CHAPTER III

MATERIALS AND METHODS

Materials

1. Chemicals and reagents

1.1 Acetonitrile, HPLC grade (Fishers)

1.2 Anthrone reagent (Sigma)

1.3 Chloroform, AR grade (Lab scan)

1.4 Dichloromethane (Lab scan)

1.5 Di-potassium hydrogen phosphate (Sigma)

1.6 Ethanol (Lab scan)

1.7 Ethyl acetate (Lab scan)

1.8 Filter paper (Whatman, Number1)

1.9 Membrane filter (sartolon, diameter 47 and 13 mm)

1.10 Methanol, AR and HPLC grade (Lab scan)

1.11 n-butanol (Lab scan)

1.12 Ortho-phosphoric acid (Lab scan)

1.13 Silica gel (E. Merck)

1.14 Sodium carbonate (Sigma)

1.15 Solid-phase cartridge, C18 500 mg 3ml (Varians)

1.16 Sulfuric acid (Lab scan)

1.17 TLC plate silica gel 60 F254 (E. Merck)

1.18 Asiaticoside, standard (Quangxi chemical, 90.0%)

1.19 Asiatic acid, standard (Quangxi chemical, 95.0%)

1.20 Madecassoside, standard (Quangxi chemical, 95.0%)

1.21 Madecassic acid, standard (Quangxi chemical, 95.0%)

1.22 Prednisolone, standard (Sigma, 98.4%)

2. Instruments

2.1 Hot air oven (OMRON)

2.2 Electric mill (Retsch Muhle)

2.3 Rotary evaporator (BUCHI)

2.4 pH meter (Consort C231)

2.5 Densitometer (Shimadzu : CS9301PC)

2.6 High-Performance Liquid Chromatography (shimadzu)

2.6.1 Pump : solvent delivery system module LC-10ADvp

2.6.2 Autosampler : SIL-10ADvp

2.6.3 Degasser : DGU-14A

2.6.4 Detector : Diode-array detector SPD-M10Avp

2.6.5 Controller : System controller SCL-10Avp

2.6.6 Software : Class VP

Methods

3.1. Collection of plant sample and storage

3.1.1 Sources of plant sample

CA sample were collected from two gardens (source A and source B) in Nongdindang district, Ampur Muang of Nakornpathom province.

Figure 3.1 Picture of CA garden in Nakornpathom province

3.1.2 Sampling time

The plant sample was collected during the middle of each month for a year (from January to December, 2003) and the collected plant were refrained from insecticide one week before harvesting of sample.

The plants were cutted about two inch above the ground. At least 10 kg of fresh plant were collected each month.

3.1.3 Storage of plant sample

Fresh collecting plant was cleaned with water to remove soil and other solid particle, and then air dried at room temperature for three days and subsequently dried by hot air oven at 50 °C for 24 hour. The dried plant samples were ground with milling machine. The dried ground plant samples were kept in 3 layers' polyethylene bag and the bag was kept in dry place.

3.2. Extraction and Isolation of triterpene glycoside

Scheme 3.1 is the diagram of extraction and isolation of triterpene glycoside. The whole dried ground plant (300 g) was macerated with three litres of a mixture of methanol-water (70:30) for three days and then filtered by vacuum suction. The filtrate collected and marc process of residue was repeated twice.

The collected filtrates were combined collecting and partitioned with dichloromethane and n-butanol, respectively. The n-butanol fraction was partition with 0.2 N sodium carbonate solution and finally with water. The combine n-butanol layer was concentrated under reduced pressure at 60 °C. Ethyl acetate was added to the concentrated n-butanol and the triterpenoid precipitate (powder A) was obtained.

3.3. Preparation of AS, AA, MS and MA as working standard

3.3.1 Preparation of AS and MS

The 100 mg of powder A was chromatographed over a silica gel column and eluted with the mixture of dichloromethane:methanol:water with increasing polarity to separate AS and MS. Pure compounds (AS and MS) were finally purified by crystallization with methanol and methanol-acetone respectively.

3.3.2 Preparation of AA and MA

Scheme 3.2 is the diagram of alkaline hydrolysis of glycosides to aglycones. The 2.5 g of powder A was dissolved in methanol 50 ml and then added 283 ml of 5% potassium hydroxide. The mixture was refluxed for four hours at 80 °C, the mixture was neutralized with glacial acetic acid and partitioned

twice with 100 ml of dichloromethane. Collected dichloromethane layer was evaporated under reduced pressured to dryness to obtain powder B.

