THE PURIFICATION OF HUMAN CHORIONIC GONADOTROPIN ON DEAE-SEPHADEX

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A number of methods have been reported for the purification of human chorionic gonadotropin (HCG)[6-8,11,16,17]. These methods are either laborious, time consuming or limited to preparation of relatively small quantities. A simple procedure for the purification of moderate quantities of HCG on DEAE-Sephadex, the immunoelectrophoretic properties of the purified material obtained and a quantitative immunoassay of HCG, form the subject of this report.

MATERIALS AND METHODS

Human chorionic gonadotropin

Commercial HCG, with a specific activity of 3000 I.U./mg, was purchased from Organon Inc., West Orange, N.J.

HCG antiserum

Albino rabbits of both sexes, weighing 3.5 kg, were used. Animals were bled from the marginal ear vein. Both the commercial HCG preparation and the highly purified immunoelectrophoretically homogeneous HCG was used for the immunization of rabbits. The immunization regimen was as follows:

1. An initial dose of HCG (500 I.U.) dissolved in saline (0.25 ml) and emulsified with an equal volume of Freund's Complete Adjuvant, was injected into the toe pads of the hind feet.

2. Booster doses of HCG (2000 I.U.), dissolved in saline (0.25 ml) and emulsified with an equal volume of Freund's Complete Adjuvant, were injected intramuscularly (hind legs) three weeks after the initial doses and every two weeks subsequently.

Fractionation

DEAE-Sephadex A-50 medium from Pharmacia, Uppsala, Sweden, was treated according to the manufacturer's instructions and then suspended in 0.005 M phosphate buffer, pH 6.0, overnight. The column (1.5 cm diameter) was prepared according to the procedure described by FLODIN[5]. The HCG sample (aqueous solution, 80 mg/ml) was then placed on the top of the column (6 cm column section per 10 mg of protein). Elutions were made either with a stepwise increasing concentration of NaCl (0.00, 0.04, 0.08, 0.12 and 0.16 M) in 0.005 M phosphate buffer, pH 6.0, or with continuous gradient elution according to LAKSHMANAN AND LIEBERMAN[9], with V_0 = 2000 ml, 0.005 M phosphate buffer, pH 6.0; C_0 = 1.5 M NaCl in 0.005 M phosphate buffer, pH 6.0; R_1 = 1.0 ml/min; R_2 = 2.4 ml/min.

Protein measurement

Protein concentration in the fractions from the column was determined by U.V. absorbancy at 280 mμ using a Beckman Model DU spectrophotometer.

Double diffusion

Double diffusion was carried out according to the OUCHTERLONY[14] technique. Agar solution (1 %) was prepared in barbital buffer (sodium barbital (5.88 g), sodium acetate (3.84 g), and sodium merthiolate (0.044 g) were dissolved in water (440 ml) and adjusted to pH 8.4 with 3*N* HCl. Stock solution was diluted 1:4 with water before use. Agar solution (12 ml) was pipetted into a petri dish (diameter 8.5) and allowed to solidify. Wells were then cut into the agar, the distance between the center well and the wells surrounding it being 3 mm. Antiserum was introduced into the center well and 10 λ of the sample was introduced into the surrounding wells. The plate was then incubated at 25° for 16 h.

Immunoelectrophoresis

Immunoelectrophoresis was carried out employing LKB 6800A immunoelectrophoresis apparatus following the standard immuno-processing technique recommended by the manufacturer. In our hands, the following set of conditions were optimal: 15 mA/tray; 5–6 V/cm; duration 1 h. The plates were incubated in a humid chamber at 25° for a sufficient period of time (2–3 days) for the precipitation bands to occur. The plate was then washed in 1% sodium chloride solution for 16 h, dried and finally stained with amido black.

Immunodiffusion in agar

Antiserum (1 ml) was added to agar solution (25 ml) at 50°, and mixed thoroughly. The mixture (10 ml) was placed in a trough (26.8 cm × 2.6 cm) and allowed to solidify. Wells (2.5 mm in diameter) were then cut into the agar at 2.5 cm apart. 6 λ of HCG solution was introduced in each well and the plate incubated in a humid chamber at 25° for 2 days for the precipitation zone to form. The diameter of the precipitation zone was measured under a B & L stereomicroscope with a sealed lens. The diameters of the precipitation zones of HCG solutions of unknown concentrations were compared with those of HCG solutions of known concentration.

