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Detection of anabolic steroid administration: ratio of urinary testosterone to epitestosterone vs the ratio of urinary testosterone to luteinizing hormone

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Our goal in this study was to determine whether the urinary ratio of testosterone to luteinizing hormone (T/LH) as an indicator of exogenous anabolic steroid (AS) use is superior to the urinary ratio of testosterone to epitestosterone (T/E). After 2 weekly placebo injections, 19 subjects were given testosterone cypionate (TC) injections of 250 or 500 mg/week for 14 weeks followed by 14 weekly placebo injections. Patients were considered to have ceased taking TC if they tested negative 9 weeks after their last injection. For detection of illicit or supraphysiological TC (AS) use, the urinary T/E ratio of ≥6 yielded a false-negative rate of 46% and a false-positive rate of 4%. However, a urinary T/LH ratio of ≥30 produced a false-negative rate of only 24% and a false-positive rate of 13%. We conclude that the urinary T/LH ratio of ≥30 is a more sensitive marker of AS use than the urinary T/E ratio of ≥6 and remains sensitive for twice as long as urinary T/E.

INDEXING TERMS: abused drugs • sports medicine • GC-MS • androgens • anabolic steroids

The primary method for detecting illicit anabolic steroid (AS) use has been the analysis of urinary steroids.[4] This methodology has been successful for the majority of steroids, especially the synthetic variety that have specific structures that are easily identified by GC-MS. However, the detection and monitoring of anabolic compounds is not fail-safe. Detection of the illicit use of testosterone (T), a naturally occurring AS, has become a difficult clinical problem. Methods for detecting administration of exogenous T depend on distortions of the normal hormone profile in the user's urine *[1]*. Attempts to identify optimal markers of exogenous T administration from untimed urine samples in male athletes have uncovered several compounds as possible indicators of T abuse. In 1982, the ratio of androgen glucuronides to epitestosterone (E; 17α-hydroxy-4-androsten-3-one) was adopted by the Medical Commission of the International Olympic Committee (IOC) in Los Angeles, with a cutoff point ≥6 being the sole test for illicit T self-administration *[2, 3]*; the expected urinary ratio of T/E among healthy subjects not using AS is ~1 *[1]*. However, analyses from all IOC-accredited laboratories in 1991 suggested that the majority of athletes who were using AS had switched from synthetic compounds to T pharmaceuticals to evade detection *[4]*. Consequently, covert AS use has become more difficult to detect.

The overall incidence of urinary T/E ≥6 in the general population of healthy males not abusing steroids is <0.8%, as evaluated by Catlin and Hatton *[5]* and confirmed by Dehennin *[4]*. In general, the increase of the T/E ratio after high-dose T administration results from increased T excretion and a decrease of E output *[6]*. However, some athletes have produced false-positives, i.e., T/E ratios ≥6 with subsequent verification that no exogenous T had been administered *[7]*. Dehennin and Matsumoto *[6]* indicated that this problem could be reduced by taking into account the sulfate excretions of E (ES) in the ratio T/(ES + E), the relevant threshold being 2.85. Accordingly, Dehennin *[4]* suggested that using a

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[4] Nonstandard abbreviations: AS, anabolic steroid(s); T, testosterone; E, epitestosterone; IOC, International Olympics Committee; ES, epitestosterone sulfate; LH, luteinizing hormone; and TC, testosterone cypionate.

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T/(ES + E) ratio of ≥3.0 would be a more sensitive marker of covert T use.

Dehennin *[7]* also noted that the joint misuse of T and E could also lead to false-negative test results, and the IOC in 1992 recommended that urinary E concentrations >150 μg/L should be noted as abnormally high and therefore suspicious. False-negative results can also be produced by stimulation of testicular steroidogenesis by administering human chorionic gonadotropin, which would result in a concomitant increase in the urinary excretion rate of T and E but with no significant change in the T/E ratio *[1]*. Dehennin and Matsumoto *[6]* confirmed earlier reports of false negatives by demonstrating that, despite their determination that an average dose of 47 mg of exogenous testosterone per week would equal or exceed the IOC cutoff, 2 of 9 subjects receiving 72 mg of testosterone (100 mg of testosterone enanthate) per week for 6 months did not produce a T/E ratio ≥6.

