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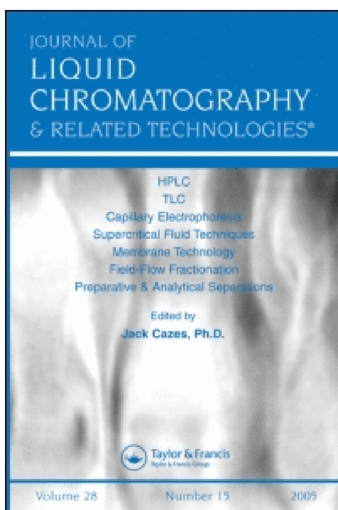

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Liquid Chromatographic Separation with Electrochemical Detection of the Phenylthiohydantoin and Methylthiohydantoin Derivatives of Amino Acids

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LIQUID CHROMATOGRAPHIC SEPARATION WITH ELECTRO-CHEMICAL DETECTION OF THE PHENYLTHIOHYDANTOIN AND METHYLTHIOHYDANTOIN DERIVATIVES OF AMINO ACIDS

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ABSTRACT

Phenylthiohydantoin (PTH) and methylthiohydantoin (MTH) derivatives of the common amino acids were examined for electroactivity on platinum and gold electrodes. All of the derivatives could be electrocatalytically oxidized at both electrodes. Constant-potential and pulsed-potential amperometry were examined for detection of thiohydantoins following reversed-phase liquid chromatography. Amperometric detection at a fixed potential of 1.2 V vs. SCE resulted in detection limits of less than 10 pmol for lysine and glycine derivatives (50-ul injection). Use of a gradient elution program with a C-18 reversed-phase column allowed separation of nearly all common amino acids as MTH derivatives.

INTRODUCTION

The degradation method of Edman (1) has been an essential tool for nearly 40 years for those seeking amino acid sequences in

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proteins and peptides. While the basic chemistry of the Edman procedure has not changed, advances in analytical techniques have resulted in improved ability to identify and quantitate the phenylthiohydantoin (PTH) adducts of amino acids produced in the reaction. Current methods (2-7) allow separation and determination of most of the common PTH amino acids by HPLC with photometric detection (UV-vis) in a single experiment of less than one hour duration. A slight modification of the Edman procedure (8,9) produces the methylthiohydantoin (MTH) adducts of amino acids, which can be determined rapidly through HPLC techniques (10) in a similar manner as for the PTH derivatives. The PTH amino acids have also been made in situations where sequencing was not needed, serving simply as derivatives for enhanced detectability of the amino acids. A simplification of the Edman procedure (11) will produce the phenylthiocarbamyl (PTC) adducts of amino acids which also can be quantitatively determined to lower concentrations than the free amino acids.

Amperometric detection of organic compounds traditionally has suffered because of problems caused by the loss of electrode surface activity, especially in the case of aliphatic compounds. The introduction of pulsed amperometric detection (PAD) has eliminated this problem and has been demonstrated to be useful for the direct detection of a wide range of analytes (12-15), including amino acids (16,17). While PAD ranks as one of the most sensitive methods for the direct detection of free amino acids, users of microsequencing techniques for sub-nanomole quantities of

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protein require even lower detection limits than available from PAD and typically will employ derivatization techniques to form the OPA (18), PTH, or PTC adducts.

No thorough examination was found in the literature of the electrochemical properties of PTH and MTH amino acids. Amperometric detection of PTC amino acids has been reported (19); however, the electrode material was not specified and very little information on the electrochemical response was provided. The purpose of this work was to determine if amperometric detection can provide sufficiently high sensitivity for the Edman products to support microsequencing work in conjunction with a commercially available HPLC column.

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EXPERIMENTAL

Reagents

Standard MTH and PTH adducts of amino acids were obtained from Sigma (St. Louis, MO). All other chemicals were reagent grade from Fisher Scientific (Fair Lawn, NJ). Water was deionized followed by purification with a MILLI-Q system from Millipore, (Bedford, MA) and filtration (0.2 um). All chromatography solvents were filtered through a 0.45-um Nylon-66 filter from Rainin, (Woburn, MA) prior to use. Solvent compositions are described in Table 1.

