Effect of temperature on the chromatographic retention of ionizable compounds. III. Modelling retention of pharmaceuticals as a function of eluent pH and column temperature in reversed-phase liquid chromatography

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ABSTRACT

We propose a general simple equation for accurately predicting the retention factors of ionizable compounds upon simultaneous changes in mobile phase pH and column temperature at a given hydroorganic solvent composition. Only four independent experiments provide the input data: retention factors measured in two pH buffered mobile phases at extreme acidic and basic pH values (e.g. at least ±2 pH units far from the analyte pKa) and at two column temperatures. The equations, derived from the basic thermodynamics of the acid-base equilibria, additionally require the knowledge of the solute pKa and enthalpies of acid-base dissociation of both, the solute and the buffer components in the hydroorganic solvent mixture. The performance of the predictive model is corroborated with the comparison between theoretical and experimental retention factors of several weak acids and bases of important pharmacological activity, in mobile phases containing different buffer solutions prepared in 25% (w/w) acetonitrile in water and at several temperatures.

Keywords

Column temperature
Ionizable solutes
Predictive model
Profens, β-blockers, alkaloids

Introduction

Ionizable compounds are very frequent analytes in the biochemical, biomedical, pharmaceutical, and environmental analytical fields, and reversed-phase high-performance liquid chromatography (RP-HPLC) is the method most frequently applied to separate and analyze this type of compounds. The technique requires the selection of a hydrophobic stationary phase, an organic modifier to control solvent strength and, finally, it is highly recommended the addition of buffers in order to fix the pH of the mobile phase. Even the use of additives such as ion-pairing reagents is sometimes needed to enhance retention of the analytes at a pH in which they are ionized. Much less attention has received the column temperature as optimization tool. This can be attributed to the combination of several theoretical and practical reasons. First, it has been early established that the solvent strength has a stronger effect than temperature on solute retention and both effects may be compensated. For instance, Bowermaster and McNair reported that about 1% increase in methanol concentration has a similar effect on retention than a 4ºC increase in temperature [1] whereas Chen and Horváth found that an 1% increase in acetonitrile concentration and a 5ºC change in column temperature have also similar effect on retention [2]. A different situation occurs with separation of large molecules, such as peptides and proteins, where the simultaneous control of gradient strength and temperature has been considered a powerful tool to modulate both retention and selectivity [3,4]. Second, the thermal differences between the incoming mobile phase and the column operated at high temperature cause serious peak broadening [5]. This loss in efficiency, which depends on the thermal mismatch, mobile-phase flow rate and viscosity, and column diameter, can be controlled by heating the incoming eluent temperature and by using narrower columns [5,6].

Practical considerations have also prevented the development of methods at higher temperatures. Some years ago column manufacturers advised on the (narrow) constraints for both eluent pH and column temperature in order to keep the column integrity. Also, the fear of solute degradation into the column at elevated temperatures with the concomitant distortion in the elution profiles has disregarded its use. Finally, some older HPLC instruments have not incorporated column heating capabilities. Today, practically all these impediments have been overcome. Recent development of new temperature stable reversed phase LC materials has resulted in commercially available columns which allow safe operation at higher temperature. On the other hand, Thompson and Carr have proposed criteria for determining those analytes which are thermally unstable during the column residence time and established that for all other solutes, the chromatographic analysis method at high temperatures is as reliable as that at room temperature [7]. Finally, nowadays column heating and mobile phase preheater devices are often used in order to obtain reproducible retention results.

From a theoretical point of view, an adequate theoretical description of the retention process leads to a successful prediction of chromatographic retention and selectivity which, ultimately, leads to a

simplified interpretation of the effect of all relevant parameters over the analytical method. Whereas the thermodynamics of phase equilibria involved in the retention of neutral molecules in RP-HPLC has been widely studied [8-12], the same is not totally true for ionizable analytes.

The aim of this study consists in the critical evaluation of the equations derived from thermodynamic considerations and proposed for the prediction of retention of compounds with acid-base properties in RPLC at any eluent pH and column temperature within a given range [13]. The proposed model conceives the own acid-base properties of the compounds used to prepare the buffer solutions, i.e. their pKa and dissociation enthalpies, in the resulting retentive behavior. This means that typical substances, widely used to prepare buffers, will lead to different retention behavior when the column is heated. All these changes can be fully predicted within the context of the proposed model which considers a unique hydrophobic retention mechanism. The target compounds were chosen by taking into account the knowledge of their aqueous pKa and as well as due to the relevance of their pharmacological action. The selection includes some profens, β-blockers, and cinchona alkaloids.

Theoretical

The retention factor of a monoprotic acid-base compound in a reversed-phase chromatographic system can be described through a sigmoidal function of the eluent pH [14-18]:

$$k = \frac{k_{HA} + k_A.w}{1 + w} \qquad (1)$$

where $w = 10^{(pH - pK_{a(an)})}$. In this function, the limiting retention factors $k_{HA} = \varphi K_{HA}$ and $k_A = \varphi K_A$ correspond to the retention factors of the fully protonated (HA) and the dissociated (A) forms of the analyte, respectively, φ is the phase ratio, K_{HA} and K_A are the equilibrium constants for the transfer between mobile and stationary phase of the protonated and unprotonated forms of the solute, respectively. $K_{a(an)}$ represents the analyte dissociation constant. Previous works have demonstrated that can be misleading to extrapolate aqueous pH data to partially aqueous solutions, thus the ${}^{s}_{w}pH$ or ${}^{s}_{s}pH$ scales should be used in order to obtain reliable predictions between experimental retention factors and mobile phase pH [16,17,19]. In both scales, the pH is measured after mixing the aqueous buffer with the organic solvent, and the electrode system used for measurements can be calibrated either with aqueous buffer (${}^{s}_{w}pH$) or with buffers prepared in the same solvent composition as the mobile phase (${}^{s}_{s}pH$). The difference between both scales (δ-parameter) depends on the primary medium effect and the residual liquid junction potential of the used electrode, and it is a constant value for each mobile phase composition at a given temperature [20]. We shall use in this study the ${}^{s}_{s}pH$ scale and thus, ${}^{s}_{s}pK_{a(an)} (= -\log K_{a(an)})$ defines the analyte dissociation constant in the specific

solvent mixture. Equation (1) will strictly apply if the dominant retention mechanism for the analyte is due to hydrophobic and/or dispersive interactions. On the contrary, the experimental data would not be described by this equation whenever other interactions between solute and solid surface are also significant.

All the implicit equilibria in Equation (1) will be affected by temperature. This influence can be considered into the equations through the thermodynamic standard enthalpies associated with each equilibrium. Assuming that these enthalpies are constant within the experimental temperature range, the van´t Hoff expression applies for each of the involved equilibrium:

$$-R\frac{\partial \ln K}{\partial(1/T)} = \Delta H^{o} \qquad (2)$$

Then, by defined integration between a reference temperature T_r and a given T, the following expressions are obtained:

$$k_{HA}(T) = k_{HA}(T_r)\,exp\{(-\Delta_t H^{o}_{HA}/R)[T^{-1} - T_r^{-1}]\} \qquad (3)$$

$$k_{A}(T) = k_{A}(T_r)\,exp\{(-\Delta_t H^{o}_{A}/R)[T^{-1} - T_r^{-1}]\} \qquad (4)$$

$$K_{a(an)}(T) = K_{a(an)}(T_r)\,exp\{(-\Delta H^{o}_{a(an)}/R)[T^{-1} - T_r^{-1}]\} \qquad (5a)$$

$$K_{a(buff)}(T) = K_{a(buff)}(T_r)\,exp\{(-\Delta H^{o}_{a(buff)}/R)[T^{-1} - T_r^{-1}]\} \qquad (6a)$$

where $\Delta_t H^{o}_{HA}$ and $\Delta_t H^{o}_{A}$ represent the enthalpies of transfer for HA and A between mobile and stationary phase, respectively, $\Delta H^{o}_{a(an)}$ is the standard enthalpy for the dissociation of the analyte and, finally, $K_{a(buff)}$ represent the acidity constant of the substance used to prepare the buffer and $\Delta H^{o}_{a(buff)}$ corresponds to its enthalpy of dissociation. According with these expressions, the final dependence of retention factors (*k*) with temperature will be dictated by the relative weight that these four standard enthalpies can have.

