Chapter 2

Literature Review
2.1 SHILAJIT: FROM ANTIQUITY TO PRESENT

Shilajit is a pale-brown to blackish-brown exudation, of variable consistency, coming out from layer of rocks in many mountain ranges of the world, especially the Himalayas and Hindukush ranges of the Indian subcontinent (Chopra, et. al., 1958; Ghosal, 1992). It is also found in Australia, Bhutan, China, Egypt, Mongolia, Nepal, Norway, Pakistan, Russia and other countries, where it is collected in small quantities from steep rock faces at altitudes between 1000 and 5000m. Shilajit samples from different regions of the world however vary in their physiological properties.

Although the name shilajit means “Winner of Rock”, shilajit itself is not a rock comprising inorganic material but is a complex mixture of organic humic substances and plant and microbial metabolites occurring in the rock rhizospheres of its natural habitat.

2.1.1 Shilajit: In ancient texts

Shilajit finds mention in a number of ancient texts of the Ayurvedic and Unani systems of medicine (Ghosal et. al., 1995e). It has been used as a rejuvenator and an adaptogen for thousands of years, in one form or the other, under the indigenous systems of medicine. It has been said that there is hardly any curable disease which cannot be controlled or cured with the aid of shilajit. Although this is a tall order, scientific studies over the last 20-25 years have shown that it is indeed a panacea of traditional medicine, effective in a number of ailments.

2.1.2 Shilajit: Synonyms

Shilajit has various synonyms (Nadkarni, 1976). In Sanskrit, it is called Silajit or Silaras. In English, it is called Asphalt, Mineral pitch or Jews pitch. In Hindi, Gujarati and Marathi, it is called Silajita; in Bengali, it is called Silajatu; in Tamilian, Perangyum while in Arabic, it is called Hajar-ul-musa. In Persian, it is called, Momiai Pajurul Yahud and in Russian, it is known as Mummiyo. Sanskrit meaning of shilajit is “Conqueror of mountain and destroyer of weakness”. Other terms like dathuras, dathusara and shiladhata have also been used in ancient medical texts like Sushruta.
samhita, Charak samhita and Rasarangini to describe shilajit. The word dhatu has been used simply to emphasize its capability as a rasayana, which increases the activity of the saptadhatus of the body.

2.1.3 Varieties of Shilajit

There are four different varieties of shilajit which have been described in charaka samhita, viz., savrana, rajat, tamra and lauha shilajit (Chopra et. al., 1926). Savrana shilajit is gold shilajit and is red in colour. Tamra shilajit is copper shilajit and is blue in colour. Rajat shilajit is silver shilajit and is white in colour while lauha shilajit is an iron containing shilajit and is brownish-black in colour. Tamra and Savrana shilajit are not found commonly but the last variety, i.e., lauha shilajit is commonly found in Himalayan ranges and is supposed to be the most effective according to the therapeutic point of view.

2.1.4 Shilajit: Origin

There are a number of hypotheses about the origin of shilajit (Tiwari and Agarwal, 2002). Shilajit is believed to have been derived from vegetation fossils that have been compressed under layers of rocks for hundreds of years and have undergone a high amount of metamorphosis due to the high temperature and pressure condition prevalent there. During warm summer months, shilajit becomes less viscous and flows out between the layers of rocks. Ancient texts of Sushruta samhita and Rasarangini also suggest that shilajit has a vegetative origin. It has been mentioned in Sushruta samhita that in the month of may-june, the sap or latex juice of plants comes out as a gummy exudation from the rocks of mountain due to strong heat of sun. Rasarangini and Dwarishtarang also claim that shilajit is an exudation of latex gum resin, etc. of plant which comes from the rocks of mountain under the presence of harsh scorching heat (Tiwari et. al, 1973).

Recent scientific work carried out on shilajit has shown that it is mainly composed of Humus – the characteristic constituents of soil together with other organic constituents (Ghosal et. al., 1992). Latex bearing plants like *Euphorbia royleana* and *Trifolium repens* which occur in the vicinity of the shilajit bearing rocks are thought to
be the most likely source of shilajit (Ghosal, et. al., 1988b). Fig. 2.1 shows the photograph of shilajit in its natural habitat as well as in the raw form.

Another recent research claims that mosses like species of Barbula, Fissidenc, Minium, Thuidium and species of Liverworts which are present in the vicinity of shilajit exuding rocks may be responsible for the formation of shilajit (Joshi et. al., 1994). The bryophytes reveal occurrence of minerals and metals in their tissue which are similar to the elements present in shilajit.

![Shilajit in its natural habitat](image1)

![Raw Shilajit](image2)

**Fig. 2.1: Photographs of Shilajit in raw form**

### 2.1.5 Shilajit: Chemical Nature

Early scientific work on shilajit in the 1930’s showed that the major organic constituents of shilajit included benzoic acid, hippuric acid, fatty acids, resin and waxy materials, gums, albuminoids and vegetable matter with benzoic acid being the active ingredient (Chopra et. al., 1926).

Extensive research in the eighties showed that the major organic mass of shilajit comprised of Humus (60-80%), benzoic acid, hippuric acid, fatty acid, ichthyol, ellagic acid, resin, triterpenes, sterol, aromatic carboxylic acid, 3,4-benzocoumarins, amino acids and phenolic lipids (Ghosal et. al., 1976; Kong, et. al., 1987; Ghosal, 1990).
2.1.6 Odour of Shilajit

Two odourous varieties of shilajit have been reported in Ayurveda: Gomutra shilajit (smell - like cow urine) and Karpurgandha shilajit (smell - like camphor) (Ghosal, 1994a). Scientific investigations using head space gas chromatographic technique revealed that the odour of Gomutra shilajit was due to the presence of a number of constituents including long chain aliphatic compounds (hydrocarbon and their corresponding derivatives) at different level of oxidation, naphtienes (alkyl-cycloalkanes), aromatic, phenolic, carboxylic acids and their N- conjugates and N- and S- heterocyclics. Besides these, abundance of cymenes and their reduced equivalent contributed to the fragrance of Karpurgandha shilajit (Ghosal, 1994b; Ghosal et. al., 1995b).

2.1.7 Shilajit: Bioactive Constituents

The activity of shilajit has been ascribed to two distinct classes of compounds (Ghosal, et. al., 1991; Ghosal, S., 2002):

1. The low molecular weight organic, compounds such as oxygenated dibenzo-\(\alpha\)-pyrones, both mono- and bis-compounds thereof, in free and metal-ion conjugated forms;

2. The medium molecular weight humic and fulvic acids which act as carriers for the bioactive principles and help in their in-vivo transportation in the body.

The humic and fulvic acids present in shilajit have a porous structure having voids into which the bioactive compounds get entrapped (Ghosal, et. al., 1991; Ghosal, S., 2003).

