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DETERMINATION OF 3-PHENYL-2-THIOHYDANTOIN-AMINO ACIDS BY MEANS OF *IN SITU* QUANTITATION OF THIN-LAYER CHROMATOGRAMS

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SUMMARY

This investigation demonstrates the applicability of the linear detector system for the quantitation of 3-phenyl-2-thiohydantoin (PTH)-amino acids on thin-layer chromatograms. This method is rapid and more sensitive than the Edman procedure. As little as 0.5 nanomoles of PTH-amino acid can be determined quantitatively. Standard calibrations are presented for a representative group of PTH-amino acids with correlation coefficients $r > 0.996$ and standard deviation $s_a < 3.14\%$.

INTRODUCTION

This investigation was undertaken in an attempt to apply the linear detector system[1] to the problem of quantitating 3-phenyl-2-thiohydantoin (PTH)-amino acids in thin-layer chromatography (TLC). It is possible to obtain unequivocal separation of the PTH-amino acids resulting from the Edman degradation of peptides on TLC plates in the solvent systems developed by Edman and Sjöquist[2] and Pataki[3]. The only difficulty had been the cumbersome and reliable method of quantitation. The method of Edman[4] consists of removal of the spot from the plate, extraction with the proper solvent, and analysis by spectrophotometry. Although gas chromatographic analysis[5] of silylated PTH-amino acids has provided a quick and reliable method of determination and quantitation, the programming of the apparatus for the proper column conditions that give adequate separation is a time-consuming process. The method here proposed, the application of a reflectance spectrophotometer equipped with the linear detector system, has an advantage since the same apparatus and conditions which are used in routine analyses of steroids, sulfonamides, and other pharmacological compounds can be directly adapted. Changing the wavelength of the scanning apparatus is the only adjustment that is necessary.

A few years ago a new function[6,7] had been developed for direct spectrophoto-

metric quantitative analysis of thin-layer chromatograms. The function is given in formula 1

$$K_x c = K_R \left(\frac{I_0}{I_x} + \frac{I_x}{I_0} - 2\right) + K_T \ln \frac{I_0}{I_x} \quad (1)$$

where K_x, K_R and K_T are constants, c is the concentration of the substance in the chromatographic spot, I_0 is the light intensity of the background (maximal light intensity), and I_x is the light intensity of the chromatographic spot ($0 \leqq I_x \leqq I_0$). This function consists of a combination of Beer's law and the special case of the Kubelka–Munk function[8], which respectively describe spectrophotometry for homogeneous and heterogeneous media. In the two extreme possible cases the function may revert back to either Beer's law ($K_R = 0$) or to the above-mentioned case of the Kubelka–Munk function ($K_T = 0$).

This new theory for the mathematical description of densitometry provided excellent linearity between instrument response and the quantity of the light-absorbing substance in the chromatographic spot for a variety of steroid[1] and sulfonamide[9] derivatives. At the same time the nonlinearity of other commercially available instruments have recently been shown to be significant[9]. Standard calibrations were determined with straight-line correlation coefficients of $\leqq 0.998$ with slopes that had standard deviations of $\leqq 2.3\%$. These results were achieved over a wide concentration range and with μg quantities. An analog computer device manufactured by Amlab has made this method applicable to any densitometer due to the wide voltage range of its input.

MATERIAL AND EQUIPMENT

The following instruments were used: a Zeiss chromatogram spectrophotometer (Carl Zeiss, D-7082 Oberkochen, G.F.R.), an analog computer device (Amlab, Nynäshamn, Sweden), a potentiometer recorder, Servogor, F, Type RE 541 (Goerz Electro, Vienna, Austria) and a Hewlett-Packard 9100 calculator/printer (Hewlett-Packard, Loveland, Colo., U.S.A.).

Merck pre-coated silica gel 60 TLC plates were used. The layer thickness was 0.25 mm and the support glass had a size of 20 × 20 cm (E. Merck, Darmstadt, G.F.R.).

The 3-phenyl-2-thiohydantoin-amino acid derivatives were obtained from Pierce, Rockford, Ill., U.S.A.

