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Estradiol Fatty Acid Esters Occur Naturally in Human Blood

Abstract. *Treatment of nonpolar ether extracts of human female blood with mild alkali produced more immunoassayable estradiol than the unhydrolyzed extract. Analysis of the serum extracts showed that the substance which released immunoreactive estradiol after hydrolysis has chromatographic properties identical to those of fatty acid esters of estradiol esterified at carbon 17. The physiological role of these previously unknown endogenous esters might be inferred from their structural similarity to synthetic drugs used therapeutically for their prolonged estrogenic action.*

For over a half-century, alkyl and aryl esters of estradiol have been known as biologically potent steroids, generally considered to be "long-acting" estrogens (*1–3*). These esters, such as estradiol benzoate, propionate, cyclopentylpropionate, and valerate are synthetic drugs still used for prolonged estrogen stimulation (*4–6*). The most potent natural steroidal estrogen, 17β-estradiol (E_2), exists not only as the "free" steroid, but in conjugated form. However, the conjugates are exclusively ionic, extremely water-soluble polar steroids linked to glucuronic acid or sulfate moieties (*7, 8*). To our knowledge, the natural occurrence of estrogenic steroids similar to the pharmacologically active, nonpolar alkyl esters of estradiol has not previously been shown. We now present evidence that alkyl esters of estradiol normally circulate in human blood and thus exist endogenously in nature.

An unusual nonpolar metabolite of estradiol was produced in our laboratory by in vitro incubations of several tissues (*9*). This compound, which could be converted back into estradiol by mild hydrolytic treatment, was named the lipoidal derivative of estradiol (LE_2) to underscore its unique hydrophobic nature and uncertain structure. Subsequently, we isolated and identified LE_2 as a family of fatty acid esters of estradiol esterified exclusively at carbon 17 (*10*). The experiments described here were designed to determine whether LE_2 is present in human blood. Using immunochemical and chromatographic techniques, we found that a nonpolar compound with properties identical to those of LE_2, the carbon 17 esters of estradiol, exists in serum.

We reasoned that if LE_2 is present in human blood, mild alkaline hydrolysis of an ether extract of serum should produce more estradiol than the untreated extract. Unlike LE_2, other known conjugates of estradiol, such as sulfates and glucuronides, are neither extractable with ether (*11*) nor hydrolyzable with a weak base (*12*). Estradiol was measured by radioimmunoassay with an antiserum highly specific for estradiol (*13*). Esters representative of LE_2, such as estradiol-17-stearate and estradiol-17-arachidonate, did not cross-react in this assay. Serum samples (*14*) from males, cycling

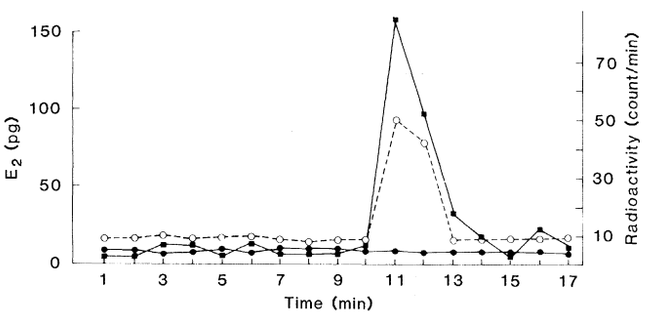

Fig. 1 (left). High-pressure liquid chromatography of the LE_2 fraction from human serum. A serum sample (2.0 ml) obtained from an HMG-treated subject was extracted with ether after [^{3}H]estradiol-17-stearate (1000 count/min) had been added. The ether was evaporated and the residue was purified on a silica gel column and by HPLC (Table 2). Fractions of 1 ml were collected and 200-μl portions were counted for tritium. One half of the remainder of each fraction was analyzed for estradiol by radioimmunoassay in duplicate. The remaining half of each fraction was hydrolyzed overnight in alkali. Estradiol was radioimmunoassayed in duplicate in these hydrolyzed fractions. The control values were obtained from the first four fractions, which elute from the HPLC column before the solvent front. The E_2 values shown are those obtained after subtraction of the controls (< 10 pg before and 79 pg after hydrolysis). Symbols: (○) tritium, (●) E_2, and (■) E_2 after alkaline hydrolysis.

