Identification of Amino Acid Thiohydantoin Directly by Thin-Layer Chromatography and Indirectly by Gas–Liquid Chromatography after Hydrolysis

By MINNIE RANGARAJAN and ANDRÉ DARBRE

Department of Biochemistry, University of London King's College, Strand, London WC2R 2LS, U.K.

(Received 20 November 1974)

A method is described for the identification of amino acid thiohydantoin by two-dimensional t.l.c. An indirect method for the determination of amino acid thiohydantoin is described by which, after hydrolysis, the corresponding amino acids are determined by g.l.c.

The sequential degradation of peptides from the C-terminus by the method of Stark (1968) requires the identification of the cleaved amino acid thiohydantoin. This can be achieved qualitatively by using two-dimensional t.l.c. directly, or, after hydrolysis, by determination of the amino acids quantitatively by g.l.c.

The preferred method for determining the sequence of amino acids in proteins is that of Edman (1949), in which the N-terminal amino acids are converted into their phenylthiohydantoin derivatives (Edman & Begg, 1967; Pisano & Bronzert, 1969; Laursen, 1971; Pisano *et al.*, 1972; Peterson *et al.*, 1972). Methods for sequencing from the C-terminus are less successful and are mostly restricted to the determination of the terminal amino acid by hydrazinolysis (Akabori *et al.*, 1956) or by ³H labelling (Matsuo *et al.*, 1966), or of a limited number of residues by using carboxypeptidase (Ambler, 1967*a,b*).

The method originally proposed by Schlack & Kumpf (1926) for the formation of C-terminal peptidyl-thiohydantoin was shown to have application as a sequencing method (Stark, 1968). The amino acid thiohydantoin cleaved from the C-terminus of the peptide was not determined directly, but indirectly by difference analysis (Stark, 1968). In later work the amino acid thiohydantoin was identified by t.l.c. (Cromwell & Stark, 1969; Yamashita, 1971) and by g.l.c. and mass spectrometry (Rangarajan *et al.*, 1973; Rangarajan & Darbre, 1974). Because we were unable to identify arginine we developed a rapid two-dimensional t.l.c. separation on pre-coated polyamide plates for all the common amino acid thiohydantoin. Also, we report on the hydrolysis of the thiohydantoin to their constituent amino acids, so that these may be determined by g.l.c.

Materials and Methods

Polygram polyamide-6/UV₂₅₄ and Polygram polyamide-6 pre-coated (0.1 mm) plastic sheets (20 cm × 20 cm) and a Desaga UVIS lamp with emission at 254 nm were purchased from Camlab (Cambridge, U.K.). Chromatography was carried out with solvent system 1, dichloroethane–acetic acid (45:8, v/v), 2, chloroform–95% (v/v) ethanol–acetic acid (20:10:3, by vol.), 3, toluene–*n*-heptane–acetic acid (12:6:5, by vol.), 4, acetic acid–water (7:13, v/v), 5, acetic acid–water (1:3, v/v). Butyl-PBD [5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole] was purchased from Intertechnique Ltd., Portslade, Sussex, U.K. This was used at a concentration of 0.025% (w/v) in solvent systems 1, 2 and 3 when plates not incorporating a fluorescent indicator were used. Amino acid thiohydantoin were prepared as described by Rangarajan *et al.* (1973). Amino acids were determined as their trifluoroacetylated methyl ester derivatives (Darbre & Islam, 1968; Islam & Darbre, 1972). Acid hydrolyses were carried out in Rotaflo stopcocks (Darbre, 1971).

Results and Discussion

Thin-layer chromatography

One-dimensional t.l.c. of amino acid thiohydantoin on silica-gel plates was described by Cromwell & Stark (1969) and Yamashita (1971). We were unable to resolve unambiguously many of these compounds and therefore developed a two-dimensional separation on polyamide pre-coated plates, as reported previously for methylthiohydantoin amino acids (Rabin & Darbre, 1974).

In Table 1 the *R_F* values (×100) are reported for the one-dimensional separation of 19 amino acid thio-

Table 1. $R_F (\times 100)$ values for amino acid thiohydantoin in one-dimensional separations on polyamide plates

The solvent systems are described in the Materials and Methods section. Results are averages of four determinations. Abbreviation: CmCys, carboxymethylcysteine.

Amino acid thiohydantoin	Solvent system	...	$100 \times R_F$				
			1	2	3	4	5
Ala			57	76	31	72	64
Arg			20	80	5	90	87
Asn			18	33	2	63	75
Asp			63	49	1	70	59
Cys			76	50	39	57	36
CmCys			4	33	4	43	21
Glu			12	68	5	64	53
Gly			37	65	16	75	68
His			18	75	4	93	92
Ile			75	85	49	56	41
Leu			86	90	65	56	39
Lys			49	88	9	83	75
Met			65	84	32	61	47
Phe			83	84	51	45	29
Ser			45	87	27	58	55
Thr			40	76	25	57	44
Trp			34	67	8	27	16
Tyr			81	60	1	45	28
Val			77	88	56	63	51

thiohydantoin in five different solvent systems. The chromatography tank was used without equilibration of the atmosphere and the solvents could be used without change of R_F values for up to 1 week. The polyamide plates incorporating a fluorescent indicator were most convenient. When viewed under u.v., dark-purple spots were seen on a green fluorescent background. In the absence of this indicator, butyl-PBD was used as an external indicator, but it was soluble only in solvent systems 1, 2 and 3. With plates incorporating the fluorescent indicator, the lower third of the plate was very dark when viewed under u.v. after chromatography in solvent systems 4 and 5.

