Evaluation of Assays Available to Measure Free Testosterone

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This study compared the results of various testosterone assays in a cross-sectional study of 50 male subjects age 28 to 90 years. The purpose of the study was to determine the relationship of the various testosterone assays to one another. In addition, we determined week-to-week variability in testosterone and bioavailable testosterone in 16 subjects. The following assays were undertaken: total testosterone (T), free testosterone by equilibrium dialysis (FT_D), bioavailable testosterone (BT), free testosterone by ultracentrifugation (FT_U), and direct estimation of serum free T by an analog ligand radioimmunoassay (FT_A). In addition, using total T and sex hormone-binding globulin (SHBG), we calculated the free androgen index (FAI = T/SHBG) and the free testosterone index (FTI) using the method of Vermeulen. In a second study, we measured the week-to-week variation in T and BT in a group of older males. Lastly, we demonstrated excellent stability of serum stored at –70°C for up to 7 years for T and BT. Correlations of the various assays to increasing age were significant for all assays except total T (r = -.126). The best correlation was found with BT (r = -.744, P < .001). All measures were statistically significantly correlated with FT. The best correlations were FTI (r = .807, P < .007) and BT (r = .670, P < .001). If T was used to classify hypogonadism in comparison to BT, it resulted in misclassification in 42% of cases. In addition, we demonstrated a marked week-to-week variability in T and BT in older men with the T and BT being in the eugonadal range some weeks and hypogonadal on other occasions. This occurred in 8 of 16 men for T and 10 of 16 men for BT. Based on these data, we suggest that the FTI or BT are the most practical methods to determine hypogonadism. There is a need for increased awareness that marked week-to-week variability within a single individual can occur for measurements of both T and BT.

OVER THE LAST decade, it has become clear that the assays available to measure the tissue available amounts of circulating testosterone compared with total testosterone produce discrepant results regarding the percentage of the population that is hypogonadal. This is particularly true in older males in whom sex hormone-binding globulin (SHBG) tends to increase with age.[1] In older men, it has been suggested that bioavailable testosterone (free and albumin-bound testosterone) may be a better measure of hypogonadism and correlate better with clinical symptoms than does total testosterone.[2-6] While the terms bioavailable or tissue-available testosterone have become most commonly used, the term non-SHBG is more accurate, because the role of SHBG in testosterone "bioavailability" in all target tissues remains controversial.

At present, a number of assays are available to measure circulating levels of testosterone. These include total testosterone (T), free testosterone (by dialysis and by ultracentrifugation), bioavailable testosterone (BT), direct measurement of free testosterone by an analog immunoassay and 2 calculated free testosterone indices viz free androgen index (FAI), and the calculated free testosterone index (FTI). Measurement of free testosterone by equilibrium dialysis (FT_D at 37°C has been considered to be the best physiologic method for the estimation of free testosterone.[7] It is, however, a time-consuming procedure and thus methods that are equally exact, but less laborious, would be preferable for clinical use.

In the study reported here, we have compared the results of the different available testosterone assays in a cross-sectional study over the lifespan in males. We have performed these assays on the same single serum specimens in order to examine systematic differences in assay techniques for tissue-available testosterone. For the reason stated above, we elected to use the FT_D as the "gold standard". We have also examined serum testosterone variation using a single assay system for samples at different times from the same individual. The purpose of the study was to allow clinicians to make a logical, data-driven choice of the most appropriate approach to measure gonadal status. This represents the most comprehensive direct comparison of assays to be published.

METHODS

Fifty male subjects age 28 to 90 years, whose blood had been drawn for previous studies on gonadal function from healthy ambulant men and stored in a –70°C freezer, were studied. Samples were drawn between 8:00 AM and 10:00 AM. At the time of blood draw, none of the subjects were known to be hypogonadal. None had disease or took medications that produce a decline in testosterone. None had an elevated luteinizing hormone level. No exclusion was made based on the serum SHBG level.