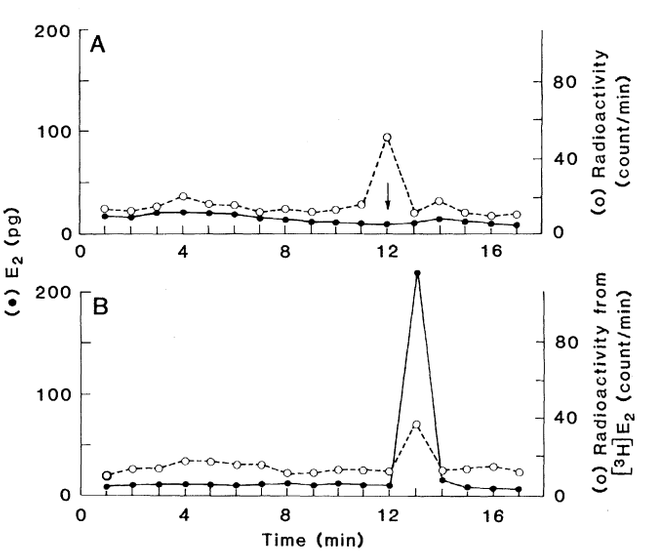

Fig. 2 (right). (A) High-pressure liquid chromatography of LE_2 fraction isolated from human serum. Tritiated estradiol-17-stearate was added to serum obtained from a patient receiving HMG. LE_2 was extracted and purified by silica gel chromatography and HPLC (Fig. 1). Fractions of 1 ml were collected and tritium was counted in 100-μl portions. The remainders of each fraction and one fifth of fraction 12 (arrow) were analyzed for E_2 by radioimmunoassaying duplicate portions. The control value for the radioimmunoassay, 13 pg per fraction, has not been subtracted. The quantity of E_2 in fraction 12 has been normalized to correct for the size of the portion tested. (B) High-pressure liquid chromatography of K_2CO_3-treated fraction 12. Fraction 12 of the HPLC [arrow in (A)] in which [^{3}H]estradiol-17-stearate migrated was evaporated and hydrolyzed overnight with K_2CO_3. The alkali-treated fraction was processed in the usual manner and purified by HPLC on a LiChrosorb Diol column with methylene chloride at 1 ml/min. Fractions of 1 ml were collected. Portions of 100 μl were counted for detection of [^{3}H]estradiol. The remainder of each fraction was analyzed for estradiol by radioimmunoassaying duplicate portions. The control value was < 10 pg.

females, and women treated with human menopausal gonadotropin (HMG) were assayed for estradiol before and after saponification with K_2CO_3 (Table 1). In several assays these manipulations of ether extracts of water, the controls, produced relatively high values (more than 50 pg/ml), and consequently the estradiol levels of some nonstimulated patients could not be determined with accuracy. There was little estradiol in the serum from all seven male subjects and saponification had no effect, confirming that alkaline hydrolysis of the ether extracts of serum does not nonspecifically increase radioimmunoassayable estradiol.

