International Journal of Pharmaceutics, 65 (1990) 109–114
Elsevier

IJP 02198

Effects of 3-hydrazone modification on the metabolism and protein binding of progesterone

Krishnakali Basu and Ashim K. Mitra

Department of Industrial and Physical Pharmacy, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, IN 47907 (U.S.A.)

(Received 18 October 1989)
(Modified version received 6 April 1990)
(Accepted 19 April 1990)

Key words: Progesterone; 3-Hydrazone derivative; Isolated rat liver microsomes; In vitro metabolism; Protein binding

Summary

Progesterone was rapidly metabolized with an apparent half-life of 2–2.5 min when incubated with isolated rat liver microsomes. Progesterone 3-hydrazone prodrugs, on the other hand, exhibited remarkable liver microsomal stability with apparent first-order half-lives increasing by about 35–350-fold over the parent drug. Protein binding experiments indicated significantly lower association of the prodrugs with human serum albumin as compared to progesterone. However, the combining affinities of the derivatives with α_1-acid glycoprotein are comparable to that of progesterone itself. No appreciable binding of either progesterone or its produgs with other serum proteins, i.e., fibrinogen and γ-globulin, was observed. Therefore, 3-hydrazone prodrugs may be able to enhance oral absorption of progesterone not only by increasing its aqueous solubility but by reducing its first pass liver metabolism as well.

Introduction

Progesterone (4-pregnene-3,20-dione) (I) is an extremely hydrophobic steroid with very poor oral bioavailability and short biological half-life (Whitehead et al., 1980). Poor aqueous solubility and rapid degradation by the gastrointestinal mucosa and liver (Little et al., 1966) are two major causes of its low systemic availability. Several synthetic progestins have been introduced to deal with these absorption limitations. However, these compounds have been linked to abnormal fetal development, mental depression, masculinization, headaches, etc. (Morville et al., 1982; Huff, 1988). The synthetic progestins, i.e., hydroxyprogesterone, medroxyprogesterone, norgestrel, etc. are hydroxyl, acetoxyl or ethinyl derivatives of progesterone primarily at the 17-α position having their own hormonal bioactivity. However, the present report describes the metabolism of three bioreversible 3-hydrazone derivatives of progesterone which upon bioreversion will produce the progestogenic activity.

An ideal approach to progestin therapy would be to administer natural progesterone by the oral route. Such a mode of drug delivery would necessitate the development of a nontoxic water-soluble

Correspondence: A.K. Mitra, Department of Industrial and Physical Pharmacy, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, IN 47907, U.S.A.

prodrug of progesterone with less susceptibility to first pass hepatic metabolism. In our earlier report, three highly water-soluble 3-hydrazone transient derivatives (prodrugs) of progesterone have been described (Basu et al., 1988). These compounds have enhanced the progesterone aqueous solubility in the range of four to five orders of magnitude. The rationale behind the selection of hydrazone modification of the 3-keto group in progesterone partly stems from the fact that hydrazone derivatives of other ketosteroids have not produced any noticeable signs of toxicity (Bochmühl et al., 1945; Ward, 1953). The present study was undertaken to investigate any possible inhibitory effects of 3-hydrazone modification on the liver metabolism of progesterone. Since oxido-reductive metabolic processes of progesterone mainly occur in liver microsomal subcellular fractions (Sencia et al., 1981) the current studies have been carried out using isolated rat liver microsomal preparations.

This report also describes the effects of ionic characters of the derivatives on their binding affinities with a number of human serum proteins, i.e., serum albumin, α_1-acid glycoprotein, fibrinogen and γ-globulin. Wiseman and Nelson (1964) reported rank-order correlation between the rates of in vitro metabolism of a number of sulfonamides and their degrees of protein binding. Results from the studies described in this report may be helpful in predicting the oral delivery potential of these water-soluble progesterone prodrugs.

Materials and Methods

Materials

NADH, cytochrome *c*, D-glucose 6-phosphate, β-glycerophosphate, pyruvic acid, human serum albumin, α_1-acid glycoprotein, fibrinogen and γ-globulin were obtained from Sigma (St. Louis, MO) and were used as received. All other chemicals were of analytical reagent grade. The synthesis, structural elucidation and reversion kinetics of 3-hydrazone derivatives to progesterone were reported previously from this laboratory (Basu et al., 1988). Stock solutions of progesterone and its three derivatives were prepared in methanol and kept at 4°C until further use. Adult male Sprague-Dawley rats weighing about 150–200 g were used for metabolism studies.

