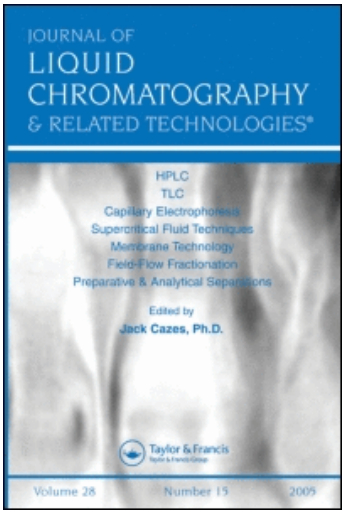

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HPLC-ANALYSIS OF PTH-AMINO ACIDS

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ABSTRACT

Some of the problems that affect the usefulness of HPLC-analysis of PTH-amino acids have been examined. Four different C_{18}-reversed phase packings have been tested, together with different mobile phases. The separation problems of the Met/Val-group, the Phe/Leu/Ile/Lys-group and of the basic amino acids His and Arg on the reversed phase packings have been examined and solutions to some of the problems have been demonstrated. The sensitivity of the quantitative analysis of amino acids from a hydrolysate was determined.

INTRODUCTION

Phenylthiohydantoin (PTH) derivatives of amino acids have found extensive use in sequencing procedures of peptides and proteins after the development of the Edman degradation method (1). The use of PTH-derivatives for total amino acid composition analysis of hydrolysates have not gained an equal acceptance,

probably due to the relatively time-consuming derivativization procedures, compared to the post-column procedures of regular amino acid analyzers. During recent years the development of reversed phase packings for high performance liquid chromatography (HPLC) have resulted in several papers on the use of HPLC for PTH-amino acid analysis. By coupling of two Zorbax-ODS columns, Bronzert and Brewer (2) demonstrated the separation of 17 PTH-amino acids by gradient elution with acetonitril-water-sodium acetate at 60°C. A similar gradient system, but at ambient temperature, was used by Margolies and Brauer (3) in an attempt to separate 22 PTH-amino acids. The unresolved pairs of Pro/Met and Trp/Phe had to be resolved by different programs. A separate program was used for His, Arg and Cysteic acid (Cys-OH) from the aqueous phase after extracting the Edman degradation mixture with ethyl acetate. At the highest sensitivity, 10 pmole of the PTH-amino acids could be detected. An alternative method, with methanol-water-acetic acid-sodium acetate, also at ambient temperature, was reported by Brown et al. (4). Met and Val did not separate, the group of Leu/Ile/Phe was poorly resolved and Arg and His were run separately. Also with a methanolic mobile phase, we later demonstrated (5) the separation of 24 amino acids, but the Met/Val pair and the Phe/Ile/Lys/Leu quartet was poorly resolved.

A very good resolution was obtained by Zimmermann et al. (6) on Zorbax-ODS, with acetonitril at 62°C. 20 PTH-amino acids were separated within 20 min. Cysteic acid and cystine were not included. The resolution was reported to be significantly lower at 50°C. Thus, there is no reason to doubt that the maximum

resolution and minimum time of analysis can be obtained only by elevated temperatures. The question that can be raised is what effects the higher temperature will have on the columns lifetime, considering the rather limited lifetime of columns used at room temperature.

In this paper some of the separation problems of the reversed phase HPLC methods will be discussed, especially with regard to the differences in properties of column packings of the same type, but of different manufacture. In addition the potential usefulness of PTH-derivativization of hydrolysates will be discussed.

EXPERIMENTAL

Apparatus.

The HPLC equipment consisted of two Waters 6000 A pumps, a Waters 660 Solvent Flow Programmer, a Waters U6K valveloop injector, a Waters 440 UV-detector and a Perkin-Elmer LC-55 variable wavelength UV-detector. The 440 detector was equipped with the dual channel accessory with filters for 254 and 280 nm.

Columns.

The columns were packed with four different C_{18}(ODS) materials. 4,6x230 mm columns were slurrypacked in methanol with Spherisorb S 5 W-ODS (5μ) from Phase Separations Ltd. 3 columns with the same packing were tested.

Two 4,6x230 mm columns were slurrypacked in methanol with ODS-Hypersil (5μ) from Shandon Southern Products Ltd.

A 3,9x300 mm column with μ-Bondapak-C_{18} (10μ) was obtained, packed by the manufacturer, from Waters

Associates. A 4,6x190 mm column filled with the same packing was partially used for control purposes.

A 4,6x190 mm column was slurrypacked as above with Spherosil XOA 600 Normatom 5μ C_{18} from Prolab, Rhone Poulenc. This column was obtained at the end of this project and was not as thoroughly tested as the other packings.

