AN ABSTRACT OF THE THESIS OF

Darawan Palalikit for the degree of Master of Science

in Pharmacy presented on March 9, 1979.

Title: Determination of Ionization Constants by Chromatography

Redacted for Privacy

Abstract Approved: _____
Professor John H. Block

Abstract

The determination of the pK_a's of several organic acids and bases using chromatography was investigated. Normal-phase buffer-impregnated paper chromatography was found to be unsuitable. TLC plates impregnated with mineral oil were examined as a possible reversed-phase method, with poor results. The use of XAD-2 copolymer as the stationary phase in a simple HPLC method gave good results. Experimentally determined pK_a values were confirmed using UV spectrophotometry with the identical solvents and buffers used in the HPLC method. Statistical comparisons using both pK_a and K_a values were performed. The advantage of using a computer method involving the second derivative to determine pK_a was illustrated.

Determination of Ionization Constants by Chromatography

by

Darawan Palalikit

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed March 9, 1979

Commencement June 1979

APPROVED:

Redacted for Privacy

Professor of Pharmacy
in charge of major

Redacted for Privacy

Dean of Pharmacy School

Redacted for Privacy

Dean of Graduate School

Date thesis is presented March 9, 1979

Typed by Cheryl E. Curb for Darawan Palalikit

ACKNOWLEDGEMENTS

I wish to give sincere acknowledgement to Professor John H. Block for the unmeasurable assistance he extended to me. Without his kind, understanding support I could not have completed my thesis.

I also wish to thank Dr. James W. Ayres and Mr. Douglas R. Henry for their helpful assistance and suggestions.

I wish to express my appreciation to Dr. Robert F. Doerge for his review and suggestions. I also wish to express my appreciation to Dr. Elliot Marvell for his encouragement.

This work is dedicated to my family, The Palalikit, whose encouragement and support enabled me to get this work done.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

DETERMINATION OF IONIZATION CONSTANTS BY CHROMATOGRAPHY

ABSTRACT

The determination of the pK_a's of several organic acids and bases using chromatography was investigated. Normal-phase buffer-impregnated paper chromatography was found to be unsuitable. TLC plates impregnated with mineral oil were examined as a possible reversed-phase method, with poor results. The use of XAD-2 copolymer as the stationary phase in a simple HPLC method gave good results. Experimentally determined pK_a values were confirmed using UV spectrophotometry with the identical solvents and buffers used in the HPLC method. Statistical comparisons using both pK_a and K_a values were performed. The advantage of using a computer method involving the second derivative to determine pK_a was illustrated.

Introduction

Several experimental methods such as spectrophotometry[1], potentiometry[2], conductometry[3], and proton magnetic resonance spectrometry[2,4] are available for the determination of the ionization constant of a compound. Each method has its advantages and disadvantages. The spectrophotometric method requires chromophores which absorb ultraviolet or visible light. Also the relevant ionic and molecular species must have different spectra. The potentiometric titration method is very commonly used, but good water-solubility with an adequate quantity of the compound is needed. The conductometric method is quite tedious and more time consuming. The proton magnetic resonance method has been proved useful

for substances whose ultraviolet spectra do not change upon ionization, but the compound must be water soluble. The limitations of the proton magnetic resonance method are the types of buffer that must be used, and the fact that at least one proton must show a significant chemical shift when going from the unionized to the ionized species. All of these four methods require a compound which is very pure. An alternative approach is to use chromatography. Advantages of this method are that (1) small quantities of compounds are required; (2) poor water-solubility need not be a serious drawback; and (3) the samples need not be pure.

There are many kinds of chromatographic methods which could be used: gas chromatography, paper chromatography, thin-layer chromatography, and high pressure liquid chromatography (HPLC). The gas chromatographic method is of limited usefulness because compounds are in the vapor phase rather than in aqueous solution. However, it has been valuable in finding the characteristics of nonideal solutions[5].

All of the liquid chromatography methods involve the use of mobile phases of different pH values. For example, paper chromatographic methods were reported[6,7]. The resulting ionization constants, when compared with previously published results using traditional techniques, were found to be in good agreement.

The determination of oil/water partition coefficients of substances has been studied by thin-layer chromatographic methods[8,9,10], but so far the determination of the ionization constants of substances has not been reported by this method. Since the partition coefficient relates to the ionization constant, it should be possible to determine the ionization constant by thin-layer chromatography.

Using the HPLC method, either ion exchange chromatography or reversed phase chromatography would appear to be appropriate. The traditional ion exchange resins have several drawbacks. They lack mechanical strength and will collapse under the pressures common to HPLC. They also swell or shrink as the counter ion changes in the mobile phase[11,12]. Both of these handicaps lead to changing column volumes and inconsistent retention times.

Many of the columns currently used in reversed phase HPLC suffer from two drawbacks. First, the columns cannot be operated above pH 8 because deterioration of the silica support becomes significant. Second, these columns have active silanol sites where no bonding of hydrocarbon to the silica has occurred. Some type of silation is usually required to cover these active sites[13,14].

Recently a series of nonionic stationary phases have been reported. They are all organic and have been shown to withstand the pressures common to low flow rates[11]. Further, those polymers are devoid of ester linkages and would appear to be usable at any pH which can be tolerated by the pumps and seals. Also, since they are nonionic polymers, they are not subject to shrinkage and swelling due to changes in the counter ions in the mobile phase. Finally, being totally organic, and nonionic, they should lack the active adsorption sites characteristic of the silica-based internal phases. It was decided to investigate the use of one of these nonionic copolymer resins, XAD-2, in the determination of pK_a's by HPLC, and to compare the results with literature values and with those obtained by UV spectrophotometry using two methods of calculation.

