Pharmacokinetics and Pharmacodynamics of Conjugated Equine Estrogens: Chemistry and Metabolism (44199)

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Abstract. **Conjugated equine estrogens (Premarin), are used extensively for estrogen replacement therapy and prevention of osteoporosis and cardiovascular disease in postmenopausal women. Premarin contains at least 10 estrogens that are the sulfate esters of the ring B saturated estrogens: estrone, 17β-estradiol, 17α-estradiol, and the ring B unsaturated estrogens: equilin, 17β-dihydroequilin, 17α-dihydroequilin, equilenin, 17β-dihydroequilenin, 17α-dihydroequilenin, and delta-8-estrone. Bioassays and estrogen receptor binding studies indicate that all 10 estrogens are biologically active. Moreover, individual components, such as equilin sulfate, delta-8-estrone sulfate, 17β-dihydroequilin sulfate and estrone sulfate, have potent estrogenic effects. Estrogen sulfates can be absorbed directly from the gastrointestinal tract; however, hydrolysis of the sulfates also occurs in the gastrointestinal tract, and the unconjugated estrogens formed are readily absorbed. After absorption, these estrogens are sulfated rapidly and circulate in this form. The pharmacokinetics of these estrogens indicate that the unconjugated estrogens are cleared from the circulation at a faster rate than their sulfate ester forms. In postmenopausal women, the 17-keto derivatives of these estrogens are metabolized to the more potent 17β-reduced products. The extent of this activation is nearly 10 times higher with some ring B unsaturated estrogens. The 17β-reduced metabolites are cleared from the blood at a slower rate than their corresponding 17-keto derivatives. In the human endometrium, equilin is metabolized to 2-hydroxy and 4-hydroxy equilin, with 2-hydroxylation being predominant. In contrast, 2-hydroxy and 4-hydroxy estradiol are formed in equal amounts. Similarly, 16α-hydroxylation occurs with both types of estrogens; however, with the ring B saturated estrogens, the 17-keto steroid 16α-hydroxy estrone was the major urinary metabolite, whereas with the ring B unsaturated estrogens, the 17β-reduced steroids, such as 16α-hydroxy-17β-dihydroequilin and 16α-hydroxy-17β-dihydroequilenin, were the major metabolites. This difference in metabolism may be important as it has been suggested that 16α-hydroxy estrone (α-ketol structure) can form covalent adducts with macromolecules and that it may be oncogenic. These types of interactions will not occur with the 16α-hydroxylated-17β-reduced metabolites of ring B unsaturated estrogens.**

Since all of the estrogens present in Premarin have estrogenic activity, the pharmacological effects of Premarin are a result of the sum of these individual activities. Therefore, preparations lacking some of these important components may not offer the same degree of beneficial effects as Premarin. [P.S.E.B.M. 1998, Vol 217]

Introduction

For more than half a century, conjugated equine estrogen preparations, such as Premarin (Wyeth-Ayerst, Philadelphia, PA), have been used extensively for estrogen replacement therapy and prevention of osteoporosis and cardiovascular disease in postmenopausal women. To date, from the pregnant mares' urine, 10 estrogens have been

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identified. These are the sulfate esters of the ring B saturated estrogens (classical estrogens), estrone (E_1), 17β-estradiol (17β-E_2), and 17α-estradiol (17α-E_2) and the ring B unsaturated estrogens, equilin (Eq; 3-hydroxy-1,3,5(10)7-estratetraen-17-one), equilenin (Eqn; 3-hydroxy-1,3,5(10)6-8-estrapentaen-17-one), 17α-dihydroequilin (17α-Eq; 1,3,5(10)7-estratetraen-3,17α-diol), 17β-dihydroequilin (17β-Eq; 1,3,5(10)7-estratetraen-3,17β-diol), 17α-dihydroequilenin (17α-Eqn; 1,3,5(10)6,8-estrapentaen-3,17α-diol), 17β-dihydroequilenin (17β-Eqn; 1,3,5(10)6,8-estrapentaen-3,17β-diol), and delta-8-estrone (delta-8E_1; isoequilin; 3-hydroxy-1,3,5(10)8-estratetraen-17-one). In keeping with the reported metabolism of ring B unsaturated estrogens in the pregnant mare (1), one would anticipate that two additional metabolites of delta-8-estrone namely delta-8-17β-estradiol (delta-8-17βE_2; 1,3,5(10)8-estratetraen-3,17β-diol) and delta-8-17α-estradiol (delta-8-17α-E_2; 1,3,5(10)8-estratetraen-3-17α-diol), would also be present in the pregnant mares' urine and therefore in the drug Premarin. However, their presence remains to be established. The structures of these 12 equine estrogens are depicted in Figure 1. Structurally, the ring B unsaturated estrogens differ from the classical estrogens by the presence of one or two additional double bonds in the ring B of the steroid nucleus.

In the pregnant mare, the ring B saturated estrogens estrone, 17β-estradiol, are formed from cholesterol by the classical pathway of steroidogenesis, while the ring B unsaturated estrogens equilin, equilenin, 17α-dihydroequilin, 17α-dihydroequilenin, 17β-dihydroequilin and 17β-dihydroequilenin, are formed by an alternate pathway not involving cholesterol and squalene, and this has been recently reviewed (2). This review will discuss the pharmacokinetics and pharmacodynamics of the individual equine estrogens with emphasis on the unique ring B unsaturated estrogens.

Biological Activity. Previous bioassay data clearly indicated that all 9 original estrogens present in the pregnant mares' urine and Premarin are biologically active estrogens (2, 3, 4). Moreover, some of the bioassay data (uterotrophic assay) indicated that some of the 17β-reduced ring B unsaturated estrogens, such as 17β-dihydroequilin sulfate (17β-EqS) was nearly 8 times more potent than estrone sulfate (E_1S) when administered orally (4). In contrast, when these estrogens in their unconjugated form are administered subcutaneously or intraperitoneally (2 μg/day for 3 days), to immature rats, all of the estrogens increased in uterine weight (Table I). The data also indicate that the potency of various estrogens depends on the route of administration.