After 2-4 weeks of daily use all the columns had their efficiency reduced to 3-5000 theoretical plates. The columns were washed with methanol after use and stored in methanol when not in use.

Chemicals.

The water was distilled twice. Methanol and acetonitrile of HPLC grade S was obtained from Rathburn Chemicals (Peeblesshire, Scotland). Ethanol (96%) was obtained from A/S Vinmonopolet, Oslo. Sodium acetate and ammonium acetate of pro analysi quality came from Merck (Darmstadt) and acetic acid p.a. from Riedel de Häen (Hannover). Amino acids, peptides and PTH-amino acids came from Sigma (St. Louis, Missouri), the triethylamine was Fluka p.a., the acetone was Baker p.a., the pyridine was Pierce (sequential grade) and the phenylisothiocyanate was Fluka (zur sequenzanalyse nach Edman).

1 M stock solutions of the salts were filtered through 0,45μ Millipore solvent filters and stored at +4°C. The 10 mM solutions were made by diluting the stock solutions. Prior to use all the HPLC solvents were degassed by 2 hours of ultrasound treatment. Stock solutions of PTH-amino acids were prepared by dissolving 2-3 mg in 10 ml methanol, and stored at -20°C.

Synthesis of PTH-Amino Acids

The synthesis of PTH-amino acids from commercial amino acids or from hydrolysates of peptides were performed in micro vials, according to Rosmus and Deyl (7). The buffer solution (pH 9) was made from water (5 ml), pyridine (5 ml) and triethylamine (0,2 ml). To the dry amino acids (or hydrolysate) the buffer solution (10-30 µl) was added first, then a 2-30 times excess of phenylisothiocyanate (PITC) dissolved in acetone (10-30 µl). The reaction mixture was bubbled with nitrogen for 15-20 seconds, capped and placed in a heating block at 40°C for 2 hours. Excess reagent and buffer was then removed by 2 hours storage in vacuum-desiccator over phosphorus pentoxide (starting at 15 torr). For more effecient removal of excess reagent and interfering byproducts an extra 10 min drying at 1 torr was added. The phenylthiocarbamyl (PTC) amino acids synthesized so far were rearranged to PTH-amino acids by adding 15 µl of water and 30 µl of acetic acid saturated with HCl, and left at room temperature for 12-14 hours. The excess HCl was then removed in a vacuumdesiccator with KOH overnight, starting at about 15 torr. Prior to use the residue was dissolved in 20-100 µl of methanol.

The test samples of mixtures of amino acids contained either 5 nmol, 1 nmol or 0,2 nmol of each amino acid. The test samples of hydrolysates from acidic hydrolysis of a few tripeptides, had the same concentrations.

Chromatographic Procedures.

The chromatographic system was washed with water, then with methanol, at the end of each day. When the columns

had been used for a few weeks, and the efficiency was declining, the inlet was opened at intervals and the first 2-3 mm of packing replaced by fresh material if a miscolouring was apparent.

All the chromatographic data obtained were based on at least 3 different injections from the same sample.

If the two detectors were coupled in series, the 440 detector, with the least band spreading, always was put in front.

The injection volumes of methanolic solutions were 5 µl or less.

All chromatograms were run at ambient temperature. For each packing the gradient conditions were varied until maximum resolution was obtained. Thus, the final gradient programs for the different packings became slightly different.

The five not naturally occurring amino acids methionine sulfone (Met-SO_2) α-aminoisobutyric acid (isob), α-aminobutyric acid (but), norvaline (Norval) and norleucine (Norleu) were included in the test mixtures in order to obtain chromatograms from which one or two suitable standards later could be selected.

RESULTS AND DISCUSSION

Solvent Effects at the Injection.

Due to the limited solubility of PTH-amino acids in aqueous solutions, methanol (or another organic component of the mobile phase) is often used as solvent, especially if relatively concentrated stock solutions

