Journal of Chromatography, 209 (1981) 149–160
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,616

pH METHOD FOR THE INTERPRETATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC BEHAVIOUR WITH NON-AQUEOUS POLAR MOBILE PHASES

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(Received November 10th, 1980)

SUMMARY

A pH method for the interpretation of the chromatographic behaviour of ionogenic substances in both reversed-phase and ion-exchange high-performance liquid chromatography is proposed. The pH determination in a particular polar mobile phase is easily performed by means of a simple potentiometric procedure. Equations are derived concerning the retention of ionogenic substances in the light of the proposed pH approach. An experimental check with two different water–organic mobile phases showed good agreement between theory and experiment. The method allows one to predict the sequence of appearance of the components in a chromatographic separation and even to change this sequence.

INTRODUCTION

Until recently, the most commonly used chromatographic technique for the separation of ionogenic substances was ion-exchange liquid chromatography (IEC)[1]. Nowadays, together with this mode, reversed-phase liquid chromatography (RPLC) seems to be the most popular technique for the separation not only of ionogenic but also of a great number of substances having various properties[2–4]. In addition to other advantages, these two effective techniques use readily available and inexpensive polar mobile phases, mainly mixtures of water with organic solvents (methanol, acetonitrile, etc.). Compared with "normal-phase" chromatography, RPLC and IEC provide a further possibility for the separation of substances (mainly ionogenic) based on appropriate pH control of the mobile phase.

Based on theoretical considerations, equations are derived[3] that have been used successfully in predicting the chromatographic behaviour of a number of ionogenic substances (organic acids and bases) as a function of pH. It must be emphasized that the interpretation of the ionization effect in non-aqueous mobile phases is difficult because of many problems connected with the pH measurements in non-

0021-9673/81/0000–0000/$02.50

aqueous solvents. For both water–methanol and water–ethanol mixtures a number of pH standards have been developed and the pH measurement is performed in a manner similar to that in water. Another method that is not so reliable but is frequently used is to calibrate the glass electrode cell with an aqueous pH standard and then to use the δ correction[4], which expresses the effect of the medium and the liquid junction changes. Unfortunately, the data for δ values are as scarce as pH standards in non-aqueous solvents.

In this paper a new approach to the pH assessment of polar mobile phases in RPLC and IEC is proposed. The method is based on the simple assumption that the mobile phases in RPLC and IEC have high dielectric constants and amphiprotic behaviour, providing a fully dissociated strong acid (*e.g.*, hydrochloric or nitric acid). Once a solution is available with a known concentration of hydrogen ions, it is easy to calibrate a galvanic cell (glass and calomel electrodes) immersed in a particular medium. The pH function is defined in terms of concentration (not activity). At constant ionic strength this pH function has a full thermodynamic significance.

THEORETICAL

Standardization and determination of pH in non-aqueous polar mobile phases used in liquid chromatography

The idea of using fully dissociated strong acids for the calibration of a glass electrode cell at constant ionic strength is not new[5]. This method has been widely used with water[6] and has been extended to water–organic mixtures[7]. The same method was used in our previous acid–base investigations in non-aqueous solutions[8] and proved to give reliable results. This method is especially appropriate for the interpretation of acid–base equilibria in LC with polar mobile phases because it is universal, being applicable to various mixtures in the presence of buffers and electrolytes.

The mobile phases used in RPLC and IEC are mostly water–organic mixtures, which are considered as individual solvents. In other words, each water–organic mixture has its individual pH scale, not specifying what proportion of the protons are hydrated by water or solvated by the organic molecules of the solvent. In this sense, by the symbol SH one means[9] a molecule of the mixed medium having specific amphiprotic properties, *i.e.*

$$SH + SH \rightleftharpoons SH_2^+ + S^- \tag{1}$$

In the proposed procedure the pH function is defined by means of the so-called lyonium ion concentration, $[SH_2^+]$:

$$pc_H^* = pc_{zH_2^+} = -\log [SH_2^+] \tag{2}$$

The definition of this practical pH scale is realized through measurements of the potential of a cell consisting of a glass and a calomel reference (aqueous) electrode immersed in a water–organic mixture with a known hydrogen ion concentration at a constant ionic strength. The potential of this galvanic cell follows the Nernst equation:

$$E = E^{0'} - 59.16 \log [SH_2^+] \tag{3}$$

where $E^{0'}$ represents the specific constant of the particular cell, including the standard electrode potential of the glass electrode, the potential of the reference electrode, activity factors and the liquid junction potential. All of these quantities are considered to be constant.

