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IN

PHARMACY

By

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1. Introduction

1.1 Peptide therapeutics

Peptides have been anticipated to be an exclusive therapeutic agent and potentially being applied for treatment of various dreadful and intractable ailments. Owing to its inherent characteristics and pharmacological profiles of peptides, they are considered as novel biological actives and regarded as excellent initiators for drug development [1]. Currently, peptides are used to treat various disorders including metabolic disorders, cancers, and cardiovascular diseases. Peptides evolved with emerging therapeutic applications in the field of infectious diseases, pain, urinary tract, gastrointestinal and respiratory disorders. Furthermore, the subtle size of peptides allows them to infiltrate deep seated tissues. Peptide-based therapeutics are highly effective, less immunogenic, less toxic, do not accumulate in body tissues and are fairly inexpensive *vis-à-vis* antibodies and recombinant proteins. Thus, in spite of possible confines, like high molecular weight, low systemic absorption, speedy hepatic and renal clearance, and lower membrane permeability, numerous peptides have been commercialized to date and have displayed noteworthy clinical outcomes against various ailments [2].

1.1.1. Oxytocin

Oxytocin, a nonapeptide containing nine amino acids, acts as a neuropeptide hormone synthesized from the hypothalamus in the mammalian brain. In females, it is primarily used for management of parturition, control of postpartum uterine bleeding, treatment of incomplete, inevitable or elective abortion, lactation, etc. [3-5]. In the brain, oxytocin works as a neurotransmitter and a neuromodulator. Oxytocin functions as a chemical transmitter [3], suppresses inflammation in neurons and acts as a neuroprotective [4] by protecting the hippocampus from excitotoxicity [4-6].

Currently, Oxytocin is marketed as 5 Units/mL to 10 Units/mL in the various regulatory markets for gynaecological and obstetrics related treatment and administered via intravenous (i.v.) or intramuscular (i.m.) route. In brain, a limited amount of oxytocin reaches when administer via i.v route. This miniatured concentration in brain is due to short half-life and hydrophilic nature of molecule. To overcome the issues related to BBB permeation, various technologies have been explored to enhance effective delivery of oxytocin in brain [7]. The strategies associated with engineered nano drug deliveries are considered imperative for brain

delivery of Oxytocin, as they are capable of minimizing its toxicity and increase therapeutic concentration in the brain [8]. While, there are several literature testimonials are available that depicts about clinical and preclinical studies related to oxytocin delivery through nasal route and overcomes the symptoms related to pathophysiology of neurodegeneration [9]. The prominent reason for selection of intranasal (i.n.) route is due to its advantage of bypassing blood–brain barrier (BBB) and direct access to brain through its anatomy and physiology. This project covers formulation approaches explored for Oxytocin in particular dosage forms that are stable and comparison of traditional and new-generation carrier systems approaches for neurological purpose.

1.1.2. Vasopressin

Vasopressin (VP), a nonapeptide is a single linear chain molecule of nine amino acids, produced from enormous precursor proteins (propeptides), which in turn were derived from "prepropeptides," and comes under the class of macromolecules [10]. Vasopressin got first recognition in individuals who demonstrated high levels of blood pressure when administered with pituitary extract intravenously in the 19th century. Today, Vasopressin has recognition in major physiological events from maintenance of arterial pressure to edema in brain to restoration of kidney functions and management of neurological disorders [11, 12]. Vasopressin or prominently antidiuretic hormone, is the peptide hormone primarily used in clinical setting for its vasoconstrictive and antidiuretic properties. It is primarily used for management of vasodilatory shocks to increase the blood pressure in adults who are in a state of severely low blood pressure (hypotension) from vasodilatory shock, to manage diabetes *insipidus* to control excessive urination and fluid loss. Vasopressin is used to prevent the bleeding in esophageal varices by constricting large blood vessel in the oesophagus. It is used as an alternative to Epinephrine in cardiopulmonary resuscitation (CPR) for cardiac arrest due to its vasoconstrictive effect. It also manages low blood pressure or bleeding after surgery. Vasopressin is used for abdominal distention that occurs after certain surgeries. It is also used as a part of hormone replacement therapy to manage organ recovery in brain-dead donors. Additionally, Vasopressin is also reported for management of various neurodegenerative disorders such as autism, Parkinson’s disease (PD), Alzheimer’s disease (AD), Schizophrenia. However, there are the limitation for delivery of VP, due to its poor physicochemical and pharmacokinetics properties. Vasopressin has a very short half-life (about 10-20 min.) that necessitates the need for continuous infusion or frequent administration. It is usually given

intravenously or intramuscular or subcutaneous injection, which can be inconvenient and painful for patient. Oral administration is ineffective due to its degradation behaviour in digestive tract. It is reported to cause vasoconstriction, leading to increase blood pressure and potentially worsening the condition such as coronary artery disease or heart failure. Common side effects of vasopressin include water retention, hyponatremia (low sodium level), headaches and abdominal cramps. Overdose can lead to severe complication like seizures and coma due to water intoxication. Prolong use can lead to receptor desensitization and decrease effeteness [12]. Therefore, precise dosing and monitoring are required in complicated treatment, especially in acute setting.

1.1.3. Renin Angiotensin system

RAS (Renin Angiotensin System) plays a crucial role in maintaining electrolyte balance, regulating body fluid volume, and controlling cardiovascular function in the peripheral circulation. The enzyme renin, which is synthesized in the kidney, catalyses the hydrolysis of angiotensinogen (AGT), a precursor synthesized in the liver, and results in Angiotensin I (Ang I) release with limited biological activity [13]. ACE (Angiotensin-converting enzyme) is an additional enzyme that facilitates the cleavage of Ang I into the active octapeptide Angiotensin II. This peptide is a crucial component of the RAS with a significant role in different physiological activities. Nonetheless, the persistent activation of RAS and elevation of Angiotensin II (Ang II) levels can potentially impact AT1R (Angiotensin II type 1 receptor), hence initiating inflammation, vasoconstriction, fibrosis, and augmented renal salt reabsorption [14].

Angiotensin, particularly Angiotensin-II, is a peptide hormone that plays a critical role in regulating blood pressure and fluid balance. It is part of the renin-angiotensin-aldosterone system (RAAS). It is primarily used for the treatment of severe hypotension, particularly in vasodilatory shock, such as septic shock, where it helps to increase blood pressure by vasoconstriction. In some cases, modulation of the RAAS, including the use of Angiotensin-II receptor blockers (ARBs) or inhibitors, can help manage heart failure by reducing blood pressure and decreasing the workload on the heart. Angiotensin-II levels can be measured to assess the function of the RAAS in certain medical conditions. Apart from above uses, Angiotensin-II is reported to use for treatment of neurological disorder such as autism, Parkinson’s disease, Alzheimer’s disease, and Schizophrenia [14].

However, Angiotensin-II has a very short half-life (about 1-2 minutes), necessitating continuous infusion for therapeutic use. It may cause excessive vasoconstriction can lead to dangerously high blood pressure. Prolonged high levels of Angiotensin-II can cause damage to the kidneys and exacerbate kidney disease. Increased levels of Angiotensin-II are associated with a higher risk of cardiovascular events, such as heart attacks and strokes, due to its role in vasoconstriction and promoting inflammation and fibrosis. The RAAS system is complex, and modulating it can have widespread effects. Balancing the therapeutic benefits while minimizing adverse effects can be challenging. Not all patients respond equally to Angiotensin-II therapy. Factors such as genetic differences, the presence of other medical conditions, and concurrent medications can influence effectiveness and safety. Close monitoring of blood pressure, kidney function, and electrolyte levels is essential during Angiotensin-II therapy to avoid complications. Prolonged use of Angiotensin II can lead to receptor desensitization and reduced efficacy over time. In summary, while Angiotensin-II has important therapeutic applications, its use is limited by its short half-life, potential adverse effects, and the complexity of the RAAS system. Improved delivery methods or formulation are crucial to maximise benefits and minimizing of risks [14].

1.2 Challenges of peptides as therapeutic agent

Although, the small-molecular drugs are currently pioneering in the therapeutic market and are the first choice of medical practitioners, peptides-based therapeutics are progressively exhibiting their huge potential in the management of various tenacious ailments owing to their higher selectivity, higher biological activity, and lesser toxicity [15,16]. However, the clinical applications of peptides could be more robust owing to some inevitable confines namely target specificity, intracellular activity and stability (Figure 1.1). Moreover, the therapeutic delivery of peptides is still an uphill struggle owing to their structural complexity.

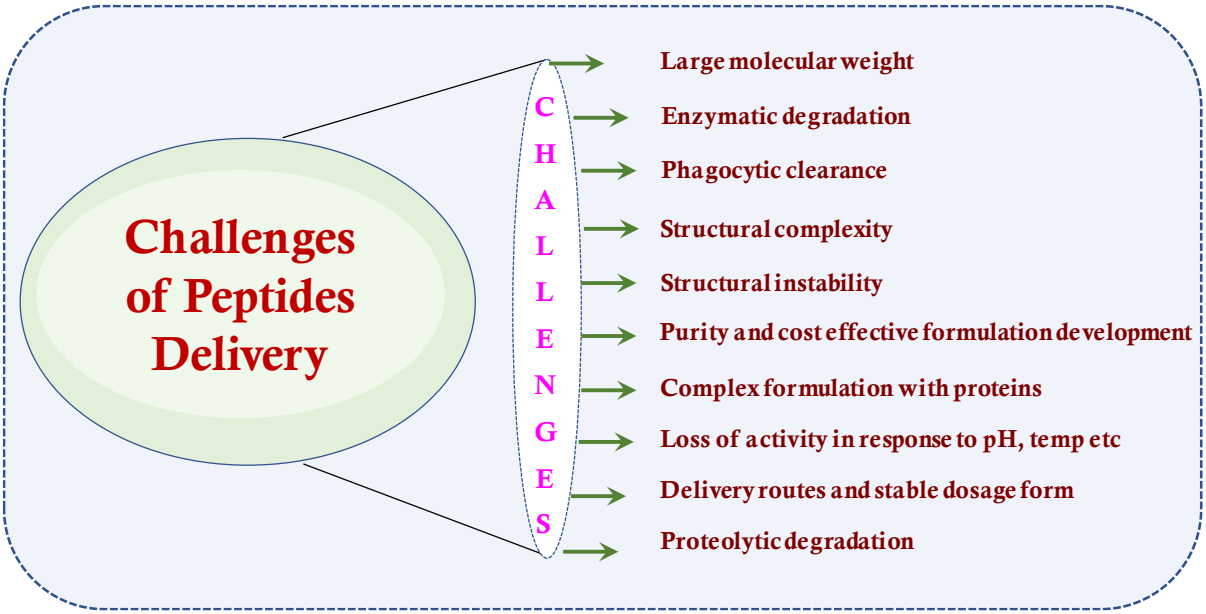


Figure 1.1 Various challenges of delivering peptides

Upon oral administration, various physiological and biological factors such as mucus layer, enzyme degradation, acidic pH of the gastrointestinal tract, presence of ingested food, low oral bioavailability of peptides hamper the efficacy of these macromolecules. Additionally, peptides are poorly absorbed through mucosal membranes and metabolise rapidly by peptidases and have short plasma half-life [17]. Therefore, most of the peptide -based formulations are administered *via* intravenous, intramuscular, or subcutaneous routes [18]. Systemic delivery of peptide suffers from various factors including short half-life owing to degradation by plasma and cellular nucleases, inadequate cellular affinity and poor intracellular trafficking [19]. Besides, plasma instability, metalloproteinase mediated enzymatic degradation, rapid renal clearance, and possible immune system responses are still inevitable even *via* parenteral administration [20]. Collectively, these factors lead to short half-life of these bioactive, resulting in frequent dosing requirement to enable their steady-state plasma level, thus lessening patient compliance and restraining their clinical uses. Therefore, ready to use formulations for selected peptide molecules are indeed needed which can infuse the drug at slower rate and would able to maintain the adequate plasma concentration of the drug.

Secondly, the intricacy of selective delivery of bioactives, which involves navigating several biological barriers, needs to be addressed before the full potential of peptide therapy is apprehended. Also, unfavourable characteristics *viz*, rapid degradation in plasma, rapid metabolism, negative-charge density and conventional administration techniques leading to poor patient compliance, restrict the clinical uses of peptide therapeutics. For incapacitating these encounters, approaches involving recombinant engineering, chemical alteration with

hydrophobic polymers, and micro/nano-encapsulation-based delivery of peptides illustrate great promises and have been extensively studied for the last 15 years [21]. These discoveries safeguard the peptide therapeutics from enzymatic degradation, prevent initial burst and uncontrollable release, increase plasma half-life, reduce dosing frequency, improve patient compliance, deliver the bioactive selectively, show lesser side effects and remain consistently effective during long-term therapy particularly for chronic diseases [22]. The fate of peptide-based therapeutics depends upon the potential of engineered delivery vehicles that offer enhanced stability of loaded bioactives from the biological environment and selectively deliver them at the desired site. Recent progress in nanoengineered lipid and polymeric-based delivery systems has broadened the scope of therapeutic targets of peptide drugs for various medical conditions.

Chemical modification is current and an effective method for producing therapeutic peptide analogues with the targeted and desired structures. After the peptide's synthesis, modifications are required through employing medicinal chemistry techniques to mimic, stabilize, or design a model secondary or tertiary structure to expand the biological activity and achieve specificity, stability, and solubility of the peptide drugs. Stabilized peptides are developed by various techniques such as: chemical synthesis of peptides, chemical modification of peptide and peptidomimetics, backbone modification of peptides, side chain modification of peptides, peptide cyclization, peptide mimicking of α-helices and stabilization, peptide mimicking of β-strands and β-sheets, peptide production by recombinant technology, peptides modification by genetic code expansion, PEGylation of peptides, covalent peptide drug developments etc. [23].

1.3 Recent therapeutic strategies for biomacromolecule delivery

1.3.1 For Ready to Infuse (RTI) dosage form

As many molecules (peptide and other sensitive chemical moieties) are unstable in aqueous and diluted form. Thus, these molecules are available in lyophilized and concentrated injection form respectively. These formulations need to be diluted in suitable diluents such as dextrose or sodium chloride or dextrose and sodium chloride injection or lactate ringer's solution or others diluents prior to administration. The dilution of injection involves aseptic manipulation, which incorporate additional stage in the delivery of medicine to the patient. This is very critical when the drug is delivered in the emergency situation. In addition, physicochemical stability of such diluted formulation is limited up to 24 h at room temperature.

1.3.2 Nanocarriers-based delivery approach

The need for potential delivery vehicles to transport the biomacromolecules at curatively appropriate amount to disease sites limits their clinical efficiency. These specially designed biocarriers (Figure 1.2) should be able to transport the macromolecules at desired target sites, minimize non-specific adverse effects and prevent systemic and local toxicities. In this context, the progression of nanotechnology has been important in overwhelming countless biopharmaceutical challenges for delivery of macromolecules and improving their pharmacological effectiveness [24].

Nanocarriers, widely used delivery vehicles for drugs and diagnostic agents, are materials with sizes ranging from 1–100 nm and have excellent characteristics *viz,* subtle size, hydrophilicity, solubility, high selectivity and apt drug-release profile. Nanocarriers also own an enhanced permeability and retention effect (EPR) which helps them to accumulate in leaky vasculature [24]. At present, numerous nanocarrier systems have been explored for the delivery of bioactive and diagnostic agents, like lipid-based, polymer-based, lipid-polymer hybrid systems, quantum dots, metallic nanoparticles, carbon nanotubes etc [25, 26].

1.3.2.1 Nanocarriers mediated delivery of peptides to Brain

1.3.2.1.1 Challenges in drug delivery to brain

Blood-brain barrier (BBB), blood-cerebrospinal fluid barrier and cerebrospinal fluid-brain barrier are three main parts to form barriers between cerebrovascular and parenchyma in brain. BBB is composed of various parts, including pericytes, astrocytes, perivascular spaces, glycocalyx, basement membrane, astrocyte end foot encircling tightly connected capillary endothelial cells surrounded by neurons, microglia, etc. The blood-cerebrospinal fluid barrier is found in arachnoid granulations that extend into veins and choroid plexus [27]. It consists of a basement membrane and closely linked epithelium. The cerebrospinal fluid-brain interface consists of the pia mater and a glial barrier membrane comprised of astrocytes. A perivascular space is created when arterioles transition into capillaries and penetrate deep into the brain parenchyma. The pia mater, which surrounds the arterioles, gradually transforms into a basement membrane within this space [28].

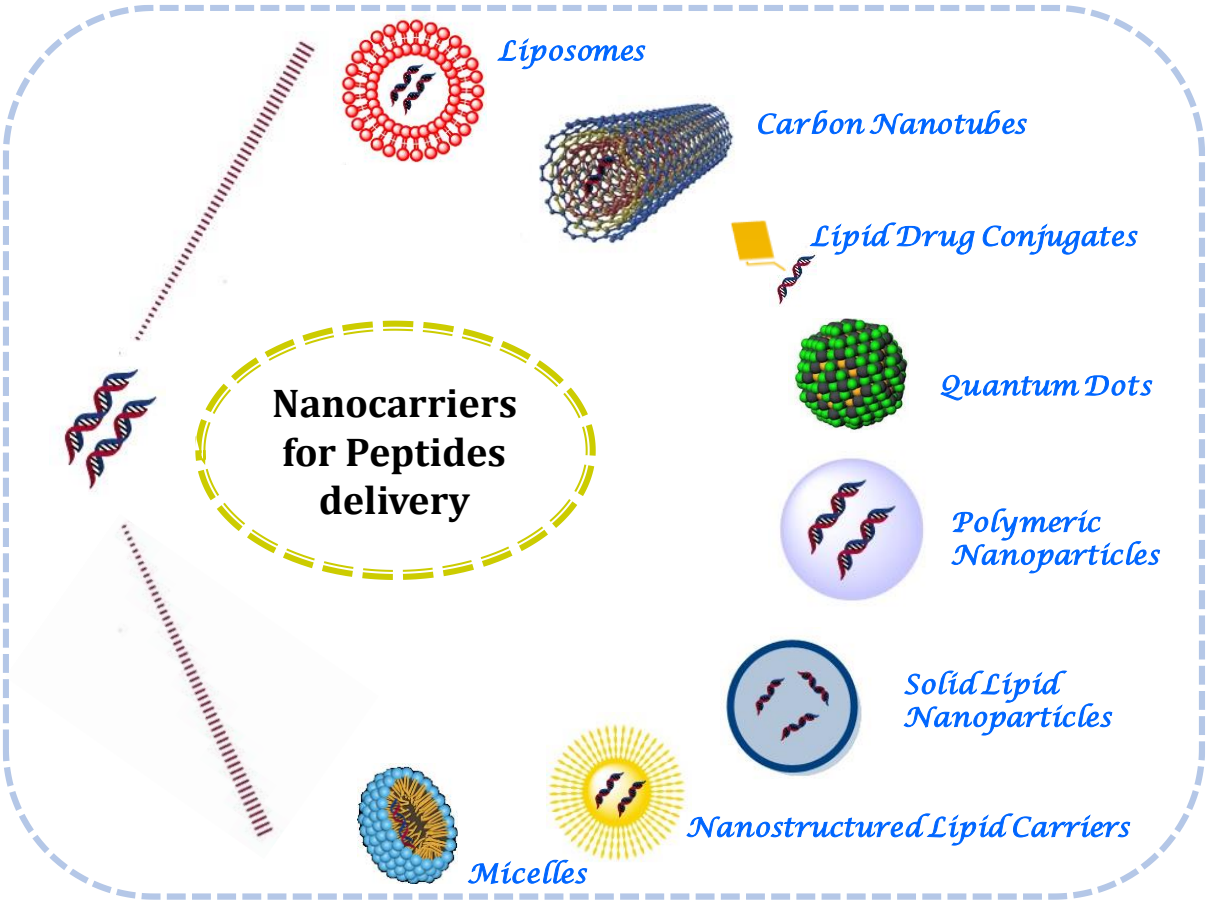


Figure 1.2: Various nanocarriers for delivery of peptides

Various remedies are used in multiple directions for management of neurological disorders. Although there is no current therapy has been found to develop a helpful model which can easily cross the BBB [29]. The BBB is an active interface junction which limits the passive drive of therapeutics and other molecules associated with blood into the brain via various pathways that include transcellular diffusion, paracellular transport, solute carrier transport, receptor-mediated transcytosis transport and adsorptive-mediated transcytosis transport. The primary function of the BBB is to serve as a barrier, stopping the majority of chemicals from entering the brain tissue from blood vessels and eliminating metabolites from brain tissue. The pathways encompass the tight junctions connecting brain endothelial cells, efflux transporters, and metabolic enzymes [28].

As discussed above, the BBB is made of close-fitting lipophilic endothelial cell connections, which allows only low molecular weight and lipophilic molecules to pass BBB to reach in brain actively. The BBB restrict the therapeutic use of molecules or medicine more than 400 Da, as

it prevents them from entering the brain through passive movement after the conventional routes of administration [30].

Although oxytocin has multiple actions in the brain and has a significant activity in neurological disorders, a major difficulty in the development of oxytocin as a neuro medication is that it is unable to cross the BBB after administration freely. Development of peptide-based formulations for treating neurological disorders in brain is are most challenging, but it also opens a new venue to develop new carrier systems for treating CNS disorders [50]. Several reports have been published on these imitations of Nano drug delivery approach that specifically discuss on the challenges and unmet needs [31-33].

1.3.2.1.2 Need for Intranasal route

In clinical development, therapeutics delivery to the brain is still the biggest challenge because of unfavourable conditions for drugs and miniscule absorbance in the brain. The most challenging task of brain delivery is the cumbersome anatomy and physiology of the BBB which deprives entrances of active molecules to the brain or CNS [34]. As per available literature data or reports published in other peer-reviewed literature, it is observed that 98% of drug molecules developed for CNS are devoid of entry by barriers on the junctions because of their physicochemical and biopharmaceutical properties. Interstitial fluid act as the primary barrier that lies within CNS and circulating blood vessels [34]. Further, BBB is incorporated with various transporter mechanisms, which directly inhibit the entry of drug molecules into the brain. The most prominent example is of P-glycoprotein efflux transporter. This limits the brain delivery and therapeutic concentration of drugs in CNS which subsequently decrease the efficacy and prolong the treatment in various CNS linked disorders like migraine, meningitis, Parkinson's, schizophrenia, Alzheimer's, brain tumour, etc [35]. For achieving therapeutic concentrations in CNS, various researchers have developed many methodologies and alternative routes including invasive and non-invasive techniques. But in the last decade, researchers have expressed their keen interest in the nose to brain route as a subsidiary of the non-invasive procedure for CNS targeting with special emphasis on trigeminal or olfactory pathways for overcoming the hurdles arising from BBB [35]. Delivery via the nose to the brain flourishes with the advantages of safer, efficacious, non-invasiveness, devoid of hepatic metabolism, convenient to administer, and last not least, capable to be self-administered. Selective regions present in the nose permits direct entry of drug molecules into the brain. Regions include the olfactory and trigeminal portions in nasal anatomy. The olfactory region

has direct entry to CNS because of the presence of olfactory receptors, its neurons and further extensions of axons into the olfactory bulb. The olfactory region is considered the only peripheral portion of the body with direct connectivity with the brain [34]. As per various pre-clinical and clinical investigations, it was concluded that the nose to brain route acts as a potential bypass for BBB and provides a non-invasive method of drug administration. Despite these advantages, nose to brain route only allows 0.1% of the delivered amount to CNS. On administration of formulation to the nose, it firstly comes in contact with the mucosa and is transported directly to CNS thus bypassing BBB. Although nose to brain delivery provides an additional advantage of dose reduction, it suffers from some demerits of limited administration of dose (25 to 200 μL), nasal-based enzymatic degradation and mucociliary clearance. Demerit in nose to brain has limited the commercial exploration of this route [34, 36]. Alternatively, to overcome these demerits, researchers are in a continuous process to make this route accessible by nurturing advanced drug delivery systems and exploring nanotechnological-based approaches.

1.3.3 Conjugation of peptide molecules to enhance the pharmacokinetics and pharmacodynamic attributes

The improved stability, selectivity and activity are the main outcomes of peptide modification. Selepressin, Liraglutide and Semaglutide are the best examples of chemical modification technique's outcome, which have been introduced in clinical applications recently. However, some chemical alterations cannot recover the proteolytic stability, selectivity and activity at the same time. Such as, the addition of D-amino acid in place of L-amino acid can usually support to extend the plasma half-life of the therapeutic peptide. On the other hand, peptides with D-amino acid modification rarely exhibit effective biological activity [37-39]. Peptide modifications allow peptides to achieve better activity and plasma stability, and become more drug-like attributes [40].

2. Aims & Objectives

2.1 Aims

With the insight of the reported problems such as poor bioavailability, short half-life, poor penetration in brain via oral/i.v. route along with associated stability issues. This work was aimed to design, develop and optimize the suitable delivery systems of Oxytocin, Vasopressin, and Angiotensin-II to fill the gap in the most appropriate manner.

2.1.1 Ready to Infuse (RTI)

The aforementioned limitation creates the need for the ready to administer formulation of the molecules which can be directly administered to the patient. Moreover, parenteral drug manufacturing of oxygen/photosensitive drug in the plastic infusion bag becomes critical due to the semipermeable nature of infusion bag, as it reacts with oxygen during manufacturing operations till it reaches to the final packing.

With the insight of the reported problems during dilution and associated stability issue, it was proposed to design, develop and optimize the suitable RTI delivery systems of Oxytocin, Vasopressin and Angiotensin-II to address the issues associated with selected peptides. RTI approach will provide a composition and process for producing injection of sensitive molecule with low concentration of active ingredient and it will also provide better compliance and safety to the patient. The maximum safety is prominent factor in RTI due to its uniqueness in zero dose administration errors and minimum cross contamination.

The proposed "Ready to Infuse" dosage form will be novel and unique in terms of strategy for administration, compositions and dosage form, which can be easily patentable and can be commercialized.

2.1.2 Nano-formulations for Nose to Brain Delivery

In this proposal, design and development of suitable nano-delivery systems for effective delivery of Oxytocin and Vasopressin in brain as repurposing for Alzheimer's disease management. It was postulated that the designed dosage form will provide a composition and process for formulating injection and delivery *via* intranasal route of these sensitive molecules in order to provide better compliance and safety for the patient.

The proposed hypothesis is unique and novel in terms of dosage form, strategy for administration, compositions of delivery systems, which enhances patentability as well as commercialization aspects and can be commercialized. In addition to above, we also proposed

the conjugation of Oxytocin with suitable molecule that might prolongs plasma half-life and bioavailability of Oxytocin in brain.

2.1.3 Chemical modification to improve half-life and bioavailability of oxytocin

Currently, chemical modification is an effective method for producing therapeutic peptide analogues with the targeted and desired structures. Among selected bioactives for the research work, Oxytocin has the very short half-life in the blood circulation (4~10 min). Therefore, it was selected as a model molecule for further modifications. On the path of addressing bioavailability and short half-life of Oxytocin, the aim of present study is to improve retention time of oxytocin in body along with enhancement in permeation of Oxytocin across BBB through conjugation of 2-(2-(2-Aminoethoxy) ethoxy) acetic acid (AEEA) with one of the amino acids of Oxytocin with improved biopharmaceutical properties of Oxytocin.

2.2 Objectives

The objectives of present work have been divided in three major sections as mentioned below:

2.2.1 Ready to Infuse (RTI)

1) To design, develop and characterize Oxytocin based RTI injection
2) To design, develop and characterize Angiotensin-II based RTI injection
3) To design, develop and characterize Vasopressin based RTI injection

2.2.2 Nano-formulations for Nose to Brain Delivery

1) To design and develop Oxytocin-loaded nanovesicles (Liposomes) for brain delivery
2) To design and develop Vasopressin-loaded nanovesicles (Liposomes) for brain delivery

2.2.3 Oxytocin-conjugates for Nose to brain delivery

1) To synthesize, characterize and evaluation of chemically modified Oxytocin to enhance half-life and bioavailability for brain delivery.

3. Plan of Work

3.1. Development and characterization of Ready to Infuse formulations

1. Pre-formulation studies.
2. Ready to Infuse formulation development for Oxytocin, Vasopressin, Angiotensin-II
3. Physicochemical characterisation of developed formulations.
4. *In vitro* evaluation
5. Stability studies

3.2. Development and characterization of Nano-formulation for Nose to brain

1. Nano formulation development for:
    - Oxytocin
    - Vasopressin
2. Physicochemical characterisation of developed formulations.
3. *In vitro* cell line studies (cellular uptake and cytotoxicity studies).
4. *In vivo* pharmacodynamics studies.

3.3. Synthesis and characterization of Oxytocin conjugate for Nose to brain delivery

1. Synthesis of Oxytocin conjugate with 2-(2-(2-Aminoethoxy) ethoxy) acetic acid
2. Physicochemical characterisation of conjugate
3. *In vitro* cell line studies (cellular uptake and cytotoxicity studies).
4. *In vivo* pharmacodynamics studies.

4. Procurement of Drugs, Excipients and Other Chemicals and Reagents

The details of materials which were used in the research work portrayed in table 4.1. The details of instruments used displayed in Table 4.2.

Table 4.1: **Various raw materials, chemicals and reagents employed in the current research work**

Items	Source
0.2 μ PVDF Capsule filter	Pall, USA
2-Hydroxypropyl-β-cyclodextrin	Roquette, France
100 mL sterile PHC bag	Hosokawa, Japan
Acetonitrile for HPLC	Spectrochem Pvt. Ltd., Mumbai, India
Advantaflex tube	Advantapure, USA
Aluminum overwrap pouch	Hosokawa, Japan
Angiotensin-II	Sun Pharmaceutical Industries Ltd, Ahmednagar
Dextrose anhydrous	J T Baker, USA
Disodium hydrogen phosphate	Merck, USA
Dextrose	J T Baker, USA
Hydrochloric acid	J T Baker, USA
Mannitol	Roquette, France
Methanol	Merck India Ltd., Mumbai, India
Methanol for HPLC	Spectrochem Pvt. Ltd., Mumbai, India
Minitulip	Sippex, France
Needles (Hypodermic-BD)	Beckton Dickinson and Co., USA
Nylon filters:	Advanced Microdevices Pvt. Ltd., Ambala, India
Octane	S.D. Fine-Chem Ltd., Mumbai, India
Orthophosphoric acid	Fisher Scientific, Mumbai, India
Oxygen Scavenger (Ageless ZPT-200MBC)	Mitsubishi, Japan
Oxytocin	Sun Pharmaceutical Industries Ltd, Ahmednagar
Potassium Bromide	SRL Pvt. Ltd., Mumbai, India
Potassium Dihydrogen Phosphate	Fuligins Fine Chemicals Ltd., Mumbai, India
Propidium iodide (PI) staining kit	eBioscience, USA
Sodium Chloride	Merck, USA

Items	Source
Sodium Citrate	Merck, USA
Sodium Hydroxide	Merck, USA
Sucrose	Merck, USA
Teflon coated rubber stopper	West, USA
Vasopressin USP	Polypeptide laboratories Pvt. Ltd., India
Water for Injection	Mili-Q
Water, for HPLC	Merck Specialties Pvt. Ltd., Mumbai, India

Table 4.2: **Details of various instruments employed during the current research work**

Instruments	Make
pH meter	Mettler Toledo
Analytical balance	Mettler Toledo
HPLC	Waters
Zetasizer	Malvern

5. HPLC Method Development of Selected Bioactives

5.1 Oxytocin

Instrumentation: The Shimadzu LC–2010CHT liquid chromatograph located in Japan is equipped with a system driver, a solvent delivery module, a low-pressure gradient pump, an online degasser, an auto injector (capable of injection volumes ranging from 5 to 100 µL), auto-samplers with a cooler, and a UV-Vis detector (refer to Table 5.1).