Experimental

APPARATUS

A liquid chromatograph (Waters Associates Model 201), a variable wavelength UV detector (Varion Model 635LC) and a sample injection system (Waters Associates Model U6K) were used for the HPLC method. A spectrophotometer (Beckman Model DB-GT) and 1 cm. matched cells were used for the spectrophotometric method.

REAGENTS

All chemicals were used as received. Solvents were of an analytical grade and purchased in glass bottles. Amberlite XAD-2 was purchased as 20-50 mesh beads. The procedures for cleaning and sizing of XAD-2 copolymers have been described previously[11,15,16,17]. Particles of 45-65 micron mesh size were used.

PROCEDURE

Paper Chromatography: Chromatographic paper (Whatman 3MM) was impregnated with inorganic phosphate buffer solution which was saturated with amyl alcohol. Methanolic solutions (0.3% w/v) of benzoic acid, phenol and ethanolamine were prepared each, and 1 μl. volumes of solutions were spotted onto the paper. The chromatography was carried out with an ascending flow of amyl alcohol saturated with buffer. The ionization constant could be determined by plotting R_f values as a function of the pH of the mobile phase[6].

Thin-Layer Chromatography: A non-aqueous stationary phase was obtained by impregnating the support (Kieselguhr G) either directly

or indirectly using a 2% v/v solution of Mineral Oil U.S.P. in an acetone-dioxane mixture[10]. Methanolic solutions (0.3% w/v) of benzoic acid, o-chlorobenzoic acid, phenol and ethanolamine were prepared, and 1 μl. volumes of solutions were spotted onto the plates. The plates were placed in chromatographic chambers that had been equilibrated for 16 hours with the mobile phase. The mobile phase, saturated with mineral oil, consisted of inorganic phosphate buffer solutions of pH range 2-9. After development, the plates were air dried prior to being sprayed with a variety of detecting reagents[18].

Hulshoff and Perrin derived a relationship between the R_m value and the dissociation constant in thin-layer partition chromatography[10].

$$R_m = \log({}_sP) + \log \frac{K_a}{K_a + [H^+]} + \log r \qquad (1)$$

Where R_m is defined as $\log(1/R_f - 1)$, ${}_sP$ is the partition coefficient expressed as the molar concentration in the stationary phase divided by the molar concentration in the mobile phase, r is the phase-volume ratio expressed as the volume of the stationary phase divided by the volume of the mobile phase, which was constant for a given chromatographic system.

High Pressure Liquid Chromatography: A short column 9 cm. long and 0.23 cm. I.D. proved satisfactory. Phosphate buffers (0.01 M.) were maintained at an ionic strength of 0.1 M. by adding an appropriate amount of KCl[19]. Sample solutions of 0.5% w/v were prepared in methanol. The column was slurry packed with XAD-2 using water as a slurry agent.

Pressures and flow rate were in the range of 600-800 psi and 1.0 ml./min. Buffer-acetonitrile mixtures were expressed as % by volume.

The pH values of solvents containing the buffer were determined by a pH meter before and after adding acetonitrile. The pH meter was standardized each day; it was used at pH 4 and pH 7. The capacity factor was evaluated from the chromatogram[20] and is defined as:

$$k' = (t_r - t_0)/t_0$$

Where t_r was the retention time for the sample component under investigation. The t_0 value was obtained from the retention time of $NaNO_2$.

It has been shown by Equation 2 that the k' is dependent on the dissociation of the compound and the pH of the mobile phase[19]. For acids:

$$k' = \frac{k_0}{1 + K_a/[H^+]} + \frac{k_{-1}}{1 + [H^+]/K_a} \qquad (2)$$

Where k_0 and k_{-1} are defined as the capacity factors for the completely unionized (protonated, HA) and completely ionized (conjugate base, A^-) forms of the acids, and K_a is its ionization constant. Equation 2 can be written as:

$$k' = \frac{k_0[H^+] + k_{-1}(K_a)}{K_a + [H^+]} \qquad (3)$$

Solving for K_a:

$$K_a = \frac{[H^+](k_0-k')}{(k'-k_{-1})} \qquad (4)$$

Taking the negative logarithm:

$$pK_a = pH + \log\frac{(k'-k_{-1})}{(k_o-k')} \tag{5}$$

Equation 3 was rewritten in a linearized form[21] as:

$$k' = k_0 - K_a(k'-k_{-1})/\,[H^+] \tag{6}$$

By plotting k' versus $(k'-k_{-1})/\,[H^+]$, the slope is $-K_a$ and the intercept is k_0.

Either Equation 5 or 6 can be used to determine pK_a provided the k_0 and k_{-1} values were known. It should be noted that in Equation 6, k' occurs on both sides of the equation.

For bases, a relationship between the capacity factor (k') and pH of the mobile phase has been shown[19] to be:

$$k' = \frac{k_1}{1 + [OH^-]/K_b} + \frac{k_0}{1 + K_b/[OH^-]} \tag{7}$$

Where k_1 and k_0 were the capacity factors for the completely ionized (protonated, BH^+) and completely unionized (conjugate base B) form of the base, respectively. Equation 7 can be rewritten as:

$$k' = \frac{k_1(K_b) + k_0[OH^-]}{K_b + [OH^-]} \tag{8}$$

Setting

$$K_b = 10^{-14}/K_a \tag{9}$$

$$[OH^-] = 10^{-14}/[H^+] \tag{10}$$

$$k' = \frac{k'\ [H^+] + k_0(K_a)}{K_a + [H^+]} \tag{11}$$

Solving for K_a:

$$K_a = \frac{[H^+](k'-k_1)}{(k_0-k')} \tag{12}$$

Taking the negative logarithm:

$$pK_a = pH + \log\frac{(k_0-k')}{(k'-k_1)} \tag{13}$$

Equation 11 was rewritten in a linearized form[21] as:

$$k' = k_1 + K_a(k_0-k')/[H^+] \tag{14}$$

By plotting k' vs. $(k_0-k')/[H^+]$, the slope is K_a and the intercept is k_1. The pK_a can be determined by either Equation 13 or 14 provided the k_1 and k_0 values were known. The same limitation applies to Equation 14 as with Equation 6.

