Depth Profiling of Penetration of Niacinamide into the Stratum Corneum: Effect of Hydration and Oil massage

A Dissertation Submitted to the Department of Pharmacy, East West University, Bangladesh, in the Conformity with the Requirement for the Degree of Masters of Pharmacy

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DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation "Depth profiling of penetration of niacinamide into the stratum corneum: Effect of hydration and oil massage'' submitted to the Department of Pharmacy, East West University, in the partial fulfillment of the requirement for the degree of Masters of Pharmacy (M.PHRM) is a genuine and authentic research work carried out by me under the guidance of Dr. Tasnuva Haque, Assistant Professor, Department of Pharmacy. The contents of this dissertation, in full or in parts, have not been submitted to any other institute or University for the award of any degree or Diploma of Fellowship.

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CERTIFICATE BY THE SUPERVISOR

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DEDICATION

This Research Paper Is Dedicated To My Honorable Research Supervisor

Dr. Tasnuva Haque, Assistant Professor, Department of Pharmacy.

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Abstract

Niacinamide is a cosmetic compound that is used in many cosmetic formulations nowadays due to its skin care properties. The present study was designed for the depth profiling of penetration of niacinamide into the skin using a control formulation. In addition, since proper amount of niacinamide penetration is necessary for skin benefit, influence of two external factors, such as skin hydration and oil massage on the penetration of niacinamide was investigated. For this study an international face cream brand was used as a test sample and the formulation was applied to eleven healthy volunteer on different sites of right and left volar forearm. The volunteers were aged between 18to 27 years. The calibration curve was formed by range of 5µg/mL to 30µg/mL. The calibration curve was found to be linear (R^2>0.999). This study is conducted by tape-striping process where stratum corneum of skin was removed and amount of niacinamide content in per tape were analyzed by using UV-Spectroscopy at 262nm wavelength. The depth profile of niacinamide from control site was compared with the skin hydration and oil massaged sites. No significant difference was observed in the amount of niacinamide at any depth of skin from control to hydration or oil massage ($p>0.05$). In addition, the analysis was extended to observe if there any difference between male and female skin. However, like the general result, male and female skin was also failed to show any significant influence of skin hydration and oil massage in penetrating niacinamide ($p>0.05$). The skin was hydrated or massaged with oil for only 2 minutes. The time may not be sufficient to show any significant difference.

Chapter-1

Introduction

1. Overview

A cosmetic should do more than only create beauty from outside as an ideal cosmetic brings out the natural beauty and unique inner radiance of every person. Now the cosmetic world strives to make sophisticated formulation for cosmetics and skin care product that awaken inner beauty and bring happiness and fulfillment into people's lives.

To fulfill the beautification purpose a model cosmetic compound niacinamide is used in many cosmetic formulations due to its skin care property such as to inhibit of sebum production as a result reduced acne, pore size and improved texture. Niacinamide also increases the production of collagen and inhibits the production of excess dermal glycosaminoglycans as a result it acts as anti-wrinkle and anti-aging agent[1].

To get full benefit from niacinamide, it is necessary to deliver proper amount of niacinamide inside the skin. This is why our study was aimed to evaluate the external factors (simple everyday practice, such as hydrating the skin or massaging the skin with oil, etc.) which may aid higher penetration of niacinamide in skin.

1.1 .The physiology of skin

The skin is the largest vital organ of the human body and the most visible covering 1.6 m^2 of surface area and accounting for approximately 16% of an adult's body weight. The main function of the skin is to act as a barrier to protect the warm moist internal environment from the essentially hostile, dry and cool external environment in which we live. The skin is not only the interface between humans and their environment but also it helps to helps to maintain essential bodily functions a) retention of moisture and prevention of permeation or loss of other molecules b) regulation of body temperature c)protection of the body from microbes and harmful external influences, and d) sensation[2].

1.1.1. The structure of skin

The human skin is composed of several layers, each with a unique structure and function. Knowledge about the mechanical behavior of these skin layers is important for clinical and cosmetic research, such as the development of personal care products and the understanding of skin diseases. Studying these layers *in vivo* is very challenging. The different length scales, ranging from μm for the stratum corneum to cm for the hypodermis, the interwoven layered structure and the inverse relation between penetration depth and resolution of non-invasive measurement techniques form major problems.
Skin generally consists of a three-layer structure: a) the epidermis which is top, the stratified layer b) dermis, the middle layer which is composed of the connective tissues and c) subcutaneous tissues which is below the dermis [3].

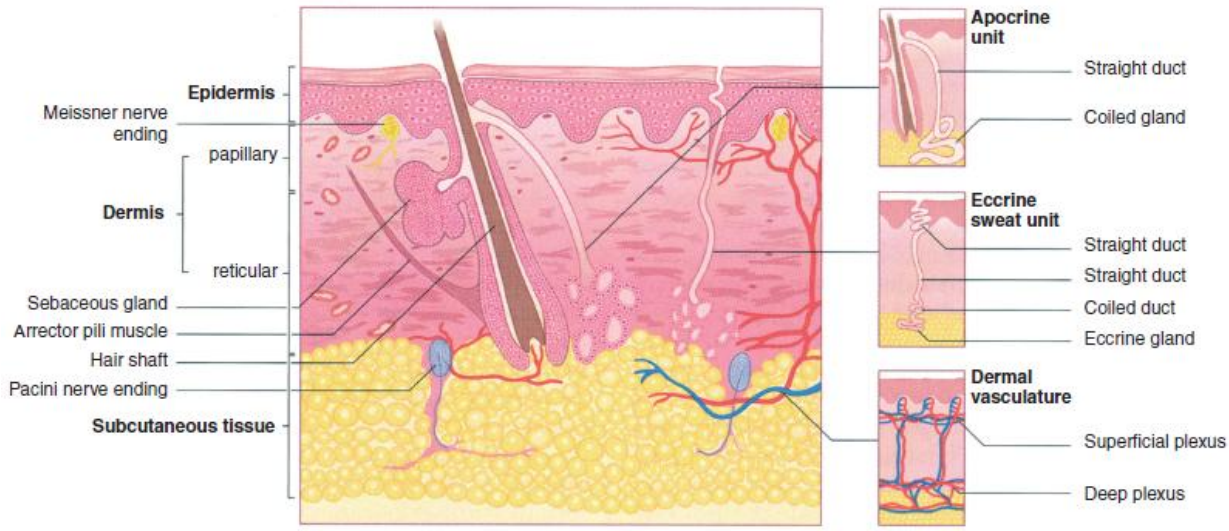


Figure1.1: Structure of skin[4]

i) Epidermis

The epidermis is composed of the outer-most layers of skin cells. Epidermis means 'upon' or 'over' dermis which is a multilayered structure whose thickness varies from 0.8 mm (on the palms and soles) to 0.06 mm (on the eyelids).It does not contain any blood vessels and therefore the cells obtain diffused oxygen from the surrounding air.The epidermis consists mainly of strafitied squamous keratinized epithelium. The keratinized epithelium cells are called keratinocytes.

From the dermis outward, epidermis consist of five layers of keratinocytes as a) The stratum basale (stratum germinativum) b) The stratum spinosum c) The stratum granulosum d) The stratum lucidum e) The stratum corneum

a) Stratum basale(Stratum germinativum): The deepest layer of the epidermis, the stratum basale, consists of a single layer of basophilic columner or coboidal cells resting on the basal lamina at the dermal epidermal junction. The cells contain a nucleus, arranged vertically and are columnar in shape. The keratinocytes are connected to the dermis with the help of collagen fibres. Desnosomes and henidesmosomes attach one cell to another and to the basement membrane.

The stratum basale is characterized by intense mitotic activity and is responsible in conjunction with the initial portion of the next layer, for constant renewal of epidermal cells. This layer also contains other cells, for example, melanocytes, Langerhans cells and Merkel cells. Melanin pigment is produced in melanocytes which help to protect skin from UV radiation. Langerhans cells play a role in the immune system of the body. Merkel cells are associated with nerve endings and are involved in sensory perception.

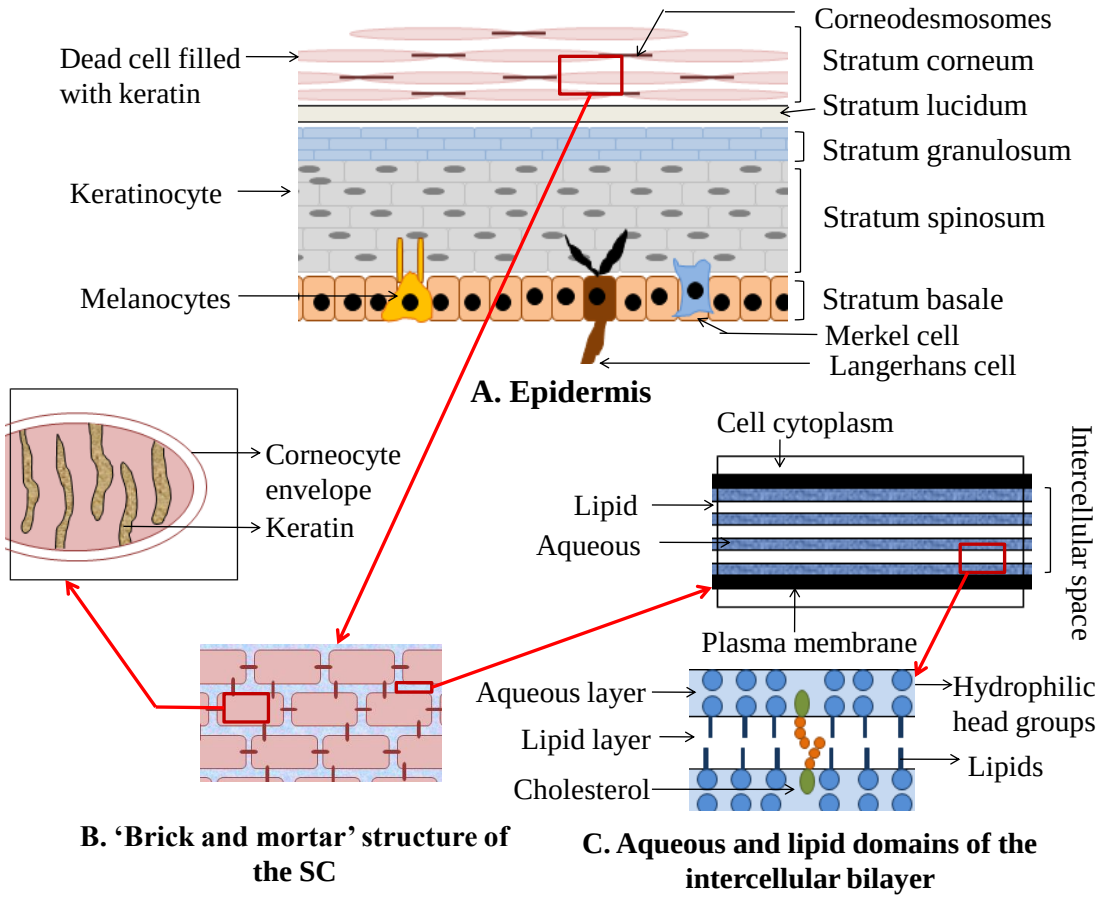


Figure1.2: Diagrammatic representation of: A. layers of human epidermis, B. 'brick and mortar' organisation of the SC and C. organisation of aqueous and lipid domains in the intercellular bilayer region. [5]

b) Stratum spinosum: This is the thickest layer of epidermis lying 2 to 6 rows of cell layers above the stratum basale. It consists of several layers of large keratinocytes that are cuboidal or slightly flattened cells with a central nucleus and a cytoplasm whose processes are filled with bundles of keratin filaments. As the cell migrates upward from the stratum basale, the shape changes from columnar to polygonal and their nuclei start to decrease in size. The cell layer is called spinosum as the cells are interconnected by intercellular bridges (desmosomes) which give the cell a spiny appearance. Keratinocytes contain membrane –coating granules and these cells are mitotically active, especially in the deeper layer. Langerhans cells also are located in this layer.

c) Stratum granulosum : It is present above the spinosum cell layer which is consisted of 3-5 layers of flattened cells whose long axis is parallel to the skin surface. The cytoplasms of these cells contain coarse basophilic granules called keratohyaline granules. Another characteristic structure in the cells of the granular layer is the membrane-coated lamellar granular. The contents of these granules are released by exocytosis, forming a layer of lipid-rich substance over the plasma membranes. The coating acts as a waterproof barrier.

d) Stratum lucidum: This layer is evident in thick skin .It is a translucent, thin layer of externally flattened eosinophilic epidermal cells. The organelles and nuclei are indistinct or absent in these cells. The cytoplasm contains a derivative of keratohyalin called eleidin .It is present in the palm of the hand and sole of the foot. The cells of this layer are flatter, compact and in the process of becoming flat, anucleate and cornified dead cells of the SC.

e) Stratum corneum(SC): It is the outermost layer of epidermis having a heterogeneous structure and composed of 70 to 80% protein (keratin) and 20% lipid. This layer is composed of 10 to 15 layers of flattened, keratinised dead cells (corneocytes) which are highly organized. Almost all of the lipids of the SC are present between the SC corneocytes. The thickness of this layer is only 10 to 20 μm when it is dry. If SC is considered as a brick wall, the corneocytes are the 'bricks' present in a 'mortar' (or intercellular lipid matrix) and desmosomes are the rivets between the cells (Figure 2A and B).

