Journal of Chromatography, 97 (1974) 289–292

CHROM. 7541

Note

Subnanomole detection of phenylthiohydantoins of amino acids after thin-layer chromatography

A. S. INGLIS and P. W. NICHOLLS
Division of Protein Chemistry, CSIRO, Parkville (Melbourne), Victoria 3052 (Australia)
(Received April 16th, 1974)

Recently it has been shown that the limits of detection of amino acid phenylthiohydantoins (PTHs) on silica gel plates could be extended in the subnanomole region by exposing the plates to iodine vapour before viewing under short wavelength UV (254 nm) radiation[1].

In two recent publications[2,3] Frei and co-workers have developed a fluorescent method for the detection of organosulphur compounds by using a mixture of calcein containing a slight molar excess of palladium(II). Displacement of the palladium from this non-fluorescent complex occurs in the presence of sulphur-containing compounds and results in the liberation of the calcein fluorophore which may be observed by its excitation under long wavelength UV (366 nm) light. We have found this procedure to be applicable to the detection of subnanomole quantities of PTHs on thin-layer plates and have observed some advantages over the iodine vapour method[1] for the detection of PTHs at this level.

EXPERIMENTAL

Reagents

Fluorescein complexone (calcein) and palladium(II) chloride were obtained from BDH (Poole, Great Britain). The spray reagent was prepared as directed by Frei *et al.*[2,3] to give a final solution containing 1.0×10^{-4} *M* palladium(II) and 8.0×10^{-5} *M* calcein in acetone–water (1:1). PTHs were purchased from Pierce (Rockford, Ill., U.S.A.) and standard mixtures (10^{-4} *M*) a, b and c of the PTHs were prepared in ethylene chloride and stored in the freezer.

Chromatography

Thin-layer chromatography was carried out on aluminium-backed plates (20 × 20 cm) coated with silica gel containing a fluorescent indicator (Kieselgel $60F_{254}$, Merck, Darmstadt, G.F.R.). Aliquots of the PTH solutions (1 to 10 μl) were loaded on to the plates with a Unimetrics multi-spotter (Joliet, Ill., U.S.A.) and were separated as described previously[4].

Photography

Photographs were taken under long wavelength UV radiation in a Chromato-

vue viewer (Ultra-Violet Products, San Gabriel, Calif., U.S.A.). Routinely a Polaroid Land Camera Model CU5 (Polaroid, Cambridge, Mass., U.S.A.) with a 5-in. lens (+ 1.5 filter) was rested on top of the filter in the Chromatovue and the film (Type 107) was exposed for 5 sec at F 16.

RESULTS AND DISCUSSION

After thin-layer chromatography[4] of the PTH standard solutions, the solvent was allowed to evaporate in a fume hood and the plates were sprayed very lightly to give a faint pink background with the palladium–calcein (PdC) reagent. Fluorescent spots, due to calcein liberated from the palladium complex in the presence of the sulphur-containing PTHs, appeared almost immediately: PTH–proline was the only exception in that the fluorescence developed very slowly and did not attain the same intensity as equivalent concentrations of the other PTHs. We observed that the background fluorescence of the chromatograms gradually increased on standing but there was no appreciable change to the eye for at least an hour after spraying (except for PTH–proline). Unlike Frei *et al.*[2,3] for qualitative work we found that humidity control was not necessary for PTHs since there was no obvious difference in stability on storing the plates under different conditions of humidity. Partial restoration of fluorescence was possible by re-applying another light spray of PdC. This behaviour is in contrast to that of the PTH–iodine complex which rapidly dissociates on removing the plate from the iodine tank and is therefore unsuitable for any subsequent quantitative measurements.

Fig. 1 clearly demonstrates the sensitivity of the PdC reagent: under long UV (366 nm) radiation PTHs could be located at the 0.1 nanomole level as yellow-green fluorescent spots. This figure also shows the much weaker response observed in the presence of PTH–proline (the fastest moving spot in standard a). This PTH is barely discernible at the 0.5 nanomole level even though the photograph was taken 2 h after spraying. Nevertheless the development of a fluorescent spot is a distinctive characteristic for the identification of PTH–proline and has been useful in this respect for samples from the protein sequenator.

The PdC spray reagent is also suitable for the detection of the basic PTHs of arginine and histidine at the 0.1 nmole range. PTH–arginine reacts in the expected way to liberate the yellow-green calcein fluorescence but PTH–histidine gives rise to a blue fluorescent entity. We later observed that PTH–histidine by itself gives rise to a violet fluorescent spot in long wavelength UV light, hence the unique fluorescence observed for PTH–histidine on spraying with the PdC reagent. This characteristic has apparently not been reported previously and should facilitate the detection and identification of histidinyl residues during protein sequencing. Moreover, we observed that both PTH–histidine and PTH–arginine could be detected at the 0.1 nmole level (which in the case of PTH–histidine represents a considerable increase in sensitivity[5]) and eliminates the need for specific tests for these basic derivatives when they are separated using the method of Inagami[5].

