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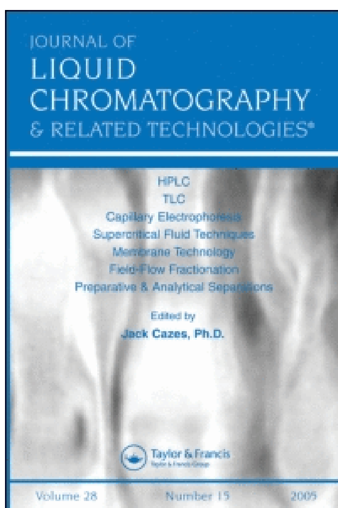

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A RAPID ISOLATION OF HUMAN CHORIONIC GONADOTROPIN AND ITS SUBUNITS BY REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A rapid isolation of human chorionic gonadotropin and its subunits from a commercially available concentrate of human urine has been achieved using reversed-phase high performance liquid chromatography. With μBondapak C_{18} columns and a gradient employing aqueous trifluoroacetic acid as one solvent and dilute trifluoroacetic acid in acetonitrile as the other, complete separation can be accomplished in one day whereas standard column chromatographic procedures take about two weeks. Specific radioimmunoassays, polyacrylamide gel electrophoresis, and amino acid analyses were used to identify and characterize chromatographic peaks.

INTRODUCTION

Human chorionic gonadotropin (hCG) (1) is a trophic hormone composed of two dissimilar noncovalently attached glycopeptide

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subunits, termed α and β. The amino acid sequences (2-4) as well as the carbohydrate sequences of both subunits have been determined (5-6). The α subunit contains 90-92 amino acid residues while the β subunit contains 145. Each subunit is extensively crosslinked by intramolecular disulfide bonds and contains about 30% carbohydrate which causes hCG to elute from gel filtration columns earlier than expected and facilitates its aggregation.

The hormone hCG is normally synthesized by trophoblastic cells of the placenta. Levels of hCG are usually highest in the urine of women in their first trimester of pregnancy. The hormone and/or its subunits are also synthesized by malignant trophoblastic cells as well as by other human cancer cells, including carcinomas, lymphomas and melanomas (7, 8).

A previous paper from this laboratory (9) described a lengthy procedure for isolating hCG and its subunits from commercially available crude material. This procedure included dialysis, ion-exchange chromatography and gel filtration and took about two weeks. Below we describe the use of reversed-phase high performance liquid chromatography (HPLC) to rapidly obtain highly purified subunits of hCG.

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EXPERIMENTAL

Chemicals

HPLC grade water was obtained by passing our standard laboratory grade water through a Critical Applications Adsorption Column (Hydro Services and Supplies, Inc., Durham, NC) which contains highly purified, activated charcoal and has a 0.2 μm polycarbonate filter (Nucleopore Corp., Pleasanton, CA) attached to its outlet. Trifluoroacetic acid (TFA) and pentafluoropropionic acid (PFPA), both Sequanal Grade, obtained from Pierce Chemical Co. (Rockford, IL), and acetonitrile purchased

from Burdick and Jackson Laboratories (Muskegon, MI), were used without further purification. Crude hCG isolated from the urine of pregnant women, was purchased from Organon, Inc. (Oss, the Netherlands). (Met) and (Leu) Enkephalins were obtained from Calbiochem-Behring Corp. (La Jolla, CA). Ribonuclease A and lysozyme were purchased from Worthington Biochemical Co. (Freehold, NJ) and insulin was obtained from Sigma Chemical Co. (St. Louis, MO).

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Apparatus

HPLC was performed with an Altex Model 312 MP (Altex Instruments, Berkeley, CA) chromatographic system which consisted of an Altex Model 420 microprocessor controller/programmer, two Altex Model 110A pumps, an Altex gradient mixing chamber, and a Rheodyne (Berkley, CA) Model 7120 syringe-loading sample injection valve equipped with a 3.5 ml sample loop. Separations were performed with 0.39 x 30 cm and 0.78 x 30 cm μBondapak C_{18} columns (Waters Associates, Milford, MA). The particle size of the column packings was 10 microns. Runs were monitored at 206 nm with a Model 970A variable wavelength detector from Tracor Instruments (Austin, TX) and an Altex Model 385 recorder. A back pressure of 150 lb/in^2 was placed on the outlet tubing with a pinch clamp to prevent solvent outgassing and maintain a stable baseline (10, 11).

