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Hydrolysis of Esters and Amides of 20R- and 20S-Dihydroprednisolonic Acid in Rat Serum and Liver Homogenate

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Abstract□The hydrolysis rates of ester and amide derivatives of 20-dihydroprednisolonic acid were measured in rat serum and liver homogenate. The hydrolysis rate of the esters in serum was found to be faster than that in liver homogenate on the basis of blood volume and liver weight, while the amide derivatives showed much slower change. And it is also found that the size of substituents at C-21 and C-20 configuration expressed considerable effects on the hydrolysis rate of these derivatives.

Keywords□Esters and amides of 20-dihydroprednisolonic acid, hydrolysis rate, rat serum and liver homogenate

For developing safer local anti-inflammatory steroids, various ester and amide derivatives have been synthesized and pharmacologically evaluated[1-3]. The rationale for the synthesis of these derivatives is based on the "antedrug" concept suggesting that the derivatives are locally active but hydrolyzed rapidly in the systemic circulation to inactive compounds, giving reduced systemic side-effects[4]. Therefore, it would be very important that the compounds having reduced systemic side-effects should have considerably rapid degradation rates in the systemic circulation. Methyl 20R- and 20S-dihydroprednisolonate were prepared and tested. It was found that the 20S-isomer showed local anti-inflammatory activity with reduced systemic side-effects[2,5]. The hydrolysis study in serum and various organs of rat revealed that serum had the highest esterolytic activity to these ester derivatives in contrast to low activity in the liver[6]. And it was also found that the 20S-isomer was more rapidly hydrolyzed by rat serum than the 20R-isomer. Because all of various ester derivatives synthesized recently showed reduced systemic effects in rat[3], it is very interesting to investigate the effects of different substituents and C-20 configuration of these ester derivatives on the hydrolysis in rat serum. For a search of the other derivatives with same property, it was expected that the amide derivatives could behave similarly by amidolytic activity in serum. Kim *et al.*[1] synthesized and found that the 20R-isomer among the amide derivatives of 20-dihydroprednisolonic acids showed higher local anti-inflammatory activity than the parent compound, prednisolone, but with significant systemic side-effects. Therefore, it is of interest to compare the hydrolysis rate of these ester and amide derivatives in order to investigate the differences in expressing systemic side-effects.

In this communication, the hydrolysis rates of the ester and amide derivatives of 20R- and 20S-dihydroprednisolonic acids were compared and the effects of various substituents at C-21 and C-20 configuration were also studied.

EXPERIMENTAL METHODS

Materials

Prednisolone was purchased from Sigma Chem. Co. (St. Louis, MO). The HPLC system (Waters Associates) consisted of isocratic pump (M-510), μ-bondapak reverse phase C_{18}-cartridge (Merck) and flatted-bed recorder (TOA electronics). The detector wavelength was set at 254 nm. The mobile phase was MeOH/water (60:40). All determinations were duplicated. All the ester and amide derivatives (Fig. 1) used in this study were prepared as

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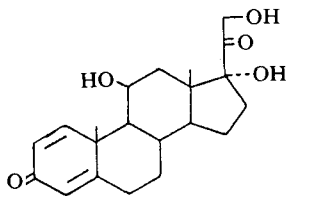
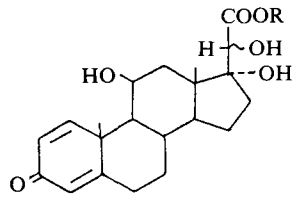
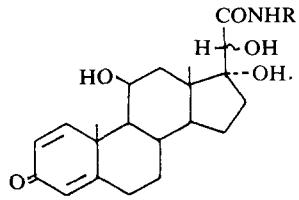


PREDNISOLONE
(I)



IIa, b (Me)
IIIa, b (Et)
IVa, b (Pr)
Va, b (Bz)
VIa, b (H)



VIIa, b (Me)
VIIIa, b (Et)
IXa, b (Pr)
Xa, b (Bz)

Fig. 1. Derivatives of 20(R,S)-dihydroprednisolonate.
(a denotes 20R-isomer, b denotes 20S-isomer.)

described in Kim *et al.*[1] and the accompaning paper[3]. For hydrolysis study, all the compounds (0.70-3.20 mg) were dissolved in acetone (1,000 μl) and DMSO (50 μl), and the appropriate amounts of each compound were incubated with various dilutions of rat serum or liver homogenate.

