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RADIOIMMUNOASSAY OF 7α-METHYL-19-NORTESTOSTERONE AND INVESTIGATION OF ITS PHARMACOKINETICS IN ANIMALS

NARENDER KUMAR, ASHOK K. DIDOLKAR, ANNA LADD, ROSEMARIE THAU, CARL MONDER, C. WAYNE BARDIN and KALYAN SUNDARAM*

Center for Biomedical Research, The Population Council, 1230 York Avenue, New York, NY 10021, U.S.A.

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Summary—A method for the measurement of 7α-methyl-19-nortestosterone (7MENT) in serum/plasma by radioimmunoassay (RIA) is described. The antiserum, raised against 7α-methyl-19-nortestosterone-3-*O*-oxime-bovine serum albumin, had a low titer (final dilution = 1:4500) and low affinity ($K_a = 1.17 \times 10^9$ l/mol) but showed little or no cross-reactivity with several of the steroids tested. The sensitivity of the RIA was 28.2 pg/ml and the mean recovery of added cold steroid was 86 to 100%. Intra- and inter-assay coefficients of variation ranged from 4.3 to 7.3% and 7.3 to 8.4%, respectively. This RIA was used to follow plasma 7MENT levels after a single i.v. injection of the steroid in rats and rabbits. The metabolic clearance rates (MCR) of 7MENT as determined from the plasma disappearance curve for rats and rabbits were 50 l/day and 336 l/day, respectively. The MCR of 7MENT in rats and rabbits lies in the same range as for testosterone. When compared to other nortestosterone derivatives such as norethisterone, 7MENT is metabolized relatively faster.

INTRODUCTION

The preferred method of androgen replacement over several decades has been the injection of long-acting testosterone esters. Such treatment is an essential part of therapy for primary and secondary hypogonadism [1]. In addition, androgen supplementation has been proposed as a component of several potential male contraceptives that involve suppression of the pituitary–gonadal axis. For example, regimens using either progestins or LHRH analogs combined with androgens can be used to suppress spermatogenesis [2, 3]. Various long-acting testosterone esters have also been used for this purpose [4, 5]. The disadvantage of these agents is that they are derivatives of testosterone and as a consequence a large mass of ester must be administered so as to deliver 5–7 mg of testosterone daily; this limits the dosage form that can be used in injectable preparations. There are a variety of synthetic androgens such as the methylated derivatives of nortestosterone that have been shown to be more potent than testosterone and dihydrotestosterone in restoring the accessory sex organs in castrated animals. If appropriate delivery systems were available, these synthetic androgens could be used for androgen therapy since much lower daily doses would be needed. For this purpose our laboratory has been investigating 7α-methyl-19-nortestosterone (7MENT) [6]. The present studies were undertaken to develop a radioimmunoassay for 7MENT and to perform pharmacokinetic studies of this androgen in rats and rabbits.

EXPERIMENTAL

Solvents and reagents

Diethyl ether (Mallinckrodt), methanol, Tris (hydroxymethyl)aminomethane (Tris), and sodium chloride were all reagent grade. Charcoal and dextran T-70 were purchased from J. T. Baker and Pharmacia Fine Chemicals, respectively. Sodium azide was obtained from J. T. Baker and gelatin from Difco Laboratories.

Steroids

The synthetic androgen, 7MENT was kindly provided by Dr John Babcock, The Upjohn

*To whom correspondence should be addressed.

Co., Kalamazoo, Mich. The 7MENT acetate was custom synthesized by Steraloids Inc. (Wilton, N.H.). The intermediate compound used to prepare [6,7-H^3]MENT, 17β-hydroxy-7-methyl-estra-4,6-diene-3-one, was synthesized by Dr Stephen A. Matlin (London, England) by arrangement with Dr Peter Crabbe of the International Organization for Contraceptive Development. [6,7-^{3}H]MENT was prepared by catalytically reducing 17β-hydroxy-7-methyl-estra-4,6-diene-3-one in dioxane with tritium over 5% Pd/$CaCO_3$ at New England Nuclear Laboratories (Boston, Mass). Purity of the labeled 7MENT was confirmed by TLC using dichloromethane/acetone (8:2, v/v) as mobile phase. The SA was 42 Ci/mmol. The other steroids used for cross-reactivity studies were obtained from Sigma Chemical Co. (St Louis, Mo.).

