2. MOTIVACIÓN

Dentro de la investigación del eje distal de la GH:IGF-I, el estudio de los receptores de la hormona del crecimiento presenta muchas dificultades debidas principalmente a la limitada accesibilidad de la mayoría de los tejidos donde se expresan. La oportunidad de obtener en sangre periférica una medida indirecta del estado de los receptores de la GH ha sido posible gracias a la identificación y caracterización de la proteína transportadora de la hormona del crecimiento (GHBP) pues esta proteína tiene una estructura homóloga al dominio extracelular del receptor de la GH del cual deriva por separación proteolítica[2,3]

Esta tesis nació por el interés de profundizar en el estudio fisiopatológico de esta proteína transportadora de la GH, como pieza importante del eje GH:IGF-I, a través de su determinación en dos áreas de investigación diferentes: los trastornos del crecimiento y las alteraciones de la nutrición. En el primer caso se escogió un grupo de niños y adolescentes con talla baja y velocidad de crecimiento disminuida de diferentes etiologías estudiando la determinación de GHBP como marcador de la insensibilidad a la acción de la GH. En segundo lugar se estudió esta proteína en un grupo de adultos con distintos estados nutricionales, profundizando en la relación de esta proteína con la leptina, una hormona también regulada por la nutrición. Sin embargo, antes de iniciar el estudio en estos dos grupos de pacientes fue necesaria la elección de un método de determinación que ofreciera las garantías analíticas necesarias para poder realizar la determinación de GHBP en estos pacientes.

Por ello, en el desarrollo de esta memoria, tras una introducción de los aspectos más destacables que se conocían sobre la GHBP al inicio del

trabajo presentado en esta tesis, se exponen los resultados obtenidos tras el desarrollo de los objetivos, divididos en tres capítulos basados en la elección del método de determinación, el estudio de la GHBP en la talla baja y el estudio de la GHBP en distintos estados nutricionales.

3. OBJETIVOS

OBJETIVOS

1. Elección de un método para determinar la actividad de la GHBP plasmática comparando las características analíticas y los resultados obtenidos con dos ensayos de unión basados en la capacidad funcional de unión de la GH a la GHBP y que utilizan dos métodos de separación distintos: cromatografía líquida de alta precisión (HPLC)-filtración en gel y adsorción con carbon dextrano.

2. Determinación de la actividad de la GHBP de alta afinidad en un grupo homogéneo de niños con talla baja y velocidad de crecimiento disminuida para descartar un defecto del receptor de la GH como posible causa de su retraso de crecimiento.

3. Estudio de la actividad de la GHBP de alta afinidad y de la concentración de leptina estandarizada en una población adulta con distintos estados nutricionales.

4. Estudio de la relación GHBP-leptina en adultos con distintos estados nutricionales: Valoración de la leptina como posible factor de unión entre nutrición y el eje GH:IGF-I .

4. RESULTADOS

4.1 ESTUDIO 1: ”Comparación de dos ensayos de unión para medir la proteína transportadora de la hormona de crecimiento (GHBP): Cromatografía líquida de alta precision (HPLC) y filtración en gel y adsorción mediante carbón dextrano”

4.1.1 Resumen

Tras el diseño inicial del proyecto de la presente tesis doctoral, la primera necesidad que surgió fue la de adaptar en nuestro laboratorio un método para la determinación de GHBP en suero. Se eligió un ensayo de unión pues queríamos utilizar un método que permitiera medir la GHBP funcionalmente activa, es decir, que tiene capacidad de unión a la GH. De entre los métodos de unión, seleccionamos dos que se diferenciaban en el modo de separar la fracción de GH-I^{125} libre de la ligada a la GHBP: separación mediante cromatografía líquida de alta precisión (HPLC)-filtración en gel y mediante adsorción y precipitación con carbón dextrano (CD).

En la cromatografía mediante HPLC y filtración en gel la separación entre la fase ligada y la libre se realiza en base a su diferente peso molecular. La fase libre tiene un peso molecular menor y queda retenida durante un mayor tiempo en la columna, mientras que la fase ligada tiene un peso molecular mayor y por ello su tiempo de retención es menor. De esta forma en el cromatograma aparecen diferentes picos que corresponden a las diferentes fases ligadas o no. Puesto que la GH-I^{125} se une tanto a la GHBP de alta como a la de baja afinidad y éstas tienen diferentes

pesos moleculares en el cromatograma, los picos correspondientes a estas fases ligadas también tienen diferentes tiempos de retención y por ello es posible distinguir entre ambas.

En el método de adsorción con carbón dextrano, el carbón activo atrapa la fase libre, mientras que los complejos GHBP:GH-I^{125} quedan en la solución. Tras una centrifugación, el carbón sedimenta en el fondo del tubo con la fase libre, mientras que la fase ligada queda en el sobrenadante que se decanta.

