CHAPTER 3

Research design and methods

3.1 Raw materials

The raw materials containing in LWDH formula were purchased from Vejpong Pharmacy Co., Ltd.; Bangkok, Thailand. A List of the raw material used in this study was as follows:

1) Cortex Moutan Radicis (Danpi/Mudanpi)
2) Fructus Corni (Shanzhuyu)
3) Poria (Fuling)
4) Redix Rehmanniae Praeparata (Shudihuang)
5) Rhizoma Alismatis (Zexia)
6) Rhizoma Dioscoreae (Shanyoa)

3.2 Chemical reagents, solvents and standards

1) 5-(hydroxyl methyl) furfural CRS	U&V Holding, Thailand
2) 95 % Ethanol	Liquor Distillaery Organization, Thailand
3) Absolute ethanol	RCI Labscan, Thailand
4) Acetone	RCI Labscan, Thailand
5) Acetonitrile HPLC grade	RCI Labscan, Thailand
6) Corn starch	CM Chemical & Lab supply, Thailand
7) Colloidal silicon dioxide	Sigma-Aldrich, Germany
8) Cyclohexane	RCI Labscan, Thailand
9) Diethyl ether	RCI Labscan, Thailand
10) Ethyl acetate	RCI Labscan, Thailand

11)	Ferric chloride	Merck, Germany
12)	Formic acid	Merck, Germany
13)	Hydrochloric acid	Merck, Germany
14)	Lactose	DMV-Fonterra Excipients, Germany
15)	Loganin CRS	U&V Holding, Thailand
16)	Magnesium stearate	Riedel-de Haen, Germany
17)	Methanol HPLC grade	Merck, Germany
18)	Microcrystalline cellulose	DMV-Fonterra Excipients, Germany
19)	Paeonol CRS	U&V Holding, Thailand
20)	Phosphoric acid	RCI Labscan, Thailand
21)	Petroleum ether	RCI Labscan, Thailand
22)	Sulfuric acid	Merck, Germany
23)	Talcum	Ilshin industrial, China
24)	Toluene	RCI Labscan, Thailand
25)	Ursolic acid	U&V Holding, Thailand