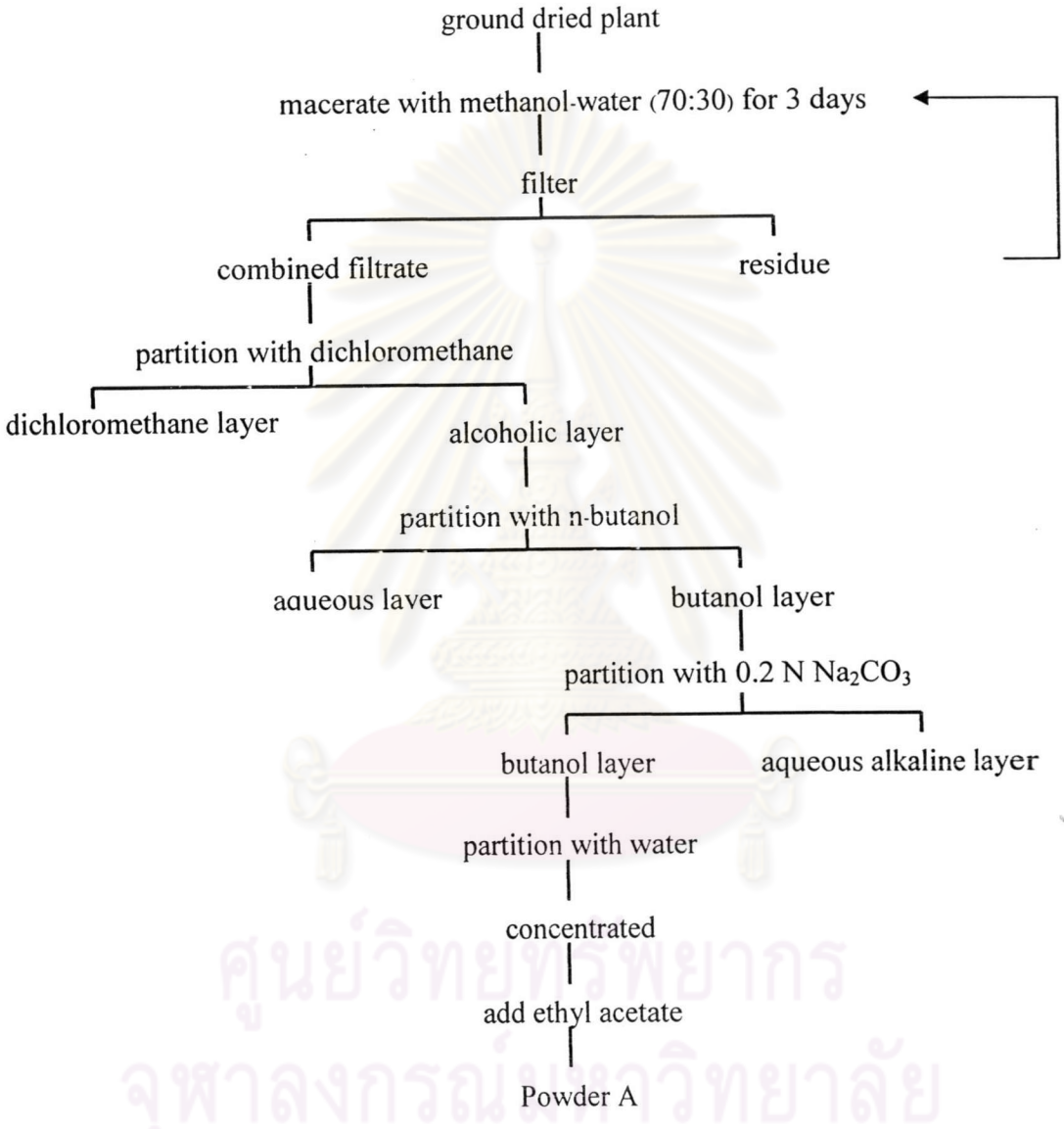
The 100 mg of powder B was loaded to a silica gel column which was eluted with the mixture of dichloromethane:methanol (9:1) to obtain AA and MA.

Pure AA and MA were obtained by crystallization with methanol.

