Spectral Determination of Microdissociation Constants

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Abstract ☐ Two new methods are given for the spectral determination of microdissociation constants. The methods were compared to the method used currently, using D,L-tyrosine and morphine hydrochloride. In both instances the two new methods gave better agreement between calculated and observed experimental points. One of the new methods, based on nonlinear regression analysis, is the method of choice if a high speed digital computer is available.

Keyphrases ☐ Microdissociation constants—determination, two methods for analyzing spectral data ☐ Nonlinear regression analysis—applied to microdissociation constant determinations ☐ Spectrophotometry—determination of microdissociation constants, two methods for analysis of data

The accurate determination of the microdissociation constants of drugs is important for a complete understanding of their chemical behavior and biological activity. Many drugs contain phenolic, sulfhydryl, or other groups whose absorbance at a particular wavelength changes as the percentage dissociation of the group increases. In most instances, the change in absorbance is not affected by the presence or ionization of other acidic groups such as the protonated amino or carboxyl group. It is not surprising, therefore, that spectral methods have been shown to be useful for estimating the microdissociation constants of such drugs. The purpose of this communication is to report two methods of analyzing spectral data which have distinct advantages over the methods presently used.

EXPERIMENTAL

Reagents—The following were used: D,L-tyrosine[1], m.p. 316° dec.; potassium chloride, reagent grade; carbonate-free 5 N potassium hydroxide; reagent grade 5 N hydrochloric acid; and distilled, deionized water which had been degassed by boiling for 30 min.
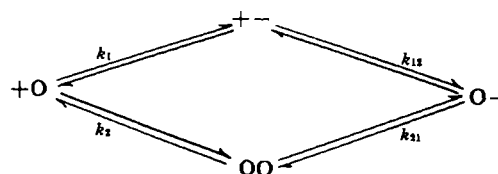
Equipment—The spectral studies were carried out at 25° using a spectrophotometer[2]. The pH measurements were made with an expanded scale pH meter[3].

Methods—Solutions containing 1.0 × 10⁻³ M D,L-tyrosine were made in a 0.1 M potassium chloride solution. Sixty milliliters of this solution was added to a 100-ml. beaker, and the pH was adjusted using 5 N potassium hydroxide or 5 N hydrochloric acid. In no instance did the amount of acid or base added exceed 0.2 ml. The absorbance of the solution was immediately read at 292 nm. *versus* a 0.1 M potassium chloride solution blank. The pH was again measured to ensure that no change in pH had taken place The absorbance thus measured was corrected for the absorbance of the completely protonated drug by subtracting the absorbance of a solution at pH 1.23. It was determined previously that above pH 12.5 the drug was completely ionized, as evidenced by no further increases in absorbance as the pH was raised.

THEORETICAL

Typical examples of chemicals whose microdissociation constants can be obtained through the use of spectral data are morphine hy-

drochloride and D,L-tyrosine. In the interest of simplification, the molecules will be represented in their most highly protonated state as +O, in which the + refers to the protonated amino group and the O refers to the protonated phenolic group. The uncharged form, representing the dissociation of the amino proton only, can be given as OO; the zwitterion form representing dissociation only of the phenolic proton can be given as +−, and the completely dissociated form as O−. Thus the entire dissociation scheme can be given as:

in which:

$$k_1 = \frac{[+-][H_2O^+]}{[+O]} \qquad k_2 = \frac{[OO][H_2O^+]}{[+O]} \qquad \text{(Eq. 1a)}$$

$$k_{12} = \frac{[O-][H_3O^+]}{[+-]} \qquad k_{21} = \frac{[O-][H_2O^+]}{[OO]} \qquad \text{(Eq. 1b)}$$

The relationships between the macro- and microdissociation constants are:

$$K_1 = k_1 + k_2 \qquad \text{(Eq. 2)}$$

$$1/K_2 = 1/k_{12} + 1/k_{21} \qquad \text{(Eq. 3)}$$

$$K_1 K_2 = k_1 k_{12} = k_2 k_{21} \qquad \text{(Eq. 4)}$$

From the set of Eqs. 1:

$$[+-] = k_1[+O]/[H_3O^+] \qquad \text{(Eq. 5a)}$$

$$[OO] = k_2[+O]/[H_3O^+] \qquad \text{(Eq. 5b)}$$

$$[O-] = k_{12}[+-]/[H_3O^+] = k_1 k_{12}[+O]/[H_3O^+]^2 = $$
$$k_2 k_{21}[+O]/[H_3O^+]^2 \quad \text{(Eq. 5c)}$$

