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On-line separation of phenylthiohydantoin derivatives of hydrophilic modified amino acids during sequencing ☆

Daniel J. Strydom

Center for Biochemical and Biophysical Sciences and Medicine and Department of Pathology, Harvard Medical School, 250 Longwood Avenue, Boston, MA 02115, USA

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Abstract

All 20 commonly occurring phenylthiohydantoin (PTH) amino acids can be separated by a new reversed-phase chromatography method without interference from breakthrough peaks that prevail in other methods, and with good resolution from the most common reagent-related background peaks. Separation is compatible with automated sequencing in a cartridge sequencer. A large chromatographic area exists between the breakthrough peaks and the first- and second-eluting common PTH-amino acids, PTH-His and PTH-Asn, and is consequently available for detection and separation of hydrophilic amino acid derivatives. Derivatives such as phosphotyrosine, β-hydroxyaspartic acid and N-glycosylated asparagine elute as readily identifiable peaks. The method uses chromatography on octadecylsilane with isocratic elution followed by a simple linear gradient from acetonitrile–methanol to isopropanol–methanol to provide complete separation in 23 min. The turnaround time of 34 min includes a period of column washing to eliminate sequencer byproducts. PTH-Amino acids are generally detected with single pmol (1 to 4 pmol) sensitivity. PTH-His and PTH-Arg are eluted earlier than conventionally with a consequent sharpening of peaks and higher practical sensitivity.

1. Introduction

The identification and quantitation of phenylthiohydantoin (PTH) amino acids are at present fundamental to the process of protein and peptide sequencing, and provide a means to both recognize known modifications and point to the existence of unknown modifications. Chemical protein sequencing is therefore often used to examine potential post-translational modification of proteins. The availability of the protein of interest is often the limiting factor in such studies and therefore individual experiments should yield as much information as possible. Successful recognition of a modified residue at a specific position in a sequence presupposes the positive identification of a chromatographic peak during sequencing, and not merely the absence or low yield of normal residues, which may be caused by conventional losses inherent to the chemistry of the sequencing process.

Many post-translational modifications give rise to hydrophilic amino acid side chains and those are most likely to be missed or confused when using the common separation systems. Such modifications may be natural, such as glycosylation, phosphorylation, sulfation, hydroxylation and carboxylation, or synthetic, due to deliberate modifications, and are usually detected by

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alternative PTH separations, which are not used routinely. As an example acidic derivatives such as β-hydroxyaspartic acid may be analyzed by using 0.1% trifluoroacetic acid (TFA) as eluent [1]. Other derivatives that have been detected, but which elute in inconvenient positions for studies with low amounts of sample, are *e.g.* phosphotyrosine [2,3], γ-carboxyglutamic acid [4], and phosphoserine/threonine [5], although the latter derivatives may not survive the Edman chemistry [6,7]. Chemically modified residues studied by Edman degradation include degraded carboxyhistidines [8], azobenzene arsonate derivatives [9] and photoaffinity-modified residues, reacted with 8-azido ATP [5]. Glycosylation sites are frequently detected as "blank" sequencer cycles (see, *e.g.* ref. 10), although partial deglycosylation of protein samples allows extraction and identification of monoglycosidic PTH-amino acids [11] and the use of covalently immobilized samples in the sequencers allow extraction and chromatography of fully glycosylated residues [12,13].

Most current cartridge-style sequencers ("gas-phase sequencers") incorporate the on-line determination of PTH-amino acids by HPLC. The separation schemes have to accommodate the short cycle times of the sequencers and as a consequence most elute the first PTHs very early, in many cases within seconds of the sample breakthrough peaks and disturbances. In addition, the most common separations operate at a pH where PTH-Asp elutes first, thereby providing the characteristic "Asp-problem" for sequencing, which is the frequent loss of information on small amounts of Asp, or at best integration problems with the Asp peak eluting on or next to baseline disturbances. Hydrophilic (early-eluting) derivatives will consequently only be recognized with difficulty, if at all, in these early parts of the chromatograms.

Other difficulties experienced in PTH-amino acid separation schemes include the broadening of PTH-His and -Arg peaks and their consequently less sensitive detection, and the existence of reagent-related background peaks which may co-elute with PTH-amino acids.

