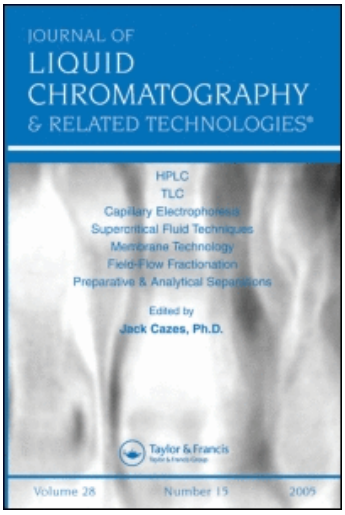

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Medium Throughput pK_a Determinations of Drugs and Chemicals by Reverse Phase HPLC with an Organic Gradient

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Abstract: A semi-automated method to determine pK_a values of drugs and chemicals by reverse phase HPLC is described. The method uses the capabilities of a crosslinked reverse phase HPLC column (Agilent extend C-18) as a separation device. The pK_a value was determined based on the different retention behaviors of the charged, multiply charged, and uncharged species at different pH conditions. The advantages of this method are low sample consumption, suitability for less soluble compounds, high purity samples not necessary, no spectral shift needed, high sample throughput, easy set up, high sensitivity, low cost, good precision, and agreement with literature values.

Keywords: Medium throughput, pK_a, Drug substances, Reverse phase HPLC, Organic gradient

INTRODUCTION

Among all the physicochemical parameters of a chemical entity, the acid dissociation constant (pK_a) is one of the most important ones. It is especially useful in the pharmaceutical industry for drug design and formulation. In 1998, it was estimated that approximately 95% of drugs on the market were

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ionizable.[1] Parameters, such as lipophilicity (logD), pH-solubility, and salt formation (different counterions) are pK_a dependent and are important to the understanding of drug absorption, drug distribution, and transport processes.

While many in-silico programs exist to calculate pK_a values, no single program has been accepted as universal to calculate the pK_a for the wide variety of chemical templates encountered in the pharmaceutical discovery setting. Measurements are required to obtain pK_a values for various templates for discovery projects. Traditionally, pK_a measurements were made by potentiometry and spectrometry in the chemical laboratory.[2] While these techniques are widely recognized, they are disadvantageous to use in a discovery setting where compound and time is limited. These methods are further confronted by parameters, such as low aqueous solubility and detection sensitivity.

Many researches have been published on the development of higher throughput pK_a methods in the pharmaceutical industry to keep up with the plethora of newly synthesized analogs.[3,4] Capillary electrophoresis has been widely accepted as a preferred method for measuring pK_a since the technique requires minimum sample, an exact concentration does not need to be determined, and the measurement does not require a UV shift corresponding to an ionization state.[5,6] However, a major drawback to the CE method is that it does not work well for compounds with poor aqueous solubility. Researchers have added organic modifiers to the assay in order to better deal with insoluble compounds, however, the throughput of the assay suffers greatly, in particular, for users with single CE systems.

Several researchers have recently published HPLC methods for pK_a determination.[7–11] These methods are based on the concept that retention time in reversed phase HPLC is strongly dependent on the degree of ionization of the analyte.[12] In reversed phase HPLC, the retention of an analyte is highly related to its hydrophobicity, the more hydrophobic the analyte, the longer it is retained. In general, ionized compounds are poorly retained in hydrophobic stationary phases, whereas nonionized compounds may be strongly retained. The ionization of a compound in the mobile phase buffer depends on the pH of the buffer and on the pK_a of the compound. For a singly charged compound, the equilibrium between charged and uncharged species (HA^z and A^{z-1}) is dictated by the K_a. The mole fraction (α) of each of the species that coexist in the mixture can be expressed as follows:

$$\alpha_{HA} = 1/(1 + 10^{pH-pKa})$$

$$\alpha_{A} = 1/(1 + 10^{pKa-pH})$$

For a neutral acid ($z = 0$), α_A is its degree of ionization $(\alpha)_A$, whereas for a neutral base ($z = +1$), $\alpha = \alpha_{HA}$. Thus, the retention time on a hydrophobic column will change as the mole fraction of the charged and uncharged

species changes. Analysis of the change in retention time of the analyte versus change in pH will give an indirect measure of the p*K*a.