In the corneocytes, there is a filamentous network of keratin. The cornified cells are surrounded by a corneocyte envelope which is a protein-lipid polymer structure (Figure 2B). This envelope provides a rigid structure for the corneocytes. It is highly resistant to external chemical assault and regulates the hydration between the intra- and extra-cellular environments of the corneocytes.

The intercellular space between corneocytes is filled with multiple lipid lamellae (Figure 2C).The major components of intercellular lipid lamellae are thirteen subgroups of ceramides (41%), cholesterol (27%), cholesterol esters (10%), cholesterol sulphate (2%) and free fatty acids (9%). Interestingly, phospholipids which are the major component of other biological membranes are not present in the SC. It has been established that cholesterol and ceramides are very important for the formation of lamellar phases. The lipid lamellae are organised in parallel to the surface of the corneocytes. This phase consists of alternating layers of lipid bilayers and water.

Intercellular lipids are the main barrier to water loss. Extracting the lipid from the SC enhanced the water loss faster compared with non-extracted skin. Thus, intercellular lipid lamellae are very important for the barrier function of the SC and also help in cohesion between corneocytes. The SC also contains approximately 15 to 20% water mainly associated with keratin and a small amount in the polar head group of the intercellular space. Besides these elements, there are also some proteolytic and lipolytic enzymes present in the SC which perform some biochemical activities, such as processing pro-barrier lipids and breaking down desmosomes.

The lower water loss (1000-fold less than other biological membranes) and higher barrier function of SC is because of the unique composition, especially because of the intercellular lipids and corneocyte envelope. The epidermis undergoes a differentiation process in which cornification or generation of SC takes place. The process starts at the stratum basale and cells migrate upwards to the SC. The total process usually takes 2 to 3 weeks to complete[6].

ii) Dermis

The dermis is the layer of the skin underlying the epidermis. It is composed of connective tissue that contains many type I collagen fibres and networks of thick elastic fibres which is about 20 to 30 times thicker (3 to 5 mm) than the epidermis. Collagen is responsible for the strength of the skin and holding skin tissue together while elastic connective tissue provides elasticity and flexibility immersed in a semi-gel matrix of mucopolysaccharide.

There are some cone-like papillae present on the surface of the dermis and underneath the epidermis which help to hold the two layers of skin together. This layer contains mast cells, macrophages, lymphocytes and melanocytes. Immune and inflammatory responses are provided by the mast cells. Blood vessels, nerves and skin appendages (sweat and sebaceous glands) are also present in this layer. Because of the structural composition, this layer does not offer the same resistance to drugs as the stratum corneum.

Dermis is divided into two layers: superficial papillary layer and deeper reticular layer though there is no distinct boundary exist between these layers.

a) Papillary layer: this thin superficial layer is composed of loose connective tissue, fibroblasts and other connective tissue cells, such as mast cells and macrophages. The fine-touch receptors meissner corpuscles are present in this layer. The papillary layer is so called because it forms the major of the dermal papillae. From this layer, special collagen fibrils called anchoring fibrils, insert into the basal dermis to the epidermis.

b) Reticular layer: this thicker layer is composed of irregular dense connective tissue and thick elastic fibres. The layer may contain pacinian corpuscles and krause end-bulbs in its deeper aspects. The elastic network of this layer is responsible for the elasticity. [6]

iii) Subcutaneous tissue

The lowermost layer of skin is the subcutaneous fat layer and is also called the 'hypodermis' meaning 'beneath the skin'. It consists of loose connective tissue, elastin and cells such as fibroblasts, macrophages and adipocytes. This layer mainly consists of fat cells (50% adipocytes) and plays an important role in our body by attaching the dermis to the muscles and bones via a special connecting tissue called septa which consists of blood vessels, nerve cells and collagen.

The subcutaneous fat layer controls the body temperature (thermoregulation) via homeostasis. Excess body heat is controlled by vasodilation and sweating, which assists cooling via evaporation. An excessively cold body temperature is controlled via vasoconstriction and converting fat directly into heat energy by the rmogenesis[7]

1.**2. Compound penetration across the skin**

The function of skin is to act as a barrier between our body and our surroundings and that is why it is challenge to penetrate compounds as it repels outside. The upper layer of skin stratum corneum efficiently limits the penetration of compounds as SC is a heterogeneous

structure that containcorneocytes which are arranged in pillars to form clusters. The barrier nature of the SC is governed by its constituents: 75%-80% are proteins, 5%-15% lipids, and 5%-10% unidentified elements. Such a composition favors the absorption of certain lipid-soluble compounds by the skin. In order to simplify the calculation of a compound's permeation profile through skin, scientists have adopted some number of models to explain the flux of compounds through SC lipid domains [8].

1.2.1. Mechanism of skin penetration

Any molecules in contact with the skin surface can penetrate by the following three potential pathways: through the sweat ducts, through the hair follicles and sebaceous glands (collectively called the shunt or appendageal route), or directly across the stratum corneum. It is considered that a follicular shunt route was responsible for the pre-steady state permeation of polar molecules and flux of large polar molecules or ions that have difficulty diffusing across the intact stratum corneum[9].

Considerable research effort has been directed towards gaining a better understanding of the structure and barrier properties of the stratum corneum which is consisted of 10 to 15 layers of corneocytes and varies in thickness from approximately 10 to 15 μm in the dry state to 40 μm when hydrated. The intercellular lipid matrix is generated by keratinocytes in the mid to upper part of the stratum granulosum, discharging their lamellar contents into the intercellular space. In the initial layers of the stratum corneum, this extruded material rearranges to form broad intercellular lipid lamellae which then associate intolipid bilayers [10]

It comprises a multi-layered 'brick and mortar'-like structure of keratin rich corneocytes (bricks) in an intercellular matrix (mortar) composed primarily of long chain ceramides, free fatty acids, triglycerides, cholesterol, cholesterol sulphate, and sterol wax esters. However, it is important to view this model in the context that the corneocytes are not brick shaped, but are polygonal, elongated, and flat (0.2 – 1.5 μm thick, 34 – 46 μm in diameter). The intercellular lipid matrix is generated by keratinocytes in the mid to upper part of the stratum granulosum, discharging their lamellar contents into the intercellular space. In the initial layers of the stratum corneum, this extruded material rearranges to form broad intercellular lipid lamellae which then associate into lipid bilayers with the hydrocarbon chains aligned and polar head groups dissolved in an aqueous layer[11].

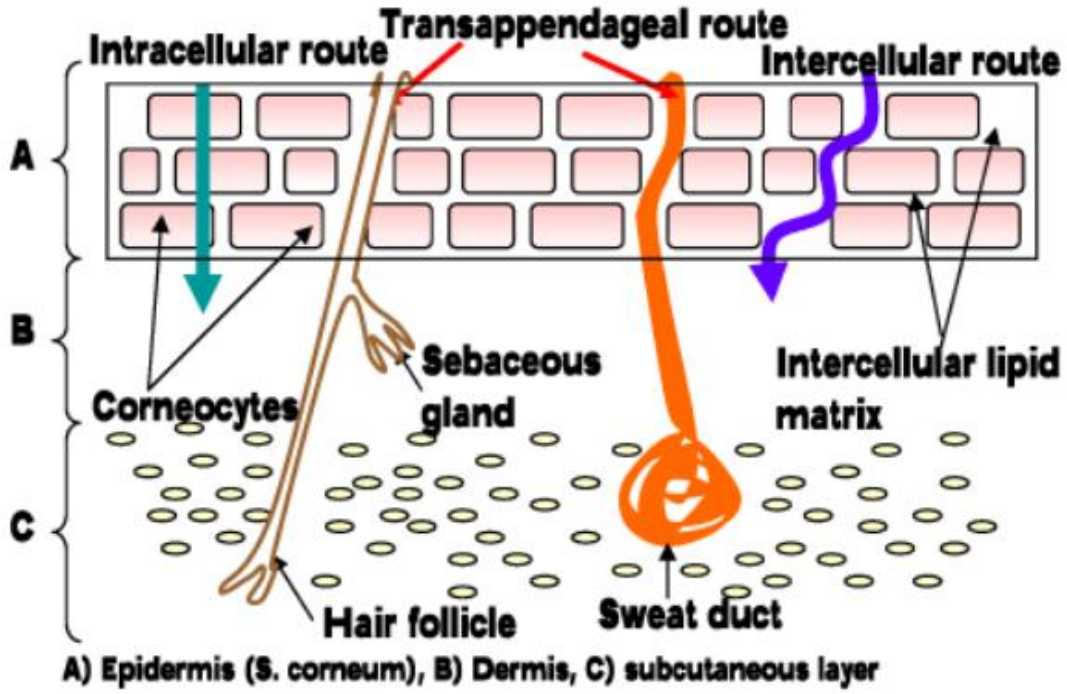


Figure1.3: The mechanism of skin penetration[12]

1.2.2. Factors Influencing Skin Permeation of compounds

The transdermal permeation of compounds is significantly affected by various factors among them, these factors are broadly classified into two groups, one is the physiological factors another is physicochemical factors.

A) Physiological Factors

The physiological factors that influence the absorption of molecules through a healthy skin include:

i) Skin Age

With the increase of skin ages some functional and structural changes take place that affect the transdermal absorption of molecules into it. Hydration plays an important role in transdermal absorption which is decreased with age as the skin loses its moisture content. The enzymatic activity in the skin also reduces with age as well as the blood flow to the skin is also decreased.

ii) Anatomical Location

The skin differs in appearance all across the body. The generalized decreasing order of absorption exists from the genitals > head and neck > trunk > arm > leg. Differences in anatomical sites are therefore indicative that the location of transdermal ointment or cosmetic products applications would affect absorption.

iii) Race

Some studies about racial differences in skin function have been reported that an increase in intracellular cohesion and higher lipid content in black skin. Although relatively limited data are available on race, indications are that racial differences do exist among white and black skins, with regards to some anatomical and physiological functions of the skin and that these differences could determine race related patterns in skin behavior, namely the response to irritant chemicals and the penetration of topically applied compounds.

iv) Temperature

The permeation of molecules across the skin is a passive process. An increase in temperature would thus result in an increase in kinetic energy of the molecules which would therefore cause the molecules to move faster through the SC. An increasing temperature would also cause structural alterations in the SC and underlying tissue and result in a faster movement of the compound through the different skin layers.

v) Skin Hydration

The water content of the skin plays a vital role in transdermal permeation. From one study it is found that hydration of the skin through soaking, moisturizing or humidity. Hydration causes the corneocytes in the SC to swell which allows molecules to permeate through that layer much easier.

vi) Skin Condition (Disease)

Disease compromises the natural barrier function of the skin which affects the transdermal absorption of therapeutic agents. The rate of transdermal absorption would increase with a disease that ravages the skin but would decrease upon self-healing of the skin

vii) Skin Metabolism

Enzymes in the skin are responsible for metabolism and for the elimination of drugs. For prodrugs which have to undergo biotransformation, enzymatic activity is therefore a rate

limiting factor in the transdermal absorption process and is reported to be the highest in the viable epidermis of the skin[13]

B) Physicochemical Factors

Physicochemical factors are the primary factors that influence the transdermal absorption of compounds. For molecules designed to pass through the SC favorable physicochemical properties are necessary which include:

i) Solubility

The SC is the most important barrier to skin permeation and therefore the rate limiting factor for
any substance to cross the skin. The SC is a lipophilic membrane and the amount of a drug that accumulates in it bears some relationship to the solubility of that drug in some organic solvents, such as the highly lipophilic hexane. Saturated solutions better permeate through the SC, because they represent maximum thermodynamic activity.

A very hydrophilic drug is unable to penetrate the skin, while a very lipophilic drug has the propensity to remain in the layers of the SC. While the SC is lipophilic in nature and favors the permeation of lipophilic drugs, the aqueous nature of the layers beneath the SC dictate that drugs should embody some hydrophilic properties to pass through them.

ii) Partition Coefficient

Penetration of the SC requires that a compound partitions into the membrane. Such partitioning is an important step in the penetration of the membrane. The partition coefficient is usually the key factor in determining which pathway a molecule would follow when passing through the SC. Hydrophilic molecules are therefore expected to pass through the intracellular routes whereas more lipophilic molecules would use the intercellular routes. Either lipophilic or hydrophilic permeants must pass through the hydrophilic and lipophilic layers respectively to achieve successful transdermal delivery.

iii) Molecular Mass

The skin is a compact membrane that is difficult to penetrate. Smaller molecules are therefore likely to permeate the skin at higher rates than larger molecules, hence the importance of molecular size in transdermal absorption. There is an inverse relationship between molecular size and transdermal permeation. Compounds with a molecular mass (MW) between 100 and 500 Daltons were found to be suitable for transdermal transport.

iv) Ionization

The lipophilic nature of the SC had led to the belief that ionized compounds would be poor candidates for transdermal delivery. As a result of the complex structure of the skin, however, compounds can cross the skin via various pathways. The transcellular route is regarded as having intermediate properties whereas the intracellular route is mainly regarded for allowing the delivery of lipophilic molecules.

