Journal of Chromatography, 329 (1985) 428–433
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17 818

Note

Monitoring of the production of D-phenylglycine from DL-phenylhydantoin by high-performance liquid chromatography*

O. LADRÓN DE GUEVARA*
Istituto de Investigaciones Biomédicas, Departamento de Biologia Molecular, Universidad Nacional Autonoma de México, P.O. Box 70-228, Mexico 04510 D.F. (Mexico)
and
R. QUINTERO and P. PADILLA
Centro de Investigación sobre Ingeniería Genética y Biotecnología, Departamento de Biotecnologia, Universidad Nacional Autonoma de México, Mexico (Mexico)
(Received April 19th, 1985)

Phenylglycine is an important starting material in the production of β-lactam antibiotics such as semisynthetic penicillins and cephalosporins. The conversion of DL-phenylhydantoin into D-phenylglycine was achieved by an enzymatic process patented by the Centro de Investigación sobre Ingeniería Genética y Biotecnología, Universidad Nacional Autonoma de México. The enzymatic hydrolysis of DL-hydantoins to D-amino acids[1] is shown in Fig. 1.

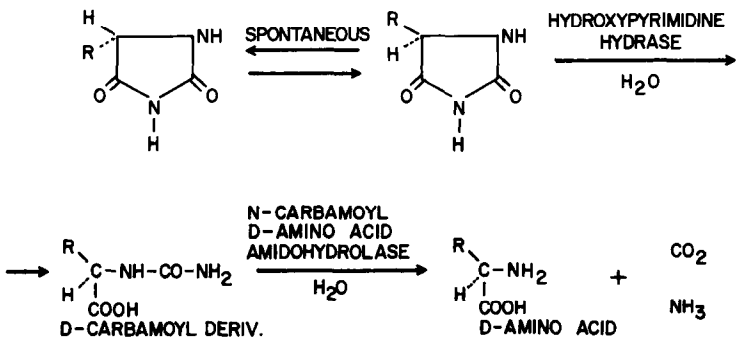

Fig. 1. Enzymatic hydrolysis of DL-hydantoins to D-amino acids.

The various approaches currently available for high-performance liquid chromatographic (HPLC) analysis of amino acids were examined[2–4]. There are two basic techniques; (a) analysis using classical ion-exchange techniques with post-column derivatization; (b) analysis using reversed-phase separation of pre-derivatized amino

* Presented at the *Fourth International Symposium on High-Performance Liquid Chromatography of Proteins, Peptides and Polynucleotides, Baltimore, MD, December 10–12, 1984.* The majority of the papers presented at this symposium have been published in *J. Chromatogr.*, Vols. 326 and 237 (1985).

0021-9673/85/$03.30

acids[2]. When a ninhydrin or OPA (*o*-phthalaldehyde) pre- or post-column reaction system is employed, it is necessary to chromatograph the samples at 60 or 30°C[3] and to use an elution time of over 80 min (*i.e.*, for the separation of the usual amino acid mixtures) with the ion-exchange techniques and of over 30 min with reversed-phase separation[4]. The need to derivatize the samples, the rather long separation times and the need to use high temperatures are not convenient for monitoring the formation of product during the reaction.

EXPERIMENTAL

Apparatus

A SP8400 variable-wavelength detector, SP8700 solvent-delivery systems, SP8750 Organizer with Rheodyne injection loop (10 μl) and SP4100 computing integrator were employed (all from Spectra-Physics). The Model 490 programmable, multiwavelength detector, Model 680 automated gradient controller, Model 730 data module, Model 590 programmable solvent-delivery system and automated switching-valve loop (10 μl) were from Waters. In both systems, the same Radial-Pak μBondapak™ C_{18} cartridge (10 cm × 8 mm, 10 μm) contained in a Z-module radial compression separation system (Waters) was used. The samples and solvents were filtered through porous membranes (0.22 μm, Millipore).

