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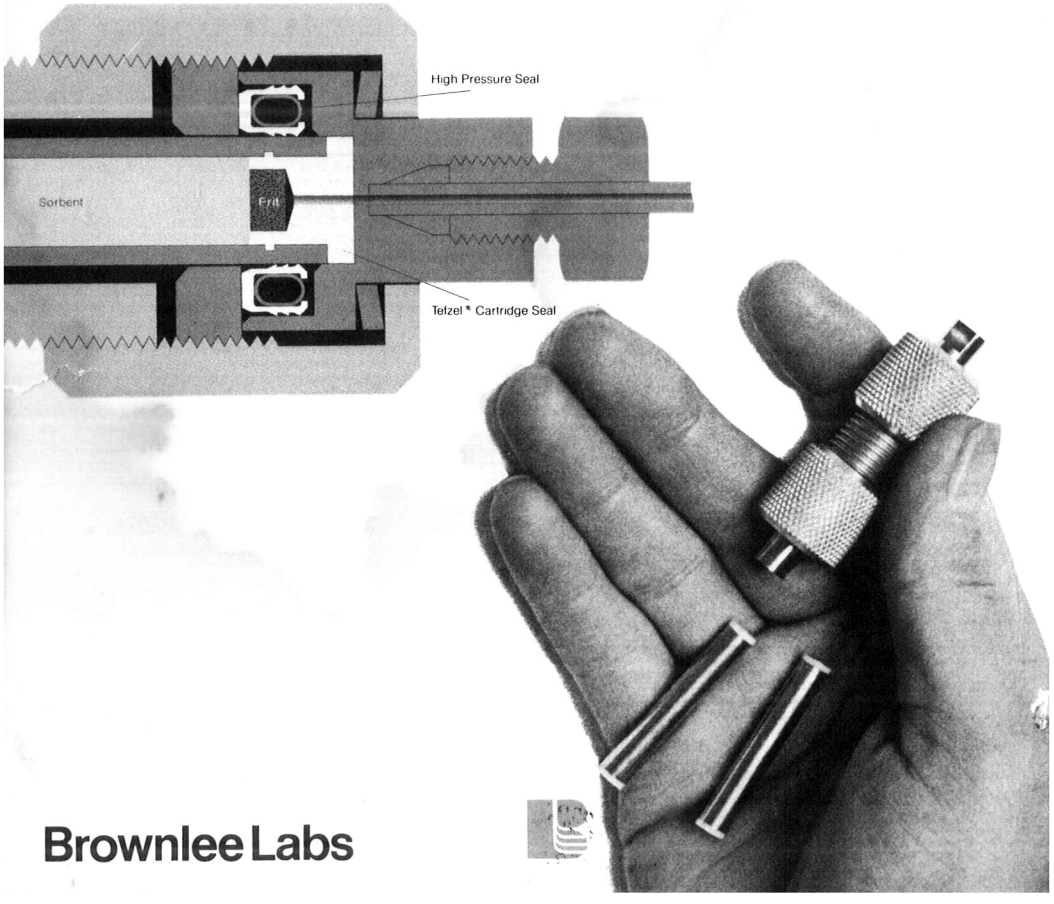

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LIQUID CHROMATOGRAPHIC ANALYSIS OF AMINO ACIDS, PEPTIDES, AND PROTEINS

Edited by M. T. W. HEARN
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Otago University Medical School
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and

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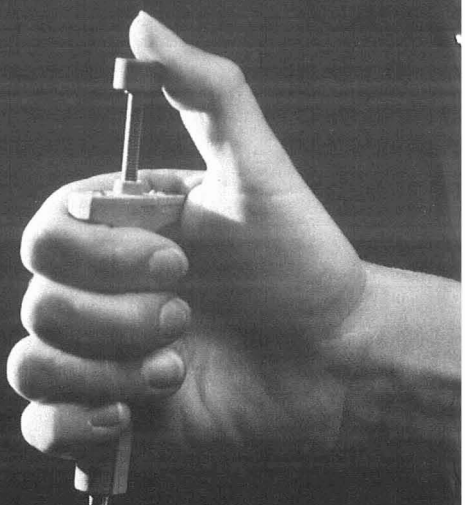

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CONTENTS

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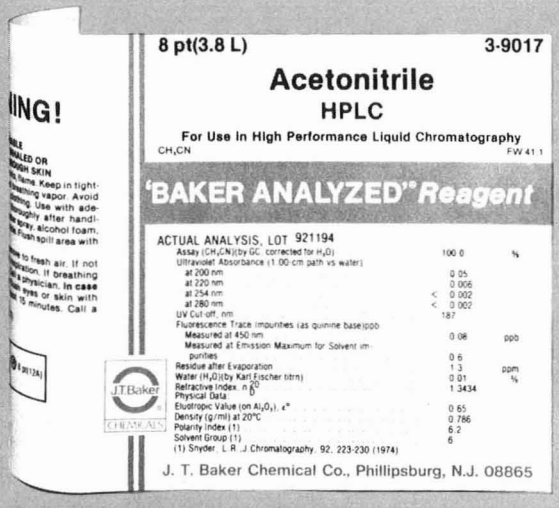

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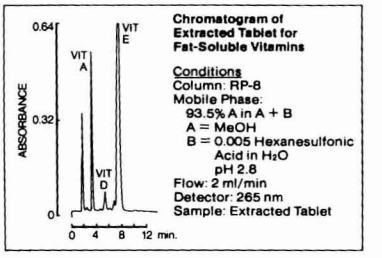

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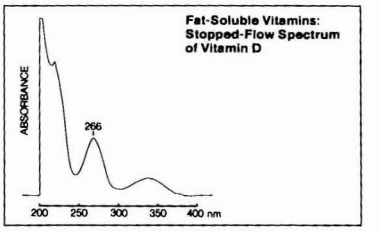

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AMINO ACID ANALYSIS AND ENZYMATIC SEQUENCE DETERMINATION OF PEPTIDES BY AN IMPROVED o-PHTHALDIALDEHYDE PRECOLUMN LABELING PROCEDURE

Barry N. Jones, Svante Pääbo and Stanley Stein

Roche Institute of Molecular Biology
Nutley, New Jersey 07110

ABSTRACT

Primary amino acids react with o-phthaldialdehyde in the presence of mercaptans to form intensely fluorescent derivatives. By the use of reverse-phase high-performance liquid chromatography, a mixture containing 26 of these derivatives was efficiently resolved with an analysis time of less than 35 minutes. The quantitation of the individual amino acids was reproducible with an average relative deviation of ± 1.4% and had a detection limit of approximately 50 femtomoles. Improvements in the stability and fluorescence response of lysine and hydroxylysine were obtained by the incorporation of sodium dodecyl sulfate in the derivatizing medium. Applications of the chromatography system involving the amino acid analysis of peptides after either acid or enzymatic hydrolysis are presented. Methods for the sequence analysis of picomole quantities of peptides by time course hydrolysis with exopeptidases were also developed, which employed the above separation procedure for the identification and quantification of the released amino acids.

INTRODUCTION

The ability to separate and quantitate amino acids is essential for the characterization and structural elucidation of peptides and proteins. This is usually accomplished by separating amino acid mixtures using classical ion-exchange chromatography (1) followed by post-column derivatization with ninhydrin (2,3) or a fluorogenic reagent (4-7). This technique resolves most amino acids with good detection limits, especially if either fluorescamine or o-phthaldialdehyde is used as the post-column derivatizing

reagent. However, this procedure generally requires specialized equipment and an analysis time greater than an hour.

In order to improve the efficiency and decrease the analysis time for the resolution of amino acid mixtures, several precolumn techniques have been developed which utilize reverse-phase high-performance liquid chromatography. Two of the most widely employed methods are the formation of dansyl (8) or phenylthiohydantoin (9,10) derivatives of the amino acids prior to high-performance liquid chromatographic analysis. Recent studies have demonstrated that o-phthaldialdehyde can also be used successfully as a precolumn derivatizing reagent. o-Phthaldialdehyde in the presence of either 2-mercaptoethanol or ethanethiol reacts rapidly with primary amino acids to form highly fluorescent, thio-substituted isoindoles (11,12). These derivatives are then analyzed with good selectivity and sensitivity by reverse-phase high-performance liquid chromatography (13-17). In this report, the use of o-phthaldialdehyde as a precolumn derivatizing reagent for primary amino acids is evaluated further. In addition, its applicability for the amino acid analysis of peptides, as well as for the determination of their amino acid sequence, is investigated.

MATERIALS AND METHODS

Apparatus

Two separate chromatography systems were employed in the present studies. One consisted of a high-pressure Milton Roy minipump (Lab Data Control), an LKB Ultrograd unit for generation of elution gradients, and a model FS970 Liquid Chromatographic Fluorometer (Schoeffel Instrument Corp.). The following fluorometer settings and characteristics were used for detection: 5 μL flow cell, excitation monochrometer at 330 nm, the emission measured with a 418 nm cut-off filter, time constant of 0.5 sec, and a sensitivity dial setting at 7.00 units. The other chromatography system consisted of two Altex Model 110A high-performance liquid chromatography (HPLC) pumps, an Altex Model 420 microprocessor

for generation of elution gradients, and a model FS950 Liquid Chromatographic Filter Fluorometer (Schoeffel Instrument Corp.). The following fluorometer settings and characteristics were used: 20 µL flow cell, a FSA 403 excitation filter and a FSA 111 lamp for excitation in the 330-375 nm range, the emission was measured with a FSA 426 (418 nm cut-off) filter, time constant of 0.5 sec., and a sensitivity dial setting at 8.00 units. The two chromatography systems gave essentially identical results with respect to the resolution of amino acid mixtures. The only major difference between the two systems was in sensitivity. Using the above fluorometer settings, the FS970 detection unit was approximately ten times more sensitive.

All sample injections were performed with a Rheodyne injection valve (Model 7120) equipped with a 20 µL sample loop. An Altex Ultrasphere ODS column (250 x 4.6 mm; particle size, 5 µ) fitted with a guard column (80 x 4.6 mm) packed with CO:PELL ODS sorbent (particle size, 40 µ) (Reeve Angel) was used for chromatographic separations. The guard column was packed downwards at a pressure of 3000 psi with a Haskel pump (DST-150). Chromatographic peaks were integrated by a Spectra-Physics Minigrator and recorded on a two-channel Linear recorder operated on the 10 mV and 100 mV scales. Gradients were formed between two degassed solvent mixtures. Solvent A was tetrahydrofuran:methanol:0.05M sodium acetate (pH 5.9) 1:19:80 and solvent B was methanol:0.05M sodium acetate (pH 5.9) 8:2. Further details of the chromatographic procedure are given in the figure legends.

Reagents and Standards

All HPLC solvents were distilled-in-glass grade (Burdick and Jackson) and used without further treatment. High purity water was obtained with a system from Hydro Service and Supplies (Durham, N.C.). Solutions of amino acid standards (2.5 µmol/mL), 2-mercaptoethanol, ethanethiol, o-phthaldialdehyde (Fluoropa), 4N methanesulfonic acid (MSA), and sodium dodecyl sulfate (SDS)

were obtained from Pierce Chemical Co. Individual amino acid standards were purchased from Sigma Chemical Co. All other chemicals were reagent grade. Enzymatic digestion buffers contained 0.01% pentachlorophenol (Pierce) as a preservative.

Peptides and Enzymes

Met-enkephalin-Arg^6-Phe^7 was synthesized by Peninsula Laboratories. Met-enkephalin, porcine glucagon, carboxypeptidase B, and carboxypeptidase Y were products of Boehringer Mannheim Co. Aminopeptidase M was purchased from Sigma Chemical Co. Oxidized Met-enkephalin was prepared according to the procedure of Savige and Fontana (18) and purified by reverse phase HPLC (19).

Preparation of Amino Acid Standards

Amino acid standard solutions were prepared in water in the following concentrations: 1.25, 6.25, 25, and 50 pmol/μL and stored at -20^{0}C. The standard solutions were stable for approximately 3 weeks of continual use. After this time, appreciable amounts of methionine were lost with a concomitant increase in the methionine sulfoxide content of the standard. The glutamine peak in the standard runs also decreased with time, presumably due to the conversion of glutamine to pyroglutamic acid. For the ammonia standard, a 50 pmol/μL solution in water was prepared from ammonium acetate.

Preparation of the o-Phthaldialdehyde/2-Mercaptoethanol Derivatizing Solution

Fifty milligrams of o-phthaldialdehyde were dissolved in 1.25 mL of absolute methanol. 2-Mercaptoethanol (50 μL) and 0.4M sodium borate (pH 9.5) (11.2 mL) were then added and the solution mixed. The mixture was flushed with nitrogen and stored in the dark. The solution was allowed to stand for 24 hours before use. This was found to reduce the background amino acids which are contributed by the derivatizing reagent. 2-Mercaptoethanol

(10 µL) was added every 2 days to help maintain the reagent strength. Using these precautions, the derivatizing reagent was usable for approximately two weeks before serious contaminants appeared in the elution profiles. The o-phthaldialdehyde/ethanethiol derivatizing solution was prepared daily by the procedure of Hill et al. (13).

Derivatization Procedure

The general procedure for derivatization was as follows: 5 µL aliquots of standards or unknown samples were mixed with 5 µL of a 2% sodium dodecyl sulfate (SDS) solution in 0.4M sodium borate (pH 9.5) and then 5 µL of the derivatizing solution added. The resulting solution was mixed thoroughly. After 1 minute, 10 µL of 0.1M potassium phosphate (pH 4.0) was added, the solution mixed, and 5-20 µL injected onto the reverse phase column.

Acid Hydrolysis of Peptides

For HCl hydrolysis, peptides (50-300 pmol) were dried in a hydrolysis tube and the tube sealed under vacuum after the addition of 200 µL of constant boiling HCl containing 0.1% thioglycolic acid. Following hydrolysis, the HCl was removed by lyophilization and the resulting residue dissolved in 25-50 µL of 2% SDS in 0.4M sodium borate (pH 9.5). Analyses were then performed as above, usually on 5 µL aliquots. For hydrolysis with methanesulfonic acid (MSA), peptides (500 pmol - 1 nmol) were hydrolyzed with 50 µL of 4N methanesulfonic acid. After hydrolysis, the acid was neutralized with 50 µL of 4N NaOH. Aliquots (5 µL) were removed, mixed with 2% SDS in borate, derivatized, and analyzed by HPLC. All acid hydrolyses were performed at 110^0C for 22 hours.

Total Enzymatic Hydrolysis of Peptides

Peptides (200 pmol - 1 nmol) were dissolved in 24 µL of 0.2M sodium phosphate (pH 7.0) buffer containing 4-aminobutyric acid (usually 10 pmol/µL) as an internal standard. To this solution,

1 µg of aminopeptidase M (1 µL) was added and the resulting mixture incubated at 37°C for 24 hours. Aliquots (5 µL) were removed and analyzed. A digest blank containing 24 µL buffer and 1 µL aminopeptidase M was also prepared and analyzed.

Hydrolysis of Peptides with Carboxypeptidase B

Peptides (20-200 pmol) were dissolved in 24 µL of 0.2M sodium phosphate (pH 8.5) buffer containing 4-aminobutyric acid (5 pmol/µL) as an internal standard. To this solution, 25 ng of carboxypeptidase B (1 µL) was added and the resulting mixture incubated at 37°C for 1 hour. Aliquots (5 µL) were removed and analyzed. As an added precaution during enzymatic hydrolyses, the peptide solution was usually analyzed for free amino acids before the addition of the enzyme. Enzyme blanks were always prepared and analyzed.

Time Course Hydrolysis with Aminopeptidase M

Peptides (200 pmol - 1 nmol) were dissolved in 49 µL of 0.2M sodium phosphate (pH 7.0) buffer containing 4-aminobutyric acid as an internal standard. To this solution, 100 ng of aminopeptidase M (1 µL) was added and the resulting mixture incubated at room temperature. Timed aliquots (5 µL) were removed, mixed with 2% SDS in borate (5 µL), and then frozen in a dry ice/methanol bath. The aliquots were stored at -20°C until analyzed.

Time Course Hydrolysis with Carboxypeptidase Y

Peptides (200 - 1 nmol) were dissolved in 49 µL of 0.05M sodium acetate (pH 5.5) buffer containing 4-aminobutyric acid as an internal standard. To this solution, 1 µg of carboxypeptidase Y (1 µL) was added and the resulting mixture incubated at room temperature. Timed aliquots (5 µL) were removed and analyzed. Carboxypeptidase Y digestions were also performed in the presence of 5M urea.

RESULTS AND DISCUSSION

Preliminary separations of the o-phthaldialdehyde/2-mercaptoethanol derivatives of primary amino acids were conducted using mixtures of methanol and sodium or potassium phosphate buffer as the mobile phase according to the procedures of Lindroth and Mopper (14). Satisfactory resolution was obtained for all derivatives tested except for glycine and threonine which co-eluted even under isocratic conditions. In an attampt to resolve these two derivatives, the pH of the mobile phase was varied between 5.0 and 7.5 and the ionic strength was varied between 0.05M and 0.2M; however, glycine and threonine did not resolve under any of these conditions. Citrate and acetate buffers were then tested but did not help to resolve these two amino acids. Following the suggestion of Hodgin (16), tetrahydrofuran was added to the mobile phase an an organic modifier; this resulted in the immediate resolution of glycine and threonine. The final conditions chosen for HPLC analysis are given in Figure 1 which illustrates the resolution obtained for a mixture of 26 amino acids plus ammonia. Excellent resolution was obtained for the components in the mixture. Of a total of 30 amino acids tested, only two were not resolved by these conditions. Norleucine co-eluted with leucine and methionine sulfone eluted as a shoulder on the threonine peak.

The stability of the o-phthaldialdehyde/2-mercaptoethanol derivatives was determined by reacting a mixture of amino acids (40 pmol each) with the derivatizing reagent for varying time periods before injection. Only the derivatives of glycine, lysine, and hydroxylysine showed appreciable decay of fluorescence after an eight minute reaction time. In addition, the lysine and hydroxylysine derivatives gave a very low response when compared to the other amino acid derivatives. According to Benson and Hare (6), the addition of the surfactant, Brij 35, to the o-phthaldialdehyde reagent enhances the fluorescence of lysine during post-column derivatization procedures. Gardner and

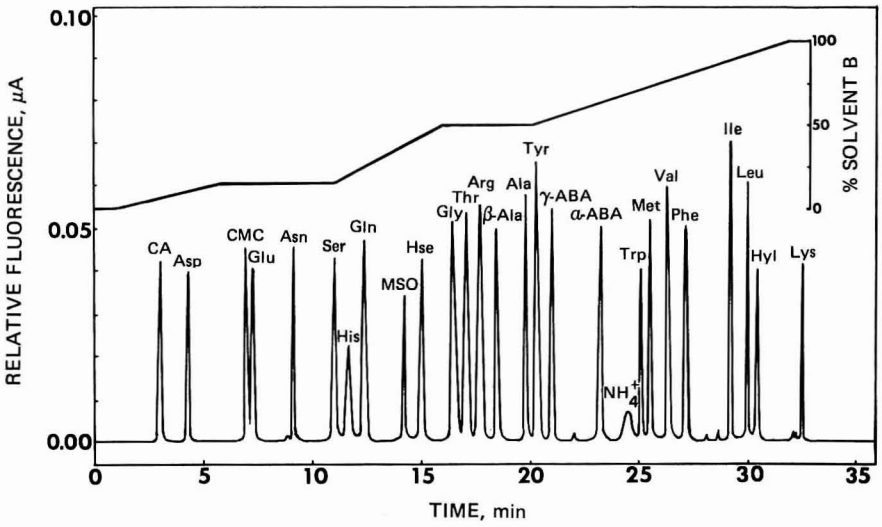

Figure 1. Elution profile of amino acid standards derivatized by the reaction with o-phthaldialdehyde/2-mercaptoethanol. Each peak represents 5.0 pmoles except for the ammonia peak which represents 20.0 pmoles. Conditions: Solvent A, tetrahydrofuran:methanol: 0.05M sodium acetate (pH 5.9), 1:19:80; Solvent B, methanol:0.05M sodium acetate (pH 5.9), 8:2; gradient program, 0%B for 1 min from the initiation of the program, linear step to 14% B in 5 min, isocratic step at 14% B of 5 min duration, linear step to 50% B in 5 min, isocratic step at 50% B of 4 min duration, linear step to 100% B in 12 min; flow rate of 1.7 mL/min; Schoeffel FS950 fluorescence HPLC detector. Non-standard abbreviations used: CA, cysteic acid; CMC, carboxymethyl cysteine; MSO, methionine sulfoxide; Hse, homoserine; γ-ABA, 4-aminobutyric acid; α-ABA, 2-aminobutyric acid.

Miller (17) found that the addition of Brij 35 also increased the fluorescence response of lysine in their precolumn derivatization procedure. In the present study, sodium dodecyl sulfate (SDS) was evaluated for its effect on the fluorescence of o-phthaldialdehyde-derivatized amino acids (Figure 2). An approximately 50% increase in the response of lysine was obtained and approximately a 40% increase for hydroxylysine. The stability of these two derivatives was also increased by the presence of SDS. These observations are in agreement with those reported by Chen

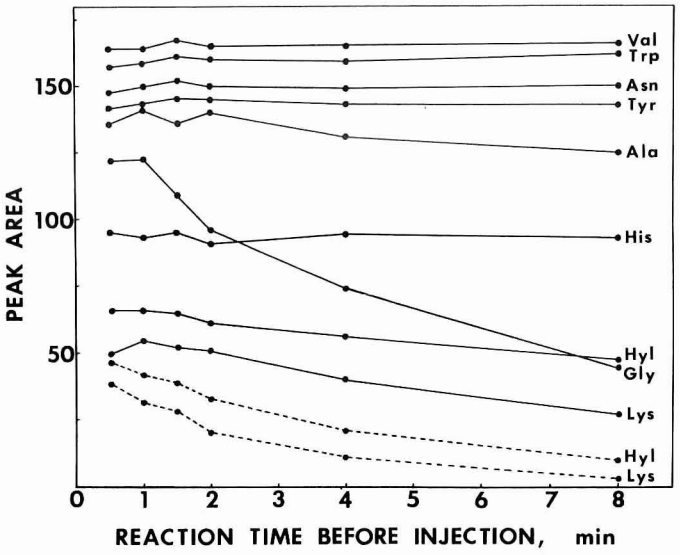

Figure 2. Influence of reaction time before injection on the fluorescent intensity of ten amino acids. Peak areas are given in arbitrary units. The effect of the presence of sodium dodecyl sulfate (SDS) on the fluorescent intensity and stability of the lysine and hydroxylysine derivatives is also shown. Derivatizations in the presence of SDS are given by the solid lines and in the absence of SDS by dashed lines.

et al. (20). No observable effect on the retention times and fluorescence response of the other amino acids tested (see Figure 1) was detected. The large decrease in the fluorescence response of glycine was minimized by injection of all derivatized samples after a 1 minute reaction time (see Figure 2),

In order for the present chromatographic system to be of value for the quantitative analysis of amino acids, the technique must yield reproducible results, possess high sensitivity, and give a linear fluorescence response in the general concentration range required for analyses. The precision of the procedure was evaluated by injection of a 22-component amino acid standard using the chromatographic conditions presented in Figure 1. Five consecutive injections were performed following a reaction time

of one minute and the resulting retention times, peak heights, and peak areas of the individual components measured. Analysis of the data indicated that the average deviation of the retention times was ± 1.1 second. The largest deviation observed was for methionine sulfoxide (± 2.6 sec.) and the smallest deviation obtained was for methionine (± 0.2 sec.). The total analysis time was 34 minutes. The average relative deviation in the measured peak heights was ± 6.4%. However, part of this deviation is due to pipetting errors that may occur during the derivatization procedure. When results from all five runs were normalized to an internal standard (4-aminobutyric acid) the relative deviation of the peak heights was calculated to be ± 1.4%. The average relative deviation of the normalized peak areas was ± 1.7%. The largest deviation in the normalized peak height values was observed for cysteic acid (± 2.8%) whereas the lowest was for arginine (± 0.4%). From the above results, the incorporation of an internal standard is recommended for a quantitative analysis of an amino acid mixture.

The detedtion limits for the chromatography system were determined by serial dilution of an amino acid standard solution followed by derivatization and analysis. A detection limit of approximately 50 fmol was obtained for the Schoeffel FS 970 detection unit whereas the Schoeffel FS950 unit displayed a detection limit of approximately 500 fmol for the individual amino acid components. The sensitivity of detection can be increased somewhat by using ethanethiol instead of 2-mercaptoethanol in the derivatizing reagent (12,13). However, in the present study, the added sensitivity was not required and the use of ethanethiol was abandoned because of its unpleasant odor. Additional sensitivity (approximately a six-fold increase) can be obtained by excitation at 229 nm instead of 330 nm (13), but interference from extraneous components may be a problem at this lower wavelength when biological fluids are examined.

The linearity of response was examined in the concentration range of 5-100 pmol for a number of amino acid derivatives

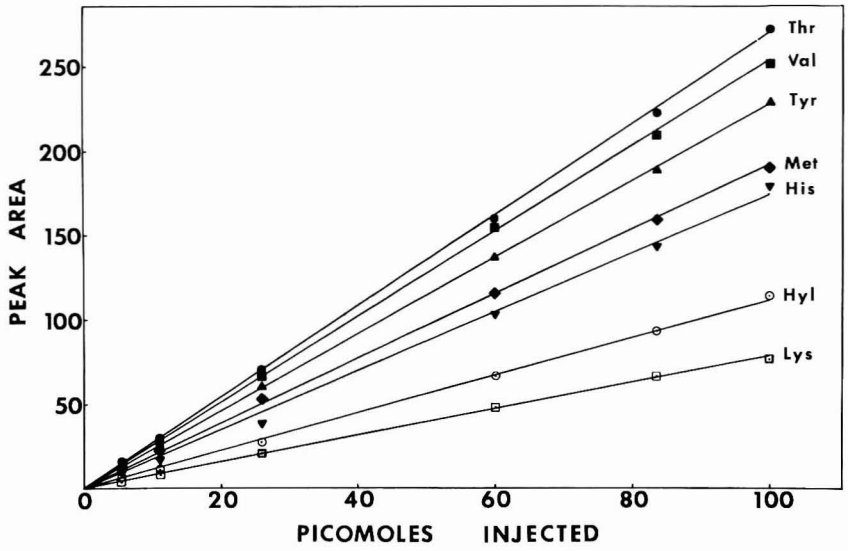

Figure 3. Linearity of response for seven amino acid derivatives. The chromatography conditions were identical to those in Figure 1.

(Figure 3). At concentration levels below 5 pmol, large deviations from linearity were obtained, especially for serine, glycine, and alanine. These deviations from linearity are most probably due to the background presence of these amino acids. This can be partially corrected by the subtraction of the background amino acids which are determined by "blank" runs. Even this procedure does not completely correct for the presence of glycine and serine whose presence vary considerably during a series of analyses. To minimize this effect when quantitative analysis was desired below the 5 pmol level, all reaction vessels (1 mL polypropylene centrifuge tubes) and pipet tips were acid washed before use. Furthermore, plastic surgical gloves had to be worn to prevent "fingerprint" contamination.

Three different types of HPLC columns were evaluated for their use in the separation of the o-phthaldialdehyde derivatives of amino acids. The three were Altex Ultrasphere columns, octadecylsilane (ODS), octadecylsilane/ion-pairing (ODS I/P.), and

cyanopropylsilane (CN). The amino acid derivatives shown in Figure 1 were resolved on the CN column, but variable peak heights and retention times were obtained and its use was discontinued. The ODS and ODS I.P. columns gave identical results. The Ultrasphere ODS column was used in all chromatographic separations used in this report. Over 600 analyses have been performed on this column without any detectable loss of resolving power. However, the column is protected with a guard column since many analyses (see below) require the injection of derivatized amino acid mixtures which contain enzymes and partially digested peptides. The guard column was replaced after every 200 injections as a precaution.

The applicability of the chromatography system for the determination of the amino acid composition of peptides was evaluated. Three different hydrolysis procedures, HCl hydrolysis, methanesulfonic acid (MSA) hydrolysis, and total enzymatic hydrolysis with aminopeptidase M, were used and found to be compatible with the o-phthaldialdehyde derivatization technique. The results obtained for the amino acid analysis of porcine glucagon using each of these hydrolysis procedures is summarized in Table I. The elution profile of the enzymatic hydrolysate is given in Figure 4. The resulting compositions are in good agreement with the reported amino acid sequence of porcine glucagon (21). It should be noted that the acid/amide content of glucagon was readily obtained by the enzymatic hydrolysis procedure. Detection and quantitation of asparagine and glutamine usually require special buffers (lithium citrate) and modified chromatography conditions (22) when analysis is performed by classical ion-exchange methods.

The quantitation of methionine sulfoxide is also a problem for many amino acid analyzer systems. However, methionine sulfoxide content of peptides is easily determined by the precolumn derivatization with o-phthaldialdehyde. This is illustrated in Figure 5 by the elution profile of a MSA hydrolysate of purified oxidized Met-enkephalin. The methionine sulfoxide elutes as a

TABLE I

AMINO ACID COMPOSITION OF PORCINE GLUCAGON

Amino Acid	HCl Hydrolysate[a]	MSA Hydrolysate[b]	Enzymatic Hydrolysate[c]	From Sequence[d]
Asp[e]	4.0	3.8	3.2	3
Glu[f]	3.0	3.1	0.1	0
Asn	---	---	1.1	1
Ser	3.8	3.6	3.8	4
His	0.9	0.7	1.1	1
Gln	---	---	3.2	3
Gly	1.2	1.1	1.0	1
Thr	2.9	2.5	3.0	3
Arg	2.0	2.1	2.0	2
Ala	1.0	1.2	1.0	1
Tyr	2.0	2.0	1.9	2
Trp	0.3	0.8	0.9	1
Met	0.9	0.9	1.1	1
Val	1.0	1.0	1.0	1
Phe	2.0	2.0	1.9	2
Ile	0.0	0.0	0.0	0
Leu	2.0	2.1	2.0	2
Lys	1.3	1.0	0.9	1

[a] 300 pmol of peptide were hydrolyzed by HCl. Results are the average of three analyses.

[b] 1 nmol of peptide was hydrolyzed with methanesulfonic acid (MSA). Results are the average of two analyses.

[c] 500 pmol of peptide were digested with aminopeptidase M. Results are the average of two analyses.

[d] The amino acid sequence used is from Bromer *et al.* (21).

[e] Asp + Asn for HCl and MSA hydrolysates.

[f] Glu + Gln for HCl and MSA hydrolysates.

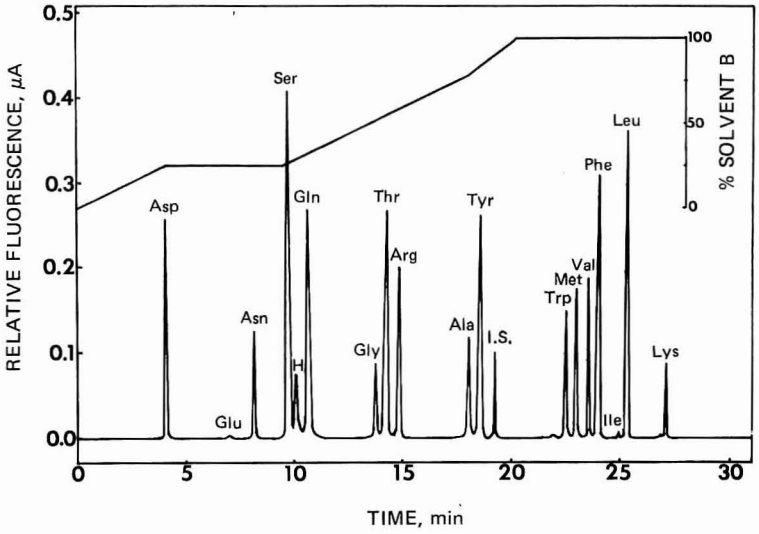

Figure 4. Elution profile of a total enzymatic hydrolysate of glucagon. Digestion was accomplished by incubating 500 pmoles of the peptide with 1 μg of aminopeptidase M. A 5 μL aliquot representing 10% of the hydrolysate was derivatized and 30% of the resulting derivatization mixture applied to the HPLC column. Conditions: Solvents A and B, same as in Figure 1; gradient program, linear step to 25% B in 4 min, isocratic step at 25% B of 5 min duration, linear step to 80% B in 9 min, linear step to 100% B in 2 min; flow rate of 2.0 mL/min; Schoeffel FS950 fluorescence HPLC detector. Internal standard (I.S.) was 4-aminobutyric acid. The single letter code, H, was used for histidine.

characteristic doublet. However, in the elution profile shown in Figure 1, it elutes as a singlet. The discrepancy appears to result from the two diastereomeric forms of methionine sulfoxide. The amino acid standards in Figure 1 were obtained from Pierce and presumably contained only one of the diastereomeric forms of methionine sulfoxide. This was further investigated by testing a methionine sulfoxide standard from Sigma tha- contained a mixture of the two diastereomers. Analysis of this standard yielded the characteristic doublet. Figure 5 also indicates that some of the methionine sulfoxide was reduced to methionine during the hydrolysis procedure.

In addition to the use of this chromatography system for amino acid analysis, its applicability in obtaining sequence data was also evaluated. The methods developed include digestion of peptides with carboxypeptidase B (CPB) plus aminopeptidase M (APM) and carboxypeptidase Y (CPY) time course hydrolysis. The use of CPB digestion is illustrated in Figure 6. In this example, two tryptic peptides were obtained from enkephalin precursor molecules that gave identical compositions, Gly_2,Met,Tyr,Phe,Arg (23). However, their elution positions were different as determined by their chromatography on reverse phase columns. In order

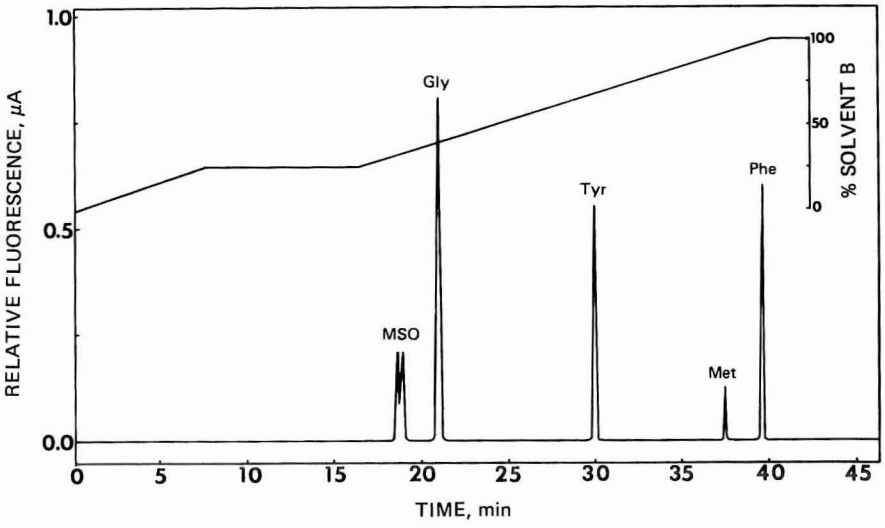

Figure 5. Elution profile of an acid hydrolysate of oxidized Met-enkephalin (Tyr-Gly-Gly-Phe-MSO). The peptide (1 nmole) was hydrolyzed in 50 μL of 4N methanesulfonic acid and then diluted to a volume of 100 μL with 4N NaOH. A 5 μL aliquot of the hydrolysate was derivatized and 20% of the resulting derivatization mixture applied to the HPLC column. Conditions: Solvents A and B, same as in Figure 1, gradient program, linear step to 25% B in 7.5 min, isocratic step at 25% B of 9 min duration, linear step to 100% B in 23 min; flow rate of 1.0 mL/min; Schoeffel FS970 fluorescence HPLC detector. Methionine sulfoxide (MSO) elutes as a doublet due to the resolution of its two diastereomeric forms. Composition was calculated to be MSO, 0.9 residues; Gly, 1.9 residues; Tyr, 1.1 residues; Met, 0.2 residues; Phe, 0.9 residues.