3.3 Equipments and Materials

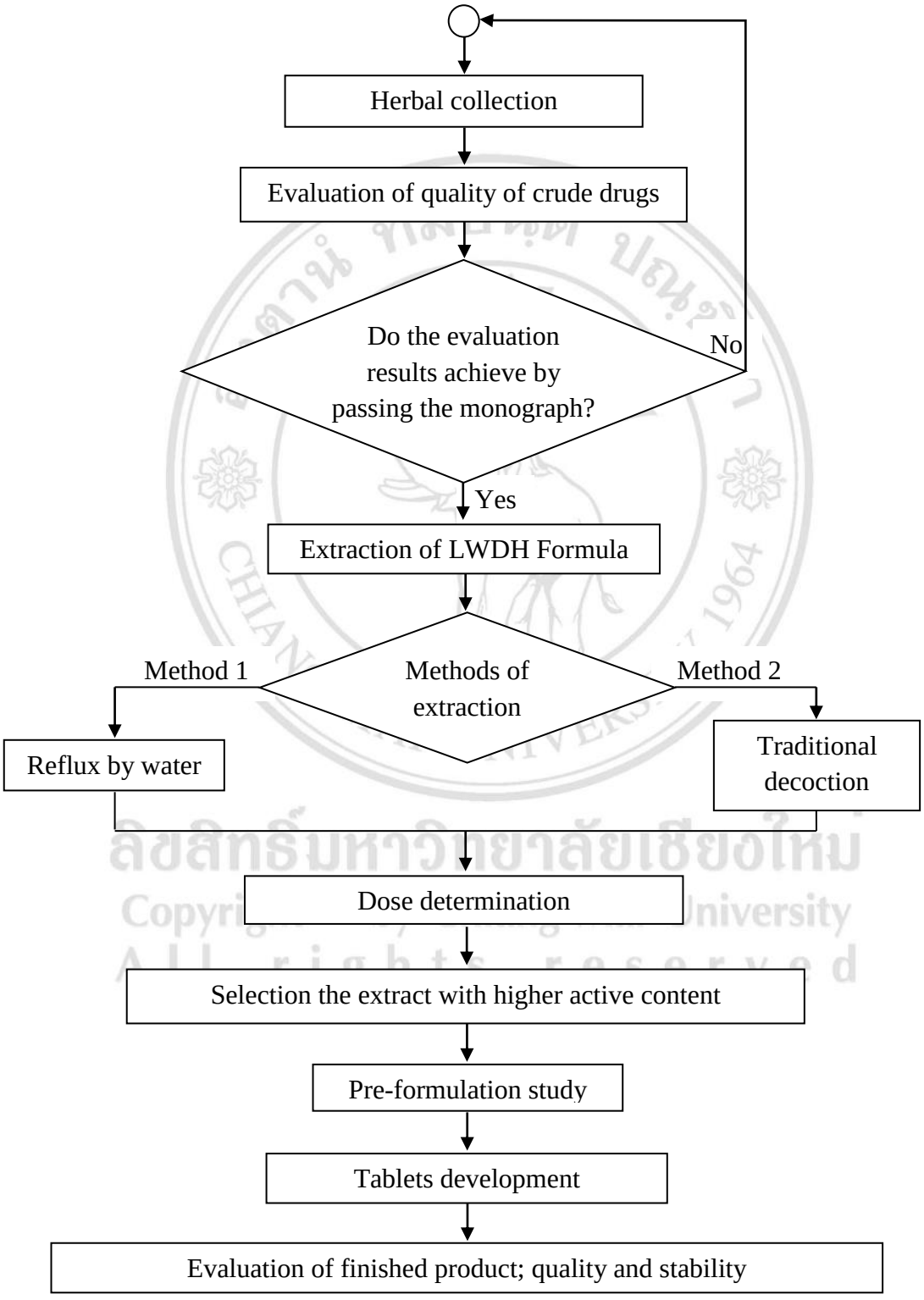
1) Tablet hardness tester (Campbell MHT-20)
2) High temperature furnace (Carbolite CEF1100)
3) Analytical balance (OHAUS ARC 120, SARTORIUS ME 2155)
4) Beaker (Schott duran, Pyrex)
5) Cylinder (Pyrex, Witeg)
6) C18 reversed phase silica gel (Inertsil ODS-3)
7) Disintegration tester (Pharmatest PTZ Auto 3)
8) Erlenmeyer flask (Schott duran, Pyrex)
9) Evaporating dish
10) Filter paper (Whatman No. 1, 41)
11) Friability test apparatus (Pharmatest PTF 20E)
12) Funnel
13) Heating mantle (ELECTROMANTLE EM 0500/C MR1, ISOPAD U2/102)

14) High Performance Liquid Chromatography (Shimadzu)

- Degasser Model DGU-20A5
- Pump Model LC-20AD
- Autosampler Model SIL-20AC
- Column oven Model CTO-20A
- Diode array detector Model SPD-M20A

15) Communication Bus Module Model CBM-20A
16) Hot air oven (BINDER ED240/E2)
17) Hydrolic Tablet Machine (Carver Laboratory press Model C)
18) Micropipet (GILSON MODEL PIPETMAN P200, P1000)
19) Microscope (OLYMPUS CHS No. 1D0199)
20) Nylon membrane filter (Whatman 0.45 micron)
21) pH universal indicator strips (Merck)
22) Rotary evaporator (Eyela N-1000)
23) Round flask (Schott duran)
24) Single stroke tableting machine (CMT 12)
25) Slide and cover slit
26) Sonicator (Elma S30H Elmasonic)
27) Soxhlet apparatus (PYREX)
28) Environment test chamber (Espec Humidity cabinet LHL-112, LHU-112)
29) TLC plate (silica gel GF_{254}) (Merck, Germany)
30) Thin layer chromatography tank
31) UV viewer (CAMAG)
32) Vials
33) Volumetric flask (Schott duran, Pyrex)
34) Water bath

3.4 Flow diagram



3.5 Research methodology

3.5.1 Evaluation of crude drugs quality

Six kinds of crude drugs containing in LWDH formula were evaluated for their quality by following the monograph of the Pharmacopoeia of the People's Republic of China (2005) [2]. Detail of the crude drugs evaluations was as follows:

1) Macroscopic character

The crude drugs were inspected for their appearance; shape, size, fracture, color, odor and taste.