Each sample had the following assays completed on it. Total serum testosterone (T) was measured by radioimmunoassay using a commercially available kit according to manufacturer's instructions (DPC, Los Angeles, CA). Recovery experiments using tritiated testosterone showed a recovery of 89% to 112% of testosterone (n = 9). Serum SHBG assay was measured by radioimmunoassay using a commercially available kit according to manufacturer's instructions (Endocrine Sciences, Calabassas Hills, CA). Endocrine Sciences measured FT_D. BT was measured by the ammonium sulfate precipitation method as previously reported by us[4,6] using a commercially available radioimmunoassay kit (DPC). The lower limit of normal for BT is 70 ng/dL (2.1 nmol/L). For conversion from ng/dL to nmol/L, divide by 28.84. Free testosterone by ultracentrifugation (FT_U) was measured using the

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Table 1. Intra- and Interassay Precision of the Various T Assays

Method	Interassay Coefficient of Variation (%)	Interassay Coefficient of Variation (%)	Normal Range*
T†	6.7	7.7	300–1,266 ng/dL (n = 20)
SHBG	6.7	8.2	0.5–1.5 ng/dL (n = 89)
FT_D	6.8	10.0	52–280 pg/mL (n = 66)
FT_A	6.3	6.5	12.4–40 pg/mL (n = 48)
FT_U	8.9	10.3	17–99 pg/mL (n = 20)
BT	7.9	7.2	70–320 ng/dL (n = 20)

*Normal ranges were calculated from healthy young men age 21 to 45 years.

†Calculated from values of 70, 312, and 1,069 ng/dL and meaned. Values from 10 separate assays.

method of Ekins[8] (with reagents obtained from Pantex, Santa Monica, CA).

Briefly, samples were centrifuged in an anglehead centrifuge at 2,500 RLF × *g*, at room temperature, for 30 minutes. An ultrafiltration membrane ws used. A total of 100 μL of ultrafiltrated testosterone tracer and rabbit-produced first antiserum was mixed and left at 37°C for 3 hours. Then 500 μL of second antiserum was added, the tube mixed, and left at room temperature for 10 minutes. Tubes were then centrifuged at 2,500 RCF × *g* for 15 minutes, the supernatant discarded, and the tubes counted.

Direct estimation of serum free T by an analog ligand radioimmunoassay (FT_A) was measured using a commercially available kit according to manufacturer's instructions (Coat-A-Count; DPC, Los Angeles, CA). The intra- and interassay precision of these assays is given in Table 1. With the exception of the FT_D, all other assays were undertaken by 1 technician.

FAI was calculated from total T and SHBG in which the FAI = (100 T/SHBG).[9] The FTI was calculated from SHBG and T using the method of Vermeulen et al[7] and using a computer program supplied by Dr Jean M. Kaufman, University Hospital Ghent, Ghent, Belgium.

In a second study, as part of a double-blind, placebo-controlled study of saw palmetto extract, we measured week-to-week variability in T and total BT in 16 men (age, 69.3 ± 1.7 years) receiving placebo. No values in persons receiving saw palmetto were included. Blood samples were obtained at 0, 1, 2, 4, 6, and 8 weeks. All samples were obtained between 8:00 AM to 10:00 AM. All samples from a single individual were measured in the same assay. The Human Subjects Committee of the St. Louis VA Medical Center approved these studies.

Stability of BT and T stored at –70°C was determined in 10 subjects whose specimens were first analyzed 3 years prior to re-analysis. Correlation of results was estimated using the methods of least square regression.

RESULTS

Comparison of the Various Testosterone Measures

Comparison of the measures of T and FT_D is shown in Fig 1. All measures were statistically significant, with the best correlation being with FTI ($r = .807$, $P < .001$) and the second best being with BT ($r = .670$, $P < .001$). Comparison of the measures of T and BT are shown in Fig 2. BT correlated best with FTI ($r = .817$), $P < .001$).