Unexpectedly, the thin-layer material was found to affect the results quite drastically: silica gel plates without a fluorescent indicator showed only a very weak calcein fluorescence, whereas plates containing a fluorescent indicator yielded the results shown in Fig. 1. According to West[6] the fluorescent intensity of calcein is

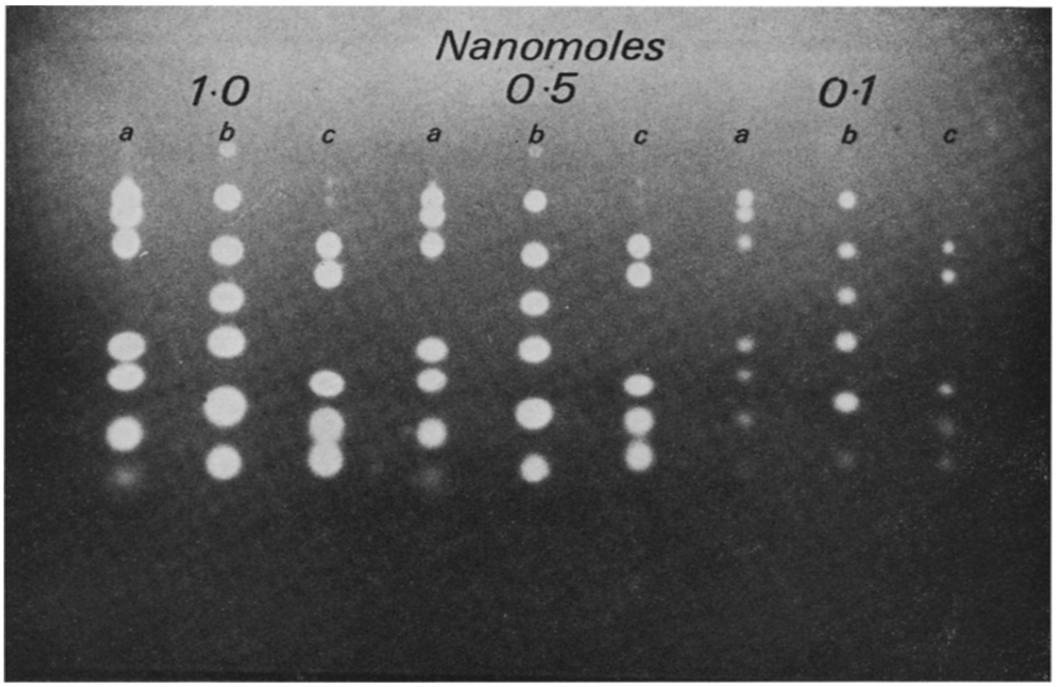

Fig. 1. Photograph under 366 nm radiation of PTHs on a thin-layer plate sprayed with the palladium–calcein reagent. Standard a contains PTHs of Asn, Ser, S-carboxymethyl-Cys, Gly, Ala, Val and Pro. Standard b contains PTHs of Gln, Thr, Tyr, N^{ε}-phenylthiocarbamyl-Lys, Met and Leu. Standard c contains PTHs of Asp, Glu, Trp, Phe and Ile.

markedly enhanced by the presence of zinc or cadmium salts and we found using atomic absorption spectrophotometry that the fluorescent indicator incorporated in the thin-layer plates was an inorganic zinc salt (most probably zinc silicate) which would explain the observed effects. The presence of the fluorescent indicator is advantageous, of course, as it may still be used to detect nanomole quantities of PTHs by their quenching of the background fluorescence under short wavelength UV (254 nm) radiation. The presence of the PdC reagents does not affect this detection method nor does it interfere with the ninhydrin reagent of Roseau and Pantel[7] for the colorimetric identification of PTHs.

The PdC reagent can therefore be used routinely following an initial examination of the plate under short wavelength UV light to detect nanomole quantities of PTHs. This approach has particular use for the detection of unstable or insensitive residues such as serine, threonine, tryptophan, S-carboxymethylcysteine and proline which may not be unequivocally identified under short wavelength UV light. Although the iodine reagent[1] can also be employed for this purpose, in practice we have found that a subsequent ninhydrin reaction[7] after the removal of iodine has not always been satisfactory because some loss in sensitivity is often observed. The fact that the PdC reagent is basically a more specific one also makes it preferable to the iodine reagent. Moreover, the basic amino acid phenylthiohydantoins from histidine and arginine can be positively identified by their differing fluorescent response in the presence of

the PdC complex. The only time we have encountered problems with the PdC reagent was during a study of the effect of mercaptoethanol additions to the reagents in the protein sequenator. Small amounts of the reductant were acceptable in the coupling buffer but otherwise the addition led to excessive background fluorescence. Presumably this will also apply to the use of other thiols, for example, dithiothreitol[8] and 1,4-butanedithiol[9], in the sequenator.

ACKNOWLEDGEMENT

The authors wish to thank Mr. Royston F. Larkin for his expert assistance with the photography.

REFERENCES

1 A. S. Inglis, P. W. Nicholls and L. G. Sparrow, *J. Chromatogr.*, 90 (1974) 362.
2 T. F. Bidleman, B. Nowlan and R. W. Frei, *Anal. Chim. Acta*, 60 (1972) 13.
3 R. W. Frei, B. L. Maclellan and J. D. MacNeil, *Anal. Chim. Acta*, 66 (1973) 139.
4 A. S. Inglis and P. W. Nicholls, *J. Chromatogr.*, 79 (1973) 344.
5 T. Inagami, *Anal. Biochem.*, 52 (1973) 318.
6 T. S. West, *Complexometry with EDTA and Related Reagents*, Broglia Press, Bournemouth, 1969.
7 G. Roseau and P. Pantel, *J. Chromatogr.*, 44 (1969) 392.
8 O. Smithies, D. Gibson, E. M. Fanning, R. M. Goodfliesh, J. G. Gilman and D. L. Ballantyne, *Biochemistry*, 10 (1971) 4912.
9 M. A. Hermodson, L. H. Ericsson, K. Titani, H. Neurath and K. A. Walsh, *Biochemistry*, 11 (1972) 4493.