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Biopharmaceutical Studies of Thiazide Diuretics. I. Determination of pK_a Values and Partition Coefficients of Thiazide Diuretics

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The overlapping pK_a values of several thiazide diuretics were determined at 25±2 °C by an improved spectrophotometric method. The absorbance of the ionized form cannot be measured directly due to the overlapping, but can be calculated from the data obtained experimentally in a region where overlapping does not occur. This method can be used to determine the pK_a values of drugs to which the usual means available for measurement of the ionization constants cannot be applied. The partition coefficients of these drugs between water and *n*-octanol were also determined at pH 2—10, and the behavior on an adsorption chromatographic column was studied. The pH profiles of both characteristics were very similar.

Keywords——overlapping pK_a; partition coefficient; hydrochlorothiazide; hydroflumethiazide; chlorothiazide; trichlormethiazide; penfluzide; cyclothiazide; polythiazide

Thiazide diuretics are commonly used for the treatment of hypertension, congestive heart failure and other edematous conditions. To carry out structure–activity studies and to determine the concentration in the body fluids by high-performance liquid chromatography, it is necessary to determine the physicochemical properties of these drugs. This need prompted us to investigate the pK_a values and partition coefficents, since these are the most important physicochemical constants in relation to medical and pharmaceutical studies.

There are a few reports[1)] dealing with the pK_a of thiazide diuretics. However, determination of the pK_a values for these diuretics is quite complicated, particularly if the ratio of the successive ionization constants is less than 1000.[2,3)] One major difficulty is the estimation of the absorbance of each ionized form, because the absorbance cannot be measured directly due to the overlapping ionizations.

Experimental

Materials——The sources of thiazide diuretics used in this work were as follows: hydrochlorothiazide (Esidrex®) from Ciba-Geige (Japan), hydroflumethiazide (Rontyl®) from Sankyo Co., chlorothiazide (Chlotride®) from Nippon Merck-Banyu Co., trichlormethiazide (Fluitran®) from Shionogi & Co., penfluzide (Brizide®) from Toyo Jozo Co., cyclothiazide (Valmiran®) from Nippon Boehringer Ingelheim Co., and polythiazide (Renese®) from Pfizer Taito Inc. Purification of the selected drugs was carried out by recrystallization to a constant melting point from ethanol or ethanol–water mixtures.

Metanilamide was prepared from metanilic acid by the method of Jacobs,[4)] giving fine white crystals, mp 141 °C. All other chemicals and solvents were of analytical reagent grade.

Ultraviolet (UV) Spectra——The UV absorption spectra were recorded with a double-beam spectrophotometer

(UV-200, Shimadzu Corporation) with a constant-temperature cell holder maintained at 25±2 °C. All spectra were measured against a blank consisting of the buffer used as the solvent for the compound, and all the curves for one compound were recorded on the same paper. Experimental solutions were prepared by diluting the drug solution with the appropriate buffer to give a concentration of 50 μM. No apparent decomposition could be observed during the course of each experiment.

Buffers and pH Measurement——Buffers were prepared with deionized and glass-distilled water, and adjusted to a constant ionic strength of 0.1 with NaCl. Components of buffers were as follows: pH 1.1—3.4, HCl and KH_2PO_4; pH 2.9—6, acetic acid and sodium acetate; pH 5—8, KH_2PO_4 and Na_2HPO_4; pH 8—11, NH_4Cl and NH_3; pH 11—13, Na_2HPO_4 and NaOH. The pH measurements were made with a pH meter (F-8, Horiba-Hitachi) at 25±1 °C.

Partition Coefficients——The drug was dissolved in a suitable buffer at a concentration of 0.5 mM. The solution (5 ml) was partitioned in an *n*-octanol–aqueous system (the organic phase having been presaturated with water) in a 25-ml glass-stoppered tube. In most cases, a 1 : 1 (v/v) ratio of *n*-octanol to buffer was used. The tube was shaken mechanically at 25±2 °C until attainment of equilibrium. The drug concentration in the aqueous phase after centrifugation was determined by UV absorbance measurement in conjunction with a standard curve obtained from duplicate samples using two separate weighings of the drug. When the concentration of hydrochlorothiazide (HCT) was measured, the colorimetric method was used in addition. There was fair agreement between the assay values obtained by the two methods.