Dehennin *[4]* suggested that when a T/E ratio of 6 to 12 is found for the first time in subjects for whom no documentation of a previously normal ratio exists, some complimentary criteria should be examined. He found that the ratio of urinary T and E glucuronides to 5-androstene-3β,17α-diol glucuronide was increased in the use of exogenous T and E use despite the T/E ratio being <6. These findings indicate a need for further study of additional markers for detecting the administration of T.

Because the secretion of T is under the control of luteinizing hormone (LH), Brooks et al. *[8]* suggested that the urinary T/LH ratio might be a potentially useful marker for detecting administration of exogenous T. Kicman et al. *[9]* observed that high-dose T administration resulted in dose-dependent suppression of both serum and urinary LH. This was confirmed by Matsumoto *[2]*, who found that the urinary LH excretion was reduced to a lesser extent than was the decrease in both E and T conjugates, such that the T/LH values were lower than those reported by Kicman et al.—also suggesting a need for more study.

Palonek et al. *[3]* reported significantly increased T/LH ratios in 11 healthy sedentary men participating in a WHO investigational program for male contraception. Each subject received 144 mg of T per week (200 mg of testosterone enanthate) for 9 months. The T/LH ratio increased from a mean of 0.052 (range 0.002–0.108) at baseline to 45.16 (1.28–252) at 3 months, 85.7 (8.3–238) at 6 months, and 71.7 (5.3–344) at 9 months. The authors indicated that, among all the different ratios or proposed markers they investigated, the urinary T/LH ratio showed the most dramatic increase (~1000-fold). Of the other markers, the increase in the serum T/LH ratio was of similar magnitude as that of the urinary T/LH ratio, whereas the urinary T/E ratio had only a 50-fold increase. The investigators also reported that 1 of the 11 subjects produced a T/E ratio below the IOC cutoff at 3 and 9 months of administration and just over the threshold at 6 months. The T/LH ratio for the same subject was above the upper reference limit at 3, 6, and 9 months. Palonek et al. concluded that increased serum and urinary T/LH ratios in the presence of a normal T/E ratio may indicate self-administration of both T and E.

Unanswered is whether the T/LH ratio might be more sensitive than the T/E ratio for identifying illicit use of AS. Thus the goal of the present study was to determine which laboratory test is most sensitive and specific for detecting the administration of exogenous T.

Materials and Methods

SUBJECTS AND STUDY DESIGN

Healthy male volunteers between ages 18 and 40 years were recruited and, after an explanation of the study, gave their signed informed consent. The study protocol was reviewed and approved by the Human Subjects Institutional Review Board and the Clinic Research Center of the University of Iowa. A standard drug history, developed by the National Institute on Drug Abuse, was administered before entry. Any subject who indicated he was currently using central nervous system stimulants other than modest amounts of caffeine (two cups of coffee per day) was excluded from the study.

Each subject received two weekly placebo doses of cottonseed oil, the vehicle for testosterone cypionate (TC). At the end of the 2-week placebo lead-in period, subjects were randomized to one of three doses of TC (100, 250, or 500 mg/week) given for 14 consecutive weeks. In our experience with AS users *[10]*, the subjects' shortest average cycle was 7 weeks, the longest 14 weeks. Thus, we decided that subjects should be administered TC for a typical 14-week AS cycle to mimic the maximum cycling interval.

Subjects functioned as their own controls. They received weekly intramuscular injections of either TC or placebo (vehicle only) for 28 consecutive weeks. For the purpose of evaluating the effectiveness of the urine T/E ratio as an indicator of recent AS use, we considered only the subjects receiving supraphysiological TC doses (250 and 500 mg/week). A 100 mg/week dose is generally regarded as a physiological replacement dose in the majority of patients.

To monitor the subjects medically, we assessed their liver-function tests, fasting lipid profiles, thyroxine-binding globulin, sex-hormone-binding globulin, 24-h urinary free cortisols, serum free and total T, estradiol, LH, follicle-stimulating hormone, thyroid-stimulating hormone, and free thyroxine—obtained at baseline, at entry into the study, after the 2-week placebo injection period, and then biweekly for the remainder of the study. All endocrine samples were collected between 0700 and 0900 to minimize the chronotropic secretion effects of these hormones. Depo®-testosterone (TC), 200 g/L (200 mg/mL), was the proprietary product utilized for the study. The diluent (0.2 mL of benzyl benzoate, 9.45 mg of benzyl alcohol, and 560 mg of cottonseed oil per milliliter) was prepared by the Pharmaceutical Services Division of the

University of Iowa College of Pharmacy (an FDA-approved manufacturing group). At the end of the 14 weeks of TC administration, the subjects were switched to diluent-only injections.

