EFFECTIVE SEPARATION OF HUMAN CHORIONIC GONADOTROPIN SUBUNITS BY HIGH PERFORMANCE GEL PERMEATION CHROMATOGRAPHY

Yoshikazu YUKI, Ryuichiro NISHIMURA and Matsuo MOCHIZUKI
Department of Obstetrics and Gynecology, Kobe University School of Medicine, Kobe

Synopsis Human chorionic gonadotropin (hCG) was effectively separated into $\alpha$ and $\beta$ subunits by treating with 8 M guanidine hydrochloride, followed by high performance gel permeation chromatography (GPC) on a 2.5 × 60 cm TSK G 3000 SW column in 6 M guanidine hydrochloride. Their molecular weights estimated by GPC were about 29,000 daltons for the $\beta$ subunit and 19,000 daltons for the $\alpha$ subunit.

Both subunits were examined for SDS-polyacrylamide gel electrophoresis, amino acid analyses and radioimmunoassay, respectively. Neither subunit showed significant binding to the hCG-receptor fraction of rat Leydig cell in vitro. However, the separated subunits easily recombined and recovered about 90% of receptor binding activity of intact hCG.

The present results indicate that the GPC technique is an effective, simple and non-denaturing method for the separation of hCG into $\alpha$ and $\beta$ subunits.

Key words: Human chorionic gonadotropin • Subunit • High performance gel permeation chromatography

Introduction

The glycoprotein hormones—luteinizing hormone (LH), follicle-stimulating hormone (FSH), thyroid-stimulating hormone (TSH) and human chorionic gonadotropin (hCG)—share a common quaternary structure, with each hormone possessing two non-identical $\alpha$ and $\beta$ subunits, with hydrophobic binding between subunits9. The non-covalently bound subunits of these hormones can be dissociated under appropriate chemical conditions, and separated, purified into subunits1018.

Separation of the subunits of hCG has been reported by Swaminathan et al.12 and Morgan et al.9. The procedures used consisted of dissociating hCG into subunits by 8M- or 10M- urea treatment and separating them by ion-exchange chromatography followed by gel filtration91012. It is well known that separated subunits of hCG were devoid of significant biological activity, which could only be obtained by recombination of the subunits under appropriate conditions91012. However, these standard column chromatographic procedures were time consuming and an effective separation of hCG into the subunits could not be obtained.

The present report described a simple and effective procedure for the separation of subunits of hCG by high performance gel permeation chromatography (GPC). Furthermore, the separated subunits were characterized by SDS-polyacrylamide gel electrophoresis, amino acid analyses, radioimmunoassay, receptor binding assay and combination studies.

Materials and Methods

Human chorionic gonadotropin (hCG)

hCG was extracted and purified from normal pregnant urine by the standard kaolin-acetone method in combination with alcohol precipitation, followed by chromatographies on DEAE-cellulose and Sephadex G-100 as described previously11. The immunoactivity and bioactivity (by ovarian weight method10) of purified hCG was 11,000 IU/mg and 10,000 IU/mg, respectively.

Separation of hCG into subunits

A high performance gel permeation chromatography (GPC) was performed with a Gilson high performance liquid chromatography system which consisted of a Gilson Model 302 pump, a Gilson Model 802 monometric module, a M & S Model 311 UV-detector and a Pantos U-228 recorder. Separations were performed on a 2.5 × 60cm TSK G3000 SW column (Toyo Soda).

Four mg of hCG dissolved in 0.3ml of 8M guanidine hydrochloride was incubated at 37°C for 2 hours and 100 µl of distilled water was added. In
one course of GPC, the aliquots of 100μl (1mg of hCG) was injected into the GPC system. The elu-
tion pattern of subunits was monitored by
absorbance at 280nm and recorded automatically.
The effluent was collected into 0.5ml fractions with
a flow rate of 0.5ml per minute. Aliquots were
removed for identification of immunoactivity by
radioimmunoassay as described below. The frac-
tions comprising each subunit were pooled, exhaus-
tively dialyzed against distilled water and lyophil-
ized. In the determination of molecular weight of
hCG subunits, the standard molecular markers
(BDH, monomer of 14,300 daltons) were injected
under the same conditions.

