Pharmaceutical Research, Vol. 5, No. 1, 1988

Report

Prodrugs for Improved Oral β-Estradiol Bioavailability

Munir A. Hussain,[1,2] Bruce J. Aungst,[1] and Eli Shefter[1]

Received May 22, 1987; accepted August 12, 1987

Prodrugs of β-estradiol (1) were prepared with the objective of improving its oral bioavailability. β-Estradiol-3-acetylsalicylate (2), β-estradiol-3-salicylate (3), and β-estradiol-3-anthranilate (4) were synthesized. With these prodrugs the 3-phenolic hydroxy group of estradiol was protected, so that first-pass conjugative metabolism could be reduced. Prodrug hydrolysis rates in dog and human plasma *in vitro* were determined. Deacetylation of estradiol-3-acetylsalicylate was much more rapid than its hydrolysis to estradiol. In dogs, oral estradiol bioavailability after administration of 2 and 4 was 17-fold and 5-fold higher, respectively, than after oral 1.

KEY WORDS: estradiol; prodrugs; bioavailability; first-pass metabolism.

INTRODUCTION

Estrogens have a valuable role in the treatment of various conditions, including relief of menopausal symptoms and prevention of the progression of osteoporosis. The most common oral dosage forms are micronized estradiol and conjugated and esterified estrogen mixtures. Orally administered estradiol is well absorbed but undergoes considerable first-pass metabolism. Systemic bioavailability in young female volunteers was only 11% of the dose, and the primary routes of first-pass metabolism were oxidation to estrone and conjugation of estradiol and estrone to the sulfates and glucuronides (1). The increased levels and accumulation of these metabolites have been implicated as causing changes in hepatic function and may significantly contribute to the undesirable side effects of chronic estradiol therapy (2,3). If oral estradiol bioavailability could be increased, it is possible that the required dose and the incidence of side effects could be reduced.

One approach to increasing oral bioavailability is through prodrugs, in which the normally metabolized functional group is blocked to prevent metabolism at that position. After the prodrug passes through the site of first-pass metabolism, the blocking substituent should be enzymatically cleaved to free the active drug. The prodrug approach to preventing oral first-pass metabolism has not been explored for estradiol. Conjugated and esterified estrogens, which presumably act as prodrugs, are commercially available products isolated from pregnant mare's urine. These preparations do not provide substantially higher serum estradiol concentrations than the micronized estradiol preparations (4). Estradiol 3-benzoate 17-cyclooctenyl ether was reported to have a prolonged duration of action orally (5), but oral bioavailability and a comparison with estradiol were not reported.

It was previously shown that prodrugs of nalbuphine, an opioid antagonist/analgesic, reduced conjugative first-pass metabolism and increased oral bioavailability in dogs (6). This prodrug approach was also successfully applied to improving the oral bioavailability of naltrexone (7). Nalbuphine, naltrexone, and estradiol are similar in that each is conjugated at the phenolic hydroxy position. In this study two estradiol prodrugs, similar to those previously described for nalbuphine, were synthesized and evaluated to see whether these types of prodrugs work for a different series of drugs.

MATERIALS AND METHODS

β-Estradiol was obtained from Sigma Chemical Company. Isatoic anhydride and 4-dimethylaminopyridine were obtained from Fluka Chemical Corporation. Acetylsalicyloyl chloride was purchased from Aldrich Chemical Company. *N,N*-Dimethylformamide and petroleum ether were obtained from J. T. Baker Chemical Company. Triethylamine, methylene chloride, methanol [high-performance liquid chromatographic (HPLC) grade], acetonitrile (HPLC grade), ethyl acetate, *n*-hexane, acetic acid, sodium acetate, and ammonium acetate were obtained from Fisher Scientific Company. d_6-Deuterated dimethylsulfoxide was purchased from Columbia Organic Chemical Co.

Melting points were determined on a Fisher–Johns melting point apparatus and are uncorrected. Proton nuclear magnetic resonance (NMR) spectra were recorded with WP200SY, Bruker (IBM). Optical rotations were recorded on Perkin Elmer 241 MC. Elemental analyses were performed by Atlantic Microlabs, Inc. (Atlanta, Ga.).

Synthesis

β-Estradiol-3-acetylsalicylate (2) (See Fig. 1)

β-Estradiol (2.72 g, 0.01 mol) was added to a solution of triethylamine (8.8 ml, 0.063 mol) in methylene chloride (120 ml) at room temperature. The mixture was stirred until a

[1] Du Pont Pharmaceuticals, Medical Products Department, Experimental Station, Building 400, Wilmington, Delaware 19898.

