CHAPTER III

MATERIALS AND METHODS

Materials

1. Methyl 4-hydroxybenzoate (Lot No. 406565/1, Fluka, Buchs, Switzerland)
2. Ethyl 4-hydroxybenzoate (Lot No. 379752/1, Fluka, Buchs, Switzerland)
3. Propyl 4-hydroxybenzoate (Lot No. 404395/1, Fluka, Buchs, Switzerland)
4. Bytyl 4-hydroxybenzoate (Lot No. 381657/1, Fluka, Buchs, Switzerland)
5. Alprazolam (Lot No. 220103, Supplied by Siam Pharmaceutical Ltd., Bangkok, Thailand)
6. Clonazepam (Lot No. C500001-271, Supplied by Siam Pharmaceutical Ltd., Bangkok, Thailand)
7. Diazepam (Lot No. R50293010, Supplied by Siam Pharmaceutical Ltd., Bangkok, Thailand
8. Lorazepam (Supplied by Siam Pharmaceutical Ltd., Bangkok, Thailand)
9. Soybean oil (Lot No. 417782/1, Fluka, Buchs, Switzerland)
10. Dimethyl isosorbide (Arlasolve® DMI, Supplied by The East Asiatic Ltd., Bangkok, Thailand)
11. Methanol HPLC (Labscan Ltd., Bangkok, Thailand)
12. Isopropanol (Labscan Ltd., Bangkok, Thailand)
13. Phospholipids (Epikuron® 200, Lucas Meyer, Germany)
14. Glycerol (Sigma Chemical, St. Louis, USA)

Equipment

1. Analytical Balance (Sartorius, A200S, Germany)
2. High pressure homogenizer (EmulsiFlex C5, Avestin, Canada)
3. High performance liquid chromatography (HPLC) instrument consisted with the following
    - System controller (SCL-10 A VP, Shimadzu, Japan)
    - Liquid chromatograph pumps (LC-10 AD VP, Shimadzu, Japan)

- UV-VIS detector (SPD-10 A VP, Shimadzu, Japan)
- Auto-injector (SIL-10 AP VP, Shimadzu, Japan)
- Degasser (DGU-14A, Shimadzu, Japan)

4. Column HPLC
    - Bondclone C18 10 μm, 3.9×300 mm, Phenomenex
    - Symmetry C8 5 μm, 150 x 3.9 mm, Waters, USA
5. Syringe filter size (13 mm diameter, 0.45 μm pore size, Orange Sci, Belgium)
6. Solid phase extraction cartridge (Extra-Sep™ H.L. C18)
7. Photon correlation spectroscopy (Zetaplus™, Brookhaven Instruments Ltd., USA)
8. Laser diffraction analyzer (Mastersizer™2000, Malvern Instrument Ltd., United Kingdom)
9. Ultracentrifuge (L 80, Beckman, USA)
10. UV visble spectrophotometer (Shimadzu, Japan)
11. Sonicator (Transsonic digital S, Germany)
12. Vacuum filtration apparatus with sinter glass fiber (Waters, USA)
13. pH meter (Thermo Orion™, Boston, USA)

Methods

I The assay of alkyl-4-hydroxybenzoate and benzodiazepine drugs

1.1 UV-spectrophotometry

UV-spectrophotometry method was performed to determine aqueous solubility of alkyl-4-hydroxybenzoate compounds and benzodiazepine drugs. The standard curve of model drug was constructed. About 100 mg of model drugs was accurately weighed and dissolved in 50 ml of methanol. Appropriate dilutions with double distilled water were made to obtain standard solutions of known concentration (2-20 μg/ml). The absorbance of these solutions was determined in a 1 cm cell at the wavelength of 256 and 254 nm for alkyl-4-hydroxybenzoate and benzodiazepine drugs (diazepam and lorazepam), respectively, with UV-spectrophotometer apparatus (Shimadzu, Japan) and using double distilled water as a blank. The absorbances

obtained versus known drug concentrations were fitted to a straight line using linear regression analysis.

