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PROTEIN MICROSEQUENCING USING HIGH-PRESSURE LIQUID CHROMATOGRAPHY OF PHENYLTHIOHYDANTOIN AMINO ACIDS

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SUMMARY

22 Phenylthiohydantoin (PTH) amino acids have been separated and quantitated using gradient elutions with acetonitrile on columns of Microbondapak C_{18}. The low signal-to-noise ratio and high sensitivity of the detection system permit quantitation of as little as 10 pmoles of phenylthiohydantoin amino acids from the sequencer. This high sensitivity has been used used to advantage in conjunction with a 0.1 *M* Quadrol sequencer program; the sequence of 2.7 nmoles of myoglobin was obtained for 32 successive cycles of Edman degradation employing high-pressure liquid chromatography as the sole method of identification of the PTH-amino acids. All of the phenylthiohydantoin amino acids are detected at 254 nm; the detection of PTH-threonine as PTH-dehydrothreonine is enhanced by detection at 313 nm.

INTRODUCTION

A necessary pre-requisite for a successful extended automatic Edman degradation is a quantitative determination of the phenylthiohydantoin (PTH)-amino acids obtained at each cycle. Gas–liquid chromatography (GLC) has made it possible to detect most PTH-amino acids[1], some at subnanomole levels. However, the sensitivity for certain PTH-amino acids is insufficient, and derivatization by silylation is necessary for certain residues, with variable results[2,3]. Hydrolysis to the free amino acids[4] does not permit distinction between the acidic amino acids and their amides, and hydrolytic yields for each residue vary. Moreover, certain pairs of PTH-amino acids yield an identical amino acid residue after hydrolysis. Thin-layer chromatography (TLC)[5,6] in several solvent systems may be used to resolve all of the PTH-amino acids, but the lack of quantitation makes it difficult to interpret extended sequences owing to the increasing background and overlap. TLC methods for the identification of PTH-histidine and PTH-arginine are problematic. Early reports of the direct identification of PTH-amino acids by high-pressure liquid chromatography (HPLC) on silica columns[7–11] suggested that polar PTH-amino acids could be detected without

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derivatization. However, non-polar derivatives were not retained, and re-equilibration was difficult following the use of protic solvents to elute the dicarboxylic PTH-amino acids[9]. In order to overcome these difficulties more than one solvent system or column packing was necessary to separate both polar and non-polar PTH-amino acids[7,9,12,13]. The identification and separation of PTH-amino acids on reversed-phase packings resulted in improved separation of most of the PTH-amino acids encountered in sequencing[12–15] including PTH-histidine and PTH-arginine. However, two groups of PTH-amino acids were difficult to resolve[9,15] the derivatives of valine, methionine and proline and the derivatives of tryptophan, phenylalanine and isoleucine.

We report here a strategy for the HPLC identification of 22 PTH-amino acids using a single reversed-phase packing and elution with acetonitrile employing one or two gradients. HPLC was used as the preferred primary method for quantitation of PTH-amino acids obtained from the sequencer. The identification of PTH-threonine from the sequencer was complemented by detection of the dehydro-derivative at 313 nm.

HPLC has been compared favorably in sensitivity to GLC and TLC[15]. It was demonstrated[13] that PTH-amino acid standards can be detected by their absorbance at 254 nm at levels less than 10 pmoles. Such sensitivity is attractive for the sequencing of proteins of biological interest available only in small amounts.

In the present study, the sensitivity of the UV detector used permits detection and quantitation not only of PTH-amino acid standards, but also of samples from the sequencer at levels as low as 10 pmoles of PTH-amino acid. The programs employed permit identification at this level without interference from contaminants arising from the sequencer, even when as much as 40% of the entire sequencer output for a given cycle is injected into the HPLC. Using only HPLC for PTH-amino acid identification, 32 successive cycles were possible from a sequencer degradation of 2.7 nmol of sperm whale apomyoglobin employing a 0.1 *M* Quadrol protein microsequence program[3].

METHODS

PTH-amino acids were obtained from Pierce (Rockford, Ill., U.S.A.) and were stored in acetonitrile at concentrations of 50 nmole/ml at −10° in the dark. The UV absorbance of PTH-histidine and PTH-arginine decreased significantly after a few days under these conditions; they were therefore stored in methanol at concentrations of 1 μmol/ml. On the day of use they were diluted 20-fold into acetonitrile. Spectral grade acetonitrile was obtained from Jackson and Burdick (Muskegon, Mich., U.S.A.). Water used for HPLC buffers was deionized and distilled twice from glass.