By applying decimal logarithm to the last two equations:

$$pK_{a(an)}(T) = pK_{a(an)}(T_r) + (\Delta H^{o}_{a(an)}/2.303R)[T^{-1} - T_r^{-1}] \qquad (5b)$$

$$pK_{a(buff)}(T) = pK_{a(buff)}(T_r) + (\Delta H^{o}_{a(buff)}/2.303R)[T^{-1} - T_r^{-1}] \qquad (6b)$$

Now, considering that the buffer is relatively concentrated, the relationship between the pKa of the buffering compound and the pH of this buffer is given by:

$$pK_{a(buff)} = pH - log\left(\frac{m_B}{m_{HB}}\right) - log\left(\frac{\gamma_B}{\gamma_{HB}}\right) \qquad (7)$$

where m_B and m_{HB} are the molalities of the components of the conjugated pair and γ_B and γ_{HB} their corresponding activity coefficients. Since the second term on the right hand of Equation (7) is

expressed in molalities, it is independent of temperature and the changes of the last term with temperature can be assumed negligible. In that case, Equation (6b) can be transformed in terms of pH as follows:

$$pH(T) = pH(T_r) + (\Delta H^o_{a(buff)} / 2.303R)[T^{-1} - T_r^{-1}] \quad (8)$$

Now, by subtracting the expression (5b) from equation (8), the following expression is obtained:

$$pH(T) - pK_{a(an)}(T) = pH(T_r) - pK_{a(an)}(T_r) + (\Delta H^o_{a(buff)} - \Delta H^o_{a(an)})(2.303R)^{-1}[T^{-1} - T_r^{-1}] \quad (9)$$

The introduction of equations (3), (4) and (9) into Equation (1) gives a final expressions which explicitly contains all the temperature dependencies:

$$k(T) = \frac{k_{HA}(T_r)\Delta k_{HA} + k_A(T_r)\Delta k_A w(T_r)\Delta w}{1 + w(T_r)\Delta w} \quad (10)$$

where Δk_{HA} and Δk_A summarize the exponential terms involving the standard enthalpies of transfer of HA and A, respectively, and,

$$w(T_r) = 10^{(pH(T_r) - pK_a(T_r))} \quad (11)$$

$$\Delta w = 10^{(\Delta H^o_{a(buff)} - \Delta H^o_{a(an)})(2.303R)^{-1}[T^{-1} - T_r^{-1}]} \quad (12)$$

Equations (11) and (12), which contain the thermodynamic properties of the buffer ($^s_s pK_{a(buff)}$ and $\Delta H^o_{a(buff)}$), clearly indicate the role that the buffer would play depending on its chemical nature. It is well known the large differences between enthalpies of dissociation of amines respect to those of carboxylic acids, both in water [21] and also in hydroorganic solvent mixtures [20,22]. These differences indicate that an increase in column temperature would cause a shift in the mobile phase pH of different magnitude depending on the buffer type (i.e. prepared from an amine or from a carboxylic acid). The analytes, which also would change their $^s_s pK_{a(an)}$ in some degree, will modify the ratio between ionized and non-ionized forms (increasing or decreasing) and, as a consequence, their averaged retention factor.

Equation (10) has been tested in the prediction of retention of several simple molecules in buffered mobile phases prepared with a fixed composition of methanol and of acetonitrile [13]. In this study, we selected larger and more complex molecules, which also have very important pharmacological properties, to test the predictive reliability of the equation. The main assumptions made in its deduction were that a single hydrophobic mechanism dominates retention, the constancy of all the thermodynamic quantities (enthalpies and entropies of dissociation and of transfer) within the studied temperature interval, as well as the independence of phase ratio with temperature.

3. EXPERIMENTAL SECTION

3.1. Instrumentation

Chromatographic measurements were conducted with a Shimadzu LC-10A instrument, equipped with helium degasser, LC-10AD pump, Sil-10A autoinjector, SPD-M10A diode array detector and a Class LC10 Chemstation. A 150x4.6 mm i.d. octadecylsilica X-Terra® MS-C18 analytical column provided by Waters was used for all the determinations. It has been proved that the hybrid silica particles of this column have very low silanol activity within the pH range 3 to 11 [23-25]. Detection wavelengths were set at 254 nm for the analytes and 200 nm for detecting potassium bromide and potassium nitrate, which were used as dead volume marker. The incoming mobile phase was pre-heated into a small-bore diameter 20-cm stainless steel capillary tube. Both, column and capillary tube were immersed into a temperature controlled thermostatic bath. Temperature was measured by using a thermometer calibrated at ±0.1ºC.

pH measurements of mobile phase solutions were conducted with a Schott Blueline combined glass electrode, connected to a 702 SM Titrino pH-meter (Metrohm) with a precision of ±0.01 pH units.

3.2. Reagents

All chemicals used here were reagent grade or better. Acetonitrile (ACN) HPLC-grade 99.9% was purchased from Mallinckrodt (Mallinckrodt, Paris, KY). HPLC water was purified by a MilliQ® deionizing system (Simplicity 185, Millipore). All other chemicals used to prepare the buffer solutions were reagent grade and they were obtained from Fluka, Merck and Baker. Phosphoric acid (Merck, 85%), potassium dihydrogen phosphate (Merck p.a. >99.5%), disodium hydrogen phosphate (Merck, >99%), 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) (Baker z.a. >99.5%), hydrochloric acid (Merck, 25% in water), 1-aminobutane (Aldrich, >99.5%), glacial acetic acid (Merck p. a., 99-100%), sodium acetate anhydrous (Merck, >99%), piperazine (Fluka, >99%) were used to prepare the buffer solutions. Analytes were purchased to Fluka and to Sigma-Aldrich; solutions prepared in 25% (w/w) acetonitrile in water were filtered and separately injected into the chromatographic system.

3.3. Procedure

The mobile phase solutions were prepared by mixing the buffer components with a fixed acetonitrile composition of 25% (w/w) equivalent to 29.8% (v/v) at 25ºC. Even though analysts are used to the molar scale concentrations, in this study the temperature independent molal scale expressions for buffer compositions were used. ${}^{s}_{w}pH$ of the mobile phase was measured after calibration of the electrode system by using aqueous standard reference buffers; ${}^{s}_{s}pH$ has been obtained as

${}^{s}_{s}pH = {}^{s}_{w}pH$ - δ [26]. where δ represents the difference between both pH scales. Information about concentrations, ionic strengths, and the measured ${}^{s}_{w}pH$ at 25ºC are reported in Table 1.

All the chromatographic measurements were taken at flow-rate of 1 mL/min. Typically, the injection volume was 5 μL with a concentration of 0.1 mg/ml of the solute dissolved in 25% (w/w) acetonitrile. The mobile phase was flushed through the column at the corresponding experimental temperature for about one hour before injection. The hold-up time was measured with both potassium bromide and potassium nitrate. Solute retention factors, k_I, were calculated by taking into account the extracolumn contributions to retention. All the results are the average of triplicate injections.