The solvents used were spectrophotometric or reagent grade.

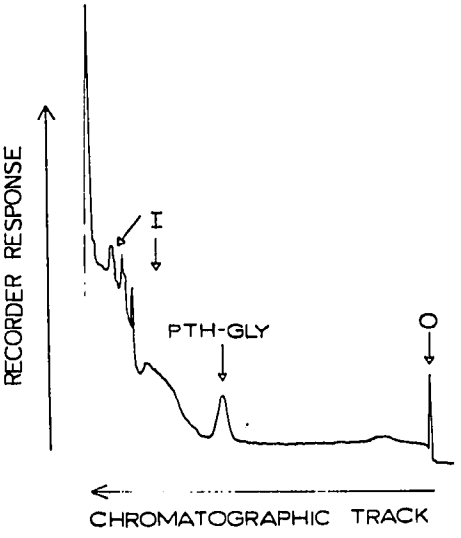
EXPERIMENTAL

Preparation of stock solutions

The necessary amounts of the PTH-amino acid derivatives were weighed into a 10-ml volumetric flask and dissolved with 1,2-dichloroethane. Dilutions were made such that 10-μl samples of the standard solutions were applied to the TLC plates giving the proper number of nmoles. A Hamilton μl syringe was used to apply the standard solutions to the TLC plates.

Chromatographic solvent systems

The three solvent systems which were used to develop the chromatograms are: (A) chloroform–dioxane–acetic acid (9:1:0.5); (B) butyl acetate–propionic acid–water[2,*]; (C) chloroform–methanol (9:1)[10]. With system B it was necessary to pre-wash the TLC plates in 5% acetic acid to remove a UV-absorbing material which interfered with the scanning (Fig. 1). All three solvent systems gave linear calibration curves. The data reported in this paper were obtained with system A (Fig. 2). With this solvent system it was not necessary to pre-wash the plates.



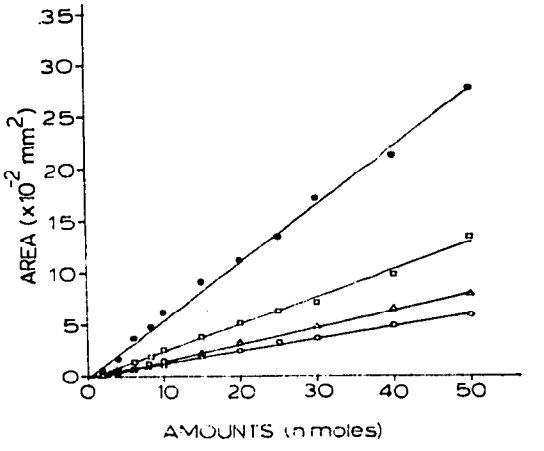


Fig. 1. Densitometric chart of 0.608 nmoles of PTH-glycine in system B. O = Origin; I = impurity (binder, etc.).

Fig. 2. Densitometric responses *versus* amounts of PTH-amino acids and the best fitting straight lines. ○—○, PTH-Glu; △—△, PTH-Gly; □—□, PTH-Leu; ●—●, PTH-N$^{\varepsilon}$-PTC-Lys.

Application of sample and development of plates

The 20 × 20 cm TLC plates were divided into eleven 17-mm tracks by scoring the silica gel plates with a spatula. The origins were placed at 2.5 cm from the bottom of the plates. With the use of a Hamilton syringe, 10-μl samples of each standard solution were spotted at the origin. The TLC plates were then developed with the freshly prepared solvent system after they had reached an equilibrium condition, allowed to dry, and were then scanned with the densitometer–linear detector system.

