# Spectrophotofluorometric Determination of Methyltestosterone in Plasma or Serum

DAVID ALKALAY▲, LAKSHMI KHEMANI, and M. FRED BARTLETT

**Abstract** □ For determination of the relative bioavailability of methyltestosterone in man, a spectrophotofluorometric procedure was developed, based on fluorescence induced by hydrochloric acid and stabilized by ascorbic acid. The method is essentially specific and capable of assaying nanograms per milliliter amounts of unchanged drug in blood plasma or serum. The variability of determinations is associated with a relative standard deviation of less than 12%. The procedure was tested in a pilot study involving two healthy human volunteers, each administered a single 10-mg. methyltestosterone tablet. Serum concentrations in these subjects attained a maximum of about 30 ng./ml. within 1–2 hr. following administration of drug and then declined biphasically, with half-lives of approximately 1 hr. initially and changing to approximately 3.5 hr. within 3–4 hr. of dosage.

**Keyphrases** □ Methyltestosterone in plasma or serum—spectrophotofluorometric analysis □ Plasma levels, methyltestosterone—spectrophotofluorometric analysis □ Serum levels, methyltestosterone—spectrophotofluorometric analysis □ Spectrophotofluorometry—analysis, methyltestosterone in plasma or serum

Methyltestosterone[1] (I), 17β-hydroxy-17-methylandrost-4-en-3-one, was first prepared by Ruzicka *et al.* (1) in 1935. It is an orally effective synthetic androgen, which has been shown to have considerably higher oral activity than testosterone (2). Levedahl and Samuels (3) demonstrated *in vitro* that methyltestosterone, unlike testosterone, is not metabolized to 17-oxosteroids, and they suggested that a slower metabolic rate of inactivation *in vivo* might account for its higher oral activity. The slower metabolic disposition of methyltestosterone in man was later confirmed (4–6), and Quincey and Gray (6) showed the major metabolic pathways to be reduction of ring A, hydroxylation, and conjugation, resulting mostly in conjugates and in highly polar hydroxylated ring A-reduced products. The partially identified human metabolites, E and F, hydroxylated at some position other than C-2 and C-16 (6), as well as 17α-methyl-5β-androstane-3α,16β,17β-triol (II), 17α-methyl-5β-androstane-3α,16α,17β-triol (III), and 3α,17β-dihydroxy-17α-methyl-5β-androstan-16-one (IV), isolated from rabbit urine (7), are products of ring A reduction and hydroxylation. Reduction alone is evident in 17α-methyl-5α-androstane-3β,17β-diol (V), 17α-methyl-5β-androstane-3α,17β-diol (VI), and 17α-methyl-5α-androstane-3α,17β-diol (VII), isolated as human and canine metabolites (6, 8, 9), and in 17α-methyl-5β-androstane-3β,17β-diol (VIII), reported as a fecal metabolite in the dog (9). Monohydroxylation without reduction in ring A has been shown to occur in the partially identified Δ⁴-3-oxosteroidal metabolite, L (6).

A search of the literature did not reveal any published human or animal bioavailability study for orally administered methyltestosterone. This may be attributed to the lack of suitably developed analytical methodology, capable of detecting and quantitating accurately nanogram amounts of this drug. Absorption spectra of methyltestosterone after treatment with sulfuric acid (10), color reactions with certain metal chlorides (11), and fluorescence resulting from treatment with dimethyl sulfate (12), sulfuric acid (13), or phosphoric acid (14) have been known for some time. The methodology for analysis of methyltestosterone, described in this study, is based on hydrochloric acid-induced fluorescence, suggested as a practical approach for the assay of blood levels by Steinetz *et al.* (15). These authors observed that methyltestosterone developed intense fluorescence in concentrated hydrochloric acid, which was stabilized by prior treatment of the hydrochloric acid reagent with ascorbic acid; they reported that intensity readings, taken 1 hr. after mixing, showed a linear relationship of concentration to fluorescence.

## MATERIALS AND METHODS

**Chemicals**—The chemicals and solvents used consistently in this study were: ascorbic acid[2]; chloroform[3] and dichloromethane[3], spectroquality; 37–38% hydrochloric acid[4], reagent; methanol[5], reagent; and I[6], XV[6], XX[6], V[7], XI[7], XIII[7], XIV[7], VII[8], IX[8], X[8], XII[8], XVI[8], XVIII[8], and XIX[8]. All steroids were of commercial quality and were used without further purification.

