the compound in our lab (borosilicate glass and polystyrene tissue culture plastic) had any effect on its stability. Interestingly, at the end of the study, no statistically significant sample loss was observed ($P > 0.05$) (**Figure 14**). So throughout the project, samples of T4 were handled in borosilicate glass and polystyrene plastic.

As shown in **Figure 11**, T4 was more stable in basic (pH 8.0 and pH 9.0) than acidic conditions (pH 5.0, pH 6.0 and pH 7.0). This observation maybe attributed to the fact that T4 has three ionisable groups and can exist as a cation, zwitterion, anion and dianion, depending on the pH of the solution. The pKa of the carboxyl, phenolic and amino group are 2.2, 6.7 and 10.1, respectively. Won (1992)[35] proposed that deiodination at acidic pH takes place due to proton attack on the anion and dianion and due to water attack at alkaline pH on the anion and dianion. As proton is a stronger electrophile than water, degradation is higher at acidic pH compared to alkaline pH. Our results seem to be consistent with published data on aqueous stability of T4[35,84].

Although T4 was more stable at pH 9.0 compared to pH 8.0, there is a possibility of the amino group ionizing in solution at pH higher than 9.0. Ionization, limits the ability of the hormone to diffuse through cell membranes. Unionized drugs are generally more lipid soluble and thus are able to pass through the lipid bilayer of cell membranes. Based on our stability data and ionization potential at pH above 9.0 we kept the pH of our working solutions at 8.0. At this pH we were able to strike a balance between solubility, ionization, stability and permeability. Furthermore, pH 8.0 is closer to the physiologically relevant pH of 7.4 and many commercially available formulations are buffered at this pH.

Degradation of T4 was directly proportional to temperature. As the temperature increased the amount of drug remaining in solution decreased. The maximum percentage decrease was 15 % which occurred at 37°C (**Figure 12).** Results obtained from this study are in line with those obtained by Won (1992)[35] who found that T4 underwent first order degradation kinetics and the possible mechanism of degradation at elevated temperatures was deamination. Stability studies at various temperatures helped us establish the storage and handling temperatures for T4 solutions. This information is of paramount importance for future respiratory and transdermal formulation development.

An attempt was made to reduce or eliminate T4 degradation in aqueous solutions. The major mechanisms of T4 degradation is deiodination and oxidation[35]. We therefore investigated the effect of additives on T4 stability. Ascorbic acid, KI and EDTA were used as antioxidant, iodide donor and chelating agent, respectively. Data from these studies showed that these compounds offered varying degrees of protection as follows: ascorbic acid > potassium iodide > EDTA (**Figure 13**). As oxidation is a major degradation pathway for T4, protective effect of ascorbic acid was observed to be higher. While T4 formulation without any protectant was the least stable, a combination of the excipients had a synergistic effect. This might be explained by the fact that the compounds acted through different mechanisms. Based on our stability enhancement data, subsequent studies were conducted with solutions augmented with a combination of potassium iodide, EDTA and ascorbic acid.

5.4 IN VITRO RESPIRATORY STUDIES

Using *in vitro* tissue culture models, it is possible to determine drug flux and permeability coefficients. These parameters give an idea of the rate and extent of drug absorption *in vivo*. *In vitro* models are also very useful for determining mechanisms of drug permeation, toxicity and absorption enhancement strategies. In this thesis Calu-3 cells were used to investigate T4 permeation across the respiratory epithelium, toxicity of T4 formulation excipients and T4 absorption mechanisms. The apparent permeability (P_{app}) of T4 transport through Calu-3 cells that was observed in both the AP-to-BL and BL-to-AP directions is listed in **Table 6**. Initial transport studies were conducted to find out if T4 passed through Calu-3 cells. The permeability coefficient in the BL-to-AP direction was higher than AP-to-BL direction (**Figure 15).** Usually cells actively take up T4 from the blood to be deiodinated to T3, the active hormone that regulates basal metabolism in the body. The uptake takes place via thyroid hormone transporters located on the serosal side of the cells. Thus higher BL-to-AP transport relative to AP-to-BL direction means that respiratory cells actively concentrate T4 from the blood. The functions of the apically located transporters are not yet known. However, transporters such as OATP 2 mediate bidirectional transport of organic anions[85]. Thyroid hormone transport was seen to decrease in rat skeletal muscle and kidney in the presence of ouabain, a specific inhibitor of sodium and potassium transport[86]. Thus, we wanted to see if absence of sodium affects T4 transport in Calu-3 cells. Based on the data obtained there was a 2-fold decrease in AP-to-BL T4 transport. However, absence of sodium had no effect on the BL-to-AP transport (**Figure 16**). The slight decrease in the AP-to-BL transport may be due to the fact that the thyroid hormone transporters expressed on the

