CHAPTER III

MATERIALS AND METHODS

Materials

Clopidogrel polymorph Form II (Lot R1-53/00438) was obtained from Dr.Reddy's Laboratories Ltd., India. HPLC grade acetonitrile and methanol were purchased from Burdick & Jackson (SK Chemicals, Korea). Analytical grade buffer salt (KH_2PO_4) was obtained from Univar (Ajax Finechem Pty Ltd., Australia). Ultrapure water was collected from ELGA (Bucks, England) water purification system.

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Instruments

1. Confocal microscopic Raman spectrometer (DXR Raman Microscope Spectrometer, Thermo Fisher Scientific Inc., USA)
2. Powder X-ray diffractometer (MiniFlex II Desktop X-Ray Diffractometer, Rigaku, Japan)
3. Differential scanning calorimeter (D244e, Mettler Toledo, Switzerland)
4. Thermogravimetric analyser (SDTA851^e, Mettler Toledo, Switzerland)
5. Spray dryer (Buchi Mini Spray Dryer B-290, Buchi, Switzerland)
6. Lyophilizer (LYO LAB, Lyophilization Systems, Inc., USA)
7. High performance liquid chromatographic analyzer (HPLC, Shimadzu, Japan)
8. Dynamic vapor sorption analyzer (Serial No. 1045, DVS Intrinsic, UK)
9. Polarized light microscope (Nikon Eclipse E200, Nikon, Japan)
10. Analytical balance (XP205, Mettler Toledo, Switzerland)
11. Analytical balance (A200S, Sartorius, Germany)

12. Sonicator (Elma S70H, Elmasonic Sonicator)
13. Shaking Incubator (Labtech Manufacturer of Lab. Ind. & Vac. Instruments)
14. Stability Incubator (Memmert D 06057, Model 100, Germany)

Experimental methods

1. Preparation polyamorphous samples of clopidogrel

Polyamorphous samples of clopidogrel were prepared from clopidogrel bisulfate polymorphic Form II by two methods, spray drying and freeze drying.

1.1. Spray drying method

The amorphous form was prepared by dissolving ten grams of clopidogrel bisulfate polymorphic Form II in 40 ml of methanol, add 300 ml of water, adjusted to volumn (400 ml) with water and spray dried using a Buchi Mini Spray Dryer B-290 (Buchi, Switzerland) with inlet-air temperature of 130°C and outlet-air temperature of 80°C. The spray rate of the solution was 1.0 ml/min and aspirator was set at 90%.

1.2. Freeze drying method

The amorphous form was prepared by dissolving ten grams of clopidogrel bisulfate polymorphic Form II in 40 ml of methanol, add 300 ml of water, adjusted to volume (400 ml) with water. Pipette 2.0 ml of the solution into each 5 ml glass vial. The vials were freeze dried using a LYO LAB (Lyophilization Systems, Inc., USA) with conditions according to Table 3.

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Table 3 Three essential steps (freezing, primary drying and secondary drying) in freeze drying of clopidogrel bisulfate solution

	Freezing Step															
Temperature (°C)	-30															
Time (Minutes)	120															
Pressure (mTorr)	600															
	Primary Drying Step															
Temperature (°C)	-30	-30	-25	-25	-20	-20	-10	-10	0	0	10	10	20	20	30	30
Time (Minutes)	0	210	0	120	0	120	0	120	0	120	0	120	0	120	0	60
Pressure (mTorr)	600															
	Secondary Drying Step															
Temperature (°C)	20															
Time (Minutes)	60															
Pressure (mTorr)	600															

2. Solid-state characterization

Polyamorphous form of clopidogrel were analyzed by polarized light microscopy, powder X-ray diffractometry (PXRD), differential scanning calorimetry (DSC), thermogravimetry (TGA), dynamic vapor sorption (DVS) analysis and confocal microscopic Raman spectrometry (Raman).

2.1. Physical appearance

Physical characteristics and color of all samples were evaluated.

2.2. Polarized light microscopy

Polarized light microscope (Nikon Eclipse E200, Nikon, Japan) equipped with 4x objective lens and 10x eyepiece lens were used. Polarized light was produced from normal light passed through polarized lens. Crystalline samples were expected to show birefringence behavior but amorphous samples will not show this phenomenon.

2.3. Powder X-ray diffractometry (PXRD)

Powder X-ray diffraction (PXRD) patterns were recorded at room temperature using MiniFlex II (Rigaku, Japan) equipped with CuKα anode (λ = 1.5406 Å), 15.0 mA, 30.0 kV and slit path 1.25°. X-ray diffraction data were collected at 1° 2θ/min using an angular step size of 0.01° 2θ. The scanning range was from 5° to 40° 2θ. Measurement was done by two replicates in continuous scan mode using quartz sample holder. All data were processed using PDXL® (Version 1.8.1.0) software.