Few clinical studies exist in which the effects of individual ring B unsaturated estrogens have been determined in postmenopausal women. Oral equilin sulfate in women was reported to be 4–8 times as potent as estrone sulfate in suppressing urinary gonadotropins (5); orally administered equilin sulfate (0.25 mg) was as effective as 0.625 mg of Premarin in the alleviation of menopausal symptoms and stimulation of the vaginal epithelium in postmenopausal women (6). Similarly, 0.3 and 0.625 mg of equilin sulfate were 1.5–8 times more potent than comparable doses of estrone sulfate and conjugated equine estrogens in stimulating the synthesis of hepatic proteins such as sex hormone binding globulin (SHBG), corticosteroid binding globulin (CBG) and angiotensinogen (7). Oral administration of 17β-dihydroequilin sulfate (0.3–0.4 mg/day) was as effective as 0.625 mg of conjugated estrogens for the control of vasomotor symptoms (8). At these doses, 17β-dihydroequilin sulfate also resulted in uterine bleeding associated

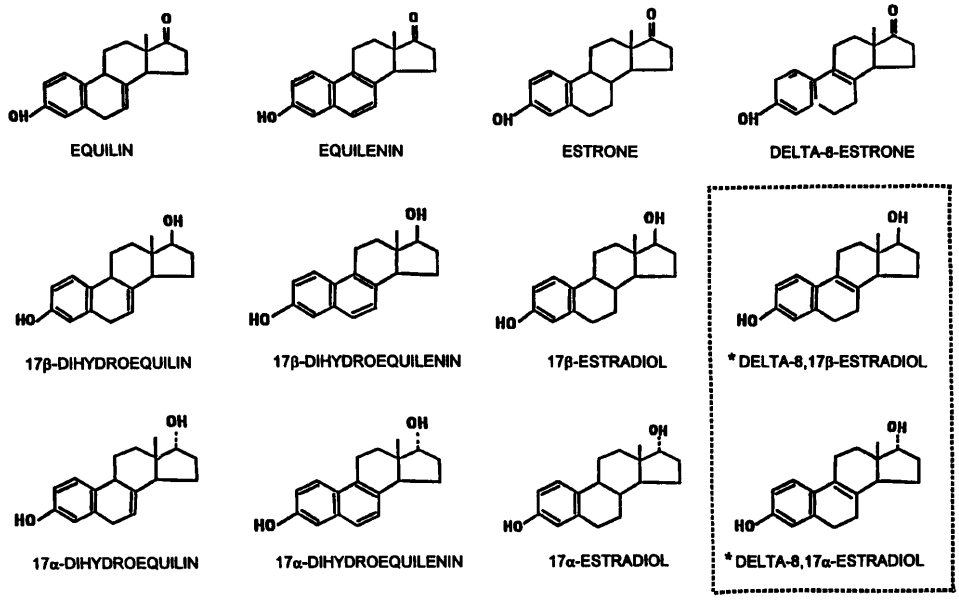


Figure 1. Some of the estrogens isolated from the urine of pregnant mares. These estrogens in their sulfate ester form are present in the drug Premarin.

*Potential Components in Premarin

Table I. Rat Uterine Response to Subcutaneous or Intraperitoneal Administered Estrogens

Estrogen	Uterine weight (mg) mean ± S.E.	
	Subcutaneous	Intraperitoneal
1) Equilin	150.8 ± 10[a]	98.8 ± 5.0[a]
2) Estrone	129.3 ± 12[a]	88.4 ± 8.0[a]
3) 17β-Estradiol	112.9 ± 4[a]	89.4 ± 5.0[a]
4) 17β-Dihydroequilin	102.3 ± 2.8[a]	98.6 ± 6.0[a]
5) Equilenin	79.8 ± 4.5[a]	52.6 ± 1.0[a]
6) 17β-Dihydroequilenin	61.3 ± 2.0[a]	51.9 ± 2.0[a]
7) 17α-Dihydroequilin	59.9 ± 2.5[a]	39.0 ± 2.0[a]
8) 17α-Estradiol	57.4 ± 3.4[a]	37.4 ± 2.0
9) 17α-Dihydroequilenin	44.8 ± 3.8	39.4 ± 2.0[a]
10) Delta-8-Estrone	ND	60.5 ± 2.0[a]
11) Delta-8-17β-Estradiol	ND	75.0 ± 3.0[a]
12) Control	37.0 ± 2	33.8 ± 1.0

ND = Not determined
[a] Different from control.
$P < 0.005$.

with proliferative and hyperplastic changes in the endometrium. More recently, daily oral administration of small doses (0.125 mg) of delta-8-estrone sulfate for 8 weeks to healthy postmenopausal women resulted in suppression of plasma FSH and urinary-n-telopeptide (bone turnover marker), a significant increase in the plasma CBG, and in the lag time for LDL_c oxidation (9). Direct comparison of the biological activity of the conjugated equine estrogen preparation Premarin with piperazine estrone sulfate (Ogen) and micronized estradiol (Estrace) in terms of a number of parameters, indicate that the potency of Premarin estrogens was 2- to 6-fold greater than that of the other two estrogens, even though the plasma levels of estrone and 17β-estradiol were similar (10). These data suggest that the presence of the unique ring B unsaturated estrogen components in Premarin are contributing to the increased activity. These combined data clearly demonstrate that the ring B unsaturated estrogens, including the more recently identified delta-8-estrone sulfate and its major *in vivo* metabolite delta-8-17β-estradiol (9), are biologically active with potency either equal to or greater than that of the classical estrogens.

Mechanism of Action. Estrogens regulate tissue function by modulating gene transcription through their interaction with estrogen receptors, which belong to the steroid-thyroid hormone receptor superfamily (11). In a comparative study, the relative binding affinities of the various estrogen components of Premarin in their unconjugated form for estrogen receptors in human endometrium and rat uterus, were measured. In both species, all of the estrogens bind with high affinity to cytosol and nuclear receptors. The relative binding affinities indicate the following order of activity: 17β-dihydroequilin > 17β-estradiol > 17β-dihydroequilenin > estrone = equilin > 17α-dihydroequilin > 17α-estradiol > delta-8,17β-estradiol > 17α-dihydroequilenin > equilenin > delta-8-estrone (12,13).

