Comparative Evaluation of Testosterone Release and its Derivatives in Adult Male Monkeys

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Abstract: The present study was undertaken to compare the release of testosterone and its derivatives in adult male bonnet monkeys by different routes. Testosterone and its derivatives such as T.propionate, T.enanthate, T.undecanoate, T.busiclate and 7α-Methyl 19-nortestosterone (MENT) were administered via different routes that includes intramuscular, Alzet pump and Silastic tubing. It was observed that 5 ng/ml testosterone concentration was maintained up to 45 days with esters like enanthate, undecanoate or buciclate when given via Alzet pump, whereas with Silastic implants, the testosterone concentration was maintained at 5 ng/ml up to 105 days in the enanthate group, 112 days in the undecanoate group and 133 days in the buciclate group. The results of the study clearly establish the efficacy of delivery by Silastic tubing, which maintained high concentration of testosterone buciclate even on Day 49 compared to 18 days by Alzet pumps or only for 13 days by intramuscular route.

Keywords: Testosterone, testosterone derivatives, half-life, pharmacokinetics, release, monkey.

INTRODUCTION

Contraceptive regimens using testosterone alone have the unique advantage, compared to other suppressors of the HPG such as progesterone and estradiol, of providing androgen replacement simultaneously with inhibition of spermatogenesis. This simplicity makes testosterone the obvious first choice as a reversible male contraceptive [1, 2].

Testosterone enanthate is the most widely used replacement androgen [3, 4]. While the clinical effectiveness and safety is well established, it has the disadvantage of requiring i.m. or s.c. administration at 1 to 3 week intervals [3, 5]. Testosterone cypionate must also be injected at similar intervals [6]. The pharmacokinetics following the administration of these preparations reveals that serum testosterone reaches supra physiological concentrations for 2 to 3 days and then depending on the frequency of injection, can decline to concentrations below normal before the next injection [7]. The need for frequent intramuscular injection and the overdose/under dose variation are a major drawback in the use of these esters. Testosterone undecanoate, an orally active testosterone ester, is absorbed by the intestine. However, the drug must be taken three times daily to maintain adequate androgen support [8]. Testosterone buciclate has been shown to maintain serum testosterone concentration in the normal range for up to 4 months in castrated cynomolgus monkeys after a single intramuscular injection of 40 mg of the ester [9].

However, in humans, there is a practical disadvantage with testosterone, in that a large dose of steroid must be delivered to replicate the endogenous production rate of 3-10 mg/day [10]. This has been partially remedied with the development of long-acting testosterone preparations, including (i) natural testosterone formulated in a slowly releasing biodegradable implant; (ii) testosterone incorporated into sustained release delivery systems such as biodegradable copolymers and (iii) long acting testosterone esters which are hydrolyzed slowly in the body to release the biologically active form over a prolonged period of time. Considering these problems, it is desirable that a suitable method of delivery of testosterone and its derivatives should be developed. This will facilitate release of the steroid in question in a sustained manner at optimal concentration for prolonged duration, thus avoiding the need for frequent injections. In the present study, an attempt has been made to compare the release of testosterone and its derivatives administered in the castrated adult male bonnet monkeys by different routes, which include intramuscular or via Alzet pump or Silastic tubing.

MATERIALS & METHODS

The bonnet monkeys (Macaca radiata) used in the present study were either caught from the wild or bred in the colony. The method used in husbandry and breeding of bonnet monkeys in the colony has been described in detail earlier [11, 12].

The bonnet monkeys were housed in rooms of 14’ x 10’ dimension. Each room housed 8 monkeys which were kept in individual cages of 3’ x 2’ x 4’ dimension. Each cage had a restraint mechanism which facilitated drawing of the animals forward for procedures like blood sampling, injections etc. All the rooms were provided with humidified
water was provided.

Fresh and purified (Aquaguard) drinking water was provided ad libidum. Monkeys were anesthetized before surgery with ketamine hydrochloride (Ketec, Parke Davis) at the dose rate of 10 mg/kg body weight. All the surgical procedures were performed in the well-equipped surgical theater attached to Primate Research Laboratory (PRL) and the procedures employed in the study were approved by the Institutional Animal Ethics Committee.