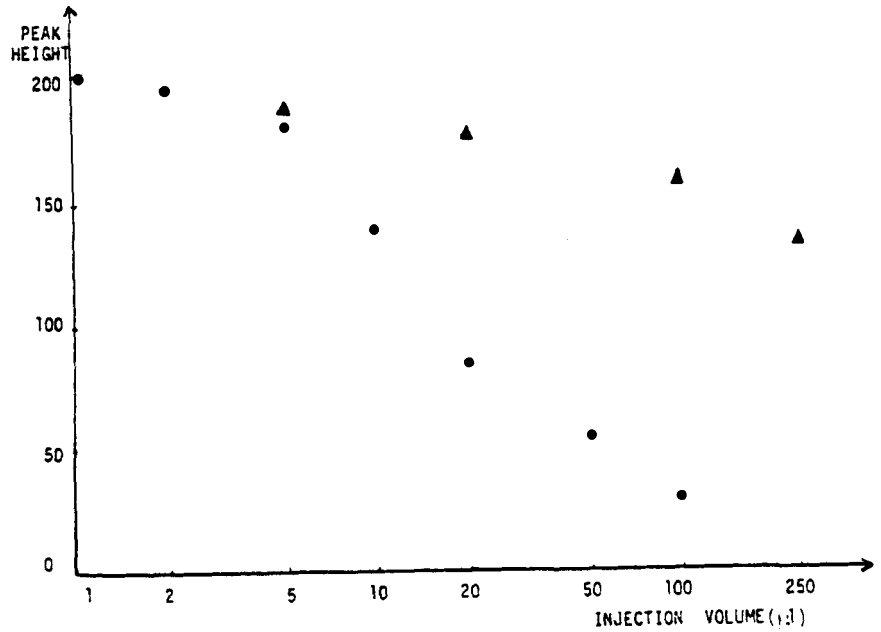

FIGURE 1. Peak height vs. injection volume of 240 ng PTH-Gly, dissolved in 100% methanol (●) and in 35% methanol (▲). Mobile phase: 35% methanol.

are made. When this was done, the injection volume had to be restricted to 5 µl or less, if serious peakbroadening should be avoided (fig. 1). Generally it must be recommended that the stock solutions should be diluted with the solvent of the starting conditions, when a gradient is used.

Purity and Stability of the PTH-Amino Acids.

The majority of the commercial PTH-amino acids were sufficiently pure to be used as standards for quantitative analysis, with the notable exception of PTH-Cys and PTH-Cys-Cys. The cysteine contained cystine and

vice versa, as well as other impurities. A preparative purification of both could be obtained by using one of the packings described in this paper in a semipreparative column with a methanolic mobile phase containing at least 50% methanol. With acetic acid in the mobile phase, the (air) oxydation of PTH-Cys to PTH-Cys-Cys was more effectively prevented.

Apart from PTH-Cys, PTH-Thr was the only amino acid that appeared to give decomposition products of any measureable quantity, by 8 weeks storage of the stock solutions (in methanol). The commercial product contained less than 10% of PTH-dehydro-Thr, but this amount increased even after 2 hours storage at room temperature. By storage at -20°C the decomposition process was delayed. Solutions that had been stored at room temperature actually contained at least 4 components, two separable diastereomers of PTH-Thr and a cis/trans-isomer mixture of PTH dehydro-Thr. The four peaks were separated by 0.01 M ammonium acetate in 45% methanol on Spherisorb ODS at 254 nm. At 313 nm the conjugated double bond of PTH-dehydro-Thr gave a strong absorbance not found in PTH-Thr.

PTH-Ser, which also may decompose by loss of water, showed no measurable decomposition in any of the solutions used.

PTH-Ile, which is another amino acid, in addition to PTH-Thr, that may give chromatographically separable diastereomers by racemization, did so too, but mainly by storage in aqeous solutions (see the comments in the chapter of the Phe/Leu/Ile/Lys group).

The PH of the Mobile Phase.

The pH of the mobile phase was controlled by using 0.01 M solutions of ammonium acetate, sodium acetate or acetic acid. The 0,01 M concentrations were chosen

to be able to handle picomol-nanomol amounts of amino acids. The best resolution of the acidic PTH-amino acids, aspartic acid (Asp), glutamic acid (Glu) and Cysteic acid (Cys-OH), was obtained with acetic acid. On Spherisorb and Spherosil, with ammonium acetate or sodium acetate, Asp and Cys-OH would not separate (fig. 2), unless a very long gradient was introduced. Apart from this, the acetic acid solutions had a tendency to cause inferior separation of many other PTH-amino acids. Solutions with ammonium acetate and sodium acetate showed negligible differences in the chromatograms. On Spherisorb the separation of the