Though the bioassay data (Table I) indicate that both equilin and estrone are more or equally as active as their 17β-reduced metabolites, 17β-dihydroequilin and 17β-estradiol, their relative binding affinities for estrogen receptors are much lower. These observations suggest that equilin and estrone in the presence of 17β-hydroxy steroid dehydrogenase are first metabolized to 17β-dihydroequilin and 17β-estradiol, and it is through these 17β-reduced metabolites that the biological effects (uterotropic activity) are exerted. These data also clearly indicate that all ring B unsaturated components present in conjugated equine estrogen preparations can interact with estrogen receptors and are therefore capable of exerting biological effects in estrogen target tissues.

Recent observations regarding the mechanisms of gene activation by estrogens and antiestrogens, particularly in nonreproductive tissues, suggest that the estrogen receptors, in combination with various estrogens, can regulate more than one DNA response element (14). Therefore, it is possible that each of the various estrogen components present in Premarin can regulate different estrogen response element(s), perhaps in a tissue-specific manner. Thus, even though the amount of some of the conjugated equine estrogens present in Premarin is relatively small, these estrogens could still have a significant clinical impact. Further studies are needed to delineate their specific role.

Interaction of Unconjugated and Conjugated Equine Estrogen With SHBG and Serum Albumin. Unconjugated equine estrogens and conjugated equine estrogens bind to SHBG and serum albumin in a manner similar to that described for the classical estrogens, estrone and estradiol, and androgens, such as testosterone and 5α-dihydrotestosterone. Thus, the sulfate esters of equilin, estrone, and estradiol do not bind with SHBG (15, 16); however, equilin sulfate and estrone sulfate do interact with serum albumin with high affinity ($0.9–1.1 \times 10^5\ M^{-1}$) (15). Low affinity binding sites were also present. Up to 74% of the total equilin sulfate and 85%–90% of estrone sulfate were bound to serum albumin (Table II) (15, 16). In contrast, the unconjugated equilin, estrone, 17β-dihydroequilin, and 17β-estradiol are only loosely bound to serum albumin, but they bind with SHBG with high affinity (Table II) (15, 17). Based on competitive Scatchard analysis, the relative affinity constants for estrone, equilin, 17β-dihydroequilin, 17β-estradiol, testosterone, and 5α-dihydro-testosterone were found to be 0.07, 0.15, 0.22, 0.29, 2.70, and 4.53 ($\times 10^9\ M^{-1}$) respectively (15). The binding affinities of both the ring B unsaturated estrogens and ring B saturated estrogens are similar. Though the 17β-reduced metabolites as expected, have a higher affinity for SHBG, these affinities are significantly lower than those observed with androgens (15–18). Thus, like other steroid hormones, conjugated and unconjugated equine estrogens circulate in the blood either bound to serum albumin, or specific serum proteins, and only a small percent of the total is present in the unbound form (free form, physiologically active form). Estrogen therapy is associated with an increase in SHBG levels, a change that would result in a greater percentage of estrogen

Table II. Percent of Unconjugated and Sulfate Conjugated Estrogens and 5α-DHT Bound with Albumin and SHBG

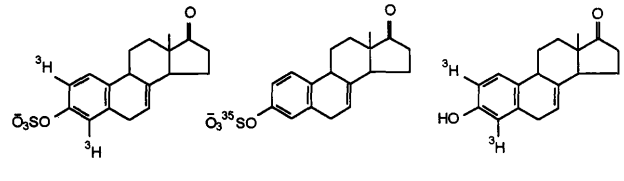
Serum samples	EqS	E_1S	Eq		E_1		E_2		5α-DHT	
	Albumin	Albumin	SHBG	Albumin	SHBG	Albumin	SHBG	Albumin	SHBG	Albumin
PMW on Premarin*	60	83	45	11	23	17	53	12	79	7
Female	63	83	26	13	9	14	26	13	73	4
Male	71	86	0	1	0	4	0	5	25	5
5% HSA	74	85	0	3	0	4	0	4	0	5

* PMW = Postmenopausal Women.
Adapted with permission from Ref. 15.

bound to SHBG (Table II) along with a concomitant decrease in the percentage of unbound estrogen. These changes can play a role in the metabolic clearance rate and the bioavailability of the estrogen. This aspect will be discussed later.

Absorption of Conjugated Equine Estrogens. Ingestion of Premarin (10 mg), containing approximately 4.5 mg of estrone sulfate and 2.5 mg of equilin sulfate, results in the gradual appearance of unconjugated estrone and equilin in the blood. Maximum levels of equilin (560 pg/ml) and estrone (1400 pg/ml) were reached after 3 hr and 5 hr respectively. These levels declined gradually; however, small amounts of both estrogens were still detectable after 24 hr (19). Following intravenous administration of 10 mg Premarin, maximum concentration of equilin (4 ng/ml) and estrone (11.2 mg/ml) were reached by 20 min (19). These results very clearly indicate that both equilin sulfate and estrone sulfate present in Premarin are hydrolyzed to unconjugated equilin and estrone fairly rapidly. Similarly, oral ingestion of estrone sulfate results in rapid appearance of unconjugated estrone and 17β-estradiol in the blood (20, 21).

In order to determine whether conjugated equine estrogens are absorbed from the gastrointestinal tract as sulfates or only after hydrolysis, mixtures of [^{3}H]- and [^{35}S]-labeled equilin sulfate and [^{3}H]equilin were administered orally (Figure 2). Following administration of a mixture of [^{3}H]equilin sulfate and equilin [^{35}S]sulfate, the equilin sulfate isolated from the plasma contained both [^{3}H] and [^{35}S] labels (Table III). These data clearly indicate that some of the equilin sulfate ingested was absorbed from the gastrointestinal tract without prior hydrolysis. Since the ^{3}H/^{35}S ratio



[2,4-^{3}H] Equilin Sulfate I | Equilin [^{35}S] Sulfate II | [2,4-^{3}H] Equilin III

Figure 2. Labeled equilin sulfate and equilin used in absorption studies (Experiment: A and B—1:1 mixture of I and II was used; Experiment C—1:2 mixture of II and III was used.

