# Determination of p$K_a$ Values by Liquid Chromatography

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## Abstract

**In this paper, we investigate the potential of a high-performance liquid chromatography technique to determine p$K_a$ values of drug candidates that show poor solubility in water. The determination of pKa values by this method is in principle not new, but it exhibits simplicity, requires lower quantities of drugs and solvents, and minimal analysis time. The method is an alternative to existing methodology, in which this determination is not readily feasible.**

## Introduction

The p$K_a$ value is a main item in the biophysical characterization of a drug and may be helpful in predicting the behavior of a drug under in vivo conditions. Because a correlation exists between the p$K_a$ value and the solubility of the drug in different media, it is possible to make predictions in the behavior of absorption in the organism and, as a result of this, the closely linked bioavailability.

Current properties of new drugs include poor solubility characteristics, so the need to generate a high-performance liquid chromatography (HPLC)-based method to predict the p$K_a$ values of such drugs was recognized. The described method is attractive because of its simplicity and ability to use a variety of isocratic HPLC systems, resulting in the requirement for minimal amounts of drug substance. In addition, because of the high quality and acceptable throughput and further information obtained for the test material, the method is attractive for the pharmaceutical industry.

To measure p$K_a$ values of acids and bases in the conventional way, titration is applied to get the p$K_a$ value at the half-neutralization point, which is ideally the point of inflection in the titration curve (Figure 1) (1–5). For acids, the following relationship exists:

$$OH^- + XH \rightarrow X^- + H_2O \qquad \text{Eq. 1}$$

The following exists for bases:

$$X + H^+ \rightarrow HX^+ \qquad \text{Eq. 2}$$

This method is applicable if the drug is acceptably soluble and stays in solution during the titration procedure. If there are differences between the protonized and the nonprotonized form, the procedure can only be used in a limited way. Furthermore, a relatively high amount of test material is needed.

To compensate for the disadvantages of titration, and considering the very small amounts of available test materials, an alternative method needs to be developed.

In principle, the use of liquid chromatography (LC) to determine p$K_a$ values is not new (6–8). However, these methods do not fulfill the requirements of a high-throughput lab, in which short retention times, employment of microgram quantities of test materials, the use of simple and reasonably priced equipment, and a variety of appropriate chromatographic columns are required.

To evaluate our method, three well-known substances were chosen to demonstrate its appropriateness: the organic acids (*a*) piretanide, (*b*) furosemide, and (*c*) glyburide (Figure 2).

The LC method is based on the different retention behavior of the protonized and the nonprotonized form of the test material. The retention time is determined in relationship to the pH-value of the mobile phase by reversed-phase HPLC. The p$K_a$ value is the point of inflection in the resulting sigmoidal curve, which can be easily achieved.

The test materials, with the exception of glyburide, are readily soluble in water and show a moderate acidic behavior (Table I).

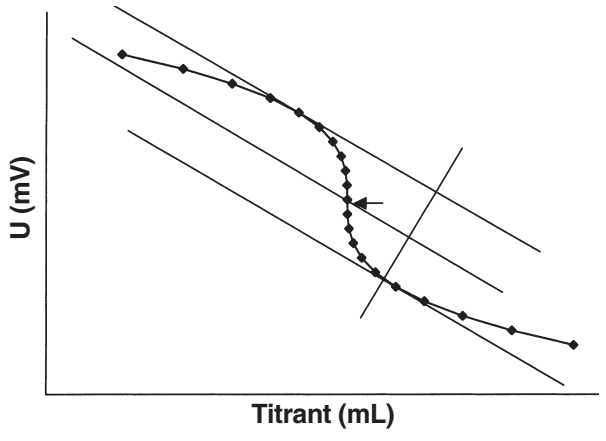


**Figure 1.** Determination of the point of inflection in the titration curve of an acid (schematic).

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## Experimental

### Instrumentation

Measurements were carried out with an isocratic HPLC system (HP 1090, Agilent, Waldronn, Germany) with a standard UV detector (Gynkotek SP-4, Germering, Germany). The detection wavelengths for piretanide, furosemide, and glyburide were 226, 232, and 227 nm, corresponding to their respective UV maxima.

