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pH/Organic Solvent Double-Gradient Reversed-Phase HPLC

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A new reversed-phase high-performance liquid chromatographic (RP HPLC) procedure has been theoretically and experimentally established. The approach consists of the simultaneous development of a gradient of pH and of the organic modifier in the mobile phase. The proposed theoretical model of the pH/organic solvent double-gradient RP HPLC allows determination of both pK_a and the lipophilicity parameter of the ionized and the nonionized form of the analyte and prediction of the retention times at specific separation conditions as well as bandwidth for all analytes. The model provides a rational basis for optimization of separation of ionizable analytes at any given chromatographic mode and analysis conditions. In addition, in the case of pH/organic solvent double-gradient RP HPLC, a compression of analyte peak and its reduced tailing can be expected.

The gradient mode of reversed-phase high-performance liquid chromatography (RP HPLC) has been employed for several years to separate mixtures of analytes difficult to separate with the standard isocratic mode.[1−5] Conventionally, "gradient HPLC" denotes a programmed change of the elution strength of the mobile phase during the chromatographic run by adding a stronger solvent B (organic) to a weaker solvent A (normally water). Recently, we developed[6−9] pH gradient RP HPLC that is realized by decreasing (in the case of basic analytes) or increasing (in the case of acids) the pH of the eluent of a constant organic solvent content, thus providing a functional increase with time of the analyte's ionization and, consequently, a decrease of its retention. After having successfully implemented the pH gradient RP HPLC, we realized that a combination of the two gradients, pH and organic solvent, could be advantageous. In particular, the derived theoretical model of pH/organic solvent double-gradient RP HPLC allows a fast and reliable determination of analyte acidity and lipophilicity parameters. The determined parameters can be used in optimization of separation of ionizable analytes in all the chromatographic modes (isocratic, organic solvent gradient, pH gradient, double pH/organic solvent gradient). Additionally, analyte peak compression occurs in pH gradient and in pH/organic solvent double-gradient RP HPLC modes. Here we report a comprehensive theoretical background of the new RP HPLC procedure, along with some illustrative experimental results.

THEORY

A fundamental equation describing gradient chromatography is[10,11]

$$\mathrm{d}x = \mathrm{d}V/V_0k_\mathrm{i} \tag{1}$$

where V denotes the volume of mobile phase passing through the inlet of the column since the start of gradient, V_0 is the void ("dead") volume (i.e., retention volume of a nonretained marker), k_i is the instantaneous retention factor referring to the isocratic retention that would be obtained at the composition of mobile phase actually at column inlet, and dx is a fractional analyte band migration through the column. Since eventually $\sum \mathrm{d}x = 1$ and the volume parameters can be replaced with the corresponding time parameters, one gets

$$\int_0^{t_\mathrm{R}'} \frac{1}{t_0}\frac{\mathrm{d}t}{k_\mathrm{i}} = 1 \tag{2}$$

where $t_\mathrm{R}' = t_\mathrm{R} - t_0$ means the measured gradient retention time, t_R, less the void time, t_0. In the combined pH/organic solvent gradient RP HPLC, both the pH and the organic modifier content, φ, change with time as programmed.

A purely empirical attempt to use a narrow-range gradient of pH in RP HPLC was reported in 1991.[12] Later on, pH gradient RP

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Table 1. Conditions of Individual Chromatographic Modes Considered To Draw Figure 1 for a Hypothetical Analyte[a]

no.	mode (analyte form)	chromatographic conditions
1	isocratic (ionized)	${}^{s}_{w}$pH 2.50, φ 20% v/v
2	organic solvent gradient (ionized)	${}^{s}_{w}$pH 2.50, φ 5−60% v/v
3	combined pH/organic solvent gradient	${}^{s}_{w}$pH 11.50−2.50, φ 5−60% v/v
4	pH gradient mode I	${}^{s}_{w}$pH 11.50−2.50, φ 5% v/v
5	organic solvent gradient (nonionized)	${}^{s}_{w}$pH 11.50, φ 5−60% v/v
6	pH gradient mode II	${}^{s}_{w}$pH 11.50−2.50, φ 15% v/v
7	isocratic (nonionized)	${}^{s}_{w}$pH 11.50, φ 20% v/v

[a] Analyte characteristics are given in the text.

Table 2. Conditions of Individual Chromatographic Modes and the Corresponding Accelerations, *p*, at the Moment of Elution of a Hypothetical Probe Analyte

no.	mode (analyte form)	chromatographic conditions	p (cm/min^2)
1	organic solvent gradient (ionized)	${}^{s}_{w}$pH 2.50, 0.05−0.60, t_G 20 min	1.234
2	combined pH/organic solvent gradient	${}^{s}_{w}$pH 11.50−2.50, t_G(pH) 10 min, φ 0.05−0.60, t_G(MeOH) 20 min	2.049
3	combined pH/organic solvent gradient	${}^{s}_{w}$pH 11.50−2.50, φ 0.20−0.60, t_G(pH) 20 min, t_G(MeOH) 20 min	5.566
4	combined pH/organic solvent gradient	${}^{s}_{w}$pH 11.50−2.50, φ 0.05−0.60, t_G(pH) 20 min, t_G(MeOH) 20 min	5.162
5	pH gradient mode I	${}^{s}_{w}$pH 11.50−2.50, φ 0.25, t_G 20 min	2.618
6	pH gradient mode II	${}^{s}_{w}$pH 11.50−2.50, φ 0.15, t_G 20 min	0.423
7	pH gradient mode III	${}^{s}_{w}$pH 11.50−2.50, φ 0.05, t_G 20 min	0.004
8	organic solvent gradient (nonionized)	${}^{s}_{w}$pH 11.50, φ 0.05−0.60, t_G 20 min	0.786

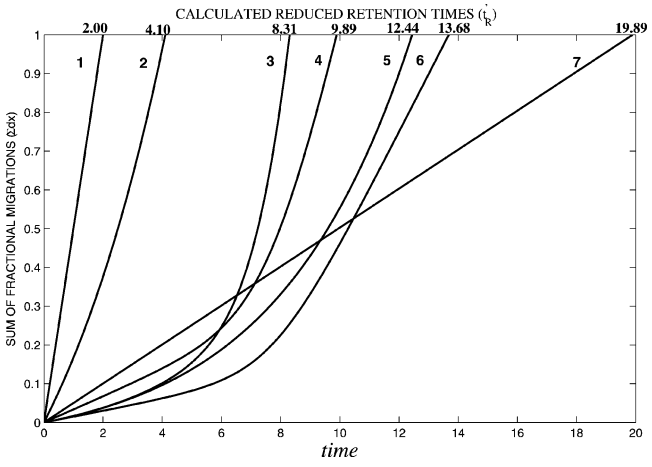

Figure 1. Fractional migration of a hypothetical analyte as a function of time. Calculations were done by eq 6, assuming the parameters of the analyte and of the chromatographic system as specified in the text. Plot numbers refer to the chromatographic conditions defined in Table 1.