Hydrolysis in rat serum

Sprague-Dawley Rats (♀ , 150-200 g) were lightly anesthetized with ether. Blood was withdrawn by cardiac puncture. After standing at 4 °C for 1 hr, it was centrifuged and serum was obtained. It was stored at -20 °C until use. On the day of the experiment, 10 μl of the compound solution and 100 μl serum were added to 90 μl buffer solution (Tris, 0.04 M, pH 7.4) at 0 °C. For esters of 20S-dihydroprednisolonic acid, it was not possible to measure precise hydrolysis rate in the above condition (50% serum) because the compounds were hydrolyzed too fast. Therefore, the rate serum was diluted as indicated in Fig. 3 for obtaining measurable hydrolysis rates. After incubation at 37 °C for the various time intervals (0-3 hrs), 28 μl of 40% TCA solution was added to precipitate proteins. The mixture was allowed to stand at 0 °C for 30 min, followed by microcentrifugation at 10,000 rpm for 20 min. The supernatant was filtered and injected to HPLC for measuring the amount of the unhydrolyzed compounds. The control experiments were done using the compound solution (10 μl) in 190 μl buffer solution at 37 °C for 0-3 hrs. To substract the effects of nonenzymatic hydrolytic activity in the rate serum, parallel experiments were performed using serum at 0 °C.

In order to study the product composition, methyl 20R- (**IIa**) and benzyl 20S-dihydroprednisolonate (**Vb**) were incubated with serum, 50% and 5% repectively for 2 and 1 hrs. After extracting the unreacted compounds with ethyl acetate, the remaining aqueous phase was acidified with 3 drops of 2N HCl and the hydrolyzed acid derivatives were extracted with ethyl acetate (500 μl) and dried under nitrogen. The dried acid derivatives were esterified with diazomethane in ether[7]. After dissolving in 20 μl of acetone, the solutions were analyzed by HPLC. For the other compounds, only the unhydrolyed portions were measured.

Hydrolysis in rat liver homogenate

Rat liver was excised from anethesized S-D rat and perfused with physiological saline at 0 °C for 10 min to remove the blood. The above Tris buffer solution (10 ml) was added to the liver (10 g) and homogenized with teflon pestle. The homogenate was filtered with 2 layers of cheese cloth and the filtrate was centrifuged at 5,000 g, 4 °C for 30 min. The supernatant was used as a liver homogenate source. Hydrolysis condition was same as described for hydrolysis in rat serum.

RESULTS AND DISCUSSION

The present investigation has been carried out to compare the hydrolysis rates of the synthesized ester and amide derivatives in rat serum and liver homogenate. The normal detection range of the derivatives in HPLC was 10-100 ng at AUFS 0.005. The retention times for the ester derivatives were reported in the accompanying paper[3] and the retention times for the amide derivatives were shown to be 7.2 min for **VIIa**, 11.3 min for **VIIb**, 16.0 min for **Xa** and 24.1 min for **Xb**. In order to figure out product composition after incubation of **IIa** and **Vb** with 50% and 5% serum in the reaction mixture, the acid portions were extracted. Because it is difficult to detect 20R-and 20S-dihydroprednisolonic acids with HPLC *in situ*, the above extracted acid portions were esterified to methyl 20R- and 20S-dihydroprednisolonate with diazomethane and analyzed with HPLC. From these experiments, it was found that 13% of **IIa** was converted after incubation for

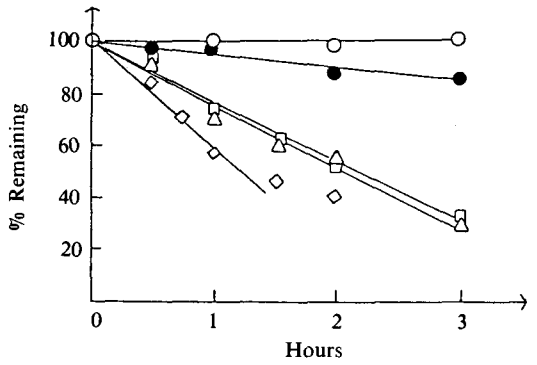


Fig. 2. Percent remaining of 20R-ester derivatives in rat serum.

