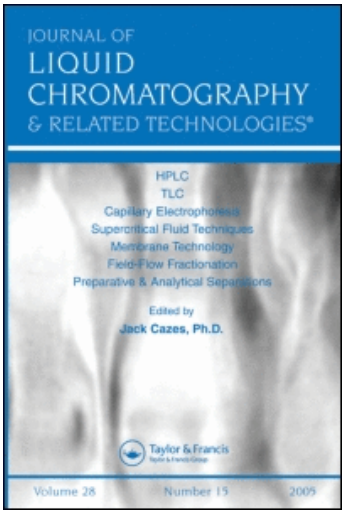

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HPLC SEPARATION AND CHARACTERIZATION OF SYNTHETIC AND NATIVE CYTOCHROME C DERIVED PEPTIDES

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ABSTRACT

The use of HPLC for the characterization of peptides used in semisynthesis of cytochrome c is discussed. HPLC provided a convenient method for assessing the purity and structure of the peptides used. HPLC was also useful in monitoring the progress of solid phase peptide synthesis and in purifying the final product.

INTRODUCTION

The preparation, purification, and characterization of relatively large peptides (30-50 residues), in particular those produced by solid phase synthesis techniques, present a considerable analytical challenge. Conventional techniques such as amino acid analysis (AAA) and peptide mapping are frequently insufficient indicators of purity and can consume excessive amounts of time and material without providing definitive answers. High performance liquid chromatography (HPLC) has proven very valuable in providing such information (1, 2), and we report on its application to the preparation of semisynthetic cytochrome c.

Harbury and co-workers (3, 4) demonstrated that horse heart cytochrome c, a 104 residue protein, could be cleaved at met-65 to produce two essential pep-

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tides, a 65 residue peptide to which a heme group is attached (HP1-65) and a non-heme peptide containing the remaining residues (NHP 66-104). As a result of the cleavage reaction with cyanogen bromide, the met-65 of the heme peptide is converted into homoserine lactone. This latter moiety will react with NHP 66-104 under appropriate conditions to reestablish a covalent linkage between residues 65 and 66. A fully biologically active protein results which differs only from the native at residue 65, now homoserine. The effect of individual residues on structure-function relationships in the intact protein may be established by making individual substitutions in the NHP 66-104 sequence followed by fragment recombination. The success of such a venture clearly depends upon the efficacy of the purification and characterization methods employed particularly in distinguishing changes in individual residues.

The HPLC has proven useful in the purification and characterization of peptides and proteins (1, 2). This is in spite of the still not completely understood mechanism for retention. Several groups (6-10) have established empirical relationships between amino acid composition and retention. Others (11-13), including this paper, have found it convenient and successful to use the values calculated by employing Rekker's hydrophobic values (5) or by Meek (6, 7) to predict elution order.

EXPERIMENTAL PROCEDURES

Materials. All reagents and buffers used were reagent grade unless otherwise specified. Reagents were obtained from the following sources: Horse heart cytochrome c (type III), cyanogen bromide from Sigma Chemical Company; HPLC grade acetonitrile, dipotassium hydrogen phosphate from Matheson, Coleman and Bell; ammonium acetate, formic acid, and 1-butanol from Fisher Scientific; t-butoxycarbonyl and g-Fluoromethyloxycarbonyl protected amino acids from Vega Biochemicals.

Methods. Heme peptides 1-80 and 1-65 were prepared by limited cyanogen bromide cleavage and were purified by chromatography on Sephadex G-75 with acetic acid:formic acid:H_2O, 40:10:50 v/v as the mobile phase (14). Non-heme peptides

66-104, 66-80 and 81-104 were prepared as described by Corradin and Harbury (3, 4), except that purfication was performed on the Sephadex G-75 column described above. The appropriate fractions were pooled, lyophilized, and then purified in a final step by gel chromatography on Sephadex G-50-F with 7% formic acid as solvent.