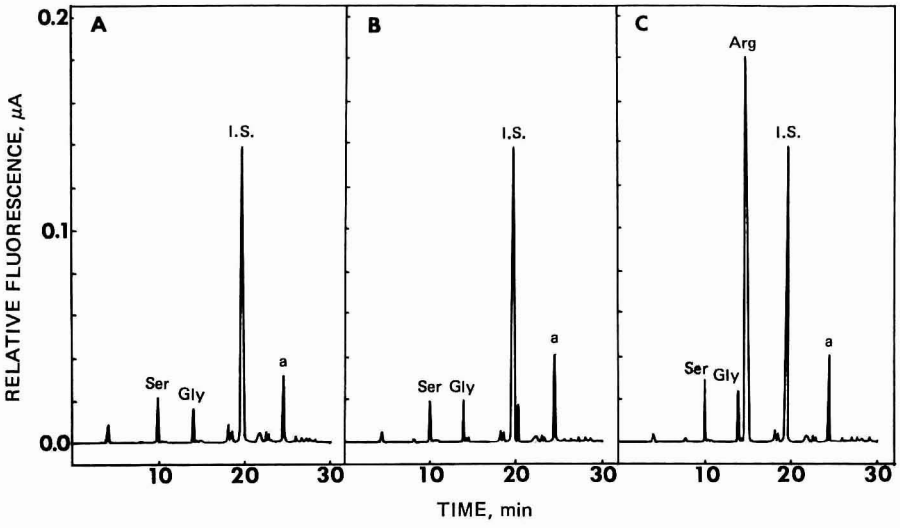

Figure 6. Elution profiles of carboxypeptidase B (CPB) digests of two Met-enkephalin-containing peptides. A, Digest blank: 25 ng CPB in 25 μL of digestion buffer, 5 μL aliquot derivatized and applied to HPLC column. B, Digest of Arg-Tyr-Gly-Gly-Phe-Met, 125 pmoles of peptide in 25 μL of digestion buffer containing 25 ng CPB, 5 μL aliquot derivatized and analyzed. C, Digest of Tyr-Gly-Gly-Phe-Met-Arg, 150 pmoles of peptide in 25 μL of digestion buffer containing 25 ng CPB, 5 μL aliquot derivatized and analyzed. The digestion buffer contained 4-aminobutyric acid (5 pmol/μL) as an internal standard (I.S.). Peak "a" is contributed by the derivatizing reagent. Conditions: identical to those in Figure 3.

to determine the structural difference in the two peptides, each was digested with CPB. For one of the peptides, no detectable amino acids were released (Figure 6B) after comparison to the analysis of the enzyme blank (Figure 6A), but CPB did release one residue of arginine from the other tryptic peptide indicating that this was the carboxyl-terminal residue. These results were later confirmed by sequence analysis which determined the amino acid sequence of each tryptic peptide (23) (see Figure 6).

Amino-terminal sequencing with APM is illustrated in Figure 7. In this example, Met-enkephalin was digested with APM and timed aliquots removed for analysis. From Figure 7, it can be seen what tyrosine is the first amino acid to be released

followed by glycine. Methionine and phenylalanine are released simultaneously. Thus, the release data are consistent with the known amino acid sequence of Met-enkephalin, Tyr-Gly-Gly-Phe-Met.

Carboxyl-terminal sequencing with CPY is illustrated in Figure 8. In this example, Met-enkephalin-Arg^{6}-Phe^{7} was digested with the enzyme and the released amino acids identified and quantitated. The data obtained from the HPLC analyses are presented in Figure 9 in the form of a time course release plot and established the carboxyl-terminal sequence of the peptide as -Gly-Phe-Met-Arg-Phe.

This chromatography system has other applications. For example, free amino acids released during tryptic digestion of peptides and proteins are readily identified and quantitated. In

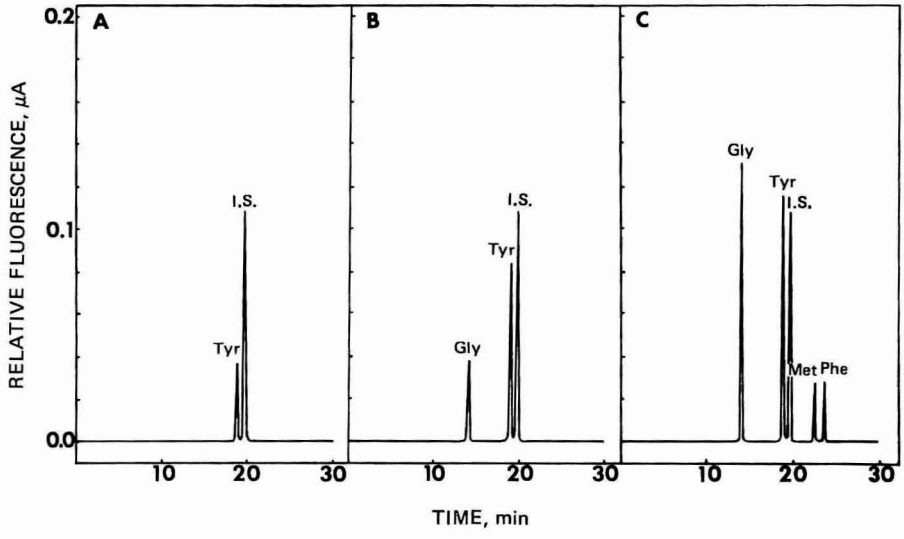

Figure 7. Elution profiles of aminopeptidase M (APM) time course hydrolysis of Met-enkephalin (Tyr-Gly-Gly-Phe-Met). Peptide (1 nmole) was dissolved in 50 μL of digestion buffer containing 50 ng APM and 20 pmol/μL 4-aminobutyric acid as internal standard (I.S.). Timed 5 μL aliquots were derivatized and 10% of the resulting derivatization mixtures applied to the HPLC column. A, 4-min aliquot; B, 30-min aliquot; C, 180-min aliquot. Conditions: identical to those in Figure 3.

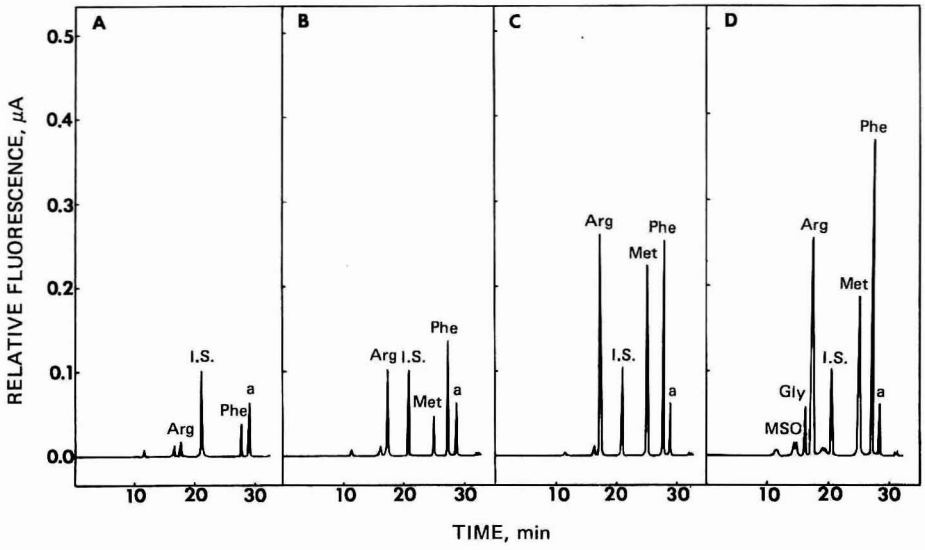

Figure 8. Elution profiles of carboxypeptidase Y (CBY) time course hydrolysis of Met-enkephalin-Arg^6-Phe^7 (Tyr-Gly-Gly-Phe-Met-Arg-Phe). Peptide (360 pmol) was dissolved in 60 μL of digestion buffer containing 500 ng CPY and 2 pmol/μL 4-aminobutyric acid as internal standard (I.S.). Timed 5 μL aliquots were derivatized and analyzed. A, 2-min aliquot; B, 8-min aliquot; C, 60-min aliquot; D, 600-min aliquot. Peak "a" is contributed by the derivatizing reagent. MSO represents methionine sulfoxide. Conditions: identical to those in Figure 1.

addition, the homoserine content of cyanogen bromide fragments is readily determined by amino acid analysis using this precolumn derivatization procedure. The method also has the potential for many other applications such as the determination of free amino acids in biological fluids (13,14). A variety of primary amines of biological importance could conceivably be identified and quantitated by this method. These include taurine, 4-aminobutyric acid, ornithine, citrulline, histamine, serotonin, and epinephrine as well as others. Several reports have already appeared describing procedures for the analysis of some of these components (13-15,24,25).

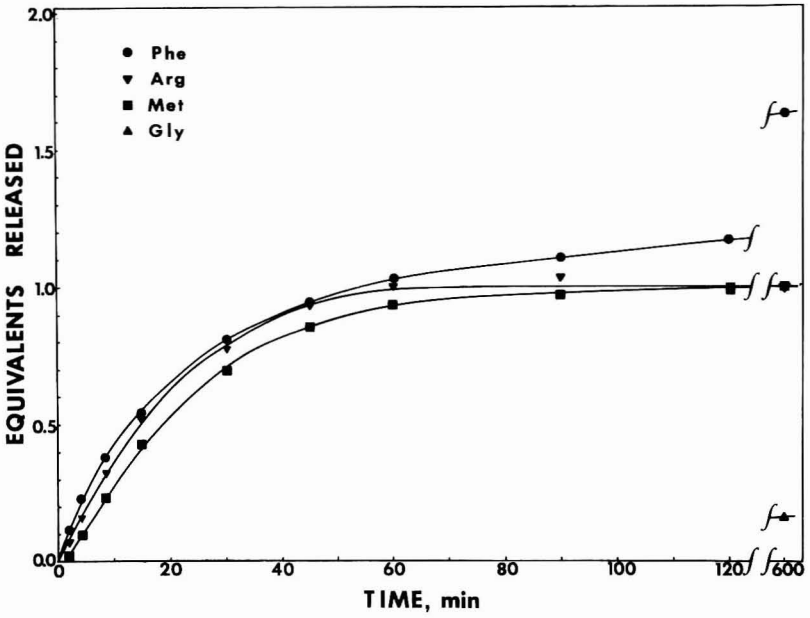

Figure 9. Time course release plot for the carboxypeptidase Y digestion of Met-enkephalin-Arg^6-Phe^7. Carboxyl-terminal sequence established by release data is -Gly-Phe-Met-Arg-Phe-COOH.

CONCLUSIONS

The use of precolumn derivatization of amino acids with o-phthaldialdehyde and their subsequent separation by reverse phase HPLC provides an excellent method for the amino acid analysis and sequence determination of peptides. The derivatization procedure is rapid, is performed in an aqueous medium, requires few transfer steps, and yields strongly fluorescent derivatives. The separation procedure is highly reproducible and rapid when compared to current amino acid analyzers. The cost per analysis is considerably less in comparison to post-column procedures in which the derivatizing reagent is added in a continuous manner.

Three major disadvantages to this procedure are the low response of cysteine, lysine, and hydroxylysine, the lack of re-

action with the secondary amino acids, proline and hydroxyproline, and the decay in fluorescence of the derivatized amino acids. The response of lysine and hydroxylysine was improved by the addition of sodium dodecyl sulfate to the derivatizing reagent. Cysteine can be readily detected as cysteic acid or carboxymethyl cysteine following performic acid oxidation or carboxymethylation (26). The problem of the detection of proline and hydroxyproline requires further investigation. The effect of fluorescence decay can be minimized by strict attention to the reaction time before injection.

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REVERSE PHASE ION PAIR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF VIRAL TRYPTIC GLYCOPEPTIDES

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ABSTRACT

Tryptic glycopeptides from influenza A/WSN (H_0N_1) and mink cell focus (MCF)-inducing (MCF-247) murine leukemia virus were subjected to high performance liquid chromatography (hplc) for mapping purposes. Hydrophilic ion-pairing was accomplished using 0.1% phosphoric acid on C_{18} μBondapack and LiChrosorb RP-18 ODS commercially available columns. The samples were eluted from the column with acetonitrile with sample recovery in the range of 70 to 80%. The advantages of hplc over the conventional techniques previously used are detailed.

INTRODUCTION

Recent studies on the importance of glycoproteins for cellular interactions has necessitated the development of analytical techniques for the molecular characterization of peptides and glycopeptides available in minute quantities. When high performance liquid chromatography was introduced as an analytical tool it was expected that this technique would allow rapid, discrete

separation of peptides and proteins. Unfortunately early analyses of underivatized peptides were not completely successful due to poor resolution, lack of reproducibility, peak broadening, long retention times and poor yields. However, the introduction of small hydrophilic ion pairing agents into the mobile phase offered a solution to these problems and made it possible to analyze and purify a wide variety of peptides.

Ion pair partition chromatography has been used effectively with a variety of compounds (1-5). Hydrophobic ion pairing agents such as heptane sulfonic acid and tetrabutyl ammonium phosphate have been used to increase the retention times of molecules. Hydrophilic ion pairing agents such as phosphoric acid (1-3), acetic acid (1), formic acid (1), picrates (6), trialkyl-ammonium phosphate (7), and ammonium acetate (10) have been used with good success for complex peptide separations. The use of hydrophilic pairing agents has greatly enhanced the scope of ion-pair reverse phase liquid chromatography. On reverse phase supports the hydrophilic modifiers increase polarity, thereby reducing the retention time of the sample components (1,11). Considerable flexibility is afforded to the chromatographer in that retention times of sample components may be manipulated allowing the resolution of complex mixtures with increased yields in the case of peptides. In addition combinations of hydrophobic and hydrophilic ion pairing agents may be used simultaneously to further resolve difficult sample mixtures.

Over the past few years this laboratory (1,3,10,12,15,21) and others (2,4,5,11,13,14) have developed techniques utilizing hplc for the rapid, discrete separation of peptide mixtures that are available only in minute quantities. Resolution was sufficient to use reverse phase hydrophilic ion pairing for peptide mapping of several biologically active proteins. Concurrent with

these efforts, this laboratory was carrying out peptide and glycopeptide mapping studies of viral proteins and glycoproteins using classical gel permeation and ion exchange techniques. These studies were time consuming and resolution of complex tryptic glycopeptide mixtures was often incomplete. We have now found that tryptic glycopeptides may be chromatographed using reverse phase ion pair partition chromatography with a hydrophilic pairing agent, phosphoric acid. The usefulness of this method for the separation of tryptic glycopeptides is demonstrated in this report.

MATERIALS AND METHODS

Chemicals and Apparatus

All work was carried out on a Waters high performance liquid chromatographic system that included two M6000 solvent delivery units, a M660 solvent programmer and a U6K injector or on a Laboratory Data Control System including Constametric IIG and III solvent delivery units and a gradient master. All samples were monitored by radioactivity with a LKB 1210 Ultrobeta Liquid Scintillation counter. All solvents were filtered using either a Millipore FA or HA 40μ solvent filters. Phosphoric acid was purchased from Fisher Chemicals and acetonitrile was purchased from Burdick and Jackson. Water was passed through a Millipore reverse osmosis Super-Q deionization System using a 0.22μ filter to approximately 13 megaohm and passed over activated charcoal cartridges prior to use on the high pressure system. The C_{18} μBondapak (Waters Associates) and LiChrosorb RP-18 (E. M. Laboratories) columns that were used in this procedure were purchased commercially. TPCK-trypsin was purchased from Millipore Corporation.

Chromatographic Procedures

All chromatograms were developed at room temperature. Peptides and glycopeptides were dissolved in the eluting solvent before injection and centrifuged in a Beckman Eppendorff centrifuge to remove any insoluble sample components. The columns were allowed to equilibrate in the appropriate mobile phase for about 120 mls prior to chromatography. The A solvent in each study was 0.1% H_3PO_4, pH 2.85, and the organic solvent was 60% acetonitrile (CH_3CN) in the A buffer.

Sample Preparation

Influenza A/WSN (H_0N_1) virions were grown in MDBK cells, labeled with ^{3}H-glucosamine, and purified as previously described (16). Virion polypeptides were solublized under reducing conditions and separated by SDS-polyacrylamide gel electrophoresis on a 10% slab gel. Following electrophoresis the slab gel was fixed and the fluorographic procedure of Bonner and Laskey (17) was applied to each gel. Fluorography on dried gels was carried out with Kodak XR-2 X-ray film at -70°. Gel segments containing viral glycoproteins were excised and rehydrated in a solution of 0.1 M ammonium bicarbonate containing 50 µg/ml of TPCK-trypsin (Millipore Corp.). The gel slices were incubated for 24 hr at 37° and the treatment was repeated at least three times. The enzymatic digests were pooled and lyophilized. The lyophilized material was rehydrated in water and three to five drops of concentrated formic acid was added to each digest to liberate residual ammonium bicarbonate. The ^{3}H-glucosamine labeled tryptic glycopeptides were then filtered through a 0.2µm Millex filter (Millipore Corp.) and relyophilized. The dried samples were then stored until analyzed.

The envelope precursors of mink cell focus (MCF) - inducing (MCF-247) murine leukemia virus (MuLV) were kindly provided by Dr. Nancy Famulari (Sloan-Kettering Cancer Center, New York).

These glycoproteins were labeled with ^{3}H-mannose in SC-1 or mink lung cells as previously described (18,19). The glycoproteins were immunoprecipitated from cell lysates and separated by SDS-polyacrylamide gel electrophoresis on 7.5% slab gels using methods previously described (18). The slab gel, following electrophoresis, was treated as decribed above and the tryptic glycopeptides of SC-1/MCF-247 and mink/MCF-247 Pr^{env} were prepared in the same manner described for the influenza viral glycoproteins.

RESULTS AND DISCUSSION

Influenza A hemagglutinin (HA) glycoproteins are located on the surface of the virions. HA glycoproteins may be present in virions as uncleaved HA or as two cleavage products, HA_1 and HA_2, which are linked by disulfide bonds. Previous studies showed that two major types of oligosaccharides are present in HA: complex (type I) oligosaccharides containing glucosamine, mannose, galactose and fucose, as well as high mannose (type II) oligosaccharides which lack galactose and fucose (16). For the A/WSN strain, HA_1 was shown to have both type I and type II oligosaccharides whereas HA_2 possessed only type I oligosaccharides (16,20).

Our laboratory is interested in determining the number and type of glycosylation sites in HA and developing a method to compare glycoproteins when only small amounts of material can be obtained. To fulfil these objectives we have analyzed the tryptic glycopeptides of A/WSN influenza HA virus after extensive digestion with TPCK-trypsin, which produces more peptides containing a larger number of amino acid residues than pronase treatment (21,22). The tryptic glycopeptides were fractionated on DE52 cellulose (Whatman Inc.) followed by further fractionation on Bio-gel P-6. These procedures are laborious and application of this method to characterization of other viral

glycoproteins is often impossible because of the limited amount of material that can be obtained. Therefore, to demonstrate the applicability of reverse phase hplc to characterization of the glycosylation sites of glycoproteins, ^{3}H-glucosamine labeled HA was digested with TPCK-trypsin and prepared for chromatography as described in Materials and Methods. The HA tryptic glycopeptides were dissolved in 0.1% H_3PO_4 and applied to a C_{18} μBondapack column. The tryptic glycopeptides were eluted from the column by a gradient containing increasing amounts of acetonitrile from 0 to 36%. Under these conditions a total of about eight glycopeptide peaks were resolved as previously described (15,21). We felt resolution would be improved by altering the hplc techniques. Previously we had noted that LiChrosorb RP-18 columns had a longer retention time and it was felt that this property might aid in the resolution of tryptic glycopeptides with low hydrophobic properties. HA glycoprotein labeled with ^{3}H-glucosamine was prepared as described in Materials and Methods. The ^{3}H-glucosamine labeled tryptic glycopeptides from HA were dissolved in 0.1% H_3PO_4 and applied to a RP-18 column. The glycopeptides were eluted by a 0 to 40% acetonitrile gradient (Fig. 1A). As can be seen from this profile a major glycopeptide eluting between fraction 38 and 50 was clearly resolved from the complex of tryptic glycopeptides that eluted at higher acetonitrile concentration. This major tryptic glycopeptide appears to be highly ionic as demonstrated by its selective binding in different ion pairing agents. When HA tryptic glycopeptides were dissolved in formic acid and applied to a C_{18} μBondapack column and eluted with 0 to 40% acetonitrile gradient the major glycopeptide eluted in the void volume of the column.

As mentioned above, HA may exist as two cleavage products, HA_1 and HA_2. HA_2 has a greater electrophoretic mobility than HA_1 and it has been shown to possess a single type I glycosylation site (16). Therefore, we prepared ^{3}H-glucosamine labeled HA_2,

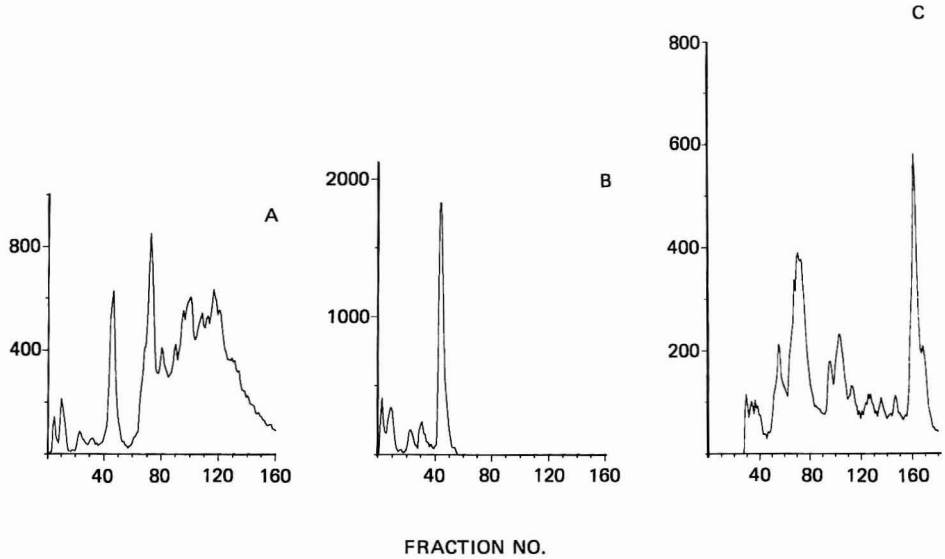

Figure 1. Chromatograms of (a) HA tryptic glycopeptides, (b) HA_2 tryptic glycopeptides and (c) HA_1 tryptic glycopeptides. Chromatographic conditions for (a) and (b): The labeled tryptic glycopeptides were applied to a RP-18 ODS column in 0.1% H_3PO_4, pH 2.85, and were eluted from the column by a linear gradient increasing in acetonitrile concentration to 40%. The sample was applied at 0% acetonotrile and allowed to run for 10 minutes before beginning the gradient. The flow rate was 2.0 ml/min. One minute fractions were collected and the radioactivity was determined by liquid scintillation counting. Chromatographic conditions for (c) are the same except the flow rate was 1.0 ml/min and 20 minutes after injection the gradient began at 12.5% acetonitrile concentration to final concentration of 35%.

digested it with TPCK-trypsin, and chromatographed it on an RP-18 column under the same conditions used for the elution of HA tryptic glycopeptides (Fig. 1B). As can be seen from Fig. 1B, the elution profile of the HA_2 tryptic glycopeptides is nearly identical to that of the first part of the HA elution profile. The major difference is an increased proportion of the material which elutes at an acetonitrile concentration of ~8%. The minor

tryptic glycopeptides in this profile may reflect slight charge differences in the amino acid portion of the tryptic glycopeptides or altered charge in the oligosaccharide part since complex type I oligosaccharides are often sulfated (16,23).

As shown in Fig. 1A, only tryptic glycopeptides associated with HA_2 were resolved under the gradient conditions used. To increase the resolution of the HA_1 tryptic glycopeptides the gradient conditions were altered. The glycopeptides were dissolved in phosphoric acid and applied to an RP-18 column, and eluted by a 12.5 to 35% acetonitrile gradient, 1 ml fractions were collected. The elution profile of the HA_1 tryptic glycopeptides is shown in Fig. 1C. As can be seen from this profile, the complex of tryptic glycopeptides that were unresolved by the conditions used for chromatography of HA appear to be resolved into five or six major tryptic glycopeptide constituents. Taken together, the results of these analyses suggest that the HA of the A/WSN influenza virus strain has six or seven glycosylation sites. These results are similar to an analysis carried out using ion exchange and open column methods that required weeks of labor. The analysis of the three glycoproteins can be accomplished in less than 12 hours with approximately a ten fold reduction in starting material. In addition, this method can be used for preparative separation of the tryptic glycopeptides for further biochemical and biological analyses.

To demonstrate further the general applicability of this method to the comparison of minute amounts of viral glycoproteins the envelope precursor proteins of MCF-247 MuLV were analyzed. Precursor proteins ($Pr75^{env}$) grown in two different cells were labeled with ^3H-mannose and prepared as described in Materials and Methods. Mink/MCF-247 and SC-1/MCF-247 $Pr75^{env}$ tryptic glycopeptides were dissolved in phosphoric acid and chromatographed on a C_{18} μBondapack column. The tryptic glycopeptides were eluted by a 0 to 36% acetonitrile gradient. A total of 160

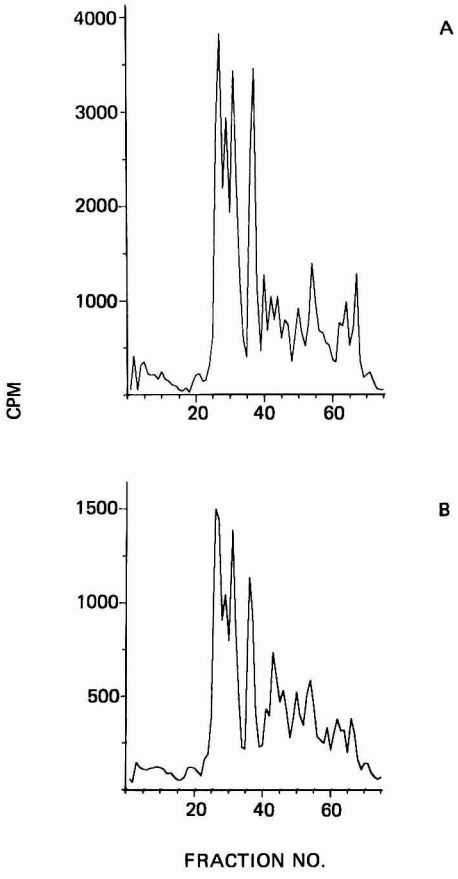

Figure 2. Chromatograms of (a) Mink/MCF-247 and (b) SC-1/MCF-247 envelope precursor tryptic glycopeptides. Chromatographic conditions were the same as described for Figure 1a and 1b except the column was a C_{18} μBondapak.

fractions were collected but all of the tryptic glycopeptides eluted at an acetonitrile concentration of less than 25% (Fraction 75). As can be seen from Fig. 2A (mink/MCF-247) and Fig. 2B (SC-1/MCF-247) the elution profiles are very similar which allows us to conclude that the precursors are identical (18). These analyses were performed on material immunoprecipitated from cell

lysates and separated by polyacrylamide gel electrophoresis. Such analyses would be nearly impossible using conventional ion exchange and open column methods due to the small amounts of material available.

In conclusion, the results described in this procedure for tryptic glycopeptides are comparable to previous work done by this laboratory (22) and other (23,24) for tryptic peptide mapping, and, as with the peptide mapping procedures, offers distinct advantages over open column conventional chromatographic techniques.

ACKNOWLEDGEMENTS

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HIGH PERFORMANCE CHROMATOGRAPHY OF AMINO ACIDS, PEPTIDES AND PROTEINS XXIII·PEPTIDE MAPPING BY HYDROPHILIC ION-PAIRED, REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE CHARACTERISATION OF THE TRYPTIC DIGEST OF HAEMOGLOBIN VARIANTS[1]

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ABSTRACT

The application of high performance liquid chromatography on reversed-phase columns for the tryptic mapping of haemoglobin variants is reported. The effect of flow rate and gradient shape on resolution and column efficiencies has been examined using acetonitrile-water-orthophosphoric acid elution systems. With these conditions it is possible to recognise sequence changes for variant haemoglobins including HbC, HbS, HbE, HbJ Cambridge and HbD Punjab.

INTRODUCTION

Rapid, sensitive, reproducible peptide mapping is now possible by means of high performance liquid chromatography (HPLC). A number of papers have demonstrated the use of ion-pair, reversed-phase HPLC to separate complex peptide mixtures generated by tryptic digestion of protein samples (1-6).

In order to fully assess the potential of HPLC for peptide mapping a well characterised protein was required with both sequence and structure/function data available. Perhaps the most widely investigated protein to date is haemoglobin, the structural characterisation of which has revealed a large number of functional and non-functional variants (7-10).

A number of well chraracterised haemoglobin variants in which the precise amino acid substitutions had been identified by classical paper fingerprinting and amino acid analysis techniques, were available for study by HPLC. In preliminary studies with the tryptic digests of a variety of haemoglobin variants, we demonstrated (11, 12) that HPLC techniques could be used to distinguish obvious differences between the tryptic peptide maps of HbA and the variants examined. The profiles could be obtained using microgram quantities of the digest, within 30 minutes of the initial injection and with excellent reproducibility.

A number of publications have subsequently confirmed the potential of similar techniques for screening and identifying haemoglobin variants (12-16).

In this paper we present further improvements in this HPLC approach utilising reversed-phase separations based on the hydrophilic ion pairing reagent, phosphoric acid. This chromatographic system was found to be capable of resolving variants which differ from HbA by a single amino acid substitution in one of the chains. Identification of the separated

mutant peptides was readily made by amino acid analysis of the recovered peaks. Excellent reproducibility and resolution have been obtained using this approach.

In addition, the flexibility of the chromatographic system allows easy and rapid manipulation of the mobile phase conditions to maximise resolution in areas of interest in the profile.

EXPERIMENTAL

Apparatus

A Waters Assoc. (Milford, Mass., U.S.A.) HPLC system was used. This consisted of two M6000A solvent delivery units, an M660 solvent programmer and a U6K universal liquid chromatograph injector coupled to two M450 variable wavelength UV spectrophotometers (Waters Assoc.) in series and an Omniscribe two-channel chart recorder (Houston Instruments, Austin, Texas, U.S.A.). μBondapak C_{18} columns (10μm, 30 cm x 4 mm I.D.) purchased from Waters Assoc. were used for all analyses. Sample injections were made using a Gastight 1010 W syringe (Hamilton).

Solvents were filtered using a Pyrex filter holder (Millipore, Bedford, Mass., U.S.A.), while peptide samples were filtered using a Swinney Filter (Millipore). Millipore HA grade, 0.45μm filters were used for aqueous solvent and sample preparation.

Chemicals

Water was glass-distilled. Acetonitrile, supplied by Fisher Scientific, was further purified by the method of Walter and Ramaley (17). Orthophosphoric acid was from May and Baker (Dagenham, Great Britain).

Sample Preparation

All the tryptic digest samples used in the analyses were prepared by standard methods (9, 10), freeze dried and stored in

a freezer until required. Samples were made up in 0.1% phosphoric acid prior to use, at concentrations of 10mg/ml.

Method

The flow rate used was 1.5, 1.7 or 3.0 ml/min. All chromatography was carried out at room temperature (ca. 22°C). Sample size varied from 50 - 250 µl. Detection was at 230nm or 254nm. The aqueous component of the solvent was degassed by vacuum aspiration for at least 30 minutes, while the acetonitrile-water mix was degassed for 0.5 minutes prior to use. Orthophosphoric acid was added to the mobile phase at a concentration of 0.1%.

RESULTS AND DISCUSSION

Figure 1 shows three chromatographic profiles developed under different separation conditions of flow rate and column length. Each chromatogram is of a tryptic digest of HbA (see Methods section). A 30 minute linear gradient of 0.1% phosphoric acid to acetonitrile - 0.1% phosphoric acid (1:1) eluted all the peptides present in the mixture within 30 minutes. It is obvious that using two columns in series has markedly improved the resolution of the constituent peptides (compare Figure 1A with 1C). Comparing Figures 1B and 1C shows that resolution is in general improved at lower flow rates, although a decrease in flow rate may also require a corresponding increase in the length of the gradient so that the same amount of mobile phase is used to develop a critical separation, i.e., the rate of change of the organic solvent modifier (%/min/cm^{-3}) is held effectively constant.

