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Lipophilicity and pK_a estimates from gradient high-performance liquid chromatography

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Abstract

The linear-solvent strength (LSS) model of gradient elution has been applied to estimate parameters of lipophilicity and acidity of a series of drugs and model chemicals. Apparent pK_a values and log k_w values for individual analytes were determined in 2–3 gradient runs. The first experiment (or first two experiments) uses a wide-range organic modifier gradient with pH chosen for suppressed ionization of the analyte. The result of this experiment allows an estimate of contents of organic modifier of the mobile phase (%B) providing the required retention coefficient, *k*, for the non-ionized analyte. The following experiment is carried out with the latter %B and a pH-gradient of the aqueous component of the eluent that is sufficient to overlap the possible pK_a-value of the analyte. The initial pH of the buffer used to make the mobile phase is selected to insure that the analyte is in non-ionized form. The resulting retention time allows an estimate of pK_a in a solvent of the selected %B. At the same time, estimates of log k_w can also be obtained. The log k_w parameter obtained from gradient HPLC by the approach proposed correlated well with the corresponding value obtained by standard procedure of extrapolation of retention data determined in a series of isocratic measurements. Correlation between log k_w and the reference parameter of lipophilicity, log *P*, was very good for a series of test analytes and satisfactory for a structurally diverse series of drugs. The approach supported with specific detection procedures can be recommended for fast screening of lipophilicity of individual components of complex mixtures like those produced by combinatorial chemistry. The values of pK_a obtained in a study were found to correlate with the literature pK_a data determined in water for a set of aniline derivatives studied. In case of a series of drugs the correlation was less than moderate if the general procedure of pK_a determination was applied.

Keywords: Hydrophobicity; Lipophilicity; pK_a; Gradient elution

1. Introduction

The use of combinatorial synthesis procedures for the development of drug candidates typically results in the production of a very large number of target compounds, often as a multicomponent mixture. It is desirable to characterize such compounds in terms of lipophilicity (hydrophobicity) and the ionization constants (pK_a values) of acidic or basic groups within the molecule. This requires procedures for determining lipophilicity parameters and pK_a values that are rapid and can be used with very small samples. Especially suitable appear to be the procedures which would provide the data required

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without prior isolation of a given component of a mixture. Chromatographic procedures are unique in that respect, especially if combined with universal analyte detection and identification methods, like mass spectrometry.

The general methodology of determination of lipophilicity by reversed-phase liquid chromatography has been the subject of several reviews [1–9]. Usually, the lipophilicity parameters thus produced correlate well with the standard reference parameters of lipophilicity, i.e. the logarithms of *n*-octanol–water partition coefficient, log *P*, introduced to medicinal chemistry by Hansch and Fujita [10]. For quantitative comparisons of relative lipophilicities of a series of drug analytes the best suited are the intercepts of the linear relationships between logarithm of retention coefficients (log k or R_M) and volume fraction of organic modifier in binary aqueous eluents. However, to obtain such normalized chromatographic parameters of lipophilicity (log k_w, R_M^0) is quite tedious because several (6–8) chromatographic runs are needed at a range of isocratic eluent compositions.

To accelerate the procedures of lipophilicity characterization, gradient elution procedures have been proposed. Valko and co-workers [11–13] reported that compound lipophilicity, as measured by values of log *P*, can be estimated from a single reversed-phase gradient run. They define the *chromatographic hydrophobicity index*, CHI, for a given compound as obtained from a linear acetonitrile/buffer gradient run. Thus, if the retention time is t_R, they define CHI as:

$$\mathrm{CHI} = At_R + B \quad (1)$$

where A and B are constants determined from the similar chromatographic separation of suitable standards. Such obtained values of CHI correlated with values of log *P* ($R = 0.92$). Thus, a single gradient experiment can yield an estimate of log *P*. The observation was confirmed by Sandi et al. [14].

In 1997, another chromatographic lipophilicity parameter was introduced based on fast gradient elution. Krass et al. [15] proposed the parameter k_g defined as follows:

$$K_g = (V_g - V_d - V_m)/V_m \quad (2)$$

where V_g is gradient volume, V_d is the equipment dwell volume and V_m is the column dead volume. The authors reported good correlation of their k_g (not log k_g) with the standard log k_w obtained in a series of isocratic measurements.

As most of the drugs in use are weak acids or bases, their distribution within body compartments and overall activity will depend on both lipophilicity and the degree of dissociation. For quite a number of established drugs, the reference log *P* and pK_a data have been compiled [16,17]. Similarly to log *P*, there is a need to elaborate a fast and convenient procedure to evaluate also the pK_a parameters of drug candidates.

The ionization of an acid can be represented by:

$$\mathrm{HA} \Leftrightarrow \mathrm{H}^+ + \mathrm{A}^- \quad (3)$$

with the fraction of non-dissociated molecules HA given by:

$$f_0 = 1/\{(K_a/\mathrm{H}^+) + 1\} \quad (4)$$

If the fraction of dissociated molecules A^- is f_-, and if the retention factor for non-dissociated and dissociated molecules is k_0 and k_-, respectively, the value of k of partially dissociated analyte will be:

$$k = f_0 k_0 + f_- k_- \quad (5)$$

The dependence of k and f_0 on pH for a monoprotic acid with p$K_a = 5$ is illustrated in Fig. 1. The study of retention factor, k, as a function of pH can be used in this way to estimate a value of pK_a for the compound in question.