2.4. Differential scanning calorimetry (DSC)

Differential scanning calorimeter (DSC) was operated under constant nitrogen

purge gas of 60 ml/min. Calibration was done using Indium as reference standard. Two replicates of each sample were accurately weighed (approximately 3-5 mg) and sealed in 40 µl aluminium DSC pan and were heated from 25 to 250°C at the rate of 10°C/min.

2.5. Thermogravimetric analysis (TGA)

Thermogravimetric analyser (TGA) was operated under constant nitrogen purge gas of 60 ml/min. Sample were placed in 70 µl aluminium oxide sample holder that was zero adjusted and heated from 25 to 250°C at the rate of 10°C/min. Each sample was evaluated in two replicates.

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2.6. Dynamic vapor sorption (DVS)

Dynamic vapor sorption (DVS) apparatus was operated under constant nitrogen purge gas of 2 bar and under isothermal temperature of 30°C. Calibration was done using 100 mg metal weight as reference standard. Two replicates of each sample were accurately weighed (approximately 3-10 mg) in the sample pan and exposed to predetermined relative humidity (%RH) of 0% to 90% (sorption cycle) and 90% to 0% (desorption cycle) at the ramping rate of 10%RH. The change in weight of not more than ±0.01 mg was considered as equilibrium. Data were collected and exported to excel spreadsheet for interpretation.

2.7. Confocal microscopic Raman spectrometry (Raman)

Confocal microscopic Raman spectrometer (Raman) consisted of diode laser source of 532 nm at 10 mW with power of 100% and an Olympus TH4-200 microscope. Each Raman spectrum was collected with 20x objective lens using an acquisition time of 2 seconds and accumulating 8 measurements at a time for both

sample and background. All Raman spectral data were collected in 10 replicates. Raman shift was determined from 60 to 3500 cm^{-1} and spectrum resolution was within the range of 2.7 and 4.2 cm^{-1}. The system was controlled by OMNIC® (Version 8.0) software. All Raman spectral data were further analyzed by Principal Component Analysis (PCA) in order to distinguish between groups of data.

3. Principal Component Analysis (PCA)

All Raman spectral data of clopidogrel were analyzed between the chosen ranges of 3200 to 2800 cm^{-1} and 1800 to 100 cm^{-1} where the most distinctive differences were observed. The discrimination of all data was achieved by Principal Component Analysis (PCA) using UNSCRAMBLER® (Version 9.8) software and Multibase (2014) software which is an Excel Add-Ins program. UNSCRAMBLER® software is used for statistical calculations, while Multibase software is used for graphical presentation. PCA is a multivariate statistical method that introduces data reconstruction and reduction. PCA will generate a new data set that can be presented on the orthogonal axes and expressed the total variability in the data set through comparison of only few principal components (PCs) (57). The maximal amount of variance in the data set and its direction are often explained by the first PC (PC1). The loading plot depicts the identification of important variables and the score plot indicates similarity or dissimilarity of samples.

4. Physicochemical evaluation of polyamorphous samples

4.1. Appearance and size

Evaluate the appearance and size of polyamorphous clopidogrel samples using scanning electron microscopy (SEM) (JEOL, JSE-6400 scanning microscope,

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Tokyo, Japan) at 100x and 500x magnifications.

4.2. Solubility

Add excess sample in 1 ml of water in a glass vial and shake continuously in shaking incubator at controlled temperature of 30°C and speed 100 rounds per minute (rpm). Sampling 10 microliter of this solution for evaluation and dilute with mobile phase (1:1000) to obtain a solution having a suitable final concentration. All samples were repeated in 3 replicates. Solubility of amorphous clopidogrel samples and clopidogrel RM were evaluated using high performance liquid chromatography (HPLC) according to USP35 on evaluating clopidogrel bisulfate content explained in the following section 4.3.

4.3. Related substances

All polyamorphous samples prepared and stored under 3 different conditions (30°C 30%RH, 40°C 30%RH and 40°C 75%RH) were analyzed using HPLC according to USP35 on evaluating clopidogrel bisulfate content. The liquid chromatography was equipped with 220 nm detector and 4.6 mm x 15 cm column that contains L57 packing (Ultron ES-OVM 150L x 4.6mm I.D., 5 micron, Serial No. 0081808, Shinwa chemical industries, LTD., Agilent technologies) (L57: A chiral-recognition protein, ovomucoid, chemically bonded to silica particles, about 5 micron in diameter, with a pore size of 120 angstroms). The injection volume was 10 microliter. All standard solution, related standard solution and system suitability solution were collected in 6 replicates while test solution was collected in 2 replicates. Related standard solution were prepared by dissolving accurately weighed quantities of clopidogrel bisulfate reference standard (RS), clopidogrel related

compound A RS, clopidogrel related compound B RS and clopidogrel related compound C RS in methanol and dilute with mobile phase to obtain a solution having final concentrations of approximately 0.5, 1, 3 and 5 microgram per milliliter. Verification of this method is shown in Appendix I.