With the precondition that the acid is fully dissociated (the lyonium ion concentration is known), $E^{0'}$ is calculated by means of eqn. 3. In fact, the method of cell calibration reduces to the determination of $E^{0'}$, because the pc_H^* value of every other solution (with the same water–organic composition and ionic strength) can be calculated from eqn. 3:

$$pc_H^* = \frac{E - E^{0'}}{59.16} \tag{4}$$

where E is the potential of the cell which contains the investigated solution.

For the determination of the pc_H^* of a water–organic mobile phase containing buffer components, a number of alternatives are proposed, which are discussed in detail under Experimental.

The pc_H^* approach in HPLC with polar mobile phases

Horváth *et al.*[3] derived equations describing the RPLC retention of ionogenic substances as a function of pH with pure water as the mobile phase. They obtained good agreement between theory and experiment, although the question of deriving activity factors from the pH measurement was not discussed. Van de Venne *et al.*[4] developed a similar model in water–methanol mobile phases and found good agreement between the derived equations for the retention of HX acids and the pH* function. They corrected their pH measurements with δ values, known for a number of mixed solvents[10], and considered the influence of activity factors in this mixed medium. Further, they found that the ion-pair mechanism plays a significant role in RPLC.

As will be shown below, when introducing the pc_H^* approach in the RPLC treatment, two essential points find a simple solution. The first is obvious and is connected with the universal method for the pH measurement, applicable to a particular polar solvent or mixture of solvents.

The second is more complicated and is connected with the influence of activity factors. As the pH measurements in both water and water–methanol solutions are mostly performed to give the activity of hydrogen ions and not concentration, often when dealing with equilibrium constants the so-called "mixed constants" are obtained. The use of such "mixed constants", especially when introduced into mass balance equations, leads to some undefined formulations. To overcome this difficulty a simple approach is proposed here, namely to use entirely the concentration scale, keeping the activity coefficients constant. This is simply achieved by keeping constant the ionic strength. In order to clarify this approach, a basic equation in RPLC will be re-derived in SH_2^+ terms, concerning the retention of an uncharged base B.

As shown schematically in Fig. 1, a number of equilibria should be considered in this system. Five equilibrium constants govern the distribution, which are thermodynamic in nature (as indicated by the superscript T). It can easily be shown, how-

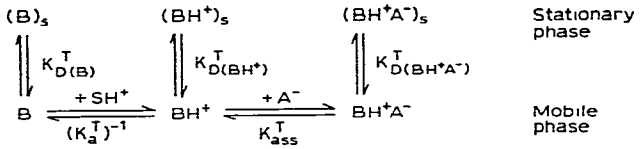

Fig. 1. Schematic representation of the equilibria in the RPLC of base B.

ever, that at constant ionic strength the activity quotient, Q, (Q is a ratio of activity coefficients; *e.g.*, $f_{B(s)}/f_B = Q_B$) is a constant in all instances and hence they should be defined in terms of concentration*:

$$\left.\begin{aligned} K_{D(B)} &= [B]_s/[B] \\ K_{D(BH^+)} &= [BH^+]_s/[BH^+] \\ K_{D(BH^+A^-)} &= [BH^+A^-]_s/[BH^+A^-] \\ K_a^* &= ([B]\,[SH_2^+])/[BH^+] \\ K_{ass} &= [BH^+A^-]/([BH^+]\,[A^-]) \end{aligned}\right\} \quad (5)$$

where K_D are the respective distribution constants**. The subscript s refers to the stationary phase and no subscript to the mobile phase; K_a^* is the acidity stoichiometric constant of B in the mixed medium and K_{ass} the association constant of the ion pair BH^+A^-, A^- being the anion from the electrolyte, supporting the ionic strength.

