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Separation of human glycoprotein hormones and their subunits by reversed-phase liquid chromatography

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ABSTRACT

Separation of human pituitary follicle-stimulating hormone (FSH), luteinizing hormone (LH) and thyroid-stimulating hormone (TSH) was effected on a micro-scale by reversed-phase high-performance liquid chromatography (HPLC) on a SynChropak C_1 column in series with a Vydac C_4 column using a linear gradient of acetonitrile in 0.1 *M* triethylamine phosphate buffer at pH 6.5. Chromatography on the C_4 column alone caused partial dissociation of FSH into its subunits, whereas LH and TSH remained intact. Good yields of the separated subunits were obtained after prior dissociation of each hormone, and the results show that reversed-phase HPLC is useful for the analytical and preparative separation of these structurally related hormones and their subunits.

INTRODUCTION

The human anterior pituitary gland synthesizes and secretes three glycoprotein hormones, follicle-stimulating (FSH), luteinizing (LH) and thyroid-stimulating (TSH) which, with human placental chorionic gonadotrophin (hCG), share certain structural features. Each hormone consists of two dissimilar subunits, α and β, and while the primary structure of the α component is virtually the same in all four hormones, the amino acid sequence of the β subunit is unique. When linked non-covalently, the inactive subunits form the hormonally active heterodimer [1].

Similarities in size, structure and considerable heterogeneity in charge [2] have caused problems in the isolation and purification of these glycoproteins, and no simple chromatographic procedure has been developed for the analyt-

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ical or preparative separation of the three pituitary hormones. Whereas reversed-phase high-performance liquid chromatography (RP-HPLC) has been successfully employed for the isolation of the subunits of glycoprotein hormones [3,4], and for the separation of individual hormones [5–7], no single procedure has been reported for the separation of FSH, LH and TSH, although its potential has been suggested [5,6]. This may reflect difficulties in the selection of optimal conditions for the reversed-phase separation of these labile heterodimers which are partially or completely dissociated in some systems [3,4]. We have examined the separation and dissociation of FSH, LH and TSH under various chromatographic conditions mainly using a silica-based, wide-pore C_4 column, with the aim of developing an RP-HPLC procedure for the separation of all three hormones and their subunits.

EXPERIMENTAL

Materials

Acetonitrile (HPLC grade), trifluoroacetic acid (TFA, reagent grade) and orthophosphoric acid (AnalaR grade) were obtained from BDH (Palmerston North, New Zealand), Triethylamine (TEA, analytical grade) was from Riedel-de Haen (Seelze-Hannover, F.R.G.). Water was purified by a Milli-Q system (Millipore, Bedford, MA, U.S.A.). Purified human FSH, LH and TSH were prepared in this laboratory by DEAE-Sephacel chromatography and RP-HPLC [8] from the Sephadex G-100 glycoprotein fraction obtained according to Chapman et al. [9]. All other chemicals were of analytical grade.

Reversed-phase high-performance liquid chromatography

A Tosoh HPLC system (Tosoh, Tokyo, Japan) consisting of a Model CCPM pump with microprocessor control and a Rheodyne 7125 injector with 2-ml sample loop was used with a Uvicord S Model 2138 UV monitor (LKB, Bromma, Sweden). Vydac C_4 (300 Å, 5 μm) columns (150 mm$\times$4.6 mm I.D. and 250 mm$\times$10 mm I.D.) (Separations Group, Hesperia, CA, U.S.A.) and a SynChropak C_1 (300 Å, 6.5 μm) RP-1 column (100 mm$\times$4.6 mm I.D.) (SynChrom, Lafayette, IN, U.S.A.) were used for separations. Cartridge columns packed with SynChropak RP-1 (10 mm$\times$4.6 mm I.D.) were supplied by Alltech (Auckland, New Zealand). Samples of each or a mixture of the three hormones were prepared in 0.1 *M* sodium phosphate buffer, pH 7.0 (1.0 mg of each hormone per ml solution), just prior to HPLC. When separating subunits, hormones were dissociated prior to HPLC as follows: FSH was treated with 0.1% TFA, pH 2.3, for 30 min at 25°C, and LH and TSH (1.0 mg/ml) were incubated for 20 h at 37°C with 6 *M* guanidine hydrochloride in 0.1 *M* sodium phosphate buffer, pH 7.0, that contained 0.01% sodium azide. Samples were chromatographed on an analytical or a semi-preparative Vydac C_4 column with and without a C_1 column in series, using various linear gradients of acetonitrile

