CHAPTER II

LITERATURE REVIEW

1. *Centella asiatica* (Linn.)Urban

Centella asiatica (Linn.)Urban (syn. *Hydrocotyle asiatica* Linn.) belonging to the family Umbelliferae (Apiaceae), is a medicinal plant that has been in use since prehistoric times. It has many common names concluding Gotu kola (U.S.A.), Indian Pennywort (English), Hydrocotyle Asiatique (French), Idrocotyle (Italian), Tsubo-kusa (Japanese), Tungchian or Luei Gong Gen (Chinese), Bua-bok (Thailand) etc. and could be found in many parts of the world such as Southeast Asia, Sri Lanka, China, in the western South Sea Islands, Madagascar, South Africa, in the southeast of U.S.A., Mexico, Venezuela and Columbia, and also in the eastern regions of South America (Brinkhaus, 1992).

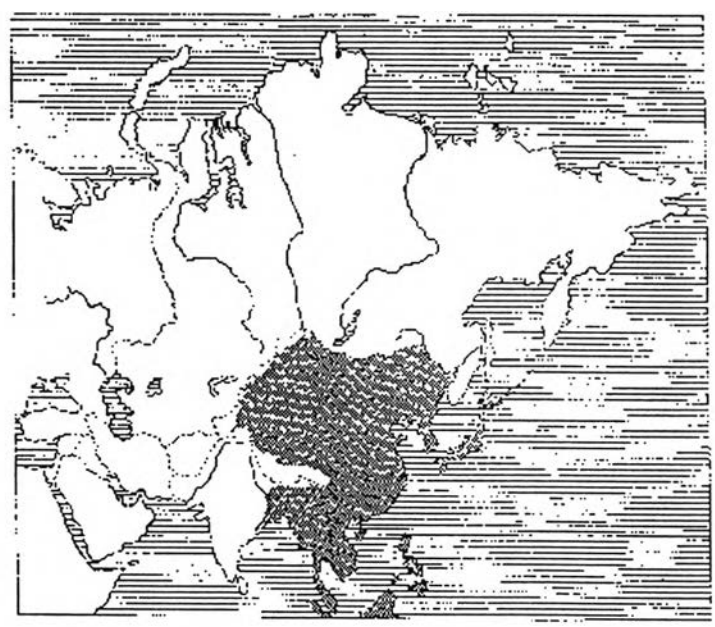


Figure 2 Area of distribution of *Centella asiatica* (Brinkhaus, 1992)

Centella asiatica is a prostrate stoloniferous plant which has long-stalked, green reniform leaves with rounded apices that have smooth texture with palmately netted veins. The stems are creeping in nature, green to reddish green in color, interconnecting one plant to another. The flowers are pinkish to red in color, born in small, rounded

bunches near the surface of the soil. Each flower is partly enclosed in 2 green bracts. The hermaphrodite flowers are minute in size (less than 3 mm), with 5-6 corolla lobes per flower. Each flower bears 5 stamens and 2 styles. The rootstock consists of rhizomes, growing vertically down. They are creamish in color and covered with root hairs. Fruits are small and flattened as shown in Figure 3.

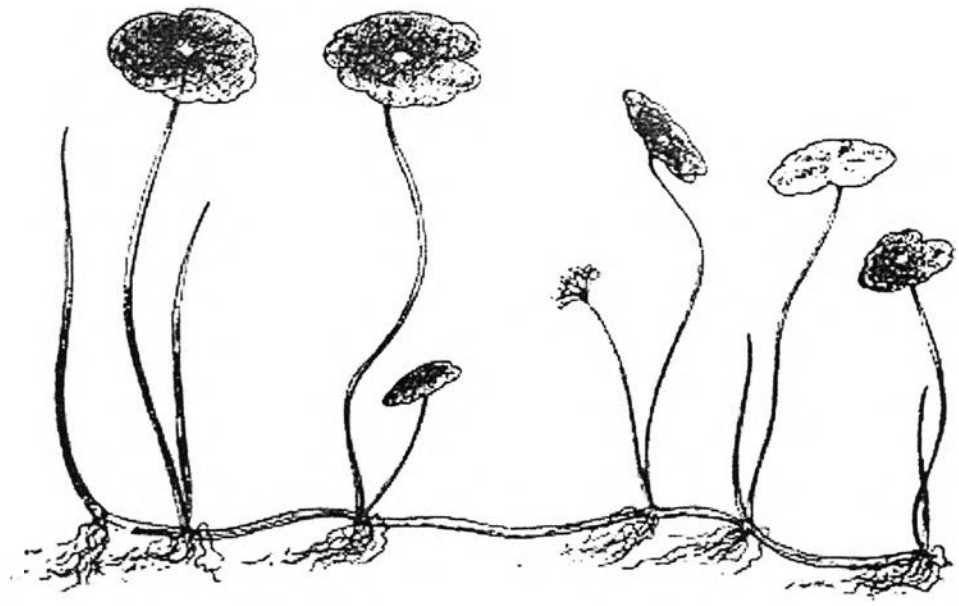


Figure 3: *Centella asiatica* (Linn.) urban (Bryan and Mark,2006)

The claimed efficacies of *Centella asiatica* in Thai traditional recipes as a poultice for accelerate the wound healing (Pramongkit, 1995). Cytotoxicity using Brain shrimp lethality test of *Centella asiatica* (whole plant) found LC_{50} (24 hr) > 1000 µg/ml (Padmaja et al, 2002).