The overall distribution coefficient D_B is a property of the system that can be indirectly observed in a chromatographic process. It is the algebraic sum of the distribution coefficients of all distributing species:

$$D_B = D_{(B)} + D_{(BH^+)} + D_{(BH^+A^-)} = \frac{[B]_s + [BH^+]_s + [BH^+A^-]_s}{[B] + [BH^+] + [BH^+A^-]} \quad (6)$$

where

$$\begin{aligned} D_{(B)} &= [B]_s/([B] + [BH^+] + [BH^+A^-]) \\ D_{(BH^+)} &= [BH^+]_s/([B] + [BH^+] + [BH^+A^-]) \\ D_{(BH^+A^-)} &= [BH^+A^-]_s/([B] + [BH^+] + [BH^+A^-]) \end{aligned}$$

At this point, attention should be paid to the fact that eqn. 6 is in essence a mass balance equation. Thus, no terms other than concentrations should be present and no substitutions are allowed with terms from thermodynamic or mixed equilibrium constants (as performed by other authors).

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* Some objections could be raised as to whether the distribution constants $K_{D(B)}$ could be defined in terms of concentration. It is true that the activity coefficients in the non-polar stationary phase cannot be assessed easily, but when the column is not overloaded they obviously have constant values very near to unity. The activity coefficients in the polar mobile phase are kept constant, and the activity quotient is therefore also a constant.

** In order to treat the RPLC process in the light of solvophobic theory, Horváth *et al.*[3] defined the distribution constant $K_{D(B)}$ as an association constant of the solute with the octadecylic part of the stationary phase, the latter functioning as a ligand. In fact, the "concentration" of this "ligand" is in a great excess and is therefore, constant.

Eqn. 6 can be simplified by neglecting the formation of ion pairs in the polar mobile phase*:

$$D_B = \frac{[B]_s + [BH^+]_s + [BH^+A^-]_s}{[B] + [BH^+]} \tag{7}$$

After combining eqn. 7 with eqn. 5 and rearrangement, one obtains the relationship

$$D_B = \frac{K_{D(B)}}{1 + [SH_2^+]/K_a^*} + \frac{K_{D(BH^+)} + K_{D(BH^+A^-)} \cdot K_{ass} [A^-]}{1 + K_a^*/[SH_2^+]} \tag{8}$$

As the ionic strength is kept constant with a strong electrolyte, the concentration of A^- is also constant**. Eqn. 8 then reduces to

$$D_B = \frac{K_{D(B)}}{1 + [SH_2^+]/K_a^*} + \frac{K'_{D(BH^+)}}{1 + K_a^*/[SH_2^+]} \tag{9}$$

where $K'_{D(BH^+)} = K_{D(BH^+)} + K_{D(BH^+A^-)} \cdot K_{ass} [A^-]$ = constant. This constant expresses the distribution of BH^+ in the particular form present.

A basic chromatographic parameter connected with the distribution coefficients and distribution constants is the capacity factor, $k' = \varphi D = \varphi K_D$, where $\varphi = V_s/V_m$ is the volume ratio between the stationary and the mobile phases (considered as a constant in a particular column). Then, from eqn. 9 we obtain

$$k' = \frac{k'_0}{1 + [SH_2^+]/K_a^*} + \frac{k'_1}{1 + K_a^*/[SH_2^+]} \tag{10}$$

where $k' = \varphi D_B$, $k'_0 = \varphi K_{D(B)}$ and $k'_1 = \varphi K'_{D(BH^+)}$.

The equation obtained is identical with that derived by Horváth *et al.* (see ref. 3, eqn. 4c), but now all terms are redefined in the concentration scale in the mixed water–organic medium.

Similar equations could be derived for other ionogenic substances, but this seems to be unnecessary as the equations derived by Horváth *et al.*[3] may be used as a basis for analogous considerations.

The second useful application of the pc_H^* approach in high-performance LC (HPLC) concerns ion-exchange chromatography (IEC) with water–organic mobile phases. As stated by many authors, the process in IEC is too complicated and "the optimal conditions are selected by using certain empirical rules and a trial and error procedure"[3]. It seems, however, that when the activity factors and the pc_H^* values are under proper control, similar relationships as in RPLC are valid. Using analogous considerations as above, a relationship was derived for the IEC retention of B as a function of pc_H^*, which is identical with eqn. 10. Now, the capacity factors have

* This assumption is correct because according to Bjerrum's theory in solvents with $\varepsilon > 40$–50, ion-pair formation is negligible.

** The electrolyte is usually ten times more concentrated than the buffer, and therefore, the influence of the buffer anions can be neglected.

different values, k'_1 being much greater than k'_0. Now, the capacity factor of BH^+ is proportional to the selectivity ion-exchange constant, which has a constant value owing to the constant metal ion concentration. The ion-pair formation in this case is neglected in both phases. The capacity factor of B has the same physical meaning as in RPLC, but it is considerably lower.

