Methods

Preformed cyclodextrin-salbutamol complexes

An amount of salbutamol base (30 mg) was weighed into 2.5 ml Chromacol® vials (Sci-VI system, USA). To each vial, 1 ml of different concentrations of cyclodextrins in water was added. The concentrations prepared were 2, 4, 6, 8 and 10 mg% of each

of the following cyclodextrins: GCD and DMCD. A blank suspension was prepared with contained 30 mg salbutamol only added to 1 ml of water. Four replicates of each solution were prepared. The suspensions in the vials were capped and shaken in a water bath shaker at 37°C for 48 h. After equilibration was attained (2 days), the solution was centrifuged at 800 g for 5 min and an aliquot of clear solution was sampled after this time and analysed for salbutamol content using a digital ultraviolet spectrophotometer (Spectronic Genesis, New York, USA).

A 100 µl of each sample was diluted with water and the volume was made up to 25 ml and the absorbance determined at 225 nm. Stability constant of 1:1 cm complexes was detected from the slope and intercept of straight line of the phase solubility diagram according to the equation of Higuchi and Connors (1965).

Cyclodextrin-salbutamol complexes

The 1:1 of drug-cyclodextrin complex for GCD and DMCD was prepared and further used for investigations. The freeze-dried

complexes were obtained by adding equimolar concentrations either of the aqueous solution of cyclodextrins to racemic salbutamol. The clear solution of a mixture was then freeze-dried for 48 h until yielding a solid cake. The solid cake was micronised under air jet microniser (Fryma jet, Switzerland) before use as an ingredient in dry powder formulation. The product was stored in environmental chamber at 25°C and 45% RH in well-closed container. In this experiment, the freeze-dried cyclodextrins and freeze-dried salbutamol alone were also prepared. The properties of the prepared complexes of salbutamol with cyclodextrin were determined by using infrared spectroscopy (IR), differential scanning calorimetry (DSC) and X-ray diffractometry (XRD).

Cyclodextrin-salbutamol characterisations

FTIR spectroscopy studies

IR spectra were recorded in the range of 4000-500 cm^{-1} with a Perkin Elmer FT-IR Model 1600 spectrophotometer (Norwalk, CT, USA) on KBr pellets using 16 scan for each sample with a

resolution factor of four. The spectra were corrected by subtracting the spectrum of a KBr blank pellet and were presented in transmittance mode.

DSC studies

Thermal analysis using DSC method was performed on a Perkin Elmer DSC 7 (Norwalk, CT, USA) with a Pyris software. A sample (5-6 mg) was weighed into an aluminium pan, which was hermetically sealed with a lid before scanning for DSC curve. An empty pan sealed in the same way, was used as a reference. All samples were heated at a scanning rate of 5° C/min under nitrogen stream between 50°C to 300°C.

XRD studies

The X-ray diffraction measurements were carried out on a Philips PW 3710 powder diffractometer with the following experimental setting; Ni filtered Cu Kα radiation (λ = 1.5418 A), tube setting 40 kV, 20 mA, time constant 4 s, angular speed 1°/ min and angular range $5° < 2\theta < 50°$.

Dry powder formulation and deposition studies

Five formulations made in-house were used and those formulations contained micronised lactose (70% of population < 10 μm) mixed with micronised salbutamol (2.77 ± 0.11 μm) in a ratio of 60:1 or cyclodextrin-salbutamol complexes (2.9 ± 0.15 μm) in a ratio of 30:1 and 60:1, respectively. The formulation was prepared by mixing drug or drug complexes with lactose in a V-shaped mixer (Mitsubishi, Tokyo, Japan) for 1 h. The powder mix was filled into 8 blister disks of Diskhaler with a drug content of 400 μg per disk. A disk loaded into Diskhaler device (Glaxo-Wellcome, Ware, UK) was aerosolised by drawing air through the twin stage impinger (TSI) at flow rate of 60 l/min for 10 s. Drug and cyclodextrin deposition on each stage of the impinger were determined by HPLC.

Salbutamol was analysed by a validated HPLC (Waters, Milford, USA) using a UV detector set at a wavelength of 225 nm. The assay conditions were: stationary phase, Zorbax ODS

(15 cm x 4.6 mm i.d.); mobile phase, 0.06 % heptanesulfonic acid adjusted with glacial acetic acid to pH 4.5: methanol (60:40); flow rate 1 ml/min; injection volume 100 µl; limit of detection 0.1 µg/ml.

Cyclodextrin was analysed by HPLC (Waters, Milford, USA) using a refractive index detector. The conditions were: stationary phase, Adsorbil NH_2 (15 cm x 4.6 mm i.d.); mobile phase, 80% acetonitrile in water; flow rate 1 ml/min; injection volume 100 µl; limit of detection 1 µg/ml.

