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BINDING OF ESTER AND AMIDE EPIMERS OF 20ξ-DIHYDROPREDNISOLONIC ACID TO CYTOSOL RECEPTORS AND THEIR ACUTE PHARMACOLOGICAL ACTIVITIES IN RATS*

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Summary—The competitive binding of two new classes of anti-inflammatory steroids, the esters and amides derived from the epimers of 20ξ-dihydroprednisolonic acid, to cytosol receptors from rat liver and thymus was studied. The relative inhibition of [^{3}H]dexamethasone binding by the steroid derivatives was the same, irrespective of the receptor source, with the following order: dexamethasone > prednisolone > methyl 17,20α-acetonidodihydroprednisolonate > methyl 17,20β-acetonidodihydroprednisolonate > *N*-propyl 20α-dihydroprednisolonamide > methyl 20α-dihydroprednisolonate > methyl 20β-dihydroprednisolonate > *N*-propyl 20β-dihydroprednisolonamide. The α-epimer of the steroids always showed a higher binding affinity than the corresponding β-epimer. In an acute pharmacological study, prednisolone induced the suppression of plasma corticosterone and an increase in tyrosine aminotransferase activity and glycogen content of rat liver. The esters and amides had no effect on these parameters except in the case of the acetonide derivatives of the steroid acid esters which slightly increased liver glycogen content.

INTRODUCTION

Glucocorticoids exert their anti-inflammatory activity by controlling the biosynthesis of certain proteins, probably as a result of alterations in gene expression in the target tissues [1, 2]. Glucocorticoids, like other steroids, are considered to bind to free cytoplasmic receptors of target cells to form a steroid–receptor complex, followed by an energy-dependent conformational change [3]. The steroid–receptor complex subsequently translocates to and interacts with nuclear acceptor sites, which trigger the biological responses. A good correlation has been observed between the pharmacological potency and binding affinity of glucocorticoids to cytoplasmic receptors *in vitro* [4]. Since the beneficial pharmacological activities as well as the undesirable effects of glucocorticoids are believed to be mediated by the same biological process, little success has been achieved in the synthesis of anti-inflammatory steroids without glucocorticoid activity.

The rationale for the synthesis of the glucocorticoid 21-oic acid esteres or amides is that these compounds have significantly local anti-inflammatory activity, but would be rapidly hydrolyzed to inactive acids and hence cause minimal systemic side effects [5, 6]. Several compounds of this type have been prepared and demonstrated to have local anti-inflammatory activity either in the vasoconstriction test in human skin [7], in the rat paw edema test [8] or in the cotton pellet granuloma assay, but not to cause pituitary-adrenal suppression, thymus involution [5, 6, 9] or skin atrophy [10]. In the present study, we have evaluated the binding affinities of these ester and amide derivatives to cytoplasmic receptors from rat liver and thymus in an attempt to relate the binding affinity with the acute pharmacological effects of the steroids.

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Steroid nomenclature and abbreviations: dexamethasone, 9α-fluoro-11β,17,21-trihydroxy-16α-methyl-pregna-1,4-dien-3,20-dione (DM); prednisolone, 11β,17,21-trihydroxypregna-1,4-dien-3,20-dione (P); methyl 20α-dihydroprednisolonate, methyl 11β,17,20α-trihydroxy-3-oxo-pregna-1,4-diene-21-oate (P4α); methyl 20β-dihydroprednisolonate, methyl 11β,17,20β-trihydroxy-3-oxo-pregna-1,4-dien-21-oate (P4β); methyl 17,20α-acetonidodihydroprednisolonate, methyl 11β-hydroxy-17,20α-isopropylidendioxy-3-oxo-pregna-1,4-dien-21-oate (P4α acetonide); methyl 17,20β-acetonidodihydroprednisolonate, methyl 11β-hydroxy-17,20β-isopropylidendioxy-3-oxo-pregna-1,4-dien-21-oate (P4β acetonide); *N*-propyl 20α-dihydroprednisolonamide, 21-propylamino-11β,17,20α-trihydroxy-3,21-dioxo-1,4-pregnadiene (P4α amide); *N*-propyl 20β-dihydroprednisolonamide, 21-propylamino 11β,17,20β-trihydroxy-3,21-dioxo-1,4-pregnadiene (P4β amide); 20α-dihydroprednisolonic acid, 11β,17,20α-trihydroxy-3-oxo-pregna-1,4-dien-21-oic acid; 20β-dihydroprednisolonic acid, 11β,17,20β-trihydroxy-3-oxo-pregna-1,4-dien-21-oic acid.