4. RESULTS AND DISCUSSION

4.1. Solute ${}^{s}_{s}pK_a$ estimations

Most of the selected ionizable solutes are compounds with some pharmacological activity; they are carboxylic acids (including a few arylpropionic acids with antiinflammatory properties) and amines such as β-blockers and cinchona alkaloids. Their chemical structures along with their acid base properties are shown in Table 2. Their water acid-base dissociation constants at 25ºC were taken from the literature [27-33]. However, dissociation constants in water-organic mixtures can be quite different from those in pure water, and the number of known ${}^{s}_{s}pKa$ values is rather limited. In several previous studies, some of us have demonstrated that a linear relationship between ${}^{w}_{w}pKa$ and ${}^{s}_{s}pKa$ for families of compounds can be established with very good correlations for both methanol-water [34,35] and acetonitrile-water mixtures [36,37]:

$$ {}^{s}_{s}pKa = a_s \, {}^{w}_{w}pKa + b_s \qquad (13) $$

where the slope (a_s) and the intercept (b_s) are common for compounds belonging to a given chemical family and they depend on the considered solvent mixture. Therefore, from the ${}^{w}_{w}pKa$ value for a given compound, these equations provide a very good estimation of the ${}^{s}_{s}pKa$ value of that compound in any methanol or acetonitrile-water mixture, which are, by far, the most usual components of RPLC mobile phases. In this work, we take the coefficients a_s and b_s for 30% (v/v) acetonitrile in water from references [36] and [37] to estimate the ${}^{s}_{s}pKa$ values for all the studied analytes. Those estimations are also given in Table 2.

4.2. Chromatographic retention

The retention behavior of the group of selected ionogenic solutes on an octadecylsilice column thermostatized at temperatures from 20 to 60°C has been measured. The mobile phases consisted in six buffer solutions, all of them prepared in 25%(w/w) acetonitrile/water mixture.

The retention factors of these compounds measured with different mobile phase pHs controlled by six buffer solutions at five column temperatures were gathered in Table 3. For computing these retention factors, hold-up times were measured with two markers: potassium bromide and potassium nitrate in all buffer solutions. Slight discrepancies in the hold-up time measurements with these two markers were observed, which could not be attributed to the ionic strength differences between these six different mobile phases. This behavior has also been observed previously [38-40]. Due to these differences found in the holdup times measurements, the values obtained with all the mobile phases at a given temperature were averaged.

Most of the solutes, in buffers B1 and B6 yielded linear van't Hoff plots allowing the evaluation of the enthalpies of transfer, which are also presented in Table 3. We can see that for those mobile phases containing a buffer at extreme pH, where a single form of each solute is dominant, the slopes of ln k versus the reciprocal of temperature are positive, indicating an exothermic equilibrium for the transfer of the corresponding analyte from the eluent to the stationary phase. Most enthalpies are in the –10 to –15 $kJ.mol^{-1}$ range for the hydrophobic neutral form of the carboxylic acids (buffer B1 pH=2.5), β-blockers which bear a positive charge have enthalpies within the range -11 to -4 $kJ.mol^{-1}$ whereas for alkaloids (ions with charge +2) the enthalpies of transfer are lower than -3 $kJ.mol^{-1}$. For neutral bases in buffer B6 (butylamine/butylamine chloride pH=11.3) the transfers are also exothermic but the enthalpies are smaller than -12 $kJ.mol^{-1}$. A practically zero slope was obtained in a few cases, where analytes are poorly retained and the errors associated to the linear regression almost exceed the slope values. Similar heats of transfer have been reported for small neutral molecules in other reversed-phase chromatographic systems using non-buffered eluents [41].

When these solutes where analyzed with mobile phases buffered at intermediate pHs (buffers B2 to B5), these analytes would be partially dissociated depending on their corresponding dissociation constant. Under these conditions, some of them exhibited an *apparent* positive enthalpy of transfer, which indicates an effective increase in retention time as column temperature is increased (see retention of β-blokers and alkaloids in buffer B4).

Carboxylic acids have somewhat similar retention factors in mobile phases B2 and B3 at the lowest temperature, i. e., at the same eluent pH at which solute dissociation is partial. Differences are small if we consider the difficulties to measure hold-up times. However, an increase in retention of phenylsuccinic acid is observed when the column temperature is increased from 20 to 60ºC and this compound is run in mobile phase buffered with piperazine/hydrochloric acid buffer (B3), whereas the opposite behavior was observed in mobile phase buffered with acetic acid/sodium acetate (B2).

Although the other carboxylic acids are less retained within the column as temperature increases independently of the buffer chemical nature, their apparent enthalpies of transfer have very different absolute values. This is illustrated in Figure 1, which comparatively shows superposed chromatograms of these carboxylic acids in both buffer solutions at the two extreme temperatures. An important change in separation factor between profens is clearly observed depending on the selected buffer used in the mobile phase.

More impressive differences are exhibited by β-blockers (aminoalcohols) when their retentive behaviors in two eluents are compared. Phosphate buffer (buffer B4) and *tris* buffer (buffer B5) solutions were prepared at exactly the same pH at 20ºC. Figure 2 shows the chromatograms of these analytes obtained at two extreme temperatures: 20 and 60ºC and under the same chromatographic conditions, but the chemical nature of the buffer. It is clearly noted that retention increases as column temperature is changed from 20 to 60ºC only when the mobile phase pH was controlled with phosphate buffer (buffer B4) whereas decreases when *tris* substitutes phosphate buffer in mobile phase (buffer B5). Moreover, a very significant difference in retention time at 20ºC was observed for all these solutes run with these two eluents buffered at the same pH but with two different buffer compositions; being retention much higher in mobile phase B5 than in mobile phase B4. This quite unexpected observation would indicate that other interaction mechanism besides reversed phase retention operates in one of the two systems. As temperature is raised to 60ºC both eluents differs in 0.98 pH-unit at this higher temperature and the differences in retention factors of these solutes between both buffered mobile phases decrease significantly.

Cinchona alkaloids show a retentive behavior pattern similar to that of β-blockers in mobile phases B4 and B5. Retention keeps almost constant between 20 and 60ºC when using buffer phosphate but decreases significantly within the same temperature interval when the used buffer is *tris*/hydrochloric acid. When data obtained at 20ºC are compared, it is observed that retention factors in buffer B5 duplicate those obtained with buffer phosphate (B4). Retention times become practically the same for these alkaloids in both mobile phases at 60ºC when both eluents differs about one pH-unit at this higher temperature. These four alkaloids have an –OH group and a tertiary nitrogen atom attached to adjacent carbon atoms. The main difference between them lies in the methoxy-group on the aromatic moiety of quinine and quinidine; this makes these solutes more hydrophobic (and also more acidic) than cinchonine and cinchonidine. Both, hydrophobicity and smaller pKa's qualitatively explain the larger retention of quinine and quinidine as compared to cinchonine and cinchonidine at 20ºC.

The experimental results show that the retention factor of these amines significantly varies from one to another buffer (e.g., *tris* buffer compared to phosphate buffer) and, apparently, there is no correlation between the pH and the retention factor. The question that arises at this point is if it does exist an additional retentive mechanism in *tris* buffer respect to phosphate buffer for all these aminoalcohols

or, on the opposite, if the presence of phosphate in mobile phase induces less retention onto the column surface. At a first glance, it is noted that ionic strength of both solutions differs in more that one concentration order and it is widely known that the ionic strength of the solution has a critical impact on the adsorption behavior of ionic species on non-polar solid surfaces. However, in case that some hydrophobic effect can be attributed to the buffer B4, which is the more concentrated, retention of analytes should increase (and not decrease) respect to buffer B5. In order to go deep insight into this issue, theoretical retention of all these solutes was estimated from the limiting retention factors and their corresponding ${}^{s}_{s}pKa$ in the acetonitrile/water mixture over a pH range.

4.3. Modelling retention.

Equation (10) was tested in its predictive capability of the retention factors of all these solutes in buffered mobile phases upon changes in both eluent pH and column temperature. An increase in temperature will affect the terms defined as Δk_{HA}, Δk_A and Δw. According to the negative values of the enthalpies of transfer, $\Delta_t H^o_{HA}$ and $\Delta_t H^o_A$, shown in Table 3, both Δk_{HA} and Δk_A will be positive but less than one. On the other hand, Δw, which takes into account the influence that the buffer can have over retention as temperature changes, can be larger or smaller than unity depending on both $\Delta H^o_{a(buff)}$ and $\Delta H^o_{a(an)}$. If $\Delta w>1$, and the second term dominates the numerator of equation (10), an increase in retention with temperature is highly probable. Under the particular case that both dissociation enthalpies equals, Δw will be zero.

