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High-performance liquid chromatography of human glycoprotein hormones

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ABSTRACT

The chromatographic behaviour of the glycoprotein hormones from human pituitary glands and of placental origin [thyroid-stimulating hormone, luteinizing hormone and chorionic gonadotropin (CG)] was studied. It was shown that hydrophobic interaction chromatography on a microparticulate packing and anion-exchange HPLC can be applied for the purification of these hormones. Reversed-phase HPLC on wide-pore C_4-bonded silica at neutral pH can be applied for the determination of the above hormones and for the isolation of pure CG and its subunits.

INTRODUCTION

The protein hormones from human pituitary glands and of placental origin include those of glycoprotein nature such as thyroid-stimulating hormone (TSH), luteinizing hormone (LH) and chorionic gonadotropin (CG), consisting of two non-covalently associated dissimilar subunits and containing up to 30% of carbohydrates with total relative molecular mass (M_r) *ca.* 30 000. The α-subunit is identical for all three hormones whereas the β-subunits differ and confer distinct biological properties on each molecule [1].

There are a number of lengthy procedures for the purification of these glycoprotein hormones, involving extraction from the corresponding sources followed by sequential chromatographic purification using supports with low efficiency [2].

It has been shown that the human glycoprotein hormones and their subunits can be purified using reversed-phase (RP) HPLC [3,4]. However, the data were considered to be controversial, especially for the retention of the biological activity [5]. Recent publications [6,7] demonstrated the real applicability of RP-HPLC and hydrophophobic interaction chromatography (HIC) to the isolation of pure biologically active human TSH, LH and follicule-stimulating hormone (FSH).

The human glycoprotein hormones are of great practical value because they are used as medical preparations, components of diagnostic kits and the substances for immunization in antibody production [8]. The development of reliable procedures for the preparation of highly purified glycoprotein hormones demands the choice of the optimum method for the determination of the content of these hormones in the fractions at various stages in their isolation and the application of the best chromatographic method for their final purification with high recovery and retention of immuno and biological activities. These aspects were the subject of this work, dealing with comparisons of the application of RP-HPLC, ion-exchange (IE) HPLC and HIC for the isolation and characterization of human TSH, CG and LH.

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EXPERIMENTAL

Materials

A CG preparation with a biological potency (b.p.) of 3500 I.U./mg and an LH sample with an immunopotency (i.p.) of 7800 I.U./mg by radioimmunoassay were purchased from Sigma (St. Louis, MO, USA). The purified TSH and LH samples were obtained from acetone-dried human pituitaries by extraction with 70% aqueous ethanol with 10% (w/v) ammonium acetate (the dry mass of the crude extract from 100 pituitaries was 65 mg with TSH i.p. = 0.9 I.U./mg), followed by chromatographic purification. The first step was purification on Sephacryl S-200 (Pharmacia, Uppsala, Sweden) with 4 m*M* ammonium acetate (pH 4.0) as eluent followed by separation on CM-Cellulose (Whatman, Maidstone, UK) with 1 *M* ammonium acetate (pH 4.0) as eluent, then Sephadex G-25 (Pharmacia) with 5 m*M* sodium glycinate buffer (pH 9.5) and finally DEAE-Sepharose (Pharmacia) with 0.2 *M* sodium glycinate–0.1 *M* NaCl to give the TSH-containing fraction. This TSH sample (6.3 mg, yield 43%) had an i.p. of 4.0 I.U./mg. The LH sample (1.5 mg, i.p. = 6300 I.U./mg) was isolated in the final stage of TSH purification as a fraction from DEAE-Sepharose, eluting before TSH with 0.2 *M* sodium glycinate buffer. The CG preparations (b.p. = 500–2500 I.U./mg) were obtained from urine from pregnant women by a modified procedure from various stages of purification [9].