A Model 332 Altex gradient liquid chromatograph was used for all HPLC applications. An Altex Ultrasphere-ODS column (5um, 4.6 mm i.d. x 25 cm, octadecylsilane bonded silica) or an Altex Ultrasil-ODS (10um, 4.6mm i.d. x 25 cm, octadecylsilane bonded silica) was used. Solvents were first filtered through 0.5 Millipore filters (type fH for organic solvents, type HA for aqueous solvents). Acetonitrile and ammonium acetate were used in all separations. 0.1 M ammonium acetate at pH 4.5 was used for most peptides while a 0.2 M solution at pH 5.0 was used for some, indicated on the individual figures. Flow rates and gradient conditions used are shown on the individual chromatograms. All peptide samples were filtered through 0.45um type HA Millipore filters prior to injection or centrifuged for 2 minutes in a Fisher micro-centrifuge model 235A.

RESULTS AND DISCUSSION

Separation of Heme Peptides. It has been our experience that the successful synthesis and recombination of peptide fragments is greatly facilitated by careful monitoring of purity at designated stages of the synthesis. Figure 1 shows the two important heme peptides which are obtained from the cyanogen bromide cleavage of cytochrome c. These correspond to attack at met-65 and met-80 to produce HP 1-65 and HP 1-80 which elute in both the open (homoserine carboxylate) (Peaks A and C) or in the synthethetically active lactone (Peaks B and D) forms. It has been previously pointed out (4) that the lactone form will open in the presence of base. Under such conditions Peaks A and C can be shown to grow at the expense of B and D thus confirming the peak assignments. This treatment does not result in modification of the heme moiety.

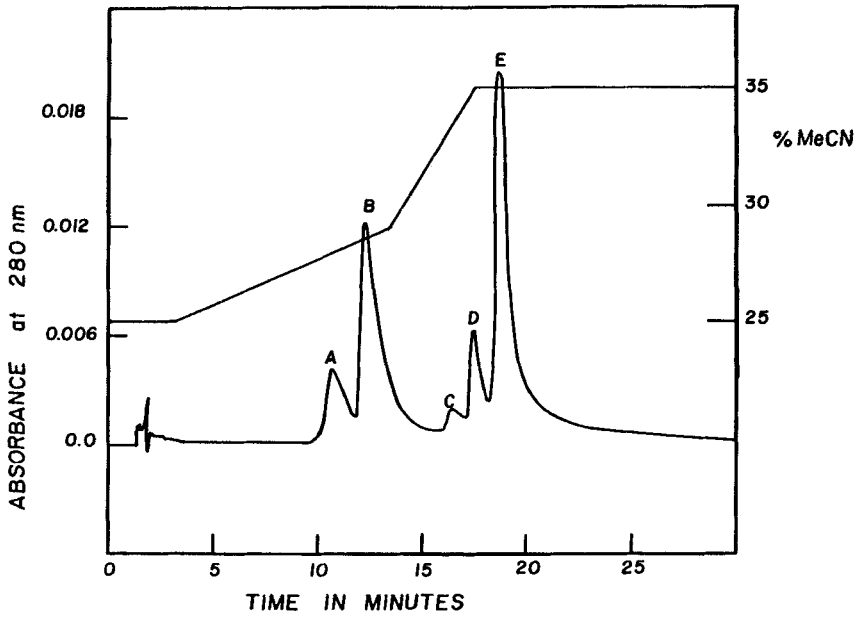

Figure 1 - HPLC of Heme Peptides from CNBr Cleavage.
A-HP1-65 homoserine; B-HP1-65 homoserine lactone; C-HP1-80 homoserine; D-HP1-80 homoserine lactone; E-native cytochrome c. Conditions: Ultrasil Column, Flow rate 2 ml/min. acetonitrile/ammonium acetate buffer gradient as shown.