Apparatus

Voltammetric data were obtained with Au and Pt rotated disc electrodes (RDE; Model AFDT07, 0.196 cm^2) in a PIR rotator under

TABLE 1

Solvent Compositions

A.	0.1 M acetate buffer (pH 4.5)/0.1 M KNO_3/30% acetonitrile.
B.	0.1 M acetate buffer (pH 4.5)/0.05 M KNO_3/8.5% acetonitrile.
C.	0.1 M acetate buffer (pH 4.5)/0.05 M KNO_3/45% acetonitrile.

potentiostatic control by a RDE3 from Pine Instrument Co. (Grove City, PA). Voltammetric data were traced with a RE0074 X-Y recorder from EG & G Princeton Applied Research (Princeton, NJ). Amperometric detection was performed with a PAD-2 from Dionex Corp., (Sunnyvale, CA) and homemade Au and Pt flow-through cells (20). A saturated calomel electrode (SCE) provided the reference potential.

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Chromatographic separations were performed in a uBondapak C-18 reversed-phase column (3.9-mm i.d. x 30 cm, 10-um resin) from Waters Associates (Milford, MA). The injection loop volume was 50 ul. Isocratic separations and flow injection analyses were performed with a CMA-1 chromatography module and an APM-1 solvent pump from Dionex Corp. Gradient separations were performed using a CHB-1 chromatography module and a GPM-1 pump and GM-2 gradient mixer from Dionex Corp.

Procedures

A flow rate of 0.9 ml min^{-1} was used for all work in flowing streams, and all experiments were done at ambient laboratory temperature (27 $\pm$ 3 ^{o}C). Voltammetric experiments and isocratic

Table 2

Mobile Phase Composition for Gradient Elution Chromatography

Time (min)	Solvent B (%)	Solvent C (%)
0	95	5
5	70	30
15	65	35
20	60	40
50	50	50
60	45	55

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chromatography employed Solvent A (see Table 1). Gradient separations of MTH amino acids mixed Solvents B and C as described in Table 2.

RESULTS AND DISCUSSION

Voltammetry

Initial investigation of the electrochemical properties of PTH and MTH amino acids employed cyclic voltammetry (CV) at rotating disc electrodes (RDE). The response for a Pt RDE in 0.5 M H_2SO_4 is shown in Fig. 1A for MTH alanine and in Fig. 1B for PTH alanine in comparison to the response for the blank solution. It

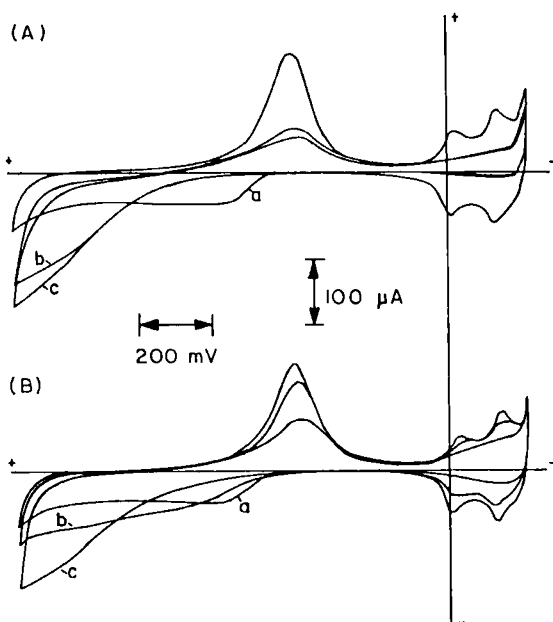

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Figure 1. Cyclic voltammetry of thiohydantoin amino acids: concentration dependence on Pt