HPLC was demonstrated experimentally after appropriate wide pH range buffers have been identified.[6−9] So far, however, a comprehensive, theoretically well founded, systematic approach to the procedure has not been proposed.

The k_i in eq 2 changes during the pH gradient elution as follows:

$$k_i = \frac{k_1 + k_2 10^{{}^{s}_{w}\mathrm{pH}(t) - {}^{s}_{w}\mathrm{p}K_a}}{1 + 10^{{}^{s}_{w}\mathrm{pH}(t) - {}^{s}_{w}\mathrm{p}K_a}} \qquad (3)$$

where pH(t) is a function describing changes of pH with time and k_1 and k_2 represent retention coefficients of individual form of the analyte. For bases $k_1 < k_2$; thus k_1 refers to the ionized and k_2 to the nonionized form of the analyte; in the case of acids, $k_1 > k_2$ and the reverse notation holds true. The left-side indexes at the pH and pK_a symbols indicate the scales in which these parameters were determined. The details of proper assessment of pH in mixed organic/water mobile phases are discussed by Rosés.[13]

At the same time, in the combined pH/organic solvent gradient RP HPLC, the changing content of the organic modifier also affects the retention of both the ionized and the nonionized form of analytes. These changes are accounted for by the well-established equation[1]

$$\log k = \log k_w - S\varphi(t) \qquad (4)$$

where subscript w denotes the retention factor in a neat water eluent; $\varphi(t)$ is a function accounting for changes of the organic modifier content with time, and S is a constant, characteristic for the analyte and the chromatographic system involved.

It must be noted here that the variations of the solvent composition during gradient elution also cause some changes in the acidity of the compounds. These changes are generally nonlinear in a wider range of organic modifier contents.[14−16]

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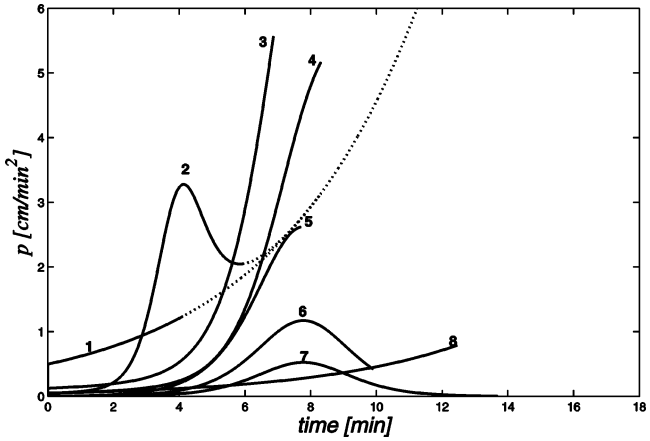

Figure 2. Acceleration, p, of a hypothetical analyte as a function of time. Calculations were done by employing eq 8 and assuming the parameters of the analyte and of the chromatographic system as described in the text. The ends of individual plots (their solid parts) fall at the reduced retention times of analytes. Plot numbers refer to the chromatographic conditions described in Table 2.

However, if the organic modifier content is less than 80%, one can assume a linear relationship to hold between the pK_a and the organic modifier content, φ:

$$ {}^{s}_{w}\mathrm{p}K_a = {}^{w}_{w}\mathrm{p}K_a + \alpha\varphi(t) \tag{5} $$

where α is a slope parameter. Now, putting eqs 3−5 into eq 2 one gets

$$ \int_0^{t'_R} \frac{1}{t_0} \frac{1 + 10^{{}^{s}_{w}\mathrm{pH}(t) - {}^{w}_{w}\mathrm{p}K - \alpha\varphi(t)}}{k_{w1}10^{-S_1\varphi(t)} + k_{w2}10^{-S_2\varphi(t) + {}^{s}_{w}\mathrm{pH}(t) - {}^{w}_{w}\mathrm{p}Ka - \alpha\varphi(t)}} \mathrm{d}t = 1 \tag{6} $$

Equation 6 is the general equation describing retention in any chromatographic mode, whether pH, organic solvent content change, or both, i.e., in isocratic, organic solvent gradient, pH gradient, and double pH/organic solvent gradient modes, providing that the organic modifier content is in the range 0−80% (v/v). A general equation for polyprotic acids and bases, as well as for zwitterionic compounds, can also be found in a similar way by using appropriate equations relating the observed retention factor to the pH of mobile phase, e.g., equations proposed by Jano et al.[17]

In Figure 1 a modeling of retention by eq 6 is illustrated for a hypothetical analyte in various HPLC modes presented in Table 1. The sum of the fractional analyte migration within the column is plotted against the time of the chromatographic run. The theoretically calculated reduced retention time, t'_R, of the probe analyte is indicated at the top of the figure for each individual HPLC mode considered. The calculations, done by numerical integration, consisted in finding a function, $y(t)$, the first derivative of which, $y'(t)$, is the integrand in eq 6. Function $y(t)$ is a sum of fractional migration times. When $y(t) = 1$ then, according to eq 6, elution of the analyte occurs at the reduced retention time, t'_R. For the hypothetical probe analyte used to draw Figure 1, a typical

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Table 3. Changes of Content of the Eluent during the pH/Methanol Double-Gradient RP HPLC Serving To Determine the Experimental Data Necessary for Eq 6[a]

eluent component	pump program no.								
	1	2	3	4	5	6	7	8	9
% B_0	5	5	5	5	5	5	5	5	5
% B_f	80	80	80	80	80	80	80	80	80
% C_0	10	19	28	37	46	56	65	74	83
% C_f	0	2	4	6	8	11	13	15	17
% D_0	85	76	67	58	49	39	30	21	12
% D_f	20	18	16	14	12	9	7	5	3

[a] Lower right index 0 denotes initial and f final content of the components B, C, and D in the eluent.

characteristic was assumed: $k_{w1} = 100$, $k_{w2} = 10$, $S_1 = S_2 = 3.5$, p$K_a = 9$, and $\alpha = 0$. At the same time, the following parameters of the model HPLC system, $t_0 = 1$ min and $t_d = 0$ min, were used in the calculations. For the sake of simplification, it has been assumed that pK_a is independent of the organic modifier content. That assumption approximates the real situation. However, our theoretical treatment rationally explains the changing migration of analytes within a chromatographic column at the various HPLC conditions and modes.

As illustrated in Figure 1, in the case of a combined pH/organic solvent gradient (curve 3), the steepness of the plot of a distance passed by the analyte within the column against time is more abrupt as compared to that observed for the organic gradient alone (curve 5). This is caused by the fact that during the former mode the pH decreases enough to induce ionization of the analyte. That is reflected by migration acceleration near the time of elution of the analyte from the column.