2) Microscopic character

The crude drugs were sliced and dried in 40-60 ^{o}C overnight, then ground and passed through the No. 60 sieve. The sieve-passed powder was prepared on slide to examine their characteristic under microscope.

3) Chemical identification

Shudihuang, Shanzhuyu and Danpi were evaluated in the topic of chemical identification with the following methods.

Shudihuang:

Sliced crude drug was weighed about 1.0 gram then was put into 25 ml Erlenmeyer flask. Absolute ethanol was filled into the flask about 10 ml then the mixture was left to macerate for 24 hours. After that, the extract was filtrated and spotted on TLC plate (silica gel GF_{254}) about 10 µl, using

5-(hydroxyl methyl) furfural 0.5 mg/ml in absolute ethanol as standard substance. TLC plate was developed in the mixture of petroleum ether and ethyl acetate (1:1). The plate was air dried then detected for spots by using 254 nm UV wavelengths.

Shanzhuyu:

Crude drug powdered was weighed about 0.5 gram then was put into 25 ml Erlenmeyer flask. The flask was filled by 10 ml of ethyl acetate and left to ultrasonicate for 15 minutes. After that, the extract was evaporated until it was dry, after that 2.0 ml of absolute ethanol was added to the dried extract. The ethanol extract was spotted on TLC plate (silica gel GF_{254}) about 5 μl, using 1.0 mg/ml of ursolic acid in absolute ethanol as standard substance. TLC plate was then developed in the mixture of toluene, ethyl acetate and formic acid (20:4:0.5). The plate was then air dried and detected for purple-red spots by using the spraying agent of 10% sulfuric acid in ethanol, and then left to incubate at 105^{o}C.

Danpi:

Crude drug powder was weighed about 1.0 gram then was put into 25 ml Erlenmeyer flask. The flask was filled by 10 ml of diethyl ether then was shaken for 10 minutes. After that, the extract was evaporated until dryness, after that 2.0 ml of absolute ethanol was added to the dried extract. The ethanol extract was spotted on TLC plate (silica gel GF_{254}) about 10 μl, using paeonol 0.5 mg/ml in acetone as standard solution. TLC plate was developed in the mixture of cyclohexane and ethyl acetate (3:1). The plate was air dried then detected for bluish-brown spots by using the spraying agent of 5% ferric chloride in ethanol, acidified by hydrochloric acid, and then left to incubate at 105 ^{o}C.

4) Determination of water

Shanzhuyu, Fuling and Danpi were determined their water content by loss on drying and the methods of toluene distillation.

Drying in oven method:

Crude drugs powdered was weighed accurately in range of 2-5 g (depending on the texture of the powder). Then, it was placed in weighing bottles which accurate weights are known. In the weighing bottles, the height of the powder measured from the bottom of the bottle was not higher than 5 mm for dense texture and 10 mm for loose texture. Then, the powder was dried in hot air oven at 105 ^{o}C for 5 hours, left to cool down by placing the bottles in desiccators for 30 minutes and then the bottles were weighed. The drying process was repeated by leaving the bottles in the oven for 1 hour, left to cool down for 30 minutes and weighed again. If the difference of the weights obtained from the 5-hour oven dry and the 1-hour oven dry was less than 5 mg, the percentage loss on drying (w/w) was calculated. If it was higher than 5 mg, the process was repeated by drying the bottle for 1 hour, cooled down and weighed until the difference was lower than 5 mg then calculated the percentage loss on drying.