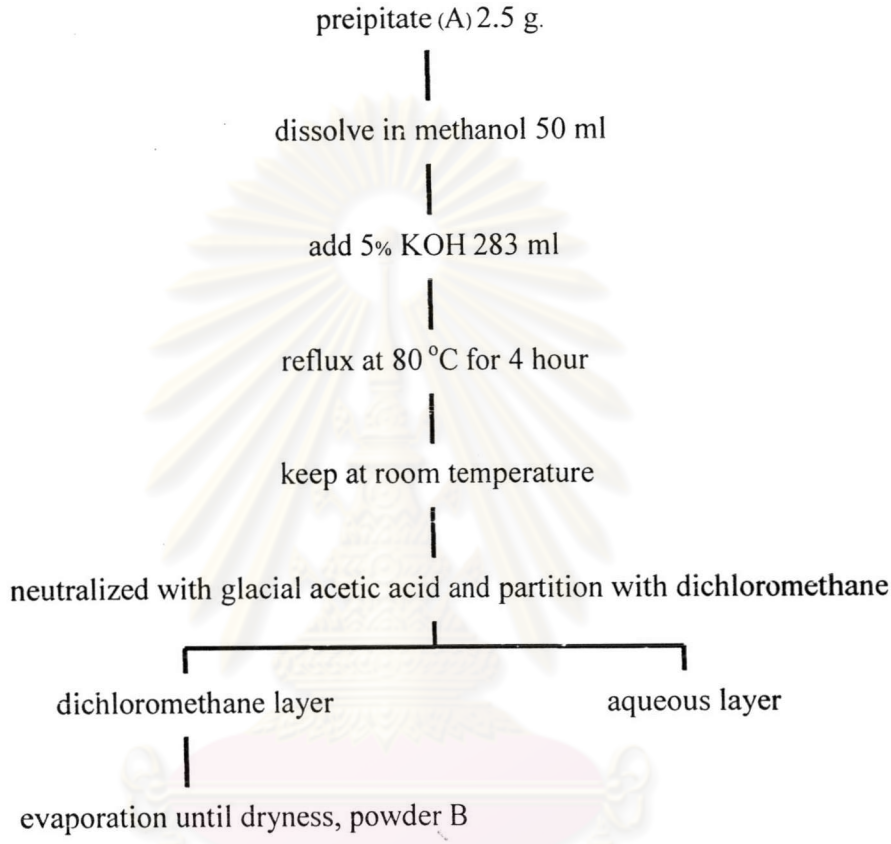
3.3.3 Identification of triterpene glycoside and its aglycone

3.3.3.1 TLC method

Each isolated compounds and each standard AS, MS, AA and MA were dissolved in methanol. The solution was applied on a silica gel plate, which was then developed in suitable mobile phase, after that, the R_f value of each compound were detected and compared to the standard AS, MS, MA and AA.



Scheme 3.1 Extraction and isolation scheme for triterpene glycoside from CA



Scheme 3.2 Diagram of alkaline hydrolysis of glycoside

3.3.3.2 HPLC method

Ten mg of each isolated compound and standard were dissolved in methanol and adjusted volume to 100.0 ml (0.1 mg/ml, concentration). The solution was injected to the HPLC system and then compared the retention time of each compounds to standard AS, MS, MA and AA.

HPLC system

Column	Hi-Q sil C18, 4.6 x 150 mm, 5 micron
Mobile phase	Acetonitrile-phosphate buffer (10 mM), pH 7.1 (29:71)
Flow rate	1 ml/min
Detector	Photo-diode array at 210 nm
Injection volume	20 µl

3.3.3.3 Spectroscopic methods

3.3.3.3.1 Infrared Spectroscopic method (IR)

Two or three mg of each isolated compound were mixed and ground with about fifty mg of previously dried potassium bromide (KBr). The solid mixture was compressed to thin film KBr disc and was scanned with IR spectrophotometer.

3.3.3.3.2 Nuclear-Magnetic Resonance (NMR)

Ten mg of each isolated compound was dissolved with deuterated DMSO in a NMR tube. The mixture was measured in the NMR machine.

3.3.3.4 Physical properties

Approximately one mg of isolated compound were packed and trapped in a capillary melting point tube. The melting points were measured by a melting point apparatus.

3.3.4 Determination of purity of isolated AS, MS, AA and MA (as working standard)

3.3.4.1 Preparation of reference standard solution

Accurately weighed about 10 mg of each reference standard and dissolved in a mixture of 50% methanol. The mixture was adjusted volume to 100.0 ml to obtain the 0.1 mg/ml of standard solution.