The ability to predict retention times based on peptide size or amino acid composition is quite important because peak assignment is a tedious process and consumes considerable time and material. It has been previously suggested (10, 16) that hydrophobic interactions with the stationary phase determine retention on ODS columns. In this case the larger peptides have longer retention times even though the overall positive charge is also higher. The striking influence of C-terminal residue modification is clearly seen in the comparison of Peaks A and B or C and D. The opening of the homoserine lactone creates a localized negative charge and reduction in the overall positive charge on the peptide. The resulting lowered retention time with otherwise constant amino acid composition does not agree with the predictions of the models (6, 7) based solely on hydrophobic interactions but suggests instead the strong individual

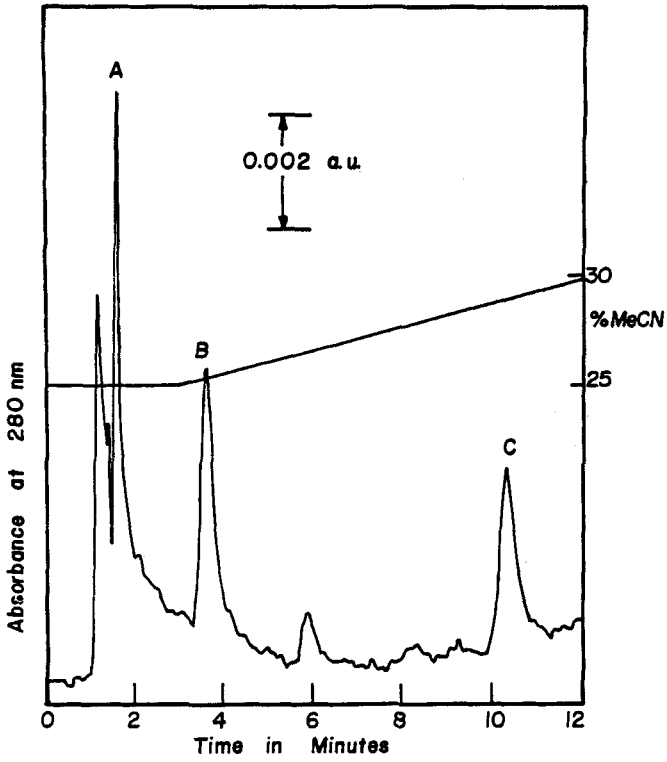

Figure 2 - HPLC of Non-Heme Peptides from CNBr Cleavage.
A-NHP66-80 homoserine lactone and NHP66-80 homoserine; B-NHP81-104; C-NHP66-104 Conditions: Ultrasphere-ODS column, flow rate 2 ml/min, Same as acetonitrile/ammonium acetate gradient as shown.

influence on retention of the charged carboxyl group In any case, the ability to quickly establish the presence of the lactone at position 65 is significant because the lactone is essential for the formation of a covalent bond between residues 65 and 66 in the recombination studies.

Separation of Non-Heme Peptides. Figure 2 shows a chromatogram of the three non-heme peptides obtained from the digestion of horse heart cytochrome c. The identity of the various peaks was established by separate injections of the various components as well as amino acid analysis of the eluted peaks. Again as in the case of the heme peptides, the elution order is determined primarily by