Chemical constituents of *Centella asiatica* depending on origin of plant material, has main groups are essential oil, flavone derivatives, sesquiterpenes, triterpenic steroids, triterpenic acids and triterpenic acid sugar esters (Table 1). The substance of therapeutic interest are saponin-containing triterpene acids and their sugar ester, the literature contains reports on the following extracts: TECA, TTFCA and TTF. The TECA (titrated extract of *Centella asiatica*) and TTFCA (total triterpenoid fraction of *Centella asiatica*)

are combination of asiatic acid, madecassic acid and asiaticoside (30 : 30 : 40) but the centella extract TTF (total triterpenic fraction) comprises asiatic acid and madecassic acid (60%) in a ratio that is not clearly defined and asiaticoside (40%)(Brinkhaus, 2000).

Table 1 Main constituents of *Centella asiatica* (Brinkhaus, 2000)

Main Groups	Constituents
Essential oil	Terpene acetate
(0.1% of the plant)	Germacrene
	Caryophyllene
	p-Cymol
	Pinene
Flavone derivatives	Quercetin glycoside
	Kaempferol,glycoside and in free
	form Astragalin
Sesquiterpenes	Caryophyllene
	Elemene and bicycloelemene
	Trans-farnesene
	Ermacrene D
Triterpenic steroids	Stigmasterol
	Sitosterol
Triterpenic acids	Asiatic acid
	6-hydroxy asiatic acid
	Madecassic acid
	Madasiatic acid
	Betulinic acid
	Thankunic acid
	Isothankunic acid
Triterpenic acid sugar esters	Asiaticoside
	Asiaticoside A
	Asiaticoside B
	Braminoside
	Brahmoside
	Brahminoside
	Thankuniside
	Isothankuniside

2. Asiaticoside

Asiaticoside was first found in *Centella asiatica*, a plant used in India and Madagascar for the treatment of leprosy and in Vietnam for liver diseases (Sung,1992). Asiaticoside was triterpene glycoside which a major compound isolated form the whole plant of *Centella asiatica*. Properties of this compound are as follow:

Chemical name : 2α, 3β, 23α-trihydroxy-urs-12-en-28-oate O-α-L-rhamno-Pyranosyl-(1→ 4) –O- β-D-glucopyranosyl-(1→ 6)-O- β-D-glucopyranose

Molecular formula : $C_{48}H_{78}O_{19}$

Molecular weight : 958

Molecular structure : Figure 4

Melting point : 230 – 232 °C (decomposition)

