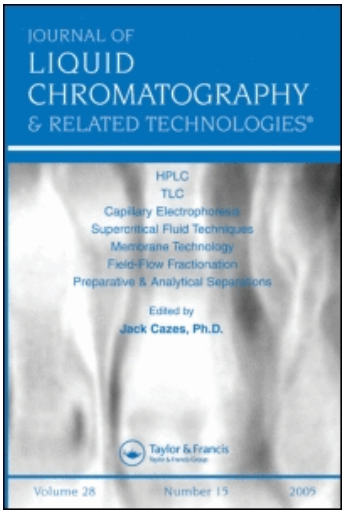

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Medium Throughput pK_a Determination of Drugs and Chemicals Using an HPLC Equipped with a DAD as a Flow Injection Apparatus

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Abstract: A semi-automated method to determine pK_a values of drugs and chemicals spectrophotometrically is described. The method uses the capabilities of a HPLC with a diode array detector (DAD) as a flow injection apparatus and a binary solvent delivery system. The advantages of this method are low sample consumption (less than 0.1 mg compound needed), high sample throughput, easy set up, high sensitivity, and low cost, precision within a 0.2 unit. Experimental pK_a values obtained for over thirty model compounds are consistent with literature values.

Keywords: Medium throughput, Drug substances, pK_a, HPLC, Flow injection, Diode array detector

INTRODUCTION

It has been widely accepted that the acid dissociation constant (pK_a) is one of the most important physicochemical parameters in the pharmaceutical industry for drug design and formulation. It was estimated that approximately 95% of drugs currently on the market are ionizable.[1] Parameters, such as lipophilicity (log D) and pH-solubility, are pK_a dependent and are important

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to the understanding of drug absorption, drug distribution, salt selection, and transport processes. In order to interface with the discovery funnel and to assist drug candidate selection since the numbers of candidates are abundant, a readily available medium or high throughput method to measure pK_a values is needed.

Historically, potentiometric and spectrophotometric methods are most commonly used for pK_a determination.[2] In the traditional potentiometer method, the requirement for sample concentration is usually greater than 10^{-4} M. For water-insoluble drugs, the pK_a values in aqueous solution were often determined in organic-water mixtures by extrapolation.[3] The glass electrode needs to be carefully calibrated using a complicated calibration procedure. The traditional spectroscopic method can determine pK_a values of compounds with large molar absorptivities to a compound concentration as low as 10^{-6} M. The sensitivity of the pK_a measurement depends on the spectral dissimilarity of the protonated and deprotonated forms of the compound. Appropriate wavelengths will have good separation at each pH and show successive/linear increase or decrease with pH. The relationship can be translated as follows:

$$A_{total}(\lambda) = X_P A_P + X_d A_d,$$

where $A_{total}(\lambda)$ is the total absorbance of the analyte at a particular wavelength and certain pH. X_P is the molar fraction of the protonated form and A_P is the molar absorbance of the portonated form. X_d is the mole fraction of deprotonated form and A_d is the molar absorbance of the deprotonated form.

Although spectrophotometry is a highly sensitive method for determining pK_a values,[1] it is generally considered that the method is very labor intensive and time consuming and it usually requires large amount of materials. In general, several buffer solutions of varying pH values have to be used to ensure an accurate determination. Flow injection analysis (FIA)[4,5] methods to determine pK_a values have been reported previously in the literature. The usage of the FIA method to determine pK_a values of weak acids with flow injection titration using potentiometric detection has also been described.[6] However, these methods are not easily automated for medium or high throughput pK_a determinations. In this study, we used a commonly available HPLC system equipped with multiple solvent delivery pump, diode array detector (DAD), and auto sampler to take advantage of the instrument's built-in automation.

Our method uses the HPLC autosampler's ability to automatically inject small volumes of sample solutions accurately into a flowing mobile phase buffer mixed by the solvent delivery system of the HPLC, and uses the DAD to rapidly obtain both spectra and absorption at specific wavelengths. Upon compiling the data, a plot of absorbance at different wavelengths vs. pH was generated. Suitable wavelengths will have good separation at each pH and show successive linear increase or decrease with pH. This method

is particularly designed to fulfill the needs of the pharmaceutical industry for medium or potential high throughput pK_a read out, while the HPLC comprises standard analytical equipment and is readily available. Other advantages include speed, ease of sample preparation, and automation via the use of an autosampler. This technique allows for examination of a wide pH range and requires preparation of only one universal buffer, in contrast to the multiple buffers required for analysis via UV-Vis spectroscopy. In addition, sample requirements are minimal at approximately 0.1 mg, making this an ideal technique for pK_a assessment in drug discovery when the compound supply is typically scarce.

