CHAPTER 2

Materials and Methods

2.1 Materials

- Piroxicam, 4-Hydroxy-2-methyl-N-(2-pyridyl)-2H-1,2-benzothiazine-3-carboxamide-1,1-dioxide (M.W. 331.32)
- Meloxicam,4-Hydroxy-2-methyl-N-(5-methyl-2-thiazolyl)-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide (M.W. 351.39), Boehringer Ingelheim, Germany; Kwizda Co., Austria.
- β-Cyclodextrin, Cavamax® W7 Pharma (M.W. 1135), Wacker-Chemie, Germany
- γ-Cyclodextrin, Cavamax® W8 Pharma (M.W. 1297), Wacker-Chemie, Germany
- Hydroxypropyl-β-Cyclodextrin, Cavasol® W7 HP Pharma (D.S. 0.62, M.W. 1378), Wacker-Chemie, Germany
- Methylated-β-Cyclodextrin, Cavasol® W7 M Pharma (D.S. ,M.W. 1311), Wacker-Chemie, Germany
- Hydroxypropyl-γ-Cyclodextrin, Cavasol® W8 HP Pharma (D.S. 0.70, M.W. 1610), Wacker-Chemie, Germany
- Ammonium hydroxide solution (32%) ExtraPure, Merck,Germany.
- Di-sodium hydrogen phosphate dodecahydrate, ExtraPure, Merck, Germany
- Hydrochloric acid (37%) Puriss, ACS reagent Grade, Sigma-Aldrich, Germany.

- Ethyl alcohol (95%),
- Potassium dihydrogen phosphate crystalline, ExtraPure, Merck, Germany.
- Sodium chloride, ExtraPure, Merck, Germany.
- Sodium di-hydrogen phosphate dehydrate ExtraPure, Merck, Germany.

2.2 Instruments

- Analytical balance, Sartorius CE95, Sartorius AG, Göttingen, Germany.
- Supermicrobalance, Sartorius, Göttingen, Germany.
- Deep freezer, Liebherr, Germany.
- Differential scanning calorimeter, Perkin Elmer DSC7, USA
- Dissolution tester, Type PTWS3C, PharmaTest GmbH, Germany.
- Freeze dryer, Christ® Beta 1-8K, with Anlagensteuerung Chirst® LMC-1, Martin, Christ GmbH, Germany.
- Spectrum 1000 FTIR spectrophotometer, Perkin Elmer, USA
- Hot air oven, Memmert, Germany.
- Incubator, Memmert Type UE500, Memmert GmbH&Co., Germany.
- Multiple magnetic stirrers, Variomag® H&P Labortechnik AG, Germany.
- Magnetic plate, MR3001K, Heidolph, Germany.
- pH meter, Microprocessor, pH539, Wissenschaftlich-Technische Werkstätten (WTW), Germany.
- NIRVIS/1004, Bühler Ltd., Switzerland.
- Rotary evaporator, Heidolph, Germany.
- Standard sieves, Analysensieb, Retsch, Germany.
- Shaking water bath, GFL 1092, Muller-Scherr, Laborausrüstungsgesellschaft

mbH&Co., Germany.

- Spectrophotometer, U-3000, Hitachi, Japan.

- Spectrophotometer, SPEKOL® 1200, Analytik Jena AG, Germany.

- X-ray diffractometer, Semen-500, Germany.

