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Rapid Determination of Ionization Constants (pK_a) by UV Spectroscopy Using 96-Well Microtiter Plates

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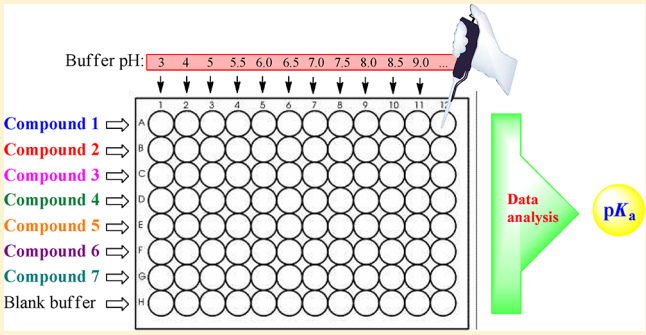
Supporting Information

ABSTRACT: We have developed a methodology that enables for the rapid measurement of ionization constants (pK_a) of series of compounds by UV spectrophotometry. This protocol, which is straightforward to set up, takes advantage of the sensitivity of UV spectroscopy and the throughput enabled by the 96-well microplate (as opposed to the use of 1 cm quartz cuvette). The compounds, in stock solutions in DMSO, are dissolved in several aqueous buffer solutions directly in the microtiter plate, allowing the simultaneous determination of the UV spectra as a function of pH. Further treatment of the data provides the pK_a values in a medium-throughput manner. The pK_a values of 11 new antitrypanosomal dibasic compounds were determined using this methodology.



KEYWORDS: *pK_a, ionization constant, imidazoline, 96-well microplate, UV spectrophotometry, buffer solution, trypanosome, sleeping sickness, dibasic compounds*

The ionization constant (pK_a) of a drug is a physicochemical parameter that significantly affects its pharmacokinetic behavior, that is, absorption, distribution, metabolism, and excretion (ADME). Lipophilicity, solubility, protein binding, and permeability of a compound are influenced by its pK_a;[1] for instance, basic compounds with p$K_a \geq 7.4$ will be charged at physiological pH and display slower diffusion rate across biological membranes such as the blood−brain barrier (BBB).

In the last years, we have been interested in the design of new antitrypanosomal dicationic compounds as potential agents against late-stage sleeping sickness.[2−4] This disease caused by subspecies of African trypanosomes is characterized by an early hemolymphatic stage that is followed by a fatal late stage due to an invasion of the CNS by the parasites. In the course of this project, we have designed new derivatives of dibasic lead compounds modulating the basicity of the nitrogen moieties with *O*-alkyl substituents to improve their BBB permeability. Thus, measuring the pK_a of these new series was a necessary task to rationalize the in vitro and in vivo biological activity findings.

Different methods exist to measure pK_a values,[5] the following ones being the most common: (a) potentiometric titration, where the pK_a is derived from the titration curve; (b) spectrophotometric titration, where a UV spectrum of the compound is taken for each point of the titration and the change in UV absorbance is plotted against the pH;[6] and (c) capillary electrophoresis in different buffer solutions with results calculated from the relative mobility of ions at different pH.[7−11] Among these, the potentiometric method requires a larger quantity of sample as compared to UV spectroscopy or capillary electrophoresis. In addition, special equipment has been developed for measuring pK_a in an automatic way using a mixed-buffer linear pH gradient system.[12,13] However, in the absence of such an apparatus, the determination of pK_a for series of compounds by traditional methods can be a time-consuming task.

In the present paper, we report an efficient medium-throughput method for the determination of ionization constants by UV spectroscopy using standard 96-well microtiter plates. This methodology, which is straightforward to set up, takes advantage of the sensitivity of UV spectroscopy (i.e., only a small amount of compound is needed, useful for sparingly soluble substances, work at high and low pH values)[5] and the throughput enabled by the 96-well microplate. It does not require complex equipment (apart from a UV spectrophotometer equipped with a microplate reader and a pH meter for the preparation of the buffer solutions) and allows for the rapid measurement of pK_a values of series of compounds.