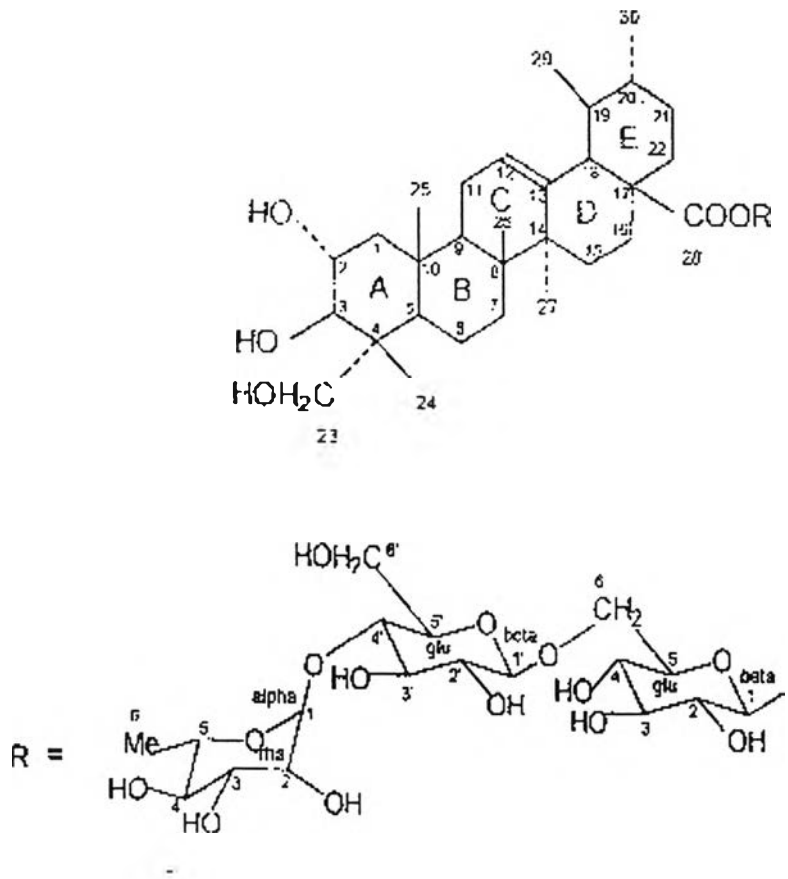


Figure 4 Structure formula of asiaticoside (Arunya, 1997)

In experiments, an in vivo transformation of asiaticoside into asiatic acid was established. It was demonstrated that the maximum plasma concentration of asiatic acid increased significantly with increasing dose administrated, while the time point of maximum plasma concentration and the elimination half-life did not significantly change with increasing dose (Brinkhaus, 2000).Some data suggest that asiatic acid is the only component responsibility for collagen synthesis stimulation and the therapeutic effects of asiaticoside may be mediated through conversion to asiatic acid (Shim,1996; Rush, Nurray and Graham, 1993). For in vitro study. Alkaline hydrolysis of asiaticoside in MeOH with 5% KOH for 3 hr. at 80°C give asiatic acid and methyl asiatate (Sung, 1992).

The therapeutic mechanism of asiaticoside were studied by various workers. It was suggested that the possible mechanism of the effect of asiaticoside on hypertrophic scars is related to its inhibitory action on fibroblast proliferation and collagen synthesis (Qi, Xie and Li, 2000) but Bonte F et.al(1995) found that asiaticoside was increased collagen type I which plays an important role in wound healing and it was increased in tensile strength and better epithelisation (Shukla et al, 1999). In addition, Asiaticoside may be effective therapeutic agents for septic shock and other inflammatory diseases by inhibit COX-2 and IL-1α(Sang-Sup, Ok-Nam and Jin-Ho, 2003).

3. Pharmaceutical Preformulation

Pharmaceutical preformulation had its birth in the very early sixties. Prior to this time, many assays that were used to assess stability (e.g., those in the USP, the United States Pharmacopoeia) simply indicated the amount of drug that originally was incorporated into the dosage form. However, stability-indicating assays method were being introduced into pharmaceutical undertaking, and more and more instances would surface where a formulator had used his skill to make a good tablet of a new compound, but half a year later that it was unstable. Then, the study of an ingredient in formulation was initiated, and that was the beginning of the branch of the pharmaceutical sciences which is known as preformulation (Cartensen, 1998).

Pharmaceutical preformulation involves numerous investigations on a drug substance in order to produce many useful information for subsequent formulation of a physicochemically stable and biopharmaceutically suitable of drug dosage form. The physicochemical and physicomechanical parameters include chemical stability, solubility, dissolution rate, dissociation constant, partition coefficient, crystallinity, polymorphism, solvate and particle size. The compatibility, drug-drug interaction or drug-excipient interaction, is also investigated and the results are useful for dosage drug design. Preliminary pharmacokinetic studies of active ingredient in animal such as absorption, metabolism, protein binding, distribution and elimination may be performed during this process. A through understanding of these properties may ultimately provide a rationale for drug formulation design (Fiese and Hagen,1987; Goto, Kim and Hirakava,1995).

The mainly goal of the pharmaceutical performulator was to investigate and characterize possible modifications in term of physicochemical properties and select a solid form of drug substance which has the best combination of desired properties to proceed to high quality formulation (Singh et al, 1998).

4. Classification of solids

Solid state characterization of a new drug substance is recognized as an essential and very important part of preformulation research (Singh et al, 1998). Solid drug substances display a wide and largely unpredictable of solid state properties. Nevertheless, application of basic physicochemical principles and appropriate analytical metrology can provide a strategy for scientific and regulatory decisions related to solid state behavior in the majority of cases. Understanding of solid state properties are fully resolve solid state issues before the critical stages of drug development. Further benefit of these preformulation studies is the development of solid state specifications, which critically describe the solid form of the drug substance (Byrn et al, 1995).

Solid chemical compound can be divided following its differential habit and crystal chemistry as shown in Figure 5 (Haleblian,1975).