Spectrophotometric method: Stock solutions of the same compounds as in the HPLC method were prepared ($5x10^{-4}$ M.). To facilitate dissolution, an acid compound was dissolved in 0.005 N. KOH and basic compounds were dissolved in 0.005 N. HCl. Methanol was also added if

the compound was not very soluble, but the alcohol content did not exceed 1% v/v in the stock solution. The stock solution was then diluted to 10^{-4} M. in buffer-acetonitrile solution to make the final solution at a specified pH. The absorbance of the final solution was then measured.

Ionization constants of acids and bases were determined by Equations 15 and 16, respectively[2].

$$pK_a = pH + \log \frac{A_i - A}{A - A_m} \tag{15}$$

$$pK_a = pH + \log \frac{A - A_m}{A_i - A} \tag{16}$$

At a specified wavelength, A is the absorbance at a certain pH, A_i is the absorbance when all of the sample is in the ionized state, and A_m is the absorbance when all of the sample is in the neutral state. The wavelength used was between 220-300 nm.

Determination of pK_a using Computer Program: A program was written in FORTRAN which would calculate the ionization constant from the second derivative of the k' vs. pH curve for the HPLC method and the absorbance vs. pH curve for the spectrophotometric method. The program was based on a program written by Isenhour and Jurs[22]. This enlarged program which is interactive is available upon request from J. H. Block and accomplishes the following:

1 Reads up to 100 data pairs.

2 Orders the data pairs that make up each point on the curve such that the X-axis will start with the lowest pH and end with the highest.

3 Decides if the curve is "uphill" or "downhill" and proceed accordingly.

4 Plots k' versus pH, the first derivative, and the second derivative.

5 Prints out data tables with appropriate labeling.

6 Prints out a limited number of error messages when the input data is incomplete or contain certain types of error

7 The pK_a obtained by taking the cross-over value in the second derivative as the equivalence point (pK_a=pH).

Results and Discussion

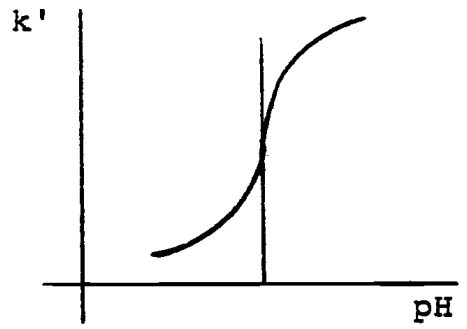
Paper Chromatography: With the experimental method that was described, unsatisfactory results were obtained. The reasons were (1) the paper was destroyed by the detecting agent, and (2) it took too long for the chromatogram to develop.

Thin-Layer Chromatography: Hulshoff and Perrin[10] derived a relationship between the R_m value and the dissociation constant in oil/water partition chromatography as shown in Equation 1. Problems were encountered with this procedure. The R_m value for several of the compounds behaved independently of the pH of the mobile phase. In most cases the mobile phase had to be significantly non-aqueous to obtain a meaningful and reproducible R_m value. While it might have been possible to extrapolate back to the R_m values in pure water, the purpose of this study was to develop rapid procedures. Finally, the thin-layer chromatography procedure suffered from the same problem as with paper chromatography; i.e., finding appropriate detecting agents. Sometimes the mineral oil seemed to "protect" the compound from the

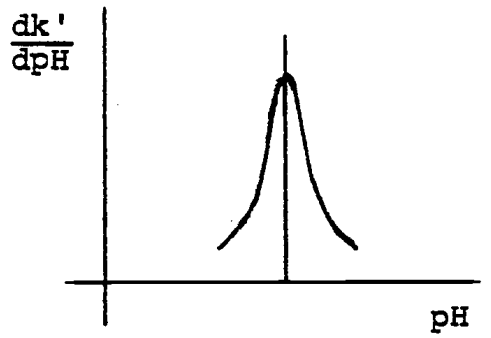
detecting reagent. At other times the mineral oil would be affected by harsh visualization reagents with subsequent darkening of the entire plate.

High Pressure Liquid Chromatography: Hovath[21] showed that a plot of k' versus pH produced a curve similar in appearance to that of a regular titration curve. He obtained dissociation constants by finding the midpoint of the curve where the concentrations of the acid form and conjugate base form were equal. His procedure suffered from two limitations. First, it was necessary to use a high concentration of acetonitrile in the mobile phase to obtain reasonable retention times for the unionized species. In addition, he needed to obtain k' values for the completely ionized form and unionized form of the compound (Equation 2-16). Thus solutes with pK_a values lower than 3 or greater than 9 could be difficult to study because of damage to the pumps, seals, and columns.