- **Marcaje de la GH**

  En los ensayos de unión la pureza del ligando marcado es un factor muy importante, esto junto con la necesidad de obtener una actividad específica relativamente alta nos llevó a marcar la GH en nuestro laboratorio. La GH recombinante se marcó con [I^{125}]Na mediante el método de cloramina-T modificado. Tras el marcaje fuenecesario realizar una purificación mediante cromatografía de filtración en gel en columnas de Sephacryl 200 HR (55 x 1.6 cm). Esta purificación se repitió semanalmente y sólo se utlizó la GH-I^{125} durante las cuatro semanas posteriores al marcaje. La actividad específica osciló entre 60-100 µCi/µg.

- **Calibración de las columnas de filtración en gel**

  Tanto las columnas de Sephadex utilizadas para purificar la GH-I^{125} como la columna para HPLC Protein Pack 300 sw se calibraron con marcadores de diferente peso molecular para averiguar los tiempos de retención correspondientes a los diferentes pesos moleculares. Para ello se utilizaron los siguientes calibradores de peso molecular conocido: azul dextrano (200 k), ß-amilasa (66 k), anhidrasa carbónica (29 k) y citocromomo C (12,4 k).

- **Determinación de la actividad GHBP**

  HPLC-Filtración en gel: La actividad GHBP se midió con HPLC-filtración en gel siguiendo el método de Tar y cols[13]. Tras incubar el suero con una cantidad de GH-I^{125}, la mezcla se inyectó en el cromatógrafo, recogiéndose el eluido en fracciones de 30 segundos, durante media hora. Se procedió al contaje manual de la radioactividad presente en cada fracción y a su representación gráfica, obteniéndose un cromatograma en el que se separaban 3 picos con diferentes tiempos de retención correspondientes a las fracciones en orden de peso molecular decreciente. El primero de ellos, con un tiempo de retención menor, correspondía a la fracción de GH-I^{125} unida a la GHBP de baja afinidad (pico I), el segundo pico (pico II) a la GH-I^{125} unida a la GHBP de alta afinidad, finalmente el tercer pico (pico III) correspondía a la GH-I^{125} libre. Para valorar la unión no específica (NSB, non-specific binding), para cada suero se

realizó paralelamente una incubación con GH-I^{125} añadiendo un exceso de GH no marcada que desplaza a la GH-I^{125} de su unión a la GHBP. El porcentaje de unión de la GH-I^{125} a la GHBP se calculó dividiendo la radioactividad presente en el pico II respecto a la suma de radioactividades de los picos I, II, III. La actividad de la GHBP presente en el suero se expresó como el porcentaje de unión específica, calculada como la diferencia entre la unión total (incubación sin exceso de GH fría) y la no la específica (incubación con exceso de GH fría).

Adsorción con carbón dextrano: La separación mediante carbón dextrano se realizó siguiendo el método de Amit y cols[29]. Tras incubar el suero con GH-I^{125} en ausencia (unión total) o presencia (unión no específica) de un exceso de GH fría, se procedió a separar la fase ligada de la libre añadiendo carbón dextrano, centrifugando y decantando el sobrenadante. La radioactividad presente en el sobrenadante representa la unión de la GH-I^{125} a la GHBP y se expresa como el porcentaje sobre el total de GH-I^{125} incubada en la muestra (radioactividad del precipitado + radioactividad del sobrenadante). El porcentaje de unión específica (unión total menos unión no específica) representa la actividad de la GHBP presente en el suero.

- **Estudio de la imprecisión**

  Como material control, se utilizaron dos mezclas de sueros correspondientes a adultos con peso normal y mujeres obesas, de este modo se obtuvieron dos controles con actividad GHBP media y alta respectivamente. Para poder comparar los coeficientes de variación de los dos métodos de separación, los resultados obtenidos en el material control se expresaron en cada método como unión específica relativa (% RSB, relative specific binding). Esta unión específica relativa se calculó dividiendo la unión específica de cada control individual (controles de actividad GHBP alta o media) procesados en cada serie por la media de los resultados obtenidos en todos los controles de actividad GHBP media analizados durante el estudio.

  En nuestro estudio se utilizó GH-I^{125} procedente de 5 marcajes diferentes y no se encontraron diferencias significativas en los coeficientes de variación interseriales totales, intra e intermarcaje de ambos métodos.

- **Estudio de la interferencia por GH endógena**

  Para cada método de separación, se realizaron curvas de desplazamiento de la unión GHBP:GH-I^{125} incubando cantidades

crecientes de GH fría con las dos mezclas de suero utilizadas como control. De este modo se obtuvo que para ambos métodos a partir de una concentración sérica de GH ≥ 7 ng/ mL el desplazamiento de la unión del GHBP por la GH-I125 era significativo. En las muestras analizadas que presentaban concentraciones de GH superiores se realizaron las correcciones oportunas.

