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M AND L FORMS OF 18-HYDROXY-11-DEOXYCORTICOSTERONE AND 18-HYDROXYCORTICOSTERONE: FACTORS INFLUENCING CONVERSION, STABILITY AND IMMUNOLOGICAL PROPERTIES

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SUMMARY

The precise mechanisms conducive to the formation of the less polar (L forms) of 18-OH-DOC and 18-OH-B from their respective, more polar (M forms) were investigated. The conversion to the L form during storage appeared to be related to the presence of organic acid impurities in the solvent. Room temperature and temperature of 37°C favored more rapid transformation to the L form than 4°C. The predicatable method of obtaining M and L forms of the steroids and their convenient separation by LH-20 column chromatography permitted further studies of their solubility and immunological characteristics. L forms of both steroids failed to show any binding to an antiserum generated against 18-OH-DOC. These studies provide important insight into the complexity of interconversions of these 18-hydroxylated steroids and will facilitate further studies.

INTRODUCTION

18-hydroxy-11-deoxycorticosterone (18-OH-DOC) is known to be unstable under certain conditions giving rise to a more polar (M) and a less polar (L) form [1]. Dominguez observed the appearance of two closely related steroids with different polarities during paper chromatography of 18-OH-DOC [2]. He suggested that these two compounds might be tautomers of 18-OH-DOC. Roy and his colleagues [1] more recently have carefully investigated the chemical nature of the two forms and have indicated that the more polar and less polar forms of 18-OH-DOC represented cyclic hemiketal and mixed ketal forms of the steroid, respectively, rather than tautomers. 18-hydroxy-corticosterone (18-OH-B) also appears to share similar properties [3–5]. This instability of these steroids has hampered attempts to measure them in biological fluids. The causes and precise conditions predisposing to the formation of the different forms of these steroids have not been fully defined. Roy, Ramirez and Ulick [1] have suggested that traces of acid impurities in organic solvents might contribute to the interconversion of the two forms of 18-OH-DOC.

We examined the role of varying concentrations of trace amounts of organic acids as well as the effect of temperature on conversion of M to L forms of the two 18-hydroxylated steroids using Sephadex LH 20 column chromatography to separate the two forms. In addition, we investigated the solubility, extractability and immunological properties of the two forms of these two steroids.

MATERIALS AND METHODS

Reagents. All solvents used were spectroquality (Matheson-Coleman & Bell). Ethanol, methanol and ethylacetate were redistilled with sodium hydroxide pellets for the purpose of eliminating any trace amounts of organic acids.

[1, 2 ^{3}H]-18-hydroxy-11-deoxy corticosterone and [1, 2(n)^{3}H]-18-hydroxy corticosterone were purchased from Amersham-Searle Co. Both labeled steroids were diluted in distilled ethanol and stored at 4°C. Aliquots of stock steroids were purified before each study by Sephadex LH 20 (Pharmacia, Inc.) column chromatography.

Non-radioactive 18-hydroxy-11-deoxycorticosterone was obtained from Steraloid, Inc. (Pauling, New York) and used without further purification.

Column chromatography. Chromatography of steroids was performed using glass columns (30 × 1 cm, Bio-Rad) containing Sephadex LH 20. Columns were washed with methanol. After packing the column with Sephadex LH 20, 20 ml of a solvent mixture containing heptane–benzene–methanol–water (65:25:10:0.1, by vol.) was run through the Sephadex

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bed. The steroids were dissolved in 0.5 ml of the same solvent mixture and applied to the top of the packed column. Eluates were collected in 1 ml fractions. A 50 μl aliquot from each fraction was counted for radioactivity.

The more polar forms of 18-OH-DOC and 18-OH-B eluted between 27 and 35 ml, and 46 and 58 ml, respectively. The less polar forms of 18-OH-DOC and 18-OH-B eluted between 17 and 22 ml, and 31 and 40 ml, respectively. The elution position of each steroid was constant in this system using the same columns and solvents.