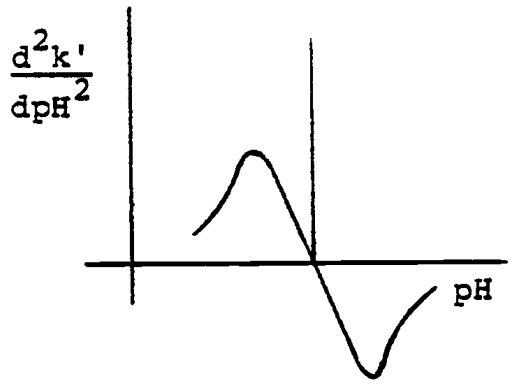
Alternatively, the pK_a could be calculated from a computer program[22] which obtains the first (Figure 1b) and second (Figure 1c) derivatives from the plot of k' versus pH (Figure 1a). Using this method it was not necessary to know the k' values for the completely ionized form and unionized form. The program inputs a set of data representing a curve as a series of points (about six pairs). It numerically calculates the first and second derivatives, and then searches for the cross-over in the second derivative. An interpolation is made to find the exact cross-over value.



1a A typical curve of k' versus pH



1b First derivative curve of k' versus pH



1c Second derivative curve of k' versus pH

Spectrophotometric Method: Because the relationship between the absorbance and pH is similar to the relationship between the capacity factor and pH (i.e., a titration curve), the pK_a's of organic acids and bases can also be calculated by using the second derivative. This is advantageous because the absorbance of the completely ionized and unionized species are not needed.

Two different comparisons were made. In one, the results obtained by the HPLC procedure were compared with those obtained spectroscopically. In the other, pK_a's calculated from the second derivative were compared with those obtained from Equations 5, 13, 15 and 16. These comparisons are summarized in Table I.

Organic Acids: The relative accuracy of the computer method versus the use of Equation 5 or 15 was dependent on whether the pK_a determinations were obtained spectroscopically or by HPLC. Examination of the residuals in Table I showed that the experimental pK_a values were consistently low for the HPLC method when determined by computer calculation and generally high when obtained by Equation 5. The results were more mixed with the spectrophotometric method. However, the computer method gave more random residuals with a mean nearly equal to zero. The use of Equation 15 gave results which tended to be high. There appeared to be a negative correlation between the residuals obtained from the computer method and a positive correlation when using Equations 5 and 15.

Ignoring whether the computer method was better than the use of Equations 5 and 15, the question of whether the HPLC method produced significantly different results from the spectrophotometric method was examined. There was little difference in the results when the computer

Table I. a) Comparison of the pK_a values of organic acids.

Compound Name	HPLC Computer	HPLC D[a]	HPLC R[b]	HPLC Equation 5	Spectrophotometer D[a]	Spectrophotometer R[b]	Spectrophotometer Computer	Spectrophotometer D[a]	Spectrophotometer R[b]	Spectrophotometer Equation 15	Spectrophotometer D[a]	Spectrophotometer R[b]	Lit. Value	Ref.
o-Chlorobenzoic Acid (10% ACN)	2.76	0.16	94.54	3.19(±0.12)[d]	-0.27	109.25	2.47	0.45	84.59	3.03(±0.10)[d]	-0.11	103.77	2.92	2, 23, 28
o-Chlorobenzoic Acid (20% ACN)	2.86	0.06	97.95	3.32(±0.16)	-0.40	113.70	2.98	-0.06	102.06	3.20(±0.09)	-0.28	109.59	2.92	2, 23, 28
Salicylic Acid (10% ACN)[c]	2.90	0.10	96.67	3.01(±0.10)	-0.01	100.33	--	--	--	3.01(±0.10)	-0.01	100.33	3.00	26, 27
p-Nitrobenzoic Acid (10% ACN)	3.22	0.22	93.60	3.50(±0.07)	-0.06	101.74	3.61	-0.17	104.94	3.47(±0.08)	-0.03	100.87	3.44	2, 23, 29
p-Nitrobenzoic Acid (20% ACN)	3.12	0.32	90.70	3.43(±0.06)	0.01	99.71	3.23	0.21	93.90	3.50(±0.11)	-0.06	101.74	3.44	2, 23, 29
Acetylsalicylic Acid (10% ACN)	4.49	0.02	99.43	3.64(±0.08)	-0.13	103.70	3.59	-0.08	102.28	3.60(±0.07)	-0.09	102.56	3.51	29
p-Chlorobenzoic Acid (20% ACN)	3.94	0.05	98.75	3.99(±0.04)	0.00	100.00	3.96	0.03	99.25	3.96(±0.06)	0.03	99.25	3.99	2, 23, 29
Benzoic Acid (10% ACN)	4.09	0.10	97.61	4.13(±0.04)	0.06	98.57	4.02	0.17	95.94	4.29(±0.08)	-0.01	102.39	4.19	2, 28
Cinnamic Acid (20% ACN)	4.54	-0.10	102.25	4.45(±0.16)	-0.01	100.23	4.85	-0.41	109.23	4.39(±0.11)	0.05	98.87	4.44	23,28
Number of Compounds	9	9	9	9	9	9	8	8	8	9	9	9		
Mean		0.10	96.83		-0.09	103.02		0.02	99.02		-0.07	102.15		
α Level of Mean		0.002	0.006		0.028	0.028		0.214	0.182		0.019	0.020		
S.D.		0.12	3.45		0.15	5.12		0.26	7.59		0.10	3.21		
C.V. = (S.D./ Mean)x100		1.17	0.04		-1.69	0.05		14.98	0.08		-1.47	0.03		
Correlation		-0.45	0.51		0.67	0.67		-0.48	-0.48		0.57	-0.59		
α Level of Correlation		0.055	0.040		0.012	0.012		0.055	0.058		0.027	0.023		

Table I. b) Comparison of the pK_a values of organic bases and summary results of acids and bases