Antisera

7MENT-3-(*O*-carboxymethyl)oxime(7MENT-CMO) was prepared according to the method of Erlanger *et al.* [7]. 7MENT-CMO was conjugated to bovine serum albumin (BSA) according to the method described by Lindner *et al.* [8]. The conjugate was analyzed as described by Erlanger *et al.* [7] by u.v. spectrophotometry. The results of the analysis revealed that the conjugate contained 32 mol of 7MENT per mol of BSA. The antisera against 7MENT-CMO-BSA conjugate were raised in rabbits. High titer antisera were pooled, aliquoted and stored at −20°C. The optimum final dilution of antiserum was 1:4500.

Extraction of 7MENT from serum

Depending on the levels of 7MENT, the required amount of plasma or serum was diluted with double distilled water to make a final volume of 0.6 ml. The diluted samples were extracted with 4 ml of diethyl ether (2 min, multi-tube vortexer). After freezing the aqueous phase in dry ice/acetone mixture, the organic phase was decanted and evaporated under a stream of nitrogen. Residues were dissolved in 0.1 ml of assay buffer containing 5% methanol. Tris–HCl buffer (0.05 M, pH 7.4) containing 0.1% Knox gelatin, 0.9% NaCl and 1% sodium azide was used as RIA buffer instead of phosphate buffer in order to accommodate the biodegradable scintillation fluid (Ready Safe™, Beckman, Calif.).

Assay procedure

All extraction and assay tubes were rinsed with 95% ethanol and dried. The final incubation volume was 0.3 ml, consisting of 0.1 ml sample or standard, 0.1 ml antiserum (dilution 1:1500) and 0.1 ml of [^{3}H]7MENT (~25,000 cpm). 7MENT concentrations in the assay standards ranged from 7.8 to 1000 pg/tube. All the tubes used for the standards contained ether-extract residue from 0.2 ml of normal rat serum pool to compensate for the serum blanks. The assay tubes were incubated at room temperature for 30 min and then at 4°C overnight. Free radioactivity was separated from the antibody-bound fraction by adsorption on dextran-coated charcoal (0.5%, w/v, charcoal and 0.05%, w/v, dextran T-70 in assay buffer). 0.5 ml of charcoal suspension (pre-chilled on ice) was added to each tube and mixed. After 15 min at 4°C, the tubes were centrifuged for 15 min at 1000 ***g*** in a refrigerated centrifuge. Supernatants were decanted into scintillation vials and 5 ml of Ready Safe™ (Beckman) scintillation fluid was dispensed into each vial. The contents of the vials were mixed for 5 min using a horizontal shaker and counted in a liquid scintillation counter (Packard) for 5 min each. Calculations were performed using Rodbard-NIH RIA computer program as modified by Dr Glen Gunsalus at the Population Council.

Affinity

The affinity constant (K_a) of the antiserum for 7MENT was estimated by Scatchard analysis [9].

Specificity, accuracy and precision

The cross-reactivity of the antiserum to various endogenous and synthetic steroids was determined according to Abraham [10]. The accuracy of the method was tested by measuring 7MENT in control serum to which known amounts (31.2–500 pg/tube) of 7MENT were added. The precision of the assay was determined by the measurement of 7MENT in 5 replicate aliquots obtained from two different rat serum pools (containing low and high concentrations of 7MENT) and in duplicate aliquots from the same serum pools in 8 different assays.

Pharmacokinetics of 7MENT

Rats. Rats were injected with 25 μg of 7MENT in 0.5 ml of 10% ethanol in saline into

the tail vein. Three rats per time period (i.e. 4, 8, 12, 16, 20, 30, 40 and 50 min after injection) were killed by decapitation under light ether anaesthesia and trunk blood collected in heparinized tubes. After 15 min, samples were centrifuged and the plasma stored at −20°C until assayed.

Pharmacokinetic studies were also performed in rats anaesthetized with ketamine. Rats were anaesthetized by injecting a mixture of ketamine (30 mg/kg) and xylazine (6 mg/kg) i.m. They were injected with 25 μg 7MENT in 0.5 ml 10% ethanol in saline into the tail vein and immediately thereafter, a butterfly (size 23G × $\frac{3}{4}$″) was inserted into the caudal artery. Blood was withdrawn 2, 5, 10, 20, 30, 60 and 90 min after injection. Plasma was separated and stored at −20°C.

