CHAPTER II

MATERIALS AND METHODS

Materials

1. Test Products

Thirteen commercial brands of furosemide, 40-mg uncoated tablets, were purchased from drugstores. The letters (A, B, ..., and M) were given to represent the brand names of products. Information of test products were accessible in Appendix A.

2. Reagents

2.1 Standard Furosemide Powder (ASSIA Chemical Lab.) Lot no. 128229

2.2 Internal Standard, 4-chloro-N-furfuryl-5-sulfamoyl-anthranilic acid methyl ester, powder (Hoechst, Frankfurt, Germany)

2.3 Working Standard Furosemide Powder, potency 98.06% (Chinoin, Budapest) Lot no. 830947

2.4 Sodium Hydroxide AR (E. Merck, Darmstadt) Lot no. 507 C 499998

2.5 Monobasic Potassium Phosphate AR (E. Merck, Darmstadt) Lot no. 440 A 877573

2.6 Acetonitrile AR (Fisher Scientific, USA) Lot no. 855916

2.7 Absolute Methanol AR (J.T. Baker, USA) Lot no. 9070-3

2.8 Ortho-Phosphoric Acid 85% (E. Merck, Darmstadt) Lot no.
     616 K 2327173

3. Apparatus

3.1 Analytical Balance (Mettler H 51 AR and Sartorius
     1615 MP)

3.2 Disintegration Tester (GC-21, Hanson Research Corp.,
     Northridge, Calif., USA)

3.3 Dissolution Apparatus (72 RL, Hanson Research Corp.,
     Northridge, Calif., USA)

3.4 Spectrophotometer (Spectronic 2000, Bausch & Lomb,
     N.Y., USA)

3.5 High Pressure Liquid Chromatography (LC-3A, Shimadzu,
     Japan)

Method

1. In Vitro Studies

There were 13 brands of furosemide, 40-mg
uncoated-tablets, evaluated by using the official and non-official
tests of U.S.P. for uncoated tablets. The tests include :

1.1 Weight Variation U.S.P. XX (28)

20 tablets from each brand of furosemide tablets were sampled and accurately weighed tablet by tablet. The average weight and standard deviation were calculated.

1.2 Standard for Content of Active Ingredient in Tablet (29)

20 tablets of each brand were weighed and powdered. A portion of this powder was accurately weighed equivalent to about 40 mg of furosemide and transferred to a 100-ml volumetric flask. Added 25 ml of 0.1 N sodium hydroxide, and allowed to stand for 30 min. with occasional shaking. Diluted this solution with water to volume and mixed. The solution was filtered, discarding the first 10 ml of the filtrate, and 2.0 ml of it was transferred to a second 10-ml volumetric flask. 0.02 N sodium hydroxide was added to volume and mixed. The absorbance of the resulted solution was measured using a spectrophotometer at 271 nm.

The actual content of furosemide in tablet was quantified utilizing a standard solution (Appendix B).

1.3 Disintegration Test (28)

The disintegration tests for 13 brands of furosemide tablets were determined according to the USP XX method for uncoated-tablets.

Procedure :

Individual tablet was introduced into each of the six tubes of the basket. A disk was then added to each tube, and the apparatus was operated using water maintained at 37±2 °C as the immersion fluid. The tablets pass the test if all six disintegrate within thirty minutes. (If any of the tablets does not disintegrate, the test is repeated on a further twelve tablets. The tablets then pass the test if not less than sixteen tablets from all eighteen tablets disintegrate within thirty minutes). The mean disintegration time of each brands was calculated.

1.4 Dissolution Test

From the compendial monograph dissolution requirement, the U.S.P. Dissolution Apparatus II (paddle) (28) is used to establish and compare dissolution profiles. A phosphate buffer, pH 5.8±0.05, is used as a dissolution medium. (Preparation of dissolution medium see Appendix C).

Dissolution rates of furosemide from six tablets of each brand were studied according to the following procedure.

Nine hundred milliliters of dissolution medium was placed in the vessel and equilibrated at 37±0.5°C. A tablet was introduced into each of the six vessel, the apparatus was then immediately operated and maintained stirring speed at 50 rpm. 5 ml of dissolution medium was taken from each vessel at 2, 5, 7, 10, 15, 20, 30, 50, 70, 90, 120, 150, 180, 210 and 240 minutes intervals and added immediately the same quantity of it after each sampling to keep the

volume of dissolution medium constant during the course of test. The absorbance of the drug dissolved in dissolution medium were measured using a spectrophotometer at 271 nm. The amount of the drug dissolved at sampling time intervals was calculated from the standard curve.

