CONCENTRATION AND PURIFICATION OF HUMAN CHORIONIC SOMATO-MAMMOTROPIN (HCS) BY AFFINITY CHROMATOGRAPHY: APPLICATION TO RADIOIMMUNOASSAY

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Summary. Columns of Sepharose-coupled antibody to HCS have been used to purify labeled and unlabeled hormone by affinity chromatography prior to radioimmunoassay. The method permits concentration and 85-95% recovery of HCS from large volumes of biologic fluid or tissue extract, resulting in a five hundredfold increase in ultimate sensitivity for the hormone (2 μμg/ml). This has enabled demonstration of previously undetected HCS in plasma and tumor extracts of certain patients with bronchogenic carcinoma.

Various methods have been used to concentrate and purify protein hormones from biologic fluids in an attempt to increase the sensitivity and specificity of biologic and immunologic assays (1). These methods have often yielded variable recovery of the hormone, and none is generally applicable to all protein hormones. It has been shown that a variety of enzymes can be purified by affinity chromatography, using inhibitors covalently linked to agarose (Sepharose) activated with cyanogen bromide (2). This method has recently been adapted to the purification of antibodies (3,4) but its use in the purification of antigens has received only limited attention (4). The present studies describe the use of affinity chromatography with Sepharose-coupled antibody to HCS in the concentration and purification of this hormone prior to radioimmunoassay.

Materials and Methods. Human growth hormone (HGH, Wilhelmi NIH-HS-1147-BC) was donated by Dr. P. Gorden and HCS was a gift from Lederle Laboratories as the purified preparation of Dr. J. R. Florini. HCS was labeled with ^{131}I (5) to specific activity 150-350 μCi/μg and partially purified by gel filtration on Sephadex G-75. Antibodies to HCS were induced in guinea pigs and selected

for both high affinity and high binding capacity. The antiserum used in all present studies displayed an average equilibrium constant of 1×10^{11} L/M and a binding capacity of 3×10^{-5}M HCS per ml in solution (6).

The radioimmunoassay of HCS was a modification of the general method of Berson and Yalow (7) using the double antibody technique (8) and will be described in detail elsewhere (9). The sensitivity of the assay was 0.05 mμg/ml and cross-reaction with HGH was not observed below 10 mμg/ml. Discrimination between the two hormones was further enhanced by the addition of 200 mμg HGH to each assay tube, resulting in no interference below 520 mμg HGH/ml (Fig. 1).

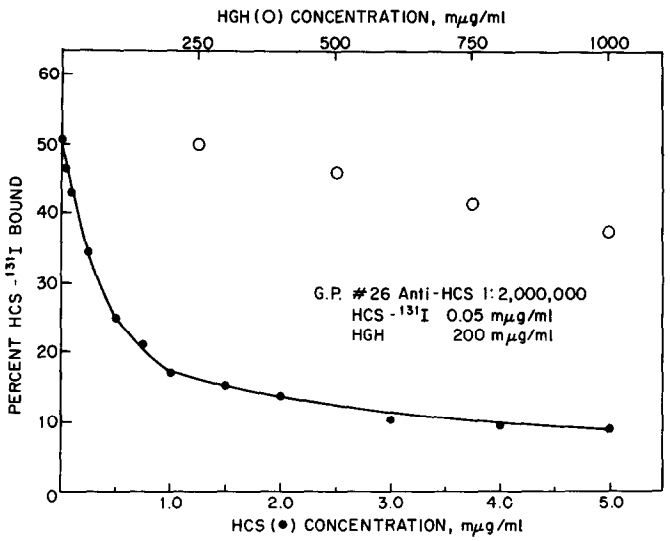

Figure 1 - Radioimmunoassay of HCS showing minimal cross reaction with increments of HGH above the 200 mμg HGH added to each 1 ml incubate.

Anti-HCS for coupling was partially purified from one ml antiserum by precipitation with 40% saturated ammonium sulfate at 4°C. This was repeated twice and the final precipitate dissolved in 0.05M sodium phosphate pH 7.4 and dialyzed against the same buffer. Greater than 99% of the original anti-HCS activity was recovered in the final solution and the specific antibody was calculated to represent approximately 10% of the total protein (16 mg).