The best two-dimensional separation was obtained with solvent system 4 (Kulbe, 1971) used for the first dimension followed by solvent system 2 (Cromwell & Stark, 1969), giving a total running time of 1 h. The schematic representation of the separation of 19 amino acid thiohydantoin is shown in Fig. 1. The origin was 1 cm from the edges of the plate, which was sectioned to limit the solvent flow to 7.0 cm from the origin, and the markers in the outer zones were tyrosine and carboxymethylcysteine (see Rabin & Darbre, 1974). Butyl-PBD was used in solvent system 2 when plates did not include an indicator. Under u.v., dark-purple spots appeared on a pale-purple fluorescent background. The limit of detection was approx. 0.5 nmol for alanine thiohydantoin. Colour differences in daylight helped identification. Histidine, tryptophan and tyrosine

thiohydantoin spots were yellow. On heating to about 80°C, the thiohydantoin of aspartic acid and asparagine turned yellow, those of glycine, serine and threonine turned pink and that of *S*-carboxymethylcysteine turned brown.

We attempted to prepare glutamine thiohydantoin, but t.l.c. with different solvents failed to distinguish between glutamine and glutamic acid thiohydantoin. It is suspected that the strong acid used during the preparation of glutamine thiohydantoin results in its hydrolysis to glutamic acid thiohydantoin. This would probably occur when the method of Stark (1968) is applied to a peptide and thus glutamine would not be distinguished from glutamic acid. No suitable peptide was available for this study. Rabin & Darbre (1974) were unable to distinguish between the methylthiohydantoin of glutamine and glutamic acid. These authors pointed out that Kulbe (1971) and Summers *et al.* (1973), although using similar solvent systems with polyamide plates, disagreed over the relative positions of glutamic acid phenylthiohydantoin and glutamine phenylthiohydantoin. Suzuki *et al.* (1973) reported that they were unable to prepare glutamine thiohydantoin.

Hydrolysis of thiohydantoin

In addition to the methods for the direct identification of amino acid thiohydantoin by g.l.c. and mass spectrometry (Rangarajan *et al.*, 1973) it was desirable to have an indirect procedure by which, after hydrolysis, the amino acids could be determined

Fig. 1. Schematic representation of two-dimensional t.l.c. of 19 amino acid thiohydantoins

Solvent system 4 was used for the first dimension and solvent system 2 for the second dimension. (See the Materials and Methods section.)

by g.l.c. Stark (1968) hydrolysed amino acid thiohydantoins under alkaline conditions with low yields of amino acids. Turner & Schmerzler (1954) reported severe decomposition of some derivatives by hydrobromic acid. Because some amino acid thiohydantoins could be hydrolysed more successfully than the corresponding thiohydantoins, Cromwell & Stark (1969) removed the sulphur from thiohydantoins by oxidative desulphuration before hydrolysing them with alkali. We studied methods of acid hydrolysis and concluded that low yields might be due to either oxidative destruction or to incomplete hydrolysis. The results in Table 2 were with 6M-HCl, containing 0.1% phenol (Li & Yanofsky, 1972) and 1 mM- β -mercaptoethanol, at 135°C for 23 h. Arginine thiohydantoin on hydrolysis gave arginine only, and not ornithine as was obtained after alkaline hydrolysis of arginine phenylthiohydantoin (Van Orden & Carpenter, 1964) and arginine thiohydantoin (Stark & Smyth, 1963). Hydrolysis of serine thiohydantoin gave alanine (approx. 30%) by g.l.c. All the amino acid

thiohydantoins studied gave single g.l.c. peaks, except those given special mention. Isoleucine thiohydantoin prepared from L-isoleucine (*allo*-free) always yielded a mixture of L-isoleucine and D-*allo*-isoleucine (Rangarajan *et al.*, 1973). Cysteine thiohydantoin gave a mixture of alanine (approx. 20%) and cysteine (approx. 13%). S-Carboxymethylcysteine thiohydantoin gave a mixture of glycine (approx. 85%) and methionine (approx. 5%). Tryptophan thiohydantoin gave a mixture of glycine (approx. 19%) and alanine (approx. 5%), although Inglis *et al.* (1971), using hydroiodic acid hydrolysis, claimed a total yield of better than 70% from the phenylthiohydantoin.

Threonine thiohydantoin was exceptional in giving three amino acids on hydrolysis, α -amino-*n*-butyric acid (3%), aspartic acid (6%) and homoserine (64%) which were identified and determined by g.l.c. When threonine thiohydantoin was hydrolysed with alkali (Baptist & Bull, 1953) the products detected by paper chromatography were α -amino-*n*-butyric acid

Table 2. *Recovery of amino acids from their thiohydantoin derivatives after acid hydrolysis*

Hydrolysis was carried out in Rotaflo stopcocks with 6M-HCl containing 0.1% (w/v) phenol and 1mM- β -mercaptoethanol at 135°C for 23 h. The amino acids were determined by g.l.c. Results of duplicate experiments are given.