Electrode: Pt RDE, 900 rpm, 6 V min^{-1}

Solution: 0.5 M H_2SO_4

(A) Samples: a) residual
b) 2.54 x 10^{-5} M MTH alanine
c) 6.01 x 10^{-5} M MTH alanine

(B) Samples: a) residual
b) 1.13 x 10^{-5} M PTH alanine
c) 3.23 x 10^{-5} M PTH alanine

is concluded, based on the marked similarity of the voltammetric data, that the substitution of a methyl group for a phenyl on the adduct does not affect the electrochemical response for these amino acid adducts. Furthermore, all corresponding MTH and PTH adducts are observed to produce virtually equivalent voltammetric response. Therefore, only one example of each adduct is presented here.

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The addition of the PTH and MTH amino acids results in the elimination of the cathodic and anodic peaks for hydrogen adsorption and desorption seen on the negative and positive potential sweep, respectively, in the potential range 0.1 to -0.3 V vs. SCE when the cell contains only supporting electrolyte. This is strong evidence that the PTH and MTH amino acids are adsorbed and, thereby, block the hydrogen adsorption sites. The onset of the anodic wave corresponding to oxide formation obtained on the positive potential sweep in the region 0.55 - 1.00 V is suppressed as well because of adsorption of the adducts.

In acidic solution, the MTH and PTH adducts are oxidized at the Pt electrode in the region of 1.10 - 1.30 V on the positive potential sweep simultaneously with the anodic formation of surface oxide. This anodic signal is observed to increase for higher rotation speeds of the electrode. However, the increase in signal for a change from 900 to 1600 rev min^{-1} is only ca. 50% of the value expected on the basis of the Levich equation for a mass transport-limited reaction. The current in the region 1.10 - 1.30 V on the positive sweep also is observed to increase nearly as a

linear function of the rate of potential sweep. This is diagnostic evidence for a surface-controlled reaction mechanism. Hence, it is concluded that the detection process is under mixed control by kinetic and mass transport processes, and probably involves the simultaneous oxide-catalyzed oxidations of adducts in the adsorbed as well as the solution state.

When a Au RDE is employed (Figure 2), it is evident that PTH glycine can be oxidized in both acidic and basic media. Current is also rotation speed dependent for the oxidative region in both media. A very favorable result of these voltammetric studies is the discovery that the thiohydantoins can be oxidized throughout a wide pH range, contrary to free amino acids which can only be oxidized in basic solution. The freedom to choose pH for oxidation allows *good* flexibility for the choice of elution conditions for optimum HPLC separation without the need for post-column addition of pH buffer.

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Several sources (2-7) describe acetate buffer eluents with acetonitrile and/or methanol as eluent modifiers for the HPLC separation of PTH amino acids. Methanol is known to be oxidized at noble metal electrodes under certain conditions. Therefore, to avoid background interference, only acetonitrile is used as an organic modifier. Some problems are encountered as the fraction of acetonitrile is increased due to high cell resistance (IR loss). Consequently, the eluent is supplemented with KNO_3 electrolyte to reach the composition of solvent A. The CV data of MTH lysine in solvent A on a Au electrode is shown in Figures 3A