- **Cálculo de la constante de afinidad y de la capacidad de unión máxima**

  El análisis de los datos obtenidos en las curvas de desplazamiento mediante la representación Scatchard, nos permitió el cálculo de la constante de afinidad y de la capacidad de unión máxima con ambos métodos, obteniéndose resultados similares. Con el método de HPLC se obtuvieron unas constantes de afinidad (Ka) de 0,65±0,06 y 0,35±0,2 $x10^9$ mol/L^{-1} en las mezclas de suero de actividad GHBP media y alta respectivamente; mientras que con el método CD se obtuvieron unas Ka de 0,51±0,2 y 0,37±0,11 $x10^9$ mol/L^{-1} para las mismas mezclas de sueros. Las capacidades de unión expresadas en nmol/L de suero, con el método HPLC fueron de 0,79±0,2 y 3,16±0,1 para las mezclas de actividad GHBP media y alta respectivamente; mientras que con el método de CD, fueron de 0,98±1,2 y 3,63±0,19 para las mismas mezclas de suero.

- **Comparación de los resultados de GHBP obtenidos con los dos métodos**

  Un total de 62 muestras de suero procedentes de niños y adultos, se analizaron con los dos métodos de separación, obteniéndose una correlación estadísticamente significativa de los resultados de GHBP obtenidos (r=0, 77, p<0,001).

  El método de separación mediante carbón dextrano no permitió cuantificar la GHBP en una paciente desnutrida, debido a la mayor unión no específica (NSB), mientras que con el método de HPLC se pudo cuantificar.

El presente estudio muestra que la determinación de GHBP por dos ensayos de unión con diferente método de separación, HPLC y adsorción con carbón dextrano da resultados comparables y tienen unas características analíticas similares. El método de HPLC es un método más caro, laborioso y precisa una instrumentación compleja mientras que el método de separación con carbón dextrano es más simple, rápido, puede ser aplicado a un número mayor de muestras en una misma serie. Sin embargo, únicamente el método de separación con HPLC y filtración en gel mide la actividad GHBP de alta afinidad de forma específica y será el que utilizaremos para obtener los resultados de los estudios posteriores.

4.1.2 Artículo

ELSEVIER

Clinica Chimica Acta 267 (1997) 167–181

Analytical performance and clinical usefulness of two binding assays for growth hormone binding protein (GHBP) measurement: high performance liquid chromatography (HPLC)-gel filtration and dextran-coated charcoal adsorption

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Received 10 March 1997; received in revised form 11 July 1997; accepted 19 July 1997

Abstract

We compared two binding assays for growth hormone binding protein (GHBP) measurements, which differ in the method of bound and free GH separation: HPLC-gel filtration or dextran coated-charcoal adsorption (DCC). Two pools of sera (high and medium GHBP activity) were used for quality-control assessment. Moreover, 62 samples from 34 children and 28 adults with different nutritional status were studied. Total, between- and intra-iodination coefficients of variation (CVs) from the two methods were not different. Although percentage binding measured

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PII S0009-8981(97)00133-2

in the pool sera significantly differed, the concentrations assessed by Scatchard plot were comparable. Results obtained by the two methods in the 62 sera were significantly correlated ($r = 0.77$, $P < 0.001$). With both methods GHBP activity correlated with chronological age and body mass index (BMI) and differed among groups with different nutritional status. Although HPLC and DCC separation methods for GHBP measurement differ in their practicability, our study demonstrates that performance and the clinical usefulness of the two methods are comparable. © 1997 Elsevier Science B.V.

Keywords: Growth hormone binding protein (GHBP); High performance liquid chromatography (HPLC); Dextran-coated charcoal adsorption; Idiopathic short stature; Obesity

1. Introduction

A specific high affinity binding protein for growth hormone (GHBP) in human serum was described and characterized in 1986 [1,2]. In humans, GHBP derives from GH membrane receptors by proteolytic cleavage and is identical to the extracellular binding domain of this receptor [3]. Thus, measurement of GHBP activity or levels in serum is of considerable clinical and physiological interest because it may reflect tissue GH receptor levels. The role of GHBP, however, is not well known. Recent data show regulatory effects of circulating GHBP on GH action, both as an enhancer, prolonging its half-life [4] and as an inhibitor by competing with tissue receptors for GH as a ligand [5]. For these reasons, the circulating GHBP is an important component of the GH distal axis.

