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p-N,N-DIMETHYLAMINOPHENYLISOTHIOCYANATE AS AN ELECTROCHEMICAL LABEL FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF AMINO ACIDS

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SUMMARY

Three aromatic isothiocyanates were surveyed as possible precolumn derivatizing agents for the electrochemical detection of amines and amino acids. From the isothiocyanates studied, *p*-N,N-dimethylaminophenylisothiocyanate (DMAPI) was chosen for further development as a label in the high-performance liquid chromatographic determination of amino acids. Amino acids reacted with DMAPI to form the corresponding substituted phenylthiohydantoins, which have been isolated and characterized, and which can be reversibly oxidized at a glassy carbon electrode at pH 2 with $E_{\frac{1}{2}}$ = 0.68 ± 0.01 V *vs.* Ag/AgCl. The derivatized amino acids were separated on a bonded C_8 column in 0.1 *M* phosphate buffer–25% acetonitrile (pH 2 or 6), and detected at a glassy carbon electrode set at 0.85 V *vs.* Ag/AgCl. A mixture of 21 amino acids has been separated with 80–98% recovery, and with a linear response from 1 to greater than 150 ng and detection limits of 0.5 to 1 ng.

INTRODUCTION

The determination of amines and amino acids plays a central role in biochemical, clinical, and environmental research, and therefore, a substantial amount of effort has been expended on the development of improved techniques for separation and detection. One of the more versatile methods of analysis uses an isothiocyanate (–N=C=S) to couple a chromophoric organic moiety to an available primary or secondary amine (Scheme I)[1]. The utilization of an isothiocyanate also permits the use of the Edman degradation of proteins involving the sequential conversion of the N-terminal amino acid to a substituted thiohydantoin (Scheme II)[2,3].

The formation of methyl- and phenylthiohydantoins has also been used as a precolumn derivatization method for high-performance liquid chromatographic (HPLC) analysis of mixtures of free amino acids[4] including several recent reports employing reversed-phase columns with bonded packings[5,6]. The eluted derivatives have been detected by UV absorption, with detection limits ranging from 0.01 to 0.1 nmol.

The selectivity and sensitivity of electrochemical detection methods in HPLC

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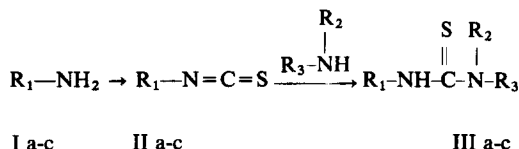
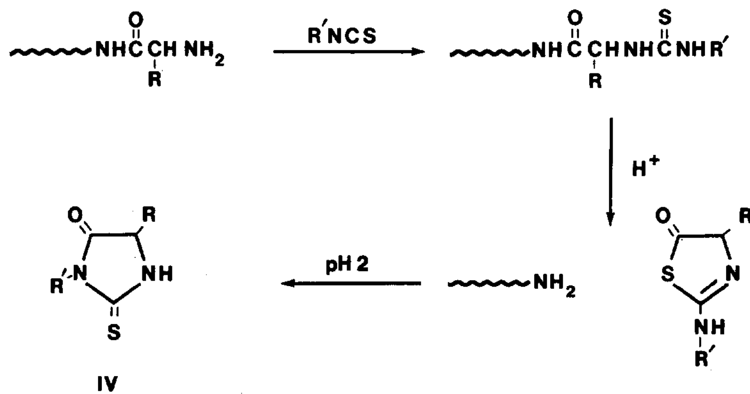


I a-c II a-c III a-c

Ia, R_1 = *p*-Dimethylaminophenyl
Ib, R_1 = 2,5-Dimethoxyphenyl
Ic, R_1 = 3,4-Dimethoxyphenyl

Scheme I.