2.3 Methods

2.3.1. Thermodynamic studies

2.3.1.1 Phase solubility studies of piroxicam-CDs inclusion complexes

The phase solubility studies of piroxicam were performed by the method described by Higuchi and Connor (1965). Accordingly, an excessive amount of piroxicam was placed in a screw-capped glass vial, to which 10 ml aqueous solution of CDs at varying concentrations was added. To avoid any loose of the solution by evaporation, the vial was tightly closed and wrapped-around the closure using paraffin film. The suspension was sonicated for 10 minutes to ensure the intimate mixing. All vials were then placed on the magnetic plate (Variomag®, H&P Labortechnik AG, Germany) kept in thermostatic chamber at 25° C, 30° C, 37° C and 45° C. The temperature of chamber was kept constant within ±0.1°C. All samples were stirred magnetically at 200 revolutions/minute for 3 days to attain the equilibrium. Then the content in each vial was filtered through 0.45µm membrane filter (Cellulose acetate filter, Sartorius, Germany). The filtrate was appropriately diluted and analyzed for the total drug content using UV-spectrophotometer at maximum wavelength of 359 nm. The corresponding CD solution at the same dilution was used as a blank. The weight of the vial before and after storage was recorded. The pH of the suspensions at initial

and the pH of the undiluted filtrates were measured. All experiments were performed in triplicate.

To investigate the effect of pH on the complex formation, the CD solutions at varying concentrations were prepared by dissolving an appropriate amount of CD in 0.1N hydrochloric acid solution, distilled water, or 0.01M phosphate buffer solution (pH 7.4) in order to obtain the pH of the suspension of 2.5, 5.5 and 7.4 respectively.

Different types of CD, the two natural cyclodextrins, BCD and GCD and two CD derivatives; HPBCD and MeBCD were used in this study. The concentration range of the natural CD was 0-15 mM whereas it was 0-100 mM for the other two CDs.

2.3.1.2 Phase solubility studies of meloxicam-CDs inclusion complexes

The phase solubility studies of meloxicam were also conducted using the same procedures previously described for piroxicam. The experimental conditions were slightly different. Briefly, an excessive amount of meloxicam was suspended in 10 ml of CDs aqueous solutions in a 25-ml stoppered-ground glass flask. The flask was tightly closed and wrapped around the closure using paraffin film. After the suspension was mixed in the ultra-sonicating bath for 10 minutes, the flasks were placed in the controlled-temperature shaking water bath. The temperature of the water bath was maintained at 25° C, 30° C, 37° C and 45° C within the range of ±0.1° C. The sample was shaken at 150 revolutions/minute and equilibrated for 5 days. At the end of the shaking time, the excessive solid drug in each flask was immediately removed by passing the suspension through 0.45μm membrane filter. After an appropriate dilution, the filtrate was analyzed for the drug content using

spectrophotometer (Spectrophotometer U-3000, Hitachi, Japan) at maximum wavelength of 362 nm. The corresponding CD solution at same dilution was used as a blank. The weight of the flask and the pH of the sample before and after the equilibration was measured and recorded. All experiments were performed in triplicate.

In the case of meloxicam, the CD solutions were prepared using acidified distilled water (pH 3.0) or 0.01M phosphate buffer solution (pH 6.0) as the medium in order to obtain the experimental pH conditions at these two pH values. The pH value of the medium was strictly controlled within ±0.01 pH unit.

Four different CD types used in the study were BCD, GCD, HPBCD and HPGCD. The concentration range was 0-15 mM for BCD and GCD and 0-100 mM for HPBCD and HPGCD respectively.

2.3.1.3 Calculation of the stability constant.

The apparent stability constant, K of the complexes was determined using the following relationship:

$$K_{1:1} = \text{slope}/So(1 - \text{slope})$$

The slope is obtained from the least square linear regression plot of the molar concentrations of the drug in solution versus the molar concentration of cyclodextrin.

So is the intrinsic solubility of the drug in the absence of cyclodextrin.