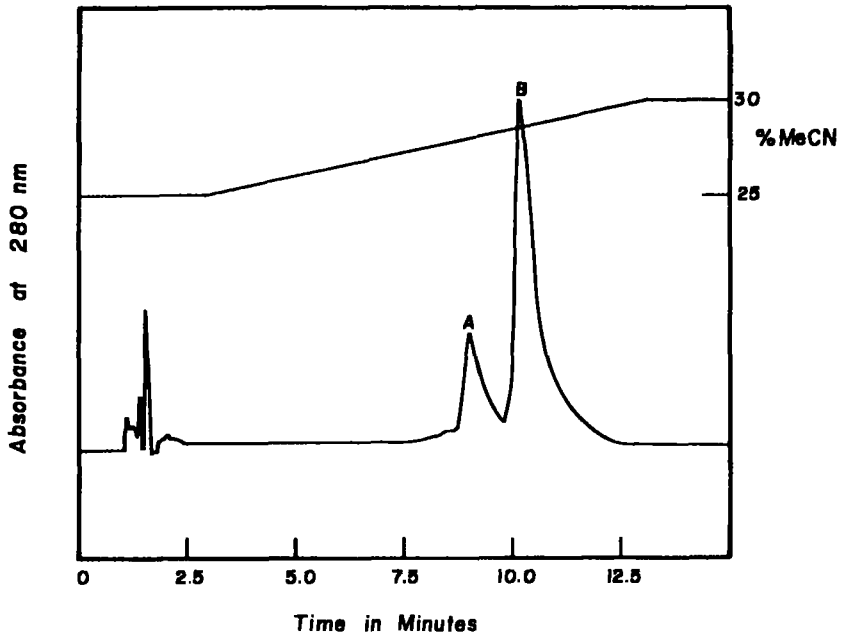

Figure 3 - HPLC of Non-Terminal Homoserine Non-Heme Peptide A-NHP66-104 (homoserine-80); B-NHP66-104(methionine-80) Conditons: Same as Figure 2, gradient as shown.

peptide length rather then by charge. If the NHP 66-80 peak is rechromatographed at a lower acetonitrile concentration (13%) the two peptides containing the C-terminal homoserine lactone and carboxylate with net charges of +2.52 and +1.52 respectively can be resolved. The elution order is analogous to that of the heme peptides. If NHP 66-104 is prepared with a 50-fold excess of CNBr instead of the normal 3-fold excess, part of the NHP 66-104 formed will possess met-80 which has been converted into homoserine without amide bond cleavage (3, 4). This is shown in Figure 3 where the distinction between these two closely related peptides is essential to the subsequent peptide reconstruction. The substitution of homoserine for methionine may render the peptide more polar thus resulting in more rapid elution.

Separation of Synthetic Cytochrome c-Derived Peptides. We have previously demonstrated the preparation of semi-synthetic cytochrome c from the native

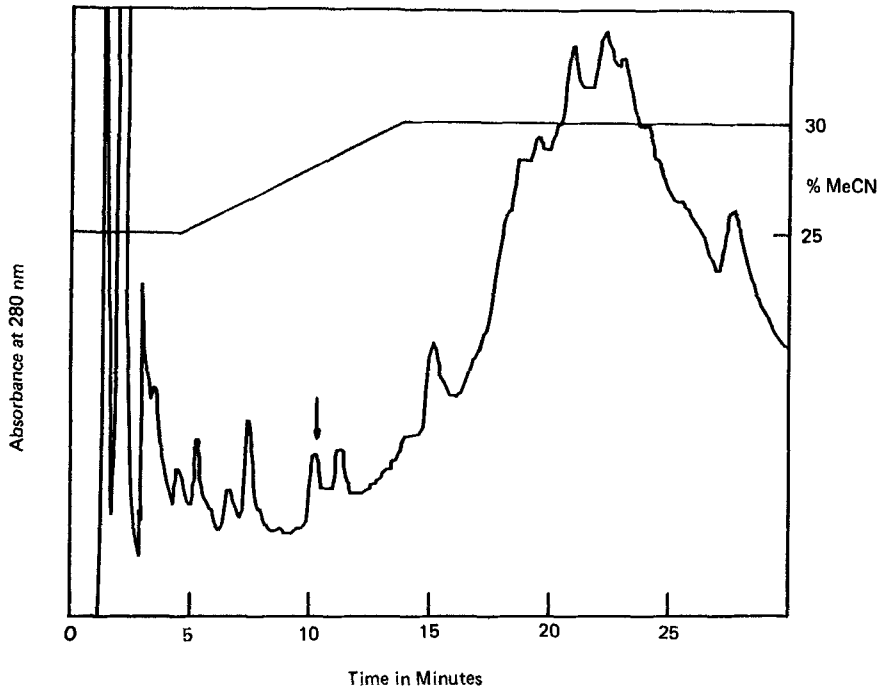

Figure 4 - HPLC of t-Boc Synthesized NHP66-104
Arrow indicates retention time of native peptide. Conditions: Same as Figure 2, gradient as shown.