EXPERIMENTAL

Apparatus

A Perkin-Elmer Model 601 liquid chromatograph with a Rheodyne Model 7105 syringe loading sample injector, a Perkin-Elmer Model LC-55 variable-wavelength detector and a Perkin-Elmer Model R-56 recorder were used. The detector was operated at 254 nm.

The potentiometric titrations were performed in a cell consisting of a glass and reference electrodes, thermostated at 25 ± 0.2°C. A Radiometer G 202 B glass electrode and a Radiometer K 401 calomel electrode were used. The potential of the cell was measured by means of a Radiometer PHM-52 digital pH meter with an accuracy of ±0.2 mV, equipped with an ABU 12 Autoburette (2.5 ml).

Columns

The RPLC experiments were carried out with Spherisorb-10 ODS (Perkin-Elmer, Norwalk, CT, U.S.A.) and IEC experiments with a Partisil PXS 1025 SCX strong cation-exchange column (Whatman, Clifton, NJ, U.S.A.), both with dimensions 25 × 0.46 cm I.D., at 25°C under isocratic elution conditions.

Reagents

Methanol (Reanal, Budapest, Hungary) and acetonitrile (Laborchemie, Apolda, G.D.R.) were used to prepare water–organic solvents. Potassium hydrogen phosphate (K_2HPO_4), sodium formate (HCOONa), sodium acetate ($CH_3COONa \cdot 3H_2O$) and potassium nitrate (KNO_3) were of analytical-reagent grade. Standard solutions of nitric acid were carefully standardized by means of a primary standard. The sample components are listed in Table II. Antipyrine, 4-aminoantipyrine and acetylsalicylic acid were of analytical-reagent grade and the pharmaceuticals dipyrone and aminopyrine (4-dimethylaminoantipyrine), of pharmacopoeial purity, were used without further purification. The other substances investigated were recrystallized.

Cell calibration and pc_H^* measurements

A 20.00-ml volume of a 0.005 *M* solution of the buffer basic component A^- (*e.g.* CH_3COO^-) in the appropriate water–organic mixture, containing 0.05 *M* KNO_3 to maintain a constant ionic strength, was titrated with a 0.05 *M* HNO_3 standard solution in the same non-aqueous solvent. The potential of the glass electrode cell was read and from the volume of HNO_3 added and E_{meas} (see Table I, columns 2 and 3), a Gran plot* (column 4) was constructed. The data after the equivalence point were

* The construction of the Gran plot is not an obligatory step if the potential break in the equivalence region is large enough. In such a case the titration should be performed in smaller steps in the vicinity of the equivalence point.

used for the cell calibration, *i.e.*, for the determination of the specific constant of the cell, $E_a^{0'}$ (see column 5, in Table I)*. Once this value is known, the pc_H^* value in every titration point can easily be calculated with eqn. 4 (see Table I, column 6). A further calculation, which is not a necessary step, is connected with the determination of the pK_a^* of the buffer-acting substance (see Table I, column 7). The latter gives valuable information concerning the buffer properties of the system, as the buffer capacity reaches a maximum at $pc_H^* = pK_a^*$ (the constancy of the pK_a^* value gives further valuable information*). After these calculations have been performed, it is an easy matter to prepare a buffer solution with a preliminarily chosen pc_H^* value. For instance, to obtain $pc_H^* = 5.091$ one has to add to 1 l of 0.005 *M* CH_3COONa 60 ml (1.20 × 50) of 0.04942 *M* HNO_3, as seen from point No. 5 in Table I. One can also use calculations based on the Henderson–Hasselbalch equation[11,13], $pc_H^* = pK_a^* + \log [A^-]/[HA]$, because from Gran plot one knows the exact concentration of $[A^-]$.

TABLE I

TITRATION OF SODIUM ACETATE IN ACETONITRILE–WATER (30:70) AND DETERMINATION OF E_a^0 AND pc_H^* VALUES

20.00 ml of 0.005 *M* $CH_3COONa \cdot 3H_2O$ titrated with 0.04942 *M* HNO_3, $\mu = 0.05$ (KNO_3); $t = 25 \pm 0.2$°C; $V_{eq} = 2.00$ ml.