Several types of assays for GHBP measurement have been reported [1,2,6–11] as well as some comparisons between them [12–15]. Assays may be divided into those measuring the functional binding capacity of sera and those measuring the protein immunoreactivity. Functional binding assays differ according to the methodology for bound and free GH separation. In this study, we measured serum GHBP activity using two binding assays with different methods of separation: gel filtration and high pressure liquid chromatography (HPLC) [6] and dextran-coated charcoal adsorption (DCC) [7]. The HPLC separation method measures the specific binding to the high affinity binding protein; however, this approach is logistically more difficult, labor intensive, expensive and requires the availability of laboratory facilities suited to the performance of HPLC. In contrast, the DCC method cannot discriminate between low and high affinity binding protein but is simple, rapid, cheaper and can be applied to a large number of specimens. To our knowledge, a comparison between these two methods has not been reported. Therefore, the aim of this study was to compare the assays' performance and the results obtained by these two different binding assays in prepubertal and pubertal children with normal or short stature, in normal weight adults and in patients with anorexia nervosa or morbid obesity.

2. Materials and methods

2.1. Subjects

Serum samples were obtained from 34 children (30 boys, 4 girls) aged 4.3–17.3 years. Twenty-four were prepubertal (Tanner stage I) and 10 were pubertal (Tanner stages II–III). Children were classified as: control group (C), seven healthy children (six boys, one girl) aged 4.8–13.3 years with mean height-SDS of −1.03 (−1.6 to −0.3) and BMI 17.1±1.59 kg/m^2 (mean±S.D.).

Idiopathic short stature (ISS): 27 short but otherwise healthy children (24 boys, three girls) aged 4.3–17.3 years. Their heights were below the 3rd percentile for age and sex and the serum GH concentration was above 7.5 ng/ml on at least one provocative GH secretion test. This group had a mean height-SDS of −2.47 (−4.5 to −1.8) and BMI 15.9±1.34 kg/m^2.

Serum samples were also obtained from 28 adults: nine normal weight adults (six women, three men) aged 16–43 years and BMI 19.1±2.78 kg/m^2; nine severely undernourished anorexia nervosa patients (seven women, two men) aged 17–27 years and BMI 15.9±0.85 kg/m^2; and 10 patients with morbid obesity (eight women, two men), aged 25–55 years and BMI 48.13±6.56 kg/m^2.

Blood samples were obtained at 09:00 h after overnight fasting, for GH and GHBP measurements. Sera were immediately frozen upon separation and stored at −40°C until assayed.

In seven of these children two serum samples were obtained on two different days to determine intra-individual interday variation of GHBP.

2.2. Materials

2.2.1. Quality-control material

Two pools of sera from normal weight and from obese women were obtained and run in each assay to assess the analytical performance at medium and high GHBP levels and to control the quality of the [^{125}I]hGH tracer. At least one pool of serum was run every day by the HPLC method and in every batch by the DCC method.

2.2.2. Chemicals

Recombinant hGH (Humatrope®) was a gift from Lilly (Eli Lilly and Co., Indianapolis, IN). Carrier free [^{125}I]Na was purchased from Du Pont de Nemours (Brussels, Belgium). Sephacryl 200 HR and dextran T-70 were purchased from Pharmacia (Uppsala, Sweden). BSA fraction V, Chloramine-T

and a gel filtration calibration kit were obtained from Sigma Chemical Co. (St. Louis, MO). Norit A Charcoal was obtained from Merck (Darmstadt, Germany).

2.2.3. Instruments

HPLC separations were performed using a liquid chromatograph (model SERIES 2, Perkin Elmer, Norwalk, CT) equipped with a sample injector (model Reodyne 7125) fitted with a 500-μl loop and an analytical Protein Pak 300 sw column (Waters, Millipore, Mildford, MA; 0.78 × 30 cm). Absorbance at 280 nm was measured with an LC spectrophotometric detector (Perkin Elmer). The eluates were collected with a fraction collector FRAC 300 (Pharmacia) and radioactivity was counted in an automatic gamma counter (GAMBYT, CR-20, DPC, Los Angeles).

2.3. Iodination of hGH

Recombinant hGH was radiolabeled with [^{125}I]Na using the chloramine-T method modified by Lesniak et al. [16] and purified by gel filtration chromatography on a Sephacryl 200 HR column (55 × 1.6 cm). The column was eluted with potassium phosphate buffer (0.05 M, pH 7.0) containing 0.1% BSA and 0.02% NaN_3. The specific activity of [^{125}I]rhGH ranged from 60–100 μCi/μg. After iodination, radiolabeled hGH was only used during 4 weeks and was repurified once weekly.

2.4. Hormone assays

Serum hGH was measured using a monoclonal antibody-based immunoradiometric assay (bioMérieux, Marcy L'Etoile, France), which uses standards calibrated against the IRP 66/217. The assay has a sensitivity of 0.1 ng/ml, with intra- and interassay coefficients of variation (CVs) of 3.0 and 5.0%, respectively.