Ionized drugs hence cross the skin through the shunt route but the amount of molecules that pass through that route is significantly less than unionized molecules that take the intracellular route. The pH range of the viable epidermis is 7.3–7.4 and that of the SC 4.2–5.6. The compounds concentration that exists in the unionized form is a function of both the dissociation constant of the drug and the pH at the absorption site.

From the Henderson-Hasselbach equation:

$$\text{Aci drug:pH} = \text{pKa} + \log\frac{(\text{salt})(\text{ionized})}{(\text{acid})(\text{unionized})}$$

$$\text{Base drug pH} = \text{pKb} + \log\frac{(\text{salt})(\text{ionized})}{(\text{acid})(\text{unionized})}$$

The fraction of the unionized drug is thus a function of the pH

v) Hydrogen Bonding

The varied skin components (lipids, proteins, aqueous regions, enzymes, *etc.*) and possible compound penetrates (weak acids/bases, ionized/unionized species, neutral molecules, etc.) suggest a multitude of potential interactions between drug substances and skin tissue. The formation of hydrogen bonds, or weak Van der Waals forces would hence influence skin penetration of a permeating drug. Lag time is an important factor being influenced by drug binding to the tissue. Bound drugs to skin tissue in the SC would result in a prolonged lag time, which would translate into a delayed onset of the therapeutic action. Since the SC is mainly a hydrogen bond acceptor an increase in the number of hydrogen bonding groups of the drug may inhibit its permeation across the layers of the SC.

vi) Melting Point

Generally organic substances with high melting points and high enthalpy of melting have lower aqueous solubility properties because solvents cannot enter the crystalline structure of such molecules to dissolve them. An indirect relationship therefore exists between the melting point and the solubility of a compound. Lowering the melting point of a compound would hence cause an increase in its solubility in the SC and ultimately in its permeation across the skin[13]

1.2.3 Different penetration enhancement technique

The various methods developed for enhancing penetration across the stratum corneum are broadly classified into following enhancement techniques-[14]

1. Chemical enhancement
2. Physical enhancement

1.2.3.1. Chemical enhancement

Chemical Penetration Enhancers are vastly present in huge numbers to facilitate the penetrationof drugs in transdermal, demagogical and cosmetic products. Incorporation of penetration enhancers facilitates the absorption of compounds by altering the barrier property of the stratum corneum. A permeation enhancer should be pharmacologically inert, nontoxic, nonirritating, nonallergic, odorless, tasteless, colorless, compatible with most compounds and excipients, inexpensive, and have good solvent properties.

Different classes of penetration enhancers including alcohols and polyols (ethanol, propylene glycol), surfactants (Tween, Span, SLS), fatty acids (Oleic acid), amines and amides (Azone, *N*-methylpyrrolidone), terpenes (limonene) sulfoxides (dimethylsulfoxide), esters (isopropylmyristate) were developed over time.

i) Sulphoxides and similar chemicals

Dimethyl sulphoxide (DMSO) is one of the earliest and most widely studied penetration enhancers. It is a powerful aportic solvent which binds with hydrogen rather than with water. Similar chemically-related material as an accelerant like dimethylacetamide and dimethylformamide (DMF) are similarly powerful aportic solvents. The mechanism of the sulphoxide penetration enhancers is widely used to denature protein and, on application to human skin, has been shown to change the intercellular keratin conformation from α helical to ß sheet[8]

ii) Azone

Azone was the first molecule specifically designed as a skin penetration enhancer. It is a colorless, odorless liquid with a melting point of -7°C and it possesses a smooth, oily but yet non-greasy feel. Azone enhances the skin transport of a wide variety of drugs including steroids, antibiotics, and antiviral agents [8]

iii) Pyrrolidones

Pyrrolidones have been used as permeation enhancers for numerous molecules including hydrophilic (e.g., mannitol and 5-flurouracil) and lipophilic (progesterone and hydrocortisone)

Permeants [8]

iv) Essential oil, terpenes and terpenoids

Terpenes are found in essential oils, and are compounds comprising of only carbon, hydrogen, and oxygen atoms. The essential oils of eucalyptus, Chenopodium and ylang-ylang have been found to be effective penetration enhancers for 5-flouorouracil transversing human skin in vivo [8]

v) Water

In general, increased tissue hydration appears to increase transdermal delivery of both hydrophilic and lipophilic permeates. Considering the heterogeneous nature of human stratum corneum, it is not surprising that water within this membrane is found in several 'states.' [8]

vi) Alcohols, fatty alcohols and glycols

Ethanol is commonly used in many transdermal formulations and is often the solvent of choice for use in patches. With water, ethanol permeates rapidly through human skin with a steady state flux of approximately 1 mg cm2/h. [8]

vii) Surfactants

Surfactant are found in many existing therapeutic , cosmetic and agrochemical preparations Usually, surfactants are added to formulations in order to solubilise lipophilic active ingredients, and so they have potential to solubilise lipids within the stratum corneum [8]

1.2.3.2. Physical penetration enhancers

i) Electroporation

Electroporation involves the application of high voltage pulses to induce skin perturbation. The technology has been successfully used to enhance the skin permeability of molecules with differing lipophilicity and size [15]

ii) Iontophoresis

This method involves enhancing the permeation of a topically applied therapeutic agent by the application of a low-level electric current, either directly to the skin or indirectly via the dosage form[8]

iii) Laser radiation and photomechanical waves

Lasers are frequently used for the treatment of dermatological conditions such as acne and to confer facial rejuvenation, where the laser radiation destroys the target cells over a short frame of time (300 ns). [8]

iv) Ultrasound

Ultrasound involves the use of ultrasonic energy to enhance the transdermal delivery of solutes either simultaneously or through pretreatment, and is frequently referred to as sonophoresis. Recent studies have shown that ultrasound can increase up to 5,000 times the ability of protein the size of insulin to penetrate the skin. [8]

v) Magnetophoresis

This method involves the application of a magnetic field which acts as an external driving force to enhance the diffusion of a diamagnetic solute across the skin. Skin exposure to a magnetic field might also induce structural alterations that could contribute to an increase in permeability.[8]

vi) Microfabrication microneedles technology

The microfabricated microneedles technology employs micron-sized needles made from silicon. Microneedles have been fabricated with different range of size, shape and materials. These microneedle arrays after insertion into the skin create conduits for transport of drug across the stratum corneum [8]

vii) Macroflux

The system incorporates a titanium microprojection array that creates superficial pathway through the skin barrier layer to allow transportation of therapeutic proteins and vaccines or access to the interstitial fluids for sampling[8]

viii) Needleless injection

It is highly sophisticated technique whereby liquid and solid particles are bombarded at supersonic speed into the skin. The technique utilizes compressed gases such as Helium or nitrogen through narrow nozzle along with the drug molecules in a jet flow[8]

ix) Microporation

Microporation involves the use of microneedles that are applied to the skin so that they pierce only the stratum corneum and increase skin permeability [8]

x) Heat

Heat enhances the skin permeation of drugs by increasing body fluid circulation, blood vessel wall permeability, rate-limiting membrane permeability, and drug solubility, thus facilitating drug transfer to the systemic circulation [8]

1.2.4. Natural approaches of penetration enhancer

An important limitation of using chemical enhancers is their efficiency and safety as they exhibit poor permeation across the SC and their activity is limited to the top few layers of the SC. In deeper layers of the SC their concentration decreases as well their activities. Higher concentration of chemical enhancers in formulation increases drug transport across skin but is proportionally related with their ability to cause skin irritation. Therefore, it is challenging to maintain an optimal balance between the safety and potency of chemical enhancers in compound permeation.

Natural approaches of penetration like massage, hydration and massage with different oils(olive oil, coconut oil, mustard oil) may be preferred over the traditionally used synthetics materials as safe and suitable permeation enhancers to promote the percutaneous absorption of a number of drugs from topical formulation into the lower skin layers. These Natural approaches of penetration effectively facilitate the permeation of both hydrophilic and lipophilic drugs with low cytotoxicity. Therefore, in these approaches compounds may be considered as a safety enhancer for drug permeation through the skin.

In our study we have used two natural approaches of skin penetration, one is hydration another is oil massage to evaluate the skin penetration of niacinamide.

1.2.4.1. Effect of hydration in skin penetration

Hydration is long known to improve the permeability of skin as it can cause microstructural changes in the stratum corneum (SC) and enable a mechanistic interpretation of biomacromolecule penetration.Hydration of skin swells corneocytes, creates intercorneocyte ruptures, and causes microstructural changes in lipid self-assembly. The implications to biomacromolecule penetration are significant, since these disruptions allow penetration through the barrier of the SC. The disruptions are reversible, as removing the hydration source easily restores the barrier. A sufficient hydration time is required before gross changes occur in the SC ultrastructure, which can be correlated to an enhancement in protein penetration. An improvement in penetration is also expected when water-based formulations are applied on the skin for an extended period of time since the skin would have been hydrated sufficiently for new penetration pathways to emerge[16]

1.2.4.2 Effect of oil massage in skin penetration

Different oil massaging may be preferred over the traditionally used synthetics materials as safe and suitable permeation enhancers to promote the percutaneous absorption of hydrophilic and lipophilic compound from topical formulation into the lower skin layers.The effect of oil massaging help to enhance the skin penetration of different compound from topical formulation into the lower skin layers using different mechanisms of action based on : (1) disruption of the highly ordered intercellular lipid structure between corneocytes in SC, what makes this layer permeable to compounds, (2) interaction with intercellular domain of protein, which induces their conformational modification and makes SC more permeable, (3) partitioning promotion –many solvents change properties of the SC and thus increase the partitioning of a compound and (4) enhancer acting on desmosomal connections between corneocytes or altering metabolic activity within the skin.

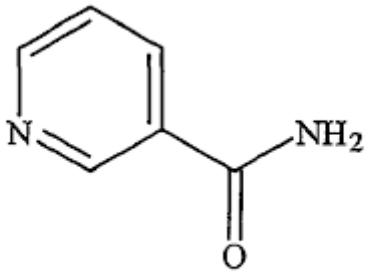
Oils massaging has been shown to impact directly on the barrier function of the skin to facilitate skin penetration, possibly by affecting the skin's lipid structure as it can increase the stratum corneum (SC) filling rate or increase follicular penetration. General enhancer altering the polar pathway causes protein conformational change or solvent swelling, whereas fatty acid enhancers increase the fluidity of the lipid portion of the SC. Some enhancers act on both polar and non-polar pathway by altering the multilaminate pathway for penetration. Different natural oils and their constituents have been widely investigated as safe and suitable skin penetration enhancers for both hydrophilic and hydrophobic drugs but mechanism of their action is not fully understood. After application to the skin oils and their components are rapidly metabolized not accumulated in the organism and fast excreted that is way it is strongly suggest that they can be successfully use as safe penetration enhancers[17].

1. 3. Niacinamide

Niacinamide is a water- soluble amide of nicotinic acid. Niacinamide is one of two principal forms of the B-complex vitamin; B3.Nicotinamide and nicotinic acid are also called niacinamide and niacin, respectively. Niacinamide is a precursor to the co-factors NAD and NADP whichisinvolved in many biochemical reactions in the skin. It helps to inhibit of sebum production, specifically reducing the content of diglycerides, triglycerides and fatty acids as result reduced acne, reduced pore size and improved texture. Stimulation of epidermal skin barrier lipids (ceramides) and proteins (keratin, involucrin,filaggrin) as a result improved skin barrier and moisture, reduced skin redness and rosacea appearance benefits.Niacinamide increases the production of collagen and inhibits the production of excess dermal glycosaminoglycans as a result it acts as anti-wrinkle and anti-aging agent[1].

1.3.1. Physicochemical propertiesof niacinamide

Chemical Name in niacinamide is 3-Pyridinecarboxamide and molecular formula is C6H6N2O. Physical state of niacinamide is solid which crystalline powder with odorless white color is. Niacinamide has melting point 127-131°C and boiling point 224°C (2000 Pa) where solubility is 691-1000 g/L. The structure of niacinamide consists of a pyridine ring with an amide group in position three.



Niacinamide

Figure 3: The structure of niacinamide

Niacinamide is a component of nicotinamide adenine dinucleotide (NAD), also known as coenzyme I, and nicotinamide adenine dinucleotide phosphate (NADP), also known as coenzyme II. These coenzymes are involved in many intracellular oxidation-reduction reactions. They participate in hydrogen transfer reactions, functioning as hydride ion carriers of biological systems[18].

1.3.2. Pharmacokinetics

The pharmacokinetics of niacinamide depends on dose, species, gender, and route of administration. Niacinamide is readily absorbed from all parts of the gastrointestinal tract. A negligible portion of niacinamide is metabolized to niacin, mostly due to bacterial activity. Peak serum concentrations are reached in humans within one hour of oral ingestion of standard preparations. Niacinamide is rapidly cleared from the circulation and distributed in all tissues. It has a high hepatic excretion ratio and plasma clearance can be reduced in patients with hepatic insufficiency[19].

1.3.3. Physiological role of niacinamide

Niacinamide acts as an antioxidant by preventing NAD depletion during DNA repair by inhibiting poly (ADP-ribose) polymerase (PARP) which also modulates major histocompatibility complex (MHC) class II expression. Niacinamide inhibits free radical formation and facilitates beta-cell regeneration in vivo and in vitro. Additional protection from macrophage toxins may be involved in prevention of type 1 diabetes.