Compound Name	HPLC Computer	HPLC D[a]	HPLC R[b]	HPLC Equation 13	HPLC D[a]	HPLC R[b]	Spectrophotometer Computer	Spectrophotometer D[a]	Spectrophotometer R[b]	Spectrophotometer Equation 1c	Spectrophotometer D[a]	Spectrophotometer R[b]	Lit. Value	Ref.
Aniline (10% ACN)	4.62	0.00	100.00	4.44(±0.12)[d]	0.18	96.10	4.57	0.05	98.92	4.56(±0.11)[d]	0.06	98.70	4.62	29
Aniline (20% ACN)	4.50	0.12	97.40	4.23(±0.08)	0.39	91.56	4.55	0.07	98.48	4.52(±0.09)	0.10	97.84	4.62	29
Pyridine (10% ACN)	4.91	0.28	94.61	5.12(±0.09)	0.07	98.65	4.97	0.22	95.76	5.11(±0.07)	0.08	98.46	5.19	2, 26
P-Nitrophenol (10% ACN)	6.91	0.24	96.64	7.06(±0.10)	0.09	98.74	6.90	0.25	96.50	7.17(±0.14)	-0.02	100.28	7.15	2, 28
P-Nitrophenol (10% ACN)	7.21	0.03	99.58	7.11(±0.03)	0.04	99.44	6.93	0.22	96.92	7.01(±0.06)	0.07	99.02	7.15	2, 28
Phenobarbital Sod. (20% ACN)	7.24	0.20	94.61	7.55(±0.13)	-0.11	98.65	7.40	0.04	95.76	7.60(±0.16)	-0.16	98.46	7.44	29
Ephedrine (10% ACN)[c]	9.19	0.41	95.73	--	--	--	9.56	0.04	99.58	9.60(±0.29)	0.00	100.00	9.60	27
Pyrilamine Maleate (20% ACN)[c]	4.13	-0.13	103.25	3.94(±0.11)	0.06	98.50	4.17	-0.17	104.25	4.01(±0.07)	-0.01	100.25	4.00	27
	8.87	0.03	99.66	--	--	--	8.70	0.20	97.75	8.93(±0.12)	-0.03	100.35	8.90	27
Quinoline (20% ACN)	5.14	-0.24	104.90	4.99(±0.06)	-0.09	101.84	4.89	0.01	99.80	4.76(±0.05)	0.14	97.14	4.90	28
Strycnnine Hydrochloride (20% ACN)[c]	6.03	-0.03	100.50	6.01(±0.08)	-0.01	100.17	6.08	-0.08	101.33	5.94(±0.10)	0.06	99.00	6.00	2b
	8.08	0.18	97.82	8.17(±0.03)	0.09	98.91	--	--	--	--	--	--	8.26	28, 29
Number of Compounds	12	12	12	10	10	10	11	11	11	11	11	11		
Mean		0.08	98.73		0.02	98.54		0.07	98.98		0.03	99.38		
α Level of Mean		0.014	0.036		0.037	0.038		0.021	0.047		0.078	0.043		
S.D.		0.20	3.02		0.14	2.94		0.13	2.39		0.08	1.40		
C.V. = (S.D./ Mean)x100		1.70	0.03		2.00	0.03		1.73	0.02		3.12	0.01		
Correlation		0.65	-0.51		-0.33	0.40		0.41	-0.31		-0.51	0.54		
α Level of Correlation		0.004	0.019		0.087	0.062		0.052	0.087		0.028	0.021		
Summary pK_a of acids and bases.														
Number of Compounds	21	21	21	19	19	19	19	19	19	20	20	20		
Mean		0.09	97.92		-0.01	100.66		0.05	99.00		-0.02	100.63		
α Level of Mean		0.001	0.002		0.222	0.134		0.064	0.100		0.123	0.079		
S.D.		0.17	3.26		0.16	4.61		0.19	5.06		0.10	2.71		
C.V. = (S.D./ Mean)x100		1.50	0.03		31.28	0.05		3.72	0.05		-6.40	0.03		
Correlation		0.38	0.02		0.34	-0.39		0.19	-0.002		0.20	-0.80		
α Level of Correlation		0.020	0.229		0.039	0.030		0.112	0.250		0.098	0.043		

(a), D = The difference between the literature values and the experimental values.

(b), R = The ratio of the experimental values to literature values times 100.

(c), Some pK_a values could not be determined because of excessive retention times or because of a lack of difference in spectra of the ionized and unionized forms.

(d), Sample Standard Deviation.

approach was used (α = 0.072). There was even less difference noted when Equations 5 and 15 were used (α = 0.12).

Organic Bases: For the HPLC method the experimental pK_a values obtained by both the computer method and using Equation 13 were lower than literature values. The results were more random using Equation 16 in the spectrophotometric method, but the computer method gave results which were consistently lower. There was a positive correlation between the residuals and the pK_a values obtained from the computer method and a negative correlation when using Equations 13 and 16.

Comparison of the HPLC method and the spectrophotometric method showed no significant difference in results obtained by either the computer method (α = 0.23) or using Equations 13 and 16 (α = 0.13).

Combining the data from the acids and bases caused a decrease in the correlations with the literature pK_a values. Also there was no significant difference between the HPLC and the spectrophotometric method either when the computer method was used (α = 0.09) or when Equations 5, 13, 15 and 16 were used (α = 0.22).

During all of the above discussions, it was important to remember that pK_a values were obtained from the negative logarithm of the ionization constant (K_a). The pK_a values were directly obtained as such by the methods previously described in the introduction and in the experimental section. Nevertheless, using a logarithmic scale could distort results when comparing literature and experimental pK_a's. For that reason the results shown using the ratio percents must be examined with some caution even though they were consistent with those obtained from the residuals.

The analyses described above were repeated for the respective K_a values and the results shown in Table II. Overall, the results parallel those found in Table I except the ratio percents are larger. Also, there appears to be a higher correlation between the residuals and the literature K_a values.

