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Brief technical note

A simplified method for the calculation of unbound or free testosterone by equilibrium dialysis of undiluted plasma

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Introduction

It has been known for some time that the physiological effects of a given circulating sex steroid is not correlated with the total plasma concentration as determined by RIA but rather with that fraction of the total which is not bound to the high affinity sex hormone-binding globulin and to the low affinity serum albumin, with the suggestion that the latter may supplement the free pool of hormone within tissues because of the rapid dissociation of steroids from albumin [1].

None of the conventional methods for the determination of the steroid hormones will allow the measurement of the free fraction of steroids in blood. Several procedures have been developed for the assay of free steroids such as equilibrium dialysis, flow dialysis, ultrafiltration, etc., all employing radiometric techniques [2].

In this report we evaluate a method of equilibrium dialysis for the measurement of free testosterone in non-diluted plasma samples.

The percent free testosterone was measured in 10 healthy females, collecting samples at mid-follicular and mid-luteal phases of their menstrual cycle. For comparison, 19 samples from healthy males were also collected.

Methods

We used the technique of equilibrium dialysis described by Kley et al [3] in which dialysis tubes, 1 cm in diameter and 15 cm long ('Spectrapor Membrane', cat. no.

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8667A, Fisher Scientific Co. (Pittsburgh, PA, USA) were washed with distilled water and left overnight in phosphate buffer, pH 7.4, 0.05 mol/l. The tubes were filled with 1 ml plasma and sealed at each end by a double knot, bent into U-shape and placed in 20-ml scintillation vials with 10 ml of phosphate buffer containing about 8,000 cpm 1,2 3H(*N*)-testosterone (spec. act. 40 Ci/mmol obtained from New England Nuclear (Boston, MA, USA), and purified before use by chromatography on Sephadex-LH 20 columns). The vials were kept in a shaking water bath at 37°C for 16 h. Under these conditions, equilibrium was reached after 10 h of incubation and remained unchanged up to 24 h. In contrast to the method of calculation proposed by Kley et al [3], only samples of 1 ml from outside the tubing were taken for measurement of radioactivity and compared to 1 ml of the original buffer containing the radioactive testosterone ('before dialysis').

For comparison with the method of Kley et al [3] the filled dialysis bags were weighed exactly before and after equilibrium dialysis and radioactivity measured in 0.3-ml portions of fluid from inside the bag after dialysis. The weighing step was necessary due to the fact that a gradient of osmotic pressure exists between the non-diluted plasma within the dialysis bag and the buffer solution outside the bag with the ensuing tendency of increase in the volume inside bag after dialysis. On the other hand, the increase in volume of the plasma phase varies greatly from sample to sample and the use of a constant correction factor for the dilution effect of the volume flowing into the inner phase is inexact.

To eliminate the weighing process (a time-consuming procedure with many possibilities of error), the equilibrium dialysis with diluted plasma was performed on a trial basis to avoid significant changes in volume of the plasma inside the bag. As suggested by several investigators [4,7], plasma samples were diluted 1:5 with phosphate buffer. Additionally, we studied the effect of successive dilutions of a sample, extrapolating to the value for undiluted plasma [4]. Thus, plasma was diluted at 1:2.5, 1:5, 1:7.5, 1:10, 1:20 in phosphate buffer. One milliliter of diluted plasma was placed inside the dialysis tubing following the same procedure indicated above.

The estimation of the free or unbound steroid fraction was derived as follows: the vial in which the equilibrium dialysis is done being a closed system, the total radioactivity initially added to the system outside the dialysing tube is distributed at the end of dialysis both inside and outside the tubing. Therefore, the difference between the total radioactivity 'before dialysis' and that found outside tube after equilibrium, should be equal to that found inside the dialysis tube:

cpm/ml plasma inside dialysis tube

= (cpm/ml buffer pre-dialysis − cpm/ml outside dialysis tube)10.

Therefore

% free = (cpm/ml outside dialysis tube/cpm/ml plasma inside dialysis tube)100.

