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Effects of Synthetic Androgens on Liver Function Using the Rabbit as a Model

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ABSTRACT: The objective of this study was to determine whether the rabbit was a suitable model to test new synthetic androgens for potential liver toxicity within a short dosing interval. Adult male rabbits were dosed orally daily on days 0–13 with 17α-methyltestosterone (MT) as a positive control and testosterone (T) as a negative control to validate this model. Synthetic androgens tested were: 7α-methyl-19-nortestosterone (MENT), dimethandrolone-undecanoate (DMAU), and 11β-methyl-19-nortestosterone-17β-dodecylcarbonate (11β-MNTDC). Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transpeptidase (GGT), and sorbitol dehydrogenase (SDH), as well as clearance of intravenous injected bromsulfonphthalein (BSP) from serum on days 0, 7, and 14, were determined. As expected, T (10 mg/kg/d) did not adversely affect BSP retention or serum liver enzymes. MT (10 mg/kg/d) increased BSP retention, and AST, ALT, GGT, and SDH levels, indicating that this model could detect androgens known to be hepatotoxic. DMAU and MENT (10 mg/kg/d) increased BSP retention and all 4 serum liver enzymes as well, but the effects were less than those observed with MT at the same dose. All parameters returned to baseline 2 weeks after cessation of dosing. 11β-MNTDC at 10 mg/kg/d did not have an effect on BSP retention or liver enzymes, but a slight increase in serum GGT levels was observed in rabbits treated with 25 mg/kg/d. For the androgens that exhibited liver toxicity at 10 mg/kg/d (MT, DMAU, and MENT), a no-observed-effect level of 1 mg/kg/d was established. Overall ranking of the synthetic androgens from most to least hepatotoxic on the basis of percent BSP retention was: MT $\gg$ DMAU $>$ MENT $>$ 11β-MNTDC. Hence, the rabbit appears to be a promising model for detection of potential liver toxicity by synthetic androgens using BSP clearance and serum liver enzyme levels as early indicators of injury.

Key words: Dimethandrolone-17β-undecanoate,11β-methyl-19-nortestosterone-17β-dodecylcarbonate, bromsulfonphthalein, hepatotoxicity.

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Previous studies have demonstrated that 17α-alkylated steroids have detrimental effects on the liver of various species, including mice, rabbits, dogs, and humans (deLorimier et al, 1965; Heywood et al, 1977; Tennant et al, 1981; Taylor and Snowball, 1984; Ishak and Zimmerman, 1987; Welder et al, 1995; Pagonis et al, 2008). In contrast, steroids that do not contain the 17α-alkyl moiety did not demonstrate the same effects on the liver (deLorimier et al, 1965; Ishak and Zimmerman, 1987; Welder et al, 1995). The primary disease states induced by 17α-alkylated steroids, which are exemplified by 17α-methyltestosterone (MT), are cholestasis and liver tumors (Moslen, 1996). These disease states evolve after continuous long-term administration. Indeed, the adverse effects of MT on the liver are the major liability prohibiting long-term use of this orally active androgen in humans (deLorimier et al, 1965; Ishak and Zimmerman, 1987; Borhan-Manesh and Farnum, 1989). Potential hepatoxicity has been one of the concerns associated with anabolic androgen abuse, as well as treatment of various disorders with synthetic androgen agonists (eg, MT), and antagonists (eg, flutamide, cyproterone acetate; Miquel et al, 2007; Pagonis et al, 2008; Sanchez-Osorio et al, 2008). Thus, determination of the potential hepatotoxicity of newly developed androgens is a primary concern in drug development because the synthetic androgens would be administered chronically to men for hormone replacement, as hormonal contraceptives, or in both capacities.

Development of model systems to detect hepatotoxicity of test compounds, particularly early in drug development, is desirable. Toward this end, investigators have been developing in vitro models using either primary liver cells or liver-derived cell lines (Welder et al, 1995; Li et al, 1999; Li, 2001; Josse et al, 2008; Sahu et al, 2008). In some instances, investigators have been able to distinguish between hepatotoxic and nonhepatotoxic compounds with selected endpoints, as was determined for 17α-alkylated steroids compared with nonalkylated steroids (Welder et al, 1995). In other

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cases, results from the rat liver–derived clone-9 cells did not completely agree with data obtained from an in vivo rat model (Sahu et al, 2008). In this direct comparison, only 3 of the 8 endpoints evaluated were concordant, suggesting that the in vitro effects were not necessarily predictive of in vivo effects. Our attempts to evaluate the potential effects of known hepatotoxic steroids, as well as several endogenous hormones in the HepG2 C3A liver cell line, were also inconclusive (our unpublished data). However, our data on potential effects of steroids on CYP 450 enzymes were in agreement with those of other investigators supporting the use of these in vitro assays as predictors of drug-induced effects on liver metabolic activity (Li et al, 1999; Li, 2001; Josse et al, 2008). Thus, some in vitro assays appear to be indicative of effects on metabolic activity of the liver, but prediction of liver toxicity on the basis of cytotoxicity of liver cells was inconclusive.