Once an area of difference has been observed between the normal and mutant haemoglobin peptide map it is then necessary to maximise resolution in this area in order to facilitate the analysis and the subsequent identification of the variant pep-

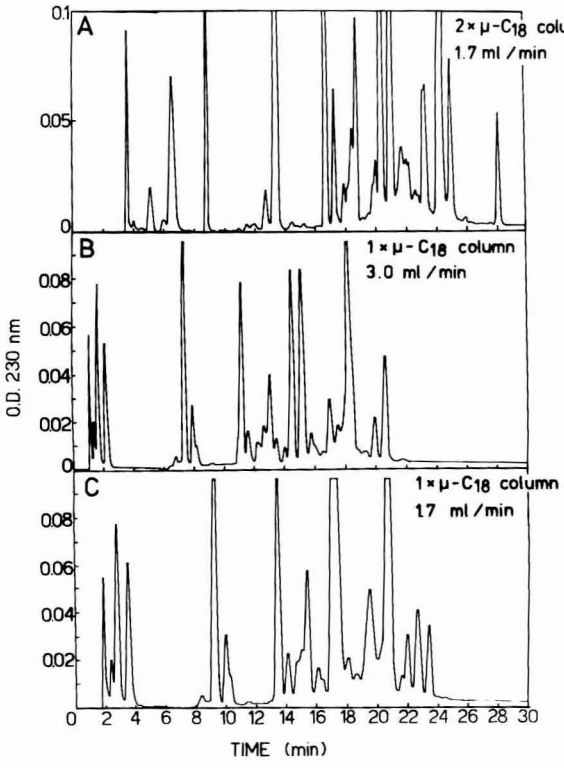

FIGURE 1

The HPLC elution profiles of a tryptic digest of HbA. Chromatographic conditions: in each case a 30 minute linear gradient was used with solvent A, 0.1% phosphoric acid, pH 2.2, and solvent B acetonitrile - 0.1% phosphoric acid, pH 2.2. (A) two μBondapak C_{18} columns connected in series with a flow rate of 1.7ml/min, (B) one μBondapak C_{18} column with a flow rate of 3.0ml/min and (C) one μBondapak C_{18} column with a flow rate of 1.7ml/min.

tide(s). One simple method for doing this, without altering the mobile phase conditions per se, is to manipulate the gradient shape. Figure 2 shows three markedly different elution profiles obtained for the tryptic digest of HbA using the same

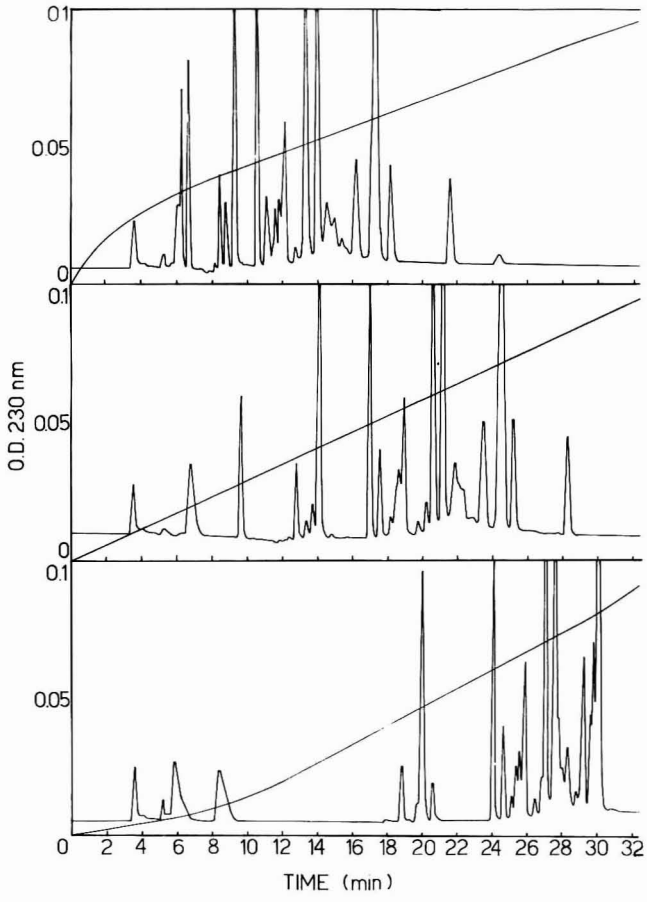

FIGURE 2

The elution profiles obtained for the tryptic digest peptides of HbA developed using three distinct gradient shapes, numbers 3, 6, and 8 on the M660 solvent programmer. In each case two μBondapak C_{18} columns were connected in series, a flow rate of 1.7ml/min was used, with the mobile phase compositions as in Figure 1.

conditions as in Figure 1A but altering the shape of the gradient. In this example the linear gradient, shown in the middle elution profile, gives optimal separation. Similar conclusions regarding the optimal gradient for acetonitrile - water systems have also been reached by Schoenmakers et al (18).

For unambiguous identification of peaks obtained within an HPLC peptide map the peaks must be collected and subjected to amino acid analysis and, if possible, other chemical procedures such as mass spectrometry. However preliminary rapid identifications can be made by simultaneous detection of the eluted peptides at two or more wavelengths, using variable wavelength UV spectrophotometry. Figure 3 shows the detection of HbA tryptic peptides at 230nm and 254nm. Only peptides containing amino acids with aromatic side chains will be observed at the higher wavelength. In addition UV detection at 210nm can be used to gain a significant increase in sensitivity (1,2,11,12). The use of low wavelength detection, however, requires the use of highly purified aqueous and organic solvents in the mobile phase. To guard against the presence of artifactual solvent peaks in the peptide map, blank gradients were always run following any alteration in chromatographic conditions.

Once suitable chromatographic conditions were established, the tryptic digests of a series of mutant haemoglobins were analysed and compared to normal adult HbA. Figure 4 shows one such comparison, between HbA and HbS. The clear difference between the chromatograms is shown by the arrows in Figure 4. A peak with a retention time (R_t) of 13.6 minutes in the Haemoglobin A profile is absent in the Haemoglobin S profile and is replaced by a peak with R_t 14.6 minutes. In order to establish that the different peak did in fact correspond to the known structure of the variant peptide, the peak was collected, freeze-dried, subjected to acid hydrolysis (6M HCl, 24 hours) and amino acid analysis. HbS has a valine residue substituted

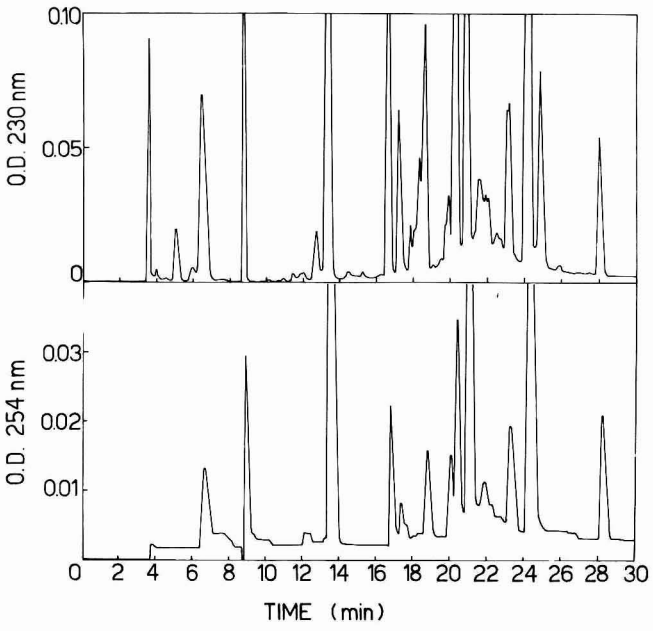

FIGURE 3

The elution profile of the HbA tryptic digest peptides using chromatographic conditions as in Figure 1A with UV detection at 230 nm and 254 nm. The chromatograms were recorded simultaneously using two variable wavelength spectrophotometers connected in series by minimum dead-volume connecting tubing.

for a glutamic acid at position 6 on the β chain of HbA. Such a substitution would make the octapeptide Val-His-Leu-Thr-Pro-Glu-Glu-Lys, (β^A chain, 1-8), in which it occurs more non-polar. This would increase the interaction between the peptide and the hydrophobic stationary phase, hence increasing the observed retention time. Amino acid analyses of the two peaks in question confirmed that the composition of the R_t 14.6 peak was in agreement with the known mutant octapeptide of haemoglobin S. The analysis results obtained were as follows. For the octa-

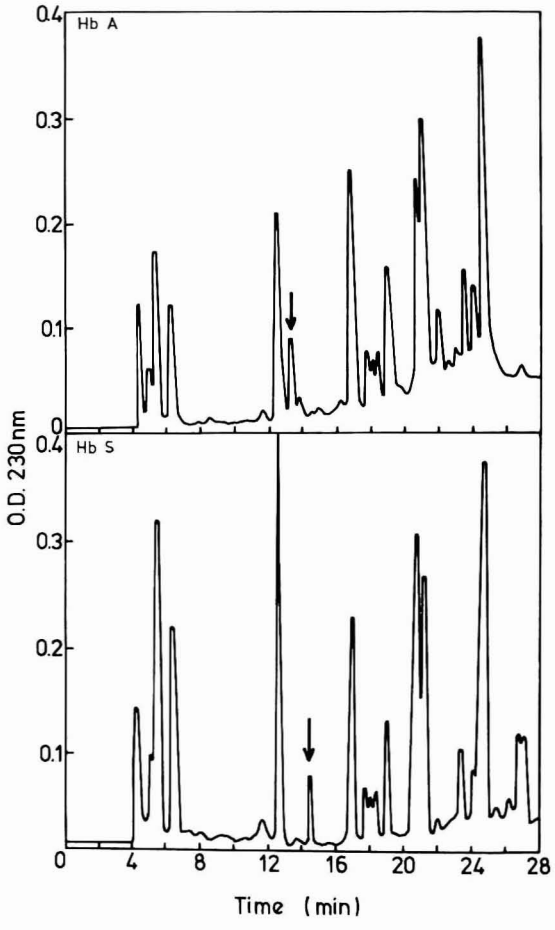

FIGURE 4

The comparative elution profiles of the tryptic digests of HbA and HbS, run using the same chromatographic conditions as in Figure 1A with the exception of the flow rate, which in both cases was 1.5ml/min. The arrow refers to the replacement (β6 Glu→Val) octapeptides.

peptide from haemoglobin A: $Thr_{0.98(1)}Glu_{2.01(2)}Pro_{1.0(1)}Val_{1.1(1.0)}Leu_{1.1(1)}His_{1.0(1)}Lys_{1.0(1)}$. For the octapeptide collected from the haemoglobin S sample: $Thr_{1.02(1)}Glu_{1.0(1.0)}Pro_{1.05(1)}Val_{2.03(2.0)}Leu_{1.15(1.0)}His_{1.08(1.0)}Lys_{1.0(1.0)}$. In general, it has been our experience that the determination of the amino acid composition of peptides, recovered from reversed-phase HPLC under the above elution conditions, is straightforward. Alternatively, the fractionated peptides can be subjected to sequence analysis by standard techniques.

This example represents a relatively major change in the polarity of the variant peptide. However, the identification of more subtle changes in peptide structure are possible by similar means, particularly when the potential flexibility of the chromatographic conditions is taken into account. It is now possible, when investigating a selected area of difference within a chromatogram, to manipulate the elution conditions such as gradient shape, gradient time, pH, type of ion pairing reagent used or temperature, and thus greatly improve resolution of overlapping peaks in that area. In order to demonstrate that these procedures were generally applicable, a range of variant haemoglobins were analysed and compared to HbA. Figure 5 shows a selection of these comparisons. The arrow on each profile indicates the position of a peptide which does not occur in the HbA analysis. Circled letters indicate peaks missing in corresponding chromatograms. The amino acid substitutions occurring in each variant peptide are also indicated.

With the conditions used in this study, excellent reproducibility was observed between analyses of the same tryptic digestion sample run at times differing by up to 6 months. As a cautionary point, however, care must be exercised in accurately reproducing these chromatographic conditions between runs since a change in any of the conditions e.g. pH, can markedly alter a profile. In general, these precautions present

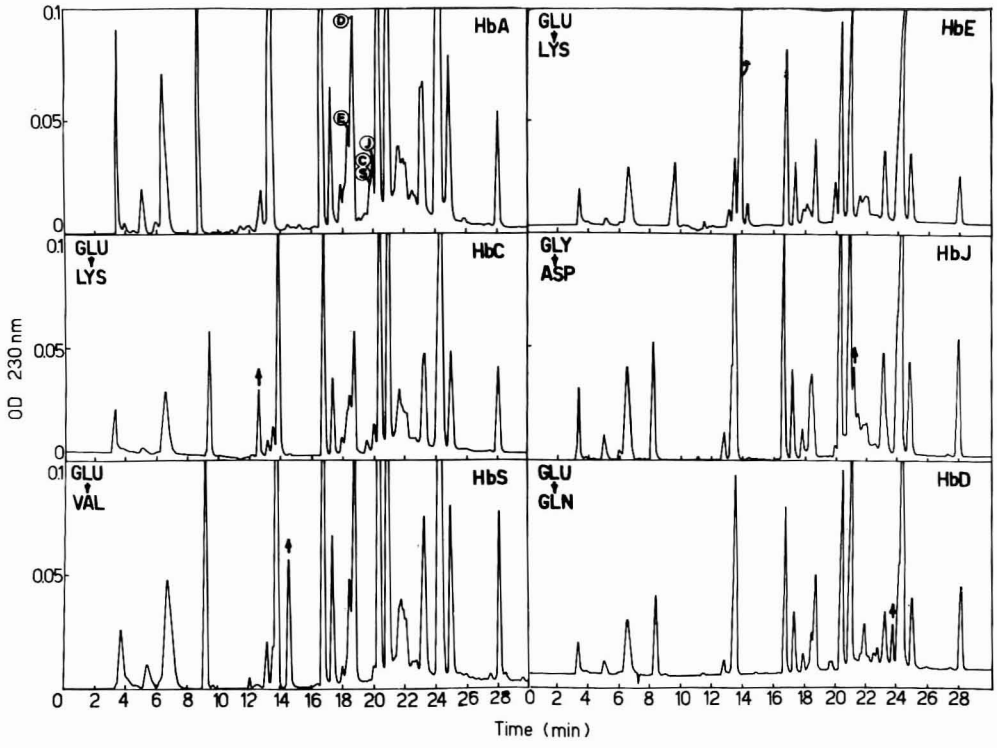

FIGURE 5

The comparative elution profiles of the tryptic digest profiles of HbA and a range of well characterised variants: HbC (β6 Glu→Lys), HbS (β6 Glu→Val), HbE (β26, Glu→Lys), HbJ Cambridge (β69 Gly→Asp), HbD Punjab (β121 Glu→Gln). Chromatographic conditions were in each case identical to those in Figure 1A. Two variants (HbC and HbE) would be expected to give an additional tryptic fragment due to the presence of an additional lysine residue. In both cases, the region which occurs at 13 to 15 minutes in the gradient analysis shows an additional peak, although the major difference in each chromatogram is indicated by an arrow.

no experimental difficulty. We have, however, noted some changes occurring in the chromatograms of haemoglobin digests obtained when different tryptic digestion conditions are used. As it is often difficult to exactly reproduce an enzymatic hydrolysis of a protein, slight variations in the extent of digestion can result in altered relative peak heights and positions. If the tryptic digestion and subsequent chromatographic separation are carried out using carefully standardised procedures, any variation should be limited to small changes in peak heights and not alter the observed order or number of peptides.

CONCLUSIONS

This publication has shown that peptide mapping of tryptic digests of haemoglobin variants is a rapid, sensitive, reproducible and extremely flexible technique.

This conclusion combined with other recent publications (12-16) suggests that reversed-phase HPLC will become an important procedure in the screening for haemoglobin variants. The technique should be particularly useful for examining variants exhibiting minor sequence differences, e.g. Gly→Ala, which may be amenable to separation by reversed-phase HPLC, but not as readily by other current chromatographic procedures. In addition the use of the hydrophilic ion pairing reagent, phosphoric acid combines excellent peak shape with low wavelength UV detection. This should be particularly useful for the analysis of haemoglobin variants which are only available in microgram amounts.

Similar peptide mapping techniques also have many potential applications for the rapid screening of homology similarities of variant proteins, a number of which are presently under investigation.

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FOOTNOTES

1. Part XXII : ref 19.
2. To whom correspondance should be addressed.

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HIGH-SPEED GEL FILTRATION OF POLYPEPTIDES IN SOME DENATURANTS

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ABSTRACT

The separation and analysis of proteins and polypeptides by use of a silica-based gel packing, G3000SW, for high-speed gel filtration are investigated. The peaks of bovine serum albumin, pepsin, trypsinogen, myoglobin and cytochrome c were completely separated in the presence of 0.2% SDS and 0.2 M sodium phosphate buffer (pH 7.0). The elution positions of native proteins, polypeptides in 8 M urea and polypeptide-SDS complexes were influenced by the concentration of sodium phosphate in eluents, though those of polypeptides in 6 M guanidine hydrochloride were little. These facts suggest the presence of the electrostatic interactions between negatively charged gel surfaces of the packing and polypeptides. Taking into account of the interactions, it is shown that the high-speed gel filtration by use of this column is available to the rapid estimation of molecular weight of polypeptides in both systems of SDS and 6 M guanidine hydrochloride.

INTRODUCTION

In recent year, high-speed liquid chromatography has been frequently applied for the separation of proteins and polypeptides. In particular, the appearance of porous glass and silica has advanced the application of high-speed gel filtration to the field of protein separation. The packing, G3000SW, for high-speed gel filtration developed by Toyo Soda Manufacturing Company (Tokyo) is a particulate silica gel covalently bonded with hydrophilic compounds and its utility for the separation of proteins has been appreciated by some authors (1-6). In previous paper, we

reported on the separation of polypeptide-SDS complexes by use of this column packing (2) and Ui showed also that this packing was applicable to rapid estimation of molecular weight of protein polypeptides in 6 M guanidine hydrochloride (3).

In this paper, we report on the separation of polypeptides in denaturants such as SDS, urea and guanidine hydrochloride. Taking into consideration of the electrostatic interaction between gel surfaces and polypeptides, the molecular weight estimation of polypeptides in some denaturants is also discussed.

MATERIALS

Bovine serum albumin, catalase, aldolase, carboxypeptidase A, chymotrypsinogen A, trypsinogen, α-chymotrypsin, trypsin, β-lactoglobulin, myoglobin, ribonuclease A, trypsin inhibitor (lima bean), glucagon and bacitracin were obtained from Sigma; ovomucoid, cytochrome c, aprotinin and insulin B and A chains (S-carboxymethylated) from Boehlinger Mannheim; ovalbumin from ICN Pharmaceuticals and pepsin from Nutrition Biochemicals. Guanidine hydrochloride and urea were purchased as specially prepared reagents from Nakarai Chemicals (Kyoto) and sodium dodecyl sulfate (SDS) was from Wako Chemicals (Osaka) as a reagent for biochemical research. These reagents were used without further purification. All other chemicals were of pure grade.

METHODS

Preparation of Cyanogen Bromide Fragments

Some polypeptides and oligopeptides were prepared by cyanogen bromide (CNBr) treatments of bovine serum albumin, aldolase, carboxypeptidase A, pepsin, chymotrypsinogen A, α-chymotrypsin, trypsin, myoglobin, lysozyme and cytochrome c. Each protein dissolved in 70% formic acid to a concentration of 10 mg/ml was reacted with 50 molar quantities of CNBr for 20 hours at room temperature, then 10 volumes of distilled water was added and the solution was lyophilized. The values of molecular weight of CNBr fragments are listed in TABLE.

TABLE
Cyanogen Bromide Fragments Used in Gel Filtration Experiments

CNBr fragment		Molecular weight	Reference
Serum albumin	CNBr I	9,860	(7)
	CNBr III	29,520	
Aldolase	CNBr I	17,710	(7)
	CNBr II	7,370	
	CNBr III	2,040	
	CNBr IV	11,900	
Carboxypeptidase A	CNBr I	2,660	(7)
	CNBr II	9,230	
	CNBr III	22,120	
Pepsin	CNBr I	8,630	(7)
	CNBr II	12,640	
Chymotrypsinogen A	CNBr I	19,180	(8)
	CNBr II	1,060	
	CNBr III	5,560	
Trypsinogen	CNBr I	9,900	(8)
α-Chymotrypsin	B chain	13,920	(8)
	CNBr I	3,580	
	CNBr III	5,560	
Trypsin	CNBr I	9,100	(8)
Myoglobin	CNBr I	6,230	(7)
	CNBr III	2,510	
Lysozyme	CNBr I	1,270	(7)
	CNBr II	10,160	
	CNBr III	2,860	
Cytochrome c	CNBr I	7,030	(7)
	CNBr II	1,780	
	CNBr III	2,780	

CNBr fragments were prepared as described in METHODS. Each fragment was numbered in the order of amino acid sequence of the protein from N-terminal. Only the fragments which observed as chromatographically distinguishable peaks were listed. The values of molecular weight of fragments were calculated from amino acid sequence data of proteins appeared in references.

Preparation of the Sample Solution for High-Speed Gel Filtration

The samples of proteins, polypeptides and CNBr fragments were dissolved in the buffers described in figure legends at the concentration of 3 to 5 mg/ml. For the chromatographic experiments in denaturants, proteins and polypeptides possessing disulfide bonds were reduced by 0.3% 2-mercaptoethanol. Since no significant differnces in chromatographic behaviours between poly-

peptides treated with 0.3% 2-mercaptoethanol and S-carboxymethylated polypeptides were detected in SDS for some dozen species of proteins, the samples were used without modification of SH groups in later experiments.

High-Speed Gel Filtration

High-speed gel filtration was carried out using a Model HLC 803 A liquid chromatograph (Toyo Soda) connected to a TSK-GEL G3000SW column (7.5 x 600 mm) with a pre-column (7.5 x 75 mm). The samples (20 µl) were injected in the column which was previously equilibrated with each eluent and eluted at the flow rate of 0.5 to 1.0 ml/min. Ultraviolet absorption (apparatus: Schoeffer Spectro Flow Monitor SF770) at 220 or 280 nm was employed for the detection of elution peaks of the samples. The distribution coefficients, K_d, were calculated from the equation of $K_d=(V_e-V_0)/(V_i-V_0)$, where V_e, V_0 and V_i were the elution volumes of the sample, Blue Dextran 2000 and 2-mercaptoethanol, respectively.

RESULTS

Chromatographic Behaviours of Native Proteins in the Absence of the Denaturants

The values of distribution coefficients, K_d, for some native proteins were plotted against logarithm of sodium phosphate concentrations as shown in Fig. 1. The salt concentration dependence of K_d values was different between acidic and basic proteins. In acidic proteins such as serum albumin, ovalbumin, β-lactoglobulin and insulin A chain, the values of K_d increased with the rise of the concentration of sodium phosphate. Basic proteins such as chymotrypsinogen A, lysozyme and cytochrome c were adsorbed with the resin and symmetries of the elution peaks were lost in low salt concentration conditions of eluents. The K_d value of myoglobin, a neutral protein, was unaltered except in extensively low salt concentration. It is obvious that the low concentration conditions of sodium phosphate below 10 mM are unsuitable for the size separation of proteins. High concentration conditions of

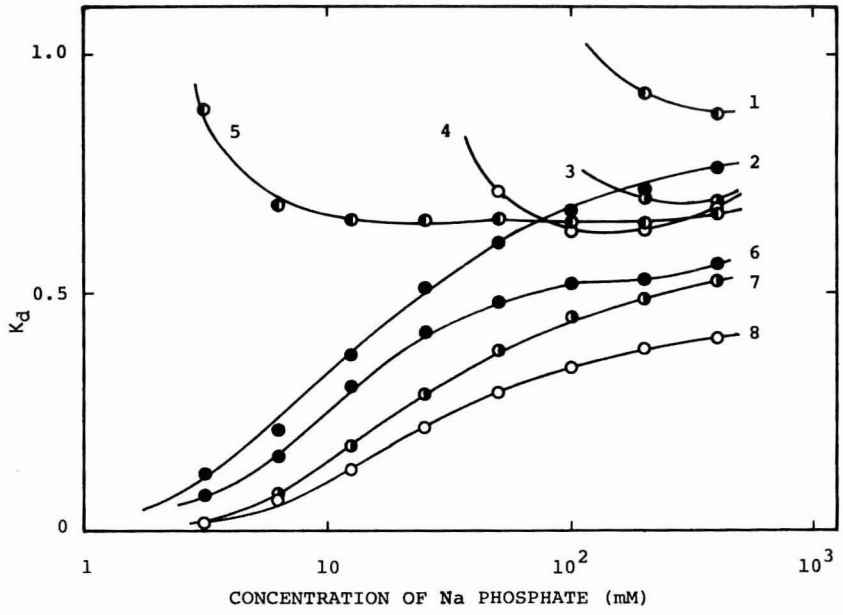

FIGURE 1 Relationships between distribution coefficients, K_d, of native proteins and sodium phosphate concentrations in the eluents. Protein samples (20 µl) dissolved in 0.05 M sodium phosphate buffer (pH 7.0) were charged in the column and eluted under various concentration conditions of sodium phosphate buffer (pH 7.0). The flow rate was 1.0 ml/min. Proteins are: 1, lysozyme; 2, insulin A chain; 3, cytochrome c; 4, chymotrypsinogen A; 5, myoglobin; 6, β-lactoglobulin; 7, ovalbumin; 8, serum albumin.

sodium phosphate such as 0.2 M may be required for the elution of highly basic proteins such as lysozyme and cytochrome c. However, the concentration conditions above 0.4 M tended to make the elution peaks broadened.

8 M Urea System

Figure 2 shows the relationships between log molecular weight and K_d of polypeptides reduced on disulfide bonds in 8 M urea. The elution condition of 0.2 M sodium phosphate gave a relatively good linearity. The plots for both highly acidic and basic polypeptides, however, slightly deviated from the line and the extent of the deviation was enhanced in lower salt concen-

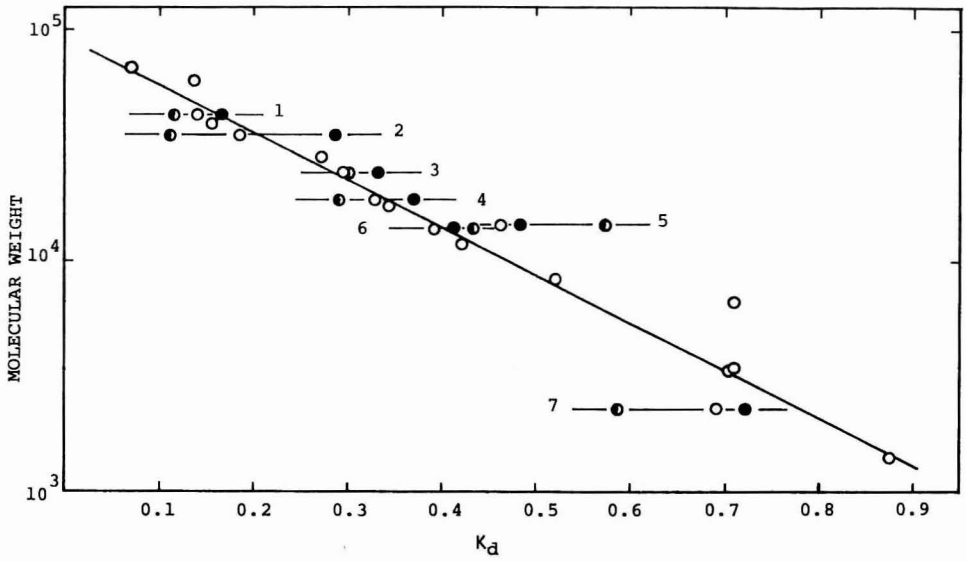

FIGURE 2 Plots for log molecular weight *versus* K_d of polypeptides in 8 M urea at various concentrations of sodium phosphate buffer (pH 7.0). Proteins dissolved in 8 M urea and 0.05 M sodium phosphate buffer (pH 7.0) containing 0.3% 2-mercaptoethanol were charged in the column and eluted at a flow rate of 1.0 ml/min. Proteins are: 1, ovalbumin; 2, pepsin; 3, trypsinogen; 4, β-lactoglobulin; 5, lysozyme; 6, ribonuclease; 7, insulin A chain. The concentrations of sodium phosphate buffer (pH 7.0) in the eluents were 0.05 M (◐), 0.20 M (○) and 0.40 M (●).

tration. At higher sodium phosphate concentration such as 0.4 M, K_d values were larger than those expected on the basis of the electric properties of polypeptides and the resolution of the peaks was lost.

SDS System

Semi-logarithmic plots of molecular weight and K_d of polypeptides in 0.1% SDS are shown in Fig. 3. The plots of log molecular weight *versus* K_d of polypeptides were significantly influenced by the concentration of sodium phosphate as previously described (2) and there was the difference in the manner of the influence of salt concentration between polypeptides having the molecular weight values higher than about 15,000 daltons and

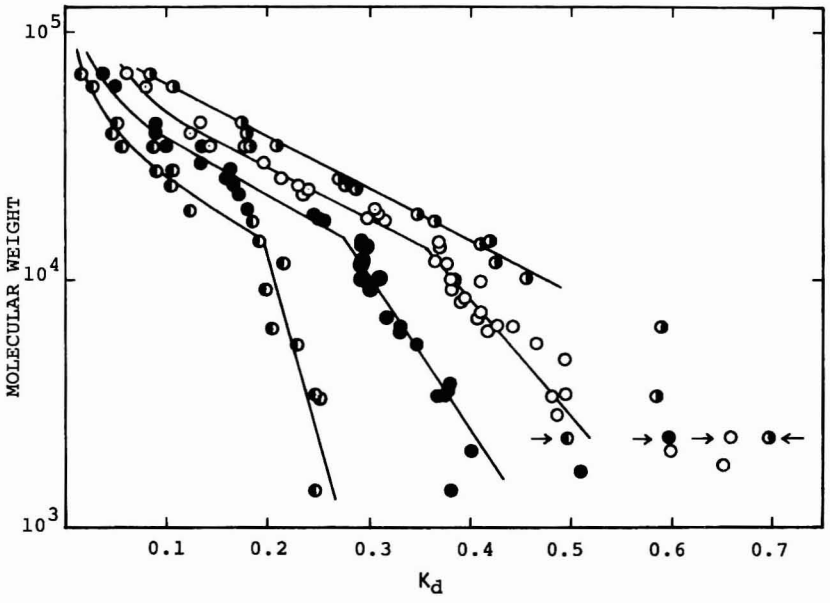

FIGURE 3 Sodium phosphate concentration dependence of the plots of log molecular weight *versus* K_d of polypeptides in SDS. The samples of proteins and polypeptides dissolved in 2% SDS and 0.05 M sodium phosphate buffer (pH 7.0) containing 0.3% 2-mercaptoethanol and incubated at 60°C for 1 hr were charged in the column and eluted at a flow rate of 1.0 ml/min. Sodium phosphate concentrations in the eluents illustrated were 0.025 M (◐), 0.05 M (●), 0.10 M (○) and 0.20 M (◑). The solid arrows represent the data for the sample of insulin A chain (S-carboxymethylated) which is probably a highly acidic oligopeptide. Only the points for insulin A chain were extremely deviated from the curves in relatively low sodium phosphate concentration.

those lower than about 15,000 daltons, *i.e.*, oligopeptides. Detailed examination for many speacies of polypeptides showed that the slopes of the plots did not change with the variation of salt concentration but only the values of K_d increased with the rise of the salt concentration. The increments of K_d values were practically suppressed at about 0.2 M and higher sodium phosphate concentration gave broadened peaks.

In oligopeptides, the slopes of the plots were steeper than those for polypeptides in low salt concentration. Thus the plots

had an inflection around about 15,000 daltons. The magnitudes of the slopes for oligopeptides approached those for polypeptides with the rise of sodium phosphate concentration. For oligopeptides, the concentration condition of 0.2 M sodium phosphate, however, gave broadened peaks and linearity of the plots was lost. In 0.2 M sodium phosphate, a higher SDS concentration than 0.1% was required for the maintenance of the linearity of the plots. The concentration condition of 0.2 M sodium phosphate containing 0.2% SDS was selected for the preparation of a calibulation curve. The plot of log molecular weight *versus* K_d of different fortyfive polypeptides and oligopeptides is shown in Fig. 4. The plot gave a good linearity, particulary for polypeptides and an

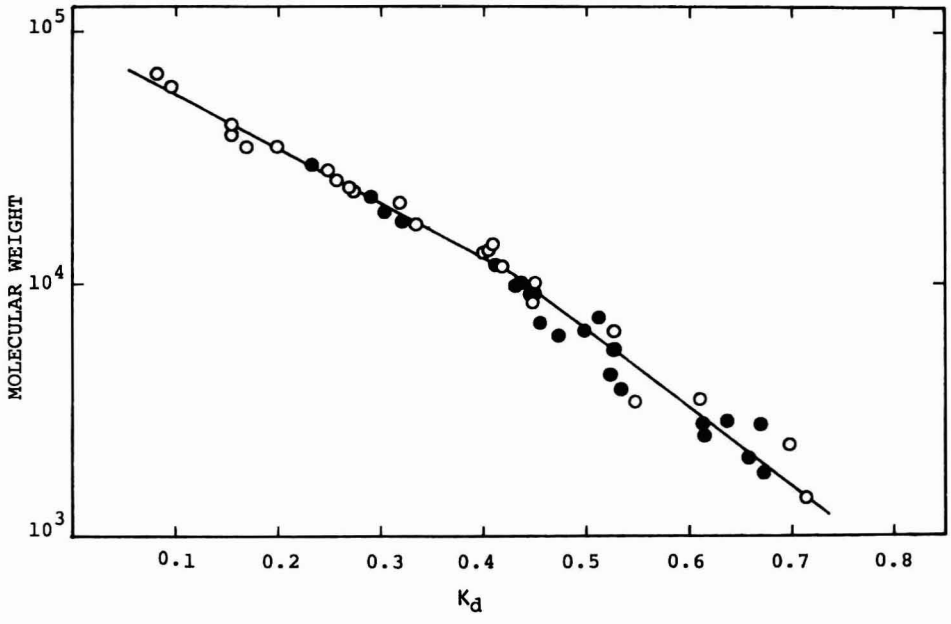

FIGURE 4 Plot of log molecular weight *versus* K_d of fortyfive polypeptides in 0.2% SDS and 0.2 M sodium phosphate buffer (pH 7.0). The polypeptide samples were incubated and charged in a similar manner as described in Fig. 3. Filled circles represent the data for CNBr fragments.

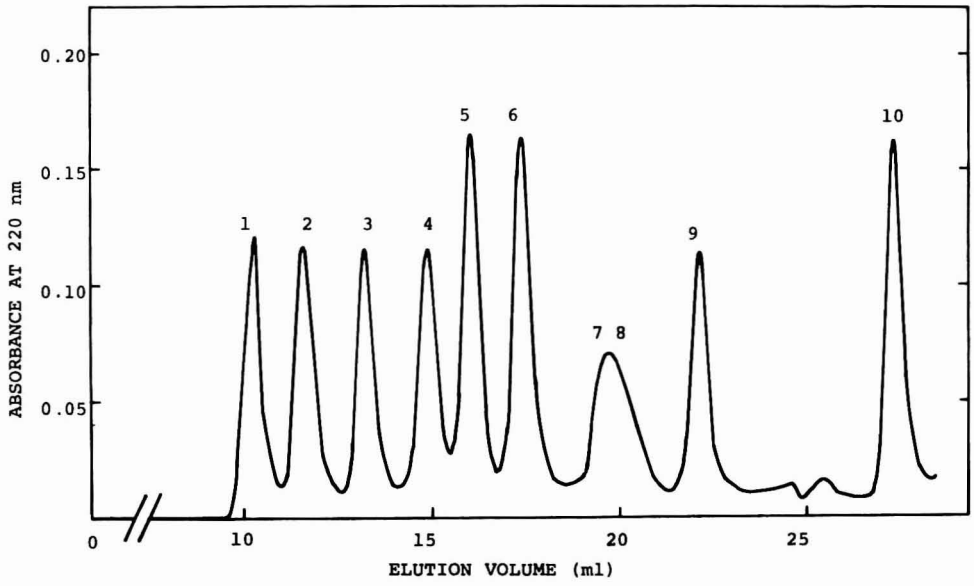

FIGURE 5 An elution profile of a mixture of polypeptides and oligopeptides from a G3000SW column. The elution was carried out in 0.2% SDS and 0.2 M sodium phosphate buffer (pH 7.0) and at a flow rate of 0.5 ml/min. Peaks are: 1, blue dextran; 2, serum albumin; 3, pepsin; 4, trypsinogen; 5, myoglobin; 6, cytochrome c; 7, aprotinin; 8, insulin B chain; 9, insulin A chain; 10, 2-mercaptoethanol. Aprotinin and insulin B chain were eluted as undistinguishable peaks under this condition. However, the peaks of both oligopeptides were completely separated under the condition of 0.1% SDS and 0.1 M sodium phosphate buffer (pH 7.0).

inflection on the curve appeared around 15,000 daltons was moderated.