Some HPLC p*K*a determination methods involve measuring retention time changes of the analyte with change in pH of the mobile phase after a series of isocratic runs.[8] The retention time versus pH is plotted and the data is analyzed by nonlinear regression to curve fit the pK_a values. One drawback is that the isocratic method times can be long for runs at pH values where the analyte is unionized and, thus, it is less suitable for higher throughput analysis. More recently, a pH gradient method has been developed where a compound is subject to increasing or decreasing pH environment and organic modifier simultaneously.[11] The Yasuda-Shedlovsky equation is incorporated to take into account the effect of organic modifier on pK_a. However, this type of method often requires separate runs of acidic and basic analytes. Upfront knowledge of the molecule's behavior and the data analysis is somewhat complicated, which makes higher throughput analysis more challenging.

The scope of this work was to develop a medium to high throughput HPLC pK_a assay, which can be implemented with minimal analysis time. This pK_a method involves 13 HPLC methods in which the analyte is subject to a given pH and the acetonitrile content is kept constant during the separation phase of the run. In order to shorten the retention time of the analyte in each method, the acetonitrile content is increased in a fast gradient fashion after the separation of the ionized forms of the molecules have already taken place. For compounds that eluted during the isocratic section of the method, the pK_a was determined by calculating the IC50 value of the pH vs retention time curve using Grafit software. For compounds that eluted past the isocratic portion of the method, two linear regression fits of the pH vs retention time curve per pK_a were used, and the pK_a value of the compound was determined by the intersection point.

EXPERIMENTAL

HPLC grade acetonitrile was obtained from Burdick & Jackson (Muskegon, MI) and reagent grade acetic acid, hydrochloric acid, citric acid, phosphoric acid, boric acid, and NaCl were obtained from EM Science (Gibbstown. N J). The HPLC system used was a Agilent 1100 Series HPLC equipped with a diode array detector (DAD) and quaternary solvent delivery system (Wilmington, DE). Several analytical columns were tested and an Agilent Zorbax Extend-C18 (5 μm, 4.6 * 50 mm) was selected and used for analysis. The water purification system used was a Millipore Milli-Q system.

Ibuprofen was obtained internally and all other chemicals were obtained from Aldrich (St. Louis, MO) and used without further purification.

A 4 nm slit width was used for the diode array detector (DAD) to collect the absorbance for each individual wavelength along with full spectrum.

A 0.5 μm KrudKatcher disposable pre-column filter was used to prevent on-line precipitation and prolong the column life. The HPLC auto injector was used to accurately inject small volumes (2 to 5 μL) of sample solution. The HPLC's DAD was used for automated spectral detection of the injected sample solution. A standard 13 μL flow cell was used and the path length of the DAD flow cell was 10 mm. The temperature of the HPLC's column thermostat was set at 25°C. These studies were carried out using the Agilent ChemStation software version A.08.03 with a 3D absorbance-wavelength-time software.

The HPLC's multiple solvent pump system was utilized to automatically prepare mobile phase buffer solutions with apparent pH values between 1.8 and 11.5. A total of four solutions were used to prepare the mobile phase buffers and gradient eluents. Solvent line A contained 100% acetonitrile, solvent line B contained a universal buffer at pH 1.9, which contained hydrochloric acid (0.01 M), citric acid (0.025 M), phosphoric acid (0.025 M), boric acid (0.03 M), and NaCl was added to increase the ionic strength to 60 mM. Solvent line C contained KOH (0.05 M) for pH adjustment, and NaCl was added to adjust the ionic strength. Solvent line D contained 100% Milli-Q water. Initially, acetic acid was used to prepare the universal buffer.[13] Later, citric acid was used to replace the acetic acid for wider buffer range, despite the ionic strength shift at pH values greater than 7 (all acidic pK_a values less than 7 (3.13, 4.76, 6.6)). For phosphoric acid, the three pK_a values have a wider distribution (2.12, 7.12, 12.32). This fact not only allows phosphoric acid to have wide buffer ranges but also makes the impact of increasing ionic strength (μ) less significant when pH is below 12. The ionic strength (μ) was calculated by $\mu = 1/2 * \sum m_i z_i^2$, where z is charge of the species and m is concentration of charged species. A thorough calculation with regard to the impact of ionic strength on the hydrogen ion activity (*f*) was conducted using the Debye-Hückel equation. It was determined that the maximum effect on the activity coefficient of the mixed buffer was at the highest pH, and the corresponding theoretical δpH is approximately 0.6 (Table 1) for 100% aqueous system. Further refinement was needed for the theoretical pH since organic solvent was used in this method. However, because this method was designed for medium or high throughput work, no further adjustment was done and only apparent pHs were used for calculations. A more accurate pK_a value can be obtained by extrapolation, which was thoroughly studied in other publications.[14]