Paper chromatography. Whatman paper No. 1 was washed with methanol for 2 days in a Soxhlet extraction apparatus. Chromatography was performed using a modified Bush B_3 solvent system (cyclohexane–benzene–methanol–water, 677:333:800:200, by vol.) and a modified Bush B_5 solvent system (benzene–methanol–water, 100:50:50, by vol.). The paper was equilibrated in the chromatographic tank for 3 h and developed for 3 h (Bush B_3) or for 12 h (Bush B_5). Radioactive steroids were identified on the paper strips by a radiochromatogram scanner (Packard Model 7201). The nonradioactive M and L forms of 18-OH-DOC were detected by Mineralight (U.V. SL 25).

Studies of solubility and extractability. The solubility of the M and L forms of these steroids were examined using several solvents listed in Tables 2 and 3 of varying polarities. Similarly the ability of various solvents to extract the tritiated M and L forms of each steroid from plasma was also investigated.

Immunological studies. Antisera were raised in rabbits by injecting the carboxymethyloxime of 18-OH-DOC conjugated with bovine serum albumin. The antiserum with the highest titer was used for the studies involving 18-OH-DOC and 18-OH-B. Both labeled and unlabeled 18-OH-DOC and labeled 18-OH-B were used in these investigations. After incubation with antiserum, the bound and free forms of the labeled steroids were separated by dextran-coated charcoal.

Other methods. The Blue tetrazolium (BT) reaction for α-ketolic steroids (20-keto, 21-hydroxyl) was performed using the method of Elliot *et al.* [6]. Steroid concentrations were read using a spectrophotometer at a wave length of 510 mμ.

Periodic oxidation of 18-OH-DOC and 18-OH-B was carried out by utilizing the method of Tait *et al.*[7]. The gamma lactone of each steroid was purified by column chromatography and later subjected to paper chromatography also.

Experiments were performed to determine the effects of prolonged storage and of temperature of incubation in the presence or absence of formic acid or acetic acid in methanol and the influence of pyridine or triethylamine (TEA) on the conversion of the M forms of 18-OH-DOC and 18-OH-B. M and L forms of each steroid were separated by Sephadex LH 20 column chromatography and identified by paper chromatography, BT reaction and U.V. light absorption (for unlabeled steroids).

EXPERIMENTS AND RESULTS

(a) *Effect of storage of 18-OH-DOC and 18-OH-B in methanol and chloroform*

Purified M and L forms of tritiated 18-OH-DOC and 18-OH-B were allowed to stand in distilled or non-distilled methanol or chloroform for 1–3 weeks at 4°C. At the end of 1 and 3 weeks, the elution positions of the radioactive steroids in distilled methanol from Sephadex columns were identical with those of the original M forms of the steroids. However, when the steroids were allowed to stand in non-distilled methanol, in addition to the peak consistent with the original M form of the steroids, a smaller, less polar peak (after one week) or a larger, less polar peak (after 3 weeks of incubation) was seen as shown in Fig. 1. Incubation at room temperature produced greater conversion to the L form than at 4°C. M and L forms of 18-OH-DOC and 18-OH-B allowed to stand in chloroform were unchanged after one and three weeks.

(b) *Effect of traces of organic acids in methanol*

The M forms of 18-OH-DOC and 18-OH-B were incubated for 15 min at 23°C with or without 0.05 M

