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Review

Free estradiol and sex hormone-binding globulin

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ABSTRACT

SHBG is a plasma protein that participates in the regulation of free estradiol and free testosterone in plasma. We discuss the concept of the nature of a free estradiol and how best to ascertain its value. It can be measured or calculated; the ways in which this can be done are explored along with the advantages and disadvantages of each.

Contents

1. Introduction

The theme of this special issue is estradiol (E_2); the purpose of this communication is to set out the role of sex hormone-binding globulin (SHBG) in relationship to free estradiol in human plasma. However, we would be remiss if we omitted mention of SHBG's multiple other functionalities. It participates in signal transduction at the plasma membrane where it has a specific binding site [1]; it is a predictor/marker of a number of important disease states [2]; and it is synthesized in a number of tissues, that are unrelated to its presence in plasma, and, in prostate, enhances the action of dihydrotestosterone [3] Thus, although this communication will deal only with SHBG's function relative to the binding of E_2, we should recall that it is a multi-functional protein whose biology is far from clearly understood.

1.1. SHBG general properties

The first descriptions of SHBG established that it was a plasma glycoprotein that circulates as a homodimer that binds a variety of sex steroids, most importantly dihydrotestosterone, testosterone (T) and estradiol. It subsequently became clear that the homodimer binds two molecules of ligand [4], and that the source of plasma SHBG is the liver [5], which synthesizes and secretes it. Its concentration in plasma is known to be increased by thyroid hormone, estrogenic hormones, and cirrhosis whereas its concentration is decreased by obesity, androgens, prolactin, puberty, progestins, insulin and IGF-1 [6].

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1.2. Free estradiol

E_2 is presumed to exist in two physical states in human plasma – that which is bound to proteins and that which is unbound (free). The two proteins that bind E_2 are SHBG and albumin. We should be aware that the concept of free estradiol concentration is a theoretical construct and that free or bound hormone is not measured directly *in vivo*, but rather examined *in vitro* by a number of techniques which attempt not to disturb the presumed equilibrium which exists *in vivo*. The theoretical construct arises from the Law of Mass Action which describes the distribution of two or more interacting molecular entities based on their molar concentration and the quantitative strength of the interaction, e.g. the association constant (Ka). The original work on Mass Action was done on chemical kinetics in simplified systems with reactants in a relatively dilute, pH-buffered, aqueous solution. In more complex environments, where bound particles may be prevented from dissociation by their environment, the model of Mass Action may not always describe the behavior of the reaction kinetics accurately. This caveat may bear on some of the inconsistencies between measured and calculated free estradiol discussed below. Fig. 1 sets out, in a formal manner, how one predicts the concentration of free estradiol if the concentration of SHBG, albumin, total estradiol and the appropriate Ka's are known. Note that this is a cubic equation which may not be familiar, as the two most common expositions of the Law of Mass Action, as applied to this circumstance, result in quadratic equations after a simplifying assumption [7,8]. The simplifications are justified and if all the same numbers are entered into the equations, they all yield the same result. Several intuitive and useful messages are inherent in these equations:

- Increases in SHBG or Ka decrease free E_2.
- Increments in an existing excess of total E_2 over SHBG all go to free E_2.
- When total E_2 is small compared to SHBG, the fraction bound depends *only* on the Ka and SHBG concentration.
- When SHBG and E_2 are more or less equal the fraction of E_2 bound depends on [total E_2], [SHBG], Ka *and* on [total E_2]/[SHBG].

2. Free E_2: calculate or measure?

2.1. Calculate

Substantially more effort has gone into examining the vagaries of free T than free E_2. Although there are distinct differences in estimating free T and free E_2, the general approaches are the same and, as appropriate, we will cite papers dealing with the approach to calculating/measuring free T. To calculate free estradiol one must first have an accurate measure of total estradiol, SHBG, albumin, and the appropriate Ka's. The difficulties with, and problems of, assaying estradiol constitute a large part of this special issue and I would simply emphasize that *without a proper assay for total estradiol, there can be no accurate determination of free estradiol.* SHBG can be measured, either by its ligand binding capacity, by immunoassay or, more recently by mass spectrometry [9]. Commercial methods are available for its immunoassay. At least some of those assays have been standardized against the binding capacity of a standard SHBG preparation obtained from the WHO. There are however problems with the standard; its binding capacity is lower than the previous standards, and its Ka for the SHBG steroid interaction is also lower [10]. Further, one need be sure that the standard's binding activity is equal to its immunologic activity as the two activities must be proven to be identical and are differentially

Table 1
Variation in reported association constants between estradiol and albumin and estradiol and SHBG (adapted from [11]).