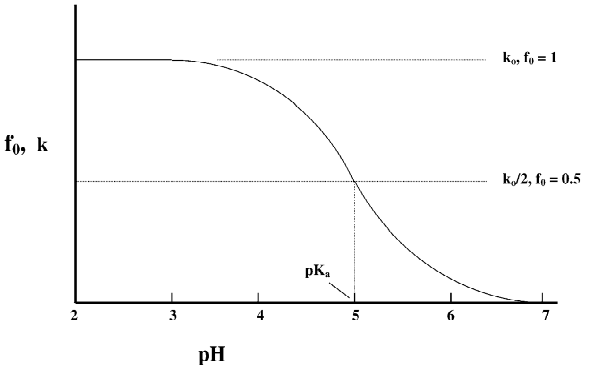

Fig. 1. Dependence of fraction f_0 of molecules of an acidic solute HA which are not ionized to A^- as a function of pH (p$K_a = 5$). Plot of f_0 and k versus pH.

Dependence of isocratic reversed-phase HPLC retention factor, k, on pH of the mobile phase has been the subject of numerous publications [18–23]. Moreover, some papers dealt with the problem in case of thin-layer chromatography [23,24]. Apart from a rigorous theoretical treatment, a few empirical modifications of Eq. (5) were proposed. The equation given by Hanai [25] has the form:

$$k = 0.5(k_0 - k_i) \tan h\,(pK_a - pH) + 0.5(k_0 + k_i) \quad (6)$$

where tan h is the tangent hyperbolic function. More recently, Hanai et al. [26] reported an approach to calculate pK_a values of phenolic and nitrogen-containing analytes based on reversed-phase HPLC retention data and Hammett's constants of substituents.

The equation for acids by van de Waterbeemd et al. [7] is as follows:

$$\log k_0 = \log k + [1 - \tan h\,(pK_a - pH + 1)] \quad (7)$$

Usually, a measurable retention of the sample requires a water–organic mobile phase. Then the value of pK_a found will apply to that mobile phase only. It is well known that values of pK_a obtained in this way (as suggested by Fig. 1) often vary with %B (for organic/water mixtures A/B). Unfortunately, all the approaches mentioned above assume the pH of the mobile phase to be the same as the pH of its aqueous fraction.

Some authors propose measuring of pH with potentiometric systems after mixing the organic and aqueous components and that procedure is recommended to obtain the correct pH value [27–30]. However, the potentiometric systems are normally calibrated with aqueous standards. Hence, the apparent pH of the mixture may be different from the actual one.

Having the above in mind, Bosch and co-workers [31–33] proposed procedures to calculate the true pH value and ionic strength of the methanol–buffer mobile phases. Those authors argue that dissociation of acids in methanol–water mixed solvents is governed by electrostatic interactions and specific analyte–solvent interactions (solvation effects). In the dissociation of acids, charges are formed. The process of dissociation is disturbed as the dielectric constant of the medium decreases with increasing content of methanol. In effect, the pK_a of a neutral or anionic acid increases if methanol concentration increases. For a cationic acid the decrease in pK_a by solvation by methanol–water is not balanced by the change in dielectric constant and the pK_a decreases with the formation of the methanol–water complex. Roses et al. [33,34] derived a rather complex equation predicting pK_a values of the acids most often used to prepare buffers for reversed-phase HPLC at different compositions of the methanol–water mobile phases. From the pK_a values and the buffer composition, the pH values are calculated for the buffer at given mobile phase composition. The dependence of retention time, t_R, for an acid HA on the true pH values in the mobile phase derived by those authors is:

$$t_R = [(t'_{R(HA)} + t_{O(HA)})y_{A^-}\, 10^{pK_a - pH} + (t'_{R(A^-)} + t'_{O(A^-)})][y_{A^-}\, 10^{pK_a - pH} + 1] \quad (8)$$

where $t'_{R(HA)}$ and $t'_{R(A^-)}$ are adjusted retention times of a neutral form of the acid and its anion, respectively; $t'_{O(HA)}$ and $t'_{O(A^-)}$ are the corresponding hold-up times; y_{A^-} is the activity coefficient of the anion and pK_a is actual acidity of the analyte in a given methanol–water mobile phase. Employing Eq. (8) one can calculate pK_a values of acid analytes. These values are increasing with increasing methanol content in mobile phase.

Lewis et al. [20] identified the main reasons for errors in predicted retention as a function of pH:

(i) interactions of analytes with exposed silanols or metal contaminants of stationary phase, whose pK_a or complexing constants can also depend on pH;
(ii) effect of ionic strength on K_a;
(iii) solvophobic effect of ionic strength on retention;
(iv) ion-pair interaction of sample ions with ionized buffer components;
(v) change in the microscopic nature and sorption properties of the hydrocarbonaceous silica as a result of changing ionization of silanols;
(vi) changes in buffer type, when more than one buffer is used to attain the required pH range.

Other authors mention additional complications, e.g. those caused by the presence of neutral, polybasic and/or amphoteric sample components [27,35]. One has to remember that the retention of neutral

forms of ionizable analytes also depends in some instances on pH, although this effect is much weaker than for ionizable species.

A pronounced effect of ionic strength of eluent on retention factors of anionic solutes chromatographed on silica-based hydrocarbonaceous stationary phases was reported by Knox et al. [36]. It appeared that ionized organic acids can be excluded from the pores of stationary phase available to the eluent. The anions are thus eluted earlier than the molecules of the mobile phase simultaneously introduced on the column. This gives negative values of the retention factor, *k*. The process of exclusion of acids becomes saturated at a higher ionic strength of the eluent (0.1–1 *M*).

