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Note

Separation of phenylthiohydantoin-amino acids by high-performance liquid chromatography

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High-performance liquid chromatography (HPLC) has emerged as the most satisfactory method for the identification of the phenylthiohydantoin-amino acids (PTH-amino acids) produced by the Edman sequence analysis of proteins and peptides. Thin-layer chromatography (TLC) and gas–liquid chromatography (GLC) have been widely used but both of these methods have shortcomings. Quantitation is difficult using TLC and, using GLC, the more polar PTH-amino acids suffer from thermal decomposition and lack of volatility which necessitates their conversion to trimethylsilyl derivatives prior to chromatography.

The separation of PTH-amino acids by HPLC has, apart from some recent work[1] using a bonded tripeptide stationary phase, been based on either chromatography on silica[2–6] or reversed-phase chromatography[7–16]. This latter technique is particularly attractive in view of its suitability for the chromatography of those PTH-amino acids which are least satisfactorily chromatographed by GLC and, furthermore, one separation has been reported[11] using this technique in which the resolution of all 20 PTH-amino acids was achieved in a single run although the authors stated that the conditions required for this were rather critical.

It is apparent that it is not easy even with reversed-phase chromatography to obtain adequate resolution in a single run, and that the acidic and the basic PTH-amino acids are often involved in this difficulty. It is also apparent that little work has been done on the eluents used, which invariably consist of acetate buffer usually adjusted to a pH value in the region of 4–5, and containing acetonitrile or, more recently, methanol. It has been stated[15] that the pH value of the eluent may be varied between 4.0 and 7.4 without effect on the separation. But the retention times of the acidic PTH-amino acids should depend on their degree of ionisation and can be expected to be quite sensitive to changes of pH in the region of pH 4. Concerning the basic PTH-amino acids, PTH-histidine and PTH-arginine, it has been shown[17] that hydrophilic amines may chromatograph as ion-pairs in reversed-phase chromatography on a hydrocarbon stationary phase which suggested the possibility of varying the retention times of these PTH-amino acids by changing the anion present in the eluting buffer. A suitable choice, therefore, of both the pH and the counter ion should facilitate the resolution in a single chromatographic run of the acidic and the basic PTH-amino acids.

EXPERIMENTAL

The chromatograph consisted of a Model 660 programmer, two Model 6000 pumps, a Model U6K injector and a μBondapak C_{18} column of 4-mm bore and 300-mm length (Waters Assoc., Hartford, Great Britain). Chromatography was performed at room temperature which was *ca.* 20°. Elution was by a gradient generated with a pair of eluents, A and B, whose composition is described below. Curve 7 on the Model 660 programmer was used, running from 5% B to 100% B in 33 min.

A CE 212 UV photometer (Cecil, Cambridge, Great Britain) fitted with a 10-μl flow cell and operated at 268 nm was used for detection.

The PTH-amino acids were dissolved in methanol and, except where otherwise stated, samples of 10 μl containing *ca.* 40 nmol of each PTH-amino acid were injected and eluted using a flow-rate of 4.5 ml/min (4000 p.s.i.).

RESULTS AND DISCUSSION

A series of buffers was prepared as shown in Table I using, as a weak base, diethylenetriamine (DETA), which has a pK_a of 4.2, together with trichloroacetic acid (TCA). Using each of these buffers in turn as eluent A, together with a buffer of the same composition except that it incorporated acetonitrile (60% v/v) as eluent B, the results presented in Fig. 1 were obtained. This shows the retention times of the acidic and the basic PTH-amino acids along with, for comparison, the retention times of several of those of the neutral PTH-amino acids which elute most closely to the acidic and the basic PTH-amino acids. It is clear that the retention times of the acidic PTH-amino acids were markedly influenced by changes in pH in the range 3.7–4.9, an increased pH causing an earlier elution of these PTH-amino acids, while the neutral and the basic derivatives were unaffected.