The limiting retention factors at two temperatures, 20 and 60ºC, were used to estimate $k_i(T_r)$and Δk_i for both forms of each analyte. Standard enthalpies of dissociation of buffer components in 25%(w/w) acetonitrile were previously measured by potentiometry [22]. Unfortunatly, data of dissociation enthalpies for these solutes in solvent mixtures or even in water are not available. Thus, these solute dissociation enthalpies were estimated by analogy with other known values of molecules with similar chemical acid base structures despite these values refer to aqueous solutions [21]. Thus, we used $\Delta H^o_{a(an)}$ = 0 for carboxylic acids, 50 kJ/mol for primary and secondary amines, 30kJ/mol for tertiary amines and 20kJ/mol for aromatic nitrogens.

Theoretical retention factors were calculated and plotted as a function of ${}^{s}_{s}pH$ at all the temperatures. The Figures 3 to 5 show some selected examples of theoretical sigmoidal curves and experimental retention factors of profens (Figure 3), β-blockers (Figure 4) and alkaloids (Figure 5) as a function of mobile phase ${}^{s}_{s}pH$ measured at 20, 40 and 60ºC. Very good agreement is observed between the theoretical curves with the experimental data points for profens. A slight shift between the curve and

data points at about pH 5 for suprofen, which could be attributed to the unaccuracy of its literature pKa value. Better predicted values are observed for ketoprofen and also for phenylsuccinic acid.

In Figure 4, the predicted and experimental points for three β-blockers at three temperatures are represented. The plots clearly indicate that the experimental points measured in *tris* buffer are well above those estimated by the theoretical model. Curves of retention factors versus ${}^{s}_{s}pH$ for the cinchona alkaloids are illustrated in Figure 5. The model applied to diprotic solutes requires retention factors at an intermediate pH (see Appendix), in which the ampholyte would be unequivocally predominant. The mobile phases used in this study did not necessarily satisfy this requisite so, for these alkaloids, the theoretical curve should be taken as approximate. Despite of this approximation, experimental retention factors in *tris* buffer are appreciably higher and the agreement is far away from the theoretical predictions.

In 1979, Melander et al. [42] coined the term of "retention modulus" to describe the ratio between retention factors obtained with two different buffers controlling exactly the same aqueous eluent pH. The discrepancies were attributed to ion-pairing complex formation between sample molecules and buffer species. In Table 4, we gathered the ratio between the retention factors of these basic analytes in buffer B5 respect to the expected values, theoretically estimated, at the same eluent pH and at all the studied temperatures. Two different trends can be noted: β-blockers, quinine and quinidine show an almost constant ratio between retention in buffer *tris* as compared with theoretical retention independently of the column temperature. Thus, it should be inferred that the additional retention mechanism depends on temperature in a similar way to that the reversed phase retention does. On the other hand, the estimated ratios for alkaloids cinchonine and cinchonidine decrease as temperature increases, suggesting that the additional interaction mechanism becomes negligible at temperatures above 50ºC.

This retentive behavior of bases when using buffer *tris* to control pH in both acetonitrile or methanol/water mixtures has not been observed in our previous studies [13]. It should be noted that in buffer B5 the aminoalcohols and alkaloids predominate as cations, thus ion-pair formation between solute and the anion (chloride) of the buffer components would be feasible. Roberts *et al*. [23] and Dai and Carr [43] studied the effect of anions with different solvation properties over retention of amines at pH below the analyte pKa, and established an order of influence of these anions over amine retention. However, the results reported by these two groups are in disagreement with respect to the effect induced by the anion chloride. This anion leads to an increase in retention factor of less than 30% as compared to retention in absence of chloride anion for the conditions studied by Roberts. Although significant, this increment in retention in presence of chloride ions is not enough to explain our up to 270% increments in retention factors (i. e. albuterol). Even more, a decrease in retention (or not effect at all) was observed by Dai and Carr for a group of amines evaluated at different chloride

anion concentrations. We thus concluded that ion-pair formation of cationic amines and chloride ions would not be the main cause of the observed retentions into the column when buffer B5 is used to control the eluent pH.

Another possible explanation for this additional retention can be inferred from the chemical structure of *tris* base, which has also an amino and –OH groups onto adjacent carbon atoms and thus, interactions with aminoalcohols through hydrogen bonds involving both groups would be feasible. Further systematic work is planned to elucidate the possible mechanisms involved in the retention of these compounds in other buffered mobile phases and other hydrophobic column types.

With the exception discussed above, the behavior of all analytes in any of the other buffer solutions is accurately modeled by Eqn. (10), as it could be appreciated from Figures 3 to 5. The residuals between predicted and experimental data were plotted against the experimental retention values at the five temperatures in Figure 6. In this plot, residuals corresponding to diprotic cinchona alkaloids were excluded due to the reason exposed above. The larger residuals correspond to suprofen and we suspect that, as mentioned, these differences are attributed to the solute pKa value obtained from the literature. As a whole, estimations obtained with equation (10) are quite accurate: the computed total error (ratio between sum of the squared residuals and squared retention values) was as low as 2.3%.

Conclusions

Retention of several ionizable compounds with pharmacological activity has been measured in acetonitrile/buffer mobile phases controlled by buffer substances of different chemical characteristics at five column temperatures. A very simple model capable to predict retention data as a function of both eluent pH and column temperature has been proposed and carefully tested with very good performance for most analytes. The model is based on a single hydrophobic retention mechanism for ionizable compounds under secondary equilibria conditions.

Our experimental results show that the retention factor significantly varies from one to another buffer (e.g., *tris* buffer compared to phosphate buffer). And, as it is expected, at mobile phase pH close to the pKa of the analyte and for buffer components which have an enthalpy of dissociation differing from that of the analyte, a special dependence of the retention on temperature would be conceived.

Finally, there is no correlation between the mobile phase pH and the retention factor of aminoalcohols (β-blockers and alkaloids) in *tris* buffer. Instead, the nature of the *tris* buffer mainly controls the retention of these analytes, based on another retention mechanism besides hydrophobicity.

Acknowledgements

Financial support from CONICET (PIP-5631) and Fundación Antorchas (Argentina), and from IFS (Sweden) is kindly acknowledged. We also thank the support of Spanish Government (Project CTQ2004-00633/BQU).

Appendix

The extension of the equations to diprotic compounds (generalized as H_2A) is as follows. The average retention factor can be written as:

$$k = \frac{k_{H_2A} + k_{HA}.w_1 + k_A w_1 w_2}{1 + w_1 w_2} \qquad \text{(A.1)}$$

where w_1 and w_2 are defined as $w_1 = 10^{(pH - pK_{a1(an)})}$ and $w_2 = 10^{(pH - pK_{a2(an)})}$, respectively, and k_{H2A}, k_{HA} and k_A represent the retention factors for the three pure forms of the analyte. Analogous to equation (3) and (4), these retention factors are related with their enthalpies of transfer ($\Delta_t H_i^o$) according to:

$$k_{H_2A}(T) = k_{H_2A}(T_r)\,exp\{(-\Delta_t H^o_{H_2A}/R)[T^{-1} - T_r^{-1}]\} \equiv k_{H_2A}(T_r)\,\Delta k_{H_2A} \qquad \text{(A.2)}$$

$$k_{HA}(T) = k_{HA}(T_r)\,exp\{(-\Delta_t H^o_{HA}/R)[T^{-1} - T_r^{-1}]\} \equiv k_{HA}(T_r)\,\Delta k_{HA} \qquad \text{(A.3)}$$

$$k_{A}(T) = k_{A}(T_r)\,exp\{(-\Delta_t H^o_{A}/R)[T^{-1} - T_r^{-1}]\} \equiv k_{A}(T_r)\,\Delta k_{A} \qquad \text{(A.4)}$$