3.3.4.2 Preparation of working standard solution

Accurately weighed about 10 mg of each working standard and dissolved in a mixture of 50% methanol. The mixture was adjusted volume to 100.0 ml to obtain the 0.1 mg/ml of working standard solution

3.3.4.3 Analysis of standard and working standard solutions

The solutions from 3.3.4.1 and 3.3.4.2 were injected to a HPLC system, the purity of each working standard were determined and calculated from the peak area ratio compared to the reference standard.

The HPLC system was the same as in 3.3.3.2

3.3.4.4 Calculation

$$\% \text{ purity} = \frac{Au \times Ws \times \%\text{purity of reference standard}}{As \times Wu}$$

Where

Au = Peak area of sample solution

As = Peak area of standard solution

Wu = Weight of sample (mg)

Ws = Weight of standard (mg)

Purity of reference AS standard	=	90.0 %
Purity of reference AA standard	=	95.0 %
Purity of reference MS standard	=	95.0 %
Purity of reference MA standard	=	95.0 %

3.4. Development of analytical method for quantitative determination of AS, MS, AA and MA

3.4.1 Development of sample preparation

3.4.1.1 Extracting solvent and refluxing time

A five gram of dried ground plant sample was refluxing in the mixture of 60, 70 and 80% methanol-water for two hours. The mixture was filtered and cooled and finally injected into the HPLC systems. The contents of each triterpenoid compound were calculated.

A five gram of dried grounded plant sample was refluxing in the mixture of 80% methanol-water for 0.25, 0.50, 0.74, 1, 1.5, 2 and 3 hours. The mixture was filtered and cooled and finally injected into the HPLC systems. The contents of each triterpenoid compound were calculated.

3.4.1.2 Sample preparation clean up by solid phase extraction

3.4.1.2.1 Impurities eluting solvent

Two millitres of sample solution was loaded onto a 500 mg C18 SPE cartridge. A three millitres of the mixture of 0%, 10%

and 15% of acetonitrile in water were used to wash the impurities from the SPE. The washed eluent was injected into the HPLC system to detected the absence of the triterpenoid compounds.

3.4.1.2.2 Analytes eluting solvent

Two millitres of sample solution was loaded onto a 500 mg C18 SPE cartridge. A three millitres of mixture of 10% of acetonitrile in water were used to wash the impurities from the SPE. Four individual cycles of 1 ml of the mixture of 45% of acetonitrile in 10 mM (pH 7.10) phosphate buffer was used to elute the analytes out of the SPE cartridge. The eluent was injected into the HPLC system to detect the completeness of the eluting of the triterpenoid compounds.

3.4.2 Development of high-performance liquid chromatographic method (HPLC method)

3.4.2.1 Criteria of the method development

Acceptance criteria of the ICH guideline for the pharmaceutical analysis were used in this research as following; Resolution of each pair

of analyte should not less than 1.50, Tailing factor of each compound should not more than 2.0 and the number of theoretical plate of column used should not less than 2000. Moreover, solvent or buffering ions in the mobile phase should not interfere and detected at the wavelength of 210 nm.

3.4.2.2 Effect of the stationary phase

Two type of the stationary phase (octadecylsilylated-(C18) and phenyl-column) were used as stationary phase in this study.

For each stationary phase, the mixture of working standard solution was injected into the HPLC system. The following parameters were determined and considered as follow; peak resolution, retention time, run time, peak shape, etc.

3.4.2.3 Effect of mobile phase

3.4.2.3.1 Ratio of organic modifier

Various ratio of acetonitrile in phosphate buffer (25-30%) was used as mobile phase to elute the analytes for optimum condition.

3.4.2.3.2 Concentration and pH of buffer

Phosphate buffer at concentration 10 and 20 mM were used in the mixture of acetonitrile in phosphate buffer, pH 7.10 (29:71) to determine the optimum HPLC chromatogram.

Phosphate buffer, 10 mM at pH 7.00, 7.10, 7.50 and 8.00 were used in the mixture of acetonitrile in phosphate buffer (29:71) to determine the optimum HPLC chromatogram.

3.4.2.4 Internal standard

Several criteria were purposed in finding the suitable internal standard as following; stable compound, no interaction with an analyte, detectable at interested wavelength (210nm), resolve from the other component in the matrix sample. The following compounds were studied in process of finding internal standard; prednisolone, betametasone, dexametasone, hydrocortisone hemisuccinate, 18β-glycyrhetic acid, oleanolic acid, dipotassium glycyrhezinate, fluocinolone acetate and propyl paraben.