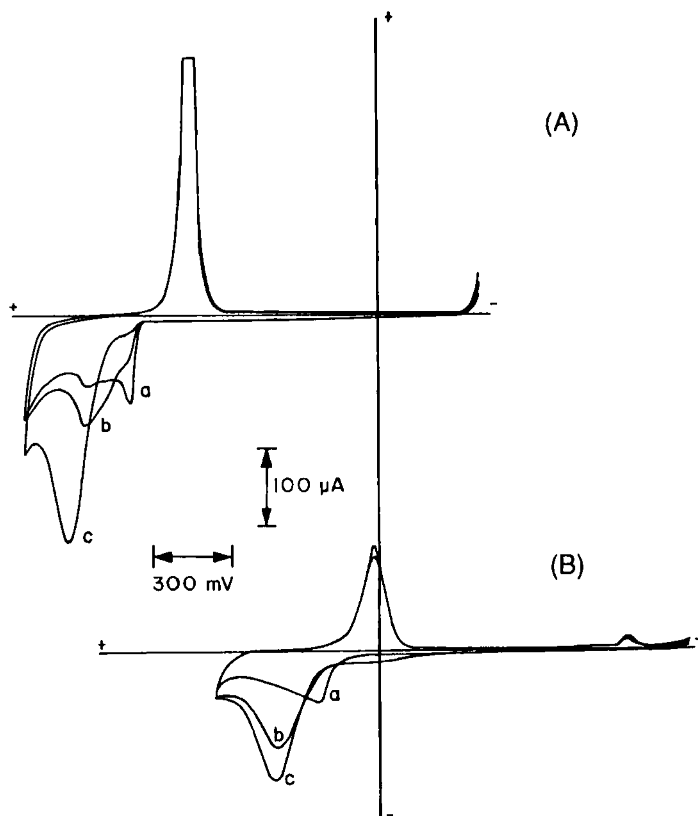

Figure 2. Concentration dependence of PTH glycine on a Au RDE

Electrode: Au RDE, 900 rpm, 6 V min^{-1}

(A) Solution: 0.5 M H_2SO_4
Samples: a) residual
b) 6.94×10^{-6} M PTH glycine
c) 1.73×10^{-5} M PTH glycine

(B) Solution: 0.25 M NaOH
Samples: a) residual
b) 5.20×10^{-6} M PTH glycine
c) 1.21×10^{-5} M PTH glycine

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and 3B as a function of analyte concentration and rotation speed. Mixed mass transport - surface control is present in this system as well, although it appears that mass transport control is more predominant than for Pt in acid.

<u>Waveform Design</u>

From experience with applications of PAD to the determination of other compounds, it is evident that a PAD waveform can be developed to determine thiohydantoin amino acids in solvent A. It had originally been assumed that DC amperometry would be unacceptable due to loss of electrode surface activity, which had been observed with other organic compounds (12,13,15,16), including amino acids (17).

However, the voltammetric response observed for the thiohydantoins differs considerably from that for free amino acids, and the CV data in acidic solutions appears to be consistent with the prediction that DC amperometry might be suitable for detection. In particular, current is not observed to decay to zero immediately on the reversal from positive to negative scan during a cyclic sweep (Figure 3A), as is typically seen for underivatized amino acids. The presence of anodic current on the negative scan is evidence that the oxidation products formed on the positive scan have not irreversibly adsorbed to the electrode surface, and electrode activity is still intact. This phenomenon appears limited to more acidic solutions for Au (Figure 2), where the thiohydantoins can be oxidized, in contrast with free amino acids, which require basic solutions.

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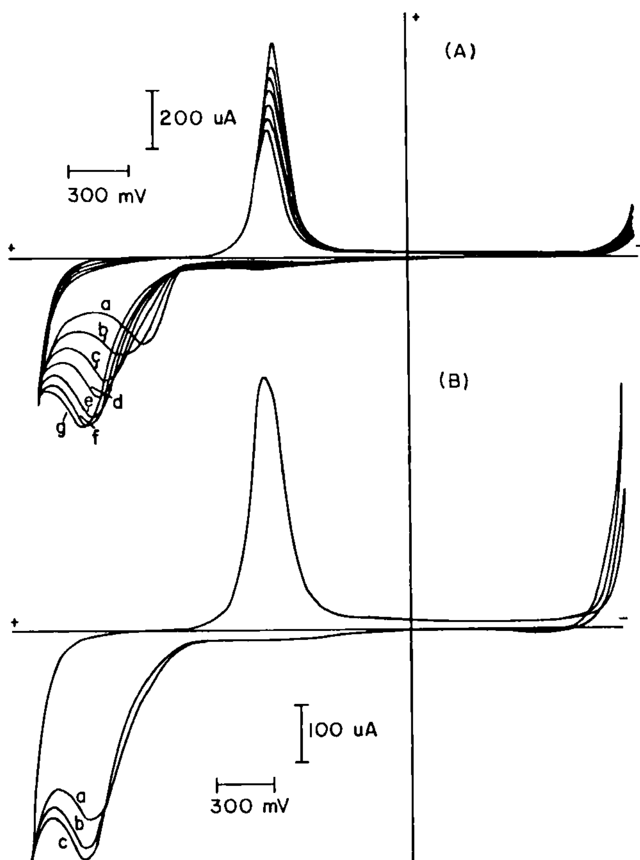