Because the in vitro assays were inconclusive for predicting liver toxicity, we decided to determine whether an in vivo model could be used for screening synthetic androgens for liver toxicity. In an initial study, we treated Sprague Dawley adult male rats with MT at 10 and 25 mg/kg/d for 14 days in an attempt to detect hepatotoxicity in this animal model after a short dosing interval. However, MT had no significant effect on body weight, serum liver enzymes, liver organ weights, or liver histology compared with vehicle-treated rats (our unpublished data). These data suggested that the rat was not a sensitive model for detection of hepatotoxicity in the short term. Therefore, we choose the rabbit as a model because it is a common laboratory animal species that is easily manipulated and appears to be sensitive to liver toxicants (Carmichael et al, 1963; Coert et al, 1975; Tennant et al, 1981). In particular, serum liver enzyme levels were elevated in the rabbit within 2 weeks of dosing with MT, suggesting that this animal model might be sensitive enough to detect toxicity after a short dosing interval (Tennant et al, 1981). The goal of the present study was to determine whether the rabbit model would be useful for detecting potential effects of newly developed synthetic androgens on the liver within a short dosing interval (2 weeks). In the previous toxicity studies, MT dosing occurred for several weeks or months. Therefore, the known hepatotoxicant MT was used as the positive control for hepatotoxicity, and the endogenous androgen, testosterone (T), was tested in the same animal model as a negative control. The experimental design of this liver toxicity protocol in rabbits was based on published studies involving clearance of the dye bromsulfonphthalein (BSP) from circulation after intravenous (IV) administration (Carmichael et al, 1963; Coert et al, 1975; Tennant et al, 1981). The liver of the untreated rabbit normally clears the BSP dye within 20 minutes after IV injection, but this is delayed when the liver is damaged. In addition, serum levels of 4 specific liver enzymes were evaluated because these enzymes are used as indicators of potential liver damage in humans. We used the lowest number of animals possible per group ($n = 3$) for statistical comparisons to determine whether we could establish significant effects for use as a screening assay. After establishment of the assay, including specificity, we evaluated the effects of 3 synthetic androgens, in development for hormonal therapy and contraception in men, in the rabbit model to predict potential liver toxicity: 7α-methyl-19-nortestosterone (MENT), 7α,11β-dimethyl-19-nortestosterone 17β-undecanoate (dimethandrolone undecanoate, DMAU), and 11β-methyl-19-nortestosterone 17β-dodecylcarbonate (11β-MNTDC).

Materials and Methods

Materials

MT was purchased from Sigma-Aldrich Inc (St Louis, Missouri), and T was purchased from Steraloids Inc (Newport, Rhode Island). MENT, DMAU, and 11β-MNTDC were synthesized at the Southwest Foundation for Biomedical Research (San Antonio, Texas) under National Institute of Child Health and Human Development (NICHD) contract NO1-HD-6-3255 and were all 99% pure on the basis of high-performance liquid chromatography (HPLC) analysis. BSP was purchased from Sigma-Aldrich, dissolved in isotonic saline at 40 mg/mL, and filter sterilized before IV injection. Needles, syringes, saline, vacutainer SST tubes, intravenous catheters, and related animal supplies were purchased from NLS Inc (Baltimore, Maryland). Neutral buffered 10% formalin, sodium hydroxide, and other reagent-grade chemicals were purchased from Sigma-Aldrich or VWR Inc (West Chester, Pennsylvania). Food-grade sesame oil (Hain, Melville, New York) was purchased from a local grocery store. Reagents for alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma glutamyl transpeptidase (GGT) assays were purchased from Randox Laboratories Ltd (Oceanside, California), and reagents for sorbitol dehydrogenase (SDH) assays were purchased from Sigma-Aldrich.

Animals

Adult male New Zealand white rabbits (HsdOkd:NZW; 2–3 kg) were purchased from Harlan (Oxford, Michigan) and housed individually in compliance with BIOQUAL's standard operating procedures. The environmental conditions of the animal rooms were maintained as recommended in the National Research Council *Guide for the Care and Use of Animals* (1996). All study protocols were approved by BIOQUAL's institutional animal care and use committee before undertaking any experiments.

Treatment of Animals

Rabbits (3/group) were dosed orally on days 0–13 with androgens dissolved in 10% ethanol/sesame oil at a dose volume of 0.5 mL/kg. The group size of 3 was the minimum number of animals that allowed statistical comparisons. This number was kept at a minimum to determine whether the assay design could detect significant effects on the liver for use as a screening test. Body weights were obtained on days 0, 7, and 14, and adjustments to doses were made on the basis of the most recent body weight. Dose groups included: MT, DMAU, and MENT at 10, 3, and 1 mg/kg/d, respectively; T at 10 mg/kg/d; and 11β-MNTDC at 10 and 25 mg/kg/d. MT, a known hepatotoxicant, was included as a positive control, whereas the natural hormone, T, was included as a negative control. The synthetic androgen MENT is being developed for use in men (Sundaram and Kumar, 2000). Because this androgen is structurally similar to the synthetic androgens released from the 17β-esters, DMAU, and 11β-MNTDC, which are being developed by the Contraception and Reproductive Health Branch of NICHD, we included them in this study. All animals were observed cage side twice daily, except weekends, for clinical signs of toxicity. A more careful physical evaluation was given during routine daily handling for technical procedures.

Serum Liver Enzyme Determinations

Before dosing on days 0, 3, 7, 10, and 14 (24 hours after the last dose), blood was collected from the central ear artery of each rabbit. Serum was harvested from clotted blood, snap frozen in liquid nitrogen, and stored at −80°C until analyzed for levels of ALT, AST, GGT, and SDH by Ani Lytics Inc (Gaithersburg, Maryland). Serum ALT and AST levels were determined according to International Federation of Clinical Chemistry and Laboratory Medicine–recommended ultraviolet (UV) methods (Karmen et al, 1955; Wroblewski and La Du, 1956a,b) on a Hitachi 717 spectrophotometer (Indianapolis, Indiana). GGT levels in serum samples were detected using the standard colorimetric method according to the European Committee for Clinical Laboratory Standards (Szasz and Bergmeyer, 1974). SDH activity in serum was determined on the basis of the reduction of D-fructose (Asada and Galambos, 1963; Wiesner et al, 1965). The level of D-fructose was detected by absorbance at 340 nm on the Hitachi 717 spectrophotometer.