[2] To whom correspondence should be addressed

0724-8741/88/0100-0044$06.00/0

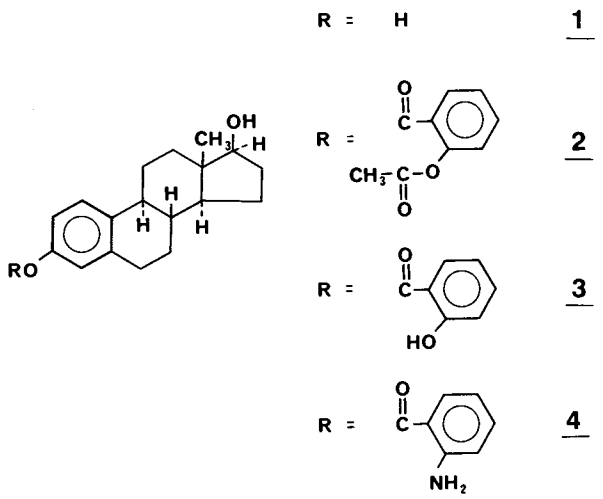

Fig. 1. Structures of estradiol (1), estradiol-3-acetylsalicylate (2), estradiol-3-salicylate (3), and estradiol-3-anthranilate (4).

clear solution resulted, which was then cooled to between 0 and 5°C. A solution of acetylsalicyloyl chloride (2.2 g, 0.011 mol) was added dropwise with stirring. After addition was complete, the ice bath was removed and the mixture was stirred at ambient temperature for 5 hr. It was washed with 10% aqueous sodium carbonate, then with water, and it was then dried over sodium sulfate, filtered, and evaporated. The residue was triturated with ether, filtered, and recrystallized from methylene chloride:*n*-hexane (1:1) to provide the title compound (70% yield) as a white solid, mp 185–188°C. ^{1}H NMR (Me_2SO-d_6): δ 0.7 (s, 3H, methyl); 1.13–3.63 (m, 16H, aliphatic); 2.25 (s, 3H, acetyl methyl); 4.53 (d, 1H, OH); 6.9–8.16 (m, 7H, aromatic). $[\alpha]_D^{25}$ = +48.5°C (c 1, Dioxane). Elemental analysis: Calc. for $C_{27}H_{30}O_5$: C, 74.63; H, 6.96. Found: C, 74.49; H, 7.02.

Estradiol-3-salicylate (3) from Estradiol-3-acetylsalicylate (2) (See Fig. 1)

Compound 2 (4.35 g, 0.01 mol) was dissolved in 500 ml of a solution of 5% concentrated hydrochloric acid in methanol and the solution was stirred at ambient temperature overnight (until all 2 was converted to 3 as observed by HPLC). The methanol was evaporated under reduced pressure. Methylene chloride (250 ml) and water (150 ml) were added and the aqueous layer pH was adjusted to pH 7.5 with saturated sodium carbonate solution. The methylene chloride layer was separated, dried over anhydrous sodium sulfate, and evaporated. The product was crystallized from ethyl acetate:*n*-hexane (1:1) to provide the title compound (70% yield) as a white solid, mp 204–207°C. ^{1}H NMR (Me_2SO-d_6): δ 0.7 (s, 3H, methyl); 1.13–3.63 (m, 16H, aliphatic); 4.53 (d, 1H; OH); 7–8 (m, 7H, aromatic); 10.5 (s, 1H, phenolic OH). $[\alpha]_D^{25}$ = +55.0°C (c 1, Dioxane). Elemental analysis: Calc. for $C_{25}H_{28}O_4$: C, 76.5, H, 7.19. Found: C, 76.4; H, 7.24.

β-Estradiol-3-anthranilate (4) (See Fig. 1)