The fraction of total phenolic group dissociated, α, can be given as:

$$\alpha = \frac{[+-] + [O-]}{C_a} \qquad \text{(Eq. 6)}$$

in which C_a is the stoichiometric concentration of drug. A mass balance on C_a gives:

$$C_a = [+O] + [+-] + [OO] + [O-] \qquad \text{(Eq. 7)}$$

Substituting the identities of Eqs. 5 into Eq. 7 and solving for [+O] give:

$$[+O] = \frac{[H_3O^+]^2 C_a}{[H_3O^+]^2 + K_1[H_3O^+] + K_1 K_2} \qquad \text{(Eq. 8)}$$

in which K_1 and $K_1 K_2$ have been introduced from Eqs. 2 and 4.

Utilizing Eq. 8 and the definitions for [+−] and [O−] from Eqs. 5 gives, after insertion into Eq. 6:

$$\alpha = \frac{k_1[H_3O^+] + K_1 K_2}{[H_3O^+]^2 + K_1[H_3O^+] + K_1 K_2} \qquad \text{(Eq. 9)}$$

which can be rearranged to the linear form:

$$\alpha([H_3O^+] + K_1) = k_1 + \frac{K_1 K_2(1 - \alpha)}{[H_3O^+]} \qquad \text{(Eq. 10)}$$

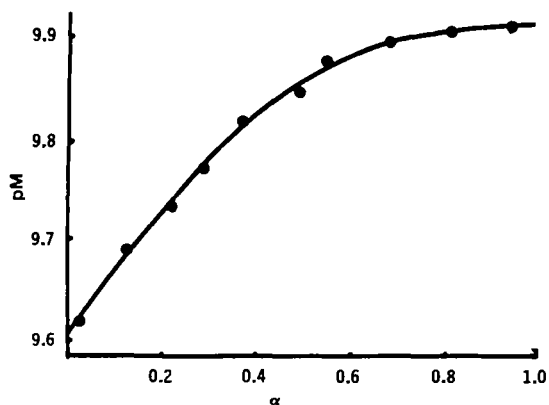[1] Nutritional Biochemical Co.
[2] Beckman DU.
[3] Corning model 10.

Figure 1—*Plot of pM versus α for D,L-tyrosine.*

The value for K_1 can be obtained by standard potentiometric titration. Thus, Eq. 10 can be plotted to give a straight line with an intercept of k_1 and a slope of K_1K_2.

The method of Edsall *et al.* (1) appears to be the method currently used for determining microdissociation constants and can be illustrated by the work of Schill and Gustavii (2) for morphine hydrochloride and Riegelman *et al.* (3) for phenylalkanolamines. In this method, a variable pM is defined as:

$$pM = pH - \log \frac{\alpha}{1 - \alpha} \qquad (Eq. 11)$$

Using the definition of α as given by Eq. 9 and the relationships between the micro- and macrodissociation constants given by Eqs. 2 and 4 yields:

$$pM = -\log \frac{k_1[H_2O^+] + k_2k_{21}}{[H_2O^+] + k_2} \qquad (Eq. 12)$$

A plot of pM *versus* α is made when α = 0, or $[H_3O^+] \to \infty$, pM = pk_1; when α = 1, or $[H_2O^+] \to 0$, pM = pk_2; and finally, when α = 0.5:

$$k_2 = \frac{[H_3O^+](k_1 - [H_3O^+])}{[H_3O^+] - k_{21}} \qquad (Eq. 13)$$

Since this method depends upon a smooth line being drawn through the data points, it is subject to investigator bias. Furthermore, this method does not yield any estimate of the standard deviation of the parameters obtained. Thus, although the use of Eq. 10 requires prior knowledge of K_1, the data can be subjected to least-squares analysis to remove possible investigator bias and to obtain estimates of the standard deviations for the constants.