in 0.1 *M* triethylamine phosphate (TEAP) buffer, pH 6.5, at ambient temperatures (about 25°C). Mobile phase A was 0.1 *M* TEAP (pH 6.5, adjusted with TEA) and mobile phase B was 0.1 *M* TEAP in 60% (v/v) acetonitrile in water (pH 6.5, adjusted with TEA). Typically, a linear gradient of 20–100% B over 60 min was used at a flow-rate of 0.8 ml/min, and column eluates were monitored at 226 nm. Detailed chromatographic conditions are described in the legends for figures. Subunit peaks were identified by amino acid analysis or by comparison of retention times with those of the hormonal subunits.

Analytical methods

Recovery of intact hormones after RP-HPLC was estimated by specific radioimmunoassays using Amerlex-M RIA kits for FSH (IM 3071), LH (IM 3081) and TSH (IM 3161) (Amersham Australia, Sydney, Australia). Each of the hormone peak fractions was collected and diluted to 3.0 ml in water. Aliquots of this solution were further diluted with 0.025 *M* Tris–HCl buffer, pH 7.4, that contained 0.9% sodium chloride and 0.1% bovine serum albumin, to yield a final concentration of 2.5–10 ng/ml. Controls were prepared by diluting unfractionated hormones (100 μg) as above. The yield of each subunit was estimated from protein concentrations determined with protein assay kits (Bio-Rad, Richmond, CA, U.S.A.). Controls were prepared as above from dissociated hormones (100 μg). Samples and controls were assayed using bovine γ-globulin as reference. The following M_r values (kD) were used to calculate yields in % by mass: α 14.6, FSH-β 18.0, LH-β 14.8, TSH-β 15.8 [10].

RESULTS AND DISCUSSION

As noted earlier, all three pituitary glycoprotein hormones are unstable in acidic solution (pH <4) at 25°C, and they readily dissociate into their subunits [11,12]; this is especially true in the case of FSH. Because of this, the pH of mobile phases used for RP-HPLC of the intact hormones is limited to the range where dissociation does not occur or where it is minimal. Various mobile phases in the pH range 7–7.8 have been employed with alkylphenyl, C_1, C_4, C_8 and C_{18} alkyl-chain bonded silica columns in studies of the behaviour of the individual hormones [5,6]. Bristow et al. [5] reported that TSH, which was stable at pH 7.0, was significantly dissociated into subunits at pH 5–6, during analysis on a C_4 column in sodium chloride-containing buffers. Because this hormone is stable in aqueous buffers at pH 5–7, the marked effect of mobile phase pH could have reflected significant pH-dependent influences of protein–ligand interactions on the association of subunits.

We initially studied the influences of various mobile phase conditions at pH 5–7 on the separation and stabilities of the three intact hormones using C_4 and C_{18} columns. It was found that these hormones were more stable on shorter

alkyl-chain packings (C_4) and that gradient elutions with acetonitrile in 0.1 *M* TEAP at pH 6–7 gave good separations of LH and TSH. The dissociation of these hormones was noticeably decreased upon increasing the mobile phase pH up to 6.5, above which no significant differences were observed. However, under these conditions FSH was very unstable, although its hydrophobic interaction with this column packing was apparently less than that of LH and TSH. Its dissociation was slightly decreased on increasing the initial concentration of acetonitrile for gradient elutions from 0 to 18%. Above this range, this hormone was not effectively retained by C_4. These preliminary results indicated that the pH of the mobile phase and the initial concentration of acetonitrile were important variables that affected the separation of the three hormones. Acetonitrile (12%, v/v) was chosen for initial gradient elution during the separation of FSH, LH and TSH, to ensure the retention of FSH, which was eluted first from the C_4 column. The elution profiles of the three hormones obtained with a Vydac C_4 column (150 mm×4.6 mm I.D.) with a linear gradient of 12–44% acetonitrile in 0.1 *M* TEAP, pH 6.5, are shown in Fig. 1a. The extent of dissociation of each intact hormone was assessed by detection of its α and/or β subunit peaks, which were clearly resolved from their respective intact hormones except in the case of FSH (Fig. 1b). TSH and LH were eluted almost as a single peak without significant formation of subunits. In contrast, FSH was eluted as two discrete peaks that corresponded in retention time to those of its subunits. These results indicated that LH and TSH were stable