Chromatographic conditions: YMC Pack ODS-A column with dimensions of 150 × 4.6 mm and a particle size of 3 µm was utilized. The mobile Phase consisted of two parts wherein phase A consisted of 13.8 grams of sodium dihydrogen phosphate monohydrate dissolved in 1 liter of water and the pH was adjusted to 3.0 with orthophosphoric acid. This solution was filtered and transferred to reservoir, labelled as A. On the other hand, mobile phase B constituted of a mixture of water and acetonitrile in 50:50 ratio v/v. This solution was also filtered in the same manner as that of mobile phase A and stored in mobile phase reservoir, designated as B. The flow rate was kept at 0.6 mL/min throughout the HPLC run and the column temperature was maintained at 40°C. The injection volume was kept as 500 µl. The mobile phase was utilized in a gradient mode and the ratio of mobile phase A and B with respect to time is represented in Table 3.3. The run time for a single HPLC run was kept 75 min

Table 5.1: Chromatographic conditions for HPLC method development for Oxytocin

Instrument	**HPLC**
Mobile Phase Preparation	Mobile Phase A: 13.8 g of sodium dihydrogen phosphate monohydrate is dissolved in 1.0 L of water. pH=3.0 adjusted with Ortho phosphoric acid Mobile Phase B: water: Acetonitrile (50:50, *v/v*)
Diluent	Potassium dihydrogen phosphate buffer preparation for diluent. Accurately weighed and dissolved 6.8 g of Potassium dihydrogen phosphate in to 1000 mL water. (Observed pH=4.5) Adjusted pH 7.0 ± 0.05 with dilute Sodium Hydroxide solution (Require approximately 6 to 8 mL for 1 L buffer). Diluent-1: Prepared a mixture of Potassium dihydrogen phosphate buffer and acetonitrile in the ratio of 80:20.
Chromatographic system	
Column	YMC Pack ODS-A (150*4.6 mm), 3 µm

Wavelength	220 nm		
Flow rate	0.6 mL/min		
Injection volume	500 µL		
Column temperature	40°C		
Run time	75 min		
Gradient	Time	%A	%B
	0	82	18
	50	45	55
	55	40	60
	65	40	60
	65.1	82	18
	75	82	18
Standard Preparation	Accurately weighed WS was transferred to a 200 mL volumetric flask, dissolved in 100 mL of water and diluted up to mark with water and mixed well. Transferred 4 mL of this solution in to another 200 mL volumetric flask and diluted up to the mark with water and mixed well. Transferred 4 mL of this solution in to a 50 mL volumetric flask and made up the volume up to the mark with diluent-1 and mixed well.		
Sample solution A	Transferred 50 mL of sample from preconditioned strata C18-E cartridge applying the 150 mm of Hg by using water's Negative pressure processor and washed the cartridge each with 1.5 mL (500 µLx3) of Milli Q water. Eluted the Oxytocin from cartridge 12 times each with 200 µL of diluent-2 and collected in a clean and dry test tube and further added 0.6 mL of Potassium dihydrogen phosphate buffer solution for diluent, mixed well. Afterwards, this solution was injected to HPLC system.		
Sample solution B	NA		
Sample solution C	NA		
System Suitability solution	NA		
Control solution	NA		

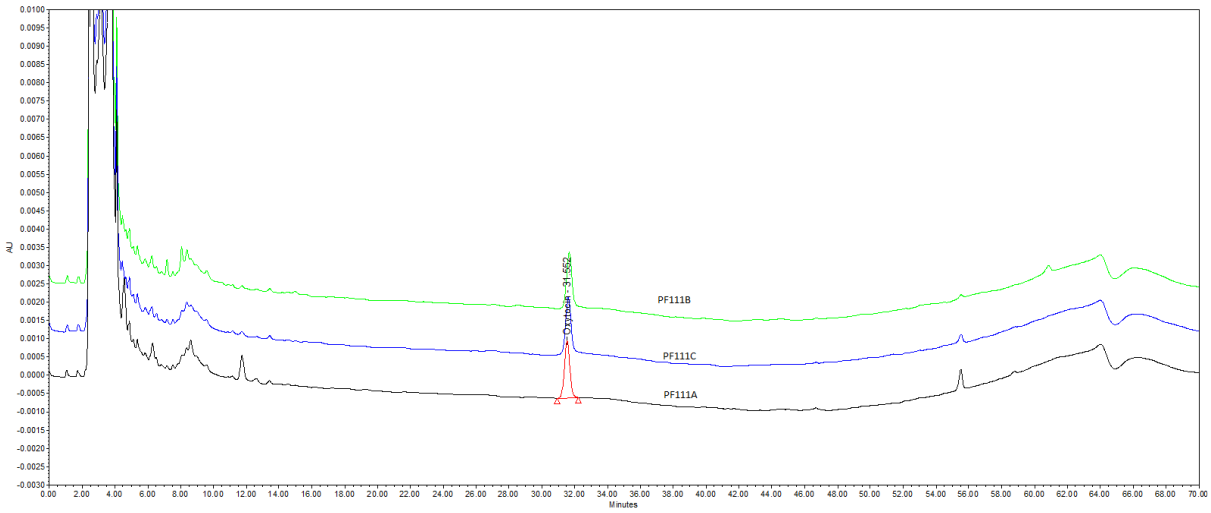


Figure 5.1: HPLC chromatogram for Oxytocin standard and samples

5.1.1 Discussion:

In all these estimations, the separate, well resolved peak of Oxytocin, without any impurity or merged peaks was appeared. The Rt of this peak was similar to the standard peak of Oxytocin i.e., 31.6 min, which can also be confirmed by seeing the overlap of all the chromatograms (Figure 5.1). This confirmed that the method is applicable for the estimation of drug in all sample matrices, with great accuracy. All other peaks of sample appeared well before 15 min, in all the cases, which helped in identifying the separate peak of Oxytocin.

5.2 Vasopressin

6. nstrumentation

The liquid chromatograph utilized in this study was the Shimadzu LC–2010CHT, a sophisticated instrument manufactured in Japan designed for precise and efficient chromatographic analysis. This system was equipped with a comprehensive setup including a system controller for overall operation, a solvent delivery module for managing solvent flow, a low-pressure gradient pump to facilitate smooth solvent transitions, and an online degasser to remove dissolved gases that could interfere with the analysis. Additionally, it featured an auto-injector, capable of handling injection volumes ranging from 5 to 100 µL, as well as an auto-sampling unit combined with a cooler to maintain sample integrity. The analysis was monitored using a UV-Vis detector to capture and quantify the light absorption of the analytes. Chromatographic separation was executed on a YMC Pack ODS-A column, measuring 250 mm in length and 4.6 mm in diameter, with a particle size of 5 µm, which played a crucial role in achieving optimal separation of components, as detailed in Table 3.7 [1,8].

Column Operation: The YMC Pack ODS-A column was meticulously operated at a controlled temperature of 40°C, ensuring consistent performance and reliable results.

Preparation of Mobile Phase Buffer: For the mobile phase buffer, precise measurements were taken: 6.6 g of di-basic ammonium phosphate was carefully weighed and then dissolved in 1000 mL of distilled water. To achieve the target pH of 3.0 ± 0.05 a critical parameter for the separation processes an appropriate amount of strong orthophosphoric acid solution was added. The resulting solution was then filtered through a 0.45 µm membrane filter to eliminate any impurities, and degassed to remove dissolved gases, utilizing Millipore-Durapore HVLP 0.45 µm filter paper (Cat No.: HVLP04700).

Composition of Mobile Phase: The mobile phase was formulated by mixing the prepared buffer with acetonitrile in an 87:13 volume ratio, creating an optimal environment for effective chromatography.

Diluent Preparation: The diluent was composed of a carefully crafted blend of the buffer solution and methanol in an 80:20 ratio, ensuring suitable conditions for sample dilution.

Standard Preparation: To create the working standard, 23.5 mg of normal vasopressin powder was accurately weighed and dissolved in 70 mL of the prepared diluent within a 100 mL volumetric flask. The flask was then filled to the calibration mark with additional diluent and thoroughly mixed to ensure homogeneity. A portion of this solution, specifically 6 mL, was subsequently transferred into a new 100 mL volumetric flask. Buffer was added until the mark was reached, and thorough mixing was performed to obtain a consistent standard solution.

Sample Preparation (1.887 µg/mL Strength): For this sample preparation, 10 g of the selected sample was placed into a 20 mL volumetric flask. Sufficient solvent was then added to fill the flask to the mark, followed by vigorous mixing to ensure the sample was fully dissolved, resulting in a final concentration of 0.94 ppm.

Sample Preparation (37.74 µg/mL Strength): In this preparation, 1.25 g of the desired sample was carefully measured and added to a 50 mL volumetric flask. The flask was filled with enough solvent to reach the calibrated mark, and the contents were mixed thoroughly to ensure a homogeneous solution was achieved for further analysis.

Table 5.2: Chromatographic conditions for HPLC method development for Vasopressin

Column	YMC pack ODS A (250 mm x 4.6 mm), 5 µ (YMC Co. Ltd Japan)
Flow rate	1.2 mL/min
Wavelength	220 nm
Column temperature	40°C
Sample temperature	10°C
Injection volume	200 µL
Run time	20 min
Needle Wash	Water: Acetonitrile (50:50)
Seal Wash	Water: Acetonitrile (50:50)

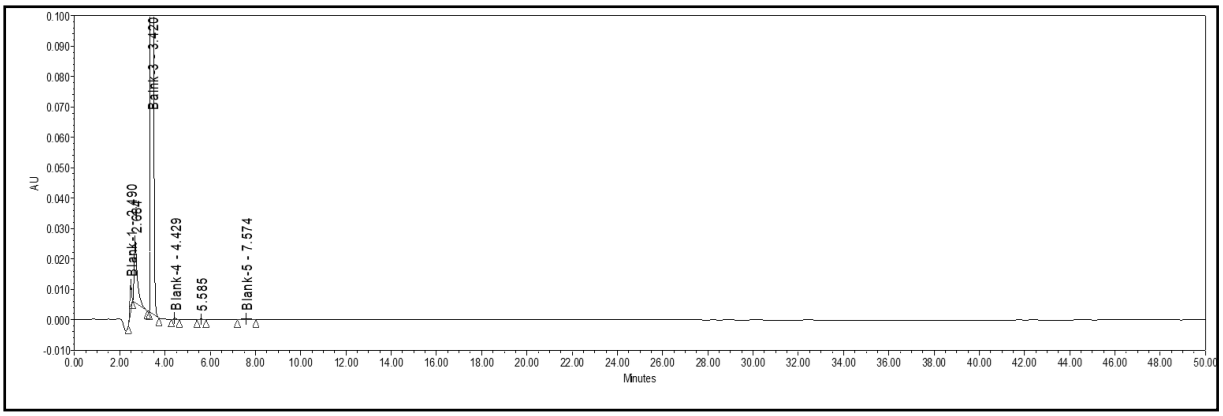


Figure 5.2 : Blank-diluent chromatogram

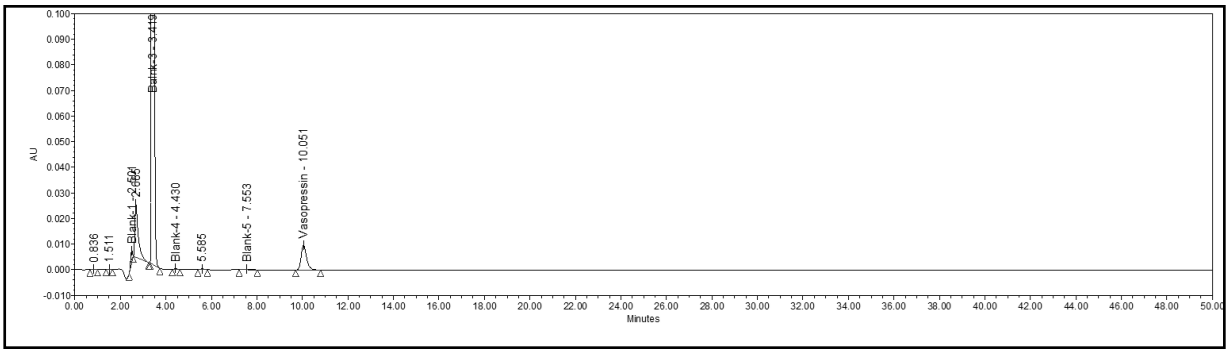


Figure 5.3: Standard chromatogram of Vasopressin

5.2.1 Discussion:

In all these estimations, the separate, well resolved peak of Vasopressin, without any impurity or merged peaks was appeared. The Rt of this peak was similar to the standard peak of Vasopressin i.e., 10.05 min, which can also be confirmed by comparing the chromatogram of Vasopressin standard and Vasopressin sample. (**Figure 5.2 and Figure 5.3**). This confirmed that the method is applicable for the estimation of drug in all sample matrices, with great

accuracy. All other peaks of sample appeared well before 8 min, in all the cases, which helped in identifying the separate peak of Vasopressin.

6.1 Angiotensin-II

Buffer solution: 2.72 g of potassium dihydrogen orthophosphate were mixed with 1000 millilitres of water and sonicated to break up the solids. It was given 30 mL of triethylamine. With orthophosphoric acid, the pH of the solution was brought down to 2.0 ± 0.05. Through 0.45-inch filter paper (Filter: MILLIPORE-Durapore HVLP 0.45 µm 047 mm, CAT No: HVLP04700) (Table 3.11).

Mobile Phase-A: buffer solution and acetonitrile were mixed in a 900:100 ratio to make it. The mixture was degassed prior to use.

Mobile Phase-B: was made by mixing 300 parts buffer solution to 700 parts acetonitrile. The mixture was degassed prior to use.

Diluent: HPLC grade water

Standard solution: Carefully measured approximately 5 mg of Angiotensin-II WS/RS and placed it into a 50 mL volumetric flask. Added around 10 mL of diluent and sonicated the mixture to ensure it was dissolved. Then, added more diluent until the solution reached the fill line. Transferred this solution into a 20-mL volumetric flask using a syringe, diluted it further with diluent to the mark, and mixed thoroughly, resulting in a final concentration of 10 ppm.

Sample preparation

Injected as such sample (10 ppm)

Table 5.3: Chromatographic condition for Angiotensin-II

Column	X-SELECT CSH Phenyl Hexyl (150 x 4.6) mm 2.5 µ (Make: Waters) Part No:186006735
Flow rate	0.6 mL/min
Wavelength	210 nm
Injection volume	100 µL
Column temperature	50ºC
Sample cooler	5ºC
Run time	50 min
Retention Time	About 17.46 min.

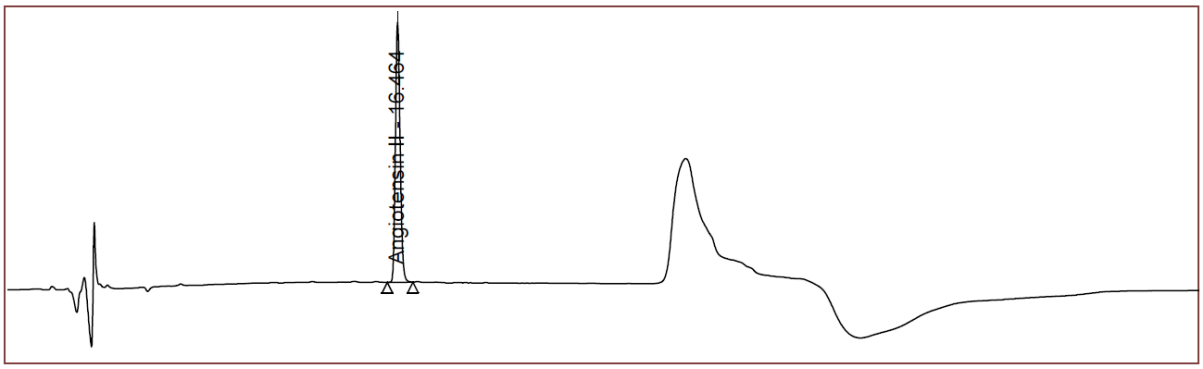


Figure 5.4 : Blank-diluent chromatogram of Angiotensin-II standard

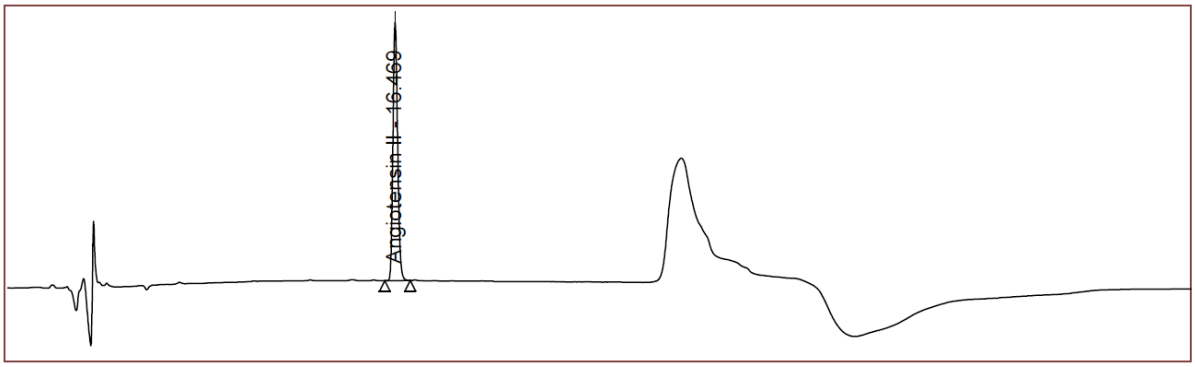


Figure 5.5 : Blank-diluent chromatogram of Angiotensin-II API

5.3.1 Results and discussion

In all these estimations, the separate, well resolved peak of Angiotensin-II, without any impurity or merged peaks was appeared. The Rt of this peak was similar to the standard peak of Angiotensin-II i.e., 17.46 min, which can also be confirmed by comparing the chromatogram of Angiotensin-II standard and Angiotensin-II sample. This confirmed that the method is applicable for the estimation of drug in all sample matrices, with great accuracy. All other peaks of sample appeared well before 15 min, in all the cases, which helped in identifying the separate peak of Angiotensin-II.

6. Formulation Development

6.1 Development and Optimization of Ready to Infuse formulation of Oxytocin

6.1.1 Preliminary Feasibility and Developmental trial with different excipients

Various formulation variables for the development of RTI formulation for oxytocin were evaluated to acquire a final formulation with assay range of 90-110% and osmolarity range of 250-350. Therefore, different buffering agents, amino acids, metal ions, osmogens and antioxidants were evaluated. The different compositions are described in below tables along with their assay and osmolarity value.

Table 6.1: Preliminary developmental trials

Formulation Code	Compositions	Sample Description	Batch No	Stage	Assay (%)	Osmolarity
FA-1	Each mL contains: Oxytocin 0.02 IU, Citric acid 2 mg, $MgCl_2$ 2.033 mg NaOH q.s. to adjust pH 4	100 mL PHC	PF015_A-4	Initial	83.13	46
		100 mL AOB	PF015_A-5	Initial	83.47	46
		100 mL TH82	PF015_A-6	Initial	83.12	46
FA-2	Each mL contains: Oxytocin 0.02 IU, Citric acid 2 mg, $MgCl_2$ 2.033 mg L-methionine 3 mg, L-arginine 1.5 mg, SMBS 3.2 mcg	100 mL PHC	PF015_B-4	Initial	62.44	63
		100 mL AOB	PF015_B-5	Initial	67.63	63
		100 mL TH82	PF015_B-6	Initial	60.77	63
FA-3	Each mL contains: Oxytocin 0.02 IU, Citric acid 2 mg, $MgCl_2$ 2.033 mg, Mannitol 50 mg, NaOH q.s. to adjust pH 4	100 mL PHC	PF015_C-4	Initial	83.56	318
		100 mL AOB	PF015_C-5	Initial	82.07	318
		100 mL TH82	PF015_C-6	Initial	72.42	318
FA-4	Each mL contains: Oxytocin 0.02 IU, Citric acid 2 mg, $MgCl_2$ 2.033 mg, Dextrose 50 mg, NaOH q.s. to adjust pH 4	100 mL PHC	PF015_D-4	Initial	61.14	327
		100 mL AOB	PF015_D-5	Initial	65.34	327
		100 mL TH82	PF015_D-6	Initial	64.27	327

$MgCl_2$: Magnesium chloride; NaOH: Sodium Hydroxide; q.s.: quantity sufficient.

6.1.2 Application of the analytical method in formulations

The developed and validated analytical method for Oxytocin was applied for its determination in developed RTI dosage forms.

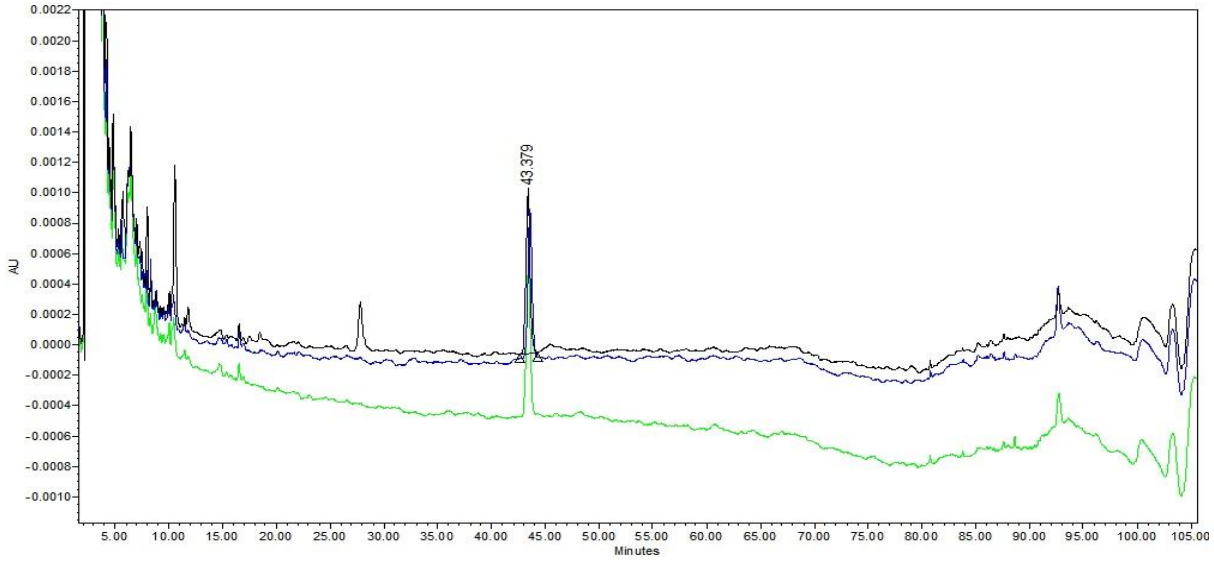


Figure 6.1: Chromatogram of oxytocin in formulation and three different packaging materials

On the basis of above results none of the composition has shown assay value as per specifications. However, composition comprised of dextrose and mannitol have shown osmolarity values in the range. Therefore, mannitol and dextrose were selected for further optimization.

6.1.3 Selection of Osmogens

In order to select the buffering agent, different formulation trials were conducted with different osmogens and in their different concentration.

Table 6.2: Development trials for selection of Osmogens for Oxytocin

Formulation Code	Compositions (Each mL contains)	Sample description	Batch No	stage	Assay (%)	pH	Osmolarity
FB-1	Oxytocin 0.02 IU, Sod Acetate 1 mg Mannitol 50 mg GAA q.s. to pH 4 WFI q.s.	TH-82	PF064-A2	INITIAL	88.27	3.96	391
		AOB	PF064-A3	INITIAL	84.26	3.96	388
FB-2	Oxytocin 0.02 IU, Sod. Acetate 1 mg Mannitol 50 mg Glycine 1 mg GAA q.s. to pH 4 WFI q.s.	TH-82	PF064-B2	INITIAL	87.32	3.99	398
		AOB	PF064-B3	INITIAL	86.94	4.0	398

Formulation Code	Compositions (Each mL contains)	Sample description	Batch No	stage	Assay (%)	pH	Osmolarity
FB-3	Oxytocin 0.02 IU, Sod. Acetate 1 mg Dextrose 50 mg GAA q.s. to pH 4 WFI q.s.	TH-82	PF065-A2	INITIAL	80.07	3.96	364
		AOB	PF065-A3	INITIAL	82.74	3.97	363
FB-4	Oxytocin 0.02 IU, Sod. Acetate 1 mg Dextrose 50 mg Glycine 1 mg GAA q.s. to pH 4 WFI q.s.	TH-82	PF065-B2	INITIAL	80.89	3.95	383
		AOB	PF065-B3	INITIAL	81.7	3.96	385
FB-5	Oxytocin 0.02 IU, Sod. Acetate 1 mg Sucrose 50 mg GAA q.s. to pH 4 WFI q.s.	TH-82	PF063-A2	INITIAL	**100.21**	3.97	280
		AOB	PF063-A3	INITIAL	100.5	3.97	279
FB-6	Oxytocin 0.02 IU, Sod. Acetate 1 mg Sucrose 50 mg Glycine 1 mg GAA q.s. to pH 4 WFI q.s.	TH-82	PF063-B2	INITIAL	**97.98**	3.98	280
		AOB	PF063-B3	INITIAL	99.67	3.97	282
FB-7	Oxytocin 0.02 IU, Sod. Acetate 1 mg Trehalose 50 mg GAA q.s. to pH 4 WFI q.s.	TH-82	PF032D	INITIAL	83.84	3.99	218
		AOB	PF032E	INITIAL	75.1	4.0	218
FB-8	Oxytocin 0.02 IU, Sod. Citrate 4.9 mg Lactose 50 mg Citric acid 6.4 mg GAA q.s. to pH 4 WFI q.s.	TH-82	PF030D	INITIAL	96.94	4.02	246
		AOB	PF030E	INITIAL	96.65	4.03	247
FB-9	Oxytocin 0.02 IU, Sod. Acetate 1 mg Sodium Chloride 4.50 mg GAA q.s. to pH 4 WFI q.s.	TH-82	PF067-A2	INITIAL	80.85	3.96	227
		AOB	PF067-A3	INITIAL	66.27	3.95	226
FB-10	Oxytocin 0.02 IU, Sod. Acetate 1 mg Sodium Chloride 4.50 mg HPβCD 1 mg GAA q.s. to pH 4 WFI q.s.	TH-82	PF063-E2	INITIAL	98.08	3.98	223
		AOB	PF063-E3	INITIAL	98.79	3.97	222

Sod. Acetate: Sodium acetate; GAA: Glacial acetic acid; WFI: Water for Injection; HPβCD: Hydroxy propyl beta cyclodextrin

All formulations were prepared with sodium acetate and citrate buffers with various stabilizers and filled in two different packaging materials (table 6.2). The assay in FB-5, FB-6, FB-8 & FB-10 was found to be above 90% and within proposed specification (90-110%). % of assay

in FB-5, FB-6 & FB-10 was found to be 98-100% in both bags (PHC & AOB), while 96 % assay was found in FB-8 in both filled bags. Formulation FB-5 and FB-6 contains sucrose as osmogen while FB-10 contains sodium chloride. The formulations which were formulated with lactose, sucrose and sodium chloride (with HPβCD) have shown assay value within the limits. However, in 4.50 mg/mL concentration of sodium chloride, the obtained value of osmolarity was slightly low. But FB-11 found osmolality with in desired value. Based on the results portrayed in Table 6.2, sodium acetate as buffer, sodium Chloride as osmogene, while HPβCD as stabilizer were found to be prominent compared to others.

Additionally, on the basis of reported literature, sodium acetate and acetic acid were found to improve stability of peptides, but after experimental outcome with these buffers in oxytocin formulations, sodium acetate buffer was found to be prominent compared to others. Therefore, sodium acetate was selected as a buffering agent to stabilize the formulation. Henceforth, we decided to select Sodium chloride as osmogene, sodium acetate as buffer and HPβCD as stabilizer for further optimization studies

6.1.4 Optimization of selected excipients

The optimization of selected excipients was done in the current study. Sodium chloride was fixed at 0.9 mg/mL as optimum osmogen, while sodium acetate and HPβCD were optimized from 0.01 and 0.5 mg/mL and 0.1 mg to 0.5 mg/mL respectively. Osmolality of solution for infusion should be isotonic and should be in range from 250 to 350mOsmo/kg, as per the literature and regulatory recommendation, while the pH of solution should be from 3 to 5. .

Table 6.3 : Development trials for selection of excipients for Oxytocin RTI

Compositions (Each mL contains)	Bag description	Form Code	Stability Station	Assay (%)	pH	Osmolarity
Oxytocin 0.02 IU, Sod acetate 0.5 mg, Sodium chloride 9 mg, HPβCD 0.5 mg Sodium Hydroxide q.s. to pH 4, WFI q.s.	PHC	F1	Initial	101.24±1.02	3.98±0.09	307±1.58
			25°C/40%RH-3M	97.76±1.01	3.97±0.08	306±1.93
	AOB		Initial	100.81±1.06	3.98±0.09	308±1.69
			25°C/40%RH-3M	97.58±1.07	4.0±0.06	305±1.13
Oxytocin 0.02 IU, Sod	PHC	F2	Initial	100.41±1.12	3.97±0.07	306±1.56

Compositions (Each mL contains)	Bag description	Form Code	Stability Station	Assay (%)	pH	Osmolarity
acetate 0.5 mg, Sodium chloride 9 mg, HPβCD 0.1 mg Sodium Hydroxide q.s. to pH 4, WFI q.s			25°C/40%RH-3M	96.57±1.31	3.98±0.08	307±1.61
	AOB		Initial	100.45±1.21	4.06±0.09	308±1.46
			25°C/40%RH-3M	97.23±1.14	4.10±0.08	311±1.63
Oxytocin 0.02 IU, Sod acetate 0.01 mg, Sodium chloride 9 mg, HPβCD 0.5 mg Sodium Hydroxide q.s. to pH 4, WFI q.s	PHC	F3	Initial	100.48±1.41	4.06±0.06	298±1.51
			25°C/40%RH-3M	97.66±1.19	4.10±0.06	299.±1.65
	AOB		Initial	99.02±1.16	4.06±0.09	296±1.87
			25°C/40%RH-3M	97.55±1.61	4.10±0.08	296±1.56
Oxytocin 0.02 IU, Sod acetate 0.01 mg, Sodium chloride 9 mg, HPβCD 0.1 mg Sodium Hydroxide q.s. to pH 4, WFI q.s	PHC	F4	Initial	100.06±1.09	3.99±0.05	294±1.34
			25°C/40%RH-3M	98.26±1.08	4.02±0.06	291±1.21
	AOB		Initial	99.48±1.27	4.02±0.06	294±1.67
			25°C/40%RH-3M	97.88±1.34	3.94±0.08	293±1.91

HCl: Hydrochloric acid

In previous section, the concentration of both sodium acetate and HPβCD was 1 mg/mL (FB-10). To optimize their concentration, we took trials for both in combination of 0.01 and 0.5 mg/mL and 0.1 mg and 0.5 mg /mL respectively. On the basis of results depicted in Table 6.3, all the formulations prepared with different concentration of HPβCD and Sod acetate combination have shown assay values within the limits in both bags at initial stage.

HPβCD was also evaluated to find its role in the stability of oxytocin. The effect can be clearly seen in the formulation with (F1-4) and without (F5, as mentioned in Table 6.3) HPβCD, where it increases the stability of oxytocin fter 3M at 25°C/40%RH. The assay of formulation

remained same when concentration of HPβCD increased from 0.1 to 0.5 mg/mL. Therefore, selection of lower concentration i.e., 0.1 mg/mL for further study was done.

