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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF HUMAN THYROID-STIMULATING HORMONE

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SUMMARY

Human pituitary thyroid-stimulating hormone (TSH) has been analysed by reversed-phase high-performance liquid chromatography on Aquapore RP-300, a 300-Å pore-size short-alkyl-chain silica packing, in sodium chloride-containing buffers. At pH 2.0, two clearly resolved peaks were observed corresponding in retention time to α and β subunit of TSH. With increasing pH, the resolution between these peaks was lost. At pH 7.0 a single major peak was observed, co-eluting with immunoreactive TSH, which could be resolved from β-subunit. *In vivo* TSH bioactivity was recovered at pH 7.0 in 58% yield. High-performance liquid chromatography of a sample of hypothyroid serum yielded a major peak of TSH immunoreactivity corresponding in retention time to that observed for purified TSH.

INTRODUCTION

Human "thyrotrophin" (TSH) is a glycoprotein consisting of two dissimilar subunits, each of *ca.* 15,000 daltons[1]. The overall carbohydrate content is 21.1% with both subunits containing carbohydrate moieties[2]. The β-subunit is unique to TSH and confers biological specificity, whereas the α-subunit is closely similar to the subunit of the related glycoprotein hormones follicle-stimulating hormone[3], human chorionic gonadotrophin[4], and luteinizing hormone[5]. Dissociation of the hormone into free subunits can be achieved at low pH[6]. Both the native hormone and the free subunits exhibit charge-based heterogeneity when subjected to electrophoresis[6,7], with up to five isohormones being observed.

Purification of TSH and its subunits has been achieved using conventional ion-exchange and gel-filtration techniques[1], immunoaffinity chromatography[8] and hydrophobic-interaction chromatography on pentyl-Sepharose[6]. In this report we describe the behaviour of human TSH on a reversed-phase high-performance liquid chromatography (HPLC) system, using a short-alkyl-chain silica, with large (300 Å) pore-size, Aquapore RP-300, eluted with sodium chloride-containing mobile phase, and gradients of acetonitrile.

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EXPERIMENTAL

Materials

Human TSH and human TSH β-subunit used in this study were prepared in the laboratory of Dr. A. F. Parlow, California, U.S.A., for the U.S.A. National Pituitary Agency Human Pituitary Program. These materials were donated to WHO by the U.S.A. National Pituitary Agency for evaluation as potential international reference preparations. The preparation of human chorionic gonadotrophin (HCG) α-subunit studied was a preparation coded 76/508. Hypothyroid serum was supplied for use in a WHO TSH collaborative assay by Professor W. S. Munro, Sheffield, U.K. HPLC grade acetonitrile was obtained from BDH (Poole, U.K.). All other reagents were of analytical grade.

High-performance liquid chromatography

HPLC was performed on a Altex liquid chromatograph (Anachem, Luton, U.K.) using a Model 420 solvent programmer. All experiments were performed using an Aquapore RP-300 column (25 × 0.46 cm I.D., Anachem) at a flow-rate of 1 ml/min. Aquapore RP-300 consists of a short-alkyl-chain hydrophobic bonded phase on a 300 Å pore size, 10-μm spherical silica base. Mobile phase A consisted of either 0.155 *M* sodium chloride–0.01 *M* hydrochloric acid or 0.155 *M* sodium chloride–0.025 *M* orthophosphoric acid, adjusted to the appropriate pH using sodium hydroxide. To prevent corrosion the equipment was passivated with nitric acid according to manufacturers instructions. Mobile phase B was acetonitrile. Linear gradients of 5–50% (v/v) or 5–60% (v/v) were employed, the details of which are given in the figure legends.

TSH immunoassay

Radioimmunoassay of TSH was performed using chloramine T-iodinated TSH tracer and a rabbit anti-TSH kindly supplied by Professor W. R. Butt (Birmingham, U.K.), and a donkey-anti-rabbit second antibody supplied by the WHO Matched Reagent Scheme. The First International Reference Preparation of hTSH for Immunoassay (68/38) was used as the standard.