Toluene distillation method:

Toluene (200 ml) and wate (2 ml) were filled into a round flask. The flask was connected to the toluene distillation apparatus (Figure 3.1) [48]. The mixture was distilled for 2 hours and left to cool down the apparatus then recorded the volume of distilled water in the receiving tube. Crude drug powdered, which was anticipated to yield about 1-4 ml water content, was weighed accurately and added into the round flask, followed by adding of glass bead then heated. When the toluene started to boil, the distill

temperature was adjusted to distill the mixture at the rate of 2 drops/second. When the water content in the receiving tube did not increase, the distill temperature was adjusted to distill the mixture at the rate of 4 drops/second and left at that rate for a while. After that the condenser was rinsed by using toluene, then continued the distillation for 5 minute, finally cooled down the apparatus. The volume of water in the receiving tube was recorded for calculation of percentage of water content (v/w).

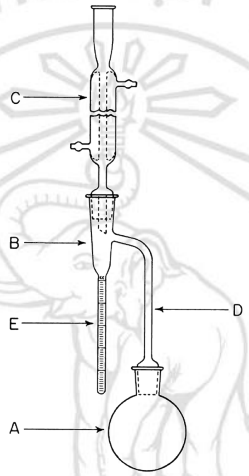


Figure 3.1: Toluene distillation apparatus; round flask (A), trap (B), condenser (C), connecting tube (D) and receiving tube (E) (picture from http://www.pharmacopeia.cn/v29240/usp29nf24s0_c921.html; accessed 10 Feb 2014)

5) Determination of ash

Total ash:

Sliced crude drug (Shudihuang) and crude drugs powder (Shanzhuyu, Zexia, Fuling and Danpi) were weighed accurately in range of 3-5 g and placed into the crucibles whose accurate weight was known, then ignited at 500-600^{o}C until they turned to white ash without carbon. The white ash was weighed and calculated for the percentage of total ash.

Acid-insoluble ash:

2 M hydrochloric acid (25 ml) was added into the crucibles that contained the white ash obtained from the first step. It was then boiled for 10 minutes and filtered by using No. 41 ashless paper. The obtained ash on the filter paper was washed with hot water to ensure neutralization. The ash and the paper were then put into crucible, ignited at 500-600 ^{o}C until white ash without carbon was shown. After that, weighed the white ash and the percentage of acid-insoluble ash was calculated.

6) Determination of extractive value

Shudihuang and Shanzhuyu were determined their water soluble extractive value. Danpi was evaluated the ethanolic extractive value.

Determination of water soluble extractive value:

Sliced crude drug (Shudihuang) and crude drug powder (Shanzhuyu) were weighed accurately for 4 g and placed into 250-300 ml Erlenmeyer flasks. Water (100 ml) was added and left the mixture to be shaken on the shaker for 6 hours, and then the flasks were left on the stand for 18 hours. The extract was filtered by using No.1 filter paper. The obtained filtrate was put into the well-known accurate weight evaporating dishs for 20 ml, then the dishs were left to evaporate on the water bath to obtain dried extract. After that, the dishs with the dried extract were placed into 105 ^{o}C hot air oven for 3 hours, and cooled down in desiccators for 30 minutes and then recorded their weight. After that, the dishs with the dried extract were placed into 105 ^{o}C hot air oven again for 1 hour, and repeated cooled down 30 minutes, and then recorded their weight. If the difference of the weights obtained from the 3-hour hot air oven and the 1-hour was less than 5 mg, the percentage of extractive (w/w) was calculated. If it was higher than 5 mg, the process was repeated by drying the dishs with the

extract for 1 hour, cooled down and weighed until the difference was less than 5 mg then calculated the percentage of extractive.