2.5. Measurement of GHBP

2.5.1. HPLC-gel filtration

GHBP activity was measured by the HPLC-gel filtration method of Tar et al. [6]. Briefly, serum (100 μl) was incubated overnight at 4°C with 100 μl potassium phosphate (0.1 mol/l; pH 7.0) buffer and 0.1% BSA containing [^{125}I]hGH (0.5 ng). A parallel incubation was carried out in the presence of a large excess of rhGH (2 μg) to evaluate non-specific binding. After filtration through a 0.45-μm Millipore minifilter, the entire incubation mixture was placed onto HPLC Protein Pack 300 sw column. Elution was performed autocratically

using a degassed buffer (0.1 mol/l Na_2SO_4 and 0.1 mol/l potassium phosphate, pH 7.0) pumped at a rate of 0.5 ml/min in order to separate bound and free [^{125}I]hGH. The column was calibrated with blue dextran, β-amylase, BSA, carbonic anhydrase and cytochrome C. The eluate was collected in 30-s fractions. The binding of [^{125}I]hGH is expressed as the radioactivity in the individual peak II (Fig. 1) divided by the sum of the radioactivity in peaks I, II, and III. The binding to the peak II-BP is given as the percentage of specific binding, calculated as the difference between total (incubation without unlabeled hGH) and non-specific binding (incubation with an excess of hGH).

2.5.2. Dextran-coated charcoal

GHBP was measured in the same samples by the dextran-coated charcoal separation method of Amit et al. [7]. In brief, [^{125}I]hGH (1 ng) was incubated with 50 μl serum in the absence (total binding) or presence (non-specific binding) of excess hGH (2 μg) in 0.1 mol/l sodium phosphate buffer containing 1% BSA and 0.075 mol/l $MgCl_2$ in a final volume of 275 μl for 20–24 h at 4°C. At the end of the incubation period, bound and free hormone were separated by adding 1 ml cold dextran-coated charcoal (0.2% dextran T-70-2% Norit-A charcoal in 0.01 mol/l phosphate buffer, pH 7.6). The assay tubes were vigorously shaken and incubated for 15 min on ice, centrifuged at 3000 × *g* for

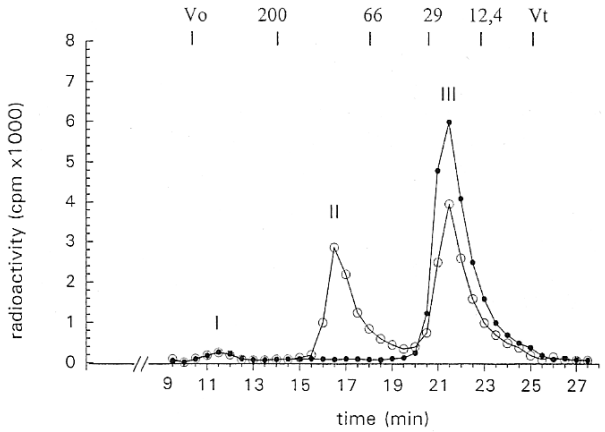

Fig. 1. Representative elution profile of the high GHBP activity pool serum incubated with [^{125}I]hGH, in the absence (○ total binding) and presence of 2 μg hGH (● non-specific binding). I, [^{125}I]hGH bound to the low affinity binding protein; II, [^{125}I]hGH bound to the high GHBP; III, free monomeric [^{125}I]hGH. At the top of the figure, the void (V_o) and total (V_t) volumes and the molecular weight of the markers used to calibrate the column are indicated (200 k, β-amylase; 66 k, BSA; 29 k, carbonic anhydrase; 12,4 k cytochrome C).

20 min at 4°C, and the supernatant decanted and counted in a gamma counter. The specific binding (total minus non-specific binding) was expressed as a percentage of the total [^{125}I]hGH cpm incubated.

For each separating method, displacement curves were performed by spiking the two pool sera with increasing amounts of GH to calculate the extent to which endogenous GH would significantly reduce the binding of [^{125}I]hGH. Scatchard analyses were also performed to determine binding affinity and capacity of the two pool sera.

2.6. Statistical analysis

2.6.1. Method comparison

All results are expressed as mean±S.E.M. Results of specific binding obtained by both methods are not comparable owing to different kinds of calculation. Thus, in order to compare assay performance, results of the two-pool sera were expressed in each method as relative specific binding (%RSB) by dividing the specific binding of each individual pool sera (high or medium GHBP level) run in each assay by mean specific binding of all medium pool sera run throughout this study. Thus, imprecision between both methods was compared using the F-test.

In our study we radiolabelled hGH on five occasions and calculated the between-iodination CVs as:

$$CV_{bi} = \frac{\sqrt{\frac{s_1^2 + s_2^2 + \cdots + s_n^2}{n}}}{\bar{x}}$$

where n = number of iodinations, $\bar{x}$ = mean GHBP activities of the pool sera (high or medium) obtained in different iodinations, and s_n = standard deviation of GHBP activities of the pool sera obtained in each iodination.

