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SHORT COMMUNICATION

Correlation of Migration Distance of Peptides in High-Performance Thin-Layer Chromatography and Pressurized Planar Electrochromatography Systems

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Abstract

In a series of our previous papers we have investigated the influence of various variables on retention/migration of peptides in various high-performance thin-layer chromatography (HPTLC) and pressurized planar electrochromatography (PPEC) systems. Here we present a correlation of the selectivity of peptide separation in similar, as well as in various HPTLC and PPEC systems investigated before. Our results show that the selectivity in similar HPTLC and PPEC systems is quite different. This results from the share of electrophoresis in separation of solutes by PPEC. The results suggest that combination of HPTLC and PPEC, with properly selected separation conditions (the same, or even better—different for each technique), may be used for efficient two-dimensional separation of peptides. The best separation can be obtained if PPEC is carried out in two opposite directions (toward the cathode and the anode) simultaneously.

Keywords Hypothetical two-dimensional separation · Peptide separation · Correlation of separation selectivity · Pressurized planar electrochromatography · High-performance thin-layer chromatography

Introduction

The pH change of the mobile phase is the most common approach to fine tuning the selectivity of peptide separation in thin-layer chromatography/high-performance thin-layer chromatography (TLC/HPTLC). It is used for two-dimensional (2D) separation of both simple [1] and relatively complex peptide mixtures [2, 3]. Chromatograms are developed with an acidic mobile phase in one dimension, and with a basic mobile phase (of relatively similar composition) in the second dimension. However, as the influence of the mobile phase pH on retention of peptides is rather moderate [4], the difference in selectivity of two systems involved in 2D separation process is also limited. In result, most of the solute zones separated are located near the diagonal of the chromatographic plate [1–3]. Better change of separation selectivity can be obtained with the use of special chromatographic plates showing different properties of adsorbent for each dimension of separation. This is obvious, as the change of selectivity of separation depends mainly on the different properties of the adsorbent. Anyway, this approach requires preparation of the special adsorbent layer [5]. A much easier approach is to use overall inversion of separation system type—from normal phase (NP) to reversed phase system (RP) or vice versa [6]. This can be obtained with commercially available chromatographic plates of mixed properties of adsorbent (e.g. HPTLC RP-18 W plates from Merck). The mechanism of retention depends on the mobile phase composition; thus selectivity of peptide separation is strictly different for each dimension of 2D separation [6, 7].

Another way to obtain significant change of selectivity of peptide separation is the use of electric field to influence the selectivity. Combining TLC/HPTLC with planar/thin-layer electrophoresis [8–10] or electrochromatography [11] has been reported to provide very good 2D separation of complex mixtures of peptides, with clearly different selectivity for each dimension. Moreover, use of electromigration techniques enables to obtain much faster separation on a longer distance, than use of standard planar techniques (TLC/HPTLC). However, planar electrochromatography in an open system can also suffer some disadvantages, making

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the separation conditions unrepeatable. These are caused by: evaporation of the mobile phase (or its components), flux of the mobile phase to the surface of adsorbent layer (dispersion of solute zones being separated), and extensive Joule's heat which is hard to control. All these disadvantages can be abolished, while using modern equipment for pressurized planar electrochromatography (PPEC) [12–17]. So PPEC seems to have sufficient potential and should be considered as an alternative for the other planar techniques used for separation of peptides.

In our previous papers we have presented detailed investigations concerning influence of particular variables on selectivity and efficiency of peptide separation in HPTLC [4, 6, 18, 19] and PPEC [4, 20] systems, under various conditions. The aim of this work was the correlation of the selectivity obtained using similar HPTLC and PPEC separation systems studied before and evaluation of potential utility of combination of these both techniques for 2D separation of peptides.

Experimental

Chemicals and Equipment

Ninhydrin (98%, analytical grade) was purchased from Fluka (Buchs, Switzerland). Trifluoroacetic acid (TFA, 99.5%, for biochemistry) was purchased from ACROS ORGANICS (Geel, Belgium). Formic acid (FA), ammonia, acetone, and methanol (all analytical grade) were provided by POCH (Gliwice, Poland). Glass-backed HPTLC RP-18 W plates were purchased from Merck (Darmstadt, Germany). Water used in all experiments was purified using HLP demineralizer from Hydrolab (Gdańsk, Poland). Horizontal DS-II-10 × 10 chambers for TLC were received from CHROMDES (Lublin, Poland). Prototype PPEC equipment, described elsewhere [21], was constructed in the Department of Physical Chemistry, Medical University of Lublin (Lublin, Poland). LINOMAT 5 semi-automatic TLC sampler, TLC Scanner 4, and TLC Visualizer with winCATS 1.4.8 software were provided by CAMAG (Muttenz, Switzerland). Liberty microwave automated peptide synthesizer was from CEM (Matthews, USA).