basolateral membrane may be sodium independent. Recently it has been postulated that some thyroid hormone transporters such as Na^+/taurocholate cotransporting polypeptide (NTCP) and apical Na^+-dependent bile acid transporter (ASBT) could be dependent on sodium for transport of T4 across the cell membrane[90]. MCT 8 transporters have also been found to sodium-dependently transport T4[87]. Decrease in temperature is known to decrease transport of molecules across cell membranes[88,89]. Low temperature corresponds to low energy, which results in a decrease in the rate at which molecules pass through the cell membrane. A decrease in T4 transport at 4°C was a classical indication that the compound was transported actively across the Calu-3 cells (**Figure 17**). To further evaluate the mechanisms of T4 transport, we evaluated the impact of concentration (25 to 100 μM) on its permeation (**Figure 18**). Data obtained from our studies indicated that within the investigated concentration range, T4 transport was non-saturable. This observation was not surprising as we could not investigate higher concentration because of poor solubility of the compound. It is likely that at higher concentrations the T4 transporters become saturated.

5.4.1 PCR Studies

Drug transport across biological membranes takes place mainly via three mechanisms: facilitated diffusion or active transport, passive transcellular transport and passive paracellular transport. Functional transport studies suggested that T4 permeated the Calu-3cell membranes via active transport systems. Currently, no information exists on the presence and localization of thyroid hormone transporters in human respiratory airway membranes. It was necessary to confirm the presence of thyroid hormone transporters[90,91] {monocarboxylate transporters (MCT-8 and MCT-10), organic anion

transporting polypeptides (OATP4A, OATP1A2) and large amino acid transporters (LAT1, LAT-2 and CD-98)} in the respiratory epithelium. These transporters were found to exist in the Calu-3 cells (**Figure 20**). This finding is of great significance as the expression of thyroid hormone transporters in Calu-3 cells implies that systemic delivery of this compound is possible via this route.

5.4.2 Toxicity Studies

An important application of the Calu-3 cells in this project was for toxicity screening. **Figure 21** and **Figure 22** show the effect of different cosolvents on mucosal toxicity using TEER decrease and formazan crystals formation after exposure to cosolvents as toxicity indices. Considering that all the cosolvent concentrations used were within the concentration ranges recommended by the FDA for respiratory delivery and the fact that buffered saline decreased the TEER significantly[92], the data points to the fact that *in vitro* models sometimes exaggerate toxicity results. Significant TEER decrease does not necessarily imply irreversible epithelial damage.This was highlighted by the MTT assay which showed no statistically significant decrease in cell viability ($p>0.05$) between control and cosolvent-treated cells. PEG and lower concentrations of polypropylene glycol (1 %) and glycerol (1 %) showed no significant effect on cell viability. Even at high cosolvent concentrations of polypropylene glycol (5 %, 10 %) and glycerol (5 %, 10 %), cell viability decreased by a maximum of 25 %. Glycerol is known to slightly decrease cell viability at concentrations of 5 % and 10 % while lower concentrations (1 %, 0.01 %) have no effect on cell viability[93]. The FDA suggests that

upto 40 % of polypropylene glycol be used for parenteral purposes. Decrease in TEER at higher concentrations of polypropylene glycol can be a temporary phenomenon. Decrease in TEER can be beneficial as temporary opening in the epithelial barriers can improve drug transport across the cell membrane. As the percentages of cosolvents used were within FDA-approved range, these excipients will be used in future formulation studies. Sometimes decrease in TEER is followed by full recovery within 24 hours following exposure to drugs and excipients[94,95,96].Previous studies have shown that there is almost a 600-fold difference between the toxicological concentrations of PEG and the doses generally recommended in biological agents[97]. Therefore, chances of PEG 600 being cytotoxic are remote. Although change in TEER was used to screen the cosolvents, it was also used in conjunction with sodium fluorescein permeation as quality control tool during the permeation studies (**Table 6**). The fact that TEER decrease was never below 400 Ω Cm^2 and sodium fluorescein permeation was less 1 % following exposure to different T4 formulations implies that the permeation data presented in this work were not compromised by epithelia damage.

Table 6 Apparent permeabilities P_{app} and percentage decrease in TEER obtained under various conditions for transport of T4 across Calu-3 cells.