Scheme II.

has gained wide acceptance[7], and the incorporation of an electroactive functionality into a chromatographic label is a natural outgrowth of this active area of reserch[8]. Fatty acids and related compounds have been detected electrochemically following derivatization with *p*-aminophenol[9], carbonyl compounds with *p*-nitrophenylhydrazine[10] and 2,4-dinitrophenylhydrazine[11]. Dimethylaminophenylisocyanate has been used to detect arylhydroxylamines[12]. Primary and secondary amines have been detected using N-(4-anilinophenyl)isomaleimide[13], and the detection of sympathomimetic amines with *o*-phthalaldehyde has also been reported[14]. Amino acids have been detected electrochemically using *o*-phthalaldehyde and 2,4,6-trinitrobenzenesulfonic acid as derivatizing agents[12]. We have synthesized three electrochemically active derivatizing agents which incorporate an isothiocyanate, and have studied their suitability as electrochemical labels for amines and amino acids. As a result of these studies, we report here on the development of *p*-N,N-diethylaminophenylisothiocyanate (DMAPI) as an electrochemical label for determination for amino acids by HPLC.

EXPERIMENTAL

Reagents

The *p*-N,N-dimethylaminoaniline (Ia) was from Sigma, the sodium octyl sulfonate from Eastman, and the 2,5-dimethoxyaniline (Ib), 4-aminoveratrole (c), pyridine, trifluoroacetic acid, thiophosgene, and the amino acids from Aldrich. HPLC

grade acetone, methanol, and acetonitrile were from Fisher Scientific. Methylene chloride (technical grade) and absolute ethanol were obtained from the University of Minnesota Chemical Storehouse. The methylene chloride was distilled before use. Water was obtained from a Milli-Q water purification system.

The pH 9.5 buffer used in derivatizations of amino acids was prepared by adding 5 ml of 0.2 *M* acetic acid and 1 ml of triethylamine to 100 ml of 50% aqueous acetone and adjusting to pH 9.5 with pyridine.

Equipment

Stability studies and pK_a determinations were performed using a Beckman Instruments Acta II UV–visible recording spectrophotometer and Fisher Scientific matched 1.0 cm silica cells. Melting points were determined using a Fisher-Johns melting point apparatus and are uncorrected. A Beckman IR 33 and a Varian EM 360 60-MHz NMR were used for structure confirmation of the products. Cyclic voltammetry experiments were carried out using a CV-1A cyclic voltammetry unit and associated cell and electrodes from Bioanalytical Systems (BAS). The three-electrode system included a glassy carbon or carbon paste working electrode, a Ag/AgCl reference electrode containing 3 *M* sodium chloride as electrolyte, and a platinum auxiliary electrode. Cyclic voltammograms were recorded on a Houston Instruments Model 100 *X–Y* recorder. Chromatographic data were obtained using a Waters Assoc. Model M6000 dual-piston pump and Model U6K injector, a Hewlett-Packard RP-8 250 × 4.6 mm reversed-phase column with 10-μm particles, a Bioanalytical Systems LC-4 amperometric controller and TL-5 glassy carbon flow-cell with Ag/AgCl reference electrode, and a Fisher Model 5000 strip chart recorder or Hewlett-Packard Model 3390A recording integrator.

Synthesis

Isothiocyanates. To 0.06 mol of the amine (Ia–c) in 100 ml of 50% aqueous ethanol was added with vigorous magnetic stirring 150 ml of an aqueous solution containing 33 g sodium carbonate and 24 g thiophosgene. The reaction mixture remained at room temperature for 2 h and the product was then extracted into methylene chloride. The organic solvents were dried, removed under vacuum, and recrystallized from isooctane. Yields were 68% for IIa,b and 80% for IIc. Melting points (°C, uncorrected): IIa, oil; IIb, 97–100; IIc, 69–70. NMR (chemical shift in ppm from TMS, multiplicity, integration): IIa (6.8, M, 3H), (3.8, s, 6H). IIc (7.2, d, 2H), (6.8, d, 2H), (2.25, s, 6H).