Solvents

A stock solution obtained by mixing 10 ml of TFA with 100 ml of purified water was used to prepare the chromatographic solvents. Solvent A was prepared by diluting 5 ml of the stock solution to 1 liter with water and solvent B was prepared by diluting 4.25 ml of the stock solution to 1 liter with acetonitrile.

Procedure

Our abbreviated program consisted of running isocratically at 0% B for 5 min and then employing a linear gradient from 0% B to 60% B in 60 min. An extended program consisted of running isocratically at 0% B for 4 min and then using a linear step gradient from 0% B to 20% B in 15 min, then 20% B to 45% B in 75 min, and finally from 45% B to 60% B in 15 min. Thus, the overall time for this program was 109 min. With both programs a flow rate of 1.0 ml/min was used. Samples to be rerun on HPLC were first placed under a stream of purified nitrogen which evaporated the acetonitrile. Aliquots were removed for identification and characterization as described below and the remaining sample was re-injected into the HPLC system.

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Other methods

The hormone hCG and its subunits were identified and peaks were characterized by means of specific RIAs, SDS-polyacrylamide gel electrophoresis and amino acid analysis as previously described (9).

RESULTS AND DISCUSSION

Standards

In earlier work (9) hCG was dissociated into its subunits by incubation in 1 M propionic acid, a procedure which did not apparently disturb immunological specificity or damage the polypeptide chains. Thus, the recently reported solvent systems for reversed-phase HPLC which contain dilute aqueous TFA as the more polar solvent (10-12) seemed ideally suited for the isolation of hCG subunits. To evaluate the effectiveness of our HPLC system, a mixture of model peptides and proteins employed by others (11) was analyzed with the abbreviated program (EXPERIMENTAL) and excellent results were obtained (Figure 1).

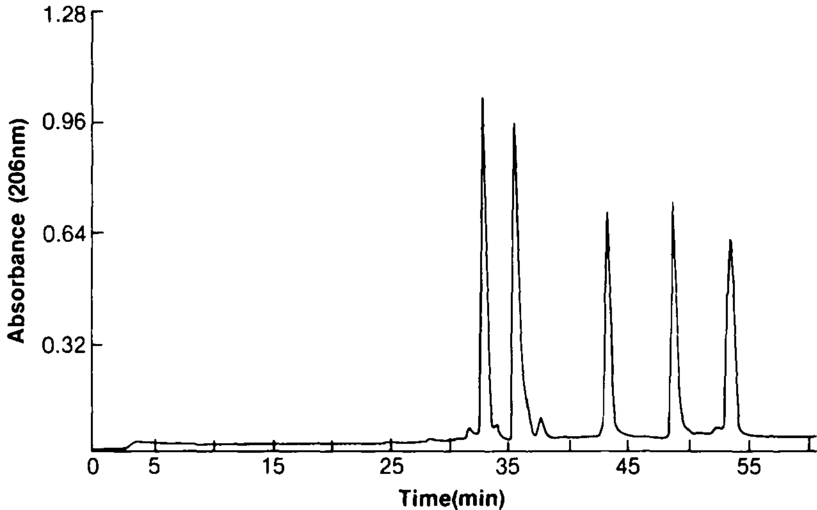

FIGURE 1: Reversed-phase HPLC chromatogram of model peptides and proteins using the abbreviated program. The order of elution is: (Met) Enkephalin, (Leu) Enkephalin, ribonuclease A, insulin, and lysozyme. Sample size: 20 μg of each component.

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To determine the location of hCG and its subunits on HPLC, we used, as a standard, a mixture of hCG and β subunit obtained by chromatographing highly purified, acid-dissociated hCG on Sephadex G-100 as previously described. (Figure 3S of reference 9 illustrates that such a mixture results from the incomplete separation of hCG from the β subunit). This mixture, having been extensively lyophilized, was incubated at 37°C in solvent A for 1 hr. Immediately after incubation the mixture was analyzed by HPLC using the abbreviated program (EXPERIMENTAL). Resolution was adequate enough to distinguish peaks; these were subsequently identified by specific RIAs for the α and β subunits and by amino acid analyses. Resolution was further enhanced by using the extended program (EXPERIMENTAL).