Subcellular fractionation

Rats were euthanized by an overdose of sodium pentobarbital. Livers were excised immediately and then washed thoroughly with ice-cold saline. The organs were homogenized in 9 volumes of buffer (0.01 M Tris-HCl, pH 7.4, 25 mM sucrose and 1 mM EDTA) and the homogenate was filtered through cheesecloth to remove any debris and unbroken tissues. The supernatant was centrifuged at $25\,000 \times g$ for 15 min to remove nuclear, mitochondrial and lysosomal fractions. Microsomes were isolated from the supernatant by subsequent centrifugation at $100\,000 \times g$ for 40 min. Isolated microsomes were then suspended in 0.25 M sucrose and stored at −20°C in small aliquots until further use. When required, the fractions were rapidly thawed prior to use. Any unused thawed quantity was never refrozen, or reused. The purity of microsomal preparation was ascertained by measuring succinate cytochrome *c* reductase (Schnaitman and Greenwalt, 1968), acid phosphatase (Chen et al., 1956), catalase (Luck, 1965), lactate dehydrogenase (Kornberg, 1955) and glucose-6-phosphatase (Nordlie and Arion, 1966) levels for the presence of mitochondria, lysosomes, peroxisomes, cytosol and microsomes, respectively. The specific activities for acid phosphatase, catalase and glucose-6-phosphatase were 0.7, 0.5 and 6.0 U/mg protein, respectively. Protein estimation was carried out according to the method of Lowry et al. (1951) using bovine serum albumin in 0.25 M sucrose as the standard.

In vitro liver microsomal metabolism

The metabolism of progesterone and its derivatives was studied according to Sencia et al. (1981) with slight modification. The reaction was carried out in 0.025 M phosphate buffer (pH 7.2, 37°C) containing different amounts of microsomal protein and a constant amount of NADH (100 μg). At various predetermined time points, the reaction was stopped by the addition of 4 volumes of ice-cold acetonitrile. After vortexing for 2 min the mixture was centrifuged ($50\,000 \times g$, 10 min) and

the supernatant was analyzed by ultraviolet spectrophotometry for the starting compounds. For control experiments, the microsomal protein was replaced by the same volume of blank buffer.

Protein binding

The binding of progesterone and its derivatives was carried out by using the equilibrium dialysis method as described by Selden et al. (1961). Phosphate buffer (0.01 M) at pH 7.4 and 0.1 M ionic strength served as the medium for the dialysis experiments. A mixture containing 1 ml of protein solution and 0.1 ml of substrate was incubated at 37 °C for 1 h before being dialysed against 25 ml of phosphate buffer containing the same amount of compound per ml. The dialysis was continued in a constant temperature water bath at 25 ± 0.1° C with continuous shaking until equilibrium was attained. Upon termination of the dialysis period, the volumes of the outside solution were measured and the substrate concentrations were determined by the analytical techniques described previously. The values were corrected by subtracting the value from a control experiment where the solution containing the compound had been replaced by the same volume of blank buffer. The compounds were found to be chemically stable in the protein dialysate or liver microsomal media during the time course of an experiment as demonstrated by constant UV absorption spectra.

Binding parameter calculation

Percent protein bound can often be expressed as the percentage of total substrate bound according to Eqn 1:

$$\% \text{ compound bound} = \frac{[S]_{bound}}{[S]_{total}} \times 100 \qquad (1)$$

where $[S]_{bound}$ and $[S]_{total}$ are the concentrations of the bound and total substrate, respectively. Since the magnitude of binding depends primarily on the binding affinity and concentration of a given protein, references to binding per unit concentration of the protein actually signify true binding affinity. A convenient expression (Daughaday, 1958) denoting such affinity (K_p) is represented by Eqn 2:

$$K_p = \frac{[S]_{bound}}{[S]_{unbound} \times [P]} \qquad (2)$$

where $[S]_{unbound}$ and $[S]_{bound}$ may be expressed as percent or molar concentrations and [P] denotes free protein concentration in molar quantity.

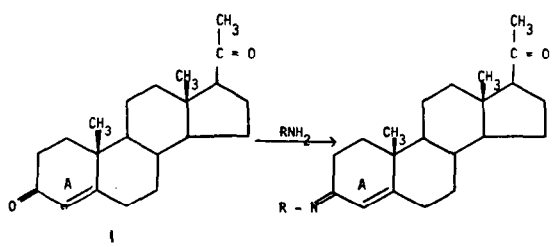
An approximate free energy of association (ΔG^0 in kcal/mol) can be calculated according to Eqn 3 (Sandberg et al., 1957):

$$\Delta G^0 = -2.303RT \log K_p \qquad (3)$$

R is the universal gas constant, T is the absolute temperature of the medium and K_p is the binding affinity constant as described in Eqn 2.