Point No.	V_{HNO_3} *(ml added)*	E_{meas} *(mV)*	$10^4\psi$[a]	$E_a^{0'}$[b] *(mV)*	pc_H^*	$pK_a^* = pc_H^* + \log \frac{[HA]}{[A^-]}$
1	0.40	+12.0			5.860	5.26
2	0.60	+26.0			5.624	5.25
3	0.80	+37.5			5.429	5.25
4	1.00	+47.5			5.260	5.26
5	1.20	+57.5			5.091	5.26
6	1.40	+68.0			4.914	5.28
7	1.60	+81.0			4.694	5.28
8	1.80	+100.0			4.373	5.28
9	2.00	+128.0				$pK_a^* = 5.26 \pm 0.01$[c]
10	2.30	+170.5	1.70	+358.5	3.181	
11	2.50	+183.5	2.84	+358.6	2.961	
12	2.70	+192.0	3.99	+358.7	2.818	
13	3.00	+201.0	5.74	+358.8	2.666	
14	3.50	+210.5	8.50	+358.5	2.505	
15	4.00	+217.5	11.39	+358.6	2.387	
16	5.00	+227.0	17.18	+358.7	2.226	
17	6.00	+233.5	23.01	+358.9	2.116	
18	8.00	+242.0	34.50	+358.8	1.973	
19	10.00	+247.5	45.75	+358.7	1.880	
				$E_a^{0'} = +358.7 \pm 0.09$[c]		

[a] The function is calculated by means of $\psi = (V_0 + V) \cdot 10^{E/59}$ [16].

[b] The specific constant of the cell is calculated by means of $E_a^0 = E_{meas} - 59.16 \log (C_{HNO_3} - C_{A^-})$, where C_{HNO_3} is the total concentration of the strong acid added (HNO_3) and C_{A^-} is the total concentration of the basic component (CH_3COO^-) of the buffer pair HA/A^-.

[c] Confidence interval ($p = 0.95$).

* A reliable proof of the accuracy of the cell calibration as a whole is the constancy of the E_a^0 value, as is the case with the data in Table I. The constancy of $E_a^{0'}$ shows that (a) the glass electrode cell has an accurate pH response, (b) the acid of the titrant is fully dissociated in this medium and (c) the liquid junction potential and the activity factors are constant.

Another strongly recommended alternative which is simpler consists in the preliminary preparation of the buffer and then performance of the described titration. This is the same experiment as presented in Table I, but now one starts from point No. 4 onwards (or some other nearby point). This alternative is recommended for a periodical check of the buffer stock solutions, taking an aliquot and performing the titration already described. As the $E_a^{0\prime}$ value tends towards a constant value, the glass electrode cell can be used for approximate pc_H^* measurements. In this way buffer solutions can be prepared adjusting their pc_H^* values by means of KOH and HNO_3 solutions (preferably concentrated). Subsequently a titration as already described is recommended.

The pK_a^* values of the solutes investigated in the chromatographic procedures were determined with a procedure similar to that described above[12].

Chromatographic procedures

The 0.005 *M* solutions of buffer components K_2HPO_4 or HCOONa or $CH_3COONa \cdot 3H_2O$ were prepared in 50:50 methanol–water and 30:70 acetonitrile–water mixtures. The mobile phases were degassed ultrasonically.

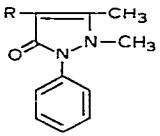
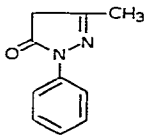
Retention times were measured from the distance between the injection point and the peak maximum on the chromatogram. The mobile phase hold-up times were measured by injecting a small amount of KNO_3 together with the sample components and the retention time of the frontal peak on the chromatogram was taken as t_0[2]. Capacity factors were calculated from the relationship $k' = (t_R/t_0) - 1$, where t_R is the sample retention time. The chromatographic conditions are stated in the figure captions.