SDS-polyacrylamide gel electrophoresis
Sodium dodecyl sulfate polyacrylamide gel
electrophoresis was performed in 7.5% polyac-
rylamide gel as described by Weber et al.14. The
samples were dissolved in 0.02M phosphate buffer,
pH7.2 containing 0.1% SDS, 2% 2-mercaptoeth-
anol, 0.05% bromophenol blue and heated at 100°C
for 2 minutes before the electrophoretic run. In
the determination of molecular weight by the elect-
rophoresis the standard molecular markers (BDH,
monomer of 14,300 daltons) was applied under the
same conditions.

Amino acid analyses
Approximately 1mg of each sample was hydrol-
yzed with 6N HCl in sealed tube at 110°C for 24
hours. After the removal of acid, the hydrolysate
was analyzed by an amino acid analyzer, JLC-6AH
(JEOL). Tryptophan was not determined.

Immunoreactivity
The radioimmunoassays of hCG, hCGα and
hCGβ were performed according to our previously
published method16. All assays were carried out by
the double-antibody technique. Highly purified
preparations of hCG (CR119), hCGα (CR123α) and
hCGβ (CR123β) were kindly provided by Dr. R.
Canfield as reference preparations.

Receptor binding activity
The radioreceptor assay in vitro was performed
according to the method as described by Catt et al.9.

Combination of α and β subunits
The ability of recombination of α and β subunits
separated by GPC was determined by radioimmu-
noassay and radioreceptor assay. The methods of
recombination study and calculation of the per-
centage of theoretical combination were performed

![Graph](image-url)

Fig. 1. Separation of subunits of hCG by GPC on a 2.5×60cm TSK G3000SW
column in 6M guanidine hydrochloride. The hCG treated with 8M guanidine
hydrochloride was injected into the GPC system. Five hundred μl fraction
were collected with a flow rate of 0.5ml/minute and immunoactivity of hCGα
and hCGβ was measured. ——, fraction pooled.
as described by Weintraub et al.\textsuperscript{15}.

Fifteen $\mu$g of $\alpha$ subunit was incubated with 23$\mu$g of $\beta$ subunit according to molecular weight. The incubation was carried out at 27°C for 48 hours in 100$\mu$l of 0.01M sodium phosphate buffer, pH7.4 in plastic tubes (1.5×20mm) sealed with parafilm. After incubation, 10$\mu$l aliquots were diluted in 4ml of cold 0.05M sodium phosphate buffer, pH7.4 containing 0.05% bovine serum albumin and assayed.

\section*{Results}

\textit{Separation of hCG into subunits}

After the treatment with 8M guanidine hydrochloride, hCG subunits were eluted by GPC as two sharp symmetrical peaks as shown in Fig. 1. The results of specific radioimmunoassay for hCG$\alpha$ and hCG$\beta$ indicated that the first and second peaks corresponded to hCG$\beta$ and hCG$\alpha$, respectively. The retention times of $\beta$ and $\alpha$ subunits were 62.6 minutes and 68.7 minutes, respectively. The total yield measured by absorbance at 280nm was 65%; the recovery of $\beta$ subunit was approximately twice as much as that of $\alpha$ subunit on a weight basis. The standard molecular markers (monomer, 14,300 daltons) were eluted with the retention time of monomer for 71.7 minutes, dimer for 63.6 minutes, trimer for 57.2 minutes, and tetramer for 53.1 minutes under the same condition. Molecular weights of $\alpha$ and $\beta$ subunits were estimated about 19,000 daltons and 29,000 daltons, respectively (Fig. 2).