Also, both solute dissociation constants would be affected by temperature according to the enthalpy of dissociation corresponding to the separation of each proton ($\Delta H^o_{a_i(an)}$). Thus,

$$pK_{a1(an)}(T) = pK_{a1(an)}(T_r) + (\Delta H^o_{a1(an)}/2.303R)[T^{-1} - T_r^{-1}] \qquad \text{(A.5)}$$

$$pK_{a2(an)}(T) = pK_{a2(an)}(T_r) + (\Delta H^o_{a2(an)}/2.303R)[T^{-1} - T_r^{-1}] \qquad \text{(A.6)}$$

By combining Eq. (8) with (A.5) and (A.6), the two following expressions are obtained:

$$pH(T) - pK_{a1(an)}(T) = pH(T_r) - pK_{a1(an)}(T_r) + (\Delta H^o_{a(buff)} - \Delta H^o_{a1(an)})(2.303R)^{-1}[T^{-1} - T_r^{-1}] \qquad \text{(A.7)}$$

$$pH(T) - pK_{a2(an)}(T) = pH(T_r) - pK_{a2(an)}(T_r) + (\Delta H^o_{a(buff)} - \Delta H^o_{a2(an)})(2.303R)^{-1}[T^{-1} - T_r^{-1}] \qquad \text{(A.8)}$$

By defining $w_1(T_r) = 10^{(pH(T_r) - pK_{a1}(T_r))}$ and $w_2(T_r) = 10^{(pH(T_r) - pK_a2(T_r))}$, and introducing expressions (A.7) and (A.8) into equation (A.1), the general equation (A.9) for diprotic compounds will be:

$$k(T) = \frac{k_{H_2A}(T_r)\Delta k_{H_2A} + k_{HA}(T_r)\Delta k_{HA} w_1(T_r)\Delta w_1 + k_A(T_r)\Delta k_A w_1(T_r)\, w_2(T_r)\,\Delta w_1\,\Delta w_2}{1 + w_1(T_r)\Delta w_1 + w_1(T_r)\, w_2(T_r)\,\Delta w_1\,\Delta w_2} \qquad \text{(A.9)}$$

where $\Delta w_i = 10^{(\Delta H^o_{a(buff)} - \Delta H^o_{a_i(an)})(2.303R)^{-1}[T^{-1} - T_r^{-1}]}$ (A.10)

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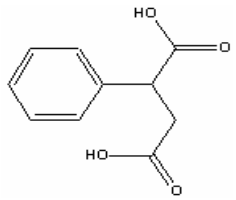
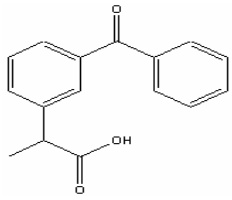
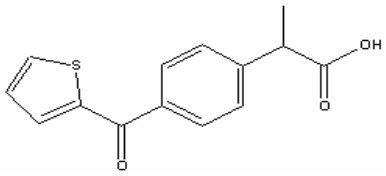
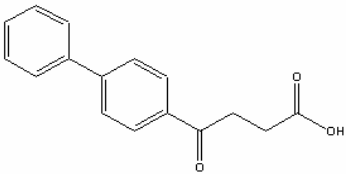
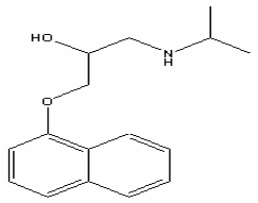
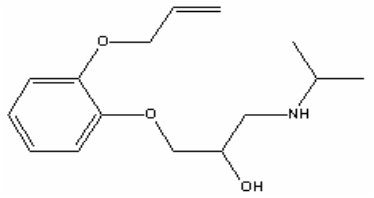
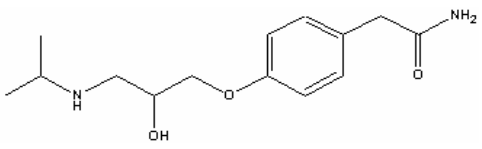
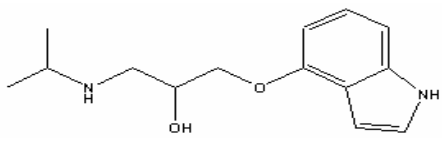
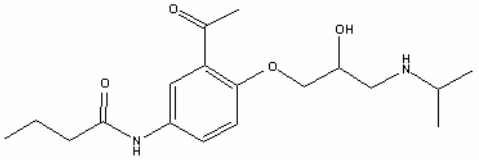
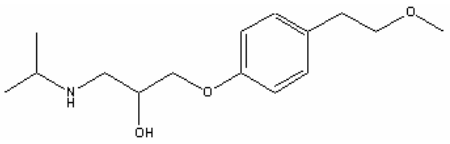
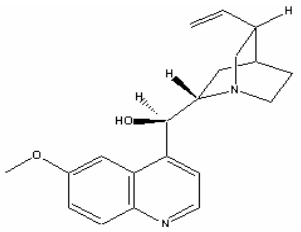
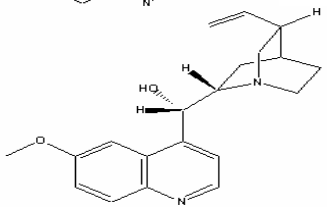
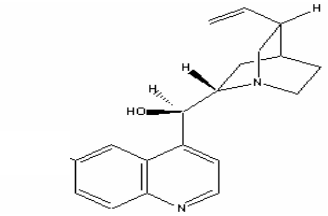
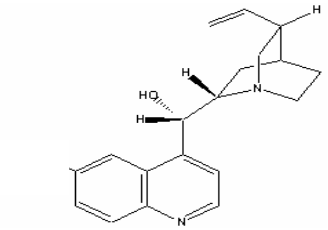
Table 1. Buffer solutions prepared in 25% *w/w* acetonitrile/water.

Buffer Solutions		Concentrations (mmolal)	Ionic strength (mmolal)	${}^{s}_{w}pH(25^{\circ}C)$
B1	H_3PO_4 KH_2PO_4	15.27 9.76	11.3	2.50
B2	Acetic Acid Sodium Acetate	17.5 7.76	7.8	4.88
B3	Piperazine + HCl	25 45.3	65	4.88
B4	KH_2PO_4 Na_2HPO_4	2.5 23.4	72.7	8.47
B5	tris-hydroxymethyl aminomethane, *Tris*+HCl	25 6.07	6.1	8.47
B6	n-butylamine + HCl	24.87 1.5	1.5	11.30

Table 2. Solute structures and acid base dissociation constants in water and in 30%(v/v) acetonitrile/water mixtures at 25ºC.

Solute	***Structure***	***Acidic group***	${}^{w}_{w}pK_a(25^{\circ}C)^{a}$	${}^{s}_{s}pK_a(25^{\circ}C)^{b}$
Phenylsuccinic acid (*PSC*)		-COOH	3.78 (pKa_1)	4.54
		-COOH	5.55 (pKa_2)	6.24
Ketoprofen (*KET*)		-COOH	4.40	5.23
Suprofen (*SUP*)		-COOH	3.91	4.68
Fenbufen (*FBF*)		-COOH	4.43	5.27
Propranolol (*PRO*)		$>NH_2^+$	9.49	9.20
Oxprenolol (*OXP*)		$>NH_2^+$	9.50	9.21
Atenolol (*ATE*)		$>NH_2^+$	9.55	9.26
Pindolol (*PIN*)		$>NH_2^+$	9.60	9.31

Acebutolol (*ABT*)		$>NH_2^+$	9.67	9.39
Metoprolol (*MTP*)		$>NH_2^+$	9.56	9.27
Quinine (*QUI*)		Quinolinic NH^+ $R_1R_2R_3NH^+$	4.33 (pKa1) 8.59 (pKa2)	3.46 8.27
Quinidine (*QDN*)		Quinolinic NH^+ $R_1R_2R_3NH^+$	4.21 (pKa1) 8.34 (pKa2)	3.34 8.01
Cinchonine (*CIN*)		Quinolinic NH^+ $R_1R_2R_3NH^+$	5.85 (pKa1) 9.92 (pKa2)	4.97 9.65
Cinchonidine (*CCN*)		Quinolinic NH^+ $R_1R_2R_3NH^+$	5.8 (pKa1) 10.03 (pKa2)	4.92 9.76

a: pKa values taken from references[27,29]
b: Estimated pKa in 30% acetonitrile (see the Results and Discussion)

Table3. Retention factors of solutes in six buffer solutions and at five temperatures.