The dissociation of a given hormone into subunits was carried out by treatment of its solution with 6 *M* guanidine hydrochloride (GH) or 10% trifluoroacetic acid (TFA) at 37°C for 1 h.

HPLC-grade acetonitrile (MeCN) was purchased from Merck (Darmstadt, Germany). All other reagents were of analytical-reagent grade. Distilled water was further purified with a Milli-Q System (Millipore, Bedford, MA, USA).

HPLC

Analytical HPLC was carried out with a Millipore–Waters liquid chromatograph consisting of two M-501 pumps, an M-680 gradient controller, a Rheodyne Model 7125 injector, an M-441 spectrophotometer set at 280 nm and an M-750 computing integrator. The volume of the hormone solutions (1 mg/ml) injected was 20–50 μl. The chromatographic conditions are given in the figure captions.

Preparative HPLC was carried out using a Gilson Auto-Prep system equipped with a Waters (Milford, MA, USA) Lambda-Max M-481 UV detector operated at 220 and 280 nm.

A Pharmacia fast protein liquid chromatography (FPLC) system was used for IE-HPLC and HIC and consisted of two P-500 syringe pumps, an MV-7 injector and a 280-nm fixed-wavelength single-path UV monitor, coupled to a two-channel REC 482 pen recorder. Gradient elution was controlled with an LCC-500 programmer.

The following prepacked columns were applied for HPLC: Vydac 214 TP, 10 μm (Separations Group, Hesperia, CA, USA) (250 × 4.6 mm I.D.), Mono Q HR 5/5, 10 μm (Pharmacia) (50 × 5 mm I.D.) and Separon HEMA BIO 1000 Phenyl, 10 μm (Tessek, Prague, Czechoslovakia) (80 × 8 mm I.D.). Packing materials such as anion exchangers, Accell QMA, 37–55 μm (Waters), and HB-IV, 10 μm (Vagos, Tallin, Estonia) (a macroporous polymer-based support with secondary and tertiary amino groups), and reversed-phase support, laboratory-made C_4-modified and end-capped LiChrospher Si 500, 10 μm (Merck) (LC-4) [10], were slurry packed according to the standard procedure.

Hormone bioassay

The immunopotencies of human LH and FSH were determined using Bio-Rad (Richmond, CA, USA) Novo Path, that of TSH with Bio Merieux (Marcy l'Etoile, France) and Boehringer (Mannheim, Germany) and that of CG with Roche (Basle, Switzerland) enzyme immunoassay kits. The biological potencies of CG samples were estimated on the basis of the increase in mass of male rat testicular glands [11].

Sodium dodecyl sulphate–polyacrylamide gel electrophoreis (SDS-PAGE) of some hormones was performed according to the standard Laemmli procedure [12].

RESULTS AND DISCUSSION

Reversed-phase HPLC at low pH

Gradient elution with a mixture of MeCN with 0.1% TFA is widely used for the RP-HPLC of protein hormones [13]. Various silica-based packings with different porosity and modified with hydrocarbon groups with various chain lengths have been applied for their separation. These sorbents are also

varied with the degree of surface modification and in the use of the end-capping process. We previously compared [14] a number of commercial packings differing in the above respects and also a domestic-made LC-4 support in order to find those most suitable for peptide and glycoprotein hormones. It was found that the end-capped packings with bonded C_4 groups and a pore size of more than 300 Å are the optimum for the analytical RP-HPLC of relatively large hormones (M_r > 20 000), including glycoproteins, whereas bare silica is more suitable for the separation of relatively small hormones.

It was subsequently shown [15] that highly pure procine calcitonin could be isolated from a crude extract of porcine thyroid glands using sequential preparative HPLC separation on RP and bare silica supports with a mixture of acetic acid and ethanol as the eluent.