Figure 3. Cyclic voltammetry of MTH lysine on a Au electrode

Electrode: Au RDE, 6 V min^{-1}

Solution: Solvent A

(A) Samples:
a) residual
b) 1.62×10^{-6} M MTH lysine
c) 3.24×10^{-6} M MTH lysine
d) 8.10×10^{-6} M MTH lysine
e) 2.11×10^{-5} M MTH lysine
f) 3.89×10^{-5} M MTH lysine
g) 6.80×10^{-5} M MTH lysine

(B) Samples:
a) 400 rpm, 2.60×10^{-6} M MTH lysine
b) 900 rpm
c) 1600 rpm

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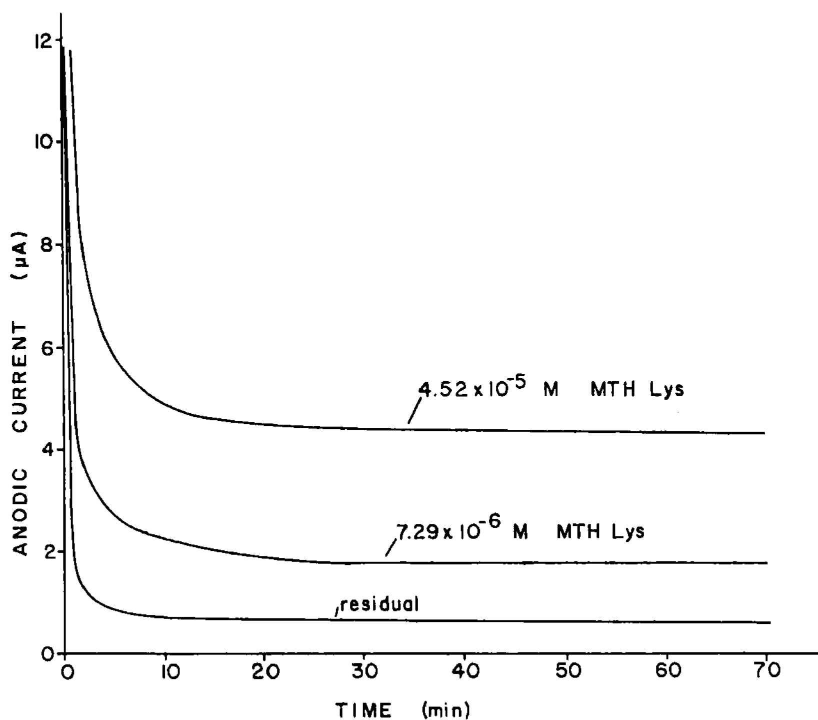

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Figure 4. Current decay with time at a fixed potential of 1.2 V vs. SCE

Electrode: Au RDE, 900 rpm

Solution: Solvent A

A current decay experiment can be performed in solvent A to explore the possibility of DC amperometric detection. The potential is swept in cyclic manner to pretreat the electrode, followed by a pulse from the negative sweep limit to 1.2 V, where oxidation of MTH lysine will take place. The potential is held at 1.2 V and the anodic current plotted versus time. Some decay is seen in the first 5 min (Figure 4), but steady-state current is