Factors Involved in the HPLC Method: There were many factors to consider when using the HPLC method. First, the stationary phase used in the HPLC procedure was completely nonionic. While there is debate in the literature concerning the mode of action of the reversed phase type of column, any adsorption due to ionic interaction should be nearly nonexistent. Therefore, the retention time could be considered proportional to the degree of ionization of the solute, and the adsorption characteristics of the packing material should not change with the pH of the mobile phase.

Second, the mobile phase ideally should be aqueous, and the buffers used should be inorganic in order to minimize any direct involvement with the stationary phase. Also, the ionic strength should be held constant throughout the pH range studied.

Third, a reference compound to measure t_0 is necessary which would not be absorbed by the stationary phase. Sodium nitrite was found to be satisfactory for this purpose.

In practice, the elution times of the unionized species were so long that the peaks were too broad to accurately measure the retention times. This problem was partially solved by using a shorter column. However, column capacity became a problem which was best handled by injecting the same volume and concentration each time. Addition of an organic solvent was necessary to obtain retention times which were

Table II.a) Comparison of the K_A values of organic acids

Compound Name	HPLC Computer	HPLC Equation 5 b^a	HPLC Equation 5 R^b	HPLC Equation 5 b^a	HPLC Equation 5 R^b	Spectrophotometer Computer	Spectrophotometer Equation 15 b^a	Spectrophotometer Equation 15 R^b	Spectrophotometer Equation 15 b^a	Spectrophotometer Equation 15 R^b	Lit. Value	Ref.
o-Chlorobenzoic Acid (10% ACN)	1.74×10^{-3}	-5.36×10^{-4}	144.54	6.46×10^{-4}	5.57×10^{-3}	53.70	3.39×10^{-3}	-2.19×10^{-3}	281.10	9.33×10^{-4}	2.69×10^{-4}	77.62	1.20×10^{-1}	2, 23, 28
o-Chlorobenzoic Acid (20% ACN)	1.38×10^{-3}	-1.78×10^{-4}	114.82	4.79×10^{-4}	7.24×10^{-4}	39.81	1.05×10^{-3}	1.55×10^{-4}	107.15	6.31×10^{-4}	5.71×10^{-4}	52.48	1.20×10^{-3}	2, 23, 28
Salicylic Acid (10% ACN)[c]	1.26×10^{-3}	-2.59×10^{-4}	125.89	9.77×10^{-4}	2.28×10^{-5}	97.72	--	--	--	9.77×10^{-4}	2.28×10^{-5}	97.72	1.00×10^{-3}	26, 27
p-Nitrobenzoic Acid (10% ACN)	6.03×10^{-4}	-2.39×10^{-4}	165.96	3.16×10^{-4}	4.69×10^{-5}	87.10	2.45×10^{-4}	1.18×10^{-4}	162.18	1.39×10^{-5}	2.42×10^{-5}	93.33	3.63×10^{-4}	2, 23, 29
p-Nitrobenzoic Acid (20% ACN)	7.59×10^{-4}	-3.96×10^{-4}	208.93	3.72×10^{-4}	-8.46×10^{-6}	102.33	5.89×10^{-4}	-2.26×10^{-4}	162.25	3.16×10^{-4}	4.69×10^{-5}	89.10	3.63×10^{-4}	2, 23, 29
Acetylsalicylic Acid (10% ACN)	3.24×10^{-4}	-1.46×10^{-5}	104.71	2.29×10^{-4}	7.99×10^{-5}	74.13	2.57×10^{-4}	5.20×10^{-5}	83.18	2.51×10^{-5}	5.78×10^{-5}	81.28	3.09×10^{-4}	29
p-Chlorobenzoic Acid (20% ACN)	1.15×10^{-4}	-1.25×10^{-5}	112.20	1.02×10^{-4}	0	100.00	1.09×10^{-4}	-7.32×10^{-6}	67.61	1.10×10^{-4}	-7.32×10^{-6}	107.15	1.02×10^{-4}	2, 23, 29
Benzoic Acid (10% ACN)	8.13×10^{-5}	-1.67×10^{-5}	125.89	7.41×10^{-5}	-9.57×10^{-6}	114.82	9.55×10^{-5}	-3.05×10^{-5}	147.91	5.13×10^{-5}	1.33×10^{-5}	79.43	6.46×10^{-5}	2, 28
Cinnamic Acid (20% ACN)	2.88×10^{-5}	7.47×10^{-6}	79.43	2.88×10^{-5}	7.47×10^{-6}	97.72	1.41×10^{-5}	2.22×10^{-5}	38.90	4.07×10^{-5}	-4.43×10^{-6}	112.20	3.63×10^{-5}	23,28
Number of Compounds	9	9	9	9	9	9	8	8	8	9	9	9		
Mean		-0.0002	131.38		0.0002	85.26		-0.0003	121.98		0.0001	87.59		
% Level of Mean		0.006	0.010		0.032	0.028		0.094	0.110		0.031	0.018		
S.D.		0.0002	37.91		0.0001	24.70		0.0008	76.20		0.0002	17.90		
C.V. = (S.D./ Mean)x100		1.06	0.29		1.76	0.29		-2.99	0.63		1.74	0.20		
Correlation		-0.67	0.13		0.81	-0.76		-0.57	0.51		0.76	-0.60		
% Level of Correlation		0.011	0.186		0.002	0.004		0.110	0.049		0.005	0.021		

Table II. b) Comparison of the K_a values of organic bases and summary results of acids and bases