ASSAYS

The urine drug screens were performed by Smith Kline Beecham Clinical Laboratories Sports Testing Center in Tucker, GA, a laboratory certified by the US Department of Health and Human Services. The initial drug screen and all subsequent screens were negative for AS (other than T), amphetamines, barbiturates, benzodiazepines, cocaine metabolites, methadone, methaqualone, opiates, phencyclidine, and propoxyphene.

Urine concentrations of T, E, LH, and creatinine were also determined in the samples (assayed by Smith Kline Beecham). The urine samples were refrigerated at 8 °C and were analyzed within 5–10 days after collection. If the T/E ratio was <6, the sample was discarded within 30 days. Urine drug screens were routinely obtained at weeks 0, 1, 4, 8, 12, 16 or 17, 20, 24, and 28; in some follow-up cases, they were obtained at weeks 40 and 92. AS screens and confirmations were performed by GC-MS on separate aliquots. Samples were initially screened for the substance abuse panel by Emit (Behring, Palo Alto, CA); all positive results were confirmed by GC-MS *[11]*. LH in urine was performed by Microparticle Enzyme Immunoassay with the Abbott Diagnostics (Chicago, IL) IMx system.

The T/E ratio was determined by GC-MS. Both free and conjugated T and E were extracted with C_{18} solid-phase extraction columns (Bond Elute LRC; Varian, Harbor City, CA), hydrolyzed with β-glucuronidase (Boehringer Mannheim, Mannheim, Germany), and detected by monitoring characteristic ions with the mass spectrometer. Quantification and identification of T and E required selected-ion mode analysis in which the presumptive positive specimens were matched with the retention times and ion ratios of known compounds. The T calibration curve was linear between 2 and 400 μg/L; that for E was linear between 2 and 500 μg/L. The CV for the T/E ratio was 13.3%. The specificity of this method is extremely high: At the time of the performance of the assays, no compounds were known to interfere with either T or E.

The urine concentration of LH was determined with the IMx system kit for serum LH as described in the 1991 IMx LH package insert. To determine that there was no matrix effect for the assay, we added known amounts LH to urine and serum samples and found that the resulting calibration curves could be superimposed on each other and were linear between 2 and 600 IU/L. The lower limit of detection for this assay is 0.5 IU/L. The CV for the serum LH assay is 8.7% at 5.37 IU/L, 6.4% at 43.2 IU/L, and 6.2% at 82.5 IU/L.

All serum samples for determining free and total T were stored at −20 °C until assay. The T concentrations were quantified with Coat-A-Count® kits (Diagnostic Products Corp., Los Angeles, CA) as described in the manufacturer's package insert (1995). The lower limits of detection were 40 ng/L for total T and 0.15 ng/L for free T. The inter- and intraassay CVs for the free T assay were 11.2% and 5.5%, respectively; those for total T were 10.4% and 8.8%, respectively.

Results

In all, 93 urine drug screen samples were obtained from the 19 subjects participating in the study who received supraphysiological doses of TC. Seven received 250 mg/week and 12 took 500 mg/week. None of the subjects was positive for exogenous AS use other than for the TC injections administered during weeks 2–15 of the study. Concentrations of free T in serum were analyzed 5–7 times between days 3 and 21 after the last TC injection for 17 of the 19 subjects. From these data, we calculated for each patient the terminal elimination rate (k_e) and the elimination half-life ($t_{1/2}$) for free T in serum. To determine k_e, we fit the T concentrations $f(t)$ and time points (t) to the following single exponential decay equation, where a is the concentration of T at time 0:

$$f(t) = ae^{-k_e t} \quad (1)$$

We determined $t_{1/2}$ as follows:

$$t_{1/2} = 0.693/k_e \quad (2)$$

A 21-day T sampling period was appropriate to determine the $t_{1/2}$ of exogenous T because gonadotropin-releasing-hormone stimulation tests indicated that the hypothalamic–pituitary–testicular axes of the subjects did not regain sufficient sensitivity to stimulate release of T until 4–6 weeks after discontinuation of the TC injections.