Antibodies and other proteins (2-20 mg) were coupled to 10 ml wet

Sepharose 4B (Pharmacia) at pH 8.0 following activation with cyanogen bromide (Eastman) (2). After coupling, the Sepharose was washed with at least 20 liters 0.5M sodium bicarbonate and 500 ml 6M guanidine-HCl until no further antibody activity was detected in the wash solution. Coupling efficiency, calculated from material recovered in the wash, was invariably greater than 95%. Each 10 ml substituted Sepharose was prepared in a small column (1.6 x 5 cm) operated at a flow rate of 30-60 ml/hour at room temperature. Solutions were applied to the column, which was then washed with 0.15M sodium chloride -0.01M sodium phosphate, pH 7.4 until no absorbancy at 280 mμ was detected. Materials adsorbed to the column were eluted with 6M guanidine-HCl, pH 3.1 and this eluate was dialyzed extensively against 0.1M sodium acetate pH 7.4 at 4°C prior to radioimmunoassay.

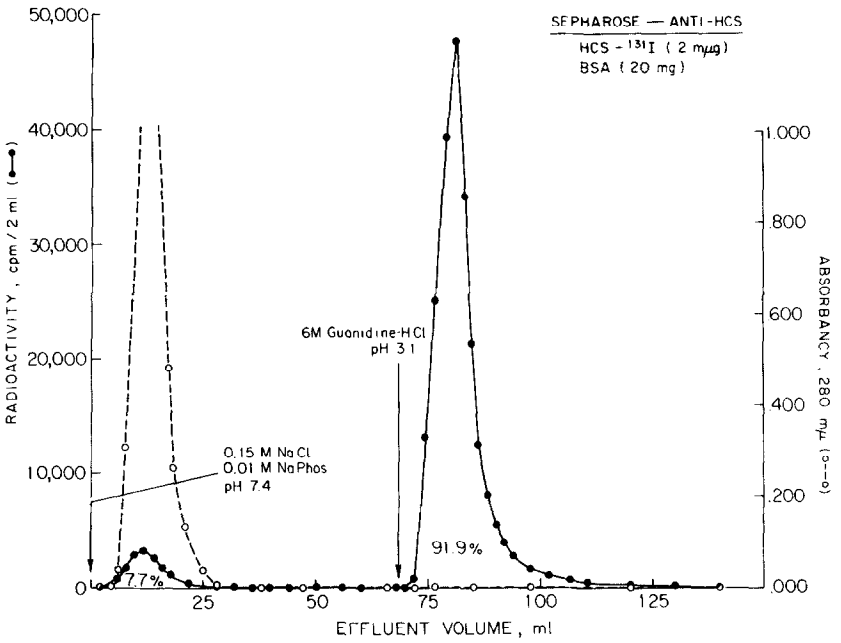

Figure 2 - Application of partially purified HCS-^{131}I and bovine serum albumin (BSA) to a column (1.6 x 5 cm) of Sepharose coupled with anti-HCS. The column was equilibrated and run with saline-phosphate buffer, and the sample applied in 2 ml of the same solution. The unretarded radioactivity was devoid of immunoreactivity in solution, while the radioactivity eluted with guanidine was fully immunoreactive.

Results and Discussion. Neither HCS nor HCS-^{131}I was significantly retarded or adsorbed when applied to columns of unsubstituted Sepharose or Sepharose coupled to nonantibody protein. Following application of HCS-^{131}I to a column of Sepharose-anti-HCS, 8% of the radioactivity was unretarded while the rest was adsorbed to the column and eluted with 6M guanidine-HCl, pH 3.1 (Fig. 2). When this eluted fraction was dialyzed against neutral buffer or diluted one hundredfold and reapplied to the column, virtually all the radioactivity was readsorbed and reapppeared in the guanidine eluate. The original labeled hormone was 88% bound by excess antibody in solution; this agreed well with the 92% bound to the column. Assay of the unretarded peak showed no bindable radioactivity, but the guanidine peak was virtually 100% immunoreactive. Even without prior gel filtration, HCS-^{131}I could be separated from unreacted ^{131}I and "damaged" products of iodination in a single step to yield greater than 95% immunoreactivity, thus providing an immunologically-defined tracer for radioimmunoassay.

Unlabeled HCS behaved similarly to immunoreactive labeled hormone upon application to Sepharose-anti-HCS (Fig. 3). No hormone was detected by radioimmunoassay of the unretarded fraction in concentrations up to 150 mμg/ml or total amounts of 1 μg. Because of limited supply of purified hormone the maximal HCS binding capacity of the entire column was not directly determined, but 0.2 ml column aliquots showed a capacity of 6 - 8 μg, indicating a total column capacity of 300 - 400 μg (50-67% theoretical capacity of antiserum coupled). It was useful to add 0.05% (w/v) human serum albumin (HSA) to the guanidine to enhance recovery of HCS; with this carrier protein 95% of the applied HCS was recovered in 25 ml of guanidine eluate and greater than 99% in 40 ml. As with labeled hormone, unlabeled HCS was restored to full immunoreactivity even if it had been exposed to 6M guanidine for as long as 5 hours prior to dilution or dialysis against neutral buffer. Eluted hormone was usually assayed after further concentration by membrane ultrafiltration (Diaflo Co., UM-10 membrane) to a volume of 2 ml, with a final recovery of 85-95%.

