Effective Purification of Human Chorionic Gonadotropin and Its Subunits from Pregnant Women's Urinary Peptides Adsorbed on Reverse-Phase Resin

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Human chorionic gonadotropin (hCG) was extracted and purified by reverse-phase resin (Sepralyte C_8 resin) adsorption and Sephadex G-100 column chromatography from urine of pregnant women. Approximately 15000 IU of hCG was recovered from 1000 ml of urine by C_8 resin adsorption. hCG from the gel-filtration step stimulated testosterone production in rat Leydig cells and had a specific activity of approximately 8000 IU/mg, a value which was higher than those of commercial hCGs. Furthermore, hCG purified to near-homogeneity was separated effectively into its subunits by reverse-phase high-performance liquid chromatography using an acetonitrile gradient in the presence of 0.1% trifluoroacetic acid with no special pretreatment for dissociation of subunits. The separated subunits were able to re-associate. The techniques used are simple and may be suitable for not only laboratory but also industrial production of hCG and its subunits.

Keywords human chorionic gonadotropin; hCG; urinary hCG; reverse-phase resin adsorption; hCG subunit; reverse-phase HPLC

Human chorionic gonadotropin (hCG) is a glycoprotein hormone consisting of noncovalently bound α- and β-subunits which are synthesized in syncytiotrophoblastic cells of the placenta. A large quantity of hCG is secreted into the blood and then the urine in early pregnancy. Partially purified hCG from urine of pregnant women is used as a clinical drug for various conditions, *e.g.*, cryptorchidism and hypogonadism secondary to pituitary failure of males and for induction of ovulation and pregnancy in females.

In the extraction of urinary peptides, it is very troublesome to exclude salts in the urine. Several previously reported methods for extraction of urinary peptides including hCG involve concentration by adsorption[1] or precipitation.[2] These procedures have been improved and applied to obtain urinary hCG from normal subjects and patients with trophoblastic diseases.[3,4] Although the precipitation method is widely used for extraction of urinary peptides, one must handle a great volume of urine increased 2- to 3-fold by addition of an organic solvent, *e.g.* acetone or ethanol.

The purpose of the present study was to develop a simple, easy and efficient method for extraction of urinary peptides and for purification of hCG from the extracts. The method which we have developed is based on the use of reverse-phase resin as the adsorbent for urinary peptides. Reverse-phase resin has been used in this manner by Ranganathan *et al.*[5] for the adsorption of transforming factor type β from normal human urine. We report here that hCG which could be used satisfactorily as a clinical drug, was obtained in only two steps, *i.e.*, adsorption of hCG from urine of pregnant women by reverse-phase resin as the first step and gel-filtration on Sephadex G-100 as the second step. Furthermore, we describe a simple procedure for the separation of subunits of purified hCG.

Experimental

Commercial hCG Commercial hCGs were obtained from Mochida Inc. (6000 IU/mg) and Organon (3430 IU/mg).

Urine Urine from pregnant women was kindly provided by obstetricians in Nagasaki city. The urine of women in early pregnancy was collected in bottles containing 0.01% NaN_3 and stored at 4 °C until transport to the laboratory. The urine was centrifuged for 30 min at 7000 × *g* to remove insoluble materials, and the supernatant was used as the material for extraction of urinary peptides.

Analyses of hCG hCG in each sample was analyzed by immunoassay and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Immunoassay was performed by the method of time-resolved fluoroimmunoassay (TR-FIA) using the hCG-DELFIA system of Pharmacia. SDS-PAGE (13% gel) was performed according to the method of Laemmli[6] under reducing and non-reducing conditions for detection of dissociated and associated subunits, respectively, as described by Schlaff[7] with some modification.[8] Gels were stained with silver using a silver stain kit (Wako Chemical Co.).