Knox et al. [36] also demonstrated that exclusion of anionic analytes from a reversed-phase material depends in a complex manner on the composition of eluent. They described U-shaped plots of *k* against water content in water–ethanol eluents for three simple organic acids. Maximal exclusion occurred at a composition of about 50% water in mobile phase. With more than 80% water, benzoic acid and salicylic acid are retained, but sulfanilic acid is still excluded. Adding a cation to the mobile phase decreases exclusion of acids [36] and decreases the retention of basic analytes [37]. This effect also depends on the kind of the cation applied.

All these effects are attributed to the strong interactions between the analytes and the residual silica hydroxyls of stationary phase. To reduce the effects of free silanols, various practical methods have been proposed. Stadalius et al. [38] suggested using a pH between 2.5 and 3.5 with higher buffer concentrations, potassium salts instead of sodium, and the addition of amine modifiers such as triethylamine or dimethyloctylamine. Unfortunately, the approach is not fully effective.

2. Background of the gradient HPLC approach to determination of log k_w and pK_a

In reversed-phase HPLC, the general Snyder–Soczewinski equation relating retention coefficients, *k*, to volume percent of organic modifier in binary aqueous mobile phase, Φ, has the form:

$$\log k = \log k_w - S\Phi \quad (9)$$

where log k_w and *S* are regression coefficients. Specifically, log k_w is interpreted as the hypothetical retention parameter corresponding to neat water (buffer) eluent. Eq. (9) usually holds precisely for relatively small ranges of Φ. Hence, errors in the estimated values of log k_w can occur when extrapolations to $\Phi = 0$ are done [5]. The log k_w parameter is a standardized retention parameter that is considered to be more reliable than any arbitrarily selected isocratic log *k*. It is known, however, that log k_w depends on the nature of organic modifier of the binary aqueous eluents employed in reversed-phase HPLC [39].

Instead of extrapolating log k_w from a series of 6–8 isocratic experiments of fixed Φ, Snyder and co-workers [40] elaborated an approach allowing approximate evaluation of log k_w from a single gradient run and its precise calculation from two gradient runs. The appropriate equation derived by Snyder and co-workers [40] is:

$$t_R = (t_0/b) \log (2.3\, k_0 b + 1) + t_0 + t_D \quad (10)$$

where:

$$b = V_m \Delta\Phi S/(t_G F) \quad (11)$$

The symbols used in Eqs. (10) and (11) are: t_R, analyte retention time (min); t_0, column dead time (min); *b*, gradient steepness parameter; k_0, value of *k* corresponding to 0% of organic modifier in eluent; t_D, gradient delay time, equal to V_D/F; V_D, equipment hold-up or 'dwell' volume (ml); $\Delta\Phi$ change in Φ during the gradient; Φ, volume fraction of strong solvent B in the mobile phase, equal to %B/100; *S*, analyte parameter equal to $\mathrm{d}(\log k)/\mathrm{d}\Phi$; V_m, column dead volume (ml), equal to $t_0 F$; t_G, gradient time (min), i.e. time from beginning to end of gradient; *F*, flow-rate (ml/min).

Using Eq. (10) and assuming a typical value of $S = 4$, one can estimate log k_w from a single gradient run. Having t_R data from two gradient runs of different t_G one gets an exact value of log k_w by solving a set of two equations of the form of Eq. (10). That can be done automatically by chromatographic software like DryLab (LC Resources, Walnut Creek, CA, USA) used in this work.

The estimation of pK_a as in Fig. 1, using either

isocratic or gradient data, typically requires several experiments to define the plot of k versus pH. An alternative procedure proposed by Snyder and co-workers [41–43] is based on a single gradient run. It is assumed that a preceding gradient run(s) for the estimation of log k_w has already been carried out.

A typical plot of log k versus mobile phase pH (other conditions constant) for an acidic solute HA is shown in Fig. 2 (solid curve). The gradient begins at $pH = pH_0$, the retention of the non-ionized form of the solute is k_0, and it is assumed that the retention of the ionized form of the solute can be approximated by $k_i = 0$. The ionization of a single acidic group within the solute molecule is further assumed, at least for a pH-range of $(pK_a - 2)$ to $(pK_a + 2)$. This plot of log k versus pH can be approximated by a linear-solvent-strength (LSS) relationship as shown by the dashed line of Fig. 1 [41]:

$$\log k = \log k_0' - 0.5(\mathrm{pH} - \mathrm{pH}_0) \quad (12)$$

Eq. (12) predicts values of k with an error of less than 11% when the pH value is within ±0.4 U of $pH = pK_a$. From Eq. (12) and Fig. 1, for $pH = pK_a$ and $k = k_0/2$, it can be seen that:

$$\log k_0' = \log(k_0/2) + 0.5[(pK_a) - \mathrm{pH}_0] \quad (13)$$

If a linear pH-gradient is assumed (other conditions constant), pH as a function of time, t, is given as:

$$(\mathrm{pH}) = \mathrm{pH}_0 + (\Delta\mathrm{pH}/t_G)\, t \quad (14)$$

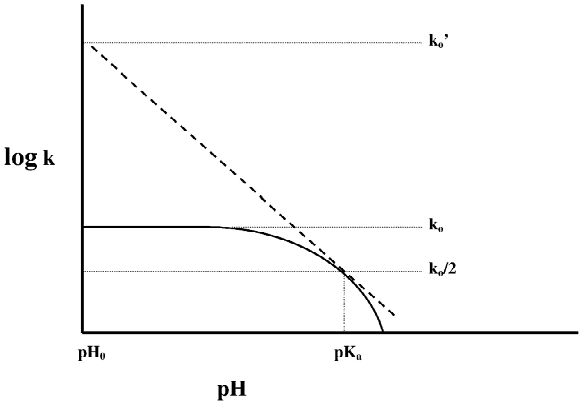

Fig. 2. Typical plot of log k versus pH. Dotted line is LSS approximation.

