CHAPTER II

REVIEW OF LITERATURE

C. asiatica (L.)or Urban (Umbelliferae) is commonly used in the Ayurvedic system of medicine to treat various diseases. ***C. asiatica*** is a small herb firstly used in India and tropical islands of the Indian Ocean where is a part of traditional medicine. It has recently become popular due to its pharmaceutical and cosmetic properties. Local populations, it has been known for more than 3,000 years that it was listed on the Indian Ayurvedic medicine. The ancient Vedas used the plant either in poultries applied directly to the skin or by oral route to help heal wounds and ugly skin lesions of various origins. After a long period of empiric use, the first scientific investigations were undertaken in Madagascar. It described in the (French Codex in 1884). The next in 1941, with the preparation of the first crystalline extract from the crude extract of ***C. asiatica*** by Bontems. Pohonsky and friends were the first who finding the structure of **asiaticoside**. (Bhagirath Singh and R. P. Rastogi, Photochemistry, 1969)

The Singhalese and the people of Asia have used this plant for hundreds of years, because they believe it contains remarkable longevity properties. The Singhalese say that ***C. asiatica*** may increase the span of life 50 years by rejuvenating brain and body cells, thus making it incapable of breaking down for a long period of time. It was mainly found in Madagascar and East Africa that ***C. asiatica*** is polymorphous, creeping plant, rooting at nodes, with sometimes-significant

taproot, cylindrical and glabrous stems. The plant has a long taproot and side shoots and the mature root form can resemble the human body, similarly to ginseng root. Some Herbalists regard it "the arthritis herb" as highly as ginseng and as having similar properties. It will grow in full sun, but will thrive in semi-shade and the leaves are much larger.

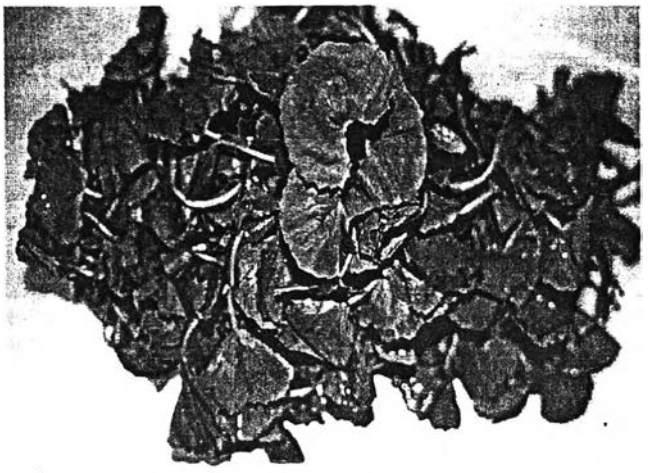


Figure 1; The appearance leaves of ***C. asiatica*** found in U.K.

C. asiatica is one of the remedies for leprosy. The **asiaticoside**, a glycoside, has given encouraging results for the treatment of leprosy. As a remedy in this disease it was first brought prominently to notice by Boileau, in 1859. Dr. A. Hunter, who tried it in the Madras Leper Hospital, came to the conclusion that it had no claim to consider ***C. asiatica*** as a specific in leprosy. He found it was most useful in ameliorating the symptoms and improving general health. Reports from Europe in 1885 confirm the use of ***C. asiatica*** for syphilitic skin diseases both internally and externally. Nowadays, ***C. asiatica*** is the active ingredient of many drugs and cosmetic preparations in Europe, United States and Japan in the field of skin care. In

traditional Chinese medicine, it was believed that ***C. asiatica*** provided longevity, and thus called it the "fountain of youth" herb. In the United States, ***C. asiatica*** is found in countless energy formulas and tonics. This herb has also been used to bring down fever and relieve congestion due to colds and upper respiratory infections. Recent studies show that ***C. asiatica*** has positive effects on the circulatory system; it seems to improve the flow of blood throughout the body by strengthening the veins and capillaries. It has been used successfully to treat phlebitis, as well as leg cramps, swelling of the legs, and heaviness or tingling in the legs. This is particularly useful for bedridden people. ***C. asiatica*** has even been used as 'food for the brain' after a nervous breakdown to rebuild energy reserves, or to prevent a nervous breakdown. It has an energizing effect on the cells of the brain, relieves high blood pressure, mental fatigue, senility, and helps the body defend itself against various toxins. It works as a blood purifier and in strengthening the heart, as well as with bowel problems, rheumatism, skin problems, and also promotes blood circulation in the lower limbs and reduces the pain and swelling due to phlebitis. Researchers have found that it contains several glycosides that exhibit wound healing and anti-inflammatory activities, and in large doses it can act as a sedative. Other researchers have shown that fresh leaves of the ***C. asiatica*** plant are effective in healing chronic skin ulcers and other wounds. ***C. asiatica*** contains a group of triterpenes called **asiaticoside** that possess strong antioxidant properties. In modern health care

C. asiatica is used primarily for venous insufficiency, localized inflammation and infection, and post-surgery recovery. ***C. asiatica*** is also used for the following:

- Skin: Open wounds, sores, ulcers, other infections and radiation ulcers.
- Confinement: Bedsores, phlebitis, and tingling, night cramps.
- Vein problems: Phlebitis, varicose veins, cellulite and edema.
- Gynecology: Lesions during pregnancy, delivery and obstetric manipulations, and episiotomies tears.

C. asiatica affects various stages of tissue development, including keratinization that *is* the process of replacing skin after sores or ulcers. Because it has **asiaticoside** that stimulates the formation of lipids and proteins which are necessary for healthy skin. ***C. asiatica*** has been found to have significant results in healing of skin, other connective tissues, lymph tissue, blood vessels *(*decreasing capillary fragility*)*, and mucous membranes. ***C. asiatica*** is believed to be a rejuvenate and restoring 'anti-aging' herb, and is naturally consumed as a preferred food by South Indian elephants which are animals with exceptional memories and longevity. In general, it is traditionally used as a cooling, soothing, relaxing, antispasmodic diuretic. Its historical applications include treatment for a sore or inflamed throat, and for skin, liver and urinary tract diseases. It is excellent for both internal and topical application. French scientists recently did some breakthrough research to show it stimulates synthesis of collagen, for powerful anti-aging effects for the skin. In addition to its intellect promoting and anxiolytic effects, the plant is also used in chronic cough, eczema, psoriasis, and boils. It is in preparations given for anemia, dyspnea, emaciation, splenic enlargement, and rheumatic joint pain. The leaves of

this swamp plant have been used around the world for centuries to treat leprosy, cancer, skin disorders, arthritis, hemorrhoids, and tuberculosis.

In normal as well as delayed-type wound healing, **asiaticoside** isolated from ***C. asiatica*** has been proven for its activity. *In vitro* and *in vivo* wound healing activity of guinea pig punch wounds topical applications of 57% solution of **asiaticoside** produced 56% increase in hydroxyproline, 57% increase in tensile strength, increased collagen content and better epithelisation. In streptozotocin diabetic rats, where healing is delayed, topical application of 0.4% solution of **asiaticoside** over punch wounds increased hydroxyproline content, tensile strength, collagen content and epithelisation thereby facilitating the healing. **Asiaticoside** was also improved the guinea pig punch wound model (A. shukla, A. M. Rasik, G. K. Jain, R. Shankar, D. K. Kulshrestha, B. N. Dhawan, 1999) by oral administration of 1 mg./kg dose. It promoted angiogenesis in the chick chorioallantoic membrane model. These results indicate that **asiaticoside** exhibits significant would healing activity in normal as well as delayed healing models and is the main active constituents of ***C. asiatica***.