The validation of our modification was analysed using the following parameters: specificity and precision. Specificity was verified assaying the different dilutions of pooled human plasma samples. Within-assay reproducibility was evaluated by assaying two pooled human plasmas containing high and medium free testosterone levels respectively, each with 8 replicates. Between-assay precision was estimated in 6 separate assays in duplicate.

Ten women (ages 23–40 yr) with body weight within the normal range according of the Metropolitan Life Insurance tables, with regular menstrual cycles of 25–30 days were studied. Blood samples were collected early in the morning after a 12-h fast in both the first and second halves of the cycles, corresponding to the mid-follicular and mid-luteal phases, respectively, confirmed by appropriate hormonal changes. Nineteen, healthy normal males (ages ranging 20–40 yr), and normal body weights had also blood collected in the fasting state.

Results

We compared the percent free fraction (%FF) of testosterone obtained from equilibrium dialysis of diluted and undiluted plasma. Ten plasma pools were dialysed at 1 : 5 dilution without weighing the dialysing bags [4,5] and compared to the values of the same undiluted pools as obtained by the method of Kley et al [3] (Table I). The means ± SD of %FF were 1.76 ± 0.60 and 2.60 ± 0.77% for diluted and undiluted plasma, respectively. Student's paired t test indicated that the results were significantly different ($t = 3.41$ and $t_{0.05} = 2.26$).

The results of the %FF obtained from undiluted plasma were compared with those resulting from multiple dilutions of the same samples. Five pooled plasma samples were studied and the results obtained from undiluted plasma were compared to the correspondent extrapolated values (to the undiluted level) (Table II). The mean ± SD were 2.66 ± 1.31 and 2.72 ± 1.02% for the extrapolated undiluted and undiluted values, respectively. There were no significant differences as shown by the paired t test ($t = 0.30$; $t_{0.05} = 2.78$).

Comparison of %FF in undiluted plasma obtained using the calculation of Kley et al [3] and the results obtained from our modification: the comparison was done in 62 paired samples of plasma showing a mean ± SD of 2.09 ± 0.76 and 2.11 ± 0.76% for the standardized method of Kley et al [3] and with the proposed modification respectively (Fig. 1). There were no significant differences by the paired t test ($t = 1.32$; $t_{0.05} = 2.00$).

The specificity of the method, evaluated by progressive dilution of the 5 pooled plasma samples, indicated a mean correlation coefficient between dilution and %FF of 0.9967, ranging from 0.9930–0.9988.

Within-assay precision gave CV values of 6.2 and 4.6% for the pool with medium (2.70) free plasma testosterone level with Kley et al method [3] and our modification, respectively. For the plasma pool with high (3.40) mean %FF, the CV values were of the order of 8.6 and 6.3% with the Kley and our modification, respectively.

In between-assay reproducibility studies, CV values were 6.0 and 5.0% for the pool of medium (2.56) %FF using the Kley and our method respectively. For plasma

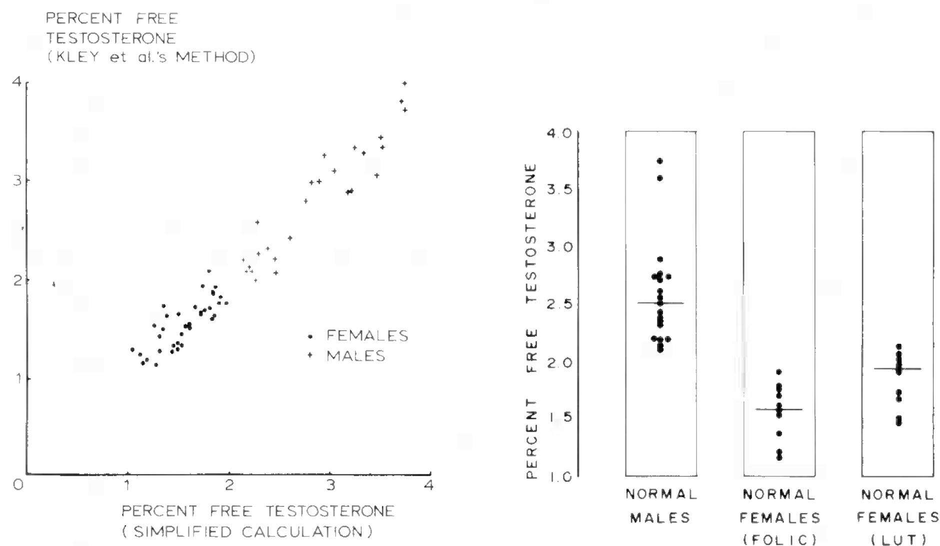

Fig. 1. Correlation between percentage of free testosterone obtained in 62 plasma samples with the proposed method of calculation and corresponding results with the method of Kley et al [3].