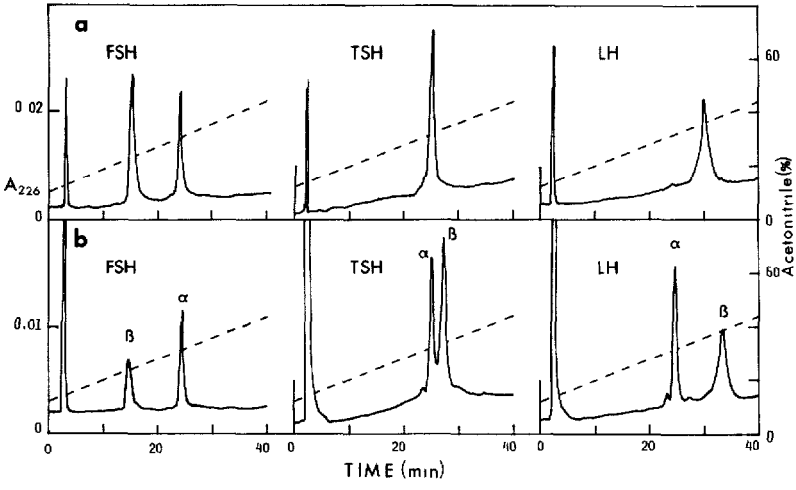

Fig. 1. RP-HPLC of FSH, LH, TSH and their subunits on a Vydac C_4 (150 mm×4.6 mm I.D.) column. Mobile phase A, 0.1 *M* TEAP (pH 6.5); mobile phase B, 0.1 *M* TEAP in 60% acetonitrile (pH 6.5). The 40-min linear gradient from 12 to 44% (v/v) acetonitrile is indicated by the broken line. Flow-rate, 0.8 ml/min. Samples: (a) intact hormones (25 μg each); (b) dissociated hormones, FSH (10 μg), LH and TSH (25 μg each) prepared as described in Experimental. The subunit peaks from each hormone are indicated.

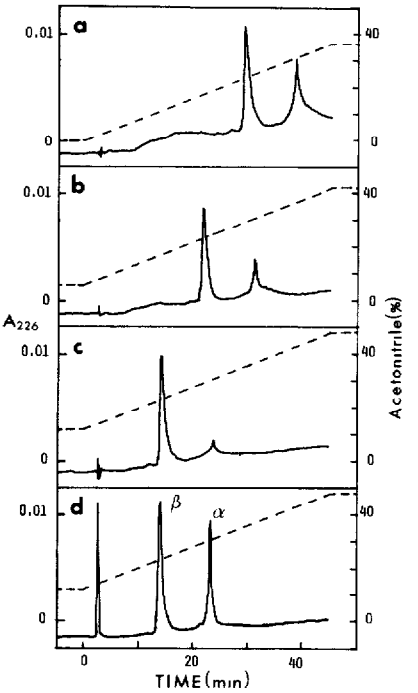

Fig. 2. Elution profiles of FSH on C_1 and C_4 columns in series. Columns: SynChropak RP-1 (10 mm×4.6 mm I.D.) and Vydac C_4 (150 mm×4.6 mm I.D.). Mobile phases A and B are the same as in Fig. 1. The 45-min linear gradient from 0% (a), 6% (b) and 12% (v/v) acetonitrile (c and d) with a slope of 0.8% (v/v) acetonitrile per min are indicated by the broken line. Flow-rate was 0.8 ml/min. Samples: (a–c) FSH, 10 μg; (d) dissociated FSH, 20 μg.

under conditions employed, whereas FSH was largely dissociated. Various unsuccessful attempts were made to reduce the dissociation of FSH by modification of mobile phase parameters such as flow-rate, gradient slope and buffer concentration. The substitution of the less eluotropic methanol for acetonitrile and the use of Na^+ as a less hydrophobic counter-cation were also ineffective. The results suggested that the C_4 column was too strongly hydrophobic for the separation of FSH by RP-HPLC.