Here, pH_0 is the pH-value at the start of the gradient, ΔpH is the change in pH during the gradient, t_G is the gradient time (during which pH changes) and t is the time after the start of the gradient. Combining Eqs. (12) and (14) yields:

$$\log k = \log k_0' - 0.5(\Delta\mathrm{pH}/t_G)\, t \quad (15)$$

which is the form of an LSS gradient as described by Snyder and Dolan [41]. Because:

$$\log k = \log k_0 - b(t/t_0) \quad (16)$$

then from Eqs. (15) and (16) one gets b:

$$b = 0.5 t_0 \Delta\mathrm{pH}/t_G = 0.5 V_m \Delta\mathrm{pH}/(t_G F) \quad (17)$$

Denoting pH of the mobile phase at elution as pH**, Snyder arrived eventually at the following equations allowing for calculation of pK_a:

$$\text{for acids: } pK_a = \mathrm{pH}^{**} - 2\log(1.15 b k_0) \quad (18)$$

$$\text{for bases: } pK_a = \mathrm{pH}^{**} + 2\log(1.15 b k_0) \quad (19)$$

The latter equation has been used in this work. Using it, one has to be cognizant of the fact that pK_a such calculated is a function of several variables (pH**, pH_0, ΔpH, t_0, t_G, k_0).

3. Experimental

3.1. Equipment

The HPLC system was an LC Module I Plus (Waters Associates, Milford, MA, USA) with a dwell volume of 4.3 ml, equipped with a pump, variable wavelength UV–Vis detector, autosampler and thermostat. Data were collected using the Waters Millenium version 2.15 software and processed with DryLab program (LC Resources, Walnut Creek, CA, USA); 1% $NaNO_3$ was used as a dead time marker. Detection at 254 nm was a standard. The following columns were employed: Inertsil ODS-3, 150×4.6 mm I.D., particle size 5 μm (GL Sciences Inc., Shinjuku-ku, Tokyo, Japan), XTerra RP_{18}, 150×4.6 mm I.D., particle size 5 μm (Waters Corporation, Milford, MA, USA), both packed with octadecyl-bonded silica, Aluspher 100 RP-select B, 125×4.0 mm I.D., particle size 5 μm (Merck, Darmstadt,

Germany) packed with polybutadiene coated alumina and PRP-1, 150×4.1 mm I.D., particle size 5 μm (Hamilton Company, Reno, NV, USA), made of cross-linked polystyrene (divinylbenzene).

Mobile phases contained either methanol or acetonitrile as organic modifiers. Water or buffers of fixed pH formed the aqueous component of the eluent.

The injected sample volume was 20 μl. All the chromatographic measurements were done at 35 °C with eluent flow-rate of 1.5 ml/min.

All the reagents and analytes employed were of highest quality commercially available.

3.2. Buffers

Universal buffer [44] consisted of parts A and B. Part A was formed by three acids, all at concentration 0.004 M: phosphoric, acetic and boric. Part B, 0.02 *M* sodium hydroxide was added to part A at amounts required to obtain the required pH. The pH of the buffers was measured at 21 °C before mixing with organic modifiers. The measurements were done with an HI 9017 pH-meter (Hanna Instruments, Bedfordshire, UK).

3.3. Determination of log k_w values by isocratic and by gradient elution

Two organic modifier gradients, 5–100% B, at gradient times t_G, equal to 20 and 60 min were carried out. Based on retention times from two gradient runs with different *b* value for each compound, log k_w values were derived by the DryLab program. Based on Eq. (9), DryLab software also predicted isocratic retention parameter, *k*, corresponding to a defined percent of given organic modifier.

A comparison of log k_w values obtained by isocratic elution to those obtained by gradient elution was done for 37 analytes listed in Table 1. In gradient elution mode the retention times derived from two gradient runs differing in gradient time served as input data and the log k_w values were derived by the DryLab program. In the case of isocratic elution, the retention coefficients, *k*, were determined at fixed compositions of the binary organic solvent–water mobile phase ranging from 90:10 to 10:90 (v/v). Methanol and acetonitrile were the organic solvents employed. Linear relationships were determined between log *k* and the volume percent of organic solvent in the eluent. On the basis of these relationships (in each case the correlation coefficient was above 0.99) the values of log k_w corresponding to 100% water were obtained by extrapolation. The log k_w values obtained by isocratic and by gradient elution on Inertsil ODS-3 and on Aluspher 100 RP-select B columns are collected in Table 1 along with logarithms of octanol–water partition coefficients, log *P*, taken from literature [17]. Corresponding data (log k_w from gradient elution and log *P* from literature) for a series of drugs are given in Table 2. For those analytes the column used was XTerra RP_{18} and methanol was the organic modifier.

