

## CHAPTER III

### EXPERIMENTAL

#### MATERIALS

##### Material

Standard material :

- Asiaticoside (Lot. No. AS030610, Changzhou Natural Products Development Co.Ltd. ,China)
- Madecassic acid (Lot. No. MA030501, Changzhou Natural Products Development Co.Ltd. ,China)
- Asiatic acid (Lot. No. AA030610, Changzhou Natural Products Development Co.Ltd. ,China)

Tablet Diluent:

- Spray dried lactose monohydrate, Super-Tab® (Lot No.2031104, The Lactose Company of New Zealand, Ltd., New Zealand)
- Pregelatinized starch, Starch 1500® (Batch No. IN505698, supply Rama Production Co.Ltd., Thailand)
- Dibasic calcium phosphate (supply by Rama Production Co.Ltd., Thailand)
- Magnesium stearate (Faci Asia Pacific PTE. Ltd.)
- Talcum (Haichen Talc Powder, China)
- Silicon dioxide (supply by Rama Production Co.Ltd.. Thailand)

Chemical :

- Distilled water
- Acetone (Analytical grade, Lab scan,Ltd., USA)
- Acetonitrile (Analytical grade, Lab scan,Ltd., USA)
- Acetonitrile ( HPLC grade, Lab scan,Ltd., USA)

- 1-Butyl alcohol (Analytical grade, Fluka Chemie AG., Switzerland)
- 2-Butyl alcohol (Analytical grade, Malinckrodt Baker, Inc., USA)
- Butyl acetate (Analytical grade, Malinckrodt Baker, Inc., USA)
- Chloroform (Analytical grade, Malinckrodt Baker, Inc., USA)
- Ethyl alcohol (Analytical grade. Malinckrodt Baker, Inc., USA)
- Ethyl acetate (Analytical grade, Malinckrodt Baker, Inc., USA)
- Isopropyl alcohol (Analytical grade, Malinckrodt Baker, Inc., USA)
- Methyl alcohol (Analytical grade, Lab scan,Ltd., USA)
- Methyl alcohol ( HPLC grade, Lab scan.Ltd.. USA)
- Methylene chloride (Analytical grade. Malinckrodt Baker, Inc., USA)
- N – Propyl alcohol (Analytical grade. Malinckrodt Baker, Inc., USA)
- Potassium carbonate (Analytical grade. E. Merck, Germany)
- Sodium chloride (Analytical grade. Lab scan,Ltd., USA)
- Sodium nitrite (Analytical grade, E. Merck, Germany)
- Sulfuric acid (Analytical grade, Malinckrodt Baker, Inc., USA)

### **Analytical Instrument**

- Oven (Mammert, Germany)
- Fourier Transformed Infrared Spectroscopy (Perkin Elmer Ltd. Model SP2000 , USA)
- X-ray Powder Diffractometer (Rigaku Model DMAX 2000, Japan)
- Thermogravimetric analysis (Mettler Model TGA/SDTA 851<sup>e</sup>, Germany)
- Differential Scanning Calorimeter (Mettler Model DSC 822<sup>e</sup>, Germany)
- Microscope with Hot stage (Nikon E200 and Mettler FP 90 central processor,Switzerland)
- Water bath with dual action shaker – (Poly Science Model 28L/B/SH/C, USA)
- Nuclear Magnetic Resonance Spectrometer (Burker-spectrospin 300)
- Gas Chromatography – (Shimadzu Model GC7AG, Japan)
- High Pressure Liquid Chromatography – Shimadzu. Japan



1. System controller : Model SCL-10A
  2. Pump : Model LC-10AD
  3. Degasser : Model DGV-14A
  4. UV-Vis Detector : Model SPD-10A
  5. Auto Injector : Model SIL-10AD
  6. Column oven : Model CTO-10AS
- Analytical Balance
1. Mettler Toledo Model FP90 and Model PB3002, Switzerland
  2. Sartorius Model A200S ,Germany

## METHOD

### 1. Recrystallization of asiaticoside in various solvents

The various methods for preparing different polymorphs of solid substances such as sublimation, crystallization from various solvent, vapor diffusion, thermal treatment, crystallization from the melt, rapid pH changing, thermal desolvation. However, crystallization from various solvent is often preferred for reasons of convenience, efficiency and relative mildness (Guillory,1999; Zhang,2002)