1.2 High Performance Liquid Chromatography (HPLC)

The high performance liquid chromatography with ultraviolet detector was utilized to determine oil solubility, partition coefficient, the content of drug in submicron emulsion preparation and the amount of drug in various phases of submicron emulsion. The standard curve of model drug was established. About 50 mg of drug was accurately weighed into 50 ml volumetric flask. Drug was completely dissolved with methanol (HPLC grade). The stock solution was diluted with mobile phase to obtain the concentration of 1-10 μg/ml. Stock solution of corresponding internal standard was added into each concentration of standard solution to the concentration of 6 μg/ml and then adjusted to volume with mobile phase. The mobile phase was freshly prepared and filtered through a 0.45 μm membrane filter and then degassed for 45 minutes before used.

The HPLC was performed by using a reverse phase column, Symmetry C8, 5 μm, 150 x 3.9 mm for paraben esters analysis and Bondclone C18, 10 μm, 300 x 3.9 mm for benzodiazepine drugs analysis.

HPLC conditions were as follows:

1.2.1 Mobile phase

- Methanol: water=60: 40 for methyl-4-hydroxybenzoate and propyl-4-hydroxybenzoate analysis
- Methanol: water=65: 35 for ethyl-4-hydroxybenzoate and butyl-4-hydroxybenzoate analysis
- Methanol: water=70: 30 for benzodiazepine drugs analysis

1.2.2 Standard solution: paraben esters or benzodiazepine drugs in mobile phase in the concentration of 1-10 μg/ml

1.2.3 Internal standard

- methyl-4-hydroxybenzoate for propyl-4-hydroxybenzoate analysis
- ethyl-4-hydroxybenzoate for butyl-4-hydroxybenzoate analysis

- propyl-4-hydroxybenzoate for methyl-4-hydroxybenzoate analysis
- butyl-4-hydroxybenzoate for ethyl-4-hydroxybenzoate analysis
- lorazepam for diazepam analysis
- diazepam for lorazepam, alprazolam and clonazepam analysis

1.2.4 Flow rate: 1 ml/ml

1.2.5 Injection volume: 20 µl

1.2.6 Wavelength: 256 nm for alkyl-4-hydroxybenzoate analysis and 254 nm for benzodiazepine drugs analysis

1.2.7 HPLC apparatus consisted of

- system controller model SCL-10 A VP
- Pump: Shimadzu model LC-10 AD VP
- Detector: Shimadzu model SPD-10 A VP UV- Visible Detector
- Auto injector: Shimadzu model SIL-10 AD VP
- Degasser DGU-14A
- Soft ware: Chromatography data system class-VP

There were 2 steps for determining the amount of drug in oily phase and submicron emulsion preparation. They were sample preparation and quantification.

1. Sample preparation

Since the components of soybean oil could interfere the analysis of submicron emulsion containing lipophilic drug, thus, in this study, solid phase extraction (SPE) was employed to extract alkyl-4hydroxybenzoate and benzodiazepine drugs from other components of submicron emulsion.

Extra-Sep™, the 3-cc and 200 mg adsorbent loading solid phase extraction cartridge was employed to extract the interesting compound from other components of oil phase or submicron emulsion. The general extraction protocols were as follows.

1.1 1×5 ml methanol (HPLC grade) was conditioned the sorbent cartridge and follow by 5×1 ml double distilled water. These two solvents had been filtered through 0.45 µm filter and degassed for 30 minutes before used.

1.2 500 µl of oil phase or submicron emulsions was loaded to the top of the cartridge. The samples were passed through the cartridge by gravity flow. In this step, drugs and unwanted components were retained in the cartridge.