2.1.8 Purification and Formulation of Shilajit

Shilajit in its natural form is often contaminated with varied amount of impurities such as mycotoxins, heavy metal ions, polymeric quinones, reactive free radical, etc. (Ghosal, et. al., 1995c). Mycotoxins are produced by mold or fungi and can cause illness or death in man. Free radicals can be harmful to cells and are believed to be a causative factor in aging. Polymeric quinones are an oxidation product of quinic acid which is found in some plants. Hence, it is necessary to purify shilajit before it is
consumed. The findings are consistent with the ancient texts which recommend purification of shilajit before consumption.

Hindi and English translations of ancient Ayurvedic texts have described two methods for the purification of shilajit (Ghosal et al., 1995c):

1. Suryatapi: heating aqueous solution of shilajit under sunlight and
2. Agnitapi: heating the solution by direct contact with fire.

The viscous creamy waxy material which comes out over the aqueous layer is collected and regarded as pure shilajit. However, the translations had missed out an important point that the essential metalo-organic humates, one of the most important components of shilajit, would be left out in the water-soluble portion by this method.

2.1.9 Shilajit: Shodhana

Distinct protocols can be found in the original Ayurvedic texts for graded purification (Shodhana) and subsequent formulation (Bhavana) of shilajit. According to these recommendations, it is necessary to remove first the water insoluble inorganic and polymeric materials from the external surface (Bahirmal: external impurities) of shilajit. Washing with water is sufficient for it. To remove the internal impurities (Antamal: internal impurities) from the void/pores of shilajit would require neutral salts (sodium pyrophosphates, ammonium chloride) and buffers (citrate, lemon juice) of mineral and organic acids. The remaining part of the impurities such as loose metal ions, polymeric quinones and other free radicals can then be removed by treatment with small tannoids such as from pistacia species or with Triphala (Ghosal et al., 1995c).

2.1.10 Shilajit: Bhavana

Ayurvedic texts also describe the process of Bhavana or Impregnation of shilajit. According to this method, the micropores of shilajit fulvic acid are made vacant by
the requirement of the specific disease. The varieties of shilajit which are sufficiently rich in bioactive substances (e.g., oxygenated dibenzo-α-pyrones) are called as Satwajacta shilajit and need not be used for the process of Bhavana/Formulation. It is the Nisatwa shilajit whose fulvic acid micropores have enough vacant spaces, which should be used for the process of Bhavana (Ghosal et. al., 1995e).

2.1.11 Marketed Preparations of Shilajit
A number of formulations of shilajit are marketed in India as well as abroad by companies like Dabur, Baidhyanath, Indian Herbs, Gurukul Kangri, etc. These formulations usually contain Shodhit shilajit which has been purified by the Shodhana process described before. The formulations are either in the form of thick paste or as powder filled into hard gelatin capsules. A few formulations also contain other herbs along with shilajit.

2.1.12 Uses of Shilajit in traditional medicine
Shilajit has an important and unique place in the traditional texts of Ayurveda, Siddha and Unani medicine. In regional folk medicine, shilajit is a reputed rasayana (a rejuvenator and immunomodulator), claimed to arrest the process of ageing and prolong life (Phillips, 1997). It is prescribed for the treatment of genitourinary disorder, jaundice, gallstone, digestive disorders, enlarged spleen, epilepsy, nervous disorder, chronic bronchitis, and anaemia (Chopra et. al., 1958). Shilajit has also been ascribed potent aphrodisiac property. According to Ayurveda, shilajit arrests the process of aging and produces rejuvenation which are two important aspects of an Ayurvedic rasayana.

Shilajit is useful for treating kidney stones, edema, piles, internal antiseptic, adiposity, to reduce fat and anorexia (Nadkarni, 1976). Shilajit is given along with milk to treat Diabetes. Shilajit is prescribed along with guggul to treat fracture. It is believed that it goes to the joints and forms a callus quickly. The same combination is also used to treat osteoarthritis and spondylysis.
2.1.13 Validation of ancient claims of Shilajit

Although not all therapeutic claims associated with shilajit have been verified by modern scientific evaluation, a number of pharmacological properties of shilajit have been proven by systematic experimentation in animals (Acharya et. al., 1988).

2.1.13.1 Anti-ulcerogenic and Anti-inflammatory activity

Shilajit is perhaps the first agent to possess both anti-ulcerogenic and anti-inflammatory activities in a single compound and this unique property of shilajit can be safely utilized in clinical practice (Goel et. al., 1990). Shilajit, at a dose of 50mg/kg was found to significantly reduce carrageenan induced hind paw edema in rats having an effect comparable to phenylbutazone (100 mg/kg, i.p.) and betamethasone (0.25 mg/kg, ip). It also increased the carbohydrate/protein ratio and decreased gastric ulcer index in rats, indicating anti-ulcerogenic activity (Ghosal et. al., 1988a).

2.1.13.2 Antioxidant activity

The uncontrolled production of oxygen-, sulphur- and nitrogen-centered free radicals have been implicated in a number of diseases and debility conditions in humans ranging from arthritis and haemorrhagic shock to AIDS, Alzheimer's disease and aging. Agents that can regulate the uncontrolled systemic production of these biogenic free radicals can presumably provide cellular protection and regress cellular damage. Processed shilajit has been shown to possess such radical scavenging and antioxidant effect against these biogenic free radicals, thus validating its claim as an important Rasayana (revitalizer) (Bhattacharya, et. al., 1995: Ghosal et. al., 1995d). Processed shilajit not only exhibited significant antioxidant activity of itself but also had the ability to regenerate (recycle) ascorbic acid after it had neutralized free radicals (Ghosal and Bhattacharya, 1996).

2.1.13.3 Learning Augmentation

The study was carried out to test the validity of use of shilajit as an Ayurvedic medha rasayana (enhancer of memory and learning) in albino rats. Processed shilajit, native shilajit and a preparation consisting of a mixture of ethyl acetate extractive and fulvic acids obtained from processed shilajit were evaluated in an active
avoidance, elevated plus-maze and open field behavior paradigms. It was found that processed shilajit and its active constituents (total ethyl acetate fraction and fulvic acids) significantly increased the learning acquisition and memory retention in old albino rats. However, native shilajit produced erratic response (both augmentive and retardative) in the above parameters (Ghosal et. al., 1993).

2.1.13.4 Antidiabetic activity
Shilajit at a dose of 100 mg/kg, PO was found to decrease streptozocin induced hyperglycaemia in rats. It also reduced the STZ-induced decrease in superoxide dismutase activity in pancreatic islet cells (Bhattacharya, 1995; Kanikkannan et. al., 1995). Importantly, it had no effect on the blood glucose level in normal rats. The results support the earlier writing of Ayurveda that shilajit can prevent maturity onset diabetes mellitus.

2.1.13.5 Antistress activity
Shilajit collected from India, Nepal, Pakistan and Russia and organic constituents isolated from them were studied for their antistress effect in albino mice. It was found that shilajit from Kumoan (India), Dolpa (Nepal) and a combination of the total ethyl acetate extract and fulvic acids extracted from Kumoan shilajit produced statistically significant improvement in forced swimming induced immobility in albino mice (Ghosal et. al., 1991).

