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TRACE ANALYSIS USING A COMPETING MODIFIER ADDED TO THE SAMPLE FOR RETENTION AND FOCUSING CONTROL IN MICROBORE COLUMN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

K. ŠLAIS*, M. KREJČÍ, J. CHMELÍKOVÁ and D. KOUŘILOVÁ
Institute of Analytical Chemistry, Czechoslovak Academy of Sciences, Leninova 82, 611 42 Brno (Czechoslovakia)
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SUMMARY

A method for trace analysis in microbore column high-performance liquid chromatography (HPLC) is developed. Solute focusing in the zone of a modifier competing with the solute sorption is proposed to increase the sensitivity and speed of HPLC. The elution mechanism of ionic solutes in the zone of the modifier is discussed on the basis of the theory of protolytic and sorption equilibria. The conditions of chromatographic separation are chosen such that the modifier concentration gradually increases. Under these conditions the gradient elution of solutes is achieved. The proposed method is used to advantage in combination with the elution of ionic solutes in the counter-ion zone. Both the modifier and counter ion are injected simultaneously with the sample. The method is applied to the analysis of catecholamine trace concentrations by microbore column HPLC with electrochemical detection. It presents possibilities of increasing the ratio of the sample volume injected to the retention volume.

INTRODUCTION

Liquid chromatography on chemically bonded non-polar stationary phases has become a widely used technique for the separation of compounds with various physico-chemical properties. For ionized solutes, charged compounds have been used as additives to the aqueous mobile phase to control the retention. Ion-pair reversed-phase high-performance liquid chromatography (IP-RPLC) is the most popular designation for a chromatographic technique under such conditions[1]. The retention and selectivity of solutes is strongly influenced by pH, ionic strength and the contents of organic solvents in the aqueous mobile phase[2]. A small amount of a non-ionized, strongly sorbed organic compound[3,4] can also be used to regulate the retention of ionized solutes in IP-RPLC.

HPLC is often used to determine trace concentrations of solutes in complex matrices. The analytical sensitivity can be improved and the "general elution problem" can be solved by use of a continuous gradient of the mobile phase composi-

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tion[5–7]. Gradient elution in IP-RPLC can be achieved, *e.g.*, decreasing the counter-ion concentration in the mobile phase.[8] The anlytical sensitivity can also be improved by solute injection in a non-eluting solvent[9–12]. A decrease in the elution power of the sample solution injected can be obtained by adding a suitable counter ion[8,13–15]. The counter ion can be injected into the column also before the solute solution[16,17]. Under certain conditions with the solute eluted in the zone of the ion-pair reagent vacancy[18], marked zone narrowing of an individual solute can be observed whereas the other solutes are eluted under isocratic conditions.

If only a small amount of the sample is available, components can advantageously be separated by microbore column HPLC[19,20]. With on-column enrichment, an important improvement in the analytical sensitivity can be obtained if the sample consumption ranges from *ca.* 0.1 to 1 ml[12,15].

We have used microbore column HPLC in our work[15] and have developed and described the on-column enrichment and ionic solute gradient-elution method. This method is based on elution of the solute in the counter-ion zone. With simultaneous injection of the solute and counter ion, the solute is retained on the ion exchanger generated dynamically by adsorption of the counter ion. On washing with the mobile phase, the counter ion is gradually lost. Thereby the ability of the stationary phase to bind the solute decreases. Thus the solute is eluted in a gradient of gradually decreasing sorption activity of the stationary phase. Solutes eluted when all the counter ion has been washed out of the microbore column are chromatographed under isocratic conditions.

In this work, we propose a new method for focusing the solute zone, based on solute elution in the competing modifier zone. The modifier is injected onto the column simultaneously with the sample. The influence of the mobile phase composition on the shape of the modifier zone and consequent changes of the retention and width of the solute peaks are demonstrated by the example of separation of catecholamines.