Thiocarbamates. Thiocarbamates of IIa–c were prepared by reaction with ammonia[15]. To 0.0056 mol of the isothiocyanate in 30 ml absolute ethanol was added 0.0056 mol of ammonia. The solution was heated in a waterbath at about 90°C for 30 min, cooled, and the product collected on a filter. Recrystallization of the product was from ethanol. The ammonia derivatives were isolated in 96–98% yield. Melting points of ammonia derivatives were (IIIa–c, $R_2 = R_3 = H$) 244–247°C (IIIa), 142–145°C (IIIb) and 205°C (IIIc).

p-N,N-Dimethylaminophenylthiocarbamates of the amino acids were prepared following the method of Chang and Creaser[16]. In a 100-mm test tube with a screw cap was dissolved 0.001 mol of the amino acid in freshly prepared pH 9.5 buffer. The system was then purged with nitrogen, 0.4 g (0.002 mol) of DMAPI (IIa)

was added, the test tube capped, and the tube heated in a waterbath at 50°C for 10–20 min. The solvent was removed under vacuum and the residue washed several times with isooctane to remove the excess iosthiocyanate. Yields and melting points of the thiocarbamates are given in Table I.

Thiohydantoins. Thiohydantoins were prepared by dissolving 0.30 g of the amino acid thiocarbamate in 5 ml of water–glacial acetic acid (1:1) saturated with hydrochloric acid[17] (or 50% aqueous trifluoroacetic acid[18]) in a 100-mm test tube with a screw cap. The test tube was purged with nitrogen, capped and heated in a waterbath at 50°C for 10–20 min and then let stand at room temperature for 20–30 min. The solvent was removed under vacuum, the product washed several times with isooctane to remove any remaining isothiocyanate, and the product isolated by freeze drying. Yields and melting points are given in Table I.

Electrochemical characterization

Cyclic voltammetry was performed on about 10^{-5} mol of the isothiocyanate, thiocarbamate, or thiohydantoin derivative dissolved in 10 ml of aqueous 0.1 *M* phosphate buffer at the desired pH value. The buffer solution was placed in a cell of 30-ml capacity and covered with a PTFE cap through which were inserted the three electrodes and a nitrogen purge tube. After bubbling with nitrogen for 10 min, the flow of nitrogen was stopped, and the cyclic voltammogram of the quiet solution was obtained.

TABLE I

YIELDS AND MELTING POINTS FOR THIOCARBAMATES AND THIOHYDANTOINS OF AMINO ACIDS PREPARED FROM DMAPI

Amino acid	*Thiocarbamate (IIIa) m.p. (°C)*	*Thiohydantoin (IVa)*	
		% yield	*m.p. (°C)*
Alanine	127–132	95	212–214
Arginine	Deliquescent	–	Deliquescent
Asparagine	Not isolated	97	198–201
Aspartic acid	Not isolated	–	Deliquescent
Cysteine	176–182	95	169–174
Cystine	Not isolated	86	210–212
Glycine	136–144	84	184–188
Glutamic acid	Not isolated	91	130–134
Glutamine	Not isolated	95	215–217
Histidine	Deliquescent	–	Deliquescent
Isoleucine	Not isolated	88	211–212
Leucine	Not isolated	87	144–150
Lysine	Deliquescent	85	Deliquescent
Methionine	Not isolated	85	160–165
Phenylalanine	115–120	89	204–206
Proline	Not isolated	89	169–199
Serine	147–150	99	215–217
Threonine	Not isolated	–	Deliquescent
Tryptophan	135–140	–	Deliquescent
Tyrosine	153–158	–	Deliquescent
Valine	187–190	90	245–247

Chromatographic conditions

The HPLC mobile phase consisted of 0.1 *M* aqueous phosphate buffer in 25% acetonitrile prepared by dissolving 13.6 g of anhydrous potassium dihydrogen phosphate in 750 ml water followed by the addition of 250 ml of acetonitrile, and sulfuric acid or potassium hydroxide was used to adjust the pH. Prior to use, the mobile phase was degassed using reduced pressure. Injections (10 μl) containing up to 340 ng of each derivatized amino acid were made using a flow-rate of 2.5 ml/min. The eluting solutes were detected by oxidation at a glassy carbon electrode set at 0.85 V *vs*. Ag/AgCl.