2.1.13.6 Antiallergic activity
The effect of shilajit and its main active constituents were studied in relation to the degranulation and disruption of mast cell against noxious stimuli. Shilajit and its active constituents provided statistically significant protection to antigen-induced degranulation of sensitized mast cells, markedly inhibited the antigen induced spasm of sensitized guinea-pig ileum and prevented mast cell disruption (Ghosal et. al., 1989). These findings are consistent with the therapeutic use of shilajit in the treatment of allergic disorders.
2.1.13.7 Immunomodulatory activity

Shilajit as an immunomodulator agent was studied in mice that were given either shilajit extract or a placebo. The white blood cell activity was studied and monitored prior to and at intervals after receiving the shilajit extract or a placebo. It was found that the white blood cell activity was increased by shilajit extract. Shilajit and its combined constituents elicited and activated, in different degrees murine peritoneal macrophages and activated splenocytes of tumor-bearing animals at early and later stages of tumor growth (Ghosal et. al., 1995a).

In another experiment, the effect of shilajit was determined on the levels of brain monoamines in rats. It was found that shilajit at a dose of 25 and 50 mg/kg i.p. for five days significantly reduced the level of 5-hydroxy tryptamine and 5-hydroxy indole acetic acid and increased the level of dopamine, noradrenaline and their metabolites in rat brain (Bhattacharya and Ghosal, 1992). These changes in neurotransmitter levels are similar to those seen in cases of increased humoral (immune) activity and hence validate its use as an Ayurvedic Rasayana.

2.1.14 Shilajit: A Yogavaha

A remarkable property of shilajit which has been described in ancient texts is that of a Yogavaha. Yogavaha is an agent which enhances the properties of other drugs. Shilajit is usually soaked in the decoction of one or more of the following plants: Shoria robusta, Bachanania lactifolia, Acacia farnesiana, Terminalia tomentosa, Catechu nigrum, Terminalia chebula and Sida cardifolia, as this is said to increase their efficacy (Nadkarni, 1976).

Although the yogavaha property of shilajit has been described in ancient literature, the reason for the same has not been described. It is possible that this property may be due to the presence of humic substances, mainly humic and fulvic acids, present in shilajit. These humic substances have an “open” flexible structure perforated by voids of varying dimensions. These voids are capable of entrapping the bioactive molecules of shilajit like the low molecular weight dibenzo-α-pyrones. Such entrainment affords a high degree of protection and stability to the entrapped molecules in their natural habitat. It has also been suggested that humic and fulvic acids act as carrier molecules for delivering these bioactive molecules at their intended site of action.
# Literature Review

## Humic Substances
(Pigmented polymers)

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<th>Fulvic acids</th>
<th>Humic acids</th>
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<td>Dark brown</td>
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<td>Grey black</td>
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- Increase in intensity of colour
- Increase in degree of polymerization

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<td>45 %</td>
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<td>48 %</td>
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<tr>
<td>1400</td>
<td>Decrease in degree of solubility</td>
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Fig. 2.2: Physico-chemical properties of humic substances (Saluja, A., 2001)
2.2 HUMIC SUBSTANCES (Schnitzer, 1978; Gaffney et. al., 1996)

Humic substances, which arise from the decomposition of dead and decaying plant and animal tissues, are ubiquitous in the environment and occur in soils, waters and sediments of the ecospheres. They are the major organic constituent of soils and sediments and occur widely in almost all terrestrial and aquatic environments. Humic substances are also present in shilajit (Ghosal et al., 1993).

Humic substances are dark coloured, predominantly acidic, chemically complex, polyelectrolyte, macromolecular materials that range in molecular weight from few hundreds to several thousands. These materials are usually partitioned into the following three main fractions based on their solubility (Aiken et. al., 1985):

**Humic acids:** The fraction of humic substances that is not soluble in water under acidic conditions (pH < 2) but is soluble at higher pH values. They are dark brown to black in colour.

**Fulvic acids:** The fraction of humic substances that is soluble in water under all pH conditions. These are light yellow to yellow-brown in colour.

**Humin:** The fraction of humic substances that is not soluble in water at any pH value and in alkali. Humin is black in colour.

Humic substances arise from the decomposition of dead and decaying plant and animal tissues and are ubiquitous in the environment. They are the major organic constituent of soils and sediments and occur widely in almost all terrestrial and aquatic environments (Stevenson, 1994). Although the exact chemical structure of humic substances has not been elucidated, it has become apparent that humic substances are not single molecules but rather association of molecules of microbiological, polyphenolic, lignin and condensed lignin origin. The principal properties of humic acids and their subsequent potential applications depend strongly on their origin (source) as well as on the isolation procedure. The chemistry of humic acids is also deeply influenced by these factors.
2.2.2 Humic acids: A profile (Schnitzer, 1978; Martin et al., 1998)

**Synonyms**: Ulmic acid

**Description**: Dark brown to black amorphous granular powder with a characteristic taste.

**Solubility**: Soluble in water above pH 3.0

**CAS No.**: 1415-93-6

**Elemental composition**: C= 41-56 %, H= 4-6 %, N= 13-20 %, O = 20-38 %

**Molecular weight range**: 3,000-100000

**Melting point**: $>300^\circ$C

Humic acids form a major portion of the humus in soils, natural waters, river, lake and sea sediments, brown and brown-black coals and other natural materials such as shilajit, as a product of chemical and biological transformations of animal and plant residues. Substantial evidence exists that humic acids consist of a skeleton of aryl/aromatic units cross-linked mainly by oxygen and nitrogen groups with the major functional groups being carboxylic acid, phenolic and alcoholic hydroxyls, ketone and quinine groups (Livens, 1991). The large number of diverse chemical functionalities contained in their polymeric nature and relatively high chemical stability favour their practical exploitation (Ghabour and Davies, 2000). Being highly aromatic, humic acids become insoluble when the carboxylate groups are protonated at low pH values.
2.2.3 Fulvic acids: A profile (Schnitzer, 1978; Martin et. al., 1998)

**Description**: Light yellow to yellowish-brown powder with a characteristic taste.

**Solubility**: Soluble in water at all pH values

**Elemental composition**: C≈ 28-39 %, H= 4-6 %, N= 1-8 %, O = 46-62 %

**Molecular weight range**: 200 - 3000

**Melting point**: >300°C

In general, fulvic acids are of lower molecular weight than humic acids and soil-derived materials are larger than aquatic materials (Stevenson, 1982). The content of C, H and N, is in general, lower in fulvic acid in comparison with humic acid, while the content of O is comparatively more.

The structures of fulvic acids are somewhat more aliphatic and less aromatic than humic acids and these are comparatively richer in carboxylic acid, phenolic and ketonic groups than humic acids. This is responsible for their higher solubility in water at all pH values. The total acidities of fulvic acids (900 – 1400 meq/100g) are considerably higher than for humic acids (400 – 870 meq/100g).
2.2.4 Formation of Humic substances in nature (Stevenson, 1982; Liven, 1991)

The formation of humic substances is one of the least understood and most intriguing aspects of humus chemistry. Studies on this subject are of long-standing and continued research can be justified on theoretical and practical grounds. A variety of synthetic routes have been suggested, based both on attempts to synthesize humic materials and on their reactions. Presently, the view is that humic substances are produced by the condensation of quinines or phenolic compounds, which are themselves formed in the biological transformation of plant residues.