The data points represented were mean of the duplicate experiments. Serum concentration was 50%. The concentration of the derivatives were 1×10^{-4}M. Prednisolone (○), **IIa** (●), **IIIa** (△), **IVa** (□), **Va** (◇)

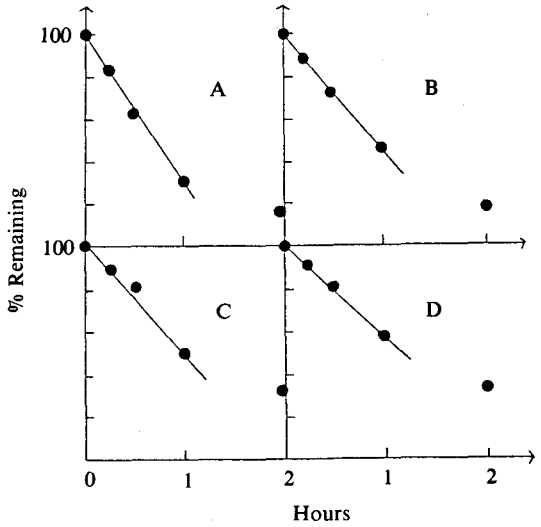


Fig. 3. Percent remaining of 20S-ester derivatives in rat serum.

A for the hydrolysis of **IIb** (serum; 50%, 1×10^{-4} M), **B** for **IIIb** (serum; 33%, 2×10^{-4} M), **C** for **IVb** (serum; 10%, 3×10^{-4} M), **D** for **Vb** (serum; 5%, 3×10^{-4} M)

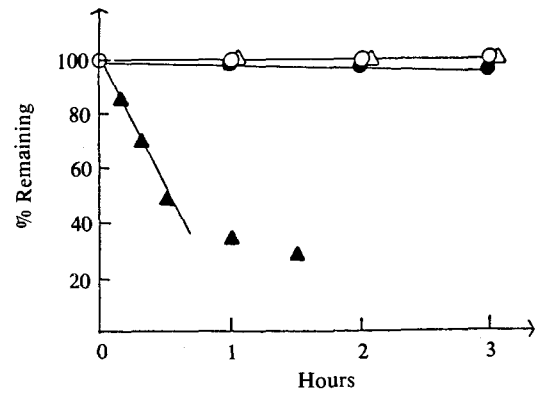


Fig. 4. Percent remaining of 20(R,S)-amide derivatives in rat serum.

Serum concentration was 33% and the concentration of the derivatives were 1×10^{-4} M. **VIIa** (○), **VIIb** (●), **Xa** (△), **Xb** (▲)

2 hrs in serum and 72% of the converted products was recovered as the original ester derivative (methyl 20R-dihydroprednisolonate, **IIa**) with diazomethane treatment. In **Vb**, 44% of the derivative was converted after incubation for 2 hrs and 88% of the converted products was recovered as methyl 20S-dihydroprednisolonate (**IIb**). These results could suggest that the primary metabolic pathway of these ester derivatives in rat serum would be the hydrolysis of C-21 ester portion and the hydrolysis occurred with retention of C-20 configuration. Fig. 2 illustrates the hydrolysis pattern of 20R-ester derivatives in 50% serum at 37 °C. Control experiment without serum at 37 °C showed no detectable hydrolysis in all derivatives including 20S-esters. Parallel experiment with rat serum at 0 °C for 3 hrs showed that all the derivatives were not hydrolyzed except **IVb** and **Vb**. It was found that 6% of propyl 20S-ester derivative (**IVb**) and 11% of **Vb** were hydrolyzed after incubation for 3 hrs. Fig. 3 represented the hydrolysis pattern of 20S-ester derivatives after correction with above parallel experiment. Because the hydrolysis rate of 20S-ester derivatives in 50% serum were too fast to measure, the appropriate dilutions of serum were used. Fig. 2 and 3 indicated that the ester derivatives having larger substituents showed the faster hydrolysis than the esters having small substituents while prednisolone showed no significant change in the same condition. It is also found that the 20S-ester derivatives always showed faster hydrolysis than the corresponding 20R-ester derivatives (Table I). Although the amount of steroid binding to serum protein was not measured in this experiment, this effect could be neglected because the concentration of the derivatives used here were far excess of the concentration of steroid in the blood. The hydrolysis of the amide derivatives in 33% rat serum is shown in Fig. 4 which revealed that 20S-amide derivatives showed the considerable change while 20R-amide derivatives did not. The calculated hydrolysis rates (nmole/min/ml serum) are represented in Table I.

Table I. The maximum hydrolysis rate in rat serum

Compounds[a]	V_{max}[b] (nmol/min/ml serum)
prednisolone (**I**)	<0.02
IIa	0.16
IIb	2.46
IIIa	0.98
IIIb	5.57
IVa	0.86
IVb	29.6
Va	1.40
Vb	52.9
VIIa	<0.02
VIIb	0.11
Xa	<0.02
Xb	3.83

[a]The amounts and serum concentation used for this experiments were represented in Fig. 4-9.
[b]These values were calculated based on first 1 hr incubation period.