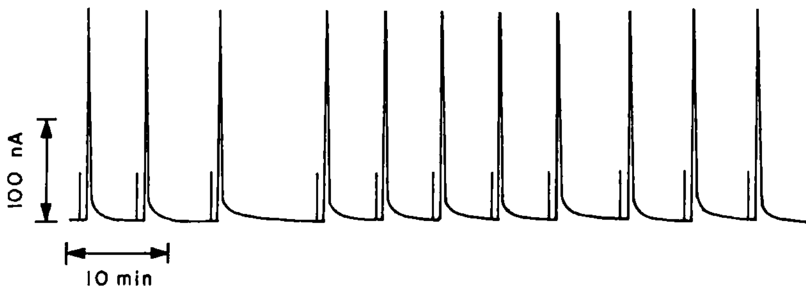

Figure 5. Flow injection analysis with reproducible DC anodic detection of PTH glycine

Electrode: Au, 1.2 V vs. SCE

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reached for MTH lysine solutions at values considerably greater than the residual value. Since electrode activity is not seen to decay to the residual value, DC amperometry should be successful under these conditions.

DC detection is tested by repetitive injections of PTH glycine into a flowing stream of solvent A. Figure 5 shows the anodic current measured versus time at a Au electrode potentiostated at 1.2 V. No loss of sensitivity (<1 %) can be detected over several hours and dozens of injections. The optimum S/N ratio is found to exist for an electrode potential in the range 1.15 to 1.25 V vs. SCE; 1.20 V became the standard value for chromatographic work. DC detection is also acceptable with stable and reproducible signals at a Pt electrode. PAD can be used as well for both Pt and Au electrodes, with the optimum waveforms on each listed in Table 3.

TABLE 3

Optimum Waveforms for Pulsed Amperometric Detection of Amino Acid Adducts

VARIABLE	ELECTRODE	
	Au	Pt
E_1 (t_1)	1.35 V (420 ms)	0.90 V (540 ms)
E_2 (t_2)	-0.35 V (240 ms)	1.10 V (120 ms)
E_3 (t_3)	1.55 V (60 ms)	-0.30 V (240 ms)

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The limits of detection (LOD) measured for four model compounds using flow injection analysis with a Au electrode are summarized in Table 4. PAD detection limits using a Pt electrode are in general 3-5 times worse than for Au, while DC results are considerably worse on Pt.

Chromatography

Separations of the thiohydantoin adducts of amino acids have been described (2-7,10) and served as the starting point for this work. However, KNO_3 added as an electrolyte to the mobile phase is found to alter the solvent polarity and, therefore, the chromatograms obtained differ greatly from those published. Solutions of Solvent A are made without KNO_3 and S/N values compared to previous results with KNO_3 present. DC amperometric

TABLE 4

Representative Values of Detection Limits

	DETECTION MODE	
ADDUCT	PAD (picomoles)	DCD (picomoles)
PTH Glycine	25	5.6
PTH Lysine	17	2.4
MTH Glycine	35	5.8
MTH Lysine	19	2.2

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detection shows only slightly worse response in the absence of KNO_3, but PAD results suffer markedly. The desire to maintain detector flexibility deems that KNO_3 supplementation shall continue, and a new separation be constructed.

For practical use with the Edman degradation procedure, it is desired that all the common thiohydantoin derivatives be separable in a single chromatographic run. The capacity factors (k') for the PTH amino acids have a very wide range of values with KNO_3 present in the eluent, making isocratic separation impossible within a reasonable period of time. The nonpolar amino acids