THEORETICAL

The retention of an ionized solute in IP-RPLC is influenced by the type and concentration of both the ionic and neutral modifiers[3,4]. In the present paper, a cationic and a neutral modifier are conjugated as acid and base. When the modifier is a weak base, cationic (BH^+) and neutral (B) forms of the modifier are present in the mobile phase. The ratio of the concentrations of these forms, $[B]/[BH^+]$, is determined by pH of the mobile phase. If the total concentration, c_B, of the modifier and of the solute is negligible in comparison with the concentration of the buffer in the mobile phase, the pH is determined by the dissociation constant of the buffering compound and by the ratio of the concentrations of the buffer forms. If the concentration c_B becomes comparable with that of the buffer, the pH of the mobile phase increases and so does the ratio $[B]/[BH^+]$. Since in RPLC the retention of the undissociated form of the modifier is considerably higher than that of the dissociated one, the retention of the modifier zone maximum increases wiht increasing concentration, c_B. Hence, the basic modifier is eluted in the form of an asymmetric zone with a slow increase and a fast decrease in c_B. If the cationic solute is eluted within such a modifier zone, both the ionized and non-ionized modifier forms compete with the solute for the accessible surface of the stationary phase. Thus conditions are established for a

continuous increase in the eluting power of the mobile phase. The steepness of the gradient produced in this way depends on the steepness of the increase in the modifier concentration, c_B, within the zone.

The steepness of the rise in c_B can be varied by the buffering capacity of the mobile phase used. The higher the buffering capacity, the slower is the increase in the ratio $[B]/[BH^+]$ with increasing c_B and thus the steeper is the increase in c_B with the volume of the mobile phase passed through the column.

The technique of simultaneous injection of the modifier and the solute proposed here is used in combination with simultaneous injection of the counter ion and the sample[15]. Hence, the solute, counter ion and modifier are injected simultaneously. The model mixture is chosen in such a way that the elution of the solutes in the modifier zone should not be influenced by the presence of the counter ion in the sample and, *vice versa,* the elution of the solutes in the counter-ion zone should not be influenced by the presence of the modifier in the sample. This is achieved by separating the zone of the modifier from that of the counter ion in the column.

As both the counter ion and the modifier are eluted in strongly asymmetric zones, their elution can be described in terms of non-linear, ideal chromatography[21]. The distance between their maxima increases with increasing concentration within the zones. This situation allows us to assume that both zones are separated from one another at the head of the column and proceed along the remaining part of the column independently of one another.

EXPERIMENTAL

Chromatography

Measurements were performed on glass microbore columns CGC (150 mm × 0.7 mm) (Laboratory Instruments, Prague, Czechoslovakia). The columns were packed by means of the viscosity technique[22] with Separon SIC 18 (particle diameter, d_p = 10 μm) (Laboratory Instruments). Its quantity (48.2 mg) was determined by weighing. The column dead volume was 42 μl. The specific surface area was measured by the dynamic desorption method[23] to be 243 $m^2\ g^{-1}$. The mobile phase was pumped into the column by a MC 100 micropump (Mikrotechna, Prague, Czechoslovakia) at 1.5 MPa. The mobile phase was a solution of 0.02 *M* sodium sulphate in distilled water to which acetic acid was added to final concentration of 1–10 m*M*. The sample was injected by a home-made six-port valve with an outer loop made of a stainless steel capillary (200 mm × 0.25 mm). Thus the sample volume injected was 0.1 ml. A cell of an EMD 10 electrochemical detector (Laboratory Instruments) was fitted on the column outlet. The platinum working electrode of this detector was polarized by a voltage of 1.2 V. The electrochemcial detector cell outlet was connected to an UV detector cell via a capillary (0.1 mm I.D. volume 7 μl). A SF 769 Z UV detector (Kratos, Ramsey, NJ, U.S.A.) with a measuring cell (volume 0.5 μl) was used. The signals of both detectors were registered by a TZ 4200 double-line recording millivoltmeter (Laboratory Instruments).