A mixture of β-estradiol (2.72 g, 0.01 mol), isatoic anhydride 1.8g, 0.011 mol), and 4-dimethylaminopyridine (1.3 g, 0.011 mol) in *N,N*-dimethylformamide (50 ml) was heated at 80°C for 4 hr. After cooling, 200 ml of 10% aqueous sodium chloride solution was added and the mixture was stirred for 10 min. The precipitate was collected by filtration, washed with water, dissolved in methylene chloride, and extracted with 10% sodium carbonate. The methylene chloride layer was then dried over sodium sulfate and treated with charcoal to remove the tan color. The methylene chloride was then evaporated and the product was recrystallized from ethyl acetate. The title compound (65% yield) was obtained as a white solid, mp 201–203°C. ^{1}H NMR (Me_2SO-d_6): δ 0.7 (s, 3H, methyl); 1.13–3.63 (m, 16H, aliphatic); 4.53 (d, 1H, OH); 6.53–7.9 (m, 7H, aromatic); 6.72 (s, 2H, NH_2). $[\alpha]_D^{25}$ = +55.8°C (c 1, Dioxane). Elemental analysis: Calc. for $C_{25}H_{29}NO_3$: C, 76.7; H, 7.47; N, 3.58. Found: C, 76.57; H, 7.49; N, 3.55.

Prodrug Hydrolysis in Plasma

The hydrolysis rates of each prodrug were determined in dog and human plasma. Human plasma was obtained from a local blood bank and was kept frozen between the time of receipt and the initiation of the experiments. The dog plasma was obtained from beagle dogs and was anticoagulated with heparin and stored frozen until the start of the experiment.

The prodrugs were dissolved in DMSO (4.6 m*M*) and added to 3 ml plasma at 37°C so that the prodrug concentration was 46 μ*M*. At various times of incubation, a 200-μl aliquot of plasma was taken and added to 200 μl of acetonitrile, which was then centrifuged. The clear supernatant was divided for separate HPLC analysis for the prodrug and estradiol. For HPLC, a reverse-phase (Zorbax C_8, Du Pont) column and UV detection at 242 nm were used. A mobile phase containing 82.5% methanol in 0.1 *M* ammonium acetate delivered at 1.45 ml/min gave retention times for 2 and 3 of 5.6 and 10 min, respectively. The mobile phase for 4 hydrolysis contained 80% methanol in 0.1 *M* ammonium acetate and was delivered at 1.6 ml/min. The retention time for 4 was 9.1 min. HPLC was used for 1 analysis in the plasma hydrolysis studies only. The detector was set at 282 nm and the mobile phase contained 50% acetonitrile in 0.1 *M*, pH 4.5, acetate buffer. At a flow rate of 1.3 ml/min, the retention time for 1 was 6 min.

Bioavailability Studies

Three male beagle dogs were administered, in a crossover fashion, equimolar oral doses (0.13 μmol/kg) of 1, 2, and 4. Doses were administered as solutions in PEG 400:water (3:1). All doses were followed by oral administration of 50 ml water. Blood (5 ml) was collected by jugular venipuncture into evacuated tubes containing sodium EDTA as the anticoagulant. Plasma was separated and stored frozen. Animals were fasted overnight prior to each experiment. Radioimmunoassay for plasma estradiol was performed by Hazelton Biotechnologies (Vienna, Va.). Briefly, 2-ml plasma samples were extracted with diethyl ether. The extracts were purified using Celite chromatography. They were then quantitated by radioimmunoassay using a specific rabbit antiserum generated against 17 β-estradiol 6-CMO:BSA. Separate experiments showed that the prodrugs did not interfere in the estradiol assay. Intact prodrugs

were not assayed in the *in vivo* studies. Individual baseline (predose) plasma estradiol concentrations were subtracted from each postdose sample value to correct for endogenous estradiol. Baseline plasma estradiol concentrations ranged from 8 to 18 pg/ml.

The estradiol half-life, $t_{1/2}$, was calculated by linear regression of the terminal portion of semilog plots of plasma estradiol concentration versus time. The area under each estradiol concentration versus time curve (AUC) was calculated using the trapezoidal method. Relative bioavailability was determined by dividing the estradiol AUC after prodrug doses by the estradiol AUC after oral estradiol doses.

RESULTS AND DISCUSSION

In vitro prodrug hydrolysis rates in plasma provide a measure of relative susceptibility to enzymatic attack. However, plasma hydrolysis rates may not be predictive of *in vivo* hydrolysis rates because of the contribution of other tissues, especially after oral dosing, and erythrocytes (6,7). *In vitro* hydrolysis rates of the estradiol prodrugs were determined using human and dog plasma. Compound **2** was very rapidly deacetylated to **3**. Subsequent hydrolysis of **3** to **1** was much slower (Fig. 2). Similar hydrolysis kinetics were observed for naltrexone-3-acetylsalicylate; deacetylation was much more rapid than hydrolysis of naltrexone-3-salicylate (unpublished). For both estradiol and naltrexone, deacetylation was faster in human plasma than in dog plasma, and hydrolysis of the salicylate prodrug had approximately the same half-life in dog and human plasma. Hydrolysis half-lives are given in Table I. Compound **4** had a very long hydrolysis half-life in plasma from either species.