HPLC analysis of crude hCG

A 2 mg batch of crude hCG was incubated for 1 hr in TFA and analyzed by HPLC using the extended program (EXPERIMENTAL). The

contents of each of the peaks noted in Figure 2 were subsequently rerun using the extended program (EXPERIMENTAL). Again, specific RIAs and amino acid analyses clearly identified the peaks but SDS-polyacrylamide gel electrophoresis revealed that each subunit was contaminated to about 5% with the other subunit. Changing the conditions for dissociating the crude material or using PFPA in place of TFA did little to improve the resolution of the subunits. An alternate approach for isolating the subunits consisted of trying to dissociate a partially purified sample of hCG for subsequent analysis by HPLC. This partially purified hCG was obtained by dissolving 2 mg of crude hCG in solvent A and immediately running the mixture on HPLC. The profile shown in Figure 3 indicates that omitting the incubation step results in the appearance of a new peak of aggregated hCG (underlined in the figure). The contents of this peak were freed of acetonitrile as described above (EXPERIMENTAL) and the mixture was incubated at 37°C for 1 hr. Immediately following incubation the mixture was run on HPLC using the extended program (EXPERIMENTAL) and the results shown in Figure 4 were obtained. SDS-polyacrylamide gel electrophoresis of each peak indicated no cross-contamination of the subunits (Figure 5), and specific RIAs of each subunit allowed their recoveries to be estimated. From 2 mg of crude hCG, 55 μg of α subunit and 90 μg of β subunit were obtained, which on a percentage basis indicates an improvement over standard liquid chromatographic procedures (9, 13, 14). This result is not surprising since our HPLC approach entails less sample manipulation with less opportunity for sample loss.

The profiles obtained in Figures 1-4 were quite reproducible when samples were repeatedly run on the same μBondapak C_{18} column or on other μBondapak C_{18} columns. The ability of reversed-phase columns containing other bonded packings or C_{18}

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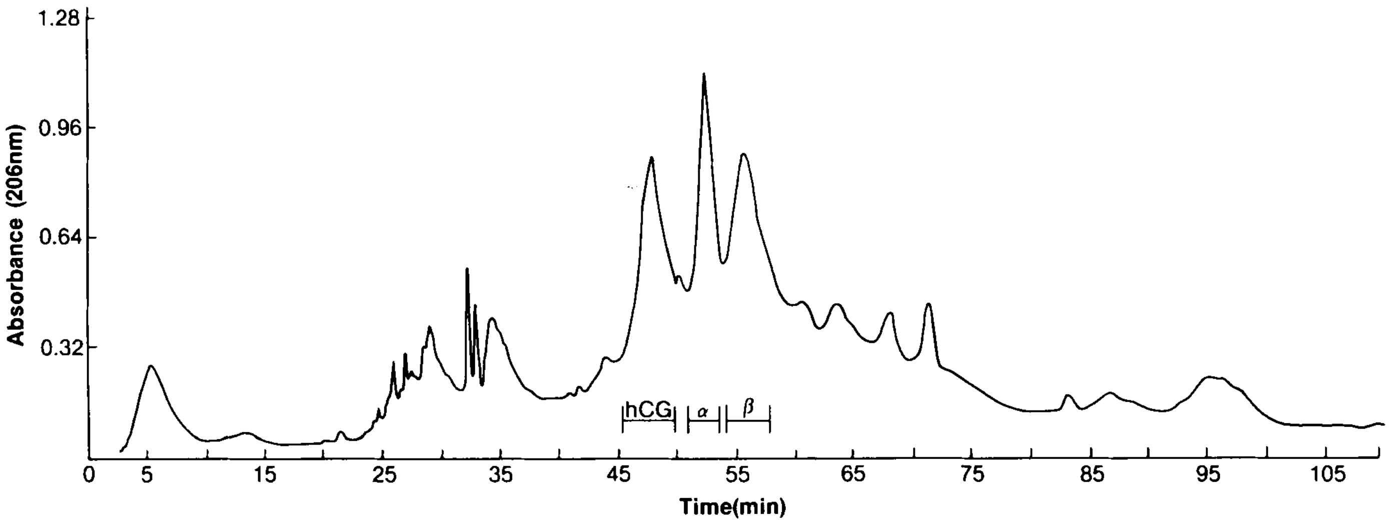

FIGURE 2: Reversed-phase HPLC chromatogram of crude hCG (2 mg) preincubated in solvent A for 1 hour at 37°C and run using the extended program.