**Standards and Samples**—Methyltestosterone and other steroid standards were prepared by dilution in methanol and/or chloroform. Samples were obtained by adding known amounts of methyltestosterone to distilled water, heparinized canine plasma, or human serum and extracting with chloroform. Standardized extracts were prepared by adding known amounts of methyltestosterone to blank plasma or serum extracts. Blood specimens were withdrawn immediately before and 0.5, 1, 2, 3, 4, 6, and 8 hr. after administration of a single 10-mg. methyltestosterone tablet[9] to healthy human volunteers[10]. The serum, obtained the same day by centrifugation, was extracted with chloroform.

**Extraction**—To 2 ml. plasma or serum placed in a 40-ml. centrifuge tube with Teflon-lined screw cap were added 2 ml. distilled water and 8 ml. chloroform. The mixture was shaken horizontally in an Eberbach shaker [240 excursions/min., 2.54-cm. (1-in.) stroke] for 10 min. Following 5 min. of centrifugation, the organic layer was transferred to a 25-ml. volumetric flask, and the extraction was repeated two more times. Enough chloroform was added to the combined extracts to adjust the volume to 25 ml.

**Development of Fluorescence**—Aliquots of standards or extracts (usually 5 ml.) were evaporated in test tubes at 45–50°, under a mild air or nitrogen current. Whenever the aliquots exceeded 2 ml., the walls of the test tubes were washed down with 1–2 ml. of dichloromethane and the solution was reevaporated. The residues were occasionally stored under nitrogen for 1–3 days by capping

[1] Metandren, Ciba-Geigy Corp.

[2] Analabs, Inc., North Haven, Conn.
[3] Matheson, Coleman & Bell, Norwood, Ohio.
[4] E. I. duPont de Nemours & Co., Inc., Wilmington, Del.
[5] J. T. Baker Chemical Co., Phillipsburg, N. J.
[6] Ciba-Geigy Corp., Summit, N. J.
[7] Mann Research Laboratories, Inc., New York, N. Y.
[8] Steraloids, Inc., Pawling, N. Y.
[9] Used commercial Metandren (P.O. 34998-A).
[10] Supplied by the Medical Division.

**Table I**—Variability of Fluorescence Readings Taken at Peak Level and at 1 hr. after Treatment of Methyltestosterone Standards with 37% Hydrochloric Acid Containing Ascorbic Acid (100 mcg./ml.)

| Methyltestosterone, ng./ml. Reagent | Readings Taken at Peak Level | | | | Readings Taken at 1 hr. | | | |
|---|---|---|---|---|---|---|---|---|
| | 2 | 10 | 100 | 200 | 2 | 10 | 100 | 200 |
| Intensities | 0.035 | 0.216 | 2.21 | 3.84 | 0.014 | 0.114 | 1.55 | 2.21 |
| | 0.030 | 0.228 | 1.985 | 3.99 | 0.011 | 0.138 | 1.43 | 2.57 |
| | 0.036 | 0.210 | 1.88 | 4.04 | 0.017 | 0.096 | 1.22 | 2.48 |
| | 0.041 | 0.225 | 2.075 | 4.19 | 0.021 | 0.108 | 1.52 | 3.19 |
| | 0.040 | 0.195 | 1.985 | 3.99 | 0.020 | 0.087 | 1.46 | 2.54 |
| | | | 2.03 | 3.89 | | | 2.00 | 3.59 |
| | | | 1.985 | 4.14 | | | 1.91 | 2.90 |
| | | | 2.12 | 4.24 | | | 1.795 | 3.95 |
| Mean intensity | 0.0364 | 0.215 | 2.034 | 4.04 | 0.0166 | 0.109 | 1.611 | 2.929 |
| Standard deviation | 0.0044 | 0.0132 | 0.101 | 0.141 | 0.0041 | 0.0195 | 0.266 | 0.603 |
| Relative standard deviation, % | 11.97 | 6.12 | 4.95 | 3.50 | 24.84 | 17.95 | 16.51 | 20.60 |