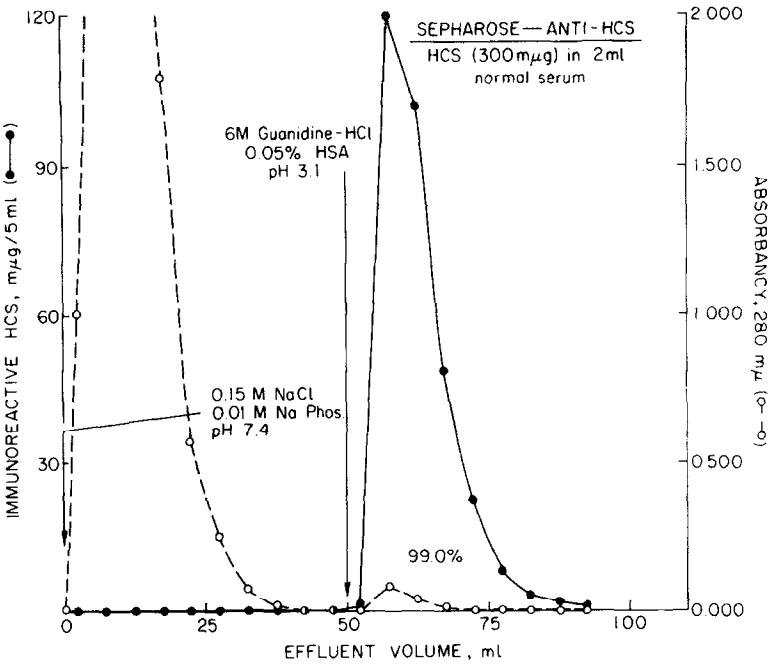

Figure 3 - **Application of HCS in 2 ml normal human serum to a column of Sepharose-anti-HCS. No hormone, as measured by radioimmunoassay, appeared in the unretarded fraction, while 99% was recovered in the guanidine eluate. Human serum albumin (HSA) was added to the guanidine solution and this served as a blank in measuring increments of absorbancy in the eluate. The small eluate peak (about 1% applied absorbancy) was not attributable to HCS and represented non-specific adsorption of serum proteins.**

Applying these techniques to the radioimmunoassay of HCS, we have detected as little as 2 μμg/ml (1×10^{-13}M) in 100 ml of plasma or tissue extracts. This represents at least a five hundredfold increase in sensitivity above most conventional radioimmunoassays in which plasma is usually diluted 1:10 or more to minimize damage to the tracer (10,11). The added sensitivity accrues not only from fiftyfold concentration of the hormone, but also because the final concentrate can be assayed with only slight dilution (8:10). Moreover, unknown samples can be compared to standards of virtually identical pH, ionic strength, and protein composition, and free of other non-hormonal factors in plasma which are known to affect radioimmunoassay (7). Employing these methods we have been able to detect ectopic production of

HCS in 4 of 8 patients with bronchogenic carcinoma, at concentrations (7-250 μμg/ml) not detected by conventional assay (12,9). HCS was not detected in concentrates of plasma from 10 control patients, indicating levels below 2 μμg/ml. We have also demonstrated previously undetected HCS in tumor extracts from two patients with bronchogenic carcinoma (90 and 125 μμg/mg dry wt.) but not in control tissues (less than 15 μμg/mg dry wt.).

Columns of Sepharose-anti-HCS are reusable for long periods even after repeated exposure to 6M guanidine-HCl. They show less than 1% non-specific adsorption of human follicle stimulating hormone (FSH), luteinizing hormone (LH), chorionic gonadotropin (HCG), hemoglobin, albumin and other plasma proteins. However Sepharose-anti-HCS does bind HGH, as does excess antibody in solution. Theoretically HCS might be separable from HGH by gradient elution, exploiting differences in antibody affinity for the two hormones, but complete separation was difficult to achieve in practice. Fortunately, under radioimmunoassay conditions the diluted antibody was highly specific for HCS and was not affected by HGH levels as high as 520 mμg/ml in the incubates - corresponding to 13 mμg/ml HGH in 100 ml original plasma (Fig. 1).

The methods described herein are generally applicable to other proteins. Unlike conventional techniques of concentration and purification, they are not dependent on unique physicochemical characteristics of the protein. Sepharose-antibody columns utilize immunoreactivity as the basis for separation and hence are particularly useful in conjunction with immunoassay. We have successfully applied these methods to the assay of FSH, LH, and HCG (13).

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