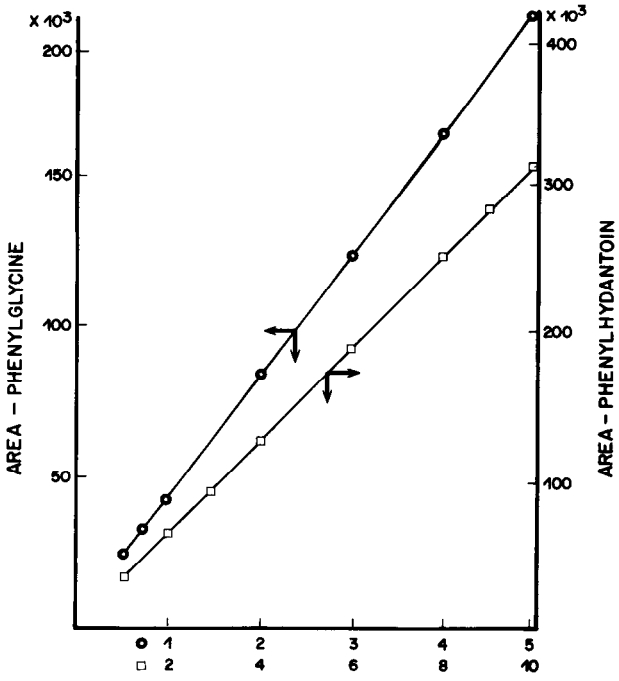

Fig. 2. Linearity of response for D-phenylglycine (○, 0.5–5.0 μg) and D-phenylhydantoin (□, 1.0–10 μg). The standards were eluted from Radial-Pak μBondapak C_{18} (10 cm × 8 mm, 10 μm). Mobile phase: convex gradient (20 to 25% methanol in phosphate buffer, pH 6.8); flow-rate 1.5 ml/min. Detection: UV, 212 nm.

Reagents

HPLC-grade water was obtained from pre-treated water with a Norganic Filter Apparatus (Cat. No. XX1504-710, Millipore). Methanol (UV grade), anhydrous Na_2HPO_4 and crystalline $NaH_2PO_4 \cdot H_2O$ were from Baker, D(−)-C-phenylglycine (Sigma A-1255) and DL-S-phenylhydantoin (Chemalog 72-31-20-00) were used as standards.

Procedure

The eluents (A, 20 m*M* sodium phosphate buffer, pH 6.8; B, methanol) were degassed by vacuum filtration through a porous membrane (0.22 μm). The standards, D-phenylglycine (2.5 μg) and DL-phenylhydantoin (5 μg), were dissolved in ultra-pure water or in methanol, respectively. Both standards were filtered through a porous membrane (0.22 μm) and chromatographed individually in order to determine their retention times. They were then mixed and chromatographed using each different system. The external standard procedure was employed to calculate the yield (Figs. 4, 5 and 7). The RF (response factor) values were determined for each set of samples. The linearity of response (Fig. 2) was confirmed for both D-phenylglycine (0.5–5 μg) and D-phenylhydantoin (1–10 μg).

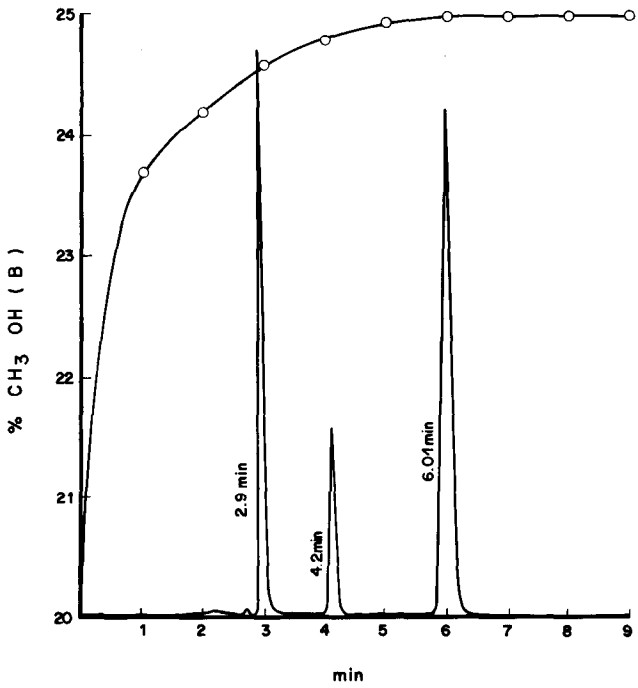

Fig. 3. Chromatogram of 10 μl of a standard mixture (2.5 μg of D-phenylglycine plus 5 μg of DL-phenylhydantoin). Spectra-Physics system: column; Radial-Pak μBondapak C_{18} (10 cm × 8 mm, 10 μm); mobile phase, convex gradient (○, 20 to 25% methanol in phosphate buffer, pH 6.8); flow-rate 1.5 ml/min; UV detection at 212 nm. Peaks: retention time 2.9 min = D-phenylglycine; 4.2 min = impurity; 6.01 min = DL-phenylhydantoin.