EXPERIMENTAL

Chemicals

[^{3}H]Dexamethasone (DM; 35 Ci/mmol) and Aquasol 2 scintillation fluid were obtained from New England Nuclear (Boston, Mass., U.S.A.). The radiochemical purity was determined by TLC with the solvent systems recommended by the supplier. Prednisolone was purchased from Upjohn (Kalamazoo, Mich., U.S.A.) and all other chemicals were reagent grade obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.), or from Mallinckrodt (Paris, Ky, U.S.A.).

Synthesis of prednisolone derivatives

Methyl 20α- and 20β-dihydroprednisolonate were prepared by oxidative rearrangement of prednisolone with cupric acetate in methanol [11, 12]. Following initial purification using silica gel 60 column chromatography employing a hexane–dichloromethane–acetone (50:20:30, v/v/v) solvent system the epimers were separated by preparative HPLC using a C_{18} (22 × 250 mm) column and methanol–water (60:40, v/v) as a mobile phase [13]. Methyl 17,20α- and 17,20β-acetonidodihydroprednisolonate were prepared by reacting methyl 20α- and 20β-dihydroprednisolonate with perchloric acid in acetone as described previously [13]. 20α- And 20β-dihydroprednisolonic acid were obtained by alkaline hydrolysis of the corresponding steroid acid esters. *N*-propyl 20α- and 20β-dihydroprednisolonamide were prepared from the respective steroid acid by activating with *N*,*N*′-dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriacole (HOBT) followed by reacting with propylamine [14] (Fig. 1). The steroids were crystallized from acetone or an appropriate solvent mixture and their structures were confirmed as previously described [13, 14].

Cytosol preparation

Male Sprague–Dawley rats (Southern Animal Farms, Pratteville, Ala, U.S.A.) were bilaterally adrenalectomized and maintained on normal saline for 3–5 days prior to sacrifice. The livers were excised, perfused with cold saline and divided into portions which were stored in liquid nitrogen until required. Thymus was prepared and stored under the same conditions. For binding experiments, the liver or thymus tissue was homogenized in 4 vol of TTES buffer (10 mM Tes, 12 mM thioglycerol, 1.5 mM EDTA and 0.25 M sucrose, pH 7.4) containing 20 mM sodium molybdate [15]. The homogenate was centrifuged at 105,000 *g* for 1 h at 4°C and the supernatant used as a receptor source. Protein concentrations of the cytosol were determined by the Lowry method [16] with bovine serum albumin as the standard.

Steroid binding assays

[^{3}H]DM was used as the labeled ligand for studies of the glucocorticoid binding to hepatic or thymus receptor and of the relative binding affinities of the ester and amide derivatives to these receptors. Unlabeled steroids and [^{3}H]DM were dissolved in ethanol and aliquots dried *in vacuo* in incubation tubes. Following the addition of 0.1 ml of cytosol preparation, the tubes were incubated at 4°C for 5 h at which time maximal binding is reached, as previously described [15]. Bound and free steroids were separated by charcoal–dextran treatment [15]. The incubation mixture was agitated briefly on a vortex mixer after adding 0.1 ml of a suspension of 10% charcoal and 1% dextran in 10 mM Tris, pH 8.0. Following centrifugation for 5 min at 3000 *g*, the supernatant (0.1 ml) was counted in 10 ml of Aquasol 2 in a Packard scintillation counter with an efficiency of ~30% for tritium. Quenching was corrected by the channels-ratio method. Non-specific binding was determined by incubating 1000-fold excess of unlabeled DM with [^{3}H]DM and subtracting from all measurements to yield specific binding. All determinations were performed in duplicate.