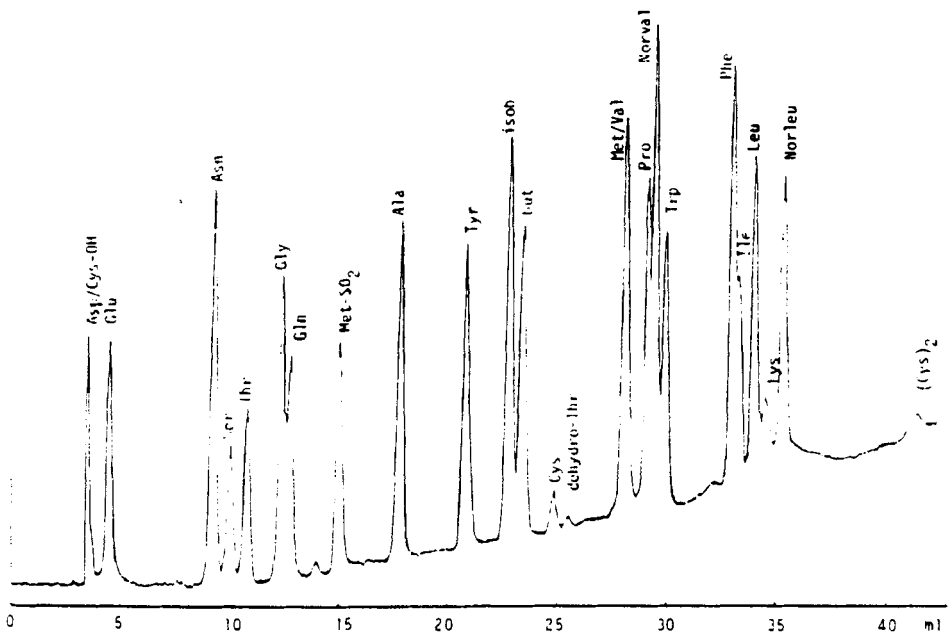

FIGURE 2. Separation of PTH-AA on Spherisorb ODS. 43 min. linear gradient with 0,01 M ammonium acetate in 30-70% methanol at 1 ml/min. UV detection at 267 nm.

phenylalanine (Phe) and isoleucine (Ile) was slightly better with sodium acetate. Since the lability of ammonium acetate was more likely to cause variation in the ionstrength by the solvent filtering procedures, sodium acetate was chosen for most purposes.

The Organic Component of the Mobile Phase

In the literature the choice between methanol and acetonintrile in reversed phase elution often seem to be a matter of coinsidence or tradition. Relevant arguments do, however, exist for using the one or the other solvent. With constant-pressure pumps the greater variation of the viscosity of the aqueous mixtures of methanol as a function of percent organic solvent, would favor the use of acetonitrile in gradients. Because of the lower viscosity of acetonitrile in gradients, the pressure drop over the column also will be lower.

The elution strength of the solvent also is different. As seen in Fig. 3, the surface tension of methanol mixtures, compared to acetonitrile mixtures, decreases more rapidly when the amount of organic solvent is increased from 30% and up. By accepting the relationship between retention and surface tension (8), this means that with a gradient of 30% - 70% of the organic compound, the selectivity could be expected to be better with methanol than with acetontrile. This also means that the solvent strength of acetonitrile is expected to be higher in mixtures up to 70%, but lower in 80% - 100% mixtures, compared to methanol. With gradient elution the stability of the baseline also must be considered an important factor, especially for quantitative measurements. Of the HPLC-grade solvents used, methanol had a lower background at 280 nm (fig. 4), while acetonitrile performed better

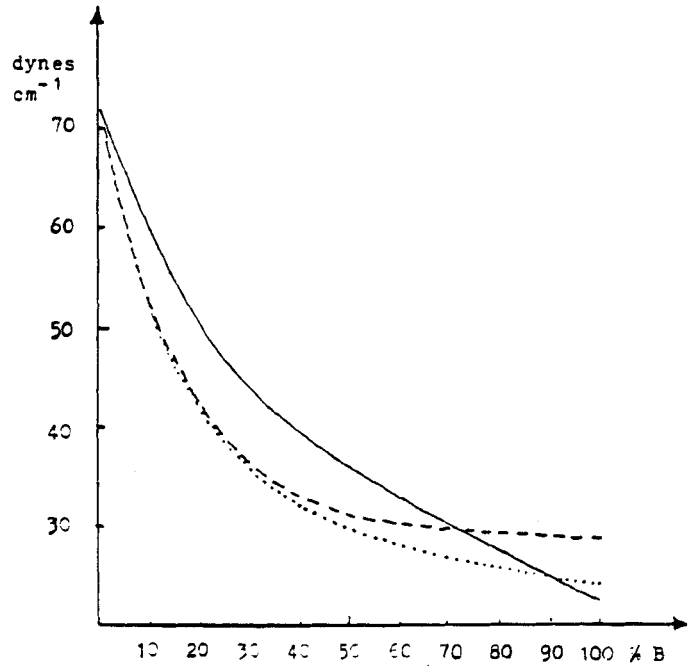

FIGURE 3. Surface tension of aqueous mixtures of methanol (-), acetonitrile (--) and ethanol (...). Based on data from Timmermans (12).

at 254 nm (fig. 4). The wavelength of highest sensitivity of the PTH-amino acids was determined to 267 nm in methanol. Bu using the filterphotometric detector, approximately the same sensitivity was however obtained at both 254 nm and 280 nm as with the spectrophotometer at 267 nm.