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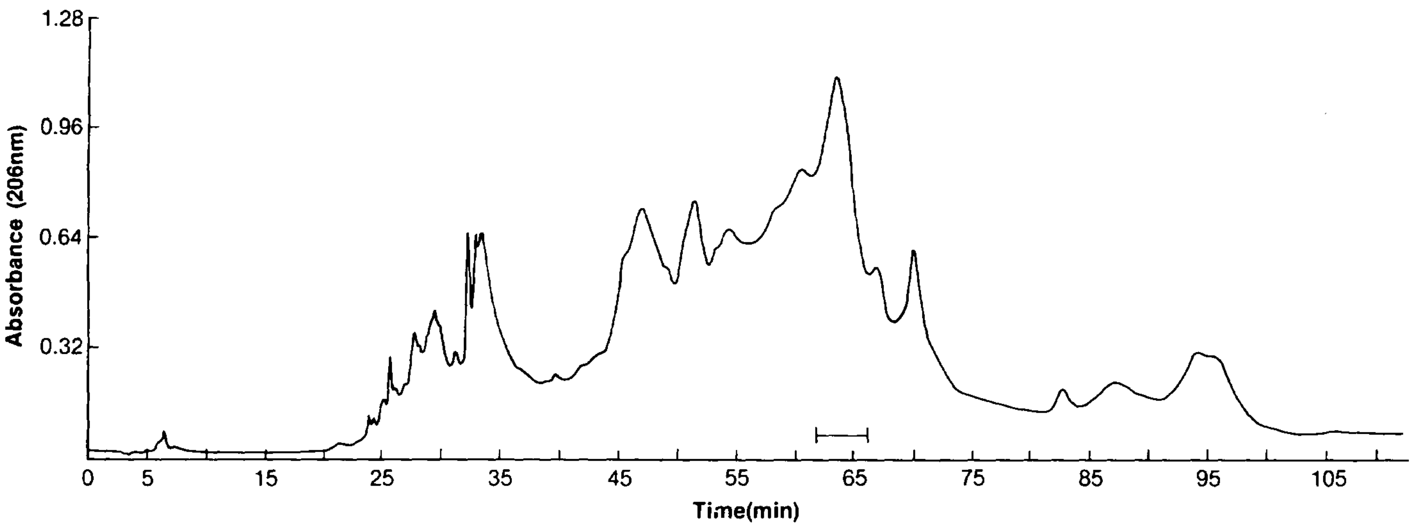

FIGURE 3: Reversed-phase HPLC chromatogram of crude hCG (2 mg) run without preincubation using the extended program. The peak underlined in the figure was subsequently incubated and rerun as described in the text.