RESULTS

The distribution of serologic activity in a series of fractions obtained by chromatography of commercial HCG, employing a stepwise increasing NaCl gradient, is shown in Fig. 1. Serologic activity was determined by immunodiffusion assay using homogeneous rabbit-HCG-antiserum. In peaks 1 and 2, there was a complete absence of serologic activity as determined by immunoelectrophoresis and immunodiffusion. In peaks 3 and 4, there were traces of serologic activity. Immunoelectrophoresis showed the presence of three antigens, in small amounts, in the α- and β-globulin regions. Most of the serologic activity was found in peak 5. Immunoelectrophoresis showed the presence of two antigens, one in the α_2-globulin region and one in the α_1–α_2-globulin region (Fig. 2b). These fractions (No. 115–145) were combined, lyophylized, and desalted. The material thus obtained was rechromatographed employing a linear

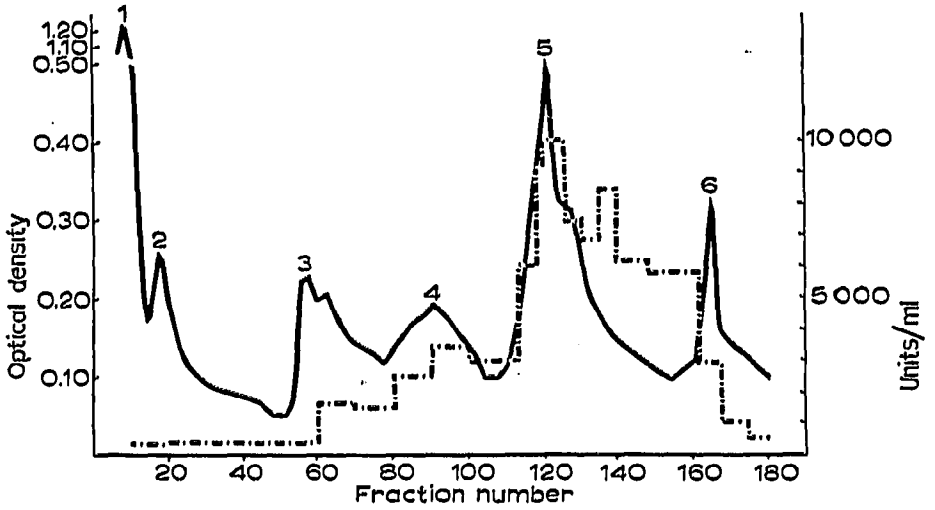

Fig. 1. Chromatography of HCG on DEAE-Sephadex, stepwise increasing concentration of NaCl (0.00, 0.04, 0.08, 0.12 and 0.16 *M*) in 0.005 *M* phosphate buffer, pH 6.0. (————) Protein measurement; (–·–·–) HCG activity (immunological).

Fig. 2. Electrophorogram of Organon HCG (a), partially purified HCG (see text) (b), and final purified HCG (see text) (c), against antiserum to Organon HCG.

gradient of NaCl. The distribution of serologic activity in a series of fractions obtained from the second chromatography is shown in Fig. 3. Fractions 98 to 123 were combined, lyophylized, and desalted. The final product was serologically homogeneous as shown by double diffusion and by immunoelectrophoresis (Fig. 2c). The specific immunologic activity of this material was 11,000 I.U./mg. The overall recovery of immunological activity was 19%. The biological activity was not assessed.

The heterogeneity of the antiserum produced by the rabbit immunized with commercial HCG is shown in Fig. 4a. A solution of commercial HCG (200 I.U./ml)

was subjected to electrophoresis, developed with homologous antiserum (*i.e.* antiserum produced by a rabbit immunized with commercial HCG) and stained with amido black. Six precipitation bands were observed: one in the α_1 region, two in the α_2 region, two in the β region and one in the γ region. The same experiment, carried out with the specific antiserum obtained from rabbit immunized with immunoelectrophoretically homogeneous HCG, showed a single precipitation line located in the α_2 region (Fig. 4b). The homogeneity of the antiserum produced in rabbits immunized with immunoelectrophoretically homogeneous HCG was further confirmed by a series of double-diffusion tests according to the OUCHTERLONY[14] technique carried out with the heterogeneous antiserum or homogeneous antiserum in the center wells and human plasma, normal female urine concentrate, pregnancy urine concentrate, commercial HCG and highly purified HCG in the wells surrounding the antisera. With the antisera produced by rabbit immunized with commercial HCG, there were five precipitation lines between commercial HCG and the antiserum, one line between urine concentrate from normal women and antiserum, two lines between pregnancy urine concentrate