Information available on the separation of mixtures containing glycoprotein hormones and their subunits using RP-HPLC at low pH even with macroporous C_4 packings was contradictory. For example, Bristow *et al.* [16] reported a better separation of TSH subunits at acidic pH, whereas Parsons *et al.* [17] demonstrated good resolution of human CG, TSH and LH subunits at neutral pH. Wilks and Butler [5] compared various mobile phases and packing materials and demonstrated that CG retained only 10–60% of its biological activity following RP-HPLC.

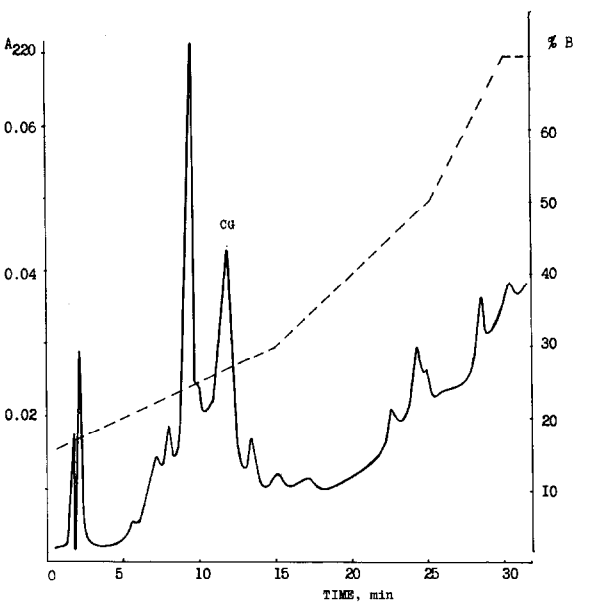

Fig. 1. Chromatography of CG (Sigma) on Vydac 214TP (250 × 4.6 mm I.D.). Mobile phase A, 10% MeCN plus 0.1% TFA; mobile phase B, 90% MeCN plus 0.1% TFA; sample loaded, 20 μl; flow-rate, 1 ml/min. The linear gradient for mobile phase B is indicated by the broken line.

In previous work [14,15] we demonstrated that RP-HPLC of glycoprotein hormones under standard acidic conditions (MeCN–0.1% TFA gradient) results in good separations and peak shapes. A serious decrease in the biological activity of the preparations obtained after purification under such conditions was observed with porcine FSH and CG. Similar results were demonstrated by other workers [5,17].

Thus RP-HPLC in acidic media, in spite of the convenience of using volatile buffers, fails to give reliable data for the isolation of pure biologically active glycoprotein hormones. Such difficulties in the isolation of active hormones at low pH may result from dissociation of the hormone subunits and probably partial elimination of terminal carbohydrate moieties.

RP-HPLC at low pH is still used for the analysis of complex mixtures containing glycoprotein hormones due to the high selectivity of separation. An example of such a separation is presented in Fig. 1 for a partially purified CG sample (Sigma). The separation profile is different from that obtained for the same sample with the same column but at neutral pH (Fig. 2a). These data demonstrate the effect of pH on the character of separation.

Reversed-phase HPLC at neutral pH

In spite of the rare application of neutral eluents for the RP-HPLC of proteins, these conditions were shown to be suitable for the analytical and preparative HPLC of human glycoprotein hormone subunits [17]. Hiyama and co-workers [6,7] clearly demonstrated that RP-HPLC on a C_4 column at neutral pH using a gradient of MeCN in 0.1 *M* triethylamine phosphate buffer (pH 6.5) is efficient for the separation of a mixture of human FSH, TSH and LH and for their final purification.