We here developed a separation of PTH-amino acids that is compatible with a 34-min turn-around on a Millipore ProSequencer and that also provides disturbance-free chromatography in the early parts of the chromatogram as well as chromatographic space for detection of hydrophilic post-translational modifications. In addition PTH-His and -Arg are eluted much earlier and with sharper peaks than conventionally, thereby providing enhanced sensitivity. Many extraneous peaks [such as diphenylthiourea (DPTU)] are separated from the common PTH-amino acids.

2. Experimental

2.1. Materials

Standard PTH-amino acids, β-hydroxyaspartic acid and phosphotyrosine were from Sigma (St. Louis, MO, USA). Diphenylurea, diphenylthiourea, phenylthiourea and aniline were from Aldrich (Milwaukee, WI, USA). HPLC-grade solvents were from J.T. Baker (Phillipsburg, NJ, USA) and water was purified on a Milli-Q system (Millipore, Bedford, MA, USA) utilizing ion-exchange and Organex-Q cartridges. A glycopeptide, V2, was derived from a *Staphylococcus aureus* V8 digest of a ribonuclease homologue (unpublished). (The peptide contains significant amounts of mannose and glucosamine, and has the characteristic Asn–X–Thr sequence for potentially N-glycosylated Asn. Only the C-terminal Asp is found by conventional sequencing of the whole peptide, which has an amino acid composition that includes two aspartic acid or asparagine residues).

2.2. Sequencer

A Millipore ProSequencer [14] was used, employing the manufacturer's standard TFA-100 protocol which elutes anilinothiazolinone (ATZ) derivatives from the cartridge membrane with a pulse of TFA. Peptides were bound to arylamine

membranes (Sequelon-AA, Millipore), by carbodiimide (EDC) coupling [15–18].

2.3. HPLC

The HPLC equipment consisted of either (i) two M6000 pumps, a M680 gradient controller and a M440 detector (Millipore/Waters) and a PE Nelson interface/Nelson analytical chromatography software (Perkin-Elmer, Norwalk, CT, USA) for data collection or (ii) a Model 62M pump, 600E system controller and M486 tunable absorbance detector (Millipore) and Maxima software, on-line to a Millipore ProSequencer. The column was a Supelcosil LC-18-DB (25 × 0.46 cm; Supelco, Bellefonte, PA, USA), with a guard column of the same material.

Solvent A contained 0.33% (v/v) triethylamine (HPLC grade, Fisher, Pittsburgh, PA, USA), 9.5% (v/v) methanol and 10.5% (v/v) acetonitrile, and the pH was adjusted with orthophosphoric acid (HPLC-grade, Fisher) to 3.55 before addition of the organic solvents. Solvent B was methanol–isopropanol–water (60:20:20). The applied gradient is given in Table 1.

Table 1
Protocol for gradient-elution of PTH-amino acids

Time (min)	Flow-ate (ml/min)	Solvent A (%)	Solvent B (%)
0	1	95	5
7	1	95	5
22	1	33	67
23	1	0	100
25	1	0	100
25.5	1.2	0	100
28	1.2	0	100
29	1.2	95	5
33	1.2	95	5
33.5	1	95	5

The gradient segments are all linear. The column was a Supelcosil LC-18-DB (25 × 0.46 cm), with a guard column of the same material. Solvent A contained 0.33% (v/v) triethylamine, 9.5% (v/v) methanol and 10.5% (v/v) acetonitrile, and the pH was adjusted with orthophosphoric acid to 3.55 before addition of the organic solvents. Solvent B was methanol–isopropanol–water (60:20:20).

3. Results and discussion

3.1. Design and implementation of the PTH separation

An octadecylsilane column and methanol and isopropanol as solvents were taken as baseline conditions for the design of an improved PTH separation, based on prior experience over more than a decade in this laboratory with such a system in conjunction with a spinning cup sequencer [19]. Relatively few studies have used isopropanol as the major eluent for achieving separation of PTH amino acids (*e.g.* refs. 19–24). All of these demonstrate the same characteristic separation of the later-eluting PTH-amino acids which was again found in this study. Thus isopropanol provides a useful selectivity for the hydrophobic amino acids, allowing facile PTH-Met/Val separation and positioning PTH-(ε-PTC; phenylthiocarbamyl)-Lys and PTH-Trp conveniently earlier than PTH-Phe, in the large open chromatographic space after PTH-Val. The viscosity of isopropanol was accommodated and the consequent high chromatographic pressures were alleviated by the addition of a large ratio of methanol to the B solvent, and raising the temperature above room temperature. Notably the change from solely isopropanol in the second solvent to methanol–isopropanol (3:1) did not markedly change the relative elution positions of the amino acids Ala through Leu. The use of triethylamine phosphate as modifier of the chromatographic solvent allowed PTH-His and -Arg to elute at much earlier times than is commonly found, and the adjustment of pH to 3.55 delayed elution of PTH-Asp and PTH-Glu until *after* both PTH-His and -Arg. A mixture of acetonitrile and methanol was needed to separate the PTH-Thr, -Gln, -Gly triplet and the ratio of these two modifiers affects the position of those derivatives relative to one another.