Peptide Standards

Synthetic peptide standards of the following sequences were used in experiments:

1. Leu-Ile-Thr-Thr
2. Asn-Ser-Tyr-Tyr
3. Asp-Glu-Lys-Arg
4. Ser-Lys-Arg
5. Ser-Glu-Asp
6. Ser-His-His
7. Gly-Ala
8. Gly-Leu-Ile
9. Leu-Val-Val-Tyr-Pro-Trp-Thr
10. Gly-Ala-Val-Ser-Thr-Ala—Necrofibrin
11. Leu-Pro-Pro-Ser-Arg—Lymphocyte activating pentapeptide
12. Tyr-Arg—Kyotorphin
13. Ala-Pro-Gly-Pro-Arg—Eneterostatin
14. c[Cys-Tyr-Phe-Gln-Asn-Cys]-Pro-Lys-Gly-NH2 (disulfide bridge between Cys1 and Cys6)—[Lys8] Vasopressin
15. Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-NH2—β-neoendorphin
16. Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-NH2—Adrenorphin
17. Asp-Arg-Val-Tyr-Ile-His-Pro-Phe—Angiotensin II
18. Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg—Bradykinin
19. Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH2—Substance P
20. Thr-Lys-Pro-Arg—Tuftsin
21. Leu-Ile-Thr(p)-Thr(p) (Thr3 and Thr4 phosphorylated)
22. Leu-Ile-Tyr(p)-Tyr(p) (Tyr 3 and Tyr4 phosphorylated)

Peptides: Leu-Ile-Thr-Thr, Asn-Ser-Tyr-Tyr, Asp-Glu-Lys-Arg, Ser-Lys-Arg, Ser-Glu-Asp, Ser-His-His, Gly-Ala, Gly-Leu-Ile, Leu-Val-Val-Tyr-Pro-Trp-Thr, Gly-Ala-Val-Ser-Thr-Ala, Leu-Pro-Pro-Ser-Arg, Tyr-Arg, Ala-Pro-Gly-Pro-Arg, were synthesized in the Department using Liberty microwave automated peptide synthesizer (CEM, Matthews, USA). Their identity was confirmed as described elsewhere [19]. Peptides: c[Cys-Tyr-Phe-Gln-Asn-Cys]-Pro-Lys-Gly-NH_2, Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-NH_2, Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-NH_2, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe, Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg, Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2, Thr-Lys-Pro-Arg, Leu-Ile-Thr(p)-Thr(p), Leu-Ile-Tyr(p)-Tyr(p), (purity ≥ 85%) were purchased from Lipopharm (Zblewo, Poland).

Chromatography and Electrochromatography of Peptides

Preparation of Chromatographic Plates

Before use, the chromatographic plates (10 × 10 cm for HPTLC and 20 × 10 cm for PPEC) were washed by dipping for 5 min in methanol. After that, they were dried at room temperature and activated in the oven at 105 °C for 15 min. Then they were stored in an desiccator. Additionally, for PPEC, edges of the plates were impregnated with special sealant as described elsewhere [22].

Application of Samples

The peptides were dissolved in the mixture composed of water/methanol (1/1 v/v) to obtain 1 μg μL^{-1} solutions. For HPTLC, 1 μL of each sample solution was applied 10 mm from the lower edge of the chromatographic plate as a spot with a diameter of 1 mm with aerosol applicator Linomat 5 at speed 70 nL s^{-1}. Analogously, for PPEC—2 μL of each sample was applied 15 mm from the lower edge of the chromatographic plate as 2 × 1 mm bands.

Development of Chromatograms and Electrochromatograms

The chromatograms were developed at room temperature, using Horizontal DS Chamber for TLC, model DS-II-10 × 10 from CHROMDES (Lublin, Poland). Before development, the chromatographic plates were conditioned for 15 min in vapors of the mobile phase. The development distance was 8 cm from the origin.

The electrochromatograms were developed using PPEC equipment described elsewhere [21]. Before development, the chromatographic plates were prewetted/conditioned for 2 min with the mobile phase. The electrical potential used was equal to 1000 V (for the mobile phase containing methanol and ammonium acetate) [4], 400 V (for the mobile phase containing methanol and TFA) or 600 V (for the mobile phase containing propanol and TFA) [20]. The development time was 15 min. Adsorbent layer of the chromatographic plate was pressurized by an external pressure equal to 20 bar. The temperature of separation was set to 25 °C. As the current PPEC equipment enables separation in one direction only in the same time—toward the cathode or the anode (sample application position is 15 cm from the lower edge of the chromatographic plate). The two groups of peptides migrating in opposite directions (here at high pH) were separated and registered in individual subsequent experiments. It was possible due to inversion of polarization of the electrodes.