The velocity of the analyte in the column at time t may be derived from eq 2, which may be treated as a classical kinematics path equation. Multiplying eq 2 by the length of the column, L, one obtains

$$ \int_0^{t'_R} \frac{L}{t_0} \frac{\mathrm{d}t}{k_i} = L \tag{7} $$

Now, it is clear that the integrand may be treated as the velocity of the analyte migration within the column. Hence, acceleration, p, as the first derivate of velocity on time, is

$$ p = (L/t_0 k_i)' \tag{8} $$

The value of p can be treated as a parameter showing how rapidly the analyte migration velocity is changing during the chromatographic run.

Changes of p with time for our hypothetical analyte in different chromatographic modes described in Table 2 are shown in Figure 2. The values of p at the moment of elution at specific RP HPLC conditions are presented in Table 2. From Table 2 it can be seen that in the case of double pH/organic solvent gradients the values of p are the highest (conditions 3 and 4). However, p values depend on the conditions of individual chromatographic modes, especially in case of the pH gradient. The value of p is highest if the analyte is eluted at a pH close to its pK_a. Of course, analyte

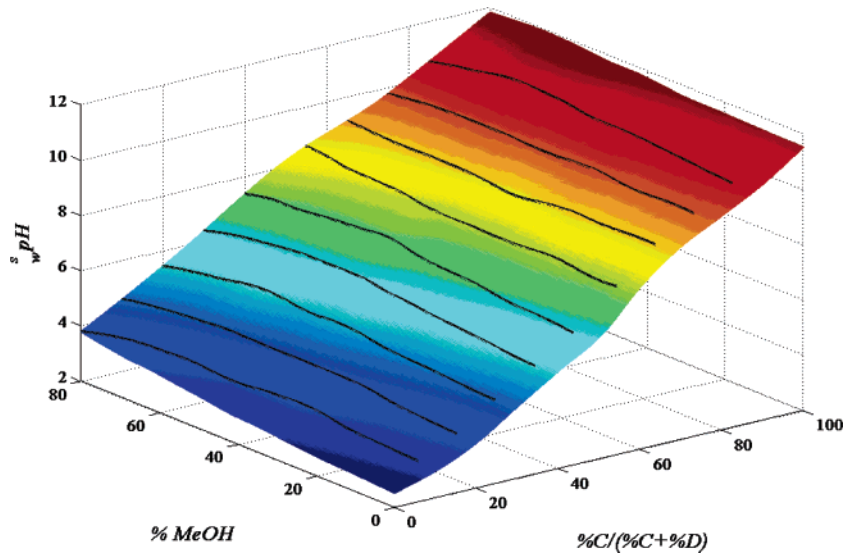

Figure 3. Relationship between $^{s}_{w}$pH, methanol content, and buffer C content, expressed as volume percent of aqueous component of eluent. The black lines show changes of $^{s}_{w}$pH during individual pH/methanol content double-gradient runs described in Table 3.

Table 4. Retention Times (in min) Obtained from pH/Methanol Content Double Gradient[a]

analyte	experiment no. 1	2	3	4	5	6	7	8	9
(A) pH/Methanol Content Double-Gradient Developed at t_G = 20 min									
aniline	9.17	9.23	9.25	9.2	9.15	8.83	7.01	4.16	3.28
2-amino-5-nitropyridine	11.09	11.09	11.07	10.53	6.72	4.85	4.64	4.53	4.36
N-methylaniline	14.56	14.59	14.61	14.53	14.48	14.24	11.81	6.29	4.93
N-ethylaniline	17.04	17.12	17.09	17.09	17.04	16.75	13.52	7.65	6.56
2,4,6-collidine	17.52	17.61	17.49	17.25	14.11	7.09	5.49	5.28	5.09
brucine	17.72	17.83	17.49	16.45	13.01	11.41	11.04	10.99	10.86
p-nitrophenol	4.67	4.96	5.92	9.39	13.09	13.65	13.76	13.81	13.84
diethylbarbituric acid	7.52	7.97	9.84	13.12	14.24	14.35	14.37	14.4	14.43
2-chloro-4-nitrophenol	8.43	8.75	9.25	9.84	12.11	15.04	17.2	17.55	17.63
2,6-dimethyl-4-nitrophenol	9.09	9.63	11.57	15.79	18.00	18.43	18.48	18.51	18.53
1-naphthylacetic acid	13.09	13.41	13.79	14.08	14.29	15.07	17.36	18.32	18.43
N,*N*-benzyldimethylaniline	18.61	18.45	17.12	12.03	6.13	5.71	5.76	5.73	5.57
(B) pH/Methanol Content Double-Gradient Developed at t_G = 60 min									
aniline	11.84	11.84	11.73	11.73	11.63	10.96	7.81	4.29	3.28
2-amino-5-nitropyridine	16.99	16.88	16.61	15.41	7.52	5.2	4.93	4.85	4.59
N-methylaniline	24.45	24.4	24.27	24.24	24.11	22.96	15.76	7.15	5.31
N-ethylaniline	32.64	32.59	32.51	32.45	32.19	30.48	18.85	9.68	8.08
2,4,6-collidine	35.92	35.81	35.53	34.16	20.16	8.59	6.37	6.11	5.79
brucine	39.07	39.2	38.56	34.35	24.96	21.52	21.01	20.88	20.59
p-nitrophenol	4.83	5.15	6.51	12.58	21.17	22.45	22.67	22.75	22.69
diethylbarbituric acid	9.65	10.48	14.37	23.12	26.72	27.09	27.15	27.17	27.12
2-chloro-4-nitrophenol	10.48	10.88	11.79	12.85	19.01	27.65	33.76	34.85	34.93
2,6-dimethyl-4-nitrophenol	12.48	13.41	17.57	29.6	37.17	38.48	38.75	38.85	38.88
1-naphthylacetic acid	23.33	24.4	24.96	25.65	26.27	28.72	36.03	39.47	39.84
N,*N*-benzyldimethylaniline	38.67	37.81	31.84	16.91	7.01	6.56	6.61	6.61	6.19

[a] Experiment number corresponds to pump program number from Table 3.

retention may be adjusted purposely by selecting an appropriate organic solvent content (in the case of pH gradient) or the rate of its change (in case of combined pH/organic solvent gradient). In Figure 2, the three pH gradient runs differ in methanol content in the mobile phase (curves 5−7). It can be seen that the retention times are shorter and the accelerations higher when the organic modifier content is increased. Curves 5−7 indicate that the highest p can be expected when the pH at the moment of elution is roughly at the analyte's pK_a. Conditions 5 in Table 2 appear optimal for acquiring a maximum acceleration in the pH gradient mode for the hypothetical analyte considered.