Colorimetric Method for Hydrochlorothiazide——This procedure is a modification of the method described by Sheppard.[5] The sample (1 ml) was put into a test tube with 1 ml of 10 N NaOH. This mixture was hydrolyzed in a boiling water bath for 60 min, then cooled to room temperature, and 5 ml of 3 N HCl and 1 ml of 0.01% $NaNO_2$ were added to diazotize the amino group. After 3 min, 1 ml of 0.5% sulfamic acid was added to destroy the excess nitrite. The mixture was allowed to stand for 5 min, then 1 ml of 0.05% Tsuda's reagent was added. The absorbance was determined at 525 nm within 15 to 45 min. The standard curve was reproducible and linear over the concentration range of 10—150 μM (3—45 μg/ml).

High-Performance Liquid Chromatograph——The high-performance liquid chromatograph consisted of a constant flow pump (Hitachi 635S, 1.0 ml/min), a multi-wavelength UV detector (Hitachi 635M, 280 nm, 0.08 AUFS) and a recorder (Hitachi 056, 10 mV, 2.5 mm/min). A stainless steel column (500×2.6 mm, i.d.) was packed with polystylene–divinylbenzene (Hitachi Gel #3010, particle size 15—20 μm). This system was operated at 25±2 °C. Each solvent was degassed with an ultrasonic apparatus just before use. Solvent A (for chlorothiazide, hydrochlorothiazide, hydroflumethiazide, and trichlormethiazide) was composed of 30% (v/v) methanol and 70% (v/v) 0.01 M phosphate buffer. The phosphate buffer consisted of Na_2HPO_4 and H_3PO_4, and the pH was adjusted in the range of 3—10. Solvent B (for trichlormethiazide, cyclothiazide, polythiazide, and penfluzide) was 60% (v/v) methanol and 40% (v/v) phosphate buffer. The drug (2 mg) was dissolved in 1 ml of methanol, and a 1—3 μl sample was injected into the chromatograph. The capacity factor k' is defined as:

$$k' = (V_r - V_0)/V_0 \tag{1}$$

where V_0 = retention volume of the solvent, and V_r = retention volume of the compound.

Results and Discussion

Overlapping pK_a

UV spectrophotometry is commonly used in the determination of pK_a values of sparingly soluble drugs. This and other techniques have been reviewed in varying detail in some excellent sources.[2]

The spectrophotometric method requires significant pH-dependent changes in the absorption spectra of a compound. The wavelength of greatest difference in absorbance between the spectra of the unionized form and the ionized form is chosen as the optimum analytical wavelength for a pK_a determination. The pK_a values are calculated using Eq. 2.[2]

$$pK_a = pH_i - \log[(A_U - A_i)/(A_i - A_I)] \tag{2}$$

where A_i = absorbance at pH_i of each buffer solution tested, A_U = absorbance of the unionized species, and A_I = absorbance of the completely ionized species. UV spectra are obtained by scanning several drug solutions in increments of 0.2—0.3 pH unit around pK_a region. From the spectra obtained, A_i is evaluated at each pH_i. In the case of benzene sulfonamide (monoprotic acid), there is only one pK_a, and all the spectra pass through an isosbestic point. Sulfanilamide and metanilamide (ampholyte) exhibit two pK_a values, but these values are separated by more than 6 pH units ($\Delta pK_a > 6$) and the spectra pass through isosbestic points. It is easy to determine the two pK_a values of such a compound.

The situation is more complicated when the pK_a values of the various groups are sufficiently close for their ionizations to overlap (*i.e.* $\Delta pK_a < 2.7$).[2b)] Equation 2 is no longer applicable and recourse to more sophisticated approaches is necessary.[3)] That is to say, the second ionization starts before the first ionization is completed. Consequently, the specra do not pass through an isosbestic point, and the A_I value cannot be determined. Some investigators[3)] tried to determine overlapping pK_a's, but they were generally unsuccessful.

Yoshioka[3f)] tried to calculate the overlapping pK_a's of sulfonamides. He selected some A_i and pH_i values, and estimated pK_a. However, because the selection of the combination of A_i and pH_i influences the estimated A_I, it was hard to obtain an accurate pK_a value.

We devised a new calculation method for overlapping pK_a's without determination of A_I. Equation 2 is rearranged by substituting the first ionization constant K_{a1}:

$$K_{a1} = [H_i^+](A_{UI} - A_i)/(A_i - A_{II}) \qquad (3)$$

and therefore

$$A_i = A_{II} + [H_i^+](A_{UI} - A_i)/K_{a1} \qquad (4)$$

A_{UI} can be measured. A_i values were obtained from the spectra which passed through the isosbestic point. $[H_i^+]$ were calculated from each pH_i. Equation 4 is a linear relationship and can be expressed as

$$y = ax + b \qquad (5)$$

in which y is A_i, x is $[H_i^+]$ $(A_{UI} - A_i)$, and a $(= A_{II})$ and b $(= 1/K_{a1})$ are constants. Using the least-squares method, we can obtain these constants, and evaluate pK_{a1}.

