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IDENTIFICATION OF AMINO ACID PHENYLTHIOHYDANTOINS BY MULTICOMPONENT ANALYSIS OF ULTRAVIOLET SPECTRA

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SUMMARY

Mixtures of amino acid phenylthiohydantoins (PTHs) can be identified and quantitated by multicomponent analysis of their ultraviolet spectra. For this analysis, the direct absorbance spectra were converted to their first derivatives. Then the multicomponent analysis routine resident in the spectrophotometer was used. The entire process requires less than one minute. Mixtures of two amino acid phenylthiohydantoins with extreme spectral similarity were accurately quantitated with the method. (We used mixtures of PTH-Asp–PTH-Glu and PTH-Ile–PTH-Leu.) The ability to identify amino acid phenylthiohydantoins by analysis of their ultraviolet spectra may provide a simple method for confirmation of the identification of the amino acid PTH obtained during protein sequencing.

INTRODUCTION

Sequencing of proteins and peptides is generally accomplished by stepwise Edman degradation coupled with high-performance liquid chromatographic (HPLC) identification of the phenylthiohydantoin (PTH) derivative of each cycle[1]. The introduction of rapid HPLC analysis of amino acid PTHs[2] was critical to advances in automated microsequencing. However, definitive separation and identification of all amino acid PTHs in a single HPLC analysis has not been routinely possible for most workers[3,4]. Routine analyses usually provide identification of most amino acid PTHs, but certain pairs or trios of PTH amino acids exhibit very similar retention times[2–5]. For example, PTH-Asp and PTH-Glu are quite sensitive to pH, while PTH-Leu and PTH-Ile are not always resolved under elution conditions used for a single analysis. In addition, carryover or "background"[6] from previous cycles of the Edman degradation may cause contamination with minor amino acid PTHs which must be distinguished from the major derivative.

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If the initial analytical run proves ambiguous, one can repeat the analysis under elution conditions designed to improve the likelihood of identification of the amino acid PTH[3,4]. Alternatively, one can use a variety of other analytical techniques[1,6]. Even if HPLC identification appears unambiguous, some workers prefer to confirm the HPLC identification with at least one other analytical method.

Ultraviolet (UV) spectroscopy has not been employed for identification of the amino acid PTHs because of the extreme spectral similarity of these compounds[6]. While the spectra of the amino acid PTHs are very similar, they are not identical. In principle one might be able to identify a particular amino acid PTH from its UV spectrum. Also in principle, one might be able to identify and even quantify the components of a mixture of amino acid PTHs. Examples of such multicomponent analyses by "deconvolution" of the UV spectrum are well known (*e.g.*, the quantitation of protein and nucleic acid in a mixture of the two).

In practice, such multicomponent analyses have not succeeded when applied to compounds of extreme spectral similarly. However, the commercial introduction of rapid-scanning spectrophotometers provided a means to obtain spectra with much improved signal-to-noise characteristics. Coupled with appropriate computer programs, these spectrophotometers make possible the qualitative and quantitative determination of mixtures of extreme spectral similarity[7–9]. The spectrum of a protein can be considered a "mixture" of the contributions from the aromatic amino acids within the protein[8]. We recently demonstrated that multicomponent analysis of protein spectra permits quantitation of the constituent phenylalanine, tyrosine, and tryptophan residues, usually with less than 5% error[8]. This demonstration suggested that UV spectroscopy might also permit qualitative (and possibly quantitative) analysis of the amino acid PTHs.

In essence, multicomponent analysis "synthesizes" a spectrum from a library of standard spectra. The synthetic mixture which best matches the actual spectrum is determined, using statistical methods to determine the best fit. The composition of the synthetic spectrum provides quantitation of the components in the actual mixture. While such an analysis can be performed with the direct absorbance spectrum, better results are usually obtained by calculating the first or second derivative of the spectrum (refs. 7 and 8 and references therein). The first derivative is the rate of change of the absorbancy with wavelength; the second derivative is the velocity of that change. A derivative spectrum enhances small dips, peaks, and shoulders compared to the direct spectrum.