Determination of ethanol soluble extractive value:

Crude drugs powder was weighed accurately in range of 2-4 g and put into a round flask. 95% ethanol with accurate volume in range of 50-100 ml was filled into the flask then the weight of the flask with the mixture was recorded. The flask was connected to the reflux apparatus (Figure 3.2)[49], boiled for 1 hour and cooled down then weighed the flask for impletion of evaporated solvent. The extracted was filtered using No.1 filter paper. Twenty five ml of the obtained filtrate was put into the evaporating dishs which accurate weight was known and left to evaporate on the water bath until the extract was dried. After that, the dishs with the dried extract were placed into the 105 ^{o}C hot air oven for 3 hours, cooled down in desiccators for 30 minutes, and then recorded their weight. After that, the dishs with the dried extract were placed into 105 ^{o}C hot air oven again for 1 hour, and repeated cooled down 30 minutes, and then recorded their weight. If the difference of the weights obtained from the 3-hour hot air oven and the 1-hour was less than 5 mg, the percentage of extractive (w/w) was calculated. If it was higher than 5 mg, the process was repeated by drying the dishs with the extract for 1 hour, cooled down and weighed until the difference was less than 5 mg then calculated the percentage of extractive.

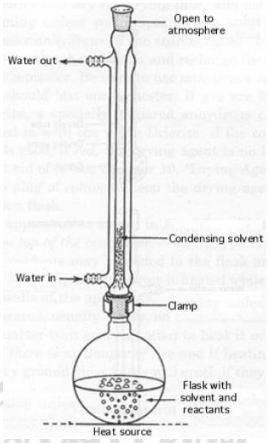


Figure 3.2: Reflux apparatus (picture from http://hazards.tees.ac.uk/rams3/documents/Solvent%20extraction%20hair.pdf; accessed 10 Feb 2014)

7) Determination of chemical composition

Shanzhuyu, and Danpi were determined the chemical content by High Performance Liquid Chromatography coupled with Photo Diode Array detector (HPLC-PDA).

Shanzhuyu:

The standard solution was 400 μg/ml of loganin in 80 % methanol. The test solution was prepared by weighing the crude drug powdered was at 1.0 g accurately and put into a round flask, after that filled the flask with 25 ml of 80 % methanol then weighed the mixture-containing flask and recorded the weight. The mixture was refluxed for 1 hour, cooled down then weighed the flask for impletion of evaporated solvent. The extract was filtered by using No.1 dried filter paper, then 0.45 micron nylon membrane to obtain test solution.

The standard and test solution were injected each 10 μl into HPLC instrument. A stationary phase of chromatographic system was C18

reversed phase silica gel without controlling the column temperature and a mobile phase was mixture of acetonitrile (line A) DI water (line D). The flow system was isocratic of 15% line A and 85% line D with 1 ml/min flow rate. Detection wavelength was 240 nm.

Danpi:

The standard solution was 100 μg/ml of paeonol in methanol. The test solution was prepared by weighing the crude drugs powdered at 0.5 g accurately, after that filled the flask with 25 ml of 80 % methanol then weighed the mixture-containing flask and recorded the weight. The mixture was extracted by ultrasonic for 30 minutes cooled down then weighed the flask for impletion of evaporated solvent. The mixture was filtered by using No.1 dried filter paper. Measure accurately 5 ml of the obtained filtrate to a 10 ml volumetric flask, added methanol to volume, mixed well then filtrated by 0.45 micron nylon membrane to obtain the test solution.

The standard and test solution were injected each 10 μl into HPLC instrument. A stationary phase of chromatographic system was C18 reversed phase silica gel without controlling the column temperature and a mobile phase was mixture of methanol (line C) and DI water (line D). The flow system was isocratic of 45 % line C and 55 % line D with 1 ml/min flow rate. Detection wavelength was 274 nm.

3.5.2 Determination of dosage of LWDH water extract

1) Method of extraction

According to the available of the data of ability to prepared LWDH formula as decoction [33], two extraction methods; traditional decoction and water reflux, were performed.