The examples for the separation of a mixture of polypeptides and oligopeptides and CNBr cleavage products from rabbit muscle aldolase under the elution condition of 0.2 M sodium phosphate (pH 7.0) containing 0.2% SDS are shown in Fig. 5 and 6, respectively. The peaks of serum albumin, pepsin, trypsinogen, myoglobin, cytochrome c and insulin A chain were completely separated. Four fragments produced by CNBr treatment of aldolase were ob-

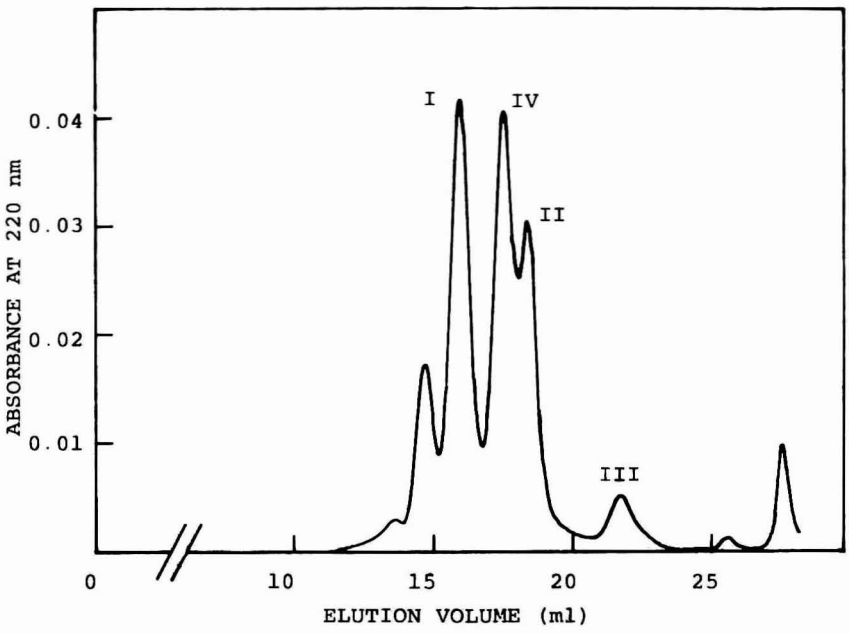

FIGURE 6 An elution profile of CNBr fragments from rabbit muscle aldolase in 0.2% SDS and 0.2 M sodium phosphate buffer (pH 7.0). The CNBr fragment sample was treated and charged in a similar manner as described in Fig. 3. The flow rate of elution was 1.0 ml/min. The values of molecular weight of CNBr fragments, I, II, III and IV are 17,710, 7,370, 2,040 and 11,900, respectively.

served as distinguishable peaks and the peak of CNBr III was completely separated from other fragments.

6 M Guanidine Hydrochloride System

Figure 7 shows the relationships between log molecular weight and K_d of polypepetides and oligopeptides in 6 M guanidine hydrochloride and 0.05 M sodium phosphate buffer (pH 7.0). A linearity was kept in the molecular weight range of 2,000 to 70,000 daltons. It was the important characteristic of 6 M guanidine hydrochloride system that the K_d values of polypeptides were little influenced by the concentration of sodium phosphate.

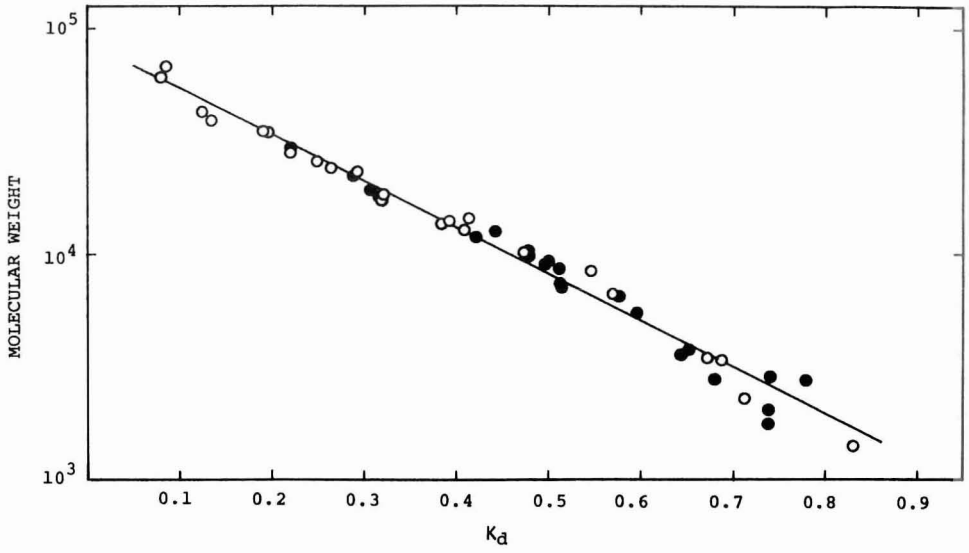

FIGURE 7 Plot of log molecular weight *versus* K_d of forty-five polypeptides and oligopeptides in 6 M guanidine hydrochloride and 0.05 M sodium phosphate buffer (pH 7.0). Charged samples were treated with 6 M guanidine hydrochloride and 0.05 M sodium phosphate buffer (pH 7.0) containing 0.3% 2-mercaptoethanol. The flow rate was 1.0 ml/min. Filled circles represent the data for CNBr fragments.

DISCUSSION

The chromatographic behaviours of native proteins in G3000SW column were influenced by the variation of sodium phosphate concentration. The K_d values of acidic proteins increased with the rise of sodium phosphate concentration. The basic proteins were adsorbed on gel surfaces in low sodium phosphate concentration. The polypeptides in 8 M urea were also similar in chromatographic behaviours to native proteins, but the effects of sodium phosphate concentrations were not so drastic as those to native proteins. The polypeptide-SDS complexes which had highly negative charges behaved in a similar manner as acidic proteins. The K_d values of polypeptides in 6 M guanidine hydrochloride, however, were little affected by the variation of sodium phosphate con-

centration. It seems that these phenomena are attributable to the interaction between polypeptides and negatively charged gel surfaces. That is to say, the electric diffuse double layers on gel surfaces and polypeptides will be compressed by high salt concentration in eluents and acidic polypeptides and polypeptide-SDS complexes may be consequently capable to penetrate into gel interior. The elution positions of basic polypeptides may be retarded by attractive interaction between positively charged polypeptides and gel surfaces. High concentration of electrolytes such as 6 M guanidine hydrochloride may virtually eliminate the electrostatic interaction between polypeptides and gel surfaces. However, high concentration of sodium phosphate above 0.4 M will not be recommended because it was observed for native proteins, polypeptides in 8 M urea and polypeptide-SDS complexes that such a high sodium phosphate concentration resulted in low resolution owing to the broadening of the peaks.

In order to systematically consider the chromatographic behaviours of native proteins and polypeptides in some denaturants, the G3000SW column may be necessary to be calibulated with the method based on the K_d values and Stokes radii of proteins and polypeptides. The method proposed by Ackers was used for this purpose (9-11). The relationships between Stokes radii, R_s, calculated from intrinsic viscosity data and $Erf^{-1}(1-K_d)$ of some proteins and polypeptides in various eluents are shown in Fig. 8. The concentration condition of 0.2 M sodium phosphate (pH 7.0) which was suitable for the separation of proteins and polypeptides was selected for the calibulation in spite of the presence or absence of the denaturants except for 6 M guanidine hydrochloride. The buffer concentration of 0.05 M sodium phosphate was used only for data in 6 M guanidine hydrochloride, since the elution positions of polypeptides in 6 M guanidine hydrochloride were independent on the sodium phosphate concentration. The plots for some native proteins and for polypeptides in 8 M urea and 6 M guanidine hydrochloride followed a common curve except for native lysozyme. The position of native lysozyme was deviated upward from

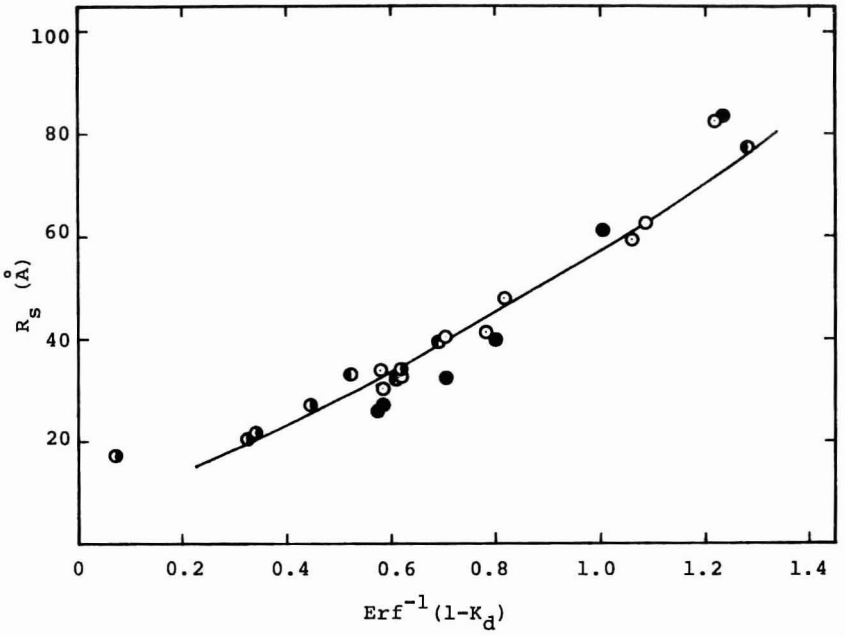

FIGURE 8 The relationships between R_s and $Erf^{-1}(1-K_d)$ of some polypeptides in various eluents. The eluents were: 0.20 M sodium phosphate (◑); 6 M guanidine hydrochloride and 0.05 M sodium phosphate (○); 8 M urea and 0.20 M sodium phosphate (◐); 0.2% SDS and 0.20 M sodium phosphate (●). The values of Stokes radii, R_s, were calculated from the intrinsic viscosity data according to the equation $[\eta]=2.5L/M(4\pi R_s^3/3)$, where L was Avogadro's constant, M was the molecular weight of polypeptide and $[\eta]$ was the intrinsic viscosity (12-14).

the curve. This fact may be due to the attractive interaction of positively charged lysozyme with gel surfaces. The plots for some polypeptide-SDS complexes which had highly negative charges substantially followed the common curve. The repulsive interaction between negatively charged polypeptide-SDS complexes and gel surfaces may be taken as negligible small in 0.2 M sodium phosphate.

When this column is tried appling for the estimation of molecular weight of polypeptides, it is necessary to be taken into account of the interaction between polypeptides and gel surfaces. There may be two ways available for this purpose; one is to make

the interaction negligible small with high electrolyte concentration such as 6 M guanidine hydrochloride, the other is to bury the intrinsic charges of the polypeptides by formation of polypeptide-detergent complexes. It had been shown with Ui that the former method was available for the rapid estimation of molecular weight of polypeptides (3). Our results examined for fortyfive polypeptides and oligopeptides also showed that the relationship between log molecular weight and K_d was linear in the range of about 2,000 to 70,000 daltons. For the latter method, the use of cationic detergent, cetyltrimethyl ammonium bromide, resulted in extremely poor resolution of the peaks. Therefore, an anionic detergent, SDS was selected. Under the condition of 0.2 M sodium phosphate buffer (pH 7.0) containing 0.2% SDS, the relationship between log molecular weight and K_d of polypeptides exhibited a good linearity in a molecular weight range of 15,000 to 70,000 daltons and the resolution of elution peaks of polypeptide-SDS complexes was satisfactory (Fig. 5). For oligopeptides less than about 15,000 daltons, on the other hand, the elution profiles and positions were affected by SDS concentration and the properties of oligopeptide-SDS complexes are so far unclear. Therefore, the application of G3000SW column to the molecular weight estimation of oligopeptides in SDS system may be accompanied by some dangers, though the plots of log molecular weight *versus* K_d of oligopeptides showed a rough linearity in the range of 2,000 to 15,000 daltons. For the molecular weight estimation of oligopeptides less than 15,000 daltons, 6 M guanidine hydrochloride system may be rather recommended.

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RAPID PURIFICATION OF THE UNSTABLE ENZYME CARBAMOYL PHOSPHATE SYNTHASE BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

Extracts of soluble proteins obtained from rat liver mitochondria by freeze-thawing and subsequent diafiltration were fractionated by HPLC on a I 250 protein column. The column was eluted either with 0.05 M phosphate buffer pH 6.85 or 0.1 M acetate buffer pH 7.15. Specific fractions obtained by elution with either phosphate or acetate buffer showed a 6.1-fold or 5.5-fold increase in the specific activity of Carbamoyl phosphate synthase when compared with that of crude mitochondrial preparations. The purification and the molecular weight of carbamoyl phosphate synthase were verified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

Introduction

Carbamoyl phosphate synthase-I (EC 2.7.2.5), which catalyses the first step in urea synthesis, is located in the matrix of hepatic mitochondria in ureotelic animals (1-5). The enzyme is extremely unstable in its unpurified state, apparently because of its susceptibility to proteolytic degradation (1,4). The rat liver enzyme has been identified with a single polypeptide chain which migrates as a single component with a molecular weight of 165,000 during sodium dodecyl sulphate (SDS)-polyacrylamide electrophoresis, but rapidly degrades, first to a component of molecular weight 155,000 and subsequently to even lower molecular

weight components during purification by conventional and time consuming methods (4).

During our investigations of this enzyme it became necessary to develop a simple, rapid method for its purification. The method, which is reported in this communication, involves extraction of soluble matrix proteins by freeze-thawing rat liver mitochondria in hypotonic buffer, the concentration of these proteins by diafiltration and fractionation by high pressure liquid chromatography (HPLC) on an I 250 column.

The combined procedures enable purification of carbamoyl phosphate synthase to near-homogeneity with high yield within 2 hours of preparing liver mitochondria. The purified enzyme migrates as a single polypeptide of molecular weight 166,000 during SDS-polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Preparation of Mitochondria

Mitochondria were isolated from random-bred Wistar rats weighing between 150 - 200 g essentially as described previously (6); the only modification involved the last two washes, which were carried out in a medium containing 250 mM-sucrose, 1 mM-4-(2-hydroxy-ethyl)-piperazine-ethanesulphonic acid (Hepes), 1 mM-EDTA, pH 7.4.

Preparation of Mitochondrial Matrix

Mitochondrial pellets were suspended in 10 mM Hepes buffer pH 7.4 at a concentration of 20 mg/ml. The suspension was frozen and thawed 3 times by alternate immersion into liquid N_2 and a waterbath at 37 ^{o}C, centrifuged for 10 min. at 10,000 g and the supernatant collected and filtered through a Millex-GS 0.22µm filter unit (Millipore Corporation, Bedford, Ma. U.S.A.) The filtrate which contained the soluble proteins of the mitochondrial matrix was then concentrated 10 times by dia-

filtration using a stirred Model 12 cell (Amicon Corp. Lexington, Mass. U.S.A.) containing a PM 30 (Amicon) membrane.

High Pressure Liquid Chromatography

HPLC was carried out with an ALC/GPC 204 Liquid chromatograph (Waters Associates, Milford, Mass. U.S.A.) using the absorbance detector with a 280 nm filter.

Up to 200 µl of concentrated mitochondrial matrix were injected into an I 250 protein column (Waters Associates) which had been equilibrated either with 0.05 M phosphate buffer pH 6.85 or 0.1 M acetate buffer pH 7.15. The column was eluted with the same buffer with which it had been equilibrated. The flow rate was either 1 ml or 2 ml per min. The elution buffer and the column were maintained at 5 - 6°C by a water jacketed cooling block and the collecting tubes were kept on ice. Fractions were collected manually by following the absorption pattern at 280 nm. For enzyme assays the fractions were used without any further treatment, for SDS-polyacrilamide gel electrophoresis the relevant fractions were concentrated by centrifugation in CF 50A Centiflo ultrafiltration membrane cones (Amicon Corp.)

Carbamoyl Phosphate Synthase I Assay

Enzyme activity was assayed by a modification of the method described by Clarke (4). Samples varying between 0.05-0.2 ml incubated in a final volume of 0.4 ml with 50 mM-ammonium acetate, 3 mM-ornithine, 10 mM-$MgSO_4$, 6.5 mM-N-acetylglutamate, 20 mM-glycylglycine, 20 mM-$NaHCO_3$, containing 130,000 d.p.m. [^{14}C] $NaHCO_3$, 10 mM-mercaptoethanol, 2.5 mM-ATP, 2.5 mM-phosphoenol-pyruvate, 4 units pyruvate kinase and 0.5 units ornithine transcarbamylase at pH 7.5 for 20 min at 37°C.
The reaction was terminated by the addition of 0.2 ml 30% trichloracetic acid and 0.5 ml portions were pipetted into scintillation vials with 0.1 ml 5 M HCl. The vials were heated for 15 min. with a 150 watt flood-light to remove unreacted bicarbonate.

After cooling, 5 ml Aquasol (New England Nuclear Corp., Boston, Mass. U.S.A.) was added to each scintillation vial. The residual radioactivity due to the conversion of ornithine to citrulline was counted in a model 2211 Packard liquid-scintillation spectrometer for at least 4000 counts and quench corrections were carried out with a quench curve in which acetone was used as a quenching agent.

Assay of Protein Concentration

The protein concentration of samples was determined by the method of Lowry *et al.* (7) with bovine serum albumin (fraction V; Sigma) as standard.

SDS-Polyacrylamide Gel Electrophoresis

Electrophoresis was carried out on 3 mm thick slab gels measuring 7 cm x 7 cm containing a 6% acrylamide mixture as described by Melnick *et al.* (8) and in a buffer consisting of 0.04 M Tris, 0.02 M sodium acetate, 0.1% SDS, 0.002 M EDTA at pH 7.4. Samples contained between 20 - 80µg protein. At the completion of the run the gels were fixed for 1 hour in a solution containing 57 g trichloracetic acid, 17 g sulphosalicylic acid, 150 ml methanol and 350 ml distilled water and stained with Coomassie blue (8). Destaining was effected in a mixture of Methanol 30: water 63: acetic acid 7. Prior to photographing the gels were swollen in 7% acetic acid. After drying in a GSD-4 Gel slab dryer (Pharmacia, Sweden) the gels were scanned.

For molecular weight determinations the rate of migration of the purified carbamoyl phosphate synthase was compared with that of several standard proteins (myosin, 220,000; heavy meromyosin, 175,000; bovine serum albumin, 68,000).

RESULTS AND DISCUSSION

The HPLC elution profile of the concentrated matrix proteins is shown in Fig. 1A and 1B using phosphate and acetate buffers,

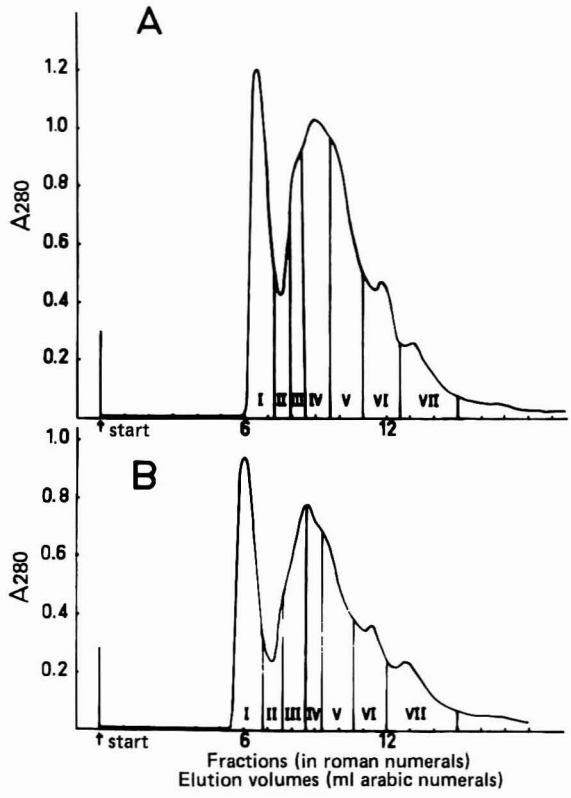

FIGURE 1

HPLC elution profiles. A. eluted with 0.05 M phosphate buffer, pH 6.85. B. eluted with 0.1 M acetate buffer, pH 7.15. Conditions as described in Materials and Methods.

respectively. Only four major peaks were observed as most proteins below a molecular weight of 30,000 had already been removed by diafiltration. Seven fractions were collected in each buffer system and the results are summarised in Table 1. Because of slightly different fraction sizes the enzyme activity was collected in differently numbered fractions in each run. The purification was similar in both buffer systems, except that phosphate clearly inhibited enzyme activity as shown in Table 1.

TABLE I

Specific Activities of Carbamoyl Phosphate Synthase after Fractionation by HPLC

Fraction	Column eluted with phosphate buffer		Column eluted with acetate buffer	
	Protein	Enzyme activity	Protein	Enzyme activity
	mg/fraction	umol/min./mg protein	mg/fraction	umol/min./mg protein
Mitochondria	----	0.276**	----	0.469
Concentrated mitochondrial matrix	5.28*	0.394**	3.96*	0.602
Fraction I	0.25	0.119	0.10	0.215
Fraction II	0.13	0.968	0.21	2.584
Fraction III	0.51	1.676	0.68	1.186
Fraction IV	1.43	0.545	0.89	0.141
Fraction V	0.94	0.099	0.54	
Fraction VI	0.39	0.045	0.36	0.075
Fraction VII	0.27	0.031	0.33	0.036

* Amount of Protein applied onto Column.

** Assayed in the presence of 0.025 M Phosphate buffer.

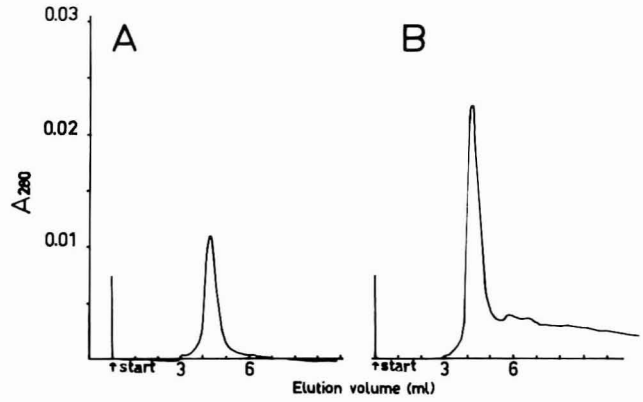

FIGURE 2

Elution profiles of carbamoyl phosphate synthase in the absence (A) and presence (B) of aldolase. Eluant was 0.05 M phosphate buffer, pH 6.85.

The specific activity of 2.58 units/mg protein for Fraction II (acetate buffer) compared favourably with previously published values of 1.3 units/mg protein for purified rat liver enzyme (4) and 2.44 units/mg protein for crystalline frog liver enzyme (5). From the results presented in Table 1 it may be calculated that on elution with phosphate buffer 74% of the protein and 91% of the carbamoyl phosphate synthase activity were recovered. The corresponding values for the elution with acetate buffer are 78% and 66%.

The fraction with the maximum specific activity rechromatographed with purified aldolase (M.W. 150,000) (Fig. 2). The purification factor of 6.1 and 5.5 from whole mitochondria and 4.8 and 4.2 from mitochondrial matrix suggests that the protein has been purified to near-homogeneity, since carbamoyl phosphate synthase comprises 14 - 18% of total mitochondrial protein and 25% of the matrix proteins (4). This is confirmed in Fig. 3, which compares the protein bands obtained by SDS-polyacrylamide gel electrophoresis of whole mitochondria, matrix proteins, Fraction II (acetate buffer) and Fraction III (phosphate buffer).

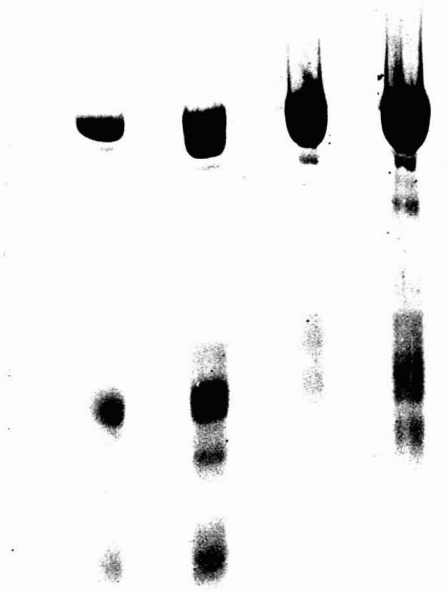

FIGURE 3

Electrophoresis of mitochondrial polypeptides. From left to right. Whole mitochondria, mitochondria matrix, Fraction III after elution with phosphate buffer, Fraction II after elution with acetate buffer.

In Fraction II (acetate buffer) more than 88% of the Coomassie Blue stain is associated with one slow-moving band, as measured by an integrating scanner. This is an underestimate, as it has been shown that Coomassie blue stain intensity increases linearly only to a maximum of 10 μg protein per band (9). This amount was exceeded in order to detect any contaminating proteins.

When compared to suitable marker polypeptides of known molecular weight this slow-moving band had a molecular weight of 166,000, which agrees well with the reported value of 165,000

also obtained by SDS-polyacrylamide gel electrophoresis (4). It is noteworthy that the undenatured enzyme showed a similar molecular weight during HPLC elution to the SDS-treated enzyme (Fig. 2), in view of suggestions that carbamoyl phosphate synthase may exist as a dimer *in vivo* (2, 10).

The results presented in this communication therefore indicate that the use of an I 250 protein column with HPLC is a suitable technique for the rapid purification of unstable proteins. Such a single step fractionation procedure is particularly appropriate when the protein under investigation comprises a large proportion of the crude protein extract, as in the case of carbamoyl phosphate synthase. However, other work currently being carried out in this laboratory (M.T. Campbell, J.K.Pollak, R. Sutton; unpublished observations) indicates that this purification technique is also applicable to the cytoplasmic precursor of carbamoyl phosphate synthase, which is reported to be 6000 daltons larger than the mature protein (10, 11) and ammount to less than 2% of cytoplasmic proteins. This precursor protein is also highly unstable (J.K.Pollak, unpublished observation). Because of the speed and convenience HPLC should be regarded as a powerful tool for protein isolation, separation and purification, especially when used in conjunction with diafiltration.

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HIGH-PERFORMANCE GEL-PERMEATION CHROMATOGRAPHY OF BOVINE SKIM MILK PROTEINS

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ABSTRACT

The separation of bovine skim milk proteins by gel-permeation high performance liquid chromatography was examined. Toya-Soda TSK-GEL (Type SW) columns were used with an eluent of .05 M phosphate buffer (pH 6.80) containing .1 M sodium sulfate at .5 ml/min. Bovine whole milk was centrifuged to remove lipids, and the resultant skim milk directly injected. A 2000SW column yielded three protein peaks: 1 = casein, IgG and BSA; 2 = β-lactoglobulins and BSA; and 3 = α-lactalbumin and BSA. A 3000SW plus 2000SW column system with a 30 µl injection volume yielded four protein peaks: 1 = minor amounts of α_{s_1}- and β-casein; 2 = casein, BSA and IgG; 3 = β-lactoglobulins; and 4 = α-lactalbumin. A 3000SW plus 2000SW column system with a 10 µl injection volume yielded five protein peaks: 1 = casein; 2 = IgG; 3 = BSA; 4 = β-lactoglobulins; and 5 = α-lactalbumin. Both the single column and dual column applications yielded three nonprotein peaks, which were dialyzed from solution. Thus, a high speed analytical separation of milk proteins was achieved according to molecular size, but this application is highly dependent on sample size.

INTRODUCTION

Gel-permeation chromatography (GPC) on Sephadex is used to separate proteins according to their molecular size. Sephadex is not mechanically

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stable under pressure resulting in relatively low flow rates and lengthy analysis time.

A number of GPC column packings have recently been developed that exhibit good resolution and can be operated under moderate pressure (1-4). Data on the applicability of these GPC columns for biological analysis is rapidly expanding, but many applications require proteins to be denatured with SDS to achieve good separation (5,6). New GPC column packings such as TSK-GEL (Type PW), consisting of microspheres of a hydrophilic polymer, have recently offered improved resolution (7-9).

This study was undertaken to establish a rapid method to separate and isolate milk proteins. Environmental toxicants such as pyrrolizidine alkaloids or aflatoxins are known contaminants in milk (10-12). These toxicants and their metabolites may be covalently bound to milk proteins, yet no direct high-speed analytical method has been developed to rapidly separate and isolate milk proteins. The application of high performance liquid chromatography (HPLC) utilizing Toya-Soda TSK-GEL columns for the separation of bovine skim milk proteins is discussed.

EXPERIMENTAL

Sample Preparation

Bovine whole milk samples were obtained from the bulk tank at the University dairy facilities. Milk was centrifuged twice at 200 x g for 10 min and 10,000 x g for 20 min to remove lipids. Total protein concentration was determined in an aliquot of the skim milk (13). The concentration of protein in the skim milk samples ranged from 2.7 to 3.5%. Whey was obtained by acid-precipitating casein from skim milk (14).

Chromatography

The chromatography system consisted of a Waters Associates 6000A pump and U6K injector, Schoeffel 770 variable-wavelength UV detector (280 nm), and Varian A-25 recorder. The following 7.5 x 300 mm columns were used: TSK-125

(Toya-Soda 2000SW, 10 μm particle size, 25 nm pore size, Bio-Rad, Richmond, CA) and MicroPak TSK 3000SW (10 μm particle size, 150 nm pore size, Varian, Sunnyvale, CA). Protein exclusion limits are approximately 100,000 daltons for the TSK-125 column and >350,000 daltons for the TSK 3000SW column (15). The eluent was 0.05 M phosphate buffer (pH 6.80) containing 0.1 M sodium sulfate. The flow rate was 0.5 ml/min with a backpressure of 100 psi per column. Ten or 30 μl aliquots of the skim milk were injected, and individual peaks collected. The peaks from individual runs were pooled, dialyzed against deionized water (1000 dalton cut-off membrane, Spectraphor, Los Angeles, CA), then lyophilized.

Electrophoresis

The proteins in each peak were characterized against milk protein standards by discontinuous polyacrylamide gel electrophoresis (16). The gel concentrations in the stacking and running gels were 3.5 and 7.5% with 7 M urea, or 5 and 9% without urea. The gels were stained with 0.05% Coomassie Blue in glacial acetic acid:methanol:water (10:25:65), and destained in glacial acetic acid:methanol:water (10:25:65).

Protein Standards

Protein standards, α-lactalbumin, cytochrome-C, pepsin, β-lactoglobulins A and B, ovalbumin, bovine serum albumin (BSA), and immunoglobulin G (IgG) were purchased from Sigma Chemical Company (St. Louis, MO.).

RESULTS AND DISCUSSION

Figure 1 shows the elution curves of various commercial milk protein standards on the 2000SW column. IgG (161,000 daltons), BSA (69,000 daltons), β-lactoglobulins A and B (dimers, 36,000 daltons), α-lactalbumin (14,150 daltons) elute according to their molecular size, and exhibited retention times of 12.1, 12.6, 14.6, 14.9 and 16.1 min. The void volume (V_o) was 5.7 ml using blue dextran (average MW = 2 x 10^6) as a marker. The total column volume (V_t) was 10.5 ml using phenylalanine (MW = 165.2) as a marker. The

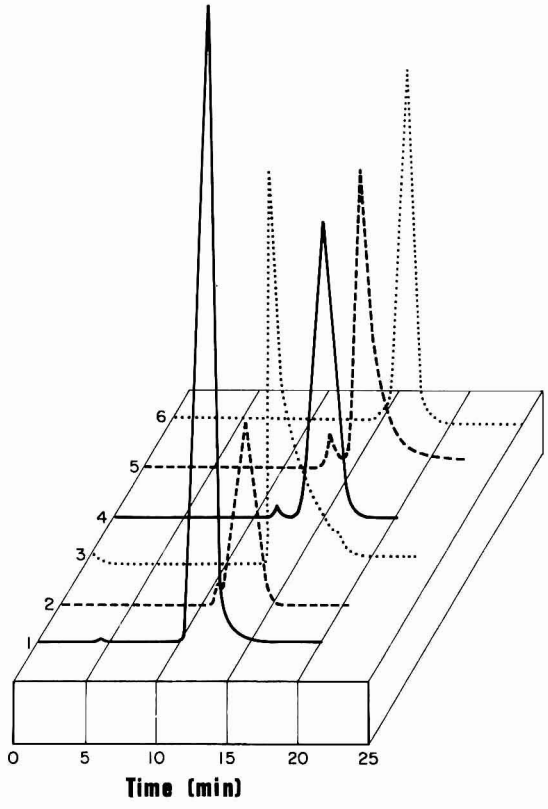

Figure 1. Elution curves of commercial milk proteins and blue dextran. Column 7.5 mm x 300 mm Toya-Soda 2000SW; Schoeffel model 770 variable wavelength uv detector (280 nm); eluent, .05 M PO_4 buffer (pH 6.80) containing .1 M Na_2SO_4; flow rate of .5 ml/min. Peaks and retention times: 1 = blue dextran (11.4 min); 2 = BSA (12.6 min); 3 = IgG (12.1 min); 4 = β-lactoglobulin A (14.6 min); 5 = β-lactoglobulin B (14.9 min); and 6 = α-lactalbumin (16.1 min).

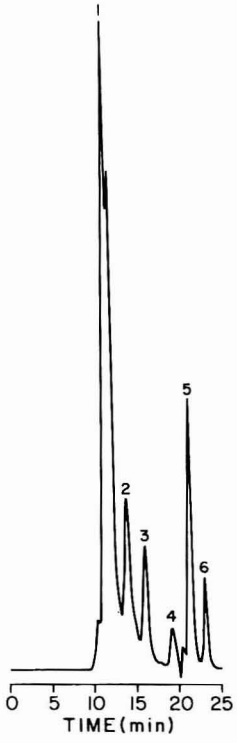

Figure 2. Elution curve of skim milk. Conditions as in Figure 1, 30 µl injected.

elution volumes (V_e) of BSA and IgG were similar, though their molecular weights are markedly different. Jones et al. (17), using a 10 nm pore C_8 reversed-phase support, found that BSA did not chromatograph as efficiently as ribonuclease (13,700 daltons), which was attributed to BSA having a lower diffusion rate and equilibrating more slowly with the stationary phase. However, this argument may not be valid with an aqueous gel-permeation system.