6.1.5 Autoclave cycle study

Injectable drugs must be sterile, as they bypass the body's natural defence against pathogens. The product may be contaminated during manufacturing or filling, or the packaging could become compromised after sterile injectables are shipped. This means that the sterile injectable manufacturing process is subject to stringent regulation, and facilities that manufacture these drugs must be specially equipped for aseptic production. Injectable drugs can be made sterile in two ways: through an end-to-end aseptic manufacturing process or through terminal sterilization. Terminal sterilization often uses a steam autoclave to expose a drug to heat, killing any pathogens. These methods are used because they sterilize both the outside container/ prominently packaging container and its contents. However, in many of cases where the formulation is heat sensitive and sterilization at standard condition is not possible, there is a recommendation to evaluate the drug product according to below mentioned sterilization decision tree.

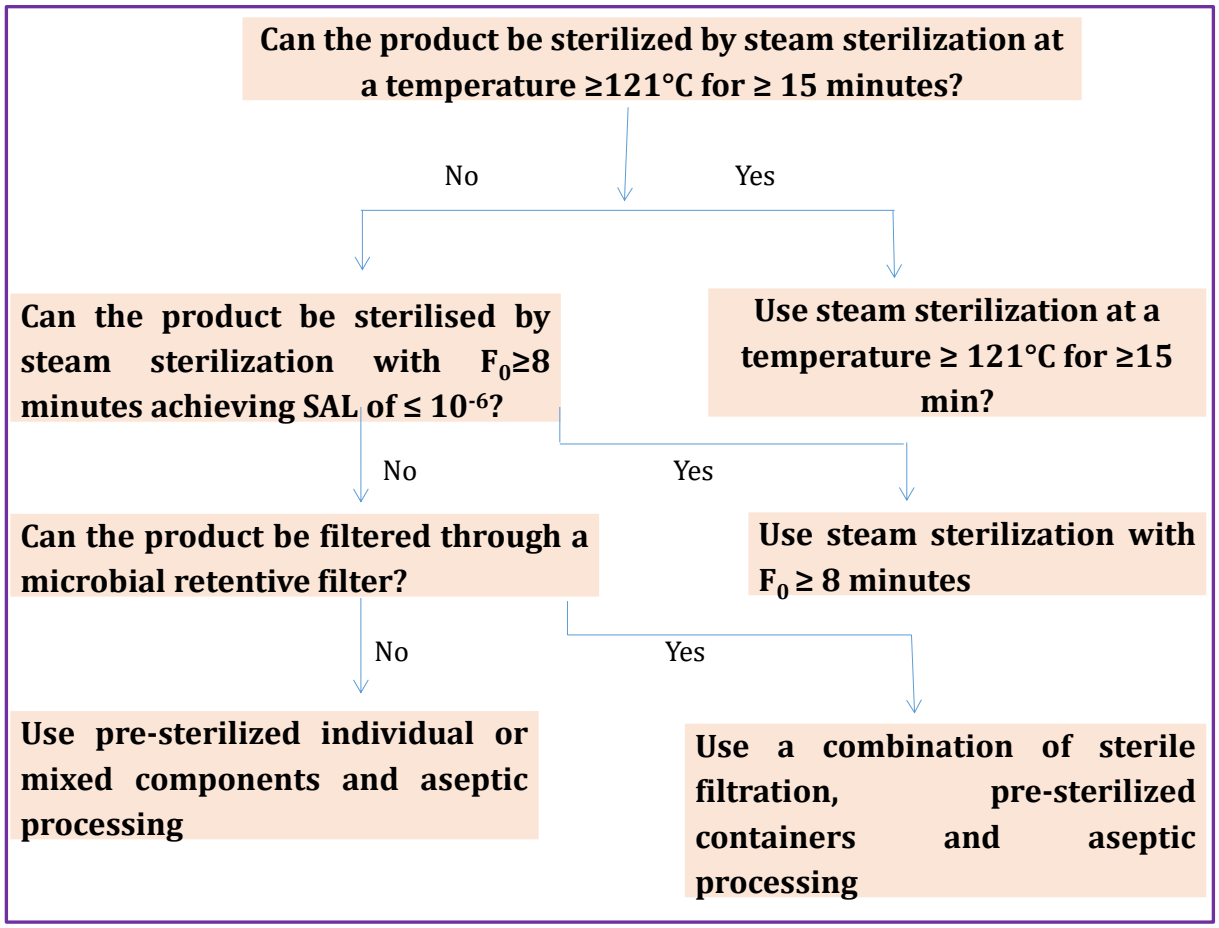


Figure 6.2: Decision tree for sterilisation choices for aqueous based products

In this regard, in order to evaluate a suitable sterilization method, Oxytocin RTI formulation was sterilized at different recommended sterilization conditions and analysed thereafter.

Table 6.4 :Effect of sterilization process on the Oxytocin RTI formulation

Compositions	Sterilization parameter	Assay (%)	pH	Osmolarity
Each mL contains: Oxytocin 0.02 IU/mL Sod Acetate 0.01 mg Sodium Chloride 9.0 mg HPβCD 0.1 mg GAA q.s. to adjusted pH WFI q.s.	**Initial (Un-autoclaved)**	100.19	4.05	295
	15 Min_121°C	64.12	4.04	295
	F0 12 Min_121°C	82.79	4.03	294
	F0 08 Min_121°C	85.56	4.06	295
	F0 08 Min_116°C	73.81	4.05	294
	F0 08 Min_111°C	30.21	4.05	295

In all sterilization conditioned recommended and mentioned above, the assay of Oxytocin in RTI formulation was found reduced. There was no effect on pH and osmolarity of the formulation. Therefore, utmost care should be taken in manufacturing, handling and packaging of Oxytocin formulation. Therefore, the process comprising sterile filtration, pre-sterilized container and aseptic processing was found suitable for further use.

6.1.6 Short term evaluation of optimized formulation

On the basis outcomes of optimization study, sucrose and sodium chloride were selected as an osmogens and sodium acetate was selected as buffering agent. Since, sodium chloride, in comparatively small concentration was able to provide optimum osmolarity, it was selected for further development. Additionally, there may be a probability of microorganism growth in the formulation over the period of time when develop with sucrose. Table 6.5 has shown the optimized composition. A short-term stability was also initiated with the formulation developed with optimized formulation.

Table 6.5: Composition of optimized Oxytocin formulation

Components	Acetate buffer with Sodium Chloride
Batch No	20202224PF097A
Oxytocin	0.02 IU/mL
Sod Acetate (anhydrous)	0.01 mg/mL
Sodium Chloride	9 mg/mL
HPβCD	0.1 mg/mL
Glacial acetic acid (GAA)	q.s. to adjust pH 4
Sodium Hydroxide	q.s. to adjust pH 4

The developed formulation was tested for different critical quality attributes till 6 months at 25 °C/40%RH and 2-8 °C. Formulation was found stable after 6M at both the storage conditions. The stability study's results are presentd in Table 6.6 and in Figure 6.3 to 6.5.

Table 6.6: Short term stability data of optimized Oxytocin RTI formulation

Condition	Assay	pH	Osmolality (mOsm/kg)	Particulate matter (PMT)		% Transmittance at 650 nm	Absorbance at 420 nm
				≥10µm	≥25µm		
Specification	**90-110**	**3-5**	**250-350**	**6000**	**600**	**>95**	**<1**
Initial	100.39±1.06	4.03	295±1.32	23.33±1.03	40.00±1.13	99.814±1.06	0.010±0.03
5 ± 3 °C_3M	99.90±1.07	4.00	290±1.41	22.66±2.81	13.00±0.41	99.937±1.07	0.011±0.004
5 ± 3 °C_6M	99.80±1.11	4.05	292±1.35	40.00±1.56	20.0±0.23	99.377±1.67	0.01±0.003
25 °C / 40%RH_1M	99.20±1.23	4.04	291±161	73.00±4.05	7.00±0.03	99.944±1.45	0.01±0.002
25 °C / 40%RH_3M	98.13±1.46	4.03	296±1.23	20.67±3.41	66.67±0.12	98.844±1.89	0.01±0.001
25 °C / 40%RH_6M	97.05±1.18	4.02	291±1.56	22.00±3.67	3.20±0.03	99.073±1.13	0.012±0.003
30 °C / 65%RH_3M	96.47±1.61	4.03	292±1.07	180.00±2.87	7.00±0.03	100.210±1.21	0.01±0.001
30 °C / 65%RH_6M	93.89±1.34	4.06	293±1.98	80.00±2.76	2.00±0.08	99.570±1.56	0.0±0.00

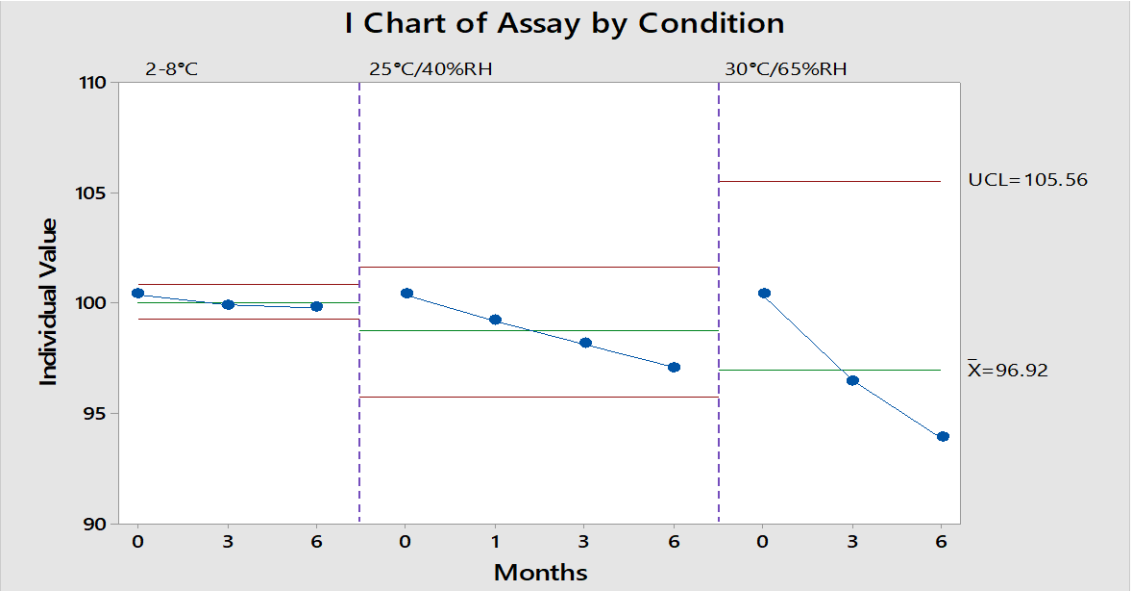


Figure 6.3: Short term Stability Plot of Assay at different conditions and at different time point for optimized Oxytocin formulation

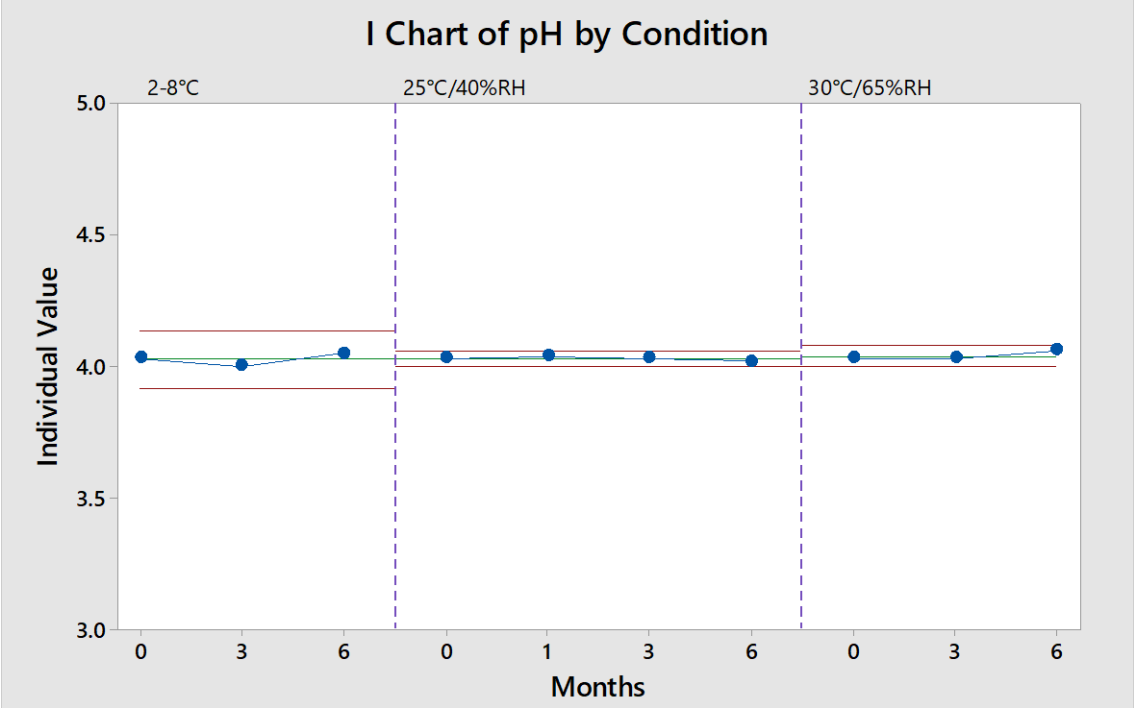


Figure 6.4: Short term stability plot of pH at different conditions and at different time point

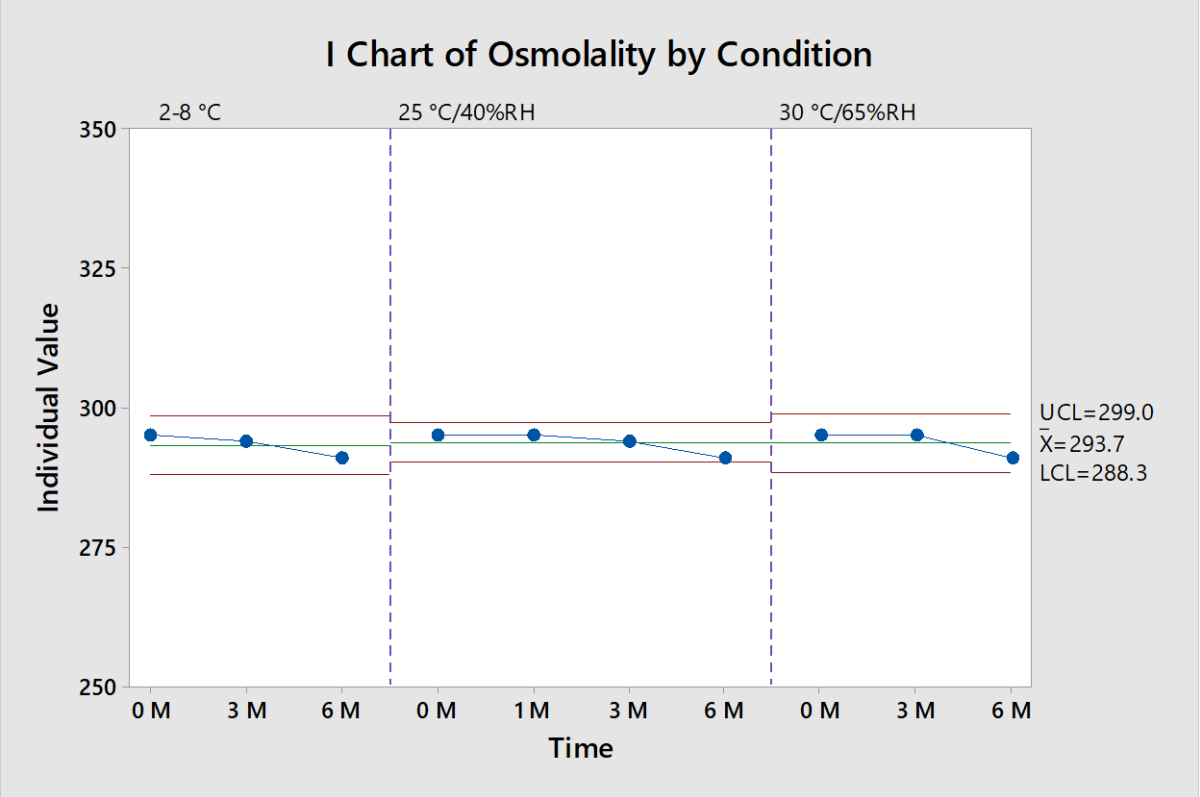


Figure 6.5 : Short term stability plot of osmolarity at different conditions and at different time point

6.1.7 pH Stability Study

Stability of any aqueous formulation depends on its pH. For a stable product, the pH of formulation should be within limit during stability studies [6]. Before initiation of long-term stability of optimized formulation and prototype finalization, a pH stability study was conducted to optimize the suitable pH of formulation. With the optimized composition, five formulations of different pH (5.0, 4.5, 4.0, 3.5 and 3.0) were prepared by method described in section 4.3.1.1. Formulations were tested at initial stage and for 3M at 25°C/40%RH. After 3M, results were compared with their respective initial results (Table 4.7; Figure 4.1 and 4.2). pH variation of formulation over the period may change the physical and chemical properties of oxytocin RTI formulation. So, as per Pharmacopeial recommendation, in addition to assay, pH and osmolality, PMT, absorbance and transmittance as CQAs in all pH stability studies were considered. Initially, there were no remarkable difference in assay values of all formulations, formulated at different pH. However, over the period of 3M at 25°C/40%RH, the assay value was decresed remarkably at pH 3.0 and 5.0. The formultion prepared at pH 4.0 had shown better stability after 3M among all tested formulations.

.

Table 6.7: Experimental trials at different pH

Formulation Code	Sample pH (Target)	Batch No	Stage	Assay (%)	pH (Observed)	Osmolarity (mOsm/kg)	PMT		% Transmittance at 650 nm	% Absorbance at 420 nm
							≥10µm	≥25µm		
Specification	3-5			90-110		250-350	6000	600	>95	<1
FD-1	5.0	FP094B	Initial	98.37±1.08	4.88±0.06	284±1.34	87.33±1.56	13.33±1.09	99.656±0.09	0.01±0.003
	5.0	FP094B	25°C/40%RH-3M	94.58±0.82	4.93±0.09	283±1.21	66.5±2.34	15.6±0.61	99.656±0.06	0.012±0.001
FD-2	4.5	FP094C	Initial	99.52±1.03	4.44±0.11	287±1.82	37.33±2.15	3.66±1.34	99.446±0.05	0.04±0.002
	4.5	FP094C	25°C/40%RH-3M	97.58±1.02	4.48±0.09	290±1.73	11.6±0.94	6.87±2.05	99.656±0.04	0.011±0.002
FD-3	4.0	FP094D	Initial	99.78±1.01	3.91±0.06	292±1.89	56.67±2.31	6.67±2.14	99.408±0.08	0.01±0.003
	4.0	FP094D	25°C/40%RH-3M	98.66±1.06	3.97±0.09	293±1.09	10.98±2.76	5.76±1.76	99.656±0.07	0.02±0.001
FD-4	3.5	FP094E	Initial	98.42±1.24	3.41±0.05	294±2.14	58.76±1.81	6.67±2.87	99.370±0.12	0.013±0.004
	3.5	FP094E	25°C/40%RH-3M	93.43±1.32	3.46±0.04	296±.2.36	12.76±2.01	11.31±2.07	98.67±0.15	0.03±0.003
FD-5	3.0	FP094F	Initial	98.25±1.16	2.92±0.02	339±2.94	30.54±1.52	14.76±3.54	99.415±0.19	0.014±0.001
	3.0	FP094F	25°C/40%RH-3M	89.5±1.11	2.94±0.03	344±2.87	56.87±2.02	6.4±2.86	99.670±0.23	0.016±0.002

Initialy, there were no remarkable difference in the assay values of all the formulation of different pH. However, over the period of 3M at 25°C/40%RH, the assay value was decresed remarkably at pH 3.0 and 5.0. The formultion prepared at pH 4.0 had shown better stability after 3M among all tested formulation.

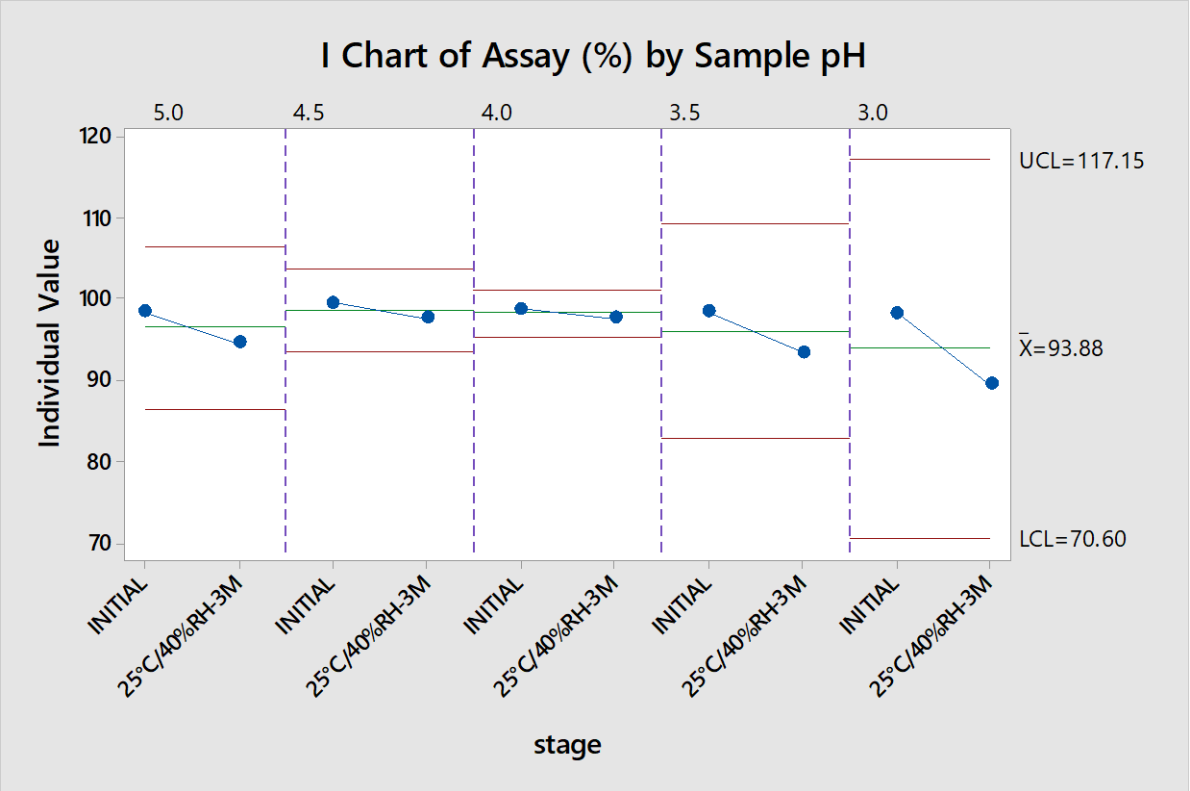


Figure 6.6: Assay of Oxytocin in RTI formulation at different pH at Initial level and after 3M at 25 °C/40% RH

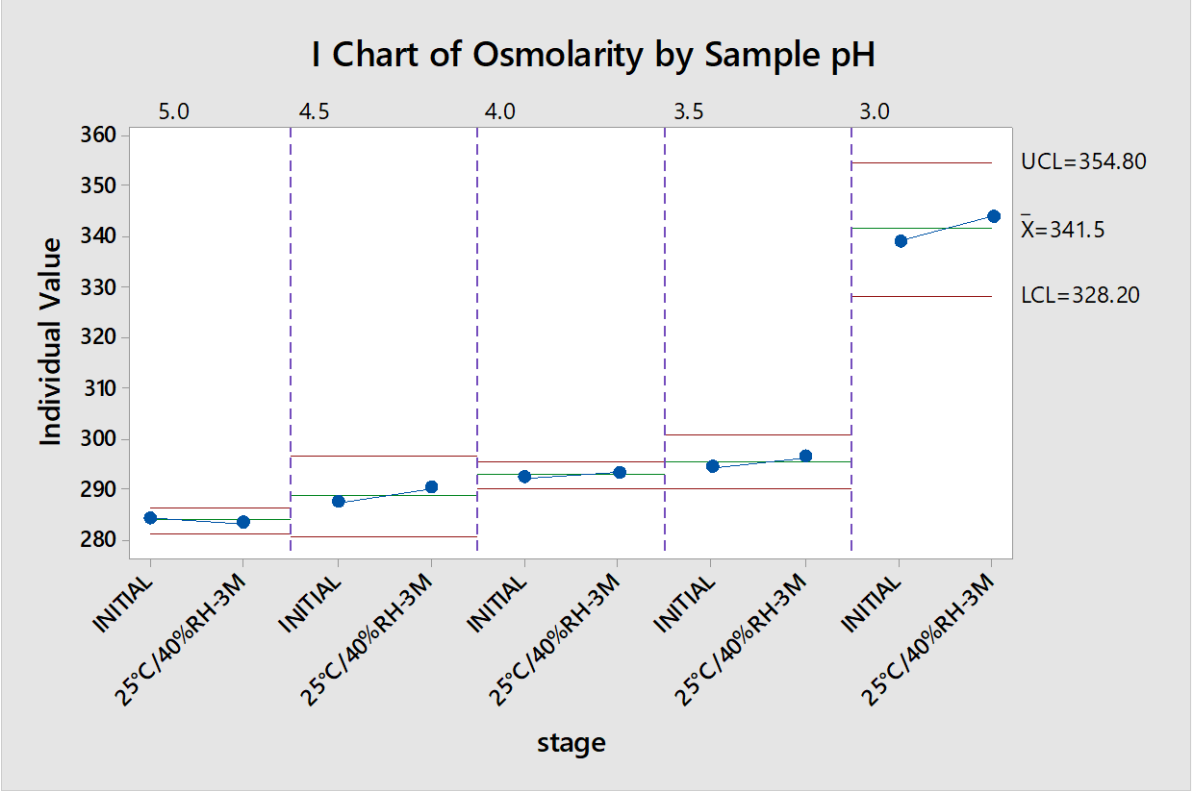


Figure 6.7: Osmolarity of Oxytocin RTI formulation at different pH at Initial level and after 3M at 25 °C/40% RH

6.1.8 Temperature cycling study

Freeze thaw study is important to find out the excursion storage condition during transport. Moreover, sometimes, products may come in contact in higher and lower temperature during storage and transport. Therefore, we need to evaluate developed products to ensure the drug product quality, efficacy, and safety are not compromised when materials are subjected to short term temperature excursions from intended storage that may occur during e.g., shipping, transport, or patient use. RTI formulations of Oxytocin were placed on stability storage at -20°C for 24 h. Samples were then transferred to 40°C / 75%RH for 24 h. Procedure was repeated for a total of 14 cycles. Samples were analysed for Critical quality attributes (CQAs), assay, pH and osmolality after 7 and 14 cycles respectively. The outcomes after 7 and 14 cycles were compared with initial results (Table 6.8 and Figure 6.8). .

Table 6.8: Experimental data of freeze thaw cycle studyFormulation Code	**Compositions (Each mL Contains)**	**Batch No**	**Stage**	**Assay (%)**	**pH**	**Osmolarity**

FD-3	Oxytocin 0.02 IU, Sod Acetate 0.01 mg, Sodium Chloride 9.0 mg, HPβCD 0.1 mg, GAA q.s. to adjust pH, WFI q.s.	PF094D	INITIAL	**98.78**	**3.9**	**292**
		PF094F	**After 7 Cycles**	97.33	3.87	-
		PF094F	**After 14 Cycles**	98.05	3.85	-

There was no remarkable difference in the assay value of the formulation after 7 and 14 days of study. This signifies the robustness and stability of developed formulation.

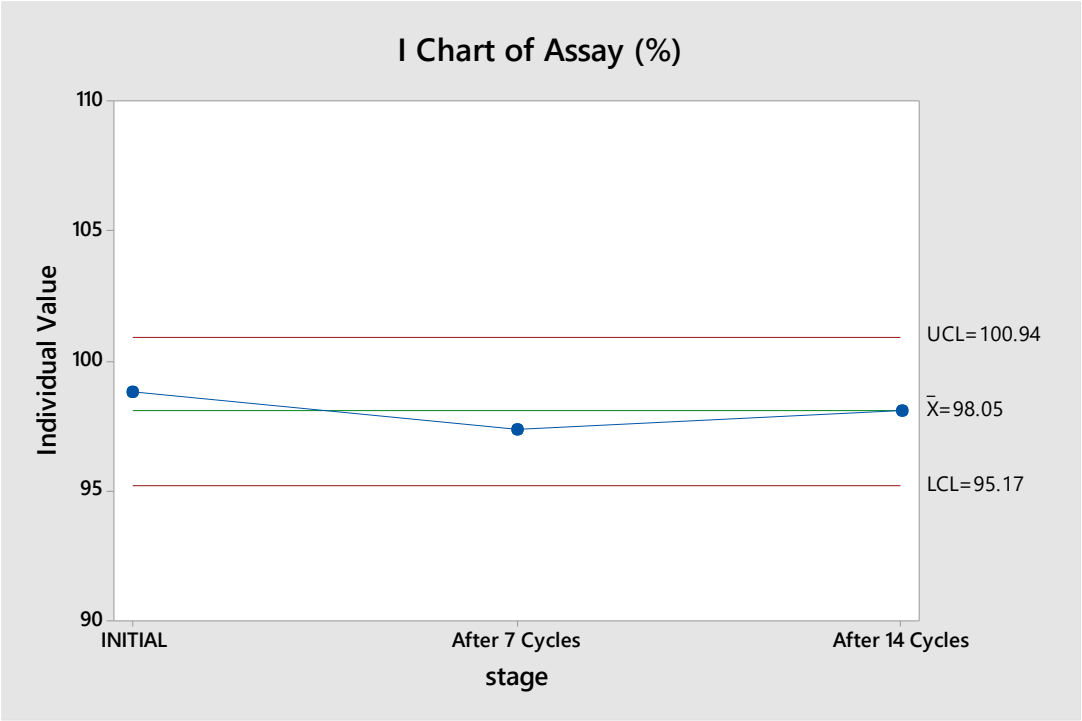


Figure 6.8: Assay of Oxytocin in RTI formulation at after different freeze (-20 °C) thaw (+40 °C) cycles

6.1.9 Oxygen Sensitivity Study

Oxygen is the second leading cause for qualitative and quantitative degradation of pharmaceuticals, water being the first. Utmost care was taken while the selection of packaging. Since packaging material is the primary resource used to protect drugs from the negative effects of oxygen. Apart from that there are things to consider during development and manufacturing to make sure excess oxygen isn't simply packaged or bottled with the drug product. During

research and development, formulations must be tested and characterized for long-term stability when exposed to oxygen and incorporated into the Critical Quality Attributes (CQAs) for the drug product. If oxygen exposure is a CQA there are ways to mitigate the impact and there are well-proven best practices that can be applied while manufacturing and liquid fill-finish. Therefore, effect of different gases was evaluated on the stability of developed RTI formulation of Oxytocin. Three formulations were prepared using aforementioned procedure and in the presence of different gases viz air, nitrogen and oxygen. The formulations were filled and packed in the respective environment they prepared in. All the formulations were tested at initial stage and were kept on stability at 25°C/40%RH for 3M. After 3M, samples were withdrawal and analysed. Results of the study were presented in the Table 6.9 and in Figure 6.9.