Results and Discussion

The chemical structures of progesterone and its 3-hydrazone derivatives are depicted in Scheme 1. Structural identification, physicochemical properties and other kinetic reversion parameters of the derivatives have been described in our earlier report (Basu et al., 1988). Although progesterone is known to be metabolized by various mechanisms (Sandberg and Slaunwhite, 1958; Fotherby, 1964), the major pathway of its biotransformation is related to the reduction of both keto groups at the 3- and 20-positions (Aufrere and Benson, 1976).



Scheme 1

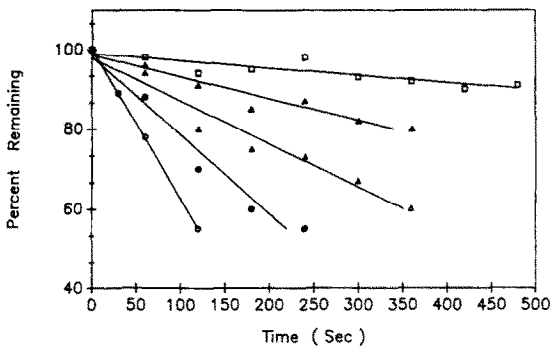


Fig. 1. Metabolism of progesterone by liver microsomal proteins. 32 nmol of progesterone was added to a solution containing different amounts of microsomal protein (ranging from 0.082 mg to 0.41 mg) and 100 μg NADH. The mixture was incubated at 37°C for various time periods and the reaction was stopped by the addition of ice-cold acetonitrile. The supernatant was analyzed for progesterone as described in the text. (□) 0.082, (▲) 0.117, (△) 0.165, (●) 0.27, and (○) 0.41 mg microsomal protein.

Reduction at position C-3 occurs at a much faster rate than at C-20. Therefore, an effort has been made to stabilize progesterone by protecting the 3-keto group in the molecule.

As illustrated in Fig. 1, progesterone was metabolized rapidly in the isolated rat liver microsomal preparations with a half-life of around 2.5 min. The rate of this metabolic reaction was dependent on the protein concentration in the microsomal preparation. A similar study has been described by Sencia et al. (1981) using different amounts of microsomal protein and radioactive progesterone.

A constant amount (0.41 mg) of microsomal protein was henceforth used in all metabolism studies. Approx. 65–70% of progesterone was metabolized by 0.41 mg protein in 2 min. However, all the 3-hydrazone derivatives particularly compounds II and IV exhibited remarkable stability in the same rat liver microsomal preparations. As Fig. 2 suggests, compound III was less stable than the other two derivatives. The same compound also had different protein binding properties as described later in this report. The rate constants and half-lives of metabolism for progesterone and its three hydrazone derivatives have been summarized in Table 1. A 35-fold increase in half-life was observed with compound III compared to 160- and 360-fold increases for compounds II and IV, respectively. The rate of disappearance of progesterone in microsomal preparations as described earlier was shown to have a $t_{1/2}$ of 2.5 min, whereas the reported half-life of intravenously injected progesterone in rats varies from 3 to 90 min (Sandberg and Slaunwhite, 1958). The UV spectrophotometric assay for each of the compounds was specific and no interference was observed with progesterone or its metabolites. As described in our earlier report (Basu et al., 1988), standard curves generated within the concentration range of 1–150 μg/ml were linear with correlation coefficients of 0.997–0.999 in all cases. Any comparison of this result to published literature values is inappropriate, since previous metabolism studies lacked a specific and sensitive assay method

TABLE 1

Kinetic biotransformation parameters of progesterone and 3-hydrazone derivatives in isolated rat liver microsomes

Compound	k (μM min^{-1})	$t_{1/2}$ (min)	Ratio
Progesterone	7.22	2.2	
Compound III	0.33	78.2	36
Compound II	0.09	357.9	163
Compound IV	0.069	797.5	363