We applied the chromatographic conditions given by Parsons *et al.* [17] using C_4 rather than C_{18}-modified macroporous silica and successfully controlled different stages of the isolation of human glycoprotein hormones such as TSH, CG and LH

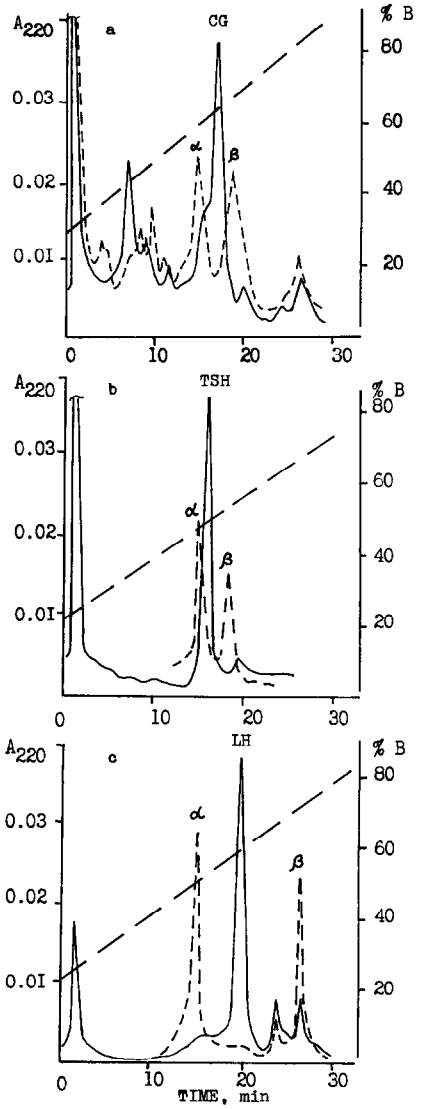

Fig. 2. Chromatography of (a) CG (Sigma), (b) TSH (i.p. = 6.2 I.U./mg) and (c) LH (i.p. = 6300 I.U./mg) on Vydac 214TP. Mobile phase A, 0.1 *M* sodium phosphate (pH 6.8); mobile phase B, 50% MeCN plus 50% mobile phase A; sample loaded, 20 μl; flow-rate, 2 ml/min. Linear gradient from 25 to 100% B in 40 min. The solid lines are intact hormone and the dashed lines are the same sample after incubation with 6 *M* GH.

and achieved the preparation of purified CG and its subunits.

The chromatographic profiles of partially purified CG and relatively pure TSH and LH are presented in Fig. 2. The peaks of the corresponding hormones are separated from the other admixtures. This facilitates the collection of the related fractions of the purified hormones. For example, we isolated relatively pure CG from the partially purified preparation (22 mg, b.p. = 2400 I.U./mg) after HPLC under the above conditions, desalting and lyophilization. The purified sample obtained (3.4 mg) had a high specific immunoactivity and its b.p. was about 11 000 I.U./mg (yield 71%). It can be also seen (Fig. 2b and c) that TSH and LH samples purified by HIC and IE chromatography, respectively, are fairly homogeneous, as also demonstrated by SDS-PAGE.

After incubation with 6 *M* GH, resulting in dissociation of the hormones into subunits, we obtained the chromatographic profiles shown as dashed lines in Fig. 2. It can be seen that the intact hormones dissociate into subunits. The positions of the LH subunits were identified by comparison with those of the Sigma LH sample. The elution volumes of TSH and LH subunits correspond to those observed by Hiyama and Renwick [6] in a similar neutral eluent. CG subunits were identified after their isolation by RP-HPLC with specific antibodies immobilized on the polymer support. We obtained 2.0