Rabbits. Rabbits were injected with 250 μg of 7MENT dissolved in 1 ml of 10% ethanol in saline into the marginal ear vein. Blood samples were collected from the median artery of the contralateral ear at 2, 4, 8, 12, 16, 20, 30, 40, 50 and 60 min after the injection. The experiment was repeated in two rabbits anaesthetized with ketamine.

Kinetic analysis

Assessment of kinetic parameters was carried out according to a pharmacokinetic model as proposed by Greenblatt and Koch-Weser [11]. The kinetic analysis of 7MENT concentration in plasma as a function of time was carried out by curve fitting using nonlinear least square regression analysis using a computer program.

RESULTS

Evaluation of 7MENT RIA

Sensitivity. The smallest amount of 7MENT which significantly differed from zero on the standard curve was 2.1 pg. The dose–response curve was linear between 7.8 and 1000 pg. A mean serum blank of 21 ± 3.6 pg/ml (±SD) was obtained in RIA. Therefore, 28 pg/ml which is 2 SD above the serum blank was considered to be the detection limit of the assay (conversion factor; 100 pg/ml = 347 pmol/l).

Specificity. Table 1 shows the cross-reaction of anti-7MENT antiserum with various synthetic and endogenous steroid hormones tested in this RIA. With the exception of testosterone (T) and dihydrotestosterone (DHT) (cross-reaction 1–2%), no significant cross-reaction was observed with any of the steroids tested.

Table 1. Cross-reactivities of various steroids with the antiserum against 7MENT-3-CMO-BSA

Steroid	Cross-reactivity (%)
7α-Methyl-19-nortestosterone	100.0
Dihydrotestosterone	1.8
Testosterone	1.1
Estradiol-17β	0.4
Estriol	0.3
Progesterone	0.3
Androstenedione	0.2
11β,17β-Dihydroxy-androst-4-ene-3-one	<0.1
3β,17β-Dihydroxy-androst-5-ene	<0.1
Androst-4-ene-3,17 dione	<0.1
19-Hydroxy-androst-4-ene-3,17-dione	<0.1
17β-Hydroxy-androst-4-ene	<0.1
20α-Hydroxy-pregn-4-ene-3-one	<0.1
3α,11β-Dihydroxy-5α-androstan-17-one	<0.1
3α,11β-Dihydroxy-5β-androstan-17-one	<0.01
11β-Hydroxy-androsterone	<0.01
3α,11β-Dihydroxy-5α androstan-17-one	<0.01
Androsterone	<0.01
Androst-4-ene-3,11,17-trione	<0.01

Affinity. The affinity constant (K_a) of anti-7MENT antiserum (at the final dilution of 1:4500) for 7MENT from displacement analysis was estimated to be 1.17×10^9 l/mol.

Accuracy and precision. The accuracy of determinations in control rat serum containing known amounts of 7MENT was between 86 and 100% of the nominal values (Table 2). The precision, determined for two different serum pools was: intra- and interassay coefficients of variation of 7.3 and 8.4% for the low concentration pool and 4.3 and 7.3% for the high concentration pool, respectively.

Pharmacokinetic parameters of 7MENT. The plasma disappearance of immunoreactive 7MENT following intravenous administration of 7MENT was analyzed by computer implemented non-linear regression analysis. The data obtained for the half-lives and metabolic clearance rates (MCR) of 7MENT in rats and rabbits are summarized in Table 3. Half-lives were calculated from the computer generated slopes, α and β and MCR from area under the curve as described by Greenblatt and Koch-Weser [11]. The MCR of 7MENT in rats and rabbits were calculated to be 49 l/day and 336 l/day, respectively.

Table 2. Recovery of non-labeled 7MENT added to normal rat serum pool

7MENT added (pg/tube)	31.2	125	250	500
7MENT measured (pg/tube)	29.4 ± 3.6[a]	117 ± 11	243 ± 10	499 ± 29
Recovery (%)	94.0	85.5	97.4	99.8

[a]Values are mean ± SEM of 8 measurements.

Table 3. Half-lives and metabolic clearance rates of 7MENT following the intravenous administration of 7MENT

	α (min^{-1})	β (min^{-1})	$t_{1/2\alpha}$ (min)	$t_{1/2\beta}$ (min)	MCR (l/day)
Rat (3)[a]	0.44 ± 0.05	0.067 ± 0.004	1.6	10.3	52
Rat (5)[b]	0.26 ± 0.01	0.035 ± 0.004	2.7	19.6	49
Rabbit (4)[a]	0.52 ± 0.03	0.041 ± 0.001	1.3	16.8	336
Rabbit (2)[b]	0.18	0.027	4.2	25.7	249

[a]Animals were not anaesthetized.
[b]Animals were anaesthetized with ketamine.
Figures in parentheses indicate the number of animals at each blood sampling time.