3.4.3 Development of thin layer chromatographic method (TLC method)

TLC silica gel plate (20x10 cm) and the mixture of chloroform:methanol: water (30:15:2) was used in this study. The developed plate was sprayed with anthrone reagent and lieberman Burchard's reagent and heated at 110 °C for 10 min. The plate was scanned with a densitometer at 525 nm every 15 min interval. Plot the degree of reflection versus time to determine the optimum time to develop stable color.

3.5. Method validation

3.5.1 Validation of HPLC method

The developed HPLC method was validated according to the ICH guideline on following parameter; accuracy, precision, specificity, quantitative limit, linearity and range.

3.5.1.1 Preparation of standard solution

3.5.1.1.1 Stock standard mixture solutions

1) Aglycone standard stock solutions

Three aglycone stock standard solutions (A_1, A_2 and A_3) were prepared according to table 3.1.

Table 3.1 Preparation of aglycone standard stock solutions for method validation

Solution	MA (mg)	AA (mg)	Volume (ml)
A_1	20.0	10.0	25.0
A_2	25.0	12.5	25.0
A_3	30.0	15.0	25.0

2) Internal standard stock solution (IS)

Accurately weigh prednisolone 110 mg and dissolved in methanol and adjusted the volume to 25.0 ml.

3) Stock mixture standard solutions

Five stock mixture standard solutions (S_1, S_2, S_3, S_4 and S_5) were prepared according to table 3.2.

Table 3.2 Preparation of stock mixture standard solutions for method validation

Items	Solutions				
	S_1	S_2	S_3	S_4	S_5
MS (mg)	7.0	13.0	27.0	33.0	40.0
AS (mg)	5.0	10.0	20.0	25.0	30.0
A_1 (ml)	1.0	2.0	4.0	-	-
A_2 (ml)	-	-	-	4.0	-
A_3 (ml)	-	-	-	-	4.0
IS (ml)	1.0	1.0	1.0	1.0	1.0
Volume (ml)	10.0	10.0	10.0	10.0	10.0

3.5.1.1.2 Working standard solutions

Five working standard solution (WS_1, WS_2, WS_3, WS_4 and WS_5) were prepared by diluting of each 1.0 ml of stock mixture

standard solutions (S_1, S_2, S_3, S_4 and S_5) to 10.0 ml with the mobile phase.

Table 3.3 Concentration of working standard solutions

Items	Concentration of working standard solutions (mg/ml)				
	WS_1	WS_2	WS_3	WS_4	WS_5
MS	0.070	0.130	0.270	0.330	0.400
AS	0.050	0.100	0.200	0.250	0.300
MA	0.008	0.016	0.032	0.04	0.048
AA	0.004	0.008	0.016	0.020	0.024

3.5.1.2 Preparation of sample solutions

3.5.1.2.1 Extracted solution

A five gram of dried ground herb was refluxed with 90 ml of the mixture of 80% methanol in water for 1.5 hour. The extract solution was cooled down to room temperature, filtered and adjusted volume to 100.0 ml with water.

3.5.1.2.2 Sample solutions

For each five sample solution, sample solutions (E_1, E_2, E_3, E_4 and E_5) were prepared by pipet 5.0 ml of extracted solution, then added 1.0 ml of each stock mixture standard solutions (S_1, S_2, S_3, S_4, S_5) and adjusted volume to 10.0 ml with mobile phase.

Another sample solution (without standard addition, E_0) was prepared by pipet 5.0 ml of extracted solution and added 0.10 ml of internal stock standard solution and adjusted volume to 10.0 ml with mobile phase.

Table 3.4 Concentration of additional working standard solutions

Items	Concentration of additional working standard solutions (mg/ml)					
	E_0	E_1	E_2	E_3	E_4	E_5
IS	0.044	0.044	0.044	0.044	0.044	0.044
MS		0.070	0.130	0.270	0.330	0.400
AS		0.050	0.100	0.200	0.250	0.300
MA		0.008	0.016	0.032	0.04	0.048
AA		0.004	0.008	0.016	0.020	0.024

5.1.2.3 Clean up of sample solution

For each sample solution (E_0, E_1, E_2, E_3, E_4 and E_5), It was loaded 2.0 ml onto a SPE cartridge (500 mg). The impurities were washed out with acetonitrile:water (1:9) 3 ml. The analytes were eluted with three cycles of 1.0 ml of the mixture of acetonitrile:phosphate buffer, 10 mM pH 7.1 (45 : 55).

