PHARMACOKINETICS OF FREE CATECHOLESTROGENS AND CATECHOLESTROGEN BENZOATES

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ABSTRACT

To provide a definite basis for studies on the biological effects of exogenously administered catecholestrogens, the time courses of the concentrations of these estrogens in serum, pituitary and CNS-tissues were studied in male rats after s.c. injection of either 150 µg of 4-hydroxyestradiol or 2-hydroxyestradiol (dissolved in 200 µl sesame oil/ethanol/ascorbic acid; 97.5/2.5/0.1; vol/vol/wt) or equimolar amounts of 4-hydroxyestradiol 3,4-dibenzoate or 2-hydroxyestradiol 2,3-dibenzoate (dissolved in 200 µl sesame oil). The injection of free catecholestrogens resulted in bolus-like elevations of the serum and tissue concentrations of the respective compound (max. values up to 9 ng/ml, half-life below 1 h) whereas the injection of catecholestrogen benzoates gave lower (max. values about 1 ng/ml) but prolonged elevations (half-life approx. 24 h and 32 h for 4-OHE_2 and 2-OHE_2) of the respective free catecholestrogen.

INTRODUCTION

The exogenous application of catecholestrogens (2- and 4-hydroxyestrogens) has often been used to test the biological effects of this class of steroids [for review cf. 1-4]. The results of these studies, however, have been quite contradictory, a fact which may be partly explained by either inadequate handling of the extremely labile catecholestrogens (CE) or the use of impure CE preparations (for review cf. 1). A major reason for the observed discrepancies may also be found in

the various modes of application used by the different authors, *e.g.* i.v., i.m., s.c. injection, i.v. infusion, s.c. implants of silastic capsules or paraffin pellets (for review cf. 1) or osmotic minipumps [4-6].

To provide a definite basis for further investigations using the exogenous application of CE, we first studied the time course of serum and tissue (pituitary, hypothalamus, parietal cortex) concentrations of 4-hydroxyestradiol (4-OHE_2) and 4-hydroxyestrone (4-OHE_1) or 2-hydroxyestradiol (2-OHE_2) and 2-hydroxyestrone (2-OHE_1) after the s.c. injection of 4-OHE_2 or 2-OHE_2 dissolved in sesame oil/ethanol/ascorbic acid (97.5/2.5/0.1; vol/vol/wt). In a second step we tried to retard the resorption of 4-OHE_2 and 2-OHE_2 from the s.c. depot, and at the same time stabilize these compounds by esterifying both of the catecholic hydroxy groups with benzoic acid, a technique well known in steroid pharmacology [for review cf. 7]. In order to check whether these new 4- and 2-hydroxyestradiol benzoates performed as expected, they were also dissolved in sesame oil and injected s.c. to monitor the time course of the resulting serum and tissue concentrations of the respective free CE.

MATERIALS AND METHODS

Steroids. 2- and 4-hydroxyestrogens were prepared according to Stubenrauch and Knuppen [8]. Their purity, checked by gas chromatography-mass spectrometry [9] was >99% (contamination with primary estrogens <0.02%).

Chemicals. All chemicals were purchased from E.Merck, Darmstadt, Germany, and were of analytical grade.

Preparation of CE benzoates.

1. 2-Hydroxyestrone 2,3-dibenzoate (2-OHE$_1$B$_2$) and 4-hydroxyestrone 3,4-dibenzoate (4-OHE$_1$B$_2$). 175 mg of 2-OHE$_1$ or 4-OHE$_1$ in pyridine (20 ml) was reacted with an excess of benzoyl chloride (room temp.,18 h). After the usual work-up procedure the material was chromatographed on silica gel columns (int. diameter 1.5 cm, fill height 50 cm) with ethyl acetate/n-hexane (1:3, vol:vol). Crystallization from ethyl acetate/n-hexane yielded 222 mg of 2-OHE$_1$B$_2$ (74%) or 237 mg of 4-OHE$_1$B$_2$ (79%).

2-OHE$_1$B$_2$: m.p. 171-172°C; IR:1745cm^{-1}(C=O),1250(C-O); UV(ethanol):λ(max)268(ε 6500),229(34900),λ(min)212(20200); ^{1}H-NMR:δ8.06m(4), 7.53m(2), 7.29m(4) benzoate protons; 7.27s(1), 7.10s(1) 1-CH, 4-CH; 2.97m(2) 6-CH$_2$; 0.92s(3) 18-CH$_3$;mass spectrum:M$^+$494, m/e 105(C$_6$H$_5$-C=O$^+$); anal.:calcd. C$_{32}$H$_{30}$O$_5$(494.59) C 77.71, H 6.11; found C 77.43, H 6.08.