Radioimmunoassay of Steroid Hormones

Radioimmunoassay methods were based on the procedures standardized in our laboratory for measuring testosterone, estradiol-17β and progesterone concentrations [13, 14]. Radioimmunoassay of MENT was carried out according to the procedure described by Kumar et al., 1990 [15].

Hormones and Chemicals

The BSA-conjugates of steroids for raising the antisera were procured from Steraloids, USA. The unlabelled testosterone was obtained from Steraloids Inc, USA. Tritiated testosterone was purchased from Radiochemical center, Amersham, UK. The specific activity of the tritiated testosterone used in the study was 70 Ci/mmol. Activated charcoal, 2,5-diphenyl oxazole (PPO) and dimethyl POPOP was obtained from Sigma Chemicals, USA. Gelatin was purchased from Difco Laboratory, USA and Dextran-T70 from Pharmacia Fine Chemicals, Uppsala, Sweden. Other chemicals like diethyl ether, methanol etc. was obtained from Sarabhai Chemicals, India.

Estimation of Monkey Serum LH

A method for LH estimation was developed using immobilization of antisera to ovine LH on fresh plastic wells through an immunochemical bridge. This type of immobilization has been shown to provide more consistent values than direct adsorption on plastic wells. This method has been used in the development of non-centrifugation radioimmunoassay [16].

Implantation of Alzet Pumps

Sterile techniques were followed during filling and handling of Alzet pumps and also during surgical implantation procedure. During filling and handling, Alzet pumps were handled with surgical gloves. Initially pump was weighed together with its flow moderator. The drug was filled in the pump with a syringe and a blunt-tipped filling tube, which was supplied with pumps. The solution was drawn into the syringe and care was taken that it was free of bubbles. Flow moderator was removed and the pump was held in an upright position, and inserted the filling tube through the opening at the end of the pump until the bottom of the pump reservoir. Plunger of the syringe was slowly pushed while holding the pump in an upright position. When the solution appeared at the outlet, filling was stopped and carefully the tube was removed.

Then flow moderator was inserted until the cap was flush with top of the pump. Filled pump was weighed again. The difference in the weights gives net weight of the solution. The fill volume adjusted to be over 90% of the reservoir volume (specified on the instruction sheet). In the present study, Alzet pump which releases 2.5 μl/h (Model 2ML4, Alza Inc, Palo Alto, USA) was used. The mean fill volume was 2 ml and the duration of release was 28 days.

Preparation of Silastic Tubing

Silastic tubing (# 602-305, Dow Corning Corporation, Midland, USA), which had 0.078 cm inner diameter and 0.125 cm outer diameter dimension, were used in the present study. They were cut into lengths of 4 cm and the drug was filled up to 3 cm but an addition of 1 cm provided in each implant to accommodate adhesive plug. Tubes were cleaned with mild soap water, deionised water, hot water and rinsed several times with alcohol. Then the tubes were dried. Using a Silastic medical adhesive, Silicone type A (# 891), one end of tube was sealed. Only 4 to 5 mm of the tube was used for plugging with adhesive and it was allowed to dry overnight. By using a cut-off glass pipette, capsules were filled with testosterone or its esters or MENT. Tubes were vortexed gently to allow the compound to settle. The compound was filled up to a final length of 3 cm. The other end of the capsule was sealed with Silastic adhesive and allowed for overnight to polymerize [17]. Excess of steroid on the capsule was washed off using alcohol. Release rate in case of testosterone was based on the earlier study [18]. A one cm of Silastic tubing releases 32 μg of testosterone/day.

RESULTS

Determination of Half-Life of Testosterone and its Esters

In adult castrated monkeys which received testosterone (10 mg i.m., n=3), the serum testosterone concentrations increased and reached a peak concentration (3033.33 ± 88.19 ng/ml) in 30 minutes. Serum testosterone concentration started declining by 30 minutes and decreased in 5 h (48.33 ± 1.66 ng/ml) and reached basal concentrations by 120 h. The half-life of testosterone during the absorption phase (t₁/₂a), was 12.05 min and during the elimination phase (t₁/₂b) was 12.41 min (Fig. 1).