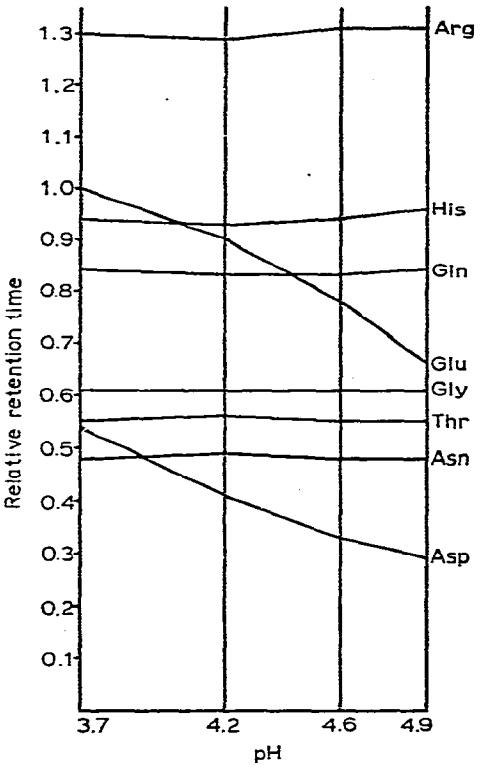
TABLE I

COMPOSITION OF DETA–TCA BUFFERS

DETA (mM)	*TCA (mM)*	*pH*
7.3	20.0	3.7
8.0	20.0	4.2
8.9	20.0	4.6
9.6	20.0	4.9

A second series of buffers was prepared all containing 8.0 m*M* DETA and of pH 4.2 but with either hydrochloric acid, perchloric acid, trifluoroacetic acid (TFA) or dichloroacetic acid (DCA), each 20 m*M*. Use of these buffers with the gradient described above of acetonitrile concentration gave the results shown in Fig. 2 which also includes the results obtained above with the TCA buffer of pH 4.2. The retention times of the basic PTH-amino acids only are clearly influenced by the nature of the counter ion present.

From these results it was apparent that both effects should combine in a DETA–DCA buffer of pH 4.2 to resolve satisfactorily the acidic and the basic PTH-



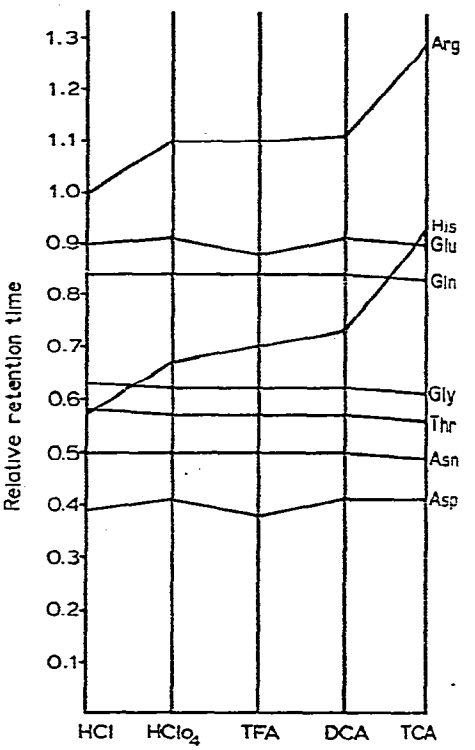


Fig. 1. Effect of pH on the relative retention times of PTH-amino acids using DETA–TCA buffers. Rentention times relative to that of PTH-alanine which was used as an internal reference and which had a retention time of 14.3 ± 0.5 min.

Fig. 2. Effect of different acids on the relative retention times of PTH-amino acids using DETA buffers of pH 4.2. Rentention times relative to that of PTH-alanine (14.3 ± 0.5 min).