We have begun studies directed at the identification of amino acid PTHs through multicomponent analysis of their UV spectra. We report here that the technique permits quantitation of mixtures containing two amino acid PTHs of extreme spectral similarity.

EXPERIMENTAL

Spectra were taken with the Hewlett-Packard Model 8450A spectrophotometer. This instrument employs an array of photodiodes as its detector, permitting parallel (simultaneous) acquisition of absorbances at 401 specific wavelengths. Measurements were made for 10 sec, during which 20 complete spectra were measured and then averaged. This generated spectra with the high signal-to-noise ratios re-

quired for multicomponent analysis of the very similar amino acid PTHs. The spectrophotometer includes software allowing rapid generation of first and second derivatives of the spectra. The spectrophotometer also implements a multicomponent analysis method which takes advantage of estimates of precision available from the averaged spectra[9].

Actual spectra were obtained after balancing the reference and sample cuvettes which had been filled with solvent (described below). The multicomponent analysis was then performed on the first derivative of the spectrum, from 250 to 275 nm. Other wavelength ranges also give good results, but narrowing the range typically improves accuracy[8]. We found that use of the first derivative gave more accurate qualitative and quantitative results than did the direct absorbance spectra; this is not unusual[7,8]. The second derivative occasionally gives even better results[8], but we noted no significant improvement in the current studies. Since each higher derivative worsens the signal-to-noise ratio, the first derivative is the optimal choice here. The entire procedure of obtaining the spectrum and the analysis required under 1 min. If desired, spectra can be obtained continously during the HPLC analysis and stored on floppy disk for later analysis. A photograph showing the instrument configured as an HPLC detector appears in ref. 10.

Amino acid PTHs were purchased from Pierce (Rockford, IL, U.S.A.). Stock solutions of about 6 m*M* concentration were prepared in methanol. Acutal concentrations were determined using published absorptivities[6]. These stock solutions were diluted 100-fold into 45% methanol, 0.5% propionic acid, pH 4.0. This solvent was chosen as representative of those which might be employed during HPLC analysis[3]. We had similar success with acetate buffered acetonitrile solutions. While the exact solvent may not be important, it is important that the spectrum of the unknown compounds be obtained in the same solvent as that of the reference standards. This practice obviates difficulties which can result from solvent perturbation effects[8]. We used reagent-grade solvents; they were not HPLC grade.

RESULTS AND DISCUSSION

The actual pairs or trios of amino acid PTHs most likely to be incorrectly identified will depend on the particular HPLC system utilized for their separation[2–5]. To study the resolving power of the multicomponent analysis, we chose two "worst case" examples. We assumed that PTH-Asp was contaminated from a previous cycle by PTH-Glu or that PTH-Leu was contaminated by PTH-Ile. These cases provide a rigorous test of the ability to identify the quantitatively dominant amino acid PTH, because the pairs have very similar UV spectra (Figs. 1 and 3). The first derivative spectra also demonstrate similarities (Figs. 2 and 4), but differences are more appreciable. For example, the direct absorbance spectra of PTH-Leu and PTH-Ile are very similar, with both exhibiting maxima at 266 nm (Fig. 3). While the first derivative spectra are also similar, the trough is at 277 nm for PTH-Ile and 276 nm for PTH-Leu (Fig. 4).

Despite close spectral similarity, the multicomponent analysis readily identified the major component, providing the qualitative identification needed for sequence work. Notably, the procedure also provided quantitative analyses which were accurate to within 5% (Figs. 5 and 6).

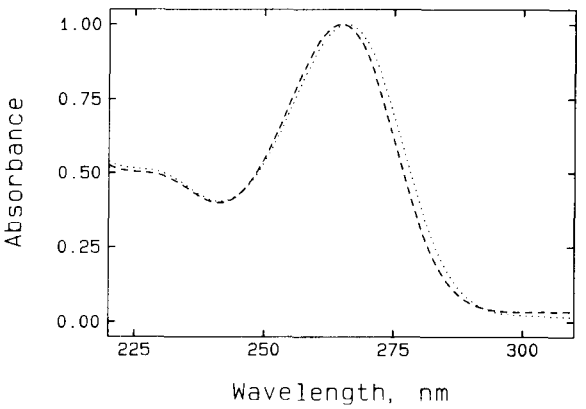

Fig. 1. Direct spectra of PTH-Asp (– – –) and PTH-Glu (· · · · ·). For ease in comparison, the spectra were normalized to an absorbance of 1 at their absorbance peaks (265 nm for PTH-Asp and 266 nm for PTH-Glu).