In adult castrated monkeys which received testosterone propionate (10 mg i.m., n=3), the serum testosterone concentration increased and reached a peak concentration by 3 h (3333.33 ± 88.19 ng/ml). The serum testosterone concentration started declining thereafter and reached basal concentrations by 744 h. The half-life of testosterone propionate during the absorption phase (t₁/₂a) was 1.20 h and during elimination phase (t₁/₂b), it was 21.9 h.

Testosterone enanthate (10 mg) was administered to adult, castrated male monkeys by intramuscular route. The serum testosterone concentration increased rapidly and reached a peak concentration by 12 h (4183±101.37 ng/ml). Following this, it started declining slowly and reached a basal concentration by 1032 h. The half-life of testosterone enanthate during absorption phase (t₁/₂a) was 3.04 h and during elimination phase (t₁/₂b), it was 111.40 h.
Testosterone undecanoate (10 mg) was given intramuscularly to 3 adult, castrated male monkeys. The serum testosterone concentration reached the peak concentration by 120 h (2750.00 ± 144.33 ng/ml) and started declining slowly. The basal testosterone concentration was seen at 1100 h. The half-life of testosterone undecanoate during absorption phase was 4.90 h and during elimination phase was 549 h.

In adult, castrated bonnet monkeys (n=3), which received testosterone buciclate (10 mg) through the intramuscular route, the serum testosterone concentration (from a 0 h concentration of 0.16 ± 0.03 ng/ml) started increasing slowly and reached the peak concentration by Day 13. Following this, the serum testosterone concentration started declining very slowly and reached the basal concentration by Day 61. The half-life of testosterone buciclate during the absorption phase (\(t_{1/2a}\)) was 13.28 h and during elimination phase (\(t_{1/2ß}\)), it was 766.3 h.

MENT given at a dose of 1 mg increased sharply in the serum and attained a peak concentration by 30 minutes (475 ± 14.34 ng/ml). Following this, it started declining and reached the basal concentration by 48 h (0.23 ± 0.03). The half-life of MENT during the absorption phase (\(t_{1/2a}\)) was 6.56 minutes and during the elimination (\(t_{1/2ß}\)) phase was 39.98 minutes and these results are in complete agreement with those obtained by Kumar et al., 1997 [19] using cynomolgus monkeys.

A comparative analysis of the half-life of testosterone and its esters in adult male bonnet monkeys presented in Table 1 reveals that the half-life of testosterone was 12.41 minutes in comparison to testosterone buciclate, which had a \(t_{1/2ß}\) of 13.28 h. Testosterone buciclate was therefore 66 times more potent than testosterone in terms of \(t_{1/2ß}\). Testosterone propionate had a half-life of 21.90 hr, 112 times more potent than testosterone. Testosterone undecanoate was more potent than testosterone enanthate, testosterone propionate and testosterone but far less potent than testosterone buciclate. The absorption half-life (\(t_{1/2a}\)) of MENT was 6.56 minutes and the half-life during elimination phase (\(t_{1/2ß}\)) was 39.98 minutes. These results clearly establish the efficacy of testosterone buciclate in terms of its half-life in circulation.

**Release of Testosterone and its Esters by Alzet Pump**

Alzet pump (2 ML4), with a release rate of 2.5 μl/h was implanted into adult, castrated bonnet monkeys (n=3). Testosterone or its esters were administered via Alzet pumps which released 2 μg/h (48 μg/day) testosterone or its esters (n=3). The pumps were removed on Day 28. Blood samples were collected at regular intervals for the estimation of serum testosterone. The serum testosterone concentration started increasing by Day 3 and attained a peak concentration by Day 9 in monkeys given testosterone compared to those that received testosterone buciclate, which attained a peak concentration on Day 18. Most importantly, all groups of animals treated with testosterone esters maintained higher serum testosterone concentrations (>10 ng/ml) than those treated with plain testosterone. Serum testosterone concentration started declining from Day 27 in testosterone-treated monkeys and in the testosterone propionate-treated group. However, the decrease in the other groups was also slow and the testosterone concentration remained high (>10 ng/ml) in testosterone buciclate-treated group even after Day 45.