Table 6.9: Effect of different gases on Oxytocin assay

Formulation Code	Compositions (Each mL Contains)	Sample description	Batch No	stage	Assay (%)	pH	Osmolarity
FD-6	Oxytocin 0.02 IU, Sod Acetate 0.01 mg, Sodium Chloride 9.0 mg, HPβCD 0.1 mg, GAA q.s. to adjust pH, WFI q.s	Nitrogen	**PF097A**	INITIAL	100.39	4.03	295
		Nitrogen	**PF097A**	25°C/40%RH-3M	98.13	4.03	NP
FD-7		Air	**PF097B**	INITIAL	101.49	4.03	290
		Air	**PF097B**	25°C/40%RH-3M	85.82	4.03	NP
FD-8		Oxygen	**PF097C**	INITIAL	100.74	4.03	288
		Oxygen	**PF097C**	25°C/40%RH-3M	46.06	4.03	NP

All the formulations were tested at initial stage and were kept on stability at 25°C/40%RH for 3M. After 3M, samples were withdrawn and analysed. Results of the study are presented in Table 6.9 and Figure 6.9.

The developed formulation was found to be stable in presence of nitrogen. However, formulations, prepared in presence of air and oxygen had shown prominent degradation with most significant degradation observed in formulation that was prepared in presence of oxygen. The outcome the current study will help to control process during formulation by continuous

N_2 purging and secondary packaging in which oxygen scavenger can be used to reduce O_2 present between primary packaging and secondary packaging material.

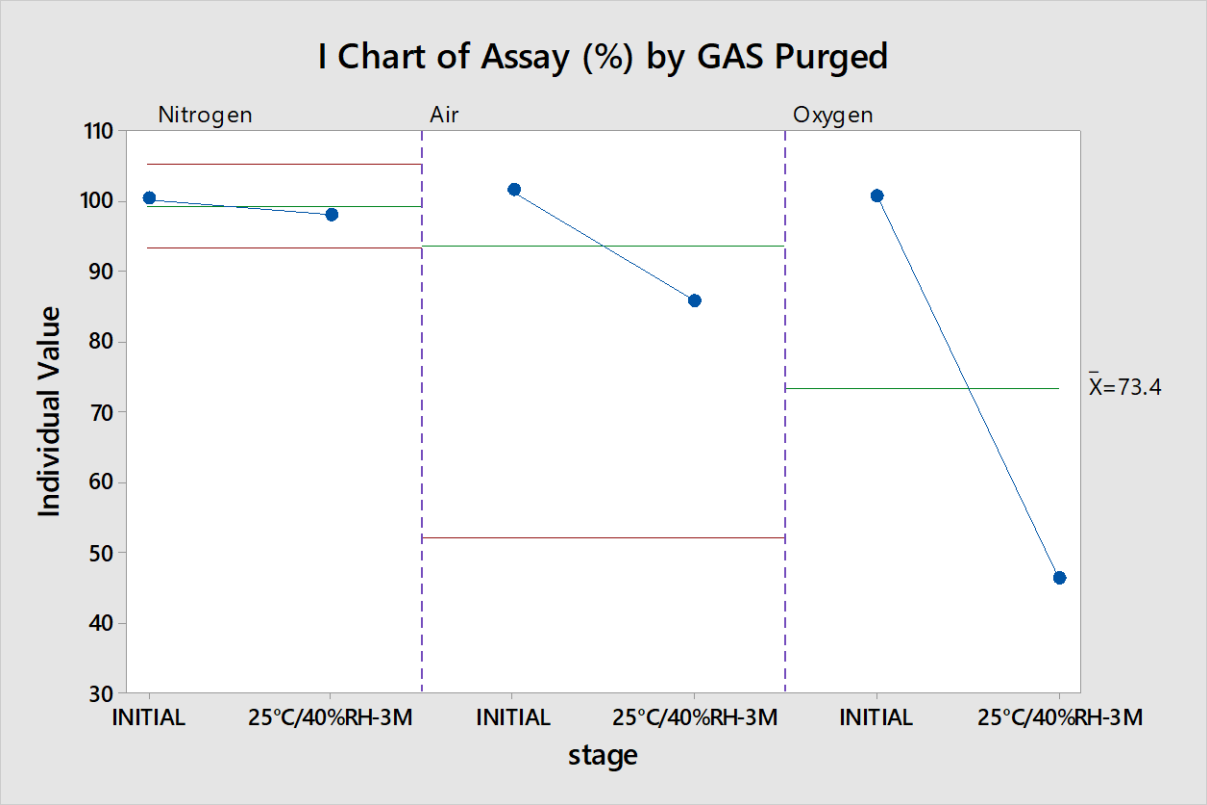


Figure 6.9: Sensitivity to gas: Assay of Oxytocin in RTI formulation after purged with different gases

6.1.10 Photostability study for Oxytocin RTI

Photostability testing was undertaken to evaluate the overall photosensitivity of the pharmaceuticals for the development and validation purposes and to provide information necessary for manufacturing, handling, packaging, and labelling. USA FDA in 1996 issued ICH guidance Q1B for industry and stated that "the intrinsic photostability characteristics of new drug substances and products should be evaluated to demonstrate that, as appropriate, light exposure does not result in unacceptable change". In this photostability testing was recommended to be carried out as Oxytocin was prone to photodegradation. In this regard a photostability study was conducted to evaluate the photosensitivity of oxytocin RTI formulation. Oxytocin RTI samples (in infusion bag, in final pack and a control sample) were placed in the photostability chamber and exposed to light of 20 million lux. After exposure, the samples were withdrawn and analyzed.

Table 6.10: Effect of light on the assay of Oxytocin

Compositions (Each mL)	Sample description	Assay (%)	pH	Osmolarity
Oxytocin 0.02 IU, Sod Acetate 0.01 mg, Sodium Chloride 9.0 mg, HPβCD 0.1 mg, GAA q.s. to adjust pH, WFI q.s.	Controlled (Infusion bag wrapped in aluminium sheet)	100.19	4.05	295
	Market pack (Infusion bag placed inside the overwrap pouch)	100.13	4.04	295
	Direct exposed (infusion bag only)	94.49	4.03	294

The assay of Oxytocin in RTI formulation was found reduced when it was exposed to light. There was no effect on pH and osmolarity of the formulation. Therefore, utmost care should be taken during manufacturing, handling and packaging of Oxytocin formulation.

6.1.11 Formulation and Stability study of optimized RTI formulation of Oxytocin

The stability of any pharmaceutical product is usually defined as the capacity of the formulation to remain within defined limits over a predetermined period of time (shelf life of the product). Durability of a product may be defined as the capability of a particular formulation in a specific container to remain within the physical, chemical, microbiological, therapeutic and toxicological specifications. Storage stability of formulation based on drug in drug product is of great concern as it is the principal requirement in the development of any formulation. Hence, a well-designed stability-testing plan is essential for the evaluation of RTI formulation of Oxytocin. The prepared RTI formulations were tested for stability on storing them at 5°C ±3°C and 25°C/40% RH.

RTI formulations were filled in non-PVC infusion bags and subsequently stoppered. Bags per then overwrapped in aluminium pouches and labelled. The packed formulations were placed in 5°C ±3°C and 25°C/40% RH stability chamber. At selected intervals the samples were analysed for %drug assay, pH, osmolarity, particulate matters, %transmittance and absorbance.

6.1.11.1 Formulation of oxytocin

I. **<u>Preparation of bulk solution and filling</u>**:

i. Appropriate quantity of water for injection (60-80% of batch size) was collected in a container, and nitrogen was purged to reduce the dissolved oxygen (DO) level below 1 ppm.

ii. Accurately weighed quantity of Sodium Acetate was added and dissolved completely, followed by measuring the pH of the solution.

iii. Accurately weighed quantity of HPβCD was added and dissolved under stirring condition with measurement of the pH.

iv. Accurately weighed quantity of Sodium Chloride was added and dissolved under stirring respectively as in above table, with measurement of the pH;

v. The pH of the solution was adjusted to 4.0 using gradual addition of glacial acetic acid (10%) or sodium hydroxide (10%).

vi. Calculated quantity of oxytocin stock was added with continuous nitrogen purging;

vii. Volume was made up to the final batch size with water for injection and mixed well under stirring.

viii. The solution was aseptically filtered through a 0.2 µm filter and filled into infusion bags.

ix. Nitrogen was continuously purged during the course of the process to maintain the DO level below 1 ppm with continuous nitrogen purging; and Stoppered bag immediately.

II. **<u>Overwrap</u>**: Infusion bags were overwrapped with nitrogen blanketing and an oxygen scavenger.

Table 6.11: Sample composition with various excipients

Components	Each ml contains
Oxytocin (USP)	0.02/0.08 IU
Sodium chloride	9 mg
HPβCD	0.1 mg
Sodium Acetate	0.1 mg
Glacial acetic acid/ Sodium Hydroxide	q.s. to pH
Water for injection	q.s

6.1.12 Stability study with lower strength (0.02 IU/mL)

Table 6.12: Stability data of Oxytocin injection RTI 0.02 IU/mL

Stage	Assay (%)	pH	Osmolality (mOsm/kg)	PMT		% Transmittance at 650 nm	Absorbance at 420 nm	Sterility
				≥10µm	≥25µm			
Specification	90-110	3-5	250-350	6000	600	>95	<1%	No evidence of microbial growth should be found
Initial	102.2±1.76	4.01±0.03	290±2.67	33.00±0.03	13.00±0.03	99.467±1.10	0.015±0.01	Complies
5 ± 3°C-3M	98.33±1.03	4.12±0.04	288±1.13	53.33±0.03	0.00±0.03	99.23±0.03	0.03±0.01	-
5 ± 3°C-6M	97.99±1.09	4.06±0.08	285±1.18	33.00±0.03	7.00±0.03	99.467±0.04	0.012±0.01	Complies
5 ± 3°C-9M	97.52±1.15	4.12±0.06	287±1.23	49.33±0.03	3.70±0.03	99.835±0.05	0.0±0.00	Complies
5 ± 3°C-12M	97.27±1.10	4.00±0.09	291±1.23	30.70±0.03	2.00±0.03	99.944±0.06	0.0±0.00	Complies
5 ± 3°C-24M	95.73±0.95	4.10±0.07	295±2.15	23.33±0.03	3.33±0.03	99.853±0.07	0.0±0.00	Complies
25°C/40%RH-1M	100.63±0.91	4.03±0.08	284±2.00	48.7±0.03	00±0.03	99.700±0.08	0.01±0.00	Complies
25°C/40%RH-3M	98.22±1.05	4.05±0.06	288±2.15	53.33±0.03	6.67±0.03	99.965±0.07	0.0±0.00	Complies
25°C/40%RH-6M	95.53±1.01	4.12±0.09	285±2.13	72.3±0.03	00±0.03	99.772±0.04	0.015±0.00	Complies
25°C/40%RH-9M	94.11±1.03	4.18±0.05	287±2.13	67.77±0.03	6.7±0.03	99.921±0.07	0.0±0.00	Complies
25°C/40%RH-12M	93.13±1.01	4.03±0.05	293±1.95	33±0.03	0.0±0.03	99.512±0.05	0.012±0.01	Complies
30°C/65%RH-3M	95.33±1.06	4.07±0.07	287±1.65	47.7±0.03	7±0.03	99.667±0.06	0.011±0.01	Complies
30°C/65%RH-6M	91.09±1.06	4.05±0.08	286±1.76	43.33±0.03	0.67±0.03	99.872±0.05	0.010±0.01	Complies
Note: Extrapolation and stability study report generated for 25°C/40%RH Container content @ initial, 3M, 6M & 12 M & 12M done and found NLT 100mL								

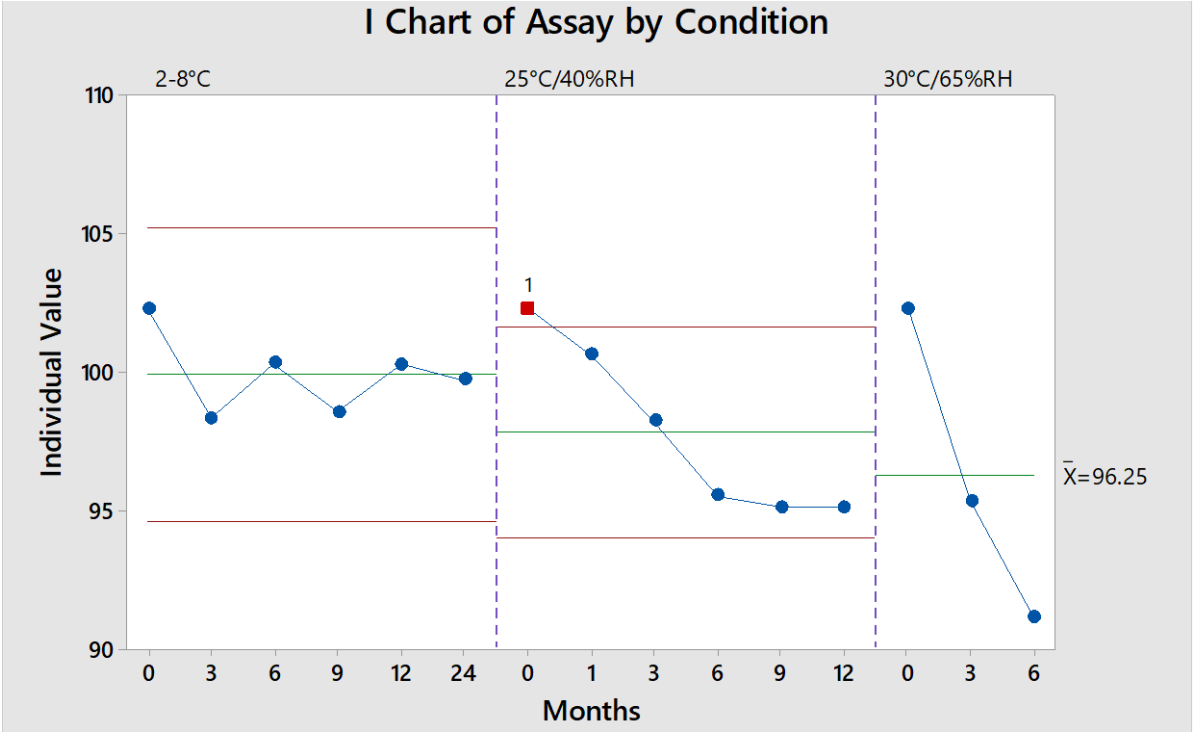


Figure 6.10: Assay of Oxytocin at different stability conditions and time points

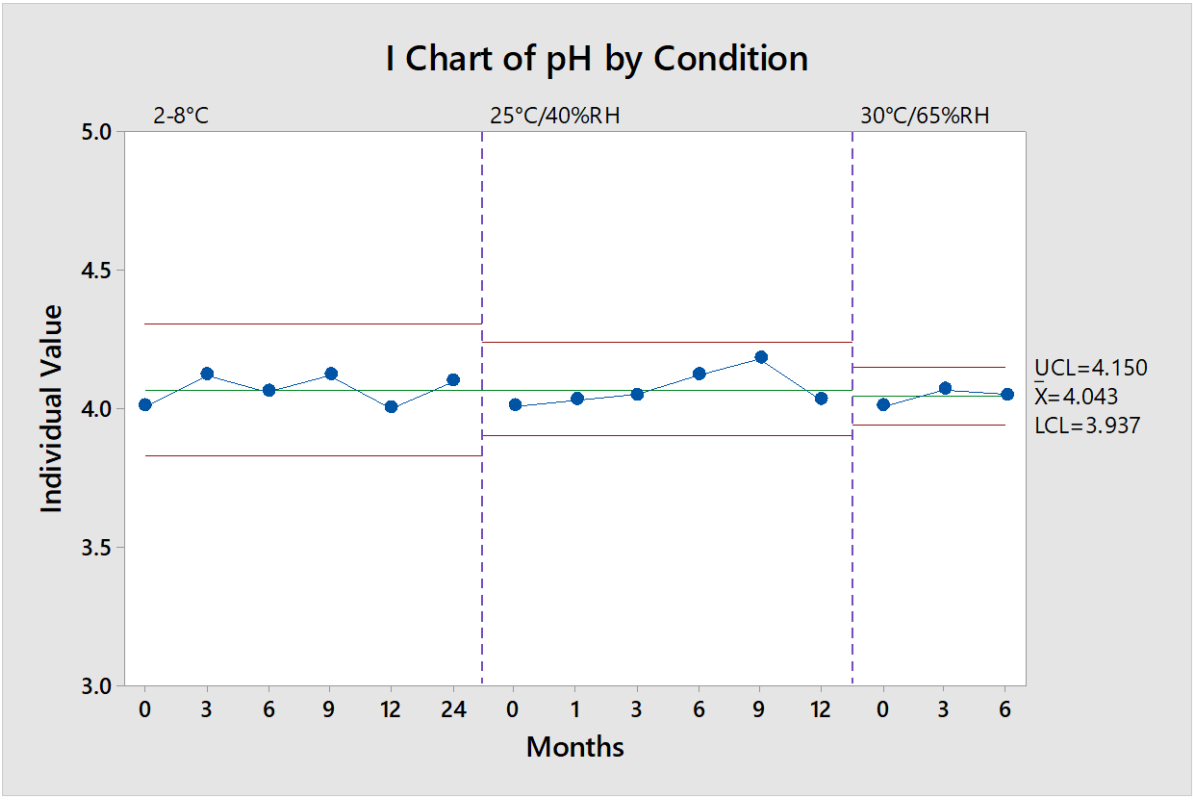


Figure 6.11: pH of Oxytocin formulation at different stability conditions and time points

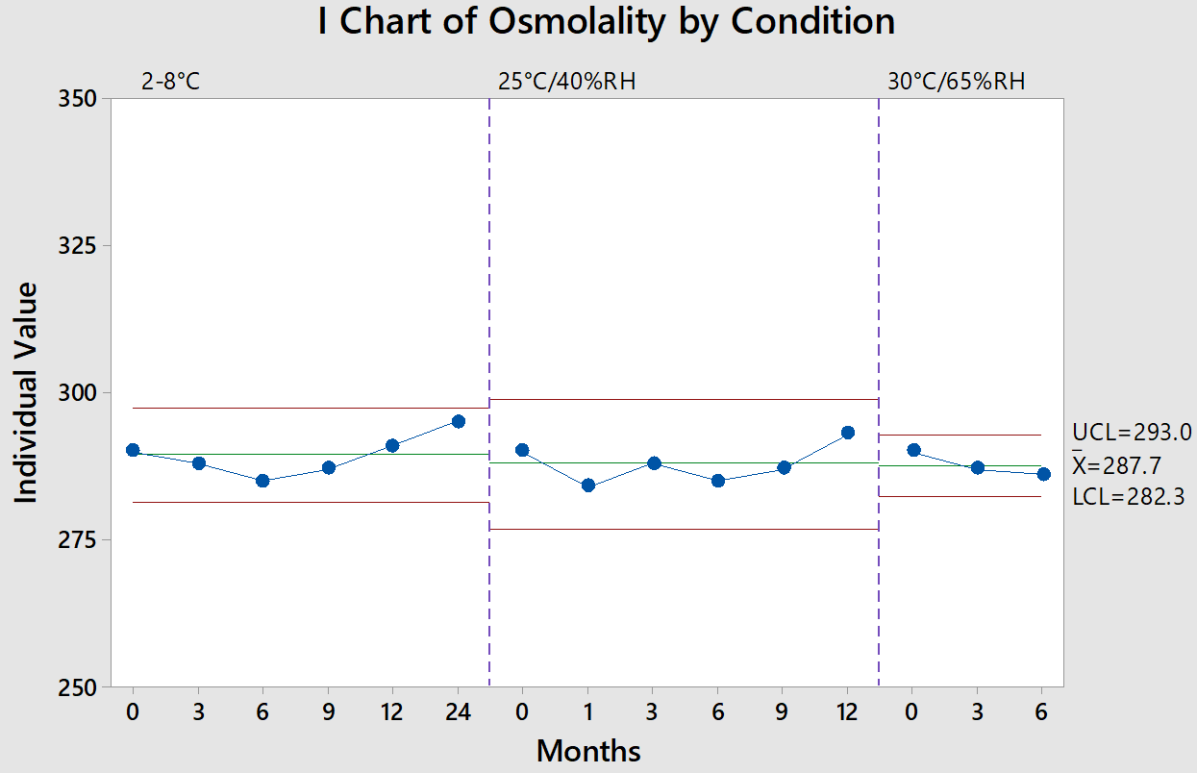


Figure 6.12: Osmolarity of Oxytocin formulation at different stability conditions and time points

The stability of the API in drug formulations is a significant concern, as it is a key requirement in the formulation development process. Therefore, a thorough stability testing plan was devised, where the manufactured RTI formulations were evaluated for storage stability at 5°C ±3°C and at 25°C/40% relative humidity, in accordance with ICH guidelines.

RTI formulations were filled in PHC bag (non-PVC infusion) and subsequently stoppered. Bags were then overwrapped in aluminium pouches followed by labelling. The packed formulations were subjected to storage stability in 5°C ±3°C and 25°C/40% RH stability chamber. At selected intervals, the samples were analysed for %drug assay, pH, osmolarity, particulate matters, %transmittance and absorbance at 420 nm. The analytical test results for RTI formulation are illustrated in Table 6.12, and Figure 6.10 to 6.12.

The stability evaluation of the oxytocin RTI formulation, set at a concentration of 0.02 IU/mL, was meticulously conducted under specific environmental conditions: 5 ± 3 °C, 25°C with 40% relative humidity, and 30°C with 65% relative humidity, all over a period of six months. The stability data gathered was subsequently subjected to comprehensive analysis using Minitab, allowing for an in-depth assessment of the formulation's integrity over time. The RTI formulations were systematically stored at the designated temperatures and humidity levels for the stability studies, during which they were rigorously tested for several critical parameters,

including assay potency, pH level, osmolality, PMT, percentage of absorbance, percentage of transmittance, and sterility. The evaluation of the formulation's storage conditions involved analysing the percentage assay at specified time intervals, revealing important insights into its stability.

Remarkably, the percentage of assay was found to decline from an initial value of 102.2±1.76 to 95.73±0.95 after 24 months at the controlled temperature of 5±3 °C. In contrast, the assay value showed a decrease to 93.13±1.01 after 12 months in the 25°C/40%RH environment, and further declined to 91.09±1.06 after just six months at the 30°C/65%RH condition. Notably, the oxytocin formulation displayed no significant changes when stored at 5 ± 3 °C, whereas the assay values at 25°C/40%RH and 30°C/65%RH indicated measurable changes over six months. Throughout the stability period, the effects of varying environmental conditions on the assay stability were closely monitored, revealing that the percentage assay consistently decreased under the higher temperature and humidity conditions of 25°C/40%RH and 30°C/65%RH. Conversely, the formulation stored at 5 ± 3 °C exhibited minimal degradation, suggesting that maintaining this temperature is effective in preserving the integrity of the formulation.

Additionally, there were no noteworthy differences in the initial baseline measurements of osmolality, PMT, percentage absorbance, percentage transmittance, and sterility when compared to the values recorded throughout the various stability conditions. Thus, the data gathered from the stability testing strongly indicated that the RTI formulations of oxytocin stored at the cooler temperature of 5±3 °C exhibited a significantly higher degree of stability over time.

6.1.13 Stability study with higher strength (0.08 IU/mL)

In the same line, stability study of Oxytocin injection of higher strength was also carried out. The analytical outcomes of 0.08 IU/mL RTI formulation of oxytocin are presented below (Table 6.13 and Figure 6.13 to 6.15).

The stability evaluation of the oxytocin RTI formulation (0.08 IU/mL) was conducted at 5 ± 3 °C, 25°C/40%RH, and 30°C/65%RH over a period of 6 months. The stability data obtained was analysed using Minitab to assess its relation to time. RTI formulations were maintained at 5 ± 3 °C, 25°C/40%RH, and 30°C/65%RH for stability analysis, and were examined for assay, pH, osmolality, PMT, % absorbance, % transmittance, and sterility. The formulation's storage conditions were evaluated by analysing the percentage of assay after designated time intervals.

The percentage of assay changed from 100.68±1.06 to 98.66±1.67 after 24 months at 5±3 °C, while it was 96.39±1.41 after 12 months at 25°C/40%RH and 93.78±1.14 after 6 months at 30°C/65%RH. Oxytocin exhibited no significant changes at 5 ± 3 °C, whereas changes in assay were noted at 25°C/40%RH and 30°C/65%RH after 6 months.

The effects of different conditions on the assay were noted throughout the stability period, while others remained unaffected. The percentage assay was observed to decline when stored at 25°C/40%RH and 30°C/65%RH. This degradation effect was minimal for the formulation kept at 5±3 °C, suggesting that maintaining the formulation at this temperature can help control degradation. There were no significant differences in the initial values of osmolality, PMT, % absorbance, % transmittance, and sterility when compared to the values noted throughout the stability testing under all storage conditions. Therefore, the stability testing results indicated that the RTI formulations of oxytocin stored at 5±3 °C exhibited greater stability

Table 6.13: Stability data of 0.08 IU/mL

Stage	Assay (%)	pH	Osmolality (mOsm/kg)	PMT (Per container)		% Transmittance	Absorbance ≥10µm	Sterility
				≥10µm	≥25µm			
Specification	90-110	3-5	250-350	6000	600	>95	<1%	No evidence of microbial growth should be found
Initial	100.68±1.06	4.02±0.04	290±1.06	53.3±0.08	0.0±0.00	99.76±0.03	0.011±0.001	Complies
5 ± 3°C-3M	98.83±1.13	4.14±0.08	291±1.06	33.3±0.13	0.33±0.01	99.65±0.03	0.012±0.001	Complies
5 ± 3°C-6M	100.38±1.51	4.11±0.06	289±1.13	66.7±1.21	6.7±0.04	100.0±0.03	0.0±0.001	Complies
5 ± 3°C-9M	97.37±1.31	4.07±0.07	287±1.91	29.3±1.34	3.67±0.02	99.63±0.03	0.015±0.001	Complies
5 ± 3°C-12M	101.80±1.21	4.13±0.08	286±1.14	70.0±1.22	10±0.01	99.44±0.03	0.016±0.001	Complies
5 ± 3°C-24M	98.66±1.67	4.13±0.09	297±1.17	23.2±1.21	7.0±0.06	99.36±0.03	0.023±0.00	Complies
25°C/40%RH-1M	101.98±1.01	4.05±0.05	289±1.18	20.0±1.81	0.67±0.03	99.69±0.01	0.010±0.00	Complies
25°C/40%RH-3M	97.39±1.09	4.08±0.07	288±2.01	33.1±1.72	0.0±0.07	99.99±0.01	0.01±0.00	Complies
25°C/40%RH-6M	97.72±1.06	4.12±0.08	286±1.12	40.0±1.62	0.33±0.00	99.27±0.01	0.019±0.00	Complies
25°C/40%RH-9M	94.26±1.32	4.11±0.06	289±1.16	47.7±1.61	0.67±0.01	99.38±0.01	0.01±0.0	Complies
25°C/40%RH-12M	96.39±1.41	4.14±0.07	290±1.15	47.7±1.82	7.0±0.02	99.24±0.02	0.01±0.00	Complies
30°C/65%RH-3M	93.00±1.21	4.09±0.05	288±1.17	33.0±1.13	0.0±0.00	99.76±0.01	0.01±0.00	Complies
30°C/65%RH-6M	93.78±1.14	4.07±0.05	286±1.18	6.77±1.13	0.33±0.01	99.98±0.03	0.01±0.00	Complies

Note: Extrapolation and stability study report generated for 5 ± 3°C & 25°C/40%RH.