A Superspher 100 $RP_{18}$ endcapped column (75-mm × 4-mm × 5-µm particle size) was used for the analysis of furosemide, a Lichrospher 60 RP select B column for piretanide, and a Lichrospher 100 $RP_8$ column for glyburide (all Merck, Darmstadt, Germany).

An SP 4270 integrator (Spectra Physics, Egelsbach, Germany) was used to obtain the chromatogram and data calculations.

To titrate the pH values of the acetonitrile buffers to 0.01 pH units accuracy, a MetrOhm 605-pH-Meter (Herisau, Switzerland) was used.

### Chemicals and reagents

Deionized water was produced using a Milli-$Q_{185}$ Plus water system (Millipore, Milford, MA) for all aqueous solutions. All chemicals and solvents were of ACS-reagent grade. Furosemide (lot no. B030), piretanide (lot no. E041), and glyburide (lot no. B008) were obtained from Aventis Pharma Deutschland GmbH (Frankfurt am Main, Germany).

The HPLC mobile phases were prepared from deionized water, acetonitrile, sodium chloride (1 g/L), phosphoric acid (85%, 2 mL/L), and sodium hydroxide (10N, to adjust the pH values, starting at pH 7.0).

The relation of organic solvent to water was chosen in a way that the substance peak did not overlap with the peak of injection and the run would result in acceptable retention times in the acidic pH range, in which the substance showed strong interaction with the column because of hampered dissociation of the corresponding acid.

The disadvantage of the use of an organic modifier and the subsequent "incorrect" p$K_a$ value can be overcome by a simple experiment.

Three elution phases differing in their amounts of organic solvent were chosen. Extrapolation to the 100% water value was performed by linear regression analysis. The generated results for the three model compounds were close to the theoretical values and substantiate the appropriateness of the applied methodology.

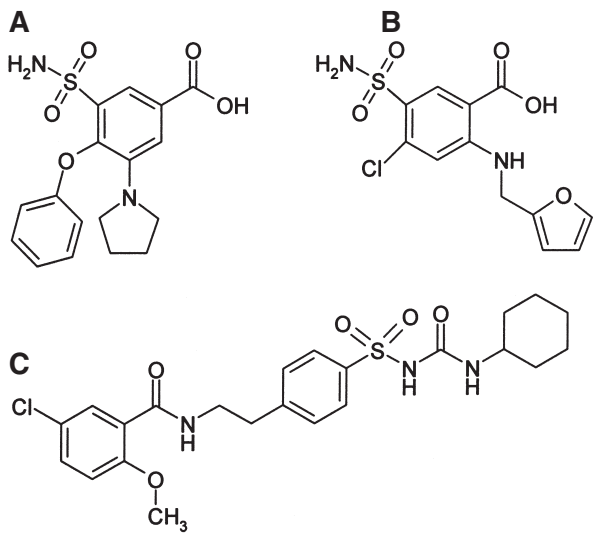


**Figure 2.** Chemical structures of the tested drugs: (A) piretanide, (B) furosemide, and (C) glyburide.

**Table I. Solubility Data of the Tested Drugs***

| Substance | Solvent | Solubility (mg/100 mL) |
|---|---|---|
| Furosemide | Water | 8.2 |
| | Buffer pH 7.5 | 2400 |
| Piretanide | Water | 5.3 |
| | Buffer pH 7.0 | 2800 |
| Glyburide | Water | 0.16 |
| | Buffer pH 7.5 | 2.4 |

* The values were taken from the *List of Pharmaceutical Substances*, 12th ed. Eschborn, Germany, 2000.