\textit{SDS-polyacrylamide gel electrophoresis}

Fig. 3 shows the results of SDS-polyacrylamide gel electrophoresis of hCG, hCG$\alpha$ and hCG$\beta$. SDS-polyacrylamide gel electrophoresis of each subunit preparation indicated no cross-contamination of the subunits. Molecular weights of $\alpha$ and $\beta$ subunits

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Molecular weight of the hCG subunits estimated by GPC on a 2.5×60cm TSK G3000SW column in 6M guanidine hydrochloride.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{SDS-polyacrylamide gel electrophoresis of hCG and hCG subunits. hCG (gel 1), the hCG$\alpha$ (gel 2), the hCG$\beta$ (gel 3).}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Molecular weight of the hCG subunits estimated by SDS-polyacrylamide gel electrophoresis. Mobilities were expressed as percentage of that of bromophenol blue used as reference.}
\end{figure}
by SDS-polyacrylamide gel electrophoresis were about 19,000 daltons and 29,000 daltons, respectively (Fig. 4).

Amino acid analyses
The results of the amino acid analyses of \( \alpha \) and \( \beta \) subunits are listed in Table 1. There were considerable differences in the composition between the subunits.

Immunooactivity
Table 2 indicates the relative immunological potencies of hCG, hCG\( \alpha \) and hCG\( \beta \) in each homologous radioimmunoassay system. Highly purified hCG (CR119), hCG\( \alpha \) (CR123\( \alpha \)) and hCG\( \beta \) (CR123\( \beta \)) were used as reference preparations. The hCG\( \alpha \) and hCG\( \beta \) subunits by GPC had almost the same immunooactivities as compared with those of each reference preparations.

Receptor binding activity
The relative receptor binding potencies of hCG, hCG\( \alpha \) and hCG\( \beta \) subunits are indicated in Table 3. The hCG\( \alpha \) (GPC) and hCG\( \beta \) (GPC) as well as subunits of reference did not reveal significant

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### Table 1. Amino acid composition of \( \alpha \) and \( \beta \) subunits separated by GPC.

<table>
<thead>
<tr>
<th>Amino acid*</th>
<th>hCG ( \alpha ) subunit</th>
<th>hCG ( \beta ) subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>6.0</td>
<td>2.9</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.1</td>
<td>0.8</td>
</tr>
<tr>
<td>Arginine</td>
<td>3.7</td>
<td>8.0</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>6.9</td>
<td>9.5</td>
</tr>
<tr>
<td>Threonine</td>
<td>8.5</td>
<td>6.2</td>
</tr>
<tr>
<td>Serine</td>
<td>9.0</td>
<td>9.3</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>10.7</td>
<td>6.8</td>
</tr>
<tr>
<td>Proline</td>
<td>7.8</td>
<td>15.0</td>
</tr>
<tr>
<td>Glycine</td>
<td>5.9</td>
<td>6.2</td>
</tr>
<tr>
<td>Alanine</td>
<td>5.5</td>
<td>5.7</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>8.4</td>
<td>8.0</td>
</tr>
<tr>
<td>Valine</td>
<td>7.5</td>
<td>7.0</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.8</td>
<td>0.5</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1.8</td>
<td>2.6</td>
</tr>
<tr>
<td>Leucine</td>
<td>4.6</td>
<td>8.0</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.5</td>
<td>1.9</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4.3</td>
<td>1.6</td>
</tr>
</tbody>
</table>

*expressed as moles/mole subunit
Tryptophan was not examined.
Table 3. Relative receptor binding potencies of hCG and hCG subunits.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Relative receptor binding potency a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCG (CR119)</td>
<td>100%</td>
</tr>
<tr>
<td>hCGα (CR123α)</td>
<td>0.2</td>
</tr>
<tr>
<td>hCGα (GPC)</td>
<td>0.3</td>
</tr>
<tr>
<td>hCGβ (CR123β)</td>
<td>0.6</td>
</tr>
<tr>
<td>hCGβ (GPC)</td>
<td>0.8</td>
</tr>
<tr>
<td>recombined hCG</td>
<td>90.0</td>
</tr>
</tbody>
</table>

a) Relative receptor binding potencies of hCG and hCG subunits were compared on the basis of 50% intercept determined from radioreceptor assay inhibition binding curves.