Automatic Edman degradation was carried out in a Beckman 890B sequencer equipped with nitrogen blowdown and an undercut cup adaptation (Beckman No. 336481) using a 0.1 *M* Quadrol protein microsequence or peptide microsequence program as described previously[3]. For some peptides a volatile buffer program employing dimethylallylamine (DMAA) was used. In certain experiments, in addition to HPLC, the identification of PTH-amino acids was also made by GLC using SP-400 (ref. 3) and by two dimensional TLC on polyamide sheets[6]. Thiazolinone amino acids were converted into the phenylthiohydantoin derivative as described previously[3]. Appropriate aliquots of PTH-norleucine were added as an internal

standard to each tube in the sequencer fraction collector. Amino acid analysis of protein samples was performed as described previously[16].

The apparatus used for liquid chromatography consisted of a Model ALC/6PC 204 liquid chromatograph (Waters Assoc., Milford, Mass., U.S.A.) equipped with a second Model 6000 A pumping system, Model 660 solvent programmer for gradient elution, Model U6K injector and Model 440 absorbance detector. Reversed-phase chromatography was performed using a single column (30 cm × 4 mm) of Microbondapak C_{18} (Waters Assoc.). All of the solvents used in the liquid chromatograph were passed through a 0.22 μm Millipore filter.

Buffer A was composed of 10% acetonitrile in 0.01 *M* sodium acetate buffer (pH 4.0); buffer B was composed of 90% acetonitrile in 0.01 *M* sodium acetate buffer (pH 4.0). Following conversion of the thiazolinone in aqueous acid, the PTH derivative was extracted twice with 1 ml of ethyl acetate. The organic phase was dried under a stream of nitrogen at 50°, and then dissolved in 50–200 μl of acetonitrile for injection in the liquid chromatograph. Samples for GC and TLC identification were redissolved as necessary in ethyl acetate. One ml of water was added to the aqueous phase which was then lyophilized. The dry residue was dissolved in 50–100 μl of acetonitrile. Between 5 and 20 μl of sample were used for injection. For the experiment employing sperm whale apomyoglobin, dried samples from conversion were dissolved in 25–50 μl of acetonitrile; 10–40% of the total sample was used for injection. Chromatography was carried out at ambient temperature (20–23°) at pressures of 1200 to 2000 p.s.i. PTH-amino acids were detected at 254 nm. In addition, PTH-threonine was detected at 313 nm (as PTH-dehydrothreonine) using a 313 nm filter.

Programs optimized for PTH-amino acid separation by variation in the flow-rate, gradient shape, time or initial and final buffer concentrations were as follows (Fig. 1): Program 1 (Fig. 1a) utilized gradient curve No. 10 of the programmer, beginning with 25% solvent B and ending with 50% solvent B, over 20 min at 2.4 ml/min. Program 2 (Fig. 1b) also utilized gradient curve No. 10 with initial conditions of 2% solvent B and final conditions of 30% solvent B, for 15 min at 2.2 ml/min. Program 3 (Fig. 1c) employed isocratic elution at concentrations of 50% solvent B over 10 min at a flow-rate of 1 ml/min. Program 4 (Fig. 1d) used for PTH-amino acids found in the aqueous phase, employs gradient No. 10 for 10 min at 2.0 ml/min. Initial conditions were 10% solvent B and final conditi ns were 30% solvent B. The attenuation of the detector used in these experiments varied between 0.005 and 0.05 absorbance units at full scale deflection of the recorder (a.u.f.s.).

After multiple successive injections using program 2, accumulated non-polar PTH-amino acids bled from the column and interfered with subsequent identifications. Therefore, after each group of injections, the final concentration of buffer B was changed to 50 or 65% for 2 min at 3 ml/min, followed by 3 min elution under the initial conditions.