The standard curve could be obtained from the preparation of series of standard solutions with known amounts of furosemide in a dissolution medium (Appendix D). Analysed this solution set by spectrophotometric method at 271 nm. using a spectrophotometer. Plotted the absorbance observed versus known concentration of drug and fitted these plots to a straight line using linear regression.

1.5 In Vitro Evaluation

Physical characteristics of 13 brands of furosemide tablets were examined and evaluated to determine whether they passed the general standard U.S.P. requirement for uncoated-tablet. Analysis of variance and multiple comparison (30) were performed to assess the differences between the original and other brands for the disintegration times and the dissolution rates. This is accomplished using a computerized statistical program SPSS (Appendix E).

2. In Vivo Studies

    2.1 Bioavailability Study

        2.1.1 Test Products

                Four brands of furosemide tablet with differences in vitro dissolution characteristics were selected. One was the original brand assigned as the reference standard against the others. The other brands had maximum, moderate and minimum dissolution values respectively.

        2.1.2 Subjects

                Eight healthy male volunteers with 25 to 39 years of ages and within 10% of their ideal body weights participated in this study. A medical history, completely physical examination and standard laboratory screen for individual subject were performed prior to the study to ensure the absence of any significant hepatic, renal disturbance and/or the gastrointestinal tract disorder. The method of the study was fully explained to all subjects and all gave their written consent before entering the study. They abstained from medications, smoking and alcohol for 1 week prior to and throughout the study.

2.1.3 Drug Administration

Subjects fasted for 9 hr. before each drug administration and 1 hr. thereafter. A single dose of 40 mg furosemide tablet was taken orally with 200 ml of water.

2.1.4 Experimental Design

The study was conducted in a randomized crossover design. Each subject received the drug in a randomized order, with a one-week 'washout' period between each administration as shown in Table 1

2.1.5 Sample Collection

Blood samples (5-7 ml) were collected from a forearm vien using a plastic syringe with immediately transfer to heparinized tubes. Blood was collected before and at 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 and 5.0 hr. after drug administration. Following centrifugation (3000 rpm. for 5 minutes), the plasma were stored in the deep-freeze until analyzed. During each study day, urine was collected immediately before drug administration and for the following periods : 0-1, 1-2, 2-3, 3-4, 4-5 and 5-6 hr. Urine volumes were recorded; aliquots were stored in the deep-freeze for electrolyte assay.

Table 1   Treatment Schedule

| Subject | Week | | | |
no.	1	2	3	4
1	A[a]	D	B	C
2	D	B	C	A
3	B	C	A	D
4	C	A	D	B
5	A	D	B	C
6	D	B	C	A
7	B	C	A	D
8	C	A	D	B

a. Each A, B, C, and D represented the brand name of furosemide
   tablets

2.1.6 Determination of Furosemide in Plasma

Concentrations of furosemide in plasma samples were determined using modified high-performance liquid chromatographic method described by Rapaka et. al. (31). The procedure was developed as follows:

Plasma sample 1 ml

— mixed with 0.5 ml of internal standard[*] (1 μg in acetonitrile 0.5 ml) for 10 seconds

— extracted with 1.5 ml acetonitrile (mixed 10 seconds then centrifuged 3000 rpm. for 10 minutes)