RESULTS AND DISCUSSION

In order to demonstrate the applicability of the proposed pc_H^* approach in LC one has to verify the validity of eqn. 10 and related equations in terms of SH_2^+ concentration. For this reason, the substances listed in Table II were chosen as

TABLE II

SUBSTANCES INVESTIGATED

Formula	*Compound*	*R*
	Antipyrine	–H
	4-Aminoantipyrine	$-NH_2$
	4-Methylaminoantipyrine	$-N(CH_3)H$
	4-Dimethylaminoantipyrine	$-N(CH_3)_2$
	4-Nitrosoantipyrine	–N=O
	4-Disodiumsulphaminoantipyrine	$-N(Na)SO_3Na$
	Dipyrone	$-N(CH_3)CH_2SO_3Na$
	1-Phenyl-3-methyl-5-pyrazolone	
	Acetylsalicylic acid	

suitable models. These substances were chosen on the basis of their pK_a^* values, lying in the range 2–7, which are considered suitable for RPLC and IEC separations. When measuring the capacity factor (k') as a function of pc_H^* one has to obtain sigmoidal curves with an inflection point where $pc_H^* = pK_a^*$. This is why these substances were investigated as described above by two different techniques —chromatographic (for the determination of k' as a function of pc_H^*) and potentiometric (for the determination of pK_a^*). The investigations were performed in methanol–water (50:50) and acetonitrile–water (30:70) as mobile phase with various buffered solutions. The results from the RPLC mode are shown in Figs. 2 and 3, where the k' *vs.* pc_H^* relationships are presented.

As predicted by the theory, sigmoidal curves are obtained, from the inflections of which the pK_a^* values of the substances investigated are read. The latter values are given in Table III and are compared with the pK_a^* values obtained by the potentiometric determination. Fairly good agreement between the pK_a^* values obtained by the two methods is observed, which demonstrates the validity of the pc_H^* approach. It is