Serum BSP Determinations

Immediately after blood collection for clinical chemistry tests on days 0, 7, and 14, rabbits were injected with 20 mg/kg BSP in the marginal auricular vein (Carmichael et al, 1963; Coert et al, 1975; Tennant et al, 1981). In the initial experiments with 10 mg/kg/d of MT, T, and DMAU, rabbits were bled from the central auricular artery of the contralateral ear at 0, 5, 10, and 20 minutes after the BSP injection. A 1-minute time point after IV injection of BSP was added to subsequent studies. Although variability in serum BSP levels at the 1-minute time point was greater than at the other time points, the absolute values were at least 2-fold greater than those at the 5-minute time point. Serum samples were stored frozen, and BSP levels were determined in 1 assay for each treatment group. The BSP concentration in the serum samples was determined on the basis of a modification of the original method described by Henry et al (1959). BSP was dissolved in normal saline, and serial dilutions were made to produce a standard curve from 0 to 200 μg/mL in 0.1 N sodium hydroxide. Serum samples were diluted 2-fold with 0.2 N sodium hydroxide. Both standards and samples were aliquoted in 96-well plates, and the optical density at 562 nm was measured with a Molecular Devices Vmax kinetic microplate reader (Sunnyvale, California). The limit of detection was the lowest BSP standard, 3.1 μg/mL. The BSP recovery was 87.1% ± 1.9% of expected, and interassay variation was 15.4%. Normal intact male rabbit serum exhibited background levels near the limit of detection, 3–4 μg/mL. If an elevation in serum BSP retention and liver enzymes was observed, additional blood samples were collected through day 28, and a BSP test was performed on day 28, 2 weeks after cessation of treatment, to assess recovery of the liver.

Analysis of Serum for Androgen Levels

T levels were determined in serum samples from rabbits dosed orally with T at 10 mg/kg/d using DPC's Coat-A-Count radioimmunoassay (RIA; Diagnostic Products Corp, Los Angeles, California). Serum samples were extracted with 1-chlorobutane, reconstituted in zero calibrator, and assayed as described in the kit insert. The EC_{90} value of the standard curve for the RIA was set as the limit of detection and was 0.08 ng/mL. The serum samples were all assayed at one time, and the intra-assay variation was 6.3%.

Serum levels of dimethandrolone (DMA) and immunoreactive metabolites were determined in rabbits dosed orally with DMAU at 1, 3, or 10 mg/kg/d using a specific RIA developed at BIOQUAL (Attardi et al, 2006). Serum samples were extracted by methanol precipitation before incubation with the primary rabbit antiserum at a final dilution of $1{:}6.0 \times 10^6$. The limit of detection, 0.093 ng/mL, was calculated as the mean + 3 SDs of the background values from 17 μL of serum per tube collected before treatment and extracted. The serum samples were all assayed in a single assay, and the intra-assay variation was 2.2%.

Serum levels of 11β-MNT and immunoreactive metabolites were determined in rabbits dosed orally with 11β-MNTDC at 10 or 25 mg/kg/d using a specific RIA developed at BIOQUAL. Polyclonal antisera to 11β-MNT were generated in rabbits by immunization with the 3-(carboxymethyl)oxime-BSA conjugate of 11β-MNT (Vaitukaitis et al, 1971; Larner et al, 2000; and Attardi et al, 2006). The 3-(carboxymethyl)oxime-histamine conjugate of 11β-MNT was iodinated with 1 mCi Na[^{125}I] (Perkin Elmer Life Sciences Inc, Waltham, Massachusetts) using chloramine-T as the catalyst. The iodinated conjugate was extracted with benzene and purified by reverse phase HPLC. Serum samples were extracted by methanol precipitation. For the RIA, an 11β-MNT standard curve, suitable dilutions of extracted serum, PBS + 7.5% methanol, and antiserum from rabbit AF-9, bleed 5, at a final dilution of $1{:}3.6 \times 10^6$, were preincubated in the assay tubes

for 1 hour at room temperature. Radioligand was added, and the tubes were incubated overnight at 2–6°C. Bound and free radioligand were separated by centrifugation after addition of dextran-coated charcoal. The supernatants were transferred to fresh tubes and counted in a Packard Cobra II γ-counter, and the raw data were exported to the RiaSmart data reduction program. A 4-parameter logistic curve fit was used to generate the standard curve and interpolate the serum concentrations of 11β-MNT. The limit of detection, 0.59 ng/mL, was calculated as the mean + 3 SDs of the background values from 1 μL of serum per tube collected before treatment and extracted. The serum samples were all assayed at one time and the intra-assay variation was 2.9%.

Because the metabolism of 11β-MNT has not been studied, we do not know whether other potential metabolites would cross-react in this RIA. However, the antiserum used in this RIA has been tested extensively for cross-reactivity with compounds closely related to 11β-MNT and has been shown to be specific for 11β-MNT. We have determined that there is negligible (<0.1%) cross-reactivity between 11β-MNTDC and the rabbit antiserum; therefore, levels of 11β-MNT in serum of rabbits administered 11β-MNTDC are only detectable after cleavage of the 17β-dodecylcarbonate moiety. Cross-reactivity with endogenous hormones (ie, T, 5α-dihydrotestosterone, 17β-estradiol, progesterone, and cortisol) was less than 1%.

Statistical Analysis

All statistical analyses were performed using SigmaStat for Windows, Version 3.5 (SPSS Inc, Chicago, Illinois). All tests were 2-tailed with significance set at $\alpha = 0.05$. Significant differences in body weight over time were determined on the basis of 1-way analysis of variance on repeated measures (ANOVA-RM) for each bioassay. Significant treatment-related effects on the serum levels of AST, ALT, GGT, and SDH, were determined by 1-way ANOVA-RM or Friedman ANOVA-RM on ranks with the Holm-Sidak method of comparison to baseline/control levels obtained on day 0. BSP levels for each study were graphed with SigmaPlot for Windows, Version 10.0 (SPSS Inc), and the area under the curve ($AUC_{0–20min}$) determined over the 20-minute interval. Significant differences in BSP AUC values were determined by ANOVA-RM followed by the Holm-Sidak comparison test. To compare the effects of the various treatments on serum BSP dye retention across multiple studies, the percentage of BSP dye retention was calculated as $AUC_{0–20min}$ on day 14 divided by $AUC_{0–20min}$ on day 0 multiplied by 100. Percent BSP dye retention at baseline (day 0) was defined as 100%. Significant difference in percent BSP dye retention across treatment groups was determined by ANOVA-RM, followed by the Holm-Sidak comparison test. The $AUC_{0–14days}$ was determined for serum levels of DMA and 11β-MNT at each dose level. Significant differences in DMA AUC values were determined by ANOVA-RM, followed by the Holm-Sidak comparison test (3 dose levels), and 11β-MNT AUC values were compared with Student's *t* test (2 dose levels).