Figure 2 illustrates the elution curve of skim milk obtained with the 2000SW column. A total of six peaks were obtained, and the percent distributions of protein in each peak were 70.7, 18.0, 7.0, 0.7, 3.2 and 0.3%. The total recovery of protein was 91.0%. The V_t was 12.5 ml as compared to 10.5 ml for

phenylalanine. Thus, some milk component(s) had absorbed onto the column. Others have found similar absorption problems with low molecular weight compounds on the Toya-Soda (Type SW) and Waters I-125 gel-permeation columns (17). The proteins in each peak were characterized electrophoretically against milk protein standards in the presence or absence of 7 M urea in the gels. Peak 1 contained the casein proteins, IgG, and BSA; peak 2 contained β-lactoglobulins; peak 3 contained α-lactalbumin; peaks 2 and 3 were contaminated with BSA; and peak 3 was contaminated with β-lactoglobubin. No protein bands were obtained with peaks 4-6, and these samples were not further analyzed. Although the molecular weights of individual casein proteins are on the order of 20,000 daltons (18), they eluted with the V_o. The milk caseins are in a micellar form, which is spherical in shape and may be on the order of 40-300 nm in diameter with particle weights of 10^6 to 3×10^9 daltons (14). Therefore, the casein micelle is probably remaining intact during the chromatographic run. In agreement, Hill and Hansen (19) and Morr et al. (20) separated skim milk proteins on Sephadex G-100, and casein proteins eluted exclusively in the first fraction.

Figure 3 shows the elution curve of whey with the 2000SW column. The elution profile of whey is directly comparable to that of skim milk, except peak 1 is a minor component and peaks 2 and 3 are the major components of the elution profile; providing further evidence that the casein micelle elutes with the V_o. Peaks 4-6 could be dialyzed (1000 dalton cut-off membrane) out of solution.

Since casein, IgG and BSA eluted in the V_o, a dual column system (3000SW plus 2000SW), was used to increase the exclusion limit. Figure 4 shows the comparison between V_e and protein molecular weight with the 3000SW and 2000SW columns. Addition of the 3000SW effectively increased the molecular weight exclusion limit and resolution over the 2000SW column alone. Also, molecular weight of proteins could be effectively estimated with these gel-permeation columns.

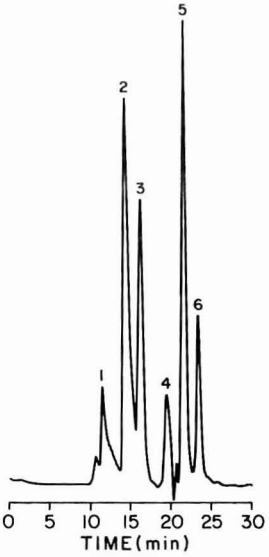

Figure 3. Elution curve of the whey fraction of skim milk. Conditions as in Figure 1, 25 µl injected.

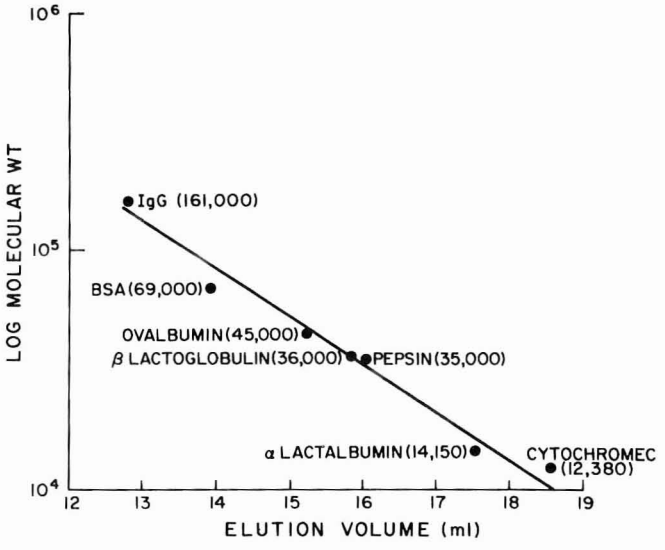

Figure 4. Protein molecular weight versus elution volume. Columns 7.5 mm x 300 mm Toya-Soda 3000SW plus 2000SW; other conditions as in Figure 1.

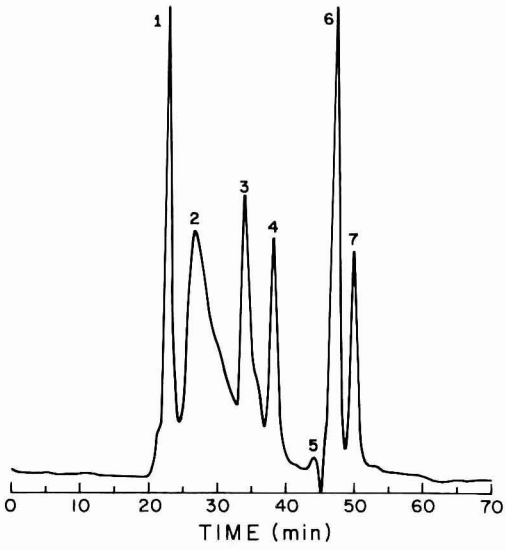

Figure 5. Elution curve of skim milk. Conditions as in Figure 4, 30 µl injected.

Figure 5 shows the elution profile of skim milk (30 µl injected) on the 3000SW plus 2000SW columns. A total of seven peaks were obtained, and the percent distributions of protein in each peak were 21.3, 32.8, 22.8, 15.4, 2.5, 4.7 and 0.5%. The total recovery of protein was 86.0%. Peak 1 contained a minor amount of α_{S_1}-and β-casein; peak 2 contained the remainder of caseins, BSA and IgG; peak 3 contained β-lactoglobulins with contamination from α_{S_1}-and β-casein; peak 4 contained only α-lactalbumin. The presence of α_{S_1}-and β-casein in peak 3 suggests that the casein micelle is not remaining intact during the chromatographic run. No proteins were present in peaks 5-7. The 3000SW plus 2000SW column system with a 30 µl injection volume did not enhance milk protein separation, since BSA and IgG eluted with casein (peak 2).

When 10 µl of skim milk was injected onto the 3000SW plus 2000SW columns, eight peaks were obtained as shown in Figure 6. The percent distributions of protein in each peak were 26.7, 3.0, 6.7, 27.4, 18.6, 3.3, 14.3 and 0%.

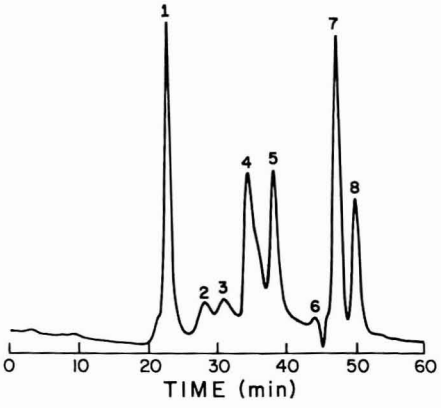

Figure 6. Elution curve of skim milk. Conditions as in Figure 4, 10 µl injected.

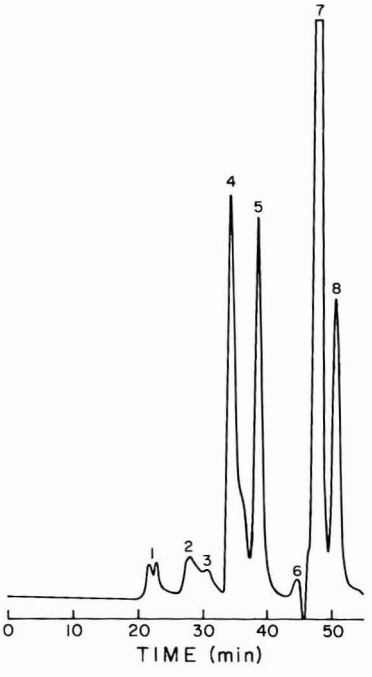

Figure 7. Elution curve of the whey fraction of skim milk. Conditions as in Figure 4, 30 µl injected.

However, total recovery of protein was $\leq$ 75%. The low protein recovery may be a result of assay error, since only .3 mg protein was injected. The proteins from this chromatogram were characterized by comparing the retention times with standards: peak 1 = casein, 2 = IgG, 3 = BSA, 4 = β-lactoglobulin, 5 = α-lactalbumin, and 6-8 = nonprotein. When casein was precipitated from the skim milk sample and an aliquot of whey injected, peak 1 was a minor component of the chromatogram (Figure 7), providing further evidence that the casein proteins eluted with peak 1 (Figure 6).

The TSK-GEL (Type SW) columns were effective in separating proteins according to molecular size in a nonpractical (Figure 4) and a practical application (Figure 6). However, separation of bovine skim milk proteins was highly dependent on sample size, as a 10 µl versus a 30 µl injection volume resulted in a better separation of casein proteins from whey proteins and IgG from BSA.

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ANALYSIS OF RAT CASEINS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The analysis of rat caseins by high performance liquid chromatography is described. These procedures have confirmed the structural similarity of the polypeptide chains of native and dephosphorylated rat β_1- and β_2-caseins as well as to demonstrate that these two related proteins are structurally unrelated to the rat α_1-casein protein. In addition, these HPLC techniques allow the analysis of the phosphorylation patterns of these rat caseins following digestion with potato acid phosphatase (E.C. 3.1.3.2).

INTRODUCTION

The caseins, the major protein components of milk, are a family of relatively low-molecular weight phosphoproteins which in milk are found associated together in the form of protein

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micelles [1]. Whole rat casein contain three major casein components [2,3], the most abundant component (designated in this study as α_1-casein) has an apparent molecular weight of 43,000 daltons and represents over 50% of the total casein proteins. Another major fraction, with an apparent molecular weight of 28,000 daltons as determined by SDS-gel electrophoresis, can be resolved on DEAE-cellulose into two components in approximately equal amounts (designated β_1 and β_2). In a detailed study on these rat caseins, Hobbs and Smith [2] have proposed that the 28,000 dalton rat caseins are probably derived from the same polypeptide, differing only in the number of bound phosphate groups per molecule, and that both these rat β-caseins are unrelated structurally to the 43,000 dalton rat α_1-casein. Recently, we reported methods [4-6] for probing protein homologies based on enzymatic mapping analysis, using reversed phase high performance liquid chromatography (HPLC). In this paper we wish to describe the application of these methods to the analysis of rat α_1-, β_1- and β_2-caseins which not only confirms the proposed differences between the α_1 and β proteins but also allows a direct comparison of the phosphorylated and dephosphorylated rat caseins.

MATERIALS AND METHODS

Rat Whole Casein Isolation and Fractionation.

Milk was collected from female rats of the Wistar strain between 9 and 14 days post partum following anaesthetisation with ketamine hydrochloride (50mg/ml, 0.5ml i.p.) and administration of oxytocin (5 I.U. i.p.). Whole casein was prepared by centrifugation at 20,000g for 1h of the diluted milk (1:2) in the presence of 75mM $CaCl_2$, the final pellet was dissolved in 100mM EDTA, pH 7.6, exhaustively dialysed against de-ionised water and stored at -20^{o}. The separation of the rat casein

components on DEAE-cellulose essentially follow procedures described [7] for bovine casein. In brief, the crude rat whole casein (100mg) was chromatographed on a DEAE cellulose column (30 x 1cm) using an initial buffer containing 6M urea, 10mM Tris-HCl, pH 8.6, 1mM EDTA and 1mM DTE. The casein components were eluted with a linear gradient of 0 to 220mM NaCl in the same buffer. Appropriate fractions were pooled, dialysed against 10mM NH_4HCO_3, lyophilised and analysed by SDS-gel electrophoresis using a modification of the procedure of Laemmli [8].

Casein Dephosphorylations.

The rat caseins were dephosphorylated by a procedure similar to that of Bingham et al. [9] using potato acid phosphatase (E.C. 3.1.3.2). The caseins were dissolved in a buffer containing 50mM imidazole-HCl, pH 7.0, at a concentration of 1-3mg/ml and the potato acid phosphatase (60units/mg) added at an enzyme/casein ration of 1:1000. The solution was applied to a Sephadex G25 column in the same buffer and incubated at 37^{o} for 2.5-3h. The proteins were then eluted and desalted on a second Sephadex G25 column equilibrated in 10mM NH_4HCO_3 and lyophilised.

Enzymatic Digestion of Caseins.

The trypsin digestions were carried out in 100mM NH_4HCO_3 pH 8.5, 1mM DTE using an enzyme (1mg/ml in 1mM HCl) to protein ratio of 1:100. After incubation for 2h at 37^{o}, a further aliquot of trypsin was added, incubation continued for a further 2h and the samples analysed immediately by HPLC or frozen at -20^{o}.

High Performance Liquid Chromatography.

The configuration of the HPLC equipment, sample and solvent preparation, which were used in this study, are essentially as described previously [4-6]. Waters μBondapak Fatty Acid

Analysis columns (30 x 0.39cm) were used throughout, at a flow rate 2.0ml/min, with a linear 60min gradient generated from water-16mM H_3PO_4 to 50% acetonitrile-50% water-16mM H_3PO_4. Peptides were detected by their absorbance at 210nm.

RESULTS AND DISCUSSION

Compared to conventional techniques of peptide separation and analysis, it is now possible to obtain superior peak resolution for both hydrophilic and hydrophobic peptides with modern reversed-phase HPLC approaches. In addition, these methods offer short elution times and, frequently, excellent recoveries. In previous papers [4-6,10,11] from this laboratory, we have exploited these advantages in a variety of studies on the analysis and isolation of peptides from synthetic and natural sources. An additional area, where the potential of these methods has become increasingly evident, is their use in the separation of peptide fragments derived from proteins following enzymatic digestion thus allowing the comparative profiling of different proteins, either by way of assessment of the homogeneity or structure, or by the detection of distinctive sequence homologies of related proteins. All of these advantageous features were apparent in the HPLC analysis of the different rat caseins.

Typical elution profiles for the 2h tryptic digests of the phosphorylated and dephosphorylated rat α_1-, β_1- and β_2-caseins are shown in Figs. 1-3 respectively. No significant changes in these elution profiles were observed when the trypsin digestion was carried out for 4h. Comparison of the elution profiles of the tryptic peptides of the dephosphorylated forms of rat β_1-casein (Fig. 2a) and β_2-casein (Fig. 3a) clearly show the close similarity between these two proteins, with nearly all the corresponding peptides showing coincidental elution times and similar peak area ratios, the only notable

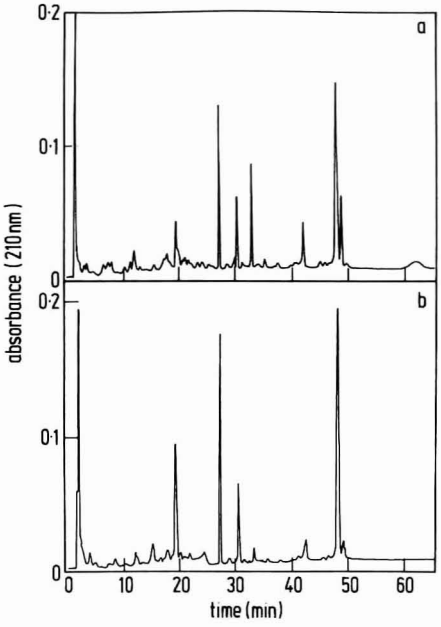

Figure 1.

Reversed phase HPLC analysis of the tryptic peptides of rat dephosphorylated α_1-casein (a) and rat phosphorylated α_1-casein (b) on a μBondapak Fatty Acid Analysis column (30cm x 3.9mm I.D.) using a linear 60min gradient from water-16mM orthophosphoric acid to 50% acetonitrile-50% water-16mM orthophosphoric acid at a flow rate of 2.0ml/min; temperature 18°, sample loading (150μg).

exception being the peptide with t_R = 44.0min, which although present in both samples, was considerably reduced in peak area in the digest of the rat β_2-casein. Comparison of both these elution profiles with that of the 2h tryptic digest of the dephosphorylated rat α_1-casein (Fig. 1a) shows that there is little structural similarity between the α_1- and β-caseins.

As can also be seen by comparison of the phosphorylated and dephosphorylated rat caseins, these reversed phase HPLC procedures can also be used to obtain information on the

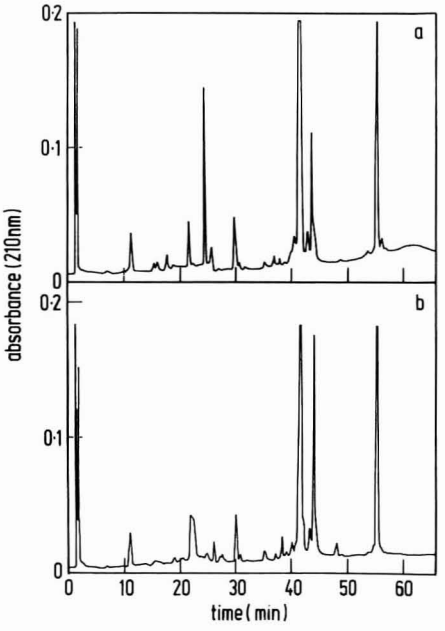

Figure 2.

Reversed phase HPLC analysis of the tryptic peptides of rat dephosphorylated β_1 casein (a) and rat phosphorylated β_1-casein (b). Chromatographic conditions as in Figure 1.

distribution of phosphate groups within the native casein molecules. For example, comparison of the tryptic peptides obtained from the dephosphorylated and phosphorylated forms of rat β_1-casein (Fig. 2a and 2b respectively) reveals that the enzymatic removal by potato acid phosphatase of the phosphate groups affects the elution times of only several characteristic peptides, i.e. the disappearance of the peptides with t_R 22.5 and 48mins present in the elution profile of the phosphorylated rat β_1-casein and the appearance of a major peptide peak at t_R 24.5 and several minor peaks at t_R 15, 18 and 56min. Comparable analyses of rat β_2-casein show similar changes in the elution profiles. It is note-

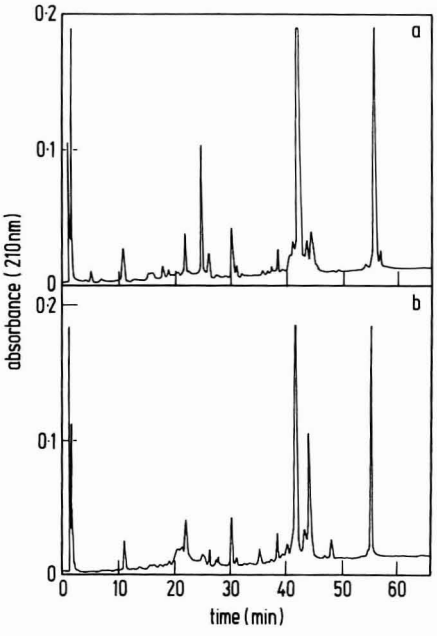

Figure 3.

Reversed phase HPLC analysis of the tryptic peptides of rat dephosphorylated β_2-casein (a) and rat phosphorylated β_2-casein (b). Chromatographic conditions as in Figure 1.

worthy that comparison of the elution profiles of the tryptic digests of the phosphorylated β_1- and β_2-caseins also reveal a close similarity except for the region which elutes near to 20-24min. Other studies [2] have shown that rat β_1-casein contains six phosphate groups and rat β_2-casein seven phosphate groups per molecule. The essentially identical profiles for the tryptic maps of these two related caseins would be in accord with the small changes in polarity resulting from these phosphorylation patterns. Since only a few peptides were affected by dephosphorylation of the β-caseins the phosphate groups must be confined to restricted regions of the polypeptide chain, as is also found in the major bovine caseins [12]. Furthermore, most

of the peptides which do not contain phosphate groups elute late in the gradient, indicative of nonpolar peptides. This suggests that rat caseins contain large hydrophobic domains, a characteristic also shown by other mammalian caseins [12]. The similarity between the β-caseins contrasts the numerous changes observed in the elution profile of the tryptic digest of rat α_1-casein following dephosphorylation (Fig. 1a,b).

CONCLUSION

In conclusion, methods are reported for the enzymatic mapping by reversed phase HPLC of rat β_1- and β_2 caseins. These methods clearly confirm the close structural similarity of the rat β-caseins and their unrelatedness to rat α_1-casein. In addition these techniques permit an analysis of the phosphorylation patters of these caseins.

ACKNOWLEDGEMENTS

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THE PREPARATIVE SEPARATION OF SYNTHETIC PEPTIDES ON REVERSED-PHASE SILICA PACKED IN RADIALLY COMPRESSED FLEXIBLE-WALLED COLUMNS.+

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ABSTRACT

The rapid and efficient separation of multigram amounts of unprotected synthetic peptides on octadecylsilica, packed in flexible walled polyethylene columns, is described. The mobile phase used was 0.05% trifluoroacetic acid with methanol as the organic modifier. An advantage of this eluant system was that the salt free peptide could be isolated simply by lyophilisation of the sample after chromatography. The following peptides were purified with this system: glycylgly-

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+ High Performance Liquid Chromatography of Peptides and Proteins XXIV. For the previous paper in this series see Ref.(25).

cine ethyl ester, glyclyglycylglutamic acid, glycylglycyllysine, pyroglutamylhistidinylglycine, prolyprolylproline, leucine-enkephalin and methionine-enkephalin, in amounts ranging from 50mg to 5gm.

INTRODUCTION

The use of peptides for research and clinical purposes has increased dramatically in the past five years, particularly in the field of neuro-endocrinology where a number of peptide mediated processes have been elucidated [1,2]. The peptides used in these studies have either been isolated from biological sources or prepared by solution or solid phase chemical synthesis techniques. Purification of peptides from both synthetic and biological sources has presented significant problems, as an extremely high degree of purity is required for biological studies. Recently 'ion-pair' reversed phase HPLC has been used effectively in the analysis of a variety of peptides [3-11]. This technique uses an aqueous mobile phase which contains small amounts of an ionic modifier which can ion-pair with the analyte and/or modify the organic coating on the silica particles of the stationary phase. The theoretical basis of the interactions which occur between the analyte, mobile phase and stationary phase are now beginning to be understood more clearly [12-19].

In an early study we described the use of radially compressed polyethylene cartridges containing octadecylsilane bonded silica particles in the purification of 10gm amounts of a model underivatised synthetic peptide [20]. In this case a homogeneous product was obtained with an elution time of twenty minutes using an aqueous solvent containing a hydrophilic ion-pairing reagent [13]. It is the purpose of this report to extend this study and demonstrate that preparative high performance liquid chromatography with radially compressed columns can be used to purify the following synthetic peptides - the pro-

tected dipeptide glycylglycyl-OEt, the tripeptides glycylglycyl-glutamic acid, glycylglycyllysine, pyroglutamylhistidinylglycine, and prolylprolylproline and the pentapeptides Leu-enkephalin and Met-enkephalin with separation times less than 60min.

MATERIALS AND METHODS

Apparatus: A Waters Assoc. (Milford, Mass., U.S.A.) HPLC system was used for the analytical separations. This consisted of two M6000A solvent delivery units, an M660 solvent programmer and U6K universal liquid chromatograph injector, coupled to an M450 variable wavelength UV Spectrophotometer and an Omniscribe two-channel chart recorder (Houston Instruments, Austin, Texas, U.S.A.). Waters μBondapak C_{18} columns (10μm, 30cm x 4mm, I.D.) were used for all analyses. Sample injections were made using a Microliter 802 syringe (Hamilton, Reno, Nev., U.S.A.).

The preparative separations were carried out on a Waters Assoc. Prep LC/System 500 instrument with a built in refractive index detector connected in series with the UV spectrophotometer and coupled to an Omniscribe two-channel recorder (Houston Instruments). A Waters Assoc. Prep PAK-500 C_{18} cartridge (75μm mean particle size, 30cm x 5.7cm) was used for the purification. Before use and after each chromatographic separation the cartridges were washed with 4 litres of methanol and the 4 litres of methanol:water (1:1) at a flow rate of 50ml/min to remove any adsorbed contaminants. Sample injections were made using a Gastight 101W syringe (Hamilton).

For both analytical and preparative work solvents were filtered using a Pyrex filter holder (Millipore, Bedford, Mass., U.S.A.) while peptide samples were filtered using a Swinney Filter (Millipore). Millipore HA grade, 0.45μm filters were used at all times for solvent and sample preparation, except

for filtration of the methanol when a Millipore FH grade filter was used.

Chemicals: Water was glass-distilled. Methanol was drum-grade (ICI, Wellington, New Zealand) and distilled before use. Trifluoroacetic acid (TFA) (Halocarbon Products, Hackensack, N.J., USA) was also distilled before use. Phosphoric acid (AR) was obtained from May and Baker. Synthetic peptides were prepared by standard solution techniques which will be described elsewhere. All amino acids were of the L-configuration except glycine.

Methods: Analytical HPLC was carried out at a flow rate of 1.5ml/min using 0.05% TFA in water, pH 2.3, as mobile phase. All chromatography was carried out at room temperature (ca. 22^{o}). Peptides were dissolved in the mobile phase at a concentration of 5mg/ml and 10-25µl volumes injected onto the columns. Similarly, 25µl aliquots from the preparative HPLC runs were analysed under identical elution conditions.

For the preparative separations, a flow-rate of 100ml/min was maintained (back pressure 100psi). The mobile phase was degassed completely by vacuum aspiration or by a helium gas purge. The crude sample was loaded in amounts between 50mg and 10g in 10ml of the eluting solvent. Immediately after collection each fraction was neutralised to pH 7 with ammonium hydroxide, concentrated on a rotory evaporator and lyophilised.

RESULTS

Figure 1 shows the analysis and preparative scale purification of a crude sample of the ethyl ester of glycyl-glycine, a simple dipeptide containing a single amide group and no side chains. Part A shows the analysis of 50µg of the peptide on a µBondapak C_{18} column using a mobile phase consisting of a 0.05% solution of trifluoroacetic acid (TFA) in water, with UV detection at 210nm. Figure 1B and 1C respectively show the purification of 50 and 200mg of the dipeptide

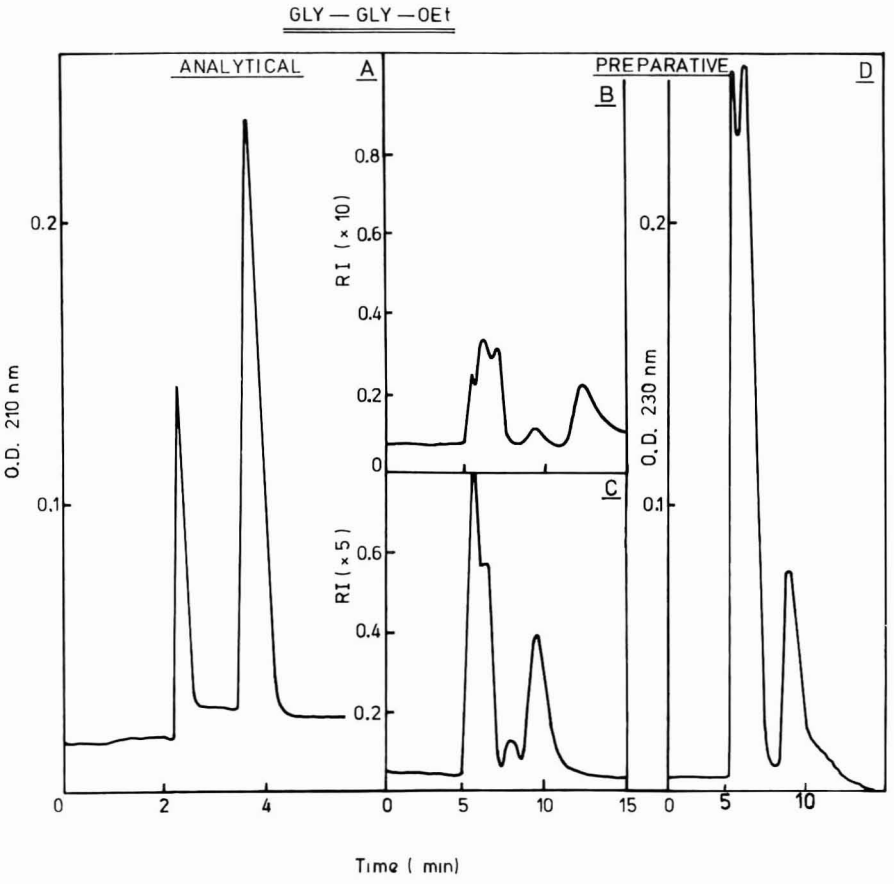


Figure 1.

The purification of Gly-GlyOEt. In part A a 50µg sample of Gly-GlyOEt was analysed on a µBondapak-C_{18} column with 0.05% TFA as the mobile phase and a flow rate of 1.5ml/min. In parts B to D the purification was carried out on a C_{18} cartridge in the Prep 500 Liquid Chromatograph with 0.05% TFA as the mobile phase and a flow rate of 50ml/min. The loadings were 0.05g and 0.2g in parts B and C respectively and the solute was monitored with a RI detector. Part D shows the monitoring of the effluent from the RI detector used in part C with an UV detector connected in series.

on the Prep 500 liquid chromatograph, using a C_{18} cartridge. The mobile phase was 0.05% trifluoroacetic acid in water, and detection was by means of a differential refractometer. The flow rate was 50ml/min. Figure 1D shows the same analysis as depicted in Figure 1C but with detection by means of a UV detector connected in series after the refractive index detector. In this separation the first peak(s) were attributed to salts and derivatives of glycine, while the later eluting peak (retention time of 4min in part A and 10min in part D) was the desired product Gly-GlyOEt, recovered in a 93% yield. The identity of this peak was confirmed by the chromatography of an authentic sample of the dipeptide.

Figure 2 shows the purification of 5gm of a crude synthetic sample of the tripeptide pyroglutamylhistidinylglycine, the

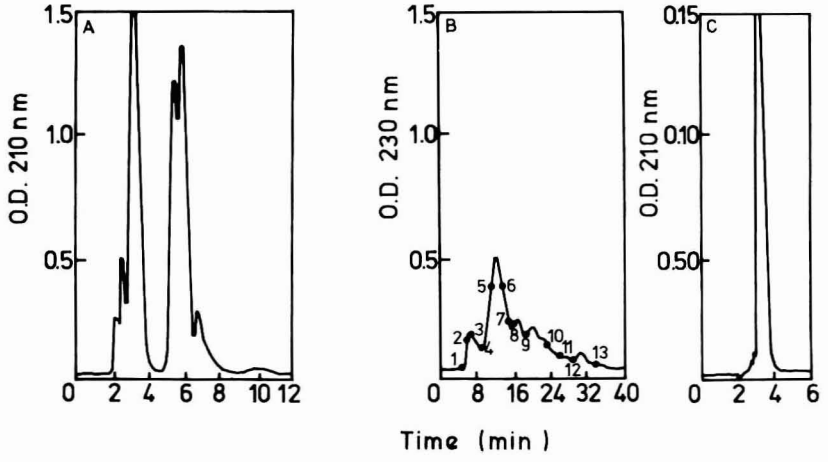


Figure 2.

The purification of Pyr-His-Gly. Parts A and C show the analytical elution profile of the crude mixture and the purified peptide respectively. The analytical system and preparative system were the same as described in Figure 1. The loading of crude mixture was 5g, and the elution profile for the preparative separation is shown in part B.

proposed anorexigenic peptide [21], with analysis before and after the purification (parts A and C). The mobile phase in each case was 0.05% TFA in water. Figure 2A shows the analytical HPLC profile of the crude sample with detection at 210nm. Figure 2B shows the preparative purification of 5gm of crude product on a C_{18} cartridge with UV detection at 230nm using a flow rate of 100ml/min. Figure 2C shows the analysis of the collected fraction which corresponded to the required product, numbered five on the chromatogram, again with detection at 210nm. Fractions 4 and 6 also contained significant amounts of the required peptide and could readily be concentrated and rechromatographed, using the recycle facility to improve their degree of purification. Following this procedure 2gm of the purified peptide was recovered and shown to be homogeneous by amino acid analysis of a hydrolysate and by analytical HPLC (Figure 2C). In addition after three lyophilisations the sample was found not to have any residual TFA, an important factor since the peptide was to be used in animal studies.

Figure 3 shows the elution profiles of the relatively polar tripeptide glycylglycylglutamic acid using a 0.05% TFA in water as the mobile phase. Figure 3A shows the analysis of crude synthetic product on the μBondapak-C_{18} column with 0.05% TFA as mobile phase. Figure 3B and 3C show the analysis of the crude and purified materials, using the same column but with 5mM perfluoropropionic acid as the mobile phase. Due to the polar nature of the peptide, little retention was obtained on the reversed phase column and therefore the center of the main peak (see Figure 3D) was recycled to improve resolution. Subsequent analysis of each fraction by analytical HPLC (see runs 1 to 7 in Figure 3) showed that the centre of this peak (fraction 5) was essentially homogeneous.

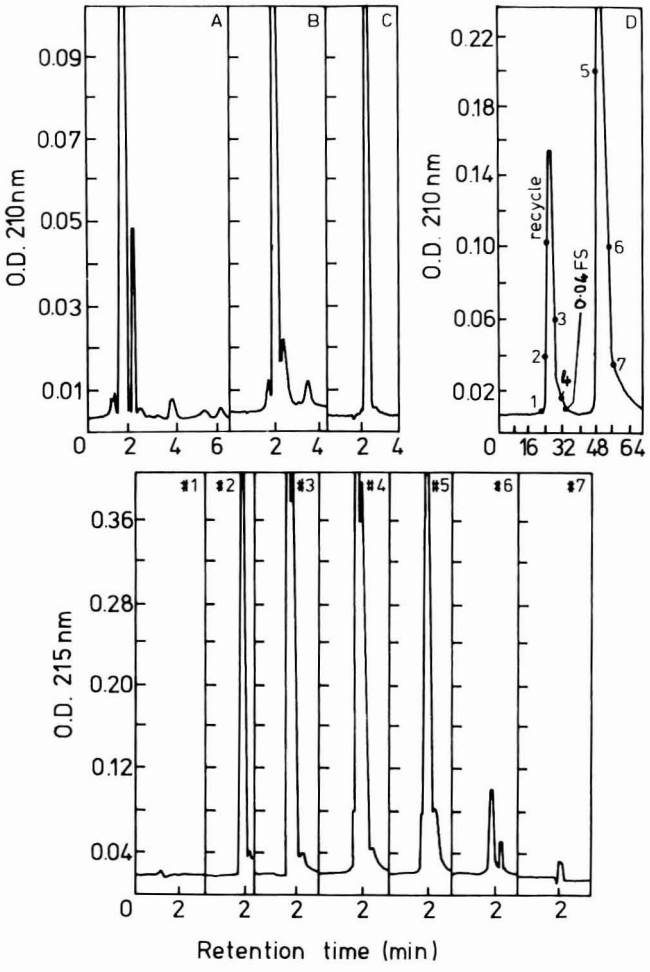


Figure 3.