To measure pK_a values by UV spectrophotometry, compounds must have a chromophore close to the ionization centers, and the spectrum (absorbance) must change as a function of ionization. Briefly, our method proceeds as follows: (1) the compounds under study are prepared as 10 mM stock solutions in DMSO; (2) the 96-well microtiter plate is filled with different buffers of constant ionic strength ($I = 0.1$ M) ranging from pH 3 to pH 12 (e.g., 2 points per pH unit); (3) a fixed amount of compound stock solution is added to each well, leaving a series

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of blank buffers (i.e., free of compound) as correction factors (note that the final amount of DMSO in the well is ≤2% v/v); (4) the UV spectra of the compounds in the range 230−500 nm are recorded; and (5) the data of the UV spectra at each wavelength as a function of pH and the pK_a are processed using the Prism program.[14]

In the present study, we have used a similar data analysis as described by Tomsho et al. for the determination of ionization constants by spectral analysis (see the Supporting Information for details).[15] Figure 1 shows an example of such an analysis for the determination of the pK_a of 4-nitrophenol. The changes observed in the UV spectrum of 4-nitrophenol depending on the species present in solution are clearly seen in Figure 1. At pH 3, the molecule is neutral (4-NO_2PhOH) with a maximum absorption at 314 nm, whereas at pH 10, only the deprotonated species is present (4-NO_2PhO$^-$) with $\lambda_{max} \approx 400$ nm. The pK_a

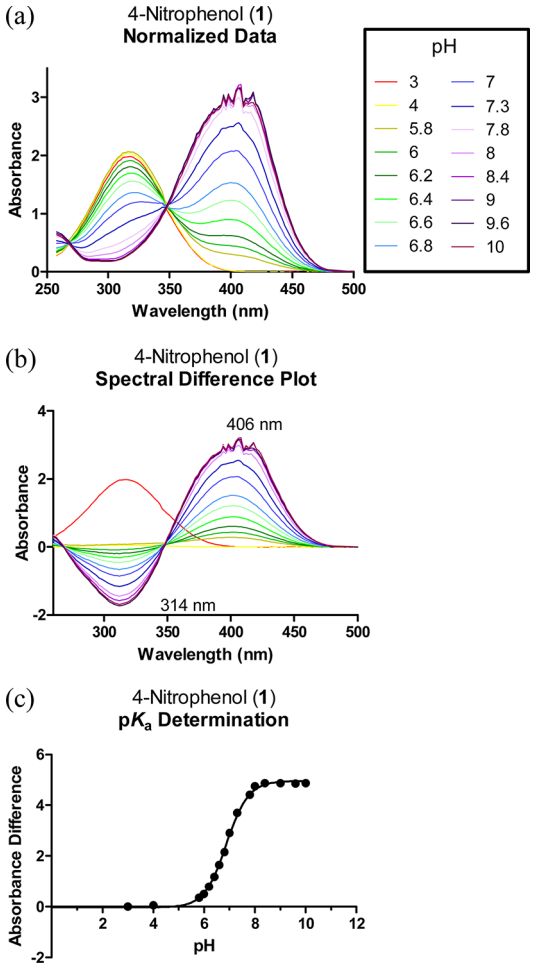
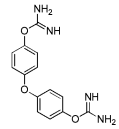


Figure 1. Spectral data analysis and pK_a determination. (a) UV spectrum (λ = 250−500 nm) of 4-nitrophenol (**1**) in different aqueous buffer solutions ranging from pH 3 to 10. The absorbances are normalized to zero for λ = 500 nm. (b) Plot of the spectral difference between different solutions of **1**. The maximum positive deviation occurred at 406 nm, while the maximum negative deviation occurred at 314 nm. (c) Plot of the total absorbance difference vs pH to determine the pK_a. The total absorbance difference is the sum of the absolute absorbance difference values at the chosen wavelengths (i.e., 314 and 406 nm). The pK_a value was worked out by nonlinear regression using eq 1 according to Tomsho et al. (see the Experimental Procedures).[15]

Table 1. pK_a Values of Monoacidic, Monobasic, and Dibasic Compounds Determined by the 96-Well UV Spectrophotometric Method

Cpd	Structure	Solvent[a]	λ (nm)[b]	Measured[c] pK_a	Mean value ± SD[d]	Lit. value[e]	Ref.
1		H_2O	318/400	7.01 7.02 7.02	7.02 ± 0.01	7.16	16
		H_2O + 2% DMSO	318/400	6.84 6.88 6.90	6.87 ± 0.03	na	na
2		H_2O + 2% DMSO	268/280	6.20 6.27 6.21 6.20	6.22 ± 0.03	6.23	17
3		H_2O + 2% DMSO	297/354	9.22 9.17 9.20	9.20 ± 0.03	9.3	18
4		H_2O + 2% DMSO	244/312	8.15 8.24 8.05	8.14 ± 0.09	8.12 8.04	19
5		H_2O + 2% DMSO	250/390	10.63 10.72 10.75	10.70 ± 0.06	10.4	15