2.3.1.4 Determination of the thermodynamic parameters

The thermodynamic parameters namely, the change in free energy (ΔG), the change in enthalpy (ΔH) and the change in entropy (ΔS) were calculated using the standard formulae:

$$\Delta G = -RT \ln K$$

$$d(\ln K)/d(1/T) = -\Delta H/R$$

$$\Delta G = \Delta H - T\Delta S$$

where T is the absolute temperature in Kelvin and R is Gas constant

2.3.2 Solid complex preparations

2.3.2.1 Piroxicam-CDs inclusion complexes

The inclusion complexes of piroxicam and BCD, GCD, HPBCD, and MeBCD were prepared at 1:1, 1:2 and 2:1 drug to CD molar ratios by the following methods:

A. Co-evaporation

The required amount of drug and CD were accurately weighed. The drug was added into the CD aqueous solution while stirring on the magnetic plate. After the uniform suspension was obtained, the ammonium hydroxide solution (32%) was added dropwise to adjust a pH value of approximately 9.0 to aid the complete dissolution of the drug. The agitation was continued until the yellow-clear solution was obtained. The clear solution was then evaporated under vacuum at temperature of 50°C using rotary evaporator (Heidolph VV2011, Germany). The solid residue was further dried at 45°C overnight. The dry mass was then pulverized, sieved and kept in

the desiccator for further investigation. The complex obtained was designated as the co-evaporated complex (COE).

B. Co-lyophilization

The method of preparing a co-lyophilized complex (COL) was similar to that of the COE complex. The clear solution of the drug and the CD obtained was frozen at -80° C in a deep-freezer overnight and then lyophilized for 48 hours (Freeze dryer, Christ® Beta 1-8K, Martin, Christ GmbH, Germany). The dry mass was pulverized and sieved and kept in the desiccator for the further experiments.

C. Kneading

The amount of the drug and the CD at any specified molar ratio were accurately weighed and mixed together in a mortar by geometric dilution technique. The small portion of 1:1 (v/v) ethyl alcohol and water mixture was added to the dry powder mixture to form a wet mass. The mass was vigorously triturated for at least 15 minutes. During the trituration, if needed, a sufficient amount of ethyl alcohol-water mixture was added to maintain the suitable consistency of the wet mass and facilitate the trituration. The kneaded complex was designated as "KN" was then dried in an oven at 45° C overnight. The dry mass was pulverized, sieved and kept in the desiccator for the further investigation.

D. Physical mixing

The drug and the CD which had been previously sieved (180μm/150μm) were accurately weighed and thoroughly blended using vortex mixer for 10 minutes. The physical mixture obtained was assigned as PM.

2.3.2.2 Meloxicam-CDs inclusion complexes

The COE, COL KN complexes and PM of meloxicam and BCD, GCD, HPBCD, and HPGCD were prepared at 1:1 drug to CD molar ratio using the same procedures as described above.

2.3. 3 Characterization of solid complexes

2.3. 3.1 Dissolution studies

In vitro dissolution studies of the intact drug, physical mixtures and the inclusion complexes were performed at 37±0.5° C using USP II, paddle method (Dissolution Tester Type PTWS3C, PharmaTest, Germany). 900 ml of distilled water or simulated gastric fluid without enzymes (pH 1.2, USP 27) maintained at 37° C was used as dissolution medium for piroxicam or meloxicam respectively. The stirring speed of the paddle was 100 revolutions/minute.

The powder sample was sieved (180 μm/150 μm) prior to the dissolution studies. An aliquot amount of the samples equivalent to one therapeutic dose (20 mg of piroxicam, 15 mg of meloxicam) was accurately weighed and spread over the surface of the dissolution medium. At appropriate time interval up to 90 minutes, an amount of 5-ml solution was taken through a filter-fitted Teflon tube. An equal volume, fresh portion of the dissolution medium kept at the same temperature was immediately

replaced. The withdrawn sample solution was suitably diluted and analyzed for the drug content using spectrophotometer (Spectrophotometer U-3000, Hitachi, Japan). The corresponding dissolution medium was used as the blank. The experiment was performed in triplicate.

The drug content was calculated using the calibration curves.

For piroxicam: $y = 0.0571x + 0.0022$; $r^2 = 0.9999$

For meloxicam: $y = 0.0426x + 0.0046$; $r^2 = 0.9998$

where y is the absorbance at maximum wavelength and x is the drug concentration in μg/ml

The drug concentration calculated was corrected for the cumulative dilution caused by the replacement of the original medium.