In the case of HCT, the UV absorption spectra are shown in Fig. 1. Based on the pH profile of partition coefficients of HCT shown in Fig. 4, the solid line (pH 1.2—6) in Fig. 1

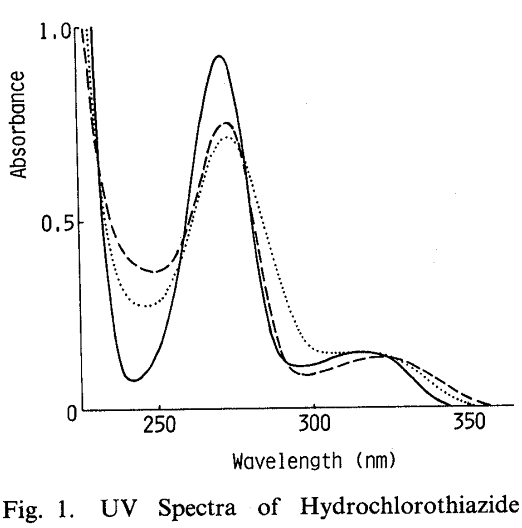

Fig. 1. UV Spectra of Hydrochlorothiazide (50 μM Solutions)
——, pH 1.2—6; · · · ·, pH 9.6; – – – –, pH 12.5—13.

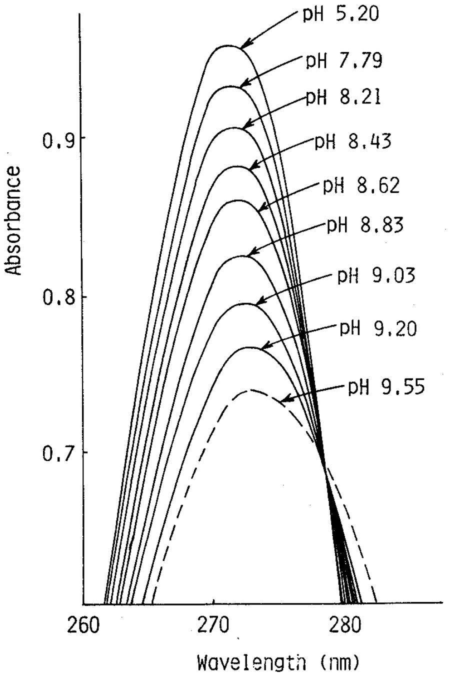

Fig. 2. UV Spectra of Hydrochlorothiazide (50 μM Solutions)

seems to be the spectrum of the unionized form. A similar curve was obtained for the methanol solution of this compound.[6] The dotted line (pH 12.5—13) in Fig. 1 is presumed to be the spectrum of the divalent form.

The region from pH 7.8 to pH 9.5 was studied in more detail by using buffers differing by increments of 0.2 pH units (Fig. 2). The UV spectra were recorded from 260 to 285 nm, the absorbances at 271 nm were determined, and the pH's were read. The curve obtained at pH 5.20 is the spectrum of the unionized form. An isosbestic point developed at 279 nm. All the absorption spectra at pH values ranging from 5.20 to 9.20 pass through the isosbestic point, but curves at more than pH 9.5 do not. This effect seems to reflect the second ionization. Absorbance values at 271 nm are listed in Table I. $[H_i^+]$ $(A_{U1}-A_i)$ was calculated from A_{U1} and A_i and is plotted against A_i in Fig. 3. The constants of Eq. 4 were obtained by the least-squares method, and Eq. 4 therefore became as follows:

$$A_i=0.672+[H_i^+]\,(0.950-A_i)\times 6.077\times 10^8 \qquad (6)$$

$$n=7,\ r=0.991$$

where n is the number of points used in the experiment and r is the correlation coefficient. The values of pK_{a1} (=8.78) and A_{I1} (=0.672) were obtained from Eq. 6.