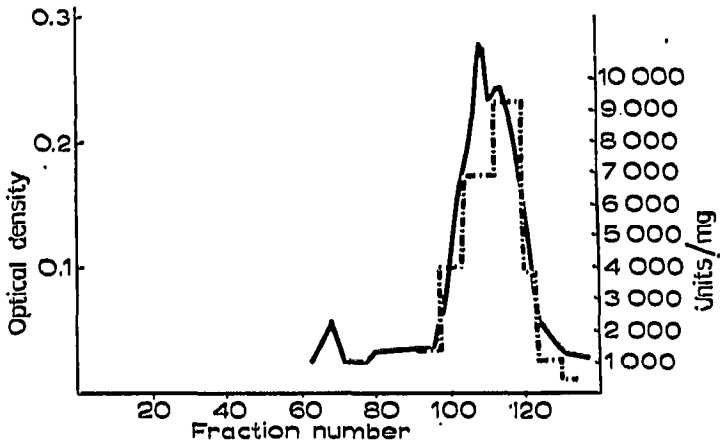

Fig. 3. Chromatography of HCG on DEAE-Sephadex, continuous gradient elution. (———) Protein measurement; (–·–·–) HCG activity (immunological).

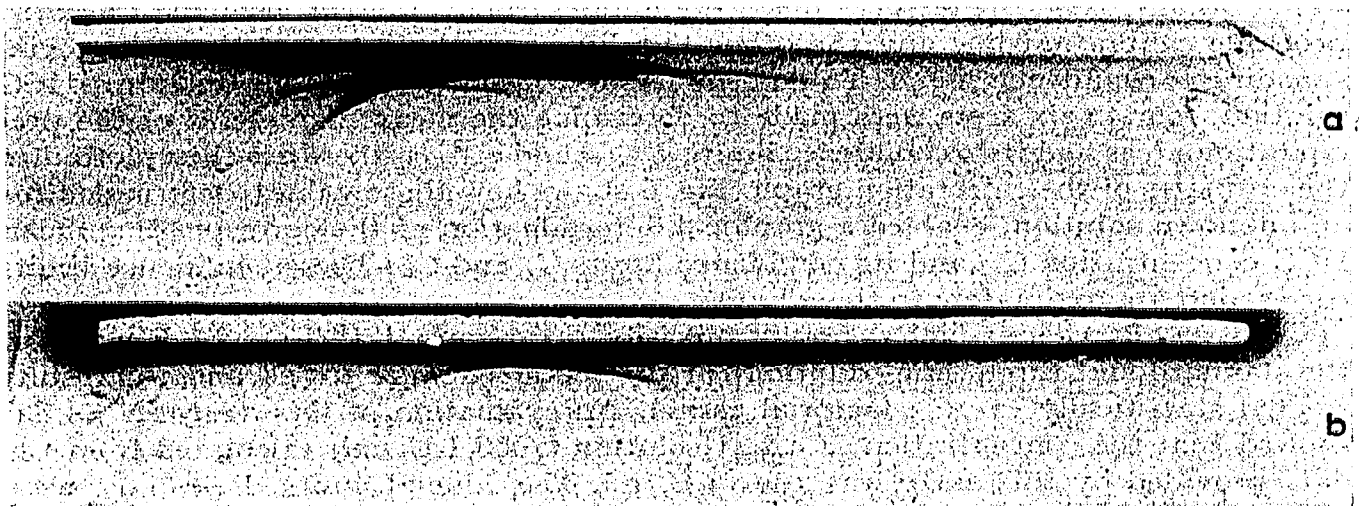

Fig. 4. Electrophorogram of Organon HCG against antiserum to Organon HCG (a) and antiserum to highly purified HCG (b).

and antiserum, and one line between highly purified HCG and the antiserum. With the antiserum produced by rabbit immunized with immunoelectrophoretically homogeneous HCG, there was no precipitation line between the wells containing either human serum or the urine concentrate from normal women and the antiserum. Only a single line was observed between commercial HCG, purified HCG and pregnancy urine concentrate and the antiserum.