In 16 of 24 HMG-treated women, serum estradiol increased significantly after alkaline hydrolysis, while in eight there was little or no increase. The increase in estradiol after alkaline treatment was highly significant for the 24 subjects [$F(1, 84) = 89.7$, $P < 0.001$; two-way analysis of variance] (*15*). Twelve of 26 females not treated with HMG had low levels of estradiol that could not be reliably assessed with the assay (*14*). None of these patients had detectable levels of saponifiable estradiol, confirming what was found with male serum and suggesting that measurable levels of plasma LE_2 are found only in women who are producing significant quantities of E_2. Of the 14 females not stimulated with HMG in whom estradiol could be detected, ten showed a significant increase in estradiol after alkaline treatment while four had little or no increase. When all 26 subjects were compared the increase in estradiol after alkaline treatment was highly significant [$F(1, 90) = 99.2$, $P < 0.001$] (*15*). Recovery experiments were performed. When estradiol (100 pg to 1 ng) was added to male serum, approximately 100 percent of the free steroid was detected by radioimmunoassay and there was no increase in estradiol after alkaline treatment. When estradiol-17-stearate (200 pg to 2 ng) was added to male serum and the water extract controls assayed at less than 50 pg/ml, no estradiol was measurable before saponification and 109 ± 13 percent (mean ± standard error, $N = 7$) was detected after saponification. In experiments in which the controls were greater than 50 pg/ml, again no estradiol was detected before saponification. But after saponification the assayable estradiol decreased to 73 ± 10 percent ($N = 6$) of the amount expected. The high control values tended to decrease the estimate of the amount of estradiol released by hydrolysis. Thus there is a sizable increase in the amount of estradiol in ether extracts of female serum after hydrolysis with K_2CO_3. If it is assumed that this increase is due to the presence of LE_2 in serum, then in most cases there is almost as much LE_2 as E_2. In fact, in some cases there was significantly more LE_2 than E_2. However, the uncertainties of this determination by difference precludes accurate quantification. Nevertheless, it is clear that a nonpolar, ether-extractable component that is convertible to estradiol with weak alkaline treatment exists in human blood.

We next showed that this compound has other properties that are identical to those of LE_2. Blood was freshly drawn from cycling women who were not receiving estrogen. Internal standards of both [^{3}H]estradiol and [^{3}H]estradiol-17-stearate were added to serum that was then extracted with ether. The radioactive internal standard, estradiol-17-stearate, which was added to correct for recovery, was chosen as a representative ester of LE_2 because it is stable and easily characterized. In the chromatographic systems used in this experiment the mobility of all the esters comprising LE_2 are identical (*10*). The residues of the ether extracts were purified on a small column of silica gel and then by high-pressure liquid chromatography (HPLC) (Table 2). The LE_2 fraction (*16*) was saponified with K_2CO_3 and rechromatographed by HPLC in a system for estradiol (*9*). In some experiments the free estradiol fraction, isolated from the silica gel column, was also purified in this HPLC system. Estradiol was quantified by radioimmunoassay as described above. As shown in Table 2, estradiol derived from the LE_2 fraction was present in statistically significant amounts in each of the ten female serum samples analyzed. In experiment A, when the

Table 1. Immunoassayable estradiol in serum extracts after alkaline treatment. The data resulted from three separate radioimmunoassays representative of a total of seven radioimmunoassays performed on serum from 24 HMG-treated females, 26 females of unknown medical history, and seven males. Tritiated estradiol (1000 count/min; 0.01 pmole) was added to 2 ml of serum and then extracted with 20 ml of diethyl ether. The organic extract was evaporated under a stream of nitrogen and the residue was dissolved in 2.0 ml of methanol. One half (1 ml) was hydrolyzed by adding 100 μl of 6 percent aqueous K_2CO_3 to the methanolic solution. The mixture was incubated overnight at 50°C, neutralized with 1 ml of 0.44 percent aqueous acetic acid, and then extracted with 20 ml of ether. The ether was evaporated and the residue was dissolved in 0.5 ml of ethanol. The nonhydrolyzed control, consisting of the remaining half of the serum extracts, was left to stand overnight in methanol, evaporated, and reconstituted in 0.5 ml of ethanol. The recovery of estradiol was corrected by measuring the amount of tritium present in portions representing 10 percent of each sample. Estradiol was measured in the remainder by radioimmunoassaying three replicates equivalent to 100, 200, and 400 μl of serum. Control values were obtained for each assay by extracting and hydrolyzing 2 ml of water, exactly as described for serum. The standard curve of the radioimmunoassay had median effective dose of 40 to 50 pg, and estradiol could be accurately measured between 10 and 400 pg. The water control values in hydrolyzed and nonhydrolyzed samples were 38 and 19 pg/ml, 4 and 0 pg/ml, and 39 and 20 pg/ml in experiments A, B, and C, respectively. The amount of estradiol was calculated by subtracting the appropriate controls and correcting for recovery of the internal standards. Samples in which more than one replicate assayed within the 95 percent confidence limits for the estimate of nonspecific binding in the radioimmunoassay were not included (only two samples from HMG-treated women were in this category). Similarly, samples that were within the 95 percent confidence limits of the water controls were considered to contain undetectable estradiol. The amount of LE_2 was assumed to be the difference calculated by subtracting the nonhydrolyzed from the hydrolyzed sample. Replicate values for each subject obtained after hydrolysis were compared to those assayed before hydrolysis by *t*-test. Subjects treated with HMG (Pergonal) were injected intramuscularly each day from day 3 to day 10 of the menstrual cycle with 150 to 225 IU of follicle-stimulating hormone and luteinizing hormone. Blood was obtained on day 10.