Specifically, niacinamide has been shown, via PARP inhibition to protect pancreatic islet-cell lysis after exposure to oxygen free radicals10 and nitric oxide. Niacinamide has also been found to stimulate GABA receptors, without binding to the receptor sites, thus creating a benzodiazepine-like effect. Anti-inflammatory action affecting neutrophil chemotaxis has been reported for niacinamide. Additionally due to its inhibition of ADP-ribosylation, niacinamide has been shown to suppress cytokinemediated induction of nitric oxide synthase in a number of cells, thus effecting interleukin-1-exposed chondrocytes resulting in decreased inflammation[20].

1.3.4. Mechanism of niacinamide in skin benefit

Since niacinamide is a precursor to the co-factors NAD(H) and NADP(H) which are involved in many biochemical reactions in the skin as it has the potential to impact a wide variety of metabolic pathways and thus impact skin functions that rely on those pathways. NADPH is a cofactor in the synthesis of fatty acids and more complex lipids such as ceramides and NADH has been observed to inhibit some of the enzymes involved in the synthesis of glycosaminoglycans (GAGs) in vitro. Thus niacinamide's precursor role appears to be important in connection with the observed increase in stratum corneum ceramides in vitro and subsequent improvement in skin barrier function and with the observed reduction in excess dermal GAGs in vitro and the improved appearance of wrinkles.

Topical formulations of niacinamide have a wide range of skin care benefit by reducing the appearance of hyperpigmented spots, redness, yellowing (sallowness), surface sebum, pore size, surface texture, and fine lines and wrinkles. Additionally, there are improvements in moisturization, stratum corneum barrier integrity and elasticity.Some recent studies showed that the ability of niacinamide to impact UV-induced changes in skin by reducing the production of PGE2 from keratinocytes when stressed with non-lethal fluencies of UVB. It is considered that niacinamide has an ability to protect overall integrity of the cellular structure from UV-induced changes. It has been hypothesized that mechanistically, niacinamide protects cellular energy metabolism in vitro. The concept of niacinamide impacting cellular metabolism is based on its rapid incorporation into both the NADH and NADPH cellular pools.[20]

1.3.5 Indication of niacinamide on skin

There are many indications of niacinamide for skin benefit that are given as bellow-

i) Niacinamide can help to normalize the nicotinamide coenzymes which is depleted with age of skin

NADH and NADPH are considered as the fundamental energy units within cells driving the metabolism of cells involved in both catabolic and anabolic processes. There is an increasing pool of evidence for a decline in systemic and intracellular concentrations of these two coenzymes with age in human and animal models. It appears that a localized

supply of niacinamide therefore can be utilized by aged cutaneous cells to restore intracellular nicotinamide coenzyme homeostasis. Nicotinic acid (niacin) produces a well-documented cutaneous vasodilatation when applied topically. [21]

ii) Niacinamide can stimulate new collagen synthesis as aged fibroblasts secrete less collagen than young cells

Some studies with collagen protein secreted from human dermal fibroblasts showed that that dermal fibroblasts from an aged donor secreted significantly ($p<0.05$) less collagen than those from a young donor and furthermore that NADPH / NADP redox ratios were also lower ($p<0.05$) in fibroblasts from the aged donor. It is also showed that supplementation of the aged cell culture with niacinamide produced significant increases in total collagen secreted (by 54%), total protein secreted (by 41%) and also in the number of cells (by 20%), relative to a vehicle control. These data suggest that treatment with niacinamide would have a positive impact on the dermal compartment, both in terms of its connective tissue and gel matrix components. These effects would be of particular significance in aged and photo-damaged skin [22]

iii) Niacinamide up-regulates epidermal ceramide synthesis with concurrent epidermal barrier benefits

Ceramides are now known to play a central role in the structural and functional integrity of the stratum corneum barrier function. A decrease in ceramide fraction has been reported in aged and atopic skin. Some studies resulted that niacinamide could induce up to a 5-fold up-regulation in ceramide synthesis ($p<0.05$) in a dose-dependent fashion. [23]

iv) Niacinamide helps prevent UV-induced deleterious molecular and immunological events

Some studies demonstrated the ability of niacinamide to protect cultured normal human keratinocytes against reactive oxygen species induced by UVC irradiation or exposure to hydrogen peroxide. Niacinamide has the ability to significantly reduce both induction of photocarcinogenesis and photoimmunesuppression[24]

v) Niacinamide inhibits transfer of melanosomes from melanocytes to keratinocytes

Studied with niacinamide observed that there is no inhibitory effect on melanocyte tyrosinase activity. So treatment of human skin in-vivo with topical niacinamide would lead to a reduction in pigmentation with time[25]

vi) Niacinamide reduces human skin hyperpigmentation

Some studies demonstrating the effect of niacinamide on skin hyperpigmentation *invivo* to do this 18 female Japanese subjects with hyperpigmented facial spots were treated for 8 weeks with a vehicle containing 5% niacinamide vs. a vehicle control in a split-face design. Pigmented spots were qualified and quantified via algorithmic analysis of high resolution digital images and subjective grading of images. Results of image analysis showed that 5% niacinamide had induced a significant ($p<0.05$) reduction in spot area atthe 4 and 8 week time-points (vs. vehicle control), accompanied by a significant reduction($p<0.05$) in graded visible spot pigmentation at 8 weeks (vs. vehicle control)[26]

vii) Regulation of sebaceous lipid and acne by niacinamide

Topical niacinamide in the form of a commercial 4% gel has been shown to provide potent anti-inflammatory activity in the treatment of acne vulgaris. One study found that after 8 weeks of usage, 82% of subjects with inflammatory acne showed an improvement in global evaluation, with a significant reduction in papules or pustules and acne severity[27]

1.4. Literature Review

1.4.1. In the Journal of Cosmetic Dermatology, Gehring, W.(2004) published his study about nicotinic acid/niacinamide and the skin.

Nicotinic acid and niacinamide are similarly effective as a vitamin because they can be converted into each other within the organism. The blanket term vitamin B3 is used for both. Topical application of niacinamide has a stabilizing effect on epidermal barrier function, seen as areduction in transepidermal water loss and an improvement in the moisture content ofthe horny layer. Niacinamide leads to an increase in protein synthesis (e.g. keratin), has a stimulating effect on ceramide synthesis, speeds up the differentiation of keratinocytes, and raises intracellular NADP levels. In ageing skin, topical application of niacinamide improves the surface structure, smoothes out wrinkles and inhibits photocarcinogenesis. It is possible to demonstrate anti-inflammatory effects in acne, rosacea and nitrogenmustard-induced irritation. Because of its verifiable beneficial effects, niacinamide would be a suitable component in cosmetic products for use in disorders of epidermal barrier function, for ageing skin, for improving pigmentary disorders and for use on skin prone to acne[28]

1.4.2. In the Journal of Cosmetic Dermatology,Kawada, Akira, *et al.* (21 October 2008) published his study about evaluation of anti-wrinkle effects of a novel cosmetic containing niacinamide

Niacinamide is known to have effectiveness on sallowness, wrinkling, red blotchiness and hyperpigmented spots in aging skin. In thestudy, it was evaluated the anti-wrinkle effects of a new cosmetic containing niacinamide. A randomized, placebo-controlled, split face

study was performed in 30 healthy Japanese females who had wrinkles in the eye areas. The tested cosmetic containing 4% niacinamide was applied on wrinkles of one side for 8 weeks, and a control cosmetic without niacinamide on another site

Anti-wrinkle effects were evaluated with two methods: (i) doctors' observation and photographs based on the guideline of the Japan Cosmetic Industry Association; and (ii) average roughness of skin surface (Ra value) using skin replica. This cosmetic showed marked and moderate improvement in 64% of the subjects with a significant difference as compared with the control site ($P < 0.001$). Wrinkle grades in the tested area significantly reduced more than pre-application ($P < 0.001$) and the control ($P < 0.001$). Reduction in Ra value on the tested area was more than pre-application ($P < 0.01$) and the control site ($P < 0.05$) with significant differences Only one subject stopped the study with minimal irritation. These results indicated that the tested lotion was well tolerated and may be an optional preparation for the treatment of wrinkles in the eye areas[29]

1.4.3. International Journal of Cosmetic Science, Otte, N., C. Borelli, and H.C. Korting (2005) studied about the nicotinamide–biologic actions of an emerging cosmetic ingredient.

Nicotinamide, the water-soluble amide of nicotinic acid, is a component of the two most important coenzymes –nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate. Thus nicotinamide is involved in numerous oxidation–reduction reactions in mammalian biological systems. Nicotinamide essentially acts as an antioxidant. Most effects are exerted via poly-adenosine diphosphate-ribose polymerase inhibition. Thus nicotinamide increasingly gains interest in the prevention and treatment of several skin diseases. It is well established in the systemic therapy of pellagra, a deficiency disease linked to nicotinic acid, but with respect to topical use there is still a need for further evidence with respect to its manifold potential uses. Currently, its local use is established in the care of acne-prone skin[30]

1.4.4. In a study conducted by Namrata and Barai (2002) to observe the effect of hydration on skin permeability

The water handling properties of the stratum corneum (SC) are keys to both the barrier function and the condition of the skin. Water in the SC can be differentiated into tightly bound water, bound water and free water. These different kinds of water are associated with the different characteristics of the skin including elasticity, permeability etc. In this study we have tried to better quantify the water content of the stratum corneum as a function of its water activity. This was done by establishing a model for water sorption in the SC based on existing data and additional data generated in our laboratory. The water content measurements by different techniques seemed to agree well. The BET isotherm for water sorption into natural polymers was used as a model to describe the water content of the SC. It has long been known that hydration of stratum corneum increases its permeability

However this effect has not been fully quantified and the mechanism is not completely understood. It was studied the transport properties of a model hydrophilic compound, niacinamide, under controlled hydration conditions. Hydration was altered using salt solutions of different water activity. By performing two complimentary studies of permeation and uptake/desorption in combination with water sorption measurements it was possible to calculate the transport parameters D (diffusivity), K (partition coefficent) along with the SC thickness H for fully hydrated and partially hydrated SC. These transport parameters were constant at low water activity and increased sharply in the regions of higher water activity (above 75% RH)[31]

1.4.5. In the journal of Skin Research and Technology Zhai, H., *et al.,(*2002) published that hydration vs. skin permeability to nicotinates in man

Prolonged skin occlusion increases stratum corneum water content and often increases skin permeability and irritant dermatitis. As skin wetness from wearing diapers is considered an important factor favoring the onset of diaper dermatitis, optimal diapering might decrease skin hyperhydration and dermatitis. The aim of this study is to define the quantitative relationship between nicotinate ester (a model penetrant) skin permeability and hydration, as measured by water evaporation rate (WER), decay curves (at individual time points) and WER-area under the curve (WER-AUC); and also to determine the level of skin hydration and skin permeability to nicotinates following a diapering simulation[32]

1.4.6. In the Journal of Young Pharmacists, Bharkatiya, M. and R. Nema (2009) published skin penetration enhancement techniques

permeation through skin layers at a controlled rate. In addition to the currently marketed formulations, new drugs are being formulated using the transdermal system because of the inherent advantage of administration by this route. It offers a noninvasive route of drug administration, although its applications are limited by low skin permeability. Innovative
research exploiting penetration-enhancing strategies, such as iontophoresis, electroporation, microneedles, and sonophoresis, holds promise for the successful use of these drugs as consumer-friendly, transdermal dosage forms in clinical practice. This review outlines promising new technologies involved in enhancing transdermal permeation[33].

1.4.7. In the journal of Dermatology, Kaymak, et al.,(2008) published their investigation about the efficacy of topical niacinamide for the treatment of mild and moderate acne vulgaris

Niacinamide is a newly-approved anti-acne drug with a potent anti-inflammatory effect. In this study, safety and efficacy of topical 4% niacinamide gel in mild and moderate acne vulgaris was investigated. Forty-one patients aged 18-25 (mean: 21.6±2.52) with

mild and moderate acne vulgarisseen in dermatology outpatient clinic were enrolled in the study. All patients were treated with niacinamide4% topical gel (Vivatinell Acnecinamide Gel) for eight weeks.
Transdermal drug delivery systems allow delivery of a drug into the systemic circulation via

The result showed that38 patients among 41 were able to complete the treatment. Decrease in the number of pustules comedones and papules were statistically significant at the end of the treatment comparedto onset of treatment ($p<0.05$). Few side effects such as pruritus (n=1) and mild burning (n=3)were observed in very small percentage of patients. Findings of this study indicate that 4% niacinamide containing gel is effective and safein alleviating symptoms of mild to moderate acne, with earlier improvement in pustules[34]

1.5. The aim of the study

The aim of the study is to investigate the effect of external factors which can improve the penetration of niacinamide in the skin when applied topically. This study was conducted by applying a renowned face cream brand containing niacinamide which is commonly available and widely used. It is important to understand how niacinamide penetration can be enhanced by implying very simple everyday-practice (such as hydrating the skin or massaging the skin with oil, etc.) to get better benefit for skin care.