The elution protocol is simple, starting with isocratic elution of the hydrophilic amino acids, followed by a single linear gradient in isopropanol–methanol, thereby allowing facile adjustment of conditions for different columns.

Two very different chromatographs were used during the development of this protocol —a single pump system with premixing of the gradient, and a dual pump system with post-delivery mixing of the solvents— and the separations are highly similar on both, suggesting ready applicability to other chromatographs.

The chromatographic separation achieved in the current studies is shown in Fig. 1. All the common amino acids are well resolved, and sequencer-derived byproducts of phenylisothiocyanate (PITC), such as DPTU and 1-phenyl-3-cyclohexylthiourea (PCHTU) (the latter derived from the scavenger, cyclohexylamine, added to wash solvents in the Millipore ProSequencer) are also separated from the PTH-amino acids (Fig. 2). Less commonly found byproducts such as aniline and phenylthiourea (PTU) elute respectively before and after PTH-His, completely separated from it. Methylphenylthiocarbamate, produced *i.a.* from methanol and PITC through incomplete drying of sequencer membranes, and sometimes seen in the first cycle of degradation, elutes midway between PTH-Met and -Val. Sequencer-derived PTH-Ile isomerizes to also yield PTH-*allo*-Ile, which elutes as a doublet with PTH-Ile. In this separation (Fig. 2) the shoulder preceding PTH-Ile indicates the elution position of PTH-*allo*-Ile, which is completely resolved from PTH-Phe. Although diphenylurea (DPU) creates difficulties for identification of PTH-Trp in some generally used systems [25], it elutes with PTH-*allo*-Ile in the present scheme. This does not prove to be a problem however, since (i) DPU, when present, is usually found only at the single picomol level, and (ii) PTH-Ile elutes as the two characteristic peaks, thereby allowing unequivocal identification and good quantitation.

The separation and identification of N-glycosylated PTH-Asn is demonstrated with the routine sequencer degradation of a peptide (V2, Arg–Arg–Asx–Met–Thr· · ·), from a digest of

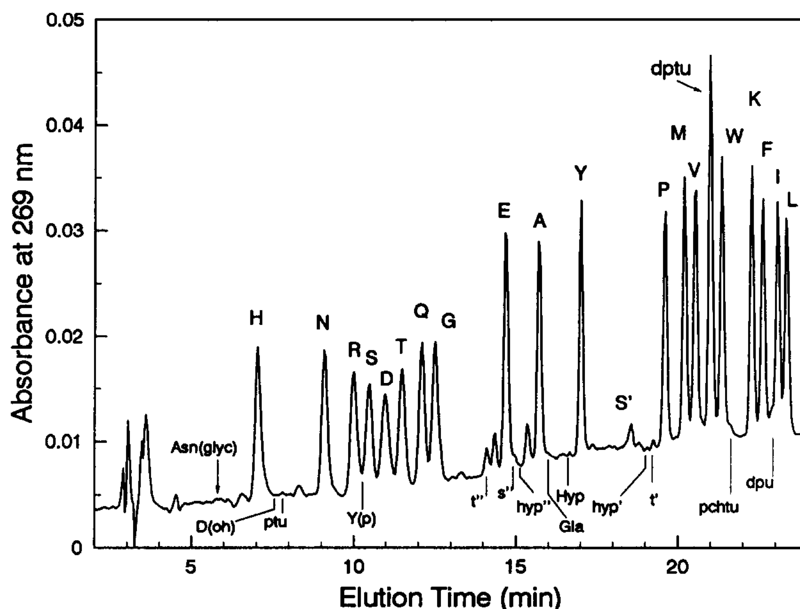

Fig. 1. Separation of 40 pmol of PTH-amino acids on a Supelcosil C_{18} column. The elution conditions are described in the text. The single letter notation for amino acids is used to identify the peaks corresponding to PTH-amino acids. Hyp = PTH-hydroxyproline, hyp' and hyp" = characteristic peaks also seen with hydroxyproline. S', s", t' and t" label the elution positions for characteristic degradation products of serine and threonine derivatives. The positions for phosphotyrosine [Y(p)], β-OH-Asp [D(oh)], γ-carboxy-Glu (Gla), glycosylated Asp and the phenylureas [diphenylthiourea (dptu), phenylthiourea (ptu), phenylcyclohexylthiourea (pchtu), diphenylurea (dpu)] are also indicated.