Compound Name	HPLC Computer	HPLC Equation 13 D[a]	HPLC Equation 13 R[b]	Spectrophotometer Computer	Spectrophotometer Equation 16 D[a]	Spectrophotometer Equation 16 R[b]	Lit. Value	Ref.
Aniline (10% ACN)	2.40×10^{-5}	0.00	100.00	2.69×10^{-5}	-2.93×10^{-6}	112.20	2.40×10^{-5}	29
Aniline (20% ACN)	3.16×10^{-5}	-7.63×10^{-6}	131.83	2.82×10^{-5}	-4.20×10^{-6}	117.49	2.40×10^{-5}	29
Pyridine (10% ACN)	1.23×10^{-5}	-5.85×10^{-6}	190.55	1.07×10^{-5}	-4.26×10^{-6}	165.16	6.46×10^{-6}	2, 26
P-Nitrophenol (10% ACN)	1.23×10^{-7}	-5.22×10^{-8}	173.78	1.26×10^{-7}	-5.51×10^{-8}	177.83	7.08×10^{-8}	2, 28
P-Nitrophenol (20% ACN)	7.59×10^{-8}	-5.06×10^{-9}	107.15	1.17×10^{-7}	-4.67×10^{-8}	165.96	7.08×10^{-8}	2, 28
Phenobarbital Sodium (20% ACN)	5.75×10^{-8}	-2.12×10^{-8}	158.49	3.98×10^{-8}	-3.50×10^{-9}	109.65	3.63×10^{-8}	29
Ephedrine (10% ACN)[c]	6.46×10^{-10}	-3.94×10^{-10}	257.04	2.75×10^{-10}	-2.42×10^{-11}	109.65	2.51×10^{-10}	27
Pyrilamine Maleate (20% ACN)[c]	7.41×10^{-5}	2.59×10^{-4}	74.13	6.67×10^{-5}	3.24×10^{-5}	67.61	1.00×10^{-4}	27
	1.35×10^{-9}	-9.00×10^{-11}	107.15	2.00×10^{-9}	7.36×10^{-10}	158.49	1.26×10^{-9}	27
Quinoline (20% ACN)	7.24×10^{-6}	5.34×10^{-6}	57.54	1.29×10^{-5}	2.93×10^{-7}	102.33	1.26×10^{-5}	28
Strychnine HCl (20% ACN)	9.33×10^{-7}	6.67×10^{-8}	93.33	8.32×10^{-7}	1.68×10^{-7}	83.18	1.00×10^{-6}	26
	8.32×10^{-9}	-2.82×10^{-9}	151.36	--	--	--	5.50×10^{-9}	28, 29
Number of Compounds	12	12	12	11	11	11		
Mean		0.00	133.54		0.00	124.15		
α Level of Mean		0.138	0.008		0.139	0.013		
S.D.		0.00001	62.77		0.00001	36.76		
C.V. = (S.D./Mean)x100		5.86	0.44		5.44	0.30		
Correlation		0.83	-0.43		0.89	-0.57		
α Level of Correlation		0.0001	0.036		0.00005	0.002		

Summary K_a of acids and bases.

	HPLC Computer	HPLC Equation 13 D[a]	HPLC Equation 13 R[b]	Spectrophotometer Computer	Spectrophotometer Equation 16 D[a]	Spectrophotometer Equation 16 R[b]
Number of Compounds	23	21	21	19	19	19
Mean		-0.00007	132.61		0.00007	105.73
α Level of Mean		0.008	0.0008		0.036	0.141
S.D.		0.00015	52.20		0.0002	42.52
C.V. = (S.D./Mean)x100		-2.05	0.39		2.87	0.40
Correlation		-0.80	-0.05		0.83	0.52
α Level of Correlation		0.00	0.209		0.00	0.006

	Spectrophotometer Computer	Spectrophotometer Equation 16 D[a]	Spectrophotometer Equation 16 R[b]
Number of Compounds	19	19	20
Mean		-0.0001	123.48
α Level of Mean		0.090	0.020
S.D.		0.0005	54.87
C.V. = (S.D./Mean)x100		-4.63	0.44
Correlation		-0.60	0.32
α Level of Correlation		0.017	0.045

	Lit. Value	Ref.
	2.40×10^{-5}	29
	2.40×10^{-5}	29
	6.46×10^{-6}	2, 26
	7.08×10^{-8}	2, 28
	7.08×10^{-8}	2, 28
	3.63×10^{-8}	29
	2.51×10^{-10}	27
	1.00×10^{-4}	27
	1.26×10^{-9}	27
	1.26×10^{-5}	28
	1.00×10^{-6}	26
	5.50×10^{-9}	28, 29
20	20	
	98.77	
	0.199	
	20.79	
	0.21	
	-0.58	
	0.002	

(a), D = The difference between the literature values and the experimental values.

(b), R = The ratio of the experimental values to literature values times 100.

(c), Some K_a values could not be determined because of excessive retention times or because of a lack of difference in spectra of the ionized and unionized forms.

short enough for accurate measurement. Table III shows the results of several solvents on the retention times of benzoic acid and sodium phenobarbital at pH 2.25. Acetonitrile proved to be the best non-aqueous solvent for the compound used in this study because acceptable peak shapes and retention times could be obtained with a minimum amount of solvent and distortion in the pH. Overall, better comparisons of the experimental values with the literature values were obtained using pH's measured before addition of the acetonitrile to the buffer.

Certain compounds could not be analyzed by the HPLC method. These included ascorbic acid, theophylline and phenylalanine, all of which were too soluble to be retained on the XAD-2 column. At the same time, only one of the two pK_a's for strychnine could be completely obtained by the spectrophotometric method because there was not a measurable difference in the UV sensitive chromophore for the other pK_a. The determinations using Equations 13 and 16 could not be utilized for ephedrine and strychnine because their pK_a's were too high to obtain k' for the completely unionized species.

