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Note

Use of ion-paired, reversed-phase thin-layer chromatography for the analysis of peptides

A simple procedure for the monitoring of preparative reversed-phase high-performance liquid chromatography

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Recent studies have demonstrated that the use of C_{18}-microparticulate silica, which is packed in flexible-walled cartridges that can be subjected to radial compression, allows the rapid and effective separation of peptide and protein mixtures[1–6]. One problem with these studies was the lack of a suitable thin-layer chromatographic (TLC) system for the monitoring of the separation. However, a number of reversed-phase TLC plates have recently become available and used for the analysis of phenols[7], benzo[*a*]pyrene metabolites[8], dyes and plant pigments[9], cephalosporin intermediates[10], bile acids[11], peptides[12–15] and amino acids[16–17]. The purpose of this study was therefore to develop a reversed-phase TLC system which allowed the efficient anlysis of high-performance liquid chromatographic (HPLC) fractions. The system described here uses Whatman $KC_{18}F$ plates with a mobile phase which contained an aqueous solution of sodium chloride (3%) and sodium dodecylsulphate (SDS, 0.2%)–acetonitrile–methanol (50:10:10, apparent pH = 2.50).

EXPERIMENTAL

Materials

The solvents used were purified as described previously[1] and the peptides studied were obtained from the sources described in Table I. The octadecapeptide was prepared by standard solid phase synthetic techniques used in this laboratory[19] The Whatman $KC_{18}F$ reversed-phase TLC plates were obtained from Whatman (Clifton, NY, U.S.A.).

TLC procedures

The TLC plates were unwrapped just prior to spotting samples, and the chromatography tanks were allowed to equilibrate in the solvent for 30–60 min prior to the development of the chromatogram. For impregnation the reversed-phase plates were immersed in ethanol–solvent II (1:1) for 30 sec and then dried overnight at room temperature or in an oven at 80°C for 5 min.

Peptides were dissolved in 50% acetic acid at a concentration of approximately

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10 mg/ml and small spots of a maximum 1.5 mm diameter (usually 0.3 μl) were introduced on the plate. After drying in a stream of air at room temperature, the plates were developed over a distance of 50 mm from the origin. The plates were then dried in an oven at 80°C, sprayed with a ninhydrin solution (1 % w/v, acetone–acetic acid, 96:4) and heated again to develop the colour. The resulting spots were of a good shape and compact (usually 3 mm diameter at an R_F of 0.5).

Solvent systems

I: water (50 ml), NaCl (1.5 g), SDS (0.1 g), acetonitrile (10 ml) and methanol (10 ml). To this mixture acetic acid was added to adjust the apparent pH to 2.5.

II: same composition as solvent I except that the amount of acetonitrile was increased to 20 ml.

III: same composition as solvent I except that the amounts of both acetonitrile and methanol were increased to 20 ml.

Equipment

A Waters Assoc. (Milford, MA, U.S.A.) HPLC system was used, which consisted of two M6000A solvent delivery units, an M660 solvent programmer and a

TABLE I

THE ANALYSIS OF PEPTIDES BY REVERSED-PHASE TLC

*Peptide**	R_F *in solvent***		
	I	*II*	*III*
Dipeptides			
Arg–Phe[1]	0.42		
Leu–Trp[1]	0.16	0.28	0.43
Phe–Ala[1]	0.54		
Gly–Phe[2]	0.52		
Tripeptides			
Arg–Phe–Ala[1]	0.44		
Leu–Trp–Met[1]	0.08	0.2	0.32
Met–Arg–Phe[1]	0.26	0.42	0.50
Gly–Leu–Ala[3]	0.50		
Gly–Leu–Tyr[4]	0.40		
Tetrapeptides			
Leu–Trp–Met–Arg[2]	0.12	0.30	0.43
Met–Arg–Phe–Ala[2]	0.26	0.42	0.52
Pentapeptide			
Leu–Trp–Met–Arg–Phe[2]	0.01	0.12	0.16
Octapeptide			
Phe–Val–Gln–Trp–Leu–Met–Asp–Thr[5]	0.00	0.13	0.16

* The peptides were obtained from the following suppliers: 1, Research Plus Lab. (Denville, NJ, U.S.A.); 2, BDH (Poole, Great Britain); 3, Nutritional Biochemicals (Cleveland, OH, U.S.A.); 4, Sigma (St. Louis, MO, U.S.A.); 5, Mann Research Labs. (New York, NY, U.S.A.).

** The TLC system used is described in the Methods section.

U6K universal liquid chromatograph injector, coupled to a Model RCM-100 Radial Compression Module equipped with a Radial-PAK-C_{18} cartridge. This system was coupled to an M450 variable-wavelength UV spectrophotometer (Waters Assoc.) equipped with a 1-cm flow-through cell, and an Omniscribe two-channel recorder (Houston Instruments, Austin, TX, U.S.A.). The sample was injected using a Microliter 825 syringe (Hamilton, Reno, NV, U.S.A.).