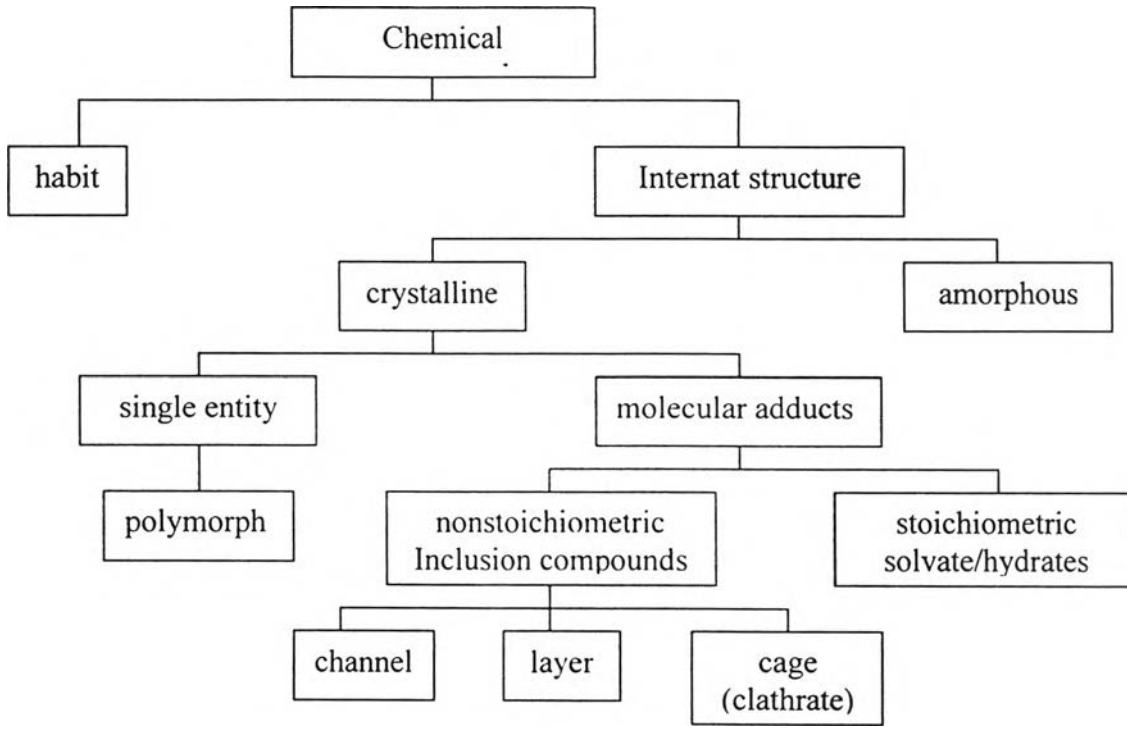


Figure 5: Outline differentiating habit and crystal chemistry of a chemical compound (Haleblian,1975)

Crystal Habits

Crystal habit is the outer appearance of a crystal. The different crystal habit will appear, if the environment of a growing crystal affects its external shape without changing its internal structure, a different habit results. These alterations are caused by the interference with the uniform approach of crystallizing molecules to the different faces of the crystal (Haleblian, 1975).

Crystal growth may be imploded by adjacent crystals growing simultaneously or contacting container walls. As a result, the development of plane faces may be inhibited or, in the case of late crystallizing crystals, an irregularly shaped crystal may occur since it is contained to occupy only the spaces left between substances already crystallized. Such irregularly shaped crystals are described as anhedral or allotriomorphic; those bound by plane faces are termed euhedral or idiomorphic as shown in Figure 6 (Hartshorne and

Stuart, 1975). Anhedral crystals, although irregularly shaped, have a regular arrangement of building units, which may be proved by x-ray diffraction (Haleblian,1975).

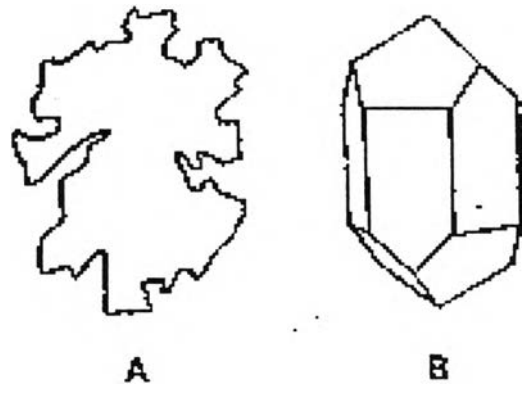


Figure 6: Morphology of anhedral (A) and euhedral (B) (Haleblian,1975)

Classification of euhedral are described in Table 2 and morphology shown in Figure 7

Table 2 Classification of crystal habits (Haleblian,1975)

Descriptor	Description
A. Tabular crystal	moderate development of a pair of parallel faces, at the expense of the others, produces a tubular crystal.
B. Platy crystal	excessive development of the parallel faces as described in the tubular habit produces a platy crystal.
C. Prismatic crystal	crystal has a columnar form.
D. Acicular crystal	Prism is elongated so much as to be needle like.
E. Bladed crystal	Acicular crystal is flattened.