[a]The use of 2% v/v DMSO as a cosolvent did not alter significantly the pK_a value of the test compounds. Working temperature = 30 °C. All pK_a values were measured at constant ionic strength (I = 0.1 M) and concentration (C = 0.2 mM). [b]Analytical wavelengths are determined at the maximum and minimum absorption values in the spectral difference plot. [c]Experiments were repeated at least three times. [d]Standard deviation. [e]Experimental pK_a values at 25 °C in water.

measured in H_2O at 30 °C (7.02 ± 0.01) was in good agreement with the reported value (7.16 at 25 °C) (Table 1).

To validate this method, the pK_a values of five compounds previously reported in the literature were measured using our protocol with an average of 16 different buffer solutions ranging ±2 pH units around the pK_a value (i.e., 16 points in the absorbance vs pH curve). As shown in Table 1, the observed pK_a values were in good agreement with the reported data. We could not discriminate two different pK_a values for the symmetrical dicarbamimidate derivative **5**. This is consistent with earlier report by Nagle et al.,[16] who measured only one ionization constant (pK_a = 10.4) for both basic groups by acid−base titration using UV spectroscopy.

We have checked that the use of 2% v/v DMSO as a cosolvent did not alter significantly the pK_a value of the tested compounds. This is an advantage as poorly water-soluble compounds can be tested using this protocol. Finally, we have observed that repeating the experiment three times for each compound gave, in most cases, satisfactory scatter values. (Scatter, as defined by Albert and Serjeant,[5] is the largest deviation between any value in the set and the value calculated by taking antilogarithms of each pK_a value in a set, averaging these, and writing down the logarithm of the average as the pK_a.)

Experimentally, the time needed to load manually the 96-well microplate (e.g., blank buffer + seven different compounds in 12 buffers or five compounds in 16 buffers) is about 45 min, whereas the reading of the plate takes approximately 40 min (e.g., with a 2 nm resolution). We have checked that the compounds solutions were stable over time and that UV spectra were not significantly affected over the course of the experiment. To do so, several readings of the same plate were repeated over a 3 h period, and the pK_a was calculated at three different time points (Figure 2).

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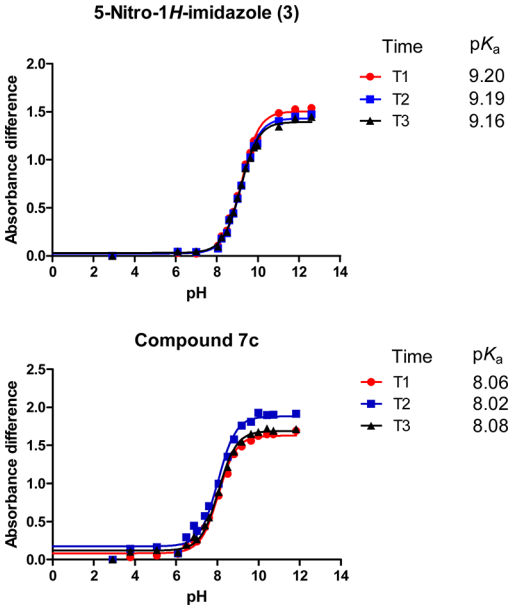
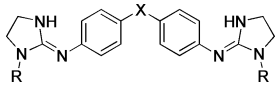


Figure 2. Study of the stability of the compounds in buffer solutions over time. The plates with the compounds dissolved in buffer solutions were read three times over a 3 h period (T1 = 40 min, T2 = 90 min, and T3 = 150 min). The pK_a was calculated for each time point.

The pK_a values of a series of 11 dibasic compounds synthesized in our group as antitrypanosomal agents were measured in triplicate using this methodology (Table 2). It should be noted that only one pK_a value could be determined, suggesting overlapping pK_a values for both imidazoline groups. This is possibly due to the second deprotonation taking place in a very small pH range so that changes in the spectra cannot be distinguished. Rozas and co-workers also failed to distinguish two discrete pK_a of similar dibasic compounds.[16,17] It should be noted that in our particular case, that is, symmetrical dibasic compounds, the scatter values were higher (0.03−0.2) than in compounds having only one basic site (0.01−0.1). This may be attributed to the fact that the bisimidazolines have overlapping pK_a values or to possible solubility problems giving rise to product precipitation in the wells. These results show that the introduction of a hydroxyl group at N1-nitrogen of the imidazoline ring reduces its ionization constant by approximately 2 pK_a units (compare **6b**/**6a** and **7b**/**7a**). Introduction of other alkoxy substituents (OMe and OEt) at the same position does not alter significantly the pK_a as compared to a hydroxyl group.