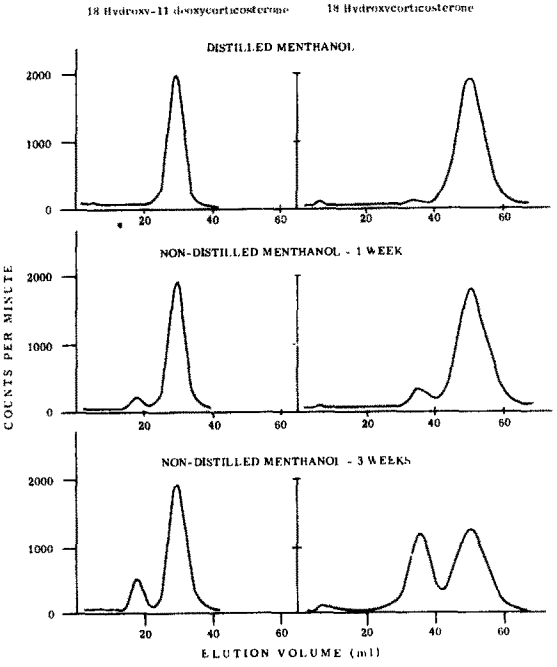

Fig. 1. Effect of storage in non-distilled methanol on M form of 18-hydroxy-17-deoxy corticosterone (18-OH-DOC) and 18-hydroxy-corticosterone (18-OH-B). Radioactivity elution profiles from LH-20 column chromatography of tritiated steroids in distilled methanol (top panel), after one week storage in non-distilled methanol (middle panel), and after three weeks of storage in non-distilled methanol (bottom panel). The major peak in each chromatogram represents the M form.

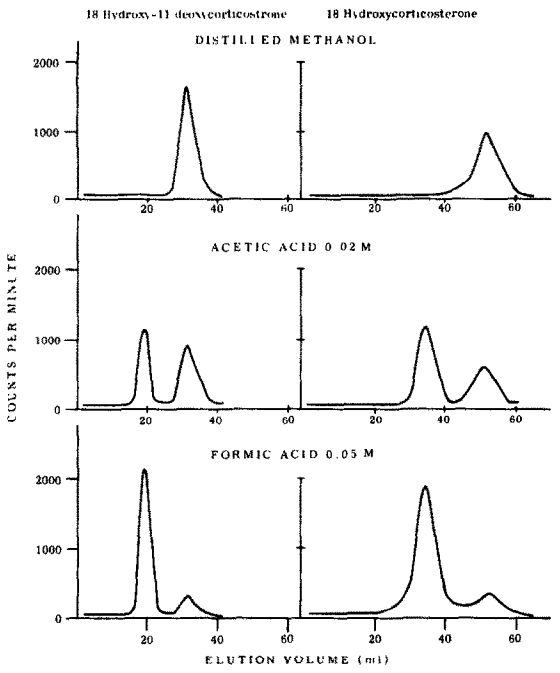

Fig. 2. Effect of trace acids in distilled methanol on M form of 18-hydroxy-11-deoxy corticosterone (18-OH-DOC) and 18-hydroxy-corticosterone (18-OH-B). Radioactivity elution profiles from LH-20 column chromatography of tritiated steroids in distilled methanol alone (top panel), distilled methanol containing 0.02 M acetic acid (middle panel) and distilled methanol containing 0.05 M formic acid (bottom panel). In distilled methanol alone only the M form of the steroid was identified. With acetic or formic acid conversion to L form was seen.

formic acid or 0.02 M acetic acid in distilled methanol. The results of the mean of 6 experiments are shown in Fig. 2. Incubation in the presence of acetic acid or formic acid produced significant amounts of the L forms of these steroids when compared with control samples not containing the acid. When M and L forms of 18-OH-DOC and 18-OH-B were incubated in distilled methanol in the presence of 0.01 N hydrochloric acid multiple peaks were identified after column chromatography.

(c) *Effects of temperature and various concentrations of formic acid on conversion of M to L forms*

The M forms of these steroids were incubated in the presence of formic acid in concentrations of 1.9×10^{-6} M, 1.9×10^{-5} M, 1.9×10^{-4} M at either 4°C or 37°C for 3 h. The mean results of 6 experiments in each group were compared with a similar number of control samples containing no acid and are shown in Table 1. It is apparent that the conversion of the M to L form was slower at the lower temperature and was also dependent on the acid concentration of the solvent.

(d) *Inhibitory effects of pyridine or triethylamine (TEA) on conversion of M to L forms*

Similar incubations containing formic acid as described above were carried out in the presence or absence of 0.01% or 0.1% of pyridine or TEA. The results of incubation (Table 1) showed that TEA in either concentration completely inhibited formation of the L form during a 3 h incubation period. Pyridine, however, was less effective, especially in the presence of a higher concentration of formic acid.