Traditional decoction method:

Six crude drugs; Shudihuang, Shanzhuyu, Shanyoa, Zexia, Fuling and Danpi were weighed in ratio of 24:12:12:9:9:9, respectively, and put into a pot. The water was added and boiled. The mixture was left for 30-60 minutes after boiling or until the volume decreased to 1/3. Then the extract was filtrated. After that, the residue was repeatly extracted 2 times. All the extracts were mixed together and filtered by using the fabric, then cotton, and then No.1 filter paper. The obtained filtrate was concentrated by using the rotary evaporator. The concentrated extract was sent to Postharvest Technology Research Institute of Faculty of Agriculture Chiang Mai University for freeze drying.

Extraction method by water reflux

Six crude drugs (Shudihuang, Shanzhuyu, Shanyoa, Zexia, Fuling and Danpi) were weighed in the ratio of 24:12:12:9:9:9, and put into a round flask. The water was added and extracted by reflux for 3 hours for 3 times. All the extracts were mixed together and filtered by using the fabric, then cotton and then No.1 filter paper. The obtained filtrate was concentrated by using the rotary evaporator. The concentrated extract was sent to Postharvest Technology Research Institute of Faculty of Agriculture Chiang Mai University for freeze drying.

2) Method of dosage determination

The method to determine the dosage was carried out by HPLC. The condition of chromatographic system was applied from the methods for analysis of LWDH formula found in the Pharmacopoeia of the People's Republic of China (2005) [2] and the research article titled “An approach based on HPLC-fingerprint and chemometrics to quality consistency

evaluation of Liuwei Dihuang Pills produced by different manufacturers" by Baogang Xie et al. [50]. The extract with lower dosage equilibrium to traditional dose was continued to the next step of tablet development.

Each LWDH extract was weighed accurately for 1.0 g and put into a 100-150 ml Erlenmeyer flask then was filled with 40 ml of 50% ethanol. The mixture was extracted by ultrasonic for 30 minutes, cooled down, refilled the evaporated solvent and then the extract was filtered by using No.1 filter paper. Measure accurately 5 ml of the obtained filtrate to a 10 ml volumetric flask, added 50 % ethanol to volume, mixed well then filtrated by 0.45 micron nylon membrane to obtain the test solution. After that analysis of loganin content was performed.

The test solution (100 μl) was injected into HPLC instrument, compared with 10 μl of 200 μg/ml loganin in 50 % ethanol (standard solution). A stationary phase of chromatographic system was C18 reversed phase silica gel with 35 ^{o}C temperature control and a mobile phase was the mixture of acetonirile (line A) and 0.015 % phosphoric acid (line B). The flow system was gradient flow system: 0-3 min (start), isocratic line A = 5 %; 3-30 min, gradient line A to 60 %; 30-33 min, gradient line A to 70 %; 33-49 min isocratic line A = 70 %; 49-50 min, gradient line A to 5 %; 50-60 min (stop), isocratic line A = 5 %, at flow rate of 1 ml/min. Detection wavelength was 236 nm. The loganin content equilibrium to traditional dose must not be lower than 4.5 mg. The approximate dose of LWDH extract, which equals to the dose of traditional dosage form, was calculated by area under the curve (AUC) comparison using absolute method.

3.5.3 Tablets formulation development

1) Adsorbent selection

Since LWDH water extract was semi-solid and sticky. Therefore the adsorbent is required to transform semi-solid into solid for developing tablet dosage form. The adsorptibility of 3 kinds of adsorbents (lactose, corn starch and Avicel® PH101); which commonly used in Chinese herbal manufacturing [51], was investigated.

Steps of study were as follows;

(1) The extract was weighed accurately for 500 mg and put into a small mortar.
(2) Adsorbent was put on a watch glass, weighed the watch glass and recorded the weight (weight A).
(3) The absorbent was added and slowly mixed with the extract until the semi-solid extract transformed into powder.
(4) The watch glass was weighed and recorded the weight again (Weight B).
(5) The quantity of the adsorbent for powder reformation was calculated by the difference of weight before and after adding the adsorbent (Weight A – Weight B).

The powders associated with adsorbent and LWDH extract were evaluated their pressure-hardness profile. The adsorbent which gave the quantity ratio for adsorption not more than 1:2 and can promote a good pressure-hardness profile was used as an excipient for development of tablets formulation.