Nonlinear Regression Analysis—One difficulty in utilizing Eq. 10 or 11 is that the experimental data are rearranged to give derived variables which are plotted. These derived variables are inherently less accurate than the raw experimental values used to obtain them. One method of eliminating this difficulty is to utilize the methods of
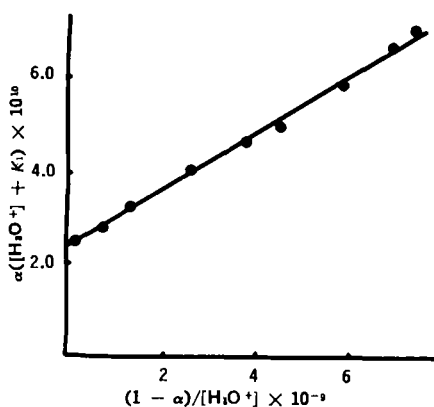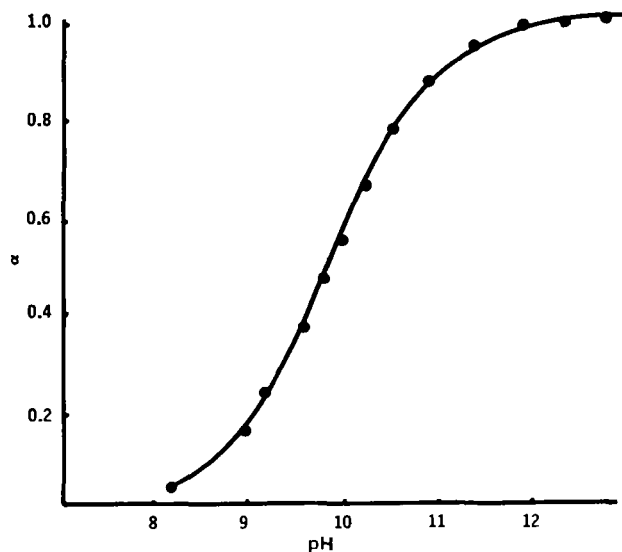

Figure 2—*Plot of Eq. 10 for D,L-tyrosine.*

Figure 3—*Comparison between experimental and calculated values for α at various pH values for the method of nonlinear regression. The solid line represents the computer-drawn curve from Eq. 9, while the solid circles represent experimental data.*

nonlinear regression analysis (4–6). To utilize nonlinear regression analysis, the partial derivatives of Eq. 9 with respect to k_1, K_1, and K_1K_2 must be obtained to give:

$$F_1 = \partial\alpha/\partial k_1 = \frac{[H_3O^+]^2 + K_1[H_3O^+]^2 + K_1K_2[H_3O^+]}{([H_3O^+]^2 + K_1[H_3O^+] + K_1K_2)^2} \qquad (Eq. 14)$$

$$F_2 = \partial\alpha/\partial K_1 = \frac{k_1[H_3O^+]^2 + K_1K_2[H_3O^+]}{([H_3O^+]^2 + K_1[H_3O^+] + K_1K_2)^2} \qquad (Eq. 15)$$

$$F_3 = \partial\alpha/\partial K_1K_2 = \frac{-([H_3O^+]^2 + K_1[H_3O^+] - k_1[H_3O^+])}{([H_3O^+]^2 + K_1[H_3O^+] + K_1K_2)^2} \qquad (Eq. 16)$$

The nonlinear regression analysis was performed on an IBM 360-75 system utilizing the computer program previously given by Niebergall *et al.* (7).

RESULTS AND CONCLUSIONS

A plot of pM *versus* α for D,L-tyrosine is shown in Fig. 1, and a plot of Eq. 10 is shown in Fig. 2. The intercepts from Fig. 1, Eq. 12, and Eqs. 2 and 4 yielded the values for the micro- and macrodissociation constants shown in Column 1 of Table I. The data used to plot Fig. 2 were analyzed using the nonlinear regression analysis and computer program described by Niebergall *et al.* (7) on an IBM 360-75 high speed digital computer. In utilizing this procedure, Eq. 10 can be represented as:

$$Y = P_1 + P_2X \qquad (Eq. 17)$$

Table I—Micro- and Macrodissociation Constants for D,L-Tyrosine at Ionic Strength of 0.1 at 25° [a]

Constant	pM *versus* α	Method — Linear Regression	Nonlinear Regression
pK_1	8.81	9.11	8.95(0.15)
pK_2	9.70	10.11	10.08(0.20)
pK_1K_2	18.51	19.22(0.04)	19.03(0.05)
pk_1	9.61	9.63(0.03)	9.57(0.03)
pk_2	8.89	9.26	9.07(0.08)
pk_{12}	9.20	9.59(0.03)	9.46(0.09)
pk_{21}	9.92	9.95	9.96(0.08)

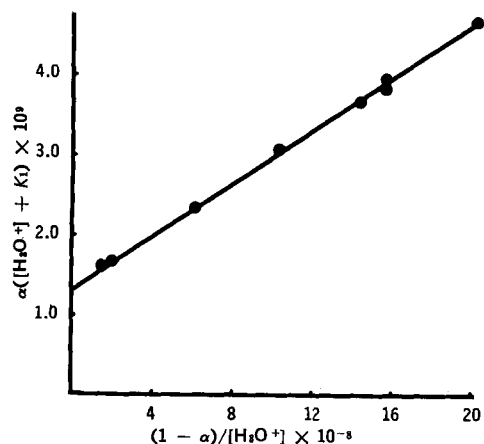[a] Figures in parentheses represent one standard deviation.