Table III. Rate of Appearance of [^{3}H]Equilin [^{35}S]Sulfate after Oral Administration of [^{3}H]Equilin [^{3}H]sulfate (Expt. A & B, ^{3}H/^{35}S = 1) or [^{35}S]Equilin and Equilin [^{35}S]Sulfate (Expt. C, ^{3}H/^{35}S = 2) to Normal Men

Time (min)	A ^{3}H/^{35}S	B ^{3}H/^{35}S	C ^{3}H/^{35}S
10	1.00	1.00	26.00
30	1.80	1.50	13.10
60	3.80	2.60	9.50
120	—	3.40	23.60
180	7.50	7.30	23.30
240	7.80	12.60	15.80
300	12.80	14.60	20.10
360	30.00	20.00	20.70
480	100.00	23.00	40.00
720	100.00	75.00	α
1440	α	α	α

Adapted with permission from Ref. 22.

progressively increased after the first 10 min (Table III), a significant portion of administered equilin sulfate was absorbed after the removal of sulfate ester by hydrolysis. The unconjugated equilin that formed after absorption was rapidly sulfated, and it circulates in this form. When a mixture of [^{3}H]equilin and equilin [^{35}S]sulfate (^{3}H/^{35}S = 2) was ingested, the ratio at all time points was greater than 2 (Table III, Expt. C). These data indicate that unconjugated equine estrogens, such as equilin, were absorbed more rapidly than the corresponding sulfates; however, after absorption, equilin was rapidly sulfated, most likely during the first pass through the liver (22). Equilin sulfate is the main circulatory form of this hormone. Similarly, estrone sulfate is the main circulatory form of estrone (20, 23).

Metabolic Clearance Rates (MCR) of Conjugated and Unconjugated Equine Estrogens

Equilin Sulfate and 17β-Dihydroequilin Sulfate. The metabolic clearance rate of equilin sulfate, 17β-dihydroequilin sulfate, and estrone sulfate have been determined by the single injection and constant infusion techniques (24). Following a single intravenous injection (25, 26), the disappearance of radioactivity from plasma as equilin sulfate and 17β-dihydroequilin sulfate can be described as a function of two exponentials (Figure 3). The

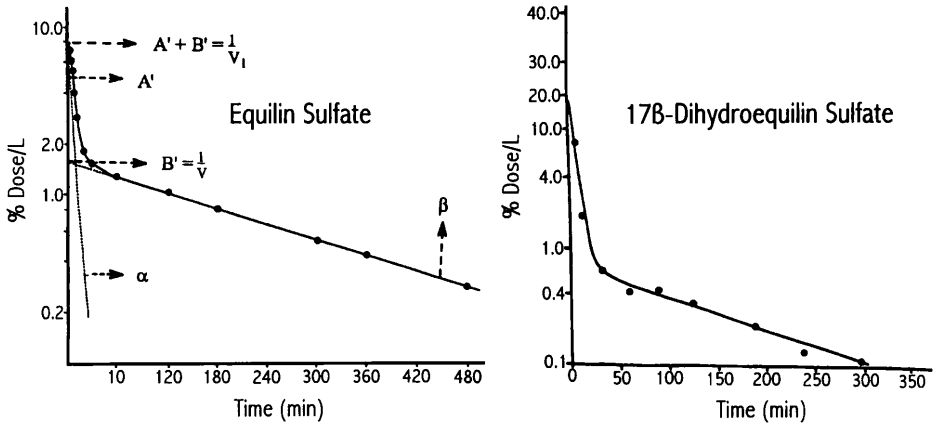


Figure 3. Disappearance of radioactivity from plasma as [^{3}H]equilin sulfate and [^{3}H]17β-dihydroequilin sulfate plotted as a percentage of the administered dose versus time of blood sampling. (Adapted with permission from Ref. 25 and 26.)

various pharmacokinetic parameters are summarized in Tables IV and Table V). The initial fast component for both estrogens had a half-life ($t^{1/2}$) of 5 min and the initial volume of distribution (V_1) was 12.4 ± 1.6 and 6.0 ± 0.5 l for equilin sulfate and 17β-dihydroequilin sulfate respectively (Tables IV and V). Similar data (23) have been previously reported for estrone sulfate (($t^{1/2}$; 3 min; V_1 = 7.2 ± 0.6). The volume of distribution of estrogen sulfate depends on their relative binding affinities to serum albumin. Since the apparent volume of distribution measured is higher than plasma volume, these estrogen sulfates bind to albumin with relatively low affinity, as has been reported (15).

The mean MCR of equilin sulfate and 17β-dihydroequilin sulfate in postmenopausal women was 176 ± 44 l/day · m^2 (Table IV) and 376 ± 53 l/day · m^2 (Table V) respectively. The MCR of estrone sulfate in men and women was 87 ± 36 to 105 ± 10 l/day · m^2 (20, 23). Based on the constant infusion technique and under steady state conditions, the MCR's for these estrogen sulfates, were similar to values given above (23, 27, 28). The half-lives of equilin sulfate, 17β-dihydroequilin sulfate, and estrone sulfate were 190 ± 23 min (25), 147 ± 15 min (26), and 300–540 min (20) respectively.

The MCR and half-life measurements, suggest that ring B unsaturated estrogens are cleared from the circulation at a faster rate than the ring B saturated estrogen estrone sulfate. However, in contrast to estrogen sulfates, androgen sulfates (29, 30) such as dehydroepiandrosterone sulfate and testosterone sulfate, have much lower (7.7, 21.5 l/day) MCR. The very low MCR of androgen sulfates is likely due to their greater binding affinity with albumin.

Unconjugated 17β-Dihydroequilin and Equilin. The mean MCR 17β-dihydroequilin in postmenopausal women was 1252 ± 103 l/day · m^2, and the disappearance of 17β-dihydroequilin from plasma also had two components. The half-lives of the fast and slow components were 5.5 ± 0.8 and 45 ± 2.0 min, respectively (Figure 4). In contrast, the disappearance of equilin from plasma was consistent with a one-compartment model (Figure 4). The half-life of equilin was approximately 19–27 min and MCR in one postmenopausal woman was 3300 l/day · m^2 and in a man was 1982 l/day · m^2 (25). The MCR of estrone has been reported to be 965 l/day · m^2–1310 l/day · m^2 (31, 32, 33), and there was no difference between males and females. 17β-estradiol has an MCR of 600–790 l/day · m^2 in females (31, 34) and 830–990 l/day · m^2 in males (31, 34). These data indicate that in the unconjugated form, both ring B unsaturated estrogens are cleared more rapidly than the corresponding ring B saturated estrogens.