supernatant

— evaporate under nitrogen gas for 10 min

sample residue

— mixed with 0.5 ml of 0.08 M Phosphoric acid

inject 100 μl of the sample solution into the HPLC column.
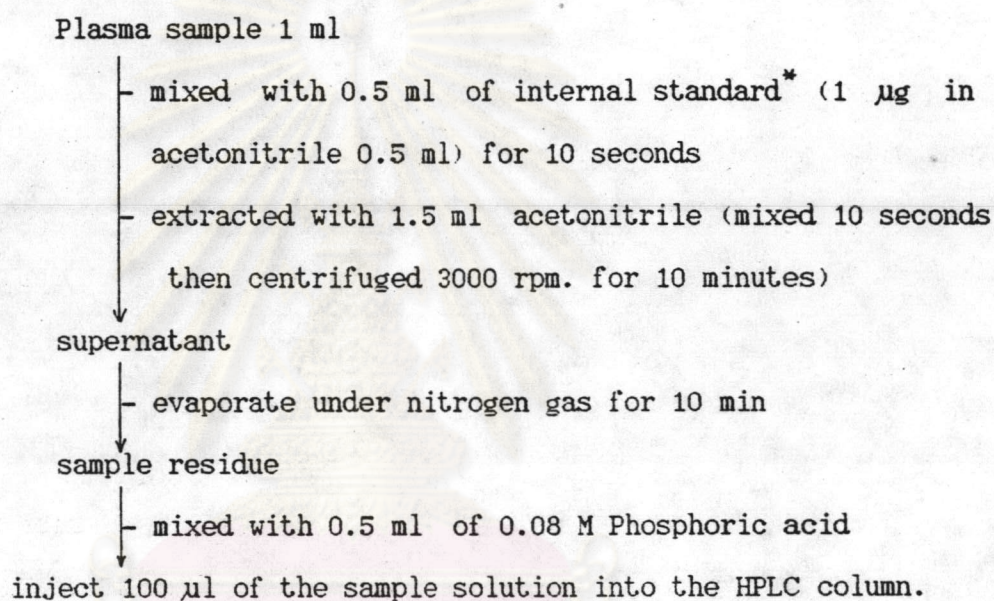
2.1.7 Operating condition

Apparatus : HPLC LC-3A, shimadzu, Japan

Column   : Sorbax ODS, stainless steel column,
            Dupont Instruments P.N. 850952 702
            pre-column 5 cm x 2.0 mm i.d.
            analysis-column 25 cm. x 4.6 mm i.d.

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[*]4-chloro-N-furfuryl-5-sulfamoyl-anthranilic acid methyl ester.

Mobile phase : acetonitrile : 0.08 M phosphoric

acid (40 : 60)

Fluorescence detector : EX-2 filter and EM-4filter

Flow rate   : 1.7 ml/min

Attenuation : 2 mv/full scale

Pressure   : 200-240 kg/cm^2

Temperature : ambient

Speed chart : 2mm/min

Injected volume : 100 μl

The furosemide concentration in plasma samples were quantified employing the standard curve (Appendix D).

2.1.8 Standard Curve

Known amounts (0.2, 0.3, 0.5, 0.6, 0.8, 1.0 and 1.5 μg) of standard furosemide and 1 μg of internal standard were added to 1 ml of pooled human plasma. These samples were analysed following the same procedure as described previously (31). The ratio of the peak height of furosemide and internal standard obtained versus the known furosemide concentrations were fitted to a straight line using linear regression (Appendix D).

2.1.9 Pharmacokinetics Analysis

Individual plasma furosemide profile from each treatment was analyzed according to a one-compartment open model with first-order absorption and elimination with lag time using the

PCNONLIN nonlinear estimation program (32), (Appendix F)

2.1.10 Statistical Evaluation for Bioavailability Results

The comparative bioavailability of the 4 brands of furosemide tablets were evaluated using the following parameters : (a) the peak plasma concentration (Cp max), (b) the time of the peak plasma concentration (tmax), and (c) the area under the plasma concentration-time curve $[AUC]_o^\infty$. Nonparametric test such as Friedman's and Wilcoxon Rank Sum Test were used to analyze for differences among and between the original brand and the selected local brands. This is accomplished using a computerized statistical program SPSS (Appendix E).

2.2 Clinical Response Study

2.2.1 Determination of Urine Output and Electrolyte Excretion

After drug administration, urine samples were collected over the intervals of 0-1, 1-2, 2-3, 3-4, 4-5 and 5-6 hr. The volume of each sample was recorded; aliquots were stored in a deep-freeze for electrolyte assay. Electrolyte in urine samples were measured by the laboratory; those electrolyte were sodium, chloride and potassium.

2.2.2 Statistical Evaluation of Clinical Response

The differences in clinical response of four brands of furosemide tablets were evaluated using the following parameters : (a) the magnitude of diuresis, e.g., urine volumes, and (b) electrolyte excretion, e.g., urinary sodium, chloride and potassium.