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A Preliminary Study Using Electron Capture Detection of Pentafluorophenylthiohydantoin Amino Acid Derivatives (39791)

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In the classical Edman degradation for amino acid sequencing (1), phenylisothiocyanate (PITC) has been used as the coupling reagent, followed by qualitative detection of end products by thin-layer chromatography (2, 3) or quantitative detection by gas chromatography (4) using flame ionization detection (FID), or back hydrolysis followed by amino acid analysis (5). These quantitative methods are generally sensitive in the nanomole range. Recent developments in protein microsequence methodology require more sensitive for studying proteins, such as cell membrane components, which usually are available only in very small amounts.

In order to deal with the problem of nanomole amounts of available protein and even picomole amounts of peptide cleavage products, many workers have explored improved detection and/or chemical methods for sequence determination. The use of radioactively labeled coupling reagent ([^{35}S]PITC) has been explored by a number of workers (6, 7), while others (8, 9) have utilized *in vivo* incorporation of radioactively labeled amino acids, followed by sequencing and quantitative detection. Both of these methods are time-consuming and expensive in comparison to direct detection methods involving gas chromatography or high-pressure liquid chromatography.

Lequin and Niall (10) previously have reported the chemical properties and gas chromatographic behavior of derivative amino acids (pentafluorophenylthiohydantoins) prepared using pentafluorophenylisothiocyanate (PFPITC) instead of PITC as the coupling reagent in the Edman degradation. They suggested that the use of this coupling reagent would facilitate the use of gas chromatographic electron capture detection (EC), which is generally three orders of magnitude more sensitive than FID. Since investigation into the feasibility of utilizing such methodology necessitates that gas chromatographic behavior be examined, this paper is a preliminary study of the elution pattern of some pentafluorophenylthiohydantoin (PFPTH) amino acid derivatives and of the sensitivity of these determinations using EC detection.

Materials and methods. Preparation of pentafluorophenylthiohydantoin (PFPTH) derivative amino acids. The basic method of Krivtsov and Stepanov (11) was used to prepare the individual PFPTH derivative amino acids with the compounds being recrystallized from the appropriate solvents. The identity of the individual derivatives was ascertained by elemental analysis (Galbraith Laboratories, Knoxville, Tenn.) and purity was ascertained by gas chromatographic analysis, using flame ionization detection.

Gas chromatography. A Varian 1800 gas chromatograph with a 6-ft × ¼-in. column containing a liquid phase of 5% SP-400 on Varaport 30 (100/200 mesh) was used to separate the PFPTH derivatives. The injector temperature was 285° and the detector oven was set at 300°. For electron capture detection on an 8-mCi ^{63}Ni source was used with applied voltage. All other parameters are specified in the individual figures.

Chemicals. Amino acids were NRC grade from Cyclo Chemical Co.: PFPITC was obtained from Pierce Chemical Co. and checked for purity by IR spectra; Tri-Sil/BSA was from Pierce Chemical Co.; SP-400 was from Supelco; Varaport 30 was from Varian; all other chemicals used were spectrochemical grade.

Results. Gas chromatographic analysis of pentafluorophenylthiohydantoin amino acids

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using flame ionization detection. The gas chromatographic separation of these derivatives on a 5% SP-400 liquid phase as detected by flame ionization is depicted in Fig. 1A. In comparison to separations obtained by Lequin and Niall (10) using 10% DC-560, the derivatives of isoleucine and leucine separate well without modification with silylating agent. In addition, serine and threonine are also detectable without further modification by silylation. As some derivatives are not eluted from the packing unless modified by silylation, treatment with Tri-Sil/BSA at 80° for 20 min gives an elution pattern of the derivative amino acids as depicted in Fig. 1B.

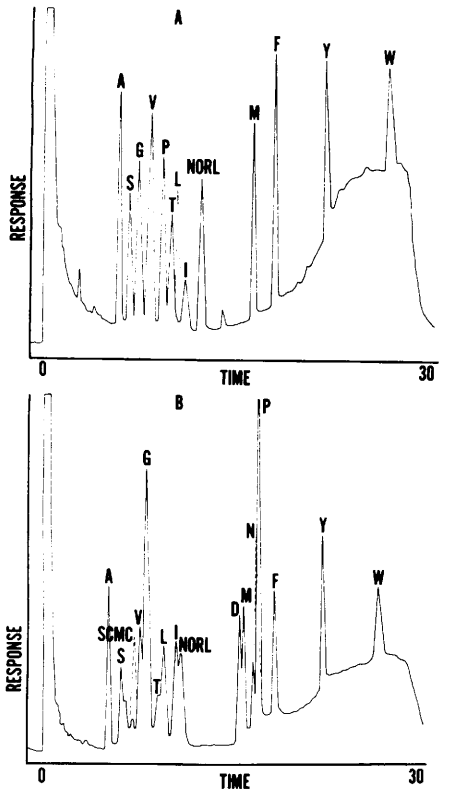

FIG. 1. PFPTH amino acid elution using FID at 8 × 10^{-10} A/mV; 1-μl sample of a 1-mg/ml solution. N_2 carrier, 50 ml/min; H_2, 50 ml/min; air, 500 ml/min. Initial temperature of 190° for 10 min; linear 8°/min increase for 10 min; final temperature of 270° for 10 min. A = alanine, S = serine, G = glycine, V = valine, P = proline, T = threonine, L = leucine, I = isoleucine, NORL = norleucine, M = methionine, F = phenylalanine, Y = tyrosine, W = tryptophan, SCMC = s-carboxymethyl cysteine, D = aspartic acid, E = glutamic acid, N = asparagine.