Amino acid thiohydantoin	Recovery (%)	
	I	II
Ala	84	88
Arg	72	74
Asn	65	65
Asp	80	82
Cys	33	33
CmCys	95	91
Glu	80	80
Gly	105	100
His	72	68
Ile	107	105
Leu	82	81
Lys	69	69
Met	66	66
Phe	90	90
Ser	29	30
Thr	75	74
Trp	24	26
Tyr	92	92
Val	102	99

and glycine. When threonine phenylthiohydantoin was hydrolysed with acid, α -amino-*n*-butyric acid, alanine and glycine were detected in varying proportions by Ingram (1953) and Levy (1954), using paper chromatography, and by Inglis *et al.* (1971), using the ion-exchange amino acid analyser. Hydrolysis with alkali yielded α -amino-*n*-butyric acid, glycine and threonine (Ingram, 1953).

No previous reports in the literature on the hydrolysis of hydantoins, thiohydantoins or their 3-alkyl derivatives involved the use of g.l.c. for qualitative or quantitative determinations.

M. R. was supported by a Tutorial Studentship from King's College. A. D. thanks the S.R.C. for support and Professor H. R. V. Arnstein for his interest.

References

- Akabori, S., Ohno, K., Ikenaka, T., Okada, Y., Hanafusa, H., Haruna, H., Tsugita, A., Sugae, K. & Matsushima, T. (1956) *Bull. Chem. Soc. Jap.* **29**, 507-518
- Ambler, R. P. (1967a) *Methods Enzymol.* **11**, 155-166
- Ambler, R. P. (1967b) *Methods Enzymol.* **11**, 436-445
- Baptist, V. H. & Bull, H. B. (1953) *J. Amer. Chem. Soc.* **75**, 1727-1729
- Cromwell, L. D. & Stark G. R. (1969) *Biochemistry* **8**, 4735-4740
- Darbre, A. (1971) *Lab. Prac.* **20**, 726
- Darbre, A. & Islam, A. (1968) *Biochem. J.* **106**, 923-925
- Edman, P. (1949) *Arch. Biochem. Biophys.* **22**, 475-476
- Edman, P. & Begg, G. (1967) *Eur. J. Biochem.* **1**, 80-91
- Inglis, A. S., Nicholls, P. W. & Roxburgh, C. M. (1971) *Aust. J. Biol. Sci.* **24**, 1247-1250
- Ingram, V. M. (1953) *J. Chem. Soc. London* 3717-3718
- Islam, A. & Darbre, A. (1972) *J. Chromatogr.* **71**, 223-232
- Kulbe, K. D. (1971) *Anal. Biochem.* **44**, 548-558
- Laursen, R. A. (1971) *Eur. J. Biochem.* **20**, 89-102
- Levy, A. L. (1954) *Biochim. Biophys. Acta* **15**, 589
- Li, S. L. & Yanofsky, C. (1972) *J. Biol. Chem.* **247**, 1034-1037
- Matsuo, H., Fujimoto, Y. & Tatsuno, T. (1966) *Biochem. Biophys. Res. Commun.* **22**, 69-74
- Peterson, J. D., Nehrlich, S., Oyer, P. E. & Steiner, D. F. (1972) *J. Biol. Chem.* **247**, 4866-4871
- Pisano, J. J. & Bronzert, T. J. (1969) *J. Biol. Chem.* **244**, 5597-5607
- Pisano, J. J., Bronzert, T. J. & Brewer, H. B. (1972) *Anal. Biochem.* **45**, 43-59
- Rabin, P. & Darbre, A. (1974) *J. Chromatogr.* **90**, 226-229
- Rangarajan, M., Ardrey, R. E. & Darbre, A. (1973) *J. Chromatogr.* **87**, 499-512
- Rangarajan, M. & Darbre, A. (1974) *Abstr. Meet. FEBS 9th* p. 348
- Schlack, P. & Kumpf, W. (1926) *Hoppe-Seyler's Z. Physiol. Chem.* **154**, 125-170
- Stark, G. R. (1968) *Biochemistry* **7**, 1796-1807
- Stark, G. R. & Smyth, D. G. (1963) *J. Biol. Chem.* **238**, 214-226
- Summers, M. R., Smythers, G. W. & Oroszlan, S. (1973) *Anal. Biochem.* **53**, 624-628
- Suzuki, T., Komatsu, K. & Tuzimura, K. (1973) *J. Chromatogr.* **80**, 199-204
- Turner, R. A. & Schmerzler, G. (1954) *Biochim. Biophys. Acta* **13**, 553-559
- Van Orden, H. O. & Carpenter, F. H. (1964) *Biochem. Biophys. Res. Commun.* **14**, 399-403
- Yamashita, S. (1971) *Biochim. Biophys. Acta* **229**, 301-309