Thus, for PTH-amino acid analysis with our equipment there was found no reason to substitute the cheaper and less toxic methanol with acetonitrile. As seen i fig. 3, ethanol may be considered a potential substitute if sufficient resolution cannot be obtained

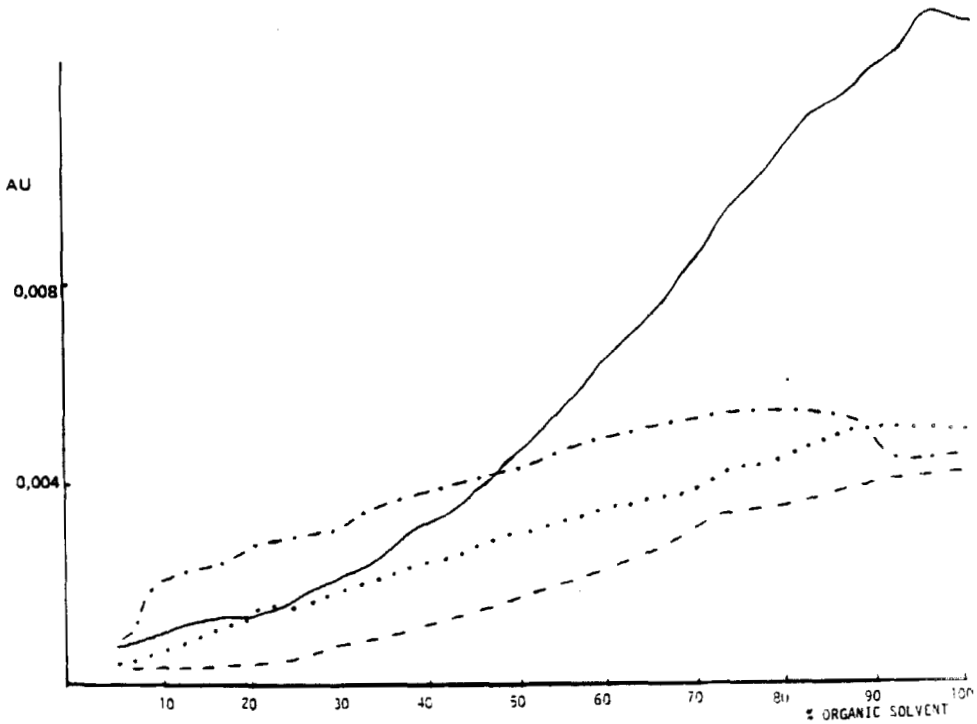

FIGURE 4. Solvent absorbance of linear aqueous gradient of 0 to 100% methanol at 254 nm (-) and 280 nm (--) and of acetonitrile at 254 nm (- . -) and 280 nm (...).

at higher percentages of the organic component. This, in fact, turned out to be one way to solve the Met/Val separation problem. The higher viscosity of ethanol did, however, create a problem with long columns packed with 5μ particles. Thus, the pressure drop over the 23 cm Spherisorb column became too high to be useful for all practical purposes. The Hypersil column could be used, and the Bondapak column performed very well with ethanol mixtures. The introduction of ethanol, however, gave little resolution of Ser and Thr, of Pro and dehydro-Thr and of the Lys/Ile/Phe group in a regular gradient (fig. 5).

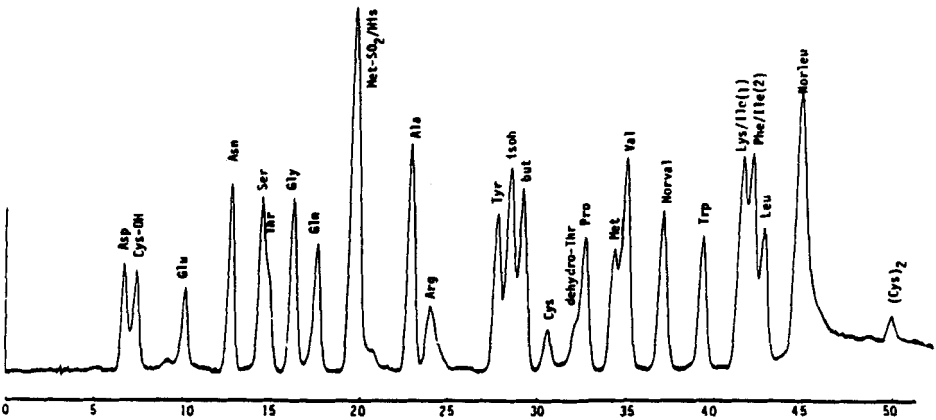

FIGURE 5. Separation of PTH-AA on Bondapak-C_{18}. 43 min. linear gradient with 0,01 M sodium acetate in 10-48% ethanol at 1 ml/min. UV-detection at 280 nm.