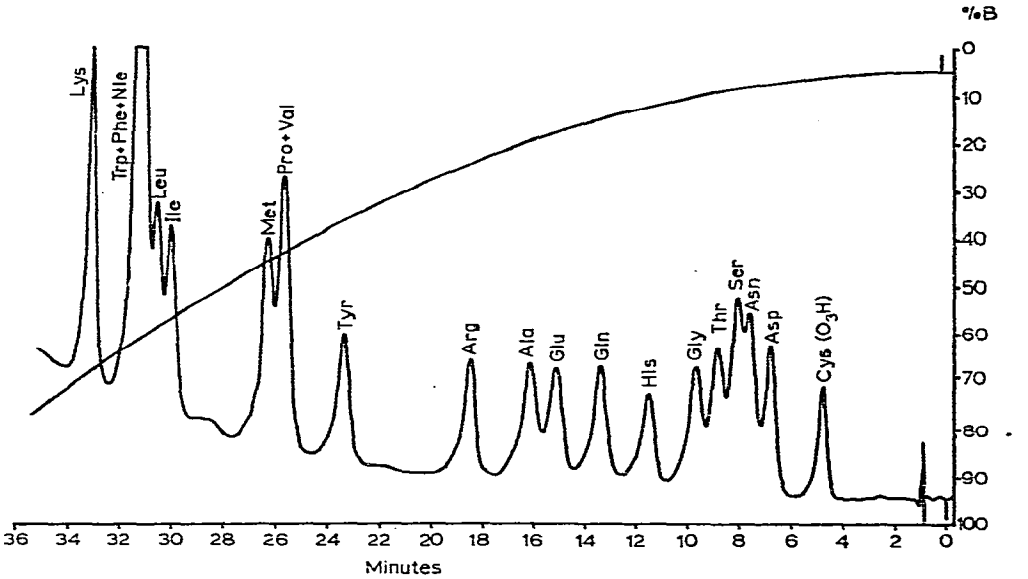


Fig. 3. Separation of PTH-amino acids using a DETA–DCA buffer of pH 4.2 with a 40-min gradient and a flow-rate of 4.0 ml/min. Sample of 14.1 μl containing 10 nmol of each PTH-amino acid. Detector sensitivity 0.2 a.u.f.s. deflection.

amino acids. This was confirmed by the chromatogram shown in Fig. 3 where, additionally, in an attempt to improve the resolution of the strongly retarded non-polar PTH-amino acids a slightly longer gradient was used. This eluent system has now been used satisfactorily for three years and it can be expected that 5-μm packing material and a column temperature raised above ambient, as used by Zimmerman *et al.*[11], would considerably increase the efficiency of the separation.

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REFERENCES

1 G. W.-K. Fong and E. Grushka, *J. Chromatogr.*, 142 (1977) 299.
2 A. P. Graffeo, A. Haag and B. L. Karger, *Anal. Lett.*, 6 (1973) 505.
3 G. Frank and W. Strubert, *Chromatographia*, 6 (1973) 522.
4 E. W. Matthews, P. G. H. Byfield and I. MacIntyre, *J. Chromatogr.*, 110 (1975) 369.
5 C. Bollet and M. Caude, *J. Chromatogr.*, 121 (1976) 323.
6 A. Devillers-Thiery and G. Blobel, *Anal. Biochem.*, 87 (1978) 66.
7 C. L. Zimmerman, J. J. Pisano and E. Appella, *Biochem. Biophys. Res. Commun.*, 55 (1973) 1220.
8 A. Haag and K. Langer, *Chromatographia*, 7 (1974) 659.
9 G. D. Lominac and H. S. Kingdon, *Arch. Biochem. Biophys.*, 173 (1976) 320.
10 C. L. Zimmerman, E. Appella and J. J. Pisano, *Anal. Biochem.*, 75 (1976) 77.
11 C. L. Zimmerman, E. Appella and J. J. Pisano, *Anal. Biochem.*, 77 (1977) 569.
12 R. Zeeuws and A. D. Strosberg, *FEBS Lett.*, 85 (1978) 68.
13 M. N. Margolies and A. Brauer, *J. Chromatogr.*, 148 (1978) 429.
14 A. S. Bhown, J. E. Mole, A. Weissinger and J. C. Bennett, *J. Chromatogr.*, 148 (1978) 532.
15 M. Abrahamsson, K. Gröningsson and S. Castensson, *J. Chromatogr.*, 154 (1978) 313.
16 J. Elion, M. Downing and K. Mann, *J. Chromatogr.*, 155 (1978) 436.
17 J. H. Knox and J. Jurand, *J. Chromatogr.*, 110 (1975) 103.