Temp (°C)	**Solute**													
	PSC	*KET*	*SUP*	*FBF*	*PRO*	*OXP*	*ATE*	*PIN*	*ABT*	*MTP*	*QUI*	*QDN*	*CIN*	*CCN*
Buffer B1														
20	1.48	15.57	12.15	10.18	2.78	1.54	0.19	0.65	0.66	0.85	0.33	0.33	0.26	0.27
30	1.29	13.17	10.14	8.81	2.61	1.47	0.18	0.58	0.66	0.83	0.32	0.30	0.27	0.26
40	1.11	10.88	8.49	7.40	2.24	1.32	0.16	0.49	0.61	0.78	0.30	0.29	0.26	0.26
50	0.98	9.12	7.11	6.07	1.95	1.17	0.15	0.43	0.57	0.73	0.29	0.28	0.26	0.26
60	0.86	7.64	5.94	4.96	1.64	1.07	0.15	0.38	0.54	0.69	0.30	0.29	0.27	0.27
${}^{s}_{w}\Delta H$	-11.2	-14.5	-14.5	-14.7	-10.9	-7.7	-5.1	-11.1	-4.2	-4.6	-2.5	0.1	-2.7	0.4
s.d.	±0.1	±0.4	±0.3	±0.9	±1.1	±0.7	±0.3	±0.3	±0.6	±0.5	±0.6	±0.5	±0.6	±0.6
Buffer B2														
20	0.54	11.83	7.07	15.93	3.31	1.78	0.14	0.71	0.71	0.93	1.43	1.53	1.12	1.03
30	0.45	9.83	5.86	14.57	3.02	1.69	0.12	0.60	0.71	0.92	1.34	1.41	1.02	0.97
40	0.37	8.09	4.92	14.09	2.77	1.64	0.14	0.57	0.72	0.93	1.29	1.34	1.02	0.95
50	0.31	6.69	4.06	14.05	2.51	1.55	0.14	0.52	0.71	0.90	1.23	1.26	0.98	0.92
60	0.26	5.48	3.36	14.42	2.24	1.46	0.14	0.48	0.66	0.89	1.14	1.18	0.93	0.90
Buffer B3														
20	0.40	10.55	6.20	16.62	3.48	1.86	0.14	0.74	0.72	0.97	1.54	1.60	1.14	1.05
30	0.49	10.84	6.62	14.60	3.30	1.84	0.15	0.67	0.76	1.00	1.42	1.48	1.08	1.02
40	0.53	10.06	6.31	11.79	2.90	1.72	0.14	0.59	0.75	0.98	1.30	1.34	0.99	0.96
50	0.56	9.18	5.93	9.76	2.58	1.60	0.14	0.52	0.74	0.95	1.18	1.20	0.91	0.89
60	0.56	8.00	5.31	7.87	2.23	1.47	0.13	0.47	0.70	0.90	1.04	1.05	0.81	0.80
Buffer B4														
20	-0.02	0.92	0.66	-[a]	6.19	2.69	0.14	0.92	0.82	1.39	7.81	7.90	6.03	5.47
30	-0.02	0.92	0.65	-	6.92	3.09	0.17	1.01	1.00	1.68	8.48	8.72	6.66	5.97
40	-0.02	0.89	0.63	-	7.82	3.61	0.24	1.15	1.24	2.08	8.67	9.06	6.83	6.18
50	-0.03	0.82	0.57	-	8.89	4.26	0.30	1.34	1.52	2.59	8.48	9.00	6.83	6.16
60	-0.04	0.76	0.53	-	9.73	4.92	0.35	1.52	1.80	3.12	7.91	8.46	6.48	5.86
Buffer B5														
20	-0.19	0.42	0.35	-	-	-	0.58	2.49	2.31	3.52	14.14	15.26	12.83	10.52
30	-0.17	0.43	0.32	-	-	-	0.57	2.19	2.26	3.44	-	12.70	10.11	8.80
40	-	0.46	0.29	-	-	-	0.47	1.72	1.99	3.00	9.90	10.43	8.29	7.29

50	-0.18	0.45	0.28	-	-	-	0.47	1.61	2.01	2.97	8.54	8.87	6.95	6.34
60	-0.16	0.41	0.26	-	-	-	0.47	1.44	1.89	2.78	7.07	7.28	5.68	5.32
Buffer B6														
20	-0.11	0.25	0.17	-	6.04	11.50	0.92	4.48	3.37	6.20	15.75	17.60	12.05	10.01
30	-0.06	0.28	0.18	-	5.50	11.45	0.97	4.24	3.51	6.50	14.30	15.50	11.30	9.72
40	-0.11	0.27	0.18	-	4.88	10.94	0.98	3.85	3.58	6.51	12.74	14.53	10.31	8.92
50	-0.11	0.24	0.16	-	4.01	9.74	0.96	3.31	3.32	5.93	10.65	11.94	8.84	7.77
60	-0.11	0.23	0.15	-	3.43	8.86	0.98	2.96	3.13	5.47	9.05	10.00	7.66	6.83
${}^{s}_{w}\Delta H$	-	-2.2	-3.1		-11.7	-5.5	1.0	-8.7	-1.6	-2.7	-11.3	-9.3	-11.2	-8.0
s.d.	-	±1.7	±1.2		±1.1	±1.2	±0.5	±1.0	±1.3	±1.5	±1.1	±1.1	±1.3	±1.2

a: data not measured.

Table 4. Ratio between retention factor in buffer B5 and theoretical predicted value.

Solute	**Temperature (ºC)**				
	20	**30**	**40**	**50**	**60**
Atenolol	2.2	2.2	1.9	2.0	2.0
Pindolol	2.5	2.4	2.1	2.3	2.3
Acebutolol	2.7	2.5	2.3	2.5	2.5
Metoprolol	2.5	2.4	2.1	2.3	2.3
Quinine	1.5	1.6	1.5	1.8	1.8
Quinidine	1.2	1.1	1.1	1.3	1.3
Cinchonine	2.0	1.5	1.3	1.0	0.9
Cinchonidine	1.9	1.4	1.2	1.0	0.9

Figure captions

Figure 1. Influence of temperature and buffer on retention. Chromatograms of phenylsuccinic acid, ketoprofen and suprofen at 20 and 60ºC. Column: MS X-Terra (15 x 0.46 cm i.d.). Mobile phase: 25% (w/w) ACN/buffer. Flow-rate: 1 mL/min. Injection volume: 5 μL. Detection at 254 nm. For buffer compositions, see Table 1: Buffer B2: Acetate/acetic acid and buffer B3: piperazine/HC, both regulated at ${}^{s}_{w}pH(25^{\circ}C)$=4.88.

Figure 2. Chromatograms of β-blockers metoprolol, pindolol, atenolol and acebutolol at 20 and 60ºC. Mobile phase 25% (w/w) acetonitrile in buffers B4 and B5 (see Table 1). Other chromatographic conditions as in Figure 1.

Figure 3. Predicted and experimental retention factors of three carboxylic acids as a function of ${}^{s}_{s}pH$ at 20, 40 and 60ºC. Mobile phase: buffer solutions in 25% w/w acetonitrile/water mixture. Symbols: circles, phosphoric acid/dihydrogen phosphate; triangles down, acetic acid/sodium acetate; squares, piperazine/hydrochloric acid; diamonds, dihydrogen phosphate/disodium phosphate; triangles up, *tris*/*tris*:HCl; hexagons, butylamine/ HCl.

