Computer Assisted Validation of Binding Assays for Sex Hormone Binding Globulin

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ABSTRACT

Accurate measurement of sex hormone binding globulin (SHBG) by 5α-dihydrotestosterone (DHT) binding assays requires the establishment of acceptable upper and lower limits of labelled DHT binding and the use of optimal quantities of radioinert DHT to displace labelled DHT. A computer assisted binding assay for SHBG is described in which upper and lower binding limits are derived mathematically from analysis of labelled DHT binding to eight concentrations of SHBG in the range 25 to 400 nM. The optimal quantity of radioinert DHT for a given SHBG concentration is also derived from these binding curves. A computer program determines whether or not, in a given sample, the binding of labelled DHT falls within these established binding limits and also calculates the concentration of SHBG. This technique reduces the number of tubes required in an assay and provides rapid validation and calculation of results.

Introduction

Estimation of sex hormone binding globulin (SHBG) in plasma has conveniently utilized techniques in which binding sites on the protein are saturated with a mixture of radiolabelled and radioinert 5α-dihydrotestosterone (DHT) of known specific activity.8,17 The steroid-protein complex is usually precipitated with ammonium sulfate,2,4,6,7,9,15,21 although other separation methods have been employed.3,12,13,14,18,19 There are several possible sources of error and imprecision in this type of assay. The fraction of unbound steroid is calculated from the difference between measured levels of labelled total and free DHT. The quantity of unlabelled steroid required to displace radiolabelled tracer varies with the concentration of SHBG. If the percentage of labelled DHT bound to SHBG (percent bound) is low, the difference between total and free labelled steroid will be small and strongly influenced by scintillation counting errors. High levels of tracer binding (associated with high levels of SHBG) may indicate...
that saturation of binding sites has not been achieved. In both cases the assay will be invalid. Thus for a given level of SHBG, the quantity of unlabelled steroid needed to displace labelled tracer and the acceptable upper and lower binding limits of labelled tracer must be defined for valid results.

This paper describes the mathematical derivation of upper and lower binding limits of labelled DHT from experimental data and the optimal quantities of radioinert DHT required for displacement of tracer for eight different SHBG concentrations in the range 25 to 400 nM. A computer program determines whether in a given sample the percent bound falls within these binding limits and also calculates the concentration of SHBG.

Materials and Methods

Reagents

5α-Dihydrotestosterone* and [1,2,4, 5,6,7-3H]DHT† were obtained. The labelled steroid was purified by chromatography on Sephadex LH-20‡ by elution with a mixture of hexane, benzene, and methanol in the proportions 100:10:5 by volume. All other chemicals and solvents were of analytical grade. Solvents were distilled before use.

Scintillation Counting

A liquid scintillation spectrometer§ was used for measurement of [3H] DHT with a scintillation cocktail containing 0.5 g of p-bis-(o-methyl-styryl)-benzene, 2.5 g of diphenyloxazole, and 20 ml of methanol per liter of toluene. An efficiency curve was generated from quench standards§ and quench correction automatically applied to sample values. All samples were counted for sufficient time to result in a standard deviation less than two percent.

Patient Samples

Plasma, obtained from normal adult males as well as non-pregnant and pregnant women, was stored at −20°C until analysis.

Binding Assay

Experimental conditions for the assay have been described previously.5

Binding Curves

Plasma pools with SHBG levels ranging from 25 to 400 nM were assayed with quantities of radioinert DHT varying from 0.5 ng to 10 ng per sample tube. At each level of SHBG, an equation was derived to describe the change in percent bound with varying concentrations of radioinert DHT. The first and second derivatives of these equations were computer plotted over the domain of DHT values. Analysis of these curves provides the region of maximum sensitivity, thus defining the upper and lower limits of percent bound for that SHBG level. From the eight sets of limits, two equations were derived to describe the upper and lower binding limits over the range of SHBG concentrations analyzed. For the assay conditions and using the equations derived for the upper and lower binding limits, tables of optimal quantities of radioinert DHT required for assaying increasing levels of SHBG were calculated (table I). A computer program was designed to solve the equations describing the binding limits, to check that the percent binding of a given sample fell within these limits and to calculate the concentration of SHBG.

