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Rapid Communication

Practical digest for the evaluation of acidity constants of drugs by reversed-phase high performance liquid chromatography

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Summary

A practical guide to evaluate acidity constants by using reversed-phase HPLC considering experimental conditions, instrumentation, theoretical respects and troubleshootings is given.

As was indicated in a recent paper (González et al., 1992a), reversed-phase high-performance liquid chromatography (RP-HPLC) is a very appropriate technique for evaluating acidity constants of acid solutes in mixed media. The advantages of this method over others are that only 1 mg or less of the compound is needed for the evaluation and that the purity of the compound is not critical when the impurity may be separated on the column. In the present work attention will be paid to the reliability of the experimental procedure in order to obtain optimal results.

Solvents, mobile phases and sample solutions: Organic cosolvents must be of HPLC quality. Distilled and deionized water should be filtered through a 0.2 μm filter in a solvent filtration apparatus. Milli-Q treated water is strongly recommended.

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Mobile phases at a given pH value are prepared by mixing a suitable volume of organic cosolvent and the corresponding volume of aqueous buffer. All these buffer solutions covering the pH range 2–12 may be prepared from potassium hydrogen phosphate (analytical grade) to give a final buffer concentration of 0.015–0.020 M. The ionic strength is kept constant by addition of potassium chloride (analytical grade) considering the buffer ionic contributions (Otto and Wegscheider, 1983). Then the pH is adjusted pH-metrically and corrected for by use of the corresponding correction factors to be made to the glass electrode (González et al., 1992b). These correction factors for a number of water-organic cosolvent mixtures have been reported in the literature: for example, Douhéret (1967), aqueous mixtures of dioxane, methanol, ethanol, and acetone; Dohuéret (1968), aqueous mixtures of dimethylsulphoxide, acetonitrile, *N,N*-dimethylformamide and tetrahydrofuran; González and Pablos (1991), dioxane-water mixtures in the

whole pH range; Asuero et al. (1993), aqueous mixtures of aliphatic amides.

Once the mobile phase is prepared, it must be filtered trough a 0.4 μm solvent filtration apparatus, and then degassed by means of a flow of He or by using an ultrasonic bath.

Sample solutions of 500 mg/l or as appropriate to obtain suitable chromatographic peaks are prepared in the corresponding mobile phase and then filtered through a 0.4 μm disposable syringe filter unit.

Instrumentation: The minimal instrumentation needed consists of (i) an isocratic pump, (ii) a Rheodyne type injector with loops of 10–25 μl, (iii) an ultraviolet-visible detector (of variable wavelength, if possible) and (iv) a suitable recorder for processing the chromatogram (a straightforward integrator like the HP 3394A model is recommended).

Columns: Owing to the disadvantages of using silica bonded materials as stationary phases (González et al., 1992a), non-ionic copolymer stationary phases are suitable materials. Some recommended columns are: (i) RSpak D Series (Waters) – RSpak columns are packed with porous polymeric particles that are stable over a pH range of 2–12 (see the Waters Chromatography Columns and Supplies Catalog, 1991); (ii) PRP-1 (Hamilton) – The stationary phase is a macroporous polymer which is stable over a wide range of pH (1–13) (Li et al., 1991); (iii) Hypercarb pH (Hypercarb) – The packing material consists of spherical microparticles of graphited coal. This material shows extraordinary features of mechanical resistance and is stable over the whole pH range.

The column must operate at constant temperature. Although there are several models of column-thermostats available, a 'low-cost' home-made water jacket built from a suitable distillation condenser which permits the column to pass through it in assembly with a simple circulator thermostat may be useful for our purposes as well as being inexpensive.

Operational conditions: Typical flow rates are within 1–2 ml/min. For the sake of comparison with tabulated values, the ionization constant should be obtained at 25°C. Thus, the column may operate at 25 ± 1°C. Throughout the experiment the mobile phase should be subjected to a slight flow of He to prevent gas bubbles.