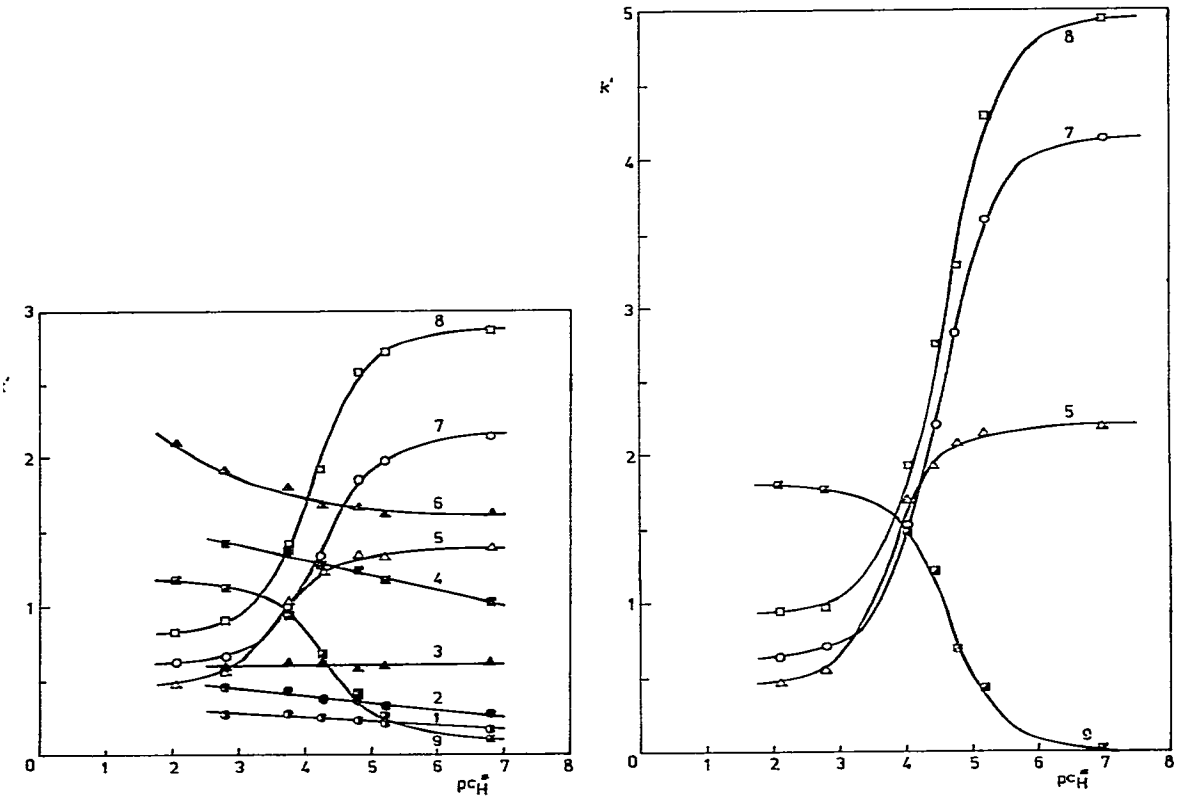


Fig. 2. RPLC: plot of (k') *vs.* pc_H^* of the eluent 0.05 *M* KNO_3 and 0.005 *M* buffers in methanol–water (50:50). Compounds: 1 = 4-disodiumsulphaminoantipyridine; 2 = dipyrone; 3 = 4-nitrosoantipyrine; 4 = 1-phenyl-3-methyl-5-pyrazolone; 5 = 4-aminoantipyrine; 6 = antipyrine; 7 = 4-methylaminoantipyrine; 8 = 4-dimethylaminoantipyrine; 9 = acetylsalicylic acid.

Fig. 3. RPLC: plot of k' *vs.* pc_H^* of the eluent, 0.05 *M* KNO_3 and 0.005 *M* buffers in acetonitrile–water (30:70). Compounds as in Fig. 2.

TABLE III

COMPARISON OF pK_a^* VALUES DETERMINED BY CHROMATOGRAPHY AND POTENTIOMETRY

Compound	*Methanol–water (50:50)*			*Acetonitrile–water (30:70)*	
	Potentiometry	*RPLC*	*IEC*	*Potentiometry*	*RPLC*
4-Aminoantipyrine	3.66	3.63	3.65	4.05	3.80
4-Methylaminoantipyrine	4.41	4.27	4.45	4.78	4.56
4-Dimethylaminoantipyrine	4.35	4.14	4.34	4.80	4.60
Acetylsalicylic acid	4.56	4.40	–	4.45	4.66

obvious that similar relationships could be obtained in other non-aqueous mobile phases.

Interesting results were obtained in the IEC mode, where even better agreement between theory and experiment was observed. As shown in Fig. 4, sigmoidal curves were obtained when k' was plotted against pc_H^* for a number of organic bases B. In agreement with theory, the reverse relationship is observed (*cf.*, Figs. 2 and 3), k_I' now being greater than k_0'. Note that above $pc_H^* = 5$ a noticeable retention is observed, which can be ascribed to a reversed-phase mechanism. Very good coincidence between the pK_a^* values determined by potentiometry and chromatography was observed, as can be seen from Table III. As far as we know, our results provide the first experimental confirmation of eqn. 10 in IEC. In our opinion, this is due to the explicitly defined pc_H^* value, introduced into LC in this investigation. It should be noted that other authors[3] have not obtained such a clear picture of the ion-exchange behaviour of ionogenic substances in HPLC. Additional investigations are desirable,

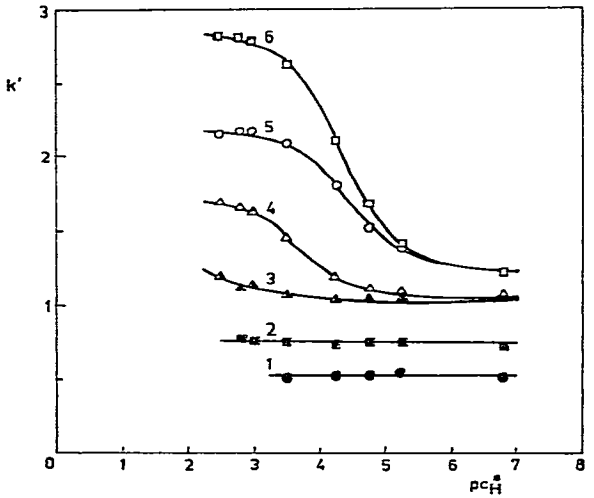


Fig. 4. IEC: plot of k' *vs.* pc_H^* of the eluent, 0.05 *M* KNO_3 and 0.005 *M* buffers in methanol–water (50:50). Compounds: 1 = dipyrone; 2 = 1-phenyl-3-methyl-5-pyrazolone; 3 = antipyrine; 4 = 4-aminoantipyrine; 5 = 4-methylaminoantipyrine; 6 = 4-dimethylaminoantipyrine.

including the re-derivation of some basic equations in the light of ion-exchange equilibria and the pc_H^* approach and more experimental data for anion-exchange.