A sequence of method programs from Chemstation controlled the 1100 Series system's pump for the preparations of buffers and eluents, and the flow rate is controlled at 1 mL/min. For the method in general, at T = 0 minutes, the buffers (line B and C) were mixed by the HPLC pump along with a fixed percentage of acetonitrile (line A) and held for two minutes (isocratic elution). At T = 2.01 minutes, the buffer was turned off and replaced with water (line D) and acetonitrile was quickly raised to 100% at T = 3.00 minutes with linear gradient, and held for 2 minutes to wash out

Table 1. Calculated ionic strength (μ) and activity coefficient (γ) for mobile phases containing 20% ACN. Listed also include pH normalized to 60 mM in ionic strength and δpH of the buffer mixture at each pH containing 20% acetonitrile

Method Number	Apparent pH with 20% ACN	Apparent pH with 100% H_2O	Ionic strength of the mobile phase with 20% ACN	Activity coefficient (γ) for H^+ or OH^-	pH normalized to 60 mM ionic strength in 100 % aqueous	δ pH
0	1.96	1.81	48	0.73	1.81	0.2
1	2.4	2.27	52	0.72	2.26	0.1
2	3.21	2.96	57	0.71	2.95	0.3
3	4.08	3.82	61	0.70	3.80	0.3
4	4.95	4.66	70	0.68	4.63	0.3
5	5.65	5.34	76	0.67	5.30	0.3
6	6.3	5.98	77	0.67	5.94	0.4
7	7.16	6.81	81	0.66	6.77	0.4
8	7.95	7.61	85	0.65	7.56	0.4
9	8.91	8.43	87	0.65	8.38	0.5
10	9.6	9.05	87	0.65	9.00	0.6
11	10.46	9.95	83	0.66	9.91	0.6
12	11.07	10.65	79	0.66	10.61	0.5
13	11.54	11.08	77	0.67	11.04	0.5

samples. Due to retention differences for the wide variety of compounds tested, acetonitrile was used at different percentages (5% or 20%) for the isocratic section in order to obtain better retention. A total of thirteen methods were created to cover the pH ranges from 1.8 to 11.5. Prior to testing the samples, for each pH, the buffers and acetonitrile were mixed by the HPLC pump, and the pH values of the mobile phase for each method were measured and calibrated to the target pH in situ using a Beckman Φ350 pH/Temp/mV meter and a Beckman Futura combination electrode. A 20% acetonitrile in water (v/v) mixture was used as the diluent for sample preparation. For compounds that were less soluble, a higher organic content diluent (or different organic solvent) may be used. However, the amount of organic should be kept as low as possible to avoid diffusion problems, which can cause peaks to split. If this was encountered, the injection volume was reduced (with the strong diluent) or switched to a weaker diluent with larger injection volume. In general, powder drug or an aliquot of DMSO stock was placed in a microcentrifuge tube with the addition of 500 μL of diluent. The resulting mixture was sonicated for five minutes and then centrifuged at 14,000 rpm for 30 minutes. An aliquot of supernatant solution was transferred into a HPLC vial and capped. The injection volume of the sample was ~2 μL. Typically, compounds were initially analyzed using the high organic methods (20% ACN), if the

resulting retention time of the compound was too short, then lower organic content methods were applied. A diluent blank sample was added to each run to obtain the background information. Mesityl oxide was added to several samples as a neutral marker to monitor the retention time shift due to the change of pH. In general, very little shift (<0.05 minutes) was observed throughout the runs.

