CHAPTER III

MATERIALS AND METHODS

Materials

Azithromycin dihydrate (AZD) was obtained from Zhejiang Guobang Pharmaceutical Co., Ltd. (Zhejiang, China) as model active pharmaceutical ingredient (API), Eudragit® E PO (EPO) from Evonik GmbH (Essen, Germany) was used as a taste-masking agent and ethanol was purchased from the Liquor Distillery Organization (Chachoengsao, Thailand) as processing solvent for Eudragit® E PO.

Instruments

1. Near-infrared spectrophotometer (Antaris II analyzer series S, Thermo Fisher Scientific Inc., USA)
2. Fourier transform infrared spectrophotometer (Nicolet iS10 with an Attenuated Total Reflectance (ATR) accessory, Thermo Fisher Scientific Inc., USA)
3. Differential scanning calorimeter (D244e, Mettler Toledo, Switzerland)
4. Thermo gravimetric analyzer (TGA/SDTA 851^e, Mettler Toledo, Switzerland)
5. Powder X-ray diffractometer (MiniFlex II Desktop X-Ray Diffractometer, Rigaku, Japan)
6. Universal Lab mixer (AR400, Erweka GmbH, Germany)
7. High speed mixer (MG15, Pharmaceuticals and Medical Supply Ltd. Part., Thailand)
8. Ultraviolet-spectrophotometer (UV1800, Shimadzu, Japan)
9. High Performance Liquid Chromatography (Shimadzu, Japan)
10. Analytical balance (XP205, Mettler Toledo, Switzerland)

11. Polarized light microscope (Eclipse E200, Nikon, Japan)

Experimental methods

1. Taste-masked azithromycin dry powder preparation

1.1 Blending in Erweka® AR400 Universal Lab Mixer

Spraying alcoholic solution containing Eudragit® E PO to azithromycin dihydrate in solid ratios of 0.1:1, 0.2:1, 0.3:1 and 0.4:1. This solution was prepared by solubilizing Eudragit® E PO 1 g in ethanol 3 mL. A model active pharmaceutical ingredient of 500 grams, was loaded to a mixer. Pre-mixing and mixing time were 10 and 30 minutes, respectively. Mixtures were monitored by using at-line NIR spectroscopy technique in pre-mixing step and mixing step at 5, 10, 15, 20 and 30 minutes.

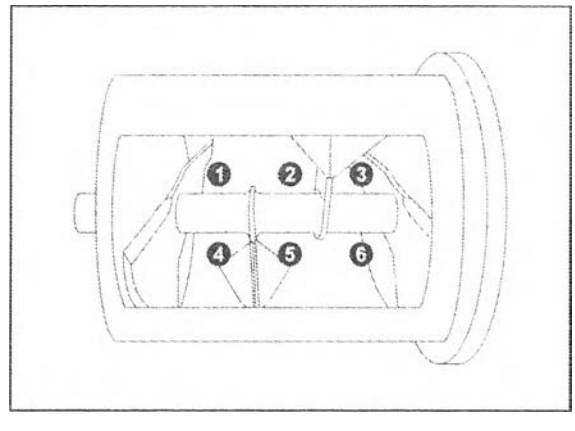

Figure 16 Schematic illustration of the blending chamber in Erweka® AR400 Universal Lab Mixer. Measurement positions using NIRs fiber optic probe are indicated by numeric symbols.

Table 1 Summary of the process settings for the continuous blending trials of taste-masked azithromycin powder by using Erweka® AR400 Universal Lab Mixer.

	Continuous blending trials of taste-masked azithromycin powder			
	B1	B2	B3	B4
EPO:AZD solid ratios	0.1:1	0.2:1	0.3:1	0.4:1
AZD (g)	500	500	500	500
Blending speed (rpm)	50	50	50	50
Sampling time (minutes)	Pre-mix (0), 5, 10, 15, 20, 30			
Sampling point	❶, ❷, ❸, ❹, ❺, ❻			
Total samples (n)	n = 108	n = 108	n = 108	n = 108

Remark: One of sampling point was collected spectra by using NIRs for triplicate.

1.2 Blending in PMS® MG15T high speed mixer

Spraying alcoholic solution containing Eudragit® E PO to azithromycin dihydrate in solid ratio of 0.4:1. This optimal proportion of Eudragit® E PO and azithromycin dihydrate derived from above blending experiment by using Erweka® AR400 Universal Lab mixer. A model API was loaded to mixer for 1,500 grams. Pre-mixing and mixing time were 5 and 10 minutes respectively. Mixtures were monitored by using at-line NIR spectroscopy technique in mixing step at every cycle after blending with Eudragit® E PO alcoholic solution.