It can be concluded that the pc_H^* approach in LC with polar mobile phases is a reliable means of interpreting equilibria in HPLC. When the pK_a^* value of a substance being investigated is known (*e.g.*, by potentiometric determination), only two experiments are needed in order to predict the chromatographic behaviour of this substance in a larger pc_H^* range. This includes the determination of k' in two buffered mobile phases having two pc_H^* values above and below the pK_a^* value. Then results like those presented in Figs. 2–4 are obtained and it is easy to choose conditions for the separation of various mixtures, predicting the sequence of appearance of the components in a chromatogram and, more interesting, permitting this sequence to be changed. To illustrate these possibilities, three chromatograms are shown in Figs. 5 and 6. Fig. 5a was obtained at $pc_H^* = 6.80$, this value being selected from Fig. 2. At this pc_H^* value the capacity factors of the eight investigated substances are different enough to provide a good separation (as confirmed in Fig. 5a). A change of the elution sequence for some pyrazolones at $pc_H^* = 3.80$ (also according to Fig. 2) is shown in Fig. 5b.

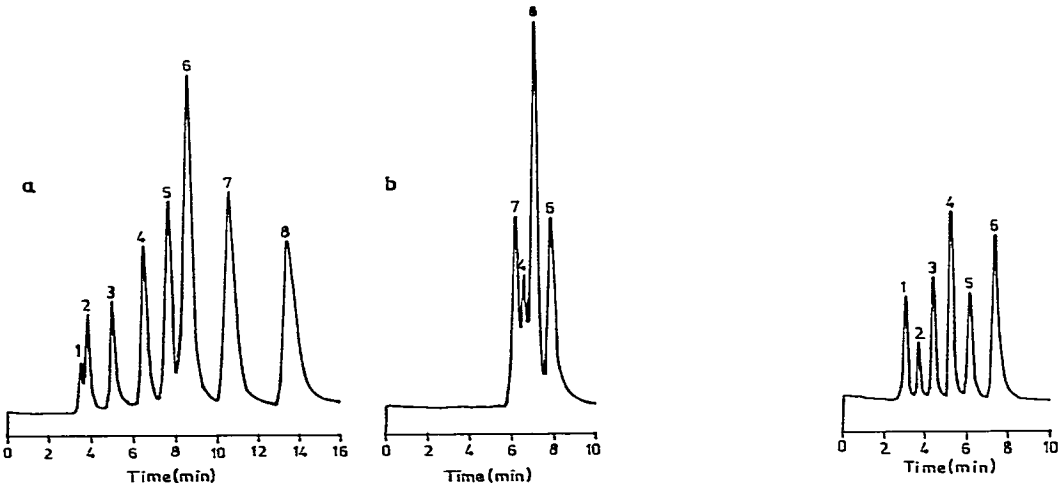


Fig. 5. RPLC: chromatograms of pyrazolone derivatives: 1 = 4-disodiumsulphaminoantipyrine; 2 = dipyrone; 3 = 4-nitrosoantipyrine; 4 = 1-phenyl-3-methyl-5-pyrazolone; 5 = 4-aminoantipyrine; 6 = antipyrine; 7 = 4-methylaminoantipyrine; 8 = 4-dimethylaminoantipyrine. Column, Spherisorb-10 ODS; eluent, 0.05 *M* KNO_3 and 0.005 *M* buffers in methanol–water (50:50); flow-rate, 1 ml/min; temperature, 25°C. (a) $pc_H^* = 6.80$ (phosphate buffer); (b) $pc_H^* = 3.80$ (formate buffer).

Fig. 6. IEC: chromatogram of pyrazolone derivatives: 1 = dipyrone; 2 = 1-phenyl-3-methyl-5-pyrazolone; 3 = antipyrine; 4 = 4-aminoantipyrine; 5 = 4-methylaminoantipyrine; 6 = 4-dimethylaminoantipyrine. Column, Partisil PXS 1025 SCX; eluent, 0.05 *M* KNO_3 and 0.005 *M* formate buffer in methanol–water (50:50), $pc_H^* = 3.50$; flow-rate, 1 ml/min; temperature, 25°C.

The separation of some ionogenic and non-ionic substances by IEC (strong cation-exchanger) will be illustrated. As follows from Fig. 4, separation of the components can be performed at any pc_H^* value up to 4, as confirmed by Fig. 6.

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