Adrenalin (A) (Sigma, St. Louis, MO, U.S.A.), dopamine (DA) (AWD, VEB Arzneimittelwerk, Dresden, G.D.R.), serotonin (5-HT) (Merck, Darmstadt, F.R.G.) and 4-methyldopamine (4-MDA) (Calbiochem, Los Angeles, CA, U.S.A.) were used as cationic solutes. Benzenesulphonate (BS) injected as its sodium salt was used as

a counter ion. 4-Ethylpyridine (EP) (Fluka, Buchs, Zwitzerland) was used as a modifier. Other chemicals were supplied by Lachema (Brno, Czechoslovakia) and were of analytical grade. Solute stock solutions in distilled water at a concentration of 10^{-3} *M* were stabilized with 0.1 *M* sodium sulphite and 0.1 *M* sulphuric acid. The stock solution of BS was prepared at a concentration of 0.1 *M* in distilled water and that of EP was 0.02 *M* in distilled water. The solutions injected into the microbore column were prepared by diluting and mixing the stock solutions to yield a final concentration of BS of 0.04 *M* and concentrations of EP in the range from 6 to 10 m*M*.

Spectrophotometric measurements

4-Ethylpyridine used as a modifier occurs in the mobile phase in both neutral and dissociated forms. The profile of the total concentration in the modifier zone can be observed only by a spectrophotometric detector at a wavelength corresponding to an isosbectic point. This wavelength was determined by a 634 D spectrophotometer (Varian, Techtron, Springvale, Australia) with the use of a cell 1 cm thick and a slit width of 1 nm. Spectra of 1 m*M* solutions of EP in distilled water and in 1 and 10 m*M* acetic acid were determined. The spectra intersect at a wavelength, $\lambda = 211$ nm. This was confirmed by discontinuous measurement of the absorbances of 1 m*M* EP solution in 1 m*M* acetic acid and in water by use of the SF 769 Z spectrophotometric detector. The detector was adjusted to this wavelength for subsequent measurements.

Measurement and calculation of the mobile phase pH

The pH of the mobile phase was measured by an OP 208 pH-meter (Radelkis, Budapest, Hungary). In the calculations, dissociation constant for acetic acid, $K_a = 1.75 \cdot 10^{-5}$ mol l^{-1}, for 4-ethylpyridine, $K_b = 1.05 \cdot 10^{-8}$ mol l^{-1} and the ionic product of water, $K_w = 1.0 \cdot 10^{-14}$ mol^2 l^{-2}. A correction for the solution ionic strength was made. Table I summarizes the measured and calculated values of the mobile phase pH.

RESULTS AND DISCUSSION

The mofidier capacity ratios, k_B, and corrected retention volumes, V'_{RB}, for the modifier are presented in Table I. These values are determined at the intersection of

TABLE I

DEPENCENCE OF THE RETENTION OF 4-ETHYLPYRIDINE AND THE pH OF THE MOBILE PHASE ON THE ACETIC ACID CONCENTRATION, c_A

V'_{RB} = Corrected retention volume of the modifier.

c_A (mM)	pH		V'_{RB} (μl)	k_B
	Calc.	Exp.		
1	3.98	4.06	1420	33.8
2	3.83	3.87	920	21.9
5	3.63	3.63	685	16.3
7.5	3.54	3.51	–	–
10	3.48	3.45	–	–

the ascending curve of the modifier zone with the baseline, if the modifier amount injected is sufficient for its elution in the asymmetric zone. The values of k_B increase with increasig pH of the mobile phase. This is caused by the increase in the ratio of the concentrations of the non-dissociated and dissociated modifier forms, $[B]/[BH^+]$, with increasing pH of the solution. The dependence of $[B]/[BH^+]$ on pH significantly influences the beginning of the modifier zone, and, as a consequence, also the beginning of the chromatogram part where the modifier competes with the solutes during their sorption on the stationary phase.