2.6.2. Clinical studies

Statistical analysis was performed with Student's t-test for difference of means and linear regression analysis for correlation. Statistical significance was assumed at a level of $P<0.05$.

3. Results

3.1. Binding-displacement curves and Scatchard analysis

For each method of separation, Scatchard analysis obtained from the hGH displacement studies with the two pool sera yielded linear plots (Fig. 2). Mean

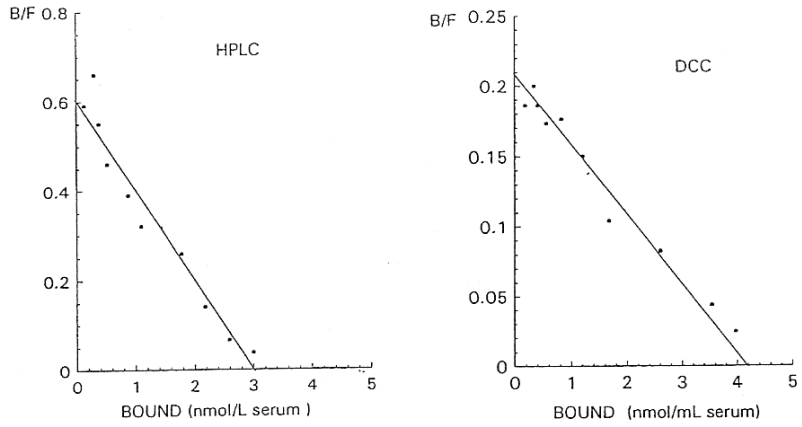

Fig. 2. Example of a Scatchard plot of the high GHBP pool serum obtained by HPLC and DCC methods.

binding parameters (affinity constants and maximum binding capacities) are presented in Table 1.

Displacement curves showed that the percent specific binding to GHBP needed to be corrected for hGH serum levels ≥7 ng/ml by both methods (data not shown). The great majority of our samples did not need correction because endogenous hGH was <7 ng/ml. Only two samples required correction. To obtain a corrected value, the measured binding was divided by the fraction bound in the pool sera of medium GHBP level at the hGH concentration found in the unknown sample.

3.2. Imprecision analysis for HPLC and DCC method

Table 2 shows the results of binding activity of the medium and high GHBP pool sera by both methods expressed both as specific binding and relative

Table 1
Binding parameters (mean±S.E.M.) derived from Scatchard analysis of the two pool sera in repeated determinations (*n*)

	Medium GHBP pool	High GHBP pool
HPLC K_a ($\times 10^9$ mol/l^{-1})	0.65±0.06	0.35±0.02
Capacity (nmol/l serum)	0.79±0.02	3.16±0.1
(*n*)	(2)	(4)
DCC K_a ($\times 10^9$mol/l^{-1})	0.51±0.02	0.37±0.11
Capacity (nmol/l serum)	0.98±1.2	3.63±0.19
(*n*)	(2)	(4)

Table 2
GHBP activities (mean±S.E.M.) of the two pool sera measured by each method, expressed as specific binding (%SB) and relative specific binding (%RSB) obtained in repeated determinations (*n*)

		Medium GHBP pool	High GHBP pool
HPLC	%SB	19.2±0.7	40.9±0.8
	%RSB	99.8±3.7	212.1±4.5
	(*n*)	(29)	(20)
DCC	%SB	8.2±0.3	17.1±0.5
	%RSB	100.1±4.2	208.2±5.8
	(*n*)	(19)	(22)

specific binding. No differences were found in %RSB between the two methods at medium and high GHBP levels.

The total interassay CVs for the high and medium GHBP sera pools measured by HPLC method were 11.3% ($n=29$) and 16.9% ($n=20$), respectively, while the CVs for the same pools measured by DCC were 12.2% ($n=19$) and 19.6% ($n=22$), respectively. During this study we radiolabeled hGH on five occasions and we calculated the between-iodination CVs, being 8.7% and 13.7% for high and medium GHBP activity pool sera by HPLC and 10.1% and 16.3% for high and medium GHBP activity pool sera by DCC. Moreover, if analysis was restricted to assays in which the same batch of radiolabeled GH was used, the interassay variability (intra-iodination CV) for both methods decreased to 8.4% ($n=6$) and 9.8% ($n=7$) for high and medium GHBP controls by HPLC, and 7.5% ($n=6$) and 14.2% ($n=8$) for the same controls by DCC.

Total interassay CVs for medium and high GHBP levels assessed by variance comparison of the %RSB showed no significant difference between the two methods: $F=1.1$ and $F=1.3$ for high and medium GHBP levels respectively (95% confidence interval for equality of variances $F=0.41$–2.51).