Chapter -2

Materials and Method

2 Materials and method

2.1 Materials used

Table 2.1: List of equipments used for the experiment

Serial No.	Equipment	Source (supplier name)	Origin
1	UV Spectrophotometer	Shimadzu UV1800	Japan
2	Electronic Balance	Shimadzu ATX 224	Japan
3	Sonicator	HWASHIN Power sonic 520	Korea

Table 2.2: List of reagents and drug used in the experiment

Reagents Name	Source (Supplier Name)
Methanol (HPLC grade)	Active Fine Chemicals
Distilled Water/De-ionized Water	RciLabscan Limited
Niacinamide	Gift sample from Eskayef Bangladesh Ltd.

2.2 List of Other Materials used

1. Offset paper (laminating afterwards)
2. Small Scotch tape with a dispenser (Name of the Scoth Tape)
3. A tweezer
4. A small scissor
5. Aluminium foil
6. Centrifuge tubes/test tube
7. Centrifuge tube holder/test tube holder
8. Big spatula
9. Gauze
10. Large Scotch tape/masking tape
11. Stop watch
12. Facial tissue
13. A marker pen/permanent marker
14. Gloves(Manufactured in Malaysia by Meditech Gloves Sdn Bhd)

2.3 Methods

2.3.1Construction of calibration curve

Construct of calibration curve involved the following steps-

a) Preparation of stock solution and different dilute solutions

b) Determination of maximum wavelength of absorbance (λ_{max}) for niacinamide
c) Construction of standard curve

a) Preparation of stock solution and different dilute solutions

Stock solution of niacinamide was prepared in distilled water as niacinamide is a highly water soluble compound. Using Electronic Balance, 5mg of niacinamide reference standard was accurately weighed and dissolved in distilled/deionise water in a 50 ml volumetric flask to make a stock solution. The concentration of stock solution was 100 µg/ml. The stock solution was diluted further to make six standard solutions of 5, 10, 15, 20, 25 and 30 µg/ml.

b) Determination of λ_{max} of niacinamide

A standard niacinamide solution of 4.96µg/mL was used to perform UV scanning to detect the maximum wavelength of absorbance (λ_{max}) from 200 to 400 nm. Scanning results are given as bellow-

Table 2.3: Absorbance values of 4.96 µg/mL niacinamide solution at different wavelengths

λ (nm)	Absorbance
214	0.716
244	0.16
262	0.24
324	0.00

As most solvents and excipients show absorbance values at around200nm, λ_{max} of niacinamide was selected at 262 nm. Highest absorbance of niacinamide was found at 214nm. However, such wavelength may not be free from interference of solvents and other formulation ingredients. This is why finally 262 nm was chosen as the λ_{max} of niacinamide.

c) Construction of standard curve

By using UV spectrophotometer, the absorbance values of all standard solutions were determined at 262 nm. Data were collected and recorded. A calibration curve was constructed based on the average absorbance value from the four repeats and the respective concentrations. Within the specific range, the absorbance values followed Beer-Lambert's law perfectly.

2.3.2 UV method validation

a) Linearity

Linearity is the ability to provide results that are directly proportional to the concentration of the analyte in the test sample. It typically refers to overall system response. The

linearity of a system is measured by the degree to which the plotted curve conforms to a straight line is a measure of system linearity. The linearity of constructed calibration curve was assessed by separately evaluating the regression coefficient (R^2), slope and intercept of four separately prepared calibration curve. R^2 value close of 1 indicates a perfect linear relationship between concentration and absorbance value[35]

b) Range

A reportable range will be established for each analyte tested. The upper limit of the reportable range will be set at the concentration of the highest standard tested which exhibited acceptable results for linearity, accuracy and precision. The lower limit of the reportable range will be set at the lowest standard tested which exhibits acceptable results, however, this concentration may not exceed the analyzer's lower limit[35].

c) Recovery

Recovery of the UV method was conducted by calculating the mean absorbance values from four sets of absorbance values. The mean absorbance values were then used to calculate the concentration of niacinamide using the calibration curve (recovered concentration).The % recovery of the concentration was calculated using the following equation:

$$\% \text{ Recovery} = \frac{\text{Recovered concentration} \times 100}{\text{Original concentration}} \quad \text{..... (Equation 1)}$$

d) Accuracy testing

According to USP accuracy testing is "The accuracy of an analytical procedure is the closeness of test results obtained by that procedure to the true value. The accuracy of an analytical procedure should be established across its range''[36]

To perform the accuracy test a known concentration of freshly prepared niacinamide solution in distilled water was prepared using reference standard. The absorbance values of niacinamide were taken using UV Spectrophotometer. From the absorbance values, concentration of niacinamide in the solution was calculated using the equation of calibration curve. Finally the percent recovery of niacinamide was calculated using Equation 1. The process was repeated by preparing and getting absorbance values of 3 concentrations in 3 times [no. of replicates (n) was (n=9)]

e) Intra-day and inter-day precision

In this step of method validation, three set of solutions of concentrations of 5, 15 and 30 µg/mL were prepared from 100µg/ml stock solution [n= 9]. The absorbance values of these solutions were examined using UV Spectrophotometer at two different times in a day (for intraday precision) and at 2 and 4day intervals (inter day precision). The

difference in absorbance values obtained within a day or between days were calculated and evaluated by relative standard deviation (RSD)[35].

f) Sensitivity

Sensitivity of the method is tested in terms of limit of detection (LOD) and limit of quantitation (LOQ) values.

i) Limit of detection (LOD)

According to USP the definition of LOQ is "The detection limit is a characteristic of limit tests. It is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. Thus, limit tests merely substantiate that the amount of analyte is above or below a certain level. The detection limit is usually expressed as the concentration of analyte (e.g., percentage, parts per billion) in the sample''[36]

LOD is the lowest detectable concentration of a sample. Here several lower concentrations of niacinamide (0.5 to 3 μg/mL) were analyzed using UV spectrophotometer the LOD against the blank. The lowest concentration of niacinamide (LOD) was determined based on the lowest detectable absorbance values.

ii) Limit of Quantitation (LOQ)

USP defines the LOQ as "The quantitation limit is a characteristic of quantitative assays for low levels of compounds in sample matrices, such as impurities in bulk drug substances and degradation products in finished pharmaceuticals. It is the lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. The quantitation limit is expressed as the concentration of analyte (e.g., percentage, parts per billion) in the sample"(Pharmacopoeia 2006)

The minimum working concentration of a sample is referred as limit of quantification or LOQ. Here several lower concentrations of niacinamide (0.5 to 4μg/mL) were analyzed using UV spectrophotometer against the blank. The lowest quantifiable concentration of niacinamide (LOQ) was determined based on the lowest absorbance value that follows the Beer- Lambert's law and with the acceptable recovery and precision limit.

2.2.3. Protocol development for tape stripping from human volunteer

2.3.3.1. Developing the informed consent form

Informed consent is an ethical and legal requirement for research involving human participants. It is the process where a participant is informed about all aspects of the trial, which are important for the participant to make a decision and after studying all aspects of the trial the participant voluntarily confirms his or her willingness to participate in a

particular clinical trial and significance of the research for advancement of medical knowledge and social welfare.

Informed consent is an inevitable requirement prior to every research involving human being as subjects for study. Obtaining consent involves informing the subject about his or her rights, the purpose of the study, procedures to be undertaken, potential risks and benefits of participation, expected duration of study, extent of confidentiality of personal identification and demographic data, so that the participation of subjects in the study is entirely voluntary.

We have developed the participant information leaflet where we descried the purpose of the study, the potential benefit and disadvantages of taking part in this investigation, confidential policy for research, exclusion criteria, prediction of study result etc. The volunteer who are interested in our study we have provided consent form where we collect the information about the volunteer's death of birth, gender, overall health condition, and skin condition. The consent form is given in the Appendix.

2.3.3.2 Developing protocol for tape striping

A) Determination the amount of solvent requires

The solvent of choice was methanol and water mixture solution in 20:80 ratio in order to dissolve maximum niacinamide from the tape strips. Due to lower sensitivity of UV Spectrophotometer compared with High Performance Liquid Chromatography (HPLC), the aim was to maximize the NIA concentration in a sample so that it can be easily quantifiable in the UV Spectrophotometer. In addition, the UV cuvette requires to be filled with certain volume of solvent in order to be able to quantify accurately.

Therefore, the least amount of solvent is requited in an UV cuvette was taken into consideration. A cuvette requires a minimum of 3.5ml of solvent for UV reading. Moreover, a test has been conducted where which showed that after tape trips when the extracting solvents were filtered through the filter paper; the paper absorbed approximately 1.1ml to 1.3ml solvent during filtration. Finally, it has been decided to use 5ml of extracting solvent to extract the content in the tape which will ultimately produce a minimum of 3.5 ml sufficient for analyzing in the UV Spectrophotometer.

B) Determination of number of tape requires

Before conducting actual test process, it is necessary to detect how many tapes are required in a single site for depth profiling. Initially, 15 tapes were taken from one site. Five tape strips were takenin 3 different test tubes. The UV absorbance values from each tube were within the optimum range of absorbance. Secondly the same procedure was repeated taking 15 tapes where3 tape strips were placed in 5 test tubes and extracted with 5 ml solvent. Similar to the previous study the absorbance was also within the optimum range. As up to 15 tape strips UV absorbance was quantified, it was decided later to take further 7 tape strips. Finally a total of 21 tape strips were decided to be taken from a site.

Seven test tubes were taken where 3 sequential tape strips were placed and extracted. This procedure was adopted to detect the deeper penetration of niacinamide inside the skin.

C) Developing a complete protocol of tape stripping to quantify niacinamide inside the skin

The final tape striping process involved the following steps-

1. Preparation of two sheets of papers with areas of 4x4 cm^2
2. A scotch tape with a dispenser, a scissor and a tweezer were arranged
3. The volunteer was given the consent form to read carefully, put their required information and sign the form if they are agreed to volunteer.
4. A pair of gloves was worn before starting the experiment to avoid tape contamination.
5. The volar forearm of the volunteer was marked placing the paper sheet on it. Two sites were allocated on the volar forearm. In order to avoid repetition of applying formulation on the same site, the two places of left hand were marked as L-1 or L-2 and the same on right hand were R-1 and R-2.
6. If the specific area on the arm has excess hair, it has to be cut with scissor carefully without damaging the skin
7. For water hydration test, a tissue paper was wet with tap water and placed on the specific site of skin to be hydrated for 2 minutes. Similarly for oil massage test, a drop of olive oil was placed on the skin and massaged with the finger tip for 2 minutes. If there any water or oil remained on the skin, it was removed by simple and light wiping with a facial tissue.
8. A big spatula it was weighed
9. A large excess of formulation was taken on the spatula and weigh it
10. A specific amount of formulation (~ 0.2 g or 200 mg) was applied on the skin and was evenly spread on the marked area of the skin with the spatula
11. Final weight of the spatula was recorded and from the difference, the exact amount applied was calculated
12. Then the skin was covered with a gauze followed by large scotch tape and left for 30 min
13. After 30 min, the formulation was gently removed from the skin with the help of a soft facial tissue by wiping, not by rubbing
14. With a marker pen, a small area was marked again inside the marked skin.

15. The weight of a approximately fixed size of scotch tape was measured using a precision balance with the help of a tweezer
16. The scotch tape was placed on the skin inside the small area (facing the glued side towards the skin)
17. A coin of 5 taka (weigh of approximately 8g) was placed on the scotch tape, lightly press the coin with finger pressure and left for 5 seconds and then lift the weight
18. The tape was removed from the skin in one go with the help of a tweezer
19. The tape was weighed again with a precision balance. The difference showedthe amount of SC component was removed with one tape. The same procedure (step 15 to 18) was repeated for the rest of 20 tapes
20. Three consecutive tapes were placed in a previously marked centrifuge tube in such a way that glued side did not face or stick to the tube wall
21. Then 5 ml of methanol: water (20:80) mixture was added to it and the tubes were capped properly
22. All the centrifuge tubes were placed in a tube holder and placed in a sonicator for 5 minutes at room temperature
23. The samples were then filtered using separate filter papers(Double Rings made in China) and the filtrate (the clear solution) was immediately analyzed using UV Spectrophotometer at 262 nm.