Acute pharmacological studies

Sprague–Dawley rats (100–120 g) were maintained on a normal diet under controlled conditions (light on from 0600 to 1800 h) for 1 week prior to use. The steroids were injected at a dose of 5 mg/kg in a saline–propylene glycol (1:1, v/v) vehicle to animals previously fasted for 24 h. Three hours later, blood was collected by cardiac puncture and the liver excised. The liver was washed in cold saline and divided into two portions. One (2 g) portion was frozen for the glycogen assay and the other homogenized in 4 vol of 0.14 M KCl containing 1 mM EDTA. The homogenate was centrifuged at 4°C for 30 min at 105,000 *g* and the supernatant stored at −20°C until required for the enzyme assays.

Tyrosine aminotransferase (L-tyrosine α-ketoglutarate aminotransferase, EC 2.6.1.5) activity was measured by the method of Diamondstone [17] and alanine aminotransferase (L-alanine α-ketoglutarate aminotransferase, EC 2.6.1.2) activity by the method of King [18]. Plasma corticosterone was determined by a fluorophotometric assay [19] and liver glycogen by the anthrone assay [20].

RESULTS

Binding of [^{3}H]DM to hepatic and thymus cytosol receptor

Figure 2 compares the levels of specific binding of [^{3}H]DM to liver and thymus cytosolic receptors after incubation for 5 h in the presence of 20 mM sodium molybdate. Linear regression analysis of the Scatchard plots [21] inset in Fig. 2 indicated binding capacities of ~520 and ~640 fmol/mg protein and apparent dissociation constants (K_d) of 3.1 and 13.2 nM for the liver and thymus receptors, respectively.

Inhibition of [^{3}H]DM binding by the ester and amide derivatives

The relative affinities of the ester and amide derivatives of 20α- and 20β-dihydroprednisolonic acid were evaluated by incubating 28 nM of [^{3}H]DM with aliquots of liver or thymus cytosol at 4°C for 5 h in the absence or presence of various concentrations of the competitors. Under these conditions no metabolism of the compounds occurred as measured by HPLC analysis. The data are expressed as percentages of [^{3}H]DM that was bound in the absence of competitors and are plotted as a function of competitor concentrations on a logarithmic scale. The data for hepatic and thymus cytosol are shown in Fig. 3 and 4, respectively. The results indicate the following sequence of relative affinities of these compounds for [^{3}H]DM binding sites in the hepatic and thymic cytosols: dexamethasone > prednisolone > methyl 17,20α-acetonidodihydroprednisolonate > methyl 17,20β-acetonidodihydroprednisolonate > *N*-propyl 20α-dihydroprednisolonamide > methyl 20α-dihydroprednisolonate > methyl 20β-dihydroprednisolonate > *N*-propyl 20β-dihydroprednisolonamide. The relative affinities obtained by comparing the concentrations of various competitors that inhibited 50% of the binding of [^{3}H]DM binding (C_{50}) are shown in Table 1.