(e) *Solubility and extractability of M and L forms of 18-OH-DOC and 18-OH-B*

To the dried residues of purified M or L forms of 18-OH-DOC and 18-OH-B were added 1 ml of *n*-hexane, benzene, dichloromethane, methanol, ethylacetate, acetone or water. They were mixed for 30 s and then an aliquot of 0.1 ml was counted. As shown in Table 2, the L forms of 18-OH-DOC and 18-OH-B

Table 1. Effect of formic acid, temperature, pyridine and TEA on the conversion of L and M forms of 18-hydroxy-deoxycorticosterone (18-OH-DOC) and 18-hydroxy-corticosterone (18-OH-B)

Incubation	Control	Formic acid Concentration		
		1.9×10^{-6} M	1.9×10^{-5} M	1.9×10^{-4} M
		(Per cent conversion of M to L form)		
4 C, 3 h				
18-OH-DOC	5.0 ± 0.5	6.7 ± 0.7	7.6 ± 0.6	26.7 ± 1.6
18-OH-B	6.9 ± 0.4	7.9 ± 0.6	12.8 ± 0.7	49.1 ± 2.0
37 C, 3 h				
18-OH-DOC	4.4 ± 0.6	9.5 ± 0.9	55.6 ± 3.9	79.7 ± 4.9
+0.01% Pyridine	1.6 ± 0.1	5.1 ± 0.2	10.1 ± 0.8	32.4 ± 1.8
+0.1% Pyridine	1.4 ± 0.2	2.1 ± 0.1	8.0 ± 0.3	30.6 ± 1.5
+0.01% TEA	3.5 ± 0.1	1.8 ± 0.1	4.5 ± 0.1	5.6 ± 0.4
+0.1% TEA	2.8 ± 0.1	1.2 ± 0.1	2.5 ± 0.1	5.2 ± 0.4
18-OH-B	4.3 ± 0.3	13.7 ± 0.8	29.1 ± 1.9	83.9 ± 2.4
+0.01% Pyridine	3.2 ± 0.3	3.2 ± 0.2	2.4 ± 0.1	58.9 ± 1.3
+0.1% Pyridine	2.1 ± 0.1	2.6 ± 0.1	2.2 ± 0.2	54.6 ± 0.8
+0.01% TEA	3.4 ± 0.4	6.2 ± 0.4	7.1 ± 0.3	9.2 ± 0.5
+0.1% TEA	2.5 ± 0.2	2.4 ± 0.1	2.8 ± 0.1	2.6 ± 0.1

TEA = triethylamine; all experiments represent mean ± S.D. of 6 observations.

Table 2. Relative solubility of M and L forms of 18-hydroxy-11-deoxycorticosterone (18-OH-DOC) and 18-hydroxy-corticosterone (18-OH-B)

	18-OH-DOC		18-OH-B	
	M	L	M	L
	(Per cent solubility of tritiated steroid)			
Hexane	6.6 ± 1.7	51.7 ± 2.2	0.8 ± 0.3	2.1 ± 0.2
Benzene	55.8 ± 2.4	97.4 ± 1.7	17.9 ± 1.6	81.3 ± 1.5
Dichloromethane	44.6 ± 3.1	89.9 ± 3.8	24.3 ± 1.6	69.2 ± 1.6
Methanol	98.7 ± 4.3	98.2 ± 1.9	87.7 ± 1.7	94.8 ± 3.7
Ethylacetate	89.6 ± 4.1	97.9 ± 1.8	86.9 ± 3.1	99.2 ± 3.8
Acetone	50.8 ± 3.0	55.4 ± 1.8	31.9 ± 1.1	47.4 ± 1.1
Water	92.3 ± 1.4	87.4 ± 1.4	83.1 ± 0.9	84.2 ± 1.2

All experiments represent mean ± S.D. of 6 observations.

Table 3. Relative extractability of M and L forms of 18-hydroxy-11-deoxy corticosterone (18-OH-DOC) and 18-hydroxy-corticosterone (18-OH-B)

	18-OH-DOC		18-OH-B	
	M	L	M	L
	(Per cent extractability of tritiated steroid)			
Hexane	1.7 ± 0.5	43.4 ± 2.0	0.04 ± 0.05	0.8 ± 0.3
Benzene	43.0 ± 1.8	89.7 ± 1.0	31.5 ± 0.3	88.3 ± 2.2
Dichloromethane	98.7 ± 2.3	86.0 ± 5.2	96.9 ± 2.5	98.1 ± 6.2
Ethylacetate	88.2 ± 3.5	86.4 ± 2.9	96.7 ± 4.2	97.7 ± 5.5