3.3. GHBP results comparison

Serum GHBP activities determined by HPLC and DCC in the 61 samples studied were highly correlated ($r=0.84$, $P<0.0001$) (Fig. 3).

In addition, in the totality of sera studied, GHBP activities measured by HPLC and DCC methods correlated significantly with BMI ($r=0.77$, $P<0.0001$ and $r=0.88$, $P<0.0001$, respectively).

In the group of 34 children and nine normal-weight adults, GHBP activity measured by HPLC and DCC methods correlated significantly with chronological age ($r=0.34$, $P=0.025$ and $r=0.72$, $P<0.0001$, respectively) (Fig. 4). No

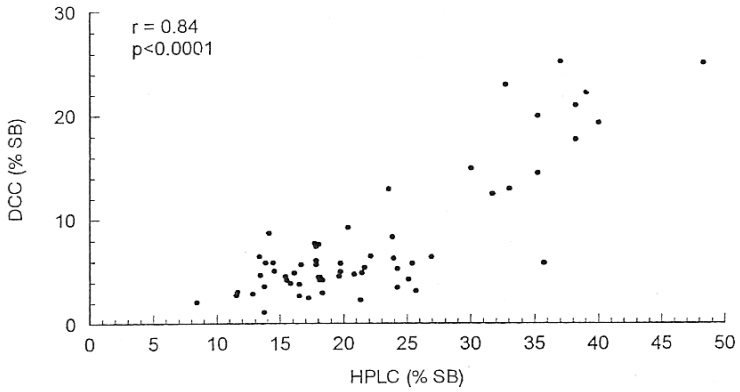

Fig. 3. Correlation between GHBP activities expressed as % of specific binding (%SB) obtained by HPLC and DCC method in 61 different serum samples.

correlation was found, for either method, between GHBP activity and height-SDS in the group of children.

Mean±S.E.M. GHBP activities obtained with both methods for the different groups of patients are shown in Table 3. No differences were found in GHBP activity between prepubertal and pubertal children in either method. In addition no differences were found in GHBP activity measured by both methods between control and ISS children (19.01±1.4 vs. 19.1±1.0 and 4.3±1.0 vs. 4.9±0.3 by HPLC and DCC methods, respectively).

GHBP activity in obese patients measured by both methods was significantly higher than in all other groups studied ($P<0.0001$). The serum GHBP levels

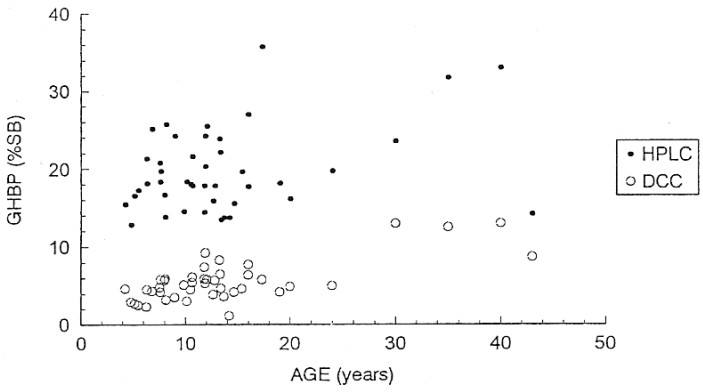

Fig. 4. Correlation of GHBP activity (expressed as percentage of specific binding, %SB) measured by HPLC or DCC methods with chronological age in serum samples from children and normal-weight adults.

Table 3
GHBP activities (mean±S.E.M.) expressed as specific binding (%SB) in the different groups studied

	n	HPLC	DCC
Prepubertal	24	18.2±0.7	4.7±0.3
Pubertal	10	21.2±2.1	5.3±0.6
Control adults	9	22.3±2.3	8.3±1.2
Anorexia nervosa	8	15.5±1.9[a]	4.6±0.7[b]
Obesity	10	37.7±1.4[c]	19.9±1.1[c]

[a] $P<0.041$ and [b] $P<0.021$ vs. control adults.
[c] $P<0.0001$ vs. control adults and vs. anorexia nervosa.

measured by both methods were lower in the anorexia nervosa patients when compared with GHBP levels in adult-control subjects ($P<0.05$ for both methods). The levels of GHBP in anorexia nervosa patients were not different from those found in prepubertal or pubertal children.

On the other hand, the DCC method was unable to detect any GHBP activity in one of the anorexia nervosa patients (on three different occasions the non-specific binding was higher than the specific binding), while with the HPLC method this serum yielded a GHBP activity of 2.9%. The result of this serum was not included in the study.