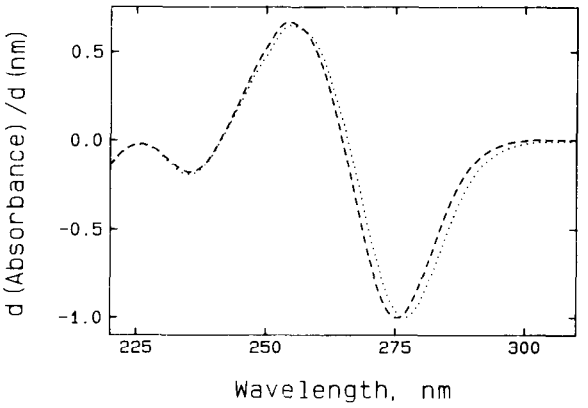

Fig. 2. First derivative spectra of PTH-Asp (– – –) and PTH-Glu (· · · · ·). These were derived from the direct spectra of Fig. 1. They were normalized to −1 at their troughs (275 nm for PTH-Asp and 277 nm for PTH-Glu).

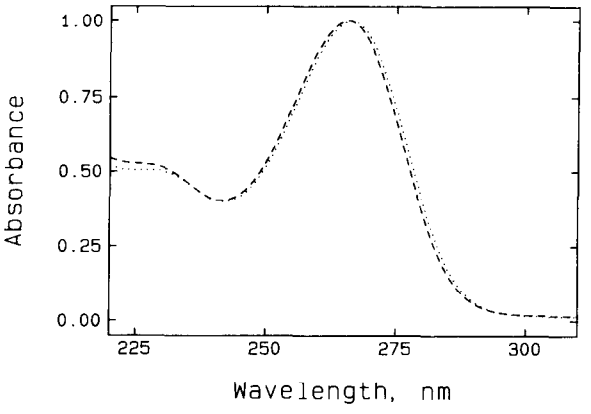

Fig. 3. Direct spectra of PTH-Leu (– – –) and PTH-Ile (· · · · ·). For ease in comparison, the spectra were normalized to an absorbance of 1 at their 266 nm peak.

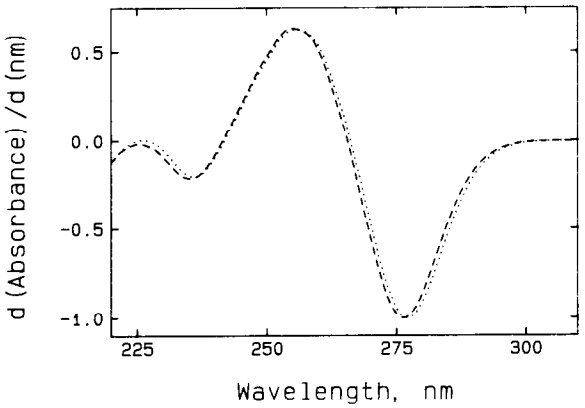

Fig. 4. First derivative spectra of PTH-Leu (— — —) and PTH-Ile (· · · · ·). These were derived from the direct spectra of Fig. 3. They were normalized to −1 at their troughs (276 nm for PTH-Leu and 277 nm for PTH-Ile).

The sensitivity of the analysis was tested by analyzing serial 1:2 dilutions of a solution which contained 90% PTH-Leu and 10% PTH-Ile. The initial solution had an absorbance of 1.04 at the 266 nm peak and was 56 μM in PTH-Leu. The spectrophotometer's multicomponent routine reported the concentration of the two amino acid PTHs along with the coefficient of variation. The latter increased as more dilute solutions were analyzed, reaching 6% when the peak absorbance was 0.037. This solution was 1.8 μM in PTH-Leu. Two additional dilutions yielded a peak absorbance of 0.0095, and a PTH-Leu concentration of 0.44 μM. Multicomponent analysis identified PTH-Leu as the major component. While this result was correct, the coefficient of variation for the PTH-Leu concentration was 22%. Thus, one might take 2 μM as a conservative, limiting concentration. If one collects fractions during the HPLC run for later analysis, then 200 μl would be required to fill a typical 10-mm microcuvette. This would permit identification of 0.5 nanomole of the amino acid PTH. If a low-volume flow-cell were used for monitoring the HPLC run, then sensitivity should improve by at least an order of magnitude.