The rate of pH increase as well as of the increase in k_B with increasing c_B in the zone are inversely proportional to the buffering capacity of the mobile phase. A change in the concentration of the weak acid, c_A, in the mobile phase causes both a change in pH and a change in the buffering capacity of the mobile phase. Thus, at an higher c_A, the beginning of the modifier zone is eluted earlier and, at the same time, the increase in c_B with the mobile phase elution volume will be steeper. Fig. 1 illustrates the injection of a sample of a constant composition into the microbore column while the column is flushed with a mobile phase containing acetic acid at various concentrations. The peaks of solutes that are not eluted in the modifier zone leave the column in unchanged elution volumes (Figs. 1 and 2) and they are equally wide (Fig. 3) independently of the concentration, c_A (*cf.*, Theoretical). On the other hand, c_A significantly influences both the retention and width of solute peaks eluted

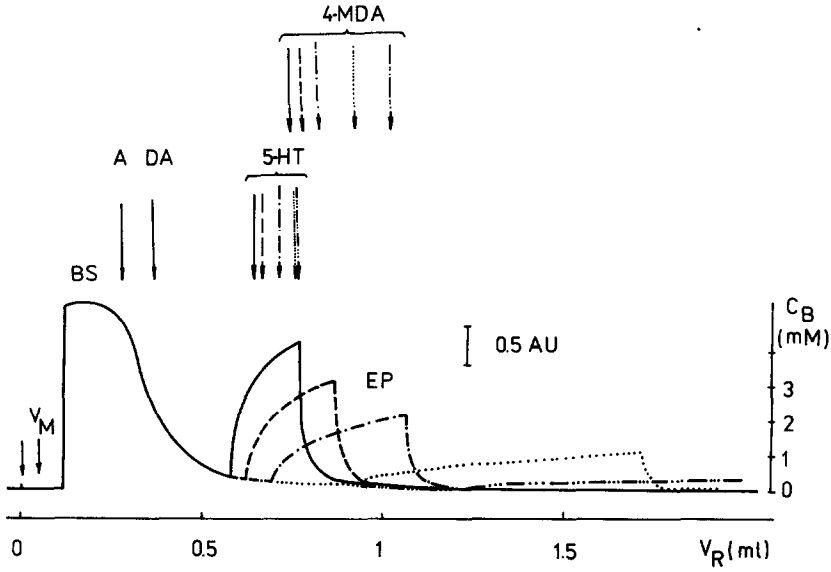

Fig. 1. The dependence of the zone shape of 4-ethylpyridine (EP) and the retention volumes of catecholamines on the acetic acid concentration, c_A in the mobile phase. Microbore column: CGC (150 mm × 0.7 mm), Separon SIC 18, d_p = 10 μm. Mobile phase: 0.2 *M* sodium sulphate in distilled water with acetic acid of concentration, c_A: ———, 10 m*M*; — — —, 7.5 m*M*; - - - - - -, 5 m*M*; · · · · · ·, 2 m*M*; - · · - · · -, 1 m*M*. c_B is the concentration of EP in the mobile phase at the column outlet. Catecholamine retention volumes are indicated by arrows: A = adrenalin; DA = dopamine; 5-HT = serotonin; 4-MDA = methyldopamine. The catecholamine peaks were registred by the EMD 10 detector with a platinum working electrode, polarization voltate +1.2 V. The zones of BS and EP were registred by a Kratos SF 769 UV detector, at a wavelength, λ = 211 nm. Sample composition: 1 μ*M* catecholamines; 6 m*M* EP; 40 m*M* BS in distilled water. Sample volume: 100 μl. V_M = Dead volume.