Figure 4. Predicted and experimental retention factors of four β-blockers as a function of ${}^{s}_{s}pH$ at 20, 40 and 60ºC. Mobile phase: buffer solutions in 25% w/w acetonitrile/water mixture. Symbols as in Figure 3.

Figure 5. Predicted and experimental retention factors of four alkaloids as a function of ${}^{s}_{s}pH$ at 20, 40 and 60ºC. Mobile phase: buffer solutions in 25% w/w acetonitrile/water mixture. Symbols as in Figure 3.

Figure 6. Residual plot. Differences between experimental and predicted *k*-values against experimental retention data.

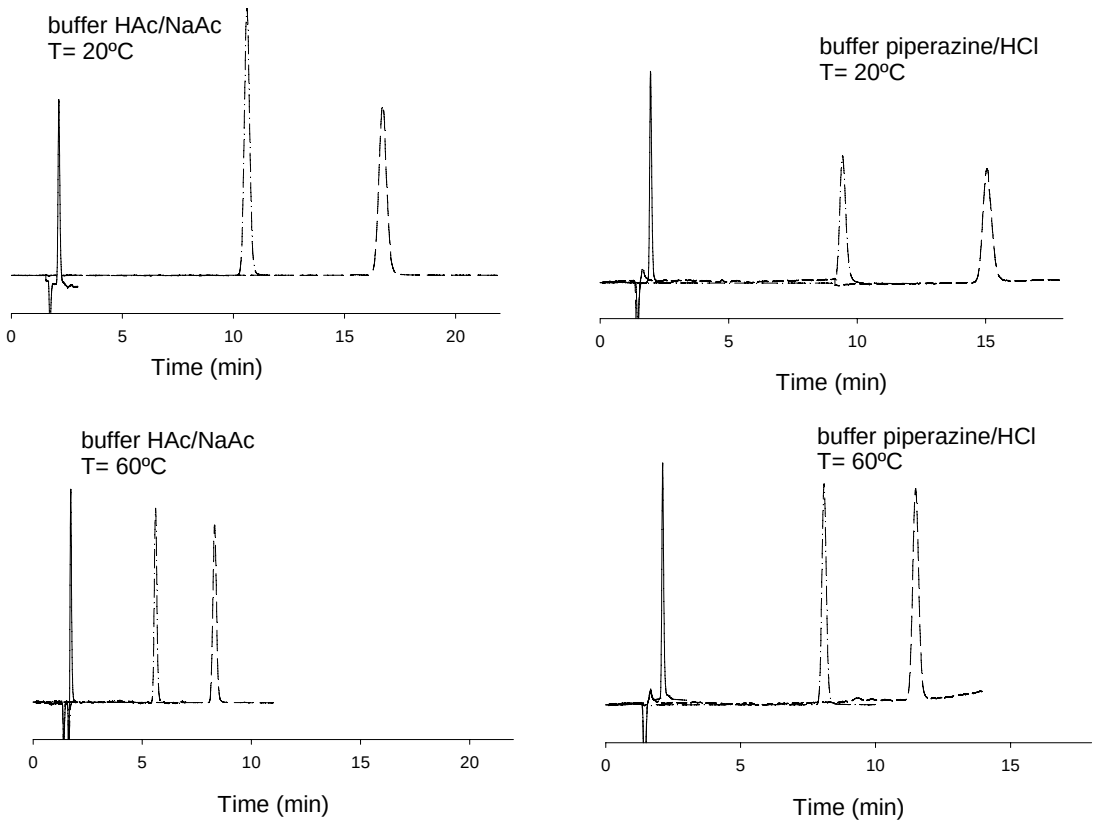

buffer HAc/NaAc
T= 20ºC
0
5
10
15
20
Time (min)
buffer piperazine/HCl
T= 20ºC
0
5
10
15
Time (min)
buffer HAc/NaAc
T= 60ºC
0
5
10
15
20
Time (min)
buffer piperazine/HCl
T= 60ºC
0
5
10
15
Time (min)

Figure 1

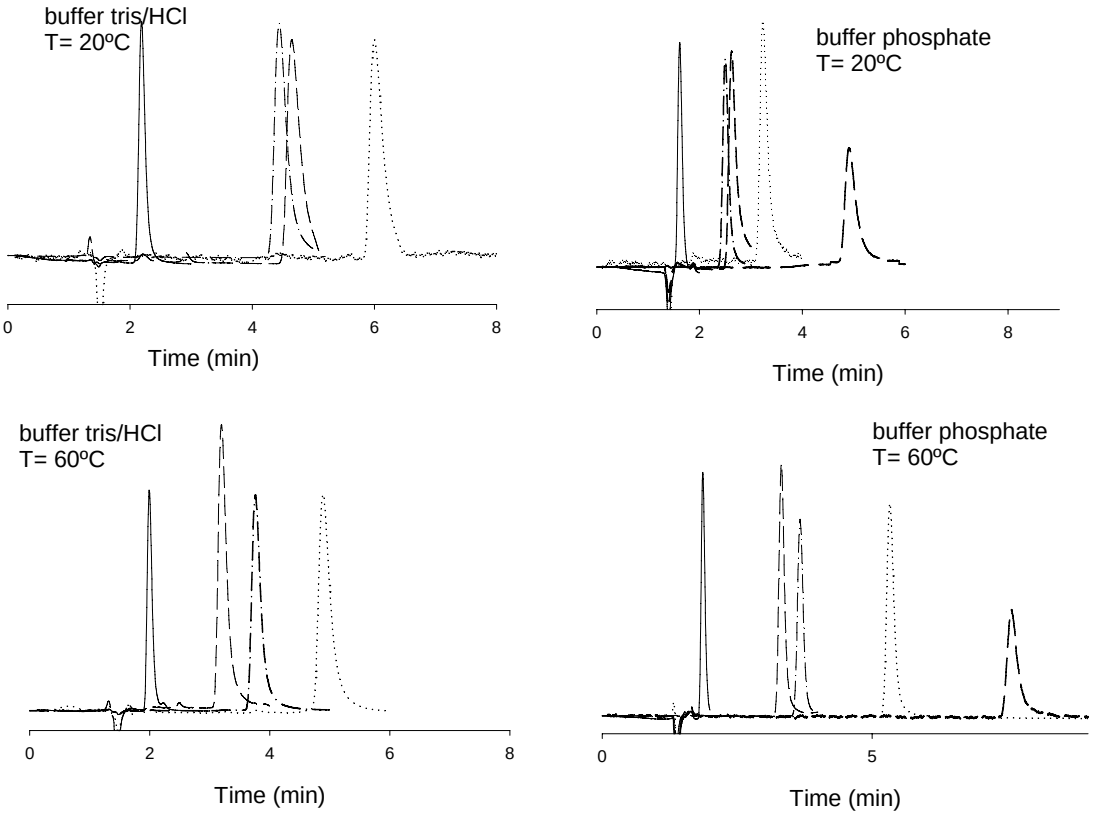

buffer tris/HCl
T= 20ºC
0
2
4
6
8
Time (min)
buffer phosphate
T= 20ºC
0
2
4
6
8
Time (min)
buffer tris/HCl
T= 60ºC
0
2
4
6
8
Time (min)
buffer phosphate
T= 60ºC
0
5
Time (min)