To determine the utility of total T in identifying hypogonadism, we compared the misclassification, ie, the number of false positives and false negatives, produced by T compared with BT and FT_D (Table 2). A cut off of 300 ng/dL for T was first selected to define hypogonadism. Using total T, 42% of patients would have been misclassified. A total of 26% with normal total T were hypogonadal by BT and 16% of those hypogonadal by T (using 300 ng/dL (10.4 nmol/L) as the lower limit of normal), were normal by BT. While lowering the definition of hypogonadal by total T from a level of 300 ng/dL to 280 ng/dL decreased the false positives, it increased the false negatives. Similar levels of false positives and false negatives were seen with the comparison between total T and FT_D. When FT_D was compared with BT, the false positive level was 6% and false negative was 30%.

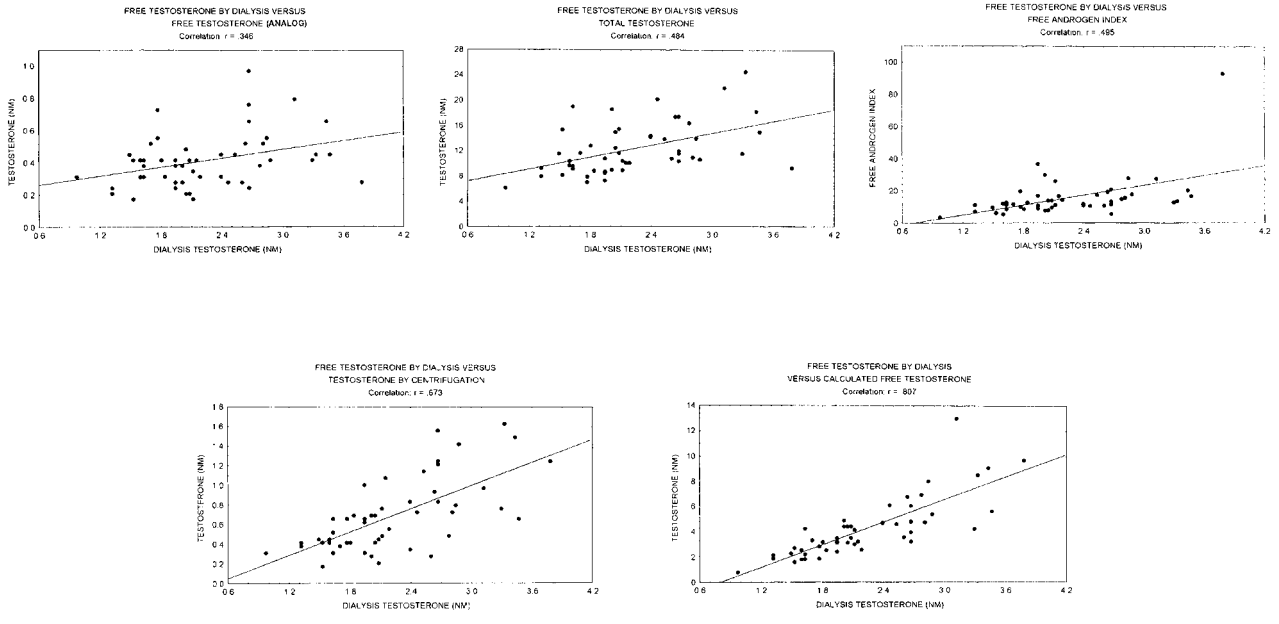

Fig 1. Comparison of T assays to free T by dialysis.