Fig. 2. Plasma percentage of free testosterone in 19 healthy males and 10 healthy females at the mid-follicular (FOLIC) and mid-luteal (LUT) phases of the menstrual cycle. The horizontal bars within the columns mark the median for the group.

pool with high (3.31) mean %FF the CV values were similar, 4.6%, for both methods of calculation.

In the healthy females the median (range) %FF of plasma testosterone was 1.58% (1.18–1.90%) and 1.93% (1.48–2.12%) in the mid-follicular and mid-luteal phases,

TABLE I

Percent free testosterone in 10 plasma pools measured after 1 : 5 dilution and undiluted

Free testosterone (%)	
1 : 5 Dilution	Undiluted plasma
1.44	2.21
1.38	2.03
1.34	1.88
1.20	3.15
2.04	4.32
2.21	2.51
2.20	2.50
1.02	1.71
1.80	3.12
2.98	2.71
Mean ± SD: 1.76 ± 0.60	2.61 ± 0.77
$t_{obs} = 3.41$; $t_{0.05} = 2.26$	

TABLE II

Percent free testosterone in 5 plasma pools determined after multiple dilutions and in undiluted plasma

Free testosterone (%)	
Multiple dilutions	Undiluted plasma
2.13	2.21
1.86	2.03
1.77	1.88
2.61	3.15
4.94	4.32
Mean ± SD: 2.66 ± 1.31	2.72 ± 1.02
$t_{obs} = 0.30$; $t_{0.05} = 2.78$	

respectively. In the healthy males, the median and range %FF of plasma testosterone were 2.51% and 2.11–3.75%, respectively (Fig. 2).

Discussion

Since no standard method for the measurement of the free steroid fraction is available, we chose the equilibrium dialysis technique of undiluted plasma presented by Kley et al [3] as a simple and practical method allowing direct measurements of non-protein-bound steroids under nearly physiological conditions taking into consideration the temperature in which the dialysis takes part, the small plasma dilution during the dialysing period and assuming that dissociation of the bound steroid does not take place during the procedure [3]. Furthermore, the technique does not require indirect parameters of steroid binding in plasma.

However, weighing of the dialysis bags in the calculation as proposed by Kley et al [3] is not only time-consuming but variable weight changes occur during the weighing process. We attempted to use plasma diluted 1 : 5 and this showed no detectable change in volume inside the bag [4]. However, the results were significantly lower than those obtained with the same undiluted samples. Multiple dilutions of the same plasma samples and extrapolation to the undiluted plasma value gave results similar to those using the Kley et al method [3] directly on the undiluted sample. The multiple dilution method is, however, too laborious for routine use in a clinical laboratory.

Since similar results were obtained from equilibrium dialysis with and without weight correction using our method of calculation, it was possible to eliminate this step allowing the performance of many samples in each assay. In effect, the within-assay CV was lower with our method of obtaining the %FF testosterone than using the Kley method of calculation [3]. For between-assay the CV values were either slightly lower (medium %FF) or the same (high %FF) with the proposed modification.

Contrary to the results described by Motohashi et al [6] using a technique similar to ours, we found an increased %FF of testosterone in the mid-luteal phase in

comparison to the mid-follicular phase. It has been suggested that this is related to progesterone displacement of testosterone from albumin [7]. The results in males were manifestly different and expectedly so in view of the great difference in total testosterone concentration.

There is no explanation for the discrepancy between our findings and those of Motohashi et al [6] except perhaps for the way in which the free fraction was calculated, a point which is not specified in their publication.

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