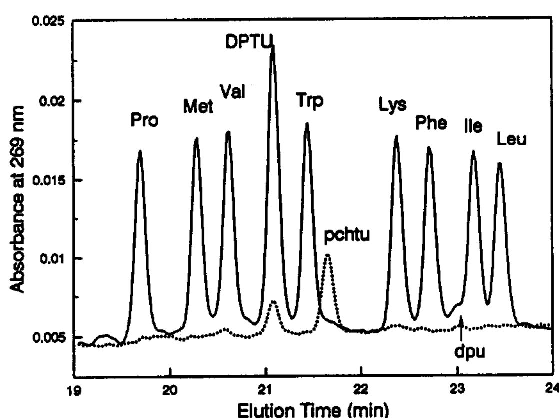

Fig. 2. Separation of the most hydrophobic PTH-amino acids on a Supelcosil C_{18} column. PTH Standards (40 pmol) and diphenylthiourea (DPTU) (solid line) are compared to a blank sequencer run (dotted line).

a ribonuclease homologue. Fig. 3 records the chromatograms of cycles 2 to 4 of Edman degradation on this peptide. The broad peak at 5.9 min is clearly the residue cleaved off in cycle 3, and is consistent with an expected N-glycosylated PTH-Asn. The asymmetry of this peak, also seen in a separate experiment on another peptide containing the same residue, probably reflects heterogeneity in the carbohydrate moiety.

The sensitivity of quantitation that can be achieved is illustrated by the separation of 4 pmol standard PTH-amino acids, dissolved in 100 μl sequencer-transfer buffer (20% acetonitrile, 0.1% acetic acid), in Fig. 4. All the PTH-amino acids are detected at the 4 pmol level, most are detectable at 1 pmol and with appropriate subtraction software the few residues such as PTH-Asn and PTH-Gly that are partly overlapped by small amounts of background compounds from the chemistry or HPLC buffers are also easily detected at 1 pmol.

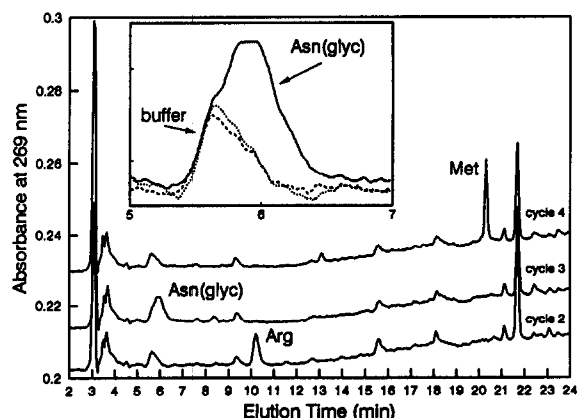

Fig. 3. Sequencer chromatograms for three cycles of degradation of a glycopeptide. Cycles 2, 3 and 4 each contain one major product which is identified as respectively Arg, an "unknown", and Met. The unknown elutes as a broad peak at 5.9 min (inset: the solid line is from cycle 3, the dashed and dotted lines are from cycles 2 and 4) which reflects its hydrophilicity and, as discussed in the text, would be N-glycosylated PTH-Asn, with heterogeneity in the carbohydrate.

3.2. Adjustment of separation

The chromatography is easily adaptable to other set-ups by consideration of a few specific aspects of the two-stage separation.