The second ionization constant K_{a2} was calculated based on an investigation from the alkaline side. Equation 2 can be rearranged by substituting the second ionization constant K_{a2}

TABLE I. Absorbance of Hydrochlorothiazide Solutions (50 μM) at 271 nm

pH	5.20	7.79	8.21	8.43	8.62	8.83	9.03	9.20
A_{271}	0.950	0.924	0.890	0.867	0.843	0.800	0.773	0.744

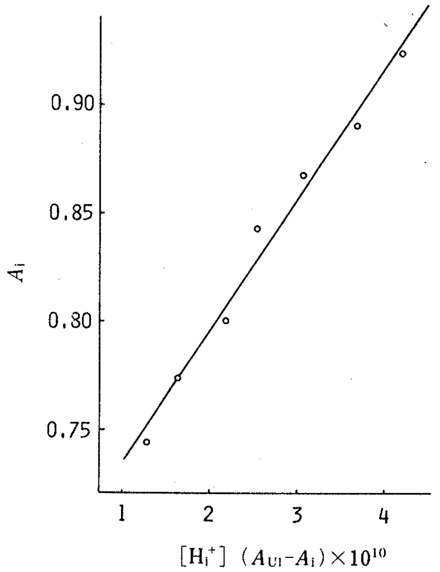

Fig. 3. Relationship between Absorbance and $[H_i^+](A_{U1}-A_i)$ for Hydrochlorothiazide

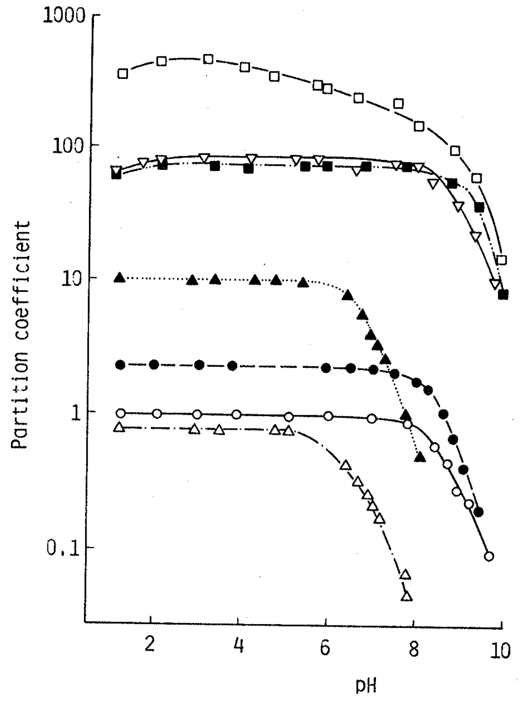

Fig. 4. pH Profile of Partition Coefficients (n-Octanol–Water) of Thiazide Diuretics

○, hydrochlorothiazide; ●, hydroflumethiazide; △, chlorothiazide; ▲, trichlormethiazide; □, penfluzide; ▽, cyclothiazide; ■, polythiazide.

$$A_i = A_{U2} + K_{a2}(A_{I2} - A_i)/[H_i^+] \tag{7}$$

A_{I2} can be measured in alkaline solution. The region from pH 12.83 to pH 9.75 was studied in detail in the same manner as for the first ionization. The curve obtained at pH 12.83 is the spectrum of the divalent form. By using 288 nm (Table II) as the analytical wavelength, $(A_{I2} - A_i)/[H_i^+]$ was calculated and the values were plotted against A_i. Constants were obtained by the least-squares method, and Eq. 7 therefore became as follows:

$$A_i = 0.460 + 4.36 \times 10^{-11}(0.190 - A_i)/[H_i^+] \tag{8}$$

$$n=7,\ r=0.984$$

The pK_{a2} and A_U values were 10.36 and 0.460, respectively.

The pK_{a1} and pK_{a2} values obtained by our method are given in Table III and compared with literature values. According to our study, the pK_a values of the sulfamoyl group of benzene sulfonamide, sulfanilamide, metanilamide were 10.20, 10.60, 10.13, respectively. Thus, it appears that the ionization constant of the sulfamoyl group is not strongly influenced by the substituent group on the benzene nucleus. The values of pK_{a2} (Table III) are about 10, and are thus presumed to be the pK_a value of the sulfamoyl group.

Polythiazide has a single pK_a (Table III). The value of its pK_a is about 10, and the UV absorption peak shifts to the short wavelength side: hence this pK_a is presumed to reflect the ionization of the sulfamoyl group. The nitrogen atom at the 2-position of polythiazide is acetylated, in contrast to that of the other thiazide diuretics, and does not ionize. Therefore, the pK_{a1} values of the other diuretics reflect the ionization of N–H at the 2-position.

This method offers a serviceable approach to determination of the ionization constants of drugs which exhibit overlapping pK_a's. It is extremely reproducible and easy to apply.