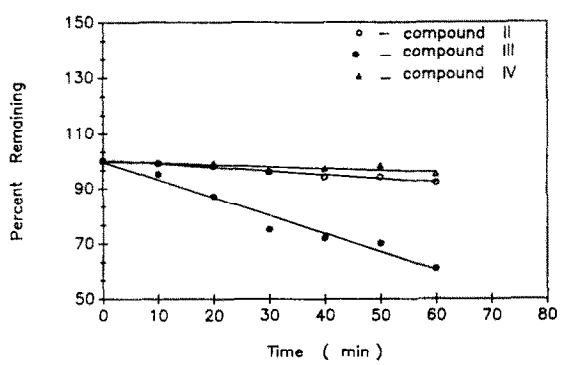


Fig. 2. Metabolism of progesterone derivatives by liver microsomes. 65, 52 and 95 nmol of compound II, III and IV, respectively, were added separately to a mixture containing 0.41 mg of microsomal protein and 100 μg NADH. The mixture was incubated at 37°C for various time periods and the reaction was stopped by the addition of ice-cold acetonitrile. The supernatant was analyzed for the compounds as described in the text.

TABLE 2

Binding of progesterone and its 3-hydrazone derivatives to different proteins in phosphate buffer pH 7.4 ($\mu = 0.1$) at 37 °C

Protein	Compound	% Binding	Combining affinity (M^{-1}) ($\times 10^{-6}$)	ΔG^0 (kcal/mol)
Human serum albumin	I	95	0.239	−7.60
	II	55	0.41	−7.96
	III	7	0.03	−6.24
	IV	45	0.27	−7.71
Fibrinogen	I	8.8	0.01	−5.70
	II	31	1.28	−8.67
	III	27	1.04	−8.54
	IV	16	0.57	−8.16
α_1-Acid glycoprotein	I	81	0.37	−7.90
	II	95	41.80	−10.80
	III	69.5	4.79	−9.47
	IV	53	2.51	−9.07
γ-Globulin	I	3	0.01	−5.30
	II	80	19.11	−10.32
	III	30	1.97	−8.92
	IV	59	6.39	−9.65

for progesterone. Nevertheless, such in vitro microsomal metabolic rates provide a measure of relative susceptibility. In vivo metabolic rates may be different because of possible contributions from other extrahepatic sites. A therapeutic (25 mg) dose of progesterone administered by intramuscular injection usually generates concentrations within the range of 10–100 ng/ml. Similar concentrations have been used in the in vitro liver microsomal experiments.

The results of interaction of progesterone and its derivatives with four different plasma proteins are presented in Table 2. Fibrinogen exhibited the weakest interaction among all proteins and the value of progesterone binding is consistent with the data reported by Westphal et al. (1961). Therefore, it appears that 3-keto modification has no effect on binding with fibrinogen. Among the four compounds studied, progesterone interacts most strongly with albumin, a fact that is consistent with the earlier results from Westphal (1955) and Westphal et al. (1955). The binding of steroid molecules with albumin is inversely related to the number of polar groups and also to the degree of aqueous solubility, a phenomenon described by Eik-Nes et al. (1954). Such a relationship has not been observed for the progesterone 3-hydrazone derivatives. Compound IV interacts strongly with albumin in spite of its highest solubility whereas compound III having an aqueous solubility intermediate between compound II and compound IV showed the least binding. Similar differences in binding affinities among the derivatives were observed with γ-globulin. With this protein, compounds II and IV exhibited a higher degree of binding than compound III. Progesterone, on the other hand, is already known for its very low affinity for γ-globulin (Westphal et al., 1961). Progesterone and all three 3-hydrazone derivatives exhibited significant binding with α_1-acid glycoprotein. Similar results were reported by Westphal et al. (1961) where α_1-acid glycoprotein interacted very strongly with progesterone. This glycoprotein contains 64% protein, 16.4% hexose, 11.9% hexosamine, and 10.6% sialic acid and such a combination might enhance binding affinity of progesterone and its derivatives. The physiological and clinical significance of this observation remains to be elucidated. Results from these studies clearly indicate that both compounds II and IV offer significant metabolic protection to progesterone and may be suitable candidates for further in vivo evaluation as orally administered progesterone prodrugs.

Acknowledgements

This work was supported in part by a Merck Faculty Development Award to A.K.M. by Merck, Sharp and Dohme Research Laboratories. Instrumentation support was provided by NIH grant NS 25284 and a biomedical research support grant RR 05586.

References

Aufrere, M.B. and Benson, H., Progesterone: An overview and recent advances. *J. Pharm. Sci.*, 65 (1976) 783–800.