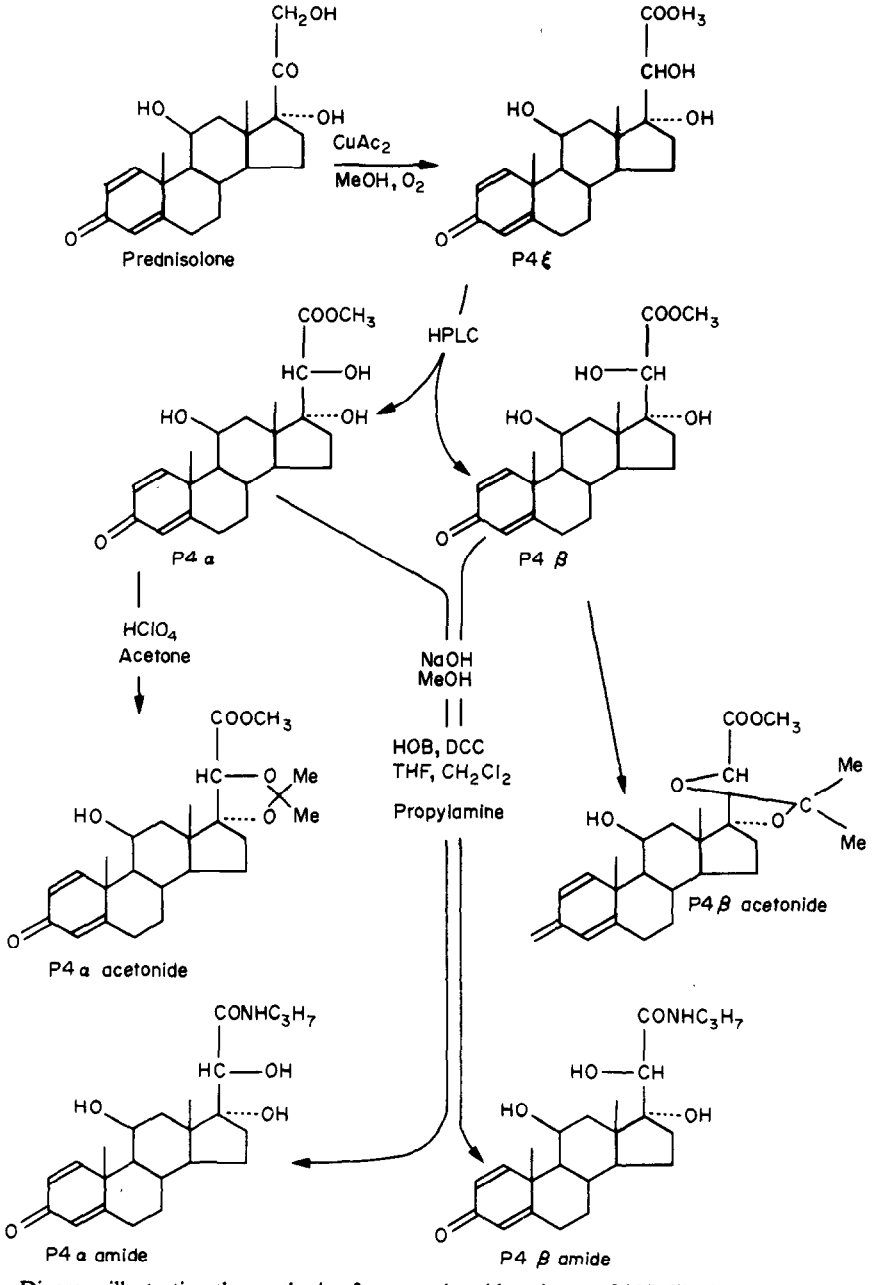

Fig. 1. Diagram illustrating the synthesis of ester and amide epimers of 20ξ-dihydroprednisolonic acid.