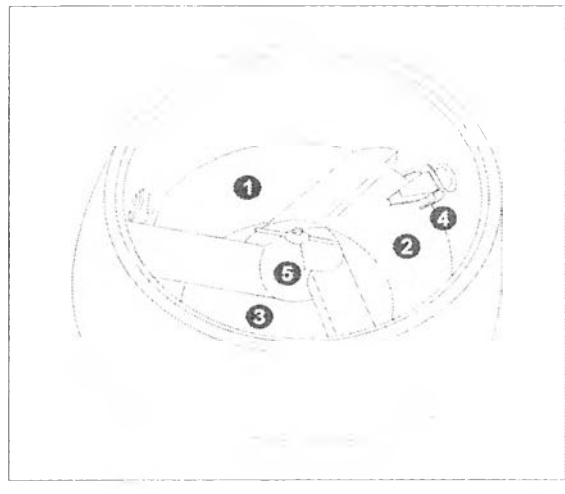

Figure 17 Schematic illustration of the blending chamber in PMS® MG15T high speed mixer. Measurement positions using NIRs fiber optic probe are indicated by numeric symbols.

Table 2 Summary of the process settings for the continuous blending trials of taste-masked azithromycin powder by using PMS® MG15T mixer.

Continuous blending trials of taste-masked azithromycin powder	Process settings (EPO:AZD ratio of 0.4:1, AZD=1.5kg)
	Eudragit® E PO solution volume (mL)
Cycle 1-2	200
Cycle 3-4	150
Cycle 5-20	100
Agitator speed	500 rpm
Chopper speed	1500 rpm

Continuous blending trials of taste-masked azithromycin powder	Process settings (EPO:AZD ratio of 0.4:1, AZD=1.5kg)
	Eudragit® E PO solution volume (mL)
Pre-blending time	5 minutes/cycle
Blending time	10 minutes/cycle
Sprayed pressure	2 bar
Sampling point	❶, ❷, ❸, ❹, ❺
Total samples (n)	n = 300

Remark: One of sampling point was collected spectra by using NIRs for triplicate.

2. Physicochemical Characterization

2.1 Powder X-ray Diffractometry (PXRD)

PXRD patterns of azithromycin dihydrate, Eudragit® E PO and final mixture of EPO and AZD were recorded by MiniFlex II equipped with CuKα anode (λ = 1.5406 Å), a current of 15.0 mA and a voltage of 30.0 kV. Diffraction data were collected at 1°2θ/min using an angular step size of 0.01° 2θ. The range used for scanning was from the range of 5°2θ - 40°2θ and signal recorded for 35 minutes. Fine powder were measured in continuous scan mode using quartz sample holder with 0.3 mm thickness. Analyses of the diffractograms were done by generating intensity integration of the selected peak by Peak Search® software.

2.2 Polarized light microscopy

Microscopic technique was carried out by a Nikon eclipse E200 microscope with polarized lens. The pictures of azithromycin dihydrate, Eudragit® E PO and final mixture of EPO and AZD were taken by a digital camera with polarized light microscopy for a magnification = 10X.

2.3 Differential Scanning Calorimetry (DSC)

The researches were operated by DSC822^e Differential Scanning Calorimeter (DSC). A standard (indium metal) was running to calibrate the instrument for cell constant and temperature. The samples of approximately 5–7 mg were weighed and sealed in aluminum DSC pans, and scanned with constant purge (60 mL/min) of nitrogen gas with a blank pan as a reference. The range of scanning was 25–250°C and heated at the rate of 10 °C/min. The DSC thermograms of azithromycin dihydrate, Eudragit® E PO and final mixture of EPO and AZD were recorded.

2.4 Thermo Gravimetric Analysis (TGA)

The studies were taken by using TGA/SDTA 851^e Thermo Gravimetric Analyzer (TGA). The substances of about 6–8 mg were weighed and placed in crucible, then scanned under nitrogen purge (60 mL/min) constantly. A heating rate of 10 °C/min was employed over a temperature range of 20–250 °C. The TGA thermograms of azithromycin dihydrate, Eudragit® E PO and final mixture of EPO and AZD were recorded.

2.5 Fourier Transform Infrared spectroscopy (FT-IR)

FT-IR spectroscopy was performed by a Thermo scientific Nicolet iS10 FT-IR spectrometer with an Attenuated Total Reflectance (ATR) accessory. The spectra was

captured and analyzed by OMNIC® software operation. The spectra of azithromycin dihydrate, Eudragit® E PO and final mixture of EPO and AZD were recorded.

2.6 Near-Infrared spectroscopy (NIRs)

NIRs spectra of the azithromycin dihydrate, Eudragit® E PO and final mixture of EPO and AZD were detected by using a NIRs Antaris II analyzer series S equipped with a fiber optic probe apparatus over the 10000–4000 cm^{1} wavenumber range. RESULT3® software was operated to capture and analyze the spectra, a 16 cm^{-1} resolution and 32 scans per spectrum. TQ Analyst 8™ software was used for spectra preprocessing.