the tubes. Twenty-four hours before usage, a solution of concentrated hydrochloric acid, containing ascorbic acid (usually 200 mcg./ml.), was prepared. Of the acid reagent, 0.4–1 ml. was added to each residue and stirred with a Vortex mixer for 10 sec. The resulting solutions were used to fill 4 × 40-mm. quartz microcells, giving a group of samples, standards, and blanks. The fluorescence of these was measured in succession, and six to eight intensity readings per sample were taken at approximately 30-min. intervals. This permitted observation of the development, peaking out, and beginning of decay of fluorescence for each sample. Results were computed after correction of blanks by comparing sample intensities to those obtained with standards. All readings were taken with spectrophotofluorometer[11], set at a maximum activation wavelength of 470 nm. and a maximum fluorescence wavelength of 530 nm. and equipped with a xenon light source, a 1P28 photomultiplier tube, 0.15- and 0.31-cm. (0.06- and 0.125-in.) excitation slits (with center slit omitted), and 0.15- and 0.31-cm. (0.06- and 0.125-in.) slits on the emission side of the cell compartment.

## RESULTS AND DISCUSSION

The development of fluorescence for varying amounts of methyltestosterone was tested with several acid reagents (hydrochloric acid in water, methanol, dioxane, and mixtures of the aforementioned—with or without ascorbic acid or other additives). Hydrochloric acid (37%) containing ascorbic acid (50–400 mcg./ml.) proved to be the reagent of choice, to which methanol or dioxane, because of their quenching effect, could be added in amounts not exceeding 10% of the total volume.

During these tests, it became apparent that the time course of induced fluorescence showed considerable variability. As a consequence, fluorescence readings taken 1 hr. after acid treatment of the samples reflected methyltestosterone concentrations rather unsatisfactorily. Peak fluorescence intensities, however, independently of the differences in time required for their attainment, bore a close linear relationship to methyltestosterone concentrations up to about 200 ng./ml. of the acid reagent. Data obtained with methyltestosterone standards are presented in Table I. They show the variability of intensities at peak fluorescence level, even at low methyltestosterone concentrations, to be associated with a relative standard deviation of less than 12%. Similar results were obtained with canine plasma samples and standardized extracts. Fluorescence readings for methyltestosterone concentrations ranging from 0.5 to 128 ng./ml., after conversion to intensities per nanogram per milliliter, are presented in Table II.

Peak level intensities show again a much more favorable distribution of random errors in comparison with 1-hr. readings and are associated with a relative standard deviation of less than 9%. A comparison of mean intensities indicates a 91% extraction recovery. Methyltestosterone recoveries in triple extractions from aqueous solutions, as well as canine plasma or human serum samples, invariably fell within the range of 88–97%. Single extractions showed somewhat lower recoveries and less reproducibility.

Heat and exposure to sunlight have been reported (15) to quench hydrochloric acid-induced fluorescence. The time and rate of mixing methyltestosterone samples with acid reagent appear to have an effect on fluorescence, which probably can be attributed to air oxidation. As can be seen from the data in Table III, even trace amounts of hydrogen peroxide quenches fluorescence effectively. Increasing

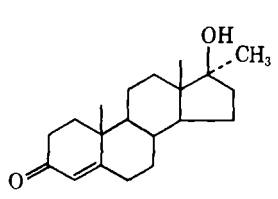
[11] Aminco Bowman.

**Table II**—Fluorescence Readings of Extracted Plasma Samples and Standardized Plasma Extracts, Taken at Peak Level and at 1 hr. after Treatment with 37% Hydrochloric Acid Containing Ascorbic Acid (100 mcg./ml.)

| Methyltestosterone Concentrations, ng./ml. | $10^3$ × Intensities, per ng./ml. Extracted Plasma Samples | | Standardized Plasma Extracts | |
|---|---|---|---|---|
| | Peak Level | 1 hr. | Peak Level | 1 hr. |
| 0.5 | 21 | 16 | 24 | 18 |
| 1 | 24 | 23 | 27 | 27 |
| 2 | 25 | 18 | 23 | 16.5 |
| 2 | 21.5 | 17.5 | 26.8 | 25.5 |
| 4 | 20.8 | 13.8 | 26.3 | 25.1 |
| 8 | 18 | 14.3 | 24.2 | 15.4 |
| 8 | 26.3 | 25.5 | 27 | 26.3 |
| 16 | 25.2 | 21.1 | 24.3 | 18.6 |
| 32 | 21.3 | 12.6 | 22.6 | 13.8 |
| 32 | 23.1 | 21.5 | 27.4 | 19.3 |
| 64 | 23 | 12.8 | 24.7 | 13.9 |
| 128 | 22.6 | 21 | 23.1 | 10.9 |
| $10^3$ × mean intensity, per ng./ml. | 22.65 | 18.08 | 25.02 | 19.20 |
| $10^3$ × standard deviation | 2.00 | 4.24 | 1.41 | 5.48 |
| Relative standard deviation, % | 8.83 | 23.5 | 5.65 | 28.5 |