3.5.1.3 Method validation of sample analysis by HPLC

3.5.1.3.1 Accuracy

Six replications of each sample solutions (E_0, E_1, E_2, E_3, E_4 and E_5) were injected into the HPLC system and calculated as accuracy by using below equation.

The accuracy was determined in term of percent recovery (%R)

$$\%R = \frac{[XE_i - XE_0] \times 100}{WS_I}$$

Where

XE_i = total analytical concentration

XE_0 = analytical concentration from extract solution (E_0)

WS_I = concentration of added standard

3.5.1.3.2 Precision

Five replications of each sample solution (E_0, E_1, E_2, E_3, E_4 and E_5) were injected into the HPLC system in the same days. The percent recoveries of each of sample solution were calculated as repeatability precision (or intra-day precision) by using below equation.

Two replications of each sample solution (E_0, E_1, E_2, E_3, E_4 and E_5) were injected into the HPLC system for three days. The percent recoveries of each of sample solution in three days were calculated as intermediate precision (or inter-day precision) by using below equation.

The precision was determined in term of percent of coefficient of variation (%CV) or relative standard deviation (%RSD).

$$\%RSD = [SD / \bar{X}] \times 100$$

Where

SD = standard deviation of percent recovery

$\bar{X}$ = mean of percent recovery

3.5.1.3.3 Linearity and range

Each of sample solution (E_0, E_1, E_2, E_3, E_4 and E_5) was injected into the HPLC system. Plot graph between observed concentration and actuated concentration and calculate coefficient of determination (r^2) in term of linearity.

The linearity was determined by a series of three injections of five sample concentrations which were demonstrated as a graph between observed concentrations by analytical found of standard solution and actual concentrations by addition of standard solution.

The range was determined in term of interval concentration which demonstrated as good accuracy (96-104%R), precision (RSD $\leq$3%) and linearity ($r^2 \geq 0.99$).

3.5.1.3.4 Specificity

The standard and sample solutions were injected into the HPLC system. The UV spectrums of all analyte peaks in sample solution were compared with UV spectrums in standard solution.

The specificity was determined by photodiode array detector. Photodiode array permit the resolving of overlapping chromatographic peaks by deconvolution techniques, and they allow the detection of impurities that are hidden within chromatographic peaks.

3.5.1.3.5 Quantitation limit

Diluting solvent was injected into HPLC system to determine the noise. Standard solution of each analyte was diluted until the peak height of signal was more than 10 times of noise. The precision of integration was calculated from five repeatable injections of standard solution at quantitation limit.

The quantitation limit was determined from signal to noise ratio 10 times. The noise was evaluated by average peak height of diluting solvent at retention time of interested chromatographic

peaks (AS, MS, AA and MA). The signal was evaluated by injection of standard. Concentration of standard that was showed peak height of standard more than 10 time of noise is quantitative limit.

3.5.1.4 System suitability test

The system suitability test were determined by using 4 parameters (precision, number of theoretical plates, tailing factor and resolution) from five replicate injections of standard solution.

3.5.1.4.1 Precision

The system precision was calculated from five replicate injections of standard solution.

The precision was determined in term of percent of coefficient of variation (%CV) or percentage relative standard deviation (%RSD) by using below equation.

$$\%RSD = [SD / \bar{X}] \times 100$$

Where

SD = standard deviation of peak area

$\bar{X}$ = mean of peak area

3.5.1.4.2 Number of theoretical plates (N)

The number of theoretical plates was calculated from five replicate injections of standard solution by using below equation.

$$N = 16 \times [t_r / W]^2$$

Where

t_r = retention time

W = peak width

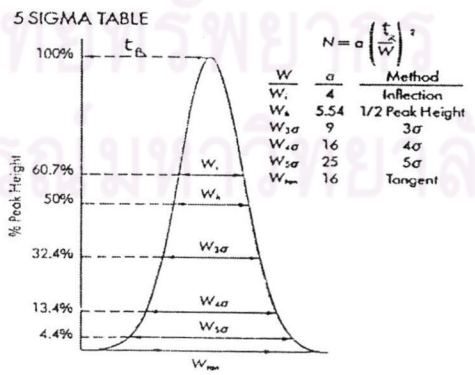


Figure 3.2 Chromatogram for calculation of Number of theoretical plates (N)

3.5.1.4.3 Tailing factor (T)

The tailing factor was calculated from five replicate injections of standard solution by using below equation.