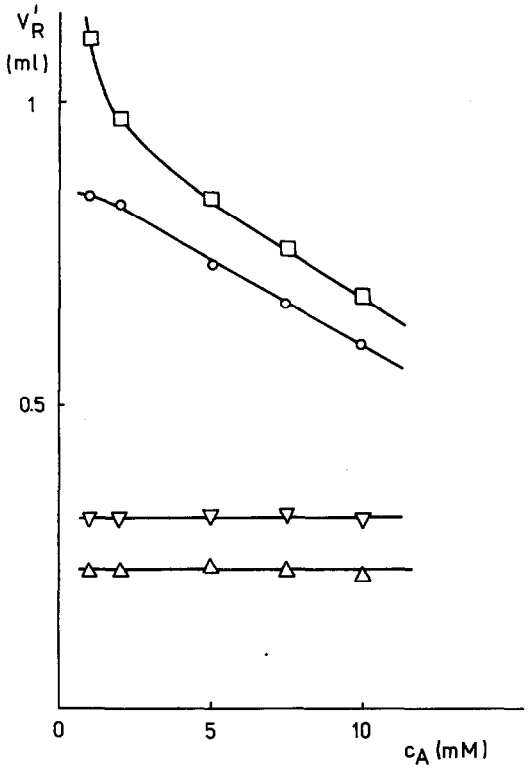

Fig. 2. Dependence of the corrected retention volumes, V'_R, of catecholamines on the acetic acid concentration in the mobile phase, c_A. Solutes: △, A; ▽, DA; ○, 5-HT; □, 4- MDA. For conditions see Fig. 1.

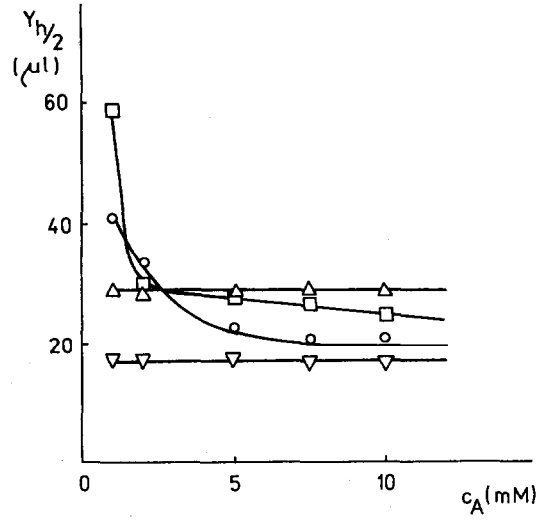

Fig. 3. The dependence of the width of catecholamine peaks expressed as the half-heigth, $Y_{h/2}$, on the acetic acid concentration in the mobile phase, c_A. Solutes as in Fig. 2. For conditions see Fig. 1.

in the modifier zone. The steeper the increase in the modifier concentration in this zone, the earlier the solute peaks are eluted (Figs. 1 and 2) and the narrower they are (Fig. 3). From this we can judge that c_A does not directly influence the retention of completely dissociated solutes, but influences both the pH and the buffering capacity of the mobile phase and, consequently, the retention and shape of the modifier zone. The higher the concentration c_B, the stronger is the modifier competition on the sorbent surface. The more rapid the increase in c_B the steeper is the gradient in the modifier zone.

The relative contribution of the modifier forms to the influence on the solute retention can be determined, for example, if we know two values of k_B at two different pH values of the mobile phase[24]. From the values presented in Table I, the contribution of the two forms is the same if the pH in the modifier zone is 3.63. Thus, as the pH of the mobile phase used in this work is about this value, the contributions of the neutral and dissociated forms of the modifier to the influence on the solute retention should be comparable.

The area under the modifier zone is proportional to the amount of modifier injected into the column. Whereas the beginning of this zone and the steepness of the increase in c_B are determined by the mobile phase composition, a change in the injected amount of modifier will manifest as a change in the retention volume of the end of the modifier zone (see Fig. 4). Regulation of the modifier amount injected with the mobile phase composition can be used for adjusting the modifier zone width. This can influence the number of solutes eluted in the gradient of increasing modifier concentration.

The technique of elution of solutes in the competing modifier zone can be used in combination with techniques of ionic solute elution in the counter-ion zone and with injection of large sample volume into a microbore column, see Fig. 5. Solute focusing during the sample injection and elution of early eluted solutes in the gradient of decreasing counter-ion concentration is maintained. Highly sorbed solutes are