- Container content @ initial, 3M, 6M & 12 M & 12M done and found NLT 100mL

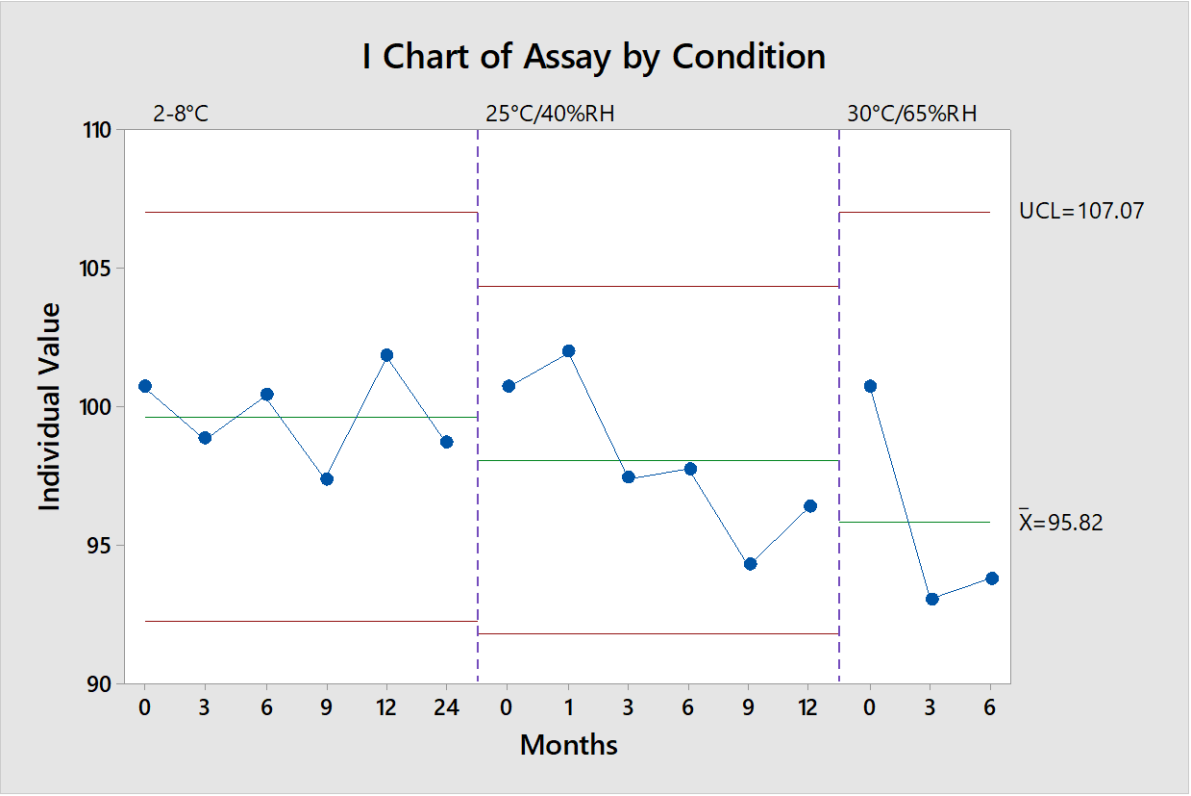


Figure 6.13: Assay of Oxytocin in final formulation at different stability conditions and time points

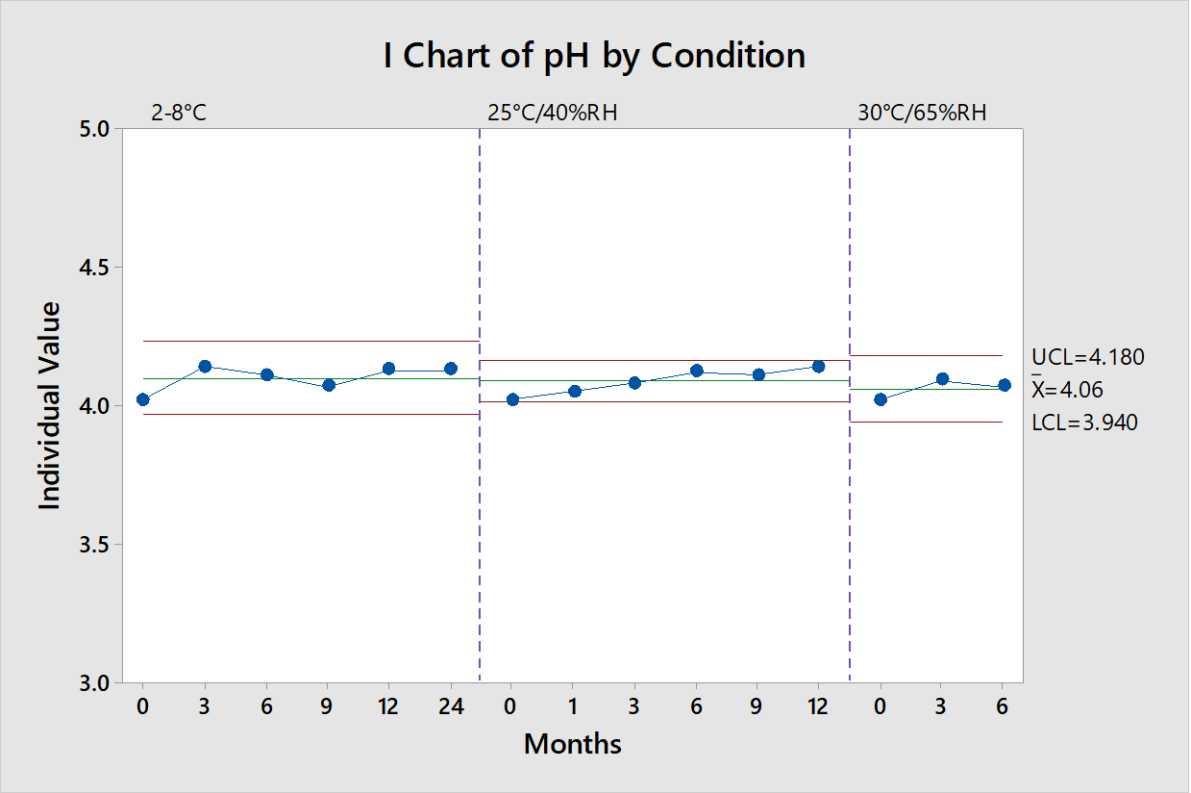


Figure 6.14: pH of final formulation at different stability conditions and time points

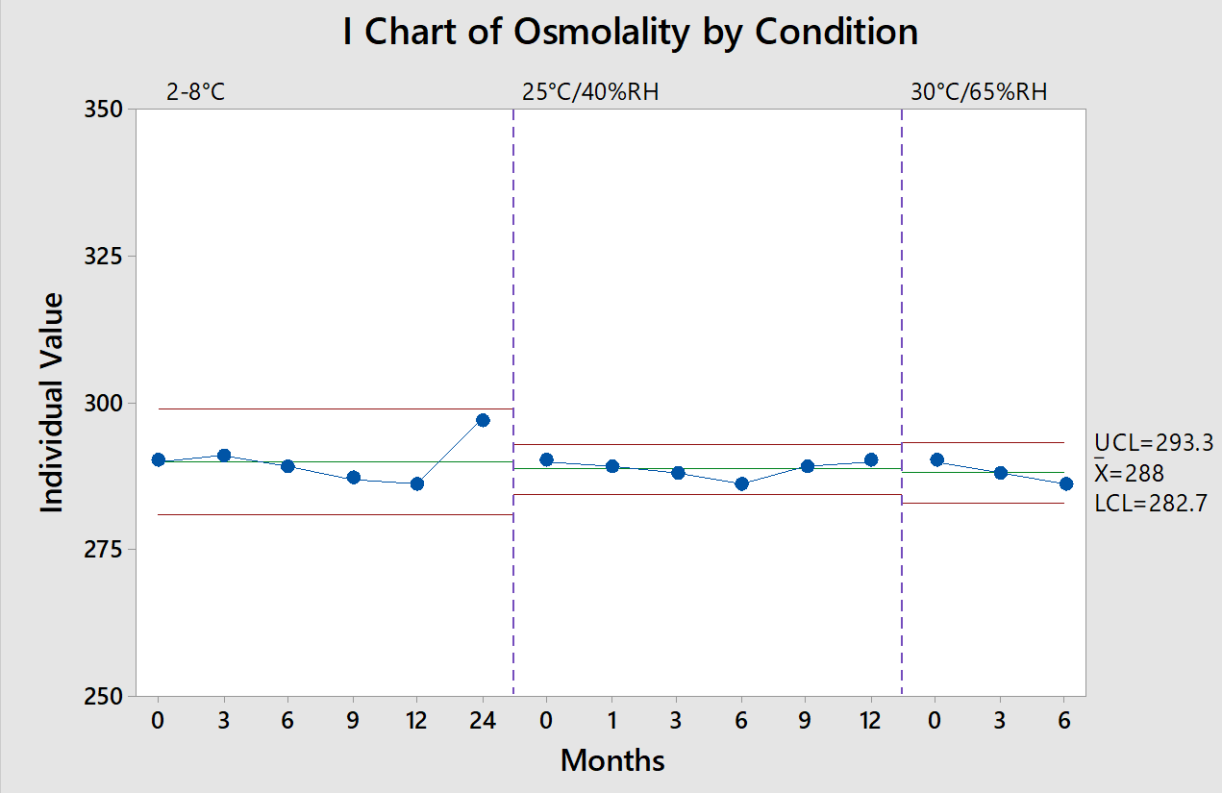


Figure 6.15: Osmolarity of final formulation at different stability conditions and time points

6.1.14 Discussion

RTI formulations were stored at 5±3°C, and 25°C/40%RH for stability studies. Optimal storage condition of the formulation was assessed by analysing the percent assay after the time intervals of 3, 6, 9, and 12M. Percent assay was found to decrease on storage at 25°C/40%RH, which may be due to instability of peptide molecules at higher temperature. This effect was least in the case of formulation stored at 5±3°C, which indicates that degradation can be controlled by storing formulation at 5±3°C. There were no remarkable differences in the initial value of pH and osmolarity when compared with the and values observed after 12M. Hence, the stability testing data indicated that RTI formulations of oxytocin stored at 5±3°C were more stable. However, it can also be stored at temperature not exceeding 25°C/40%RH till 12M without remarkable drug loss.

6.2 Development and Optimization of Ready to Infuse formulation of Vasopressin

In view of knowledge gathered during formulation development of Oxytocin RTI and considering effect of concentration of different osmogens on osmolarity of formulation, various developmental trials have been taken in order to stabilize the Vasopressin in large volume aqueous based formulations. For this purpose, osmogens such as Sodium chloride, Mannitol, Lactose, Sucrose, Trehalose and Dextrose were evaluated. Sodium acetate buffer with 0.01 mg/mL was selected due to prominent result in previous part (Oxytocin). Oxytocin and vasopressin are nonapeptides and similar in structure. Therefore, sodium acetate buffer in same concentration was selected in vasopressin RTI development. In initial screening, pH was kept 3.7 as per concentrate vasopressin data. The details of different developmental trials are presented below (Table 6.14) .

Table 6.14: Development trials for Vasopressin RTI formulation

Formulation Code	Compositions (Each mL Contains)	Sample description	stage	Assay (%)	pH	Osmolarity
FB-1	Vasopressin USP 1.887 µg, Citric acid 0.1 mg, Sodium Citrate Dihydrate 0.242 mg, Sodium Chloride 9 mg, HCl/NaOH q.s. to pH 5.5), WFI q.s.	TH-82	INITIAL	95.27	3.66	295
		AOB	INITIAL	94.26	3.71	296
FB-2	Vasopressin USP 1.887 µg Citric acid 0.1 mg, Sodium Citrate Dihydrate 0.242 mg, Mannitol 50 mg, HCl/NaOH q.s. to pH 5.5, WFI q.s.	TH-82	INITIAL	86.29	3.69	298
		AOB	INITIAL	84.14	3.70	298
FB-3	Vasopressin USP 1.887 µg, Sod Acetate 1 mg, Dextrose 50 mg, HCl/NaOH q.s. to pH 3.7, WFI q.s.	TH-82	INITIAL	90.71	3.66	293
		AOB	INITIAL	92.42	3.70	290
FB-4	Vasopressin USP 1.887 µg,	TH-82	INITIAL	**91.01**	3.71	295

Formulation Code	Compositions (Each mL Contains)	Sample description	stage	Assay (%)	pH	Osmolarity
	Sod Acetate 1 mg, Sucrose 50 mg, HCl/NaOH q.s. to pH 3.7, WFI q.s.	AOB	INITIAL	90.5	3.67	299
FB-5	Vasopressin USP 1.887 µg, Sod Acetate 1 mg, Sucrose 50 mg, Glycine 1 mg, HCl/NaOH q.s. to pH 3.7, WFI q.s.	TH-82	INITIAL	87.98	3.74	280
		AOB	INITIAL	89.67	3.65	282
FB-6	Vasopressin USP 1.887 µg Sod Acetate 1 mg, Trehalose 50 mg, HCl/NaOH q.s. to pH 3.7, WFI q.s.	TH-82	INITIAL	73.84	3.68	288
		AOB	INITIAL	76.2	3.65	288
FB-7	Vasopressin USP 1.887 µg, Sodium Chloride 9 mg, HCl/NaOH q.s. to pH 3.7, WFI q.s.	TH-82	INITIAL	100.85	3.66	297
		AOB	INITIAL	101.07	3.71	298

Among different trials taken with different osmogens and buffering agents, the formulations which were formulated with sodium chloride has shown assay value within the limits. Based on results portrayed in Table 6.14, sodium chloride was selected as osmogens for further development. With selected composition a formulation was prepared and evaluated for short term stability study at 5 ± 3°C and at 25°/40%RH.

Table 6.15: Formulation composition for development of Vasopressin RTI

Ingredients/Components	Specification
Vasopressin USP	1.887 µg/mL (1 IU/mL)
Sodium Chloride	9.0 mg/mL
Hydrochloric acid	q.s. to adjust pH 3.80
Sodium Hydroxide	q.s. to adjust pH 3.80
Water for Injection	q.s.

6.2.1 Manufacturing Process for RTI formulation of Vasopressin

i. 80% batch size of WFI was collected in SS vessel

ii. Temperature 2-8°C was achieved and maintained throughout process. N_2 was purged to achieve DO <1ppm.

iii. pH was adjusted to 3.8 with 0.1% HCl and/or 0.1% NaOH

iv. Vasopressin was added and dissolved with continuous stirring

v. Volume was made up to 100% with WFI and pH was adjusted to 3.8 with 0.1% HCl and/or 0.1% NaOH.

vi. Solution was purged to achieve DO< 1ppm

vii. Solution was diluted with 0.9% NaCl (pH adjusted to 3.8 with 0.1% HCl and/or 0.1% NaOH, DO<1 ppm) to achieve formulation concentration 1.887 µg/ml (1 IU/mL)

Overwrap: Infusion bags were overwrapped with nitrogen blanketing and an oxygen scavenger

6.2.2 Application of the analytical method in formulations

The developed and validated analytical method for vasopressin was applied for its determination in developed pharmaceutical dosage forms.

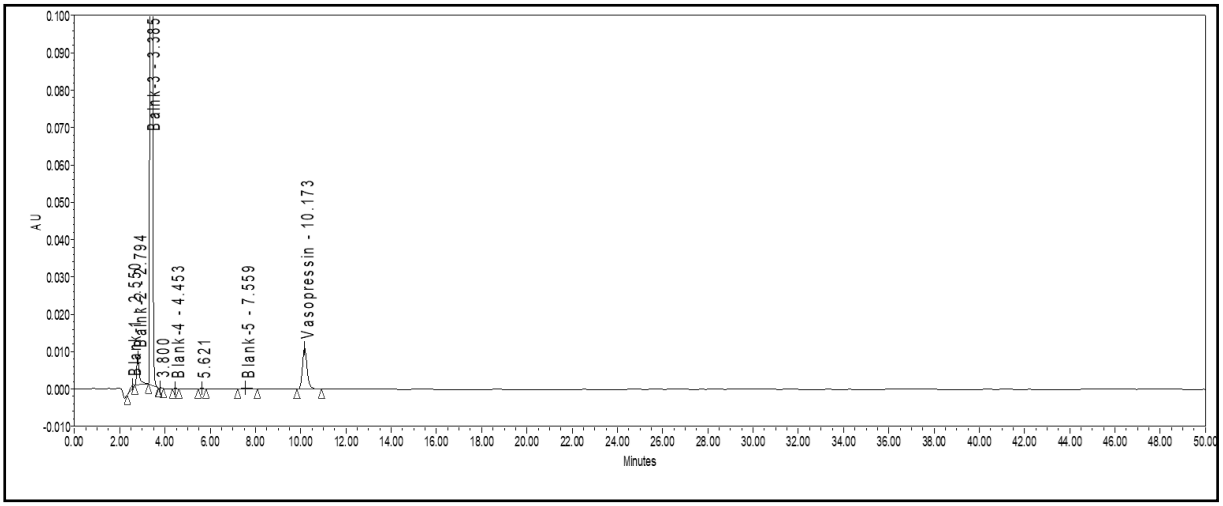


Figure 6.16: Sample chromatogram of Vasopressin (In RTI formulation)

6.2.3 Sterilization process for Vasopressin RTI

In order to evaluate a suitable sterilization method, vasopressin RTI formulation was sterilized at different recommended sterilization conditions and analyzed thereafter.

Table 6.16: Effect of sterilization process on the Vasopressin RTI formulation

Compositions (Each mL Contains)	Sterilization parameter	Assay (%)	pH	Osmolarity
Vasopressin USP 1.887 µg, Sodium Chloride 9 mg, HCl/NaOH q.s. to pH 3.7), WFI q.s.	**Initial (Un-autoclaved)**	100.32	3.69	294
	15 Min_121°C	74.21	3.70	296
	F0 12 Min_121°C	87.12	3.68	295
	F0 08 Min_121°C	88.86	3.71	291
	F0 08 Min_116°C	79.13	3.67	292
	F0 08 Min_111°C	51.21	3.69	296

In all sterilization conditioned recommended and mentioned above, the assay of Vasopressin in RTI formulation was found reduced. There was no effect on pH and osmolarity of the formulation. Therefore, care should be taken to manufacturing, handling and packaging of formulation of Vasopressin. Therefore, the process comprising sterile filtration, pre-sterilized container and aseptic processing was found suitable for further use.

6.2.4 Photostability study for Vasopressin RTI

To evaluate the photosensitivity of Vasopressin RTI, the samples (in infusion bag, in final pack and a control sample) were placed in the photostability chamber and exposed to light of 20 million lux. After exposure, the samples were withdrawn and analyzed.

Table 6.17: Effect of light on Vasopressin RTI

Compositions (Each mL Contains)	Sample description	Assay (%)	pH	Osmolarity
Vasopressin USP 1.887 µg, Sodium Chloride 9 mg, HCl/NaOH q.s. to pH 3.7), WFI q.s.	Controlled (Infusion bag wrapped in aluminium sheet)	100.32	3.69	294
	Market pack (Infusion bag placed inside the overwrap pouch)	100.24	3.67	296
	Direct exposed (infusion bag only)	96.91	4.03	295

The assay of Vasopressin in RTI formulation was found slightly reduced when it was exposed to light. There was no effect on pH and osmolarity of the formulation. Therefore, utmost care should be taken while manufacturing, handling and packaging of Vasopressin formulation.

6.2.5 Stability study of Vasopressin RTI formulation

Stability study of Vasopressin RTI was conducted for final formulation (Table 6.17) as described in section of Oxytocin RTI formulation.

Table 6.18: Stability of various formulations in infusion bags at initial stage and after 12M at different temperature conditions

Condition	Station	Assay (%)	pH	Osmolality (mOsm/kg)	PMT		% Transmittance at 650 nm	Absorbance at 420 nm
					≥10µm	≥25µm		
Initial	0	97.54	3.74	291	93.33	4.00	99.889	0.01
2° to 8° C	3M	102.73	3.78	290	23.3	13	99.912	0.0
2° to 8° C	6M	102.44	3.74	286	12..00	20.00	99.768	0.012
2° to 8° C	12M	103.40	3.74	285	67.73	07	99.961	0.0
25° /40%RH	1M	98.32	3.77	281	20..67	6.67	98.889	0.0
25° /40%RH	3M	99.43	4.03	308	22.0	00	99.673	0.023
25° /40%RH	6M	96.79	3.75	289	33.33	0.0	99.961	0.01
25° /40%RH	12M	93.64	3.86	288	67..67	0.67	98.889	0.0

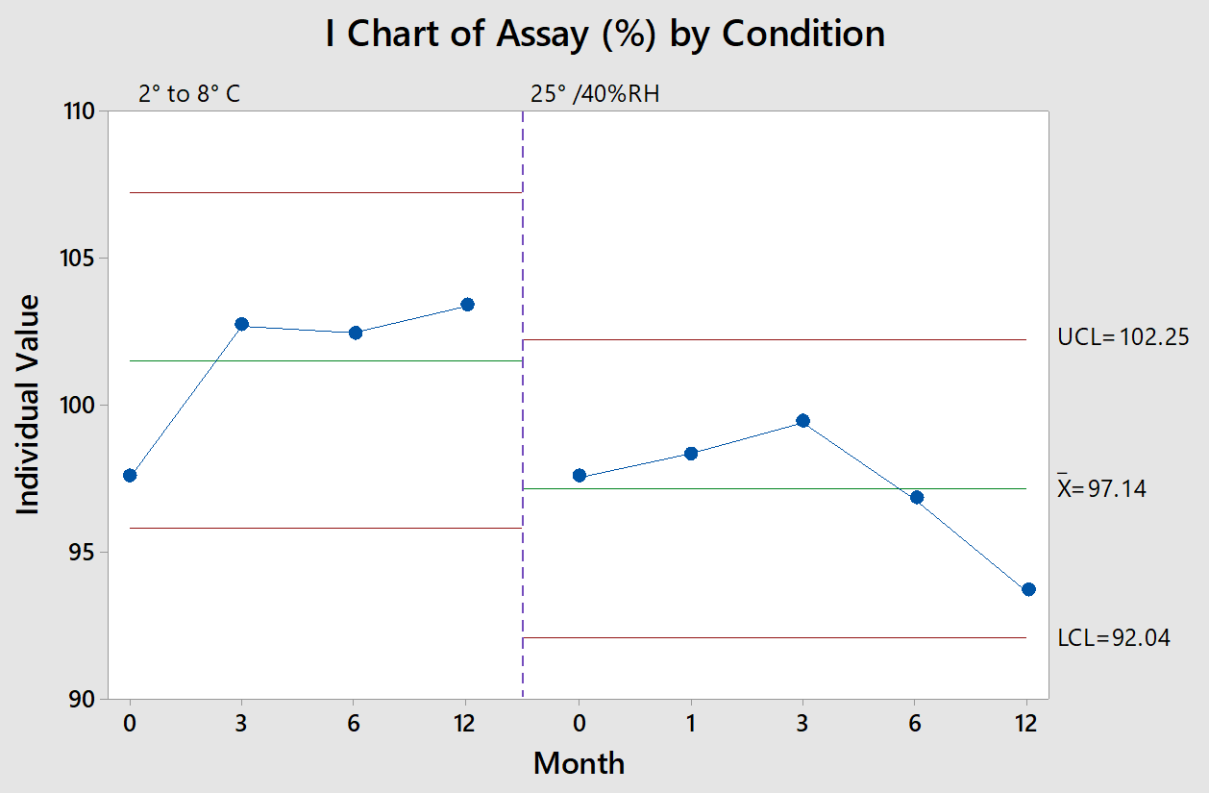


Figure 6.17: Assay of Vasopressin at different stability conditions and time points

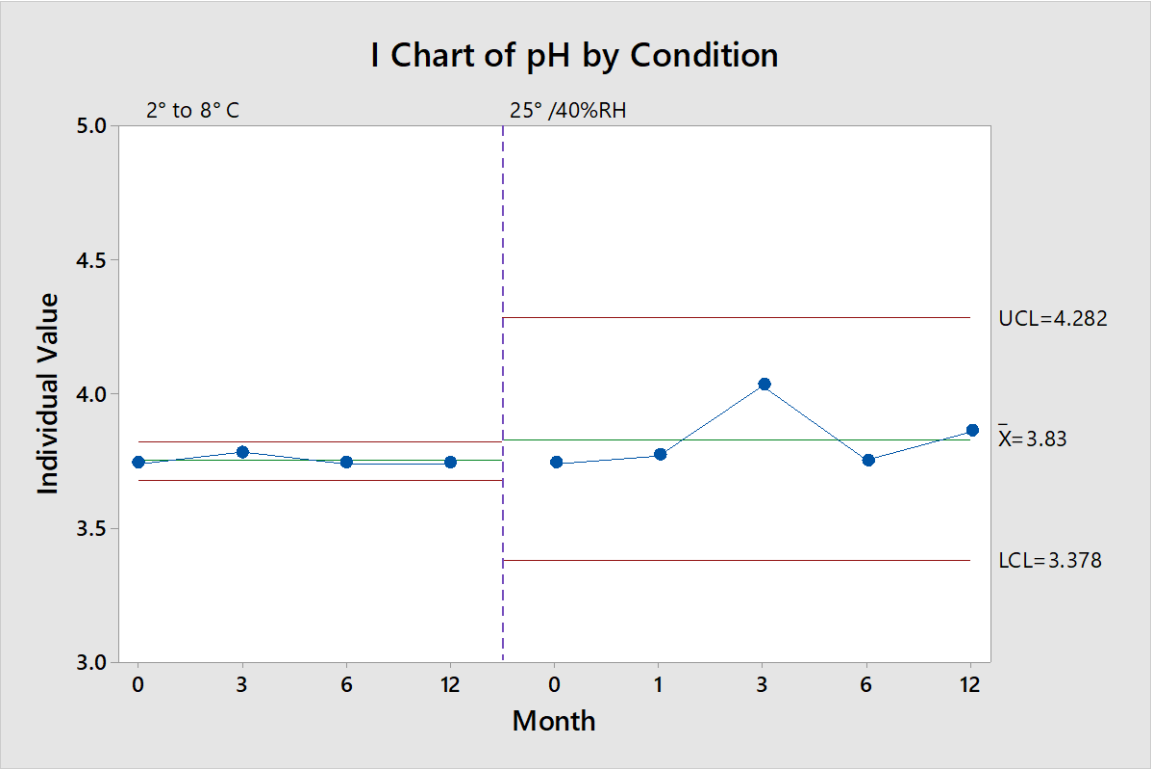


Figure 6.18: pH of vasopressin formulation at different stability conditions and time points

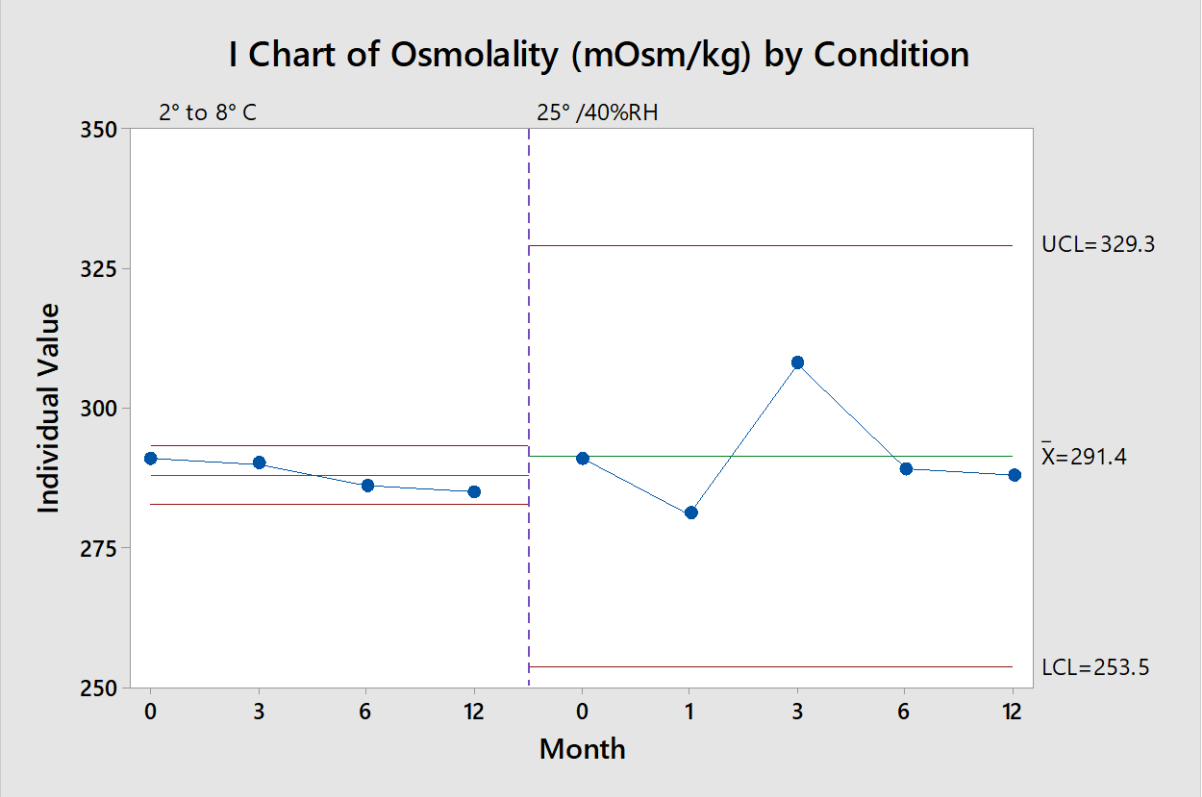


Figure 6.19: Osmolarity of vasopressin formulation at different stability conditions and time points

6.2.6 Discussion

RTI formulation of Vasopressin was stored at 5±3°C, and 25°C/40%RH for stability studies. Optimal storage condition of the formulation was assessed by analysing the percent assay after specified time intervals of 3, 6, 9, and 12M of storage. Percent assay of Vasopressin was found to decrease on storage 25°C/40%RH, which may be due to instability of peptide molecules at higher temperature. This effect was least in the case of formulation stored at 5±3ºC, which indicates that degradation can be controlled by storing formulation at 5±3ºC. There were no remarkable differences in the initial value of pH and osmolarity when compared with the and values observed after 12M. Hence, the stability testing data indicated that RTI formulations od oxytocin stored at 5±3ºC were more stable. However, it can also be stored at temperature not exceeding 25°C/40%RH till 12M without remarkable drug loss.

6.3 Development and Optimization of Ready to Infuse formulation of Angiotensin-II

In view of knowledge gathered during formulation development of Oxytocin RTI, Vasopressin RTI and considering effect of concentration of different osmogens on osmolarity of formulation, various developmental trials have been taken in order to stabilize the Angiotensin-II in large volume aqueous formulations. For this purpose, osmogens such as Sodium chloride, mannitol and Dextrose have been evaluated. The details of different developmental trials are presented below.

Table 6.19: Development trials for selection of Osmogens

Formulation Code	Compositions (Each mL Contains)	Bag description	Stage	Assay (%)	pH	Osmolarity
FB-1	Angiotensin-II USP 0.01 mg, Citric acid 0.1 mg, Sodium Citrate Dihydrate 0.242 mg, Sodium Chloride 9 mg, HCl/NaOH q.s. to pH 5.5, WFI q.s.	TH-82	INITIAL	91.39	5.45	298
		AOB	INITIAL	90.13	5.46	297
FB-2	Angiotensin-II USP 0.01 mg, Citric acid 0.1 mg, Sodium Citrate Dihydrate 0.242 mg, Mannitol 50 mg, HCl/NaOH q.s. to 5.5, WFI q.s.	TH-82	INITIAL	81.98	5.51	295
		AOB	INITIAL	83.47	5.50	294
FB-3	Angiotensin-II USP 0.01 mg, Sod Acetate 1 mg, Dextrose 50 mg, HCl/NaOH q.s. to pH 5.5, WFI q.s.	TH-82	INITIAL	88.74	5.56	291
		AOB	INITIAL	89.74	5.62	288
FB-4	Angiotensin-II USP 0.01 mg, Sod Acetate 1 mg, Sucrose 50 mg, HCl/NaOH q.s. to pH 5.5, WFI q.s.	TH-82	INITIAL	90.41	5.49	298
		AOB	INITIAL	89.15	5.47	297
FB-5	Angiotensin-II USP 0.01 mg, Sod Acetate 1 mg,	TH-82	INITIAL	87.09	5.54	289

Formulation Code	Compositions (Each mL Contains)	Bag description	Stage	Assay (%)	pH	Osmolarity
	Sucrose 50 mg, Glycine 1 mg, HCl/NaOH q.s. to pH 5.5, WFI q.s.	AOB	INITIAL	89.32	5,55	292
FB-6	Angiotensin-II USP 0.01 mg, Sod Acetate 1 mg, Trehalose 50 mg, HCl/NaOH q.s. to pH 5.5, WFI q.s.	TH-82	INITIAL	78.54	5.49	293
		AOB	INITIAL	77.92	5.52	294
FB-7	Angiotensin-II USP 0.01 mg, Sodium Chloride 9 mg, Mannitol 1 mg, HCl/NaOH q.s. to pH 5.5, WFI q.s.	TH-82	INITIAL	100.12	5.44	309
		AOB	INITIAL	100.67	5.45	310

Among different trials taken with different osmogens and buffering agents, the formulations which were formulated with Sodium chloride (9 mg/mL) and mannitol (1 mg/mL) had shown assay value within the limits. Based on results portrayed in Table 6.19, Sodium chloride was selected as osmogens for further trials.

6.3.1 Application of the analytical method in formulations

The developed and validated analytical method for Angiotensin-II was applied for its determination in developed pharmaceutical dosage forms.

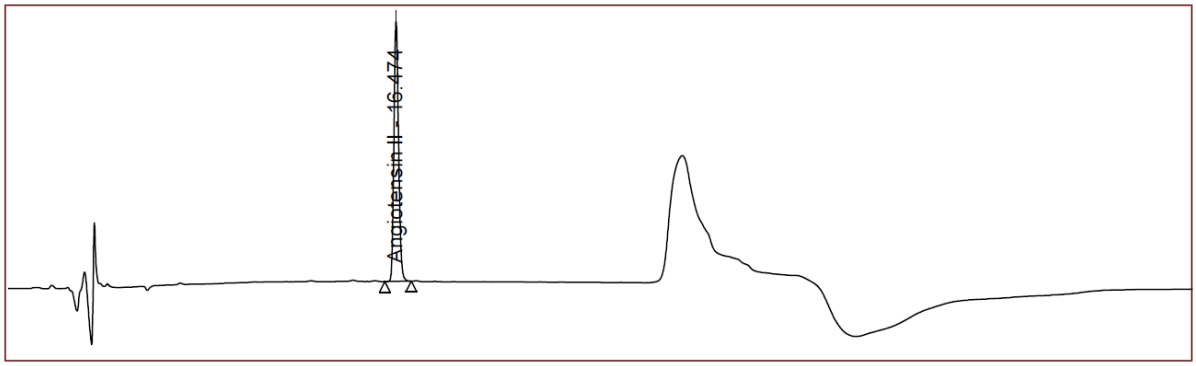


Figure 6.20: Sample chromatogram of Angiotensin-II (In RTI formulation)

6.3.2 Autoclave cycle study for Angiotensin-II RTI

In order to evaluate a suitable sterilization method, Angiotensin-II RTI formulation was sterilized at different recommended sterilization conditions and analysed thereafter.

Table 6.20: Effect of sterilization process on the Angiotensin-II RTI formulation

Compositions (Each mL Contains)	Sterilization parameter	Assay (%)	pH	Osmolarity
Angiotensin-II USP 0.01 mg, Sodium Chloride 9 mg, Mannitol 1 mg, HCl/NaOH q.s. to pH 5.5, WFI q.s.	**Initial (Un-autoclaved)**	99.97	5.55	309
	15 Min_121°C	81.98	5.53	310
	F0 12 Min_121°C	89.56	5.54	309
	F0 08 Min_121°C	90.29	5.57	309
	F0 08 Min_116°C	82.97	5.56	310
	F0 08 Min_111°C	66.91	5.54	311

In all sterilization conditioned recommended and mentioned above, the assay of Angiotensin-II-II in RTI formulation was found reduced. There was no effect on pH and osmolarity of the formulation. Therefore, utmost care should be taken while manufacturing, handling and packaging of Angiotensin-II formulation. Therefore, the process comprising sterile filtration, pre-sterilized container and aseptic processing was found suitable for further use.