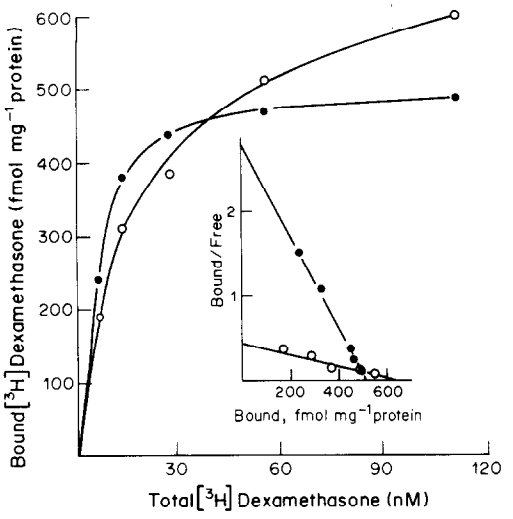

Fig. 2. Specific binding of [^{3}H]DM to rat liver or thymus glucocorticoid receptor. Various concentrations of [^{3}H]DM (7–112 nM) were incubated at 4°C for 5 h with aliquots (0.1 ml) of liver (1.66 mg protein/0.1 ml) or thymus cytosol (0.84 mg protein/0.1 ml) in the absence or presence (non-specific binding) of 1000-fold excess of unlabeled DM. The specific binding data of liver (●) and thymus (○) were estimated by subtracting non-specific binding from the corresponding total binding. Each point is the average of duplicate determinations. Insets: Scatchard analysis of the specific binding data indicates K_d-values of 3.1 or 13.2 nM and binding capacities of 520 protein or 640 fmol/mg protein for liver or thymus glucocorticoid receptor, respectively.

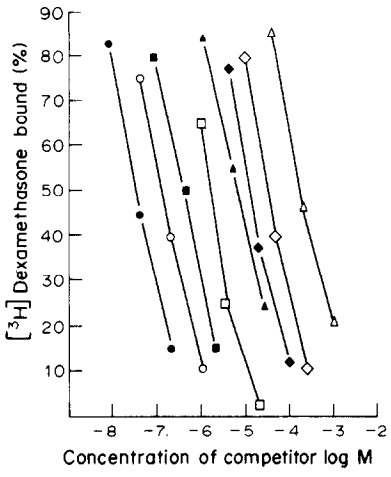

Fig. 3. Relative binding affinities of the steroid esters and amides for rat hepatic receptor. [^{3}H]DM (28 nM) was incubated with liver cytosol at 4°C for 5 h in the absence of competitors or the presence of various concentrations of DM (●), prednisolone (○), methyl 17,20α- (■) or methyl 17,20β-acetonidodihydroprednisolonate (□), methyl 20α- (○) or methyl 20β-dihydroprednisolonate (○), *N*-propyl 20α- (▲) or *N*-propyl 20β-prednisolonamide (△). Each point represents the mean of duplicate determinations, expressed as a percentage of [^{3}H]DM binding in the absence of competitors.

Competition of the steroid derivatives with [^{3}H]DM for binding to the hepatic cytosol receptor

The competitive binding of the ester and amide derivatives vs [^{3}H]DM to the hepatic receptor was measured by changing the concentration of [^{3}H]DM from 7 to 112 nM with a fixed concentration of each competitor. The concentrations of the steroids, chosen to produce appropriate slopes in the double-reciprocal plots of the binding data in a preliminary test were: DM (40 nM), prednisolone (200 nM), methyl 17,20α- (400 nM) and methyl 17,20β-acetonidodihydroprednisolonate (25 μM), methyl 20α- (20 μM) and methyl 20β-dihydroprednisolonate (50 μM), *N*-propyl 20α- (5 μM) and *N*-propyl 20β-dihydroprednisolonamide (200 μM). Double-reciprocal plots of the binding data are presented in Fig. 5. All the ester and amide derivatives were found to intercept at the same point on the ordinate axis, indicating that the derivatives bind competitively with DM to the hepatic receptor. The binding capacity of the receptor for the steroids was ~520 fmol/mg protein which is consistent with the data from the preceding assay.