All experiments represent mean ± S.D. of 6 observations.

were generally more soluble in the less polar organic solvents (such as *n*-hexane, benzene and dichloromethane) than the M forms of the same steroids. But the M and L forms of these steroids were highly soluble in water, ethylacetate and methanol. Known quantities of tritiated M or L forms of 18-OH-DOC and 18-OH-B were added to 1 ml aliquots of pooled human plasma and mixed. Then 10 ml of either *n*-hexane, benzene, dichloromethane or ethylacetate were added to each aliquot of plasma. The mixture was vortexed, washed with 0.1 N sodium hydroxide and water. An aliquot from the organic solvent was counted. There was little difference in the ability of dichloromethane or ethylacetate to extract the M and L forms of these steroids as shown in Table 3. But, the extractability of benzene was different for M and L forms. Hexane was extremely inefficient in extracting the more polar of the steroids including the M form of 18-OH-DOC.

(f) *Immunological studies*

Antiserum dilution curves using the tritiated M form of 18-OH-DOC were examined. A dilution of anti-serum (1:150,000) which resulted in 50–60% binding of the labeled M form of the steroid was chosen for further studies. Labeled and unlabeled L form was prepared by incubating the M form with methanol containing formic acid, and was used for the immunologic studies. Antibody dilution curves utilizing the same concentrations of tritiated L form as that of the M form showed no significant binding of L form to the above-mentioned antiserum (Fig. 3). The standard curve of 18-OH-DOC using the same antibody with 2.5, 5, 10, 20, 25, 50, 75, 100, 250, 500 and 1000 pg of unlabeled M or L form of the steroids is shown in Fig. 4. While the addition of the unlabeled M form resulted in 50% displacement of the labeled M form of 18-OH-DOC from the initial binding at about 170 pg and 85% displacement occurred at 500 pg of the steroid; the binding of the L form was strikingly different. Up to a concentration of 250 pg of L form there was insignificant binding to the antibody. Only at a concentration of 1000 pg did the L form displace slightly greater than 50% of the labeled M form of the steroid from the antibody. Similar differences in the binding of labeled 18-OH-B M or L forms were observed using the same 18-OH-DOC antiserum, which cross-reacted with 18-OH-B, at a lower dilution. The antiserum, at a dilution of 1:10,000, resulted in 50% binding of tritiated 18-OH-B M form while tritiated L form of 18-OH-B showed no binding (See Fig. 5).

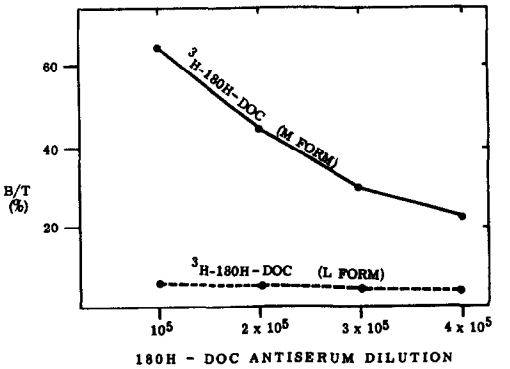

Fig. 3. Different binding characteristics of M and L forms of [^{3}H]-18-hydroxy-11-deoxy corticosterone ([^{3}H]-18-OH-DOC) to the antiserum are shown.

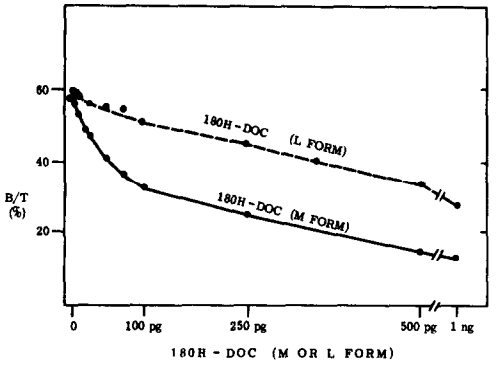

Fig. 4. The standard curves using M and L forms of 18-hydroxy-deoxy corticosterone (18-OH-DOC) are demonstrated showing a difference in the binding characteristics of the steroids.