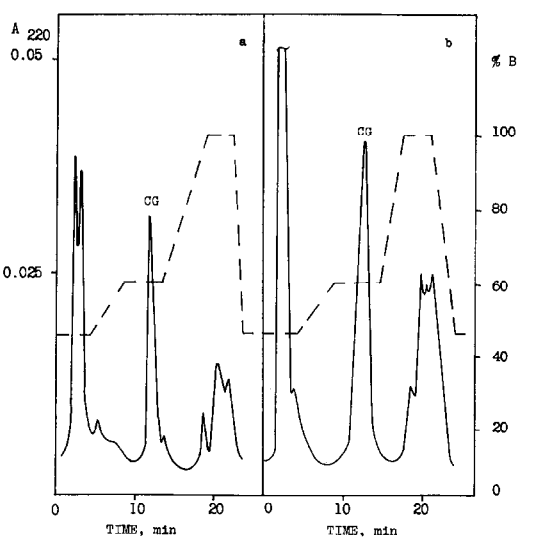

Fig. 3. Chromatography of CG preparation (a) from Sigma and (b) analysed sample on Vydac 214TP with specific gradient profile. Mobile phases and flow-rate as in Fig. 2. Sample loaded, (a) 20 μl and (b) 60 μl. The gradient for mobile phase B is indicated by the broken line.

mg of α- and 2.8 mg of β-subunit from 8 mg of intact hormone (yield 60%). The latter data demonstrate the possibility of isolating fairly pure subunits of the above hormones with a reasonable yield after HPLC.

The elution volumes (V_e) of human CG, TSH and LH subunits correlate with their structures each containing identical α-subunits (V_e = 29 ml) and β-subunits specific for each hormone (V_e = 39, 37 and 53 ml for CG, TSH and LH, respectively). The presence of two peaks instead of one in the chromatogram after incubation of the hormone preparation with GH may also be an indirect identification of the elution volume of the intact hormone if the corresponding standard is absent.

We also obtained the specific gradient profile for the HPLC of CG-containing samples (see Fig. 3). Such conditions allow the main peak to be separated from all admixtures and thus to the peak area of the intact hormone be determined and then normalized to a given amount of CG (611 arbitrary units per 20 μg in Fig. 3b). This permits an approximate determination of the biological potency of the sample to be obtained by comparison of the CG peak area with that of the sample (*e.g.*, Sigma) with known b.p. = 3500 I.U./mg (peak area = 1070 AU in Fig. 3a). Hence the found b.p. of the unknown sample was about 2000 I.U./mg. Biological analysis of the above sample gave 2200 I.U./mg. We performed such determinations on ten samples with a relative error of about 20%. This determination, although approximate, makes the HPLC control of the CG isolation process easier and facilitates the realization of the biological analysis where the linearity range is limited and dependent on the selected dose [11].

Similar results for the isolation and determination of CG were obtained on application of domestic LC-4 supports under similar chromatographic conditions.

Anion-exchange HPLC

Ion-exchange chromatography is a traditional method for the separation and purification of protein hormones [1,8]. With regard to human glycoprotein hormones, IE-HPLC has been successfully applied to the investigation and isolation of CG, TSH and FSH [15,18,19]. It has been demonstrated that HPLC on silica-based IE packings did not always give reproducible results, probably owing to the presence of residual silanol groups [18]. In fact, a high recovery on isolation with retention of biological potency and reliable analytical data on IE-HPLC of glycoprotein hormones were obtained using mainly either a polymer-based anion exchanger, Mono Q [19], a cation exchanger, Protein Pak SP 5PW (Waters) [18], or macroporous silica covered with a polymer layer with bonded ionogenic groups, Accell QMA [15]. In the last study we demonstrated that bovine FSH with the required biological potency and high recovery can be obtained from a crude extract from bovine pituitary glands with a high throughput (load up to 80 mg of extract per gram of sorbent).

Good separations of the components of a crude precipitate containing CG (b.p. = 400 I.U./mg) and a crude extract containing TSH (i.p. = 0.9 I.U./mg) were obtained using the macroporous anion exchanger HB-IV and Mono Q, respectively. The chromatographic profiles obtained are presented in Figs. 4 and 5. Good selectivity can be seen and the fractions containing the purified hormones (hatched zones) after desalting and lyophilization exhibit a ninefold increase in b.p. for CG (1.3 mg,