HP1-65 and NHP 66-104 prepared by solid phase peptide synthetic techniques described elsewhere (15). Conventional t-butoxycarbonyl (t-Boc) derivatives were used for α-amino group protection (17, 18). Standard amino acid analysis of synthetic NHP 66-104 samples give the expected results within experimental error. Yet, as shown in Figure 4, HPLC analysis of the same peptide shows a high degree of heterogeneity. Indeed very little synthetic peptide is eluted at the retention time of the corresponding native peptide. Enzymatic hydrolysis of the synthetic material followed by amino acid analysis showed that the large very broad chromatographic peak (Figure 4) was due primarily to incomplete removal of blocking groups at the conclusion of the synthesis. A variety of conditions were examined for the resin-peptide cleavage step following synthesis

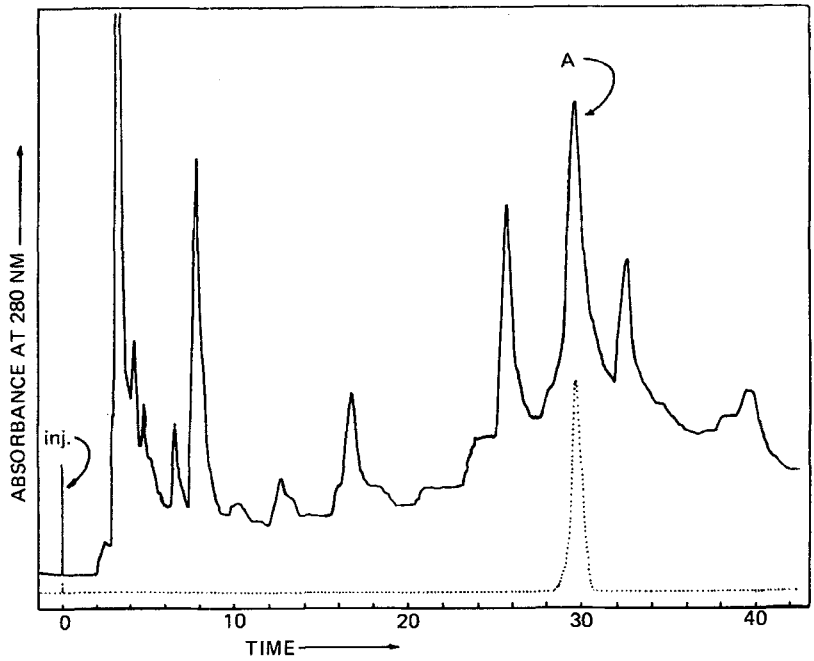

Figure 5 - HPLC of F-moc Synthesized NHP66-104
Arrow indicated retention time of native peptide. Conditions: Ultrasphere ODS column, flow rate 1ml/min, acetonitrile/ammonium acetate gradient as shown. Curve A - HPLC after HF cleavage, Curve B - HPLC after preliminary HPLC purfication.

that also removes side-chain blocking groups. It was not possible to achieve complete deblocking in anhydrous HF without destruction of the peptide. Further, the constant washing of the growing peptide with mild acids in the α-amino deprotection step can also have detrimental effects. This has encouraged the use of the base labile α-amino protecting group 9 fluoromethyloxycarbonyl (F-moc) (19). Figure 5 Curve A shows the chromatogram of F-moc synthesized NHP 66-104 before HPLC purification. The presence of a small number of well-defined peaks supports a "cleaner" higher yield synthesis. The fraction eluting at ca. 29 minutes was rechromatographed, giving Curve B. The characteristics of this latter fraction were identical to those of the native peptide.