Conversion Ratios for Interconversions Between Ring B Unsaturated Estrogens Under Steady State Conditions

In earlier study, it was demonstrated that in the pregnant mare, equilin was metabolized to the ring B unsaturated estrogen: equilenin, 17β-dihydroequilin, 17β-dihydro-

Table IV. Pharmacokinetic Parameters of Equilin Sulfate in Postmenopausal Women

Subject no.	Sex/age (year)	V_1 (L)	$t_{1/2}$ components (minutes)		MCR (L/day)	MCR (L/day.m^2)	MCR/kV_1
			1	2			
1	F,38	8.7	9.8	170	168	93	0.215
2	F,60	18.0	4.0	270	582	342	0.155
3	F,57	13.3	4.4	180	199	117	0.083
4	F,51	11.4	4.1	150	303	168	0.156
5	M,58	10.8	4.0	180	323	162	0.179
Mean ± SEM		12.4 ± 1.6	5.2 ± 1.2	190 ± 23	315 ± 73	176 ± 44	0.158 ± 0.02

Adapted with permission from Ref. 25.

Table V. Pharmacokinetic Parameters of 17β-Dihydroequilin Sulfate in Postmenopausal Women

Subject no.	Age (year)	V_1(L)	$t_{1/2}$ components (minutes)		(L/day)	MCR (L/day.m^2)	MCR/kV_1
			1	2			
6	56	6	5	110	832	475	0.45
7	51	5	5	145	510	300	0.30
8	53	6	4	140	635	385	0.35
9	56	8	5	200	802	502	0.35
10	61	5	5	140	331	220	0.30
Mean ± SEM		6 ± 0.5	5.0 ± 0.2	147 ± 15	622 ± 93	376 ± 53	0.35 ± 0.02

Adapted with permission from Ref. 26.

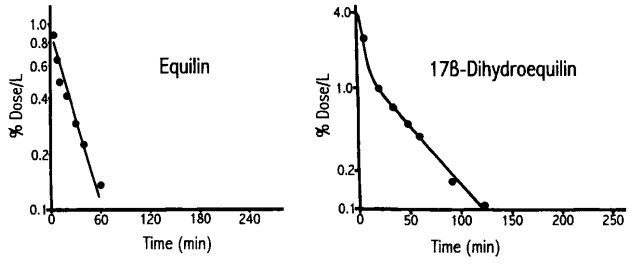


Figure 4. Disappearance of radioactivity from plasma as [^{3}H]equilin and [^{3}H]17β-dihydroequilin plotted as a percentage of the administered dose versus time of blood sampling. (Adapted with permission from Ref. 26.)

equilenin, 17α-dihydroequilin, and 17α-dihydroequilenin (1). With the exception of the 17α-reduced metabolites, the remaining three metabolites of equilin were also formed in the human (35–36). Similarly, in the above pharmacokinetic studies, following pulse injections of equilin sulfate and equilin, small amounts of 17β-dihydroequilin sulfate, 17β-dihydroequilin, equilenin sulfate, equilenin, 17β-dihydroequilenin sulfate, and 17β-dihydroequilenin were formed (25). Along with equilin sulfate and equilin, the above metabolites were also formed following pulse injections of 17β-dihydroequilin sulfate and 17β-dihydroequilin (26).

The precise amounts of each metabolite of the parent estrogen equilin sulfate have been determined, under steady state conditions, following a constant infusion of [^{3}H]equilin sulfate (27). The mean conversion ratios for equilin sulfate to its various metabolites, indicate the following order of formation: 17β-dihydroequilin sulfate > equilenin sulfate > 17β-dihydroequilenin sulfate > 17β-dihydroequilin > equilin > equilenin > 17β-dihydroequilenin. In both the sulfate-conjugated form and the unconjugated form, the most abundant metabolite formed was the more potent estrogen 17β-dihydroequilin. This conversion is analogous to the conversion of estrone sulfate to 17β-estradiol; however, the latter conversion is approximately 10 times lower (20, 29). The conversion of equilin sulfate to equilin (0.016) was similar to the conversion of estrone sulfate to estrone (0.015) (20, 27). The transfer constants or ρ values for the conversion of equilin sulfate to equilin and 17β-dihydroequilin were 0.25 and 0.15 respectively (27). The corresponding values for the conversion of estrone sulfate to estrone and 17β-estradiol were 0.15–0.21 and 0.014–0.03 respectively (20, 23). Since 17β-dihydroequilin and 17β-estradiol are the active metabolites of equilin sulfate and estrone sulfate respectively, the extent of this activation by 17β-reduction, is several-fold higher for the ring B unsaturated estrogens, and this is depicted in Figure 5.

Preliminary results also indicate that 17β-dihydroequilin sulfate under steady state conditions, was rapidly metabolized to equilin sulfate and other ring B unsaturated estrogens (28). Thus, even though the amount of 17β-dihydroequilin sulfate originally present in Premarin is small, the pharmacokinetics and pharmacodynamics of equilin sulfate and 17β-dihydroequilin sulfate and the extensive interconversions between these estrogens, support the hypothesis that increased estrogenic activity, associated with Premarin, compared to single component drugs such as

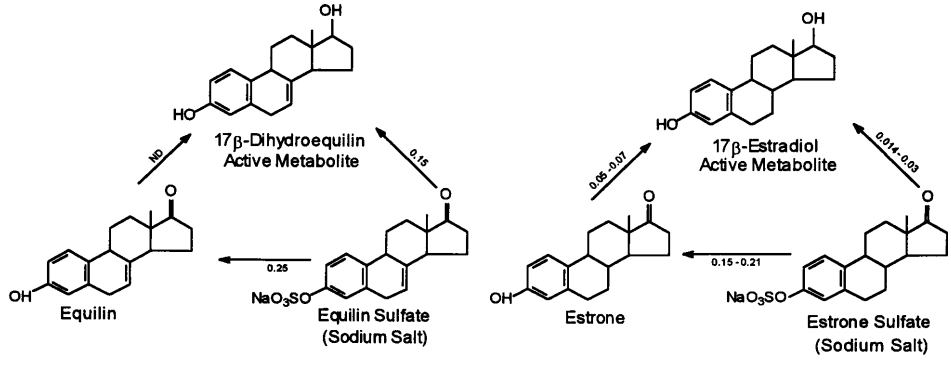


Figure 5. Extent of *in vivo* activation of the two main components of conjugated equine estrogen preparations by 17β-hydroxysteroid dehydrogenase. The values shown are transfer constants.

piperazine estrone sulfate and micronized estradiol, may in fact be due to the increased formation of the ring B unsaturated 17β-reduced metabolites.