Subject	Assay	Estradiol (pg/ml)			Percent increase after hydrolysis
		Before hydrolysis	After hydrolysis	Difference	
Female (HMG)	A	622	583	−39	−7
Female (HMG)	A	913	1545	631	69*
Female (HMG)	A	935	1149	214	23*
Female (HMG)	A	2048	3197	1148	56*
Female (HMG)	C	1115	1760	645	58*
Female (HMG)	C	1362	1771	409	30
Female (HMG)	C	2238	2014	−224	−10
Female	B	89	140	51	57*
Female	B	130	530	400	307*
Male	B	< 10	< 10	< 10	0

*Significant at $P < 0.05$.

control value was low the presence of LE_2 was readily apparent. Again, there was little if any LE_2 in the male serum. In experiments B and C, in which free estradiol was also measured, the combination of relatively high control values and low LE_2 values hindered the accurate measurement of LE_2 levels, and thus comparison of LE_2 and E_2 is not possible in these subjects. Nevertheless, Table 2 shows that in both experiments B and C the female serum contained more LE_2 than male serum or water extracts ($P < 0.01$).

Further studies were performed to confirm that the substance in blood which was converted to estradiol by alkali had properties identical to those of LE_2. Serum from an HMG-treated patient was extracted with ether and the nonpolar LE_2 fraction was obtained by chromatography on a silica gel column. After evaporation to dryness the resulting residue was chromatographed by HPLC. Portions of every fraction were assayed for estradiol by radioimmunoassay. As expected, no estradiol could be detected. Every fraction was further subjected to hydrolysis with K_2CO_3 and then assayed for estradiol. Estradiol was detectable after saponification in those fractions in which the tritiated internal standard migrated (Fig. 1). This experiment was repeated two more times, with identical results. Thus in this HPLC system the nonpolar component of blood that is immunoreactive in the estradiol assay after hydrolysis migrates exactly as LE_2.

Finally, we showed that the immunoreactive substance released by hydrolysis had properties identical to those of estradiol. An ether extract of serum from an HMG-treated subject was chromatographed first on a silica gel column and then by HPLC. Portions of every fraction of the latter chromatogram were assayed for estradiol by radioimmunoassay, and no estradiol was detected (Fig. 2A). The fraction in which LE_2 migrates was then saponified and rechromatographed by HPLC in a more polar system. Every fraction from this chromatogram was assayed for estradiol. As shown in Fig. 2B, the only fraction that contained immunoassayable estradiol was the one in which estradiol migrated. Therefore the nonpolar immunoassayable substance had chromatographic properties identical to those of LE_2 and, as would be expected, after saponification its chromatographic mobility was the same as that of estradiol.