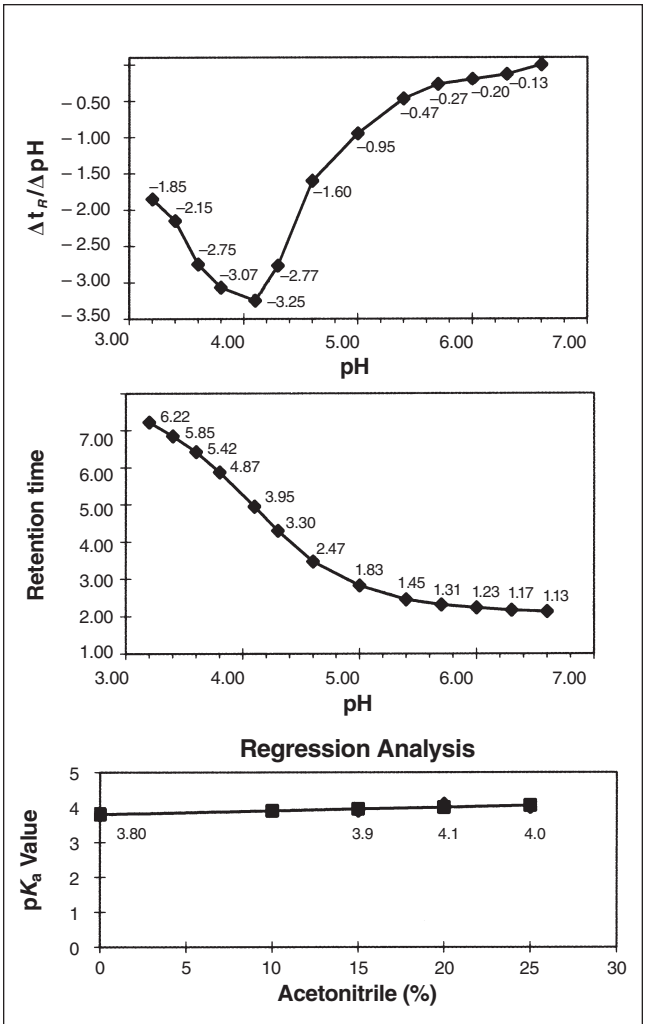


**Figure 3.** Example for the analysis of the obtained data: furosemide with 20% acetonitrile as organic modifier.

**Procedure (sample preparation)**

One milligram or less of each test material was dissolved in acetonitrile to obtain a solution having a concentration of 0.1 µg/10 µL. The column was equilibrated by rinsing with the mobile phase for 5 min, at 3.0 mL/min flow.

We have used a flow rate of 1.0 mL/min and injected 10 µL of sample solution until two retention time values were equal. In this way, we established the relationship between the retention time of each substance and the pH value (Figure 3).

The used pH values ranged from 2.0 to 7.0; measuring started at pH 7.0 in steps of 0.3 units.

**Table III. Comparison of p$K_a$ Values***

| | Furosemide | Piretanide | Glyburide |
|---|---|---|---|
| Literature | 3.9 | 3.9 | 5.3 |
| Found after regression | 3.8 | 4.0 | 5.5 |

* The literature values are taken from reference 9.

## Results and Discussion

In the conventional way, p$K_a$ values of drugs are detected is by titration (2–4). However, when the drug is only poorly soluble or does not stay in the solvents during the titration procedure, this procedure can be used only in a limited way. In this study, we used the LiChroCART 25-4 column cartridge system, which is available for the resins Superspher 100 $RP_{18}$ endcapped,