3.4. Determination of pK_a values by gradient elution

The pH gradient of the aqueous component of the mobile phase was programmed for the selected *k* value of each compound. Percent of organic modifier was kept constant during chromatographic run, while composition of buffer components was linearly changed during a programmed gradient time. For basic compounds, pH was 10.5 at the start and 3.00 at the end of gradient (ΔpH 7.5). For acidic compounds reverse pH gradient was applied.

4. Results and discussion

In Table 1, log k_w values are collected as determined by isocratic and by gradient method for structurally diversified analytes at pH conditions providing suppression of ionization. Apparently, the log k_w values produced by the two methods are closely similar. That is the case for both columns (Inertsil ODS-3 and Aluspher 100 RP-select B) and the two organic modifiers (methanol, acetonitrile) studied. A higher correlation (Fig. 3) is observed for the methanol-containing mobile phases ($R>0.98$). Nevertheless, the correlations found for the acetonitrile-containing mobile phases remain high ($R>0.95$).

Correlation analysis demonstrates distinctive prop-

Table 1
The log k_w values obtained by gradient and by isocratic elution on Inertsil ODS-3 and on Aluspher 100 RP-select B columns for a series of test analytes

No.	Analyte	log *P*	Inertsil ODS-3				Aluspher 100 RP-select B			
			Methanol-containing mobile phase		Acetonitrile-containing mobile phase		Methanol-containing mobile phase		Acetonitrile-containing mobile phase	
			log k_w (gradient)	log k_w (isocratic)	log k_w (gradient)	log k_w (isocratic)	log k_w (gradient)	log k_w (isocratic)	log k_w (gradient)	log k_w (isocratic)
1	Aniline	0.90	1.11	1.10	1.09	1.04	0.03	−0.09	0.11	−0.01
2	Phenol	1.46	1.34	1.43	1.32	1.31	0.11	0.11	0.28	−0.01
3	2-Chloropyridine	1.22	1.51	1.41	1.26	1.23	0.06	−0.02	0.24	−0.12
4	Anisole	2.11	2.36	2.30	2.12	1.92	0.89	0.72	1.16	0.64
5	Benzamide	0.64	1.22	1.07	1.09	0.62	−0.19	−0.28	−0.03	−0.45
6	Benzene	2.13	2.25	2.27	1.74	1.95	0.86	0.71	1.18	0.71
7	Benzonitrile	1.56	1.92	1.86	1.76	1.73	0.37	0.42	0.59	0.32
8	Benzyl chloride	2.30	3.00	2.77	2.62	2.26	1.38	1.12	1.83	1.31
9	Biphenyl	3.98	4.39	4.05	3.09	2.56	3.05	2.86	2.88	2.48
10	4-Cyanophenol	1.60	1.64	1.55	1.50	1.43	0.21	0.24	0.47	0.25
11	2,2′-Dinaphthyl ether	6.40	6.35	6.26	3.94	3.74	4.65	4.86	3.10	3.39
12	Indazole	1.77	1.95	1.89	1.73	1.49	0.60	0.43	0.73	0.42
13	Indole	2.14	2.13	2.16	2.30	1.76	0.86	0.90	1.12	0.84
14	Naphthalene	3.30	3.66	3.38	2.82	2.37	2.35	2.11	2.42	1.96
15	2-Naphthol	2.70	2.80	2.56	2.41	1.77	1.47	1.40	1.82	1.26
16	1-Naphthylacetonitrile	2.74	3.19	2.72	2.76	2.30	1.80	1.57	2.12	1.53
17	Phenanthrene	4.46	4.70	4.34	3.16	2.69	3.46	3.09	2.82	2.54
18	Pyrene	4.88	5.01	4.68	3.23	2.93	3.06	3.70	2.74	2.95
19	Coumarin	1.39	1.98	2.00	1.72	1.76	0.49	0.56	0.66	0.68
20	Phthalimide	1.15	1.61	1.65	1.47	1.15	0.16	0.47	0.30	0.42
21	Phthalonitrile	0.99	1.67	1.71	1.74	1.70	0.17	0.47	0.41	0.50
22	1,4-Naphthoquinone	1.71	2.24	2.11	1.97	1.49	0.74	0.81	0.92	1.22
23	Toluene	2.73	2.92	2.94	2.53	2.21	1.32	1.44	1.66	1.46
24	Phenylacetylene	2.53	2.93	3.08	2.63	1.82	1.50	1.49	1.72	1.46
25	Ethylbenzene	3.15	3.57	4.12	2.79	2.48	2.14	2.18	2.27	1.94
26	Carbazole	3.72	3.63	3.45	3.12	2.70	2.56	2.36	2.68	2.06
27	Cumene	3.66	4.31	3.81	2.98	2.61	2.64	2.56	2.61	2.22
28	1-Bromonaphthalene	4.06	4.47	3.97	2.98	2.59	3.11	2.60	2.71	2.14
29	*n*-Propylbenzene	3.69	4.25	3.89	2.92	2.69	2.80	2.73	2.69	2.32
30	*n*-Butylbenzene	4.38	4.92	4.42	2.88	2.90	3.25	3.19	2.92	2.23
31	9,10-Anthraquinone	3.39	3.43	3.20	2.65	2.44	2.35	2.24	2.44	3.29
32	Xanthene	4.23	4.23	2.92	3.06	2.36	3.09	2.98	2.88	2.28
33	*n*-Amylbenzene	4.90	5.86	4.95	3.49	3.16	3.63	3.71	3.03	2.81
34	*n*-Hexylbenzene	5.52	6.38	5.81	3.65	3.52	3.94	4.32	3.08	3.01
35	Hexachlorobutadiene	4.78	5.30	4.80	3.26	3.43	3.56	3.56	2.77	2.92
36	Anthracene	4.45	4.70	4.75	3.07	3.09	4.04	3.17	2.87	2.42
37	1,3,5-Triisopropylbenzene	6.36	6.98	6.47	4.28	3.96	4.24	4.72	3.28	3.71