Peak symmetry was determined in term of tailing factor.

$$T = W_{0.05} / 2 \times f$$

Where

$W_{0.05}$ = peak width at peak 5% height position

f = width of first half of peak (start to top) at the position of 5% peak height

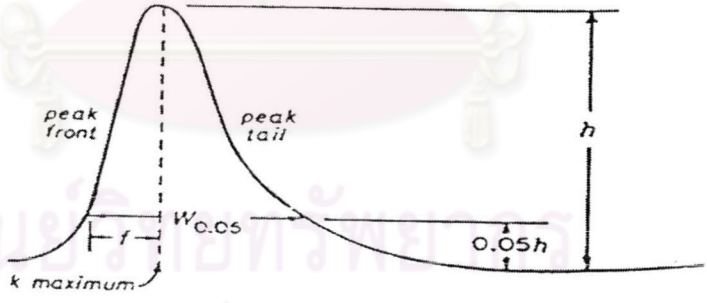


Figure 3.3 Chromatogram for calculation of tailing factor

3.5.1.4.4 Resolution (Rs)

The resolution was calculated from five replicate injections of standard solution by using below equation.

Resolution represents an ability of HPLC system to resolve analyte peak from other peak in the matrix.

$$Rs = 2 \times [t_{r2} - t_{r1}]/[W_1 + W_2]$$

Where

t_{r1} = retention time of the first peak

t_{r2} = retention time of the second peak

W_1 = peak width of the first peak

W_2 = peak width of the second peak

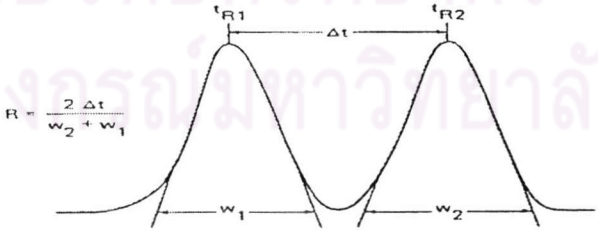


Figure 3.4 Chromatogram for calculation of resolution

3.5.2 Validation of TLC method

3.5.2.1 Preparation of standard solutions

Accurately weigh 25.0 mg of MS and 19.0 mg of AS and dissolved in 10.0 ml of methanol (2.5 mg/ml of MS and 1.88 mg/ml of AS). The mixture was pipeted 1.0 ml and diluted to 10.0 ml with methanol (0.25 mg/ml of MS and 0.19 mg/ml of AS).

3.5.2.2 Preparation of sample solutions

Five sample solutions were prepared according to table 3.5. Extract powder was contained 49.46% of MS and 35.23 % of AS which was assayed by HPLC method.

Table 3.5 Preparation of sample solutions for TLC method validation

Items	Solutions				
	1	2	3	4	5
Extract (mg)	10.0	20.0	100.0	150.0	200.0
methanol (ml)	10.0	10.0	10.0	10.0	10.0
Concentration of extract (mg/ml)	1.00	2.00	10.0	15.0	20.0

3.5.2.3 Spraying reagent

100 mg of anthrone was dissolved in 5 ml of concentrated sulfuric acid and diluted with 50 ml of ethanol.

TLC condition

Stationary phase	silica gel plate GF_{254} 10 x 20 cm
Developing solvent	chloroform : methanol : water (30 : 15 : 2)
Applied	2 µl

Densitometer parameter

Photo mode	Reflection
Scan mode	Linear
Set zero mode	At start
Beam size	0.4 x 1.0 mm.
Wavelength	525 nm.

The developed plate was sprayed with anthrone reagent and heated at 110 °C for 10 min. The plate was kept at room temperature for 30 min before determining the absorbance of AS and MS with densitometer at the wavelength of 525 nm.

3.5.2.4 Method of validation of sample determination by TLC

3.5.2.4.1 Accuracy

Six replications of each sample solution (E_0, E_1, E_2, E_3, E_4 and E_5) were applied to the TLC plate and calculated the accuracy by using below equation. The accuracy was determined in term of percentage recovery (%R)

$$\%R = [\text{analytical found} / \text{Actual amount}] \times 100$$

3.5.2.4.2 Precision

Six replications of each sample solution (E_0, E_1, E_2, E_3, E_4 and E_5) were applied to the TLC plate and calculated the precision.