Procedure for analysis of amino acid samples

To a prepared sample solution of amino acids in a 15 × 100 mm screw-cap tube containing 10–1000 ng of each amino acid, was added 2 ml of freshly prepared pH 9.5 buffer generated by mixing 50 ml water, 50 ml acetone, 5 ml of 0.2 *M* acetic acid, 1 ml triethylamine, and enough pyridine to bring the pH to 9.5. A volume 5 ml of a 10-ppm solution of DMAPI in acetone was added (or a minimum of a 2-fold excess of DMAPI by weight) and the tube purged with nitrogen. The test tube was covered and heated at 50°C in a water bath for 20 min. At the end of this heating period the solution was acidified by adding 5 ml of a solution consisting of one part glacial acetic acid saturated with hydrochloric acid and one part water. After purging with nitrogen, the mixture was again heated at 50°C for 20 min, and then set aside for an additional 20 min. The solvent was removed with a vacuum pump and the resulting solid product washed several times with isooctane to remove the excess isothiocyanate. The purified product was then dissolved in a measured volume of 0.1 *M* phosphate buffer (pH 2) and 10 μl injected into the chromatographic column.

Protein sequencing procedure

The peptide (100 ng) was dissolved in 2 ml of 0.1 *M* triethylamine–acetate buffer (pH 9.5) and added to a 2-fold excess of isothiocyanate in 0.2 ml of acetonitrile at 40°C and reacted for about 1 h. After evaporation *in vacuo* to remove the solvents, the excess reagent (DMAPI) was extracted using isooctane. The thiocarbamate formed was then treated at 40°C with 0.1 ml of 60% glacial acetic acid saturated with hydrochloric acid. After 15 min the solvents were evaporated *in vacuo*, and the product extracted using ethyl acetate. After removal of ethyl acetate *in vacuo*, the product was dissolved in the mobile phase (0.1 *M* potassium dihydrogen phosphate–25% acetonitrile, pH 6.0) and 10 μl of the sample (containing about 40 ng of product) was introduced onto the column.

RESULTS AND DISCUSSION

All three isothiocyanate reagents (IIa–c) react rapidly and quantitatively with ammonia to give the corresponding thiocarbamates (IIIa–c, $R_2 = R_3 = H$). An examination of the voltammogram of each derivative quickly focused our attention on the *p*-dimethylamino substituent because of the observed electrochemical reversibility. Cyclic voltammetry of the 2,5- and 3,4-dimethoxyphenylisothiocyanates (IIb, IIc) and their derivatives in aqueous solutions of acetonitrile and sodium sulfate yielded complex patterns whose major features include a large anodic peak with shoulder

around +0.4–0.6 V *vs*. Ag/AgCl. Some of the derivatives showed only slight solubility in aqueous systems and these compounds were therefore not investigated further.

The reaction of amino acids with *p*-N,N-dimethylaminophenylisothiocyanate (IIa, DMAPI), occurs readily to generate thiocarbamates (III). However, the difficulty in isolating an amino acid thiocarbamate free from cyclization products dictated that the mixture of products be completely converted to the thiohydantoins.

Spectrophotometric examination of buffered aqueous solutions of amino acid thiocarbamates and thiohydantoins before and after aeration indicated good stability at pH 2, but with some decomposition at pH 12. Standard solutions of the derivatives were therefore made slightly acidic, and the solid derivatives and solutions were kept in the refrigerator when not in use.

The acid dissociation constant of the aromatic dimethylammonium ion of the ammonia DMAPI derivative was estimated by UV absorption spectrophotometry. Absorbance measurements at 250 nm yielded a value for the pK_a of 4.4 ± 0.4.

Cyclic voltammograms of DMAPI (IIa) and of several of its derivatives are shown in Fig. 1. At pH 2–4 all of the derivatives undergo oxidation with varying degrees of reversibility at carbon paste or glassy carbon electrodes with anodic peak