Gas chromatographic analysis and quantitation of pentafluorophenylthiohydantoin amino acids using electron capture detection. Analysis of the derivatives using electron capture detection is shown in Fig. 2. The response shown was obtained with 1 ng (1–3 pmole) of each derivative. This is three orders of magnitude more sensitive than the corresponding response using FID. The response of the initial derivatized amino acids is dependent upon the log of amount of sample injected, and is shown over a 10-fold range in Fig. 3.

The later eluting derivatives cannot be determined using our EC system, as the increase in baseline upon temperature programming obviates any sensitivity in this region. This problem should be circumvented by differential column balancing with two EC detectors, which effects subtraction of background noise between columns and results in a normalized baseline.

Discussion. The results of these experiments indicate that the PFPTH derivatives

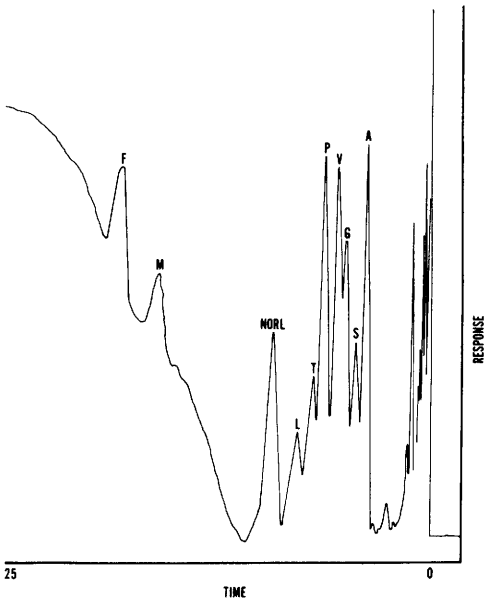

FIG. 2. PFPTH amino acid elution using EC at 2 × 10^{-10} A/mV; 1-μl sample of a 1-μg/ml solution. N_2 carrier, 50 ml/min. Initial temperature of 190° for 10 min; linear increase at 8°/min for 10 min; final temperature of 270° for 10 min. Abbreviations are the same as those used in Fig. 1.

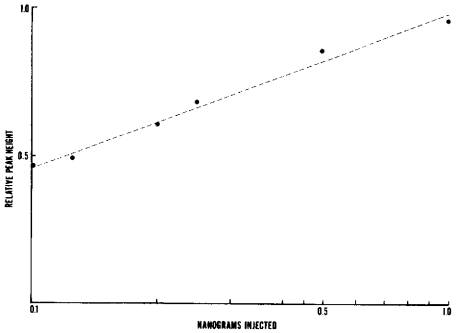

FIG. 3. One-microliter aliquots of PFPTH valine were analyzed using the same gas chromatographic conditions as those in Fig. 2. Slope = 0.510, correlation coefficient = 0.994.

can be detected, using electron capture, with a 1000-fold increase in sensitivity over flame ionization. The utilization of differential column balancing should implement detection of the later eluting derivatives, thereby giving a full range of analytical utility for amino acid sequence determination.

Although the exact chemical and physical parameters for automated use of this coupling reagent have yet to be defined, this method of detection for sequence derivatives provides an alternative means of scaling down the required sample size. Clearly, available analytical methods have become an important limiting factor in contemporary sequence analysis.

Summary. Pentafluorophenylthiohydantoin amino acids can be detected using electron capture analyses. The sensitivity of the method is in the picomole range and is comparable to the more expensive and laborious radioactive detection techniques.

1. Edman, P., Acta Chemica Scand. **4,** 283 (1950).
2. Edman, P., and Begg, G., Europ. J. Biochem. **1,** 80 (1967).
3. Summers, M. R., Smythers, G. W., and Oroszlan, Anal. Biochem. **53,** 624 (1973).
4. Pisano, J. L., and Bronzert, T. L., J. Biol. Chem. **244,** 5597 (1969).
5. Smithies, O., Gibson, D., Fanning, E. M., Goodfliesh, R. M., Gilman, J. G., and Ballantyne, D. L., Biochemistry **10,** 4912 (1971).
6. Jacobs, J. W., and Niall, H. D., J. Biol. Chem. **250,** 3629 (1975).
7. Bridgen, J., Biochem. **15,** 3600 (1976).
8. McKean, D. J., Peters, E. H., Waldby, J. I., and Smithies, O., Biochemistry **13,** 3048 (1974).
9. Silver, J., and Hood, L., Nature (London) **256,** 63 (1975).
10. Lequin, R. M., and Niall, H. D., Biochem. Biophys. Acta **257,** 76 (1972).
11. Krivtsov, V. F., and Stepanov, V. M., J. Gen. Chem. U.S.S.R. **35,** 556 (1965).

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