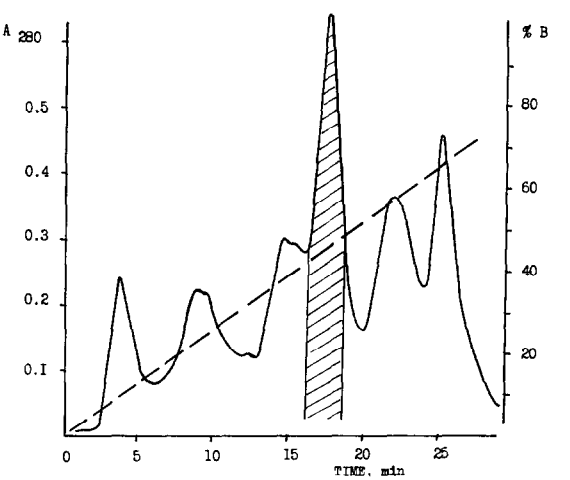

Fig. 4. Chromatography of CG samples (b.p. = 400 I.U./mg) on HB-1V anion-exchange column (140 × 4.6 mm I.D.). Mobile phase A, 0.01 *M* ammonium hydrogencarbonate buffer (pH 8.75); mobile phase B, 0.2 *M* ammonium hydrogencarbonate buffer (pH 8.75). The linear gradient of from 0 to 100% B in 40 min is indicated by the broken line. Sample loaded, 16 mg in 1 ml.

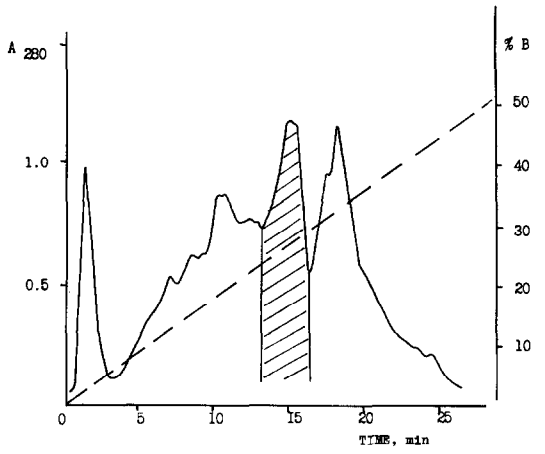

Fig. 5. Chromatography of TSH sample (i.p. = 0.9 I.U./mg) on a Mono-Q column. Mobile phase A, 20 m*M* Tris–HCl (pH 8.1); mobile phase B, 20 m*M* Tris–HCl (pH 8.1)–1 *M* NaCl. Linear gradient from 0 to 60% B in 35 min. Sample loaded, 15 mg in 1 ml. Flow-rate, 1 ml/min.

yield 55%) and a fourfold increase in i.p. for TSH (2.6 mg, yield 70%). Both supports have five times lower loading capacities than Accell QMA but allow fairly pure hormones to be obtained especially after rechromatography. The efficiency of separation of CG and TSH samples with Accell QMA was markedly lower, mainly owing to the larger particle size of the packing. The purification steps were controlled by RP-HPLC at neutral pH and by immunoenzyme analysis.

Hence anion-exchange HPLC on polymer-based macroporous supports, in spite of its lower efficiency in comparison with RP-HPLC, is a more promising method for primary purification of the crude extracts and for the isolation of those glycoprotein hormones which are required in relatively large amounts, owing to the much lower cost of the eluents and the higher loading capacity.

Hydrophobic interaction chromatography

HIC is now widely used for the purification of various proteins, including glycoproteins [20]. Its popularity resulted from the low price of the eluents and the suitability of the salt solutions for the preservation of biological activity of labile proteins. HIC was first applied to the separation of TSH subunits using low-efficiency pentyl-Sepharose 4B [21]. Recently, Hiyama *et al.* [7] demonstrated that HIC on a similar support, phenyl-Sepharose CL-4B, can be successfully employed to prepare highly purified biologically active human LH and TSH. These hormones were obtained with good yields although the separation procedure was long (more than 4 h).