TSH bioassay

In vivo bioassay of TSH was performed using a two-day crossover modification of the method of McKenzie[9], in which the release of thyroidal radioiodine in mice is measured in response to graded doses of TSH. The method used in this studies compared three concentrations of test with three concentrations of standard using two-fold dilution increments, with eight animals per dosage point and has been described elsewhere[10]. Data were analysed by the method of Gaines-Das and Hughes[11]. In order to assess the *in vivo* bioactivity recovered in the chromatogram eluate the following experiment was performed. TSH (180 μg) was weighed and dissolved in 50 μl of mobile phase A (0.155 *M* sodium chloride–0.025 *M* sodium phosphate, pH 7.0). The volume containing 150 μg was fractionated on Aquapore RP-300 (for conditions see Fig. 3). The remaining solution containing 30 μg of TSH was used as the bioassay standard. Following chromatography, the material eluting at 30–40 min was pooled, 0.4 ml of 0.2% bovine serum albumin added, and this solution was freeze-dried.

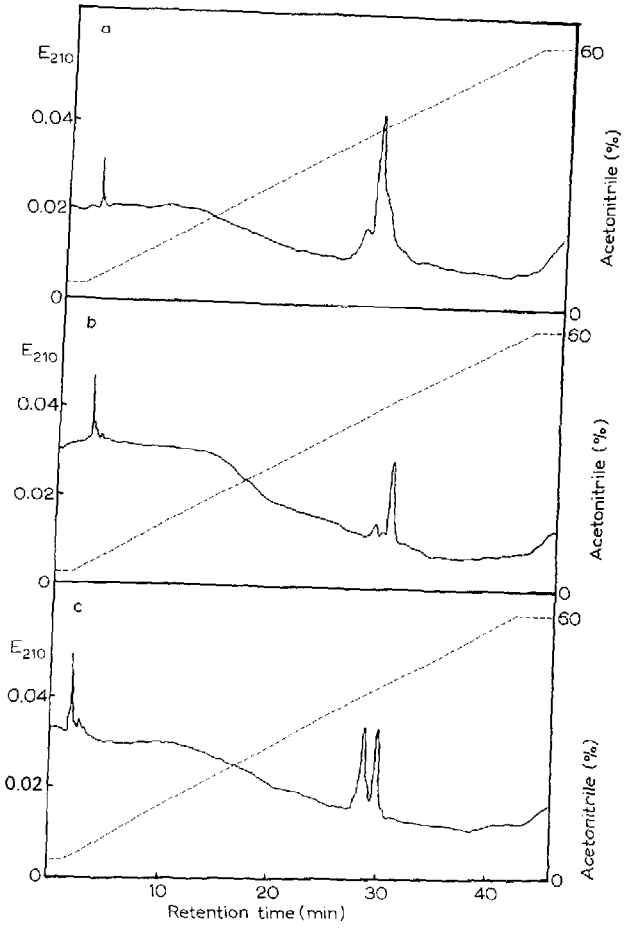

Fig. 1. Chromatography of TSH-β (a), HCG-α (b) and TSH (c) on Aquapore RP-300. Mobile phase A, 0.155 *M* sodium chloride–0.01 *M* hydrochloric acid, pH 2.0; mobile phase B, acetonitrile; sample loaded in each case, 10 μg. The linear gradient of 5–60% (v/v) acetonitrile is indicated by the broken line.

Using the *in vivo* bioassay the potency ratio of this fraction to the non-chromatographed "load" fraction was estimated.

RESULTS

Following chromatography at pH 2.0 in the 0.155 *M* sodium chloride–0.01 *M* hydrochloric acid system, TSH eluted as two well-resolved components (Fig. 1c). The retention time of the earlier-eluting peak corresponded to that of β-subunit of TSH (Fig. 1a) whilst the retention time of the later-eluting peak corresponded to that of α-subunit of hCG (Fig. 1b). This result showed that under the conditions used in this chromatogram, TSH dissociated to give TSH-α and TSH-β which were resolved. Both peaks were asymmetric, suggesting multiple components in each subunit. In five experiments under the conditions described in Fig. 1c, the mean retention times of the two major peaks were 29.12 $\pm$ 0.12 min and 30.34 $\pm$ 0.14 min.