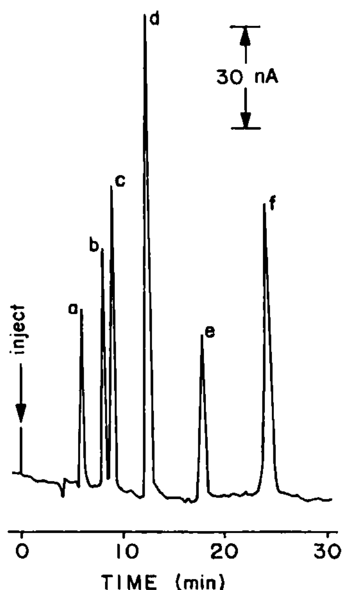

Figure 6. Separation and DC anodic detection of PTH amino acid derivatives

Electrode: Au, 1.2 V vs. SCE

Column: uBondapak C-18

Solution: Solvent A, 0.9 ml min^{-1}

Samples: a) PTH aspartic acid (4.33 nmol)
b) PTH asparagine (4.35 nmol)
c) PTH arginine (3.30 nmol)
d) PTH glycine (6.07 nmol)
e) PTH alanine (8.08 nmol)
f) PTH tyrosine (3.07 nmol)

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elute very late using pure solvent A; the higher percentage of organic modifier (acetonitrile) needed to decrease retention time causes co-elution of many of the more polar derivatives. For a limited separation of PTH derivatives with similar polarities, isocratic elution can be useful, as illustrated in Figure 6. The

MTH amino acids are more polar than the PTH derivatives, yet they also have k′ values distributed over a wide range. Isocratic elution again lacks resolving power to separate all of these compounds; a partial separation can be seen in Figure 7. The MTH amino acids appear to have a smaller range of k′ values than the PTH adducts, and application of a more efficient column may permit an acceptable isocratic separation.

Small variations in the acetonitrile fraction are found to affect large changes in the k′ values of the MTH amino acids, suggesting application of an acetonitrile gradient *in lieu* of a pH gradient. Acetonitrile is not electroactive under the experimental conditions used, but its apparent adsorption at a Au electrode surface will slow the kinetics of reactions at the electrode surface. This adsorption causes the oxidation wave of the analyte to be shifted slightly to more positive potentials. The major portion of the shift occurs during the addition of the first few percent of acetonitrile (14), so a gradient is attempted avoiding this composition range. Experimental data confirms that attempts to span a wide range of acetonitrile concentration will result in a large baseline shift. Gradients covering a smaller range are found to produce only a slight baseline drift and are deemed to be acceptable as long as this range is covered slowly. The gradient selected varies the acetonitrile fraction from 10.3% to 28.6% over 60 min (see Tables 1 and 2). A sample containing the adducts of 19 amino acids at ca. 20 ppm each studied using this gradient gave the chromatogram shown in Figure 8. MTH

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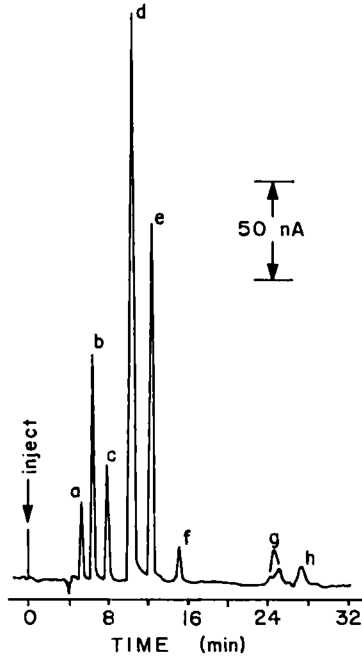

Figure 7. Separation and DC anodic detection of MTH amino acid derivatives

Electrode: Au, 1.2 V vs. SCE

Column: uBondapak C-18

Solution: Solvent A, 0.9 ml min^{-1}

Samples:
a) MTH glutamic acid (2.96 nmol)
b) MTH glycine (5.75 nmol)
c) MTH alanine (5.70 nmol)
d) MTH lysine (3.54 nmol)
e) MTH tyrosine (3.48 nmol)
f) MTH valine (4.77 nmol)
g) MTH isoleucine (5.60 nmol)
h) MTH leucine (6.40 nmol)