Results

General Toxicity

None of the rabbits in these studies exhibited overt clinical signs of toxicity in response to treatment with androgen. Body weights were not suppressed by any androgen treatment (not shown). Slight, but significant, increases in body weight were observed in some rabbits over time. In rare cases, rabbits developed necrotic tissue around the marginal auricular vein from BSP entering the subcutaneous space surrounding the vein during venipuncture. The few rabbits with an affected ear were treated with topical antibiotics, and the tissue healed without incident.

Effects of T and MT on the Liver: Model Validation

Before treatment (day 0), serum levels of BSP dye returned to background within 20 minutes after IV injection, suggesting that the BSP was essentially cleared from the circulation within this time, and these data agree with previous investigations (Carmichael et al, 1963; Coert et al, 1975; Tennant et al, 1981). T was administered orally at 10 mg/kg/d to serve as a negative control. Indeed, treatment with T resulted in a significant ($P < .05$) reduction in BSP retention ($AUC_{0–20\ min}$) on days 7 and 14 compared with baseline (day 0; Table). T treatment had no effect on the liver enzymes ALT and GGT. However, a slight, but significant ($P < .05$), increase in serum SDH and AST levels was observed on day 14, and AST levels were transiently, but significantly, decreased ($P < .05$) on day 7 of T treatment (Figure 1A). Serum T levels were extremely variable among the 3 intact male rabbits before treatment (day 0). This variability continued throughout the treatment interval (samples obtained 24 hours after the previous oral dose), and there were no obvious increases or decreases in circulating T levels after initiation of treatment (data not shown).

Rabbits were dosed orally with MT, an androgen with known hepatotoxic properties, as a positive control. After oral dosing of MT at 10 mg/kg/d for 7 or 14 days, all 3 rabbits demonstrated elevated serum BSP levels up to 20 minutes after the IV injection compared with BSP levels before oral dosing. Significant increases ($P < .05$) in the BSP $AUC_{0–20\ min}$ were observed on days 7 and 14 compared with baseline (day 0; Table). These returned to baseline 2 weeks after cessation of treatment (day 28). AST, ALT, GGT, and SDH were all significantly increased ($P < .05$) on days 7, 10, and 14 compared with baseline in serum of rabbits dosed with MT (Figure 1B). By day 28, the serum enzyme levels had returned to baseline values.

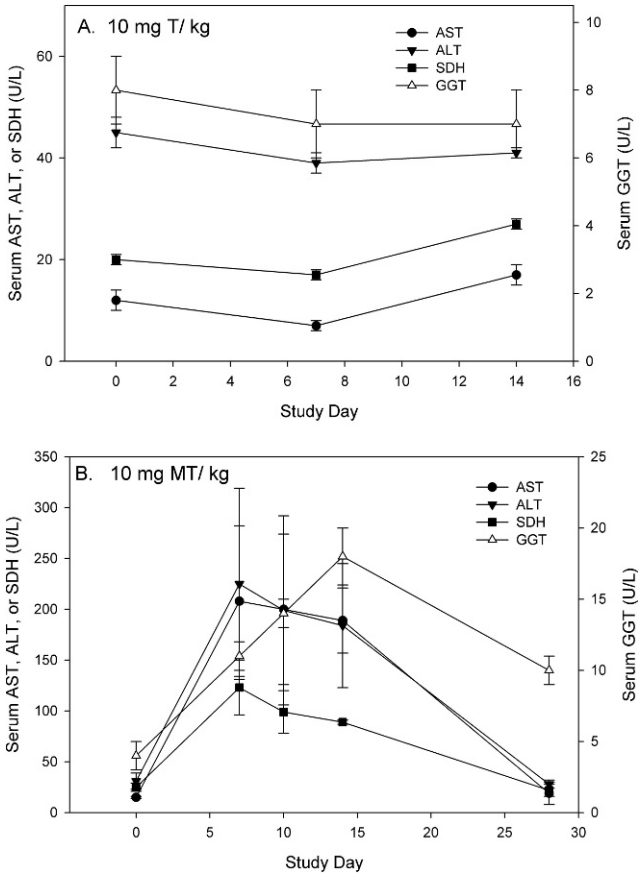

Figure 1. Serum levels of the liver enzymes AST, ALT, GGT, and SDH in rabbits dosed orally with 10 mg/kg/d of T **(A)** or MT **(B)** for 14 days. Data points represent mean ± SE (n = 3). ALT indicates alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma glutamyl transpeptidase; MT, 17α-methyltestosterone; SDH, sorbitol dehydrogenase; T, testosterone.

Earlier time points were evaluated to determine whether MT-induced increases in BSP dye retention and serum liver enzyme levels could be detected before day 7. Baseline BSP clearance and serum liver enzyme levels were determined on day −7. BSP dye retention appeared to be increased on days 3 and 7, but unlike the previous results, there was no significant effect on $AUC_{0–20\ min}$ ($P > .05$; data not shown). Likewise, serum liver enzyme levels were not significantly different from baseline levels on days 1, 3, 4, or 7 ($P > .05$; not shown). These data suggested that the assay was not necessarily a reliable predictor of toxicity when dosing was less than 14 days.

To determine a no-observed-effect level (NOEL) on liver function for MT, we repeated the study at lower daily doses. At 3 mg/kg/d of MT, significant increases ($P < .05$) in BSP dye retention ($AUC_{0–20min}$) were observed on days 7 and 14 (Table). In addition, serum GGT levels were significantly increased by day 10 of treatment (data not shown). Serum levels of AST, ALT, and SDH tended to increase during the treatment interval, but these values were not significantly different ($P > .05$; data not shown) from baseline (day 0). At 1 mg MT/kg/d, there was no increase in BSP dye retention ($AUC_{0–20min}$); rather a slight, but significant, decrease was observed on day 7 (Table). Serum liver enzyme levels also were not affected, except for a slight, but significant, transitory increase in serum GGT levels on day 7 (data not shown), establishing 1 mg/kg/d as the NOEL.

