CHAPTER 3

EXPERIMENTS

MATERIALS

Chemicals

- 2-propylpentanoic acid (Valproic acid)
  (Sigma Chemical Co., USA, Lot. 67H 1126)
- Distilled water
- Acetone (E. Merck, Germany)
- Acetonitrile (Malinckrodt Baker, Inc., USA)
- Benzene (AJAX Laboratory Chemicals, Australia)
- Chloroform (E. Merck, Germany)
- Diethyl ether (AJAX Laboratory Chemicals, Australia)
- Ethanol absoluted anhydrous (Malinckrodt Baker, Inc., USA)
- Ethyl acetate (Malinckrodt Baker, Inc., USA)
- Heptane (E. Merck, Germany)
- Hexane (Malinckrodt Baker, Inc., USA)
- Isopropyl alcohol (Malinckrodt Baker, Inc., USA)
- Methyl alcohol anhydrous (Malinckrodt Baker, Inc., USA)
- Methylene chloride (Malinckrodt Baker, Inc., USA)
- Sodium carbonate (E. Merck, Germany)
- Sulfuric acid concentration (Lab-scan, Ltd., Ireland)
- Urea (Fluka Chemie AG., Switzerland)
- Sodium chloride (Riedel-deHaën, RdH Laborchemikalien GmbH & Co., Switzerland, Lot. 83140)
- Sodium sulphate anhydrous (E. Merck, Germany)

All solvents are of analytical grade.

Analytical Instruments

- Oven (Mammert, Germany)
- Fourier Transform Infrared Spectroscopy – Spectrum 2000 (Perkin Elmer Ltd., Germany)
- X-ray Diffractometer for qualitative analysis – JDX-3530 Diffractometer (Jeol, Japan)
- X-ray Diffractometer for quantitative analysis – Philips PW3710 (Philips)
- Thermogravimetric analysis – STA 409 C Thermische Analyse (NETZSCH, Germany)
- Differential Scanning Calorimeter – DSC 200C Thermische Analyse (NETZSCH, Germany)
- Scanning Electron Microscope – JSM-5410LV (Jeol, Japan)
- Water bath with dual action shaker – 28L/B/SH/C (Poly Science, USA)
- Ultraviolet Spectrophotometer – Spectronic 2000 array (Milton Roy, USA)
- Analytical balance for quantitative analysis – AG245 (Mettler Toledo, Switzerland)

Miscellaneous

- Rotaevaporator – Rotavapo (Büchi, Switzerland)
- TLC plate – plastic sheet silica gel 60 F_{254} (E. Merck, Germany)
- Filter paper No. 1 (Whatman International Ltd., England)
- Parafilm (America National Can., USA)
- Aluminium foil (MMP Packaging, Thailand)

METHOD

I SYNTHESIS OF N-(2-PROPYLPENTANOYL) UREA

2-propylpentanoic acid (VPA) (14.42 g, 0.10 mole) was dropwisely added to thionyl chloride (8.0 ml, 0.11 mole), then the mixture was stirred under reflux condition for approximately three hours. Excess thionyl chloride was removed by distillation to yield a crude acid chloride, which was further purified by distillation under reduced pressure. The pure acid chloride was then added dropwise into a mixture of urea (12.01 g, 0.2 mole) and potassium carbonate (16.59 g, 0.12 mole) in dry benzene (30 ml). The mixture was stirred under reflux condition for six hours, then filtered immediately. Precipitate was collected. Benzene in filtrate was removed under reduced pressure to yield dry residue, which then dissolve in ethyl acetate together with the early precipitate. Then the organic phase was extracted with water, dried over magnesium sulfate anhydrous and evaporated under reduced pressure to yield crude VPU.

The crude VPU was further purified by dissolving crude VPU in hot benzene. Then, hexane was slowly added in to the solution and allowed to stand in the refrigerator overnight. The separated crystals were then filtered off and allowed to dried at room temperature to yield final VPU product. The product was identified by TLC and FTIR as mentioned below. The synthetic pathway of VPU is shown in Figure 3.