The highest values of acceleration in Figure 2 are for the combined double pH/organic solvent gradients (curves 2−4). The values of p in this mode are several times higher than in the organic solvent alone gradient mode. In the case of conditions 2 (curve 2), it can be seen that the pH gradient accelerates the analyte movement as long as the pH is relatively high, i.e., if the organic solvent content in the mobile phase is low. Maximum acceleration occurs at the time when the eluent pH approaches the analyte's pK_a. After that, the migration of the analyte is less and less influenced by the pH gradient. Eventually, the migration of the ionized form of the analyte depends solely on the organic

Table 5. ${}^{w}_{w}pK_a$, α, k_1, k_2, S_1, and S_2 Parameters Obtained by Nonlinear Fitting to Eq 6[a]

analyte	log k_1	S_1	log k_2	S_2	${}^{w}_{w}pK_a$	α	${}^{w}_{w}pK_{alit}$
aniline	0.1475	4.6086	1.0505	2.6217	4.57	0.0504	4.63
2-amino-5-nitropyridine	0.7021	7.6669	1.4462	3.4756	7.40	−2.5788	7.22
N-methylaniline	0.6517	7.5187	1.6717	2.7553	4.86	−0.4371	4.85
N-ethylaniline	1.0878	7.3918	2.1172	3.1734	5.32	−0.7674	5.12
2,4,6-collidine	0.7706	5.5581	2.4737	3.8209	7.54	−1.3646	7.43
brucine	2.4450	7.4991	3.2924	5.4369	7.68	0.6424	8.26
p-nitrophenol	1.6293	2.8834	0.5039	3.3592	7.15	0.6623	7.15
diethylbarbituric acid	2.1288	4.1571	1.0526	4.2978	7.61	1.2044	7.43
2-chloro-4-nitrophenol	2.2621	3.2836	0.9746	2.2537	5.76	−0.4950	5.45
2,6-dimethyl-4-nitrophenol	2.6512	3.9115	1.1569	3.2326	7.13	1.1498	7.07
1-naphthylacetic acid	3.0254	4.6549	1.9609	3.9972	4.11	2.5463	4.26
N,*N*-benzyldimethylaniline	0.8094	5.5187	2.5130	3.5549	8.76	−0.0568	8.91

[a] ${}^{w}_{w}pK_{alit}$ denotes literature pK_a values[24].

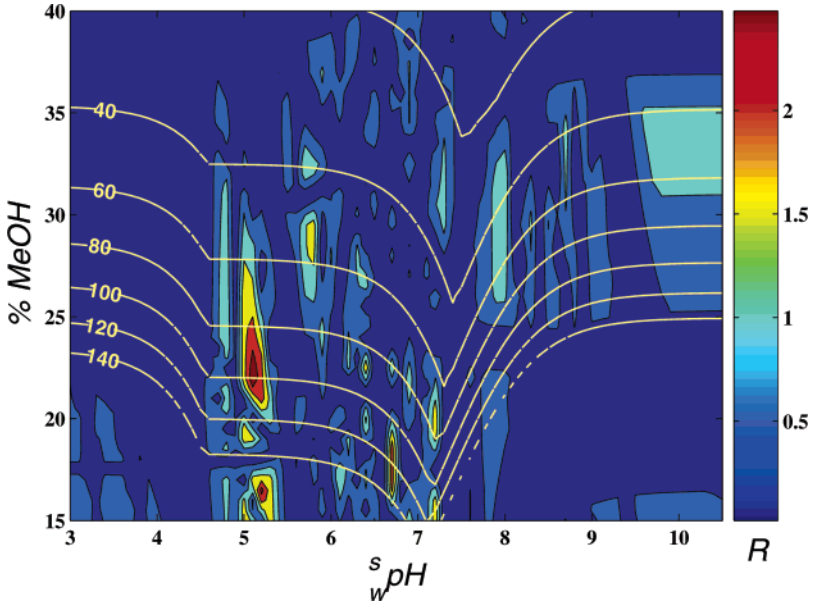

Figure 4. Resolution map for isocratic conditions. Color bar represent resolution, *R*. Isotherms show retention times of maximally retained compound.

solvent gradient. Now, the acceleration curves (dashed parts) for the analyte in the double pH/organic solvent gradient (curve 2) and for the ionized form of the analyte in the organic solvent gradient (curve 1) overlap.

The implication of a high analyte acceleration just before elution from the column is peak compression, which also leads to an increase of the peak height. Mechanistically, peak compression in gradient elution may be explained as follows.[18] During the elution, at any site in the column, the analyte molecules passing through it earlier are exposed to a weaker eluent than the molecules that pass through it later on. A stronger eluent for bases (lower pH, higher methanol content) pushes the analytes faster than a weaker eluent preceding it (higher pH, lower methanol content). Thus, the "tail" is permanently being pushed back into the main peak and peak widening is reduced. The combined double pH/organic solvent gradient RP HPLC here proposed should strengthen that effect.

Usage of eq 6 for retention modeling must be proceeded by obtaining several analyte and RP HPLC system parameters, namely: ${}^{w}_{w}pK_a$, α, k_1, k_2, S_1, and S_2. Obviously, the number of necessary parameters increases with the number of ionizable groups present in the analyte.

Once determined, necessary parameters can be used for prediction of retention times at any defined chromatographic conditions by means of eq 6. That, in turn, allows optimization of separation of ionizable analytes. However, a separate problem with double pH/organic solvent gradient RP HPLC is prediction of bandwidth, especially when nonlinear changes of pH are to occur. Knowledge of the bandwidth is required to predict resolution.

In the case of isocratic conditions the bandwidth, *W*, can be estimated from

$$W = 4N^{-0.5}t_0(1 + k) \tag{9}$$

In the case of organic gradient elution, the respective relationship is

$$W = 4GN^{-0.5}t_0(1 + k_f) \tag{10}$$

where k_f denotes the retention coefficient, which would correspond

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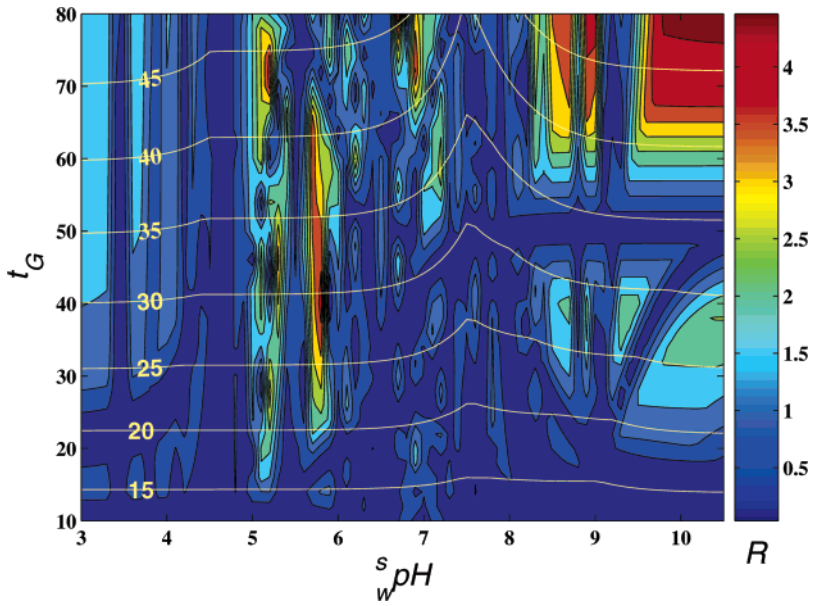