Testing of Synthetic Androgens for Potential Adverse Effects on the Liver

DMAU—DMAU at 10 mg/kg/d, significantly increased ($P < .05$) BSP dye retention on days 7 and 14, and all 4 serum liver enzyme levels on days 7, 10, and 14 (Table;

Table. *Serum bromsulfonphthalein (BSP) levels presented as area under the curve (AUC) in rabbits dosed orally with androgens for 14 days*

Daily Dose of Androgen, mg/kg/d	Study Day	Serum BSP $AUC_{0–20min}$, μg/ml·min[a]			
		T	MT	DMAU	MENT
10	0	997 ± 57 a	646 ± 58 a	1048 ± 193 a	1301 ± 29 a
	7	630 ± 60 b	1558 ± 174 b	2251 ± 124 b	1985 ± 132 b
	14	529 ± 52 b	1774 ± 84 b	1766 ± 62 c	2139 ± 40 b
	28		933 ± 222 a	864 ± 25 a	1415 ± 59 a
3	0		1252 ± 61 a	1596 ± 132 a	1516 ± 59
	7		1869 ± 56 b	2105 ± 169 b	1797 ± 156
	14		1763 ± 198 b	2390 ± 324 b	1711 ± 116
1	0		1782 ± 91 a	1486 ± 136	1833 ± 247
	7		1540 ± 48 b	1603 ± 274	2241 ± 266
	14		1686 ± 21 ab	1452 ± 291	1893 ± 81

Abbreviations: DMAU, dimethandrolone-undecanoate; MENT, 7α-methyl-19-nortestosterone; MT, 17α-methyltestosterone; T, testosterone.

[a] AUC values represent mean ± SE (n = 3), except for day 7 of the 10 mg/kg/d MENT group (n = 2) and, therefore, mean ± SD. Means with different letters within a dose level are significantly different from one another ($P < .05$).

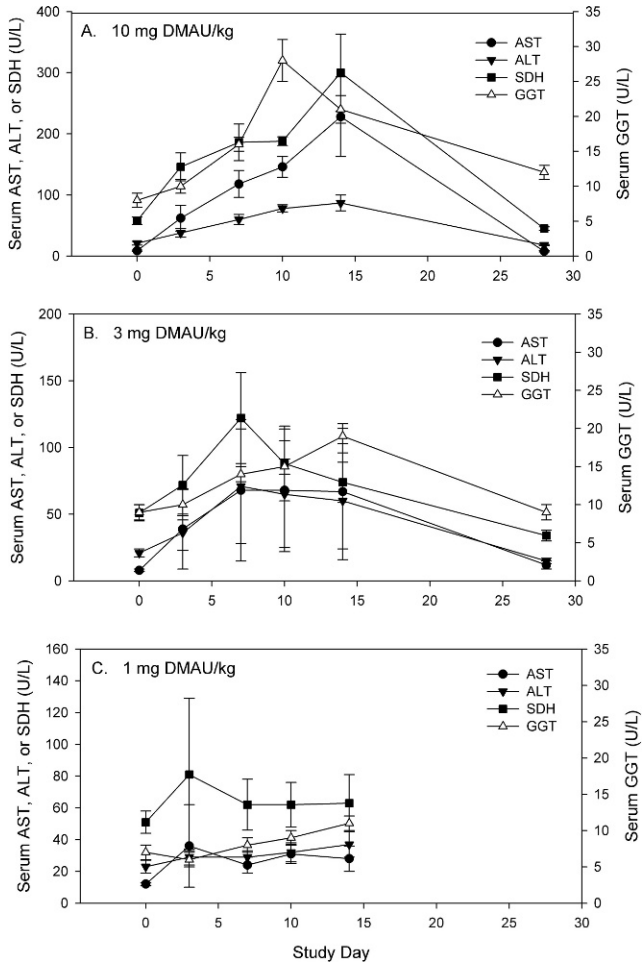

Figure 2. Serum levels of the liver enzymes AST, ALT, GGT, and SDH in rabbits dosed orally with **(A)** 10, **(B)** 3, or **(C)** 1 mg/kg/d of DMAU for 14 days. Data points represent mean ± SE (n = 3). ALT indicates alanine aminotransferase; AST, aspartate aminotransferase; DMAU, dimethandrolone-undecanoate; GGT, gamma glutamyl transpeptidase; SDH, sorbitol dehydrogenase.

Figure 2A, respectively). BSP dye retention and serum liver enzyme levels returned to baseline after cessation of treatment (day 28). At 3 mg DMAU/kg/d, serum BSP dye retention was significantly elevated above baseline on days 7 and 14 ($P < .05$) but returned to baseline on day 28 ($P > .05$; Table). Although serum levels of AST, ALT, GGT, and SDH tended to increase during the course of treatment, significant increases ($P < .05$) were observed only in serum levels of GGT on days 10 and 14 and SDH levels on day 7 (Figure 2B). No significant increases in serum BSP dye retention were observed in rabbits dosed orally with 1 mg DMAU/kg/d ($P > .05$; Table). Slight, but significant ($P < .05$), increases in serum ALT and GGT levels were observed on days 10 and 14 at this dose, but there were no significant elevations ($P > .05$) in the serum levels of AST and SDH (Figure 2C).

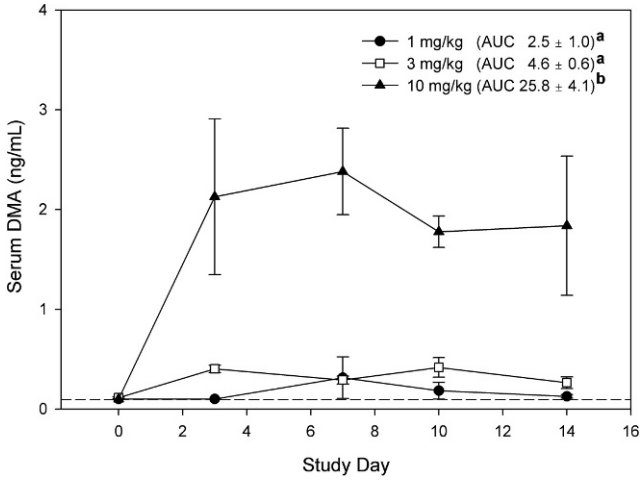

Figure 3. Serum levels of DMA in rabbits dosed orally for 14 days with DMAU. The $AUC_{0–14days}$ for each dose level is presented (ng/mL·d). Means with different letters are significantly different from one another ($P < .05$). Data points and AUC values represent mean ± SE (n = 3). AUC indicates area under the curve; DMA, dimethandrolone.