Several lots of synthesized VPU were recrystallized together by the same method mentioning above to yield crystals, which were identified by TLC and FTIR. The crystals obtained will be referred to as a reference form of VPU in future studies including of solid phase screening, solid state stability and solubility measurement.

The reference VPU was characterized by SEM, XRPD, TGA and DSC.

II SOLID PHASE SCREENING OF N-(2-PROPYLPENTANOYL) UREA

2.1. Preparation of polymorphs, hydrates, solvates or amorphous forms of VPU

Attempts were made to treat reference VPU by various techniques in order to obtain its polymorphs, hydrates, solvates or amorphous forms. All treated products were identified by TLC and FTIR and characterized by XRPD to determine solid forms. The methods were as follows.

Method I – Recrystallization by rapid solvent evaporation

The reference VPU was dissolved in solvents, including acetone, acetonitrile, benzene, chloroform, diethyl ether, ethanol absoluted anhydrous, ethyl acetate, heptane, hexane, isopropyl alcohol, anhydrous methyl alcohol and methylene chloride, at boiling point of each solvent. The solution at the boiling point was then filtered. Because of theirs low boiling points, diethyl ether and methylene chloride were removed by placing the solution, contacting with steam, over water bath to evaporate the solvents until almost dried out. The other solvents were removed in a rotary evaporator under vacuum until almost dried out. Then, the residues were dried at room temperature.

The residues recrystallized from heptane and diethyl ether were further characterized by TGA/DSC.

Method II – Recrystallization by temperature change

The reference VPU was dissolved in solvents, including benzene, heptane, hexane and water, at the boiling point of each solvent. The solution at the boiling point was filtered and allowed to recrystallized by spontaneous cooling to room temperature and was then allowed to stand in the refrigerator overnight. The separated crystals were filtered off. The crystals were collected in two conditions.

a) The crystals were allowed to dry at room temperature and collected in dry form.
b) The crystals were collected as damp mass in order to obtain solvate or hydrate forms.

All of the dried form products were examined by XRPD, the dried forms obtained by hexane, heptane and water were examined by DSC. The dry product obtained from water was also determined by TGA. All of the damp mass products were examined by DSC, the damp mass products obtained from hexane, heptane and benzene were examined by XRPD summarized in Table 4. The dried crystals obtained were gently ground in agate mortar before determined by XRPD.

Method III – Recrystallization by slow evaporation

The reference VPU was dissolved in boiling diethyl ether. Then, the boiling solution was filtered. The solution was allowed to stand at room temperature until all the solvent was evaporated. Then the crystallized residue was collected. The product was determined by DSC.

Method IV – Recrystallization from binary mixture of solvents

100 mg of the reference VPU was dissolved in 50 ml of anhydrous ethanol, and then 350 ml of distilled water was slowly added into the solution with stirring at ambient temperature. The separated crystals were filtered off and allowed to dried under reduced pressure at room temperature.

Method V – Evaporation crystallization

The reference VPU was placed in an aluminium cup and heated on a hot-plate to allow melting. With an increased in temperature, the sample evaporated. When the evaporated VPU reached the cooler rim of the aluminium cup, it recrystallized. Then, the crystals were collected.

Method VI – Immediate solidification of the melt

The reference VPU was melted in an aluminium cup on hot-plate and immediately cooled down with liquid nitrogen or ethanol bath filled with dried ice. The residues were collected.

Method VII – Thermal treatment

Dried VPU recrystallized from benzene and heptane by method II and from diethyl ether by method III were placed into open 5 ml amber glass vials, which were then heated in a hot air oven maintained at 100°C, 10 hours for three consecutive days. The residues were collected. All residues were examined by DSC. The products obtained from benzene and heptane were characterized by XRPD.