Table 1. Elimination half-life of free testosterone in 19 subjects.

Subject	TC dose, mg/week	Free T elimination half-life, days
2455	250	8.2
2908	250	6.0
3058	250	6.4
3626	250	4.8
5361	250	6.4
9298	250	6.4
0030	250	14.1
1078	500	6.0
2166	500	5.0
3415	500	7.3
4008	500	7.8
4045	500	4.9
4249	500	4.8
5340	500	7.4
6218	500	Not available
6534	500	6.8
9338	500	5.5
0153	500	4.2
0012	500	Not available

The individual elimination half-life data are presented in Table 1. There was no difference in half-life values between the weekly TC doses of 250 and 500 mg (Mann–Whitney $U = 25.0$, $P = 0.37$). The overall mean ± SD elimination half-life for free T in serum after administration of TC was 6.6 ± 2.3 days. Based on these data, an 11-day $t_{1/2}$ would be 2 SD from the mean. Given that 97% of the exogenous T was excreted in 5 half-lives (i.e., 11-day half-life × 5 half-lives = 55 days, or 8 weeks) and that pituitary sensitivity to gonadotropin-releasing hormone returned within 4–6 weeks of the last TC injection, subjects were assumed to have ceased taking ("be off") exogenous T by the time of the urine drug screen performed 9 weeks after the last TC injection.

The AS urine drug screen findings indicated that the urinary T/E ratio cutoff of ≥6, the traditional laboratory marker to determine the use of exogenous T and used as such by the National Collegiate Athletic Association and the IOC, although quite specific for determining nonuse of T, is not a sensitive indicator for detecting illicit T usage. Table 2 illustrates this. Although the T/E ratio of >6 had 96% specificity in identifying our subjects as being off steroids by 9 weeks after their last dose, it was correct only 54% of the time for identifying our subjects as being on steroids during the 14 weeks of TC injections and in the 9 subsequent weeks when they received sham injections. As a practical matter, these data suggest that one of every two subjects using injectable TC will, both during injection periods and for 9 weeks afterwards, give a false-negative urine drug screen. Receiver operating characteristic (ROC) analysis of these data *[12]* identified a urinary T/E ratio of ≥1.2 as the cutoff value that provided optimum sensitivity and specificity for indicating use or nonuse of T. Resorting the data in Table 2 illustrates that use of a T/E ratio of ≥1.2 for a T-positive urine improves the sensitivity to 83% and the specificity decreases only somewhat, to 77%.

The potential usefulness of the urine T/LH ratio as an indicator of T use and nonuse is illustrated in Table 3. These data suggest that to maintain 100% specificity requires a threshold T/LH ratio of ≥74, although the sensitivity at this cutoff is only 52%. However, by ROC analysis (data not shown), the urinary T/LH ratio cutpoint that optimizes sensitivity and specificity is ≥30. As Table 3 shows, use of a urinary T/LH ratio ≥30 increases sensitivity to 76% but decreases specificity to 87%.

Table 2. Contingency table for various urinary T/E ratios used as the threshold ratio for anabolic steroid use (TC 250 or 500 mg/week).

	No. of subjects	
T/E ratio	On TC	Off TC[a]
≥6[b]	25	2
<6[b]	21	45
≥1.2[c]	38	11
<1.2[c]	8	36

[a] Subjects who tested negative 9 weeks after their last TC injection.
[b] $\chi^2 = 28.312$, $P < 0.0001$, sensitivity = 54%, specificity = 96%.
[c] $\chi^2 = 32.689$, $P < 0.0001$, sensitivity = 83%, specificity = 77%.

Table 3. Contingency table for urinary T/LH ratios used as the threshold ratio for anabolic steroid use (TC 250 or 500 mg/week).