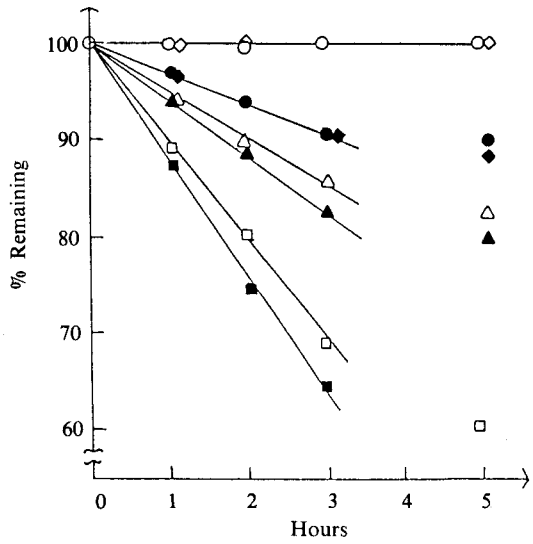


Fig. 5. Percent remaining of 20(R,S)-ester derivatives in liver homogenate.
The concentration of homogenate was 50% and 1×10^{-4} M of compounds were used. **IIa** (○), **IIb** (●), **IIIa** (△), **IIIb** (▲), **IVa** (□), **IVb** (■), **Va** (◇), **Vb** (◆)

Ingeneral, the hydrolysis rates of the ester derivatives was found to be far faster than the amide derivatives and the 20S-isomer was always rapidly hydrolyzed than the corresponding 20R-isomer in both derivatives. From these results, it is suggested that hydrophobicity and C-20 configuration are the essential factor governing the hydrolysis rate of both derivatives in rat serum. The similar findings were previously observed by O'Neill *et al.*[8] who showed the increasing hydrolysis rate with the increased size of carbon side chains of steroidal-21-ol ester when incubated with rat skin homogenate. Hattori *et al.*[9] found that the most active sites in rat organs for hydrolysing steroidal-21-ol ester were liver, intestine and duodenum. When the ester derivatives were incubated with rat liver homogenate for the esterolytic activity source, the hydrolysis rates of the ester derivatives were found to be much lower than those in serum, based on blood volume and liver weight, but the hydrolysis pattern (Fig. 5) was similar with the hydrolysis in serum except benzyl esters (**Va,b**). These results are well correlated with the findings of Kumari *et al.*[6] who reported that rat serum showed the highest esterolytic activity to methyl 20R-and 20S-dihydroprednisolonate (**IIa,b**), while the liver homogenate showed very low hydrolyzing activity to these esters. They further studied the inhibitory activities of various esterase inhibitors. Diisopropylfluorophosphate (1×10^{-6}M) showed 18% inhibition of the esterolytic activity and bis-p-nitrophenyl phosphate (1×10^{-6}M) inhibited 26-48% of the esterolytic activity. Depending on the substrate used (steroid-21-ol ester or steroid-21-oic acid ester), inhibition degree were varied. Therefore, it is suspected that this discrepancy of the hydrolysis of esters of steroidal-21-oic acid and steroidal-21-ol ester might come from the different esterolytic enzyme source.

Pharmacological evaluation revealed that the 20S-isomer of the ester derivatives showed higher local anti-inflammatory activity than the respective 20R-isomer while 20R-isomer of the amide derivatives showed the higher local anti-inflammatory activity than the corresponding 20S-isomer. In respect to systemic side-effects, the ester derivatives did not possess systemic effects in contrast to the amides having significant systemic effects[1,3]. On the basis of "antedrug" concept, the compounds showing reduced systemic side-effects should have reasonably rapid hydrolysis rates in the systemic circulation. From the results of present investigation, it was found that 20S-esters with reduced systemic effects showed rapid hydrolysis in serum, but 20R-amides having significant systemic side-effects showed no detectable change at the same condition. These findings suggested that systemic side-effects

expressed by the amide derivatives might be partly, if not all, due to the slow conversion rate of the derivatives to the inactive compound in the systemic circulation and these results may support the "antedrug" concept. And the hydrolysis rate may be the important factor for the "antedrug" design in this series of compounds.

CONCLUSION

Using rat serum and liver homogenate, the hydrolysis rate of various ester and amide derivatives of 20-dihydroprednisolonic acid were compared. In general, the ester derivatives showed the faster hydrolysis in rat serum than the amide derivatives and 20S-isomer of both derivatives showed the faster hydrolysis than the corresponding 20R-isomer. Es-Especially, 20S-esters having reduced systemic side-effects were found to be hydrolyzed rapidly while 20R-amides having considerable side-effects was not significantly hydrolyzed, which strongly supports the "antedrug" concept.

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