RESULTS AND DISCUSSION

Following conversion, all of the PTH-amino acids from the organic phase may be detected within 20 min using program 1 (Fig. 1a). In the course of identifying unknown PTH-amino acids from automated Edman degradation, this program is used for an initial evaluation. If the PTH derivatives of alanine, tyrosine, valine, isoleucine,

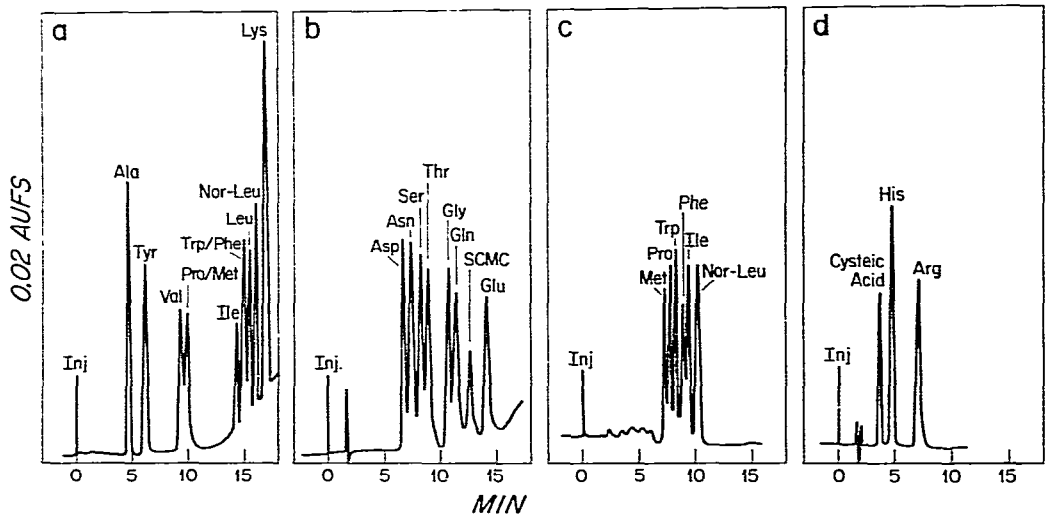

Fig. 1. The identification of PTH-amino acids by HPLC. (a) Program 1 employs gradient curve No. 10 at a flow-rate of 2.4 ml/min. Initial conditions, 25% buffer B; final conditions, 50% buffer B. 0.5 nmole of a mixture of standard PTH-amino acids were separated within 20 min. The unresolved pairs PTH-proline–methionine and PTH-tryptophan–phenylalanine may be resolved by isocratic elution using 50% buffer B (Fig. 1c). (b) Program 2 employs gradient No. 10 at a flow-rate of 2.2 ml/min. Initial conditions, 2% buffer B; final conditions, 30% buffer B. 0.5 nmole of a mixture of standard PTH-amino acids were separated in 15 min. The PTH-amino acids that are separated using program 2 are all eluted within the first 4 min using program 1. (c) Program 3 resolves the PTH-amino acid pairs unresolved by program 1, using isocratic elution at 50% buffer B and a flow-rate of 1.0 ml/min. 250 pmoles of PTH-amino acids were injected. The pairs PTH-proline–methionine not resolved by program 1 are distinguished. During isocratic elution, PTH-valine is coeluted with PTH-methionine. Similarly PTH-tryptophan and PTH-phenylalanine not resolved by program 1 are separated here by isocratic elution. However, under these conditions PTH-phenylalanine is coeluted with PTH-lysine; PTH-leucine is coeluted with the internal standard (PTH-norleucine) and PTH-alanine is coeluted with PTH-tyrosine (not shown in the Figure). (d) Program 4 is used for PTH-amino acids from the aqueous phase. They were separated within 10 min using gradient curve No. 10 at a flow-rate of 2.0 ml/min. Initial conditions, 10% buffer B; final conditions, 30% buffer B.

eucine or lysine are present they may be quantitated from this run. If the PTH-derivatives of aspartic acid, asparagine, serine, threonine, glycine, glutamine, S-carboxymethylcysteine or glutamic acid are present, they elute within the first 4 min on program 1 as closely spaced peaks (see Fig. 3b,f). They may be unequivocally confirmed and quantitated using program 2 (Fig. 1b), which separates all of them in 15 min using a gradient of lower concentration of acetonitrile than does program 1.

Two pairs of PTH-amino acids are not resolved using gradient elution in program 1: PTH-proline–methionine and PTH-tryptophan–phenylalanine. However, they may be resolved, within 10 min by isocratic elution using program 3 in which the separation is facilitated by the lower flow-rate (Fig. 1c).