Table 6.21: Formulation composition for development of Angiotensin-II Injection

Composition	Quantity for each mL
Angiotensin-II (acetate)	0.01 mg
Mannitol	100 mg
Sodium Chloride	90 mg
Hydrochloric acid	adjust pH 5.5
Sodium Hydroxide	adjust pH 5.5
Water for Injection	q.s.

6.3.3 Photostability study for Angiotensin-II RTI

To evaluate the photosensitivity of Angiotensin-II RTI, the samples (in infusion bag, in final pack and a control sample) were placed in the photostability chamber and exposed to light of 20 million lux. After exposure, the samples were withdrawn and analysed.

Table 6.22: Effect of light on Angiotensin-II RTI

Compositions	Sample description	Assay (%)	pH	Osmolarity
Angiotensin-II USP 0.01 mg/mL, Sodium Chloride 9 mg, Mannitol 1 mg, HCl/NaOH q.s. to pH 5.5, WFI q.s.	Controlled (Infusion bag wrapped in aluminium sheet)	99.97	5.55	309
	Market pack (Infusion bag placed inside the overwrap pouch)	100.04	5.57	310
	Direct exposed (infusion bag only)	97.12	5.53	308

The assay of Angiotensin-II in RTI formulation was found slightly reduced when it was exposed to light. There was no effect on pH and osmolarity of the formulation. Therefore, care should be taken to manufacturing, handling and packaging of formulation of Angiotensin-II.

6.3.4 Manufacturing process for RTI formulation of Angiotensin-II

Manufacturing process followed for drug product manufacturing in given below:

1. WFI (Approx. 80% of batch size) was collected in SS vessel.
2. Temperature 2-8ºC was achieved and maintained throughout process.
3. N_2 was purged to achieve DO <1ppm.
4. Mannitol was added and dissolved with continuous stirring and purging.
5. pH was adjusted to 5.5 using 0.1%w/v HCl and/or 0.1%w/v NaOH.
6. Purging was stopped. Angiotensin-II acetate was added and dissolved with continuous stirring.
7. Sodium chloride was added and dissolved with continuous stirring and purging.
8. pH was again adjusted to 5.5 with 0.1% HCl and/or 0.1% NaOH.
9. Volume was made up to 100% with WFI.
10. Solution was purged to achieve DO< 1ppm.
11. Filtration was performed using 0.2 µ PES capsule filter and non-siliconized tubing.
12. Solution was filled in container and suitably stoppered.
13. Containers were then overwrapped in aluminium pouch with Nitrogen blanketing and oxygen scavenger.

Table 6.23: Stability of final formulation in infusion bags at initial stage and after 12M at different temperature conditions

Condition	Months	Assay	pH	Osmolality	PMT		% Transmittance at 650 nm	Absorbance at 420 nm
					≥10µm	≥25µm		
Initial	0	102.12	5.44	285	20	0.67	99.951	0.00
2-8 ºC	1	102.04	5.35	284	33.3	3.33	99.241	0.02
2-8 ºC	3	100.45	5.6	285	67.67	0.33	99.875	0.01
2-8 ºC	6	100.14	5.46	284	39.33	0.67	99.888	0.0
2-8 ºC	12	99.87	5.43	285	80	10	98.881	0.01
25°C/60%RH	1	102.51	5.39	282	6.67	0.67	99.779	0.023
25°C/60%RH	3	100.54	5.55	284	33.33	0.0	99.961	0.01
25°C/40%RH	6	97.72	5.23	286	67..67	0.67	98.889	0.0
25°C/40%RH	12	95.76	5.32	288	NP	NP	NP	0.0
Note: Stability data for final formulation. Each mL contains Angiotensin-II USP 0.01 mg/mL, Sodium Chloride 9 mg, Mannitol 1 mg,HCl/NaOH q.s. to pH 5.5, WFI q.s.								

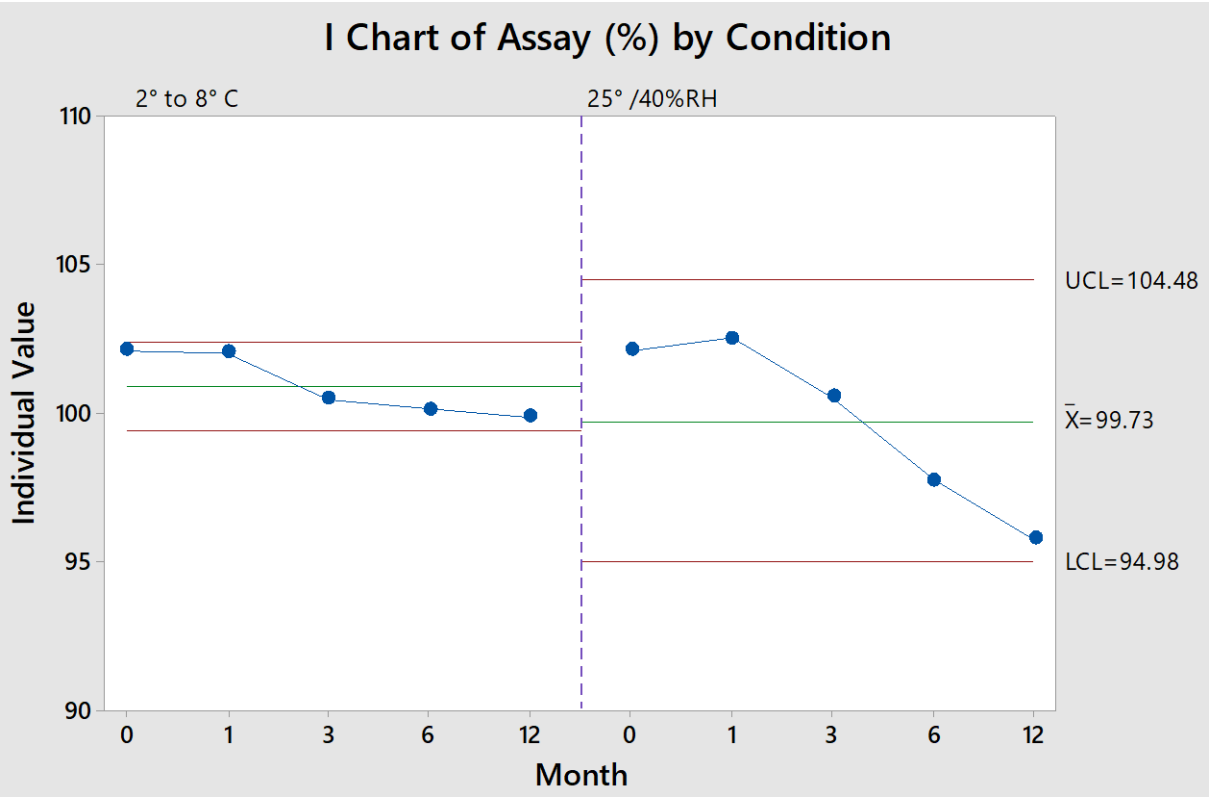


Figure 6.21: Assay of Angiotensin-II at different stability conditions and time points

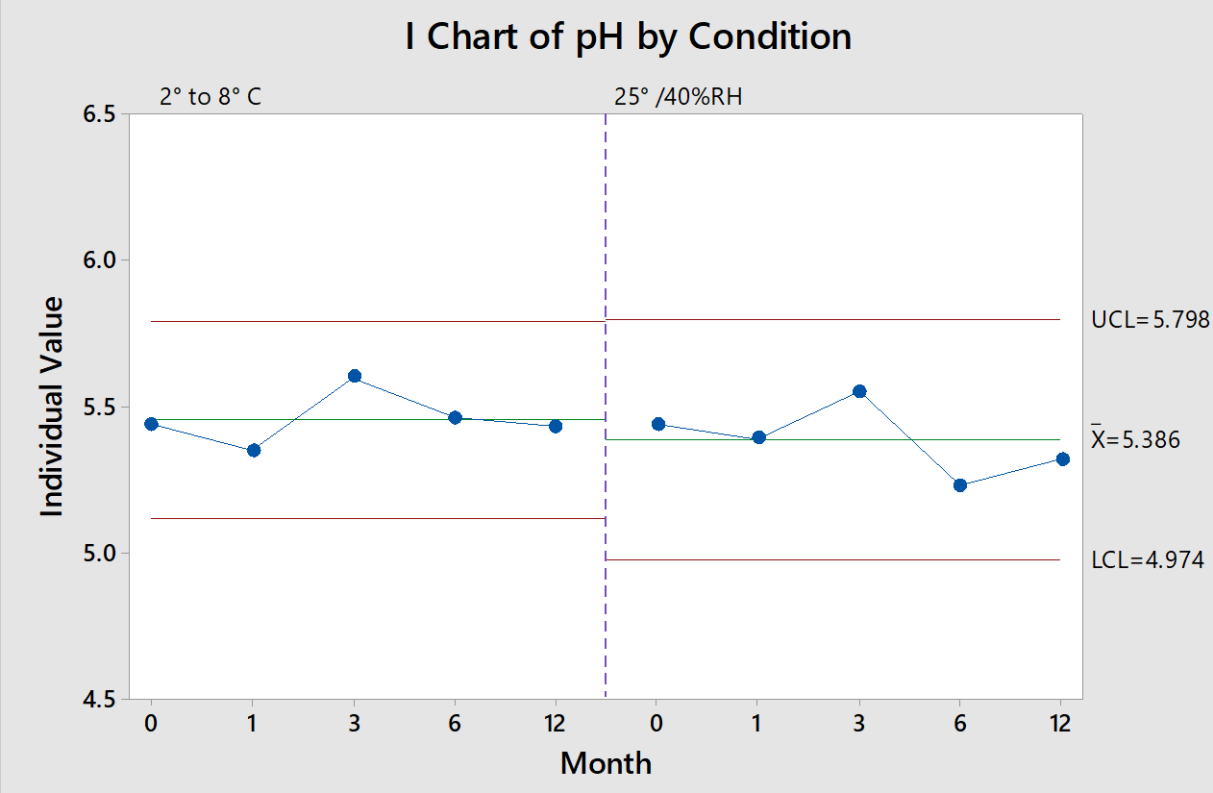


Figure 6.22: pH of Angiotensin-II formulation at different stability conditions and time points

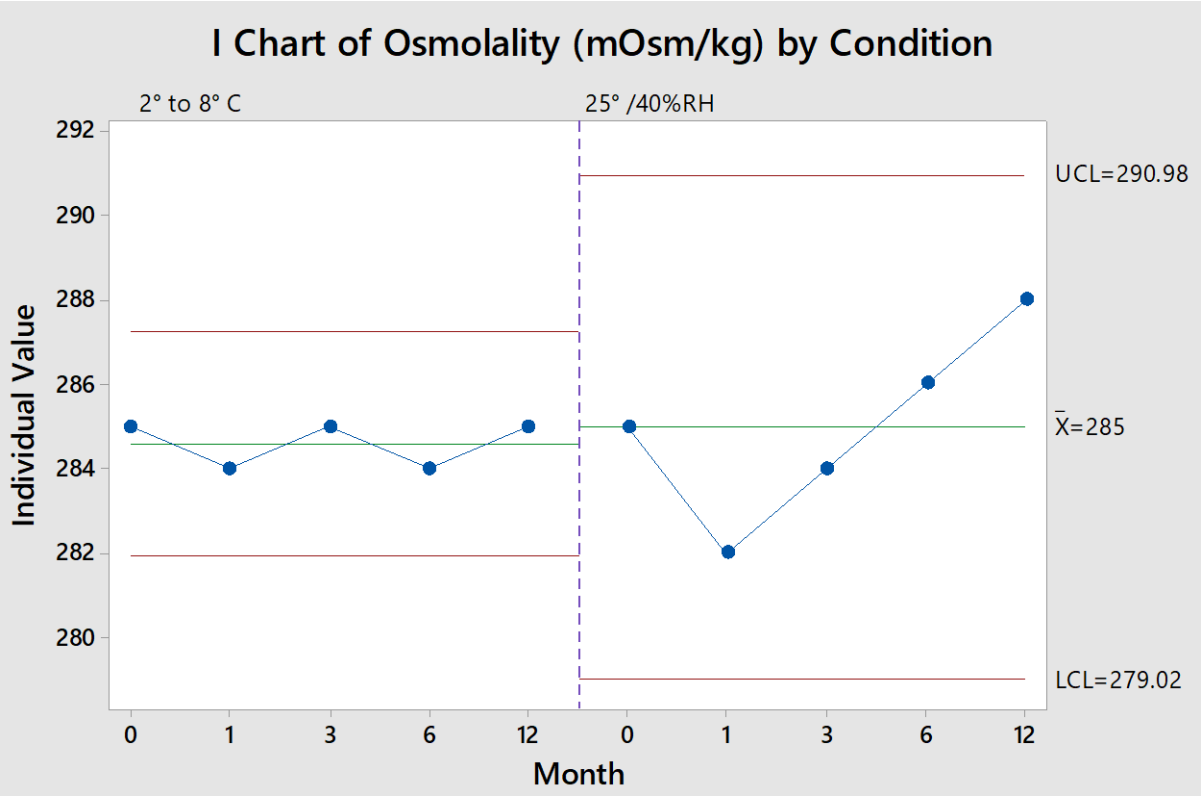


Figure 6.23: Osmolarity of Angiotensin-II formulation at different stability conditions and time points:

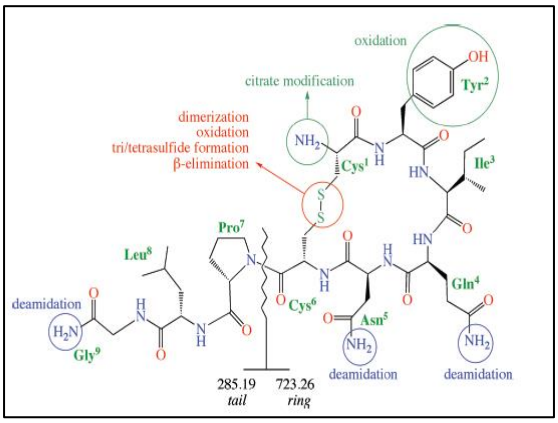
6.3.5 Discussion:

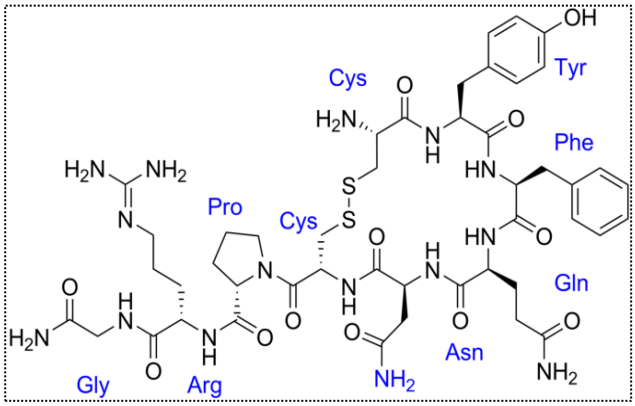
RTI formulation of Angiotensin-II was stored at 5±3°C, and 25°C/40%RH for stability studies. Optimal storage condition of the formulation was assessed by analysing the percent assay after specified time intervals of 3, 6, 9, and 12M. Percent assay of Angiotensin-II was found to decrease on storage 25°C/40%RH, which may be due to instability of peptide molecules at higher temperature. This effect was least in the case of formulation stored at 5±3^{o}C, which indicates that degradation can be controlled by storing formulation at 5±3^{o}C. There were no remarkable differences in the initial value of pH and osmolarity when compared with the and values observed after 12M. Hence, the stability testing data indicated that RTI formulations od oxytocin stored at 5±3^{o}C were more stable. However, it can also be stored at temperature not exceeding 25°C/40%RH till 12M without remarkable drug loss.

Section B: (Nanovesicles for nose to brain delivery)

7. Preparation and characterization nanovesicles (liposomes)

7.1. Physico-chemical characteristics of studies of selected bioactives





Oxytocin (Nonapeptide) **Vasopressin (Nonapeptide)**

Figure 7.1: Structure of Oxytocin and Vasopressin

Table 7.1: Physicochemical properties of Oxytocin and Vasopressin

Property Name	Oxytocin	Vasopressin
Molecular Weight	1007.193 g/mol	1084.239 g/mol
XLogP3-AA	-2.6	-1.4
CAS No	50-56-6	113-79-1
Colour/Form	White powder	White to off-white amorphous powder
Solubility	Soluble in water, butanol in water, 1.2X10+4 mg/L @ 25 ˚C	Freely water soluble

7.2. Fabrication of Nanovesicles for Oxytocin and Vasopressin

Lipid nanovesicles were used as a novel drug delivery system for pharmaceutical drugs in various application routes and therapy. They also represent a promising carrier system for

cosmetic active ingredients due to their numerous advantages over existing conventional formulations [41, 42], which are as follows-

- The protection of labile compounds against chemical degradation.
- Depending on the produced lipid nanovesicles-type, controlled release of the active ingredients is possible.
- Lipid nanovesicles can be modified by attaching suitable ligand and may act as targeted vehicles.
- Nanovesicles of many other drugs have been prepared & they have provided better therapeutic efficacy as well as improved patient compliance.

These vital things fabricate them to serve as potent carrier for the delivery of therapeutic peptide. Hence it was envisaged to formulate nanovesicles of oxytocin and vasopressin for the management of neurological disorder.

7.2.1 Preparation and optimization of nanovesicles for Oxytocin

The lipids including HSPC, DMG-PEG-2000 and cholesterol were dissolved in 15 mL absolute ethanol followed by Oxytocin at 40 °C for 15 minutes. The lipid solution was injected into Phosphate buffer saline (PBS at pH 4.0) with continuous stirring (250 rpm). The solution was kept for stirring for 30 mints. The formed liposomes were extruded step-wise through 400/200 nm, 200/200 nm. Whatman filters (Instruchemie, Delfzijl, the Netherlands) to reduce and unify the particle size.

7.3. Methodology for Characterization of nanovesicles:

7.3.1 Size, Zeta Potential and Surface morphology

The average vesicle size, polydispersity index (PDI) and zeta potential (surface charge) were analysed by photon correlation spectroscopy using Malvern zetasizer Nano-ZS 90 (Malvern ZS Nano Instrument, Worcestershire, United Kingdom). Sample was placed in the cuvette and particle size of the prepared vesicles was determined using dynamic light scattering (DLS) technique. DLS measures the diffusion of particles which are moving under Brownian motion, and converts into size and its distribution by the Stokes–Einstein relationship. As zeta potential holds promise to predict the in vivo stability of the nanovesicles, the same instrument was employed to determine the zeta potential of liposomal formulations too. The zeta potential of the prepared nanosystems was determined in folded capillary cells by laser Doppler

anemometry. The zeta potential was measured on samples well-dispersed in deionized water at temperature of 25 ± 0.1°C and electric field of 15.24 V/cm. All samples were analysed in triplicate [34].

The morphology of formulated vesicles was confirmed by High resolution (HR) and cryo transmission electron microscopy (TEM). A drop of liposomal suspension was placed and adsorbed on microscopic carbon coated grids. These grids were subsequently negatively stained with 1% (*w/v*) aqueous solution of phosphotungstic acid, dried, and viewed under TEM at suitable magnifications, operating at an acceleration voltage of 200 kV [25].

7.3.2 Percentage drug entrapment (PDE)

Percentage drug entrapment (PDE) was calculated by direct lysis method [43]. In brief, PDE of nano-vesicular systems was determined in triplicate by centrifugation method. The liposomal suspension of Oxytocin and Vasopressin were centrifuged (M/s REMI CPR 24) separately at 10000 rpm at 4°C for 10 min. Clear supernatants as well as the vesicular sediments were collected. Sediments were individually lysed with n-propanol and samples were collected. The appropriate dilutions of samples were made and analysed to quantify Oxytocin and Vasopressin by the validated HPLC method. PEE of drug was calculated using following equation:

$$PEE = \frac{(T-C)}{T} X\ 100 \ldots\ldots\ldots \text{eq. 7.1.}$$

Whereas, T is the total amount of drug that is detected both in the supernatant and sediment, and C is the amount of drug detected only in the supernatant

7.4. Optimization of liposomes for Oxytocin

Various formulation and process variables for the preparation of liposomes were optimized while keeping the amount of drug constant to acquire a final formulation with small particle size (<100 nm), narrow polydispersity index (PDI; <0.5), and maximum drug loading with high entrapment efficiency. During preparation of liposomes the operational temperature was kept ≥ 40°C to safeguard the peptide molecules from higher temperature.

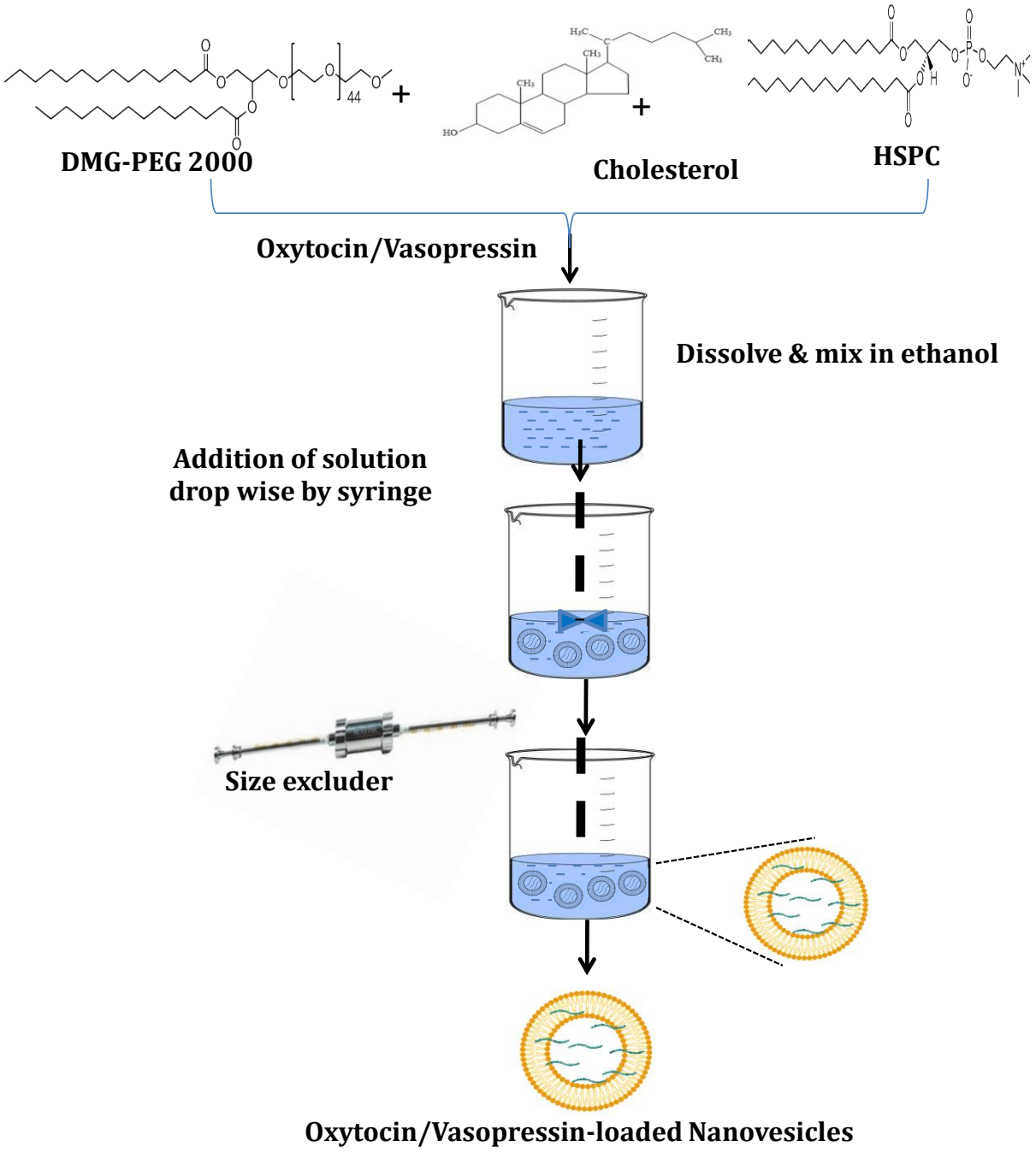


Figure 7.2: Schematic presentation of fabrication of liposomes

7.4.1 Optimization of lipids ratio

Liposomes formulation were prepared by varying the ratio (w/w/w) of HSPC, DMG-PEG-2000 and cholesterol while keeping the amount of HSPC constant and other parameters were kept constant (stirring speed- 300 rpm, stirring time-45 minutes). Optimization was done on the basis of average particle size and polydespersity index (PDI) of Liposomes which was determined by using Malvern Zetasizer (Table 7.2).

Table 7.2: Table: Optimization of ratio of lipids

Formulation code	HSPC:DMG-PEG-2000: Cholesterol (molar)*	Particle size (nm)*	Polydispersity index (PI)*
F1	4:0.5:0.5	146.6±8.1	0.342±0.06
F2	4:1:0.5	108.6±7.2	0.251±0.09
F3	4:1.5:0.5	168.6±6.3	0.342±0.04
F4	4:1:1	**95.06±4.86**	**0.115±0.002**
F5	4:1:1.5	142.8±9.8	0.412±0.05

**Data are shown as mean± SD (n=6)*

7.4.2 Optimization of process variables:

Stirring speed:

Stirring speed was varied from 100 to 400 rpm for liposomes preparation using the same optimized formulation parameters. The particle size and percent drug entrapment of prepared liposomes were determined for further optimization.

Table 7.3: Optimization with respect to stirring speed

Formulation code	stirring speed (RPM)*	Particle size (nm)*	Polydispersity index (PI)*	Entrapment efficiency (%)*
F6	100	189.2±16.23	0.75±0.09	51.1±2.2
F7	200	159.7±15.11	0.67±0.10	57.5±2.4
F8	**300**	**95.06±0.86**	**0.115±0.002**	**64.03±0.54**
F9	400	87.7±1.42	0.18±0.04	53.4±2.5

**Data are shown as mean± SD (n=6)*

Stirring time:

For the optimization of stirring time, formulation F8 was selected while other process variables were kept constant. The liposomes dispersions were prepared with different stirring time (*viz.* 30, 45, 60 min.). Particle size and percent drug entrapment were determined for selection of optimized parameters.

Table 7.4: Optimization of stirring time

Formulation code	stirring time (min)*	Particle size (nm)*	Polydispersity index (PI)*	Entrapment efficiency (%)*
F10	30	118.7±5.71	0.51±0.09	58.1±1.92
F11	**45**	**95.06±4.8**	**0.115±0.002**	**64.03±0.54**
F12	60	84.7±5.23	0.13±0.04	57.9±0.65

***Data are shown as mean ± SD (n=6)*

7.4.3 Optimized parameters

From the optimization studies the liposomes formulation (**F11)** was selected as optimized formulation and the parameters used for the preparation of this optimized formulation are recorded in below Table 7.5.

Table 7.5: Optimized parameters for Liposomes for Oxytocin

Parameters	Optimized values
HSPC:DMG-PEG-2000: cholesterol	4:1:1 (w/w/w)
API	10 mg/mL formulation
Stirring speed	300 RPM
Stirring time	45 min

7.5. Optimization of liposomes for vasopressin

Vasopressin and Oxytocin are two closely related neuropeptides, both consisting of nine amino acids that only differ at the 3rd and 8th position respectively. Both the peptides have molecular weight in approximately similar range. Therefore, similar optimization strategy was evaluated for the development of liposomes of vasopressin.

7.5.1 Optimization of lipids ratio

Liposomes formulation for Vasopressin were prepared by varying the ratio (w/w/w) of HSPC, DMG-PEG-2000 and cholesterol while keeping the amount of HSPC constant and other parameters were kept constant (stirring speed- 300 rpm, stirring time-45 min). Optimization

was done on the basis of average particle size and polydespersity index (PDI) of Liposomes which was determined by using Malvern Zetasizer (Table 7.6).

Table 7.6: Optimization with respect to ratio of lipids used cholesterol ratio

Formulation code	HSPC:DMG-PEG-2000: Cholestrol (molar)*	Particle size (nm)*	Polydispersity index (PI)*
F1	4:0.5:0.5	152.3±1.3	0.368±0.07
F2	4:1:0.5	113.1±6.4	0.581±0.06
F3	4:1.5:0.5	156.9±5.3	0.489±0.05
F4	**4:1:1**	**99.06±1.01**	**0.121±0.004**
F5	4:1:1.5	157.5±11.3	0.701±0.06

***Data are shown as mean±SD (n=6)*

7.5.2 Optimization of process variables:

Stirring speed:

Stirring speed was varied from 100 to 400 rpm for liposomes preparation using the same optimized formulation parameters. The particle size and percent drug entrapment of prepared liposomes were determined for further optimization.

Table 7.7: Optimization with respect to stirring speed

Formulation code	stirring speed (RPM)*	Particle size (nm)*	Polydispersity index (PI)*	Entrapment efficiency (%)*
F6	100	178.2±17.1	0.69±0.08	50.2±1.9
F7	200	145.8±16.2	0.61±0.09	52.95±2.1
F8	**300**	**99.06±1.01**	**0.121±0.004**	**62.59±0.39**
F9	400	89.2±1.8	0.19±0.05	46.1±1.9

***Data are shown as mean±SD (n=6)*

Stirring time:

For the optimization of stirring time, formulation F8 was selected while other process variables were kept constant. The liposomes dispersions were prepared with different stirring time (*viz.* 30, 45, 60 min). Particle size and percent drug entrapment were determined for selection of optimized parameters.

Table 7.8: Optimization of stirring time

Formulation code	stirring time (min)*	Particle size (nm)*	Polydispersity index (PI)*	Entrapment efficiency (%)*
F10	30	145.9±6.21	0.51±0.09	59.2±2.12
F11	**45**	**99.06±1.01**	**0.121±0.004**	**62.59±0.39**
F12	60	79.7±1.13	0.13±0.04	55.2±0.75

***Data are shown as mean ± SD (n=6)*

7.5.3 Optimized parameters for liposomes of vasopressin

From the optimization studies the liposomes formulation (**F11)** was selected as optimized formulation and the parameters used for the preparation of this optimized formulation are recorded in below Table 7.9.

Table 7.9: Optimized parameters for Liposomes for Vasopressin

Parameters	Optimized values
HSPC:DMG-PEG-2000: cholesterol	4:1:1 (*w/w/w*)
API	0.133 mg/mL formulation
Stirring speed	300 rpm
Stirring time	45 min

7.6. Stability study of liposomes formulations:

The stability of drug (Oxytocin and Vasopressin) loaded liposomal dispersions were studied at 2-8°C, 25±2°C/60±5% and 40±2°C/75±5% RH for 90 days with an interval of 15 days. The samples were withdrawn every 15 days and evaluated for vesicles size and % assay.