Chromatographic conditions

The Spectra-Physics system was programmed to provide a stepwise convex gradient elution in order to separate the impurities present from enzymatic hydrolysis products (Fig. 3). In the Waters system, the Model 680 automatic gradient controller was used to achieve the convex gradient (curve No. 5). In both systems the mobile phase consisted of eluents A and B, the convex gradient being from 20 to 25% B.

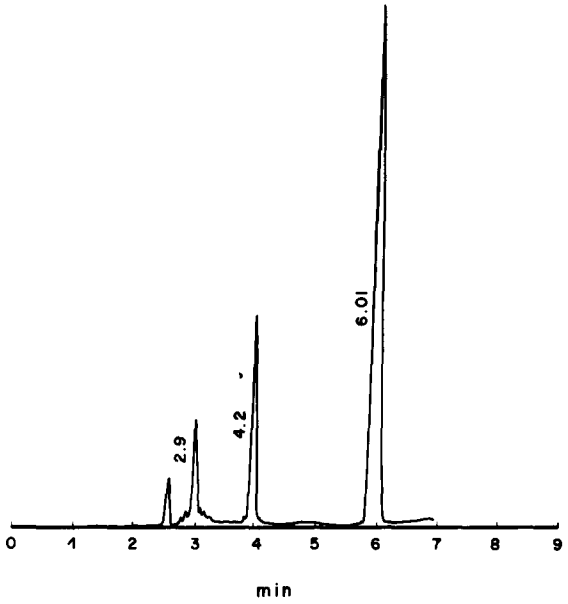

Fig. 4. Chromatogram taken at the start of the enzymatic reaction. Conditions and peaks as in Fig. 3.

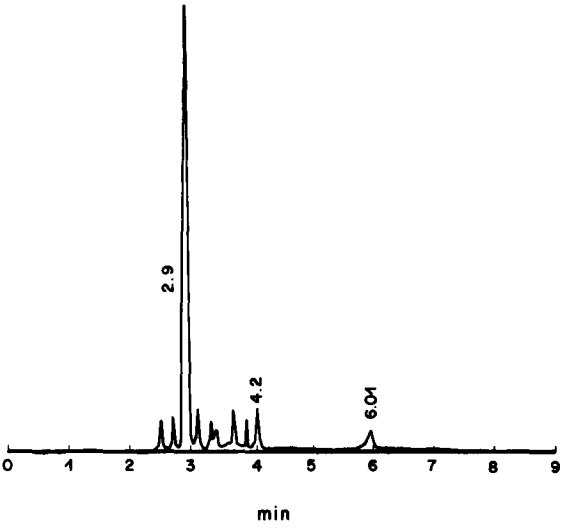

Fig. 5. Chromatogram taken near the end of the enzymatic reaction. Conditions and peaks as in Fig. 3.

The mobile phase flow-rate was 1.5 ml/min and the column effluent was monitored at 212 nm. Chromatography was performed at room temperature in a μBondapak C_{18} cartridge. The volume of sample injected was 10 μl.

RESULTS AND DISCUSSION

Chromatograms showing the separation of D-phenylglycine and D-phenylhydantoin standards are shown in Figs. 3 and 6. Well resolved peaks were obtained despite the short elution time: in the Spectra-Physics system, the retention time for D-phenylglycine was 2.9 min and for phenylhydantoin was 6.01 min; in the Waters system the retention time for D-phenylglycine and phenylhydantoin were 2.37 and 7.37 min, respectively. That the peak corresponding to D-phenylglycine in a sample did in fact contain this compound was determined by measuring the specific rotation of the recovered material.

The impurities in the samples are given in Figs. 4, 5 and 7. Because of these impurities, it was necessary to use a convex gradient[5] to achieve resolution.

Statistical calculations of the accuracy, reproducibility and repeatability[6,7] were made to determine the confidence limits that could be placed on the analytical