Fig. 2.3: Mechanisms of formation of Humic substances (Stevenson, 1982)

Several pathways exist for the formation of humic substances during the decay of plant and animal remains in soil (Fig. 2.3). The classical theory, popularized by Waksman, is that humic substances represent modified lignins (pathway 1) but the majority of present-day investigators favor a mechanism involving quinones (pathway 2 and 3). In practice, all four pathways must be considered as likely mechanisms for the synthesis of humic and fulvic acids in nature, including sugar-amine condensation (pathway 4).
Literature Review

2.2.5 Molecular and chemical structure of humic substances

The structures of humic substances (humic and fulvic acids) are at present ill defined despite many decades of research, although numerous tentative structures have been proposed. The main task that confronts researchers in this field today is to develop a valid concept of the chemical structure of humic materials. Humic substances are believed to consist of molecules ranging in molecular weight from a few hundred to several hundred thousand. Individual fractions such as humic and fulvic acid merely represent a particular part of this molecular weight range (Livens, 1991). Nevertheless, since they are derived from chemically similar starting materials, all the molecules of humic substances share some structural and chemical characteristics.

A number of techniques such as NMR (Frund and Ludemann, 1989; Simpson et al., 2002), mass (Novotny and Rice, 1995), x-ray (Rice et al., 1999), spectroscopy (Spiteller and Schnitzer, 1983; Shin et al., 1999) and a number of other allied techniques (Grasset and Amblès, 1998; Avena et al., 1999) have been employed to investigate the detailed structure of humic substances. Fig. 2.4 shows a model structure of humic acid proposed by Stevenson, 1982.

Fig. 2.4: Model structure of humic acid proposed by Stevenson (1982)
In the broadest terms, the structures can be described as assemblies of covalently linked aromatic and aliphatic residues carrying carboxyl, phenolic and alkoxy groups cross linked mainly by oxygen and nitrogen groups although sulphate esters, alanine moieties, semiquinone, phosphate ester and hydroquinone groups have been proposed to exist in some humic isolates. With time, it has become more apparent that humic substances are not single molecules but rather association of molecules of microbiological, polyphenolic, lignin and condensed lignin origin. X-ray analysis and viscosity measurements of humic substances have shown them to have an "open" flexible structure perforated by voids of varying dimensions that can trap or fix organic or inorganic compounds like carbohydrates and proteins, besides others, that fit into the voids provided that the charges are complimentary (Schultze, 1978).

Another model structure for humic acids proposed by Kickuth (1972) is shown in Fig. 2.5.

Fig. 2.5: Model structure of humic acids proposed by Kickuth (1972)
Ghosal, S. (2003) has also proposed a polymeric structure for fulvic acid present in shilajit, consisting of repeat units of 3,8-oxygenated dibenzo-α-pyrones (Fig. 2.6).

![Fig. 2.6: Model Structure of Fulvic Acids of Shilajit](image)

A cyclic structure for fulvic acid has also been proposed by Narayana, D.B.A. (2001) (Fig. 2.7). It is hypothesized that drug molecules can get complexed with the various reactive groups of the fulvic acid and get entrapped into the hydrophobic voids provided by it.

![Fig. 2.7: Cyclic Structure of Fulvic Acids of Shilajit](image)
2.2.6 Colloidal characteristics of Humic substances

The colloidal state represents a phase intermediate between true solutions, where species are of ionic or molecular dimension, and suspended particulates, where species are sufficiently large to settle under the force of gravity. The colloidal range is considered to extend from 0.001 to 1 \( \mu \text{m} \). Chemical and physical reactions are generally enhanced in colloidal systems due to the large surface area of colloidal particles. The ranges of molecular size for the majority of humic and fulvic acids place them in the colloidal range when in aqueous solution (Gaffney et al., 1996).

Humic colloidal material is thought to consist of coiled, long chained or three-dimensional cross-linked macromolecules with electrical charges variously distributed on the particle. The presence of charged sites arising from ionized groups, result in mutual repulsion and causes maximum expansion of the macromolecule (Stevenson, 1982). The factors most important in controlling the molecular conformation of humic materials are concentration of the humic materials, pH and ionic strength of the system. At high sample concentration (>3.5 g/L), low pH (<3.5) and high electrolyte concentration (>0.05 M), the humic materials are rigid uncharged colloidal particles. At low concentrations, high pH and low electrolyte concentrations, humic and fulvic acids exist as flexible linear polyelectrolytes.

2.2.7 Surfactant properties of Humic substances

As mentioned earlier humic acids are predominantly hydrophilic (except at lower acidic pH) but they also contain a substantial concentration of aromatic rings, fatty acid esters, aliphatic hydrocarbon and other hydrophobic substances, which together with the hydrophilic groups account for the surface activity of these materials. The hydrophilic oxygen containing functional groups (COOH, C=O, OH) are thought to play a significant role in lowering the surface tension of water and in so increasing aqueous wettability of hydrophobic materials. Tschapek and Wasowski (1976) were amongst the first to demonstrate the surfactant properties of humic substances.
It has also been recognized that presence of even a small amount of humic acid in an aqueous solution can significantly enhance the water solubility of a hydrophobic organic compound (Gaffney et. al., 1996). This solubilization in solutions is often attributed to the presence of micelles. The structure of humic acids is such that it allows them to function as surfactants with the ability to bind both hydrophilic and hydrophobic materials. This function in combination with their colloidal properties makes humic acids effective agents in transporting both organic and inorganic materials in the environment.

Humic acids being highly aromatic as compared to fulvic acids become insoluble at low pH values when the carboxylate groups become protonated that may also lead to formation of intramolecular "pseudomicelles", as opposed to intermolecular micelles, due to coiling and contraction of humic acid chains (Wandruszka et. al., 1998). Pseudomicelles are submicroscopic aggregates of humic acid molecules that are analogous to the micelles formed by soaps and other surface active compounds. As such, they have nonpolar cores, comparable to miniature oil drops, and polar surfaces that make them water compatible. Their structure in humic acid is less defined than it is in synthetic detergents, due to variations in molecular size and composition of humic acid. The effects, however, are similar. It is found that humic acid pseudomicelles can form by both intra- and intermolecular processes. In the intramolecular case, humic acid polymers coil and fold to create molecular domains that may be linked to knots in a string.

2.2.8 Complexation properties of Humic substances

Since many of the functional groups identified as components of humic substances contain suitable atoms, particularly nitrogen and oxygen, these are capable of acting as ligands (Livens, 1991). Given the variety of functional groups in humic molecules and the various ways in which they can interact with metals, a near infinite number of metal-humic complexes are possible in principle. It has long been recognized that natural organic matter is involved in the geochemical transport and concentration process of metal ions in the environment.
Extensive studies have shown that not much of the humic substances in soil are in free state but are mainly bound to colloidal clay (Nayak et al., 1990). Humic acids combine in many ways with different fractions of naturally occurring matter and minerals:

1. As salts of low - molecular weight organic acids (acetate, oxalate, lactate and others).
2. As salts of humic substances with alkaline cations – Humates
3. As chelates with metal ions.
4. As substances held on clay mineral surfaces:

The interaction of organic substances with clay has a multitude of consequences that are reflected in the physical, chemical and biological properties of the soil matrix.