**Table III**—Effect of Hydrogen Peroxide and Ascorbic Acid on Hydrochloric Acid-Induced Fluorescence of Methyltestosterone

| Hydrogen Peroxide, $10^{-11}$ × moles/ml. | Relative Fluorescence of Methyltestosterone (16 ng./ml.) Induced by 37% Hydrochloric Acid, Containing: No Additives | Ascorbic Acid 50 mcg./ml. | Ascorbic Acid 100 mcg./ml. |
|---|---|---|---|
| None | 100% | 100% | 100% |
| 0.88 | 67% | 95% | 102% |
| 3.5 | None | 115% | 100% |
| 4.4 | None | 102% | 102% |
| 13 | None | 66% | 107% |
| 22 | None | 71% | 100% |



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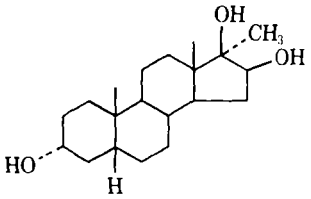

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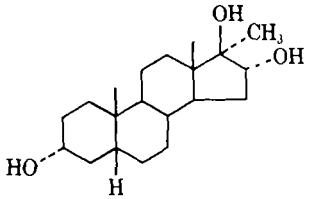

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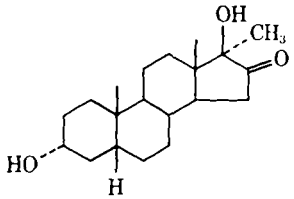

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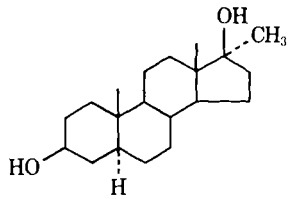

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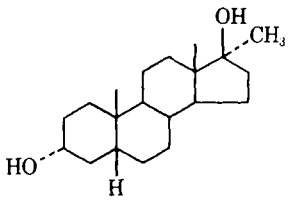

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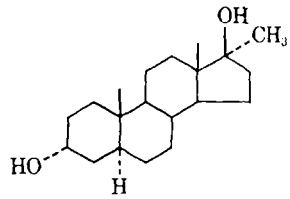

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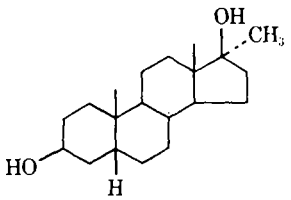

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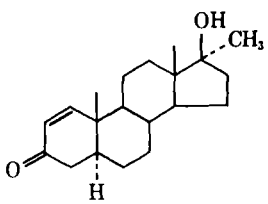

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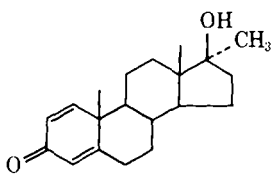

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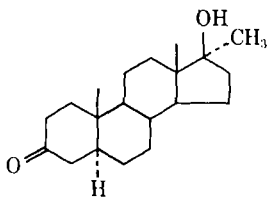

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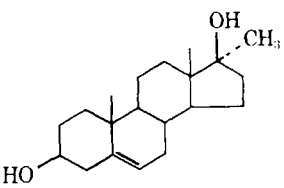

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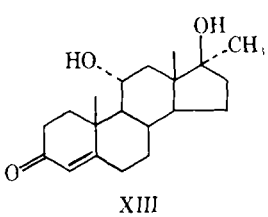

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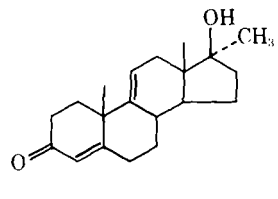