4-OHE$_1$B$_2$: m.p. 96-97.5°C; IR:1750cm^{-1}(C=O),1255(C-O); UV(ethanol):λ(max)269(ε 3500),229(23400),λ(min)212(14800); ^{1}H-NMR:δ8.05m(3), 7.72-7.17m(9) 1-CH, 2-CH,benzoate protons; 0.92s(3) 18-CH$_3$; mass spectrum:m/e 389 (M$^+$-105), m/e 105 (C$_6$H$_5$-C=O$^+$); anal.:calcd. C$_{32}$H$_{30}$O$_5$(494.59) C 77.71, H 6.11; found C 77.51, H 6.05.

2. 2-Hydroxyestradiol 2,3-dibenzoate (2-OHE$_2$B$_2$) and 4-hydroxyestradiol 3,4-dibenzoate (4-OHE$_2$B$_2$). 80 mg of 2-OHE$_1$B$_2$ or 4-OHE$_1$B$_2$ in tetrahydrofuran (10 ml) was stirred with an excess of NaBH$_4$ (room temp., 3 days). The solvent was evaporated *in vacuo*, the residue suspended in water and extracted with methylene chloride. After washing with water and evaporation *in vacuo* the residue was chromatographed with ethyl acetate/n-hexane (1:1, vol:vol) on silica gel columns (intern. diameter 1.5 cm, fill height 14 cm), yielding 53 mg of 2-OHE$_2$B$_2$ (66%) or 55 mg of 4-OHE$_2$B$_2$ (69%).

2-OHE$_2$B$_2$: m.p. 151-154°C; IR:1750(C=O),1250(C-O); UV(ethanol):λ(max)269(ε 6500),230(35200),λ(min)214(22100); ^{1}H-NMR: δ8.05m(4), 7.53m(2), 7.36m(4) benzoate protons;7.26s(1), 7.07s(1) 1-CH, 4-CH; 3.74dd(1) 17-CH; 2.92m(2) 6-CH$_2$; 0.77s(1) 18-CH$_3$; mass spectrum (trimethylsilylether):M$^+$568, m/e 553 (M$^+$-15), m/e 105 (CH$_5$-C=O$^+$); anal.: calcd. C$_{32}$H$_{32}$O$_5$ (496.61) C 77.39, H 6.50; found C 76.85, H 6.51.

4-OHE$_2$B$_2$: m.p. 59-60.5°C; IR:1750cm^{-1}(C=O),1250(C-O); UV(ethanol):λ(max)274(ε 2800),228(22100),λ(min)212(15600); ^{1}H-NMR:δ8.03m(4), 7.56-7.14m(9) 1-CH, 2-CH, 17-C-OH, benzoate protons; 3.73dd(1) 17-CH; 0.76s(3) 18-CH$_3$; mass spectrum (trimethylsilylether): m/e 465 (M$^+$-105), m/e 105(C$_6$H$_5$C=O$^+$); anal.: calcd. C$_{32}$H$_{32}$O$_5$ (496.6) C 77.39, H 6.50; found C 76.89, H 6.66.

Assessment of the purity of the CE benzoates. All CE benzoates were treated with NaBH$_4$ in methanol to liberate the steroid moieties and to reduce the 17-oxo groups. The concentration of E$_2$ in the steroid moieties as determined by gas chromatography-mass spectrometry [9] was found to be <0.02%.

Miscellaneous: Melting points were determined with a microscope hot-stage and are uncorrected. Ultraviolet spectra were recorded with a Beckman Acta III spectrophotometer. Infrared spectra were taken with a Beckman Acculab 4 spectrophotometer using 0.5-1% carbon tetrachloride solutions in 0.5 mm KBr-cells. Mass spectra were recorded on a LKB 2091 gas chromatograph-mass spectrometer (electron potential 70 eV). Nuclear magnetic resonance spectra were run with a Bruker WH 270 spectrometer in deuterochloroform solutions with tetramethylsilane as internal standard. Acidic protons were detected by exchange with tetradeuteromethanol. Elemental and molecular weight analyses were carried out by Dr. F. Pascher and E. Pascher, Mikroanalytisches Laboratorium, Bonn, Germany.