Conclusion

HPLC can be used to measure pK_a's with an accuracy very close to that of the common spectrophotometric methods. TLC methods possessed several limitations which included a lack of retention of water soluble compounds and interference by the mobile-phase with the detecting reagents. HPLC analysis has the advantages that the compounds can have a very low solubility, need not possess a UV chromophore, and need not be pure. Due to its stability, the XAD-2 column can be used with broader pH range than the silica based octadecylsilane columns.

Table III. Comparison of organic solvent used in buffer solution (pH = 2.25).

Solvent	Percent of Solvent	pH[3]	k' of Benzoic Acid	k' of Sodium Phenobarbital
Acetonitrile	5	.10	39.00	VBP[2]
	10	.13	14.50	VBP[2]
	15	.13	8.60	13.60
Dioxane[1]	10	.08	27.00	VBP[2]
	20	.19	7.75	18.16
Methanol	20	.14	No Peak	No Peak
	30	.25	VBP[2]	VBP[2]
	40	.30	21.00	36.90
Tetrahydrofuran	10	.11	24.00	VBP[2]
	20	.24	10.25	16.08

1. Pressure increased up approximately 300 psi.

2. A very broad peak.

3. Change in pH after addition of the acetonitrile to pH 2.25 buffer.

Direct calculation of pK_a's by Equations 5, 13, 15 and 16 and by computer interpolation gave similar results, but the former has the disadvantage of requiring knowledge of the capacity factors for the spectrophotometric method, and the absorbance of the fully ionized and unionized forms of the compound. These are difficult to obtain for substances with very high or very low pK_a values.

References

1. J. C. Rossotti and H. Rossotti, "The Determination of Stability Constants," McGraw-Hill, New York, 1961.

2. A. Albert and E. P. Serjeant, "The Determination of Ionization Constants and Laboratory Manual," Chapman and Hall, Ltd., 1971.

3. F. L. J. Sixma and H. Wynberg, "A Manual of Physical in Organic Chemistry," John Wiley and Sons, Inc., New York, 1964.

4. D. L. Rabenstein and T. L. Sayer, Anal. Chem., 48, 1141 (1976).

5. A. G. Vitenberg, B. V. Ioffe, Z. St. Dimitrova, and T. P. Strukova, Translated from Doklady Akademii Nauk, SSR, 230, 849 (1976).

6. D. E. Dykhovichnaya, B. N. Bondarenko, R. P. Illarionova, and Yu. V. Rann, Translated from Khimiko-Farmatsevtichsekit Zhurnal, 5, 56 (1971).

7. S. B. Padhye and B. A. Kulkarni, J. of Sci. and Techno., 44, 45 (1973).

8. G. L. Biagi, A. M. Barbaro, M. F. Gamba, and M. C. Guerra, J. of Chromatogr., 41, 371 (1969).

9. A. E. Bird and A. C. Marshall, J. of Chromatogr., 63, 313(1971).

10. A. Hulshoff and J. H. Perrin, J. of Chromatogr., 120, 65(1976).

11. D. J. Pietrzyk and C. H. Chu, Anal. Chem., 49, 860(1977).

12. "Materials, Equipment and System for Chromatography, Electrophorgsis, Immunochemistry and Membrane Filtration," Bio-Rad Laboratories, Richmond, California, 1977.

13. J. M. McCall, J. Med. Chem., 18, 549(1975).

14. S. H. Unger, J. R. Cook, and J. S. Hollenberg, J. Pharm. Sci., 68, 1364(1978).

15. C. H. Chu and D. J. Pietrzyk, Anal. Chem., 46, 330(1974).

16. D. J. Pietrzyk and C. H. Chu, Anal. Chem., 49, 757(1977).

17. M. D. Grieser and D. J. Pietrzyk, Anal. Chem., 45, 1348(1973).

18. E. Stahl, "Thin-Layer Chromatography, A Laboratory Handbook," Translated by M. R. F. Ashworth, Springer-Verlag, New York, Second Edition, 1969.

19. D. J. Pietrzyk, E. P. Kroeff, and T. D. Rotsch, Anal. Chem., 50, 497(1978).

20. C. Horvath and W. Melander, Amer, Lab., 10, 24(1978).

21. C. Horvath, W. Melander, and I. Molnar, Anal. Chem., 49, 142(1977).

22. T. L. Isenhour and P. C. Jurs, "Introduction to Computer Programming for Chemists," Allyn and Bacon, Inc., Boston, 1972.

23. G. Kortum, W. Vogel, and K. Andrussow, "Dissociation Constants of Organic Acids in Aqueous Solution," Butterworths, London, 1961.

24. J. H. Perrin, "Dissociation Constants of Organic Bases in Aqueous Solution," Butterworths, London, 1965.

25. J. H. Perrin, "Dissociation Constants of Organic Bases in Aqueous Solution," Supplement, Page Bros. (Norwich), Ltd., Norwich, 1972.

26. The Merck Index, Merck & Co., Inc., Eighth Edition, 1968.

27. C. O. Wilson, O. Gisvold, and R. F. Doerge, "Textbook of Organic Medicinal and Pharmaceutical Chemistry," J. B. Lippincott Co., Phila., Seventh Edition, 1977.

28. Handbook of Chemistry and Physics 52nd Edition, The Chemical Rubber Co., Cleveland, Ohio, 1971-1972.

29. Handbook of Biochemistry and Molecular Biology, 3rd Edition, The Chemical Rubber Company, Cleveland, Ohio, 1975.