If no new PTH-amino acid is found in a sequencer sample using program 1, then the aqueous phase is next examined using program 4 (Fig. 1d). This program resolves the PTH derivatives of cysteic acid, histidine and arginine within 8 min. Thus, in order to quantitate and separate 22 PTH-amino acids including PTH-norleucine, one or a maximum of two injections are necessary. We found it convenient

to reduce the total analysis time by initially examining all of the samples using program 1. Even when programs 1 and 2 are combined into a more prolonged run over an extended acetonitrile gradient, the triplets PTH-proline–valine–methionine and PTH-tryptophan–phenylalanine–isoleucine are still incompletely resolved, and program 3 is nonetheless required. All of the four programs employ the same reversed-phase packing and a single buffer system for the detection of samples from the sequencer.

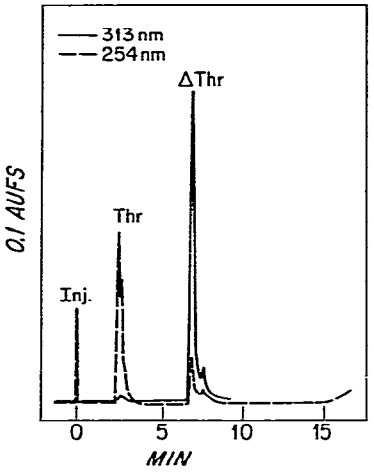

Fig. 2. PTH-threonine (5 nmole) was subjected to the conditions for conversion in aqueous acid (see text) and chromatographed using program 1 (see Fig. 1a), with detection at 254 nm and also at 313 nm. The major species is PTH-dehydrothreonine (ΔThr, ———) which is eluted at a position just following tyrosine in program 1 (compare Fig. 1a) and is detected at 313 nm. PTH-threonine (---------) is barely detectable at 313 nm, but is the major PTH-threonine species detected at 254 nm.

During Edman degradation in the automatic sequencer, the unstable PTH derivatives of threonine are recovered as PTH-threonine and PTH-dehydrothreonine[2]. Although PTH-threonine may be identified by HPLC in program 1 (Fig. 2) or 2 (Fig. 1b), the major product from the sequencer is usually PTH-dehydrothreonine. Since the absorbance spectrum of PTH-dehydrothreonine is shifted to higher wavelengths[5], this fact was used to advantage to complement the identification of threonine from sequencer samples. Samples of standard PTH-threonine (5 nmoles) were subjected to the conditions of conversion (see Methods) and examined by HPLC at 254, 280 and 313 nm. While solutions of standard PTH-threonine gave a single peak eluting early in program 1 (see Fig. 2), after conversion two peaks were founded. The additional peak due to PTH-dehydrothreonine eluted following PTH-tyrosine (Fig. 2) in program 1 and had an absorption maximum at 313 nm. At this wavelength, the absorption of PTH-threonine was nil. Samples from the automated sequencer behaved in a similar fashion, with a major peak (PTH-dehydrothreonine) best detected at 313 nm. At this wavelength all the other PTH-amino acids (except perhaps the unstable PTH derivative of dehydroserine for which recovery by HPLC was unreliable) have little absorption. Thus, the estimation of threonine as PTH-

dehydrothreonine at 313 nm is a more sensitive and quantitative measure of threonine from the sequencer than detection at 254 nm. The quantitative distribution of a given sequencer sample between PTH-threonine and PTH-dehydrothreonine found by HPLC paralleled the distribution between these two peaks as measured by GLC[3].

The only PTH-amino acid which could not be reliably recovered was PTH-serine (as PTH-dehydroserine) resulting from the sequencer, despite the fact that it could be detected by GLC at levels of *ca.* 40–60% as compared to the stable PTH-amino acids in the same sequencer run.

With continuous use, the columns employed have maintained resolution and sensitivity for between 1000 and 3000 injections. We encountered variations in the resolution of non-polar PTH-amino acids using successive columns, and thus the programs used (especially program 3) must be optimized for each new column by adjusting the flow-rate and the initial and final solvent concentrations. Fluctuations in the retention time due to changes in ambient temperature were not encountered.

Contaminants from sequencer degradations using 0.1 *M* Quadrol programs were eluted prior to any PTH-amino acid (see Fig. 5d,g,k). Similarly, all of the contaminants obtained from the sequencer aqueous phases were eluted prior to PTH-cysteic acid and did not therefore interfere with the identification. Samples from sequencer degradations employing the volatile DMAA buffer program for peptides typically demonstrated one or two contaminant peaks eluting between PTH-proline and PTH-isoleucine in program 1 (Fig. 1a).