**Table II. Summary of the Collected Data for Furosemide, Piretanide, and Glyburide***

| | Concentration of organic modifier (acetonitrile) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Furosemide ($\Delta t_R/\Delta$pH) | | | Piretanide ($\Delta t_R/\Delta$pH) | | | Glyburide ($\Delta t_R/\Delta$pH) | | |
| pH value | 15% | 20% | 25% | 15% | 20% | 25% | 30% | 35% | 40% |
| 7.0 | 0.00 | | 0.00 | –0.98 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 6.7 | | | –0.07 | | | –0.17 | | | |
| 6.6 | | 0.00 | | | | | | –2.23 | –1.10 |
| 6.5 | –0.14 | | | –1.44 | –0.24 | | –5.32 | | |
| 6.4 | | | –0.03 | | | -0.20 | | | |
| 6.3 | | –0.13 | | | | | | –3.20 | –1.30 |
| 6.1 | | | | | | –0.23 | | | |
| 6.0 | –0.28 | –0.20 | –0.02 | –3.16 | –0.56 | | –8.32 | –3.47 | –1.17 |
| 5.8 | | | | | | –0.53 | | | |
| 5.7 | | –0.27 | –0.13 | | | | | –2.67 | –0.87 |
| 5.5 | | | | –8.22 | –1.52 | –0.87 | –6.98 | –2.25 | |
| 5.4 | –0.70 | –0.47 | | | | | | | 0.79 |
| 5.3 | | | –0.21 | | | | –6.40 | –1.75 | |
| 5.2 | | | | | | –1.47 | | | |
| 5.1 | | | | | | | | –1.60 | –0.47 |
| 5.0 | –1.93 | –0.95 | –0.44 | –19.74 | –4.14 | | –4.90 | | |
| 4.9 | | | | | | –2.73 | | | |
| 4.8 | | | | | | | –0.87 | –0.30 | |
| 4.7 | –3.50 | | | | | | | | |
| 4.6 | | –1.60 | | | | 3.90 | | | |
| 4.5 | | | –0.82 | –32.63 | –9.30 | | | –0.73 | –0.23 |
| 4.4 | –5.30 | | | | | | | | |
| 4.3 | | –2.77 | | | | –5.00 | | | |
| 4.2 | | | –1.10 | –39.27 | –13.87 | | | –0.53 | |
| 4.1 | –6.87 | –3.25 | | | | | | | |
| 4.0 | –1.25 | –5.40 | | | | | | | |
| 3.9 | –7.87 | | | –37.40 | –14.30 | | | | |
| 3.8 | | –3.07 | –1.15 | | | | | | |
| 3.7 | –7.70 | | | | –13.60 | –7.60 | | | |
| 3.6 | | –2.75 | –1.00 | 0.00 | | | | | |
| 3.5 | –8.15 | | | | –12.00 | –8.45 | | | |
| 3.4 | | –2.15 | | | | | | | |
| 3.3 | | | | | –11.55 | | | | |
| 3.2 | –6.50 | –1.85 | –0.90 | | | | | | |
| 3.0 | | | –0.75 | | | | | | |

* Obtained with different concentrations of organic modifier in dependence of the pH value.

Lichrospher 60 RP select B, and Lichrospher 100 $RP_8$ (Merck). As mobile phases, water–acetonitrile mixtures with different pH values were applied with a flow rate of 1.0 mL/min and detection at appropriate wavelengths.

We calculated the retention time divided by the pH value and plotted the resulting ratio in a coordinate system with the pH value on the abscissa. The resulting curve had a U-shaped form, in which one can estimate the p$K_a$ value at the minimum of the curve. We estimated the p$K_a$ value for the tested drugs by regression analysis of the p$K_a$ values found at the different acetonitrile concentrations (Table II), which corresponded to values reported in literature and thus appeared readily acceptable (Table III) (9–11). Using the three selected drugs having similar chemical structures, we could show that one can easily measure the p$K_a$ values despite different solubilities. Other advantages of this method are the short retention time, which leads to a short run time that results in a high-quality output (2–3 compounds/day) and a minimum in drug and solvent requirements with resulting low-running costs.

By regression analysis, the influence of the modifier was considered. Further positive side effects are that one can get additional valuable information of the drug concerning its chromatographic behavior (i.e., the peak shape for the investigated stationary and mobile phases).

By connecting the p$K_a$ values, a hockey-stick-shaped curve was obtained (12). If one uses the data that resulted from measurements at higher amounts of the organic modifier to perform the regression analysis, one will get slightly lower p$K_a$ values that will not lay on the straight line resulting from the data obtained from measurements at smaller amounts of organic solvent (12). Therefore, the investigator should use the smallest amounts of organic solvent and the shortest columns possible. Reduction in the organic solvent concentration will lead to a prolonged retention time, and the time needed to perform a single run will increase.

## Conclusion

This paper investigated the potential of an analytical method to determine the p$K_a$ values of drug candidates that show poor solubility. The advantages of this method are its simplicity, low need for drug and solvent, and low time requirement. As a conclusion, one could suggest it as a convenient alternative to the conventional methods.

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