3.5.2.4.3 Linearity and range

The linearity was determined by a series of three spots of five sample concentrations which were demonstrated as a graph between amount of analytical found and actual amount.

The range was determined in term of interval concentration which demonstrated good accuracy (96-104 %R), precision (%RSD $\leq$ 3) and linearity ($r^2 \geq 0.99$).

3.6. Determination of MS, AS, MA and AA in CA

3.6.1 HPLC method

3.6.1.1 Preparation of standard solutions

3.6.1.1.1 Stock standard mixture solutions

1) Acid standard solutions

Two aglycone stock standard solutions (A_1 and A_2) were prepared according to table 3.6.

Table 3.6 Preparation of acid standard solutions for sample analysis

Solution	MA (mg)	AA (mg)	Volume (ml)
A_1	20.0	10.0	25.0
A_2	30.0	15.0	25.0

2) Stock standard mixture solutions

Three stock standard mixture solutions (S_1, S_2 and S_3) were prepared according to table 3.7.

Table 3.7 Preparation of stock standard mixture solutions for sample analysis

Items	Solutions		
	S_1	S_2	S_3
MS (mg)	7.0	27.0	40.0
AS (mg)	5.0	20.0	30.0
A_1 (ml)	1.0	4.0	-
A_2(ml)	-	-	4.0
Volume (ml)	10.0	10.0	10.0

3) Internal standard solution

Prednisolone 10.0 mg was dissolved in methanol and adjusted volume to 25.0 ml.

3.6.1.1.2 Working standard solutions

1.0 ml of each stock standard mixture solution (S_1, S_2 and S_3) and internal standard solution were mixed together and adjusted volume to 10.0 with mobile phase as working standard solutions WS_1, WS_2 and WS_3, as sequentially.

Table 3.8 Concentration of working standard solutions

Items	Concentration of working standard solutions (mg/ml)		
	WS_1	WS_3	WS_5
MS	0.070	0.270	0.400
AS	0.050	0.200	0.300
MA	0.008	0.032	0.048
AA	0.004	0.016	0.024

3.6.1.2 Preparation of extract sample solutions

3.6.1.2.1 Extracted solution

Dried ground herb 5 g was refluxed with 90 ml of 80% methanol for 1.5 hour. After the extract solution was cooled down to

room temperature, the extract solution was filtered and was adjusted to volume 100.0 ml with water.

3.6.1.2.2 Sample solutions

5.0 ml of extracted solution and 1.0 ml of internal standard solution were mixed together and adjusted volume to 10.0 ml with mobile phase.

3.6.1.2.3 Clean up of sample solution

Sample was loaded 2.0 ml onto a SPE cartridge (500 mg). The impurities were washed out with acetonitrile:water (1:9) 3 ml. The analytes were eluted with three cycle of 1.0 ml of the mixture of acetonitrile:phosphate buffer, 10 mM pH 7.1 (45 : 55).

3.7.1.2 Preparation of sample solution (extract powder)

20.0 mg of sample was dissolved with 50.0 ml of mobile phase.10.0 ml of internal standard solution was pipetted to this solution and mixed together. After that, it was adjusted to 100.0 ml with mobile phase.

3.7.1.3 Stability of extract powder

200 mg of extract powder were packed in many glass bottles and kept in humidity/temperature control oven at 50 °C and 75%RH for 3 months. They were sampled every month to analyze.

20.0 mg of sample was dissolved with 50.0 ml of mobile phase. 10.0 ml of internal standard solution was pipetted to this solution and mixed together. After that, it was adjusted to 100.0 ml with mobile phase.

3.7.1 TLC method

3.7.1.1 Standard preparation

MS 30.0 mg and AS 20.0 mg were dissolved with methanol in volumetric flask 10.0 ml.

3.7.1.2 Sample preparation

5.0 ml of extracted solution was adjusted volume to 10.0 ml with water.

3.7.1.3 Pretreatment of sample solution

Sample was loaded 2.0 ml onto a SPE cartridge (500 mg). The impurities were washed out with acetonitrile:water (1:9) 3 ml. The analytes were eluted with methanol 1 ml for three cycles. After that, it was evaporated to dryness with nitrogen gas. The residue was dissolved with methanol 200 µl.