7.7. *In vitro* cell line study:

% Cell viability study

Percent cell viability study was performed in accordance with the previously reported methods [44]. The SH-SY5Y cells were seeded with 5×10^3 cells/well cell density in a 96 well microtiter plate using Dulbecco′s Modified Eagle′s Medium - high glucose (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotic solution. This study was done by MTT assay

on the plain drug (Oxytocin and Vasopressin), drug (Oxytocin and Vasopressin) loaded liposomes and plain liposomes (Placebo) using PBS treated cells as control. The cells were cultured for 24 hours in an incubator with maintaining CO_2 at 5°C ± 0.5°C % concentration and humidified with saturated Copper sulphate solution. After 24 h, the media was removed, cells were washed with PBS 7.4 and replaced with complete media containing 10% FBS and 1% antibiotic. Then the cells were treated with different formulations and exposed for 12 and 24 h. After given exposure time, the wells were washed with PBS 7.4 and added 20 µL of MTT dye (5 mg/mL) solution in each plate. After 4 hrs, the medium in wells were replaced with 100 µL of DMSO to dissolve formazan crystals. The intensity of formazan crystals was determined using BIORAD microtiter plate reader (Biorad, California) at 570 nm. The absorbance values of cells treated with PBS were taken as 100 % cell viability and absorbances of other treated wells were compared to it. Triton X 100 and PBS 7.4 were used as negative and positive control respectively [45].

In vitro* cell permeation study (Cell uptake*)

SH-SY5Y cells were incubated for 24 h with FITC-loaded liposomes (50 and 100 µg/mL) or plain liposomes as auto-fluorescence control. Thereafter, cells were washed with PBS, fixed for 15 min with 4% paraformaldehyde and rinsed in PBS. To visualize the nuclei, material was mounted with Seebright mounting medium with diamidino-2-phenylindole (DAPI, Merck). DAPI Dye has a maximum excitation wavelength in the ultraviolet range (359 nm) and the dye can be optimally detected in the blue channel with an emission maximum of 461 nm. Fluorescein isothiocyanate (FITC) has an excitation peak at 495 nm and an emission peak at 519 nm.

Genomic microarray (qRT-PCR) analysis

SH-SY5Y cells were purchased from National Centre for Cell Science, Pune, India. These cells were maintained in RPMI-1640 medium supplemented with 10% inactivated fetal calf serum (FCS) and 50 µg/mL gentamycin (Invitrogen, USA).

SH-SY5Y cells were seeded in 96-well plates at 4×10^4 cells per well. After culture for 24 h, 100 nM PMA (Wako, Japan) was added for differentiation into macrophages. SH-SY5Y cells were further cultured for 48 h and cells were incubated with plain drug and drug loaded vesicles for 2 h, subsequently stimulated with 10 ng/mL ultrapure Lipopolysaccharide (LPS) for 24 h. The analysis of gene expression in SH-SY5Y cells treated with formulations was performed

using reverse transcription PCR (RT-PCR). Total RNA was isolated from cells treated with formulations by RNeasy kit. The cDNA was synthesized by reverse-transcription reaction (Applied biosystems). The qualitative RT-PCR was performed on TaqMan array fast plates using TaqMan fast universal PCR master mix (2x). The fold regulations were calculated using $2^{-\Delta\Delta Ct}$ method. The fold regulation of gene TNF-α, IL-6, MMP1, CD44, IL-1β, IL-10, iNOS, FOXO1, BCL2 and BCL2l1 in culture medium treated with formulations was determined relative to their untreated control. The custom-designed PCR array plates procured from Life Technologies, USA were used for qRT-PCR.

Annexin-V staining

Annexin V staining, paired with 7-aminoactinomycine (7-AAD), Life technologies, USA or PI is widely used to identify apoptotic stages by flow cytometry. The SH-SY5Y cells induced with LPS were analysed for Annexin V-FITC and propidium iodide using a cell death detection kit for flow cytometry following manufacturer protocol.

Immunofluorescence assay (IFA)

IFA was carried out on cells treated with various formulations following manufacturer's recommendation. Briefly, cells were plated at 37°C for 4 min at 1000 rpm onto slide by cytospin. The cells were fixed in 4% paraformaldehyde in PBS (10 min at room temperature). The cells were stained with 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) to estimate % apoptosis. Before mounting cells were washed with PBS. Cover slips were mounted with fluorescence mounting medium. The microscopy was performed with DAPI channel.

Immunoblotting

To analyse total and phosphorylated proteins, SH-SY5Y cells (2.4×10^6), both adhered and in suspension were solubilized in ice cold cell lysis buffer (100 µL) mixed with 1mM PMSF, incubated on ice for 5 min sonicated, briefly, following manufacturer's recommandations. The lysates were clarified by centrifugation at 14000 rpm for 10 min and pellets were discarded. The supernatant was used to perform immunoblot. After extraction, all samples were separated by SDS-PAGE and immunoblotted. For the immunoblot analysis, samples fractionated by SDS-PAGE were electro-transferred to membranes. After blocking with 5% nonfat milk protein in Tris-Buffered Saline and Tween 20 (TBST) or phosphate buffer saline and Tween 20 (PBST) (0.1% or 0.2% Tween-20), membranes were incubated with antibodies dissolved in TBS plus 0.1 or 0.2% Tween-20 solution and 5% BSA. Subsequently, the membranes were

incubated with suitable peroxidase-conjugated secondary antibodies (Cell signalling and Abcam) and immune reactive bands were visualized using ECL reagents (Pierce), USA.

7.8. Neurological activities of developed formulations in experimental animals

7.8.1 Rationale for *In vivo* studies

While *In vitro* assessments of drug release and targeting are crucial, neurological disorders are multifactorial, complex diseases involving many physiological processes. For example, epilepsy, neurodevelopmental disorders, and neuropsychiatric disease are difficult to model in vitro. For this reason, it is important to examine both the ability of Oxytocin-loaded liposomes and Vasopressin-loaded liposomes to successfully deliver therapeutics to the brain and the resulting effects on disease symptoms and pathology in animal models of neurological and neuropsychiatric disease. Currently, there are limited examples of intranasal nanocarrier-based delivery of peptides in CNS disease models, and so, evidence of the systemic biocompatibility of Oxytocin- and Vasopressin-loaded liposomes, as well as the efficacy of drug delivery following intranasal administration, are highlighted here.

For this purpose, male Balb/C mice weighing between 25 and 40 g were selected as the subjects of the in vivo experiments. The mice were housed in groups of 6 in a temperature and humidity-controlled room. Animals had access to food (Laboratory Rodent Diet) and water ad libitum. Mice were housed in rooms maintained in a 12- hour light/dark cycle. The lights were turned off at 6 pm every evening, and turned back on at 6 am every morning. All animals employed in this study were treated according to protocols evaluated and approved by Institutional Animal Ethical Committee of National Institute of Occupational Health (NIOH), Ahmedabad.

Morris water maze test

The MWM test was performed to compare the free drug solution-based intranasal and intravenous route in comparison to drug loaded nanoparticles administered via the intranasal and intravenous route [46]. First, the trials were done for 4 days in which mice were trained to reach the hidden platform in the 4th quadrant. After treatment, a final acquisition trial was done.

In the MWM test, the parameters governed were the time to reach the hidden platform and the time spent by mice in the fourth quadrant. The earlier the reach time on the hidden platform

determines that the mouse retains its memory. The less time spent in quadrant fourth determines loss in memory.

The comparison will be made with the naive group and another Scopolamine (SCP) treated group only. It will be observed that the mice that received drug solution or nanoparticles via intranasal route will retain the memory and compared with the control (naive) versus the SCP-treated group.

These studies will clearly depict the trial runs of mice for four days and the acquisition trial after treatment. The earlier time to reach the hidden platform in the 4th quadrant depicts the retention of memory by mice and can be observed in heat map and comparable graphs when restructured using Graph Pad Prism software.

Elevated plus maze (EPM) test

In the EPM study, the mice reaching latency in a closed loop in seconds was observed. Transfer latency was studied for 1, 48, and 96 h to determine the efficacy of nanoparticles via the intranasal and intravenous routes in comparison to free drug solution administered via intranasal and intravenous route.

The order of transfer latency of the respective groups was analysed after 1 h of treatment. The earlier time to reach closed depicts the retention of memory by mice and can be observed in heat map and comparable graphs when restructured using Graph Pad Prism software.

Gene expression studies

To confirm molecular effectiveness of above treatment, gene expression studies are prominent for the same. Gene expression studies of brain homogenate samples was studied for AIF-1 (Allograft inflammatory factor 1), BDNF (brain-derived neurotrophic factor), CD68 (cluster of differentiation), GFAP (glial fibrillary acidic protein), IL-6 (interleukin), Nrf 2 (nuclear factor erythroid), HO-1 (heme oxygenase), NF-KB, MyD88 (myeloid differentiation factor) analysis.

AIF-1 is a prominent neurodegeneration marker linked to calcium binding adaptor molecule-1 with its genesis from macrophages, microglial cells, and neutrophils. Thus, AIF-1 gene expression studies demark the efficacy studies of best treatment groups [47].

BDNF expression explains the antioxidant and cholinergic transmission. The Nrf-2 expression is about cellular resistance to oxidants. Antioxidant presence is directly proportional to Nrf-2 expression [47].

HO-1 expression represents the Nrf-2 downstream production and levels tend to decrease in the SCP-treated group. The IL-6 expression represents the nerve cell loss and synapse in neurodegeneration and is a prominent marker for neuroinflammation especially linked to cholinergic inflammation [47].

MyD88 reportedly activates NF-KB expression, and NF-KB is expressed as a prominent inflammatory biomarker.

The expression of GFAP and CD68 is studied due to its linkage with amyloid theory and its neuroinflammation.

Brain Histopathological evaluation

The histopathological evaluation of hippocampus observations demarks the comparative difference between the formulation's efficacies proposed in the current research work. The higher the density of neurons, more efficacy and best will be the treatment.

7.8.2 Methodology for Pharmacodynamics studies

Male BALB/c mice (8−10 weeks aged, 28 ± 3 g) were housed at the animal facility of ICMR-National Institute of Occupational Health, Ahmedabad, Gujarat. Mice were fed normal chow and water ad libitum. All the experimental protocols were approved by the IAEC of ICMR-NIOH, Ahmedabad, with the IAEC approval number (IAEC/ICMR-NIOH/2022-23/26/01) and the experiments were conducted in accordance with the guidelines provided by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). All the experiment treatments and cognitive function assessments (Transfer latency in Elevated Plus Maze (EPM) and Learning and memory task performances in Morris Water Maze (MWM)), gene expression studies and brain hippocampal studies were performed.

Experimentation Treatments and Cognitive Function Assessments

Mice were randomized based on their weights for having initial similar group weights. They were allocated to the following groups:

(a) control or untreated mice (n = 12);

(b) Scopolamine (2 mg/kg), intraperitoneal (IP) or positive control (n = 12), and

Mice undergone various treatment or intervention group after scopolamine (2 mg/kg), IP. Injection viz.;

(c) Donepezil: 1 mg/kg per (*n* = 12), peroral

(d) Vasopressin drug solution intranasal (*n* = 12),

(e) Vasopressin loaded nanoparticles intranasal (*n* = 12),

(f) Vasopressin loaded nanoparticles intravenous (*n* = 12),

(g) Vasopressin drug solution intravenous (*n* = 12),

(h) Oxytocin drug solution intranasal (*n* = 12),

(i) Oxytocin loaded nanoparticles intranasal (*n* = 12),

(j) Oxytocin loaded nanoparticles intravenous (*n* = 12),

(k) Oxytocin drug solution intravenous (*n* = 12)

The following behaviour tests were conducted in mice to assess the memory function and learning behaviour.

Transfer Latency in EPM

Mice (*n* = 6 per group) were treated for 14 days in various treatment groups and after the first 7 days of housing in their home cage, the first dose of scopolamine was given to all the mice except the control group. A training trial of 90 s for the situation of a closed arm in an EPM was conducted, while placing the mice on either of the open arms and facing the central zone of the maze. If the mice were unable to reach the closed arm, they were guided for the same and kept there for 10 s. After the 30 min interval, all the mice were administered the respective treatment as mentioned above. After 1 h, the acquisition trail was conducted, and transfer latency was recorded for mice reaching the closed arm. A retention trial was conducted after 24, 48, and 96 h for memory consolidation. Mice were then sacrificed for molecular investigations.

Learning and Memory Task Performances in MWM

To assess the long-term learning and memory function in the MWM test after the scopolamine and other treatments, mice (n = 6 per group) were trained for four days in various quadrants and guided to reach the hidden or submerged platform in the water maze. Escape latency or time to reach the hidden platform were noted. On the fifth day, an acquisition trial was performed and time to reach the hidden platform and time spent in the same quadrant were

recorded. After the behavioural tests, the mice were sacrificed, and biological samples were collected, as described in the EPM test.

Gene Expression Studies

Gene expression analyses were performed in half of the brain (sagittal whole section of each mouse). Using the Trizol reagent (Sigma Aldrich, MA, USA), total RNA was extracted from these tissues. For appropriate downstream processing, an initial quality assessment for the quantity and purity of isolated RNA was carried out. Following the instructions provided by the manufacturer, a cDNA synthesis kit (HiMedia Lab Pvt. Ltd. Mumbai, India) was used to create a complementary strand of DNA for amplification from 800 ng of total RNA. Using SYBR green dye and appropriate primers (Table), qRT-PCR (Step One Plus Real-Time PCR system, Applied Biosystem)-based relative gene expressions for various genes were carried out in these tissues. The qRT-PCR conditions used were followed by 40 cycles of 95 and 60°C for one minute each. Data were analysed using the $2^{-\Delta\Delta Ct}$ method and values are expressed as fold change relative to the control group.

Table 7.10: PCR Primers for Genes

Gene	forward (5′−3′)	reverse (5′−3′)
AIF-1	TCCGAGGAGACGTTCAGCTA	CGTGTGACATCCACCTCCAA
GFAP	GTGCAGAGATGATGGAGCTC	ACTGTTGGCCGTAAGCTGGT
BDNF	CGGCGCCCATGAAAGAAGTA	TCGTTGGGCCGAACCTTCT
CD68	GGGGCTCTTGGGAACTACAC	GTACCGTCACAACCTCCCTG
IL6	GATGGATGCTACCAAACTGGA	GAGCATTGGAAATTGGGGTA
Nrf 2	TTTGTAGATGACCATGAGTCGC	CCTGCTGCTTGTTTTCCGTA
HO-1	GTAAAGCGTCTCCACGAGGT	AACTTGGTGGGGTTGTCGAT
NF-kB	AAGCCTGTAGCCCACGTCGTA	GGCACCACTAGTTGGTTGTCTTTG
MyD88	CATACCCTTGGTCGCGCTTA	CCAGGCATCCAACAAACTGC

Hippocampus Brain Histopathology

The brain was extracted and subsequently submerged in a fresh solution of formaldehyde for a duration of 24 h. Following this, the organs underwent a dehydration process involving ethanol, with a concentration of 70% for a period of 24 h, 90% for 1 h, and finally 100% for 1 h. Subsequently, the organs were subjected to a cleansing procedure utilizing xylene and ultimately embedded within paraffin. Coronal sections were prepared using a microtome with a thickness of 5 μm. These sections were then affixed onto glass slides and subjected to staining using the standard hematoxylin and eosin technique.

7.9. Results and discussion on liposomal system of selected bioactives

7.9.1 Physicochemical Attributes

The average particle size of Oxytocin loaded liposomes (Oxy-NVs) and Vasopressin loaded liposomes (Vas-NVs) was found in the range of 50–100 nm with a unimodal distribution. Both the Nano-formulations were fabricated with 4:1:1 ratio of HSPC, DMG-PEG-2000/Cholesterol having average particle size of 95.06±0.86 nm for Oxy-NVs & 99.06±1.01 for Vas-NVs and PDI of 0.115±0.002 for Oxy-NVs & 0.121±0.004 for Vas-NVs were selected as the optimum. The mean diameters of liposomes for the formulation were less than 100 nm as confirmed by TEM images of liposomes formulations of oxytocin and vasopressin. The optimized Oxy-NVs and Vas-NVs formulation exhibited zeta potential of -22.1±0.12 mV and -21.8±0.17, respectively. A number of different combinations of HSPC, DMG-PEG-2000 and cholesterol were employed to prepare liposomes for both the APIs with an aim to achieve higher value of %EE. The lipid liposomes prepared with 4:1:1 ratio of HSPC, DMG-PEG-2000 and cholesterol exhibited high entrapment efficiency (64.03±0.54 for oxy-NVs and 62.59±0.39 for Vas-NVs) as compared to other combinations. The high entrapment efficiency with HSPC, DMG-PEG-2000 and cholesterol can be ascribed to more compatibility selected peptides in the lipidic mixture.

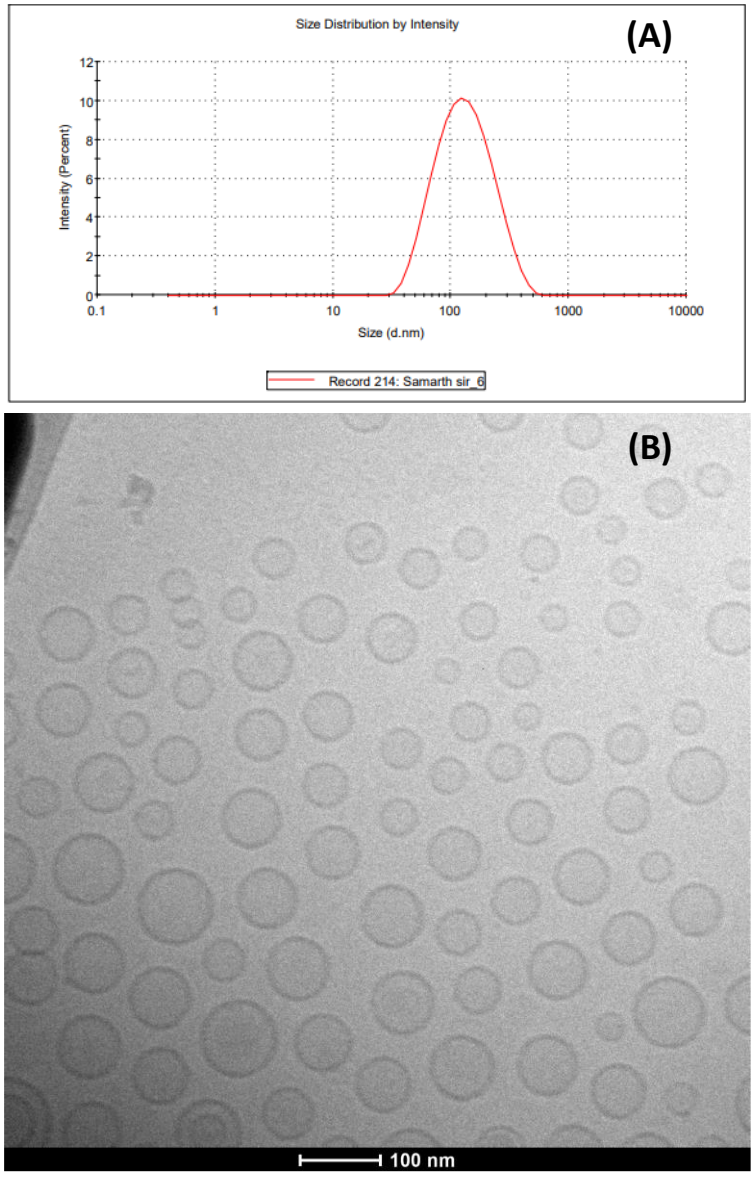


Figure 7.3: Graph of Particle size analysis (A), and HR-TEM image of liposomes of Oxytocin (B)

Table 7.11: Particle size, PDI, Zeta potential and % Entrapment Efficiency of Oxytocin and Vasopressin loaded liposomes

Nano-formulation for	HSPC, DMG-PEG-2000: Cholesterol	Particle size (nm)	PDI	Zeta Potential (mV)	%Drug Entrapment (PDE)
Oxytocin	4:1:1	95.06±0.86	0.115±0.002	-22.1±0.12	64.03±0.54
Vasopressin	4:1:1	99.06±1.01	0.121±0.004	-21.8±0.17	62.59±0.39

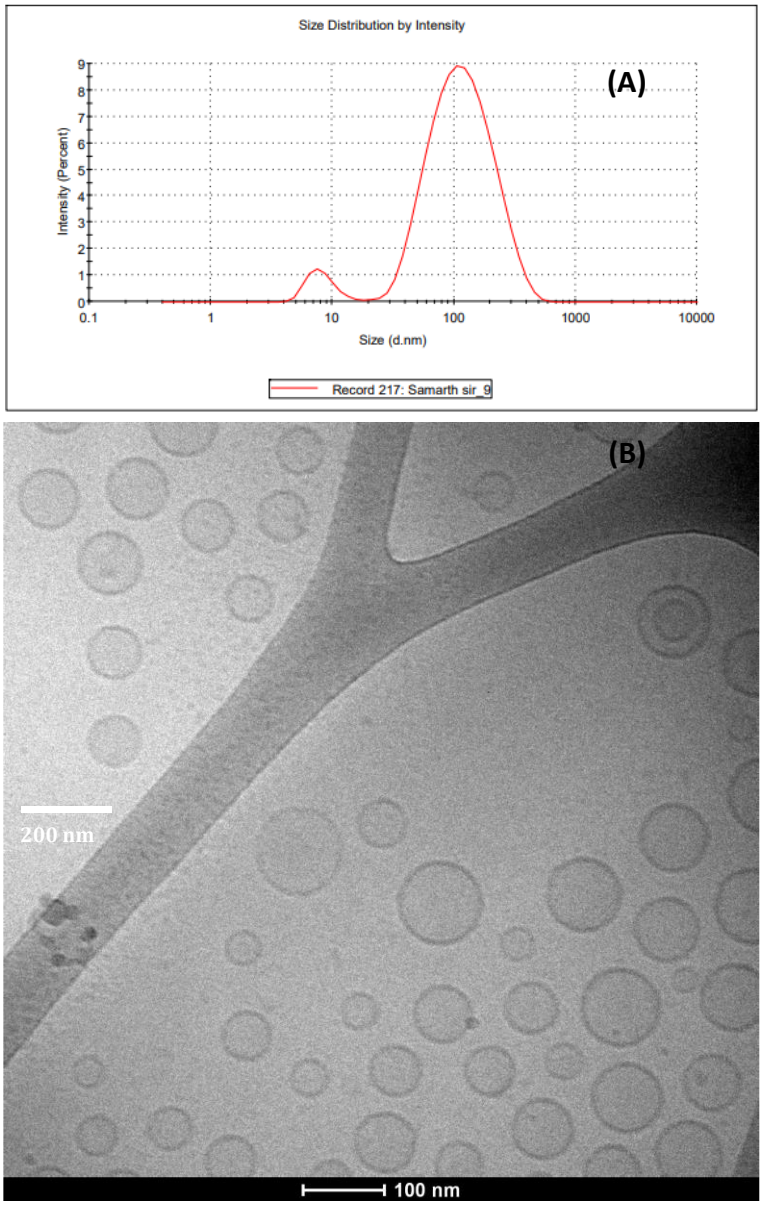


Figure 7.4: Graph of Particle size analysis (A), and HR-TEM image of liposomes of Vasopressin

7.9.2 Gene transcription analyses of inflammatory and apoptotic markers

The primary aim of study was to investigate impact of oxytocin, vasopressin, OXT-Lipo, and Vaso-Lipo on transcriptional profiles of various apoptotic and inflammatory immune markers. These markers included caspase-3, caspase-8, caspase-9, Akt1, NF-kβ, BCL-2, CD40, Bim, Bak, and IL-6, all of which play significant roles in cell death and inflammation.

There was a notable decrease in expression levels of pro-apoptotic markers' caspase-3 and caspase-8 in hTHP-I cells that were treated with all formulations of vasopressin and oxytocin (as depicted in Fig 7.5). In striking contrast, the expression of caspase-9 was elevated in treated

group, indicating a complex interplay of signals. However, it is important to note that these variations in pro-apoptotic markers did not attain statistical significance. Moreover, a reduction in levels of inflammatory markers NF-kB and IL-6 was recorded in all treated cells exposed to the various formulations of vasopressin and oxytocin. Cells that were treated with Lipo infused with vasopressin and oxytocin showed a significant reduction in BCL-2 expression, an important regulator of cell survival, in comparison to those that received standard drug treatment. Additionally, changes in expression of co-stimulatory markers were observed. The expression of CD40, implicated in regulating programmed cell death, along with pro-apoptotic gene Bak, was significantly reduced across all treated hTHP-I cells. Conversely, cells treated with oxytocin formulation exhibited an increase in expression levels of Bim, another pro-apoptotic marker.

The hTHP-I cells that received treatment with oxytocin displayed a highly significant reduction in expression of IL-6, a key pro-inflammatory cytokine, compared to both the cells treated with vasopressin and the untreated control group. This suggests that oxytocin may play a vital role in modulating inflammatory responses in addition to its effects on apoptosis

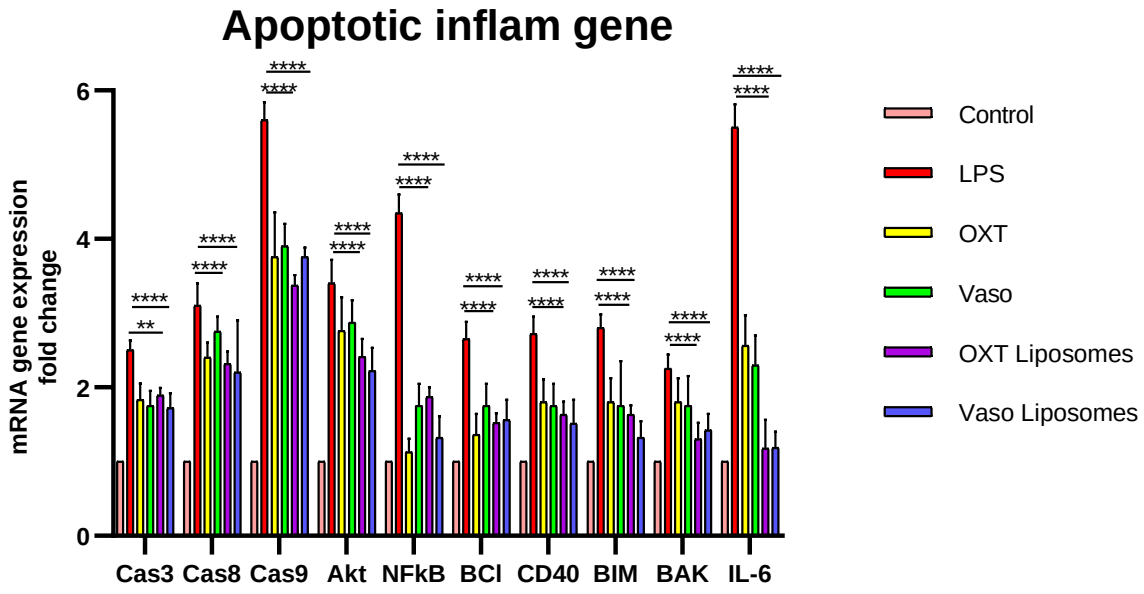
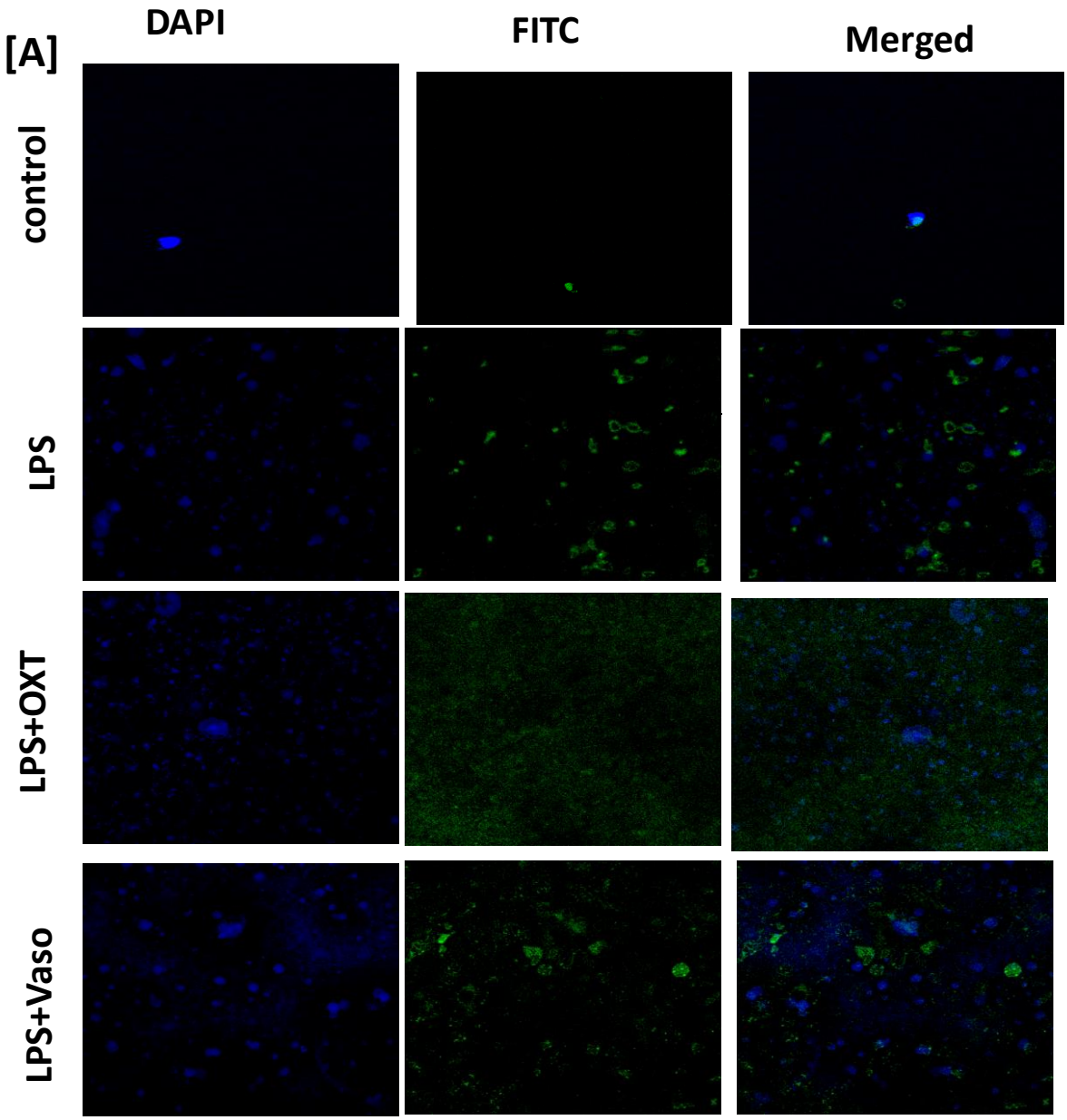


Figure 7.5: Determination of transcriptional gene expression of inflammatory, signalling and apoptotic markers

7.9.3 Confocal microscopy-based immunofluorescence assay (IFA)

The primary aim of this investigation was to establish a cell line model to enable us to explore the effects of various Lipo on the transcription factor FOXO1. This factor, in its phosphorylated state, is rendered inactive and plays a crucial role in regulating apoptotic pathways. Specifically, FOXO1 works in conjunction with the pro-apoptotic factor Bim, and their regulation is tightly controlled by overexpression of co-stimulatory molecule CD40. This interaction mediates the signalling pathways of PI3-Akt within human THP-1 (hTHP-I) cells.

The confocal microscopy assessed qualitative expression levels of FOXO1 in hTHP-I cells subjected to LPS stimulation. After 1 h post treatment with LPS, analysis revealed difference in FOXO1 expression when compared with LPS-stimulated cells to their unstimulated counterparts (as shown in Fig. 7.6). Moreover, quantification of fluorescence intensity of FOXO1 across a variety of cellular fields indicated a marked increase in FOXO1 expression when compared with the unstimulated control group. Notably, the liposome-encapsulated drug demonstrated superior outcomes compared to the non-encapsulated version. Among all formulations tested, the conjugation of oxytocin with AEEA yielded the most promising results, likely due to enhanced cell permeability, facilitating greater drug uptake.