After oral administration of DMAU at 3 or 10 mg/kg/d for 14 days, DMA and immunoreactive metabolites were detected in the serum samples for the full 14 days (Figure 3). In contrast, levels of DMA were near the limit of detection (0.10 ng/mL) in serum samples from rabbits dosed orally with DMAU at 1 mg/kg/d. Overall, there was a dose-dependent increase in serum levels of DMA over the 14-day interval ($AUC_{0–14days}$). In all cases, DMA and immunoreactive metabolites were nondetectable in serum samples on day 28.

MENT—At 10 mg/kg/d, MENT resulted in increased serum BSP dye retention ($AUC_{0–20min}$) on days 7 and 14 ($P < .05$; Table). Serum levels of AST, ALT, GGT, and SDH were all significantly increased ($P < .05$) on days 7, 10, and 14 in MENT-treated rabbits (Figure 4A). Both serum BSP retention and liver enzyme levels returned to baseline by day 28. At an oral dose of 3 mg/kg/d, MENT resulted in intermediate effects on the liver. Serum BSP dye retention tended to increase with the treatment interval, but the $AUC_{0–20min}$ for serum BSP levels on days 7 and 14 were not significantly different ($P > .05$) from baseline (day 0; Table). Serum levels of AST, ALT, GGT, and SDH were all significantly elevated by day 10 of treatment, with 3 mg/kg/d of MENT ($P < .05$; Figure 4B). At 1 mg/kg/d, MENT did not significantly affect serum BSP dye retention or serum liver enzymes levels ($P > .05$), except for a slight, but significant ($P < .05$), increase in serum GGT levels on days 10 and 14 (Table; Figure 4C, respectively).

11β-MNTDC—At 10 mg/kg/d, 11β-MNTDC did not have any effect on serum BSP dye retention on day 7 or 14. Likewise, serum levels of AST, ALT, and SDH were

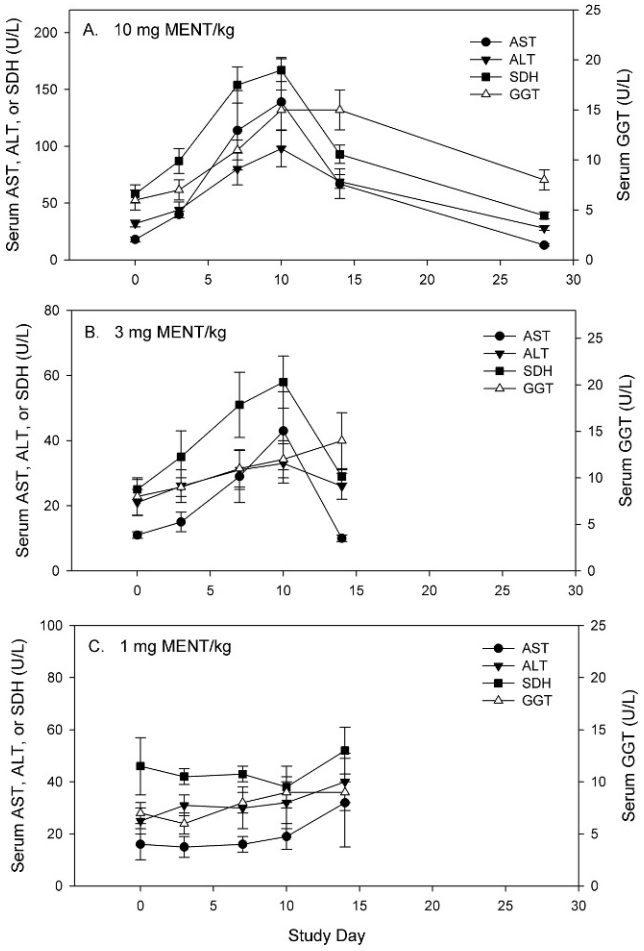

Figure 4. Serum levels of the liver enzymes AST, ALT, GGT, and SDH in rabbits dosed orally with **(A)** 10, **(B)** 3, or **(C)** 1 mg/kg/d of MENT for 14 days. Data points represent mean ± SE (n = 3). ALT indicates alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma glutamyl transpeptidase; MENT, 7α-methyl-19-nortestosterone; SDH, sorbitol dehydrogenase.