Animals and experimental protocol. Male Wistar rats (240 ±15 g) were obtained from Zentralinstitut für Versuchstierzucht, Hannover, Germany, kept in a constant temperature room, lights on from 0530-1930 h, Altromin chow and water ad libitum. At time zero these rats were injected s.c. with either 150 µg of 4-OHE_2 or 2-OHE_2, dissolved in 200 µl sesame oil/ethanol/ascorbic acid (97.5/2.5/0.1; vol/vol/wt), or with equimolar amounts (259 µg) of 4-OHE_2B_2 or 2-OHE_2B_2 dissolved in 200 µl of sesame oil. At defined times (0.25-64 h after injection, cf. Figures 1 and 2) 5 animals each from the different treatment groups were decapitated, the trunk blood collected and the pituitaries, whole hypothalami (including the preoptic area) and slices of parietal cortex removed and frozen (-70°C) immediately.

Determination of the CE concentrations in serum and tissue samples. Sera and tissue homogenates [10] were extracted with ether. The extracts were subjected to column chromatography on Sephadex LH-20 to remove major impurities and to separate 4-OHE_1 and 4-OHE_2 or 2-OHE_1 and 2-OHE_2 [for details cf. 10]. These estrogens were then measured radioimmunologically as described previously [10,11,12].

RESULTS

The data compiled in Fig.1 and 2 (lower sections) indicate that the injection of free CE dissolved in sesame oil/ethanol/ascorbic acid (97.5/2.5/0.1; vol/vol/wt) leads to an extremely sharp increase of the serum levels of the respective compound, with an apparent half-life of less than 1 h. The injection of an equimolar amount of 4-OHE_2B_2 or 2-OHE_2B_2 results in a smooth and long-lasting elevation of the serum levels of free 4-OHE_2 or 2-OHE_2 (cf. Fig.1 and 2, lower sections). The

upper sections of the Figures show that the concentrations of free catecholestrogens found in the pituitary run in parallel Similar concentration/time patterns, though on a slightly lower level, were found in the hypothalamus and the parietal

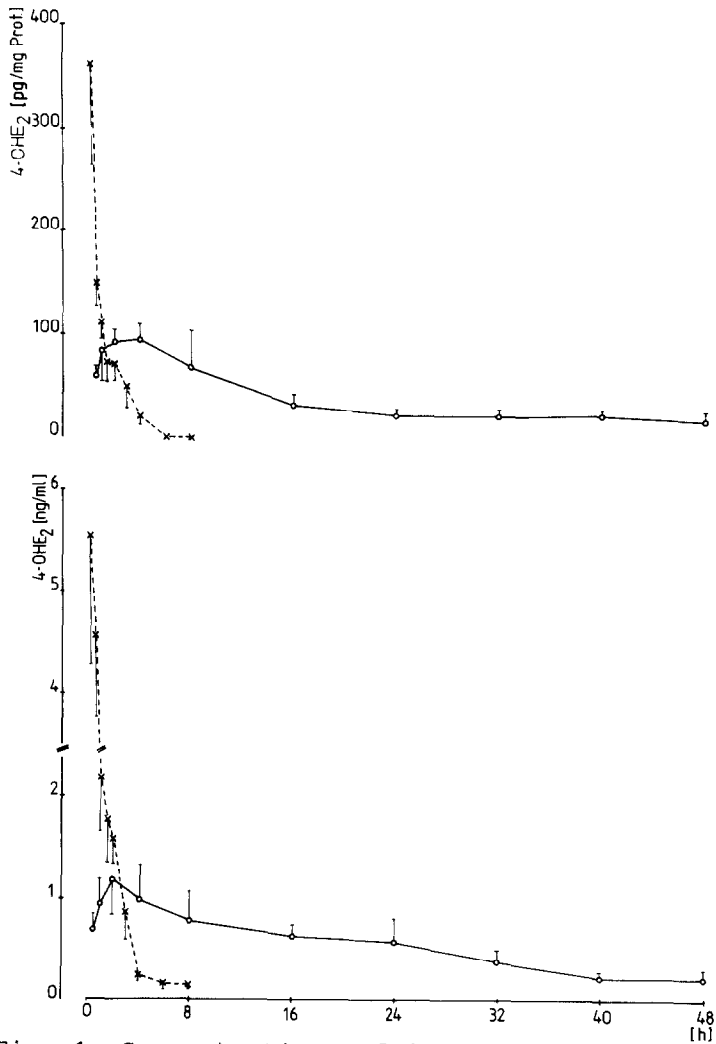

Fig. 1: Concentrations of 4-OHE_2 (mean ± SD) found in the serum (lower section) or the pituitaries (upper section) of male rats at different times after the injection of 150 µg of 4-OHE_2 (x--x--x) or 259 µg of 4-OHE_2B_2 (o—o—o).

cortex, so that for these tissues only the ratios between tissue- and serum concentrations are given here (cf.Table 1).