Basu, K., Kildsig, D.O. and Mitra, A.K., Synthesis and kinetic

stability studies of progesterone derivatives. *Int. J. Pharm.,* 47 (1988) 195–203.

Bochmühl, M., Schaumann, O., Bartholmaus, E. and Leditschike, H., Derivatives of 2-alkyl-1,4-naphthaquinone. *U.S. Patent* 2,372,655, *Chem. Abstr.,* 39 (1945) 4723.

Chen, P.S., Jr, Toribara, T.Y. and Warner, H., Microdetermination of phosphorus. *Anal. Chem.,* 28 (1956) 1756–1758.

Daughaday, W.H., Binding of corticosteroids by plasma protein. III. The binding of corticosteroid and related homone by human plasma and plasma protein fractions as measured by equilibrium dialysis. *J. Clin. Invest.,* 37 (1958) 511–519.

Eik-Nes, K., Schellman, J.A., Lumry, R. and Samuels, L.T., The binding of steroids to proteins. I. Solubility determinations. *J. Biol. Chem.,* 206 (1954) 411–419.

Fotherby, K., The biochemistry of progesterone. *Vitam. Horm.,* 22 (1964) 153–204.

Huff, B.B., *Physician Desk References*, Medical Economic, Oradell, NJ, 1988, pp. 411–420.

Kornberg, A., Lactic dehydrogenase of muscle. *Methods Enzymol.,* 1 (1955) 441–443.

Little, B., Tait, J.F., Tait, S.A.S. and Erlenmeyer, F., The metabolic clearance rate of progesterone in males and ovariectomized females. *J. Clin. Invest.,* 6 (1966) 901–912.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., Protein measurement with the folin phenol reagent. *J. Biol. Chem.,* 193 (1951) 265–275.

Luck, H., In Bergmeyer, H.U. (Ed.), *Methods of Enzymatic Analysis,* 2nd Edn, Academic Press, New York, 1965, pp. 885–894.

Morville, R., Dray, F., Reymier, J. and Barrat, J., Biodisponsibility de la progesterone naturelle administres par voie oral. Measure des concentrations du steroide dans le plasma, l'endometre et le tissue mammarie. *J. Gynecol. Obstet. Biol. Reprod.,* 11 (1982) 355–363.

Nordlie, R.C. and Arion, W.J., Glucose-6-phosphatase. *Methods Enzymol.,* 9 (1966) 619–625.

Sandberg, A.A., Slaunwhite, W.R., Jr and Autoniader, H.N., Binding of steroids and steroid conjugates to human plasma protein. *Recent Prog. Horm. Res.,* 13 (1957) 209–267.

Sandberg, A.A. and Slaunwhite, W.R., Jr, The metabolic rate of progesterone-C^{14} in human subjects. *J. Clin. Endocrinol. Metab.,* 18 (1958) 253–265.

Schnaitman, C. and Greenwalt, J.W., Enzymatic properties of the inner and outer membrane of rat liver mitochondria. *J. Cell. Biol.,* 38 (1968) 158–175.

Sencia, I.R., II, Rahl, S. and Dey, A.C., Metabolism of progesterone by phosphate buffer extracts of rabbit liver microsomes. *J. Steroid Biochem.,* 14 (1981) 473–479.

Ward, W.C., 5-Nitrofuraldehyde derivatives. *U.S. Patent,* 2,626,258, *Chem. Abstr.,* 47 (1953) 11254.

Westphal, U., Interaction between hydrocortisone-4-C^{14} or progesterone-4-C^{14} and serum albumin, demonstrated by ultracentrifuge and electrophoresis. *Endocrinology,* 57 (1955) 456–465.

Westphal, U., Frischein, H.H. and Pearce, E.M., Binding of hydrocortisone-4-C^{14} and progesterone-4-C^{14} to serum albumin demonstrated by paper electrophoresis. *Science*, 121 (1955) 601–602.

Westphal, U., Ashley, B.D. and Selden, G.L., Steroid-protein interactions. VII. Interaction of progesterone and corticosteroids with human plasma proteins determined by multiple equilibrium dialysis. *Arch. Biochem. Biophys.,* 92 (1961) 441–448.

Whitehead, M.I., Towensend, P.T., Gill, D.K., Collins, W.P. and Campbell, S., Absorption and metabolism of oral progesterone. *Br. Med. J.,* 280 (1980) 825–827.

Wiseman, E.H. and Nelson, E., Correlation of In vivo metabolism rate and physical properties of sulfonamides. *J. Pharm. Sci.,* 53 (1964) 992.