Several mechanisms are involved in the interaction of humic substances by clay minerals, the main ones being:

1. Van der Waals' forces
2. Bonding by cation bridging
3. Hydrogen bonding
4. Adsorption by association with hydrous oxides
5. Adsorption on interlamellar spaces of clay minerals

Humic substances have also been implicated as the fundamental factor controlling the fate and transport of hydrophobic organic contaminants in soil and the subsurface environment (Bhandari et al., 1996). The solubilization in water by humic substances of organic compounds, which are otherwise water insoluble is a matter of considerable interest. Wershaw et al (1969) have shown that solubility of DDT in aqueous sodium humate solution is at least 20 times greater than that in water.

Chien (1997) examined the "membrane micelle" model of humic substances in which micelle like aggregates with hydrophobic interiors exist, into which non-polar organic compound partition. Atrazine, labeled with trifluoromethane group on the ethylamino side chain, was solubilized in aqueous solutions of humic acid and F-19 NMR relaxation of atrazine induced by paramagnetic probes to humic acid solution was
observed. The results confirmed that atrazine solubilized by humic acid occupies a domain accessible only to neutral hydrophobic molecules and confirmed the existence of hydrophobic domains. It was suggested that atrazine resides in the interiors of the humic acid micelles.

2.2.9 Role of Humic Substances in Environment

Humic substances are surface active, by virtue of the hydrophobic and hydrophilic moieties coexisting in a single molecule, and have a tendency to form micelles in solutions at and above their critical micelle concentration. This gives them the ability to play important role in the solubilization and transport of hydrophobic chemical entities in nature. The presence of humic material can also promote the solubilization of non-polar hydrophobic compounds. Such ability can affect not only the mobility of the bound molecule but also the rate of chemical degradation, photolysis, volatilization and biological uptake (Gaffney et. al., 1996).

In the environment humic acids can bind metal ions from dissolved rocks and minerals, can interact with other soil components such as clay particles and can bind pollutants and biocides used for agricultural purposes. Humic acids play a major role in the geocycling of metal ions and in the transport of pollutants and biocides in the environment. It has also been frequently suggested that humic substances play a major role in controlling the behaviour and mobility of metals in the environment by forming complexes with them (Livens, 1991).

Humic substances specially humic and fulvic acids present in soil are known to increase nutrient uptake, drought tolerance and seed germination in plants. They increase the availability of nutrients that are already in the soil and naturally aerate the soil from inside. Researchers have also recognized their ability to complex metals and radionuclides and to interact with free radicals.

2.2.10 Role of Humic Substances in Shilajit

Humic acids and fulvic acids present in shilajit have an “open” flexible structure perforated by voids of varying dimensions. These voids are capable of entrapping bioactive molecules like the low molecular weight dibenzo-α-pyrones present in
Such entrapment affords a high degree of protection and stability to the entrapped molecules in their natural habitat. It has also been suggested that humic and fulvic acids act as carrier molecules for delivering these bioactive molecules at their intended site of action.

2.3 BIOAVAILABILITY

Bioavailability is a pharmacokinetic term that describes the rate and extent to which the active drug ingredient is absorbed from a drug product and becomes available at the site of drug action. Since pharmacologic response is generally related to the concentration of drug at the receptor site, the availability of a drug from a dosage form is a critical element of a drug product's clinical efficacy. However, drug concentrations usually cannot be readily measured directly at the site of action. Therefore, most bioavailability studies involve the determination of drug concentration in the blood or urine. This is based on the premise that the drug at the site of action is in equilibrium with drug in the blood. It is therefore possible to obtain an indirect measure of drug response by monitoring drug levels in the blood or urine. Thus, bioavailability is concerned with how quickly and how much of a drug appears in the blood after a specific dose is administered. The bioavailability of a drug product often determines the therapeutic efficacy of that product since it affects the onset, intensity and duration of therapeutic response of the drug. In most cases one is concerned with the extent of absorption of drug, (that is, the fraction of the dose that actually reaches the bloodstream) since this represents the "effective dose" of a drug. This is generally less than the amount of drug actually administered in the dosage form. In some cases, notably those where acute conditions are being treated, one is also concerned with the rate of absorption of a drug, since rapid onset of pharmacologic action is desired. Conversely, there are instances where a slower rate of absorption is desired, either to avoid adverse effects or to produce a prolonged duration of action.

2.3.1 Biopharmaceutic Considerations in Drug Product Design

Drugs are generally given to a patient as a manufactured drug product (finished dosage form) that includes the active drug and selected ingredients (excipients) that
make up the dosage form. Common pharmaceutical dosage forms include liquids, tablets, capsules, injections, suppositories, transdermal systems, and topical drug products. The formulation and manufacture of a drug product requires a thorough understanding of the biopharmaceutics.

2.3.2 Biopharmaceutics of Oral Route

The successful transposition of a drug from an oral dosage form into the general circulation can be described as four step process:

1. Disintegration of the drug product
2. Dissolution of the drug in the fluids at the absorption site
3. Movement of the dissolved drug through the membranes of the GI tract
4. Movement of the drug away from the site of absorption into the general circulation.

Any factor that affects any of these steps can alter the drug's bioavailability and thereby its therapeutic effect.

2.3.3 Factors affecting Bioavailability

2.3.3.1 Drug Substance Physicochemical Properties

1. Particle size
2. Crystalline or amorphous form
3. Salt form
4. Hydration
5. Lipid/Water solubility
6. pH and pKa

2.3.3.2 Formulation Factors

1. Pharmaceutical dosage form
   - Solutions
   - Suspension
   - Capsules
   - Tablets
   - Coated tablets
   - Controlled release formulations
2. Pharmaceutical Ingredients
   - Fillers
   - Binders
   - Coatings
   - Disintegrating agents
   - Lubricants
   - Suspending agents
   - Surface active agents
   - Stabilizing agents

3. Disintegration rate (tablets)

4. Dissolution time of drug in dosage form

5. Product age and storage condition

2.3.3.3 Physiologic Factors and Patient Characteristics

1. Gastric emptying time
2. Intestinal transit time
3. Gastrointestinal abnormality or pathologic Condition
4. Gastric contents
   - Other drugs
   - Food
   - Fluids
5. Gastrointestinal pH
6. Drug metabolism (Gut and during first passage through liver)
2.4 BIOPHARMACEUTICS CLASSIFICATION SYSTEM

The Biopharmaceutics Classification System (BCS) is a scientific framework for classifying drug substances based on their aqueous solubility and intestinal permeability into one of the following four possible classes (Amidon et al., 1995):