	No. of subjects	
T/LH ratio	On TC	Off TC[a]
≥74[b]	24	0
<74[b]	22	47
≥30[c]	35	6
<30[c]	11	41

[a] Subjects who tested negative 9 weeks after their last TC injection.
[b] $\chi^2 = 33.051$, $P < 0.0001$, sensitivity = 52%, specificity = 100%.
[c] $\chi^2 = 37.814$, $P < 0.0001$, sensitivity = 76%, and specificity = 87%.

Discussion

From a medical-legal standpoint the most worrisome finding of these data is the false-positive tests. Table 4 characterizes the false positives—i.e., a test result that is not negative at 9 weeks after the last TC injection—for the various testing schemes. Nine weeks is equivalent to the amount of time required to clear 97% of the exogenous TC. For the urinary T/E ratio of 6, only two subjects did not meet this criteria, whereas for the urine T/LH ratio of 30, four subjects did not meet this criteria. All six patients who tested "positive" were actually tested 9–25 weeks after their last TC injection. When contrasted with the half-life data, this suggests that the normalization of LH concentrations may lag behind the rate at which the exogenous T clears from the body. Moreover, in reality there are no truely false-positive test results. However, in no case did a subject's urine screen test positive before the start of the TC injections.

The mean ± SD urinary T/LH and T/E ratios before the start of the TC injections were 3.8 ± 2.4 and 0.8 ± 1.3, respectively. Other than when the subjects were receiving T injections, the only time there was a significant difference between pre-TC injection urinary T/E and T/LH ratios and the ratios after the start of the TC injections was 2 weeks after the last injection. For the urinary T/LH ratio, the mean difference between the baseline value and the ratio 2 weeks after the last injection was 29.8 ($t = 2.829$, $P < 0.02$, $df = 16$); for the urine T/E ratio, the mean difference was 14.9 ($t = 2.703$, $P < 0.02$, $df = 16$).

As suggested in Table 4, the urinary T/LH ratio of ≥30 is the screen most likely to detect AS use the longest, i.e., as long as 25 weeks after the last injection. Using the urinary T/LH ratio ≥30 as a marker showed that 4 of 19 (21%) subjects tested positive 9–25 weeks after their last injection of T. In contrast, use of the urinary T/E ratio ≥6 found only 2 of 19 (11%) patients positive for steroid usage at 9 weeks after their last T injection. Fig. 1 chronologically contrasts the mean urine T/LH and T/E ratios. Inspection of Fig. 1 suggests that the urinary T/LH

Table 4. False-positive rates for use of supraphysiological doses of anabolic steroids in different urine testing schemes.

	False-positive rates		Subject description		
Test cutoff	%	No. of subjects	TC dose, mg/week	No. of weeks since last inj.	Ratio
T/E ≥6	4	2	500	9	7.3
			500	9	6.5
T/LH ≥30	13	4	500	9	73
			500	9 & 13[a]	65 & 35[a]
			250	13	64
			250	13 & 25[a]	47 & 34[a]

[a] Same subject, two times.

ratio returns to baseline at a slower rate than the urinary T/E ratio does, thereby explaining the greater number of false-positive results for T/LH in this group. To prove this point, we regressed the mean T/E and T/LH ratios against their timepoints at weeks 17, 20, 24, 28, and 40 and fit this as a monoexponential decay curve. The regression line intersects the critical T/LH ratio of 30 at 7.9 weeks after the last TC injection (T/LH ratio = $90.2\, e^{-0.14(\text{week})}$, $r^2 = 0.86$). However, the T/E ratio fitted to the exponential equation (T/E ratio = $12.3\, e^{-0.1916(\text{week})}$, $r^2 = 0.80$) intersects the ratio of 6 at 3.7 weeks. Both models, therefore, demonstrate why more subjects test positive for a longer time when assessed with the T/LH ratio.

It is not uncommon for nonpower athletes (e.g., distance runners, swimmers, tennis players, soccer players) to utilize physiological doses of T (i.e., TC 100 mg/week) to counter the catabolic effects of stress and exercise on muscle. We measured urine T/E and urine T/LH in seven subjects who were administered TC at 100 mg/week. Monoexponential regression equations for the T/E and T/LH ratios to return to baseline values were based on the mean ratios measured in these subjects at weeks 17, 20, and 24 after cessation of TC injections. The urinary T/E ratio, when fitted as an exponential decay equation