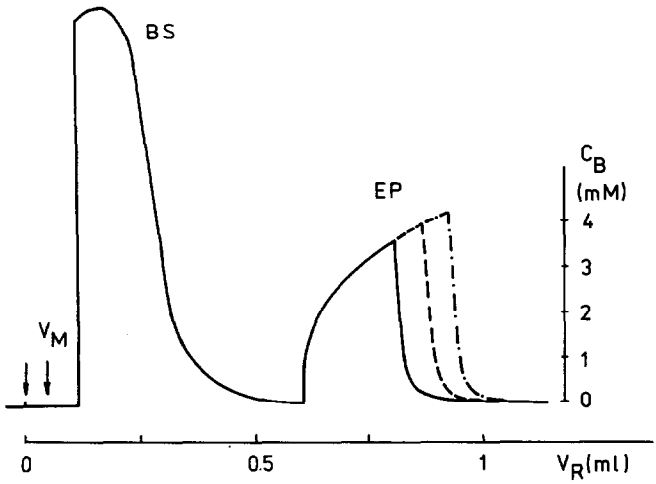

Fig. 4. The dependence of the zone shape of 4-ethylpyridine (EP) on its injected amount: ———, 0.6 μmol; – – –, 0.8 μmol; – · – · – · –, 1.0 μmol (c_A = 7.5 m*M*). For other conditions see Fig. 1.

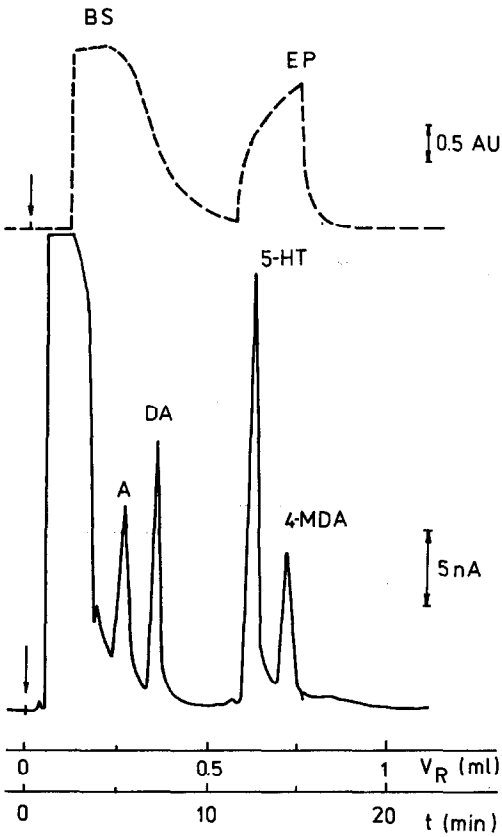

Fig. 5. An example of a chromatogram of catecholamines by combination of elution in the counter-ion zone and the competing modifier zone technique. ———, Electrochemical trace; – – –, UV detector trace. Peaks: A = adrenalin; DA = dopamine; 5-HT = serotonin; 4-MDA = 4-methyldopamine, BS = benzenesulphonate; EP = 4-ethylpyridine. Concentrations in the sample: 1 μM catecholamines; 40 mM BS; 6 mM EP. Sample volume: 100 μl, Mobile phase flow-rate: 50 μl/min. Acetic acid concentration in the mobile phase, c_A = 10 mM. For other conditions see Fig. 1.

eluted in the zone of the competing modifier and leave the column earlier, their peaks being narrower than for injections performed without the modifier. Thus the analysis time becomes significantly shorter and the sensitivity of the analysis is higher.

CONCLUSIONS

The elution of solutes in the competing modifier zone was studied. The analysis of the interactions of the modifier with the mobile phase components, the stationary phase and the solute, and the experiments performed, lead to the following conclusions that allow one to influence both the retention and peak width of solutes eluted in the modifier zone:

(1) If the solute is a cation, the modifier must behave as a base present in the mobile phase as both neutral and dissociated forms.

(2) The elution volume of the beginning of the modifier zone increases with increasing pH of the mobile phase.

(3) The steepness of the increase in modifier concentration in the mobile phase increases with increasing buffering capacity of the latter.

(4) The modifier zone width increases with the amount of modifier injected and decreases with increasing buffering capacity of the mobile phase.

The technique of elution of solutes in the zone of the competing modifier is suitable for increasing the sensitivity during trace analysis by HPLC. It allows gradient elution conditions to be achieved even on small-bore columns where other ways of gradient formation can be realized only with difficulties.

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