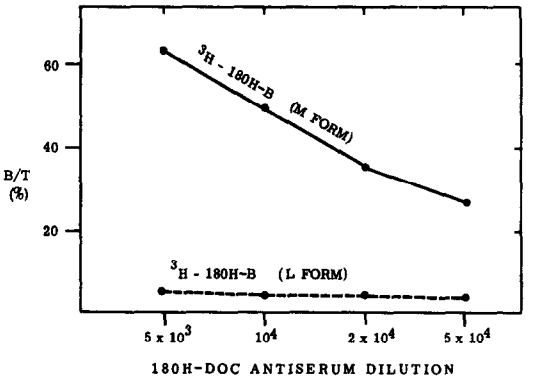

Fig. 5. The contrasting binding characteristics of M and L forms of [^{3}H]-18-hydroxy-corticosterone ([^{3}H]-18-OH-B) to the 18-hydroxy-11-deoxy corticosterone (18-OH-DOC) antiserum are shown.

DISCUSSION

Our results confirm the role of organic acid in the conversion of M to L forms of 18-OH-DOC as suggested by Roy *et al.*[1]. We have also examined the effect of temperature, which seems to influence the rate of conversion significantly. Our studies were extended to 18-OH-B as well, showing similar conditions influencing conversion of the M to L form of that steroid. Lantos and his associates [3, 4] also observed conversion of M form to L form of 18-OH-B in methanol and by treatment with hydrochloric acid. It was further demonstrated in our studies that TEA was a better agent than pyridine in stabilizing the M form of 18-OH-DOC or 18-OH-B in the presence of acid concentrations shown to predispose to the formation of the L form. In our column chromatographic system, the two steroids appeared to be stable, but during paper chromatography conversion of M to L forms was consistently observed.

Differences in other properties of the M and L forms of each steroid were observed in this study. The solubility characteristics of M and L forms were dependent on the polarity of the solvent. As expected, M forms were more soluble in the more polar solvents than L forms. But, the result of extraction from plasma of M and L forms by various solvents showed that, contrary to solubility studies, there was no significant difference in the ability of dichloromethane or ethyl acetate in extracting either M or L forms from plasma. However, benzene was less efficient in extracting the M form of 18-OH-DOC and 18-OH-B than the L form of the same steroids. The results of these studies indicate that the solubility of the steroids in solvents cannot be equated with their extractability.

The immunological studies, suprisingly, showed significantly different binding of M and L forms of the two steroids to an antiserum generated against 18-OH-DOC. The antiserum seemed unable to recognize the L forms of the steroids compared to the M forms, as judged by the binding of labeled steroids or the displacement of the tritiated M form by the unlabeled M or L forms. These observations have obvious implications in the measurement of these steroids.

Considerable interest has been generated about the role of 18-OH-DOC in recent years. Several years ago two groups [8, 9] demonstrated the production of 18-OH-DOC *in vitro* by human adrenal gland. DeNicola and Birmingham[9] and Rapp[10] have suggested a role for 18-OH-DOC in experimental hypertension. More recently, Melby and his colleagues[11] measured 18-OH-DOC in human subjects and suggested that low renin hypertension might be mediated by excessive 18-OH-DOC secretion. They also have proposed that 18-OH-DOC might be an intermediate in aldosterone biosynthesis in some situations [12]. Since then, several investigators have attempted the measurement of 18-OH-DOC *in vivo*. Very limited studies related to the measurement of 18-OH-B in blood have been undertaken. The known instability of these steroids has impeded the development of methods of measuring them. Some investigators [12, 13] have circumvented the problem by converting these steroids to their gamma lactone prior to measurement. Others have measured the steroid without prior chemical alteration. Our studies, showing significant influence of solvent impurities and temperature on the relative proportion of M and L forms that may be produced in a given sample, and the important observation that the M and L forms of the two steroids did not bind to the antibody to the same degree, have important bearing on the measurement of these steroids by radioimmunoassays. The predictable means of stabilizing the steroids and the convenient and reliable method of chromatographic separation described in this report will facilitate further studies of these steroids.

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