C. asiatica

Latin:	***Centella asiatica*** Linn.
English names:	Tiger herbal, Indian Pennywort, Centella, Gotu kola
Thai name:	Bua Bok

Japanese name: Tsubokura

Sanskrit name: Mandukaparni, Brahmi, Mandukig, Divya

Hindi names: Brahma-manduki, Khulakhudi, Mandookaparni

Family: Umbelliferae

Botanical: *Hydrocotyle asiatica* (Linn.) Urban

Synonyms: Indian Pennywort, Marsh Penny, White Rot, Thick-leaved, Pennywort

Part Used: Whole plant, Leaves.

Habitat: Asia and Africa.

Description: A small umbelliferous plant growing in Southern Africa and India, indigenous to the Southern United States. The special characteristics of the leaflets are petiolate, reniform, crenate, seven nerved and nearly glabrous.

Constituents: An oily volatile liquid called vellarin (which has a strong smell reminiscent of the plant, and a bitter, pungent, persistent taste) and tannic acid.

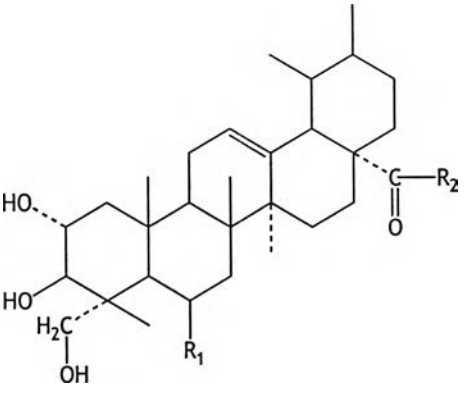
Pharmacological Action: Tonic, sedative, alterative, anxiolytic.

Medicinal Action and Uses: A valuable medicine for its diuretic properties; has long been used in India as an aperient's or alterative tonic, useful in fever and bowel complaints and a noted remedy for leprosy, rheumatism and ichthyosis; employed as a poultice for syphilitic ulcers. In small

doses it acts as a stimulant, in large doses as a narcotic, causing stupor and headache and with some people vertigo and coma.

The Structure of **Asiaticoside**

Chemical Name	**asiaticoside**
Molecular Formula	$C_{48}H_{78}O_{19}$
Molecular Weight	959.13
Structure	Stereo compound
Compound Type	Heterocyclic
Melting point	235-237°c



R_1 = H; R_2 = OH : Asiatic acid

R_1 = OH; R_2 = OH : Madecassic acid

R_1 = H; R_2 = glucose-glucose-rhamnose : **Asiaticoside**

Figure 2; The structure of **asiaticoside**

The component of ***C. asiatica*** has 4 groups.-

1. Flavonoid glycoside is kaempferol-3-glucoside and guercentin-3-glucoside.
2. Free amino acids found in the leaf and stem are glutamate, serine and alanine. Amino acids mostly found in the root are aspartate, glutamate, serine, threonine, alanine, lysine, histidine and amino butylate.
3. Polyacetylenic compounds.
4. Terpenoid compounds.

Table 1; Active ingredients of ***C. asiatica***

Name of the extract	Chemical composition		Current areas of application
ASIATIC ACID	2 α, 3 β, 23-trihydroxyursa-2-ene-28-oic acid	96%	*Cosmetology*: - Body (draining, slimming)
	Others (non identified)	4%	
ASIATICOSIDE	1[0-∝-L-rhamnopyranosyl-(1-4)-0-β-D-glucopyranosyl(1-6)]-0-β-D-glucopyranose asiatate	~95%	*Cosmetology*
	Other (non identified)	<5%	
HETEROSIDES of *C.asiatica*	Madecassoside	~56%	*Cosmetology:* -Face (tensioning serum) -Body (Slimming)
	Asiaticoside	~14%	
	Other (non identified)	~30%	

Table 1 (continue)

Name of the extract	Chemical composition		Current areas of application
Purified Extract of *C.asiatica* (P.E.C.A.)	**Asiaticoside**	~17%	*Cosmetology:*
	Madecassoside	~16%	-Face (anti-
	Asiatic Acid	~5%	wrinkle, anti-
	Madecassic Acid	~4%	ageing)
	NaCl	~20%	-Body
	Other (non identified)	~30%	(Slimming)
AMEL GENINS	Asiatic Acid	~25%	*Cosmetology :*
	Madecassic Acid	~60%	-Body
	Other (non identified)	~20%	(Slimming)

C. asiatica is a medicinal plant that has been in use since prehistoric times. Its active constituents include pentacyclic triterpene derivatives. Studies have been conducted in particular to investigate the **asiaticoside.** In common with most traditional phytotherapeutic agents, ***C. asiatica*** is used in folk medicine to treat a wide range of indications. In contrast to other medicinal plants, however, ***C. asiatica*** has been subjected to quite extensive experimental and clinical investigations. Studies done in accordance with standardized scientific criteria have shown it to have a positive effect in the treatment of insufficiency and striate gravidarum. ***C. asiatica*** also appears to be effective in the treatment of wound healing disturbances. However, the therapeutic potential of this plant in terms of its efficacy and versatility is such that further detailed research would appear worthwhile.

It is a common plant in South East Asia and Australia. ***C. asiatica*** has a long tradition in South East Asian and Indian (Ayurvedic) medicine as a healing and tonic herb. Poultices are used for contusions, boils, sprains and fractures. In India the plant is used for all sorts of skin problems- eczema, psoriasis, abscess, ulcerations- it is said to stimulate the growth of skin, hair and nails. ***C. asiatica*** is a home remedy for skin problems, rheumatism, piles, inflammations swellings, fever, dysentery, and children's bowel complaints, mental weakness and to improve memory. A powder of the leaves, dried in the shade is given in doses of 5-10 grains three times a day to adults; for children the dose is one to four grains. Juice and decoctions (strong tea using one to two ounces of the herb) are also used. There are many studies examine the anti-tumor effect of the crude extract of ***C. asiatica*** as well as its partially purified fractions from chromatographic procedures by both in vitro short and long term chemo sensitivity and in vivo tumor model test systems.

High Resolution LC-MS with Electrospray Ionisation

High Resolution LC-MS analyses insufficiently volatile and thermally unstable components of *C. asiatica* to permit volatilisation prior to ionisation. The initial complication in combining LC and MS were the liquid at atmospheric pressure. The interface process also had to ensure ionisation of the analyses present in the mobile phase. The sample is collected from the drain of HPLC sample which will determine the component of the ethanol extract and hexane extract and the result is as follow in figure 24-30 from F3 which find that the range of asiaticoside for *C. asiatica* fresh leave F1 and dried finely powdered of *C. asiatica*

extraction for CA1 were dissolved in methanol in the range of 0.5 – 5 mg./ ml. Extracts of *C. asiatica* was examined by High resolution LC-MS.