Reference SHBG	KE2 L/mol	Reference Albumin	KE2 L/mol
Anderson (1974) [24]	6.40E + 08	Anderson (1974)	5.55E + 04
Rosner and Smith (1975) [25]	2.20E + 08	Rosner and Smith (1975)	
Dunn (1981) [26]	6.80E + 08	Dunn (1981)	6.00E + 04
Moll (1981) [27]	5.00E + 08	Moll (1981)	3.50E + 04
Sodergard (1982) [8]	3.14E + 08	Sodergard (1982)	4.21E + 04
N	5	*N*	4
Mean	4.71E + 08	Mean	4.82E + 04
SD	2.00E + 08	SD	1.16E + 04
CV (%)	42.6	CV (%)	24.1

$E_2 + SHBG + Alb \leftrightarrow (E_2 \bullet SHBG) + (E_2 \bullet Alb)$

$K_{SHBG \bullet E2} = (E_2 \bullet SHBG) / (E_2)(SHBG)$; $K_{Alb \bullet E2} = (E_2 \bullet Alb) / (E_2)(Alb)$

Let X= all the E_2: $E_2 + (E_2 \bullet SHBG) + (E_2 \bullet Alb)$

Let Y = all the albumin: $Alb + (E_2 \bullet Alb)$

Let = all the SHBG: $SHBG + (E_2 \bullet SHBG)$

Then from the law of mass action the following cubic equation, given X, Y, and Z, can be solved for Free E_2 (FE_2).

$$(FE_2)^3 + (FE_2A_2)^2 + FE_2A_1 + A_0 = 0$$

FE_2 = Free Estradiol

Alb = Albumin

SHBG = Sex hormone-binding globulin

Fig. 1. Calculation of free estradiol from the Law of Mass Action without simplifying assumptions regarding the binding of E2 to albumin.

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sensitive to denaturation. Estimation of the proper association constants for E_2-SHBG and E_2-albumin are problematic (Table 1). As can be seen, there is a >3-fold difference in the highest vs. the lowest Ka for estradiol-SHBG and an almost 2-fold difference in the highest vs. the lowest Ka for estradiol–albumin. Thus, depending on the choice of Ka, widely discrepant values would be calculated. Finally, it is possible for a variety of other steroids to compete for binding with E_2. This would raise the concentration of free estradiol, but is not ordinarily taken into account in calculated values. For the most part, this does not appear to be a problem in non-pregnant women (in whom competing steroids do not circulate in sufficiently high concentrations to increase free estradiol) but can yield incorrect results in the presence of unsuspected competitors or in known situations, such as male plasma, where the concentration of T far exceeds that of estradiol and displaces it from SHBG binding sites. If competitors are present and measured, the overall binding equations can be altered and appropriate corrections can be made [11]. Despite all these caveats, calculation is the prevalent way that free E_2 is estimated. For those agreeing on the various measurements, and using the same Ka's, the results can be comparable, thus giving the false impression of "accuracy by the majority." Finally, it has been suggested that the binding of ligands to SHBG is allosteric and that the two binding sites on the homodimer do not have the same Ka. Occupancy of one of the sites on the homodimer is posited to alter the Ka of the other. Modeling based on this concept is said to result in calculated values in better agreement with careful measurements of free estradiol than previous models [12].

2.2. Measure

As stated above, free E_2 in plasma is a theoretical construct that follows from the Law of Mass Action which was formulated by chemists in the late 1800's and "explains and predicts behaviours [sic] of solutes in dynamic equilibrium." Acceptance of the Law of Mass Action does not necessarily imply that all the components of an equilibrium mixture can be accurately measured or, indeed, how it is to be done. Whatever method is used ideally should not: disturb the equilibrium that exists *in vivo*; change the environment that exists *in vivo* (ionic strength pH, etc); and be accomplished at the same temperature that exists *in vivo*. The two methods that best approximate these criteria are equilibrium dialysis and centrifugal ultrafiltration (see Table 2).