The Basic PTH-Amino Acids

In the Edman degradation procedures the basic amino acids stay in the aqeuos phase after extraction with ethyl acetate from an acidic solution. If the total amino acid composition, and not the sequence, is to be determined, a similar extraction step could be included. The easiest procedure would, however, be to determine all the PTH-derivatives directly after hydrolysis. When PTH-Lys, His and Arg were included in the test mixtures, PTH-Lys showed a chromatographic behaviour similar to the neutral amino acids. PTH-His and Arg, on the other hand, gave broad peak shapes on Spherisorb, mediocre peaks on Hypersil and satisfactory peak shapes on Bondapak and Spherosil. The peak shape was not consistent, but dependent on the column age.

After one month of daily use in the pH-area between 2 and 7, the Bondapak column showed a bump instead of a peak for PTH-His.

In addition to the aging problem, assumed to be caused by appearance of more silanol groups in the packing, an adsorption-saturation phenomenon also was detected with PTH-His. With neutral mobile phases (ammonium acetate and sodium acetate) the first injections on a previously cleaned column could be partially or even fully adsorbed (fig. 6). An initial injection of at least 3 µg of PTH-His was found sufficient to saturate the column. With acetic acid in the mobile phase, no such adsorption was evident.

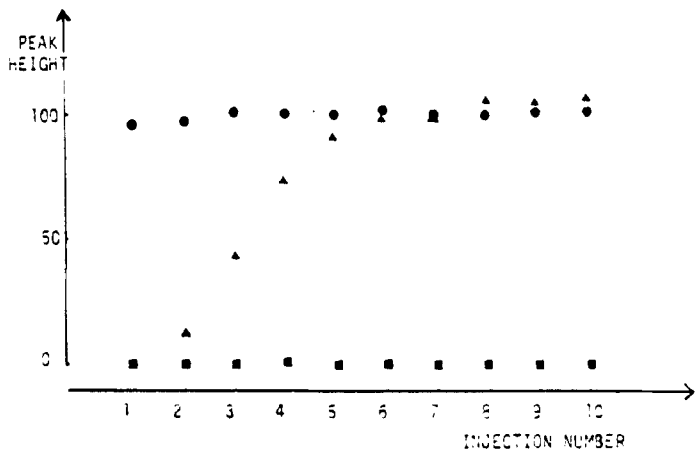

FIGURE 6. Adsorption to cleaned columns demonstrated by the peak height of repeated injections of PTH-His (320 ng) on ODS-Hypersil (●), Spherisorb-ODS (▲) and Bondapak-C_{18} (■). Mobile phase: 0,01 M sodium acetate in 50% methanol.

TABLE 1

Retentionvolume (ml) of PTH-His with Two Different Mobile Phases on 3 ODS-Columns

Column	0,01 M NaOAc in 50% MeOH	0,01 M HOAc in 50% MeOH
Hypersil	4,2	37
Spherisorb	5,6	not eluted
Bondapak	7,3	5,3

With acetic acid in the mobile phase, the retention of PTH-His changed remarkably (table 1). On Hypersil and Spherisorb the retention was strongly increased, while on Bondapak it decreased. This apparently inconsistent behaviour is difficult to explain. The different packings may contain free silanol groups in different amounts as well as of different kind and acidity. Infrared spectra of the three packings, in KBr tablets, showed a stronger band at 1650 cm^{-1} of the Spherisorb packing, compared to Hypersil and Bondapak, indicating the presence of more free silanol groups (9). The infrared spectra did not give any information on possible differences in the bonding structure of the silanol groups. Since such differences are known to exist (10) and since some silanol groups of porous silica are known not to be fully protonized before pH 2, the existence of ionic interactions (even in acetic acid) as well as concerted bonding and sample localization (11) cannot be excluded. A full explanation of the strange behaviour of PTH-His seems impossible until a detailed study of the surface structure

of the packings has been performed. PTH-Arg showed a similar behaviour, with even more peakbroadening than PTH-His.

The Spherosil column with its 20% carbon content, compared to 7-10% C for the other packings, was assumed to contain less free silanol groups. Preliminary results also indicated sharper peaks and better stability versus His and Arg, but the column was obtained too recently to be completely tested. Of the three other packings, the combination of Bondapak and an acetic acid containing solvent seemed to produce the best results, especially in regard to sensitivity, since sharper peaks were obtained in the acidic solvent. The combination of Hypersil and sodium acetate also could be used, by conditions such as isocratic elution with the final mixture of the gradient program. Since the column age could affect the peak shape as well as the retention of PTH-His and Arg, a one-step program for analysis of these two together with the other amino acids cannot be recommended unless a strict control with ageing effects is implemented.