In seven children, GHBP activity measured in two serum samples from different days yielded a mean CV of 13.7% (7.2–23%) by HPLC and of 15.6% (1.2–38.3%) by DCC separation.

4. Discussion

Since the first method was described for detection of GHBP [1,2], other methods also based on binding studies have been developed. In them, after incubation of the sample with radiolabeled GH, separation of bound and free GH can be effected by several means: classically gel filtration chromatography has been used on columns of varying dimensions and composition [1,2,17]. Newer variants of these include HPLC and FPLC [6,18]. Other means of separation include charcoal adsorption [7] and immunoprecipitation with anti-receptor antibody [19].

In the literature there are many studies published in which GHBP activity is measured either with the HPLC or the DCC method of separation but to date no study had compared these two methods. In the report of Amit et al. [7], the first describing the DCC method, a comparison was made between the binding parameters obtained with this method and those reported in the literature, using various chromatographic techniques, except the HPLC method.

These methods do not measure exactly the same analyte. While HPLC can measure the specific binding to the high affinity binding protein, the DCC method cannot discriminate between low and high affinity binding proteins. In our study we found different absolute percentages of specific binding in the same samples because these methods also differ in the way to calculate the percentage of specific binding. However maximum binding capacities and affinity constants derived from Scatchard analysis yield similar results for both levels of GHBP pool sera.

With respect to the assays' characteristics, in our study we have calculated three different types of interassay CVs: total, between-iodination and within-iodination. When we compare our results of assay performance with those reported in the literature using the same methods of separation, we found interassay CVs similar to our within-iodination CVs. In this way, with HPLC as the method of separation the following interassay CVs have been published: 8% ($n=17$) at the level of 32.2% [6] or 8% ($n=9$) at the level of 25.1% and 18% ($n=10$) at the level of 3.6% [20]. The same occur with the DCC method: Amit et al. [7] found an interassay CV of 4.63% ($n=8$) in normal human sera, Menon et al. [21] found it to be 10% ($n=7$) in a pool of normal sera and Kratzsch et al. [10] found a value of 9% with an unspecified number of determinations. In these reports, however, it is not mentioned whether the interassay CV has been calculated between- or within-batch of radiolabeled GH. Variation in the quality of tracer is an important factor contributing to between-assay measurement errors [14]. Thus, we found that the between-iodination and total interassay CVs were higher than the within-iodination CV.

In our study, in spite of the apparently higher interassay CVs for the DCC method, when we compared the imprecisions after the results of the pool sera were expressed as %RSB, we could not find any significant difference between the two methods.

Our data confirm the results previously published according to which adiposity and nutritional status are significant determinants of GHBP levels [17,22–27]. In this way, in our series we found a significant correlation between BMI and GHBP activity measured with both methods and also GHBP levels in obese were significantly higher than in normal weight adults, anorexic patients and prepubertal or pubertal children. Moreover, GHBP levels observed in our group of anorexia patients were lower than in normal weight adults, in agreement with data from the literature [22,24,25], but did not differ from those in pubertal children, in contradiction to Hochberg et al. [22].

In our series, we have found a significant correlation between chronological age and GHBP activity measured by both methods in the group of normal weight children and adults. Previous studies have given conflicting data with regard to correlation with chronological age. An age-related increase in GHBP has been shown in several reports [6,7,20,26,28,29] which show that GHBP

activity is very low in neonates with a rapid increase during the first 2 years of life, followed by a progressive and slower rise throughout childhood, but other authors [30,31] did not find this relationship.

In our population of children no correlation was found between serum GHBP levels measured by either method and height-SDS. This finding is in agreement with the data from some reports [32,33] but are in contrast with the data of Massa et al. [31] and Silbergeld et al. [26] reporting a positive relationship between GHBP levels and height-SDS in normal children.

Recently, it has been published that some children with ISS have low levels of GHBP [33–39] suggesting a partial GH insensitivity. In our study we could not demonstrate this finding, but this may be due to the small number of patients.

In relation to the intrasubject interday variability we found lower results than those reported in the literature. Martha et al. [40] found a mean intrasubject CV of 30% but they studied 11 boys during 4–5 years, obtaining samples every 4 months, while we have obtained only two samples.

In summary, we have demonstrated that GHBP activities obtained by HPLC correlate well with those derived from the DCC method and that the same significant correlations between GHBP activities and BMI or chronological age are found, and that the differences observed between groups of patients were similar.

In conclusion, the present study shows that serum GHBP determinations obtained by two binding assays with different methods of separation, HPLC and dextran-coated charcoal adsorption, give comparable results and have similar performance characteristics. The DCC method is faster and cheaper than the HPLC method but it has been unable to measure GHBP activity in one of the low samples. On the other hand, HPLC is more specific and permits separation of the high and low affinity GHBPs.

Acknowledgements

This work received the financial support of Lilly SA, Spain.

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