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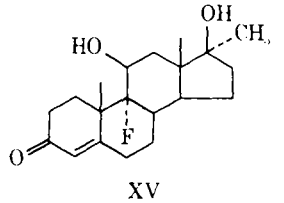

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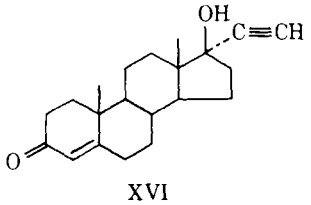

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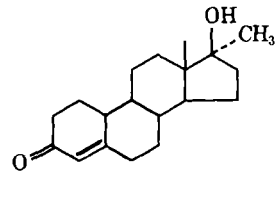

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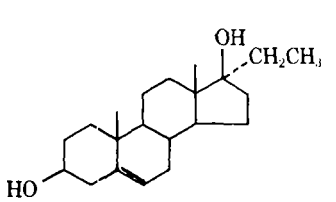

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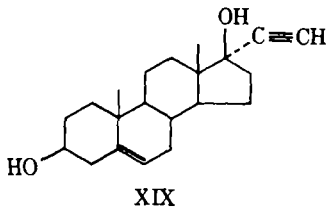

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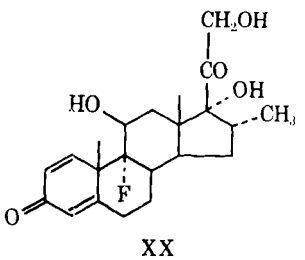

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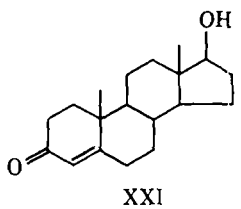

XXI

**Table IV**—Fluorescence of Steroids in 37% Hydrochloric Acid, Containing Ascorbic Acid (200 mcg./ml.), Relative to that of Methyltestosterone

| Steroid | Compound Number | Percent Fluorescence |
|---|---|---|
| Methyltestosterone | I | 100 |
| Metabolite V | V | 0.1 |
| Metabolite VII | VII | 0.1 |
| 1,(5α)-Androsten-17α-methyl-17β-ol-3-one | IX | 0.6 |
| 1,4-Androstadien-17α-methyl-17β-ol-3-one | X | 0.1 |
| 5α-Androstan-17α-methyl-17β-ol-3-one | XI | 1 |
| 5-Androsten-17α-methyl-3β,17β-diol | XII | 0.6 |
| 4-Androsten-17α-methyl-11α,17β-diol-3-one | XIII | ~57 |
| 4,9(11)-Androstadien-17α-methyl-17β-ol-3-one | XIV | ~80 |
| Fluoxymesterone | XV | 0.2 |
| 4-Androsten-17α-ethinyl-17β-ol-3-one | XVI | 5 |
| 17α-Methyl-19-nortestosterone | XVII | ~4[a] |
| 5-Androsten-17α-ethyl-3β,17β-diol | XVIII | 0.3 |
| 5-Androsten-17α-ethinyl-3β,17β-diol | XIX | 0.2 |
| Dexamethasone | XX | 0.2 |
| Testosterone | XXI | ~0.03[a] |

[a] Based on previously reported data (15).

amounts of hydrogen peroxide can be tolerated, however, by raising correspondingly the concentration of ascorbic acid in the reagent. Apparently, ascorbic acid acts as a stabilizing antioxidant.

Acid reagents with varying concentrations of ascorbic acid were tested with samples prepared from human serum containing known amounts of drug, and a concentration of 200 mcg./ml. of reagent was found to be optimal.

The effect of storage upon recoveries of unchanged drug was also investigated. Canine plasma samples, containing known amounts of methyltestosterone, were kept at 4° for 9 days. No changes in drug concentrations were detected within the limits of experimental error. Organic extracts from canine plasma and human serum samples, as well as solutions of methyltestosterone, were stored at room temperature for up to 6 weeks without any apparent harm.