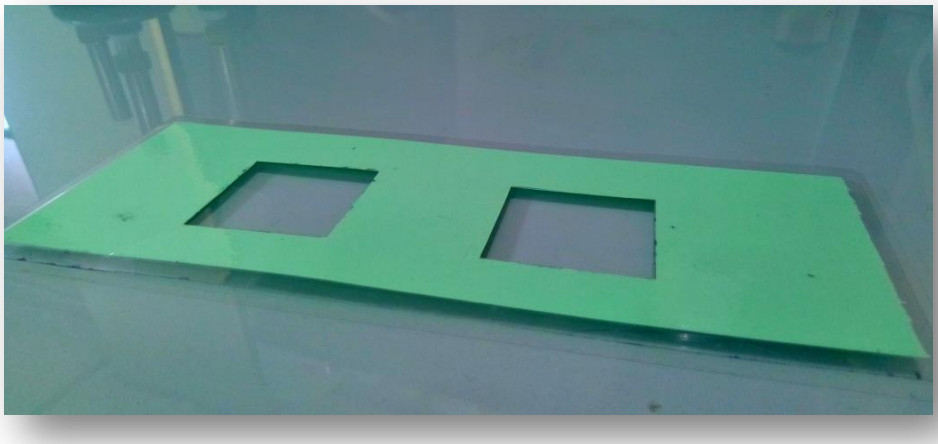


Figure2.1: This paper used to mark the specific site of hand where areas of 4x4 cm

Figure 2.2: Marked the specific site of hand where sample was applied

Figure 2.3: Initial weigh of the tape was taken before use on skin

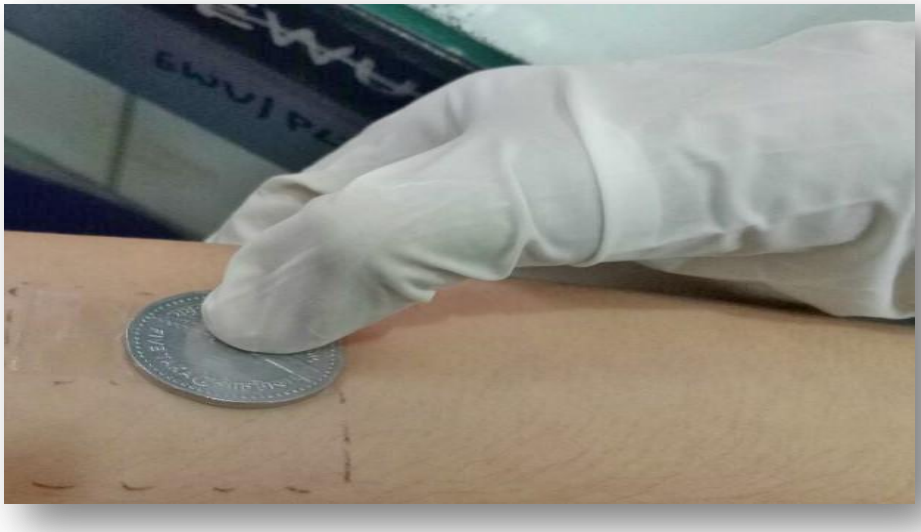


Figure 2.4: Applied gentle pressure by using coin that is weighted 8g on the tape

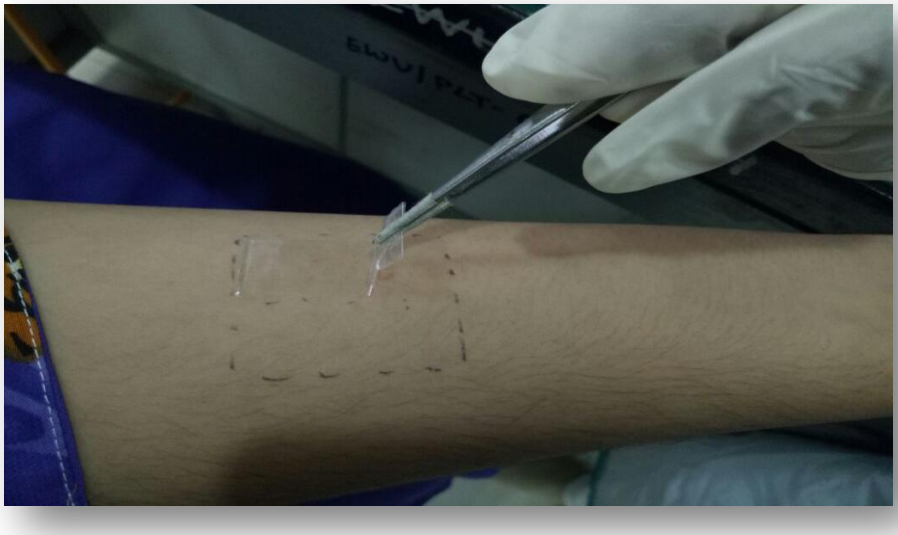


Figure 2.5: Applied tape was picked to weigh the final weight

Figure 2.6: Final weight of tape was taken after using on skin

Figure 2.7: Tape strips were sonicated to extract the niacinamide from the tape sonicator

Figure2.8: Filtration of solution after sonicating though five minutes

Figure 2.9: Samples were finally analyzed using the UV Spectrophotometer

D) Quantification of blank tape strips

Before analyzing the sample it was necessary to quantify any materials (SC components) that comes out with a tape strip and also gives UV absorbance besides niacinamide. Keratin or other skin lipid may also give absorbance values at 262nm which is the maximum wavelength of absorbance of niacinamide.

Therefore, a blank test was conducted following the steps mentioned in the protocol (2.2.3.2 C) without using any formulation on the skin. It is to be mentioned that before conducting the blank experiment, it was not allowed to use any cream or lotion on the skin for at least 3 days before the experiment. After analyzing the blank tape strips, it was found that the samples showed some absorbance values at 262 nm. The blank absorbance values were then deducted from the formulation containing tape strips values in order to get actual amount of niacinamide.

2.4. Statistical analysis

All the calculations were performed using MS Excel 2007.All the values were represented as mean±SD (standard deviation) or mean±SEM (standard error of mean). Some values were evaluated using the term relative standard deviation (RSD). RSD and SEM were calculated using Equation 2 and 3, respectively (n= no. of volunteers)-

$$RSD=\frac{SD\times 100}{Mean} \quad \text{.....(2)}$$

$$SEM=\frac{SD}{\sqrt{n}} \quad \text{..... (3)}$$

The comparison between the sites was conducted using unpaired t-test, where control was compared with hydration and control with oil massage. To observe any difference of external factors for niacinamide penetration on the basis of gender, it was analyzed by

using unpaired t-test where if the P values less than 0.05(at 5% significance level) indicated statistically significant difference between groups.

Chapter -3

Results and Discussion

3.1. Quantitative estimation of niacinamide using UV spectroscopic method

i) Determination of maximum wavelength of absorbance (λ_{max}) for niacinamide

The first step was to determine the λ_{max} of niacinamide. To determine λ_{max} of niacinamide, a standard NIA solution of 4.96µg/mL was used to perform UV scanning to detect the λ_{max} from 200 to 400 nm. As most solvents and excipients show absorbance values at around200nm, λ_{max} of niacinamide was selected at 262 nm. Highest absorbance of niacinamide was found at214nm. However, such wavelength may not be free from interference of solvents and other formulation ingredients. This is why finally 262 nm was chosen as the λ_{max} of niacinamide.

ii) Construction of standard curve

Calibration curve was constructed with 5, 10, 15, 20, 25 and 30µg/ml standard solutions of niacinamide. By using UV spectrophotometer, the absorbance values of all standard solutions were determined at 262nm.Data were collected and recorded.

A calibration curve was constructed based on the average absorbance value from the four repeats and the respective concentrations. Within the specific range, the absorbance values followed Beer-Lambert's law perfectly (Figure 3.1).

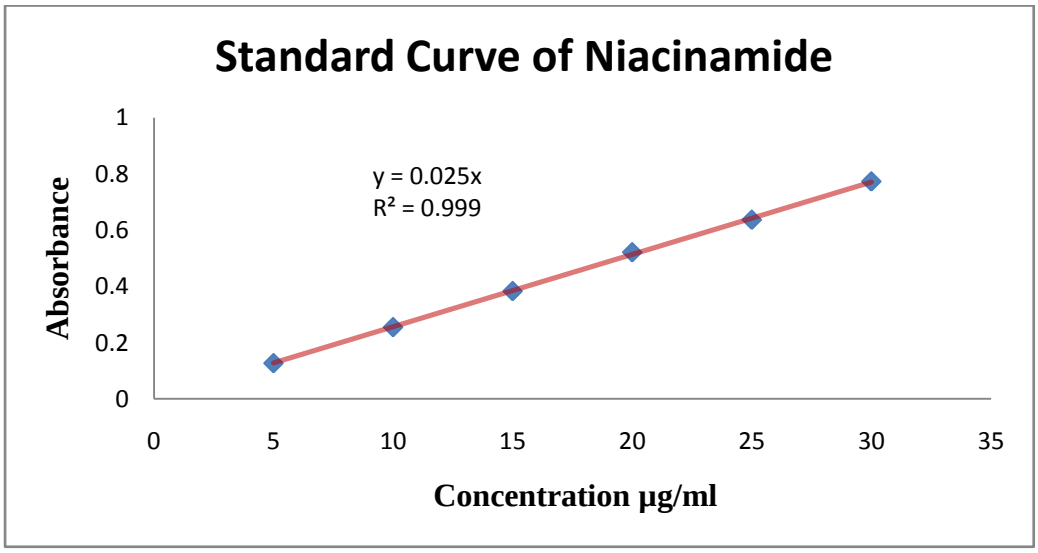


Figure 3.1: Standard curve of niacinamide at 262 nm

iii) Linearity and Range

From the standard curve it is found that the calibration curve was linear (R^2>0.999) and follows Beer-Lambert's law from 5 µg/mL to 30 µg/mL concentration. The Table 3.2 shows the slope and intercept of the four sets of standard curves.

The linearity of constructed calibration curve was assessed by separately evaluating the regression coefficient (R^2), slope and intercept of four separately prepared calibration curve. R^2 value close of 1 indicates a perfect linear relationship between concentration and absorbance value.

In our study, we found a R^2 value of 0.9997 which indicates the good linearity of the calibration curve (Table 3.2). The RSD value of R^2 and slope were 0.07 and 2.06%, respectively which indicate the linearity of the UV method. The intercept value was 0.0014±0.009. The range for NIA in this analytical procedure is between 5µg/ml to 30 µg/ml.

Table 3.1: Linearity of UV spectrophotometric method of niacinamide

	Standard 1	Standard 2	Standard 3	Standard 4	Average	SD	RSD
R^2	0.9998	0.9998	0.9984	0.9992	0.9993	0.001	0.07
Slope (m)	0.03	0.03	0.03	0.03	0.03	0.001	2.06
Intercept	0.00	-0.01	-0.01	0.01	-0.0014	0.009	

iv) Recovery testing

Recovery of niacinamide in the UV method was conducted according to the method stated in Chapter 2, section2.2.2.The results are given in Table 3.3. The table shows that a recovery of 100.06±0.83% applied niacinamide was recovered using the UV method. The RSD value was found to be 0.83% which is less than 2%.

Table3.2: Recovery studies of Niacinamide

Conc. (µg/mL)	Standard 1	Standard 2	Standard 3	Standard 4	Observed concentration (ug/mL)	%Recovery	Mean (% Recovery)	SD (% Recovery)	RSD
5	0.125	0.125	0.125	0.132	4.99	99.81	100.06	0.83	0.888883
10	0.255	0.249	0.253	0.262	9.97	99.71			
15	0.384	0.381	0.385	0.382	14.96	99.74			
20	0.521	0.521	0.521	0.521	20.33	101.65			
25	0.64	0.638	0.62	0.648	24.82	99.30			
30	0.764	0.777	0.793	0.748	30.04	100.13			

v) Accuracy testing

Accuracy testing was conducted according to the method stated in Chapter 2, section2.2.2.The accuracy results are shown in Table 3.4. From the table, it can be seen that 95.85±7.82% of niacinamide was recovered in this method which fell within 80-120% range. It also showed a higher RSD value (8.15%) which is higher than 5%

Table3.3: Accuracy testing of Niacinamide

Absorbance	Observed conc. (µg/mL)	Original conc. (µg/mL)	% Recovery	Mean	SD	RSD
0.24	9.64	10	96.40	95.85	7.82	8.15
0.29	11.64	10	116.40			
0.233	9.36	10	93.60			
0.462	18.52	20	92.60			
0.464	18.60	20	93.00			
0.46	18.44	20	92.20			
0.665	26.64	29	91.86			
0.673	26.96	29	92.97			
0.678	27.16	29	93.66			

vi) Intra-day and inter-day precision

In this step of method validation, three set of solutions of concentrations of 5, 15 and 30 µg/mL were prepared from 100µg/ml stock solution [n= 9]. The absorbance values of these solutions were examined using UV Spectrophotometer at two different times in a day (for intraday precision) and at 2 and 4-day intervals (inter-day precision). The method was stated in detail in Chapter 2 Section2.2.2.[35]

Table 3.5 (a), (b) and (c) show intra and Inter-days precision values of UV spectrophotometric method. The RSD of intra-day precision was slightly higher than 2% (2.54%). However, at 2 days intervals there were no significant variability found in the absorbance values (RSD= 1.42%) Whereas, at 4 days interval, theRSD value was found to be highest (4.67%). This indicates that niacinamide samples should be analyzed within 2 days of preparation.