The purification of Gly-Gly-Glu. Parts A and B show the analytical elution profile of the crude mixture and Part C the profile for the purified peptide. The analytical procedure was the same as described in Figure 1 except that the mobile phase used in Part A was 0.05% TFA and in Parts B and C perfluoropropionic acid (5mM). The preparative run (loading 4gm) is shown in Part D and the same conditions as Figure 1 were used. The centre of the peak was recycled and the elution profile of the recycled peak is shown at a greater OD sensitivity. The amount of material in the major fractions was as follows, 2, 1.1gm; 5, 1.28gm; 3 and 4, 0.6gm. The analytical HPLC of the fractions is shown in the bottom part of the figure using the conditions as described in Figure 1.

Figure 4 shows the analysis and purification of HPLC of the tripeptide glycylglycyllysine using 0.05% TFA in water as eluant. Figure 4A and 4C show the analysis of the crude sample and the purified product respectively. The product was analysed after fraction 3 from the preparative run had been neutralised, concentrated and lyophilised. Figure 4B shows the UV trace obtained during the preparative run of 4gm of the crude sample at a flow rate of 100ml/min. The numbers on the chromatogram refer to fraction numbers collected. The profiles numbered 1-4 are analytical HPLC chromatograms obtained by analysing the fractions as they were collected during the preparative run. In each case a 25µl sample was withdrawn and injected directly onto the analytical HPLC system in the same solvent.

Figure 5 shows the analysis and purification of the tripeptide prolylprolylproline. Figure 5A is the analysis of 0.5mg of the crude sample whilst Figure 5B is the preparative scale separation of 5gm of the crude sample obtained with a flow rate of 100ml/min. The mobile phase used in this separation was 20% methanol-water, 0.05% TFA. Again the numbers refer to the collected fractions, while the solvent eluted between fractions 3 and 4 was diverted to waste. The numbered chromatograms relate to the collected fractions from the preparative run, and were achieved by direct injection of aliquots from the relevant fraction, prior to neutralisation. Identical conditions were used for the analysis of both crude and purified fractions. The desired peptide was identified in the last eluting peak (t_R 4min and 22min in the analytical and preparative runs respectively) by quantitative amino acid analysis.

Figure 6 shows the analytical and preparative chromatogram of the pentapeptide tyrosylglycylglycylphenylalanylmethionine, methionine-enkephalin. In this separation 0.4gm of the crude

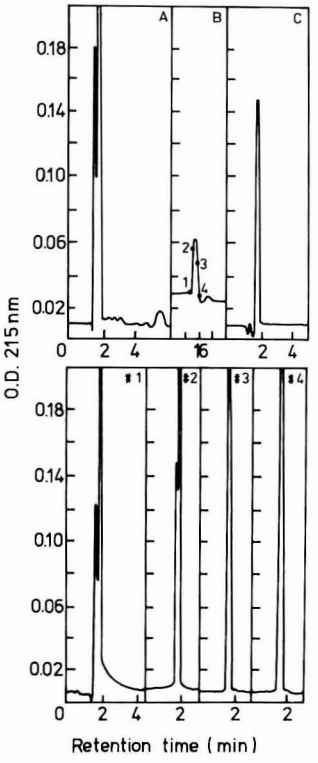
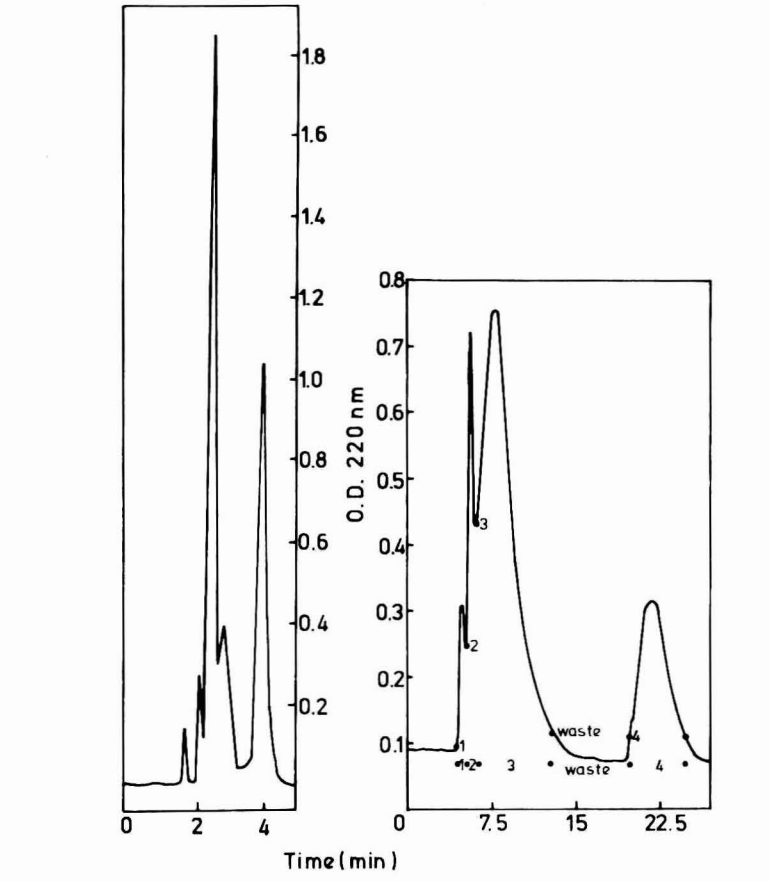


Figure 4.

The purification of Gly-Gly-Lys. Parts A and C show the analytical elution profile of the crude mixture and the purified peptide respectively, while part B shows the profile obtained for the preparative run. All chromatographic conditions were the same as described for Figure 2. The analysis of the fractions from the preparative run is shown in the bottom Figure. The loading for the preparative run was 4gm.

Figure 5.

The purification of $(Pro)_3$. Part A shows the elution profile of a 0.5mg samples of the crude product on a μBondapak-C_{18} column with a mobile phase of 0.05% TFA/H_2O-20% CH_3OH at a flow rate of 1.5ml/min. Part B shows the corresponding preparative run which was carried out on a 5gm sample and with a mobile phase of 0.05% TFA/H_2O-25% CH_3OH and a flow rate of 100ml/min. The bottom part of the Figure shows the analysis of fractions from the preparative run.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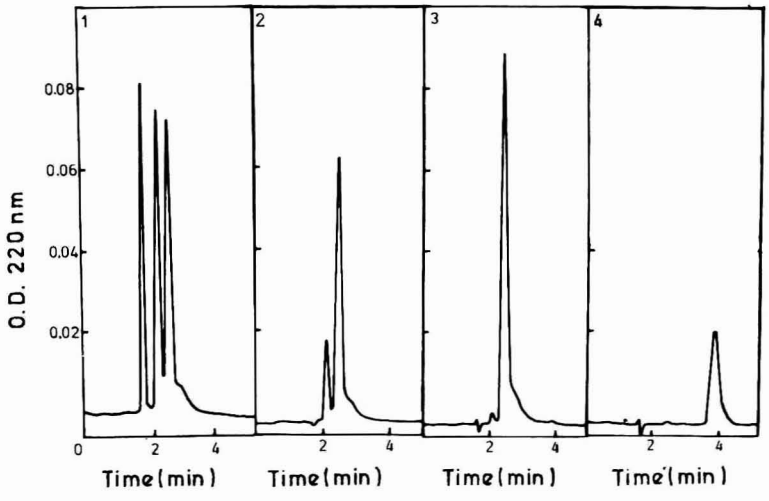
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0.08
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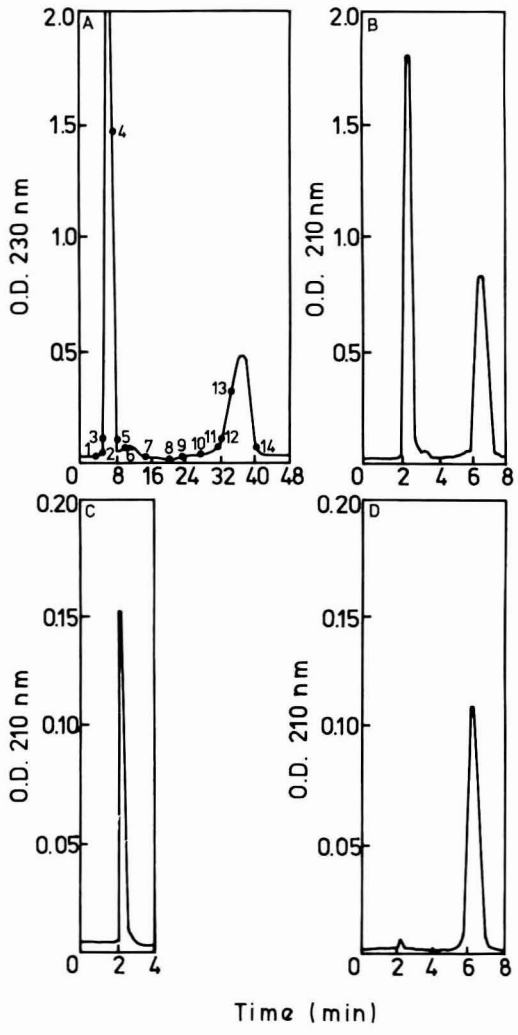

Figure 6.

The purification of methionine-enkephalin (0.4gm). The chromatographic conditions were as described for Figure 2 except that 0.05% TFA/H_2O-25% CH_3OH was used as the mobile phase. Part A shows the elution profile for the preparative run, Part B, C, D are analytical profiles of the crude mixture and the two major fractions from the preparative run.

sample was loaded in 28ml of the mobile phase, which was 25% methanol-water, 0.05% TFA. Figure 6A represents a UV trace of the preparative separation carried out at 100ml/min. The numbers represent fractions collected. Figure 6B shows the analytical separation of the crude sample. Figure 6C is the analysis of 25µl of fraction 4 and Figure 6D the analysis of 25µl of fraction 13. The analyses were carried out immediately after collection and prior to neutralisation. Again the analysis of the crude and purified fractions were carried out in the same mobile phase, namely 25% methanol-water, 0.05% TFA. Amino acid analysis of an acid hydrolysate of an aliquot of each peak identified the desired pentapeptide as the late eluting peak.

Figures 7A and 7B show the preparative scale purification of a crude sample of the pentapeptide, tyrosylglycylglycyl-

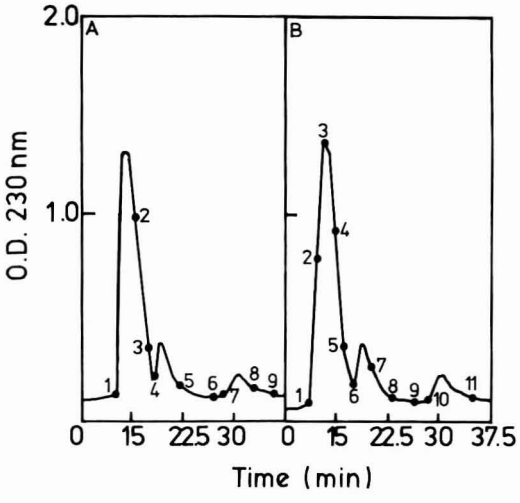

Figure 7.

The purification of leucine-enkephalin. The chromatographic conditions were as described for Figure 2, except that the mobile phase was 0.01% phosphoric acid/H_2O-25% CH_3OH. Parts A and B show the elution profiles obtained for two duplicate preparative runs on 2gm of crude mixture.

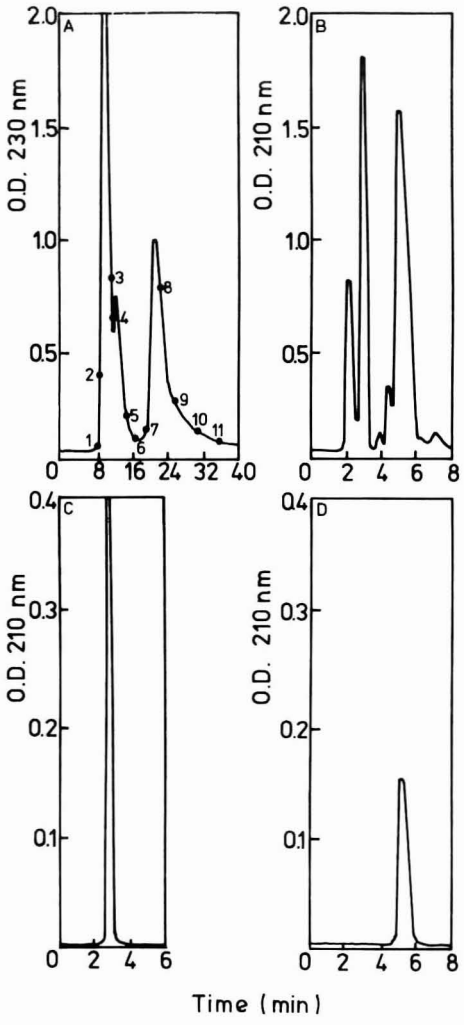

Figure 8.

The purification of leucine-enkephalin. The chromatographic conditions used were as described in Figure 2 except that the mobile phase was 0.05% TFA/H_2O-25% CH_3OH. Part A shows the preparative run, while parts B, C, D are analytical runs on the crude mixture and the two major fractions from the preparative run.

phenylalanylleucine, leucine-enkephalin, with a mobile phase consisting of 25% methanol-water, 0.1% phosphoric acid. In these separations 2gm of the sample was loaded in 10ml of the mobile phase. Again the numbered fractions were collected and an aliquot of each analysed by HPLC in 20% methanol-water, 0.1% phosphoric acid prior to neutralisation, concentration and lyophilisation.

Figure 8A shows the preparative separation of Leu-enkephalin using 30% methanol-water, 0.05% TFA as mobile phase. 1gm of the crude sample was loaded in 10ml of the mobile phase, and the flow rate maintained at 100ml/min. Figure 8B shows the analysis of a sample of the crude product while Figures 8C and 8D show analysis of fractions collected during the preparative run which corresponds to the major peaks in Figure 8B. Amino acid analysis studies of the late eluting peak (retention time of 23min in part A and 5.3min in parts B to D) showed that the desired pentapeptide was contained in this peak.

DISCUSSION

Recently there has been increasing interest in the use of preparative high performance liquid chromatography for the isolation of compounds of biological or synthetic origin [22]. It is now clear that the capacity of a particular column can vary considerably for different biological compounds. At this stage of development, three categories of column dimensions are used for peptide purification by HPLC: (1) sample size up to 1mg, analytical columns, e.g. 25 x 0.4cm I.D.; (2) sample size between 1-100mg, semipreparative columns, e.g. 60 x 0.7cm I.D. stainless steel columns or 10 x 0.8cm I.D. radial compression columns, and (3) sample size 100mg-1gm and upwards, preparative columns, e.g. 30 x 5.7cm I.D. With current technology, nearly all columns available either commercially or

packed in-house would fit in these ranges. The great majority of analytical columns function most efficiently in the microgram to low milligram range. Recently, some manufacturers have introduced colums with greater internal diameter and length, packed with materials essentially identical to those available for analytical chromatography [23]. Such columns probably have a maximum capacity of approximately 100-500mg for solutes such as peptides which contain a number of ionisable groups. Fully preparative HPLC has effectively extended this range, as is clearly demonstrated in the present report on the rapid and high efficiency purification of eight synthetic peptides. The good resolution obtained with the radially compressed, flexible-walled cartridges can be seen in preparative separations shown in Figures 1 to 8, where the elution profile of the preparative run is similar to that of the analytical run. It should be remembered that these preparative cartridges are packed with 75μm particles and the analytical columns with 10μm particles. The improvement in column efficiency on radial compression of flexible cartridges has been related to the formation of highly efficient chromatographic beds without wall effects [24].

As described in a previous report on the preparative separation of a model tetrapeptide [21], trifluoroacetic acid (TFA) was chosen as the hydrophilic ion pairing reagent on account of its volatility and transparency at the low wavelengths required to monitor elution of these solutes. The effectiveness of this volatile ionic modifier can be assessed in the comparative study shown in Figures 7 and 8 where leucine-enkephalin is purified with a mobile phase which consists of 0.1% phosphoric acid and 0.05% TFA respectively. Clearly the volatile acid allows an equally effective separation as the involatile phosphoric acid. The volatility of TFA allows the facile isolation of the peptide by lyophilis-

ation, after the pH of the sample is adjusted to 7 with ammonium hydroxide. It has been our experience that it is important to carry out the neutralisation step as soon as possible after collection since some peptides are unstable when exposed to the acidic aqueous-organic solvent mixtures for extended periods of time.

At the higher sample loadings used in preparative separations, refractive index detection can be used to monitor the purification of ca. 50mg of a simple peptide (see Figure 1). Also, as is apparent by comparison of parts C and D of Figure 1, the use of a UV detector linked in series with the refractometer can identify certain differences in the chromatogram. In addition UV detection in the preparative separation can be more readily related to the analytical system, where UV detection is often the method of choice due to the relative insensitivity of the refractometer to peptides.

Figure 7 shows that the preparative separation is extremely reproducible as in this Figure two identical loadings of leucine-enkephalin gave exactly the same elution profile when chromatographed under the same chromatographic conditions. Similar reproducibility was obtained with mobile phases containing either trifluoroacetic or phosphoric acid. In the separations described in this report the loading limit of the purification was not established, as the amount of sample available was always less than the expected maximum capacity of the cartridge. In a previous study however, it was found that 10gm of a model tetrapeptide could be chromatographed without loss of separation efficiency [20]. The samples used in this new study were deliberately limited to crude synthetic products to test the resolving power of the chromatographic techniques. In each case it was possible to

demonstrate that the isolated pure peptide was the major component of the crude mixture. For example, in the purification of Pyr-His-Gly (Figure 2), 5gm of a crude sample were loaded with 2gm of pure product being obtained despite the fact that fraction 5 was a conservative cut. In our earlier study it was demonstrated that rechromatography of a purified tetrapeptide gave essentially quantitative recovery [20].

In conclusion, this paper as well as several related studies [20,26-29] have shown that preparative reversed phase HPLC, when used in conjunction with radially compressed columns in the Prep 500 Liquid Chromatograph or with conventional HPLC columns, is a promising technique for the large scale purification of synthetic peptides, under conditions which allow the facile and rapid isolation of the desired compound in excellent yield. As a consequence, these reversed phase HPLC methods should gain wide popularity for the large scale isolation of peptides from natural and synthetic sources and compliment existing capabilities [30,31] for the preparative separation of protected peptides by liquid-solid adsorption HPLC.

ACKNOWLEDGEMENTS

We wish to thank Dr G.G. Midwinter for the amino acid analyses. This investigation was supported in part by the Medical Research Council of New Zealand and a National Heart Foundation of New Zealand Award No.200. We also wish to acknowledge the generous equipment loan may by Waters Assoc. (New Zealand) which made this work possible.

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REVERSED-PHASE, ION-PAIR SEPARATION OF L-METHIONINE AND L-METHIONINE DIPEPTIDES COMPLEXED WITH PALLADIUM (II)

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ABSTRACT

A high performance liquid chromatographic (HPLC) method is described for separation of L-methionine and L- methionine dipeptides complexed with palladium (II). Under isocratic conditions, at room temperatures, with the appropriate selection of counter-ion (cetyltrimethylammonium or trioctylmethylammonium), it was possible by ion-pairing reversed phase chromatography to resolve the palladium (II) complexes studied. Stainless steel and polythylene columns were used. The chromatograms from both the two different materials made columns indicate about the same ratio of capacity factor of the palladium (II) complexes.

INTRODUCTION

Reversed-phase HPLC using the metallic cations Cd(II) and Zn(II) in mobile phase has allowed a good separation of amino-acids and dipeptides (I). The same is true for ion-pair reversed-phase HPLC using anionic and cationic reagents (2, 3). However, no example of the ion-pair formation method for metallic complexes of amino-acids and dipeptides has yet been reported in the literature.

In previous circular dichroism studies (4, 5) of complexes between palladium (II) and sulfur of S-S-group containing amino-acids and peptides, we showed that strong interaction occurred between the sulfur and the metal and we described the different kinds of complexes which were formed via these interactions. The present work is part of an extensive HPLC:CD

study on complexes between Pd(II) and the thioether group (6) and describes how chromatographic conditions have been found in order to achieve good separation of complexes of Pd(II) with L-methionine, L-methionyl-L-alanine and L-alanyl-L-methionine in acidic aqueous medium.

MATERIALS AND METHODS

High performance liquid chromatography equipment is composed of a M6000 A solvent delivery system (Waters Assoc., USA), a U 6K universal liquid chromatograph injector, a SF-770 UV-Vis variable wavelength monitor (Schoeffel, USA) coupled a chart recorder (Meci, France). The stainless steel μ Bondapak-C_{18} (10 μm, 30 cm x 4 mm I.D.) column and polyethylene μ Bondapak-C_{18} column (C_{18}-radial Pak and RCM 100) or radially compressed system of separation (RCSS) were obtained from Waters Assoc. Counter-ions used are : cetyltrimethylammonium bromide, formulated by CH_3-$(CH_2)_{15}$-$N(CH_3)_3$ Br or CTAB (Eastman-Kodak, USA), trioctylmethylammonium chloride, formulated by $(CH_3$-$(CH_2)_7$-$)_3$ NCH_3Cl or Adogen 464 (Serva, W. Germany). All solvents are of analytical grade, pure water obtained from Milli Q system (Millipore, USA), Solvent systems were filtrated on Millipore 0.45 μm and degazed by ultra-sound, 15 min., before use. L-methionine or (M) (Merck, W. Germany), L-methionyl-L-alanine or (MA) and L-alanyl-L-methionine or (AM) (Schwarz-Mann, USA) were dissolved in water (10 mM). Palladium (II) was used as sodium tetrachloropalladate Na_2PdCl_4, 35 % Pd (Prolabo, France) in aqueous solution (100 mM). Fresh stoechiometric complexes were preformed before their injection in the column : 10 μM Pd (II) was added to 1 ml water containing 10 μM of ligand, pH was adjusted to 2.0 by HCl(N). 10 μl was injected at once with precision-syringe (Hamilton, Switzerland). Palladium (II) chelates were detected by absorbance at 380 nm, maximum wavelength of the lowest energy d-d band of the complexed metals ; the non-complexed metal has a weak molar extinction coefficient. The chromatography was performed in isocratic conditions : 20°C, 40 % methanol in water in presence of 2 to 25 mM of counter-ion. Solvent systems were adjusted to pH 3 concentrated H_3PO_4 before use.

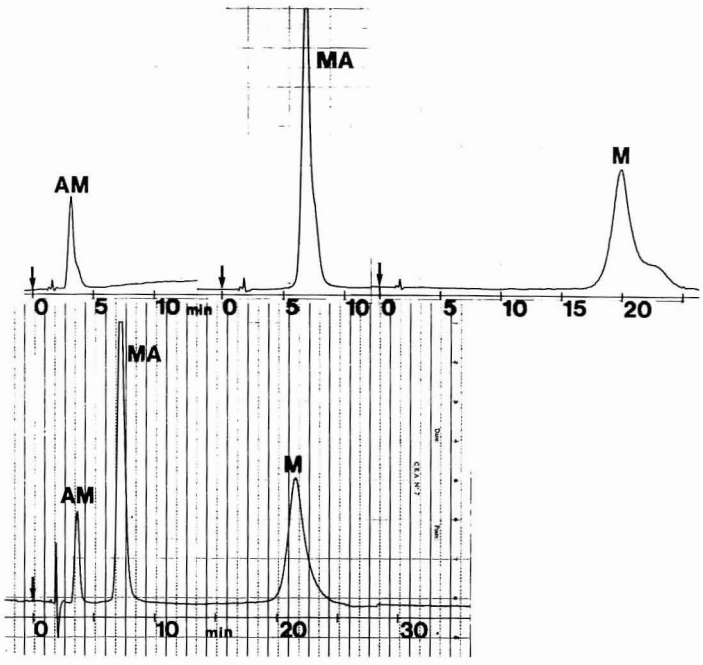

FIGURE 1

Ion-pair separation by reversed-phase chromatography of L-methionine (M), L-methionyl-L-alanine (MA) and L-analyl-L-methionine complexed with $PdCl_4^{-2}$ or Pd(II). Stainless steel column μ Bondapak-C_{18} ; flow rate 2 ml/min ; t = 20°C ; eluent 40 % methanol in water - 2 mM CTAB ; pH adjusted to 3 by concentrated H_3PO_4. Absorbance unit full scale (A.u.f.s.) at 380 nm : 0.040. 10 μl or 0.1 μM of complexes injected. Top : complexes analyzed separately, bottom : mixture of 3 complexes.

RESULTS AND DISCUSSION

Optimal separation of the L-methionine-Pd(II), L-methionyl-L-alanine-Pd(II) and L-alanyl-methionine-Pd(II) complexes was achieved by using a solvent system of 40 % methanol in water in presence of 2 mM CTAB or Adogen 464 as counter-ion acidified to pH 3 by H_3PO_4 (Figs. 1 and 2). A shoulder effect was observed on the M peak (Fig. 1, top), which however occurs only when an old solution (> 24 hours in open tubes) is used. The effect does

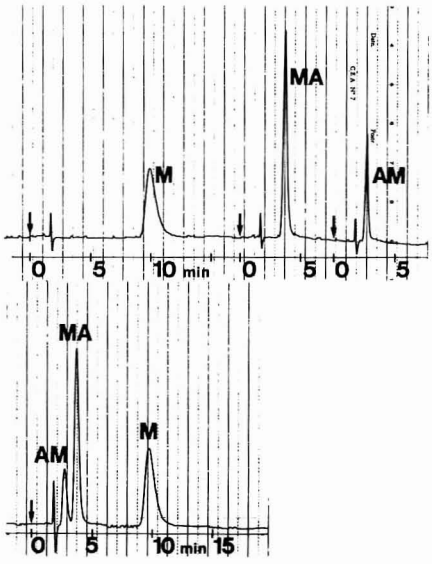

FIGURE 2

Ion pair separation by reversed phase chromatography of L-methionine (M), L-methionyl-L-alanine (MA) and L-alanyl-L-methionine complexed with $PdCl_4^{-2}$ or Pd(II). Experimental conditions as in fig. 1 except for the counter-ion used : Adogen 464.

not exist for freshly performed complexes (Figs. 1 bottom and 2) which indicates that the tailing is caused by the presence of L-methionine sulfoxyde in the medium, arising from the tendency of L-methionine to be oxidized by air even in the absence of metallic cations (7).

Acetonitrile as solvent in the mobile phase was not inert towards $PdCl_4^{-2}$ since a slight precipitate was observed probably corresponding to a complex $Pd(CH_3CN)_2Cl_2$ (8). Stainless steel tubing and column are not altered by several injections of 0.1 µM of the complexes but by 2 mM $PdCl_4^{-2}$ alone in the mobile phase, as might be expected from the electromotive series.

To check the first observation, separation of preformed complexes were achieved on the C_{18}-polyethylene column of the RCSS-Waters system. The chromatograms obtained from the stainless steel column and the polyethylene column (not shown) indicate about the same ratio of capacity factor of the complexes for the two solvent systems used. Therefore, the metal (if any) from the stainless steel column does not interfere with palladium (II) in the complexation process between Pd(II) and M, MA or AM. On the other hand, the anionic reagent, sodium dodecyl sulfate (SdS) did not yield separation of Pd(II) chelates. The result clearly indicates that Pd(II) chelates and SdS adsorbed on the stationary phase do not form ion-pairs (9), and suggests that in these conditions both palladium (II) complexes and SdS were charged negatively.

pH effects corroborate the role of the sign of the electrical charges on the separation of complexes. In the solvent system consisting of 40 % methanol in water and 2 mM CTAB at pH 5.6, under the dynamic conditions of ionization in the column, palladium (II) chelates should have their carboxylic groups completely deprotonated and therefore should be retained. When H_3PO_4 is added to the mobile phase for a decrease in pH to 3, the observed retention time of complexes is now compatible with a good separation (Figs. 1 and 2). This result could be explained by the partial ionization at pH 3 of the carboxylic group (X-ray studies on the L-methionine-Pd(II) complex show that the carboxylic group is free of all interaction) (10), by the minimal interaction of $H_2PO_4^{-2}$ with the stationary phase and the hydrophilic mechanism of ion-pairing of $H_2PO_4^{-2}$ with palladium (II) chelates (11).

Chromatographically, we can demonstrate the complexation effect of palladium (II) with L-methionine, L-methionyl-L-alanine and L-alanyl-methionine. Injection of $PdCl_4^{-2}$ alone in the (40 % methanol in water, 2 mM CTAB, pH 3), solvent system leads to elution which does not show any absorbance at 380 nm. $PdCl_4^{-2}$ is completely retained and can be eluted only when 10 mM trichloroacetate was used instead of 2 mM CTAB in the mobile phase (6). In contrast, successive injections of $PdCl_4^{-2}$ alone and then of ligand L-methionine or L-methionyl-L-alanine

or L-analyl-L-methionine yield a chromatogram similar to the one of the preformed complex. Ammonium counter-ion e.g. CTAB with a long alkyl chain is known to be adsorbed on the C_{18}-stationary phase (11), a dynamic ion-exchange mechanism should be proposed according to the previous observation :

$$2\ CTAB^{+}_{stat.phase} + PdCl_4^{-2} \rightleftharpoons (2\ CTAB^{+} - PdCl_4^{-2})_{stat.phase}$$

$$(2\ CTAB^{+} - PdCl_4^{-2})_{stat.phase} + M \rightleftharpoons 2\ CTAB^{+} + (M\text{-}PdCl_4^{-2})_{mob.phase}$$

Experiments were also carried out with different concentrations of cationic hydrophobic ion-pairing reagents. A plot

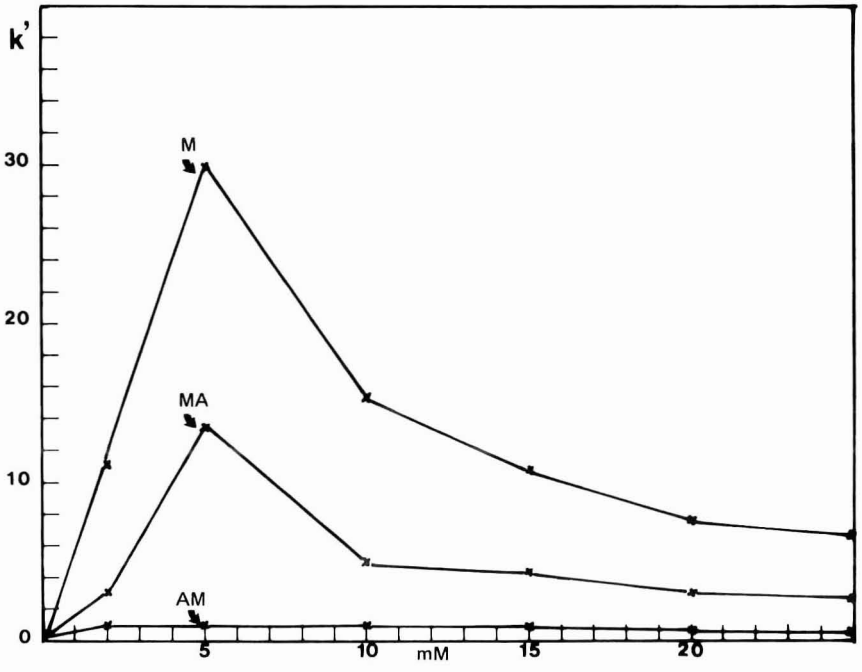

FIGURE 3

Dependence of the capacity factor L-methionine (M), L-methionyl-L-alanine (MA), L-alanyl-L-methionine (AM) complexed by Pd(II) on the concentration of CTAB in the mobile phase. Experimental conditions as in fig. 1.

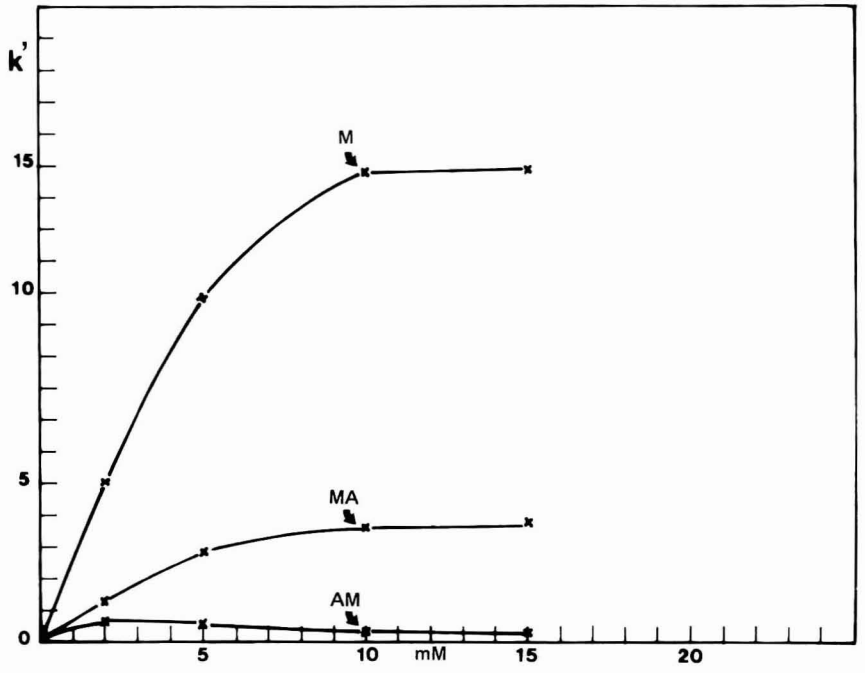

FIGURE 4

Dependence of the capacity factor of L-methionine (M), L-methionyl-L-alanine (MA), L-analyl-L-methionine (AM) complexed by Pd(II) on the concentration of Adogen 464 in the mobile phase. Experimental conditions as in fig. 1.

of the capacity factor of palladium (II) chelates versus increasing concentration of cationic reagents shows an optimum at 5 mM CTAB and 10 mM Adogen 464 (Figs. 3 and 4). The difference of retention time of complexes at the same concentration of counter-ion (2 mM) should be explained by the difference of hydrophobic area between the two counter-ions (C_{16} for CTAB and $3XC_8$ for Adogen 464) but also by the steric hindrance on the quaternary ammonium. The decrease of the capacity factor of complexes with increasing concentration of counter-ion (Fig. 3) could be interpreted, within the ion-pairing mechanism (2, 3), as being due to the lack of balance of the hydrophobic interactions :

$$(\text{counter-ion})_{\text{mob.phase}} - \text{Stat. phase}$$

$$> (\text{counter-ion-complex})_{\text{mob.phase}} - \text{Stat. phase}$$

However this cannot be true for the Adogen 464 counter-ion (Fig. 4) owing to the difference of the hydrophobic area between CTAB and Adogen 464.

We think that this method of dynamic ion-exchange is useful for rapid and efficient separation of metal chelates of amino-acids or oligopeptides in the analytical range.