The more hydrophilic phenylthiohydantoins PTH-His, -Asn, -Arg, -Ser, -Asp, -Thr, -Gln and -Gly, are in effect eluted isocratically. PTH-Arg is positioned between PTH-Asn and -Ser by modifying the ionic strength of the A solvent

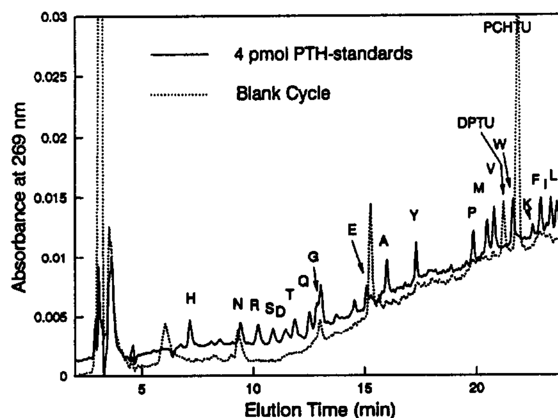

Fig. 4. Separation of 4 pmol of PTH-amino acids under conditions described in the text. A blank sequencer cycle (dotted line) is included to illustrate the background seen with a specific set of sequencer reagents.

(higher triethylamine concentrations allow earlier elution of PTH-Arg). The initial conditions are then adjusted to elute PTH-His at 4 ± 0.2 min after the solvent breakthrough. Changes in the initial state (2 to 7% B; lower amounts of B cause the PTHs to elute later) and/or temperature (higher temperatures cause earlier elution) should be used to position PTH-His. PTH-Gln is centered between PTH-Thr and -Gly by adjusting the methanol–acetonitrile ratio in solvent A. Thus ratios of 0.8 to 1.2 will move PTH-Gln from elution with PTH-Thr to elution with PTH-Gly in the specific system discussed here. Likewise, on changes of 0.15 to 0.2 pH unit from pH 3.55, PTH-Asp will coelute either with PTH-Ser (at higher pH) or with PTH-Thr (at lower pH) (as an example, with one new column we changed the pH to 3.70 to center PTH-Asp between PTH-Ser and -Thr; the rest of the separation remained the same). The start of the second stage, a linear gradient to high amounts of solvent B, is set to coincide with the elution of PTH-Arg. Thus if PTH-Arg elutes 7 min after the solvent breakthrough, the gradient portion of the elution should start at 7 min into the elution protocol. These gradient settings accommodate varying instrumental holdup volumes. Once this set of hydrophilic PTHs separates well the subsequent gradient can be adjusted to separate the later-eluting group.

The gradient portion of the protocol yields a robust separation of all the relevant PTH-amino acids and allows at least a 20% variation in gradient slope to be tolerated. The precise elution positions of DPTU and PCHTU however depend critically on the gradient and their positioning relative to PTH-Val and -Trp may require adjustments in the gradient slope. Additionally, lower temperatures or lower ratios of acetonitrile to methanol in the A solvent will move PCHTU further from DPTU, without much relative movement of the PTH-amino acids.

The washing cycle is important in the repeated analyses of sequencing cycles and since the organic content of the A solvent is high, reequilibration after washing is rapid, within 5 to 7 ml.

4. Conclusions

The primary requirements for a good PTH-amino acid separation system which will be compatible with on-line detection of sequencer products are: (i) a short turn-around time to accommodate sequencer cycle times; (ii) toleration of large injection volumes, compatible with automated transfer from the sequencer; (iii) a robust separation that can be installed and maintained with ease; (iv) separation of known reagent- and solvent-related peaks from common amino acids; (v) chromatographic space in which to elute and recognize known and unknown modifications; and (vi) detection sensitivity at low picomol level.

The separation developed here fulfills these requirements. The use of triethylamine phosphate as modifier of the elution solvent for PTH-amino acids provides very early elution of PTH-His and -Arg and, consequently, an opportunity to design a separation of PTH-amino acids that provides more chromatographic space early in the separation than conventionally used with on-line sequencers. This separation is easy to install and maintain. The very early elution of PTH-His and -Arg furthermore increases the sensitivity of detection of these usually problematic residues. Additionally, the sequencer byproducts DPTU, PTU, PCHTU and aniline are well separated from all common PTH-amino acids, including PTH-Trp. The extra chromatographic space is useful for the detection of hydrophilic post-translational modifications of proteins. Such modified residues of course need to be eluted from the sequencer cartridges under conditions that require covalent immobilization of the parent proteins [2,3,12,13]. The rapidly developing mass spectrometric sequencing technologies [26,27] are slated to supercede the traditional Edman chemistries in due course. The present high cost of the specialized instrumentation and the problems inherent in any youthful technology will, however, preclude many from immediate conversion to these technologies. The new capability of analyzing hydrophilic post-translational modifications of proteins during conventional

sequencing should therefore be immediately useful during this transition between methodologies.

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