Acute pharmacological effects of the steroids

The acute pharmacological effects of the steroids on plasma corticosterone, liver glycogen and liver enzyme activities are shown in Table 2. Administration of prednisolone caused a marked decrease (89.1%) in plasma corticosterone levels. Furthermore, prednisolone increased liver glycogen deposi-

Table 1. The relative binding affinity of steroid esters and amides to the cytosolic receptors of rat liver and thymus

	Liver		Thymus	
Compound	Relative affinity	C_{50}[a]	Relative affinity	C_{50}[a]
DM	100	32 nM	100	138 nM
Prednisolone	27	117 nM	39	355 nM
P4α acetonide	9	347 nM	20	708 nM
P4β acetonide	2	1.6 μM	5	2.8 μM
P4α	0.3	11.5 μM	0.2	63.1 μM
P4β	0.1	31.6 μM	0.1	89.1 μM
P4α amide	0.5	6.3 μM	0.6	22.4 μM
P4β amide	0.02	166.0 μM	0.02	891.3 μM

[a]Concentration required for 50% inhibition of the specific binding of 28 nM [^{3}H]DM to hepatic and thymus cytosol (data from Figs 3 and 4).

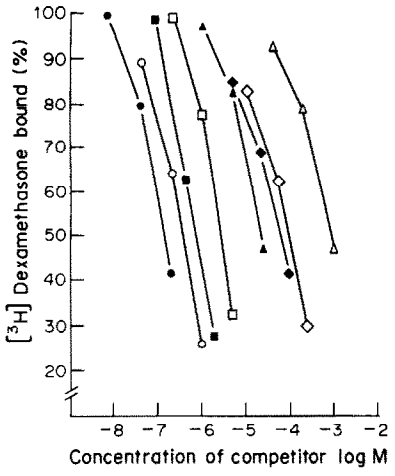

Fig. 4. Relative binding affinities of the steroid esters and amides for rat thymus receptor. The binding affinities were measured using the method described for the hepatic receptor (cf. legend for Fig. 2): DM (●), prednisolone (○), methyl 17,20α- (■) or methyl 17,20β-acetonidodihydroprednisolonate (□), methyl 20α- (○), or methyl 20β-dihydroprednisolonate (○), *N*-propyl 20α- (▲) or *N*-propyl 20β-dihydroprednisolonamide (△). Each point represents the mean of duplicate determinations, expressed as a percentage of [^{3}H]DM binding without competitors.

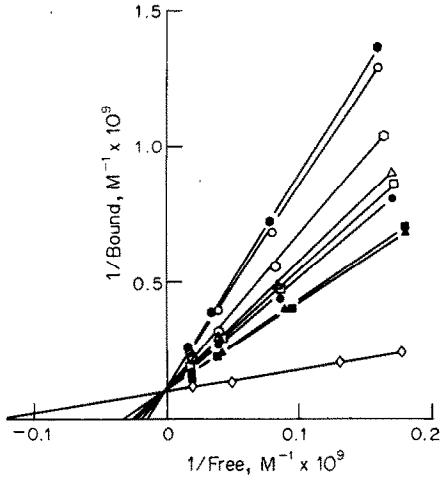

Fig. 5. Competitive binding of the steroid esters and amides vs [^{3}H]DM for rat hepatic glucocorticoid receptor. Various concentrations of [^{3}H]DM (7–112 nM) were incubated with liver cytosol in the absence of competitor (◇) or presence of fixed concentration of DM (40 nM, ●), prednisolone (200 nM, ○), methyl 17,20α- (400 nM, ■) or methyl 17,20β-acetonidodihydroprednisolonate (25 μM, □), methyl 20α- (20 μM, ○) or methyl 20β-dihydroprednisolonate (50 μM, ○), *N*-propyl 20α- (5 μM, ▲) or *N*-propyl 20β-dihydroprednisolonamide (200 μM, △).

tion and tyrosine aminotransferase activity of liver cytosol by 2.7 and 3.7 times, respectively, compared with control levels in this acute experiment. Alanine aminotransferase activity was not affected by prednisolone.

There were no significant changes in any of the parameters measured in the animals treated with the ester or amide derivatives, with the exception of methyl 17,20α-and methyl 17,20β-acetonidodihydroprednisolonate which showed a slight increase in liver glycogen content compared with the controls.