Pharmacokinetics of Delta-8-Estrone

This novel ring B unsaturated estrogen (conjugated double bond in the B ring) is the most recent component identified in Premarin. Approximately 3.5% of the total estrogens, present in Premarin, consists of delta-8-estrone sulfate. This estrogen in its radioactive form is not available for pharmacokinetic studies of the type carried out with equilin sulfate and 17β-dihydroequilin sulfate. However, as discussed above, these types of estrogen sulfates are readily converted to unconjugated estrogens; therefore, pharmacokinetics of unconjugated delta-8-estrone were determined in both postmenopausal women and men (13). The mean MCR of delta-8-estrone was 1711 ± 252 l/day · m^2 (Table VI), and the disappearance of radioactivity as delta-8-estrone also had at least two components (Figure 6). The 2-fold difference between the two men and five postmenopausal women was most likely due to normal individual variations in fractional turnover rates, volumes of distribution, and hepatic and renal blood flow. Similar differences have been reported for other estrogens. The V_1 (19.8 l ± 2.0) for delta-8-estrone was much larger than the plasma volume, and this suggests relatively low binding affinity for SHBG and albumin. The MCR of delta-8-estrone is approximately 1.5 times lower than that of equilin but higher than that of 17β-dihydroequilin and similar to that of estrone. The results from these investigations also indicated that delta-8-estrone was fairly rapidly metabolized to the more active estrogen, delta-8-17β-estradiol, delta-8-17β-estradiol sulfate, and delta-8-estrone sulfate (Figure 6).

The pattern of delta-8-estrone metabolism appeared to be unique since the major metabolite in both the unconjugated and sulfate-conjugated form was the more active 17β-reduced product. Very small amounts of radioactivity remained unaccounted for. The rate of formation of delta-8-estrone sulfate and delta-8-17β-estradiol sulfate indicates that peak levels of these two metabolites were attained in 60–90 minutes and that nearly equal amounts of the two metabolites were formed (Figure 6). Though the extent of 17β-reduction (i.e., formation of delta-8-17β-estradiol and its sulfate) supports observations with other ring B unsaturated estrogens, the extent of this activation of this estrogen is far greater. Whether or not this is due to the novel structure of delta-8-estrone, remains to be determined. Since both delta-8-estrone and delta-8-17β-estradiol can interact with estrogen receptors (13), and delta-8-estrone sulfate has been shown to have clinical effects (9), this estrogen can contribute to the overall *in vivo* biological effects of Premarin even though it is present in relatively small amounts.

In Vitro Metabolism of Ring B Unsaturated Equine Estrogens

Metabolism of Equilin in Human Endometrium. Equilin is extensively metabolized by various types of normal and malignant human endometrium, including adenocarcinoma grown in athymic nude mice (37). In these tissues, equilin was metabolized to equilenin, 17β-dihydroequilin, and 17β-dihydroequilenin. Equilenin was the most abundant metabolite formed by both normal and malignant endometrium. The highest level of the two 17β-reduced metabolites was formed by the secretory endometrium. Similar observations have been made for the conversion of estrone to 17β-estradiol (38, 39). These findings are in keeping with the several-fold higher activity of 17β-hydroxysteroid dehydrogenase reported in the secretory endometrium (39). The formation of 17β-dihydroequilin in the endometrium may be of importance, as this estrogen is several times more potent a uterotropic agent than equilin and estrone.

Metabolism of Equilin to Catechol Estrogens. It has been suggested that the oncogenic potential of the classical estrogens, estrone and 17β-estradiol, and synthetic estrogen depends on the extent of their metabolism to extremely labile catechol estrogens (40, 41). In the Syrian hamster kidney mode, 17β-estradiol, estrone, and equilin induced kidney tumors in the majority of animals. Interestingly, 2-hydroxy estrone and 2-hydroxy estradiol were inactive whereas 4-hydroxy estradiol induced kidney tumors in 100% of the animals (42). In contrast, ring B unsaturated estrogen equilenin in pig liver formed only 4-hydroxy equilenin, which was devoid of any carcinogenic activity in the

Table VI. Pharmacokinetic Parameters of Delta-8 Estrone in Postmenopausal Women

Subject no.	Sex/age year	V_1(L)	$t_{½}$ components (minutes)		MCR (L/day)	MCR (L/day.m^2)	MCR/kV_1
			1	2			
1	M,59	22.0	6	50	2133	1066	0.50
2	M,66	9.0	6	60	1261	630	0.76
3	F,47	25.0	5	35	3580	1990	0.65
4	F,83	25.0	5	40	3458	2034	0.60
5	F,70	20.0	5	35	3595	2055	0.74
6	F,37	21.0	5	28	4069	2543	0.59
7	F,67	17.0	5	35	3150	1660	0.72
Mean ± SEM		19.8 ± 2.0	5 ± 0.2	40.4 ± 4	3035 ± 380	1711 ± 252	0.65 ± 0.04