We developed a procedure for the purification of CG and TSH using a column packed with the microparticulate packing Separon HEMA BIO 1000 Phenyl (10 μm) and a descending gradient of ammonium sulphate. The chromatographic conditions used for the HIC of partially purified CG and of TSH purified according to the traditional scheme (see Experimental) are presented in Fig. 6. As these hormones behave like hydrophobic compounds we add 10% ethanol to buffer B. It is interesting that the glycoprotein hormones studied are distinctly

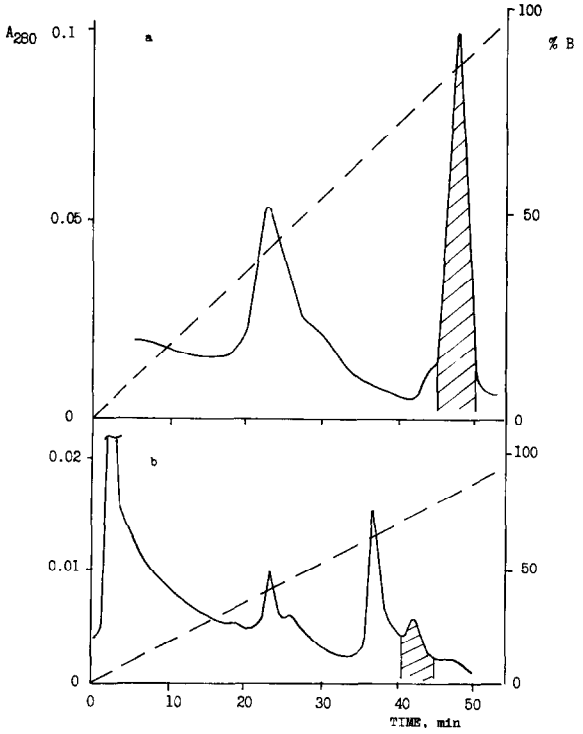

Fig. 6. Chromatograms of (a) TSH (i.p. = 4 I.U./mg) and (b) CG (b.p. = 1800 I.U./mg) samples on a Separon HEMA BIO 1000 phenyl column. Mobile phase A, 0.1 *M* ammonium acetate (pH 6.8)–2 *M* ammonium sulphate; mobile phase B, 0.1 *M* ammonium acetate–10% ethanol. Linear gradient from 0 to 100% B in 60 min. Sample loaded, (a) 15 mg and (b) 5 mg, both in 1 ml. Flow-rate, 1 ml/min.

separated from most of the admixtures and eluted similarly at the end of the gradient. The fractions containing the purified hormones (Fig. 6, hatched zones) were analysed by RP-HPLC at neutral pH using a C_4 column and by immunoenzyme assay. The i.p. of the TSH sample obtained (4.5 mg, yield 48%) was 6.3 I.U./mg and the contamination of this sample with FSH and LH was less than 0.05% and 2.3%, respectively. The b.p. of the CG sample (0.9 mg, yield 54%) was 5500 I.U./mg.

It seems possible to use HIC for the purification of mixtures containing small amounts of the glycoprotein hormones in question and for their final purification. The loading capacity of this column (80 × 8 mm I.D.) was shown to be high, *e.g.*, about 100 mg of partially purified CG per column. Thus the application of HIC may provide new possibilities for the creation of an inexpensive and efficient scheme for the isolation of human glycoprotein hormones, *e.g.*, like that proposed by Hiyama *et al.* [7].

CONCLUSIONS

The results obtained from these comparative studies of the application of various modes of HPLC to the human glycoprotein hormones CG, TSH and LH demonstrated that RP-HPLC at neutral pH, in contrast to that under acidic conditions, can be used for the isolation and characterization of human glycoprotein hormones and their subunits.

For the preparative isolation of the glycoprotein hormones from partially purified mixtures, IE-HPLC on macroporous polymer-based anion exchangers and HIC on phenyl-modified polymeric microparticulate supports can be applied.

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