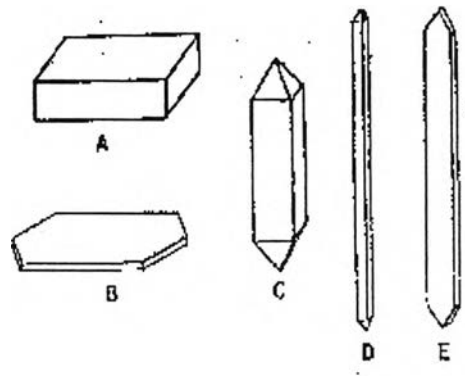


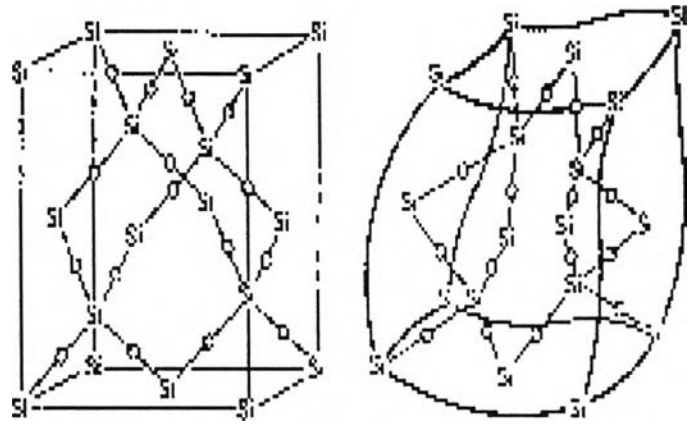
Figure 7: Different habits of crystals (Haleblian,1975)
(A-tabular, B-platy, C-prismatic, D-acicular, E-bladed)

Internal structure of solid

In general, Internal structures are divided into two categories: (a) amorphous, where there is no regularity in the structure, and (b) crystalline, where the atoms are arranged in regular array (Haleblian, 1975).

Amorphous form

Amorphous solids consist of disordered arrangements of molecules and therefore possess no distinguishable crystal lattice nor unit cell and have zero crystallinity. In amorphous forms, the molecules display no long-range order, although the short-range intermolecular forces give rise to the short-range order typical of that between nearest neighbors (Figure 8). The amorphous solid absent the stabilizing lattice energy causes the molar internal energy or molar enthalpy of the amorphous form to exceed that of the crystalline state. Furthermore, the lower stability and greater reactivity of the amorphous form indicates that its molar Gibbs free energy exceeds that of the crystalline state (Grant, 1999).



(a) (b)

Figure 8: Two forms of silica:(a) crystobalite crystalline and(b)glass amorphous (Grant,1999)

The high internal energy and specific volume of the amorphous state relative to the crystalline state can lead to enhanced dissolution and bioavailability, but can also create the possibility that during processing or storage the amorphous state may spontaneously convert back to the crystalline state (Hancock and Zografi,1997).

In considering the importance of the amorphous state in pharmaceutical systems. It may separate in two main situations. In the first, a material may exist intrinsically in the amorphous state or it may be purposefully rendered amorphous and we would like to take advantage of its physical chemical properties. Under these condition we usually want to develop strategies to prevent physical and chemical instability of the amorphous sample. In the second case, we may dealing with a crystalline material that has been inadvertently rendered amorphous during processing (Hancock and Zografi, 1997).

For example: Cefoxitin sodium in crystalline form is thus both chemically and physically more stable than the amorphous form (Oberholtzer and Brenner,1979).

Crystalline

Crystalline is different from amorphous where the atoms in molecule are arranged in regular array. Crystalline can divided into 2 categories by molecule in crystal lattice : (a) single-entity crystalline solids (b) molecular adducts as shown in Figure 5

Single-entity crystalline solids – Polymorph

Many pharmaceutical solids exhibit polymorphism, which is frequently defined as the ability of a substance to exist as two or more crystalline phases that have different arrangements and/or conformations of the molecules in the crystal lattice. Thus, in the strictest sense, polymorphs are different crystalline forms of the same pure substance in which the molecules have different arrangements and/or different unit cells and hence display different physical properties, including those due to packing, and various thermodynamic, spectroscopic, interfacial, and mechanical properties (Haleblian, 1975; Hartshorne and Stuart, 1975; Grant, 1999). In general, it should be possible to obtain different crystal forms of a drug and thus modify the performance properties for solid compound (Handcock and Zografi, 1997).