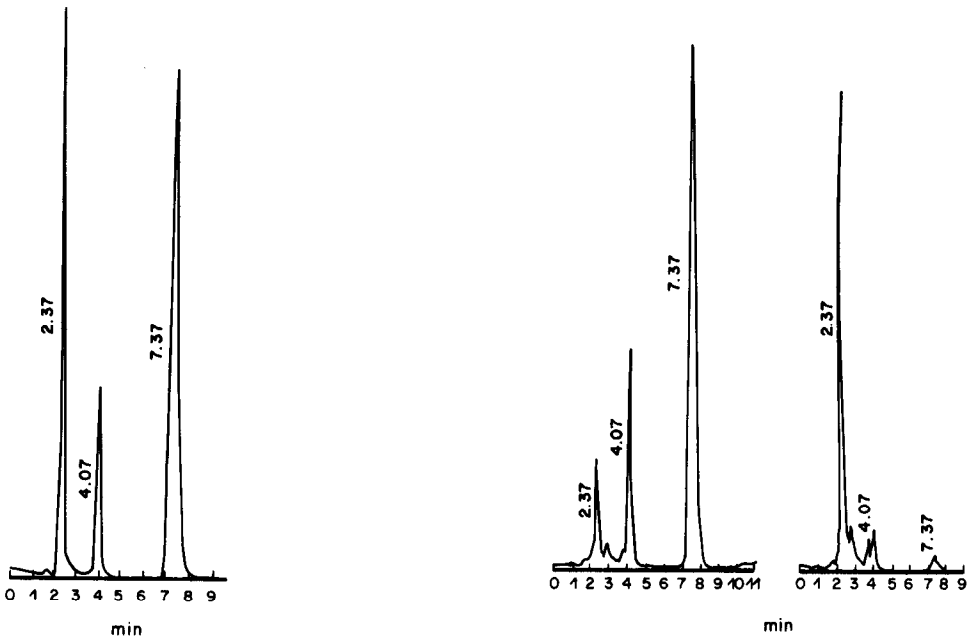

Fig. 6. Chromatogram of 10 μl of a standard mixture (2.5 μg of D-phenylglycine plus 5 μg of DL-phenylhydantoin). Waters Modular system: column; Radial-Pak μBondapak C_{18} (10 cm × 8 mm, 10 μm); mobilije phase; convex gradient (curve No. 5 in Waters Model 680 automated gradient controller) from 20 to 25% methanol in phosphate buffer, pH 6.8; flow-rate 1.5 ml/min; UV detection at 212 nm. Peaks: 2.37 min = D-phenylglycine; 4.07 min = impurity; 7.37 min = DL-phenylhydantoin.

Fig. 7. Chromatogram taken at the start (A) and near the end (B) of the enzymatic reaction. Conditions and peaks as in Fig. 6.

TABLE I

STATISTICAL RESULTS OBTAINED WITH THE WATERS AND SPECTRA-PHYSICS MODULAR SYSTEMS

S = Standard deviation; S^2 = variance; $\bar{X}$ = average value; C = coefficient of variation; CL = confidence limits; α = separation factor.

Compound	*Parameter*	*System*	
		Waters	*Spectra-Physics*
D-Phenylglycine	S	0.01164	0.0536
	S^2	0.000122	0.0029
	$\bar{X}$	2.5024	2.5228
	C	0.465	2.12
	CL	±0.0083	±0.066
D-Phenylhydantoin	S	0.0419	0.095
	S^2	0.00158	0.009
	$\bar{X}$	5.02837	1.85
	C	0.83327	1.85
	CL	±0.03	±0.11
	α	4.69	5.024

data obtained from experiments carried out over the course of 14 months. The data were accurate since a high number of replicate analyses agreed very well. Repeatability refers to the precision of a single operator repeating the analysis on the same apparatus; reproducibility refers to the precision with the same method used for different systems. High reproducibility and repeatability were obtained in our experiments (Table I). Since this simple, quick and efficient analytical method allowed us to monitor the enzymatic conversion of DL-phenylhydantoin into D-phenylglycine, it is now used at the Centro de Ingeniería Genética y Biotecnología, UNAM, as a standard method.

REFERENCES

1 R. Oliveri, E. Fassetti, L. Angeloni and L. Degene, *Enzyme Microbiol. Technol.*, 1 (1979) 201–204.
2 R. P. Feizer, R. Karol, J. Korpi, R. Burgoyne and D. Mc Court, *Amer. Lab.*, March (1983) 78–87.
3 R. P. Feizer and D. W. Hill, *Advan. Chromatogr.*, 22 (1983) 37–69.
4 P. B. Hamilton, *Advan. Chromatogr.*, 2 (1966) 4.
5 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, New York, 1979, p. 669.
6 W. J. Dixon and F. J. Massey, *Introduction to Statistical Analysis*, McGraw-Hill, New York, 1957.
7 N. Hadden, F. Baumann and F. MacDonald, *Basic Liquid Chromatography*, Varian Aerograph, Walnut Creek, CA, 1972, pp. 8–16, 8–18.