- **Class I**: High Solubility - High Permeability
- **Class II**: Low Solubility - High Permeability
- **Class III**: High Solubility - Low Permeability
- **Class IV**: Low Solubility - Low Permeability

When combined with the dissolution of the drug product, the BCS takes into account three major factors that govern the rate and extent of drug absorption from immediate release solid oral dosage forms:

1. Dissolution,
2. Solubility
3. Intestinal permeability.

Recently, Dressman and Reppas (2000) have described the characteristics of the above four classes of drugs as per the BCS. According to them, being highly soluble and permeable, Class I drugs should be more than 90% absorbed. Drug examples in this class include diltiazem, captopril, propranolol, labetalol, enalapril, phenylalanine, caffeine, etc. Class II drugs are those with solubility too low to be consistent with complete absorption, even though they are highly membrane permeable. A few instances of Class II drugs are flurbiprofen, diclofenac, naproxen, piroxicam, ketoprofen, phenytoin, verapamil, etc. Class III is the mirror image of Class II. Hence, Class III drugs, despite having good aqueous solubility, are unable to permeate through the gut wall quickly and completely. The examples of Class III drugs include famotidine, nadolol, atenolol, cimetidine, ranitidine, etc. Class IV compounds have neither sufficient solubility nor permeability for absorption to be complete, hence are problematic for product development pharmacist (Amidon et al., 1995).
2.4.1 Classification of Drugs as per BCS

Lindenberg et al., 2004 has classified a number of drugs appearing in the WHO essential medicines into one of the four BCS classes based on available literature as follows:

**BCS Class I Drugs**
- Amiloride hydrochloride
- Chloroquin phosphate
- Cyclophosphamide
- Diazepam
- Digoxin
- Doxycycline
- Fluconazole
- Levonogestrel hormone
- Metronidazole
- Phenobarbital
- Phenoxyethyl penicillin potassium
- Prednisolone
- Primaquine
- Propranolol
- Pyrazinamide
- Riboflavin
- Salbutamol
- Stavudine
- Theophylline
- Zidovudine

**BCS Class II Drugs**
- Carbamazepine
- Dapsone
- Griseofulvin
• Ibuprofen
• Nifedipine
• Nitrofurantoin
• Phenytion
• Sulphamethoxazole
• Trimethoprim
• Valproic acid

BCS Class III Drugs
• Abacavir
• Acetylsalicylic acid
• Acyclovir
• Allopurinol
• Ascorbic Acid
• Atenolol
• Captopril
• Chloramphenicol
• Cimetidine
• Sodium cloxacillin
• Codeine phosphate
• Colchicine
• Ergotamine tartrate
• Hydralazine hydrochloride
• Hydrochlorothiazide
• Levothyroxine sodium
• Metformin hydrochloride
• Methyldopa
• Paracetamol
• Penicillamine
• Promethazine hydrochloride
• Propylthiouracil
2.4.2 Solubility Determination for BCS

A drug substance is considered highly soluble when the highest dose strength is soluble in 250 ml or less of aqueous media over the pH range of 1-7.5 at 37 ±1°C.

The number of pH conditions for a solubility determination can be based on the ionisation characteristics of the test substance. For example, when the pKa of a drug is in the range of 3 - 5, solubility should be determined at pH = pKa+1, pH = pKa-1, pH = 1 and pH = 7.5. A minimum of three replicate determinations of solubility in each pH condition is recommended.

Concentration of the drug substance in selected buffers (or pH conditions) should be determined using a validated stability indicating assay that can distinguish the drug substance from its degradation products. The solubility class should be determined by calculating the volume of an aqueous medium sufficient to dissolve the highest dose strength in the pH range of 1 - 7.5. A drug substance should be classified as highly soluble when the highest dose strength is soluble in ≤ 250ml of aqueous media over pH range of 1-7.5.

2.4.3 Permeability Determination for BCS

In the absence of evidence suggesting instability in the GI tract, drug absorption is considered to be highly permeable when the extent of absorption in humans is
determined to be 90% or more of an administered dose based on a mass balance
determination or in comparison to an intravenous.

For the purpose of BCS, the permeability class of a drug substance can be
determined by one of the following methods:

2.4.3.1 Pharmacokinetic Studies in Human

2.4.3.1.1 Mass Balance Studies
Pharmacokinetic mass balance studies using unlabeled, stable isotopes or a
radiolabeled drug substance can be used to document the extent of absorption of a
derug.

2.4.3.1.2 Absolute Bioavailability Studies
Oral bioavailability determination using intravenous administration as a reference
can be used.

2.4.3.2 Intestinal Permeability Methods
Any of the following methods can be used to determine the permeability of a drug
substance from the GI tract:

1. In vivo intestinal perfusion studies in human
2. In vivo or in situ intestinal perfusion studies using suitable animal models.
3. In vitro permeation studies using excised human or animal intestinal tissues.
4. In vitro permeation studies across a monolayer of cultured epithelial cells.

2.4.4 Dissolution determination for BCS
For the purpose of BCS, dissolution testing should be carried out in USP apparatus
I at 100 rpm or Apparatus II at 50 rpm using 900 ml of the following solution
media:

1. 0.1N HCl or Simulated Gastric Fluid USP without enzymes.
3. A pH 6.8 buffer or Simulated Intestinal Fluid USP without enzymes.
For capsules and tablets with gelatin coating, Simulated Gastric and Intestinal Fluids USP (with enzymes) can be used. A minimum of 12 dosage units of a drug product should be evaluated. Samples should be collected at a sufficient number of intervals to characterize the dissolution profile of the drug product.

When comparing the test and reference products, dissolution profiles should be compared using a similarity factor \( f_2 \). Two dissolution profiles are considered similar when the \( f_2 \) value is 0.50. To allow the use of mean data, the coefficient of variation should not be more than 20% at the earlier time points (e.g. 10 minutes), and should not be more than 10% at other time points. Note that when both test and reference products dissolve 85% or more of the label amount in all the three dissolution media recommended above, the profile comparison with an \( f_2 \) test is unnecessary.

### 2.5 BIOAVAILABILITY ENHANCEMENT

Generally drugs showing poor bioavailability by the per-oral route are the ones showing one of the following characteristics:

1. Poor aqueous solubility and/or slow dissolution rate in the biologic fluids.
2. Poor stability of the dissolved drug at the physiologic pH.
3. Inadequate partition coefficient and thus poor permeation through the biomembrane.
4. Extensive presystemic metabolism

Any of the approaches which can alter these characteristics should help in improving the bioavailability of drugs. Generally, three major approaches are followed for overcoming the bioavailability problems.

#### 2.5.1 The Pharmaceutical approach

It involves the modification of the formulation, manufacturing process or the physicochemical properties of the drug without changing the chemical structure. The dissolution rate, solubility and/or permeability is generally targeted by this method.
2.5.2 The Pharmacokinetic approach
It involves the modification of chemical structure to alter the pharmacokinetic behaviour of the drug.