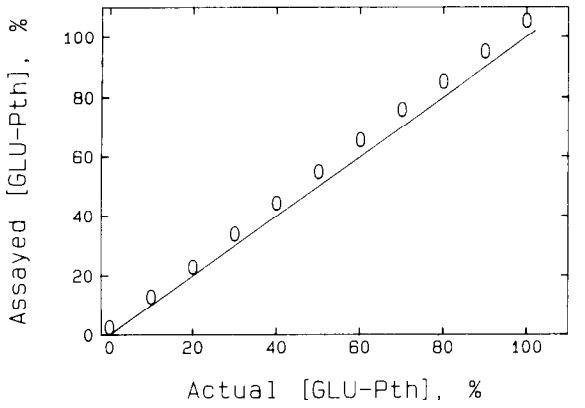

Fig. 5. Multicomponent analysis of mixtures of PTH-Asp and PTH-Glu. Stock solutions of approximately equal concentrations were mixed to give the mixtures (by volume) shown on the abscissa. Spectra of these mixtures were recorded. Then the multicomponent analysis was performed on the first derivative, from 250 to 275 nm, using pure solutions of PTH-Asp and PTH-Glu as reference standards for the multicomponent analysis routine of the spectrophotometer.

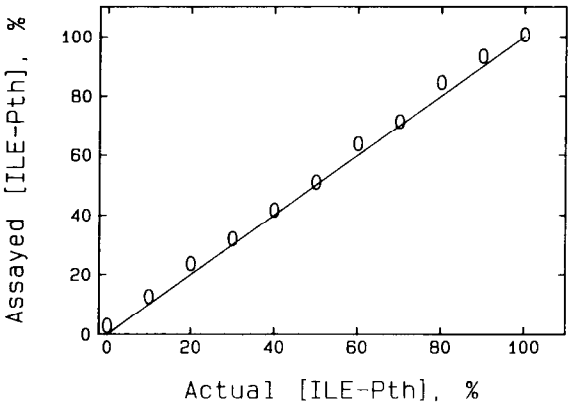

Fig. 6. Multicomponent analysis of mixtures of PTH-Leu and PTH-Ile. The analysis was performed as described for Fig. 5, using PTH-Leu and PTH-Ile as standards.

Of course, in most cases the major amino acid PTH will not be contaminated by other amino acid PTHs because the contaminants are separated by HPLC. The identity of the amino acid PTH is ambiguous only because its retention time is not sufficiently different from other amino acid PTHs. In this setting, multicomponent analysis can be performed using standard spectra of the two or three amino acid PTHs which could elute in that region. Our preliminary results demonstrate that the spectrophotometer identifies the major amino acid PTH, even if contaminated by 20% of one of four other amino acid PTHs. In this case, the five amino acid PTHs were Asp, Glu, Gln, Ser, and Thr. The spectra of the dehydration products of PTH-Ser and PTH-Thr are distinctive[6], making the analysis less demanding than for a mixture of five amino acid PTHs with extreme similarity. Given a sufficiently large number of related spectra, the instrument's multicomponent analysis routine will begin to make errors in identification.

We have demonstrated that binary mixtures of very similar amino acid PTHs can be quantitated by multicomponent analysis of their UV spectra. However, additional studies must examine the actual performance during HPLC analysis of amino acid PTHs from automated sequenators. Hopefully, one could utilize the multicomponent analysis to provide a rapid, simple confirmation of the identity established from the HPLC retention time. The spectrophotometer can replace the standard ultraviolet detector[10], so one can decrease the total cost of dedicating the spectrophotometer to the HPLC system. In addition, the technique could be useful in the identification of these derivatives after manual Edman degradation[6].

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