Extraction of Urinary Peptides Sepralyte resins C_8 (10 g, Analytical International) pre-swollen with a minimum volume of acetonitrile were added to 1000 ml of urine and the mixture was stirred overnight at 4 °C. The resins were collected on a glass filter. The resins were washed extensively with distilled water and adsorbed peptides including hCG subunits were eluted with 50 ml of 30% acetonitrile then lyophilized.

Sephadex G-100 Column Chromatography The lyophilized materials were dissolved in 5 ml of 0.1% ammonium bicarbonate (pH 8.1) and the peptides were fractionated (6 ml fractions) on a Sephadex G-100 column (2.5 × 95 cm). Absorbances of the eluted peptides were measured at 280 and 220 nm. hCG in each fraction was analyzed by TR-FIA and SDS-PAGE. Fractions containing hCG were pooled and lyophilized. The lyophilized hCG preparation was analyzed for biological activity (activity to stimulate testosterone production in rat Leydig cells) and was used as the material for further purification of hCG and its subunits.

Assay of Testosterone Leydig cells from rat testes were suspended in medium 199 containing 10 mM *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (Hepes) and 0.1% bovine serum albumin (pH 7.4). After preincubation at 37 °C for 60 min in a 5% CO_2 incubator, the Leydig cells (8×10^5 cell/0.2 ml) were incubated with samples at 37 °C under 5% CO_2–95% O_2 with shaking at 100 stroke/min. After a 2 h incubation, an aliquot of an incubation mixture was transferred onto ice and then centrifuged at 3000 rpm for 2 min. Testosterone in the supernatant was determined by radioimmunoassay using a Testosterone assay kit (Amersham).

Separation of hCG Subunits Further purification of hCG from Sephadex G-100 fractions was performed by Con A Sepharose 4B, Sephadex G-100 and diethylaminoethyl (DEAE) cellulose chromatographies as described by Manjunath and Sairam.[9] Purified hCG (14000 IU/mg, 200 μg) dissolved in water (200 μl) was applied to a reverse-phase high-performance liquid chromatography (HPLC) column (Wakosil 5C18-200, 4.6 × 150 mm). The α- and β-subunits were separated with a gradient of 24 to 30% acetonitrile in 0.1% trifluoroacetic acid (90 min) at a flow rate of 1 ml/min. Peptide content was monitored by measuring absorbance at 220 nm. Fractions were collected, and hCG subunits were

analyzed by SDS-PAGE. The fractions containing hCG subunits were lyophilized.

Assay of Subunit Association Purified α- and β-subunits, which were dissolved in 0.1 M ammonium bicarbonate (pH 8.1) at a concentration of 0.4 mg/ml, were mixed at a ratio of 1:1, 3:1 and 7:1 by weight and incubated for 48 h at 37 °C. At the indicated time, aliquots of reaction mixture were analyzed by SDS-PAGE under non-reducing conditions.

Protein Assay Amounts of proteins were determined as the sum of amino acids in samples hydrolyzed by 6 N HCl for 24 h at 110 °C.