The sample solutions at different pH are injected (ensuring that the loop is filled) and the chromatogram recorded. Retention times are taken from the recorder report. Triplicate measurements should be performed in order to determine realistic values for the standard deviations of pK_a values.

Calculation of acidity constants: The theory needed for evaluating the acidity constant K_a^* (in the standard state of the aqueous organic solvent) of a monoprotic acid solute HA is given in a previous paper (González et al., 1992a). The fundamental formula is

$$K_a^* = H\frac{(t_{HA} - t)}{(t - t_A)} \tag{1}$$

where t_{HA} and t_A are the limiting retention times when the pH* (the pH corrected in the mobile phase considered) is either low enough or high enough to ensure that the solute species is the pure acid HA or the pure conjugated base A, respectively, and H is

$$H = 10^{-pH^*}\frac{f_A^*}{f_{HA^*}} \tag{2}$$

f^* being the activity coefficient of the corresponding species (Glab and Hulanicki, 1981).

It is advisable to deal with capacity factors rather than retention times. When the hold-up time of the column, t_0, is known (from an unretained solute), the capacity factor is readily obtained from the retention time, $k = (t - t_0)/t_0$. In RP-HPLC, a typical unretained solute is sodium nitrite (Palalikit and Block, 1980). Thus, replacing retention times by capacity factors, Eqn 1 becomes

$$pK_a^* = -\log H - \log\frac{k_{HA} - k}{k - k_A} \tag{3}$$

So, for each experimental point H_i, k_i, a value of $pK_{a\,i}^*$ is obtained. The final result is the averaged

value. The pK_a^* value may be also graphically evaluated, by plotting $\log(k_{HA}-k)/(k-k_A)$ vs– $\log H$ and taking pK_a^* as the value of $-\log H$ where the straight line intercepts the x-axis.

It should be noted that if the pK_a^* lies near to the extremes of the pH scale, the accuracy of the method will decrease owing to the error introduced by the pH measurement itself as well as the uncertainty associated with the evaluation of the limiting capacity factors. Accordingly, if either k_{AH} or k_A cannot be measured, several graphical techniques may be useful. These are outlined below:

When k_{HA} is unknown, K_a^* may be evaluated by using the following equations

$$k = k_{HA} + K_a^* \frac{k_A - k}{H} \tag{4}$$

$$\frac{1}{k-k_A} = \frac{1}{k_{HA}-k_A} + \frac{K_a^*}{(k_{HA}-k_A)H} \tag{5}$$

$$\frac{1}{k} = \frac{1}{k_{HA}} + \frac{K_a^*}{k_{HA}} \frac{k-k_A}{kH} \tag{6}$$

For graphical evaluation the ionization constant is evaluated from the intercept (Eqn 4) or from the ratio between the slope and intercept (Eqns 5 and 6). The standard deviation for the pK_a^* may be obtained by applying the law of propagation of the variance taking into consideration the variances of the slope and intercept as well as the mutual covariance (Asuero and González, 1989).

Similarly, when k_A is unknown, we have

$$k = k_A + \frac{1}{K_a^*} H(k_{HA} - k) \tag{4'}$$

$$\frac{1}{k-k_{HA}} = \frac{1}{k_A-k_{HA}} + \frac{H}{(k_A-k_{HA})K_a^*} \tag{5'}$$

$$\frac{1}{k} = \frac{1}{k_A} + \frac{1}{k_A K_a^*} \frac{(k-k_{HA})H}{k} \tag{6'}$$

For graphical evaluation the ionization constant is evaluated from the intercept (Eqn 4') or from the ratio between the intercept and slope (Eqns 5' and 6').