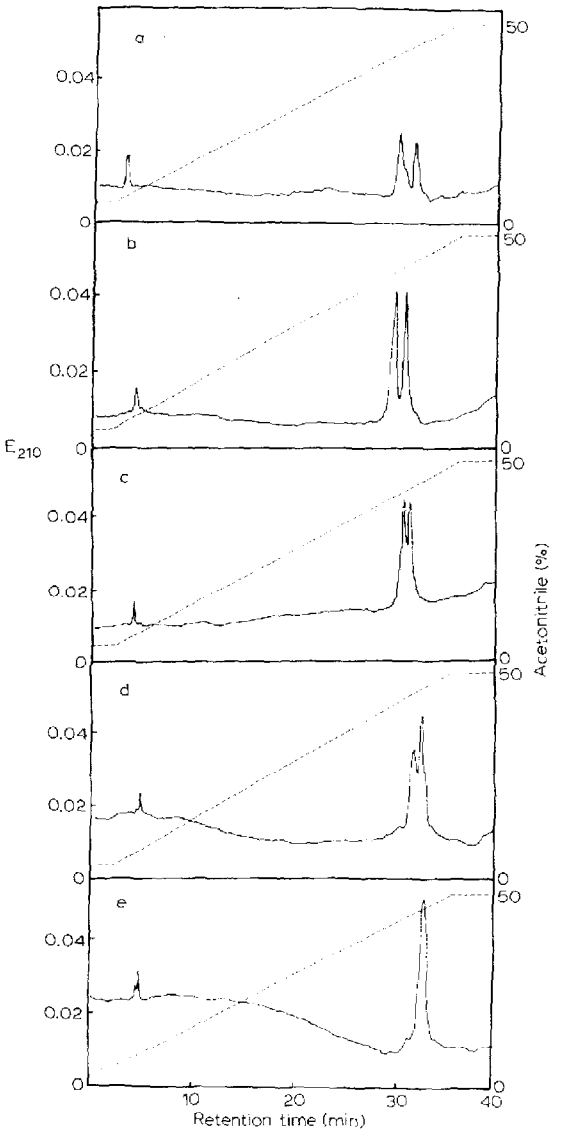

Fig. 2. Chromatography of TSH on Aquapore RP-300. Mobile phase A, 0.155 *M* sodium chloride 0.025 *M* orthophosphoric acid, adjusted with sodium hydroxide to pH 2.0 (a), pH 3.0 (b), 4.7 (c), 6.0 (d) and 7.0 (e); mobile phase B, acetonitrile; samples loaded in each case, 10 μg. The linear gradient of 5 50% (v/v) acetonitrile is indicated by the broken line.

The effect of increasing the pH of the eluent on the behaviour of TSH on Aquapore RP-300 is shown in Fig. 2. In 0.155 *M* sodium chloride 0.025 *M* orthophosphoric acid, pH 2.0 (Fig. 2a), resolution of TSH-α and TSH-β was similar to that seen in Fig. 1c. At increasing pH values of 3.0 (Fig. 2b), 4.7 (Fig. 2c), 6.0 (Fig. 2d) and 7.0 (Fig. 2e) an apparent loss of resolution between the α and β subunit peaks was observed. A pH 7.0, a single major asymmetric peak was seen (Fig. 2e), which may have corresponded either to free α and β subunits, which no longer re-