Table3.4 (a): Intra-day precision

Concentration (µg/mL)	Absorbance (obtained in the morning)	Absorbance (obtained in the evening)	Conc. (obtained in the morning)	Conc. (obtained in the evening)	Average conc. in the morning)	Average conc. in the evening)	Mean conc.(between morning & evening)	SD of conc.(between morning & evening)	RSD	Average RSD
	0.869	0.857	33.87	33.40						
	0.863	0.861	33.64	33.56						
30	0.867	0.867	33.79	33.79	33.77	33.59	33.68	0.13	0.38	
	0.434	0.417	16.95	16.28						
	0.438	0.434	17.10	16.95						
15	0.448	0.442	17.49	17.26	17.18	16.83	17.00	0.25	1.46	
	0.106	0.109	4.18	4.30						
	0.116	0.106	4.57	4.18						
5	0.117	0.097	4.61	3.83	4.46	4.11	4.28	0.25	5.79	2.54

Table3.4 (b): Inter-day precision (2 days interval)

Concentration (µg/mL)	Absorbance (Day 0)	Absorbance (Day 2)	Conc. (Day 0)	Conc. (Day 2)	Average conc in Day 0	Average conc in Day 2	Mean conc (between Day 0 & Day 2)	SD of conc (between Day 0 & Day 2)	RSD	Average RSD
30	0.808	0.797	31.50	31.07	31.65	31.30	31.48	0.25	0.79	1.42
	0.811	0.809	31.61	31.54						
	0.817	0.803	31.85	31.30						
15	0.391	0.398	15.27	15.54	15.79	15.29	15.54	0.36	2.30	
	0.408	0.379	15.93	14.81						
	0.414	0.397	16.17	15.51						
5	0.135	0.133	5.31	5.23	5.51	5.42	5.46	0.06	1.18	
	0.143	0.144	5.62	5.66						
	0.142	0.136	5.58	5.35						

Table3.4 (c): Inter-day precision (4 days intervals)

Concentration (µg/mL)	Absorbance (Day 0)	Absorbance (Day 4)	Conc. (Day 0)	Conc. (Day 4)	Average conc in (Day 0)	Average conc in (Day 4)	Mean conc (between Day 0 & Day 4)	SD of conc (between Day 0 & Day 4)	RSD	Average RSD
30	0.808	0.786	31.50	30.64	31.65	30.63	31.14	0.72	2.33	4.67
	0.811	0.775	31.61	30.21						
	0.817	0.796	31.85	31.03						
15	0.391	0.392	15.27	15.31	15.79	14.86	15.32	0.66	4.31	
	0.408	0.366	15.93	14.30						
	0.414	0.383	16.17	14.96						
5	0.135	0.123	5.31	4.84	5.51	4.96	5.23	0.39	7.36	
	0.143	0.13	5.62	5.12						
	0.142	0.125	5.58	4.92						

vii) Sensitivity

The sensitivity of the method was tested in terms of limit of detection (LOD) and limit of quantitation (LOQ) values. Niacinamide solutions of different minimum concentrations were prepared and analyzed in UV spectrophotometer at 262nm. The values are given in Table 3.6.

Table 3.5: Sensitivity of UV spectrophotometric method

Conc.(µg/Ml)	Absorbance	LOQ/LOD
0.5	0.15	**LOD**
1	0.042	
1.5	0.062	
2	0.065	
2.5	0.087	
3	0.079	
4	0.113	**LOQ**

The table shows 0.5µg/ml is the lowest concentration that was detectable in the UV spectrophotometer. This is why it is the LOD in this method. Whereas 4µg/ml is the lowest concentration that follows Beer-Lambert's lay, recovery values (99.81%), intra-day precision (5.79%) and inter-day precision values (5.12 and 2.73%, respectively at 2 and 4 days interval). This is why the concentration was considered as the LOQ.

viii) Summary of validation UV spectroscopic method for analyzing niacinamide

The method validation parameters of niacinamide in UV spectrophotometer, such as range, linearity, accuracy testing, recovery testing, precision and sensitivity, were presented in Table 3.

Table3.6: Summary of validation parameter of NIA in UV spectroscopic method

Parameter	Unit		
	µg/mL	**mean±SD**	**RSD (%)**
Range	5 to 30		
Linearity (n=3)			
R^2		0.9993±0.001	0.07
Slope		0.03±0.001	2.06
Intercept		(-)0.0014±0.009	
% Recovery		100.06±0.83	0.83
Accuracy (n=9)		95.85±7.82	
Precision (n=9)			
Intraday			1.13
Interday (2 days variation)			1.42
Interday (4 days variation)			4.67
Sensitivity			
LOD	0.5		
LOQ	4		

3.2. Protocol development for tape stripping

Protocol development included the number of tape strips should be taken, how many tape strips should be extracted at a time, how much extracted solvent will fill up the UV quartz cell, blank tape analysis, etc. After taking written consent from the participants who wished to take part in the research work, one of them was selected for the protocol development study. The procedure for developing protocol was given in Chapter 2, section2.2.3.

A total of 15 tapes were taken from the formulation applied skin from the volunteer and three of the formulation containing tapes were extracted together and analyzed in the UV method. The results are given in Figure 3.2 and 3.3.It can be seen from Figure 3.2 that while tape stripping deeper higher cumulative amount of SC was removed. However, Figure 3.3, it shows that with the increased number of tape strips, niacinamide content decreased.

As higher amount of NIA removed from a total of 5 tape strips, the number was reduced to 3 tape strips while tape stripping the volunteers. At the same time as NIA was still quantified up to 15 tape strips, the number to total tape strips was increased to 21in the volunteers in order to track niacinamide deeper into the skin.

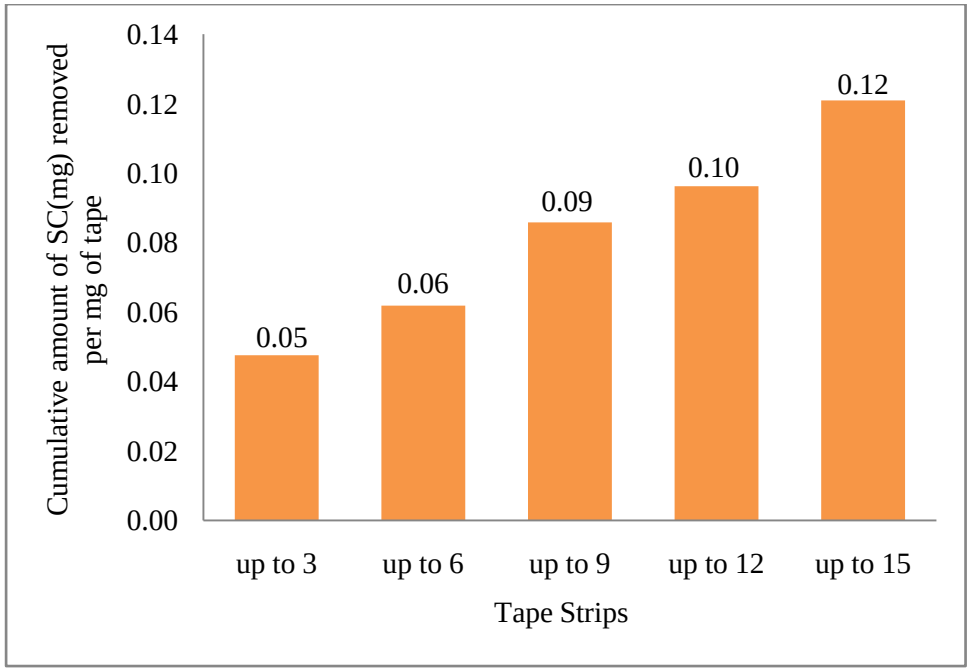


Figure 3.2: Cumulative amount of SC (mg) removed per mg of tape in protocol development procedure(n=1)

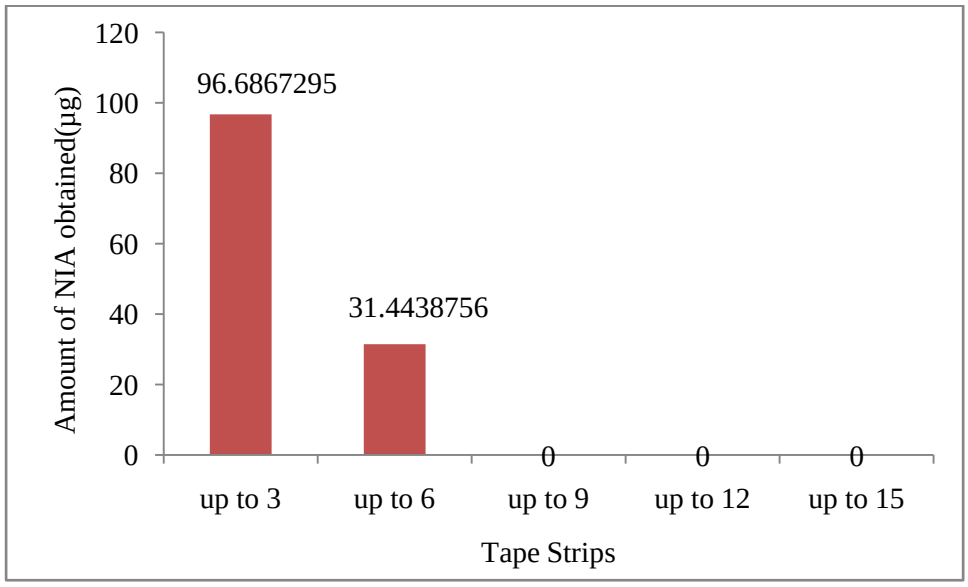


Figure 3.3: Amount of niacinamide obtained during the protocol development process

At the same time, blank measurement was performed by tape striping the volunteers without applying formulation (a total of 21 tapes) and 3 of the skin containing tapes were analyzed together(Chapter 2, section2.2.3.2 C). Surprisingly the blank tape strips showed some absorbance values, which may be due to different skin components which absorb UV light in UV Spectrophotometer at 262 nm. The amount of SC (mg) removed per mg of tape along with the absorbance values from the blank tape strips are given in Table 3.8.

Table 3.8 Cumulative amount of SC removed and mean absorbance values of per mg of tape in blank tape strips

No. of tapes	Cumulative amount of SC removed (mg) per mg of tape	Mean absorbance value	Mean absorbance value per mg of tape
1 to 3	0.03	0.21	0.01
4 to 6	0.05	0.26	0.01
7 to 9	0.06	0.23	0.01
10 to 12	0.09	0.27	0.01
13 to 15	0.10	0.25	0.01
16 to 18	0.12	0.27	0.01
19to 21	0.13	0.26	0.01

This table shows that an increased cumulative amount of SC removed per mg of tape with the increased tape numbers. An average of 0.01 absorbance value per mg of tape was also observed. The mean absorbance value per mg of tape was deducted from the niacinamide containing tape strips obtained from volunteers in order to avoid interference from the SC components.

3.3. Tape stripping from volunteer's volar forearm and quantitative analysis of niacinamide

The study was conducted on 11 participants consisting of 7 female and 4 male volunteers. To determine the skin penetration of niacinamide of an international brand to evaluate the effect of external factors like hydration and oil massage. The formulation was applied according to the protocol and absorbance values from the tapes stripped were utilized to calculate the amount of niacinamide. Four sites were selected in the lower and upper portion of left and right volar forearm and assigned as L-1, L-2, R-1, and R-2 respectively. About 200mg of formulation was applied in the site each time.

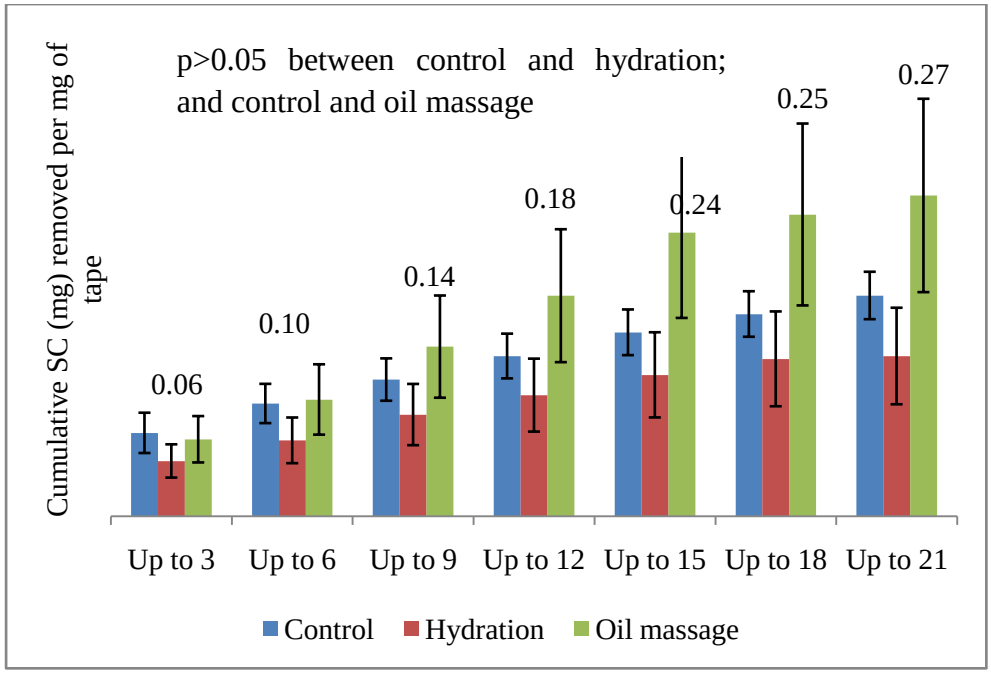


Figure3.4: Cumulative thickness of SC (mg) removed with tape strips from the sites of control, hydration and oil massage (mean±SEM, n=11).

From this figure it is observed that the cumulative thickness of SC removed from the site of oil massage was higher while hydration showed the lower value compared with the control. However, no statistical significant difference was observed (p>0.05).