2.2.1. Equilibrium dialysis [13,14]

A detailed discussion of this methodology is set out in a recent review and is recommend to the interested reader [15]. In this method diluted plasma is placed on one side of a dialysis membrane (permeable to salt, water, and other small molecules (specifically E_2)), and a buffered aqueous salt solution on the other. The conglomerate is allowed to come to equilibrium at 37 °C and the E_2 in the aqueous solution, which is equal to the concentration of free E_2 in the plasma is measured. Until quite recently, the concentration of free estradiol was too small to be measured and a more indirect method is most commonly used. Tritiated (^{3}H) E_2 is added to the dialysis mix and, at equilibrium, is measured in the plasma and the dialysate. Because the mass of the tritiated steroid is tiny compared to the endogenous estradiol there is no disturbance of the equilibrium. The percent of free $^3HE_2$ is then multiplied by the separately determined concentration of total estradiol to arrive at the concentration of free estradiol. The major difficulties with equilibrium dialysis have been succinctly set out for T and apply equally for estradiol:

> "Although the use of equilibrium dialysis has no insuperable obstacles, it requires a high degree of technical expertise, exquisite attention to temperature control (if the temperature of the sample increases, the proportion of unbound T [or unbound E_2] will increase), and particular care in the maintenance of dialysis cells. In addition, an impure label [$^3HE_2$] results in reduced binding of SHBG, leading to a spuriously high free T [E_2] result. Moreover, the dilution factor, due to the addition of a small volume of tritiated T [E_2] to the sample (7% of the total serum volume) is not taken into account routinely in equilibrium dialysis and may be a further source of error." [16]

In spite of these difficulties, both practical and theoretical objections have been fairly addressed in the literature. Thus, equilibrium dialysis is widely acknowledged as the "gold standard." It gains this designation not because there is experimental evidence to support the designation, but because it adheres most closely to the theoretical framework of the Law of Mass Action.

2.2.2. Centrifugal ultrafiltration [17–19]

In this method undiluted plasma is placed in an apparatus which allows the plasma to be centrifuged, at a controlled temperature, in an apparatus with a semipermeable membrane at the apex of a tube which allows the collection of a small amount of ultrafiltrate. So long as the ultrafiltrate is "small" the equilibrium mixture of estradiol and its binding proteins is not disturbed and the estradiol in the ultrafiltrate represents the concentration of free E_2 in the plasma. As in equilibrium dialysis, one can either measure E_2 in the filtrate directly (when a sufficiently sensitive method is available) or add $^3HE_2$ to the plasma and make the calculation as in equilibrium dialysis.

2.2.3. Choose a method

The choice is guided by both convenience/ease and the desire to obtain the answer most likely to coincide with the true value of free E_2. Whichever method one chooses, E_2 must be accurately and reproducibly measured; this is required whether one calculates or measures. Once having conceded the ability to measure E_2, then the calculation offers the advantages of simplicity and economy. SHBG and albumin are easily measured and the calculation is automated. On the negative side: there is disagreement about the proper Ka's and SHBG is not properly standardized/harmonized. In spite of these potential hazards, a number of communications have claimed excellent agreement between calculated and measured values for free testosterone [13,16,20] and free estradiol [13]; however, a number of publications find disagreement between measured and calculated values [21–23]. In addition the calculation of free E_2 has been compared to measured values, and found to be satisfactory, in only a small sample of postmenopausal patients [13]; the authors caution against using the calculated value in other populations. Moreover, it should be recalled that E_2 is bound to SHBG 2–3-fold less tightly to SHBG than is testosterone, rendering the assumption of a fixed value for albumin unacceptable until the appropriate calculations and experiments are at hand. This is still an unsettled issue and no irreversible recommendations as to the best way to proceed can be issued at this time.

Table 2
Distribution of estradiol in the plasma of men and postmenopausal women..

	Total (pM, pg/ml)	Unbound (%, pM)	SHBG-bound (%, pM)	Albumin-bound (%, pM)
Women	92, 25	1.85, 1.70	44.4, 40.8	53.7, 49.5
Men	92, 25	2.56, 2.35	23.1, 20.3	74.2, 69.3

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