2.5.3 The Biologic approach
It involves the change in the route of administration of the drug.

2.6 PHARMACEUTICAL APPROACHES FOR BIOAVAILABILITY ENHANCEMENT

2.6.1 Solubility/Dissolution Enhancement
1. Micronization
2. Use of surfactants
3. Use of salt forms
4. Modifications of the crystal habit
5. Alteration of pH of the drug microenvironment
6. Polymorphs and pseudopolymorphs
7. Complexation/solubilization
8. Eutectic mixtures
9. Solid dispersions and solid solutions
10. Selective adsorption on insoluble carriers

2.6.2 Permeability Enhancement
1. Synthetic surfactants
   • Sodium lauryl sulphate
   • Polysorbate 20 and 80
   • PEG-8 laurate
   • Sorbitan laurate
   • Glycerol monolaurate
   • Saponins
2. Bile salts
   • Sodium cholate
   • Sodium deoxycholate
3. Fatty acids and derivatives
   - Oleic acid
   - Palmitic acid
   - Lauric acid

4. Chelators
   - Di sodium EDTA
   - Citric acid
   - Salicylates

5. Complexes
   - Non-cyclodextrin complexes
   - Cyclodextrin complexes

6. Other agents
   - Piperine
   - Polycarbophil
   - Chitosan

2.7 THE COMPLEXATION PHENOMENON

A "complex" is a species formed by interaction of two or more molecules or ions. The following definitions are relevant in this context.

A "Substrate" $S$ is the interactant whose physical or chemical properties are observed experimentally.

A "Ligand" $L$ is the second interactant whose concentration may be varied independently in an experimental study.

A complex is a species with a definite substrate to ligand stoichiometry, which can be formed in an equilibrium process, in solution and also may exist in solid state.

$$mS + nL \leftrightarrow SmLn$$

Substrate Ligand Complex

*Types of complexes:* The definition of a complex lead to a classification into two groups based on chemical bonding.
**Co-ordination complexes:** These are formed by coordinate bonds in which transfer of a pair of electrons takes place e.g. metal ion coordination complexes between metal ions and bases.

**Molecular Complexes:** These are formed by non-covalent interactions between the substrate and the ligand such as electrostatic induction and dispersion interactions.

The molecular complexes may be classified according to the:

1. Type of bonding or interaction e.g. charge transfer and hydrogen binding complexes.
2. Type of structure of interaction: enzyme substrate complex, drug-receptor complex.
3. Type of structure of complex: Self-associated aggregate, micelles, inclusion complexes and clathrates.

**Self-association:** Complexation of a molecule with others of its own species e.g. benzene forms dimers.

**Micelle:** A special form of self aggregated complex in which interactant is a surfactant.

**Clathrate:** Host molecule forms a crystal lattice containing spaces into which guest molecules can fit.

### 2.6.1 The Inclusion Complex

An inclusion complex is formed when a macrocyclic compound possessing an intramolecular cavity of molecular dimension, interacts with a small molecule that can enter the cavity (Loftsson & Brewster, 1996). The macrocyclic molecule is called the “host” and the small included molecule is called the “guest”. Synthetic macrocyclic hosts are exemplified by crown ethers while natural macrocyclic hosts are exemplified by cyclodextrins (cycloamyloses or Schardinger dextrins) which are oligosaccharides, formed by action of certain enzymes on starch. They consist of α-D glucopyranose units joined with (α-1,4) glycosidic (ether) linkages and are water soluble polymers (Baboota, et. al., 2001). The binding of “guest” molecules within
the "host" Cyclodextrin is not fixed or permanent but rather is submitted to a dynamic equilibrium thereby affording an ease of assembly and disassembly. Binding strength depends on how well the "host-guest" complex fits together and on specific local interactions between surface atoms (Baboota, et. al, 2003).

Complexes can be formed either in solution or in the solid state and while water is typically the solvent of choice, inclusion complexation can be accomplished in co-solvent systems and with some non-aqueous solvents. Since most of the work reported in the literature regarding inclusion complexation relates to cyclodextrins, the following information is more applicable to complexation of drugs with cyclodextrins.

2.6.2 Factors affecting Complexation

A number of factors can affect complexation of drugs by cyclodextrins:

2.6.2.1 Molecular dimension of the drug

Cyclodextrins can form complexes with drugs which have a size that is compatible with the dimensions of the cyclodextrin cavity. Complexation of larger molecules is also possible but in such cases only certain groups or side chains of the drug penetrate the cyclodextrin cavity. Derivatization of the hydroxyl groups of natural cyclodextrins for preparation of water soluble derivative also imparts certain steric requirements on the part of the drug molecule to be able to form complexes. The geometry and structure of the cyclodextrin is given below:
2.6.2.2 Charge and Charge Density

The charge and charge density on the cyclodextrin molecules exerts a profound impact on the complexing ability. Ionic cyclodextrins are capable of forming complexes with neutral hydrophobic drugs, if the ionic charge is not directly attached to the carbohydrate backbone of the cyclodextrin. Changing the ionization state of a drug may affect its binding to the cyclodextrin.

2.6.2.3 Temperature

Inclusion complexation is an equilibrium process and the strength of association is affected by the temperature of the system. The solubility of a drug in the cyclodextrin solution may increase with an increase in temperature even though the binding constant is decreasing, because the increase in temperature improves the solubility of the free drug. (Hoshino et al., 1993; Menard et al., 1990).

2.6.2.4 Solvents

Organic solvents typically tend to reduce the complexation of the drug in the cyclodextrin by competing for the hydrophobic cavity (Pitha et al., 1992).

2.6.2.5 Cosolubilizers

Water soluble polymers, in low concentration, have recently been shown to increase the complexing abilities of cyclodextrins and enhance the availability of drugs in aqueous cyclodextrin solution (Loftsson et al., 1998). Enhancement of complexation efficacy and increased drug availability in cyclodextrin solutions are usually obtained by heating aqueous solutions containing a water soluble polymer, cyclodextrin and drug in an autoclave (e.g., 120 to 140°C for 20 to 40 minutes) or by heating in a sonicator (e.g., 70°C for one hour). Simply adding the polymers to the solutions without heating does not enhance the complexation or the drug availability. In aqueous solutions the polymers reduce the mobility of the cyclodextrin molecules and enhance the solubility of the complexes formed.

2.6.3 Methods for Evaluating Inclusion Complexation

The most common and widely used method to evaluate the ability of the cyclodextrin to complex a drug is the phase solubility studies. Higuchi and Connors
(Higuchi et al., 1965) have classified the solubility behaviour seen during complex formation (in following graph) as A-type (a soluble inclusion complex is formed) or B-type (an inclusion compound of finite solubility is formed).

![Theoretical Phase Solubility diagram]

Theoretical Phase Solubility diagram

The equilibrium binding or association constant (k) for the 1:1 complex can be determined from the slope of linear portion using the following relationship, where $S_o$ is the intrinsic solubility of the drug under the conditions studied.

$$K_{ab} = \frac{\text{Slope}}{S_o \left(1 - \text{slope}\right)}$$

There are a number of other methods available to determine these association or stability constants like spectroscopy [UV(Qi et al., 1994), fluorimetry (Duran-Meras et al., 1994), NMR (Djedaini et al., 1991), potentiometry (Valsami et al., 1992), microcalorimetry (Tong et al., 1991), freezing-point depression studies (Suzuki et al., 1993), HPLC (Thuad et al., 1990) and TLC techniques (Csabai et al., 1993)].