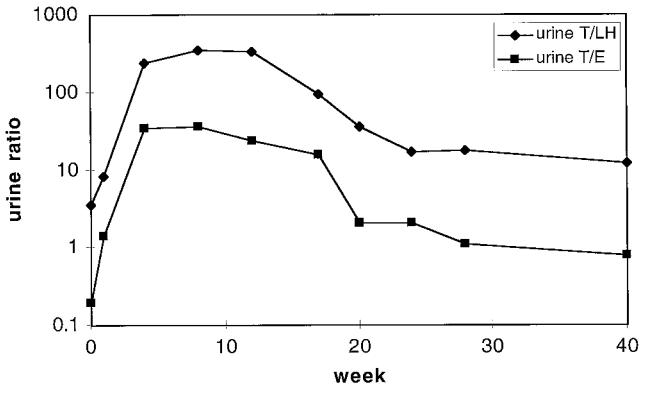

Fig. 1. Urine T/LH ratios and T/E ratios in 19 subjects receiving TC, 250 or 500 mg/week.

(T/E ratio = $8.3\, e^{-0.2072(\text{week})}$, $r^2 = 0.82$), intersects the ratio of 6 at 1.6 weeks, whereas the urinary T/LH ratio, fitted to the equation T/LH ratio = $48.9\, e^{-0.2148(\text{week})}$ ($r^2 = 0.99$), intersects the ratio of 30 at 2.3 weeks. These data suggest that is debatable whether TC at 100 mg/week is actually a physiological replacement dose: Some athletes may test positive even at this small a dose of T.

In conclusion, we find that the urinary T/LH ratio is a more sensitive and specific test for a longer time for investigating AS use than is the urinary T/E ratio. Supporting this finding is the fact that, unlike the case for E, there are no commercially available FDA-approved LH products. This advantage alone makes the urinary T/LH ratio a considerably more practical screening test than the urinary T/E ratio.

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References

1. Kicman A, Oftebro H, Walker C, Norman N, Cowan DA. Potential use of ketoconazole in a dynamic endocrine test to differentiate between biological outliers and testosterone use by athletes. Clin Chem 1993;39:1798–803.
2. Matsumoto AM. Effects of chronic testosterone administration in normal men: safety and efficacy of high-dosage testosterone and parallel dose-dependent suppression of luteinizing hormone, follicle-stimulating hormone, and sperm production. J Clin Endocrinol Metab 1990;70:282–7.
3. Palonek E, Gottlieb C, Garle M, Björkhem I, Carlstrom K. Serum and urinary markers of exogenous testosterone administration. J Steroid Biochem Mol Biol 1995;55:121–7.
4. Dehennin L. Detection of simultaneous self-administration of testosterone and epitestosterone in healthy men. Clin Chem 1994;40:106–9.
5. Catlin DH, Hatton CK. Use and abuse of anabolic and other drugs for athletic enhancement. Adv Intern Med 1991;36:399–424.
6. Dehennin L, Matsumoto A. Long-term administration of testosterone enanthate to normal men: alterations of the urinary profile of androgen metabolites potentially useful for detection of testosterone misuse in sport. J Steroid Biochem Mol Biol 1993;44:179–89.
7. Dehennin L. On the origin of physiologically high ratios of urinary testosterone to epitestosterone: consequences for reliable detection of testosterone administration by male athletes. J Endocrinol 1994;142:353–60.
8. Brooks R, Jeremiah G, Webb W, Wheeler M. Detection of anabolic steroid administration to athletes. J Steroid Biochem 1979;11:913–7.
9. Kicman A, Brooks R, Collyer S, Cowan DA, Nanjee MN, Southan GJ, Wheeler MJ. Criteria to indicate testosterone administration. Br J Sports Med 1990;24:253–64.
10. Perry PJ, Andersen KH, Yates WR. Illicit anabolic steroid use in athletes: a case series analysis. Am J Sports Med 1990;18:422–8.
11. Sample RHB, Baenziger JC. Gas chromatography/mass spectrometric detection of anabolic steroids. In: Deutsch J, ed. Analytical aspects of toxicology. New York: JR Wiley and Sons, 1989:247–72.
12. Somoza E, Mossman D. Neuropsychiatric decision making: designing nonbinary diagnostic tests. J Neuropsychiatry Clin Neurosci 1991;3:197–200.