[A]
DAPI
FITC
Merged
control
LPS
LPS+OXT
LPS+Vaso

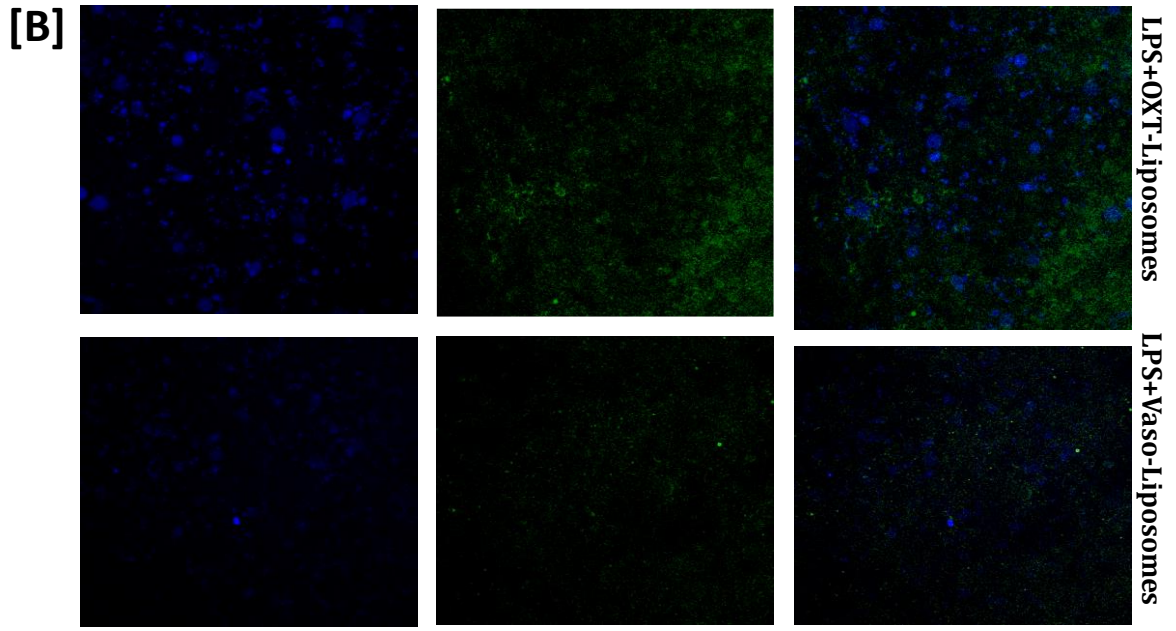


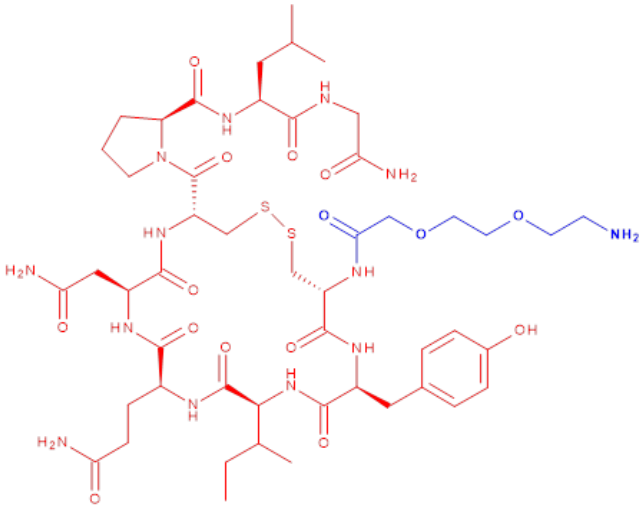
Figure 7.6: [A] & [B]: Expression of FOXO-1 gene following the LPS after various formulations treatment

8. Synthesis and Characterization of AEEA-Oxytocin

The aim was to synthesize, characterize and in vitro evaluation of conjugation of oxytocin with 2-(2-(2-Aminoethoxy) ethoxy) acetic acid to enhance half-life and bioavailability of for brain delivery

8.1. Synthesis of AEEA-Oxytocin

Structure

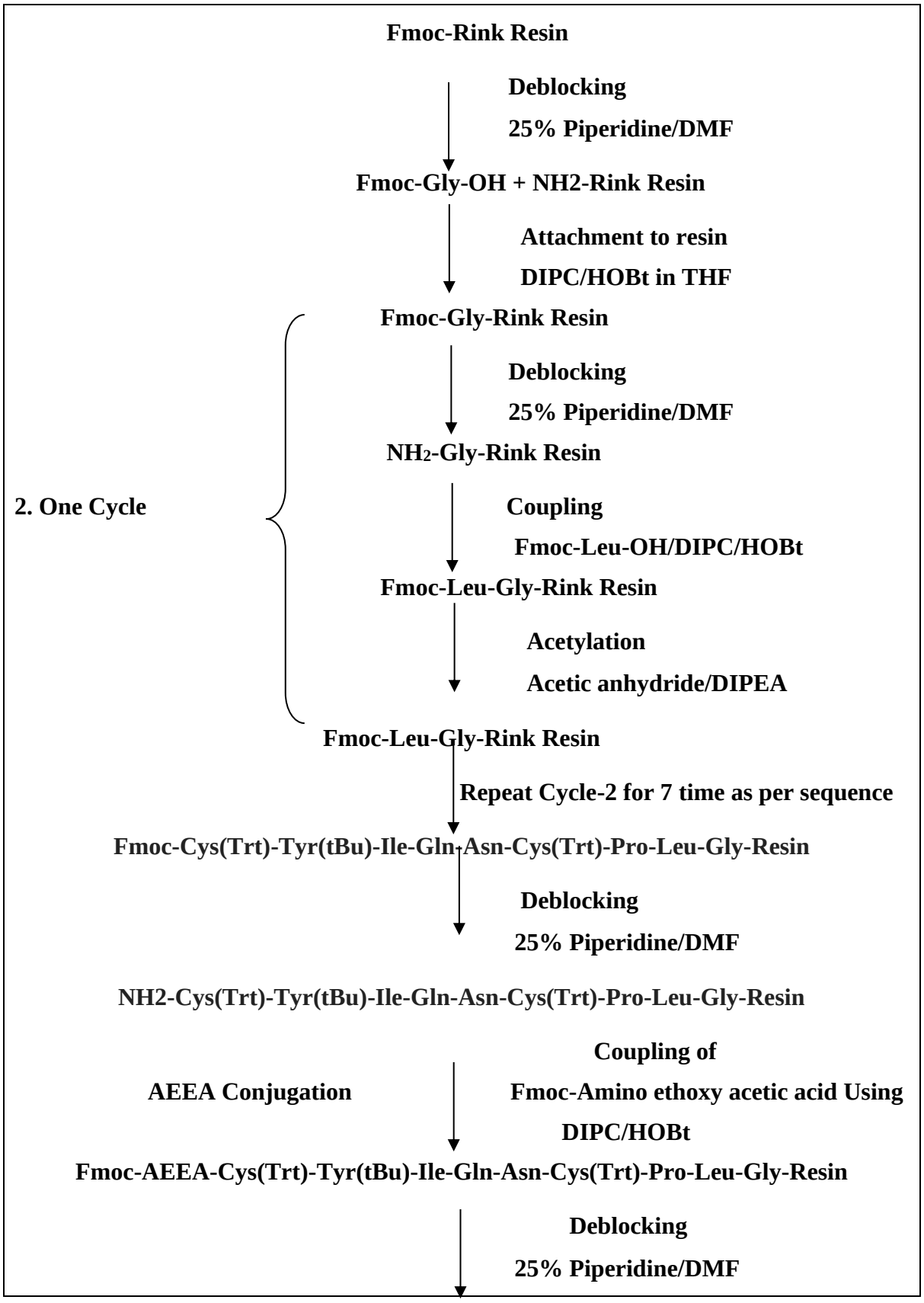


Formula Weight : 1152.34(4)
Exact Mass : 1151.5103502(2)
Formula : $C_{49}H_{77}N_{13}O_{15}S_2$
Composition : C 51.1% H 6.7% N 15.8% O 20.8% S 5.6%

Figure 8.1: Structure of prepared oxytocin conjugate

Sequence: AEEA-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH2 (2-7)-disulfide

Chemical Name: AEEA-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparagyl-L-cysteinyl-L-prolyl-Lleucyl-glycin (2-7)-disulfide


Fmoc-Rink Resin
Deblocking
25% Piperidine/DMF
Fmoc-Gly-OH + NH2-Rink Resin
Attachment to resin
DIPC/HOBt in THF
Fmoc-Gly-Rink Resin
Deblocking
25% Piperidine/DMF
NH2-Gly-Rink Resin
2. One Cycle
Coupling
Fmoc-Leu-OH/DIPC/HOBt
Fmoc-Leu-Gly-Rink Resin
Acetylation
Acetic anhydride/DIPEA
Fmoc-Leu-Gly-Rink Resin
Repeat Cycle-2 for 7 time as per sequence
Fmoc-Cys(Trt)-Tyr(tBu)-Ile-Gln-Asn-Cys(Trt)-Pro-Leu-Gly-Resin
Deblocking
25% Piperidine/DMF
NH2-Cys(Trt)-Tyr(tBu)-Ile-Gln-Asn-Cys(Trt)-Pro-Leu-Gly-Resin
AEEA Conjugation
Coupling of
Fmoc-Amino ethoxy acetic acid Using
DIPC/HOBt
Fmoc-AEEA-Cys(Trt)-Tyr(tBu)-Ile-Gln-Asn-Cys(Trt)-Pro-Leu-Gly-Resin
Deblocking
25% Piperidine/DMF

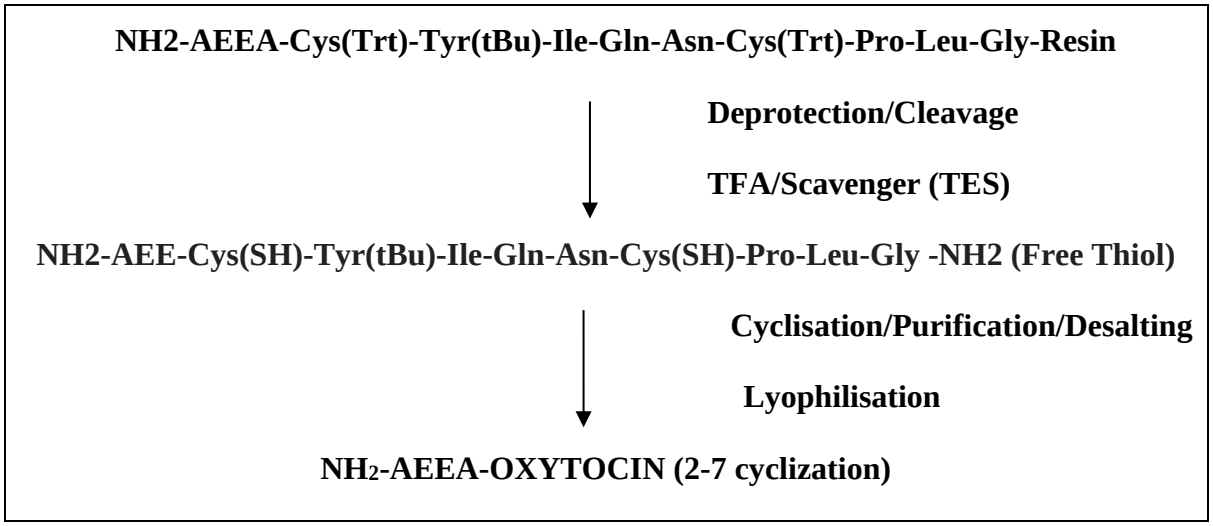


Figure 8.2: Schematic presentation of synthesis of NH_2-AEEA-OXYTOCIN

Figure 8.3: Schematic presentation of synthesis of NH_2-AEEA-OXYTOCIN

Stage–I: Manufacture of AEEA-Oxytocin-Stage-H-(01-10)-Resin

Fmoc-Rink amide AM resin used as solid support for the synthesis of **Oxytocin-Stage-H-(01-09)- Resin.** Fmoc protection was removed by using 25% piperidine/DMF. Fmoc-Gly-OH was attached to the H-Rink amide AM Resin using Diisopropyl carbodiimide and HOBt to generate in-situ intermediate Fmoc-Gly-Resin. Selective de-blocking of Fmoc group followed by the attachment of second amino acid (Fmoc-Leu-OH) using DIPC/HOBt at 20-25°C and Tetrahydrofuran as a coupling solvent gives Fmoc-2PP (08-09)-RESIN. Acetic anhydride and Diisopropylethyl amine were used to terminate the uncoupled amino groups after coupling of every amino acid. Rest of 7 amino acids were attached using DIPC/HOBt to synthesise Fmoc-(01-09)-RESIN. Finally, Fmoc group was removed using 25% piperidine/DMF to further conjugation of AEEA. Cycle 2 was repeated and Amino ethoxy ethoxy acetic acid was used in step of coupling to get the modified oxytocin from solid support

Stage–II: Manufacture of Oxytocin-AEEA

The final step involves cleavage and de-protection of protecting group from AEEA-**Oxytocin-solids.** (Intermediate Stage). The deprotection has carried out in Trifluoroacetic acid (TFA) as cleavage and de-protecting reagent and Triethyl silane as scavenger during the de-protection of protecting groups to form crude AEEA-Oxytocin free thiol. Further crude was dissolved and

cyclised using Iodine solution followed by purification, desalting and lyophilisation to synthesise Oxytocin-EEA final.

8.2. Characterization

FTIR was employed to furnish information about the groups present in the AEEA-Oxytocin and also for exploration of potential interactions. FTIR of plain Oxytocin and AEEA-Oxytocin was done to confirm the modifications in functional group of Oxytocin after conjugation with AEEA. FTIR analysis of samples (10-15 mg) was carried out by KBr pellet method, using Perkin Elmer Spectrum (Version 10.03.08) spectrometer operated at a resolution of 4 cm^{-1} in the range of 400–4000 cm^{-1}.

The sample was further analysed by NMR. NMR spectra were acquired on Bruker DPX-300 MHz, HD-300, and HD-400 spectrometers with samples prepared in deuterated solvents (D_2O) and chemical shifts were reported in parts per million (ppm) with reference to solvent residual peaks. D_2O NMR spectra for unreacted oxytocin and the reaction products with butyraldehyde were acquired at 298 K on a Bruker Avance III 600 NMR spectrometer operating at 500.13 MHz for 1H nuclei using the reaction mixture 15 mg in 0.6 mL D_2O for field frequency lock.

The mass of conjugated molecule was further confirmed by direct mass method using HRMS (Thermo Scientific, Q Exactive plus). Xcaliber software *Ver*. 4.4.16.44 was used to interpret data. Sample was prepared by dissolving AEEA-Oxytocin to get 1000 ppm concentration. 10 µL was injected in the instrument.

8.2.1. FTIR Spectroscopy

The conjugation of AEEA with oxytocin was investigated using FT-IR spectroscopy in order to determine the conjugation efficiency. The FT-IR spectra were recorded using IR affinity −1S Spectrometer (Shimadzu Corporation, Japan). FTIR spectra of plain oxytocin and AEEA-oxytocin were obtained in the range of 4000 to 600 cm^{-1}. Spectral output was recorded by the transmittance as a function of wave number.

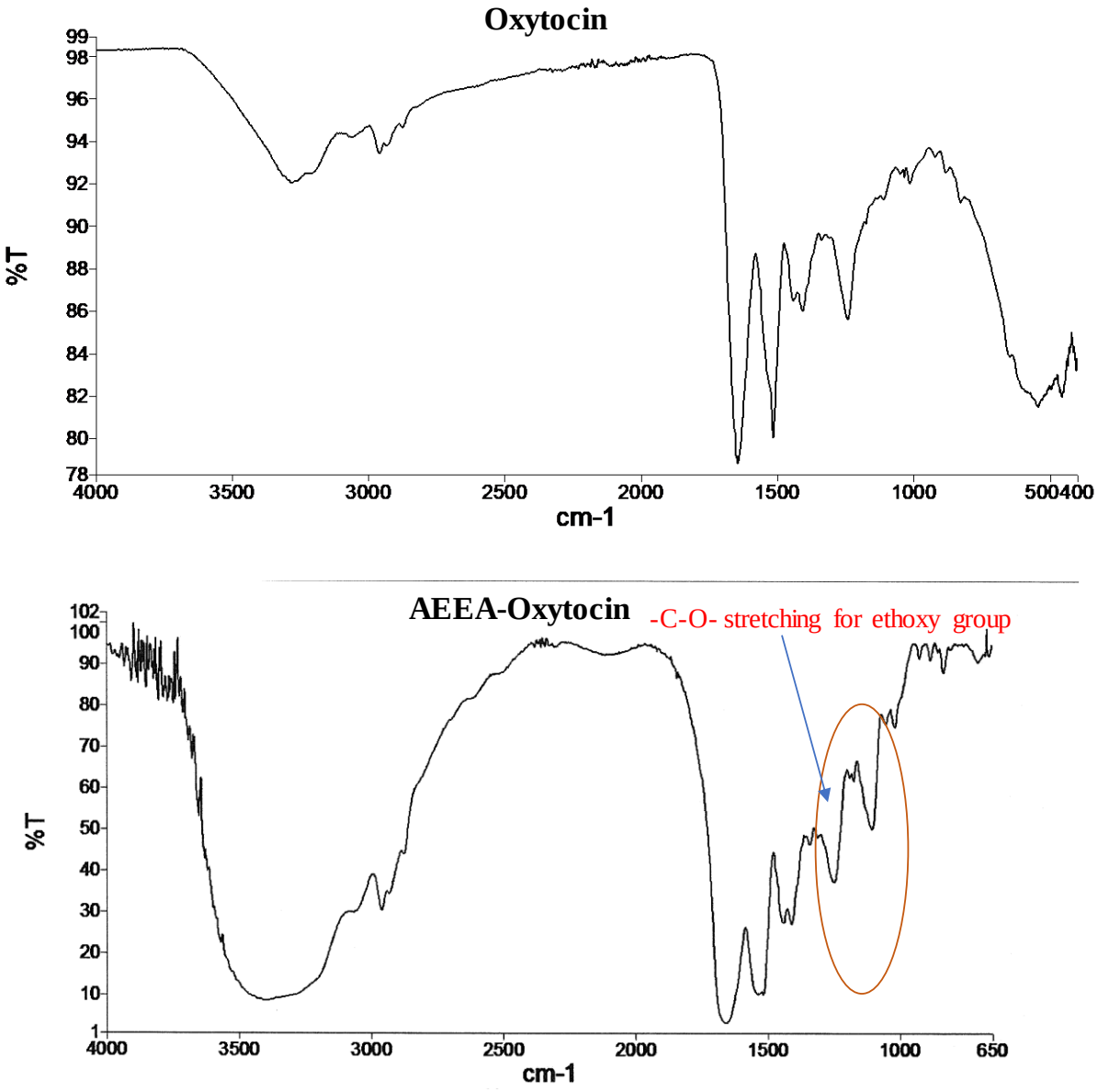


Figure 8.4: FT-IR Spectra of AEEA-Oxytocin

8.2.2. NMR Spectroscopy

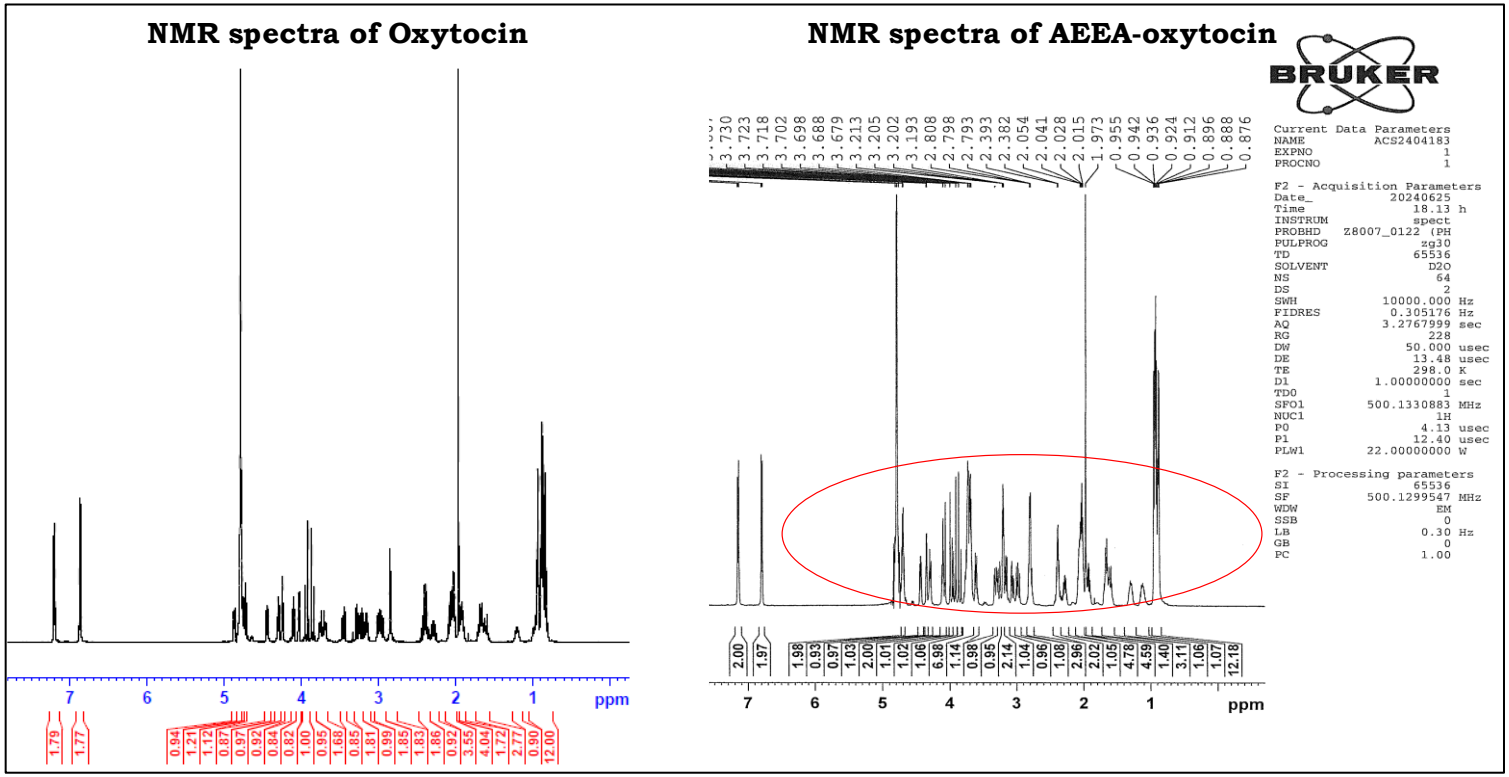


Figure 8.5: NMR spectra of AEEA-Oxytocin

Table 8.1: Observations from NMR spectra

S. No.	Chemical Shift ppm	No. of Proton	Assignment of Proton	Remarks
1	0.87-0.95	12	CH_3	Isoleucine/Leucine
2	1.09-1.32	2	CH	Isoleucine/Leucine
3	1.26-3.77	32	CH_2	AEEA and CH_2 of all amino acid
4	3.83-4.83	10	CH_2(1) CH (8)	CH_2-Glycine CH of 8 amino acid (Chiral)
5	6.78-7.15	4	CH (Aromatic)	Tyrosine
	Total	60		
Remark: -		Solvent peak observed: - 1.97-2.01ppm (Acetic acid),		
		No peak for -OH/NH_2/NH observed in spectrograph as it is an exchangeable proton. (Total = 17H)		

8.2.3. Mass spectroscopy

Figure 8.6: Direct mass spectra of AEEA-oxytocin

Table 8.2: Major Peaks observed in MS spectra

m/z	Rel. Abundance, %	Fragment
1151.509 [Mono isotopic mass]	100.00	$[C_{49}H_{77}N_{13}O_{15}S_2]+$

8.3. Results and discussion

2-(2-(2-Aminoethoxy) ethoxy) acetic acid was successfully conjugated with oxytocin and confirmed by FTIR, NMR and Mass spectroscopy. Additional -C-O- stretching band (1050-1150 cm^{-1}) observed (Figure 8.3) compared to plain oxytocin IR spectra which confirms the conjugation, and the number of protons presence (Figure 8.4 & Table 8.2 & 8.3) in AEEA-Oxytocin was further confirmed by NMR spectra. The total mass (Figure 8.5) of conjugated molecule is confirmed by direct HR-Mass.

9. Ongoing work

***In vitro* evaluation of Oxytocin, and Vasopressin loaded liposomes on SH-SY5Y cells**

- % Cell viability study
- *In vitro* cell permeation study (Cell uptake)
- Annexin-V staining
- Immunoblotting

***In vivo* evaluation of developed Oxytocin and Vasopressin loaded liposomes in experimental animals**

- Morris water maze test
- Elevated plus maze (EPM) test
- Gene expression studies
- Brain Histopathological evaluation

***In vitro* evaluation of Conjugated Oxytocin on cell lines**

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ANNEXURE-I

LIST OF PUBLICATIONS

Articles Published

1. **Samarth Kumar et al**. Nanocarriers mediated delivery of methotrexate is instrumental in treating auto-immune diseases and cancer. **Journal of Drug Delivery Science and Technology**, 2023, 88, p.104969. **(Impact factor 5.0)**
2. **Samarth Kumar et al**. Emerging Therapeutic Landscape on Delivery of Oxytocin to Brain for Treating Neurological Disorders**. Journal of Drug Delivery Science and Technology,** 2024, 92, p. 105370 **(Impact factor 5.0)**
3. **Samarth Kumar et al**. Glucagon: Delivery Advancements for Hypoglycemia Management. **International Journal of Pharmaceutics,** 2024, 652, p. 123785. **(Impact factor 5.8)**
4. **Samarth Kumar et al**. Novel discoveries and clinical advancements for treating onychomycosis. **Advanced Drug Delivery Reviews**, 2024, 205, p. 115174 **(Impact Factor 16)**
5. **Samarth Kumar et al**. USFDA-approved parenteral peptide formulations and excipients: Industrial perspective. **Journal of Drug Delivery Science and Technology,** 2024, 95, p. 105589 **(Impact factor 5.0)**
6. **Samarth Kumar et al**. Recent Progress in Macromolecules: From Current Therapeutic Strategies to Theranostic Applications**. Journal of Drug Delivery Science and Technology.** 2024, 96, p. 105664 **(Impact factor 5.0).**
7. **Samarth Kumar et al**. Influence of interaction of milk fat on release, characteristics and absorption of theophylline. **Indian drugs** 2022 | (59) | Issue No.1 | Page No. 28-33.
8. **Samarth Kumar et al.** Application of Sphingolipid based nanocarrier in drug delivery: An Overview. **Therapeutic delivery**.

Book chapter Published

1. Toward Eco-friendly Nanotechnology-based Polymers for Drug Delivery Applications. In Sustainable Nanotechnology: Strategies, Products, and Applications**. ISBN No. 978-1-119-65029-4 John Wily & Sons Inc.**
2. Pharmacokinetics and Pharmacodynamics of Liposomal Nanoparticles. In Pharmacokinetics and Pharmacodynamics of Nanoparticulate Drug Delivery Systems. **ISBN No.978-3-030-83395-4 Springer Nature Switzerland AG 2022.**
3. Nanoparticle Properties Affecting the Drug Release, Absorption, and Pharmacokinetics of Nanoparticulate Drug Delivery Systems. In Pharmacokinetics and Pharmacodynamics of Nanoparticulate Drug Delivery Systems. **ISBN No.978-3-030-83395-4 Springer Nature Switzerland AG 2022.**
4. Current Application of CRISPR/Cas9 Gene-Editing Technique to Eradication of HIV/AIDS. In Gene Delivery Systems: Nano delivery Technologies **ISBN No.9781003186083 CRS Press, Taylor & Francis Group.**
5. CRISPER Gene Therapy Recent Trends and Clinical Applications. In Gene Delivery Systems: Nano delivery Technologies. **ISBN No. 9781003186083 CRS Press, Taylor & Francis Group.**
6. Breast Cancer: Diagnostic Landscape. In Diagnostics landscape (Volume 2), **ISBN No. 9780443338496, Elsevier International. (In press)**
7. Breast Cancer: Delivery System Landscape. In Drug delivery systems landscape - DDS (Volume 1), **ISBN No. 9780443291685, Elsevier International. (In press).**

Under consideration

8. Vasopressin in the management of Brain Disorders: Current Status and Future Perspective.
9. Renin Angiotensin-II system: Haleness of Angiotensin-II in neurological disorders.
10. Application of lyophilization in pharmaceutical injectable formulation: An industrial and regulatory perspective

ANNEXURE-II

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Contents lists available at ScienceDirect

Journal of Drug Delivery Science and Technology

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Review article

Emerging therapeutic landscape on delivery of oxytocin to brain for treating neurological disorders

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Neurological disorders
Blood brain barrier (BBB)
Brain targeting nanocarriers

ABSTRACT

Neurological ailments constitute a substantial cause of disability and death. There are several treatments to manage neurological disorders, employing both synthesized molecules and biomacromolecules. Oxytocin is a promising neuropeptide molecule for the management of various neurological disorders. Oxytocin receptors are expressed in various regions of the brain, including the hypothalamus, amygdala, and *nucleus accumbens*. They are involved in the pathophysiology of various neurodegenerative and neuropsychiatric ailments such as Parkinson's disease, Alzheimer's disease, major depressive disorder, anxiety, mood disorders, autism, schizophrenia etc. Various pre-clinical and clinical reports advocate oxytocin to be a promising remedy for neurodisorders. However, limitations to cross the blood-brain barrier (BBB), frequent dose regime due to fast metabolism, elimination, the unwanted side effects lead to limited brain exposure to this neuropeptide in neuro ailments. This review presents an overview of the newer and advanced discoveries which have been enabled effective delivery of oxytocin across BBB. Numerous facts on nanocarrier-based medicine and routes of oxytocin administration for the treatment of neuro disorders have been recapitulated with ex vivo, preclinical, and clinical technologies explored so far to manage various neurological disorders with oxytocin.

1. Background

Oxytocin (OXT), a nonapeptide containing nine amino acids, acts as a neuropeptide hormone synthesized from the hypothalamus in the mammalian brain. In females, it is primarily used for management of parturition, control of postpartum uterine bleeding, in the treatment of incomplete, inevitable or elective abortion, lactation, etc. [1–3]. In the brain, OXT works as a neurotransmitter and a neuromodulator. Peptides associated with neurotransmitters have been established as latent therapeutics in neurological disorders and revealed their intense effectiveness in brain-associated disorders in preclinical studies. Amongst various neuropeptides, OXT functions as a chemical transmitter [1], also suppresses inflammation in neurons and acts as a neuroprotective [2] by protecting the hippocampus from excitotoxicity in brain [2–4].

The OXT receptor (OXTR) is a G protein-coupled receptor (GPCR), which binds to GTP and excites the cascade activity of Phospholipase-C enzyme. OXTR is present in various organs in humans. It is overexpressed in central nucleus of the amygdala, ventromedial nucleus of the hypothalamus, the head of the caudate-putamen and the hippocampus in central nervous system (CNS) [5]. Therefore, OXT acts as a neuromodulator and controls a wide range of CNS functions in both males and females.

The role of OXT in the CNS has now become an interesting area of investigation, wherever numerous preclinical and clinical studies specify that neurotransmission could be associated with the regulation of various neurological disorders. OXT has found a positive role in various neuropsychiatrics and degenerative disorders, including social [6] and non-social [7] behavior, sexual activity and mating [8], emotional [9] and sexual [5], affiliative [10], epilepsy [11], schizophrenia [12], mood disorders [13], obsessive-compulsive disorder

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