The products obtained by all methods were identified by TLC, FTIR. XRPD. Morphology study of the products was conducted by SEM.

Table 4: Characterization methods used in solid phase screening of VPU

Methods	**Solvents**		**Characterization methods**
Method I	diethyl ether, heptane		XRPD, TGA/DSC, SEM
	acetone, acetonitrile, chloroform, ethanol, hexane, methyl alcohol, methylene chloride, ethyl acetate, isopropyl alcohol		XRPD, SEM
	benzene		XRPD
Method II	Damp mass	Water	DSC
		hexane, heptane	XRPD, DSC
		benzene	XRPD, DSC, SEM
	Dry forms	Water	XRPD, TGA/DSC, DSC
		benzene, hexane, heptane	XRPD, DSC
Method III	diethyl ether		XRPD, DSC
Method IV	ethanol/water		XRPD, SEM
Method V	---		XRPD, SEM
Method VI	---		XRPD, SEM
Method VII	Products obtained from diethyl ether (method III)		DSC
	Products obtained from benzene and heptane (method II)		XRPD, DSC

2.2. Method for Identification of VPU products

VPU samples were identified using TLC and FTIR.

2.2.1 Fourier Transform Infrared Spectroscopy

The solid state FTIR was used to determined identity of all VPU products compared with IR pattern of VPU achieved by Wicharn Janwitayanuchit (5).

FTIR can be used to indicate differences in crystal structures for some compounds but in general often found just slightly different among polymorph. In addition, many FTIR pattern can be altered when solvent molecules are incorporated in crystal lattice (51).

A Perkin Elmer Fourier Transform Infrared Spectroscopy (Model spectrum 2000) was used to acquire the infrared data. The dried sample (about 1 mg) was mixed with potassium bromide (KBr, about 250 mg) in an agate mortar by geometric dilution technique, then press using hydrolic press to a thin disc. The KBr pellet was then measured within a wavenumber range 400-4000 cm^{-1}

2.2.2 Thin layer chromatography

TLC was used to ensure chemical identity and purity of all VPU samples by comparing with TLC patterns of the VPU samples previously identified by FTIR. TLC pattern was acquired using TLC plastic sheet silica gel 60 F_{254} (Merck) and ethyl acetate was used as a mobile phase.

The samples were dissolved in a smallest volume possible of ethyl acetate. Then, the solutions were applied to the TLC plate by capillary tube drawn out to a fine point. The spots were located by spraying with Dragendorff reagent (72). The orange spot of VPU occurred after heating with a blow dryer.

2.3. Methods for Characterization of Solid phase.

2.3.1 Qualitative X-ray Powder Diffraction analysis

XRPD was used to determine solid phase of VPU samples obtained from all methods since XRPD pattern of each crystalline form is unique, while those of amorphous forms exhibit a broad halo between 2 and 20° 2θ and expected to have no peaks (34).

Sample preparation

The solid form of VPU obtained by all methods mentioned previously were packed onto a holder made of quartz plates containing rectangular window. The samples were smoothed-out and pressed using a microscope slide until it was at the same level as the frame of the holder. Only dried VPU recrystallized from benzene, hexane and heptane by method II were gently ground in an agate mortar before packing because of its large monoclinic habits.

The powder x-ray diffraction patterns of various solid samples were determined at ambient temperature and atmosphere using a Jeol X-ray Diffraction Refractometer (JDX-3530) at 30 mA and 30 kV with CuK_{α} radiation. The samples were scanned, with the diffraction angle increasing from 5° to 40°, 2θ, with a step size of 0.04° and count time of 0.5 second.

2.3.2 Thermogravimetric Analysis

Thermogravimetric analysis (TGA) was used to study the desolvation process and compound decomposition. TGA and DSC curves were obtained together using a NETZSCH (STA 409 C). A heating rate of 15°C/minute was employed under nitrogen atmosphere at temperature range from 20-250°C.