An example of the application of the strategy described for identification of PTH-amino acids by HPLC in the course of sequencer degradation is illustrated in Fig. 3. A 46-residue cyanogen bromide peptide (75 nmole) obtained from the heavy chain of a homogeneous rabbit antibody was subjected to Edman degradation using a 0.1 *M* Quadrol peptide sequencer program. The results from six successive cycles are shown. All of the identifications were made initially using program 1 which was sufficient to identify and quantitate PTH-alanine at cycles 26 (Fig. 3a) and cycle 29 (Fig. 3d), and PTH-lysine at cycle 30 (Fig. 3e). PTH-asparagine at cycle 27 (Fig. 3b) and PTH-glycine at cycle 31 (Fig. 3f) could be identified from program 1 alone, but are best quantitated thereafter with a second injection using program 2 (Fig. 3g,h). The distinction between PTH-phenylalanine and PTH-tryptophan at cycle 28 (Fig.3c) was made by a second injection using isocratic elution (see Fig. 1c, program 3). At cycle 32, no new organic phase PTH-amino acid was found; therefore the aqueous phase was examined using program 4, revealing 140 pmoles of PTH-arginine (Fig. 3j). This may be compared to the nackground level of PTH-arginine from the aqueous phase shown in cycle 31 (Fig. 3i). All of the PTH-amino acids in this experiment were detected at an attenuation of 0.01 a.u.f.s. The unlabeled peaks shown are due to the background and overlap expected for a sequencer degradation using a peptide program. The detector noise was minimal. The chromatograms shown in Fig. 3 represent 140–800 pmoles of PTH-amino acid injected.

Using PTH-amino acid standards, at maximal detector sensitivity (0.005 a.u.f.s.), as little as 10–30 pmoles of PTH-amino acid may be detected. Similar sensitivity was found when between 1 and 10% of each sequencer sample from degradations of 50–150 nmoles of peptide or protein were examined, a situation in which noise due to contaminants from the sequencer is expected to be low. In order to determine whether such sensitivity as determined for PTH-amino acid standards