RESULTS AND DISCUSSION

Brinkman and De Vries[18] found that Whatman $KC_{18}F$ TLC plates developed rapidly with aqueous–organic solvent mixtures and, relative to other reversed-phase TLC plates, demonstrated superior wettability with mobile phases which contained more than 20–40% water. Also these authors found that mobile phase with proportions of water of over 40% must contain 3% NaCl to avoid flaking of the precoated reversed-phase layers. In our studies 3% NaCl was always added to the mobile phases, as it had been previously demonstrated that a high concentration of an electrolyte suppresses unwanted interactions between silanol groups present in the reversed-phase and ammonium groups present in the peptide[5].

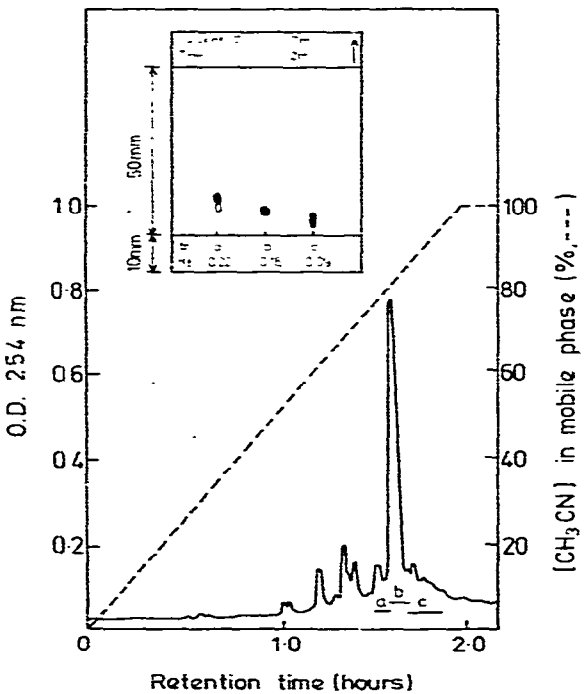

Fig. 1. The semipreparative purification of a synthetic octadecapeptide. Leu–Glu–Ser–Phe–Leu–Lys–Ser–Trp(CHO)–Leu–Ser–Ala–Leu–Glu–Gln–Ala–Leu–Lys–Ala. The chromatographic conditions were described in the Methods section. Solvent A consisted of aqueous triethylammonium phosphate (1.5 m*M*, pH 3.2) and solvent B of propan-2-ol–acetonitrile–aqueous triethylammonium phosphate (7.5 m*M*), (40:40:20, v/v/v). The flow-rate was 1.0 ml/min and the gradient (A to B) shown by the dotted line was used. A 5-mg sample of the peptide dissolved in buffer A (0.2 ml) was used. The inset shows the corresponding reversed-phase TLC separation of the fractions.

Several authors[7,8,13,15] have found that a suitable hydrophobic ion-pairing reagent must be added to the mobile phase before polar samples can be satisfactorily chromatographed on a reversed-phase TLC plate. The ion-pairing reagent is particularly useful in reducing the mobility of polar solutes and in minimizing spreading of the spots. For example, Lepri *et al.*[7,15,16] used dodecylbenzenesulphonic acid in the TLC of amino acids and peptides. In this study we found that a significant improvement in the shape of the spots we obtained by the addition of SDS (0.2%) to the mobile phase, and by decreasing the apparent pH to 2.5. SDS was found to give better results than octylnaphthyl- or dodecylsulphonic acid or the triethylammonium phosphate that was used in the analytical reversed-phase HPLC separation shown in Fig. 1. At higher concentrations of SDS (up to 0.6%) a small decrease in R_F was observed, a result which agreed with concentration effects described by Lepri *et al.*[7]. The size of the spots was minimised and resolution was improved if the $KC_{18}F$ plates had been previously impregnated with the mobile phase (see Methods section).

The separation of a number of peptides with the TLC system is shown in Table I. As is often observed in reversed-phase HPLC[20], mobile phases which contained acetonitrile were found to give more efficient separations of peptides in reversed-phase TLC, than was achieved with methanol. As was found in earlier studies[15], the presence of hydrophobic and basic residues in the peptide lead to increased interaction of the sample with the stationary phase and thus lower R_F values. Conversely an increase in the amount of methanol or acetonitrile in the mobile phase (see solvent systems II and III in Table I) resulted in a higher R_F value.

Fig. 1 shows use of the reversed-phase TLC system to follow the semipreparative purification of a synthetic octadecapeptide, Leu–Glu–Ser–Phe–Leu–Lys–Ser–Trp(CHO)–Leu–Ser–Ala–Leu–Glu–Gln–Ala–Leu–Lys–Ala. The TLC results provided a rapid check of the HPLC separation and also verified that the optical density peaks actually consisted of peptidic material (ninhydrin reactive). This peptide contained a large number of hydrophobic residues and therefore it was strongly retained on a reversed-phase TLC plate even with solvent III as the mobile phase. However, satisfactory results were obtained when tetrahydrofuran was added to the mobile phase (2 ml of THF to 7 ml of solvent II) and the narrow spots shown in Fig. 1 were obtained. Despite differences in the mobile phases there is a clear correlation between the HPLC and TLC systems, for example the early eluting material in pool A of the HPLC fractions gave the highest R_F in the reversed-phase TLC system.

In conclusion, it was found that a reversed-phase TLC system based on Whatman $KC_{18}F$ plates and a mobile phase which contained 3% sodium chloride and 0.2% sodium dodecylsulphate, allowed the efficient separation of both peptide standards and fractions from a HPLC separation.

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