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SEPARATION OF COBALT (III) BIS(ETHYLENEDIAMINE) AMINO ACID COMPLEXES BY REVERSED PHASE HPLC

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ABSTRACT

Methods for the rapid analysis of amino acid cobalt (III) bis(ethylenediamine) complexes by reversed phase high performance liquid chromatography (HPLC) are described with mobile phases containing the pairing ions, p-toluenesulphonate and hexanesulphonate. Under these conditions, the amino acid cobalt (III) bis(ethylenediamine) complexes elute in order of the relative hydrophobicities of the parent amino acids which suggests that the amino acid side chain makes a significant contribution to the retention mechanism. At high sample loadings, these complexes shows a concentration dependent peak splitting effect divergent to that normally experienced with inadequate buffering capacity of the pairing ion reagent.

INTRODUCTION

Over the last several years, reversed phase high performance liquid chromatography (HPLC) has become established as a powerful technique for the rapid separation and analysis of amino acids and peptides. A feature of these advances has been

the development of a variety of novel elution systems which allow adequate control over selectivity and column efficiencies for the separation of these polar amphoteric solutes on microparticulate chemically-bonded hydrocarbonaceous silicas. Our previous experiences [1-3] with these techniques has now led us to investigate the application of similar methods as a means of separating and identifying charged metal complexes of the classical 'Werner' type, particularly as they apply to the monitoring of cobalt (III)-induced synthesis of small peptides [4]. Charged metal complexes of this type have conventionally been separated by ion-exchange or thin layer chromatographic methods [5-7]. It was anticipated that the reversed phase HPLC techniques would allow considerably shorter analysis times, improved resolution and excellent reproducibility if suitable elution systems, particularly those which could exploit pairing ion interactions between the metal complexes and components in the mobile phase, could be developed. In this paper we wish to described the separation of a series of amino acid Co(III) bis (ethylene-diamine) complexes, $[Co(en)_2AA]X_2$, under such conditions.

MATERIALS AND METHODS

All solvents were AnalaR grade and purified as reported earlier [8]. Orthophosphoric acid was from May and Baker Ltd., p-toluenesulphonic acid was from BDH, sodium hexanesulphonate was prepared according to published procedures [9]. Water was de-ionised by reverse osmosis and distilled. The bis (ethylenediamine) cobalt (III) chelates of the amino acids were prepared by established methods and the individual diasteriomeric pairs isolated and resolved as described previously [10-13]. All amino acids except for glycine were of the S-configuration.

Optical rotation measurements were carried out on a Perkin Elmer 141 polarimeter using 1dm cells ($\pm$ 0.004^{o}).

All chromatograms were carried out at room temperature (ca. 20^{o}) using isocratic or linear gradient elution conditions with a Waters High Performance Liquid Chromatographic System (two M6000A solvent delivery units, M660 solvent programmer, U6K universal injector) coupled to a M450 variable wavelength UV-visible monitor and a Rikadenki dual channel chart recorder. The μBondapak C_{18} columns (10μm, 30cm x 3.9mm I.D.) were purchased from Waters Associates (N.Z.), Auckland. All columns were equilibrated to new solvent conditions, or following gradient elution to the initial mobile phase conditions, for at least 30min. Flow rate were maintained between 2.0 and 2.5ml min^{-1}, as indicated in the text. Detection of the cobalt complexes was generally at 480nm; in some cases detection was also carried out at 350nm and/or 254nm depending on the nature of the sample and the mobile phase. Sample injections were made with Pressure Lok liquid syringes, Series B110, from Precision Sampling (Baton Rouge, La., U.S.A.).

RESULTS AND DISCUSSION

The pseudo-octahedral bisethylenediamine cobalt (III) complexes of α-amino acid derivatives contain chiral centres about both the metal atom (Δ- or Λ-) and the α-carbon atom of the amino acid (R- or S-). Consequently, these metal complexes exist as diastereoisomers. In order to simplify the analysis, only metal complexes with amino acids of the S-configuration were used, ie. only the diastereoisimeric pairs Δ(S), Λ(S) (Fig.1a,b) were employed. Since these metal complexes exist as cationic 2-plus ions in solution (counterion is often I^{-}, Cl^{-} or ClO_4^{-}), it is not surprising that on octadecylsilica with neat aqueous eluents over a variety of pH

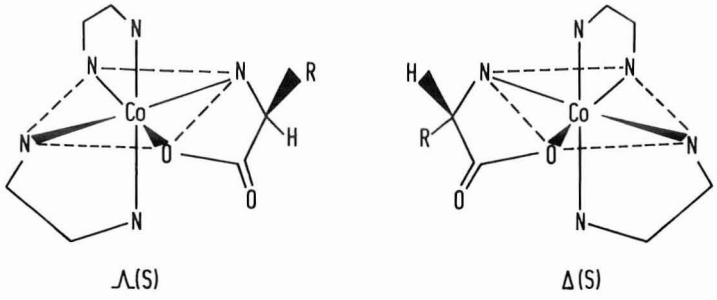

Figure 1.

Two diastereoisomers of the cobalt (III) bisethylene-diamine amino acid complexes.

conditions, they showed no, or little, retention, ie. k′ values <1. In order to obtain satisfactory retention and selectivity recourse was made to the use of low concentrations, of the anionic pairing ions, p-toluente sulphonate and hexane-sulphonate. With chemically bonded alkylsilicas, hydrophobic anionic reagents of this type are known to act as surface active ions as revealed by Freundlich adsorption isotherms [2,8,14-16]. The effect of these reagents on amino acid and peptides retention to alkylsilicas has been discussed [2,3,8] in terms of pairing ion:dynamic liquid-liquid ion-exchange interactions with both hyperbolic and parabolic dependencies of k′ versus pairing ion concentration being evident. A discussion of this concentration dependency, and the manner it can be used to obtain improved selectivity in the separation of a wide range of charged Co(III) complexes will be deferred to later [17]. In the present study 5mM of the pairing ions was chosen for most experiments on the basis of this analysis.

Figure 2 shows a typical separation of the amino acid complexes Δ-$[Co(en)_2AA]^{2+}$ (AA= gly,pro,val,leu, phe) whilst the Table illustrates the effect of pairing ion structure

Figure 2.

Gradient elution profile of the Δ[Co(en)$_2$AA]I$_2$ complexes using 5mM p-toluenesulphonate as the pairing ion. The elution order for the complexes was: 1, gly (t_R 5.6min); 2, pro (t_R 6.8min); 3, val (t_R 8.8min); 4, leu (t_R 10.8min); 5, phe (t_R 13.8min). Chromatographic conditions: column, µBondapak C_{18} ; flow rate, 2.5ml min^{-1}; temperature, 18^o; elution conditions, 15min. linear gradient from 0 to 100% methanol, containing 5mM p-toluene sulphonate, pH 3.5, sample volume 10µl containing 367 nmol complexes.

on retention. As can be seen, the elution order of the cobalt complexes is the same with both the p-toluenesulphonate and the hexanesulphonate pairing ion system, although to achieve comparable k´ values at a pairing ion concentration of 5mM, the percentage methanol had to be increased approximately ten-fold from 2.5% with the p-toluenesulphonate system to 24% with the hexanesulphonate system. Similar

Table

Comparison of Capacity Factors for the $[Co(en)_2AA]$ Complexes with two Different Pairing Ions.

Co(III) complex AA =	5mM Pairing Ion	
	p-Toluenesulphonate*	Hexanesulphonate†
	$\underline{k'}$	$\underline{k'}$
-Gly	1.67	1.67
-Ala	1.66	1.83
-Pro	2.11	2.00
-Val	4.00	3.56

* Mobile phase: 2.5% methanol-water, pH 3.5.

† Mobile phase: 24% methanol-water, pH 3.5.

effects have been noted [2,3,8,18] previously with peptide separations under a variety of pairing ion and organic modifier conditions. Compared to traditional methods of analysis, these HPLC separations show excellent control over selectivities, peak shape and short elution times, eg. the above five cobalt (III) complexes could all be eluted reproducibly within 15min of injection. The elution order of these charged metal complexes, eg. k' Gly<Pro<Val<Leu<Phe, which is the same as that observed [19,20] for the free amino acids when chromatographed on alkylsilicas and similar to that found [21] for these complexes when separated by partition chromatography on cellulose, clearly indicates that there is a preferred orientation of the charged complex with regard to the hydrocarbonaceous stationary phase, such that the amino acid side chain participates significantly in the retention mechanism.

This feature can be exploited for the separation of Δ-S and Λ-S or Δ-R and Λ-R diastereoisomeric mixtures [17]. As anticipated the nature of the cobalt complex salt counterion, $[Co(en)_2AA]X_2$ X = iodide, chloride, perchlorate, did not affect the resolution.

During the course of these studies, we observed an unusual chromatographic effect which is dependent on the association of two (or more) different cationic species and is distinguished from related phenomena normally experienced with distribution coefficient perturbation arising from an inadequate buffering capacity of the pairing ion reagent. Figure 3 summarises these observations. At constant pairing ion conditions but increasing sample loading of two charged cobalt complexes, eg. the Λ-Gly and Λ-Pro complexes, each component peak was progressively split into two (Fig. 3a-d). This peak splitting could also be achieved by lowering the pairing ion concentration at fixed solute concentration but was clearly distinguished from conventional 'overloading' phenomena when the buffer capacity of the pairing ion is inadequate by increasing loadings of only one cobalt-amino acid complex, eg. Λ-Gly complex. In this case the development of a broad asymmetic preview peak, preceding a sharper peak and typical of conventional overloading effects [22-24] was observed. Furthermore the achiral complexes such as $[Co(NH_3)_5X]^{2+}$, X = $CH_3CO_2^-$, $C_2H_5CO_2^-$, showed a similar peak splitting effect as the sample loading was increased.

That this splitting effect must involve an interaction between two (or more) complex ions, the pairing ion, and the stationary phase was demonstrated by the following experiments. Firstly, the Λ-Gly complex was loaded onto the column following the Δ-Val complex after a 10 second delay and 30 second delay (Fig. 4a,b). Peak splitting was evident when both complexes were coinjected or when the Λ-Gly was injected within 10 seconds

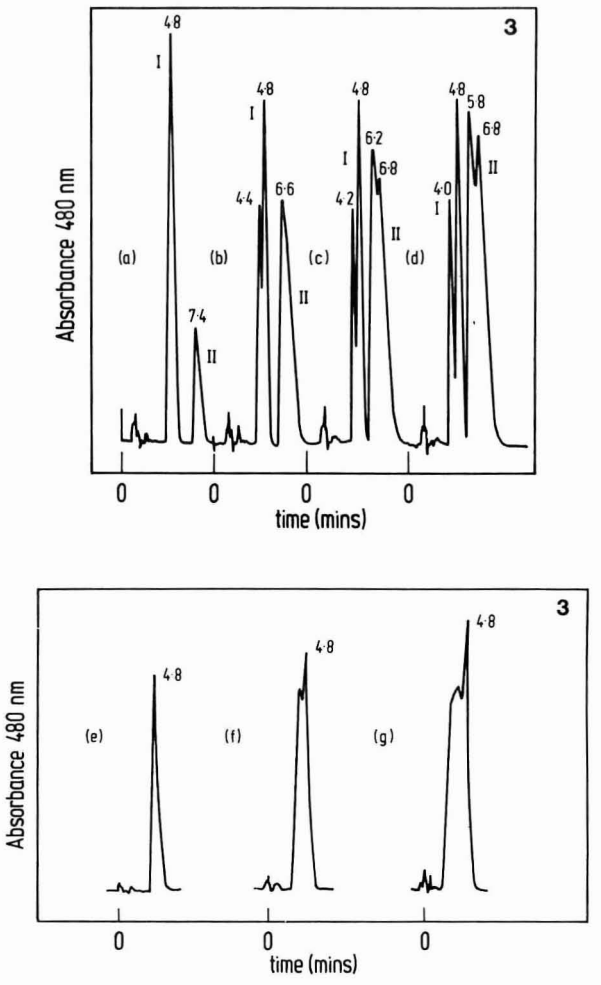

Figure 3.

Effect of increasing sample load with mixtures of the $[Co(en)_2AA]$ complexes, AA = Gly (I) and Pro (II), (a-d) and the individual complex $[Co(en)_2Gly]$ (e-g). Chromatographic conditions: column, μBondapak C_{18}; flow rate, 2.0ml min^{-1}; temperature 18°; mobile phase, 2.5% methanol-water-5mM p-toluenesulphonate, pH 3.5; sample concentrations: (a-d) Λ-$[Co(en)_2Gly]$ complex 438nmol in 30μl plus Λ-$[Co(en)_2Pro]$ complex at (a) 270 nmol in 5μl, (b) 1080nmol in 20μl, (c) 1620nmol in 30μl (d) 2160nmol in 40μl concentration and (e-g) Λ-$[Co(en)_2Gly]$ complex alone at (e) 591nmol in 30μl, (f) 1182nmol in 60μl and (g) 1970 nmol in 100μl concentration. The t_Rs for the peak maxima are given in minutes.

Figure 4.

Elution profiles obtained by (a) loading the Λ[Co(en)$_2$Gly] complex (1) 10 seconds after the Δ[Co(en)$_2$Val] complex (2) and (b) loading the Λ-[Co(en)$_2$Gly] complex (1) 30 seconds after the Δ-[Co(en)$_2$Val] complex (2) Chromatographic conditions as for Fig.3.

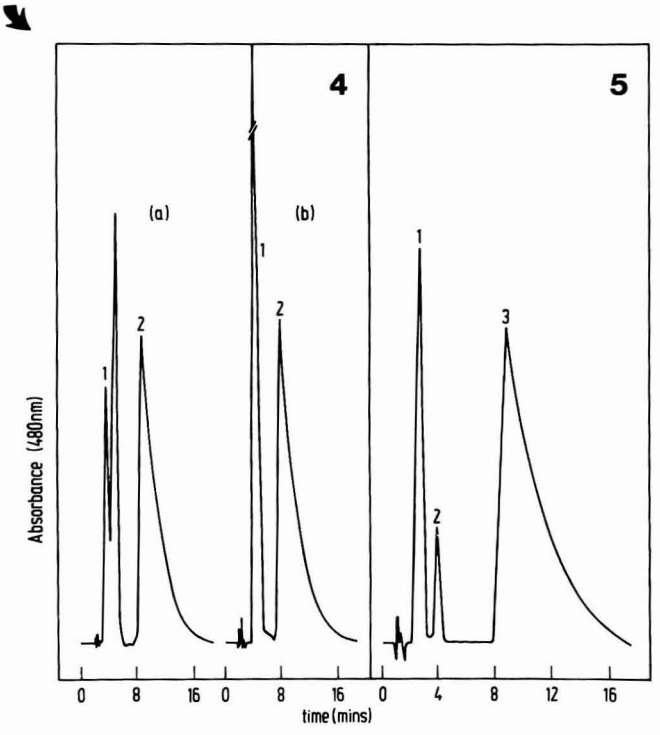

Figure 5.

Elution profile obtained for a mixture of Λ-[Co(en)$_2$Gly] and Δ-[Co(en)$_2$Val] complexes on a μBondapak C_{18} column. Chromatographic conditions as in Fig. 3. After recovery and desalting on Sephadex SP-25, the optical rotations (deg.mol^{-1}.dm^3.cm^{-1}) of the three peak components were: 1, (+ 1.43 ± 0.03) x 10^3; 2, (+ 1.48 ± 0.03) x 10^3; 3, (-2.11 ± 0.04) x 10^3; the values for the optically pure Λ-[Co(en)$_2$Gly] and Δ-[Co(en)$_2$Val] complexes are (+ 1.45 ± 0.03) x 10^3 and (-2.12 ± 0.04) x 10^3 respectively.

of the Δ-Val complex, but did not arise with a longer delay time, despite the fact that the Λ-Gly complex still overtakes the Δ-Val complex on the column. Secondly, it was possible to base-line resolve and recover the peaks for the Λ-Gly complex generated in the presence of the Δ-Val complex (Fig.5). Optical rotation measurements were carried out on these recovered peaks, following desalting on Sephadex SP-25. The optical rotation values obtained were: peak 1, (+ 1.45 ± 0.03) x 10^3 $deg.mol^{-1}.dm^3.cm^{-1}$; peak 2, (+1.48 ± 0.03) x 10^3 $deg.mol^{-1}.dm^3.cm^{-1}$; peak 3, (-2.11 ± 0.04) x 10^3 $deg.mol^{-1}.dm^3.cm^{-1}$. The values for the optically pure Λ-Gly complex and Δ-Val complex are (+ 1.45 ± 0.03) x 10^3 and (-2.12 ± 0.04) x 10^3 $deg.mol^{-1}.dm^3.cm^{-1}$ respectively. This result clearly indicates that both peaks 1 and 2 in Fig. 5 are due solely to the Λ-Gly complex. This was confirmed by re-injecting the recovered peaks under analytical conditions. It thus appears that this type of splitting of individual complexes into multiple peaks on the column arises, in part, due to a co-operative effect between different complexes in the mixture, and is not a property of the loading or the loading zone of an individual complex.

These experiments show that there must be an interaction between two (or more) complex ions which influences their respective distribution ratios, in addition to the normal pairing ion depletion effect observed at high sample concentrations. Under these conditions, interactions between the complex cations and the pairing ion apparently lead to species of different counterion stoichiometry or geometry which have sufficient lifetime on the matrix of the stationary phase to allow separation as discrete entities. This effect can be easily avoided for analytical purposes, eg. for the p-toluene-sulphonate-2.5% MeOH mobile phase, peak splitting can be prevented if the solute load (nmoles) to the pairing ion

concentration (mM) ratio is less than 100:1, but could lead to serious misinterpretation with regard to peak assignments, especially when large amounts of these metal complexes are used in preparative studies.

In summary, two aspects of the reversed phase HPLC separation of water soluble Werner type cationic complex ions using mobile phases containing hydrophobic anionic pairing ions have been examined. Conditions for the chromatographic resolution of $[Co(en)_2AA]X_2$ complexes have been developed. These procedures also allow the preparative recovery of individual complexes with, in many cases, elution times under 30min. The application of these techniques to the separation of peptide complexes is under investigation.

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RESOLUTION OF THE OPTICAL ISOMERS OF UNDERIVATISIZED AMINO ACIDS ON CHEMICALLY BONDED CHIRAL PHASES BY LIGAND EXCHANGE CHROMATOGRAPHY.

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ABSTRACT

A method for the direct separation of racemates by HPLC is described. A chiral stationary phase is synthesized, suitable for ligand exchange chromatography. L-proline is chemically bonded to silica gel via 3-glycidoxypropyltrimethoxysilane. The bonded support is loaded with Cu(II) ions as a complexing agent. Complete resolution of amino acid racemates can be obtained in less than ten minutes. α-values up to 3.5 are observed.

INTRODUCTION

Chromatographic resolution of optical isomers is based either on the formation of diastereomers or on application of chiral stationary or mobile phases. A number of reports appear in the literature, describing attempts to separate racemates using natural and later synthetic optically active polymeric sorbents (1-5).

Recently, ligand exchange chromatography was introduced for the separation of enantiomers. Davankov et al. (6-13), Snyder et al. (14), and later Josefonvicz et al. (15) used polystyrenedivinylbenzene resins, containing L-aminoacids as chiral groups. They described complete separations of DL-aminoacids with Cu(II) ions as a complexing agent. The time taken for separation, however, is about ten hours. Shorter separation times are obtained by Lefebvre et al. (16-18), using acrylamide polymers substituted by L-amino acids. The synthesis of such polymers in HPLC-quality, however, is very complicated and difficult to reproduce. Another approach, namely the addition of chiral metal chelates to the mobile phase was chosen by several authors (19-23).

In a previous paper, we reported the synthesis of a chemically bonded chiral phase for the separation of DL-amino acids by ligand exchange chromatography (24). The stationary phase consists of L-proline, chemically bonded to silica gel and loaded with Cu(II) ions. This paper deals with the optimation of this method and its application to the separation of a great number of racemic amino acids.

EXPERIMENTAL

Reagents:

Silica gel, LiChrosorb Si 100, 10 µm, was obtained from Merck (Darmstadt, Germany).

3-Glycidoxypropyltrimethoxysilane was purchased from Serva (Heidelberg, Germany).

Amino acids were obtained from Sigma chemical company (St.Louis Missouri, USA).

Potassium dihydrogen phosphate and disodium hydrogen phosphate were obtained from Merck (Darmstadt, Germany).

All solvents were of reagent grade.

Instrumentation:

The liquid chromatograph consisted of a Perkin-Elmer Series 2 pump equipped with a Rheodyne 7105 injector, a Perin-Elmer LC 55 UV detector and a Perkin-Elmer 023 recorder.

Stainless steel columns of 5, 10 and 25 cm length and 0.46 cm I.D. were used.

Volumes of 20 µl containing about 10 µg amino acid were injected. Detection was carried out at 220 nm.

Synthesis:

10 g Silica gel, 10 µm, dried for 2 hours at 150° are suspended in 50 ml dry benzene. After the addition of 5 ml 3-glycidoxypropyltrimethoxysilane, the suspension is refluxed for 6 hours. The reflux condenser is kept at 65° in order to remove the formed methanol from the mixture (25). After cooling, the benzene is removed by filtration and the product is suspended in methanol. 9 g sodium prolinate are added and the mixture is shaken for 48 hours at room temperature. The bonded support is then filtered, washed with methanol and dried.

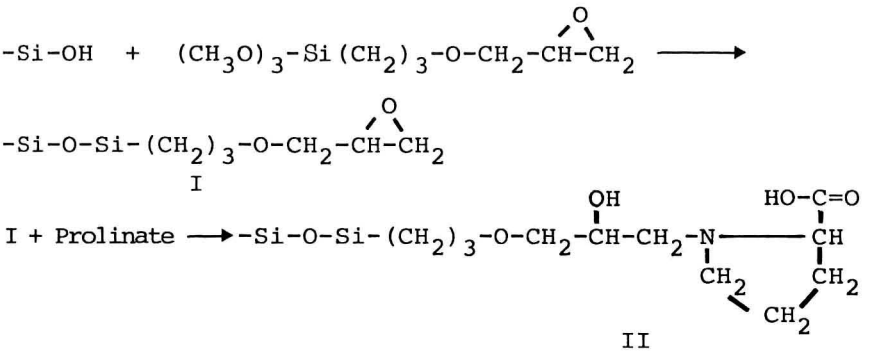
The complex formation is either subsequently carried out by treatment of the material with aquous copper nitrate solution or by passing copper nitrate solution through the column.

Columns are packed by the ascending slurry technique (26).

RESULTS AND DISCUSSION

Various silanes were tested for their suitability to form appropiate reaction intermediates with silica for the attachement of L-α-amino acids as chiral components. 3-Glycidoxypropyltrimethoxysilane, known as reagent in the synthesis of "diol"-phases (27), was found to be a suitable one.

The synthesis of the support is described in the following reaction scheme:



3-Glycidoxypropyltrimethoxysilane is bonded to silica gel in the first step. L-Proline (as sodium salt) is attached then to the epoxyd I in the second step to give product II. As a complexing agent Cu(II)ions were used. The loading of the support with metal ions can be carried out before packing and "in situ" after packing. Better results were achieved by "in situ" loading.

Repeated synthesis of the material resulted in products with well conformable elementary analysis and reproducible chromatographic properties. The elementary analysis of the final product showed 8.1% ± 1% C, 1.8% ± 0.2% H, 0.9% ± 0.1% N and 1.5% ± 0.2% Cu.

Per 2 mole L-proline 1 mole copper is bonded, indicating that a bidentate complex is formed (Fig. 1).

The material showed a good stability in the tested pH range from 4 to 8. The columns were used for several weeks without loss of selectivity. After longer use, they can be regenerated by passing a copper solution through the column. The number of theoretical plates, determined with D-valine, was about 2500/m. Fig. 2

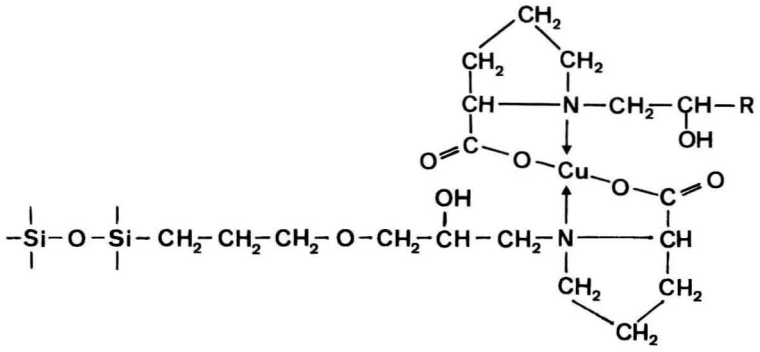


Figure 1 : Complex on the stationary phase

shows the dependence of the height equivalent of theoretical plates (HETP) on the linear flow.

The principle of ligand exchange chromatography was discussed by Davankov et al. (5).

$$R - Me - R + D(L)R' \overset{K_{D(L)}}{\rightleftharpoons} R - Me - D(L)R'$$

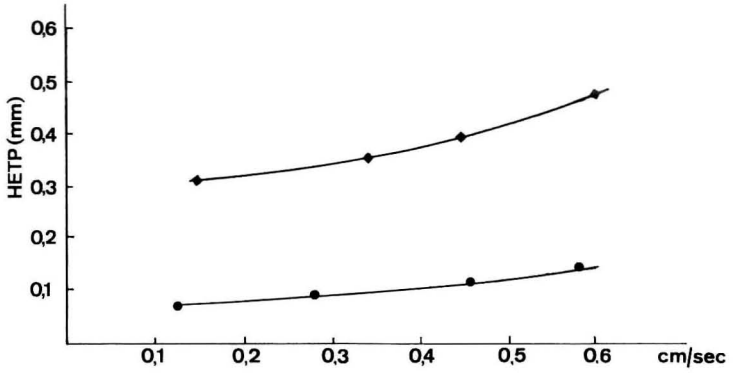


Figure 2 : Dependence of HETP on the linear flow.
D-Valine (□□□), $NaNO_3$ (ooo)
Column: 25 x 0.46 cm; mobile phase: 0.05 M KH_2PO_4, pH 4.6;
Temperature: 50°

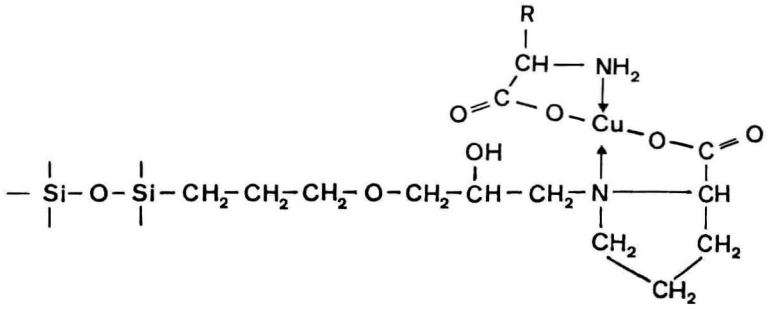


Figure 3 : Structure of the mixed complex between the fixed ligand and D- or L-amino acid

A ligand (R = L-proline), fixed on the stationary phase is replaced by a mobile ligand (R' = D and L-amino acid, respectively) to give the mixed complex, shown in Fig. 3.

The stereoselectivity depends greatly on the structure of the stationary phase.

For optimal resolution it seems to be necessary for the carboxy group to be free for complex forming with the metal ion. These observations have been confirmed by the results of Foucault et al., who bonded L-proline to silica via an amide bonding, using 3-triethoxysilylpropylamine as the coupling component. They described only partial resolution with this sorbens (28).

For the optimation of the conditions of separation, the influence of temperature, pH and ionic strength of the mobile phase have been thoroughly investigated. The increase in temperature results in a significant reduction of HETP.

A significant improvement of selectivity can be observed. Using a 0.05 M KH_2PO_4 solution, pH 4.6, at 50° a complete resolution of a great number of amino acids can be obtained within 2 - 15 minutes. Typical results of separation are shown in Fig. 4 and 5.

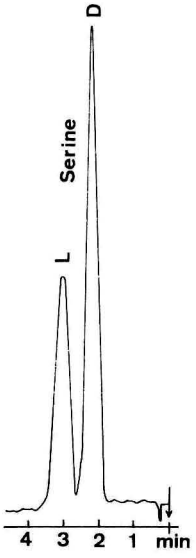


Figure 4 : Separation of D,L-serine
Column: 25 x 0.46 cm
Mobile phase: 0.05 M KH_2PO_4-buffer, pH 4.6
Temperature: 50°
Flow rate: 5 ml/min

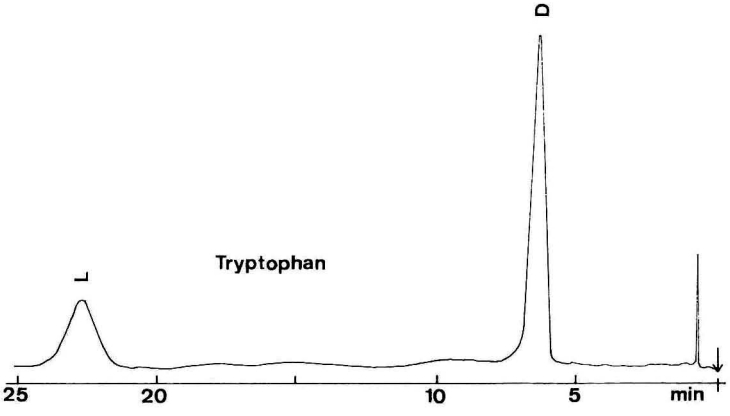


Figure 5 : Separation of D,L-tryptophan
Conditions as in figure 4

Amino acids with aromatic substituents are more strongly retarded and show high α-values. The α-value for DL-tryptophan, for example, is 3.5. By using a short column (4 cm) and a high flow rate, a rapid resolution of DL-tryptophan is achieved within 3 minutes. The K' and α-values of some DL-amino acids are given in Table 1.

The identification of the separated enantiomers was carried out by comparing their retention times with those of reference substances. Furthermore, fractions were collected and the optical rotation was measured.

An enzymatic method was also used as proof. L-Tyrosinedecarboxylase, for example, destroys only the L-form of tyrosine and forms tyramine (Fig. 6).

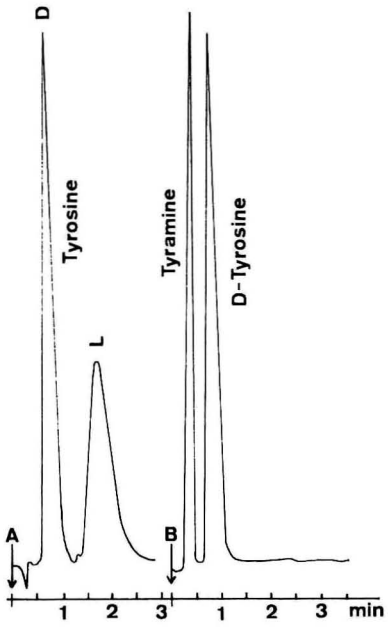


Figure 6 : Enzymatic cleavage of D,L-tyrosine with L-tyrosinedecarboxylase
A : Injection of the racemate before treatment
B : After treatment

TABLE 1

K'-values and relative retention ($\alpha = K'_{(L)}/K'_{(D)}$) for DL-amino acids.

Column: 25 x 0.46 cm; mobile phase: 0.05 M KH_2PO_4, pH 4.6; flow rate: 2 ml/min.

DL-amino acid	$K'_{(D)}$	$K'_{(L)}$	α
Valine	2.5	3.8	1.5
Serine	2.0	3.2	1.6
Proline	2.4	1.4	0.6
Histidine	6.7	12.1	1.8
Phenylalanine	3.2	9.4	2.9
Tyrosine	3.3	10.2	3.1
Tryptophan	7.8	27.4	3.5

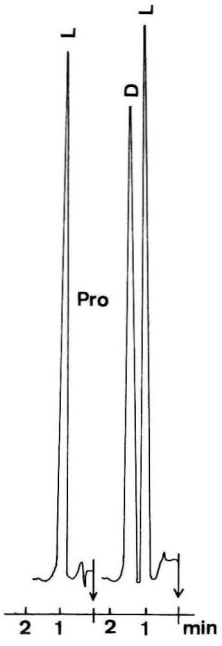


Figure 7 : Separation of D,L-proline
Conditions as in figure 4

On columns containing L-proline as a fixed ligand, the L-enantiomers constantly appeared with higher K' values. An exception was proline, where a reversed sequence was observed (Fig. 7).

An application of this method on a preparative scale is also possible. On an analytic column of 25 cm length and 0.46 cm I.D. up to one and a half mg amino acid racemate can be separated without significant change in K'-values and resolution.

CONCLUSION

Ligand exchange chromatography on chemically bonded chiral phases offers new possibilities for rapid separation of racemic compounds on an analytical or preparative scale. The use of a special bonded phase combines the selectivity of ligand exchange chromatography and the efficiency of HPLC. Further work needs to be done in order to clear some theoretical aspects. The influence of various bonded amino acids and also of the fixed metal ions, on the stereoselectivity, is now beeing studied.

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This work is dedicated to Univ.-Prof.Dr.G.Zigeuner on his 60th birthday.

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ASSAY OF ARGININE-ESTERASE ACTIVITIES BY REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Application of a high performance liquid chromatographic technique to assay of arginine-esterase activities is presented. Enzyme reaction was carried out with benzoyl-L-arginine ethylester as a substrate and analysis was performed on a reversed phase chromatographic system using a μ Bondapak C_{18} or a Radial-PAK A column and buffered aqueous methanol as the mobile phase. The enzyme activities were determined by the peak height of cleaved product (benzoyl-L-arginine). The minimum detection limit for benzoyl-L-arginine was 0.02 nM on each column. The generality of this method was demonstrated by its application to determination of plasmin activity, and so it might be suitable for both kinetic studies and routine assays of plasmin-like esterases.

INTRODUCTION

Several methods are available at present for the determination of plasmin (EC 3.4.21.7)-like esterases such as kallikrein (EC 3.4.21.8), trypsin (EC 3.4.21.4) and other arginine-esterases using various arginine derivatives such as benzoyl-L-arginine ethylester (BAEE) and tosyl-L-arginine methylester (TAME) as substrates.

In these assays, enzyme activities are measured by following the increase in the optical density due to the hydrolyzed product (1,2), or by direct electrometric titration of the carboxyl groups liberated (3,4), by colorimetric determination of hydroxamate-ferric complex (5,6) or the color developed by chromotropic acid reagent (7,8), and also by following the oxidation of alcohol liberated with alcohol dehydrogenase (9).

Recently a new sensitive method for the rapid determination of arginine-esterases using high performance liquid chromatography (HPLC) to separative determination of hydrolyzed product of TAME or BAEE has been reported by Matsumoto et al. (10). However, under the conditions used in their experiments the substrates were retained on the column, and the column condition became worse by repeated injection of incubation mixture.

We now report a rapid and sensitive method for the assay of plasmin using BAEE as a substrate, the product(s) of the reaction being analyzed by reversed phase system of HPLC. This method, as demonstrated by its successful application to plasmin (11) and kallikrein (12) in our laboratory, appears to be of wide utility.