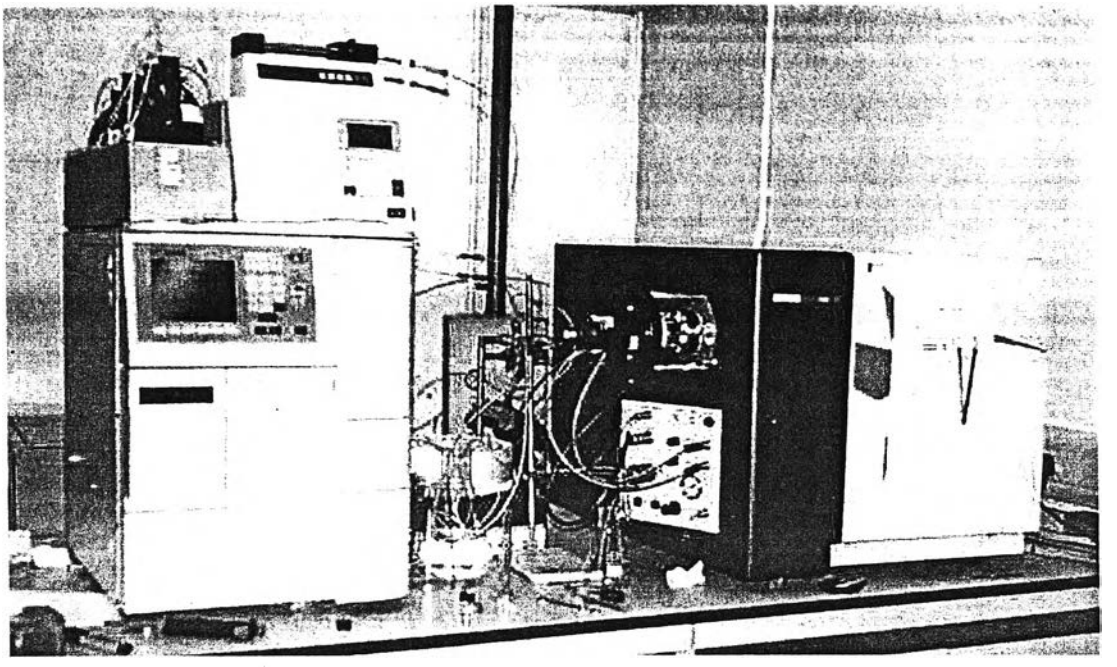


Figure3; High resolution LC-MS with electrospray ionisation.

Ion Optics

Ions generated in the Z-spray source are transferred to the orthogonal time of flight analyzer via the two independently pumped RF lenses. After entering the analyzer the ion beam is focussed into the pusher by the acceleration, focus, steer and tube lenses. The pusher then pulses a section of the beam towards the reflector, which then reflects ions back to the detector.

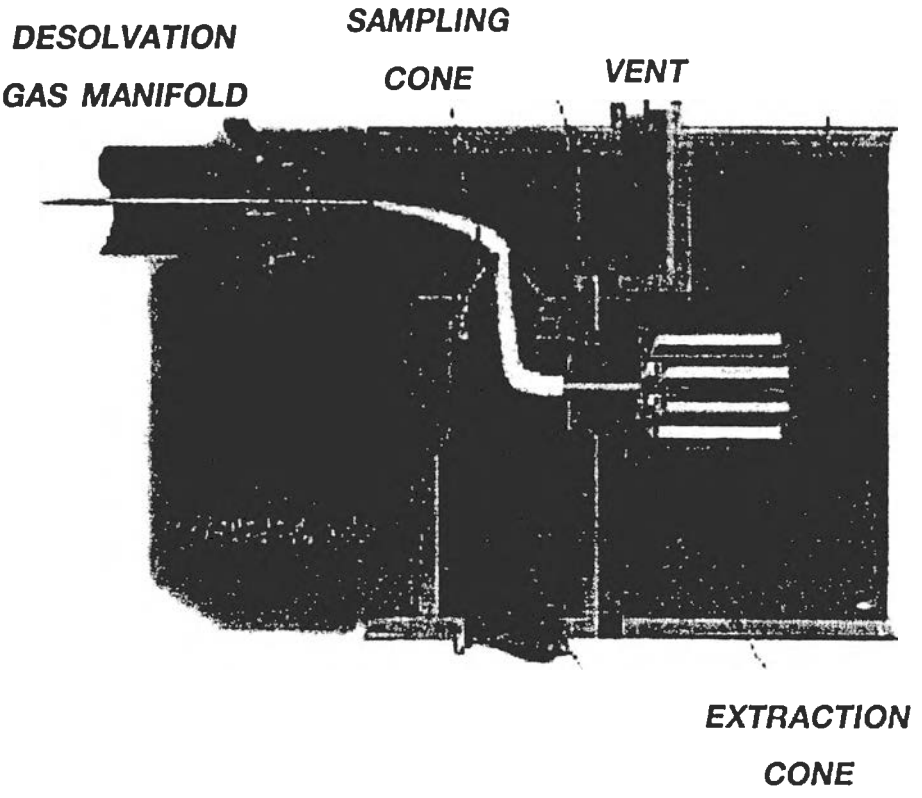


Figure 4; Schematic of Z-spray source design in electrospray ionisation.

As ions travel from the pusher to the detector they are separated in mass according to their flight times, with ions of the highest mass to charge ratio ($^{m}/z$) arriving later in the spectrum. The best outstanding of high resolution LC-MS and electrospray ionization is the z type direction in the inner chamber. The rated at repetition frequencies of up to 20 kHz, resulting in a full spectrum being recorded by the detector every 50 microseconds. Each spectrum is summed in the histogram memory of the time to digital converter until the histogrammed spectra are transferred to the host PC.

If the user has requested an acquisition rate of 1 spectrum/second, each spectrum viewed on the host PC will be the result o summing up to 20,000 individual spectra recorded at the detector.

Unlike scanning instruments, the TOF performs parallel detection of all masses within the spectrum at very high sensitivity and acquisition rates. This characteristic is of particular advantage when the instrument is coupled to fast chromatography, since each spectrum is representative of the sample composition at that point in time, irrespective of how rapidly the sample composition is changing.

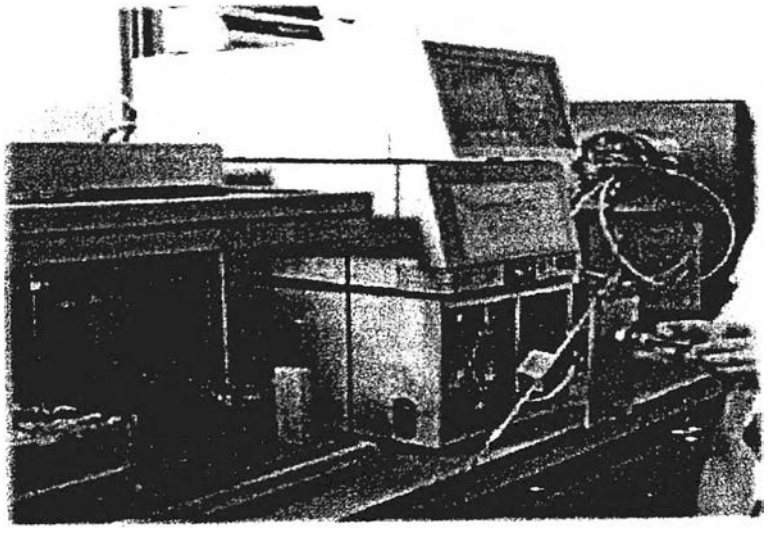


Figure 5; Electrospray ionization instrument.

The isolation and characterisation of **asiaticoside** obtained from ***C. asiatica*** using HPLC & High Resolution LC-MS and Electrospray Ionisation give both rapid analysis and sensitive detection. **Asiaticoside** is the active ingredient, and by purification it may give more powerful biochemical effects. (Hollas. J. M. 1998)

High resolution spectroscopy has been developed in 1979. (Mauri, J. et al.2000) Infrared spectroscopy, which use in high resolution spectroscopy, although started in 1966, and now for were commercial instruments. But the first laser for spectroscopy and the ruby laser were produced in 1960, this proved to be of limited use in spectroscopy. It was not until the development of more flexible, particularly tunable, lasers. Much high resolution spectroscopy has been done with resolution limited by the line width imposed by the effect. In 1969, the first spectra were obtained: these were in the microwave region. More recently, laser techniques such as the use of a skimmed supersonic jet and, in two-photon spectroscopy, counter propagating laser beams, have been devised for obtaining resolution in other regions of the electromagnetic spectrum. To take advantage of the capability for the laser bandwidth must be small compared with the line width. This is readily achieved with, for example, a ring dye or diode laser.

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laser beams, have been devised for obtaining resolution in other regions of the electromagnetic spectrum.

Calibration for Accurate Mass

The elevated resolution and inherent stability of the calibration law of orthogonal TOF instruments allow accurate mass measurements to be performed.

$$\sqrt{m} = Q + Pt \qquad \text{——————} \quad (1)$$

Where the charge state is assumed to be 1.