Figure 5. Resolution map for organic gradient conditions. The MeOH concentration changes from 5 to 80%. The pump program consist in six steps giving approximately constant $^{s}_{w}$pH during the whole run. Color bar represent resolution, *R*. Isotherms show retention times of maximally retained compound.

to eluent composition reaching the column's inlet at the moment when the analyte comes out of the column, and *G* is the so-called band-narrowing coefficient, which can be estimated from the equation

$$G^2 = \frac{1 + p + 1/3p^3}{(1 + p)^2} \quad \text{where} \quad p = \frac{k_0}{k_0 + 1} b \qquad (11)$$

where b is the steepness parameter of the organic modifier gradient and k_0 is retention coefficient corresponding to the initial composition of the mobile phase.[19,20]

In the case of simultaneous changes of pH and organic modifier content, it can be demonstrated that bandwidth can be calculated from eq 10 with factor *G* determined by

$$G^2 = \frac{\int_0^1 \left(\frac{k_i + 1}{k_i}\right)^2 dx}{\left(\frac{k_f}{k_f + 1}\right)^2} \qquad (12)$$

where x is fractional movement of analyte within the column. The equation similar to eq 12 was previously derived by Poppe.[19]

Having bandwidth determined, a resolution between two neighboring peaks can be calculated from

$$R = 2(t_2 - t_1)/(W_1 + W_2) \qquad (13)$$

The resolution of separation is the parameter best describing quality of separation, and it should be used in designing optimal RP HPLC separation conditions.

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Table 6. Pump Programs Designed To Provide Optimal Separation Conditions in Isocratic, Methanol Content Gradient, and Double pH/Methanol Content Gradient RP HPLC Modes

mode	pump program			
	t (min)	% B	% C	$^{s}_{w}$pH
isocratic	0.00	23.00	25.00	5.09
	100.00	23.00	25.00	5.09
methanol content gradient	0.00	5.00	80.00	10.21
	15.60	20.00	66.00	10.11
	31.20	35.00	54.00	10.22
	46.80	50.00	40.00	10.08
	62.40	65.00	28.00	10.23
	78.00	80.00	16.00	10.24
double pH/methanol content gradient (condition I)	0.00	16.00	32.00	5.47
	12.00	23.00	15.00	3.98
	24.00	28.00	4.00	3.09
	36.00	43.00	42.00	9.35
	48.00	51.00	27.00	7.76
	60.00	64.00	19.00	7.81
double pH/methanol content gradient (condition II)	0.00	13.00	51.00	7.62
	12.00	20.00	14.00	3.69
	24.00	33.00	34.00	7.03
	36.00	37.00	41.00	8.45
	48.00	39.00	40.00	8.52
	60.00	75.00	5.00	5.23
double pH/methanol content gradient (condition III)	0.00	10.00	36.00	5.55
	6.00	12.00	37.00	5.87
	12.00	14.00	12.00	3.38
	18.00	37.00	2.00	3.10
	24.00	43.00	41.00	9.21
	30.00	70.00	8.00	5.68
double pH/methanol content gradient (condition IV)	0.00	16.00	42.00	6.75
	6.00	18.00	7.00	3.09
	12.00	47.00	6.00	3.85
	18.00	56.00	33.00	9.71
	24.00	57.00	26.00	8.38
	30.00	68.00	3.00	4.18

Table 7. Retention Times Obtained Experimentally, $t_{r\ exp}$, and Calculated Theoretically, $t_{r\ clcd}$, in Isocratic, Organic Methanol Content Gradient, and Double pH/Methanol Gradient RP HPLC Modes[a]

		isocratic mode		methanol content gradient		double pH/methanol content gradient							
						condition I		condition II		condition III		condition IV	
no.	analyte	$t_{r\ clcd}$	$t_{r\ exp}$	$t_{r\ clcd}$	$t_{r\ exp}$	$t_{r\ clcd}$	$t_{r\ exp}$	$t_{r\ clcd}$	$t_{r\ exp}$	$t_{r\ clcd}$	$t_{r\ exp}$	$t_{r\ clcd}$	$t_{r\ exp}$
1	aniline	5.11	4.93	12.28	12.12	7.21	6.59	9.13	8.76	10.30	9.40	7.19	6.96
2	2-amino-5-nitro-pyridine	1.88	2.03	18.51	18.09	2.46	2.53	7.94	7.19	3.50	3.41	4.89	4.25
3	*N*-methylaniline	13.75	11.51	27.18	26.65	13.33	11.65	15.21	14.43	15.25	14.67	9.00	8.72
4	*N*-ethylaniline	20.60	17.64	37.83	37.19	14.52	12.64	16.06	15.24	16.15	15.72	9.45	9.33
5	2,4,6-collidine	2.58	2.35	42.45	42.01	4.47	3.47	10.79	10.08	7.44	5.40	6.55	6.00
6	brucine	10.32	9.52	47.67	47.28	16.56	15.92	20.58	19.92	20.21	20.13	12.68	12.49
7	*p*-nitrophenol	16.31	15.63	5.03	4.71	19.29	18.40	18.22	18.13	21.70	21.48	14.64	14.43
8	diethylbarbituric acid	25.38	23.96	10.31	10.13	26.74	25.73	26.64	25.92	23.90	23.80	15.52	15.35
9	2-chloro-4-nitrophenol	42.49	44.25	11.47	10.63	34.89	35.03	24.54	25.29	25.92	26.23	17.96	18.16
10	2,6-dimethyl-4 nitrophenol	91.46	84.52	13.44	13.21	38.68	39.25	36.95	37.87	27.81	28.65	20.18	20.35
11	1-naphthylacetic acid	54.22	74.69	28.41	27.32	36.69	37.13	29.08	29.60	26.93	27.45	18.67	18.91
12	*N*,*N*-benzyldimethyl-aniline	2.17	2.19	45.51	44.93	3.03	3.11	7.27	6.59	4.63	4.57	4.12	3.80
RMSE		6.3710		0.5529		0.9352		0.6829		0.7439		0.3152	

[a] The applied pump programs are characterized in Table 6.