The ratios found between 4-OHE_1 and 4-OHE_2 varied from 0.11 to 0.48, those between 2-OHE_1 and 2-OHE_2 from 0.095 to

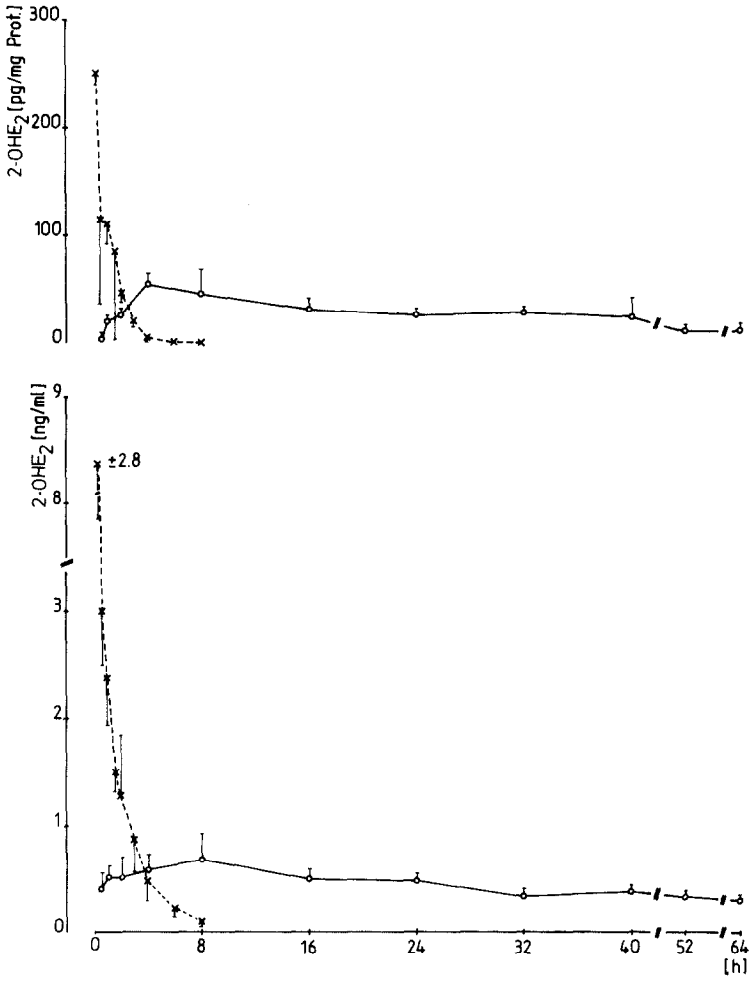

Fig. 2: Concentrations of 2-OHE_2 (mean ± SD) found in the serum (lower section) or the pituitaries (upper section) of male rats at different times after the injection of 150 µg of 2-OHE_2 (x--x--x) or 259 µg of 2-OHE_2B_2 (o—o—o).

Table 1: Ratios (mean ± SD) between tissue concentrations (ng/g of tissue) and serum concentrations (ng/ml serum found after the injection of CE or CE benzoates.

Tissue	Steroid injected			
	4-OHE_2	$4\text{-OHE}_2\text{B}_2$	2-OHE_2	$2\text{-OHE}_2\text{B}_2$
Pituit.	5.4±1.7	7.3±2.3	3.8±1	5.3±2.7
Hypoth.	3.5±1.6	2.7±2.7	2.5±0.9	2.5±0.1
Cortex	3.3±0.9	2.5±0.8	2.5±0.7	2.5±1.2

0.41. Neither significant time patterns nor differences between the various tissues and serum were found for these ratios.