The specific rabbit-HCG-antiserum (*i.e.* antiserum produced by a rabbit immunized with electrophoretically homogeneous HCG) was utilized in the quantitative assay of various HCG preparations. An actual diffusion plate is shown in Fig. 5. In this experiment, twelve dilutions were made from a known solution of biologically standardized HCG and 6 λ of each solution was placed in a well in the agar plate impregnated with HCG antiserum. The plate was stained with amido black.

A standard curve was constructed by carrying out fourteen similar experiments each involving six dilutions of a standard sample. The mean diameter of precipitation areas and the mean square deviation of the individual values from the mean were calculated for each HCG concentration. Plotting the resulting mean values $\bar{d}$, $\bar{d} + \sigma$ and $\bar{d} - \sigma$ as a function of logarithm of the HCG concentrations, three calibration lines

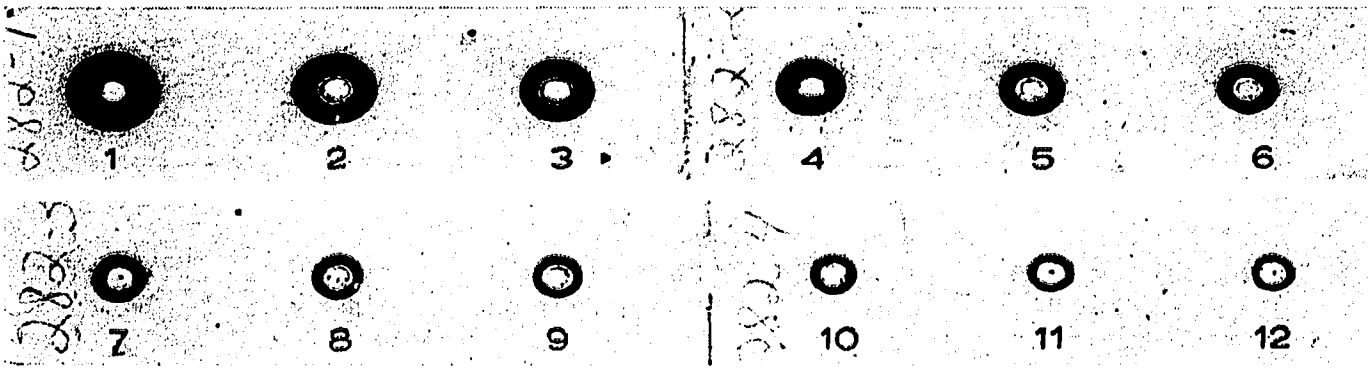

Fig. 5. Quantitative diffusion plate. (1) 19.05 I.U.; (2) 15.12 I.U.; (3) 12.00 I.U.; (4) 9.52 I.U.; (5) 7.56 I.U.; (6) 6.00 I.U.; (7) 4.76 I.U.; (8) 3.78 I.U.; (9) 3.00 I.U.; (10) 2.38 I.U.; (11) 1.89 I.U.; (12) 1.50 I.U.

were obtained (Fig. 6); of these lines, the middle (solid) line is considered the most probable calibration line and the other two lines may be interpreted as the corresponding σ confidence limits. To determine the concentration of an unknown HCG solution, four to six tests were made to determine the mean diameter $\bar{d}$ of the precipitation areas and its σ confidence limits $\bar{d} + \sigma$ and $\bar{d} - \sigma$; these are then plotted as the ordinates of the calibration graph. Fig. 6 shows how these values, determined for an unknown solution, enabled a graphical determination of the corresponding mean HCG concentration C_m and its confidence limits C_1 and C_u. These confidence limits correspond approximately to the σ limits.

In assays where a high degree of precision was required, three serial dilutions of the standard and three serial dilutions of the unknown were tested and the results treated statistically (3 $\times$ 3 factorial assay). An evaluation of this assay ($N = 84$) showed that the concentration of a test solution (1008 I.U./ml) calculated from the data provided by this assay was 1001 I.U./ml. The fiducial limits, $P = 0.95$, were 857.8 to 1184.4 I.U./ml. The calculated slope, b, was 89.8. The value for λ, the index of precision, was 0.1308.