3. Process Analytical Technology (PAT) of at-line applications in taste-masked azithromycin dry powder preparation

The goal of these application is to obtain deeper comprehension in pharmaceutical processing, replacing pragmatic approaches by knowledge-based methods. Thus, a control strategy based on the selected multivariate real-time analysis was required by PAT. Taste-masked azithromycin dry powder blending process was analyzed by conventional pharmaceutical analysis for primary method and NIRs multivariate analysis for secondary method. Homogeneity end-point was described by chemometric as follows.

3.1 Qualitative analysis

Qualitative analysis refers to classify the samples in processing according to their NIRs spectra from taste-masked azithromycin dry powder blending process by using Erweka® AR400 Universal Lab Mixer.

3.1.1 Primary method

The homogeneity end-point for taste-masked blending process could be monitored by polarized light microscopy and infrared spectroscopy for physical and chemical characterization subsequently. The studies about physical morphology and chemical spectra were performed by 2.2 and 2.5.

3.1.2 Secondary method (Principal Component Analysis: PCA)

- **Spectral Acquisition and Pretreatment**

Spectral pretreatment was applied to separate the chemical signature contained in the signal from the physical interferences due to differences in the geometries and measurement conditions. The spectra were recorded with the RESULT$^{3®}$ software from FT-NIR spectrophotometer within the 10000–4000 cm^{-1} range, a 16 cm^{-1} resolution and 32 scans per spectrum. For every measurement positions, 18 recorded spectra were integrated. As a results, the integration time for one spectrum in one position was roughly 30 seconds. The pretreatment was Norris derivative filter with 1st derivative.

- **End-point determined by the qualitative multivariate analysis method**

The identifications of NIRs are depended on pattern recognition procedures. Regarding the unsupervised classification, samples are categorized without a prior knowledge, except the spectra. Then the researcher needs to make the explanation to these clusters. PCA was obtained on mean centered spectra for B1, B2, B3 and B4 trials, over the 10000 – 4000 cm^{-1} wavelength range. The blending of taste-masked azithromycin dry powder process, which could be related with the different taste-

masking agent to API solid ratio and blending time used the PCA tool for clusters and gradients detection. Multibase 2014® software was used for chemometric analysis as principal component analysis. Before analysis with this software, all spectra were pretreated and transformed from .spa to .csv files, then classified by selected factors. Put the data through Multibase 2014®, that is an add-in to Microsoft Excel program and open form in tab of this program. The PCA results were generated by "method and preparation" function, after that were chosen the set variable range and the set sample range for next step to calculate the score and loading plot in each principal component (PC). The stated method was to find the principal component that affected most to factors, blending time and solid ratio of EPO:AZD. The resulting principal components were only used for the taste-masked blending process by using Erweka® AR400 Universal Lab Mixer.

3.2 Quantitative analysis

Once the classification of samples has been achieved, knowing more precise in what extend samples are different could be useful. Therefore, the quantitative model development appears to be essential. The samples from taste-masked azithromycin dry powder blending process by PMS® MG15T high speed mixer was analyzed for quantitative analysis.

3.2.1 Primary method

The sensitive and reproducible for azithromycin determination in samples with low background interference were described by the High Performance Liquid Chromatography (HPLC) method. An analytical method has been made to improve and verify to confirm their precision, accuracy and other analytical method

validation factors. For example, the development for determination of HPLC method was based on the using of a HPLC column as C-18, with a well mobile phase, without the using of any internal standard.

- **HPLC method validation**

Method validation was performed on the best determined stationary phase i.e. C18 column, 5 µm, 250 mm x 4.6 mm.

(1) Linearity

Six concentrations constructed the calibration (simultaneously prepared) from ranging of 50 to 500 µg/mL for azithromycin dihydrate. Each concentration level was prepared and analyzed in triplicate. Calibration curves were constructed by plotting the compounds concentration versus responded peak area. The linearity was evaluated by the least square linear regression method.

(2) Precision

Repeatability (intra-day) and intermediate precision (inter-day) determined the precision of the method with (intra-day) and expressed as RSD (%). Six replicate injections of the standard solutions of azithromycin dihydrate at concentrations from 50 to 500 µg/mL range prepared as described above.

(3) Accuracy

The accuracy of the method was tested by replicate analysis of different samples of azithromycin dihydrate at known concentrations and then compared with the true concentration of it. Accuracy was assessed by the percentage of recovery.

(4) Determination and quantitation limits (sensitivity)

During the evaluation of the calibration curve linear range, Limit of quantification (LOQ) was determined and defined as the lowest concentration yielding a precision (%CV) and accuracy (% recovery) within their acceptable range with a peak area of three times the limits of detection (LOD).