When other ligands such as C_1, alkylphenyl and cyanopropyl silicas were examined with the view of replacing C_4, only C_1 showed a noticeable decrease in the dissociation of FSH under the conditions used in Fig. 1. However, its lower selectivity did not permit the effective separation of the three hormones (results not shown). Because the magnitude of solvent–ligand interactions is expected to decrease with less retentive ligands and with increasing organic solvent modifier in the mobile phase, we inserted a small C_1 cartridge column in series with the C_4 column. Fig. 2 shows the effects of a coupled C_1 column (10 mm×4.6 mm I.D.) on the elution of FSH, which was examined at various

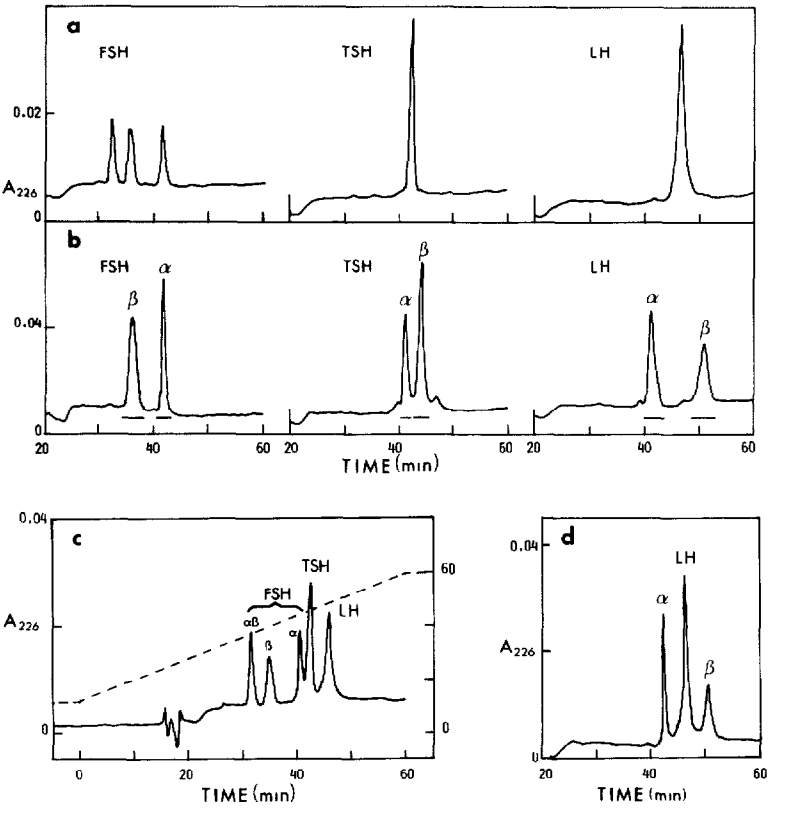

Fig. 3. Separation of FSH, LH and TSH and their subunits on a Vydac C_4 column (250 mm × 10 mm I.D.). Mobile phase A, 0.1 *M* TEAP (pH 6.5); mobile phase B, 0.1 *M* TEAP in 60% acetonitrile. The 60-min linear gradient of 12–60% (v/v) acetonitrile (20–100% B) is exemplified by the broken line in (c). The flow-rate was 0.8 ml/min. Samples: (a) intact hormones: FSH (25 μg), LH (50 μg) and TSH (30 μg); (b) dissociated hormones FSH, LH and TSH (100 μg each); (c) a mixture of FSH, LH and TSH (25 μg each); (d) a mixture of intact and dissociated LH (30 μg). The two major subunit peaks from each hormone were collected as indicated.

initial concentrations of acetonitrile (0–12%) without changing the solvent system and gradient slope as in Fig. 1. Although these effects were small at 0% acetonitrile, they became more prominent at higher concentrations of acetonitrile (Fig. 2a–c); at 12% acetonitrile, FSH was eluted as a major peak at the position of its β subunit, and only a small α subunit peak was detected (Fig. 2c and d). This showed that the rapid subunit dissociation of this hormone was greatly reduced and that the intact hormone was eluted with its β-subunit. The results suggested that this method would be effective for the separation of all three hormones by RP-HPLC without dissociation.