EXPERIMENTAL

Materials and Methods

HPLC grade acetonitrile was obtained from Burdick&Jackson (Muskegon, MI) and reagent grade acetic acid, phosphoric acid, boric acid, and NaCl were obtained from EM Science (Gibbstown. NJ). The HPLC system used was a Hewlett Packard HP 1100 HPLC equipped with a diode array detector (DAD) (Palo Alto, CA). 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 were used without further purification.

A 6 nm slit width was used for the diode array detector (DAD) to collect the absorbance for each individual wavelength along with full spectrum. A 0.05×5 cm peek tubing was used to allow for equilibration of the injected solutions and to increase the time from injection to peak elution. Several other devices were evaluated for the same purpose, and it was found that the above one performed the best. The 0.5 μm KrudKatcher disposable pre-column filter was used both pre- and post- the peek tubing to prevent the potential on-line precipitation. The HPLC auto injector was used to accurately inject small volumes of sample solution into a continuous flow mobile phase. Typical injection volumes and flow rates were 1 μL and 2.0 mL/min, respectively, unless otherwise noted. 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 HP ChemStation software version A.08.03 with a 3D absorbance-wavelength-time software.

The HPLC's multiple solvent pump system was used to automatically prepare mobile phase buffer solutions with pH values between 1.9 and 12.5. Two solutions were used to prepare the mobile phase buffers. Solvent line A contained a universal buffer[4] at pH 1.9 which contained acetate (0.025 M), borate (0.025 M), and phosphate (0.025 M–0.03 M), and NaCl

was added to increase the ionic strength. Reservoir B contained NaOH (0.05 M) for pH adjustment and NaCl was added to adjust the ionic strength. Initially, citric acid was used to prepare the universal buffer instead of acetic acid for better buffer ranges; however, it was found that the ionic strength shifted too much when pH values are greater than 7 due to all of its acidic pK_a values being below 7 (3.13, 4.76, and 6.6); thus, the acetic acid was used instead. For the phosphoric acid, its three pK_a values are widely apart (2.12, 7.12, and 12.32); this fact not only allowed phosphoric acid to have wide buffer ranges but also made the impact of increasing ionic strength μ less significant when pH is below 12. The ionic strength $\mu$was calculated based on the full strength of the universal buffer by $\mu = 1/2 * \sum m_i z_i^2$, where z is charge of the species and m is the concentration of charged species. A thorough calculation with regard to the impact of ionic strength on the hydrogen ion activity f was conducted by using the Debye and Hückel equation and found the maximum effect of activity coefficient of the universal buffer was at the highest pH and the corresponded δpH is approximately 0.15 (Table 1). In the mixed buffer system, the ionic strength value would be lower due to the dilution, thus, its impact on the pH would be less significant. Since this method was designed for medium or high throughput work, no further justification was done.

A sequence of method programs from the HP ChemStation controlled the HP 1100's pump for the preparations of buffers with known pH. Since no chromatographic column was used, elution times were short (ca. 0.1 min) and run times per injection were nominally 0.5 min. The buffers were mixed by the HPLC pump, and the pHs of the mobile phase buffers for each

Table 1. Calculated ionic strength (μ) activity coefficient (γ), normalized against common ionic strength, δpH of the full strength uiversal buffer at each pH

pH measured	NaCl added to adjust ionic strength (mMol)	Ionic strength (μ) of the buffer (mMol)	Activity coefficient (γ) for H^+ or OH^-	pH Normalized against 60 mM ionic strength	δpH
1.90	50	59	0.70	1.90	0.00
2.00	50	61	0.70	2.00	0.00
3.00	50	73	0.68	2.98	0.02
4.00	50	79	0.67	3.98	0.02
5.00	50	91	0.64	4.96	0.04
6.00	50	102	0.63	5.95	0.05
7.00	50	119	0.61	6.94	0.06
8.00	50	145	0.58	7.91	0.09
9.00	50	162	0.56	8.90	0.10
10.00	50	173	0.55	9.89	0.11
11.00	50	179	0.54	10.89	0.11
12.50	50	236	0.50	12.35	0.15

mixing/method were measured and calibrated to desirable pH in situ by 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 sample diluent for sample preparation. In general, approximately 0.1 mg of drug was placed in a micro centrifuge tub with the addition of 250 μL of diluent. The resulting mixture was sonicated by a ultrasonicator for five minutes and then centrifuged at 14,000 rpm for a period of 30 minutes. An aliquot of supernatant solution was transferred into a HPLC vial with insert and capped. The injection volume of the sample is 1 μL. Diluent blank samples were run three times to obtain the background information for correction.