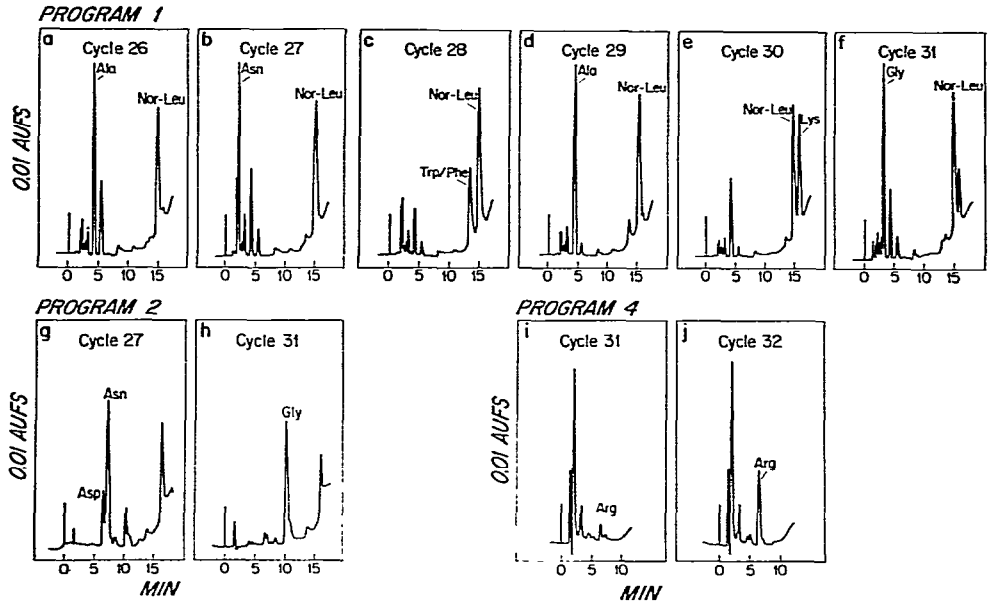

Fig. 3. The identification of PTH-amino acids from an Edman degradation of 75 nmoles of a 46-residue cyanogen bromide peptide from a rabbit antibody heavy chain using a 0.1 *M* Quadrol peptide sequencer program[3]. The overlap was 2% per cycle. Cycles 26–32 were initially examined using program 1 (a–f). PTH-norleucine was added as an internal standard to each tube in the fraction collector. PTH-asparagine at cycle 27 (b) and PTH-glycine at cycle 31 (f) identified from program 1 were confirmed using program 2 (g,h). The pair PTH-phenylalanine–tryptophan at cycle 28 (c) were resolved using program 3 (see Fig. 1c). No new PTH-amino acid was found using program 1 at cycle 32. Therefore the aqueous phase was examined using program 4. The amount of PTH-arginine found at cycle 32 (j) was 140 pmoles. Note the low PTH-arginine background at cycle 31 (i). The peaks eluting earlier than PTH-cysteic acid in program 4 are contaminants in the aqueous phase and increased during the course of sequencer degradations.

can be utilized in practice in situations where a larger portion of the converted sequencer sample is injected, thereby increasing the ratio of contaminants to PTH-amino acid, we studied the identification of PTH-amino acids by HPLC from the degradation of only 2.7 nmoles of sperm whale apomyoglobin using a 0.1 *M* Quadrol program. The results of 32 successive cycles of Edman degradation are shown in Fig. 4. The initial yield was 63% and the repetitive yield was 93.2%. We previously demonstrated that alteration of liquid phase sequencer programs using 0.1 *M* Quadrol makes it unnecessary to employ a carrier polypeptide in the spinning cup cup to reduce mechanical and chemical losses of small quantities of protein from the cup[3]. When using this program previously on 3 nmoles of myoglobin with PTH-amino acid identification by GLC and TLC, we were able to obtain the sequence for 20 cycles[3]. By comparison, in the present study using HPLC as the sole method of identification, the sequence could be interpreted for 32 cycles. Elution profiles of a mixture of PTH-amino acid standards used for quantitation in this experiment are shown at high detector sensitivity in Fig. 5a (program 1, 250 pmoles, 0.01 a.u.f.s.) and 5b (program 2, 100 pmoles, 0.005 a.u.f.s.). The organic phase from each cycle was first examined by using program 1. As noted for cycle 6 (Fig. 5c,d) and cycle 8 (Fig. 5f,g),

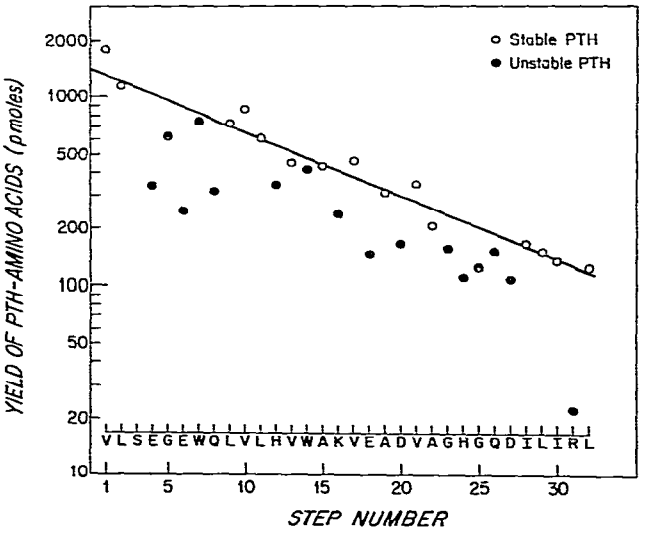

Fig. 4. The yields of PTH-amino acids found by HPLC from a sequencer run using a 0.1 *M* Quadrol microsequence program on 2.7 nmoles of sperm whale apomyoglobin. The initial yield was 1.7 nmoles. Amino acids are given in the one-letter code[20]. Stable PTH-amino acids are indicated by open circles; relatively unstable PTH-amino acids are indicated by closed circles.

PTH-amino acids which elute early using program 1 were confirmed by reinjection using program 2. It is to be noted that PTH-glutamic acid and PTH-glutamine may be quantitated from the same chromatogram (Fig. 5f,g). The extent of deamidation was 35%. Inspection of Fig. 5d,g,k reveals that, using program 2, the major UV-absorbing contaminants eluted earlier than any PTH-amino acid. Despite the fact that as much as 40% of the converted sequencer sample was injected, contaminants did not interfere with identification using program 1 (Fig. 5j,l–n).