4.4. Dynamic Vapour Sorption

Dynamic vapor sorption (DVS) apparatus was operated under constant nitrogen purge gas of 2 bar and under isothermal constant temperature of 30°C. Calibration was done using 100 mg metal weight as reference standard. Two replicates of each sample was accurately weighed (approximately 3-10 mg) in the sample pan and exposed to predetermined relative humidity (%RH) of 0% to 90% (sorption cycle) and 90% to 0% (desorption cycle) at the ramping rate 10%RH. The change in weight of not more than ±0.01 mg was considered to be reached equilibrium. Data were collected and exported to an excel spreadsheet for interpretation.

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5. Effect of temperature and humidity on recrystallization of polyamorphous samples

5.1. Effect of temperature

Study effect of temperature using isothermal DSC method operated under constant nitrogen purge gas of 60 mL/min. Calibration was done using Indium as reference standard. Each sample was accurately weighed (approximately 3-5 mg) and sealed in 40 µl aluminium DSC pan and was heated at a constant temperatures of 40°C, 60°C and 80°C for 24 hours (1440 min).

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Table 4 Abbreviations of spray dried and freeze dried samples prepared and stored at 3 different conditions (30°C 30%RH, 40°C 30%RH and 40°C 75%RH) for 0, 1, 2, 3, 5, 7, 30, 60 and 90 days

Spray Drying Method (SP)									
Condition	Day 0	Day 1	Day 2	Day 3	Day 5	Day 7	Day 30	Day 60	Day 90
30°C 30%RH	$SP^{0}_{30/30}$	$SP^{1}_{30/30}$	$SP^{2}_{30/30}$	$SP^{3}_{30/30}$	$SP^{5}_{30/30}$	$SP^{7}_{30/30}$	$SP^{30}_{30/30}$	$SP^{60}_{30/30}$	$SP^{90}_{30/30}$
40°C 30%RH	$SP^{0}_{40/30}$	$SP^{1}_{40/30}$	$SP^{2}_{40/30}$	$SP^{3}_{40/30}$	$SP^{5}_{40/30}$	$SP^{7}_{40/30}$	$SP^{30}_{40/30}$	$SP^{60}_{40/30}$	$SP^{90}_{40/30}$
40°C 75%RH	$SP^{0}_{40/75}$	$SP^{1}_{40/75}$	$SP^{2}_{40/75}$	$SP^{3}_{40/75}$	$SP^{5}_{40/75}$	$SP^{7}_{40/75}$	$SP^{30}_{40/75}$	$SP^{60}_{40/75}$	$SP^{90}_{40/75}$
Freeze Drying Method (FZ)									
Condition	Day 0	Day 1	Day 2	Day 3	Day 5	Day 7	Day 30	Day 60	Day 90
30°C 30%RH	$FZ^{0}_{30/30}$	$FZ^{1}_{30/30}$	$FZ^{2}_{30/30}$	$FZ^{3}_{30/30}$	$FZ^{5}_{30/30}$	$FZ^{7}_{30/30}$	$FZ^{30}_{30/30}$	$FZ^{60}_{30/30}$	$FZ^{90}_{30/30}$
40°C 30%RH	$FZ^{0}_{40/30}$	$FZ^{1}_{40/30}$	$FZ^{2}_{40/30}$	$FZ^{3}_{40/30}$	$FZ^{5}_{40/30}$	$FZ^{7}_{40/30}$	$FZ^{30}_{40/30}$	$FZ^{60}_{40/30}$	$FZ^{90}_{40/30}$
40°C 75%RH	$FZ^{0}_{40/75}$	$FZ^{1}_{40/75}$	$FZ^{2}_{40/75}$	$FZ^{3}_{40/75}$	$FZ^{5}_{40/75}$	$FZ^{7}_{40/75}$	$FZ^{30}_{40/75}$	$FZ^{60}_{40/75}$	$FZ^{90}_{40/75}$

5.2. Effect of humidity

Study the effect of humidity using DVS apparatus according to section 4.4. Results from each sample will be shown as sorption/desorption isotherms.

6. Stability evaluation

Stability study of polyamorphous samples stored in 3 different conditions (30°C 30%RH, 40°C 30%RH and 40°C 75%RH) were evaluated for their possible solid-state conversion. Effect of temperature can be evaluated from sample stored between 30°C 30%RH and 40°C 30%RH condition. Furthermore, Effect of humidity can be evaluated from sample stored between 40°C 30%RH and 40°C 75%RH condition. On the contrary, no samples stored at 30°C 75%RH condition for testing because of want to reduce amount of sample for testing. Physicochemical characterization according to section 2, were done for samples stored at 0, 1, 2, 3, 5, 7, 10, 20, 30, 60 and 90 days and were later identified and referred to according to Table 4.

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7. Solid-state characterization in physical mixture

Physical mixture of each polyamorphous sample with lactose in proportion of 1:2, 1:1 and 2:1 were prepared. Evaluate the mixed samples using DSC, PXRD and Raman, according to section 2.