Plasma estradiol concentration versus time profiles in dogs orally dosed with **1**, **2**, or **4** are shown in Fig. 3. After oral **1** doses, the peak plasma concentrations were at the first sampling time, 10 min, indicative of rapid absorption. After oral prodrug doses, maximum plasma estradiol concentrations were observed within 1 hr. Presumably, this rapid formation of estradiol (compared to in plasma *in vitro*) is a result of intestinal or hepatic prodrug hydrolysis and would not have been predicted based on plasma hydrolysis rates. It was previously shown that nalbuphine-3-anthranilate was rapidly hydrolyzed in dog intestine and liver homogenates *in vitro* (6).

Table I. Prodrug Hydrolysis Half-Lives in Plasma *in Vitro*

	Hydrolysis half-life (hr)[a]	
	Human plasma	Dog plasma
Estradiol-3-acetylsalicylate deacetylation	0.07	0.31
Estradiol-3-salicylate hydrolysis	7.50	8.00
Estradiol-3-anthranilate hydrolysis	57[b]	10% in 31 hr[b]

[a] Half-lives represent reactant disappearance, average of two experiments.
[b] Disappearance was followed only for 31 hr.

Relative bioavailability of estradiol was significantly improved when administered in prodrug form. A 17-fold increase was observed for **2**, and for **4** the increase was approximately 5-fold (Table II). Although absolute oral bioavailability of estradiol is not reported here, it was previously reported to be very low in dogs administered radiolabeled estradiol, and the major metabolites were estrone and estradiol glucuronides (8). Increased bioavailability should result in lower metabolite levels, which may lower the incidence of side effects. Another advantage is apparent when examining the interanimal variability in AUC. The relative standard deviation (100% × SD/mean) was 49% after oral **1** but only 9% after **2**.

The terminal half-life of plasma estradiol concentration was prolonged after the administration of either prodrug. Half-lives are reported in Table III. Several explanations for prolonged half-lives can be proposed. First, estradiol clearance could be nonlinear; at higher plasma concentrations the half-life would be longer. This does not appear to be the case since at the same plasma concentrations (for example, 100–10 pg/ml) the decay slopes are different. Another hypothesis is that a portion of the prodrug dose escapes intestinal and hepatic hydrolysis during absorption, and then its hydrolysis could be the rate-determining step in estradiol elimination. One inconsistency is that *in vitro* the acetylsali-

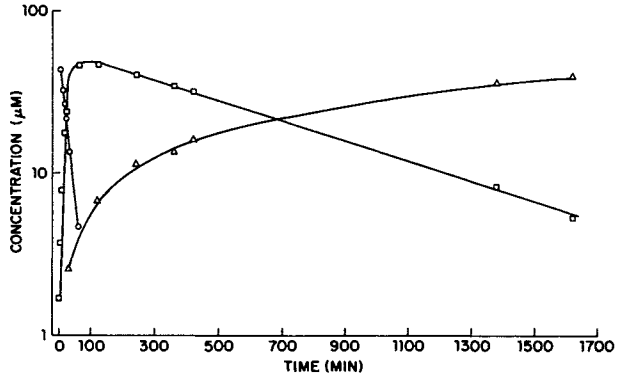

Fig. 2. Hydrolysis of estradiol-3-acetylsalicylate (○) in dog plasma with appearance and subsequent disappearance of estradiol-3-salicylate (□) and appearance of estradiol (△).

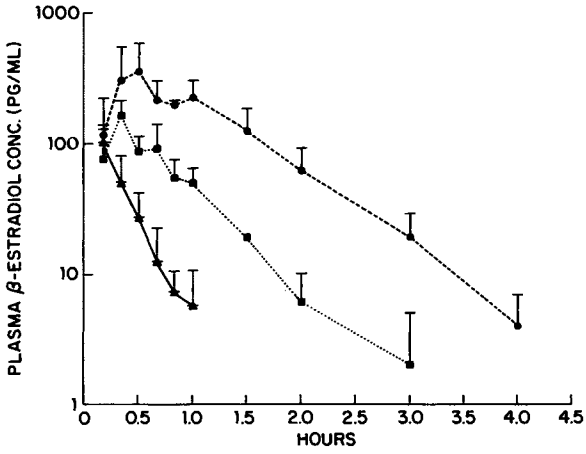

Fig. 3. Plasma estradiol concentrations in dogs administered equimolar doses of estradiol (▲), estradiol-3-anthranilate (■), or estradiol-3-acetylsalicylate (●).