#### Method I - Recrystallization by slow evaporation

Asiaticoside raw material was dissolved until saturated in acetone, acetonitrile, 1-butyl alcohol, 2-butyl alcohol, butyl acetate, chloroform, ethyl alcohol, ethyl acetate, isopropyl alcohol, methyl alcohol, methylene chloride, N – propyl alcohol. The saturated solution was filtered, and was allowed to stand at room temperature in 250 ml beaker until all solvent were evaporated.

#### Method II – Recrystallization from binary mixture of solvent

Asiaticoside raw material was dissolved in 10 ml of methyl alcohol(a), 40 ml of water or acetonitrile was slowly added into the solution(a) with stirring at ambient temperature. The precipitates were filtered and allowed to dry at room temperature.

### 2. Identification of asiaticoside

The solid samples were identified by TLC and FTIR.

#### 2.1 Thin layer chromatography

Thin layer chromatography (TLC) was used for chemical identification of asiaticoside and degraded substances of all asiaticoside products by comparing TLC pattern.

The samples were dissolved in methyl alcohol. Then, the solutions were applied to the TLC plate (Silica gel 60 F254, Merck) by capillary tube drawn out to a fine point. Ethyl acetate, Methyl alcohol and water (8:2:1) were used as a mobile phase. The spots were located by spraying with 10% H<sub>2</sub>SO<sub>4</sub> in methyl alcohol. The purple spot occurred after heating 15 minute with a blow dryer.

## 2.2 Fourier Transformed Infrared Spectroscopy

FTIR can be used to indicate differences in crystal structures for some compounds but in general often found just slightly different among polymorphs. In addition, many FTIR pattern can be altered when solvent molecules are incorporated in the crystal lattice.

The dried sample was mixed with potassium bromide in a mortar, then it was compressed using hydrolic press to a thin disc. The KBr pellet was measured within a wave number range 400 – 4000 cm<sup>-1</sup>

## 2.3 C<sup>13</sup> Nuclear Magnetic Resonance

Nuclear Magnetic Resonance is traditionally used for structure elucidation. NMR pattern of crystallized product will identify with Asiaticoside raw material by comparing NMR pattern in the same condition. The dried sample was dissolved in dimethyl sulfoxide (DMSO). After that analysed by using Bruker-spectrospin 300 MHz for C<sup>13</sup> pattern.

# 3. Solid State Characterization

## 3.1 Microscopy

A small amount of sample was viewed using a scanning electron microscope (SEM) to observed in their morphology. The selected samples were mounted onto the slide by mineral oil. After that the morphology of crystals were investigated using Nikon E200 microscope at 400x. The melting point and the temperature where the

solvent was released were observed by hot stage microscope from 30 – 300 °C at an increasing rate of 5°C per minute.

### 3.2 X-ray Powder Diffractometry

XRPD pattern of each crystalline form is unique, while those of amorphous form exhibit a broad halo and expected to have no peaks. Thus, XRPD was used to determine the solid phase of asiaticoside products obtained from I.

Asiaticoside samples were packed onto a holder made of quartz plates with rectangular window of 1.5 x 1.0 c.m.. The samples were smoothed-out and pressed until it was at the same level as the frame of the holder.

The X-ray powder diffraction pattern of various asiaticoside solid samples were determined at ambient temperature and atmosphere using Rigaku X-ray Diffraction Refractometer at 30 mA and 40 kV with  $\text{CuK}\alpha$  radiation. The samples were scanned with the diffraction angle increasing from 5° to 50°,  $2\theta$ , with a step size of 5° per minute.

### 3.3 Thermogravimetric analysis

Thermogravimetric analysis (TGA) was used to study the desolvation process and decomposition of compound. TGA curve were determined by Mettler TGA/SDTA 851<sup>e</sup> using heating rate of 5°C/minute under nitrogen atmosphere at temperature ranged from 25 - 300°C

### 3.4 Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) was used to determine melting points, solid phase transformation and solvate or hydrate forms of sample. DSC thermograms were obtained using Mettler DSC 822<sup>e</sup>. The sample was accurately weighed into an aluminium pan crimped with 1 pinhole. The samples were heated at the rate of 5°C/minute from 25 – 300 °C under nitrogen atmosphere.