There are several researches studies on different physical properties among various polymorphs e.g. molar volume and density, dissolution rate, solubility, compaction, habit, solid state stability, bioavailability, activity, quality of product (Lowes et al, 1987; Chikaraishi, 1994; Chikaraishi, Otsuka and Matsuda, 1996; Jozwaiakowski, 1996; Griesser, Burger and Mereiter, 1997; Liggins, Hunter and Burt, 1997; Phadnis and Suryanarayanan, 1997; Ttros de Iladuya et al, 1997; Nichols and Frampton,1998; Bettinetti et al, 1999; Kimura, Hirayama and Uekama,1999; Sun and Grant, 2001; Dong et al, 2002; Schinzer,2002; Sohn and Kim,2002; Zhang, 2002)

For example: Lamivudine crystal in bipyramidal form is more stable in solid state than acicular form (Jozwaiakowski, 1996).Cabamazepine can exist in monoclinic or trigonal form which has different intrinsic dissolution rate (Lowers et al,1987). Tolbutamide has 4 polymorphs: Form I, FormII, FormIII and FormIV. The dissolution

characteristics of Tolbutamide polymorphs were reflected in the oral adsorption behavior in dogs (Kimura, Hirayama and Uekama,1999).

Sometimes only one polymorph is stable at all temperatures below the melting point, with all other polymorphs being therefore unstable. These polymorphs are said to be monotropes, and the system of the two solid phases is called monotropic. If one polymorph is stable (i.e.,has the lower free energy content and solubility over a certain temperature range and pressure),while another polymorph is stable (has a lower free energy and solubility over a different temperature range and pressure), the two polymorphs are called enantiotropes, and the system of the two solid phases is said to be enantiotropic. For an enantiotropic system a reversible transition can be observed at a definite transition temperature (Byrn et al, 1995). For example, Sulfamerazine polymorphs were found to be enantiotropic system. The thermodynamic transition temperature lies between 51 and 54°C, with polymorph II stable at lower temperature(Zhang et al, 2002).

The important quality attributes of a solid product include stability, dissolution, bioavailability, appearance, manufacturability, density, hardness, etc., all of which may be influenced by phase transformation. The manufacturing processes may be lead to transformation of polymorphs such as particle size reduction, granulation, drying, compression, coating etc. so potential for process induced solid phase transformation must be evaluated during design and development of formulations and manufacturing process for good quality pharmaceutical products (Brittain and Fiese, 1999; Byrn, Pfeiffer and Stowell,1999; Zhang, 2004).

For example, Two polymorphs of aspartame hemihydrate can transform from Form II to Form I during ball-milling or on heating for 30 min. at 160 °C in the presence of steam(Leung et al,1998).

Stoichiometric adducts-solvates

During crystallization process form a solution, Crystals may consist of a pure component or be a molecular compound. Molecular compounds may contain two or more

constituents that have completely satisfied classical " valent forces" and are crystallized together as a new single entity. Solvate are molecular complexes that have incorporated the crystallizing solvent molecule in their lattice. When the solvent incorporated in the solvate is water, it is called a hydrate (Haleblian, 1975).

The solid state characterization of rifampicin when recrystallization form various solvent systems were investigated and found a rifampicin monohydrate, a rifampicin dihydrate, two amorphous forms, a 1:1 rifampicin:acetone solvate and 1:2 rifampicin:2-pyrrolidone solvate were isolated. The crystal forms were relatively unstable, except for the 2-pyrrolidone solvate, because all hydrated and solvated forms changed to amorphous form after desolvation (Henwood et al, 2001).

Nonstoichiometric adducts-clathrates

Clathrates are inclusion compounds. They were given this name by Powell since the guest is enclosed or protected by crossbars of a grating. According to one source, a clathrate is a single-phased solid with two distinct components; the host and the quest. The quest is retained in closed cavities provided by the crystalline structure of the host. Generally, a cage and its enclosed molecule(s) are taken as a unit cell (Haleblian, 1975).

5. Generation of polymorphs, hydrates, solvates, and amorphous solids

Methods employed to obtain unique polymorphs. solvates, hydrate and amorphous forms including of sublimination, crystallization from a single solvent, evaporation from binary mixture solvents, vapor diffusion. thermal treatment, crystallization from the melt, rapidly changing solution pH to precipitate acidic or basic substances, removal of solvent from crystalline solvates or hydrates, growth in the present of additive, spray drying, solidification of the melt, lyophilization and grainding(Guillory,1999).

However, Crystallization form solution employing various solvents and temperature regimens is often preferred for reasons of convenience, efficiency, and relative mildness. The necessary super saturation for crystallization may be achieved by solvent evaporation, cooling or heating solution, addition of a poor solvent(s), chemical

reaction, or pH adjustment (Zhang, 2002). The solvents selected for recrystallization should include any with which the compound will come into contract during synthesis, purification, and processing, as well as solvents having a range of boiling points and polarities (Guillory, 1999). Example of solvents routinely used for such work and boiling point are listed in Table 3.