At step 12 (Fig. 5h) no new PTH-amino acid was detected. Therefore the aqueous phase was examined using program 4, revealing a new PTH-histidine (Fig. 5i). PTH-alanine at cycle 22 (Fig. 5j) and PTH-glycine at cycle 25 (Fig. 5k) may be easily distinguished from the background. The sequence isoleucine–leucine–isoleucine at cycles 28–30 could be unequivovally confirmed (Fig. 5l–n) despite the fact that the total working sequence at these cycles was only 100–150 pmoles (Fig. 4). At these levels the distinction between PTH-leucine and PTH-isoleucine cannot be made by GLC (requiring silylation) nor by back-hydrolysis to the free amino acid detected on an unmodified high sensitivity amino acid analyzer. The results of this experiment using myoglobin demonstrate that HPLC may be used to extend the limit of detection during Edman degradation, and is particularly useful when limited amounts of material are available for sequencing.

The results reported here compare favorably with those of Niall[17] using [^{35}S]phenylisothiocyanate (PITC) in coupling, a carrier in the cup to prevent extractive losses and semiquantitative detection of PTH-amino acids by TLC and autoradiography. The identification of 17 cycles of degradation on 800 pmoles of myoglobin was accomplished[17]. Successful sequencer degradation of subnanomole amounts of intrinsically labeled protein is also possible using back-hydrolysis and quantitation