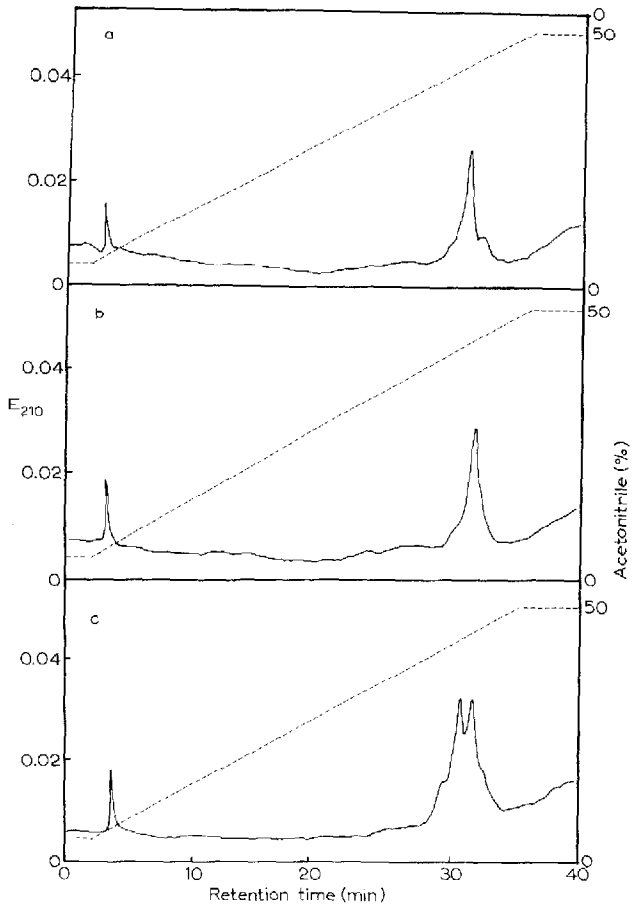

Fig. 3. Chromatography of TSH (a), TSH-β (b) and TSH plus TSH-β (c) on Aquapore RP-300. Mobile phase A, 0.155 *M* sodium chloride 0.025 *M* orthophosphoric acid adjusted to pH 7.0 with sodium hydroxide; mobile phase B, acetonitrile; sample loaded in each case, 10 μg. The linear gradient of 5–50% (v/v) acetonitrile is indicated by the broken line.

solved at this pH, or to native TSH. In four experiments under the conditions described in Fig. 2, the major peak eluted at a retention time of 31.98 ± 0.23 min.

Co-chromatography of TSH and TSH-β under the conditions of Fig. 2e, (Fig. 3), suggested that any free subunit would be expected to separate from native TSH at pH 7.0, although the resolution between the α and β subunits at pH 7.0 (not shown) was considerably less efficient than at pH 2.0. Nevertheless it appears that the single asymmetric peak observed at pH 7.0 represented native hormone.

In a further experiment, immunoassays of the column effluent following chromatography at pH 7.0 (Fig. 4) revealed a major peak of TSH immunoreactivity, corresponding in retention time to that of the major UV-absorbing peak, with a slight displacement to the right caused by the dead space between the detector and the fraction collector. The recovery of immunoreactivity estimated by comparison with an unchromatographed fraction of the load was 109%.

In order to assess the recovery of biological activity, TSH (150 μg) was frac-

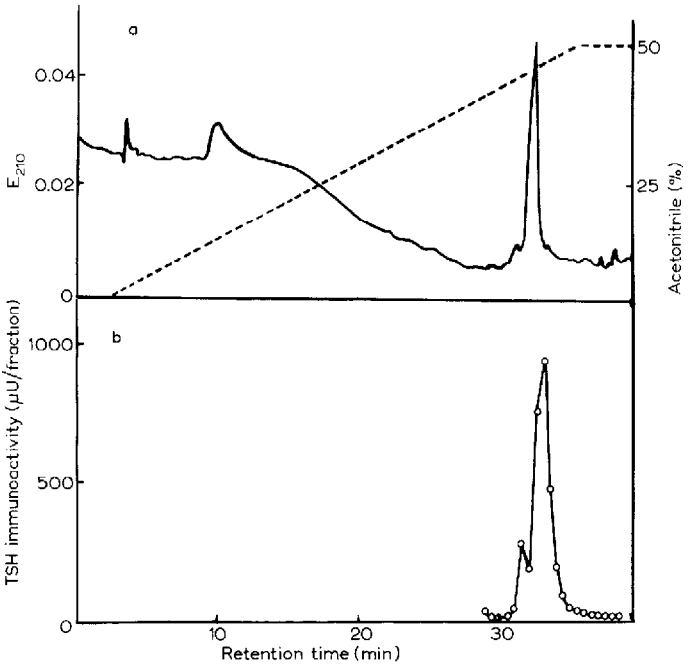

Fig. 4. Recovery of immunoreactive TSH from Aquapore RP-300. Mobile phase A, 0.155 *M* sodium chloride–0.025 *M* orthophosphoric acid adjusted to pH 7.0; mobile phase B, acetonitrile; sample load, 10 μg. The linear gradient of 5–50% (v/v) acetonitrile is indicated by the broken line. Fractions (0.5 ml) were collected into 2 ml of phosphate-buffered saline containing 0.1% bovine serum albumin. Fractions were then radioimmunoassayed as described at a further ten-fold dilution. To assess recovery of TSH from the column a further 10 μg of the sample was treated identically without chromatography. Each point is the mean of triplicate determinations.

tionated at pH 7.0 as described in the Experimental section. The estimated recovery of biological activity was 57.7% with 95% confidence limits of 40.7% to 95.9%.