MATERIALS AND METHODS

Reagents

Benzoyl-L-arginine (BA) and BAEE·HCl were purchased from Protein Research Foundation, Osaka, Japan. Human plasmin (25 CU/vial) was obtained from AB KABI, Stockholm, Sweden. High-purity methanol was of Katayama Chemical Industry Co., Ltd., Osaka, Japan. All other chemicals were of analytical grade.

Chromatographic system

Analyses were carried out on a liquid chromatograph consisting of a solvent delivery system (Model

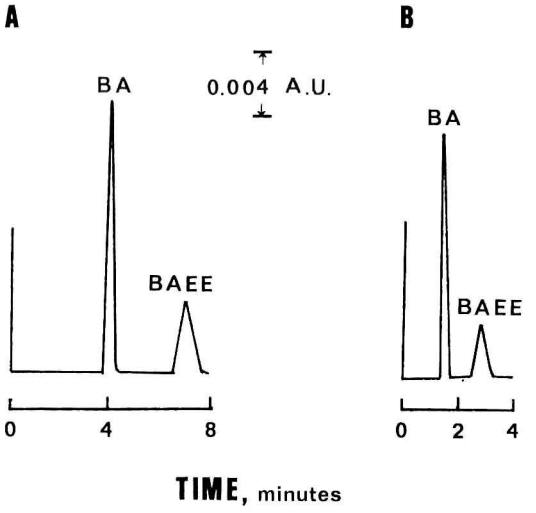

FIGURE 1

Chromatograms of a standard mixture of BA and BAEE (12.5 nM each).
Column: A) μ Bondapak C_{18} (4 mm ID x 30 cm)
B) Radial-PAK A^{18} (8 mm ID x 10 cm)
Solvent: A) 0.01 M ammonium acetate, pH 6.8/methanol (50 : 50, v/v)
B) 0.02 M sodium sulfate and acetic acid, pH 4.6/methanol (20 : 80, v/v)
Flow rate: A) 1.0 ml/min. B) 4.0 ml/min.

6000 A, Waters Associates, Milford, Mass.), an injector (Model U 6K, Waters Associates), a reversed phase analytical column (μ Bondapak C_{18} or Radial-PAK A, each from Waters Associates), an absorbance detector (Model 440, Waters Associates) operated at 254 nm, and a 10 mV chart recorder (Type 056, Hitachi, Co., Ltd., Tokyo, Japan). The solvent systems were as indicated in Figure 1. The chromatographic system was operated at room temperature and the flow rates for μ Bondapak C_{18} and Radial-PAK A columns were set at 1.0 ml/min. and 4.0 ml/min., respectively.

Procedure

Enzyme activity was measured by the following procedure. Fifty µl of enzyme solution was added to 250 µl of BAEE solution (10 µM/ml in 0.05 M Tris-HCl buffer to a final volume of 400 µl. The resulting mixture was incubated at 37°C for 30 minutes. After incubation, the reaction was stopped by the addition of 100 µl of 10 % (w/v) trichloroacetic acid, and the solution was filtrated through a 0.45 µm filter (Millipore Corp. Bedford, Mass.). Five µl of the filtrate was then injected into the chromatograph.

RESULTS AND DISCUSSION

Chromatograms of BA and BAEE standards were shown in Figure 1 A and B. These solute peaks were completely resolved and were symmetrical. The minimum detection limits on each column were 0.02 nM for BA and 0.1 nM for BAEE. The apparent differences in elution profiles and recoveries of the two materials were not observed on both columns, but the retention times of them on µ Bondapak C_{18} were shortened within 3.0 minutes by using a Radial-PAK A column. Therefore, the following procedures were carried out on the Radial-PAK A column.

A series of standard samples containing 0 to 5 µM each of BA and BAEE per ml were prepared and 5 µl aliquots were injected. Chromatographic results showed that the peak height versus concentration plots were linear up to 25 nM for each solute (Figure 2). These standard curves were utilized as a calibration curve of enzyme activities.

Practical enzyme assay was done according to the procedure described in MATERIALS AND METHODS. Enzyme activity was determined by measuring the peak height of BA. The column condition was checked by the determina-

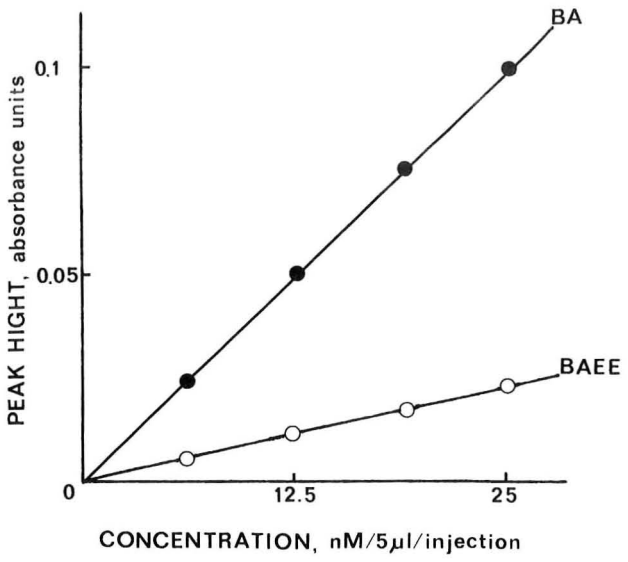

FIGURE 2

Plots of peak height as a function of analytical concentrations of BA and BAEE. Column: Radial-PAK A.

tion of the peak height ratio of BA and BAEE. Figure 3 shows the results of determination of plasmin activity. A good correlation of concentration of the cleaved product (BA) to plasmin activity in the range of 0.0015 - 0.06 CU of plasmin was obtained.

As results, this method might be useful not only for the microdetermination of plasmin, but for the routine assay of it. Therefore, it would be successfully applied to the determination of small amounts of other arginine-esterases in physiological or pathological investigations.

Finally, wide applications of a reversed phase HPLC to chemical assays of the other enzymes by the use of synthetic substrates with ultraviolet absorption are recommended.

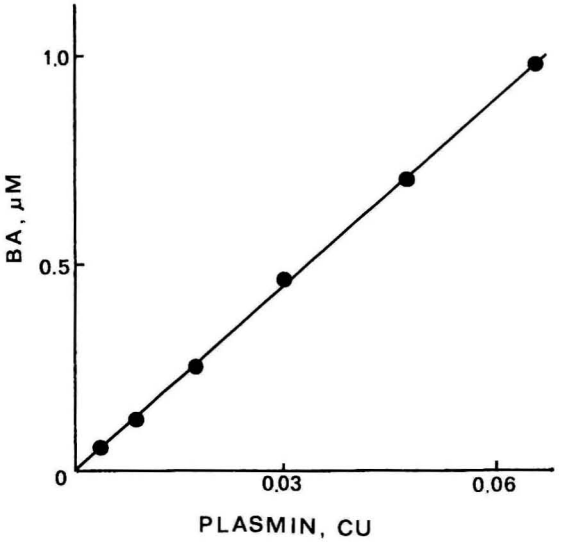

FIGURE 3

Assay of plasmin activity. Column: Radial-PAK A.

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TRIETHYLAMINE FORMATE BUFFER FOR HPLC-FIELD DESORPTION MASS SPECTROMETRY OF OLIGOPEPTIDES

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ABSTRACT

A triethylamine-formate buffer system for the HPLC analysis of mixtures of oligopeptides is described. The volatility of TEAF facilitates buffer removal for subsequent field desorption-mass spectral qualitative and quantitative analyses. TEAF permits femtomole quantification of somatostatin, is UV transparent and enables high resolution separation of oligopeptide mixtures.

INTRODUCTION

Reverse phase (RP) high performance (pressure) liquid chromatography (HPLC) is assuming an increasingly pivotal role in resolution of mixtures of biologic oligopeptides (1-20). Quantification of underivatized oligopeptides by field desorption mass-spectral (FD-MS) techniques at the pmol level signals a need for volatile HPLC buffers (21). Requirements for a volatile buffer in our research program include ultraviolet transparency down to 190 nm, high resolution (22) and sensitivity to less than one ng peptide. This paper describes for the first time development of a triethylamine-formic acid (TEAF) buffer system for RP-HPLC which meets these requirements.

Formic acid-pyridine buffers were utilized in separation of opioid peptide mixtures (23,24), while formic acid-methanol was used for endorphin purification (25). Trifluorocetic and formic acids were compared in separation of opioids and opioid peptides (26). Rivier mentions use of TEAF both in isolation of somatostatin from pigeon pancreas (27) and while describing trialkylammonium phosphate buffers in HPLC (9), but problems due to lack of sensitivity in the 200-230 nm region were noted. However, high concentrations (0.25M) TEAF were utilized in those studies. Data from our laboratory using a triethylamine-phosphate (TEAP) buffer system indicated excellent resolution of oligopeptides, down to five ng SS, and speed of separation (22). However, this buffer system is not volatile and interferes with subsequent FD-MS analysis.

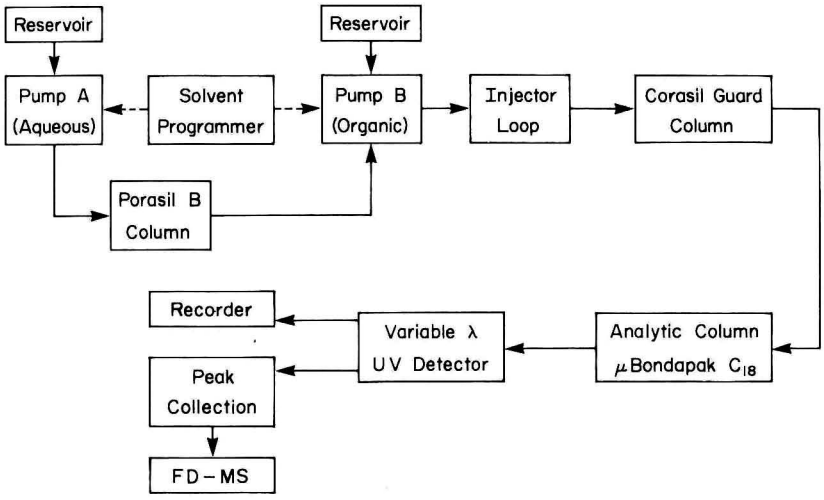
Ammonium acetate-acetonitrile was used to separate enkephalins, endorphins and analogs on a μ-alkylphenyl column (28). Trifluorocetic acid (0.1%) optimized separation of peptides containing two to 32 amino acids (29). An optimum RP-HPLC system was evaluated and 10-20 ng somatostatin (SS) was determined using 210 nm as detection wavelength (30). A detailed explanation of UV detection at 191-194 nm underlines the importance of removing chloride which also absorbs strongly in that region (31). A recent publication outlines a proposed retention mechanism for RP-HPLC wherein it is established by conductometric measurement that ion-pair formation does not occur (32).

This paper describes 0.04M TEAF as an appropriate buffer for eventual use in RP-HPLC-FD-MS quantification of underivatized oligopeptides. This buffer system is volatile, UV transparent,

resolves peptides within minutes and permits determination of peptides down to the 600 femtomole (fmol) level.

MATERIALS AND METHODS

A Waters (Milford, MA) HPLC system was employed (see schematic). This system was outfitted with a U6K injector, 660 solvent programmer, two 6000A pumps and a 450 variable wavelength UV detector. A guard column packed with 37-50μ Bondapak C_{18}-Corasil was inserted in-line after the solvent pumps and before the analytic column to protect the latter when injecting biologic extracts. A 30 cm column packed with 37-75μ Porasil B was placed between the solvent pumps to absorb any TEAF impurities that might be present.



SCHEMATIC

Schematic representing sample and buffer flow through chromatograph illustrating pumps, programmers, aqueous clean-up column, injector, guard and analytic columns, UV detector, peak collection and recorder.

A Laboratory Data Control (Riviera Beach, FL) Spectromonitor III variable wavelength UV detector was employed during high sensitivity measurements of this study. This detector has higher sensitivity specifications (0.005 AUFS) compared to the Waters 450 detector (0.01 AUFS).

A Waters μBondapak C_{18} column (registry no. 105142) was employed and a Precision Sampling (Baton Rouge, LA) Pressure-Lok series B-110 25 μl syringe used for sample injection. The column was washed each night with either CH_3OH or CH_3CN. Unsilanized glassware was employed.

Acetonitrile (Lot AE397) was purchased from Burdick and Jackson (Muskegon, MI), formic acid (Lot FL12A979) from Matheson, Coleman & Bell (Cincinnati, OH) and triethylamine (Lot 59C-0352) from Sigma Chemical Company (St. Louis, MO). Triethylamine (150 ml) was redistilled before use and the fraction distilling at 87.5°C was collected and stored under nitrogen in screw-top vials before use. Laboratory deionized water was employed.

Somatostatin (SS) was purchased from Bachem (Torrance, CA), while bradykinin, luteinizing releasing hormone (LRH), neurotensin, met-enkephalin, angiotensin II, leu-enkephalin, substance P and eledoisin-related peptide were purchased from Sigma. Buffer was prepared by titrating 0.04M formic acid with triethylamine to pH 3.15. Other experimental conditions where otherwise noted: 200 nm; -5 offset; flow rate = 1.5 ml/min; pressure 1200-1300 p.s.i. at 25-26% CH_3CN and 1100 p.s.i. at 34-35%.

Mobile phases were aspirated through 0.47μ cellulose acetate filters (HAWP04700, Millipore, Bedford, MA) and organic solutions through 0.5μ fluorocarbon filters (FHUP04700). Chart speed 0.2 cm/min.

Factory-packed small disposable cartridges (Sep-Pak$^{(R)}$) filled with Bondapak C_{18} (70μ) were purchased from Waters. Time equivalent to the void volume (t_o) was determined by measuring the time between injection and the first baseline disturbance or by using the formula t_o=LD/1.57 (33) where d is the column i.d. (0.39 cm), F is solvent flow rate (1.5 ml min^{-1}) and L = 30 cm. A value of t_o = 2.01 is calculated and corresponds to the measured t_o = 1.6 min.

Analog UV detector output was recorded on a Houston Instrument Omniscribe Recorder Model B5217-1 (Houston, TX) dual-pen strip-chart recorder 10 mV FS.

RESULTS AND DISCUSSION

Figure 1 contains an HPLC chromatogram illustrating isocratic 26% CH_3CN resolution of a mixture of seven oligopeptides - bradykinin, angiotensin II, leu-enkephalin, eledoisin-related peptide, met-enkephalin, substance P and somatostatin. Good resolution and speed of separation are observed in this chromatogram. Additional peptides are easily and completely resolved whenever necessary by either simply adjusting the isocratic elution conditions or by running a solvent gradient.

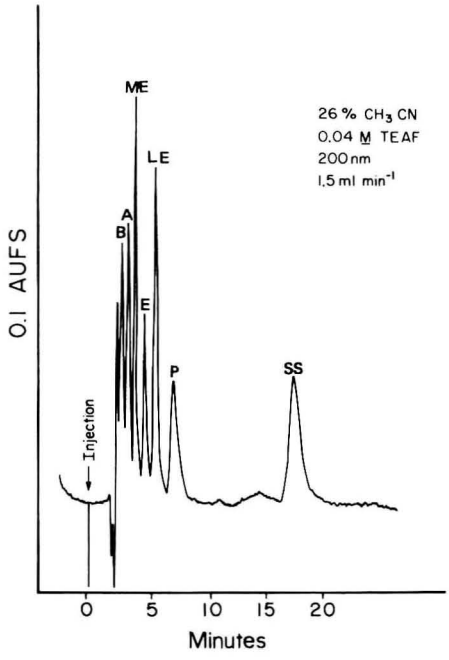


FIGURE 1

HPLC separation of mixture of seven oligopeptides with TEAF buffer: 500 ng each of bradykinin (B), angiotensin II (A), methionine-enkephalin (ME), eledoisin-related peptide (E) and leucine-enkephalin (LE); 1 µg each of substance P (P) and somatostatin (SS). 26% CH_3CN: 74% TEAF; 200 nm; 0.1 AUFS; 1.5 ml min^{-1}; 1200 psi.

Linear variation of the logarithm of the capacity factor (k') for several peptides versus percentage of organic modifier is shown in Figure 2. Extensive data are shown for LRH and bradykinin, while limited data are given for the other oligopeptides. Visual inspection shows all slopes are generally equal.

Data in Figure 3 represent the linear relationship between peak height of an HPLC peak and amount of somatos-

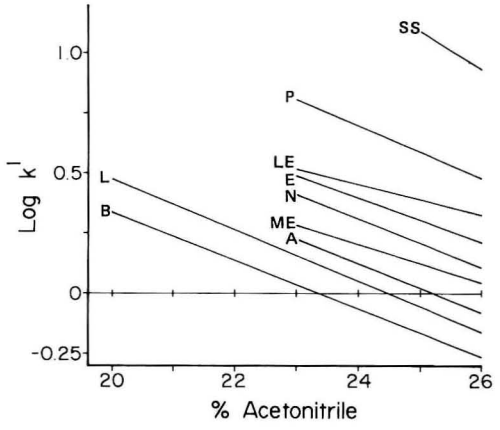


FIGURE 2

Linear variation of logarithm of k' versus percentage of organic modifier.

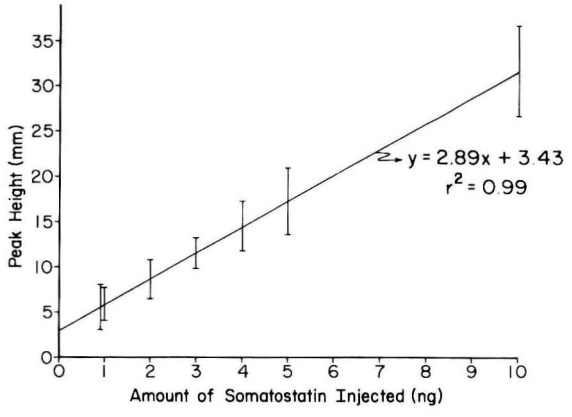


FIGURE 3

Linear relationship observed between recorded peak height (mm) and amount (ng) of injected somatostatin (34% CH_3CN). Linear regression and correlation coefficient are shown. Other experimental parameters are given in the text. Vertical lines indicate standard deviations. Number of determinations are: 7, 10, 7, 9, 7, 5 and 4 for 10 ng, 5, 4, 3, 2, 1 and 0.9, respectively.

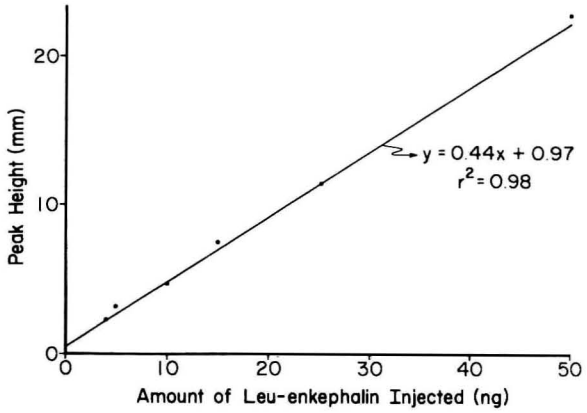


FIGURE 4

Linear relationship observed between recorded peak height (mm) and amount (ng) of leu-enkephalin injected (26% CH_3CN). Linear regression and correlation coefficient are shown. Other experimental parameters are given in the text.

tatin injected. Linearity is observed from below one ng to ten ng. The correlation coefficient for this regression line is 0.99. Vertical lines represent $\pm$ standard deviation obtained from multiple injections (see legend). The lowest amount of SS injected producing a peak having the appropriate retention time and approximately a 1:1 signal-to-noise ratio is represented by 0.9 ng or 552 fmol. By comparison, the amount of SS in one rat hypothalamus is 40 ng (34).

The linear relationship between HPLC peak height and amount of injected leu-enkephalin is given in Figure 4 and for met-enkephalin in Figure 5. Linearity is observed in both cases from one to 50 ng.

Data in Figure 6 represent original recordings of sequentially lower amounts of somatostatin injected. Good peak shapes are

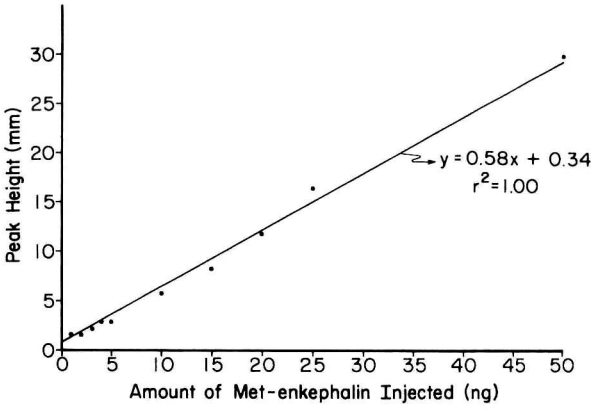


FIGURE 5

Linear relationship observed between recorded peak height (mm) and amount (ng) of met-enkephalin (26% CH_3CN). Linear regression and correlation coefficient are shown. Other experimental parameters are given in the text.

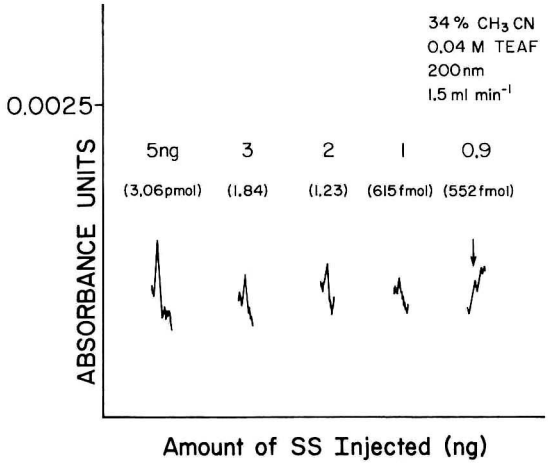


FIGURE 6

Original traces of 5, 3, 2, 1 and 0.9 ng injected SS. 34% CH_3CN: 66% 0.04M TEAF; 200 nm; 0.005 AUFS; 1.5 ml min^{-1}; 1100 psi. Arrow on 0.9 ng injection denotes known SS retention time.

observed. These data illustrate femtomole sensitivity obtainable with a 0.04M TEAF buffer system. In the more dilute solutions used in this study vis-a-vis more concentrated solutions (0.25M) of other studies, it can be seen UV transparency, sensitivity and resolution properties of the current TEAF buffer are excellent.

CONCLUSIONS

A volatile TEAF buffer system capable of high resolution and femtomole sensitivity is described. Regression lines were obtained enabling quantification of endogenous levels of biologic oligopeptides. The ability to remove TEAF buffer by lyophilization permits analysis of underivatized oligopeptides by FD-MS where three pmol of leu-enkephalin have been quantified recently (21).

Work is in progress in our laboratory with extraction, purification and quantification of biologic oligopeptides in hypothalamic, dental and brain tissue. After protein precipitation with perchloric acid, samples are prepurified with RP Sep-Paks (R) (35-38). Samples may be directed to either FD-MS quantification or, on the other hand, to further chromatographic purification and/or quantification with HPLC. HPLC eluates can also be collected and subjected to FD-MS quantification. This research program is being undertaken to provide an independent assay method to verify radioimmunoassay (RIA) results (39). FD-MS quantitative data are based upon intact molecular structure of the peptide while RIA may be sensitive only to a portion of the

peptide. Development of the described TEAF buffer system is an obligatory component in this overall research scheme. TEAF buffer has been shown to possess sufficient resolving power, speed of analysis, sensitivity, volatility and UV transparency. Equipment and columns are long-lasting and no degradation of resolution is observed even after injection of several biologic samples. Fmol sensitivity is obtainable and no derivatization is required as UV detection at 190-210 nm is universal for peptide bonds.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 4(4), 735-736 (1981)

LC NEWS

SAFETY BOTTLE COVER FOR HAZARDOUS LIQUIDS to protect gallon glass bottles and minimize risks of handling dangerous materials fits over the bottle without interfering with pouring. It reduces breakage and contains spills for safe and proper disposal. Burdick and Jackson Laboratories, Inc., JLC/81/4, 1953 South Harvey Street, Muskegon, MI, 49442, USA.

HIGH FLOW-RATE PUMPS are offered for preparative scale liquid chromatography. Liquid ends are easily interchangeable with analytical-scale liquid ends. LDC Corp., JLC/81/4, P. O. Box 10235, Riviera Beach, FL, 33404, USA.

WATER ANALYSIS BY HPLC describes on-line trace enrichment using a pre-column or reverse phase analytical column. Trace enrichment is an improvement over classical techniques such as freeze drying, extraction, and steam distillation. Very low levels of priority pollutants can be determined. Varian Instrument Group, JLC/81/4, 10060 Bubb Road, Cupertino, CA, 95014, USA.

POLYACRYLAMIDE GEL ELECTROPHORESIS MANUAL (72 pp.) describes gradient, disc, and 2-dimensional techniques, specific staining procedures, and several applications including determination of DNA fragments and protein molecular weights. Pharmacia Fine Chemicals, Inc., JLC/81/4, 800 Centennial Avenue, Piscataway, NJ, 08854, USA.

PRODUCTS FOR CHROMATOGRAPHY CATALOG describes materials for TLC, HPTLC, HPLC, and Prep LC. Included are columns for protein and enzyme analysis, pre-concentrating zone TLC-HPTLC and PrepTLC, sorbents for dry column chromatography, and microslides for TLC. MCB Mfg. Chem., Inc., JLC/81/4, 2909 Highland Avenue, Cincinnatti, OH, 45212, USA.

TRIGLYCERIDE ANALYSIS is described in a recent technical bulletin. Separations can be made on the basis of degree of unsaturation and acyl chain length in one step, by elution

from a high efficiency column with a non-aqueous mobile phase. Supelco, Inc., JLC/81/4, Supelco Park, Bellefonte, PA, 16823, USA.

LC COLUMN & PACKING GUIDE contains specifications and performance data for a complete line of products. In this new edition are recently announced preparative columns designed for operation at 40-50 cm^3/min. They are available in SIL, ODS, C-8, and NH_2 types. DuPont Company, JLC/81/4, Anal. Inst. Div., McKean Building-Concord Plaza, Wilmington, DE, 19898, USA.

JOURNAL OF LIQUID CHROMATOGRAPHY, 4(4), 737-739 (1981)

LC CALENDAR

1981

March 29-April 3	"National Am. Chem. Soc. Meeting", Atlanta, GA, USA. Contact: Am. Chem. Soc., 1155 Sixteenth Streeet, NW, Washington, DC 20036, USA.
March 29-April 3	"Advances in Separation Technology", Nat'l ACS Meeting, Atlanta, GA, USA. Contact: N. Li, Exxon Res. & Eng. Co., P. O. Box 8, Linden, NJ 07036, USA.
March 29-April 3	"Chromatographic Separations of Coal-Derived Materials", "Nat'l ACS Meeting, Atlanta, GA USA. Contact: L. Taylor, Chem. Dept., Virginia Polytechnic Inst. & State Univ., Blacksburg, VA 24601, USA.
March 29-April 3	"Standardized Materials for Chromatography", Nat'l ACS Meeting, Atlanta, GA USA. Contact: L. S. Ettre, Perkin-Elmer Corp., Main Avenue, Norwalk, CT 06856, USA.
April 28	"Detectors in Chromatography", Chrom. & Electrophoresis Grp. The Royal Society of Chemistry, College of Technology, Southend, U.K. Contact: Dr. D. Simpson, Anal. for Industry, Bosworth House, High Street, Thorpe-le-Soken, Essex CO 16 OEA, U.K.
May 11-15	"5th International Symposium on Column Liquid Chromatography", Avignon, France. Contact: G. Guiochon, Lab de Chim. Anal. Phys., Ecole Polytechnique, Rte. de Saclay, 91128 Palaiseau, France.
May 17-19	"Symposium on Environmental and Industrial Applications of LCEC and Voltammetry", Indianapolis, Indiana, USA. Contact: LCEC Symposium, 1205 Kent Avenue, West Lafayette, IN 47906, USA.
May 18-20	"11th Annual Symposium on the Analytical Chemistry of Pollutants", Jekyll Island, Georgia, USA. Contact: Mrs. E. McGarity, U.S.E.P.A., Environmental Research Lab, College Station Road, Athens, GA 30613, USA.
May 20-22	"Symposium on the Anal. of Steroids", sponsored by The Hungarian Chemical Society, Eger, Hungary. Contract: Prof. S. Gorog, Hungarian Chem. Soc., 1061 Budapest VI, Anker koz 1, Hungary.
June 4-5	"4th World Chromatography Conference", Aerogolf Sheraton Hotel, Luxembourg. Contact: V.M. Bhatnagar, Alena Enterprises of Canada, P.O. Box 1779, Cornwall, Ontario, K6H 5V7, Canada.
June 22-26	"4th Int'l Symposium on Affinity Chromatography and Related Techniques", Katholieke Universiteit, Nijmegen, The Netherlands. Contact: Dr. T.C.J. Gribnau, Organon Scientific Development Group, P.O. Box 20, 5340 BH OSS, The Netherlands.
July 13-17	Workshop: "Checking Foodstuffs for Trace Organics", Guildford, U.K. Contact: Dr. E. Reid, Wolfson Bioanalytical Unit, Robens Inst., Univ. of Surrey, Guildford, GU2 5XH, U.K.
July 20-24	"Second International Flavor Conference", National Hellenic Research Foundation, Athens, Greece. Contact: Dr. S.J. Kazeniac, Campbell Institute for Food Research, Campbell Place Camden, N.J. 08101, USA.

August 23-28 "National Am. Chem. Soc. Meeting", New York, NY, USA. Contact: Am. Chem. Soc., 1155 Sixteenth Street, NW, Washington, DC 20036, USA.

August 30-September 5 "XI Int'l Congress, IV European Congress of Clinical Chemistry", Vienna, Austria. Contact: 11th Int'l Congress of Clinical Chem., P. O. Box 105, A-1014 Wien, Austria.

September 7-10 "4th Int'l Bioanalytical Forum", Guildford, U.K. Contact: Dr. E. Reid, Wolfson Bioanalytical Unit, Robens Inst., Univ. of Surrey, Guildford, GU2 5XH, U.K.

September 10-12 Workshop: "Some Approaches to the Anal. of Biological Specimens", Guildford, U.K. Contact: Dr. E. Reid, Wolfson Bioanalytical Unit, Robens Inst., Univ. of Surrey, Guildford, GU2 5XH, U.K.

September 15-19 Workshop: "Introduction to Determination of Drugs in Biological Fluids", Guildford, U.K. Contact: Dr. E. Reid, Wolfson Bioanalytical Unit, Robens Inst., Univ. of Surrey, Guildford, GU2 5XH, U.K.

September 20-25 "8th Annual FACSS Meeting", Philadelphia, PA USA. Contact: R. A. Barford, USDA, 600 E. Mermaid Lane, Philadelphia, PA 19118, USA.

October 1-2 "Japan Conference on Chromatography", Palace Hotel, Tokyo, Japan. Contact: V.M. Bhatnagar, Alena Enterprises of Canada, P.O. Box 1779, Cornwall, Ontario, K6H 5V7, Canada.

October 4-9 "Symposium on Novel Separation Processes", at the 2nd World Congress of Chemical Engineering, Montreal, Canada. Contact: Congress Secretariat, 151 Slater Street, Suite 906, Ottawa, Ont., Canada, K1P 5H3.

October 12-15 "EXPOCHEM '81", Houston, TX. Contact: Dr. A. Zlatkis, Chem. Dept., University Houston, Houston, TX 77004, USA.

November 19-20 "1981 International Chromatography Conference", Carillon Hotel, Miami Beach, Florida, USA. Contact: V.M. Bhatnagar, Alena Enterprises of Canada, P.O. Box 1779, Cornwall, Ontario, K6H 5V7.

1982

March 28-April 2 "National American Chem. Soc. Meeting", Las Vegas, NV USA. Contact: A. T. Winstead, Am. Chem. Sco., 1155 Sixteenth St., NW, Washington, DC 20036, USA.

April 14-16 "12th Annual Symposium on the Anal. Chem. of Pollutants", Amsterdam, The Netherlands. Contact: Prof. R. W. Frei, Congress Office, Vrije Universiteit, P. O. Box 7161, 1007-MC Amsterdam, The Netherlands.

June 28-30 "Analytical Summer Symposium", Michigan State Univ., East Lansing, MI USA. Contact: A. I. Popov, Chem. Dept., Michigan State Univ., East Lansing, MI 48824, USA.

July 12-16 "2nd Int'l Symposium on Macromolecules", - IUPAC, University of Massachusetts, Amherst, Massachusetts, USA.

August 15-21 "12th Int'l Congress of Biochemistry", Perth, Western Australia. Contact: Brian Thorpe, Dept. of Biochemistry, Faculty of Science Australian National University, Canberra A.C.T. 2600, Australia.

September 12-17 "National American Chem. Soc. Meeting", Kansas City, MO USA. Contact: A. T. Winstead, American Chem. Soc., 1155 Sixteenth St., NW, Washington, DC 20036, USA.

1983

March 20-25 "National American Chem. Soc. Meeting", Seattle, WA USA. Contact: A. T. Winstead, American Chem. Soc., 1155 Sixteenth St., NW, Washington, DC 20036, USA.

The Journal of Liquid Chromatography will publish announcements of LC meetings and symposia in each issue of the Journal. To be listed in the LC Calendar, we will need to know: Name of meeting or symposium, sponsoring organization, when and where it will be held, and whom to contact for additional details. You are invited to send announcements for inclusion in the LC Calendar to Dr. Jack Cazes, Editor, Journal of Liquid Chromatography, P. O. Box 127, Hopedale, Massachusetts 01747, USA.

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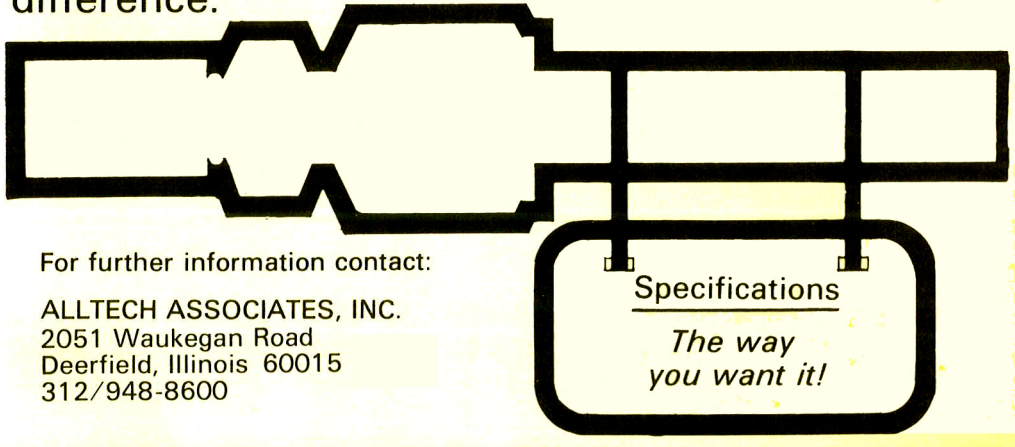

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