### 3.5 Karl Fischer titration

Karl Fischer was used to determine water content in sample. The Karl Fischer titer of Karl Fisher reagent was calibrated with water. After that the sample was accurately weighed into Karl Fisher titrator and collect data after end-point was found.

### 3.6 Gas Chromatography

Gas chromatography in this study was used to identify volatile solvent which may be absorb on crystal surface. Asiaticoside I and Asiaticoside II about 300 mg were contain in sealed 10 ml vial and heat at 50°C about 20 minute. Inject vapor upper sample in to gas chromatography. Chromatographic condition are as follow :

Mobile phase	Nitrogen
Column	Porapak QS , 3.3 mm x 2 m
Flow rate	40 ml/min
Detector	FID 230 °C
Injection volume	1 µl
Injection temperature	230 °C

## 4 Physicochemical Characterization

### 4.1 Solubility

Excess amounts of asiaticoside were added to 150 ml water in 250 ml flat-bottomed flask. The samples were placed in  $37 \pm 2^\circ\text{C}$  water bath and shake at 150 rpm. At suitable time intervals the sample was withdrawn, filtered and determined for its concentration by high pressure liquid chromatography.

### 4.2 Stability studies of asiaticoside

Objective of this study was to evaluate the polymorphic transformation and chemical degradation of asiaticoside by temperature or humidity. The effect of thermerature was studied by placing asiaticoside I and II into separate open amber

glass bottles and then placed in a desiccator containing saturated sodium nitrite ( $\text{NaNO}_2$ ) to maintain the relative humidity between 55 – 65 % RH . The oven was used to maintain constant temperature at 40, 50 and 60°C. For effect of humidity test, the sample was placed into amber glass bottles and placed in a desiccator containing saturated potassium carbonate ( $\text{K}_2\text{CO}_3$ ), sodium nitrite ( $\text{NaNO}_2$ ), or sodium chloride ( $\text{NaCl}$ ) to produce the required relative humidity (42%RH, 62%RH and 75%RH) at 40°C. The sample of both experiments were collected at 2, 4, 6, 8, and 18 week interval. The solid state integrity was determined by X-ray powder diffraction analysis and chemical integrity was determined by high pressure liquid chromatography.

### 4.3 Incompatibility studies

This experimental was to study about incompatibility between asiaticoside and tableting diluent as follow : spray-dried lactose, pregelatinized starch, dibasic calcium phosphate, talcum, silicon dioxide and magnesium stearate. The asiaticoside formI and formII were mixed with the diluent in mortar at different ratio , 1:20 for spray-dried lactose, pregelatinized starch. dibasic calcium phosphate and 1:1 for talcum, silicon dioxide and magnesium stearate (Cartensen,1998). The mixtures were tested for transformation in various temperature by differential scanning calorimetry , solid state integrity was determined by X-ray powder diffraction analysis and chemical integrity was determined by high pressure liquid chromatography. After that the sample was placed in a desiccator containing saturated sodium chloride at 40°C. The samples were collected at 2, 4. 6, 8 and 18 week interval to evaluate solid state integrity (determined by X-ray powder diffraction analysis) and chemical integrity (determined by high pressure liquid chromatography).

HPLC method for determination of asiaticoside as following details:

Standard preparation: Asiaticoside 25 mg was accurately weighed into 25 ml volumetric flask dissolved and adjusted to volume with methanol.



Sample preparation: The sample equivalent to asiaticoside 25 mg was accurately weighed into 25 ml volumetric flask dissolved and adjusted to volume with methanol.

Procedure: The standard and sample solution were injected into the HPLC column using the following chromatographic conditions :

Mobile phase	Acetonitrile : Water ( 30 : 70 )
Column	C18, 5 $\mu$ m , 250 x 4.6 mm (Thermo Hypersil-Keystone BDS batch no.5887)
Flow rate	1.0 ml/min
Detector	UV 210 nm
Injection volume	20 $\mu$ l