HPLC fractionation of a sample of serum from a hypothyroid patient is shown in Fig. 5. Although an UV-absorbing peak corresponding in retention time to TSH was not seen, since the sample only contained *ca.* 5 ng of TSH, a major peak of the TSH immunoreactivity was observed, well-separated from most of the serum proteins, and corresponding in retention time to purified pituitary TSH (Fig. 4). Recovery of TSH in this peak was estimated by immunoassays as 76%.

DISCUSSION

The use of large (300 Å) pore-size, short-alkyl-chain packings for reversed-phase HPLC of proteins has recently received much attention. O'Hare *et al.*[12] reported high efficiency of chromatography and recovery of proteins on large-pore-size silica using a mobile phase containing 0.155 *M* sodium chloride, pH 2.1. Chromatography of TSH under these conditions resulted in good resolution of α and β subunits, suggesting dissociation of the subunits at low pH. Two lines of evidence suggest that the single major peak observed at pH 7.0 corresponded to native, un-

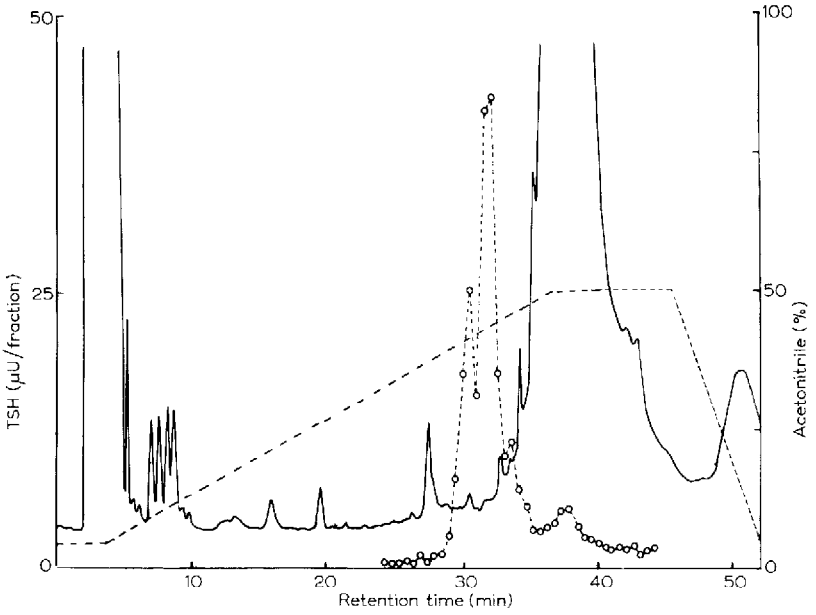

Fig. 5. Chromatography on Aquapore RP-300 of hypothyroid serum. Mobile phase A, 0.155 *M* sodium chloride–0.025 *M* orthophosphoric acid, adjusted to pH 7.0; mobile phase B, acetonitrile. The linear gradient of 5–50% (v/v) acetonitrile is indicated by the broken line. The freeze-dried residue of 1 ml of serum derived from an initially hypothyroid patient was reconstituted in 1 ml of mobile phase A. This was applied to the column, previously equilibrated at 5% B, in 4 × 250 μl aliquots before commencing the gradient. Fractions (0.5 ml) were collected and radioimmunoassayed as described in Fig. 4.

dissociated TSH. Firstly, TSH-β clearly separated from TSH at pH 7.0 (Fig. 3). The presence of significant amounts of free subunit would have been shown by the peaks being split. Secondly, *in vivo* TSH bioactivity was recovered in reasonable yield (57.7%). The subunits of TSH are biologically inactive, although the possibility of subunit recombination[5] cannot be entirely excluded.

Whilst it is known that TSH is dissociated at low pH, high concentrations of ethanol are routinely used in the extraction and purification of pituitary TSH[1,3], and the observation that at neutral pH, TSH is undissociated at high concentrations of organic modifier, is consistent with the known properties of the hormone.

Both the free subunits at pH 2.0 and the native hormone at pH 7.0 showed asymmetry of chromatogram peaks in this study. It is known that up to five charge variants of TSH and its subunits can exist[7], and it is tempting to suggest that this is related to the observed peak asymmetry. In this study however, no further resolution into discrete peaks was achieved with the use of shallower gradients (results not shown).

The recovery of TSH in good yield, well separated from most of the serum proteins, following chromatography of a complex biological material, the hypothyroid serum (Fig. 5), indicates that reversed-phase HPLC will be useful for the isolation of TSH and perhaps other related glycoproteins.

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