Table 2. Ionization Constants of New Dibasic Antitrypanosomal Compounds Determined by the 96-Well UV Spectrophotometric Method at 30 °C and Constant Ionic Strength (I = 0.1 M)



compd[a]	X	R	λ (nm)[b]	pK_a[c]
6a	NHCO	H	258/308	9.29 ± 0.07[d]
6b		OH	258/298	7.43 ± 0.10
6c		OMe	258/312	7.27 ± 0.09
6d		OEt	258/308	7.34 ± 0.03
7a	CH_2CH_2	H	260/310	10.71 ± 0.10
7b		OH	238/264	7.97 ± 0.13
7c		OMe	236/262	8.01 ± 0.12
7d		OEt	238/262	8.01 ± 0.21
8a	NHCONH	H	260/292	10.34 ± 0.04
8c		OMe	240/270	7.95 ± 0.05
8d		OEt	258/296	8.27 ± 0.07

[a]The compounds were dissolved in DMSO (stock solution) and diluted with the corresponding buffers to reach a final concentration of 0.2 mM in the well (the final quantity of DMSO is 2% v/v). [b]Analytical wavelength. [c]pK_a of the aminoimidazoline groups; only one pK_a could be calculated for both imidazolines. [d]The pK_a values are the mean of three or four (for **7c** and **8d**) independent measurements ± SDs.

In summary, we present here a very convenient method to measure ionization constants by UV spectrophotometry in a medium-throughput manner. Satisfactory pK_a values can be obtained with a minimum of three repeats for each experiment. We trust that this methodology will be of interest to medicinal chemists when assessing pK_a values of medium-size compound libraries for the study of their structure−activity relationships.

EXPERIMENTAL PROCEDURES

The buffer solutions used here were a follows: (a) AcOH/AcONa, covering the pH range 3.0−5.0; (b) KH_2PO_4/K_2HPO_4, covering the pH range 6.0−8.0; (c) Borax/HCl, covering the pH range 8.2−9.0; (d) Borax/NaOH, covering the pH range 9.2−10.8; (d) Na_2HPO_4/Na_3PO_4, covering the pH range 10.8−12; and (e) glycine/NaOH for pH 12.6. All of these buffer solutions were prepared with the same ionic strength (I = 0.1 M) by adding KCl (see the Supporting Information for details). The stock solutions of the compounds were prepared at 10 mM concentration in DMSO. Standard solutions were prepared adding 4 μL of the stock solution in 196 μL of buffer solution in each well of the microplate getting a final concentration of 0.2 mM. In some cases where saturated spectra were observed, 0.1 mM analyte solutions were used (e.g., 2 μL of stock solution in 198 μL of buffer). UV spectra were recorded on a THERMO Multiskan Spectrum apparatus between 210 and 400 nm (230−500 nm for compound **1**) at 2 nm resolution. The data were processed with the Excel program. The pK_a values were worked out by nonlinear regression (Prism program) using eq 1 as reported.[15]

$$\text{absorbance total} = \frac{\varepsilon_{HA} - \varepsilon_{A^-} \times [10^{(pH-pK_a)}]}{1 + 10^{(pH-pK_a)}} \times [S_t] \quad (1)$$

ε_{HA} and ε_{A^-} are the extinction coefficients of the acid and base forms of the compound, respectively (i.e., the minima and maxima of the absorbance difference curve, respectively), and $[S_t]$ is the total compound concentration. See the Supporting Information for a detailed description of the experimental protocols and data analysis.

ASSOCIATED CONTENT

Supporting Information

Detailed description of the experimental procedures: preparation of buffer solutions, loading of the 96-well microtiter plate, data analysis, multiwavelengths spectra, and pK_a determination plots for compounds **2**−**8**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. C.D. and C.H.R.M. designed the experiments and analyzed the data. C.H.R.M. performed all of the experimental work.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

BBB, blood−brain barrier; ADME, absorption, distribution, metabolism, and excretion

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