Transport study	**P_{app} AP-to-BL X 10^{-6} (cm/s) Mean ± SD**	**P_{app} BL-to-AP X 10^{-6} (cm/s) Mean ± SD**	**TEER decrease Mean ± SD (%)**
Control	19.76 ± 4.89	43.45± 5.29	20.80 ± 12.30
Effect of sodium	10.27 ± 1.80	52.18 ± 9.45	5.51 ± 12.32
Effect of temperature	3.30 ± 2.36	4.14 ± 1.53	22.43 ± 8.56
Effect of concentration (25 µM)	17.86 ± 3.48	29.33 ± 6.70	19.71 ± 4.74
Effect of concentration (50 µM)	21.78 ± 6.93	28.23 ± 1.75	21.82 ± 8.73
Effect of concentration (75 µM)	18.87 ± 1.09	37.74 ± 2.18	25.61 ± 7.48
Effect of concentration (100 µM)	15.28 ± 6.49	34.46 ± 1.34	23. 22 ± 6.27
Effect of pH 8 → 5.5	16.36 ± 3.32	20.08 ± 1.60	32.41 ± 4.76
Effect of pH 8 → 7.4	21.46 ± 1.14	13.61 ± 5.41	28.04 ± 4.22

Mean percent sodium fluorescence rate was less than 0.8 % in all the transport conditions.

5.4 TRANSDERMAL STUDIES

Caffeine is a low molecular weight (~194) compound with a log *P* of -0.63. It is easily absorbed through skin via passive diffusion and is one of the test compounds recommended by the OECD Guideline 428 for validation of human skin for *in vitro* absorption testing. The test method was optimized according to parameters mentioned in the OECD Guideline 428 and the method described by van de Sandt et al. (2004)[98].The maximal absorption rate and time to maximal rate we observed for caffeine permeation was 6.58 ± 0.86 µg/cm^2-h and 2.06 ± 0.92 h, respectively. The values obtained were in line with the values observed by van de Sandt *et al.* (2004)[98] for maximal absorption rate

and time to maximal absorption in rat and human skin. Based on our in-house validation data, we used the Strat-M® as skin model for T4 permeation studies.

As mentioned in the methods section, initial T4 permeation was carried out in 0.9 % saline. No transport was seen over a 24-hour period (**Figure 24**). Further experiments with various concentrations of absorption enhancers (PEG 600, polypropylene glycol and Vitamin E-TPGS) also resulted in no transport (**Figure 25**). The lack of T4 permeation can be explained by the fact that T4 is a relatively large molecular weight compound (776.87 g/mol) with molecular weight twice the cut-off for transdermal delivery[99]. Furthermore, although the compound is lipophilic, it has a net negative meaning that it is unlikely to permeate the *stratum corneum* lipid bilayer. In epithelial cells, T4 are transported by T4 transporters that are not expressed in dead *stratum corneum* layer. The results of T4 permeation across Strat-M® was in agreement with the data published by Padula *et al* (2008).The authors found that T4 permeated through the skin barrier into the receptor fluid only after skin stripping (*stratum corneum* removal). Even then the amounts that passed through were too low to show any systemic activity.

CHAPTER 6 CONCLUSIONS

The objective of this study was to determine if levothyroxine permeates through when applied to the respiratory mucosa and artificial skin membranes at a reasonable rate and quantity to possibly elicit systemic effect. The apparent permeability coefficient values indicated that T4 can significantly permeate the respiratory mucosa. One of the significant findings emerging from this study is that specific thyroid hormone transporters are expressed in the respiratory mucosa which was responsible for the passage of the compound. The involvement of the transporters was confirmed by transport polarity, effect of temperature and sodium ions, which suggests a possible role of sodium-dependent thyroid hormone transporters. Based on our stability data, T4 stability was dependent on pH and temperature. The hormone was more stable at higher pH and lower temperature, respectively. Stability enhancers (KI, EDTA and AA) worked to a reasonable extent in stabilizing the compound. Solubility studies revealed that T4 solubility was highest in deionized water compared to PBS and HBSS without Ca^{2+} and Mg^{2+} due to possible interactions with carbonate and phosphate ions. Cosolvents (PEG-600 and PEG 400) were effective in enhancing T4 solubility without killing the cells. Although T4 was not found to permeate through artificial skin membranes, even after addition of transdermal absorption enhancers (PEG 600, polypropylene glycol, Vitamin E TPGS), the result was in agreement with other published studies on the permeation of T4 through animal skin models. This suggests that Strat-M® is a viable model for screening skin drug permeation. Considering the Log *P*, molecular weight and net charge of T4 at

physiological pH, successful transport of the compound across the skin requires a strategy that physically and reversibly compromises the *stratum corneum* (e.g. iontophoresis, sonophoresis, electroporation and microneedles).

Taken together, the data generated from this project suggest that the respiratory route is a possible route for non-invasive delivery of levothyroxine for treatment of hypothyroidism. Factors affecting stability such as pH and temperature should be taken into account during formulation development.

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