* Sigma Chemical Company, St. Louis, MO, USA.
† Amersham, Surry Hills, Australia.
‡ Pharmacia, North Ryde, Australia.
§ Packard Tri Carb Model 2650, Downers Grove, IL, USA.
TABLE I
Unlabelled 5α-dihydrotestosterone and Sex Hormone Binding Globulin Ranges

<table>
<thead>
<tr>
<th>DHT (ng)</th>
<th>SHBG Range (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lower Limit</td>
</tr>
<tr>
<td>0.5</td>
<td>13</td>
</tr>
<tr>
<td>0.75</td>
<td>21</td>
</tr>
<tr>
<td>1.0</td>
<td>28</td>
</tr>
<tr>
<td>1.5</td>
<td>44</td>
</tr>
<tr>
<td>2.5</td>
<td>79</td>
</tr>
<tr>
<td>5.0</td>
<td>204</td>
</tr>
</tbody>
</table>

SHBG results were calculated for these points using the formula

\[
SH = \frac{A - B}{A} \times \frac{10^6 \text{ NG}}{V \times MW}
\]

where

- \(SH\) = SHBG (nM)
- \(A\) = total counts
- \(B\) = free counts (supernatant)
- \(V\) = volume of sample per assay tube (μl)
- \(NG\) = weight of DHT per assay tube (ng)
- \(MW\) = molecular weight of DHT

Computing

Programs were written in Applesoft BASIC and run on an Apple II Plus* microcomputer equipped with two Apple II disk drives and controller board and interfaced to an NEC Model 7710 Spinwritert via a Supercom II‡ interface board. Graphical analyses were performed using a Curve Fitter§ program.

Results

The binding curve for a plasma pool with an SHBG concentration of 100 nM is shown in figure 1. A polynomial of degree 5 was computed by least squares techniques to describe the curve in figure 1.

\[
y = -0.92x^5 + 11.76x^4 - 57.98x^3 + 139.16x^2 - 171.19x + 108.61 \\
(r = 0.998)
\]

Equations for the first and second derivatives were derived and from computer plots, conservative upper and lower percent bound limits of 30 and 15 percent, respectively, were estimated for figure 1. These values represent the limits for the maximum rate of change of slope for the curve since this delineates the region resulting in highest sensitivity. Similar curves were constructed from seven other plasma pools with SHBG concentrations ranging from 25 to 400 nM. The sets of limits obtained were correlated with the range of SHBG levels assayed (figure 2).

From a series of functions, a cubic and quadratic relationship were chosen as best describing the upper and lower bound limits respectively in figure 2 and the corresponding equations were computed to be:

\[
PBH = 1.54 SH^3 - 3.87 SH^2 + 0.23 SH + 13.78 \\
(r = 0.986)
\]

and

\[
PBL = -3.70 SH^2 + 0.04 SH + 9.44 \\
(r = 0.952)
\]

where \(PBH\) = percent bound upper limit and \(PBL\) = percent bound lower limit.

These percent bound limits were then used to tabulate the corresponding upper and lower bounds for the amounts of unlabelled DHT required to assay various SHBG levels. These tables are represented graphically in figure 3 and are based on solutions of the equation:

\[
NG = \frac{SH \times V \times MW}{FB \times 10^6}
\]

where \(FB\) = fraction bound, either for the upper or the lower limit.

* Cupertino, CA, USA.
† Tokyo, Japan.
‡ CED Distributors, Auckland, New Zealand.
§ Interactive Microware Inc., State College, PA, USA.
 VALIDATION OF SHBG BINDING ASSAYS

Discussion

Although binding limits for SHBG assays have previously been described, these have been derived from normal male and pregnant and nonpregnant female plasma pools at single levels of SHBG. In pathological states, SHBG concentrations can vary widely. Levels are reduced up to 50 percent in hirsute and hyperprolactinemic females and estrogen therapy can increase SHBG as much as three-fold.

During pregnancy, the level of SHBG rises progressively from 8 to 10 weeks gestation with an approximate total five fold increase. Following delivery, the level subsides and at 10 days postpartum, a mean decrease from 367 to 111 nM was shown in a study of seven patients.

With these large variations from the normal range, an assay relying on the use of single binding limits derived from normal subjects may well be invalid. No single level of radioinert DHT is suitable for the ranges of SHBG found in males or pregnant and nonpregnant females. However, from the computed table of concentrations of radioinert DHT (table I) and a knowledge of the clinical details of the patient, the optimum range of DHT to be added can be selected. In some instances, only one level of DHT may be required for the assay. At very low SHBG levels, the binding assay requires very small quantities of unlabelled steroid to maintain a displacement within acceptable limits (figure 3) and samples of this type are more accurately and easily analyzed by using a larger sample size.

For each SHBG level, percent bound

![Figure 1. Binding characteristics of a sex hormone binding globulin pool (100 nM).](image1)

![Figure 2. Upper and lower percentage bound limits for varying SHBG concentrations.](image2)

![Figure 3. Computed 5α-dihydrotestosterone concentrations corresponding to percentage bound limits for varying sex hormone binding globulin concentrations.](image3)
limits are derived from continuous functions based on experimentally obtained data, thus removing the discontinuities in the binding criteria of reported assays. With computer assessment of radioinert DHT concentrations which fulfill binding limit requirements and the rapid performance of complex calculations, there is a considerable saving in time and the removal of a potential source of imprecision and inaccuracy in the binding assay.

References