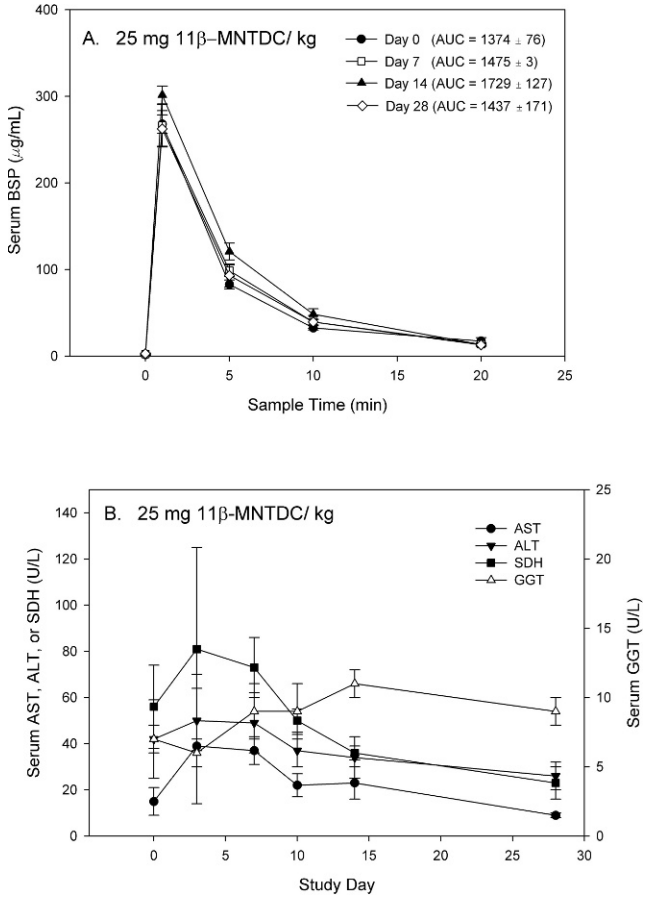

Figure 5. Serum BSP **(A)** and liver enzyme **(B)** levels in rabbits dosed orally with 25 mg/kg/d of the synthetic androgen 11β-MNTDC for 14 days. The $AUC_{0-20min}$ (μg/mL·min) for BSP levels are presented in Panel A. Data points and AUC values represent mean ± SE (n = 3). ALT indicates alanine aminotransferase; AST, aspartate aminotransferase; AUC, area under the curve; BSP, bromsulfonphthalein; GGT, gamma glutamyl transpeptidase; SDH, sorbitol dehydrogenase; 11β-MNTDC, 11β-methyl-19-nortestosterone-17β-dodecylcarbonate.

not affected by treatment with 11β-MNTDC. A slight, but significant, increase in serum GGT was observed on days 10 and 14 ($P < .05$). These results suggested that, in contrast to the other synthetic androgens tested in this assay, 10 mg/kg/d was a NOEL. Hence, we tested this compound at a higher daily dose. At 25 mg/kg/d, 11β-MNTDC resulted in a slight increase in serum BSP dye retention on day 14, but this was not significant ($P > .05$; Figure 5A). Serum levels of AST, ALT, and SDH were not affected by treatment with 11β-MNTDC at 25 mg/kg/d (Figure 5B). However, a slight, but significant, increase in serum GGT was observed on day 14 ($P < .05$). By day 28, serum GGT levels were not different from those obtained on day 0.

After oral administration of 11β-MNTDC at 10 and 25 mg/kg/d for 14 days, 11β-MNT and immunoreactive metabolites were detected in the serum samples for the full 14 days (Figure 6). Although there was a 2.5-fold increase in dose level, there was only a slight, but not significant, increase in serum levels of 11β-MNT over the 14 day interval ($AUC_{0-14days}$) at 25 mg/kg/d compared with 10 mg/kg/d of 11β-MNTDC (Figure 6). In all cases, serum levels of 11β-MNT and immunoreactive metabolites were nondetectable at day 28.

Comparison of All 5 Androgens

Because all 5 androgens were tested at 10 mg/kg/d for 14 days, percent BSP dye retention was determined for comparison with relative heptatotoxicity (Figure 7). The percent BSP dye retention after oral administration of T was below baseline (100%, defined), whereas the synthetic androgens MT, DMAU, and MENT resulted in significant increases in percent BSP retention ($P < .05$). In contrast, the synthetic androgen 11β-MNTDC at 10 mg/kg/d did not increase percent BSP dye retention

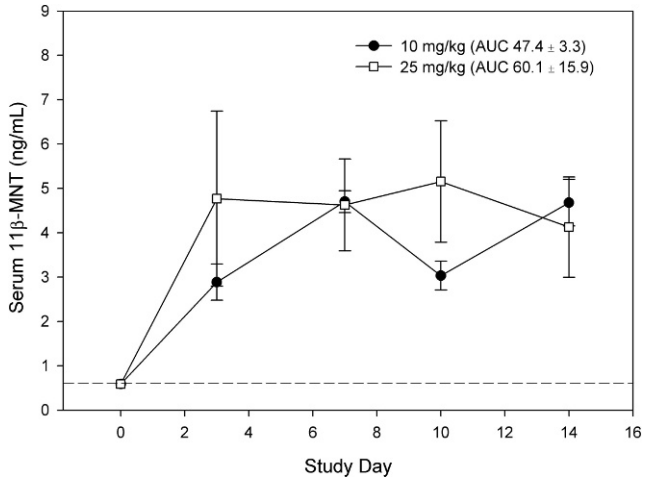

Figure 6. Serum levels of 11β-MNT in rabbits dosed orally for 14 days with 11β-MNTDC. The $AUC_{0-14days}$ (ng/mL·min) for each dose level is presented. Data points and AUC values represent mean ± SE (n = 3). AUC indicates area under the curve; 11β-MNTDC, 11β-methyl-19-nortestosterone-17β-dodecylcarbonate.

significantly. On the basis of these data, the overall ranking of the synthetic androgens from most to least hepatotoxic is MT ≫ DMAU > MENT > 11β-MNTDC.

Discussion

The evaluation of BSP clearance from circulation and the determination of serum liver enzymes in treated adult male rabbits appear to be useful markers for detection of potential liver toxicity of synthetic androgens over a short dosing interval. Our data support the results of previous investigators, indicating that MT has hepatotoxic properties (deLorimier et al, 1965; Heywood et al, 1977; Tennant et al, 1981; Taylor and Snowball, 1984; Ishak and Zimmerman, 1987; Welder et al, 1995). In contrast, the endogenous hormone T did not increase BSP retention and had minimal effects on serum liver enzyme levels (slight increases in serum SDH and AST). The data indicated that significant effects, as would be required for a screening assay, were obtainable with a small sample size (n = 3). Likewise, the duration of dosing, 14 days, was considerably shorter than that of the previous toxicology studies. In addition, since this is a nonterminal study, rabbits could potentially be used to test additional compounds after an adequate recovery interval. Baseline BSP retention and serum liver enzymes would be reestablished for testing of new synthetic steroids in the same animals.