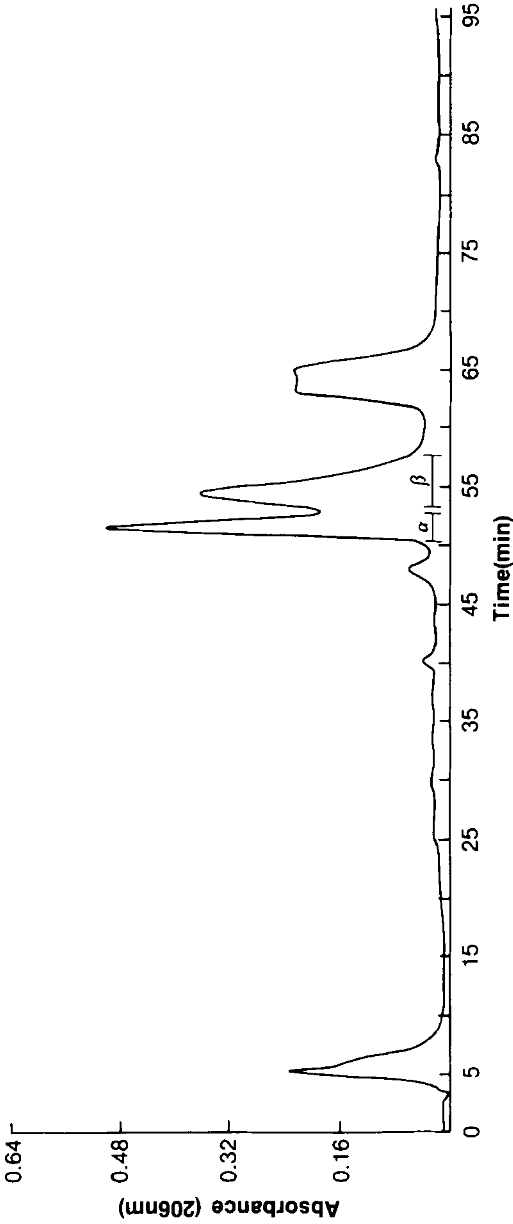

FIGURE 4: Reversed-phase HPLC chromatogram of the peak underlined in Figure 3. Sample was preincubated in Solvent A for 1 hour at 37°C and run using the extended program.

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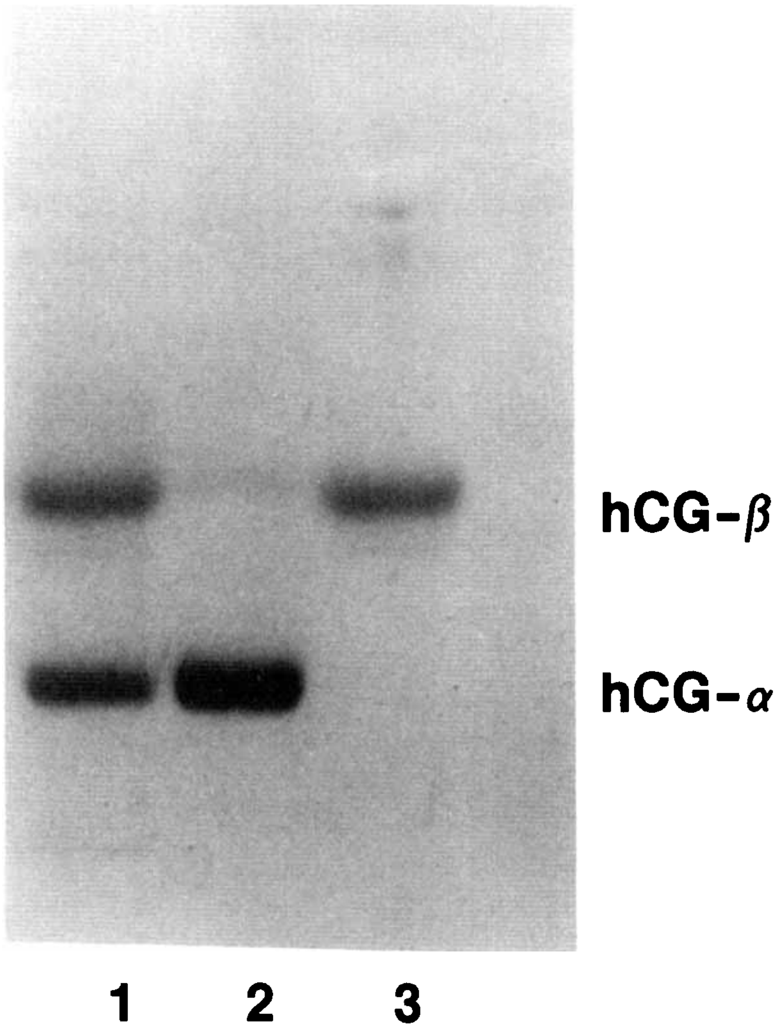

FIGURE 5: SDS-polyacrylamide gel electrophoresis of samples shown in Figures 3 and 4. Electrophoresis was performed with a 3% stacking gel and 13.5% running gel. Staining was with Coomassie blue. Lane 1: aliquot from peak underlined in Figure 3. Lanes 2 and 3: α and β subunits obtained as shown in Figure 4.

columns prepared by other manufacturers to separate hCG and its subunits remains to be determined.

Although this report has described the analysis of only 2 mg of crude hCG on 0.39 x 30 cm columns, similar or even better results have been obtained by running 5 mg of crude hCG on a 0.78 x 30 cm column of μBondapak C_{18}. The only change made in our programs in converting to the larger column consisted of doubling the flow rate to 2 ml/min. With the larger column the profile obtained for the sample without incubation indicates some subunit dissociation (probably due to the slower flow rate per column cross-sectional area). Peaks corresponding to free α and free β were observed, with α shown to be essentially pure and β slightly contaminated with α (Figure 6). This free α subunit can be combined with the α obtained following incubation and HPLC of the aggregated hCG. The free β subunit fraction can be mixed with the aggregate prior to acid dissociation and HPLC thereby giving even better yields than were obtained with the smaller column.