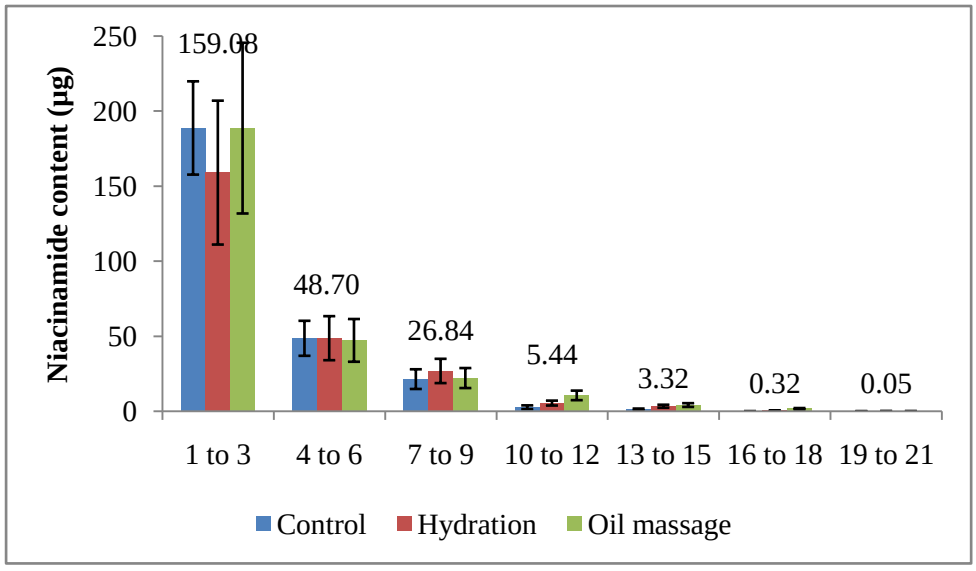


Figure3.5: Amount of niacinamide obtained in the tape strips from the sites of control, hydration and oil massage (mean±SEM, n=11).

From Figure 3.5, it can be seen that highest amount of niacinamide was visible from the three sites from 1-3 tape strips as niacinamide can be present in the outer most layer of the skin. Permeation of niacinamide was observed up to 21 tape strips for hydration and

oil massage. However, from control site no niacinamide was quantified after 15 tape strips.

Therefore, it can be said that hydration and oil massage helped niacinamide to penetrate further into the skin compared with the control. Up to 9 tape strips, similar quantities of niacinamide penetration was observed from the three sites. On the otherhand, after 9 tape strips, oil massaging proved to increase the penetration of niacinamide in higher quantities compared with hydration. However, there was not statistically significant difference ($p>0.05$).

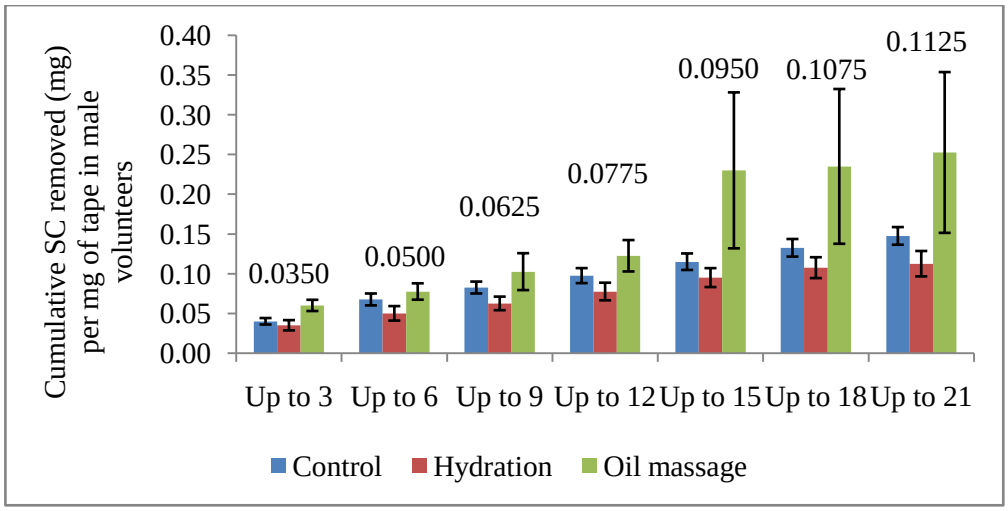


Figure 3.6: Cumulative SC removed (mg) per mg of tape in male volunteers (mean±SEM, n=4).

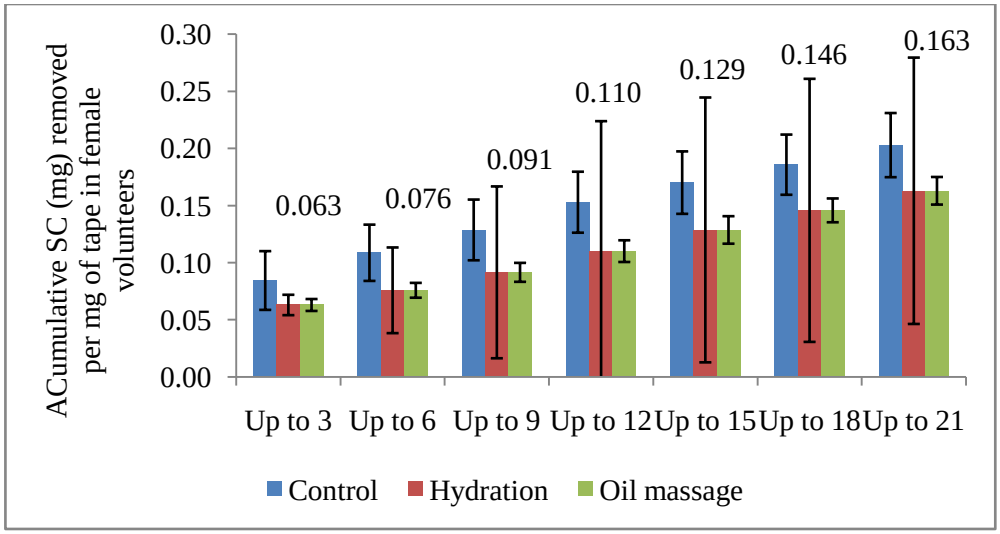


Figure 3.7: Cumulative SC removed (mg) per mg of tape in female volunteers (mean±SEM, n=7).

From figures 3.6 and3.7; it is seen that cumulative SC(mg) removed from per mg of tape, where male volunteers experienced more SC removed during oil massage while female

volunteer inform the control site. In case of female volunteers, cumulative SC removed from hydration and oil massage sites were less than control site at any depth.

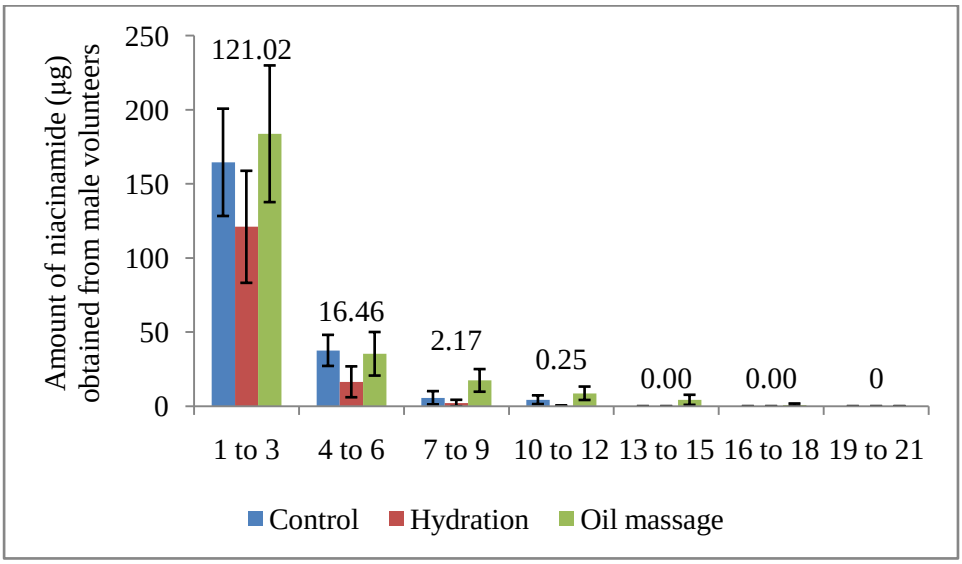


Figure 3.8: Amount of niacinamide (µg) obtained from the male volunteers (mean±SEM, n=4).

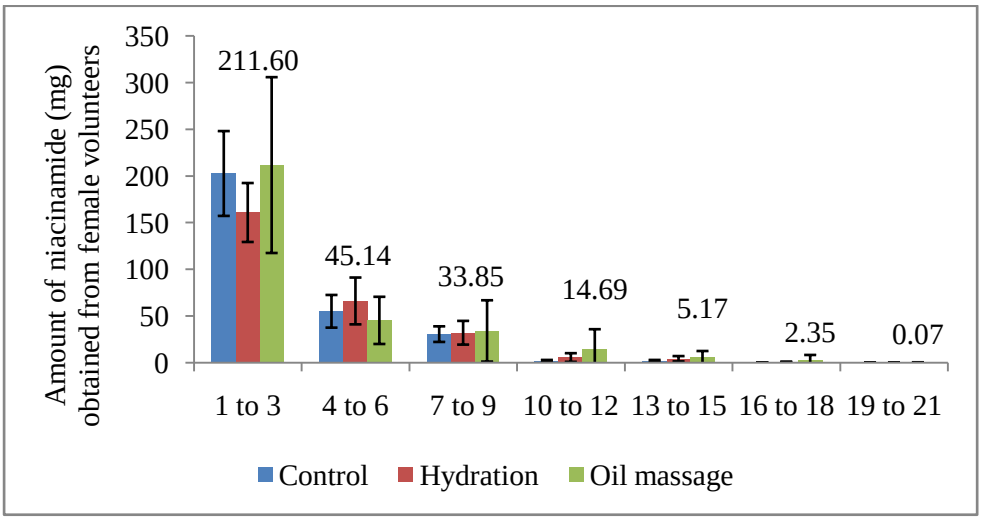


Figure 3.9: Amount of niacinamide (µg) obtained from the female volunteers (mean±SEM, n=7).

From Figure 3.8 and 3.9 it can be seen that in both male and female skin oil massage helped niacinamide to penetrate deeper into the skin in higher quantities than control. However, there was no statistically significant difference ($p>0.05$).In male volunteers, oil massage helped niacinamide to reach up to 21 tape strips whereas, from control and hydration niacinamide penetrated up to 12 tape strips. On the contrary, skin hydration aided niacinamide penetration deeper into the skin (up to 18 tape strips) in case of female volunteers. At 18 tape strips, in female volunteers, no niacinamide was quantified in case

of control. However, ~0.5 and 2.38µg of niacinamide were present in 15 to 18 tape strips from hydration and oil massage in respect in female volunteers.

3.4. Conclusions

Niacinamide has several beneficiary effects on skin and this is why it is used in many cosmetic formulations. The skin benefit is obtained by penetration of required amount of niacinamide into the skin. As hydration of skin helps to increase the pore size and oil acts as natural enhancer of skin penetration, this study was conducted to evaluated effect of these external factors. The factors may aid higher penetration of niacinamide in the skin.

The study method was developed by construction of calibration curve, UV method validation, and protocol development for tape striping. By using niacinamide reference sample, calibration curve was constructed where the curve was linear (R^2>0.999) and the range of niacinamide concentration was 5 µg/mL to 30 µg/mL. UV method was developed by determining λ_{max} of niacinamide which was 262nm.Recovery test, accuracy test and sensitivity test(LOD and LOQ) were also determined. The LOD and LOQ values were 0.5 µg/mL and 4 µg/mL respectively. The accuracy value was in between 95.85±7.82%.The protocol was developed by determining amount of solvent, number of tape and procedure of conducting test.

We have used 11 healthy volunteers. Sample was applied on different sites of left and right volar forearm. Tape striping helped to remove the SC of the skin along with niacinamide that penetrated to the specific depth. Finally, niacinamide content in tape strips was analyzed by measuring the absorbance at 262 nm using UV Spectroscopy. The data of hydration and oil massage were compared with the control data by using unpaired t-test. No significant difference was observed in the amount of niacinamide at any depth of skin from control to hydration or oil massage ($p>0.05$). In addition, the analysis was extended to observe if there any difference between male and female skin. However, like the general result, male and female skin was also failed to show any significant influence of skin hydration and oil massage in penetrating niacinamide ($p>0.05$). The skin was hydrated or massaged with oil for only 2 minutes. The time may not be sufficient to show any significant difference.

Chapter-4

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Patient Identification Number for this trial:

CONSENT FORM

Title of Project: Penetration of niacinamide (vitamin B3) in human skin from commercially available creams

Date of birth: Age: Gender:

Your overall health: Skin condition:

Please initial box

☐ 1. I confirm that I have read and understand the information sheet for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.

☐ 2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

☐ 3. I agree to take part in the above study.

_____	_____	_____
Name of Participant	Date	Signature
_____	_____	_____
Researcher	Date	Signature