Logarithms of octanol–water partition coefficient, log *P*, are taken from Ref. [17].

erties of the alumina-based reversed-phase Aluspher 100 RP-select B washed with methanol–buffer eluent. For that HPLC system, the correlation between log k_w isocratic versus gradient is high and standard deviations low. Also, the slope of the relationship is close to unity and the intercept is close to zero. In other words, on Aluspher 100 RP-select B, the log k_w values determined by the proposed gradient method are strictly equivalent to those obtained by standard isocratic procedure. The reason is the absence of specific interactions of analytes with support material of alumina, as op-

Table 2
Hydrophobicity parameters from gradient HPLC, log k_w, and reference lipophilicity parameters, log P for a series of drugs

No.	Drug	Log k_w (gradient)	Log P (literature)[a]
1	Acetylsalicylic acid	1.28 (pH 3.00)	1.19
2	Antipyrine	0.93 (pH 10.50)	0.38
3	Atenolol	1.65 (pH 10.50)	0.16
4	Bromazepam	2.21 (pH 10.50)	2.05
5	Caffeine	1.15 (pH 10.50)	−0.07
6	Chlorothiazide	−0.46 (pH 10.50)	−0.24
7	Cimetidine	1.39 (pH 10.50)	0.4
8	Clonidine	1.35 (pH 10.50)	1.59
9	Desipramin	3.29 (pH 10.50)	4.9
10	Diazepam	2.85 (pH 10.50)	2.8
11	Dilevalol	2.11 (pH 10.50)	3.09
12	Hydrochlorothiazide	−0.45 (pH 10.50)	−0.07
13	Ibuprofen	3.36 (pH 3.00)	3.5
14	Ketoprofen	2.86 (pH 3.00)	3.12
15	Labetalol	2.18 (pH 10.50)	3.09
16	Metoprolol	2.43 (pH 10.50)	1.88
17	Nadolol	2.23 (pH 10.50)	0.71
18	Naloxone	2.14 (pH 10.50)	2.09
19	Oxprenolol	2.54 (pH 10.50)	2.18
20	Phenytoin	1.23 (pH 10.50)	2.47
21	Pindolol	2.14 (pH 10.50)	1.75
22	Practolol	1.81 (pH 10.50)	0.79
23	Propranolol	3.12 (pH 10.50)	3.65
24	Quinidine	3.01 (pH 10.50)	3.44
25	Salicylic acid	1.21 (pH 3.00)	2.26
26	Scopolamine	1.93 (pH 10.50)	1.2
27	Sotalol	0.74 (pH 10.50)	0.24
28	Theophylline	−0.40 (pH 10.50)	−0.02
29	Timolol	2.54 (pH 10.50)	1.91
30	Trimethoprim	1.87 (pH 10.50)	0.91
31	Warfarin	3.03 (pH 3.00)	2.52

[a] From Ref. [46].

posed to not fully controllable interactions of analytes with free silanols on typical silica-based reversed-phase columns.

Table 3 demonstrates that the chromatographic parameters of lipophilicity and log k_w, correlate very well with log P of analytes for which reliable log P data are available. Again, the correlation is best for the alumina-based column and methanol as organic modifier. That observation once more confirms the unique suitability of alumina-based stationary phases for quantification of hydrophobicity as has been observed years ago [45].