The Met/Val Separation Problem

In the majority of published papers on reversed phase elution of PTH-amino acids, the separation of Met and Val is regarded a rather serious problem. Thus it could hardly be considered a big surprise that neither Spherisorb (with methanolic mobile phase), Bondapak (with methanolic mobile phase) nor Spherosil (with acetonitrile) gave any resolution of the two PTH-amino acids. On Hypersil (fig. 7) a poor resolution was obtained, but after 2 weeks the column efficiency was sufficiently reduced to destroy separation. The problem

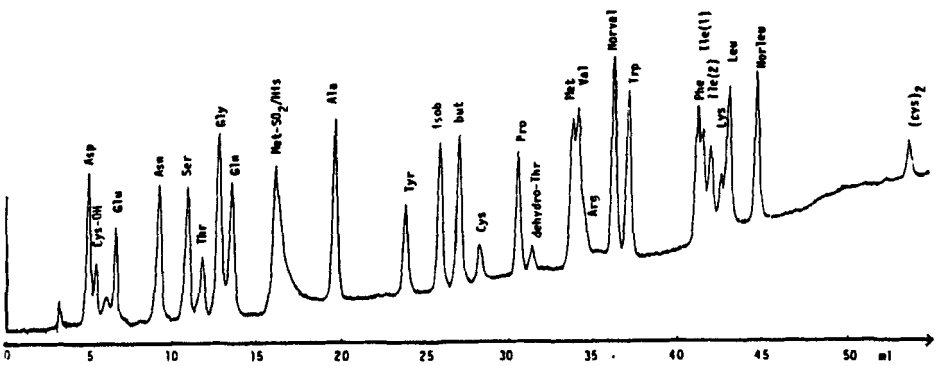

FIGURE 7. Separation of PTH-AA on ODS-Hypersil. 50 min. gradient with 0,01 M sodium acetate in 27-65% methanol at 1 ml/min. UV-detection at 254 nm.

could, however, be handled by using ethanolic mobile phases. On Bondapak (fig. 5) as well as on Hypersil a reasonable resolution was obtained. An even better separation could be obtained by using a less steep gradient from 37 to 48% ethanol (fig. 8).

The Phe/Leu/Ile/Lys Group

In addition to the Met/Val problem, there is also a general problem of poor resolution of the four PTH-amino acids Phe, Leu, Ile and Lys, when gradients for separating 20 or more amino acid-mixtures are used. If one of the four amino acids was excluded from a test mixture, a satisfactory resolution usually could be obtained relatively easy. Also, the group of four amino acids could be resolved if the other amino acids were of no concern, even on mediocre columns, as shown in fig. 9. The conditions in fig. 9 were found to give no interferences from any of the other amino acids included in

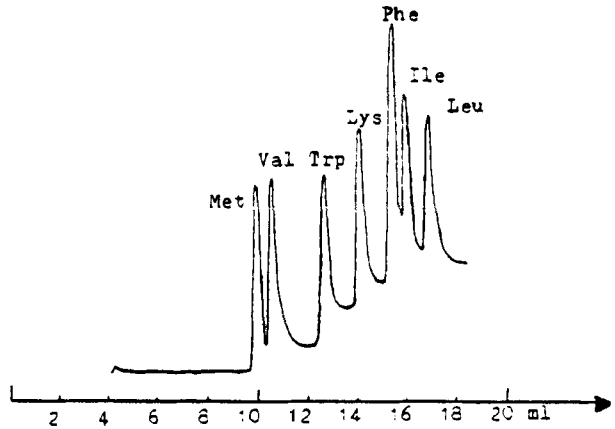

FIGURE 8. Separation of the PTH-amino acids Met/Val and Phe/Leu/Ile/Lys on ODS-Hypersil. 30 min. gradient with 0,01 M sodium acetate in 37-48% ethanol at 1 ml/min. UV-detection at 254 nm.

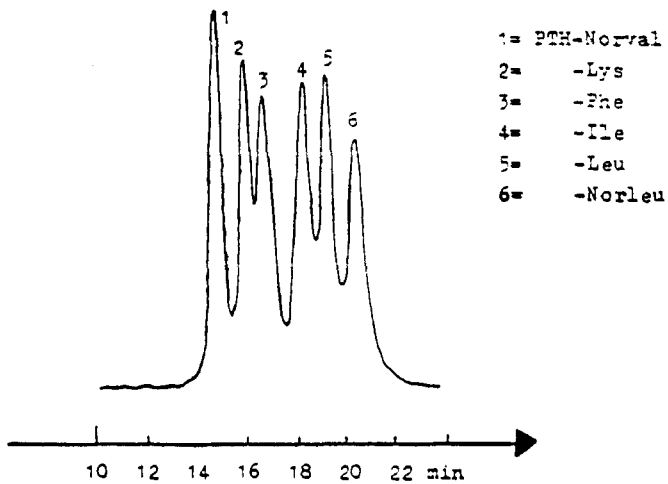

FIGURE 9. Isocratic separation of PTH-Lys/Phe/Ile/Leu on ODS-Hypersil with 0,01 M acetic acid in 60% methanol at 0,4 ml/min.

the test programs. An acceptable resolution also was obtained by the ethanol gradient used for separating Met and Val (fig. 8).