2.3.3 Differential Scanning Calorimetry

The differential scanning calorimetry (DSC) was used to determine melting point, solid phase transformation and solvate or hydrate forms of samples. DSC thermograms for various systems were determined using a NETZSCH DSC 200. Approximately 2 mg of sample was accurately weighed into a crimped aluminium pan with 1 pinhole. The samples were heated using heating rate of 10°C/minute for all samples. The temperature used was from room temperature to 300°C under nitrogen atmosphere.

2.3.4 Scanning Electron Microscopy

The morphology of various solid samples was investigated using a Jeol JSM-5410LV Scanning Electron Microscope. The dry powder was mounted onto metal stubs using a piece of double-sided conductive adhesive tape and vacuum-coated with gold.

III SOLID-STATE STABILITY

Solid state reaction can be either chemical reactions or physical transformation (37). Objective of this study was to evaluate the susceptibility of VPU to physicochemical transformation such as polymorphic transformation by temperature. Solid state transformation affects the physicochemical properties of compound.

The solid-state thermal degradation of VPU was studied by placing the reference VPU samples of known weight (approximately 1g) into 5 ml open amber glass vials. The vials were then, placed in a desiccator containing silica gel to maintain 0% RH dryness. A Mammert® oven was used to maintain constant temperature at 50, 60, 70 and 80°C (±2°C). Samples were collected at two and four weeks interval. Morphology of the samples at four weeks interval were then examined by SEM. The chemical integrity of samples were determined by TLC and FTIR as mentioned previously and the solid state integrity was determined by a quantitative XRPD assay.

Quantitative X-ray Powder Diffraction analysis

XRPD can be used for the quantitative determination of phase composition. Quantitative XRPD analysis with sodium chloride as an internal standard was used in this study.

Determination for the Optimal Proportion of Sodium Chloride

The reference VPU was mixed with sodium chloride at concentration of 5, 10, 20, 30 and 40% w/w. The mixtures were prepared and characterized by the method described in the next section.

Sample preparation

VPU samples obtained from stability study were mixed with sodium chloride (10% w/w) as an internal standard. To ensure uniformity of the mixture and reduce a mixing problem from the static property of VPU, sodium chloride was dissolved in a few drops of water in a glass mortar, and then the VPU sample was added by geometric dilution technique (approximately 300 mg total weight). The mixtures were allowed to dry at room temperature under reduced pressure. Then, the mixtures were gently ground in an agate mortar. The fine mixtures were filled into a sample holder made of aluminium consisting of rectangular central cavity.

The mixtures were filled from the back of the holder to reduced preferred orientation (53). First, the face of holder was covered with a glass slide bonded with tape. Excess amount of loose powder was loaded from the back, and then the surplus powder was tamped gently and slice off with edge of the spatula. The back of holder was then covered with aluminium plate. Finally, the glass slide was removed to yield smooth surface.

The experiment was repeated three times for each sample.

The powder x-ray diffraction patterns of the samples were acquired at ambient temperature and atmosphere using a Philips Analytical X-ray Diffractometer (type PW3710) at 30 mA and 40 kV with CuK_{α} radiation. The samples were scanned continuously, with the diffraction angle increasing from 5° to 40° 2θ, with a step size of 0.02° and count time of 1 second to be more accurate.

IV SOLUBILITY MEASUREMENTS

The reference VPU and the VPU obtained from stability study at 80°C for four weeks were selected for solubility study. The samples were ground gently in a glass mortar to reduce size variation between samples. Excess amounts of VPU powder were added to 150 ml distilled water in sealed 250 ml flat-bottomed flask. Then, the samples were placed in 37°C (±2°C) water bath, consisting of agitator, shaken at 150 rpm. At suitable time intervals the samples were withdrawn, filtered and diluted with acetonitrile at ratio sample solution : acetonitrile of 7 : 3. Then, the samples were assayed for drug concentration by UV spectrophotometer (Milton Roy Spectronic 2000 array) at λ = 225 nm (n=3).