1.3 The cartridge was washed with 1 ml of hexane fraction to remove the interfering components from the cartridge.

1.4 Drug was eluted from the cartridge with 5× 1 ml of methanol.

2. Quantification

After the appropriate volume of internal standard was added and then adjusted to volume with mobile phase. The samples were injected into the HPLC column and the peak area of the drug and its corresponding internal standard were quantified. The drug concentration was calculated from the calibration curve which was the relationship between peak area ratio of drug to its corresponding internal standard and drug concentrations.

Validation characteristics

1. System suitability

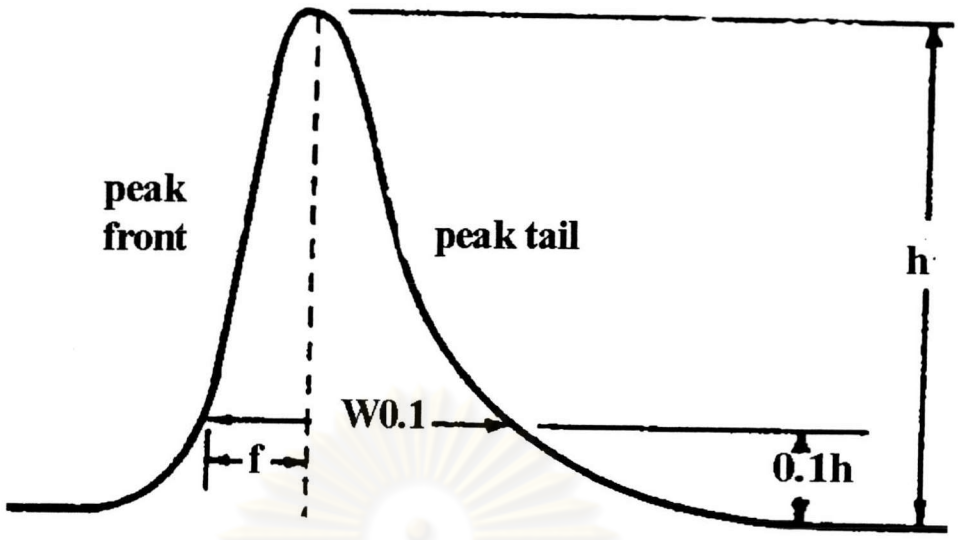
System suitability tests were used to verify that the resolution and repeatability of the chromatographic system were adequate for the sample analysis.

1.1 Tailing factor

Tailing factor was obtained by collecting data from injection of standard solution. This test was determined by the equation

$$T = \frac{W_{0.1}}{2f}$$

In which $W_{0.1}$ is the width of the model drug peak and the corresponding internal standard at 10% height and f is the distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 10% of the peak height from the baseline.

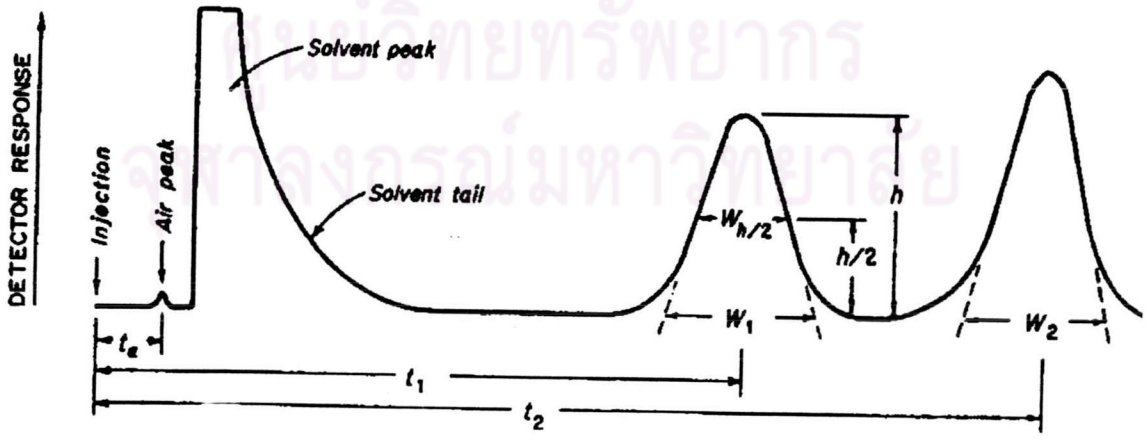


1.2 Resolution

The resolution was a function of column efficiency and was specified to ensure that model drugs were resolved from corresponding internal standard. The resolution, R, was determined by the following equation