HPLC has also been used to monitor the course of the solid phase reactions. This is quite important for the synthesis of relatively large peptides because

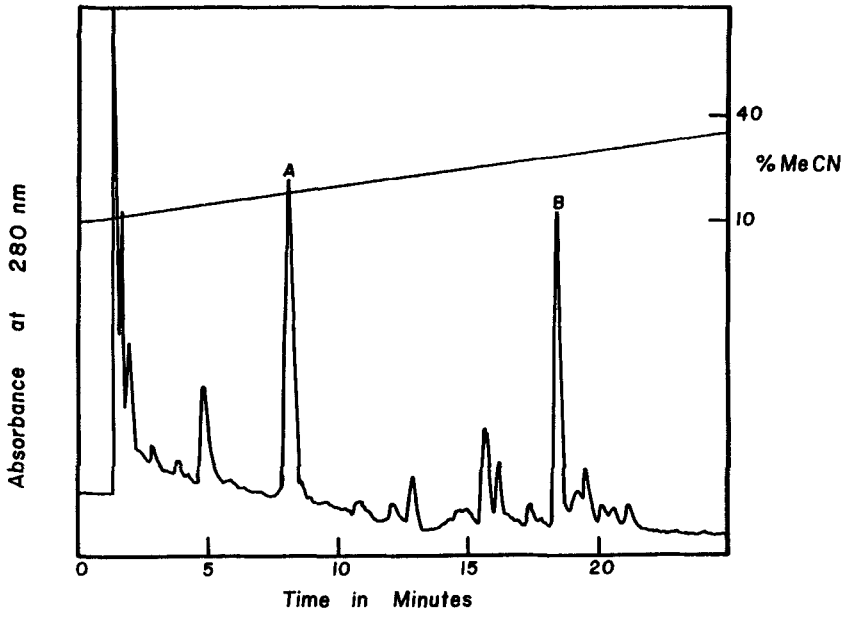

Figure 6 - HPLC of F-moc Synthesized NHP81-104
A-Truncated Peptide; B - NHP81-104 Conditions: Same as Figure 2, gradient as shown.

errors occurring early in the sequence will result in low yields and complicated mixtures which are time consuming and expensive to evaluate and purify. Figure 6 shows the chromatogram of synthetic NHP 81-104. Peak B is identical to native NHP 81-104. leaving Peak A as a major contaminant. It can be presumed to be a shorter or truncated peptide resulting from incomplete reaction. By comparing the peptide product at various stages of synthesis with native peptides derived from cytochrome c by limited enzymatic hydrolysis, it is possible to establish the step at which extraneous peaks appear. In this case, it was established that truncation occurs in the Arg-91 region. It has been suggested that resin support collapse is the cause of the problem (21). Correction of the factors causing this problem now makes possible synthesis of the NHP 66-104 peptide in reasonable yields.

Retention times are reproducible on a single column (within 4%), however, columns of the same type from the same manufacturer do not give identical reten-

tion behavior. This makes the availability of suitable standards essential. The retention of cytochrome c derived peptides is very sensitive to the acetonitrile concentration of the mobile phase. Changes as small as 5% can result in the extremes of complete retention or non-retention on the column.

It is of interest in the present study to be able to qualitatively predict retention times, or at least retention order for eluting peptides. Such predictions do not eliminate the need for peptide isolation and identification, but they can help considerably in the optimization of elution conditions. The peptides in question are relatively large (15-104 residues) and the separations are carried out at around pH 4.5. This means that the peptides are not in an open chain configuration and consequently not all residues are freely accessible for interaction with the stationary phase. The simplest approach is to consider the retention order to be determined by peptide size (number of residues). We find that for our peptides this latter method works as well as those of Rekker (5), Meek (6, 7) and Sasagawa (10). These methods all produce about 10% uncertainty in the prediction retention time which becomes quite important when peptides of comparable size but different sequence must be separated. In some cases the effect of individual amino acid substitutions can be correctly predicted (66-104 vs 66-104 homoserine) but this approach cannot be described as totally reliable.

The ability of HPLC for the separation, purification and identification of cytochrome c-derived peptides has been demonstrated. Separation of relatively large peptides with single residue modification has been achieved. The monitoring of the growing peptide product during solid phase synthesis has enabled the detection of synthesis error thus saving considerable time and expense.

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