The immunoassay we used is specific for estradiol (*13*), and the HPLC system has been extensively tested in this laboratory and is capable of separating estrogens with great resolution (*17*). Nevertheless, there is some uncertainty about the concentration of LE_2 in blood. This is caused both by the inherent inaccuracy of measuring LE_2 by the difference between saponified and nonhydrolyzed serum extracts and by the elevated control values in the estradiol radioimmunoassay that are not found when ether extracts of water are analyzed directly. While LE_2 could be readily detected, these elevated control values precluded the accurate quantification of LE_2 in serum. Thus many questions relating to the physiological and pathological control of LE_2 secretion must await the development of a sensitive and accurate assay of LE_2 in blood.

Our finding that alkyl esters of estrogens circulate in human blood raises numerous questions about the physiological role of these unusual steroids. The pharmacologic use of similar synthetic esters for their prolonged estrogenic properties indicates that LE_2 may be a family of naturally occurring, "long-acting" estrogens.

Table 2. Immunoassay of LE_2 in serum after chromatographic purification. Tritiated estradiol (1000 count/min) and [^{3}H]estradiol-17-stearate were added to 2 ml of serum and then extracted with 20 ml of ether. The [^{3}H]estradiol-17-stearate was synthesized from [2,4,6,7-^{3}H]estradiol (110 Ci/mmole) (*10*). The ether extracts were evaporated and chromatographed on silica gel 60 columns (70 to 230 mesh, 0.6 by 7.5 cm; Merck) equilibrated with benzene. LE_2 was eluted with 20 ml of 1 percent ethyl acetate in benzene, after which estradiol was eluted with 15 ml of benzene and ethyl acetate (70:30). The LE_2 fraction was further purified by HPLC in a LiChrosorb Diol (Merck) column (250 by 4.6 mm) with methylene chloride and isooctane (60:40) at a flow rate of 1 ml/min. The fractions containing LE_2, identified by detection of the tritiated internal standard, were combined, evaporated, and hydrolyzed with K_2CO_3 as described in the legend to Table 1. Both the estradiol fraction from the silica gel column and the alkali-treated LE_2 fractions of the HPLC were chromatographed by HPLC on the LiChrosorb Diol column with methylene chloride at a flow rate of 1 ml/min. The estradiol fractions were combined and evaporated and portions were counted for tritium to correct for recovery. The average recovery of LE_2 as hydrolyzed estradiol was approximately 50 percent. Estradiol was determined by radioimmunoassay in duplicate portions as described in the legend to Table 1. The control values were obtained from extracts of 2 ml of water that were processed exactly like the serum. Every assay had two water controls, each performed with two replicates. The control values for the estradiol assay were 28 pg/ml (experiment B) and 24 pg/ml (experiment C); estradiol was not assayed in experiment A. The control values for LE_2 were 17, 44, and 63 pg/ml in experiments A, B, and C, respectively.

Subject	Experiment	E_2 (pg/ml)	LE_2 (pg/ml)*
Cycling females	A		586
	A		500
	A		224
	A		133
	A		195
	B	109	41
	B	351	33
	B	170	53
	C	84	73
	C	63	44
Males	A		< 10
	B	< 10	< 10
	B	< 10	< 10
	C	21	< 10

*Shown as molar equivalents of estradiol.

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14. Sera were random samples obtained from the Gynecologic Endocrine Laboratory in this department. This laboratory assays blood from patients with a wide variety of endocrine conditions and these sera are not representative of a normal population of cycling women.
15. Results were analyzed by two-way analysis of variance after natural log transformation to eliminate inhomogeneity of variance present in the raw data. Analyses were performed on an Apple II microcomputer with STATS-PLUS (Human Systems Dynamics).
16. To eliminate the possibility of cross-contamination during HPLC, the injector, tubing, and column were reserved specifically for this purpose and were never used with large amounts of steroid standards, including radioactive compounds.
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18. We thank N. MacLusky for his help with the statistical analyses, B. Shaywitz for providing the statistical programs, and H. Behrman for discussions and suggestions. This work was supported in part by grant CA29591 from the National Institutes of Health.

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