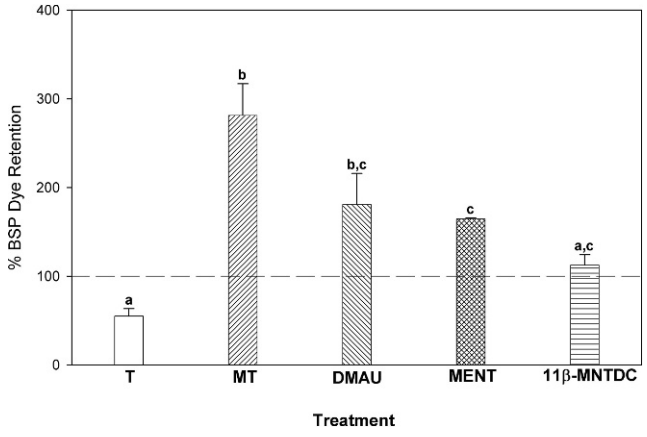

Figure 7. BSP dye retention in rabbits dosed orally for 14 days with 10 mg/kg/d of T, MT, DMAU, MENT, or 11β-MNTDC. Percent BSP retention was calculated as $[(\text{day14 } AUC_{0-20\ min})/(\text{day 0 } AUC_{0-20\ min})] \times 100$. Bars with different letters are significantly different ($P < .05$) from one another; bars and brackets represent mean ± SE (n = 3). Dashed line represents baseline, which was defined as 100%. BSP indicates, bromsulfonphthalein; DMAU, dimethandrolone-undecanoate; MENT, 7α-methyl-19-nortestosterone; 11β-MNTDC, 11β-methyl-19-nortestosterone-17β-dodecylcarbonate; MT, 17α-methyltestosterone; T, testosterone.

Treatment effects on BSP clearance were observed within a short dosing interval, 7 to 14 days of oral dosing, in the rabbit. However, our attempt to shorten the dosing interval to less than 14 days indicated that the assay was not sufficiently reliable at shorter time points because the observed effects were not consistently statistically significant with the small sample size. Thus, we recommend a dosing interval of at least 14 days for evaluation of potential effects. BSP retention appears to be a sensitive and rapid indicator of synthetic androgen-mediated adverse effects on the liver and could be useful for evaluation of other classes of steroids as well. BSP is primarily eliminated by the liver through the biliary system, requiring uptake by hepatocytes, conjugation with glutathione, translocation across the bile canalicular membrane, and excretion in bile (Chuttani et al, 1965; Colon et al, 1974; Yam et al, 1976; Molino et al, 1982; Snel et al, 1995). Thus, compounds that interfere with BSP clearance could have a cholestatic effect; however, other types of hepatotoxicants, including cadmium and carbon tetrachloride, can interfere with BSP elimination (Molino et al, 1982; Soto et al, 2002). Because cholestasis is typically observed only after long-term or chronic treatment, evaluation of BSP retention might be a useful indicator of potential cholestatic agents early in the drug development process. Renal elimination of BSP and BSP conjugates is normally negligible (Yam et al, 1976; Snel et al, 1995). However, secondary renal elimination of BSP is observed in rats with surgically obstructed bile ducts, which is associated with increased renal organic anion transporting peptide 1 and bilitranslocase and is most likely an adaptation in response to the injury to the liver (Brandoni and Torres, 2009; Brandoni et al, 2010). Chronic renal failure can

also impair liver uptake of BSP (Sun et al, 2006), but it is unlikely that these conditions would have an effect on the results of a short-term screening assay in healthy adult rabbits. Further evaluation of this rabbit model with known steroidal hepatotoxicants versus those considered safe (eg, endogenous steroid hormones) will determine the value of this model system for predicting potential synthetic steroid-induced hepatotoxicity as a screening method.

Although 3 of the synthetic androgens, MT, DMAU, and MENT, had the same NOEL, 1 mg/kg/d, the severity of the dose-dependent effects on serum liver enzyme levels, and BSP retention differed. In particular, BSP retention was quantified and compared across all the androgens at the 10 mg/kg/d dose, which was then used to rank potential hepatotoxicity. MT clearly exhibited the greatest activity. DMAU and MENT both increased BSP dye retention, but the effect of MT was still 2-fold greater than that of either DMAU or MENT. Additionally, DMAU has been shown to be more potent than MT in terms of both oral androgenic (stimulation of ventral prostate and seminal vesicles growth) and anabolic (stimulation of levator ani muscle growth) activity—approximately 3- and 20-fold, respectively—suggesting a favorable therapeutic index (Attardi et al, 2006). 11β-MNTDC did not exhibit liver toxicity, except for a slight effect on serum GGT levels at a higher daily dose, 25 mg/kg/d. However, serum levels of the active androgen, 11β-MNT, were not significantly increased despite a 2.5-fold increase in dose. This suggests that absorption of 11β-MNTDC, cleavage of the dodecylcarbonate ester by esterases, or both was maximal, and higher oral doses would not result in greater circulating levels. Alternatively, the interference of a metabolite in the 11β-MNT assay could account for the apparent lack of dose-dependent increases in serum levels. Because 11β-MNT only differs from DMA by the methyl group at the 7α-position, and this 7α-methyl group is common to both DMA and MENT, it is interesting to speculate that the addition at the 7α-position could be a contributing factor to the observed effects of DMAU and MENT on the liver. Additional studies would be needed to support this initial conjecture.

In conclusion, the adult male rabbit model used in this study, in which BSP clearance as well as serum liver enzyme levels are compared before and during treatment, appears to be promising for detecting potential liver toxicity of synthetic androgens over a short dosing interval. In particular, this model could be useful for identifying drugs (eg, synthetic steroids) with potential cholestatic effects early in the drug development process. Additional testing is required to determine the full value of this model as a screening tool for liver toxicity of other synthetic steroids (estrogen or progestin agonists and antagonists). On the basis of the results of this study, we were able to rank the synthetic androgens from most to least hepatotoxic: MT $\gg$ DMAU $>$ MENT $>$ 11β-MNTDC. Further development of these new synthetic androgens for use as hormone replacement, male contraception, or both in humans will depend on their efficacy and safety profile.

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