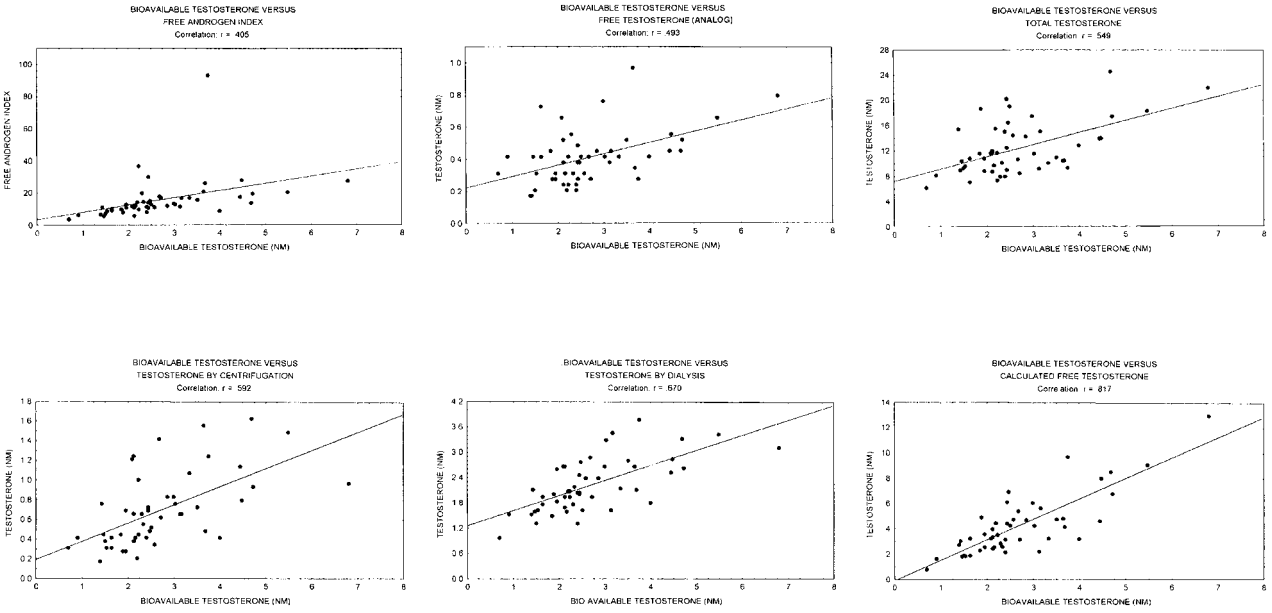

Fig 2. Comparison of T assays to BT.

Age and Testosterone Estimates

The correlation between increasing age and the different testosterone estimates is shown in Fig 3. The best correlation was found with BT (r = -.744, $P < .001$). All the correlations were significant except for total T (r = -.126).

Week-to-Week Variability in T and BT

Figures 4A and B and 5A and B demonstrate the week-to-week variability in total T and BT. In the case of total T over the 8-week period, 8 of the 16 men would have been considered hypogonadal at 1 time point in the study and eugonadal at a second point. For BT, 10 of the 16 men would have been considered hypogonadal at 1 time point in the study and eugonadal at a second point.

Stability of BT Stored at –70°C

The correlation between the original T level and one measured 3 years later was 0.9312. The correlation between the original BT level and one measured 3 years later was 0.9019.

Table 2. Prevalence of Low and Normal Values of Non-SHBG T (BT) and Free T by Equilibrium Dialysis (FT_D) at Various Levels of Total T

	BT		FT_D	
Total T, mg/dL (nmol/L)*	False Positive† (%)	False Negative‡ (%)	False Positive† (%)	False Negative‡ (%)
300 (10.4)	16	26	18	10
280 (9.7)	12	28	14	12
250 (8.7)	6	42	6	22

*Values equal to or less than.

†False positive represents the number of persons hypogonadal by T who were eugonadal by BT or FT_D.

‡False negative represents the number of persons eugonadal by T who were hypogonadal by BT for FT_D.

DISCUSSION

The studies reported here support the concept that some measure of free T (FT_D, FT_U, or FTI) or BT should be used to identify hypogonadism in men across the lifespan. Total T misclassifies hypogonadism in a third of cases and thus seems an inappropriate measure to use. In view of the large week-to-week variability in BT, it would seem reasonable to get a second level 1 to 2 weeks apart in a male with hypogonadal symptoms who has a normal value on the first measurement.