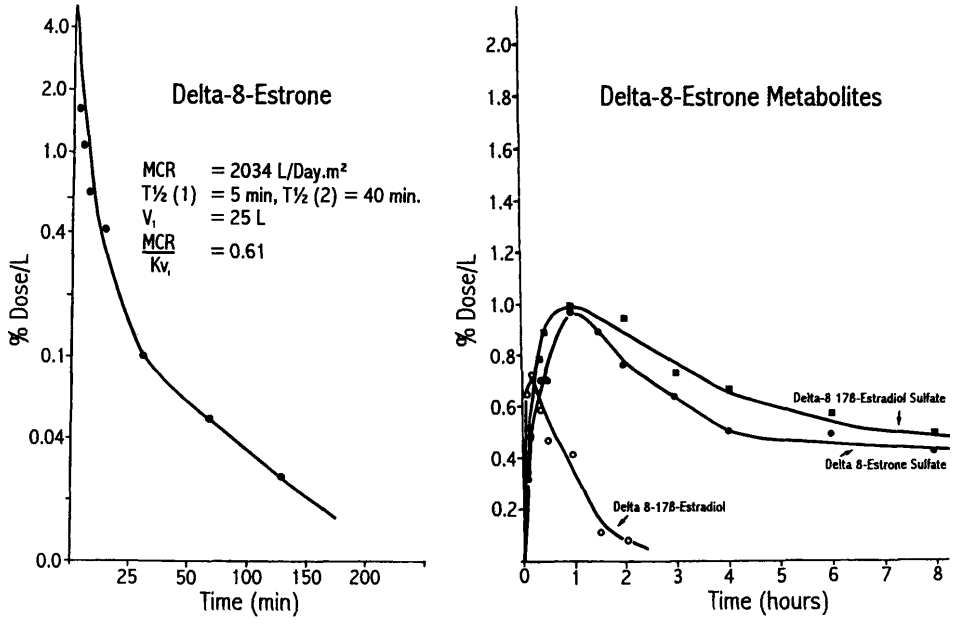


Figure 6. Pharmacokinetics of delta-8-estrone and its three metabolites, after administration of [^{14}C]delta-8-estrone.

hamster kidney (42). However, conflicting results have recently been reported by the same investigator (43).

We have recently (44), demonstrated that human endometrial preparations can form 2- and 4-hydroxy equilin, with 2-hydroxylation being predominant (2.5 times higher than 4-hydroxylation). In contrast, 2-hydroxy estradiol and 4-hydroxy estradiol in the proliferative human endometrium were formed in equal amounts (45). Thus, the extent of 2- and 4-hydroxylation of an estrogen may be dependent on the structure. The role of catechol derivatives of ring B unsaturated estrogens in postmenopausal women has not been studied. These catechol derivatives can serve as substrates for redox cycling and generation of free radicals that may result in adduct formation and subsequent cell damage. Alternatively, these equilin metabolites could play a protective role in the endometrium as free radical scavengers and antioxidants. Moreover, the formation of these catechol estrogens in the human endometrium can also decrease the amount of equilin available for metabolism to the more potent 17β-reduced products.

Urinary Excretion of Ring B Unsaturated Estrogens

In the pregnant mare, following administration of [^{3}H]equilin, more than 90% of the administered dose was excreted in the urine within 2 hr. Over 84% of this total was present in the sulfate fraction whereas only 5.6% and 1.7% were found in the glucoronide and unconjugated fraction respectively (1). The major metabolite of equilin excreted in the urine was equilin sulfate, followed by 17α-dihydroequilin sulfate. In contrast, following administration of [^{3}H]equilin sulfate (or equilin) and [^{3}H]17β-dihydroequilin to post-menopausal women and men (22, 35, 36), less than 50% of the administered dose was excreted in the urine. The bulk (63%–74%) of the radioactive metabolites excreted were in form of glucuronides (Table VII), whereas 16%–17% and 1%–2% were found in the sulfate and unconjugated fractions respectively. From these fractions, equilin, equilenin, 17β-dihydroequilin, and 17β-dihydroequilenin were identified. No 17α-reduced metabolites were formed. However, the bulk of the radioactivity was present in the form of two very polar metabolites (1, 2, 22, 35, 36). Recently, these two metabolites were identified (46) as 16α-hydroxy-17β-dihydroequilin (1,3,5(10)7-estratetraen-3,16α,17β-triol) and 16α-hydroxy-17β-dihydroequilenin (1,3,5(10)6,8-estrapentaen-3,16α,17β-triol) depicted in Figure 7. Previous studies (47, 48) have discussed the potential role of 16α-hydroxylated estrogens in oncogenic and other disease processes. More importantly, it was demonstrated that 16α-hydroxyestrone, which is a major urinary metabolite of 17β-estradiol and estrone in humans, can form stable covalent adducts with macromolecules, which may be involved in diseases such as breast cancer (49). The covalent adduct formation between 16α-hydroxy estrone and macro-

Table VII. Distribution of Urinary Radioactivity in Various Fractions after Intravenous Administration of ^{3}H-Equilin Sulfate and ^{3}H-17β-Dihydroequilin to Postmenopausal Women

Fraction	% of total recovered in the XAD-2-extract of urine (mean ± SD)	
	Equilin sulfate (n = 4)[a]	17β-Dihydroequilin (n = 5)[b]
Unconjugated	1.3 ± 0.5	1.7 ± 0.5
Sulfates	16.9 ± 8.0	16.4 ± 5.0
Glucuronides	73.6 ± 6.6	63.0 ± 10

[a] % dose excreted in urine in 3 days 39.75 ± 8.6; XAD-2 extractable 87.75 ± 4.6.
[b] % dose excreted in urine in 3 days 46.2 ± 10.5; XAD-2 extractable 78.8 ± 6.6.