When both k_{HA} and k_A are unknown, the following procedure may be applied:

Assume a guess value for k_A (or k_{HA}) and according to Eqn 6 (or 6'), apply the regression technique. Because the model equation depends on the quantity $k_A - k$ (or $k - k_{HA}$), any error in k_A (or k_{HA}) will lead to curvature in the plot. Only the value of k_A (or k_{HA}) which is correct will yield a plot which is straight. The procedure consists of calculating the correlation coefficient for each trial value of k_A (or k_{HA}). The best value for k_A (or k_{HA}) is defined as that which gives the optimum correlation coefficient (very close to unity) which corresponds to a minimum value for the regression variance. Once it is attained, the slope and intercept of this best plot correspond to the true values of K_a^* (or $1/K_a^*$) and of k_{HA} (or k_A). This technique is based on a former similar procedure devised by Ramette (1967) for evaluating donor-acceptor equilibrium constants from absorbance data.

Extrapolation back to 0% organic cosolvent should be considered with caution. Only when the plot of pK_a^* against the mole fraction or volume fraction of the organic cosolvent is nearly linear in the 'rich water zone' would the extrapolation be carried out. The technique of extrapolation is always dangerous.

Chromatogram troubleshooting: We will consider here retention-time anomalies only. Dolan (1992) in his excellent paper on HPLC troubleshooting gives a general HPLC problem guide which would be of interest for the workers in this frame.

The study of reproducibility of retention times when using a given mobile phase at a fixed pH is carried out for by injection replication under fixed experimental conditions. Then one may be faced with some anomalies:

(i) Changes in the hold-up time, t_0: The problem is related to the flow rate (assuming no changes in the chromatographic system). Longer retention times can result from leaks or air bubbles in the pump. Shorter retention times occur when the flow rate setting is too high. Then reset the pump.

(ii) The retention time has changed but t_0 has not changed: If the composition in the mobile phase has not been modified, the change may be due to a variation in the column temperature.

Mobile phase changes tend to cause retention to drift in one direction. A common source for this variation is the buffer content. Buffer should be 20 mM to ensure reproducible results. In addition, there is a typical error during the preparation of mobile phases, consisting of adding a volume of one solvent to a volumetric flask and then to fill the flask to the mark with the another component. This procedure is error-prone because the solvent mixture is generally non-ideal and is subject to change in volume. On the other hand, for measuring the pH of the mobile phase, the glass electrode should be previously soaked in a solvent mixture of the same composition as the mobile phase (except buffer). The pH is recorded after the stabilization has been reached (in organic cosolvents stability may be attained after some minutes).

(iii) Temperature variations: Retention times decrease 1–2% per 1°C increase. The column as was indicated above must be thermostated, and the transfer lines between both the injector and column and the column and detector should be insulated.

(iv) Broad peaks: Check for sample overload by diluting the sample by a factor of 10 and reinjecting. A sharper peak and perhaps a shift to somewhat retention time comfirms this. Apart from this source of broadening, when the substance is, by nature, considerably retained, the elution time for a species is so long that the peak is too broad to accurately measure the retention time. In this case, partial troubleshooting consists of using a shorter column, but the column capacity becomes a problem. The best way of improving the evaluation of the acidity constant is to use a mobile phase more rich in organic cosolvent.

Prediction of drug retention: The retention characteristics of many drugs at a given pH when a mixed solvent is used as mobile phase can be predicted on the basis of their pK_a value and chemical structure. This may be extended for predicting retention data of potential drug metabolites through use of the functional group contribution (FGC) approach (Law, 1990). The basis of this theory is that a given chemical change in a molecule will produce a characteristic change in chromatographic retention regardless the structure of the whole molecule. For each metabolic transformation a FGC value τ is obtained from

$$\tau = \log \frac{k_m}{k_p} \tag{7}$$

where k_p and k_m are the capacity factors of the parent compound and the metabolite, respectively. As Law (1990) pointed out, in certain instances, the FGC approach fails. However, it is a useful and simple approach which can be used as an aid to drug metabolism, allowing tentative identification of drug metabolites. Its application requires nothing more than the ability to calculate capacity factors. It is particularly useful in the early stages of drug development, where a full investigation of metabolism is impractical or unwarranted.

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