Table 8. Band Widths at Half Peak Heights Determined Experimentally, $W_{1/2exp}$, and Calculated Theoretically, $W_{1/2clcd}$, in Isocratic, Organic Methanol Content Gradient, and Double pH/Methanol Gradient RP HPLC Modes[a]

		isocratic mode		methanol content gradient		double pH/methanol content gradient							
						condition I		condition II		condition III		condition IV	
no	analyte	$W_{1/2exp}$	$W_{1/2clcd}$	$W_{1/2exp}$	$W_{1/2clcd}$	$W_{1/2exp}$	$W_{1/2clcd}$	$W_{1/2exp}$	$W_{1/2clcd}$	$W_{1/2exp}$	$W_{1/2clcd}$	$W_{1/2exp}$	$W_{1/2clcd}$
1	aniline	0.159	0.170	0.301	0.308	0.185	0.196	0.249	0.257	0.307	0.306	0.120	0.114
2	2-amino-5-nitro-pyridine			0.378	0.350	0.120	0.082	0.120	0.0970	0.150	0.117	0.117	0.067
3	*N*-methylaniline	0.348	0.458			0.173	0.183	0.113	0.108	0.111	0.093	0.085	0.067
4	*N*-ethylaniline	0.521	0.686	0.422	0.466	0.149	0.154	0.114	0.122	0.131	0.100	0.108	0.080
5	2,4,6-collidine			0.372	0.409	0.154	0.127	0.100	0.093	0.227	0.265	0.086	0.078
6	brucine	0.357	0.344	0.413	0.302	0.335	0.295	0.268	0.218	0.138	0.086	0.125	0.074
7	*p*-nitrophenol	0.461	0.543	0.194	0.154	0.386	0.486	0.384	0.553	0.196	0.216	0.148	0.160
8	diethylbarbituric acid	0.760	0.845	0.289	0.240	0.4688	0.544	0.351	0.347	0.224	0.197	0.139	0.108
9	2-chloro-4-nitro-phenol	1.161	1.414	0.246	0.305	0.1625	0.156	0.394	0.384	0.102	0.088	0.160	0.114
10	2,6-dimethyl-4-nitro-phenol	1.800	3.044	0.339	0.308	0.1430	0.088	0.250	0.251	0.120	0.068	0.130	0.087
11	1-naphthylacetic acid	1.243	1.805			0.1860	0.159	0.315	0.260	0.157	0.130	0.115	0.078
12	*N*,*N*-benzyldimethyl-aniline	0.160	0.170	0.591	0.436	0.258	0.101	0.172	0.103	0.261	0.150	0.161	0.100
RMSE		0.4694		0.0700		0.0636		0.0575		0.0444		0.0369	

[a] The applied pump programs are characterized in Table 6.

MATERIALS AND METHODS

Experiments were done using a Merck-Hitachi LaChrome (Darmstadt, Germany and San Jose, CA) apparatus of the dwell volume, V_d, of 2 mL, equipped with a diode array detector, autosampler, and thermostat. Chromatographic data were collected using a D-7000 HPLC System Manager, version 3.1 (Merck-Hitachi). Numerical analysis and data processing were done with Matlab Software version 7.0 (The MathWorks, Inc., Natick, MA).

An XTerra MS C-18 column, 150 × 4.6 mm i.d., particle size 5 μm (Waters Corp., Milford, MA), with a low silanol activity was used.[21] The mobile phases contained buffer and methanol as the organic modifier (solvent B). A buffer of programmed pH formed the aqueous component of the eluent (solvent A). A 1% urea solution was injected to determine the column dead volume, V_o, which was 1.60 ± 0.02 mL. The estimated plate number is N = 5000. Chromatographic measurements were done at 25 °C with an eluent flow rate of 1.0 mL/min. All the reagents and the analytes employed were of a highest commercially available quality. Buffers of ${}^{w}_{w}$pH = 2.50 (buffer D) and ${}^{w}_{w}$pH = 11.50 (buffer C), mixed in various proportions, formed solvent A. The ${}^{w}_{w}$pH of the buffers was measured at 25 °C. The measurements were done with an HI 9017 pH meter (Hanna Instruments, Bedfordshire, U.K.). The base buffer solution was formed using three compounds, each at a concentration of 0.008 M: citric acid, tris(hydroxymethyl)aminomethane, and glycine. Buffer D was

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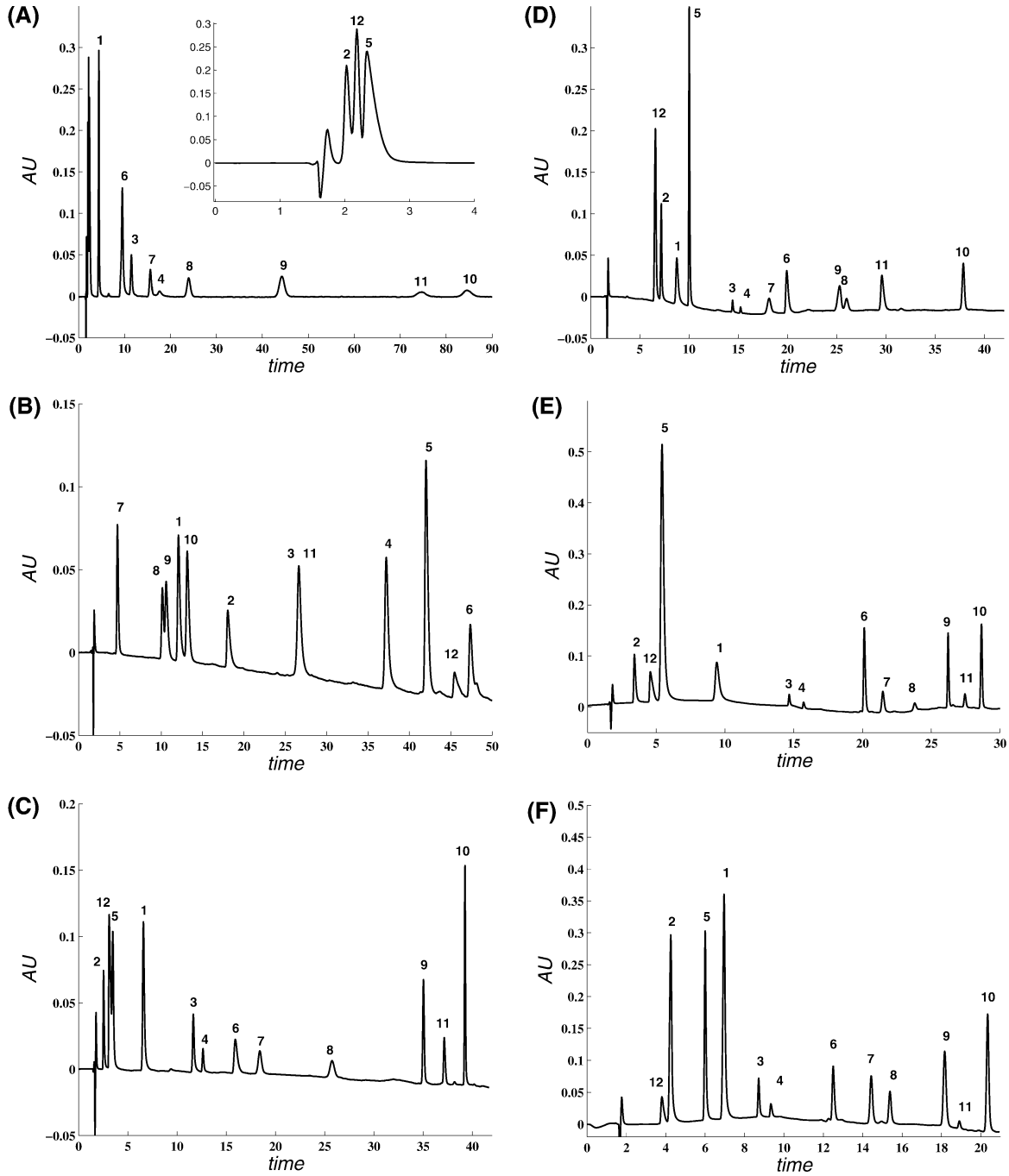