Interestingly, in experiments using Alzet pumps, it was observed that no significant (p<0.05) differences in either the time taken to reach peak serum concentration or in the peak concentrations of testosterone were observed among different groups.
concentration per se. Following testosterone buciclate administration, higher serum testosterone concentration was noted when compared to plain testosterone (Fig. 2).

It can also be seen from results presented in Table 2 that while the peak concentration of 4183 ng of testosterone was seen in the testosterone enanthate administered group via intramuscular route by 12 h and with other testosterone derivatives, the peak concentration was generally around 3000 ng. However, the time at which the peak concentration was seen varied quite a bit ranging from 30 min for testosterone to 13th day in the case of buciclate. In contrast, release of testosterone or its derivatives via Alzet pumps or Silastic tubing resulted in sustained released although the peak concentration was much lower than achieved by intramuscular route.

**Release of Testosterone and Esters Via Silastic Implants**

Silastic implants of one cm length, (number 602-305, Dow Corning) which can hold 10 mg testosterone or its esters and capable of releasing 30 μg/day, were implanted in castrated monkeys (n=3). These pumps were removed on Day 90. Peak serum testosterone concentrations were seen by Day 21 and remained at that level till Day 75 and thereafter started to decline. Serum testosterone concentration attained peak concentration on Day 21 (6.90 ± 0.49 ng/ml) in the testosterone propionate-treated group, on Day 42 (8.90 ± 0.05 ng/ml) in the testosterone enanthate-treated group, on Day 42 (9.36 ± 0.08 ng/ml) in the testosterone undecanoate-treated group and by Day 49 (9.60 ± 0.47 ng/ml) in the testosterone buciclate-treated group. However, serum testosterone concentration reached the basal concentration by Day 119 in the testosterone group, by Day 133 in the testosterone propionate-treated group, by Day 147 in the testosterone enanthate group, by Day 175 in testosterone undecanoate-treated group and by Day 189 in the testosterone buciclate-treated group. Even though the implants were removed on Day 90, serum testosterone levels above 10 ng/ml were maintained with the testosterone undecanoate-treated and buciclate-treated groups up to Day 133 (Fig. 3).

It is very clear from the results presented in the Table 3 that testosterone concentration reached a peak level slowly in

<table>
<thead>
<tr>
<th>Drug</th>
<th>$t_{a}$</th>
<th>Fold Difference</th>
<th>$t_{e}$</th>
<th>Fold Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>12.05 min</td>
<td>-</td>
<td>12.41 min</td>
<td>-</td>
</tr>
<tr>
<td>T. propionate</td>
<td>01.20 h</td>
<td>06.00</td>
<td>21.90 h</td>
<td>112</td>
</tr>
<tr>
<td>T. enanthate</td>
<td>03.04 h</td>
<td>15.20</td>
<td>111.40 h</td>
<td>580</td>
</tr>
<tr>
<td>T. undecanoate</td>
<td>04.90 h</td>
<td>24.50</td>
<td>549.00 h</td>
<td>2858</td>
</tr>
<tr>
<td>T. buciclate</td>
<td>13.28 h</td>
<td>66.40</td>
<td>766.30 h</td>
<td>3990</td>
</tr>
<tr>
<td>MENT</td>
<td>6.56 min</td>
<td>-</td>
<td>39.98 min</td>
<td>-</td>
</tr>
</tbody>
</table>

$t_{a}$: Half-life of the compound during the absorption phase.  
$t_{e}$: Half-life of the compound during the elimination phase.
animals that had Silastic implants compared to those that had Alzet pumps. Peak testosterone concentrations were seen on Day 9 (15.16 ± 0.60 ng/ml) in animals given testosterone via Alzet pump but reached peak concentration on Day 21 (6.10 ± 0.05 ng/ml) when administered via Silastic implants. In case of testosterone buciclate, serum testosterone concentration reached peak on Day 18 (26.83 ± 0.44 ng/ml) when administered via Alzet pump and reached peak concentration on Day 49 (9.60 ± 0.47 ng/ml) when administered via Silastic implants.
A 5 ng/ml testosterone concentration was maintained up to 45 days with esters like enanthate, undecanoate or buciclate when given via Alzet pump, whereas with Silastic implants, the testosterone concentration was maintained at 5 ng/ml up to 105 days in the enanthate group, 112 days in the undecanoate group and 133 days in the buciclate group (Table 4).