Results and Discussion

hCG was extracted from urine obtained from pregnant women by adsorption on reverse-phase resin. Urine which had been prepared without any special pretreatment, *e.g.*, acidification, but had been centrifuged to remove insoluble materials, was mixed with reverse-phase resin and stirred overnight at 4 °C. The ability of various resins ($C_{1,2,8 \text{ and } 18}$) tested to adsorb urinary peptides did not vary significantly, judging from the separation profiles of peptides eluted with 30% acetonitrile on SDS-PAGE and reverse-phase HPLC. We chose C_8 resin for adsorption of hCG on the basis of recovery. Immunological activity of hCG but not individual subunits in each sample was determined by the hCG-DELFIA system which is based on a sandwich immunoassay using anti-β-subunit and anti-α-subunit antibodies as the first and second antibodies. The immunological activity was calculated in terms of international units (IU) using World Health Organization (WHO) hCG as the standard. As shown in Table I, approximately 27% of hCG in urine was recovered by C_8 resin adsorption. The single hCG peak fraction detected by the hCG-DELFIA system on Sephadex G-100 chromatography (Fig. 1) was collected and characterized by comparing it with commercial hCGs. Each sample equivalent to 2 IU of hCG was analyzed by SDS-PAGE (Fig. 2). The separation profile of the Sephadex G-100 preparation under non-reducing conditions (lane 3 of A) showed that this preparation contained hCG (the associated form of hCG subunits), predominantly, since dissociated subunits were detected as the major components under reducing conditions (lane 3 of B). Apparently uncombined β- and α-subunits were also detected as minor bands in lane 3 of A. The appearance of uncombined subunits may be due to dissociation of a small part of hCG during storage or electrophoresis. These minor bands of uncombined subunits were detected in commercial hCG as well (lanes 4 and 5 of A). However, contaminating smaller-molecular-weight peptides detected in commercial hCGs were not found in the Sephadex G-100 preparation. The specific activity of the hCG from Sephadex G-100 chromatography was approximately 7500 IU/mg, indicating that the immunological potency of the present hCG preparation was higher than, rather than equivalent to, those of commercial hCGs (Table I). To determine if hCG prepared by the present method maintains its biological activity, we investigated whether the present hCG preparation has the ability to stimulate testosterone production in rat Leydig cells. hCG obtained by the present method stimulated testosterone production, dose-dependently, in the same manner as commercial hCGs (data not shown), indicating that the present hCG preparation can be used as a clinical drug.

To isolate and purify each subunit of hCG, the preparation from Sephadex G-100 was purified to near-

TABLE I. Immunological Activity and Recovery of hCG from 1000 ml of Urine

	Total protein (mg)	Total activity (IU)	Recovery (%)	Specific activity (IU/mg)
Urine	1800	59000	100	33
Reverse-phase resin (C_8)	31.4	15700	27	500
Sephadex G-100	1.9	14400	24	7580

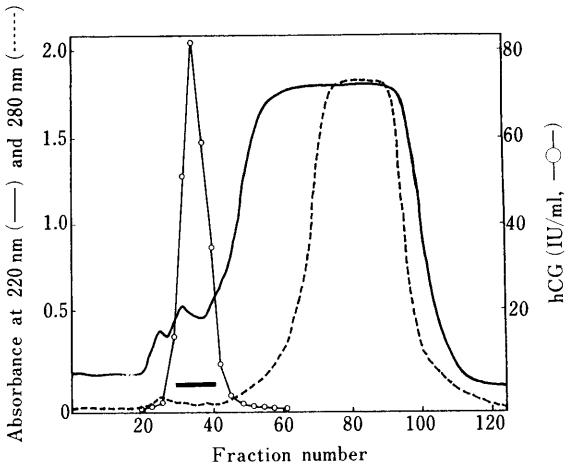

Fig. 1. Separation Profile of Extracted Peptides by C_8 Resin Adsorption on Sephadex G-100

Every third fraction was analyzed for hCG by the hCG-DELFIA system. Fractions indicated by a bar was pooled and lyophilized.

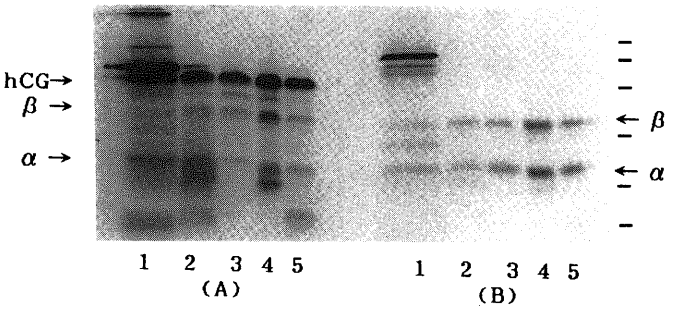

Fig. 2. Separation of Peptides in Each Preparation on SDS-PAGE

Each preparation equivalent to 2 IU of hCG was analyzed under non-reducing (A) and reducing (B) conditions. Lane 1, urine from pregnant women; lane 2, urinary peptides extracted by C_8 resin; lane 3, Sephadex G-100 preparation; lanes 4 and 5, commercial hCG from Mochida and Organon, respectively. Bars indicate positions of molecular weight standards (94000, 67000, 43000, 30000, 20000 and 14400).