Figure 6. Example chromatograms obtained at optimized conditions. (A) Isocratic; (B) methanol gradient; (C) double pH/methanol gradient, condition I; (D) double pH/methanol gradient, condition II; (E) double pH/methanol gradient, condition III; (F) double pH/methanol gradient, condition IV. The corresponding pump programs are given in Table 6. The analytes are numbered as in Table 7.

made by adding 1 M HCl to the base solution to obtain the desired pH. Buffer C was made by adding the necessary amounts of 3 M NaOH. During the pH gradient run buffers D and C were mixed in a mixing chamber together with a fixed content of methanol.

RESULTS AND DISCUSSION

The validation of eq 6 must be preceded by determination of ${}^{w}_{w}pK_a$, α, k_1, k_2, S_1, and S_2 parameters. This was done for 12 test analytes (7 bases and 5 acids) by nonlinear least-squares curve fitting. The appropriate set of retention times determined at diffrent pH and organic modifier (methanol) content in eluent were collected. To reduce total time of experiments, a set of methanol concentration gradients at different eluent pHs and different gradient times was carried out. Because the organic modifier gradient at constant pH is difficult to perform due to changes of pH accompanying increases in content of organic modifier in the eluent, the only choice was a double pH/methanol gradient with a wide organic modifier concentration range and a small variation of pH. The advantage of such a procedure is that the conditions applied provide a high resolution, and hence, the analytes can be analyzed simultaneously. The number of analytes that can be analyzed jointly depends only on analyte identification potency of

Table 9. Quantitative Characteristics of Chromatograms Presented in Figure 7[a]

		chromatogram A methanol content gradient			chromatogram B double pH/methanol content solvent gradient		
no.	analyte	t_R (min)	h (AU)	w (min)	t_R (min)	h (AU)	w (min)
1	codeine	16.27	0.112	0.22	14.48	0.144	0.19
2	brucine	16.52	0.324	0.23	14.92	0.546	0.18
3	2,4,6-collidine	17.00	0.355	0.26	15.35	0.749	0.17

[a] Symbols: t_R, gradient retention time; w, peak width at the baseline; h, peak height in absorbance units.

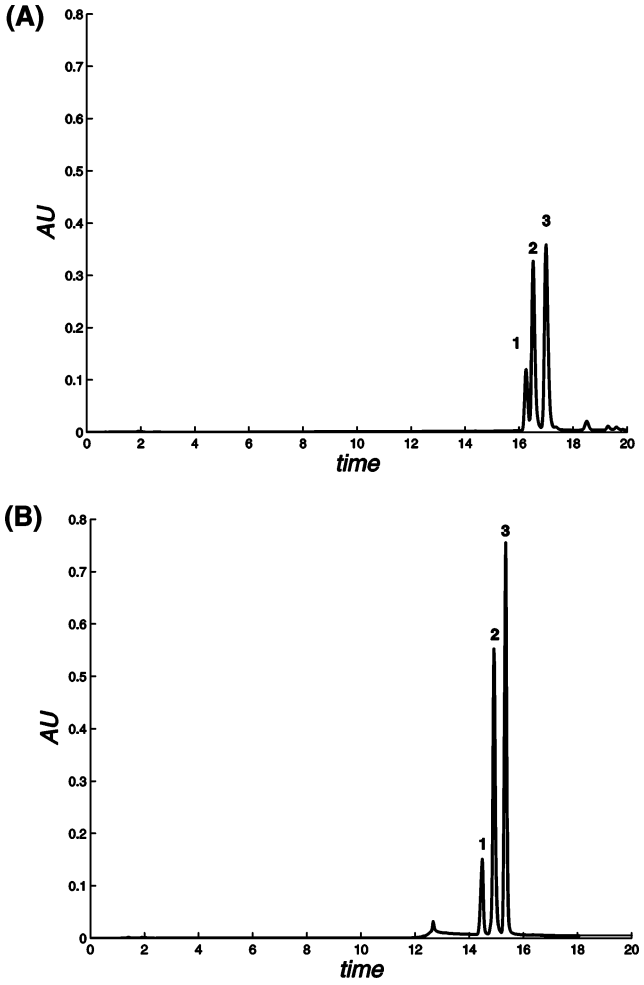

Figure 7. Chromatograms of test analytes characterized in Table 9, as obtained under the following conditions: (A) methanol content gradient B from 5 to 100% (v/v) at ${}^{w}_{w}$pH 10.50, t_G 20 min; (B) double linear pH/methanol content gradient, ${}^{w}_{w}$pH from 10.50 to 3.50 and B from 5 to 50% (v/v), t_G 10 min. Analyte numbers are as in Table 9.