The term P is a "gain" which comes from the instrument geometry (path lengths and voltages).

Q is an offset, and comes from propagation delays through the electronics (detector rise time and delays of trigger signals through cables).

If a data file is acquired from the instrument with no calibration applied then it is assumed that the offset is zero and the gain P is calculated from the instrument geometry.

Nominal Mass Accuracy

It is important that the path lengths are set up to give at least nominal mass accuracy. Nominal mass measurement is achieved on the LCT by adjustment TOF.

The procedure to set up a nominal mass calibration is described in calibration. With no calibration applied, then the data displayed on the spectrum based upon instrument geometry. Because of the inherent relationship between mass and time shown above it is prudent to generate higher order calibration coefficients that are applied to the square root of the nominal masses: where the terms A, B, C, D… are calculated by fitting a polynomial to acquired mass spectral data.

$$\sqrt{M_c} = A + B\sqrt{M_n} + CM_n + DM_n^{3/2} + ... \quad \text{(2)}$$

Mc is the calibrated displayed mass.

If a polynomial of order 1 is requested the values for A & B are calculated and the higher terms are set to zero. For a polynomial of order 5 (the highest supported in Mass Lynx) there will be six terms generated.

When calibrating over a large mass range (>500Da) it is advisable to use a higher order polynomial as the deviations from the straight-line fit become more appreciable.

Once a calibration has been generated from a reference compound such as PEG it should be used as an "instrument calibration" to be applied to all subsequently acquired data.

Microemulsion Gel (Nimmannit, U., 2001)

Microemulsions are transparent, isotropic and thermodynamic stable, dispersions of water and oil, usually stabilized by a surfactant. Capric/caprylic triglyceride, Soy bean oil were used as an oil component. The surfactants used in this study were Brij 97® , Tween 80® , and Myrj52® . Microemulsions are multicomponent fluids composed of water, oil and amphiphiles. The characteristic properties of microemulsion include spontaneous formation; optically clear appearance, large interfacial area, low interfacial tension, large solubilization capability and low viscosity. The particle size of droplets was between 10-100 nm. Microemulsion forms under a wide range of surfactant concentrations, water in oil ratios, temperature etc. Phase boundaries in phase diagram of polyoxyethylene (10) oleyl ether : water : capric / caprylic triglyceride were investigated and composition were shown in figure 6. Phase boundaries of polyoxyethylene sorbitan monooleate : water : capric / caprylic triglyceride were also shown in figure 7

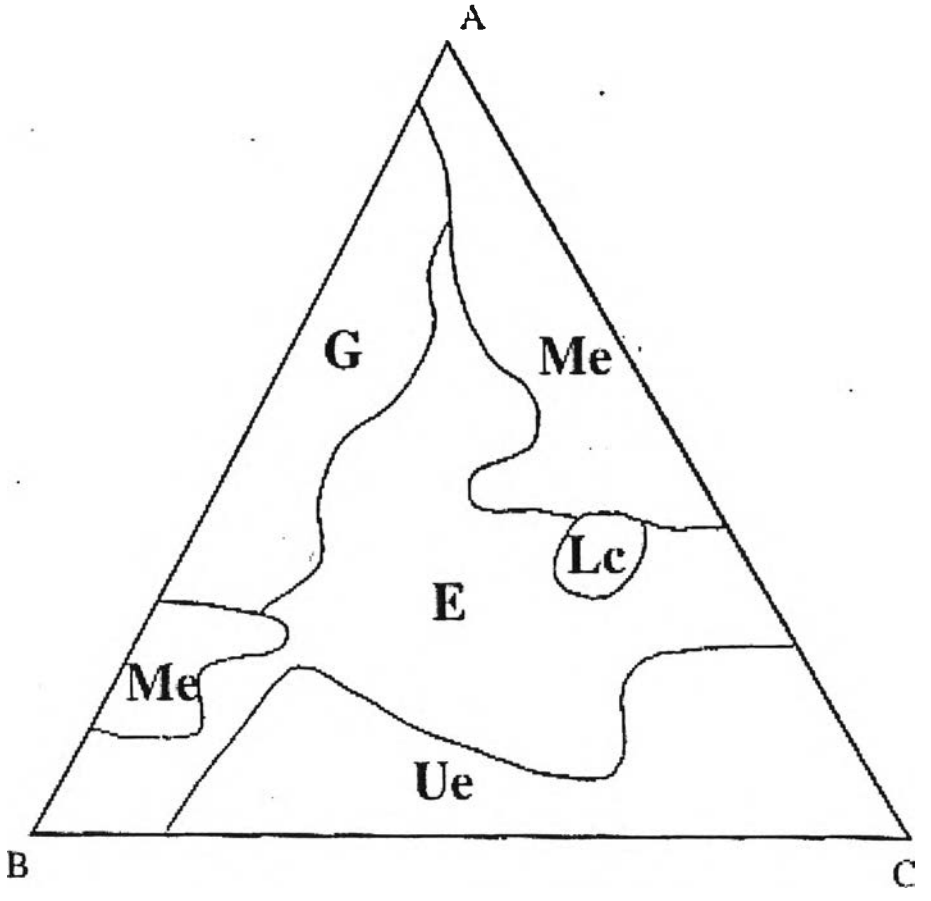

Figure 6; Phase diagram of polyoxyethylene (10) oleyl ether(A):water (B) -capric/caprylic triglyceride (C) Ue: unstable emulsion (2 phase) E : emulsion (1 phase), Lc: liquid crystal, G : microemulsion gel, Me: microemulsion

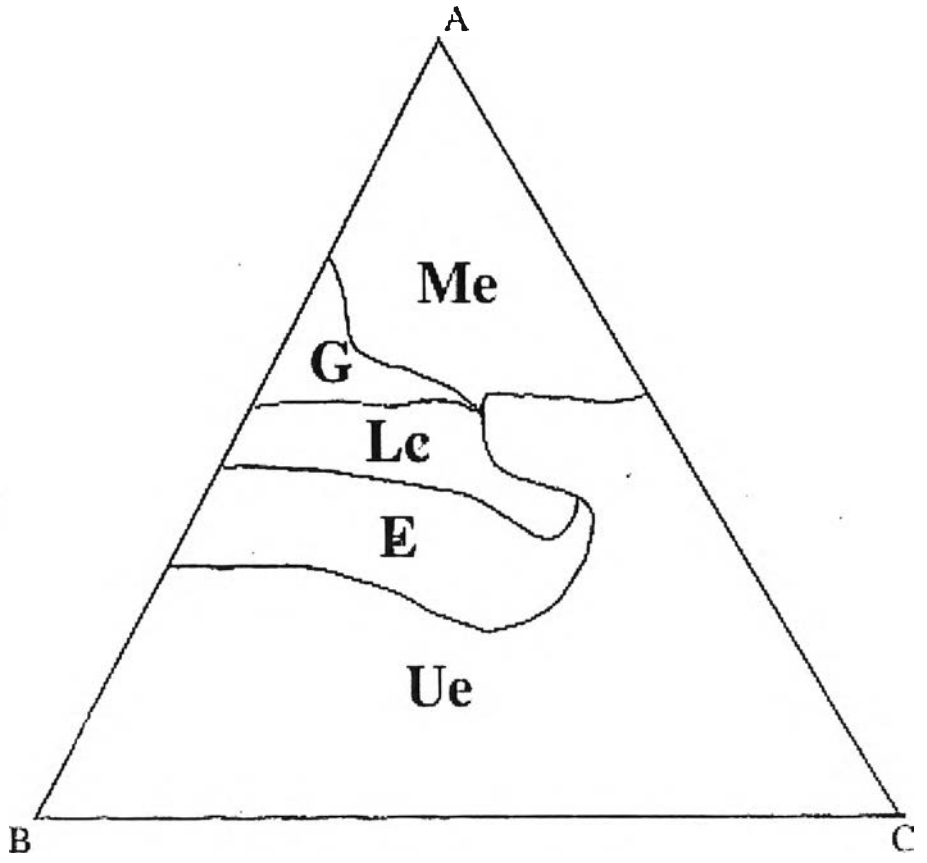

Figure 7 Phase diagram of polyoxyethylene sorbitan monooleate. (A):water (B) -capric/caprylic triglyceride (C) Ue: unstable emulsion (2 phase) E : emulsion (1 phase), Lc: liquid crystal, G : microemulsion gel, Me: microemulsion