DISCUSSION

With the availability of many synthetic agonists and antagonists of steroid hormones, a large number have been tested for their potential usefulness as contraceptives and in hormone replacement therapy. Even though there are many synthetic androgen derivatives which are more potent than testosterone, not much is known about their structure–function relationships. Methylation of 19-nortestosterone at positions 7 and 17 is known to increase its androgenicity several fold over that of testosterone [12]. We are interested in investigating the potential of using 7MENT as a replacement androgen in various situations. We have previously demonstrated in rhesus monkeys that, following suppression of androgen production and spermatogenesis with an LHRH agonist, administration of 7MENT acetate via Silastic® implants led to a restoration of ejaculatory function [6].

A RIA for 7MENT was developed to measure its serum levels and to study its pharmacokinetics in animals. The RIA was specific for 7MENT. Testosterone and dihydrotestosterone cross-reacted only minimally in this assay. Cross-reactivity of 7MENT antiserum with 7MENT metabolites could not be ascertained because of their unavailability at present. The sensitivity of the RIA was improved by compensating the standard curve tubes with solvent extracted serum residue as described by de la Pena *et al.* [13]. The compensation with the serum residue became necessary because of the low affinity and titer of the anti-7MENT antiserum which resulted in high serum blanks. The sensitivity of this RIA was comparable to the RIAs developed for some of the synthetic steroids such as ethynylestradiol [13], ST 1435 [14], and gestodene [15].

Using the above RIA, pharmacokinetic studies were performed in rats and rabbits. The half lives and metabolic clearance rates of 7MENT (Table 3) are comparable to those of testosterone in both the species studied. The MCR of the latter has been shown to be 55.8 l/day in rats and 229–289 l/day in rabbits by Wang *et al.* [16]. A 5 and 25% decrease in the MCR of 7MENT in rats and rabbits, respectively, in the anaesthetized compared to conscious animals was observed in our studies. A slight decrease (15%) in MCR of estrogen was observed in the human under ketamine anaesthesia [17] while in the rat a substantial decrease (38%) in the MCR of progesterone was reported under similar conditions [18]. It has been suggested that moderate changes in hepatic blood flow are unlikely to cause changes in the hepatic clearance of steroids [19]. But if the enterohepatic extraction of the steroid is substantial, then lowering of the hepatic blood flow may cause a proportionate fall in enterohepatic clearance and the overall metabolic clearance rate [20]. Anaesthesia has been shown to cause a decrease in the cardiac output [21], thereby decreasing the MCR, especially for compounds with high hepatic extraction [22]. The total hepatic extraction for testosterone has been shown to be 60–70% [23].

Based on the pharmacokinetic parameters of 7MENT found in this study, it would seem that this synthetic androgen is metabolized relatively fast compared to other synthetic steroids. For example norethisterone, which is also a 19-nortestosterone derivative, has a terminal half-life of 3.7 h in the rat [24]. The increased bioactivity of synthetic steroid derivatives can either be due to their increased terminal half lives *in vivo*, i.e. slower metabolism, or higher binding affinity to its specific receptors in the target tissues. The *in vitro* metabolism of 7MENT by rat liver, prostate and epididymis was compared with testosterone and 19-nortestosterone [25]. It was shown that 7MENT is metabolized more slowly by rat liver microsomes and cytosol than testosterone or 19-nortestosterone. Additionally, 7MENT does not undergo 5α-reduction and thus acts *per se*

at the target organs (ventral prostate and epididymis). The binding affinity of 7MENT to the androgen receptors was shown to be 5–6 times greater than that of testosterone [26]. The biological potency of 7MENT metabolites has not yet been established; however, Segaloff [12] has shown that the 7α-methyl-estr-4-ene-3,17-dione is biologically more active than the corresponding diones derived from testosterone and 19-nortestosterone. Thus the increased bioactivity of 7MENT compared to testosterone may involve one or more of the above discussed factors.

In summary, a sensitive RIA has been established for 7MENT which was utilized to evaluate some of the pharmacokinetic parameters in rats and rabbits. These developments will facilitate further biological studies of this potent androgen.

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