Table 3 Solvents often used in the preparation of polymorphs (Pasharin, 2000)

Solvent	Boiling point (°C)
Dimethylformamide	153
Acetic acid	118
Water	100
1-Propanol	97
2-Propanol	83
Acetonitrile	82
2-Butanone	80
Ethyl acetate	77
Ethanol	78
Isopropyl ether	68
Hexane	69
Methanol	65
Acetone	57
Methylene Chloride	40
Diethyl ether	35

6. Method for the characterization of Polymorphs, Hydrates, Solvate and Amorphous solids

Of all the methods available for the physical characterization of solid materials, it is generally agreed that crystallography, microscopy, thermal analysis, solubility studies, vibrational spectroscopy, and nuclear magnetic resonance are the most useful for

characterization of polymorphs and solvates. All other methodologies cannot identify for the existence of polymorphism by themselves alone (Brittain, 1999).

X-ray powder diffraction (XRPD)

Diffaction is a scattering phenomenon. When x-rays are incident on crystalline solids, they are scattered in all directions. In some of these directions, the scattered beams. Bragg's law describes the conditions under which this would occur. It is assumed that a perfectly parallel and monochromic x-ray beam, of wavelength λ, is incident on a crystalline sample at an angle θ. Diffraction will occur if

$$n\lambda = 2d \sin\theta$$

where d = distance between the planes in the crystal, expressed in angstrom units
n = order of reflection (an integer)

X-ray powder diffractometry is widely used for the identification of solid phases. The x-ray powder pattern of every crystalline form of a compound is unique, making this technique particularly suited for the identification of different polymorphic forms of a compound. The technique can also be used to identify the solvated and the unsolvated (anhydrous) forms of a compound, Provided the crystal lattices of the two are different (Suryanarayanan, 1995). X-ray powder diffraction method is an excellent method to determining the existence of an amorphous form since they usually exhibit a broad hump between 2 and 20° 2θ. An amorphous form is expected to have no peaks in the powder diffraction pattern (Byrn, 1995).

The X-ray powder method is experimentally simple and does not require large single crystal but instead can readily be applied to any powdered sample. This method is an effective method of distinguishing solid phases having different internal structure (Byrn, Pfeiffer and Stowell,1999).

Amorphous Quinapril hydrochloride (QHCl) was prepared by grinding of crystalline QHCl-CH_3CN. Figure 9 shown the gradual transition from the crystalline form

to amorphous form. The intensities of the crystalline peaks decreased with an increase in grainding time, and a halo pattern of the typical amorphous form was observed after 5 minutes of grainding (Guo, Byrn and Zografi, 2000).

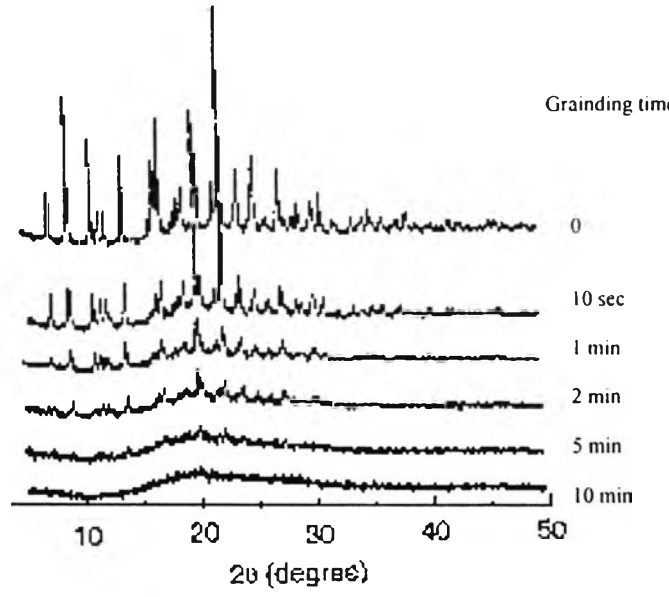


Figure 9 PXRD pattern of sample obtained from grainding for different time interval (Guo. Byrn and Zografi, 2000)

Thermal methods of analysis

Measurement of thermal analysis are conducted for the purpose of evaluating the physical and chemical changes that may take place in a heated sample, requiring that the operator interpret a thermogram in terms of plausible reaction processes. Thermal reactions can be endothermic such as melting. boiling, sublimination, vaporization, desolvation, solid-solid phase transitions, chemical degradation, etc. or exothermic such as crystallization, oxidative decomposition, etc. (McCauley and Brittain, 1995).

Differential scanning calorimetry

In the DSC method, the sample and reference are kept at the same temperature and the heat flow required to maintain the equality in temperature is measured. This achieved by placing separate heating elements being controlled and measured. The plots from DSC are obtained as the differential rate of heating (in units of watts/second, calories/second, or Joules/second) against temperature. The area under a DSC peak is directly proportional to the heat absorbed or evolved by the thermal event, and integration of these peak areas yields the heat of reaction (in units of calories/second.gram or Joules/second.gram).