Figure 2

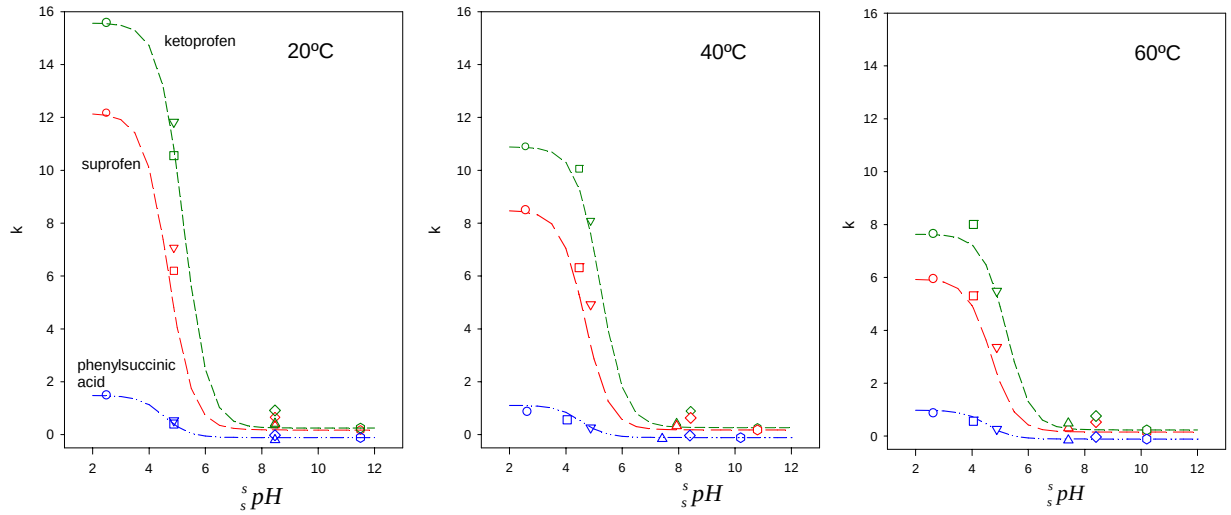

ketoprofen
suprofen
phenylsuccinic acid
20ºC
40ºC
60ºC
k
$_s^s pH$

Figure 3

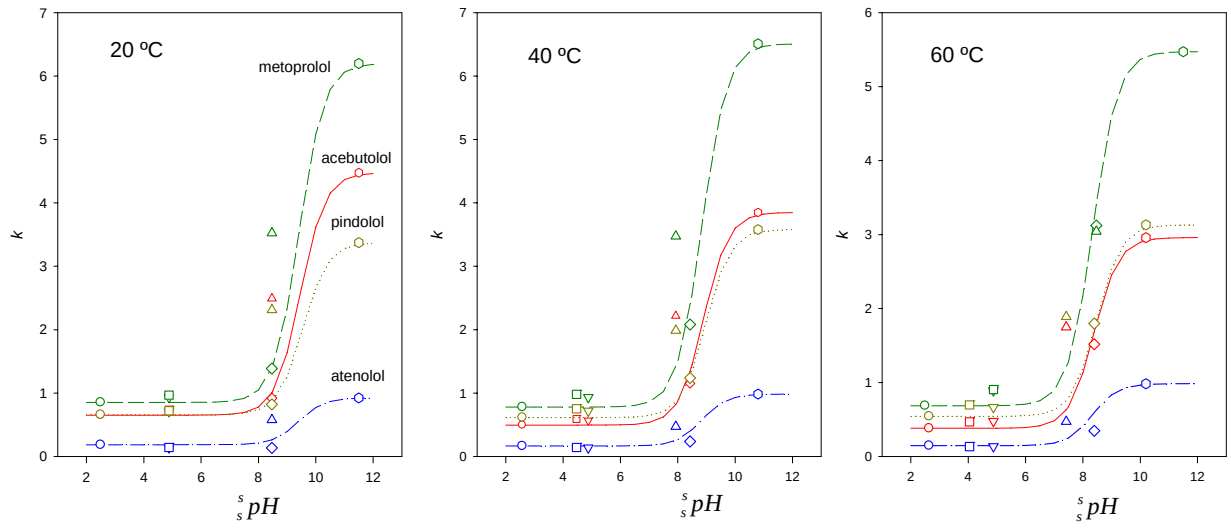

20 ºC
40 ºC
60 ºC
metoprolol
acebutolol
pindolol
atenolol
k
s
s pH

Figure 4

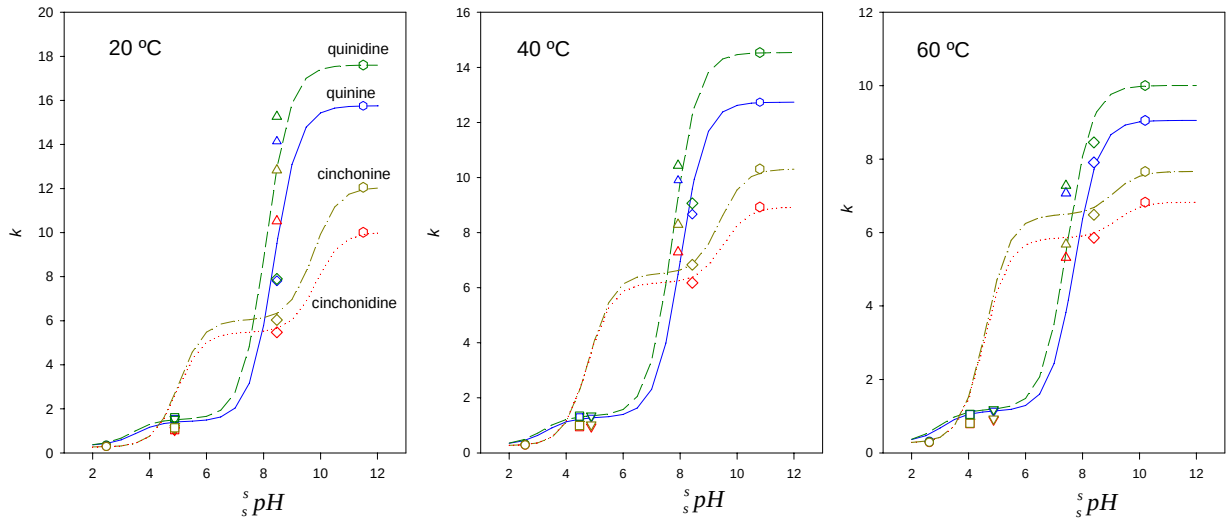

20 ºC
40 ºC
60 ºC
quinidine
quinine
cinchonine
cinchonidine
k
$_s^s pH$

Figure 5

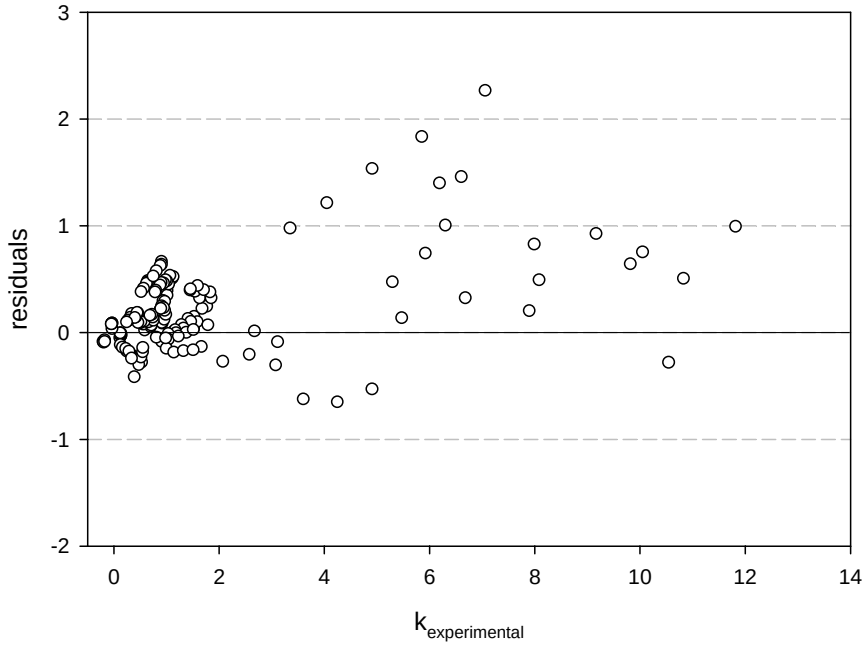


Figure 6