2) Tablets formulation development

After obtaining the desired adsorbents, the suitability of tableting must be investigated. The suitability is based on 2 properties; friability and disintegration. The steps of the study are listed below;

(1) The extract was mixed with the adsorbents to gain wet mass appearance by geometric dilution technique using mortar and pestle.
(2) The wet mass was passed through No.8 sieve to produce wet granules.
(3) The wet granules were dried in a hot air oven at 50^{o}C until loss on drying was not more than 5% to produce dry granules.
(4) The dry granules were passed through No.12 sieve and weighed. The weight was recorded.
(5) Talcum, magnesium stearate and Cab-osil® (tableting excipients) were weighed with the ratio of 1% of the granules weight.
(6) The granules and tableting excipients were mixed by tumbling for 1 minute.
(7) The mixture was compressed by using a single stroke tableting machine. The finished tablets were collected.

The finished tablets were evaluated their parameter of friability and disintegration time for preliminary study of effect of adsorbents to the parameters. The evaluation results were used for development of the suitable formulation.

3) Quality control of LWDH tablets

The suitable tablets formulation was scaled up to evaluate quality. The steps are as follows;

3.1) Physical appearance

The appearance of the finished tablets (shape, diameter, thickness, color, odor, and taste) was observed and recorded.

3.2) Weight variation test

(1) 20 tablets were picked up randomly.
(2) Each tablet was weighed by analytical balance. The weight was recorded.
(3) Average weight, Standard Deviation (SD) and percentage of variation were calculated.

3.3) Friability test

(1) 20 tablets were picked up randomly and weighed. The weight was recorded.
(2) The tablets were put into the friability test apparatus. The apparatus was allowed to circulate for 100 rounds in 4 minutes.
(3) 20 tablets were observed for capping and laminating, and then weighed again.
(4) The percentage of the friability was calculated.

3.4) Hardness test

(1) 10 tablets were picked up randomly.
(2) Each tablet was tested for its hardness by hardness tester. The hardness was recorded.
(3) Average hardness was calculated.

3.5) Disintegration time

(1) 6 tablets were picked up randomly.
(2) Each tablet was put into the basket in "Basket-Rack" assembly disintegration tester with 37.5^{o}C water as a medium.
(3) The tester was run for 30 minutes.
(4) After 30 minutes, the fragments of tablets in the basket were observed.

3.6) Assay of active marker

(1) 10 tablets were picked up randomly and weighed.
(2) The average weight of 1 tablet was calculated.
(3) 10 tablets were ground into granules by using the mortar and pestle.
(4) The ground granules were weighed for their average weight and put into Erlenmeyer flasks.
(5) 40 ml of 50% ethanol was added into the flasks.
(6) Methods of preparation of test solution, standard solution and condition of HPLC assay were the same as the topic 2) of 3.5.2.
(7) The percentage of the label amount was calculated.

3.7) Contamination test

200 tablets were put into the laminate packages, sealed with heat and submitted to the Regional Medical Science Center 1 Chiang Mai for contamination testing. The first 100 tablets were tested for heavy metal contamination test and the others for microbial contamination test.

The tablets whose quality reached the test standard would be applied for the further step of stability study.

3.5.4 Test of tablets stability

Tablets were packed by 2 packaging methods as follows;

- The tablets were put into a glass bottle with light prevention (container B).
- The tablets were packed in the laminate package and sealed by heat. Then, the package was stored in a glass bottle with light prevention (container L).

The packages of tablets were stored the stability testing cabinets under 2 conditions; 30ºC/65%RH (standard condition) and 40ºC/75%RH (accelerated condition). The stability test was carried out on day 0, day 30 (1 month) and day 90 (3 months) in order to investigate physical appearance, friability, hardness, disintegration time and assay of active marker. Tablet's shelf-life labeling was calculated by investigating the difference of the results from day 0 and the day of testing (30 and 90 days).