The difference in the dissolution profiles was characterized by the dissolution parameters, the dissolution efficiency up to 30 minutes, DE30 and the time required to dissolved 50 % of the drug, $t_{50\%}$ (Khan, 1975). The statistical analysis of DE30 values was conducted using one-way analysis of variance (ANOVA) with the Least significant difference (LSD) for multiple comparison at a significant level of $\alpha<0.05$.

2.3.3.2 Differential scanning calorimetry (DSC)

DSC thermograms of the samples were recorded using differential scanning calorimeter (Perkin-Elmer, DSC7, USA). The instrument was calibrated with standard indium (99.99%; m.p.156.60°C,ΔHf 28.45 J/g) and standard zinc (m.p.419.47 °C , ΔHf 108.37 J/g). The sample approximately 2.5-3.0 mg was accurately weighed and placed in an aluminum pan with holes. The pan was hermetically sealed and scanned at the heating rate of 10° C/minute over the temperature range of 50-280° C under

nitrogen gas (flow rate 20 ml/minute). The empty pan of the same capacity was used as the reference.

2.3.3.3 X-ray powder diffraction

The X-ray diffraction patterns of the samples were obtained using diffractometer (Semen-D500, Germany). The measurement was performed at room temperature according to the following conditions: target, Cu-K∝ (Ni-filter) radiation (λ= 1.5418 °A); voltage, 20kV; current, 20mA; time constant, 0.5 second; at the scanning speed of 4°/minute over the 2θ angle range of 10-60 (where θ is the scattering angle).

2.3.3.4 Fourier Transform Infrared (FTIR) absorption spectroscopy

The IR spectra of the intact drug, physical mixtures and the inclusion complexes were recorded using Infrared spectrophotometer (Model Hitachi 295). The samples were prepared as potassium bromide disks and scanned over the absorbance of 4000-600 cm^{-1}.

2.3.3.5 Near infrared (NIR) spectroscopy

The NIR spectra of the all samples were obtained using NIRVIS/1004, Buhler, Switzerland. The data were processed using software NIRCal version 4.21,build 389.

2.3.4. Storage-stability studies

The complexes were stored in thermostatic cabinet at specified temperature for definite time interval. The complexes of piroxicam-CDs were stored at 30°C for 1 year whereas meloxicam-CDs complexes were kept at 45°C for 4 month. After the end of storage time, the complexes were characterized by powder X-ray diffractometry, DSC and dissolution studies.

2.3.5 Molecular Modeling

2.3.5.1 Construction of the quantitative structural property relationships (QSPR) predictive model

The stability constants, K of 44 guest-HPBCD inclusion complexes were collected from published papers. These experimental values were selected on the basis of 1:1 complex stability constant performed at 25° C irrespective to the method of the determination.

The molecular structures of the guests were constructed using Hyperchem 7.04 software. Prior to the descriptor calculations, the structures were optimized by adding explicit hydrogen atoms and performing energy minimization using MMFF94X force field parameter set to an RMS gradient less than 0.1.The built structures were imported to the MOE 2004.03 (Molecular Operating Environment software, Chemical Computing Group Inc.)

All 216 molecular descriptors available in MOE were used in the initial stage of the descriptor selection.

The guest compounds were divided into two groups. The first 22 compounds, assigned as training set, were used to generate the QSPR model and another 22

compounds were reserved as a test set on which the prediction ability of the model was evaluated.

The model was constructed using partial least square (PLS), by algorithmic steps of the descriptor selection. The best model was obtained with respect to the high correlation coefficient, r^2 value and the correlation coefficient obtained by successively leave-one-out cross validation, r^2cv value.

2.3.5.2 Conformational studies of piroxicam-BCD complexes

Energy optimization of piroxicam and piroxicam-BCD complex was performed with GAUSSIAN 98. The semiempirical methods, PM3 and AM1 calculations were applied compared to the Density Function Theory (DFT) calculation using GAUSSAIN B3LYP/6-31G** basis sets.