DISCUSSION

The results of this study clearly demonstrate that the s.c. injection of free CE dissolved in oil/ethanol (97.5/2.5, vol/vol) cannot be looked upon as a depot application form but leads to a bolus-like increase of the 4-OHE_2 or 2-OHE_2 serum levels. The short half-life of these elevations is probably due to the high metabolic clearance rate of CE's which has been recently demonstrated for 2-OHE_1 [13,14] and 2-OHE_2 [15].

The application of the new CE benzoates, however, results in a smooth and long-lasting elevation of the serum levels of the respective free CE, indicating that $4\text{-OHE}_2\text{B}_2$ and $2\text{-OHE}_2\text{B}_2$ are useful depot forms for 4-OHE_2 or 2-OHE_2. An interesting observation is that the serum levels of 4-OHE_2 seem to show a faster and higher increase after the injection of $4\text{-OHE}_2\text{B}_2$ than those of 2-OHE_2 after the injection of $2\text{-OHE}_2\text{B}_2$ [cf. Fig. 1 and 2]. This phenomenon might be explained by the fact that the benzoyl group in position 4 of $4\text{-OHE}_2\text{B}_2$ seems to be chem-

ically much more readily cleaved than the 3-benzoyl group of this compound or both the 2- and 3-benzoyl groups of the $2\text{-OHE}_2\text{B}_2$-molecule [Grühn-Schultek, unpublished results].

Comparing the serum levels of 4-OHE_2 or 2-OHE_2 obtained by the injection of the respective CE benzoates with those obtained for E_2 by the injection of estradiol 3-benzoate (E_2B) [16] it is obvious that the increase of E_2 in serum is much higher than that of 4-OHE_2 or 2-OHE_2 even if one corrects for the body weights of the animals used. One explanation for this observation might be that the steroid moiety is liberated to a lesser extent from catecholestrogen benzoates than from E_2B. Another explanation which is favoured by us is that the liberated 4-OHE_2 or 2-OHE_2 is more rapidly cleared from serum than estradiol [cf. 13-15] thus keeping the CE serum levels relatively low. As to the time pattern, the curves obtained for the serum concentrations of 4-OHE_2 or 2-OHE_2 after the injection of the respective benzoate are comparable with those of E_2 after the injection of E_2B. Catecholestrogen benzoates, therefore, seem to be a useful tool to compare the effects of CE with those of E_2B, if one keeps in mind that higher molar amounts of CE-benzoates have to be injected to obtain free CE serum concentrations comparable with the E_2 serum concentrations after the injection of E_2B.

The application of free CE or CE benzoates results in significant elevations of CE concentrations in CNS and pituitary tissues. As the ratios of the CE tissue concentrations versus CE serum concentrations for the hypothalamus and the cortex

are greater than 1 (cf. Table 1), we conclude that CE do not only cross the blood brain barrier, but are also enriched in these tissues. The highest enrichment, however, was found in the pituitary which may be due to the higher concentration of estrogen-specific cells or the absence of a special barrier between the blood and this organ.

The rather low amounts of 4-OHE_1 or 2-OHE_1 found after the injection of the respective 17-hydroxy compound might indicate that either the oxidation at C-17 is not of major quantitative importance and/or that the 4-OHE_1 or 2-OHE_1 molecules formed from 4-OHE_2 or 2-OHE_2 are rapidly converted into other metabolites, *e.g.* methyl ethers, glucuronides or sulfates.

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ABBREVIATIONS AND TRIVIAL NAMES

E_2 = estradiol = 1,3,5(10)-estratriene-3,17β-diol

E_2B = estradiol 3-benzoate

$4\text{-}OHE_1$ = 4-hydroxyestrone = 3,4-dihydroxy-1,3,5(10)-estratrien-17-one

$4\text{-}OHE_2$ = 4-hydroxyestradiol = 1,3,5(10)-estratriene-3,4,17β-triol

$4\text{-}OHE_1B_2$ = 4-hydroxyestrone 3,4-dibenzoate

$4\text{-}OHE_2B_2$ = 4-hydroxyestradiol 3,4-dibenzoate

$2\text{-}OHE_1$ = 2-hydroxyestrone = 2,3-dihydroxy-1,3,5(10)-estratrien-17-one

$2\text{-}OHE_2$ = 2-hydroxyestradiol = 1,3,5(10)-estratriene-2,3,17β-triol

$2\text{-}OHE_1B_2$ = 2-hydroxyestrone 2,3-dibenzoate

$2\text{-}OHE_2B_2$ = 2-hydroxyestradiol 2,3-dibenzoate