DISCUSSION

In previous studies, using the cotton pellet granuloma bioassay [22, 23], methyl 20β-dihydroprednisolonate, *N*-propyl 20β-dihydroprednislonamide and methyl 17,20α-acetonidodihydroprednisolonate exhibited local anti-inflammatory activity equivalent to that of the parent compound, prednisolone, but their corresponding epimers showed far less activity. In the present investigation the inhibition by these compounds of [^{3}H]glucocorticoid binding to receptors in rat liver and thymus cytosol was evaluated. Since the order of relative affinities of the steroids was the same with both receptor sources the data would support previous suggestions that glucocorticoid receptors from various target tissues are closely related [24–26]. The higher affinity of the α-epimers also suggest that orientation of the hydroxyl group at C-20 plays an important role in the binding to glucocorticoid receptors. Initial studies using DM gave rise to binding capacities and K_d-values in accord with those previously reported [15, 22, 23, 25, 27, 28]. There is a discrepancy between receptor affinities and pharma-

Table 2. Acute pharmacological effects of steroid esters and amides

	Plasma corticosterone (μg/100 ml)	Liver glycogen (mg/g)	Tyrosine amino transferase (nmol/10 min per mg protein)	Alanine amino transferase (mU/mg protein)
Control	21.1 ± 6.8	3.0 ± 0.3	90.3 ± 26.0	988 ± 113
Prednisolone	2.3 ± 0.2*	8.1 ± 0.8**	214.3 ± 20.3**	1012 ± 123
P4α	22.2 ± 5.2	2.9 ± 0.3	46.9 ± 8.8	1132 ± 83
P4β	19.2 ± 4.4	4.0 ± 0.6	73.6 ± 17.8	1101 ± 310
P4α acetonide	16.9 ± 6.8	6.2 ± 1.1*	135.4 ± 12.2	1098 ± 165
P4β acetonide	16.2 ± 6.2	4.7 ± 0.6*	101.9 ± 15.4	1021 ± 168
P4α amide	25.1 ± 5.4	3.1 ± 0.4	93.2 ± 19.2	1300 ± 165
P4β amide	21.4 ± 6.3	5.0 ± 1.1	82.4 ± 11.4	1154 ± 134
Normal fed	20.2 ± 4.5	32.0 ± 1.2***	82.4 ± 17.2	1275 ± 94

Animals were sacrificed 3 h after the injection of steroids (i.p., 5 mg/kg) to rats previously fasted for 24 h. Data represents the mean ± SE of 6 animals.
Significantly different from control by $^*P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$ using a one-way ANOVA.

cological responses of the steroid derivatives. The physiological effectiveness of steroids depends on several properties including: intrinsic affinity for the receptors, ability to traverse membranes and rates of metabolism and excretion. A combination of these factors may account for the differences noted in this study.

General properties of glucocorticoids include pituitary-adrenal suppression, induction of liver transaminases, and liver gluconeogenesis [27, 29]. As expected in the acute pharmacological experiment, administration of prednisolone significantly suppressed plasma corticosterone levels and increased tyrosine aminotransferase activity and glycogen deposition in the rat liver. The ester and amide derivatives of the steroid acids did not alter these parameters, except in the case of methyl 17,20α- and methyl 17,20β-acetonidodihydroprednisolonate which slightly elevated the liver glycogen content. This data, together with the previous pharmacological studies from this laboratory, indicate that the low systemic pharmacological activity of the ester compounds may be partially due to the lower affinity of the compounds to the steroid receptor. Moreover, the rapid biotransformation in the systemic circulation to pharmacologically inactive steroid acids which failed to bind to liver cytosol receptor as previously reported [22, 28] may be important. In addition the data would suggest that methyl 20β-dihydroprednisolonate, *N*-propyl 20β-dihydroprednisolonamide and methyl 17,20α-acetonidodihydroprednisolonate could be potentially useful steroids for topical or local use since they have anti-inflammatory potency approaching that of the more traditional steroids such as prednisolone, but are without their adverse effects such as pituitary-adrenal suppression, thymus involution and skin atrophy [10].

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