A simple alternative to these assays could be the analog ligand immunoassay procedure (FT_A). However, while FT_A correlates with free T and BT measurements, it has been previously shown not to be a reliable measure of FT_D, representing a variable fraction (20% to 60%) of FT_D.[7,10,11] In addition, the FT/FT_D ratio is SHBG-dependent.[7,12]

FAI, while correlated with FT_D, also is a problematic measure of true free T. Both Vermeulen et al[7] and Kapoor et al[9] found that the ratio of FAI/FT_D will be high when the number of occupied binding sites is small related to the SHBG capacity, and conversely, will be low when a substantial number of binding sites are occupied. Therefore, FAI does not appear to be a valid assay for adult males.

As found by Vermeulen et al,[7] we found excellent correspondence between FTI and FT_D and BT. This suggests that FTI is a reliable index of unbound T. We agree with Vermeulen et al[7] who stated that "calculation of FT from total T and immunoassayable SHBG represents a simple method . . . (that) yielded values very close, if not identical to those obtained by equilibrium dialysis". This value may be less accurate at very low albumin levels.

BT has been suggested to be the assay of choice in older persons in which SHBG increases and substantial variation of albumin levels may occur.[13] This is supported by the

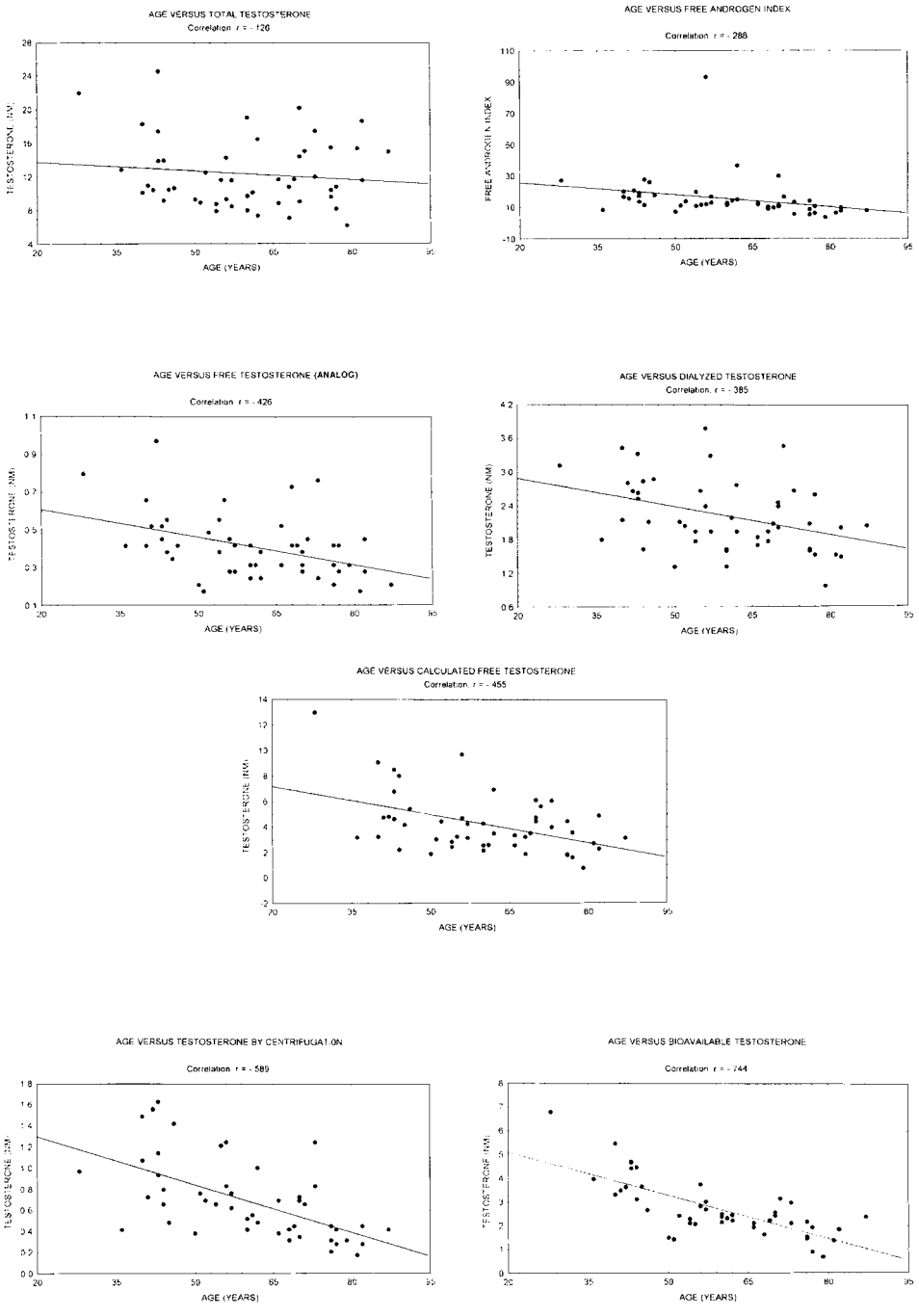

Fig 3. Effect of age on T levels using various assays.

evidence that at least part of the albumin bound T is bioavailable.[14] This study supports the utilization of the ammonium sulfate precipitation technique for measuring BT, a relatively simple assay, as a useful measure of circulating T levels. A number of previous studies have found a decline in BT with aging.[15-24] When BT levels are measured, more older subjects are classified as hypogonadal than when total T is used.[15,17] This would also appear to be the case when free T by dialysis levels are compared with total T levels. Studies have demonstrated an association between BT levels and libido,[25] mood,[26,27] memory,[28] coronary artery disease,[29,30] bone mineral density,[31] muscle strength,[32] and function.[32] T replacement studies using a low BT as an entry criteria have demonstrated a decrease in leptin,[33] increase in upper arm muscle strength,[33,34] and increased bone mineral density.[35]

Previous studies have suggested that with aging there is a marked decline in the circadian variation of T.[36] This has led to the suggestion that in older persons a single BT sample is sufficient to make the diagnosis of hypogonadism. However,

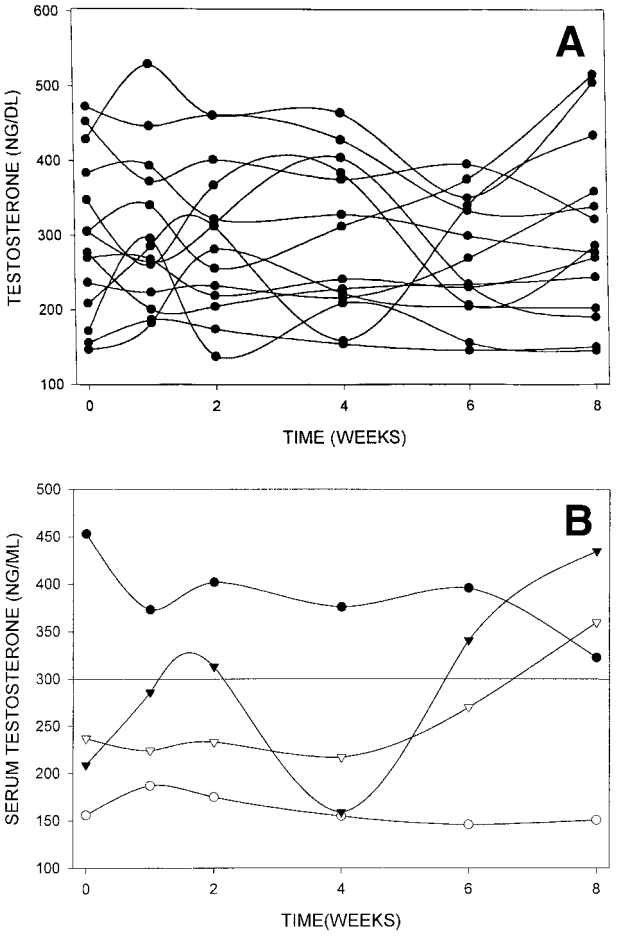

Fig 4. Weekly variability of T. (A) All subjects and (B) examples of the different response patterns.