For the data collection, a total of five different wavelengths (225, 254, 280, 320, and 360 nm) were collected for each pH method. These five wavelengths were selected to cover absorbance of most analytes and the neutral marker, while keeping within the low background noise region. For each run, retention time of selected wavelengths was entered into an excel spreadsheet and plotted against pH to determine the pK_a of the analyte. Typically, when a compound was ionized at a certain pH, it became less retained by the column and eluted earlier than its nonionized form at other pH values. Assuming no other factors affect the retention of the compound, the retention time of a compound should be inversely proportional to the charge it carries. Thus, when retention time of a compound was plotted against the pH, a sigmoidal shaped curve was often obtained. The pK_a value was determined from the "intersection" of two linear regressions of the sigmoidal curve. Each individual linear regression was established by using the data points closest to the inflection point of the curve. An example of pKa determination for Metoprolol is illustrated in Figure 1.

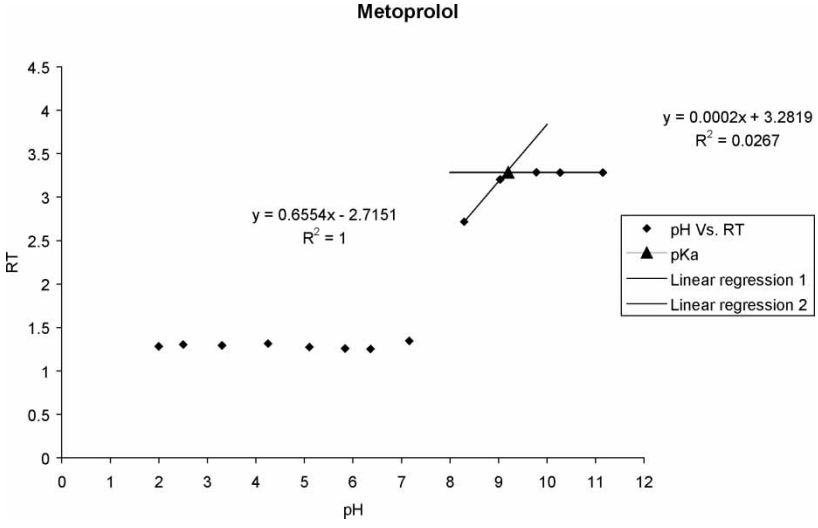


Figure 1. Determination of pKa for Metoprolol from the intersection of two linear regression lines in the retention vs. pH plot.

RESULTS AND DISCUSSION

The effects of pH on the % unionized form of Quinine and on its retention time are depicted in Figure 2. The upper curve was obtained by plotting the retention time of Quinine studied under our method and plotted against the apparent pH. The pK_a1 and pK_a2 were determined to be 4.5 and 8.1. A total of forty-four compounds were tested using this procedure and the results are presented in Table 2. In general, pK_a values determined via the current method for a wide variety of compounds were similar to the literature values, and the average δpK_a was within 0.4 units of the literature values.

This method is based on the separation of the charge or charges of the analytes that was achieved during the isocratic part of the method. The analytical time was shortened by switching the mobile phase to 100% acetonitrile at T = 3.0 minutes to accelerate or flush sample out of the column. The influence on the retention time by the acceleration of 100% acetonitrile follows a simple equation:

$$AF \, \alpha \, X_U$$

AF is the acceleration factor induced by switching the mobile phase from a quick gradient to 100% acetonitrile and X_U is the molar fraction of the lower or uncharged species. Thus, the following assumption was made: if the acceleration of retention time follows the above equation, a "reconstructed" retention time vs. pH plot can be created as if only isocratic