- **HPLC quantitative analysis**

HPLC method is validated by using the following conditions

(1) Chromatographic conditions

Column : C-18, 250 mm x 4.6 mm, 5 µm

Flow rate : 1.0 mL/min

Wavelength : 210 nm

Column temperature : 25 °C

Injection volume : 20 µL

Run time : 20 minutes

Diluent : Mobile phase; Phosphate buffer pH 7.5:Acetonitrile (40:60)

Elution : Isocratic

*Suitability of system requirements from standard stock solution (Tailing factor: Not more than 2.0)

(2) Preparation of mobile phase

The preparation of mobile phase was filtered and degassed the mixture that was containing phosphate buffer pH 7.5 and acetonitrile in the ratio of 40:60v/v.

(3) Preparation of azithromycin standard stock solution

Weighed and transferred 30 mg of azithromycin powder into 100 mL

of volumetric flask added 60 mL of diluents and sonicated. Further filtered the solution through 0.45 µm filter paper and made up with diluent.

(4) Sample solution preparation

Took 20 cycle samples, each containing 25 mg of cycle sample were weighed and transferred to 25 mL of volumetric flask. Dissolved the content by shaking rapidly and made up the volume with diluent and mixed well, and filtered solution through 0.45 µm filter paper.

3.2.2 Secondary method (Partial Least Squares regression: PLS)

- **Spectral Acquisition and Pretreatment**

The regressions calculation is least squares linear algorithms. A linear relationship between two matrices, the spectral data X and the reference values Y is the aim of the PLS regression. The spectra were recorded with the RESULT[3®] software from NIR spectroscopy within the 10,000-4.000 cm^{-1} range, a 16 cm^{-1} resolution and 32 scans per spectrum. For every measurement positions, 100 recorded spectra were integrated. The integration time for one spectrum in one position was roughly 30 seconds. The first step was to choose a proper pretreatment method. The method tested included baseline correction, first and second derivative for data format setting. The spectra data were performed about smoothing method by Savitzky-Golay filter or Norris derivative filter.

- **Calibration and validation model construction by the quantitative multivariate-analysis method**

From analysis type in TQ Analyst 8[TM] software, All spectral data were computed by quantitative analysis as Partial Least Squares (PLS). The PLS model

could be constructed by TQ Analyst 8™ software. The calibration model derived from the standard PLS method. Add the components in mixture of EPO and AZD into components table of program. Input the standard spectral data with usage calibration for calibration model and validation for validation model. All spectra will be pretreated by data format, smoothing and multipoint baseline correction methods. Define the region type and baseline type by TQ Analyst 8™ software suggestion. The last process for model building were data normalization and fit value algorithm, and then click the calibrate tab, that produced the calibration model and the significant values, such as correlation coefficient (r) and root mean square error (RMSE). The validation model was also operated by the same procedure.

Table 3 Conclusion of at-line PAT in taste-masked blending process analysis.

Process analysis	Primary method(s)	Secondary method
Qualitative analysis	FT-IR, Microscopy	NIRs (PCA)
Quantitative analysis	HPLC	NIRs (PLS regression)

3.3 Critical sampling points evaluation

In each mixing step, all mixtures were brought for pharmaceutical analysis that followed by measurement points map. These derived from literature reviews and the manufacturer's information about blending process and mixing equipment. Quality risk management tools were used for critical sampling points assessment. The determination of sampling points was applied to pull samples and confirm a suitable state of homogeneous mixing (De Beer 2011, Scheibelhofer 2013,

Burggraeve 2013). The critical sampling locations from literature reviews and quality risk tool can be conclude as following:

(1) Segregation area, (2) Gap density area, (3) Dead space/spot. (4) Center area of mixing bed. (5) Recommendation area from specialist

Sampling points in each mixer were determined by the critical sampling locations. All of the sampling points must cover all critical area and represent total final product as sampling.

4. In vitro evaluation of taste-masked azithromycin dry powder

We developed taste masked evaluation from the studies of Mahore's team (2010). Build the calibration curve by standard preparation within range 10, 20, 40, 60, 80 and 100 µg/mL. Final mixtures (from two blending procedures equivalent to 500 mg of azithromycin dihydrate) were placed in a test tube with 10 mL of phosphate buffer pH 6.8 to simulate the human saliva and shaken for 5 minutes. The mixture was filtered by 0.45µm cellulose acetate filter paper. 1 mL of filtrate was taken to a 100 mL of volumetric flask and dilute with phosphate buffer solution pH 6.8 to volume and mix. This solution was analyzed for azithromycin dihydrate concentration as a bitter taste from product. The solution was also detected at wavelength 213 nm by UV-visible spectrophotometer and compared the data with three commercial products in Thailand.