Microemulsion Theory (Solan, C. 1997)

The formation and thermodynamic stability of microemulsions were the issues that attracted most of the interest. It was to realize that a reduction of the interfacial tension by three to four orders of magnitude is a requirement for the stability of these systems. This view is a natural consequence of their experimental approach to microemulsion formation. A typical experiment consists of adding a medium chain length alcohol to an emulsion consisting of water, oil and soap as the emulsifier. At a certain concentration of alcohol transition soap takes place spontaneously from a turbid emulsion to a transparent microemulsion. The spontaneous formation and thermodynamic stability of microemulsions were attributed to a further decrease of interfacial tension between water and oil by the effect of added alcohol, up to negative values. Different groups examined the requirement for transient negative interfacial tensions for microemulsion formation theoretically and experimentally.

The understanding of the basis for the thermodynamic stability of microemulsions was an improved considerably with the development of several thermodynamic theories. Ruckenstein and Chi considered the free energy of formation of microemulsions to consist of three contributions:

(1) Interfacial free energy

(2) Energy of interaction between droplets

(3) Entropy of dispersion.

Analysis of the thermodynamic factors showed that the contribution of the interaction energy between droplets was negligible and that the free energy of formation can be zero or negative if the interfacial tension is very low (of the order of 10^{-2}-10^{-3} mN/m) although not necessarily negative. So microemulsions are thermodynamically stable because the interfacial tension between oil and water is low enough to be compensated by the entropy of dispersion.

Surfactants with well-balanced hydrophile-lipophile (H-L) properties have the ability to reduce the interfacial tension to the values required for microemulsion formation. Surfactants with unbalanced H-L properties are unable to reduce the oil-water interfacial tension to values lower than about 1 mN/m.

Considering microemulsions directly related to micellar solutions rather than to emulsions was a significative contribution to elucidation of the problem of the formation of microemulsions may take place on increasing the amount of oil added to a micellar solution without a phase transition. Furthermore, phase behavior studies of nonionic surfactant systems as a function of temperature showed that the hydrophile-lipophile properties of nonionic surfactants are highly temperature dependent. Shinoda and Saito introduced the concept of HLB temperature of phase inversion temperature (PIT) as the temperature at which the hydrophile-lipophile properties of the surfactants are balanced. At this temperature, maximum solubilization of oil in water and ultra low interfacial tensions are achieved. Further studies showed that the effects produced by temperature nonionic surfactant systems

were produced by salinity in ionic surfactant systems. The study of the phase behavior of surfactant systems has made it possible to rationalize the formation of microemulsions (Ubonthip N., et al.2001). Two main general structures have been proposed and are accepted: discrete microemulsions and bicontinuous microemulsions. A schematic picture of a ternary phase diagram at constant temperature corresponding to typical water/ surfactant/ oil ternary system is investigated. Microemulsions poor in either water or oil have a globular structure. Microemulsions containing similar amounts of oil and water and relatively high amounts of surfactant present bicontinuous structures. Frequently, liquid crystalline phases are also demonstrated in the phase diagrams.

Discrete microemulsions consist of domains of one of the psuedophases (water or oil) dispersed in the other pseudophase. These structures are generally found when the main component of one of the pseudophases (water or oil) is present in a higher proportion than the main component of the other psuedophase and little surfactant is present. The structures of this type of microemulsion resembles that of emulsions in that one phase is dispersed in another phase. However, as already stated, they are essentially different in many aspects, in particular, for concerning their stability. The structure of emulsions depends on their history and they evolve with time, whereas microemulsions are thermodynamically stable and their structure is independent of their preparation history. Moreover, other differences arise from other aspects. Emulsion droplets are spherical or nearly spherical; this form minimizes the interface, which gives a highly energetic term because of the interfacial tension. In microemulsions, because of

the very low interfacial tension, the energetic term related to the interfacial tension and total surface is of less importance and therefore nonspherical droplets can be present without a large energy contribution. Because of the small size of the droplets and the low contribution of total surface to the total energy, the geometry of the surfactant molecules at the interface plays an important role.

For microemulsions it is useful to consider the so-called critical packing parameter. This concept, put forward by Israelachvilli et al., considers that the amphiphile molecules can be regarded as a two-piece structure: polar head and hydrophobic tail. The possible geometry of a film formed by the amphiphile molecules depends on their intrinsic geometry. The critical packing parameter (CPP) is calculated as CPP= V/a_0l where a_0 is the optimal area of the polar head, l the length of the hydrophobic tail, and V its volume. The area per polar head is usually measured at an air-water or oil-water interface using the Gibbs isotherm. The length of the hydrophobic tail can be calculated from the values obtained by Tanford, and the volume of the hydrocarbon tail can be calculated from the density of bulk hydrocarbon. Critical packing parameters lower than 1/3 give a tendency to form globular structures, values around ½ favor cylindrical structures, and values close to 1 favor planar layers. Attention should be paid to the indiscriminate use of this parameter evaluates the natural geometry of the amphiphile by itself. In microemulsions, hydrocarbon penetration and cosurfactant presence may completely change the structure from the natural tendency. Oil penetration in the hydrocarbon tail produces an increase in the apparent hydrophobic volume and thus an increase in the critical packing parameter. Cosurfactants, such as medium-chain alcohols, absorb at

the interface, producing an overall reduction of the critical packing parameter. The concentration of surfactant and the ratio of pseudophase play important roles in the structure as well. High amounts of ionic surfactant produce a high ionic strength with a subsequent reduction of the polar head area and reduction of the critical packing parameter. High amounts of the internal pseudophase may produce phase separation if the total concentration of surfactant is low. Other variables that influence the natural curvature of the amphiphile are electrolyte concentration (mainly for ionic surfactants, although they influence nonionic as well) and temperature (mainly affecting nonionic surfactants).

In contrast to the discrete microemulsion structure, which is relatively easy to treat theoretically, the structure of bicontinuous microemulsions is more difficult to visualize and therefore this theoretical treatment is complicated. In a bicontinuous microemulsion both the aqueous and oil phases are continuous. This continuity means that it is possible to go from one extreme of the system to the other by either an oil path or an aqueous path. This structure has an extremely large interfacial area, which is possible because of an extremely low interfacial tension, close to zero. Contrary to what some authors have maintained, in a microemulsion there is not a negative interfacial tension because this would mean production of energy as the interface increases. This could be the case in microemulsion formation but not at equilibrium. Near-zero interfacial tension implies, at the same time, that the interfaces are unstable, forming and disappear without an energy increase. Interfacial energy of the order has been considered to be a condition for the formation of bicontinuous structures. Conditions for the formation of bicontinuous structures

are a ratio of oil and water pseudophases close to one, large amounts of surfactant (enough to cover the interface), and zero natural curvature of the interface.

The theoretical treatment of a bicontinuous structure is complicated. Since Scriven proposed this structure, several models have been proposed. The " random lattice " theory of Talmon and Prager are based on a tessellation of the space by a Voronoi structure; the cells of this tessellation are occupied by either oil or water in a random way. De Gennes and Taupin, whose model is based on a cubic lattice, improved this model. The cubes are occupied by either water or oil in a random way. There is a critical water/oil ratio for which percolation occurs; that is, an infinite path is possible in both phases.