2.6.4 Methods of preparing cyclodextrin inclusion complexes

Several methods have been described in literature for preparing cyclodextrin complexes of drugs. By only trial and error one can find a method, which will give the best results for a given drug. These methods are described below:
2.6.4.1 Grinding (Szejti, 1988; Arias et al., 1997)
Cyclodextrin inclusion complexes can be prepared by simply grinding the guest with cyclodextrin. This is a very slow process for making inclusion complex and degree of complexation achieved is also very low.

2.6.4.2 Solid dispersion/Co-evaporated dispersion (Kumar, et. al., 2003)
The drug is dissolved in ethanol and cyclodextrin is either dissolved in alcoholic solution or dissolved separately in water or other suitable medium. The cyclodextrin solution is then added to the drug solution or vice-versa and stirred to attain equilibrium. The resulting solution is evaporated to dryness preferably under vacuum.

2.6.4.3 Neutralization Method (Martin & Udupa, 1995)
Martin and Udupa (1995) reported this method for various fluoroquinolones. In this method equimolar concentrations of drug and cyclodextrin are separately dissolved in 0.1 N NaOH, mixed and stirred for about half an hour, pH is recorded and 0.1 N HCl is added dropwise with stirring until pH reaches 7.5, where upon complex precipitates. The residue is filtered and washed until free from Cl-. It is dried at 25°C for 24 hours and stored in a desiccator.

2.6.4.4 Kneading (Otero-Espinar et al., 1992; Palmieri et al., 1997)
In this method cyclodextrin is not dissolved but kneaded like a paste, with small amount of water to which the guest component has been added. Guest component can be added without a solvent or in small amount of ethanol in which guest has been suspended. Several hours of grinding of paste in mortar results in evaporation of solvent and formation of powder like complex.

2.6.4.5 Precipitation (Sanghavi et al., 1995)
The guests which show Bs type phase solubility curve are suitable for this method of complex formation. In this method the drug (guest) and cyclodextrin are dispersed in water and the solution is heated to obtain concentrated, viscous and translucent liquid. The solution is left to give a precipitate of inclusion complex. Precipitate obtained is separated and dried to get solid inclusion complex.
2.6.4.6 Spray Drying (Bietti et al., 1992; Piel et al., 1997)
In this method first a monophasic solution of drug and cyclodextrin is prepared using a suitable solvent (generally hydroalcoholic solutions are used). The solution is then stirred to attain equilibrium following which the solvent is removed by spray drying.

2.6.4.7 Freeze-drying (Becirevic-Lacan et al., 1996; Singh & Agarwal, 2002)
Freeze drying method is similar to spray drying method except that in this method, after attaining the equilibrium, the solvent is removed by freeze-drying.

2.6.4.8 Preparation in Suspension (Szejtli, 1988)
Cyclodextrin need not be dissolved. Simply stirring the guest in an aqueous suspension of cyclodextrin can achieve complexation within 2-24 hrs at ambient temperature. This is a recommended method for industrial application.

2.6.4.9 Melting (Szejtli, 1988)
Complexes can be prepared by simply melting the guest, mixed with finely powdered cyclodextrin. In such cases there has to be large excess of guest, and after cooling this excess is removed by careful washing with a weak complex forming solvent or by vacuum sublimation. The latter is preferred method and is used to sublimate guests such as menthol.

2.7 CHARACTERIZATION OF INCLUSION COMPLEXES
The formation of inclusion complex can be studied and characterized in two ways. (Szejtli, 1988)

2.7.1 Characterization in Solid State
2.7.1.1 Differential Scanning Calorimetry (DSC)
DSC is the measurement of rate of heat evolved or absorbed by the sample, during a temperature programme. The DSC curve of β-cyclodextrin generally shows an endotherm near 100°C which signifies removal of water. The DSC curve of the guest molecule shows a sharp intense peak (endotherm) at its melting temperature (m.p.) and when it starts decomposing. In DSC curve of cyclodextrin-guest inclusion complex, these peaks are either diminished or absent. Partial complex
formation may be shown by varying patterns e.g. small exotherm adjacent to the melting endotherm of guest molecule. The DSC curve of simple mixture would resemble, the combination of curves of pure substances i.e. guest and cyclodextrin.

2.7.1.2 Powder X-ray Diffraction (XRD)
This is an important technique for determination of three dimensional structure of molecule and distinguishing between amorphous and crystalline forms. The diffraction pattern is characteristic of a substance. The crystalline substance has sharp intense peaks in its powder diffraction pattern whereas amorphous substance shows only undefined, broad, diffused peaks of low intensities. The pure drug in its free form is represented by sharp, intense peaks (crystalline nature) whereas complex has an amorphous nature i.e. broad, undefined peaks with low intensities.

2.7.1.3 Fourier Transform Infra-red Spectroscopy (FTIR)
It is another useful technique to verify complex formation. The guest molecules within the cyclodextrin cavity show shifts in its peaks or show peaks of less intensity. Basically, peaks, which lie in the fingerprint region, and peaks due to C-O or O-H stretching are affected (shifted or intensity is changed). FTIR technique is known to have superior sensitivity and resolution, absolute wavelength accuracy and higher precision of measurement than conventional IR technique.

2.7.1.4 Scanning Electron Microscopy (SEM)
SEM is done to observe crystal structure of the samples. SEM studies help to observe changes that occur in crystal structure during or after the preparation procedure.

2.7.2 Characterization in Solution
2.7.2.1 Solubility Studies and dissolution tests
An increase in solubility of a potential guest with increasing cyclodextrin concentration indicates complex formation in solution. When the assumed complex is dispersed in water, a very rapid dissolution and in most cases an enhanced solubility is observed.
2.7.2.2 TLC (Thin Layer Chromatography)
TLC may also be useful for verification of complex formation, since the Rf values are altered considerably. Rf values are usually diminished provided the complex is sufficiently stable in solvent mixture used.

2.7.2.3 Proton Nuclear Magnetic Resonance (H-NMR)
It is useful not only for verification of complex formation but also to guess how the guest is geometrically aligned in the cyclodextrin cavity. The inclusion of a guest molecule into the cyclodextrin cavity clearly induces some changes in the chemical shift values. The chemical shift values are also indicative of the interactions, if any between protons of cyclodextrin and guest. (Piel et al., 1997; Moyano et al., 1997c).

2.7.2.4 UV Studies
A constant amount of host is added to increasing concentration of cyclodextrin. UV spectra are taken and absorbance is recorded at $\lambda_{\text{max}}$. The UV spectra shifts are attributed to a partial shielding of the excitable drug electrons into the β-cyclodextrin cavity. For example a bathochromic shift along with a decrease in absorption on addition of β-cyclodextrin may refer to complex formation. (Moyano et. al., 1997a).