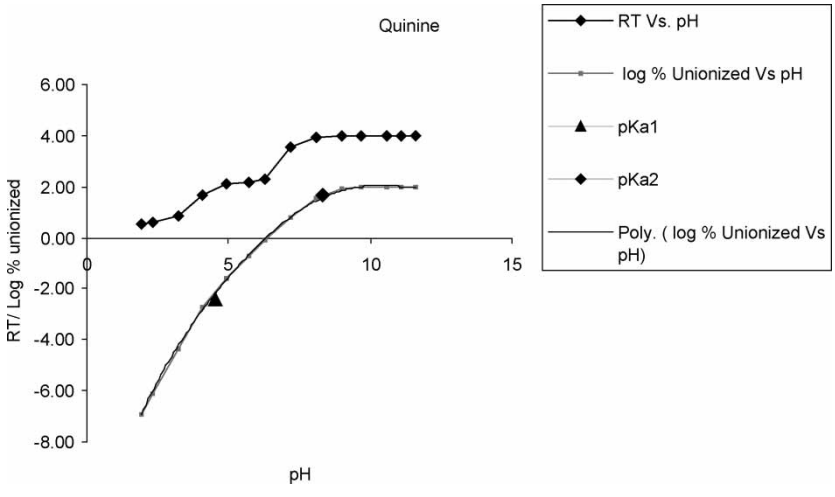


Figure 2. Determination of pKa for Quinine from the retention time vs. pH plot. Both the retention time and log percent unionized are being plotted as a function of pH.

Table 2. Comparsion of pKa obtained by current method vs. literature values

	Compound Name	Average obtained method pKa by values current	Literature values (pKa between 2 and 11)	δpKa
1	Aspirin	3.2	3.3	0.1
2	Ranitidine	2.7	2.7	0.0
2	Ranitidine	9.2	8.2	1.0
3	Metoprolol	9.2	9.5	0.3
4	Terfenadine	10	9.6	0.4
5	Probencid	3.7	3.7	0.0
6	Ketoprofen	4.2	4.2	0.0
7	Benzoic Acid	3.9	4.2	0.3
8	Naproxen	4.9	4.5	0.4
9	Dipyridomale	6.6	6.1	0.5
10	Resorufin	5.1	6.0	0.9
11	Tolnaftate	None	None	0
12	Amino salicyclic acid	3.1	3.2	0.1
12	Amino salicyclic acid	<2.5	1.8	—
13	Benzocaine	2.6	2.5	0.1
14	Amiodarone	6.8	7.0	0.2
15	Atenolol	9.8	9.5	0.3
16	Theophylline	8.1	8.6	0.5
17	Indomethycin	4.8	4.5	0.3
18	Tamoxifin	9.3	8.9	0.4
19	Lidocaine	8.4	8.0	0.4
20	Acetaminophen	10.2	9.5	0.7
21	Phenytoin	8.0	8.30	0.1
22	Sulfaphenazole	6.2	6.50	0.3
22	Sulfaphenazole	<2.5	1.70	—
23	1-Aminopyrene	3.3	3.6	0.3
24	Diclofenac	4.7	4.2	0.5
25	Fluorescein	5.6	6.4	0.8
26	Allopurinol	9.5	9.60	0.1
27	Hypoxanthine	<2.4	2.00	—
27	Hypoxanthine	9.3	8.90	0.8
28	Quinine	4.5	4.30	0.2
28	Quinine	8.1	8.40	0.3
29	Tenoxicam	4.6	5.3	0.7
29	Tenoxicam	<2.5	1.1	—
30	Warfarin	4.7	4.20	0.5
31	Fenofibrate	None	None	0
32	2,5 Dichlorobenzoic acid	2.6	2.50	0.1

(*continued*)

Table 2. Continued

	Compound Name	Average obtained method pKa by values current	Literature values (pKa between 2 and 11)	δpKa
33	Ketoconazole	6.6	6.5	0.1
34	Caffeine	None	None	0
35	Indoleacetic acid	3.9	4.1	0.2
36	Flurbiprofen	4.6	4.2	0.4
37	Fluvoxamine	9.6	9.5	0.1
38	Propranolol	9.5	9.4	0.1
39	Ibuprofen	4.7	4.4	0.3
40	Cefadroxil	2.3	2.4	0.1
40	Cefadroxil	7.5	7.3	0.2
40	Cefadroxil	10.1	9.8	0.3
41	Acycloguansine	2.4	2.3	0.1
41	Acycloguansine	9.7	9.3	0.4
42	Cimetidine	7.1	6.9	0.2
43	Minoxidil	5.3	4.6	0.7
44	Salbutamol	10.1	9.7	0.4
44	Salbutamol	10.7	10.1	0.6
			Average	0.32