$$R = \frac{2(t_2 - t_1)}{W_2 + W_1}$$

In which t_2 and t_1 is the retention time of the model drug and the corresponding internal standard, respectively. W_2 and W_1 is the corresponding widths at the bases of the peaks obtained by extrapolating the relatively straight sides of the peak to the baseline as shown in the following figure:



1.3 Repeatability

Repeatability expresses the precision under the same operating condition. The repeatability is displayed as the %RSD and determined by multiple injections of a homogeneous sample under the analytical conditions.

2. Accuracy

Accuracy was evaluated by adding known quantities of standard solution to the placebo (soybean oil medium and submicron emulsion base). After adding those standard solutions to the both test mediums, the mixtures were mixed for 30 minutes with magnetic stirrer. The samples were cleaned by using solid phase extraction technique and subsequently analyzed by HPLC method in which previously described. The percentage of analytical recovery of each standard solution was calculated.

3. Precision

The precision was determined by analyzing three sets of the five standard solutions of model drugs. Peak area ratios of model drugs to the corresponding internal standards were compared and the percentage coefficient of variation (%CV) for each concentration was determined.

4. Linearity

Linearity was determined by calculating a regression line by method of least squares of peak area ratios of model drugs to corresponding internal standard and concentrations of model drugs. The slope, intercept and coefficient of determination (R^2) were performed.

II Physicochemical properties of drug determination

2.1 Solubility determination

The solubilities of drugs in both oil and aqueous media were determined. An excess of drugs was added to each tested medium and the mixtures were shaken at 25±1 °C for 7 days. After the equilibration period, the mixtures were filtered through 0.45 μm membrane filters. The samples, then, were appropriately diluted with double distilled water and it was used as a blank. Drug concentrations in the aqueous sample were assayed by UV-spectrophotometry at a wavelength of 256 nm for alkyl-4-hydroxybenzoate and 254 nm for diazepam and lorazepam and by high performance liquid chromatography (HPLC) for alprazolam and clonazepam. Drug concentrations in the oily samples were assayed by HPLC. The solubility determination was carried out in triplicate.

2.2 Oil-water partition coefficient determination

Soybean oil was saturated in double distilled water (1:1) by shaking at 25±1 °C for 24 hr. After that, the aqueous phase was harvested and the model drug was dissolved in it given the approximate concentration of 50 μg/ml. 5 ml of each aqueous drug solution and the oily phase were gently transferred to a 25 ml erlenmeyer flasks and shaken at a speed of 100 rpm at 25±1 °C for 7 days. The amount of alkyl-4-hydroxybenzoate in aqueous phase was examined using UV-Spectrophotometry at the wavelength of 256 nm and double distilled water was used as a blank. The amount of drug in oil phase was a difference between the total amount and the analyzed amount in aqueous phase. Benzodiazepine drug concentration in each phase was assayed using HPLC. The determination was done in triplicate.