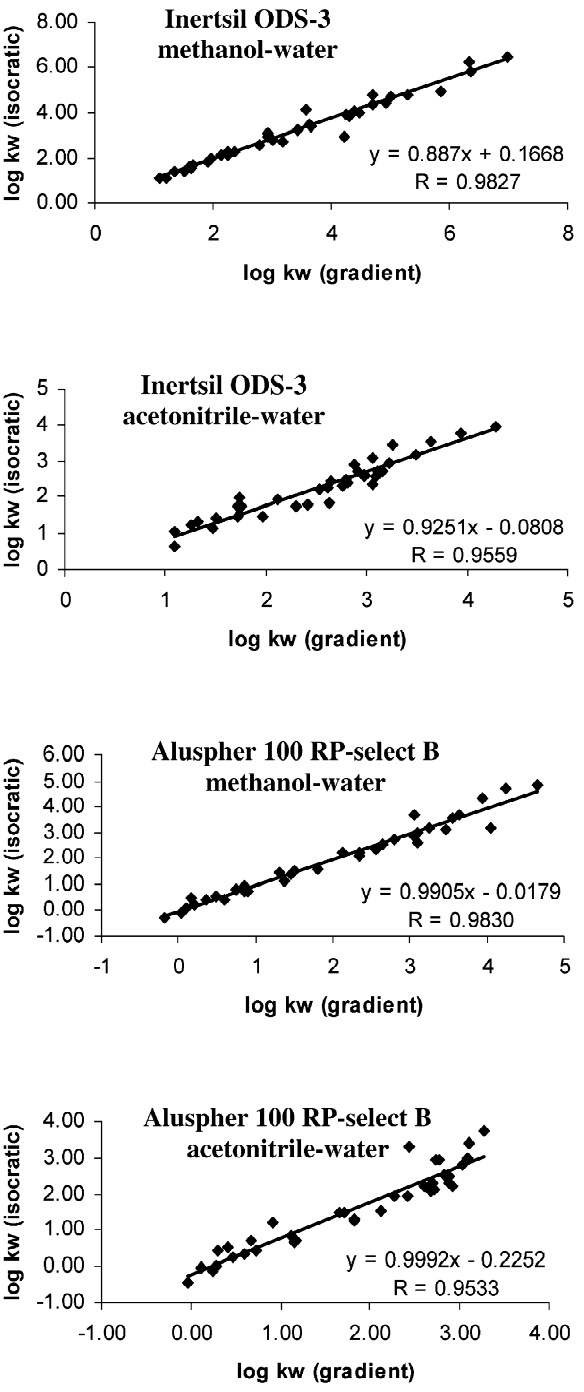

Fig. 3. Correlation between log k_w determined by gradient versus isocratic method for a series of analytes from Table 1.

Table 3
Linear relationships log $k_w = k_1 + k_2$ log P between log k_w data obtained by isocratic and by gradient method and the logarithm of *n*-octanol/water partition coefficient, log P

log k_w	k_1	k_2	R	s
Inertsil, MeOH, gradient	0.2904	1.0184	0.9860	0.2714
Inertsil, MeOH, isocratic	0.4135	0.9069	0.9727	0.3405
Inertsil, ACN, gradient	0.9769	0.5039	0.9609	0.2288
Inertsil, ACN, isocratic	0.7555	0.4882	0.9620	0.2184
Aluspher, MeOH, gradient	−0.8641	0.9084	0.9812	0.2814
Aluspher, MeOH, isocratic	−0.9496	0.9245	0.9911	0.1961
Aluspher, ACN, gradient	−0.1725	0.6532	0.9433	0.3619
Aluspher, ACN, isocratic	−0.5050	0.6879	0.9478	0.3646

k_1 and k_2 are regression coefficients, R is correlation coefficient, s is the standard error of estimate.

In Table 2, log P and log k_w data are given for a series of drugs as determined by gradient method on XTerra RP_{18} column with methanol as organic modifier. The correlation between log k_w and log P is relatively low ($R = 0.804$). That may be due to the uncertainty of the reported log P data for the drugs studied.

The results of this study indicate that the use of the LSS model for deriving log k_w values by gradient elution can be recommended as a fast, reliable, convenient and efficient means to obtain chromatographic parameter of hydrophobicity. The gradient method is certainly less time-consuming than the conventional isocratic mode.

The relationships derived by Snyder [42,43] to determine pK_a by gradient HPLC (Eqs. (12)–(19)) identify several factors affecting the final result. Apparent pK_a depends, among others, on percent of organic modifier in the mobile phase whose pH is changed according to a program during elution. Such changes are difficult to predict as illustrated in Fig. 4. Extrapolation of the relationship between apparent

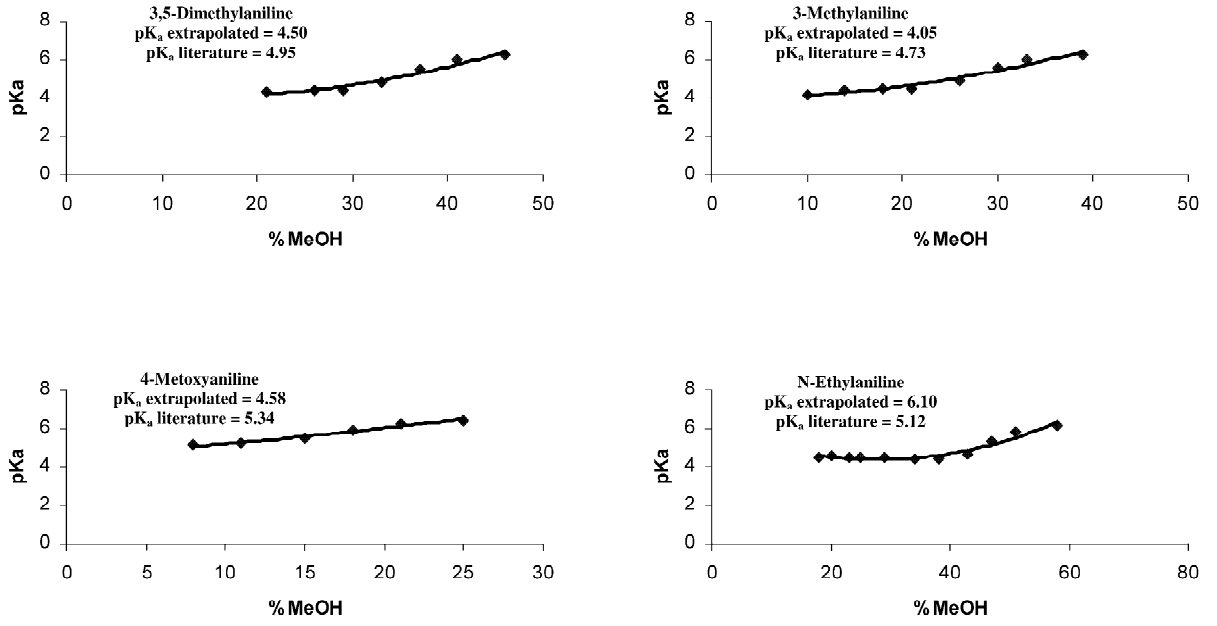

Fig. 4. Relationship between apparent pK_a and percent of organic modifier in mobile phase: column PRP-1, citrate buffer, starting pH 6.5, ΔpH 3.50; t_G of pH gradient 5.00 min.

pK_a^* and the percent of organic modifier to the value corresponding to pure buffer does not produce the expected reference value of pK_a.