Figure 4—*Plot of Eq. 10 for morphine hydrochloride. Data taken from Schill and Gustavii (2), Table 2.*

in which Y is equal to $\alpha([H_3O^+] + K_1)$, X is equal to $(1 - \alpha)/[H_3O^+]$, P_1 represents k_1, and P_2 represents K_1K_2. The partial derivatives of Eq. 16 with respect to P_1 and P_2 are:

$$F_1 = \partial Y/\partial P_1 = 1.0 \qquad \text{(Eq. 18)}$$

$$F_2 = \partial Y/\partial P_2 = X \qquad \text{(Eq. 19)}$$

Initial estimates of P_1 and P_2 were taken to be zero which, in effect, reduces the nonlinear regression analysis to a linear regression analysis. The results are shown in Column 2 of Table I. The regression analysis gives standard deviations for k_1 and K_1K_2 directly, and the standard deviation for k_{12} can be obtained as shown by Niebergall et al. (7). Standard deviations for the other constants cannot be obtained since the standard deviation for K_1, which was taken from the literature, was not given. Had the standard deviation for K_1 been available, the standard deviations for all other constants could have been obtained.

The nonlinear regression analysis on Eq. 9 using Eqs. 14–16 gave the results shown in Column 3 of Table I.

One criterion which may be used to judge which of the three methods gave the best results would be to calculate $\alpha_{calc.}$ from the various sets of micro- and macroconstants using Eq. 9 and to compare the $\alpha_{calc.}$ to the experimental α at each pH. The "best" set of constants was chosen as that set which gave a minimum for the sum of squares of residuals, SS:

$$SS = \Sigma(\alpha - \alpha_{calc.})^2 \qquad \text{(Eq. 20)}$$

A plot of $\alpha_{calc.}$ versus pH for the method of nonlinear regression is shown in Fig. 3 to illustrate the good fit that can be obtained using this method. The SS using the pM versus α method was 8.59×10^{-2}; this was reduced to 3.75×10^{-4} through the use of Eq. 10 and finally to 1.69×10^{-4} by using nonlinear regression on Eq. 9. Thus, in addition to giving estimates of the standard deviations which are not possible from the pM versus α method, the two methods presented in this article also improve the agreement between experimental and

Table II—Micro- and Macrodissociation Constants for Morphine Hydrochloride Using the Data of Schill and Gustavii (2)[a]

Constant	pM versus α[b]	Method Linear Regression	Nonlinear Regression
pK_1	8.31	8.29	8.32(0.04)
pK_2	9.51	9.51	9.52(0.05)
pK_1K_2	17.82	17.80(0.004)	17.84(0.05)
pk_1	8.87	8.86(0.005)	8.87(0.01)
pk_2	8.45	8.43	8.46(0.05)
pk_{12}	8.95	8.94(0.005)	8.97(0.04)
pk_{21}	9.37	9.37	9.37(0.05)

[a] Figures in parentheses represent one standard deviation. [b] Taken from Schill and Gustavii (2), Table 2.

calculated values of α. Finally, the method of nonlinear regression analysis did appear to give the best results.

To test further the utility of the methods presented, the data of Schill and Gustavii (2) for morphine hydrochloride were also analyzed using Eqs. 9 and 10. A plot of their data according to Eq. 10 is shown in Fig. 4. The values obtained using Eqs. 9 and 10 are compared to the values obtained by Schill and Gustavii (2), using the method of pM versus α, in Table II. The SS using the pM versus α method was 1.04×10^{-4}; this was reduced to 9.07×10^{-6} through use of Eq. 10 and finally to 8.22×10^{-5} using nonlinear regression.

It appears that the linear plot of Eq. 10, which is capable of linear regression analysis, is superior to the pM versus α method, but it does require the prior knowledge of K_1. If a high speed digital computer is available, the method of choice would be nonlinear regression analysis using Eqs. 9 and 14–16.

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