III Submicron emulsion base and drug containing submicron emulsion preparation

In this study, three methods of drug incorporation in submicron emulsion were investigated. They are de novo emulsification, extemporaneous addition and shaking methods. The protocol of each method was described as following:

3.1 De novo emulsification

The soybean lecithin (1.2 %w/w, Epikuron®200) was dispersed in a mixture of glycerol (2.2 %w/w) and distilled water (86.6 %w/w) and acted as an aqueous phase while the model drug was dissolved in soybean oil (10.0 % w/w). The amount of model drugs incorporated in the oil phase were at a concentration of 30%, 40%, 50%, 60% and 70% of saturated oil solubility as previously determined. Both phases were separately heated up to 80-85 °C and aqueous phase was added to an oil phase and then mixed together. The mixture was passed through the high pressure homogenizer (Emulsiflex™-C5, Avestin, Canada) at a pressure of 1000 psi for 1 cycle and a coarse emulsion was formed. Subsequently, a coarse emulsion was again passed through the high pressure homogenizer for 6 cycles at a pressure of 15,000 psi in order to achieve the submicron sized emulsion.

3.2 Extemporaneous addition

A submicron emulsion base was prepared and the method of preparation was similar to that of de novo emulsification method. The submicron emulsion base consisted of 10.0 % w/w soybean oil, 1.2 % w/w soybean lecithin, 2.2 % w/w glycerol and 86.6 %w/w distilled water. Each model drug was dissolved in dimethyl isosorbide (0.45% w/w and 0.44%w/w for alkyl-4-hydroxybenzoate and benzodiazepine drugs, respectively) and then submicron emulsion base was added and stirred by magnetic stirrer at ambient temperature for 2 hours. The final concentration of drug containing submicron emulsion was similar to that of preparing by de novo emulsification.

3.3 Shaking

The excess model drug powder was added to the submicron emulsion base and shaken at 25 °C for 24 hours. Submicron emulsion was then filtered through 0.45 μm nylon membrane filter.

After preparing, all preparations which prepared by different methods were characterized through the topics as will be described below. Consequently, they were kept at ambient temperature for seven days before further study.

IV The characterization of submicron emulsion base and drug containing submicron emulsion preparation

The physical properties of submicron emulsion base and drug containing submicron emulsion, droplet size and size distribution, zeta potential and pH, were examined. The measurements were performed at the initial and after being stored for seven days.

4.1 droplet size and size distribution

The mean droplet size of submicron emulsions was determined by photon correlation spectroscopy method. The analyses were carried out on a Zetaplus™ which covered the size range of 20 nm to 1000 nm with a wavelength of 670 nm and detection under a fixed angle of 90°. Submicron emulsions were diluted 1000 folds in order to obtain the optimum scattering intensity with double distilled water which was filtered through a 0.22 μm membrane before used and then transferred to small glass cuvettes. Each sample was assessed in triplicate at 25 °C. The data were shown in terms of effective mean diameter and polydispersity index (PI) that is an expression of the width of particle size distribution.

The larger particles were detected by a laser diffraction analyzer (Mastersizer 2000™) which covered the size range of 20 nm to 20000 nm. The

droplet size of submicron emulsion preparations were expressed in terms of the volume mean diameter (D[4,3]) and span value which indicated the size distribution.

4.2 Zeta potential determination

The charge on submicron emulsion droplets were measured by Zetaplus™. Submicron emulsions were diluted 1000 folds with double distilled water which had been passed through 0.22 μm membrane filter before used. Each sample was recorded 5 times at 25 °C.

4.3 pH determination

The pH value of all submicron emulsions was measured by pH meter at 25 °C. The calibration standard solution pH 4.0 and 7.0 were used to calibrate the pH meter prior to making a measurement.

V Phase separation by ultracentrifugation

After keeping the drug containing submicron emulsion preparations for seven days, they were fractionated by using ultracentrifugation technique. Submicron emulsion was accurately weighed into polycarbonate tubes and centrifuged using a 90 Ti type fixed angle rotor at 223171 g (60,000 rpm) at 25 °C for 6 hrs. After centrifugation, the supernatant (a clear yellowish oil phase) was collected with a needle and syringe. The subnatant, creamy layer and aqueous phase, was gently poured into a container and then the aqueous phase was withdrawn by needle and syringe. The mesophase remained stuck in the bottom of the centrifuge tube.