The possibility was further examined with larger C_4 and C_1 columns, because of the limited capacity of the C_1 cartridge in preparative applications. The

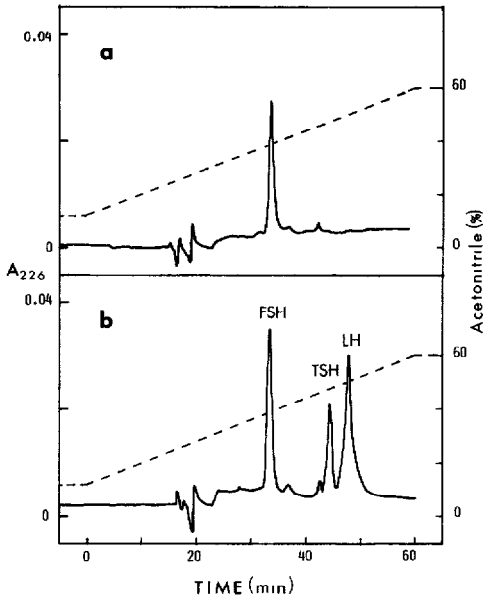

Fig. 4. Separation of FSH, LH and TSH on C_1 and C_4 columns in series. Columns: SynChropak RP-1 (100 mm × 4.6 mm I.D.) and Vydac C_4 (250 mm × 10 mm I.D.). The elution conditions were the same as those in the legend to Fig. 3. Samples: (a) FSH (25 μg); (b) FSH (25 μg), LH (50 μg) and TSH (15 μg).

semi-preparative Vydac C_4 column (250 mm × 10 mm I.D.) gave similar or even better results when the intact hormones were chromatographed without changing the gradient programme and flow-rate as in Fig. 1. As shown in Fig. 3a, FSH gave three peaks, two of which corresponded in retention time to those of its α and β subunits. Because the first peak was completely converted to the subunit peaks after dissociation (Fig. 3b), it was identified as the intact hormone; the resolution of FSH and its β subunit was not attained on the analytical C_4 column.

TSH and LH were eluted as major single peaks. The absence of subunit peaks indicated that both hormones were almost entirely undissociated (Fig. 3a and b). Fig. 3c shows that, because of the improved resolution of FSH-α and TSH, it was possible to separate all three intact hormones with partial dissociation of FSH. Despite this limitation, this procedure will be useful for the separation of intact LH and TSH. While the three α subunits, which are virtually identical in their amino acid sequences, were eluted very closely as previously noted [4], the separation of all three β subunits was readily obtained. Furthermore, excellent separation of the subunits of all three hormones (Fig. 3b) suggests that this procedure will be of value for the preparation of hormonal subunits. The average yield of subunits from approximately 100 μg of each hormone pretreated as described in Experimental was as follows: FSH-

α 69%, FSH-β 67%; LH-α 52%; LH-β 71%; TSH-α 63%, TSH-β 76%. In the case of LH, the intact hormone and its subunits were almost completely separated from each other (Fig. 3d). Thus, this method may provide a convenient means of preparing reassociated hormones, including hybrids consisting of LH-β and α subunits from other hormones, since the chromatographic behaviour of such hormones can be expected to be similar to that of LH.

The effect of a coupled C_1 column (SynChropak RP-1, 100 mm$\times$4.6 mm I.D.) in reducing the partial dissociation of FSH on the C_4 column (250 mm$\times$10 mm I.D.) is shown in Fig. 4a. This C_1 column was used because it gave appropriate retention of the three hormones without the need for changing the gradient elution conditions. This result was dependent upon flow-rate, and the dissociation of the labile hormone was increased at flow-rates higher than 0.8 ml/min. When a mixture of FSH, TSH and LH was chromatographed, all three hormones were separated from each other without significant loss in resolution (Fig. 4b), indicating that this modified procedure was effective for the separation of all three intact hormones. The average recovery of FSH, TSH and LH (100 μg each) from the columns was 56, 66 and 76%, respectively, by radioimmunoassay. This procedure has been found useful for the micro-scale isolation of each pituitary hormone from partially purified preparations that contained the three glycoproteins in various amounts [8]. Although no attempts were made to determine the effect of RP-HPLC on biological activity in this study, we have recently confirmed that LH and TSH retained high biological activity after purification on a C_4 column, according to the procedure described in this paper [13]. We have yet to determine the effect on FSH. These results show the effectiveness of RP-HPLC for the rapid separation of intact pituitary glycoprotein hormones and their subunits.

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