Detection of Peptides and Determination of Their Retardation Factor/Migration Distance

After development, chromatographic plates were dried at room temperature, then dipped (for about 2 s) in 2% (w/v) ninhydrin solution in acetone/methanol/glacial acetic acid (125/125/10 v/v/v) as described elsewhere [23]. After that, the plates were dried and kept in darkness at room temperature till distinct peptide zones appeared (usually, for good detection—till the next day). Then they were scanned with TLC Scanner 4 at 520 nm wavelength and imaged using TLC Visualizer from CAMAG (Muttenz, Switzerland). Migration distance values were obtained automatically using winCATS software. Mean R_f and migration distance values have been calculated on the basis of the results obtained in three independent experiments. For HPTLC, the majority of the data obtained was in the range ± 1% of mean R_f value; however, the maximum error was ± 3%. For PPEC, majority of the data obtained was in the range ± 2% of mean migration distance and the maximum error was ± 7%.

Results and Discussion

On the basis of our previous research, we have selected a few separation systems of relatively good separation selectivity of the peptides with HPTLC [4, 6, 19] and PPEC [4, 20]. Figure 1 presents a correlation of the migration distances of the same solutes separated in similar HPTLC and PPEC systems. The diagrams can be considered as hypothetical 2D chromatograms/electrochromatograms. The results show that separation selectivity obtained with PPEC is clearly different than the one obtained with HPTLC. Comparison of the NP-HPTLC and NP-PPEC systems shows that the differences in selectivity are quite significant, despite the difference between extreme (the highest and the lowest) migration distances of solutes is rather moderate (about 50 mm for HPTLC and about 35 mm for PPEC; Fig. 1a). Only one point of peptide no. 14 is located on the diagonal of hypothetical chromatogram, while the remaining peptide points are distributed outside the diagonal. The comparison of the RP-HPTLC and RP-PPEC systems with methanol as the mobile phase component shows greater difference between the extreme migration distances (about 70 mm for HPTLC and 45 mm for PPEC); however, the difference in selectivity between the systems is somewhat smaller (more peptide points are distributed near the diagonal; Fig. 2b). Relatively great difference between the extreme migration distances (65 mm for HPTLC and 65 mm for PPEC) and significant change of selectivity (no peptide points are on the diagonal) can be obtained in the RP systems, when propanol is used instead of methanol as the mobile phase component (Fig. 2c). The greatest difference between the extreme migration distances (60 mm for HPTLC and 75 mm for PPEC) and significant difference in selectivity can be also observed when the NP-HPTLC and NP-PPEC systems with the mobile phase of high pH (11.0), Fig. 1d, are compared. Here, good separation selectivity of PPEC results from the fact that at high pH some peptides migrates toward the cathode, while some others migrate toward the anode—against the electroosmotic flow of the mobile phase [4].

The results suggest that the combination of the HPTLC and PPEC systems mentioned may be used for efficient 2D separation of peptides. However, as we have shown before [20], the extensive tailing of peptide zones occurred, when the mobile phase of low pH (addition of ion-pairing acid)