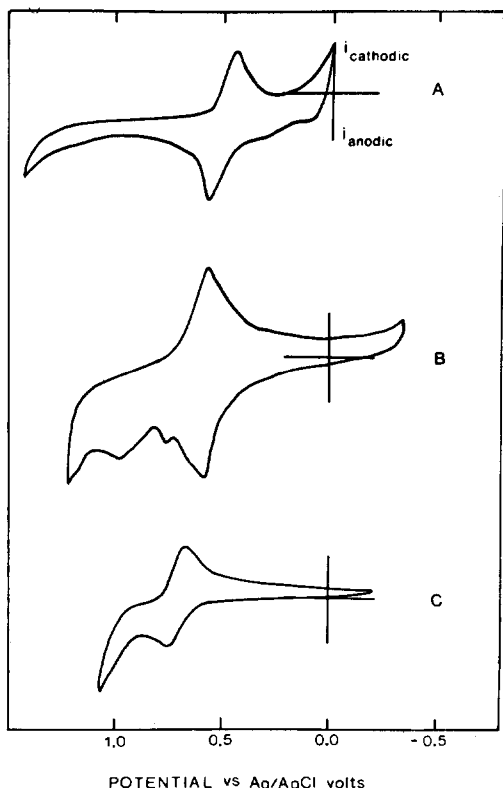


Fig. 1. Cyclic Voltammograms of (A) DMAPI (IIa), pH 4.0, (B) the thiocarbamate of DMAPI (IIIa, $R_2=R_3=H$), pH 4.0 and (C) the glycine thiohydantoin of DMAPI (IVa), pH 2.0. Conditions: carbon paste working electrode, scan 300 mV/sec; 0.1 *M* sodium sulfate in 2% acetonitrile.

TABLE II

CYCLIC VOLTAMMETRY DATA FOR DMAPI AND ITS DERIVATIVES

*Compound**	*pH*	*Working electrode*	$E_{p,a}$	$E_{p,c}$	*Current ratio***
DMAPI (Ia)	4.0	Carbon paste	0.56	0.44	1.0
Ammonia derivative (III)	4.0	Carbon paste	0.56, 0.75, 0.95	0.55, 0.45	–
Thiohydantoin–glycine (IV)	2.0	Glassy carbon	0.72	0.65	1.0
Thiohydantoin–arginine (IV)	2.0	Glassy carbon	0.72	0.64	1.1
			0.96	0.90	–

* Dissolved in 2% acetonitrile to give approximately 0.1 m*M* solutions in 0.1 *M* Na_2SO_4 as supporting electrolyte.

** Current ratio = anodic peak current/cathodic peak current.

potentials ($E_{p,a}$) in the range from +0.56 to 0.72 V *vs.* Ag/AgCl. Representative peak potentials and current data are listed in Table II.

The isothiocyanate IIa (DMAPI), shows a well-defined reversible wave at pH 4.0 with $E_{p,a}$ = 0.56 V *vs.* Ag/AgCl with the ratio of anodic to cathodic peak current equal to unity. The peak separation of 0.12 V indicates less than complete reversibility. This behavior was characteristic of all DMAPI derivatives studied in this work.

The ammonia derivative of DMAPI (IIIa) showed a characteristic anodic peak at 0.56 V, due to the oxidation of the aromatic dimethylamine at pH 4.0, plus additional peaks at 0.75 and 0.95 V, and several cathodic peaks on the reverse scan (see ammonia derivative, Table II). The cyclic voltammetry became more complex at higher pH values, with all peaks shifting to more negative potentials. The additional peaks are apparently due to oxidation of the thiourea portion of the molecule[19].

The thiohydantoin derivatives of the amino acids (IVa) yielded well-defined reversible waves in pH 2.0 phosphate buffer at glassy carbon with anodic peak potentials of 0.72 ± 0.01 V for all derivatives. The difference in potential between the cathodic and anodic peaks was 70–80 mV, and the $E_{\frac{1}{2}}$ values were 0.67–0.69 V. Some derivatives showed additional peaks at more positive potentials, which were attributed to oxidation of the amino acid portion of the molecule.

For the glycine derivative, the ratio of anodic to cathodic peak currents was unity at scan rates up to 1000 mV/sec, indicating that a reversible electron transfer is involved[20].

The presence of both polar and non-polar groups on thiohydantoins of the amino acids suggested that separation of the derivatives could be based on the use of a reversed-phase alkyl-bonded silica column. In addition, there is the possibility to control the ionization of various functional groups by the adjustment of pH. The derivatives also have sufficient solubility in aqueous acetonitrile and aqueous methanol for HPLC analysis.