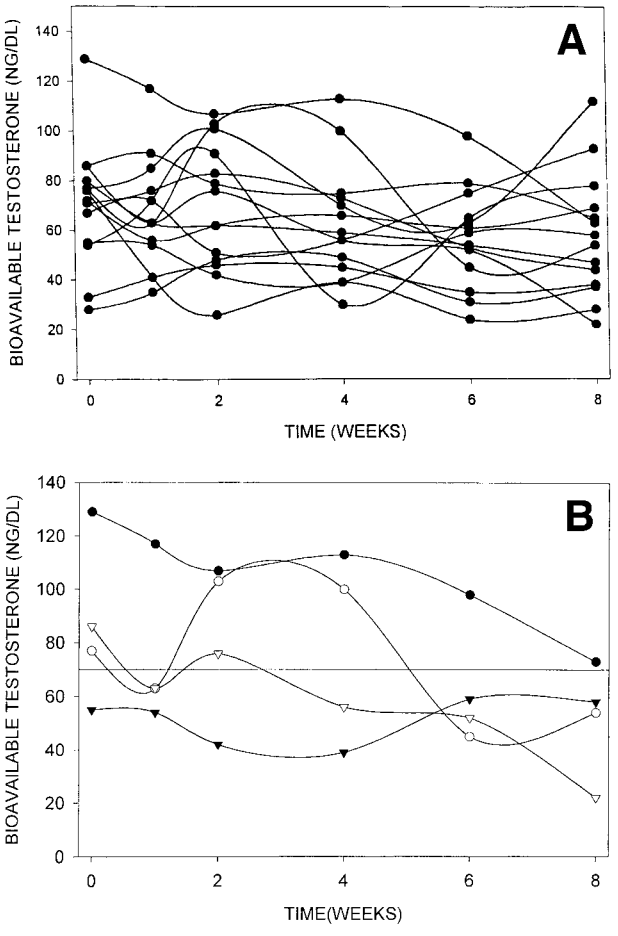

Fig 5. Weekly variability of BT. (A) All subjects and (B) examples of the different response patterns.

the marked week-to-week variability in both T and BT concentrations suggest the need to obtain a second sample at least a week apart in an older person who has symptoms of hypogonadism. Vermeulen and Verdonck[37] studied 169 males aged 40 to 80 years who had T levels measured 8 times over a 50-week period. The mean coefficient of variation for T over the year was 16.9% ± 8.4%, with 9 subjects having a variability of over 25%. As the initial sample was highly correlated to the mean, they concluded a single level was adequate for epidemiologic purposes. Our study clearly suggests that this is not the case for making the diagnosis of hypogonadism in an individual. Recently, we have developed and validated a simple 10-symptom questionnaire for androgen deficiency in aging males that has excellent sensitivity and specificity and may be useful in identifying males in need of hormonal measurements for hypogonadism.[6]

It should be noted that the development of the normal controls, which was performed separately, compared different number of samples ranging from 20 to 66. This unequal number of samples may introduce bias into the results.

In conclusion, this study has identified a number of assays that appear to be appropriate measurements of circulating T viz FT_D, FT_U, FTI, and BT. Of these, FTI and BT appear to be the simplest assays to perform. BT would appear to be the approach of choice in which marked variability in albumin can be predicted to occur, such as in the older male with concomitant illnesses. In addition, marked week-to-week variability of T and BT in older males occurs. This utility of a single value in making the diagnosis of T deficiency is problematic.

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