Determination of MENT in the Serum Samples of Bonnet Monkeys

A study was carried out to determine the MENT in serum samples of normal bonnet monkeys. The MENT (100 µg/day) was given to 3 adult male bonnet monkeys via Alzet pump. Blood samples were collected on days 0, 3, 5, 10 and 15 at 10 AM and 10 PM. It is very clear from the results presented in Fig. (4) that serum MENT concentration increased significantly (p<0.05) on Day 3 (5458.00 ± 744.0 pg/ml) at 10 AM and (3801.0 ± 583.0 pg/ml) at 10 PM. The serum concentration of MENT was maintained nearly at 3000 pg till Day 25. There was no significant difference in the serum concentrations of MENT either at 10 AM or at 10 PM till the end of the study and the Day 0 value represent due to minimal cross reaction of antiserum to MENT with serum testosterone.

Table 4. The Blood Serum Testosterone Concentration (>5 ng/ml) in Monkeys Treated with Various Esters of Testosterone Via Alzet Pumps or Silastic Implants

<table>
<thead>
<tr>
<th>Compound</th>
<th>Serum Testosterone Concentration (&gt; 5 ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alzet pump (Days)</td>
</tr>
<tr>
<td>Testosterone</td>
<td>33</td>
</tr>
<tr>
<td>T.propionate</td>
<td>39</td>
</tr>
<tr>
<td>T.enanthate</td>
<td>45</td>
</tr>
<tr>
<td>T.undecanoate</td>
<td>45</td>
</tr>
<tr>
<td>T.buciclate</td>
<td>45</td>
</tr>
</tbody>
</table>

Table shows the duration (in days) of serum testosterone concentration maintained more than 5 ng/ml.

Fig. (4). Levels of MENT in serum samples of bonnet monkeys administered with 100 µg of MENT
Table 5. Serum Concentration of LH in Adult Male Bonnet Monkeys Following Administration of MENT

<table>
<thead>
<tr>
<th>Serum LH ng/ml</th>
<th>Before Castration</th>
<th>7 Days After Castration</th>
<th>21 Days After Castration</th>
</tr>
</thead>
<tbody>
<tr>
<td>LH was estimated by radioimmuno assay using a macaque LH kit</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>MENT (100 μg/day) was administered via mini osmotic pump 7 days after castration, till Day 21.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>* Significantly different from value before castration and after castration (p&lt;0.05).</td>
<td></td>
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</tr>
</tbody>
</table>

**Efficacy of 7α-Methyl 19-Nortestosterone (MENT) to Inhibit the Hypothalamo-Pituitary-Gonadal Axis**

To ascertain the ability of MENT to inhibit serum LH levels, castrated monkey model was used. Five adult male monkeys were castrated and blood samples were collected before castration, 7 and 21 days after castration. A 100 μg of MENT was administered via mini osmotic pump from Day 7 after castration till Day 21. Serum LH was estimated by radioimmuno assay using macaque LH kit. Serum LH concentration before castration was 2.5 ± 0.6 ng/ml, which increased to 13 ± 2.0 ng/ml by 7 days after castration (p<0.05) and following administration of MENT serum LH concentration decreased significantly (p<0.05) by Day 21 (Table 5). In this connection, it is pertinent to note that suppression of gonadotrophin in human subjects was achieved after implantation of five pellets containing a total of 1200 mg of crystalline testosterone releasing 9 mg/day [20]. Considering this, the dose used in the monkeys in the present study is nearly 100 times less than the dose used in humans.