In addition to using HPLC to obtain subunits of hCG originating in human urine, we have also employed this technique to isolate the subunits from the media of JAR choriocarcinoma cells pulsed with $[^{35}S]$methionine (9). Preliminary results suggest that HPLC may be as effective as immuno-precipitation techniques in studying the synthesis, processing, and secretion of these subunits by cultured tumor cells (R. W. Ruddon, R. Hartle, M. B. Spear and G. J. Putterman, personal communication).

In conclusion, our results have demonstrated the effectiveness of reversed-phase HPLC in isolating small batches of hCG and its subunits. These products were characterized by comparing their retention times with products obtained by more "classical" approaches (9), by specific radioimmunoassays with well characterized antisera (9), by SDS-polyacrylamide gel electrophoresis

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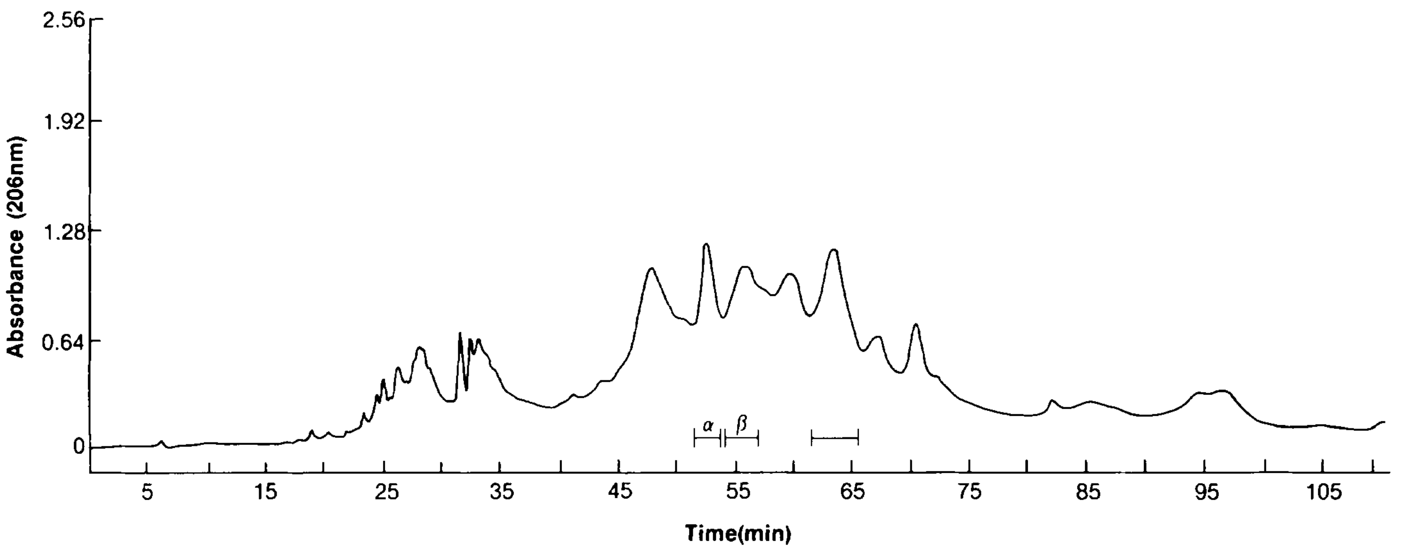

FIGURE 6: Reversed-phase HPLC chromatogram of crude hCG (5 mg) using a 0.78 x 30 cm column and extended program modified as described in the text.

and by amino acid analyses. Although no attempt has been made to subject the isolated hCG or hCG prepared by combining the isolated subunits to bioassay, the results described above regarding the isolation of the hormone and its subunits and the success observed in effectively increasing the sample load by means of a somewhat larger column encourage the use of preparative HPLC apparatus and extensive characterization of the biological properties of the isolated products. Meanwhile, analytical HPLC appears to be a rapid and convenient method of obtaining fresh hCG and its subunits to be used in immunological assays of these medically important molecules.

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