Partition Coefficient

Thiazide diuretics must be extracted from the body fluids to be assayed. It was

TABLE II. Absorbance of Hydrochlorothiazide Solutions (50 μM) at 288 nm

pH	12.83	11.39	11.02	10.80	10.64	10.42	10.24	9.91
A_{288}	0.190	0.212	0.241	0.259	0.283	0.319	0.350	0.383

TABLE III. pK_a Values of Thiazide Diuretics

Diuretic	pK_{a1}		pK_{a2}	
	Author[a]	Literature	Author[a]	Literature
Hydrochlorothiazide	8.78	7.0,[b] 8.80,[c] 8.7,[d]	10.36	9.2[b]
Hydroflumethiazide	8.59	8.9,[b] 8.45,[c] 8.5,[d]	10.33	10.7[b]
Chlorothiazide	6.85	6.83[c]	9.57	
Trichlormethiazide	6.80	6.9[d]	9.99	
Penfluzide	8.50		10.39	
Cyclothiazide	8.67	9.1[e]	10.49	10.5[e]
Polythiazide	—		9.58	9.1[d]

a) At 25±2 °C, $\mu=0.1$.
b) Reported in reference 1*d*).
c) Reported in reference 1*a*).
d) Reported in reference 1*b*).
e) Reported in reference 1*c*).

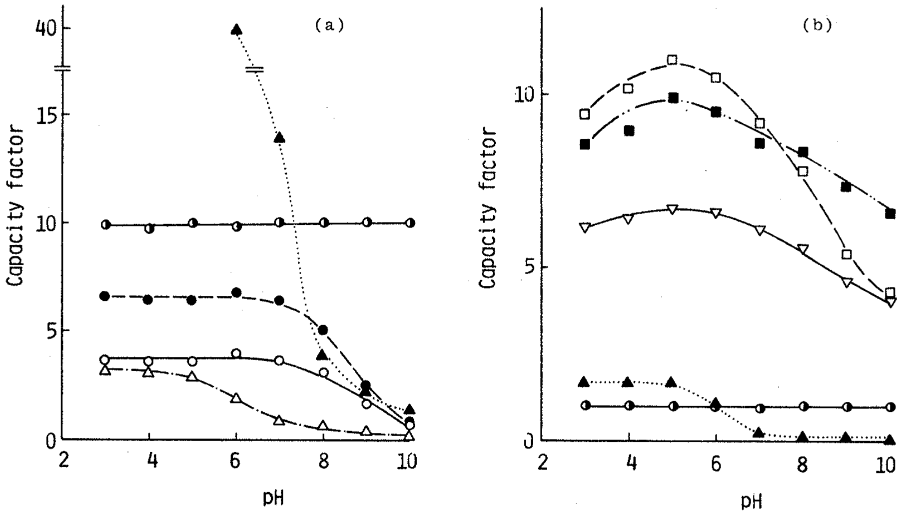

Fig. 5. pH Profile of Capacity Factors of Thiazide Diuretics

○, hydrochlorothiazide; ●, hydroflumethiazide; △, chlorothiazide; ▲, trichlormethiazide; □, penfluzide; ▽, cyclothiazide; ■, polythiazide; ◑, acetyl aminoantipyrine.
Column: Hitachi Gel #3010, 2.6 × 500 mm.
Eluent: (a), solvent A; (b), solvent B.
Flow rate: 1.0 ml/min.

anticipated that the maximal partition coefficient would be obtained for the unionized form. A definite pH dependency of the partition coefficient was observed (Fig. 6). Generally, diuretics which are given orally at a small dosage[7] and have a substituent group at the 2-position are considered to be lipophilic.

Effect of pH on High-Performance Liquid Chromatography

Figure 5 shows the effect of pH on the retention volumes (capacity factors) of the thiazide diuretics. The pH has no effect on the elution of a non-ionized compound (*N*-acetyl-4-aminoantipyrine, $pK_a=0.5$[8]), but with thiazide diuretics, the elution volume falls as the pH rises. The pH values of half-retention for the unionized form are very close to the pK_{a1} values of the compounds. The thiazide diuretics with a substituent group at the 2-position are held much more strongly on the polymer gel than the thiazide diuretics without a substituent group at the 2-position. When we compare Fig. 5 with Fig. 4, it is evident that the unionized molecules are retained, but the anions are not.

In the case of unionized molecules, a large partition coefficient results in an increase of capacity factor. A linear relationship between partition coefficients and capacity factors was obtained for seven diuretics, as follows:

$$\log(k'/k_A') = -0.421 + 0.651 \log P \qquad (9)$$

$$n=7,\ r=0.986,\ s=0.126$$

where k_A' = capacity factor of acetyl aminoanitpyrine, P = partition coefficient at pH 5 (pH range of unionized form), and s = standard deviation.

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