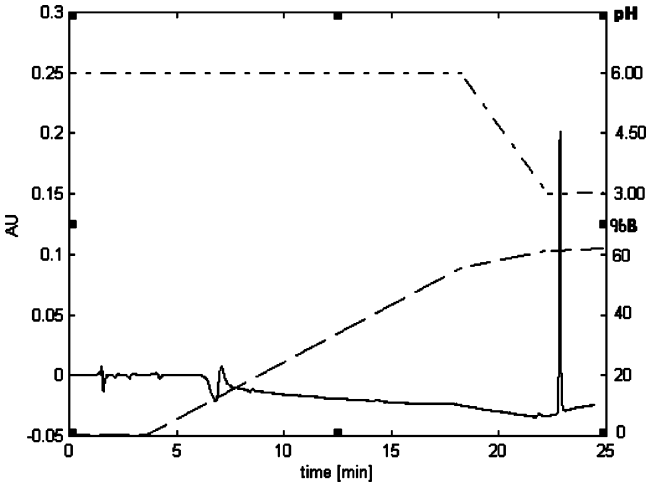

Figure 8. Chromatogram of opipramol sample obtained in double pH/methanol content gradient RP HPLC. The dash−dotted line shows the changes in eluent pH and the dashed line shows the changes in methanol content in eluent (%B v/v), at the column outlet.

the kind of detector used. Using diode array detector tens and in the case of mass spectrometric detection even hundreds of analytes can be simultaneously analyzed and identified.

In this work, 18 RP HPLC experiments in total were performed at different pH/organic solvent gradient conditions. Two series of nine experiments each were carried out. The series differed in gradient time: t_G was 20 min for the first series and 60 min for the second series. The range of changes of each eluent component content was the same in both series of experiments (Table 3). The exact changes of ${}^{s}_{w}$pH during each experiment must be known before calculations. A curve, representing variation of ${}^{s}_{w}$pH with varying eluent composition, was experimentally determined. From that curve the changes of ${}^{s}_{w}$pH during gradient course for any eluent composition can be determined. Changes of ${}^{s}_{w}$pH, for each of the nine double pH/methanol gradient conditions described in Table 3, are presented in Figure 3.

The retention times obtained in 18 RP HPLC experiments are collected in Table 4. Using these data, the values of ${}^{w}_{w}p\mathit{K}_a$, α, k_1, k_2, S_1, and S_2 were calculated by means of the Matlab lsqcurvefit function from the optimization toolbox. The results obtained are presented in Table 5. There is a very good agreement between the literature and the experimentally determined ${}^{w}_{w}p\mathit{K}_a$ data. Thus, our approach to determination of ${}^{w}_{w}p\mathit{K}_a$ is precise enough being at the same time able to provide ready ${}^{w}_{w}p\mathit{K}_a$ data for many analytes, not necessarily of high purity. The method is time saving if a large number of analytes is analyzed simultaneously. Here it took 75 min/analyte.

Reliability of eq 6 was confirmed by determination of optimal separation conditions for isocratic, methanol gradient, and double pH/methanol gradient modes and comparison of the theoretically and the experimentally obtained retention times and bandwidths. In the case of isocratic and organic solvent gradient conditions, the optimal conditions were found from resolution maps presented in Figures 4 and 5, respectively. The resolution maps were calculated from eq 13 and bandwidths from eq 9 for isocratic conditions and from eq 11 for methanol concentration gradient conditions. In the case of the double pH/methanol gradient, optimal conditions were found by the genetic algorithm method. It consists of maximizing the resolution of separation obtained in

a six-step gradient program. Another criterion was retention of the maximally retained peak and total duration of the gradient. The pump programs for optimal isocratic conditions, organic solvent gradient conditions, and four double pH/methanol gradient conditions, which differed in duration of the gradient, along with the maximal retention of the most retained analyte, are presented in Table 6. The obtained retention times, bandwidths, and chromatograms are given in Tables 7 and 8 and in Figure 6, respectively. From observed data it can be noted that only when simultaneous changes in pH and methanol content are applied, is the complete separation of the analytes achieved. In addition, the separation can be completed fast: here in 20 min. The predicted retention times (Table 7) and bandwidths in half peak heights (Table 8) are reliable and can be of value to enhance separation at any defined chromatographic conditions.

There is a variation of peak width with organic modifier content changes as well as with pH changes. A marked analyte band compression can be expected when simultaneous variations in organic modifier content and in pH occur. Of importance is the direction of the pH changes. In the case of basic analytes, the pH should decrease before elution, whereas in the case of acidic analytes, the pH should increase before elution. To get a narrow peak, pH changes should cover the region near the ${}^{s}_{w}pK_a$ of the analyte. Another course of changes of pH results in peak tailing. At each double pH/methanol content gradient conditions presented in Figure 6, very narrow peaks are observed.

Generally, for a complex mixture of analytes, it is difficult to find the conditions allowing at the same time a good separation of analytes and a maximum reduction of peak tailing (due to the pH gradient). The problem is that, to obtain maximum peak compression, the analyte should be eluted after the eluent attains the pH that mostly affects retention. Therefore, the quality of the separation depends on the steepness of the eluent pH gradient and, even more, on the rate of change of the organic modifier content. On the other hand, the conditions can be identified relatively easily, which allows one or more compounds of a mixture to be separated with maximum sensitivity. That was done for three compounds characterized in Table 9.

In Figure 7A, it can be seen that in the methanol gradient mode the analytes are well separated. However, an improvement is achieved when the combined pH/methanol gradient is applied (Figure 7B).

In Table 9, one can note a narrower peak width: a 1.58-fold for 2,4,6-collidine, a 1.28-fold for brucine, and a 1.15-fold for codeine when the combined pH/methanol gradient is used instead of the single methanol gradient. The steepness of the organic solvent gradient was the same in both modes. The retention times are shorter in the case of double gradient. Thus, Figure 7 clearly shows that pH gradient really helps to separate ionizable analytes.

A special problem arising with the pH gradient mode might be the change in the UV spectrum of analytes due to the changes of eluent pH, especially when the analytes are eluted at a pH near their pK_a. Varying UV absorbance can cause poor reproducibility of the pH gradient method. That can be prevented by an appropriate programming of the changes of pH and the organic modifier content. It was done here in the example of a drug analyte: opipramol (pK_a values: 7.80 and 4.16 [22]). Figure 8 presents a chromatogram of the opipramol sample obtained in a combined pH/methanol gradient run. In the figure, the programmed changes of pH and methanol concentration (% B) in the eluent are also indicated. These changes were programmed in such a way as to provide elution of the analyte at an eluent pH in which analyte is completely ionized but immediately after that rapid change in pH occur. The analyte elution at isocratic conditions provides a good reproducibility, whereas the prior changes in pH cause peak compression. Due to this new method, it was possible to quantitatively determine opipramol in tissues of suicide victims, at concentrations otherwise not attainable by standard RP HPLC procedures.[23]

CONCLUSIONS

Theoretical and experimental principles were established for a new procedure of simultaneous double pH and organic solvent content gradient RP HPLC providing improved separation of ionizable analytes and a convenient method of determination of their lipophilicity and acidity parameters. The combined pH/organic solvent gradient mode can be carried out with standard HPLC equipment. It offers a simple means to increase the quality of chromatographic separations, which is especially important when dealing with basic analytes. The method possesses the features that appear to be advantageous over the standard gradient procedure, but certainly further studies must precede its routine usage.

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