The amounts of drug and cyclodextrin depositing in the upper and lower stage of the TSI were expressed as a percentage of the nominal dose. Each formulation was tested six times.

Toxicity studies of preformed cyclodextrin-salbutamol

Male Wistar rats used in this study were obtained from the animal house, Faculty of Science, Prince of Songkhla University

Hat-Yai, Thailand. They were housed for at least one week in the laboratory animal room prior to testing. They (weight 190-210 g) received either salbutamol sulphate (200 μg/ml), GCD (2 mg/ml, 1 ml), DMCD (2 mg/ml, 1 ml) and normal saline solution (1 ml) by intraperitoneal injection. The rats were fasted overnight with water *ad lib* before blood and urine collections. At 0 h (before drug administration), 24 h, 7 days and 1 month after drug administration, the rats were anesthetized with ether and blood was collected from retro-orbital plexus by capillary action for determination of blood chemistry. Plasma was isolated by centrifugation of the whole blood and collected with microsyringe. The plasma urea level was determined for each experimental group of 6 rats. The urine samples were also collected at the same time as that of plasma for creatinine determination.

Plasma urea nitrogen determination

Blood urea nitrogen (BUN) determination employed the method reviewed by Matinek (1969). BUN concentration was measured by coupled enzyme reactions involving urease and glutamate dehydrogenase (BUN reagent). By adding 10 µl of plasma into sample cuvette containing 1 ml of BUN reagent then the cuvette was covered and gently mixed inversely for 30 s. The spectrophotometer was set at a wavelength of 340 nm to read absorbance at exactly 30 and 90 s, respectively. The water was used as a blank. The difference of absorbance at 90 and 30 s, which correlated with BUN concentration linearly, was used to prepare a standard curve.

Urinary creatinine determination

The detection method for urinary creatinine was adapted from Sigma catalogue. 100 µl urinary creatinine reacted with picric acid (10 µl) under alkaline conditions (adding 1 ml of 0.1 N NaOH) to form a yellow orange complex. The color is derived

from creatinine as well as certain non-specific substance likely to be present in the sample. Upon addition of acid (1 ml of 0.1 HCl), the color contributed by creatinine is destroyed while the product of non-specific substance remains. Thus, the difference in color intensity, measured at 500 nm before and after an acidification is proportional to creatinine concentration. The standard curve was prepared in a range of 1-50 mg%.

Red blood cell hemolysis studies

Red blood cells were isolated from whole human blood by centrifugation at 1000 G for 10 min. The plasma was removed and the red blood cells were resuspended in buffered normal saline. The cells were washed and diluted 250 times with buffered normal saline. Solutions of 0.5, 1, 2, 3 and 5 mg% of DMCD and GCD, saline solution and 200 μg/ml salbutamol were prepared by dissolving an accurately weighed amount of the respective compound in buffered normal saline and diluting to volume. All solutions were stored at 37 °C before using them

to dilute red blood cells followed by gentle inversion. The samples were incubated for 5 min at 37 °C, after which time, the red blood cells were counted and viewed microscopically under hemocytometer (Olympus, Tokyo, Japan). The degree of hemolysis was reported as a percentage of red blood cells count incubated in different medium as compared to that in buffered normal saline solution (Hirayama et al., 1999).

Preformed cyclodextrin-salbutamol release studies

A dissolution study was conducted using a condition described by the British Pharmacopoeia for testing salbutamol tablet formulations with the paddle method. The apparatus was set up according to the Pharmacopoeia, the dissolution medium was 200 ml of water set at 37 °C. The paddle rotated at 50 rpm. A semipermeable membrane approximately 3 cm in diameter which was obtained from Spectrum (Spectrapor, Intermedicell, UK) with a molecular weight cut-off from 1,000 was employed in this study. The membrane was prepared by cutting

approximately 6 cm lengths of membrane which was soaked in water for 1 h prior to use. The freeze-dried complex (200 mg) was weighed into this membrane sac. The sac was then clipped at both sides to avoid leakage and placed into dissolution chamber. Samples were taken for each of the two formulations every 5 min up to 30 min. Each experiment was performed at least 6 times and the mean was calculated in each case. The samples were analysed by capillary electrophoresis under the following conditions.

Salbutamol was analysed by employing capillary electrophoresis (Bio-Rad, California, USA). The cartridge was fused silica (70 cm x 75 μm i.d.). The capillary column was first cleaned with 0.1 N NaOH for 3 min then equilibrated with 10 mM phosphate buffer pH 2.4 for 3 min before a sample loaded under pressure injection for 5 s. The detector was set at 214 nm and applied voltage was 10 kV and run time was set for 20 min. Standard concentration was 0.02-0.1 mg/ml.

Statistical analysis of data was performed by one-way ANOVA. Differences were considered to be statistically significant at $p < 0.05$.