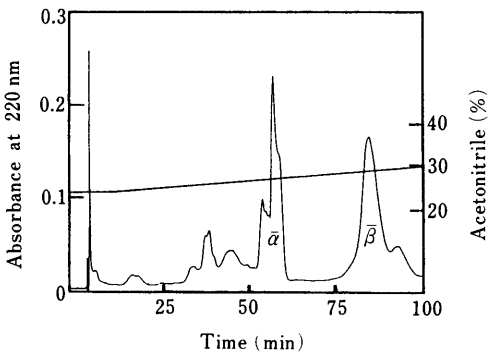

Fig. 3. Separation Profile of hCG Subunits on Wakosil 5C18-200

Experimental details are described in Experimental.

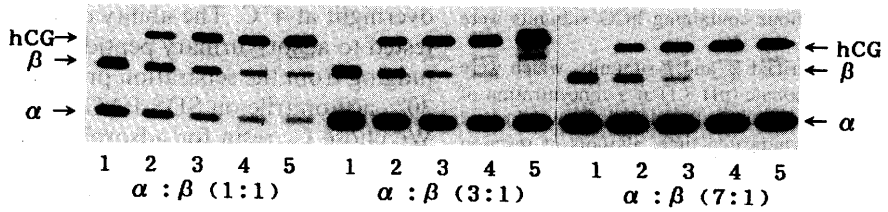

Fig. 4. Reassociation of hCG Subunits

Subunit association was assayed using SDS-PAGE under non-reducing conditions after incubating the mixed subunits for 0, 3, 12, 24 and 48 h (lanes 1 to 5).

homogeneously. In previous studies,[10,11] hCG was dissociated into its subunits by incubation in protein-denaturing reagents, *e.g.* 6 M guanidine hydrochloride or 8 M urea, and subunits were separated by high-performance gel permeation chromatography or DEAE-Sephadex chromatography in the presence of reagents. Although hCG subunits were effectively separated by these methods, it is sometimes troublesome to exclude salts from the separated subunit preparations. Subunits of hCG were also dissociated by incubation in acid, *e.g.* 1 M propionic acid[12] and approximately 0.05% trifluoroacetic acid,[13] and then the dissociated subunits were separated by reverse-phase HPLC. Recently, we found that the rate of dissociation of hCG subunits in 0.1% trifluoroacetic acid is very fast and the extent of dissociation approaches 100% almost immediately. In the present study, hCG dissolved in water was subjected to reverse-phase HPLC without preincubation with acid and the subunits were separated by an acetonitrile gradient containing 0.1% trifluoroacetic acid. As shown in Fig. 3, hCG subunits were separated effectively with retention times of 56 and 80 min for α- and β-subunits, respectively. The shoulder peak of retention time at 58 min was the asialo form of α-subunit, judging from its sensitivity to sialidase (data not shown). Thus, hCG subunits adsorbed on the column seem to be dissociated immediately by washing with the initial solvent containing trifluoroacetic acid.

To determine the integrity of separated subunits, the α- and β-subunits were associated by incubating them at 37 °C for the indicated time in 0.1 M ammonium bicarbonate, and reassociated hCG in the solution was analyzed of SDS-PAGE under non-reducing conditions. This analyzing system of SDS-PAGE under non-reducing conditions does not dissociate the reassociated subunits, or intact hCG. As shown in Fig. 4, reassociation of subunits occurred time-dependently at various mixing ratios. This result indicates that each subunit obtained by the present procedure was in the native state.

In conclusion, the present procedure for the purification of hCG from urine of pregnant women and for separation of its subunits is a simple, effective and non-denaturing method.

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