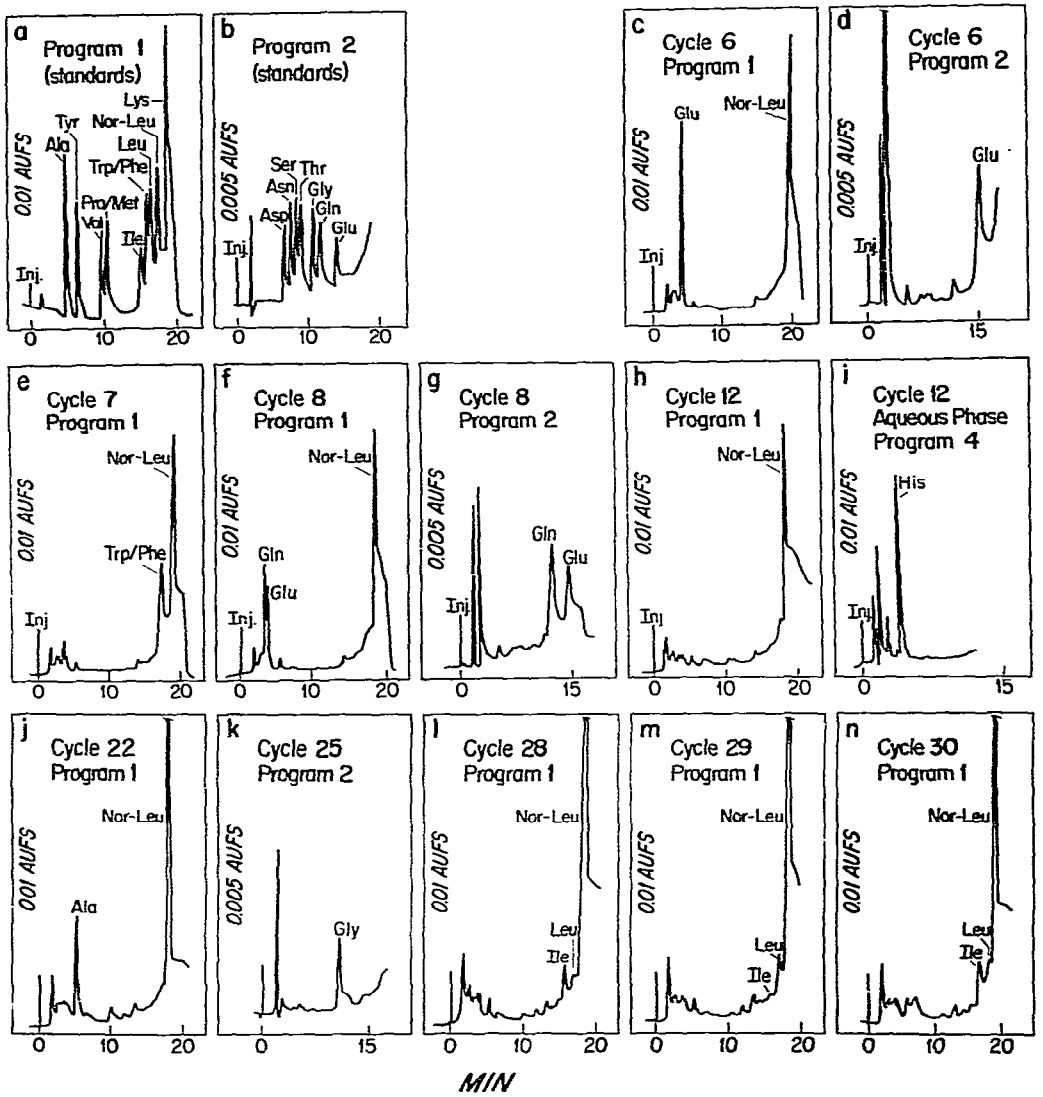

Fig. 5. Elution profiles of PTH-amino acids obtained by HPLC from sequencer degradation of 2.7 nmoles of sperm whale apomyoglobin. Between 10 and 40% of the entire sample from the sequencer was injected following conversion. (a) 250 pmoles of a mixture of PTH-amino acid standards program 1, 0.01 a.u.f.s. (b) 100 pmoles of a mixture of PTH-amino acid standards, program 2, 0.005 a.u.f.s. (c,d) PTH-glutamic acid at cycle 6 is detected on program 1 (c) and confirmed by using program 2 (d). Large peaks eluting within the first 4 min using program 2 are non-PTH-contaminants (see also g,k). (e) PTH-tryptophan at cycle 7 was detected using program 1 and was distinguished from PTH-phenylalanine using program 3 (compare Fig. 1c). (f,g) PTH-glutamine at step 8 produces a doublet using program 1 (f) due to deamidation. Both forms (PTH-glutamine and PTH-glutamic acid) are confirmed and quantitated by repeat injection using program 2 (g). The extent of deamidation was *ca.* 35%. (h) At step 12 no new PTH-amino acid was found in the organic phase using program 1. Note the very low background due to contaminants from the sequencer. (i) Examination of the aqueous phase from step 12 revealed PTH-histidine. (j,k) PTH-alanine, using program 1, was clearly distinguished from the background at cycle 22 (j) as was PTH-glycine using program 2 for cycle 25 (k). (l–n) Despite a total sequencer yield of only 100–150 pmoles, the sequence isoleucine–leucine–isoleucine may be distinguished at cycles 28–30 using program 1.

of radioactive free amino amino acids after correction for hydrolysis losses[18]. With recent improvements in separation by HPLC it is possible to collect and quantitate radiolabeled PTH-amino acids following the addition of cold carrier PTH-amino acids[19]. The ability to quantitate radiolabeled PTH-amino acids from the automated sequencer without derivatization is an attractive alternative for obtaining extended Edman degradations on subnanomole amounts of protein; this approach is equally applicable using an extrinsic label [^{35}S]-PITC or to intrinsically labeled proteins.

The ability to quantitate all of the PTH-amino acids using a single method is a distinct advantage of HPLC in comparison to GLC where certain PTH-amino acids cannot be identified and the sensitivity is lower. An additional advantage of HPLC is a 10 to 50-fold greater sensitivity for PTH-amino acids than GLC[3]. Direct identification of PTH-amino acids by HPLC makes it unnecessary to derivatize or hydrolyze to the free amino acid with concomitant variation in yields. In addition, for certain PTH-amino acids, HPLC is clearly superior. PTH-histidine and PTH-arginine heretofore could only be quantitated after hydrolysis to the free amino acid. The detection of amides cannot be done following back-hydrolysis and is difficult and of low sensitivity by GLC. The distinction between aspartic acid and its amide and glutamic acid and its amide may easily be made by HPLC (program 2) including quantitation of the extent of deamidation. In these experiments using the 0.1 *M* Quadrol sequencer program the extent of deamidation varied between 25 and 35%. The distinction between PTH-leucine and PTH-isoleucine is also most easily made by HPLC even at low levels (Fig. 5l–n).

The results reported here, and previously by other workers[13,15], indicate that HPLC is now the most versatile single method for quantitative identification of PTH-amino acids obtained from automatic sequencing*. We have taken advantage of the high sensitivity of UV detection of PTH-amino acids, in combination with a 0.1 *M* Quadrol sequencer program which obviates extractive losses of protein from the cup, to obtain extended sequence analysis on amounts of protein in the range of 1–10 nmoles, without the necessity of using a radiolabel.

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