The bicontinuous structure is consistent with most of the experimental observations on these systems. For instance, the self-diffusion coefficients of oil, water, and surfactant are well explained. In these systems the self-diffusion coefficients of oil wand water are close to the self-diffusion coefficients of these molecules in the pure liquids and the self-diffusion coefficient of the surfactant is about one order of magnitude lower. This indicates "free" diffusion for water and oil, i.e., infinite domains, and the lower diffusion coefficient of the surfactant is related to the positioning of the molecules at the interface (see more information below).

A useful picture of the structural transitions can be obtained by considering a surfactant solution in water with an increasing amount of oil.

Surfactants above the critical micelle concentration form micelles in which some oil can be solubilized. The limit of solubility in the micelles depends on the nature of the surfactant and the number of micelles. Ionic surfactants usually have large head groups and have a strong tendency to form spherical aggregates in water. The incorporation of oil increases the size of the aggregates and therefore reduces the curvature. To reach a large amount of solubilizate the oil must penetrate the surfactant tail effectively, a cosurfactant should be added, the temperature should be changed, or a combination of the four. On increasing the amount of oil pseudophase the percolation point will be reached and a bicontinuous structure formed. As said before this point is reached. Further increase of the oil pseudophase will make the system reach the percolation threshold for the water domains and discrete water domains will be formed.

Microemulsions have been studied using a great variety of techniques. This suggests that characterization of microemulsions is a rather difficult task. This is due to their complexity, namely the variety of structures and components involved in these systems, as well as the limitations associated with each technique. Therefore, complementary studies using a combination of techniques are usually required to obtain a comprehensive view of the physicochemical properties and structure of microemulsions. Some of the most common methods and techniques used to identify and characterize microemulsions are briefly described.

A. Phase Behavior

Phase behavior studies, with phase diagram determinations, are essential in the study of surfactant systems. They provide information on the boundaries of the different phases as a function of composition variables and temperature, and, more important, structural organization can be also inferred. In addition, phase behavior studies allow comparison of the efficiency of different surfactants for a given application. It is important to note that simple measurements and equipment are required in this type of study. The boundaries of one-phase regions can be assessed easily by visual observation of samples of known composition. However, long equilibration times in multiphase regions, especially if liquid crystalline phase are involved, can make these determinations long and difficult.

The phase behavior of interest for microemulsion studies involves at least three components: water, surfactant, and oil. Although most of the formulations of practical interest consist of more than three components, study of simple systems with the basic three, our, etc. components from which they are formulated is a prerequisite to understanding the behavior of complex systems. The phase behavior of three-component systems at fixed temperature and pressure is best represented by a ternary diagram and by a triangular prism if temperature is considered as a variable. Other useful ways of representing the phase behavior are to keep constant the concentration of one component or the ratio of two components. As the number of components increases, the number of experiments needed to define the complete phase behavior becomes extraordinary large and the representation of phase behavior

is extremely complex. One approach to characterizing these multicomponent systems is by means of pseudoternary diagrams that combine more than one component in the vertices of the ternary diagram.

Most of the phase studies concerning microemulsions have limited to the determination of one-liquid-isotropic phase boundaries. However, information about the number and compositions of the coexisting phases in equilibrium is of the utmost interest in characterizing these systems.

B. Scattering Techniques

Scattering methods have been widely applied in the study of microemulsions. These include small-angle X-ray scattering (SAXS), small-angle neutron scattering (SANS), and static as well as dynamic light scattering. In the static scattering techniques, the intensity of scattered radiation $I(q)$ is measured as a function of the scattering vector q, $q = (4\pi/\lambda)\sin\theta/2$, where θ is the scattering angle and λ the wavelength of the radiation. The general expression for the scattering intensity of monodispersed spheres interacting through hard sphere repulsion is $I(q) = nP(Q)S(q)$, where n is the number density of the spheres, $P(q)$ is the form factor, which expresses the scattering cross section of the particle, and S (q) is the structural factor, which takes into account the particle-particle interaction. P (q) and S (q) can be estimated by using appropriate analytical expressions. The lower limit of size that can be measured with these techniques is about 2 nm. The upper limit is about 100 nm for SANS and SAXS and a few micrometers for light scattering. These methods are very

valuable for obtaining quantitative information on the size, shape, and dynamics of the components. There is a major difficulty in the study of microemulsions with the use of scattering techniques: dilution of the sample, to reduce interparticle interaction, is not appropriate because it can modify the structure and the composition of the pseudophases. Nevertheless, successful determinations have been achieved by using a dilution technique that maintains the identity of droplets and extrapolating the results obtained at infinite dilution to obtain the size, shape, etc., or by measurements at very low concentrations.

Small-angle X-ray scattering techniques have long been used to obtain information on droplet size and shape. Using synchrotron radiation sources, with which sample-to-detector distances are bigger (4 m instead of 30-50 cm as with laboratory-based X-ray sources), significant improvements have been achieved. With synchrotron radiation more defined spectra are obtained and a wide range of systems can be studied, including those in which the surfactant molecules are poor X-ray scatterers.

Small-angle neutron scattering (SANS) allows selective enhancement of the scattering power of the different microemulsion pseudophases by using protonated or deuterated molecules (contrast variation technique). Therefore, this technique allows determination of the size and shape of the droplets as well as the characteristics of the amphiphilic layer without great perturbation of the system.

Static light scattering techniques have also been widely used to determine microemulsion droplet size and shape. In these experiments the intensity of scattered light is generally measured at various angles and for different concentrations of microemulsion droplets. At sufficiently low concentrations, provided that the particles are small enough, the Fayleigh approximation can be applied. Droplet size can be estimated by plotting the intensity as a function of droplet volume fraction.

Dynamic light scattering, also referred to as photon correlation spectroscopy (PCS), is used to analyze the fluctuations in the intensity of scattering by the droplets due to Brownian motion. The self-correlation function is measured and gives information on the dynamics of the system. This technique allows the determination of *z*-average diffusion coefficients, *D*. In the absence of interparticle interactions, the hydrodynamic radius of the particles, R_h, can be determined from the diffusion coefficient using the Stokes-Einstein equationm:$D=kT/6\pi\eta R_h$, where *k* is the Boltzmann constant, *T* is the absolute temperature, and η is the viscosity of the medium. Although dynamic light scattering measurements are relatively easy and fast, extrapolation of results to infinite dilution is not possible in most microemulsion systems and R_h values obtained should be corrected because of interparticle interaction.

C. Nuclear Magnetic Resonance

Nuclear magnetic resonance techniques have been used to study the structure and dynamics of microemulsions. Self-diffusion measurements using

different tracer techniques, generally radioactive labeling, supply information on the mobility of the components (self-diffusion coefficient). A limitation of this technique is that experiments are time-consuming and the use of labeled molecules in multicomponent systems such as microemulsions is not practical. However, the Fourier transform pulsed-gradient spin-echo (FT-PGSE) techniques, in which magnetic field gradients are applied to the sample, allows simultaneous and rapid determination of the self-diffusion coefficients (in the rage of 10^{-9} to $10^{-12}m^2s^{-1}$), of many components. In water-in-oil microemulsions, water diffusion is slow and corresponds to that of the droplets (of the order of $10^{-9}m^2s^{-1}$ oil diffusion is high (of the order of $10^{-11}m^2s^{-1}$), and the diffusion of surfactant molecules, located at the interface, is of the same order as that of the droplets. In contrast, in oil-in-water microemulsions the diffusion coefficients of water are higher than that of oil. In bicontinuous microemulsions the diffusion coefficients of water and oil are both high (of the order of $10^{-9}m^2\ s^{-1}$) and the diffusion coefficient of the surfactant has been found to be intermediate between the value of no associated surfactant molecules and the value for a droplet-type structure (of the order of $10^{-10}\ m^2\ s^{-1}$).