Scanning

The two absorption maxima of each PTH-amino acid were determined on the TLC plate and were found to be the same as those reported in ethanol solution. The two maxima were at 245 and 269 nm. Since the maximum at 269 nm is larger, the scannings of the standard series were performed at this wavelength. The linearity of

* 60 ml of butyl acetate saturated with water are shaken with propionic acid (1.8 ml) and formamide (8 ml). The upper phase is used.

the calibration curves was empirically estimated using the following K_T values: 0.25, 0.30, 0.35, 0.40, 0.45, and 0.50. The K_R value was kept at 1.00. The best linearity was obtained with a K_T value of 0.45 (Table I, Fig. 2). In contrast to other data available, neither Beer's law[11] ($K_R = 0$) nor the special case of the Kubelka–Munk function[8,12] ($K_T = 0$) alone resulted in linear calibrations in our cases. For each peak, the area was calculated by means of the formula given below (formula 2)

$$F = HW_{1/2} \tag{2}$$

where F is the peak area, H is the peak height, and $W_{1/2}$ is the peak width at the half-height. The areas plotted against the corresponding amounts provided the calibration curves (Fig. 2). The reliability of the method is characterized by the statistical calculations in Table I.

RESULTS AND DISCUSSION

The linear detection system for quantitation of the chromatograms was used to determine standard calibration curves for a group of representative PTH-derivatives of amino acids. The group consisted of two neutral, one acidic, and one basic PTH-amino acid. The specific derivatives are listed in Table I. Despite the fact that manual integration was used, the calibrations of the standards (Fig. 2) show an outstanding linearity between the densitometer response and the amount of PTH-amino acid in the chromatographic spot for all derivatives up to 60 nmoles. Amounts greater than 50 nmoles were not determined because the sensitivity of the method does not require greater amounts. The straight-line correlation coefficients for the four compounds were $r \geqslant 0.996$ and the relative standard deviations of the slopes, s_a, were $\leqslant 3.14\%$ (Table I). Straight-line correlation coefficients $\geqslant 0.999$ can be obtained with these systems if an electronic integrator is used[1] to determine the peak areas resulting from the densitometer scan. This linearity was obtained with the analog computer adjusted to $K_T = 0.45$ (Beer's law) and $K_R = 1.00$ (Kubelka–Munk function). The sensitivity of this method is comparable with or even superior to the other available quantitative methods. As little as 0.5 nmoles of PTH-glycine can be determined quantitatively (Fig. 1) in contrast to about 4 nmoles with other methods[5,13]. Thus, it

TABLE I

CHROMATOGRAPHIC AND PHOTOMETRIC PROPERTIES OF PTH-AMINO ACIDS AND STATISTICAL PARAMETERS OF THE CALIBRATION CURVES

Abbreviations: r = correlation coefficient; b = y-intercept; a = slope; $\pm s_a$ = standard deviation of the slope; $\varepsilon_{\lambda 269}$ = molar absorptivities of the phenylthiohydantoins measured in ethanolic solution. Solvents A–C, see text.

Substance	R_F *values*			r	b	a	$\pm s_a$ (%)	$\varepsilon_{\lambda 269}$
	A	*B*	*C*					
PTH-Glu	0.26	0.21	0.07	0.9967	16.68	11.78	2.72	15,900
PTH-Gly	0.48	0.46	0.51	0.9987	−18.49	16.26	3.14	14,900
PTH-Leu	0.86	0.90	0.65	0.9974	−24.24	26.34	2.42	16,700
PTH-N$^{\varepsilon}$-PTC-Lys	0.56	0.76	0.59	0.9966	19.66	54.88	2.10	29,000

is possible to diminish the initial amount of peptide subjected to the Edman degradation.

In view of the characteristics of this method, it is possible to carry out Edman degradation of peptides or proteins which are slightly impure and to surmount problems associated with overlapping, reagent byproducts, and impurities. An additional advantage of this method is the ability to calculate the R_F values of all spectrophotometric spots in the chromatogram from the peaks on the recording charts. The charts carry the densitometer scan, data on the sample, the experimental conditions, and can be stored and re-examined at any time (Fig. 1). With the particular linear detection system used in this study, Beer's law and the special case of the Kubelka–Munk function can be chosen separately or in any ratio required by the photometric medium. This versatility combined with the simplicity and high resolution of TLC (up to 1500 theoretical plates have been reported in Programmed Multiple Development[14]) initiated this investigation for the utilization of this technique for the analysis of PTH-derivatives of amino acids resulting from Edman degradation of peptides.

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