For the spectrophotometric data collection, a total of ten different wavelengths (two separated collections) and total spectrum were collected for each pH point. They were 375, 355, 335, 315, 295, 280, 270, 260, 250, and 230 nm. The ten wavelengths were selected to cover most of $n \rightarrow \pi^*$, $\pi \rightarrow \pi^*$, and $n \rightarrow \sigma^*$[8–11] absorbance shift due to the protonation or deprotonation of the analyte, while keeping within the low background noise region. For each run, area counts of each wavelength were entered into an excel spreadsheet as absorbance, and plotted against λ and pH in order to pick the best wavelengths.[10] A pH vs. absorbance plot of the chosen wavelengths was used to determine the pK_a of the analyte. The equation described by Albert and Serjeant was used for pK_a determination:

For pK_a of an Acid

$$pK_a = pH + \log |(Aion - Aobs)/(Aobs - Am)|;$$

for pK_a of a Base

$$pK_a = pH + \log |(Aobs - Am)/(Aion - Aobs)|$$

where Am and Aion, are the absorbances of the unionized and ionized species at the analytical wavelength, respectively. Aobs is the observed absorbance of the sample at the measured mobile phase pH and analytical wavelength.

RESULTS AND DISCUSSION

Figure 1 shows a 3D plot of trimethyoprim obtained by plotting area counts (as absorbance) of each wavelength as a function of pH via an Excel spreadsheet for choosing the best wavelengths. In this case two wavelengths were selected (260 and 295 nm) to conduct the calculation. A pH vs. absorbance plot of the chosen wavelengths was reconstructed and illustrated as Figure 2.

A total of thirty three compounds were tested in triplicate by using the above procedure and results are presented in Table 2. In general, pK_a values determined via our method for a wide variety of compounds were very close to the literature values and the average δpK_a is less than 0.2. Theoretically, for compounds that have pK_a values less than 3.8 or greater than

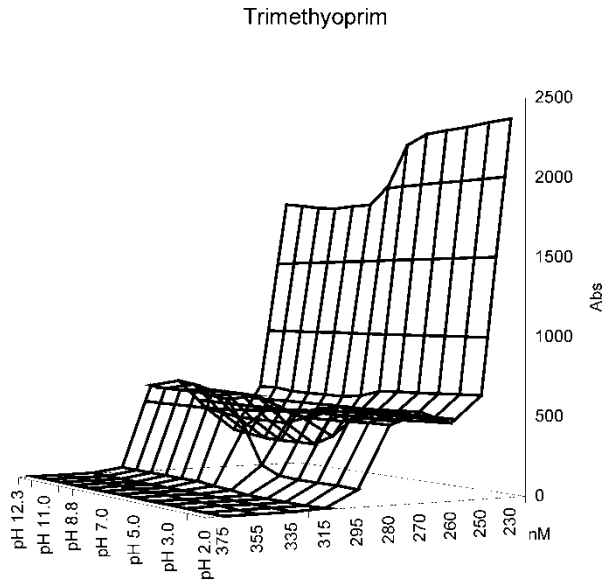

Figure 1. 3 Dimensional plot of trimethyoprim (absorbance, wavelength, and pH).

10.5, our method could only provide an approximate number due to the upper and lower pH limit setting of our buffer system. For compounds that have pK_a values between 2.8 and 3.8 or 10.5 and 11.8 we typically observed that the spectrums at extreme pHs (1.8 and 12.5) were nearly plateaued. Such phenomenon was due to the near completion of the protonation or deprotonation

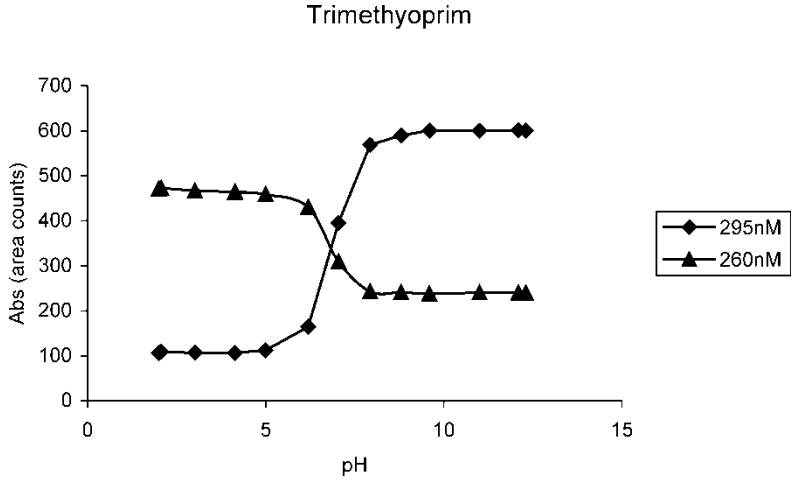

Figure 2. The pH vs. absorbance plot of the chosen wavelengths for trimethyoprim.