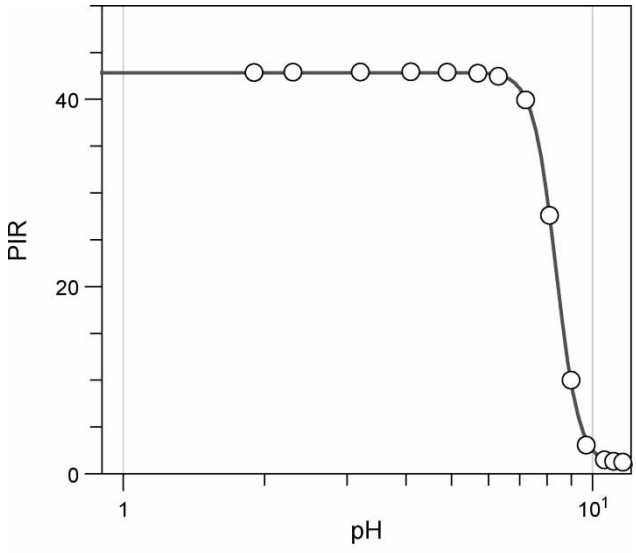
methods were used, and the pK_a can be calculated from the inflection point of the curve (or from IC50 plot via Grafit software).

To verify the above assumption, the retention time of several compounds were "reconstructed" by assigning a constant C as the acceleration factor and the X_U was calculated based on literature values at different pHs. The pseudo- isocratic retention time (*PIR*) was calculated by the following equation:

$$PIR = RT\,(1 + C{*}X_U)$$

The reconstructed PIRs of select compounds were plotted as a function of pH and the pK_a values of those compounds were calculated from the IC50 plot by utilizing the Grafit software, instead of from the intersection of two linear regressions. The calculated pK_a values matched the literature values reasonably well providing good evidence of the validity of our theory. As an illustration, pK_a analysis of phenytoin based on the *PIR* versus pH using the Grafit software is presented in Figure 3.

A limitation of this method is that it is not suitable for compounds that are either not retained or are strongly retained by the column. Compounds, such as short chain carboxylic acid or simple amines often elute too quickly, even under the low organic condition. However, the pK_a measurements of such structurally simple compounds are less complex and their pK_a values



Parameter	Value	Std. Error
Y Range	41.9254	0.1783
IC 50	8.3573	0.0105
Slope factor	17.8461	0.3450
Background	0.9125	0.1472

Figure 3. Analysis of pKa for Phenytoin from the PIR vs pH plot by using the Grafit software (pKa is estimated as the IC 50).

usually can be obtained by either a simple acid/base potentiometric titration or estimated by computational modeling with a high degree of confidence. For those compounds that are overly retained due to excessive interaction with the stationary phase, different columns/stationary phases are being evaluated and the progress will be addressed in future publications.

An additional limitation encountered with this method is that the compound has to have UV absorption within the detection range. Compounds such as Perhexiline, Pseudoconhydrine, and Psuedotropine are not suitable for our method of detection. More detection methods such as LC/MS are currently being explored to further expand this method. Furthermore, due to the aim of a higher throughput of the assay, the ionic strengths of the final mixtures of mobile phase were left unadjusted, which could cause a deviation in activity coefficients. This issue would cause measurement errors, which could contribute to the differences of experimental values compared to the literature values.

CONCLUSIONS

A medium throughput HPLC assay has been developed and validated for pK_a measurement. It combines high sensitivity, selectivity, and convenience by utilization of an automated HPLC system. It has been demonstrated that this assay is capable of measuring multiple pK_a values over a wide range, as illustrated with more than 40 compounds measured and compared to literature values. The accuracy of this assay is within 0.4 pK_a units (at 95% confidence level). Duplicate or triplicate pK_a measurements are easily conducted by multiple injections at different pH values utilizing the capabilities of a HPLC autosampler and, thus, making pK_a determinations for a large number of compounds feasible. In addition, the minimal amount of compound needed (0.1 mg) for these determinations truly makes this an ideal assay for discovery setting laboratories requiring a higher throughput level. Although, there are many other assays available and commonly used for pK_a determinations today in the pharmaceutical lab, none have this combination of accuracy, ease of use, simplicity of calculation, time and cost effectiveness, and increased capacity for screening of pharmaceuticals with diverse chemical structures.

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