Acetonitrile as a mobile phase modifier at pH 2 was found to yield shorter retention times than did the same concentration of methanol. Acetonitrile was selected for use because a lower proportion of organic could be used, thereby avoiding the precipitation of salts added to the mobile phase to provide for control of pH and

for the elctrolytic conductivity necessary for electrochemical detection. Retention times decreased with increasing acetonitrile concentration, but the retention times of the different amino acid derivatives were affected to varying degrees by changing the acetonitrile–water ratio. This phenomenon led to the observation that a decrease in acetonitrile concentration did not necessarily result in better resolution. A mobile phase containing 25% acetonitrile gave the best compromise between resolution and overall separation time.

The chromatograms obtained on C_8 and C_{18} columns were similar, with the C_{18} column yielding longer overall retention times for the same mobile phase. As there was no significant difference in the resolution obtained with the two columns, the C_8 column was selected to minimize the separation time. A chromatogram of 21 amino acid derivatives on a C_8 column using a mobile phase of 0.1 M phosphate buffer at pH 2.0 and 25% acetonitrile is shown in Fig. 2. The separation is complete in less than 80 min at a flow-rate of 2.5 ml/min. The resolution of most peaks is sufficient to give quantitative information, although the groups serine–glutamic acid–glutamine and histidine–alanine–arginine show significant overlap. Performing the separation at pH 6 gave resolved peaks for glutamic acid, histidine, alanine, and arginine, but the pairs glutamine–serine, tryptophan–lysine, and cystine–leucine were unresolved, and the separation of 21 amino acids required 95 min (Fig. 3).

Standard curves were constructed using prepared solutions of the purified thiohydantoins (IVa). A linear response for both peak current and peak area was observed over the range of 1 to 150 ng injected. The peak height sensitivity for

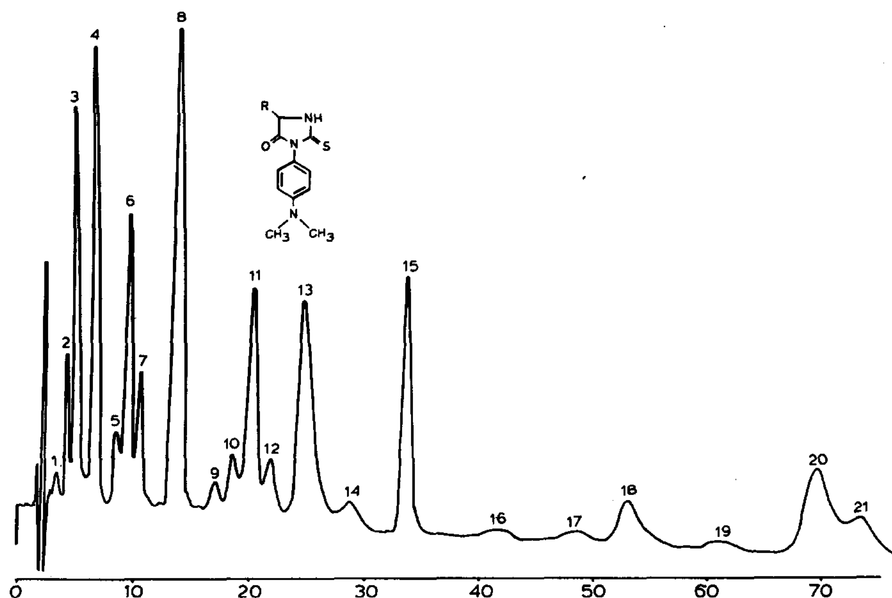


Fig. 2. Chromatogram of 21 p-dimethylaminophenylthiohydantoins. Conditions: column, 250 × 4.6 mm RP-8; mobile phase, 0.1 M potassium dihydrogen phosphate in 25% acetonitrile, pH 2; electrochemical detection at 0.85 V. Peaks: 1 = Cys, 2 = Asp, 3 = CyssCy, 4 = Asn, 5 = Ser, 6 = Glu, 7 = Gln, 8 = Thr, 9 = Gly, 10 = His, 11 = Ala, 12 = Arg, 13 = Met; 14 = Pro, 15 = Val, 16 = Trp, 17 = Phe, 18 = Tyr, 19 = Lys, 20 = Ile, 21 = Leu.