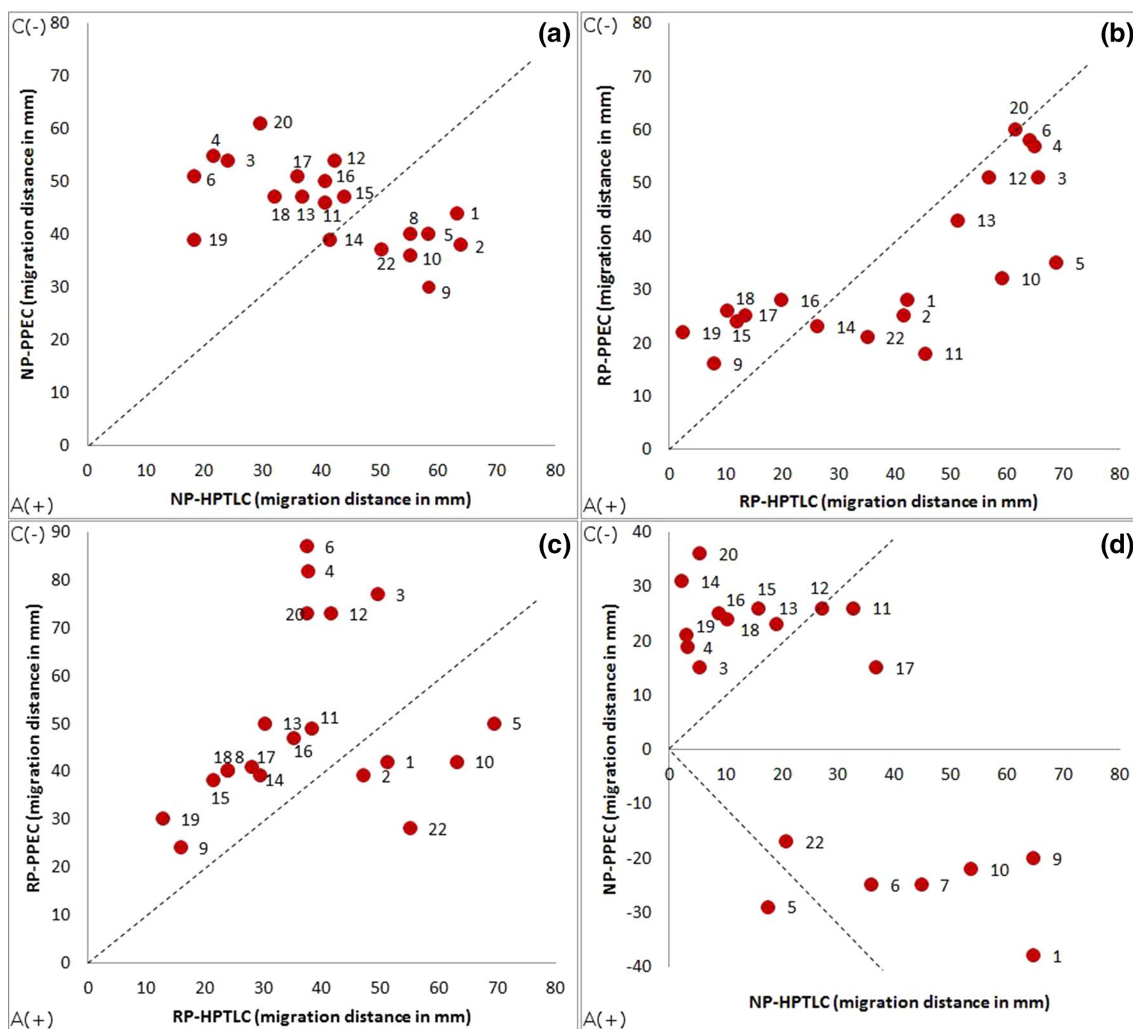

Fig. 1 Correlation of peptide migration distance in similar HPTLC and PPEC separation systems. HPTLC chromatograms' development distance was 80 mm. HPTLC RP-18 W plates from Merck were used. Mobile phases used were: **a** water/methanol 1/9 (v/v) with 25 mM TFA, **b** water/methanol 7/3 (v/v) with 100 mM TFA, **c** water/propanol 8/2 (v/v) with 100 mM FA (HPTLC) or 100 mM TFA (PPEC), and **d** water/methanol 1/9 (v/v) with 100 mM ammonium formate (pH 11.0)

was used. Contrary to HPTLC, in PPEC reduction of this tailing requires very high concentration of ion-pairing reagents (e.g. about 500 mM of TFA) in the mobile phase. This may be a serious problem, especially in RP-PPEC, because high concentration of both: water and ion-pairing reagent results in high electric current and extensive Joule's heating, as discussed elsewhere [4]. Therefore, despite the good selectivity, the use of the 2D separation systems considered (Fig. 1a–c) cannot guarantee to obtain high resolution of final separation. On the contrary, both: HPTLC and PPEC systems compared in Fig. 1d provide high separation efficiency (narrow peptide zones [4]). Therefore, combination of HPTLC and PPEC with such separation conditions can be supposed to provide efficient 2D separation of peptides.