Another complication, especially from a quantitative point of view, was the occasional appearance of a second peak from PTH-Ile. This amino acid is one of the few that contain two assymetric centers and therefore have the ability of giving diastereomers which are chromatographically separable. The production of diastereomers as a function of the rate of racemization naturally will depend on treatment and storage conditions. Since the resolution of the four amino acids already is problematic, the introduction of a fifth in the same group does not improve the total resolution. Thus, the second PTH-Ile peak was only occasionly seen in the gradient programs, as in fig. 7 on Hypersil. With optically pure amino acids and fresh solutions of PTH standards, qualitative determinations should not be significantly disturbed. With quantitative measurements, performed after conditions which may enhance racemization, an open eye should be kept for the possible appearance of new peaks, and conclusions then be carefully drawn.

With the regular gradients none of the four packings tested gave a satisfactory resolution of the components of this group (see figures 2,5 and 7). An additional run, such as in fig. 8 or fig. 9, must therefore be recommended.

Interferences after Derivativization.

The chromatograms presented so far have been based on commercial standards of reasonable purity (with the exception of PTH-cysteine and cystine). Another question is whether the derivativization procedure

could produce byproducts interfering in the chromatograms.

The synthetic yields of PTH-amino acids are generally known to be good, and the same observation was made by us! The question of interfering byproducts was examined by running blanks without amino acids, blanks to which PTH-amino acids later was added, test mixtures of amino acids reacted with phenylisothiocyanate in 2-30 fold excess and hydrolysates of peptides with 2-30 fold excess of PITC.

When the blanks, after drying, were chromatographed, 9 different peaks could be seen. One was unreacted PITC, and two came from pyridine in the buffer and disappeared when the sequential grade of pyridine was used. The 6 other peaks were not identified. When the excess of the PITC reagent was increased, the amount of the byproducts also increased. With 1 nanomole of amino acides and a 5-fold excess of PITC, the interferences of the byproducts with the PTH-amino acids gave serious problems especially in quantitative analysis. The problem was strongly reduced when a 10 min. drying period at 1 torr of the phenylthiocarbamyl (PTC) intermediates was introduced, after the initial drying in vacuumdesiccator at about 15 torr. When 200 picomole of two different tripeptides were hydrolyzed, derivatized and 30% of the total amount injected on a column of medium efficiency, peaks of 5-10 times the noise level were obtained and could be determined, qualitatively as well as quantitatively. Detection at 280 nm gave less noise than detection at 254 nm. With amounts of amino acids much less than the 60 picomole above, the interferences could give serious problems. Another additional problem then is the danger of losses by adsorption to glass walls. When amino acids as well as PTH-amino acids in the low picomole range was added to

and transferred from the micro vials, neither acid washing nor silanization of the glasses could prevent large inconsistencies to appear.

CONCLUSION

By reversed phase chromatography on ODS packings more than 20 amino acids could be identified. The majority of the amino acids could be determined qualitatively as well as quantitatively by one gradient run. With Spherisorb-ODS, adsorption of the basic amino acids became a problem. The best combination for solving the Met/Val and the Phe/Leu/Ile/Lys separation problems was a α-Bondapak-C_{18} column with isocratic or gradient elution with ethanol in the mobile phase. A definitive determination of each amino acid thus would require one additional isocratic or gradient run.

Five internal standards were included in the regular gradient runs. Depending on the column used and the mobile phase chosen, one or two of these standards could be selected and used in qualitative and quantitative analysis. Approximately 50 picomole of each amino acid was considered a realistic detection limit, even if smaller amounts could be detected, by UV-detection at 280 nm or (less preferably) at 254 nm. Thus, the speed and the sensitivity of the method could favourably compete with the post-column ninhydrin techniques of regular amino acid analyzers.

The main obstacle in using this method for routine amino acid analysis of hydrolysates is the time-consuming preparation of the derivatives. In our opinion the PTH-method would primarily be of interest to institutions or groups who do not already possess a sensitive

amino acid analyzer, but do possess HPLC-equipment, and who occasionally see the need for amino acid analysis as well as peptide/protein sequencing and then could use the same analytical procedure.

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