The reassociated hCG after recombination recovered about 95% of immunological activity and about 90% of receptor binding activity of the intact hCG (Table 2, Table 3).

Discussion

The technique of gel filtration on agarose gel in 6 M guanidine hydrochloride has been evaluated by Fish et al. as means of estimating protein or protein subunit molecular weights. For the study of hCG subunits this technique has a conceivable pitfall, a possibility of irreversible denature of isolated subunits due to a long process (about one week) of gel filtration in 6 M guanidine hydrochloride at room temperature. In the present study we have succeeded in rapid separation of hCG into subunits by GPC.

hCG could be effectively separated into subunits by treating with 8 M guanidine hydrochloride, followed by GPC on a 2.5 × 60 cm TSK G3000SW column in 6 M guanidine hydrochloride. By this procedure, hCG subunits are automatically separated within 80 minutes and their molecular weights are estimated simultaneously.

The molecular weights of hCG subunits estimated by GPC were equal to those estimated by SDS-polyacrylamide gel electrophoresis (Fig. 2, 4) and are consistent with previous report. SDS-polyacrylamide gel electrophoresis of each subunit by GPC also indicated no cross-contamination of the subunits. The result of amino acid analyses showed that our preparations are practically identical to those reported previously. Furthermore, the cross-reactivities of α and β subunits by GPC showed similar immunological potencies in hCG, hCGα and hCGβ radioimmunoassay when compared with reference preparations (Table 2). These results demonstrate that the chemical composition of our preparations are in good agreement with the preparations of Swaminathan et al. and Morgan et al.

On the other hand, Vaitukaitis et al. reported that isolated and purified subunits are essentially devoid of significant intrinsic biological activity. The minimal biological activity present in purified subunit preparations can be attributed to cross-contaminations with either intact hormone or complementary subunit. The result of receptor binding activity indicated that our preparations did not reveal significant binding activities in vitro (Table 3).

Aloj et al. demonstrated that dissociation of the glycoprotein hormones including hCG initially results in reversible denaturation, then dissociation. However, complementary α and β subunits isolated may be recombined with restitution of a significant portion of the nature hormone's initial biological activity. Although no attempt has been made to subject the hCG prepared by combining the isolated subunits to bioassay in vivo, the recombined hCG after incubation of α and β subunits at 27°C for 48 hours recovered about 90% of binding activity of intact hCG (Table 3).

The present GPC technique is an effective, simple and non-denaturing method for the separation of hCG into the subunits. The GPC technique may also be possibly used to effectively separate pituitary glycoprotein hormones, LH, FSH and TSH into subunits.

Acknowledgments

Human chorionic gonadotropin (CR119), α subunit (CR123α) and β subunit (CR123β) were generously supplied by the Center for Population Research of the National Institute of Child Health and Human Development of the National Institutes of Health.
概要　Human chorionic gonadotropin（hCG）を8M塩酸アシモニウム処理後、6M塩酸アシモニウム中で2.5×60cm TSK G3000SW column を用いる高performance gel permeation chromatography（GPC）を実施した結果、hCGを効果的にαおよびβsubunitに分離できることがわかった。αおよびβsubunitのGPCにおける分子量はそれぞれ約19,000daltonsおよび29,000daltonsであった。両subunitをSDS-polycacrylamide gel電気泳動、アミノ酸分析およびradioimmunoassayによりそれぞれ検討したところ、subunitへの分離がほぼ完全に行われていることが確認できた。両subunitは単独では両ともrat Leydig cellのhCG-receptorには有意に結合を示さなかったが、両subunitを再結合させると、intact hCGの90％までreceptor結合能を回復した。以上の結果からGPC法は、簡便かつ効果的で、しかも変性を伴わないhCGのsubunit分離法であることが明らかになった。

References
don, 1974.
9. Morgan, F.J. and Canfield, R.E.: Nature of the subunits of human chorionic gonadotropin. Endocri-

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