D.Electron Microscopy

Several electron microscopic techniques have been attempted for the characterization of microemulsions. Because of the high labiality of the samples and the danger of artifacts, electron microscopy used to considered a misleading technique in microemulsion studies. However, images showing clear evidence of the microstructure have been obtained. Freeze fracture electron microscopy, a well

established method in the biological field has been successfully applied to microemulsions. Careful control of the temperature of the sample before freezing and ultra rapid cooling followed by fracture and replication of the fracture face yield images of the microstructure of these systems.

E.Other Methods

Interfacial tension measurements are useful in the study of the formation and properties of microemulsions. Ultralow values of interfacial tensions are correlated with phase behavior, particularly the existence of surfactant phase or middle-phase microemulsions in equilibrium with aqueous and oil phase. Ultralow interfacial tensions can be measured with the spinning-drop apparatus. Interfacial tensions are derived from the measurement of the shape of a drop of the low-density phase, rotating in a cylindrical capillary filled with the high-density phase.

Electrical conductivity has widely used to determine the nature of the continuous phase and to detect phase inversion phenomena. The distinction between O/W (high-conductivity) and W/O (low-conductivity) emulsions is quite straightforward. However, in microemulsions the behavior is more complex. A sharp increase in conductivity in certain W/O microemulsion systems was observed at low volume fraction. This behavior was interpreted as an indication of a "percolative behavior" or exchange of ions between droplets before the formation of bicontinuous structures. When the conductivity of nonionic surfactant systems is measured, water is generally replaced by an electrolyte solution. If the electrolyte concentration is kept

low (10^{-2}-10^{-3} M), no effect on the structure is produced. Dielectric measurements have also been used in the determination of structural aspects of microemulsions and results similar to those obtained from conductivity measurements have been obtained.

Viscosity measurements as a function of volume fraction have been used to determine the hydrodynamic radius of droplets, as well as interactions between droplets and deviations from spherical shape by fitting the results to appropriate models. Some microemulsions show Newtonian behavior, and their viscosities are similar to that of water. For those microemulsions, the hydrodynamic volume of the particles can be calculated from Einstein's equation for the relative viscosity (ηr)

$$\eta r = 1 + 2.5\phi \quad \text{(3)}$$

ϕ = The particle volume fraction is lower than ~0.1 or from modifications of this equation if it is higher.

Structure and Function of skin

Skin cover the entire body and protects it from various types of external stimuli and damage as well from moisture loss. The surface area of the skin of an adult person is about 1.6 m^2. The thickness of skin varies with age, sex and location. Generally, the skin of men is thicker than women. However, women have a

thicker subcutaneous fat layer. In general, the skin of the thinnest and that of the soles of the foot is the thickest.

The outer skin is divided into three layers called the epidermis, the dermis, and the subcutaneous tissue. Various appendages, such as hair, nails, and glands (sweet and sebaceous), are also found in the skin

The epidermis is composed of several cell layers about 0.1-0.3 mm thick. From the external surface inwards, these layers are called the horny layer (stratum corneum), granular layer, spinous layer, and basal layer. The basal layer is formed of a single layer of columnar cells (basal cells) abutting against the basement membrane, which is in contact with the dermis.

The dermis is composed of connective tissue below the epidermis. The convoluted surface of the dermis is in contact with the epidermis and the areas where the epidermis protrudes downward into the dermis are called epidermis ridges. The area of the dermis near the epidermal protrusions is called the papillary dermis, and the deeper dermis is called the reticular dermis. Unlike epidermal cells, many of the dermal cells, are not in tight cellular connect with each other, and there are many extracellular spaces. This part of the skin that has a macromolecular network structure is called the extracellular matrix. The basic materials comprising the extracellular matrix consist of glycosaminoglycans, or acidic mucopolysaccharides, and fibrous proteins. Glycosaminoglycan has different forms depending on the type of saccharide from which it is formed and depending upon the position of the sulfate

group. In skin, however, hyaluronic acid and dermatan sulfate are the most common forms. Glycosaminoglycans exist as proteoglycans, combining protein, and can contain large quantities of water forming a gel. The fibrous proteins are embedded in this gel. The water in the gel transfer nutrients, metabolic products and hormones between the blood vessels and the cell tissues. The fibrous protein is composed of collagen and elastin for constructive purposed as well as fibronectin and laminin for connective purposes. Collagen is the principal protein of the extracellular matrix and maintains the form of the tissues. Elastic fibers are connected to each other, forming cross-links to maintain tissue elasticity. As a result of this construction. The dermis plays a large role in the elasticity and tension of the skin. The dermis also contains blood vessels, nerves, hairs, hair-erector muscles, sweat glands, and sebaceous glands. Beneath the dermis, there is subcutaneous tissue, which contains many adipose cells in and between the connective tissue. The boundary of the dermis is not very clear. The main role of the subcutaneous fat is to regulate temperature. Subcutaneous fat is generally better developed in women than in men and in children than in adults.

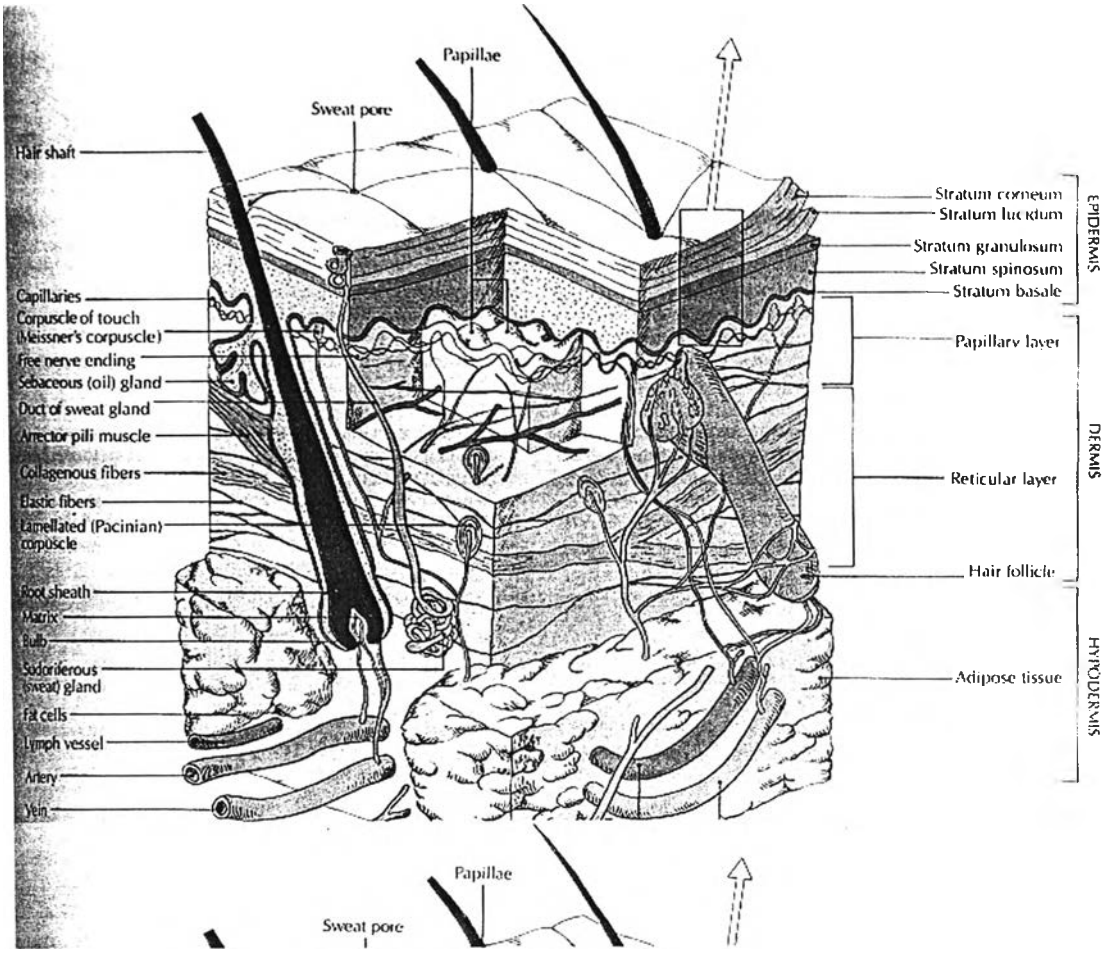

Figure 8 The structure of skin

Design of In Vitro Skin Permeation Apparatus

Various types of in vitro apparatus for measuring drug permeation profiles across the skin have been reported in the literature.