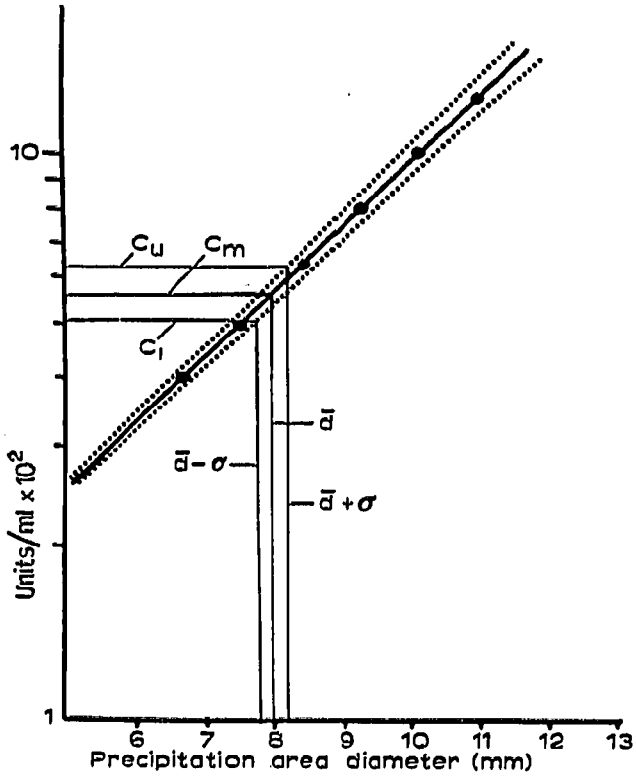

Fig. 6. Standard curve.

DISCUSSION

Although homogeneous samples of HCG have been isolated[6,7], repeated attempts have been made[12] to develop more convenient and less time consuming methods for the purification of HCG on a preparative scale. Crude HCG in gram quantities can easily be purified by the method here described. The simplicity of the method and economy in time and labor present further advantages. The purified material obtained by this method was shown to be immunoelectrophoretically homogeneous. The specificity of the HCG thus obtained was further demonstrated by the production of specific antiserum to HCG by rabbits immunized with this material.

Until recently only biological tests were used for the assessment of HCG in body fluids. These bioassay techniques for gonadotropin activity were developed on the basis of acceleration of gonadal growth and development. As such, the tests lack specificity, and they are inadequate to test the efficacy of separation procedures for isolating different gonadotropic hormones, or to assess the HCG activity in a sample containing other gonadotropic hormones. Furthermore, existing bioassays yielding quantitative information are laborious and time-consuming. Recently serological methods for the determination of HCG were introduced by BRODY AND CARLSTRÖM[1,2] and LUNENFELD *et al.*[10]. These methods were based on the finding that HCG is able to elicit antibody production in rabbits[1,15], and that the hormone can be detected and assayed by the technique of complement fixation[1-3] or by the inhibition of the agglutination reaction of HCG-coated blood cells[19] or HCG-coated latex particles[8]. Precision and specificity can be achieved with these methods, but each has its limitations. Haemagglutination, for example, could not be performed directly

on untreated serum or amniotic fluids[13,18], and the endpoint was often hard to read.

To measure a particular antigen in a mixture of antigens by any serologic technique, it is necessary to employ a specific antiserum. The antigen employed in our work to produce antiserum to HCG was serologically homogeneous. The specificity of the antiserum obtained was established by immunoelectrophoresis and double-diffusion (Fig. 4a and 4b).

The immunodiffusion method reported here is a modification of Hyland's immunodiffusion plates. It is highly specific, quantitative, sensitive, and extremely simple in operation. An evaluation of this assay ($N = 84$) showed that the concentration of a test solution (1008 I.U./ml) calculated from the data provided by this assay was 1001 I.U./ml (95 % confidence limits 857.8–1184.4). The calculated slope, b, was 89.8. The value for λ, the index of precision, was 0.1308. The sensitivity of the method depends upon the titer of the antiserum employed. With an antiserum having a titer of 1/600, 1 I.U. of HCG can be measured. Greater sensitivity can be achieved by employing antiserum of higher titer. For routine estimation, a standard curve may be set up and the concentrations of an unknown HCG solution may be read off directly from the standard curve. The method can be applied to the determination of HCG in urine and serum without any prior treatment of the sample.

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SUMMARY

A simple procedure for the purification of commercial human chorionic gonadotropin (HCG) by chromatography on DEAE-Sephadex is described. The method is reproducible and suitable for application on a preparative scale. The HCG thus purified had a specific immunological activity of 11,000 I.U./mg and was homogeneous by immunoelectrophoresis. The overall recovery of immunological activity was 19 %. A simple, specific quantitative assay for HCG employing the technique of immunodiffusion is described. The precision, accuracy, and advantages of the method are discussed.

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