Figure 1 presents comparison of similar HPTLC and PPEC systems. Naturally, it is possible to combine various HPTLC and PPEC systems. As we have shown in our previous papers [6, 7], it is possible to obtain significant change of selectivity of peptide separation with the inversion of separation system type (NP/RP). With such inversion, applying the electric field may result in farther/additional change of separation selectivity. As both separation systems presented above, RP (Fig. 1b) and NP (Fig. 1d), provide high efficiency of separation [4, 6] their combination can be supposed to provide also efficient 2D peptide separation. Figure 2a presents hypothetical 2D separation (or correlation of migration distances) of the solutes investigated with the systems mentioned. Figure 2b presents the comparison of migration distances of the solutes under similar conditions, however, when methanol is replaced with propanol in RP-HPTLC. This provides some additional change of selectivity with respect to the one presented in Fig. 2a. The results show

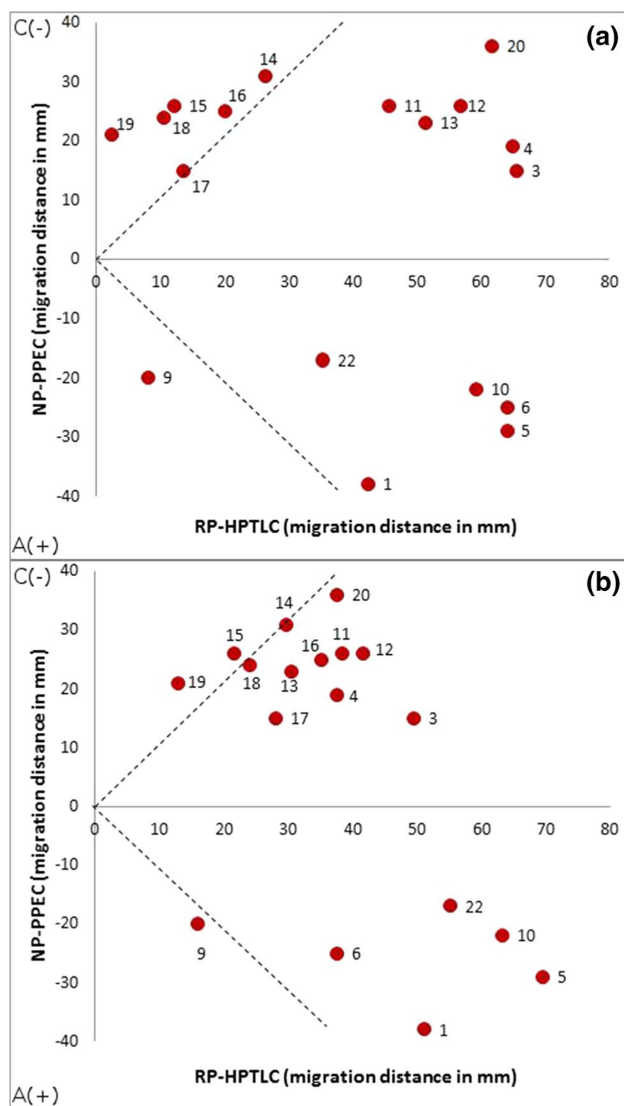

Fig. 2 Hypothetical 2D separation of peptides obtained with combination of RP-HPTLC and NP-PPEC. HPTLC chromatograms' development distance was 80 mm. HPTLC RP-18 W plates from Merck were used. Mobile phases used for HPTLC were: **a** water/methanol 7/3 (v/v) with 100 mM TFA and **b** water/propanol 8/2 (v/v) with 100 mM FA. Mobile phase used for PPEC was water/methanol 1/9 (v/v) with 100 mM ammonium formate (pH 11.0)

that combination of various HPTLC and PPEC systems may provide even greater change of selectivity, and even better 2D separation of peptides, than combination of the similar or identical systems. Unfortunately, for now, we are unable to obtain such separations, as our current PPEC equipment does not enable to separate solutes in two opposite directions (toward the anode and toward the cathode) simultaneously. In our current equipment samples can be applied only along the start line 15 cm from the lower edge of the plate. This results from the construction of PPEC chamber—especially from the position of the partition covering the starting sample spots during prewetting of the adsorbent layer, and from the position of the cover pressurizing the chromatographic plate [21]. We have suggested usefulness of the construction change of the equipment before in this regard [4].

Conclusions

The same HPTLC and PPEC systems provide quite different selectivity of peptide separation. This is due to the share of electrophoresis in PPEC system. Combination of similar HPTLC and PPEC systems may be used for 2D separation of peptides, with good overall selectivity. Even better selectivity of separation may be obtained by combining of various HPTLC and PPEC systems (e.g. NP/RP). However, combination of the same HPTLC and PPEC systems in 2D separation should be used with care (or should be avoided) because under some conditions the later can provide lower efficiency than the former. Our results also suggest that the best final selectivity of peptide separation (and to our current knowledge—also the highest efficiency of separation) can be obtained if PPEC separation is carried out at high pH of the mobile phase, in the two opposite directions (toward the anode and toward the cathode) simultaneously. Anyway, such separation requires construction of a special PPEC equipment.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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