For studying the release profiles of drugs from a transdermal drug delivery system, the effect of the hydrodynamic diffusion layer (existing on the drug-releasing surface) on the rate of drug release must be carefully investigated. Generally speaking, the mechanism of agitation in the in vitro diffusion cell should be designed to maintain a hydrodynamic condition such that the thickness of the diffusion boundary layer is at a minimum, while the primary convective flow toward the drug-releasing surface also is minimized. It is, therefore, more desirable to have the in vitro diffusion cell designed in such way that the agitation element will rotate at 9 plane paralle! to the drug-releasing surface. If this is not the case, the intensity of agitation must be controlled within an appropriate range.

On the other hand, the effect of the hydrodynamic diffusion layer on the rate of skin permeation may not be substantial, since the principal resistance to drug transport in the transdermal delivery system is often the drug permeation process through the stratum corneum. This is because the diffusivity of a drug across the stratum corneum normally ranges form 10^{-8} to 10^{-3} cm^2/sec, as compared to the intrinsic diffusivity of 10^{-5} to 10^{-6} cm^2/sec in the elution medium, Therefore, the agitation in this skin-limited permeation study is often important only for maintaining

uniform temperature and drug distribution throughout the in vitro skin permeation cell. However, if the barrier function of the stratum corneum is markedly reduced, as in the case of increased skin permeability, the effect of the diffusion layer on the rate of skin permeation could become rather significant.

There are several designs for *in vitro* membrane of permeation in apparatus, whose hydrodynamic characteristics have been fully investigated and discussed in the sections that will be following.

Franz Diffusion Cell (Chien, Y. W. 1987.)

The vertical-type skin permeation system developed by Franz and commercialized by Crown Glass has been frequently used for studying the kinetics of percutaneous absorption. The cell has a receptor compartment with an effective volume of approximately 10-12 ml and an effective surface area for permeation varying form 1.57 to 4.71 cm^2. A rod-shaped magnet driven by a 3-W synchromous morter stirs the solution in the receptor compartment. The stirring magnet rotates at a speed of 600 rpm in a low viscosity receptor solution such as saline solution, the temperature in the bulk of the solution can be maintained at a constant level by circulation thermo stated water through the water jacket surrounding the receptor compartment. However, the temperature near the upper opening, at which the skin will be positioned, varies as the surrounding temperature varies. The observed variation in receptor solution temperature results because the donor compartment is

not thermally controlled. The hydrodynamic characteristics of the Franz diffusion cell recently were established.

Modified Franz Diffusion Cell

Another vertical-type skin permeation cell as shown in Figure 3 was recently developed in response to the observation that the Franz cell has rater poor solution hydrodynamics as a result of inefficient mixing. The results are a significant temperature gradient in the diffusion cell and an inhomogeneous drug concentration in the receptor solution. Recognizing these deficiencies (Keshary and Chien 1984) set forth to modify the Franz diffusion cell to improve its efficiency of fluid mixing. The modified cell (the K-C cell) has an effective receptor solution volume of 12 ml and a skin surface area of 3.14 cm^2. The receptor solution is stirred by a star-head magnet rotating at a constant speed of 600 rpm by the same driving unit originally designed for Franz diffusion cell. The hydrodynamic characteristics of the modified Franz cell were recently investigated.

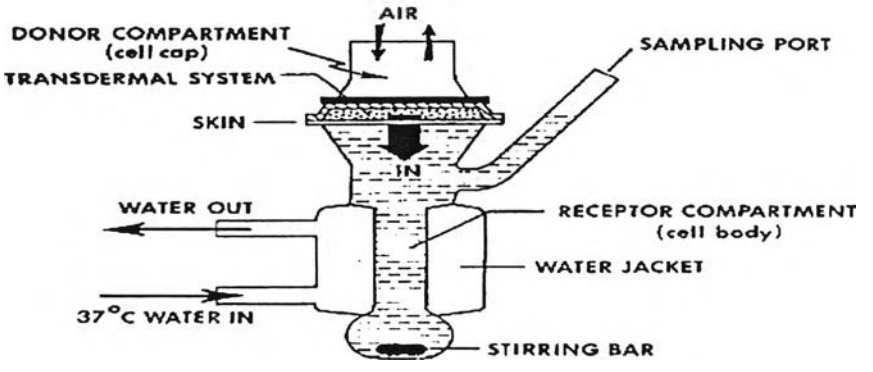

Figure 9 ; Modified Franz diffusion cell

There are general considerations that apply for the enhancement of permeation across any membrane. By permeation, one really means flux, and one is thus concerned with the problem of increasing the flux across membranes. For any region within a membrane the flux, J, can be written as follows:

$$J = -D \frac{dC}{dX} \qquad \text{(4)}$$

For flow in one dimension, hear D is the diffusion coefficient, C is the permeant concentration, and X is the spatial coordinate. Although the solution for J with various boundary conditions and membrane heterogeneities can be very complex (1), the basic concepts regarding flux enhancement can be found in equation (4). The concentration of gradient is thermodynamic in origin, and the diffusion coefficient is related to the size and shape of the permeant and to the energy required to make a hole for diffusion. Thus enhancement of flux across membranes reduces to considerations of:

1. Thermodynamics (Lattice energies, distribution coefficients).
2. Molecular size and shape.
3. Reducing the energy required to make a molecular hole in the membrane.

Although these basic concepts can be used to develop systems for enhanced transport, such practical considerations as vehicle evaporation and recipient interactions make useful systems somewhat evasive. (Cooper, E. R. 1987)

Shed Snake Skin (Itoh, 1990)

A variety of model membranes has been used for transdermal research, such as human cadaver skin, hairless mouse skin, and synthetic membranes. Although human skin is the best model membrane, the cost and limited availability put a limitation on the use of human skin. Also, the permeability through human skin varies up to 10-fold depending on the body site. On the other hand, it is easy to obtain animal skins of the same species with the same line and age. However, the time for experimental use of some animal skin in *in vitro* penetration studies is limited because of deterioration of membrane integrity after prolonged use. Moreover, most animal skins are more permeable than human skin partly because of a larger number of hair follicles. The use of artificial membranes in transdermal research is limited because they lack keratinized proteins and lipids which are primary components in the stratum corneum of mammalian skins.

Shed snake skin is the model membrane which is a nonliving pure stratum corneum with no hair follicles. Snakes shed their skins periodically, leaving their old stratum corneum behind, which makes it possible to obtain multiple shed skins from the same individual snake. Unlike human stratum corneum, which consists of 10-20 layers of an alpha-keratin-rich intracellular layer and a lipid-rich intercellular layer, shed snake skin consists of three distinctive layers. These are the beta-keratin-rich outermost beta layer, alpha-keratin- and lipid-rich intermediate mesos layer, and alpha-keratin-rich innermost alpha layer. Further, the mesos layer shows three to five layers of multiplayer structure with cornified cells surrounded by intercellular lipids,

which is similar to human stratum corneum. This mesos layer is also a major depot of lipids, and the mesos layer and alpha layer are considered to be the main barrier to water penetration through the skin. Further, water permeability was found to be very similar between normal and scaleless skins. This indicates that the existence of scales may not affect significantly the permeability of compounds through shed snake skin.