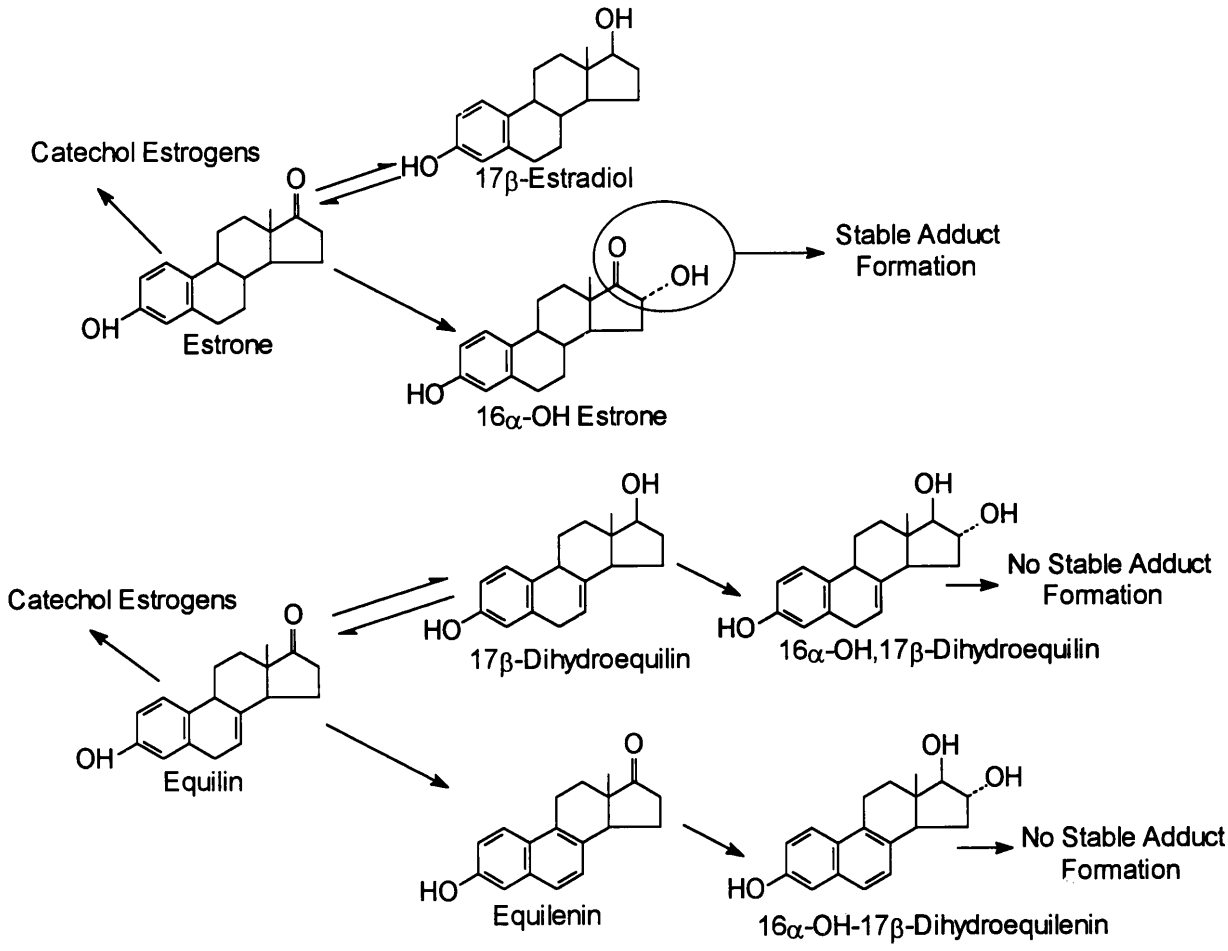


Figure 7. *In vivo* 16α-hydroxylation of estrone and the ring B unsaturated estrogen equilin, and the potential for forming adducts.

molecules occurs because of the presence of the D-ring α-ketol (i.e., 16α-hydroxy-17-ketone structure (Figure 7)) (50, 51). Since the two 16α-hydroxylated metabolites of equilin lack this α-ketol, it is highly unlikely that these metabolites of ring B unsaturated estrogens can form the potentially carcinogenic stable adducts, by the proposed mechanisms. The absence of 17-keto-16α-hydroxylated derivatives of equilin in human urine following the administration of equilin and 17β-dihydroequilin, supports the observations that 17β-reduction occurs at a much higher extent than with the classical estrogen estrone, as discussed above (Figure 6).

Summary of Key Points

1. All 10 known components of Premarin are biologically active estrogens.
2. These estrogens can be absorbed as sulfates from the gastrointestinal tract.
3. Estrogen sulfates do get hydrolyzed to some extent in the gastrointestinal tract. The unconjugated estrogens are absorbed more readily than their corresponding sulfate forms.
4. The unconjugated estrogens after absorption are rapidly sulfated (First Pass Metabolism) and circulate in this form.
5. The sulfate forms of these estrogens can bind to albumin with a higher affinity than their unconjugated form.
6. The 17β-reduced forms bind to SHBG with high affinity.
7. The 17-keto components of conjugated equine estrogen preparations, such as estrone sulfate, equilin sulfate, and delta-8-estrone sulfate, are metabolized by postmenopausal women to the more potent 17β-reduced products.
8. The extent of this activation (17β-reduction) is nearly 10 times higher with the ring B unsaturated estrogens. This activation can occur in target tissues such as the human endometrium.
9. Both types of estrogens can form the 2- and 4-hydroxy catechol derivatives.
10. 16α-hydroxylation occurs with both types of estrogens; however, 16α-hydroxy-17β-dihydroequilin and 16α-hydroxy-17β-dihydroequilenin cannot bind cova-

lently with proteins or other macromolecules as proposed for 16α-hydroxy estrone.

11. Individual components of conjugated equine estrogens, such as equilin sulfate, delta-8-estrone sulfate, 17β-dihydroequilin sulfate, and estrone sulfate have potent clinical (estrogenic) effects.
12. Since all of the conjugated equine estrogens have estrogenic activity, the pharmacological effects of Premarin are a result of the sum of these individual activities. Therefore, preparations lacking some of these components may mimic only some of the pharmacological effects of Premarin. Whether or not these types of preparations can offer the same degree of beneficial effects as Premarin has not been demonstrated.

Conclusions

In 1988, we reviewed (2) the literature regarding the biosynthesis of the ring B unsaturated by the pregnant mare in a paper entitled "The Saga of the Ring B Unsaturated Equine Estrogens." Since then, we have gained insight into the pharmacokinetics and pharmacodynamics of these unique groups of estrogens. Moreover, there is now a large body of evidence clearly indicating that conjugated equine estrogens, such as Premarin, not only provide relief of the vasomotor symptoms in postmenopausal women but also prevent osteoporosis, decrease the risk of cardiovascular disease, and improve the quality of life in these women. More recently, preliminary data suggest that these estrogens may also delay or prevent Alzheimer's disease and the aging process in general. Further research and clinical trials are needed to substantiate these important initial observations.

Since Premarin expresses its effects through all of its components, and because of its complexity, it is difficult to envision how a synthetic mixture of some of these estrogens is going to exert the same biological and clinical effects associated with this drug. Recent work dealing with the mechanism of how an estrogen expresses its effects strongly suggests that some of the Premarin components may have tissue selective effects even when present in small concentrations. Thus, identification of estrogen components that may be involved, for example, in cardioprotective effects offers the opportunity for development of new drugs that may be of therapeutic use not only in postmenopausal women, but also in men. Hence, the "Saga of the Ring B Unsaturated Estrogens Continues."

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