Table V—Methyltestosterone Serum Levels following Administration of a Single 10-mg. Tablet

| Subject | Determination | Concentration, ng./ml., at Hour 0.5 | 1 | 2 | 3 | 4 | 6 | 8 | Area under the Curve, ng. hr. ml.$^{-1}$ |
|---|---|---|---|---|---|---|---|---|---|
| L.J.M. | 1 | 1.8 | 7.2 | 23.0 | 10.9 | 6.4 | 5.1 | 2.9 | |
| | 2 | 1.8 | 7.2 | 24.3 | 11.4 | 6.6 | 4.1 | 3.1 | |
| | Mean | 1.8 | 7.2 | 23.7 | 11.2 | 6.5 | 4.6 | 3.0 | 60 |
| R.G. | 1 | 8.3 | 35.6 | 21.8 | 8.5 | 7.1 | 6.3 | 3.9 | |
| | 2 | 9.7 | 39.9 | 24.9 | 10.4 | 8.1 | 7.6 | 3.7 | |
| | Mean | 9.0 | 37.8 | 23.4 | 9.5 | 7.6 | 7.0 | 3.8 | 94 |

To evaluate the specificity of this analytical procedure for methyltestosterone, hydrochloric acid-induced fluorescence of several analogs, including two known metabolites (V and VII), was compared to that of I. The results, expressed as percent fluorescence relative to that of methyltestosterone, are presented in Table IV. It appears from these results that even minor changes in the methyltestosterone molecule, such as reduction (V, VII, XI, and XII), shifting of the conjugation (IX), increased unsaturation of ring A (X), absence of a methyl group at C-19 (XVII) or C-17 (XVI and XXI), or the presence of an $\alpha$-substituent at position 9 (XV), can result in a very significant weakening of the otherwise unusually strong fluorescence. The gross decrease in intensity of fluorescence, associated with reduction of ring A, is of particular interest since nine of the 10 metabolites isolated so far (E, F, and II–VIII) have been shown to have a reduced ring A.

Hydroxylation of methyltestosterone at position 11 results in a derivative (XIII) exhibiting strong fluorescence, which is even enhanced by dehydration (XIV). Metabolite L, a minor human urinary metabolite, has been shown to be a $\Delta^4$-3-oxosteroid, hydroxylated at position 1, 2, 4, 5, 7, 15$\alpha$, or 16$\beta$ (6). It is not identical with analog XIII; but to the extent that it might be produced and extracted with methyltestosterone, it could contribute to fluorescence.

In the study of Quincey and Gray (6), the radioactivity of extracted steroids in one patient was shown (by chromatographic separation) to be due only to radioactive methyltestosterone within the limits of experimental error. With this independent evidence, along with the data reported herein, it is concluded that the procedure described in this report can be considered an essentially specific method for assaying nanogram amounts of methyltestosterone in plasma or serum.

To validate the analytical methodology, a pilot study involving two healthy human volunteers, each receiving a single 10-mg. methyltestosterone tablet[9], was carried out. The results are presented in Table V. The relative standard deviation[12] of 6.3%, associated with determinations in the pilot study, is in good agreement with the variability encountered in fluorometric determinations throughout this investigation. Serum levels for the two subjects attained a maximum (24–39 ng./ml.) within 1–2 hr. and subsequently declined biphasically. The initial decay, characterized by a half-life of approximately 1 hr., ended about 3–4 hr. after administration of the drug. The final exponential decline exhibited a half-life of approximately 3.5 hr., which is somewhat longer than the average biological half-life of 2.7 hr. found in a comprehensive bioavailability study, the results of which are to be published[13].

## CONCLUSIONS

The spectrophotofluorometric procedure described in this study is capable of detecting and quantitating nanogram amounts of methyltestosterone in blood plasma or serum, and storage of samples or their extracts for limited periods of time has no measurable effect upon the recoveries of unchanged drug. The precision of the method is good, and random errors, as measured by standard deviation, should not influence results by more than 12%. The specificity of the assay is supported by published evidence and by data generated in this laboratory. It is concluded, therefore, that this method can be used advantageously for human bioavailability studies.

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[12] The relative standard deviation was calculated from:

$$S_{rel}(x) = 100\sqrt{\frac{2\Sigma(^1/_2d/\bar{m})^2}{n-1}}$$

where $d$ is the difference between duplicates, $m$ is the arithmetic mean of each duplicate determination, and $n$ is the number of determinations.

[13] D. Alkalay, L. Khemani, W. E. Wagner, Jr., and M. F. Bartlett, to be published.