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

	Compound name	Average pK_a values obtained by our method (in triplicates)	SD	Literature values	δpK_a
1	Benzoic acid	4.3	0.0	4.20	0.1
2	Trimethyoprim	7.2	0.1	7.10	0.1
3	Indomethacin	4.4	0.0	4.50	−0.1
4	Ketoconazole	3.2	0.1	3.20	0
5	Minoxidil	4.5	0.2	4.60	−0.1
6	Ketoprofen	4.3	0.0	4.23	0.07
7	Diclofenac	4.3	0.1	4.0–4.2	0.2
8	Diazepam	3.5	0.1	3.40	0.1
9	Naproxen	4.3	0.2	4.3–4.5	−0.1
10	Tolbutamide	5.5	0.0	5.30	0.2
11	Resorufin	6.4	0.1	6.00	0.4
12	Acetominophen	10.0	0.2	9.5–9.7	0.3
13	Amino salicyclic acid	3.1	0.2	3.25	−0.15
14	Benzocaine	<2.5	0.1	2.50	0
15	Niacin	4.7	0.0	4.85	−0.15
16	Aspirin	3.7	0.0	3.50	0.2
17	Ibuprofen	4.7	0.0	4.43	0.27
18	Indole acetic acid	3.9	0.0	4.10	−0.2
19	Aniline	4.5	0.0	4.60	−0.1
20	Norfloxacin	6.5	0.0	6.30	0.2
20	Norfloxacin	>8.7	0.0	8.80	0.1
21	Sulfasalazine	<2.7	0.2	2.70	0.0
21	Sulfasalazine	>11.8	0.1	11.70	0.1
22	Theophylline	9.1	0.0	8.80	0.3
23	Phenytoin	8.2	0.2	8.30	−0.1
24	Bromothymol Blue	7.4	0.1	7.10	0.3
25	Sulfaphenazole	6.3	0.2	6.50	−0.2
25	Sulfaphenazole	<2.5	0	1.70	0.8
26	Allopurinol	9.6	0.3	9.60	0
27	Hypoxanthine	ND	—	2.00	—
27	Hypoxanthine	9.2	0.1	8.90	0.3
27	Hypoxanthine	>11.7	0	12.10	−0.4
28	Quinine	4.2	0	4.30	−0.1
28	Quinine	8.5	0	8.40	0.1
29	Tenoxicam	5.5	0	5.3 & 1.1	0.2
30	Warfarin	4.3	0	4.20	0.1
31	Fenofibrate	NO		NO	0
32	2,5 Dichlorobenzoic acid	<2.6	0	2.50	0.1
33	Dipyridamole	6.2	0	6.10	0.1
				Average	0.2

(greater than 90%). Thus, the absorbance values at extreme pHs were used in the calculation in lieu of Am of Aion, and estimated numbers were given.

The biggest limitation of our method is that UV detection can only cover certain transitions. For a compound that contains a functional group, such as aliphatic amine, the UV shifts caused by protonation or deprotonation were out of our detection range.[3] Such difficulty was encountered on several in house candidates (data not included). However, the measurement of pK_a of such simple functional groups are less complex compared with compounds that contain functional groups that result in conjugation or de-conjugation upon protonation or deprotonation. Thus, their pK_a values usually can be obtained by either a simple acid/base potentiometric titration or estimated by computational modeling with a high degree of confidence.

One other limitation encountered with this method is the compound solubility. For compounds that are not soluble enough in the diluent, pK_a can not be measured. Compounds such as felodipine, clotrimazole, and di-chlorofluorescin, which have solubility less than 0.1 μM in the diluent, pK_a can not be determined. Such incidents can be easily identified by comparing the area counts of all wavelengths verses blank injection. The difference between those two are often very small, thus, a solubility limited detection conclusion can be drawn in such cases. Furthermore, due to the intention of higher throughputs of the assay, the ionic strength of the final mixes of mobile phase was left unadjusted, which could cause changes in activity coefficients of the ions. Such matters would cause measurement errors, which could contribute to the deviation of our experimental values verses the literature values. Further efforts are underway, and the progress will be addressed in future publications.

CONCLUSIONS

A medium throughput HPLC assay has been developed and validated for pK_a measurement. It combines the high sensitivity of the spectrophotometric method and convenience of an automated HPLC system. The accuracy of this assay is within 0.2 pK_a units (at 95% confidence level) and compound consumption is less than 0.1 mg. This assay can measure multiple pK_a values over a wide pK_a range. Duplicate or triplicate determinations are easily conducted by using the multiple injections at different pH values and the capabilities of an automated HPLC auto-sampler make pK_a determination for large number of compounds feasible. The quaternary HPLC system can be easily switched back and forth for both conventional column chromatography and our assay, since no modification was made and only two solvent lines were used. A simple column switch valve can be used to further simplify the system. Thus, no adjustments will be needed to switch the system to conventional column chromatography usage if only two solvent lines are needed. It has been demonstrated that this assay is accurate, easy to use, cost effective,

and capable of handling large volumes for pK_a screening of pharmaceuticals with diverse chemical structures.

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