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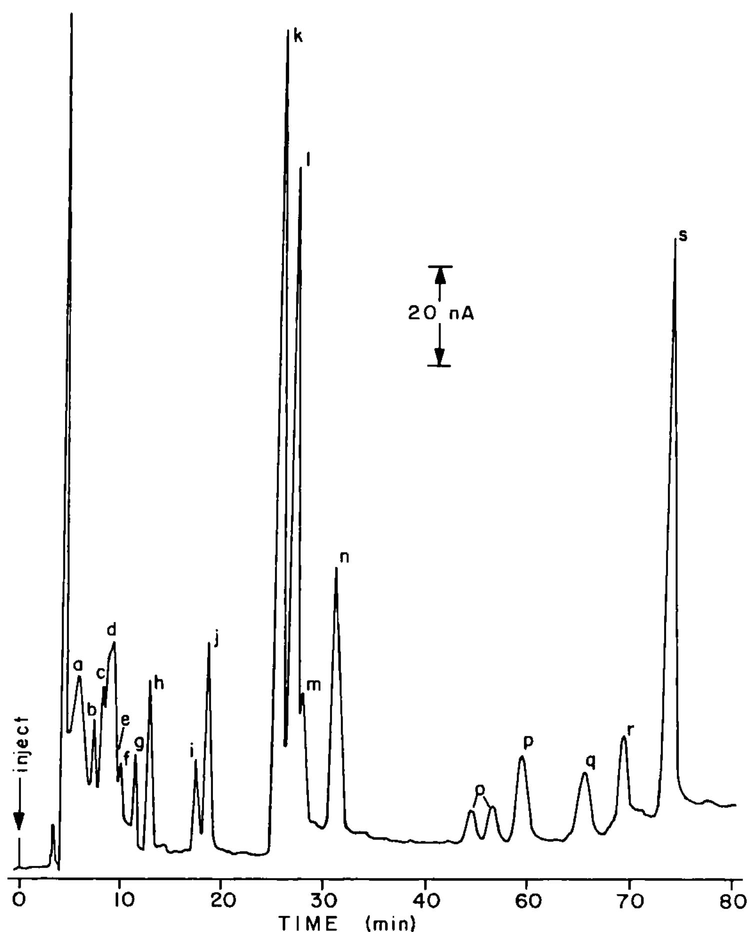

Figure 8. Separation of MTH amino acids using gradient elution HPLC

Electrode: Au, 1.2 V vs. SCE

Column: uBondapak C-18

Solution: described in Table 1

Samples:
a) aspartic acid
b) asparagine
c) histidine
d) glycine
e) glutamine
f) glutamic acid
g) arginine
h) alanine
i) aminoisobutyric acid
j) aminobutyric acid
k) lysine
l) tyrosine
m) valine
n) methionine
o) isoleucine
p) norleucine
q) leucine
r) phenylalanine
s) tryptophan

glycine and MTH glutamine coelute, but all other compounds are resolved.

PAD response to the gradient employed in Figure 8 is similar to that for DC amperometry. Improvements in the separation are expected if a more efficient column were to be utilized. Application of a faster gradient to allow more rapid analysis is possible if background subtraction methods are available.

CONCLUSIONS

The electrochemistry of the MTH and PTH amino acids differs greatly from that of the free amino acids, and it is likely that the thiocarbonyl group is oxidized rather than the amino acid nitrogen. Theoretical treatment of thiocarbonyl oxidations has been reviewed by Polta and Johnson (21).

Gradient elution chromatography can be performed with amperometric detection, and its application allows a single-injection analysis of an Edman Degradation sample with detectability to concentrations less than the 10-pmol value desired by modern microsequencing techniques (7). No problems with reproducibility are encountered using the gradient (4,22). The application of amperometric detection to thiohydantoins is not limited to the pH range used, and can be employed with other stationary phases should a separation be developed.

It should be emphasized that although PAD results are inferior to DC amperometry, PAD should not be discarded automatically for separations of the thiohydantoin adducts of

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amino acids. DC amperometry may suffer surface poisoning from extraneous material within a more complex sample matrix than the relatively "clean" samples used in this work. PAD has great resistance to electrode fouling and may prove to be the best detector choice for applications with these types of samples.

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