**DISCUSSION**

Ever since the demonstration that the mammalian hypothalamo pituitary axis can be regulated by gonadal steroids in the female and the development of the female pill, efforts have been in progress to apply the same principle in human males up to 13 days. However, while these are effective in selected population, there appears to exist a variety of androgens [23-26]. It should be noted that we reported the observation that a sustained release of testosterone can be achieved by delivery via Silastic tubing, which maintained high concentration of testosterone buciclate even on Day 49 compared to 18 days by Alzet pumps or only for 13 days by intramuscular route. It is also to be noted that the duration up to which greater than 5 ng of testosterone concentration is maintained is considered and delivery by Silastic implants is the most effective one in that it maintains up to 133 days.

An important outcome of the present study is the observation that a sustained release of testosterone can be achieved by delivery via Silastic tubing [22]. Our study confirms the findings of Rajalakshmi and Bajaj 1990 [23] who have made an exhaustive study on evaluation of variety of androgens [23-26]. It should be noted that we reported the delivery of MENT via Silastic tubing resulted in suppression of nocturnal serum testosterone levels by 3 days. In the present study, we have demonstrated that in adult male bonnet monkeys administered 100 μg of MENT, serum MENT concentrations on Day 3 was in the range of 0.4 – 0.5 μg/ml. Similar results were obtained by Cummings, et al., 1998 [27] who reported that complete suppression of LH was achieved with a minimum of 0.3 mg/day MENT compared to 3.0 mg/day testosterone delivered via osmotic mini pump in M. fascicularis. In contrast, the concentration of serum testosterone on Day 3 with any testosterone derivatives tested in the present study was in the range of 3000 ng/ml. An important outcome of this study is a finding that it is possible to maintain 5 ng/ml testosterone concentration up to 133 days in the monkeys administered testosterone buciclate which is much longer than the plain testosterone. This observation provides a basis for designing better methods of the delivery of testosterone buciclate. However, it is to be emphasized that this study is limited only to the establishment of release rate of various testosterone analogues through different routes of administration.

It has been demonstrated that 7α methylated androgens including MENT can maintain muscle mass and normal gonadotrophin levels in androgen deficient without hyperstimulating prostate [19]. The results of the present study also establish the efficacy of MENT in suppressing HPG axis (as assessed by decreased in serum LH levels) at a much lower dose compared to testosterone.

As a male contraceptive, steroid use of MENT has several advantages. In a structure activity comparative study, MENT was found to be the most potent steroids followed by dihydrotosterone, 19-nortestosterone, 7α-methyl-19-nortestosterone, testosterone and 7α-cyano-19-NT. Because...
of steric hindrance from the 7α methyl group, MENT does not get 5α reduced and consequently androgenic potency of MENT is not amplified as in the case of testosterone. Thus, it has been concluded that MENT is 10 times more potent than testosterone with regard to clinically desirable end points with minimal adverse effect on prostate. In addition, it is aromatized to 7α-methyl oestradiol, which can prevent osteopenia. More importantly, as reported earlier as well as in our own studies, only a low dose of MENT need to be administered. In fact, MENT acetate which was administered by subdermal implant to deliver 500 μg/day to healthy men for 28 days maintained serum MENT concentration for the duration of treatment resulting in a dose dependent decrease in gonadotropin levels, although no studies were carried out on its effect on spermatogenesis. No serious adverse reactions were reported. In a subsequent study, MENT acetate, administered to human volunteers at a dose of 400 μg/day was able to cause a dose dependent decrease in serum testosterone LH, FSH and sperm counts. However, no fertility studies were carried out even in this study. However, we have reported (Ramachandra, et al., 2002) that administration of a low dose of 50 μg MENT and 50 pg estradiol resulted in azoospermia in all the adult male bonnet monkeys and the monkeys were infertile. These effects were seen even when none of the animals treated with MENT showed muscle wasting, loss of body weight or testicular volume, which are commonly seen when testosterone concentrations are decreased. It should be pointed out that ours is the only study where in reversible antifertility effects of MENT has been demonstrated in non-human primates.

CONFLICT OF INTEREST

None declared.

ACKNOWLEDGEMENT

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