Table II. Estradiol Bioavailability After Oral Estradiol-3-acetylsalicylate (2) and Estradiol-3-anthranilate (4), Relative to Oral Estradiol Doses

	Relative bioavailability (estradiol = 1)	
	Estradiol-3-acetylsalicylate	Estradiol-3-anthranilate
Dog 1	7.0	1.4
Dog 2	10.8	4.0
Dog 3	33.1	8.3
Mean (SD)	17.0 (11.5)	4.6 (2.8)

cylate prodrug had a much shorter hydrolysis half-life for conversion to estradiol than the anthranilate ester, but *in vivo* they were similar. A further possibility for estradiol-3-acetylsalicylate is that it is deacetylated rapidly after oral dosing, and then estradiol-3-salicylate is subject to conjugation on the phenol. Hydrolysis of conjugated estradiol-3-salicylate could be rate-limiting, and this conjugate could also undergo enterohepatic recycling, prolonging the plasma estradiol half-life. A similar scheme of sequential first-pass hydrolysis and conjugation was proposed for cascade esters of terbutaline, which also prolonged its half-life (9).

Estradiol bioavailability after estradiol-3-salicylate dosing was not determined but would be expected to be similar to estradiol-3-acetylsalicylate because of its rapid deacetylation. This similarity was demonstrated previously for the acetylsalicylate and salicylate esters of naltrexone (unpublished). An easy method for the synthesis of estradiol-3-salicylate from estradiol-3-acetylsalicylate was described. An additional potential advantage of a salicylate prodrug is that the phenolic hydroxy group of the salicylate moiety provides a chemical handle for further derivatization to improve water solubility, e.g., with an amine ester.

Table III. Terminal Half-Life of Plasma Estradiol Concentrations After Oral Estradiol or Oral Prodrug

Dose	Estradiol half-life (min, mean ± SD)
Estradiol	10.0 ± 2.8
Estradiol-3-acetylsalicylate	36.2 ± 8.2*
Estradiol-3-anthranilate	27.2 ± 13.8

* Significantly ($P < 0.05$) greater than estradiol-dosed group by paired t test.

In conclusion, a prodrug approach, previously shown to increase successfully the oral bioavailability of opioid antagonists and analgesics, was useful for improving oral estradiol bioavailability.

ACKNOWLEDGMENT

The technical assistance of Christopher A. Koval is greatly appreciated.

REFERENCES

1. C. Longcope, S. Gorbach, B. Goldin, M. Woods, J. Dwyer, and J. Warram. *J. Steroid Biochem.* 23:1065–1070 (1985).
2. M. S. Powers, L. Schenkel, P. E. Darley, W. R. Good, J. C. Balestra, and V. A. Place. *Am. J. Obstet. Gynecol.* 152:1099–1106 (1985).
3. B. DeLignieres, A. Basdevant, G. Thomas, J. -C. Thalabard, C. Mercier-Bodard, J. Conrad, T. -T. Guyene, N. Mairon, P. Corvol, B. Guy-Grand, P. Mauvis-Jarvis, and R. Sitruk-Ware. *J. Clin. Endocrinol. Metab.* 62:536–541 (1986).
4. C. A. Mashchak, R. A. Lobo, R. Dozono-Takano, P. Eggena, R. A. Nakamura, P. F. Brenner, and D. R. Mishell, Jr. *Am. J. Obstet. Gynecol.* 144:511–518 (1982).
5. G. Falconi, F. Galletti, G. Celasco, and R. Gardi. *Steroids* 20:627–631 (1972).
6. B. J. Aungst, M. J. Myers, E. G. Shami, and E. Shefter. *Int. J. Pharm.* 38:199–209 (1987).
7. M. A. Hussain, C. A. Koval, M. J. Myers, E. G. Shami, and E. Shefter. *J. Pharm. Sci.* 76:356–358 (1987).
8. C. Longcope, D. W. Yesair, K. I. H. Williams, M. M. Callahan, C. Bourget, S. K. Brown, M. S. Carraher, C. Flood, and P. C. Rachwell. *J. Steroid Biochem.* 13:1047–1055 (1980).
9. O. A. T. Olsson and L. -Å. Svensson. *Pharm. Res.* 1:19–23 (1984).