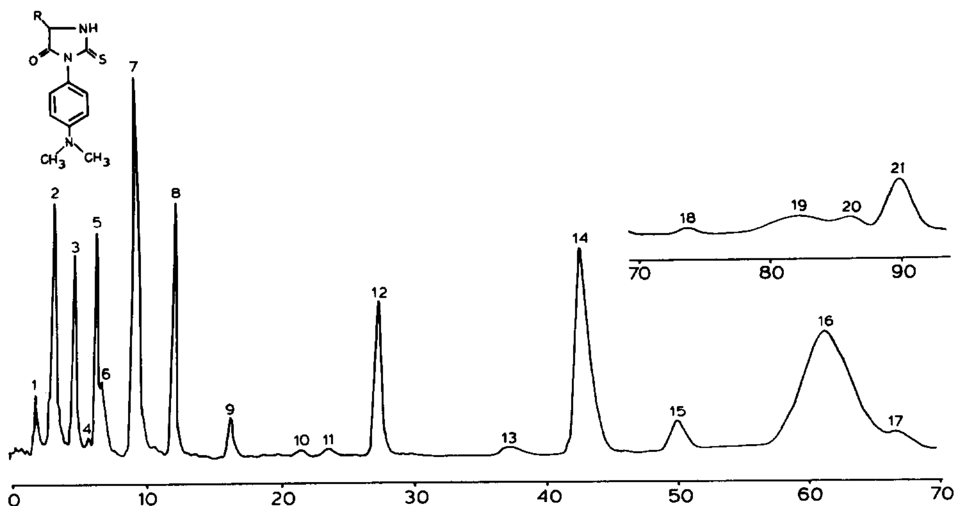


Fig. 3. Chromatogram of 21 *p*-dimethylaminophenylthiohydantoins. Conditions as in Fig. 2 except pH 6. Peaks: 1 = Cys, 2 = Asp, 3 = Glu, 4 = Asn, 5 = Gln, 6 = Ser, 7 = His, 8 = Pro, 9 = Ala, 10 = Gly, 11 = Arg, 12 = Tyr, 13 = Val, 14 = Met, 15 = Phe, 16 = Trp, 17 = Lys, 18 = Thr, 19 = CyssCy, 20 = Leu, 21 = Ile.

glutamine, with a short tetention time, was 0.33 nA/ng of derivative. For the valine derivative, which is more strongly retained, the sensitivity was 0.088 nA/ng.

The reproducibility in peak height for a series of seven injections of 20 ng of the alanine derivative over a 50-h period was excellent. The average peak height was 2.20 nA with a freshly polished glassy carbon electrode. (Detection limits signal-to-noise ratio = 2) were 0.2 ng for derivatives with short retention times (*e.g.* aspartic acid and glutamic acid), and 0.6 ng for derivatives with long retention time (*e.g.* methionine). There was no significant degradation in electrode response over a period of several weeks as long as injection of samples containing large amounts of analyte (greater than 100 ng) was avoided.

The percent recovery for ten of the amino acids ranged from 80% for alanine to 98% for glutamic acid. The values listed in Table III were determined by com-

TABLE III

PERCENT RECOVERY FOR THEN AMINO ACIDS

Amino acid	*% Recovery ± S.D.*
Alanine	80 ± 2
Asparagine	94 ± 0
Aspartic acid	96 ± 3
Glutamic acid	98 ± 4
Glutamine	96 ± 4
Isoleucine	85 ± 2
Leucine	87 ± 2
Methionine	85 ± 2
Tyrosine	90 ± 1
Valine	86 ± 4

parison of the peak area for a freshly derivatized sample with the response on the regression line indicated for the same amount of standard. Two derivatizations were carried out for each amino acid sample, and three injections were made for each run.

The ability of DMAPI to sequence proteins was tested using a di- and a tripeptide. In each instance the Edman degradation[3] occurred readily with the analysis indicating the presence of the appropriate N-terminal amino acid. Under the non-optimized conditions used, there was present 10–25% of the second amino acid resulting from some carry-over of DMAPI after the initial cleavage.

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