DSC can be used to obtain useful and characteristic thermal and melting point data for crystal polymorphs or solvate species. This information is of great importance to the pharmaceutical industry since many compounds can crystallize in more than one structure modification. Although the primary means of polymorph or solvate characterization is centered around x-ray diffraction methodology, in suitable situations thermal analysis can be used to advantage (McCauley and Brittain,1995).

Thermogravimetric analysis (TGA)

Thermogravimetry is a measure of the thermally induced weight loss of a material as a function of the applied temperature. Thermogravimetric analysis is restricted to studies involving either a mass gain or loss, and it is most commonly used to study desolvation processes and compound decomposition. Thermogravimetric analysis is a very useful method for the quantitative determination of volatile content of solid, and it can be used as an adjunct to Karl Fischer titration for the determination of moisture. Desolvation processes or decomposition reactions must be accompanied by weight changes, and they can be thusly identified by a TG weight loss over the same temperature range. On the other hand, solid-liquid or solid-solid phase transformations are not accompanied by any loss of sample mass and would not register in a TG thermogram (McCauley and Brittain, 1995).

Karl Fischer titration

Karl Fisher titration is a widely used analytical method for quantifying water content in a variety of product. The fundamental principle of this method based on the reaction between iodine and sulfur dioxide in an aqueous medium. The rate of reaction depends on the pH value. When pH is lower than 5, the titration rate is very slow. On the other hand, when pH is higher than 8, titration rate is fast, but only due to an interfering esterification side reaction which produces water. The result is a vanishing end-point. Thus, the optimum pH range for Karl Fischer reaction is from 5 to 8.

Infrared absorption spectroscopy (IR)

Most solid state investigrations of bulk drug material involve the identification and quantitation of polymorphic and pesudopolymorphis system. Since different polymorphic forms of a drug substance exhibit different three-dimension structures, the vibrational motion for each polymorphic form is potentially different, whence the ability to investigate polymorphism by vibrational spectroscopy techniques. Infrared absorption spectroscopy (IR) is one of vibrational spectroscopy techniques which used as an identification assay for various intermediates and final bulk drug products (Bugay and Williams, 1995).

The near-IR technique has been used very successfully for moisture determination, whole tablet assay, and blending validation. These methods are typically easy to develop and validate, and far easier to run than more traditional assay methods. Using the overtone and combination band of water, it was possible to develop near-IR methods whose accuracy was equivalent to that obtained using Karl-Fischer titration (Brittain, 1995).

Nuclear magnetic resonance spectroscopy (NMR)

Nuclear magnetic resonance spectroscopy (NMR) in pharmaceutical research has been used primarily in a classical, organic chemistry framework. Typical studies have included the structure elucidation of compound, investigating chirality of drug substances,

the determination of cellular metabolism, and protein studies, to name but a few. Form the development perspective; NMR is traditionally used again for structure elucidation, but also for analytical applications. In each case, solution-phase NMR has been utilized. It seems ironic that although ~ 90% of the pharmaceutical products on the market exist in the solid form, solid state NMR is in its infancy as applied to pharmaceutical problem solving and methods development (Bugay, 1995).

Microscopy

A variety of concerns require a determination of all possible crystalline forms of a given compound that might be encountered under different conditions. When only small amounts of material are available, crystallization form a variety of solvents can still be effected, and a full microscopic examination (paying critical attention to the optical crystallography of the sample) is used to observe any possible differences in crystal habit or structural class. Should a compound be capable of exhibiting polymorph and provide determinations as to the physical interconvertibility of these (Brittain, 1995).

Thermal microscopic is conducted by mounting the sample in a system whose temperature can be accurately controlled and monitored, which is usually terms a hot stage or cold stage depending on the type of thermal control employed. Most cold stage systems operate form room temperature down to approximately - 50°C, while most hot-stage systems are functional between room temperature and 300 – 350 °C. The dehydration, desolvation, or decomposition temperature of a compound can also be evaluated form an examination of discontinuities in the optical properties. These processes are most effectively monitored if the sample is immersed in an oil, since the evolution of gas is evident in the generation of gaseous bubbles form solid under study (Newman and Brittain, 1995).

Thin-layer Chromatography

Thin-layer Chromatography (TLC) is used for separation and identification of compounds. It is a method of chromatography in which a mobile phase moves by capillary action across a uniform thin layer of finely divided stationary phase bound to the plate (Moffat, 1986).The detection reagent is usually applied post chromatography by spraying or dipping the layer, or the reagent may be preimpregnated into the layer period to spotting and chromatography. Volatile reagents may be applied by exposing the plate to their vapors. Reagent that have been used on C-18 layers include iodine , 10% phosphatemolypdic acid in ethanol with heating 120°C(for lipids), 10% sulfuric acid in ethanol with heating for 2-3 min (general reagent), and fluorescamine (amino acids)(Sherma, 1991).