By the trial and error method, we arrived at experimental conditions providing pK_a values for a series of aniline derivatives which are in accordance with the literature pK_a data (Table 4). As evident from the table each analyte requires different starting conditions for pH gradient. Having such condition adjusted individually for seven anilines, their pK_a values could be determined. For five of them, literature pK_a values are available. Correlation between the two types of pK_a is given in Fig. 5.

Attempts were undertaken to determine pK_a of a series of drugs employing the elaborated condition of pH-gradient procedure. The data obtained are given in Table 2. Unfortunately, although there is agreement in a few cases, there is no general correlation between the determined and the reference pK_a values.

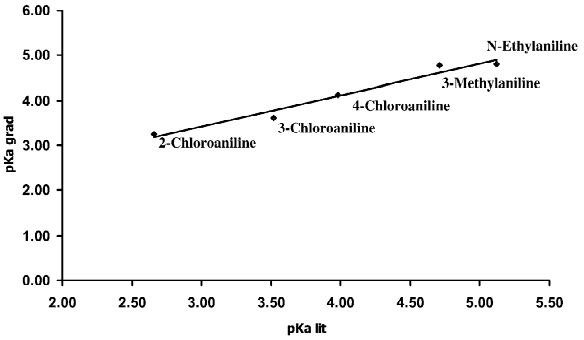

Fig. 5. Correlation between pK_a data determined by pH-gradient HPLC and reference data pK_a for a series of aniline derivatives.

Table 4
Conditions for determination of pK_a of a series of anilines by pH-gradient HPLC

No.	Analyte	Starting conditions for pH-gradient			pH-gradient program[a]				pH**[b]	pK_a (gradient)	pK_a (literature[c])
		% MeOH	k_0	t_R	pH 10.50		pH 3.00				
					%A	%C	%A	%C			
1	Atenolol	18	6.9	6.70	43.4	38.6	71.0	11.0	4.9	6.9	9.6
2	Cimetidine	12	6.8	7.08	46.5	41.5	76.2	11.8	4.4	6.3	6.8
3	Clonidine	17	7.3	7.07	43.9	39.1	71.9	11.1	4.4	6.3	8.05
4	Diazepam	47	6.8	8.96	28.0	25.0	45.9	7.1	0.9	2.8	3.3
5	Dilevalol	24	6.7	6.98	40.2	35.8	65.8	10.2	4.6	6.5	9.45
6	Metoprolol	38	6.7	7.35	32.8	29.2	53.7	8.3	3.8	5.7	9.7
7	Nadolol	32	6.3	6.98	36.0	32.0	58.9	9.1	4.3	6.2	9.39
8	Naloxone	31	5.9	7.15	36.5	32.5	59.8	9.2	4.1	5.9	7.9
9	Oxprenolol	42	7.0	7.26	30.7	27.3	50.2	7.8	3.9	5.8	9.5
10	Phenytoin	9	6.3	6.69	48.1	42.9	78.8	12.2	5.1	6.9	8.3
11	Practolol	22	7.0	6.76	41.2	36.8	67.6	10.4	4.8	6.7	9.5
12	Prazosin	39	6.4	7.37	32.3	28.7	52.8	8.2	4.0	5.9	6.5
13	Propranolol	52	6.4	7.48	25.4	22.6	41.6	6.4	3.5	5.3	9.45
14	Scopolamine	25	6.3	7.09	39.7	35.3	65.0	10.0	4.2	6.0	7.75
15	Timolol	41	6.8	7.09	31.2	27.8	51.1	7.9	4.2	6.1	9.21
16	Trimethoprim	21	6.7	7.27	41.8	37.2	68.4	10.6	4.0	5.9	7.13
					pH 3.00		pH 10.50				
					%A	%C	%A	%C			
17	Acetylsalicylic acid	13	6.6	5.71	75.4	11.6	46.0	41.0	6.5	4.6	3.5
18	Glyburide	57	6.3	6.86	37.3	5.7	22.7	20.3	7.4	5.6	5.3
19	Ketoprofen	45	6.5	6.69	47.6	7.4	29.1	25.9	8.2	6.3	4.6
20	Warfarin	49	6.4	6.55	44.2	6.8	27.0	24.0	8.4	6.6	5.1

Column Xterra, t_G of pH gradient 3.75 min; ΔpH 7.50; value of parameter $b = 1.15$.
[a] Universal buffer composed of part A and B (see Experimental section).
[b] pH at elution.
[c] From Ref. [46].

5. Conclusions

Results of this study can be summarized as follows:

- gradient HPLC can be used for rapid screening of hydrophobicity of analytes including drugs and drug candidates;
- two gradient runs suffice to obtain a reliable measure of analyte lipophilicity;
- estimates of pK_a of analytes can be obtained from two gradient runs: one with modifier gradient and the other with pH gradient; however, the conditions must be adjusted to individual analytes;
- further experiments are required to apply gradient as a routine method of determination of acidity constants.

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